

Overexpression and unique rearrangement of VH2 transcripts in immunoglobulin variable heavy chain genes in ankylosing spondylitis patients

Yeon Joo Kim^{1*}, Nayoung Kim^{1*}, Min-Kyung Lee²,
Hyo-Jin Choi², Han Joo Baek^{2,3} and
Chang-Hoon Nam^{1,3}

¹Korea Institute of Science and Technology -
Europe Forschungsges. mbH
Saarbücken, Germany

²Department of Rheumatology
Gachon University School of Medicine
Gil Hospital

Incheon 405-760, Korea

³Corresponding authors: Tel, 49-681-9382-416;

Fax, 49-681-9382-279; E-mail, chang@kist-europe.de (C.H. Nam),

Tel, 82-32-460-8426; Fax, 82-32-469-4320;

E-mail, baekhj@gilhospital.com (H.J. Baek)

*These authors contributed equally to this work.

DOI 10.3858/emm.2010.42.5.030

Accepted 10 February 2010

Available Online 22 February 2010

Abbreviations: AS, ankylosing spondylitis; C, constant region; CDC42 BPB, CDC42 binding protein kinase beta; D, diversity gene segment; H, heavy chain; HuPo, human acidic ribosomal protein; J, joining gene segment; L, light chain; Q-PCR, quantitative real-time PCR; RSS, recombination signal sequences; V, variable region; VH, variable heavy chain

Abstract

To identify immunoglobulin variable heavy chain (VH) gene usages in Korean ankylosing spondylitis (AS) patients, expression level of VH2 genes from peripheral blood mononuclear cells (PBMCs) of 8 AS patients and 9 healthy donors was analysed by quantitative real-time PCR (Q-PCR). Q-PCR results demonstrated VH2 genes were overexpressed in AS patients (Relative amount of mRNA of VH2 genes to a house-keeping gene, 7.13 ± 7.77 vs, 0.68 ± 0.55 ; $P < 0.0001$). The sequence analysis revealed the majority of them contained CDC42 binding protein kinase beta (CDC42 BPB) genes. The insertion of CDC42 BPB gene was confirmed by PCR with primers corresponding CDC42 BPB and CH genes. Our study revealed VH2 overexpression and unique rearrangement in Ig VH genes from peripheral blood of AS patients. This may

imply aberrant immunoglobulin gene rearrangement in B cell occurs in Korean AS patients, which requires further investigation.

Keywords: spondylitis, ankylosing; gene rearrangement, B-Lymphocyte, heavy chain; immunoglobulin variable region; reverse transcriptase polymerase chain reaction

Introduction

Ankylosing spondylitis (AS), a prototype of spondyloarthritis (SpA), is a chronic inflammatory arthritis that mainly affects the sacroiliac joints and the spine. It is characterized by peripheral arthritis, enthesitis, and extraskeletal features, such as uveitis and inflammatory bowel disease. The pathogenesis of AS/spondyloarthritis is not fully elucidated, but much has been learned regarding this condition in recent decades. Human leukocyte antigen (HLA)-B27, an MHC class I allele, is a key genetic factor in AS/spondyloarthritis (Brewerton *et al.*, 1973; Schlosstein *et al.*, 1973). Enteric bacteria may also play a role in causing the diseases as an environmental component (Taurog *et al.*, 1994; De Keyser *et al.*, 1998). Populations of immune cells have been implicated in the pathogenesis of AS/spondyloarthritis. T cells, particularly CD4⁺ T cells, are required for developing the disease in rats (Brebant *et al.*, 1996; May *et al.*, 2003). There has been evidence that macrophages participate in the pathogenesis of the disease (Baeten *et al.*, 2002; McGonagle *et al.*, 2002). Recent data have demonstrated that Th17 cells, IL-17-producing effector T helper cells, contribute to AS/ spondyloarthritis inflammation (Jandus *et al.*, 2008; Shen *et al.*, 2009). However, specific autoantibodies have not yet been identified in patients with AS/spondyloarthritis, and the role or relevance of B cells in the pathogenesis of the disease is unclear.

Analyses of immunoglobulin variable region (V) gene usages have revealed differences in the basic Ig V repertoire of patients with B cell- and/or T cell-mediated autoimmune diseases, including systemic lupus erythematosus (Isenberg *et al.*, 1993; Stevenson *et al.*, 1993), myasthenia gravis (Sims *et al.*, 2001), rheumatoid arthritis (Robbins *et*

al., 1990; Ermel *et al.*, 1997), and Sjögren syndrome (Dorner *et al.*, 2002), compared to healthy controls. Such differences could be derived from intrinsic abnormalities in the generation of Ig V genes or B cell development and functions (Foreman *et al.*, 2007). Thus, the analysis of Ig V gene usages can offer new insights into possible pathogenic role of B cells in diseases.

