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NOVEL DISEASE SEVERITY FACTORS IN SYSTEMIC LUPUS ERYTHEMATOSUS: A PROFILE OF MANNOSE BINDING LECTIN GENE POLYMORPHISMS, NEPHRIN AUTOANTIBODIES AND BRAIN-REACTIVE AUTOANTIBODIES

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NATIONAL UNIVERSITY OF SINGAPORE

2005

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SUMMARY

The aim of this post graduate thesis is to study novel disease severity factors in Systemic Lupus Erythematosus (SLE) relating to infections, and involvement of the kidney and brain. Although the pathogenesis of SLE is not fully understood, the management of SLE has dramatically improved over the last decade with use of various therapeutic agents. Lupus patients are now expected to live longer compared to those of earlier days. However, the number of hospitalizations of lupus patients is still significant. The predominant causes of hospitalizations were infections and major organ complications.

It is reasonable to conclude that more lupus patients are seen with infections because of the use of immunosuppressive drugs in lupus management. In innate defense mechanisms, complement proteins are generally well regarded, as "important proteins" against pathogens. They also play a role in clearance of immune complexes and complement deficiencies are associated with SLE. Mannose Binding Lectin (MBL) is from the lectin pathway of complement system. Its deficiencies were associated with infections in both children and adults. It was also interesting to note that MBL plays an important role against infections in patients on immunosuppressive therapy. We therefore studied the different variants of genotypes and haplotypes of MBL, especially structural mutants and MBL low-producing haplotypes, to determine its predisposition to infection. Recurrent infections worsen disease severity in lupus patients on immunosuppressive therapy.

Proper management of lupus patients on immunosuppressive drugs can reduce the disease severity and increase the survival rate of lupus patients. However organ damages are still significant morbidity and mortality issues facing lupus patients. Among many suggested

mechanisms causing organ-system damages, autoantibodies against structural systems in the kidneys and brain are thought to play important roles. However it is not clear how these autoantibodies are involved in the pathogenesis of such organ involvement.

The significant finding of renal involvement in lupus is proteinuria, which is widely been used as a diagnostic, prognostic and disease-monitoring tool in the management of lupus nephritis. Recently a monoclonal antibody (mAb 5-1-6), which caused immediate transient and massive proteinuria in animal models upon intravenous injection, was reported as targeting against nephrin, a structural protein of slit diaphragm. We therefore studied nephrin autoantibodies in lupus patients to determine the role these autoantibodies play in disease severity in lupus nephritis patients. The role of brain reactive autoantibodies in lupus has been studied. However no significant autoantibodies that can be used in diagnosis and management of neuropsychiatric lupus are yet defined. We looked into autoantibodies against the neuronal membrane proteins in unselected lupus patients to determine their roles in neuropsychiatric lupus and disease severity.

Together with those finding (genetic MBL variants with protein deficiencies, anti-nephrin antibodies and brain reactive autoantibodies), the damage index in SLE patients (SLICC/ACR score) were studied if these factors predispose to severity in Systemic Lupus Erythematosus. The structural MBL mutant alleles and MBL low-producing promoter haplotypes are found to be associated SLE and correlated with infections during immunosuppressive therapy. Anti-nephrin antibodies are highly associated with lupus disease, but their association with renal involvement was seen only in lupus mouse models. Brain Reactive Autoantibodies are highly associated with lupus patients and correlated with psychosis or seizure disorders.

The data suggests that the MBL variants play a role in SLE pathogenesis and the usefulness of anti-nephrin antibodies and BRAA need further exploration before their clinical usefulness can be determined.

LIST OF PUBLICATIONS, ABSTRACTS AND POSTERS

I. INTERNATIONAL JOURNALS

1. Extraction of high quality genomic DNA: A simple and efficient method to overcome the effect of storage time

Tin SK, Fong KY

APLAR Journal of Rheumatology 1998; 1 (3): 198-200

2. The sensitivity and specificity of autoantibodies to the Sm antigen in the diagnosis of systemic lupus erythematosus

Pan LT, Tin SK, Boey ML, Fong KY.

Ann Acad Med Singapore. 1998 Jan;27(1):21-3.

3. A novel susceptibility locus on chromosome 2 in the (New Zealand Black x New Zealand White) F1 hybrid mouse model of systemic lupus erythematosus.

Rahman ZS, Tin SK, Buenaventura PN, Ho CH, Yap EP, Yong RY, Koh DR.

J Immunol. 2002 Mar 15; 168(6):3042-9.

4. PCR-RFLP genotyping for exon 1 and promoter region mutations of Mannose Binding Lectin (MBL) gene

Tin SK, Lee LY, Thumboo J, Koh DR, Fong KY.

J Immunol Methods. 2005 Aug;303(1-2):148-51.

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5. Novel Brain Reactive Autoantibodies: Prevalence in Systemic Lupus Erythematosus and Association with Psychoses and Seizures.

Tin SK, Xu Q, Thumboo J, Lee LY, Tse C, Fong KY

J Neuroimmunol. 2005 Dec;169(1-2):153-60.

6. Genetic polymorphisms of the Mannose Binding Lectin gene in Chinese and Myanmar individuals by PCR-RFLP approach

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II. MANUSCRIPTS IN PREPARATION.

1. Association of MBL-deficient Genetic Variants with Infections and Damage Index in SLE Patients under Immunosuppressive Therapy

III. ABSTRACTS AND POSTERS

1. Polymorphism of Mannose Binding Protein in Chinese patients with Systemic Lupus Erythematosus and correlation with ACR criteria

SK Tin, KY Fong, DR Koh, ML Boey, PH Feng

6th Tan Tock Seng Hospital Scientific Meeting
14 November 1998 Singapore.

2. The polymorphism of Mannose Binding Protein gene in Chinese SLE patients in Singapore

SK Tin, DR Koh, KY Fong

9th APLAR Congress of Rheumatology
21-26 May 2000, Beijing, China.

