SKEWED X-CHROMOSOME INACTIVATION IN JUVENILE IDIOPATHIC ARTHRITIS AND RHEUMATOID ARTHRITIS

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ABSTRACT

SKEWED X-CHROMOSOME INACTIVATION IN JUVENILE IDIOPATHIC ARTHRITIS AND RHEUMATOID ARTHRITIS

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There is a female predominance in most of the autoimmune diseases, and it is thought to play an important role in identifying the etiological factors. Sex hormones, microchimerism and environmental factors are thought to be responsible. Nowadays, it is proposed that a disturbance in mosaicism of females may cause autoimmune disease development. Recently, in our lab, an association between extremely skewed X-chromosome inactivation (XCI) patterns and female predisposition to autoimmunity was identified in Turkish population. In this study, we hypothesized that skewed XCI might play a role in the disease development of Juvenile idiopathic arthritis (JIA) in Turkish, and Rheumatoid Arthritis (RA) in French population. Therefore, XCI status of healthy individuals and patients diagnosed with JIA and RA were genotyped by analyzing androgen receptor (AR) locus by methylation sensitive *Hpa*II digestion followed by PCR. Extremely skewed XCI was observed in a significant proportion of JIA (OR: 11.33; P=0.0008) in Turkish population, and RA (OR: 7.6; P=0.005) in French population. In conclusion, our results suggest that extremely skewed XCI may play an important role in autoimmune disease pathogenesis.

ÖZET

JÜVENİL İDİYOPATİK ARTRİT VE ROMATOİD ARTRİT HASTALIĞINDA X KROMOZOMU İNAKTİVASYONU SAPMASI

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Dünya çapında en sık rastlanan hastalık olan otoimmün hastalıkların çoğu kadınlarda daha sık görülmektedir. Bunun nedeni olarak cinsiyet hormonları, mikrokimerizm ve çevresel etkenler sorumlu tutulmaktadır. Son zamanlarda X kromozomu inaktivasyonu (XCI) sapması da buna neden olarak gösterilmektedir. Yakın zamanda laboratuarımızda gerçekleştirilen çalışmalarda, Türk popülasyonunda, X kromozomu inaktivasyonu sapması ve kadınların otoimmün hastalıklara yatkınlığı arasında bağlantı kurulmuştur. Bu çalışmada X kromozomu inaktivasyonuna bağlı mozaik yapının bozulmasının otoimmün hastalık etiolojisinde rol alabileceği hipotezi test edilmiştir. Bu nedenle Türk popülasyonunda jüvenil idiyopatik artrit (JIA) ve Fransız popülasyonunda romatoid artrit (RA) hastaları ve sağlıklı bireyler genotiplenmiştir. XCI statüsünü belirlemek için androjen reseptörü (AR) geni metillemeye duyarlı *Hpa*II enzimi ile analiz edilmiştir. Türk popülasyonunda JIA (OR: 11.33; P=0.0008) ve Fransız popülasyonunda RA (OR: 7.6; P=0.005) hastalarında XCI'nda aşırı sapma gözlenmiştir. Sonuçlarımız XCI ile otoimmün hastalık gelişimi arasında bir ilişki olabileceği görüşünü desteklemektedir.

To my Family

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ABBREVIATIONS

ACR	American college of rheumatism
Abs	Antibodies
AIRE	autoimmune regulator
AITD	Autoimmune thyroid disease
ANA	Antinuclear antibodies
APC	Antigen presenting cell
APS-1	autoimmune polyendocrine syndrome
APS	Ammonium persulfate
AR	Androgen Receptor
ARA	American Rheumatology Association
ASP	Affected sibling pair
Bisacrylamide	N, N, methylene bisacrylamide
Bp	base pair
BTK	Bruton tyrosine kinase
CCP	cyclic citrullinated peptide
CrR	Corrected ratio
CTLA4	Cytotoxic T lymphocyte antigen 4
ddH_2O	deionized water
DNA	deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
EDTA	ethylenediaminetetraacetic acid
ER	Estrogen receptors
ERA	Enthesitis-related arthritis
EtBr	Ethidium bromide
EtOH	Ethanol
EULAR	European league against rheumatism

FHL-1	4.5 LIM domain 1
FOXP3	forkhead box P3
G6PD	glucose 6-phosphate dehydrogenase
GD	Grave's disease
HLA	Human leukocyte antigen
HT	Hashimoto's thyroiditis
IAS	intraarticular corticosteroids
IDS	iduronate-2-sulfatase
IL	Interleukin
ILAR	International league against rheumatism
IPEX	immune dysregulaton, polyendocrinopathy, enteropathy, X-linked syndrome
JAS	Juvenile ankylosing spondylitis
JCA	Juvenile chronic arthritis
JIA	Juvenile idiopathic arthritis
JRA	Juvenile rheumatoid arthritis
Kb	Kilobase
kDa	Kilodalton
LyP	lymphoid specific phosphatase
MAS	Macrophage Activation Syndrome
$MgCl_2$	Magnesium chloride
MHC	major histocompatibility complex
mM	Millimolar
ml	Milliliter
µl	Microliter
MPP1	p55
MS	multiple sclerosis
MTX	methotrexate
ng	nano gram
NSAID	non-steroidal anti-inflammatory drugs
PAGE	polyacrylamide gel electrophoresis
PAMP	pathogen-associated molecular patterns
PCR	Polymerase chain reaction

PRED	prednisolone
PTP	Protein tyrosine phosphatase
PTPN22	protein tyrosine phosphatase, non-receptor type 22
R	Arginine
RA	Rheumatoid Arthritis
RE	restriction enzyme
RF	Rheumatoid factor
pmol	picomole
SAZ	sulfasalazine
SDS	sodium dodecyl sulphate
SLE	systemic lupus erythematous
SNP	Single nucleotide polymorphism
SSc	systemic sclerosis
STAT4	Signal transducer and activator of transcription 4
T1D	Type-1 diabetes
TAE	tric-acetic acid-EDTA
TCR	T cell receptor
TEMED	N, N, N, N-tetramethyl-1-2, diaminoethane
TNF	Tumor necrosis factor
TSIX	XIST antisense
Xa	Active X
Xi	Inactive X
XIST	X-inactive specific transcript
XITE	X-inactivation intergenic transcription element
XCI	X-chromosome inactivation
Xic	X-inactivation center
W	Tryptophan

CHAPTER I

INTRODUCTION

1.1. Immune System

The immune system protects the body from infectious agents and the damage that they cause, with the help of variety of effector cells and molecules, which are able to differentiate self from non-self antigens. There are two mechanisms that immune system use for determination of foreigners, innate and adaptive immunity. Innate immunity is evolutionarily old and present in both plants and animals (Hoffman et al., 1999). The pattern recognition receptors in the innate immunity recognize the conserved molecular patterns, pathogen-associated molecular patterns (PAMPs), that are shared by many of the organisms (Medzhitov and Janeway, 2000). However, adaptive immunity provides specific recognition of pathogens by pathogen-specific adaptor proteins. Although the adaptive immune system can recognize the foreign antigens with the help of rearrangement of large variety of receptor gene segments, it is also responsible for allergy, rejection of tissue grafts, and autoimmunity (Janeway and Medzhitov, 2002).

1.2. Autoimmunity

The immune system is able to discriminate self antigens and foreign antigens. While lymphocyte development, lymphocytes are tested against self tolerance by two mechanisms, central tolerance and peripheral tolerance. The immature lymphocytes that recognize self antigens are eliminated by apoptosis in the central lymphoid organs, which are thymus for T cells, and bone marrow for B cells. The tolerance induced in this stage is called central tolerance. If this lymphocyte escapes from test of self tolerance, it can be still removed in peripheral tissues. There are three mechanisms of elimination of self reactive lymphocytes: 1) deletion, lymphocytes are killed by induction of apoptosis; 2) anergy, functional unresponsiveness; and 3) suppression by regulatory T cells (Goodnow *et al.*, 2005). Upon activation of self reactive lymphocytes that could escape from the tolerance, an immune response that causes autoimmune disease development is induced against its own cells and tissues (Rioux and Abbas, 2005).

Autoimmune diseases affect 3-5% of the population in US, Asia and Europe (Cooper *et al*., 2009). Autoimmune diseases are classified by clinicians either systemic, or organ specific. In systemic autoimmune diseases, such as systemic lupus erythematosus (SLE), multiple organs might be affected, while in organ specific, such as hashimoto's thyroiditis (HT), only one organ might be affected (Table 1.1).

Table 1.1 Systemic and organ specific autoimmune diseases

Type	Name of the disorder	Affected organs/tissues
Systemic	Rheumatoid arthritis	Joints, skin, less commonly lung
	SLE	Skin, joints, kidneys, heart, brain, red blood cells
	Scleroderma	Skin, intestine, lung
	Sjogren's syndrome	Salivary glands, tear glands, joints
Organ specific	Type I diabetes mellitus	Pancreas islets
	Hashimoto's thyroiditis, Grave's disease	Thyroid
	Celiac disease, Crohn's disease	GI tract
	Primary biliary cirrhosis	Liver
	Vitiligo	Skin

1.2.1. Causes of autoimmunity

The mechanisms of autoimmune diseases remain to be fully elucidated. There are multiple factors that are thought to play role in autoimmune disease development, such as genetic susceptibility, environmental factors, and infectious agents. Nowadays epigenetic factors are also thought to play role in autoimmunity.

Different human leukocyte antigen (HLA) alleles, which are encoded by major histocompatibility complex (MHC), were found to be associated with autoimmune diseases. However, upon linkage analyses and gene association studies, new candidate genes – non-HLA genes, such as *AIRE*, *FOXP3*, *PTPN22*, *CTLA4*, and *STAT4* were identified (Gregersen and Behrens, 2006).

For a long time, infections were proposed to be an environmental factor for autoimmune disease induction. The pathogens might induce autoimmunity by several mechanisms such as, molecular mimicry, cell apoptosis or necrosis, polyclonal activation of autoreactive lymphocytes (Kivity *et al.*, 2009). Upon infection, cell apoptosis is induced, and the deficiency of the clearance of the apoptotic cell leads to nuclear material accumulation, which may cause autoimmunity (Schulze *et al.*, 2008).

1.2.1.1. Genes associated with autoimmunity

1.2.1.1.1. AIRE

AIRE (autoimmune regulator) is a transcription factor that regulates the expression of some self antigens in thymic epithelial cells, which play role in the negative selection of central tolerance. It was first found to be mutated in autoimmune polyendocrine syndrome (APS-1), in which autoantigens attack multiple organs and the skin (Bjorses et al., 1998).

When the AIRE is mutated, the self antigens are not presented by MHC molecules, leading to the escape of T cells specific for these, and attack the target tissues (Liston *et al*., 2003; Anderson *et al*., 2005).

1.2.1.1.2. CTLA4

CTLA4 (Cytotoxic T lymphocyte antigen 4) is an inhibitory receptor, which regulates T cell proliferation. Association of *CTLA4* with several autoimmune diseases, such as Grave's disease (GD), type 1 diabetes (T1D), and rheumatoid arthritis (RA) was reported (Ueda *et al.*,2003, Gregersen and Behrens, 2006). *CTLA4* function both negatively and positively. It inhibits T cell activity upon binding to its ligands CD80 and CD86 on

antigen presenting cells (APC), while activates regulatory T cells, which play an important role on autoimmunity (Gregersen and Behrens, 2006).

1.2.1.1.3. FOXP3

FOXP3 (forkhead box P3) encodes a transcription factor of the forkhead family. It was shown that inhibition of *Foxp3* in mouse induces systemic autoimmune disease because of the absence of regulatory T cells (Fontenot *et al*., 2003). Similar to the mouse model, *FOXP3* mutation causes IPEX (immune dysregulaton, polyendocrinopathy, enteropathy, X-linked syndrome) in humans (Wildin *et al.*, 2002).

1.2.1.1.4. PTPN22

PTPN22 (protein tyrosine phosphatase, non-receptor type 22) encodes a lymphoid specific phosphatase (LyP). It was shown that a SNP 1858C→T, which changes the amino acid from an arginine (R) to a tryptophan (W) at codon 620, is associated with T1D, RA, SLE and GD (Begovich et al., 2004; Bottini et al., 2004, Gregersen and Behrens, 2006).

1.2.1.1.5. STAT4

STAT4 (signal transducer and activator of transcription 4) encodes a transcription factor that play role in the expression of genes that are important in immune response. An association of an intronic SNP and SLE and RA was first found by Remmers and colleagues in 2007.

1.2.1.2. Molecular Mimicry

One of the environmental factors that cause autoimmunity is molecular mimicry. In molecular mimicry, the foreign peptide that share similar sequence with self-antigen can cause cross-reaction. Upon activation of autoreactive T or B cells, this may cause to break of self-tolerance, causing autoimmunity. Molecular mimicry was shown to play role in some autoimmune diseases, such as SLE and systemic sclerosis (SSc) (Doria *et al.,* 2008; Randone *et al.,* 2008; Reviewed in Lin *et al*., 2011).

1.2.1.3. Epigenetics

Although higher disease concordance of autoimmune diseases in monozygotic twins relative to dizygotic twins or other family members indicates a genetic contribution, the incomplete concordance of the disease in some monozygotic twins indicate that some additional factors of environment also play a role. Recently, it was shown that environmentally-induced epigenetic changes may alter the DNA methylation pattern, resulting in the loss of self-tolerance because of aberrant gene expression (Strickland *et al.,* 2008; reviewd in Hewagama and Richardson, 2009).

1.2.2. Female predominance in autoimmunity

For a long time, it is well known that there is a female predominance in most of the autoimmune diseases (Whitacre, 2001). In Table 1.2, female:male ratio is represented in several autoimmune diseases. The reasons of this predominance remain to be fully elucidated, but there are several explanations such as hormonal differences, reproductivity, microchimerism and skewed X-chromosome inactivation (XCI).

Table 1.2. Female to male ratio for selected autoimmune diseases (Selmi, 2008)

Disease	female:male ratio
Addison's disease	0.8-2.4:1
Autoimmune chronic hepatitis	7:1
Graves' disease	7:1
Hashimoto's disease	5-18:1
Multiple sclerosis	2:1
Primary biliary cirrhosis	10:1
Rheumatoid arthritis	2:1
Sjogren's syndrome	9:1
Systemic lupus erythematosus	9:1
Systemic sclerosis	5:1

1.2.2.1. Hormones

T and B cells express estrogen receptors (ER) and androgen receptors (AR), indicating the possible role of sex hormones in immune response. Also, it was indicated that immune response in females varies as their hormonal status changes. In rheumatoid arthritis (RA) and multiple sclerosis (MS) symptoms of the disease are decreased during the pregnancy where estrogen level is high, while in SLE, they are increased or remain same (Nalbandian and Kovats, 2005). However, studies have failed to demonstrate direct role of the hormone – but not disregarding their importance, indicating some other factors on gender difference (Invernizzi *et al.*, 2009).

1.2.2.2 Microchimerism

Another difference between males and females is the reproductivity. During pregnancy, maternal and fetal cells are exchanged, leading to microchimerism in the mother. Microchimerism was first found to be associated with systemic sclerosis (SSc) by Nelson *et al.*, in 1998 and confirmed by Artlett *et al*., in 1998. Although the mechanism is not clear, it cannot explain the female predominance, because of the patients that do not give birth (Invernizzi *et al*., 2009).

1.2.2.3. Skewed X-chromosome inactivation

The sex chromosomes differ in males and females. Males are hemizygous for X-chromosome, while females inactivate one of their X-chromosomes for dosage compensation. Therefore, females are mosaics for X-chromosome inactivation (XCI), as a result of random inactivation of one of the X-chromosomes. Disturbed XCI was used to explain the female predominance in autoimmune diseases by Kast in 1977 and developed by Stewart in 1998. According to Kast and Stewart, the disturbance of the mosaicism could lead to differences of the antigen presentation, causing autoimmunity. Although Chitnis *et al*. was failed to show the association of several autoimmune diseases with skewed XCI in 2000, the association was found with SSc, autoimmune thyroid disease (AITD), and pre-eclampsia (Ozbalkan *et al*., 2005; Ozcelik *et al*., 2006; Uz *et al.,* 2007; Uz *et al.*, 2008).

1.3. X-inactivation

1.3.1. History

X-chromosome inactivation was first proposed by Lyon in 1961 to be the dosage compensation mechanism in mice. Upon several unexpected results in her analysis of mutations affecting the coat color of female mice, she suggested that one of the two X-chromosomes in each cell of a female is inactivated randomly leading females to be mosaics. She suggested that the X-inactivation event occurred early in development that causes formation of large patches of different color (Lyon, 1961).

After these proposals, a hypothesis came from Ohno and colleagues, who demonstrated, first in mice then in humans, each Barr body (Barr *et al*., 1949) was a single X-chromosome, and the other X was euchromatic like the autosomes (Ohno *et al*., 1960, Ohno *et al*., 1961).

1.3.2. Mechanism

There are different mechanisms for sex development in different species. The presence of a Y chromosome is necessary for male development in mammals, while in fruit flies and worms, it is dependent on the ratio of the X-chromosome to autosomal chromosomes. In order to compensate the dosage problem between males and females, different species have developed different mechanisms. In *Drosophila melanogaster*, XY males increase the expression of their single X-chromosome, while in *Caenorhabditis elegans,* XX hermaphrodites reduce each X chromosome's expression by half, in order to achieve similar transcription levels in XO males. However, in mammals, females inactivate one of the X chromosomes (Pontier and Gribnau, 2011).

There is a random XCI in eutherian mammals, while in marsupials, X-chromosome inactivation is imprinted - the X-chromosome coming from the father is always inactivated (Cooper *et al*., 1971). However, it was shown that eutherian mammals also have imprinted XCI, which is limited to extra-embryonic tissues-the placenta. In four-cell stage of mouse embryos, paternally-derived X-chromosomes undergo an early imprinted inactivation, leaving the maternal X-chromosome active only. The extraembryonic tissues retain this early imprinted inactivation, but in the early blastocyst, initial imprinted X-inactivation is reversed in the inner cell mass that give rise to the embryo. Each of these cells then independently and randomly inactivates one of the X-chromosomes irreversibly, leading to mosaicism (Huynh and Lee, 2004). The existence of imprinted XCI in humans remains controversial. Recently, it was shown that XCI in human extraembryonic tissues is also random (Moreira de Mello *et al.*, 2010).

X-inactivation is a multistep process including counting, choice, silencing and maintenance. In the counting step, the X-chromosome number relative to autosomes is determined, while in choice, X-chromosome that remain active (Xa) is chosen. Silencing is then initiated and spread on the inactive X-chromosome (Xi), and maintained in daughter cell lineages (Chow *et al*., 2005).

XCI is regulated by the *X-inactivation center* (*XIC*), which is mapped to Xq13. *XIC* consists of several genes such as, *XIST*, *TSIX*, and *XITE* (Brown *et al.*, 1991; Lee *et al.*, 1999; Kalantry 2011). *XIST* (X-inactive specific transcript), which is a non-coding RNA, is expressed from the future Xi. During the inactivation process, *XIST* is regulated by *TSIX*, which is the antisense complementary transcript of *XIST*. *TSIX*, which is regulated by *XITE*, is expressed from Xa and prevents *XIST* expression in the Xa (Kalantry. 2011).

1.4. Autoimmune disorders that were selected in this study

1.4.1. Rheumatoid Arthritis (RA)

Rheumatoid arthritis (RA) is an inflammatory disease that causes joint destruction, resulting in pain, stiffness and swelling of peripheral joints (Aletaha *et al.,* 2010). RA affects 1% of the population, worldwide, with a female predominance (Gabriel and Michaud, 2009). There are multicellular inflammation in the joint, including infiltration of lymphocytes and granulocytes into the articular cartilage, and proliferation of macrophages. These processes not only cause pain and stiffness but also joint destruction and reduction of bone density (Nandakumar and Holmdahl, 2006).

1.4.1.1. Classification

In 1987, criteria for RA were developed by the American Rheumatology Association (ARA). According to these criteria four of the seven features are needed to be present: morning stiffness, arthritis of 3 or more joint areas, arthritis of hand joints, symmetric arthritis, rheumatoid nodules, positive serum rheumatoid factor (RF) and radiographic changes (Arnett *et al.*, 1988). Recently, new criteria was developed and introduced by American College of Rheumatology/European League Against Rheumatism (ACR/EULAR). According to the new criteria, patients that have a point score of 6 or higher are called RA patient. The point scores were developed by ACR/EULAR. The points are given according to four areas of diagnosis: joint involvement, serological parameters, acute-phase reactants and duration of symptoms (Aletaha *et al.,* 2010).

1.4.1.2. Prevalence, incidence

During last two decades, several incidence and prevalence studies of RA have been reported. According to these studies, the frequency changes with the different ethnic and racial groups. The prevalence of RA is between 0.5% and 1% and the incidence of RA decreased from 61.2 per 100,000 population in the period 1955–1964 to 32.7 per 100,000 in the period 1985–1994 (Gabriel and Michaud, 2009). In south European countries, the occurrence of RA is relatively lower according to north European and north American countries (Alamanos and Drosos, 2005).

1.4.1.3. Causes

The cause of the RA is not well known, but genetic and environmental factors are thought to be involved. Smoking is the most reported risk factor for RA (Silman et al. 1996, Harrison 2002). It is reported that genetics determines 50-60% of susceptibility, severity and phenotype of RA (MacGregor *et al.,* 2000; Deighton *et al.,* 1989). Monozygotic twins have concordance rates of 15%, while dizygotic twins have rates of 4% (Silman *et al.,* 1993; Aho *et al.,* 1986).

1.4.1.3.1. Associated genes

In 1978, *HLA-DRB1* was first to be associated with RA (Stastny, 1978). Later on, shared epitope common to all *HLA-DRB1* alleles were found to be associated with RA in different populations (Imboden, 2009).

In a large scale screening of 16,000 non-synonymous SNPs, *PTPN22* R620W SNP was found to be associated with RA (Begovich *et al.,* 2004).

Like *PTPN22*, *CTLA-4* negatively regulates T-cell activation (Sharpe and Freeman, 2002). A SNP of *CTLA-4* was found to be associated with RA in German (Seidl *et al.,* 1998), Japanese (Yanagawa *et al.,* 2000), and British (Vaidya *et al.,* 2002) populations. Recently *STAT4* was also found to be associated with RA in different populations (Remmers *et al.,* 2007; Korman *et al.,* 2008).

1.4.1.3.2. Antibodies

RA is characterized by the presence of autoantibodies, such as rheumatoid factor (RF) and anti-cyclic citrullinated peptide (CCP) antibody. Although RF was never proved to cause arthritis in experimental systems, it was the only antibody that is used for classification of RA (Arnett *et al.,* 1988). However, anti-CCP was found to be present in 70% of RA patients, and rare in healthy poeple (Agrawal *et al.,* 2007). In the new classification criteria, anti-CCP is also included for diagnosis of RA (Aletaha *et al.,* 2010).

1.4.2. Juvenile Idiopathic Arthritis (JIA)

Juvenile idiopathic arthritis (JIA), which is the inflammation (cellular damage) of the synovium (the lining of joints), is the most prevalent pediatric rheumatic disease that is seen in children with onset before 16 years of age. JIA patients have swollen, painful joints, which may be stiff and difficult to move. (Cuccurullo, 2004).

1.4.2.1. Classification

Juvenile idiopathic arthritis (JIA) term (Petty *et al*., 1998; Petty *et al*., 2003; Petty *et al*., 2004) was first proposed in 1994 and later revised in 1997.It is now used instead of the American term 'juvenile rheumatoid arthritis' (JRA) as defined by American College of Rheumatology (ACR) and the European classification 'juvenile chronic arthritis' (JCA) as defined by the European League Against Rheumatism (EULAR) (Wood *et al*., 1978). The American and European classifications of the disease were confusing (Table 1.3), and was difficult to use them interchangeably (disease duration is 6 weeks for ACR, while it is 12 weeks for EULAR). In order to improve research and treatment, the International League Against Rheumatism (ILAR) unified the criteria, using the term 'juvenile idiopathic arthritis'. The word 'idiopathic' means 'of unknown cause'.

Table 1.3. Comparison of the classification systems of arthritis in children

Classification	ACR	EULAR	ILAR
Designation	JRA	JCA	JIA
Types	Systemic Pauciarticular Polyarticular	Systemic Pauciarticular RF-negative polyarticular RF-positive polyarticular Psoriatic JAS	Systemic Oligoarticular RF-negative polyarthritis RF-positive polyarthritis Psoriatic Enthesitis-related Undefined

ACR=American College of Rheumatology; EULAR=European League against Rheumatism; ILAR=International League of Associations for Rheumatology; JRA= juvenile rheumatoid arthritis; JCA= juvenile chronic arthritis; JIA= juvenile idiopathic arthritis; JAS= juvenile ankylosing spondylitis (Petty *et al*., 2003).

1.4.2.2. Types

According to ILAR, the subtypes of JIA are oligoarticular JIA, which may be persistent or extended, polyarticular rheumatoid factor (RF)–negative JIA, polyarticular RF-positive JIA, systemic JIA, enthesitis-related arthritis (ERA), psoriatic JIA, or a classification of "other JIA" when the criteria for more than one subtype of JIA or none of the criteria were met (Petty *et al*., 1998).

1.4.2.2.1. Oligoarticular JIA

Oligoarthritis, which is the most common type that affect about 50% of all children with JIA, is mostly seen in females. This term is used when there are four or fewer affected joints during the first 6 months of disease. According to the ILAR classification, children who have psoriasis/a family history of psoriasis, a human leukocyte antigen (HLA) B27-associated disease in a first-degree relative, and a positive rheumatoid factor (RF) test are excluded from the oligoarthritis category (Petty *et al*., 1998).

This form of JIA is the only one that is not seen in adults, and characterized by asymmetric arthritis, early age of onset (before 6 years of age), female predominance, and high frequency of positive antinuclear antibodies (ANAs).

According to the ILAR classification, oligoarthritis subtype is divided to persistent oligoarthritis, in which the disease consists of four or fewer joints, and extended oligoarthritis, in which arthritis extends to more than four joints after the first 6 months of disease (Petty *et al*., 1998; Ravelli *et al*., 2007).

1.4.2.2.2. Polyarticular JIA

Polyarticular arthritis affects 35% children with JIA, more girls than boys. This kind of JIA usually involves small joints of the hands and feet. Polyarticular JIA is often symmetrical. There are two types of polyarticular JIA: rheumatoid-factor-positive and rheumatoid-factor-negative polyarthritis (Cuccurullo, 2004; Ravelli *et al*., 2007).

1.4.2.2.2.1. Rheumatoid-factor-positive polyarthritis

This disease, comprises 10% of all patients with JIA, and is characterized by age of onset greater than 11 years of age with female predominance (Cuccurullo, 2004). Although it is same as adult RF-positive rheumatoid arthritis, the disease phenotype between children and adults is different as the children's skeleton is still growing. It is mainly seen in adolescent girls (Cuccurullo, 2004; Ravelli *et al*., 2007).

It is characterized as a symmetrical polyarthritis and affects small joints of the hands and feet (Ravelli *et al*., 2007).

1.4.2.2.2.2. Rheumatoid-factor-negative polyarthritis

It is the most heterogeneous subtype (Ravelli *et al*., 2007). It affects 25% of all patients with JIA (Cuccurullo, 2004). There are at least three subsets of RF-negative polyarthritis. The first form resembles early-onset oligoarticular juvenile idiopathic arthritis because of the asymmetric arthritis, and early age of onset (Martini, 2003; Ravelli *et al*., 2007). The second subset is similar to adult onset RF-negative rheumatoid arthritis, because of the characteristics of symmetric synovitis of large and small

joints. The third form is known as dry synovitis. This subset is often poorly responsive to treatment and could follow a destructive progress (Ravelli *et al*., 2007).

1.4.2.2.3. Systemic JIA

It usually begins in early childhood. Researchers sometimes call this Still's disease. This type accounts for about 10-20% of cases of JIA. It affects both boys and girls almost equally. It is characterized by suddenly occurring fever. Anemia and weight loss may also occur (Ravelli *et al*., 2007).

1.4.2.2.4. Enthesitis-related arthritis

Enthesitis-related arthritis, which is characterized by the association of enthesitis (inflammation of the entheses) and arthritis, mainly affects male patients after the age of 6 years. The joints of the lower extremities are affected. It resembles oligoarthritis because of the hip involvement (Petty *et al*., 1998; Petty *et al*., 2003; Ravelli *et al*., 2007).

1.4.2.2.5. Psoriatic arthritis

According to ILAR, in order to diagnose juvenile psoriatic arthritis, arthritis and psoriatic rash need to be present. If a rash is absent, dactylitis (sausage-shaped swelling of the fingers and toes that can be painful), nail pitting and the presence of psoriasis in a first degree relative must be present. The symptoms are similar to the subset of RF-negative polyarthritis, and oligoarthritis (Petty *et al*., 1998; Ravelli *et al*., 2007).

1.4.2.2.6. Undifferentiated arthritis

This subtype includes the patients that do not fulfil the inclusion criteria for any category (Petty *et al*., 2004; Ravelli *et al*., 2007).

1.4.2.3. Prevalence and incidence

The incidence and the prevalence of the disease differ among different ethnicity. It has an incidence of 2–20 cases per 100 000 population and a prevalence of 16–150 cases per 100 000 population (Ravelli *et al*., 2007).

The frequency of the subtypes differs. There is female predominance, except in the systemic and enthesitis-related arthritis (Table 1.4). In systemic arthritis female-male ratio is equal. In enthesitis-related arthritis, there is male predominance.

Table 1.4 International League of Associations for Rheumatology (ILAR) categories of juvenile idiopathic arthritis

	Frequency	**Onset age**	**Sex ratio**
Systemic arthritis	4-17%	Throughout childhood	F=M
Oligoarthritis	27-56%	Early childhood	F>>>M
Rheumatoid factor (+) polyarthritis	2-7%	Late childhood	F>>M
Rheumatoid factor (-) polyarthritis	11-28%		F>>M
Enthesitis related arthritis	3-11%	Late childhood	M>>F
Psoriatic arthritis	2-11%		F>M
Undifferentiated arthritis	11-21%		

Adopted from (Ravelli *et al*., 2007)

1.4.2.4. Causes

Although the cause of JIA is not well-understood, it is believed that JIA is caused by a combination of genetic and environmental factors such as viral or bacterial infections that may trigger the autoimmune process (Cuccurullo, 2004).

1.4.2.4.1. Associated Genes

There are both MHC-associated and Non-MHC genes that are found to be associated with JIA. The class I gene, HLA-B27, was the first HLA association found in JIA. It is found that HLA-B27 is a risk factor for oligoarthritis, particularly in older male patients (Rachelefsky *et al*., 1974; Reviewed in Borchers *et al*., 2006).

SNPs in PTPN22 and CTLA4 were also found to be associated with JIA in different populations (reviewed in Phelan *et al.*, 2006).

1.4.2.4.2. Antibodies

Although wide variety of autoantibodies has been described in JIA patients, RF and antinuclear antibodies (ANA) are routinely used to provide serological support for the diagnosis of JIA.

ANA are detected in ~30–50% of patients with JIA (Berntson *et al*., 2003) the prevalence vary from 38% to 85% in oligoarthritis, ~30–50% in polyarthritis and 0–17% in systemic onset disease (Al-Matar *et al*., 2002; Moroldo *et al*., 2004).

In more recent studies, anti-CCP antibodies were reported in 77% of patients with JIA overall, 93% in RF-negative polyarthritis, 84% in oligoarthritis and 62% in systemic arthritis (Borchers *et al*., 2006).

1.5. Aim and Strategy

Most of the autoimmune diseases have high female predominance (Whitacre, 2001). Although the female prevalence is often attributed to the effect of estrogen, it is stated that other sex differences might have as much or more relevance to autoimmune disease, that is X-inactivation (Stewart, 1998). Recently, it has been shown that high proportion of scleroderma, AITD and pre-clampsia patients has extremely skewed X-inactivation in their blood cells (Ozbalkan *et al*., 2005; Ozcelik *et al*., 2006; Uz *et al.*, 2007; Uz *et al.*, 2008).

RA is an autoimmune disease, with unknown cause. Like other autoimmune diseases there is female predominance. Hormonal levels and pregnancy-related microchimerism was previously hypothesized to play role in autoimmunity. However our group and others observed that skewed XCI could be a factor (Ozbalkan *et al*., 2005; Brix *et al.*, 2005; Ozcelik *et al*., 2006; Uz *et al.*, 2007; Uz *et al.*, 2008). Therefore we included the pediatric form of RA, JIA, in which the onset of the disease is before puberty, therefore hormonal status would not be effective. There is also a female predominance in JIA.

Here we hypothesize that skewed XCI might play a role in the pathogenesis of JIA and RA. In order to test our hypothesis, we analyzed the methylation status of a highly polymorphic CAG repeat in the androgen receptor (AR) gene. In this study we used JIA patients within the subgroups that have female predominance: oligoarthritis and polyarthritis from Turkish population, and RA from French population.

CHAPTER II

MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. PEDIATRIC SAMPLES

2.1.1.1. Turkish Children Control Samples

In this study, 211 healthy, unrelated Turkish children with no history of autoimmune disorders or cancer were involved. The mean age at analysis was 13±4 (mean±SD). Informed consent was obtained from all subjects (or legal guardians of subjects who had not reached age of majority). The ethics committee of the participating institutions approved the study protocol.

2.1.1.2. Turkish Juvenile Idiopathic Arthritis Patients

Caucasian children diagnosed with juvenile idiopathic arthritis (n=81) were included in this study. All patients fulfilled the International League of Associations for Rheumatology diagnostic criteria for juvenile idiopathic arthritis (Petty *et al.* 2004). The mean±SD age of the patients was 10±5

years, and the mean age at the time of disease onset was 6±4 years. 21 of the patients were diagnosed with polyarthritis, while 60 were having oligoarthritis. Informed consent was obtained from all subjects (or legal guardians of subjects who had not reached age of majority). The ethics committee of the participating institutions approved the study protocol.

2.1.2. ADULT SAMPLES

2.1.2.1. French Control Samples

Healthy, unrelated French females (n=100) with no history of autoimmune disorders or cancer were involved in this study. Informed consent was obtained from all subjects. The ethics committee of the participating institutions approved the study protocol.

2.1.2.2. French Rheumatoid Arthritis Patients

French females diagnosed with rheumatoid arthritis (n=86) were involved. Informed consent was obtained from all subjects. The ethics committee of the participating institutions approved the study protocol.

2.1.2.3. Turkish Adult Control Samples

For determination of frequency of *PTPN22* genotypes in Turkish population 68 unrelated healthy women were genotyped. Informed consent was obtained from all subjects. The ethics committee of the participating institutions approved the study protocol.

2.1.2.4. Turkish Systemic Sclerosis Patients

For determination of frequency of *PTPN22* genotypes in Turkish population 71 SSc patients were enrolled in the study. The mean age of the patients was 47 years. Informed consent was obtained from all subjects. The ethics committee of the participating institutions approved the study protocol.

2.1.2.5. Turkish Autoimmune Thyroid Disease Patients

In order to determine the frequency of *PTPN22* genotypes 104 AITD patients were enrolled in the study. The mean±SD age of the patients was 49±14 years. Informed consent was obtained from all subjects. The ethics committee of the participating institutions approved the study protocol.

2.1.3. CHEMICALS, REAGENTS AND ENZYMES

2.1.3.1. *Primers*

The primers used in polymerase chain reaction (PCR) were purchased from IONTEK (Istanbul, Turkey).

