ANALYSIS OF X CHROMOSOME INACTIVATION IN PRIMARY AND SECONDARY SJOGREN SYNDROME

A THESIS SUBMITTED TO THE DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS AND THE INSTITUTE OF ENGINEERING AND SCIENCE OF BILKENT UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

BY MELDA KANTAR

AUGUST, 2008

I certify that I have read this thesis and that in my opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

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Prof. Tayfun Özçelik

I certify that I have read this thesis and that in my opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

_____

Assist. Prof. Ali O. Güre

I certify that I have read this thesis and that in my opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

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Assist. Prof. Hüseyin Boyacı

Approved for the Institute of Engineering and Science

_____

Director of Institute of Engineering and Science
Prof. Dr. Mehmet Baray

ABSTRACT

Lack of evidence for the role of skewed X chromosome inactivation in the female predisposition to Sjogren Syndrome

Melda Kantar
M.S. in Molecular Biology and Genetics
Supervisor: Prof. Dr. Tayfun Özçelik
August 2008, Pages 91

Sjogren Syndrome is an autoimmune disease with one of the highest prevalences and unknown etiology. The majority of the patients (~90%) are female similar to several other autoimmune diseases. Based on this observation, a hypothesis was proposed stating that X chromosome inactivation (XCI) could be involved in female predisposition to autoimmunity. XCI is a physiological mechanism which takes place early in development resulting in the transcriptional silencing of one of the pair of X chromosomes at random in each cell. A significant deviation from a random distribution of two cell populations with paternal and maternal X chromosome inactive is called skewed XCI. Skewing in the dendritic cell population involved in tolerance induction in the thymus was proposed to cause escape of autoreactive lymphocytes and result in autoimmunity (Immunol Today, 19, 352-7, 1998). Skewed XCI was observed in scleroderma (Arth Rheum 52, 1564-70, 2005) and autoimmune thyroiditis (Eur J Hum Genet 14, 791-7, 2006). But this observation is not true for all autoimmune diseases. For example, the XCI profiles of primary biliary cirrhosis patients are similar to normal controls (Hepatol Res 37, Suppl 3, 384-8, 2007). The aim of this study is to determine the XCI profiles of patients diagnosed with primary Sjogren Syndrome, manifesting exocrinopathy or secondary Sjogren Syndrome displaying additional systemic features. DNA was isolated from the peripheric blood samples of 78 Sjogren syndrome patients and 160 controls. XCI profile was determined by the genotyping of a polymorphism in the androgen receptor (AR) gene. For this analysis, restriction enzyme *HpaII* was used which does not cut methylated regions. Analysis was done with Genescan Abi Prism 310 or 8% polyacrylamide gel electrophoresis and densitometric analysis. Extreme skewing (>90%) of XCI was observed in 3 (5.9%) patients and 3 controls (2.4%) samples ($P = 0.3651$). Our findings do not support a role for skewed XCI in Sjogren Syndrome.

ÖZET

Sjogren sendromu ile X inaktivasyonu bozukluğu arasında bir ilişki bulunmuyor

Melda Kantar
Moleküler Biyoloji ve Genetik Yüksek Lisans
Tez Yöneticisi: Prof. Dr. Tayfun Özçelik
Ağustos 2007, Sayfa 91

Sjogren sendromu etiyolojisi bilinmeyen ve prevalansı en yüksek olan otoimmün hastalıklardan biridir. Pek çok otoimmün hastalıkta olduğu gibi hastaların büyük bir çoğunluğu (~%90) kadındır. Bu gözlemden yola çıkarak, bozuk X inaktivasyonunun otoimmun hastalıkları tetikleyebileceği hipotezi ileri sürülmüştür (Immunol Today, 19, 352-7, 1998). X inaktivasyonu kadınlarda erken gelişme döneminde hücrelerdeki X kromozomu çiftinden rastgele birinin susturulmasıyla sonuçlanan normal bir fizyolojik olaydır. Hücrelerin önemli bir kısmında aynı X kromozomunun susturulması bozuk X inaktivasyonu olarak adlandırılır. Timusda, tolerans oluşumunda rol alan dendritik hücrelerdeki X inaktivasyonu bozukluğunun otoreaktif lenfositlerin kaçmasına ve otoimmüniteye neden olabileceği hipotezi ileri sürülmüştür. Skleroderma (Arth Rheum 52, 1564-70, 2005) ve otoimmün tiroiditis (Eur J Hum Genet 14, 791-7, 2006) hastalıklarında bozuk X inaktivasyonu gözlenmiştir. Fakat bu gözlem tüm otoimmün hastalıklar için geçerli değildir. Örneğin primer biliyer siroz hastalarının X inaktivasyonu profilleri normal kontroller gibidir (Hepatol Res 37, Suppl 3, 384-8, 2007). Bu çalışmanın amacı sadece salgı bezi patolojisi olan primer Sjogren Sendromu ve aynı zamanda başka sistemik patolojiler içeren sekonder Sjogren Sendromu tanısı alan hastalarda X-inaktivasyonu profillerinin belirlenmesidir. 78 hasta ve 160 kontrolün periferik kanından DNA izole edilmiştir. X inaktivasyon profili androjen reseptör geninde bulunan bir polimorfizmin genotiplemesi ile belirlenmiştir. Bu inceleme için metilli bölgeleri tanımayan bir restriksiyon enzimi olan *HpaII* ile kesim yapılmış ve hedef bölge çoğaltılmıştır. İnceleme Genescan Abi Prism 310 ya da %8 poliakrilamid jel elektroforezi ve densitometrik ölçümle yapılmıştır. İleri derecede (>%90) X inaktivasyonu bozukluğu 3 (%5.9) hasta ve 3 kontrol (%2.4) örneğinde gözlenmiştir (P = 0.3651). Bulgularımız X inaktivasyonu bozukluğu ile Sjogren Sendromu arasında bir ilişkinin varlığını desteklememiştir.

TO MY FAMILY,
FOR THEIR LOVE AND SUPPORT

ACKNOWLEDGEMENTS

It is my pleasure to express my deepest gratitude to my advisor Prof. Dr. Tayfun Özçelik for his scientific guidance and support all through my thesis. I especially want to thank him for introducing me to research..

I would like to adress my special thanks to Zeynep Özbalkan for obtaining the blood samples and coordinating the clinical studies.

I would like to thank Scientific and Technological Research Council of Turkey for the funding.

I would like to express my thanks to Elif Uz and Çiğdem Aydın Mustafa for guiding me in every step of my research and always being there for my questions and for their endless support in every obstacle I was faced to. A special thank to Elif Uz for her help in determining the XCI status of Sjogren Syndrome patients. I would like to thank all my group members Emre Onat, Şafak Çağlayan, Süleyman Gülsuner for their incredible help and support. I would like to thank Mustafa Gökhan Ertosun for helping me with the format of my thesis.

I would like to thank all MBG members for making Bilkent Molecular Biology Department an incredible place to live and work.

I would also like to thank my friends Hülya Budunoğlu, Hande Erkut, Cansu Aksu, Banu Demir, Emre Onat, Fuat Yağcı, Elif Yaman, Hande Kocak, Pelin Telkoparan, Esen Oktay, Gizem Tinçer, Ceren Sucularlı and all the others I have not mentioned for listening to my every problem during my academic and personal life. I want to thank my boyfriend, Onur Karakuş for sharing my life.

Lastly, I want to express my sincere love and thanks to my family for being there whenever I needed help and support. I want to thank my mother, Nujan Kocagöz and my father, Elvend Kantar for helping me through every struggle and especially my dear sister, Selda Kantar without whom life would be unbearable.

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ABBREVIATIONS

ANA	Antinuclear antibodies
Abs	Antibodies
AID	Autoimmune disease
AIDS	Acquired immunodeficiency disease
AITD	Autoimmune tyroid disease
AIRE	Autoimmune regulator
ALPS	Autoimmune lymphoproliferative syndrome
AQP	Aquaporin
APC	Antigen presenting cell
AR	Androgen receptor
BAFF	B cell survival factor
bp	Base pair
BCR	B cell receptor
Csk	c-src tyrosine kinase
CTLA-4	Cytotoxic T lymphocyte antigen 4
DEMS	Dry eyes and mouth symptoms
ddH_2O	Deionized water
dNTP	Deoxynucleotide triphosphate
DNA	Deoxyribonucleic acid
EBV	Epstein barr virus
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium bromide
EtOH	Ethanol
G6PD	Glucose 6-phosphate dehydrogenase
GVHD	Graft versus host disease
HBV	Hepatitis B virus
HLA	Human leukocyte antigen
HIV	Human immunodeficiency virus
HSV-1	Herpes simplex virus Type I

HTLV	Human T lymphotrophic virus
HIGM1	Hyper-IgM syndrome type I
Ig	Immunoglobulin
IgVλ	Immunoglobulin variable λ
IFN-γ	Interferon γ
IL	Interleukin
IPEX	Immune dysregulation, polyendocrinopahty, enteropathy
L1	LINE1
Mb	Megabase
MCTD	Muscle connective tissue disease
$MgCl_2$	Magnesium chloride
MHC	Major histocompatibility complex
MBL	Mannose binding lectin
mM	Millimolar
ml	Milliliter
μl	Microliter
MS	Multiple sclerosis
kb	Kilobase
L1	Line1 elements
NF-κB	Nuclear factor kappa B
PAGE	Polyacrylamide gel electrophoresis
PI3K	Phosphatidylinositol-3-kinase
PBC	Primary biliary cirrhosis
PCR	Polymerase chain reaction
PcG	Polycomb-group proteins
PI3K	Phosphoinositide 3 phosphatide
PLP	Proteolipid protein
PKC	Phosphokinase C
RA	Rheumatoid arthritis
RE	Restriction enzyme
RF	Rheumatoid factor

SCID	Severe combined immunodeficiency syndrome
SCLE	Subacute cutaneous lupus erthematosus
SDS	Sodium dodecyl sulphate
SLE	Systemic lupus erythramatosus
SNP	Single nucleotide polymophism
SH3	Src homology 3
SSc	Systemic sclerosis
T1D	Type 1 diabetis
TAE	Tric-acetic acid-EDTA
TCR	T cell receptor
TEMED	N, N, N, N-tetramethyl-1-2, diaminoethane
Th1	T helper 1
Th2	T helper 2
TLR	Toll like receptor
TNF	Tumour necrosis factor
Tris	Tris aminomethan
WAS	Wiskott-Aldrich syndrome
Xa	Active X
XCI	X chromosome inactivation
Xce	X chromosome controlling element
Xi	Inactive X
Xic	X-inactivation center
Xist	X-inactive specific element
Xite	X-inactivation intergenic transcription element
XEDA-ID	X-linked recessive anhydortic ectodermal dysplasia with immunodeficiency
XLA	X-linked agammaglobulinemia
XLP	X-linked lymphoproliferative syndrome

CHAPTER I: INTRODUCTION

1.1. Sjogren Syndrome

Sjogren Syndrome is a chronic and systemic autoimmune disease characterized by progressive lymphocytic infiltration of polyglandular tissue and subsequent tissue destruction. This results in the functional impairment of salivary and lacrimal glands, and causes keratoconjunctivitis sicca, which refers to dryness of the eyes and xerostomia meaning dryness of the mouth. The ease and safety of a minor salivary gland biopsy enables study of the molecular biology of this autoimmune exocrinopathy. With microscopic examination, the lymphocytic replacement of the epithelium and lymphoepithelial lesion can be observed. It is an inflammatory rheumatic disease with main features in the eyes and mouth (Ramos-Casals, 2005; Kassan, 2004; Schwartz, 2007; Fox, 2000).

1.1.1 History

Sjogren Syndrome is a relatively newly identified disease. Its histology was described and a link with arthritis was reported in the late 19th and early 20th centuries as single case reports. In 1933, Henrick Sjogren reported the association of xeropthalmia meaning dry eyes and xerostomia with polyarthritis in 19 cases. In the early years, research was mainly on the opthalmology of Sjogren Syndrome. The clinical spectrum of Sjogren Syndrome in 1956 was defined in 62 subjects. The rheumatologic aspect of Sjogren Syndrome was heavily emphasized when the distinction between primary and secondary Sjogren Syndrome was first described in 1965. In 1970s, autoantibodies characteristic of Sjogren Syndrome were described. In 1990s, the diagnostic tests became a routine procedure and research on disease-modifying drugs started (Venables, 2004). Table 1.1 shows the timeline of advances in delineating Sjogren Syndrome.

Table 1.1 Timeline of important advances in delineating Sjogren Syndrome (Venables *et al.*,2004)

Year	Progress
1888	Case reports of Hadden and Rowlands
1892	Case report of Miculicz (histology described)
1926	Description of three patients by Gougerot
1928	Case report of Houwer (link with arthritis)
1933	Description of nineteen patients with keratoconjuctivitis sicca by Sjogren
1946	Translation of Sjogren thesis to English
1953	"Sjogren syndrome" established as a term in literature
1965	Primary and secondary Sjogren Syndrome distinguished
1970	Anti-Ro (SS-A) and anti-La (SS-B) in Sjogren Syndrome is determined
1980	Extraglandular symptoms described
1990	Routine application of diagnostic tests
1990	Trials of disease-modifying drugs
2002	Consensus diagnosis criteria

Sjogren Syndrome is a milder disease in comparison to rheumatoid arthritis (RA) or systemic lupus erythematosus (SLE). There was not a strict classification criteria for the syndrome until lately. Its low mortality and underdiagnosis prevented the disease from being a primary research area and caused Sjogren patients to be underdiognosed and undertreated (Venables, 2004).

1.1.2 Symptoms

The symptoms in relation to the eyes are more serious than the symptoms of the mouth. Underlying collagen can be revealed as a result of erosions in the conjunctiva leading to the appearance of filamentary keratitis. More dental care is a sign of xerostomia. Parotid swelling occurs in half of the patients. Other exocrine glands can be involved effecting skin, vagina, gastrointestinal tract, colon and rectum. 70% of the patients complain of fatigue.

Systemic features include renal involvement, neurological involvement, vascular involvement, cutenous involvement, pulmonary involvement, muscuskeletal involvement, gastroenterologic involvement, congenital heart block and lymphoma (Venables, 2004; Kassan, 2008; Ramos-Casals, 2005).

1.1.3 Criteria for diagnosis

Until recently, a consensus international diagnosis criteria for Sjogren Syndrome was unavailable. Because of its heterogeneous nature, Sjogren Syndrome is hard to diagnose. It has been hard to achieve a consensus because there are diseases that mimic Sjogren Syndrome such as age-related atrophy, chronic anxiety, chronic fatigue syndrome, fibromyalgia and dry eyes and mouth symptoms (DEMS). Previously, there were `Copenhagen`, `Californian` and `European` criteria. The revised American-European consensus criteria was established in 2002 (Vitali, 2002). The application of this criteria was essential both for clinical diagnosis and research.

The current criteria is as designated in Table 1.2. Four of the listed observations including either the fourth or sixth one is needed to diagnose a patient as Sjogren Syndrome. The observations include objective signs and symptoms of dryness. Evaluation of symptoms is based on a short questionaire. Ocular signs are evaluated based on Schirmer`s test or Rose Bengal staining. Oral signs are evaluated with scintiagraphy, sialography or a test for salivary flow rate. One observation that indicates the inflammatory nature of the disease is the

characteristic appearance of a minor salivary gland biopsy with focal inflammation. Focal lymphocytic infiltrates are expected in the minor salivary gland biopsy resulting in a focus score of more than 1 (A cluster of 50 or more lymphocytes is called a focus). Another observation that indicates inflammation is the presence of characteristic autoantibodies, rheumatoid factor or anti-nuclear antibodies in the serum. To prevent confusion with dry eyes and mouth symptoms (DEMS), these observations for determining the inflammatory nature of the disease are necessary (Venables, 2004).

Table 1.2 Current Criteria for Sjogren Syndrome diagnosis (Venables *et al*, 2004)

	Ocular symptoms
I	1. Have you had daily, persistance, troublesome dry eyes for more than 3 months 2. Do you have a recurrent sensation of sand and gravel in the eyes 3. Do you use a tear substitutive more than 3 times a day
	Oral symptoms
II	1. Have you had a daily feeling of dry mouth for more than 3 months 2. Have you had recurrently or persistantly swollen salivary glands as an adult 3. Do you frequently drink liquids to aid in swallowing dry foods
III	Positive Schirmer`s I test or Rose Bengal score
IV	Abnormal lower lip biopsy (focus score >= 1)
V	Positive result for unstimulated whole salivary flow (<= 1.5ml in 15 min)
VI	Antibodies to Ro (SS-A) or La (SS-B), or both

In diagnosis, it is important to show that the salivary dysfunction roots from an inflammation. Salivary dysfunction can also be caused by drugs, infection, head and neck radiation treatment, acquired immune deficiency syndrome (AIDS), preexisting lymphoma, sarcoidosis, graft versus host disease (GVHD), use of anticholinergic drugs and the autonomic nervous system. These are exclusions to diagnosis (Venables, 2004).

1.1.4 Treatment

Until recently, the treatment was only topical and symptomatic to improve moisture; decrease inflammation and prevent damage. These include performing general measures and using tear and saliva substitutes and mucolytic agents. Recently, research on disease-modifying drugs have been intensified with the recognition that Sjogren Syndrome is a disease of considerable morbidity. Currently a more aggressive approach to therapy is applied with topical and systemic treatment. The disease-modifying drugs include secretagogues and immunomodulatory drugs.

If Sjogren Syndrome is a consequence of the destruction of glands, secretagogues can not be a rational form of treatment. However there is currently increasing evidence that hypofunction of the glands is caused by inflammation and dryness is caused by this hypofunction. Sialogogues, pilocarpine and cevimeline hydrocholorine are both cholinergic agents with muscarinic agonist activity and are of proven benefit for Sjogren Syndrome (Vivino, 2001 ; Fife, 2002).