A previous study of heavy chain variable segment (VH) germline gene usages in synovial B cells from AS patients demonstrated that the majority of rearranged Ig VH genes belonged to the VH3 genes (Voswinkel *et al.*, 2001). However, it did not include a direct comparison to healthy donors and some of the VH germline genes were overlooked due to the incomplete VH PCR primer sets. Therefore we investigated VH germline gene usages of patients with AS compared to healthy donors using PCR with additional primer sets.

Results

Profile of subjects

The demographic and clinical characteristics of patients and controls are shown in Table 1. The mean age of AS patients was older than controls (38.6 and 30.2 years, respectively), but it was not statistically significant.

Overexpressed VH2* genes in AS patients

To analyze VH2 gene usages in detail, Q-PCR was performed with RNA from PBMC in AS patients. After we checked out that primer sets used in previous studies (Voswinkel *et al.*, 2001) can cover VH germline sequence from Ig Blast databases, we realized that 8 sequences of IGHV2 subfamilies (IGHV2-5*01, 5*04, 5*05, 5*06, 5*07, 5*10, 70*09 and 70*12) and 10 sequences of IGHV4 subfamilies (IGHV4-34*01, 34*03, 34*04, 34*05, 34*06, 34*07, 34*08, 34*11, 34*12 and 59*10) were not covered with those primer sets (Cowell *et al.*, 1999; Van Esch *et al.*, 2003) (Table 2). Therefore additional primers were designed to cover complete VH germline genes and designated them as VH2*a and VH4*a.

The results from reverse transcriptase PCR with individual samples and from Q-PCR with pooled samples demonstrated that there were no significant differences in PCR profile between AS patients and control group, that was produced by PCR primer sets for VH1a-VH6a and for VH4*a (Figures 1A and 1B). The results confirmed the previous study showing VH3a was dominantly expressed (Voswinkel *et al.*, 2001). Interestingly,

Table 1. Demographic and clinical characteristics of subjects.

	AS (n = 8)	HC (n = 9)
Age (years)*	38.6 ± 13.1	30.2 ± 2.3
Sex (M:F)	7:1	8:1
Disease duration (years)	11.4 ± 7.1	
History of uveitis	3 (37.5%)	
History of enthesitis	2 (25%)	
BASDAI*	4.2 ± 2.3	
BASFI*	2.6 ± 2.2	
ESR (mm/h)*	37.0 ± 35.8	
CRP (mg/dl)*	1.7 ± 2.2	
HLA-B27 positivity	8 (100%)	

AS, ankylosing spondylitis; HC, healthy controls; BASDAI, Bath Ankylosing Spondylitis Disease Activity Index; BASFI, Bath Ankylosing Spondylitis Functional Index. *Mean ± SD.

VH2* genes represented by VH2*a forward primer were overexpressed exclusively in AS patients (patient sample numbers 4, 5, 6, 7, and 8 as shown in Figure 1A). Thus, Q-PCR with individual samples was performed to quantify the amount of VH2* transcripts. Significant difference was shown in the level of expression of VH2* genes between healthy donors and AS patients (relative amount of mRNA of VH2* genes to human acidic ribosomal protein (HuPo), 0.68 ± 0.55 [mean ± SD] and 7.13 ± 7.77 , respectively; $P < 0.0001$; Figure 1C). There were no correlations of the expression level of VH2* with clinical variables such as sex, age, disease duration and inflammatory parameters (data not shown).

Gene structure of VH2* PCR products

The PCR products from samples demonstrating high expression level of VH2* genes were cloned and sequenced. Sequence analysis revealed a short fragment from CDC42 BPB genes incorporated into major part of cloned VH2* PCR products from patient sample number 4, 5, 6, 7, and 8. This is an intron fragment located in the region encompassing 252 bps (36096-36348) of 125-kb of CDC42 BPB which maps to 14q32.32 (Figure 2) (Moncrieff *et al.*, 1999) and is assumed to be incorporated in-between a short stretch of VH2 and DH6.

Another PCR using primers corresponding to sequences in the middle of the intron of CDC42 BPB gene and Ig constant regions (C μ , C γ and C ϵ) was set up to confirm that the intron fragment from CDC42 BPB genes was indeed incorporated into Ig heavy chain gene segments (Figure 1D). The results demonstrated the expected band size, about 330 bps, which appeared exclusively by a C ϵ primer. Therefore these results imply that an intron

Table 2. Oligonucleotide primers used to evaluate VH gene usage and confirm CDC42BPB inserted gene structure.

