CTLA4 gene polymorphism and autoimmunity

By

Haneen Alakhras

Advisors

Dr . Tamer Essawi

Prof. Dr. Jan Phillipe

2014

CTLA4 gene polymorphism and autoimmunity

By

Haneen Alakhras

Advisors

Dr . Tamer Essawi

Prof. Dr. Jan Phillipe

This thesis was submitted in partial fulfillment of the requirements of the master's degree in clinical laboratory sciences from the faculty of graduate studies at Birzeit University, Palestine.

November / 2014

CTLA4 gene polymorphism and autoimmunity

By

Haneen Alakhras

This thesis was successfully defended and approved on Thursday 27\11\2014.

Examination committee	**Signature**
Tamer Essawi, PhD (Advisor)	_____
Jan Phillipe, PhD (Advisor)	_____
Wael Hammouda, M.D. (External examiner)	_____
Mohhamad Farraj, PhD (Internal examiner)	_____

Dedication

I would like to dedicate this work to my father Radwan Al-Akhras, my mother Rawheya, my brother Mohammad and my sisters.

Acknowledgements

I would like to acknowledge my advisors Dr. Tamer Essawi, the Dean of the Faculty of Nursing Pharmacy and Health professions at Birzeit University, and Professor Dr. Jan Philipe from Gent University in Belgium. They provided me with guidance, support and all I needed to accomplish this project.

I would like also to thank Dr. Mohammad Farraj for his support throughout this work. Israr Sabri was a big help, I would like to thank him for his efforts.

HRA

Table of contents

List of Tables

List of Figures

Abstract

Autoimmunity is the process where auto-reactive T- lymphocytes or auto-antibodies produced by B-cells react against self antigens. The prevalence of autoimmune diseases has been estimated to be 3-5% which makes it one of the major health concerns.

A total of 120 samples were collected from Patients with different autoimmune diseases from hospitals and clinics throughout the West Bank and 82 from normal controls. The aims of this study were focusing on the presence of two point mutations on the CTLA4 gene; CT60 and AG49, and to associate between their presence and susceptibility to autoimmune diseases in the Palestinian population living in the West Bank, Palestine. Both mutations were tested using RFLP-PCR on all cases and control samples.

There was a significant difference in the AG49 point mutation between the controls (29.6%) and patients (60%) for the G allele ($P<0.05$). Homozygous A allele was clearly much higher in controls (70.4%) as compared to patients (40%). For point mutation in the CT60 allele, there was no significant association between the sample tested and autoimmune diseases ($P>0.05$). Results reflect that the G allele doesn't play a role in susceptibility to autoimmune diseases.

الملخص

المناعة الذاتية هي عملية يحدث خلالها مهاجمة خلايا الجسم من خلاياه الليمفاوية T او الاجسام المضادة المصنعة داخل الجسم.

ويقدر انتشار أمراض المناعة الذاتية أن تكون 3-5 ٪ . التي تجعل منها واحدة من المخاوف الصحية الرئيسية.

في هذه الدراسة نحن نركز على اثنين من الطفرات من الجين CTLA4، CT60 و AG49 ، لنربط بين وجودها و التعرض للأمراض المناعة الذاتية في فلسطين تحديدا منطقة الضفة الغربية.

تم اختبار كل من الطفرات باستخدام RFLP -PCR على المرضى من مختلف أمراض المناعة الذاتية وعينات من اشخاص سليمين للمقارنة .

جمعنا 120 عينة من المرضى الذين يعانون من أمراض المناعة الذاتية المختلفة من المستشفيات والعيادات في أنحاء الضفة الغربية و 82 عينة من اشخاص سليمين .

كان هناك اختلاف كبير في طفرة AG49 بين عينات الاشخاص السليمين (29.6 ٪) والمرضى (60٪) للاليل G (P< 0.05) .A متماثل كان اعلى بشكل واضح فى عينات الاشخاص السليمين (70.4 ٪) بالمقارنة مع المرضى (40٪) .بخصوص طفرة في أليل CT60 ، لم يكن هناك ارتباط كبير بين العينة المختبرة و أمراض المناعة الذاتية (P> 0.05) .

وتعكس النتائج أن أليل G لا يلعب دورا في التعرض ل أمراض المناعة الذاتية .

Chapter I

Introduction

The concept of self vs. non-self discrimination was known since 1949 (1). Autoimmunity or breakdown of self tolerance can be defined as imbalance between immunity and self tolerance where T-helper cell function excessively increased and T-cell suppression become deficient. In other words, autoimmunity is the process where auto-reactive T- lymphocytes or auto-antibodies produced by B-cells react against self antigens.

It may be part of the physiological immune response (natural autoimmunity) or pathologically induced, which may lead to development of clinical abnormalities (autoimmune diseases).(2)

Many different autoimmune diseases can occur due to various factors such as genetic predisposition, viral infections, environmental factors, stress, aging, hormones and pregnancy. All autoimmune diseases are characterized by the excessive immune response against self-antigens, leading to chronic inflammation, tissue destruction, and/or dysfunction. Recent evidence revealed the presence of more than 60 diseases that are strongly suspected to be of autoimmune origin. (2)

Autoimmune diseases are usually more common in women than men. The prevalence of all autoimmune diseases combined is high. The prevalence in the general population has been estimated to be 3-5% emphasizing their importance in public health. Due to difficulties in diagnosing autoimmune diseases, designing and standardizing epidemiological studies, limited data are available, and the prevalence may actually be underestimated.(3)

Etiology of Autoimmune Diseases

Autoimmune diseases usually occur in families indicating a genetic susceptibility. Two thirds of the affected patients are women which may indicate some additional environmental, stress or hormones. Following are the major causes that may individually or combined act in the development of autoimmune diseases:

Intrinsic factors (genetics, hormones, gender, and race)

- ## Genetics

Autoimmune diseases are good examples of multigenic diseases. These genes induce the transcription of proteins involved in key pathogenetic pathways, including apoptosis and clearance of apoptotic material or immune complexes, function of innate and adaptive immunity, production of cytokines, chemokines, or adhesion molecules (4)

The genetic involvement in autoimmune disease has been investigated. Such investigations have reported that identical twins for example will suffer from the same disease. Furthermore, emphasis was put on relatives of autoimmune patients are at higher risk of developing the same autoimmune disease. It was also observed the predominance of certain autoimmune diseases among certain races or ethnic groups (3).

The genetics of autoimmune diseases does not follow Mendelian genetics and allele segregation as seen in autosomal recessive genetic disorders or the presence of single gene mutation as seen in cystic fibrosis. The inherited genes involved in autoimmune diseases do not individually lead to the development of the disease but in combination may predict the susceptibility of carriers to these diseases (3).

Gender

Studies showed that autoimmune diseases affect females more than males, 65%of autoimmune diagnosed patients are females (5). Theories were suggested to explain female susceptibility; one theory indicates that women are genetically predisposed to abnormal autoimmune function, because the X chromosome may confer susceptibility towards tolerance breakdown.

Another theory suggests that microchimerism (transfer of cells between mother and fetus during pregnancy, twin-to-twin transfer in utero, from organ transplantation, and blood transfusion). may play an important role in the immunologic tolerance of the fetal semi-allograft, female preponderance may be understood as a consequence of increased allogeneic cell traffic in females . In view of increased exposure to cell traffic, women would be expected to pay a higher price, reflected in more autoimmunity (6)

- **Hormones**

Research has focused on the relation between sex hormones and autoimmune diseases. Studies reported that estrogens promote antibody production-enhancing Th2 response that increases the risk towards abnormal autoimmune function (6). This may explain that Sex hormones influence the normal differentiation, maturation, and migration of lymphocytes.(7).

- **Race**

Some ethnic groups may be at higher risk for some autoimmune diseases and lower risk for others (5). Studies conducted in the United States showed that autoimmune disease are more common among certain ethnic groups as seen that African Americans are at higher risk than Caucasians for developing systemic lupus erythematosus and scleroderma. However, the same study showed that African Americans are at lower risk for type 1 diabetes and multiple sclerosis.

