Association Study of Interleukin-15 (IL-15) Gene Polymorphisms in Rheumatoid Arthritis (RA)

By

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2008-NUST-BS V&I-30

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Applied Biosciences

Project Supervisor: Dr. Peter John

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National University of Sciences and Technology (NUST)

H-12, Islamabad, Pakistan

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National University of Sciences and Technology (NUST)

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Certificate

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I dedicate the outcome of my efforts to my parents Mr. and Mrs. Jahangir Ayub and my siblings Waqas and Arooj. I also dedicate my thesis to Mr. and Mrs. Shafqat, to whom I am greatly thankful for their encouragement and support.

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Nothing is deserving of worship except ALMIGHTY 'ALLAH', the most merciful, the most gracious. All praises for Him, because He is the creator of all that is in this universe. I am really thankful to ALLAH ALMIGHTY for all His Blessings and to His Prophet (P.B.U.H) for he (P.B.U.H) guided us to the right path: to the path of our creator.

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Sidrah Jahangir

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LIST OF ACRONYMS

American College of Rheumatology	ACR
Anti-Citrullinated Protein Antibody	ACPA
Atta-ur-Rahman School of Applied Biosciences	ASAB
Base pair	bp
Chemokine Ligand 21	CCL21
Cluster of Differentiation	CD
Complement Component 5-TNF Receptor-1 Associated Factor 1	C5-TRAF1
C-Reactive Protein	CRP
CXC-Chemokine Ligand 13	CXCL13
Cytokine T Lymphocyte Antigen 4	CTLA4
Deoxyribonucleic Acid	DNA
Deoxyribonucleotide Triphosphates	dNTPs
Disease-Modifying Anti-Rheumatic Drugs	DMARDs
Ethylenediaminetetraacetic Acid	EDTA
Figure	Fig.
Magnesium Chloride	$MgCl_2$
Major Histocompatibility Complex Class II	MHCII
Messenger Ribonucleic Acid	mRNA
Microliter	µl

Millilitre	ml
Millimeter	mm
Millimolar	mM
Nanogram	ng
Non-Steroidal Anti-Inflammatory Drugs	NSAIDs
Nuclear Factor-Kappa-B Inhibitor Like Protein 1	NFκBIL1
Peptidyl Arginine Deiminase, Type IV	PAD14
Percent	%
Picomolar	pm
Polymerase Chain Reaction	PCR
Protease Activated Receptor 2	PAR2
Rheumatoid Arthritis	RA
Rheumatoid Factor	RF
Signal Transducer And Activator of Transcription 4	STAT4
Single Nucleotide Polymorphisms	SNP
Sodium Dodecyl Sulphate	SDS
Statistical Practices for Social Sciences	SPSS
Transforming Growth Factor-β	TGFβ
Tris Borate EDTA	TBE
Tumor Necrosis Factor	TNF
Untranslated Region	UTR

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ABSTRACT

Rheumatoid arthritis is a chronic inflammatory disorder with a worldwide distribution involving all racial and ethnic groups. The incidence and prevalence of rheumatoid arthritis varies across different geographic regions of the world and across different time periods. It affects 0.5-1.0 per cent of the general population worldwide. In Pakistan it affects 0.1-0.2 per cent of the population. The disease can cause inflammation of one or more joints of the body. A number of cytokines regulate key inflammatory pathways that are involved in the progression of the disease. Interleukin-15 is one of the key cytokines in the pathogenesis of rheumatoid arthritis, which is secreted by synovial macrophages and is involved in the recruitment of inflammatory cells in the synovium, differentiation of osteoclast progenitors into preosteoclasts and regulation of the levels of other immunoregulatory molecule. The genetic polymorphisms, located in the regulatory regions of interleukin-15 gene might play a role in rheumatoid arthritis. The important role being played by interleukin-15 signifies the candidacy of this gene for disease pathogenesis. In this study, the association of two SNPs rs4956403 (SNP-1) and rs3806798 (SNP-2) located in the 5' UTR region of interleukin-15 gene was studied in 40 rheumatoid arthritis patients from Pakistani population. Allele specific PCR was employed for the analysis of these polymorphisms and the results were analyzed statistically for any significant association of these polymorphisms with rheumatoid arthritis. The results of our study showed that SNP-1 and SNP-2 in interleukin-15 gene are not associated with rheumatoid arthritis in our patients. However, more molecules implicated in the inflammatory pathway of rheumatoid arthritis need to be investigated for the genetic association with disease pathogenesis and other single nucleotide polymorphisms (SNPs) located in the regulatory and untranslated regions of inteleukin-15 gene may have significant association towards rheumatoid arthritis, for which large scale data is required.

Chapter 1

INTRODUCTION

Chapter 1

INTRODUCTION

Rheumatoid arthritis (RA) is a systemic chronic inflammatory disease. The disease principally targets synovial membranes, bones and joints. It has significant morbidity and mortality. The prevalence of the disease varies considerably across different geographic regions and time periods. It affects 0.5-1 per cent of the general population (Silman and Hochberg, 2001). The prevalence of RA in Pakistani population is 0.1-0.2 per cent (Baig, 2003). In 1997, RA accounted for almost 22 per cent of all the deaths caused by other forms of arthritis and other rheumatic diseases (Sacks *et al*., 2004). It is more common among females than among males (Myasoedova *et al*., 2010).

RA is a multifactorial disease and a number of factors contribute to the development and pathogenesis of the disease. The disease as a whole is the result of interrelated contributions from number of genetic and environmental factors. According to an estimate, the contribution of the genetic factors towards RA ranges from 50-60 per cent. (Seldin *et al*., 1999; MacGregor *et al*., 2000).

Some of the chromosomal regions that have been found to be significantly associated with RA include *1p13, 1q41-43, 6p, 6q16, 16p* and *18q* (Gregersen, 2005). The most important genetic risk factor for RA is Human leukocyte Antigen (HLA) locus. This locus accounts for almost 30-60 per cent of the RA genetic susceptibility (Bowes and Barton, 2008; Imboden, 2009).

Recently carried out Genome Wide Association Studies (GWAS) have emphasized on the association of single nucleotide polymorphisms (SNPs) with RA. These SNPs are particularly useful as every individual is uniquely characterized by a set of at least 3 million common SNPs that are distributed across the genome. These SNPs, in turn, can determine the response of an individual towards different environmental factors.

Major environmental factors that contribute to increased risk of RA include smoking, diet and hormones. Smoking, however, is usually considered the most important risk factor. Smoking can lead to 1.3-2.4 times increased risk of RA (Silman and Hochberg, 2001).

The early diagnosis of RA can lead to a better management of the disease. However, early diagnosis of the disease is difficult, as the early symptoms of RA are usually non-specific. These early symptoms include sudden weight loss, weakness, malaise, low-grade fever, muscle soreness and fatigue (Chan *et al*., 1994). The diagnosis of RA is usually made on the basis of a set of clinical, serological and radiological criteria. The American College of Rheumatology developed certain criteria that were revised in 2010. These criteria are mostly used for the diagnosis and classification of RA (Aletaha *et al*., 2010). The most important factors considered in this criteria are:

- Morning stiffness in and around the affected joints
- Arthritis of 3 or more joint areas
- Arthritis of hand joints
- Symmetric arthritis
- Rheumatoid nodules
- Serum rheumatoid factor (RF)
- Radiographic changes

Various drugs are being used for the treatment of RA. These drugs can reduce the pace of progression of the disease and can help in relieving the pain and inflammation. Corticosteroids/Non-steroidal anti-inflammatory drugs (NSAIDs) have been historically used for the treatment of RA. The patients are switched to non-biologic or biologic disease-modifying anti-rheumatic drugs (DMARDs) if no response is shown to NSAIDs. Some new drugs are now being tested that might have a better treatment efficacy than the already available drugs. These new drugs usually have molecular targets and are more specific in their action.

Deregulated immune system, leads to progressive inflammation and destruction of the joints in RA. In the first phase of the disease, which precedes the clinically detectable phase, rheumatoid factors are produced in the body of the patient. This phase can last for years and is sometimes referred to as pre-articular phase. This is followed by articular localization and articular synovitis which subsequently leads to clinical presentation of RA. This phase is referred to as articular phase. After the onset of articular phase, the synovial membrane becomes hyperplastic that comprises an interstitial zone containing a massive infiltrate of cells, and a superficial lining layer of synovial fibroblasts and macrophages. Adjacent cartilage is than invaded by the inflamed synovium and articular destruction is promoted. This further leads to the involvement of underlying bone marrow which exhibits inflammatory infiltrate. Articular damage probably generates a rich source of neo-antigens which further promote autoimmunity. Angiogenesis is a characterized feature of these rheumatoid joints and the articular environment is mainly hypoxic (Maini and Taylor, 2000; Firestein, 2003; McInnes *et al*., 2005, Jimenez-Boj *et al*., 2005).

Cytokines are important component of the immune system. These cytokines basically integrate the immune regulatory pathways that ultimately lead to different autoimmune diseases including RA. The key pro-inflammatory cytokines, involved in RA include Interleukin-1 (IL-1) and Tumor Necrosis Factor-alpha (TNFα). These cytokines although play key role in the pathogenesis of the disease, alone they do not provide a complete model of RA. T-helper cell (T_H cell) cytokines are the most important of all the cytokines that might play a role in RA (Firestein, 2003).

Interleukin-15 (IL-15) is an important T cell cytokine that shares structural similarity to interleukin-2 (IL-2). The receptor for these two cytokines is the same and is a complex composed of IL-12/IL-15 receptor beta chains (Cluster of differentiation-122, CD-122) and a common gamma chain (Cluster of Differentiaiton-132, CD-132). It is primarily secreted by mononuclear phagocytes. It regulates T- and natural killer cell activation and proliferation (Fig. 1.1).