It seems logical to use immunomodulatory drugs because most of the morbidity is caused by widespread disruption of the immune system. There is supportive evidence for the use of hydroxycholoroquine sulphate. These can be effective to cure most disabling features such as fatique (Fox, 1996).

There is limited research on the use of biological agents for the treatment of Sjogren Syndrome. There are trials of a recombinant interferon-alpha, anti-tumour necrosis factor antibody Infliximab and anti-B cell antibodies such as anti-CD20 antibody Rituximab (Cummins, 2003; Serge, 2002; Bradley, 2003).

Anti-inflammatory agents and cytotoxic drugs are used to treat systemic complications (Venables, 2004).

1.1.5 Epidemiology

The prevalance of Sjogren Syndrome have been controversial based on the lack of a consensus diagnostic criteria (Thomas, 1998). An estimated 2 to 4 million people in the United States have this syndrome. The heterogeneity of its clinical manifestations is a barrier against its diagnosis in some cases. Approximately 1 million people have an established diagnosis of Sjogren Syndrome. No racial predisposition to the syndrome is known. Females are affected more than males in a 9:1 ratio. It is a disease of middle-aged or elderly women although it can occur at any age. It has a low pediatric prevelance. Approximately 60% of the Sjogren patients have secondary syndromes. Secondary Sjogren Syndrome results in death, especially in cases with myelopathy (Schwartz, 2007; Kassan, 2004).

1.1.6 Classification as primary and secondary Sjogren Syndrome

The presence of autoimmune inflammatory exocrinopathy and sicca symptoms alone is designated as primary Sjogren Syndrome. The occurence of keratoconjuctivitis sicca and xerostomia in association with another connective tissues diseases is designated secondary Sjogren Syndrome. These associated autoimmune diseases could be rheumatoid arthritis, systemic lupus erythematosus, scleroderma (SSc), muscle connective tissue disease (MCTD), subacute cutaneous lupus erythematosus (SCLE), polymyositis, sarcoidosis, systemic vasculitides and antiphospholipid syndrome (Ramos-Casals, 2007). The prevelance of Sjogren Syndrome in other autoimmune diseases is shown in Table 1.3.

Table 1.3 Prevalence of sicca features and associtated Sjogren Syndrome in systemic autoimmune diseases (Ramos-Casals *et al*, 2007)

Diseases	Sicca features (%)	Sjogren Syndrome (%)
Systemic lupus erythematosus	18-34	9-19
Rheumatoid arthritis	30-50	4-31
Systemic sclerosis	67-68	14-20
Sarcoidosis	9	3
Cryoglobulinemic vasculitis	42	-

Sjogren Syndrome patients have a 44 times increased risk of developing B cell lymphoma (Kassan, 1978). Lymphoma is the cause of death in one of five Sjogren patients (Ioannidis, 2002; Bolstad, 2002). The prevalence of malignant lymphoma in Sjogren Syndrome patients is 4.3%, being mostly low-grade marginal zone B-cell lymphoma. Whether this lymphoma is primary or secondary to Sjogren Syndrome is unknown. (Yamamoto, 2002).

1.1.7. Pathophysiology and Etiology

The etiology of Sjogren Syndrome is unknown. It is a complex disease, in other words, both genetic predisposition and environmental factors have a role in the pathogenesis. The aetiopathogenesis is probably sequential leading to selective dysfunction of target organs caused by migration of lymphocytes (Ramos-Casals, 2005). In a minor salivary biopsy, the infiltrated lymphocytes can be observed as in Figure 1.1.

Sjogren Syndrome is generally accepted a T-cell mediated disease. The infiltrating cells in the glands are mostly T cells. T helper cells (CD4+) are more commonly seen than cytotoxic T cells (CD8+) in a ratio of 5:3. In labial salivary glands of Sjogren Syndrome patients, 2% of the infiltrating mononuclear cells were reported to be dendritic cells (Xanthou, 1999). Additionally, ductal and acinar epithelial cells of Sjogren Syndrome patients express B7.1 and B7.2

co-stimulatory molecules (Monoussakis, 1999). The majority of the T cells in the glands express CD45RO, which is a feature of activated or memory cells. T helper (Th1) cytokines such as interleukin 2 (IL-2), interleukin 10 (IL-10) are produced in much higher amounts in comparison to normal controls. There is also an increase in T helper 2 (Th2) cytokines such as interleukin 4 (IL-4) and interleukin 5 (IL-5). There is additionally an increase in cytokines interleukin 1 (IL-1), interleukin 6 (IL-6), interleukin 10 (IL-10), tumor necrosis factor (TNF) and interferon gamma (IFN-γ) expressed by the tissue (Fox, 1994, Ohyama, 1996). A few T cells proliferate in the area. It is possible that memory T cells are semiactivated and the other T cells are probably suppressed in response to the strong immune response (Yamamoto, 2003).

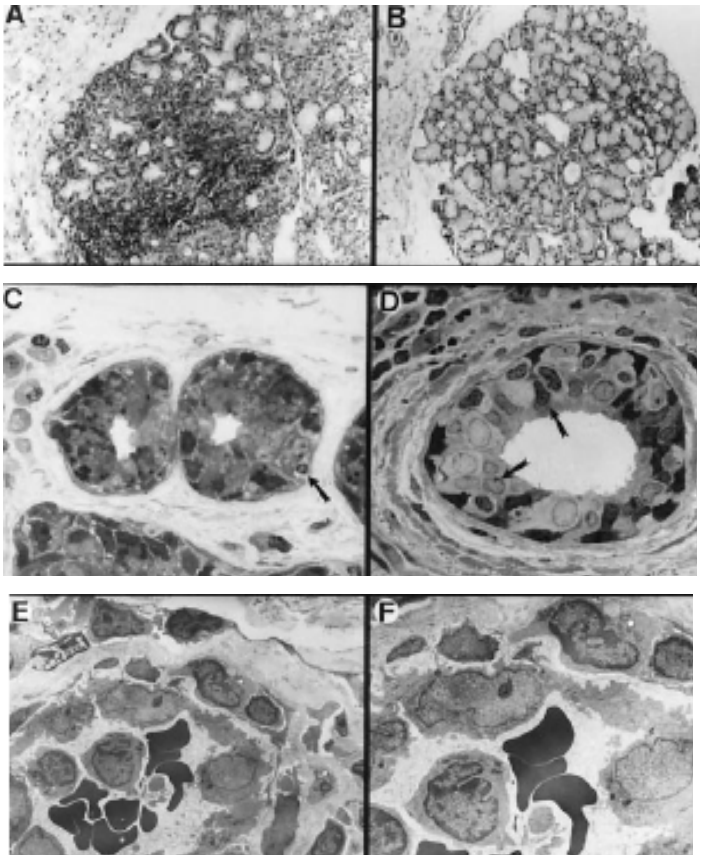

Figure 1.1 Minor salivary gland biopsy photomicrographs A) 50X, Sjogren Syndrome B) 50X, normal C) D) 200X, Sjogren Syndrome E) F) 500X, Sjogren Syndrome (Fox *et al*., 2000)

It seems likely that Sjogren Syndrome results from glandular destruction so infiltrating cytotoxic T cells are candidates for the pathogenesis. Both apoptosis with perforin/granzyme B and Fas/Fas ligand pathways were implicated with the etiology. It was shown that cytotoxic T cells expressing integrin localize around acinar epithelial cells that express E-cadherin (Fujihare, 1999). However the findings in relation to glandular apoptosis in Sjogren Syndrome patients in comparison to controls is controversial (Ohlson, 2001;Yammato 2003).

Several features of the disease can result from B cell stimulation and hyperglobulinemia. 20% of the infiltrating population consists of B cells. Antigen-driven, germinal center-type B cell response takes place within the salivary glands of Sjogren syndrome patients. Immunoglobulin G (IgG) isotype is extraordinarily seen more than immunoglobulin A (IgA) in the glands and in the serum. Immunoglobulin variable λ (IgVλ) light chain usage in primary Sjogren Syndrome was researched and it was found that there were differences in V-J recombination in Sjogren Syndrome patients from controls. It was concluded that there are defects in B-cell selection and maturation; immunoglobulin receptor editing and mutational targetting. There is B cell clonal expansion with antigen stimulation (Scott, 1998;Yammato, 2003).

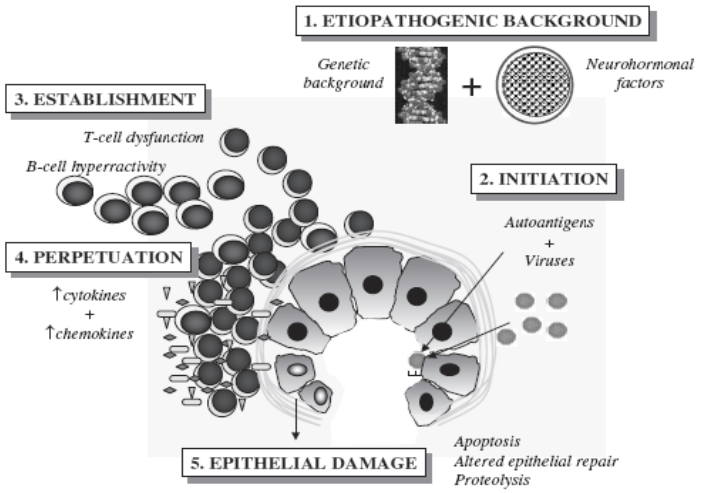

Figure 1.2 Steps of the Sjogren Syndrome pathogenesis according to epithelithis model (Ramos-Casals *et al,* 2005)

The current aetiopathogenic hypothesis of Sjogren Syndrome is autoimmune epithelitis. According to this theory, intrinsic influences such as genetic make-up and/or extrinsic influences such as environmental factors form the background for the pathogenesis. The initiation occurs as a result of an altered immune system incapable of discriminating between 'foreign', 'self' molecules and /or altered self antigens of the gland epithelium. This results in autoimmunity. The establishment process includes an abnormal immune response with abnormal T cell dysfunction and B cell hyperreactivity. This results in lymphocytic destruction forming histopathological lesions of acinar and ductal epithelial cells. It may also cause change in cytokine and chemokine secretion leading to perpetuation of the response. Then mechanisms of tissue damage such as apoptosis are activated leading to chronic inflammation with fibroses and loss of secretory function. Other mechanisms involved can be enhanced proteolytic mechanisms or altered epithelial repair. Apoptosis is thought to be related to formation of further abnormal autoantigens (Ramos-Casals, 2005). An overview of this model is given in Figure 1.2.

There are several potential mechanisms that are related to the etiology of Sjogren Syndrome: (1) Unsuccessful deletion of autoimmune T cells in the thymus. (2) Expression of increased levels of cell adhesive molecules in high endothelial venules leads to homing of autoimmune lymphocytes to glands. (3) Increase in expression of human leukocyte antigens (HLAs) in aiding increased presentation of antigens to lymphocytes. (4) In the gland, lymphocyte activation by their interaction with HLA-DR, cell adhesion molecules, and co-stimulatory factors. (5) Circulating autoantibodies against the ribonucleoproteins Ro and La (6) Inflamatory response is perpetuated by the secretion of pro-inflammatory cytokines by lymphocytes and epithelial cells. (7) Anti-apoptotic markers such as *Bcl-2* and *Bcl-x* in the lymphocytes and apoptotic markers such as *Fas* and *Bax* in the epithelium are upregulated (Fox, 2000).

The role of apoptosis in autoimmune diseases including Sjogren Syndrome has been a major research area. Apoptosis has been proposed to have two aspects in the Sjogren Syndrome pathogenesis: (1) Increase in apoptosis in the ductal epithelial cells of the salivary glands was suggested to cause salivary decrease. (2) Accumulation of lymphocytes and displacement of functional acinar cells result from lymphocytes escaping apoptosis because of the defects of in the pathway.

Another major hypothesis on the etiology of Sjogren Syndrome is autonomic dysfunction. It was seen that half of the acinar cells remain histologically intact when patient biopsies were observed. So hypofunction rather than destruction of glandular epithelial cells is proposed as a theory for the secretory problem in Sjogren Syndrome. In one model, the gland can not receive enough neural signals from medulla. This can result from decreased neural innervation. Decreased neural axon-specific protein 9.5 and synaptophysin was observed with immunohistology (Konttinen, 1992). Release of acetylcholine is needed for glandular secretion. Cytokines can be toxic to the nerves and prevent the release of acetylcholine. It was shown in vitro and in transgenic mice that interleukin 1 and tumor necrosis factor α are toxic to nerves (Main, 1993). Another model suggests that acinar cells respond in a limited manner to neural

input, in other words there are problems in post signal transduction. Decrease of protein kinase C (PKC) isoforms involved in secretory response were reported for epithelial and myoepithelial cells (Törnwall 1997; Campbell, 1995). Antibodies against muscarinic M3 receptors were also found in Sjogren Syndrome patients (Borda, 1996; Venables, 2004; Fox, 2000).

Interactions of ductal and acinar cells with matrix may be important in the etiology of the disease. These interactions mediate response to cytokines, growth signals and hormones; they aid in homeostasis, regeneration and function of the cells. It was reported that the receptors on epithelial cells and some cytokines are expressed at higher amounts in these patients (Fox, 1994, Ohyama, 1996). It was also reported that cell-matrix interactions are important for secretory functions of to glandular cells in response muscarinic M3 agonists (Laurie, 1996).

The higher prevalence of Sjogren Syndrome in perimenopausal women suggest that hormonal factors can have a role in the disease etiology. The research on this field is controversial (Ramos-Casals, 2005).

1.1.7.1 Genetic predisposition

Based on animal models, familial aggregation and candidate gene association studies, a genetic predisposition to Sjogren Syndrome was suggested. Several families with more than two cases of Sjogren Syndrome have been identified and presence of multiple autoimmune diseases in families and in an individual have frequently been reported (Lichtenfeld, 1976; Koivukangas, 1973; Mason, 1971; Boiling, 1983, Sabio, 1983; Tanaka, 2001). Approximately 35% of Sjogren Syndrome patients have relatives with other autoimmune diseases. Limited number of case reports is present on twin concordance of primary Sjogren Syndrome. Reported twins have very similar phenotypes including clinical presentation, serological data, specificity of immune response to Ro/SSA and biopsy focus score (Scofield, 1997; Besena, 1991; Kogo, 1980; Bolstad, 2000; Bolstad, 2002).

1.1.7.2. Associated genes

Many genes were implicated in Sjogren Syndrome etiology and it is a polygenic disease. The best documented genetic risk factors of autoimmune diseases are polymorphic major histocompatibility complex (MHC) genes. For Sjogren Syndrome, MHC class II genes are of importance, especially HLA-DR and HLA-DQ alleles. HLA class II haplotypes, the DRB*03-DQB1*02 were frequently reported to be linked with the disease (Kang, 1993). These distinct polymorphisms of HLA alleles were associated with differences in autoantibody repertoire, especially anti-Ro (SS-A) and anti-La (SS-B) antibodies (Gottenberg, 2003; Davies, 2001). Link with MHC class I genes was also reported. The higher frequency haplotype found was HLA-A24 (Loiseau, 2001).

Lately, research has focused on finding association between Sjogren Syndrome and polymorphic genes that encode molecules involved in the immune system, especially cytokines. Cyokines mediate and regulate immune and inflammatory responses and they were suggested to have a role in autoimmune diseases including Sjogren Syndrome pathogenesis. Polymorphism of cytokines researched are interleukin 10, interleukin 4, interleukin 6, tumor necrosis factor α and tumor growth factor β (Ramos-Casals,2005; Hulkkonen, 2001; Gottenberg, 2004; Pertovaara, 2004; Youn, 2000).

Additional candidate gene studies were also undertaken. Sjogren Syndrome was associated with the polymorphisms of mannose binding lectin (MBL), Fas/FasL and receptor mononuclear cell attracting chemokine receptor, CCR5. (Tsutsumi, 2001, Wang, 2001, Bolstad, 2000, Petrek, 2002; Ramos-Casals,2005). Table 1.4 lists the non-MHC genes reported to be associated with Sjogren Syndrome.

Table 1.4 Polymorphisms of interleukins and other genes associated with Sjogren Syndrome

Polymorphic gene	**Altered polymorphic distibution**	**Citation**
Cytokine genes		
IL10	increase in G at position -1082 , C at position -819 , C at -592	Hulkkonen, 2001; Font, 2002 ; Gottenberg, 2003
IL4R	altered IL4R allele	Youn, 2000
TNFα	increase in A at position -308	Gottenberg, 2003; Pertovaara, 2004
Other genes		
MBL	increase in wild type codon 54	Tsutnami, 2001; Wang, 2001
CCR5	decrease in delta 32 genotype	Petrek, 2002
Fas/FasL	altered Fas alleles	Bolstad, 2002

There are recently investigated candidate antigens for a role in Sjogren Syndrome etiology. The most important of these is aquaporins, plasma membrane transporters of water. There is controversial results on the aberrant cytoplasmic localization of aquaporin 5 (AQP-5) channel proteins in this disease (Tsubota, 2001; Steinfeld, 2002; Beroukas, 2001). There is one report on the reduced expression of aquaporin 3 (AQP-3) in acinar cells in primary Sjogren Syndrome patients (Waterman, 2003 ; Ramos-Casals, 2005).

