Titel

Development of early diagnostic systems for Ankylosing Spondylitis

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"If we knew what it was we were doing, it would not be called research, would it?"

Albert Einstein

CONTENTS

ABBREVIATIONS

AID – Autoimmune disease

Anti CCP – anti-cyclic citrullinated protein antibodies

AS – Ankylosing Spondylitis

ASAS – Assessment of SpondyloArthritis international Society

BASDAI – Bath AS Disease Activity Index

BCR – B-cell receptor

CD – Cluster of Differentiation

CDC42BPB - CDC42 binding protein kinase beta

CRP – C-reactive protein

CT – Computed tomography

Ct – Cycle threshold

CTL – Cytotoxic T lymphocytes

DH – Immunoglobulin diversity region gene

DZ – Dizygotic

ERAP1 – Endoplasmic reticulum aminopeptidase 1

ESR – Erythrocyte sedimentation rate

ESSG – European Spondylarthropathy Study Group

HC – Healthy control

HLA – Human Leukocyte Antigen

HLA-B27 – Human Leukocyte Antigen B27

HuPo – Human acidic ribosomal protein

IBD – Inflammatory Bowel Disease

Ig – Immunoglobulin

IgC – Immunoglobulin constant domain

IgH – Immunoglobulin heavy chain

IgV – Immunoglobulin variable domain

IgVH – Immunoglobulin variable heavy chain

IL – Interleukin

JH – Immunoglobulin joining region gene

LMP – Large multifunctional protease subunits of the proteasome

MG – Myasthenia Gravis

MHC – Major Histocompatibility Complex

MRI – Magnetic resonance imaging

MS – Multiple sclerosis

MZ – Monozygotic

NSAIDs – Non-steroidal anti-inflammatory drug

PBC – Primary Biliary Cirrhosis

PBMC – Peripheral blood mononuclear cell

PCR – Polymerase chain reaction

PsA – Psoriatic Spondylitis

RA – Rheumatoid Arthritis

RAG – Recombination activating genes

ReA – Reactive arthritis

RF – Rheumatoid factor

RSS – Recombination signal sequence

SLE – Systemic Lupus Erythematosus

SNP – Single-nucleotide polymorphism

SS – Sjogren's syndrome

SpA – Spondyloarthropathy

SPARCC –Spondyloarthritis Research Consortium of Canada Magnetic Resonance Imaging Index for Assessment of Spinal Inflammation in AS

TAP – Transporters associated with antigen processing

TCR – T cell receptor

Th17 – T helper type 17 cells

TNF – Tumor necrosis factor

Type1 diabetes – insulin-dependent diabetes mellitus

ZUSAMMENFASSUNG

Spondylitis ankylosans (AS), die häufigste Form der Spondylarthritis (SpA), ist eine chronisch entzündliche Autoimmunerkrankung (Arthritis). AS betrifft hauptsächlich die sarkrolitischen Gelenke und die Wirbelsäule.

In der vorliegenden Arbeit wurden erstmals die variablen Segmente der schweren Kette (IgVH) von Immunglobulin in peripheren mononukleären Blutzellen (PBMCs) von AS Patienten analysiert, um eine frühe Diagnostik für AS zu entwickeln. Wir haben drei Primersätze (PR1, PR2 und PR3) designed, die Homologien zu spezifischen Stellen in dem VH-Segment aufzeigen. Die Ergebnisse der quantitative PCR (Q-PCR) zeigten, dass die in AS Proben einmalig reorganizierten VH2 Transkripte etwa zehnmal staerker überexprimiert wurden, als Kontrolproben von gesunden Spender. Dies führte uns zu der Hypothesen, dass die parazentrische Inversion auf Chromosom 14 und / oder ein Klassenwechseldefizit (class switch rekombination deficiency) mit AS Anfälligkeit in Verbindung gebracht werden könnte. Desweiteren hat die Überexpression des CD40L-Gen unsere Hypothese erhaertet. Der quantitative Vergleich zwischen den relativen Genexpressionlevels bei axialer AS, RA-Patienten und gesunden Spendern zeigte, dass die neu entwickelten Primerpaare in der Systementwicklung der Früherkennung des AS angewendet werden können. Zukünftige Studien werden sich auf die weitere Untersuchungen der Diagnose von AS mit den entwickelten Primernsätzen und die Relevanz unsere bisherigen Ergebnisse für diePathogenese des AS konzentrieren.

SUMMARY

Ankylosing spondylitis (AS), a prototype of spondyloarthritis (SpA), is a chronic inflammatory arthritis that mainly affects the sarcroiliac joints and the spine.

In this study, in order to develop an early diagnosis system for AS, which is known as an autoimmune disease, immunoglobulin variable heavy chain (IgVH) segments were analyzed in peripheral blood mononuclear cells (PBMCs) of patients with AS. We designed three sets of primers (PR1, PR2 and PR3) targeting the unique VH segment in AS patients. Quantitative PCR (Q-PCR) results revealed that uniquely rearranged VH2 transcripts were about ten times over expressed in AS samples as compared to healthy donor controls. This led us to the hypothesis that the paracentric inversion on chromosome 14 and/or a class switch recombination deficiency might be associated with AS susceptibility, and this is supported by the overexpression of CD40L.The comparison between relative gene expression amounts in axial AS, healthy donors, and RA patients suggests that newly designed primers could be employed in early diagnosis system development. Future studies will focus on establishing a general AS diagnostic scheme using developed primer sets and the relevance of our findings to AS pathogenesis.

List of Figures and Tables

segments encoding the V, D, and J regions of Ig. According to the 12/23 rule, the arrangement of RSSs in the Ig heavy chain gene segments precludes direct V to J joining (M amino acid sequence A or C).

Figure 27. V region gene segments are joined by the recombination process. Recombinational signal sequences allow taking place. The joining of V and J segments creates a functional V region exon. Binding together the RSSs through 12-23 rules creates DNA looping ((1) to (4)), after which DNA intervening is repeated and a functional V region exon is generated (5).

Figure 28. Sequence analysis. 252bp of CDC42 BPB sequences identified a heptamer-12bp spacer-nonamer RSS motif in CDC42 BPB genes.

Figure 29. Unique antibody rearrangement may occur in patients with AS. The sequence analysis and homology search proposed that possible paracentric inversion of CDC42 BPB intron fragment to VH2 genes. The numericals indicate the gene fragments are: 2) VH2, 3) CDC42BPB, 4) DH, 5) JH.

Figure 30. Class switching is preceded by transcriptional activation of heavy chain C-region genes; A) naive B cell-transcribed heavy chain μ and δ at low rate, B) IL-4/IL-13 signal and B cell-activating signals, such as CD40-CD40L interaction, T cell and bacterial infection, give rise to Cε transcription.

Figure 31. T- and B-cell interaction and signals for IgE production: (1) presentation, MHC class II molecules and recognition by the TCR, (2) CD40L expression. CD40L-induced aggregation of CD40 then triggers expression (3) of B7 (CD80), (4) amplifies cytokine synthesis, (5) produces IL-4 in conjunction with CD40 ligation (signal 2), and triggers the IgE isotype switch, B-cell proliferation, and expansion of the IgE-producing clone.

Figure 32. Genes on chromosome 14q3. The distance between CDC42BPB and IgVH2 is around 1.5K to 2K.

Figure 33. Human antibody VH region genes are constructed from variable gene segments. Normal antibody formation (Right panel). Expected AS patient antibody formation (Left panel). Abnormal rearrangement of V gene segments might affect the rest of the Ig production process resulting in: 1) autoimmune antibody production in AS patients, 2) abnormalities in AS patients and 3) failure to produce complete Ig molecules

Table 1. Classification and diagnosis criteria for SpA

Table 2. Immunoglobulin variable gene usage skewed in particular autoimmune diseases.

Table 3. Genes on chromosome14 and associated diseases.

Table 4. Demographics and clinical characteristics of subjects

Table 5. Primer sets for investigation of immunoglobuline VH region usage

Table 6. Secondary PCR primers for bacterial clonning

Table 7. House-keeping gene primer for Q PCR

Table 8. Primer sets for confirmation of CDC42BPB incorporation

Table 9. The demographic and clinical characteristics of patients and HC

Table 10. Primer sets to investigate CD40L expression

Table 11. Relative transcript values for individual samples of healthy controls AS patients

Table 12. Normalized expression value relative to patients $2^{(-\Delta\Delta Ct)}$

1. INTRODUCTION

1.1. Introduction of AS

1.1.1. What is SpA?

In white population, spondyloarthropathy (SpA) prevalence is 1.9% (USA population only: 0.6-0.9%) and it is the most frequently developed rheumatoid disease (Braun *et al*., 1998). About 30 years ago, SpA was separated from the range of rheumatoid arthritis (RA) because it has particular common features such as inflammatory back pain (Moll *et al*., 1974; Davis *et al*., 2003). SpA is composed of ankylosing spondylitis (AS), reactive arthritis (also called Reiter's syndrome), arthritis with inflammatory bowel disease (IBD or Crohn's disease), and arthritis with psoriasis (Braun and Sieper J, 2007).

The main link between SpA subtypes is associated with the HLA-B27 gene and the populations with positive HLA-B27 have a 20 times higher risk developing SpA (Braun *et al*., 1998). However, there has been still remaining questions for the role of HLA-B27 in SpA. In the general population, fewer than 5% of HLA-B27 positive individuals develop SpA, although 20% in AS patients' relatives who are positive with HLA-B27 develop SpA (Linden *et al*., 1983). Epidemiology studies of patients in Middle East, West Africa and of African-Americans have shown that there is low association between the presence of HLA-B27 and SpA development in those areas (Reveille and Maganti, 2009; Gunal *et al*., 2008; López-Larrea *et al*., 2002; Díaz-Peña *et al*., 2008). These data implicate that other genetic and/or environmental factors are concurrent on SpA development.

The entire MHC genes effect on SpA is estimated at about 50% (Brown *et al*., 1997; Brown

et al., 2000) and the overall risk of HLA-B27 for SpA is only about 40% (Brown *et al*., 1997; Reveille and Maganti, 2009). These estimations imply that the other MHC genes play an important role on SpA.

1.1.2. Pathogenesis of AS

The estimated prevalence of AS in worldwide ranges from 0.3 up to 0.9% (Braun *et al*., 1998; Calin A, 1998) and 0.5% in white Europeans is (Thomas and Brown, 2010). Among the SpA subtypes, AS has the highest frequency and it also shows the higher severity of clinical symptoms (Braun *et al*., 1998; Braun and Sieper J, 2007). It is also known as highly heritable (Brown, 2008). In 1997, twin studies demonstrated 97% heritability (Brown *et al*., 1997) and more recent studies have suggested that the heritability of AS is greater than 90% (Brown, 2000).

For the last 40 years, HLA-B27 gene is considered as the most significant genetic factor on AS pathogenesis (Reveille and Maganti, 2009). About 90% of AS patients are identified as HLA-B27 positive (Reveille and Maganti, 2009). As compared to other types of SpA, value of HLA-B27 gene proportion with positive in AS patient is relatively high: reactive arthritis (70% in white people), psoriatic spondylitis (60-70%), acute anterior uveitis (50%), peripheral PsA (25%), peripheral enteropathic arthritis (no association at all) (Reveille, 2009). However, although HLA-B27 is essential in AS, this cannot give a clear explanation on genetic epidemiology and pathogenesis of AS (Reveille, 2009). Studies with HLA-B27 positive monozygotic (MZ) and dizygotic (DZ) which have concluded that AS is developed significantly less in DZ (12.5%) than in MZ (63%) (Brown *et al*., 2000). These results implicate that AS is considerably influenced by other genetic factors.

Two main hypotheses have received widespread acceptance for explaining etiology of AS.

One assumes that AS is an autoimmune disease and this is supported by many possible genetic predispositions (Arnett, 1993). Several genes in AS patients with a presumable higher risk of predisposition have been suggested which can contribute to AS pathogenesis, including HLA-B27 (Reveille and Maganti, 2009). The other one is related to the infectious etiology. This is based on the clinical association between AS and other SpA subgroups which had already established as an infectious pathogenesis, such as reactive arthritis (Mielants *et al*., 1987; Bauer and Engleman, 1942; Paronen, 1948). Furthermore, the close link between inflammatory bowel disease and AS also support the latter hypothesis of an infectious etiology (Mielants *et al*., 1987).

1.1.2.1. Accepted hypothesis for the pathogenesis of Ankylosing Spondylitis 1 – Autoimmune disease (AID)

1.1.2.1.1. Introduction of AID

AID is a clinical syndrome which is initiated by a loss of immunologic tolerance to self-antigens (Anaya, 2010). These defective immune systems are thought to be multifactorial because these are derived from the combination of genetic factors and various other factors (e.g. environment or viral infection) (Vyse and Todd, 1996; Davidson and Diamond, 2001). Several genes in the major histocompatibility complex (MHC) coding region which are represented into the class1 (HLA-A, B, C) and the class 2 (la, HLA-D) are known to contribute the most of autoimmune disease susceptibilities (Tiwari and Terasaki, 1981) and this is the reason why AS has been considered as a kind of autoimmune disease.

1.1.2.1.2. Examples of AID

More than 40 autoimmune diseases are classified in nature and 4-7% of the population suffer from it worldwide (Vyse and Todd, 1996; Sinha *et al*., 1990). Rheumatoid arthritis (RA), Graves' disease, insulin-dependent diabetes mellitus (Type1 diabetes), pernicious anemia, systemic lupus erythematosus (SLE) and Multiple sclerosis (MS) represent about half of 40 or more autoimmune diseases (Wandstrat and Wakeland, 2001). Except for a few special cases like autoimmune lymph proliferative syndrome, multiple susceptibility genes are required to the occurrence of autoimmune diseases (Davidson and Diamond, 2001; Encinas and Kuchroo, 2000; Becker, 1999).

MHC-class1 related autoimmune diseases are divided into two main categories (Sinha *et al*., 1990). The first group is HLA-B27 related spondyloarthropathies, which includes ankylosing spondylitis, Reiter's syndrome and reactive arthropathy. The second group is represented by psoriasis vulgaris (chronic stationary psoriasis) and diseases which are included in this group are related with HLA-B13, 16 and 17 (Sinha *et al*., 1990).

The MHC-class 2 associated group is much bigger than the class 1 group. This group comprises organ specific autoimmune diseases and systemic autoimmune diseases (Sinha *et al*., 1990). Organ specific autoimmune diseases are distinguished by autoantibody pattern. Insulin-dependent diabetes mellitus and MS are affiliated with it (Sinha *et al*., 1990). On the other hand, the systemic disease group is generally affected by the interaction between various autoantibodies and protoplasmic molecules involved in protein production (via DNA replication, DNA transcription and mRNA translation) (Sinha *et al*., 1990).

Although in most autoimmune diseases it has already reported that there is a particular linkage with MHC class 1 or 2 (Klein and Sato, 2000), it is insufficient to explain completely

the occurrence of autoimmune disease (Sinha et al., 1990; Klein and Sato, 2000). To lead a self-tolerance destruction and outbreak of autoimmune disease, other genes such as TNF-alpha or complements are necessary (Davidson and Diamond, 2001). Mice genetic engineering studies have suggested that at least 25 different genes can contribute to the predisposition of autoimmunity (Davidson and Diamond, 2001). Most of these genes are related with cytokines, antigen co-receptors, co-stimulatory molecules and apoptosis promoter (Davidson and Diamond, 2001).

From the beginning to the chronic stage of an autoimmune disease, it is often detected that the increase number of B and T cells targetng to autoantibodies (Moudgil and Sercarz, 1994; Lanzavecchia, 1995) and subsequently result in the change of participant cells, cytokines or other inflammatory mediators (Davidson and Diamond, 2001). Once B cells acquire auto-reactivity by somatic mutation during a germinal center response, they act as an antigen presenting cell and generate novel peptides (Vanderlugt *et al*., 2000, Liang and Mamula, 2000). These peptides activate T cells and this response activates the B cell again (Vanderlugt *et al*., 2000, Liang and Mamula, 2000). Additional auto reactive B cells present epitopes until the numerous autoantigens are produced (Davidson and Diamond, 2001). Acquired or inherited immune deficiency like the existence of MHC susceptibility allele or an abnormal function of T cells or B cells, are known as a crucial determinant for autoimmune disease susceptibility (Davidson and Diamond, 2001).

Until now, it has not yet been established that a certain antigen becomes an autoimmunity targeted antigen (Davidson and Diamond, 2001). Furthermore, the target antigens have not been identified in most of autoimmune diseases (Davidson and Diamond, 2001). Therefore, up to now most researches have concentrated on the lymphocytes specific activity under the autoimmune disease condition (Davidson and Diamond, 2001) and numerous B and T cell self epitopes have been identified (Sinha *et al*., 1990).

In B cells and T cells, self and non-self discerning methods are completely different (Davidson and Diamond, 2001; Sinha *et al*., 1990).

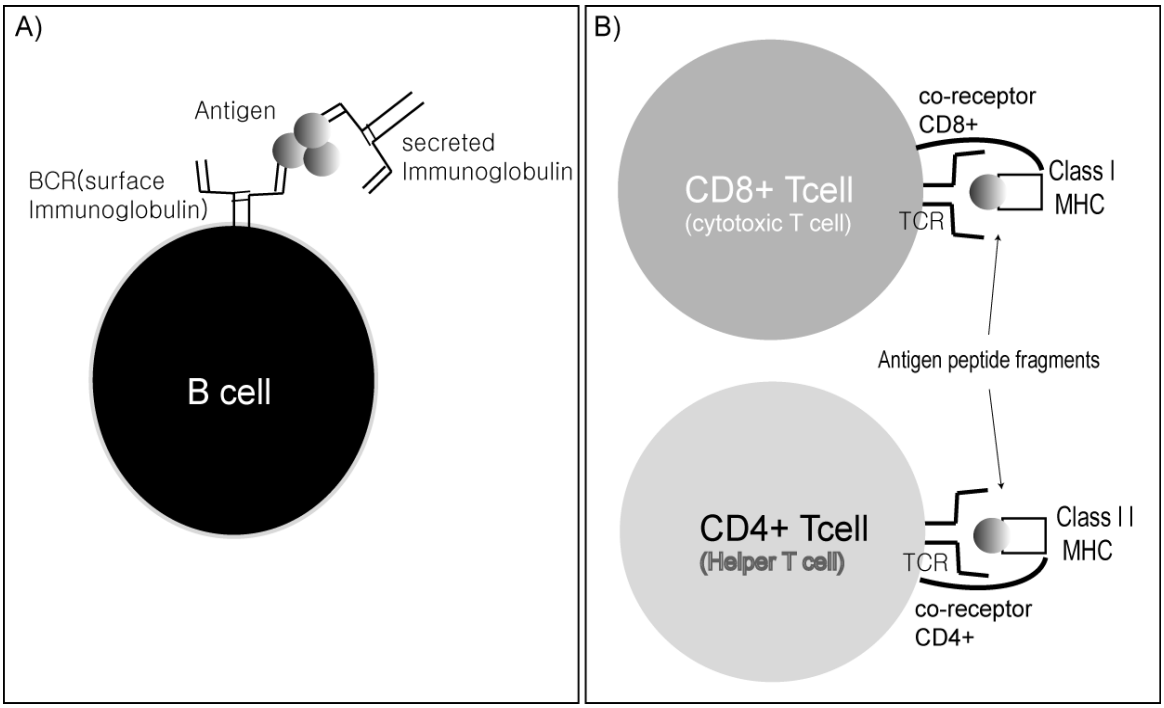


Figure 1. Antigen recognition by B cell and T cell receptors are completely different. A) B cells directly bind to antigens using B cell receptors . B Cells produce antibodies as both cell surface receptors and secreted Ig molecules. B) T cells use their antigen-specific T-cell receptors (TCR) and co-receptors (CD4, CD8) to recognize antigens. The CD8+ T cell subset recognizes antigens presented by MHC class I molecules and the CD4+ T cell subset recognizes antigens presented by MHC class II molecules.

As seen in Figure 1a, when B cells are activated by foreign antigens, they directly bind to these indigene antigens by using membrane located immunoglobulin molecules which are called B cell receptors (BCR) (Healy *et al*., 1997). In case of T cells, indigene antigens are concealed (Babbitt *et al*., 1985, Townsend *et al*., 1985). To be recognized by T cells, antigens must split into simple peptide fragments which then interact with MHC class 1 or class 2 associated antigen-presenting cells (Figure 1b) (Bjorkman *et al*., 1987; Brown *et al*., 1993; Scott *et al*., 1998). After the MHC class 1 or 2 molecules receive those antigen signals, they become a target for CD8+ (cytotoxic) and CD4+ (helper) T cells (Babbitt *et al*., 1986). T cell

coreceptors CD4 and CD8 are T cell surface glycoproteins binding to the same MHC peptide as the T cell receptors (TCR). As the key components of the acquired immunity, there is little doubt that both B and T cells have an essential role on auto-immunity. However, up to now the underlying mechanisms of B and T cells mediated auto-immunity is not fully understood. Although some autoimmune antibodies were identified and it is clear that they have a pathological role on disease effectors phase (e.g. rheumatoid factors), these antibodies alone could not to explain the autoimmunity development (Sinha *et al*., 1990). Instead, some evidences have been suggesting that the activation of CD4+ T cells induce most immune responses (Sinha *et al*., 1990). And a more recent study has suggested that the prevention of B cell responses by the destruction of T cell regulation toward antigens might develops autoimmune diseases (Foreman *et al*., 2007).

1.1.2.1.3. Correlation between AS and AID

In the Annals P. T. 1977, Fan *et al*. reported a marked decline of T lymphocytes percentage in AS patient's peripheral blood as compared with healthy donor's (Fan *et al*., 1977). It can be supported by the fact that T lymphocytes are negatively associated with C reactive protein (CRP) (Toussirot *et al*., 2000) and the elevation of CRP level is a common feature of AS patient (Mortensen and Gewurz, 1976). However, this is controversial issue because many of AS patients have showed no increase of CRP (Sonkar *et al*., 2008). Most recent research has suggested that bacteria or auto-antigen may act to be a stimulatory factor for Th1 cell, however the relevance of Th1 cell and AS progression is unclear and certain antigen has not yet been detected (Appel *et al*., 2009).

The identification of interleukin-23 receptor (IL-23R), endoplasmic reticulum aminopeptidase 1 (ERAP1) and IL-1 draw attention to T cell concerning on AS development

(Colbert *et al*., 2010). In 2007, WTCCC and TASC confirmed the association with ERAP1 and AS by association scanning (WTCCC and TASC, 2007). In 2009, association study on 992 AS cases from Canada (Maksymowych *et al*., 2009) and family based association testing (Tsui *et al*., 2010) gave further confirmation of the strong association between AS and ERAP1 and between AS and IL-23R as well.

ERAP1 and IL-23R have been known to act as an enhancer of Th17 cells which support Th17 cells survival and also stimulate IL-17 production (Colbert *et al*., 2010). In 2007, Wendling observed significantly increased IL-17 level in the AS patients serum (Wendling *et al.*, 2007). In addition, the increasing number of IL-17 producing Th17 cells in synovial fluid and tissues from spine of AS patients were detected (Appel *et al*., 2011). However, many questions are still unanswered because a closed link between IL-23 and AS has been confirmed in several studies for Spain (Rueda *et al*., 2008), Canada (Rahman *et al*., 2008) and United Kingdom (Karaderi *et al*., 2009) but for some ethnic groups were failed (e.g. Korean (Sung *et al*., 2009)) or not yet been proved (Caucasian) (Appel *et al*., 2011).

B cells contribute to the pathogenesis of autoimmune diseases in a various way such as autoantibody production, cytokine release, and antigen presentation to auto reactive T cells (Baeten *et al*., 2008). Although specific autoantibodies for AS have not yet been identified, mice model studies have suggested that humoral autoimmunity is involved in AS pathology (Shi *et al*., 2003; Zhang *et al*., 1998). Autoimmune arthritis induced mice model study by O'Neill et al (O'Neill *et al*., 2005), recently demonstrated that B cells contribute to the pathology of arthritis both by production of specific antibodies and by presentation of autoantigen and subsequent activation of T cells.

Some studies showed the increase of spontaneous production of immunoglobulin in patient of AS (Wendling *et al*., 1996; Veys and Leare, 1973). B cells hyperactivity and increased proportions of peripheral B cells in AS patient were detected (Byrom *et al*., 1979). And

immunoblast have been reported in AS patients' lymphocytes (Eghtedari *et al*., 1976). However, the notions of B cells involvement in AS is still controversial (Veys and Leare, 1973; Eghtedari et al., 1976; Byrom et al., 1979) and further investigations are needed.

1.1.2.2. Accepted hypothesis for the pathogenesis of Ankylosing Spondylitis 2 – Infectious disease

SpA includes several related disorders: AS, reactive arthritis (ReA), psoriatic arthritis, arthritis related to inflammatory bowel diseases and undifferentiated SpA (Kim *et al*., 2005). Each subgroup of SpA are sharing certain clinical features such as eye involvement and enthesopathy and also having a strong association with HLA-B27 (Kim *et al*., 2005; Dougados and Baeten, 2011).

A possible mechanism in HLA-B27 related autoimmune disease pathogenesis, the molecular mimicry principle has been suggested by López-Larrea *et al* in 1998. They suggested that those antibodies induced by bacterial infection might cross react with HLA-B27 and this reaction could provoke autoimmune diseases (López-Larrea *et al*., 1998). This suggestion is supported by the sequence homology between peptides from HLA-B27 and from a part of peptides of enterobacteria (Scofield *et al*., 1995), Chlamydia (Ramos *et al*., 2002) and cytokeratin (Wildner *et al*., 2002).

Although ReA is well known that triggered by gram-negative bacteria infection such as *Yersinia enterocolitica*, *Salmonella*, *Shigella*, *Campylobacter* or *Chlamydia trachomatis* (Kim *et al*., 2005; López-Larrea *et al*., 1998) and transgenic animal experiments confirmed that the environmental trigger is necessary for the AS provocation (López-Larrea *et al*., 1998), the relationship between antecedent infection and AS remains still unclear (Kim *et al*., 2005).

1.1.2.2.1. Brief introduction of infection and autoimmune disease

Infectious diseases are resulted from the growth and action of viruses, bacteria, and mycoplasma in the body (Cohen and Williamson, 1991). Within the subject, each antibody could recognize only a single type of antigen (Cohen and Williamson, 1991). Therefore, if the level of certain antibodies against specific agent is significantly increased and it is considered that the infection is caused by that agent (Ershler *et al*., 1984). As regard the discrepancy of the autoimmune disease rate between monozygotic twins (in case of major autoimmue diseases, 50 to 70 or even 80% of monozygotic twins are disagreement), there are a number of studies have been devoted to the search for triggering factors and most of the studies have targeted infectious factors (Bach, 2005).

Three sets of representative mechanisms for which explain the relation between infection and autoimmune disease have been proposed (Kim *et al*., 2005). First, polyclonal B or T cell activation was observed in some autoimmune disease especially some forms of systemic lupus erythematosus (Shlomchik *et al*., 1990; Bach *et al*., 1998). The second is antigen molecule mimicry which is derived by sequences homology between bacterial or viral proteins and self protein. The similarities between a numbers of sequences from bacterial or viral proteinsand autoantigen sequences have already been identified (Fujinami and Oldstone, 1989; Oldstone, 1998). Homology was found between streptococcal proteins and heart autoantigens in rheumatic fever patients (Dale and Beachey, 1985). Strong homology between lipo-oligosaccharide and *Campylobacter jejuni* was also found. *Campylobacter jejuni* is a very well known cause of intestinal infections in the Guillaine Barre´ syndrome patients, and sequence homology with lipo-oligosaccharide implies that those infections can be an induction factor of the Guillaine Barre´ syndrome (Neisser *et al*., 2000). The third mechanism is associated with infection-mediated inflammation by which the immunogenicity

of target organ increases against autoantigens (Kim *et al*., 2005). Mice model study using *Coxsackie B3* virus infection showed that the cardiomyositis by *Coxsackie B3* was developed in two phases, first viral and second autoimmune response (Wolfgram *et al*., 1985). However, the cases of human autoimmune disease which are related with infection-mediated inflammation mechanism have not yet been identified (Bach, 2005).

1.1.2.2.2. Examples of infectious arthritis

Yersinia enterocolitica infection is known to be associated with ReA patients from Finland, other Scandinavian countries, Europe and North America (Dumonde, 1986). In the part of Germany and Canada, *Salmonella* and *Shigella* are usually known as a cause of acute enteritis (Marks *et al*., 1980) and it was also reported that *Chlamydia trachomatis* infection lead to ReA in the UK (Keat, 1983). Aho K *et al* demonstrated that 80% of patients with *Yersinia*-related arthritis were positive for HLA-B27 (Aho *et al*., 1981). Therefore, it has been assumed that the molecular mimicry between HLA-B27 and *Yersinia enterocolitica* might play a role on HLA-B27 associated arthritis (Welsh *et al*., 1980).

It has already confirmed that *Chlamydia* organisms are the commonest triggering agents in ReA. In synovial tissues and peripheral blood of ReA patients, *Chlamydia* DNA, mRNA, rRNA, and intact *Chlamydia*-like cells were identified (Gérard *et al*., 1998; Kuipers *et al*., 1998; Schumacher *et al*., 1988).

And it has been reported that the major component of the outer membrane of Gram-negative bacteria, lipopolysaccharide, in synovial tissue can be act as a potential macrophage stimulator and also can induce inflammatory cytokines (Colmegna *et al*., 2004). Macrophage stimulation by lipopolysaccharide seems to be induced by inflammatory erosion in a joint via synovial fibroblast (Kim *et al*., 2005). Recently, Zhang *et al* (2004) observed that the

synovial fibroblast infection by *Salmonella typhimurium* mediates osteoclast differentiation and activation (Zhang *et al*., 2004).

Infectious pathogenesis of AS was originally addressed in support with the similarities of the clinical and genetic aspects between ReA and AS (Sieper *et al*., 2002). The etiology of ReA was established that the gastrointestinal or genitourinary *Shigella flexneri* infection precedes this syndrome (Bauer and Engleman, 1942; Paronen, 1948). In which the pathogenesis of AS, *Klebsiella pneumonia* was suggested by serologic studies. A high prevalence of *Klebsiella pneumonia* and *Klebsiella* antibodies were detected in AS patients (Sieper and Braun, 1995; Benjamin and Parham, 1990; Brown, 1997; Ebringer, 1992) but several attempts have been failed to confirm this hypothesis (Stone *et al*., 2004).

1.1.2.2.3. Correlation between AS and Infectious disease

The idea that bacterial infections are involved in the AS (or SpA) pathogenesis is based on the possibility that certain bacteria activates only the immune system of HLA-B27 positive individuals but not that of HLA-B27 negative individuals (López-Larrea *et al*., 1998) and this might be induced by sequences similarities between self and non-self protein. These sequences homologies so called molecular mimicry has been often detected (Uksila and Toivanen, 1996) and if the sequence homologies (e.g, homology between HLA-B27 and *Klebsiella pneumoniae* nitrogenase (Ewing *et al*., 1990), *Escherichia coli* OmpA protein (Yu *et al*., 1991) or bacterial outer membrane protein YadA (Yersinia adhesin, previously called Yop1) (Lahesmaa *et al*., 1991)) are existed, this can lead B cells or T cells antigenic cross reactivity and subsequently autoimmune diseases are occured (López-Larrea *et al*., 1998). This molecular mimicry theory between host protein and viral protein sequences has been assumed to be one of causes of autoimmune disease and it also offers the most plausible

explanation of a role for HLA-B27 and bacteria in the pathogenesis of AS (Fujinami *et al*., 1983; Fujinami and Oldstone, 1985).

In addition to genetic factors, environmental factors are important because autoimmune disease can be induced from the ectopic expression of MHC class 2 molecules by nonspecific inflammation or the molecular mimicry which is attributed by sequences homology between bacterial or viral proteins and self protein (Davidson and Diamond, 2001). Although a direct relation has not yet been established, HLA-B27 protein sequence shares five or six amino acids with a *Klebsiella pneumoniae* nitrogenase protein (Schwimmbeck *et al*., 1987; Sinha *et al*., 1990) and *Klebsiella pneumonia* infections in AS are specified example of molecular mimicry (Schwimmbeck *et al*., 1987).

1.1.2.3. Correlation between hypothesis 1 and hypothesis 2 on AS pathogenesis

HLA-B27 associated diseases are often regarded as an autoimmune disease (Sieper and Braun, 1995; Benjamin and Parham, 1990; Brown, 1997; Ebringer, 1992). However, even if HLA-B27 gene families are directly related with AS, it alone is insufficient to explain the AS pathogenesis (Calin A, 1988). So it has been suggested that the molecular mimicry between viral protein and HLA-B27 may play an important role on the AS pathology (Ringrose, 1999) and to support this, various theories have been postulated to explain the possible functions of HLA-B27 on the molecular mimicry (Ringrose, 1999).