The primer sequences for *AR* are:

RS6, 5'- GTCCAAGACCTACCGAGGAG -3';
RS7, 5'- CCAGGACCAGGTAGCCTGTG -3'

The primer sequences for *PTPN22* are:

PTPN22F, 5'-GATAATGTTGCTTCAACGGAATTTA -3';
PTPN22R 5'- TCACCAGCTTCCTCAACCACA -3'

2.1.3.2. *Enzymes*

Taq DNA polymerase enzymes and the restriction digestion enzymes *Xcm*I, *Rsa*I and methylation sensitive *Hpa*II were purchased from MBI Fermentas (Amherst, NY, USA).

2.1.3.3. Thermal cyclers

For PCR reactions, the thermal cycler The GeneAmp System 9600 and 2400 (Perkin-Elmer, USA) were used.

2.1.3.4. Standard Solutions and Buffers

1X TAE (Tris-acetic acid-EDTA):	40mM Tris-acetate, 2 nM EDTA, pH 8.0
Ethidium bromide:	10mg/ml in water (stock solution) 30 ng/ml (working solution)
Agarose gel loading buffer (6X):	15% ficoll 0.05% bromophenol 0.05% xylene cyanol
Acrylamide:bisacrylamide (30%):	29.5 gr acrylamide 0.44 gr bisacrylamide ddH_2O to 100 ml

2.1.3.5. Chemicals and Reagents

Table 2.1. Chemicals, reagents, and kits used in this study

Reagent/Chemical	Company
Agarose	Basica LE, EU
Acetic acid	Sigma, St Lois, MO, USA
Acrylamide	Sigma, St Lois, MO, USA
Ammonium persulfate	Carlo Elba, Milano, Italy
Bisacrylamide	Sigma, St Lois, MO, USA
Bromophenol blue	Sigma, St Lois, MO, USA
dNTPs	MBI Fermentas, Amherst, NY, USA
EDTA	Carlo Elba, Milano, Italy
Ethanol	Merck, Frankfurt, Germany
Ethidium bromide	Sigma, St Lois, MO, USA
Nucleospin® Blood Kit	Macherey-Nagel Duren, Germany
TEMED	Sigma, St Lois, MO, USA
Tris-Base	Bio-Rad, CA, USA

2.1.3.6. Nucleic Acids

As a DNA marker, pUC Mix8 was used, which is purchased from MBI Fermentas (Amherst, NY, USA) (Figure 2.1).

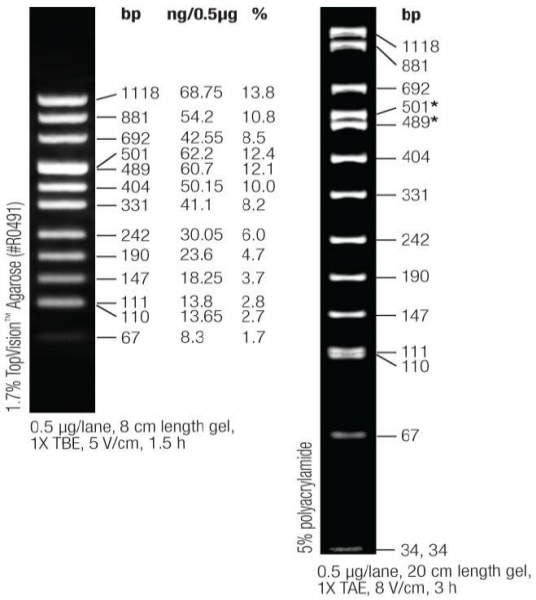

Figure 2.1. Sizes of the fragments of PUC mix marker, 8, and appearance on both agarose and polyacrylamide gel electrophoresis (MBI Fermentas web site)

2.2. METHODS

2.2.1. Sample Collection

Venous blood that was obtained from controls and patients were collected in EDTA-containing tubes. They were divided into 1 ml aliquots in 1.5 ml eppendorf tubes. 200 µl of blood was used for DNA isolation; the remaining bloods were stored at -80^{o}C for later use.

2.2.2. Determination of X chromosome inactivation status

For dosage compensation in mammals, females randomly inactivate one of their X-chromosome, maternal or paternal (Lyon *et al.,* 1961). The inactivation initiates upon transcription of *XIST* RNA from the X-chromosome that will be inactivated. The untranslated *XIST* RNA coat the X-chromosome that it is produced and with the help of histone modifications, such as hypermethylation of CpG islands (Wolf *et al.,* 1984; Heard and Disteche, 2006). *Hpa*II is a methyl sensitive restriction enzyme, which recognizes CpG dinucleotides. Therefore, X-linked genes that contain *Hpa*II recognition sites and silences during inactivation process can be used for determination of X-chromosome inactivation. Allen *et al.* first used androgen receptor (*AR*), which has highly polymorphic CAG repeats, in order to determine the XCI pattern (Allen *et al*., 1992). In this assay, the DNA is digested with *Hpa*II and the region that contains highly polymorphic CAG repeats flanked by restriction enzyme recognition site is amplified by PCR (Figure 2.2). After amplification only the inactive allele would give a product as the active one would be digested by *Hpa*II. Therefore, in women informative for CAG repeat, methylation patterns of maternal and paternal X chromosomes can be identified.

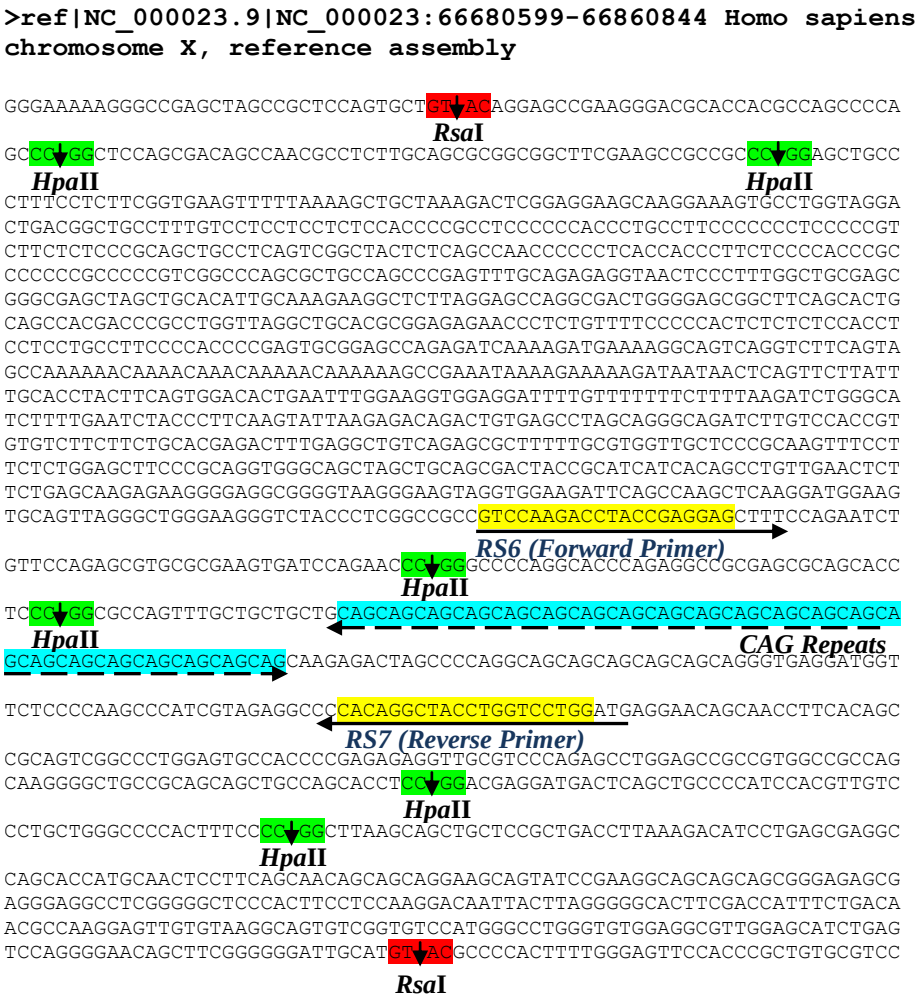

Figure 2.2. The sequence of *AR*, exon 1, downloaded from http://www.ncbi.nlm.nih.gov. The region amplified by PCR, the *Hpa*II recognition sites and CAG repeat regions are shown.

2.2.2.1. DNA Isolation

The DNA isolation was carried out from 200 µl venous blood via Nucleospin® Blood kit (Macherey-Nagel Inc., PA, USA) according to manufacturer's instructions. The remaining bloods were stored at -80^{o}C for later use. After isolation, DNA was stored at 4 ^{o}C. The quantity of DNA samples were measured using nanodrop. Ratio of 260/280 reading of 1.8 ± 0.2 was accepted as high quality DNA. The integrity of DNA samples were controlled by using 1% agarose gel electrophoresis.

2.2.2.2. Restriction Enzyme Digestion

Two sets of reactions were prepared for each subject in restriction digestion protocol. Both sets contain 150-250 ng genomic DNA, 1X reaction buffer and 2 Units of *Rsa*I. *Rsa*I is a restriction enzyme with the recognition sites outside the PCR product. It digests active and inactive X alleles equally, and was used to improve the PCR. The only difference between these two sets is the presence of methylation sensitive restriction enzyme *Hpa*II in the tube labeled as "digested (D)" (8 Units per 20 μL reaction). The tubes that do not contain *Hpa*II were labeled as "undigested (U)". Both reaction sets were completed to a final volume of 20 μL with deionized water. Restriction digestion reaction tubes were incubated at 37°C overnight. One control male sample that was cytogenetically verified as 46, XY was involved in this study as a control for complete digestion.

2.2.2.3. Polymerase Chain Reaction (PCR)

For each sample, two PCR were performed. Tubes that contain restriction digestion product of undigested reaction (without *Hpa*II) as a template were labeled as "U", and the tubes containing the product of digested reaction as a template were labeled as "D". PCRs were carried out in a total volume of 25 μL. Restriction digestion products were amplified with 10 pmol of each primers, 0.12 mM of dNTP, 1X *Taq* polymerase buffer, 1.0 mM $MgCl_2$, and 1 Unit *Taq* polymerase.

In order to amplify the desired region on *AR* locus, 94°C 5 min initial denaturation step was followed by 30 cycles of 94°C for 30 sec (denaturation), 58°C for 30 sec (annealing), 72°C for 30 sec (extension) amplification step. Final extension was performed at 72°C for 5 min.

2.2.2.4. Agarose Gel Electrophoresis

PCR products were run in the 1.5% agarose gel by using 1X TAE. Agarose was completely dissolved in 1X TAE electrophoresis buffer to required percentage in microwave and ethidium bromide was added to final concentration of 30ng/ml. The samples were loaded onto agarose gel with 1/5 volume of loading buffer. The gel was run in 1X TAE at 100V for 30 minutes at room temperature.

2.2.2.5. Polyacrilamide Gel Electrophoresis (PAGE)

The working PCR products were resolved by using 8% PAGE. In order to prepare PAGE, 30% acrylamide:bisacrylamide solution (29:1) was mixed with 10X TAE, 10 % Ammonium persulfate (APS), and TEMED and ddH_2O. 10 µL from each sample was loaded and each gel was run at 15W for 2 hours. The gels were stained with EtBr for 5 min and destained in ddH_2O for 5 min.

2.2.2.6. Densitometric Analysis

Densitometric analyses of the bands were performed twice for each sample using the MultiAnalyst version 1.1 software. The skewing ratio was calculated according to the formula in figure 2.3. A corrected ratio (CrR) was calculated by dividing the ratio of the predigested sample (upper/lower allele) by the ratio of the non-predigested sample for normalization of the ratios that were obtained from the densitometric analyses. The use of CrR compensates for preferential amplification of the shorter allele when the number of PCR cycles increases (Delforge *et al.*, 1995). A skewed

population is defined as a cell population with greater than 80% expression of one of the AR alleles. This corresponds to CrR values of <0.25 or >4.

$$\text{Skewing ratio} = \frac{D_1(U_1+U_2)}{2U_1(D_1+D_2)}$$

U_1=intensity of upper allele of undigested sample
U_2=intensity of lower allele of undigested sample
D_1= intensity of upper allele of digested sample
D_2= intensity of lower allele of digested sample

Figure 2.3. The formula for calculation of skewing ratio

2.2.3. Determination of frequency of *PTPN22* genotypes

PTPN22 encodes lymphoid protein tyrosine phosphatase (LYP), which suppresses T-cell activation by bind to negative regulatory kinase Csk. More common allele 1858C can binding Csk, while 1858T does not (Bottini *et al.*, 2004). 1858C→T SNP, which is in the first proline-rich motif in human LYP, is on exon 14 of *PTNP22* gene. The C > T transition creates a restriction site for *Xcm*I (figure 2.4). Therefore, in order to identify the polymorphism, PCR amplified fragment is digested by *Xcm*I.

>ref|NG_011432.1| Homo sapiens protein tyrosine phosphatase, non-receptor type 22 (lymphoid) (PTPN22), RefSeqGene on chromosome 1

TTATTATTATTATTTTTTGAGACGGAGTGTCCTGCTGCCACCCAGGCTGGAATGCAGTGGTGCAATCTCGGCTCAC
TGCAAGCTCTGCCTTCCGGGTTCACGCCATTCTCCCACCTCAGCCTCCCGAGTAGCTGGGACTACAGGGGCCCGCC
ACCACGCCCGGCTAATGTTTTGTGTTTTTAGTAGAGACACGGTTTCACCACGTTAGCCAGGATGGTTTCGATCTCC
TGACCTTGTGATCTGCCCGCCTCGGCCTCCGAAAGTGCTGGGATTACAGGCGTGAGCCACCGCGCCCAGCCCTACT
TTTGAGCTTTTAAAACAATAAATGTTAAAGAATAAGCAAAAACCTCCTGGGTTTGTACCTTAAGAGAATTTATTTT
GCTTTTTCCTTGAATGAACAAGTGTCAACTTTACTGATAATGTTGCTTCAACGGAATTTAAATATAAATTATGGTA

PTPN22 F

AATTTATATTTAATATTAGAATATAAGAATTTCCTTTGGATTGTTCTAATTAACAATTGTTACAATATTTTTGACA
TTTTGGATAGCAACTGCTCCAAGGATAGATGATGAAATCCCCCCTCCACTTCCTGTACGGACACCTGAATCATTT

*Xcm*I

ATTGTGGTTGAGGAAGCTGGTGAGTACAGTTCAGTAAGTATAAAATAAAGTGTGGGATGGGCATGGTGGCTCATGC

PTPN22 R

CTTTAATTCCAGCACTTTGGGAAGCTGATGTGTGAGCCTTGAGTTTGAGGAGTTCATTGAGGCCAGGAGTTCAAGA
CTAGCCTGCGCAACATAGTGAGACCTCATCTCTAATTTTTTTTTTTTAATTTAGCAGAGCAATAGCAGCATGCATG
TGTAGTCCCAACTATTTGGATGGTGGAGGTGAGAGGATCACTTGAGCCCAGGAGTTGGGGGCTGCAGTAAGCCATG
ATTGTGCCACTGCACTCCAGTCTGGGTGACAGAGCAAGACCCTGTCTCAAAAA

Figure 2.4. The sequence of *PTPN22*, exon 14, downloaded from http://www.ncbi.nlm.nih.gov. The region amplified by PCR, and the *Xcm*I recognition sites are shown. The C > T transition at nucleotide 1858, shown in red, creates in the T allele a restriction site for *Xcm*I.

2.2.3.1. Polymerase Chain Reaction (PCR)

PCRs were carried out in a total volume of 25 µL. DNAs products isolated from venous blood were amplified with 10 pmol of each primer, 0.2 mM of dNTP, 1X *Taq* polymerase buffer, 2.0 mM $MgCl_2$, and 1 Unit *Taq* polymerase.

In order to amplify the desired region on *PTPN22* locus, 95°C 5 min initial denaturation step was followed by 35 cycles of 95°C for 30 sec (denaturation), 59°C for 30 sec (annealing), 72°C for 40 sec (extension) amplification step. Final extension was performed at 72°C for 5 min.

PCR products were resolved on a 1.5% agarose gel by using 1X TAE. Ethidium bromide was added to final concentration of 30ng/ml. The samples were loaded onto agarose gels with 1/5 volume of loading buffer. The gels were run in 1X TAE at 100V for 30 minutes at room temperature.

2.2.3.2. Restriction Enzyme Digestion

The PCR amplified fragments are digested by *Xcm*I, which recognizes the T allele. The sets contain PCR fragments, 1X reaction buffer and 2 Units of *Xcm*I. Reaction sets were completed to a final volume of 20 μL with deionized water and were incubated at 37°C overnight. Each digestion was resolved on 3% agarose gel by using 1X TAE. Ethidium bromide was added to final concentration of 30ng/ml. The samples were loaded onto agarose gel with 1/5 volume of loading buffer, and the fragments were visualized by U.V. When the individual is homozygous for C allele, the product is 215 bp, while the T/T homozygous individuals have the bands of 169, and 46 bp.

2.2.4. Statistical Analysis

The results from case and control groups were compared by Fisher's Exact Test. In addition, odd's ratio value for control and case groups were performed in 95% confidence interval.

CHAPTER III

RESULTS

Only the samples whose alleles were adequately resolved were labeled as 'informative'. Extremely skewed XCI was described as the inactivation of one allele more than 90%. If one of the alleles was preferentially inactivated more than 80%, the XCI pattern was named as 'skewed'.

3.1. PCR-based X inactivation study of peripheral blood of Turkish pediatric control samples

XCI was found to be informative in 155 of 211 Turkish pediatric controls (73.5%). Skewed XCI was observed in 11 of 155 informative controls (7.1%) and extremely skewed XCI was observed in only 2 of 155 control individuals (1.3%). Extremely skewed XCI is a very rare event in the healthy individuals. Our results displayed consistency with the previously published results (Amos-Landgraf *et al.,* 2006). The gel image of representative control samples is exhibited on Figure 3.1.

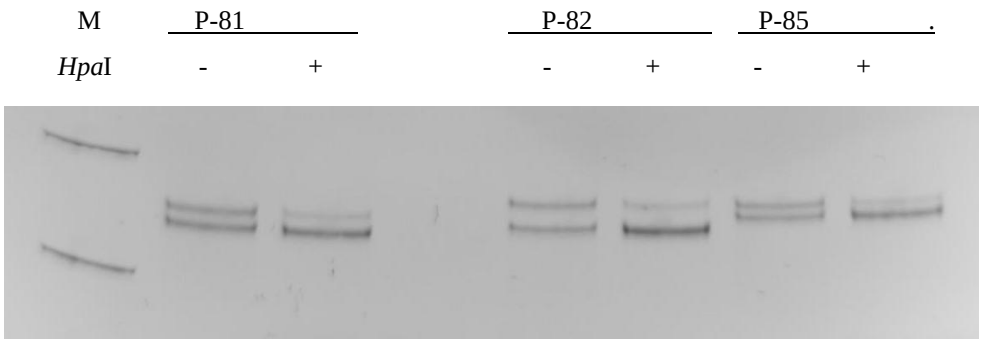

Figure 3.1. Gel image of XCI patterns of 3 controls. Polymerase chain reaction products from the androgen receptor methylation assay demonstrate X chromosome inactivation patterns in samples P-81 (allele ratio: 75%:25%), P-82 (85%:15%), and P-85 (67%:33%). For each sample, DNA was either undigested (-) or digested (+) with the methylation-sensitive restriction enzyme *Hpa*II. Marker M; pUC mix, 8. 331-bp and 242- bp fragments are visible.

3.2. PCR-based X-inactivation study of peripheral blood of Turkish juvenile idiopathic arthritis patients

81 children diagnosed with JIA were genotyped for XCI pattern. 62 of them display informative XCI (76.5%). Extremely skewed XCI was observed in 8 of the 62 informative patients (12.9%). The same pattern was observed in only 2 of 155 informative pediatric controls (P=0.0008; odds ratio=11.33 (95%CI: 2.62-48.48). Skewed XCI was observed in 14 of 62 informative patients (22.6%) and only in 11 of 155 control group (7.1%) (P=0.0036; odds ratio=3.81 (95%CI: 1.65-8.83). The gel image of representative JIA samples is exhibited on Figure 3.2. The overall XCI pattern of JIA samples and control group children are summarized in Table 3.1. Skewed and extremely skewed pattern difference between JIA patients and control individuals were statistically significant. To the best of our knowledge this is the first study that investigates XCI pattern in the blood cells of the pediatric samples.

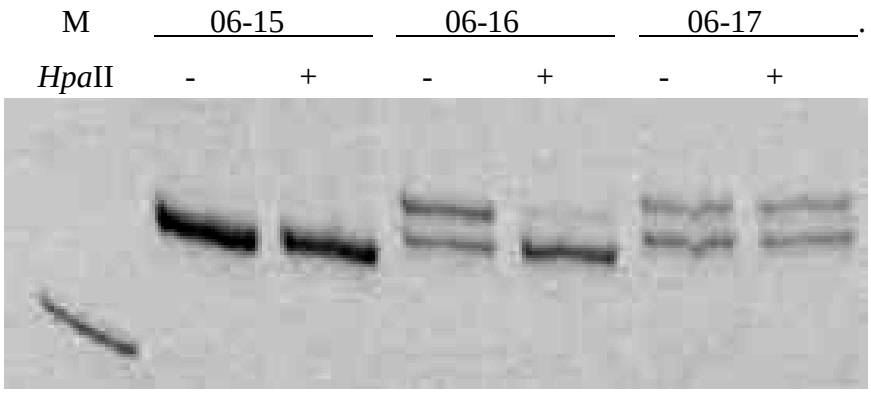

Figure 3.2. Gel image of XCI patterns of 3 JIA patients. Polymerase chain reaction products from the androgen receptor methylation assay demonstrate X chromosome inactivation patterns in samples 06-15 (allele ratio: not informative), 06-16 (93%:7%), and 06-17 (51%:49%). For each sample, DNA was either undigested (-) or digested (+) with the methylation-sensitive restriction enzyme *Hpa*II. Marker M; pUC mix, 8. 242- bp fragment is visible.

Table 3.1 Proportion of the JIA patients and controls with skewed XCI

Degree of skewing (%)	No. (%) observed with skewing	
	JIA patients (n=62)	**Controls (n=155)**
90+	8 (12.9)*	2 (1.3)
80-89	6 (9.7) *	9 (5.8)
70-79	13 (21.0)	29(18.7)
60-69	18 (29.0)	39 (25.2)
50-59	17 (27.4)	76 (49.0)

* $P < 0.005$ versus controls

For comparison by Fisher's Exact Test, For ≥80% skewing, P= 0.0036 (odds Ratio=3.81 [95% CI 1.65-8.83]); for ≥90% skewing, P=0.0008 (odds ratio=11.33 [95% CI: 2.62-48.48]),

The clinical data of JIA (Table 3.2) was used in order to construct a correlation between skewed XCI and any clinical state of the disease. The only attractive correlation was established between nonrandom XCI and the clinical classification of JIA. It was interesting that only one patient was polyarticular in the group with skewed XCI pattern (7.1%) whereas 12 in 48 patients with random XCI pattern were polyarticular (25.0%). Although the number of patients with skewed XCI was small, most probably skewed XCI may play a role more in the etiology of oligoarthritis JIA.

Previously it has been reported that after years of immunosuppressive agent exposure, skewed XCI may occur in feline hematopoietic cells (Abkowitz *et al*. 1993). Therefore, we investigated the correlation between XCI ratios and the treatment of patients. At the time of sample collection, all of the patients were receiving some treatment. Fourteen were receiving nonsteroidal anti-inflammatory drugs (NSAIDs), 11 were receiving methotrexate (MTX), 10 were receiving MTX plus NSAIDs, and 9 were receiving intraarticular corticosteroids plus NSAIDs, while the remaining 18 were receiving various combinations of these and other drugs. Seven (50%) of the patients that had skewed XCI had received treatment with immunosuppressive agents for 6–10 years, and 1 (7%) had received immunosuppressive treatment for 2 years. The remaining 6 had received antiinflammatory treatment alone. Twenty-seven (56.3%) of the patients with random XCI had received immunosuppressive treatment for more than 2 years. These results indicate that it is unlikely that immunosuppressive therapy caused skewed XCI in the patients.

Table 3.2 Clinical characteristics and XCI status of informative JIA patients

	Code	XCI	DoB	OoD (y)	Clinical Class	Affected Joint(s)	Immunosuppressive
90+							
1	06-029	100	1996	3.5	O	3	MTX
2	05-069	100	1990	11	O	4	NSAID;MTX
3	05-188	95	1989	7	O	2	NSAID
4	06-014	95	1993	14	O	2	NSAID
5	06-013	95	1983	16	EO	8	NSAID; MTX; PRED; anti-TNF
6	06-016	93	2002	2	O	1	NSAID;IAS
7	06-030	92	1999	2	O	3	MTX
8	06-008	90	1994	2	O	1	NSAID;IAS
80-89							
9	05-179	88	2000	3	P	6	NSAID;MTX
10	06-026	84	2001	2	O	2	MTX
11	07-1078	83	2004	3	O	1	NSAID
12	05-064	80	1994	7	O	3	NSAID;IAS; MTX
13	06-020	80	2003	2	O	1	NSAID;IAS
14	07-1080	80	2003	3	P	5	NSAID;MTX
70-79							
15	05-170	78	1991	10	P	7	NSAID;MTX
16	07-1087	78	1998	9	O	2	NSAID
17	06-012	76	1986	10	O	1	NSAID
18	05-071	75	1995	1	P	10	NSAID;MTX,PRED,Cyc
19	05-178	75	1996	8	P	10	NSAID
20	06-021	75	2002	2	O	1	NSAID;IAS
21	06-052	74	2002	2.5	O	2	MTX,IAS
22	05-187	73	1990	10	O	2	NSAID
23	05-183	72	1999	5	O	1	NSAID;IAS
24	06-009	71	1996	7	O	2	IAS;NSAID
25	06-043	71	1998	2	O	2	MTX;SAZ;IAS
26	05-065	70	1990	2	P	10	MTX;NSAID;SAZ
27	05-063	70	1991	11	O	3	MTX;NSAID
60-69							
28	05-070	69	1994	5	P	13	NSAID;MTX;PRED
29	06-025	69	2000	4	O	2	NSAID,MTX,IAS

30	07-1079	65	1987	3	P	14	Anti-TNF,NSAID
31	05-601	64	2001	4	O	3	NSAID
32	05-173	63	2001	2	O	1	NSAID,
33	05-182	63	1994	9	EO	7	NSAID;PRED;MTX
34	06-024	62	1999	4	O	1	NSAID;PRED
35	06-037	62	1996	4	O	3	MTX,SAZ
36	06-044	62	1991	7	O	2	NSAID;IAS
37	05-602	61	2003	2	O	1	NSAID
38	06-028	61	2001	1.5	O	2	MTX
39	05-073	60	1995	4	P	7	NSAID;IAS
40	05-072	60	1988	1	P	10	NSAID;MTX
41	05-062	60	1984	11	O	3	MTX;NSAID
42	06-051	60	2000	3	O	2	MTX
43	05-066	60	1998	3	O	1	NSAID
44	07-1084	60	1998	9	O	1	NSAID
45	07-1086	60	1995	10	O	2	NSAID
50-59							
46	05-168	58	1990	9	P	9	NSAID;MTX;anti-TNF;PRED
47	06-034	57	1999	3	O	2	MTX
48	05-167	56	2002	9	EO	8	NSAID;MTX;PRED
49	05-169	56	1987	4	P	6	NSAID;MTX
50	06-038	56	1998	3	O	2	MTX
51	05-177	55	1992	10	P	5	NSAID;MTX
52	06-019	54	2000	4	O	1	NSAID;IAS
53	06-027	53	1999	7	O	2	MTX;NSAID
54	06-031	53	2000	2	O	2	MTX
55	06-048	52	2002	1	O	2	MTX
56	05-166	51	1980	13	P	12	MTX, PRED,SAZ, Leflunomid
57	05-185	51	1998	6	O	2	NSAID;MTX;PRED;
58	06-046	51	2005	1.5	O	2	MTX
59	06-045	50	2002	2	O	2	MTX
60	06-053	50	1998	2	O	1	SAZ
61	07-1081	50	2002	5	O	2	NSAID
62	07-1085	50	1998	9	O	1	NSAID,SAZ

DoB: Date of birth, OoD: Onset of disease, y: year, O: oligoarticular, P: polyarticular, EO: extended oligoarticular, Anti-TNF: Tumor necrosis factor antagonist , MTX: methotrexate, NSAID: non-steroidal anti-inflammatory drugs, PRED: prednisolone, IAS: intraarticular corticosteroids, SAZ: sulfasalazine.

3.3. PCR-based X-inactivation study of peripheral blood of French control samples

Among 100 French controls, 69 were informative at AR locus. This number represents 69.0% of the control population. 12 of 69 informative control cases displayed skewed XCI (17.4%). Only 2 of 69 informative control women display extremely skewed XCI pattern (2.9%). The gel image of representative control samples is exhibited on Figure 3.3.

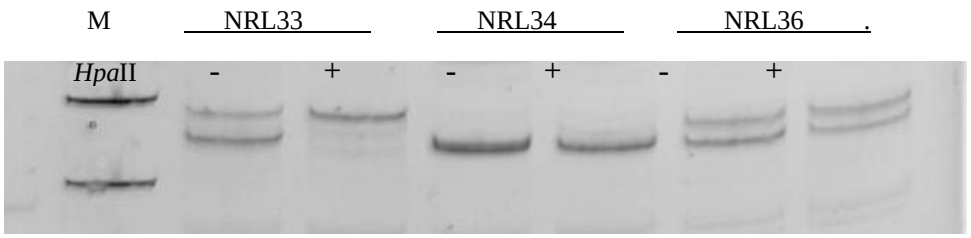

Figure 3.3. Gel image of XCI patterns of 3 controls. Polymerase chain reaction products from the androgen receptor methylation assay demonstrate X chromosome inactivation patterns in samples NRL33 (allele ratio: 90%:10%), NRL34 (not informative), and NRL36 (50%:50%). For each sample, DNA was either undigested (-) or digested (+) with the methylation-sensitive restriction enzyme *Hpa*II. Marker M; pUC mix, 8. 331-bp and 242- bp fragments are visible.

3.4. PCR-based X-inactivation study of peripheral blood of French rheumatoid arthritis patients

Informative XCI pattern was observed in 54 of 86 French RA patients (62.8%). Extremely skewed XCI was observed in 10 of 54 informative patients (18.5%). The same pattern was observed in 2 of 69 informative French controls (2.9) (P=0.005, odds ratio=7.6 with 95%CI between 1.59 and 36.42). Skewed XCI was present in 17 of 54 informative patients (31.5%) while in 12 of 69 informative controls (17.4%) (P=1.00; OR: 0.9,

95%CI=0.3-2.5). The results were significant for extremely skewed XCI, but not for skewed XCI. The overall XCI pattern of RA samples and control group are summarized in Table 3.3. Representative gel image of XCI pattern of French RA patients was displayed in Figure 3.4. The XCI patterns for all of French RA patients are listed in Table 3.4.

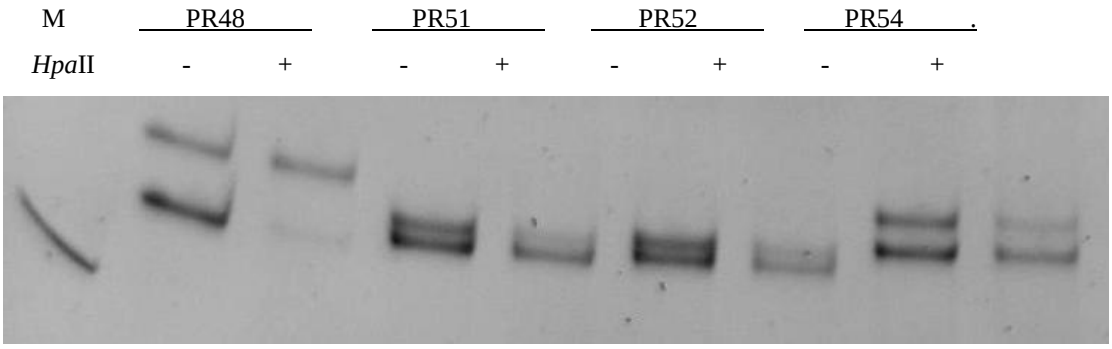

Figure 3.4. Gel image of XCI patterns of 4 RA patients. Polymerase chain reaction products from the androgen receptor methylation assay demonstrate X chromosome inactivation patterns in samples PR48 (allele ratio: 90%:10%), PR51 (80%:20%), PR52 (50%:50%), and PR54 (60%:40%). For each sample, DNA was either undigested (-) or digested (+) with the methylation-sensitive restriction enzyme *Hpa*II. Marker M; pUC mix, 8. 242- bp fragment is visible.

Table 3.3 Proportion of the French RA patients and controls with skewed XCI

Degree of skewing (%)	No. (%) observed with skewing	
	RA patients (n=54)	**Controls (n=69)**
90+	10 (18.5)*	2 (2.9)
80-89	7 (13.0)	10 (14.5)
70-79	9 (16.7)	10 (14.5)
60-69	9 (16.7)	12 (17.4)
50-59	19 (35.2)	35 (50.7)

* P< 0.05 versus controls

For comparison by Fisher's Exact Test, For ≥80% skewing, P= 1.00 (odds Ratio=0.9 [95% CI 0.3–2.5]); for ≥90% skewing, P=0.005 (odds ratio=7.6 [95% CI 1.59–36.42]),

Table 3.4. XCI patterns of French RA patients.

		Code	XCI
90+	10/54 (18,5%)		
1	1	PR 1	90
2	2	PR 19	90
3	3	PR 48	90
4	4	PR 64	90
5	5	PR 76	90
6	6	PR 165	90
7	7	PR 179	90
8	8	PR 184	90
9	9	PR 188	90
10	10	PR 211	90
80-89	7/54(13,0%)		
11	1	PR 134	85
12	2	PR 177	85
13	3	PR 194	85
14	4	PR 2	80
15	5	PR 3	80
16	6	PR 51	80
17	7	PR 136	80
70-79	9/54(16,7%)		
18	1	PR 13	75
19	2	PR 158	75
20	3	PR 197	75
21	4	PR 10	70
22	5	PR 40	70
23	6	PR 71	70
24	7	PR 185	70
25	8	PR 161	70
26	9	PR 202	70
60-69	9/54(16,7%)		
27	1	PR 37	65
28	2	PR 5	60
29	3	PR 54	60
30	4	PR 58	60
31	5	PR 160	60
32	6	PR 172	60
33	7	PR 190	60
34	8	PR 195	60
35	9	PR 200	60
50-59	19/54(35,2%)		
36	1	PR 79	55
37	2	PR 82	55
38	3	PR 141	55
39	4	PR 155	55
40	5	PR 159	55
41	6	PR 52	50
42	7	PR 62	50
43	8	PR 63	50
44	9	PR 66	50
45	10	PR 92	50
46	11	PR 105	50
47	12	PR 145	50
48	13	PR 154	50

49	14	PR 169	50
50	15	PR 170	50
51	16	PR 193	50
52	17	PR 198	50
53	18	PR 205	50
54	19	PR 212	50
Not inform.	30/84(35,7%)		
55	1	PR 20	NI
56	2	PR 21	NI
57	3	PR 24	NI
58	4	PR 31	NI
59	5	PR 47	NI
60	6	PR 59	NI
61	7	PR 60	NI
62	8	PR 65	NI
63	9	PR 77	NI
64	10	PR 78	NI
65	11	PR 85	NI
66	12	PR 88	NI
67	13	PR 93	NI
68	14	PR 110	NI
69	15	PR 131	NI
70	16	PR 135	NI
71	17	PR 138	NI
72	18	PR 139	NI
73	19	PR 146	NI
74	20	PR 149	NI
75	21	PR 151	NI
76	22	PR 164	NI
77	23	PR 167	NI
78	24	PR 187	NI
79	25	PR 191	NI
80	26	PR 192	NI
81	27	PR 203	NI
82	28	PR 206	NI
83	29	PR 209	NI
84	30	PR 213	NI
Not Worked			
85	1	PR 148	
86	2	PR 162	

XCI: X chromosome inactivation pattern

3.5. Determination of frequency of *PTPN22* genotypes in Turkish control samples

As SNP frequencies may vary across populations, we investigated the *PTPN22* polymorphism in 68 Turkish healthy unrelated individuals. The risk allele, T, was found to be present in 8 of 136 chromosomes (5.9%): 6

C/T and 1 T/T. The genotypes were also confirmed by high-density microarray analysis (Appendix J).

