Quantitative Measurement of HLA-B27 mRNA in Patients with Ankylosing Spondylitis — Correlation with Clinical Activity

SU-QIN LIU, HUI-CHUN YU, YONG-ZHANG GONG, and NING-SHENG LAI

ABSTRACT. ***Objective.*** To determine quantitative expression of HLA-B27 in patients with ankylosing spondylitis (AS) and the relationship to clinical disease activity.

Methods. Quantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed using specific primers to amplify the post-splicing mRNA of HLA-B27 gene in patients with AS (N = 15), B27+ healthy controls (N = 20), and B27+ unaffected family members (N = 30). Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) scores and expression of B27 mRNA were determined simultaneously. Serial measurements of B27 mRNA in one AS patient within a 9-month period were analyzed to determine the relationship between BASDAI scores and clinical therapy. For further validation of the significance of increased HLA-B27 mRNA expression in AS patients, extended experiments were conducted in another 10 patients with definite AS possessing HLA-B27/B60 (B*40012). Quantitative expression of HLA-B27 and B60 mRNA were determined simultaneously by RT-PCR.

Results. Expressions of HLA-B27 mRNA in HLA-B27+ AS patients were significantly higher compared to unaffected B27+ family members and B27+ controls. mRNA for HLA-B27 was observed to be present in higher amounts than for other HLA class I alleles. It was also noted that the BASDAI data were correlated with HLA-B27 mRNA estimated as $2^{\Delta\Delta CT}$ in patients with definite AS. From a longitudinal case analysis, data on quantitative measurement of HLA-B27 mRNA was shown to be valuable for patient's management.

Conclusion. The expression of mRNA of HLA-B27 determined with quantitative RT-PCR correlated with clinical disease activity in patients with AS. (J Rheumatol 2006;33:1128–32)

Key Indexing Terms:
ANKYLOSING SPONDYLITIS QUANTITATIVE REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION HLA-B27
BATH ANKYLOSING SPONDYLITIS DISEASE ACTIVITY INDEX

Ankylosing spondylitis (AS) is a chronic systemic inflammatory disorder that mainly affects the axial bone. Typically, presentation is with low back pain of insidious onset, with a prominent pain at rest, especially in the morning after waking. The major human histocompatibility antigen HLA-B27 is strongly associated with this inflammatory disease[1,2]. Direct involvement of HLA-B27 in pathogenesis was strongly suggested in data from HLA-B27+ transgenic rats[3]. The expression of HLA-B27 is therefore thought to play a critical role in the development of AS. Methods for the detection of B27 include the microlymphocytotoxicity test, flow cytometry[4,5], and immunomagnetic separation with an enzyme-linked immunosorbent assay (IMS-ELISA)[6]. The flow cytometric B27 assay is a convenient screening test. It failed to demonstrate correlations between the quantitative expression of B27 and clinical features, such as age, disease duration, Schober test results, chest expansion, white blood cell count, erythrocyte sedimentation rate, and C-reactive protein[7]. In our study, a real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) platform was established using specially designed primers for amplification of post-splicing HLA-B27 mRNA. We investigated the relationship between expression of B27 mRNA and clinical disease activities of patients with AS.

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MATERIALS AND METHODS

Total blood cells were obtained from patients with definite AS (N = 15), unaffected HLA-B27+ family members (N = 30), and healthy HLA-B27+ controls (N = 20). All patients fulfilled the modified New York diagnostic criteria[8]. Unaffected family members, by definition, showed no involvement of sacroiliac joints. Some individuals within this group had clinical manifestations of limitations of motion of the lumbar spine, low back pain, or limitation of chest expansion, however. HLA-B27+ controls were recruited from persons appearing for annual physical examinations. They had no clinical symptoms or radiographic abnormalities. Mononuclear cells were harvested with Ficoll-Hypaque and were adjusted to 2×10^7/ml. Total RNA was extracted with an RNA isolation kit (QIAamp RNA blood mini-kit) according to the instruc-

tions provided. For further validation of the significance of B27 gene expression in AS patients, 10 patients found to have B27+/HLA-B60 (B*40012) were selected for evaluation of B27 mRNA relevant to other HLA class I alleles. Quantitative expression of post-splicing mRNA of HLA-B27 and HLA-B*40012 was determined simultaneously.

