X-CHROMOSOME INACTIVATION IN FEMALE PREDISPOSITION TO AUTOIMMUNITY

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THE DEGREE OF DOCTOR OF PHILOSOPHY

BY

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MAY, 2008

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ABSTRACT

X-CHROMOSOME INACTIVATION IN FEMALE PREDISPOSITION TO AUTOIMMUNITY

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The high female preponderance is thought to be important in identifying the etiological factors. Sex hormones, pregnancy related microchimerism, and environmental factors are investigated as likely candidates. Disturbed X-chromosome inactivation (XCI) is another candidate, which may contribute to the break-down of self-tolerance. In this study, we tested the hypothesis that "loss of mosaicism" for X-linked gene expression may contribute to autoimmune disease etiology. Therefore, XCI status of healthy individuals and patients diagnosed with scleroderma (SSc), autoimmune thyroiditis (AITDs), Sjogren's syndrome (SICCA), and juvenile idiopathic arthritis (JIA) in the Turkish population were analyzed by genotyping the methylation status of a CAG polymorphism in the androgen receptor (*AR)* gene. Extremely skewed XCI was observed in a significant proportion of SSc (OR: 38.9; P<0.0001), AITDs (OR: 9.6; P<0.0001), and JIA (OR: 4.4; P=0.0022). Further genotyping of AITDs in Tunisian and SSc in the US population supported the initial observations (OR: 3.8; P=0.0046; OR: 3.8; P<0.0001) respectively. Analysis of rheumatoid arthritis (RA) in the Tunisian population suggests that extremely skewed XCI (OR: 6.7; P<0.0001) could be involved in disease pathogenesis. Moreover, pre-eclampsia, a disease in which autoimmunity may be important, skewed XCI was observed (OR; 11.7; P=0.0005). However, in SICCA random patterns of XCI was observed suggesting that extreme skewing is not a common feature of all female prevalent autoimmune disorders. In conclusion, our results suggest that extremely skewed XCI may be important factor in autoimmune disease pathogenesis.

Keywords: autoimmune diseases, X-chromosome inactivation, female predisposition, HUMARA assay.

ÖZET

X-KROMOZOMU İNAKTİVASYONU VE OTOİMMÜN HASTALIK İLİŞKİSİ

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Doktora Tezi, Moleküler Biyoloji ve Genetik
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Otoimmün hastalıklar dünya çapında en sık rastlanan hastalık gruplarından biridir. Hastalıklara göre değişmekle birlikte kadınlarda daha sık rastlanmaktadır. Nedeni tam olarak bilinmemekle birlikte cinsiyet hormonları, hamileliğe bağlı olarak gelişen mikrokimerizm ve çevresel faktörler kadınlarda sık görülme ile ilişkili olabilir. X-kromozomu inaktivasyonu (XCI) sapması önemli bir etyolojik faktör olabilir. Bu çalışmada XCI'na bağlı mozaik yapının bozulmasının otoimmün hastalık etiolojisinde rol alabileceği hipotezi test edilmiştir. Bu nedenle Türk populasyonunda skleroderma (SSc), otoimmün tiroiditis (AITDs), Sjogren sendromu (SICCA) ve juvenil idiopatik artrit (JIA) hastaları ve sağlıklı bireyler genotiplenmiştir. Androjen reseptör (*AR*) genindeki CAG polimorfizminin metillenme durumu incelenerek XCI statüsü belirlenmiştir. XCI'da aşırı sapma SSc (OR: 38.9; P<0.0001), AITDs (OR: 9.6; P<0.0001), ve JIA (OR: 4.4; P=0.0022) hastalarında görülmüştür. Buna ek olarak Tunus populasyonunda AITDs (OR: 3.8; P=0.0046) ve Amerikan populasyonunda SSc (OR: 3.8; P<0.0001) hastalarının genotiplenmesi ile bulgularımız desteklemiştir. Tunus populasyonunda romatoid artrit (RA) hastaları üzerinde yapılan inceleme sonucunda XCI'nun bu hastalıkta da aşırı saptığını göstermiştir (OR: 6.7; P<0.0001). Otoimmünitenin, hastalık etyolojisinde etkin olduğu düşünülen pre-eklampsi hastalarında da XCI sapması gözlenmiştir (OR: 11.7; P=0.0005). SICCA grubunda yürütülen incelemelerde ise XCI oranlarının kontrol grubu ile benzer olduğu saptanmıştır. Bu gözlem XCI oranlarının sapmasının tüm otoimmün hastalıklarda görülmediğini ortaya koymuştur. Sonuçlarımız XCI ile otoimmün hastalık gelişimi arasında bir ilişki olabileceği görüşünü desteklemektedir.

Anahtar kelimeler: otoimmün hastalıklar, X-inaktivasyonu, kadınlarda sık görülme, HUMARA

To my Family...

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ABBREVIATIONS

AIRE	autoimmune regulator
AITD	autoimmune thyroiditis
AKA	anti-keratin antibodies
ANA	antinuclear antibodies
APS	ammonium persulfate
APS1	autoimmune polyendocrine syndrome
AR	androgen receptor
AT1	angiotensin receptor
BBC1958	British birth cohort 1958
BSA	bovine serum albumin
bp	base pair
CCP	anti-cyclic citrullinated peptide
CD	cluster of differentiation
CI	confidence interval
CrR	corrected ratio
CTLA4	cytotoxic T lymphocyte antigen 4
DMSO	dimethyl sulfoxide
dNTP	deoxyribonucleotide triphosphate
DZ	dizygotic
EDTA	ethylenediaminetetraacetic acid
EtBr	ethidiumbromide

FMc	fetal microchimerism
FOXP3	forkhead box P3
G6PD	glucose-6-phosphate dehydrogenase
g	gram
GD	Grave's disease
GI	gastrointestinal
GVHD	graft versus host disease
h	hours
HCL	hydrochloric acid
HELLP	hemolytic anemia, elevated liver enzymes and low platelet count
HIV	human immunodeficiency syndrome virus
HLA	human leukocyte antigen
HPRT	hypoxanthine phosphoribosyl transferase
HT	Hashimoto's thyroiditis
HTLV-I	T-leukemia retrovirus-I
HUMARA	human andrgogen receptor assay
IL	interleukin
ILAR	The International League against Rheumatism
IPEX	immunodysregulation, polyendocrinopathy, and enteropathy, X-linked syndrome
JIA	juvenile idiopathic arthritis
IDDM	insulin dependent type I diabetes mellitus
MAGE	melanoma antigen family
MHC	major histocompatibility complex
mg	miligram

min	minutes
mM	millimolar
MMc	maternal microchimerism
mL	milliliter
MZ	monozygotic
µL	microliter
NDDM	non-insulin dependent diabetes mellitus
ng	nanogram
OMIM	online Mendelian inheritance in man
OR	odds ratio
PAGE	polyacrylamide gel electrophoresis
PAR	pseudoautosomal region
PBC	primary biliary cirrhosis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDC-E2	pyruvate dehydrogenase complex
PEE	pre-eclampsia
PIM	primary idiopathic myxodemia
PGK	phophoglyceraldehyde kinase
PTPN22	protein tyrosine phosphatase, non-receptor type 22
RA	rheumatoid arthritis
RF	rheumatoid factor
RFLP	restriction fragment length polymorphism
rpm	rotation per minute
RSA	recurrent spontaneous abortion

SD	standard deviation
sec	seconds
SICCA	Sjogren's syndrome
SLE	systemic lupus erythematosus
SNP	single nucleotide polymorphism
SRY	sex determining region Y
SSc	scleroderma
T3	triiodothyronine hormone
T4	thyroxine hormone
T1D	type 1 diabetes mellitus
TAE	tris-acetic acid-EDTA buffer
TEMED	N, N, N, N-tetramethyl-1-2, diaminoethane
TNF	tumor necrosis factor
TPO	thyroid peroxidase
TR	Turkish
TSIX	complementary transcript of XIST
TSH	thyroid stimulating hormone
TUN	Tunisian
UK	United Kingdom
US	United States
V	volt
VNTR	variable number tandem repeat
W	watt
WHO	World Health Organization
XCI	X-chromosome inactivation

XIC	X-inactivation center
XIST	X-inactive spesific transcript
XITE	X inactive transcript element

CHAPTER 1

INTRODUCTION

1.1 Immune system

The human body faces millions of pathogens and foreign invaders every day. Our immune system detects and eliminates antigens and pathogens by differentiating self antigens from foreigns. Immune system recognizes these foreigners by two mechanisms: Innate immune system and adaptive immune system. The former defends the host organism from infection by other organism in a non-specific manner. Innate immune system specifically recognizes antigenic structures of microbes via pattern recognition receptors. It is known that innate immune system is present both in animals and plants (Litman *et al.* 2005). In addition, the recognition is highly conserved during evolution among invertebrates and vertebrates (Klein 1999; Medzhitov&Janeway 2000). However, the specialized cells of adaptive immune system eliminate pathogen in a specific manner. Those cells (termed as T- and B cells) are equipped with receptors that provide the immune system with the ability to recognize and remember specific pathogens. This system is called as "adaptive", because a small number of products of genes are capable to recognize, eliminate and remember a vast number of different antigen receptors. The cells of this system achieve this process via somatic hypermutation and V(D)J recombination.

1.2 Self tolerance and autoimmunity

Immune system is body's defense system against "foreign" invaders. This system is programmed so that it can recognize and attack to the bacteria, viruses, antigens and proteins. Interestingly, immune system does its function with the ability of discriminating between self-and nonself antigens. Normal individuals are tolerant of their own antigens and this phenomenon is called self tolerance. It is the fundamental property of the immune system. Immunologic tolerance was recognized in the 1950s through a set of experiments performed by Peter Medawar and colleagues. In these studies, they observed that adult mouse of one type of strains rejected a skin graft from an allogeneic mouse of different strain. These two mice differ from each other at the major histocompatibility complex (MHC). They further continued the experiments by injecting the lymphocytes of mice of different strain, but this time during neonatal life. Interestingly, the injected cells were not rejected this time because the neonate became immunotolerant. After the neonate became an immunocompetent adult, skin grafts from all mouse strains whose MHC was different than of the strain whose lymphocytes were injected at neonatal stage were rejected (Medavar 1957). These experiments lead us to accept the concept that early exposure of developing lymphocytes to foreign antigens induces tolerance. A great effort has been dedicated to explain the mechanisms and to find a therapeutic improvements for inducing tolerance to prevent the rejection of organ allografts and xenografts. Moreover, understanding the basis of tolerance induction is important in treatment of autoimmune and allergic diseases. In mature lymphocytes, the mechanism of tolerance to foreign antigens is similar in many ways to those of self-tolerance. Differentiation between self-nonself proteins is called as "self tolerance". Self tolerance is divided into two classes: (1) central tolerance, in which immature lymphocytes recognize self antigens in generative lymphoid organs (bone marrow and thymus), and (2) peripheral tolerance in which mature lymphocytes were encountered to self antigens in peripheral lymphoid organs (spleen, lymph nodes). Central tolerance occurs in the generative lymphoid organs because the only antigens present in these organs at high titer are the self antigens. Normally foreign antigens that enter into the body should be already attacked by the peripheral lymphoid organs, therefore during generation of lymphocytes in the thymus or bone

marrow they normally encounter only self antigens. In this process, which is called as “negative selection”, lymphocyte clones that recognize self antigens with high affinity are eliminated. Peripheral tolerance is observed in peripheral tissues after the mature lymphoids leave the generative organs. When lymphocytes recognize antigens with a low level of costimulators in peripheral tissues, peripheral tolerance is induced. This type of unresponsiveness is necessary against self antigens that are expressed in peripheral tissues but not in generative lymphoid organs.

Tolerance against self antigens is provided by means of three types of mechanisms: (1) apoptotic cell death, also called as only “deletion”. This process is used mainly in central tolerance, (2) anergy; functional inactivation of lymphocytes without cell death, and (3) suppression of lymphocyte activation and effector function of lymphocytes. In peripheral tolerance, all of the three types of mechanisms are used. If those self-reactive lymphocytes that escape from tolerance cannot be eliminated, autoimmune disorders may develop (Goodnow *et al.*2005).

1.3 Autoimmune diseases

Autoimmune diseases are known to affect approximately 5 percent of the population in US and Europe (Sinha *et al*. 1990; Jacobson *et al.* 1997; Eaton *et al*. 2007). Clinicians classify autoimmune disorders as systemic and organ specific. In organ specific autoimmune diseases one organ is affected, whereas in systemic autoimmune disorders multiple organs or systems may be affected. Some of the examples of these two types of autoimmune diseases are indicated in Table 1.1

Table 1.1 Examples of systemic and organ specific autoimmune diseases.

Type	Name of Disorder	Affected Organ(s)
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

Genetic susceptibilities, environmental factors, and infectious agents may trigger autoimmune reactions. Epidemiological studies show that genetic susceptibility have a role in the formation of autoimmune disorders.

In simple diseases, the causative gene determines the disease state. However, like in autoimmune disorders, more than one gene may have role in the formation of complex diseases. There are only a few of the genetic traits that are associated with autoimmune disorders. *AIRE, CTLA4, FOXP3, and PTPN22* are the genes that are known to be involved in the formation of autoimmunity in *Homo sapiens* (Rioux&Abbas 2005).

AIRE (autoimmune regulator) gene encodes a 545-amino acid protein, responsible for the thymic expression of some antigens. Those antigens have high expression level in different peripheral tissues. Mutation in *AIRE* results in autoimmune polyendocrine syndrome (APS-1) (OMIM #240300) (Nagamine *et al* 1997; Björses *et al.* 2000). The autoimmune attacks are observed against multiple organs.

FOXP3 (forkhead box P3) gene encodes a transcription factor that belongs to the forkhead/winged-helix family. It was shown by Brunkow *et al.* that a frameshift mutation in *Foxp3* gene results in a protein lacking the forkhead domain in mice (scurfy mice). These mice are characterized by overproliferation of $CD4^{+}CD8^{-}$ cells, increased level of cytokines and multiorgan infiltration. In addition to the mouse model, a human disease known as IPEX (Immunodysregulation, Polyendocrinopathy, and Enteropathy, X-Linked Syndrome; OMIM #304790) has been shown to be caused by mutation in *FOXP3* gene (Benett *et al.* 2001; Wildin *et al.* 2001)

CTLA 4 (cytotoxic T lymphocyte antigen 4) protein, also known as CD152 is a member of immunoglobulin superfamily and expressed in activated T cells. CTLA 4 protein is an inhibitory receptor and binds to B7-1 and B7-2 on antigen presenting cells. It was reported by Ueda *et al* in 2003, that *CTLA-4* gene is associated with autoimmune diseases including Grave's disease, autoimmune hypothyroidism, and type 1 diabetes mellitus.

PTPN22 (protein tyrosine phosphtatase, non-receptor type 22) is also known as Lyp. The gene that codes for PTPN22 is located on chromosome 1 (1p13.3-p13.1). PTPN22 protein is involved in T-cell activation (Cohen *et al.* 1999). It is reported that R620W polymorphism on *PTPN22* increases the susceptibility in various autoimmune disorders including type I diabetes (Bottini *et al.* 2004), rheumatoid arthritis (Begovich *et al.* 2004), SLE (Kyogoku *et al.* 2004), and Grave's disease (Velaga *et al.* 2004).

In simple diseases, the causative gene is deterministic in disease state. Consequently, genome wide linkage studies demonstrate sharing of alleles between affected members of families, and are used to identify the causal genetic variant. However, the case in complex diseases is more complicated. Disease state is determined via interactions of multiple genotypes together with the environmental factors. Autoimmune disorders are a group of complex diseases. Even though a number of causative alleles are associated with certain autoimmune disorders, environmental factors are thought to have impact on disease susceptibility.

Molecular mimicry is one of the environmental factors. It is mainly based on the similarities between foreign and self antigens that are sufficient to produce an immune response. The mechanism remains puzzling. Bacteria, viruses, xenobiotics and chemicals are candidates for the initiation of autoimmune disorders by molecular mimicry. One example is in primary biliary cirrhosis (PBC). E2 component of human pyruvate dehydrogenase complex (PDC-E2) is the major autoantigen in PBC. It was proposed that exposure to a microorganism that express PDC similar to human PDC-E2 could act as a trigger of autoimmune reaction in PBC. *Novosphingobium aromaticivorans* was reported to posses a PDC-E2 like protein with high degree of homology to human PDC-E2. Exposure to this organism has been reported to a predisposing factor in PBC (Kaplan 2004).

Viral and bacterial infections are the other type of environmental factors. Local immune responses that recruit leukocytes into the tissues may be induced by infections of particular tissues by viruses or bacteria. This recruitment may result in the expression of costimulators on tissue APCs and finally in breakdown of self tolerance (Abbas&Lichtmann 2003).

1.4 Sex differences in autoimmune disorders

The high female:male prevalence is known to be associated in most autoimmune disorders. This ratio ranges from 2:1 in multiple sclerosis to 10:1 in autoimmune thyroid diseases (AITDs) (Chitnis 2000, Hernandez-Molina 2007). Some of the sex ratio according to the disease types is displayed on Table.1.2

Table 1.2 Gender prevalence ratios for selected autoimmune disorders (Whitacre 2001, Gleicher&Barad 2007, Hernandez-Molina *et al*. 2007).

Disease	Female:Male ratio
Autoimmune thyroid diseases	10:1-50:1
Primary biliary cirrhosis	9:1
Sjogren's syndrome	9:1
Systemic lupus erythamosus	8:1-9:1
Scleroderma	3:1-4:1
Rheumatoid arthritis	3:1
Multiple sclerosis	2:1
Myasthenia gravis	2:1
Type I Diabetes mellitus	1:1-2:1

Females produce higher immunoglobulin levels than age-matched males in response to infection or immunization. Moreover, it was noted that women have higher absolute number of $CD^{+}4$ lymphocytes compared to men. The tendency towards autoimmunity in females can be ascribed at first glance to hormonal differences. Hormones are distinctive chemicals between two sexes. It was shown that the acquired immune system of females differs from that of males. Estrogen stimulates immunologic reactions driven by $CD4^{+}$ T_H2 cells and B cells. However androgens perform this reaction through enhancement of $CD4^{+}$ T_H1 and $CD8^{+}$ cells (Whitacre *et al.* 1999, Beagley & Gockel 2003). In multiple sclerosis and rheumatoid arthritis, where female predominance has been observed, disease activity decreases during pregnancy, especially in the third trimester, where estrogen and progesterone levels are the highest (Nelson&Ostensen 1997, Confavreux *et al* 1998). In contrast, disease activity worsens or remains unchanged in SLE during pregnancy. Another important difference between two sexes is the pregnancy. Maternal cells remained in the blood of fetus or *vice versa* are called microchimerism. Presence of fetal cells is detected in the blood of a mother years after delivery (Bianchi *et al.* 1996). This may be

evidence that fetal cells are not effectively eliminated from maternal blood. But even microchimerism alone cannot explain the female preponderance in autoimmune diseases because there are many patients that give no birth. Moreover, the pediatric forms of certain autoimmune disorders cannot explain the female predominance due to hormones or microchimerism. X-chromosome inactivation is a biological regulation, which is observed only in females, and this may explain the female preponderance of autoimmune diseases.

1.5 Kast and Stewart Hypothesis

In 1977, Richard Kast offered an explanation for the high female/male ratio in many autoimmune diseases for the first time. In his publication, Kast listed autoimmune diseases with high female preponderance. He noticed that besides the environmental, hormonal and microchimerism effects, a novel mechanism other than classical patterns of inheritance may have a role in female predisposition to certain autoimmune diseases. One such mechanism is the disturbance in X-chromosome inactivation process. He hypothesized that any disturbance in female X-heterochromatinisation might have the potential to influence of the occurrence of autoimmune disorders with high incidence in women. Later in 1998, Jeffrey Stewart developed this hypothesis. In his publication, where he sets the similar hypothesis in systemic lupus erythematosus (SLE), Stewart argued that differences in the self-antigen presentation profiles to the immune system due to XCI mosaicism may be one of the factors that lie behind the female preponderance of the disease. Two different cell classes, differing in a subset of transcribed genes are present in females due to the XCI process. One cell population presents X-encoded genes inherited from the father, whereas the other half of the cell population transcribes those X-encoded genes inherited from mother. This hypothesis is the art of this study. Even though there are exceptions to this rule (escape from XCI), the self antigen proteins expressed from X-chromosome differ. Negative selection occurs in the thymus and mediated by antigen presenting cells (APCs), particularly the dendritic cells. If a T cell is autoreactive to an X-encoded antigen, and is tolarized only by one type of dendritic cell due to extremely skewed X chromosome inactivation pattern, that

autorective cell escape from negative selection and enter the circulation. In this case T cells may maturate and enter periphery without having been negatively selected to antigens that are encoded from predominantly inactive X chromosome. This situation is illustrated in Figure 1.1.

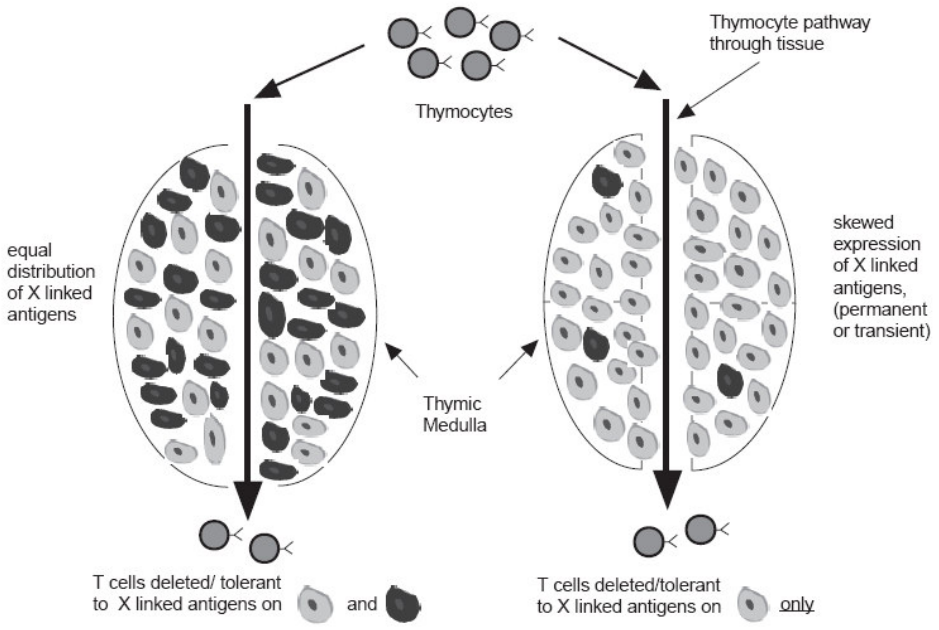

Figure 1.1 Skewed XCI and its consequences on tolerance induction in the thymus (Chitnis *et al.* 2000).

XCI patterns were examined in the blood cells of female patients with systemic lupus erythematosus, juvenile diabetes, multiple sclerosis, and juvenile rheumatoid arthritis. However no significant difference in XCI patterns between subjects and controls was observed (Chitnis *et al.* 2000). There may be two possible explanation: first the number of subjects and controls are relatively small, and second, there was only one control group for comparing XCI results of both adult and juvenile onset of autoimmune diseases. Subsequently, a case-control study on female twins

discordant for AITDs and healthy female twins was conducted in Danish population. The frequency of skewed XCI in AITDs twins was found to be higher than in the control group (Brix *et al.* 2005). In 2007, Miozzo *et al.* performed a similar study in primary biliary cirrhosis patients and difference in XCI patterns in mononuclear cells of patients and controls was not statistically significant (Miozzo *et al.* 2007). Moreover, X-chromosome monosomy has been manifested a common mechanism for autoimmune diseases (Invernizzi *et al.* 2005)

Previous studies with *AR* assay have reported that skewed XCI ratio increase with the age (Busque *et al.*1996, Sharp *et al.* 2000). These observations suggest that skewed XCI can be acquired with age. The extremely skewed XCI incidence was 1.9% in neonates, 4.5% in young women (28-32 years old), and 22.7% in old women (>60 years old) (Busque *et al.* 1996). Similar increase in the incidence of skewed XCI was observed with age. In 2000, Sharp *et al.* performed a similar study and observed an increase in the incidence of both skewed and extremely skewed XCI in women more than 60 years old.

1.6 X- Chromosome inactivation

1.6.1. Dosage compensation

Sex determination is evolved in different ways among species. Sex was determined in response to environmental stimuli in reptiles (Western&Sinclair 1999). However in majority of species sex determination is based on chromosome-based pathways. Chromosome-based pathways differ between species. Presence of a Y chromosome is sufficient in mammals do develop a male organism. However, in flies and worms, the ratio of the number of X chromosome per haploid autosome set is necessary for determining sex. Therefore, XX becomes female, and XY or XO becomes male in flies and worms respectively (Bridges 1921, Madl&Herman 1979). In mammals,

presence of Y chromosome leads to the formation of male organism due to the presence of sex determining region Y (*SRY*). In addition to *SRY*, Y chromosome harbours not more than 50 genes, whereas X chromosome encodes for approximately 1500 genes (Lahn *et al.* 2001, Ross *et al.* 2005). Due to the gene content differences between the two chromosomes and sex determination pathway, dose difference of X chromosome gene product levels arise between the two sexes. Dosage compensation mechanisms based on chromosome-wide regulation. They were developed to compensate for the dose differences. Strategies of dosage compensation evolved in several different ways (Figure 1.2). Flies increase the expression level of X chromosome twofold (Park&Kuroda 2001), while worms halved the transcription levels of each of X chromosomes (Meyer&Casson 1986, Blackwell&Walker 2002). Transcriptional silencing of one of the two X chromosome occurs in female cells of mammals (Lyon 1961).

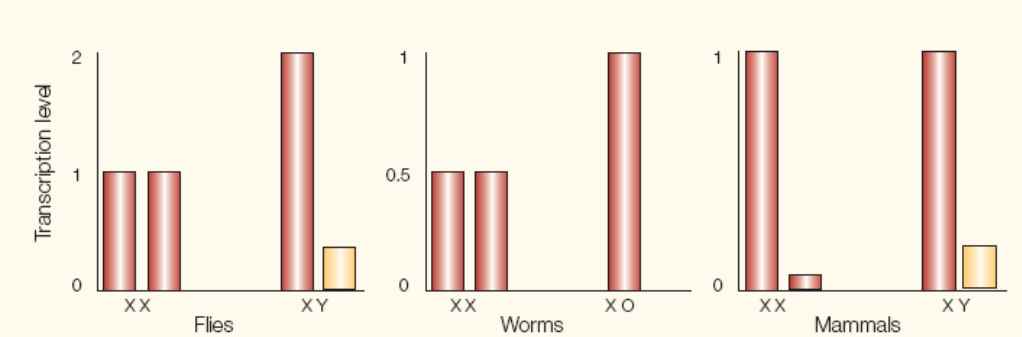

Figure 1.2 Strategies of dosage compensation. In *Drosophila melanogaster*, male (XY) X-linked expression increases twofold. In *Caenorhabditis elegans*, hermaphrodite (XX) transcription from each of the two X chromosomes decreases twofold. In mammals, one of the two X chromosomes in females is inactivated (Huynh&Lee 2005).

1.6.2. Mechanism of X-Chromosome inactivation

In mammals, dosage compensation for X-encoded gene products between females and males is achieved by silencing of the two X-chromosomes in female cells and first hypothesized by Lyon in 1961. The inactivation takes place during early

development. In female eutherians, paternal and maternal X chromosomes were randomly affected from X-Chromosome inactivation (XCI) process. This inactive state gives rise to female adults that are mosaic for two cell types expressing one of the X chromosomes and stably inherited. Inactive X chromosome is observed as 'Barr body' during interphase under microscope (Barr 1949).

Recent experiments show that XCI occurs as early as the four cell stage of the embryo but is variable and leaky and does not become fixed until after implantation but before differentiation of ES cells (Huynh&Lee 2003, Okamoto *et al* 2004). There are two consequences of X dosage compensation in humans: (1) only one X chromosome functions in all cells of both sexes, irrespective of the number of X chromosomes; and (2) females are cellular mosaics. Those mechanisms are observed in eutherians. The mosaic pattern is not observed in marsupials. They inactivate only paternal X (VandeBerg *et al.* 1987). One of the interesting finding is the pattern observed in mice. Even though the XCI pattern is random in fetus, only paternal X is inactivated in extra embryonic tissues (Takagi&Sasaki 1975, Cooper 1971).

How does XCI work? Scientists have dealed with this question for decades. Even though the detailed mechanisms for all steps are not known entirely, it is well established that four steps are crucial: counting, selection, silencing and maintenance. For X inactivation to occur cells must posses at least two XCI center (*XIC*). This locus is located in Xq13 in humans and has syntheny with murine and mouse. Studies on mouse models reported that *Xic* regulates counting, selection and silencing. The first step begins with the determination of the number of X chromosomes per cell (counting). Only a single X chromosome per diploid autosome set will remain active and the remaining extra copies being inactivated. *Xic* is involved in selection process. The choice of either paternal or maternal X chromosome for subsequent inactivation is random. Recent studies in mice showed that three non-coding loci are necessary for the XCI process. These are *Xist, Tsix*, and *Xite.* They are located within *Xic* (Brown *et al.* 1992). These three elements act in *cis* during inactivation. From these elements, *Xist* is necessary for *cis* inactivation of X chromosome (Clemson *et al.* 1996). Experiments showed us that if *Xist* is deleted, X chromosome cannot be inactivated in mice. Those data show us that *Xist*

is primarily necessary for inactivation. Even though the exact mechanism is not known entirely, a non-coding RNA transcribed from Xist coats the future inactive X chromosome. In order to test the crucial role of *Xist* in inactivation, Lee and colleagues (Lee *et al.* 1996) transfected it onto an autosomal chromosome in mouse embryonic stem cells at a critical time and observed that it inactivated the autosome. How does *Xist* alone determine inactivation of a chromosome? Recent studies show that *Xist* alone is not involved in inactivation. An antisense strand, called *Tsix* encodes also a non-coding RNA that is complementary to *Xist*. *Tsix* is transcribed from the active X. Due to this feature of *Tsix*, *Xist* is inactivated in the subsequent active X chromosome (Lee *et al.* 1999). Any targeted deletion/insertion that abolishes *Tsix* transcription, results in *Xist* RNA accumulation. This demonstrates that *Tsix* has a repressive effect on *Xist*. However, it is still not clear whether *Tsix* transcript, or the act of transcription, or both, that are involved in inactivation process (Nesterova *et al.* 2003). *Tsix* expression is regulated by bipartite enhancer, including *Xite* and *DXPas34*.Tsix and *Xite* mediate together counting and choice of XCI. After determination, they regulate *Xist* expression (Xu *et al.* 2006). *DXPas34* is a polymorphic minisatellite region and has been shown to enhance *Tsix* expression (Stavropoulos *et al.* 2005). The regulating elements and their interaction in mouse X-chromosome are illustrated in Figure 1.3.

Whatever the mechanism is, *Xist* has a primary role in coating and inactivating the whole X chromosome except pseudo autosomal regions (PAR). Those regions contain genes that have homologous pair in Y chromosome and have a role in X-Y pairing during mitosis. Not all genes that escape inactivation have Y homologs. One of the most striking features of PARs is that they are localized at the two distal parts of an X chromosome, PAR1 in Xp and PAR2 in Xq. Carrel *et al*. presented a comprehensive XCI profile of the human X chromosome in 2005. They achieve this in a fibroblast-based test system with human-rodent hybridoma cells. About 15% of total X-linked genes to be analyzed escape inactivation to some degree. Interestingly, the location of the genes that escape inactivation differs. The molecular mechanism and profile of how and which of those genes escape inactivation is currently unknown. Failure of spreading of silencing by *Xist* may involve in escape, or any reactivation of those genes may cause escaping. Even

though Ke & Collins in 2003 displayed in their research that CpG islands seem to be less abundant at the 5' end of genes escaping inactivation, these genes are seem to be the ones in Carrel's study showing variable inactivation patterns.

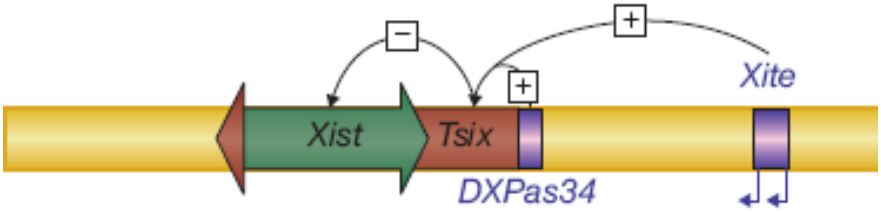

Figure 1.3 The illustrative scheme of known genes and regulatory elements in the *Xic* region on mouse X-chromosome. The *Tsix* gene initiated 16 kb downstream of *Xist* is transcribed as antisense of *Xist* and negatively regulate its transcription together with *Xite* and *DXPas34* (Morey&Bickmore 2006

1.7. Autoimmune disorders that were selected for this study

In this study, autoimmune diseases with high female preponderance were selected. Disorders both from systemic and organ specific autoimmune diseases were included. Scleroderma, Sjogren's syndrome, rheumatoid arthritis and juvenile idiopathic arthritis were selected as systemic autoimmune disorders. Type I diabetes mellitus and autoimmune thyroiditis were included as organ specific diseases.

1.7.1. Scleroderma (SSc)

The name scleroderma was derived from the Greek word "skleros", which means hard. "Derm" means skin. "Systemic sclerosis" is also used in nomenclature. It is a chronic autoimmune disorder of unknown etiology characterized with severe and progressive cutaneous and visceral fibrosis, pronounced alterations in the microvasculature, and numerous cellular and humoral immunological abnormalities. Thickening, hardening, or tightening of the skin, blood vessels and internal organs

are the major symptoms observed in systemic sclerosis. Clinical forms of scleroderma are heterogeneous ranging from limited to diffuse types. Limited cutaneous scleroderma involves limited skin fibrosis with minimal systemic alterations. In diffuse cutaneous scleroderma severe internal organ diseases are observed together with diffuse skin lesions, which can rapidly progress to hardening after an early inflammatory phase (LeRoy *et al*. 1988). The pathologic changes in scleroderma are observed in three steps: 1) accumulation of collagen and other connective tissue components in the extracellular matrix, which causes severe tissue fibrosis; 2) chronic inflammatory process characterized by infiltration of mononuclear cells, mostly of T cell lineage; 3) development of microvascular forms resulting in intimal proliferation, narrowing and thrombosis of the vessel lumen. Progression of vascular and fibrotic changes with a decrease in the inflammatory components leads to end-stage fibrosis and atrophy of the affected organs (Jimenez&Derk 2004).

The prevalence of scleroderma may vary by ethnic background and the geographical region. However an estimate of mean prevalence around 150 cases per million in Europe (Le Guern *et al.* 2004, Alamanos *et al*. 2005) and about 250 cases per million in US (Mayes *et al.* 2003) has been reported. Japan has the lowest prevalence with 20-50 cases per million (Tamaki *et al.* 1991). Systemic sclerosis is known to be at least three times more common in women than in men and two times more common in Blacks than in Whites (Laing *et al,* 1997).

The possible cause(s) of scleroderma has remained elusive despite numerous studies. It has been proposed that many chemical and physical agents may be involved in the pathogenesis. Infectious agents are one of the possible causes of scleroderma since the autoantibodies that are produced in SSc are thought to be the result of a response caused by molecular mimicry (Oldstone 1987). Self antigens that contain epitopes structurally similar to viral or bacterial proteins induce formation of autoantibodies due to the molecular mimicry. Other than molecular mimicry, retrovirus (Jimenez *et al.* 1995), cytomegalovirus (Pandey&LeRoy 1998) and parovirus (Magro *et al.* 2004) etiologies have been suggested. Viral infections may trigger initially autoimmune reaction or they may have a role in the

maintenance of the chronicity of the autoimmune process. Environmental agents have also been associated with the pathogenesis of scleroderma. Aromatic hydrocarbons, such as toluene, xylene, vinyl chloride, benzene and silica have been shown to relate to SSc (Haustein&Herrmann 1994, Garabrant *et al* 2003). The contribution of genetic factors have also been considered in the development of scleroderma because; (1) there are cases with a family history, (2) differences exist in prevalence and clinical manifestation among different ethnic groups, and (3) there is increased prevalence of certain HLA and MHC alleles among the disease classes with different types. They differ also among different ethnic groups (Derk&Jimenez 2003). Even though the concordance of SSc among monozygotic twins is 4.2% and dizygotic twins 5.9%, the presence of specific autoantibodies is higher (Feghali-Bostwick *et al.* 2003). Moreover, certain HLA classes II antigens-mostly HLA-DQ types- have associations in SSc susceptibility (Arnett 1995). In 1997, Artlett and colleagues analyzed the inheritance pattern of HLA class I and II type haplotypes in the families of 37 SSc patients and 42 control individuals. They found that 70% of SSc patients but only 21% of controls had HLA class II alleles compatible with either their offspring or mother. Based on these observations, they proposed that there are clinical similarities between systemic sclerosis and graft-versus-host-disease (GVHD) induced by the presence of persisting fetal cells. The hypothesis of involvement of fetal microchimerism in scleroderma was first proposed by Black&Stevens (1989) based on the pathological similarities between SSc and GVHD. Subsequently, Bianchi *et al.* reported presence of male fetal cells in a normal woman 27 years after the birth of her son and published certain evidence of involvement of fetal cells in the pathogenesis of scleroderma. Allogeneic fetal and maternal cells can cross the placenta during pregnancy and may persist in the blood and/or tissues. It has been proposed that these cells may become activated upon stimulation by an environmental effect. The identification of Y-chromosome specific sequences in the blood and tissues of female SSc patients that give birth to a male offspring strengthen the hypothesis of presence of a relationship between microchimerism and scleroderma (Artlett *et al*. 1998). At the same time, Nelson and colleagues (1998) found similar results by investigating involvement of microchimerism in the pathogenesis of scleroderma. Using quantitative PCR specific for Y-chromosome specific sequences, they compare presence of fetal cells

between SSc patients and control women. Fetal DNA was found significantly more in the blood of women with SSc. Moreover, women with scleroderma had given birth to an HLA class II compatible child more often than controls. All these results support the possibility of relationship between SSc and microchimerism.

1.7.2. Sjogren's Syndrome (SICCA)

Sjogren's syndrome is an autoimmune disorder of unknown etiology. The main manifestations of this syndrome include keratoconjunctuvitis sicca (dry eye), xerostomia (dry mouth) and other extraglandular abnormalities. Lymphocytes that infiltrate to lacrimal and salivary glands cause drying of eyes and mouth. Sjogren's syndrome is primarily classified into two categories: primary and secondary SICCA. Primary Sjogren's syndrome is defined as the lack of presence of another type of connective tissue disease (rheumatoid arthritis, SLE, or SSc) associated with gland inflammation. Secondary Sjogren's syndrome progresses in the presence of another type of connective tissue disease (Venables 2004).

The classification of Sjogren's syndrome has some difficulties due to lack of single disease-specific diagnostic criteria. Recently modified European classification criteria are the most functional one (Vitali *et al.* 2002) with new revisions and exclusions.

One of the major feature of Sjogren's syndrome is its high prevalence among women rather than in men (women:men ratio is 9:1). The epidemiological data about SICCA is very poor due to the ambiguity in classification criteria. It is known that it occurs worldwide and in all ages. A recent epidemiological study about prevalence of SICCA was reported that primary Sjogren's syndrome affects 0.4-3.1 million adults (Helmick *et al.* 2008).