3.6. Determination of frequency of *PTPN22* genotypes in Turkish systemic sclerosis patients

71 individuals diagnosed with SSc were genotyped for *PTPN22* C1858T SNP. 4.2% of individuals with SSc were heterozygous with respect to 1858T, compared with 8.8% of the healthy controls, and none of individuals with SSc were homozygous with respect to 1858T, compared with 1.4% of the healthy controls. The 1858T allele frequency was 2.11% compared to 5.88% in controls. These results suggest that the *PTPN22* allele 1858T is not associated with SSc in Turkish population. The overall genotype frequencies of *PTPN22* are summarized in Table 3.5. The genotypes were also confirmed by high-density microarray analysis (Appendix J).

Table 3.5 Frequency of PTPN22 genotypes in individuals with SSc and controls

	No. (frequency) of *PTPN22* genotype	
	Controls	SSc
Genotypes	(n=68)	(n=71)
C/C	61 (89.7%)	68 (95.8%)
C/T	6 (8.8%)	3 (4.2%)
T/T	1 (1.5%)	0 (0%)
T allele frequency	8/136 (5.88%)	3/142 (2.11%)
P=0.13, OR: 0.34 (95% CI: 0.089-1.33)		

3.7. Determination of frequency of *PTPN22* genotypes in Turkish autoimmune thyroid disease patients

104 individuals diagnosed with AITD were genotyped for *PTPN22* C1858T SNP. 7 (6.7%) of individuals with AITD were heterozygous with respect to 1858T, compared with 8.8% of the healthy controls, and one (0.96%) of individuals with AITD was homozygous with respect to 1858T, compared with 1.4% of the healthy controls. The 1858T allele frequency was 4.33% compared to 5.88% in controls. These results suggest that the *PTPN22* allele 1858T is not associated with AITD in Turkish population. The overall genotype frequencies of *PTPN22* are summarized in Table 3.6. The genotypes were also confirmed by high-density microarray analysis (Appendix J).

Table 3.6 Frequency of PTPN22 genotypes in individuals with AITD and controls

	No. (frequency) of *PTPN22* genotype	
	Controls	AITD
Genotypes	(n=68)	(n=104)
C/C	61 (89.7%)	96 (92.3%)
C/T	6 (8.8%)	7 (6.7%)
T/T	1 (1.5%)	1 (0.96%)
T allele frequency	8/136 (5.88%)	9/208 (4.33%)
P=0.61, OR: 0.72 (95% CI: 0.27-1.92)		

3.8. High-density microarray analysis

In order to test the hypothesis that disturbances in XCI may cause female predominance in autoimmune disorders, we developed a research strategy in which we conducted a comprehensive genomic study by using high-density microarray analysis. For the custom made microarray, all known nonsynonymous, synonymous and intronic SNPs on X chromosome were listed using NCBI and Ensembl databases. Redundant and nonappropriate SNPs were eliminated. In the end, 1618 nonsynonymous, 1091 synonymous, and 2802 intronic SNPs on X chromosome were included on the array. 166 SNPs from other autosomal chromosomes that are known to be associated with several autoimmune disorders were added. The exonic SNPs cover 783 genes and the intronic SNPs correspond to 160 genes. 56 SSc, 82 AITDs, 48 PEE, 20 JIA, 2 SICCA, 7 pediatric SSc, and 250 control samples (total 465 samples) were used in this study.

After the analysis of the data, the candidate alleles, which have OR>1.0 and $p<0.05$, were found for JIA (Table 3.7).

Table 3.7. JIA Allelic Association Results (OR>1.0, p<0.05)

Chr	SNP	A1	Aff	UnAff	A2	X2	P	OR	L 95 c.i.	U 95% c.i.
23	rs15943	G	0.08	0.01	C	11.23	0.0008	8.35	1.92	9.56
23	rs2235708	A	0.06	0.01	G	5.74	0.0166	6.09	1.14	9.23
23	rs6324	T	0.05	0.01	C	4.91	0.0267	5.43	1.02	5.88
23	rs1123773	A	0.05	0.01	G	4.55	0.0329	5.15	0.97	5.83
23	rs5030868	T	0.05	0.01	C	4.52	0.0335	5.13	0.96	36.38
23	rs1199470	G	0.08	0.02	A	6.34	0.0118	4.93	1.25	5.30
23	rs7063566	T	0.10	0.02	G	8.19	0.0042	4.88	1.48	8.52
23	rs6624109	G	0.08	0.02	T	5.36	0.0206	4.33	1.13	6.27
23	rs12395804	T	0.23	0.06	C	13.4	0.0003	4.19	1.84	6.09
23	rs17327496	G	0.23	0.07	C	12.78	0.0004	4.06	1.78	6.09
23	rs6653146	G	0.08	0.02	A	4.54	0.0331	3.86	1.02	0.69
23	rs1414368	A	0.08	0.02	G	4.39	0.0361	3.75	1.00	5.90
23	rs7050548	C	0.22	0.07	G	9.76	0.0018	3.62	1.54	0.73
23	rs16990617	T	0.08	0.02	C	4.03	0.0447	3.56	0.95	7.41
23	rs5964770	A	0.08	0.02	G	4.03	0.0447	3.56	0.95	0.75
23	rs6524876	T	0.08	0.02	A	4.03	0.0447	3.56	0.95	0.75
23	rs12006627	C	0.08	0.02	A	4.03	0.0447	3.56	0.95	16.09
23	rs12012307	G	0.20	0.07	A	7.91	0.0049	3.18	1.37	0.78
23	rs12836780	C	0.55	0.29	A	12.21	0.0005	3.06	1.59	4.49
23	rs12009120	T	0.30	0.12	G	9.68	0.0019	3.03	1.46	0.81
23	rs331369	T	0.58	0.31	G	11.86	0.0006	3.03	1.57	0.82
23	rs217994	A	0.73	0.47	G	9.66	0.0019	2.98	1.46	4.33
23	rs217992	G	0.73	0.47	C	9.66	0.0019	2.98	1.46	19.35
23	rs17281349	A	0.13	0.05	G	4.58	0.0323	2.93	1.05	4.62
23	rs9697983	G	0.13	0.05	T	4.54	0.0331	2.91	1.04	4.97
23	rs5955897	C	0.32	0.14	A	8.48	0.0036	2.84	1.37	0.84
23	rs5939385	T	0.48	0.25	C	9.9	0.0017	2.76	1.44	4.46
23	rs1202985	T	0.18	0.08	C	4.86	0.0276	2.61	1.08	0.84
23	rs17311504	A	0.15	0.06	C	4.07	0.0438	2.55	1.00	0.86
23	rs5982867	A	0.18	0.08	G	4.4	0.0359	2.50	1.04	0.91
23	rs5955838	T	0.18	0.08	A	4.29	0.0383	2.46	1.02	0.86
23	rs5955593	C	0.30	0.15	T	6.11	0.0135	2.42	1.18	4.84
23	rs7052290	A	0.28	0.14	G	5.47	0.0193	2.37	1.13	32.61
23	rs7051930	G	0.30	0.15	A	5.74	0.0166	2.36	1.15	4.15
23	rs1016625	A	0.50	0.30	C	6.96	0.0084	2.35	1.23	4.52
23	rs1091269	T	0.68	0.47	C	6.13	0.0133	2.33	1.17	4.96
23	rs6524628	A	0.65	0.45	G	5.99	0.0144	2.28	1.16	nan
23	rs12688102	G	0.53	0.33	C	6.38	0.0115	2.27	1.18	16.69
23	rs6617216	T	0.66	0.46	A	5.57	0.0183	2.26	1.13	4.25
23	rs7885068	A	0.28	0.15	T	4.71	0.0300	2.22	1.06	4.25
23	rs5955882	T	0.24	0.12	C	3.97	0.0464	2.20	1.00	4.03
23	rs5923521	T	0.65	0.46	G	5.28	0.0216	2.17	1.11	4.18
23	rs6524627	A	0.65	0.46	T	5.27	0.0217	2.17	1.11	4.08
23	rs5987015	G	0.48	0.29	A	5.64	0.0176	2.17	1.13	4.21
23	rs1988916	G	0.66	0.47	C	4.9	0.0268	2.16	1.08	4.07
23	rs5927083	C	0.28	0.15	T	4.33	0.0374	2.15	1.03	0.91
23	rs6623640	T	0.65	0.46	C	5.16	0.0232	2.15	1.10	4.05
23	rs5986916	C	0.55	0.37	T	5.26	0.0218	2.15	1.10	4.05

Chr	SNP	A1	Aff	UnAff	A2	X2	P	OR	L 95 c.i.	U 95% c.i.
23	rs2520324	A	0.60	0.42	G	5.17	0.0230	2.12	1.10	3.97
23	rs2192371	G	0.53	0.34	A	5.27	0.0217	2.11	1.10	4.03
23	rs2078865	A	0.60	0.42	G	5.11	0.0238	2.11	1.09	28.99
23	rs2464544	T	0.60	0.42	G	5.05	0.0246	2.10	1.09	0.93
23	rs2238928	T	0.60	0.42	C	5.05	0.0246	2.10	1.09	4.31
23	rs2704831	T	0.60	0.42	C	4.97	0.0258	2.09	1.08	6.30
23	rs723119	G	0.48	0.30	A	5.02	0.0250	2.08	1.08	3.99
23	rs2854426	C	0.60	0.42	G	4.84	0.0278	2.07	1.07	0.94
23	rs5936218	G	0.63	0.45	T	4.7	0.0302	2.06	1.06	0.94
23	rs7051365	T	0.43	0.27	C	4.72	0.0299	2.05	1.06	3.96
23	rs5952296	T	0.30	0.17	C	3.92	0.0479	2.03	0.99	4.65
23	rs5935984	C	0.43	0.27	T	4.58	0.0324	2.03	1.05	0.94
23	rs5930931	A	0.63	0.45	C	4.38	0.0364	2.01	1.03	4.00
23	rs5975721	T	0.63	0.45	C	4.38	0.0364	2.01	1.03	3.91
23	rs5975720	A	0.63	0.45	T	4.38	0.0364	2.01	1.03	0.94
23	rs4829829	G	0.63	0.45	A	4.38	0.0364	2.01	1.03	0.95
23	rs5977755	G	0.63	0.45	C	4.38	0.0364	2.01	1.03	8.16
23	rs723556	C	0.60	0.43	T	4.38	0.0364	2.00	1.03	0.95
23	rs12847225	C	0.50	0.33	A	4.5	0.0338	1.99	1.04	0.95
23	rs2181307	A	0.63	0.46	G	4.28	0.0386	1.99	1.03	0.95
23	rs1152198	T	0.43	0.27	A	4.31	0.0378	1.99	1.03	0.95
23	rs5973488	A	0.55	0.38	G	4.23	0.0398	1.96	1.02	0.95
23	rs2428147	A	0.58	0.41	G	3.93	0.0475	1.95	1.00	1.11
23	rs1410962	G	0.55	0.39	A	4.14	0.0418	1.94	1.02	27.42
23	rs1410961	C	0.55	0.39	T	4.14	0.0418	1.94	1.02	0.95
23	rs4829392	C	0.55	0.39	T	4.14	0.0418	1.94	1.02	8.13
23	rs12852223	G	0.55	0.39	A	3.89	0.0485	1.93	0.99	27.31
23	rs4492499	A	0.55	0.39	G	3.89	0.0485	1.93	0.99	14.63

* Chromosome 23 denotes the X-chromosome

CHAPTER IV

DISCUSSION

Our immune system can react to any foreign structure with the help of receptors on the surface of B and T cells, while it is tolerant to its own self antigens. The immune system mediates self tolerance by killing self-reactive lymphocytes by apoptosis, or by silencing them by the anergy mechanism (Goodnow *et al.*, 2005). When self antigens escape from these mechanisms, autoimmune disorders develop. Although how these molecules escape from tolerance are not entirely known, a combination of genetic variants, environmental factors like pathogen exposure, pregnancy, smoking and diet are thought to have a role in the etiology of autoimmune diseases.

A female predominance is the characteristic of the most autoimmune diseases (Whitacre, 2001). For several years candidate mechanisms that could be important in pathogenesis of autoimmunity have been uncovered. The main differences between males and females are hormonal status, pregnancy and sex chromosomes. Hormones are good candidates for the female predominance in autoimmune disorders. However they fail to explain the pediatric type of autoimmune disorders in the children before puberty. Another possible mechanism could be the pregnancy related microchimerism. Although there are studies that show association of microchimerism and scleroderma (Artlett *et al*., 1998; Nelson *et al*., 1998), these reports cannot explain why women with no history of pregnancy and children are subject to autoimmune disorders. Third difference is sex

chromosomes. Females have two X chromosomes, and for dosage compensation, one of them is randomly inactivated (Lyon, 1961), therefore, females are mosaic. It was reported that disturbances in the XCI pattern may result in inadequate X-linked antigen representation in the thymus (Stewart, 1998).

In this study, our goal was to investigate the role of XCI in the pathogenesis of autoimmune disorders that show female predominance. RA is an autoimmune disease with unknown cause. JIA is the pediatric form of the RA. We have chosen JIA also to exclude the possible reasons of microchimerism and hormones, as the disease onset is before puberty. In this study, JIA patients and children controls form Turkish population, and RA patients and controls from French population were investigated. We have chosen polyarticular and oligoarticular subtypes of JIA as they have female predominance.

We demonstrated skewed XCI patterns (>80:20) in peripheral blood mononuclear cells of a significant proportion (22.6%) of females with JIA in Turkish (OR: 3.81(95%CI: 1.65-8.83); P=0.0036), and RA (31.5%) in French populations (OR:0.9 (95%CI:0.3-2.5); P=1.00). The effect is more pronounced for patterns of extremely skewed XCI in the Turkish JIA [12.9%, OR: 11.33 (95% CI 2.62-48.48); P=0.0008] and the French RA [18.5%, OR: 7.6 (95% CI 1.59-36.42); P=0.005] patients. In Turkish female pediatric controls 7.1% demonstrate skewed and 1.3% demonstrate extremely skewed XCI. In French controls 17.4% demonstrate skewed and 2.9% demonstrate extremely skewed XCI. These ratios are in accordance with the XCI profiles of other diverse control populations from US (Chitnis *et al*. 2000, Amos-Landgraf *et al*. 2006).

It has been reported that after several years of immunosuppressive treatment, skewed XCI may occur (Abkowitz *et al.* 1993). Therefore, we investigated whether the treatments of the patients play a role on the XCI ratios. Along with previous observations (Ozbalkan *et al*., 2005; Ozcelik *et*

al., 2006), our results do not indicate that immunosuppressive treatment cause skewed XCI.

There are several mechanisms that are used in order to determine XCI pattern, such as protein isoforms and transcription based methods. A variety of genes are used to determine XCI status including *G6PD* (Prchal and Guan, 1993), *IDS* (iduronate-2-sulfatase) (Gregg *et al*., 2000; el-Kassar *et al*., 1997), *MPP1* (also known as p55) (Luhovy *et al*., 1995), *BTK* (Bruton tyrosine kinase), and *FHL-1* (4.5 LIM domain 1) (Liu *et al*., 2003). In this study we used X chromosome inactivation assay, which is based on DNA methylation and tandem CAG repeats. This assay is more widely used because of the variable number of CAG nucleotide repeats, which are informative in most of the patients. In our study we had a limited number of patients, as we used only specific subtypes that have female predominance. Therefore, it is important to have most of the patients informative for the assay.

Our results suggest that a significant proportion of juvenile idiopathic arthritis and rheumatoid arthritis patients have extremely skewed XCI. There are mainly two reasons for skewed XCI: primary and secondary. Primary cause, which results from a mutation in *XIST* (X-inactive- specific transcript) and *Xic*, is very rare (Plenge *et al.,* 1997; Puck, 1998). The secondary causes are deleterious X-linked mutations, X-chromosome rearrangements, aging, twinning, or monoclonal expansion of cells (Brown, 1999). Based on observations that skewed XCI cosegregates with the disease and recurrent spontaneous abortions are elevated in skewed group (Ozcelik *et al.,* 2006), we propose that as a secondary cause, a putative lethal mutation on the X-chromosome may result in a cell-survival disadvantage. Cells that carry a putative lethal mutation in their active X do not survive because of the mutation, causing loss of mosaicism. The self antigens on the inactive X of these cells are not presented in the thymus or in other peripheral sites. Because of an unknown mechanism, at a later stage

of the life, these self antigens encounter the lymphocytes. Therefore, these self antigens are recognized as non-self and cause autoimmunity.

In order to determine whether skewed XCI is specific to blood or observed in other tissues as well, previously in our group buccal mucosa, hair and affected tissues were collected for the patients that have skewed XCI. In both AITD and PEE patients, skewed XCI was shown in buccal cells (ectodermal origin) of the patients that have skewed XCI in their blood (measodermal origin) (Ozcelik *et al.,* 2006; Uz *et al.,* 2007). As the degree of skewing is at the extreme profiles (>95%) in most patients, we believe that the self antigens escape from presentation because of skewing. If the skewing was not the cause but the result of the autoimmune disease, we would observe more patients in the milder ranges (80:20 or 90:10).

Another evidence for our hypothesis is the maternally inherited skewed XCI cosegregates with the disease in AITD patients (Ozcelik *et al.,* 2006). We also showed that recurrent spontaneous abortions increase in the skewed group (Ozcelik *et al.,* 2006; Bagislar *et al.,* 2006; Uz *et al.,* 2007). Lethal X-linked mutation can be compatible in females because of the XCI, while is lethal in hemizygous males.

Although extremely skewed XCI is rare in general population, the ratio increases as the age increases (Sangha *et al.* 1999). Previously in our group, it was shown that the age alone is unlikely to cause skewed XCI in AITD patients (Ozcelik *et al*., 2006). Here we again show that the age of the patients do not play a significant role in skewed XCI as there is extremely skewed XCI in a significant proportion of JIA patients, which is the pediatric form of RA.

Autoimmune disorders are complex diseases with unknown causes. Several genes, such as *PTPN22*, are shown to be associated with several diseases. The frequency of the C1858T SNP of *PTPN22* changes in different populations, therefore we investigated the frequency of the *PTPN22*

genotype in Turkish population. For this purpose 104 AITD, 71 SSc patients and 68 unrelated healthy individuals were included. Unlike in North American and European populations, we did not observe an association between C1858T SNP and AITD and SSc in Turkish population. The T allele frequency is 2.11% in SSc, and 4.33% in AITD compared to 5.88% in controls. It was also shown in Japanese population that this SNP was not associated with AITD (Ban *et al.*, 2005). Later on, it was shown that another SNP on *PTPN22* promoter (1123G>C) was associated with T1D in Japanese and Korean populations (Kawasaki *et al.*, 2006). It might also be the case for the Turkish population. Therefore, other SNPs on *PTPN22* must be investigated.

In this study we also investigated which X-linked genes which might be associated with skewed XCI. Therefore, we conducted a comprehensive genomic study by using high-density microarray analysis. After elimination of redundant and nonappropriate SNPs from NCBI and Ensembl databases, 1618 nonsynonymous, 1091 synonymous, and 2802 intronic SNPs on X chromosome were included on the array. In addition, 166 SNPs from other autosomal chromosomes that are known to have association with certain autoimmune diseases were added. The exonic SNPs cover 783 genes and the intronic SNPs correspond to 160 genes. After the analysis of the data we identified the following SNPs to be associated with JIA: PDHA1, MCF2, MAOB, SRPX, and G6PD ($OR>1.0$ and $p<0.05$; please see Table 3.7 for a complete list).

Future Perspectives

In summary, there is a female predominance in the majority of the autoimmune diseases. We showed that skewed XCI is associated with autoimmune diseases including JIA and RA. We hypothesized that a putative X-linked lethal mutation may result in a cell-survival disadvantage causing skewed XCI. In order to find the culprit X-linked genes that might be associated with skewed XCI, we conducted a custom made X-linked SNP study. We examined the X-linked SNP mapping and disease association and determined the SNPs that have association with JIA with $OR>1.0$ and $p<0.05$. As a future perspective, in order to validate our findings, we plan to conduct a replication study by selecting the significantly correlating SNPs in additional case-control series. The strongly associated coding SNPs would suggest that corresponding genes would be further studied to understand their role in the pathogenesis of JIA. First, the genes' association with immune system should be studied. The proteins, which are encoded by these genes, should also be searched whether they interact with other proteins that play role in the immune sytem. According to the findings, fuctional analysis can be done.

In this study, we selected common X-linked SNPs with 0.4-0.5 heterozygosity rate in order to find diseases association. With the discovery of novel high-throughput sequencing techniques, called next generation sequencing, X-linked lethal mutations can be directly identified. By using targeted sequencing approaches, the coding regions in the X chromosome, which constitute a total of 1.47 Mb, can be completely sequenced and functional variations can be evaluated whether they have a lethal effect. This high-throughput technique would be cost-effective and powerful compared to Sanger sequencing. This technique also would be advantageous with respect to identification of the variants by using less number of patients in a short period of time.

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CHAPTER VI

APPENDICIES

Appendix A. The list of the healthy Turkish children and their XCI pattern

Number		Sample	XCI Pattern
90+	**n=2/155(1.3%)**		
1	1	05-332	100
2	2	06-072	100
80-89	**n=9/155(5.8%)**		
3	1	05-583	88
4	2	09-006	87
5	3	05-370	85
6	4	05-369	85
7	5	09-056	85
8	6	06-056	85
9	7	06-071	82
10	8	05-588	82
11	9	09-023	80
70-79	**n=29/155(18.7%)**		
12	1	06-057	79
13	2	06-070	78
14	3	09-027	77
15	4	09-079	75
16	5	09-111	75
17	6	05-595	75
18	7	06-077	75

19	8	05-587	75
20	9	05-348	75
21	10	05-359	75
22	11	05-599	75
23	12	06-068	75
24	13	09-106	75
25	14	09-109	75
26	15	09-014	75
27	16	09-009	75
28	17	09-038	74
29	18	05-375	73
30	19	05-372	73
31	20	06-066	73
32	21	05-342	72
33	22	09-045	71
34	23	06-059	70
35	24	05-354	70
36	25	06-069	70
37	26	09-067	70
38	27	09-073	70
39	28	09-110	70
40	29	09-091	70
60-69	**n=39/155(25.2%)**		
41	1	05-347	68
42	2	09-031	68
43	3	05-331	67
44	4	05-585	66
45	5	05-360	65
46	6	09-058	65
47	7	05-592	65
48	8	09-047	65
49	9	09-008	65

50	10	09-010	65
51	11	09-049	65
52	12	09-080	65
53	13	09-043	65
54	14	09-026	65
55	15	09-100	65
56	16	05-357	64
57	17	05-366	64
58	18	05-364	63
59	19	05-596	63
60	20	05-356	62
61	21	05-581	62
62	22	06-074	62
63	23	06-081	62
64	24	05-350	61
65	25	05-374	61
66	26	05-377	61
67	27	06-075	61
68	28	09-018	61
69	29	09-017	60
70	30	06-061	60
71	31	05-591	60
72	32	05-339	60
73	33	09-048	60
74	34	09-050	60
75	35	09-084	60
76	36	09-101	60
77	37	09-114	60
78	38	09-095	60
79	39	09-042	60
50-59	**n=76/155(49.0%)**		
80	1	05-341	58

81	2	05-358	58
82	3	05-367	58
83	4	05-582	58
84	5	05-594	58
85	6	06-058	58
86	7	05-333	57
87	8	05-351	57
88	9	05-346	57
89	10	06-078	56
90	11	06-060	55
91	12	05-337	55
92	13	05-355	55
93	14	05-368	55
94	15	05-586	55
95	16	05-338	55
96	17	05-336	55
97	18	06-067	55
98	19	09-003	55
99	20	09-015	55
100	21	09-002	55
101	22	09-036	55
102	23	09-032	55
103	24	09-052	55
104	25	09-055	55
105	26	09-062	55
106	27	09-064	55
107	28	09-065	55
108	29	09-068	55
109	30	09-070	55
110	31	09-085	55
111	32	09-087	55
112	33	09-093	55

113	34	09-096	55
114	35	09-102	55
115	36	09-105	55
116	37	09-108	55
117	38	09-115	55
118	39	09-117	55
119	40	09-120	55
120	41	09-022	55
121	42	05-349	54
122	43	05-584	54
123	44	05-329	53
124	45	05-593	53
125	46	09-034	53
126	47	09-024	53
127	48	09-028	52
128	49	09-005	51
129	50	09-037	51
130	51	05-363	51
131	52	06-080	51
132	53	05-590	50
133	54	05-343	50
134	55	05-598	50
135	56	06-079	50
136	57	09-030	50
137	58	09-016	50
138	59	09-035	50
139	60	09-007	50
140	61	09-041	50
141	62	09-051	50
142	63	09-060	50
143	64	09-061	50
144	65	09-066	50

145	66	09-072	50
146	67	09-075	50
147	68	09-086	50
148	69	09-088	50
149	70	09-089	50
150	71	09-094	50
151	72	09-097	50
152	73	09-099	50
153	74	09-112	50
154	75	09-113	50
155	76	09-119	50
NI	**n=56/211(26.5%)**		
156	1	05-330	NI
157	2	05-344	NI
158	3	05-345	NI
159	4	05-361	NI
160	5	05-362	NI
161	6	05-365	NI
162	7	05-371	NI
163	8	05-373	NI
164	9	05-376	NI
165	10	05-379	NI
166	11	05-589	NI
167	12	05-600	NI
168	13	06-055	NI
169	14	06-062	NI
170	15	06-065	NI
171	16	06-073	NI
172	17	06-076	NI
173	18	05-353	NI
174	19	05-378	NI
175	20	05-352	NI

176	21	09-046	NI
177	22	09-004	NI
178	23	09-013	NI
179	24	09-040	NI
180	25	09-039	NI
181	26	09-044	NI
182	27	09-020	NI
183	28	09-025	NI
184	29	09-029	NI
185	30	09-021	NI
186	31	09-019	NI
187	32	09-012	NI
188	33	09-033	NI
189	34	09-011	NI
190	35	09-053	NI
191	36	09-054	NI
192	37	09-057	NI
193	38	09-059	NI
194	39	09-063	NI
195	40	09-069	NI
196	41	09-071	NI
197	42	09-074	NI
198	43	09-076	NI
199	44	09-077	NI
200	45	09-078	NI
201	46	09-081	NI
202	47	09-082	NI
203	48	09-083	NI
204	49	09-090	NI
205	50	09-092	NI
206	51	09-098	NI
207	52	09-103	NI

208	53	09-104	NI
209	54	09-107	NI
210	55	09-116	NI
211	56	09-118	NI

Appendix B. The list of French controls and their XCI pattern

Number		Sample	XCI Pattern
90+	2/69(2.9%)		
1	1	NRL 33	90
2	2	NRL 110	90
80-89	10/69 (14.5%)		
3	1	NRL 76	85
4	2	NRL 77	85
5	3	NRL 121	85
6	4	NRL 46	80
7	5	NRL 7	80
8	6	NRL 24	80
9	7	NRL 30	80
10	8	NRL 28	80
11	9	NRL 22	80
12	10	NRL 125	80
70-79	10/69 (14.5%)		
13	1	NRL 40	75
14	2	NRL 41	75
15	3	NRL 105	75
16	4	NRL 124	75
17	5	NRL 126	75
18	6	NRL 56	70
19	7	NRL 72	70
20	8	NRL 83	70
21	9	NRL 85	70
22	10	NRL 122	70
60-69	12/69 (17.4%)		
23	1	NRL 66	65
24	2	NRL 9	60
25	3	NRL 29	60
26	4	NRL 37	60

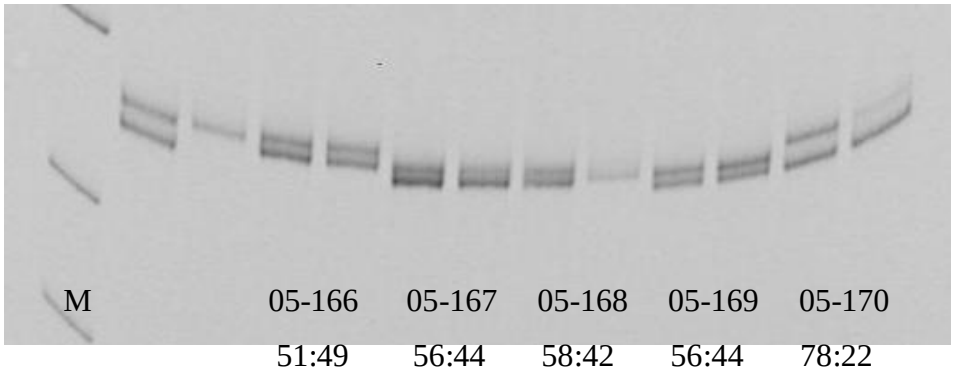
27	5	NRL 58	60
28	6	NRL 68	60
29	7	NRL 73	60
30	8	NRL 84	60
31	9	NRL 100	60
32	10	NRL 101	60
33	11	NRL 107	60
34	12	NRL 123	60
50-59	35/69(50.7%)		
35	1	NRL 10	55
36	2	NRL 25	55
37	3	NRL 27	55
38	4	NRL 31	55
39	5	NRL 57	55
40	6	NRL 95	55
41	7	NRL 97	55
42	8	NRL 103	55
43	9	NRL 115	55
44	10	NRL 130	55
45	11	NRL 135	55
46	12	NRL 136	55
47	13	NRL 139	55
48	14	NRL 4	50
49	15	NRL 13	50
50	16	NRL 36	50
51	17	NRL 38	50
52	18	NRL 42	50
53	19	NRL 53	50
54	20	NRL 59	50
55	21	NRL 69	50
56	22	NRL 70	50
57	23	NRL 78	50

58	24	NRL 81	50
59	25	NRL 82	50
60	26	NRL 96	50
61	27	NRL 111	50
62	28	NRL 114	50
63	29	NRL 118	50
64	30	NRL 127	50
65	31	NRL 128	50
66	32	NRL 129	50
67	33	NRL 133	50
68	34	NRL 137	50
69	35	NRL 142	50
NI	31/100(31%)		
70	1	NRL 3	NI
71	2	NRL 6	NI
72	3	NRL 12	NI
73	4	NRL 14	NI
74	5	NRL 15	NI
75	6	NRL 23	NI
76	7	NRL 32	NI
77	8	NRL 34	NI
78	9	NRL 43	NI
79	10	NRL 44	NI
80	11	NRL 45	NI
81	12	NRL 47	NI
82	13	NRL 61	NI
83	14	NRL 80	NI
84	15	NRL 87	NI
85	16	NRL 98	NI
86	17	NRL 99	NI
87	18	NRL 102	NI
88	19	NRL 104	NI

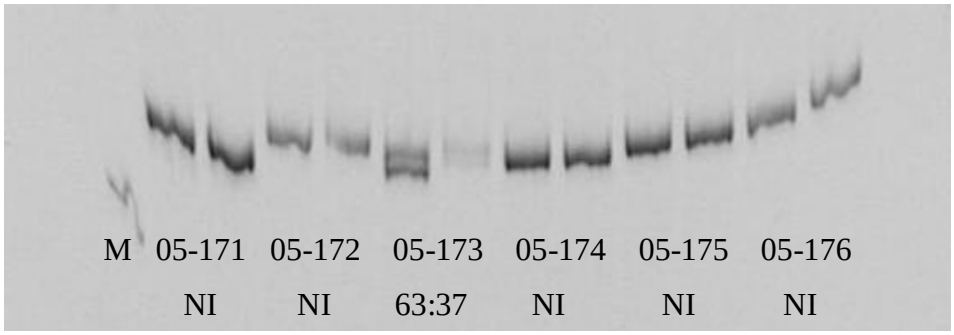
89	20	NRL 106	NI
90	21	NRL 109	NI
91	22	NRL 112	NI
92	23	NRL 116	NI
93	24	NRL 131	NI
94	25	NRL 132	NI
95	26	NRL 138	NI
96	27	NRL 140	NI
97	28	NRL 141	NI
98	29	NRL 74	NI
99	30	NRL 75	NI
100	31	NRL 113	NI