1.1.7.3 Associated antibodies

Table 1.5 Autoantibodies described in Sjogren Syndrome patients and their corresponding prevalences (Nakamura *et al*, 2006)

Autoantibodies	**Molecular weight of the corresponding Antigen**	**Prevalence in Sjogren patients**
Anti-SSA/Ro Ab	SSA/Ro (52-kd / 60-kd)	25-36%
Anti-SSB/La Ab	SSB/La (48-kd)	13-48%
anti-centromere Ab	centromere protein B (80-kd) centromere protein C (140-kd) centromere protein H (33-kd)	2-19.9%
anti-alpha-fodrin Ab	alpha-fodrin (120-kd)	73-93%
anti-type-3 muscarinic acethylcholine receptor Ab	type-3 muscarinic acethylcholine receptor (75-kd)	90%

Sjogren Syndrome is an autoimmune disease, and a wide variety of auto-antibodes have been described in these patients (Table 1.5).

In Sjogren Syndrome, especially anti-Ro/SS-A and to a lesser extent anti-La/SS-B and anti-centromere antibodies are seen. These are organ non-specific antibodies. Antigens towards these antibodies are 52 kd SSA/Ro, 60 kd SS-A/Ro and 48 kd SS-B/La. Anti-Ro/SS-A and to a lesser extent anti-La/SS-B are frequently also found in relatives of the patients. Anti-Ro/SS-A antibodies are found in 65% ; anti-La/SS-B is found in 50% of the patients. Anti-Lo/SS-A is restricted to Sjogren Syndrome but anti-Ro/SS-B can be found in systemic lupus erythematosus, rheumatoid arthritis or polymyositis. The presence of these autoantibodies is related to density of lymphocytic infiltrates, the severity of

extraglandular symptoms, parotid gland enlargement, early disease onset and longer disease duration (Yamamato, 2002).

Rheumatoid factor is found both in primary and secondary Sjogren Syndrome, but the presence of rheumatoid factor is a general feature of autoimmune diseases.

Alpha-fodrin is an apoptotically cleaved form of fodrin, which is a membrane skeletal protein found in many tissues. Antibodies against alpha-fodrin is found in Sjogren Syndrome sera. It is not known whether it is a primary or secondary event to the disease.

Based on one theory, these antibodies are produced in the salivary glands (Tengner, 1998). The abnormal localization of anti-Lo and anti-Ro was reported several times in disease and stress states. Surface expression of an antigen reactive to these antibodies was shown after irradiation of keratocytes. This raises the possibility that altered localization of antigens after apoptosis can be the immunogen in autoimmune responses (Lefeber,1984). Another theory on the production of these antibodies is molecular mimicry. Sequence similarities of SS-A/Ro and SS-B/La determinants with retroviral antigens have been reported (Kohsaka,1990;Yamamato, 2002).

Anti-M3 antibody can particularly have a role in the pathogenesis because it can cause exocrine dysfunction by blocking neurotransmission (Nakkamura, 2006; Venables, 2004).

1.1.7.4 Viruses

Viral infection is one of the most important proposed environmental factors. Viruses of the herpes family are implicated in Sjogren Syndrome pathogenesis. Cytomegalovirus was shown to induce sialadenitis with Sjogren Syndrome antibodies in a mouse model (Fleck, 1998). Epstein barr virus (EBV) commonly infects the salivary glands and induces strong T cell responses.

Hepatitis C Virus (HCV) infected people present sialadenitis and lymphocytes in the gland without other phenotypes of Sjogren Syndrome (Haddah, 1992). Retroviruses, human immunodeficiency virus (HIV) and human T lymphotrophic virus (HTLV-1) were also impilicated in this syndrome because they are known to infect cells of the immune system and cause immune dysregulation and present lymphcytosis. HIV infected people were shown to exhibit Sjogren like syndromes more frequently (Kordossis, 1998). Sjogren syndrome patients were shown to have HTLV-1 antibodies (Terada, 1994). Novel retroviral genome was found in the salivary glands of Sjogren Syndrome patients (Griffiths, 1997). These were treated in an antiretroviral manner and improvement in sicca symptoms was observed (Steinfeld, 1999;Yamamato, 2002).

1.1.8. Research to relate microchimerism and Sjogren Syndrome

Microchimerism is the presence of a small population of genetically distinct cells in an individual. Fetal microchimerism is the presence of fetal cells in the maternal blood due to their transfer during pregnancy. There is intense research on a possible contribution of fetal microchimerism in Sjogren Syndrome pathogenesis, but the results are controversial. A preliminary research concluded that circulating fetal cells are uncommon among the peripheral blood mononuclear cells of Sjogren Syndrome patients (Toda, 2000). Carlucci *et al*. or Aractingi *et al.* could not find significant correlation between the disease and salivary gland microchimerism (Carlucci, 2001; Aractingi, 2002) Further research identified fetal cells in salivary glands of Sjogren Syndrome patients. In the same article, no difference between patients and controls in relation to microchimerism in their peripheral blood was reported (Endo, 2001). In another research fetal cells were found in the salivary glands and bronchoalveolar lavage fluid of Sjogren Syndrome patients (Kuroki, 2002).

1.2. X Chromosome Inactivation

1.2.1 X Dosage compensation

Sex dosage compensation is a mechanism to equalize X chromosome expression in females and males. The term was first used by Hermann Muller, a Drosophilia geneticist (Muller, 1947). X dosage compensation is achieved through different mechanisms in D. melanogaster, C. elegans, birds and mammals. In fruit flies, it is provided by doubling of male X chromosome expression. In roundworms, expression from each of the X chromosome in females is halved. In mammals, dosage compensation takes place by chromosome silencing. X chromosome inherited from either parent is silenced at random, and normal women are thus a mosaic of 2 cell populations (Willard, 2006; Migeon, 2007).

1.2.2 Discovery of X chromosome inactivation

Murray Barr and Evart Bertram observed that in cat nerve cells, there was a dotlike body that stained like chromatin, found near the nucleus, which they named “nucleolar satellite”. They noticed the female specific presence of this “sex chromatin body” or “Barr body” and extended their research showing that this body was present in a variety of species including humans (Barr, 1949). This female specific feature was used in research of abnormal sexual development. By 1954, it was shown that Turner patients had no Barr body, but Klinefelter patients had one. In 1956, it was possible to examine chromosomes directly and it was obvious that the number of Barr bodies in an individual was one less than the number of X chromosomes. At the time, researchers hypothesized that Barr body was formed by the crossing of two X chromosomes. Susumo Ohno proposed that Barr body was derived from a single X chromosome showing that there was a single condensed chromosome in liver cells from female rats and mice. This chromosome was female specific like the Barr body (Ohno, 1959). Liane Russell and Ernest Beutler also proposed that only one X was active in female cells. Russell came to this conclusion based on her observations of mice with aberrant

sex chromosomes (Russell, 1961). Beutler`s proposal was based on his observations that heterozygotes for glucose 6 phophate dehyrdrogenease (G6PD) deficiency had mixtures of normal and G6PD deficient blood cells (Beutler, 1961).

Mary Lyon who studied the genetic effects of radiation noticed unexpected findings in relation to a X-linked coat color gene in mice (Lyon, 1953). The mutation of this gene resulted in death in males and white spotting on the coat of female heterozygotes. This was unusual when compared with other coat color genes. After other relevant findings accumulated, Lyon suggested that only one active X is necessary in each female cell. She proposed that one X is inactivated early in embryogenesis and take the appereance of a condensed chromosome like the one observed by Ohno. She suggested that the females are mosaics since either the paternal or maternal X is inactivated in each cell. She reasoned the large color patches on the coat of female mice correspond to clones of cells with one parental X inactivated early in development. She later extended her hypothesis to other mammals including humans (Lyon, 1962). She also predicted that this mottling effect should be present for all other X-linked coat color genes. She also hypothesized that this effect should be present in XXY males since they are also mosaics (Lyon, 1962). She noticed heterozygous females with the ocular albinism gene mutation partially manifested the disease. She also hypothesized that there were regions on X chromosome which were not subject to X chromosome inactivation (XCI) meaning pseudoautosomal regions and symptoms of Turner Syndrome result from loss of normal two fold dosage of these genes. She also reasoned the unexpected viability of individuals with multiple X chromosomes with XCI. Because it is known that more than two copies of a chromosome is not viable (Lyon, 1962). Lyon performed heterozygous mouse breeding experiments to show that only one X-linked coat color gene was inactivated in each cell. The most obvious proof that single X was active in each somatic cell and this inactivation was stably inherited came from single cloning experiments of skin cells heterozgous for G6PD. Clones of single

cell dilutions derived from these cells expressed either G6PD A or G6PD B (Davidson, 1963 ; Migeon, 2007).

1.2.3. Features of Inactive X

One feature of the inactive X is that it is condensed during interphase and named as Barr body in other words sex chromatin mass, although it looks the same as its active homologue during metaphase. The first indicator of an inactive X is that it`s late replicating before cell division, which can be determined by labeling the chromosomes using a tagged version of thymidine (Morishima, 1962). Another feature of inactive X is that it is heterochromatic so trancriptionally inactive except its pseudoautosomal regions. It has underacetylated H3 and H4 histones, which is demonstrated with acetylated histone specific antibodies. Inactive X chromosome has a characteristic letter ”C” shape with a bend placing the ends of the two arms closer to each other (Walker, 1991). This shape is mostly distinguished in the interphase. The genes on the inactive X have methylated CpG islands in their promoter regions. Latest research has shown that active X has twofold methylation at intergenic CpGs (Hellman, 2007). One of the most important features of inactive X is that it transcribes X-inactive-specific transcript (*XIST).*

1.2.4. Clonal Nature of XCI

The inactivation pattern is clonally inherited and irreversible in somatic cells. XCI occurs and is reversible during a limited time durindg embryogenesis and then becomes irreversible as shown in stem cell models (Wutz and Jaenisch, 2000). Adding X chromosomes with hybridization/selection experiments or removing *Xist* with silencing RNA after the critical developmental stage has no effect. The reactivation of silent X only occurs in oocytes during their maturation from ovarian germ cells. Reversal of XCI can be induced to occur also in placental cells (Migeon,1986; Migeon, 2005).

1.2.5. Timing of XCI

The exact timing of XCI is not known. There are three proposed models for XCI. These models are as shown in Figure 1.3.

The classical model supports that the X chromosomes are transmitted from gametes to embryo fully active and XCI occurs during blastocyst stage coupled with differentiation. This model doesn`t explain how embryo can tolerate twofold imbalance of X-linked gene expression. Paternal X in the sperm is inactived by a process of sex chromosome body in other words XY body inactivation. So another hypothesis called the preinactivation hypothesis defends that inactive X of the sperm is transmitted to the embryo and and more global silencing occurs in the trophoblast while reactivation and random inactivation occur in the epiblast (Huhyn, 2001). It is supported by the finding that in the early cleavage steps of embryogenesis, there is some expression from both Xs and the expression from the paternal X is increasing (Huhyn, 2003; Mak., 2004). The de novo inactivation hypothesis states zygote inherits active X chromosomes and inactivation starts at four-eight cell stage. In the epiblast, reactivation occurs following random XCI. This is based on the observation that two-cell stage embryos have some features of active X (Huhyn, 2004; Migeon 2007).

Imprinted XCI is known to take place in marsupial cells (Cooper, 1971; Namekawa 2007) and placental cells of some eutherians (Takagi, 1975). So it seems likely that XCI evolved as an imprinted phenomenon, which was later modified in eutherians (Graves, 1996).

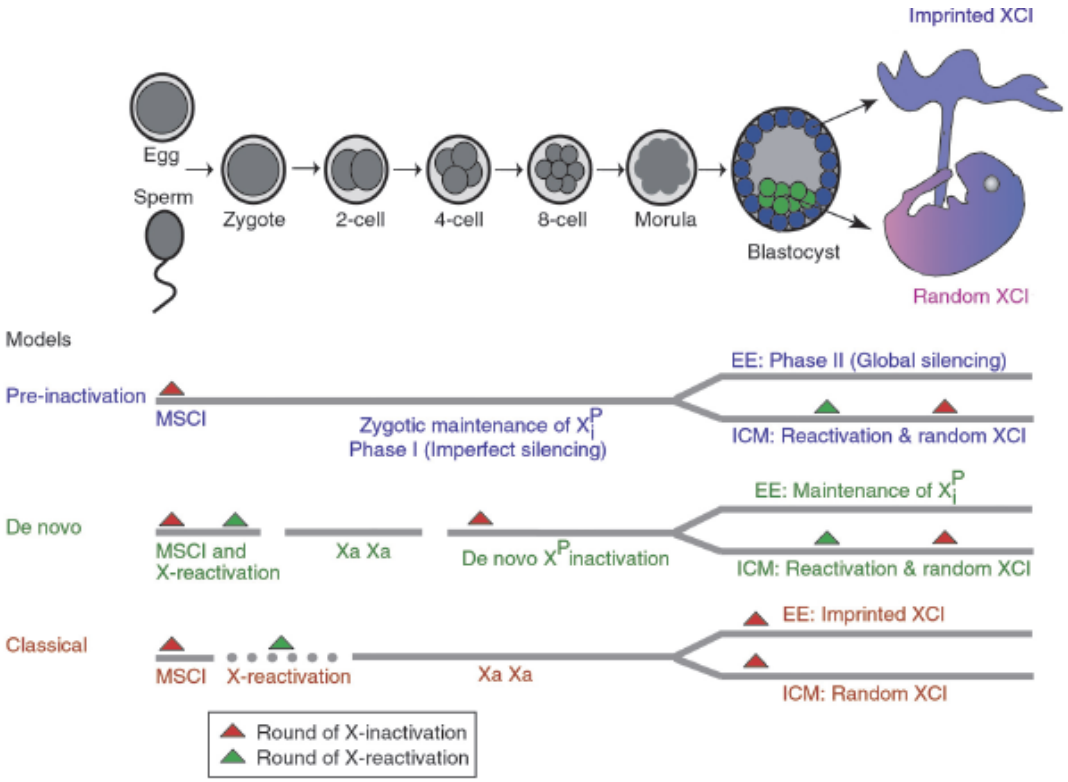

Figure 1.3 Models in relation to the XCI timing in the embryo a) pre-inactivation b) de novo inactivation c) classical model (Huhyn, 2004)

1.2.6. Master control region of XCI and its components

Russell was the first to hypothesize that there is a region on X chromosome from which XCI initiates and spreads bidirectionally (Russell, 1963). In both mice (Russell, 1965) and humans (Therman, 1974, 1979), the master control region of XCI was identified observing X-autosome translocations. The X-inactivation center (*XIC)* was first mapped to band Xq13 in humans and D region of the mouse X chromosome. The region was further defined by transfecting parts of the putative region into mouse embryonic stem cells to see if they could function (Lee, 1999; Heard, 1999: Lee, 1996; Migeon, 1999). *XIC* is poor in protein-coding genes and it includes many repeats (Ross, 2005). Several genes involved in XCI were identified in *XIC* (Figure 1.4).

The most crucial gene within is *Xist* in mouse and *XIST* in humans. It is larger than 17 kb and it codes for a regulatory RNA transcript. It was identified in many eutherians including mice and humans (Brown, 1992; Brockdorff, 1992).

However it was not identified in monotremes or marsupials (Duret, 2006). The sequence homology among *Xist* from different species is less than 50% (Chureau, 2002). However genomic organization, promoter region, transcription start sites, tandem repeat and most intron-exon boundries are conserved among species. When male mouse embryonic stem cells were transfected with a YAC transgene containing *XIST,* it induced random inactivation in male transgenic mice derived from these cells. (Migeon, 1999) This noncoding RNA is expressed from both male and female Xs partially before XCI. The significance of this low expression is not known (Ray, 1997). It continues to be fully expressed after XCI, so it is hypothesized that it can have a function in maintanance of XCI. Its different isoforms exist, which may have a role in regulation (Ma, 2005). Its deletions do not reactivate the inactivated X since there are other mechanisms of maintanance (Brown, 1994). Induced *Xist* deletions in mouse, spontaneous human *XIST* mutations such as seen in the cases of people with ring chromosomes and *XIC* transgenes have shown that role of *Xist* is XCI.

Another gene within *XIC* is *Tsix* which is antisense to *Xist*. Mouse *Tsix* is a 40-kb RNA originating 15 kb downstream of *Xist* and transcribed across the *Xist* locus (Lee, 1999). Deleting a 65-kb region downstream of *Xist* results in constitutive *Xist* expression and X inactivation. Before the onset of X inactivation, *Tsix* is expressed from both X chromosomes. At the onset of X inactivation, Tsix expression becomes monoallelic, is associated with the future active X and persists until *Xist* is turned off. *Tsix* is not found on the inactive X once cells enter the X inactivation pathway. Human *TSIX* produces a >30-kb transcript that is expressed only in cells of fetal origin. Differences in the structure of human and murine genes indicate that human *TSIX* was truncated during evolution. Human *TSIX* can not repress *XIST* and is coexpressed with it through embryonic development (Migeon, 2001). It was implicated that *Tsix* and X-inactivation intergenic transcription element (*Xite)* mutations effect choice and counting (Lee,2005; Migeon, 2002).

A cis element called *Xite* was found and shown to downregulate *Tsix* at the onset of XCI. This was suggested as a candidate for X chromosome

controlling element (*Xce)* (Ogowa, 2003). Before the discovery of *Xist*, the best candidate for XCI was *Xce* because it effects the randomness of XCI (Johnston, 1981; Simmler, 1993).