3. Association of anti-nephrin antibodies with Proteinuria in Lupus Mice Model; Preliminary Finding

SK Tin, KY Fong, DR Koh

6th NUS-NUH Annual Scientific Meeting
16-17 August 2002, Singapore.

4. Brain reactive autoantibodies in systemic lupus erythematosus patients: Prevalence and association with neuropsychiatric manifestations

SK Tin, Q Xu, C Tse, LY Lee, J Thumboo, KY Fong

SingHealth Scientific Meeting 2004
15 – 17 October 2004, Singapore

5. Fast and inexpensive novel genotyping of promoter region and exon 1 structural polymorphisms of Mannose Binding Lectin

Tin SK, Lee LY, Thumboo J, Fong KY

5th HUGO Pacific Meeting and 6th Asia-Pacific Conference on Human Genetics
17-20 November 2004, Singapore

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LIST OF ABBREVIATIONS:

ACR	American College of Rheumatology
AP	alkaline phosphatase
BRAA	Brain reactive autoantibodies
CNS	Central Nervous System
EDTA	Ehtylene Disodium Tetraacetic Acid
dNTP	deoxy nucleotide triphosphate
IPTG	isopropyl-beta-D-thiogalactopyranoside
LB	Luria Broth (bacterial growth media)
NBT/BCIP	nitro blue tetrazolium chloride/2-bromo-4-chloro-3-idolyl phosphate
Ni-NTA	nickel-nitrilotriacetic acid
NP-Lupus	Neuropsychiatric lupus
OD (OD_{600})	optic density (optic density at wave length 600nm)
PBS	phosphate buffer saline
PBS-T	phosphate buffer saline with 0.05% Tween 20
PCR	polymerase chain reaction
RT	Room Temperature
SDS	sodium dodecyl sulphate
SLE	systemic lupus erythematosus
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TBS	tris buffer saline
TBS-T	tris buffer saline with 0.05% Tween 20
PMSF	Phenyl Methyl Sulfonyl Fluoride
RFLP	Restriction Fragment Length Polymorphism

CHAPTER 1

INTRODUCTION

1.1. Systemic Lupus Erythematosus

Systemic Lupus Erythematosus (SLE) is a chronic and potentially fatal autoimmune disease, characterized by exacerbations and remissions with diverse clinical manifestations. SLE was first described with systemic manifestation in 1895 with the proposed pathophysiology based on the inflammation of blood vessels or vasculitis:- which previously was considered a destructive skin disease (Rothfield, 1989). SLE was later associated with other pathogenic lesions such as glomerulonephritis, heart valve vegetations, arthritis and pericarditis (Baehr et al., 1935). The first diagnostic test for SLE, the lupus erythematosus cell preparation (LE cell prep), was described by Hargraves et al., in 1948 and it was followed by an increase in the frequency of detection of SLE. The discovery of the LE cell prep opens up the understanding of immunological basis for SLE. With better immunologic and molecular biologic tools, the understanding of the pathogenesis of SLE has further improved and has resulted in better management of SLE. However the actual etiology of SLE still remains a mystery.

Clinical manifestations range from minor skin lesions to severe internal organ system derangement. Many organ-systems such as joint, skin, kidney, brain, lung, heart and gastrointestinal tract are involved during the disease course. Therefore treatment of patients with SLE is related to the severity of the disease and may be straightforward for mild disease such as skin lesions, or highly complex for severe, life-threatening complications such as nephritis or cerebritis.

1.1.1. Prevalence of Systemic Lupus Erythematosus

SLE is most common in women of reproductive age, although it has been reported in both extremes of life (i.e. it can occur in childhood or later in life). Females are predominantly affected during their reproductive age (female: male = 9:1), and the basis of this unequal sex ratio is not clear. According to the Lupus Foundation of America, more than 16,000 Americans develop lupus each year and it is estimated that 500,000 to 1.5 million Americans had been diagnosed with lupus. It was documented that the worldwide incidence is 50 per million population, the incidence varies hugely depending on ethnicity, geographical variation and socioeconomic status. For example, SLE is three times more common in African Americans than American Caucasians. The ethnic group at greatest risk is African Caribbeans. SLE is also more common in Asian Chinese.

1.1.2. Pathogenesis of Systemic Lupus Erythematosus

The pathogenesis of SLE is complex and a unifying mechanism has yet to be confirmed (Boumpas et al., 1995). Although the disease is homogenous and has predictable incidence and course in many lupus mouse models, the autoimmune response in human lupus patients is diverse. Furthermore, other factors such as genes, sex hormones, environment and microbes may contribute to SLE pathogenesis.

Evidence for genetic predisposition includes increased concordance for disease in monozygotic compared with dizygotic twins, a higher susceptible risk for persons with more than one affected family members, and correlation of certain genes

(especially MHC class II and III) with disease and autoantibodies. C4AQ0, a defective class III allele that fails to encode a functional C4A protein, is the most common genetic marker associated with SLE in many ethnic groups. Certain extended haplotypes, such as B8.DR3.DQw2.C4AQ0, predispose to SLE in some populations. The strongest single gene associations occur between HLA class II, especially DQB, and autoantibodies, which define clinical subsets of lupus. For example, high titers of IgG anti-dsDNA antibodies are associated with lupus nephritis and with DQB1*0201, *0602, and *0302 associated with either DR2 or DR3. Antibodies to Ro/La (SS-A/SS-B) are associated with the dermatitis of subacute cutaneous lupus and with certain DQA and DRB genes associated with DR3. The lupus anticoagulant, correlated with clotting, is associated with DQB*0301, *0302, *0303, and *0602 associated with DR4 or DR7. Family studies suggest that genes not linked to HLA antigens also participate in susceptibility and that females are more likely than males to express the autoimmune manifestations of their genotypes. The more susceptibility genes one has, the higher is the relative risk for development of SLE.

