UNIVERSITI PUTRA MALAYSIA

INTERLEUKIN-21 GENE POLYMORPHISMS IN IRANIAN MULTIPLE SCLEROSIS SUBJECTS

SALMA AHMADLOO

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By

SALMA AHMADLOO

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

March 2012

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DEDICATIONS

I would like to dedicate this thesis to:

my dear parents for their unconditional love, trust and support

and

my dearest husband, who sacrificed his good times because of my progress and giving the hope and energy during working on this thesis.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Master of Science

INTERLEUKIN-21 GENE POLYMORPHISMS IN IRANIAN MULTIPLE SCLEROSIS SUBJECTS

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March 2012

Chairman: Professor Patimah Ismail, PhD
Faculty: Medicine and Health Sciences

Multiple Sclerosis (MS) as an autoimmune inflammatory disease which affects the central nervous system (CNS), is reportedly the second most widespread culprit for neurological disability among young adults. Ranging between 2 and 150 per 100,000, MS is prevalent everywhere in the world. In recent years, the increasing incidence of MS in Iran has been a matter of interest for investigators. Even though MS cannot be claimed to be directly hereditary, genetics variants are reportedly related with MS. Cytokines play an important role in the initiation and maintenance of autoimmune diseases including MS. As a new pro-inflammatory cytokine, interleukin-21 (IL-21) has recently been observed as a target of intervention in many autoimmune diseases such as MS. On the other hand, IL-21 is a cytokine that is produced by T helper 17 (Th17) cells and functions as a growth factor for Th17 cells, considering the importance of IL-21 in Th17 cells development as a new central pathogen in MS disease also new finding on the role of IL-21 in MS disease, association of IL-21 gene polymorphisms (C5250T and G1472T) with MS disease in Iranian subjects were investigated. In this study 301 Iranian MS patients (224 females and 77 males) with mean age of 33.26 +/- 8.74 Years and 201 healthy controls (149 female and 52 male) with mean age of 32.4 +/-8.64 were used to determine the G1472T polymorphism in the second intron (rs2055979) and C5250T polymorphisms in the Exone 3 (rs4833837) of IL-21 gene. Among patients under study, information related to disease type, EDSS score, Progression Index and age of disease onset were obtained. IL-21 G1472T polymorphism was identified by RFLP-PCR method while the IL-21 C5250T polymorphism was genotyped via allele specific oligonucleotide PCR (ASO-PCR). Genotype and allele frequencies were compared between patients and controls by chi-square. Association of polymorphisms with disease types, age at disease onset, EDSS score and progression index were also investigated.

Genotype and Allele frequencies at +1472 G/T position were compared between normal population and patients group. On evaluation of genotypes and allele frequency at this position, there was a significant difference between cases and controls (p=0.003). Also, association of type of disease with G1472T polymorphism was analyzed and a significant difference in genotype frequency was observed between patients and controls group (p=0.0001). The association of G1472T polymorphism with EDSS, progression index and onset age were not significant (p=0.8, p=0.35, p=0.16, respectively). At +5250 C/T position among 301 patients under study, genotype distribution was determined Differences between cases and controls was not significant (p=0.95). Of the 310 patients studied at +5250 C/T position, type disease was determined in 223 patients. Statistical analysis showed no significant difference between genotype frequency and disease type (p=0.71). The association of C5250T with progression index, EDSS and age at disease onset, were not significant (p=0.8, p=0.35 and p=0.16, respectively).

