HLA-B27 Typing Using Simple Allele Specific PCR in Suspected Spondyloarthritis Patients Visiting Different Hospitals of Kathmandu

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Abstract

Background: Human leucocyte antigens-B27 is products of genes found at the human major histocompatibility complex class I molecule, associated with a several rheumatic disease. There is a marked variation of association between spondyloarthritis and HLA B27 surface antigen. This association encouraged in testing of HLA-B27 antigen as one of the diagnostic tools in this inflammatory disease. The study was conducted with the aim to perform HLA-B27 typing by means of simple allele specific PCR in suspected spondyloarthritis (SpA) patients.

Methods: The whole blood samples of the suspected SpA patients were collected from different hospitals and DNA was extracted. HLA-B27 Typing was performed by sequence specific primer PCR (SSP-PCR).

Results: A total 112 and 25 sample of suspected spondyloarthritis and healthy population were involved in this study, Out of 112 suspected spondyloarthritis patients 23.2% (n=26/112) were HLA-B27 positive; similarly in 25 healthy specimens 4% (n=1/25) were positive for HLA-B27. HLA-B27 were positive in 69.2% and 30.8% (8/26) in the 15-40 and >40 years age group respectively (p=0.54), and 76.9% and 23.1% in male and female respectively (p=0.15). Clinical characteristics; inflammatory low back pain, morning stiffness and enthesitis of the patient were significantly associated with HLA-B27 Typing (p<0.05).

Conclusion: Present research output confirmed the significance of HLA-B27 allele as a novel and rapid molecular marker for diagnosis of spondyloarthritis also in Nepalese patients.

Keywords: Spondyloarthritis (SpA) • HLA-B27 • Sequence specific primer (SSP-PCR)

Introduction

Human leucocyte antigens-B27 is products of genes found at the human major histocompatibility complex class I molecule, which is associated with various rheumatic diseases such as ankylosing spondylitis (AS), Reiter's syndrome, acute anterior uveitis, and inflammatory bowel disease [1]. First time in 1973 the association of HLA-B27 with AS was reported and confirmed with related spondyloarthritis later by many other investigators [2-4].

The analysis of spondyloarthritis is based on clinical symptoms, radiological findings (X-ray) and laboratory investigation. Some of the most common symptoms of AS are pain and morning stiffness of spine and sacral areas with or without associated inflammation in other tendons, joints and organs [5]. Techniques generally used in the laboratory for the routine testing of HLA-B27 cell surface antigens by antibodies are the microlymphocytotoxicity test (MLCT) and flow cytometry (FC) [6,7] which need technical expert and costly. There is almost 90% association of HLA-B27 with AS, so that HLA-B27 typing is used as a diagnostic tool in inflammatory diseases [8,9].

HLA-B27 does not seem to influence the severity of AS but the detection of HLA-B27 can be helpful in a patient having inflammatory low back pain, sacroiliitis without any radiological signs and asymmetrical oligoarthritis but without other symptoms of spondyloarthritis [10].

The applications of molecular technique in histocompatibility and immunogenetics have been increases in last two decades. HLA typing is fast replacing conventional method which has been regarded as the gold standard for SA typing by molecular based technique such as PCR sequence specific; priming (PCR-SSP), oligonucleotide probing (PCR-SSOP), and microtiter well assay [11]. Molecular technique proved as rapid and accurate method for typing HLA-B alleles and HLA typing by serology may not be sufficiently reliable [12].

There has been number of publications on HLA-B27; describing the geographical prevalence, distribution of its subtypes in different populations worldwide. To our knowledge the published report on HLAB-27 distribution among the Nepalese population is not available. Hence, HLA-B27 typing can also be used in Nepal as a screening procedure to make an earlier diagnosis and gain more experience in treating the patients with DMARDs such as TNF blockers [13]. Therefore, the aim of this study was to investigate the frequency of HLA-B27 allele among suspected SpA patients visiting the different hospital of Kathmandu.