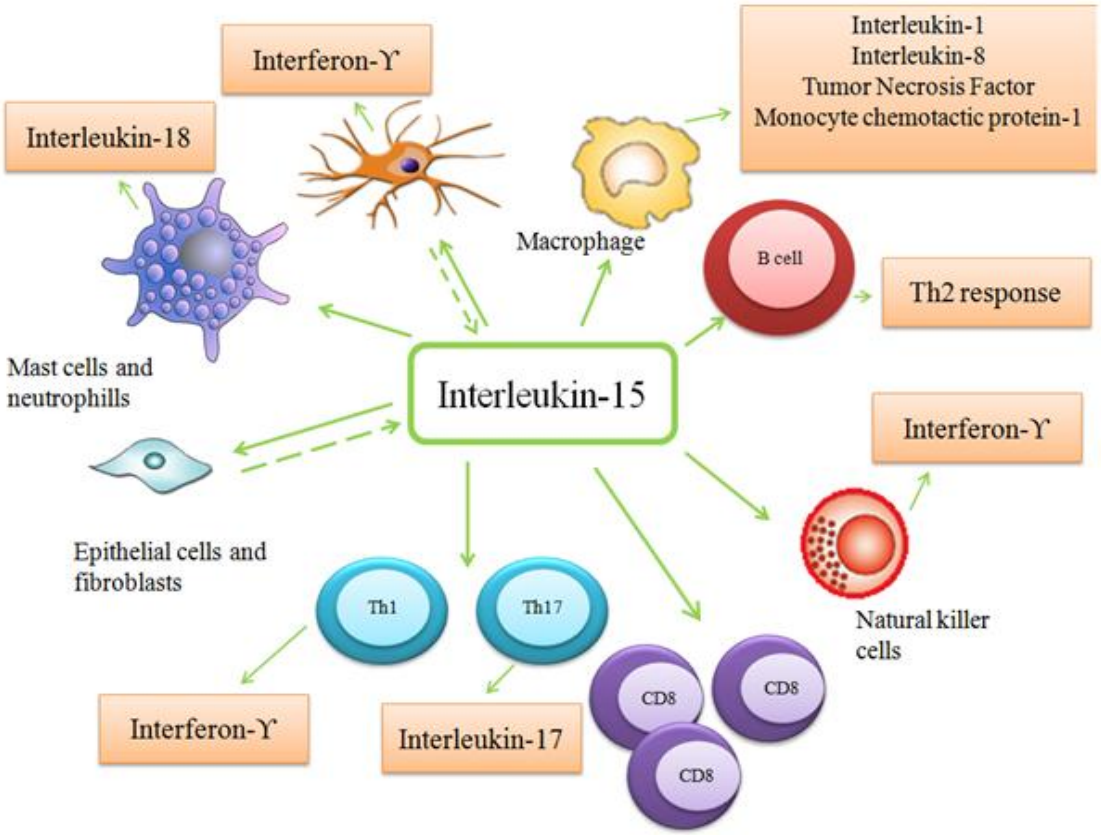


Figure 1.1: Schematic representation of target cells of interleukin-15 (IL-15): IL-15 plays an important role as mediator of several immune responses. It acts on a wide range of cells. These cells might in turn regulate the production of other cytokines.

The levels of IL-15 have been observed to be increased in RA as compared to other rheumatic diseases (Thurkow *et al*., 1997). It is produced by fibroblasts and macrophages in the inflamed synovial membrane during RA (McInnes *et al*., 1997). Increased levels of this cytokine may lead to the recruitment and activation of synovial T-cells (McInnes *et al*., 1996). It leads to the differentiation of the osteoclast progenitors and this is key step leading towards the bone deterioration (Ogata *et al.*, 1999). So, IL-15 plays a major role in the pathogenesis of RA.

IL-15 is produced by IL15 gene that is located at *4q31* chromosomal region. The expression of IL-15 is regulated at both transcriptional and post-transcriptional levels (McInnes and Liew, 1998). Genetic polymorphisms in the genes of the cytokines can lead to the variations in the levels of the proteins in the body (Ollier, 2004). Thus different genetic polymorphisms located in the regulatory regions of IL15 gene might play a role in different diseases of the immune system.

We therefore propose that the single nucleotide polymorphisms located in the regulatory regions of IL15 gene might play a significant role in the development and pathogenesis of RA in Pakistani population. Allele specific PCR was hereby employed, for determining any possible association of two different SNPs, located in the regulatory region of IL15 gene, in RA susceptibility and severity, in Pakistani population. The determination of the association between IL-15 gene polymorphisms and RA can lead to a better understanding of the pathogenesis of RA. The study will aid in the development of novel biomarkers that could assist in the early diagnosis of the disease. This study will also provide new therapeutic insights directed against the biomolecules, involved in the disease pathogenesis.

Chapter 2

REVIEW OF LITERATURE

Chapter 2

REVIEW OF LITERATURE

Rheumatoid arthritis (RA) is a chronic systemic inflammatory arthropathy. It primarily affects small diarthrodial joints of the hands and feet. RA is an autoimmune disease that mainly targets synovial membrane, cartilage and bone. The disease leads to the inflammation and destruction of the joints of the body, ultimately limiting the physical activity. RA is responsible for immature mortality and compromised lifestyle of the affected individuals (Brooks, 2006).

The incidence and prevalence of RA varies across different geographic regions of the world and across different time periods. It affects 0.5-1.0 per cent of the general population worldwide (Silman and Hochberg, 2001). In Pakistan it affects 0.1-0.2 per cent of the population (Baig, 2003). The occurrence of the disease is variable among different ethnic groups. (Lawrence *et al*., 1998; Gabriel *et al*., 1999; Alamanos and Drosos, 2005). RA can affect an individual at any stage of life, but the incidence increases with age and peaks between fourth and sixth decade of the life (Black and Hawks, 2005). It is usually more common among females than males. The prevalence of RA among women is 9.8 per 1000 but among men is 4.1 per 1000 (Myasoedova *et al*., 2010).

American College of Rheumatology (ACR) has developed certain criteria that are usually used for the diagnosis and classification of RA. The patients that are considered for further classification are those possessing at least 1 joint with definite clinical synovitis (swelling) that is not better explained by another disease. Scores are given on the basis of different criteria. The disease of the patient is then further classified on the basis of the overall score given (**Table 2.1**) (Aletaha *et al*., 2010).

The serological diagnosis is made on the basis of certain laboratory tests. These laboratory tests usually are based on the detection of definite serological markers that indicate the pathogenesis of the disease. The most important of these makers is rheumatoid factor (RF). This factor is present in very high amounts in most of the

patients. Anti-citrullinated protein antibodies (ACPAs) are likewise used as serological markers. Erythrocyte Sedimentation Rate (ESR) and C-reactive protein (CRP) test indicate inflammation and are also used for the diagnosis of RA.

RA is being treated by certain drugs, that actually slow down the progression of the disease. Disease-modifying anti-rheumatic drugs (DMARDs) are the most important drugs in this regard. Methotrexate, a DMARD, is commonly used for this purpose due to its low cost and affectivity. The drugs are well tolerated in many RA patients (Strand *et al*., 1999). More recently biologic agents are being focused due to their specificity and affectivity. The targets of biologic agents are usually the interactions occurring between the immune effector cells and the signaling molecules involved in their activation. These interactions, in case of RA, are in turn responsible for inflammation and structural damage in affected joints. The first approved biologic agents for the treatment of RA were directed against tumor necrosis factor (TNF). However, anti-TNF agents are not effective in all patients. There is still need of more exploration for developing of such targeted molecules that specifically but effectively treat the disease (Maini *et al*., 1999; Weinblatt *et al*., 1999; Weinblatt *et al.*, 2003).

RA is a multifactorial disease and it results from the interaction between different environmental and genetic factors. The contribution of the genetic elements has been suggested by familial aggregation, twin studies and segregation analysis (Cornelis *et al*., 1998). The disease is primarily characterized by incomplete penetrance, genetic heterogeneity and multiple disease genes (Cornelia and Jorg, 2000).

Human Leukocyte Antigen (HLA) locus is a well-established RA susceptibility locus. Association studies have suggested the involvement of the shared epitope in disease progression. This shared epitope, carried by vast majority of RA patients, is a five amino acid sequence motif in third allelic hypervariable region of the HLA-DRβ chain. The polymorphisms in HLA gene have been shown to play an important role in shaping the T cell repertoire and the repertoire abnormalities might in turn lead to susceptibility and severity of RA (Gregersen and Silver, 1987; Goronzy *et al*., 1998; Walser-Kuntz *et al*., 1995).

Table 2.1: 2010 American College of Rheumatology (ACR) classification criteria for rheumatoid arthritis (RA).

Criteria	Score Allotted
A. Joint involvement	
1 large joint	0
2-10 large joints	1
1-3 small joints (with or without involvement of large joints)	2
4-10 small joints (with or without involvement of large joints)	3
>10 joints (at least 1 small joint)	5
B. Serology (at least 1 test result is needed for classification)	
Negative RF *and* negative anti-citrullinated protein antibodies	0
Low-positive RF or low-positive anti-citrullinated protein antibodies	2
High-positive RF or high-positive anti-citrullinated protein antibodies	3
C. Acute-phase reactants (at least 1 test result is needed for classification)‡‡	
Normal C-reactive protein levels *and* normal Erythrocyte sedimentation rate	0
Abnormal C-reactive protein levels *and* normal Erythrocyte sedimentation rate	1
D. Duration of symptoms	
<6 weeks	0
≥6 weeks	1

Cytokine T lymphocyte antigen 4 (CTLA4) plays an important role in down regulation of T-cell activation and a noncoding variant located in 3' end of the gene encoding for CTLA4 has been frequently investigated in relation to RA (Rodriguez *et al.*, 2002; Vaidya *et al*., 2002).

Nuclear factor-Kappa-B Inhibitor like protein 1 (NFκBIL1) has been proposed to be involved in transcription of the pro-inflammatory cytokines involved in RA. The gene for NFκBIL1 is located near TNF gene and has been shown to be expressed in rheumatoid synovium. The protein is involved in messenger ribonucleic acid (mRNA) processing (Greetham *et al*., 2007). The association of a single nucleotide polymorphism (SNP) located in the promoter region of NFκBIL1 gene with RA has been reported (Okamoto *et al.*, 2003).

Signal transducer and activator of transcription 4 (STAT4) gene basically encodes for a transcription factor that is important in signal transmission in response to several key cytokines in RA (Joosten, 1997; Watford *et al*., 2004).

Peptidyl arginine deiminase, type IV (PADI4) on chromosome *1p35-36* was identified in Japanese population as a second risk factor for RA (Suzuki *et al*., 2003). The protein is involved in the conversion of arginine to citrulline. The gene that encoded, PADI4 has been shown to contribute towards RA susceptibility in Asian population (Ikari *et al*., 2005).

Complement component 5-TNF receptor-1 associated factor 1 (C5-TRAF1) on chromosome *9q33-34* was shown to be associated with RA susceptibility in a study performed in Netherland (Kurreeman *et al*., 2007). The involvement of complement pathway in the pathogenesis of RA has been suggested for more than 30 years. C5-TRAF1 might act through C5 and positive regulating the complement pathway.

Tumor necrosis factor alpha receptor 2 (*TNFR2*) gene, located at chromosomal position *1p36* has been implicated in many different studies for RA. Tumor necrosis factor alpha (TNFα) is a key cytokine in pathophysiology of RA, which suggests the candidacy of TNFR2 in RA. Tumor necrosis factor alpha induced protein 3 (TNFAIP3) is involved in the termination of TNF-induced signals. The

single nucleotide polymorphisms located near locus of TNFAIP3, *6q23*, have been reported to be associated with RA (Plenge *et al*., 2009).

Other genes, that have been shown to contribute significantly towards susceptibility and severity of RA include Fc receptor –like protein-3 (FCRL3) located at chromosomal position *1q21-22*, cluster of differentiation 24 (CD 244) gene located at *1q23.3*, angiotensin-converting enzyme (ACE) gene located at *17q23.3* and macrophage migration inhibitory factor (MIF) gene located at *22q11.23* (Cobankara *et al*., 2000; Baugh *et al*., 2002; Kochi *et al*., 2005; Bhat *et al*., 2006).