Studies on genetic variations and various susceptibilities in ethnic groups to certain autoimmune diseases have focused on genetic differences that may contribute to variations in disease risk, including genes affecting immune response and metabolism(3)

- **Age**

Several studies have reported increasing rates of some autoimmune diseases such as Type 1 diabetes mellitus over periods ranging from 10 to 40 years, However, rheumatoid arthritis appears to have declined rates during this period.(5)

Environmental factors (infections, diet, drugs, environmental chemicals) may influence induction, development, and progression of autoimmune diseases.(2)

Environmental exposure may lead to epigenetic regulation via biochemical modifications such as acetylation, methylation or other mechanism (4).

Figure 1 shows the interaction of genes and environmental factors that can lead to autoimmune pathogenesis. The role of the environmental factors in autoimmunity is summarized in Table 2.

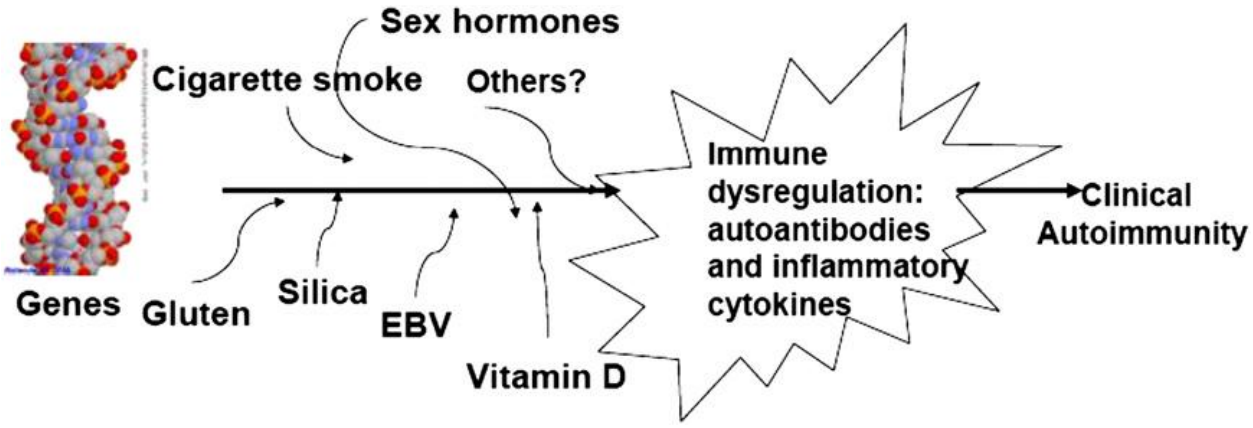

Fig. 1. Interaction of genes and the environment in the pathogenesis of autoimmune diseases (4)

It has been reported that Infectious agents may play a role in inducing autoimmunity by a mechanism called "molecular mimicry". This is mainly due to the presence of amino acid sequences on these agents that may resemble self antigens. In addition, infectious agents have the ability to induce inflammation and release of tissue antigens that can be recognized by the immune system as foreign (8).

Classification of autoimmune diseases

Autoimmune diseases are classified according to the following criteria: (9)

- **Systemic:** such as systemic lupus erythematosus, or SLE which is mediated by the production of autoantibodies which are not tissue specific.
- **Organ-specific:** such as Graves disease which is mediated by the production of antibodies against a specific organ or tissue antigens.

Involvement of immune system in autoimmune diseases mechanisms

The main function of the immune system is distinction between self and non-self antigens. This process is well regulated depending on the interaction between Antigen Presenting Cells (APCs) and effector T and B cells and products to ensure tolerance to self elements(2).

Tolerance to self antigens is mainly mediated by the acquired immune system. This system relies on the complements of B and T lymphocytes and their membrane- bound antigen specific molecules . In addition, the acquired immune system may require the help of certain elements of the innate immune system which participate in the pathogenesis of autoimmune diseases (2).

The acquired immune system is stimulated by signals derived from mononuclear leukocytes, particularly lymphocytes. Both spontaneous and induced autoimmune diseases are promoted primarily by CD4 positive T-lymphocytes of the T-helper (Th) class that are especially potent by releasing a broad spectrum of cytokines that promote the actions of other immune cells. Multiple classes of Th-lymphocytes are involved in this process. Th1 class acts as a booster to immune cell activity; Th2 class stimulates the humoral (antibody) response and the Th17 class secretes factors to recruit and stimulate neutrophils (10).These Th-cell phenotypes are activated by direct contact with APCs (dendritic cells and occasionally mitogen-stimulated B-cells) that express major histocompatability complex (MHC) type II as well as a co-stimulatory molecule (B7 [CD80/86).

Lymphocyte activation occurs in one case only if the T-cell forms an immune synapse with an APC simultaneously using three signals: 1- the primary T-cell receptor (TCR) binding to MHC II, 2- the T-cell co-stimulatory receptor (CD28) linking with the APC's co-stimulatory molecule, and 3- APC secreted cytokines interacting with T-cell receptors in a paracrine fashion.(2)

Stimulation by a single receptor-ligand modality is not sufficient to prime the

Lymphocyte. For B-cell activation, surface-anchored immunoglobulin (Ig) is required for this purpose. In AIDx, autoreactive T- and B-lymphocytes engage in mutually assisted positive feedback to be responsible for the disease over time.

The main defect in autoimmune diseases is the loss of self-tolerance, which renders immune cells unable to distinguish between ‘‘self’’ and ‘‘non-self’’ antigens. In this regard, the auto-reactive immune system is functioning ‘‘within normal limits’’ to eliminate threats—except for its unfortunate choice of target (2). The strategies for maintaining immune system self-tolerance are shown in Table 1.

The three main cell-oriented means for preventing autoimmunity are deletion (removal), anergy(relaxation), and suppression (restraint). Deletion, involves irreversible pruning of self-reactive T-cells (11). This process of ‘‘central tolerance’’ (occurring in a core immune organ) occurs mainly in the thymus, the primary lymphoid organ for lymphocyte production. In the thymic cortex, naive lymphocytes (Th0 class) that learn during development to ignore self elements as potential targets are positively selected for continued survival, while T-cell precursors that exhibit any affinity for self-antigens are eliminated via apoptosis. The survivor cells migrate to the thymic medulla where they are exposed to self-antigens in the presence of MHC type I receptors (present on most body cells as a demonstration of ‘‘self’’ status) but not MHC type II receptors (the form on APCs) or co-stimulatory molecules. Any T-cells that will bind self-antigens with high affinity in the absence of MHC type II and a co-stimulatory signal are negatively selected and diverted into an additional round of TCR-mediated apoptosis. Together, these two rounds of programmed cell death confer central tolerance. If needed, additional apoptosis is undertaken in secondary lymphoid organs. The programmed cell death

responsible for this central tolerance is controlled by interactions between Fas and Fas ligand. The absence of these molecules results in reduced pruning of auto-reactive T-cells during development, which promotes systemic autoimmunity. The mechanisms for self-tolerance breakdown in autoimmune diseases due to lymphocyte lapses are described in Table 2.

The second cell-based alternative for preventing autoimmunity is anergy. The main means by which T-cell anergy is induced is for an APC to present a self-antigen as a target in the absence of a co-stimulatory signal .The final cell-based mechanism is clonal suppression, where anti-self responses are quenched by factors derived from other leukocytes (CD4 CD25 regulatory T-cells [Treg] or CD8suppressor [Ts] cells). This repression may be mediated by cytokines with negative feedback activity (transforming growth factor-b [TGF-b]; by protein cross-linking to neutralize surface receptors with affinity for a given antigen, or by the genesis of anti-auto-antibodies that recognize the antigen receptors on autoreactive lymphocytes. Xenobiotics can interfere with T-cell suppression by modifying the cytokine profile produced by newly recruited leukocytes (2).

TABLE 1. Strategies for maintaining immune system self-tolerance (2).

