Journal Homepage: -www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH (IJAR)

Article DOI:10.21474/IJAR01/19929
DOI URL: http://dx.doi.org/10.21474/IJAR01/19929

RESEARCH ARTICLE

MOLECULAR TYPING OF HLA-B*27 SUB-ALLELES REVEALS AN ASSOCIATION WITH ERAP1 AND DISEASE SUBSET OF ANKYLOSING SPONDYLITIS IN NORTH-INDIAN (KASHMIR) POPULATION

Nasia Ismail[1], Roohi Rasool[1], Zafar A. Shah[1], Muneer Ahmad Baba[2], Fayaz A. Kanjwal[3], Nahida Majid[1], Rabia Rakhshan[4], Muzaffar Ahmad Bindroo[3] and Sakeena Ayub[1]

1. Departrment of Immunology and Molecular Medicine, Sher-i- Kashmir Institute of Medical Sciences (SKIMS) Srinagar, J&K India.
2. Department of Medical Gastroenterology, GMC Srinagar, J&K India.
3. Department of Rheumatology, Sher-i- Kashmir Institute of Medical Sciences(SKIMS)Srinagar, J&K India.
4. Department of Nano-Technology, University of Kashmir, J&K India.

Manuscript Info

Manuscript History
Received: 14 September 2024
Final Accepted: 24 October 2024
Published: November 2024

Key words:-
Human Leukocyte Antigen (HLA), Ankylosing Spondylitis, Polymerase Chain Reaction, Spondyloarthropathy, Association, Polymorphism, Subtyping, Genotyped

Abstract

Background: Human Leukocyte Antigen(HLA)-B*27 is significantly linked to Ankylosing spondylitis(AS) and its presence aids in the diagnosis of the disease. Identification of HLA-B*27 allele plays an important role in the clinical monitoring,diagnosis and therapy of this spondyloarthropathy because of high HLA polymorphism and differential contribution of alleles and molecules encoded by them. HLA-B*27 is also present in general population 7-8%.We evaluated the presence and correlation of various HLA-B*27 alleles with clinical parameters and ERAP1 (SNP's rs27434, rs27529 and rs26510) of Ankylosing spondylitis in Kashmiri Population.

Methods: This study involved total 200 cases (100 Ankylosing Spondylitis patients and 100 healthy controls). Genomic DNA was extracted by phenol-chloroform method. All subjects were genotyped for HLA-B*27 subtyping by Polymerase Chain Reaction(PCR)-Sequence Specific Primer(SSP) method.

Result: HLA-B*27 were present in 90% of cases and in 20% of controls OR 36.0(15.91-81.48);P= <0.0001. HLA-B*27:02 OR 9.12(2.63-31.6); p=<0.0001, CAFRW OR 12.61(4.73-33.9);p= <0.0001 and HLA-B*27+CAFRS OR 45.07(2.64-759.4);p=0.0001 were showing significant association with AS disease.

Conclusion: The current study indicates that a majority of Kashmiri AS patients are associated with HLA-B*27 alleles. In addition we found that HLA-B*27 associated AS patients presented with more severe axial manifestation.

Introduction:-

AS is a genetically predisposed systemic disorder. The genetics of AS have been linked to the major histocompatibility complex (MHC) locus on chromosome 6p and other non-MHC loci. Brewerton et al, described the substantial link between HLA–B*27 and AS in 1973**[Brewerton DA et al.,1973]**. HLA B*27 is a surface

Corresponding Author:- Zafar A. Shah
Address**:-**Departrment of Immunology and Molecular Medicine, Sher-i- Kashmir Institute of Medical Sciences (SKIMS) Srinagar, J&K India.

antigen of the human leukocyte antigen class I that delivers antigenic peptides to CD-8^+ T-cells. It is encoded by Major Histocompatibility Complex gene(MHC- gene) **[Brewerton D A., 1976; Khan MA et al.,2002].** The mature HLA-B*27 complex comprises a three-part quaternary structure. HLA-B*27 function depends on its correct structure and folding in the ER. After being generated as free heavy chains, HLA-B*27 is non-covalently coupled and folded with 2m and antigenic peptide, and then transported to the cell surface as a trimolecular complex **[Colbert, R. A et al.,2014].** HLAB*27, on the other hand, shows a proclivity for misfolding and the formation of dimers and even multimers**[Colbert, R. A et al.,2009],** which could be due to cysteine (C) at sites 67 (C67), 101 (C101), 164 (C164), and 325 (C325) (C325)**[Chen, B. et al].** HLA-B*27 would be synthesized and transferred to the cell surface as HLA-B*27 homodimers with heavy chains if the folding was wrong. Several genetic variables have been linked to AS susceptibility in previous research **[Colbert, R. A et al.,2009].** HLA-B*27 folds slower than other HLA alleles for a variety of reasons, and without appropriate folding, these faulty HLA-B*27 proteins continue to assemble in the ER. HLA-B*27 proteins that are incorrectly folded concentrate in the ER and activate autophagy and the interleukin (IL)-23/IL-17 pathway **[Colbert, R. A.,2009; Brown M A.,1997].** More importantly, these misfolded molecules can disrupt ER function, causing ER stress and activating the pro-inflammatory endoplasmic reticulum unfolded protein response (ERUPR), which activates the IL-23/IL-17 pathway further**[Turner, M. J. et al.,2005].**However, conflicts exist regarding whether the HLA-B*27-activated ERUPR occurs in AS patients. In macrophages from AS patients, enhanced IL-23 production was seen without significant ERUPR induction. Although disease-related SNPs in the ERAP1 or HLA-B*27 loci did not affect the severity of ER stress in AS, **[Kenna, T. J. et al.,2014]**it remained contentious in further research. One possibility is that misfolding of HLA-B*27 causes autophagy and activates the IL-23/IL-17 pathway rather than ERUPR. More research is needed to show the link between ERUPR and HLA-B*27 during the development of AS. The disulfide bonds of the cysteine at C-67 in HLA-B*27 heavy chains tend to form homodimers without 2m**[Chen, B et al.,2013].** The HLA-B*27 dimeric complexes are usually detected in patients' guts and synoviums, and they may have a role in the development of AS and other SpAs. HLA-B*27 dimers seen on antigen-presenting cells may induce IL-17 production in IL-23 receptor T lymphocytes**[Ranganathan, V et al.,2017].** HLA-B*27 dimers may play an essential role in the development of AS, according to the notion of cell-surface HLA-B*27 homodimer production. HLA-B*27 homodimers have been related to receptors expressed on natural killer (NK) immunocytes, myelomonocytes, and lymphocytes. Killer cell immunoglobulin-like receptors (KIRs) and leucocyte immunoglobulin-like receptors (LILRs) mediate the binding, which has a role in autoimmune disorders **[Allen, R. L et al.,2001].**