The environmental factors can also play a significant contribution to RA. Smoking, diet and hormones are the risk factors that are usually associated with RA (Oliver and Silman, 2006). Caffeine and decaffeinated coffee have been reported to increase the case of RA in most of the cases; however, the tea plays a protective role (Heliovaara *et al*., 2000; Mikuls *et al*., 2002). Vegetables, oil-rich fish and olive oil are believed to decrease the risk towards RA. Vitamin D is also known to play a protective role (Pattison *et al*., 2004; Merlino *et al*., 2004). Smoking has been known to be an important risk factor to RA that also contributes towards the disease severity and disease activity. Cigarette smoking has been linked to increased risk of RA. Heavy cigarette smoking is believed to increase the risk over 13-fold (Hutchinson *et al*., 2001; Papadopoulos *et al*., 2005).

The interplay between various genetic and environmental factors leads to onset of RA. The initial phases of the disease might not be clinically detectable. Autoimmunity that manifests as the production of rheumatoid factors is one of the hallmarks of RA. The inflammatory pathways involved in RA manifest mediators from, both, innate and adaptive immunity. The imbalance between the two leads to cellular damage, which in this case manifests itself as bone and joint destruction. Aberrant systemic selection, activation of T cells or both are driven via antigens presented by Major Histocompatibility Complex Class II (MHC II). Class II MHC is strongly associated with the risk of RA (Mulcahy *et al*., 1996). T cells are usually placed at the forefront of RA. It is believed that several SNPs in the regulatory and coding regions of various immunological mediators might lead to

the onset and progression of disease. Several antibodies, directed against immunoglobulin G (IgG) and cyclic citrullinated peptides have been detected in the serum of RA patients (Firestein, 2003). The presence of these antibodies indicates a loss of self-tolerance, which might form the basis of the disease. Autoimmunity combined with the intervention of several other factors leads to the clinically presentable stage of RA. During this phase, the synovial membrane (that lines the joints) becomes hyperplasic. This hyperplasic synovial membrane is characterized by a layer of synovial fibroblasts and macrophages that lines the interstitial zone. The interstitial zone contains a high density of cells including synovial fibroblasts, mast cells, macrophages, natural killer (NK) cells, T-cells, B-cells and plasma cells. The inflammation of the synovium then progresses to cartilage. This leads to the activation of osteoclasts, chondrocytes and synovial fibroblasts, causing articular damage. Bone marrow is subsequently involved and inflamed. The environment of a RA joint is generally hypoxic (Maini and Taylor, 2000; McInnes *et al*., 2005).

Cytokines play a key regulatory role in the pathogenesis of RA (Fig. 2.1). Generally the cytokines involved in rheumatoid arthritis can be divided into two main groups, the pro-inflammatory and anti-inflammatory cytokines. Among the pro-inflammatory cytokines, tumor necrosis factor (TNF) and interleukin-1 (IL-1) are key cytokines that play regulatory roles in chronic inflammation of the joint and associated cartilage and bone changes. TNFα is an inflammatory mediator and is arthritogenic even in membrane bound form (Georgopoulos S, 1996). IL-1 is a crucial cytokine in both arthritis and cartilage destruction. These cytokines have been potentially targeted in many studies for gaining therapeutic insights into RA.

RA is considered as a T_H1-associated disease. T-cell cytokines also play significant role towards susceptibility and severity of RA. Among these cytokines interleukin-15 (IL-15) occupies a very important place (Miossec and Berg, 1997; McInnes *et al*., 1997). Another important cytokine in this regard is interleukin-17 (IL-17) whose expression has been demonstrated in RA synovial tissue and is known to enhance the production of interleukin-6 (IL-6) (Chabaud *et al*., 1998; Chabaud *et al*., 1999). IL-17 might lead to osteoclastic bone resorption via receptor activator of

nuclear factor κB ligand (RANKL), a key regulator of osteoclastogenesis. Interleukin-12(IL-12) and interleukin-18 (IL-18) are potent Th1-driving cytokines that might play significant contribution towards inflammation in RA. These cytokines can induce the production of key pro-inflammatory cytokines in a T cell independent way. Interleukn-18 (IL-18) plays a pivotal role in the development of TH1 responses. IL-18 is IL-1 family protein and has been demonstrated in RA synovium and known to be expressed by articular chondrocytes and osteoblasts (Kotake, 1999; Kong *et al*., 1999; van Bezooijen *et al*., 1999; Gracie *et al*., 1999).

IL-15 is an innate response cytokine. It is a 15 kilo Dalton cytokine that consist of four alpha helices. IL-15 shares many biologic activities with T cell cytokine IL-2. IL-15 has broad functional pleiotropic and binds to a heterotrophic IL-15 receptor (IL-15R). The receptor consists of IL-15 receptor alpha chain (IL-15Rα), IL-15/ IL-2 receptor beta chain (IL-15/ IL-2Rβ) and a common gamma chain (Grabstein *et al.,* 1994; Bamford *et al*., 1994; Giri *et al.,* 1995). IL-15 is known to recruit and activate T-cells, maintain T cell memory, activate neutrophils, induce B-cell maturation, induce isotype switching and retard apoptosis of fibroblast-like synoviocytes (FLS) and endothelial cells (Waldmann and Tagaya, 1999; Zhang *et al.,* 1998; Lodolce *et al.,* 1998; McInnes and Liew, 1998). IL-15 is produced in considerable amounts by macrophages and fibroblasts in the rheumatoid synovial membrane. IL-15 has novel activity to stimulate the differentiation of osteoclast progenitors into preosteoclasts which is an important part of the pathogenesis of RA (McInnes *et al*., 1997; Ogata *et al*., 1999). IL-15 also recruits and activates CD45RO+ memory T cell subset in the synovial membrane. It thus induces TNFα production in RA and also triggers the production of interleukin-17 (IL-17) in RA (Fig. 2.2) (McInnes and Liew, 1998; Ziolkowska *et al*., 2000).

IL-15 is produced by IL-15 gene located at chromosomal position 4q31. The expression of this gene is regulated both at transcriptional and post-transcriptional levels (McInnes and Liew, 1998). Generally, it has been shown for many different cytokines that the level of expression of these cytokines is affected by different genetic polymorphisms located in the regulatory regions of these cytokines (Ollier, 2004). Thus, the genetic polymorphisms located in the regulatory regions of IL-15

might play a significant contribution towards determining the expression levels of this cytokine. These polymorphisms can lead to the heterogeneity in levels of IL-15 levels that have been observed in different individuals.

IL-15 thus plays a key role in the pathogenesis of RA. The increased levels of this cytokine in RA patients indicate the important role being played by this cytokine. This cytokine regulates the levels of many other cytokines as discussed earlier. There is a possibility that genetic polymorphism located in the regulatory regions of this cytokine might affect its expression. The altered expression might in turn determine the disease susceptibility and severity of RA.

The association of two different polymorphism located in the 5' UTR region of IL15 gene with Pakistani population was analyzed in Pakistani population. Allele specific PCR was used for this purpose and the result were then statistically analyzed for the determination of any significant association between the polymorphisms and RA.

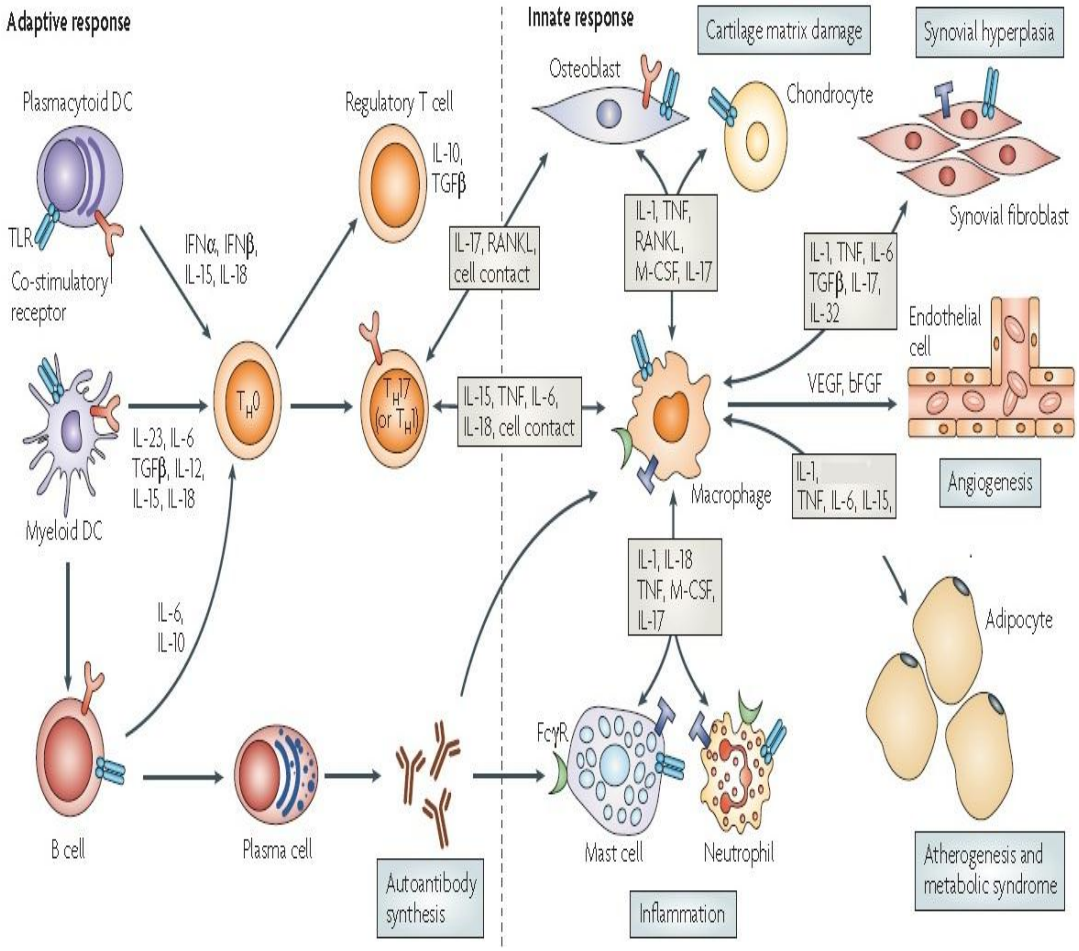


Figure 2.1: An outline of cytokine-mediated regulation of synovial interactions: Interplay between various cytokines leads to overall synovial damage, in rheumatoid arthritis (RA). Key cytokines are shown over here that play the key regulatory roles in RA. APRIL, a proliferation-inducing ligand; BAFF, B-cell activating factor; bFGF, basic fibroblast growth factor; CCL21, CC-chemokine ligand 21; CXCL13, CXC-chemokine ligand 13; FcγR, Fc receptor for IgG; IFN, interferon; IL, interleukin; LTβ, lymphotoxin-β; M-CSF, macrophage colony-stimulating factor; PAR2, protease activated receptor 2; RANKL, receptor activator of nuclear factor-κB (RANK) ligand; TGFβ, transforming growth factor-β;TH, T helper; TLR, Toll-like receptor; TNF, tumor-necrosis factor; VEGF, vascular endothelial growth factor (McInnes and Schett, 2007).