Material and Method

Patients and Samples

The ethical approval was taken from Ethical Review Committee of the Nepal Health Research Council and written consent with patient permission was obtained. Hospital based cross-sectional study was conducted at Annapurna Research Center, Kathmandu and various hospital of Kathmandu from September 2016 to September 2017. Ethical Approval was obtained from Institutional Review Board of Nepal Health Research Council (NHRC) (Ref Ref No. 444/2016). Written informed consent was obtained from all patients.

A total number of 112 suspected patients of spondyloarthritis and 25 healthy individuals were enrolled in the study. The cases include the patients having symptoms which include inflammatory back pain, morning stiffness and improvement with exercise but not with rest, alternating buttock pain, enthesitis and dactylitis. Patients with back pain of other etiologies and no symptoms specified in inclusion criteria i.e. back pain, arthritis, were excluded from the study.

DNA Extraction

DNA was extracted by using Wizard® Genomic DNA Purification Kit (Promega Corporation, US). Briefly, 300 µl whole blood was taken to a clean 1.5 ml tube and 900 µl of cell lysis solution was added. Then, mixed well and incubated for 10 min at room temperature followed by centrifugation for 1min at 14,000 rpm. The supernatant was removed and 300 µl of nucleic lysis solution was added to pellet. Then, the reaction mixture was incubated at 30ºC for 10 min. After that 100 µl of protein precipitation solution was added and tube was vortex vigorously for 10-20 s. The tube was centrifuged at 14,000 rpm for 3 min where a dark brown pellet was visible. Next, the supernatant was transferred to a 1.5 ml eppendorf tube containing 300µl of isopropanol and was mixed until the white thread like strands of DNA were visible. Again, the tube was centrifuged for 2 min at 14000 rpm then the supernatant was discarded and 300 µl of 70% ethanol was added. The ethanol was aspirated after centrifugation at 14000 rpm using a pipette and the pellet was dried overnight. Finally, 100 µl of DNA rehydration solution was added and perseved at -30ºC until used.

Genotype Screening by SSP-PCR

HLA-B27 specific primers were used the sequences which correspond to exon 2 of HLA-B were as Forward 1-5'GCTACGTGGACGACACGCT-3, Reverse 1-5'CTCGGTCAGTCTGTGCCTT 3', and Reverse 2-5'TCTCGGTAAGTCTGT GCCTT 3'. The control primers included HgH Forward 5' TGCCTTCCCAACCATTCCCTTA 3' and HgH Reverse 5' CCACTCACGGATTTC TGTTGTGTTTC 3' as used earlier [14,15].

PCR was carried out on 25 µl PCR reaction mixture of master mix, primers, while final volume was adjusted by adding molecular grade water in above solution. The PCR mix contained tube; 2 µl of DNA template for sample, DNA of positive control and molecular grade water as negative controls. The thermal cycle (Techne, UK) parameters for PCR initial denaturation at 94ºC for 5 min followed by 35 cycles of denaturation at 94ºC for 1min; annealing of primers at 65ºC for 2 min and primer extensions at 72ºC for 1 min and final extension at 72ºC for another 10 min.

1.5% agarose gel was prepared and 0.1 µl Ethidium Bromide (EtBr) was added. At the second phase, agarose gel was poured into the gel tray with the comb in placed and was allowed it to solidify completely. Then, the gel box was filled with 1X TBE until the gel was covered. Loading dye and DNA ladder was mixed at a ratio of 3:1 and 0.8µl of it was loaded in first lanes. Similar to it, PCR products was also loaded to the respective lane. After that, the gel was run for 30 min at 80 V or approximately 75-80% of the way down the gel. Finally, the gel was visualized under gel documentation system (UV Cambridge, USA) and photographed and analyzed for HLA-B27 allele.

Statistical analysis

The HLA-B27 allele frequencies and percentage in patients with suspected spondyloarthritis and healthy control population were calculated. The pearson's chi-square test was used to assess at 95% confidence interval for intergroup significance.