Females are more prone to develop SLE compared to males. However it seems unlikely that the X chromosome is involved because SLE does not follow a sex-linked pattern. Sex distribution are almost equal in those developing SLE in children or when older than 50 years. It is generally accepted that female sex hormones, not sex chromosome, are permissive for development of SLE. Sanchez-Guerrero et al.(1995), reported that menopausal women treated with hormone replacement therapy had increased risk for development of SLE compared to those without hormone replacement therapy. He further reported in 1997 that women using estrogen-containing oral contraceptives also had increased risks for SLE. Similarly, the

increased frequency of SLE in patients with Klinefelter's syndrome suggests that androgens may serve a protective function. Klinefelter's syndrome patients have low testosterone but higher estrogen levels. Femaleness is thought to be a susceptible factor in pathogenesis of SLE.

The environmental factors that induce or cause flares in SLE are largely unknown, with the exception of ultraviolet (UV) light, drugs and dietary modulation. Exposure to UV light can exacerbate SLE disease. It is postulated that UV light alters the normally non-antigenic dsDNA to become antigenic. UV light may also cause local skin damage that allows immune complex formation or deposition. Certain drugs, such as hydralazine and procainamide, induce lupus-like disease by possibly altering nuclear antigens, especially histones. Drug induced lupus has notable clinical and autoantibody differences compared to spontaneous lupus. Preliminary data have been suggested that diet may modulate the expression of SLE. For example, Hurd and Gilliam demonstrated in 1981 that NZB/NZW F1 mice fed a diet deficient in essential free fatty acids had a prolonged survival together with a decreased severity of their glomerulonephritis and inhibition of their anti-native DNA antibody response when compared to controls. And the lupus disease process in primates fed alfalfa sprouts is characterized by the development of anti-Sm and dsDNA antibodies, cutaneous and glomerular lesions (Bardana et al., 1982).

Microbial factors might also play roles in the pathogenesis of SLE. Bacterial liposaccharides (potent polyclonal B-cell activator) administered to animals induce the formation of circulating immune complexes and, subsequently antibodies to both single- and double-stranded DNA. Viruses are postulated to play major roles in both

murine and canine models of SLE. Theoretically, the chronic infection of lymphocytes with a virus might account for many of the immunologic aberrations present in SLE. Indirect evidence for a persistent viral infection in SLE includes increase in antibodies to multiple DNA and RNA viruses; the presence of electron-dense paramyxovirus-like cytoplasmic inclusions; and reports of type C virus oncornaviruses in involved renal and skin tissue. However attempts to isolate viruses from SLE tissue by hybridization and co-cultivation techniques have been largely unsuccessful. Thus the hypothesis, of viral infection as disease inducer, is inconclusive.

An association of the SLE with apoptosis was proposed. Apoptosis is regulated by several genes, comprising genes that inhibit (i.e. *Bcl*-2 gene) or promote (i.e. apoptosis-1/*Fas* gene) (Rose et al., 1994). The abnormal expression of apoptosis-related genes (e.g. the over-expression of Bcl-2 or defect in the *Fas* gene) is associated with the development of lupus –like systemic autoimmune diseases in animals. A soluble form of the *Fas* protein, an apoptosis-signaling receptor molecule on the surface of lymphocytes, was found on the sera of some lupus patients (Singh., 1995). Normal mice injected with this soluble form of the *Fas* protein resulted in the inhibition of apoptosis and the appearance of autoimmune features. Accumulation of nucleosomes formed during apoptosis by cleavage of chromatin has to be cleared. Failure of clearance by mutant DNAse I has been proposed as a factor in the pathogenesis of SLE (Napirei et al., 2000).

A healthy immune system produces antibodies, which help to fight and destroy viruses, bacteria, and other foreign substances. In lupus, the immune system fails to

recognize “self” from “non-self” and “mistakenly” produces antibodies against self-tissues. This leads to counterproductive inflammation and damage to various organs and tissues. In addition, some autoantibodies react with substances from the body's own cells or tissues to form immune complexes. Clinical manifestations are mainly due to the effects of this impaired immune system. Impaired immune responses include sustained production of pathogenic subset of autoantibodies and immune complexes.

The antibodies to host antigen (self-antigen), particularly nucleic acid-protein complexes such as the nucleosome made up of DNA and histones, are the predominant antibodies produced. These antibodies combine with their corresponding antigens to form antigen-antibody complexes. The immune complexes are eliminated by the reticuloendothelial system (RES). Defect in the clearance of antibody-coated erythrocytes, removed through Fc receptor-mediated phagocytosis in the liver and spleen, have been demonstrated in SLE patients. Conceivably, the uncontrolled formation of immune complexes leads to a saturation of receptor-binding sites, resulting in a functional blockade of the RES and an accumulation of circulating immune complexes.

When the circulating immune complexes are not cleared adequately, the circulating immune complexes are deposited in the subendothelial layers of vascular basement membranes of multiple organs. The sites of deposition and pathologic potential of immune complexes in skin, kidney, choroid plexus, or serosal surfaces are dictated in part by physiochemical properties of antigen or antibody such as size, charge, molecular configuration, immunoglobulin class and complement-fixing properties.

Once deposited, the immune complexes initiate a localized inflammatory response involving activation of complements, emigration of neutrophils, release of kinins and prostaglandins and antigen-antibody cell mediated tissue injury.

1.1.3. Clinical manifestations and diagnosis of Systemic Lupus Erythematosus

Genetic susceptibility, environmental triggers, hormonal influences and defective immune system are generally considered as major contributing factors in pathogenesis of SLE. SLE patients can present with different clinical presentations and many sub-sets have been observed. Furthermore there is no single diagnostic test or investigation method for diagnosis of SLE. The diversity and heterogenicity of the disease, require a set of clinical manifestations and investigations to establish a diagnosis of SLE.