In conclusion this study showed that IL-21 gene polymorphism (G1472T) was significantly associated with MS development. Other studies to clarify the association of this polymorphism with MS susceptibility in other populations would be desired.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Sarjana Sains

POLIMORFISME GEN INTERLEUKIN-21 DALAM SUBJEK MULTIPLE SCLEROSIS WARGA IRAN

Oleh

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Sklerosis Berbilang (MS), satu penyakit keradangan autoimun yang menjejaskan sistem saraf pusat (CNS), dilaporkan sebagai penyebab kecacatan neurologi kedua paling meluas di kalangan belia. MS lazim diseluruh dunia, dengan kadar kelaziman di antara 2 hingga 150 per 100,000. Dewasa ini, peningkatan insidens MS di Iran amat diminati para penyelidik. Walaupun MS bukanlah penyakit keturunan semata-mata, variant genetik telah dikaitkan dengan MS. Sitokin memainkan peranan penting dalam permulaan dan penyelenggaran penyakit autoimun, termasuk MS. Baru-baru ini, sebagai sitokin pro-radang baru, didapati interleukin-21 (IL-21) adalah sasaran kaedah intervensi dalam banyak penyakit autoimun seperti MS. Walaubagaimanapun, IL-21 adalah sitokin yang dihasilkan oleh sel-sel pembantu T 17 (Th17) dan berfungsi sebagai faktor pertumbuhan untuk sel-sel T17. Dengan kepentingan IL-21 dalam pembentukan sel-sel Th17 sebagai patogen utama baru dalam MS, serta penemuan baru peranan IL-21 dalam MS, kaitan antara polimorfisme gen IL-21 (C5250Tdan G1472T) dengan MS dalam subjek-subjek warga Iran telah dikaji, Dalam kajian ini, 301 pesakit warga Iran (224 wanita dan 77 lelaki) dengan min umur sebanyak 33.26 +/-8.74 tahun serta 201 subjek kawalan yang sihat (149 wanita dan 52 lelaki) dengan min umur sebanyak 32.4 +/-8.64 tahun telah digunakan untuk menentukan poliformisme G1472T dalam intron kedua (rs2055979) dan poliformisme C5250Tdalam exon 3 (rs4833837) gen IL-21. Di antara para pesakit yang dikaji, maklumat berkaitan dengan jenis penyakit, skor EDSS, Indeks Kemajuan dan umur pada waktu serangan penyakit telah diperolehi. Poliformisme IL-21 G1472T telah dikenalpasti melalui kaedah RFLP-PCR, manakala poliformisme IL-21 C5250T telah digenotip melalui PCR oligonukleotida alel spesifik (ASO-PCR). Frekuensi genotip dan alel telah dibandingkan antara pesakit dan kawalan dengan menggunakan ‘chi-square’. Kaitan antara poliformisme dengan jenis

penyakit, umur pada waktu serangan penyakit, skor EDSS dan indeks kemajuan juga telah dikaji.

Frekuensi genotip pada kedudukan G1472T telah dibandingkan antara populasi lumrah dan kumpulan pesakit. Terdapat perbezaan signifikan (p=0.003) antara kes dan kawalan dalam penilaian frekuensi genotip pada kedudukan ini, Di antara 301 pesakit MS yang dikaji, jenis penyakit telah ditentukan dalam 223 orang. Kami telah mendapati 158 subjek dengan RR-MS, 53 pesakit dengan SP-MS dan 12 pesakit dengan PP-MS. Apabila kami menganalisis kaitan antara jenis penyakit dengan G1472Tpoliformisme, terdapat perbezaan signifikan antara frekuensi genotip antara kumpulan pesakit dan kawalan (p=0.0001). Tiada kaitan signifikan antara SNP G1472T dengan EDSS (p=0.8), indeks kemajuan (p=0.35) dan umur pada waktu serangan penyakit (p=0.16). Pengagihan genotip pada kedudukan C5250T telah ditentukan di antara 301 pesakit yang dikaji. Tiada perbezaan signifikan antara kes dan kawalan (p=0.95). Daripada 310 pesakit di mana C5250T telah dikaji, jenis penyakit telah ditentukan dalam 223 pesakit. Analisis statistik tidak menunjukkan perbezaan signifikan di antara frekuensi genotip dan jenis penyakit (p=0.71). Tiada kaitan signifikan di antara C5250T dengan indeks kemajuan (p=0.8), EDSS (p=0.35) dan umur pada waktu serangan penyakit (p=0.16).