Appendix C. The PAGE figures of XCI patterns of Turkish JIA patients

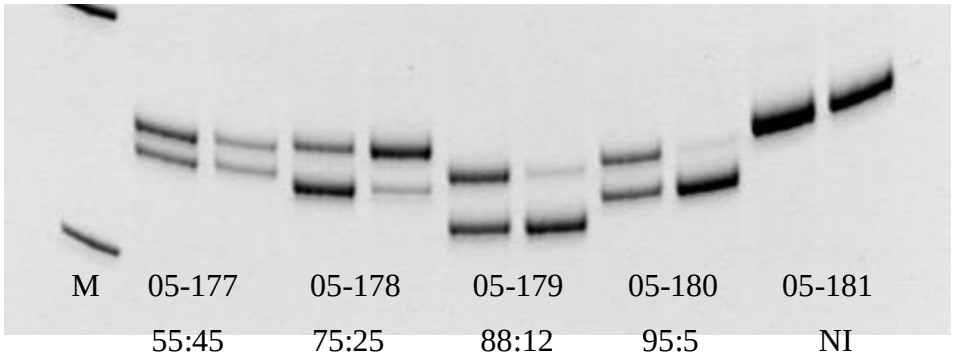
GEL1



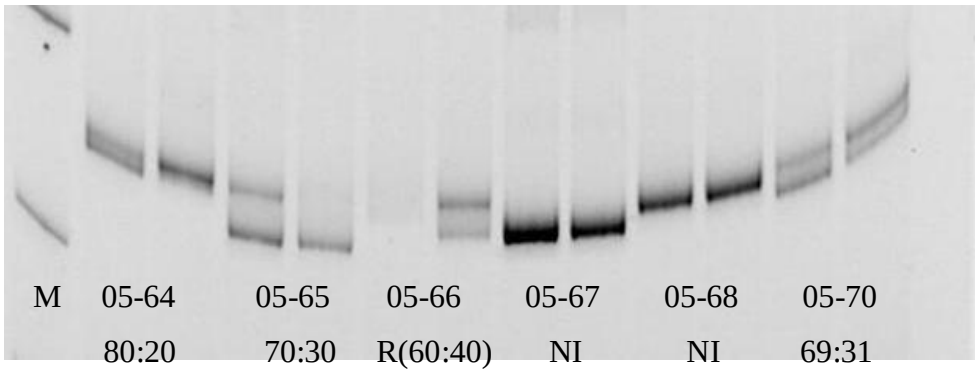
GEL2



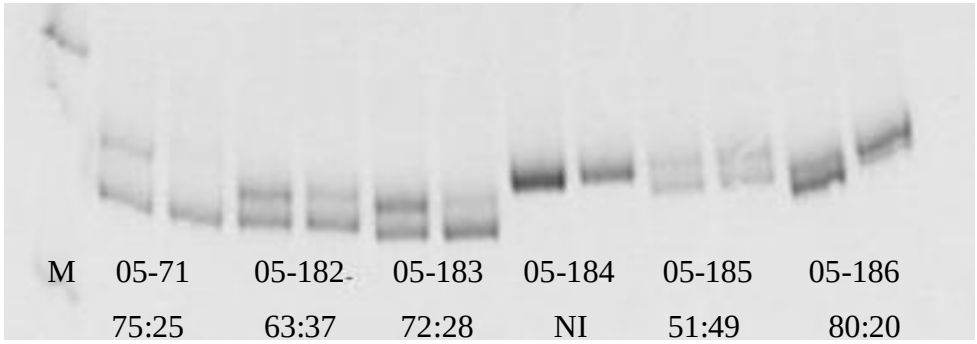
GEL3



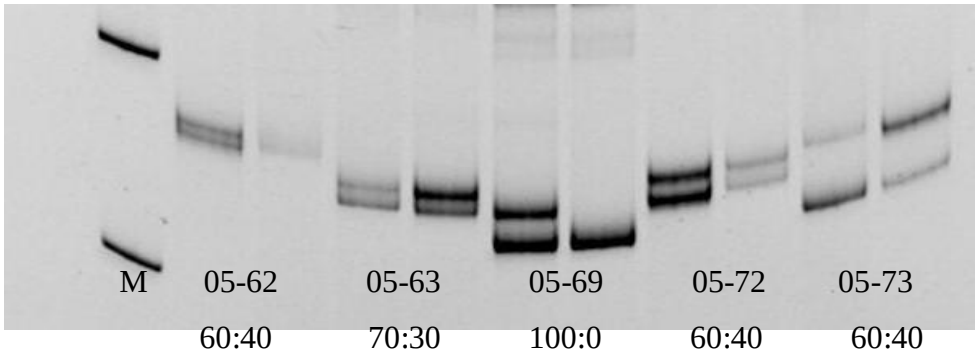
GEL4



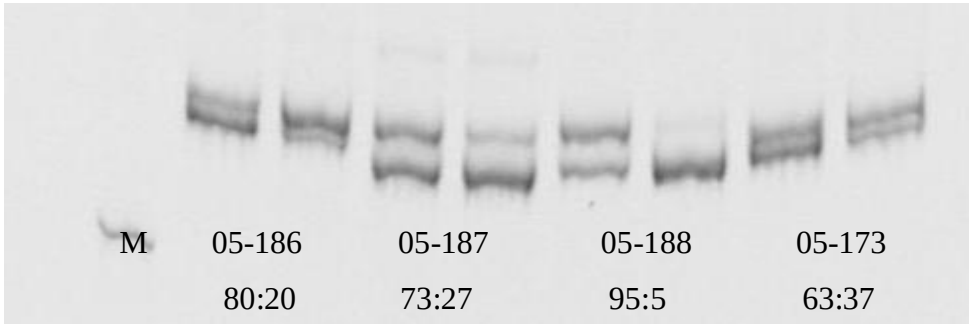
GEL5



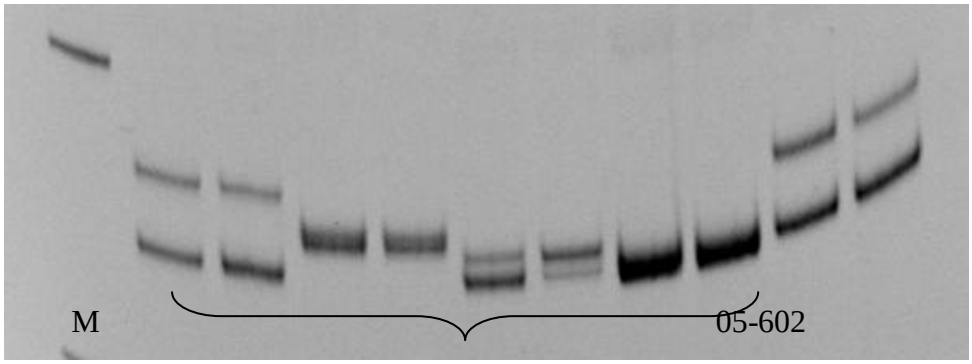
GEL6



GEL7

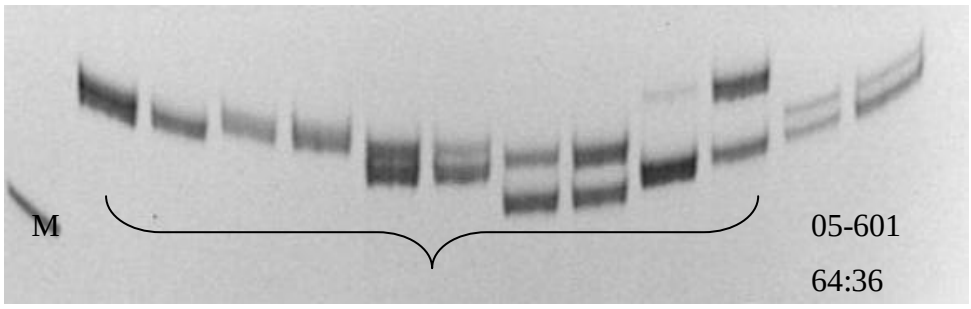


GEL8



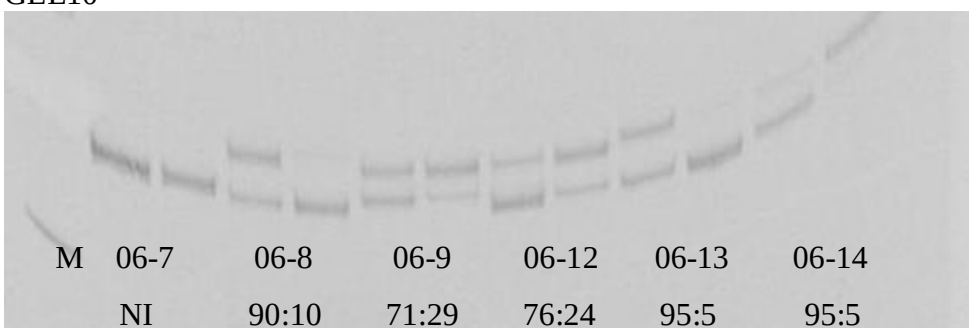
JIA controls

GEL9

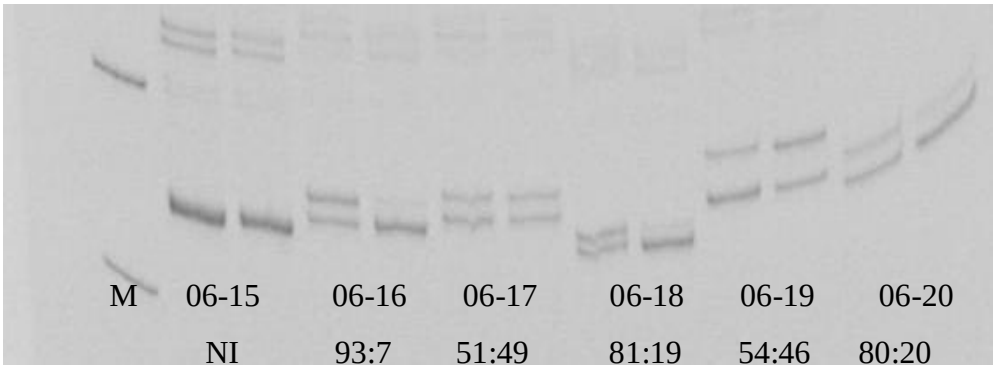


JIA controls

GEL10

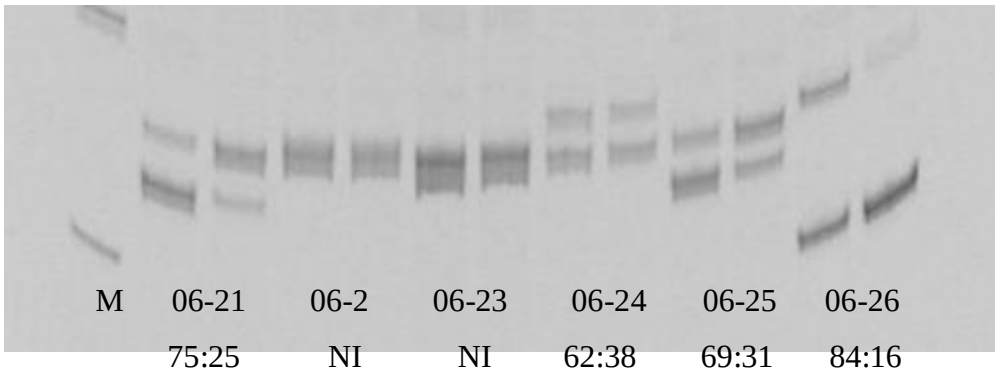


GEL11



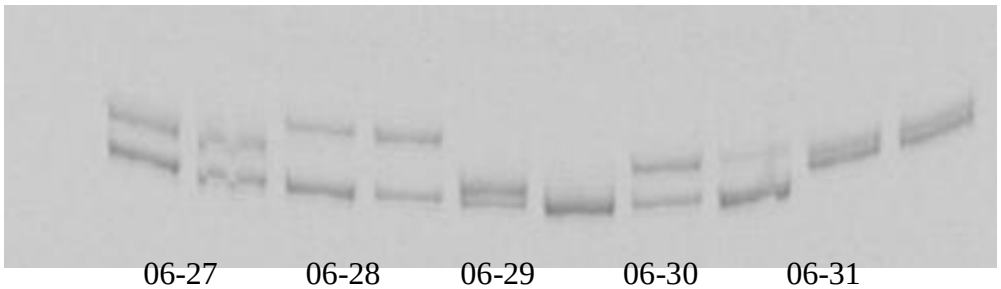
M	06-15	06-16	06-17	06-18	06-19	06-20
	NI	93:7	51:49	81:19	54:46	80:20

GEL12



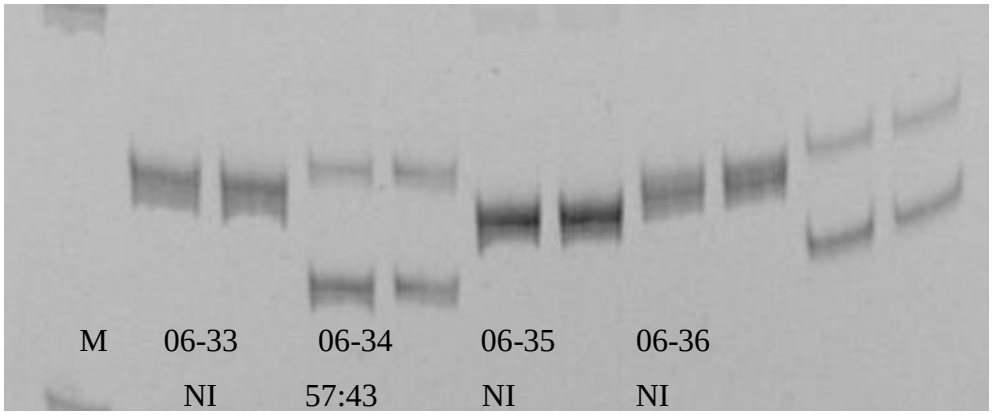
M	06-21	06-2	06-23	06-24	06-25	06-26
	75:25	NI	NI	62:38	69:31	84:16

GEL13

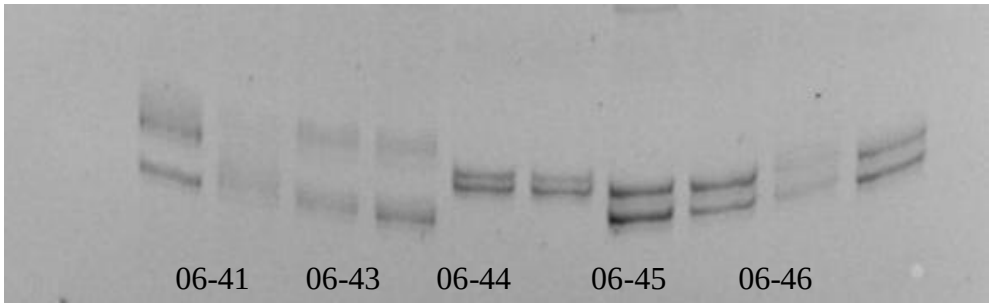


06-27	06-28	06-29	06-30	06-31
53:47	61:39	100:0	92:8	53:47

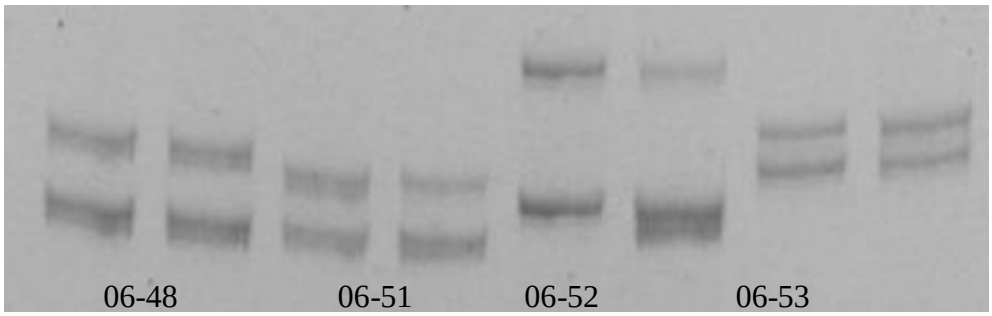
GEL14



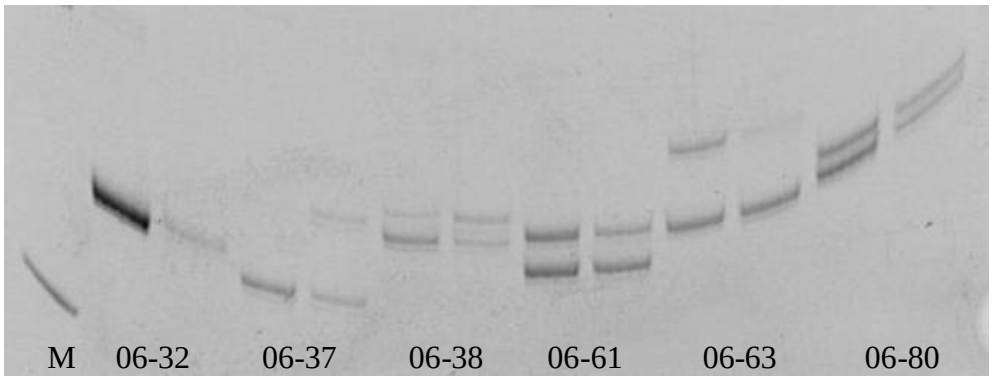
GEL15



GEL16

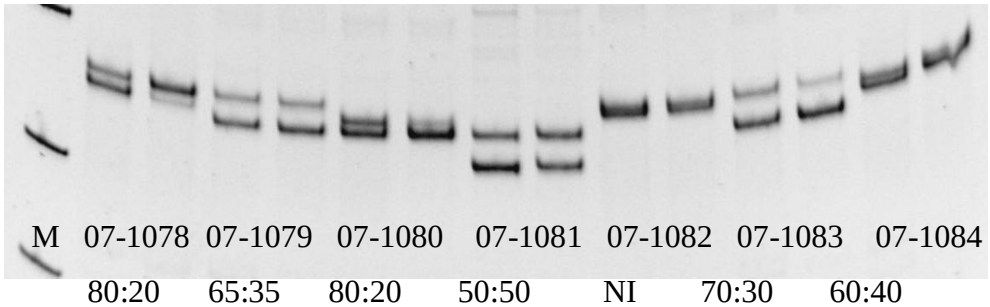


GEL17



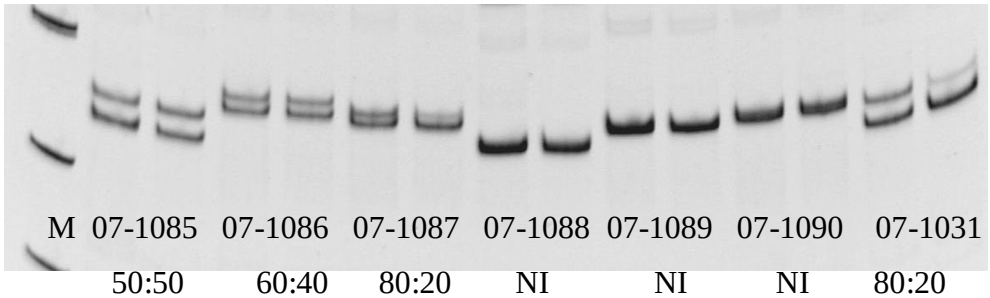
NI 62:38 56:44 JIA controls

GEL18



80:20 65:35 80:20 50:50 NI 70:30 60:40

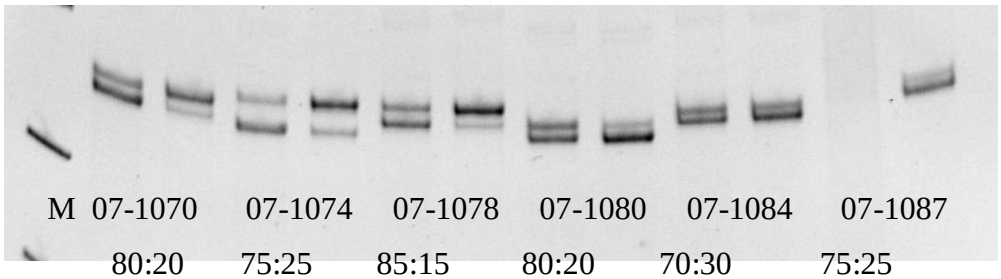
GEL19



50:50 60:40 80:20 NI NI NI 80:20

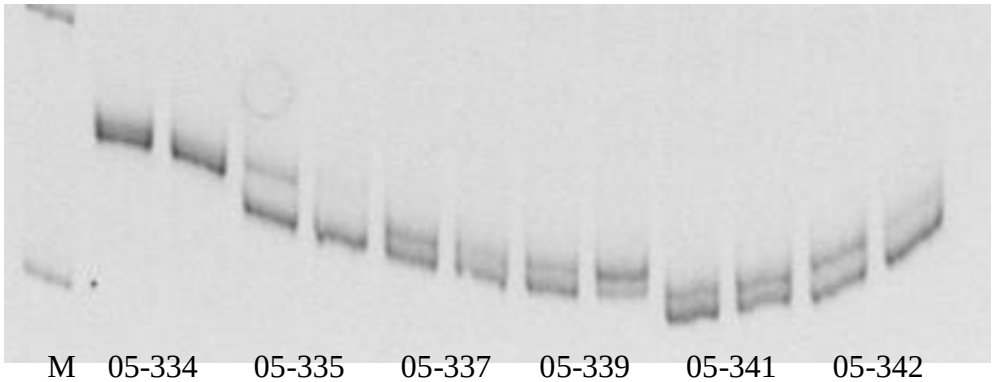
SSc

GEL20


M 07-1070 07-1074 07-1078 07-1080 07-1084 07-1087
80:20 75:25 85:15 80:20 70:30 75:25

Appendix D. The PAGE figures of XCI patterns of Turkish controls

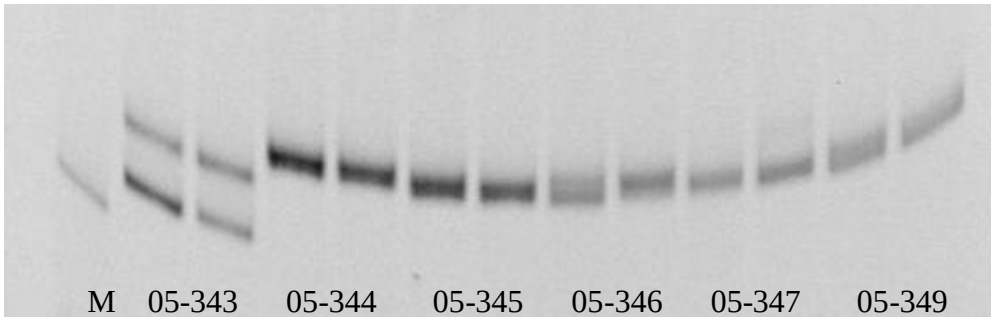
GEL1



M 05-334 05-335 05-337 05-339 05-341 05-342

NW NW 55:45 60:40 58:42 72:28

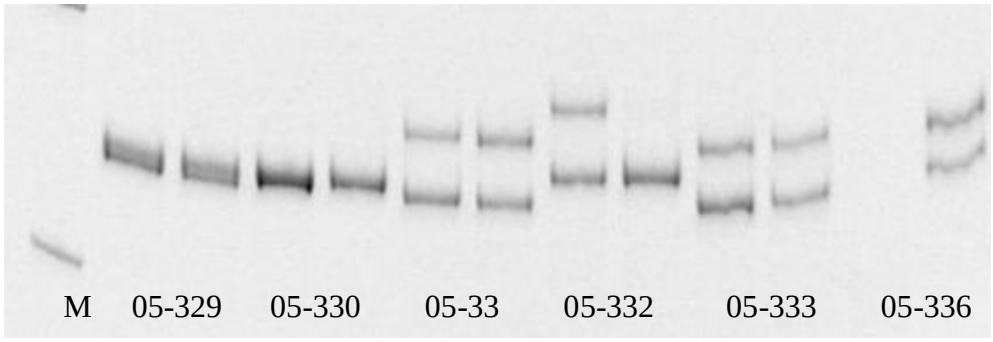
GEL2



M 05-343 05-344 05-345 05-346 05-347 05-349

50:50 NI NI 57:43 68:32 54:46

GEL3

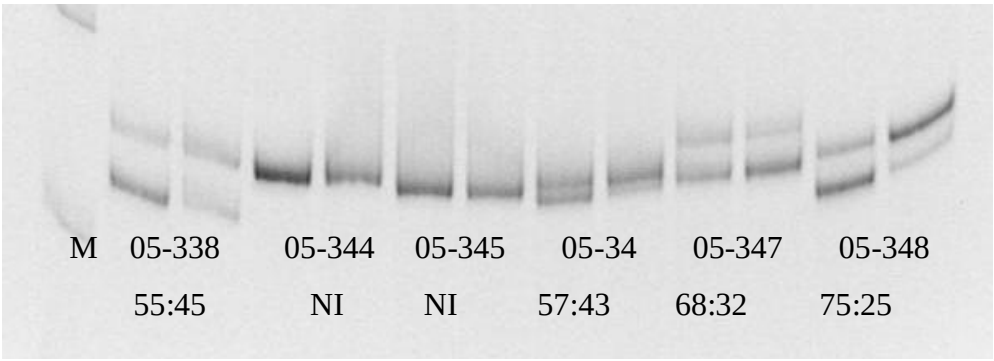


M 05-329 05-330 05-33 05-332 05-333 05-336

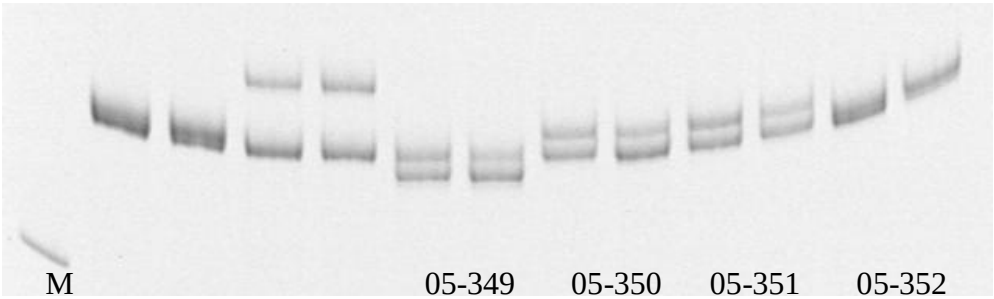
53:47 NI 67:33 100:0 57:43 NW

R(55:45)

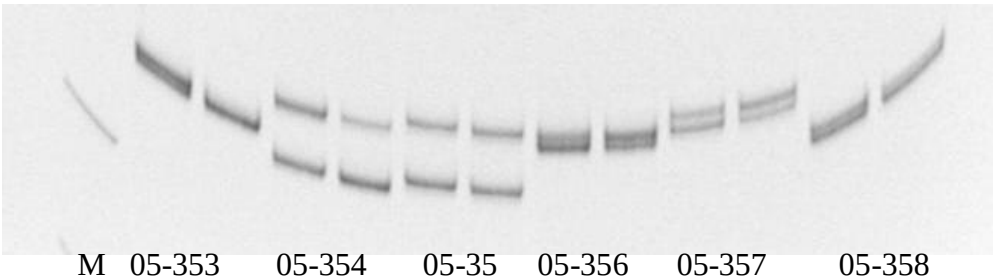
GEL4



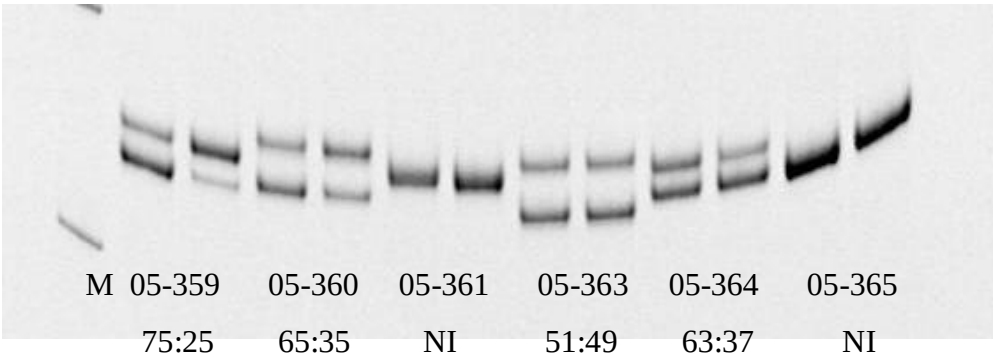
GEL5



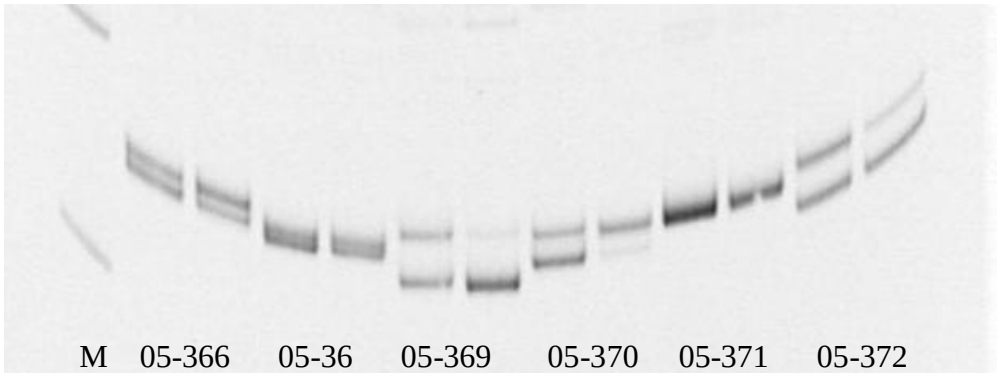
GEL6



GEL7

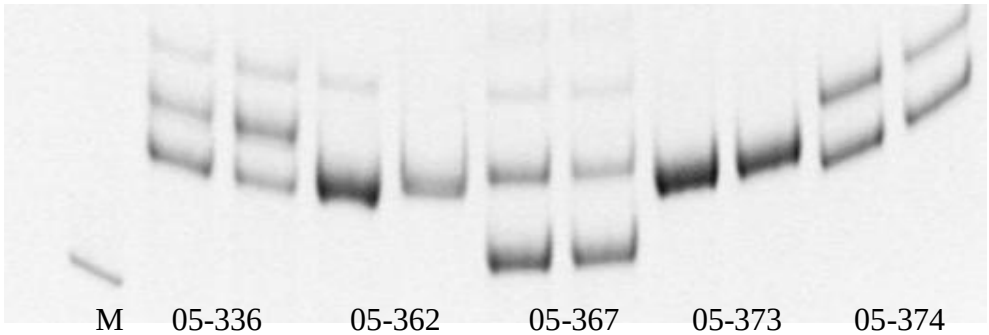


GEL8



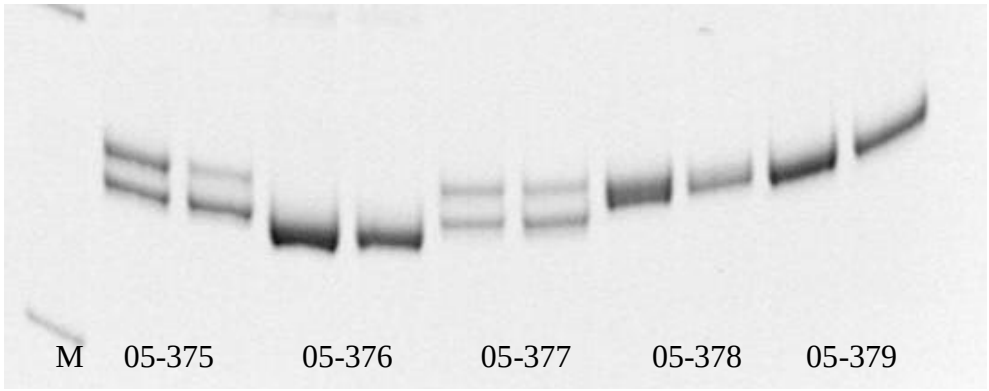
M	05-366	05-36	05-369	05-370	05-371	05-372
	64:36	55:45	85:15	85:15	NI	73:27

GEL9



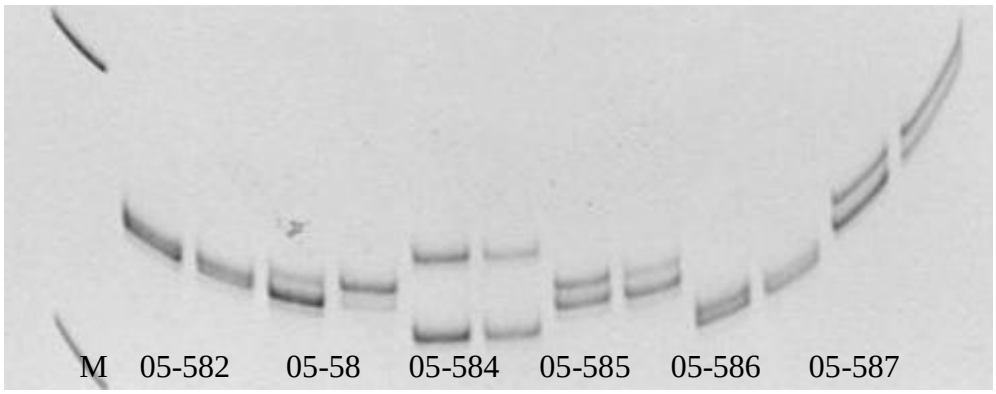
M	05-336	05-362	05-367	05-373	05-374
	55:45	NI	58:42	NI	61:39