Familial clustering of SICCA has been reported in several publications (Reveille *et al* 1984, Boling *et al.* 1983, Moriuchi *et al.* 1986). However, the lack of large twin

studies does no permit determination of concordance rate in primary Sjogren's syndrome. Only a few case reports have been published describing twins in primary SICCA (Scofield *et al*. 1997, Bolstad *et al.* 2000). Interestingly, familiar clustering of different autoimmune diseases and co-association of more than one type of autoimmune disorders in the family members has frequently been reported. In addition, presence of other autoimmune diseases in the relatives of Sjogren's syndrome probands has been widely reported (Foster *et al.* 1993, Tanaka *et al.* 2001, Anaya *et al.* 2006).

Sjogren's syndrome is considered as a complex disease with autoimmune manifestations. Susceptibility to the disease may vary from environmental factors to the viruses and genetic contributions. Since the MHC genes are the best documented genetic risk factors for the development of autoimmune disorders, they have been analyzed detailed in Sjogren's syndrome. Even though patients from different ethnic origins exhibit different HLA types, most specifically HLA-DR and DQ alleles are associated with SICCA. This HLA-mediated risk seems more strongly linked to the anti-SS-A/Ro antibody rather than the disease itself. A stronger correlation between anti Ro/SSA autoantibodies and HLA-DR3/DR2 has been reported recently (Arnett *et al.* 1989, Bolstad *et al.* 2001). Other than HLA genes, polymorphisms o the promoter of IL-10 ha been recently reported in Finnish and Italian population (Hulkkonen *et al.* 2001, Font *et al.* 2002).

Among the environmental factors, viruses are the most prominent candidates because salivary glands are a site of latent infection by them. HIV, hepatitis C virus and T-leukemia retrovirus-I (HTLV-I) have been reported as the strongest candidates in the pathogenesis of Sjogren's syndrome (Kordossis *et al.* 1998, Haddad *et al.* 1992, Terada *et al*. 1994).

1.7.3. Rheumatoid Arthritis (RA)

Rheumatoid arthritis is a chronic inflammatory autoimmune disease characterized by the presence of autoantibodies, like rheumatoid factor (RF), anti-cyclic citrullinated peptide (CCP), and anti-keratin antibodies (AKA). It leads to destruction of joints causing pain, swelling and stiffness. These criteria are developed by the American Rheumatology Association in 1988 (Arnett *et al.* 1988). The clinical symptoms are results of cascade of multicellular changes including infiltration of lymphocytes and granulocytes into the articular cartilage, proliferation of synovial fibroblasts and macrophages and neovascularization of the synovial lining surrounding the joints. Subsequently, many cellular components including macrophages, dendritic cells, fibroblast-like synoviocytes, mast cells, eosinophills, neutrophills, T cells and B cells are recruited in the joints.

Epidemiologic studies indicated that RA occurs worldwide and affects about 1% of the population in US and UK (Alamanos&Drosos 2005, Symmons *et al* 2002). A less prevalence has been reported in African population (Adebajo&Davis 1994). Disease occurs at any age but onset of disease increases in the elderly people with a female preponderance of 2.5 fold (Lee&Weinblatt 2001).

Genetic and environmental factors are suggested to play a role in the etiology of RA. Smoking is the most reported risk factor for RA (Silman *et al.* 1996, Harrison 2002). The genetic contribution in the susceptibility of RA in Finnish and UK patients was reported between 53% and 65% (MacGregor *et al* .2000). Monozygotic twins have concordance rate around 15%, whereas dizygotic twin rates fall around 3.5% in Europe and Australia (Aho *et al.* 1986, Bellamy *et al.* 1992, Silman *et al.* 1993).

Among the genetic factors, HLA locus was the first candidate region for RA due to the polymorphic immunological role of their products. Association of RA and HLA-DRB1 was first established in 1978 by Stastny. This particular locus account for nearly half of the genetic component of susceptibility to the disease and confirmed in different populations (Gregersen *et al.* 1987, Wordsworth&Bell 1991). In a recent study, candidate gene association was analyzed in 2370 RA patients and

1757 controls from the North American Rheumatoid Arthritis Consortium (NARAC) and Swedish Epidemiological Investigation of Rheumatoid Arthritis (EIRA) cohorts. Strong association between RA and *PTPN22, CTLA4,* and *PADI4* was documented (Plenge *et al*. 2005).

1.7.4. Juvenile Idiopathic Arthritis (JIA)

Juvenile idiopathic arthritis is a childhood onset autoimmune disease with unknown etiology. It is characterized by arthritis observed in the patients with less than 16 years old and persistent for at least 6 weeks. The term juvenile idiopathic arthritis (JIA) was first proposed in 1995 and then revised in 1997 (Fink 1995, Petty *et al*. 1998) instead of the term used by European League Against Rheumatism (EULAR) as juvenile chronic arthritis (JCA) (Wood 1978) and by American College of Rheumatology (ACR) as juvenile rheumatoid arthritis (JRA) (Brewer *et al*. 1977). The International League against Rheumatism (ILAR) classifies JIA and overcome disagreements between the two criteria sets (Petty *et al.* 2004). The general categorization of JIA is based on the number of joints involved and the presence of systemic symptoms:

1. Oligoarticular JIA: It is the only form of JIA that is not present in adults. This type of arthritis affects up to four joints. Affected joints are usually the large types (particularly lower limbs), notably the knee. Symmetric joint involvement is observed in less than one third of the cases (Al-Matar *et al.* 2002, Guillaume *et al.* 2000). Oligoarticular JIA is divided in two subcategories: (a) Persistent oligoarthritis in which no more than four joints were affected throughout the disease course, (b) extended oligoarthritis which affects a cumulative total of five or more joints after the first 6 months.
2. Polyarticular JIA: This form of JIA affects five or more joints, most commonly metacarpophalangeal joints and wrists and is subdivided in two major categories according to the presence of rheumatoid factor: RF(+) and RF(-). Both large and small joints are affected and symmetric joint

involvement is highly observed. The adult equivalent form of polyarticular JIA is the rheumatoid arthritis (Johnson 2006).

3. Systemic JIA: This form of JIA includes children affected from arthritis with systemic features. Systemic JIA is characterized by spiking fever which occurs daily for at least 2 weeks together with evanescent rash, lymphadeonopathy, hepato/splenomegaly or serotosis. It clinically resembles adult onset Still's disease and most patients later develop a polyarticular form with involvement of both large and small joints (Hofer&Southwood 2002).
4. Psoriotic JIA: Psoriosis together with arthritis is observed at least with two of the following symptoms: (i) dactylitis, (ii) nail pitting or onycholysis, (iii) family history of psoriasis in a first degree relative (Stoll *et al.* 2006).
5. Enthesitis related JIA: Enthesitis is the inflammation at the insertion of tendons into bones. This subgroup of JIA is characterized by arthritis together with enthesitis and at least two of the following features: (i) sacroiliac joint tenderness and/or inflammatory spinal pain, (ii) acute anterior uveitis (iii) presence of HLAB27 or presence of familial history HLAB27 positive disease. This type of JIA mainly affects boys after the age of 8 years (Davidson 2000).
6. Other JIA: This group of the disease is composed of the children that do not meet any criteria above.

JIA is one of the most common childhood rheumatic diseases. The incidance of JIA changes between 7 and 21 per 100,000 in US and Europe (Peterson *et al.* 1996, Kaipiainen-Seppänen&Savolainen 1996, von Koskull *et al*. 2001, Berntson *et al.* 2003). It was reported that cumulatively more females than males are affected by JIA (male:female ratio is 1/2 or 1/3). However this unequal distribution varies with disease subtype. Female predominance is observed in the oligo-and polyarticular subgroup, whereas male predominance in enthesitis related JIA (Borchers *et al.* 2006).

A wide variety of autoantibodies has been detected however rheumatoid factor (RF) (Minden *et al.* 2000), and antinuclear antibodies (ANA) (Berntson *et al.* 2003, Flato *et al.* 1998, Serra *et al.* 1999) are routinely used in the serological diagnosis of the patients. JIA rarely manifests familial occurrence, therefore family studies are uncommon or based on small numbers. Another parameter for genetic contribution is twin studies. In a study performed among Finnish monozygotic twins with JIA, the λs was calculated near to 20 (Savolainen *et al.* 2000). A different study from US indicated λs as 15, a value similar to type I diabetes mellitus (Moroldo *et al.* 1997). HLA-B27 was first and most strongly associated genetic risk factor in older and male JIA patients (Rachelefsky *et al.* 1974). A study that was performed on 521 British JIA patients and 537 matched controls displayed that aarious subgroups of JIA were reported to associate with distinct HLA profiles. (Thomson *et al* 2002). Studies about non-MHC loci reported polymorphisms on TNF, IL-10, interferon regulatory factor 1 (IRF-1), and macrophage migration inhibitory factor (MIF) that display strong associations with JIA (Date *et al.* 1999, Zeggini *et al.* 2002, Crawley *et al.* 1999, Vencovsky *et al.* 2001, Meazza *et al.* 2002).

1.7.5. Autoimmune Thyroid Diseases (AITDs)

Autoimmune thyroid diseases are a combination of a group of immunological responses, which target the thyroid gland. Although classical AITDs include mainly Grave's disease (GD) and Hashimoto's thyroiditis (HT), postpartum thyroiditis (PPT), atrophic autoimmune thyroid diseases, and thyroid associated ophthalmopathy (TAO) were counted as minor variants of AITDs. However, in this study, only HT and GD were included in the context of AITDs. Both GD and HT involve infiltration of the thyroid by B and T cells, giving reaction against thyroid antigens. The reaction causes production of thyroid autoantibodies, subsequently causing hypothyroidism in HT and hyperthyroidism in GD. Hypothyroidism in HT is associated with thyroid peroxidase and thyroglobulin autoantibodies. Hyperthyroidism in GD is associated with thyroid-stimulating hormone receptor autoantibodies (McLachlan *et al.* 2007).

AITDs are one of the most common autoimmune disorders and affects women more than men. The prevalence of hyperthyroidism is 2.5% in females and 0.6 % in males and the prevalence of hypothyroidism is 4.8% in females and 0.9 in males in a large study of north European population (Bjoro *et al.* 2000). Another recent study performed among US population reported prevalence of hyperthyroidism was found 0.5% and hypothyroidism 3.7% (Aoki *et al.* 2007). Even though many environmental factors, like iodine intake, may affect the prevalence, virtually all studies report the increased hypothyroidism in women with advancing age (Canaris *et al.* 2000).

The etiology of AITDs has not been fully understood, but there are numerous studies supporting the effects of genetic factors. The most powerful method to decide the presence of genetic predisposition to complex diseases is twin studies. Two main studies that search large twin populations with GD gave information about the concordance rates. First study is from Denmark by Brix *et al.* (2001) and reported that the concordance rate was 35% in monozygotic (MZ) twins and 3% in dizygotic (DZ) twins. Moreover, they suggested that genetic factors are responsible for 79% of the susceptibility to develop GD. The same group found that the frequency of skewed XCI in female twins with AITDs was 34%, which was higher than the prevalence in the corresponding control populations (Brix *et al.* 2005). Another study from California confirmed the results of the Danish group. The concordance rate among US population was found 17% in MZ twins, whereas 1.9% in DZ twins (Ringold *et al.* 2002).

Brix and colleagues (2000) performed a similar study in Danish HT twin population and statistically significant results were reported. Concordance rate was found 55% and 0% in MZ and DZ twins respectively. In addition, the concordance rates for thyroid autoantibodies were found to be higher in MZ twins compared to DZ twins.
Several methods exist to determine whether complex disease susceptibility is influenced by genetic factors. One of them is calculating λs (siblings ratio), which is a quantitative measure of genetic contribution to the disease if the value of the ratio is greater than 5. In AITDs case, the λs was calculated 16.9 for AITDs, 11.6 for GD, and 28.0 for HT. This study was performed on 155 patients (Villanueva *et al.* 2003).

Several scientists have reported the familial occurrence of AITDs from different geographical regions. In 1967, Hall&Stanbury reported a family whom 33% of siblings of patients with GD or HT developed AITDs. Later, in 2001, the largest family whose members exhibit AITDs together with other types of autoimmune disorders (The Akr Family) was reported by Maalej *et al.* This family has more than 200 members. More than 60 individuals were affected with AITDs. Subsequently, a Chinese-American family with 8 members affected from AITDs over 3 generations was reported (Villanueva *et al.* 2002).

The genes that are thought to be involved in the pathogenesis of AITDs can be categorized in two; those that are involved in immune system regulation and those involved in thyroid physiology. Among the immune related genes, the human leukocyte antigen genes on MHC region are the first candidates because they are highly polymorphic and contain many genes that have role in immune responses. An association between HLA-DR3 and GD has been conferred as a risk factor (Ban&Tomer 2003). Among the HLA types, the strongest association in the pathogenesis of HT has been shown on HLA-DR4 (Tandon *et al* 1991, Jenkins *et al.* 1992) and HLA-DQw (Badenhoop *et al.* 1990, Wu *et al.* 1994). Another important immune system regulation molecule is the cytotoxic T lymphocyte antigen-4 (CTLA-4), which down regulates T cell activation by competing for the binding of B7 to CD28. A considerable high number of studies have been carried out and numerous results support the association between AITDs and *CTLA-4* (Jacobson&Tomer 2007).

1.7.6. Type I Diabetes Mellitus (T1D)

Diabetes mellitus is a group of disorder of multiple etiology characterized by chronic hyperglycemia together with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin action, secretion or both. The first agreement about diagnostic criteria and classification system in diabetes mellitus was achieved in 1980 by World Health Organization (WHO 1980) and then revised

in 1985 (WHO 1985). The report in 1980 categorized the disease into two major classes: IDDM (insulin dependent diabetes mellitus) or Type I, and NIDDM (non-insulin dependent diabetes mellitus) or Type II. In 1985, according to the revised categorization, 4 types of the disease classes were accepted: Type I, Type II, gestational diabetes mellitus, and other types.

Briefly, Type I diabetes occurs as a result of destruction of pancreatic islet beta cells. Due to the loss of beta cell function, insulin cannot be produced. Type II includes the form of diabetes which results from defect(s) in insulin secretion. In this type, the mass of pancreatic beta cells and their functions are preserved to some extent. Gestational diabetes mellitus occurs for the first time during pregnancy and the symptoms disappear after delivery or birth. In the other type of diabetes, the disease occurs either as a result of specific mutations in the genes related to the mechanisms of insulin action or as a manifestation of various pathologic conditions. Type I diabetes occurs as a result of autoimmune destruction of insulin-producing beta cells in pancreas by $CD4^+$ and $CD8^+$ T cells and macrophages infiltrating the islets. Disease progression causes insulin deficiency and if not treated may result in coma and death. T1D has been classified as an organ-specific autoimmune disease with unclear etiology. Autoantigens that are mainly detected in T1D patients are proinsulin, glutamic acid decarboxylase (GAD65 and 67), and the protein thyrosine phosphatase (IA2) (Lieberman&DiLorenzo 2003). Even though the female:male ratio is not more than one in T1D patients, there are subgroups of the disease that exhibit high female preponderance. Thyroid antibodies were present in approximately 15-30% of T1D patients and about half of these patients progress to clinical AITDs (Golden *et al.* 2005).

Type I diabetes accounts about 10% of all cases of diabetes. It was reported that T1D occurs most commonly in Europe and North America. There are differences between the incidence rates and the geographical variation. One of the lowest incidence rate is observed in China (0.57 cases per 100,000 population in China whereas 18-20 cases per 100,000 in UK and 48 cases per 100,000 in Finnland) (Karvonen *et al.* 2000, DIAMOND project group 2006).

The family history of patients with T1D is not common; however there is clear genetic predisposition for the disease with first degree relatives having a lifetime risk of developing T1D of 6.0 vs 0.4% in the general population (Harjutsalo *et al* 2006). In addition, the twin studies reported that the concordance rate in MZ twins is approximately 50% (Kyvik *et al.* 1995, Redondo *et al.* 1999).

Susceptibility is inherited largely in the HLA region. The HLA locus is thought to play a role about 50% of the genetic susceptibility. Among them, HLA-DR and HLA-DQ subtypes predominantly are suggested to have a role in genetic susceptibility. In fact, the HLA locus on chromosome 6 was the first locus shown to be associated with the disease (Nerup *et al.* 1974). Moreover this region is considered to contribute about half of the familial basis of type I diabetes (Todd 1995). Candidate gene studies reported another non-MHC gene, insulin on chromosome 2q as risk factors contributing 10 % of susceptibility to T1D (Bell *et al.* 1984). *CTLA-4* and a variant of *PTPN22* are two other most powerful candidate genes that are reported to have association with T1D (Ueda *et al.* 2003, Bottini *et al*. 2004).

1.8. Pre-eclampsia (PEE)

Previous reports indicated that sera from patients diagnosed with pre-eclampsia include greater amounts of Th1-type cytokines. Although the etiology and patophysiology of pre-eclampsia remain unsolved, association with poor placentation, endothelial dysfunction and excessive inflammation was revealed (Saito&Sakai 2003). Since autoimmune components were suggested to be involved in pre-eclampsia, it was selected for investigating the role of XCI in the etiology of the disease. Pre-eclampsia is characterized by hypertension (more than 140/90 mmHg) and proteinuria (more than 0.3 g/L) in previously normotensive non-proteinuric pregnant women. PEE is the leading cause of maternal and fetal mortality and morbidity. It occurs in 3-7% of pregnancies and about 8,370,000 cases per year have been estimated over the world (Sibai *et al.* 1993). The presence of

placenta is necessary for appearance of symptoms, because delivery of fetus and placenta causes disappearance of them. Besides hypertension and proteinuria, edema, visual disturbances, headache and vomiting can be observed in patients as clinical features. If those symptoms become more severe, like elevated liver enzymes, hemolysis, and thrombocytopenia, the syndrome is called as HELLP (**h**emolytic anemia, **e**levated **l**iver enzymes, and **l**ow **p**latelet count) and was observed in 5-8% of pre-eclamptic women (Noris *et al.* 2005).

The etiology of pre-eclampsia is still not completely clear, however genetic, immunologic and environmental factors (oxidative stress) are suggested to play an important role in the pathogenesis. There are several publications that indicate genetic factors predisposing to PEE. In familial cases of pre-eclampsia, linkage studies indicated different candidate loci on different regions. Arngrimsson *et al.* performed a genome-wide scan and found a susceptibility locus on 2p13 in Iceland population (1999). Subsequently in 2001 by Lachmeijer *et al.* and then in 2004 by Oudejans *et al.* two candidate loci on 12q and 10q22 were reported in Dutch population. Lastly, a study in Finnish population indicated 2p13 locus as a candidate region (Laasanen *et al.* 2003).

Immune mechanisms are suggested to be involved in the patogenesis of PEE. It was reported that angiotensin receptor (AT1) agonistic antibodies were raised in the blood of pre-eclamptic women whereas they were rarely observed in normotensive pregnant women. These autoantibodies have the capability of activating the angiotensin AT1 receptor and support the classification of PEE as a pregnancy-specific autoimmune disorder (Wallukat *et al.* 1999, Xia *et al.* 2002).

1.9. AIM OF THE STUDY

Autoimmune diseases affect 5-8 percent of the population. An important characteristic of this group of diseases is the female predominance in certain autoimmune disorders. Even though the exact mechanisms that cause this unequal distribution between sexes are not entirely known, environmental factors, pregnancy related microchimerism, and sex hormones are likely candidates. Disturbed XCI is another candidate mechanism in the contribution of breakdown of self-tolerance. In this study, the hypothesis that 'loss of mosaicism' in the female cells may have a potential role in the pathogenesis of female predominant autoimmune diseases was tested. Methylation status of CAG repeat polymorphism of androgen receptor (*AR*) gene was genotyped to determine XCI status of patients and controls. Patients diagnosed with scleroderma (n=70), autoimmune thyroiditis (n=110), Sjogren's syndrome (n=78), juvenile idiopathic arthritis (n=81) and pediatric scleroderma patients (n=13) and their age matched controls (n=160 for adult and n=183 for pediatric samples) in the Turkish population were genotyped. Moreover, XCI status of autoimmune thyroiditis (n=145) and rheumatoid arthritis (n=106) patients and controls (n=97) in the Tunisian population and scleroderma patients (n=125) in the US population were genotyped. Independently, patients diagnosed with pre-eclampsia (n=67) in which autoimmunity may be an important component, were genotyped for *AR* gene polymorphism.

Subaim 1: Microchimerism was documented to associate with scleroderma, therefore the relationship between microchimerism and skewed XCI was investigated in Turkish and US scleroderma patients.

Subaim 2: In order to investigate the parental origin of inactive X-chromosome, US scleroderma patient (n=19) and their mothers were genotyped for *AR* gene polymorphism.

CHAPTER 2

MATERIALS AND METHOD

2.1. ADULT SAMPLES

2.1.1. *Turkish control samples*

Caucasian females comprised of 160 Turkish healthy unrelated volunteers were involved as controls in our studies. The mean age at analysis was 46±10.0 (mean±SD) for Turkish controls. There was no clinical evidence or family history of autoimmune disease and inflammatory joint disease. The results of XCI profile of this group were involved in comparison of XCI pattern of patients with Turkish scleroderma, Turkish AITDs, US scleroderma, and Turkish Sjogren's syndrome. Turkish control women were involved in determination of XCI psttern of Tunisian patients together with Tunisian control group.

2.1.2. *Turkish scleroderma patients*

Caucasian women (n=70) diagnosed with scleroderma and healthy control women (n=160) with no history of autoimmune disease or cancer were included in this study. All patients fulfilled the American College of rheumatology diagnostic criteria for scleroderma (Subcommittee for Scleroderma Criteria of the American Rheumatism Association Diagnostic and Therapeutic Criteria Committee, 1980). The ethics review boards at the participating institutions approved the study protocol. Informed consent was obtained from all subjects.The mean (±SD) ages were 49 (±14) years for SSc patients and 46 (±10) years in controls. The duration of symptoms (Raynaud's phenomenon, puffy hands, esophagitis, or dyspnea) was 10

(±7) years among the SSc patients and the mean age at diagnosis was 39 (±12) years. Among 70 patients, five had the limited cutaneous form of disease, and sixty five patients had diffuse disease. XCI pattern was determined in the cells of blood obtained from patients and control women. In order to test whether the same skewed pattern in XCI was observed in the cells from different tissue types, samples from skin biopsy, buccal wash and hair specimens were requested from the patients whose XCI pattern in blood cells was more than 80%.

2.1.3. *US scleroderma patients*

DNA samples were obtained from 125 scleroderma patients and 160 control women. Clinical characteristics of the patients have been published elsewhere (Loubiere *et al.* 2005). The ethics review boards at the participating institutions approved the study protocol. Informed consent was obtained from all subjects. The maternal and fetal microchimerism status of the patients was available. In order to determine the paternal origin of the inactive X chromosome, from 19 patient-mother pair, whose DNA samples were enough in quantity for the analysis, were included in this study.

2.1.4. *Turkish autoimmune thyroid diseases patients*

In this study, 110 Caucasian women diagnosed with AITDs (81 were diagnosed with HT and 29 with GD), and 160 healthy female controls with no history of autoimmune disease and cancer were included. All the patients were selected randomly. The mean ages were 44.8±14.1 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 (standard deviations were displayed in parantheses). 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 clinical investigations described in this study 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. In order to test whether the same skewed pattern in XCI is observed in the cells from different tissue types, samples from thyroid biopsy, buccal wash and hair specimens were requested from the patients whose XCI pattern in blood cells was more than 80%.

2.1.5. *Turkish Sjogren's syndrome patients*

Caucasian women diagnosed with Sjogren's syndrome (n=78) and 160 healthy Caucasian female were included and genotyped to determine the XCI patterns. The ethics review board of the participating institutions approved study protocol.

2.1.6. *Combined group of Turkish and Tunisan control samples*

Caucasian females comprised of 97 Tunisian and 160 Turkish healthy unrelated volunteers served as controls in our studies. The mean age at analysis was 43.5±15.3 (mean±SD) years for the Tunisian and 46±10.0 (mean±SD) for Turkish controls. There was no clinical evidence or family history of autoimmune disease and inflammatory joint disease.

2.1.7. *Tunisian Akr family*

Akr family is a large family with more than 400 members. The importance of examining this family is that 76 members over 3 generations were diagnosed with AITDs. 242 members from this family were sampled. 40 of them diagnosed with GD, 13 with HT, and 23 with PIM, and 166 unaffected. The ratio of female to male is 1 in GD, 3.3 in HT, and 4.2 in PIM patients. The age of patients at the onset of disease ranged between 12 and 58 years. The mean age was 35 years. 44 women members from this family were included in this study. Among these 44 individuals,

20 were diagnosed with AITDs and 24 were healthy women. Clinical characteristics of the members of Akr family have been reported by Maalej *et al.* (2001, 2008).

2.1.8. *Tunisian autoimmune thyroid diseases patients*

Caucasian women diagnosed with AITDs (n=145) were included in this study. Among those patients, 58 were diagnosed with Grave's disease (GD), 40 with Hashimoto's thyroiditis (HT), and 47 with primary idiopathic myxodema (PIM). The mean age was 46.5±14.5 (mean ±SD) years for AITDs patients (57.2±13.1 years in PIM patients, 43.3±13.2 years in the Hashimoto patients and 44.6±14 years in Graves' patients). The duration of the symptoms was 7.5±4.6 years for the AITDs patients (6.5±5 years in PIM patients, 7.2±4 years in Graves' patients, and 7.7±4.6 years in the Hashimoto patients). The mean age of diagnosis was 37.9±15.1 years. Presence of biochemical hyperthyroidism by a decrease of TSH, an increase of T4 levels and positive TSH receptor antibody, in association with diffuse goiter or the presence of exophthalmos was the diagnostic criteria for GD. For the diagnosis of HT, presence of thyroid hormone replaced primary hypothyroidism, defined as a TSH level above the upper limits associated with or without positive titters of thyroid autoantibodies (anti-thyroglobulin and/or anti-thyroid peroxydase) and a palpable goiter were the criteria. PIM was diagnosed by the presence of hypothyroidism requiring T3 or T4 replacement. Patients with PIM have an atrophic gland.

2.1.9. *Tunisian rheumatoid arthritis patients*

Caucasian women affected with RA (n=106) were enrolled in this study. All patients fulfilled the 1987 American College of Rheumatology criteria for RA (Arnett *et al.* 1988). All clinical data were reviewed by a rheumatologist university fellow. The mean age was 47.6±13.4 (mean ±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-cyclic

citrullinated peptides (anti CCP) positive (66%), 15 presented nodules (14.1%), and 70 presented with erosive disease (66%). Presence of another auto-immune diseases (Sjögren's syndrome, Type I diabetes, Thyroid auto-immune diseases) were observed in 15 patients.

2.1.10. *Turkish pre-eclampsia patients*

Caucasian women (n=67) diagnosed with preeclampsia, and 130 apparently healthy Caucasian female controls were included in this study. The control group women were selected from the individuals of Turkish control group so that the age of subjects was matched with the healthy women. For this reason the number of control group of pre-eclampsia patients is 130. The ethics review board of the participating institutions approved study protocol. Pregnancy history, age, and disease information accompanied by informed consent was obtained from all subjects. In the control group (124/130) women gave birth to at least one child. None of the control women had a history of pregnancy loss, autoimmune disease or cancer. In the patient group all women had singleton deliveries. The birth registry at the Etlik Maternity and Women's Health Teaching Hospital in Ankara was used to contact the pre-eclamptic cases. The mean ages were 29.8 ± 5.7 years (mean ± SD; range = 21– 42 years old) for the cases, and 31.6 ± 5.6 (range = 21– 47 years old) for the controls. Development of hypertension and proteinuria within 7 days of each other after the twentieth week of gestation in women was defined as pre-eclampsia. Hypertension was defined as diastolic blood pressure of at least 90 mm Hg that occurs on at least two occasions, 4-168 h apart. If the blood pressure was more than 140 mm Hg, the condition was defined as severe hypertension. Proteinuria was defined as urinary protein excretion of more than 300 mg in 24 h. In the presence of severe hypertension and proteinuria, HELLP syndrome (haemolysis, elevated liver enzymes, low platelets) or eclampsia, pre-eclampsia was considered severe. The patients with a previous history of chronic hypertension, renal disease or diabetes mellitus were excluded. In order to test whether the same skewed pattern in XCI is observed in the cells from different tissue types, samples from buccal wash specimens were requested from the patients with various degree of XCI.

2.2. PEDIATRIC SAMPLES

2.2.1. *Turkish pediatric control samples*

Helathy children with no autoimmune disorders or cancer (n=92) and newborn babies (n=91) were included in this study as the control group of pediatric autoimmune diseases. The ethics review board of the participating institutions approved study protocol.

2.2.2. *Turkish juvenile idiopathic arthritis patients*

Eighty one Caucasian children diagnosed with JIA were included in this study. All patients fulfilled the American College of rheumatology diagnostic criteria for juvenile idiopathic arthritis (Petty *et al.* 1998). The mean age of the patients at the onset of disease was 5.6±3.8 (mean±SD) years for JIA. Among 81 JIA patients, 21 were diagnosed with polyarthritis, 3 were with extended oligoarthritis, and 57 were with oligoarthritis JIA. The ethics review board of the participating institutions approved study protocol.

2.2.3. *Turkish pediatric scleroderma patients*

Thirteen children diagnosed with pediatric scleroderma were included in this study. The ethics review board of the participating institutions approved study protocol.

2.2.4. *British cell line samples of type I diabetes mellitus (TPO+&TPO-) patients and BBC1958 control individuals*

Type I diabetes mellitus was selected as an organ specific pediatric onset autoimmune disease. For this reason collaboration with Dr John Todd from

Cambridge University was proposed. However the samples from patients and controls have been preserved as established cell lines. 453 DNA samples of cell lines of T1D (TPO-) patients and 354 DNA samples of cell lines of T1D (TPO+) patients were involved in this study. 466 DNA samples of cell lines of BBC 1958 cohort were involved in this study. Clinical characteristics about the patients and controls have been reported elsewhere (Wellcome Trust Case Control Consortium; Australo-Anglo-American Spondylitis Consortium (TASC) 2007).

2.3. DNA ISOLATION

2.3.1. *DNA isolation from venous blood*

DNA from venous blood was isolated by using MN Nucleospin Blood Kit (Germany) according to the protocol of the manufacturer. In this protocol, 200µL venous blood was mixed with 25µL proteinase K and 200µL B3 solution of the kit. This solution was incubated at 70°C for 15 min. During the incubation period, the solution was vortexed minimum two times. Since B3 solution lyses the cells, mixing by vortexing enhance disruption of membrane of the cells. At the end of this incubation procedure, 210µL (96-100%) ethanol was added and vortexed. The lysate was loaded into the column provided in the kit package and centrifuged at 13,000 rpm for 1 min. During this step genomic DNA was attached to the column resin. The flow-through was discarded and 500µL of buffer BW was added into the column. These columns were centrifuged at 13,000 rpm for 1 min and the collecting tube with flow-through was discarded. The column was placed in a new collecting tube and 600 µL of buffer B5 was added. Both BW and B5 buffers are used to wash the cloumn and discard the residual parts and salts. The tube was centrifuged at 13,000 rpm for 1 min and the flow-through was discarded. Buffer B5 contain ethanol. In order to remove residual ethanol on the membrane of column, it was centrifuged one more time at 13,000 rpm for 1 min. The tube with flow-through was discarded and the column was placed in a 1.5 mL eppendorf tube. 100µL of pre-heated (at 70°C) elution buffer was added on the membrane of the column and

incubated at room temperature for 1 min. The tube was centrifuged at 13,000 rpm for 1 min. Genomic DNA atached on the membrane of the column was eluted into the eppendorf tube. The column was discarded and the DNA samples were preserved at 4°C. The quantity of DNA samples were measured using spectrophotometry or nanodrop. 5µL of DNA sample was diluted in 95µL ddH_2O and the mixture was loaded into the column of spectrophotometry apparatus. The integrity of DNA samples were controlled by using 1% agarose gel electrophoresis. Runs were performed at 120 Volt for 30 min.

2.3.2. *DNA isolation from buccal wash specimen*

Buccal wash (25 mL in H_2O) from patients were centrifuged at 2000 rpm for 10 min. The supernatant was discarded and the procedure in section 2.3.1 was performed for further DNA isolation.

2.3.3. *DNA isolation from hair specimen*

Chelex resin was used to isolate DNA from hair specimen (Walsh *et al.* 1991). Rooted hair was cut (approximately 0.5 cm) and washed with deionized water. The hair was incubated in 200 µL 5% Chelex (BioRad, CA, USA) at 56°C overnight. The mixture is then mixed and boiled for 8 min. After boiling the tubes were centrifuged at 13000 rpm for 3 min. The hair and chelex resin were accumulated in the pellet. 10 µL from the supernatant was taken and used as a template for restriction digestion reaction.

2.3.4. *DNA isolation from skin biopsy specimen*

Skin biopsy specimen was mechanicaly disrupted via a blade in the plate and then put into an eppendorf. The particules were washed in 1XPBS and then vortexed. The vortexed specimen was washed with 1XPBS and put into a new eppendorf. 25

µL proteinase K and 200 µL of Buffer3 of DNA Isolation Kit were put on the mixture. The tubes were incubated at 70°C for 15 min and the kit protocol in section 2.3.1 was procedured.

2.3.5. *DNA isolation from thyroid biopsy specimen*

The thyroid biopsy specimen was put into an eppendorf and 200µL of 1XPBS was added. The specimen was homogenized. 65µL of proteinase K and 200µL of B3 Buffer of the kit were added and incubated at 70°C overnight and the kit protocol in section 2.3.1 was procedured.

2.4. HUMARA ASSAY

The inactivation of one of the X chromosomes starts with accumulation of noncoding RNA of *XIST*. Next step is chromatin changes such as histone modification and methylation of DNA in the 5' region of the genes (Heard&Disteche 2006). Methylation at the deoxycytosine residues in CpG dinucleotides was especially noted in this process (Wolf *et al.*1984, Keith *et al.* 1986). *Hpa* II and *Hha* I are two restriction enzymes both of which contain CpG dinucleotides in their recognition sites and do not have the capability of cutting the DNA if the target site is methylated. The genes that contain *Hpa* II or *Hha* I sites in their 5' region can be analyzed to determine their methylation status, however the two alleles originating from two parents should be different from each other. Highly polymorphic CAG repeats in the first exon of androgen receptor (*AR*) gene was genotyped to asses the XCI pattern first by Allen *et al*. in 1992. Human *AR* gene was prefered in this assay because it has been shown to be highly affected by inactivation (Meyer *et al*. 1975). In this assay, highly polymorphic CAG repeats flanked by restriction sites of both methylation specific enzymes were amplified by PCR. By this way, methylation patterns of maternally and paternally derived X chromosomes can be identified. In this study, *Hpa* II was used for digestion of

DNA. Oligonucleotide primers were designed to flank both the methylation-sensitive restriction-enzyme sites and the CAG repeat simultaneously (Figure 2.1). Only the region on inactive X chromosome between the primer sets can be amplified because *Hpa* II digestion is failed due to methylated CpG dinucleotides.

2.4.1. *Restriction enzyme digestion*

The protocol that was described first by Allen *et al*. was used in HUMARA assay. Two sets of reactions were prepared for each subject in restriction digestion protocol. Both sets contain 150-250 ng genomic DNA, 1X reaction buffer (33mM Tris-acetate (pH 7.9), 10 mM magnesium acetate, 66 mM potasium acetate, 0.1 mg/mL BSA), and 2 Units of *Rsa*I (a restriction enzyme with sites outside the PCR product, which is not sensitive to methylation and thus digests active and inactive X alleles equally). *Rsa*I was used to improve the PCR and to mae the DNA more accessible. 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 rection tubes were incubated at 37°C overnight. Restriction digestion products were incubated at 70°C for 15 min in order to inactivate the enzymes. One control male sample that was cytogenetically verified as 46, XY was involved in this tudy as a control for complete digestion.

2.4.2. *Polymerase chain reaction (PCR)*

For each sample, two PCR were performed. The only difference between these two rection was the template. Tubes were labeled as "U" if the template was the restriction digestion product of undigested reaction (without *Hpa*II), and labeled as "D" if the template is the product of digested reaction. PCRs were carried out in a total of 25 µL volume. Restriction digestion products were amplified with 10 pmol of each primers, 0.12 mM of each dNTPs, 1X *Taq* polymerase buffer (750 mM Tris-

HCl (pH 8.8 at 25°C), 200 mM $(NH_4)_2SO_4$, 0.1% (v/v) Tween 20), 1.0 mM $MgCl_2$, and 1 Unit *Taq* polymerase. The sequence of primers were as follows: RS6 (forward) 5'-GTC CAA GAC CTA CCG AGG AG-3' and RS7 (reverse) 5'-CCA GGA CCA GGT AGC CTG TG-3'. 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 30 sec, 58°C 30 sec, 72°C 30 sec. amplification step. Final extension was performed at 72°C 5 min. The sequence of polymorphic region on *AR* gene is illustrated in Figure 2.2. Amplicons were controlled in 1.5% Agarose Gel. The gel was prepared by adding agarose in 1X TAE and 1 μL of ethidium bromide (10mg/mL). Runs were performed in 1X TAE at 120 V for 20 min.