First theory assumed that HLA-B27 gene has a T cell restricting activity. The presentation of bacterial peptide to B27 or this presentation itself could restrict CD8+ CTL response or induce counteraction to self peptides presented by B27 (Blaauw *et al*., 1990; Scofield *et al*.,1993; Scofield *et al*.,1995). Second theory assumed that HLA-B27 could be a antigenic peptide. HLA-B27 itself acts as a source of antigens and MHC class 2 were presented to

bacterial activated CD4+ T cell and this may produce autoimmune signals (Davenport, 1995; Parham, 1996). Thirds theory considered that HLA-B27 could be either a T cell restricting agent or an antigen. A certain bacteria may induce antibodies and activated antibodies operate against HLA-B27 because of the sequence similarity and this can be a trigger of autoimmune response (Schwimmbeck and Oldstone, 1987). However, there is still considerable ambiguity with regard to the role of HLA-B27 on autoimmune disease as well as AS pathogenesis.

1.2. Diagnosis of AS

The delays in diagnosis of the AS in HLA-B27+ patients and HLA-B27- patients are respectively estimated as 8.5 and 11.4 (Feldtkeller *et al*., 2003). The onset age of AS is 24.8 years in B27+ patients and 27.7 years in B27- patients however AS has been usually diagnosed at the average age of 33.2 years and 39.1 years respectively (Feldtkeller *et al*., 2003; Guillemin *et al*, 1990). For the females patients, the average onset age of AS is 24.2 years, and it is slightly earlier than in the male patients (25.7 years) (Khan, 2002). The delay in diagnosis of the disease is 11.4 years for HLA-B27- population and 8.5 years for HLA-B27+ population, respectively (Feldtkeller *et al*., 2003).

There are a number of possible reasons for the long delay in a diagnosis of AS. The main reason is the lack of specificity of the most common represents symptom, low-back-pain (Rostom *et al*., 2010). In addition, the radiographic changes which are mainly using for diagnosis are developed very lately in the course of AS (Rostom *et al*., 2010). However, the most of the existing criteria for diagnosis of AS apply this lately detected radiographic changes as a critical criterion. Therefore, using these criteria to the diagnosis of AS is inappropriate at an early stage (Khan, 2002; O'Shea *et al*., 2007). Furthermore, most of using criteria for diagnosis were firstly set up for the purpose of the classification so that the proper

diagnosis cannot be ensured (Dougados and Gossec, 2007).

1.2.1. Current criteria

1.2.1.1. Traditional criteria - Amor's criteria and ESSG criteria

Two representative criteria have been used to classify SpA, Amor's criteria (Amor *et al*., 1990) and European Spondylarthropathy Study Group (ESSG) criteria (Dougados *et al*., 1991). Amor's criteria listed clinical symptoms, radiographic findings, genetic background and response to treatment. Each signs has the contribute point (1 or 2) and a patient who scored 6 or more is classified as SpA patient (Rostom *et al*., 2010; Amor *et al*., 1990). In the case of ESSG criteria, a patient who have at least one major and one minor criterion is classified as having SpA on the premise that he/she has a inflammatory low back pain and/or peripheral arthritis (Rostom *et al*., 2010; Dougados *et al*., 1991). Modified New York criteria can be also used to classification of SpA (Van der Linden *et al*., 1984; Rostom *et al*., 2010). This criteria classified patients on the basis of sacroiliac joint involvement and if a patient is radiological detected bilateral sacroiliitis grade 2 or higher or unilateral sacroiliitis grade 3 or 4 is classified as having SpA (Van der Linden S *et al*., 1984) (Table 1).

Table 1. Classification and diagnosis criteria for SpA

Criteria		Ref
Amor's criteria **Clinical symptoms** Lumbar or dorsal pain at night or morning stiffness in the lumbar and dorsal region (1) Asymmetric oligoarthritis (2) Buttock pain, unspecified (1) Alternating buttock pain (2) Sausage digit (2) Heel pain or other enthesopathy (2)	The total number of points is 6 or more than the patient as having SpA	Amor(1990)

Iritis (2) Nongonococcal unethritis or cervicitis within 1 month before the onset of arthritis (1) Acute diarrhea within 1 month before the onset of arthritis (1) Past or current psoriasis and/or balanitis (2) **Radipgraphic findings** Sacroiliitis (bilateral grade 2 or unilateral grade 3) (3) **Genetic background** Presence of HLA-B27 and/or family history of SpA/AS/IBD (2) **Response to treatment** Clear cut improvement within 48h after NSAID intake or rapid lapse of the pain (within 48h) after NSAID discontinuation (2)		
ESSG **Major criteria** Inflammatory back pain Synovitis that is asymmetric or that predominates in the lower limbs **Minor criteria** Family history of SpA, Psoriasis Urethritis, cervicitis or acute diarrhea within 1 month before arthritis oneset Chronic inflammatory bowel disease Alternating buttock pain Enthesopathy Radiographic sacroiliitis, bilateral grade ≥2 or unilateral grade ≥3	Patient who have at least one major and one minor criterion is considered to SpA patient	Dougados(1991)
New York modified criteria **Clinical criteria** Low back pain: present from for more than 3 months, improved by exercise and not relieved by rest Limitation of lumba spine motion in the sagittal and frontal planes Limitation of chest expansion relative to normal values for age and sex **Radiographic criteria** Bilateral sacroiliitis grade ≥2 or unilateral sacroiliitis grade 3 or 4	Definite AS: radiographic criterion plus at least one clinical criterion Probable AS: all three clinical criteria or only the radiographic criterion	Linden(1984)

*Brackets refer to points

1.2.1.2. Recently developed criteria and other diagnosis methods

In 2009, Assessment of SpondyloArthritis international Society (ASAS) criteria were developed and it does not essentially demand the presence of radiographic sacroiliitis (Rudwaleit *et al*., 2009). According to the ASAS criteria in 2009, patient has a chronic (more than three months) back pain and age at onset less than 45 years can be classified as an axial SpA either in the presence of sacroiliitis (both definite radiographic sacroiliitis and active inflammation of sacroiliac joints on MRI screening) or without radiographic detection but in

the presence of HLA-B27 plus at least two other SpA features (Rudwaleit *et al*., 2009). The features of SpA include i) Clinical symptoms: Inflammatory back pain, uveitis, psoriasis, arthritis, enthesitis, dactylitis etc, ii) Genetic background: HLA-B27 and family history, iii) Response to treatment: NSAIDs and iv) Elevated CRP (Rudwaleit *et al*., 2009). In the diagnosis of AS, sacroiliac joints and the spine are most important sites (Van der Heijde *et al*., 2007).

To identify sacroiliitis (sacroiliac joint involvement), radiographic imaging is inevitable (O'Shea *et al*., 2007). Current available imaging methods to identify sacroiliitis are plain radiography, quantitative SI scintigraphy, computed tomography (CT), and magnetic resonance imaging (MRI) (O'Shea *et al*., 2007; Inanc *et al*., 2005). However, those modalities have also difficulties in diagnosis. By using plain radiography, the anatomy of SI joints is too complicate to imaging sacroiliitis (Inanc et al., 2005). Quantitative SI scintigraphy is quite sensitive but it has low specificity due to the bone turnover (Braun *et al*., 1998; Braun *et al*., 2000; Khan, 2002). CT could be useful for the bony change detection but MRI is superior to CT to identify abnormalities caused by inflammation (O'Shea *et al*., 2007; Braun *et al*., 1998; Braun *et al*., 2000; Khan, 2002).

The use of magnetic MRI is the most effective way on the basis of the combination with different criteria, symptoms and laboratory test results for example erythrocyte sedimentation rate (ESR) or CRP level (Rudwaleit *et al*., 2004; Rindfleisch and Muller, 2005). There have been used 3 available scoring for the MRI based assessment, which are the ASspiMRI-a, the Berlin method and SPARCC (the Spondyloarthritis Research Consortium of Canada Magnetic Resonance Imaging Index for Assessment of Spinal Inflammation in AS), however it has not yet been selected one preferred method and more studies are needed (Van der Heijde *et al*., 2007). In addition, MRI is often limited to patients with metal implants, with pacemarker or with claustrophodia and it is also time consuming and high priced technique

(O'Shea *et al*., 2007).

1.2.2. Potential DNA markers for AS diagnosis

1.2.2.1. Reported DNA markers for AS diagnosis

Several studies have reported that the total immunoglobulin level was elevated in AS patients (Cowling *et al*., 1980; Franssen *et al*., 1985; Collado *et al*., 1987; Sanders *et al*., 1987; Hocini *et al*., 1992). The changes in various inflammatory cytokine levels are also detected. Cytokine profiling study in AS patient serum showed that IL-17, TNF-α, IL-12p40 and IL-6 were significantly over expressed than controls and IL-1β, IL-4, IL-15 and IFN-γ were increased, which corresponds with the inflammatory symptom severity (Hueber *et al*., 2007). In 2007, ERAP1 and IL-23R were identified as an excellent biological candidate by WTCCC/TASC genome-wide scanning (WTCCC and TASC, 2007). ERAP1 is involved in MHC Class I presentation by its peptides trimming function and it induces the down-regulation of inflammatory cytokines such as IL-1 (Cui *et al*., 2003), IL-6 (Cui *et al*., 2003) and TNF receptor 1 (TNFR1) (Cui *et al*., 2002).

The association of IL-23R with AS has been supported by recent evidence of the Th17 cell involvement in AS (Thomas and Brown, 2010). Additionally, it has been reported that IL-23R also engaged in not only AS (Cui *et al*., 2002) but also the other disease which are frequently occur with AS patient or in their family for example inflammatory bowl disease (IBD) (Rahman *et al*., 2008; Dubinsky *et al*., 2007), psoriasis (Cargill *et al*., 2007) and psoriatic arthritis (Rahman, 2011). Following complete genome wide study by TASC confirmed the association between AS and SNPs in IL-23R at chromosome 1p23 and ERAP1 at chromosome 5p15 (Reveille *et al*., 2010). In 2008, Sims et al conducted a meta-analysis in

2675 AS cases and the result revealed that IL-1α implicated in susceptibility to AS (Sims *et al*., 2008) and the most recent study confirmed the association between IL-1α and AS (Kim *et al*., 2008).

There are several reports about the possible involvement of other genes to AS susceptibility and disease development (López-Larrea *et al*., 1998). Among the MHC genes, the large multifunctional protease subunits of the proteasome (LMP2 and LMP7) and the transporters associated with antigen processing (TAP1 and TAP2) were suggested. Through the genome wide screen in 1998, the putative non-MHC gene clusters which located on 16q snd 4q chromosomes were mentioned (López-Larrea *et al*., 1998).

1.2.2.2. Limitation of HLA B27 and the necessity of DNA markers

About 4 decades have passed since Caffery and James (1973) reported the linkage between HLA-B27 and AS, which is generally known now. A great deal of studies has been devoted to show their explicit interaction, but it has not been determined yet how HLA-B27 contributes to the pathogenesis of AS (Reveille *et al*., 2009). Up to now, 69 kinds of HLA-B27 alleles have been found (Reveille and Maganti, 2009). Recent studies suggest some specific alleles seem to be more closely related in particular population in AS susceptibility (B*2702 in Cocasians and Turkei; B*2704 in East Asians; B*2705 in East Asia and Cocasians; B*2707 and B*2714 in central and western Asians etc (Kamanli *et al*., 2009; Ben Radhia *et al*., 2008). However, there exist a contradiction in this issue because most recent studies also demonstrate many other possible candidates such as HLA-B14*03 or HLA-B*5703 (López-Larrea *et al*., 2002; Díaz-Peña *et al*., 2008).

So far, four outstanding theories to explain AS and HLA-B27 association have been presented (Reveille *et al*., 2009). The first theory is related to the arthritogenic peptides

(Ramos and Lopez, 2002). It assumes that HLA-B27 bind to the unique peptide which had been provided by triggers such as microorganisms or self antigenic proteins (Ramos and Lopez, 2002). Over ten years, many studies were keen on searching these peptides but to date no such peptide has been detected (Reveille *et al*., 2009).

The second and third theory is based on HLA-B27 homodimer formation by the unique cystein 67 residue in extracellular α1 domain (El-Zaatari *et al*., 1990). BiP (the major chaperone protein) kept HLA-B27 in endoplasmic reticulum and the heavy chains of HLA-B27 were misfolded and this may derives to produce of type-1-IL and also may induce proinflammatory unfolded/misfolded protein responses (Reveille, 2011). In addition, if the HLA-B27 homodimer is expressed on the surface of the cell, it could have functioned as ligands for natural killer cells or related cell surface receptors. But these do not seem to be adequate because the most of HLA-B27 positive individuals are not suffering from AS and SpA (Reveille *et al*., 2009).

The fourth theory based on the idea that HLA-B27 positive individuals have deficient intracellular invasion or microorganism killing ability (Reveille, 2011). It demonstrates that HLA-B27 positive individuals have less efficiency for handling some bacterial infection such as *Salmonella, Shigella,* and *Clamydia* etc, which have been suspected to be involved in AS pathology. Although, this can be important to suggest the environmental triggering possibility on AS but it is not enough to describe the pathogenesis of AS (Reveille, 2011).

1.3. Therapeutic against AS

1.3.1. Current protocol – conventional therapy

Historically, pharmacotherapy for SpA has been relatively limited and it hasmainly targeted

toward symptom control (Ritchlin and Daikh, 2001). Although, recent decade several therapeutics are trial to improve AS (Sieper *et al*., 2009), continuous versus treatment of non-steroidal anti-inflammatory drug (NSAIDs) is still the only way to reduce radiographic progression in patients with AS (Wanders *et al*., 2005). However, evidence for a positive effect of NSAIDs on radiographic progression is controversial and long term treatments of NSAIDs are seemed to be danger because it could increase risk of the NSAIDs adverse effects such as gastrointestinal effect (Miceli-Richard and Dougados, 2007).

Currently, the treatment of AS has been supported by some kinds of TNF-α antagonist (McLeod *et al*., 2007; Zochling *et al*., 2006). Through an immunohistochemical study of inflammatory lesion of AS patient, TNF-α was identified as an essential cytokines to mediate inflammation (Zochling *et al*., 2006), and it draw the TNF-α antagonist trial in AS patients (Braun and Sieper, 2007; Brandt *et al*., 2000). At present, 5 different TNF-α antagonists are using for AS, which are Etanercept, Infliximab, Adalimumab and Golimumab (McLeod *et al*., 2007; Zochling *et al*., 2006). Recently, several researches have already been going ahead to set proper criteria for their usage (McLeod *et al*., 2007; Zochling *et al*., 2006).

Clinical studies have demonstrated that adalimumab, infliximab and etanercept had showed therapeutic efficacy (Schett *et al*., 2011). However discontinuing the medications induced a relapse of AS (Baraliakos *et al*., 2005; Brandt *et al*., 2005). Moreover, although there are strong evidences that TNF-α antagonists have anti-inflammatory effect on AS, there seem to be ineffective for new bone formation of AS (Van der Heijde *et al*., 2008; Van der Heijde *et al*., 2009; Lories *et al*., 2007). New bone formation induces bony spur formation and it is a typical characteristic of AS, which can be distinguished from other similar diseases such as RA and PsA (Schett *et al*., 2011).

1.3.2. Why early diagnostics is important for the therapy of AS?

With the introduction of TNF-α inhibitor therapy for AS treatment, correct and effective early diagnostics development has becomes more important (Braun *et al*., 2002; Baraliakos *et al*., 2007; Son and Cha, 2010). Recent studies on TNF-α inhibitors in AS patients have found strong evidence that if it is treated before the radiographic sacroiliitis emerge, TNF-α inhibitors not only can control the inflammation but also can restore inflammatory lesions (Braun *et al*., 2002; Baraliakos *et al*., 2007; Son and Cha, 2010).

1.4. Immunoglobulin gene and chromosomal abnormality

1.4.1. Brief introduction of the immunoglobulin heavy chain locus

Antibodies, also known as immunoglobulins (Ig) are produced by B cells to detect and neutralize foreign substances called antigens. Immunoglobulins are Y-shaped proteins and each Ig molecule is composed of two heavy chains and two light chains connected by disulfide bonds. The Ig heavy chain (IgH) locus consists of approximately 40 functional variable region segments (V_H1-40), 25 diversity segments (D_H1-25), 6 joining segments (J_H1-6) and a large cluster of constant (C) genes including Cμ, Cδ, Cγ, Cα and Cε. During the process of antibody production, V, D and J genes are rearranged to produce variable region (V) exons. (Snapper and Finkelman FD, 1999).

IgVH regions are inherited as sets of gene segments, each encoding a part of the V region of one of the immunoglobulin polypeptide chains. Unique combinations of V_H, D_H and J_H segments become joined by somatic gene rearrangement to produce various VH segment pairs providing a unique receptor for each lymphocyte. To complete Ig generation, VH segments have to join with the constant mu region gene (Cμ) first. Through class switch

recombination, Cμ is replaced by other types of C region genes, Cδ, Cγ, Cα and Cε, which allows the formation of Ig isotypes ranging from IgM to IgD, IgG IgA and IgE (Snapper and Finkelman FD, 1999).

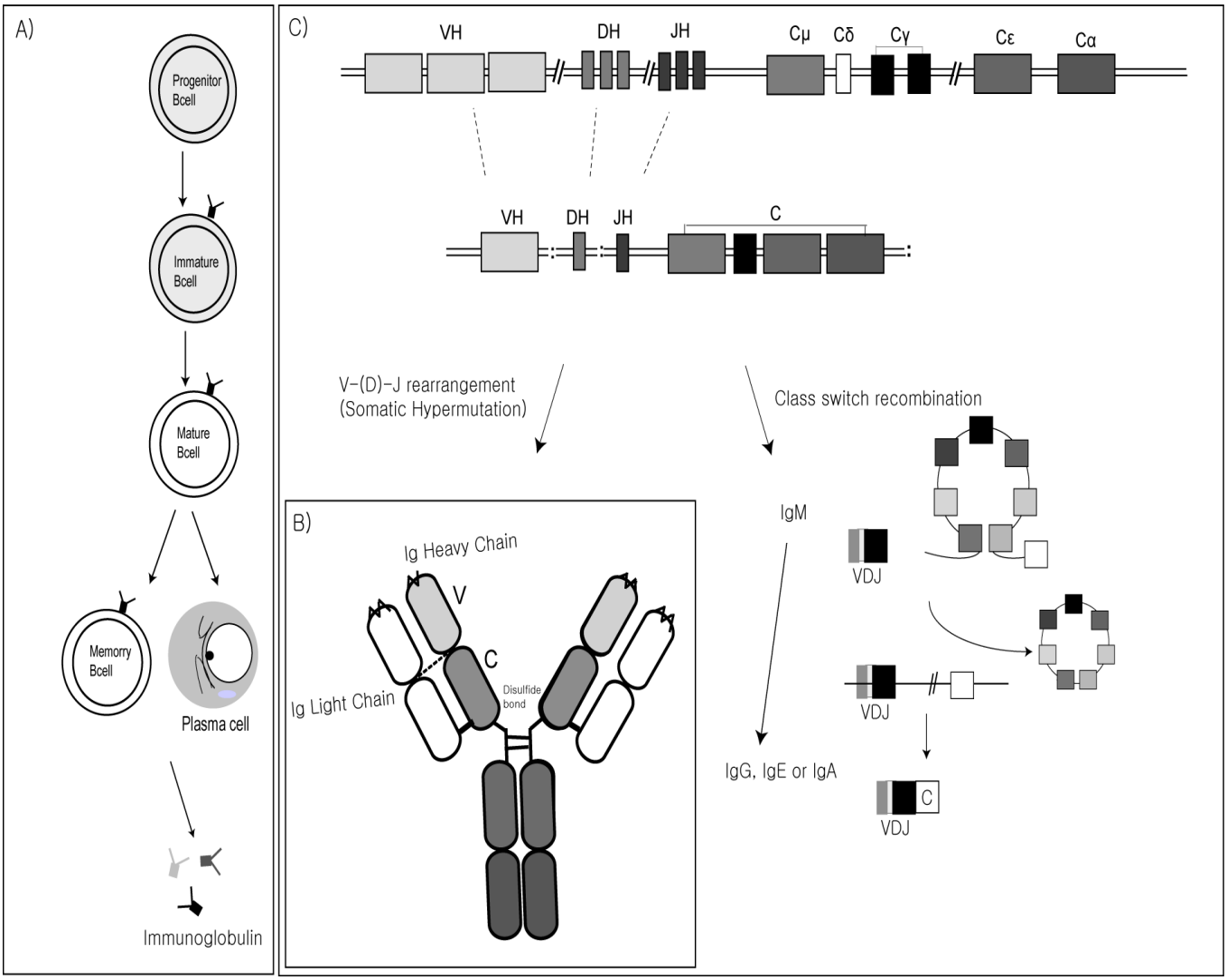


Figure 2. Stages of B cell maturation and structure of an immunoglobulin molecule. A) B cell lineages in B cell development and maturation process. B) Immunoglobulins are Y-shaped proteins. Each Ig molecules have two heavy chains and two light chains connected by disulfide bonds (V: variable region, C: constant region), C) Immunoglobulin variable region (V) exons are produced by V-(D)-J rearrangement. Subsequently, the VH segments join with the constant (C) region genes. The different Ig isotypes are generated through class switch recombination. (IgM to IgG, IgA and IgE)

According to Lossos (2000), the process of B cell development and maturation is divided into four stages. First, before the onset of V-(D)-J recombination, B cells are in the progenitor

stage. The second stage is the generation of naive B cells which have not encountered antigen, following V-(D)-J recombination. Usually in this stage, B cells are considered to be immatur because V exons produced through V-(D)-J recombination are not yet mutated. After somatic mutation of V region genes, mature B cells are generated.

The third stage takes place in the germinal center microenvironment and in this stage mature B cells and memory B cells are produced. In this stage, B cells undergo somatic mutation, clonal expansion, antigen driven selection, and class switch recombination. Through the class switch recombination process B cells obtain functional diversity of Ig by replacing Cμ with other types of C region genes, Cδ, Cγ, Cα and Cε. At the fourth stage of B cell maturation, B cells become either memory B cells or plasma cells which produce high affinity antibodies (Tsai and Lieber, 2010; Swanson, 2004; Ganesh and Neuberger, 2011; Lossos *et al*., 2000).

1.4.1.1. Germ line gene structure

Human variable heavy chain region genes can be divided into seven families, VH1-VH7 (Cook and Tomlinson, 1995). These families contain 60 to 70 VH gene segments and and all of these genes are likely to attend to the immunoglobulin rearrangement (Stewart and Schwartz, 1994; Matsuda *et al*., 1993; Cook *et al*., 1994). Each of the VH gene family, VH1-VH7, is comprised of different subfamilies and nucleotide sequence homology between them is known as at least 80% (Kodaira *et al*., 1986; Lee *et al*., 1987; Shen *et al*., 1987; Berman *et al*., 1988; Willems *et al*., 1993; Tomlinson *et al*., 1992).

During the process of B cell development, the combinational rearrangement of three distinct groups of functional gene segments, variable (VH), diversity (DH) and joining (JH) region genes (Early *et al*., 1980; Reth *et al*., 1986), forms the heavy chain variable region gene of human immunoglobulin molecule (Tonegawa, 1988). Among the 60 to 70 functional VH

gene segments, the majority of these functional gene segments are located on chromosome 14q32.3 (Cox *et al*., 1982; McBride *et al*., 1982). The Ig heavy chain gene locus on 14q32.3 has 46 to 52 VH segments (includes at least 46 functional VH) (Matsuda *et al*., 1993; Cook *et al*., 1994; Gallardo *et al*., 2008), 30 DH gene segments, and 6 JH gene segments (Kirsch *et al*., 1982; Berman *et al*., 1988; Siebenlist *et al*., 1981; Ichihara *et al*., 1988; Ravetch *et al*., 1981; Buluwela *et al*., 1988). Previously, some of the sequences of VH and DH genes were found on chromosome 15 (15q11.2) and 16 (16p11.2) (Matsuda *et al*., 1990; Cherif and Berger, 1990; Chung *et al*., 1984), but the number of segments and their sequences have not yet been determined (Tomlinson *et al*., 1994).

1.4.1.2. V-(D)-J recombination

Developing B cells and rearrangement of VH genes are dependent on expressing RAGs (recombination activating genes; RAG1 and RAG2) (Chun *et al*., 1991; Mombaerts *et al*., 1992; Early *et al*., 1980; Reth *et al*., 1986). RAGs expression is prominent in early stage B cells and it is gradually restricted or absent in matured B cells (Bories *et al*., 1991; Ma *et al*., 1992; Li *et al*., 1993; Stiemholm and Berinstein, 1993). In later stages of B cell maturation, the expression of RAGs genes gradually decreases while Ig rearrangement is accomplished, (Stewart and Schwartz, 1994).

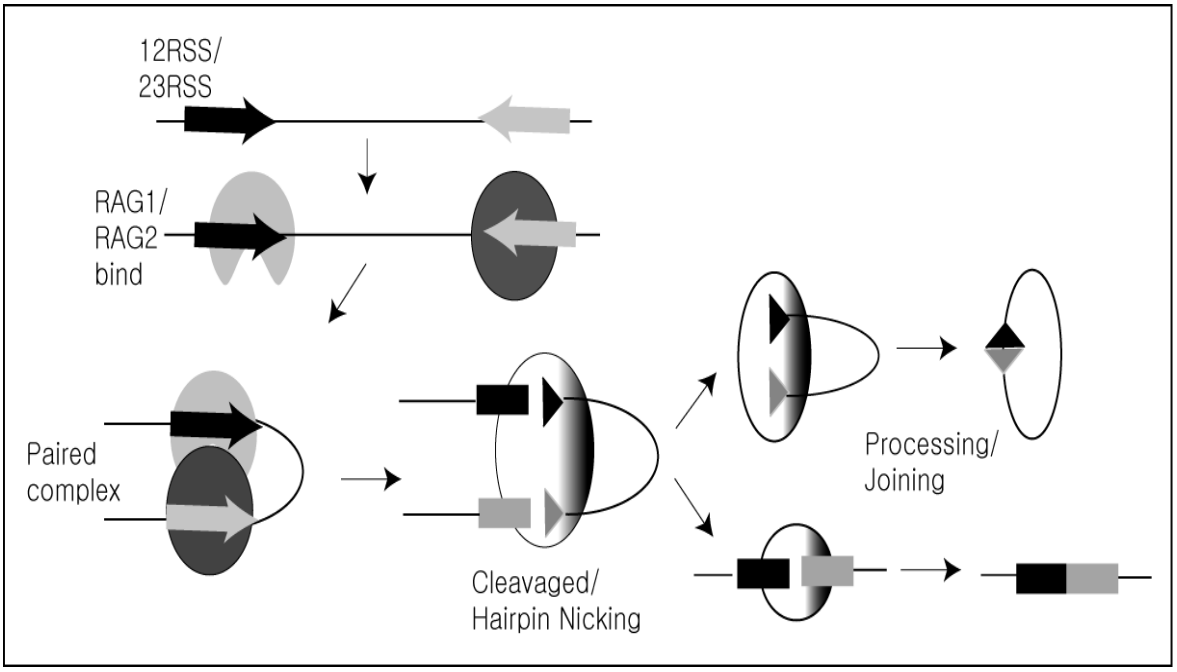


Figure 3. RAG mediated V(D)J recombination mechanism. V(D)J recombination is initiated by RAGs, and RRS is conservative heptamer or nonamer sequences and it guides recombination mechanism (RAGs: recombination activating genes, RSS: recombination signal sequence)

V-(D)-J recombination is initiated by RAGs (Oettinger *et al*., 1990; Schatz *et al*., 1989) by introducing double strand DNA breaks between variable heavy chain gene encoding regions and recombination signal sequence (RSS) (Fugmann *et al*., 2000; Jung and Alt, 2004). (Figure 3)

RRS is a conservative hepta or nonamer sequence which guides the V-(D)-J recombination mechanism (Mombaerts *et al*., 1992). After the rearrangement of one DH and one JH gene, one of the VH genes is fused and V-(D)-J segments are constructed (Yancopoulos and Alt, 1986; Altet *et al*., 1984). During the RAG-mediated V-(D)-J recombination process, one V, D and J are selected and assembled into variable domain by deleting the intervening segments, B lymphocytes use the variable domainof Ig to recognize antigens (Bassing *et al*., 2002; Jung *et al*., 2006) and bind to antigens (Bassing *et al*., 2002).

The V-(D)-J recombination process is fundamentally important, because it ensures antibody diversity. (Bassing *et al*., 2002).

The recombination process is governed by the diversified ways at the immunoglobuline heavy chain locus (Jung *et al*., 2006). A defect recombination process can cause an imbalance between immature and mature B cell subpopulations. Imbalance of B cell subpopulations has been detected in various immune responses and diseases. Moreover, it has been suggested that immunedeficiencies, autoimmune disease and cancer might be affected by defective processing of V-(D)-J recombination (Bassing *et al*., 2002).

1.4.1.3. Class switch recombination (CSR) mechanism

The V-(D)-J recombination process occurs site specifically in immunoglobulin and TCR (T cell receptor) gene segments during the lymphocyte maturation process from respective conserved recombination signal sequences (Bassing *et al*., 2002). After completion of the V-(D)-J recombination process, immunoglobulin gene segments undergo further matured and diversified processes called class switch recombination, somatic hypermutation and gene conversion (Tsai and Lieber, 2010; Swanson, 2004; Ganesh and Neuberger, 2011). (Figure 4)
In vivo, class switch recombinations are induced by antigen driven B cell activation, and through this process IgG, IgA and IgE are produced from IgM and IgD (Dudley *et al*., 2005) by the change in the constant portion of the Ig heavy chain. The CSR process results in high-affinity binding antibodies and allows the antibodies to to penetrate extravascular parts of the body such as the respiratory system and the gastrointestinal tract (Tsai and Lieber, 2010).

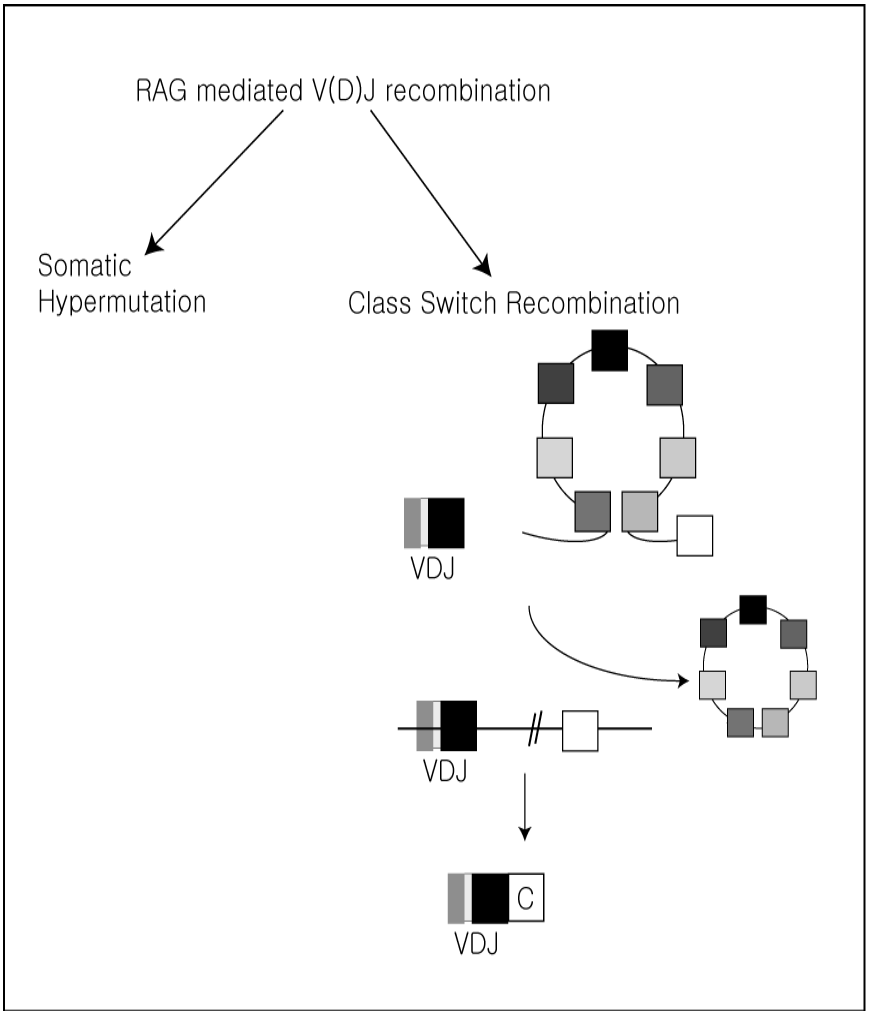


Figure 4. Further maturing and diversifying process of Ig. After the RAG-mediated V(D)J recombination process is completed, Ig gene segments undergo further maturation and diversified processes. In vivo, class switch recombinations induce B lymphocyte activation, producing IgG, IgA, and IgE from IgM and IgD.