Primer set for Q-PCR	
Human VH forward primers	
HuVH1a for	5' - CAG GTG CAG CTG GTG CAG TCT GG-3'
HuVH2a for	5'-CAG GTG AAG TTA AGG GAG TCT GG-3'
HuVH3a for	5'-GAG GTG CAG CTG GTG GAG TCT GG-3'
HuVH4a for	5'-CAG GTG CAG CTG CAG GAG TCG GG-3'
HuVH5a for	5'-GAG GTG CAG CTG TTG CAG TCT GC-3'
HuVH6a for	5'-CAG GTA CAG CTG CAG CAG TCA GG-3'
HuVH2*a for	5'-CAG ATC ACC TTG AAG GAG TCT GG-3'
HuVH4*a for	5'-CAG GTG CAG CTA CAG CAG TGG GG-3'
Human JH reverse primers	
HuJH1-2 rev	5'-TGA GGA GAC GGT GAC CAG GGT GCC-3'
HuJH3 rev	5'-TGA AGA GAC GGT GAC CAT TGT CCC-3'
HuJH4-5 rev	5'-TGA GGA GAC GGT GAC CAG GGT TCC-3'
HuJH6 rev	5'-TGA GGA GAC GGT GAC CGT GGT CCC-3'
HuJH7 rev	5'-TGA CCG TGG TCC CTT GGC CCC AGA-3'
HuPo (House-keeping gene)	
HuPo for	5'-CCA TTC TAT CAT CAA CGG GTA CAA-3'
HuPo rev	5'-AGC AAG TGG GAA GGT GTA ATC C-3'
Primer set for second PCR	
Human VH2*a forward primers with NcoI restriction sites	
HuVH2*a-Nco1	5'-AGC CGG <u>CCA TGG</u> CCG CAG ATC ACC TTG AAG GAG TCT GG-3
Human JH reverse primers with XhoI restriction sites	
HuJH1-2-Xho1	5'-TCC ACC <u>GCT CGA</u> GAC TGA GGA GAC GGT GAC CAG GGT GCC-3'
HuJH3-Xho1	5'-TCC ACC <u>GCT CGA</u> GAC TGA AGA GAC GGT GAC CAT TGT CCC-3'
HuJH4-5-Xho1	5'-TCC ACC <u>GCT CGA</u> GAC TGA GGA GAC GGT GAC CAG GGT TCC-3'
HuJH6-Xho1	5'-TCC ACC <u>GCT CGA</u> GAC TGA GGA GAC GGT GAC CGT GGT CCC-3'
HuJH7-Xho1	5'-TCC ACC <u>GCT CGA</u> GAC TGA CCG TGG TCC CTT GGC CCC AGA-3'
Primer set to confirm CDC42BPB insert	
HuCDC42 BPB Forward primer	
HuCDC42-FOR	5'-GAG CAC TGG CCA AGC ACT A-3'
Human C _μ , C _γ and C _ε reverse primer	
Hu C _μ rev	5'-TCC AGG AGA AAG TGA TGG AG-3'
Hu C _γ rev	5'-GTC TTG GCA TTA TGC ACC TC-3'
Hu C _ε rev	5'-CGG ATG GGC TCT GTG TGG-3'

fragment of the CDC42 BPB gene, suggested by sequence analysis, was incorporated in between VH2 and DH6-JH3-C_ε of rearranged Ig genes (Figure 1D).

Discussion

Immunoglobulins consist of the light (L) and heavy (H) chains, each of which has variable and constant regions. The human VH segments are located in three loci; chromosome 14, 15 and 16 but only the chromosome 14 locus contains the JH segments that are essential for somatic generation of the VH genes.

As for VH germline gene usages in AS patients, a previous report demonstrated over-representation of VH5 and under-representation of VH4 from the AS synovial B lymphocytes compared with the germline representation (Voswinkel *et al.*, 2001). In

this study, we used a novel primer set including VH2*a, representing some of the IGHV2 genes in the VH2 germline gene family, which had been absent in the previous study. VH2* genes were overexpressed only in AS patients and the level of expression was significantly higher in PB of AS patients compared to those of healthy donors. It suggests that restricted germline gene family may be selected in PB of AS patients.

The Ig V gene repertoire in PB was not compared with either synovial tissues or synovial fluid in the same patients in this study. The Ig V gene repertoire in PB may be different from that in inflammatory joints. PB contains a population of recirculating memory B cells that have encountered a wide diversity of antigens over the patient's lifetime, whereas inflammatory joint tissues may have the subset of B cells responding to antigen and undergoing antigen-driven response. Thus comparing Ig contents of synovial B cells with