The prevalence of HLA-B*27 varies by area, however, it is about 8% of the overall population. HLA-B*27 is present in 6.1 percent of the general population in the United States, but it is present in 9.2 percent of the general population in New Zealand**.** In the African population, HLA-B*27 is uncommon, which is consistent with a low illness incidence **[Benjamin R et al.,1990].** HLA-B*27 is a highly polymorphic gene with a number of subtypes that have been found **[Khan M A.,2017].** The HLA-B*27:02, B*27:04, and B*27:05 all show a strong link to AS**.** Polymorphisms in the HLA-B*27 gene are found throughout the world, but their prevalence varies. In the white British population, HLA-B*27:05 is the most prevalent variant among HLA-B*27 carriers **[Hermann E et al.,1993].** In Chinese populations, however, a combination of B*27:04 and B*27:05 is the most common subtype **[Scofield RH et al.,1995].**HLA-B*27 disease-related structures, such as HLA-B*27:05, HLA-B*27:04, and HLA-B*27:02, have been reported to have a slightly lower rate of correct folding processes than HLA-B*27:06 and HLA-B*27:09, which are not typically thought to be associated with AS**[Reveille JD.,2014].**

Patients and Methods:-

This study was a hospital based study conducted in the Department of Immunology and molecular medicine, in collaboration with the Division of Rheumatology, Sheri-Kashmir Institute of Medical Sciences (SKIMS), J&K (North India) and was approved by the institutional ethical committee IEC/SKIMS RP-66/2020. We included 100 newly diagnosed AS patients. 100 Control blood sample were taken from the healthy individuals. who visited the outpatient clinic of Department of Rheumatology, SKIMS, between March 2019 and March 2022. Diagnosis of AS followed the 1984 modified New York criteria **[Van der Linden S.,1984].**The clinical data included age, sex, family history, onset age, peripheral arthritis, hip joint involvement, enthesitis, erythrocyte sedimentation rate (ESR), and C-reactive protein (CRP). The patients who were taking immunesupression drugs and have any other autoimmune disease were excluded from the study.Participants were informed about the study, and the proper consent were taken from all the participants before taking the blood sample for the study, which were approved by ethics committee of SKIMS, Srinagar.

Genotyping:
Genomic DNA was extracted from all the blood sample by manually using phenol/chloroform extraction method (**Blin N & Stafford DW, 1976**). We design different set of primers against each SNP. The primers used for HLA-B*27 typing by the PCR-SSP reaction were constructed according to the sequences described by Duangchanchot et al. (2009) and are shown in Table 1. Two primer mixes (SC1 and SC2) were used to assess the presence of HLA-B*27 allelic group and nine mixes were used to identify the alleles.

Table 1:- Primer used for sequence specific PCR is given below:

Mix	Primers	Amplicon size
DRB1	**F:5-TGCCAAGTGGAG CACCCA-3** **R:5- GCATCTTGCTCTGTGCAGAT-3**	**736bp**
SC1	**F:** 5-**GCT ACG TGG ACG ACA CGC T-3** **R:** 5-**GTC TGT GCC TTG GCC TTG C-3**	**142bp**
SC2	**F: 5-GAC GCC GCG AGT CCG AGA-3** **R:** 5-**CAC GTC GCA GCC ATA CAT AT-3**	**436bp**
MIX 2	**F:** 5-**ACC GAG AGA ACC TGC GGA T-3** **R: 5-CAC GTC GCA GCC ATA CAT AT-3**	**330bp**
MIX 3	**F:5-GCT ACG TGG ACG ACA CGCT-3** **R:**5-**GTG TCT CCC GGT CCC AAT G-3**	**117bp**
MIX 4	**F:** 5-**GGT CTC ACA CCC TCC AGAA-3** **R:** 5-**CTC TCA GCT GCT CCG CCT-3**	**201bp**
MIX 5	**F:** 5-**ACC GGG AGA CAC AGA TCT G-3** **R:5-CTT GCC GTC GTA GGC GTC-3**	**423bp**
MIX 10	**F:5-GCA CAG ACT GAC CGA GAGG-3** **R:**5-**CAC GTC GCA GCC ATA CAT AT-3**	**340bp**

bp: base pair, F:forward, R:reverse.

Used few internal controls to monitor results. Include negative, positive and DRB1 were used as an internal control. SSP PCR was carried out to genotype. This PCR-SSP method is based on the principle that only primers with completely matched sequences to the target sequences result in amplified products under controlled PCR conditions. The presence of amplified DNA fragment is a positive indication of the existence of allele specific sequence in the genomic DNA. On the other hand, mismatched primers do not generate amplicons. In addition to sequence specific primers, an internal control primer pair, which amplifies a conserved region of the house keeping gene, is included in every PCR reaction mix. The PCR product of the internal control primer pair serves as an indication of the integrity of PCR reaction. DNA amplification was performed by using 25µl reaction volume, containing genomic DNA 2.5µl, 2.5µl PCR buffer, 0.5µl of dNTPs, 1µl Taq pol, sense and antisense primers 1µl, $Mgcl_2$0.5 µl and 17.5µl sterile water by heating the reaction volume at different temperatures (time and annealing temperature given in Table 2). The reaction sequence used for genotyping of HLA-B*27 allelic varients is given in figure.

Table 2:- The following temperature profile was used for amplification:-

Cylcles	Denaturation	Anealing	Extension
1cycle	96°Cfor 2 min	-	-
5 cycles	96°C for 30 sec	68°C for 60 sec	72°C for 40 Sec
21 cycles	96°C for 30 sec	65°C for 60 sec	72°C for 40 sec
4 cycle	96°C for 30 sec	55°C for 75 sec	72°C for 120 sec
1 cycle	-	-	72°C for 10min

Agarose gel electrophoresis:
The PCR-SSP has direct diagnostic use, the reason is that it doesn't require hybridization steps. The results are run on 2% agarose gel, ideally under proper agarose gel running conditions. Along with, add a 100 bp molecular marker. The gel electrophoresis technique is sufficient to separate different alleles and evaluate results.