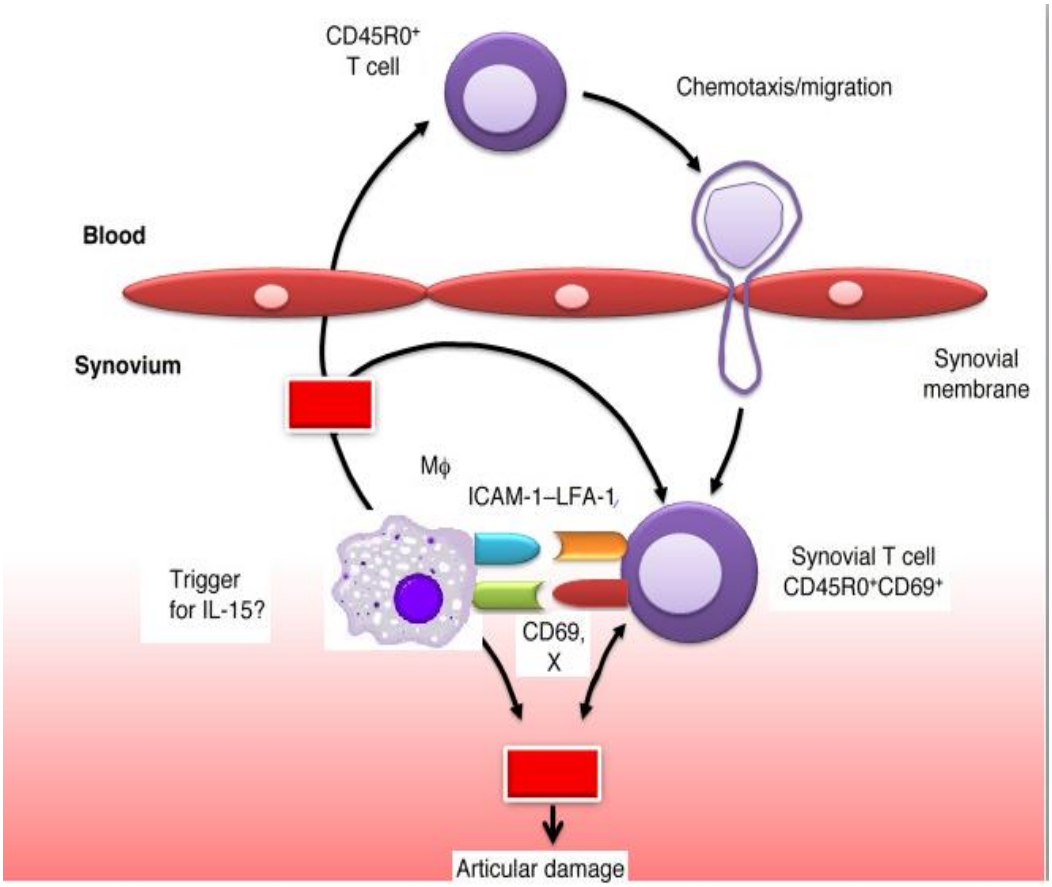


Figure 2.2: Interleukin-15 (IL-15) induced Amplification of synovitis in rheumatoid arthritis (RA): IL-15 mediates the recruitment and activation of T cells which eventually leads to the production of TNF-α. ICAM-1, intercellular adhesion molecule 1; IFN-γ, interferon γ; IL, interleukin; LFA-1, leukocyte function-associated molecule 1; Mφ, macrophage; TNF-α, tumor necrosis factor α (McInnesa, 1999).

Chapter 3

MATERIALS AND METHODS

Chapter 3

MATERIALS AND METHODS

3.1. Study subjects:

The study designed was a case control study. The individuals, recruited in the study, were from three different hospitals of Rawalpindi and Islamabad. The individuals included in the case group were clinically diagnosed patients of RA. The criterion used was American Criteria of Rheumatology and the confirmation was made on the basis of serological diagnosis. The individuals with any overlap with other rheumatic disease were excluded from the study. All study participants were included in the study after giving written informed consent. The characteristics of the individuals included in the study for SNP analysis have been summarized in Table 3.1.

3.1.1. Characteristics of Study Subjects for rs4956403 (C→T)/ SNP-1:

Percentage of the females and males included in case group for the analysis of SNP-1 was 80 per cent and 20 per cent respectively. The average age of the individuals included in the study was 46.38 ± 10.67 years.

The percentage of females, in the control group, was 77.5 per cent. The percentage of males, in this group, was 22.5 per cent. The average age of the healthy individuals included in the study was 32.68 ± 5.79.

3.1.2. Characteristics of Study Subjects for rs3806798 (T→A)/ SNP-2:

The percentage of females, in the case group for the analysis of SNP-2, was 77 per cent. The percentage of males, in this group, was 23 per cent. The average age of the RA patients included in the study was 46.9 ± 11.14.

Percentage of the males and females included in control group was 19 per cent and 81 per cent respectively. The average age of the healthy individuals included in the study was 31.6 ± 5.71 years.

Table 3.1: Summarized characteristics of rheumatoid arthritis (RA) patients included in the study:

SNP ID no.	Age (yrs)	Gender (percentage)		Age of onset			Duration of Symptoms			Duration of Morning Stiffness		Migrat--ory arthritis	Disease severity		
		Females	Males	<20 yrs	20-40 yrs	>40 yrs	>6 weeks	>6 months	>1 year	> ½ an hour	>1 hr		Minor	Moder ate	Severe
rs4956403	46.38 ± 13.11	80 %	20 %	25 %	43%	32 %	8 %	12 %	80 %	40 %	60 %	57%	48%	35%	17%
rs3806798	46.63 ± 13.46	77 %	23 %	24 %	40 %	36 %	7 %	13 %	80 %	40 %	60 %	57%	47%	33%	20%

3.2. Blood Sample Collection:

Venous blood samples were collected from the affected and normal individuals with the help of 5 ml (BD 0.6 mm X 25 mm, 23 G X 1 TW) and 10 ml syringes (BD 0.8 mm X 38 mm, 21 G X $1^{1/2}$ TW,). The samples were then collected in 10 ml Ethylenediaminetetraacetic acid (EDTA) tubes (BD vacutainer TM, Frankin Lakes, New Jersey, USA). EDTA tubes were then appropriately labeled. The label included identification (ID) number of the sample; name and age of the individual from which sample was being collected and the date and time of sample collection.

3.3. Sample Storage and Transport:

The samples were immediately dispatched to Immunogenetics laboratory (IGL), Atta-ur-Rahman School of Applied Biosciences - ASAB), National University of Sciences and Technology NUST), Islamabad, Pakistan. The samples were stored in EDTA tubes at 4 degree Celsius (˚C) before any further processing and genomic deoxyribonucleic acid (DNA) extraction.

3.4. Genomic DNA Extraction for Association Analysis:

For DNA extraction, the blood samples, stored in EDTA tubes, were incubated at room temperature for a very short interval of time. 750 µl of blood was then added into a 1.5 ml centrifuge tube (Axygen, California, USA). 750 µl of solution A was then added to the blood. The centrifuge tube was tightly closed and inverted 4-6 times and then incubated at room temperature for 5-10 minutes. The mixture of blood and solution A was centrifuged for 1 minute at 13,000 revolutions per minute (rpm) in a microcentrifuge (spectrafuge 24D Labnet, Edison, New Jersey, USA). Supernatant was carefully discarded and the nuclear pellet was re-suspended in 400 µl of solution A. The re-suspended pellet was centrifuged again for 1 minute at 13,000 rpm. The supernatant was discarded and the nuclear pellet was re-suspended in 400 µl of solution B, 12 µl of 20 % sodium dodecyl sulphate (SDS) and 5 µl of proteinase K. The mixture was incubated in a shaking incubator at 37 °C overnight.

Next day, a fresh mixture of equal volume of solution C and solution D was made and 500 µl of this mixture was added into it. These tubes were then centrifuged at 13,000 rpm for 10 minutes. After centrifugation, the aqueous phase was collected in a new tube. 500 µl of solution D was then added to the aqueous layer and was centrifuged again at 13,000 rpm for 10 minutes. The aqueous layer was collected again in a new, appropriately labeled, microfuge tube. DNA was precipitated by the addition of 55 µl sodium acetate (3M, pH 6.0) and 1000 µl of Ethanol. The tubes were then inverted several times to precipitate the DNA. The mixture was centrifuged again for 10 minutes at 13,000 rpm. The supernatant was discarded. 200 µl of 70% ethanol was added to the DNA pellet and centrifuged at 13,000 rpm for 7 minutes. The ethanol was then discarded The DNA pellet was dried for almost half an hour by keeping the microfuge tubes at 37 °C and then dissolved in 100 µl of TE buffer.

3.5. Solutions:

For DNA extraction, four different types of solutions named A, B, C and D were prepared (Table 3.2).

3.5.1. Solution A:

Solution used for the lysis of white blood cells (WBCs) was made by the addition of 0.32 M Sucrose, 10 mM Tris (pH 7.5) and 5 mM magnesium chloride ($MgCl_2$) to distilled water. More distilled water was then added to make up to the desired volume of the solution. This solution was then autoclaved and Triton X-100 (1% volume by volume) was added.

3.5.2. Solution B:

Solution B is used for the precipitation if DNA and separation of proteins was made by the addition of 10 mM Tris (pH 7.5), 400 mM sodium chloride (NaCl) and 2 mM Ethylenediaminetetraacetic acid (pH 8.0) to distilled water. More distilled water was then added to make the desired volume of the solution.

3.5.3. Solution C:

Phenol was used as solution C which helps in the separation of DNA into a separate layer.

3.5.4. Solution D:

Solution D was made as a mixture of chloroform and isoamyl alcohol in a volumetric ratio of 24:1. This solution is used for the purification of DNA

3.5.5. 1X Tris Borate Ethylenediaminetetraacetic (TBE):

1X tris borate EDTA (TBE) buffer was used for the stabilization and maintaining the pH during agarsoe gel electrophoresis that was initially prepared as a stock solution of 10X concentration. For the preparation of 10X TBE 890 mM of tris base, 890 mM of boric acid and 20 mM of EDTA (pH 8.0) were dissolved in distilled water. More distilled water was added until the desired volume was achieved. 1X TBE was then prepared from this stock solution. The final concentrations of tris base and boric acid in 1X TBE were 89 mM, 89 mM. The concentration of EDTA was 2 mM.

3.6. Primer Designing for Allele Specific Polymerase Chain Reaction (PCR):

SNPs selected for the association studies were located in 5' UTR region of IL15 gene. Primers were then manually designed for each SNP (Table 3.3). For each SNP, three primers were designed. Two forward primers contained the polymorphic nucleotide towards its 3' end, and amplification was only done in case of a perfect match. One of the possible nucleotides of polymorphism was included in one forward primer, while the other was included in the second primer. A common reverse primer was also designed for each SNP.

3.6.1. SNP-1:

This polymorphism is located in 5' UTR region of IL15 gene at position -831, +1 location being assigned to first base pair of first exon of IL15 gene.

Table 3.2: Recipes of Solutions used in DNA extraction:

Solution A	Solution B	Solution C	Solution D
0.32 M Sucrose 10 mM Tris (pH 7.5) 5 mM magnesium chloride (MgCl2) Triton X-100 1% (v/v)	10 mMTris (pH 7.5) 400 mM sodium chloride (NaCl) 2 mM Ethylenediaminetetraacetic acid (pH 8.0)	Phenol	Chloroform (24 volume) Isoamyl alcohol (1 volume)

Table 3.3: Primers designed for SNP-1 and SNP-2 located in the regulatory region of interleukin-15 (IL-15) gene. The mutated nucleotide has been indicated in red color.