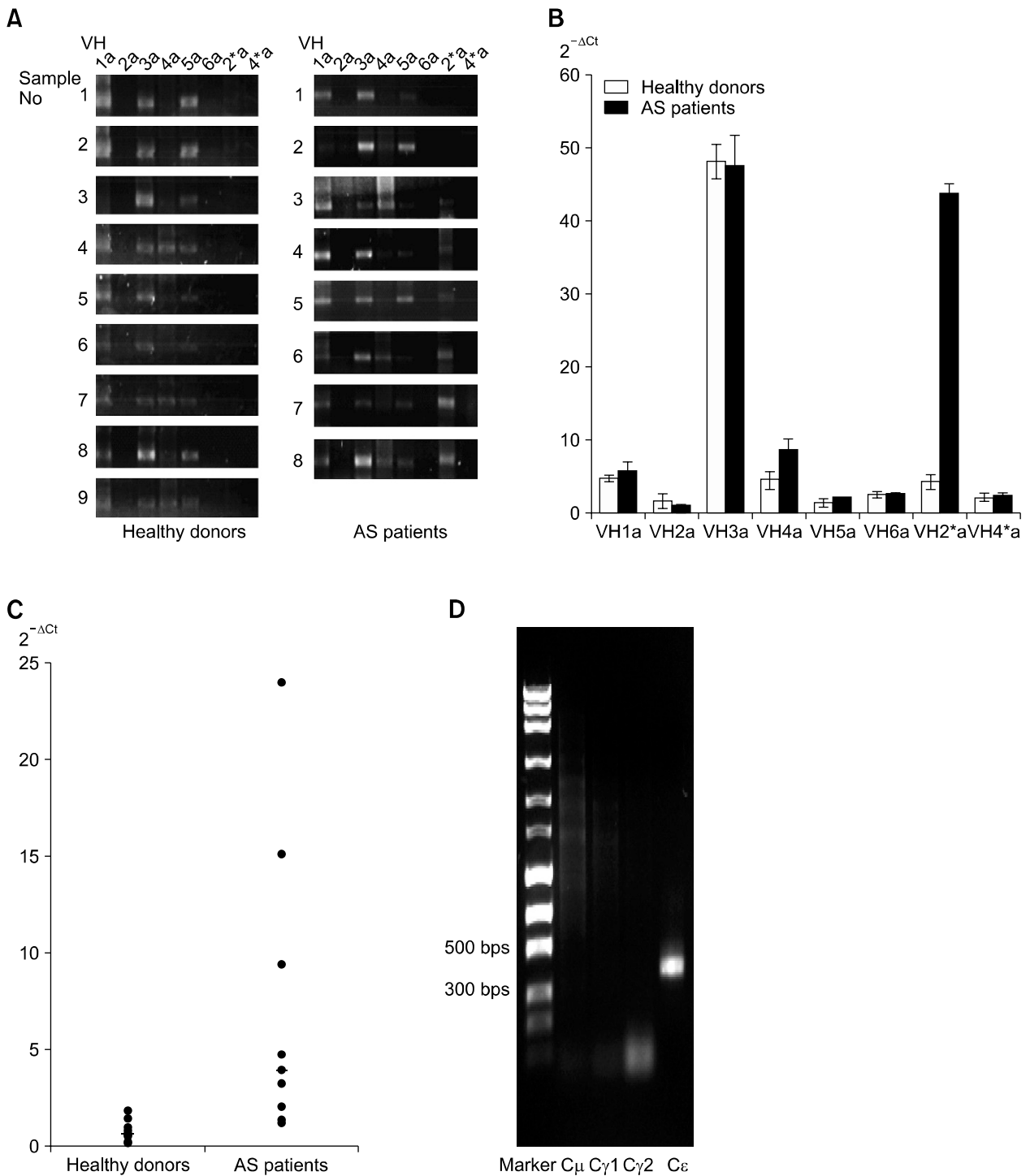


Figure 1. VH2 genes were overexpressed in AS patients. (A) Comparison of VH gene usages in PBMCs from healthy donors and AS patients. Reverse transcriptase PCR was performed with RNA from PBMCs of healthy donors and AS patients using primers shown in Table 2. VH2* genes were overexpressed exclusively in AS patients (patient sample number 4, 5, 6, 7 and 8). $n = 9$ of healthy donors and 8 of AS patients. (B) The relative expression levels of patients and healthy donors' each VH gene. Q-PCR was performed with pooled cDNA from PBMC of healthy donors and AS patients using primers specific for VH1a-6a genes. The relative amount of transcripts of target genes compared to those of a housekeeping gene was calculated as follows; $\Delta Ct = Ct_{(experimental)} - Ct_{(housekeeping)}$, $R = 2^{-(\Delta Ct)}$. The data shows the means and standard deviations of PCR amplification in triplicate. (C) Q-PCR was performed with individual cDNA from each PBMC of healthy donor and AS patient using primers specific for VH2* genes. The relative amount of mRNA of VH2* genes to HuPo was calculated as above. The relative amount of mRNA of VH2* genes to HuPo in AS patients was significantly higher compared to healthy donors ($P < 0.0001$). $n = 9$ of healthy donors and 8 of AS patients. (D) CDC42 BPB intron sequences were inserted in Ig genes in AS patients. PCR was performed with cDNA from PBMCs of healthy donors and AS patients using primers specific for CDC42 BPB genes and Ig C epsilon genes.