Polymerase chain reactions of the SSc, AITDs and Control samples were performed via radioactively labeled nucleotides. The reaction contents were the same as explained above, except 0.1 μCi of α-33P-dCTP (NEN, Boston, MA) per reaction was added. PCR conditions were the same with the cold reaction.

gaggccggcccggtgggggcgggacccgactcgcaaactgttgcatttgctctccacctcccagcgc
cccctccgagatccccgggggagccagcttgctgggagagcgggacggtccggagcaagcccagaggca
gaggaggcgacagagggaaaaagggccgagctagccgctccagtgct**gtac**aggagccgaagggacg
caccacgccagccccagccccggctccagcgacagccaacgcctcttgcagcgcggcggcttcgaagc
cgccgccccggagctgccctttcctcttcggtgaagttttaaaagctgctaaagactcggaggaagc
aaggaaagtgcctggtaggactgacggctgcctttgtcctcctcctctccaccccgcctcccccac
cctgccttcccccctccccgtcttctctcccgcagctgcctcagtcggctactctcagccaaccc
ccctcaccacccttctccccacccgcccccccgcccccgtcggcccagcgctgccagcccgagtttg
cagagaggtaactccctttggctgcgagcgggcgagctagctgcacattgcaaagaaggctcttagg
agccaggcgactggggagcggcttcagcactgcagccacgacccgcctggttaggctgcacgcggag
agaaccctctgttttcccccactctctctccacctcctcctgccttccccaccccgagtgcggagcc
agagatcaaaagatgaaaaggcagtcaggtcttcagtagccaaaaaacaaaacaaacaaaaacaaaa
aagccgaaataaaagaaaaagataataactcagttcttatttgcacctacttcagtggacactgaat
ttggaaggtggaggatttgttttttttctttaagatctgggcatcttttgaatctacccttcaagt
attaagagacagactgtgagcctagcagggcagatcttgtccaccgtgtgtcttcttctgcacgaga
ctttgaggctgtcagagcgctttttgcgtggttgctcccgcaagtttccttctctggagcttcccgc
aggtgggcagctagctgcagcgactaccgcatcatcacagcctgttgaactcttctgagcaagagaa
ggggaggcggggtaagggaagtaggtggaagattcagccaagctcaaggATGGAAGTGCAGTTAGGG
CTGGGAAGGGTCTACCCTCGGCCGCC**GTCCAAGACCTACCGAGGAG**CTTTCCAGAATCTGTTCCAGA
GCGTGCGCGAAGTGATCCAGAACCCGGGCCCCAGGCACCCAGAGGCCGCGAGCGCAGCACCTCCCGG
CGCCAGTTTGCTGCTGCTG*CAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAG*
*CAGCAGCAGCAGCAGCAG*CAAGAGACTAGCCCCAGGCAGCAGCAGCAGCAGCAGGGTGAGGATGGTT
CTCCCCAAGCCCATCGTAGAGGCCC**CACAGGCTACCTGGTCCTGG**ATGAGGAACAGCAACCTTCACA
GCCGCAGTCGGCCCTGGAGTGCCACCCCGAGAGAGGTTGCGTCCCAGAGCCTGGAGCCGCCGTGGCC
GCCAGCAAGGGGCTGCCGCAGCAGCTGCCAGCACCTCCGGACGAGGATGACTCAGCTGCCCCATCCA
CGTTGTCCCTGCTGGGCCCCACTTTCCCCGGCTTAAGCAGCTGCTCCGCTGACCTTAAAGACATCCT
GAGCGAGGCCAGCACCATGCAACTCCTTCAGCAACAGCAGCAGGAAGCAGTATCCGAAGGCAGCAGC
AGCGGGAGAGCGAGGGAGGCCTCGGGGGCTCCCACTTCCTCCAAGGACAATTACTTAGGGGGCACTT
CGACCATTTCTGACAACGCCAAGGAGTTGTGTAAGGCAGTGTCGGTGTCCATGGGCCTGGGTGTGGA
GGCGTTGGAGCATCTGAGTCCAGGGGAACAGCTTCGGGGGGATTGCATGTACGCCCCACTTTTGGGA
GTTCCACCCGCTGTGCGTCCCACTCCTTGTGCCCCATTGGCCGAATGCAAAGGTTCTCTGCTAGACG
ACAGCGCAGGCAAGAGCACTGAAGATACTGCTGAGTATTCCCCTTTCAAGGGAGGTTACACCAAAGG
GCTAGAAGGCGAGAGCCTAGGCTGCTCTGGCAGCGCTGCAGCAGGGAGCTCCGGGACACTTGAACTG
CCGTCTACCCTGTCTCTCTACAAGTCCGGAGCACTGGACGAGGCAGCTGCGTACCAGAGTCGCGACT
ACTACAACTTTCCACTGGCTCTGGCCGGACCGCCGCCCCCTCCGCCGCCTCCCCATCCCCACGCTCG
CATCAAGCTGGAGAACCCGCTGGACTACGGCAGCGCCTGGGCGGCTGCGGCGGCGCAGTGCCGCTAT
GGGGACCTGGCGAGCCTGCATGGCGCGGGTGCAGCGGGACCCGGTTCTGGGTCACCCTCAGCCGCCG
CTTCCTCATCCTGGCACACTCTCTTCACAGCCGAAGA

Figure 2.1 The sequence of intron1 and exon1 of AR gene downloaded from http://www.ensembl.org . Underlined sequences are primers. Sequences that highlighted with dark gray are restriction sites of *Hpa* II, light gray are restriction sites of *Rsa* I, and italic sequences are polymorphic CAG repeat sites

2.4.3. *Polyacrylamide gel electrophoresis (PAGE)*

Radioactively labeled PCR products were separated on 8% denaturing PAGE. Acrylamide:bisacrylamide solution (29:1) was mixed with 10XTAE and 8M urea was dissolved in this solution. TEMED and 10% Ammonium persulfate were added for polymerization. 5 μL of stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol) was added into PCR products and denatured at 94°C for 5 min. and then put immediately on ice. 4μL from the denatured products were loaded and the gel was run at 60W for 4 hours. The gel was dried and autoradiographed on medical film CP-BU (Agfa, Mortsel, Belgium). Each hot product was confirmed on 4% MetaPhor agarose gel electrophoresis (FMC Bioproducts, Rockland, ME).

Cold 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. 10 μL from each sample was loaded and each gel was run at 15W for 2-4 hours according to the length of the gel. The gels were stained with EtBr for 10 min and destained in ddH_2O for 5 min. Densitometric analysis of the bands were performed 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 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.

2.4.4. *Statistical analyses*

The results from case and control groups were compared by χ^2 test withYate's Correction and Fisher's Exact Test. In addition, odd's ratio value for control and case groups were performed in 95% confidence interval.

2.5. Y CHROMOSOME STUDY

Microchimerism was reported to be associated with scleroderma; therefore fetal microchimerism in the blood of women with scleroderma was analyzed. In this study Y chromosome specific sequences were analyzed by PCR described elsewhere (Kogan *et al*. 1987; Patri *et al.* 1994). The total volume of reactions was 15µL. Approximately, 60-100 ng of DNA was amplified with 6.0 pmol of each primers, 0.2 mM of each dNTPs, 1X *Taq* polymerase buffer (750 mM Tris-HCl (pH 8.8 at 25°C), 200 mM $(NH_4)_2SO_4$, 0.1% (v/v) Tween 20), 1.0 mM $MgCl_2$, 10% DMSO (dimethyl sufoxide) and 1 Unit *Taq* polymerase. The sequence of primers were:
Y1(F): 5'-5'-TCCACTTTATTCCAGGCCTGTCC-3'; and
Y2(R): 5'-TTGAATGGAATGGGAACGAATGG-3'

2.6. HAPLOTYPE ANALYSIS OF AITDs FAMILY

2.6.1. *PCR*

It is determined that, the mother and the sister of the patient 04-445, whose XCI ratio was above 90%, were diagnosed with AITDs. Peripheral blood from all the members of the family (including the parents, affected and non-affected siblings) were obtained and DNA isolations were performed as indicated in section 2.3.1. In order to screen the X chromosome, Human MapPairs Version 10 was purchased from Research Genetics (currently available by Invitrogen, CA, USA) (Table 2.1). PCRs were performed in a total of 25 µL volume. 90-150 ng of DNA was amplified with 10 pmol of each primers, 0.6 mM of each dNTPs, 1X *Taq* polymerase buffer (750 mM Tris-HCl (pH 8.8 at 25°C), 200 mM $(NH_4)_2SO_4$, 0.1% (v/v) Tween 20), 1.5 mM $MgCl_2$, 10% DMSO (dimethyl sufoxide) and 1 Unit *Taq* polymerase. PCR conditions were as follows: 94°C 5 min initial denaturation step was followed by 30 cycles of 94°C 30 sec, 55°C 30 sec, 72°C 30 sec. amplification step. Final extension was performed at 72°C 5 min.

2.6.2. *Denaturing PAGE and silver staining*

PCR products were separated on 6% denaturing-PAGE. The gel was prepared by mixing 25 gr urea, 10 mL acrylamide:bisacrylamide solution (38:2), 6mL TAE (10X), 300 µL APS (10%) and TEMED. Total volume of the gel was 60 mL. Amplicons were denatured at 95°C for 5 mins and put immediately on ice. 5 µL of stop solution was mixed with the PCR products and approximately 4 µL was loaded on the gel. The gel was run at 60W for 2-3 hours according to the size of the amplicons.

Silver staining was performed according to the modifications of Bassam *et al.* First, the gel was incubated in fixation solution (10% glacial acetic acid) for 15 mins. Then it was washed with ddH_2O and shaked in $AgNO_3$ solution (0.1% $AgNO_3$, 2.5 mL formaldehyde (37%)) for 20 mins. Then the gel was incubated by shaking in developing solution (3% $NaCO_3$, 1.5 mL formaldehyde (37%), 0.02% sodium thiosulfate) until the bands become visible. The gel was immediately put into fixation solution.

Table 2.1 Primers used in X-chromosome screening for haplotype analysis.

CODE	FORWARD (5'→3')	REVERSE (5'→3')	AMPLICON SIZE (bp)
DXY218	GTCCTATGATTCATGATTG	GGCTGAGCTTCCTACAGCA	164
DXS1071	CCCAGATCGCNCCATT	ATGGCTCTGAGGCGGG	234-254
DXS9895	TTGGGTGGGGACACAGAG	CCTGGCTCAAGGAATTACAA	140
DXS8051	ACCAGAAATGAGCGATTATTG	TTTTTGAACTAAGAACCTGGAG	145
DXS8022	CTGTCACAGAAGTCCCATTTTA	GGAAACTAATGCAGCATGTC	160
DXS987	GTTGAGATAATGAGGCCAGT	ACNTTAAAAGCCTGGTTCTTCTAAA	206-244
DXS9902	TGGAGTCTCTGGGTGAAGAG	CAGGAGTATGGGATCACCAG	182
DXS8036	ATACAAACTGCCCCACTTCC	CTCTGGNTCCTGTCCTGG	125-135
DXS8019	TTCATTAAAAGCCGTCTTTG	TGTTGAGTTTCCTCACAGC	156
DXS999	GCTAACAACCTAGACTTCAACC	CAGTTTCACAATCTCTGCC	260-276
DXS7163	GACCATTATATNCGACTAACTTTTA	AGCTTTGGTGCAGTTTTTG	240-254
DXS6795	TGTCTGCTAATGAATGATTTGG	CCATCCCCTAAACCTCTCAT	284
DXS9896	CCAGCCTGGCTGTTAGAGTA	ATATTCTTATATTCCATATGGCACA	195
DXS8014	GGCAAAGTTGTCAGAGGC	CAAATGGCTTGTTCCAGTT	265-283
DXS6810	ACAGAAAACCTTTTGGGACC	CCCAGCCCTGAATATTATCA	209
GATA144D04	AGCCAGAAGGAGGAGGATTA	ATCTTTCTGTACTTGCAGTGAGG	241
DXS7132	AGCCCATTTTCATAATAAATCC	AATCAGTGCTTTCTGTACTATTGG	284
DXS6800	GTGGGACCTTGTGATTGTGT	CTGGCTGACACTTAGGGAAA	206
DXS6799	ATGAATTCAGAATTATCCTCATACC	GAACCAACCTGCTTTTCTGA	252
DXS9893	TGTCACGTTTACCCTGGAAC	TATTCTTCTATCCAACCAACAGC	111
GATA172D05	TAGTGGTGATGGTTGCACAG	ATAATTGAAAGCCCGGATTC	
GATA165B12	TATGTATCATCAATCATCTATCCG	TTAAAATCATTTTCACTGTGTATGC	133
DXS8078	TGCATCCCCATAGTAATTGGT	CAAATGGCAGGATTTCCC	202
GATA31E08	AGGGGAGAAGGCTAGAATGA	CAGCTGACAGAGCACAGAGA	248
DXS998	CAGCAATTTTTCAAAGGC	AGATCATTCATATAACCTCAAAAGA	113-119

2.7. CHEMICALS, REAGENTS AND ENZYMES

2.7.1. *Enzymes*

All the restriction digestion enzymes and *Thermus aquaticus* polymerase enzymes were purchased from MBI Fermentas (Amherst, NY, USA).

2.7.2. *Thermal Cyclers*

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

2.7.3. *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 100 ml with ddH_2O
Phosphate buffered saline (10XPBS):	*per liter*: 80 g NaCl, 2 g KCl, 14.4 g Na_2HPO_4, 2.4 g KH_2PO_4, pH: 7.4

2.7.4. *Chemicals and reagents*

Table 2.2 List of chemicals and reagents used in this study

Reagent or 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
Chelex 100	BioRad, CA, USA
dNTPs	MBI Fermentas, Amherst, NY, USA
DMSO	Sigma, St Lois, MO, USA
Nucleospin® Blood Kit	Macherey-Nagel Duren, Germany
EDTA	Carlo Elba, Milano, Italy
Ethanol	Merck, Frankfurt, Germany
Ethidium bromide	Sigma, St Lois, MO, USA
Formaldehyde	Sigma, St Lois, MO, USA
MetaPhor	FMC Bioproducts, Rockland, ME
pUC Mix 8 (DNA size marker)	MBI Fermentas, Amherst, NY, USA
TEMED	Sigma, St Lois, MO, USA
Tris-Base	Bio-Rad, CA, USA
Urea	Sigma, St Lois, MO, USA

2.7.5. *Oligonucleotides*

Primers were purchased from Iontek (Istanbul, Turkey). Only the primers that were used for haplotype analysis, Human MapPairs Version 10 was purchased from Research Genetics (currently available by Invitrogen, CA, USA)

CHAPTER 3

RESULTS

3.1. ADULT SAMPLES

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'.

3.1.1. *PCR-based X inactivation study of peripheral blood of Turkish control samples*

Among 160 controls, 124 were informative at *AR* locus. This number represents 77.5% of the control population. 10 of 124 informative control cases displayed skewed XCI (8.1%). Only 3 of 124 informative control women display extremely skewed XCI pattern (2.4%). It was well documented that extremely skewed XCI is a rare event in various group of control females (Busque *et al.* 1996, Sharp *et al.* 2000, Amos-Landgraf *et al.* 2006). Our results are consistent with these previous reports.

3.1.2.1 *PCR-based X inactivation study of peripheral blood of Turkish scleroderma patients*

XCI status was informative in 55 of 70 SSc patients (78.6%). Among informative patients, skewed XCI was observed in 35 of the 55 informative patients (63.6%), while in 10 of 124 control women (8.1%). Extremely skewed XCI ratio was observed in 27 of 55 informative patients (49.1%) and in 3 of 124 control women (2.4%) (P<0.0001 for both patterns). Odds ratio for skewed pattern of XCI was calculated as 20.0 with 95% confidence interval (CI) between 8.5 and 46.6. Considering only the extremely skewed pattern, the odds ratio was calculated as 38.9 (95% CI= 11.0-137.3). A representative gel image is displayed on Figure 3.1. Since the data for SSc patients is bimodal, the distribution of XCI profiles according to age was plotted (Figure 3.2). A shift toward the skewed range in older patients and controls was not observed.

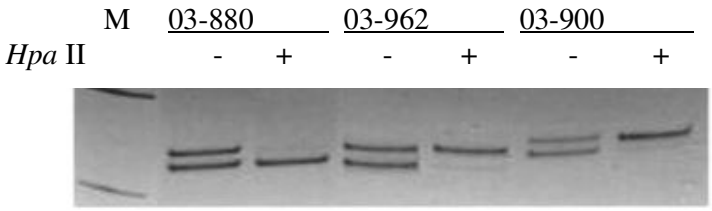

Figure 3.1 Gel image of XCI patterns of 3 scleroderma patients. Polymerase chain reaction products from the androgen receptor methylation assay demonstrate extremely skewed X chromosome inactivation patterns in samples. For each sample, DNA was either undigested (-) or digested (+) with the methylation-sensitive restriction enzyme *Hpa* II. Marker (M; pUC mix 8 [331-bp and 242-bp]) fragments are visible.

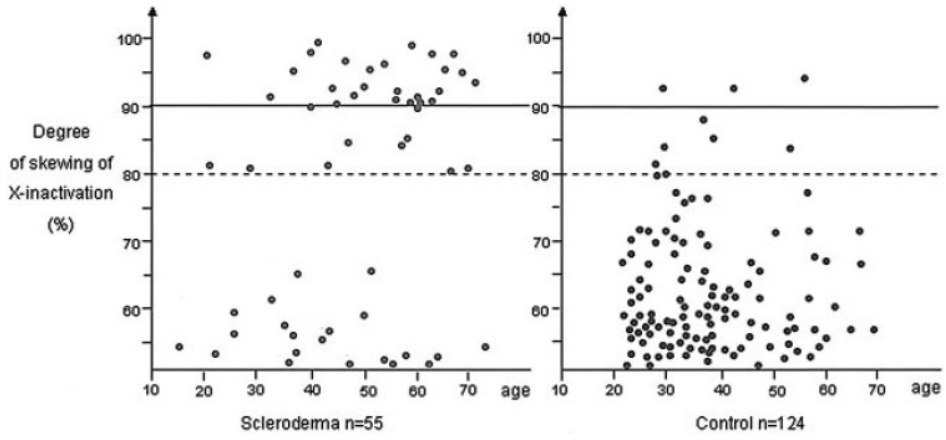

Figure 3.2 Distribution of X inactivation patterns according to age in scleroderma patients and control subjects.

3.1.2.2 *PCR-based X inactivation study of skin biopsy, buccal mucosa, and hair follicle samples of Turkish scleroderma patients*

From the patients whose XCI pattern of blood cells was above 90%, skin biopsy, buccal wash and hair samples were obtained in order to compare the XCI patterns of affected tissue and various tissue types from different embryonic cell lineages. Skin biopsy samples were obtained from the site of clinically detectable cutaneous changes in 5 patients (03-894, 03-899, 03-956, 03-963, and 03-1101). Buccal mucosa and hair follicle specimens were also collected from the same patients, excluding the patients 03-963. The skin biopsy, buccal mucosa, and hair follicle samples revealed random XCI pattern in all patients analyzed. The only exception was the XCI profile of buccal mucosa of 03-894 (Table 3.1). The gel image of blood and skin biopsy samples were represented in Figure 3.3.

Table 3.1 Blood, skin biopsy, buccal mucosa, and hair follicle XCI patterns of five Turkish SSc patients

Sample	**03-894**	**03-899**	**03-956**	**03-963**	**03-1101**
Blood	98:2	96:4	95:5	95:5	95:5
Skin biopsy	51:49	53:47	51:49	65:35	55:45
Buccal mucosa	95:5	71:29	57:43	-	65:35
Hair follicle	68:32	55:45	61:39	-	54:46

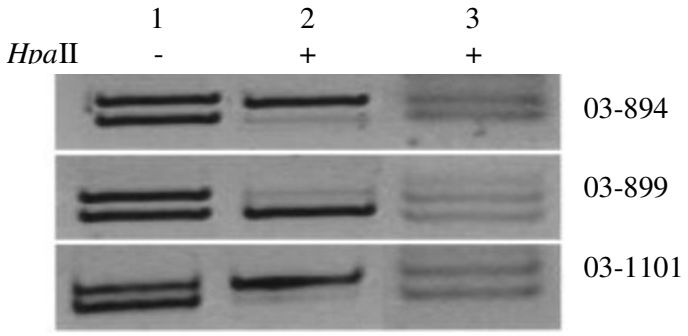

Figure 3.3. Skewed X chromosome inactivation in samples. Polymerase chain reaction products of undigested (−) (lane 1) and Hpa II–digested (+) DNA from blood (lane 2) and skin biopsy samples (lane3) of patients 03-894, 03-899, and 03-1101 are shown.

3.1.2.3 *Pregnancy history and Y chromosome analysis*

Clinical characteristics of the informative patients were displayed on Table 3.2. Since microchimerism in the blood of scleroderma patients was reported, Y chromosome sequences were investigated in the blood of scleroderma patients and control subjects. This analysis was performed in all patients without prior knowledge of the pregnancy history of the individuals. Among 39 patients who were

known to have at least one son Y chromosome specific DNA was detected in 16 patients (41%). No history of pregnancy was in 23 patients. In the control group 31 women were known to have at least one son and the Y chromosome specific sequence was detected only 2 of them (6.5%) (P=0.0026). The results of Y chromosome specific sequence analysis were summarized in Table 3.3. Difference in persistence of fetal DNA in peripheral blood of patients and control group is statistically significant and consistent with the previous reports (Nelson *et al.* 1998, Artlett *et al.* 1998). However, distribution of Y chromosome specific DNA was not significantly different between the women with skewed and random XCI

Table 3.2 Clinical characteristics of Turkish SSc patients with skewed XCI.

No	Code	DOB	YDO	PH	Sex and birth year of children
>90% skewing					
1	03-894	1945	1989	G0, P0	-
2	03-963	1938	1998	G0, P0	-
3	03-879	1966	1991	G0, P0	-
4	03-1123	1950	1990	G0, P0	-
5	03-951	1986	2003	G0, P0	-
6	03-881	1971	1990	G0, P0	-
7	03-892	1965	1993	G0, P0	-
8	03-1112	1963	1997	GII, PI	F 1998
9	03-899	1953	1998	GI, PI	F 1977
10	03-948	1959	1989	GII, PII	M 1971, M 1975
11	03-954	1965	2000	GII, PII	M 1998, F 1999
12	03-886	1960	1994	GII, PII	M 1986, F 1989
13	03-1120	1950	1983	GII, PII	M 1970, F 1973
14	03-890	1956	1989	GII, PII	M 1989, F 1992
15	03-895	1941	1984	GIV, PII	M 1959, F 1969
16	03-880	1967	2002	GII, PII	F 1987, M 1990
17	03-888	1952	1996	GII, PII	M 1973, F 1977
18	03-962	1946	1993	GIII, PIII	F 1968, F 1970, M 1973
19	03-878	1945	2000	GIII, PIII	F 1968, F 1971, M 1977
20	03-893	1936	1984	GIX, PIV	F 1953, M 1954, F 1956, M 1962
21	03-1110	1932	1993	GVI, PIV	F 1952, F 1954, M 1962, M 1964
22	03-956	1956	2001	GV, PIV	F 1973, F 1975, M 1977, M 1980
23	03-953	1941	1995	GIV, PIV	M 1967, M 1969, F 1974, F 1976
24	03-884	1938	1994	GVI, PIV	M 1965, M 1969, F 1973, F 1975
25	03-900	1940	1984	GVII, PV	M 1955, F 1960, F 1962, M 1966, M 1974
26	03-1101	1941	1993	GV, PV	F 1957, F 1959, M 1963, M 1968, M 1969
27	03-952	1938	1984	GVI, PV	M 1960, M 1964, F 1969, F 1971, F 1973
80–90% skewing					
1	03-877	1954	1999	GII, P0	-
2	03-1104	1956	1983	G0, P0	-
3	03-949	1975	2000	G0, P0	-
4	03-1128	1983	2002	G0, P0	-
5	03-943	1942	1999	GII, PII	M 1965, M 1969
6	03-950	1936	1982	GIV, PIV	M 1953; M 1955, M 1964, F 1969
7	03-1122	1949	1993	GVIII, PV	M 1966, F 1968, M 1970, M 1978, F 1982
8	03-885	1933	1993	GVII, PVII	F 1960, F 1961, F 1963, F 1964, F 1966, M 1968, M 1971
60-69% skewing					
1	03-1113	1965	1994	GVI, PV	M 1990, F 1991, M 1992, F 1996, M 1998
2	03-1126	1951	1993	G0, P0	-
3	03-958	1976	1993	G0, P0	-
4	03-957	1970	1991	G0. P0	-
5	03-1111	1952	1991	G2, P2	M 1980, M 1983
50-59% skewing					
1	03-1114	1967	1995	G3, P3	M 1996, F 2000, M 2001
2	03-1132	1977	1993	G1, P1	F 2002
3	03-947	1961	1988	G0, P0	-
4	03-1115	1961	1989	G3, P2	M 1986, F 1991
5	03-1118	1966	2000	G5, P4	F 1980, M 1985, F 1989, M 1995
6	03-1131	1929	1999	G0, P0	-
7	03-944	1945	1987	G7, P6	M 1963, M 1969, M 1972, F ?
8	03-955	1947	1996	G3, P2	M 1969, F 1973
9	03-887	1946	1989	G2, P2	M 1965, M 1969
10	03-1103	1940	1993	G2, P2	M 1961, F 1966
11	03-945	1979	2003	G1, P0	M 2000
12	03-898	1967	1998	G0, P0	-
13	03-1117	1956	2001	G1, P1	F 1990
14	03-1125	1940	1978	G2, P2	M 1962, F 1965
15	03-1127	1967	2000	G0, P0	-

DOB: Date of Birth, YDO: Year of Disease Onset, PH: Pregnancy History, G:Gravida, P: Para, F: female, M: male.

Table 3.3 Distribution of Y chromosome sequences in Turkish SSc patients and controls who gave birth to male children

Gorup	Positive no (%)	Negative no (%)	Total no
SSc patients	16 (41.0)	23 (59.0)	39
Skewed XCI	9 (40.9)	13 (59.1)	22
Random XCI	4 (36.4)	7 (63.6)	11
Control females	2 (6.5)	29 (93.6)	31
Skewed XCI	1 (25.0)	3 (75.0)	4
Random XCI	1 (4.16)	23 (95.8)	24

3.1.3.1. *PCR-based X inactivation study of peripheral blood of US scleroderma patients*

Among 125 Scleroderma patients, 94 were informative (75.2%). Extremely skewed ratio was observed in 17 of 94 informative patients (18.1%). When these results were compared with Turkish control group, the ratio was 2.4% (only 3 samples in 124 informative controls). One of the representative gel images is depicted in Figure 3.4.

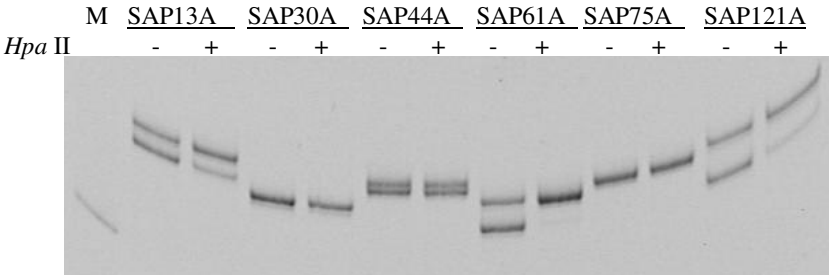

Figure 3.4 Gel image of XCI patterns of 6 US scleroderma patients. Polymerase chain reaction products from the androgen receptor methylation assay demonstrate X chromosome inactivation patterns in samples SAP13A (allele ratio:78%:22%), SAP30A (not informative), SAP44A (52%:48%), SAP61A (100%:0%), SAP75A (not informative), and SAP121A (88%12%). For each sample, DNA was either undigested (-) or digested (+) with the methylation-sensitive restriction enzyme *Hpa* II. Marker (M; pUC mix 8 [only 242-bp]) fragment is visible.

These results are statistically significant, since P<0.0001 and odds ratio=8.9 (95%CI is between 2.5 and 30.6). If skewed XCI pattern was taken into account, the skewing ratio was observed in 32 of 94 informative patients, yet only in 10 of 124 informative control (34.0% and 8.1% respectively) (P<0.0001) (odds ratio=5.9, CI 95%=2.7-12.8). These results were consistent with the results of Turkish scleroderma patients. XCI patterns of US and Turkish patients and their combined results were indicated in Table 3.4.

Table 3.4 Proportions of scleroderma patients and controls with skewed XCI.

	No. (%) observed with skewing			
Degree of skewing (%)	**US SSc patients (n=94)**	**TR SSc patients (n=55)**	**Combined (n=149)**	**Control (n=124)**
+90	17 (18.1%)	27 (49.1%)	44 (29.5%)	3 (2.4%)
80-89	15 (16.0%)	8 (14.5%)	23 (15.4%)	7 (5.6%)
70-79	15 (16.0%)	0 (0.0%)	15 (10.1%)	22 (17.7%)
60-69	24 (25.5%)	5 (9.1%)	29 (19.5%)	29 (23.4%)
50-59	23 (24.5%)	15 (27.3%)	38 (25.5%)	63 (50.8%)

For comparison by $\chi 2$, P<0.0001(both >80% skewing and >90% skewing)

3.1.3.2. *PCR-based X inactivation study of peripheral blood of US scleroderma patient-mother pairs*

To determine the parental origin of inactive X chromosome, 19 patients with skewed XCI profile for whom maternal DNA was available were analyzed. 10 in 19 patient-mother pairs were informative for the *AR* gene polymorphism. In the remaining 9 pair both the patients and their mothers were heterozygous for the *AR* gene polymorphism however no difference between allele sizes was observed. By this way it was not possible to determine the parental origin of inactive X chromosome. In 10 informative mother-daughter pairs, the inactive X chromosome was maternal origin in 8 patients and paternal origin in 2 patients (P=0.055). This result is on the border of statistical significance. Unfortunately the number of available samples from patient-mother pairs is small. XCI pattern was skewed in 3 mothers of patients (Table 3.5). Interestingly, the same allele was skewed in both the mothers and daughters. One of these mothers had been diagnosed with temporal arteritis, which is an autoimmune condition.

Table 3.5 Parental origin of the inactive X chromosome in SSc patients with skewed XCI

Sample	Degree of skewing	Parental origin of the inactive X chromosome
Patient 1	95	Maternal
Mother 1	95	
Patient 2	95	Maternal
Mother 2	95	
Patient 3	86	Maternal
Mother 3	88	
Patient 4	100	Maternal
Mother 4	Not informative	
Patient 5	90	Maternal
Mother 5	Not informative	
Patient 6	90	Maternal
Mother 6	Not informative	
Patient 7	85	Maternal
Mother 7	67	
Patient 8	85	Maternal
Mother 8	58	
Patient 9	85	Paternal
Mother 9	70	
Patient 10	80	Paternal
Mother 10	70	

3.1.3.3. *Evaluation of skewed XCI for correlation with microchimerism*

Pregnancy-related microchimerism has been reported in scleroderma (Artlett *et al.* 1998, Nelson *et al.*1998). Therefore US patients for whom microchimerism data was available were investigated for correlation of skewed XCI with fetal and maternal microchimerism. Among the patients with skewed XCI pattern, 12 patients were tested for maternal microchimerism (MMc), 14 patients were tested for fetal

microchimerism (FMc). Positive MMc was observed in 7 of the 12 skewed patients (58.3%), and positive FMc was observed in 8 of the 14 skewed patients (57.1%) (Table 3.6). Same analyses were performed for patients with random XCI pattern. Among 15 patients, 10 of them were positive for MMc (66.7%), and 14 of 25 patients were positive for FMc (56.0%) (P=1.0). Neither maternal nor fetal microchimerism was found to be correlated with skewed XCI pattern.

Table 3.6 Proportion of maternal (MMc) and fetal (FMc) microchimerism in US SSc patients

	MMc			FMc	
Skewed (>80 %)	12 tested	58.3% positive for MMc	**Skewed (>80 %)**	14 tested	57.1% positive for FMc
	7 positive 5 negative			8 positive 6 negative	
Random (<80 %)	15 tested	66.7% positive for MMc	**Random (<80 %)**	25 tested	56.0% positive for FMc
	10 positive 5 negative			14 positive 11 negative	

3.1.4.1. *PCR-based X inactivation study of peripheral blood of Turkish autoimmune thyroid diseases patients*

XCI results were informative in 83 of the 110 AITDs patients (75.5%). Skewed XCI was observed in 28 of 83 informative patients (33.80%). Extremely skewed XCI was found in 16 of 83 informative patients (19.3%). When the results of AITDs patients were compared with the results of Turkish control group, a significant difference was observed (P<0.0001 for both type of patterns) (Table 3.7). Odds ratio is 5.8 (95% CI=2.6-12.8) for skewed XCI and 9.6 (95%CI=2.7-34.3) for extremely skewed XCI. When the XCI profiles of patients diagnosed with Hashimoto's and Grave's diseases- were analyzed independently, the difference in XCI patterns

between patients and controls was still statistically significant. In 23 of 67 informative Hashimoto's patients (34.3%; P<0.0001) and 5 of 16 informative Graves' patients (31.3%; P=0.0167) skewed XCI was observed. The data in Turkish AITDs patients is bimodal, therefore a distribution of the XCI profiles according to age was plotted. However, a shift towards the skewed range in older patients and controls was not observed (Figure 3.5).

Table 3.7 Proportion of Turkish AITDs patients and controls with skewed XCI

Degree of skewing (%)	No. (%) observed with skewing	
	AITDs patients (n=83)	Control (n=124)
+90	16 (19.3%)	3 (2.4%)
80-89	12 (14.5%)	7 (5.6%)
70-79	6 (7.22%)	22 (17.8%)
60-69	16 (19.3%)	29 (23.4%)
50-59	33 (39.8%)	63 (50.8%)

For comparison by χ^2, P<0.0001(both >80% skewing and >90% skewing)

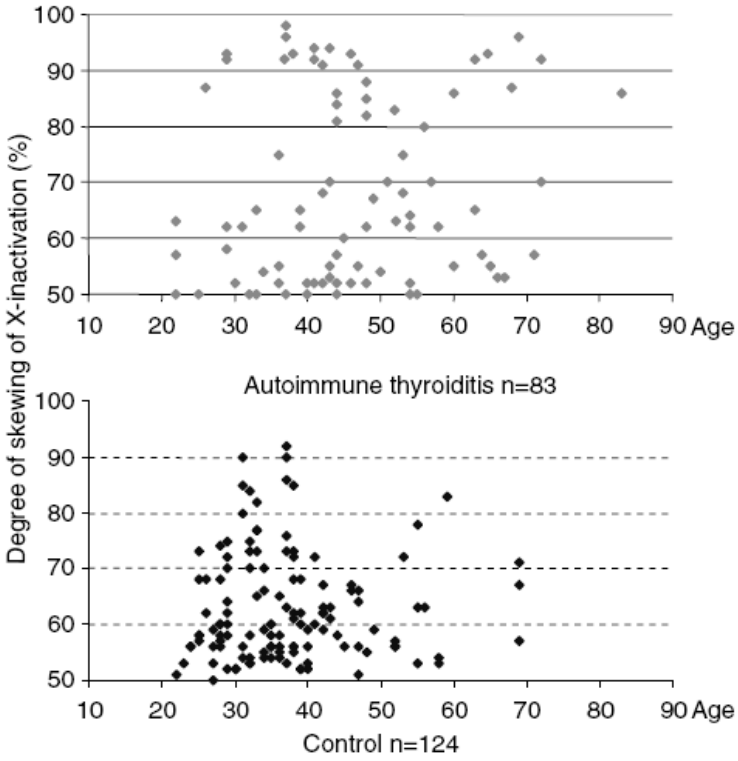

Figure 3.5 Distribution of X-inactivation patterns according to age in AITDs patients and control subjects.

3.1.4.2. *PCR-based X inactivation study of thyroid biopsy, buccal mucosa, and hair follicle samples of Turkish autoimmune thyroid diseases patients*

In order to analyze the XCI profile of affected tissue, thyroid biopsy specimen in this case, and other tissue types, DNA from buccal mucosa, thyroid biopsy, and hair follicle were obtained from five patients (04-121, 04-198, 04-214, 04-221, and 04-225). These patients were randomly selected. Their blood XCI profile displayed skewed XCI (Figure 3.6). Five patients displayed skewing in the same direction for thyroid biopsy and buccal mucosa. The skewing was less marked in the thyroid than in blood and buccal mucosa cells. Hair follicles showed random XCI pattern in all patients that were analyzed (Table 3.8).

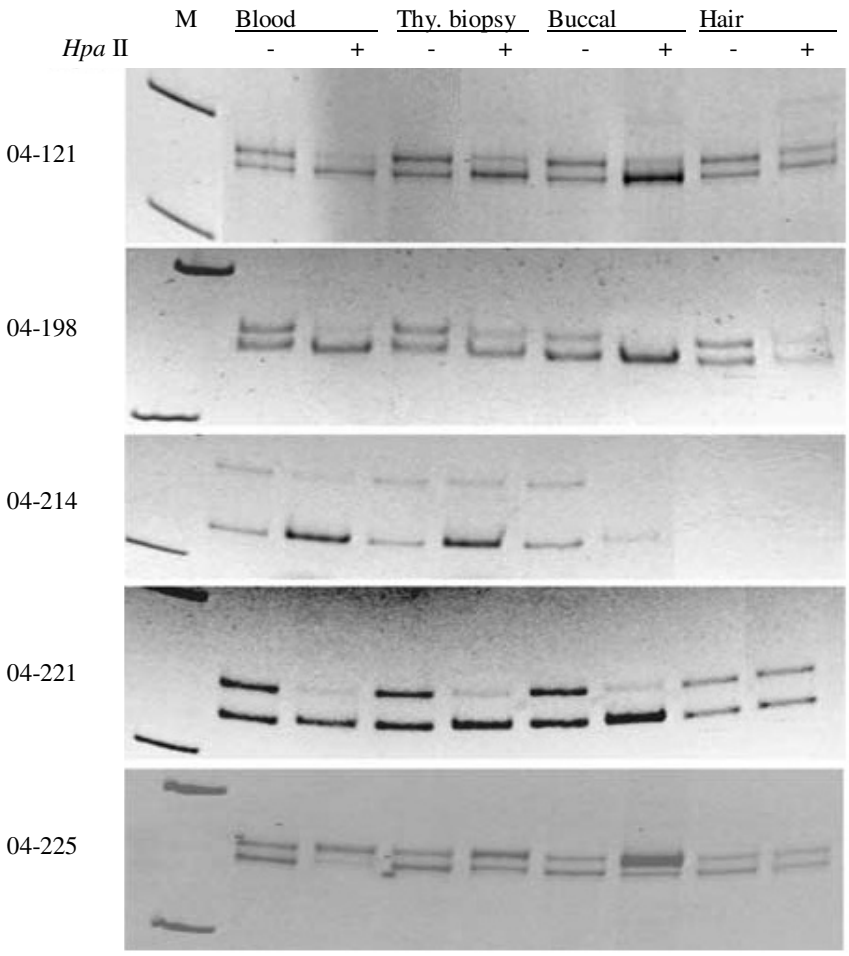

Figure 3.6 X- inactivation analysis of androgen receptor locus in five AITDs patients. PCR products of undigested (-) and *Hpa* II digested (+) DNA from peripheral blood, thyroid biopsy, buccal mucosa, and hair follicle samples were depicted.

Table 3.8 Blood, thyroid biopsy, buccal mucosa, and hair follicle XCI patterns of five Turkish AITDs patients

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

3.1.4.3. *Pregnancy history and pedigree analysis*

Characteristics of informative AITDs patients were displayed in Table 3.9. Only the patients with a complete pregnancy and family history were included. There were two features that differ in the pedigrees of AITDs patients with skewed and random XCI. The first one was recurrent spontaneous abortion (RSA) which is defined as three or more pregnancy losses. It was reported that RSA has been associated with skewed XCI (Sangha *et al.* 1999, Pegoraro *et al.* 1997). In 4 of 25 (16%) AITDs patients with skewed XCI, RSA was occurred, whereas a history of RSA was negative for both AITDs patients with random XCI and control women (P=0.0199). The etiology of RSA in AITDs is still unknown, however two-four fold of increase in the miscarriages were reported in the first trimester of pregnancy of women with thyroid antibodies (Stagnaro-Green&Glinoer 2004, Prummel&Wiersinga 2004). The second feature was the positive family history which was present in 12 of the 25 (48%) probands with skewed XCI and in 10 of 39 (25.6%) patients with random XCI. In order to extent the XCI study in the family members contact was set up with all of the 12 probands. Initially, a positive response was received from 3 families, however only the blood samples from the family members of two probands could be obtained (04-445; proband of family 1 and 04-298; proband of family 2). In both families, only the affected individuals displayed skewed XCI patterns and the nonaffected members display random XCI pattern. In family 1, the inactive X chromosome is of maternal origin. In family 2, the XCI pattern of the affected sister

of proband 04-298 was skewed however; her mother was not informative for the *AR* polymorphism and the inactive X chromosome was of paternal origin. Therefore, only the members of family 1 were investigated for haplotype analysis.