Database Identification no.	Gene Location	Change	Forward Primers	Reverse Primers	Product size (base pairs)
rs4956403	5' UTR	C -> T	GGAAGACATCATTTTCTCTGAACC GGAAGACATCATTTTCTCTGAACT	ACAGGCTGCAAGAAGGAAACTTT	461
rs3806798	5' UTR	T -> A	AACATTTCCCCTAGTTGGACA AACATTTCCCCTAGTTGGACT	AAAGAGAAAGAGCCGGGAG	470

3.6.2. SNP-2:

This polymorphism is located in 5' UTR region of IL15 gene at position -475, +1 location being assigned to the first base pair of the first exon of IL15 gene.

3.7. Amplification by Allele Specific Polymerase Chain Reaction (PCR):

Allele specific PCR was carried out to check the presence of polymorphisms in the study population. For each SNP, two PCR reactions were carried out separately for each sample. Each reaction mixture contained one of the two forward primers but same common reverse primer. This allowed the amplification from the primer only when the target nucleotide was present.

The reaction mixture (25 µl) for PCR was prepared in 0.2 ml tubes (Axygen, California, USA) by adding 1 µl of sample DNA (50 ng/ µl), 2.5 µl (10X) of PCR buffer (Fermentas, Burlington, Canada), 1.5 µl of 25 mM Magnesium chloride ($MgCl_2$) (Fermentas, Burlington, Canada), 1.5 µl of 2 mM deoxyribonucleotide triphosphates (dNTPs) (Fermentas, Burlington, Canada), 1 µl of each forward and reverse primers (20 pm/µl), 0.2 µl of *Taq* DNA polymerase (1 unit) (Fermentas, Burlington, Canada) and 16.3 µl of PCR water. The reaction mixture was centrifuged at 8000 x g for 30 seconds for thorough mixing. Air bubbles were removed by gentle tapping.

The reaction mixture was then processed through thermocycling conditions in a 2720 thermal cycler (Applied Biosystem, Foster City, USA). Thermocycling conditions consisted of a template denaturation at 95 ˚C for 5 minutes followed by 35 cycles of PCR amplification. Each PCR cycle further consisted of 3 steps: 1 minute at 95 ˚C for the denaturation of template DNA strands into single strands, 45 seconds at 55 ˚C to allow the annealing of the primers to their respective target sites on DNA and 1 minute and 20 seconds for extension of the complementary DNA strand from the annealed primers. These 35 cycles were followed by synthesis of any unextended strands by *Taq* polymerase for 10 minutes at 72 ˚C. The PCR samples were then stored at -20 ˚C.

3.8. Analysis of Polymerase Chain Reaction (PCR) Products by Agarose Gel Electrophoresis:

Agarose gel electrophoresis was carried out for the analysis of extracted DNA and for PCR products. 1 per cent (w/v) agarose gel was prepared by melting 0.5 g of agarose in 50 ml of 1X TBE buffer in microwave oven for 2 minutes. Ethidium bromide solution (0.5 µg/ml final concentration) was then added for staining of DNA. The gel was allowed to solidify at room temperature in a gel cast tray. After that, the gel was placed in the buffer tank of electrophoretic apparatus. Extracted DNA was mixed with loading dye (0.25 per cent bromophenol blue in 40 per cent sucrose solution). The mixture was then careful loaded in the wells of agarose gel. Electrophoresis was performed at 100 Volts for almost half an hour in 1 X TBE buffer. DNA was analyzed by placing the gel in UV Transilluminator (Biometra, Goettingen, Germany). For the analysis of PCR products 2 per cent (w/v) agarose gel was prepared by melting 1 g of agarose in 50 ml of 1X TBE buffer in microwave oven for 2 minutes. Ethidium bromide solution (0.5 µg/ml final concentration) was then added for staining DNA. The gel was allowed to solidify at and then placed in the buffer tank of electrophoretic apparatus containing 1 X TBE as running buffer. PCR product was mixed with loading dye at a ratio of 1:6 (PCR product to the loading dye). The mixture was then careful loaded in the wells of agarose gel. Electrophoresis was performed at 100 Volts for almost 20 minutes in 1 X TBE buffer. DNA was analyzed by placing the gel in UV Transilluminator (Biometra, Goettingen, Germany).

3.9. Statistical Analysis:

Statistical analysis was performed using Statistical Practices for Social Sciences (SPSS) and Graphpad PRISM. Fischer exact test was applied for association analysis of the polymorphisms in the study population.

Chapter 4

RESULTS

Chapter 4

RESULTS

Association analysis of two different SNPs, located in 5' UTR region was carried out. RA patients and the healthy individuals were screened for the presence of the polymorphisms by allele specific PCR. The resulting data was then statistically analyzed for any significant association of these polymorphisms with rheumatoid arthritis.

4.1. Association Studies of SNP-1:

SNP-1 is located in the 5' UTR region of IL15 gene. 40 patients (cases) and 40 healthy individuals (controls) were analyzed for this polymorphism by allele specific PCR (Fig. 4.1 and Fig. 4.2). All the possible combinations of alleles were observed in the study subjects (Fig. 4.3). The observed allele frequencies and genotype frequencies have been illustrated in Table 4.1. Allele frequencies for RA patients were not significantly different from healthy control individuals. The frequency of all the possible genotypes didn't also show any significant difference in the patients and the healthy individuals

Hardy-Weinberg equilibrium (HWE) calculations were made for each group (Table 4.2). There was no significant difference between observed values and the expected values. *p*-value for the control group was found to be 0.24 and showed to be in HWE. *p*-value for case group was a bit higher, 0.36, however was in HWE and acceptable. This indicated that there is no significant drift in the observed allele frequencies of patients, when compared with the allele frequency of random healthy population. Both the groups were suitable for further analysis and association studies.

Chi square ($\chi 2$) test was used for the association analysis of rs4956403 with rheumatoid arthritis. Two tailed analysis was performed. The data was analyzed by Prism software. The value of any significant association was found to be 0.06 with a probability of error (p-value) of 0.97. The observed results, as descripted in Table

4.3, indicated no statistically significant association between the SNP and rheumatoid arthritis. Thus, the data nullified any significant association of the studied IL-15 gene polymorphisms with RA in Pakistani population.

The polymorphisms located in the regulatory regions of IL15 gene might play a significant contribution towards different disease attributes. The characteristics features of the disease, presented by the individuals recruited in the study (Fig. 4.4) were therefore analyzed for any association with this SNP. Age of the onset of RA, duration of the symptoms, duration of morning stiffness, presence of migratory arthritis and the disease severity were analyzed for any possible association with rs4956403. Each characteristic feature was categorized into different groups or ranks. The genotypes might influence the disease outcome differently in different genders; therefore the possible association of the genotype of RA patients and their sex was determined. The association analysis showed that the genotype of the individual was not linked to any of the disease attribute or the gender of the individual (Table 4.4).

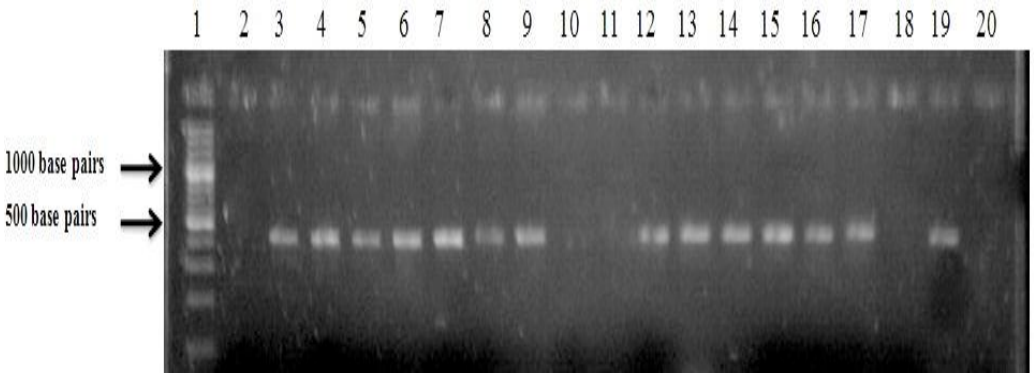
4.2. Association Studies of SNP-2:

SNP-2 is located, downstream to SNP-2, in the 5' UTR region of IL15 gene. 30 patients (cases) and 30 healthy individuals (controls) were analyzed for this polymorphism by allele specific PCR (Fig. 4.5 and Fig. 4.6). All the possible genotypes were observed in the study subjects (Fig. 4.6).The observed allele frequencies and genotype frequencies have been illustrated in Table 4.5. Allele frequencies for RA patients were not significantly different from healthy control individuals. The frequency of all the possible genotypes didn't show any significant difference between the patients and the healthy individuals.

Hardy-Weinberg equilibrium (HWE) calculations were made for each of the case group and the control group (Table 4.6). There was no significant difference between observed values and the expected values for both of the groups. *p*-value for the control group was found to be 0.26 and showed to be in HWE. Both the groups were suitable for further analysis and association studies.

Chi square (χ^2) test was used for the association analysis of SNP-2 with rheumatoid arthritis. Two tailed analysis was performed. The data was analyzed by Prism software. The value of any significant association was found to be 1.37 with a probability of error (*p*-value) of 0.51. The observed results, as shown in Table 4.7, indicated no statistically significant association between the SNP and rheumatoid arthritis. Thus, the data showed no significant association of the studied IL-15 gene polymorphisms with RA in Pakistani population.

The characteristics features of the disease, presented by the individuals recruited in the study (Fig. 4.8) were also analyzed for any association with this SNP. Age of onset of RA, duration of the symptoms, duration of morning stiffness, migratory arthritis and the disease severity were analyzed for any possible association with rs3806798. The association of different genotypes with different genders in the patients was also analyzed. The association analysis showed that the genotypes of the individuals were not linked to any of the disease attribute or the gender of the individuals (Table 4.8).