GEL10

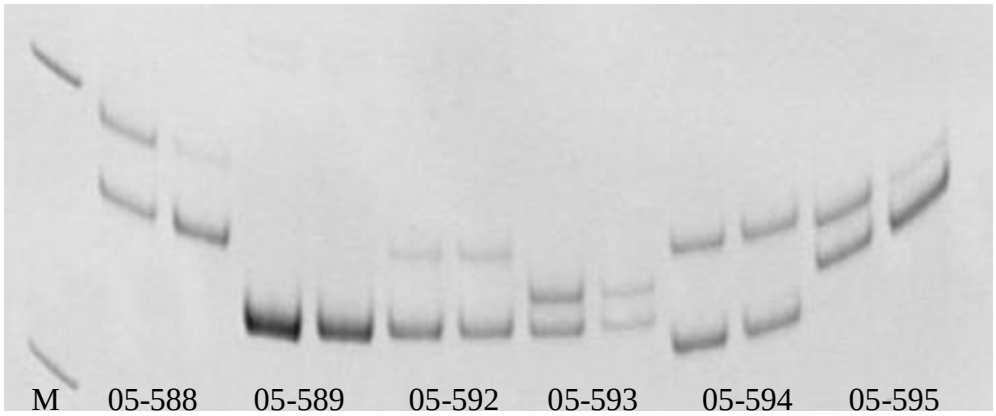


M	05-375	05-376	05-377	05-378	05-379
	73:27	NI	61:39	NI	NI

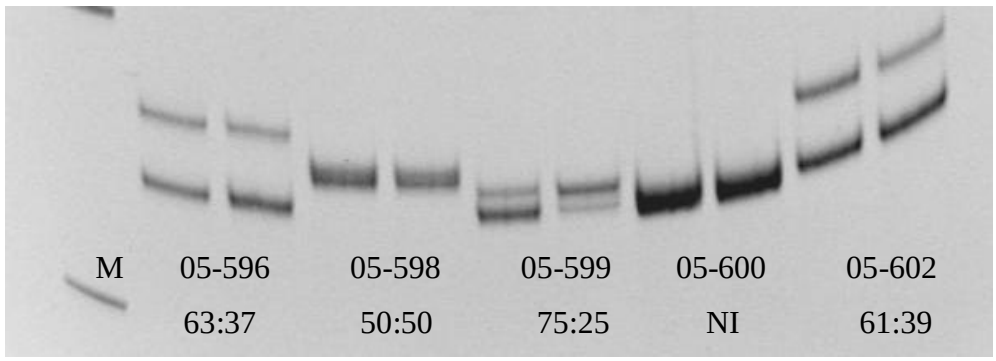
GEL11



GEL12

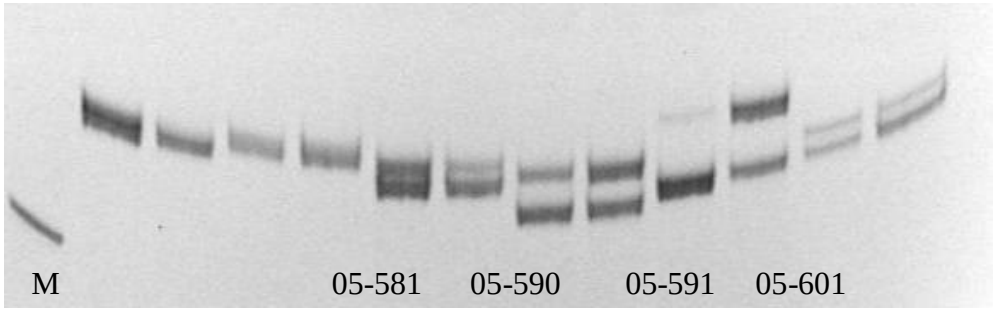


GEL13



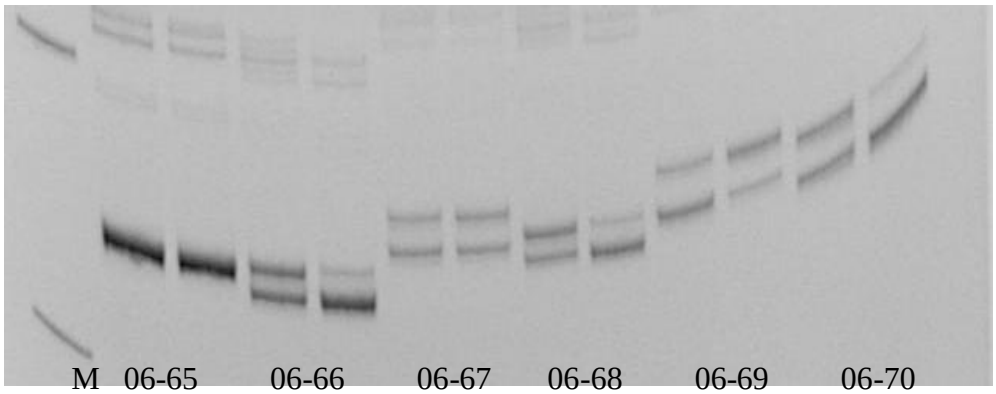
JIA patient

GEL14

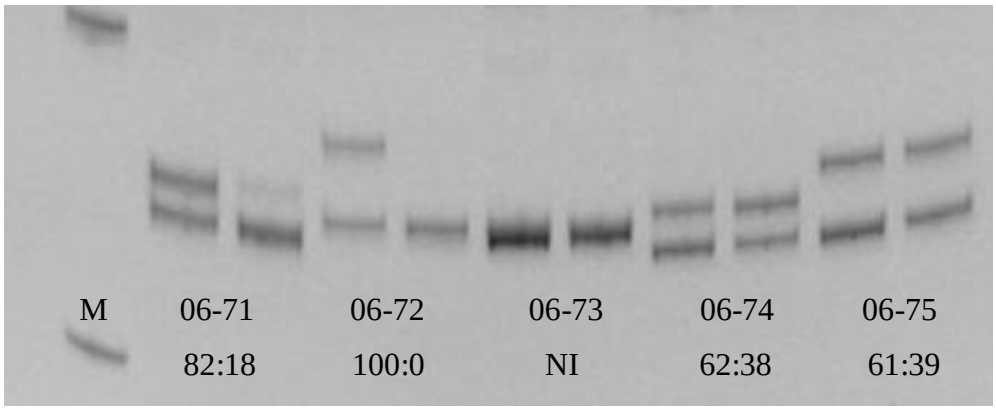


JIA patient

GEL15



GEL16



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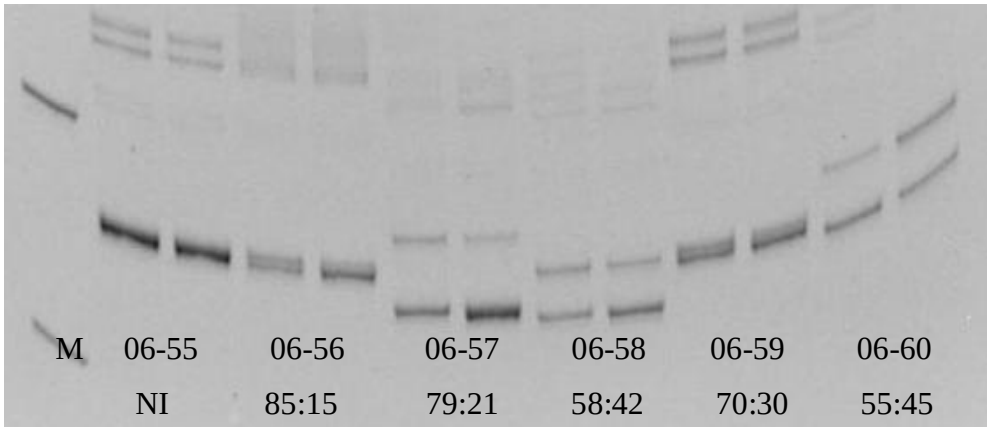
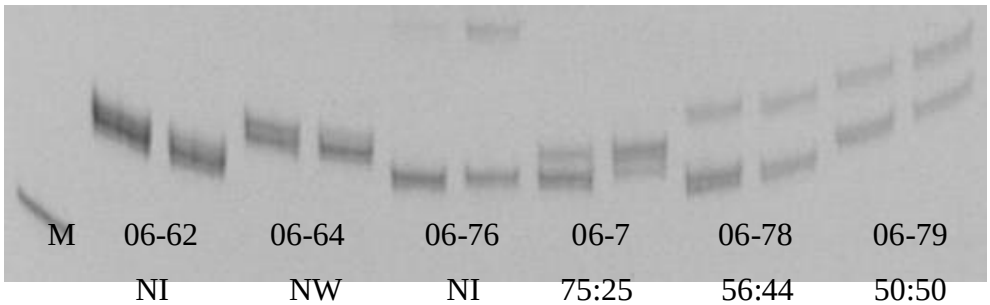
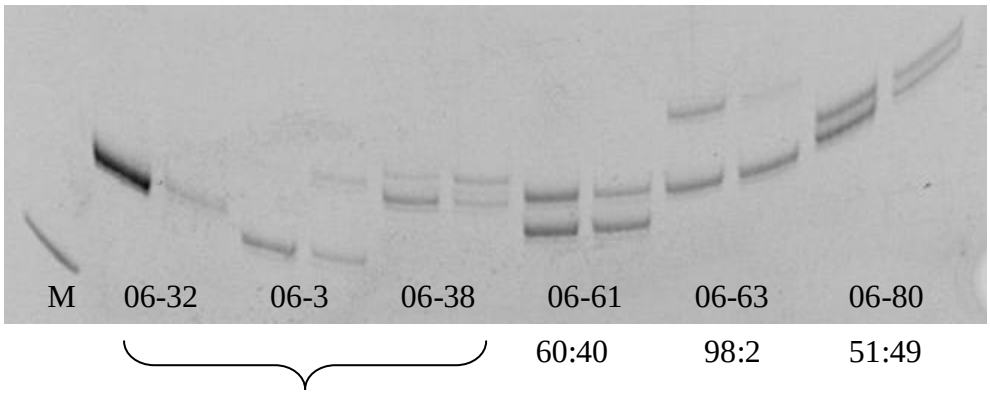


GEL18

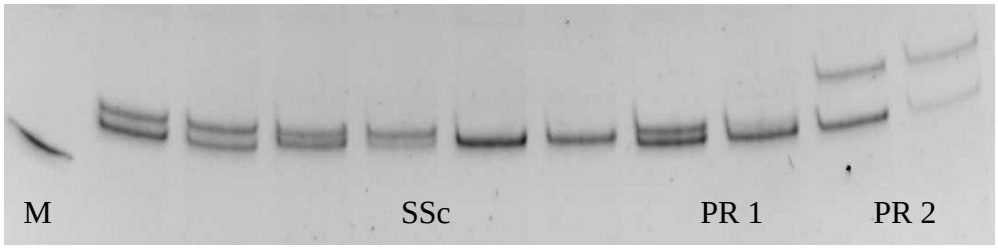


GEL19

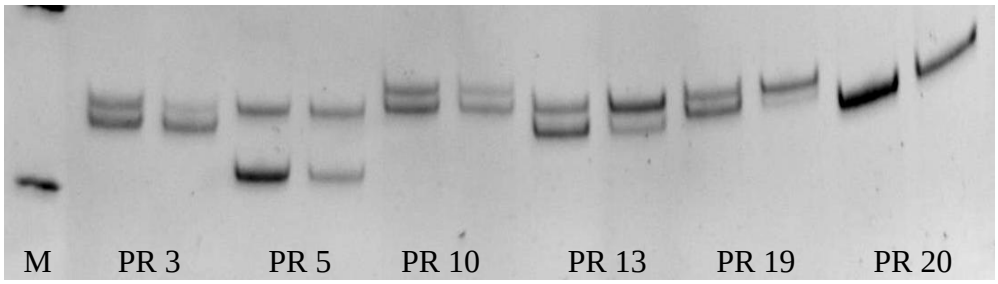


Appendix E. The PAGE figures of XCI patterns of French RA patients

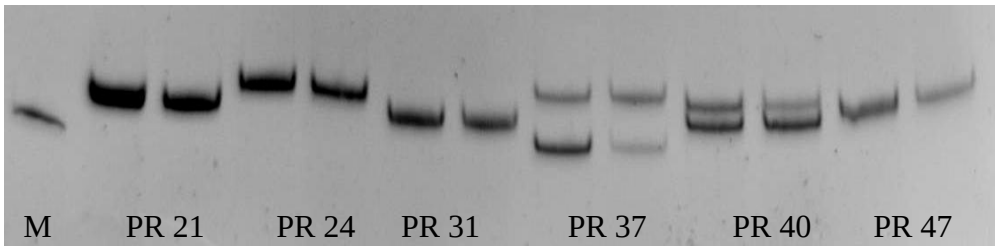
GEL1



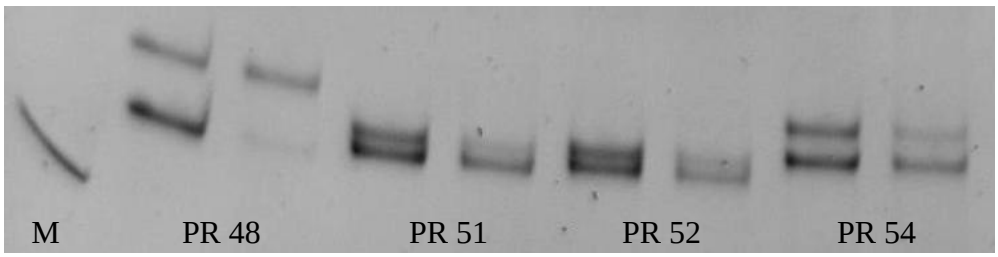
GEL2



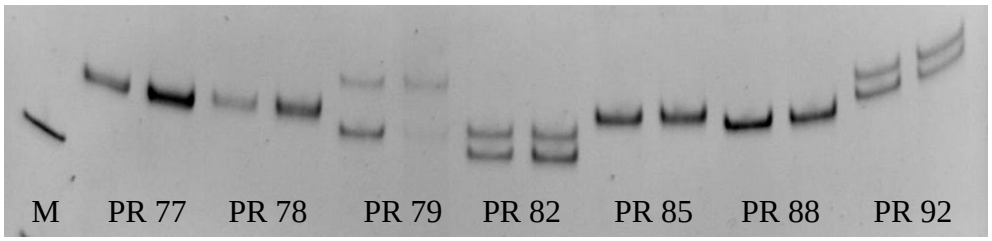
GEL3



GEL4

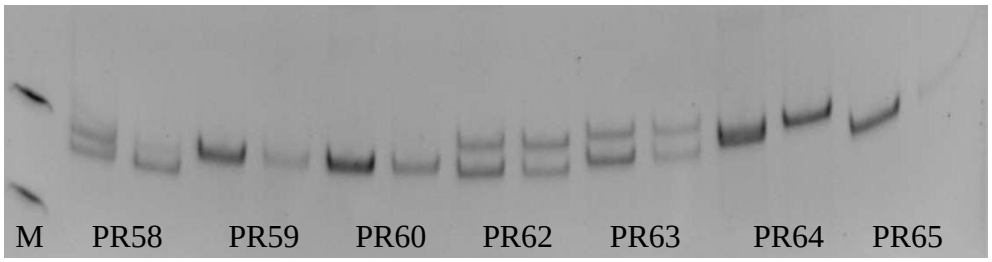


GEL5



M	PR 77	PR 78	PR 79	PR 82	PR 85	PR 88	PR 92
	NI	NI	80:20	55:45	NI	NI	50:50

GEL6



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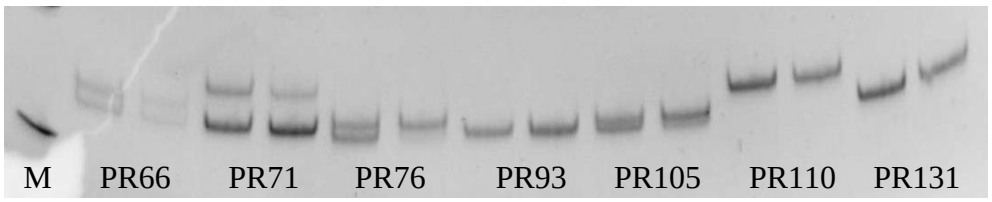
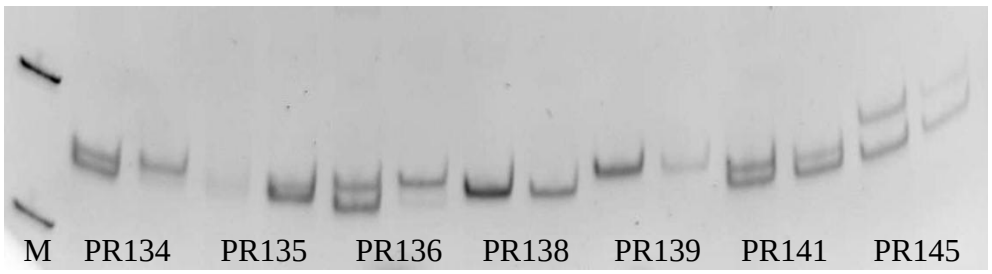
GEL7



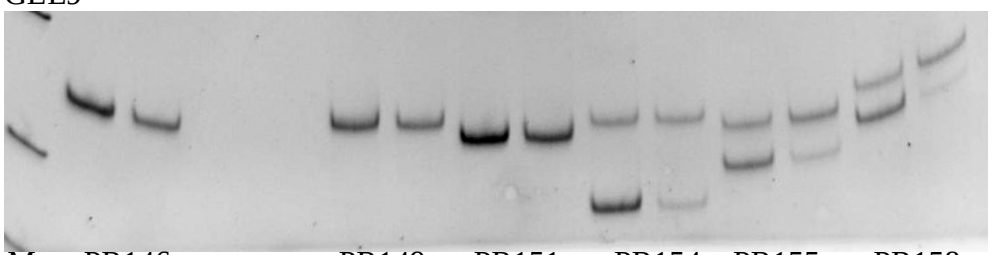
M	PR66	PR71	PR76	PR93	PR105	PR110	PR131
	50:50	70:30	90:10	NI	50:50	NI	NI

GEL8



M	PR134	PR135	PR136	PR138	PR139	PR141	PR145
	90:10	NI	90:10	NI	NI	55:45	50:50

GEL9



M	PR146	PR149	PR151	PR154	PR155	PR158
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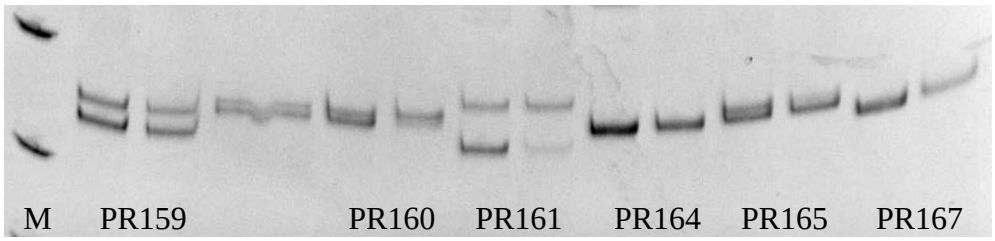
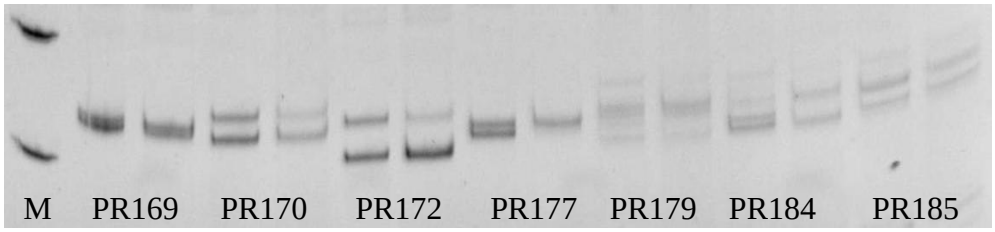
GEL10



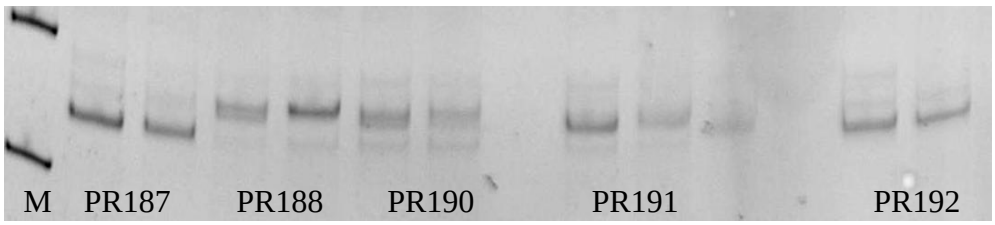
55:45 60:40 70:30 NI R NI

GEL11



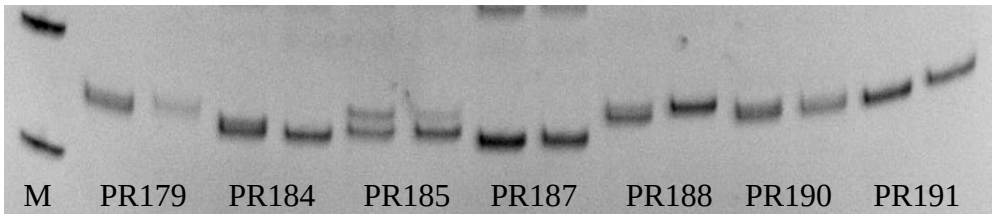
? 50:50 60:40 90:10 ? ? ?

GEL12



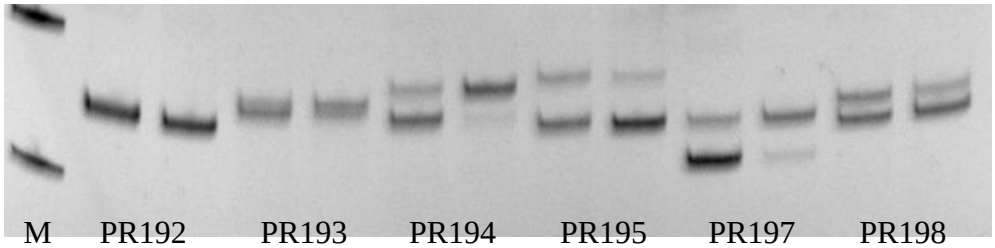
NI 90:10 90:10 ? NI

GEL13



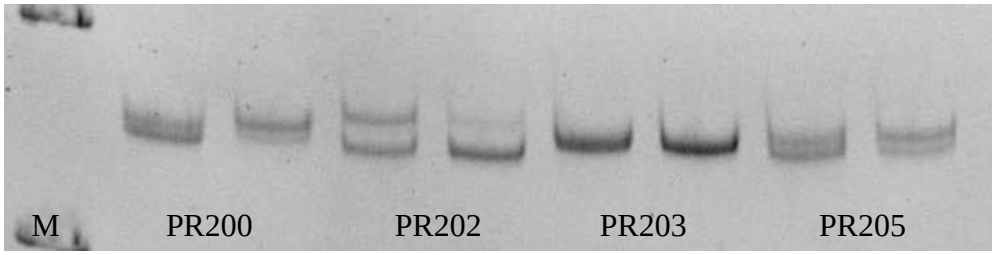
? 90:10 90:10 NI 90:10 ? NI

GEL14

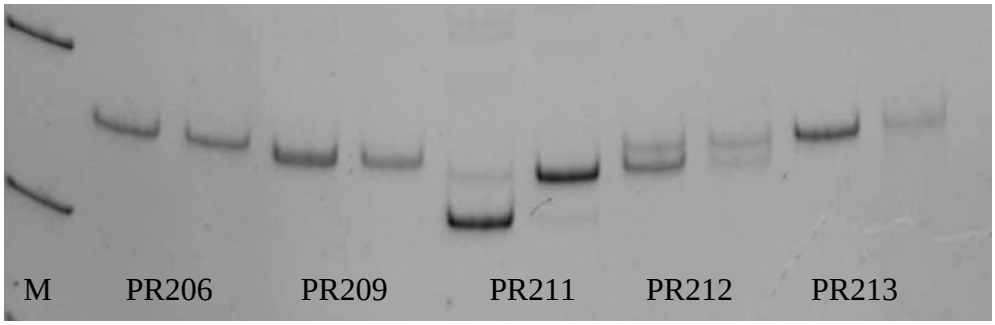


NI 50:50 90:10 60:40 80:20 50:50

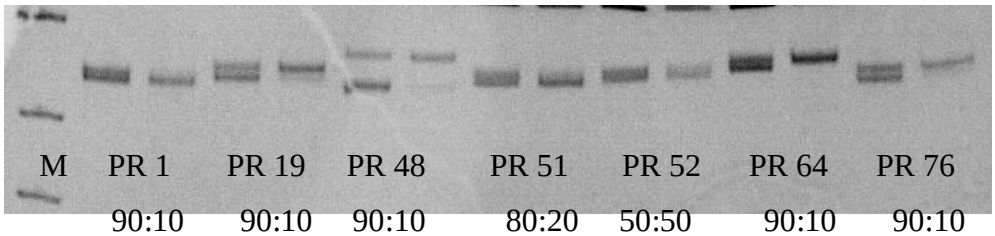
GEL15



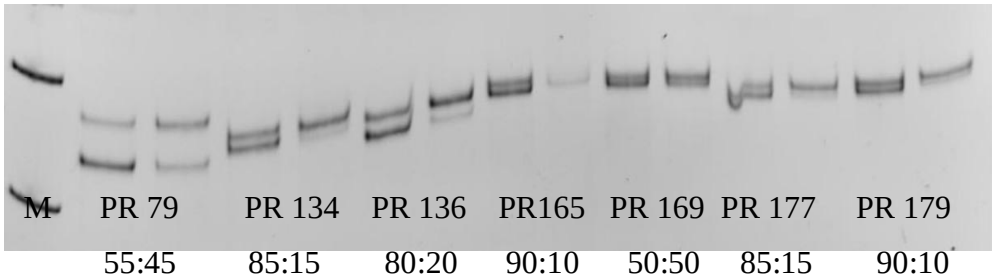
GEL16



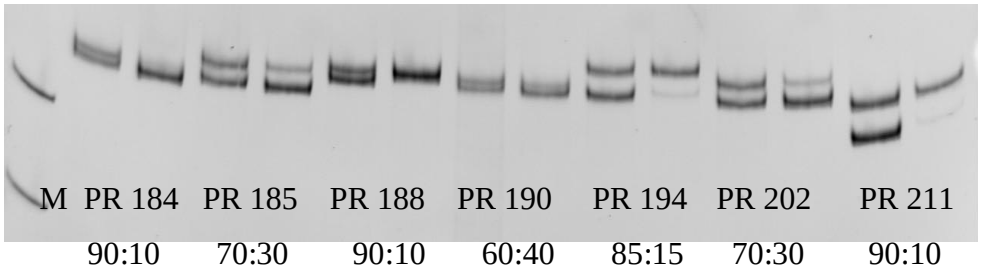
GEL17



GEL18

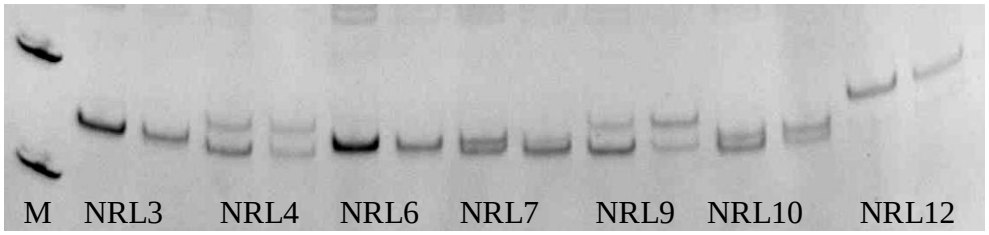


GEL19


M
PR 184
90:10
PR 185
70:30
PR 188
90:10
PR 190
60:40
PR 194
85:15
PR 202
70:30
PR 211
90:10

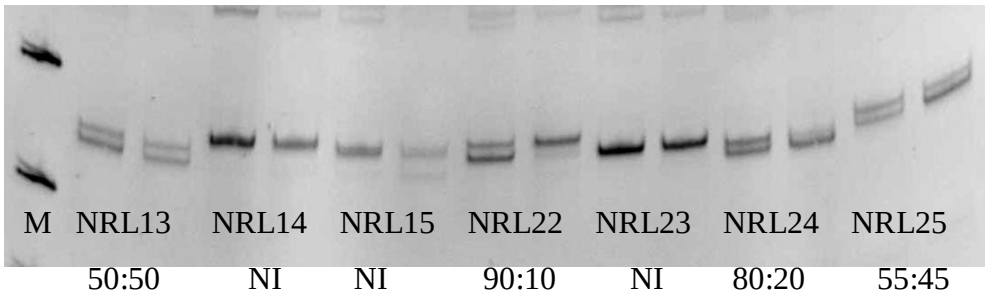
Appendix F. The PAGE figures of XCI patterns of French controls

GEL1



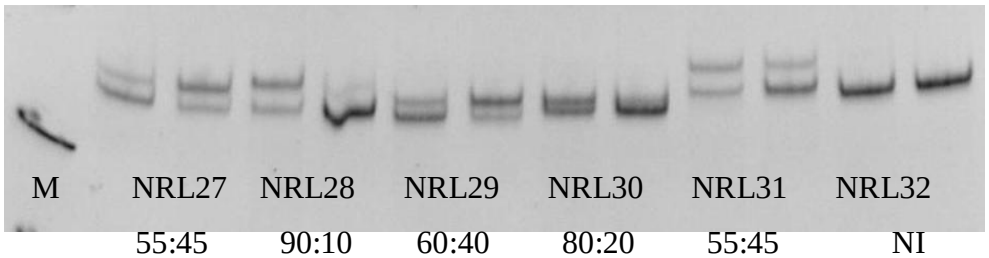
NI 50:50 NI 80:20 60:40 55:45 NI

GEL2



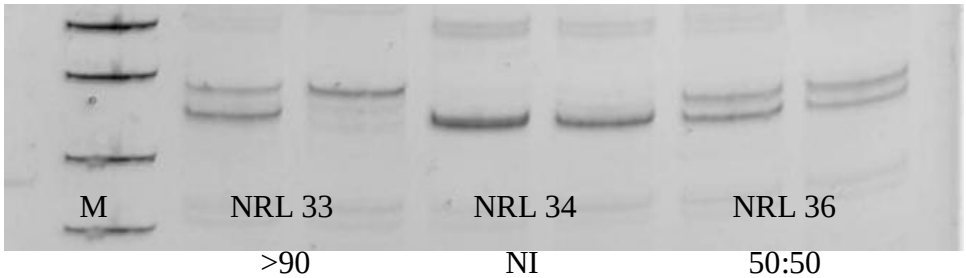
50:50 NI NI 90:10 NI 80:20 55:45

GEL3



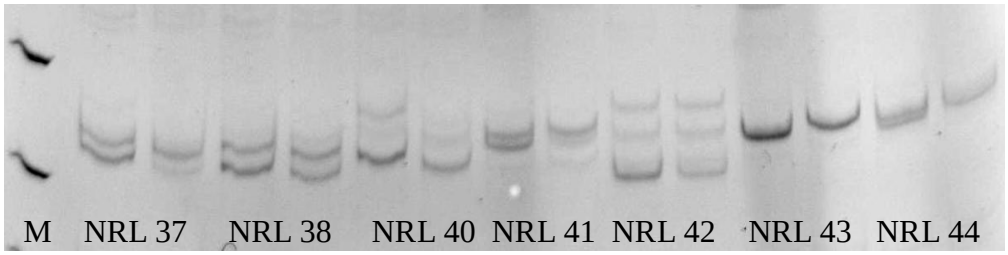
55:45 90:10 60:40 80:20 55:45 NI

GEL4



>90 NI 50:50

GEL5



60:40 50:50 NI 55:45 50:50 NI NI

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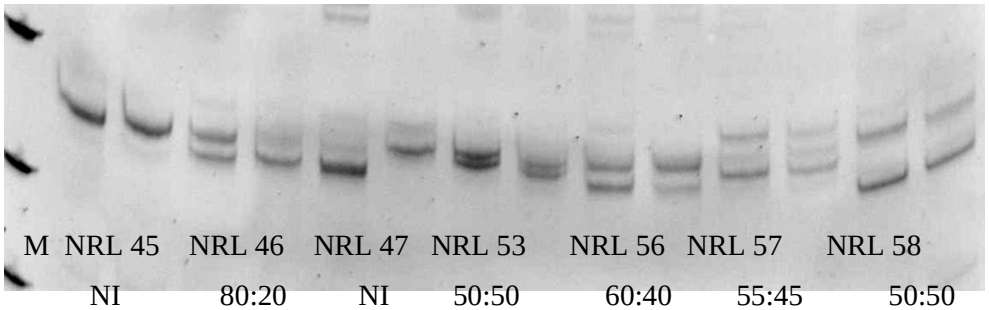

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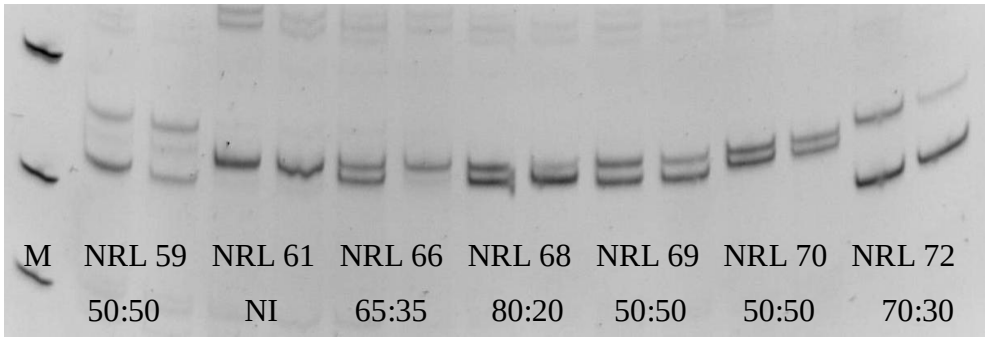

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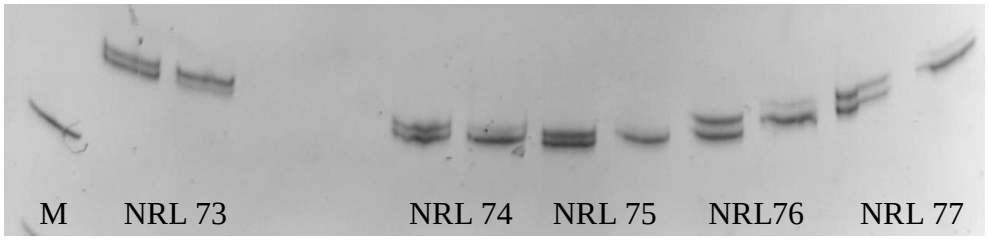

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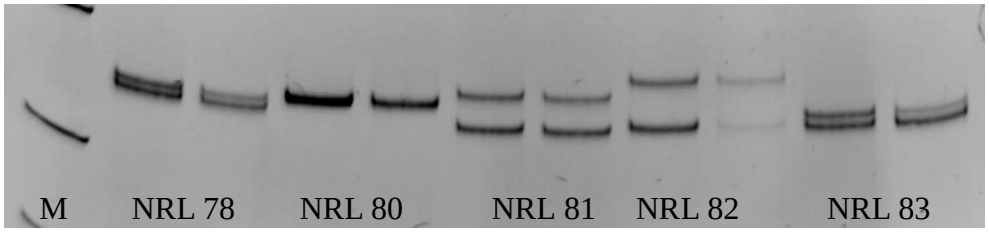

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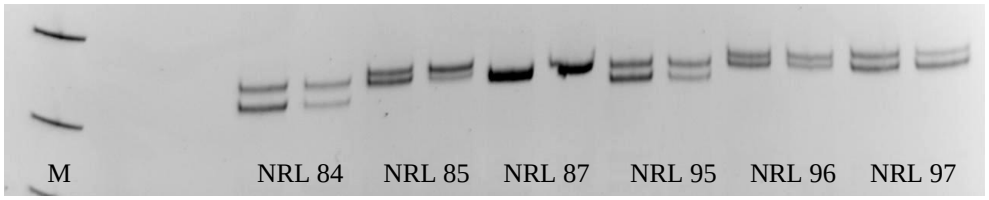

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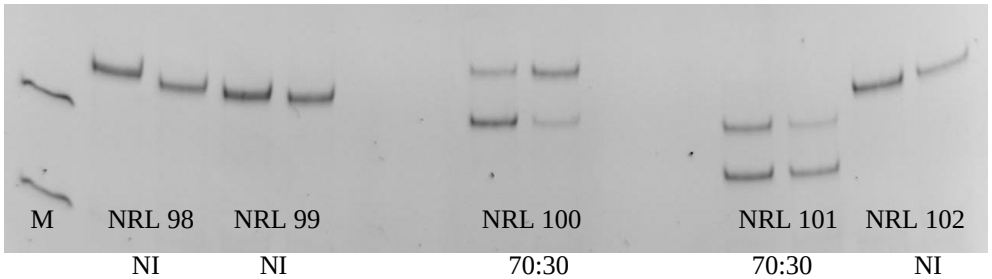
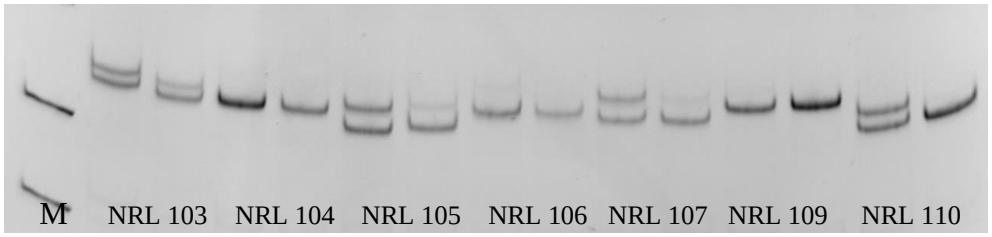


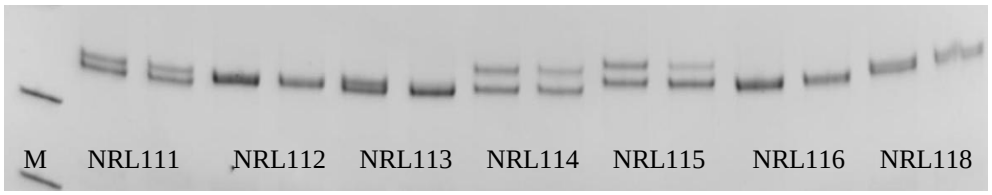
NI NI 70:30 70:30 NI

GEL12



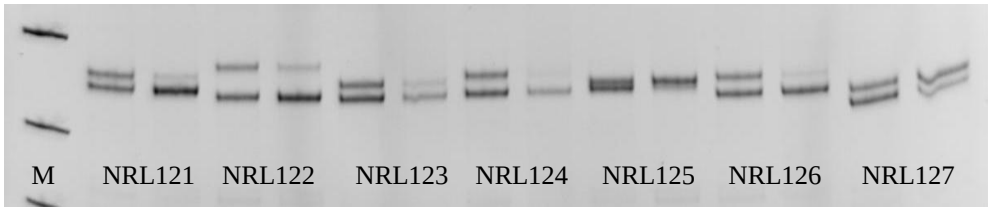
55:45 NI 90:10 NI R NI >90

GEL13



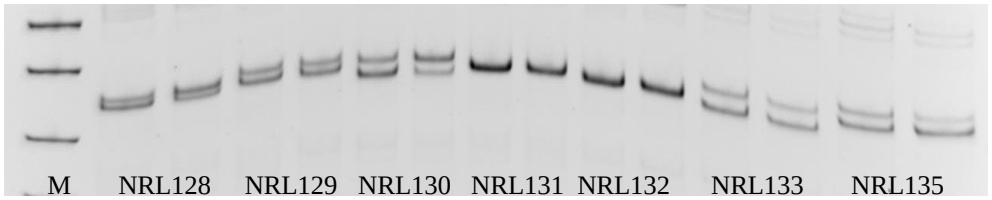
50:50 NI NI 50:50 55:45 NI 50:50

GEL14



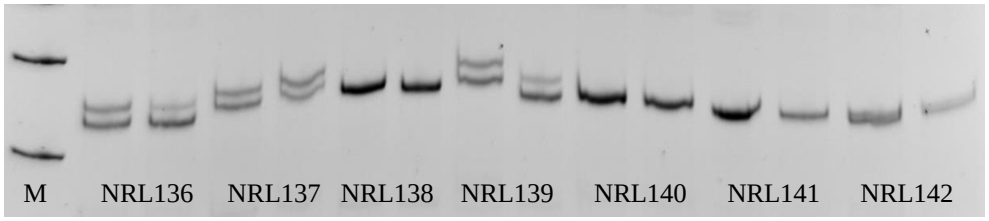
85:15 70:30 60:40 75:25 80:20 75:25 50:50