Kesimpulannya, kajian ini menunjukkan bahawa kaitan poliformisme gen IL-21 (G1472T) dengan pembentukan MS dalam pesakit warga Iran adalah signifikan. Kajian-kajian lain diperlukan untuk menjelaskan kaitan antara poliformisme ini dengan kerentanan MS dalam populasi lain.

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I certify that a Thesis Examination Committee has met on 6 March 2012 to conduct the final examination of Salma Ahmadloo on her thesis entitled "Interleukin-21 Gene Polymorphisms in Iranian Multiple Sclerosis Subjects" in accordance with the Universities and University College Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The committee recommends that the student be awarded the Master of Science.

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This thesis was submitted to the Senate of University Putra Malaysia and has been accepted as fulfillment of the requirements for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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DECLARATION

I declare that the thesis is my original work except for quotations and citations, which have been duly acknowledged. I also declare that it has not been previously and it is not any other institution concurrently, submitted for any other degree at Universiti Putra Malaysia or other institutions.

SALMA AHMADLOO
Date:

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LIST OF ABBREVIATIONS

AID	Autoimmune Disease
ASO	Allele Specific Oligonucleotide
A-T	Adenine-Thymine
BBB	Blood Brain Barrier
Bp	Base Pair
CD	Cluster of Differentiation
CD	Crohn's Disease
CNS	Central Nervous System
CSF	Cerebrospinal Fluid
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotriphosphate
EAE	Experimental Autoimmune Encephalomyelitis
EDSS	Expanded Disability Status Scale
EDTA	Ethylen Diamine Tetraacetic Acid
G-C	Guanine-Cytosine
HHV-6	Human Herpes Virus 6
HLA	Human Leukocyte Antigen
IL	Interleukin
INF	Interferon
Kb	Kilo Base
MBP	Myelin Basic Protein
MOG	Myelin Oligodendrocyte Glycoprotein
MRI	Magnetic Resonance Imaging
MS	Multiple Sclerosis
NK	Natural Killer
OD	Optical Density
PCR	Polymerase Chain Reaction
PI	Progression Index
PLP	Proteolipid Protein
PP	Primary Progressive
PR	Progressive Relapsing
RA	Rheumatoid Arthritis
RBC	Red Blood Cells
RFLP	Restriction Fragment Length Polymorphisms
RNA	Ribonucleic Acid
RR	Relapsing Remitting
SDS	Sodium Dodecyl Sulfate
SLE	Systemic Lupus Erythematosus
SNPs	Single Nucleotide Polymorphisms
SP	Secondary Progressive
Th	T Helper
Tm	Temperature Melting
TNF	Tumor Necrosis Factors
UV	Ultraviolet

WHO	World Health Organization
α	Alpha
β	Beta
µl	Microlitre

CHAPTER 1

INTRODUCTION

1.1 Background of the study

The pathologies of many autoimmune diseases are influenced by the profiles of cytokines. Inter individual differences in cytokine profiles appear to be due, at least in part, to allelic polymorphism within regulatory regions of cytokine gene. Many studies have examined the relationship between cytokine gene polymorphism, cytokine gene expression in vitro, and the susceptibility to and clinical severity of diseases (Bidwell *et al.*, 1999).