Lane 1: Ladder Lane 2: negative control Lane 3 and 4 : Positive control

Lane 5 and 6: Sample 1 (Heterozygous)

Lane 7 and 8: Sample 2 (Heterozygous)

Lane 9 and 10: Sample 3 (Homozygous for C)

Lane 11 and 12: Sample 4 (Homozygous for T)

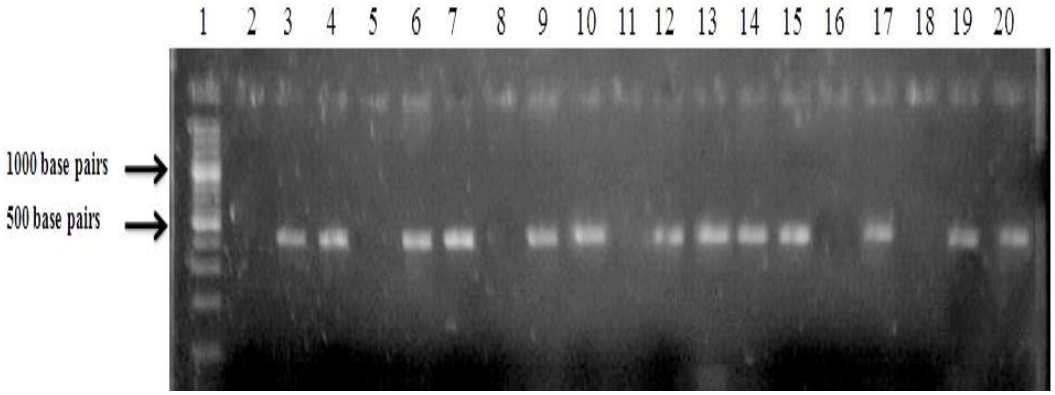
Lane 13 and 14: Sample 5 (Heterozygous)

Lane 15 and 16: Sample 6 (Heterozygous)

Lane 17 and 18: Sample 7 (Homozygous for C)

Lane 19 and 20: Sample 8 (Homozygous for T)

Figure 4.1: Electropherogram of ethidium bromide stained 2 per cent agarose gel for normal study subjects of SNP-1: The pattern of genotypes observed in the patient population has been shown for SNP-1. First band for each sample indicates C allele, while the second band indicates T allele.



Lane 1: Ladder Lane 2: negative control Lane 3 and 4 : Positive control

Lane 5 and 6: Sample 1 (Homozygous for T)

Lane 7 and 8: Sample 2 (Homozygous for C)

Lane 9 and 10: Sample 3 (Heterozygous)

Lane 11 and 12: Sample 4 (Homozygous for T)

Lane 13 and 14: Sample 5 (Heterozygous)

Lane 15 and 16: Sample 6 (Homozygous for C)

Lane 17 and 18: Sample 7 (Homozygous for C)

Lane 19 and 20: Sample 8 (Heterozygous)

Figure 4.2: Electropherogram of ethidium bromide stained 2 per cent agarose gel for affected study subjects of SNP-1: The pattern of genotypes observed in the normal healthy individuals has been shown for SNP-1. First band for each sample indicates C allele, while the second band indicates T allele.

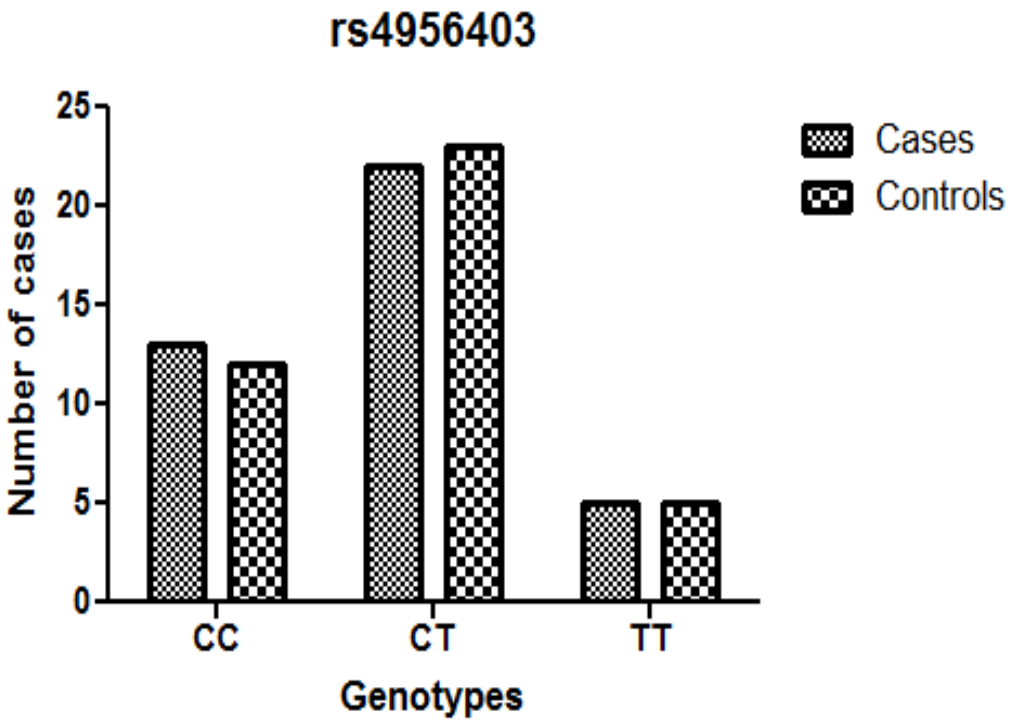


Figure 4.3: Distribution of the study subjects across different genotypes for SNP-1: Cases indicate rheumatoid arthritis (RA) patients and controls indicate healthy individuals.

Table 4.1: Allele and genotype frequencies for study subjects of SNP-1.

Cases/Controls	**Allele frequencies (%)**		**Genotype frequencies (%)**		
	C	T	CC	CT	TT
RA patients (*n = 40*)	60	40	32.50	55	12.50
Healthy controls (*n = 40*)	58.75	41.25	30	57.50	12.50

Table 4.2: Hardy-Weinberg equilibrium (HWE) calculations for the test population of SNP-1.

	Observed Genotype Counts			Observed Allele Counts		Expected Genotype Counts under HWE			χ^2	*p*-value
	CC	CT	TT	C	T	CC	CT	TT		
RA Patients (cases)	13	22	5	60%	40%	14.40	19.20	6.4	0.85	0.36
Healthy Individuals (controls)	12	23	5	59%	41%	13.80	19.40	6.80	1.39	0.24
Total	25	45	10	59%	41%	28.20	38.60	13.20	2.20	0.14

Table 4.3: Statistical association between SNP-1 and Rheumatoid Arthritis.

Statistical association	df	p-value
0.06222	2	0.9694

Table 4.4: The association of SNP-1 gene polymorphisms with different clinical features of rheumatoid arthritis (RA) and sex of the patient.

Serial number	Characteristic feature	Ranks/ Groups	Genotype			Value of statistical association	*p*-value
			CC	CT	TT		
1	Age of onset	<20 years 20-40 years >40 years	13	22	5	4.857	0.3022
2	Duration of symptoms	>6 weeks >6 months >1 year	13	22	5	3.334	0.5035
3	Duration of morning stiffness	>1 hour >1/2 an hour	13	22	5	0.7343	0.6927
4	Presence of Migratory arthritis	Yes No	13	22	5	1.037	0.5955
5	Disease severity	Minor Moderate Severe	13	22	5	1.584	0.8116
6	Sex	Male Female	21	15	4	4.107	0.1283

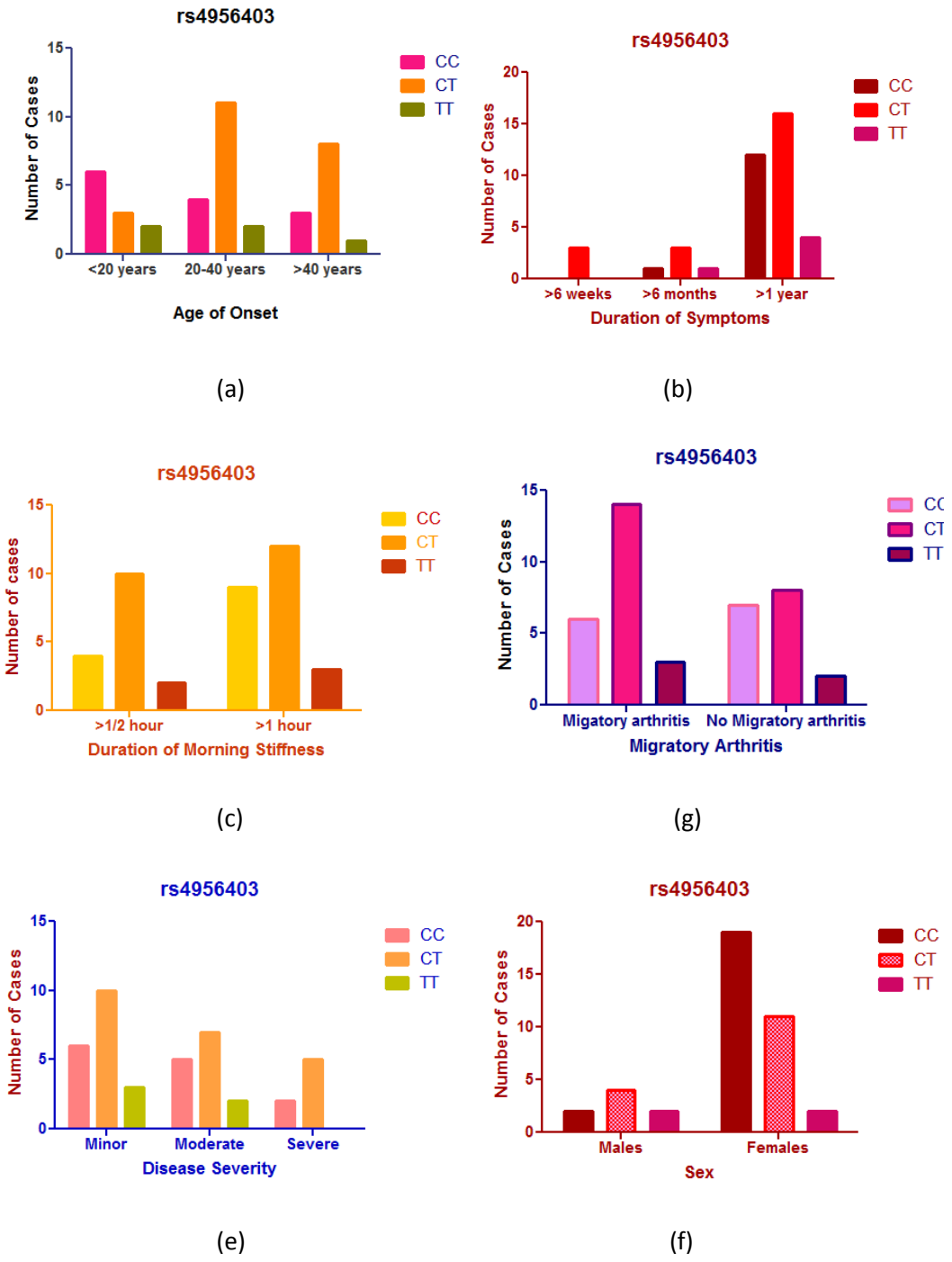
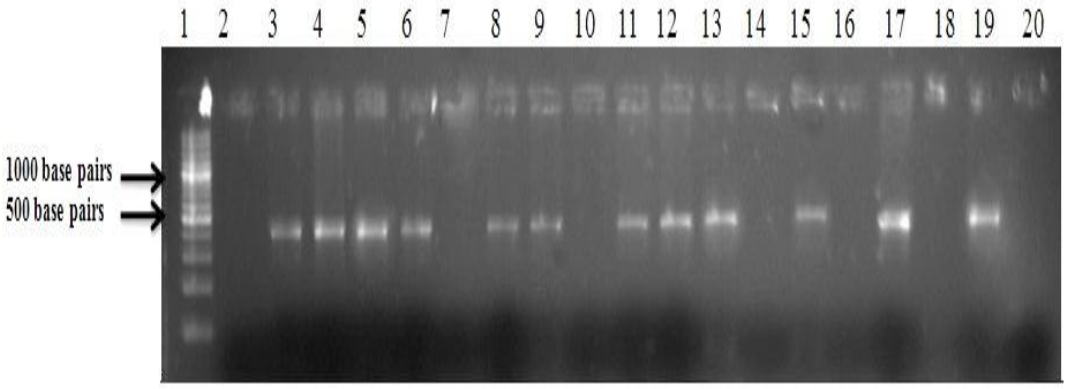


Figure 4.5: Distribution of different genotypes with respect to SNP-1 across different disease attributes.