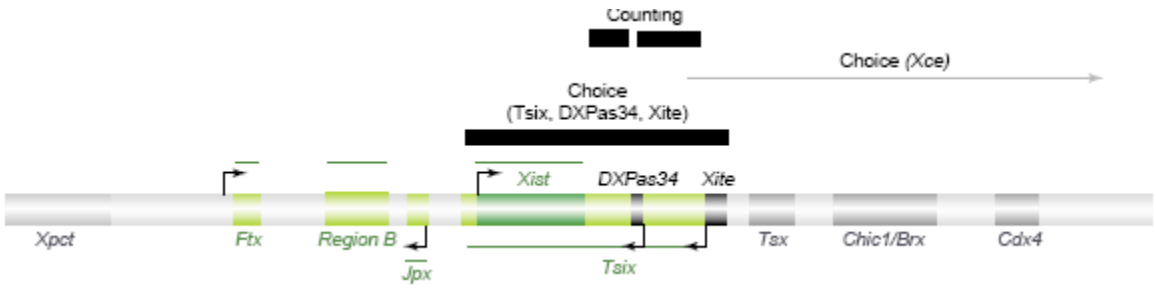

Figure 1.4 Elements of *Xic* The *Xic* center that is determined by deletion and trangene studies. Green parts demonstrate genes that give rise to non-coding transcripts. Grey parts show protein-coding genes with unknown function in relation to XCI. Black parts indicate parts involved in counting and choice determined by targeted deletions. Mapped *Xce* implicated in choice is also shown (Heard *et al*, 2004).

1.2.7. Steps of XCI

Steps of XCI include counting, choice, inactivation, spreading and maintenance. In all mammalian cells, all X chromosomes are inactivated but one remains active. The mechanism of XCI seems to depend on determining an active X and repressing all the other X chromosomes by a default pathway. Silencing is chromosome wide. Cis inactivation is mediated by a noncoding RNA molecule transcribed from *Xist* in *XIC*. The molecule spreads along the chromosome from which it is expressed and transcriptionally silences it by changing the underlying chromosome.

Counting is the essential first step in general dosage compensation mechanims. A transient interaction is seen between X chromosomes at their *Xic* at the onset of inactivation in mouse embryonic fibroblasts (Xu, 2006; Bacher, 2006). Whether this transient interaction is a chromosomal cross-talk in relation to XCI and relate to counting is not known (Carrel, 2006). Recently, an X chromosome pairing region was found >100kb upstream of *Xist* (Augui, 2007).

Choice is the second step. It is hypothesized that inactivation is the default pathway and *XIC* on the future active X should be repressed somehow. *Xist* has the features of a housekeeping gene and silencing X chromosomes individually is a more complex program than silencing *Xist*-expressing chromosomes since there is only one active X in each diploid cell. Based on this view, *XIC* should receive repressory signals for the *Xist* gene on the future active X. This regulation can be done through DNA elements in the vicinity of *Xist* acting as enhancers or binding sites for trans-acting factors. The idea of a blocking factor that protects the active X from inactivation was proposed (Lyon 1972). The support for the existence of an autosomal *Xist* repressor comes from observations that triploid males with 69,XXY chromosomes can have two active Xs in contrast to Klinefelter males (Migeon, 2008).

Another important step in XCI is the spreading of inactivation by modifying chromatin bidirectionally. Spreading and silencing should be distinguished and achieved by different domains of *Xist* (Wutz, 2002). The specific binding of *Xist* depends on conserved tandem repeat sequences within its first exon (Beletskii, 2001). These sequences can form stem loop structures which are recognized by binding factors. Silencing occurs only at a critical window of development. This was suggested to depend on the different processing of Xist RNA during development (Ma, 2005).

The observation that inactivation spreading is better in X chromosomes than autosomes have raised the possibility that there are ``way stations`` that enhance spreading (Riggs, 1985; Lyon, 1998). Line 1 (L1) elements were suggested by Mary Lyon as candidates. Studies of X-autosome translocations supported the hypothesis showing the extend of silencing into the autosomes varies. The maximum spreading to autosome observed is 45 Mb from the translocation break point. The spreading can be either continuous or discontinuous so the ability of autosomal parts to maintain the inactive state can be different (Sharp, 2002).

Silencing of X requires its heterochromatinization. After *Xist* accumulation, transient enrichment of Polycomb-group (PcG) protein complex

around the future inactive X occurs. Than all histone modifications specific to active chromatin are lost. Hypoacetylation occurs, especially globally for H4. During spreading of *Xist*, histones H3 and H4 undergo lysine modificatons specific to inactive X. Lysine of H2B is ubiquitinated before H3 methylation takes place. The signatures specific to active X are acetylation of H3K9 and di-tri methylation of H3K4. The signatures specific to inactive X are methylation of H3K9 and di-methylation of H3K4. Variants of histone H2 accumulates and is also ubiquitinated. A PcG protein accumulates around future inactive X and have a methylase activity for H3K9 and H3K27 methylation. BRCA1 was shown to localize around inactive X. It has a ubiquitin ligase acitivity, so it is possible that it has a role in H2B ubiquitination (Heard, 2004).

The last event in the silencing is CpG island methylation, which is thought to play a role in the maintanance of XCI. Methylated CpGs bind many methyl binding proteins which bind transcriptional silencing complexes such as methyl CpG binding protein 2 (MeCP2) (Lyon, 2007; Plath, 2002).

1.2.8. Genes escaping XCI

All the genes on the inactive X chromosome are not subject to full inactivation. 15% of the genes escape inactivation based on expression patterns of 95% of X assayable genes in fibroblast systems (Carrel, 2005). These escapees are variable among species. With the exception of pseudoautosomal region, most genes expressed from the inactive X have less than 15% activity. It has been hypothesized that these sequences are skipped by the spreading of inactivation or failure of maintanance (Flippova, 2005 ; Lingenfelter, 1998).

1.2.9.Primary and secondary causes of skewed XCI

The choice of the active X is not dependent on parental origin of the chromosome or its gene content. Exceptions include marsupials and nonhuman placental tissues in which exclusively paternal XCI takes place which is called imprinted XCI. In normal females, approximately half of the cells inactivate their paternal X while the others choose it as active. When the ratio of the two cell populations deviates significantly from a 1:1 distribution, it is called skewed XCI. The causes of skewed XCI is classified depending on whether skewing occurs primary or secondary to the inactivation event. Any alteration in the germline *XIC* can hypothethetically cause bias in the initial choice of which X chromosome will be inactivated in the germline and these causes of skewing are called primary causes (Puck&Willard, 1998). Secondary causes include lethal X-linked mutations, X-autosome translocations, aging, twinning, or monoclonal expansion of cells. In the presence of a deleterious mutation on one of the X chromosomes, the cells in which this X remains active dies. In X-autosome translocations, the cells in which the translocated X is inactivated dies. Skewing was observed to be increased in twins raising several explanations. One of them is twining reduces the number of cells contributing to the embryo increasing the chance of skewing. Skewing can occur as a result of monoclonal expansion of cells with selection for cells that carry the mutant X chromosome (Belmont, 1996; Brown, 2000).

1.3.Self tolerance and Autoimmunity

1.3.1 Immune system

1.3.1.1 Clonal theory of Jerne and Burnett

According to Burnet and Jerne`s theory of clonal selection theory of antibody formation, in a certain point of development, clones of lymphocytes are produced in central lymphoid organs as a result of heritable changes such as a somatic mutation. Lymphocytes have receptors similar to antibodies in structure so antigens stimulate lymphocytes; the corresponding clones of lymphocytes are stabilized in secondary lymphocytic tissue and increase in number. There is a developmental process in the primary lymphoid tissue when any clones of lymphocytes which carry reactive sites corresponding to body determinants are eliminated (Jerne, 1955 ; Burnet, 1957). This theory is important not only for the clonal selection it proposes, but also for the discovery of immunological tolerance.

1.3.1.2 Self tolerance

When antigen binding to T cell receptors (TCR) and B cell receptor (BCR) occur, they turn on cell growth and survival pathways leading to clonal lymphocyte proliferation and immunity. These pathways include nuclear factor kappa B (NF-κB) activation; Myc activation and phosphoinositide 3 phosphate (PI3K) activation of AKT and Ras activation of ERK. The growth induced by antigens is blocked by several mechanisms conferring self-tolerance (Goodnow, 2008). These mechanisms are designated in Figure 1.5.

Mechanisms of self-tolerance can be listed as 1) Autoreactive cells can be deleted with apoptosis. 2) Autoreactive cells can gain a different receptor by receptor editing. 3) The cell can be intrinsically made functionally unresponsive,

which is termed anergy. 4) The cell is extrinsically shut off by limiting the supply of co-stimuli, cytokines etc. The cell can also be suppressed by regulatory T cells. Tolerance mechanisms that take place in generative lymphoid organs when thymocytes are still immature are called central tolerance. The tolerance mechanisms in peripheral lymphoid organs are called peripheral tolerance (Goodnow, 2005).

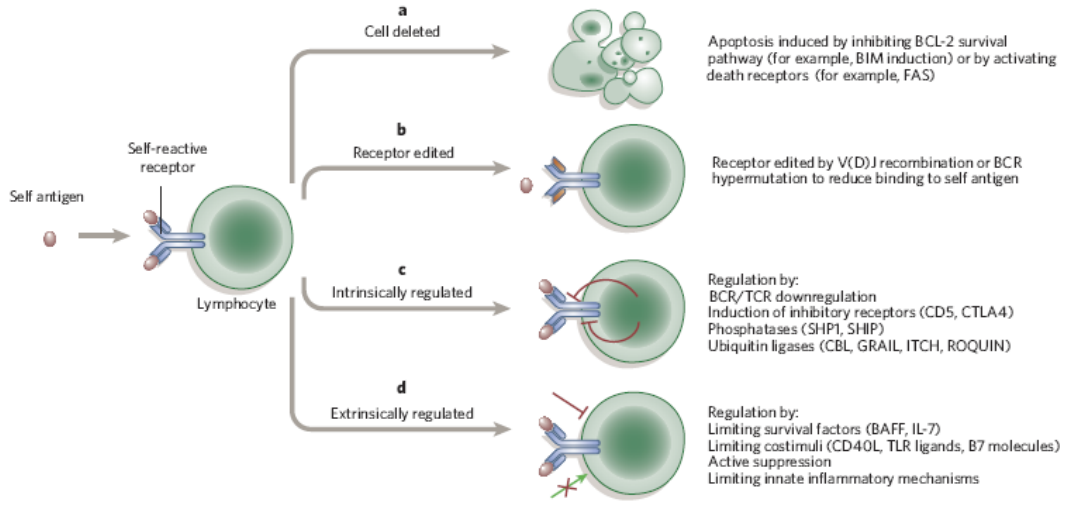

Figure 1.5 Different mechanisms of self tolerance a) T cell deletion through BIM induced or FAS activated apoptosis b) Receptor editing by V(D)J recombination or by BCR hypermutation c) Receptor downregulation, role of inhibitory receptors on T cells; role of phosphatases in increasing the threshold of B cell-activation and ubiquitin ligases to tag TCR-CD28 or cytokine receptors to interfere with signalling d) B cell require B cell survival factor (BAFF); self-reactive receptors on T cells require survival factor, IL-7. Costimulation with Toll like receptors (TLRs) for B cells; costimulation through CD28 for T cells; costimulation of B cells with CD40L on T cells are needed for activation (Goodnow *et al*, 2005).

1.3.2 Autoimmunity

Autoimmune diseases are a diverse group of diseases including more than 80 conditions with an estimated prevalence of 5% to 8% in the United States, effection 14-22 million people. The common feature of autoimmune diseases (AIDs) is that the patients have a defect which leads to the attack of the host`s immune system to self-tissues and organs. There are two subgroups of

autoimmune diseases, which are systemic and organ specific. Autoimmunity is thought to be a complex disease resulting from several genetic sequence variants and environmental triggers including infections and stochastic events. Familial clustering and twin concordance studies have shown that AIDs have a genetic basis. Cooccurence of AIDs in individuals and the occurence of different AIDs in families support the view that AIDs have common predisposing genetic elements and pathogenic pathways (Eaton, 2007; Fox, 2007).

1.3.2.1 Genes associated with autoimmunity

Studies with sib-pairs, isolated populations and comparing patients and controls have shown that there are genetic risk factors for autoimmune diseases (Feltkamp, 1999). In simple diseases, the disease state is determined by a single gene. Common diseases result from a combination of susceptibility alleles. Genome-wide associations, linkage studies and candidate gene based approaches were performed and several MHC and non-MHC genes were found to contribute to predisposition to autoimmunity (Maier, 2008; Welcome Trust Case Control Consortium & Autralo-Angolo-American Spondylitis Consortium, 2007). Mutations in a single gene can cause autoimmunity, but most autoimmune diseases are associated with several sequence variants. Below, there is brief information on some simple genetic traits associated with human autoimmunity (Rioux&Abbas 2005).

Autoimmune regulator (AIRE) was identified as a gene mutated in systemic autoimmune disease, autoimmune polyendocrine syndrome (APS-1). Patients with this syndrome show autoimmunity against endocrine organs, skin and other tissues. AIRE is a transcription factor and normally it is expressed at high levels in several central and peripheral organs and blood cells. Studies with knock out mouse showed that AIRE gene plays a role in the thymic expression of organ specific antigens. This supports a role for AIRE in central tolerance in the thymus.

Cytotoxic T lymphocyte antigen 4 (CTLA-4) is an inhibitory receptor on T cells, which binds to B7-1 and B7-2 on APCs to prevent their binding with CD28 on T cells. This prevents the costimulation necessary for activation of T cells and promotes anergy. CTLA4 knockout mouse develops features of systemic autoimmunity including enlargement of lymphoid organs and organ lympocytic infiltrates. CTLA4 polymorphism was associated with Graves disease, type 1 diabetes (T1D), autoimmune hypothyroidism and other endocrinopathies.

Forkhead box P 3 (FOXP3) encodes a transcription factor of the forkhead family and its expression commits naive T cells to become regulatory T cells that aid in tolerance induction. Mouse studies have shown FOXP3 mutations cause lack of regulatory T cells and lead to systemic autoimmune disease. FOXP3 is also associated with immune dysregulation, polyendocrinopathy, enteropathy and X-linked inheritance (IPEX) in humans. FOXP3 is located within Xp11.23. FOXP3 polymorphisms are associated with autoimmune tyroid disease susceptibility (Ban, 2007).

Protein tyrosine phosphatase, non-receptor type 22 (PTPN22) encodes a lymphoid tyrosine phosphatase which aids in T cell signalling and activation. A polymorphism in PTPN22 was identified in type 1 diabetis and also associated with rheumatoid arthritis and systemic lupus erythematosus.

1.3.2.1.1 X-linked genes associated with immune system

Observation of several immune related pathologies including immunodeficiences and immunoproliferative disease associated with the X chromosome is critical in understanding the role of X chromosome genes in the immune system. These diseases include XLP syndrome (X-linked lymphoproliferative syndrome), X-linked recessive combined immunodeficiency syndrome (SCID), Hyper-IgM syndrome type I (HIGM 1), Wiskott-Aldrich syndrome (WAS) which affect T cells and X-linked agammaglobulinemia (XLA) and X-linked recessive anhydortic ectodermal dysplasia with immunodeficiency (XEDA-ID) which effect B cells (Murchinick, 2006).

As above mentioned, FOXP3, implicated in immune dysregulation syndrome characterized by dysfunction of regulatory T cells, is X-linked. A number of non-MHC loci found to be associated with AID susceptibility also maps to X chromosome (Becker, 1998). Further, there are reports on X chromosome abnormalities implicated in autoimmune diseases (Chagnon, 2006).

1.3.2.2 Molecular Mimicry

It`s observed that there is an association of infectious agents with autoimmune diseases. One of the mechanisms proposed to explain the role of infectious agents in autoimmune etiology is molecular mimicry.

Molecular mimicry is the cross-reactivity of immune reagents with host self antigens and microbial determinants because of shared structures. It was first observed during monoclonal antibody generation that the phosphoprotein of measles virus and a protein of Herpes Simplex virus type 1 (HSV-1) cross-reacted with an human intermediate filament protein (Fujinami,1983). 5% of 800 monoclonal antibodies made against 15 viruses cross-reacted with host self determinants (Bahmanyar, 1987; Oldstone, 1987). By observing symptoms after injecting Hepatitis B virus (HBV) proteins that share sequence similarity with myelin basic protein which is known to cause allergic encephalomyelitis, in vivo

evidence of molecular mimicry was obtained. In this way molecular mimicry was proposed to have a role in autoimmune pathogenesis (Fujinami, 1985). Several diseases were implicated to have a molecular mimicry in their etiology (Oldstone, 1998). Molecular mimicry is demonstrated in animal models but has not been clearly shown in humans (Fairweather, 2004).

There are other mechanisms explaining the role of viruses in the loss of self-tolerance. One of them is epitope spreading, meaning autoimmune response is targeted to a secondary self-antigen after tissue-damage. Another one is the activation of bystander T cells in an antigen-non-specific manner. This enhances the effects of epitope spreading and molecular mimicry. As well as viruses, drugs are also implicated in causing autoimmunity with the drug metabolism forming a neo-self (Christen, 2004; Bach, 2005). Another proposed theory is that microorganisms expose self-antigens to immune system by damaging tissues (Fairweather, 2004).

1.3.2.3. Female predominance in autoimmunity

Most of the rheumatic diseases with autoimmune features exhibit higher female incidence. In Table 1.6, AIDs and corresponding female:male ratios are listed. In Figure 1.6, the incidences of selected of AIDs in females and males is designated. The reasons why AID occurs predominantly in women remains to be eluciaded.