GEL15



50:50 50:50 55:45 NI NI 50:50 55:45

GEL16



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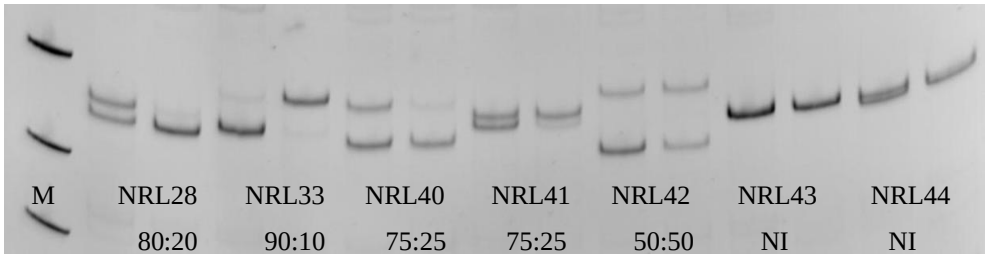

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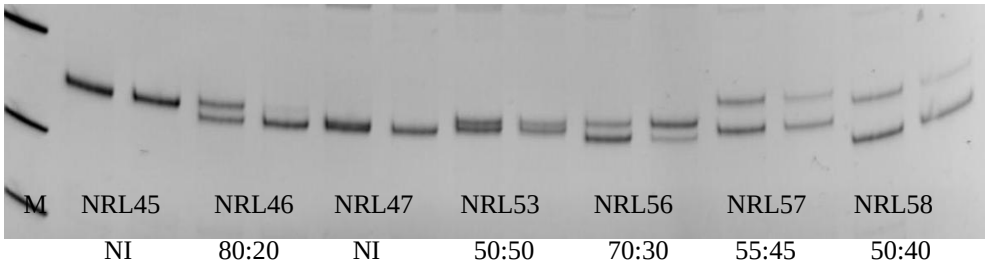

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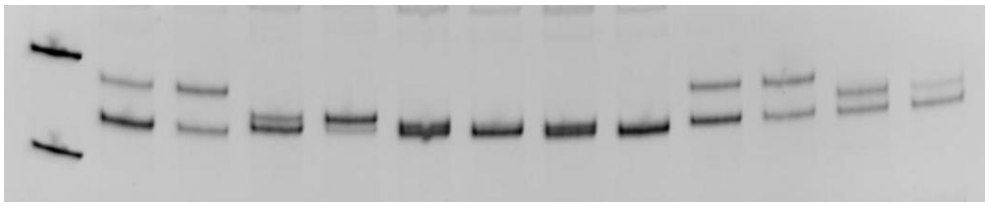
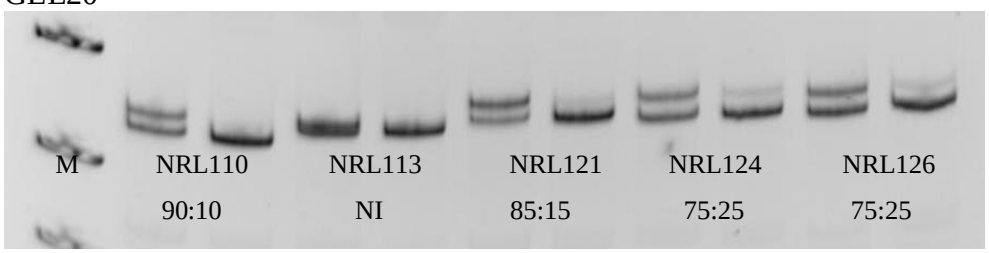


GEL20



Appendix G. Frequency of PTPN22 genotypes in individuals with SSc

		Code	XCI	PTPN22	DoB	OoD
Skewed						
1	1	03-894	98	C/C	1945 (58)	1989
2	2	03-900	97	C/C	1940 (63)	1984
3	3	03-899	96	C/C	1953 (50)	1998
4	4	03-1110	>95	C/C	1932 (71)	1993
5	5	03-962	>95	C/C	1946 (57)	1993
6	6	03-954	>95	C/C	1965 (38)	2000
7	7	03-1112	>95	C/C	1963 (40)	1997
8	8	03-963	>95	C/C	1938 (65)	1998
9	9	03-1101	>95	C/C	1941 (62)	1993
10	10	03-1123	>95	C/C	1950 (53)	1990
11	11	03-879	>95	C/T	1966 (37)	1991
12	12	03-951	94	C/C	1986 (17)	2003
13	13	03-948	92	C/C	1959 (44)	1989
14	14	03-893	92	C/C	1936 (67)	1984
15	15	03-881	>90	C/C	1971 (32)	1990
16	16	03-953	90	C/C	1941 (62)	1995
17	17	03-895	90	C/C	1941 (62)	1984
18	18	03-886	90	C/T	1960 (43)	1994
19	19	03-1120	90	C/C	1950 (53)	1983
20	20	03-890	90	C/C	1956 (47)	1989
21	21	03-878	88	C/C	1945 (58)	2000
22	22	03-892	87	C/C	1965 (38)	1993
23	23	03-880	87	C/C	1967 (36)	2002
24	24	03-888	86	C/C	1952 (51)	1996
25	25	03-952	85	C/C	1944 (59)	1984
26	26	03-884	85	C/C	1944 (59)	1994
27	27	03-1122	80	C/C	1949 (54)	1993
28	28	03-885	80	C/C	1933 (70)	1993
29	29	03-950	80	C/C	1936 (67)	1982
30	30	03-943	80	C/C	1942 (61)	1999
31	31	03-1104	80	C/C	1956 (47)	1983
32	32	03-949	80	C/C	1975 (28)	2000
33	33	03-1128	80	C/C	1983 (20)	2002
Random						
34	1	03-944	50	C/C	1945 (48)	1987
35	2	03-1113	65	C/C	1965 (38)	1994
36	3	03-1114	55	C/C	1967 (36)	1995
37	4	03-955	50	C/C	1947 (46)	1996
38	5	03-887	50	C/C	1946 (47)	1989
39	6	03-1103	50	C/C	1940 (43)	1993
40	7	03-1132	55	C/C	1977 (26)	1993
41	8	03-945	50	C/C	1979 (24)	2003
42	9	03-898	50	C/C	1967 (36)	1998
43	10	03-1117	50	C/C	1956 (47)	2001

44	11	03-947	55	C/C	1961 (42)	1988
45	12	03-958	60	C/C	1976 (27)	1993
46	13	03-957	60	C/C	1970 (33)	1991
47	14	03-1111	60	C/C	1952 (49)	1991
48	15	03-1115	55	C/C	1961 (42)	1989
49	16	03-1118	55	C/C	1966 (37)	2000
50	17	03-1125	50	C/C	1940 (63)	1978
51	18	03-1126	65	C/C	1951 (52)	1993
52	19	03-1127	50	C/C	1967 (36)	2000
53	20	03-1131	55	C/C	1929 (74)	1999
Not informative						
54	1	03-1105	NI	C/C	1937	1998
55	2	03-1116	NI	C/C	1969	2000
56	3	03-942	NI	C/C	1969	1998
57	4	03-1130	NI	C/C	1932	1963
58	5	03-897	NI	C/C	1931	1982
59	6	03-1102	NI	C/C	1947	2002
60	7	03-946	NI	C/C	1945	1989
61	8	03-1129	NI	C/C	1949	1998
62	9	03-883	NI	C/C	1945	1991
63	10	03-882	NI	C/C	1964	1996
64	11	03-961	NI	C/C	1959	1993
65	12	03-960	NI	C/C	1968	1993
66	13	03-1124	NI	C/C	1956	1991
Not Worked						
67	1	03-889	NW	C/C	1947	1995
68	2	03-896	NW	C/C	1946	1991
69	3	03-1119	NW	C/C	1949	1969
70	4	03-1121	NW	C/C	1955	1990
71	5	03-1106		C/T		

Appendix H. Frequency of PTPN22 genotypes in individuals with AITD

		Code	XCI	PTPN22	DoB	OoD
Skewed						
1	1	04-121.	>90	C/C	1957	1988
2	2	04-127.	>90	C/T	1975	2003
3	3	04-198.	>90	C/C	1935	2004
4	4	04-221.	>90	C/C	1958	2000
5	5	04-226.	>90	C/C	1963	2004
6	6	04-250.	>90	C/C	1967	1996
7	7	04-254.	>90	C/C	1941	2004
8	8	04-128.	>90	C/C	1960	2001
9	9	04-138.	>90	C/C	1962	1980
10	10	04-205.	>90	C/C	1927	2003
11	11	04-233.	>90	C/C	1967	1990
12	12	04-244.	>90	C/C	1963	1993
13	13	04-136.	>90	C/C	1975	2004
14	14	04-214.	85-90	C/C	1921	2001
15	15	04-225.	85-90	C/C	1936	1975
16	16	04-98.	85-90	C/C	1956	2000
17	17	04-132.	85-90	C/C	1960	2002
18	18	04-131.	85-90	C/C	1944	2002
19	19	04-105.	85-90	C/C	1978	1999
20	20	04-218.	80-85	C/C	1941	1991
21	21	04-108.	80-85	C/C	1952	1999
22	22	04-107.	80-85	C/C	1948	1998
23	23	04-208.	80-85	C/C	1960	1999
24	24	04-223.	80-85	C/C	1956	1988
25	25	04-110.	80-85	C/C	1960	1998
Random						
26	1	04-137.	70	C/C	1932	1981
27	2	04-203.	70	C/C	1961	2004
28	3	04-213.	70	C/C	1947	1998
29	4	04-228.	70	C/C	1953	2001
30	5	04-103.	68	C/C	1962	1986
31	6	04-248.	68	C/C	1951	1978
32	7	04-92.	65	C/C	1971	1998
33	8	04-251.	65	C/C	1941	1984
34	9	04-253.	65	C/C	1965	2001
35	10	04-199.	64	C/C	1950	2002
36	11	04-256.	63	C/C	1982	2003
37	12	04-240.	62	C/C	1975	2003
38	13	04-257.	62	C/C	1973	2004
39	14	04-112.	62	C/C	1952	1999
40	15	04-139.	60	C/C	1959	2002
41	16	04-99.	60	C/C	1961	1997
42	17	04-95.	58	C/C	1975	2004
43	18	04-96.	57	C/C	1939	1996

44	19	04-239.	57	C/C	1951	2003
45	20	04-242.	57	C/C	1960	1999
46	21	04-119.	57	C/C	1982	2001
47	22	04-238.	55	C/C	1939	2002
48	23	04-246.	55	C/C	1961	1996
49	24	04-134.	55	C/C	1968	2004
50	25	04-117.	55	C/C	1944	2004
51	26	04-200.	54	C/C	1954	1992
52	27	04-237.	54	C/C	1970	2004
53	28	04-197	53	C/C	1961	1976
54	29	04-229.	53	C/C	1938	?
55	30	04-123.	52	C/T	1963	1998
56	31	04-196.	52	C/C	1956	1999
57	32	04-231.	52	C/C	1964	2003
58	33	04-247.	52	C/C	1962	2002
59	34	04-255.	52	C/T	1974	1993
60	35	04-102.	50	C/C	1964	2003
61	36	04-118.	50	C/C	1972	2000
62	37	04-129.	50	C/C	1982	1998
63	38	04-204.	50	C/C	1949	2002
64	39	04-215.	50	C/C	1950	2002
65	40	04-202.	75	C/C	1968	2001
66	41	04-220.	67	C/C	1955	1998
67	42	04-206.	62	C/C	1946	1964
68	43	04-211.	52	C/C	1950	2002
69	44	04-212.	52	C/C	1958	2002
70	45	04-93.	52	C/C	1960	2003
71	46	04-88.	50	C/C	1979	2004
72	47	04-201.	50	C/C	1971	2001
73	48	04-224.	62	C/C	1950	2001
Not informative						
74	1	04-89.		C/C	1927	2002
75	2	04-90.		C/C	1984	2001
76	3	04-94.		C/C	1960	2001
77	4	04-100.		C/T	1965	2001
78	5	04-106.		C/C	1961	2002
79	6	04-113.		C/C	1978	2004
80	7	04-115.		C/C	1960	2001
81	8	04-122.		C/C	1948	1999
82	9	04-124.		C/T	1982	2004
83	10	04-126.		C/C	1955	1994
84	11	04-130.		C/C	1960	2002
85	12	04-209.		C/C	1971	2003
86	13	04-216.		C/C	1954	2003
87	14	04-217.		C/C	1948	2003
88	15	04-227.		C/C	1965	2003
89	16	04-234.		C/C	1971	2003

90	17	04-236.		C/C	1954	1994
91	18	04-241.		T/T	1948	1994
92	19	04-104.		C/C	1977	2004
93	20	04-207.		C/C	1952	1990
94	21	04-97.		C/C		
95	22	04-109.		C/C		
96	23	04-219.		C/C		
97	24	04-232.		C/T		
98	25	04-235.		C/C		
99	26	04-101.		C/C		
100	27	04-125.		C/C		
101	28	04-133.		C/C		
102	29	04-135		C/C		
103	30	04-245.		C/T		
104	31	04-249		C/C		

Appendix I. Frequency of PTPN22 genotypes controls

	SAMPLE ID	PTPN22
1	03-733	C/C
2	03-742	C/C
3	03-745	C/C
4	03-771	C/C
5	03-772	C/C
6	03-777	C/C
7	03-732	C/C
8	03-734	C/C
9	03-738	C/C
10	03-740	C/C
11	03-760	C/C
12	03-762	C/C
13	03-767	C/C
14	03-768	C/C
15	03-769	C/C
16	03-773	C/C
17	03-776	C/C
18	03-778	C/C
19	03-813	C/C
20	03-816	C/C
21	03-817	C/C
22	03-821	C/C
23	03-822	C/C
24	03-823	C/C
25	03-826	C/C
26	03-830	C/C
27	03-832	C/C
28	03-836	C/C
29	03-839	C/C
30	03-840	C/C
31	03-846	C/C
32	03-847	C/C
33	03-850	C/C
34	03-852	C/C
35	03-855	C/C
36	03-856	C/C
37	03-860	C/C
38	03-861	C/C
39	03-862	C/C
40	03-864	C/C
41	03-815	C/C
42	03-818	C/C
43	03-819	C/C
44	03-825	C/C

45	03-827	C/C
46	03-828	C/C
47	03-829	C/C
48	03-834	C/C
49	03-835	C/C
50	03-837	C/C
51	03-838	C/C
52	03-842	C/C
53	03-843	C/C
54	03-848	C/C
55	03-849	C/C
56	03-851	C/C
57	03-853	C/C
58	03-854	C/C
59	03-857	C/C
60	03-859	C/C
61	03-863	C/C
62	03-735	C/T
63	03-747	C/T
64	03-833	C/T
65	03-824	C/T
66	03-844	C/T
67	03-845	C/T
68	03-841	T/T

Appendix J. Confirmation of PTPN22 genotypes by microarray analysis

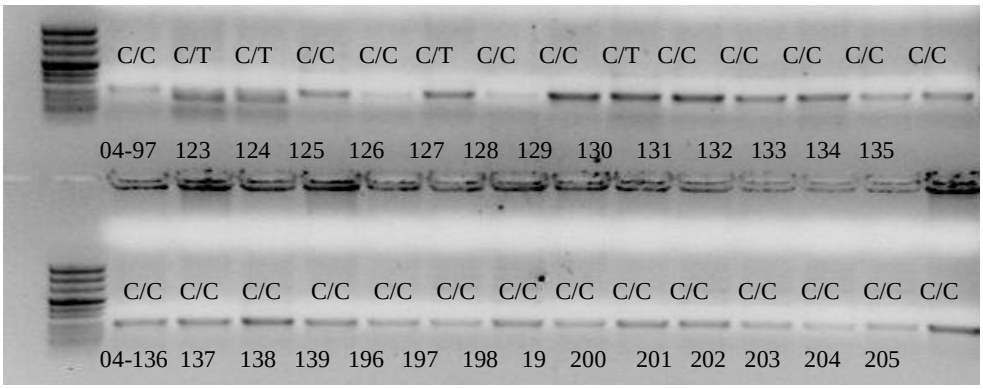
Code	PTPN22	
04-88.	C/C	AITD
04-92.	C/C	AITD
04-93.	C/C	AITD
04-95.	C/C	AITD
04-96.	C/C	AITD
04-98.	C/C	AITD
04-99.	C/C	AITD
04-102	C/C	AITD
04-103	C/C	AITD
04-105	C/C	AITD
04-107	C/C	AITD
04-108	C/C	AITD
04-110	C/C	AITD
04-112	C/C	AITD
04-117	C/C	AITD
04-118	C/C	AITD
04-119	C/C	AITD
04-121	C/C	AITD
04-123	C/T	AITD
04-127	C/T	AITD
04-128	C/C	AITD
04-131	C/C	AITD
04-132	C/C	AITD
04-134	C/C	AITD
04-136	C/C	AITD
04-137	C/C	AITD
04-138	C/C	AITD
04-139	C/C	AITD
04-196	C/C	AITD
04-197	C/C	AITD
04-198	C/C	AITD
04-199	C/C	AITD
04-200	C/C	AITD
04-201	C/C	AITD
04-202	C/C	AITD
04-203	C/C	AITD
04-204	C/C	AITD
04-205	C/C	AITD
04-206	C/C	AITD
04-208	C/C	AITD
04-211	C/C	AITD
04-212	C/C	AITD
04-213	C/C	AITD
04-214	C/C	AITD
04-215	C/C	AITD

04-218	C/C	AITD
04-220	C/C	AITD
04-223	C/C	AITD
04-224	C/C	AITD
04-226	C/C	AITD
04-228	C/C	AITD
04-229	C/C	AITD
04-233	C/C	AITD
04-237	C/C	AITD
04-238	C/C	AITD
04-239	C/C	AITD
04-242	C/C	AITD
04-244	C/C	AITD
03-890	C/C	SSc
04-129	C/C	AITD
04-225	C/C	AITD
04-231	C/C	AITD
04-240	C/C	AITD
03-878	C/C	SSc
03-880	C/C	SSc
03-881	C/C	SSc
03-884	C/C	SSc
03-885	C/C	SSc
03-886	C/T	SSc
03-887	C/T	SSc
03-888	C/C	SSc
03-892	C/C	SSc
03-893	C/C	SSc
03-893	C/C	SSc
03-895	C/C	SSc
03-898	C/C	SSc
03-899	C/C	SSc
03-900	C/C	SSc
03-943	C/C	SSc
03-944	C/C	SSc
03-945	C/C	SSc
03-947	C/C	SSc
03-1117	C/C	SSc
03-1118	NW	SSc
03-1120	C/C	SSc
03-1122	C/C	SSc
04-246	C/C	AITD
04-247	C/C	AITD
04-248	C/C	AITD
04-250	C/C	AITD
04-251	C/C	AITD
04-253	C/C	AITD

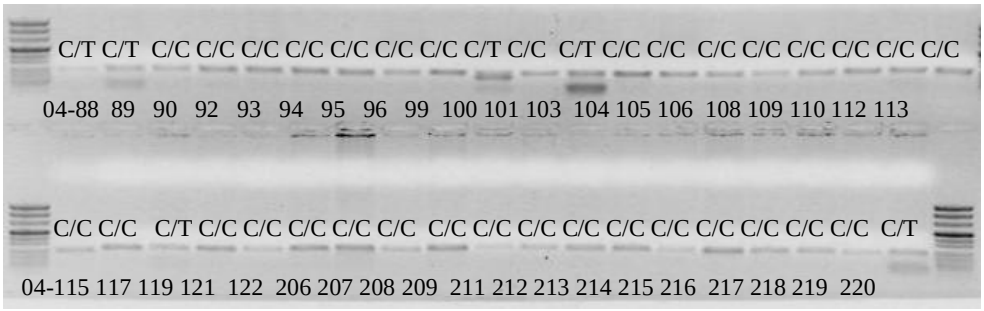
04-254	C/C	AITD
04-255	C/T	AITD
04-256	C/C	AITD
04-257	C/C	AITD
03-747	C/T	Control
03-760	C/C	Control
03-762	C/C	Control
03-767	C/C	Control
03-768	C/C	Control
03-769	C/C	Control
03-773	C/C	Control
03-776	C/C	Control
03-778	C/C	Control
03-948	C/C	SSc
03-949	C/C	SSc
03-950	NW	SSc
03-951	C/C	SSc
03-952	C/C	SSc
03-954	C/C	SSc
03-955	C/C	SSc
03-958	C/C	SSc
03-962	C/C	SSc
03-963	C/C	SSc
03-1101	C/C	SSc
03-1103	C/C	SSc
03-1104	C/C	SSc
03-1110	C/C	SSc
03-1111	C/C	SSc
03-1112	C/C	SSc

Appendix K. The gel figures of frequencies of *PTPN22* genotypes

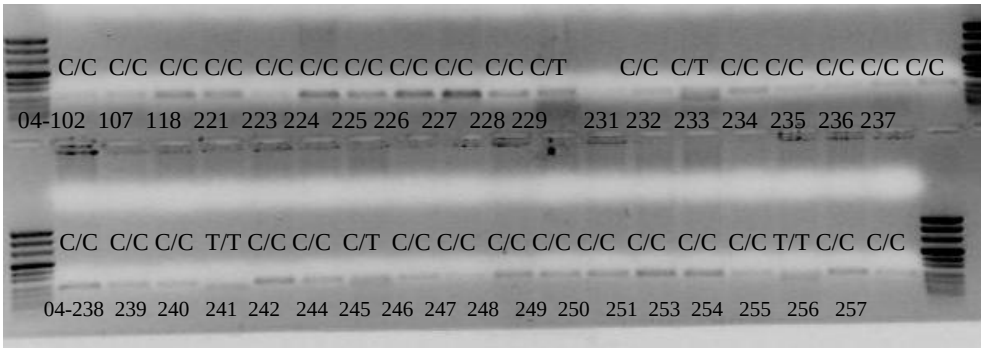
GEL1 DIGESTION OF PPTN22 LOCUS WITH XcmI OF HT



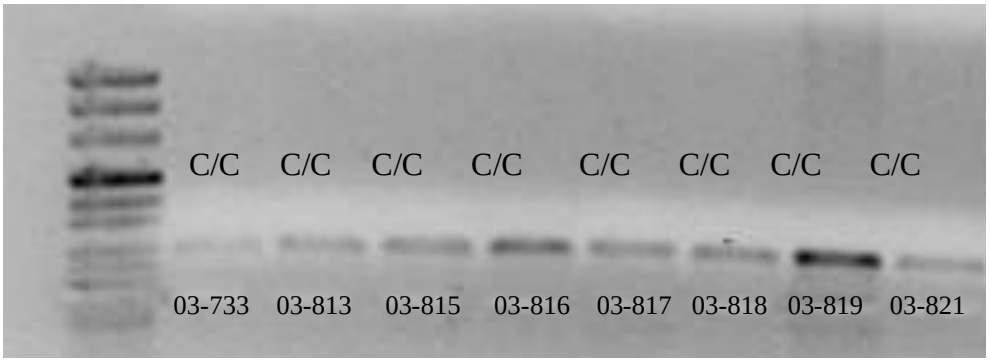
GEL2 DIGESTION OF PPTN22 LOCUS WITH XcmI OF HT



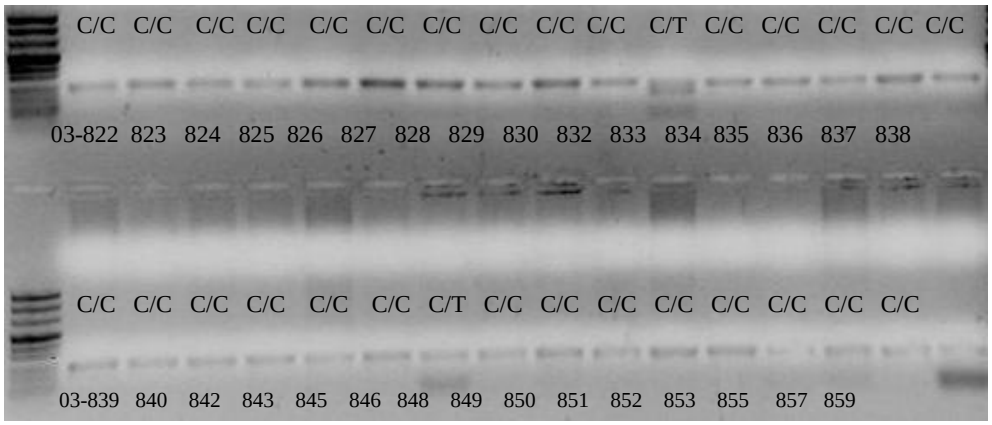
GEL3 DIGESTION OF PPTN22 LOCUS WITH XcmI OF HT



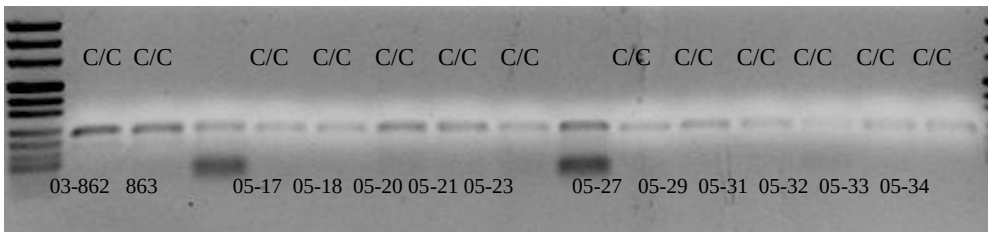
GEL4 DIGESTION OF PPTN22 LOCUS WITH XcmI OF CONTROL



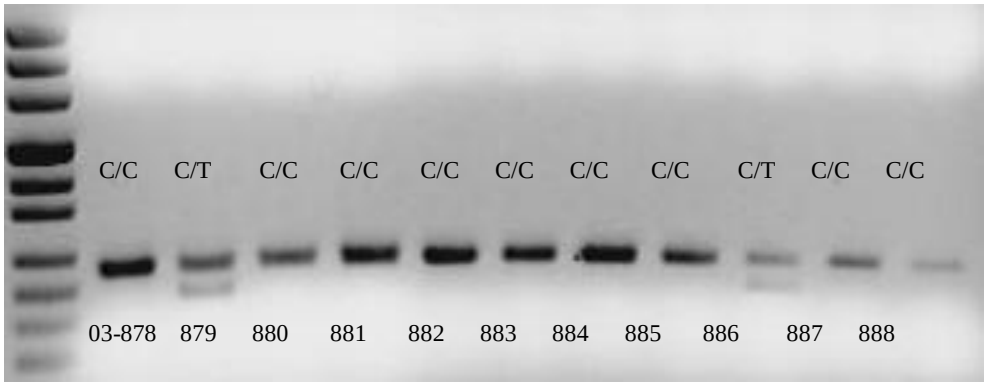
GEL5 DIGESTION OF PPTN22 LOCUS WITH XcmI OF CONTROL



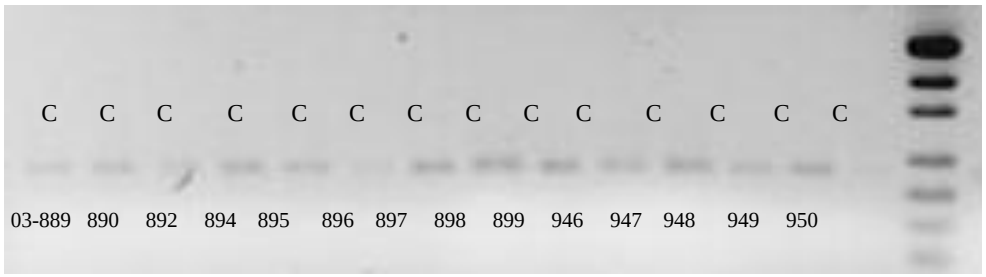
GEL6 DIGESTION OF PPTN22 LOCUS WITH XcmI OF CONTROL



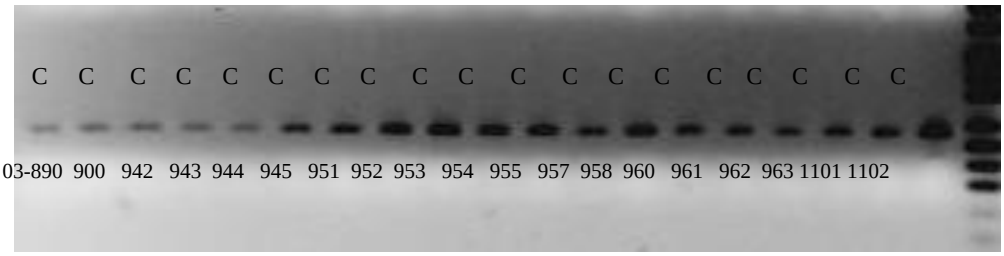
GEL7 DIGESTION OF PPTN22 LOCUS WITH XcmI OF SSC



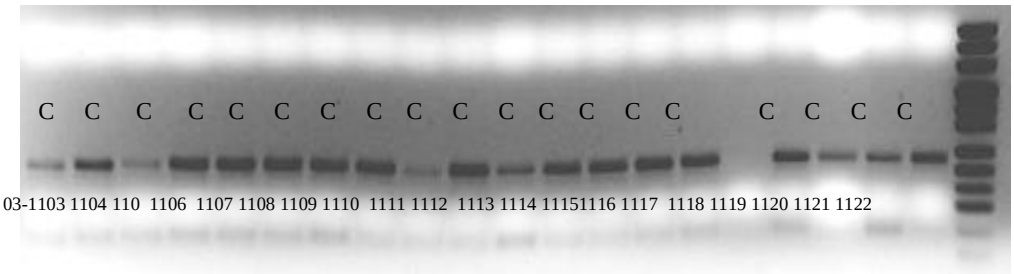
GEL8 DIGESTION OF PPTN22 LOCUS WITH XcmI OF SSC



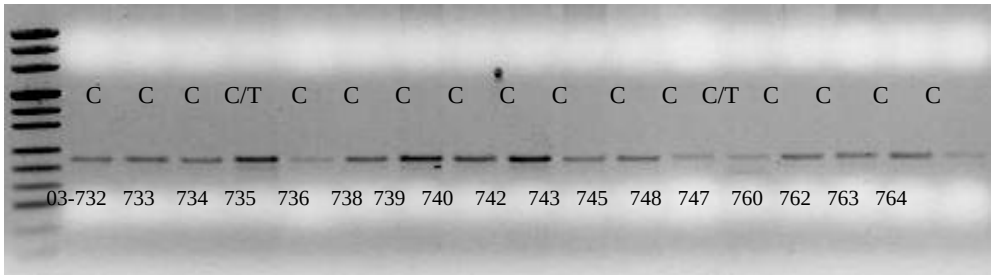
GEL9 DIGESTION OF PPTN22 LOCUS WITH XcmI OF SSC



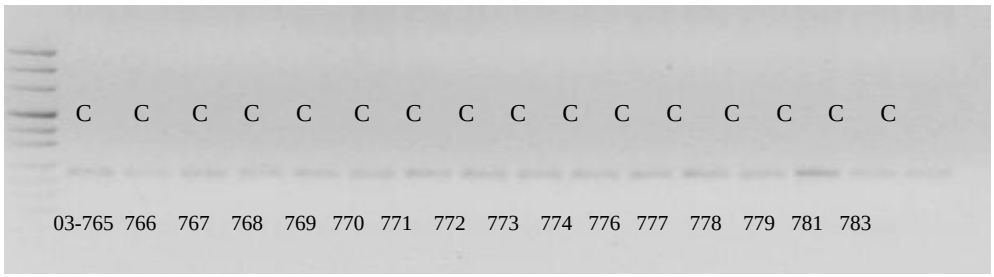
GEL10 DIGESTION OF PPTN22 LOCUS WITH XcmI OF SSC



GEL11 DIGESTION OF PPTN22 LOCUS WITH XcmI OF CONTROLS



GEL12 DIGESTION OF PPTN22 LOCUS WITH XcmI OF CONTROLS



PUBLICATIONS

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Increased Frequency of Extremely Skewed X Chromosome Inactivation in Juvenile Idiopathic Arthritis

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Objective. **Juvenile idiopathic arthritis (JIA) is a childhood rheumatic disease of unknown etiology. Two subgroups of JIA, i.e., oligoarticular and polyarticular, are thought to have an autoimmune component, and show a higher female:male ratio. Skewed X chromosome inactivation (XCI) has previously been shown to be associated with scleroderma and autoimmune thyroiditis, 2 autoimmune disorders occurring predominantly in females. This study was undertaken to extend the analysis to the pediatric age group and to determine the XCI profiles of patients with JIA.**

Methods. **A polymorphic repeat in the androgen receptor gene was genotyped to determine XCI status in 81 female patients with JIA (21 with polyarticular disease and 60 with oligoarticular disease) and 211 healthy female controls. DNA obtained from venous blood samples was used for this analysis.**

Results. **Informative data were obtained on 62 JIA patients and 155 controls. Skewed XCI was observed in 14 patients (22.6%) and 11 controls (7.1%) ($P = 0.0036$), and extreme skewing was apparent in 8 patients (12.9%) and 2 controls (1.3%) ($P = 0.0008$).**

Conclusion. **Our findings in the present study indicate that skewed XCI may be a risk factor for the occurrence of autoimmune disorders, including JIA.**

Juvenile idiopathic arthritis (JIA) is a broad term for a group of diseases characterized by chronic inflammation of 1 or more joints persisting longer than 6 weeks. It affects children before the age of 16, and its etiology is unknown. The International League of Associations for Rheumatology (ILAR) criteria set is the most recent accepted classification scheme (1). It is based on the number of affected joints and the presence of systemic symptoms. According to the ILAR classification, JIA is heterogeneous, with at least 6 subgroups: 1) systemic arthritis, 2) polyarthritis (rheumatoid factor positive and rheumatoid factor negative), 3) oligoarthritis (extended and persistent), 4) psoriatic arthritis, 5) enthesis-related arthritis, and 6) undifferentiated arthritis. Autoimmunity is particularly associated with the polyarthritis and oligoarthritis subgroups, and girls are more frequently affected than boys (female:male ratio 2–3:1) (2). Interestingly, an association between Turner's syndrome and juvenile arthritis has been reported (3).

It has long been recognized that hormone levels and pregnancy-related fetal–maternal microchimerism may influence the occurrence of autoimmunity (4,5). Recently, our group and others observed that skewed X chromosome inactivation (XCI) could be a novel predisposition factor (6–8). Among these factors, XCI could be considered a particularly attractive etiologic candidate in JIA, since the onset of the disease is before puberty, excluding the risks associated with hormone levels and fetal microchimerism. In order to test the hypothesis that XCI may play a role in the pathogenesis of JIA, we determined the X chromosome inactivation profiles of patients with JIA and control subjects.