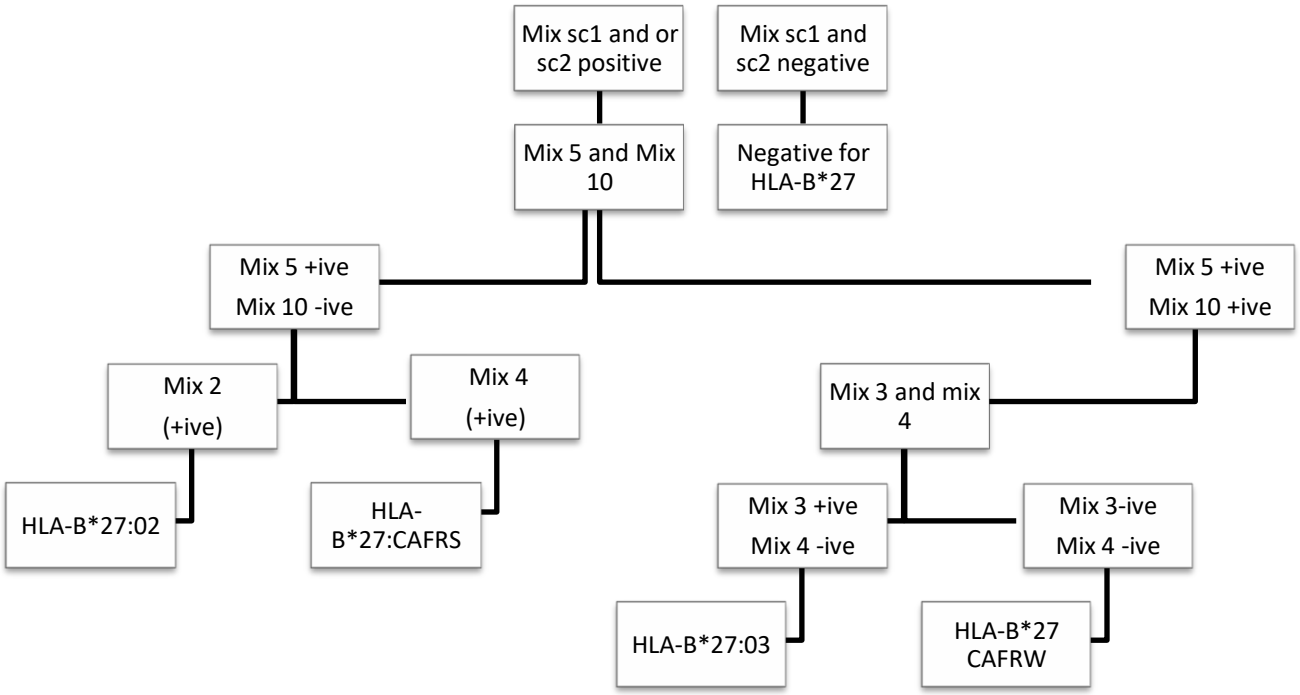
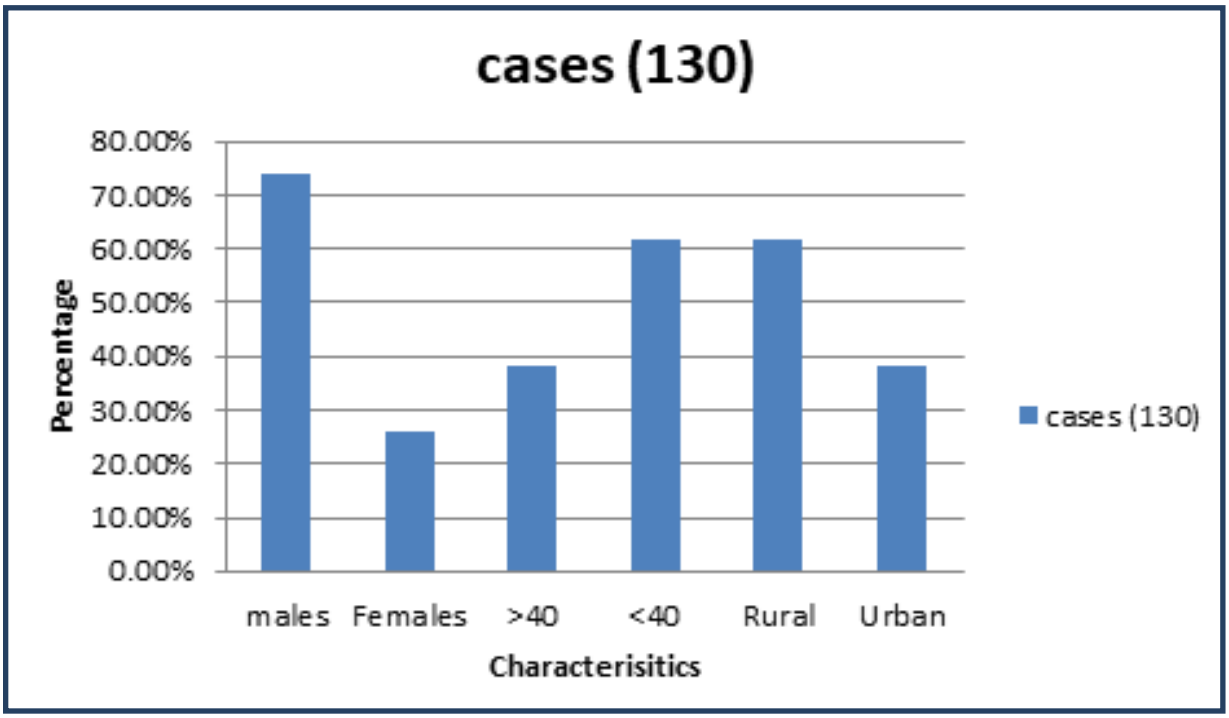


Fig. 1:- The reaction sequence used for genotyping of HLA-B*27 allelic varients.
CAFRS: 04/15/25
CAFRW: 05/13/16/17/28/37/38/39/42

Statistical analysis:
The distribution of the genotypes in controls was compared with that expected by the chi square ($\chi 2$) test. Odds ratios (ORs) and their 95% confidence intervals (CIs), were calculated by Fisher's exact test/ Chi square test. All reported P values were based on two-sided tests. Significance level was taken at $p \leq 0.05$. Statistical tests were performed using the software SPSS 16.0 (SPSS software and Graph Pad Prism 5).

Result:-

Characteristics of AS patients are shown in figure 2 and 3.The HLA-B*27 subtyping was analyzed by extraction of high molecular weight DNA from blood of 100 AS cases and 100 normal controls.PCR amplification was carried out by using a set of primer pairs that amplified the region of HLA-B*27 gene (SC1 & SC2) containing the sub alleles. The amplicon sizes were 142 and 436 bp.DRB*1 was used as an internal control having 736 bpsize. The representative HLA-B*27 PCR amplification gel picture is presented in figure 4.