Table 1.6 Ratio of female:male occurence of autoimmune disorders (Kast, 1977)

Disease	**Female:Male ratio**
Sjogren Syndrome	9:1
Autoimmune thyroiditis (Hashimato`s & Graves)	9:1
Primary biliary cirrhosis	9:1
Systemic lupus erythematosus	8:1
Chronic active hepatitis	4:1
Rheumatoid arthritis	3:1
Chronic idiopathic thrombocytopenic purpura	3:1
Polymyositis	3:1
Dermatomyositis	2:1
Polymyalgia rheumatica	2:1
Idiopathic Addison`s disease	2:1
Progressive systemic sclerosis (scleroderma)	2:1
Idiopathic acquired auto-immune hemolytic anemia	3 : 2
Myasthenia gravis	3 : 2
autoimmune gastritis (adult penicious anemia with achlorhydria)	3 : 2
Ulcerative colitis	3 : 2
Chron`s disease (granulomatus colitis)	lower female incidence 2 : 3
Goodpasture`s disease	lower female incidence 1 : 4
Preiarteritis nodosa (vasculitides)	lower female incidence 1 : 2
Wegener`s granulomatosis (vasculitides)	lower female incidence 2 : 3

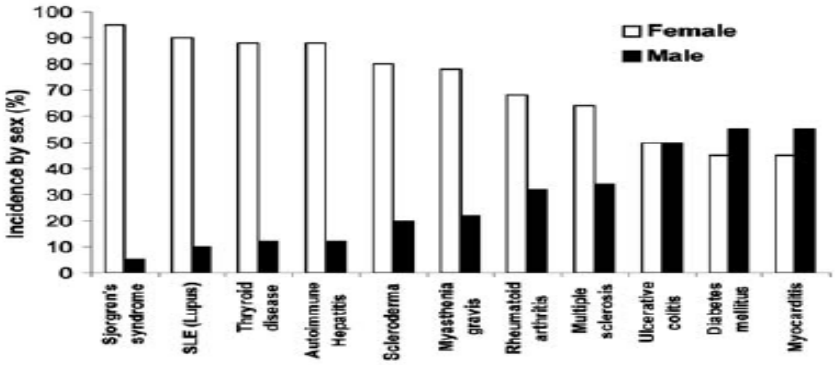

Figure 1.6 Incidence of autoimmune disorders by sex a) white bars show incidence in woman b) black bars show incidence in men (Fairweather *et al*, 2004)

1.3.1.3.1. Hormones

Hormonal differences between the two sexes have been proposed to contribute to the female predominance of autoimmunity. This was based on several observations : 1) Autoimmune diseases are more prevelant in women and one of the most distinguishing features of males and females is sex hormones. 2) Immune response is sexually dimorphic. Females show higher immune response after infection or vaccination than men. 3) Men who develop autoimmune diseases show more severe symptoms than women. In vitro experiments show that these hormones have a modulatory effect on the immune system. Androgenic hormones trigger T helper 1 response and estrogenic hormones stimulate T helper 2 response. Pregnancy is a T helper 2 dominant state and failure of this response was implicated in pregnancy loss. Disease status of autoimmune patients changes during pregnancy. Multiple sclerosis, rheumatoid arthritis patients are affected positively, in contrast systemic lupus erythematosus worsens. High estrogen states are observed in women with systemic lupus erythematosus and androgenization decreases the disease. In vivo and in vitro experiments give contradictory results on the effect of sex hormones. Sex steroids and immune

system seem to have a dialogue. There are hormone receptors on immune cells and cytokine receptors on hormone producing cells.

Some cytokines can stimulate the hypothalamus-pitiutary-adrenal axis to secrete homones. Some researchers belive the disease severity is observed as a consequence of varying exposure to hormones (Lockshin, 2002; Whitacre, 2001; Fairweather, 2004;Gleicher 2006).

1.3.1.3.2. Microchimerism

Microchimerism refers to presence of a small number of cells or DNA of an individual present in another individual and its possible causes are blood transfusions; passage of cells or DNA from the twin during embryogenesis or cell passage during pregnancy. Fetal microchimerism refers to fetal cell passage to mother during pregnancy or elective termination. Microchimerism was suggested to be critical in female predominance of autoimmunity: 1) It is an important immunological challenge being a period of semi-allogeneic graft transplantation similar to graft vs. host disease. 2) The incidence of autoimmune diseases are high in females and certain autoimmune diseases show increased incidence after pregnancy. The presence of fetal DNA in the blood, mononuclear cells and skin of scleroderma patients were investigated and the HLA relationship of the mother and the fetus was determined (Arlett, 1998; Nelson, 1998). There are variable reports, mostly favoring the presence of microchimerism in sclerosis. It was also shown that sclerosis patients without male pregnancies have microchimerism in their circulation (Lambert, 2007). Microchimerism in skin was studied for patients with polymorphic eruption and positive results were obtained. It was studied in liver biopsies of primary biliary cirrhosis (PBC) and thyroid biopsies of autoimmune thyroiditis patients and the results were variable. There are accumulated data on microchimerism in both patients with autoimmunity, people with non-immune conditions and controls. There is also data of microchimerism in both blood and other tissues. Maternal microchimerism refers to the presence of maternal cell in an individual. This kind of microchimerism was detected in myopathies (Arlett, 2000). It`s not known whether microchimerism is primary or

secondary to autoimmunity. It was hypothesized that microchimeric cells can become activated and proliferate in response to an environmental trigger and cause autoimmunity. When a mouse model was treated with a known scleroderma causing agent, vinyl chloride, increased levels of microchimeric cells were observed in blood. (Christner, 2000). Fetal cells were detected in the bone marrow and ribs of women with male pregnancies, but not in the controls (O`Donoghue, 2004). Another alternative hypothesis for the role of microchimeric cells is that they are beneficial aiding in tissue repair of the damaged organ. Microchimeric cells can aid in pathogenesis, repair, or they can be normal remnants of pregnancy (Gleicher, 2006).

1.3.1.3.3. X chromosome Monosomy

X chromosome monosomy is a form of aneuploidy with the presence of only one X chromosome in an individual`s karyotype and partial monosomy occurs when only a portion of cells have one X chromosome. Recent findings show that X chromosome abnormalities can have a role in autoimmunity. This is supported by the following observations. 1) Many genes involved in immune response are on the X chromosome. 2) Patients with X chromosome abnormalities such as Turner`s syndrome show autoimmune features. 3) Mutations in some X-linked genes cause immunodeficiency diseases and display autoimmune features (Invernizzi, 2008).

It was shown that patients with primary biliary cirrhosis, scleroderma and autoimmune tyroid disease have enhanced X monosomy rate in their peripheral blood mononuclear cells, especially in lymphocytes (Invernizzi, 2004; Invernizzi, 2005). Monosomy rates were not found to be related to disease severity, but to age. This high level of X monosomy in blood cells is not observed in systmic lupus erythematosus (Invernizzi, 2007). It was also shown that X chromosome loss was preferental in primary biliary cirrhosis (Invernizzi, 2007).

1.4 Skewed X inactivation and autoimmunity

1.4.1 Kast and Stewart hypothesis

Kast proposed an explanation for the high female incidence of autoimmune diseases based on the forbidden clone elimination stated in Burnet-Jerne`s theory. According to this theory, lymphocytes are negatively selected in the thymus, MHCs being the major survival determinants (Burnet 1959, Jerne, 1971). Some activity as survival determinants was also proposed for any antigen at a generative site (Kast, 1975). Kast proposed a mechanism other than classical patterns of inheritance, disturbance in XCI, may have a role in female predisposition to autoimmunity (Kast, 1977). Stewart developed this hypothesis in systemic lupus erythematosus by stating that differences between sexes in the self-antigen presentation profiles to the immune system due to XCI mosaicism can account for the female preponderance (Stewart, 1998). According to this theory, skewed XCI in the thymus can lead to the escape of autoreactive lymphocytes specific for self antigens expressed from one parental X and this can lead to autoimmunity (Kast, 1977; Stewart, 1998). This results because naive T cells can not be selected for alleles expressed from one X chromosome because there is XCI skewing in the dendritic cells that are localized on the path the naive lymphocyte follows during negative selection (Stewart, 1998). This hypothesis is illustrated in figure 1.7. High frequency of skewed XCI was reported in the blood of autoimmune patients: scleroderma, autoimmune tyroid disease, pre-eclampsia patients (Ozbalkan, 2005; Ozcelik, 2006, Yin, 2007; Uz, 2006). No significant correlation was found between skewed XCI and the occurence of primary biliary cirrhosis, systemic lupus erythematosus, type 1 diabetis and multiple sclerosis (Miozzo, 2007; Huang, 1997; Chitnis, 2000).

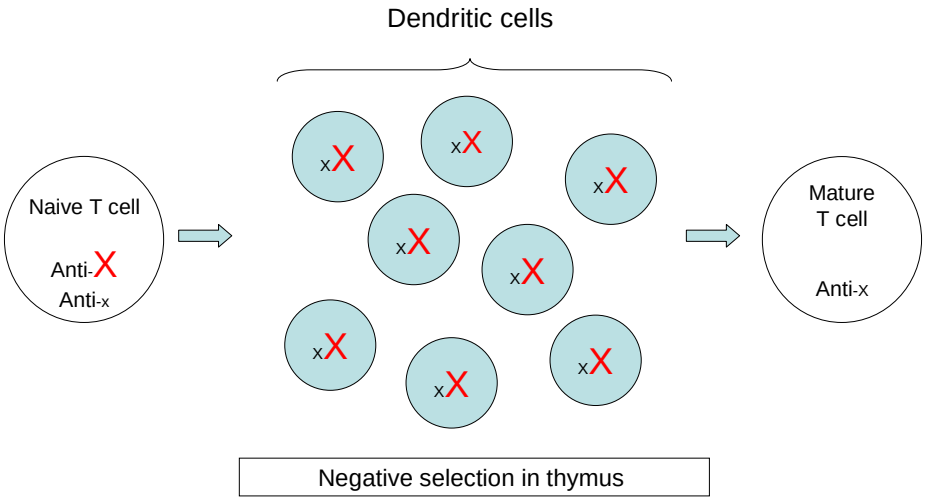

Figure 1.7 Consequences of skewed XCI of thymic cells. The thymus cells displaying skewed XCI are demonstrated. As a normal physiological event, during tolerance induction of naive T cells with dendritic cells exhibiting random XCI, naive T lymphoctyes are tolerized for genes expressed from both X chromosomes. However when skewing of XCI occurs in dendritic cells, naive T cells which are reactive to antigens from one X are deleted. However T cells reactive to antigens from the other X, which is exclusively or almost exclusively inactivated by the dendritic cells, exit the thymus nontolerized and autoreactive to self antigens.

1.5. Aim and Strategy

It was proposed that skewing of XCI in the hematopoietic cells may cause failure in representation of antigens of one X by the antigen presenting cells (APC) in the thymus and result in autoimmunity. This was hypothesized based on the higher female incidence of most of the autoimmune diseases and XCI being a major sex difference (Kast,1977; Stewart,1998). A positive correlation between skewed XCI and a number of autoimmune diseases is demonstrated (Ozbalkan, 2005; Ozcelik, 2006; Yin, 2007; Uz, 2006). Sjogren Syndrome is an autoimmune disease with unkown etiology and it shows one of the highest female prependorances. To test whether disturbances in XCI may play a role in the pathogenesis of Sjogren Syndrome, we genotyped a polymorphic repeat in the human androgen receptor *(AR)* gene to determine XCI profiles of Sjogren patients and controls. (Allen, 1992).

CHAPTER II: MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1 Samples

2.1.1 Control Samples

Caucasian females comprised of 160 Turkish healthy unrelated volunteers is the control group in this study. The mean age for Turkish controls at analysis was 46±10.0 (mean±SD). There was no clinical evidence or family history of autoimmune or inflammatory joint disease. The XCI profiles of this group was used to compare with those of Sjogren Syndrome patients (Ozbalkan, 2005; Ozcelik, 2006).

2.1.2 Sjogren Syndrome Patient Samples

Caucasian women (n=78) diagnosed with Sjogren Syndrome were referred to Bilkent University, Facutly of Science, Molecular Biology and Genetics Department (Ankara, Turkey) by Zeynep Özbalkan at the Ankara Numune Education and Research Hospital, Rheumatology Clinic (Ankara, Turkey). Their venous blood samples were transferred in EDTA collection tubes. Informed consent forms were obtained from all subjects. These samples were genotyped and their XCI patterns were determined.

2.2 Materials

2.2.1 Primers

The sequences of the primers used in polymerase chain reaction (PCR) are : primer 1, 5'- GTC CAA GAC CTA CCG AGG AG-3'; and primer 2, 5'-CCA GGA CCA GGT AGC CTG TG -3'. The primers were synthesized by IONTEK (Istanbul, Turkey). The region amplified by the primers is as designated in figure 2.1.

2.2.2 Enzymes

Thermus aquaticus (Taq) DNA polymerase was used in the PCR reactions. Restriction digestion enzymes, *RsaI* and metylation sensitive *Hpa*II were used in the digestion reactions. All enzymes were purchased from MBI Fermentas Inc. (Amh, NY, USA). The recognition sites of restriction digestion enzymes are as designated in figure 2.1.

CGAGATC**CCGG**GGAGCCAGCTTGCTGGGAGAGCGGGACGGT**CCGG**A
GCAAGCCCAGAGGCAGAGGAGGCGACAGAGGGAAAAAGGGCCGAGC
TAGCCGCTCCAGTGCT**GTAC**AGGAGCCGAAGGGACGCACCACGCCAG
CCCCAGC**CCGG**CTCCAGCGACAGCCAACGCCTCTTGCAGCGCGGCGG
CTTCGAAGCCGCCGC**CCG**

...

AACTCTTCTGAGCAAGAGAAGGGGAGGCGGGGTAAGGGAAGTAGGTG
GAAGATTCAGCCAAGCTCAAGGATGGAAGTGCAGTTAGGGCTGGGAA
GGGTCTACCCTCGGCCGCC**GTCCAAGACCTACCGAGGAG**CTTTCCAG
forward primer (RS6)
AATCTGTTCCAGAGCGTGCGCGAAGTGATCCAGAAC**CCGG**GCCCCAG
***HpaII* restriction site**
GCACCCAGAGGCCGCGAGCGCAGCACCTC**CCGG**CGCCAGTTTGCTGC
***HpaII* restriction site**
TGCTG**CAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGC**
CAG repeat
AGCAGCAGCAGCAGCAGCAGCAGCAGCAAGAGACTAGCCCCAGGCA
GCAGCAGCAGCAGCAGGGTGAGGATGGTTCTCCCCAAGCCCATCGTA
GAGGCCC**CACAGGCTACCTGGTCCTGG**ATGAGGAACAGCAACCTTC
reverse primer (RS7)

ACAGCCGCAGTCGGCCCTGGAGTGCCACCCCGAGAGAGGTTGCGTCC
CAGAGCCTGGAGCCGCCGTGGCCGCCAGCAAGGGGCTGCCGCAGC

...

GCTGTAGCCCCCTACGGCTACACTCGGCCCCCTCAGGGGCTGGCGGGC
CAGGAAAGCGACTTCACCGCACCTGATGTGTG**GTAC**CCTGGCGGCAT
GGTGAGCAGAGTGCCCTATCCCAGTCCCACTTGTGTCAAAAGCGAAAT
GGGCCCCTGGATGGATAGCTACT**CCGG**ACCTTACGGGGACATGCG

Figure 2.1 *AR* genotyping amplicon (287 bp). Sequence of Homo sapiens *AR* exon 1 on the X chromosome between 66680599-66860844 bp. is shown. Reference assembly is NC_000023.9 in NCBI and ENSE00001326500 in Ensembl. *HpaII* digestion sites and CAG repeat regions are designated (Allen, 1992).

2.2.3 Oligonucleotide

The length of DNA fragments were estimated by comparing to known molecular weight standards that had been run on the same gel. pUC Mix Marker 8 (#SM0303) (0.1µg/µl) and Massruler DNA Ladder (#SM0403) Mix were used as markers and purchased from MBI Fermentas (Amh, NY, USA) The sizes of the fragments are given in figure 2.2.

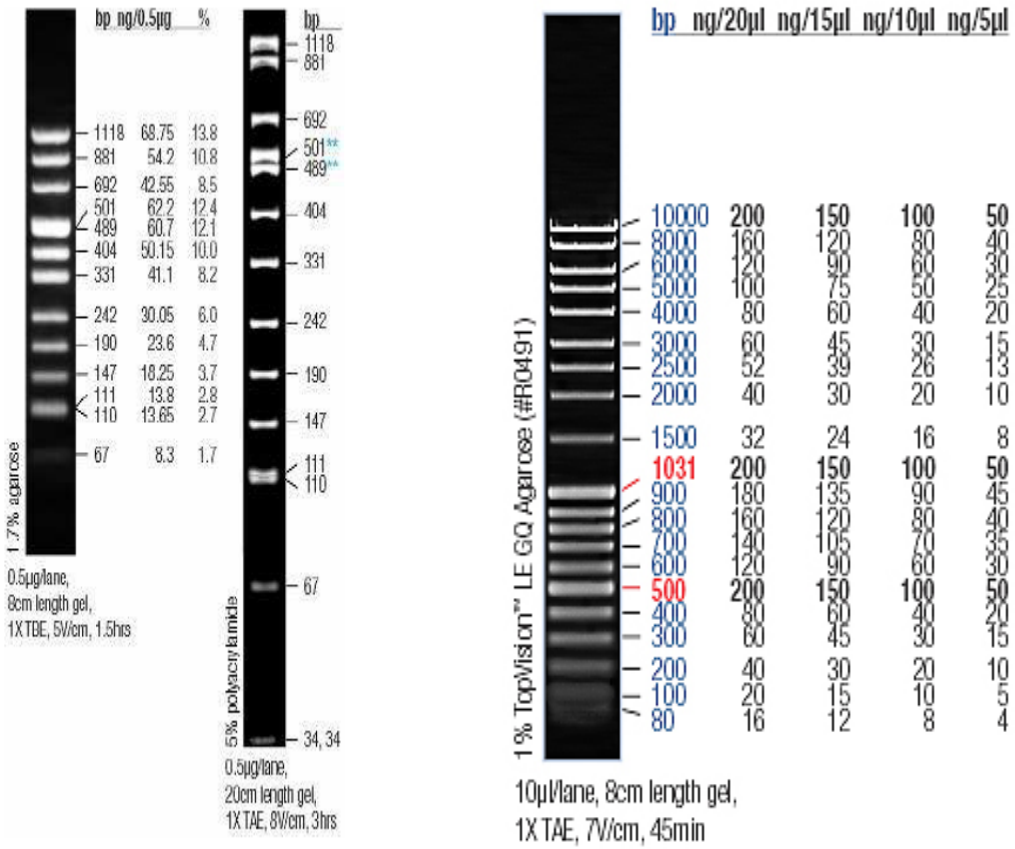

Figure 2.2 Sizes of the fragments of pUC Mix Marker 8 and MassRuler DNA Ladder Mix and appearance on gel electrophoresis.