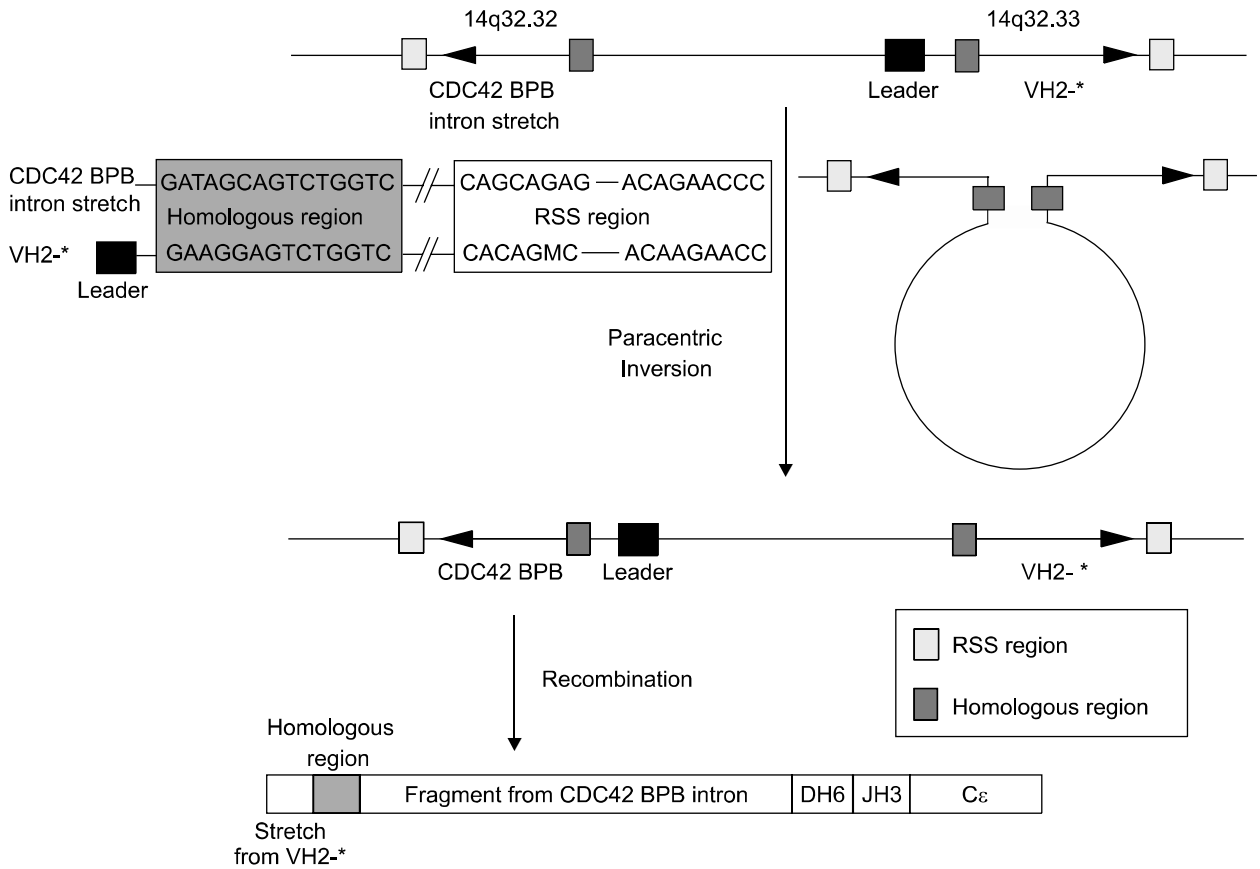


Figure 2. Proposed gene structure of rearranged VH2* genes in AS patients. CDC42 BPB intron fragment could be paracentrically inverted into VH2 genes. Both genes are located in chromosome 14q32. The sequence homology search revealed possible RSS sequences close to the inserted CDC42 BPB intron fragment. The resulted rearranged Ig gene has a part of the VH2 genes, CDC42 BPB intron sequences, DH6, JH3, and C ϵ in order.

those of PB should be interesting.

Strikingly the sequence analysis and homology search of overexpressed VH2* PCR products revealed unexpected features of VH gene structure. In the sequences of VH2* PCR products, a short stretch of CDC42 BPB intron gene was found (Figure 2). It is worth noting that this intron segment is located in chromosome 14q where human Ig VH gene locus is present (Cook *et al.*, 1994). Ig gene rearrangement requires recombination signal sequences (RSS) that consist of a heptamer, 23 nucleotides (nts), and a nonamer in this order (Jung *et al.*, 2006). A short stretch of intron sequences of CDC42 BPB was found between VH and DH genes. The 5' end of this fragment includes homologous region (15 nucleotides) to that of the VH2 germline gene (Figure 2). Interestingly, further analysis of CDC42 BPB genomic sequences identified a nonamer of RSS, which is located following an insertion site of this fragment by 18 nucleotides, exists in CDC42 BPB genes. In addition, a heptamer-like sequence (CAGCAGAG), which has one G base added

feature to generally known heptamer of RSS (CACAGAG), also appears in the end of the inserted CDC42 BPB intron genes, as seen in Figure 2. This gene structure implies that unusual gene rearrangement or recombination may occur during construction of VH gene segments during B cell development in some of the AS patients. Paracentric inversion following recombination activating gene (RAG)- based recombination might be suggested to explain unique rearranged VH2* gene structure (Figure 2). However, it should be mentioned that this hypothesis does not perfectly fit in with the norm of RAG-based recombination as the distance between the heptamer and the nonamer in CDC42 BPB gene is shorter and the heptamer-like sequence in CDC42 BPB needs to have one base deletion to become proper heptamer for recombination. The questions how these unfitted features could be modified in some AS patients and whether the unique rearrangements were found only in Korean AS patients are still remaining. Further study is required to address these questions.