Multiple sclerosis (MS) is a chronic inflammatory autoimmune disease which affects the central nervous system (CNS) (Herndon, 2003; Korn, 2008). The migration of inflammatory immune cells through the brain blood barrier (BBB) leads to the accumulation of these cells from blood (Compston & Coles, 2008). The way this migration is regulated is different from that of inflamed organs that are outside the CNS (Viglietta *et al.*, 2004). Chronic brain inflammation is the culprit for the destruction of myelin sheaths that in turn reduce influx transmission and result in function loss (Gold *et al.*, 2006). When MS relapses occurs, pieces of white matter in the CNS turn inflamed and lose their myelin (Franklin, 2008). These demyelination patches, also referred to as lesions, commonly consist of tracts of myelinated neurons (Compston & Coles, 2008; Morales *et al.*, 2006).
MS is reportedly the second most widespread culprit for neurological disability among young adults (Pender & Greer, 2007). Ranging between 2 and 150 per 100,000, MS is prevalent everywhere in the world (Rosati, 2001).

Males are less prone to suffer from MS as compared with females as it is the case in most autoimmune diseases, which suggests that genetic or hormonal factors are traceable in this malady. A ratio of about 2:1 has been reported for females to males suffering from MS (Orton *et al.*, 2006; Whitacre, 2001).

Commonly the parts of the CNS that have been drastically myelinated indicate the symptoms of MS; however, these signs are often changeable. Patients usually fail to remember or find it hard to describe some of the signs like drowsiness, tingling sensations on the skin, or visual tracking problems (Noort, 2005).

MS patients may have one or several symptoms ranging between moderately and highly intense as well as between short and long in duration. Some of the most

typical signs of MS are as follows: hypoesthesia (changeable sensations), weak muscle, abnormal spasms of muscle (making it hard for the patient to move), coordination and balance problems, dysarthria (speech problems), dysphagia (swallowing difficulties), nystagmus, optic neuritis, phosphenes or diplopia (visual problems), feeling tired and syndromes of acute or chronic pain, problems with bladder and bowel, cognitive problems, or emotional symptomatology (commonly major depression) (van den Noort, 2005; Whitlock & Siskind, 1980).

The diagnosis of MS is based on both clinical parameters, such as medical history and neurological exam, and paraclinical parameters such as Magnetic resonance imaging (MRI), Cerebrospinal fluid (CSF) oligoclonal banding. MS cannot be detected based on a specific immune-based assay test (McDonald *et al.*, 2001; Miller, 1998).

Even though MS cannot be claimed to be directly hereditary, genetics variants are reportedly related with MS (Dyment *et al.*, 2004). It suggests the relatives of a patient suffering from MS are more probably prone to acquire the disease in comparison to the general population. This is particularly true about the patient's siblings, parents, or children. The risk of acquiring MS raises about 20 fold for the first degree family over the general population (Ebers *et al.*, 1986; Thorpe *et al.*, 1994). For more than three decades, scientists have been aware of the effect of genetic factors on MS ever since the discovery of the association with the HLA-DR2 locus (Olerup & Hillert, 1991). Additionally, research findings have recently identified the different types of genes that are positively related to MS (Hoffjan & Akkad, 2010).

A number of genetic studies looked for associations between MS and polymorphic alleles of candidate genes which were selected mainly on the basis of their involvement in the autoimmune pathogenesis and include immunorelevant molecules such as cytokines, cytokine receptors, immunoglobulins, T cell receptor subunits and myelin antigens (Matesanz *et al.*, 2004).

Cytokines play an important role in the initiation and maintenance of autoimmune diseases including MS (Illes *et al.*, 2008; Imitola *et al.*, 2005). On the other hand, Cytokine gene polymorphisms may affect their transcription, influence their level of production, and may be implicated in inducing susceptibility or resistance to the diseases (Trejaut *et al.*, 2004).

As a new pro-inflammatory cytokine, interleukin-21 (IL-21) has recently been observed as a target of intervention in many autoimmune diseases such as MS (Ettinger *et al.*, 2008a). In 2000, IL-21 was introduced as a novel subset of the type I four-α-helical-bundle cytokine family. The receptor of IL-21 has the typical

γ chain and signals via the Janus kinase (JAK)/ Signal Transducers and Activators of Transcription (STAT) pathway (Spolski & Leonard, 2008). Recent study showed that IL-21 play a vital role in the development of many autoimmune diseases including MS (Vollmer *et al.*, 2005).