A deficiency in CSR can cause humoral immune deficiency, which may result in an increased susceptibility for bacterial infections (Swanson, 2004). Intrinsic defects of B cells (Catalan *et al*., 2003) and a defect in the CD40 signaling pathway (Korthäuer et al., 1993; Durandy *et al*., 1993; Agematsu *et al*., 1998) can generate CSR deficiency

In this regard, it has been assumed that 20% of patients suffering from auto-immune disease might be affected by CSR deficiency (Quartier *et al*., 2004). However, this assumption is still controversal and even the regulation of class switch recombinations has not yet been established (Swanson, 2004; Quartier *et al*., 2004).

1.4.1.3.1. CD40L – CD40 interaction

CD40 ligand (CD40L) is a ligand of CD40 and both of them belong to TNF alpha superfamily (Bishop *et al*., 2007). CD40 is expressed on antigen presenting cells, including B cells. Upon stimulation via CD40, CD40L is temporarily expressed on the surface of activated CD4+ T cells (Gauchat *et al*., 1993; Schönbeck *et al*., 2000). The CD40L is necessary for B cell activation, B cell differentiation, class switch recombination as well as T cell costimulatory molecule expression (Howard and Miller, 2004). In 1993, Nonoyama *et al* reported that class switch recombination is initiated by CD40L which is involved in B and T cells interaction (Nonoyama *et al*., 1993).

The interaction between CD40L and CD40 is required for the the activation of B-cells by T cell dependent antigen (Manis *et al*., 2002; Stavnezer, 2000). In 2009, Hassen *et al* suggested that the aberrant interaction between CD40L and CD40 could initiate inflammatory and non-inflammatory diseases (Hassan *et al*., 2009). Recent studies have demonstrated that CD40L was over-expressed on various cell types under autoimmune disease conditions (Grammer *et al*., 2003; Katsiari *et al*., 2002) and the expression level of CD40L is also significantly increased in patient with RA (MacDonald *et al*., 1997), MS (Gerritse *et al*., 1996), autoimmune thyroiditis (Faure *et al*., 1997), IBD (Liu *et al*., 1999) and ulcerative colitis (Liu *et al*., 1999).

CD40L and CD40 interaction is known to be related with several immunological processes such as B cell proliferation, immunoglobulin production, class switch recombination, germinal center formation, memory B cell generation (Foy *et al*., 1996) and T cell dependent humoral immune responses (Yazdany and Davis, 2004). IgE secretion by isotype switching was increased when CD40 was immobilized by CD40L (Ballantyne *et al*., 1998). Successive signals from CD40L and CD40 interaction completes IgE secretion by B cells, and a recently

accepted model describes that CD40 is involved in V-(D)-J fragment and C epsilon gene recombination (Pate *et al*., 2010). Most recent research suggests that CD40L and CD40 interaction could induce the differentiation of T helper type 17 (Th17) cells (Iezzi *et al*., 2009).

As a consequence of deficiency of interaction between CD40L and CD40, numerous abnormal cellular immune responses are expected. If the CD40L and CD40 interaction occurs on the surface of dendritic cells, Th1 cytokines and IL-12 were over-synthesized (Koch *et al*., 1996). If the interaction is impaired, dendritic cells are not able to mature properly. Successively dendritic cells produce IL-12 and finally macrophages are damaged (Lougaris *et al*., 2005). This can also affect T cell maturation and provoke abnormal immune responses (Lougaris *et al*., 2005).

1.4.1.4. Analysis of immunoglobulin repertories usage

The main purpose of the immunoglobulin usage analysis in the autoimmune disease is to clarify whether specified gene families or genes involved in basic antibody production are diverged in patients as compared to healthy controls (Foreman *et al*., 2007). Several researches have investigated the variable heavy chain gene (hereafter VH gene) usage in various autoimmune disease patients (Roben *et al*., 1996; Stevenson *et al*., 1993; Sims *et al*., 2001; Sblattero *et al*., 2000; Ermel *et al*., 1997; Robbins *et al*., 1990; Dörner *et al*., 2002; Voswinkel *et al*., 2001). Phage display libraries constructed from B cells and the B cell hybridomas using technique have been applied for the analysis of VH gene usage (Foreman *et al*., 2007). Nevertheless, none of the differences in immunoglobuline gene usage can be regarded as a main factor of pathogenesis of autoimmune disease (Foreman *et al*., 2007). (Table 2)

Table 2. Immunoglobulin variable gene usage skewed in particular autoimmune diseases.

Autoimmune disease	Methods	Sampling condition	VH skewed	Related isotype	Ref.
SLE	Phage display library		VH5 overexpressed	IgG	Roben(1996)
	Hybridoma	Splenocyte, blood follicular cell	VH4-21	IgM, IgG	Stevenson(1993)
MG		Thymus tissue	VH3	N/A	Sims(2001)
Celiac disease	Phage display library	Peripheral blood, lymphocyte	VH4	IgA	Sblattero(2000)
RA	Human/Mouse heterohybridoma	Rheumatoid synovial tissue	VH4	IgM	Ermel(1997)
	Hybridoma	Rheumatoid synovial tissue	VH1 (9&16 AA long in CDR3)	IgM	Robbins(1990)
Sjogren's syndrome		Glandular rearrangements	VH CDR3 shortest		Dörner(2002)
Ankylosing Spondylitis	DNA/ PCR results	Rearranged synovial membrane	VH5 overexpressed, VH4 underexpressed		Voswinkel(2001)

1.4.2. Brief introduction of chromosomal abnormality

1.4.2.1. Types of chromosomal abnormality

Four types of chromosomal aberrations, i) duplications, ii) deletions, iii) inversions and iv) translocations, are well known which could induce chromosomal abnormalities. All of these four kinds of unbalanced structural autosomal aberrations can cause hereditary diseases, including severe cases of mental retardation (Rehder and Fritz, 2005).

1.4.2.2. Chromosome 14 abnormality and related disease

More than 500 genes were identified on Chromosome 14. It contains the T cell receptor

cluster at 14q11.2 and the immunoglobulin heavy chain gene cluster at 14q32.3 (López, 1998). Impaired rearrangement processes in the immunoglobulin heavy chain gene cluster at 14q32 3 are often thought to be associated with B cell lymphomas (López, 1998). Up to now, more than 30 genes were confirmed to be associated with 21 sorts of specific diseases. In addition, the association between these 30 kinds of genes and 13 sorts disease cases are confirming now, and more than 20 loci were proved to be related with tumor (López, 1998) (Table 3).

Table 3. Genes on chromosome14 and associated diseases

Map location	Gene	Disease	OMIM number
14q11.1-q11.2	NRL (Neural retina leucine zipper)	Retinitis pigmentosa	162080
14q11.2	SLC7A7 (Solute carrier family 7, member 7)	Lysinuric protein intolerance	603593
14q11.2	TGM1 (Transglutamase-1 type I)	Congenital ichthyosiform erythroderma	190195
14q11.2-q12	unidentified (possible)	Distal myopathy 1	160500
14q11.2-q13	PAB2 (Poly(A) binding protein, nuclear 1)	Oculopharyngeal muscular dystrophy	602279
14q11.2-q24.3	unidentified (possible)	Spastic paraplegia type 3A	182600
14q12	MYH7 (Myosin, heavy polypeptide 7)	Familial hypertrophic cardiomyopathy	160760
14q12	unidentified (possible)	Deafness, autosomal recessive 5	600792
14q12-q13	COCH (Cochlin)	Deafness, autosomal dominant 9	603196
14q12-q22	unidentified (possible)	Arrhythmogenic right ventricular dysplasia 3	602086
14q13	PAX9 (Paired box homeobox gene 9)	Autosomal dominant oligodontia	167416
14q13	unidentified (possible)	Idiopathic basal ganglia calcification	213600
14q13.1	NP (Nucleoside phosphorylase)	Nucleoside phosphorylase deficiency	164050
14q13-q21	unidentified (possible)	Hereditary benign chorea	118700
14q21	MGAT2 (Mannosyl(alpha 1,6) glycoprotein beta 1,2-Nacetylglucosaminyl-transferase)	Carbohydrate deficient glycoprotein syndrome type II	602616
14q21-q22	PYGL (Glycogen phosphorylase, liver)	Glycogen storage disease VI	232700
14q21-q22	unidentified (possible)	Deafness, autosomal dominant, non-syndromic, sensorineural 23	605192
14q22-q23.2	SPTB(Spectrin, beta, erythrocytic)	Elliptocytosis	182870
14q22.1-q22.2	GCH1 (GTP cyclohydrolase 1)	Dystonia DOPA responsive	600225
14q23	GPH (Gephyrin)	Molybdenum cofactor deficiency type C	603930
14q23-q24	unidentified (possible)	Arrhythmogenic right ventricular dysplasia 1	107970
14q24	unidentified (possible)	Leber congenital amaurosis type III	604232
14q24.3	CHX10 (C elegans ceh-10 homeo domain containing homologue)	Micropthalmia, cataracts, and iris abnormalities	142993
14q24.3	PSEN1 (Presenilin 1)	Alzheimer disease3	104311

14q24.3	ALDH6A1 (Aldehyde dehydrogenase 6 family, member A1)	Methylmalonate semialdehyde dehydrogenase deficiency	603178
14q24.3-q31	SCA3 (Spinocerebellar ataxia 3)	Machado-Joseph disease	109150
14q24.3-q31	unidentified (possible)	Insulin dependent diabetes mellitus	601208
14q31	GALC (Galactosylceraminidase)	Krabbe disease	245200
14q31	TSHR (Thyroid stimulating hormone receptor)	Graves disease	603372
14q32	unidentified (possible)	Autosomal recessive microphthalmia	251600
14q32	unidentified (possible)	Usher syndrome type 1A	276900
14q32.1	AACT (Alpha-1-antichymotrypsin)	Alpha-1-antichymotrypsin deficiency	107280
14q3	unidentified (possible)	Multinodular goitre 1	138800

Catalogues of genes and/or diseases of human chromosome 14 are available at Online Mendelian Inheritance in Man (www.ncbi.nlm.nih.gov/Locuslink), Gene Cards (bioinformatics.weizmann.ac.il/cards) and Genome Database (gdbwww.gdb.org).

1.4.2.3. Paracentric inversion

1.4.2.3.1. The mechanism of paracentric inversion

Chromosomal inversions are divided into two major categorie, pericentric inversion and paracentric inversions according to the relative position of the two breakpoints and the centromere (Thomas *et al*., 2008). A pericentric inversion has one breakpoint in each chromosome and the inverted segment includes the centromere (Thomas *et al*., 2008). In case of a paracentric inversion, both breakpoints located in the same chromosome arm and the inverted segment in one chromosome arm (Madan, 1995). With the exception of some cases of one or more breakpoints disarranged specific genes (Beiraghi *et al*., 2003; Iida *et al*., 2000; Saito-Ohara *et al*., 2002; Sood *et al*., 2004; Tadin-Strapps *et al*., 2004), inversions are less likely to generate phenotypic abnormality (Thomas *et al*., 2008) and inversion has been assumed one of the most usual forms of chromosome polymorphism (Dobzhansky, 1951).

However, in the same time the detection of inversion is extremely difficult because inversions do not vary of a chromosome arm-ratio (Madan, 1995).

So far, 22 autosomes involving pericentric and paracentric cases have been reported although they are commonly regarded as variants and have no direct effect on phenotypic abnormality (Thomas *et al*., 2008). As has been showed in 184 cases reviewed by Madan (Madan, 1995), in particular chromosomes 1, 3, 5, 7, 11 and 14 are vulnerable to paracentric inversions. Paracentric inversion on chromosome 14 was represented in 11 cases among the 184 cases (Madan, 1995).

1.4.2.3.2. Related disease

In 2004, Youings *et al* suggested that paracentric inversions on chromosome 14 not only produce unbalanced gametes through meiotic cross-over between the normal and inverted homologues but also generate reproductive difficulties (Youings *et al*., 2004). In addition, although in heterozygotes paracentric inversion rarely results in genetic abnormality, the risk for an insertion heterozygote is high (about 15%) (Youings *et al*., 2004) and the paracentric inverted insertion is indistinguishable from paracentric inversion (Madan and Nieuwint, 2002). (Figure 5). Therefore, the risks of the actual genetic abnormality from paracentric inversion could be increased to 15% of individuals and more careful interpretation is needed to evaluate paracentric inversions (Madan and Nieuwint, 2002).

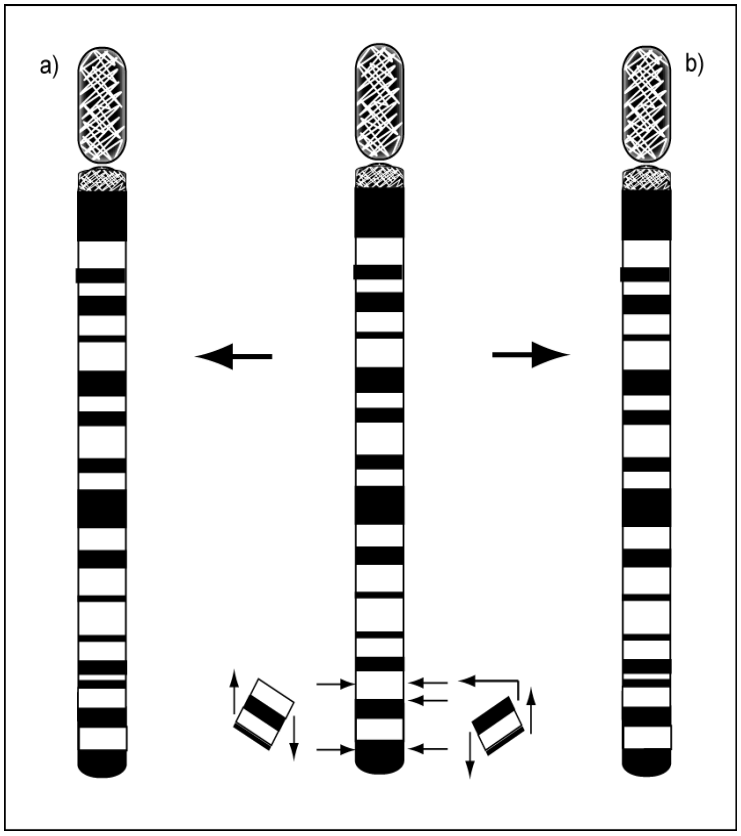


Figure 5. Paracentric inversion product (a) and paracentric inverted insertion product (b). The paracentric inverted insertion is indistinguishable from paracentric inversion, and if these are paracentric inverted insertion heterozygotes, the risk of the actual genetic abnormality occurrence increases to 15% of individuals.

1.5. Diagnosis using Q-PCR

1.5.1. Brief introduction of Q-PCR

Quantitative PCR (hereafter Q-PCR) followed by the classical PCR is the technique of choice to quantify specific DNA segments in tissues or cells (Pfaffl, 2001). From fundamental molecular biology to biotechnology, Q-PCR is a widely used for routine tool (Pfaffl, 2001; Karlen *et al*., 2007; Heid *et al*., 1996; Livak and Schmittgen, 2001) and researchers have developed several protocols for Q-PCR and these methods usually allow us to validate small changes in target gene expression in DNA sample with fast, reliable and high-throughput

manner (Pfaffl, 2001). Q-PCR using SYBR Green 1 fluorescence dye is the simplest and most commonly using detection method (Pfaffl, 2001). During the PCR using SYBR, the quantities of newly synthesized products are monitored by specific binding affinity of SYBR to the minor groove of double stranded DNA (Pfaffl, 2001; Rasmussen *et al*., 1998). When the SYBR dye is included in the PCR reacting mixture, the fluorescence is increased proportionally with the produced double stranded DNA (Ståhlberg *et al*., 2005).

Two different quantification analysis methods have been generally used: i) Absolute quantification based on internal or external standard curve and it determines the absolute transcript copy number; ii) Relative quantification ratio of the target gene expression as compared to the reference gene expression (Pfaffl, 2001; Livak and Schmittgen, 2001).

1.5.2. Some examples of Q-PCR diagnostics

For microarray and various genomic approaches to which demand prompt and reliable validation of small changes in biological samples, PCR based diagnostic technique have been commonly employed (Karlen *et al*., 2007). At recent, PCR based diagnostic is a fundamental part of the several disease diagnosis which caused by pathogenic bacteria (Song, 2005; Schaad *et al*., 2003; Henson and French, 1993; Liet *et al*., 2008; Schaad *et al*., 1999), viruses (Schaad *et al*., 1999; Boonham *et al*., 2002), fungus (Schaad *et al*., 1999) and other infectious pathogens (Mullis and Faloona, 1987).

Currently improved PCR techniques are allowed to detect nucleic acid amplification in real time and combined with other molecular techniques, which has been used to therapeutic drug invention and personalized drug response monitoring (Monis and Giglio, 2006; Kalow, 2006). Also for the diagnosis of the complicated diseases such as cancer, real time PCR technique is considered as most sensitive and effective (Monis and Giglio, 2006; Kalow, 2006). Numerous

studies have adopted PCR based diagnostics to development of an early diagnosis for various diseases like leukemia (Menskin *et al*., 1998; Pongers-Willemse *et al*., 1998; Preudhomme *et al*., 1999; Khalil, 2005), lymphoma (Luthra *et al*., 1998; Rambaldi *et al*., 2005; Eckert *et al*., 2000; Pennings *et al*., 2001), breast cancer (Aerts *et al*., 2001), human herpesvirus (Boivin *et al*., 2002), neroblastoma (Cheung *et al*., 2003), prostate cancer (Jiang *et al*., 2004), lung cancer (Lewis *et al*., 2005), thyroid cancer (Hesse *et al*., 2005), human papilomavirus (Molijn *et al*., 2005), hippel-lindau disease (Hoebeeck *et al*., 2005) and many others. In addition,the detection of chromosomal aberrations and Ig heavy chain locus rearrangements deficiencies are also common target for PCR based approach (Yashima *et al*., 2003; Donovan *et al*., 2000; Uchiyama *et al*., 2003; Lee *et al*., 2004).

1.5.3. Cons and pros of Q-PCR analysis – comparison with other strategy of diagnostics

In 1984, PCR was introduced by Kary Mullis. Since then it has been considered as a revolution (Mullis and Faloona, 1987). The PCR technique has provided many advantages which could not be provided by traditional and immunological methods (Ferre, 1992). The most important advantages of PCR based diagnostics are in the great specificity and time saving (Schaad, 2003). However, although it could offer a powerful tool, the proper quantification of PCR is imperative (Raeymaekers, 1995) and it is also essential to design a proper controls to quantify target gene expression (Ferre, 1992). Researchers have developed various methods for quantification of PCR (Heid, 1996) and have devoted to search or evaluate proper controls (Beillard *et al*., 2003; Dheda *et al*., 2004).

The quantitative fluorescent polymerase chain reaction method (QF-PCR or Q-PCR) is widely used to quantify a selected nucleotide sequences (Kubista *et al*., 2006; Nolan *et al*., 2006; Bustin, 2000). The sequence selection based on the primers' and probes' uniqueness

could ensure the specificity of PCR results (Schaad and Frederick, 2002). Properly designed primers or probes which encode interested gene regions can amplify very little number of target gene to a certain amount by polymerase chain reaction and amplified fragments can be detected accurately by using quantitative fluorescent dye (Kubista *et al*., 2006; Nolan *et al*., 2006; Bustin, 2000). And by using the fluorescence dye with the PCR mixture, quantitative approach was facilitated. In the process of reaction, increased signal from emitted fluorescence reflects the increased number of DNA fragmentsand the kinetics of amplification also visualize in a sigmoid plot (Tichopad *et al*., 2010). Emitted signal is monitored once per cycle and the cycle of quantification is recorded at which the signal reached to certain point. This point defined as threshold (Ct) and it is based on the mechanical computing and qualified decisions (Stolovitzky and Cecchi, 1996; Rosner, 2000). However, it still be challenged that the PCR results could be influenced by inhibition or generation of undesiable side products such as primer dimmers (Tichopad *et al*., 2010). Side products are the most important factors which makes difficulty of the quantification of PCR results, although the ability of amplification from a small quantity of nucleic acids is one of the strongest factors of this technique (Ferre, 1992; Mullis and Faloona, 1987; Mullis *et al*., 1986). It is also crucial to use the correct and validated interpretation methods to adopt PCR based diagnosis (Khot and Fredricks, 2009). To obtain valid and credible data, quality assurances and controls of the data are essential (Bustin *et al*., 2009) because the variety of factors can affect on PCR data validation. Therefore the one should consider sensitivity, specificity, accuracy, precision, reproducibility and linear range (United States Pharmacopeia, 1990; Paul, 1991). And for the successful use of PCR based diagnostics, optimization of amplification step (Ferre, 1992), better sample preparation techniques and the inclusion of appropriate controls are imperative (Mott *et al*., 1997).

1.6. Aim of the Study

Analyses of Ig VH gene usages have revealed differences in the basic Ig V repertoire of patients with B cell and/or T cell mediated autoimmune diseases compared to healthy controls. Such differences could be derived from intrinsic abnormalities during the generation of Ig V genes or B cell development and function. Thus, the analysis of Ig V gene usages can offer new insights into possible pathogenic role of B cells in autoimmune diseases. AS is a chronic autoimmune inflammatory disease more commonly affecting young males between the ages of 20 and 40. Enthesitis, stiffness of joints, and pains often led to functional disorders and weakness which have a negative impact on the quality of life.

Since our knowledge of B cells` involvement in AS pathogenesis is based on very limited data, in this study we aim to investigate the role of B cells in AS and to suggest a novel approach for developing an AS diagnosis system.

Previous studies of VH germline gene usages in synovial B cells from AS patients demonstrated that the majority of rearranged Ig VH genes belonged to VH3 genes. However, it did not include a direct comparison with 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. On the basis of gene usage results, we hypothesized that the aberrant Ig gene rearrangement could be associated with AS pathology.

To develop a simple and effective diagnosis system, Ig VH gene usage analysis was conducted by using quantitative PCR with PBMCs. Successively, our further studies were performed with early stage AS and rheumatoid arthritis patient samples to evaluate the specificity and applicability of our AS diagnosis technique on early-stage of AS. Following the AS diagnosis analyses, CD40L gene expression was studied additionally in order to get

more supportive evidence for our hypothesis of aberrant Ig gene rearrangement association with AS pathology.

2. MATERIALS and METHODS

2.1. The investigation of VH gene usage on AS patients

2.1.1. Subjects

To analyze immunoglobulin variable heavy chain gene usage, peripheral blood mononuclear cell (PBMC) was collected from 9 healthy controls (HC) and from 8 patients with AS who visited the rheumatology clinic at Gachon University Gil Hospital in Korea. The patients with AS met the Modified New York Criteria (Rudwaleit *et al*., 2009) and they were taking non-steroidal anti-inflammatory drug (NSAIDs) and sulfasalazine regularly. Age, sex, disease duration, erythrocyte sedimentation rate (ESR), serum C reactive protein (CRP) and HLA-B27 positivity were assessed (Table 4).

Table 4. Demographics and clinical characteristics of subjects

	AS (n=8)	Healthy Controls (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/hr)*	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; ESR (erythrocyte sedimentation rate); CRP (serum C reactive protein); * mean ± SD

The mean age of AS patients was older than controls (38.6 and 30.2 years, respectively), but it was not statistically significant. The validated Korean versions of Bath AS Disease Activity Index (BASDAI) (Garrett *et al*., 1994) were calculated for AS patients when their PBMC 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

2.1.2. cDNA preparation and PCR primers

PBMCs were obtained from nine healthy control's and eight patient's whole blood by density centrifugation on Histopaque (Sigma, UK). Used reagents for PBMC preparation are as following; Heparin (Green cross, KOREA), D-PBS (Welgene, KOREA), Trypan blue (Fluka, UK) and the figure below shows the protocol of this step.

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 (Eppendorf, Germany) were performed with primers as shown in Table 5. All primers were produced by Eurofins MWG Operon (Ebersberg, Germany). In order to amplify VH regions, a set of 5'VH primers specific for each of the eight human VH gene families in combination with the mixture of five human JH gene families primers were used (Voswinkel J *et al*., 2001). The sequence of VH and JH mRNA sequences are available at Online Ig Blast databases (http://www.ncbi.nlm.nih.gov/igblast). Through the active sequence search, three of VH and JH primers added (forward primers for HuVH2 and HuVH4, reverse primer for HuJH7). Using these primers in combination with previously published primers, we were

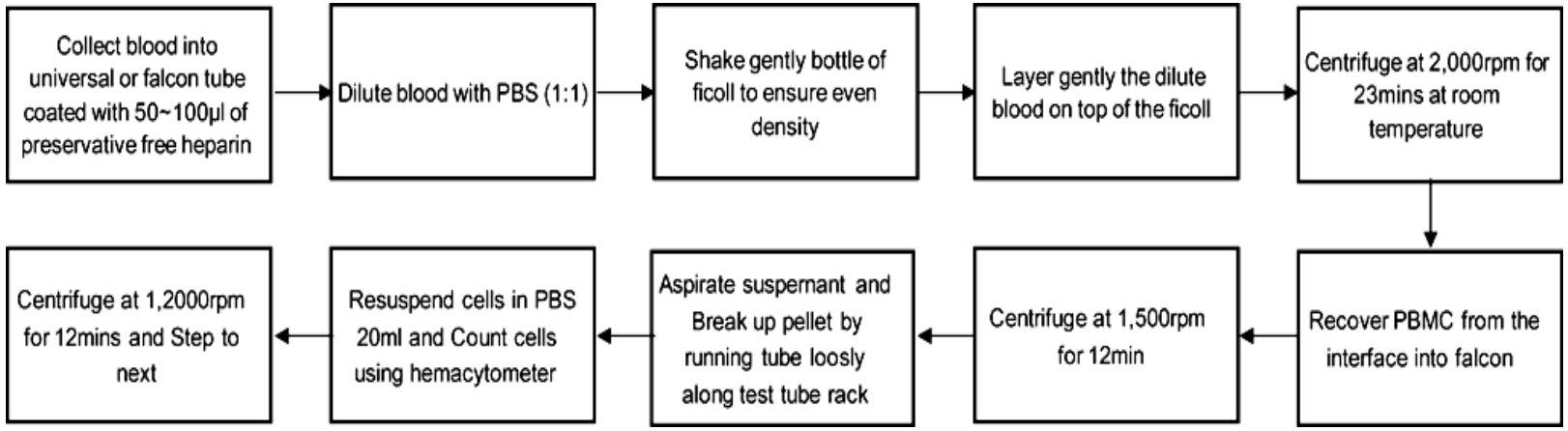


Figure 6. The isolation process of PBMCs from blood samples. PBMCs were isolated by Ficoll-Paque density centrifugation from nine healthy donor and eight patient blood samples.

able to amplify broadened range of rearranged antibody genes than previous studies.

Table 5. Primer sets for investigation of immunoglobuline VH region usage

Primer	Sequences	Ref
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'	Cowell(1999), Van Esch (2003), Dheda(2004)
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^{ab} for*	5' – CAG ATC ACC TTG AAG GAG TCT GG – 3'	Kim(2010)
HuVH4^{ab} for*	5' – CAG GTG CAG CTA CAG CAG TGG GG – 3'	Kim(2010)
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'	Cowell(1999), Van Esch (2003), Dheda(2004)
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'	Kim(2010)

*added primers
_enzyme restriction site

The variable heavy (VH) chains were amplified using the Pfu Ultra II Fusion HS DNA polymerase (Stratagene) and the following cycling conditions: an initial denaturation at 95℃ for 15min followed by 30 cycles of 94℃ for 30 s, 60℃ for 30 s and 72℃ for 1min with a final elongation at 72℃ for 10 min. The PCR products were examined by electrophoresis on 1% agarose gel first. Restriction enzymes NcoI and XhoI were subsequently introduced to the VH2^{ab} PCR product to clone it into the plasmid by using PCR amplification.

2.1.3. VH2^{ab} library construction

Tomlinson library were obtained from the Medical Research Council (MRC) Cambridge England and it provided 1.37×10^8 diversified human synthetic phage displayed scFv library (I+J:http://www.geneservice.co.uk/products/proteomic/datasheets/tomlinsonIJ.pdf) (Cook *et al*., 1994, Marcu *et al*., 2006).

The Escherichia coli strains TG1 were stored in the KIST-Europe department and phagemid pIT2 were obtained from the Medical Research Council (MRC) Cambridge England. Isolation of the pIT2 phagemid vector was performed using the Plasmid Isolation Kit (Qiagen, Hilden, Germany). The phagemids were sequenced by Eurofins MWG Operon (Ebersberg, Germany) using the LMB3 (5'-CAG GAA ACA GCT ATG AC- 3') sequence primer (Cook *et al*., 1994, Marcu *et al*., 2006).

HuVH2^{ab} forward primer and a total of five kinds of reverse primers (JH1-2, 3 4-5, 6 and 7) were used for the construction of the variable heavy chain 2^{ab} library. After the first PCR analysis using primers in Table 5, obtained PCR products for each sample were isolated from agarose gel using Gel Extraction Kit (Qiagen, Hilden, Germany) and purified using PCR product purification kit (Qiagen, Hilden, Germany) for the next step, The PCR products from the first PCR analysis are then used as template in a second PCR, using secondary PCR primers in Table 6.

Table 6. Secondary PCR primers for bacterial clonning

Primer	Sequences
Secondary PCR primers	
HuVH2^{ab}for*- NcoI	5' – AGC CGG CCA TGG CCG CAG ATC ACC TTG AAG GAG TCT GG – 3'
HuJH1-2-Xho1	5' –TCC ACC GCT CGA GAC TGA GGA GAC GGT GAC CAG GGT GCC–3'

HuJH3-Xho1	5’ –TCC ACC GCT CGA GAC TGA GGA GAC GGT GAC CAG GGT GCC–3’
HuJH4-5-Xho1	5’–TCC ACC GCT CGA GAC TGA AGA GAC GGT GAC CAT TGT CCC–3’
HuJH6-Xho1	5’–TCC ACC GCT CGA GAC TGA GGA GAC GGT GAC CAG GGT TCC–3’
HuJH7-Xho1	5’–TCC ACC GCT CGA GAC TGA GGA GAC GGT GAC CGT GGT CCC–3’

The final products were digested with restriction enzymes (Fermetas) XhoI, NcoI and cloned into the phagemid pIT2. Plasmid DNA was isolated using the Qiaprep spin midiprep kit (Qiagen, Hilden, Germany). Transformed TG1 E.coli colonies were screened for inserts, by colony PCR, using the vector specific primer, LMB3 (5’-CAGGAAACAGCTATGAC-3’) (Ziegler *et al*., 1995).

Colony PCR (94 ℃ 4 min, then 94 ℃ 1 min, 58 ℃ 30 s, 72 ℃ 2 min for 30 cycles, and final extension at 72 ℃ for 10 min) was carried out to check individual clones for the presence of $VH2^{ab}$ insert (300 bp) (GOTaq, Promega). Theses PCR products were examined by electrophoresis on 1% agarose gels. Totally, 60-80 colonies were proved to contain $VH2^{ab}$ inserts. These colonies were cultured overnight in LB medium and plasmids were isolated using the Qiaprep spin miniprep kit (Qiagen, Hilden, Germany). Plasmid DNA was purified using a Qiagen mini-prep kit and nucleotide sequence was determined using the primer LMB3 by MWG (Ebersberg, Germany) (Ziegler *et al*., 1995). A schematic outline describing the $VH2^{ab}$ library construction is shown in Figure 7.