Results

HLA-B27 specific allele positivity was indicated by the presence of both 149 bp specific band and 434 bp control band on the 1.5% agarose gel (Figure 1). In this study HLA-B27 allele was observed in 23.2% (n=26/112) suspected spondyloarthritis patients and 4% (1/25) of the healthy subjects were found positive for the HLA-B27 allele, while the rest were negative. HLA-B27 were positive in 69.2% (18/26) and 30.8% (8/26) in the 15-40 years, >40 years age group respectively (p>0.05). Among the HLA-B27
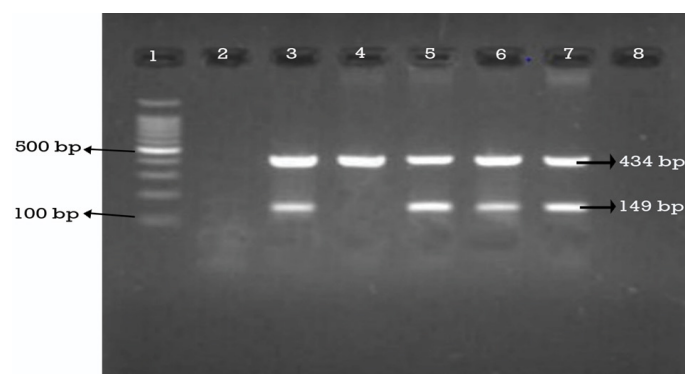

Figure 1. Gel photograph showing HLAB-27 specific band with molecular marker (L1), positive control band (L3); Negative Control (L2); Negative Sample (L4); Positive Sample (L5, L6, L7).

Table 1. Age and Sex Wise Distribution of HLA-B27 Typing in Spondyloartharitis suspected Patient.

	HLA-B27 allele		
	Positive	**Negative**	**P-value**
Age Group			
15-40	18 (69.2%)	54 (62.8%)	0.548
>40	8 (30.8%)	32 (37.2%)	
Sex			
Male	20 (76.9%)	53 (61.6%)	0.151
Female	6 (23.1%)	33 (38..4%)	

Table 2. Comparison of Clinical Characteristics of Spondyloartharitis Patient with HLA-B27 Typing.

		HLA-B27 allele		
Clinical Symptoms		**Positive**	**Negative**	**P-value**
Inflammatory low back pain	Yes	21 (33.3%)	42 (66.7%)	0.04
	No	5 (10.2%)	44 (89.8%)	
Morning stiffness	Yes	18 (35.3%)	33 (64.7%)	0.006
	No	8 (13.1%)	53 (86.9%)	
Dactylitis	Yes	11 (29.7%)	26 (70.3%)	0.251
	No	15 (20.0%)	60 (80%)	
Enthesitis	Yes	20 (47.6%)	22 (52.4%)	0
	No	6 (8.6%)	64 (91.4%)	

suspected patients higher positive frequency was observed in male than female as 76.9% (20/26) and 23.1% (6/26) respectively (P>0.05) (Table 1). On the basis of clinical symptoms HLA-B27 allele was 33.3% (21/63) and 66.7% (42/63) positive and negative in the inflammatory low back (p<0.05), 35.3% (18/51), 64.7% (33/51) negative and positive in Morning stiffness (p<0.05), 29.7% (11/37), 70.3% (26/97) negative and positive in dactylitis (p>0.05) and 47.6% (20/42), 52.4% (22/42) negative and positive in enthesitis (p<0.05) (Table 2).

Discussion

HLA-B27 Allele shows an association with AS and related SpA and plays a major role in disease pathogenesis and has association with HLAB27 antigen in 90−95% of patients with SpA [16]. In this study we found 23.2% of suspected SpA patients were positive while 76.8% were negative for HLA-B27 allele. The finding is also similar with other various studies [11,17]. The higher number of HLA-B27 allele negative found in this study that might be due to the effect of genes outside the MHC molecule: ARTS1, IL-23R and IL-1 [17]. Similarly in the study of Sonkar et al. the role of HLAB-27 was found 43.6% in seronegative spondyloarthropathy patients [18]. Therefore, the proper diagnosis had better based on combination assessment of symptoms, the findings of physical examination, radiographic imaging and laboratory investigation in daily practice [19].