PATIENTS AND METHODS

Patients. Eighty-one girls diagnosed as having JIA according to the ILAR criteria and 211 healthy girls with no

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Dr. Uz and Ms Mustafa contributed equally to this work.

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Table 1. Proportion of the juvenile idiopathic arthritis (JIA) patients and controls with skewed X chromosome inactivation*

% skewing	JIA patients (n = 62)	Controls (n = 155)
≥90	8 (12.9)	2 (1.3)
80–89	6 (9.7)	9 (5.8)
70–79	13 (21.0)	29 (18.7)
60–69	18 (29.0)	39 (25.2)
50–59	17 (27.4)	76 (49.0)

* Values are the number (%). For ≥80% skewing, $P = 0.0036$ (odds ratio 3.81 [95% confidence interval 1.65–8.83]); for ≥90% skewing, $P = 0.0008$ (odds ratio 11.33 [95% confidence interval 2.62–48.48]), by Fisher's 2-tailed exact test.

history of autoimmune disorders or cancer (control group; mean ± SD age 13 ± 4 years) were included in the study. The medical files of the parents were not available for analysis. The mean ± SD age of the JIA patients was 10 ± 5 years, and the mean age at the time of disease onset was 6 ± 4 years. Twenty-one of the JIA patients had polyarticular disease and 60 had oligoarticular disease. The following clinical data were obtained on all patients: age, age at diagnosis, type of JIA, disease duration, and treatment regimens. Informed consent was obtained from all subjects (or legal guardians of subjects who had not reached age of majority). The ethics committee of Hacettepe University approved the study protocol.

X chromosome inactivation assay. The highly polymorphic CAG repeat on the first exon of the androgen receptor gene was genotyped, as described elsewhere (6,8,9), to determine the XCI status of the patients and controls.

Statistical analysis. The significance of differences in results between the JIA patients and the control subjects was determined by Fisher's exact test.

RESULTS

Findings on XCI status were informative in 62 of the 81 JIA patients (76.5%) and in 155 of the 211 controls (73.5%). The individuals whose alleles could not be distinguished adequately were not included in the densitometric analysis and were categorized as having noninformative results. Skewed XCI (≥80% skewing) was observed in 14 of the 62 patients with informative results (22.6%), and in 11 of the 155 controls with informative results (7.1%) ($P = 0.0036$). Extremely skewed XCI ratios (≥90% skewing) were seen in 8 of the 62 patients (12.9%), but in only 2 of the controls (1.3%) ($P = 0.0008$) (Table 1). Studies in large cohorts have firmly established that extremely skewed XCI is rare in the general population (10).

It has been reported that years after exposure to an immunosuppressive agent, XCI ratios in feline hematopoietic cells may be skewed (11). We therefore investigated correlations between XCI ratios and treatment regimens in the patients. All of the patients were receiving some treatment at the time of sample collection. Fourteen were receiving nonsteroidal antiinflammatory drugs (NSAIDs), 11 were receiving methotrexate (MTX), 10 were receiving MTX plus NSAIDs, and 9 were receiving intraarticular corticosteroids plus NSAIDs. The remaining 18 were receiving various combinations of these and other drugs. Clinical details on the 62 patients are shown in Supplementary Table 1, available on the *Arthritis & Rheumatism* Web site at http://www3.interscience.wiley.com/journal/76509746/home. Among the patients with skewed XCI, 7 (50%) had received treatment with immunosuppressive agents for 6–10 years, and 1 (7%) had received immunosuppressive treatment for 2 years. The remaining 6 had received antiinflammatory treatment alone. Among the patients with random patterns of XCI, 27 (56.3%) had received immunosuppressive treatment for >2 years. At any stage of their treatment, suspended leukopenia or bone marrow ablation was not observed in any of the patients. These results, along with previous observations (6,8), indicate that it is unlikely that immunosuppressive therapy caused skewed XCI in the patients.

DISCUSSION

Autoimmune disorders are complex and affect ~5% of the world population (12). A diverse group of autoimmune diseases, including rheumatoid arthritis and JIA, affects females more frequently than males (2). In this study, we observed skewed XCI patterns in peripheral blood mononuclear cells of a significant proportion of female subjects with JIA, with 22.6% in the ≥80% skewing range (versus 7.1% of controls) and 12.9% in the ≥90% skewing range (versus 1.3% of controls). These results support the hypothesis that loss of XCI mosaicism in females may constitute a risk factor for the development of JIA.

At present, the nature of the association between skewed XCI and breakdown of self tolerance, as exemplified in this study on JIA and previous observations on scleroderma (6) and autoimmune thyroiditis (7,8,13), is not known. We propose that deleterious X-linked mutations could influence the survival of cells that inactivate a normal X chromosome and leave the mutant X transcriptionally active. Since cells that inactivate the mutant X would be immune to the deleterious effects of the putative mutations, females could tolerate them by losing their mosaic status for X-linked gene expression (14,15). An interesting aspect of extremely skewed XCI is that it does not lead to the breakdown of self tolerance

in all females. This suggests that breakdown of self tolerance may require 2 distinct events on the X chromosome: first, a lethal mutation leading to loss of mosaicism, and second, "heterozygosity" for the nonsynonymous variants of the putative critical genes. To the best of our knowledge, this is the first study that has shown an association between skewed XCI and a pediatric form of an autoimmune disease, i.e., JIA.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Ozcelik had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Ozcelik.

Acquisition of data. Uz, Mustafa, Topaloglu, Bilginer, Dursun, Kasapcopur, Ozen, Bakkaloglu.

Analysis and interpretation of data. Uz, Mustafa, Topaloglu, Bilginer, Dursun, Kasapcopur, Ozen, Bakkaloglu, Ozcelik.

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EDITORIAL

The Price of Silence

Nathalie C. Lambert

Female predisposition to autoimmunity

It has long been recognized that the prevalence of autoimmune diseases is greater in women than in men. This gender gap is often ascribed to hormonal differences and gender distinction in immune responses (i.e., higher number of absolute CD4+ cells in women than in men) (for review, see ref. 1). Fetal microchimerism, an unavoidable sequel of pregnancy which results in persistence of fetal cells in the mother, is also often discussed and described as a potential mechanism of "auto"-immunity in women, particularly in systemic sclerosis (for review, see ref. 2). While all the above possibilities are of importance, another gender difference, of relevance for autoimmune disorders, is X chromosome inactivation (3,4).

Silencing of 1 X chromosome: a female particularity

X chromosome inactivation (XCI) is a dosage compensation mechanism used by mammals to ensure that X chromosome gene expression is equalized in XX females and XY males (3). Early in female embryonic life, 1 of the 2 X chromosomes is randomly silenced, except in ~15% of genes including those corresponding to functional X and Y homologs (5). XCI depends on the presence of the X-inactive specific transcript (XIST) gene, which codes not for a protein but for a functional RNA expressed exclusively in cells with >1 X chromosome. At cell interphase, XIST RNA coats the X chromosome on which it is produced, to randomly inactivate either the paternal or the maternal X chromosome (6). As a consequence, females are a mosaic of 2 cell lines, 1 expressing maternal X-linked and the other expressing paternal X-linked genes, with a ratio close to 50:50 when XCI is random.

Skewed silencing and immunologic consequences

However, skewing, defined as a deviation from the 50:50 ratio, can occur. The most common technique used to analyze XCI patterns is genotyping of a highly polymorphic CAG repeat in the human androgen receptor (AR) gene. *Hpa* II and *Hha* I enzyme restriction sites, located <100 bp from this polymorphic short tandem repeat, are methylated on the inactive X chromosome and nonmethylated on the active X chromosome. Polymerase chain reaction (PCR) amplification across this region permits the distinguishing of 2 amplicons with different sizes, corresponding to maternal and paternal X alleles (among informative subjects heterozygous for AR) (7). After enzyme digestion, PCR amplification is possible only for the methylated (uncut) X chromosome. Densitometric analysis of the 2 alleles indicates the inactivation status of 1 allele compared with the other and reveals the percentage of XCI skewing. A skewed result is defined as 1 allele being inactivated at >75%. Extreme skewing represents an inactivation of >90%.

Women with extremely skewed XCI host a large population of cells with one X chromosome inactivated and a small population with the other X chromosome inactivated. Under such circumstances, rare cells from the smaller population would fail to be sufficiently expressed in the thymus, and this would lead to the escape of X-linked self antigens from presentation. This represents an interesting explanation for autoimmunity, that was first speculated by Stewart as a possible mechanism of predisposition to systemic lupus erythematosus (SLE) (4). This hypothesis has been tested by several research groups in different autoimmune disorders. Contrary to expectations, however, the frequency of skewing of XCI is not significantly increased among women with SLE (8). However, scleroderma and autoimmune thyroiditis are among the top 10 diseases for

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which skewed XCI is significantly increased (9–12). Most studies have been conducted on adult women, and analyses of pediatric cohorts are rare.

Bias of XCI in juvenile idiopathic arthritis

In this issue of *Arthritis & Rheumatism*, Uz and colleagues describe a bias in XCI in peripheral blood mononuclear cells of patients with juvenile idiopathic arthritis (JIA), as determined by AR genotyping (13). The analysis of a young cohort is of interest because reduction in sex ratio (male:female) is a characteristic of autoimmune disorders not only with adult onset, but also with juvenile onset (7).

A prior study of patients with pediatric autoimmune diseases conducted by Chitnis et al in 2000, as part of a large study of female patients with a variety of autoimmune diseases, did not show a bias of XCI in patients with juvenile rheumatoid arthritis (JRA) (8). One possible explanation for the difference in results between that study and the one by Uz et al is that clinical definitions were not necessarily concordant. Moreover, only 18 patients with JRA were tested in the study by Chitnis and colleagues.

XCI bias increases with age

Although skewed XCI is sometimes observed in the general population, extreme skewing is rarely seen. Uz and colleagues' finding that 12.9% of patients with JIA had extreme skewing, compared with 1.3% of controls, is of great importance. Comparison with a group of pediatric controls reinforces their study results.

Indeed, several studies have shown that skewing of XCI increases significantly in the blood cells of females from the neonatal period to old age. Controversy surrounding this issue, due to the use of different methods of analysis, appears to have been resolved in a recent study in which the age effect was confirmed with the use of independent methodologies (14). The incidence of skewing triples from birth to older age and raises the question of whether it may be an indicator of hematopoietic malignancies in the elderly (14). Most of the "war" for choosing which X chromosome to inactivate takes place in early embryonic life and results in a stable mosaic status. However, correlation of skewing with age suggests that this status is not so stable, and that some "battles" are still readjusting this choice in late adult life, at least in the blood.

XCI and hormones

Because the onset of JIA occurs before puberty, it seems unlikely that the sex ratio could be explained by hormone differences. Then, could other genetic predispositions make sense, bearing in mind the influence of age on the outcome of genotyping results? Unfortunately, there is, to my knowledge, no analysis of XCI skewing in female subjects grouped according to hormone levels, e.g., prepuberty, puberty, adults before menopause, and adults after menopause. Although the age of a child does not distinguish pre- from postpuberty with certitude, it provides an indication, and the very detailed supplemental table in the report by Uz et al (13) does not reveal notable differences in skewing according to age since, among the 8 patients found to have extreme skewing, some had very early JIA onset and others a postpubertal onset.

XCI is not influenced by treatment

Another issue addressed in the study by Uz and colleagues is whether exposure to immunosuppressive agents could influence the XCI profile, as previously reported to occur in feline hematopoietic cells (15); in humans, immunosuppressive agents do not seem to be responsible for the extreme skewing observed in some autoimmune diseases. Indeed, this is not the first attempt by this group to assess the effect of immunosuppressive drugs on XCI: in an earlier study showing XCI bias in women with scleroderma, Ozcelik et al demonstrated that patients with rheumatoid arthritis who were receiving similar treatment regimens did not exhibit similar skewing (9). In the present study (13) they again excluded a possible effect of immunosuppressive therapy by showing that both the skewed and the random XCI profiles were represented among the patients who had received immunosuppressive therapies.

Rupture of tolerance to X-encoded genes

Ozcelik and colleagues' team has been studying mosaicism in autoimmunity for several years now. Like other investigators, they have considered a potential mechanism through which lack of exposure to self antigen could occur in women with loss of mosaicism. This mechanism implies that defective tolerance from skewed XCI should be directed at X-linked antigens that are polymorphic and heterozygous. This also suggests that extreme skewing does not necessarily lead to autoimmunity in individuals homozygous for such genes. But

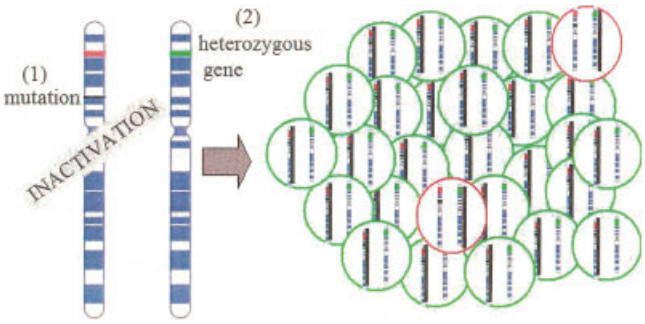

Figure 1. Proposed mechanisms by which heterozygosity and monoallelic expression of X-linked genes lead to autoimmunity. 1, A deleterious X-linked mutation (represented in black) would lead to extreme inactivation of the X chromosome carrying such a mutation and lead to loss of mosaicism. Inactive X chromosomes are schematically coated with a black bar. 2, Presence of a polymorphic X-linked gene (alleles represented in green and red) would trigger a lack of thymic presentation of the rare cells in which the allele represented in red is active. Only women heterozygous for that gene, and not homozygous women, would have autoimmune disorders.

the reason XCI is biased in some autoimmune diseases is still unknown. There are numerous causes of skewed XCI, such as X-linked mutation affecting cell survival as initially found in female carriers of specific X-linked lethal mutations, XIST mutations, and balanced X/autosome translocations perturbing the mechanism of XCI (for review, see ref. 16).

Nevertheless, although extremely skewed XCI is rare and is associated with autoimmune diseases, it does not lead to the breakdown of self tolerance in all women. Uz et al (13) propose that breakdown of self tolerance requires the coinheritance of 2 distinct events on the X chromosome. First, an X-linked mutation affecting cell survival would lead to the extreme inactivation of the X chromosome carrying such a mutation and lead to "loss of mosaicism" (Figure 1). Then X chromosomes would carry an allelic variant of the putative critical gene, leading to autoimmunity because of the loss of mosaicism through lack of thymic exposure.

Such a scheme would explain why extensive genome screening during the past decade has resulted in the identification of several susceptibility genes for different autoimmune diseases, but often with weak odds ratios. Females with loss of mosaicism may still exhibit, in their genome, all that is necessary to avoid susceptibility to autoimmunity, and therefore, an X genome scan would not necessarily reveal their disadvantage.

Would the phenotype and the origin of inactivated cells be of importance?

Our understanding of XCI is still incomplete, and several essential questions remain. For example, although several groups have demonstrated that the greatest amount of X chromosome skewing is often observed in peripheral blood, no detail on the phenotype of cells has been reported. In prior studies on X monosomy, cells losing their X chromosome were more often from the adaptive immune system (B and T cells) than from the innate system (natural killer cells, monocytes, polymorphonuclear cells) (17). Would this observation hold for XCI, and if so, what would be its significance? Moreover, is the paternal or maternal X chromosome preferentially active? In a recent study on scleroderma, Uz et al showed that maternal-origin X chromosome is more often inactive than that of paternal origin (10). Although the number of individuals tested was small, this observation warrants further investigation and emphasizes the need to determine the parental origin of the inactivated X chromosome to investigate the role of imprinting. Interestingly, several genes known to be crucial for the maintenance of immune tolerance and hormone levels are located on the X chromosome. This might close the loop regarding the gender gap in prevalence of autoimmunity.

Other nonexclusive mechanisms involving X chromosome

The finding of extremely skewed XCI in juvenile idiopathic arthritis provides an incentive to further investigate the X chromosome field. For example, haploinsufficiency of X-linked genes is an attractive candidate and has its roots in the aneuploidy of the X chromosome, as in Turner's syndrome and the recently described X chromosome monosomy, both associated with increased susceptibility to autoimmune disorders (17,18). The reported association of Turner's syndrome with juvenile arthritis (18) suggests that, reciprocally, these patients exhibit a higher rate of monosomy, as observed in patients with scleroderma and autoimmune thyroid disease. Fluorescence in situ hybridization studies in JIA patients with noninformative AR genotyping results may reveal that the lack of information is due to X monosomy rather than to homozygosity for the CAG repeat. This has not yet been investigated and merits further attention.

Losing an advantage

Females have the privilege of having 2 X-linked alleles at the same locus, rendering a copy in reserve on 1 X chromosome in case of a gene mutation on the other. This is an advantage, but only when mosaicism is retained. No single unifying hypothesis can explain the pathogenesis of JIA, but the newly reported findings by Uz et al (13) make us aware that biased X chromosome silencing likely contributes to autoimmunity. This is the price of silence.

AUTHOR CONTRIBUTIONS

Dr. Lambert wrote and revised the article, and approved the final version to be published.

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Research article

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Analysis of skewed X-chromosome inactivation in females with rheumatoid arthritis and autoimmune thyroid diseases

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Abstract

Introduction The majority of autoimmune diseases such as rheumatoid arthritis (RA) and autoimmune thyroid diseases (AITDs) are characterized by a striking female predominance superimposed on a predisposing genetic background. The role of extremely skewed X-chromosome inactivation (XCI) has been questioned in the pathogenesis of several autoimmune diseases.

Methods We examined XCI profiles of females affected with RA (n = 106), AITDs (n = 145) and age-matched healthy women (n = 257). XCI analysis was performed by enzymatic digestion of DNA with a methylation sensitive enzyme (*Hpa*II) followed by PCR of a polymorphic CAG repeat in the androgen receptor (*AR*) gene. The XCI pattern was classified as skewed when 80% or more of the cells preferentially inactivated the same X-chromosome.

Results Skewed XCI was observed in 26 of the 76 informative RA patients (34.2%), 26 of the 100 informative AITDs patients (26%), and 19 of the 170 informative controls (11.2%) ($P < 0.0001$; $P = 0.0015$, respectively). More importantly, extremely skewed XCI, defined as > 90% inactivation of one allele, was present in 17 RA patients (22.4%), 14 AITDs patients (14.0%), and in only seven controls (4.1%, $P < 0.0001$; $P = 0.0034$, respectively). Stratifying RA patients according to laboratory profiles (rheumatoid factor and anti-citrullinated protein antibodies), clinical manifestations (erosive disease and nodules) and the presence of others autoimmune diseases did not reveal any statistical significance ($P > 0.05$).

Conclusions These results suggest a possible role for XCI mosaicism in the pathogenesis of RA and AITDs and may in part explain the female preponderance of these diseases.

Introduction

It is postulated that the paternal and maternal antigens will be recognized by the immune system within the thymus, and T cells that have a high affinity for such antigens will be deleted by apoptosis [1-3]. The lack of exposure to a self-antigen in the thymus may lead to the presence of autoreactive T cells and increase the risk of autoimmunity [4]. In female mammalian cells, one of the two X-chromosomes is inactivated in early embryonic life [5]. Thus, females are mosaics for two cell populations, cells with either the paternal or the maternal X in the active form. X-chromosome choice is assumed to be random, and the result is generally 50% of cells expressing the paternal and the remaining 50% expressing the maternal genes [6]. A skewed X-chromosome inactivation (XCI) is a deviation from this ratio and is arbitrarily defined, for example, as a pattern where 80% or more of the cells inactivate the same X-chromosome [7]. This deviation may be the result of chance or genetic factors involved in the XCI or a selection process [8]. The

ACPA: anti-citrullinated protein/peptide antibodies; AITDs: autoimmune thyroid diseases; AR: androgen receptor; CrR: corrected ratio; ELISA: enzyme-linked immunosorbent assay; GD: Graves' disease; HT: Hashimoto's thyroiditis; IL: interleukin; PCR: polymerase chain reaction; RA: rheumatoid arthritis; RF: Rheumatoid factor; SD: standard deviation; TSH: thyroid stimulating hormone; XCI: X-chromosome inactivation.

existence of XCI in females offers a potential mechanism where by X-linked self-antigens may escape presentation in the thymus or in other peripheral sites that are involved in tolerance induction [9,10]. This has become an attractive candidate mechanism for breakdown of self-tolerance in autoimmune diseases. An association between skewed XCI and scleroderma was recently reported [11]. A higher frequency of a skewed XCI pattern was found in patients affected with autoimmune thyroid diseases (AITDs) compared with healthy controls, indicating that skewed XCI may be associated with a predisposing factor for the development of AITDs [12-14]. It was therefore of interest to study if there is an association between skewed XCI and rheumatoid arthritis (RA) as a non-organ-specific and AITDs as an organ-specific autoimmune disease. We investigated the peripheral blood XCI patterns of 106 females affected with RA, 145 females affected with AITDs and 257 controls in the Tunisian and Turkish populations. Extremely skewed XCI was found in the blood samples of female patients affected with RA and AITDs supporting the role of skewed XCI in female predisposition to autoimmune diseases.

Materials and methods

Patients and controls

RA sample

One hundred and six Tunisian women affected with RA were recruited into the study. All patients fulfilled the 1987 American College of Rheumatology criteria for RA [15]. A rheumatologist university fellow (ZB) reviewed all clinical data. The mean age was 47.6 ± 13.4 (mean ± standard deviation (SD)) years. The duration of the symptoms was 15 ± 8.9 years. The mean age of diagnostic was 40.3 ± 12 years. Among 106 RA patients, 65 were rheumatoid factor (RF) positive (61.3%), 70 were anti-citrullinated protein/peptide antibodies (ACPA) positive (66%), 15 presented with nodules (14.1%), and 70 presented with erosive disease (66%). Fifteen patients had another accompanying autoimmune diseases such as Sjögren's syndrome, type 1 diabetes, or autoimmune thyroid diseases.

AITDs sample

One hundred and forty-five Tunisian women affected with AITDs were included in the study. There were a total of 58 patients with Graves' disease (GD) and 87 patients with Hashimoto's thyroiditis (HT), which include 40 patients with the goitrous form. The mean age was 46.5 ± 14.5 years for AITDs patients (49.3 ± 13 years in HT patients and 44.6 ± 14 years in GD patients). The duration of the symptoms was 7.5 ± 4.6 years among the AITDs patients (6.8 ± 4.8 years in HT patients and 7.2 ± 4 years in GD patients). The mean age of diagnosis was 37.9 ± 15.1 years. The diagnosis of GD was based on the presence of biochemical hyperthyroidism as indicated by a decrease of thyroid-stimulating hormone (TSH), an increase of T4 levels, and positive TSH receptor antibody, in association with diffuse goiter or the presence of exophthalmos. The diagnosis of HT was based on the presence of thyroid hormone replaced primary hypothyroidism, defined as a TSH level above the upper limits associated with positive titers of thyroid autoantibodies (anti-thyroglobulin and/or anti-thyroid peroxidase) and with or without a palpable goiter.

Control group

Caucasian females, comprised of 97 Tunisian and 160 Turkish healthy unrelated volunteers, served as controls in our studies. The mean (± SD) age at analysis was 43.5 ± 15.3 years and 35 ± 9.9 years for Tunisian and Turkish controls, respectively. There was no clinical evidence or family history of autoimmune disease and inflammatory joint disease.

All individuals (patients and controls) provided informed consent. The ethics committee of the Centre Hospitalo-Universitaire Hédi Chaker de Sfax, Tunisie, and the Bilkent University, Ankara, Turkey approved the study protocol.

Methods

Autoantibodies analysis

In AITDs patients, thyroid autoantibodies (anti-thyroglobulin and anti-thyroid peroxydase) were measured by ELISA and indirect immunofluorescence using commercially available kits

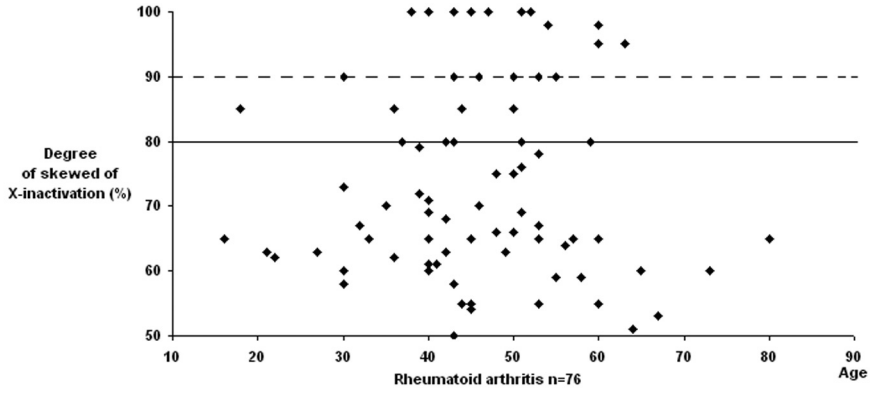
Table 1

Proportion of RA and AITDs patients and controls with skewed X-chromosome inactivation

	Number (%) observed with skewed		
Degree of skewing (%)	RA (n = 76)	AITDs (n = 100)	Control females (n = 170)
90+	17 (22.4)	14 (14)	7 (4.1)
80 to 89	9 (11.8)	12 (12)	12 (7.1)
70 to 79	11 (14.5)	23 (23)	29 (17.1)
60 to 69	28 (36.8)	22 (22)	36 (21.2)
50 to 59	11 (14.5)	29 (29)	86 (50.6)

For comparison by chi-squared $P < 0.0001$ and $P = 0.0015$ (> 80% skewing); $P < 0.0001$ and $P = 0.0034$ (90+% skewing) for patients with rheumatoid arthritis (RA) and autoimmune thyroid diseases (AITDs), respectively.

Figure 1



Distribution of X-chromosome inactivation patterns according to age in patients with rheumatoid arthritis.

(BINDAZYME™ Human EIA kits, Binding site Ltd, Birmingham, UK) with the respective normal ranges of 0 to 100 and 0 to 70 IU/mL.

The sera of RA patients obtained at the time of diagnosis were examined for RF by nephelometry and for ACPA by ELISA (second-generation test; Euro-Diagnostica, Arnhem, the Netherlands).

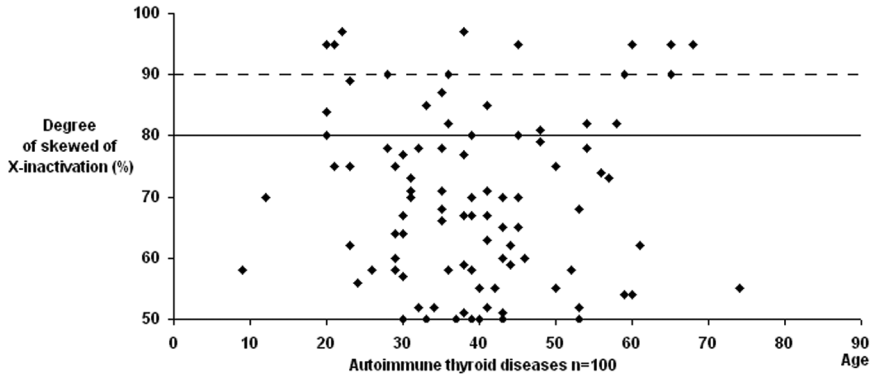
X-chromosome inactivation study

Genomic DNA was extracted from 10 ml of peripheral blood lymphocyte of patients and controls using standard methods [16]. Genotyping of a polymorphic site in the androgen receptor (*AR*) gene was performed and quantified to assess the XCI patterns as described [17]. The degree of skewing was estimated by an assay based on a methylation-sensitive *HpaII* restriction site located in exon 1 of the *AR* gene. This site is methylated on the inactive X, and unmethylated on the active X-chromosome. When the genomic DNA is cleaved with *HpaII* prior to PCR, only the methylated *AR* allele, which represents the inactive X-chromosome, is amplified. A polymorphic CAG repeat located within the amplified region is used to distinguish between the two alleles. For each patient and control two separate PCRs, with or without *Hpa*II treatment, were performed using the same set of primers. Densitometric analysis of the alleles was performed at least twice for each sample using the MultiAnalyst version 1.1 software (Bio-rad, Hercules, California, USA). A corrected ratio (CrR) was calculated by dividing the ratio of the predigested sample (upper/lower allele) by the ratio of the non-predigested sample for normalization of the ratios that were obtained from the densitometric analyses. The use of CrR compensates for preferential amplification of the shorter allele when the number of PCR cycles increases [18]. A skewed population is defined as a cell population with greater than 80% expression of one of the AR alleles. This corresponds to CrR values of less than 0.33 or more than three.

Statistical methods

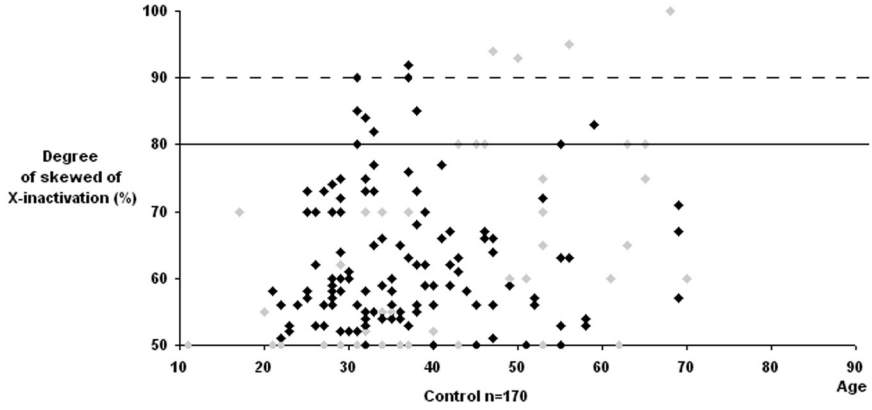
The results from control and test groups in XCI studies were compared by chi-squared test with Yate's correction. Fisher's exact test was used when one cell had an expected count of

Figure 2



Distribution of X-chromosome inactivation patterns according to age in patients with autoimmune thyroid diseases.

Figure 3



Distribution of X-chromosome inactivation patterns according to age control subjects. The control subjects were plotted according to geographic origin. Gray diamonds represent Tunisian controls and black diamonds represent Turkish controls.

less than one, or more than 20% of the cells had an expected count of less than five. *P* values of 0.05 or less were considerate to be significant. Significance of *P* value was assessed using a Bonferroni correction at 5% (a *P* value less 0.05/9 = 0.005) is considered significant.

Results

XCI status was found to be informative in 76 of the 106 RA patients, 100 of the 145 AITDs patients and 170 of the 257 controls. Only those individuals whose alleles resolve adequately for densitometric analysis were included in the study. Skewed XCI (> 80% skewing) was observed in 26 of the 76 RA patients (34.2%), 26 of the 100 AITDs patients (26%), and 19 of the 170 controls (11.2%; $P < 0.0001$ and $P = 0.0015$). More importantly, the frequency of extremely skewed XCI (> 90% skewing) was 22.4% (17 of 76) in RA and 14.0% (14 of 100) in AITDs. These frequencies are both significantly higher than that of the control population, which is 4.1% (7 of 170; $P < 0.0001$ and $P = 0.0034$; Table 1). Subdividing AITDs patients according to clinical phenotype revealed that the frequency of skewed XCI was 35% (14 of 40, $P = 0.0001$) and 20% (12 of 60, $P = 0.04$) in GD and HT, respectively. Conversely, stratifying RA patients according to RF status, ACPA status, clinical manifestations (erosive disease and nodules) and others autoimmune diseases did not reveal a statistically significant difference ($P > 0.05$). Additionally, the comparison according to geographic origin showed a skewed XCI of RA patients compared with Tunisian controls (34.2% versus 19.5%; $P = 0.03$). However, difference was non-significant for AITDs subgroup ($P > 0.05$).

Extremely skewed XCI have been reported in 1 to 2% of 20 to 40 year old women, and in 2 to 4% of 55 to 72 year old women [19]. The data for RA and AITDs patients is strikingly bimodal, we plotted the distribution of the X inactivation profiles according to age. However, we did not observe a shift toward the skewed range in older patients and controls (Figures 1, 2 and 3). Characteristics of the RA and AITDs patients with skewed XCI are shown in Tables 2 and 3.

At the time of sample collection, 66 patients affected with RA were being treated with immunosuppressive therapies (methotrexate 10 to 15 mg once a week, n = 33; D-penicillamine 300 mg/day, n = 17; plaquenil 400 to 600 mg/day, n = 16). Among 76 informative patients, 46 were received immunosuppressive agents (61%). A major concern with the observed XCI patterns among RA patients was that concomitant immunosuppressive therapy could influence the results, as has been observed in feline hematopoietic cells [20]. Analysis of the data on XCI patterns according to immunosuppressive therapy did not reveal a statistically significant association between RA patients treated with methotrexate and controls ($P = 0.52$).

Discussion

The majority of human autoimmune diseases are characterized by female predominance. RA and AITDs have a female:male ratio of approximately 3:1 and 9:1, respectively [21]. Sex hormone influences have been suggested to explain this phenomenon because the X-chromosome contains a considerable number of sex and immune-related genes such as *AR*, IL2 receptor gamma chain, *CD40* ligand and *FOXP3* [22,23]. These genes are essential in determining sex hormone levels and, more importantly, immune tolerance [24]. The contribution of genetics to sex differences in autoimmune diseases is currently unexplored. An alternative explanation for the female predominance has been recently proposed with the finding of an enhanced skewed XCI in peripheral bloods cells of female patients with autoimmune diseases [11-14]. The present study tests the hypothesis that skewed XCI would be more prevalent in females affected with autoimmune diseases than in female control individuals. Therefore, we simultaneously examined skewed XCI in 106 patients affected with RA and 145 patients affected with AITDs. The control group consisted

Table 2

Characteristics of the patients with rheumatoid arthritis and skewed X-chromosome inactivation

Patient	Birth date	Disease onset	Pregnancy history	RF status	ACPA status	Other autoimmune disease	immunosuppressive therapy
90+% skewing							
1	1949	48	G7, P4, A3	+	+	GSG	MXT
2	1954	42	G5, P4, A1	+	+	-	Plaquenil
3	1945	52	G7, P7, A0	-	-	GSG	-
4	1946	40	G3, P2, A1	+	-	-	-
5	1956	30	G2, P2, A0	-	-	-	-
6	1946	40	G3, P2, A1	-	+	GSG	-
7	1945	40	G4, P4, A0	+	-	-	-
8	1945	39	G5, P5, A0	-	+	-	-
9	1941	49	G7, P5, A2	+	+	-	MXT
10	1947	49	G4, P3, A1	+	-	GSG	-
11	1945	58	G4, P2, A1	-	+	GSG	MXT
12	1950	40	G3, P2, A1	+	+	GSG	MXT
13	1943	53	G3, P3, A0	+	-	-	-
14	1961	35	G2, P1, A1	+	-	-	-
15	1937	38	G4, P4, A0	-	-	-	-
16	1941	45	G5, P3, A1	+	+	-	-
17	1947	43	G3, P2, A0	-	+	GSG	-
80 to 89% skewing							
18	1959	42	G5, P5, A0	+	+	-	MXT
19	1940	62	G0, P0, A0	-	-	-	-
20	1938	60	G9, P8, A1	+	+	GSG	MXT
21	1954	27	G0, P0, A0	+	+	GSG	-
22	1957	37	G5, P5, A0	+	+	GSG	-
23	1948	55	G9, P7, A0	+	-	-	MXT
24	1948	55	G0, P0, A0	-	-	-	-
25	1937	50	G3, P2, A1	-	+	GSG	-
26	1985	14	G0, P0, A0	+	-	-	-

A = spontaneous abortions; ACPA = anti-citrullinated protein/peptide antibodies; G = number of pregnancies; GSG = Sjögren's syndrome; MTX = methotrexate; P = para (pregnancies carried to term and delivered); RF = rheumatoid factor.

of 170 female age-matched healthy individuals. We have demonstrated a significantly higher prevalence of extremely skewed XCI in blood cell of females affected with RA and AITDs compared with the control group ($P < 0.0001$; $P = 0.0015$, respectively), indicating a possible role of XCI in the etiology of autoimmune diseases, and in the female preponderance of RA and AITDs.