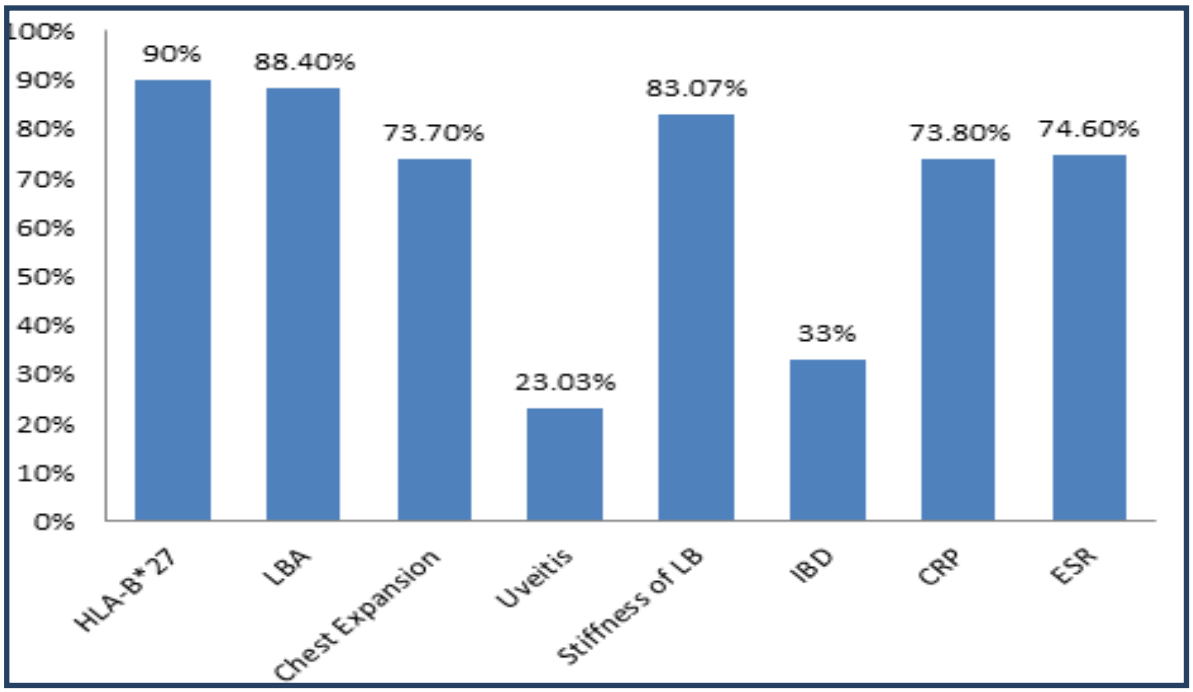


Fig 2:- Bar-graph showing age, dwelling and gender of AS casesFig 3 Bar-graph showing clinical characters of AS patients.

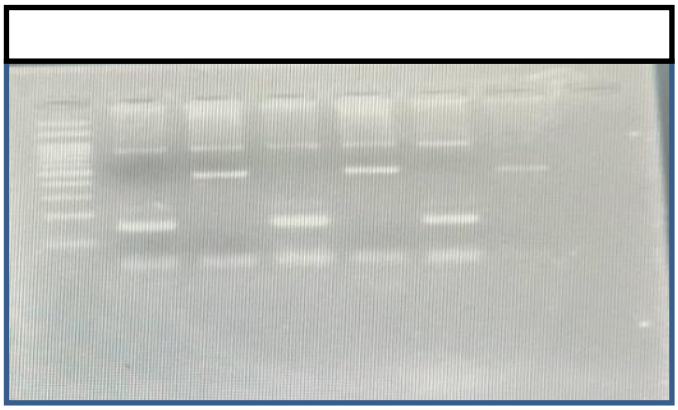


Fig.4:- Representative gel picture showing HLA-B*27 amplicons
Lane M= 100bp ledder, Lane 1,3,5=SC1 :142bp + DRB1: 736bp (internal control)
Lane 2,4,6=SC2: 436bp +DRB1:736bp.

When these samples were positive for HLA-B*27, these samples were genotyped by using primers Mix5 and Mix 10 having 423bp and 340 bp size shown in figure 5

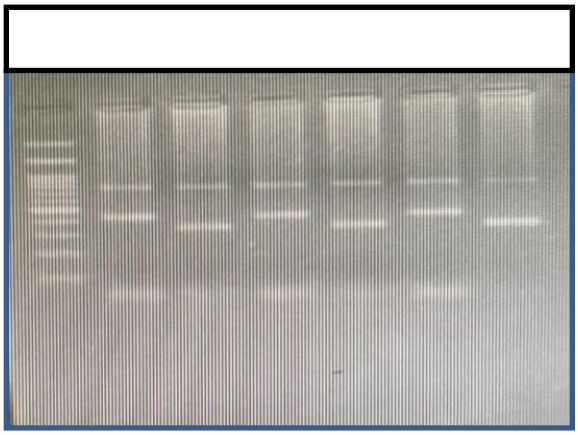


Fig.5:- Representative gel picture showing Mix5 and Mix 10.
Lane M= 100bp ledder, Lane 1,3,5= MIX 5 :423bp + DRB1: 736bp (internal control)
Lane 2,4,6=MIX 10 : 340bp +DRB1:736bp.

When these samples were positive for Mix 5 and Mix 10, these samples were genotyped by using primers Mix 2, Mix 3 and Mix 4 having 330bp, 117bp and 201 bp size shown in figure 6.

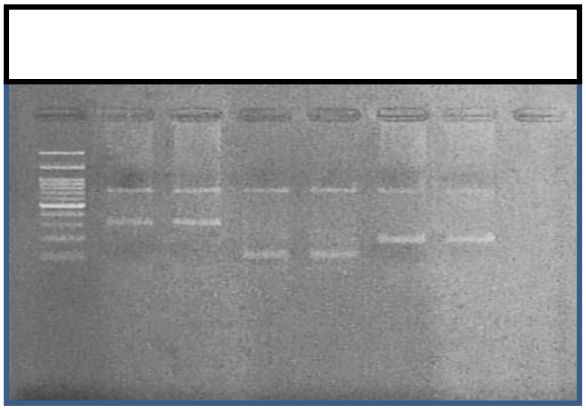


Fig.6:- Representative gel picture showing Mix2, Mix3 and Mix4 amplicons.