*Quantitative real-time measurement of post-splicing HLA-B27 mRNA and HLA-B60 (B*40012) mRNA.* RT-PCR was carried out using the ABI sequence detection system (ABI 5700) for measurement of binding of SYBR Green I fluorescence dye to double-stranded DNA. The PCR reaction was set up in a microcapillary tube in a total volume of 25 μl. Twenty nanograms of mRNA was mixed with 2× Quantitect SYBR Green RT-PCR master mix 12.5 μl (Qiagen QuantiTect SYBR Green RT-PCR; Qiagen GmbH, Hilden, Germany) and 1 μM of primers. The reactions were at reverse transcription at 50°C for 30 min, initial activation at 95°C for 15 min, then incubation at 94°C for 15 s to activate the polymerase, followed by 45 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s. From published data for HLA-B27 gene sequences[9], there are 4 exons in the gene structure, i.e., sequence 58–130 of exon 1, 259–528 of exon 2, 773–1048 of exon 3, and 1624–1899 of exon 4. To specifically amplify the post-splicing HLA-B27 mRNA with minimal DNA contamination, the sequences across the tail of exon 1 and the beginning of exon 2, but not that of the original DNA nucleotide sequence, were selected to form distinct primers. Thus, the primer sequences were with sense: 5'-CCT GGG CTG (sequence 122–130 in exon 1) GCT CCC ACT C (sequence 259–268 in exon 2)-3' and with antisense: 5'-CAC GTA GCC CAC GGT GAT G-3' (sequence 323–341 in exon 2) with a product size of 90 bp. It was therefore unique to measure the post-splicing HLA-B27 mRNA. The primer sets of the internal control of the G6PDH gene were sense: 5'-GAA CAT CAT CCC TGC ATC CA-3' and antisense: 5'-CCA GTG AGC TTC CCG TTC A-3' with a product size of 73 bp. The primer sequences for HLA-B*40012 mRNA were sense: 5'-CAT GAG GTA TTT CCA CAC CGC CA-3' (sequence 11–33 in exon 2) and antisense: 5'-TCG CTG TCG AAC CTC ACG AAC-3' (sequence 95–115 in exon 2)[10].

Relative quantification method. Before using the $\Delta\Delta C_T$ method for quantification, we performed a validation experiment to confirm that the amplification efficiencies of the target HLA-B27 gene and the internal control G6PDH gene were approximately equal (data not shown). With this confirmed, we used the $\Delta\Delta C_T$ calculation for relative quantification of the target without running standard curves on the same plate. The mean C_T value (the threshold cycle) of the replicate wells were run for each sample. The difference (ΔC_T) represents the difference between mean C_T values of the samples for target wells (HLA-B27) and those of the endogenous control wells (G6PDH). The expression $\Delta\Delta C_T$ denotes the difference between the ΔC_T values of the samples for each target and the mean C_T value of the calibrator for that target. Therefore, the amount of target, normalized to an endogenous reference and relative to a calibrator, is expressed as $2^{-\Delta\Delta CT}$, as described[11]. It does not stand for an absolute copy number of the gene, but a unit for quantitative comparison with an internal control of selected housekeeper genes. Thus, a doubling of value equates to a doubling of mRNA transcripts in the respective subject. This rather abstract terminology may be somewhat difficult for the clinician to understand, but it is indeed a useful standard in RT-PCR when absolute copies are not available.

AS disease activity. Disease activity was determined using scores from the Bath AS Disease Activity Index (BASDAI), measured with a 10 cm visual analog scale. A disease severity score is determined on a scale from 0 (no disease activity) to 10 (very severe disease) in each of following categories: fatigue, spine pain, pain and/or swelling of peripheral joints, localized tenderness, and severity and duration of morning stiffness.

Statistics. ANOVA and t tests were used for comparisons between patients and controls. A value of $p < 0.05$ was assumed to be statistically significant.

RESULTS

Higher expression of HLA-B27 mRNA in patients with definite AS. The post-splicing mRNA of B27 was expressed as $2^{-\Delta\Delta CT}$. According to arithmetic calculation, the value would be close to 1 (i.e., $2^0 = 1$) if expression of the target HLA-B27 gene was approximately equal to the internal control G6PDH gene. A variety of expressions ($2^{-\Delta\Delta CT}$ from 1.22 to 7.32, mean 3.64, N = 15) were found in patients with definite AS (Figure 1). Expressions of the B27 gene in AS patients were significantly higher, compared to those of B27+ unaffected family members (N = 30, mean = 1.86; p = 0.002) and B27+ controls (N = 20, mean 1.57; p = 0.0006). There was also a significant difference between the latter 2 groups (p = 0.014). In the group of unaffected family members, some members had a high level of B27 gene expression ($2^{-\Delta\Delta CT}$ as high as 3.45). It was interesting that low back pain and enthesopathy soft tissue pain were present in some of these family members. There were no radiographic abnormalities of sacroiliitis and enthesopathy changes, however. Whether these unaffected family members may become candidates for early onset AS will require further observations.