2.2.4.Chemicals, Reagents, Kits

The chemicals and reagents used in this project and the suppliers are listed in table 2.1.

Table 2.1 List of chemicals and reagents used in this study

Experiment	Reagent or Chemical	Company
DNA Isolation	Nucleospin® DNA Isolation Kit (Buffers B3, BE, BW, B5)	Macherey-Nagel Inc., PA,USA
	Proteinase K	Appligene-Oncor, USA
	100% Ethanol (EtOH)	Merck, Frankfurt, Germany
Agarose Gel Preparation	Agarose	Basica LE, EU
Polyacrylamide Gel Preparation	Bisacrylamide	Sigma, St. Louis, MO
	Acrylamide	Sigma, St. Louis, MO
	APS	Carlo Erba, Milano, Italy
	TEMED	Carlo Erba, Milano, Italy
Gel Electrophoresis	6X MassRuler™ DNA Loading Dye	MBI Fermentas, Amherst, NY, USA
	Ethidium Bromide (EtBr)	Sigma, St. Louis, MO, USA
	EDTA (pH 8.0)	Carlo Erba, Milano, Italy
	Tris	Merck, USA
	100% glacial acetic acid	Sigma, USA
Restriction Digestion	Tango Buffer (10X)	MBI Fermentas, Amherst, NY, USA
PCR	dNTP (10 mM)	MBI Fermentas, Amherst, NY, USA
	MgCl2 (25 mM)	MBI Fermentas, Amherst, NY, USA
	Buffer (10X)	MBI Fermentas, Amherst, NY, USA

2.2.5 Standard solutions and buffers

50X TAE (Tris-acetic acid-EDTA): 242gr Tris, 57.10 ml acetic acid, 37.2 gr EDTA. The volume was adjusted to 100 ml by adding ddH_2O and pH is adjusted to 8.0

1X TAE (Tris-acetic acid-EDTA): 40mM Tris-acetate, 2 nM EDTA, pH 8.0.

Acrylamide:Biacrylamide Stock Solution (30%): 29.5 gr acrylamide, 0.44 gr bisacrylamide. The volume was adjusted to 100 ml by adding ddH_2O.

APS solution (10%): Prepared freshly with 0.1gr ammonium persulfate in 1mL ddH_2O.

Ethidium bromide: 10mg/ml in water (stock solution) 30 ng/ml (working solution)

6X MassRuler™ DNA Loading Dye : 10mM Tris-HCl (pH 7.6) 0.03% bromophenol blue, 0.03% xylene cyanol FF, 60% glycerol 60mM EDTA.

pUC Mix 8 Marker : 1 volume of pUC Mix 8 Marker, 1 volume of 6X MassRuler™ DNA Loading Dye and 4 volumes of ddH_2O.

2.3 Methods

2.3.1 Sample collection

Venous blood samples obtained from Sjogren Syndrome patients and controls were transported in EDTA tubes. Their entry to the lab was recorded. They were seperated as 1ml aliquots to eppendorf tubes (1.5 ml). The bloods (1-3 eppendorfs) were stored at -80°C. 200 μl blood was seperated for DNA isolation and stored at -20°C.

2.3.2 DNA Isolation from venous blood

DNA from venous blood was isolated by using Nucleospin™ Blood kit (Macherey-Nagel Inc., PA, USA) (#740 951.250) according to the protocol of the manufacturer. The starting blood sample was 200 μl and the resulting material was 100 μl elution buffer containing DNA. The efficiency of this kit is typically 40-60ng/µl genomic DNA with an A260/280 value of 1.6-1.9.

Blood samples preequilibrated to room temperature were lysed with proteinase K and lysis buffer vortexing and heating at 70 °C. Adjusting of DNA binding was performed with ethanol (100%). DNA was bound to silica membrane which was then washed with buffers. With centrifugation residual ethanol was removed. Using preheated 70 °C elution buffer, DNA was eluted.
The concentrations and purity values of DNA were measured with spectrophotometric reading using NanoDrop™ ND-1000 UV-Vis Spectrophotometer.

In the process, 2 µl of the sample was used for measurement. 2 µl elution buffer was used as blank. The integrity of the DNA samples were controlled with 1% agarose gel electrophoresis.

2.3.3 *AR* genotyping

To determine the XCI status of individuals, androgen receptor *(AR)* genotyping was performed. (Figure 2.3) *AR* locus spans a region larger than 90kb and it has been mapped to Xcen-Xq13. Its cDNA has an open reading frame of 2,751 nucleotides and its protein has 917 amino acids. In its first exon there is an inframe CAG repeat that codes for 11-31 glycine residues.

A 280 bp region (nucleotides 229-508) of the *AR* is amplified. (Figure 2.3) This region includes a highly polymorphic trinucleotide repeat (20 alleles, 90% heterozygosity) and *HpaII* and *HhaI* sites less than 100 bp away from this short tandem repeat (STR). The proximity of STR to enzyme target sites gives an opportunity to distinguish between maternal and paternal alleles and to determine their XCI status.

HpaII and *HhaI* are methylation sensitive enzymes which have target sites with CpG. Cytosine residues in CpG islands, which are clustered at the 5` of genes are methylated in the inactive X. *AR* genotyping relates methylation of *HpaII* and *HhaI* sites in the *AR* locus with XCI. (All enzyme sites are consistently methylated on the inactive X, but one or more enzyme sites are unmethylated on the active X.) In *AR* genotyping, DNA is first digested with *HpaII* and/or *HhaI* and than a region including the enzymes site and the repeat is amplified. The enzyme target sites on the active X chromosome, which have unmethylated cytosines will be cleaved and no amplicon will be observed for the active X. Since they are methylated, the sites on the inactive X will not be cleaved, so an amplicon will be observed for the inactive X. The X inactivation patterns can be determined in females who are informative at their CAG repeat (Allen, 1992).

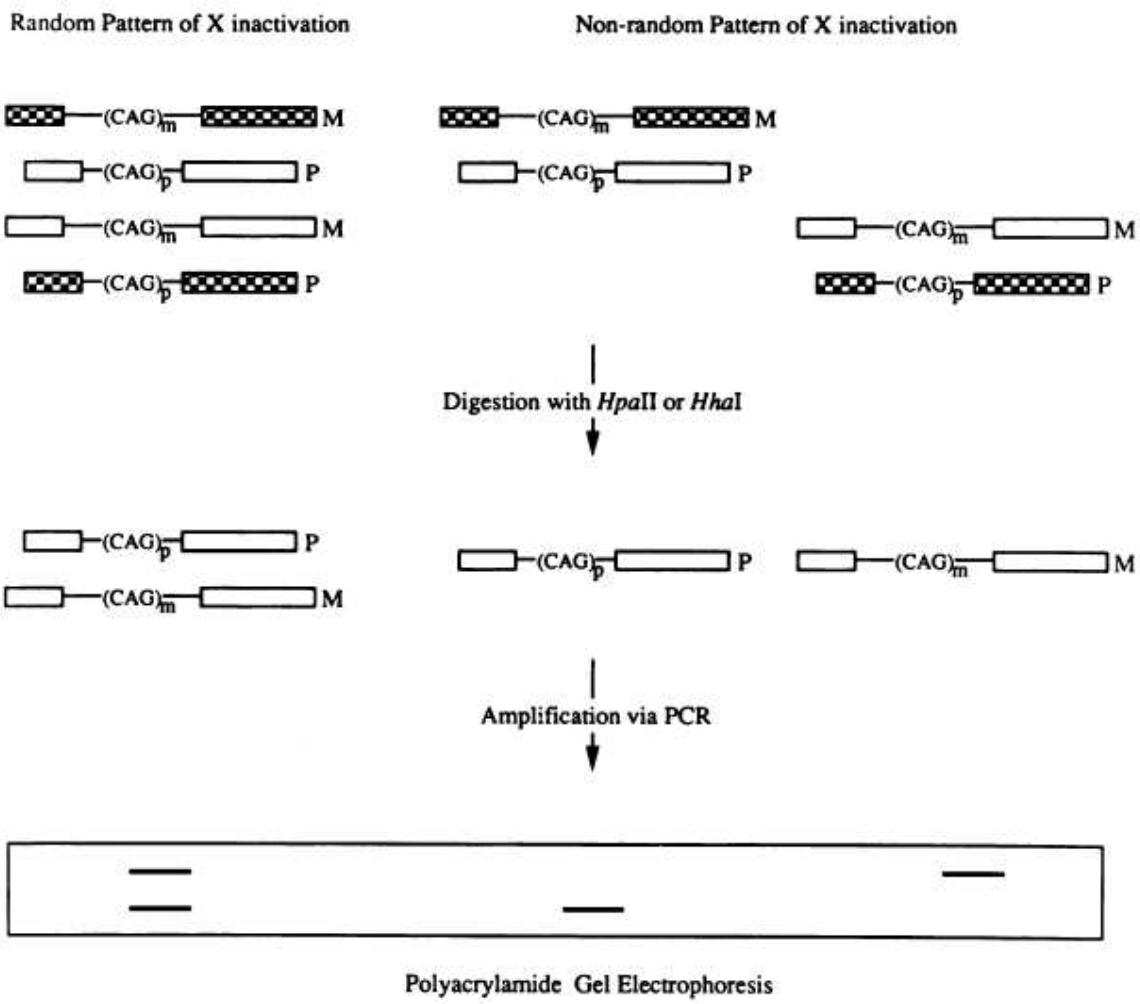

Figure 2.3 Establishing random and skewed XCI with *AR* genotyping

2.3.3.1 Restriction Enzyme Digestion

For each sample, two restriction enzyme digestion mixtures were prepared, each of which have a total reaction volume of 20 μl. The amount of DNA was adjusted to 150-250 ng in the digestion mixture. 2 µl of Y tango buffer with BSA (#BY5) (33mM Tris-acetat (pH 7.9), 10mM magnesium acetate, 66 mM ptoasium acetate, 0.1 mg/mL BSA) (10X) was added to adjust the final buffer in the mix to 1X. 0.2 µl *RsaI* (#ER1122) (10U/µl) (5`-GT↓AC-3`) was included in both mixes to adjust its concentration to 2U in the final mix. *RsaI* has target sites outside the PCR product and it makes the DNA more accessible. 0.8 µl *HpaII* (#ER0512) (10U/µl) (5`-C↓CGG-3`), which is methylation sensitive, was included in the second mix to adjust its concentration to 8U in the final mix. The reactions were incubated at 37°C in the incubator overnight. Heat inactivation of the reactions was performed at 70°C for 10-15 minutes in the heater. Restriction digestion products were stored at 4°C for further use. First digestion is used for *AR* genotyping, the second digestion was used to assess XCI pattern. Negative controls were performed without DNA and the positive controls were performed with male DNA.

2.3.3.2 Polymerase chain reaction (PCR)

The digest amount was adjusted to 50ng-100ng in the PCR mix. The final concentrations in the PCR mix were 10mM for MgCl2 (25 mM), 0.12 for dNTP (10mM), 10pmol for the primers (10pmol/µl), 1U for the Taq polymerase (#EP0402) (5U/ µl), 1X for the buffer (10X) (750mM Tris-HCl (pH 8.8 at 25^{o}C),200 mM $(NH_4)_2SO_4$, 0.1% (v/v) Tween 20). In order to amplify the desired region, 94^{o}C 5 min initial denaturation step was performed. PCR reactions were performed in Techne™ Techgene thermal cycler. This was followed by the amplification step including 30 cycles of 94^{o}C 30 sec denaturation, 58^{o}C 30 sec annealing, 72^{o}C 30sec extension. Final extension was performed at 72^{o}C for 5 min. Amplicons were controlled with 1.5% gel electrophoresis.

2.3.3.3 Visualization

2.3.3.3.1 Agarose gel electrophoresis

1% agarose gel electrophoresis was performed to check whether total human genomic DNA was isolated. 2 μl of MassRuler DNA Ladder mix (#SM0403), 2 μl DNA with 1 μl 6X MassRuler™ DNA Loading Dye (#R0611) (103 ng/µl) was loaded.

To visualize the PCR products for the determination of the success, 1.5% agarose gel electrophoresis was performed. 4 µl of pUC Mix Marker (#SM0301) (0.5 µg/µl) and 5 μl of sample with 6X MassRuler™ DNA Loading Dye (5:1 ratio) were loaded.

The gels were prepared by dissolving required amount of agaose in 1X TAE. Required amount of EtBr (10mg/mL) was added. The gel was run in 1X TAE for the required amount and time. UV transilluminator was used during visualization and photography.

2.3.3.3.2 Polyacrylamide gel electrophoresis (PAGE)

The maternal and paternal alleles were resolved using 8% PAGE (polyacrylamide gel electrophoresis). 8% PAGE gel solution for pouring two gels (60mL) was prepared by adding the reagents in the following order: in order to prepare 16 ml acryamide: bisacrylamide solution (30%), 6 ml 10X TAE, 500 µl 10% APS, 40 µl TEMED and 37,5 ml ddH2O were included.

The solution solidifies for 1 hour and the gels are attached to the vertical setting. PCR product with the loading dye is loaded. pUC Mix Marker is used as a marker. The gels were run at 20 Watt per gel in 1X TAE buffer for 2 hours. They were stained in buffer containing EtBr for 5 minutes, washed in ddH_2O for another 5 minutes.

2.3.3.4 Densitometric and Statistical Analysis

Multi-Analyst software version 1.1 (Bio-Rad, Hercules, CA) was used for the densitometric analysis. Densitometric analyses included normalization of the ratios based on the undigested samples. Normalization was performed by dividing the allele ratio of the digested sample by the allele ratio of the undigested sample. This calculation is performed as a correction for the preferential amplification of the shorter allele. The results of the Sjogren Syndrome and control group were compared by χ^2 test with Yates` correction and Fisher`s exact test. In addition, odds ratio value was calculated in the 95% confidence interval. Power analysis was performed to estimate the sample size that is needed to make accurate statistical judgements. The minimum effect size was determined based on the results of XCI status studies of our group for other autoimmune diseases (Ozbalkan, 2005; Ozcelik, 2006 ; Uz, 2006). The significance level was set as $\alpha=0.05$ and the power was set as $\beta=0.1$.

CHAPTERIII: RESULTS

Androgen receptor assay was performed for XCI status determination of Sjogren Syndrome patients and healthy controls. Methylated inactive X chromosome is resistant to digestion by methylation specific *HpaII* enzyme, while unmethylated X chromosome is prone to digestion. There are highly polymorphic triplet repeats adjacent to *HpaII* methylation sites in the androgen receptor gene, which provide a method to distinguish the two alleles and their methylation status.

Figure 3.1 demonstrates the gels of uninformative, random and skewed XCI patterns. Uncut (U) refers to DNA amplified prior to *HpaII* digestion, genotyping for the polymorphic repeat. Cut (C) refers to DNA amplified after *HpaII* digestion, giving information on the XCI status. The individuals, who are not polymorphic in the CAG repeat within the amplified region of *AR* gene were uninformative and not included in the densitometric and statistical analysis. For informative samples, densitometric analysis was performed as indicated in the methods section. 04-474 and 04-476 are examples of informative samples, 04-473 demonstrating random and 04-476 demonstrating skewed XCI.

Skewed XCI refers to a concentration difference of more than 80% between the two alleles and extremely skewed XCI refers to a concentration difference of more than 90%. (Naumova *et al*., 1996).

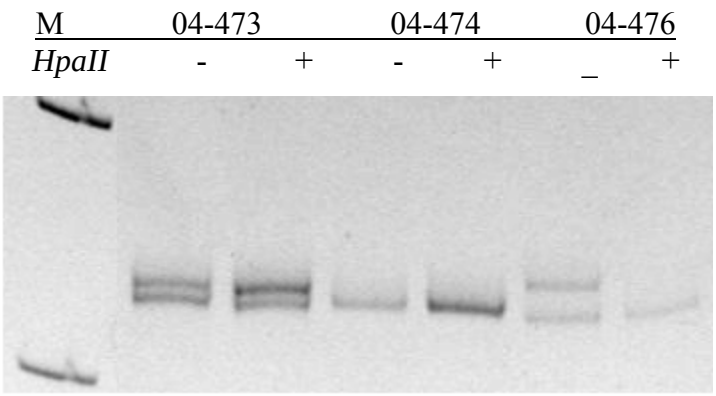

Figure 3.1 XCI inactivation status in Sjogren Syndrome patients. Polymerase chain reaction products from the androgen receptor methylation assay demonstrate random X chromosome inactivaton pattern in sample 04-473 (allele ratio 64% : 36%) and skewed pattern in sample 04-476 (100% : 0%). Sample 04-474 is not informative for the androgen receptor polymorphism. For each sample, DNA was either undigested (-) or digested (+) with the methylation-sensitive restriction enzyme *HpaII*. Marker (M; pUC mix 8[331-bp and 242-bp])

XCI patterns of 78 Sjogren Syndrome patients were studied and 51 of 78 patients were informative (65.4%). The control group comprised of females with no autoimmune condition. XCI status of 160 controls were studied and 124 were informative (77.5%) Extreme skewing (>90:10) was observed 3 out of 51 informative patients (5.9%) and in 3 of 124 (2.4%) informative controls. Skewing (>80:20) was observed in 10 of (8.1%) 124 informative controls and in 8 of 51 informative patients (15.6%). Statistical verification of the significance of correlation was assesed with the odds ratio and P value. The probability value was calculated with Fisher`s exact test. The odds ratio and P-value for extreme skewing is P=0.3592 and OR=2.5208 with 95% CI: 0.4915-12.9289. The odds ratio and P-value for skewing is P=0.17 OR=2.1209 with 95% CI: 0.7852-5.7289. These results favor that there is not a statistically significant correlation between skewed XCI and Sjogren Syndrome.