When samples were analyzed in age groups and gender wise, the positivity rate was maximum 69.2% and 76.9% observed among 15-40 year group and male. Similarly higher frequency was also reported from the study made on 1500 suspected SpA Bangladeshi patients with B27 positivity rate of 49.3% with higher prevalence among male patients [20]. In this study the higher prevalence of HLA-B27 was observed in male (76.9%) than female (23.1%) patients. Similarly in the other study higher prevalence up to 81.81% of HLA-B27 was reported in male [21]. In this study we found the clinical parameter inflammatory low back pain, morning stiffness, enthesitis were clinical significant with the HLA-B27allele positivity (P<0.05).

In the initial phase clinical symptom is usually a dull pain then it developed deep in the buttock and/or in the lower lumbar regions and which is further followed by morning stiffness [22]. Enthesitis, inflammation of the site where tendon or ligaments enter into the bones mostly the Achilles tendon is the main characteristic findings of the SpA. Dactylitis or "sausage digits" is another typical finding of the SpA which was not significantly associated with the HLA-B27 allele positivity (P>0.05) in this study [23,24].

In this study we first time reported 4% (4/25) of the healthy subjects were found positive for the HLA-B27 allele in Nepal. The prevalence of HLA-B27 among healthy individuals was found to be 18-50% in American Indians, 10-16% in Scandinarians, 6-9% in Western Europe, 2-6% in Southern Europe, 6-8% in Pakistanis, 2-6% in Indians, 1% in Japanese, and 1% in Africans, 10.7% in Bangladeshians [20,25-27]. Our study observed 0% prevalence of HLA-B27 in the control population.

Conventional technique like MLCT and FC had been used for HLA-B27 typing which have number of disadvantages such as requirement of viable cells, cross reactive nature of HLA antigens, unavailability of B27 specific antisera, costly reagents, better trained personnel [28]. HLA-B27 typing by PCR are more sensitive and specific than conventional serological tests [29]. Family members of patients with AS who are HLA-B27 positive have a 16 fold increase in the risk of developing AS themselves if they are also

HLAB27 positive [30]. Our study was based on molecular method which was simple, convenient and cost-effective than MLCT and FC, which can be routinely applied for HLA-B27 typing. This technique solely relies on the detection of the HLA-B27 specific DNA sequences and is thus an ideal test for HLA-B27.

Conclusion

PCR-SSP techniques can be used as the rapid diagnostic marker better understanding and treatment of spondyloarthropathiesm also in Nepalese patients. In order to start the proper drug it is necessary to find out the HLAB 27 type at the onset of disease.

Conflict of interest

The authors declare no any conflict of interest.

Acknowledgments

References

1. López-Larrea, C., et al. "The role of HLA-B27 polymorphism and molecular mimicry in spondylarthropathy". *Molecular medicine today* 4.12(1998): 540-549.

2. Brewerton, D., et al. "Ankylosing spondylitis and HL-A 27". *The Lancet* 301. 7809(1973): 904-907.

3. Kanga, U., et al. "Seronegative spondyloarthropathies and HLA-B27 subtypes: a study in Asian Indians". *Clinical Rheumatology* 15.1(1996): 13-18.

4. Khan, M. "HLA-B27 polymorphism and association with disease". *British Journal of Ophthalmology* 84.4(2000): 413-416.

5. Doward, L., et al. "Development of the ASQoL: a quality of life instrument specific to ankylosing spondylitis". *Annals of the rheumatic diseases* 62.1(2003): 20-26.

6. Terasaki, P.I., & McClelland, J.D. "Microdroplet assay of human serum cytotoxins". *Nature* 204(1964): 998.

7. Pei, R., et al. "A monospecific HLAB27 fluorescein isothiocyanate conjugated monoclonal antibody for rapid, simple and accurate HLA B27 typing". *HLA* 41.4 (1993): 200-203.

8. Seipp, M.T., et al. "HLAB27 typing: Evaluation of an allelespecific PCR melting assay and two flow cytometric antigen assays". *Cytometry Part B: Clinical Cytometry* 63.1 (2005): 10-15.

9. Middleton, D,. "HLA typing from serology to sequencing era". *Iranian Journal of Allergy, Asthma and Immunology* 4.2(2005): 53-66.