*Higher expression of HLA-B27 mRNA compared with HLA-B60 (B*40012) in AS patients.* Ten patients diagnosed as having definite AS with HLA-B27/B*40012 were selected, and post-splicing mRNA of B27 and B*40012 was determined simultaneously and expressed as $2^{-\Delta\Delta CT}$. The relative amounts of mRNA between 2 different HLA-B loci (B27 and B*40012) were expressed as a ratio of $2^{-\Delta\Delta CT}$ (B27)/$2^{-\Delta\Delta CT}$ (B60/B*40012) (Figure 2). Higher expression of HLA-B27 mRNA was observed in AS patients compared to HLA-B*40012 (ratios of 64.0, 17.2, 16.8, 16.0, 8.9, 8.2, 6.4, 1.2, 1.2, and 1.0, respectively, in these patients). Although having

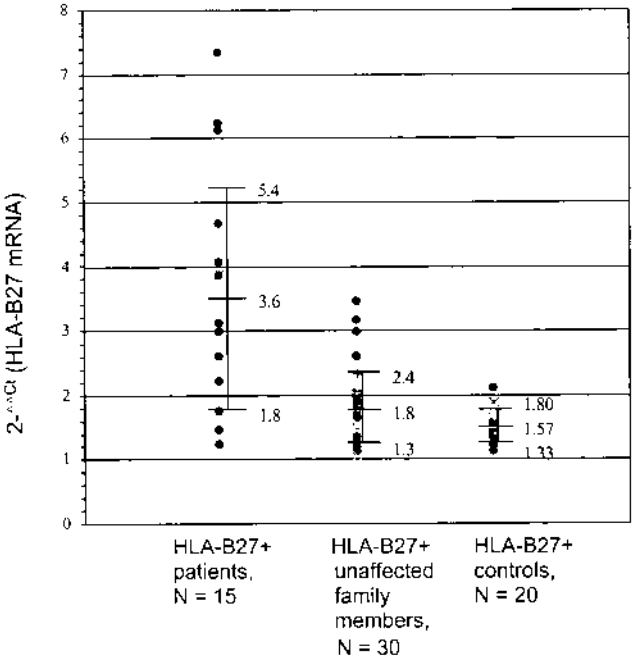

Figure 1. Quantitative expression of post-splicing B27 mRNA in patients with AS, B27+ unaffected family members, and B27+ controls was determined by RT-PCR and expressed as $2^{-\Delta\Delta CT}$.

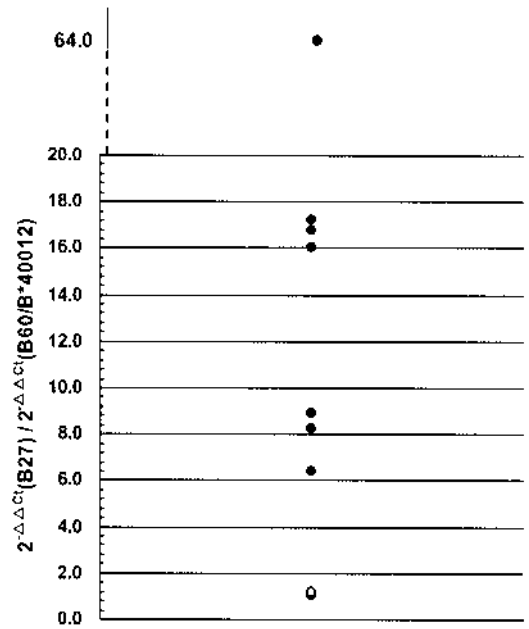

Figure 2. Quantitative differences between HLA-B27 mRNA and HLA-B60 (B*40012) mRNA, expressed as $2^{-\Delta\Delta CT}$ (B27)/$2^{-\Delta\Delta CT}$ (B60/B*40012), was determined in patients with AS by RT-PCR and expressed as $2^{-\Delta\Delta CT}$.