Strategy	Mechanism	Location
Central Central suppression	Pre-activation deletion	Primary lymphoid organs
Peripheral Antigen sequestration Anergy (clonal) Deletion (clonal)	Physical exclusion of immune effector cells Induced unresponsiveness via insufficient co-stimulation Extirpation of autoreactive lymphocytes	Peripheral organs Secondary lymphoid organs Secondary lymphoid organs
Suppression (clonal)	Repression of autoreactive lymphocytes by treg-cells	Secondary lymphoid organs Sites of inflammation
Cytokine substitution	Alternative differentiation to an anti-inflammatory phenotype	Secondary lymphoid organs Sites of inflammation

TABLE2.Selected mechanisms for self-tolerance breakdown in autoimmune diseases.(2)

Strategy	Mechanism
Environmental effects	
Hormonal balance	Gender-linked (hypothalamic-pituitary-gonadal) endocrine effects
Stress	Mental/psychological-based (cortical perception hypothalamic-pituitary-adrenal endocrine effects)
Xenobiotic exposure	Multiple (mainly altered receptor-mediated signaling and molecular cross-linking)
Fresh foes	
Altered antigens	Self-antigen alteration by attachment of a hapten (chemical or metal) to make a neo-antigen
Molecular mimicry	Structural/chemical resemblance of self-antigens to foreign (especially microbial) antigens
Unmasking	Exposure of previously sequestered self-molecules
Genetic predisposition	
Antigen-presenting cell (APC) haplotype	Certain major histocompatability complex (MHC) II variants enhance autoreactivity
T-cell receptor (TCR) haplotype	Certain TCR versions promote autoimmunity
Lymphocyte lapses	
Cytokine profile	Heightened production of lymphocyte clones with a pro-inflammatory phenotype
Forbidden clone	Loss of antigen-specific T-suppressor lymphocytes permits activation of their autoreactive Th-targets
Hyperactivity	Excessive function and/or numbers of Th-lymphocytes
Polyclonal activation	Pathogen-induced mitogenesis of autoreactive B-cells, which can then stimulate autoreactive T-cells
Premature senescence	Accelerated loss of telomeres

Role of innate immune mechanisms

Autoimmunity is often considered to be an acquired response, but innate immune cells play important roles in moderating self-tolerance. Activated dendritic cells autoreactive T cells. Natural killer T (NKT) lymphocytes, suppresses or exacerbate autoimmunity depending on a constellation of animal-specific factors. While they have potent immune -modulatory properties, NKT cells are not participated in the adaptive immune response.

In the innate immune system, three endosomal Toll-like receptors (TLR) considered to be major participants in some autoimmune diseases. These molecules are highly conserved across species, have evolved as receptors to recognize specific forms of microbial (viral) nucleic acid: TLR-3 for double-stranded (ds) RNA, TLR-7 for single-stranded RNA, and TLR-9 for ds DNA. Binding of the appropriate nucleotides induces a pro-inflammatory signal. Unluckily, these TLR have also been shown to recognize certain human antigens. The expression patterns for these three TLR are often cell type–specific; B-cells bear TLR-7 and TLR- 9, dendritic cells binds either TLR-3 alone or both TLR-7 and TLR-9, and fibroblasts carry only TLR-3. These TLRs are thought to involve in autoimmune diseases by directing the cells that express them to attack self-molecules and enhance their expression of pro inflammatory cytokines. In addition, they can induce initial B-cell activation (especially TLR-9) in the absence of T-cell support, so that B-cell clones break tolerance first. Activated B-cells then in turn activate naive T-cells, which is necessary for initiation of developed autoimmune diseases (2).

CTLA-4

CTLA4 gene was identified in 1991as a second receptor for the T cell Co-stimulation ligand B7. The specific function of this important gene was not clarified until 1995 when it was confirmed to inhibit T cell activation (12).

Activation of T-cell is the main event in the organization of effective cellular and humoral immune responses. Activated T cells are essential for provision of T-cell help, promoting the development of high-affinity antibody production and the generation of cytotoxic T-cell responses. That leads to the fact that, defects in proteins required for T-cell activation give rise to significant infectious pathology and malignancies. While the Defects in proteins involved in regulating activated T-cell therefore tend to lead to autoimmunity (13). So the major challenge faces the regulating of T cell responses is to provide enough reactive T cells against foreign antigens while keeping T-cells unresponsive towards self-antigens and that cannot be done without special mechanisms for self –tolerance .

In the thymus large numbers of potentially `self-reactive' T cells are eliminated during negative selection in a process termed central tolerance. However, the process of positive selection that permits the expansion of T cells with low avidity for self major histocompatibility complex (MHC) interactions must also lead to a degree of self-reactivity which is presumably tolerable in peripheral T cells. A number of proteins have been identified that may serve the function of `quality controlling' peripheral T-cell activation. In this study we will focus on one of those proteins, CTLA-4 and its role in autoimmunity.

Cytotoxic T lymphocyte antigen-4 (CTLA-4) or CD152 is a trans membrane protein member of the immunoglobulin gene superfamily containing a single extracellular `V-like' domain , a transmembrane domain, and a cytoplasmic tail. Levels of CTLA-4 expression in most resting T cells are extremely low (or probably absent), and predominantly appears following T-cell activation. The T cell attack can be turned on by stimulating the CD28 receptor on the T cell. The T cell attack can be turned off by stimulating the CTLA4 receptor, which acts as an "off" switch. CTLA4 is similar to the T-cell co-stimulatory protein, CD28, and both molecules bind to

CD80 and CD86, also called B7-1 and B7-2 respectively, on antigen-presenting cells. CTLA4 transmits an inhibitory signal to T cells, whereas CD28 transmits a stimulatory signal. Intracellular CTLA4 is also found in regulatory T cells and may be important to their function. T cell activation through the T cell receptor and CD28 leads to increased expression of CTLA-4, an inhibitory receptor for B7 molecules (13). T cell activation and T cell tolerance is shown in Figure 2.

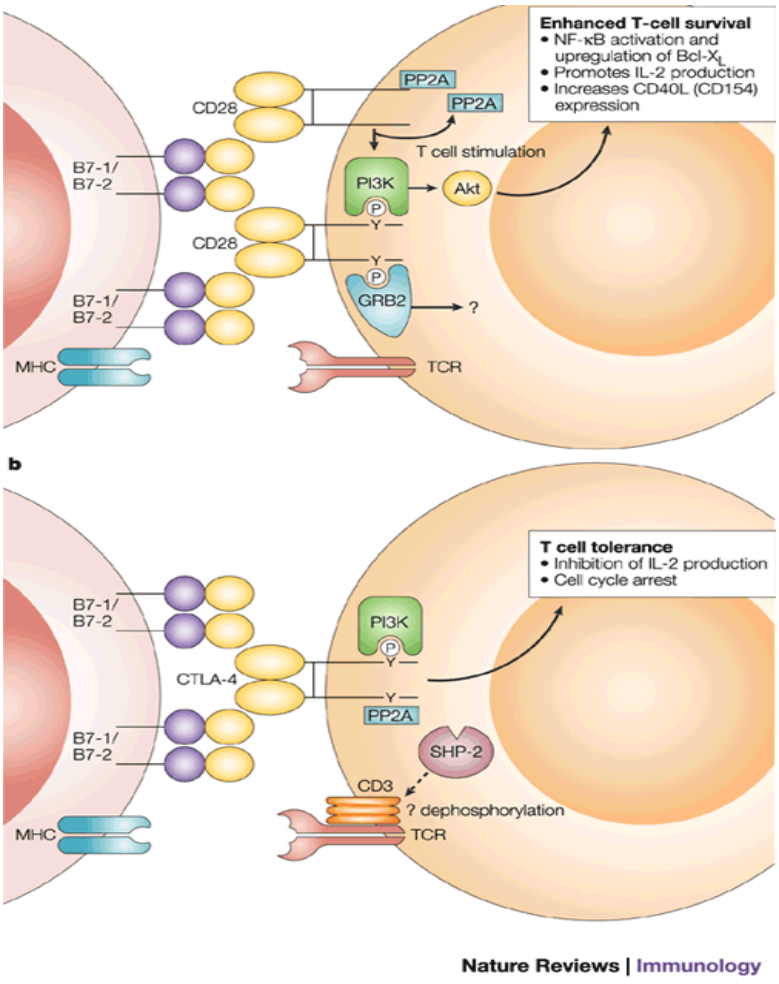

Fig 2: T cell activation and T cell tolerance (14).