Lane 1: Ladder Lane 2: negative control Lane 3 and 4 : Positive control

Lane 5 and 6: Sample 1 (Heterozygous)

Lane 7 and 8: Sample 2 (Homozygous for A)

Lane 9 and 10: Sample 3 (Homozygous for T)

Lane 11 and 12: Sample 4 (Heterozygous)

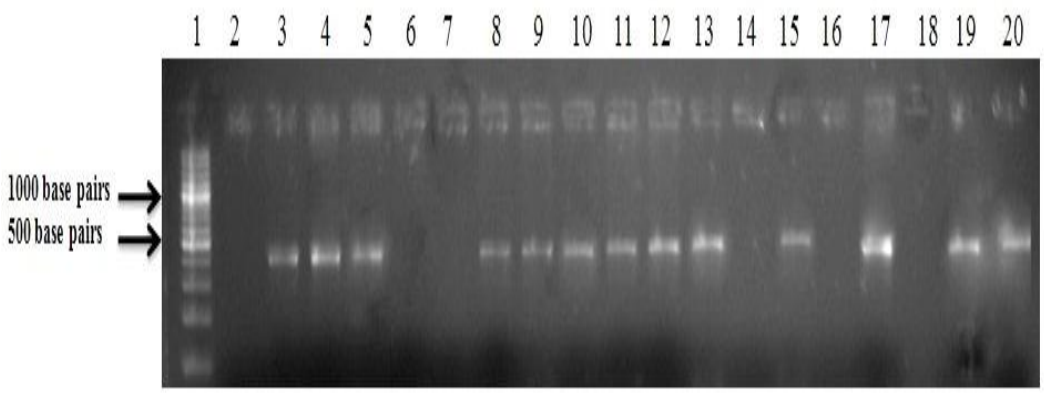
Lane 13 and 14: Sample 5 (Homozygous for T)

Lane 15 and 16: Sample 6 (Homozygous for T)

Lane 17 and 18: Sample 7 (Homozygous for T)

Lane 19 and 20: Sample 8 (Homozygous for T)

Figure 4.5: Electropherogram of ethidium bromide stained 2 per cent agarose gel for normal study subjects of SNP-2: The pattern of genotypes observed in the patients included in the study has been shown for SNP-2. First band for each sample indicates C allele, while the second band indicates T allele.



Lane 1: Ladder Lane 2: negative control Lane 3 and 4: Positive control

Lane 5 and 6: Sample 1 (Homozygous for T)

Lane 7 and 8: Sample 2 (Homozygous for A)

Lane 9 and 10: Sample 3 (Heterozygous)

Lane 11 and 12: Sample 4 (Heterozygous)

Lane 13 and 14: Sample 5 (Homozygous for T)

Lane 15 and 16: Sample 6 (Homozygous for T)

Lane 17 and 18: Sample 7 (Homozygous for T)

Lane 19 and 20: Sample 8 (Heterzygous)

Figure 4.6: Electropherogram of ethidium bromide stained 2 per cent agarose gel for affected study subjects of SNP-2: The pattern of genotypes observed in the normal healthy individuals has been shown for SNP-2. First band for each sample indicates C allele, while the second band indicates T allele.

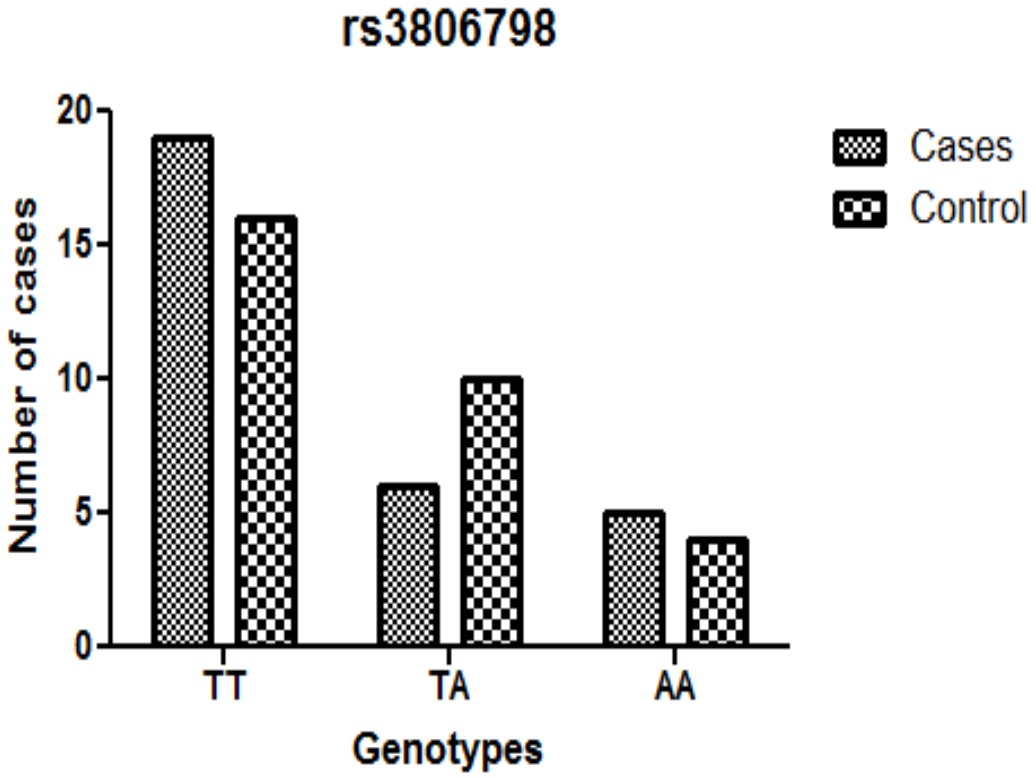


Figure 4.7: Distribution of the study subjects across different genotypes for SNP-2: Cases indicate rheumatoid arthritis (RA) patients and controls indicate healthy individuals have been shown by separate bars.

Table 4.5: Allele and genotype frequencies for study subjects of SNP-2.

Cases/Controls	Allele frequencies (%)		Genotype frequencies (%)		
	T	A	TT	TA	AA
RA patients (*n = 40*)	73.33	26.67	63.33	20	16.67
Healthy controls (*n = 40*)	70	30	53.33	33.33	13.33

Table 4.6: Hardy-Weinberg equilibrium (HWE) calculations for the test population for SNP-2.

	Observed Genotype Counts			Observed Allele Counts		Expected Genotype Counts under HWE			χ^2	*p*-value
	TT	TA	AA	T	A	TT	TA	AA		
RA Patients (cases)	19	6	5	73.33 %	26.67 %	18.60	6.80	0.60	0.34	0.56
Healthy Individuals (controls)	16	10	4	70 %	30 %	14.70	12.60	2.70	1.28	0.26
Total	35	16	9	71.67 %	28.33 %	30.80	24.40	4.80	7.07	0.01

Table 4.7: Statistical association between SNP-2 and Rheumatoid Arthritis (RA).

Statistical association	df	p-value
1.37	2	0.51

Table 4.8: The association of SNP-2 with different clinical features of rheumatoid arthritis (RA) and sex of the patient.

Serial number	Characteristic feature	Ranks/ Groups	Genotype			Value of statistical association	*p*-value
			TT	TA	AA		
1	Age of onset	<20 years 20-40 years >40 years	19	6	5	3.96	0.41
2	Duration of symptoms	>6 weeks >6 months >1 year	19	6	5	4.85	0.30
3	Duration of morning stiffness	>1 hour >1/2 an hour	19	6	5	2.69	0.28
4	Migratory arthritis	Yes No	19	6	5	5.08	0.08
5	Disease severity	Minor Moderate Severe	19	6	5	5.80	0.21
6	Sex	Male Female	19	6	5	3.02	0.22

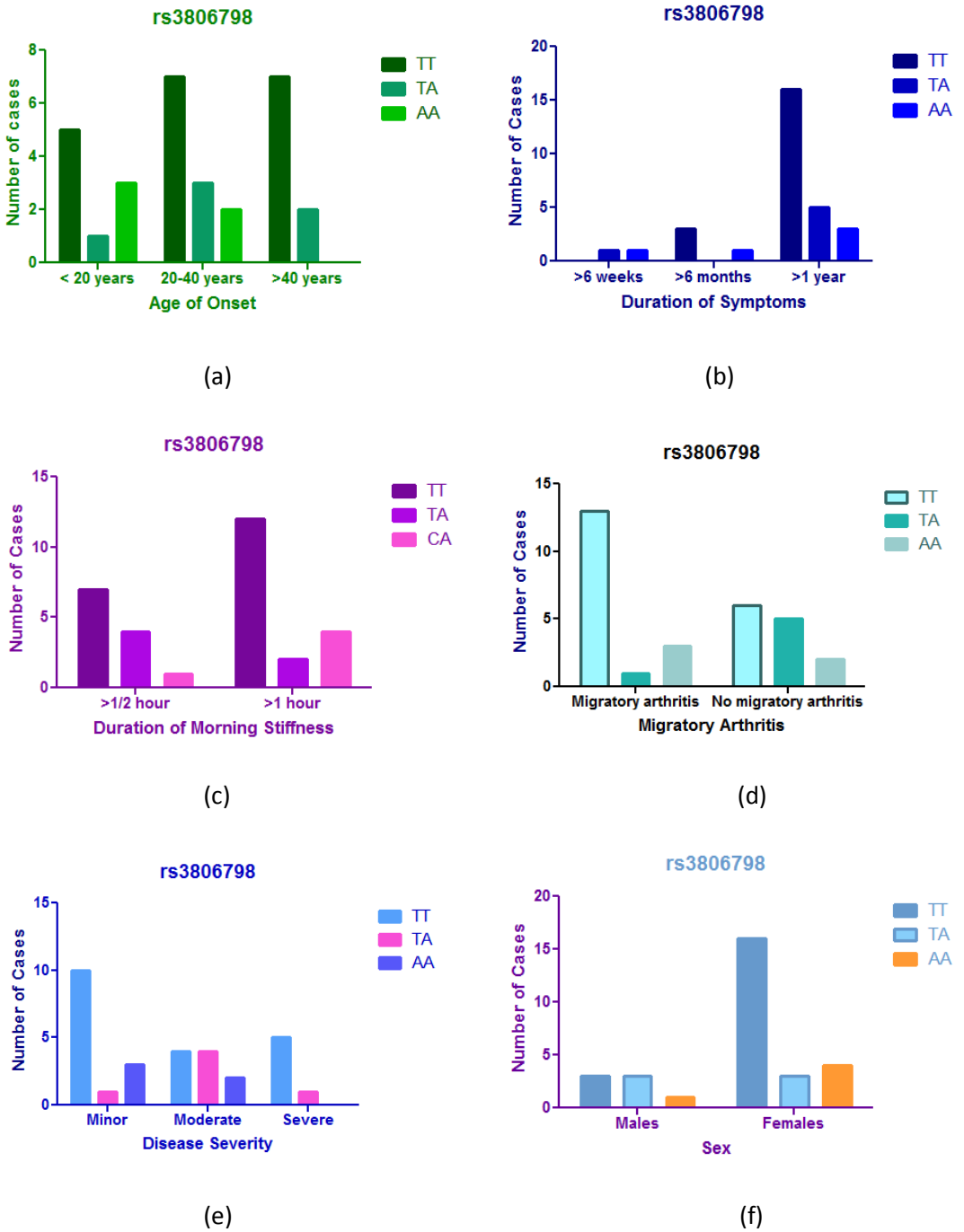


Figure 11: Distribution of different genotypes with respect to SNP-2 across different disease attributes.