10. Sheehan, N.J. "The ramifications of HLA-B27". *Journal of the Royal Society of Medicine* 97.1 (2004): 10-14.

11. Sharma, N., et al. "Usage of conventional PCR technology for the detection of HLA-B27 allele: a significant molecular marker of ankylosing spondylitis". *Indian Journal of Clinical Biochemistry* 28.2 (2013): 189-192.

12. Bunce, M., et al. "Molecular HLA typing-the brave new world". *Transplantation* 64.11(1997): 1505-1513.

13. Sieper, J., & Rudwaleit, M. "How early should ankylosing spondylitis be treated with tumour necrosis factor blockers?" *Annals of the rheumatic diseases* 64(2005): 61-64.

14. Kulkarni, B., et al. "Frequency distribution of human platelet antigens in the Indian population". *Transfus Med* 15.2 (2005): 119-124.

15. Parasannanavar, D.J., et al. "Application of a simple in-house PCR-SSP technique for HLA-B* 27 typing in spondyloarthritis patients". *Arthritis* 2013(2013): 1-4.

16. Oostveen, J., & Prevo, R. "Early detection of sacroiliitis on magnetic resonance imaging and subsequent development of sacroiliitis on plain radiography. A prospective, longitudinal study". *The Journal of Rheumatology* 26.9 (1999): 1953-1958.

17. Vojvodić, S., et al. "Human leukocyte antigen-B27 and disease susceptibility in Vojvodina, Serbia". *Balkan Journal of Medical Genetics* 15.2 (2012): 55-60.

18. Sonkar, G.K., & Usha. "Role of HLA B27 in diagnosis of seronegative spondyloarthropathies". *Indian journal of pathology & microbiology* 50.4 (2007): 908-913.

19. Van, T.A., & Weber, U. "Diagnosis and classification in spondyloarthritis: identifying a chameleon". *Nature Reviews Rheumatology* 8.5 (2012): 253.

20. Nessa, A., et al. "HLA-B27 antigen frequency among suspected Spondyloarthropathy patients attaining a tertiary level hospital of Bangladesh". *Bangladesh Medical Research Council Bulletin* 40.3 (2015):102-106.

21. Mishra, M.N., & Singai, V. "Human leukocyte antigen B27 in 453 Asian Indian patients with seronegative spondyloarthropathy". *Iranian Journal of Immunology* 7.4 (2010): 252-256.

22. Underwood, M., & Dawes, P. "Inflammatory back pain in primary care". *Rheumatology* 34.11 (1995): 1074-1077.

23. Khan, M.A,. "Update on spondyloarthropathies". *Ann Intern Med* 136.12 (2002): 896-907.

24. Puhakka, K.B., et al. "Magnetic resonance imaging of sacroiliitis in early seronegative spondylarthropathy. Abnormalities correlated to clinical and laboratory findings". *Rheumatology* 43.2 (2003): 234-237.

25. Thomas, R., et al. "Association of an extended haplotype of HLA class I alleles and their flanking microsatellites with spondyloarthropathies in South Indian patients". *Human immunology* 67.4 (2006): 318-323.

26. Ma, H.J., Hu, F.P. "Diversity of human leukocyte antigenB27 alleles in Han population of Hunan province, southern China". *Tissue Antigens* 68.2 (2006): 163-166.

27. Khan, A. "A worldwide overview: the epidemiology of HLA-B27 and associated spondyloarthritides". *The Spondyloarthrides* 17(1998): 256-268.

28. Neumüller, J., et al. "Evaluation of four monoclonal antibodies against HLAB27 for their reliability in HLAB27 typing with flow cytometry (FC): Comparison with the classic microlymphocytotoxic test (MLCT)". *Cytometry Part A* 26.3 (1996): 209-215.

29. Schaffer, M., & Olerup, O. "HLAAB typing by polymerasechain reaction with sequencespecific primers: more accurate, less errors, and increased resolution compared to serological typing". *HLA* 58.5 (2001): 299-307.

30. Sjeper, J., et al. "Ankylosing spondylitis: an overview". *Ann Rheum Dis* 61(2002): 72.