To date, evidence that B cells are involved in AS/spondyloarthritis pathogenesis has been relatively scant. Nevertheless, B cells and plasma cells are consistently present in inflammatory lesions (McGonagle *et al.*, 2002; Appel *et al.*, 2006). In a recent open label phase-II clinical trial, rituximab, a chimeric monoclonal antibody against CD20 on the surface of B cells, showed significant efficacy in TNF-naïve patients with AS (Song *et al.*, 2009). Our study, along with Voswinkel's work (Voswinkel *et al.*, 2001), showed abnormal molecular event in B cells from AS patients. Taken together, these suggest that B cells may play a role in developing AS. Since the autoantibodies are not identified in AS, B cells might contribute to AS/spondyloarthritis pathogenesis by (auto) antigen presentation to T cells or cytokine release rather than by antibody production. In a mouse model of proteoglycan-induced arthritis, B cells contributed to the disease by presentation of autoantigen and subsequent T cell activation as well as by producing specific antibodies (O'Neill *et al.*, 2005). Additional sequence analysis of rearranged VH2* gene structure is currently being performed, and it is planned to screen binding partners from serum of AS patient against this aberrant gene product. It would be also informative to determine whether the rearranged Ig actually produces protein. However, it is possible that the aberrantly rearranged Ig genes are not functional, thus no protein could be produced. Aberrant Ig transcripts could still affect other Ig protein production or composition in a way or another. Further investigations are required to verify that distinct rearrangement of Ig VH genes are involved in B cell development or function in terms of AS pathogenesis.

In conclusion, this study revealed VH2 overexpression and unique rearrangement in Ig VH genes from PB of AS patients. This may imply aberrant immunoglobulin gene rearrangement in B cell occurs in Korean AS patients, which awaits further investigation.

Methods

Subjects

Peripheral blood (PB) was collected from 9 healthy controls and from 8 patients with AS who visited the rheumatology clinic at Gachon University Gil Hospital. The patients with AS met the Modified New York Criteria (van der Linden *et al.*, 1984) and were taking non-steroidal anti-inflammatory drug and sulfasalazine regularly. Age, sex, disease duration, erythrocyte sedimentation rate (ESR), serum C reactive protein (CRP) and HLA-B27 positivity were assessed (Table 1). The validated Korean

version of Bath AS Disease Activity Index (BASDAI) (Garrett *et al.*, 1994) for AS patients were calculated when their PB was collected. The study was approved by Local Research Ethical Committee of Gachon University Gil Hospital and written informed consent was obtained from all the patients and healthy volunteers.

Quantitative real-time PCR (Q-PCR)

PB was collected into sterile, heparinized tubes and PB mononuclear cells (PBMCs) were isolated from whole blood using a Ficoll-Hypaque gradient (Cho *et al.*, 2008). Total RNA was isolated from PBMCs by RNAeasy mini kit (Qiagen, Hilden, Germany) and cDNA was synthesized by Maxime™ RT PreMix (Oligo(dT)15 primer) Kit (Intron Biotechnology, Korea) following manufacturers' instructions. PCR and Q-PCR were performed with primers as shown in Table 2 (Cowell *et al.*, 1999; Van Esch *et al.*, 2003; Dheda *et al.*, 2004). For thorough representation of VH germline genes, two novel VH forward primers, which we refer to as VH2*a and VH4*a forward primers for Q-PCR, were added to VH PCR primer sets used in previous studies (Voswinkel *et al.*, 2001). The sequences of VH2a and VH4a forward primers previously used were different from those for VH2*a and VH4*a, as shown in Table 1. Primers for human acidic ribosomal protein (HuPo) gene, as a house-keeping gene, were also used since it is known to be more reliable than β -actin gene as control for the Q-PCR in PBMC (Dheda *et al.*, 2004). PCR mixture without DNA was included in each experiment as negative control. The amplification condition was 95°C for 30 s, 50-60°C for 1 min, and 72°C for 1 min for 30-40 cycles. Primers were produced by MWG (Ebersberg, Germany). Reagents for Q-PCR were purchased from Stratagene (La Jolla, USA). PCR products were analyzed by gel electrophoresis or by MxPro™ QPCR software (Stratagene, La Jolla). The relative amount of transcripts of target genes compared to those of a housekeeping gene was calculated as follows; $\Delta tCt = Ct_{(experimental)} - Ct_{(housekeeping)}$, $R = 2^{-(\Delta Ct)}$.