At onset, SLE may involve only one organ system, which can progress on to multisystemic manifestations. Sometimes patients may have multisystemic involvement at onset. Clinical features in SLE patients can be divided into two broad categories; namely generalized and specific presentations. While generalized signs and symptoms, such as fatigue, malaise, fever, anorexia and weight loss, are common in SLE, specific presentations are based on the organ systems involved. The two most frequently involved organs/organ-systems in SLE causing major morbidity and mortality are the kidneys and brain. However, signs and symptoms presented by other systems are not uncommon. There can be vasculitic rashes in the skin, arthritis, pericarditis, pleuritis, pneumonitis, etc. The diagnosis has to be established using a set of clinical presentations and laboratory investigations. The most useful

investigations are hematological and immunological investigations. Decreased red blood cells, white blood cells and platelets are common findings in hematological tests and a phletora of autoantibodies are the hallmark of SLE. Among them, immunological tests, such as antinuclear antibodies, anti-dsDNA antibodies, anti-Sm antibodies provide strong supportive evidence for the classification of SLE.

The American College of Rheumatology (ACR) has presented criteria for classification of SLE in 1982 (Tan EM et al., 1982) (**Table 1.1**). According to advanced diagnostic technology and discovery of clinical association of antiphospholipid antibodies in SLE patients, ACR updated them in 1997 (Hochberg MC, 1997) (**Table 1.2**). The diagnosis of SLE can be established if a patient fulfils 4 or more of the 11 criteria according to the 1997 updated ACR criteria.

1.1.4. Prognosis of Systemic Lupus Erythematosus

SLE was considered a fatal disease in the past, however 5- and 10- year survival rates, especially in the western countries, have been increased dramatically since the introduction of steroids in disease management. It was noted that a 5-yr survival rate of only 50% in the 1950s (Merrell and Shulman., 1955) changed to a 10-yr survival rate of nearly 90% in the 1990s (Gripenberg and Helve., 1991; Pistiner et al., 1991). However, poor survival rate of SLE is still reported in certain ethnic groups such as Indians (Kumar et al., 1992), African Caribbeans (Nossent., 1993) and Hispanics (Lopez-Acuna et al., 1982). Developing countries reported 10-year survival rates as between 50 to 80%. (Kumar et al. 1992 and Massardo et al. 1994) The difference in these studies might be related to socio-economic conditions of these countries.

Table 1.1. The 1982 Revised Criteria for Classification of Systemic Lupus Erythematosus (Tan et al. 1982)

Criterion	Definition
1. Malar rash	Fixed erythema, flat or raised, over the malar eminences, tending to spare the nasolabial folds
2. Discoid rash	Erythematous raised patches with adherent keratotic scaling and follicular plugging; atrophic scarring may occur in older lesions
3. Photosensitivity	Skin rash as a result of unusual reaction to sunlight, by patient history or physician observation
4. Oral ulcers	Oral or nasopharyngeal ulceration, usually painless, observed by physician
5. Arthritis	Nonerosive arthritis involving 2 or more peripheral joints, characterized by tenderness, swelling, or effusion
6. Serositis	a) Pleuritis--convincing history of pleuritic pain or rubbing heard by a physician or evidence of pleural effusion (OR) b) Pericarditis--documented by ECG or rub or evidence of pericardial effusion
7. Renal disorder	a) Persistent proteinuria greater than 0.5 grams per day or grater than 3+ if quantitation not performed (OR) b) Cellular casts--may be red cell, hemoglobin, granular, tubular, or mixed
8. Neurologic disorder	a) Seizures--in the absence of offending drugs or known metabolic derangements; e.g., uremia, ketoacidosis, or electrolyte imbalance (OR) b) Psychosis—in the absence of offending drugs or known metabolic derangements, e.g., uremia, ketoacidosis, or electrolyte imbalance
9. Hematologic disorder	a) Hemolytic anemia--with reticulocytosis (OR) b) Leukopenia--less than 4,000/mm^3 total on 2 or more occasions (OR) c) Lyphopenia--less than 1,500/mm^3 on 2 or more occasions (OR) d) Thrombocytopenia--less than 100,000/mm^3 in the absence of offending drugs
10. Immunologic disorder	a) Positive LE cell preparation (OR) b) Anti-DNA: antibody to native DNA in abnormal titer (OR) c) Anti-Sm: presence of antibody to Sm nuclear antigen (OR) d) False positive serologic test for syphilis known to be positive for at least 6 months and confirmed by Treponema pallidum immobilization or fluorescent treponemal antibody absorption test
11. Antinuclear antibody	An abnormal titer of antinuclear antibody by immunofluorescence or an equivalent assay at any point in time and in the absence of drugs known to be associated with "drug-induced lupus" syndrome

The proposed classification is based on 11 criteria. For the purpose of identifying patients in clinical studies, a person shall be said to have systemic lupus erythematosus if any 4 or more of the 11 criteria are present, serially or simultaneously, during any interval of observation.