Power analysis was performed to estimate the sample size that is needed to enable accurate statistical judgements. The estimated sample size needed to test a difference of 0.18 of extreme skewing between patient and control samples with a power of 0.9 at a significance level of 0.05 is 47 Sjogren Syndrome and 113

control samples. The estimated sample size needed to test a difference of 0.27 of skewing between patient and control samples with a power of 0.9 at a significiance level of 0.05 is 36 Sjogren Syndrome and 87 control samples. The analysis shows that the sample size used for this experiment is adequate to make accurate statistical judgements.

Table 3.1 Proportion of Sjogren Syndrome patients and controls with skewed XCI

Degree of skewing (%)	No. (%) observed with skewing	
	Sjogren Syndrome patients (n=51)	Controls (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 χ^2, $P = 0.2$ (>80% skewing); $P = 0.4$ (>90% skewing)

CHAPTER IV: DISCUSSION

Female predominance is a characteristic of most autoimmune diseases. (Whitacre, 2001). In recent decades, candidate mechanisms that could be important in autoimmune disease pathogenesis have been studied. Currently there are explanations for the female predominance in autoimmune diseases. These include genetic traits associated with autoimmunity (Rioux and Abbas, 2005) and pregnancy related microchimerism (Arlett, 1998; Nelson, 1999). Another sex specific feature of females is XCI and it is known that skewed XCI can influence manifestations of X-linked disorders in females (Lyon, 2002). The hypothesis that skewed XCI in the thymus can cause defects in negative selection and lead to autoimmunity was proposed. (Kast, 1977; Stewart, 1998)

Thymocytes are negatively selected in the thymic parencyma and the selecting elements appear to be derived from the bone marrow and are dendritic cells. If the thymic dendritic cell population exhibits skewed XCI, T cells specific for certain X antigens will not be deleted. This hypothesis assumes that the initial breakdown in tolerance involves an X-linked autoantigen expressed in peripheral site of the body. In a recent report, lack of expression of one splice variant of proteolipid protein (PLP) protein in the thymus was shown to be related to susceptibility to autoimmunity (Klein, 2000). This illustrates a role of defective thymic negative selection in autoimmunity. Since proteolipid protein is X-linked, it also accounts for the hypothesis stated above.

Loss of mosaicism based on X chromosome skewing was shown to correlate with autoimmunity in several AIDs (Ozbalkan, 2005; Ozcelik, 2006; Uz, 2006; Yin, 2007). These reports supported that factors associated with loss of mosaicism based on X chromosome skewing could account for a significant proportion of these autoimmune diseases.

Currently, the relationship between skewed XCI and AIDs is not known. Skewing can be either secondary to breakdown of self-tolerance or it can be a causative factor in the breakdown of self-tolerance. We currently believe the second view is more promising since skewing ratios of patients are not mild.

XCI skewing can result either from primary or secondary reasons. Whether skewing of XCI patterns are transmitted to offspring in humans was researched on the hypothesis that an X-linked locus can alter initial random inactivation. No supporting evidence was obtained (Minks, 2008; Bolduc, 2008). The secondary reasons of XCI include X-linked lethal mutations, X-autosome translocations, aging, twining or monoclonal expansion (Brown, 1999). We believe that deleterious X-linked mutations can account for skewing leading to autoimmunity. This view is supported by the observation that recurrent spontaneous abortions are increased in AIDs and skewed XCI was associated with recurrent spontaneous abortions (Bagislar, 2006; Shelton, 1994). If the cause of skewing is indeed a X-linked deleterious mutations as hypothesized, this should be inherited from the mother unless they occur de novo in gamategenesis. An X-linked lethal mutation causes skewing in the mother and it is lethal for a male infant. This kind of skewing was reported in X-linked immunodeficiency where males have a survival disadvantage and heterozygous females have XCI skewing (Puck&Willard, 1998). We believe deleterious X-linked mutations and their differential expression patterns could lead to skewing, conferring a disadvantage on blood cells (Uz, 2007 ; Ozcelik, 2008).

Although extremely skewed XCI is rare in normal population, it does not always lead to the development of disease. To provide an estimate of the frequency of individuals in the general population, XCI status of 1,005 unaffected individuals was determined. The percentage of population with skewed XCI was found to be 8.8% and with extremely skewed XCI was found to be 1.8% (Amos-Landgraf, 2006). There are also other proposed factors for autoimmune etiology other than correlation with skewing. A subsequent event such as environmental exposure to viral, chemical and other agents may trigger autoimmunity. The co-inheritance of genetic susceptibility factors for example functional variants of molecules involved in immune tolerance may exacerbate the effects of skewed XCI leading to autoimmunity.

Sjogren Syndrome is an autoimmune disorder with one of the highest female prependorance and unknown etiology. It has been associated with genetic factors such as HLA-DR/HLA-DQ, cytokine and other gene polymorphisms (Hulkkonen, 2001; Gottenberg, 2004; Pertovaara, 2004; Youn, 2000; Tsutsumi, 2001, Wang, 2001, Bolstad, 2000, Petrek, 2002). Environmental factors, especially viral infections have been reported as causative factors (Fleck, 1998; Haddack, 1992; Kordossis, 1998;Tereda, 1994;Giffiths, 1997). There are also controversial reports on the role of microchimerism in Sjogren Syndrome (Toda, 2000; Kuroki, 2002; Endo, 2001). In this research, a potential correlation between skewed XCI and Sjogren Syndrome was investigated. Patients showed skewing patterns comparable to that of controls in their blood cells. Our results did not support a correlation between Sjogren Syndrome and skewed XCI based on the P value and odds ratio calculated. Our sample size was adequate to make a true statistical verification based on hypothesis testing.

Although the role of X mosaicism in breakdown of self tolerance caught attention, X inactivation patterns comparable to those of control women were observed in the blood from females with primary biliary cirrhosis, multiple sclerosis, juvenile diabetes and systemic lupus erythematosus (Miozzo, 2007; Knudsen, 2007; Chitnis, 2000) These results display that other reasons than XCI may have role in the pathogenesis of these diseases. For example, molecular mimicry is known to have a role in the initiation of the primary biliary cirrhosis (Kaplan, 2005). Based on our results, we can conclude other factors can trigger Sjogren Syndrome, other than skewed XCI based mosaicism.

The tissues of an organism are accepted to reflect the same XCI pattern since inactivation process takes place very early during development. However there can be exceptions to this. It has been showed that although there is a significant association of the X inactivation ratios between each tissue in younger individuals, there are significant variations in elder individuals (Sharp, 2000; Busque, 1996). It was observed that there can be variation in XCI patterns among tissues (Rosemary, 1994). These results emphasize the role of secondary selection events leading to skewing. We can suspect that the patterns of skewing

in blood cells does not demonstrate the skewing pattern of dendritic cells in the thymus. However this is unlikely because it was observed tissues of mesodermal origin do not display differences in X inactivation patterns (Chitnis, 2000). Another consideration is that tissue microenvironments may exhibit skewed XCI because of a small number of founder cells even though the tissue as a whole is not skewed. If this is the case, this can not be reported by working with blood cells. Our group studied buccal mucosa, hair and effected tissues of SSc and AITDs. We can further confirm our findings by studying other tissues such as thymic tissue sections. (Chitnis, 2000).

In a recent study, the X chromosome skewing status of multiple sclerosis patients were assessed and compared in two subgroups of multiple sclerosis. They concluded there was a significantly higher degree of skewing in progressive multiple sclerosis than relapse remitting subgroup. (Knudsen, 2007) Our data can further be used to extrapolate the differences in the degree of X chromosome skewing in primary and secondary Sjogren Syndrome patients.

X inactivation assay we used in this research is *AR* genotyping. There are various assays to determine X inactivation pattern based on a marker. Assays based on variant proteins or common exonic polymorphisms to assess expression are direct assays. *AR* genotyping is an indirect assay which utilizes a DNA repeat polymorphism and is based on the presumption that CpG island which is the target of *HpaII* is always methylated in the inactive X. These kinds of DNA assays are highly informative because the polymorphisms in the nontranscribed regions of the genome can be used. They are also more rapid and suitable for research in which establishing large informative number is more important than determining slight changes in X inactivation status. *AR* locus has a heterozygosity of >90% and a high degree of accuracy with a variance on repeated assays is around 4%. (Chen, 2007; Ozcelik, 2007; Migeon, 2007)

CHAPTER V: FUTURE PERSPECTIVES

In this research, we tested the hypothesis that disturbances in XCI may have a role in the etiology of Sjogren Syndrome. However our results does not support a correlation between skewed XCI and Sjogren Syndrome. The results of our group for two other autoimmune diseases, scleroderma and autoimmune thyroid disease favor a role of XCI in the loss of tolerance. The view of our group is that X-linked mutations can have a role in skewed XCI based on secondary selection events. A research strategy of our group was to conduct a high-density microarray analysis including 1618 nonsynonymous, 1091 synonymous and 2802 intronic SNPs on X chromosome and 166 SNPs on autosomal chromosomes that are known to be associated with autoimmune diseases. Exonic SNPs corresponded to 783 genes and intronic ones corresponded to 160 genes. Experimental part included 465 AIDs and 2 Sjogren Syndrome patient samples with extremely skewed XCI was included in this study. This study was designed to raise putative genes involved in AIDs by determining allelic frequencies and copy number variations.

CHAPTER VI: REFERENCES

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CHAPTER VII. APPENDICIES

Appendix A The list of Sjogren Syndrome patients and their XCI pattern

Number		Sample	XCI Pattern
90+	5.9%		
1	1	04-476	100
2	2	07-1057	95
3	3	07-1054	90
80-89	9.9%		
4	1	05-543	86
5	2	07-1059	85
6	3	04-472	84
7	4	04-479	80
8	5	07-1041	80
70-79	27.5%		
9	1	05-279	76
10	2	05-545, 05-571	78,7
11	3	07-1062	75
12	4	07-1064	75
13	5	07-1066	75
14	6	07-1068	75
15	7	05-008	74
16	8	04-488	73
17	9	05-567, 07-1046	73, 75
18	10	05-005	71
19	11	05-007	71
20	12	04-491	70
21	13	07-1043	70
22	14	07-1063	70
60-69	17.7%		
23	1	04-477	68
24	2	04-480	66
25	3	07-1051	65
26	4	04-473	64
27	5	04-487	63
28	6	05-544, 05-565	60, 59
29	7	05-282	60
30	8	07-1048	60
31	9	07-1061	60
50-59	39.2%		
32	1	05-276	59
33	2	04-492	58
34	3	04-485	57
35	4	05-563	57

36	5	05-564	57
37	6	04-489	55
38	7	05-540	55
39	8	05-561	55
40	9	07-1045	55
41	10	07-1056	55
42	11	05-546, 05-566	56, 53
43	12	05-547	54
44	13	05-570	54
45	14	07-1050	53
46	15	05-281	53
47	16	05-001	52
48	17	05-004	51
49	18	06-219	50
50	19	07-1060	50
51	20	07-1055	
NI	34.6%		
52	1	04-482, 05-569	NI,NI
53	2	04-490, 05-568	NI, NI
54	3	04-474	NI
55	4	04-475	NI
56	5	04-478	NI
57	6	04-481	NI
58	7	04-483	NI
59	8	04-484	NI
60	9	04-486	NI
61	10	04-493	NI
62	11	05-002	NI
63	12	05-003, 05-277,05-539, 05-572	NI
64	13	05-278	NI
65	14	05-280	NI
66	15	05-541, 05-573	NI
67	16	05-542	NI
68	17	05-562	NI
69	18	05-574	NI
70	19	07-1040	NI
71	20	07-1042	NI
72	21	07-1044	NI
73	22	07-1047	NI
74	23	07-1049	NI
75	24	07-1065	NI
76	25	07-1053	NI
77	26	07-1058	NI
78	27	07-1069	NI

Appendix B The list of healthy controls and their XCI pattern

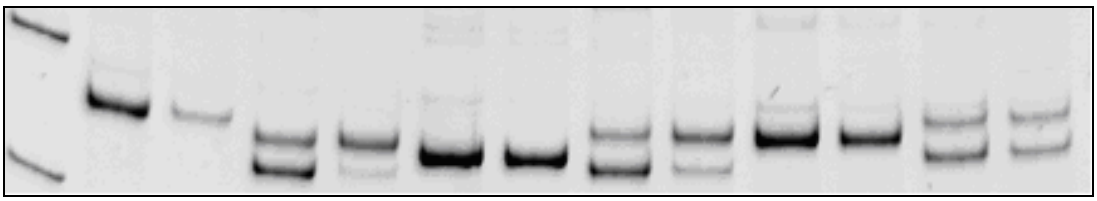
Number		Sample	XCI Pattern
90+	2.4%		
1	1	03-733	92
2	2	03-715	90
3	3	03-745	90
80-89	5.6%		
4	1	03-698	85
5F	2	03-772	85
6	3	03-771	84
7	4	7462	83
8	5	03-777	82
9	6	03-723	80
10	7	5713	80
70-79	15.8%		
11	1	03-856	77
12	2	03-861	77
13	3	NK-036	77
14	4	03-826	76
15	5	03-727	75
16	6	03-840	75
17	7	03-858	74
18	8	03-711	73
19	9	03-726	73
20	10	03-738	73
21	11	03-847	73
22	12	NK-001	73
23	13	03-846	72
24	14	GK-11	72
25	15	GK-19	71
26	16	03-708	70
27	17	03-841	70
28	18	03-850	70
29	19	03-712	70
30	20	03-839	70
31	21	03-852	70
32	22	03-862	70
60-69	31.6%		
33	1	7977	68
34	2	03-833	67
35	3	GK-01	67
36	4	NK-053	67
37	5	2670	66
38	6	7688	66
39	7	GK-22	66
40	8	NK-018	66

41	9	03-701	65
42	10	GK-02	65
43	11	03-836	64
44	12	1828	64
45	13	1883	63
46	14	2718	63
47	15	NK-003	63
48	16	NK-052	63
49	17	1717	62
50	18	03-363	62
51	19	03-366	62
52	20	03-709	62
53	21	03-746	62
54	22	03-778	62
55	23	03-864	62
56	24	03-731	61
57	25	03-813	61
58	26	03-703	60
59	27	03-720	60
60	28	03-728	60
61	29	03-760	60
50-59	52.6%		
62	1	03-821	59
63	2	GK-23	59
64	3	GK-??	59
65	4	03-713	59
66	5	4396	59
67	6	GK-05	59
68	7	NK-015	59
69	8	03-364	58
70	9	03-706	58
71	10	03-717	58
72	11	03-729	58
73	12	03-747	58
74	13	03-768	58
75	14	03-855	58
76	15	7127	58
77	16	03-775	57
78	17	03-822	57
79	18	GK-16	57
80	19	NK-012	57
81	20	1944	56
82	21	03-365	56
83	22	03-705	56
84	23	03-718	56
85	24	03-737	56
86	25	03-740	56
87	26	03-761	56
88	27	03-773	56
89	28	03-780	56

90	29	03-816	56
91	30	03-860	56
92	31	4610	56
93	32	6096	56
94	33	GK-08	56
95	34	NK-039	56
96	35	03-700	55
97	36	03-708	55
98	37	03-735	55
99	38	03-769	55
100	39	03-830	55
101	40	2290	54
102	41	03-722	54
103	42	03-744	54
104	43	03-817	54
105	44	NK-040	54
106	45	7264	53
107	46	03-730	53
108	47	03-734	53
109	48	03-762	53
110	49	03-814	53
111	50	03-832	53
112	51	8268	53
113	52	GK-24	53
114	53	03-704	52
115	54	03-714	52
116	55	03-725	52
117	56	03-732	52
118	57	03-741	52
119	58	03-776	52
120	59	03-823	52
121	60	03-767	51
122	61	03-820	51
123	62	03-702	50
124	63	03-	50
NI	22.5%		
125	1	03-699	NI
126	2	03-707	NI
127	3	03-710	NI
128	4	03-719	NI
129	5	03-721	NI
130	6	03-724	NI
131	7	03-743	NI
132	8	03-763	NI
133	9	03-764	NI
134	10	03-765	NI
135	11	03-766	NI
136	12	03-770	NI
137	13	03-774	NI
138	14	03-779	NI

139	15	03-781	NI
140	16	03-782	NI
141	17	03-783	NI
142	18	03-815	NI
143	19	03-818	NI
144	20	03-819	NI
145	21	03-824	NI
146	22	03-825	NI
147	23	03-827	NI
148	24	03-828	NI
149	25	03-835	NI
150	26	03-837	NI
151	27	03-838	NI
152	28	03-842	NI
153	29	03-843	NI
154	30	03-844	NI
155	31	03-845	NI
156	32	03-848	NI
157	33	03-849	NI
158	34	03-851	NI
159	35	03-853	NI
160	36	03-854	NI