Sequence analysis

The second PCR was performed with Q-PCR products from selected VH2* overexpressed patient samples (number 4, 5, 6, 7, and 8) as a template to introduce *NcoI* and *XhoI* site at 5' and 3' end of first PCR products, respectively. The PCR products were cloned into pT2 vectors (Enever *et al.*, 2005) and 60-80 colonies were selected to confirm inserted genes by PCR screening using primer set for second PCR (Table 1). Approximately 20 samples of each group were analyzed by sequencing (MWG, Germany). The sequence results were analyzed by homology comparison with Ig Blast databases (<http://www.ncbi.nlm.nih.gov/igblast>) and IMGT databases (<http://imgt.cines.fr>).

To identify incorporation of CDC42 BPB intron fragments, PCR amplification was performed with primers for CDC42 BPB intron gene and human Ig constant region (C) genes (Table 1). Reaction conditions were initial denaturation at 95°C for 15 min, followed by 30 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 1 min, and then 72°C for 10 min for final extension. PCR products were

analyzed by 2% agarose gel electrophoresis.

Statistics

A non-parametric statistic method, two tailed Mann-Whitney U test was performed to test the significance of differences in mRNA amount of individual VH gene families between AS patients and healthy volunteers (SPSS, Ver. 12.0. Chicago, IL). To test for correlations between the expression level of VH2**a* genes and clinical variables, Spearman's rank correlation coefficient was used.

Acknowledgements

We thank Dr. T.H Rabbitts (LIMM, United Kingdom) for helpful discussion on chromosomal inversion. This study was funded by Korea Institute of Science and Technology-Europe basic research program. HJB was supported by Korean Institute of Medicine.

References

- Appel H, Kuhne M, Spiekermann S, Ebhardt H, Grozdanovic Z, Kohler D, Dreimann M, Hempfing A, Rudwaleit M, Stein H, Metz-Stavenhagen P, Sieper J, Loddenkemper C. Immunohistologic analysis of zygapophyseal joints in patients with ankylosing spondylitis. *Arthritis Rheum* 2006; 54:2845-51
- Baeten D, Demetter P, Cuvelier CA, Kruihof E, Van Damme N, De Vos M, Veys EM, De Keyser F. Macrophages expressing the scavenger receptor CD163: a link between immune alterations of the gut and synovial inflammation in spondyloarthritis. *J Pathol* 2002;196:343-50
- Breban M, Fernandez-Sueiro JL, Richardson JA, Hadavand RR, Maika SD, Hammer RE, Taurog JD. T cells, but not thymic exposure to HLA-B27, are required for the inflammatory disease of HLA-B27 transgenic rats. *J Immunol* 1996;156:794-803
- Brewerton DA, Hart FD, Nicholls A, Caffrey M, James DC, Sturrock RD. Ankylosing spondylitis and HL-A 27. *Lancet* 1973;1:904-7
- Cho ML, Jung YO, Kim KW, Park MK, Oh HJ, Ju JH, Cho YG, Min JK, Kim SI, Park SH, Kim HY. IL-17 induces the production of IL-16 in rheumatoid arthritis. *Exp Mol Med* 2008;40:237-45
- Cook GP, Tomlinson IM, Walter G, Riethman H, Carter NP, Buluwela L, Winter G, Rabbitts TH. A map of the human immunoglobulin VH locus completed by analysis of the telomeric region of chromosome 14q. *Nat Genet* 1994;7: 162-8
- Cowell LG, Kim HJ, Humaljoki T, Berek C, Kepler TB. Enhanced evolvability in immunoglobulin V genes under somatic hypermutation. *J Mol Evol* 1999;49:23-6
- De Keyser F, Elewaut D, De Vos M, De Vlam K, Cuvelier C, Mielants H, Veys EM. Bowel inflammation and the spondyloarthropathies. *Rheum Dis Clin North Am* 1998; 24:785-813, ix-x
- Dheda K, Huggett JF, Bustin SA, Johnson MA, Rook G, Zumla A. Validation of housekeeping genes for normalizing RNA expression in real-time PCR. *Biotechniques* 2004;37: 112-4, 116, 118-9
- Dorner T, Hansen A, Jacobi A, Lipsky PE. Immunoglobulin repertoire analysis provides new insights into the immunopathogenesis of Sjogren's syndrome. *Autoimmun Rev* 2002;1:119-24
- Enever C, Tomlinson IM, Lund J, Levens M, Holliger P. Engineering high affinity superantigens by phage display. *J Mol Biol* 2005;347:107-20
- Ernel RW, Kenny TP, Wong A, Chen PP, Malyj W, Robbins DL. Analysis of the molecular basis of synovial rheumatoid factors in rheumatoid arthritis. *Clin Immunol Immunopathol* 1997;84:307-17
- Foreman AL, Van de Water J, Gougeon ML, Gershwin ME. B cells in autoimmune diseases: insights from analyses of immunoglobulin variable (Ig V) gene usage. *Autoimmun Rev* 2007;6:387-401
- Garrett S, Jenkinson T, Kennedy LG, Whitelock H, Gaisford P, Calin A. A new approach to defining disease status in ankylosing spondylitis: the Bath Ankylosing Spondylitis Disease Activity Index. *J Rheumatol* 1994;21:2286-91
- Isenberg D, Spellerberg M, Williams W, Griffiths M, Stevenson F. Identification of the 9G4 idiotope in systemic lupus erythematosus. *Br J Rheumatol* 1993;32:876-82
- Jandus C, Bioley G, Rivals JP, Dudler J, Speiser D, Romero P. Increased numbers of circulating polyfunctional Th17 memory cells in patients with seronegative spondylarthritides. *Arthritis Rheum* 2008;58:2307-17
- Jung D, Giallourakis C, Mostoslavsky R, Alt FW. Mechanism and control of V(D)J recombination at the immunoglobulin heavy chain locus. *Annu Rev Immunol* 2006;24:541-70
- May E, Dorris ML, Satumira N, Iqbal I, Rehman MI, Lightfoot E, Taurog JD. CD8 alpha beta T cells are not essential to the pathogenesis of arthritis or colitis in HLA-B27 transgenic rats. *J Immunol* 2003;170:1099-105
- McGonagle D, Marzo-Ortega H, O'Connor P, Gibbon W, Hawkey P, Henshaw K, Emery P. Histological assessment of the early enthesitis lesion in spondyloarthritis. *Ann Rheum Dis* 2002;61:534-7
- Moncrieff CL, Bailey ME, Morrison N, Johnson KJ. Cloning and chromosomal localization of human Cdc42-binding protein kinase beta. *Genomics* 1999;57:297-300
- O'Neill SK, Shlomchik MJ, Glant TT, Cao Y, Doodles PD, Finnegan A. Antigen-specific B cells are required as APCs and autoantibody-producing cells for induction of severe autoimmune arthritis. *J Immunol* 2005;174:3781-8
- Robbins DL, Kenny TP, Coloma MJ, Gavilondo-Cowley JV, Soto-Gil RW, Chen PP, Larrick JW. Serologic and molecular characterization of a human monoclonal rheumatoid factor derived from rheumatoid synovial cells. *Arthritis Rheum* 1990;33:1188-95
- Schlosstein L, Terasaki PI, Bluestone R, Pearson CM. High association of an HL-A antigen, W27, with ankylosing