Table 1.2. The 1997 updated Criteria for Classification of Systemic Lupus Erythematosus (Hochberg. 1997)

Criterion	Definition
1. Malar rash	Fixed erythema, flat or raised, over the malar eminences, tending to spare the nasolabial folds
2. Discoid rash	Erythematous raised patches with adherent keratotic scaling and follicular plugging; atrophic scarring may occur in older lesions
3. Photosensitivity	Skin rash as a result of unusual reaction to sunlight, by patient history or physician observation
4. Oral ulcers	Oral or nasopharyngeal ulceration, usually painless, observed by physician
5. Arthritis	Nonerosive arthritis involving 2 or more peripheral joints, characterized by tenderness, swelling, or effusion
6. Serositis	a) Pleuritis--convincing history of pleuritic pain or rubbing heard by a physician or evidence of pleural effusion (OR) b) Pericarditis--documented by ECG or rub or evidence of pericardial effusion
7. Renal disorder	a) Persistent proteinuria greater than 0.5 grams per day or grater than 3+ if quantitation not performed (OR) b) Cellular casts--may be red cell, hemoglobin, granular, tubular, or mixed
8. Neurologic disorder	a) Seizures--in the absence of offending drugs or known metabolic derangements; e.g., uremia, ketoacidosis, or electrolyte imbalance (OR) b) Psychosis--in the absence of offending drugs or known metabolic derangements, e.g., uremia, ketoacidosis, or electrolyte imbalance
9. Hematologic disorder	a) Hemolytic anemia--with reticulocytosis (OR) b) Leukopenia--less than 4,000/mm^3 total on 2 or more occasions (OR) c) Lyphopenia--less than 1,500/mm^3 on 2 or more occasions (OR) d) Thrombocytopenia--less than 100,000/mm^3 in the absence of offending drugs
10. Immunologic disorder	a) Anti-DNA: antibody to native DNA in abnormal titer (OR) b) Anti-Sm: presence of antibody to Sm nuclear antigen (OR) c) Positive finding of antiphospholipid antibodies based on 1. an abnormal serum level of IgG or IgM anticardiolipin antibodies, 2. a positive test result for lupus anticoagulant using a standard method, or 3. a false-positive serologic test for syphilis known to be positive for at least 6 months and confirmed by Treponema pallidum immobilization or fluorescent treponemal antibody absorption test.
11. Antinuclear antibody	An abnormal titer of antinuclear antibody by immunofluorescence or an equivalent assay at any point in time and in the absence of drugs known to be associated with "drug-induced lupus" syndrome

The proposed classification is based on 11 criteria. For the purpose of identifying patients in clinical studies, a person shall be said to have systemic lupus erythematosus if any 4 or more of the 11 criteria are present, serially or simultaneously, during any interval of observation.

Appropriate care of patients with SLE will lead to fewer hospitalizations due to uncontrolled disease exacerbations, less severe renal disease with fewer patients developing end-stage renal disease requiring chronic dialysis, fewer episodes of avascular necrosis requiring total joint replacements, and less severe osteoporosis and fractures.

On the other hand, the longer SLE patients live, the morbidity and/or disability due to SLE-related illnesses will rise. All morbidity and complications of SLE are probably due to the consequences of disease itself or drugs used in disease management. At the same time, low socioeconomic status or inadequate psychological support (Ward et al., 1999) will aggravate morbidity.

The American College of Rheumatology developed and validated the damage index to estimate morbidity in SLE in 2000 (Gladman et al., 2000). The Systemic Lupus International Collaborating Clinics/American College of Rheumatology Damage Index (SLICC/ACR damage index) is issued to estimate the irreversible damage, regardless of cause, in 12 organ-systems related to SLE, its treatment or intercurrent illness. It was validated to identify the changes in damage seen in the patients with both active and inactive diseases.

Lupus patients present with different types of nephritis on biopsy. The exact pathogenesis for renal involvement is not clear in SLE; it is widely accepted that circulating autoantibodies and immune complexes are the main causes for lupus nephritis. Improved management of SLE over the years has helped to reduce the complications of renal involvement in SLE.

A positive relationship has been demonstrated, in a longitudinal study, to exist between persistently raised anticardiolipin antibody levels and neuropsychological performance in patients with systemic lupus erythematosus (Menon et al., 1999). Lupus patients were evaluated by neuropsychological assessments and cognitive deficits, like impaired attention, memory, deficits in language understanding and psychomotor speed were noted**.** They found that neurocognitive deficits are more frequent in SLE patients than in controls and these deficits appear in lupus patients without overt CNS manifestations and independent of disease activity.

Cardiovascular diseases and cerebrovascular diseases have also been recognised as a significant cause of morbidity in SLE. The risk of hospitalization for acute myocardial infarctions, congestive heart failures or cerebrovascular accidents is increased in young women with lupus compared to those without SLE. The authors suggested the importance of preventive measures to reduce risk factors for cardiovascular and cerebrovascular diseases in the management of SLE patients (Petri et al., 1992)

Mortality in SLE is an issue in many countries, especially developing countries with less funding for health care management. The factors contributing to mortality in SLE are divided into two categories; the social and demographic factors and disease-related factors (Drenkard and Alarcon-Segovia, 2000). However, factors such as age at onset, race, gender, socioeconomic status are controversial and the use of these factors as predictors of mortality is difficult to interpret. It is better to predict mortality in SLE using disease-related factors such as renal involvement, CNS involvement, antiphospholipid syndrome and persistent active disease process.

Almost all SLE patients have multiple organ-systems involvement during their disease course. The commonest major organ-systems involved in SLE are the kidneys (lupus nephritis) and brain (neuropsychiatric lupus). Proper management can reasonably control SLE disease activity and morbidity. The commonest causes of death are infectious complications, renal failure, or severe central nervous system disease. Early mortality of SLE is often due to complications related to active disease process while vascular events and end organ failures contribute to late mortality (Abu-Shakra et al., 1995.).

1.1.5. Management concerns in SLE

Several studies have documented substantial improvements in the survival of patients with SLE. Two factors possibly contribute to this improvement; ANA testing which is widely available now, had helped in early diagnosis, and the use of corticosteroids in control of disease activity. Despite advances in diagnosis and management, complications attributable to SLE or its therapy continue to cause substantial morbidity. In a prospective university-based study, the incidence of hospital admissions for patients with lupus was found to be 0.69 admission per patient-year. (Petri and Genovese, 1992). Complications such as lupus nephritis, nervous system involvement and infections are still major concerns in all societies.