Genetics of CTLA-4

CTLA-4 gene belongs to the immunoglobulin super family. It encodes the protein which inhibits the T cells activation. There are three domains to the CTLA-4 protein, V domain, a transmembrane domain, and a cytoplasmic tail. There are two different forms of this protein due to alternative transcriptional splice. The homodimer which is a membrane-bound isoform interconnected by a disulfide bridge and the monomer which is the soluble isoform**.** The structure of chromosome 2 and the specific position of the CTLA4 gene is shown in Figure 3. The protein is 223 amino acids and a molecular weight of 24656 Da. The exact position of the gene on chromosome 2 is located between s*tart:* **204,732,509** bp from *pter End:* **204,738,683** bp from *pterSize:***6,175** bases *Orientation:***plus** strand (15)

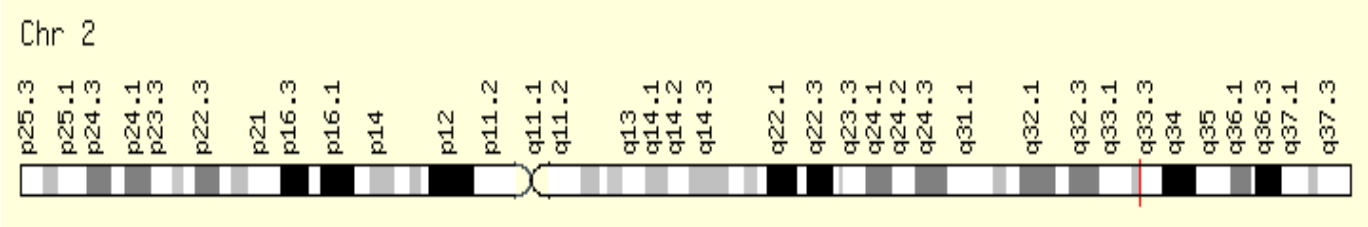

Fig 3. Structure of chromosome 2 and the location of CTLA4 gene on q33.3 (15).

Genetics of autoimmune diseases

Most of autoimmune diseases may be described as genetic abnormalities in thymic selection. Quantitative differences in the expression of the selecting self- antigens and the responsiveness of the thymocytes to these antigens can have a major effect on the selection process. Defective thymic deletion can be caused by reduced or altered expression of MHC molecules. Thymocytes, like mature T cells, require MHC molecules for presentation of (self) antigens to the TCR. If the relevant MHC molecule is absent or expressed at a low level in the thymus, thymocytes will not

be activated and will not undergo apoptosis. Indeed, this process is sensitive to the level of MHC molecule expression, and to the specific MHC alleles which are expressed in the thymus. This is the major mechanism by which HLA molecules regulate the peripheral T-cell repertoire and overall T-cell immune responsiveness (16).

Inadequate expression of self- antigens in the thymus can also cause or predispose to autoimmune disease. Large number of organ-specific self- antigens is expressed in the thymus. The absence of this expression can result in an escape from thymic deletion and the release of self-reactive T cells into the periphery.(16)

Figure 4 explains the selection process starting from bone marrow to thymus ending in peripheral.

Genetic defects in TReg cell functions demonstrated by X- linked recessive disorder IPEX (immune dysregulation, poly-endocrinopathy, enteropathy, X-linked syndrome) and is caused by mutations in FOXP3.

TReg cells are totally dependent on IL2 for its proliferation and function , which means any lack if IL2 or its receptors can lead to autoimmunity .

The activation of peripheral T cells is also controlled by genetic defects in T-cell co-stimulatory molecules located on the surface of T-cells , and work as a modulator for the e activation of T-cells through the TCR(16).

These co-stimulatory molecules are CD28, the inducible T-cell co-stimulator (ICOS) and the cytotoxic T-lymphocyte-associated protein 4 (CTLA4),these are members of the immunoglobulin super family ,expressed on T cells and bind homologous ligands on APCs.s. CD28 and ICOS provide positive signals while CTLA4 generally work as a negative regulator of T-cell activation.

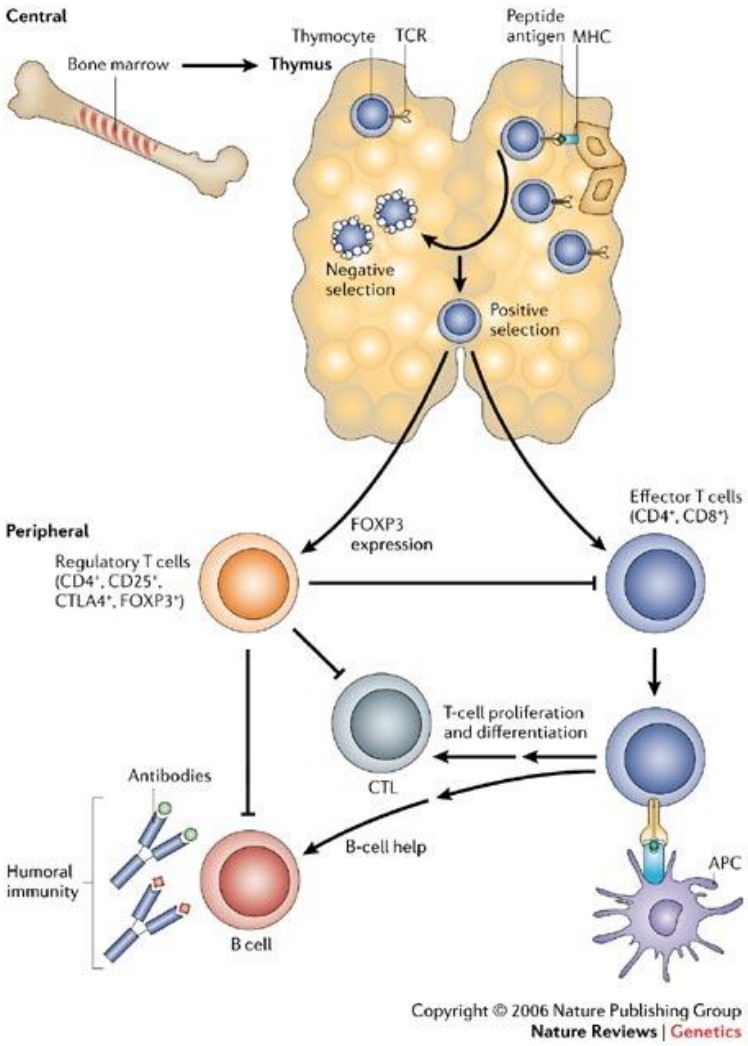

Figure 4 Central and peripheral tolerance mechanisms(16)

T cells are activated through stimulation of the T-cell receptor (TCR). Co-stimulatory and co-inhibitory molecules on the T-cell surface role, and regulate the degree of activation as well as the development of peripheral T-cell tolerance.

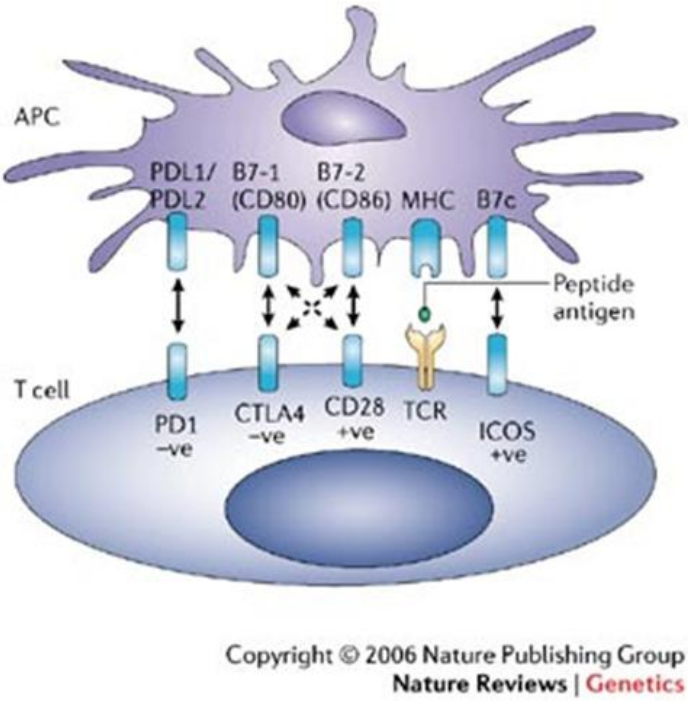

Figure 5 Co-stimulatory and co-inhibitory molecules role in T-cell activation. (16)