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Chapter 5

DISCUSSION

Chapter 5

DISCUSSION

Rheumatoid arthritis is an inflammatory disease and various cytokines are involved in the regulation of key pathways during the disease pathogenesis. IL-15 is a T_H1 cytokine and is produced by synovial macrophages. It belongs to four α-helix bundle family of cytokines. It plays a very important role in the development of various autoimmune and allergic disorders (Waldmann and Tagaya, 1999; Fehniger and Caligiuri, 2001). The levels of this cytokine are known to increase significantly in RA patients (Thurkow *et al.*, 1997). IL-15 is encoded by IL15 gene located at 4q31 and the expression of this cytokine is known to be regulated at both pre-transcriptional and post-transcriptional levels. SNPs located in the regulatory regions of this cytokine can therefore play a significant contribution towards pre-disposition towards RA.

IL-15 has been studied for genetic association with a number of autoimmune and allergic disorders. IL-15 has been long recognized as a strong candidate gene for psoriasis that is an autoimmune disease. SNPs located in IL15 gene have been shown to be associated with the pathogenesis of the disease, probably by altering the levels of inflammatory cytokines in the lesions of psoriasis (Zhang, 2007).

IL15 gene and IL15Rα gene polymorphisms have been shown to be associated to different aspects of diseases and phenomenon, including skeletal muscle, responses to resistance exercise, obesity, CD4+ T cell activity and liver graft rejection (Warle *et al*., 2002; Kurz *et al*., 2004; Riechman *et al*., 2004; Christensen *et al*.,2006; Di Renzo *et al*., 2006; Haas *et al*., 2006). The SNPs located in IL15 gene might affect the characteristic clinical features of the disease as these polymorphisms can lead to altered levels of this cytokine. These altered levels can in turn determine the extent of damage caused by the effector cell in the inflamed synovium and rheumatoid joints. These polymorphisms can thus determine the clinical presentation of RA. The association of these polymorphisms was therefore studied in relation to different characteristic features of RA. These characteristic

features included age of onset of the disease, duration of symptoms, presence of migratory arthritis, the duration of morning stiffness and the severity of the disease. The association analysis of the observed genotypes and the occurrence and extent of these clinical features indicated that there was no significant association between these clinical feature of the disease and IL15 gene polymorphisms being studied. This indicates that the actual genetic determinants of the clinical features of RA are located either at other positions in IL15 gene or they might be located in the genes of other candidate genes for RA.

The SNPs located in IL15 gene have been shown to be associated with predictors of metabolic syndrome; phenotypes of muscle, bone and strength and the response to resistance training. IL-15 polymorphisms are associated with these in a sex dependent manner (Emidio *et al*., 2008). Sex-specific association of rs3806798 has also been found in handgrip strength (Dato, 2010). RA is more common among females. The polymorphisms that affect the regulation of key regulatory molecules involved in the pathogenesis of RA might contribute towards RA in a sex-dependent manner. The association of rs4956403 and rs3806798 has therefore been analyzed in RA patients in relation to the sex of the patient, in our study. The results of the statistical analysis showed there was no significant difference between RA affected females and males in relation to the occurrence of these polymorphisms. This indicated that the studied polymorphisms do not affect the levels of IL-15 in a sex dependent manner in case of RA. Therefore, there is no link of these polymorphisms to the increased incidence of RA among females.

IL-15 is a key cytokine involved in the inflammatory pathways that ultimately lead to the inflammation and subsequent destruction of the joints in RA. However, the polymorphisms located in 5'-UTR region didn't show any significant association to the disease. The results of this study and previously carried out studies in relation to RA indicate that other key cytokines, that regulate the levels of IL-15, are genetically linked to RA. The polymorphisms located in the regulatory regions of these cytokines, must therefore be investigated, for any possible association with the RA. Moreover, the polymorphisms located in the introns and 5' UTR region of

IL15 gene needs to be investigated for any possible association with RA, in Pakistani population.

RA is basically an ethnic disease. The prevalence of the disease has been shown to be different in different geographic regions of the world and in different time periods (Lawrence *et al*., 1998; Gabriel *et al*., 1999b; Alamanos and Drosos, 2005). The genetic determinants of RA in one population might be different from the genetic determinants of the disease in other populations. Therefore, in spite of the fact that IL15 gene polymorphisms are associated with RA in Spanish population and Pakistani population, these polymorphisms can be studied for any possible association with RA in European population.

In this study, the association of two SNPs rs4956403 and rs3806798 located in the 5' UTR region of IL15 gene was studied in relation to RA. Allele specific PCR was employed for the analysis of these polymorphisms in Pakistani population and the results were then analyzed statistically for any significant association of these polymorphisms with RA.

The results of our study indicated no significant association of any of the studied IL15 gene polymorphisms in RA patients of Pakistani origin. These results are consistent with a previous association study conducted in Spanish population, where 13 different SNPs were analyzed in association to RA. These 13 SNPs included six SNPs located in 5' UTR region, three SNPs located in the intronic region and four located in 3'UTR region. The resulting data indicated no significant difference in the distribution of the observed genotypes in RA patients and healthy individuals. rs4956403 and rs3806798 located in the 5' UTR region of IL15 gene, were also analyzed in that study and were not found to be significantly associated with RA (Rueda, 2007).

5.1. Conclusions and Future Prospectus:

In summary, the results of our study showed that SNP1 and SNP2 in IL15 gene are not associated with RA in studied Pakistani patients. However, other molecules implicated in the inflammatory pathway of RA, need to be investigated for genetic

association with RA pathogenesis. Other polymorphisms of IL-15 located in the regulatory and untranslated regions may have significant association towards RA, for analysis of which large scale data is required.

Chapter 6

REFERENCES AND APPENDIX

Chapter 6

REFERENCES

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APPENDIX

APPENDIX I: Detailed characteristics of rheumatoid arthritis (RA) patients included in the study.

S. no.	Sample ID	SNP-1	SNP-2	Age	Gender	Age of onset	Duration of symptoms	Duration of morning stiffness	Migratory arthritis	Degree of disease severity
1	RA102	CC	AA	50	Female	< 20 years	>1 year	>1 hour	No	Moderate
2	RA103	CC	TT	35	Female	20-40 year	>1 year	>1 hour	Yes	Minor
3	RA104	CT	TT	33	Male	< 20 years	>1 year	>1 hour	Yes	Minor
4	RA105	CT	TT	55	Female	>40 years	>1 year	>1/2 hour	Yes	Severe
5	RA107	TT	TT	40	Female	20-40 years	>1 year	>1 hour	Yes	Moderate
6	RA187	CT	TT	46	Female	20-40 years	>1 year	>1 hour	yes	Minor
7	RA112	CC	TT	75	Female	30-40 years	>1 year	>1 hour	No	Severe
8	RA108	CT	TT	30	Female	<20 years	>1 year	> ½ hour	yes	Severe
9	RA120	CC	TT	39	Female	< 20 years	>1 year	>1/2 hour	No	Moderate
10	RA119	CT	TT	35	Female	20-40 years	>1 year	> 1 hour	No	Severe
11	RA110	CT	TA	40	Female	20-40 years	>6 weeks	> ½ hour	No	Moderate
12	RA113	CT	TT	75	Male	>40 years	>1 year	> 1 hour	Yes	Minor
13	RA114	CC	TT	23	Female	< 20 years	>1 year	>1 hour	No	Minor
14	RA111	CC	TA	62	Male	>40 years	>1 year	>1/2 hour	No	Moderate
15	RA118	CT	AA	35	Female	20-40 years	>6 weeks	>1 hour	Yes	Minor
16	RA116	CT	TT	60	Female	>40 years	>1 year	>1/2 hour	No	Minor
17	RA124	CT	-	35	Female	20-40 years	>1 year	>1 hour	Yes	Minor
18	RA180	CC	TT	65	Female	>40 years	>1 year	>1 hour	Yes	Minor
19	RA74	CT	-	30	Female	<20 years	>1 year	>1 hour	Yes	Severe
20	RA184	CT	-	56	Male	>40 years	>6 weeks	>1 hour	No	Moderate
21	RA2	TT	-	45	Female	>20 years	>1 year	>1 hour	No	Moderate
22	RA183	CT	-	43	Female	20-40 years	>1 year	> ½ hour	Yes	Minor
23	RA109	CT	-	33	Female	20-40 year	>1 year	> ½ hour	yes	Moderate
24	RA9	CT	-	70	Female	>40 years	>6 months	> 1 hour	No	Minor
25	RA7	CT	-	59	Female	>40 years	>1 year	>1/2 hour	Yes	Minor
26	RA75	CT	-	45	Female	20-40 years	>1 year	>1 hour	Yes	Moderate
27	RA15	CT	-	40	Female	20-40 years	>1 year	> ½ hour	No	Minor
28	RA101/	CC	TT	35	Female	< 20	>1 year	>1 hour	Yes	Modera

	11					years				te
29	RA1	TT	TT	46	Male	>40 years	>6 months	>1/2 hour	Yes	Minor
30	RA10	CT	AA	62	Female	20-40 years	>6 months	>1/2 hour	No	Modera te
31	RA8	CC	TT	65	Female	>40 years	>1 year	> 1 hour	No	Minor
32	RA191	CC	TA	45	Female	20-40 years	>1 year	>1 hour	No	Severe
33	RA188	TT	TA	43	Male	20-40 years	>1 year	>1/2 hour	No	Minor
34	RA3	CC	TA	40	Female	>20 years	> 1 year	> ½ hour	yes	Modera te
35	RA5	CC	TT	33	Female	20-40 years	>6 months	> ½ hour	Yes	Minir
36	RA12	CT	TT	50	Female	20-40 years	>1 year	> 1 hr	Yes	Severe
37	RA6	CT	TT	55	Female	>40 years	> 6 months	>1/2 hoyrs	Yes	Modera te
38	RA16	CC	AA	33	Male	< 20 years	> 1 year	> 1h	Yes	Minor
39	RA20	TT	AA	44	Female	< 20 years	>1 year	>1 hour	Yes	Minor
40	RA122	CT	TA	50	Male	>40 years	>1 year	> 1 hour	No	Modera te