Infections are common cause of death or first to second cause of hospitalization in lupus patients. Susceptibility of lupus patients to infection is considered because of altered immune system, which is affected by the disease itself or immunosuppressive drugs used in lupus management. Immunosuppressive drugs used in lupus

management may alter the immune system function of lupus patients. Innate immunity might play an important role in functional asplenic state.

The most serious organ systems involved in SLE are the kidneys (lupus nephritis) and the nervous system (neuropsychiatric lupus) (Cameron JS. 1999, West SG et al 1995). A significant number of all lupus patients presented with nephritis, which was confirmed with renal histology. Approximately 75% of renal biopsy results are reported with at least one abnormality in lupus patients. Cytoxic drugs are more effective than corticosteroids in controlling the clinical signs of nephritis. The use of these drugs, whether intermittent pulse therapy or long term therapy, can lead to different outcomes. Currently, significant renal involvements in lupus patients are started on immunosuppressive therapy.

It was estimated that between 31%-70% of SLE patients have significant neuro-psychiatric (NP) manifestations, in both retrospective and prospective studies (West et al., 1995; Kaell et al., 1986; Futrell et al., 1992; Sibley et al., 1992; Rood et al. 1999). The spectrum of NP manifestations in SLE includes both neurologic and psychiatric features, many of which are subjective to both patients and clinicians. They vary from overt neurologic dysfunctions due to psychoses and seizures, to subtle abnormalities in neurocognitive functions such as impaired memory, reduced intellectual capacity (Hanly and Hong 1993). They cannot be easily identifiable because lupus itself or drugs used in lupus management or other associated pathological condition may contribute to the cognitive deficits. Early diagnosis that can lead to earlier intervention has been hampered by the lack of an accurate marker for NP involvement.

1.2. Complement system and its role in SLE

The complement system, an important part of the innate immune system, plays a critical role in the defense against invading microorganisms. Initially the complement system comprised of two pathways, namely the classical and alternative pathways. Over the decade, Mannose Binding Lectin (MBL) has been accepted as the third complement pathway. Three separate pathways can now activate the complement cascade. The pathways are as follow:

- the classic pathway (C1q, C1r, C1s, C2, C4),
- the alternative pathway (C3, factor B, properdin) and
- the lectin pathway (MBL)

These 3 pathways converge centrally at component protein C3 and the terminal complement pathway consists of all proteins activated after C3; the most notable protein is the C5-C9 group of proteins known collectively as the membrane attack complex (MAC). The MAC exerts powerful "killing" activity by causing perforations in cellular membranes.

When the complement system is activated without the presence of specific antibodies, the invading microorganisms will be removed by opsonization, phagocytosis and the action of MAC. Deficiencies in complement proteins predispose patients to infection, leading to recurrent infectious episodes and severe sepsis.

In addition to playing an important role in host defense against infections, the complement system is a mediator in the clearance of immune complexes. In autoimmune disorders with high production of autoantibodies, the complement system aids clearing these autoimmune complexes. Failure to do so could result in accumulation and deposition of these complexes in organ tissues leading to organ and tissue damage. Specific complement deficiencies such as C2 and C4 are also associated with an increased risk of developing autoimmune diseases, such as SLE (Walport., 1993) (**Table 1.3**).

There is an important clinical association of hypocomplementaemia in SLE. Patients with chronic hypocomplementaemia are at particular risk of developing serious infections with encapsulated organisms such as Streptococcus pneumoniae and Neisseria meningitidis. These patients can be considered to be "functionally asplenic" because the hypocomplementaemia, in addition to causing defective opsonization and local phagocytosis, also results in reduced splenic clearance of these organisms (Davies, 1995). There is a case that such patients, analogous to a post-splenectomy state, receive prophylactic penicillin therapy and be considered for both pneumococcal and meningococcal vaccination.

Therefore the complement system plays important roles in both innate immunity protecting against different microorganisms and aiding in the clearance of immune complexes. Since the lectin pathway is part of the complement system, it is important to review if MBL deficiencies contribute to infectious episodes in SLE.

Table 1.3. The common complement deficiencies, their associated diseases, detection methods and genetic basis

Component	Disease condition	Detection methods	Genetic basis
C1q, s	collagen vascular disease, SLE, bacterial infections	CH50, RID	Point mutations acquired form due to C1q autoantibodies
C2	Neisserial infections, Respiratory infections, SLE (often symptomless)	CH50, RID, electrophoresis	*type I:* 28-bp deletion at exon/ intron junction 6 leading to splicing error; linked to HLA -A25 -B18 -C2Q0 -C4A4,B2 -DR2; *type II:* impaired C2 secretion
C4A / B	Collagen vascular disease, SSPE, Autoimmune diseases (SLE, PBC, autoimmune hepatitis, scleroderma)	CH50, RID, SDS-PAGE	1. Complete gene deletion; 2. Gene conversion (isotype change) 3. Nonexpression due to 2-bp insertion >> stop codon

CH50 - complement haemolytic activity; AP50 - alternative pathway haemolytic activity; RID - radial immunodiffusion

1.2.1. Complement deficiencies in systemic lupus erythematosus.

A variety of genetic factors have been linked to SLE and, among these, the deficiencies in components of the classical pathway of complement carry stronger association (Pisetsky, 1991). SLE develops in most individuals with genetic deficiencies in C1q, C1r, C1s, C2 and C4 (Hauptmann et al., 1988; Morgan and Walport, 1991; Walport et al., 1997). Hereditary deficiencies of C1s and C1r are rarer than that of C1q (Loos and Heinz, 1986; Chevailler et al. 1994). The majority of C1q deficient individuals are strongly associated with SLE (Bowness et al., 1994) and selective C1s deficient individuals have been reported in association with SLE (Suzuki et al. 1992).