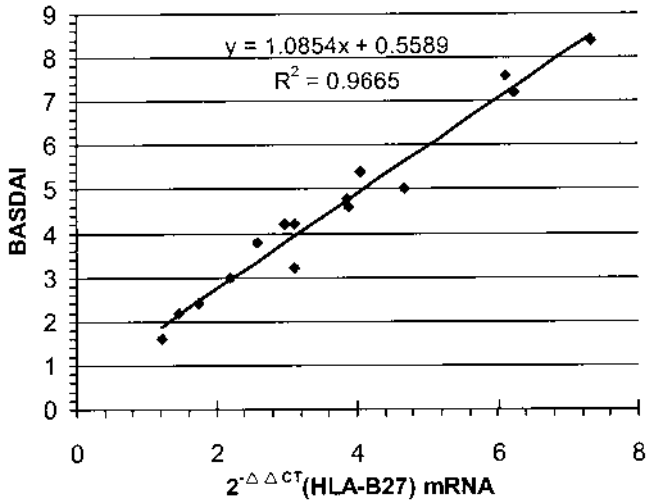

Figure 3. Correlation between quantitative expression of HLA-B27 mRNA and disease activity (BASDAI) in patients with AS. Amount of HLA-B27 mRNA was expressed as $2^{-\Delta\Delta CT}$.

high expression of HLA-B27 gene, the expression of HLA-B*40012 mRNA in some patients was even lower than for the G6PDH ($\Delta\Delta CT > 0$). The ratio of 64.0 in the current figure means there were 64 times as many copy numbers of HLA-B27 transcripts compared to HLA-B*40012.

Expression of HLA-B27 mRNA correlated with BASDAI in AS patients. BASDAI is a useful index for clinical evaluation of disease activity in AS patients. We observed that patients' BASDAI scores correlated with mRNA expression of HLA-B27, expressed as $2^{-\Delta\Delta CT}$ ($R^2 = 0.9665$; Figure 3). It was therefore deduced that clinical manifestations of pain and peripheral joint involvement in AS patients were related to increased expression of HLA-B27.

Expression of HLA-B27 mRNA was useful in guiding clinical interventions. Expression of post-splicing HLA-B27 mRNA ($2^{-\Delta\Delta CT}$) in one patient with AS was recorded monthly for a period of 9 months (Figure 4). The patient received methylprednisolone pulse therapy in November 2001 and March 2002, due to active clinical symptoms and with a $2^{-\Delta\Delta CT}$ value. Disease expression was successfully suppressed after methylprednisolone pulse therapy, with a decrease of $2^{-\Delta\Delta CT}$ thereafter.

DISCUSSION

Ankylosing spondylitis is a human leukocyte antigen (HLA)-associated disease. The association with HLA-B27 was first reported in 1973[12]. The odds ratio in excess of 100 associated with AS remains the highest observed in this inflammatory disorder. The greater expression of HLA-B27 molecules is a further predisposing factor for the development of AS[13]. Therefore, quantitative measurement of B27 molecules on the cell surface was hypothesized to play an important role in the pathogenesis of the disease.

In our study, the post-splicing mRNA of the HLA-B27 gene was determined quantitatively by one-step RT-PCR. The primer sequences used for measurement of HLA-27 mRNA were S: 5'-CCT GGG CTG (sequence 122–130 in exon 1) GCT CCC ACT C (sequence 259–268 in exon 2); and A: 5'-GTG CAT CGG GTG CCA CTA C-3' (sequence 323–341 in exon 2). The sequence 259–341 was totally identical for the 2701 to 2723 subtypes of HLA-B27. The sequence 122–130 was identical among the subtypes 2702, 2703, 2704, 2706, 2708, 2709, 2711, 2713, 2718, 2722, 2752, and 2754. This covered the major DNA subtype of HLA-B27 in patients with AS in Taiwan (i.e., 2704, which represents 94% in patients with AS and 85% in B27-positive healthy subjects) and other minor subtypes of 2705[14]. Further, reference to nucleotide data banks indicated the post-splicing transcripts being amplified were indeed those of HLA-B27 and nothing else.

It is important to define the heterozygosity or homozygosity of B27 in study subjects more clearly because this could have affected our results. Only one of our 65 subjects was a B27/B27 homozygote (a healthy control with $2^{-\Delta\Delta CT} = 1.22$). The majority of our subjects in all study groups (patients, unaffected relatives, healthy controls) were compared based on B27 heterozygosity.