Table 3.9 Clinical characteristics and XCI status of Turkish AITD patients

No	Patient	SOD	DOB	OOD	PH	S&BDOC	FH
+90% skewing							
1	04-136	GD	1975	2004	G0, P0, A0	-	-
2	04-127	HT	1975	2003	G0, P0, A0	-	-
3	04-138	HT	1962	1980	G7, P0, A7	-	2 sisters
4	04-298	HT	1979	2003	G1, P1, A0	F03	Mother, 1 sister
5	04-445	HT	1961	2000	G1, P1, A0	F88	Mother, 1 sister
6	04-198	HT	1935	2004	G2, P2, A0	M68, M72	-
7	04-221	HT	1958	2000	G4, P2, A2	M91, F94	2 sisters
8	04-250	HT	1967	1996	G7, P2, A5	M89, M93	1 son
9	04-226	HT	1963	2004	G3, P3, A0	M83, F88, M98	1 sister
10	04-233	GD	1967	1990	G4, P3, A1	M88, F94, M01	-
11	04-121	HT	1957	1988	G7, P4, A3	F78, M83, F91, F94	-
12	04-205	HT	1927	2003	G6, P5, A1	M47, M50, F52, F53, M55	-
13	04-225	HT	1936	1975	G6, P6, A0	F56, F58, F60, F62, M64, M66	1 daughter
80-89% skewing							
14	04-132	HT	1960	2002	G0, P0, A0	-	Mother, 1 sister
15	04-223	HT	1956	1988	G0, P0, A0	-	Mother, 1 sister
16	04-105	GD	1978	1999	G5, P1, A4	M98	-
17	04-131	HT	1944	2002	G3, P2, A0	M69, M71	-
18	04-120	HT	1956	1994	G3, P3, A0	F75, M77, F78	-
19	04-107	HT	1948	1998	G4, P3, A1	F70, F72, F76	-
20	04-98	HT	1956	2000	G8, P3, A1	F79, F81, F87	2 daughters
21	04-218	HT	1941	1991	G4, P3, A1	M61, F63, F67	-
22	04-108	HT	1952	1999	G5, P3, A2	F77, F78, M83	-
23	04-208	HT	1960	1999	G5, P3, A0	F83, F85, M88	Mother
24	04-110	GD	1960	1998	G4, P4, A0	M80, M83, M85, F96	-
25	04-214	HT	1921	1999	G9, P8, A1	F44, M45, F47, F48, M54, F56, F58, M60	1 daughter
70-79% skewing							
26	04-203	HT	1961	2004	G3, P1, A0	M82	-
27	04-230	HT	1951	1999	G2, P2, A0	M77, M86	1 son
28	04-213	HT	1947	1998	G7, P3, A0	F67, M68, M71	-
29	04-228	HT	1953	2001	G3, P3, A0	M71, M73, M82	-
30	04-137	HT	1932	1981	G5, P4, A0	F50, F53, M55, F59	-
60-69% skewing							
31	04-206	GD	1946	1964	G1, P0, A1	-	-
32	04-92	HT	1971	1998	G3, P1, A0	F96	Mother
33	04-240	HT	1975	2003	G2, P1, A0	M00	-
34	04-139	HT	1959	2002	G1, P1, A0	M97	-

35	04-257	HT	1973	2004	G3, P2, A1	M95, M01	-
36	04-112	HT	1952	1999	G2, P2, A0	F72, F77	Mother
37	04-220	GD	1955	1998	G3, P2, A0	M74, M78	-
38	04-103	HT	1962	1986	G6, P2, A1	F82, M92	-
39	04-251	HT	1941	1984	G5, P3, A2	M60, M63, M65	2 sisters
40	04-99	HT	1961	1997	G6, P3, A2	F81, F85, F87	Mother, 1 sister
41	04-224	GD	1950	2001	G6, P5, A1	M69, M72, F73, M75, F78	-
50-59% skewing							
42	04-96	HT	1939	1996	G0, P0, A0	-	-
43	04-242	HT	1960	1999	G0, P0, A0	-	-
44	04-129	HT	1982	1998	G0, P0, A0	-	Mother
45	04-196	HT	1956	1999	G6, P1, A0	F93	-
46	04-231	HT	1964	2003	G1, P1, A0	M93	-
47	04-201	GD	1971	2001	G2, P1, A1	F93	Mother
48	04-95	HT	1975	2004	G3, P2, A0	F98, M00	-
49	04-239	HT	1951	2003	G3, P2, A0	M68, M75	-
50	04-246	HT	1961	1996	G2, P2, A0	M78, F81	-
51	04-200	HT	1954	1992	G5, P2, A0	M73, M75	3 sisters
52	04-237	HT	1970	2004	G2, P2, A0	M93, F97	-
53	04-102	HT	1964	2003	G3, P2, A1	F95, F87	-
54	04-204	HT	1949	2002	G4, P2 A0	F71, M73	-
55	04-93	GD	1960	2003	G2, P2, A0	M91, F94	-
56	04-116	GD	1960	2003	G4, P2, A0	F86, M89	1 brother
57	04-197	HT	1961	1976	G6, P3, A2	M82, M84, M98	-
58	04-229	HT	1938	1980	G3, P3, A0	M61, M63, F65	1 sister
59	04-255	HT	1974	1993	G3, P3, A0	F92, M96, F01	-
60	04-212	GD	1958	2002	G4, P3, A0	M76, F80, M84	-
61	04-238	HT	1939	2002	G6, P4, A0	M61, M65, F67, M72	-
62	04-117	HT	1944	2004	G6, P4, A0	M60, M64, F66, F67	-
63	04-211	GD	1950	2002	G6, P6, A0	F66, F67, F72, M84, M85, M86	-
64	04-243	GD	1937	1994	G12, P7, A1	M57, M59, F60, F62, M65, M67, F68	-

SOD: state of disease, DOB: date of birth, OOD: onset of disease, PH: pregnancy history, S&BDOC: sex and birth date of children, FH: family history, HT: Hashimoto's thyroiditis, GD: Grave's disease, G: gravida, P: para; A: abortion, M: male, F: female

3.1.4.4. *Haplotype analysis*

Haplotype analysis was performed in the members of family 1 by using polymorphic X-chromosomal markers to determine possible segregation between the disease and marker alleles because XCI segregated as a heritable trait associated with AITDs in two generations. The image of haplotype structure was displayed in Figure 3.7. The size of the family was not large enough to prove linkage yet it still

provided valuable information about the exclusion area on X chromosome. A minimal critical region on X chromosome 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 and discordance among the nonaffected individuals indicating positive segregation between the disease and marker alleles. Unfortunately, Formal acceptance of linkage to any loci could not be achieved mainly due to the small size of the family (Abecasis. *et al.* 2002).

The genes located on the mininmal critical regions were listed. BioMart tool (http://www.ensembl.org) was used to list the candidate genes. Only the known protein coding genes were included in this analysis. In the region between the markers DXS8051 and DXS8036 on Xp22 48 known protein coding genes were listed. 183 genes were listed in the region between the marker DXS8014 and the *AR* gene on Xp11-q13. Among these candidate genes listed the ones that code proteins with antigenic property were searched. A variety number of genes that encode proteins that belong to cancer-testis antigen (*XAGE, GAGE, SSX*) and melanoma antigen (*MAGE*) family were present on only Xp11-q13 region. The MAGE gene encoded proteins are members of cancer-testis antigen group. They are reported to be potential targets for tumor immunotherapy (Scanlan *et al.* 2004). Interestingly, Forkhead Box P3 (*FOXP3*) a member of forkhead/winged-helix family of transcriptional regulators. A frameshift mutation in *Foxp3* gene results failure of coding the protein lacking the forkhead domain. Brunkow *et al.* (2001) reported that defective *Foxp3* was present in 'scurfy' (sf) mice and this gene is essential for normal immune homeostatis. Marson *et al.* (2007) reported that many of the target proteins of FOXP3 suppress the activation of target genes on T cell stimulation. Suppression of target genes of FOXP3 was reported for the normal function of regulatory T cells since the overexpression of these target genes were to be documented to be associated with autoimmune diseases. An arg397-to-trp mutation in the FOXP3 gene was to be reported to be the cause of immunodysregulation, polyendocrinopathy, and enteropathy, X-linked (IPEX) syndrome (Wildin *et al.* 2001).

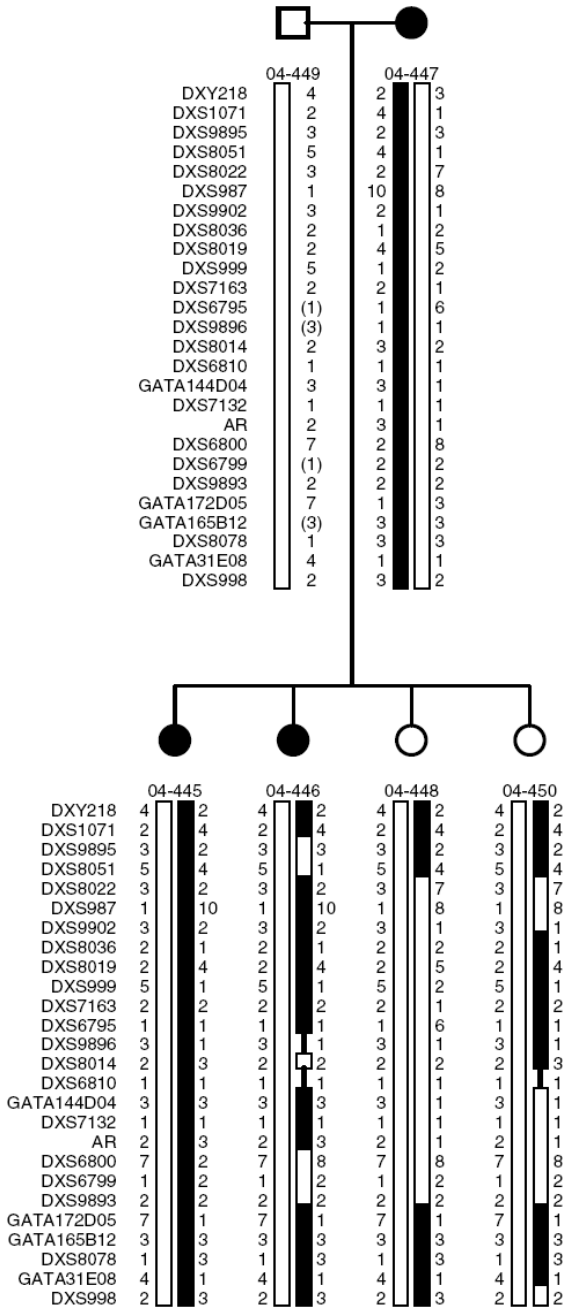

Figure 3.7 Haplotype structure of AITDs Family. Patient 04-445 was arbitrarily selected to construct the haplotype. Maternally inherited haplotype was highlighted with solid black bar. Haplotypes of the remaining sibs were compared with the reference individual (04-445), and shared portions were also 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.

3.1.5. *PCR-based X inactivation study of peripheral blood of Tunisian autoimmune thyroid diseases patients*

Informative XCI pattern was observed in 100 of 145 AITDs patients (69.0%). Extremely skewed XCI was observed in 14 of 100 informative patients (14.0%). The same pattern was observed in 4 of 46 informative Tunisian controls (8.7%). Since the number of informative control individuals was not enough (n=46), the Turkish and Tunisian control samples were combined and XCI results of Tunisian patients were compared with this combined group (n=170). Extremely skewed XCI was observed in 7 of the 170 (4.1%) informative controls in combined cohort (P=0.0046, odds ratio=3.8 with 95%CI between 1.5 and 9.8). Skewed XCI was present in 26 of 100 informative patients (26.0%) while in 19 of 170 informative controls (11.2%). These results were statistically significant (*P*=0.0022; OR:2.8, 95%CI=1.5-5.4), and were concordant with XCI results of Turkish AITDs patients. Representative gel image of XCI pattern of Tunisian AITDs patients was displayed in Figure 3.8.

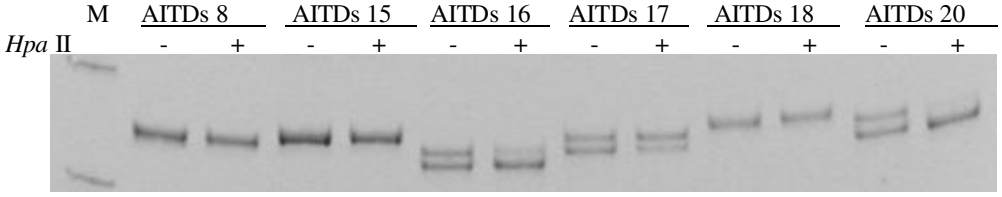

Figure 3.8 Gel image of XCI patterns of 6 Tunisian AITDs patients. Polymerase chain reaction products from the androgen receptor methylation assay demonstrate X chromosome inactivation patterns in samples AITDs 8 (allele ratio: not informative), AITDs 15 (not informative), AITDs 16 (78%:22%), AITDs 17 (68%:32%), AITDs 18 (not informative), and AITDs 20 (90%10%). 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]) fragment is visible.

Tunisian AITDs patients were categorized according to their clinical phenotype. Among 100 informative AITDs patients, 46 were positive and 54 were negative for thyroid autoantibody status. Skewed XCI in those patients (elevated anti-thyroglobulin and/or anti-thyroid peroxydase) was 39.1% (18 of 46) (*P*=0.01). The frequency of extremely skewed XCI was 21.7% (10 of 46) (*P*=0.03). When the patients who were negative for thyroid autoantibodies in their blood were considered, the skewed XCI pattern was observed in 8 of 54 informative subjects (14.8%, *P*=0.48) and extremely skewed XCI was observed in 4 of 54 informative patients (*P*=0.3). These results indicated that in the subgroup of patients who had elevated anti-thyroglobulin and/or anti-thyroid peroxydase in their blood skewed there might be association between the disease and XCI. The results of skewed XCI in AITDs patients, and control subjects are summarized in Table 3.10 according to the autoantibodies they contain in serum. Subdividing according to the clinical phenotype of AITDs patients with positive autoantibodies revealed that the frequency of skewed XCI was 38.8% (7 of 18) in GD, 40% (4 of 10) in PIM and 38.8% (7 of 18) in HT. These results were not significantly different from the female controls (*P*=0.05, *P*=0.09 and *P*=0.05) respectively.

Table 3.10 Correlation of XCI patterns and thyroid autoantibodies in Tunisian AITDs patients

Degree of skewing (%)	No. (%) observed with skewed		
	Autoantibodies (+) (n = 46)	Autoantibodies (-) (n = 54)	Control females (n=170)
90+	10 (21.7)	4 (7.4)	7 (4.1)
80-89	8 (17.4)	4 (7.4)	12 (7.1)
70-79	9 (19.5)	14 (25.9)	29 (17.1)
60-69	8 (17.4)	14 (25.9)	36 (21.2)
50-59	11 (24)	18 (33.4)	86 (50.6)

For comparison by $\chi 2$, $P = 0.01$ (>80% skewing); $P = 0.03$ (>90% skewing) (Autoantibodies+)
$P = 0.48$ (>80% skewing); $P = 0.3$ (>90% skewing) (Autoantibodies+)

3.1.6. *PCR-based X inactivation study of peripheral blood of Tunisian rheumatoid arthritis patients*

Informative XCI was observed in 76 of the 106 RA patients (71.7%). Skewed XCI was observed in 26 of the 76 informative patients (34.2%) whereas the same pattern was observed in 19 of 170 controls (11.2%). More importantly, extremely skewed XCI was present in 17 of the 76 patients (22.4%) while only in 7 of the 170 controls (4.1%) (P<0.001 for both skewed and extremely skewed patterns). The results of XCI patterns of RA patients and controls were summarized in Table 3.11. These results are statistically significant since odds ratio is 6.7 (95%CI=2.7-17.0) for extremely skewed XCI and 4.1 (95%CI=2.1-8.1) for skewed XCI patterns. A representative gel image of XCI pattern of Tunisian RA patients was displayed in Figure 3.9. Age alone is unlikely to influence the strikingly bimodal data in Tunisian RA patients (Figure 3.10). We did not observe a shift towards the skewed range in older patients and controls.

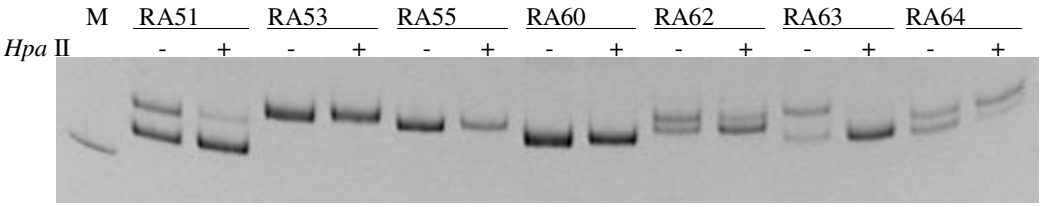

Figure 3.9 Gel image of XCI patterns of 7 Tunisian RA patients. Polymerase chain reaction products from the androgen receptor methylation assay demonstrate X chromosome inactivation patterns in samples RA51 (allele ratio: 80%:20%), RA53, RA55, RA60 (not informative), RA62 (69%:31%), RA63 (100%:0%), RA64 (80%:20%). For each sample, DNA was either undigested (-) or digested (+) with the methylation-sensitive restriction enzyme *Hpa* II. Marker (M; pUC mix 8 [only 242-bp]) fragment is visible.

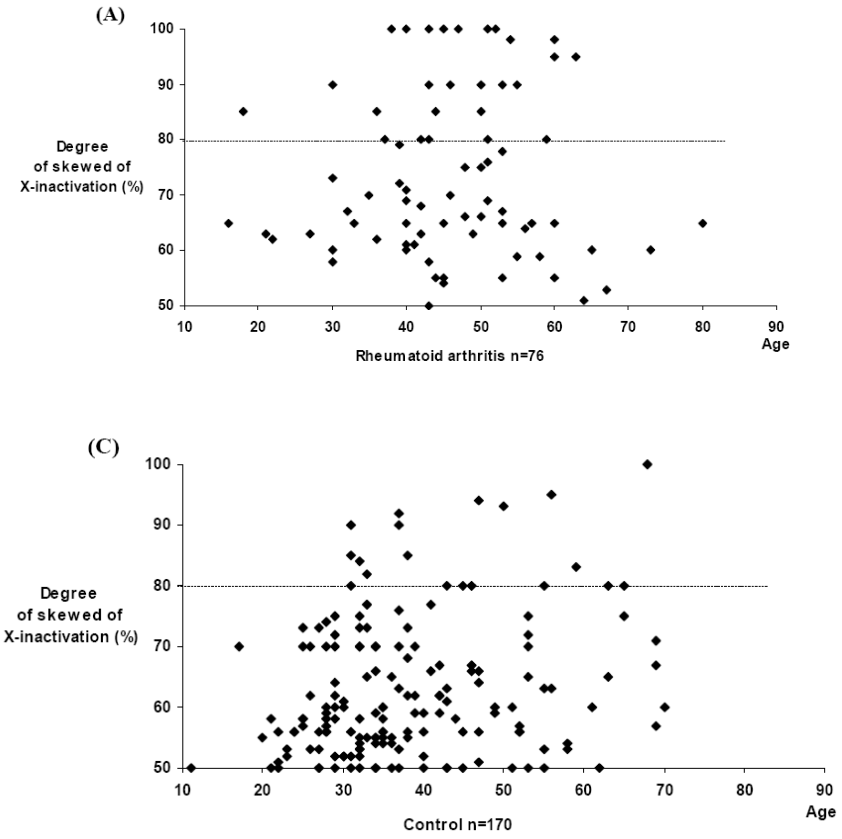

Figure 3.10 Distribution of X-inactivation patterns according to age in Tunisian RA patients and control subjects.

Table 3.11 Proportion of Tunisian RA patients and controls with skewed XCI

Degree of skewing (%)	No. (%) observed with skewed	
	Rheumatoid arthritis (n = 76)	Control females (n = 170)
90+	17 (22.4)	7 (4.1)
80-89	9 (11.8)	12 (7.1)
70-79	11 (14.5)	29 (17.1)
60-69	28 (36.8)	36 (21.2)
50-59	11 (14.5)	86 (50.6)

For comparison by $\chi 2$, $P<0.0001$(both >80% skewing and >90% skewing)

3.1.7. *PCR-based X inactivation study of peripheral blood of members of Tunisian Akr family*

Informative XCI was observed in 28 of 44 members of Akr Family (63.6%). Interestingly, in none of the members of Akr Family extremely skewed XCI was observed. The pedigree of subfamilies of Akr Family was represented in Appendix. A representative PAGE image is displayed on Figure 3.11. The skewed XCI was observed only in 3 members of the 28 informative subjects (10.7%) and in 19 of 170 controls (11.2%) (*P*=1.0). This value is not statistically significant. Among the 44 women members of Akr Family, 20 were diagnosed with AITDs. When only the patients were considered, 11 of 20 subjects were informative. Segregation between the disease and skeweded XCI pattern was not observed in the members of this family. Skewed XCI profile was observed only in one of the 11 informative patients. The summary of the results of XCI patterns in Akr Family can be found in Table 3.12.

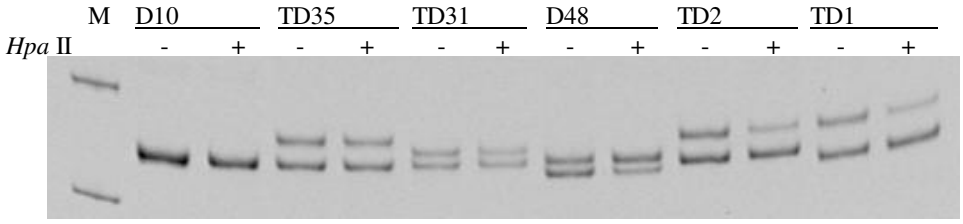

Figure 3.11 Gel image of XCI patterns of 6 members of Tunisian Akr Family. Polymerase chain reaction products from the androgen receptor methylation assay demonstrate X chromosome inactivation patterns in samples D10 (allele ratio: not informative), TD35 (55%:45%), TD31 (56%:44%), D48 (71%:29%), TD2 (70%:30%), and TD1 (73%:27%). 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]) fragment is visible.

Table 3.12 Proportion of Tunisian Akr Family members and controls with skewed XCI

Degree of skewing (%)	No. (%) observed with skewed	
	Akr Family (n = 28)	Control females (n = 170)
90+	0 (0.0)	7 (4.1)
80-89	3 (10.7)	12 (7.1)
70-79	12 (42.9)	29 (17.1)
60-69	5 (17.9)	36 (21.2)
50-59	8 (28.6)	86 (50.6)

For comparison by χ^2, P=1.0 (both >80% skewing and >90% skewing)

3.1.8. *PCR-based X inactivation study of peripheral blood of Turkish Sjogren's syndrome patients*

Informative XCI was observed in 51 of 78 SICCA patients (65.4%). Among 51 informative patients, extremely skewed XCI was detected only in 3 patients (5.9%). This percentage is very close to the percentage of control women with extremely skewed XCI pattern (3 in 124; 2.4%) (*P*=0.4). Skewed XCI was observed in 8 of 51 informative SICCA patients (15.7%) and in 10 of 124 informative control women (8.1%) (P=0.2 and odds ratio=2.1 with 95% CI: 0.8-5.7). The gel image of representative SICCA samples is exhibited on Figure 3.12. The XCI patterns of SICCA samples and control women were summarized in Table 3.13. Skewed XCI in blood cells of SICCA patients were not different than of control group. This result displayed that other than XCI may have role in the pathogenesis of Sjogren's syndrome. Environmental factors like molecular mimicry or viruses may have role in initiation of failure in self tolerance since lack of association was reported in primary biliary cirrhosis (Miozzo *et al.* 2007).

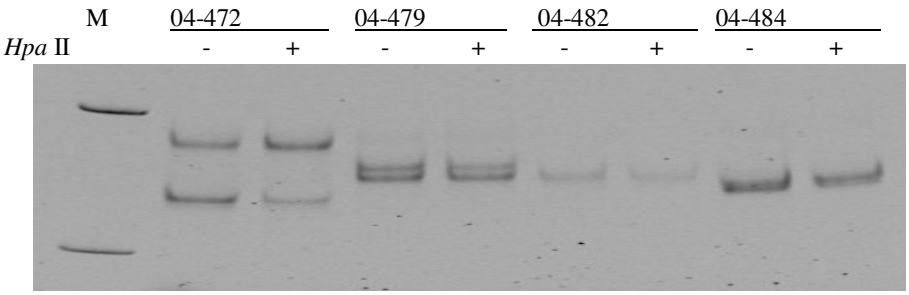

Figure 3.12 Gel image of XCI patterns of 4 SICCA patients. Polymerase chain reaction products from the androgen receptor methylation assay demonstrate X chromosome inactivation patterns in samples 04-472 (allele ratio: 84%:16%), 04-479 (70%:30%), 04-482, and 04-484 (not informative). For each sample, DNA was either undigested (-) or digested (+) with the methylation-sensitive restriction enzyme *Hpa* II. Marker (M; pUC mix 8 [331-bp and 242-bp]) fragment is visible.

Table 3.13 Proportion of SICCA patients and controls with skewed XCI

Degree of skewing (%)	No. (%) observed with skewed	
	SICCA patients (n = 51)	Control (n=124)
90+	3 (5.9)	3 (2.4)
80-89	5 (9.9)	7 (5.6)
70-79	14 (27.5)	22 (17.8)
60-69	9 (17.7)	29 (23.4)
50-59	20 (39.2)	63 (50.8)

For comparison by $\chi 2$, $P = 0.2$ (>80% skewing); $P = 0.4$ (>90% skewing)

3.1.9.1. *PCR-based X inactivation study of peripheral blood of Turkish Pre-eclampsia patients*

Informative XCI pattern was observed in 46 of 67 pre-eclampsia patients (68.7%). Control group (n=130) for pre-eclampsia patients are chosen among the Turkish Control cohort so that the mean ages were almost the same for patient and control. Among 130 healthy control women, informative XCI status was observed in 86 cases (66.2%). Extremely skewed XCI was observed in 21.7% of informative patients (10 in 46), whereas this ratio was observed only in 2 of 86 informative

healthy women (2.3%, P=0.0005). The odds ratio was calculated as 11.7 (95%CI:2.4-56.0). Skewed XCI was observed in 16 of 46 informative patients (34.8%) whereas in 8 of 86 informative controls (9.3%, P=0.0006) (Table 3.14). The odds ratio was calculated as 5.2 (95%CI:2.0-13.4). Aging is highly unlikely to be involved because of the relatively young ages of the patients. A shift towards the skewed range in older patients and controls was not observed (Figure 3.13). A gel image that display PEE patients with skewed XCI pattern can be observed in Figure 3.14.

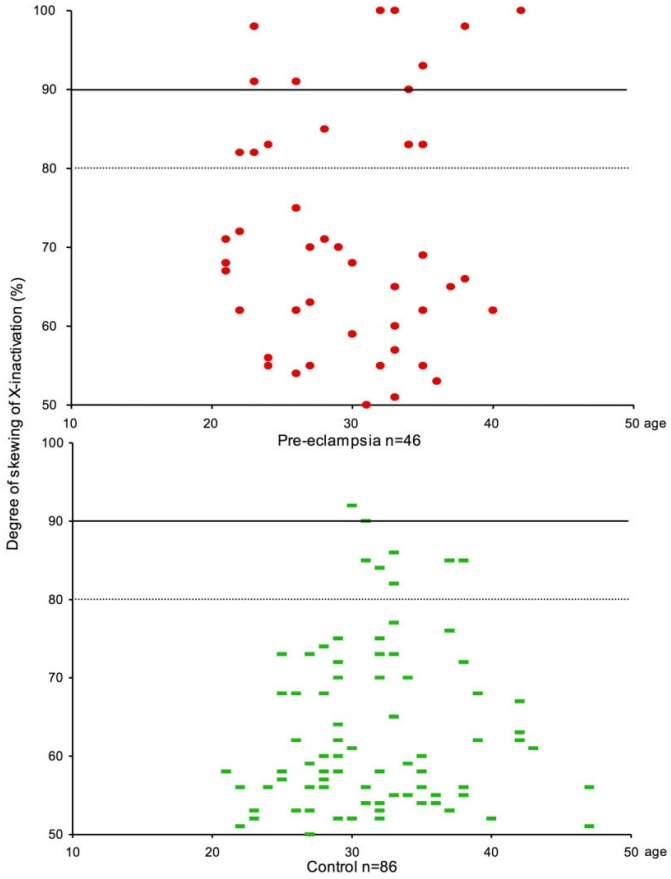

Figure 3.13 Distribution of X-chromosome inactivation patterns according to age in pre-eclampsia patients and control subjects

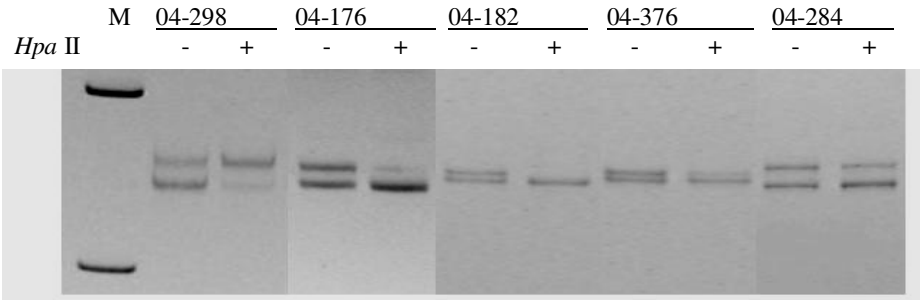

Figure 3.14 Gel image of XCI patterns of 5 PEE patients. Polymerase chain reaction products from the androgen receptor methylation assay demonstrate X chromosome inactivation patterns in samples 04-298 (allele ratio: 98%:2%), 04-176 (91%:9%), 04-182 (100%:0%), 04-376 (83%:17%), and 04-284 (76%:34%). 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]) fragment is visible.

Table 3.14 Proportion of PEE patients and controls with skewed XCI

Degree of skewing (%)	No. (%) observed with skewed	
	PEE patients (n = 46)	Control females (n = 86)
90+	10 (21.7)	2 (2.3)
80-89	6 (13.0)	6 (7.0)
70-79	6 (13.0)	15 (17.4)
60-69	13 (28.3)	20 (23.3)
50-59	11 (23.9)	43 (50.0)

For comparison by $\chi 2$, $P = 0.0006$ (>80% skewing); $P = 0.0005$ (>90% skewing)

3.1.9.2. *PCR-based X inactivation study of buccal mucosa specimen of Turkish pre-eclampsia patients*

In order to analyze the XCI pattern of mesodermal tissue (in this case blood) with the other tissue type, buccal mucosa (ectodemal origin) was obtained from 7 patients randomly. The XCI patterns of buccal mucosa cells were very close to blood cells. The results were displayed in Table 3.15.

Table 3.15 Blood and buccal mucosa XCI patterns of seven PEE patients

Sample	04-176	04-182	04-190	04-298	04-192	04-284	04-289
Blood	91:9	93:7	90:10	98:2	83:17	71:29	70:30
Buccal	90:10	90:10	90:10	100:0	80:20	69:31	66:34

3.1.9.3. *Pregnancy history*

The clinical characteristics of informative patients were displayed in Table 3.16. The differences in the pregnancy details between women with extremely skewed and random pattern were important because a common background for pre-eclampsia and recurrent spontaneous abortion has been questioned (Christiansen *et al.* 1990, Wilczynski 2006). Although the numbers were too small to reach a conclusion, recurrent spontaneous abortions were three times more common in women with extreme skewing (3/10) than in women with random patterns of XCI (3/30).

Table 3.16 Clinical characteristics and XCI status of PEE patients

Patient	DOB	Diagnosis	Pregnancy History	GW of Disease	GW of Delivery	IUGR/ Birth Weight(gr)	Sex of Child
≥90 percent skewing							
04-176*	1977	HELLP	G3, P1	24	30	-/1,200	F
04-182*	1972	MP	G9, P4	32	40	-/3,000	M
04-187	1969	SP	G6, P4	35	38	-/2,400	F
04-190*	1980	SP	G1, P1	29	29	+/850	M
04-287	1970	SP	G1, P1	30	30	-/1,550	M
04-291	1966	SP	G6, P5	35	37	-/2,350	F
04-298*	1980	SP	G1, P1	39	39	-/3,000	F
04-378	1962	MP	G3, P3	40	40	-/3,350	M
06-237	1972	MP	G3, P3	35	38	+/2,500	M
06-241	1968	MP	G6, P6	36	38	-/3,650	F
80-89 percent skewing							
04-192*	1979	SP	G1, P1	29	33	+/1,500	M
04-368	1981	SP	G1, P1	26	41	-/2,400	F
04-372	1969	SP	G3, P3	36	39	-/2,800	M
04-376	1970	MP	G4, P3	36	40	-/3,500	M
04-401	1982	SP	G2, P1	34	36	-/2,450	M
06-242	1978	MP	G1, P1	36	38.5	-/3,600	M
70-79 percent skewing							
04-186	1985	SP	G1, P1	32	33	+/1,100	F
04-284*	1975	HELLP	G4, P3	32	33	-/2,100	M
04-289*	1975	SP	G2, P2	38	38	+/2,450	M
04-365	1982	SP	G1, P1	32	36	-/2,500	M
06-238	1980	SP	G1, P1	26	34	+/1,000	F
06-239	1979	SP	G3, P2	18	20	?/500 (ex)	F
60-69 percent skewing							
04-178	1985	SP	G1, P1	34	35	-/2,050	M
04-181	1977	HELLP	G2, P2	33	33	+/1,600	M
04-184	1967	MP	G6, P4	39	40	?/3,300	F
04-285	1960	SP	G3, P1	16	20	-/400	M
04-288	1972	SP	G1, P1	23	34	+/1,250	F
04-364	1964	SP	G6, P5	24	25	?/900	F
04-370	1966	MP	G3, P3	39	39	-/2,850	F
04-374	1977	SP	G1, P1	39	40	-/2,800	F
04-402	1983	E	G1, P1	38	38	-/3,000	M
04-403	1969	SP	G4, P4	34	34	?/1,800	F
06-236	1973	MP	G1, P1	36	38	-/3,300	M
06-245	1973	MP	G3, P3	32	37	+/2,350 (ex)	F
06-246	1984	SP	G1, P1	26	34	+/1,150	F
50-59 percent skewing							
04-180	1976	MP	G4, P4	34	35	-/2,100	F
04-188	1975	SP	G2, P2	31	32	-/1,800	M
04-189	1977	SP	G1, P1	27	33	-/1,400	M
04-292	1966	SP	G1, P1	32	37	-/2,000	F
04-293	1970	SP	G3, P3	32	32	-/1,350	F
04-367	1974	SP	G3, P3	33	33	-/2,000	F
04-373	1972	SP	G1, P1	36	39	-/2,800	M
04-405	1971	E	G4, P2	34	34	-/1,700	F
06-234	1971	SP	G1, P1	30	38	+/1,900	M
06-235	1982	SP	G1, P1	24	28	+/850 (ex)	F
06-244	1982	MP	G1, P1	37	39	-/3,300	M

DOB: date of birth, GW: gestational week, IUGR: intra-uterine growth retardation, M: male. F: female, MP: mild pre-eclampsia, SP: severe pre-eclampsia, E: eclampsia, HELLP: hemolytic anemia-elevated liver enzymes and low platelet count syndrome G: gravida, P: para

3.2. PEDIATRIC SAMPLES

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

XCI was found to be informative in 124 of 183 controls (67.8%). Skewed XCI was observed in 12 of 124 informative controls (9.7%) and extremely skewed XCI was observed in only 4 of 124 control individuals (3.2%). Extremely skewed XCI is a very rare event in the newborn individuals. Our results displayed consistency with the previously published results (Amos-Landgraf *et al*. 2006).

3.2.2. *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 4 of 124 informative pediatric controls (P=0.002; odds ratio=4.4 (95%CI: 1.3-15.4). Skewed XCI was observed in 14 of 62 informative patients (22.6%) and only in 12 of 124-control group (9.7%) (P=0.02; odds ratio=2.7 (95%CI: 1.2-6.3). The gel image of representative JIA samples is exhibited on Figure 3.15. The overall XCI pattern of JIA samples and control group children are summarized in Table 3.17. 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.

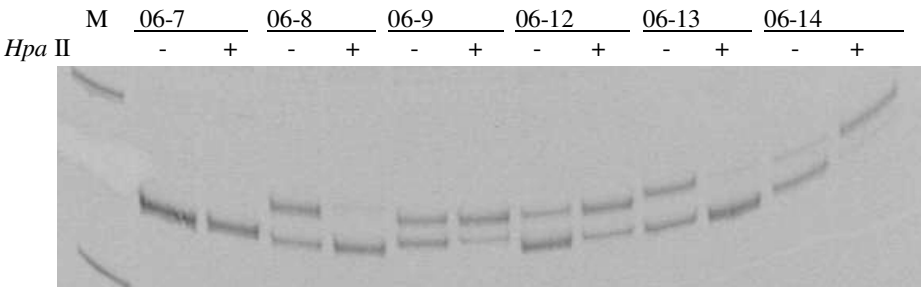

Figure 3.15 Gel image of XCI patterns of 6 JIA patients. Polymerase chain reaction products from the androgen receptor methylation assay demonstrate X chromosome inactivation patterns in samples 06-7 (allele ratio: not informative), 06-8 (90%:10%), 06-9 (71%:29%), 06-12 (76%:24%), 06-13 (95%:5%), and 06-14 (95%:5%). 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]) fragment is visible.

Table 3.17 Proportion of JIA patients and controls with skewed XCI

Degree of skewing (%)	No. (%) observed with skewing	
	JIA patients (n=62)	**Controls (n=124)**
90+	8 (12.9)	4 (3.2)
80-89	6 (9.7)	8 (6.5)
70-79	13 (21.0)	28 (22.6)
60-69	18 (29.0)	37 (29.8)
50-59	17 (23.5)	47 (37.9)

For comparison by χ^2, $P = 0.024$ (>80% skewing); $P = 0.022$ (>90% skewing)

The clinical characteristics about JIA patients were listed in Table 3.18. The information 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.