The role of C1q in suppressing autoimmunity in mice was demonstrated by Botto et al. in 1998. C1q-deficient ($C1qa^{-/-}$) mice on a mixed B6/129 genetic background spontaneously produced high titers of ANA, whereas wildtype controls generated low levels of autoantibodies. Furthermore, C1q-deficient mice had increased mortality and 25% of them also showed glomerulonephritis with immune complexes. This is consistent with the hypothesis that C1q deficiency causes autoimmunity by impairment of the clearance of apoptotic cells.

While almost all patients with C4 deficiency are associated with severe SLE, only 33% of those with C2 deficiency are associated with a milder form of SLE (Walport, 1993). Two tandemly duplicated genes, C4A and C4B, encode C4 and they are highly polymorphic with variants including non-expressed gene or null alleles for which no protein product is identifiable. There is a strong association between C4AQ*0 null

alleles and SLE in all different ethnic groups of SLE patients (Christiansen et al., 1983; Dunckley et al., 1987 Kumar et al. 1991). Although C2 null alleles were reported to be associated with SLE in 1976 (Glass et al., 1996), later studies did not demonstrate similar strong associations with SLE. Instead the C2 null alleles are linked with other HLA class I and C4 gene haplotype (Awdeh et al., 1981; Hauptmann et al. 1982). The C2 component of complement is probably a minor susceptible factor in the development of SLE (Pickering and Walport, 2000).

Chen et al. reported the role of C4 in autoimmunity in 2000. In their study, high titers of ANA were observed in all female and almost all male $C4^{-/-}$ mice (deficient of C4) in B6/129 genetic background after 10 months of age. However the titers of ANA of $CR2^{-/-}$ mice (deficient of CR1 and CR2) never rose above those of normal controls. Therefore C4 deficiency causes spontaneous lupus through a mechanism which is independent of CR1/CR2.

Acquired deficiencies in complement components are common in SLE patients (Walport et al., 1997). The measurement of complement protein levels and functional activity in serum is widely used as markers of disease activity in SLE. During periods of active disease, serum complement activity is usually reduced. Typically levels of the classical pathway of complement component (C1q, C2, C4) are low in patients with severe disease, may be accompanied by reduction in C3 levels. However, the levels of complement components usually correlate with disease activity, except C4 may remain low when the patient is clinically inactive (Lloyd and Schur, 1981).

Determinants of protein level are not well understood yet. Decrease in synthesis of complement components may lead to reduced concentrations in SLE (Sliwinski and Zvaifler, 1972). These acquired deficiencies are also thought to result from over-consumption of complement proteins by circulating immune complexes (De Bracco and Manni, 1974, Sturfelt et al. 1983). Low concentrations of complement components may also be due to a series of autoantibodies to complement components. They include anti-C1q autoantibodies (Antes et al., 1988), anti-C1 autoantibodies (Ordi-Ros et al., 1997), classical pathway nephritic factors autoantibodies (an autoantibody to the classical pathway C3 convertase, C4b2a) (Daha et al., 1983) and C3 nephritic factor autoantibodies (Daha et al., 1976).

1.2.2. The Third Complement pathway: Mannose Binding Lectin (MBL)

Mannose Binding Lectin (MBL) is a calcium-dependent serum lectin secreted by the liver as an acute phase protein (Wild et al., 1983; Thiel et al., 1992). Normal MBL is structurally analogous to C1q and is a multimeric molecule, comprising up to 6 of 96 kD subunits, each consisting of identical 32-kD polypeptide chains (Taylor et al., 1989). The MBL is now considered a member of the complement protein family.

MBL contributes to immune defense in two ways. Firstly it can directly bind the pathogens' membranes containing carbohydrate, opsonise the pathogens and finally enhance the phagocytosis of invading pathogens (Sheriff et al., 1994). Secondly, it can also activate the complement system via lectin complement pathway using two serine proteases, MBL-associated serine proteases (MASP)-1 and –2 (Matsushita and

Fujita, 1992; Thiel et al., 1997). The MASP complex shares many conserved features with C1r2s2. It can cleave complement protein C2 to initiate the complement cascade.

The association of hereditary deficient alleles of early complement components have been described earlier, the hierarchy of susceptibility and severity of lupus, according to missing classical pathway proteins (C1q>C4>>C2) suggest that the early part of classical pathway plays a key protective role against SLE (Pickering and Walport, 2000). Since one of the major roles of the classical pathway is that of host defense against infectious diseases, deficiency of classical pathway components may predispose to SLE because of impaired resistance to infectious trigger (Pickering and Walport, 2000). MBL is analogous to C1q, one of the early components of classical pathway, hence dysfunctional plasma MBL due to mutant structural alleles may impair defense mechanism against invading microorganisms such as viruses, bacteria, fungi etc. Thus deficiency in MBL is possibly a predisposing factor to SLE.

Complement proteins concentrations are low not only in hereditary causes, but also in acquired conditions in SLE patients. Decreased synthesis of complement components (Sliwinski and Zvaifler, 1972), consumption of complement by accumulated circulating immune complexes (De Bracco and Manni, 1974, Sturfelt etal. 1983) and autoantibodies to complement components (Ordi-Ros et al., 1997; Daha et al., 1983; Daha et al., 1976) have been reported in SLE patients. C1 inhibitor deficiency is also an important factor resulting in decreased complement components (Cicardi et al., 1998). In normal circumstances, this inhibitor, a serine proteinase inhibitor (sepin), binds and inactivates enzymatically active C1r and C1s. Deficiency results in uncontrolled fluid phase classical pathway activation and consequently reduced levels

of both C2 and C4. Therefore, the complement component may be decreased due to many acquired factors. SLE patients can be in a chronic hypocomplementaemia state as disease progresses. MBL might play a role in innate immunity against infectious pathogens by enhancing phagocytosis through binding to C1qR (C1q Receptor) (Nepomuceno et al., 1997). Thus MBL can help prevent infections in SLE disease.