The oligonucleotides for amplification of B*40012 in our study were all within the exon 2 region, not like those of the B27 primers designed specifically for post-splicing mRNA. Although we used an RNA isolation kit (QIAamp RNA mini-kit) to prevent possible DNA contamination, it would be possible to amplify the DNA present in the preparation. Nevertheless, the signals for HLA-B*40012 in patients with AS were still significantly lower than those for HLA-B27

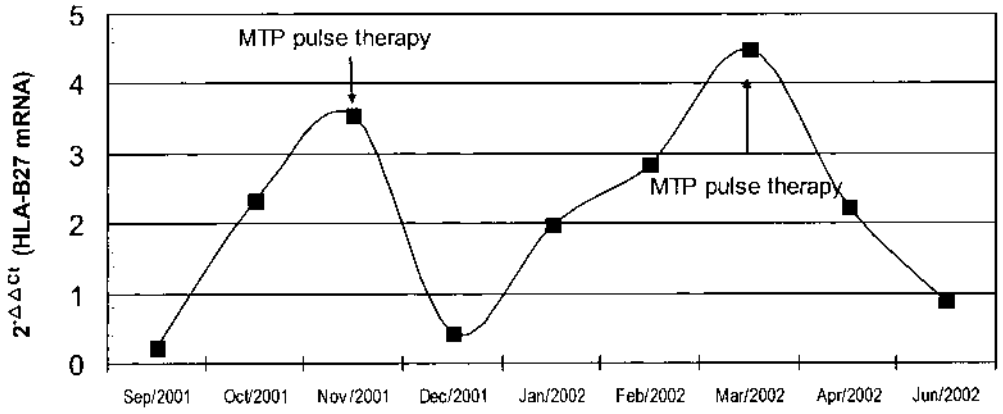

Figure 4. Expression of post-splicing HLA-B27 mRNA ($2^{-\Delta\Delta CT}$) in a patient with AS was recorded monthly for a period of 9 months. The patient received methylprednisolone (MTP) pulse therapy in November 2001 and March 2002.

(Figure 2). As there are few differences in the sequences of HLA-B*40012 and HLA-B27 in the region where the oligonucleotides specific for HLA-B*40012 are located, it is important to verify their specificity in amplification. Nine patients who were identified as HLA-B27-positive/B*40012-negative were selected, and there were no detectable signals in any samples with B*40012-specific primers (data not shown). We also tested the specificity of oligonucleotides for HLA-B27 amplification. Another 12 patients identified as HLA-B27-negative/B*40012-positive were selected, and no signals were detected in any samples with B27-specific primers (data not shown). It was therefore believed that the oligonucleotides were specific for HLA-B27 mRNA and B*40012 PCR amplifications, although they share a large scale of the same sequences.

The expression of B27 mRNA in B27-positive patients with AS in our study was significantly higher than in B27-positive unaffected family members and healthy controls. Further, a relationship between B27 gene expression and patients' clinical disease activity was also found. Data comparing the fluorescence intensity of HLA-B27 with patients' clinical characteristics[7] have shown that the fluorescence intensity of HLA-B27 was significantly higher in patients with AS than in patients with other spondyloarthropathies. However, there were no significant correlations between clinical features and quantitative expressions of B27 by flow cytometer[7]. Similar data[13] were reported using molecules of an equivalent soluble fluorochrome, in which HLA-B27 expression did not correlate with indexes of disease activity (the BASDAI and the Bath AS Functional Index). Data differences between our study and previous reports[7,13] might arise from limitations of the respective methods used. In a flow cytometry measurement, significant variation in intensity with B27 antibody[15], the choice of antibody[16], an endoplasmic reticulum stress response or aberrant heavy-chain folding and disulfide bond formation[17], and the time lag in protein translation and surface expression are all limitations. Studies on transgenic rats expressing HLA-B27 and human $ß_2$-microglobulin reported that most of the B27-positive, $ß_2$-microglobulin–/– mice developed ankylosis[18]. The $ß_2$-microglobulin-free HLA-B27 homodimers in one study were reactive with HC10 antibody[16]. Indeed, a high level of surface expression of heavy chains (by HC10 antibody) in monocytes of patients with AS was found[19]. Current data suggest that aberrant assembly, transport, and expression of HLA-B27 molecules may dispose an individual to development of disease. Ideally, quantification of mRNA combined with various antibodies may reveal clues to disease pathogenesis.