Table 3.18 Clinical characteristics and XCI status of JIA patients

	Code	DoB	OoD (y)	Clinical Class	Clinical Severity	Affected Joint(s)	Pregnancy History	ANA	RF	Uveitis
90+										
1	06-029	1996	3.5	O	Active	3	A:3	+	-	-
2	05-069	1990	11	O	remission	4	G:3,P:3,A:0	-	-	-
3	05-188	1989	7	O	remission	2	G:3, P:3; A:0	+	-	+
4	06-014	1993	14	O	remission	2	G:2,P.1, A:1	-	-	-
5	06-013	1983	16	EO	Active	8	G:3, P:3; A:0	+	-	-
6	06-016	2002	2	O	remission	1		-	-	-
7	06-030	1999	2	O	Active	3		+	-	-
8	06-008	1994	2	O	remission	1		+	-	+
80-89										
9	05-179	2000	3	P	remission	6	G:5, P:3,A:2	+	-	+
10	06-026	2001	2	O	Active	2		+	-	-
11	07-1078	2004	3	O	Active	1	G:4,P:3,ectopik:1	-	-	-
12	05-064	1994	7	O	Active	3	G:5,P:5,A:0	+	-	+
13	06-020	2003	2	O	remission	1	G:4, P:4, A:0	-	-	-
14	07-1080	2003	3	P	remission	5	G:3,P:3,A:0	-	-	-
70-79										
15	05-170	1991	10	P	remission	7		-	-	-
16	07-1087	1998	9	O	remission	2	G:3,P:2,anembryonic:1	-		
17	06-012	1986	10	O	remission	1	P:2,G:2, A:0	-	-	-
18	05-071	1995	1	P	Active	10	G:2,P:2,A:0	-	-	-
19	05-178	1996	8	P	Active	10	G:5,P:5,A:0	-	-	-
20	06-021	2002	2	O	remission	1	G:3,P.3,A:0	-	-	-
21	06-052	2002	2.5	O	Active	2	A:2	+	-	-
22	05-187	1990	10	O	remission	2	G:3,P:2,A:1	-	-	-
23	05-183	1999	5	O	remission	1		+	-	-
24	06-009	1996	7	O	remission	2		+	-	-
25	06-043	1998	2	O	Active	2	A:2	+	-	-
26	05-065	1990	2	P	Active	10	G:4,P:3,DC:1,Ex:1	-	+	-
27	05-063	1991	11	O	remission	3		+	-	-
60-69										
28	05-070	1994	5	P	Active	13	G:2,P:2,A:0	-	-	-
29	06-025	2000	4	O	Active	2	G.1,P:1,A:0	+	-	-
30	07-1079	1987	3	P	Sekel	14	G:4,P:3,A:1	-	-	-
31	05-601	2001	4	O	remission	3	G:1,P:1,A:0	-	-	-
32	05-173	2001	2	O	remission	1	P:2,G:2, A:0	+	-	-
33	05-182	1994	9	EO	Active	7	G:5, P:2, DC:3	-	-	-
34	06-024	1999	4	O	remission	1	G:4,P.2,A:1,DC:1	+	-	+
35	06-037	1996	4	O	remission	3	A:2	+	-	+
36	06-044	1991	7	O	remission	2	A:1	-	-	-
37	05-602	2003	2	O	remission	1		+	-	-
38	06-028	2001	1.5	O	remission	2		+	-	-
39	05-073	1995	4	P	remission	7	G:2,P:2,A:0	+	-	+
40	05-072	1988	1	P	remission	10	G:3, P:3; A:0	-	-	-
41	05-062	1984	11	O	remission	3	G:4,P:4,A:0	-	-	-
42	06-051	2000	3	O	remission	2	A:1			
43	05-066	1998	3	O	remission	1	G7,P6,A1	-	-	-

44	07-1084	1998	9	O	remission	1	G:1,P:1,A:0	-	-	-
45	07-1086	1995	10	O	remission	2	G:2,P2,A0	+	-	-
50-59										
46	05-168	1990	9	P	Active	9		-	-	-
47	06-034	1999	3	O	remission	2				
48	05-167	2002	9	EO	Active	8		-	-	-
49	05-169	1987	4	P	remission	6		-	-	-
50	06-038	1998	3	O	Active	2	A:1	-	-	-
51	05-177	1992	10	P	remission	5		-	+	-
52	06-019	2000	4	O	remission	1		-	-	-
53	06-027	1999	7	O	remission	2	G:3, P:3; A:0	+	-	-
54	06-031	2000	2	O	remission	2				
55	06-048	2002	1	O	remission	2				
56	05-166	1980	13	P	Active	12	G:2,P:2,A:0	-	-	-
57	05-185	1998	6	O	Active	2	G.2,P:2,A:0	+	-	+
58	06-046	2005	1.5	O	remission	2	A:1			
59	06-045	2002	2	O	remission	2	A:1			
60	06-053	1998	2	O	remission	1				
61	07-1081	2002	5	O	remission	2	G:1;P:1	+	-	-
62	07-1085	1998	9	O	remission	1	G:3,P.3,A:0	+	-	-

DoB: Date of birth, OoD: Onset of disease, y: year, O: oligoligoarticular, P: polyarticular, EO: extended oligoligoarticular, F: female, M: male G: number of pregnancies, P: para (pregnancies carried to term and delivered), A: spontaneous abortions, MTX: methotrexate, NSAID: non-steroidal anti-inflammatory drugs, PRED: prednisolone, IAS: intraarticular corticosteroids, SAZ: sulfasalazin, SAL: saline, +: present, -: absent.

3.2.3. *PCR-based X inactivation study of peripheral blood of Turkish pediatric scleroderma patients*

9 of 14 pediatric scleroderma patients were informative (64.3%) for *AR* gene polymorphism. 2 of the 9 informative patients displayed extremely skewed XCI (22.2%). Skewed XCI was observed in 3 of the 9 informative patients (33.3%). The number of patients is very small to make a conclusion. However the incidence of pediatric SSc is too low, therefore it may take more time to reach a significant population size. A representative PAGE image of one patient is in Figure 3.16. The results were summarized in Table 3.19.

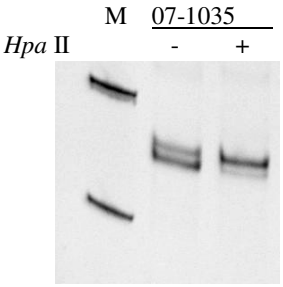

Figure 3.16 Gel image of XCI patterns of one pediatric scleroderma patient. Polymerase chain reaction products from the androgen receptor methylation assay demonstrate X chromosome inactivation patterns in samples 07-1035 (allele ratio: 85%:15%). For the sample, DNA was either undigested (-) or digested (+) with the methylation-sensitive restriction enzyme *Hpa* II. Marker (M; pUC mix 8 [331-bp and 242-bp]) fragment is visible.

Table 3.19 Proportion of pediatric SSc patients and controls with skewed XCI

Degree of skewing (%)	No. (%) observed with skewing	
	Pediatric SSc patients (n=9)	**Controls (n=124)**
90+	2 (22.2)	4 (3.2)
80-89	1 (11.1)	8 (6.5)
70-79	1 (11.1)	28 (22.6)
60-69	5 (55.5)	37 (29.8)
50-59	0 (0.0)	47 (37.9)

3.3. CELL LINE SAMPLES

3.3.1 PCR-based X inactivation study of cell line samples of British type I diabetes mellitus (TPO+) patients

XCI status was found to be informative in 88.1% cases (312 in 354). Skewed XCI pattern was observed in 115 of 312 informative samples (36.9%). Extremely skewed XCI was observed in 88 samples (28.2%). PAGE image of T1D(TPO+) samples were displayed in Figure 3.17 and the overall results were summarized in Table 3.20.

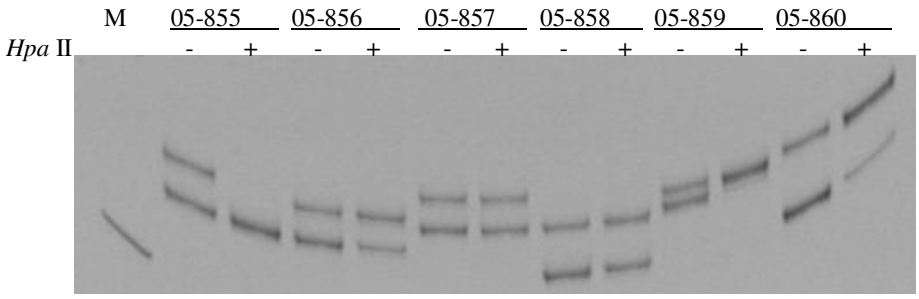

Figure 3.17 Gel image of XCI patterns of 6 cell lines of T1D(TPO+). Polymerase chain reaction products from the androgen receptor methylation assay demonstrate X chromosome inactivation patterns in samples 05-855 (allele ratio: 100%:0%), 05-856 (55%:45%), 05-857 (55%:45%), 05-858 (50%:50%), 05-859 (95%:5%), and 05-860 (75%:25%). For each sample, DNA was either undigested (-) or digested (+) with the methylation-sensitive restriction enzyme *Hpa* II. Marker (M; pUC mix 8 [only 242-bp]) fragment is visible.

3.3.2 PCR-based X inactivation study of cell line samples of British type I diabetes mellitus (TPO-) patients

XCI status was informative in 349 of 453 cell lines of T1D(TPO-) patients (77.0%). Extremely skewed XCI was observed in 84 of the 349 informative cell lines (24.1%). Skewed XCI was observed in 123 of 349 informative cell lines (35.2%). The gel image was represented in Figure 3.18, and the summarized results in Table 3.20.

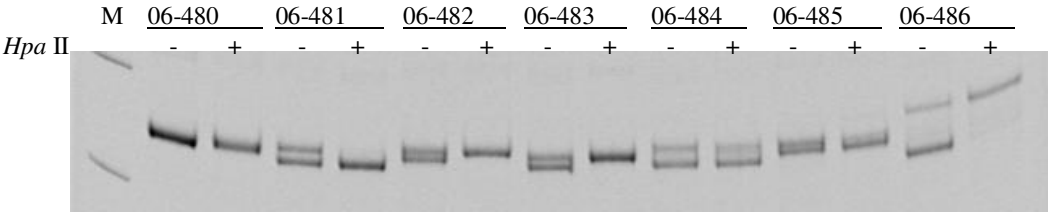

Figure 3.18 Gel image of XCI patterns of 7 cell lines of T1D(TPO-). Polymerase chain reaction products from the androgen receptor methylation assay demonstrate X chromosome inactivation patterns in samples 06-480 (allele ratio: not informative), 06-481 (100%:0%), 06-482 (100%:0%), 06-483 (100%:0%), 06-484 (63%:37%), 06-485 (85%:15%), and 06-486 (98%:2%). 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]) fragment is visible.

3.3.3 PCR-based X inactivation study of cell line samples of British control (BBC1958) individuals

British Birth Cohort 1958 samples were collected from the individuals that were born in 1958 in UK. 311 of 466 samples were informative in XCI status (66.8%). 65 of 311 (20.9%) informative samples have extremely skewed XCI patterns whereas 97 (31.2%) of them have skewed XCI patterns. A representative gel image of XCI results of BBC58 samples were displayed in Figure 3.19. All of the results of 3 groups of British cell lines were summarized in Table 3.20.

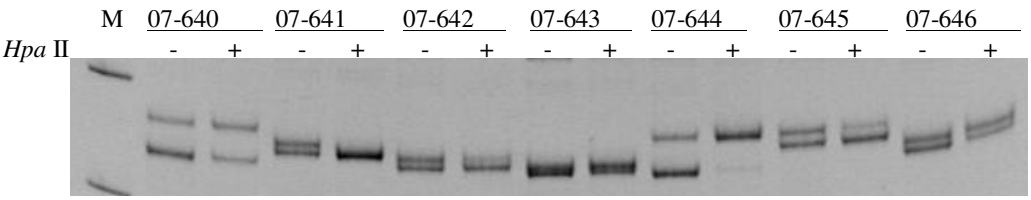

Figure 3.19 Gel image of XCI patterns of 7 cell lines of BBC1958. Polymerase chain reaction products from the androgen receptor methylation assay demonstrate X chromosome inactivation patterns in samples 07-640 (allele ratio: not informative), 07-641 (100%:0%), 07-642 (100%:0%), 07-643 (100%:0%), 07-644 (63%:37%), 07-645 (85%:15%), and 07-646 (98%:2%). 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]) fragment is visible.

Table 3.20 Skewed XCI profiles in cell line DNA of British T1D (TPO+ and TPO-) patients and controls

Degree of skewing (%)	No. (%) observed with skewed		
	T1D(TPO+) (n = 312)	T1D(TPO-) (n = 349)	Control (BBC58) (n=311)
90+	88 (28.2)	84 (24.1)	65 (20.9)
80-89	27 (8.7)	39 (11.2)	32 (10.3)
70-79	49 (15.7)	59 (16.9)	33 (10.6)
60-69	52 (16.7)	50 (14.3)	51 (16.4)
50-59	96 (30.8)	117(33.5)	130(41.8)

For comparison by χ^2, P = 0.15 (>80% skewing); P = 0.04 (>90% skewing) for T1D(TPO+) samples and P = 0.28 (>80% skewing); P = 0.35 (>90% skewing) for T1D(TPO+) samples

The difference in XCI status between cell lines of typeI diabetes mellitus patients and controls were stasitically not significant. It was an expected result bevause previous reports (Migeon *et al.* 1988) documented that lymphoblastoid cell lines were monoclonal. The XCI results of cell lines of all type I diabetes mellitus, BBC1958, and individuals of all Turkish controls were represented in Figure 3.20. There was still a significant difference in XCI patterns of Turkish control samples (for which DNA was isolated from peripheral blood) and BBC1958 control cohort (for which DNA was isolated from established cell lines). These results indicated that cell line samples were not ideal for XCI studies and expression studies.

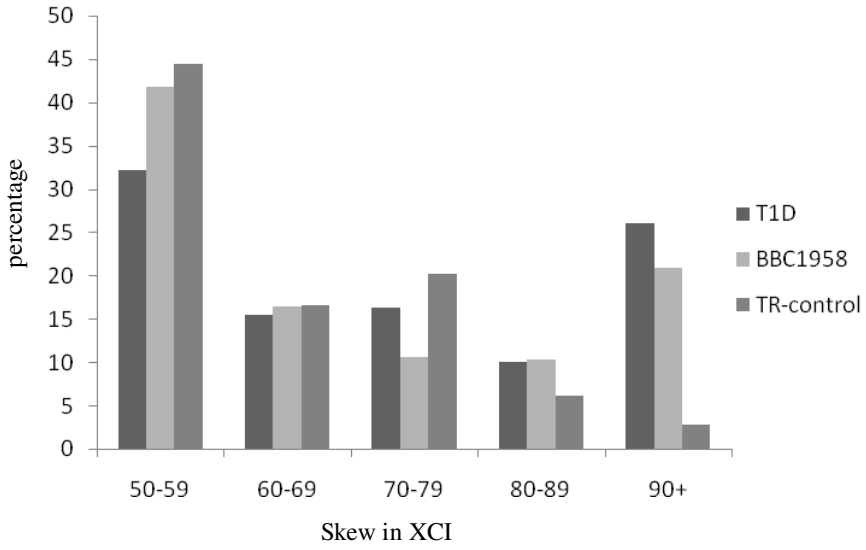

Figure 3.20 Distribution of XCI in T1D, BBC1958 cell lines and Turkish control (DNA extracted from peripheral blood).

CHAPTER 4

DISCUSSION

All individuals are tolernant to their own 'potential' antigenic molecules. Self reactive lymphocytes are deleted or made unresponsive. Autoimmune disorders develop when self antigens escape from tolerance and are activated. The exact mechanisms of how these molecules escape from tolerance are not entirely known. However, a combination of genetic variants, environmental factors like pathogen exposure, pregnancy, smoking and diet are thought to have a role in the eiology of autoimmune diseases.

One of the interesting features of autoimmune disorders is that they are mainly the problem of the female gender. Striking differences between the two sexes are observed both in systemic (i.e., scleroderma, Sjogren's syndrome and systemic lupus erythamatosus), and in organ specific autoimmune diseases (i.e., autoimmune thyroid diseases). In other types of autoimmune disorders, like rheumatoid arthritis, multiple sclerosis and myasthenia gravis the female predominance is still apparent. There are certain diseases, such as type I diabetes mellitus, in which, subgroups characterized by distinct clinical manifestations may have a female preponderance. Hormonal status, pregnancy and sex chromosomes are the main differences between the two sexes. Steroid hormone differences between the two sexes make them a good candidate for the female predominance of autoimmunity. Even though both estrogens and androgens exist in females and males, their levels are different. This difference is a quantitative but not qualitative feature. In multiple sclerosis and rheumatoid arthritis, where female predominance holds, disease activity decreases during pregnancy, especially in the third trimester, where estrogen and progesterone levels are at peak (Nelson&Ostensen 1997, Confavreux *et al* 1998). However,

presence of pediatric forms of female prevalent autoimmune diseases raises questions on the validity of this subject. Pregnancy related microchimerism is another difference between the two sexes which may have role in female predisposition to autoimmunity. In fact, there are reports that display increased fetal cells in maternal blood and skin lesions of women with scleroderma (Artlett *et al.* 1998, Nelson *et al.* 1998). However, these reports cannot explain alone why the non-pregnant women and children under the age of puberty are subject to autoimmunity. The third difference is X-Chromosome inactivation (XCI), a physiological compensation for the X-linked dosage between the two sexes (Lyon, 1961). XCI is an epigenetic event that occurs early in development leading to transcriptional silencing of one of the X chromosome pairs (Huynh&Lee 2005). The silencing process is random; therefore normal females are mosaic of two cell populations. Disturbances in the XCI patterns may result in inadequate training for X-encoded self antigens in the thymus (Stewart, 1998).

The main goal of this study was to investigate the role of skewed XCI, especially in hematopoietic stem cells, in the pathogenesis of female predominant autoimmune disorders. The diseases that were investigated for this purpose represent both the systemic and the organ specific autoimmune disorders. Scleroderma (SSc), Sjogren's syndrome (SICCA), juvenile idiopathic arthritis (JIA), and rheumatoid arthritis (JIA) are systemic, while autoimmune thyroid diseases (AITDs) and type I diabetes mellitus (T1D) are organ specific autoimmune disorders.

We first demonstrated skewed XCI patterns in peripheral blood mononuclear cells of a significant proportion of women with SSc in the Turkish [64%, OR: 20.0 (95%CI: 8.5-46.6); $p<0.0001$] and the US [34%, OR: 5.9 (95% CI 2.7-12.8); P<0.0001] populations. The effect is more pronounced for patterns of extremely skewed XCI in the Turkish SSc [49.1%, OR: 38.9 (95% CI 11.0-137.3); P<0.0001] and the US SSc [18.1%, OR: 3.8 (95% CI 1.9-7.6); P<0.0001] patients. Next, skewed XCI patterns was obsevred in peripheral blood mononuclear cells of a significant proportion of female subjects with AITDs in the Turkish [33.8%, OR: 5.8 (95% CI 2.6-12.8; P<0.0001] and the Tunisian [26%, OR: 2.8 (95% CI 1.5-5.4; P=0.0022] population. Turkish AITDs patients displayed 19.3% (OR: 9.6 (95% CI 2.7-34.3); P<0.0001) and Tunisian AITDs probands showed 14% [OR: 3.8 (95% CI

1.5-9.8); P=0.0046] extremely skewed XCI patterns in their blood cells. Third, a high proportion of skewed [34.2%, OR: 4.1 (95% CI 2.1-8.1); P<0.0001] and extremely skewed [22.4%, OR: 6.7 (95%CI 2.7-17.0); P<0.0001] XCI patterns was observed in a group of Tunisian RA patients. In thepediatric age groups, approximately 23% of JIA patients demonstrated skewed XCI patterns [OR: 2.7 (95% CI 1.2-6.3); P=0.0024]. We showed extremely skewed XCI patterns in mononuclear blood cells of a significant proportion [12.9% OR: 4.4 (95% CI 1.3-15.4); P=0.0022] of JIA patients. Lastly, in a high proportion of PEE patients, skewed XCI patterns was observed [34.7%, OR: 2.7 (95% CI 1.2-6.3); P=0.0024] in blood cells. With respect to the extremely skewed XCI ratios, the proportion is still high [21.7%, OR: 11.7 (95% CI 2.4-56.0); P=0.0005] in PEE patients. In Turkish female controls approximately 8% demonstrate skewed and 3% demonstrate extremely skewed XCI. Similar proportions were observed in the blood cells of pediatric control individuals (9.7% skewed and 3.2% extremely skewed). 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). All of the results were summarized in Table 4.1.

Skewed XCI is not a common finding in all female prevalent autoimmune disorders. For example, in SICCA, a statistically significant result was not observed (Table 4.1). Likewise in primary billiary cirrhosis (PBC) a recent publication reports random patterns in blood cells (Miozzo *et al.* 2007). Lack of association is not an unexpected finding because initiation of autoimmunity in PBC is most probably due to molecular mimicry (Kaplan&Gershwin 2005). In light of known causes of SICCA, initiation of breakdown of self-tolerance could be other factors such as to viral infections (Kordossis *et al.* 1998, Haddad et al. 1992, Terada et al. 1994). We agree with the hypothesis that a subsequent event, such as environmental exposure to viral, chemical, or other agents, may trigger a cascade that results in certain types of autoimmune disorders.

Table 4.1 Summary of the results of XCI patterns of the autoimmune diseases analyzed in this study

Disease	90+	80-89	50-79	NI	OR for 90+ skewing (95% CI and P)	OR for 80+ skewing (95% CI and P)
AITDs-TR (n=110)	16 (19.3)	12 (14.5)	55 (66.2)	27 (24.6)	9.6 (95% CI 2.7-34.3; P<0.0001)	5.8 (95% CI 2.6-12.8; P<0.0001)
AITDs-SCN. (n=40)*	5 (16.0)	6 (18.8)	21 (66.0)	8 (20.0)	7.4 (95% CI 1.7-33.2; P=0.0215)	6.0 (95% CI 2.3-15.8; P<0.0001)
AITDs-TUN (n=145)	14 (14.0)	12 (12.0)	74 (74.0)	45 (31.0)	3.8 (95% CI 1.5-9.8; P=0.0046)	2.8 (95% CI 1.5-5.4; P=0.0022)
AITDs-TR&TUN (n=255)	30 (16.4)	24 (13.1)	129 (70.5)	72 (28.2)	4.6 (95% CI 2.0-10.5; P<0.0001)	3.3 (95% CI 1.9-5.9; P<0.0001)
AITD-TR&SCN.&TUN(n=295)	35 (16.3)	30 (14.0)	150 (69.8)	80 (27.1)	4.5 (95% CI 2.0-10.5; P<0.0001)	3.4 (95% CI 2.0-6.0; P<0.0001)
RA-TUN (n=106)	17 (22.4)	9 (11.8)	50 (65.8)	30 (28.3)	6.7 (95%CI 2.7-17.0; P<0.0001	4.1 (95% CI 2.1-8.1; P<0.0001)
AKR-TUN (n=44)	0 (0)	3 (10.7)	25 (89.3)	16 (36.4)	P=1.0	P=1.0
SSc-TR (n=70)	27 (49.1)	8 (14.5)	20 (36.4)	15 (21.4)	38.9 (95% CI 11.0-137.3; P<0.0001)	20.0 (95% CI 8.5-46.6; P<0.0001)
SSc-US (n=125)	17 (18.1)	15 (16.0)	62 (66.0)	31 (24.8)	3.8 (95% CI 1.9-7.6; P<0.0001)	5.9 (95% CI 2.7-12.8; P<0.0001)
SSc-TR&US (n=195)	44 (29.5)	23 (15.4)	82 (55.1)	46 (23.6)	8.6 (95% CI 5.1-14.7; P<0.0001)	9.3(95% CI 4.5-19.2; P<0.0001)
PEE-TR (n=67)	10 (21.7)	6 (13.0)	30 (65.2)	21 (31.3)	11.7 (95% CI 2.4-56.0; P=0.0005)	5.2 (95% CI 2.0-13.4; P=0.0006)
JIA-TR (n=81)	8 (12.9)	6 (9.7)	48 (77.4)	19 (23.5)	4.4 (95% CI 1.3-15.4; P=0.0022)	2.7 (95% CI 1.2-6.3; P=0.0024)
SICCA-TR (n=78)	3 (5.9)	5 (9.9)	43 (84.3)	27 (34.6)	P=0.4	P=0.2
C-Children-TR (n=92)	2 (2.8)	6 (8.3)	64 (88.9)	20 (21.7)		
C-Newborn-TR (n=91)	2 (3.8)	2 (3.8)	48 (92.3)	39 (42.9)		
C-Newborn-US (n=590)**	4 (0.7)	29 (4.9)	557 (94.4)	?? (?)		
C-Adult-TR (n=160)	3 (2.41)	7 (5.6)	114 (91.9)	36 (22.5)		
C-Adult-US (n=415)**	22 (5.3)	59 (14.2)	334 (80.5)	?? (?)		
C-Adult-TUN (n=97)	4 (8.7)	5 (10.9)	37 (80.4)	51 (52.6)		

UK: United Kingdom, TR: Turkey, SCN: Scandinavia, TUN: Tunisia, US: United States, T1D: Type 1 diabetes, AITDs: Autoimmune thyroid diseases, SSc: Scleroderma, RA: Rheumatoid arthritis, JRA: Juvenile rheumatoid arthritis, PEE: Pre-eclampsia, SICCA: Sjogren' syndrome, CI: confidence interval, NI: not informative

* (Brix *et al.* 2005)

** (Amos-Landgraf *et al.* 2006)

Our results suggest that a significant proportion of female patients with autoimmune disorders have extremely skewed XCI. Skewing could be the result of a primary or a secondary event. The former is bias in the initial choice of which X chromosome is inactivated due to germline XIST (Xinactive- specific transcript) mutations (Puck&Willard 1998). The secondary causes are deleterious X-linked mutations, X chromosome rearrangements, aging, twinning, or monoclonal expansion of cells (Brown, 1999). We believe that deleterious X-linked mutations or X chromosome rearrangements and their differential expression patterns could provide a disadvantage to blood cells and lead to skewed XCI.

In an attempt to determine whether skewed XCI is specific to blood or observed in other tissues as well, we collected samples from buccal mucosa, hair and affected tissues of a selected group of patients in the SSc and AITDs groups. In both groups, only those patients with extreme skewing were included. As shown in Figure 3.3 and 3.6 extreme skeweing is not observed in the skin biopsy samples of SSc patients. Likewise, thyroid biopsy specimens of AITDs patients do not display a pronounced skewing of XCI. Again, differential expression patterns of X-linked mutations could account for this observation. These results suggest that the putative X-linked antigens may be presented to the extremely skewed immune system cells of these patients.

X-linked lethal mutations, which would be compatible with life in females because of X-inactivation mosaicism, is an appealing causative mechanism in autoimmune diseases. If skewing is the result of X-linked mutations, these should be inherited from the maternal lineage unless they occur *de novo* during gametogenesis. Therefore, we analyzed the parental origin of the inactive X chromosome in 19 patient-mother pairs in US SSc subjects. Of the 10 pairs with informative genotyping results, inactive X was found to be of maternal origin in eight pairs. This result is on the border of statistical significance (P=0.055). Assuming this result holds, we will be left with an interesting puzzle. Why are maternally inherited X chromosomes more likely to be inactivated in highly skewed patients? One possibility is that some X chromosomes confer a selective disadvantage to both the organism itself and the peripheral cells within the organism. This precise situation

has been documented in X-linked immunodeficiency, where heterozygous (female) carriers have high X-inactivation skew (because of natural selection within the organism), while affected males have a survival disadvantage (Puck&Willard 1998). When an X-encoded genotype is selectively disadvantageous to cells, skew is an outcome. When an X-encoded genotype is selectively disadvantageous to an organism, preferential maternal inheritance is an outcome. Together, this leads us to the hypothesis that some highly skewed patients have an X chromosome that would confer a selective disadvantage on homozygotic women and on males.

Another evidence for our hypothesis could be the increase in recurrent spontaneous abortions in the patients with extremely skewed XCI pattern in scleroderma, autoimmune thyroiditis and pre-eclampsia in this study. It has been documented that recurrent spontaneous abortion was associated with extremely skewed XCI (Lanasa *et al.* 1999, Sangha *et al.* 1999, Kim *et al.* 2004, Bagislar *et al.* 2006). Deleterious X chromosome mutations that are associated with cell-growth disadvantage could be tolerated in females due to the XCI process. Cell death occurs early in development if the mutation is carried on the active X chromosome and the XCI pattern becomes skewed. However in hemizygous males the putative mutations become a male lethal trait and may contribute to the etiology of recurrent spontaneous abortion.

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 Xp22 and Xp11q13 regions of the X-chromosome in this family. Although there are publications that report examples of skewed XCI segregating with a trait (Pegoraro *et al.* 1997, Bicocchi *et al.* 2005), this is the first example in AITDs to the best of our knowledge. In a recent publication on a three generation kindred, extremely skewed XCI profile was documented in three female subjects who have hemophilia A (Bicocchi *et al.* 2005). The authors concluded that skewing in the family resulted from an abnormality in the initial choice process, because the inactive X was always of paternal origin in affected female subjects. This process prevented the X chromosome, which carried the mutant FVIII allele, from being an inactive X. The inactive X chromosome was of paternal origin in our Family 2 with two affected sisters. 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.

Since scleroderma is the first disease in which pregnancy related microchimerism has been documented, we investigated a subset of Turkish and US SSc patients to see whether a correlation exist between skewed XCI and microchimerism. Neither maternal nor fetal microchimerism were found to be correlated with skewed X-inactivation. In conclusion, the two types of female mosaicism, which are skewed X-inactivation and maternal/fetal microchimerism, appear to be independent risk factors in scleroderma. One possible explanation for these data is that mosaicism itself is an underlying cause of scleroderma and, by extension, female-prevalent autoimmune disease in general.

The cause of autoimmune diseases could be the cause of the skewing, or the skewing of XCI could be the cause of autoimmunity. If the the secondary selection is the result of autoimmune reaction, the disease would cause the skewing. Alternatively, if the disease is at least partially caused by an X-encoded gene, the disease allele could increase or decrease the fitness of cells inactivating the wild type or mutant allele of this gene, leading to secondary selection for immune cells inactivating one or both X-chromosomes. This would mean that both the disease and the skewing is the result of the X-linked gene. We believe that escape from self tolerance could be the result of skewing because the degree of skewing is at the extreme profiles (>95%) in most patients. If the skewing was due to aging or result of an autoimmune reaction, we expect to observe more patients in the milder ranges (80:20 or 90:10).

The location of these putative X-linked mutations is a key point. Our proposal is that they do not need to be at a certain gene or locus on the chromosome. On the contrary, any mutation that affects the viability of the cell could lead to skewed XCI. In addition, X/autosomal translocations which fall within and outside genes or other X chromosome aberrations could affect XCI ratios. Therefore, a variety of X chromosomal events including a single mutation in a very rich repertoire of genes could be critical for female predisposition to autoimmunity. This may also explain why despite the extensive linkage genome scans, X chromosome is not clearly

implicated in familial cases of autoimmune thyroid diseases, (Imrie *et al.* 2001) or why a major locus for autoimmunity proves to be so difficult to find in the genome (Wellcome Trust Case Control Consortium, 2007). "Loss of mosaicism" for X-linked gene expression as the first step of the cellular events that lead to breakdown of self-tolerance in females may be considered at this point.

Lymphoblastoid cell lines (LCL), which have been immortalized by infection with Epstein Barr Virus (EBV), are being actively and extensively used to examine the expression of specific genes and genome-wide expression profiles. In this study, using X chromosome inactivation (XCI) as a measure of the degree of clonality, we confirm and quantify widespread near monoclonality in two independent sets of control and T1D cell lines.Our results on British T1D and control lymphoblastoid cell line (LCL) pauciclonality, indicate that expression data from LCLs are not well reliable to detect correlations between SNPs and gene expression. When the expression of a gene is affected by methylation patterns or other epigenetic meiotically stable factors (Gimelbrant *et al.* 2007, Migeon *et al.* 1988), the expression measurement will not be representative of the in vivo cell population in LCLs. From the random inactivation of the same allele in the small number of clones that constitute the LCL strong allelic imbalance can result. This may result in increased false positive and false negative rates. In fact, by the random subsampling of a small number of clones in a LCL any gene expression measurement that is variable across cells in vivo can be significantly altered By this way, the power of genome-wide studies or specific gene studies to detect association between SNPs and expression traits may be affected by this additional measurement noise in LCLs. Consequently, we recommend where possible the use of bulk, non cell line, ex vivo cells when measuring gene expression, and in particular when focusing on allele-specific expression.

CHAPTER V

FUTURE PERSPECTIVES

In this study we tested the hypothesis that disturbances in XCI may cause female preponderance in autoimmune disorders. This hypothesis depends on two events: First, an X-linked mutation leading to “loss of mosaicism”, and second “heterozygosity for allelic variants of the putative critical genes”. For the future, we developed a research strategy in which we are going to conduct a comprehensive genomic study by using high-density microarray analysis. All known nonsynonymous, synonymous and intronic SNPs on X chromosome were listed using NCBI and Ensembl databases. After elimination of redundant and non-appropriate SNPs, 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. The array chips were produced and the experimental part was completed with 56 SSc, 82 AITDs, 48 PEE, 20 JIA, 2 SICCA, 7 pediatric SSc, and 250 control samples (total 465 samples).

In the analysis part of these experiments, the allelic frequency, their heterozygosity status and copy number variations will be searched. For each disease, the candidate alleles will be selected.

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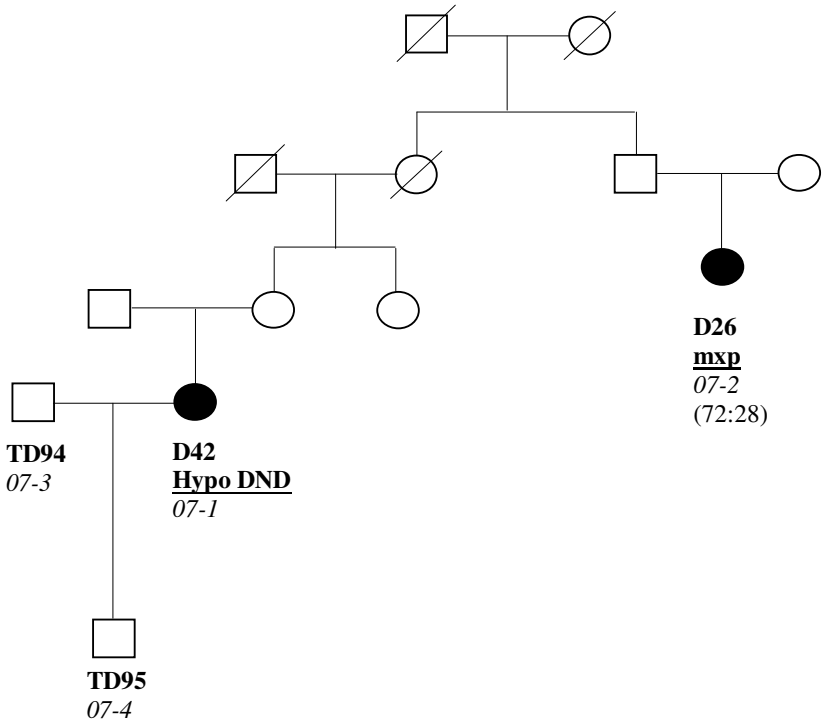
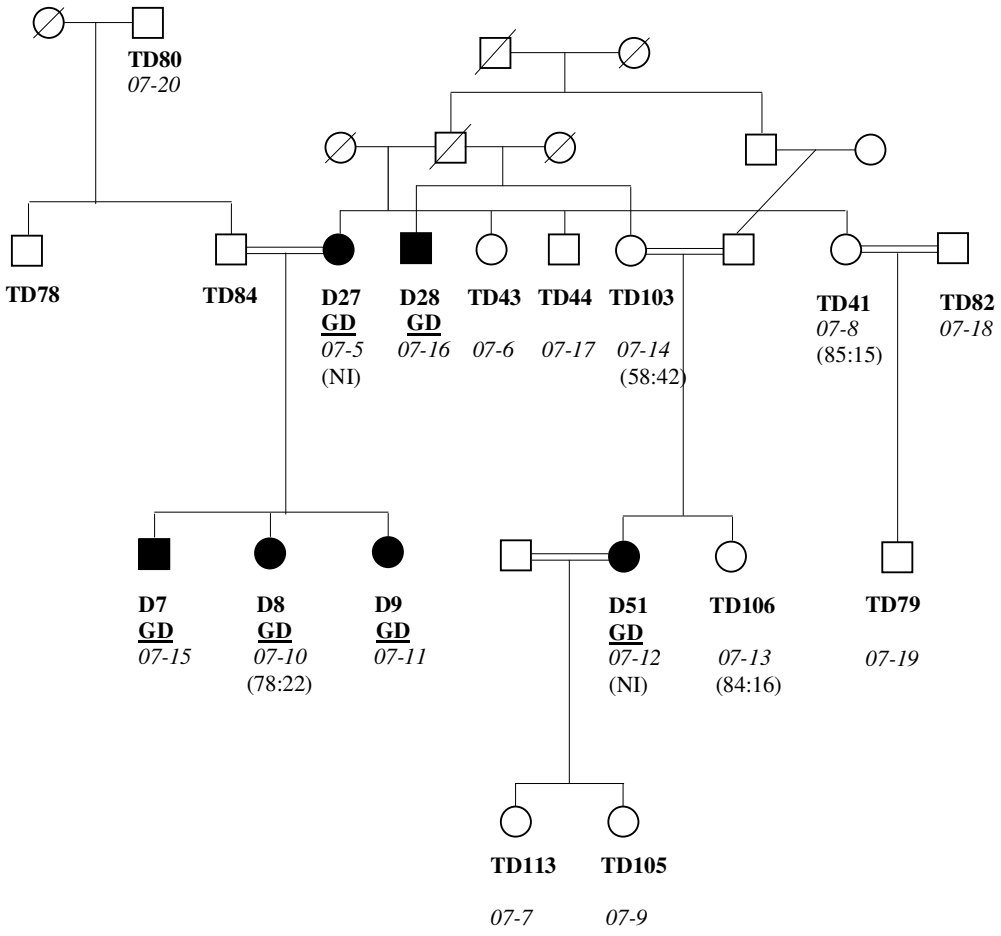
APPENDIX

Pedigree of subfamilies of Akr Family. GD: Grave's disease, HT: Hashimoto's thyroiditis. Numbers in bold: Tunisian sode number of members, italic numbers: Turkish code numbers of members, numbers in parantheses: XCI status of members



FAMILY 1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D27
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D28
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07-17
TD103
07-14
(58:42)
TD41
07-8
(85:15)
TD82
07-18
D7
GD
07-15
D8
GD
07-10
(78:22)
D9
GD
07-11
D51
GD
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TD79
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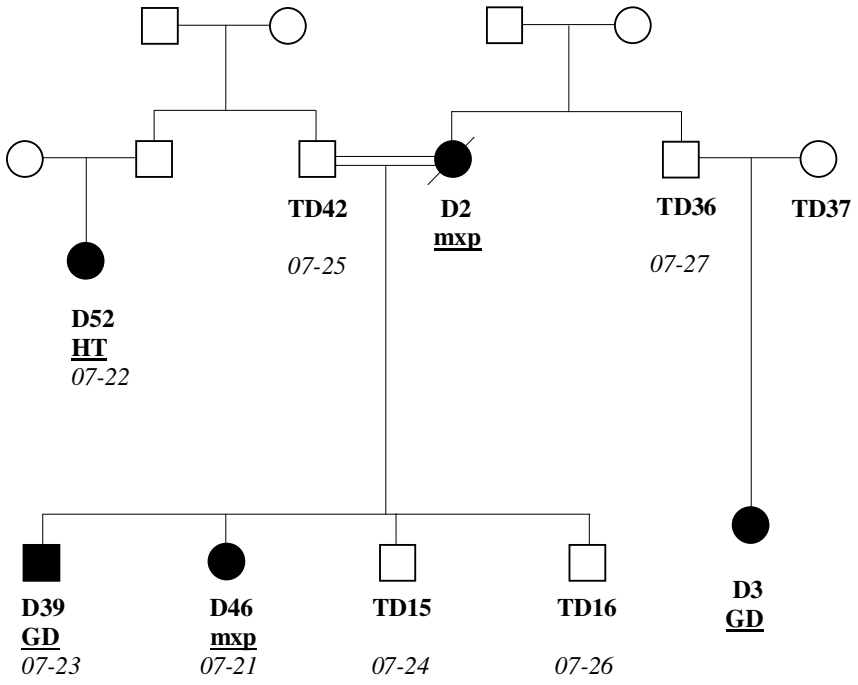
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D3
GD