Skewed XCI was more commonly expected in peripheral blood mononuclear cells due to the very high rate of turnover of blood cells compared with other solid tissues [25]. Then, we have examined XCI in peripheral blood mononuclear cells of patients affected with RA and AITDs, and we found a higher incidence of skewed XCI in those patients. We also tested the relationship between XCI and AITDs phenotypes (GD and HT). A skewed XCI was associated with both GD and HT ($P = 0.0001$ and $P = 0.04$). Although, our results suggest the involvement of XCI in female predisposition to RA and AITDs, this hypothesis still to be confirmed in specific tissue, because our analysis was performed in DNA from blood, and this may

Table 3

Characteristics of the patients with autoimmune thyroid diseases and skewed X-chromosome inactivation

Patient	Birth date	Disease onset	Pregnancy history	Diagnostic	Auto antibodies
90+% skewing					
1	1978	22	G1, P1, A0	HT	+
2	1933	65	G11, P5, A0	HT	+
3	1969	20	G1, P1, A1	HT	+
4	1938	60	G3, P3, A0	GD	+
5	1943	45	G2, P2, A0	GD	+
6	1972	21	G2, P2, A0	HT	+
7	1964	36	G2, P2, A0	HT	+
8	1924	65	G9, P9, A0	HT	+
9	1940	59	G10, P10, A0	HT	+
10	1969	28	G4, P4, A0	GD	+
11	1979	20	G0, P0, A0	GD	-
12	1931	68	G13, P13, A0	HT	-
13	1943	59	G1, P1, A0	GD	-
14	1946	42	G2, P2, A0	GD	-
80 to 89% skewing					
15	1969	23	G2, P2, A0	HT	+
16	1950	41	G3, P2, A1	GD	+
17	1980	20	G0, P0, A0	HT	+
18	1945	54	G4, P3, A0	HT	+
19	1962	36	G7, P2, A5	HT	+
20	1954	48	G3, P3, A0	GD	+
21	1984	20	G0, P0, A0	GD	+
22	1952	45	G2, P2, A0	GD	-
23	1941	58	G5, P5, A0	HT	-
24	1953	39	G2, P2, A0	GD	-
25	1947	48	G1, P1, A0	HT	-
26	1969	43	G2, P1, A1	GD	-

A = spontaneous abortions; G = number of pregnancies; GD = Graves' disease; HT = Hashimoto's thyroiditis; P = para (pregnancies carried to term and delivered).

not be a representative tissue for all autoimmune diseases [26,27] and there may exist locally skewed XCI in the thymus. Moreover, this study can be complicated by existing differences in peripheral blood mononuclear cells constituents in RA versus healthy controls. The XCI distribution in both Tunisian and Turkish controls (Figure 3) according to age showed that 19.5% (9 of 46) have a skewed XCI in Tunisian controls which have a mean age of 43.5 years, whereas only 8% (10 of 124) in Turkish controls with a younger mean age (35 years). This result suggests the importance of age in the difference of XCI skewing.

Our results are in agreement with those reported by Ozçelik and colleagues on 110 unrelated Turkish female AITDs patients and 160 female controls that showed a greater proportion of a skewed pattern of XCI (34%) than in controls (8%; $P < 0.0001$) [13]. Indeed, supporting data have been reported by Brix and colleagues, which assessed that the prevalence of skewed XCI in female twins affected with AITDs was 34% but

only 11% in controls ($P = 0.003$) and by Yin and colleagues ($P = 0.004$) [12-14]. Similar positive result was described in other autoimmune diseases such as scleroderma [11]. In addition, our results are the first report that describes a significant association between extremely skewed XCI and RA. Conversely, examination of XCI pattern of 58 Caucasian female patients affected with multiple sclerosis, 46 with systemic lupus erythematosus, 18 with juvenile RA and 45 with type 1 diabetes mellitus and 30 healthy women did not reveal skewed XCI patterns [28]. Despite extensive efforts of XCI analysis in different autoimmune diseases and populations, this hypothesis remains to be confirmed because there is no apparent autoimmunity directed against protein antigens encoded on the X chromosome and the fact that, for many autoimmune diseases, we found a female predominance in inbred mice models having two identical X chromosomes and therefore no 'foreign' antigens from the XCI [29].

In humans, it was reported that XCI process was genetically controlled by genes located on X chromosome [30]. It has also been suggested that genes on the X chromosome might show linkage with AITD and RA [31,32]. Thus, the observed association between skewed XCI and AITD and RA is not causal but could be explained by linkage disequilibrium between mutation responsible for XCI process and AITD and RA susceptibility polymorphisms. In addition, numerous environmental risk factors such as tobacco smoking, hormones, diet, drugs, toxins and/or infections are important in determining whether an individual will develop autoimmune diseases [33]. In fact, environmental agents are able to amplify autoimmunity in genetically susceptible individuals and to break tolerance in genetically resistant individuals, there by increasing the risk of developing autoimmune diseases [34]. The interaction between genetic and environmental factors remains to be achieved in order to evaluate the involvement of each component in the development of such autoimmune reactions.

Conclusions

We suggest a possible role of XCI mosaicism in the pathogenesis of RA and AITDs. However, the process of XCI needs to be considered as a potential factor in the predominance of females in most autoimmune diseases. It would also be of interest first to study the XCI pattern in females affected with other autoimmune diseases and second to test the XCI patterns of many cell types.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

GC carried out the molecular genetic study, performed the statistical analysis and wrote the manuscript. EU participated in the experimental work and the statistical analysis. AM participated in the design of the study and helped to draft the manuscript. AR participated in the statistical analysis. MM made pathological diagnosis and performed clinical data analyses. CAM participated in the molecular genetic study. ZB made pathological diagnosis, conducted sampling procedures, and performed clinical and rheumatological data analyses. TO conceived of the study, and participated in its design and coordination and helped to draft the manuscript. HA participated in the coordination of the study and revised the manuscript. All authors read and approved the final manuscript.

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ARTICLE

Evidence from autoimmune thyroiditis of skewed X-chromosome inactivation in female predisposition to autoimmunity

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The etiologic factors in the development of autoimmune thyroid diseases (AITDs) are not fully understood. We investigated the role of skewed X-chromosome inactivation (XCI) mosaicism in female predisposition to AITDs. One hundred and ten female AITDs patients (81 Hashimoto's thyroiditis (HT), 29 Graves' disease (GD)), and 160 female controls were analyzed for the androgen receptor locus by the *Hpa*II/polymerase chain reaction assay to assess XCI patterns in DNA extracted from peripheral blood cells. In addition, thyroid biopsy, buccal mucosa, and hair follicle specimens were obtained from five patients whose blood revealed an extremely skewed pattern of XCI, and the analysis was repeated. Skewed XCI was observed in DNA from peripheral blood cells in 28 of 83 informative patients (34%) as compared with 10 of 124 informative controls (8%, $P<0.0001$). Extreme skewing was present in 16 patients (19%), but only in three controls (2.4%, $P<0.0001$). The buccal mucosa, and although less marked, the thyroid specimens also showed skewing. Analysis of two familial cases showed that only the affected individuals demonstrate skewed XCI patterns. Based on these results, skewed XCI mosaicism may play a significant role in the pathogenesis of AITDs.

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Keywords: X chromosome inactivation; autoimmune thyroid disease; female predisposition to autoimmunity

Introduction

Hashimoto's thyroiditis (HD) and Graves' disease (GD) are autoimmune thyroid diseases associated with multiple genetic factors. Although the pathogenesis is poorly understood, a widely accepted model suggests an inherited background, which predisposes the subjects to autoimmunity. Additional intrinsic and extrinsic factors such as hormones and the environment may ultimately trigger or contribute to the development of the disease phenotype.[1] Extensive linkage genome screens during the past decade have resulted in the identification of several thyroid-specific susceptibility genes and/or loci, but confirmation through multiple population studies is still awaited for the majority of these loci.[1,2] A common feature of autoimmune diseases, including autoimmune thyroid diseases (AITDs), is an increased prevalence in women when compared with men. The most striking sex differences are

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observed in AITDs, scleroderma, Sjögren's syndrome, and systemic lupus erythematosus, which are diseases where over 80% of the patients are females.[3]

It has been demonstrated that risk of autoimmunity could be increased by a lack of exposure to self-antigens in the thymus and the presence of autoreactive T cells.[4–6] Disturbances in the X-chromosome inactivation (XCI) process provide a potential mechanism whereby the lack of exposure to self-antigens could occur,[7,8] including AITDs.[9,10] X-chromosome inactivation is a physiologic process that takes place in early female development and results in the transcriptional silencing of one of the pair of X chromosomes.[11] As a result of this epigenetic regulation, a random inactivation of the X chromosome inherited from either parent occurs and normal female subjects are thus a mosaic of two cell populations. It is therefore an attractive hypothesis that skewed XCI could lead to the escape of X-linked self-antigens from presentation in the thymus or in other peripheral sites that are involved in tolerance induction, inadequate thymic deletion, and finally loss of T-cell tolerance. Indeed, we recently observed skewed XCI in blood cells of women with scleroderma.[12]

Based on our observation that an association exists between skewed XCI and female predisposition to autoimmunity, we hypothesized that skewed XCI may be involved in the pathogenesis of AITDs, particularly in the hematopoietic compartment. We observed extremely skewed XCI in the blood samples of a significant proportion of female patients with AITDs.

Methods

Patients and pedigree analysis

Caucasian women diagnosed with AITDs ($n=110$), and healthy female controls with no history of autoimmune disease and cancer ($n=160$) were included in the study. Among the patients, 81 were diagnosed with HT and 29 with GD. The mean ages were 44.8 ± 14.1 (mean $\pm$ SD) years for AITDs (46 ± 14.2 years in the Hashimoto patients, and 40.6 ± 13.2 years in the Graves' patients), and 46 ± 10 for controls. The duration of the symptoms was 5.7 ± 7.4 years among the AITDs patients (5.7 ± 7 years in the Hashimoto patients and 6 ± 8.5 years in the Graves' patients). The mean age of diagnosis was 39 ± 12 years. All of the patients had attended the outpatient clinics of the Endocrinology and Metabolic Diseases Department of Ankara University School of Medicine for at least 1 year since the onset of disease. Patients were randomly chosen for the study.

All clinical investigations described in this manuscript were conducted in accordance with the guidelines in the Declaration of Helsinki (http://www.wma.net). The ethics review board of the participating institutions approved the study protocol. Informed consent was obtained from all subjects.

The diagnosis of HD was made by the existence of a firm goitre in combination with elevated thyroid auto-antibodies (thyroglobulin and/or thyroid peroxidase), a low ultrasonographic echogenity of the gland, and demonstration of lymphocytic infiltration by fine-needle aspiration biopsy and/or biochemical hypothyroidism. The diagnosis of GD was based on biochemical hyperthyroidism, and a diffuse symmetrical goitre in combination with positive thyroid antibodies (thyroglobulin, thyroid peroxidase or TSH receptor). In addition, thyroid ophthalmopathy and/or diffuse hyperplasia on an isotope scan or ultrasonography demonstrating homogenous echo texture may accompany the clinical picture.

Following the XCI studies, a complete pedigree analysis was carried out for 64 individuals informative for the AR polymorphism with medical follow-up of reported AITDs among family members when possible. Owing to emigration or unwillingness to contribute family information, data could not be obtained from the remaining 19 participants. Family history of AITDs was determined by reviewing the probands' pedigree to determine the number of relatives affected by these autoimmune diseases. Only first- and second-degree relatives were counted. A positive family history was noted if one additional AITD was documented by medical review.

X-chromosome inactivation analysis

Genotyping of a highly polymorphic CAG repeat in the androgen-receptor (*AR*) gene was performed to assess the XCI patterns as described elsewhere.[12,13] Densitometric analysis of the alleles was performed at least twice for each sample using the MultiAnalyst version 1.1 software. A corrected ratio (CrR) was calculated by dividing the ratio of the predigested sample (upper/lower allele) by the ratio of the nonpredigested sample for normalization of the ratios that were obtained from the densitometric analyses. The use of CrR compensates for preferential amplification of the shorter allele when the number of PCR cycles increases.[14] A skewed population is defined as a cell population with greater than 80% expression of one of the *AR* alleles. This corresponds to CrR values of <0.33 or >3.

Haplotype analysis

Human MapPairs Version 10 purchased from Research Genetics (currently available by Invitrogen, CA, USA) was used to screen the X chromosome. Site-specific PCR, 6% polyacrylamide gel electrophoresis, and silver staining techniques were used for genotyping the individuals. Gels were manually pictured and genotyped. Cyrillic program (version 2) was used to generate the haplotypes. A total of 27 X-chromosome-specific DNA markers from the MapPairs Panel were genotyped. Map order and physical positions (Mb) of the additional polymorphic DNA markers were obtained from USCS genome browser (The University of California Santa Cruz, CA, USA http://genome.ucsc.edu/).

Statistical methods

The results from control and test groups in XCI studies were compared by χ^2 test with Yate's correction.

Results

PCR-based X-inactivation study of peripheral blood

XCI status was informative in 83 of the 110 AITDs patients and in 124 of the 160 controls. Some heterozygous individuals were considered uninformative since only those whose alleles resolve adequately for densitometric analyses were included in the study. Skewed XCI (>80% skewing) was observed in 28 of the 83 patients (34%), and 10 of the 124 controls (8%) ($P<0.0001$). When the data for the two groups of AITDs patients was analyzed independently, 23/67 (34.33%, $P<0.0001$) of the Hashimoto's patients and 5/16 (31.25%, $P=0.0167$) of the Graves' patients were found to display the skewed XCI in blood. More importantly, extremely skewed XCI, defined as >90% inactivation of one allele, was present in 16 patients (19%), and in only three controls (2.4%, $P<0.0001$) (see Table 1). Extremely skewed XCI is a rare event in the general population. It has been reported in only 1–2% of women aged 20–40 years, and in 2–4% of women aged 55–72 years.[15,16] The distribution of XCI skewing in the general population is thought to be mainly due to chance deviations from 50:50 as a result of the limited number of embryonic cells present (4–20) at the time of XCI.[17] Age alone is unlikely to influence the strikingly bimodal data in our AITDs patients (Figure 1). We did not observe a shift towards the skewed range in older patients and controls.

PCR-based X-inactivation study of thyroid biopsy, buccal mucosa, and hair follicle specimens

Thyroid biopsy, buccal mucosa, and hair follicle specimens were obtained from five patients (04-121, 04-198, 04-214, 04-221, and 04-225). Their blood XCI profile displayed almost exclusive representation of only one allele of the *AR* polymorphism in their methylation-sensitive PCR assay, which indicates extremely skewed XCI. Five randomly selected patients showed skewing in the same direction for all tissues, except hair follicle, that in the thyroid being less marked than blood and buccal cells (Figure 2). Hair follicle specimens had a random XCI pattern. The allele ratios are given in Table 2.

Table 1 Proportion of patients and controls with skewed X-chromosome inactivation

Degree of skewing (%)	*No. (%) observed with skewing*	
	Autoimmune thyroiditis (n = 83)	*Control females (n = 124)*
90+	16 (19.27)	3 (2.41)
80–89	12 (14.45)	7 (5.64)
70–79	6 (7.22)	22 (17.74)
60–69	16 (19.27)	29 (23.38)
50–59	33 (39.75)	63 (50.80)

For comparison by χ^2, $P<0.0001$ (>80% skewing); $P<0.0001$ (90+% skewing).

Pregnancy history and pedigree analysis

Characteristics of the AITDs patients with skewed and random XCI are shown in Table 3. Only those patients with a complete pregnancy and family history are included in this table. The pedigrees of many AITDs probands with skewed XCI *versus* those with random XCI were interesting in two aspects. First, recurrent spontaneous abortions (defined as three or more pregnancy losses), which have been shown to be associated with skewed XCI,[16,18] occurred in four of 25 (16%) of our AITDs probands with skewed XCI. Conversely, a history of recurrent spontaneous abortions was negative both in the patients with random XCI and in the control group subjects ($P<0.0199$). Although the etiology of recurrent abortions in thyroid autoimmunity remains unknown, women who present with thyroid antibodies in the first trimester of pregnancy have a two- to four-fold increase in their miscarriage rates.[19]

Second, a positive family history, particularly in the skewed group, was apparent (12/25, 48% in the skewed; and 10/39, 25.6% in the random groups). We therefore contacted all of the 12 probands in an attempt to extend the X-chromosome inactivation studies to other family members. Initially, a positive response was received from three families, but blood samples could be obtained from

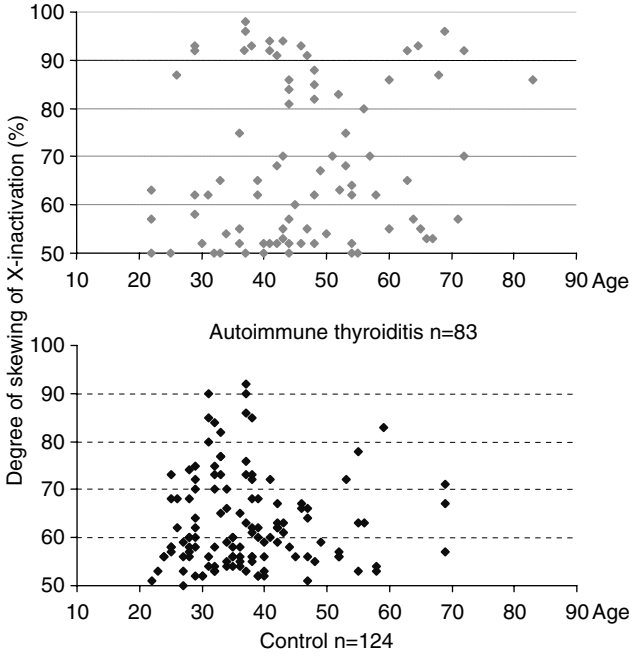

Figure 1 Distribution of X-inactivation patterns according to age in AITDs patients and control subjects.

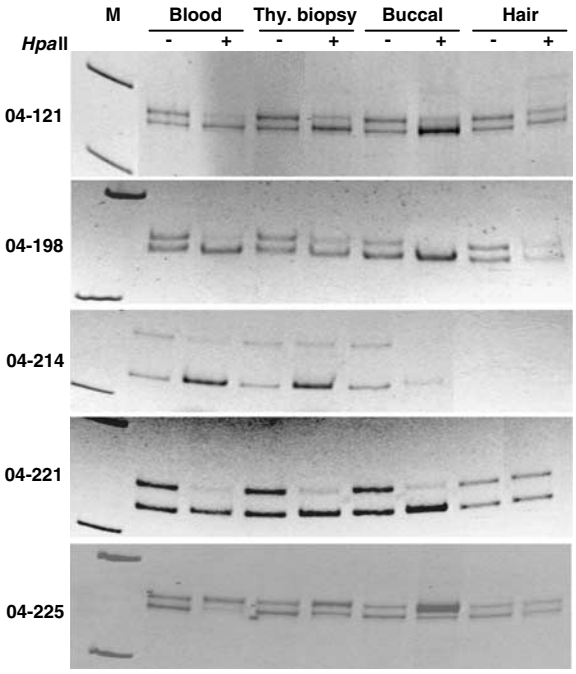

Figure 2 X-inactivation analysis of androgen receptor locus. PCR products of undigested (−) and *Hpa*II-digested (+) DNA from peripheral blood, thyroid biopsy, buccal, and hair follicle samples of AITDs patients 04-121, 04-198, 04-214, 04-221, and 04-225 are shown. Two alleles are seen in undigested samples, whereas a single allele resulting from extremely skewed XCI is clearly visible in all peripheral blood samples. Allele ratios are given in the text and in Table 2. M: marker (pUC mix 8), 331 and 242 bp fragments are visible.

the family members of only two probands (04-445, Family 1; and 04-298, Family 2). An important observation emerges from a study of these families: only the affected individuals demonstrate skewed XCI patterns. For example, XCI is extremely skewed in the affected sister and mother of 04-445 (Family 1), but random in the two unaffected sisters. The inactive X chromosome here is of maternal origin. In patient 04-298 (Family 2), skewing in the 80–89% range is noted for her affected sister, but unfortunately her mother was not informative for the AR polymorphism. Interestingly, the inactive X chromosome appears to be of paternal origin in Family 2 (Supplementary Figures 1 and 2).

Haplotype analysis

Because XCI segregates as a heritable trait associated with the disease in two generations of Family 1, we performed haplotype analysis by using polymorphic X-chromosomal markers to determine possible segregation between the

Table 2 X-chromosome inactivation patterns in blood, thyroid, buccal mucosa, and hair follicle specimens

Sample	*04-121*	*04-198*	*04-214*	*04-221*	*04-225*
Blood	94:6	91:9	84:16	92:8	91:9
Thyroid	72:28	79:21	76:24	74:26	64:36
Buccal	86:14	97:3	87:13	89:11	82:18
Hair	60:40	50:50	(−)	59:41	52:48

Table 3 Characteristics of the patients who are informative for X-chromosome inactivation status

Patient	*Birth date*	*Disease onset*	*Pregnancy history*	*Sex and birth date of children*	*Family history of first-degree relatives*
90+% skewing					
1 04-136[a]	1975	2004	G0,P0,A0	(−)	(−)
2 04-127[b]	1975	2003	G0,P0,A0	(−)	(−)
3 04-138[b]	1962	1980	G7,P0,A7	(−)	Two sisters
4 04-298[b]	1979	2003	G1,P1,A0	F03	Mother, one sister
5 04-445[b]	1961	2000	G1,P1,A0	F88	Mother, one sister
6 04-198[b,c]	1935	2004	G2,P2,A0	M68,M72	(−)
7 04-221[b,c]	1958	2000	G4,P2,A2	M91,F94	Two sisters
8 04-250[b]	1967	1996	G7,P2,A5	M89,M93	One son
9 04-226[b,c]	1963	2004	G3,P3,A0	M83,F88,M98	One sister
10 04-233[a]	1967	1990	G4,P3,A1	M88,F94,M01	(−)
11 04-121[b,c]	1957	1988	G7,P4,A3	F78,M83,F91,F94	(−)
12 04-205[b]	1927	2003	G6,P5,A1	M47,M50,F52,F53,M55	(−)
13 04-225[b,c]	1936	1975	G6,P6,A0	F56,F58,F60,F62,M64,M66	One daughter
80–89% skewing					
14 04-132[b]	1960	2002	G0,P0,A0	(−)	Mother, one sister
15 04-223[b]	1956	1988	G0,P0,A0	(−)	Mother, one sister
16 04-105[a]	1978	1999	G5,P1,A4	M98	(−)
17 04-131[b]	1944	2002	G3,P2,A0	M69,M71	(−)
18 04-120[b]	1956	1994	G3,P3,A0	F75,M77,F78	(−)
19 04-107[b]	1948	1998	G4,P3,A1	F70,F72,F76	(−)
20 04-98[b]	1956	2000	G8,P3,A1	F79,F81,F87	Two daughters
21 04-218[b]	1941	1991	G4,P3,A1	M61,F63,F67	(−)
22 04-108[b]	1952	1999	G5,P3,A2	F77,F78,M83	(−)
23 04-208[b]	1960	1999	G5,P3,A0	F83,F85,M88	Mother

Table 3 (*Continued*)

Patient	*Birth date*	*Disease onset*	*Pregnancy history*	*Sex and birth date of children*	*Family history of first-degree relatives*
24 04-110[a]	1960	1998	G4,P4,A0	M80,M83,M85,F96	(−)
25 04-214[b,c]	1921	1999	G9,P8,A1	F44,M45,F47,F48,M54,F56,F58,M60	One daughter
70–79% skewing					
26 04-203[b]	1961	2004	G3,P1,A0	M82	(−)
27 04-230[b]	1951	1999	G2,P2,A0	M77,M86	One son
28 04-213[b]	1947	1998	G7,P3,A0	F67,M68,M71	(−)
29 04-228[b]	1953	2001	G3,P3,A0	M71,M73,M82	(−)
30 04-137[b]	1932	1981	G5,P4,A0	F50,F53,M55,F59	(−)
60–69% skewing					
31 04-206[a]	1946	1964	G1,P0,A1	(−)	(−)
32 04-92[b]	1971	1998	G3,P1,A0	F96	Mother
33 04-240[b]	1975	2003	G2,P1,A0	M00	(−)
34 04-139[b]	1959	2002	G1,P1,A0	M97	(−)
35 04-257[b]	1973	2004	G3,P2,A1	M95,M01	(−)
36 04-112[b]	1952	1999	G2,P2,A0	F72,F77	Mother
37 04-220[a]	1955	1998	G3,P2,A0	M74,M78	(−)
38 04-103[b]	1962	1986	G6,P2,A1	F82,M92	(−)
39 04-251[b]	1941	1984	G5,P3,A1	M60,M63,M65	Two sisters
40 04-99[b]	1961	1997	G6,P3,A2	F81,F85,F87	Mother, one sister
41 04-224[a]	1950	2001	G6,P5,A1	M69,M72,F73,M75,F78	(−)
50–59% skewing					
42 04-96[b]	1939	1996	G0,P0,A0	(−)	(−)
43 04-242[b]	1960	1999	G0,P0,A0	(−)	(−)
44 04-129[b]	1982	1998	G0,P0,A0	(−)	Mother
45 04-196[b]	1956	1999	G6,P1,A0	F93	(−)
46 04-231[b]	1964	2003	G1,P1,A0	M93	(−)
47 04-201[a]	1971	2001	G2,P1,A1	F93	Mother
48 04-95[b]	1975	2004	G3,P2,A0	F98,M00	(−)
49 04-239[b]	1951	2003	G3,P2,A0	M68,M75	(−)
50 04-246[b]	1961	1996	G2,P2,A0	M78,F81	(−)
51 04-200[b]	1954	1992	G5,P2,A0	M73,M75	Three sisters
52 04-237[b]	1970	2004	G2,P2,A0	M93,F97	(−)
53 04-102[b]	1964	2003	G3,P2,A1	F95,F87	(−)
54 04-204[b]	1949	2002	G4,P2,A0	F71,M73	(−)
55 04-93[a]	1960	2003	G2,P2,A0	M91,F94	(−)
56 04-116[a]	1960	2003	G4,P2,A0	F86,M89	One brother
57 04-197[b]	1961	1976	G6,P3,A2	M82,M84,M98	(−)
58 04-229[b]	1938	1980	G3,P3,A0	M61,M63,F65	One sister
59 04-255[b]	1974	1993	G3,P3,A0	F92,M96,F01	(−)
60 04-212[a]	1958	2002	G4,P3,A0	M76,F80,M84	(−)
61 04-238[b]	1939	2002	G6,P4,A0	M61,M65,F67,M72	(−)
62 04-117[b]	1944	2004	G6,P4,A0	M60,M64,F66,F67	(−)
63 04-211[a]	1950	2002	G6,P6,A0	F66,F67,F72,M84,M85,M86	(−)
64 04-243[a]	1937	1994	G12,P7,A1	M57,M59,F60,F62,M65,M67,F68	(−)

G, number of pregnancies; P, para (pregnancies carried to term and delivered); A, spontaneous abortions.
[a]Graves' disease.
[b]Hashimoto's thyroiditis.
[c]Patients from whom thyroid biopsy samples were obtained.

disease and marker alleles. Although the size of this family is not large enough to prove linkage, it still provides valuable information about the exclusion area on the X chromosome. This helps to define a minimal critical region on the X chromosome, which might be associated with AITDs. Xp11-q13 (GATA144DO4, DXS7132, and AR) and Xp22 DNA markers (DXS8022, DXS987, and DX9902) showed concordance among the affected individuals indicating positive segregation between the disease and marker alleles. The haplotype structure is shown in Figure 3. However, lod score[20] analysis did not allow formal acceptance of linkage to any loci mainly due to the small size of the family.

Discussion

The autoimmune diseases include more than 70 chronic disorders that affect approximately 5% of the population. A reduction in sex ratio (male:female) is characteristic of most such diseases, including AITDs.[3] Even though the

female prevalence of autoimmune diseases has been recognized for over a hundred years, candidate mechanisms that could be important in pathogenesis have been uncovered only during the past two decades. These include genetic traits associated with autoimmunity,[21] pregnancy-related microchimerism,[22] and disturbances in XCI mosaicism in female subjects.[12] In this study, we demonstrate skewed XCI patterns in peripheral blood mononuclear cells of a significant proportion (34%) of female subjects with AITDs. Approximately 8% of female control subjects demonstrate skewed X-inactivation patterns $\geqslant$80:20, which is consistent with previous estimates.[16,18,23] The effect is more pronounced at patterns of X-inactivation $\geqslant$90:10; nearly 20% of AITDs patients show such skewing (Supplementary Figure 3), compared with only a few percent of female control subjects. Our results show that factors associated with extremely skewed XCI could account for a significant proportion of female patients with AITDs.

Skewed XCI is a result of primary or secondary causes. The former is bias in the initial choice of which X chromosome is inactivated due to germline *XIST* (X-inactive-specific transcript) mutations.[24] The secondary causes are deleterious X-linked mutations, X chromosome rearrangements, aging, twinning, or monoclonal expansion of cells (for a review, see Brown[25]). We believe that deleterious X-linked mutations or X chromosome rearrangements and their differential expression patterns could provide a disadvantage to blood and buccal cells, and possibly to thyroid cells in AITDs patients, and lead to skewed XCI. This has been supported by our observation that maternally inherited skewed XCI profile accompanies the disease phenotype for our AITDs Family 1. We observed segregation between the disease and marker alleles with the DNA markers residing on the distal short arm and pericentromeric regions of the X chromosome in this family. Although examples of skewed X-inactivation segregating with a trait have been reported previously,[18,26] this is the first example in AITDs to the best of our knowledge. In a recently published study on a three-generation kindred, extreme skewing of X inactivation was documented in three female subjects who have hemophilia A.[26] Since the inactive X was always of paternal origin in affected female subjects, the authors concluded that skewing in the family resulted from an abnormality in the initial choice process. This prevented the X chromosome, which carried the mutant *FVIII* allele, from being an inactive X. In our Family 2 with two affected sisters, the inactive X chromosome was of paternal origin like the

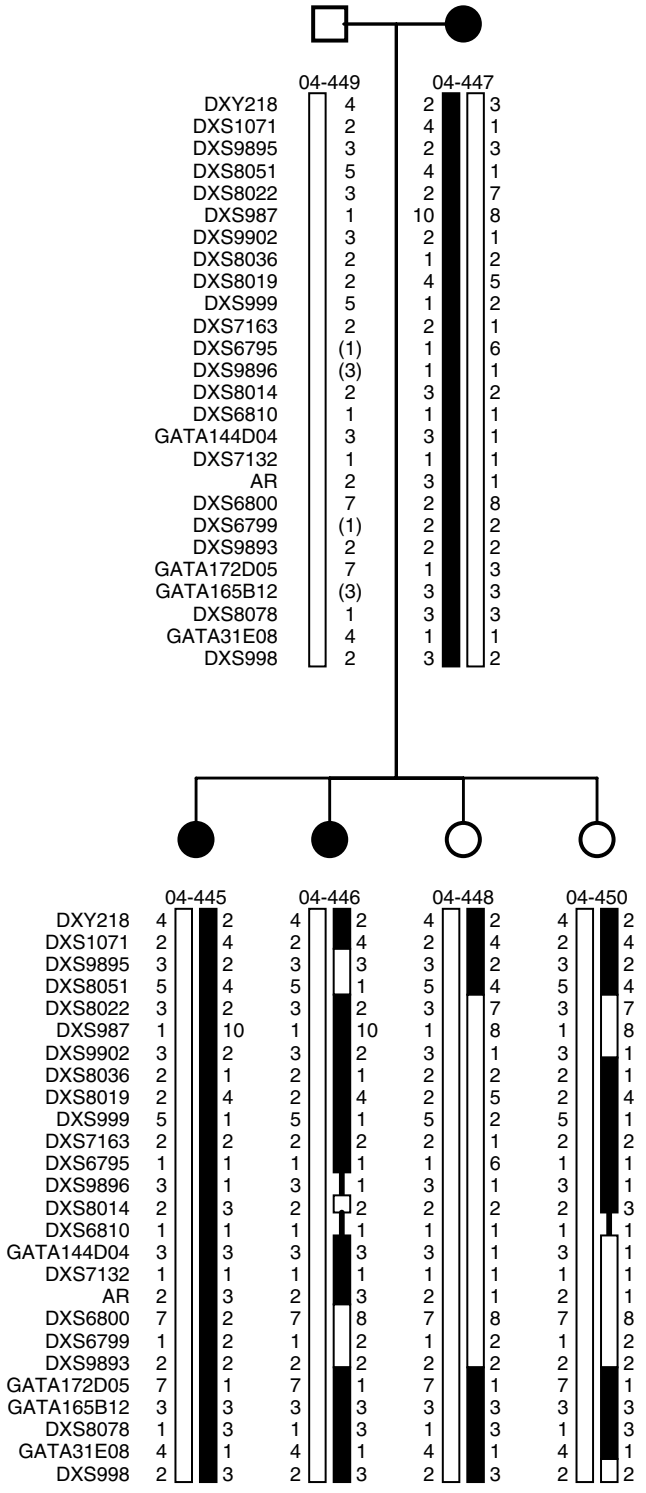

Figure 3 Haplotype structure of Family 1. Patient 04-445 was arbitrarily selected to construct the haplotype. Maternally inherited haplotype was highlighted with solid black bar. Haplotypes of the remaining sibs were compared with the reference individual (04-445), and shared portions were also marked with solid bars. Noninformativeness in the crossover regions were demonstrated with thin bars. The regions between the DNA markers DXS8051 and DXS8036 as well as DXS8014 and AR regions on Xp22 and Xp11-q13 regions, respectively, were not excluded since positive segregation between the disease and marker alleles was observed.

hemophilia A family. Extension of both the XCI and linkage studies to large cohorts with familial AITDs cases could prove to be very rewarding in understanding the relation between skewed XCI and autoimmune thyroidites.

Studies that aim to delineate the medical consequences of skewed X-inactivation have shown that clinical manifestation of X-linked disorders in female subjects could be influenced by disturbances in the XCI process.[27] In addition, it has been hypothesized that skewed XCI could be a factor that influences female predisposition to autoimmunity.[7,8] Now that we have demonstrated skewed patterns of XCI in a significant proportion of female AITD patients, deviation from the physiological range of XCI mosaicism could be considered as a potential mechanism contributing to disease pathogenesis. This is further supported by the recently reported observation that female twins with AITDs have a high frequency of skewed XCI.[28]

Although extremely skewed XCI is rare, it does not always lead to the development of AITDs. A subsequent event, such as environmental exposure to viral, chemical, or other agents may trigger a cascade that results in AITDs. In addition, the co-inheritance of genetic susceptibility factors, such as functional variants in vital negative regulatory molecules of the immune system,[29,30] may exacerbate the effects of skewed XCI and contribute to the development of autoimmune diseases including AITDs.

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Supplementary Information accompanies the paper on European Journal of Human Genetics website (http://www.nature.com/ejhg)