Lane M= 100bp ledder
Lane 1,2= MIX 2 :330bp + DRB1: 736bp (internal control)
Lane 3,4=MIX 3: 117bp +DRB1:736bp.
Lane 5,6= MIX 4:201bp+ DRB1 :736bp

Overall, genotype and distribution of allele frequencies in patients and controls for HLA-B*27 are displayed in Table 3. showed that HLA-B*27:02,CAFRW and HLA-B*27+CAFRS were significantly associated with AS. Most of AS patients were positive for HLA-B*27 shown in figure 7. The frequency of allelesHLA-B*27:02, CAFRW and HLA-B*27+CAFRSare significantly higher in cases of AS shown in figure 8. HLA-B*27:02 is present in 22% of AS patients and only 3% in controls (OR= 9.12(2.63-31.6) 95% CI; p = <0.0001). CAFRW is present in 40% of cases and 5% in controls (OR =12.61(4.73-33.9)95% CI; p = <0.0001). HLAB*27:02+CAFRS is present in 18% of cases and we did not find this allele in controls (45.07(2.64-759.4) 95% CI; p = <0.0001).We evaluated HLA-B*27 subtyping in AS patients and we found90% of patients were positive for HLA-B*27(P=<0.0001). 22% of patients and 3% of controls were positive for HLA-B*27:02(P=<0.0001).2% ofpatients and 9% of controls were positive for HLA-B*27:03.8% of patients and 3% of controls were positive for CAFRS (4/15/25).40% of patients and 5% of controls were positive for CAFRW (05/13/16/17/28/37/38/39/42) (P=<0.0001).18% of patients were positive for HLA-B*27+CAFRS. We found a significant association between HLA-B*27:02 and CRP levels of AS patients (P=0.003) shown in table 4. Other alleles did not show any significant association with rest of other parameters.The relationship between HLA-B27 subtypes and ERAP1 single nucleotide polymorphisms (SNPs) was examined to better understand how genetic variations may contribute to ankylosing spondylitis (AS) susceptibility. As shown in table 5 , our analysis revealed a significant association between the CAFRW subtype of HLA-B27 and the rs27434 A>G SNP (p = 0.03). This suggests that the CAFRW subtype may be linked to the rs27434 A>G variant in the context of AS. However, no significant associations were observed between other HLA-B27 subtypes and the ERAP1 SNPs investigated in this study. These results indicate that the association between HLA-B*27 subtypes and ERAP1 SNPs may be subtype-specific, with the CAFRW subtype showing a notable link to rs27434 A>G in AS patients.

Table 3:- Frequency distribution and association of HLA-B*27 subtypes in AS patients with controls:

Alleles	Cases (100)	Controls (100)	OR(95%CI)	P. Value
HLA –B*27 Positive Negative	 90 (90%) 10 (10%)	 20 (20%) 80 (80%)	36.0(15.91-81.48)	<**0.0001**
HLA-B*27:02 Present Absent	 22 (22%) 78 (78%)	 03 (3%) 97 (97%)	9.12(2.63-31.6)	<**0.0001**
HLA-B*27:03 Present Absent	 02 (2%) 98 (98%)	 09 (9%) 91 (91%)	0.20(0.04-0.98)	0.52
CAFRS Present Absent	 08 (8%) 92 (92%)	 03 (3%) 97 (97%)	2.81(0.72-10.9)	0.21
CAFRW Present Absent	 40 (40%) 60 (60%)	 05 (5%) 95 (95%)	12.61(4.73-33.9)	<**0.0001**
HLA- B*27:02+CAFRS Present Absent	 18 (18%) 82 (82%)	 0 100 (100%)	45.07(2.64-759.4)	<**0.0001**

OR: odds ratio, HLA: human leukocyte antigen, CAFRS: 04/15/25 CAFRW: 05/13/16/17/28/37/38/39/42

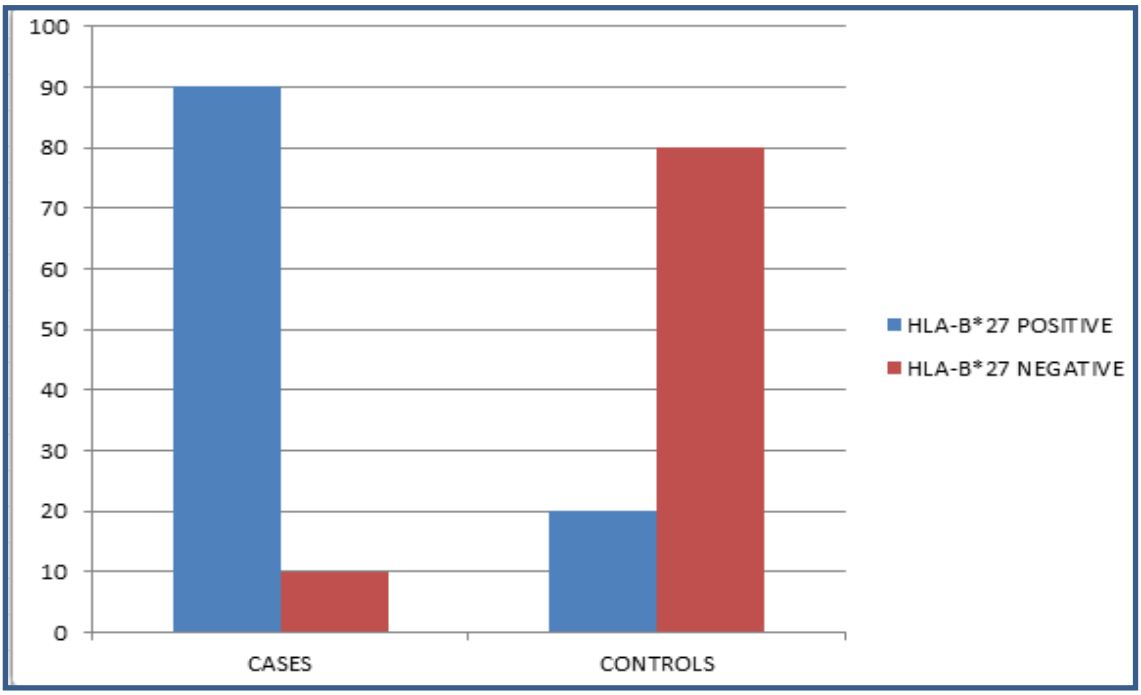


Fig.7:- Bar Graph showing HLA-B*27 positive and negative in cases and controls.