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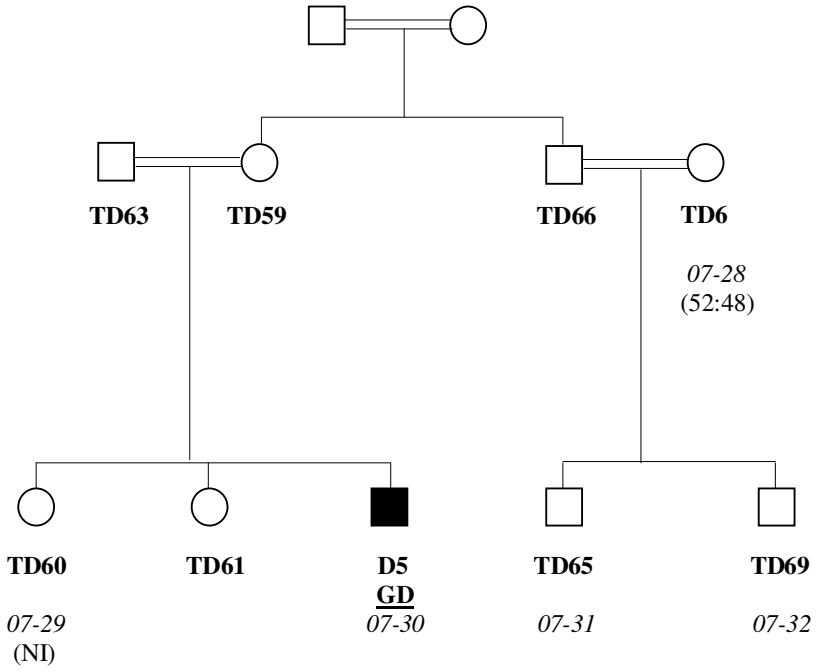
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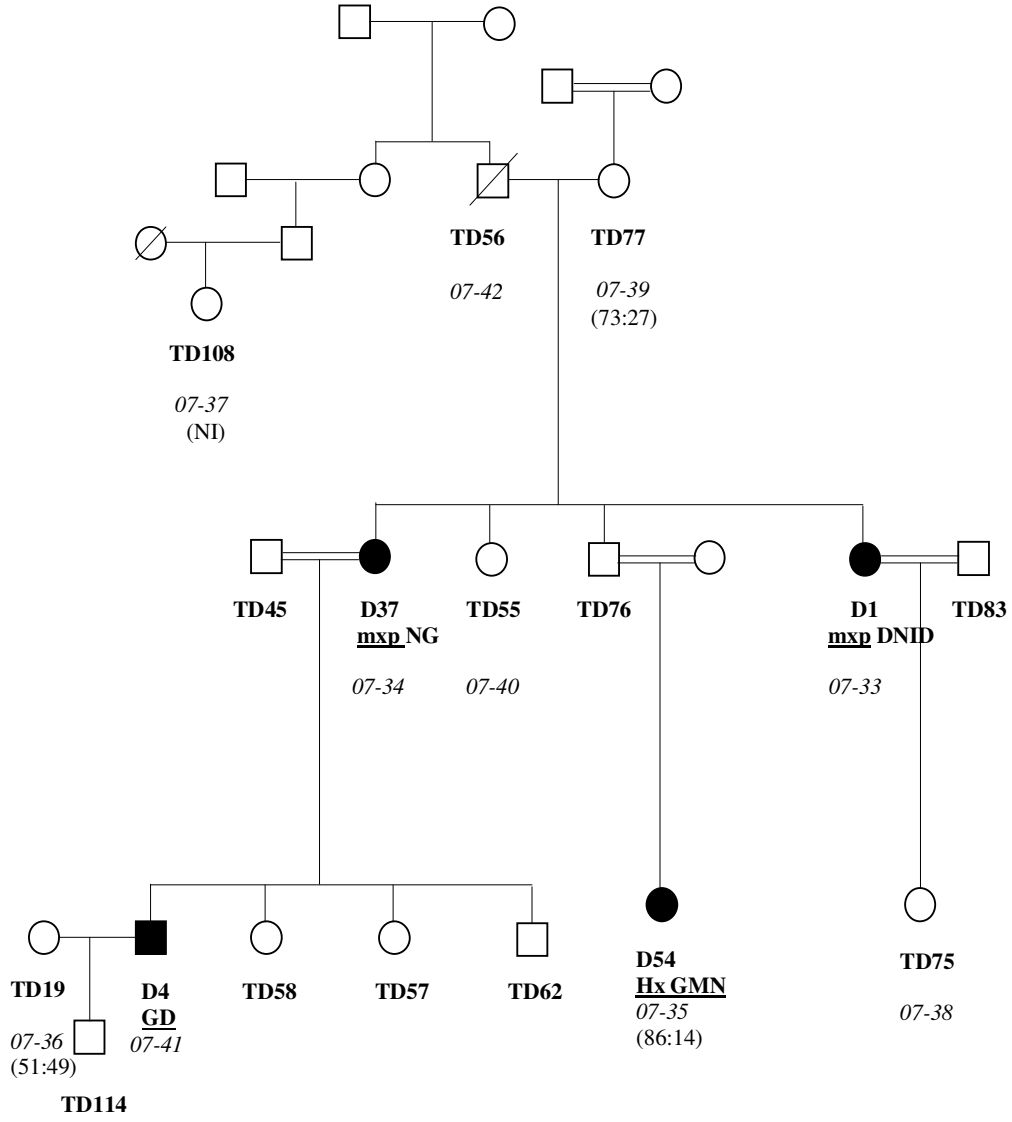
FAMILY 6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TD55
07-40
TD76
D1
mxp DNID
07-33
TD83
TD19
07-36
(51:49)
TD114
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TD57
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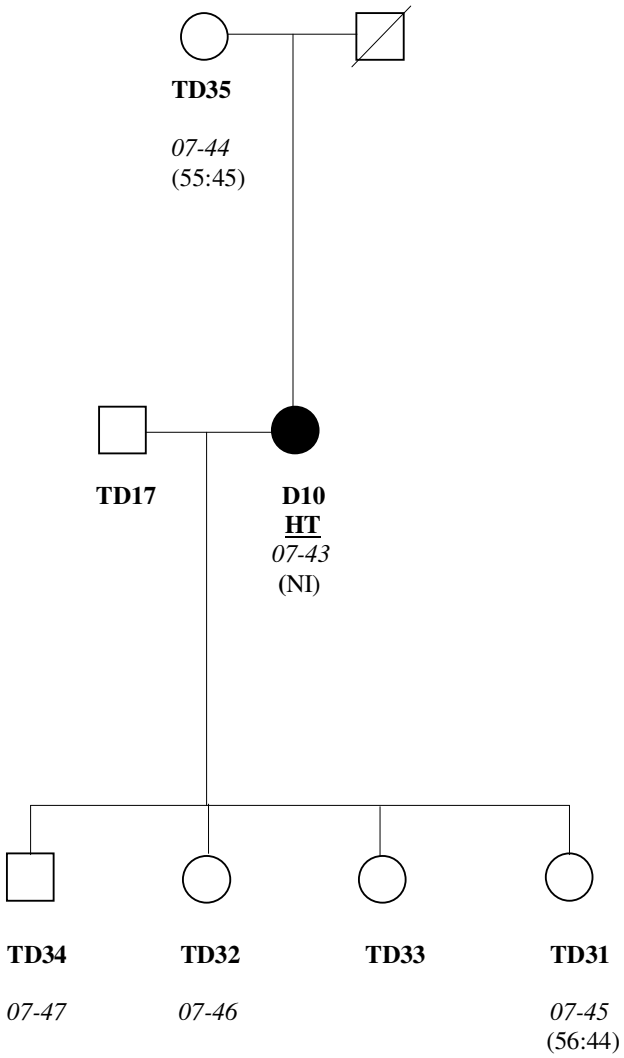
07-38

FAMILY 7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D10
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(NI)
TD34
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TD32
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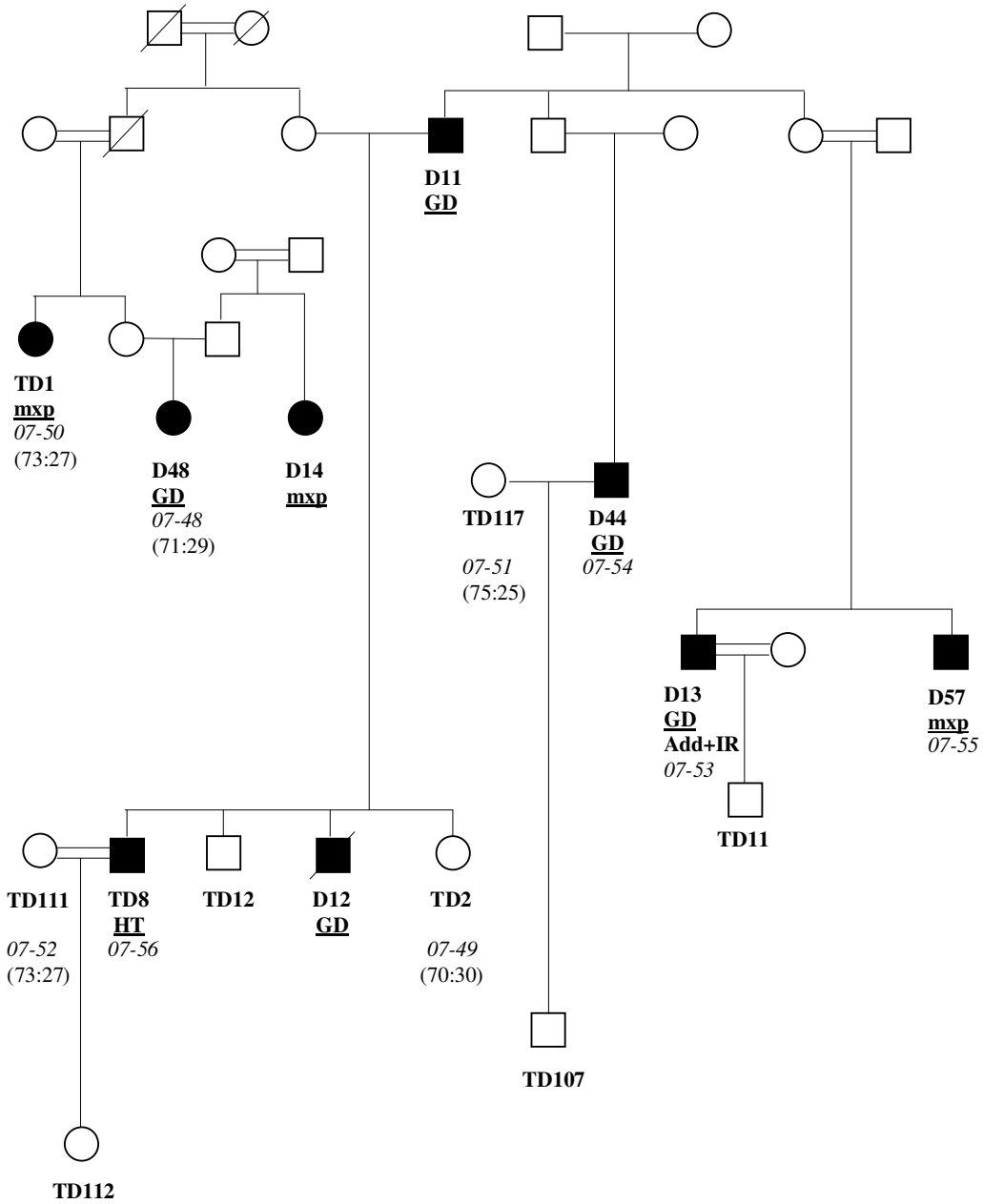
TD33
TD31
07-45
(56:44)

FAMILY 8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D44
GD
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D13
GD
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07-53
TD11
D57
mxp
07-55
TD111
07-52
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07-49
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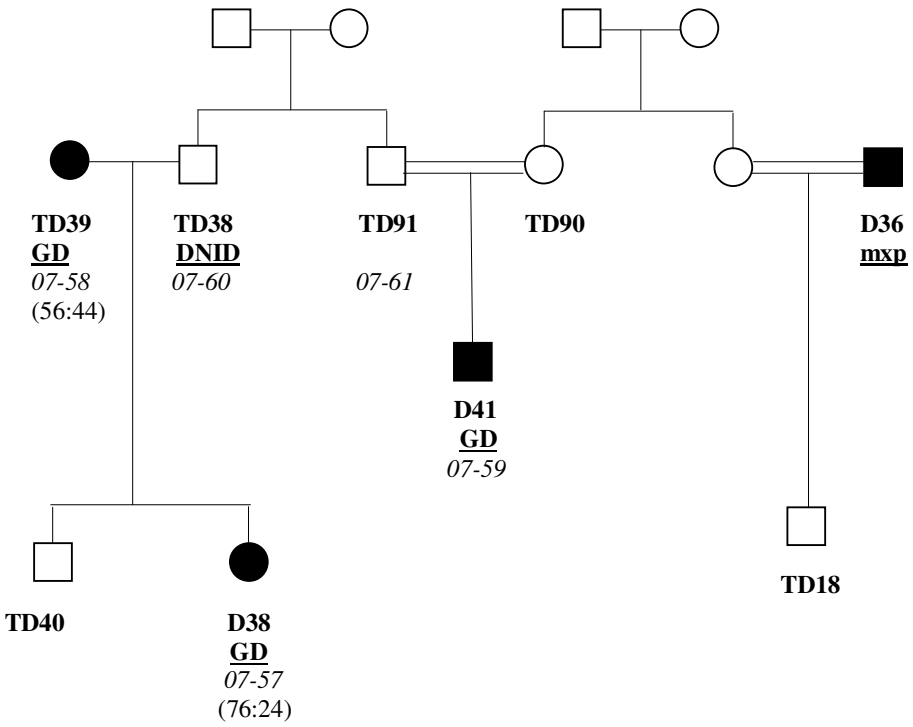
TD107
TD112

FAMILY 9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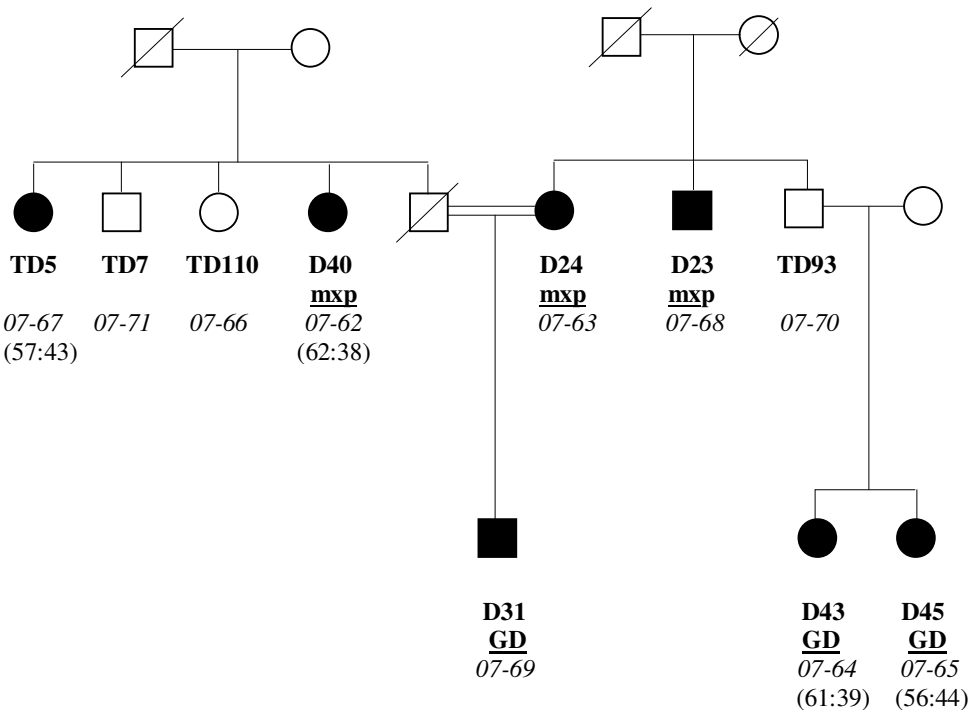
FAMILY 10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07-62
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D24
mxp
07-63
D23
mxp
07-68
TD93
07-70
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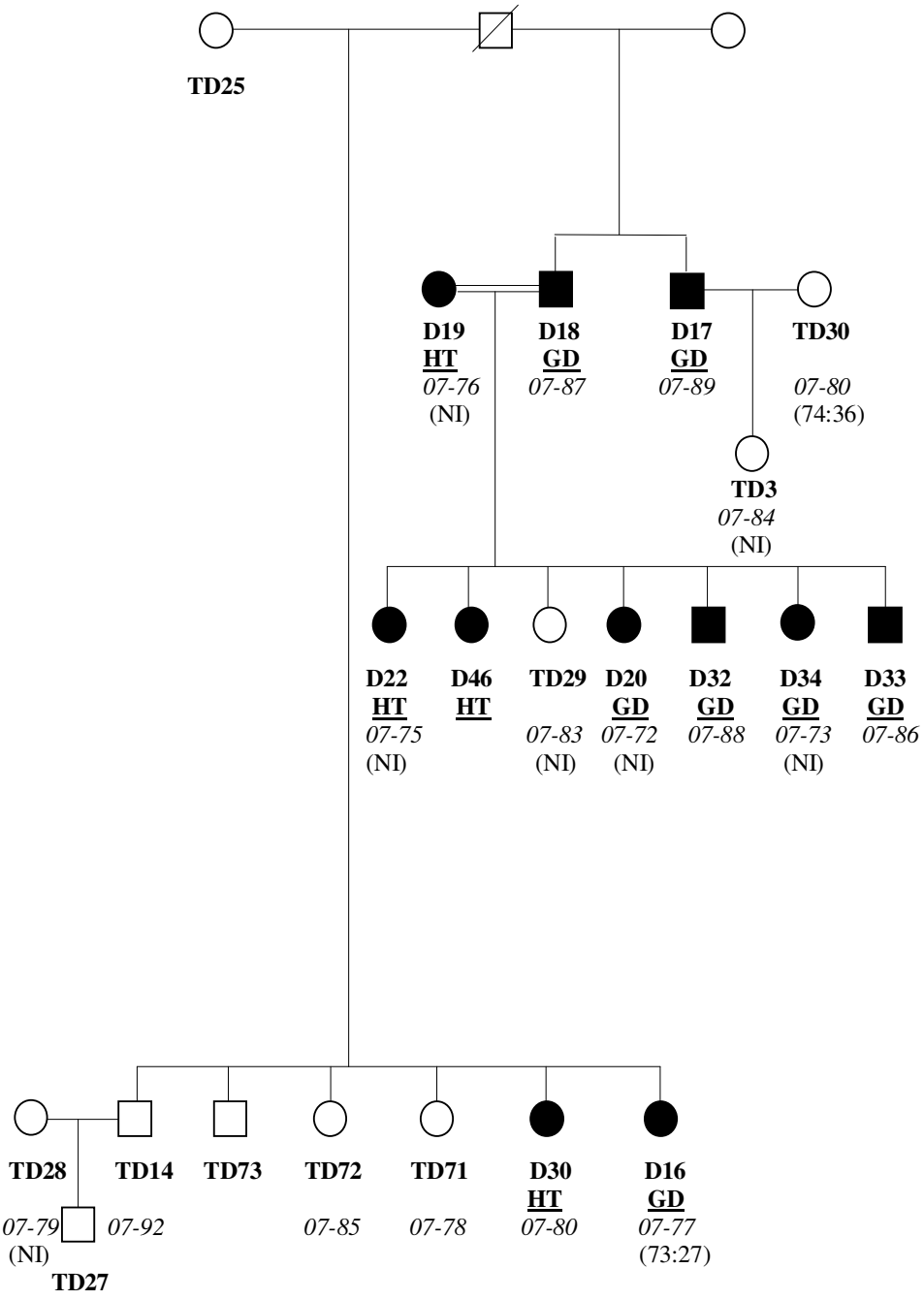
07-69
D43
GD
07-64
(61:39)
D45
GD
07-65
(56:44)

FAMILY 11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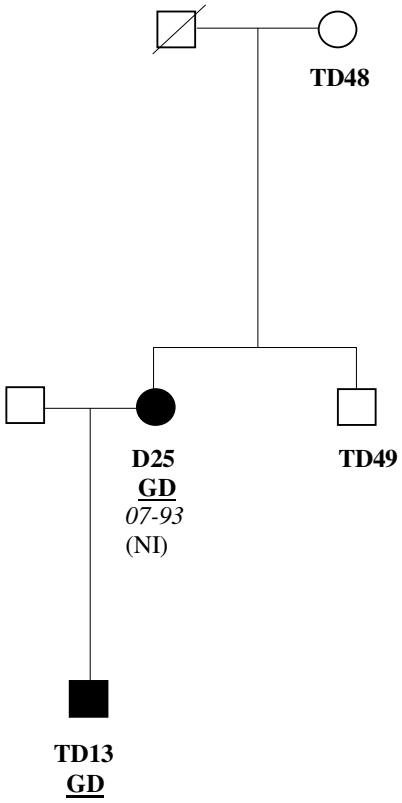
FAMILY 12



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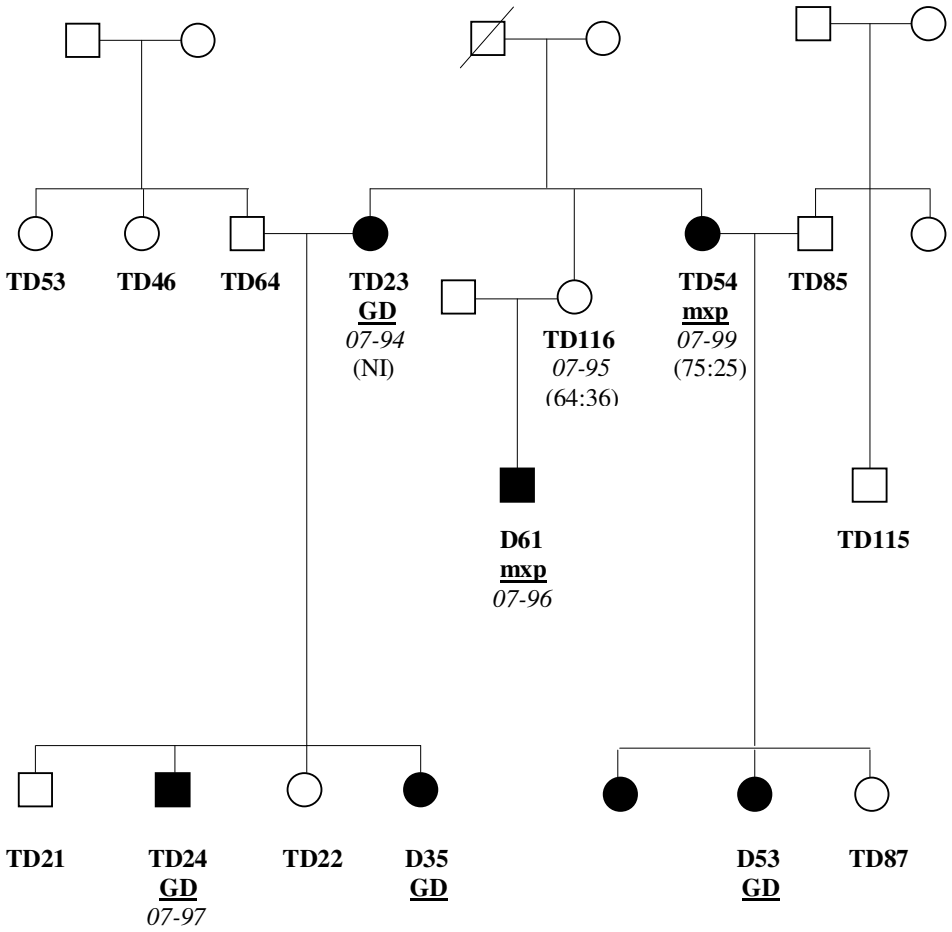
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07-99
(75:25)
TD85
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TD115
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07-97
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GD
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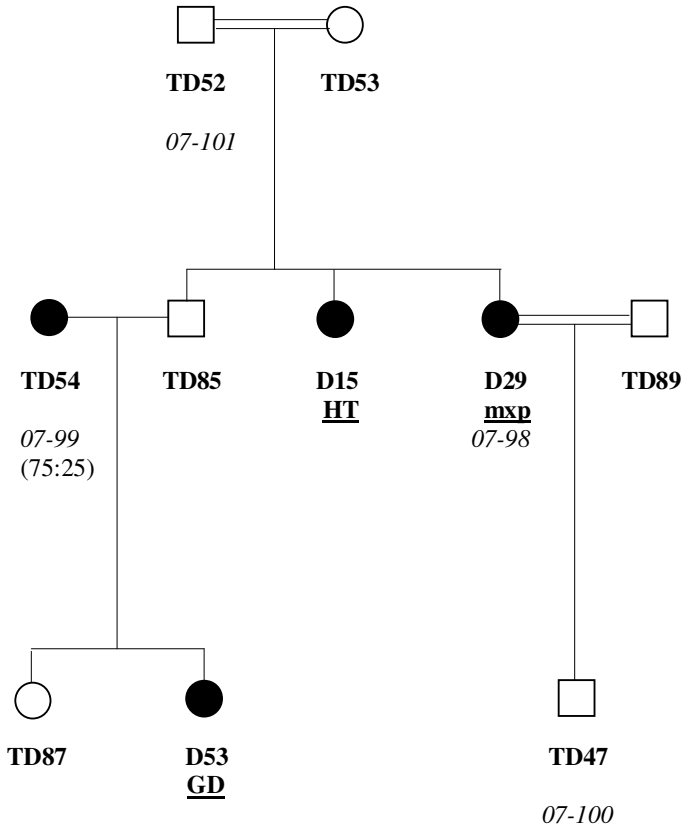
GD
TD87

FAMILY 14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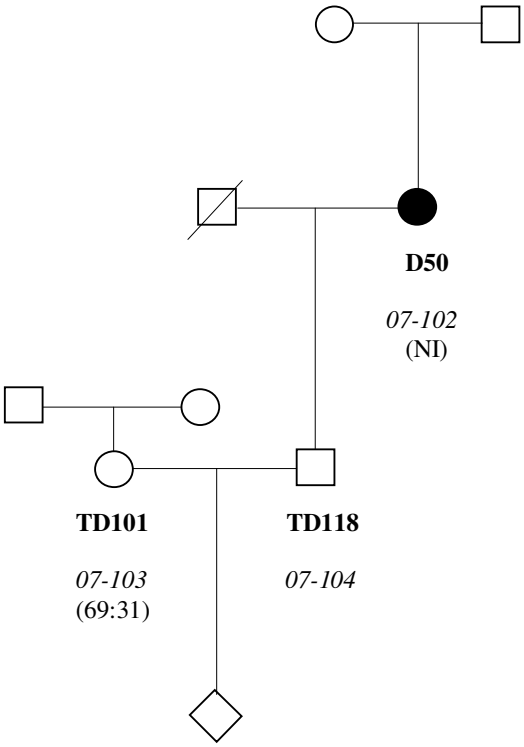

FAMILY 16

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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

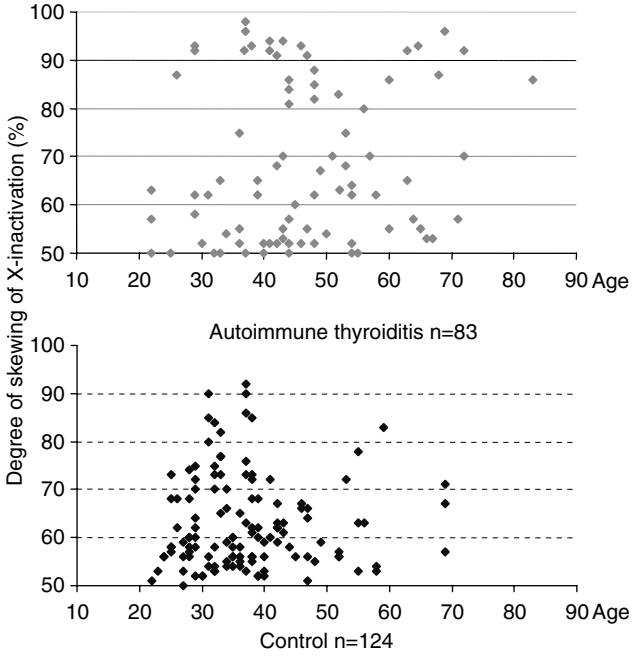

Figure 1 Distribution of X-inactivation patterns according to age in AITDs patients and control subjects.

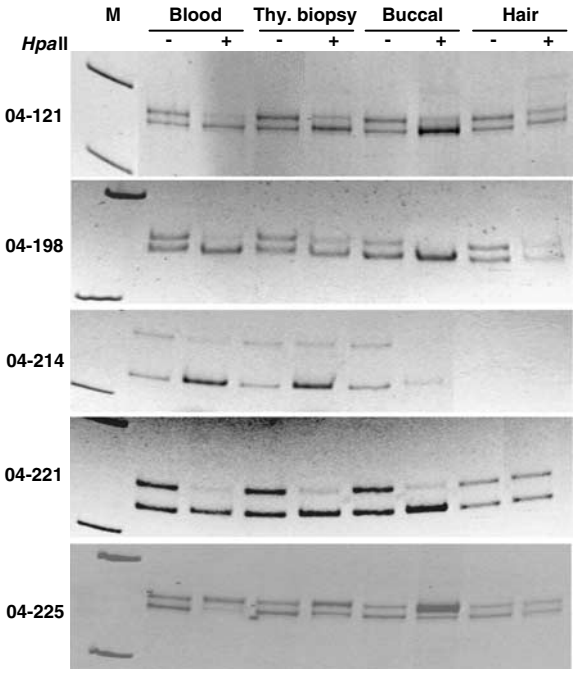

Figure 2 X-inactivation analysis of androgen receptor locus. PCR products of undigested (−) and *Hpa*II-digested (+) DNA from peripheral blood, thyroid biopsy, buccal, and hair follicle samples of AITDs patients 04-121, 04-198, 04-214, 04-221, and 04-225 are shown. Two alleles are seen in undigested 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

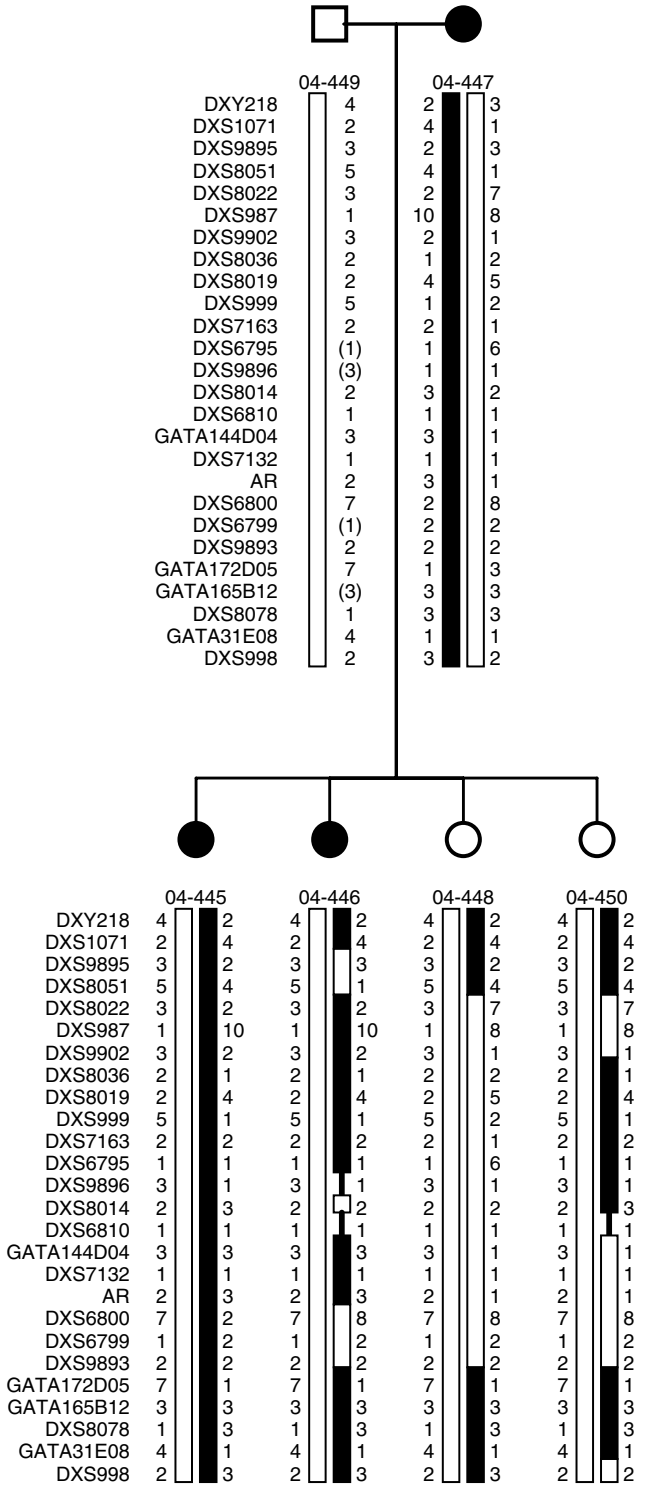

Figure 3 Haplotype structure of Family 1. Patient 04-445 was arbitrarily selected to construct the haplotype. Maternally inherited haplotype was highlighted with solid black bar. Haplotypes of the remaining sibs were compared with the reference individual (04-445), and shared portions were also marked 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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ORIGINAL INVESTIGATION

Extremely skewed X-chromosome inactivation is increased in pre-eclampsia

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Abstract Pre-eclampsia is a disorder that affects approximately 5% of pregnancies. We tested the hypothesis that skewed X-chromosome inactivation (XCI) could be involved in the pathogenesis of pre-eclampsia. Peripheral blood DNA was obtained from 67 pre-eclampsia patients and 130 control women. Androgen receptor (AR) was analyzed by the *Hpa*II/polymerase chain reaction assay to assess XCI patterns in DNA extracted from peripheral-blood cells. In addition, buccal cells were obtained from seven patients, and the analysis repeated. Extremely skewed XCI was observed in 10 of 46 informative patients (21.74%), and in 2 of 86 informative controls (2.33%, $P = 0.0005$; χ^2 test). Our findings support a role for the X-chromosome in the pathogenesis of pre-eclampsia in a subgroup of patients.

Introduction

Pre-eclampsia is a pregnancy-specific syndrome of unknown etiology. It is the leading cause of maternal and perinatal mortality, characterized by increased blood pressure and proteinuria (Roberts and Cooper 2001; Broughton Pipkin 2001). The disease occurs only in the presence of a placenta and resolves with the removal of the placenta. Studies that aim to identify susceptibility loci with significant linkage to familial cases of pre-eclampsia (OMIM 189800) resulted in the identification of four chromosome regions: 2p13 (Iceland) (Arngrimsson et al. 1999), 2p25 and 9p13 (Finland) (Laivuori et al. 2003), and 10q22 (The Netherlands) (Lachmeijer et al. 2001; Oudejans et al. 2004). The Dutch pre-eclampsia locus shows maternal segregation, and recently *STOX1* (storkhead box 1; also called C10orf24), was identified as a candidate gene for this locus (van Dijk et al. 2005).

Maternal endothelial dysfunction and failure of the tolerance system as evidenced by Th-1 type immunity are early pathophysiological modifications in pre-eclampsia (Saito and Sakai 2003). Failure of the tolerance system is associated with autoimmune diseases. A negative selection system against potentially self-reactive T-cells in the thymic medulla and cortex-medulla junction is critically important in establishing T-cell tolerance. The negative selection process is mediated primarily by the dendritic cells, which participate in antigen presentation (Speiser et al. 1989; Laufer et al. 1996). Lack of exposure to self-antigens in the thymus and the presence of autoreactive T-cells have been shown to increase the risk of autoimmunity (Klein et al. 2000).

It was hypothesized that disturbed X-chromosome inactivation (XCI) could be a mechanism whereby lack of exposure to self-antigens may occur in females (Kast 1977; Stewart 1998). X-inactivation is an epigenetic modification in females that results in the transcriptional inactivation of one of the pair of X-chromosomes at

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random (Lyon 1961). Normal females are thus mosaics of two cell populations. Four recently published studies, one by a Japanese group on primary ovarian failure (Sato et al. 2004), one by us on scleroderma (Ozbalkan et al. 2005), and two on autoimmune thyroid diseases (Brix et al. 2005; Ozcelik et al. 2006), raised the possibility that skewed XCI could play an important role in the pathogenesis of autoimmune diseases. Since the disease may have an autoimmune component, we hypothesized that skewed XCI could be involved in the pathogenesis of pre-eclampsia as well.

We observed that the number of women with extreme skewing of XCI is significantly higher in the pre-eclampsia group than in healthy control women with no history of pre-eclampsia, autoimmune diseases or cancer. The cause of skewed XCI mosaicism in autoimmune diseases and pre-eclampsia is not yet known. Possible mechanisms include skewing of XCI as cause of the disease, or alternatively the cause of pre-eclampsia could also be the cause of the skewing.

Materials and methods

Patients

Sixty-seven Caucasian women diagnosed with pre-eclampsia, and 130 apparently healthy Caucasian female controls were genotyped to determine whether women with pre-eclampsia have a greater frequency of extremely skewed XCI than control subjects. The ethics review board of the participating institutions approved study protocol. Pregnancy history, age, and disease information accompanied by informed consent was obtained from all subjects. In the control group (124/130) women gave birth to at least one child, and none had a history of pregnancy loss, autoimmune disease or cancer. The birth registry at the Etlik Maternity and Women's Health Teaching Hospital in Ankara was used to contact the pre-eclamptic cases. All women in the patient group had singleton deliveries. The mean ages were 29.8 ± 5.7 years (mean $\pm$ SD; range = 21–42 years old) for the cases, and 31.6 ± 5.6 (range = 21–47 years old) for the controls.

Pre-eclampsia was defined as the development of hypertension plus proteinuria within 7 days of each other after the twentieth week of gestation in women with no proteinuria at baseline. Hypertension and severe hypertension were defined as diastolic blood pressures of at least 90 and 110 mm Hg, respectively, occurring on at least two occasions, 4–168 h apart. Urinary protein excretion of >300 mg in 24 h was indicative of proteinuria. Pre-eclampsia was considered severe in the presence of severe hypertension or severe proteinuria, HELLP syndrome (haemolysis, elevated liver enzymes, low platelets), or eclampsia. Those subjects with a previous history of chronic hypertension, renal disease or diabetes mellitus were excluded.

X-chromosome inactivation analysis

DNA was extracted from 10 ml venous blood samples with NucleoSpin Blood kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol. After ethanol precipitation, DNA was dissolved in distilled water and stored at 4°C.