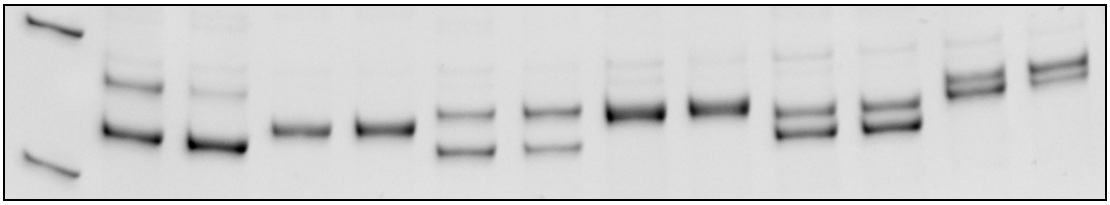
Appendix C The PAGE figures of XCI patterns of Sjogren Syndrome patients



M U C U C U C U C U C U C

07-1040 07-1041 07-1042 07-1043 07-1044 07-1045

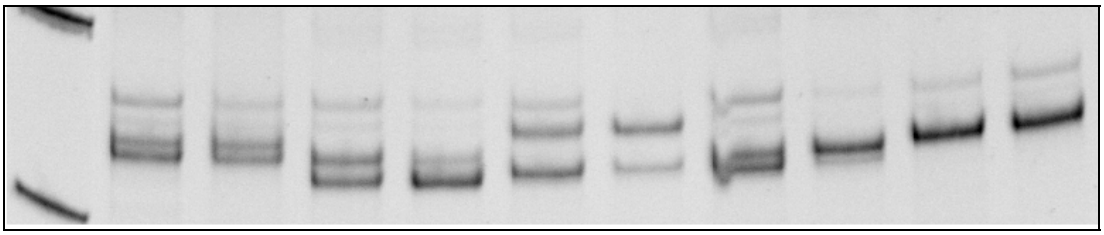
NI 80:20 NI 70:30 NI 55:45



M U C U C U C U C U C U C

07-1046 07-1047 07-1048 07-1049 07-1050 07-1051

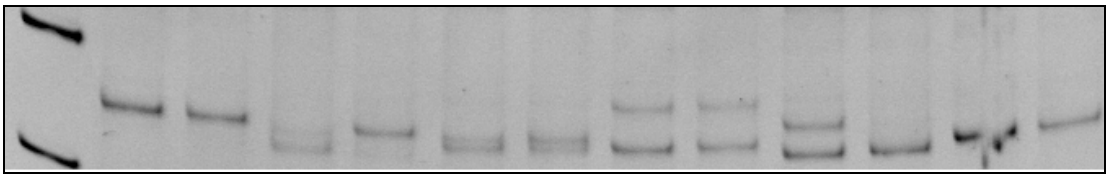
75:25 NI 60:40 NI 53:47 65:35



M U C U C U C U C U C

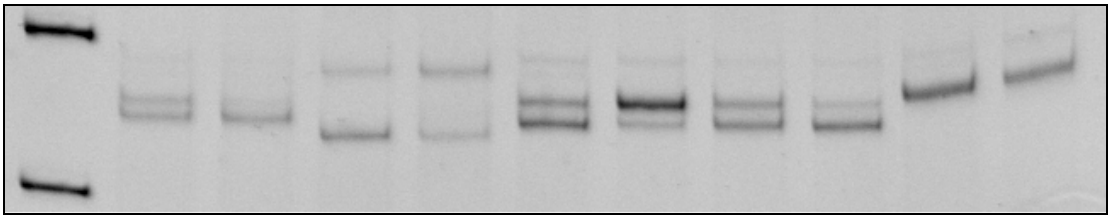
07-1060 07-1062 07-1063 07-1064 07-1065

50:50 75:25 70:30 75:25 NI



M U C U C U C U C U C U C U C

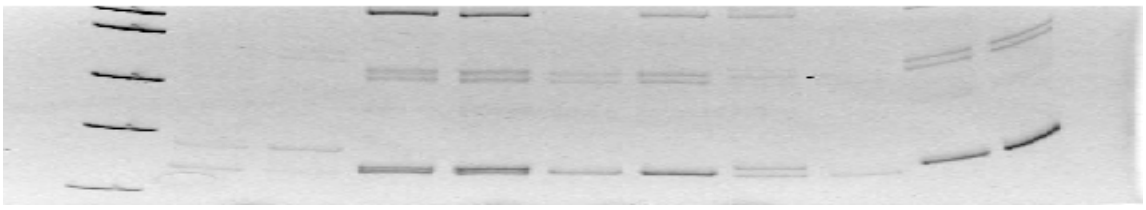
NI 90:10 50:50 55:45 95:5 NI



M U C U C U C U C U C U C

07-1059 07-1061 07-1066 07-1068 07-1069

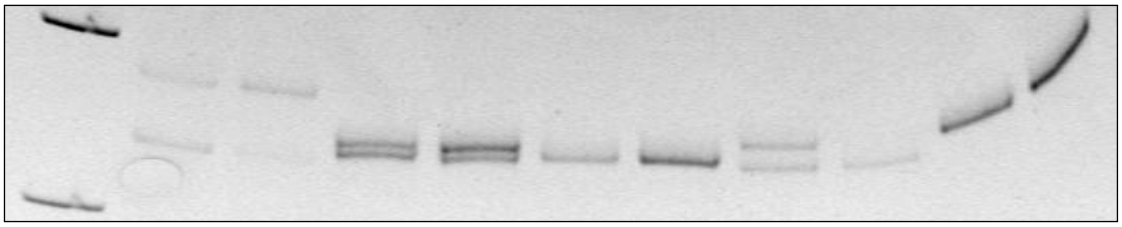
85:15 60:40 75:25 75:25 NI



M U C U C U C U C U C U C

04-472 04-473 04-474 04-475 NI

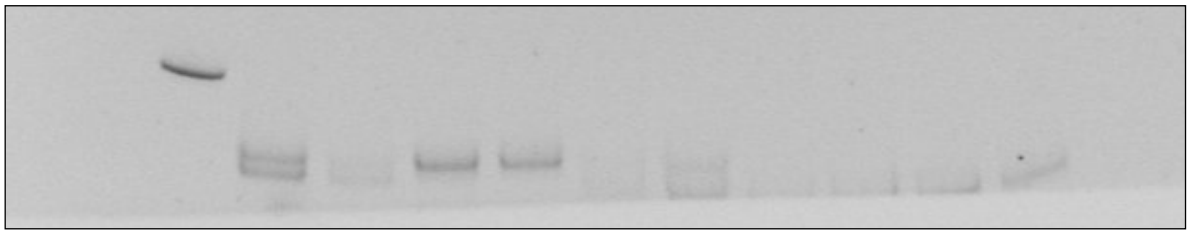
84:16 64:36 NI 100:0 NI



M U C U C U C U C U C

04-472 04-473 04-474 04-476 04-478

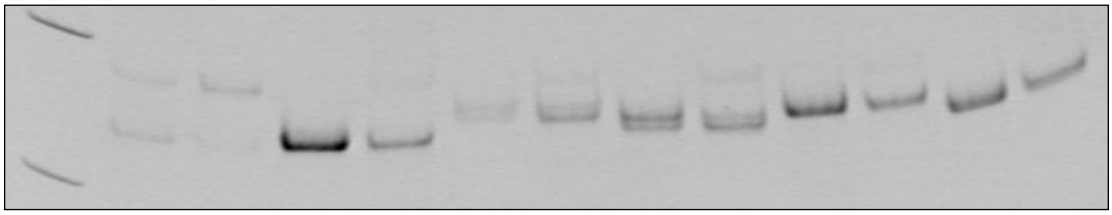
84:16 64:36 NI 100:0 NI



M U C U C U C U C U C

04- 479 04-483 04-487 04-488 04-489

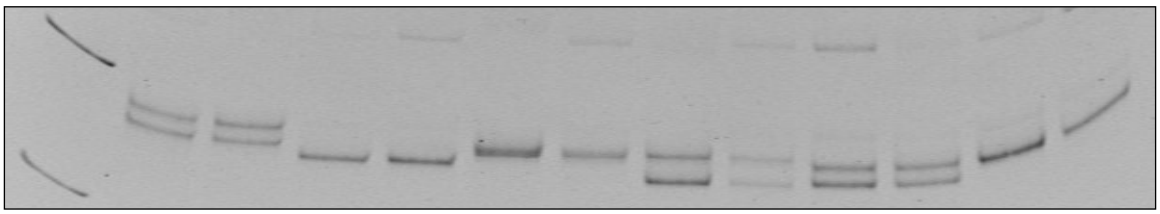
80:20 NI



M U C U C U C U C U C U C

04-472 04-475 04-477 04-480 04-481 04-484

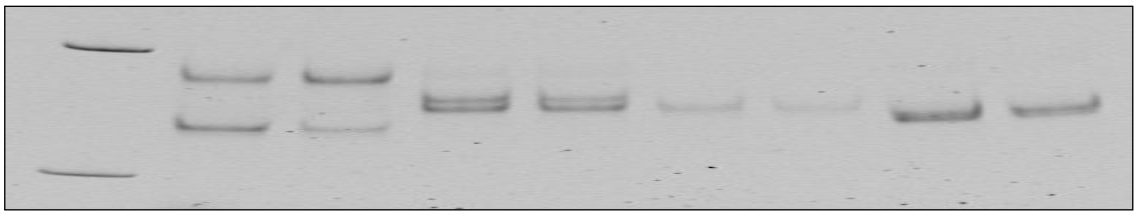
84:16 NI 68:32 66:34 NI NI



M U C U C U C U C U C U C

04-485 04-486 04-490 04-491 04-492 04-493

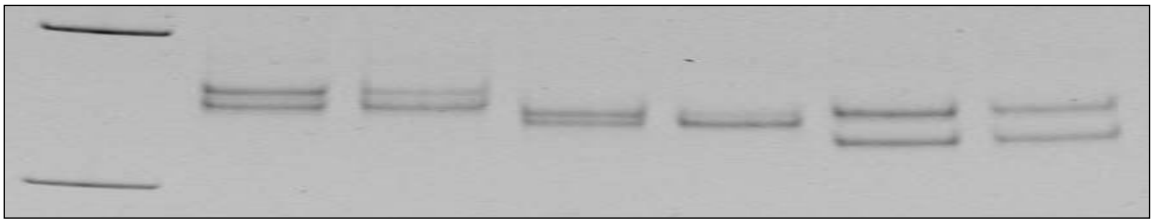
57:43 NI NI 70:30 58:42 NI



M U C U C U C U C

04-472 04-479 04-482 04-484

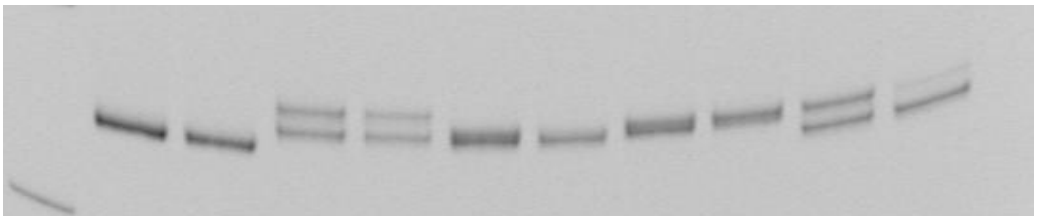
84:16 80:20 NI NI



M U C U C U C

04-487 04-488 04-489

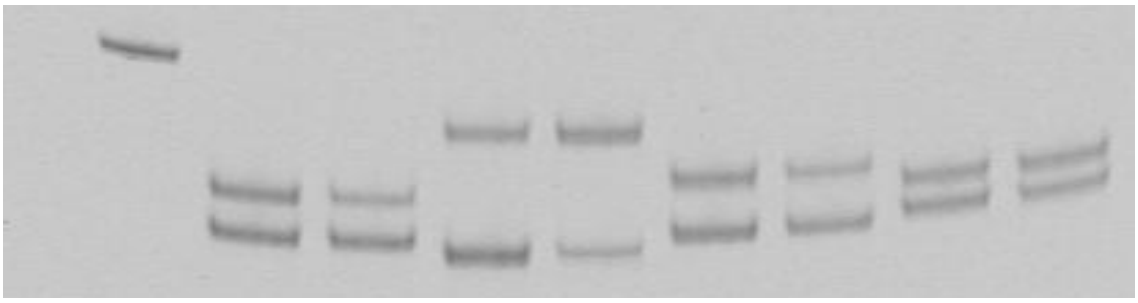
63:37 73:27 55:45



M U C U C U C U C U C

05-539 05-540 05-541 05-542 05-543

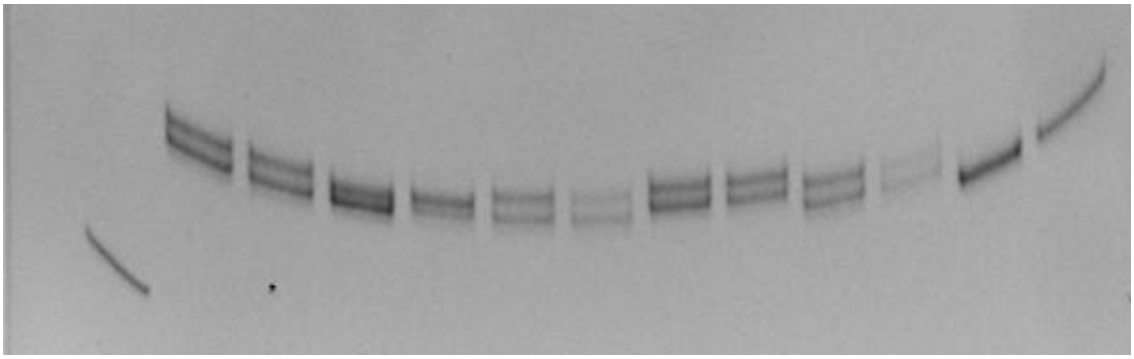
NI 55:45 NI NI 86:14



M U C U C U C U C

05-544 05-545 05-546 05-547

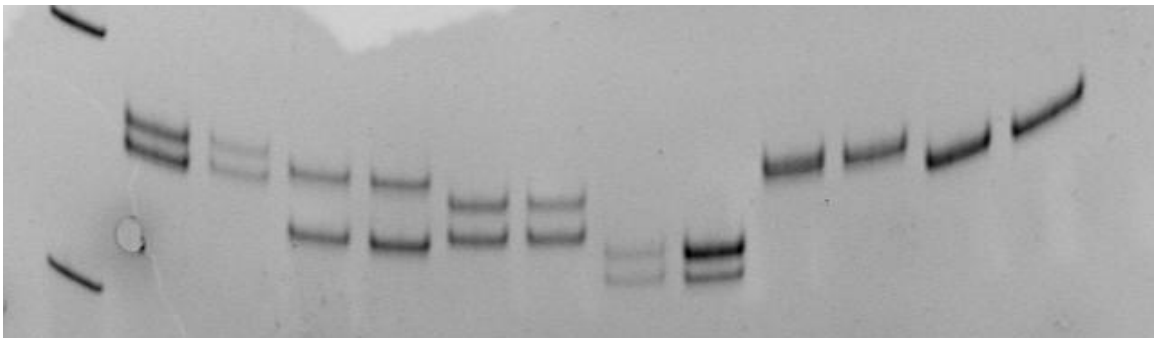
60:40 78:22 56:44 54:46



M U C U C U C U C U C U C

05-4 05-5 05-276 05-281 05-561 05-562

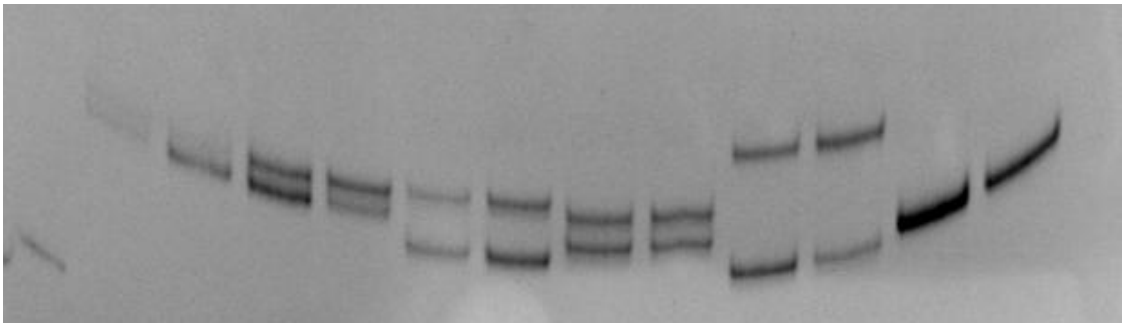
51:49 71:29 59:41 53:47 55:45 NI



M U C U C U C U C U C U C

05-563 05-564 05-565 05-567 05-568 05-569

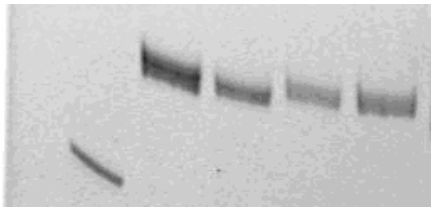
57:43 57:43 59:41 73:27 NI NI



M U C U C U C U C U C U C U C

05-7 05-8 05-566 05-570 05-571 05-572

71:29 74:26 53:47 54:46 70:30 NI



M C U C

05-573 05-574