CD28 and ICOS are activating receptors that bind members of the B7 family of ligands on APCs. CTLA4 is inhibitory on most T cells except regulatory T cells, and, like CD28, can bind CD80 and CD86, but with varying affinities. That leads to cross competition for CD28 co-stimulation. The programmed cell death 1 (PD1) protein is also inhibitory for T-cell activation, and can bind two related ligands, PDL1 and PDL2.(16)

Literature review

Paul Ehrlich in 1901, was the first scientist to introduce the concept of immunological self/non-self discrimination. He enunciated his misinterpreted doctrine of *horror autotoxicosus,* where he used this expression to refer to the extremely dangerous situation which may result from the formation of autotoxins or autoantibodies. After some experiments on animal models ,in which he tried to immunize animals with self and non self red blood cells, he came up with a conclusion that it's impossible to form antibodies against self antigens and that was proven later to be misinterpretation(17) .

Later in 1904, Donath and Landsteiner discovered the first known autoantibody which is now carrying their names. This autoantibody was found to cause haemoglobinuria *a frigore* which were recurrently seen in syphilis patients. This autohemolysin is not only able to react with human red blood cells from different individuals, but also with those of the patient himself, both *in vitro* and *in vivo.*

During 1900 to 1915, immunologists continued their efforts to induce anti-tissue antibodies, but rarely could they relate those antibodies with any accompanying disease (17).

In 1933, Rivers *et al.,* published a group of studies on the induction of neurological diseases in experimental animals through immunization with nervous tissue broths or extracts, which is known now as experimental autoimmune encephalomyelitis (AEA), and considered as the model for the study of some human demyelinating diseases such as multiple sclerosis and post vaccination encephalitides.

In 1945, the use of adjuvants came in use to enhance experimental induction of autoimmune diseases, which led to the recognition of 'lupus mice'. This has been followed in unexpected discoveries in clinical investigation on important human diseases(18).

By 1953, Witebsky collected adequate data for representing the experimental production of thyroiditis in rabbits injected with thyroid tissue, he was unable to admit the validity of his own experiments, and spent three years searching for the errors he thought he had made. Unfortunately, he tried to explain the presence of errors rather than publish the results of his brilliant investigations. Later in 1965, autoimmunity was accepted as real and a wide range of autoimmune diseases became recognized in the 20th Century Medicine.(18)

CTLA4 genotype correlation to Treg frequency

The A allele of CT60 is located in the 3' untranslated region of CTLA4 , it was considered to be single nucleotide polymorphism. Previous studies found that homozygosity of this A allele is associated with decreased risk of autoimmune diseases , as the CT60 A\A genotype found to be associated with elevated level of Treg frequency in blood by 30-40%(19)

Heterozygosity of CT60A\G allele found to be associated with intermediate risk of autoimmune diseases while Treg frequency didn't have big difference than homozygous CT60 G\G allele really.

Meanwhile there was no approved correlation between AG49 polymorphism which located in exon 1 with Treg frequency in peripheral blood.(19)

Homozygosity for A allele in both CT60 and AG49 polymorphisms found to be in related disequilibrium that means all cases who was homozygous for A allele for CT60 was also homozygous for A allele for AG 49 , reverse is not true .(19)

Hypothesis

Single nucleotide polymorphisms (SNPs) on the CTLA4 gene increase the susceptibility to autoimmune diseases.

Objectives

The aims of this study are:

1. Determine the presence of the most commonly encountered SNPs in the CTLA 4, specifically; A/G 49 and C/T 60 by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)
2. Determine the presence of significant association between CTLA4-SNPs and susceptibility to autoimmune diseases in Palestinian population living in the West Bank.

Chapter II

Materials and methods

Study subjects

A total of 120 samples were collected from Patients with different autoimmune disease and 82 normal controls from hospitals and clinics throughout the West Bank. The population size was limited to the known available patients with autoimmune diseases treated in hospitals and clinics in the West Bank, Palestine.

Details on the type of autoimmune diseases for the selected patients are shown in Table 3 below.

Autoimmune disease	Number of patients
Rheumatoid arthritis	74
Autoimmune hepatitis	1
Crohn's disease	3
Behcet's disease	3
Systemic lupus erythematosus (SLE)	12
Mixed connective tissue disease	1
Scleroderma	3
Spondylarthritis	6
Diabetes type I	17
	120

Table 3: Distribution of autoimmune diseases for patients participating in the study.

The guidelines of the Central Ethical Review Committee at Birzeit University were followed. The project and potential risks were explained to each subject prior to obtaining his/her oral consent to participate. Participation was the free choice of each participant. A questionnaire was filled by each participant containing information related to clinical history and demographic data such as: Age, Sex and Family history for specific autoimmune diseases of the participant. Other autoimmune diseases inherited in the family. A copy of the questionnaire is included in Annex 1.

Sample Collection

Whole blood was collected from each participant following aseptic techniques in EDTA tubes stored at -20^{o} C. DNA was extracted using the salting out method according to (16); the purity and quality of the isolated DNA was checked on 1.5% agarose gel electrophesresis and viewed on the Gel-Doc system (BioRad, USA).

DNA extraction

DNA was isolated from whole blood using the salting out method. Details of the procedure are summarized below:

I. Buffers

All buffers are prepared and sterilized before storage. Buffer A was autoclaved prior to addition of Triton-X-100. Sterilization by filtration was occasionally used instead of autoclaving because it is recommended.

1. Buffer A (Red blood cell lysis buffer)

0.32 M sucrose

10 mM Tris HCl

5 mM $MgCl_2$

0.75% Triton-X-100

Deionized water

Adjust pH to 7.6

2. Buffer B (Proteinase K buffer)

20 mM Tris-HCl

4 mM Na_2EDTA

100 mM NaCl

Deionized water

Adjust pH to 7.4

Procedure

One volume of blood (1 ml) of whole blood was mixed with 2 volumes (2 ml) of ice cold buffer A, mixed gently by inverting the tube 6-8 times and left on ice for 2-3 minutes. The mixture was then centrif`uged for 15 minutes at 3500 rpm at 4^{o}C. The supernatant was discarded into a container with 2.5% bleach solution. The pellet was then suspended pellet in 2 ml of buffer A and 6 ml of water, vortexed briefly and centrifuged at 3500 rpm for 15 minutes at 4^{o}C. This lysis step can be repeated until a white to cream pellet is obtained(20).

An amount of 5 ml of Buffer B and 500 µl of 10% SDS was added to the pellet, vortexed vigorously for 30-60 seconds followed by adding 50 µl of freshly made and cooled Proteinase K solution (20mg/ml). Ice cold Proteinase K solution should be made fresh prior to use. The tubes were then incubated for two hours at 55^{o}C in a water bath followed by cooling to room

temperature or placing them for 2-3 minutes on ice. Salting out of all cellular components was sedimented by adding 4 ml of 5.3 M NaCl solution. After gentle vortexing for 15 seconds, the tubes were centrifuged at 4500 rpm for 15-20 minutes at 4^{o}C. Finally, the off supernatant was carefully removed and placed in a sterile microcentrifuge tube. An equal volume of ice cold isopropanol was then added, the tube gently inverted 5-6 times to precipitate DNA. The tube was then placed in the freezer at -20^{o} C overnight followed by centrifugation at 4, 000 rpms for 20 minutes. The supernatant was then carefully removed, the pellet is washed twice with ml of 70% ethanol and allowed to dry. The pellet was then suspend in 300-400 µl of Tris HCl, pH 8.and Left to dissolve overnight at 37^{o} C(20). Isolated DNA was stored at -20^{o} C for subsequent PCR and restriction (PCR-RFLP).