Genotyping of a highly polymorphic CAG repeat in the androgen-receptor (AR) gene was performed to assess the XCI patterns (Allen et al. 1992). Patients with ≥90% representation of one allele were classified as extremely skewed XCI. The DNA was divided into two identical aliquots, one of which was incubated overnight at 37°C with methylation-sensitive restriction enzyme *Hpa*II (MBI Fermentas, Vilnius, Lithuania) for the digestion of unmethylated (or active) alleles. A second restriction enzyme, *Rsa*I (MBI Fermentas, Vilnius, Lithuania), which recognizes a four base pair sequence not present in the amplified region of the *AR* locus was also included in the reaction to facilitate the *Hpa*II digestion process. The other aliquot of the DNA was digested with *Rsa*I alone as a control. Male DNA with cytogenetically verified 46XY karyotype was used as a control for incomplete digestion.

After restriction enzyme digest, residual DNA was amplified by using the primers 5′-GTC CAA GAC CTA CCG AGG AG-3′ and 5′-CCA GGA CCA GGT AGC CTG TG-3′. Amplicons were labeled by including a radioactive nucleotide (α-[^{33}P] -dCTP) (NEN, Perkin Elmer Life Sciences, Boston, Massachusetts) in the polymerase chain reaction (PCR). PCR products were separated on 8% denaturing 29:1 acrylamide/bisacrylamide gel for 5 h at 60 W. Gels were dried and autoradiographed on Medicalfilm CP-BU (Agfa, Agfa-Gevaert AG, Belgium). Densitometric analysis of the alleles was performed at least twice for each sample using the appropriate software (MultiAnalyst version 1.1; Bio-Rad, Hercules, California).

In addition, cold PCR followed by electrophoretic separation of the alleles in 4% MetaPhor agarose (FMC BioProducts, Rockland, Maine) for 4 h 30 min at 80 V was carried out. Since the number of cycles could be critically important for the outcome of densitometric analyses, samples were subjected to 25 and also 30 cycles of amplification during the hot and the cold PCR. Products were visualized by ethidium bromide

staining, and densitometric analysis of the alleles was performed at least twice for each sample using the MultiAnalyst version 1.1software. 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 <0.33 or >3.

Statistical methods

The results from control and test groups were compared by the Fisher's exact test.

Results

XCI status was found to be informative in 69% of the cases (46/67) and 66% of the controls (86/130). Only those individuals whose alleles resolve adequately for densitometric analysis were included in the study. Extremely skewed XCI was present in ten (21.74%) cases and two (2.33%) controls ($P = 0.0005$). It is well established that extremely skewed XCI is a rare event in a diverse group of control females (Busque et al. 1996; Naumova et al. 1996; Sharp et al. 2000; Amos-Landgraf et al. 2006). When XCI values between 80 and 89% were also considered, skewed XCI was observed in 16 of the 46 (34.78%) informative cases, and 8 of the 86 (9.30%) controls ($P = 0.0006$) (Table 1). DNA from buccal cells was analyzed in seven patients with various degrees of skewing. They were chosen randomly to assess whether the results from blood, a mesodermal tissue, is comparable to other tissue types such as buccal cells, which have an ectodermal origin. Similar XCI patterns were observed (Table 2). Since it would be of interest to identify differences in the pregnancy details between those of women with extreme skewing, and women with normal X-inactivation, we obtained clinical characteristics of the pre-eclampsia patients informative for XCI status, and the controls (Supplemental Tables 1, 2). Although the numbers are too small to reach a conclusion, it is interesting that recurrent spontaneous abortions is three times more common in women with extreme skewing (3/10) than in women with random patterns of XCI (3/30) (Supplemental Table 3). It is well established that the frequency of skewed XCI is increased in recurrent spontaneous abortions (Lanasa et al. 1999; Sangha et al. 1999; Bagislar et al. 2006). Interestingly, a common genetic background for pre-eclampsia and recurrent spontaneous abortions has been questioned in the medical literature (Christiansen et al. 1990).

Table 1 Proportion of the patients and controls with skewed XCI

Degree of skewing (%)	No. (%) observed with skewing	
	Pre-eclampsia patients ($n = 46$)	Control females ($n = 86$)
90+	10 (21.74)	2 (2.33)
80–89	6 (13.04)	6 (6.98)
70–79	6 (13.04)	15 (17.44)
60–69	13 (28.26)	20 (23.26)
50–59	11 (23.91)	43 (50.00)

For comparison by χ^2, $P = 0.0006$ (>80% skewing); $P = 0.0005$ (≥90% skewing)

Table 2 X-chromosome inactivation patterns in blood and buccal mucosa specimens

Sample	04–176	04–182	04–190	04–298	04–192	04–284	04–289
Blood	91:90	93:70	90:10	98:20	83:17	71:29	70:30
Buccal	90:10	90:10	90:10	100:0	80:20	69:31	66:34

Discussion

We observed skewed XCI patterns in peripheral-blood mononuclear cells of a significant proportion (35%) of females with pre-eclampsia. Approximately 9% of female control subjects demonstrated skewed XCI patterns ≥80:20. The effect is also pronounced at patterns of XCI ≥90:10; nearly a quarter of pre-eclampsia patients showed such skewing, compared with only 2% of control subjects. Although skewed XCI patterns ≥80:20 could be as high as 19.5% (Amos-Landgraf et al. 2006) or 21.6% (Naumova et al. 1996) in phenotypically normal females, skewing in the range of ≥90:10 or ≥95:5 is quite rare with only 3.6 and 1.7% of the population, respectively (Amos-Landgraf et al. 2006). With respect to the relatively low percentage of controls in the ≥80:20 range in our study, this result may be a reflection of the pregnancy histories. An overwhelming majority (124/130) had experienced a normal pregnancy period with healthy deliveries.

Clinical manifestation of X-linked disorders in females could be influenced as a consequence of disturbances in the XCI process (Lyon 2002). High frequency of skewed XCI has also been observed in

recurrent spontaneous abortions (Lanasa et al. 1999; Sangha et al. 1999), X-linked mental retardation (Plenge et al. 2002), breast and ovarian cancers (Kristiansen et al. 2002), and in mothers of homosexual men (Bocklandt et al. 2006). In addition, it has been hypothesized that skewed XCI could be a factor that influences female predisposition to autoimmunity: recently skewed XCI has been reported in three autoimmune disorders, scleroderma (Ozbalkan et al. 2005), autoimmune thyroid diseases (Brix et al. 2005; Ozcelik et al. 2006), and premature ovarian failure (Sato et al 2004).

Skewed XCI could be the result of a bias in the initial choice of which X-chromosome is inactivated (primary cause), or selection against cells in which a given X-chromosome has been inactivated (secondary cause) [for review see Puck and Willard 1998; Brown 1999]. At present, we do not know the nature of the relationship between XCI patterns and pre-eclampsia; except that aging (a secondary cause) is highly unlikely to be involved because of the relatively young ages of the patients (mean age of diagnosis at 29.8 ± 5.7 years for all the patients, 30.3 ± 6.2 years for patients with skewed XCI, and 29.4 ± 5.5 years for patients with random XCI) (Fig. 1). However, two possibilities could be considered: The cause of pre-eclampsia could also be the cause of the skewing, or the skewing of XCI could be the cause of pre-eclampsia. If the autoimmune reaction is the cause of the secondary selection, the disease would cause the skewing. Alternatively, if the disease is at least partially caused by an X-chromosome gene, the disease allele could increase or decrease the fitness of cells inactivating the wild type or mutant allele of this gene, leading to secondary selection for immune cells inactivating one or both X-chromosomes. This would mean that the X-linked gene causes both the disease and the skewing. Since both, pre-eclampsia and skewed XCI may be heritable traits, the women with pre-eclampsia and skewed XCI may share the same mutation(s) in one or more X-linked genes. This implies that it would be particularly important to establish if any of the women who are included in the study were related. However, they were neither related, nor were they from the same geographical location.

Skewed XCI patterns are now demonstrated in a significant proportion of a subgroup of pre-eclampsia patients. Therefore, disturbed XCI mosaicism could be considered as a contributing factor to disease pathogenesis. Further studies, such as haplotype analysis of X-linked markers in mother-child pairs of pre-eclampsia patients, and a search for X-chromosomal aberrations in pre-eclampsia patients by microarray analysis (Larrabee et al. 2004), could be critically important in understanding the genetic etiology.

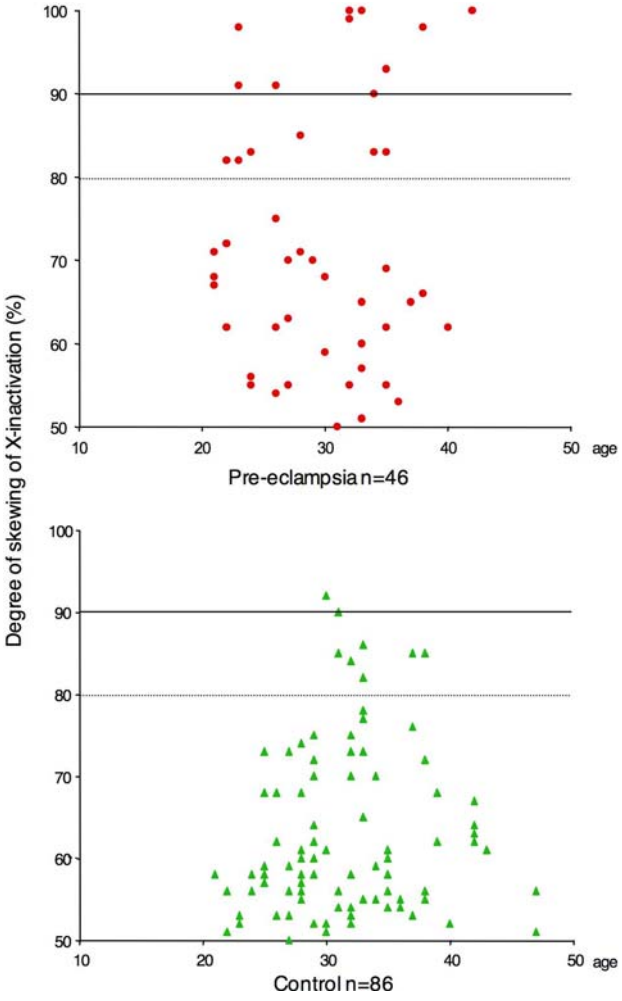

Fig. 1 Distribution of X-chromosome inactivation patterns according to age in pre-eclampsia patients and control subjects

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Skewed X-Chromosome Inactivation in Scleroderma

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Abstract Scleroderma is a female-prevalent autoimmune disease of unclear etiology. Two fundamental gender differences, skewed X-chromosome inactivation (XCI) and pregnancy-related microchimerism, have been implicated in scleroderma. We investigated the XCI patterns of female scleroderma patients and the parental origin of the inactive X chromosome in those patients having skewed XCI patterns (>80%). In addition, we investigated whether a correlation exists between XCI patterns and microchimerism in a well-characterized cohort. About 195 female scleroderma patients and 160 female controls were analyzed for the androgen receptor locus to assess XCI patterns in the DNA extracted from peripheral blood cells. Skewed XCI was observed in 67 (44.9%) of 149 informative patients and in 10 of 124 healthy controls (8.0%) [odds ratio (OR)=9.3 (95% confidence interval (CI) 4.3–20.6, $P<0.0001$)]. Extremely skewed XCI (>90%) was present in 44 of 149 patients (29.5%) but only in 3 of 124 controls (2.4%; OR=16.9; 95% CI 4.8–70.4, $P<0.0001$). Parental origin of the inactive X chromosome was investigated for ten patients for whom maternal DNA was informative, and the inactive X chromosome was of maternal origin in eight patients and of paternal origin in two patients. Skewed XCI mosaicism could be considered as an important risk factor in scleroderma.

Keywords X-inactivation · Microchimerism · Mosaicism · Scleroderma

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Introduction

Scleroderma (systemic sclerosis, SSc) is an autoimmune disease characterized by fibrosis and alterations in the microvasculature [1]. Scleroderma is three to ten times more prevalent in women than in men [2]. Most autoimmune diseases are more prevalent in females than in males [3], and fundamental differences between male and female biology, such as hormone status [4], pregnancy [5], and X-chromosome inactivation [6, 7] have been proposed as the underlying pathophysiological mechanisms leading to the female prevalence of autoimmune diseases. Indeed, both pregnancy-related microchimerism [8–11], and skewed (i.e., not the expected 50:50 balance) X-inactivation mosaicism [12] have been shown to be associated with scleroderma.

This study was performed to investigate the X-inactivation patterns of female scleroderma patients along with the parental origin of the inactive X chromosome in those patients with extremely skewed X-inactivation. In addition, the correlation between skewed X-inactivation and both maternal and fetal microchimerism was examined in a well-characterized cohort of scleroderma patients.

Materials and Method

DNA samples were obtained from 195 scleroderma patients and 160 control women. Clinical characteristics of the patients and controls have been published elsewhere [12, 13]. The ethics review boards at the participating institutions approved the study protocol. Informed consent was obtained from all subjects. The X-chromosome inactivation status of the patients and controls was determined by genotyping a highly polymorphic CAG repeat in the first intron of the androgen receptor as previously described [12, 14]. Depending on the definition and quantitative accuracy of the measurement method, a few percent to nearly one fifth of apparently healthy women display skewed (i.e., non-random) patterns of X-inactivation. Whereas ratios in the range of 50–79% are usually regarded as normal variation, deviation from this range in 80–89% of cells is defined as skewed, and above 90% of cells as extremely skewed X-inactivation. [15–17] Fisher's exact test was used for statistical analyses.

Results

X-Inactivation Ratios

Our data show that skewed X-chromosome inactivation (>80%) was associated with disease. X-chromosome inactivation status was informative in 94 of the 125 scleroderma patients (75%) and in 124 of the 160 controls (78%). Only the individuals whose alleles resolved adequately were included in the subsequent densitometric analysis; thus, some of the heterozygous individuals were considered as uninformative. Skewing in the range of 80–89% was observed in 15 of the 94 patients (16.0%) but in only 7 of the 124 controls (5.6%; $P<0.0001$; Table 1). Extremely skewed X-inactivation (>90%) was observed in 17 of the 94 patients (18.1%) but in only 3 of the 124 controls (2.4%; $P<0.0001$). When the data from the present and the previously published [12] studies were combined (Table 1), of the total of 195 patients, 149 were informative for the androgen receptor polymorphism. Skewed X-inactivation was observed in 67 of 149 patients (44.9%) but in only 10 of 124 healthy controls (8.0%). Expressed as a risk factor for scleroderma, the odds ratio (OR) was 9.3 [95% confidence interval (CI) 4.3–20.6; $P<.0001$]. Numerous studies conducted in different control populations indicate that extremely skewed X-inactivation is a rare event not exceeding 3–5% [15–17]. For the current study, extremely skewed X-inactivation was present in 44 of 149 patients (29.5%) but in only 3 controls (2.4%), and the OR was 16.9 (95% CI 4.8–70.4, $P<0.0001$).

Parental Origin of the Inactive X Chromosome

Nineteen patients with skewed X-inactivation for whom maternal DNA was available were further analyzed to determine the parental origin of the inactive X chromosome. For this analysis, DNA samples from mothers of patients were analyzed for the androgen receptor gene polymorphism, and informative results were obtained for ten patient–mother pairs. For the remaining nine pairs, both the mother and the daughter were heterozygous for the androgen receptor polymorphism, thus providing no difference in allele sizes. Therefore, it is not possible to determine the parental origin of the alleles in the absence of information regarding the paternal genotype. The inactive X chromosome was of maternal origin in eight patients and of paternal origin in two patients. In three mothers of patients studied, X-inactivation pattern was also skewed (Table 2). Interestingly, the same allele was skewed in both the mothers and the patients, and one mother had been diagnosed with an autoimmune condition, namely temporal arteritis.

Table 1 Proportions of scleroderma patients and controls with skewed X-inactivation

Degree of skewing (%)	No. (%) observed with skewing			
	Present study (n=94)	Previous study [12] (n=55)	Combined (n=149)	Control (n=124)
+90	17 (18.1%)	27 (49.1%)	44 (29.5%)	3 (2.4%)
80–89	15 (16.0%)	8 (14.5%)	23 (15.4%)	7 (5.6%)
70–79	15 (16.0%)	0 (0.0%)	15 (10.1%)	22 (17.7%)
60–69	24 (25.5%)	5 (9.1%)	29 (19.5%)	29 (23.4%)
50–59	23 (24.5%)	15 (27.3%)	38 (25.5%)	63 (50.8%)

For comparison by χ^2, $P<0.0001$(both >80% skewing and >90% skewing)

Table 2 Parental origin of the inactive X chromosome in scleroderma patients with skewed X-inactivation

Sample	Degree of skewing	Parental origin of the inactive X chromosome
Patient 1	95	Maternal
Mother 1	95	
Patient 2	95	Maternal
Mother 2	95	
Patient 3	86	Maternal
Mother 3	88	
Patient 4	100	Maternal
Mother 4	Not informative	
Patient 5	90	Maternal
Mother 5	Not informative	
Patient 6	90	Maternal
Mother 6	Not informative	
Patient 7	85	Maternal
Mother 7	67	
Patient 8	85	Maternal
Mother 8	58	
Patient 9	85	Paternal
Mother 9	70	
Patient 10	80	Paternal
Mother 10	70	

Evaluation of Skewed X-Inactivation for Correlation with Microchimerism

Maternal and fetal microchimerism was investigated as described previously [18]. We examined X-inactivation ratios for correlation with microchimerism in a limited number of patients for whom microchimerism data was also available. We observed that among patients with more than 80% skewing, 7 of 12 (58.3%) tested positive for maternal microchimerism and 8 of 14 (57.1%) for fetal microchimerism (Table 3). In patients with random patterns of X-inactivation, 10 of 15 (66.7%) were positive for maternal microchimerism, and 14 of 25 patients were positive for fetal microchimerism (56%). Neither maternal (P=0.7) nor fetal microchimerism (P=1.0) correlated with skewed X-inactivation.

Discussion

Autoimmune disorders affect more than 5% of the population, and a high female prevalence is characteristic of most autoimmune diseases including scleroderma, autoimmune thyroid diseases, systemic lupus erythematosus, and Sjögren's syndrome [3]. Pregnancy-related microchimerism [10] and skewed X-inactivation [12] have been proposed as potential contributors to the pathophysiology of scleroderma. In this study, we observed skewed X-inactivation patterns in a significant proportion (34%) of women with scleroderma. Of these women, approximately 18% displayed extreme skewing (>90:10). This result is consistent with our previous study [12] and indicates that skewed X-inactivation could be a common finding in different population groups.

We do not know the cause of skewed X inactivation in scleroderma, and probable mechanisms have been discussed in the accompanying manuscript [19]. Among them, X-linked lethal mutations, which would be compatible with life in females because of X-inactivation mosaicism, are appealing causative mechanism. If the cause of skewing is indeed X-linked mutations, these should be inherited from the maternal lineage unless they occur de novo during gametogenesis. We therefore analyzed the parental origin of the inactive X chromosome in 19 patient–mother pairs, and of the 10 pairs with informative genotyping results, inactive X was found to be of maternal origin in 8 pairs. This result is on the border of statistical significance (P=0.055). Assuming this result holds, we will be left with an interesting puzzle. Why are maternally inherited X chromosomes more likely to be inactivated in highly skewed patients? One possibility is that some X chromosomes confer a selective disadvantage to both the organism itself and the peripheral cells within the organism. This precise situation has been documented in X-linked immunodeficiency, where heterozygous (female) carriers have high X-inactivation skew (because of natural selection within the organism), whereas affected males have a survival disadvantage [20]. When an X-encoded genotype is selectively disadvantageous to cells, skew is an outcome. When an X-encoded genotype is selectively disadvantageous to an organism, preferential maternal inheritance is an outcome. Together, this leads us to the hypothesis that some highly

Table 3 Proportion of maternal (MMc) and fetal (FMc) microchimerism in scleroderma patients

	MMc		FMc	
Skewed (>80%)	12 tested 7 positive 5 negative	58.3% positive for MMc	14 tested 8 positive 6 negative	57.1% positive for FMc
Random (<80%)	15 tested 10 positive 5 negative	66.7% positive for MMc	25 tested 14 positive 11 negative	56.0% positive for FMc

skewed patients have an X chromosome that would confer a selective disadvantage on homozygotic women and on males.

Because scleroderma is the first disease in which pregnancy-related microchimerism has been documented, we investigated a subset of patients for whom microchimerism data were also available for correlation of skewed X-inactivation with microchimerism. Neither maternal nor fetal microchimerism was found to be correlated with skewed X-inactivation. In conclusion, the two types of female mosaicism—skewed X-inactivation and maternal/fetal microchimerism—appear to be independent risk factors in scleroderma. One possible explanation for these data is that mosaicism itself is an underlying cause of scleroderma and, by extension, female-prevalent autoimmune disease in general.

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Skewed X inactivation in an X linked nystagmus family resulted from a novel, p.R229G, missense mutation in the *FRMD7* gene

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ABSTRACT

Aims: This study aimed to identify the underlying genetic defect of a large Turkish X linked nystagmus (NYS) family.

Methods: Both Xp11 and Xq26 loci were tested by linkage analysis. The 12 exons and intron–exon junctions of the *FRMD7* gene were screened by direct sequencing. X chromosome inactivation analysis was performed by enzymatic predigestion of DNA with a methylation-sensitive enzyme, followed by PCR of the polymorphic CAG repeat of the androgen receptor gene.

Results: The family contained 162 individuals, among whom 28 had NYS. Linkage analysis confirmed the Xq26 locus. A novel missense c.686C>G mutation, which causes the substitution of a conserved arginine at amino acid position 229 by glycine (p.R229G) in exon 8 of the *FRMD7* gene, was observed. This change was not documented in 120 control individuals. The clinical findings in a female who was homozygous for the mutation were not different from those of affected heterozygous females. Skewed X inactivation was remarkable in the affected females of the family.

Conclusions: A novel p.R229G mutation in the *FRMD7* gene causes the NYS phenotype, and skewed X inactivation influences the manifestation of the disease in X linked NYS females.

Congenital nystagmus (NYS) is an ocular oscillatory movement disorder caused by a motor instability that can manifest with or without afferent visual system dysfunction. This entity is defined both clinically and by electronystagmography. Certain clinical features usually differentiate congenital NYS from other oscillations. NYS may be irregular, but is always conjugate and horizontal, though very rarely vertical. There may be a torsional component. Some patients with this abnormality also show head oscillations, which tend to increase when the patient attends to an object, an effort that also increases the NYS. Therefore, it seems probable that both the head tremor and the ocular oscillations are the consequence of a common disordered neural mechanism.[1]

The underlying defect in congenital NYS remains elusive. The Eye Movement Abnormalities and Strabismus Working Group proposed the term, infantile nystagmus syndrome, for the NYS that is known as congenital NYS. Although there is some controversy concerning classification of NYS in infancy, it has been suggested that NYS with an onset before 6 months of age can be divided into three categories:[2] (1) congenital idiopathic NYS in which no visual or associated neurological impairment can be found; (2) sensory deficit NYS in which there is a visual abnormality, such as ocular albinism, optic nerve hypoplasia, congenital stationary night blindness and blue cone monochromatism; (3) neurological NYS, which is associated with a neurological disorder. Category 1 is also called congenital motor NYS, which is presumed to have a primary defect in the part of the brain responsible for ocular motor control.[3]

Congenital motor NYS is a genetically heterogeneous disorder. Autosomal dominant (MIM 164100), recessive (MIM 257400) and X linked (MIM 310700) patterns of inheritance are described for this disorder. X linked inheritance is the most common form of NYS (NYS1). An irregular dominant pattern of X linked inheritance has been frequently reported, although some pedigrees support X linked recessive inheritance.[4] The penetrance is full in males and approximately 50% to 29% in females.[5 6] Large intrafamilial variance in waveforms can be observed. Two distinct loci, one on the Xq26–q27[3] and the other on the Xp11.4[7] regions have been reported as the likely loci for X linked dominant NYS. The locus on the long arm (Xq26) has been confirmed by subsequent reports of various ethnic populations.[8–10] To the best of our knowledge, there are only two reports supporting the Xp11.4 locus.[7 11] The pattern of inheritance and clinical profile of Xp11-linked families are not different from Xq26-linked pedigrees. X linked recessive NYS has also been mapped to the Xq26 region, which harbours the X linked dominant NYS locus.[10] Recently, mutations in the *FRMD7* (FERM domain-containing 7) gene have been reported as a molecular cause in Xq26-linked families.[6] Little is known about the function of the *FRMD7* gene; however, the restricted expression pattern of this gene in the human embryonic brain and developing neural retina suggests a role in eye movement and gaze stability.[6]

Herein, we report an extensive NYS pedigree, including 162 individuals across six generations from southeastern Turkey. The mode of inheritance is clearly X linked, demonstrating a reduced penetrance in female obligate gene carriers. Genetic linkage analysis confirmed the Xq26–q27 locus, and further mutation analysis identified a novel p.R229G missense mutation in the *FRMD7* gene that causes this disorder. We also detected a predisposition to skewed X inactivation in affected

females, suggesting that the X inactivation mechanism might have a role in manifestation of the disease in females.

METHODS

Clinical evaluation

The family was identified during fieldwork to study a large craniosynostosis pedigree in Antakya, Turkey. The complete pedigree structure contains over 427 individuals, including a separate branch of congenital idiopathic NYS. Only the NYS branch is included in this study, and the pedigree structure is shown in fig 1. Pedigree formation was completed in the field by NA, IV and SK. Neurological evaluation of 23 individuals was conducted by YK, a neurologist and member of the family. The recruitment criteria were NYS noted at birth or during the first 3 months of life, and no abnormalities of the ocular or neural visual pathway. Visual acuity of all the patients was measured by Snellen card. Eye movements were recorded by a video camera in 16 patients. Video recordings were further reviewed by a neuro-ophthalmologist (TK). Peripheral blood samples were collected from 48 individuals with informed consent for further molecular studies. The Hacettepe University Ethics Committee approved the study (FON 02/6-13)

Genetic linkage analysis

The family was tested for linkage to two reported loci on chromosome regions Xp11[7] and Xq26.[3] Map order and physical positions (Mb) of the polymorphic markers were obtained from the University of California Santa Cruz (USCS), Genome Bioinformatics Center (http://genome.ucsc.edu). Oligonucleotide samples were purchased from MWG-Biotechnology (Ebersberg, Germany). Site-specific PCR, 7% polyacrylamide gel electrophoresis and silver staining technique were used in genotyping the individuals. Gels were manually pictured. The Cyrillic program was used to generate haplotype and input files for linkage analysis. Two-point linkage was performed with the MLINK component of the LINKAGE package, assuming X linked dominant inheritance with 0.36 penetrance in females. Mutant allele frequencies were kept as 0.0001. Equal marker allele frequency of DNA markers was assumed.

Mutation analysis

We designed primers to amplify all 12 protein-coding exons and adjacent intronic sequences of the *FMRD7* gene by PCR using standard conditions (supplementary table). Due to its size, exon 12 was amplified in three overlapping fragments. Subsequent to amplification, PCR fragments were purified, with QIAquick spin columns (Qiagen), and directly sequenced using the corresponding forward primers with the ABI PRISM® BigDye™ Terminator v2.0 cycle sequencing ready reaction kit and an ABI PRISM® 3100 genetic analyser (Applied Biosystems). We re-sequenced all identified mutations in independent experiments, tested for co-segregation within the families, and screened 120 Turkish control individuals for the c.686C>G mutation by PCR and subsequent restriction digestion using *Bcc*I (MBI Fermentas, Germany).

Evaluation of X chromosome inactivation (XCI)

Genotyping of a highly polymorphic CAG repeat in the androgen-receptor (*AR*) gene was performed to assess the XCI patterns.[12] DNA was divided into two identical aliquots, one of

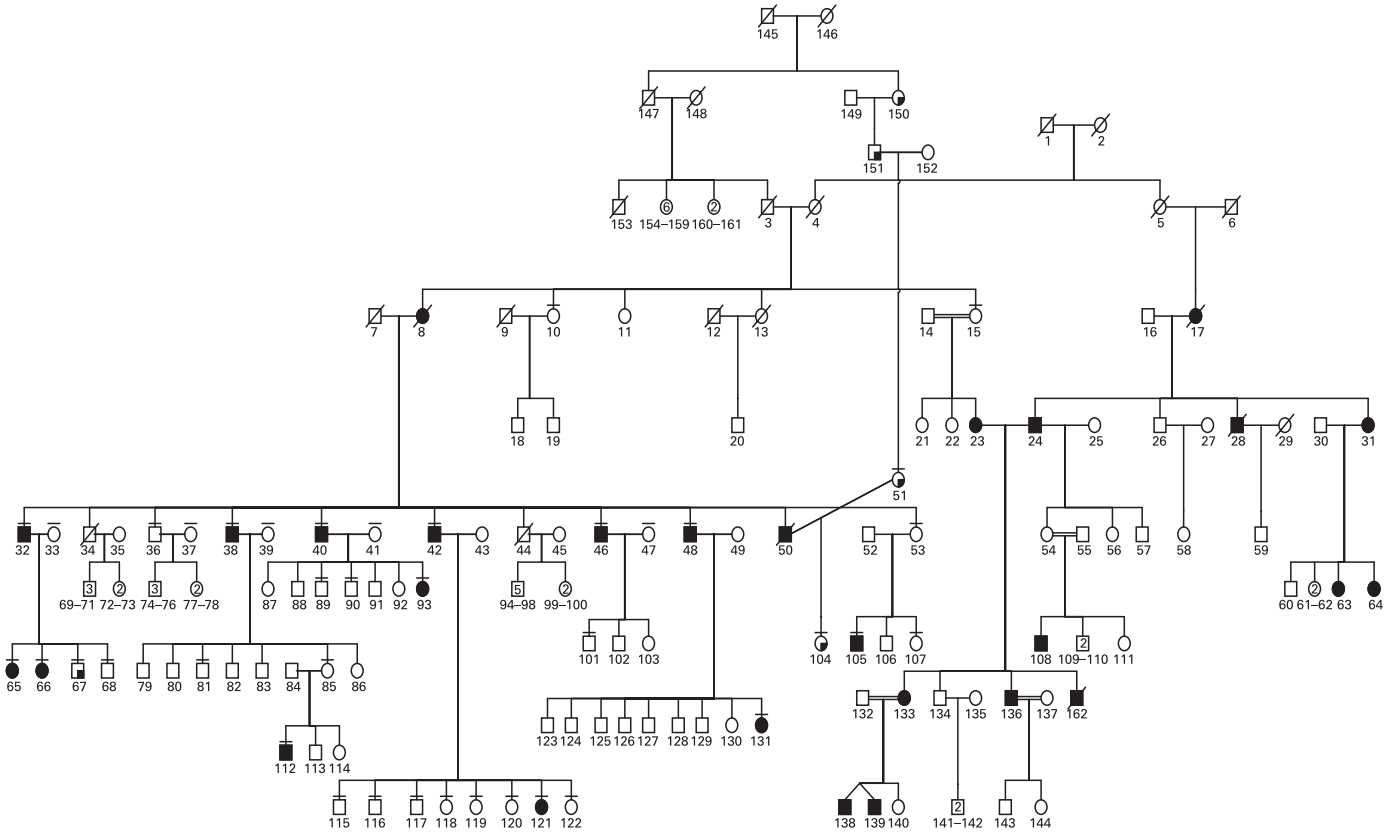

Figure 1 Complete pedigree structure of X linked idiopathic congenital NYS. Full shaded symbols indicate the NYS phenotype. Quarter shaded symbols demonstrate individuals with craniosynostosis. The craniosynostosis branch (relatives of individuals 150, 151 and 51) was not included in this study.

Table 1 Examination findings of the family members

PID	Gender	DOB	Nystagmus	Head oscillations	DM type II	Obesity	Other findings
23	F	1939	Pendular	+	–	–	–
24	M	1929	Pendular	+	+	+	Asthma, corneal opacity on the left, hypertension
32	M	1961	Pendular + jerk	+	+	+	Diabetic neuropathy, cataract, hypertension
38	M	1950	Pendular	–	+	+	Diabetic neuropathy, amyloid lichenosis
40	M	1945	Pendular	+	+	+	–
42	M	1943	Pendular	+	+	+	Diabetic neuropathy, psoriasis, hypertension
46	M	1939	Pendular	+	+	+	Diabetic neuropathy, ptosis on the left
48	M	1930	Pendular	+	+	+	Diabetic neuropathy, hypertension, ptosis on the right
65	F	1981	Pendular + jerk	+	–	–	–
66	F	1982	Pendular + jerk	–	–	–	Strabismus
93	F	1980	Pendular	–	–	–	–
105	M	1986	Pendular + jerk	+	–	+	Febrile convulsion
108	M	1985	Pendular	+	–	–	–
112	M	1998	Pendular	–	–	–	–
121	F	1971	Pendular	+	–	+	–
131	F	1965	Pendular	+	–	–	–
133	F	1969	Pendular	–	–	–	–
136	M	1968	Pendular	+	–	+	–
138	M	1993	Pendular	–	–	–	–
139	M	1993	Pendular	–	–	–	–
36	M	1957	–	–	+	+	–
39	F	1951	–	–	–	+	–
43	F	1951	–	–	–	+	–
53	F	1959	–	–	+	+	Psoriasis, hypertension
54	F	1960	–	–	–	+	–
69	M	1978	–	–	–	+	–
79	M	1971	–	–	–	+	–
80	M	1974	–	–	–	+	–
81	M	1976	–	–	–	+	–
82	M	1989	–	–	–	+	–
87	F	1970	–	–	–	+	–
91	M	1969	–	–	–	+	–
94	M	1989	–	–	–	+	Allergic conjuctivitis
95	M	1976	–	–	–	+	–
107	F	1983	–	–	–	+	–
115	M	1975	–	–	–	+	–
116	M	1979	–	–	–	+	–
117	M	1980	–	–	–	+	Asthma bronchiale
118	F	1970	–	–	–	+	–
119	F	1978	–	–	–	+	Asthma bronchiale
120	F	1984	–	–	–	+	–
122	F	1986	–	–	–	+	–
123	M	1967	–	–	–	+	–
124	M	1968	–	–	–	+	–
125	M	1969	–	–	–	+	–
126	M	1960	–	–	–	+	–
134	M	1968	–	–	–	+	–

DM, diabetes mellitus; DOB, date of birth; PID, Personal Identification Number presented in fig 1.

which was incubated overnight at 37°C with the methylation-sensitive restriction enzyme *Hpa*II (MBI Fermentas, Vilnius, Lithuania), for the digestion of unmethylated (or active) alleles. A second restriction enzyme, *Rsa*I (MBI Fermentas, Vilnius, Lithuania), which recognises a four-base-pair sequence not present in the amplified region of the *AR* locus, was also included in the reaction to facilitate the *Hpa*II digestion process. Male DNA with a cytogenetically verified 46,XY karyotype was used as the control for complete digestion. The other half of the DNA was treated similarly, but without *Hpa*II. After restriction-enzyme digestion, residual DNA was amplified using the primers 5′-GTC CAA GAC CTA CCG AGG AG-3′ and 5′-CCA GGA CCA GGT AGC CTG TG-3′. PCR products were separated on 10% denaturing 29:1 acrylamide-bisacrylamide gel for 5 h at 20 W. Gels were stained with ethidium bromide and visualised under UV light. Densitometric analysis of the alleles was performed at least twice for each sample using the appropriate software (MultiAnalyst v1.1; Bio-Rad, Hercules, CA). 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 normalisation of the ratios that were obtained from the densitometric analyses. The use of a CrR compensates for preferential amplification of the shorter allele when the number of PCR cycles increases.[13] A skewed population is defined as a cell population with >80% expression of one of the AR alleles. This corresponds to CrR values of <0.25 or >4.0.

Table 2 Two point LOD scores with the DNA markers selected from Xp11 and Xq26 regions

	Recombination fraction (θ) cM					
Marker	**0.00**	**0.01**	**0.05**	**0.10**	**0.20**	**Exclusion (cM)**
Xp11						
DXS6810	−∞	−5.35	−2.64	−1.54	−0.58	7
GATA144D03	−∞	−5.19	−2.50	−1.43	−0.52	6
DXS7132	−∞	−5.32	−2.62	−1.53	−0.57	7
Xq26						
GATA172D05	−∞	−1.78	−0.53	−0.11	0.12	–
GATA165B12	−∞	0.54	1.07	1.16	1.00	–
DXS1047	−∞	1.08	1.58	1.62	1.37	–
DXS8068	−∞	0.38	0.93	1.03	0.91	–
DXS8072	3.74	3.68	3.44	3.12	2.45	–
DXS8071	0.82	0.80	0.72	0.62	0.43	–
DXS1187	3.37	3.32	3.10	2.81	2.21	–
DXS8033	3.68	3.62	3.38	3.07	2.41	–
DXS8094	4.04	3.98	3.72	3.38	2.65	–
DXS1062	2.55	2.51	2.33	2.09	1.59	–
DXS1192	0.48	0.47	0.43	0.38	0.29	–
DXS984	3.74	3.68	3.44	3.12	2.45	–
GATA31E08	3.37	3.32	3.10	2.81	2.21	–

Exclusion area = recombination fraction (cM) at which the LOD score was $\leqslant -2$. Significant LOD scores were obtained with the DNA markers from the Xq26 region.

RESULTS

Clinical presentation and formal genetics of the family

The most striking findings of this pedigree were, 1, NYS and, 2, obesity and type 2 diabetes in some family members (table 1). Other findings included craniosynostosis and allergic complaints, such as bronchial asthma. In all, 20 patients had rhythmic pendular type NYS, with varying frequencies and amplitudes. Video recordings of 16 individuals supported the clinical observations. On lateral gaze, four individuals had horizontal jerk NYS, whereas six patients had spontaneous head oscillations, and 13 had oscillations during fixation (table 1). Visual acuity among the patients varied from 20/20 to 20/100. Direct ophthalmoscopic findings were unremarkable. In all, seven NYS patients had diabetes, and 10 were obese. Among the non-NYS family members, 27 were obese (two of them with diabetes) (table 1) suggesting independent segregation of the two disorders (NYS and obesity and/or type 2 diabetes) in the same family. Regarding the NYS phenotype, male-to-male transmission was not observed. This finding supported X linked inheritance. There were 33 obligate gene carrier females in this family, and only 12 of them developed NYS (fig 1); therefore, the penetrance of the NYS phenotype in females was estimated to be approximately 36% for this family.

Linkage studies

The family was tested for a linkage to both Xp11.4-p11.3[7] and Xq26–q27[3] loci. The NYS phenotype was previously mapped between the DNA markers DXS8015 and DXS1003 on the Xp11.4 locus. The DNA markers DXS6810, GATA144D04 and DXS7132 were used in this study, and the physical positions of these markers are: DXS8015 (39.44 Mb)-DXS6810, (42.57 Mb)-GATA144D04 and (44.67 Mb)-DXS1003 (46.19 Mb)-DXS7132 (64.33 Mb). Four NYS patients were recombinant for the entire region (data not shown). Negative LOD scores were also obtained. The excluded region was 6–7 cM for each DNA marker (table 2).

Significant LOD scores were obtained for the entire region, with a maximum of 4.04 at $\theta = 0$ cM for the DNA marker DXS8094 (table 2) in the Xq26–q27 region (fig 2). A single cross-over event in an NYS patient (person 40) positioned the disease gene telomeric to the DNA marker DXS8068. We also observed the first homozygote female case of the NYS phenotype (fig 1, person 133). No phenotypic differences were observed among the females, in terms of homozygote and heterozygote states (table 1).