In our study, the measurements of HLA-B27 post-splicing mRNA were both quick and reliable. With the establishment of such a platform for analysis of HLA-B27 gene expression in patients with AS, research into genetic loads and their complex interactions in disease pathogenesis, the immune regulation of cytokines, and the influence of other candidate genes will be facilitated.

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REFERENCES

1. Brewerton DA, Hart FD, Nicholls A, Caffrey M, James DC, Sturrock RD. Ankylosing spondylitis and HLA-B27. Lancet 1973;1:904-7.
2. Burmester GR, Daser A, Kamradt T, et al. Immunology of reactive arthritis. Ann Rev Immunol 1995;13:229-50.
3. Hammer RE, Maika SD, Richardson JA, Tang JP, Taurog JD. Spontaneous inflammatory disease in transgenic rats expressing HLA-B27 and human beta-2m: an animal model of HLA-B27 associated human disorders. Cell 1990;63:1099-112.
4. Linggenfelter B, Fuller TC, Hartung L, Hunter J, Wittwer C. HLA-B27 screening by flow cytometry. Cytometry 1995;22:146-9.
5. Chou SJ, Lai NS, Su JP, Wu JL, Lan JL. Two color analysis of HLA-B27 antigen by flow cytometer — a comparative study by conventional microlymphocytotoxicity, DNA genotyping polymerase chain reaction and flow cytometric measurement. J Clin Lab Anal 1997;11:369-73.
6. Chou CT, Tsai YF, Liu J, et al. The detection of the HLA-B27 antigen by immunomagnetic separation and enzyme-linked immunosorbent assay — comparison with a flow cytometric procedure. J Immunol Methods 2001;255:15-22.

7. Lee YH, Choi SJ, Yoon SY, Lee KN, Ji JD, Song GG. Quantitative analysis of HLA-B27 by flow cytometry using CD3 gating in seronegative spondyloarthropathies. Clin Exp Rheumatol 1999;17:191-5.
8. 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.
9. Witter K, Lau M, Zahn R, Scholz S, Albert ED. Identification of a novel HLA-B2722 allele from a Filipino cell. Tissue Antigen 2001;58:263-8.
10. Vargas-Alarcon G, Gomez-Casado E, Martinez-Laso J, et al. Description of a new HLA-B40 allele (B*4011) found in a Mexican individual of Nahua (Aztec) descent. Immunogenetics 1997;46:359-60.
11. GeneAmp 5700® sequence detection system user manual. Toroed, Norway: AME Bioscience A/S: 1999:A1-A30.
12. Brewerton DA, Hart FD, Nicholls A, Caffrey M, James DC, Sturrock PD. Ankylosing spondylitis and HLA-B27. Lancet 1973;1:904-7.
13. Cauli A, Dessole G, Fiorillo MT, et al. Increased level of HLA-B27 expression in ankylosing spondylitis patients compared with healthy HLA-B27-positive subjects: a possible further susceptibility factor for the development of disease. Rheumatology Oxford 2002;41:1375-9.
14. Chou CT, Chen JM, Hsu CM, Chen SJ. HLA-B27 and its subtypes in 4 Taiwanese Aborigine tribes: a comparison to Han Chinese patients with ankylosing spondylitis. J Rheumatol 2003;30:321-5.
15. Chen JC, Davis BH, Bigelow NC, et al. Flow cytometric HLA-B27 typing using CD3 gating and molecules of equivalent soluble fluorochrome (MESF) quantitation. Cytometry 1996;26:286-92.
16. Kollnberger S, Bird LA, Roddis M, et al. HLA-B27 heavy chain homodimers are expressed in HLA-B27 transgenic rodent models of spondyloarthritis and are ligands for paired Ig-like receptors. J Immunol 2004;173:1699-710.
17. Dangoria NS, DeLay ML, Kingsbury DJ, et al. HLA-B27 misfolding is associated with aberrant intermolecular disulfide bond formation in the endoplasmic reticulum. J Biol Chem 2002;277:23459-68.
18. Khare SD, Luthra HS, David CS. Spontaneous inflammatory arthritis in HLA-B27 transgenic mice lacking beta 2-microglobulin: a model of human spondyloarthropathies. J Exp Med 1995;182:1153-8.
19. Tsai WC, Chen CJ, Yen JH, et al. Free HLA class I heavy chain-carrying monocyte — a potential role in the pathogenesis of spondyloarthropathies. J Rheumatol 2002;29:966-72.