IL-21, a relatively new member of the common gamma chain signaling class of cytokines, has attracted a great deal of interest in view of its multiple effects on the immune system. It shares the gamma chain subunit with a range of other cytokines (including IL-2, IL-4, IL-7, IL-9, IL-13 and IL-15), while specific interactions are mediated through its own receptor (IL-21R). IL-21 shares sequence identity with IL-15 and, more distantly, with IL-4 and IL-2 (Parrish-Novak *et al.*, 2000).

IL-21R is expressed by multiple cell types, including B and T lymphocytes, NK and NKT cells, dendritic cells, epithelial cells, endothelial cells, and fibroblasts (Asao *et al.*, 2001).

The human IL-21 gene is located on the 4q26–27 locus, together with the IL-2 gene and the mRNA product is 616 nucleotides long. This region has been linked to a range of inflammatory conditions by recent genome-wide association studies. It is a 8432-bp-long gene encoding for a 162-amino-acid polypeptide precursor forming a fully processed mature protein of 133 amino acids having a molecular weight of 15 kDa. It consists of five exons of which all are coding exons (Mehta *et al.*, 2004; Parrish-Novak *et al.*, 2002).

1.2 Problem Statement

Despite the prevalence of MS and increasing rate of the disease (as the second most common cause of neurological disability in young adult) and its clinical significance, the etiology of MS remains largely unknown. In recent years, the increasing incidence of MS in Iran has been a matter of interest for investigators. Iran has a medium-high prevalence of MS patients. Many studies have focused on the candidate gene studies to identify genes involved in susceptibility to MS. The role of cytokine gene polymorphisms in MS, as a chronic immune-mediated neurodegenerative disease, has been previously reported. Considering the importance role of IL-21 in MS pathogenesis, IL-21 gene could be considered as a candidate gene for susceptibility to MS. Therefore, this research was performed in order to probe whether there is any association in IL-21 [rs4833837 (C5250T) and rs2055979 (G1472T)] gene polymorphisms in Iranian population with MS.

1.3 Objectives

The main objective is:

To investigate whether IL-21 gene polymorphisms [rs4833837 (C5250T) and rs2055979 (G1472T)] are associated with susceptibility and clinical forms of MS.

The specific objectives are:

1. To assess the association of IL-21 gene polymorphisms [rs4833837 (C5250T) and rs2055979 (G1472T)] with clinical forms of disease, onset age, EDSS and PI in Iranian MS patients.

2. To determine the alleles and genotypes frequency of IL-21 gene polymorphisms [rs4833837 (C5250T) and rs2055979 (G1472T)] in Iranian subjects with MS and control group.

1.4 Hypothesis

The hypothesis in this study:

Polymorphisms of IL-21 gene [rs4833837 (C5250T) and rs2055979 (G1472T)] increase the risk of MS in Iranian population.

1.5 Significance of Study

The search for MS-related genes is important because their discovery will provide vital information on which biologic mechanisms influence the disease. This will lead to a better understanding of what causes MS and to the development of new approaches to treatment and prevention. In recent years, the number of confirmed disease-associated genes in MS is increasing, and many of them are shared with other autoimmune conditions. As it can be inferred, in most autoimmune diseases, like MS, common pathways play a role in their pathogenesis. Recently, increasing evidence suggests that the role of IL-21 gene polymorphism with multiple autoimmune diseases such as type 1 diabetes, rheumatoid arthritis (RA), Systemic lupus erythematosus (SLE) and atopic asthma.

This study determined the genotypic and allelic frequency of the IL-21 gene polymorphisms [rs4833837 (C5250T) and rs2055979 (G1472T)] in MS patients

among Iranian subjects. This genotypic and allelic information may be used as a genetic marker in Iranian subjects with MS before they develop the disease.

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