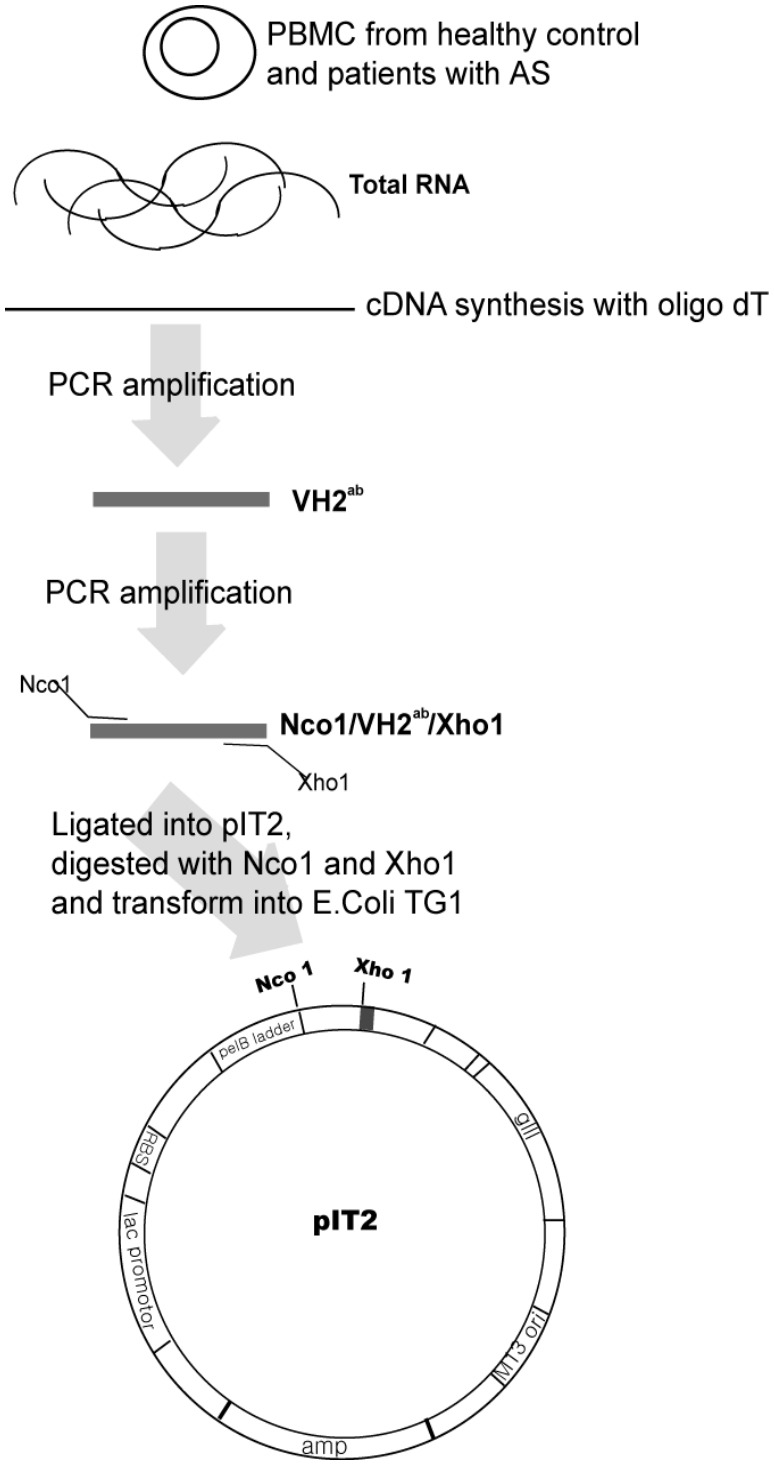


Figure 7. Schematic outline of the strategy used for the construction of the VH2^{ab} library. The total RNA was extracted from PBMC of 8 patients with AS. The locations of PCR primers on the variable region genes are shown. The list of all primers used for the construction of the library is given in Table4. Three-step PCR reactions were performed. The first PCR step comprised 30 reactions for amplification of V gene repertoire, which were cloned into pIT2 phagemid.

2.1.4. Sequence analysis

Approximately 20 successfully cloned colonies' DNA of each patient $VH2^{ab}$ libraries (in total 100 colonies were selected from colony PCR analysis) were analyzed by sequencing (MWG, Germany) using the vector specific primers, LMB3 (5'-CAGGAAACAGCTATGAC-3'). The sequence results were thoroughly analyzed by homology comparison with Immunoglobulin Blast databases (http://www.ncbi.nlm.nih.gov/igblast) and IMGT databases (http://imgt.cines.fr). Sequence alignments were performed using ClustalW2 (http://www.ebi.ac.uk/clustalw/), available at the European Bioinformatics Institute (EBI) Web server.

2.1.5. Quantitative PCR (Q-PCR)

Quantitative PCR (hereafter Q-PCR) followed by the classical PCR is the technique of choice to quantify specific DNA region (Pfaffl, 2001). The most important advantages on PCR based diagnostics are in the great specificity and time saving (Ståhlberg, 2005).

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 and all experiments were performed in triplicate. The amplification condition were 95℃ for 30 sec, 50-60℃ for 1 min, and 72℃ for 1 min for 30-40 cycles. Amplification of 10ng/$\mu\ell$ of DNA was performed using Stratagene's Brilliant SYBR Green Q-PCR core reagent kit (Catalogue nos 600546, 929546, La Jolla, CA, USA) on Stratagene's MX3000p. Final products were analyzed by MxPro™ QPCR software (Stratagene, La Jolla, USA). Reagents for Q-PCR were purchased from Stratagene (La Jolla, USA). All primers

were produced by MWG (Ebersberg, Germany) and Q-PCR was performed with primers as shown in Table 5 and Table 7 (Cowell, 1999; Kim *et al*., 1999; Van Esch, Reparon-Schuijt *et al*., 2003; Dheda, Huggett *et al*., 2004).

Table 7. House-keeping gene primer for Q PCR

Primer	Sequences	Ref
HuPo(House-keeping gene)		
HuPo forward	5'-CCA TTC TAT CAT CAA CGG GTA CAA-3'	Dheda(2004)
HuPo reverse	5'-AGC AAG TGG GAA GGT GTA ATC C-3'	

HuPo: human acidic ribosomal protein gene

In general, two different quantification analysis methods have been used: i) Absolute quantification based on internal or external standard curve and it determines the absolute transcript copy number; ii) Relative quantification ratio of the target gene expression as compared to the reference gene expression (Pfaffl, 2001; Livak and Schmittgen, 2001). In this study, we chose the relative quantification method on account of the fact that report the relative change in gene expression is suffice rather than determine absolute copy number of transcripts.

Relative quantification describes the change in expression of the target gene relative to a reference group such as the housekeeping gene; here we used human acidic ribosomal protein. The relative amount of transcripts of target genes compared to those of a housekeeping gene was calculated as follows (Ct: Cycle threshold, R: Relative amount of transcript);

$$\Delta \mathrm{Ct} = \mathrm{Ct}(\mathrm{experimental}) - \mathrm{Ct}(\mathrm{housekeeping}),$$

$$\mathrm{R} = 2^{-(\Delta \mathrm{Ct})}$$

, and

$$\Delta\Delta \mathrm{Ct} = \Delta \mathrm{Ct}(\mathrm{target\ sample}) - \Delta \mathrm{Ct}(\mathrm{reference\ sample})$$

$$\text{Amount of target} = 2^{-(\Delta\Delta Ct)}$$

ΔCt for each sample (experimental and housekeeping) is calculated by subtracting the Ct number of the experimental gene from that of the housekeeping gene (Dheda *et al*., 2004). The $2^{-(\Delta Ct)}$ method was used to analyze relative gene expression data when only one gene is being studied as compared with the housekeeping gene expression (Dheda *et al*., 2004). The average of ΔΔCt for each sample was calculated by subtracting the ΔCt number of patient samples from that healthy control samples (Pfaffl, 2001). Amount of target gene expressions could be normalized by calculating $2^{-(\Delta\Delta Ct)}$ value. The normalized values for different samples can then directly be compared (Pfaffl, 2001). The $2^{-(\Delta\Delta Ct)}$ values were also analyzed to show fold differences in gene expression level between healthy control and AS patient.

2.1.6. PCR to identify incorporation of CDC42 BPB intron fragments

Through library construction and sequence analysis, CDC42 BPB gene intron fragment was found between the VH2 and heavy chain joining region (JH) (section 3.1.5, Figure 13), and the region was located near to 125-kb of CDC42 BPB intron segment.To confirm that the intron fragments from CDC42 BPB genes were indeed incorporated into Ig heavy chain gene segments, other primers were designed to analysis of the region from Ig variable heavy chain leader to constant genes. All primers used to confirm the CDC42BPB incorporation are shown in Table 8.

Table 8. Primer sets for confirmation of CDC42BPB incorporation

Primer	Sequences	Ref
HuCDC42 BPB Forward primer		Kim(2010)

HuCDC42-FOR	5'-GAG CAC TGG CCA AGC ACT A-3'
Human Cμ, Cγ and Cε reverse primer	
Hu Cμ rev	5'-TCC AGG AGA AAG TGA TGG AG-3'
Hu Cγ rev	5'-GTC TTG GCA TTA TGC ACC TC-3'
Hu Cε rev	5'-CGG ATG GGC TCT GTG TGG-3'
VH Leader Forward primer	
VH L-For1	5' – CR CTC CTG CTG CTG ACC A – 3'
VH L-For2	5' – CR CTG AGC TGG RTT TTC CT – 3'
VH L-For3	5' – KR CTY YGC YGG SYY YYY CT – 3'
CDC42BPB reverse primer	
CDC42BPB REV	5' – TG CTC TGT AGT GTC AA – 3'

*R=A or G, K=G or T, Y=C or T

Reaction conditions were the following; initial denaturation at 95℃ for 15 min, followed by 30 cycles of 94℃ for 30 sec, 58℃ for 30 sec and 72℃ for 1 min, and then 72℃ for 10 min for final extension. PCR products were analyzed by 2% agarose gel electrophoresis.

2.1.7. Primer locations and Hypothetical gene structure

Based on our experiments and our findings, we designed three primer sets to detect the CDC42BPB incorporated Ig VH2^{ab} gene. The hypothetical gene structure in AS patients and the location of each primer sets to detect VH2^{ab} gene transcripts are demonstrated in Figure 8. Primer 2 (PR2) and primer 3 (PR3) were designed successively according to the Ig gene structure and further PCR investigation was performed.

Figure 8. Hypothetical VH2 gene structure in patients with AS. Three sets of primers, based on the Ig gene sequence analysis, were designed for PCR and Q PCR. Open triangles, PR1; closed triangles, PR2; grey triangles, PR3.

Figure 9 shows the expected aberrant Ig gene structure in AS patients. The location of each primer which we used for Q-PCR analysis is also described. The nucleotide sequences of each primer are displayed in Table 5 and PR 1 is designed to cover the whole VH2 germ line genes.

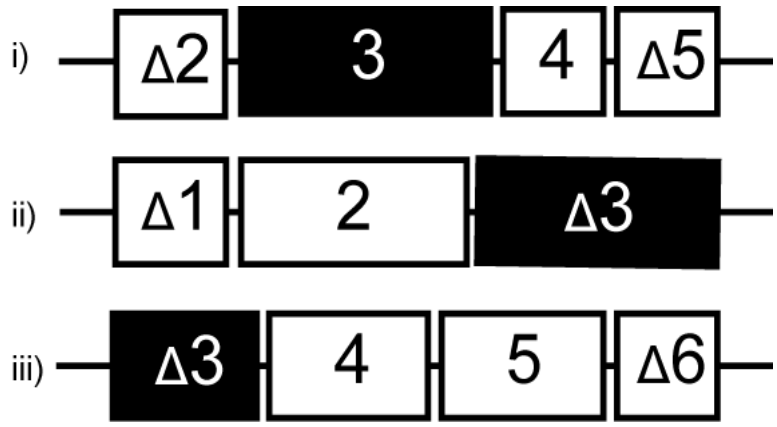


Figure 9. Schematic diagrams of expected PCR products. Expected band sizes were: i) 252bp using PR1, ii) 300bp using PR2, and iii) 330bp using PR3. The numbers indicate that the gene fragments are: 2) VH2*, 3) Inserted CDC42BPB fragment, 4) DH, 5) JH, and 6) C epsilon

2.1.8. Statistics

A non-parametric statistic method, two tailed Mann-Whitney U test was performed to test the significance of differences in mRNA amounts of individual VH gene families between AS patients and healthy controls (SPSS, Ver. 12.0. Chicago, IL). To test for correlations between the expression level of $VH2^{ab}$ genes and clinical variables, Spearman's rank correlation coefficient was used. To evaluate the correlation between two indespendent variables, Spearman's rank correlation coefficient is popular and useful tool (Gauthier, 2001).

2.2. The investigation of abnormal VH gene features on AS, SpA and RA patients

2.2.1. Study subjects

PBMC was collected from 23 HC, 26 patients with axial SpA and 18 patients with RA who visited the rheumatology clinic at Gachon University Gil Hospital. The patients with axial SpA met the Assessment of SpondyloArthritis international Society (ASAS) classification criteria for axial SpA (Rudwaleit *et al*., 2009). Among them, 17 patients satisfied the modified New York criteria for AS (Van der Linden *et al*., 1984). Thus, the other 9 patients had axial SpA without evidence of radiographic sacroiliitis and are designated as pre-AS. RA was diagnosed using the American Rheumatism Association 1987 revised criteria (Arnett, 1992).

When PBMC was collected, the age and gender of patients and disease duration were assessed (Table 9). Each patient was studied for ESR (erythrocyte sedimentation rate) and serum CRP (C reactive protein). Rheumatoid factor (RF) and anti-cyclic citrullinated protein antibodies (anti-CCP) for RA and HLA-B27 for axial SpA were also tested. The patients with axial SpA were questioned about Bath AS Disease Activity Index (BASDAI) (Garrett *et al*., 1994). Lumbar flexion mobility was measured in axial SpA patients, using modified Schober test (Macrae and Wright, 1969). 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 HC.

Table 9. The demographic and clinical characteristics of patients and HC

	HC	RA	axial SpA	pre-AS	AS
	N=23	N=18	N=26	N=9	N=17
Age (years)*	30.4 ± 3.8	49.6 ± 8.2	32.6 ± 9.2	28.3 ± 9.6	34.9 ± 8.4
Male: Female	2.8 : 1.0	1.0 : 2.0	7.7 : 1.0	3.5 : 1.0	16.0 : 1.0
Disease duration (years)*		9.6 ± 7.0	9.4 ± 9.2	3.8 ± 3.9	12.4 ± 9.9
BASDAI*			4.5 ± 1.8	4.1 ± 1.1	4.7 ± 2.0
Modified Schober test (cm)*			4.4 ± 2.6	5.1 ± 2.8	4.0 ± 2.5
ESR (mm/hr)*		25.9 ± 26.1	27.3 ± 33.5	36.7 ± 35.6	22.4 ± 32.4
CRP (mg/dL)*		1.5 ± 2.4	2.3 ± 3.6	2.7 ± 2.8	2.0 ± 4.1
RF (%)		88.9			
Anti-CCP (%)		75.0			
HLA-B27 (%)			96.2	88.9	100.0

*mean ± standard deviation
AS= ankylosing spondylitis, HC= healthy controls
BASDAI= Bath Ankylosing Spondylitis Disease Activity Index
BASFI= Bath Ankylosing Spondylitis Functional index
ESR (erythrocyte sedimentation rate)
CRP (serum C reactive protein)

2.2.2. Quantitative PCR (Q-PCR)

Using a MaximeTM RT PreMix (Oligo (dT) 15 primer) Kit (Intron Biotechnology, Korea), cDNA was synthesized from PBMCs of 23 healthy control, 26 patients with axial SpA and 18 patients with RA. The cDNA sample preparation requires the MaximeTM RT PreMix (Oligo (dT) 15 Primer) Kit (Intron biotechnology, Korea) and each sample was prepared following the manufacturer's instructions. PCR and Q-PCR were performed with primer sets as shown in Tables 4, 5 and 6 (Kim *et al*., 2010; Cho *et al*., 2008; Kamanli *et al*., 2009; Cowell *et al*., 1999; Van Esch *et al*., 2003).

As a house-keeping gene, HuPo (human acidic ribosomal protein) gene was used since it is known to be more reliable than β-actin gene as a control for the Q-PCR in PBMC (Dheda *et al*., 2004). When the stability of 12 different housekeeping genes was analyzed, the human acidic ribosomal protein gene was shown to be one of the most stable genes with a low

variation in the cycle threshold (Ct) value (He *et al*., 2008). All reactions were performed in triplicate and a negative control was included in each experiment. The amplification condition was 95℃ for 30 s, 50-60℃ for 1 min, and 72℃ for 1 min for 30-40 cycles and all primers were produced by MWG (Ebersberg, Germany). PCR products were analyzed by agarose gel electrophoresis or by MxProTM Q-PCR software (Stratagene, La Jolla, CA). The relative amount of transcripts of target genes compared to those of a housekeeping gene was calculated as follows; ΔCt = Ct (experimental) - Ct (housekeeping), $R = 2^{-(\Delta Ct)}$ or $2^{-(\Delta\Delta Ct)}$.

2.2.3. The investigation of CD40L expression in AS, SpA and RA patients

PCR analysis using Ig constant regions (Cμ, Cγ and Cε) primer, we detected that uniquely rearranged $VH2^{ab}$ gene structure is joined with constant epsilon (Cε) gene. Through bacterial cloning and sequence analysis, we hypothesized that Ig class switch deficiency might be influenced on AS pathogenesis. To get more supportive evidence, CD40L gene expression was investigated. CD40L is an inducer of Ig class switching, and it has also been reported that impaired CD40L expression can cause a defective Ig class switch recombination. To get more supportive data for this hypothesis, additional Q-PCR was performed with a primer set for CD40L gene. HuPo primers were also used as a house keeping gene. The amplification condition was 95℃ for 30 sec, 60℃ for 1 min, and 72℃ for 1 min for 40 cycles. PCR primer sequences for investigations on CD40L are shown in Table 10 (Haifa *et al*., 2001).

Table 10. Primer sets to investigate CD40L expression

Primer	Sequences	Ref
HuPo(House-keeping gene)		
HuPo forward	5'-CCA TTC TAT CAT CAA CGG GTA CAA-3'	
HuPo reverse	5'-AGC AAG TGG GAA GGT GTA ATC C-3'	
CD40L expression primer		
CD40L FOR	5' – CAC CTT CTC TGC CAG AAG ATA CCA TTT CAA –3'	Haifa(2001)

CD40L REV	5’ – TTA TGA AGA CTC CCA GCG TCA CGT CCA CCA –3’

2.2.4. Statistics

To analyze the profile of subjects, Mann-Whitney U test was performed. To analyze $VH2^{ab}$ and CD40L gene expression, Kruskal-Wallis test (non-parametric ANOVA) and Dunn’s multiple comparison test are performed using GraphPad In-Stat version 3.10 for Windows (GraphPad Software, San Diego, CA). To assess the correlation of $VH2^{ab}$ and CD40L expression in axial SpA patients, Spearmann test was performed. *P<0.05, **P<0.01, and ***P<0.001.

Reagents:

Pfu Ultra II Fusion HS DNA polymerase (stratagene, Germany), restriction endonucleases (Xho1 and NcoI), T4DNA polymerase, T4DNA ligase, plasmid DNA purification kit, PCR product purification kit (Qiagen, Hilden, Germany), Gel Extraction Kit (Qiagen, Hilden, Germany) SDS-PAGE low molecular weight standard proteins, QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany), DNA marker, Coomassie brilliant blue R-250, diaminobenzidine (DAB) (Sino-American Biotechnology Co., Ltd), The Escherichia coli strains TG1 were stored in the department. Phagemid pIT2 (MRC HGMP Resource Centre, Cambridge, UK), Brilliant SYBR Green QPCR core reagent kit (Catalogue nos 600546, 929546, La Jolla, CA, USA), RNAeasy mini kit (Qiagen, Hilden, Germany), Maxime RT PreMix (Oligo (dT)15 primer) Kit (Intron Biotechnology, Korea), GOTaq,(Promega), Stratagene MX 3000p instruments, Histopaque (Sigma, UK), Heparin (Green cross, KOREA), D-PBS (Welgene, KOREA), Trypan blue (Fluka, UK), LB medium, Heparin (Green cross, KOREA), D-PBS (Welgene, KOREA), Trypan blue (Fluka, UK)

3. RESULTS

Unusual V region repertoires and biased use of particular VH segments have been identified in several autoimmune diseases. It is commonly assumed by researchers that about 20 percent of autoimmunity patients are affected by class switch recombination deficiencies. In this study, in order to develop an early diagnosis system for AS which is known as an autoimmune disease, VH segments usage was analyzed in PBMCs of patients with AS. Subsequent sequencing analyses showed that the detected uniquely over-expressed VH segment in AS patients include a short stretch of DNA span from an adjacent gene CDC42BPB (CDC42 binding protein kinase beta)) in between V_H2 and J_H3 and this segment turned out to be joined with Cε in the process of Ig production. Identification of the uniquely assembled VH segment combined with constant epsilon (Cε) region gene led us to hypothesize that class switch recombination deficiency caused by paracentric inversion during the process of V-(D)-J gene rearrangement is associated with AS susceptibility based on the published data showing that IgVH and CDC42BPB genes have an adjacent chromosomal interval on the chromosome 14.

The next sets of experiments were conducted to evaluate sensitivity and specificity of developed primers PR1, PR2 and PR3 for diagnosing AS. Q-PCRs were performed with an increased number of samples (9 early stage AS patients having similar symptoms as those with AS but do not have X-ray evidence of structural damage in their joints, 17 AS, 18 RA (rheumatoid arthritis) patient samples and 23 HC (healthy controls)). In addition, CD40L gene expression was studied to get more supportive evidence for our hypothesis and Q-PCR analyses revealed that CD40L gene was expressed two fold higher in AS patients as compared with healthy controls as well as RA patients. In order to take more reliable data, Q-PCR analyses with highly increased numbers of smaples (49 AS, 50 RA and 50 HC) were

conducted.

3.1. Results of VH gene usage assays of patients with AS

In order to develop an early diagnosis system for AS, Ig repertoties usages in patients with AS were analyzed using PCR analysis and bacterial cloning technique. Through PCR analyses, over-expression of $VH2^{ab}$ transcripts was detected and DNA sequence analysis of $VH2^{ab}$ harbouring plasmid showed that the impaired Ig class switch was occurred in AS patient.

The first set of our experiments was conducted with PBMCs of 8 AS patients and 9 healthy donors. We designed three sets of primers (PR1, PR2 and PR3) targeting the unique VH segment. Quantitative PCR (Q-PCR) results revealed that uniquely rearranged VH2 transcripts were about ten times over expressed in AS samples as compared to healthy donor controls.

3.1.1. PCR results of VH gene usage analysis

IgVH gene usage in patients with AS were analysed using PCR method. Figure 10 shows comparative profiles of agarose gel electrophoresis of VH1-$VH4^{ab}$ PCR products. The results from PCR with individual samples demonstrated that there were no significant differences in the PCR profile between AS patients and the control group that was produced by PCR primer sets for VH1a-VH6a and for $VH4^{ab}$. All primer sequences are presented in Table 4 and in order to amplify each VH regions, VH1-VH6 and $VH4^{ab}$ forward primers were used in combination with the mixture of five human JH reverse primers.

Interestingly, $VH2^{ab}$ genes represented by $VH2^{ab}$ forward primer were over-expressed

exclusively in AS patients (patient sample numbers 3, 4, 5, 6, 7, and 8 as shown in Figure 10). Except for the negative control, all VH PCRs led to a product of the expected length (330 bp). All sequences reported in the present paper are accessible on the EMBL database.

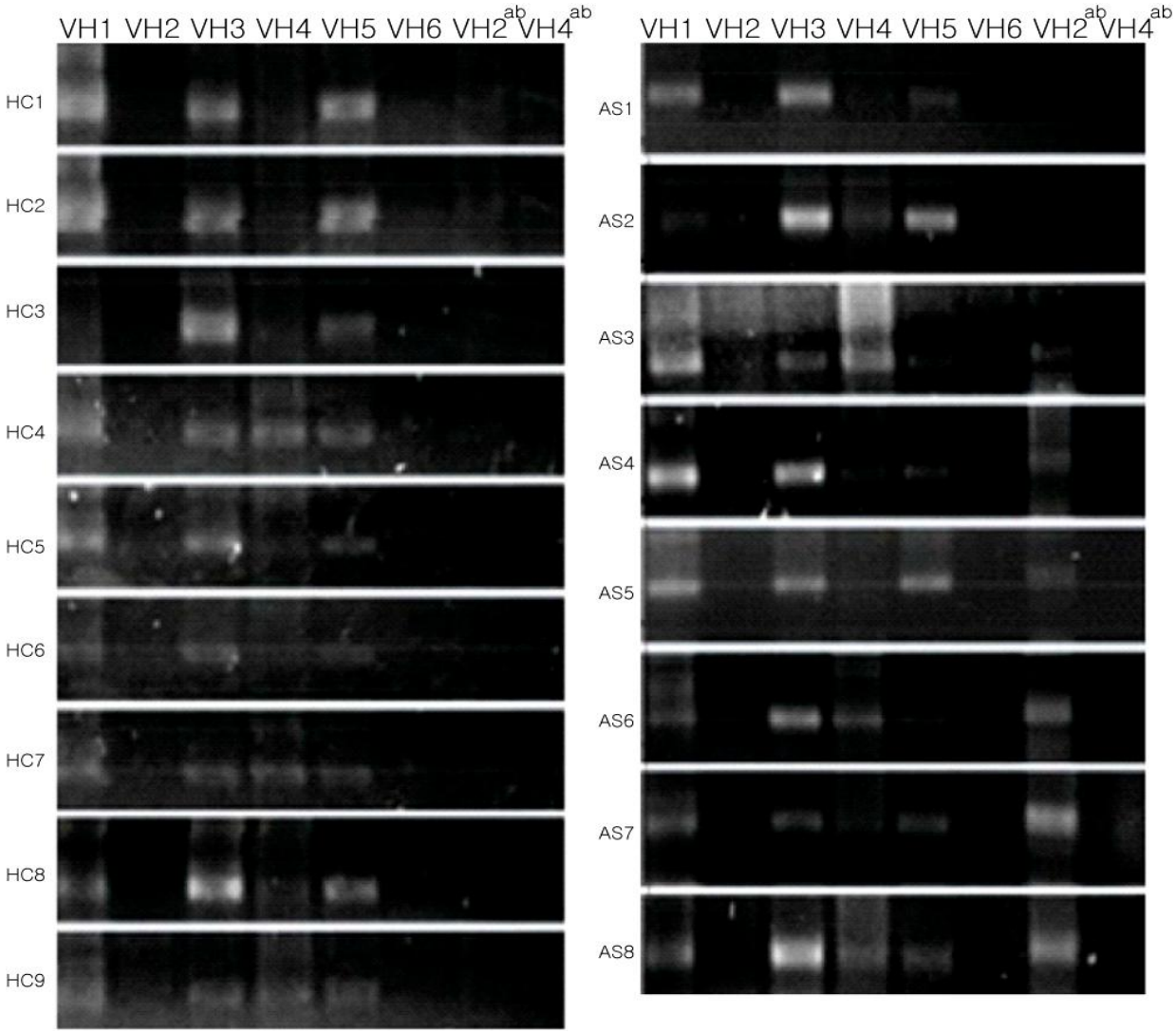


Figure 10. VH2^{ab} region was overexpressed in patients with AS. In comparison with VH gene expression in PBMCs samples from healthy donors and AS patients, VH2^{ab} genes were overexpressed exclusively in AS patients (patient sample numbers 3, 4, 5, 6, 7, and 8); N= 9 of healthy donors and 8 of AS patients.The results confirmed the previous study showing VH3 was dominantly expressed (HC: healthy control; AS: ankylosing spondylitis patient; VH: variable heavy chain)

3.1.2. Confirmation of previous VH usage experiments

In order to ensure our primers' efficiency Q-PCR with pooled samples for each group (healthy control and AS patients) was conducted with PCR primer sets for VH1-VH6, VH2^{ab} and VH4^{ab} in combination with the mixture of five JH reverse primers (Table 5, 7). The results showed that dominant expression of VH3 (Figure 11).

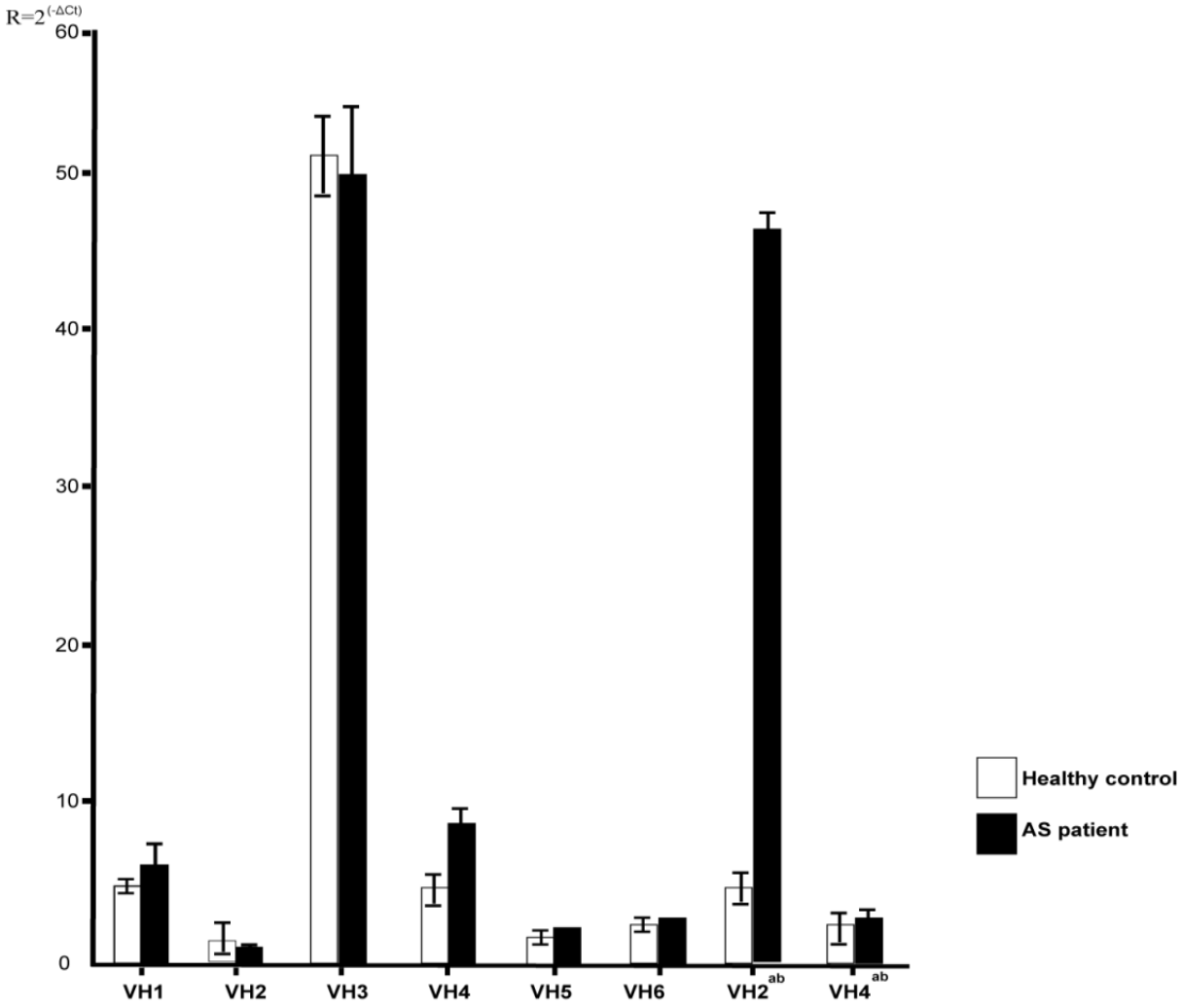


Figure 11. The relative expression levels of VH gene in AS patients and healthy controls. The relative amount of transcripts of target genes compared with those of a housekeeping gene (HuPo: human acidic ribosomal protein) was calculated as follows; ΔCt=Ct(experimental)-Ct(housekeeping), R=2-(ΔCt). The data show the mean and standard deviations of triplicate PCR amplications.

The relative expression levels of VH1-VH6, $VH4^{ab}$ and $VH2^{ab}$ in patients with AS and healthy control are displayed on Figure 11. Q-PCR was performed with pooled cDNA from PBMC of healthy donors and AS patients. The relative amount of transcripts of target genes compared to those of a housekeeping gene (HuPo: human acidic ribosomal protein) was calculated. The data shows the mean and standard deviation of triplicate PCR amplifications. Our data is in consistent with the results of Voswinkel (2001) and coworkers which examined VH usage in AS patients using DNA from inflamed tissue (Voswinkel *et al.*, 2001). However, $VH2^{ab}$ genes were over-expressed exclusively in AS patient sample pool. It could be interpreted that the newly designed primer, $VH2^{ab}$ forward, has mediated AS patient specific gene amplification. To quantify the amount of $VH2^{ab}$ transcripts in each sample, Q-PCR with individual samples was performed.

3.1.3. Q-PCR with individual samples

Q-PCR was performed with individual cDNA from of PBMC of healthy donors and of 8 AS patients using primers shown in Table 5 and Table 7. The relative amount of mRNA of genes to HuPo was calculated.

Significant differences were shown in the level of expression of $VH2^{ab}$ genes between healthy donors and AS patients (relative amount of mRNA of $VH2^{ab}$ genes to HuPo, 0.68 ± 0.55 [mean±SD] and 7.13 ± 7.77, respectively; $p<0.0001$, 9 of HC and 8 of AS patients, Figure 12). There were no correlations of the expression level of $VH2^{ab}$ with clinical variables such as sex, age, disease duration and inflammatory parameters.