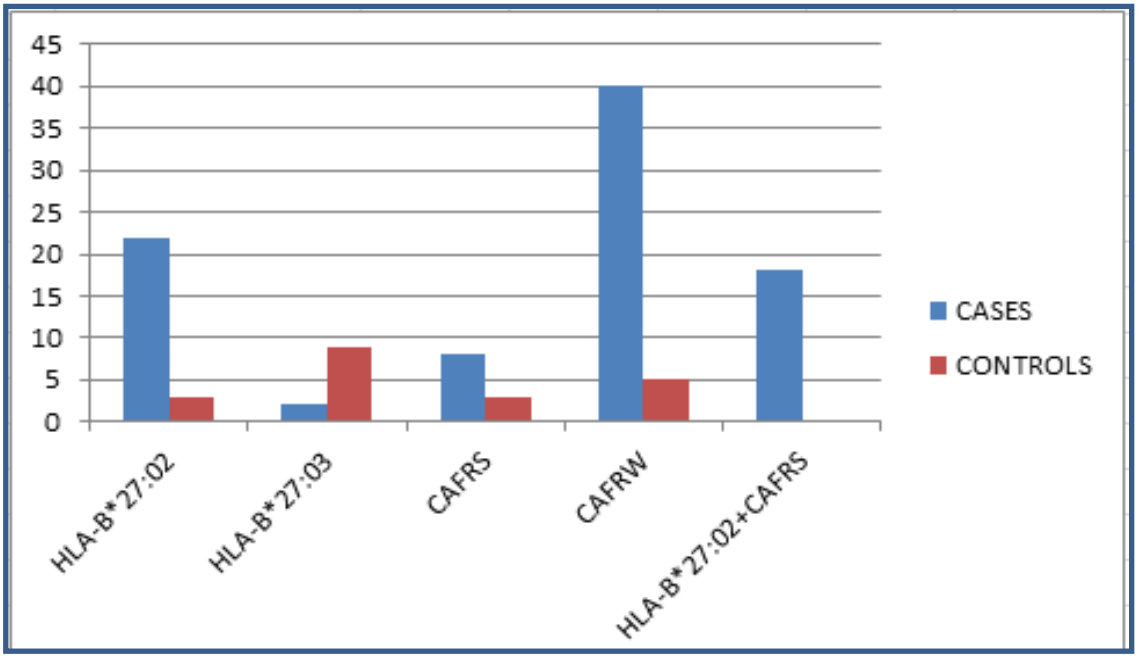


Fig.8:- Bar Graph showing HLA-B*27 sub-alleles in cases and controls.

Table 4:- Association between HLA-B*27 sub-alleles and clinicalcharacterisitics.

clinical characters												
HLA-B*27 ALLELE (Cases 100)					HLA-B*27:02 ALLELE				HLA-B*27:03 ALLELE			
Variables	HLA-B*27 +IVE	HLA-B*27 -IVE	OR (95%CI)	P value	HLA-B*27:02+IVE	HLA-B*27:02-IVE	OR	P VALUE	HLA-B*27:03+IVE	HLA-B*27:03-IVE	OR	P VALUE
Lower Back Pain												
Present(89)	80	9	0.88(0.10-7.77)	1	20	69	1.30(0.26-6.53)	1	2	87	0.65(0.02-14.57)	1
Absent(11)	10	1			2	9			0	11		
Chest Expansion												
Present(78)	71	7	1.60(0.37-6.79)	0.68	20	58	3.44(0.73-16.09)	0.14	1	77	0.27(0.01-4.59)	0.39
Absent(22)	19	3			2	20			1	21		
Uveitis												
Present(21)	18	3	0.58(0.13-2.48)	0.43	5	16	1.14(0.36-3.55)	0.77	0	21	0.72(0.03-15.60)	1
Absent(79)	72	7			17	62			2	77		
Stiffness of back												
Present(82)	74	8	1.15(0.22-5.96)	1	16	66	0.48(0.15-1.48)	0.21	2	80	1.14(0.05-24.97)	1
Absent(18)	16	2			6	12			0	18		
CRP												
Elevated	67	7	1.24(0.29-5.23)	0.71	11	63	0.23(0.08-0.65)	**0.003**	2	72	0.07(0.003-1.57)	0.07
Normal	23	3			11	15			0	26		
ESR												
Elevated	64	9	0.27(0.03-2.27)	0.27	15	58	0.73(0.26-2.07)	0.59	2	71	0.07(0.003-1.56)	0.08
Normal	26	1			7	20			0	27		
Variables	CAFRS+IVE	CAFRS-IVE	OR (95%CI)	P value	CAFRW+IVE	CAFRW-IVE	OR	P VALUE	02+CAFRS +IVE	02+CAFRS-IVE	OR	P VALUE
Lower Back Pain												
Present(89)	8	81	2.39(0.12-44.3)	0.59	36	53	1.18(0.32-4.36)	1	14	75	0.32(0.08-1.26)	0.1
Absent(11)	0	11			4	7			4	7		
Chest Expansion												
Present(78)	5	73	0.43(0.09-1.98)	0.36	30	48	0.75(0.28-1.95)	0.62	15	63	1.50(0.39-5.77)	0.75
Absent(22)	3	19			10	12			3	19		
Uveitis												
Present(21)	1	20	0.51(0.05-4.43)	1	7	14	0.69(0.25-1.91)	0.61	5	16	1.58(0.49-5.09)	0.52
Absent(79)	7	72			3	46			13	66		
Stiffness of back												
Present(82)	5	77	0.32(0.06-1.50)	0.15	36	46	2.73(0.83-9.04)	0.11	15	67	1.11(0.28-4.36)	1
Absent(18)	3	15			4	14			3	15		
CRP												
elevated	8	66	6.77(0.37-121.7	0.1	33	41	2.18(0.81-5.84)	0.16	13	61	0.89(0.28-2.81)	1
normal	0	26			7	19			5	21		
ESR												
elevated	7	66	2.75(0.32-23.54	0.67	29	44	0.95(0.38-2.35)	1	11	62	0.50(0.17-1.48)	0.24
normal	1	26			11	16			7	20		