PCR and Single Nucleotide polymorphism

CT60 Polymorphism

A total of 120 cases and 82 controls were analyzed in this study. PCR was conducted as follows: The CT60 SNP was determined by PCR-RFLP. The primers used in this step are forward: 5'-CTTCATGAGTCAGCTTTGCACCAGC-3'and reverse: 5'-AGCTGAGAAAGCAGGCGGTAAGAAA-3'(21).

The PCR reaction was carried out in 25 ul volumes consisted of 12.5 ul master mix 2x (source), 0.4 ul of each primer, 3.5 ul DNA templates and 8.2 ul sterile dH2O. The PCR program was as follows: initial denaturation for 6 min at 95°C; followed by 40 cycles of denaturation for 45 sec at 95°C; annealing for 45 sec at 60 °C; and elongation for 45 sec at 72°C ,followed by a final extension of 5 min at 72°C. The PCR product for the samples was placed on 2% agarose

containing 1uM ethidium bromide and compared for their specific size with 100 bp DNA ladder (gene direx 100 bp ladder RTU).

To detect the presence of SNP, 5 ul of the 200-bp PCR product was incubated with 5 units of HpyCH4 IV restriction edonuclease for 1 hr at 37°C. The restriction enzyme will cut the PCR product in the following manner:

1. No cut will show a 200 bp band in the gel.

2. Two bands of 100 bp and 200 bp indicate a heterozygous mutation.

3. One band of 100 bp indicates a homozygous mutation.

These results are shown in Figure 6

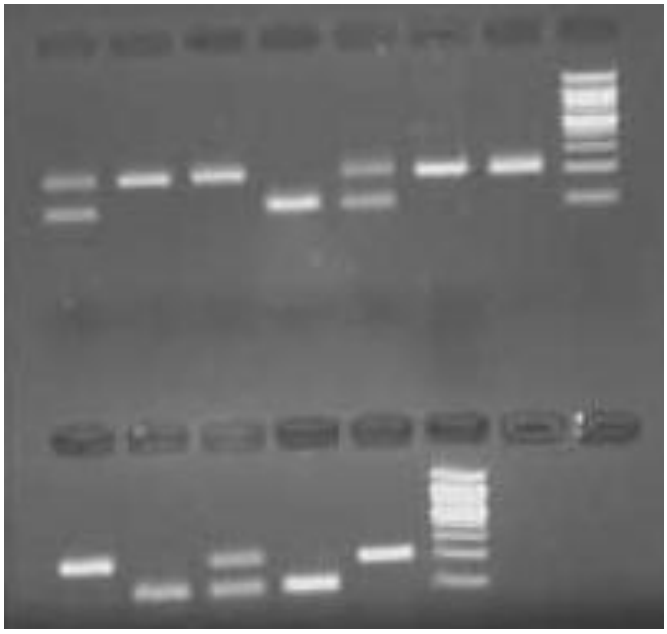

Figure 6: Agarose gel electrophoresis showing lane 1: 100 bp ladder, lanes 2,3,6 and 7 normal, 4 and 8 heterzygous, lane 5 homozygous.

A/G 49 Polymorphism

A total of 120 cases and 82 controls were analyzed in this study. PCR was conducted as follows: The A/G 49 SNP was determined by PCR-RFLP. The primers used in this step are forward: 5'-AAGGCTCAGCTGAACCTGGT-3'; reverse: 5'- CTGCTGAAACAAATGAAACCC-3' (22)The PCR reaction was carried out in 25 ul volumes consisted of 12.5 ul master mix 2x (source), 0.4 ul of each primer, 3.5 ul DNA template and 8.2 ul sterile dH2O. The PCR program was as follows: initial denaturation for 7 min at 95°C; followed by 40 cycles of denaturation for 30 sec at 95°C; annealing for 45 sec at 57 °C; and elongation for 45 sec at 72°C ,followed by a final extension of 5 min at 72°C. The PCR product for the samples was placed on 2% agarose containing 1uM ethidium bromide and compared for their specific size with 100 bp DNA ladder (gene direx 50 bp ladder RTU).

To detect the presence of SNP, 5 ul of the 152 bp PCR product was incubated with 5 units of BstEII restriction edonuclease for 1 hr at 37°C(22). The restriction enzyme will cut the PCR product in the following manner:

1. No cut, will show a 152 bp band indicating polymorphism.
2. one band of 130 bp indicates a normal allele.

These results are shown in Figure 7

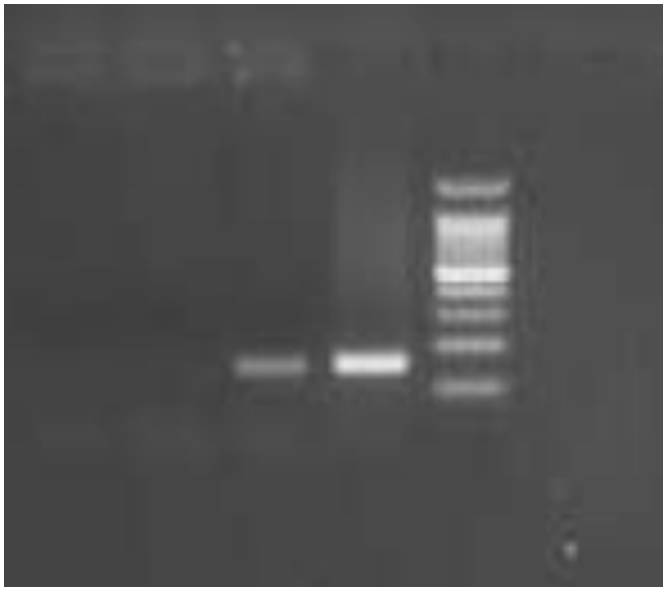

Figure 7: Agarose gel electrophoresis showing restriction results of A/G 49 mutation, lane 1: 50 bp ladder, lanes 2, 152 bp, lane 3: 130 bp.

*All used reagents were prepared manually in Berziet University lab.

Chapter III

Results

This project was conducted on 120 patients with autoimmune disease and 81 normal controls. Whole blood was aseptically collected and DNA was purified. The quality of the isolated DNA was checked on 1.5% agarose gel electrophoresis. PCR was then performed using primers to detect mutations on the CTLA4 gene at CT60 and AG49. The results obtained are summarized in Table4

Sample	CT60 polymorphism			AG49 polymorphism	
	A/A Normal (%)	A/G Carrier (%)	G/G Disease (%)	A/A Normal (%)	G/G Disease (%)
Patients	29 (24.2%)	65 (54.2)	26 (21.6)	48 (40%)	72 (60%)
Controls	19 (23.2)	41 (50.6)	22 (27.2)	58 (70.7%)	24 (29.6%)

Table 4: Summary of the results obtained for the RFLP-PCR for CT60 and AG49 for cases and controls.

1. CT60 mutation

RFLP-PCR was used to determine the presence of the mutation CT60 on the CTLA4 gene that can trigger autoimmunity in cases as compared to controls. Restrictions of the PCR products can give 3 fragments of different sizes depending on the allelic combinations that may be present. These fragments were resolved on agarose gel electrophoresis. The three allelic combinations are formed from the normal A allele and the abnormal or mutated G allele. The results of the RFLP for this mutation are shown in Figure 8.

Our results showed that 21.6% (26/120) of the patients carried two mutated alleles G/G , 24.2% (29/120) carried the wild type or normal A/A genotype and 54.2% (65/120) were carriers A/G.

Allelic combinations for the control group were 27.2% (22/81) G/G, 22.2% (18/81) were A/A and 50.6% (41/81) were A/G. There was no significant difference (P>0.05) between the cases and controls.