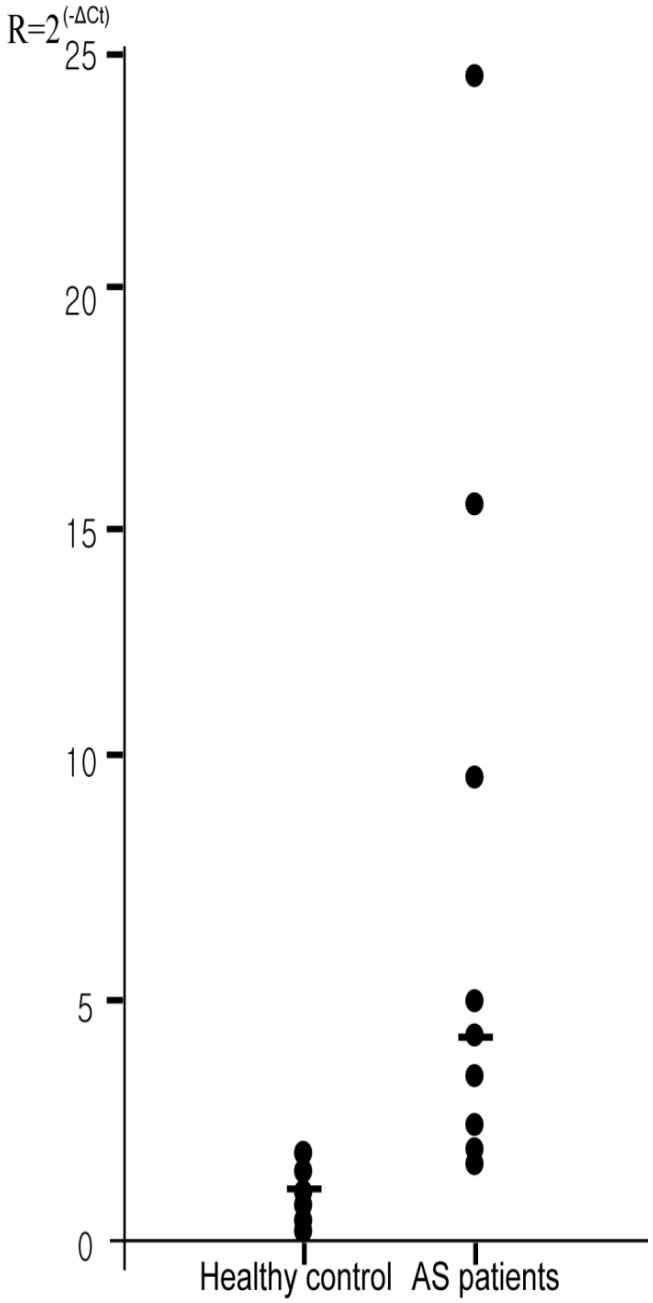


Figure 12. Q-PCR was performed with individual cDNA from each PBMC of healthy donors and AS patients, using primers specific for $VH2^{ab}$ genes. The relative amounts of mRNA of $VH2^{ab}$ genes to HuPo were calculated as above. The relative amounts of mRNA of VH2ab genes to HuPo in AS patients were significantly higher compared to healthy donors (P<0.0001); N= 9 of healthy donors and 8 of AS patients.

The average relative transcript values for individual samples are described in Table 11. The relative amount of transcripts of target genes compared to those of a housekeeping gene was calculated. The data shows the mean and standard deviation of triplicate PCR amplifications.

Table 11. Relative transcript values for individual samples of HC and AS patients

Sample	R(average)± S.D	Sample	R(average)± S.D
HC1	0.097±0.100	AS1	1.084±0.821
HC2	0.037±0.019	AS2	1.931±1.017
HC3	0.526±0.273	AS3	1.245±0.506
HC4	0.855±0.720	AS4	9.322±0.893
HC5	1.722±0.244	AS5	3.127±2.346
HC6	0.688±0.858	AS6	15.054±7.534
HC7	1.311±0.734	AS7	3.839±2.077
HC8	0.470±0.351	AS8	23.971±9.634
HC9	0.390±0.351	AS9	4.641±2.860

*****HC: healthy control, AS: ankylosing spondylitis patient, R: relative transcript, S.D: standard deviation

3.1.4. VH2^{ab} fragment bacterial cloning

Through PCR analysis and followed Q-PCR analysis, over-expression of VH2^{ab} transcripts was detected. To analyse these over-expressed PCR products, VH2^{ab} library was constructed by cloning VH2^{ab} PCR products. PCR products using VH2^{ab} primer of the expected 330 base pair (bp) length were detected by standard agarose gel electrophoresis. VH2^{ab} PCR products were purified using the Qiaprep spin midiprep kit (Qiagen, Hilden, Germany). The purified DNA template was bacterially subcloned into *E.coli* followed by plasmid isolation and sequencing (section 2.1.3 to 2.1.5). The second PCR was performed with PCR products from selected VH2^{ab} overexpressed patient samples (number 4, 6, 7, 8, and 9) as a template. In this process, each of the first-PCR purified products NcoI and XhoI restriction enzyme sites were introduced by using secondary PCR primers (Table 6).

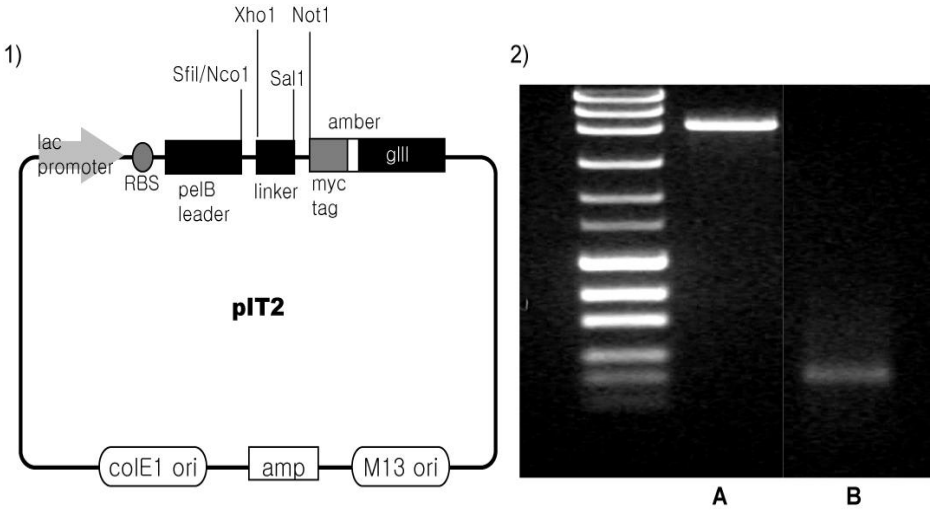


Figure 13. Vector map of pIT2 and cloning; 1) Vector map of pIT2, 2) The purified DNA template was bacterially subcloned into bacteria; A: cut out of pIT2 phagemid with Nco I and Xho1 B: VH2ab region PCR purified product (expected length: 330bp).

Plasmid DNA was isolated by using the Qiaprep spin midiprep kit (Qiagen, Hilden, Germany). The PCR products were digested with restriction enzymes (Fermetas) XhoI, NcoI and cloned into the phagemid pIT2 (Figure 13). The ligation mixtures were transformed into *E. coli* TG1 cells and plated onto LB agar plates. After colony counting, colonies were screened for inserts, by colony PCR and positive clones were confirmed with a colony PCR method (GOTaq, Promega) (Figure 14). In total, 100 colonies were selected from colony PCR analysis.

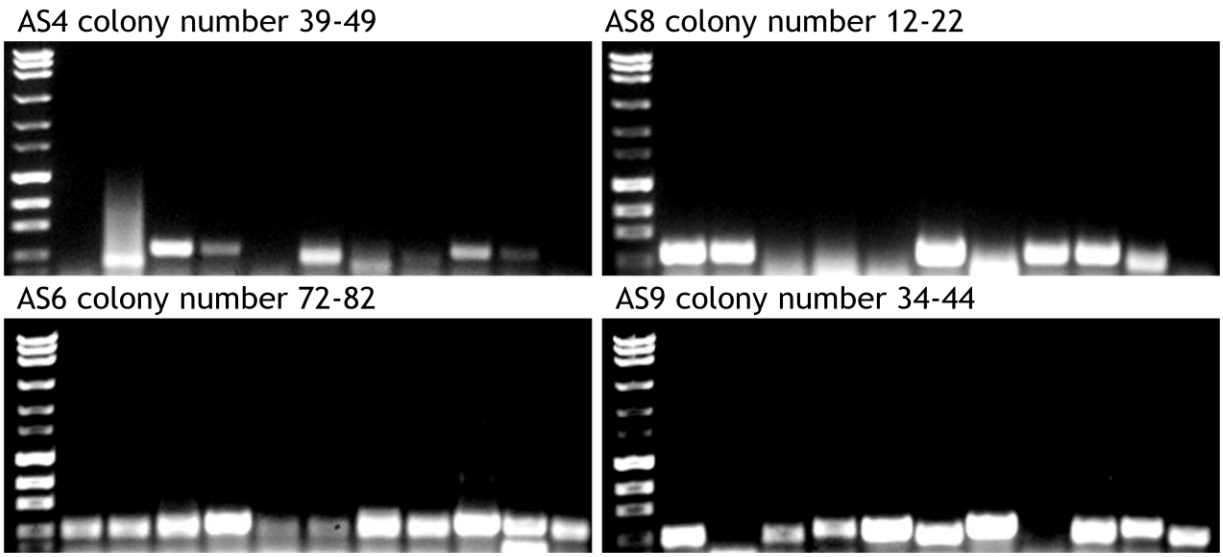


Figure 14. Examples of colony PCR products were cloned into pIT2 vectors and 100 successful VH2^{ab}-inserted colonies were selected by colony PCR analysis using VH2^{ab} primer.

3.1.5. Sequence analysis of positive clones

Selected clones were grown overnight at 37℃ in LB medium containing 100 µg/ml ampicillin. DNA was extracted from the positive clones and purified using the QIAprep Spin Miniprep Kit and sequenced.Approximately 20 samples of each patient were analyzed by sequencing (MWG, Germany), and a total of 100 sequences were obtained. The sequencing results were analyzed by homology comparison with Ig Blast databases (http://www.ncbi.nlm.nih.gov/igblast), GenBank gene databases and IMGT databases (http://imgt.cines.fr).

According to Ig Blast database analyzing results, obtained sequence data was fallen into four categories. Among 100 sequences, 48% of sequences revealed that a short fragment from CDC42 BPB gene was incorporated into the major part of cloned VH2^{ab} PCR products.

This short fragment is an intron fragment located in the region encompassing 252 bps (36096-36348) of 125-kb CDC42 BPB which maps to 14q32.32 (Moncrieff et al., 1999) and is found in between a short stretch of VH2 and JH3 (Figure 15).

Figure 15. The sequence analysis of VH2^{ab}. This short fragment (252bps) from CDC42 BPB was found in between VH2 and JH3

Other 41 sequences showed VH2 rearrangements. All were potentially functional and > 95% homologous to the respective gl gene. Less than 10% of cases (9 sequences) a clonal expansion within the VH3 genes was observed, represented by the VH3-DH3-JH4 rearrangements sequences. The rest of 7 sequences indicated other immunoglobulin variable heavy chain gene family. Sequence alignment assay of 48 CDC42 BPB genes incorporated sequence is presented in Figure below. The alignment result was analyzed by nucleotide alignment analysis with ClustalW2-Multiple Sequence Alignment program which provide by EMBL databases (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and multiple alignment editor programs (http://www.jalview.org/).

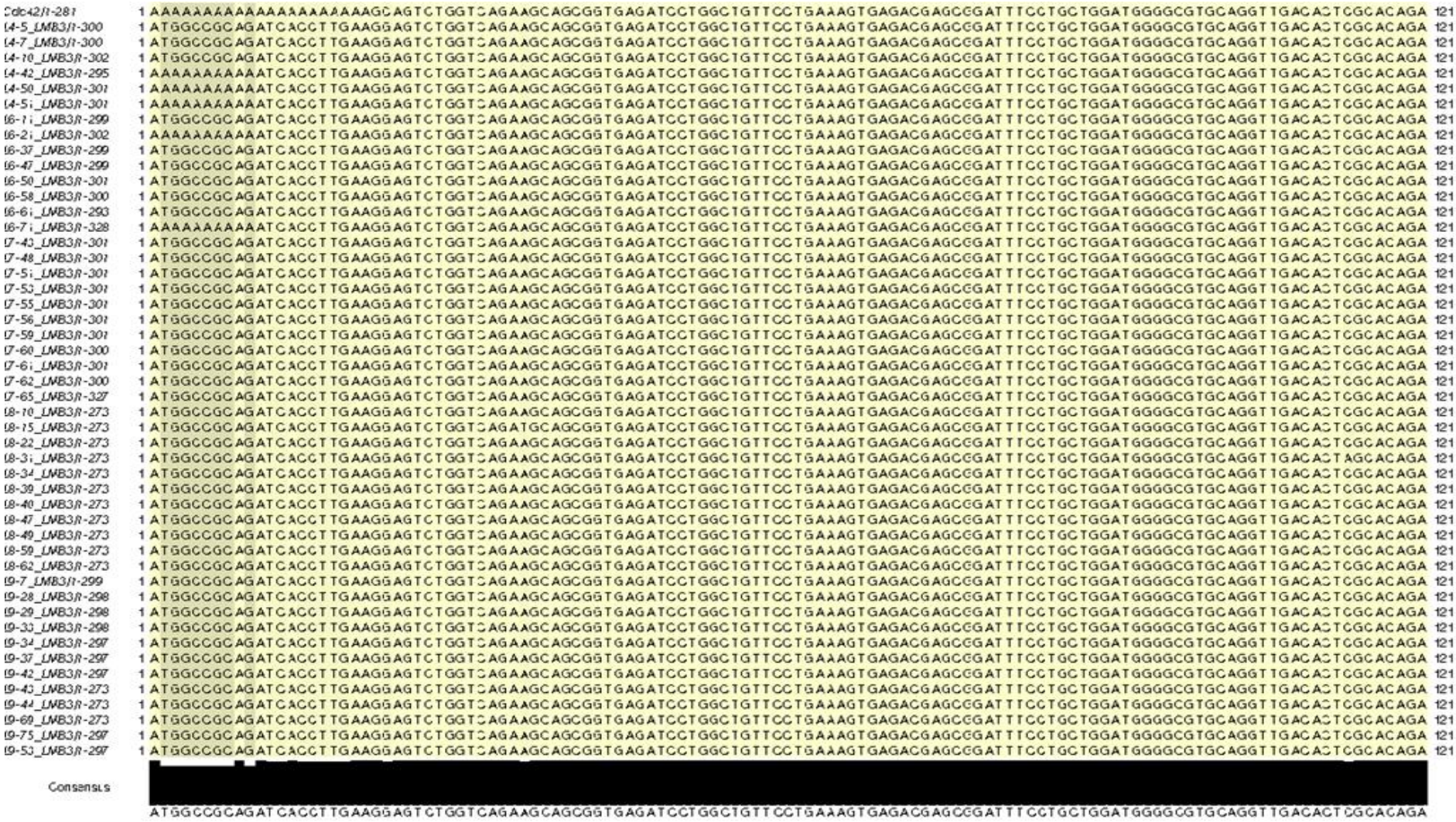
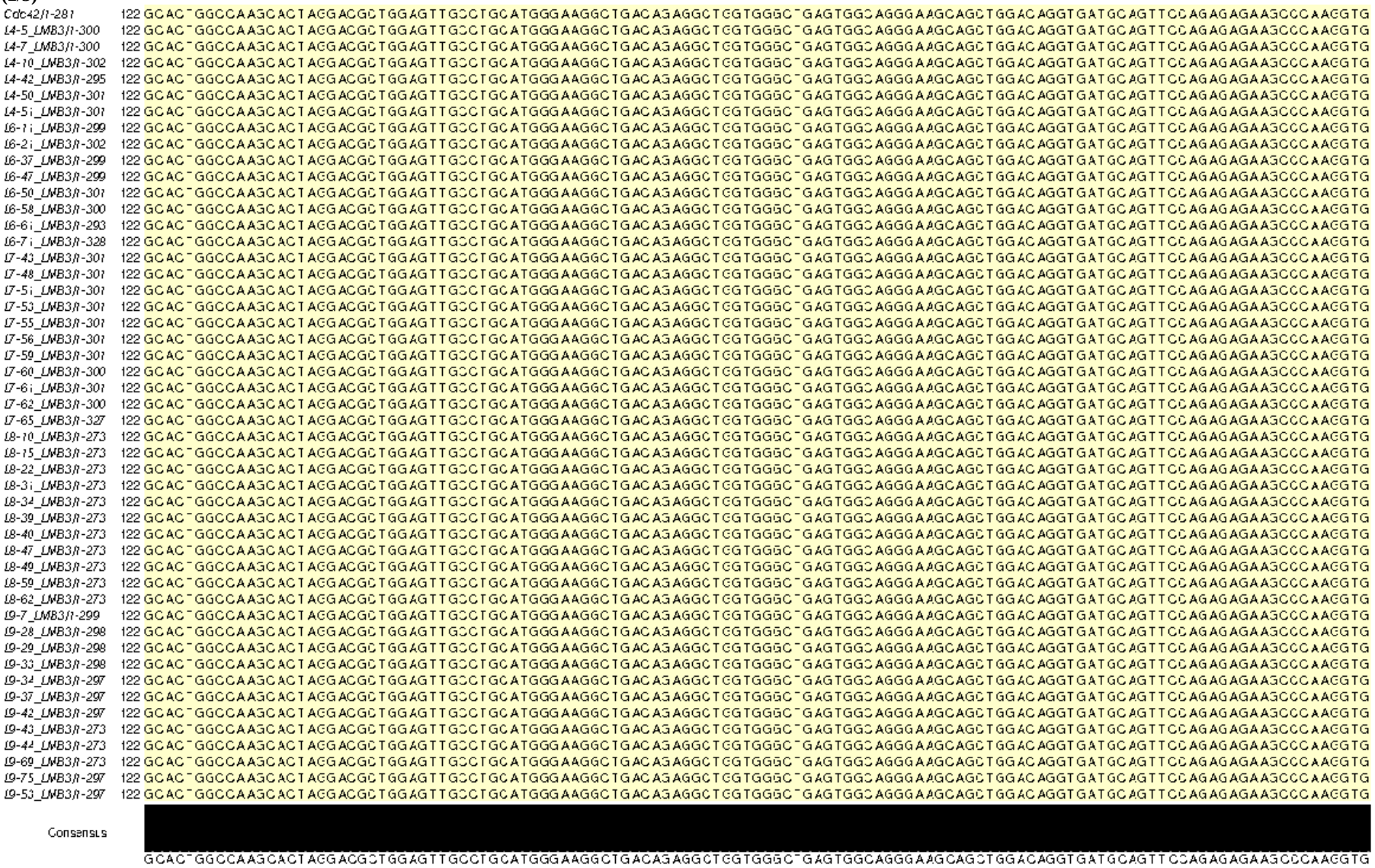


Figure 16. Sequence alignment. Among 100 sequences, 48% revealed a short fragment from CDC42 BPB genes incorporated into major parts of cloned VH2[ab] PCR products. Sequence mismatch is indicated by the shade of gray (yellow indicates no difference; darker color reflecting less matched region; L: library; number: colony number)

(2/3)


Cdc42/1-281 122 GCAC-GGCCAAGCACTAGGACGCTGGAGTTGCCTGCATGGGAAGGCTGACAGAGGCTCGTGGGC-GAGTGGCAGGGAAGCAGCTGGACAGGTGATGCAGTTCCAGAGAGAAGCCCAACGTG 242
L4-5_LMB3/1-300 122 GCAC-GGCCAAGCACTAGGACGCTGGAGTTGCCTGCATGGGAAGGCTGACAGAGGCTCGTGGGC-GAGTGGCAGGGAAGCAGCTGGACAGGTGATGCAGTTCCAGAGAGAAGCCCAACGTG 242
L4-7_LMB3/1-300 122 GCAC-GGCCAAGCACTAGGACGCTGGAGTTGCCTGCATGGGAAGGCTGACAGAGGCTCGTGGGC-GAGTGGCAGGGAAGCAGCTGGACAGGTGATGCAGTTCCAGAGAGAAGCCCAACGTG 242
L4-10_LMB3/1-302 122 GCAC-GGCCAAGCACTAGGACGCTGGAGTTGCCTGCATGGGAAGGCTGACAGAGGCTCGTGGGC-GAGTGGCAGGGAAGCAGCTGGACAGGTGATGCAGTTCCAGAGAGAAGCCCAACGTG 242
L4-42_LMB3/1-295 122 GCAC-GGCCAAGCACTAGGACGCTGGAGTTGCCTGCATGGGAAGGCTGACAGAGGCTCGTGGGC-GAGTGGCAGGGAAGCAGCTGGACAGGTGATGCAGTTCCAGAGAGAAGCCCAACGTG 242
L4-50_LMB3/1-301 122 GCAC-GGCCAAGCACTAGGACGCTGGAGTTGCCTGCATGGGAAGGCTGACAGAGGCTCGTGGGC-GAGTGGCAGGGAAGCAGCTGGACAGGTGATGCAGTTCCAGAGAGAAGCCCAACGTG 242
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L6-1i_LMB3/1-299 122 GCAC-GGCCAAGCACTAGGACGCTGGAGTTGCCTGCATGGGAAGGCTGACAGAGGCTCGTGGGC-GAGTGGCAGGGAAGCAGCTGGACAGGTGATGCAGTTCCAGAGAGAAGCCCAACGTG 242
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L6-58_LMB3/1-300 122 GCAC-GGCCAAGCACTAGGACGCTGGAGTTGCCTGCATGGGAAGGCTGACAGAGGCTCGTGGGC-GAGTGGCAGGGAAGCAGCTGGACAGGTGATGCAGTTCCAGAGAGAAGCCCAACGTG 242
L6-6i_LMB3/1-293 122 GCAC-GGCCAAGCACTAGGACGCTGGAGTTGCCTGCATGGGAAGGCTGACAGAGGCTCGTGGGC-GAGTGGCAGGGAAGCAGCTGGACAGGTGATGCAGTTCCAGAGAGAAGCCCAACGTG 242
L6-7i_LMB3/1-328 122 GCAC-GGCCAAGCACTAGGACGCTGGAGTTGCCTGCATGGGAAGGCTGACAGAGGCTCGTGGGC-GAGTGGCAGGGAAGCAGCTGGACAGGTGATGCAGTTCCAGAGAGAAGCCCAACGTG 242
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L7-59_LMB3/1-301 122 GCAC-GGCCAAGCACTAGGACGCTGGAGTTGCCTGCATGGGAAGGCTGACAGAGGCTCGTGGGC-GAGTGGCAGGGAAGCAGCTGGACAGGTGATGCAGTTCCAGAGAGAAGCCCAACGTG 242
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L8-62_LMB3/1-273 122 GCAC-GGCCAAGCACTAGGACGCTGGAGTTGCCTGCATGGGAAGGCTGACAGAGGCTCGTGGGC-GAGTGGCAGGGAAGCAGCTGGACAGGTGATGCAGTTCCAGAGAGAAGCCCAACGTG 242
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L9-28_LMB3/1-298 122 GCAC-GGCCAAGCACTAGGACGCTGGAGTTGCCTGCATGGGAAGGCTGACAGAGGCTCGTGGGC-GAGTGGCAGGGAAGCAGCTGGACAGGTGATGCAGTTCCAGAGAGAAGCCCAACGTG 242
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Consensus
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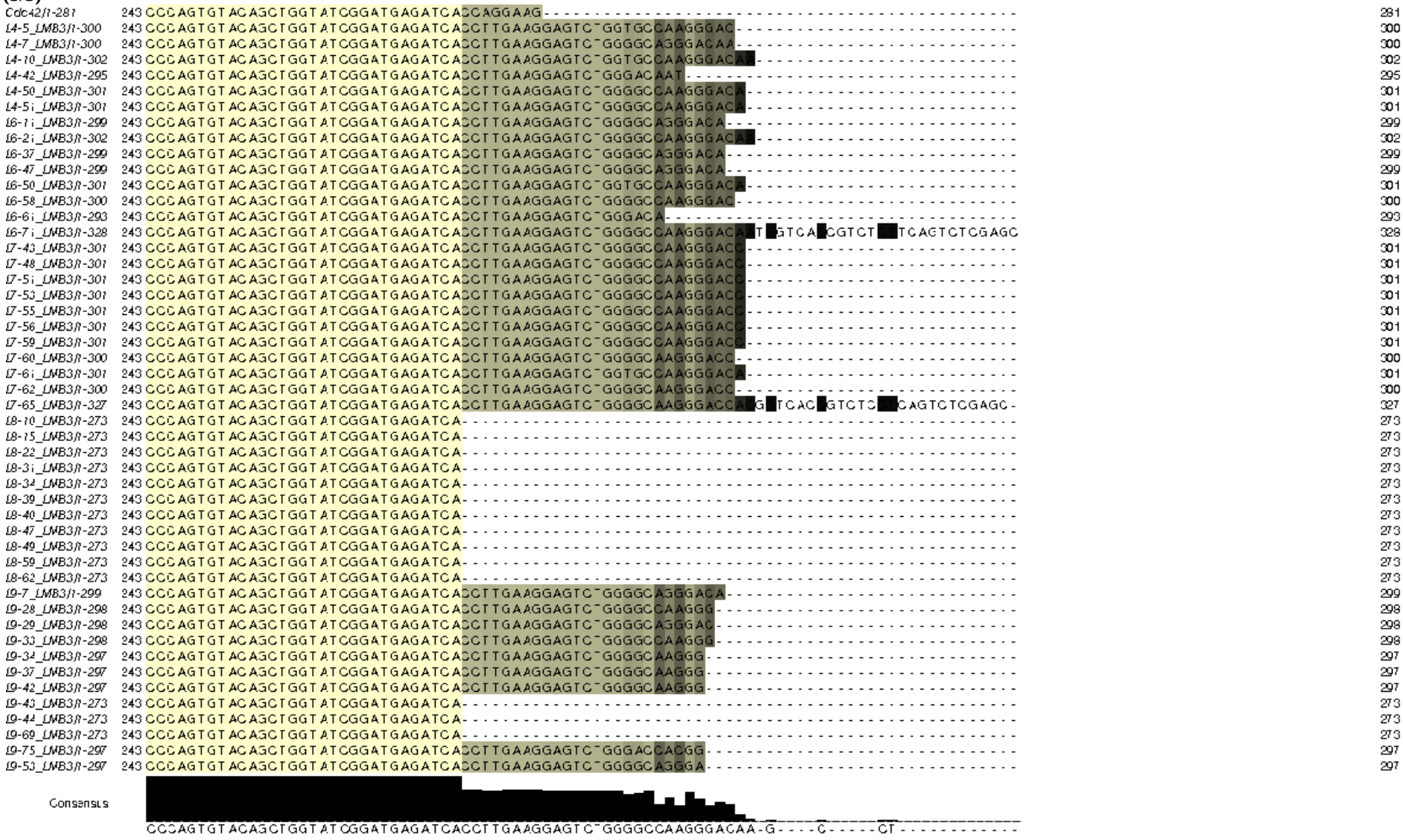
(3/3)


Cdc42/i-281 243 CCCAGTGTACAGCTGGTATCGGATGAGATCACCAGGAAG 281
L4-5_LMB3/i-300 243 CCCAGTGTACAGCTGGTATCGGATGAGATCACCTTGAAGGAGTC-GGTGCCAAGGGAC 300
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L9-75_LMB3/i-297 243 CCCAGTGTACAGCTGGTATCGGATGAGATCACCTTGAAGGAGTC-GGGACCACGG 297
L9-53_LMB3/i-297 243 CCCAGTGTACAGCTGGTATCGGATGAGATCACCTTGAAGGAGTC-GGGGCAGGGA 297
Consensus
CCCAGTGTACAGCTGGTATCGGATGAGATCACCTTGAAGGAGTC-GGGGCCAAGGGACAA-G----C-----CT----------

3.1.6. PCR analysis to identify the incorporation of CDC42 BPB intron fragments

Through library construction and sequence analysis, CDC42 BPB gene intron fragment was found between the VH2 and heavy chain joining region (JH) (Figure 15, 16), and the region was located near to 125-kb of CDC42 BPB intron segment, which maps to 14q32.32.

To confirm that the intron fragments from CDC42 BPB genes were indeed incorporated into Ig heavy chain gene segments, another PCR was performed by using primers corresponding to sequences in the middle of the intron of the CDC42 BPB gene and Ig constant regions (Cμ, Cγ and Cε) (Table 8). The results demonstrated the expected band size, about 350 bps, which appeared exclusively by using the Cε primer (Figure 17).

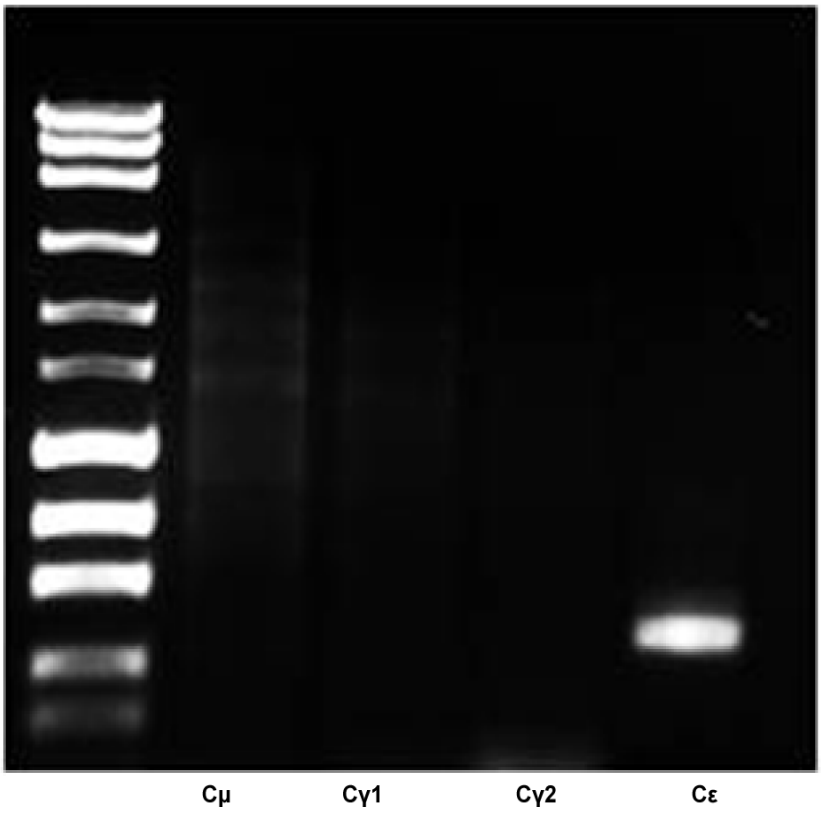


Figure 17. CDC42 BPB intron sequences were inserted into Ig genes in AS patients. PCR was performed with cDNA from PBMCs of healthy donors and AS patients, using primers specific for CDC42 BPB genes and Ig C epsilon genes (No bands were detected in healthy controls)

Therefore these results imply that an intron fragment of the CDC42 BPB gene, suggested by sequence analysis, was incorporated in between VH2 and DH6-JH3-Cε of rearranged Ig genes. During the Ig production process, switching from Cμ to Cε occurs only after B cells have been stimulated by antigens, but certain antigens for AS have not yet been identified. Furthermore, IgE, antibody produced by Cε, is present at very low levels in serum (0.03 μg/mL serum) and only research was conducted for showing the effect of change in serum IgE levels on AS. Therefore, this aberrant V segment seems more likely to affect other Ig protein production by impaired class switch recombination or induction changes in total Ig composition rather than producing rearranged Ig protein.

3.1.7. Unusual VH gene rearrangement hypothesis in patient with AS

We compared Ig VH gene usages in cDNA from PBMCs of the healthy controls and of the AS patients. The PCR analysis results in Figure 10 and Q-PCR analysis results in Figure 12 show that the $VH2^{ab}$ transcripts were exclusively over-expressed in AS patients. To get more data, we constructed $VH2^{ab}$ and anlaysed the sequence of over-expressed products (Figure 16). Strikingly the sequence analysis and homology search of overexpressed $VH2^{ab}$ PCR products revealed unexpected features of VH gene structure. In the sequences of $VH2^{ab}$ PCR products, a short stretch of CDC42 BPB intron gene was found between VH and DH genes (Figure 15). The 5' end of this fragment includes homologous region (15 nts) to that of the VH2 germline gene. 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). Interestingly, further analysis of CDC42 BPB genomic sequences identified a nonamer-like sequence and a

heptamer-like sequence of RSS, which is located following an insertion site of CDC42 BPB genes.