Mutation results

We sequenced all 12 coding exons of the recently described X linked NYS gene *FRMD7*, which was located in the critical region in two affected male individuals of the family, and identified the c.686C>G mutation in exon 8 of the gene (fig 3A). The mutation co-segregated with the disease in the family (fig 3B) and was not observed in 120 healthy individuals of the same ethnic background. The novel c.686C>G mutation predicted a substitution of a conserved arginine at amino acid position 229 in the functionally important FERM-C domain of the protein by glycine (p.R229G). Interestingly, three other missense mutations in the FERM-C domain of FRMD7 have been described in the original gene identification paper[6] (fig 3C), suggesting an important role for this domain in the pathogenesis of congenital NYS.

X chromosome inactivation results

Since at least seven females in the pedigree had NYS, we analysed the XCI patterns to verify if skewed XCI could be responsible for the clinical manifestation of the disease. Skewed patterns with ratios of 81:19 per cent in individual 65, 85:15 per cent in 121, and 80:20 per cent in 131 and 133 was apparent, while individual 23 was not informative, and only individuals 93 and 66 displayed random XCI with ratios of 57:43 per cent and 62:38 percent, respectively. Among the non-NYS females, XCI status was analysed in 18 individuals. With the exception of individual 35, who had a skewed XCI (93:7) pattern, and four more non-informative females (individuals 41, 43, 54 and 86), all women displayed random XCI profiles (table 3). Skewed X inactivation was significantly increased in the NYS females when compared with the non-NYS females in the family (odds ratio was 26:1; 95% CI = 1.83 to 367.7).

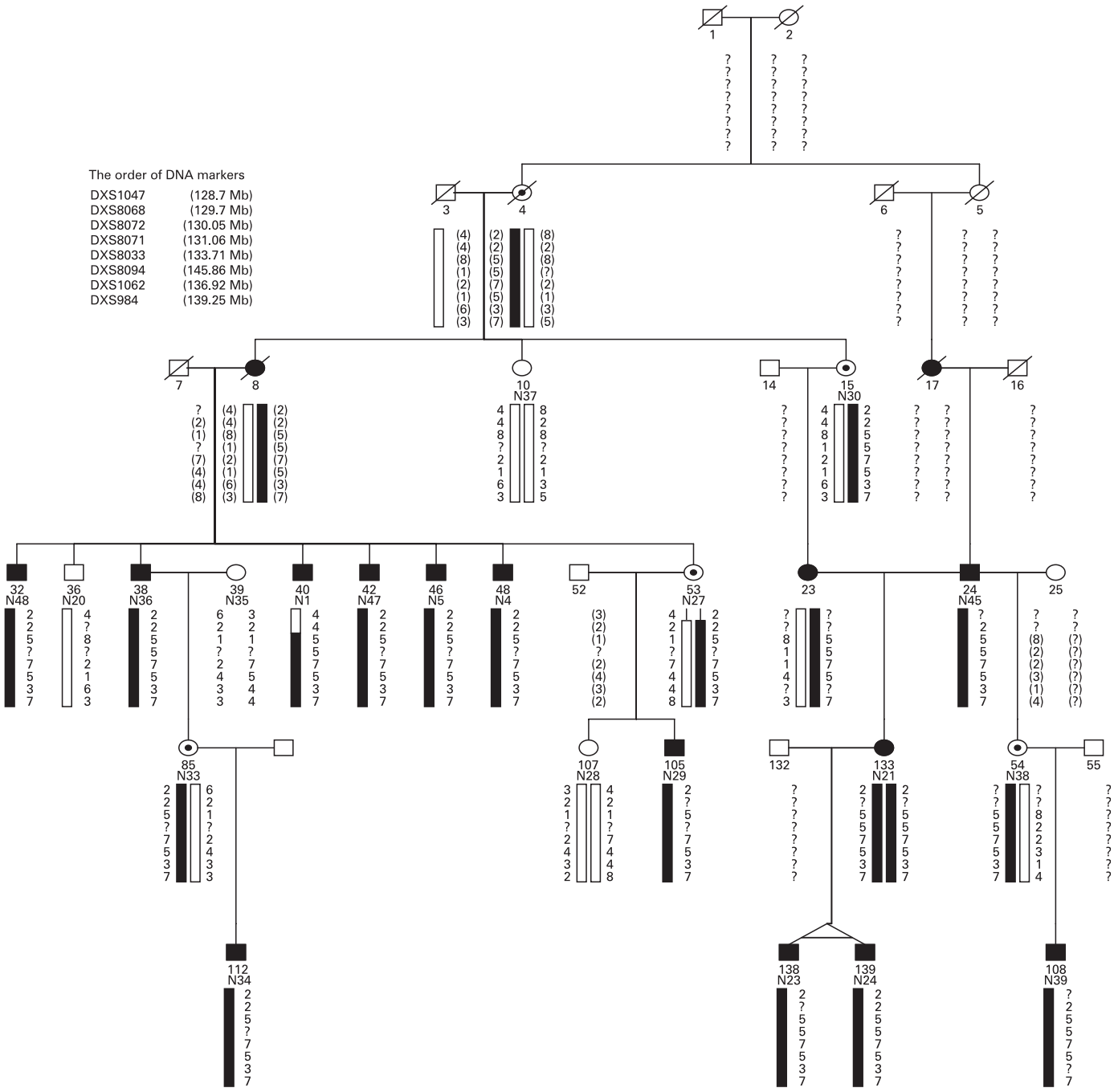

Figure 2 Haplotype structure between the NYS phenotype and the DNA markers selected from Xq26. The order and the physical locations (Mb) of the DNA markers are shown to the upper left. A single recombination event in affected individual 40 positioned the disease gene telomeric to DXS8068 containing the *FRMD7* gene (130.93 Mb).

DISCUSSION

In this study, we described a large family with X linked congenital NYS. The disorder was completely penetrant in males; however, reduced penetrance was observed in females. Various penetrance rates, ranging from 54% to 29%, are suggested to be due to family size.[3 5 6] Our study also supported 36% penetrance in females. This extremely low penetrance rate in females might challenge the mode of inheritance estimations. For genetic counselling purposes, it might be difficult to distinguish X linked recessive and dominant patterns in small family trees. Genetic linkage analysis provided strong evidence of linkage to the previously established locus on Xq26–27, which is the major locus for X linked NYS (NYS1). A recombination event in a single affected male positioned the disease gene telomeric to the DNA marker DXS8068 (fig 2). Combining these data with previous studies, the 8 Mb region residing between DNA markers DXS8068 and DXS1211 containing the recently reported *FRMD7* gene[6] was critical for this family.

We identified the novel p.R229G missense mutation in the *FRMD7* gene in the study family. The following points support the disease-causing nature of this mutation: (i) p.R229G co-segregated with the disease in this family; (ii) our control studies excluded p.R229G as a non-synonymous polymorphism

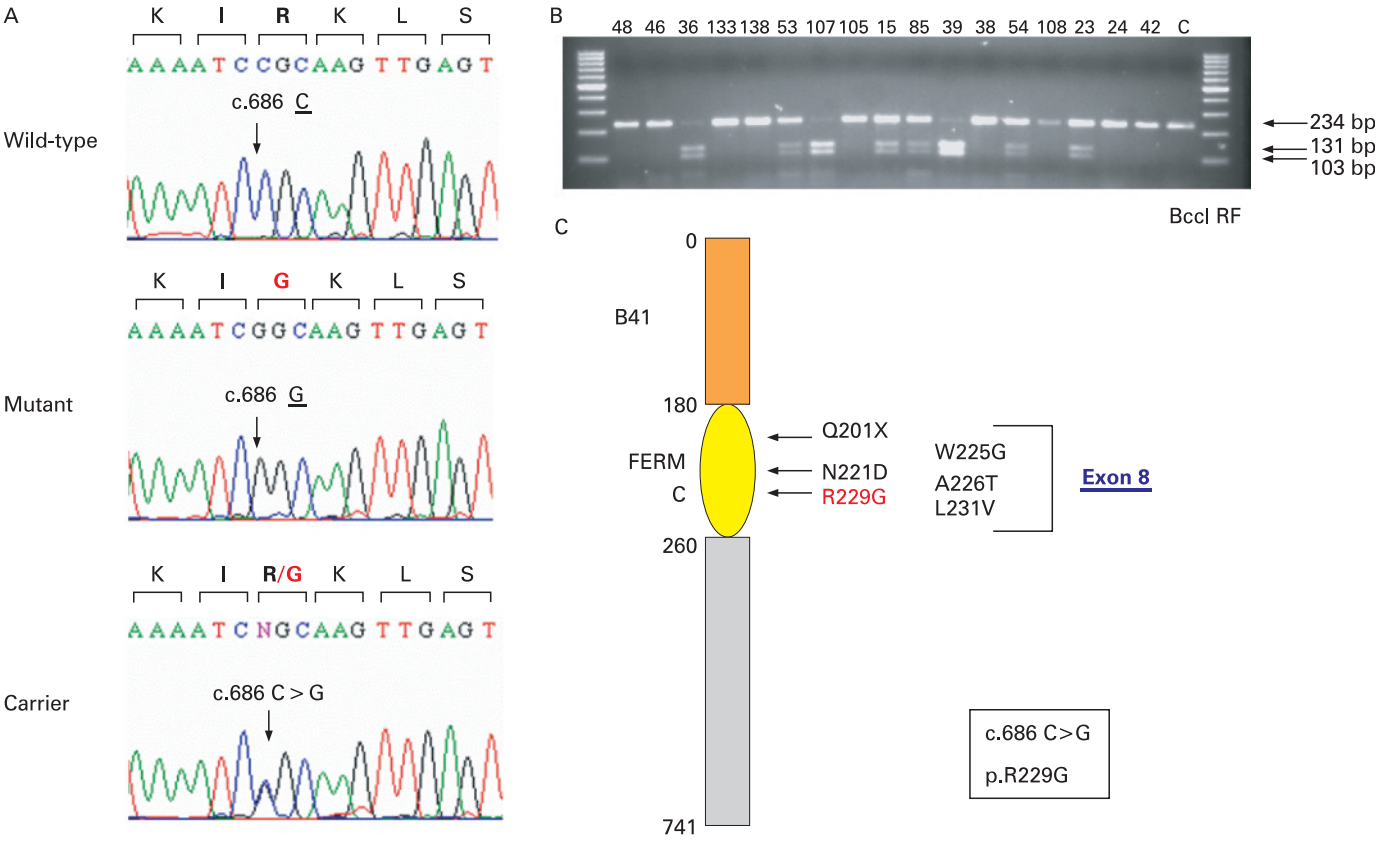

Figure 3 Identification of a novel missense mutation in the *FRMD7* gene. (A) Representative sequence chromatograms of the identified c.686C>G (p.R229G) mutation in an affected male (mutant), a carrier female (carrier) and an unaffected male (wild-type). (B) Co-segregation of the mutation in the family. Results of *Bcc*I restriction fragment analysis presenting an undigested 234 bp PCR-fragment in affected male individuals, a fully digested PCR fragment in unaffected males (two fragments 131 bp and 101 bp in size), and one undigested and one digested allele in carrier females (three fragments 234 bp, 131 bp and 101 bp in size). A non-digested sample, labelled as C, was included as a control (C) schematic view of conserved *FRMD7* domains and locations of described mutations located in the FERM-C domain.

Table 3 Distribution of skewed x-inactivation between nystagmus and normal members of the family

	PID	DNA no.	Status	Skewing degree
Skewed				
1	35	N42	Normal	93:7
2	121	N46	Affected	85:15
3	65	N31	Affected	81:19
4	131	N40	Affected	80:20
5	133	N21	Affected	80:20
Random				
1	33	N18	Normal	72:28
2	45	N11	Normal	71:29
3	107	N28	Normal	71:29
4	72	N41	Normal	69:31
5	15	N30	Normal	67:33
6	11	N37	Normal	67:33
7	53	N27	Normal	63:37
8	66	N16	Affected	62:38
9	93	N26	Affected	57:43
10	118	N13	Normal	56:44
12	99	N10	Normal	54:46
13	122	N14	Normal	54:46
14	119	N43	Normal	53:47
15	120	N12	Normal	51:49
16	Spouse of 129	N7	normal	50:50

PID reflects personal identification numbers on fig 1. A total of five individuals (fig 1, individuals 23, 41, 43, 54 and 86) were not informative. The disease status of non-informative individuals was normal except person 23.

in the Turkish population; (iii) the mutation is located in the FERM-C domain in the surrounding of previously described missense mutations in families with congenital NYS.[6 14]

The functional role of *FRMD7* remains to be elucidated. The *FRMD7* transcript is abundantly expressed in most tissues, and a localised and restricted expression was found to be involved in embryonic development in regions affecting motor control of eye development.[6] Homologies of the B41 and FERM-C domains of *FRMD7* to other proteins, such as *FARP1* and *FARP2*, which are involved in neurite branching of cortical neurons, led to the hypothesis that *FRMD7* could also be involved in the development of similar neuronal pathways.

Skewed X inactivation has consistently been suggested as a mechanism that may influence the penetrance of X linked NYS in females;[3 7 8 9] however, except for only one study,[7] an X inactivation pattern was not previously studied in NYS families. No correlation between X inactivation patterns and the NYS phenotype was observed in this sole study.[7] Nevertheless, NYS in the family used in that study was linked to the Xp11 region rather than the major NYS locus on Xq26–q27. Our Xq26-linked NYS family implies that skewed XCI could be a factor that influences the clinical manifestation of NYS in females. It is well established that skewed XCI is a rare event in a diverse group of control females.[15 16] In agreement with this prior observation, we only observed skewed X inactivation in a single healthy spouse (individual 35). None of the obligate gene carrier females and none of the remaining healthy spouses demonstrated skewed X inactivation (table 3); however, increased susceptibility to skewed X inactivation was apparent in the clinically affected females. On the other hand, we observed random X inactivation in two affected females, individuals 66 and 93, with XCI scores of 62:38 and 57:43, respectively. This finding may be a reflection of tissue mosaicism, which has been clearly shown in women.[16] To the best of our knowledge, inactivation status of the *FRMD7* gene has not been studied. Further investigations of the X inactivation status of *FRMD7* might help contribute to an understanding of the irregular pattern of inheritance of NYS1.

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Competing interests: None.

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Mutations in the very low-density lipoprotein receptor *VLDLR* cause cerebellar hypoplasia and quadrupedal locomotion in humans

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Quadrupedal gait in humans, also known as Unertan syndrome, is a rare phenotype associated with dysarthric speech, mental retardation, and varying degrees of cerebrocerebellar hypoplasia. Four large consanguineous kindreds from Turkey manifest this phenotype. In two families (A and D), shared homozygosity among affected relatives mapped the trait to a 1.3-Mb region of chromosome 9p24. This genomic region includes the *VLDLR* gene, which encodes the very low-density lipoprotein receptor, a component of the reelin signaling pathway involved in neuroblast migration in the cerebral cortex and cerebellum. Sequence analysis of *VLDLR* revealed nonsense mutation R257X in family A and single-nucleotide deletion c2339delT in family D. Both these mutations are predicted to lead to truncated proteins lacking transmembrane and signaling domains. In two other families (B and C), the phenotype is not linked to chromosome 9p. Our data indicate that mutations in *VLDLR* impair cerebrocerebellar function, conferring in these families a dramatic influence on gait, and that hereditary disorders associated with quadrupedal gait in humans are genetically heterogeneous.

genetics | Unertan syndrome

Obligatory bipedal locomotion and upright posture of modern humans are unique among living primates. Studies of fossil hominids have contributed significantly to modern understanding of the evolution of posture and locomotion (1–5), but little is known about the underlying molecular pathways for development of these traits. Evaluation of changes in brain activity during voluntary walking in normal subjects suggests that the cerebral cortices controlling motor functions, visual cortex, basal ganglia, and the cerebellum might be involved in bipedal locomotor activities (6). The cerebellum is particularly important for movement control and plays a critical role in balance and locomotion (7).

Neurodevelopmental disorders associated with cerebellar hypoplasias are rare and often accompanied by additional neuropathology. These clinical phenotypes vary from predominantly cerebellar syndromes to sensorimotor neuropathology, ophthalmological disturbances, involuntary movements, seizures, cognitive dysfunction, skeletal abnormalities, and cutaneous disorders, among others (8). Quadrupedal locomotion was first reported when Tan (9, 10) described a large consanguineous family exhibiting Unertan syndrome, an autosomal recessive neurodevelopmental condition with cerebellar and cortical hypoplasia accompanied by mental retardation, primitive and dysarthric speech, and, most notably, quadrupedal locomotion. Subsequent homozygosity mapping indicated that the phenotype of this family was linked to chromosome 17p (11). Thereafter, three additional families from Turkey (12–14) and another from Brazil (15) with similar phenotypes have been described, and video recordings illustrating the quadrupedal gait have been made (10–12). Here, we report that *VLDLR* is the gene responsible for the syndrome in two of these four Turkish families and report additional gene mapping studies that indicate the disorder to be highly genetically heterogeneous.

Fig. 1. Phenotypic (*A*) and cranial radiologic (*B*) presentation of quadrupedal gait in families A and D. (*A*) Affected brothers VI:20 and VI:18 and cousin VI:25 in family A (*Upper*) and the proband II:2 in family D (*Lower*) display palmigrate walking. This is different from quadrupedal knuckle-walking of the great apes (2). The hands make contact with the ground at the ulnar palm, and consequently this area is heavily callused as exemplified by VI:20. Strabismus was observed in all affected individuals. (*B*) Coronal and midsagittal MRI sections of VI:20, demonstrating vermial hypoplasia, with the inferior vermial portion being completely absent. Inferior cerebellar hypoplasia and a moderate simplification of the cerebral cortical gyri are noted. The brainstem and the pons are particularly small (*Left* and *Center*). Similar findings are observed for II:2 (*Right*).

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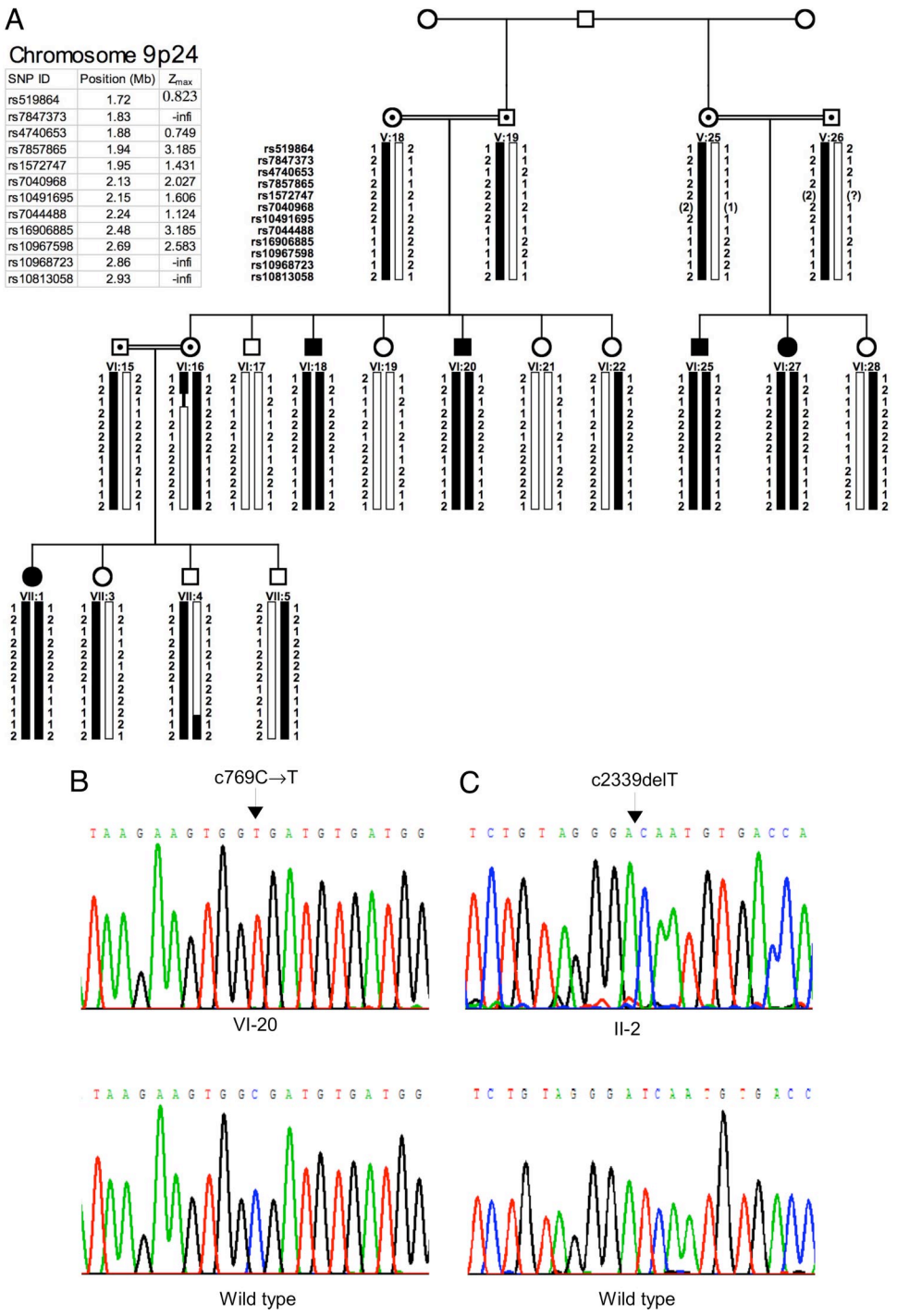

Fig. 2. Homozygosity mapping of cerebellar hypoplasia and quadrupedal locomotion to chromosome 9p24 (*A*) and identification of the *VLDLR* c769C → T mutation in family A (*B*) and of the *VLDLR* c2339delT mutation in family D (*C*). (*A*) Pedigree of family A; filled symbols represent the affected individuals. Squares indicate males, and circles indicate females. Black bars represent the haplotype coinherited with the quadrupedal phenotype in the family. Recombination events in individuals VI:16 (obligate carrier) and VII:4 (normal sibling) positioned the disease gene between markers rs7847373 and rs10968723. Physical positions and pairwise lod scores for each marker are shown on the upper left. Z_{max} represents the maximum lod score obtained at $\theta = 0.00$ cM. (*B* and *C*) Sequences of critical regions of *VLDLR* for wild-type and homozygous mutant genotypes.

Results

The proband of Family A (12) is a 37-year-old male with habitual quadrupedal gait (Fig. 1*A Upper Left* and Fig. 2*A*, VI:20). He did not make the transition to bipedality during his childhood despite the efforts of his healthy parents. He has dysarthric speech with a limited vocabulary, truncal ataxia, and profound mental retardation. He was not aware of place or of the year, month, or day. His MRI brain scan revealed inferior cerebellar and vermial hypoplasia, with the inferior vermial portion being completely absent. Whereas corpus callosum appeared normal, a moderate simplification of the cerebral cortical gyri accompanied by a particularly small brainstem and the pons was observed (Fig. 1 *B Left* and *Center*). Subsequently, we studied the proband's affected brother and cousin (Fig. 1*A Upper Center* and

Table 1. Physical, radiological, and genetic characteristics of the Turkish families in this study and of Hutterite family DES-H (27)

Characteristic	Family A	Family B	Family C	Family D	DES-H
Chromosomal location	9p24	17p	Not 9p or 17p	9p24	9p24
Gene and mutation	*VLDLR* (c769C → T)	Unknown	Unknown	*VLDLR* (c2339delT)	Deletion including *VLDLR* and *LOC401491*
Gait	Quadrupedal	Quadrupedal	Quadrupedal	Quadrupedal	Bipedal
Speech	Dysarthric	Dysarthric	Dysarthric	Dysarthric	Dysarthric
Hypotonia	Absent	Absent	Absent	Absent	Present
Barany caloric nystagmus	Normal	Cvs defect	Pvs defect	Not done	Not done
Mental retardation	Profound	Severe to profound	Profound	Profound	Moderate to profound
Ambulation	Delayed	Delayed	Delayed	Delayed	Delayed
Truncal ataxia	Severe	Severe	Severe	Severe	Severe
Lower leg reflexes	Hyperactive	Hyperactive	Hyperactive	Hyperactive	Hyperactive
Upper extremity reflexes	Vivid	Vivid	Vivid	Vivid	Vivid
Tremor	Very rare	Mild	Present	Absent	Present
Pes-planus	Present	Present	Present	Present	Present
Seizures	Very rare	Rare	Rare	Absent	Observed in 40% of cases
Strabismus	Present	Present	Present	Present	Present
Inferior cerebellum	Hypoplasia	Hypoplasia	Mild hypoplasia	Hypoplasia	Hypoplasia
Inferior vermis	Absent	Absent	Normal	Absent	Absent
Cortical gyri	Mild simplification	Mild simplification	Mild simplification	Mild simplification	Mild simplification
Corpus callosum	Normal	Reduced	Normal	Normal	Normal

Cvs, central vestibular system; Pvs, peripheral vestibular system.

Upper Right and Fig. 2*A*, VI:18 and VI:25) and other branches of the family living in nearby villages in southeastern Turkey. All affected individuals were offspring of consanguineous marriages (Fig. 2*A*). With the exception of one female (VII:1), who was an occasional biped with ataxic gait, all affected persons in family A had quadrupedal locomotion.

The proband of family D (14) is a 38-year-old male (Fig. 1*A Lower Left* and *Center*). Like all other quadrupedal individuals in these families, he did not make the transition to bipedality during his early childhood. He is profoundly retarded and exhibits dysarthric speech along with truncal ataxia. His MRI brain scan images are consistent with moderate cerebral cortical simplification and inferior cerebellar and vermial hypoplasia (Fig. 1*B Right*). The 65-year-old aunt and 63-year-old uncle of the proband are both mentally retarded and continue to walk on their wrists and feet despite their advanced ages. The family is consanguineous; all relatives were raised in neighboring villages on the western tip of the Anatolian peninsula.

All patients in these four families had significant developmental delay noted in infancy (Table 1). They sat unsupported between 9 and 18 months, and began to crawl on hands and knees or feet. Whereas normal infants make the transition to bipedal walking in a short period, the affected individuals continued to move on their palms and feet and never walked upright. All patients had severe truncal ataxia affecting their walking patterns. They can stand from a sitting position and maintain the upright position with flexed hips and knees. However, they virtually never initiate bipedal walking on their own and instead ambulate efficiently in a quadrupedal fashion. All patients had hyperactive lower leg and vivid upper extremity reflexes. Normal tone and power were observed in motor examination. All affected persons were mentally retarded to the degree that consciousness of place, time, or other experience appeared to be absent. However, no autistic features were expressed. The affected individuals all had good interpersonal skills, were friendly and curious to visitors, and followed very simple questions and commands. Additional clinical information on families A and D is provided in supporting information (SI) Table 2.

To identify the chromosomal locale of the gene or genes responsible for this phenotype, we carried out genome-wide linkage analysis and homozygosity mapping in families A–C (see *Materials and Methods* below). Although the families lived in isolated villages 200–300 km apart and reported no ancestral relationship, the rarity of the quadrupedal gait in humans led us to expect a single locus shared by affected individuals in all families. Instead, the trait mapped to three different chromosomal locales. In family A, linkage analysis and homozygosity mapping positioned the critical gene on chromosome 9p24 between rs7847373 and rs10968723 in a 1.032-Mb region (Fig. 2*A* and SI Fig. 4). In family B, the trait mapped to chromosome 17p13, confirming a previous study (11). In family C, highly negative logarithm of odds (lod) scores were obtained for both chromosomes 9p24 and 17p13 (SI Figs. 5 and 6); gene mapping in this family is ongoing. In family D, polymorphic markers from the critical intervals of chromosomes 9p24 and 17p13 were genotyped, and homozygosity was detected with markers on 9p24. Together, these results indicate that the syndrome including quadrupedal gait, dysarthric speech, mental retardation, and cerebrocerebellar hypoplasia is genetically heterogeneous.

The chromosome 9p24 region linked to the trait in families A and D includes *VLDLR*, the very low-density lipoprotein receptor. We hypothesized that a gene involved in neural development, cell positioning in brain, and cerebellar maturation could be involved in the pathogenesis of quadrupedal gait. In addition, cerebellar hypoplasia with cerebral gyral simplification was shown to be associated with a genomic deletion that includes *VLDLR* (16). We therefore considered *VLDLR* (17) to be a prime positional candidate for our phenotype and sequenced the gene in genomic DNA from probands of the four families (SI Table 3). The *VLDLR* sequence of affected members of family A was homozygous for a nonsense mutation in exon 5 (c769C → T; R257X) (Fig. 2*B*). The *VLDLR* sequence of the proband of family D was homozygous for a single-nucleotide deletion in exon 17 resulting in a stop codon (c2339delT; I780TfsX3) (Fig. 2*C*). *VLDLR* sequences excluded the possibility of compound heterozygosity in families B and C (SI Fig. 7). In families A and D, homozygosity for the *VLDLR* mutations was perfectly coinherited with quadrupedal gait (SI Figs. 8 and 9). Both mutations were absent from 100 unaffected individuals who live in the same local areas of southeastern and western Turkey as families A and D (SI Fig. 10).

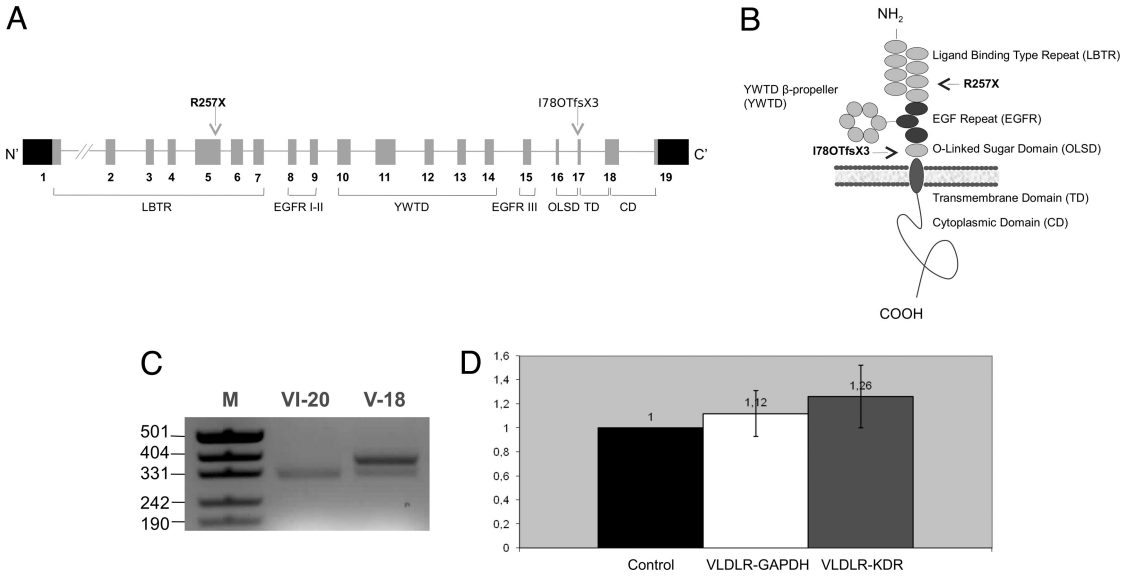

Fig. 3. Functional domains of *VLDLR* with positions of the mutations relative to the exons (*A*), domains (*B*), and the analysis of VLDLR transcript (*C* and *D*). (*A*) The gene consists of 19 exons. Arrows indicate the locations of the mutations. (*B*) VLDLR consists of ligand-binding type repeat (LBTR), epidermal growth factor repeat (EGFR) I–III, YWTD β-propeller (YWTD), *O*-linked sugar domain (OLSD), transmembrane domain (TD), and cytoplasmic domain (CD) (34) (www.expasy.org/uniprot/P98155). (*C*) Restriction-based analysis with HphI revealed the presence of only the mutant (347 bp) and both the mutant and wild type (396 and 347 bp; please note that the 49-bp fragment is not visible) VLDLR transcripts in patient VI:20 and carrier V:18 (both from family A), respectively. M is a DNA size marker. (*D*) Quantitative RT-PCR analysis of VLDLR transcript from peripheral blood samples of all probands in families A and D and controls was performed. Relative expression ratios were normalized according to the housekeeping gene *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) and the endothelial marker *KDR* (kinase insert domain receptor). ΔCt values were calculated from duplicate samples and were converted to linear scale (35). Control denotes ''VLDLR expression in controls,'' VLDLR-GAPDH denotes ''VLDLR expression in patients normalized to GAPDH,'' and finally VLDLR-KDR denotes ''VLDLR expression in patients normalized to KDR.''

VLDLR_R257X is in the ligand-binding domain, and *VLDLR_I780TfsX3* is in the *O*-linked sugar domain of the VLDLR protein (Fig. 3 *A* and *B*). Mutant VLDLR transcripts were expressed in endothelial cells from blood of affected individuals (Fig. 3*C*), and in these cells, levels of mutant and wild-type transcript expression appeared approximately equal (Fig. 3*D*; please also see *SI Text*). Because the stop codons of both mutations are located in the extracellular domain of VLDLR (Fig. 3*B*), the encoded mutant proteins could not be inserted into the membrane and could not function as receptors for reelin.

We propose *VLDLR*-associated Quadrupedal Locomotion (VLDLR-QL) or Unertan Syndrome Type 1 to describe the phenotype of families A and D.

Discussion

The identification of these *VLDLR* mutations provides molecular insight into the pathogenesis of neurodevelopmental movement disorders and expands the scope of diseases caused by mutations in components of the reelin pathway (18). Reelin is a secreted glycoprotein that regulates neuronal positioning in cortical brain structures and the migration of neurons along the radial glial fiber network by binding to lipoprotein receptors VLDLR and APOER2 and the adapter protein DAB1 (19). In the cerebellum, reelin regulates Purkinje cell alignment (20), which is necessary for the formation of a well defined cortical plate through which postmitotic granual cells migrate to form the internal granular layer (21). Homozygous mutations in the reelin gene (*RELN*) cause the Norman–Roberts type lissencephaly syndrome, associated with severe abnormalities of the cerebellum, hippocampus, and brainstem (OMIM 257320) (22). Mutation of *Reln* in the mouse results in the *reeler* phenotype and disrupts neuronal migration in several brain regions and gives rise to functional deficits such as ataxic gait and trembling (23). In contrast, mice deficient for *Vldlr* appear neurologically normal (24), but the cerebellae of these mice are small, with reduced foliation and heterotopic Purkinje cells (17).

In humans, homozygosity for either of two *VLDLR* truncating mutations leads to cerebrocerebellar hypoplasia, specifically vermial hypoplasia, accompanied by mental retardation, dysarthric speech, and quadrupedal gait. In the Hutterite population of North America, homozygosity for a 199-kb deletion encompassing the *VLDLR* gene leads to a form of Disequilibrium Syndrome (DES-H, OMIM 224050), characterized by nonprogressive cerebellar hypoplasia with moderate-to-profound mental retardation, cerebral gyral simplification, truncal ataxia, and delayed ambulation (16). The designation Disequilibrium Syndrome was originally given to cerebral palsy characterized by a variety of congenital abnormalities, including mental retardation, disturbed equilibrium, severely retarded motor development, muscular hypotonia, and perceptual abnormalities (25, 26). Neither DES-H nor other disequilibrium syndromes have been reported to include quadrupedal gait. The movement of most DES-H patients was so severely affected that independent walking was not possible. Those who could walk had a wide-based, nontandem gait (27).

The neurological phenotypes in the Turkish families and in the Hutterite families appear similar, with the most striking difference being the consistent adoption of efficient quadrupedal locomotion by the affected Turkish individuals (Table 1). In our view, the movement disorder described for the Hutterite patients may be a more profound deficit, with the patients perhaps lacking the motor skills for quadrupedal locomotion. The 199-kb deletion in DES-H encompasses the entire *VLDLR* gene and part of a hypothetical gene. *LOC401491*, the hypothetical gene, is an apparently noncoding RNA that shares a CpG island and likely promoter with *VLDLR*, and is represented by multiple alternative transcripts expressed in brain. It has been suggested that the DES-H phenotype could be the result of deletion of *VLDLR* or both *VLDLR* and the neighboring gene (16).

It has been suggested that in the Turkish families, lack of access to proper medical care exacerbated the effects of cerebellar hypoplasia, leading to quadrupedality. Although it may be true that family B lacked proper medical care, families A and D had consistent access to medical attention, and both families actively sought a correction of quadrupedal locomotion in their affected children. An unaffected individual in family A is a physician who was actively involved in the medical interventions. In family D, the proband's mother sought a definitive diagnosis and correction of the proband's quadrupedal locomotion from private medical practices and from two major academic medical centers. The parents in family A discouraged quadrupedal walking of their affected children, but without success. We conclude that social factors were highly unlikely to contribute to the quadrupedal locomotion of the affected individuals.

In conclusion, we suggest that *VLDLR*-deficiency in the brain at a key stage of development leads to abnormal formation of the neural structures that are critical for gait. Given the heterogeneity of causes of quadrupedal gait, identification of the genes in families B and C promises to offer insights into neurodevelopmental mechanisms mediating gait in humans.

Materials and Methods

Study Subjects. Parents of patients and other unaffected individuals gave consent to the study by signing the informed consent forms prepared according to the guidelines of the Ministry of Health in Turkey. The Ethics Committees of Baskent and Cukurova Universities approved the study (decision KA07/47, 02.04.2007 and 21/3, 08.11.2005, respectively).

Genome-Wide Linkage Analysis. Linkage analysis was performed by SNP genotyping with the commercial release of the GeneChip 250K (NspI digest) or 10K Affymetrix arrays as described (28). In addition, genotype data were analyzed by hand to identify regions of homozygosity. The parametric component of the Merlin package v1.01 was used for the multipoint linkage analysis assuming autosomal recessive mode of inheritance with full penetrance (29, 30). The analysis was carried out along a grid of locations equally spaced at 1 cM. Haplotype analysis was performed on chromosomal regions with positive lod scores (Fig. 2*A* and SI Figs. 4–6). Pairwise linkage was analyzed by using the MLINK component of the LINKAGE program (FASTLINK, version 3) (31–33). Markers D17S1298 (3.51 Mb) and D9S1779 (0.4 Mb), D9S1871 (3.7 Mb) were used to test for homozygosity to chromosomes 17p13 and 9p24, respectively.

Mutation Search. Sequencing primers were designed for each *VLDLR* exon by using Primer3, BLAST, and the sequence of NC_000009. DNA from all of the probands was sequenced in both directions by using standard methods. The mutations in exons 5 (c769C → T) and 17 (c2339delT) were detected in all affected (homozygous) and carrier (heterozygous) individuals of families A and D, respectively. The c769C → T mutation creates a restriction site for the enzyme HphI (5′-GGTGA(N)8 ↓ 3′), and the c2339delT mutation abolishes a restriction site for the enzyme MboI (5′-G↓ ATC-3′). Assays using these restriction enzymes were developed to test for the mutations in all four families and in 200 healthy controls from the Turkish population. Restriction based mutation and quantitative RT-PCR analyses of *VLDLR* transcript in patients and controls was also performed (please see *SI Text* relating to Fig. 3 *C* and *D*).

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