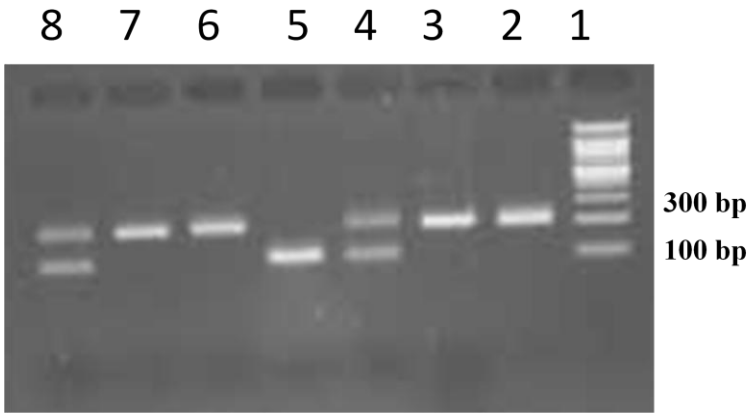

Figure8: Gel electrophoresis of RFLP-PCR for the CT60 mutation.
Lane 1: 100 bp ladder, lanes 2, 3, 6 and 7: normal (A/A) alleles ,
lanes 4 and 8 mutated (A/G) allele, lane 5 double mutation (G/G) allele

2. A/G 49 mutation

RFLP-PCR was also used to investigate the role of the A/G 49 mutation on the CTLA4 gene in triggering autoimmunity. Two types of allelic combinations of different sizes with A/A or A/G genotypes were resolved by agarose gel electrophoresis. Our results showed that 60% (72/120) of patients carried the mutated genotype G/G while 40% (48/120) carried normal A/A allelic combination. As for the controls, 29% (24/81) had the G/G genotype while 70.4% carried the normal A/A alleles. There was significant difference between the cases and the controls (P<0.05). The results of the RFLP for this mutation are shown in Figure 9.

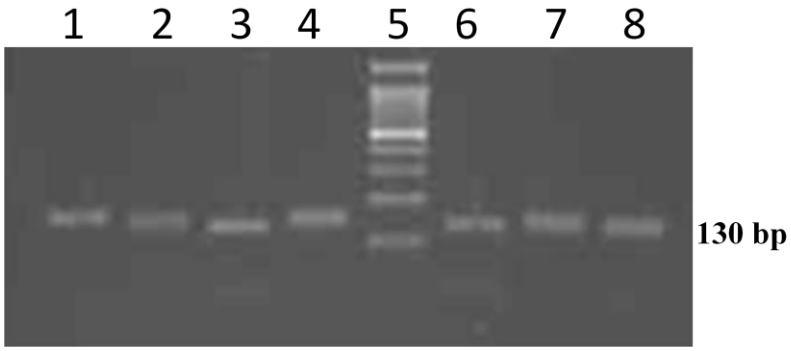

Figure 9: Gel electrophoresis of RFLP-PCR for the AG49 mutation. Lane 5, 100 bp ladder, lanes 1 and 4 are 152 bp (mutated G/G alleles). Lanes 2, 3, 6 ,7 and 8 normal (A/A) alleles.

Chapter 4

Discussion

The aim of this study was to determine the presence of association between two mutations on the CTLA4 gene; A/G49 and C/T 60; and susceptibility to autoimmune diseases among Palestinian patients living in the West Bank.

For A/G49 alleles, we detected only two homozygous alleles, A/A and G/G. Statistical analysis for our data showed a significant difference between the controls (29.6%) and patients (60%) for the G allele ($P<0.05$). Several studies has been conducted to determine the presence of association between the homozygous G allele and susceptibility to autoimmune diseases. An association between several autoimmune diseases such as Grave's disease, Hashimoto's thyroiditis, multiple sclerosis and Type I diabetes mellitus, have been associated with the G allele (23). An association has been established between polymorphism of the CTLA4 gene, specifically the G allele at position 49 and Rheumatoid arthritis (24). These findings agree and confirm the results of this study since most of the sample collected for this study has been obtained from Rheumatoid arthritis patients. It has been reported that G allele polymorphism affects cell surface expression of CTLA4 in response to T cell activation (25). Another interpretation for the significant association between the G allele and autoimmunity is the impaired control of T cells proliferation (26). However, the homozygous A allele was clearly much higher in controls (70.4%) as compared to patients (40%). This may indicate that the A allele is associated with decreased risk of susceptibility to autoimmune diseases. Furthermore, the heterozygous A/G allele was more common among controls than patients (26) emphasizing

the protective role associated the A allele against autoimmune diseases. In this study, we used RFLP-PCR technique with restriction enzymes that can only detect homozygous A and G alleles. For C/T60 polymorphism, we did not find significant association between the sample tested and autoimmune diseases (P>0.05). We determined polymorphism in patients and controls for the 3 alleles; AA, GG, AG. Our results as shown in Table 4 reflect that the G allele doesn't play a role in susceptibility to autoimmune diseases. It has been reported among Taiwanese patients that the G/G allele is the most prevalent (21). This may indicate the presence of geographical distribution of the CTLA4 gene polymorphism. Interestingly, it has been reported that decreased CT60 polymorphism has been associated with risk of Rheumatoid arthritis (24) and slightly increased risk of coeliac disease among the Dutch population (23).

The outcome of this study may contribute to better and faster diagnosis of autoimmune diseases. However, not all alleles of the CTLA4 gene are useful for this purpose. Our results indicated the presence of significant difference between cases and controls for the A/G49 allele. In addition, the carriers of this allele have an increased probability of developing Rheumatoid arthritis while the carriers of the other allele C/T60 have decreased risk of this condition. Therefore, testing the presence of these two alleles my complement the diagnostic procedures of autoimmune diseases on one hand and provide valuable information to the physician on the other. Furthermore, it may guide the treating physician on the best way to treat the patient or not.

We feel this study has been limited to only two alleles of the CTLA4 gene. Although this are important alleles that contribute to the development of autoimmune diseases, we feel it is also important to investigate more alleles on this important gene that may give a better picture of the genetics and pathogenesis of autoimmune diseases.

References

1. Burnet, F. M.; Fenner, F.: The Production of Antibodies. Monograph of the Walter and Eliza Hall Institute, Melbourne. 1949.
2. Bolon,B. : Cellular and molecular mechanisms of autoimmune diseases. Toxicologic Pathology, 40: 216-229, 2012.
3. U.S. department of health and human services: autoimmune diseases research plan. NIH Publication No. 03-5140, December 2002.
4. Costenbader KH, Gay S, Alarcón-Riquelme ME, Iaccarino L, Doria A.: Genes, epigenetic regulation and environmental factors: which is the most relevant in developing autoimmune diseases? Autoimmun Rev. 11(8):604-9, 2012.
5. Cooper GS, Stroehla BC.: The epidemiology of autoimmune diseases. Autoimmun Rev., 2(3):119-25. 2003.
6. Gleicher N, Barad DH.: Gender as risk factor for autoimmune diseases. JAutoimmun. 28(1):1-6. 2007.
7. Ansar A. S., Penhale WJ, Talal N.: Sex hormones, immune responses, and autoimmune diseases. Mechanisms of sex hormone action. Am J Pathol. 121(3):531-51, 1985.
8. Ercolini A. M., Miller S.D.: The role of infections in autoimmune disease. Clin Exp Immunol., 155(1): 1–15., 2009.
9. Davidson A., Diamond B.,: Autoimmune diseases . N Engl J Med, Vol. 345, No. 5, 2001.
10. Bachmann M.F., and Kopf M.: On the Role of the Innate Immunity in Autoimmune Disease. J Exp Med. 193(12): f47–f50, 2001

11. Goodnow C.C., Sprent J., de St Groth B.F., Vinuesa C.G,.: Cellular and genetic mechanisms of self tolerance and autoimmunity. Nature **435**, 590-597, 2005.

12. Bashyam H.: CTLA-4: From conflict to clinic. J Exp Med. 204(6): 1243, 2007

13. Sansom D M: CD28, CTLA-4 and their ligands: who does what and to whom? Immunology, 101(2): 169–177. 2000.

14. Sharpe AH, Freeman GJ.: The B7-CD28 superfamily. Nat Rev Immunol. 2(2):116-26, 2002.

15. http://www.genecards.org/cgibin/carddisp.pl?gene=CTLA4

16. Gregersen PK, Behrens TW.: Genetics of autoimmune diseases--disorders of immune homeostasis. Nat Rev Genet. 7(12):917-28, 2006

17. Maslloréns F. : Autoimmune diseases and physiological autoimmunity : recognition of self. Alergol Inmunol Clin;15:5-12, 2000.