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 $VH2^{ab}$ gene structure (Figure 18).

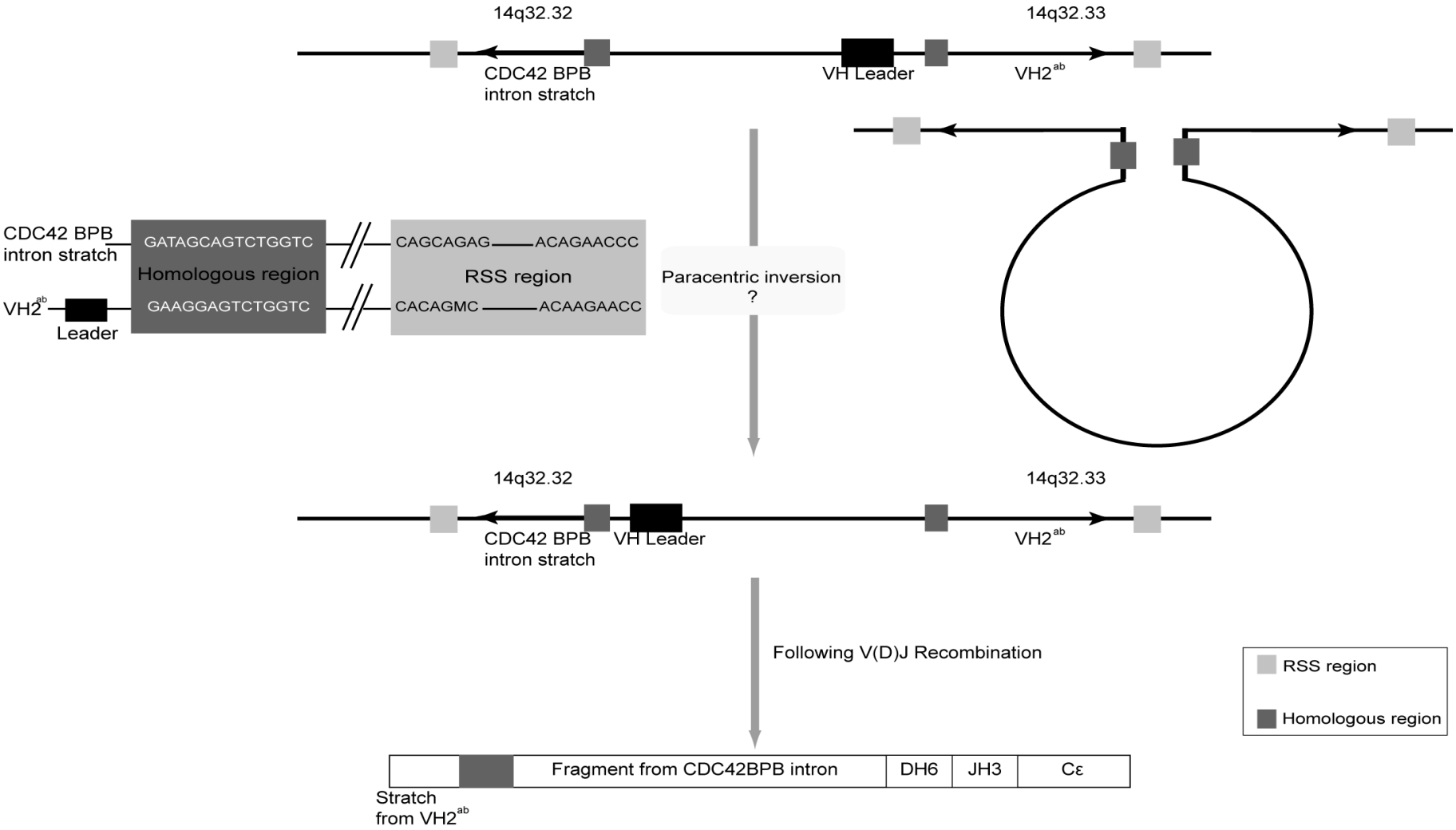


Figure 18. Proposed gene structure of rearranged VH2^{ab} genes in AS patients. CDC42 BPB intron fragments could be paracentrically inverted into VH2 genes. Both genes are located in chromosome 14q32. The sequence homology search revealed possible RSS sequences close to the inserted CDC42 BPB intron fragment. The resulting rearranged Ig gene contains part of the VH2 genes, CDC42 BPB intron sequences, DH6, JH3, and Cε in this order

3.2. Comparative investigation of patients with axial SpA (pre-AS and AS) and RA patient

On the basis of our results in 3.1.1 to 3.1.6, we established a hypothesis in Figure 18. Our hypothesis proposed that CDC42 BPB intron fragments which are located adjacent to Ig gene on chromosome 14q could be paracentrically inverted into VH2 genes in AS patients. PCR analysis using $VH2^{ab}$ forward primer and JH1-7 reverse primer mixture, over-expression of $VH2^{ab}$ transcripts were detected in AS patients. Following $VH2^{ab}$ library construction and sequence analysis results reveled that uniquely rearranged VH gene segment was existed in AS patients' PBMC. To develop early diagnostics for AS using $VH2^{ab}$ transcripts, total three sets of primers were designed (PR1, PR2 and PR3). PCR products which produced by all these primer sets are described in Figure 8 and 9.

Subsequently, comparative investigation was performed with an increased number of samples (9 early stage AS patients having similar symptoms as those with AS but do not have X-ray evidence of structural damage in their joints, 17 AS, 18 RA (rheumatoid arthritis) patient samples and 23 HC (healthy controls)). The aim of this comparative investigation is to evaluate sensitivity and specificity of developed primers PR1, PR2 and PR3 for diagnosing AS. The relative amounts ($R=2^{-(\Delta Ct)}$) of uniquely assembled $VH2^{ab}$ gene expression were determined to be approximately 28 fold higher in AS patients and 6 fold higher in early AS patients than HC by using PR1. In addition, PR1 was proven to have 96 % sensitivity and 95 % specificity in diagnosing early stage AS.

3.2.1. CDC42 BPB intron fragments expression on SpA patients

Following experiments were performed to evaluate sensitivity and specificity of developed

primer sets PR1, PR2 and PR3 for diagnosing AS. Q-PCRs were performed with an increased number of samples (9 early stage AS patients having similar symptoms as those with AS but do not have X-ray evidence of structural damage in their joints, 17 AS, 18 RA (rheumatoid arthritis) patient samples and 23 HC (healthy controls)).

Comparative assays of patients with axial SpA (pre-AS and AS) and RA patients as well as in healthy controls were performed. This study aimed that the conformation of previous result with different samples and to compare them with other types of diseases.

The results from Q-PCR with individual samples (supplement 1, 2 and 3) demonstrated that Ig $VH2^{ab}$ expressions detected by PR1, PR2 and PR3 were significantly higher in axial SpA patients (pre-AS and AS patients), as compared with healthy controls as well as with RA patients ($P<0.01$ for RA vs axial SpA by PR3; $P<0.001$ for HC vs axial SpA by PR1, 2, and 3 and for RA vs axial SpA by PR1 and 2). The relative amounts of $VH2^{ab}$ gene expression detected by PR1 are described in Figure 19.

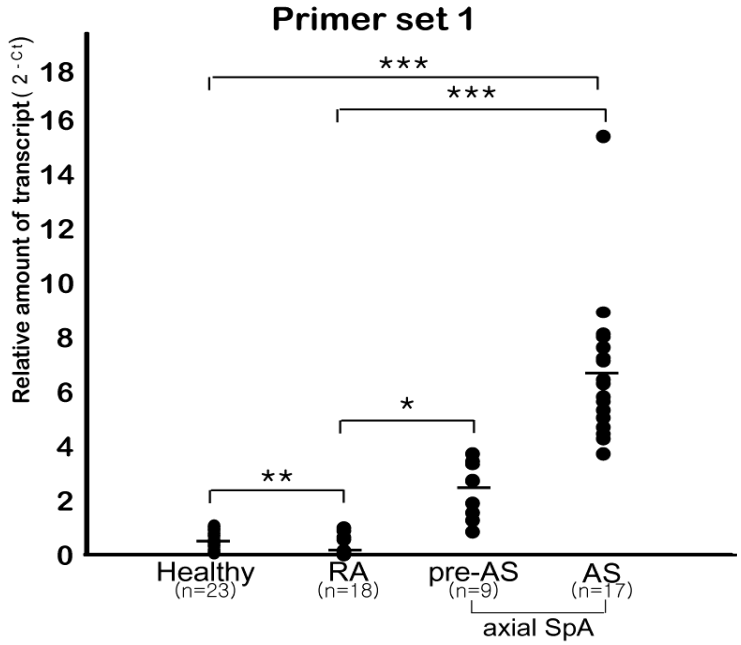


Figure 19. Results from Q-PCR products with individual samples by primer set 1 (PR1). PR 1 covers the VH2 germ line region relative amount of $VH2^{ab}$ transcript, which was significantly higher in axial SpA patients. Axial AS group contains early stage AS and AS patients. * p<0.01, **p<0.05, ***p<0.001 (Kruskal-Wallis test (non-parametric ANOVA) and Dunn's multiple comparison test)

The relative amount of transcripts of target genes compared to those of a housekeeping gene was calculated as follows (Ct: Cycle threshold, R: Relative amount of transcript);

$$\Delta\mathrm{Ct} = \mathrm{Ct}(\mathrm{experimental}) - \mathrm{Ct}(\mathrm{housekeeping}),$$

$$\mathrm{R} = 2^{-(\Delta\mathrm{Ct})}$$

The relative amounts of $VH2^{ab}$ gene expression detected by PR1 were 0.41 ± 0.36 (mean ± SD) for HC, 0.25 ± 0.33 for RA patients, 2.37 ± 1.03 for pre-AS patients, and 7.07 ± 3.16 for AS patients (Figure 19). $VH2^{ab}$ gene expression was approximately 28 fold higher in AS patients and 6 fold higher in pre-AS patients than in HC. $VH2^{ab}$ gene expression detected by PR1 was significantly higher in pre-AS patients than in HC and RA patients ($P<0.05$ and $P<0.01$, respectively).

A comparison between healthy controls, RA patients as well as pre-AS and AS patients (axial SpA) PCR product expression levels using PR1, relative amount of $VH2^{ab}$ transcript was significantly high in axial SpA patient. It is also incorporated with previous result. Notable is that the expression value of pre-AS and AS patients are obviously different. Relative amounts in AS patients were over three times higher on average than those of pre-AS patient samples. On the basis of those results, it is presumed that the $VH2^{ab}$ expression level could reflect the AS progression.

The Q-PCR results detected by PR2 and PR3 are described in the Figure below. PR2 primer produces PCR products which include the region from VH2 leader to CDC42BPB. The PR3 primer contained the CDC42BPB region and the C epsilon (immunoglobulin constant epsilon) region.

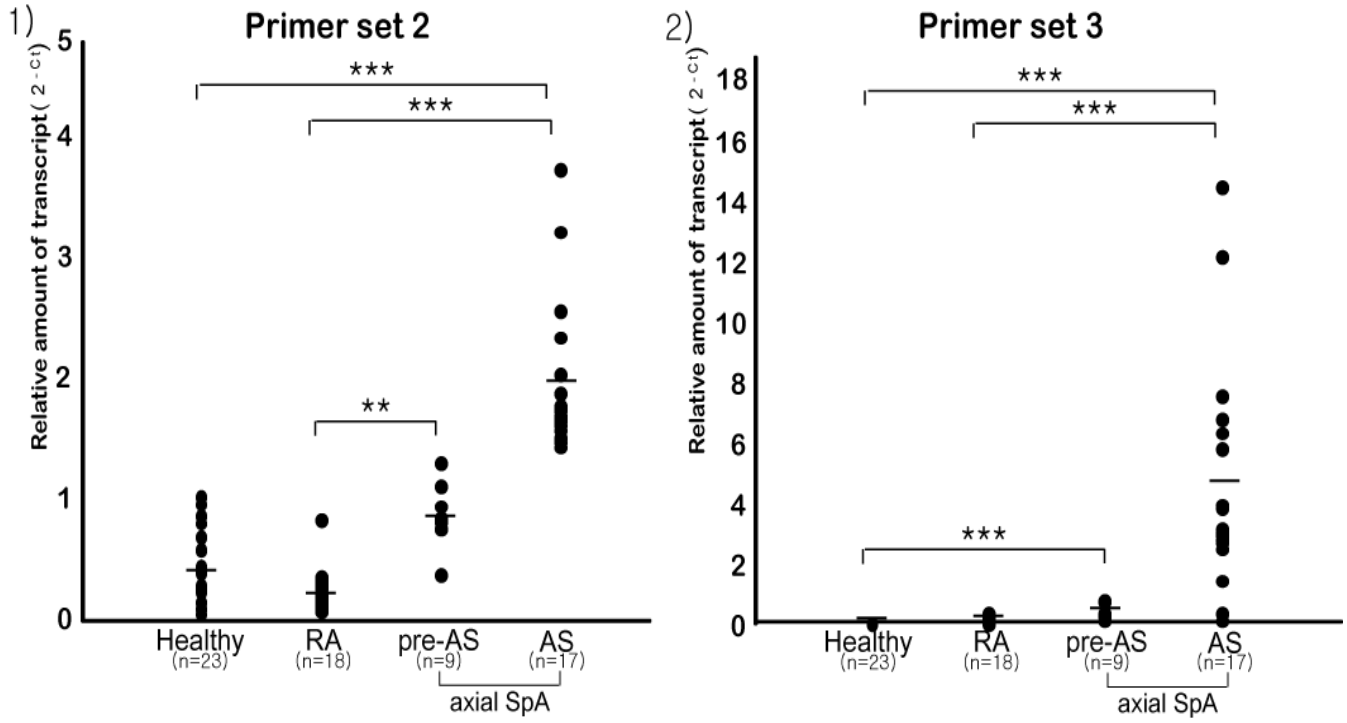


Figure 20. Q-PCRs using PR2 and PR3. The relative amounts of PCR products using primer set 2 (PR2) and primer set 3 (PR3) were significantly higher in axial SpA patients compared with HC, RA patients. * p<0.01, **p<0.05, ***p<0.001 (Kruskal-Wallis test (non-parametric ANOVA) and Dunn's multiple comparison test)

$VH2^{ab}$ gene expression detected by PR2 and PR3 was also higher in axial SpA patients. The relative amounts of PCR products using PR2 were 0.40 ± 0.28 for HC, 0.20 ± 0.18 for RA patients, 0.83 ± 0.26 for pre-AS patients, and 1.95 ± 0.64 for AS patients (Figure 20; not significant for HC vs pre-AS; P<0.01 for RA vs pre-AS). The relative amounts of PCR products using PR3 were 0.01 ± 0.01 for HC, 0.13 ± 0.11 for RA patients, 0.37 ± 0.22 for pre-AS patients, and 5.67 ± 4.46 for AS patients (Figure 20; P<0.001 for HC vs pre-AS; not significant for RA vs pre-AS).

The normalized PCR product expression values were calculated by equations follow (Ct: Cycle threshold); $\Delta Ct = Ct(\text{using PR1, PR2 or PR3}) - Ct(\text{using HuPo primers})$

$$\Delta\Delta Ct = \Delta Ct(\text{RA, pre AS and AS sample}) - \Delta Ct(\text{HC sample})$$

$$\text{Amount of target} = 2^{-(\Delta\Delta Ct)}$$

The average of ΔΔCt for each sample is calculated by subtracting the ΔCt number of patient samples from that healthy donor samples. The $2^{-(\Delta\Delta Ct)}$ are presented as the fold in gene expression amount compared to control group. Amount of PCR products expressions using PR1, PR2 and PR3 were normalized by calculating $2^{-(\Delta\Delta Ct)}$ value and compared directly (Table 12).

Table 12. Normalized expression value relative to patients

	Normalized PCR products amount relative to patients		
	PR1 (fold*)	**PR2 (fold)**	**PR3 (fold)**
HC (n=23)	1.0	1.0	1.0
RA (n=18)	0.9	0.9	1.1
Pre-AS (n=9)	3.9	1.3	1.3
AS (n=17)	81.7	3.0	23.6

* Fold values are obtained by calculating $2^{-(\Delta\Delta Ct)}$

3.2.2. Q-PCR to investigate CD40L expression

The PCR products from PR3 contained the human immunoglobulin epsilon heavy chain constant region. Considering up-regulated IgE level in AS patients and the role of CD40L in Ig isotype switching, we hypothesized that the expression of CD40L be might increased in AS patients. As seen in Figure 21, elevated CD40L expression was detected in axial SpA patients.

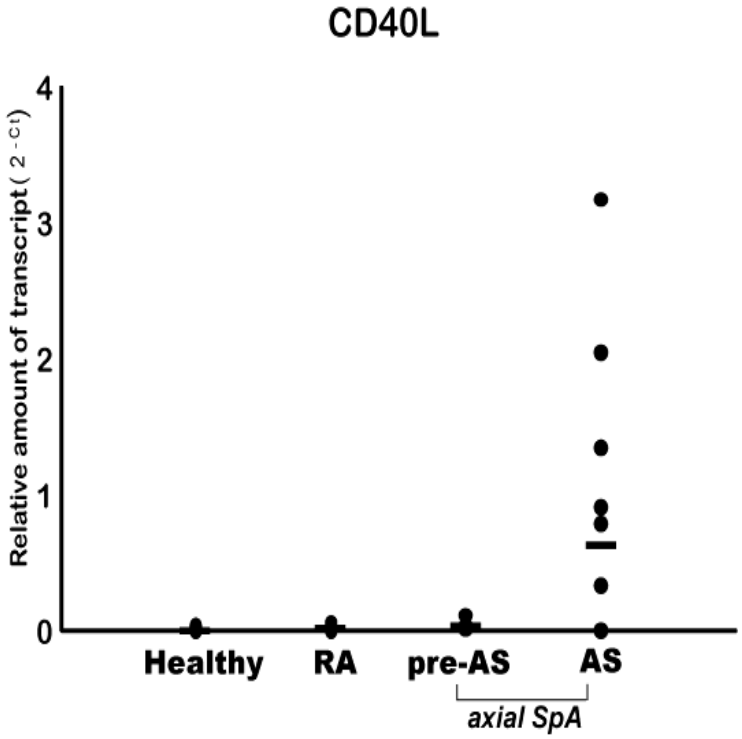


Figure 21. The relative amounts of PCR products of CD40L were significantly higher in patients with AS, ΔCt for each sample (experimental and housekeeping) is calculated by subtracting the Ct values of the experimental gene (CD40L) from that of the housekeeping gene HuPo.

The relative amount of CD40L transcripts were 0.30 ± 0.21 (mean ± SD) for HC, 0.62 ± 0.36 for RA patients, 1.48 ± 0.47 for pre-AS patients and 2.51 ± 0.23 for AS patients. The up-regulation of CD40L was significant in axial SpA, pre-AS and AS patients, compared with HC ($P<0.001$, and $P<0.05$, respectively; Kruskal-Wallis test (non-parametric ANOVA) and Dunn's multiple comparison test**).** However, there were no significant differences between axial SpA, pre-AS or AS and RA patients. Importantly, the correlation of $VH2^{ab}$ and CD40L over-expression in axial SpA patients was significant ($P<0.05$ for PR2 and PR3; $r=0.56$ for PR3). To assess the correlation of $VH2^{ab}$ and CD40L expression in axial SpA patients, Spearmann test was performed.

In AS patients, the relative amounts of PCR products which inclded CD40L were higher than in other groups. The normalized PCR product expression amounts for each group were also calculated. The average of ΔΔCt for each sample was calculated by subtracting the ΔCt

number of patient samples from that healthy control samples. Amount of CD40L transcript expressions were normalized by calculating $2^{-(\Delta\Delta Ct)}$ value and compared directly (Table 13).

Table 13. Normalized expression values relative to patients

	Normalized PCR products amount relative to patients $2^{(-\Delta\Delta Ct)}$			
	HC(n=23)	**RA (n=18)**	**Pre-AS (n=9)**	**AS (n=17)**
CD40L expression (fold*)	1.0	3.4	9.8	9.9

* Fold values are obtained by calculating $2^{-(\Delta\Delta Ct)}$

The $2^{-(\Delta\Delta Ct)}$ values mean that the average fold difference in CD40L gene expression between the AS, pre AS, RA and control group using Q-PCR analysis. Q-PCR analyses revealed that CD40L gene was expressed about ten fold higher in AS patients as compared with healthy controls and three fold higher than RA patients. CD40L is an inducer of Ig class switching, and it has also been reported that impaired CD40L expression can cause a defective Ig class switch recombination. These results suggest the potential involvement of CD40L in isotype switching during AS pathogenesis.

3.2.3. The individual results using PR3

The reverse primer for primer set 3 includes C epsilon regions. If isotype switching rearrangement has a crucial role in AS pathogenesis, PR3 results could reflect that feature. As shown in previous results, the relative amounts of PCR products using PR3 were significantly higher in AS patients than in healthy controls. Therefore, our result might imply that IgE has a certain role for AS pathogenesis. PCR products which are produced by primer set 3 of each patient were analyzed in a 1% agarose gel. The relative amounts of PCR product by PR3 for each sample are shown in Figure 22. (Individual Q-PCR data for PR1, PR2 and PR3 primer

see supplementary materials, pp 138-140)

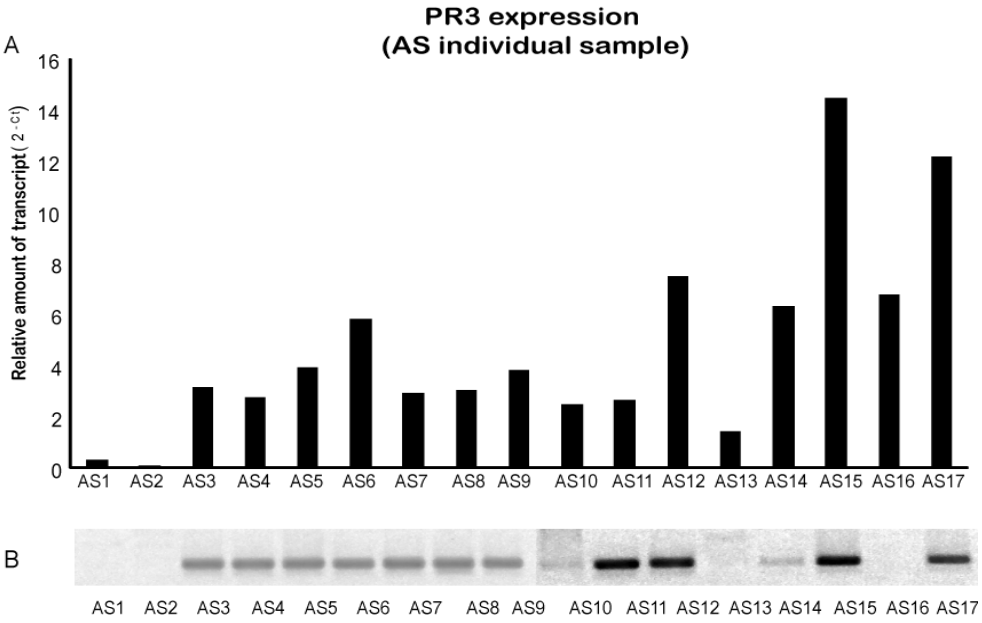


Figure 22. The result of PCR and Q-PCR using primer set 3: A) relative amounts of transcripts using PR3 for individual samples and B) electrophoresis on a 1% agarose gel.

The application plot of PR3 results for each groups, using analysis by Mx300P software, are described in Figure 23. In AS patient, the Ct values of target region (CDC42BPB forward to C epsilon backward) were clearly different with HC, RA and SpA results.

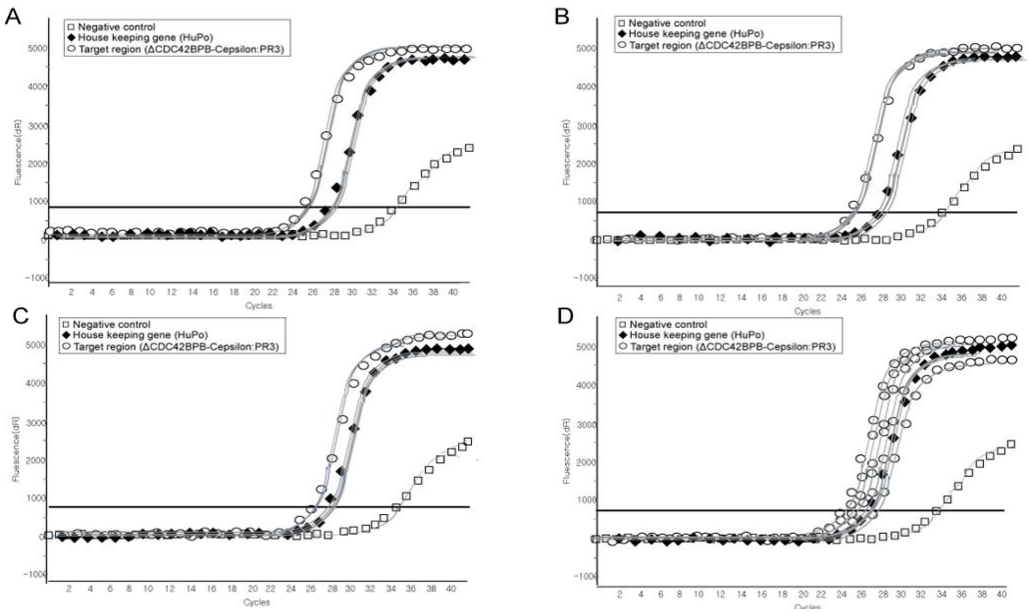


Figure 23. Amplification plots of PR3 results, using analysis by Mx300P software; A) HC sample, B) RA patient sample, C) pre- AS sample, 4) AS patient sample

$VH2^{ab}$ over-expression detected by all 3 primer sets was significant in AS patients, compared with both HC and RA ($P<0.001$ for all comparisons). Notably, $VH2^{ab}$ over-expression was specific to axial SpA (pre-AS and AS) patients, but not to RA patients. The results suggest that $VH2^{ab}$ over-expression could be used as early diagnostic tool for axial SpA. These results also support the inversion of CDC42 BPB gene into Ig gene.

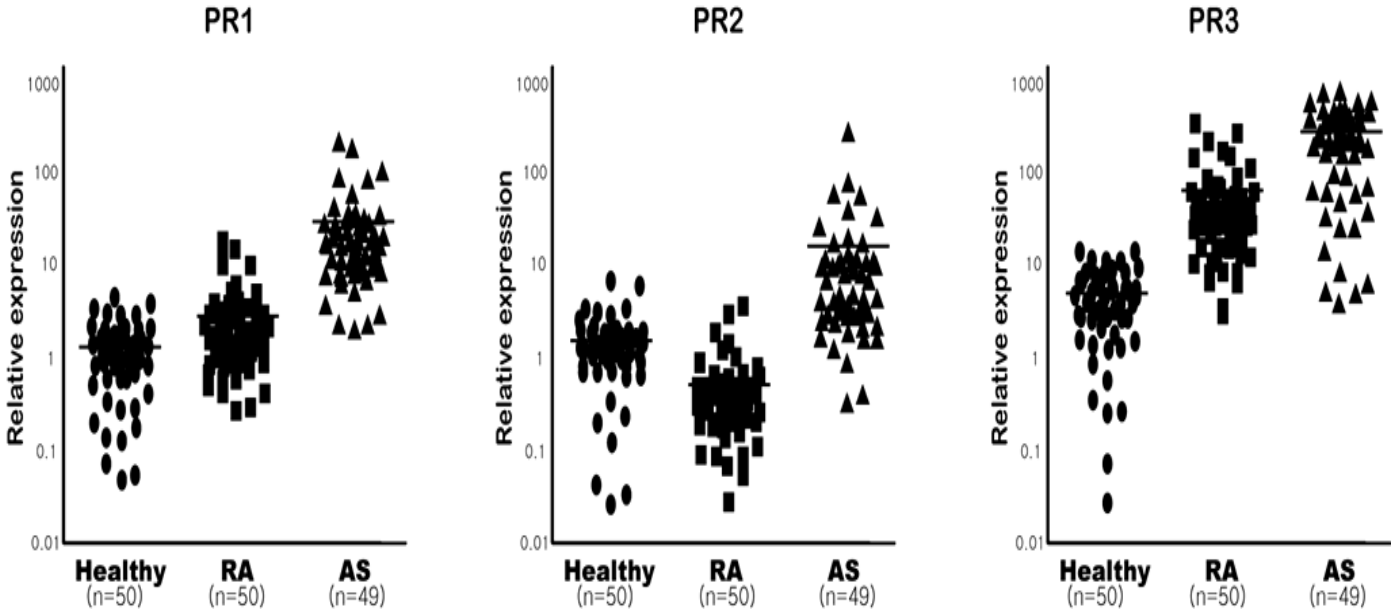
3.3. Investigation of $VH2^{ab}$ gene and CD40L gene expression in increased sample

In order to confirm and extend our previous results, we conducted other experiments with highly increased numbers of smaples (49 AS, 50 RA and 50 HC). Samples were collected from Hanyang University College of Medicine and Eulji University Hospital. The condition of sample collection and cDNA preparation were almost the same as in previous research. However, this time the SYBR® Green Realtime PCR Master Mix (TOYOBO, Japan) and Bio-Rad CFX96 Real Time PCR detection system were used. The statistical significances were determined using a one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. Table 14 and Figure 23 show the relative expression of $VH2^{ab}$ transcripts. In all cases, by using PR1, PR2 and PR3, relative expression levels are significantly higher in AS patients than in RA or HC and those results are in consistence with our previous data.

Table 14. Comparison of AS, RA and control by $VH2^{ab}$ transcript level ($R=2^{(-\Delta\Delta Ct)}$)

	Transcript level			
	AS (n=49)	**RA (n=50)**	**HC (n=50)**	**P value[a]**
PR1	33.99±46.84[b]	3.10±3.99	1.52±1.16	0.000
PR2	16.46±41.30[b]	0.48±0.63	1.52±1.25	0.001
PR3	245.8±231.61[b]	40.19±55.60	1.93±1.72	0.001

[a)] Statistical significances were tested by oneway analysis of variances among groups



[b] Significant diffenece between groups based on Tukey's multiple comparison tests

Figure 24. Comparison of AS, RA and HC (healthy control) using VH2[ab] transcript levels (samples: 49 AS, 50 RA and 50 HC)

In addition, to get more supportive evidence of our hypothesis in section 2.2.3, which suggests Ig class switch deficiency might be influenced on AS pathogenesis, we investigated the expression of CD40L as well as CD40 expression. CD40 is a costimulatory protein and CD40L is its counter receptor. CD40 is known to be related with a pathogenesis of various autoimmune diseases (e.g. IBD, SLE and RA etc) the defected CD40 signaling pathway (Korthäuer et al., 1993; Durandy *et al.*, 1993; Agematsu *et al.*, 1998) could generate the deficiency of class switch recombinations. Moreover, the interaction between CD40 and CD40L triggers Ig class switch recombination to IgE and this interaction has an important role on B cell activating (Vercelli, 1995; Oettgen and Geha, 2001).

Analysis on the expression level of CD40L and CD40 transcript were conducted with the same samples (49 AS, 50 RA and 50 HC).

Table 15. Comparison of AS, RA and control by CD40L and CD40 transcript level ($R=2^{(-\Delta\Delta Ct)}$)

	Transcript level			
	AS (n=49)	**RA (n=50)**	**HC (n=50)**	**P value[a]**
CD40L	1.43±1.9[b]	0.92±0.57	0.74±0.38	0.000
CD40	0.85±0.39[b]	0.52±0.29	0.60±0.30	0.001

[a)] Statistical significances were tested by oneway analysis of variances among groups
[b)] Significant diffenece between groups based on Tukey's multiple comparison tests

The SYBR® Green Realtime PCR Master Mix (TOYOBO, Japan) and Bio-Rad CFX96 Real Time PCR detection system was also used.