clinical characters												
HLA-B*27 ALLELE (Cases 100)					HLA-B*27:02 ALLELE				HLA-B*27:03 ALLELE			
Variables	HLA-B*27 +IVE	HLA-B*27 -IVE	OR (95%CI)	P value	HLA-B*27:02+IVE	HLA-B*27:02-IVE	OR	P VALUE	HLA-B*27:03+IVE	HLA-B*27:03-IVE	OR	P VALUE
Lower Back Pain												
Present(89)	80	9	0.88(0.10-7.77)	1	20	69	1.30(0.26-6.53)	1	2	87	0.65(0.02-14.57)	1
Absent(11)	10	1			2	9			0	11		
Chest Expansion												
Present(78)	71	7	1.60(0.37-6.79)	0.68	20	58	3.44(0.73-16.09)	0.14	1	77	0.27(0.01-4.59)	0.39
Absent(22)	19	3			2	20			1	21		
Uveitis												
Present(21)	18	3	0.58(0.13-2.48)	0.43	5	16	1.14(0.36-3.55)	0.77	0	21	0.72(0.03-15.60)	1
Absent(79)	72	7			17	62			2	77		
Stiffness of back												
Present(82)	74	8	1.15(0.22-5.96)	1	16	66	0.48(0.15-1.48)	0.21	2	80	1.14(0.05-24.97)	1
Absent(18)	16	2			6	12			0	18		
Psoriasis												
Present(17)	16	1	1.94(0.22-16.47	1	5	12	1.68(0.50-5.22)	0.52	0	17	0.93(0.04-20.28)	1
Absent(83)	74	9			17	66			2	81		
CAFRS					CAFRW				HLA-B*27:02+CAFRS			
Variables	CAFRS+IVE	CAFRS-IVE	OR (95%CI)	P value	CAFRW+IVE	CAFRW-IVE	OR	P VALUE	02+CAFRS +IVE	02+CAFRS-IVE	OR	P VALUE
Lower Back Pain												
Present(89)	8	81	2.39(0.12-44.3)	0.59	36	53	1.18(0.32-4.36)	1	14	75	0.32(0.08-1.26)	0.1
Absent(11)	0	11			4	7			4	7		
Chest Expansion												
Present(78)	5	73	0.43(0.09-1.98)	0.36	30	48	0.75(0.28-1.95)	0.62	15	63	1.50(0.39-5.77)	0.75
Absent(22)	3	19			10	12			3	19		
Uveitis												
Present(21)	1	20	0.51(0.05-4.43)	1	7	14	0.69(0.25-1.91)	0.61	5	16	1.58(0.49-5.09)	0.52
Absent(79)	7	72			3	46			13	66		
Stiffness of back												
Present(82)	5	77	0.32(0.06-1.50)	0.15	36	46	2.73(0.83-9.04)	0.11	15	67	1.11(0.28-4.36)	1
Absent(18)	3	15			4	14			3	15		
Psoriasis												
Present(17)	2	15	1.71(0.31-9.07)	0.62	7	10	1.06(0.36-3.06)	1	2	15	0.55(0.11-2.69)	0.73
Absent(83)	6	77			33	50			16	67		

OR:oddsratio,CAFRS: 04/15/25, CAFRW: 05/13/16/17/28/37/38/39/42

Table 5:- Association between HLA-B*27 sub-types and ERAP1 SNPs:

	HLAB*27			HLA-B*27:02			HLA-B*27:03			CAFRS			CAFRW			02+CAFRS		
SNPs	Present	Absent	P value	Present	Absent	P value	Present	Absent	P value	Present	Absent	P value	Present	Absent	Pvalue	Present	Absent	P value
rs27434																		
AA	14	2	0.26	6	10	0.25	0	16	0.21	2	14	0.76	2	14	**0.03**	4	12	0.46
AG	34	6		8	32		2	38		3	37		16	24		5	35	
GG	42	2		8	36		0	44		3	41		22	22		9	35	
rs27529																		
AA	16	1	0.37	4	13	0.14	0	17	0.77	1	16	0.93	6	11	0.61	5	12	0.39
AG	32	2		11	23		1	33		3	31		12	22		5	29	
GG	42	7		7	42		1	48		4	45		22	27		8	41	
rs26510																		
CC	33	5	0.68	12	26	0.19	1	37	0.65	2	36	0.54	13	25	0.63	5	33	0.51
CT	30	3		5	28		1	32		4	29		14	19		6	27	
TT	27	2		5	24		0	29		2	27		13	16		7	22	

SNP: single nucleotide polymorphism, CAFRS: 04/15/25, CAFRW: 05/13/16/17/28/37/38/39/42

Discussion:-

In our study, we evaluatedHuman leukocyte Antigen-B*27 (HLA-B*27) subtypes in Ankylosing Spondylitis and correlation of different HLA-B*27 subtypes with clinical manifestation of disease in Kashmiri population. We found that HLA-B*27:02, CAFRW (**05/13/16/17/28/37/38/39/42**) and B*27:02+ CAFRS (**04/15/25**) had a significant association with AS in our population and had some role in disease, consistent with previous reports. We found a significant association between CAFRW and SNP rs27434.The studies related to HLA-B*27 association with AS in Indian population is very few, and earlier research reported a broad range of frequency from 18% to 94%. B*27:05 (51%) and B*2704 (40%) are predominant in Northern India. In Western India, the main subtypes reported are B*2704, B*2705, and B*2707. Other alleles such as B*2702, B*2708, and B*2714 are also noticed in minor proportions. Most population studies around the world have reported the association of B*27:01, B*27:05, B*27:02, B*27:04, and B*27:07 with AS. B*27:01 and B*27:08 have not been identified in Indian population**[Shankarkumar U et al.,2003;Akkoc N et al.,2016].**

In Chinese population, HLA-B*27:04 was reported as the major susceptibility allele in AS patients, and was followed by HLA-B*27:05. In addition to these subtypes, B*27:02, B*27:03, B*27:06, B*27:10, B*27:13, B*27:15 and B27:24 also were reported in Chinese AS patients **[Zhang L et al.,2011; Zhen W et al.,2009].** Various alleles have been linked to the AS, including HLA-B*27:05, B*27:02, B*27:04, and B*27:07 **[Lopez-Larrea C et al.,1995]**. It is generally known that HLA-B*27 subtypes have different ethnic distributions, possibly due to genetic and geographical origins; nonetheless, the B*27:05 subtype is found in practically all groups surveyed globally, and is over-represented in Eur-asia and North America's circumpolar and subarctic areas.B*27:04, on the other hand, is almost exclusively found in Oriental and Polynesian groups. In Asian populations, B*27:07 has been discovered **[Lopez-Larrea C et al.,1998].**B*27:08, an uncommon subtype, has been found in people from the Azores, the United Kingdom, and western Indianfrom the Jains, Maratha, and Gujarathi caste groups. B*27:14, a newly discovered subtype, has been found in North American Indians, Siberians, Western Indians, and Kunbi caste members in India. B*27:05 might be the primordial B*27 subtype from which all others have originated, perhaps by genetic mechanisms of reciprocal recombination, point mutation, or antigen-driven gene conversion, based on amino acid variance across subtypes and their distribution in world populations. Furthermore, the HLA-B*27 allele has been linked to hemophilia and persistent synovitis in individuals**[Liu X et al.,2010].**

In conclusion, our study demonstrated that HLA-B*27 were significantly associated with AS in the Kashmiri population,suggesting that HLA-B*27 might confer genetic risk for AS in Kashmiri population through the common mechanism shared by different populations. Those found positive for the HLA-B*27 can be properly counseled about the signs and symptoms of the disease and the need for the medication and prophylaxis can also be assessed.