18. Mackay IR.: Travels and travails of autoimmunity: a historical journey from discovery to rediscovery. Autoimmun Rev. 9(5):A251-8, 2009.

19. Atabani SF, Thio CL, Divanovic S,etal.: Association of CTLA4 polymorphism with regulatory T cell frequency. Eur J Immunol. 35(7):2157-62, 2005.

20. Helms, C. :Salting out Procedure for Human DNA extraction. http://hdklab.wustl.edu/lab_manual/dna/dna2.html, 1999

21. Weng YC[1], Wu MJ, Lin WS.: CT60 single nucleotide polymorphism of the CTLA-4 gene is associated with susceptibility to Graves' disease in the Taiwanese population. Ann Clin Lab Sci. ;35(3):259-64,2005

22. Zaidan Sh.: Polymorphism of Human (*CTLA4*) Gene and Insulin-Dependent Diabetes Mellitus Associated with Obesity in Iraqi Population, Research Journal of Medical Sciences, 5(5):286-288, 2011

23. van Belzen MJ, Mulder CJ, Zhernakova A, etal.: CTLA4 +49 A/G and CT60 polymorphisms in Dutch coeliac disease patients. Eur J Hum Genet. 12(9):782-5.2004

24. Li X , Zhang C, Zhang J, etal. : Polymorphisms in the CTLA-4 gene and rheumatoid arthritis susceptibility: a meta-analysis , J Clin Immunol. 32(3):530-9. 2012.

25. Kamesh L, Heward JM, Williams JM, etal. : CT60 and +49 polymorphisms of CTLA 4 are associated with ANCA-positive small vessel vasculitis. Rheumatology (Oxford). 48(12):1502-5.2009.

26. Kucharska AM, Gorska E, Wasik M, etal. : Expression of CD152 (CTLA-4) in children with autoimmune thyroiditis and +49 A/G polymorphism of exon 1 of the CTLA-4 gene. J Physiol Pharmacol. 5:77-80.2009

ANNEXES

Annex I

CTLA4 gene polymorphism and autoimmunity
الاستعداد الجيني لامراض المناعة الذاتية

كافة المعلومات الواردة فى الاستبيان ستستعمل لغرض الدراسة فقط و سيتم التعامل معها بمنتهى المهنية و السرية .

اقر انا الموقع ادناه بموافقتى التامة بالمشاركة فى هذة الدراسة لاغراض بحثية و عدم ممانعتى لاستخدام عينتى او معلوماتى لاغراض هذا البحث .

التوقيع :

Patient No./code:

Age:

Sex:

Diagnosis:

……

……

……

……

……

……

Other autoimmune diseases diagnosed for the same patient

……

……

……

……

……

……

Other inherited autoimmune diseases in family

……

……

……

……

Notes:

……

……

……

……

AnnexII

Patients			Controls		
sample	**CT60**	**AG49**	**Control**	**CT60**	**AG49**
1	A/G	A/A	**C1**	A/G	G/G
2	A/G	A/A	C2	A/A	A/A
3	A/G	A/A	C3	A/G	A/A
4	A/G	G/G	C4	A/A	A/A
5	A/G	G/G	C5	G/G	G/G
6	A/G	A/A	C6	G/G	G/G
7	A/G	A/A	C7	A/G	G/G
8	G/G	G/G	C8	A/G	A/A
9	A/G	G/G	C9	G/G	G/G
10	A/G	A/A	C10	A/G	A/A
11	A/A	G/G	C11	A/A	G/G
12	A/G	A/A	C12	A/G	G/G
13	G/G	G/G	C13	G/G	A/A
14	A/A	A/A	C14	A/G	A/A
15	A/G	A/A	C15	A/A	A/A
16	G/G	A/A	C16	A/A	A/A
17	G/G	A/A	C17	A/A	A/A
18	A/A	G/G	C18	G/G	G/G
19	A/G	A/A	C19	G/G	G/G
20	A/G	G/G	C20	G/G	G/G
21	A/G	A/A	C21	A/G	A/A
22	A/G	G/G	C22	A/A	A/A
23	A/A	A/A	C23	G/G	A/A
24	A/A	G/G	C24	G/G	A/A
25	A/G	G/G	C25	G/G	G/G
26	A/A	G/G	C26	G/G	G/G
27	A/G	A/A	C27	G/G	G/G
28	G/G	A/A	C28	A/G	G/G
29	G/G	G/G	C29	G/G	A/A

30	A/A	A/A	C30	A/G	A/A
31	A/G	G/G	C31	A/G	A/A
32	A/G	G/G	C32	A/G	A/A
33	A/A	G/G	C33	A/G	A/A
34	G/G	G/G	C34	G/G	A/A
35	A/G	G/G	C35	A/A	G/G
36	A/A	G/G	C36	A/G	G/G
37	A/G	G/G	C37	A/G	G/G
38	A/A	G/G	C38	A/A	G/G
39	A/A	G/G	C39	G/G	A/A
40	G/G	G/G	C40	A/G	G/G
41	A/G	A/A	C41	A/G	A/A
42	A/A	G/G	C42	A/G	A/A
43	A/A	G/G	C43	G/G	A/A
44	A/A	G/G	C44	G/G	A/A
45	G/G	G/G	C45	A/A	A/A
46	A/G	G/G	C46	A/G	A/A
47	A/G	G/G	C47	G/G	G/G
48	G/G	A/A	C48	A/G	A/A
49	A/A	A/A	C49	A/A	G/G
50	A/G	G/G	C50	A/G	G/G
51	A/G	A/A	C51	A/G	G/G
52	A/G	G/G	C52	A/G	A/A
53	A/A	A/A	C53	A/G	A/A
54	A/A	G/G	C54	G/G	A/A
55	A/G	A/A	C55	A/G	A/A
56	A/A	G/G	C56	A/G	A/A
57	A/G	G/G	C57	A/A	A/A
58	G/G	A/A	C58	A/A	A/A
59	A/G	G/G	C59	A/G	G/G
60	A/G	G/G	C60	A/G	A/A
61	A/G	A/A	C61	G/G	A/A
62	A/G	A/A	C62	A/G	A/A

63	G/G	G/G	C63	A/G	A/A
64	A/G	A/A	C64	A/A	A/A
65	A/G	G/G	C65	A/G	A/A
66	A/G	G/G	C66	A/A	A/A
67	G/G	G/G	C67	A/G	A/A
68	A/G	A/A	C68	A/A	A/A
69	A/G	G/G	C69	A/G	A/A
70	A/A	A/A	C70	A/G	A/A
71	A/A	A/A	C71	G/G	A/A
72	A/A	G/G	C72	A/G	A/A
73	G/G	G/G	C73	A/A	A/A
74	A/A	G/G	C74	A/G	A/A
75	A/G	A/A	C75	A/A	A/A
76	A/A	A/A	C76	A/G	A/A
77	G/G	G/G	C77	A/G	A/A
78	A/G	G/G	C78	A/G	A/A
79	A/G	G/G	C79	A/G	A/A
80	A/G	G/G	C80	G/G	A/A
81	A/G	G/G	C81	A/G	A/A
82	A/G	A/A	C82	A/A	A/A
83	G/G	G/G			
84	A/A	A/A			
85	A/G	G/G			
86	G/G	A/A			
87	A/G	G/G			
88	A/A	A/A			
89	A/G	A/A			
90	A/A	A/A			
91	A/G	G/G			
92	A/G	G/G			
93	A/G	G/G			
94	A/G	G/G			
95	A/G	G/G			

96	A/G	G/G			
97	G/G	A/A			
98	G/G	A/A			
99	A/G	A/A			
100	A/A	A/A			
101	A/G	G/G			
102	A/G	A/A			
103	A/A	G/G			
104	G/G	G/G			
105	A/G	A/A			
106	A/G	G/G			
107	A/G	G/G			
108	A/G	A/A			
109	G/G	G/G			
110	G/G	G/G			
111	A/A	A/A			
112	A/G	G/G			
113	G/G	G/G			
114	A/G	A/A			
115	G/G	A/A			
116	A/G	G/G			
117	A/G	G/G			
118	A/G	G/G			
119	G/G	G/G			
120	G/G	G/G			