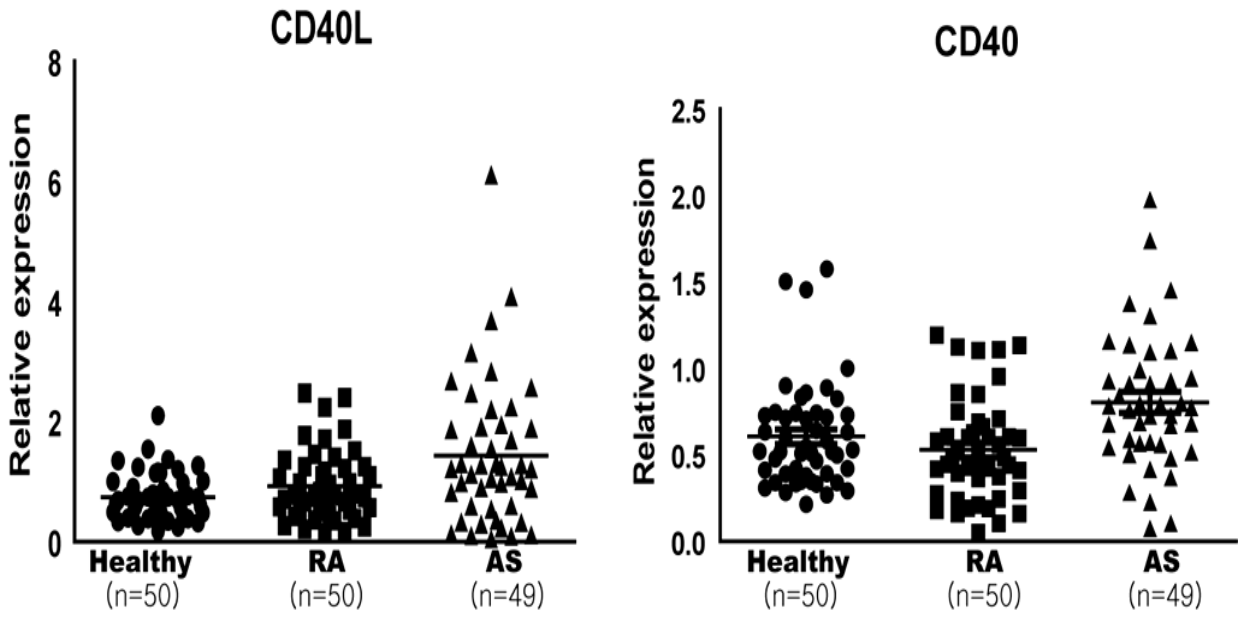


Figure 25. Comparison of AS, RA and control by CD40L and CD40 transcript levels (samples: 49 AS, 50 RA and 50 HC)

Theexpression levels of CD40L were about two times higher in AS than RA or HC, and those of CD40 were also slightly increased. The statistical significances were also determined using an one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests and the results from AS patient was significant.

4. DISCUSSION

(Summary)

Although recent studies have suggested that B cells play an important role in AS, the molecular and cellular mechanisms of B cell-associated pathogenesis of AS remain unclear. The disproportion of B cell subsets in PBMC of AS might affect disease progression. However, no data have been reported on the composition of B cells in PBMC of AS patients, and evidence for the role of B cells on AS remains limited. Through the analysis of VH region repertoires usage, uniquely rearranged VH2 transcripts were found ($VH2^{ab}$). Our findings suggest that abnormal molecular events in B cells might be associated with AS pathogenesis. This led us to the hypothesis (Figure 18, section 3.1.7.) involving the paracentric inversion on chromosome 14 and/or class switch recombination deficiency, which might be associated with AS predisposition. To test this hypothesis and examine it in-depth, we studied the $VH2^{ab}$-CDC42BPB-JH3 expression in cDNA from PBMC of axial SpA (pre-AS and AS, see section 3.2).

We provide evidence that $VH2^{ab}$-CDC42BPB-JH3 is overexpressed in AS patients (Figure 10, Figure 12, and Figure 15). The comparable amounts of PCR product using PR1 in AS, as compared with healthy donors and RA patients (Table 12 and Figure 19, 24) suggest that this phenomenon is closely related to AS susceptibility and not simply a product of Ig clonal expansion. This is further supported by additional primer sets (PR2 and PR3) that encoded surrounding regions. The overexpression of the C-epsilon -included product (Table 13 and Figure 20) implicates impaired class switch recombination. To collect stronger evidence, we investigated CD40L gene expression which regards as an inducer of Ig class switching. CD40L gene was over-expressed in AS patients as compared with healthy controls as well as

RA patients (Figure 21 and Table 13). This result suggests the potential involvement of CD40L in Ig class switching during AS pathogenesis implying that there is a plausible link between immunoglobulin production deficiency and AS.

In order to confirm our previous results, and to evaluate primer specificity, further investigation with highly increased numbers of smaples (49 AS, 50 RA and 50 HC) were conducted and the results were in good agreement with our previous data (Figure 24, 25 and Table 14, 15).

These findings provide a plausible link between Ig abnormalities and inflammatory disease in human AS, suggesting that the dysregulation of Ig genes may explain the unclear features of AS. Moreover, newly designed VH usage screening primer sets could be applied to an effective diagnosis system development.

4.1. Unusual immunoglobulin (Ig) gene rearrangement and recombination

Ig gene rearrangement requires recombination signal sequences (RSS) composed of a heptamer, 12/23 nucleotides (nts) spacer, and a nonamer in this order (Schatz *et al*., 1989) and theses RRS motifs are conservative sequence which guides the V-(D)-J recombination mechanism (Fugmann *et al*., 2000; Jung and Alt, 2004)..

Recombination signal sequences with 23bp spacer always rearrange to a 12bp spacer RSS motif. This is called 12/23 rule (Meek *et al*., 1989), and it is maintained in all seven Ig loci (Tonegawa, 1988; Alt *et al*., 1986; Ramsden *et al*., 1984). A VH region having 23bp RSS motifs is joined only to 12bp spacers. DH gene segments are flanked by signal sequences with 12bp spacers, and these can be joined with JH and VH segments (Figure 26). These recombination processes produce V-D (23-12) and D-J (12-23) rearranged segments and through this rule, V-J (23-23) or D-D (12-12) is precluded (Meek *et al*., 1989; Ramsden *et al*.,

1984) .

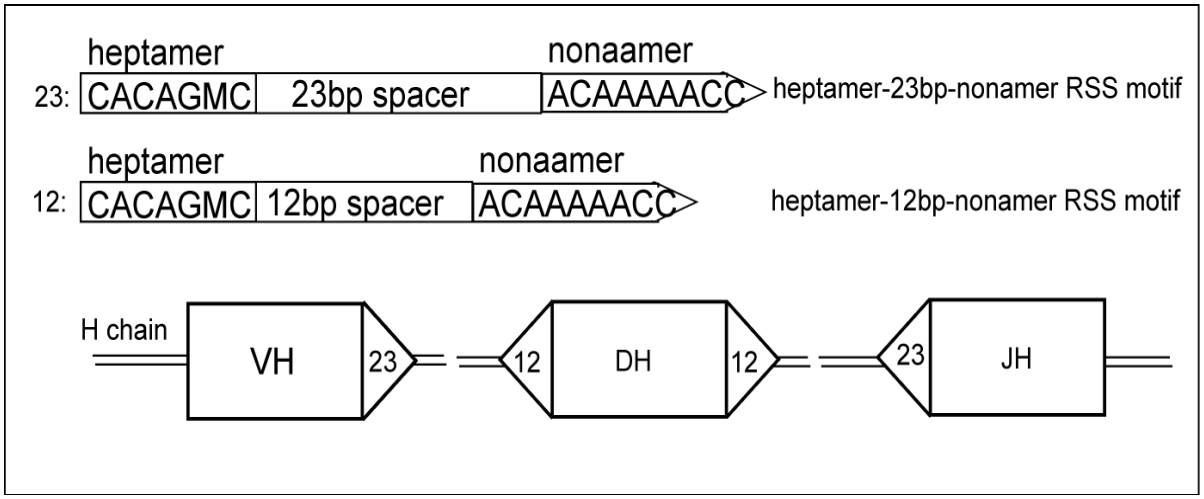


Figure 26. Recombination signal sequences are conserved heptamer and nonamer sequences that flank the gene segments encoding the V, D, and J regions of Ig. According to the 12/23 rule, the arrangement of RSSs in the Ig heavy chain gene segments precludes direct V to J joining (M amino acid sequence A or C).

In order to generate a functional V region, two separated recombination events are required. Recombination occurs at the ends of the RSSs and undergoes the looping out and deletion of the DNA. The specific details of the recombination process to generate V exon are described in Figure 27. As shown below, the V, D and J gene segments are initially oriented in opposite transcription directions. Binding together the RSSs through 12/23 rule creates DNA looping, after which DNA intervening is repeated and a functional V region exon is generated (Figure 27).

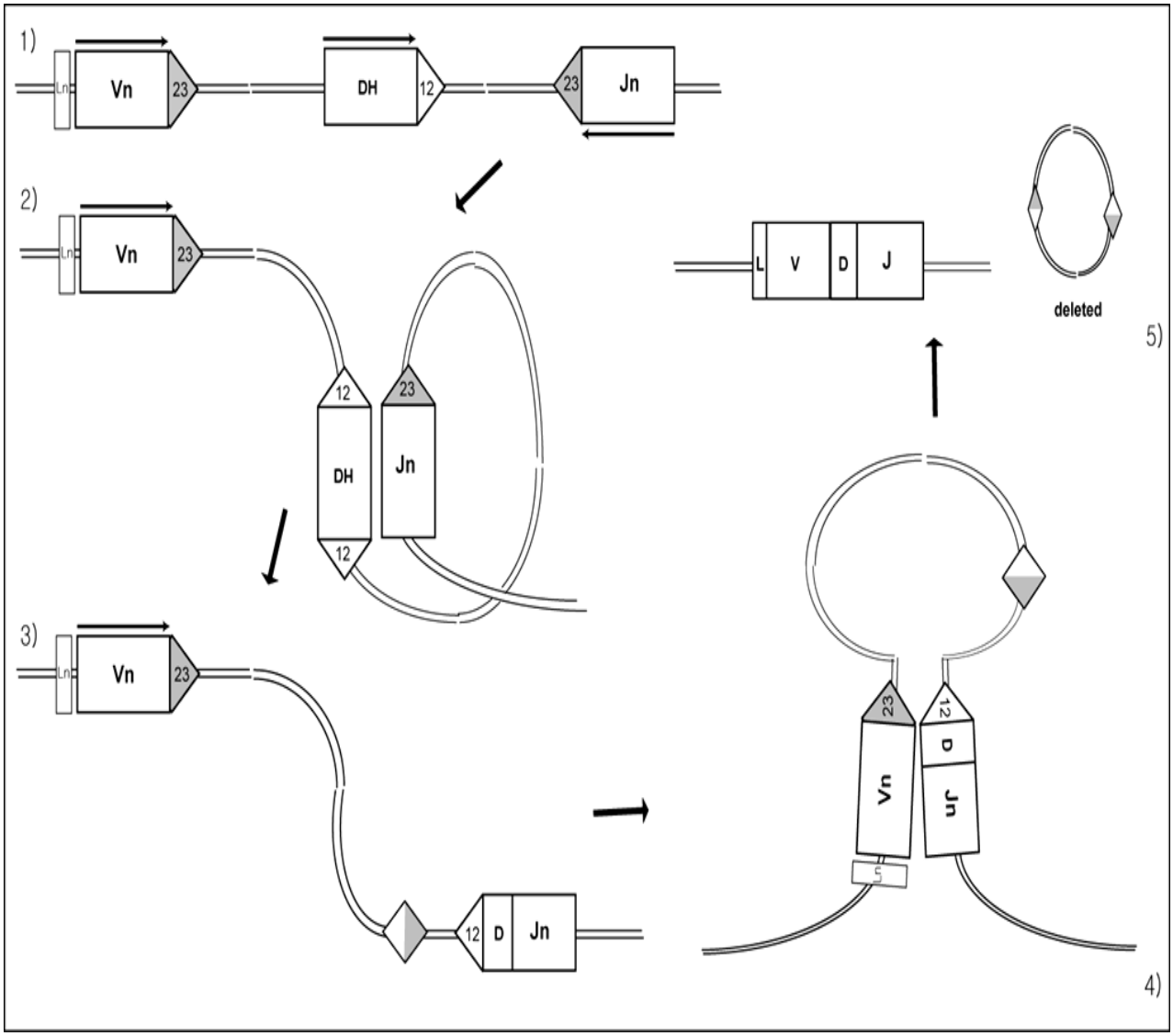


Figure 27. V region gene segments are joined by the recombination process. Recombinational signal sequences allow taking place. The joining of V and J segments creates a functional V region exon. Binding together the RSSs through 12-23 rules creates DNA looping ((1) to (4)), after which DNA intervening is repeated and a functional V region exon is generated (5).

A short stretch of intron sequences of CDC42 BPB has been found between VH and JH genes in the antibody repertoire of an AS patient (section 3.1.5, Figure 15 and 16). Among 100 sequences of positively cloned colonies, 48 of sequences revealed that a short fragment from CDC42 BPB gene was incorporated into the $VH2^{ab}$ .The most 5' end of this fragment includes a region (15 nts) homologous to that of the VH2 germline gene (IGHV2*70-12) (Figure 28).

Further analysis of inserted sequences helped identify a V-D junction and a DH6 sequence. Interestingly, the analysis of CDC42 BPB genomic sequences has led to the identification of a heptamer-12bp spacer-nonamer RSS motif which, following an insertion site of this fragment by 18 nts, has been found to exist in the CDC42 BPB genes. In addition, a heptamer-like sequence (CACAGAG), which has a single nucleotide base, changed feature to a generally known heptamer of RSS (CACAGTG). Nonamer-like sequences also appeared after 12 base pair spaces in the middle of the inserted CDC42 BPB intron genes (Figure 28).

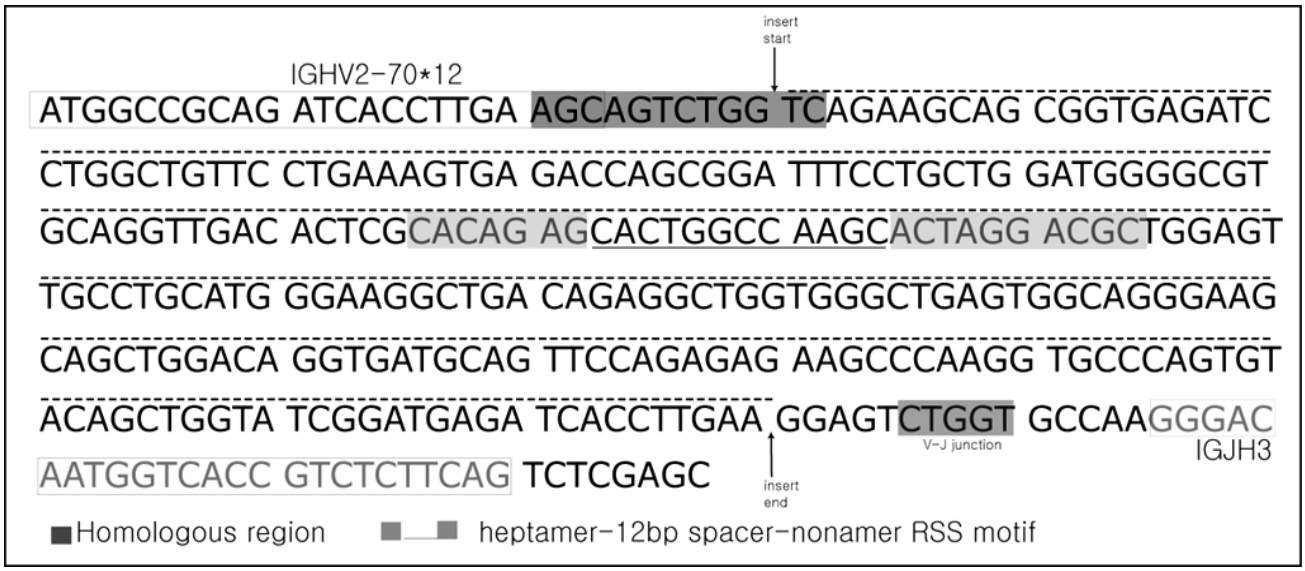


Figure 28. Sequence analysis. 252bp of CDC42 BPB sequences identified a heptamer-12bp spacer-nonamer RSS motif in CDC42 BPB genes.

This gene structure implies that unusual gene rearrangements or recombinations may occur during the construction of VH gene segments in the process of B cell development in some of the AS patients. Our sequence analysis result in Figure 28 shows that uniquely arranged V exon, including heptamer-12bp spacer-nonamer RSS motif, in between VH2 and JH3. Based on this sequencing analysis result, we assumed that 252bp of CDC42 BPB gene including 12bp-RSS motif like sequence was inserted after D-J recombination process. Although further investigations are required to verify how these unfitted nucleotides were inserted in

AS patients' antibody sequences, this unique rearranged $VH2^{ab}$ gene structure might be explained by paracentric inversion following D-J recombination (Figure 29). A paracentric inversion heterozygote rarely causes actual genetic abnormality. However, it is indistinguishable from the paracentric inverted insertion heterozygote and in this case the risk for genetic abnormality is increased up to 15% of individuals (see 1.4.2.3).

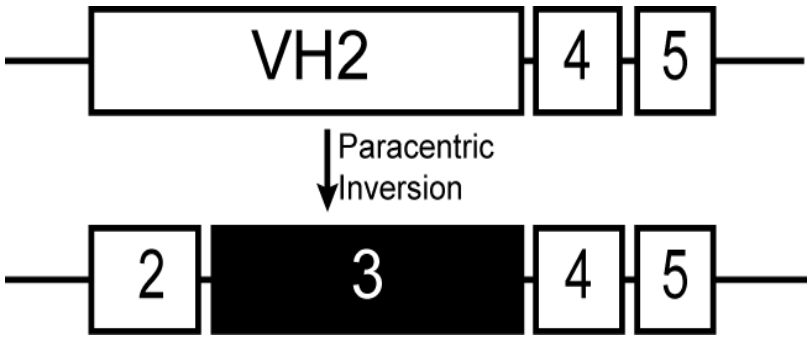


Figure 29. Unique antibody rearrangement may occur in patients with AS. The sequence analysis and homology search proposed that possible paracentric inversion of CDC42 BPB intron fragment to VH2 genes. The numericals indicate the gene fragments are: 2) VH2, 3) CDC42BPB, 4) DH, 5) JH.

In order to validate the assumption that the incorporation of the intron fragment of CDC42 BPB into Ig heavy chain gene segments, PR2 and PR3 were designed (each primer sequences present in Table 5 and 8). PR2 could use to detect the VH2 leader region and the middle of the intron of CDC42 BPB gene which, according to our assumption, inverted into Ig VH2 regions in AS patients. On the basis of PCR result in section 3.1.6 (in Figure 17), PR3 was designed in the middle of the CDC42 BPB intron and Cε region (Ig constant region of epsilon isotype: IgE).

The combined use of PR1, PR2 and PR3 allows us to detect abnormally over-expressed $VH2^{ab}$ transcript in AS patients. Because of limited sample numbers (9 of healthy controls and 8 of AS patients), Figure 12 result could not show statistical significant. However, further

experiments (in Figure 19, 20) used highly increased sample size and we could measure the statistical significance (using Kruskal-Wallis test and Dunn's multiple comparison test) of the expression of $VH2^{ab}$ transcript in AS patient patients patients ($P<0.001$ for HC vs axial SpA by PR1, 2, and 3 and for RA vs axial SpA by PR1 and 2 and $P<0.01$ for RA vs axial SpA by PR3).

4.2. Ig class switch recombination (Ig CSR) deficiencies

Antigen-specific Ig classes are categorized by different heavy chain constant regions (Geha *et al*., 2003). Five major isotypes, IgM, IgD, IgG, IgE and IgA, are conjugated with Cμ, Cδ, Cγ, Cε and Cα, respectively (Wurzburg *et al*., 2000). For the assembly of a functional IgG, IgE and IgA gene, isotype switching is required after B cell activation (Oettgen and Geha, 2001). As regards serum total Ig level, it has been suggested that total IgA levels are increased in AS patients (Cowling *et al*., 1980; Franssen *et al*., 1985; Collado *et al*., 1987; Sanders *et al*., 1987; Hocini *et al*., 1992; Calin, 1993; Mackiewicz *et al*., 1989). IgA represents mucosal humoral immunity (Franssen *et al*., 1985; Collado *et al*., 1987; Sanders *et al*., 1987; Granfors and Toivanen, 1986), which manifests in the clinical and biological activity of AS, such as gastrointestinal inflammation. However, results as to the association between AS and IgA are still contradictory (Collado *et al*., 1987; Sanders *et al*., 1987; Trull *et al*., 1984).

Concerning IgE isotype, it is common knowledge that IgE is associated with atopic condition and anti-parasitic defense mechanism (Geha *et al*., 2003). A great variety of cytokines produced by T-cells and other cells are related to the regulation of IgE, and this regulation is known to be controlled rigidly (Chowdhury, 1995) even under severe atopic conditions (i.e., typical levels of IgE to allergens are lower than 100 ng/mL in patient body fluid) (Ownby, 1993). There is some evidence indicating that IgE may have a role in the pathogenesis of

immune mediate diseases. High concentration of total IgE has already been detected in RA patients' serum (Grennan and Palmer, 1979; Hunder and Gleich, 1974). In addition, Gruber *et al* conducted an investigation on synovial mast cells in RA patients and suggested that IgE may contribute to joint inflammation and may even have a crucial role in RA pathogenesis (Gruber *et al*., 1986). However, the potential role of IgE in RA as well as AS has yet to be established. In 2009, a clinical study for HLA-B27 positive SpA patients showed that IgE levels against some allergens were significantly higher than those for controls (Kamanli *et al*., 2009). A previous study reported elevation of IgE concentrations in 16% out of 45 central AS patients and in 40% out of 50 peripheral AS patients (Guseĭnov, 1991). However, more studies are required to determine IgE and SpA relationship.

Interactions among cytokines, such as interleukin-4 (IL-4), IL-13 and CD40-CD40L are well-known important factors in the classical pathway of IgE production (Geha *et al*., 2003; Oettgen and Geha, 2001) (Figure 30). IL-4 and IL-13 are commonly discussed inducers of constant epsilon germline transcription (Oettgen and Geha, 2001).

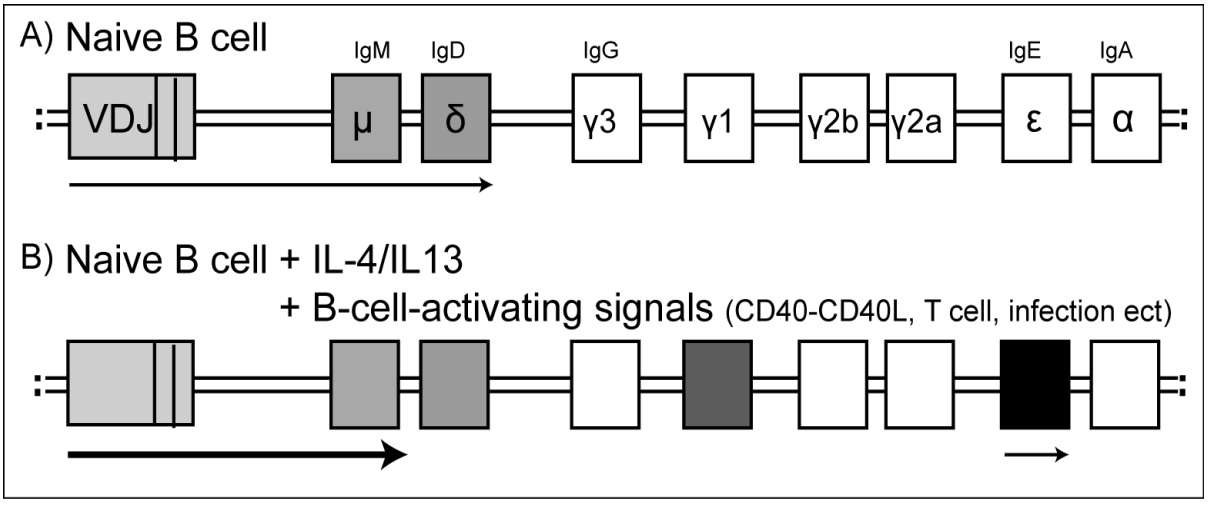


Figure 30. Class switching is preceded by transcriptional activation of heavy chain C-region genes; A) naive B cell-transcribed heavy chain μ and δ at low rate, B) IL-4/IL-13 signal and B cell-activating signals, such as CD40-CD40L interaction, T cell and bacterial infection, give rise to Cε transcription

However, IgE switching could not be completed without secondary B-cell-activating signals, including CD40-CD40L interaction (Vercelli, 1995), which arise from the T cell surface (Oettgen and Geha, 2001). T and B cell interaction and IgE production mechanisms are presented in Figure 31. The antigen-antibody complex present in the MHC II molecule is recognized by the T-cell receptor (TCR), leading to CD40L expression by Th2 cell. The CD40-CD40L interaction triggers class switch to IgE and expands the IgE-producing clone in the B cell, thus inducing B cell proliferation (Oettgen and Geha, 1999).

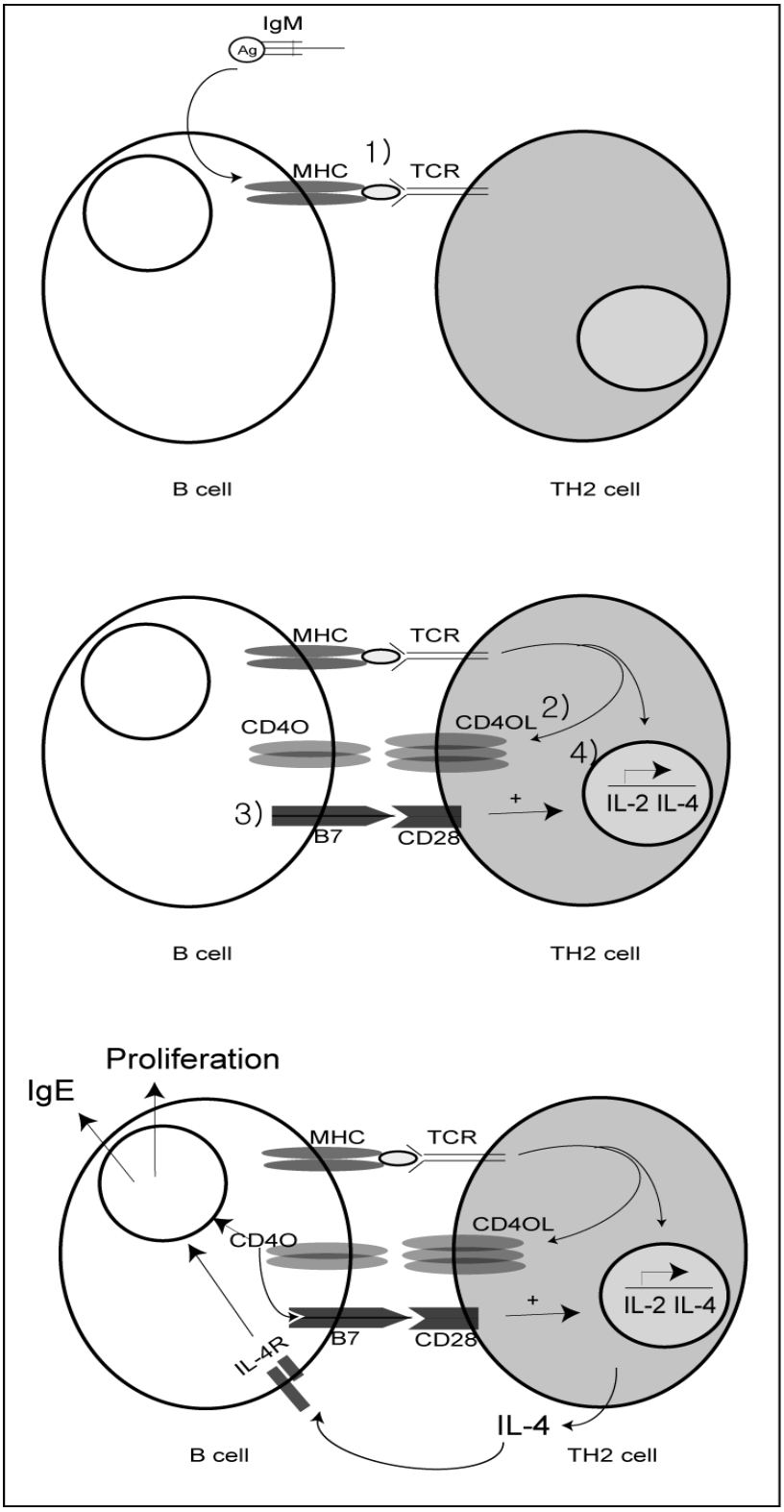


Figure 31. T- and B-cell interaction and signals for IgE production: (1) presentation, MHC class II molecules and recognition by the TCR, (2) CD40L expression. CD40L-induced aggregation of CD40 then triggers expression (3) of B7 (CD80), (4) amplifies cytokine synthesis, (5) produces IL-4 in conjunction with CD40 ligation (signal 2), and triggers the IgE isotype switch, B-cell proliferation, and expansion of the IgE-producing clone.

In 2009, Ghada S *et al*. determined that the aberrant of CD40L and CD40 interaction and their signaling pathways can affect the initiation of inflammatory and non-inflammatory diseases (Ghada *et al*., 2009). The CD40 activation directly contributes to isotype switching of Ig genes and the introduction of somatic mutations particularly in B cell (Hu *et al*., 1997). It is also well-known direct inducer of various cytokines (IL-6, IL-10, TNF-α), costimulatory receptors (Inter-Cellular Adhesion Molecule (ICAM), CD23, B7.1/CD80, B7.2/CD86), and MHC class I, MHC class II, and TAP transporter by B cells (Khanna *et al*., 1997). It has been reported that CD40-CD40L interaction is related to the regulation of B cell proliferation, Ig production, Ig class switching, and B cell protection from cell death, germinal center formation, and memory B cell generation (Ballantyne *et al*., 1998).

The encoding region of primer set 3 is included C epsilon and this region is related with IgE. The antibody gene structure which we observed implies that the unusual gene rearrangement or recombination, including IgE region, may occur during construction of VH gene segments during B cell development in some of the AS patients (Kim *et al*., 2010). This line of thought is supported by the result from CD40L expression. The results in section 3.2.2, Table 15 and Figure 21, 25, show that CD40L is increased in AS patients as compared with both HC and RA patients. These results could support our hypothesis; the impaired Ig class switch recombination might have influenced on the pathogenesis.of AS.

4.3. B cells are involved in AS/SpA pathogenesis

Despite many reports that have already been described regarding the disturbances of B cells in a number of autoimmune diseases (Baeten *et al*., 2008; Shi *et al*., 2003; Zhang *et al*., 1998; O'Neill *et al*., 2005), it is still unclear whether or not B-cells have an important role in AS pathogenesis. Unfortunately, previous studies have been limited in elucidating the role of B

cells in AS, mainly because these studies have focused more on T cells. Nevertheless, further investigation is required as to whether B cells in PBMC of AS patients are actually misbalanced. In the studies of systemic lupus erythematosus and RA patients, CD19+CD27+ B cell expression is significantly disturbed, and it has been determined that B cell is clearly associated with both diseases (Jacobi *et al*., 2003; Odendahl *et al*., 2000; Lindenau *et al*., 2003). Lin *et al.* investigated the imbalance of B cell subsets in PB samples of AS patients and suggested that CD19+B cells might play a significant role in AS pathogenesis (Lin *et al*., 2009). 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*., 2010).

Our study, along with Voswinkel's work (Voswinkel *et al*., 2001) showed abnormal molecular event in B cells from AS patients. Taken together, these results suggest that B cells may play a role in developing AS. Specifically, since the autoantibodies are not identified in AS, B cells might contribute to AS/SpA 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 through the presentation of autoantigen and subsequent T cell activation as well as by producing specific antibodies (O'Neill *et al*., 2005). Through bacterial cloning and sequence analysis, we found that impaired rearranged VH segment which include CDC42BPB intron fragment. Following experiment results reaveled that this impaired segment is over-expressed in AS patient compared with HC, as well as RA patient. These imply the possible involvement of B cells in AS pathogenesis in an alternative way. In addition, our study provides newly designed primer sets which can be used for the development of early diagnostics for AS.