Ethics Statement:
This study involving human participants were approved by the Institutional Ethics Committee of Sher-i-Kashmir Institute of Medical Sciences (SKIMS), Srinagar. Written informed consent were taken from all the patients.

Acknowledgements:-
We acknowledge all the AS patients who participated in this study.

Funding:
This research did not receive any specific grant from funding agencies in the public, commercial, or not for profit sectors.

Author Contribution:
Nasia Ismail: conceptualization, data curation, investigation, methodology, visualization. **Zafar Amin:** Project administration, resources.**Muneer Ahmad Baba**: Validation, Formal analysis, **Fayaz A. Kanjwal**: Resources, Formal analysis. **Nahida Majid**: Visualization. **RabiaRakhshan**: Formal analysis. **Muzaffar AhmadBindroo**: Resources, Validation and **SakeenaAyub:**Validation.

Conflict of interest:
The authors declare no conflict of interest.

References:-
1. Akkoc N, Khan MA. ASAS classification criteria for axial spondyloarthritis: Time to modify. ClinRheumatol (2016)
2. Allen, R. L., Raine, T., Haude, A., Trowsdale, J. & Wilson, M. J. Cutting edge: leukocyte receptor complex-encoded immunomodulatory receptors show differing specificity for alternative HLA-B27 structures. J. Immunol. 167, 5543–5547 (2001).
3. Benjamin R, Parham P. Guilt by association: HLA-B27 and ankylosing spondylitis Immunol(1990)
4. Brewerton DA, Hart FD, Nicholls A, Caffrey M, James DC, Sturrock RD. Ankylosing spondylitis and HL-A 27. Lancet (1973)
5. Brewerton D A. HLA-B27 and the inheritance of susceptibility to rheumatic diseases.Arthritis Rheum (1976)
6. Brown M A, Kennedy LG, Mac Gregor A J, Darke C, Duncan E, Shatford J L, Taylor A, Calin A, Wordsworth P. Susceptibility to ankylosing spondylitis in twins: the role of genes, HLA, and the environment. Arthritis Rheum(1997).
7. Chen, B. et al. Role of HLA-B27 in the pathogenesis of ankylosing spondylitis (review).Mol. Med. Rep. 15, 1943–1951 (2017).
8. Chen, B., Li, D. & Xu, W. Association of ankylosing spondylitis with HLA-B27 and ERAP1: pathogenic role of antigenic peptide. Med Hypotheses 80, 36 –38 (2013).
9. Colbert, R. A., Tran, T. M. &Layh-Schmitt, G. HLA-B27 misfolding and ankylosing spondylitis. Mol. Immunol.57, 44 –51 (2014).
10. Colbert, R. A., DeLay, M. L., Layh-Schmitt, G. &Sowders, D. P. HLA-B27 misfolding and spondyloarthropathies.Adv. Exp. Med Biol. 649, 217–234 (2009).
11. Colbert, R. A., DeLay, M. L., Layh-Schmitt, G. &Sowders, D. P. HLA-B27 misfolding and spondyloarthropathies.Adv. Exp. Med Biol. 649, 217–234 (2009).
12. Hermann E, Yu DT, Meyer zumBüschenfelde KH, Fleischer B. HLA-B27-restricted CD8 T cells derived from synovial fluids of patients with reactive arthritis and ankylosing spondylitis. Lancet (1993)
13. Kenna, T. J. et al. Disease-associated polymorphisms in ERAP1 do not alter endoplasmic reticulum stress in patients with ankylosing spondylitis. Genes Immun. 16, 35–42 (2014).
14. Khan MA, Ball E J. Genetic aspects of ankylosing spondylitis. Best Pract Res ClinRheumatol(2002)
15. Khan M A.An update on the genetic polymorphism of HLA-B*27 with 213 alleles encompassing 160 subtypes (and still counting)CurrRheumatol Rep (2017)
16. Liu X, Hu LH, Li YR, Chen FH, Ning Y, Yao QF.The association of HLA-B*27 subtypes with ankylosing spondylitis in Wuhan population of China.RheumatolInt 30: 587-90(2010).
17. Lopez-Larrea C, Sujirachato K, Mehra NK et al. HLA B27 subtypes in Asian patients with ankylosing spondylitis. Evidence for new associations.Tissue Antigens (1995).

18. Lopez-Larrea C, Gonzaley S, Martinez-Borra J. The role of HLA B27 polymorphism and molecular mimicry in spondyarthropathy.Mol Med Today (1998).
19. Ranganathan, V., Gracey, E., Brown, M. A., Inman, R. D. & Haroon, N. Pathogenesis of ankylosing spondylitis—recent advances and future directions. Nat. Rev. Rheumatol. 13, 359–367 (2017).
20. Reveille JD.An update on the contribution of the MHC to AS susceptibility.ClinRheumatol. (2014)
21. Scofield RH, Kurien B, Gross T, Warren WL, Harley JB. HLA-B27 binding of peptide from its own sequence and similar peptides from bacteria: implications for spondyloarthropathies. Lancet; 345: 1542-1544(1995)
22. Shankarkumar U. HLA-B27 allele diversity in Indians: Impact of ethnic origin and the caste system. Br J Biomed Sci ;60:223-6 (2003).
23. Turner, M. J. et al. HLA-B27 misfolding in transgenic rats is associated with activation of the unfolded protein response. J. Immunol. 175, 2438–2448 (2005).
24. Van der Linden S, Valkenburg HA, Cats A. Evaluation of diagnostic criteria for ankylosing spondylitis: a proposal for modification of the New York criteria. Arthritis Rheum (1984);27:361-8.
25. Zhang L, Liu JL, Zhang YJ, Wang H. Association between HLAB*27 polymorphisms and ankylosing spondylitis in Han populations: a meta-analysis. ClinExpRheumatol ; 29: 285- 92(2011).
26. Zhen W, Yikun M, Zhiming L, Jinxian H, Qiujing W, Jieruo G. HLA-B27 polymorphism in Han Chinese patients with ankylosing spondylitis: a distinctive disease association for B*2715 in a multiplex family. J Rheumatol 36: 2849-50(2009).