spondylitis. *N Engl J Med* 1973;288:704-6

Shen H, Goodall JC, Hill Gaston JS. Frequency and phenotype of peripheral blood Th17 cells in ankylosing spondylitis and rheumatoid arthritis. *Arthritis Rheum* 2009; 60:1647-56

Sims GP, Shiono H, Willcox N, Stott DI. Somatic hypermutation and selection of B cells in thymic germinal centers responding to acetylcholine receptor in myasthenia gravis. *J Immunol* 2001;167:1935-44

Song I, Heldmann F, Rudwaleit M, Listing J, Appel H, Bogdan M, Braun J, Sieper J. Major clinical response of rituximab in active TNF-blocker-naive patients with ankylosing spondylitis but not in TNF-blocker-failure patients- an open label clinical trial. *Ann Rheum Dis* 2009;68(suppl 3):74

Stevenson FK, Longhurst C, Chapman CJ, Ehrenstein M, Spellerberg MB, Hamblin TJ, Ravirajan CT, Latchman D, Isenberg D. Utilization of the VH4-21 gene segment by anti-DNA antibodies from patients with systemic lupus erythematosus. *J Autoimmun* 1993;6:809-25

Taurog JD, Richardson JA, Croft JT, Simmons WA, Zhou M,

Fernandez-Sueiro JL, Balish E, Hammer RE. The germfree state prevents development of gut and joint inflammatory disease in HLA-B27 transgenic rats. *J Exp Med* 1994;180: 2359-64

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

Van Esch WJ, Reparon-Schuijt CC, Hamstra HJ, Van Kooten C, Logtenberg T, Breedveld FC, Verweij CL. Human IgG Fc-binding phage antibodies constructed from synovial fluid CD38+ B cells of patients with rheumatoid arthritis show the imprints of an antigen-dependent process of somatic hypermutation and clonal selection. *Clin Exp Immunol* 2003;131: 364-76

Voswinkel J, Weisgerber K, Pfreundschuh M, Gause A. B lymphocyte involvement in ankylosing spondylitis: the heavy chain variable segment gene repertoire of B lymphocytes from germinal center-like foci in the synovial membrane indicates antigen selection. *Arthritis Res* 2001;3:189-95