4.4. Chromosome 14q32

Ig consists 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, and of these, only the chromosome 14 locus contains the JH segments essential for somatic generation of the VH genes. In chromosome 14, a total of 95 VH gene segments have been reported and only half of these are known to be functional (Cook *et al*., 1994; Matsuda *et al*., 1993). It has been reported that the individual VH gene usage for recombination process was altered by B cell neoplasms and by autoantibodies; moreover, it induced certain VH genes repetition on patients' antibody repertories (Scott *et al*., 1989; Pascual and Capra, 1991; Cuisinier *et al*., 1989; Schroeder *et al*., 1987; Yancopoulos *et al*., 1984; Stevenson *et al*., 1993; Kipps *et al*., 1990; Kipps *et al*., 1987; Kipps *et al*., 1988). 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 devised novel primer sets to detect $VH2^{ab}$ transcript which belong to the VH2 germline gene family but had been absent in the previous study. It could be clearly shown that $VH2^{ab}$ genes were overexpressed only in AS patients and the level of expression was significantly higher in PBMC of AS patients compared with those of healthy donors. This suggests that the restricted germline gene family may be selected in AS patients. The Ig V gene repertoire in PBMC was not compared with either synovial tissues or synovial fluid in the same patients in this study. The Ig V gene repertoire in PBMC may be different from that in inflammatory joints. Specifically, PBMC 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 a subset of B cells responding to antigen and undergoing antigen-driven response. Thus, some

interesting results can be found by comparing Ig contents of synovial B cells with those of PB. Interestingly, the sequence analysis and homology search of overexpressed $VH2^{ab}$ PCR products revealed unexpected features of VH gene structure. For example, a short stretch of CDC42 BPB intron gene was found in the sequences of $VH2^{ab}$ PCR products (Figure 15, 27). CDC42BPB is a member of the serine/threonine protein kinase family. A previous study has reported that isoforms of myosin light chain kinase (MRCK) have an effect on CDC42 cellular structure during the process of cytoskeletal reorganization (Leung *et al*., 1998). CDC42BPB is also known to be a potent transducer of diacylglycerol signals; therefore, it might have a carcinogenesis activity (Choi *et al*., 2008). The location of CDC42BPB is chromosome 14q32 (Figure 32), and this region is a common chromosomal translocation-induced area. This region is also closely related with inherent susceptibility of B cell lymphoma (Cigudosa *et al*., 1999). Among the four types of chromosome abnormality (i.e., duplications, deletions, inversions and translocations), inversion occurs at low frequencies but it is detectable (Broman *et al*., 2003). Using standard cytogenetic analysis, paracentric inversion (centromer excluded) is detected, and has 1 to 5 per 10,000 individual frequencies (Pettenati *et al*., 1995). Although the submicroscopic inversions are not yet detected, inversion might be a cause of specific heritable disease (Bondeson *et al*., 1995; de Kok *et al*., 1995; Jobling *et al*., 1998; Lagerstedt *et al*., 1997; Lakich *et al*., 1993). Clinically, inversion events are quite noteworthy because if recombination occurs within the inverted region, it can produce segmental aneusomies or accompanying abnormalities (Broman *et al*., 2003).

Through our study, paracemtric inversion products were detected in AS patients' antibody. As can be seen Figure 5, paracentric inversion product is indistinguishable from paracentric inverted insertion product. Moreover, it has been already reported that if these are paracentric inverted insertion heterozygotes, the risk of the actual genetic abnormality occurrence increases to 15% of individuals. Although the chromosomal analysis in patient with AS

wasn't performed in present study, sequence analysis as described in Figure 15 and 28 refer to the possible occurrence of the paracentric inversion between Ig VH2 and CDC42BPB site on AS patient's chromosome 14. Because of the short distance between CDC42BPB and IgVH2 (Figure 32), this assumption could be a possible explanation on our hypothesis; the aberrant $VH2^{ab}$ segment could influence on AS pathogenesis.

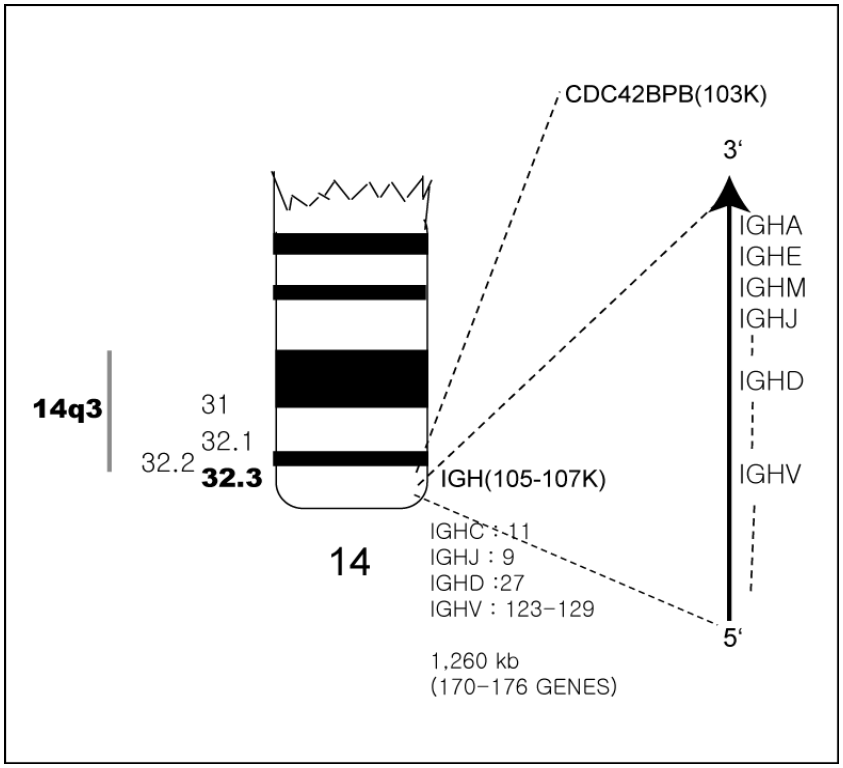


Figure 32. Genes on chromosome 14q3. The distance between CDC42BPB and IgVH2 is around 1.5K to 2K.

4.5. Unique VH region genes are construct unique V segment in AS patient

Based on our experiments and findings discussed above, our hypothetic model of VH construction in AS is described on Figure 33. The right panel depicts the classical pathway to construct the VH region. VH regions are constructed from three kinds of gene segments: variable (V), diversity (D), and constant (C).

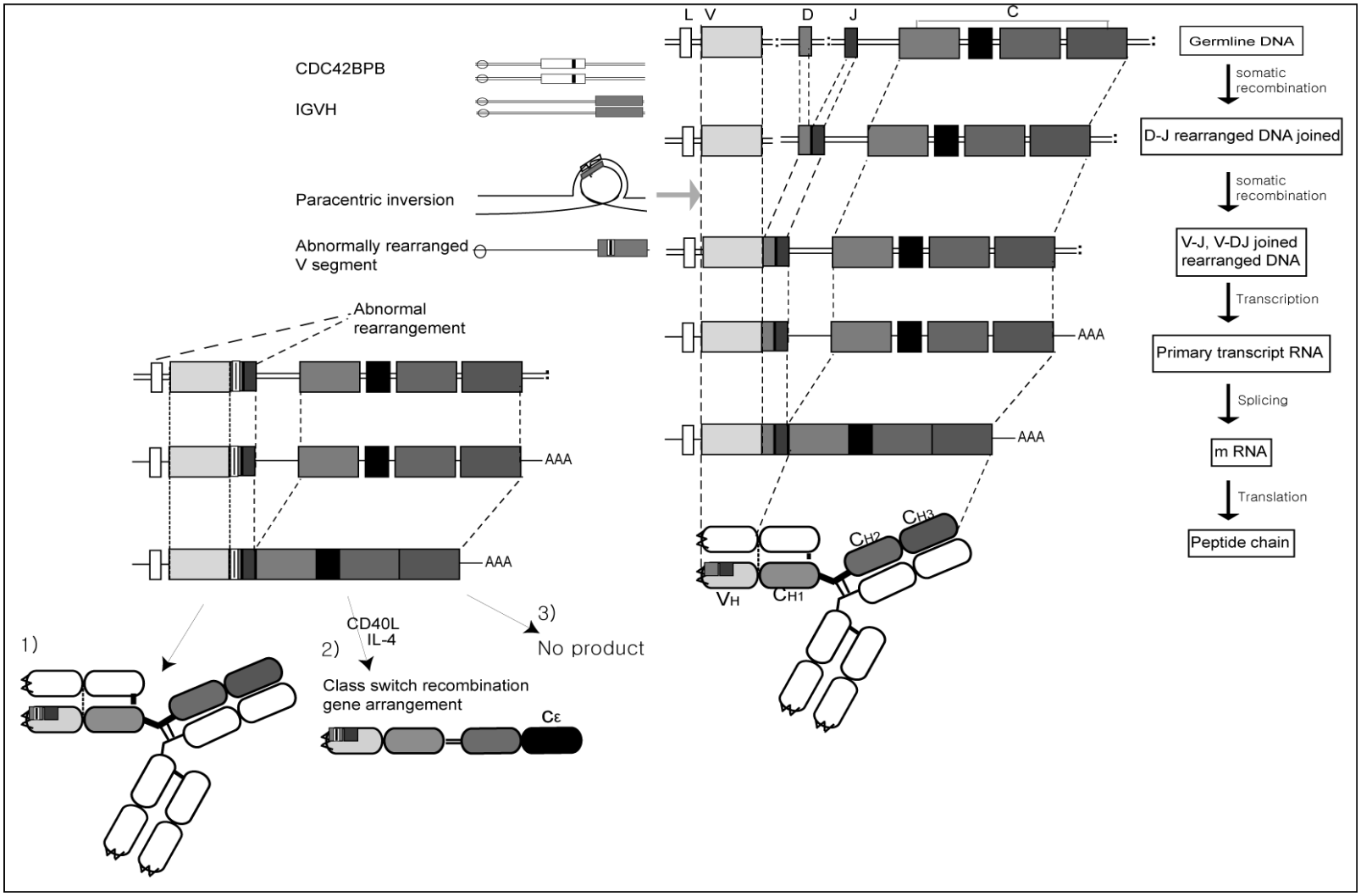


Figure 33. Human antibody VH region genes are constructed from variable gene segments. Normal antibody formation (Right panel). Expected AS patient antibody formation (Left panel). Abnormal rearrangement of V gene segments might affect the rest of the Ig production process resulting in: 1) autoimmune antibody production in AS patients, 2) abnormalities in AS patients and 3) failure to produce complete Ig molecule

In our hypothetical model, following the joining of D and J gene segments, V segment combination takes place completing the VH exon formation. In subsequent heavy chain RNA transcription process, C region exons are spliced and after translation, disulfide bonds connect the polypeptide chains generating the V segment. Upon identifying the CDC42BPB gene, which included the 12bp RSS motif in DH and JH regions, it is assumed that paracentric inversion might occur after the D-J rearranged DNA joining. As the result of abnormal rearrangement of V gene segments, a unique V-(D)-J segment is generated. Usage of this rearranged V segment in the rest of antibody production process might result inabnormalities sin AS patients (Figure 33- left panel). Our gene analysis results obtained by using the PR3 primer set suggests that in the rest of the process, V-(CDC42BPB)-J segment is combined with the C epsilon gene. Thus, CD40L overexpression may have acted as an inducer for class switch recombination.

As depicted in Figure 33 left panel below, this might have an influence on AS pathogenesis in several different pathways. First, abnormal rearrangement segments complete V region construction and produce antibodies and possible auto-antibodies. However, certain auto-antigens have yet been found in AS patients (Appel *et al*., 2009). Binding partner screening from the serum of AS patients against this aberrant gene product would be informative as it can help to determine whether or not the rearranged Ig actually produces protein. However, it is possible that the aberrantly rearranged Ig genes are not functional, making them incapable of producing protein. Nevertheless, aberrant Ig transcripts can still affect other Ig protein production or composition in one way or another. If abnormal rearrangement segments fail to form complete antibodies, it can be translated to produce etiological or disease progression signals. Moreover, even if it also failed to be translated to polypeptides, it might still be able to induce misbalanced antibody population or disturb the innate immune system. This means that abnormal rearrangement segments might refer to AS susceptibility. In light of this

possibility, this would be applicable to early AS diagnosis system development.

4.6. Possible application of the obtained results

The traditional diagnostics for SpA and AS consist of patient's clinical history, symptoms, physical signs, and radiographic findings (Elyan and Khan, 2006). Depending on radiographs, the disease is often diagnosed at a later stage because radiographic changes are detected when the disease is in an advanced stage (Khan, 2002; O'Shea *et al*., 2007). The importance of formulating a set of early diagnosis ASAS classification criteria, including sacroiliitis by MRI findings and presence of HLA-B27 has been emphasized in a recent study (Davis *et al*., 2003). Although MRI is sensitive in detecting early inflammatory change in sacroiliac joints and spine, it is a very expensive tool and one that is not widely available. HLA-B27 is a key genetic factor in SpA and is observed in around 90% of patients with axial SpA (Thomas and Brown, 2010). It can help to diagnose SpA when it is used in combination with the relevant clinical, laboratory, or imaging parameters. The diagnostic value of HLA-B27 is dependent on its prevalence in populations. Nevertheless, the ASAS classification criteria need further validation in various ethnic populations in order to further confirm its usefulness and limitation. Thus, there are urgent needs to develop novel diagnostic tools for early AS. It has always been a challenge to come up with an accurate and early diagnosis of AS. Recently, it has become increasingly important, because some effective new treatments have now become available (Baraliakos *et al*., 2005; Braun *et al*., 2002; Baraliakos *et al*., 2007; Son and Cha, 2010).

Our experimental results showed aberrant gene rearrangements and up-regulation of Ig $VH2^{ab}$ genes in PBMC of AS patients. Through the comparison between RA and AS patient sample, we investigated the specificity of the $VH2^{ab}$ gene over-expression. Results from pre-AS

(patients without evidence of radiographic sacroiliitis among the patients with axial SpA) samples show the possibility of application of designed primer sets for early diagnosis of AS. Ig $VH2^{ab}$ gene expression from patients with axial SpA significantly increased, compared with patients with RA, another inflammatory disease, and healthy controls with excellent sensitivity and specificity. Patients with early axial SpA without radiographic sacroiliitis still revealed increased Ig $VH2^{ab}$ gene expression compared with those with RA and healthy controls. When the amount of $VH2^{ab}$ transcript determined by PR1 is compared with house-keeping gene expression, the subjects are dissected as below (Table 17).

Table 17. Number of subjects in terms of levels of Ig $VH2^{ab}$ gene expression

Relative amount of $VH2^{ab}$ expression	Number of subjects		
	axial SpA	RA or HC	sum
>1.0	25	2	27
<1.0	1	39	40
sum	26	41	67

Ig $VH2^{ab}$ gene over-expression has been proven to have 96% sensitivity and 95% specificity in diagnosing axial SpA. Thus, it might play a role as a valuable tool in facilitating early diagnosis of axial SpA and differentiating axial SpA from other inflammatory diseases, such as RA. The comparison between traditional diagnostic factors and our novel approach is described in Table 18. The amount of Ig $VH2^{ab}$ gene expression from AS patients also increased compared with those in pre-AS and RA patients, although the level of acute phase reactants, such as CRP and ESR, did not show significant differences between them. Additionally, this data suggest that $VH2^{ab}$ gene over-expression might be related to disease progression rather than inflammatory process, although further investigations among other ethnic groups would be needed.

Table 18. Comparison between traditional diagnostic factors and novel approach

	Factors	RA N=18	pre-AS N=9	AS N=17
traditional tool	ESR (mm/hr)*	25.9 ± 26.1	36.7 ± 35.6	22.4 ± 32.4
	CRP (mg/dL)*	1.5 ± 2.4	2.7 ± 2.8	2.0 ± 4.1
	HLA-B27 (%)		88.9	100.0
novel approach	Relative expression ratio**			
	PR1	0.61	5.78	16.37
	PR2	0.50	2.08	4.88
	PR3	13	37	470

*mean ± standard deviation.
**expression ratio (experimental group/healthy control)
(PR: primer set)

CD40L expression, which is involved in IgE secretion caused by isotype switching, is also up-regulated (Figure 21 and 25). Guseĭnov *et al*. reported that the IgE concentrations increased in patients with SpA, and that the difference became more discernable in accordance with the AS progression from central to peripheral (Guseĭnov, 1991).

The activation of CD40L and the unusual rearrangement of IgVH genes could have many implications. First, CD40L activation is an indication of aberrant T cell stimulation. This provides a new perspective for understanding how aberrant CD40L can affect the initiation of inflammatory diseases through the induction of CD40L and CD40 interaction and disturbances in their signaling pathway. More specifically, CD40 induction by CD40L activation might directly contribute to isotype switching of Ig genes and the introduction of somatic mutations, particularly in B cells. On the premise that the CD40L and CD40 interaction relates with the regulation of B cell proliferation and Ig gene production, this observation may contribute in providing an alternative explanation to the possible involvement of B cells in AS pathogenesis. More generally, the aberrant CD40L and CD40 interactions could directly affect various cytokines and the B cells' role on the pathogenesis of general autoimmune disease included AS.

4.7. Outlooks

In this study, in order to develop an early diagnosis system for AS, VH segments usage was analyzed in PBMCs of patients with AS. We objected that a uniquely assembled VH segment ($VH2^{ab}$) is over-expressed in AS patient. Through the following experimental confirmation, we provided primer sets for AS diagnostics and a hypothetic model of VH construction in AS patients. Relative expression levels of uniquely assembled VH segments were determined to be approximately 28 fold higher in AS patients and 6 fold higher in early AS patients than HC by using PR1. In addition, PR1 was proven to have 96 % sensitivity and 95 % specificity in diagnosing early stage AS. Q-PCRs conducted with PR2 and PR3 showed similar relative expression levels of uniquely assembled VH segments as obtained with PR1. For the further confirmation of effectiveness of PR1, PR2 and PR3 for diagnosing AS, further Q-PCR experiments were carried out with lager sample sizes (49 AS, 50 RA and 50 HC). The results were in good agreement with our previous data (Figure 19, 20 and 24).

Nevertheless, further investigations are required to verify whether the distinct rearrangements of Ig VH genes are involved in B cell development and functioning in AS pathogenesis. It should also be mentioned that our hypothesis does not perfectly fit to the norm of RAG-based recombination in CDC42 BPB gene. Although the CDC42 BPB gene had a proper RSS motif (heptamer-12 bp spaces-nonamer), one and three different nucleotides were detected in RSS motif's heptamer- and nonamer-like domains respectively (Figure 28). Questions on how these unfitted nucleotides could be inserted in CDC42 BPB genes of some AS patients and whether or not these unique rearrangements can only be found in Korean AS patients still remain. Further study is required to answer these questions.

According to our antibody gene expression results, IgE antibody production may also contribute to the pathology of AS. This could be supported by Kamanli et al (2009) who

determined that IgE concentrations against some allergens are significantly higher than those for controls in patients with SpA (Kamanli et al., 2009). However, their conclusion only applies to a specific subtype of HLA allies (HLA-B*2701 and B*2702) (Kamanli et al., 2009). To our general knowledge, the relation difference between the presence of AS and the type of HLA-B27 lies not only on SpA disease varieties but also races and geographical differences. The B*2701 is a rare subtype in Asian as well as in Caucasian individuals (Ball and Khan, 2001). In this regard, our present results might be interpreted that the abnormal expression of IgE can reflect AS susceptibility covering various subtypes of HLA-B27. At present, however, 24 different proteins coded by 27 subgroups of HLA-B27 have been found and related subtypes are shown to be fairly different among various ethnic groups (Kamanli et al., 2009; Sieper et al., 2006). Therefore, further investigations among other ethnic groups are required in order to determine the corresponding HLA-B27 subgroups.

Furthermore, in this work, Ig V gene repertoire in PBMC was not compared with either synovial tissues or synovial fluid of the same AS patients. The Ig V gene repertoire in PBMC could be different than that in inflammatory joints. PBMC contains a population of recirculating and memory B cells that have encountered a wide diversity of antigens over the patient's lifetime, whereas inflammatory joint tissues may have a subset of B cells responding to antigens and undergoing an antigen-driven response. Thus comparing Ig contents of synovial B cells with those of PBMC could be interesting. It would also be remarkable to examine uniquely assembled V segment expression in various B cell subsets at different stages of B cell development. If it is found that a particular B cell subset is involved in this genetic abnormality, it will allow us to unravel the pathogenesis of AS.

Taken together, combinatorial detection of uniquely assembled $VH2^{ab}$ segments and CD40L transcripts with newly designed primer sets could be a promising diagnostic tool for the early stage AS although further studies are needed to verify that the findings in cDNA are relevant

to clinical prognosis. These results can also be useful for future investigations on the role of B lymphocytes as hidden players in AS pathogenesis.

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6. APPENDIX

Publications

Overexpression and unique rearrangement of VH2 transcripts in immunoglobulin variable heavy chain genes in ankylosing spondylitis patients. *Exp Mol Med*. 2010 May 31;42(5):319-26. Kim YJ, Kim NY, Lee MK, Choi HJ, Baek HJ, Nam CH.

Unique over-expression of aberrant VH2 genes in peripheral blood from patients with axial spondyloarthritis. (Submitted)

Conferences

Overexpression and Unique Rearrangement of VH2 Transcripts in Immunoglobulin Variable Heavy Chain Genes in Ankylosing Spondylitis Patients (oral presentation) EKC 2010 (138): 113-122 , July20, 2010

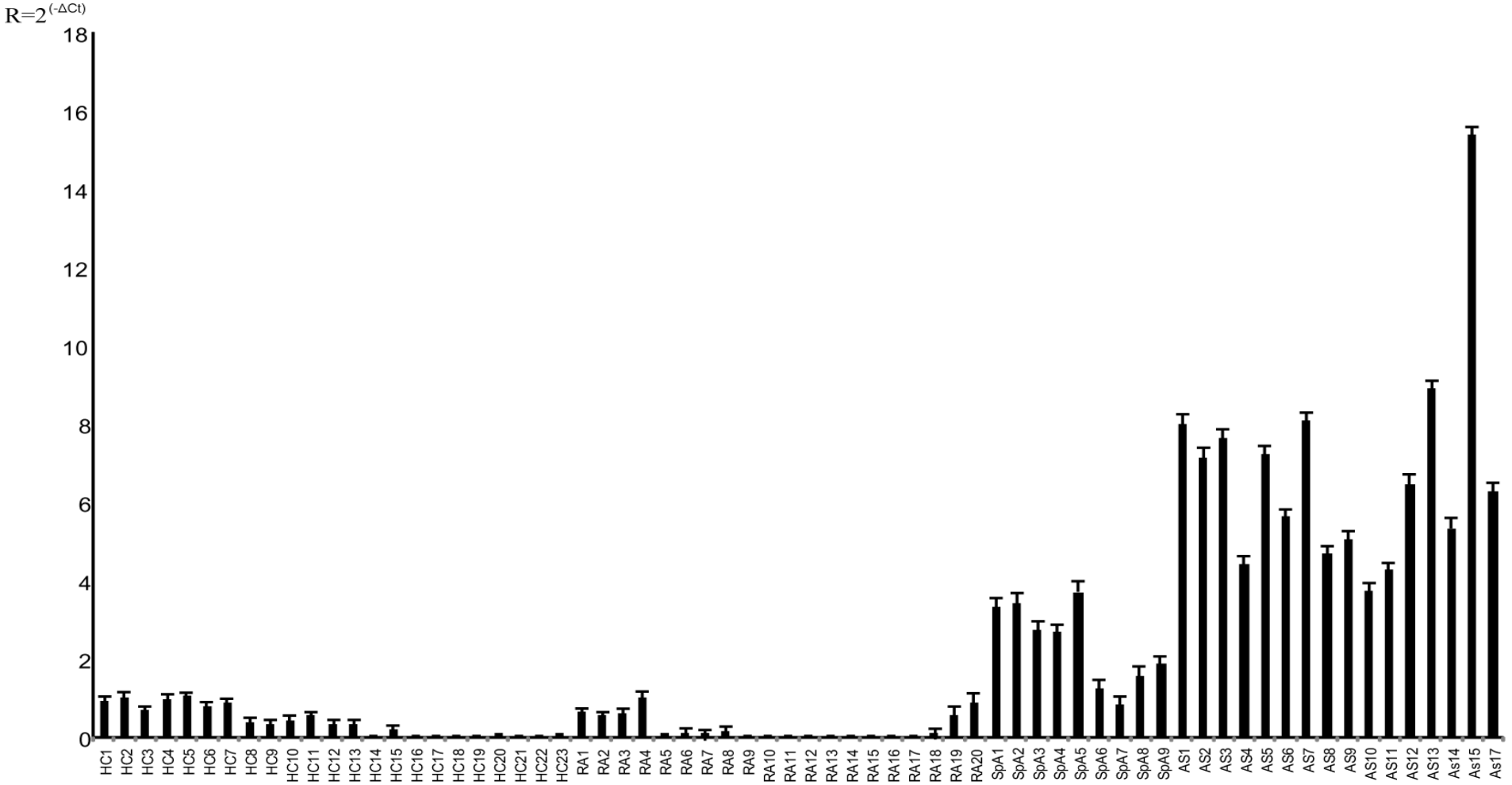
Unique Rearrangement in Immunoglobulin Variable Heavy Chain Gene in Ankylosing Spondylitis Patients (poster) EKC 2011 July23, 2011

OVER-EXPRESSION OF ABERRANT VH2 GENES AS A POTENTIAL DIAGNOSTIC TOOL FOR AXIAL SPONDYLOARTHRITIS Autoimmunity Congress 2012 Granada, Spain, May 9-13, 2012

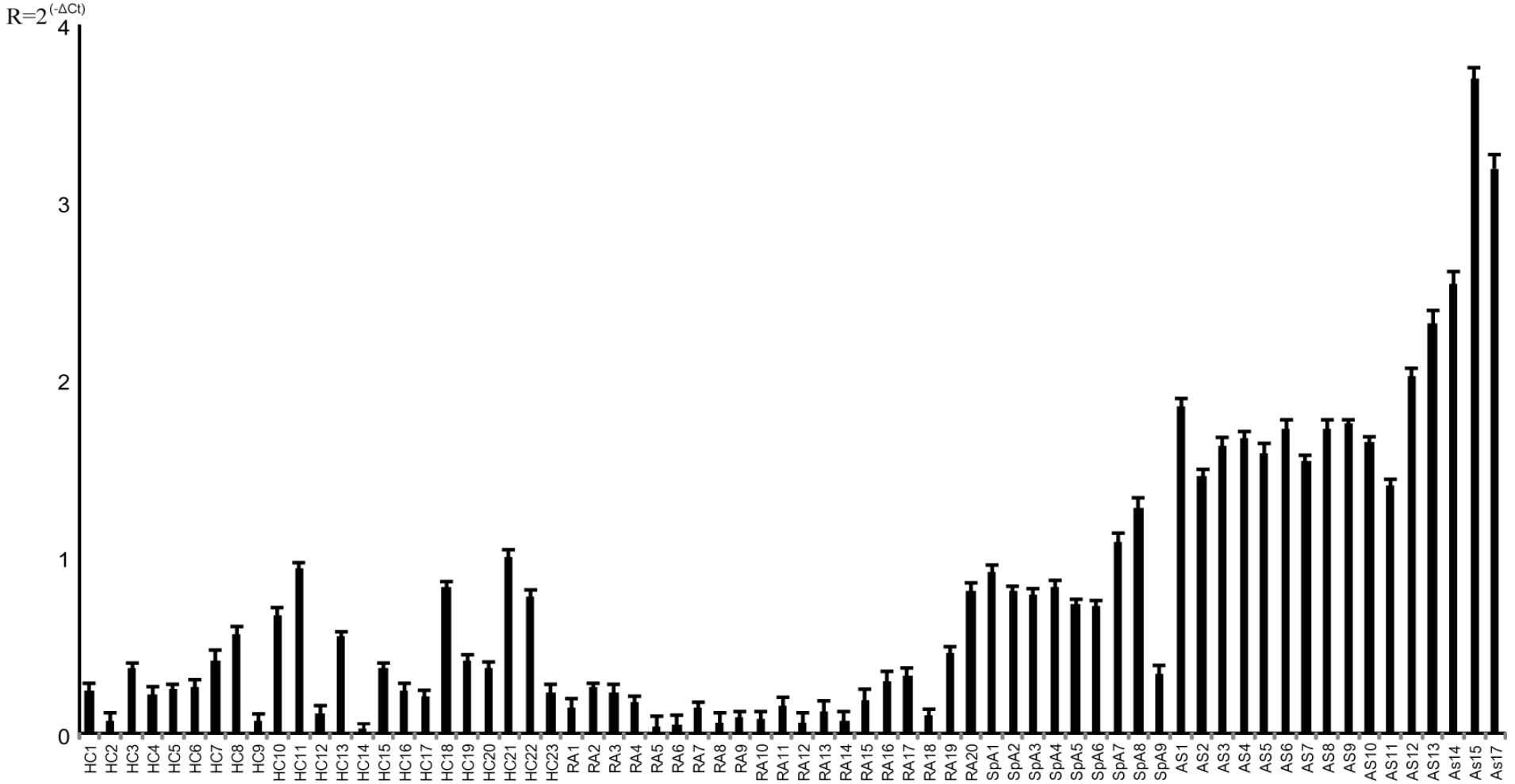
Patents

PCT/KR2011/000095 PRIMERS FOR DIAGNOSING ANKYLOSING SPONDYLITIS, AND METHOD FOR DIAGNOSING ANKYLOSING SPONDYLITIS USING THE SAME 6 Jan 2011 CH Nam, YJ Kim, HJ Baek. (Technology transfer to company RexBio was achieved based on this patent in 2011)

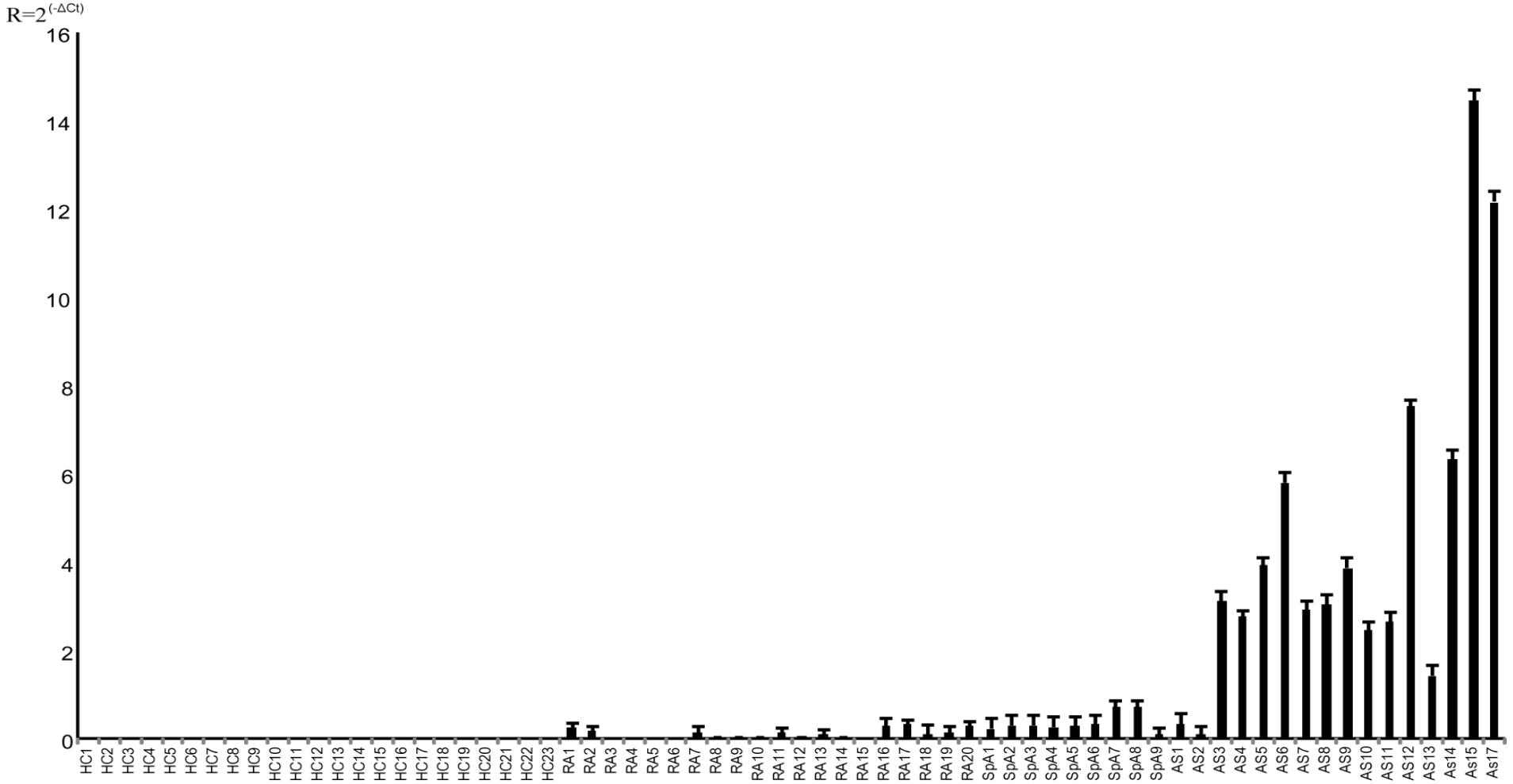
(Suppl. 1 Relative gene expression amounts for individual samples, primer set 1)



(Suppl. 2 Relative gene expression amounts for individual samples, primer set 2)



(Suppl. 3 Relative gene expression amounts for individual samples, primer set 3)



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