1.2.3 Mannose Binding Lectin (MBL) in SLE

Three point mutations, encoding 3 different structural variants of MBL, have been found in the human MBL gene (Sumiya et al., 1991; Madsen et al., 1994; Lipscombe et al., 1992), located at codons 52, 54 and 57 of exon 1 and are associated with low serum levels of MBL (Lipscombe et al., 1995; Garred et al., 1992a and 1992b). Additional polymorphisms also exist at position –550 (H/L variants) and –221 (X/L variants) in the promoter region of the gene (Madsen et al., 1995). Like structural mutations in exon 1, the variants at promoter region of MBL influence serum MBL levels. Madsen et al., reported that HY haplotype is associated with the highest plasma levels of MBL, LY haplotype with intermediate levels and LX haplotype with lowest circulating serum level of MBL (Madsen et al., 1995).

The LX haplotype, associated with lowest-producing serum MBL, was significantly associated with Chinese SLE patients and reported as risk factor for SLE (Ip et al., 1998; Huang et al., 2003). This haplotype association was not found in Danish SLE patients, instead homozygous structural mutations in exon 1 of MBL gene were strongly associated with complicating infections. (Garred et al., 1999; Garred et al., 2001). However, studies from Hong Kong, UK and Spain did not report significant

findings. (Davies et al.,1997; Davies et al., 1995; Lau et al., 1996; Garcia-Laorden et al., 2003). Interestingly, there are two conflicting reports, with one stating no significant findings on mutations of structural MBL gene related to development of SLE, and another with significant linkages with SLE. (Horiuchi et al., 2000; Tsutsumi et al., 2001).

The issue of MBL as a predisposing factor to SLE development is controversial. There are no reported studies on whether MBL haplotypes confer risk to susceptibility of infectious agents in SLE patients, resulting in more severe disease and worse outcome. MBL is thus studied to clarify its role in severe lupus disease.

1.2.4 Therapeutic uses of MBL in clinical trials

MBL has been reported as playing an important role in innate immunity and recurrent severe infections are associated with its mutant alleles. The MBL protein, purified from pooled human sera and recombinant technology, was used in clinical trials for subjects with frequent infections. The results were promising, with good control of infections in patients and more trials are planned. Affinity purified MBL from pooled fresh human plasma (pMBL) has been used to treat patients with frequent and chronic infections with promising initial results. A 2-year old girl with recurrent infections since the age of 4 months remains free of abnormal infections for eight years since she received pMBL infusions. No adverse clinical or immunological reaction was observed in both patient and healthy volunteers (Valdimarsson et al., 1998; Valdimarsson, 2003). Recent production of recombinant MBL (rMBL) was reported and clinical trials were expected to start using rMBL (Jensenius et al., 2003).

Summerfield suggested that MBL infusion may potentially be a therapeutic option for patients with uncontrolled sepsis (Summerfield, 2003).

1.3. Autoantibodies and their roles in SLE

The production of antibodies against one's own tissue has been known for years and medical textbooks had documented that 10% of the normal population have at least one autoantibody. The production of autoantibodies could be considered a normal phenomenon, assisting in the removal of "damaged" tissues. It was documented that the levels of ANA dramatically increased immediately after an ischemic event which however normalizes over a few days. This showed that antibody producing cells (B cells) produce ANAs upon stimulation by nuclear materials released after an ischemic event.

Physiologically, detectable autoantibodies indicate the presence of tissue or cellular damages. However, the persistent presence of excess autoantibodies may be an indicator of abnormal tissue damage (acute or chronic inflammations) or over-expression of these autoantibodies without tissue damages. When these accumulated autoantibodies become pathogenic factors that target specific organs, tissues or cells, they manifest clinically by two possible mechanisms. Firstly, autoantibodies mediate the tissue injury by an immune-complex-mediated inflammatory response, as can be seen in glomerulonephritis caused by deposition of antigen-antibody complexes. Secondly, autoantibodies can also mediate cellular dysfunction or destruction by binding to specific autoantigens. Autoimmune hemolytic anemia is mediated through this mechanism. Cellular destruction (haemolysis) of red blood cells is due to

antibodies formed to components on the surface of the red blood cells. These mechanisms are not mutually exclusive, but can explain the actions of pathogenic autoantibodies. For example, pathogenic anti-dsDNA antibodies usually result in immune-complex-mediated renal disease, but a subset of these antibodies may also penetrate renal cells, bind to nuclei and induce glomerular disease.

It is important to note that more than one self-antigen may drive an autoantibody response and that an autoantibody nominally directed to one self-antigen may arise from stimulation of another. Anti-dsDNA antibodies appear to be the product of an antigen-driven, T-helper-cell-dependent immune response. Although DNA is the target antigen, recent studies have suggested that anti-dsDNA antibodies may result from autoimmunization with chromatin (the complex of DNA, histones, and other proteins found in the nucleus) or nucleosome (the basic structural subunit of chromatin) rather than naked DNA. Likewise, anti-dsDNA antibodies may bind both DNA and components of the small nuclear ribonucleoprotein, a characteristic attributed to anti-Smith antibodies.

Generally, SLE has a profile of multiple autoantibodies during their disease course. However their pathogenicity and specific relationships with organs or tissues are still largely unknown. Circulating pathogenic autoantibodies directed against different tissues are present in several autoimmune states. The pathogenic roles of many autoantibodies are not clear in autoimmune disorders. In acquired myasthenia gravis, specific acetylcholine receptor autoantibodies alter the physiological state of neuromuscular end-plate by reducing impulse transmission to the post-synaptic membrane of neuromuscular end-plate. Similarly, calcium channel autoantibodies

play a role in altering the physiological state of synaptic gap junction in Lambert-Eaton myasthenic syndrome. They reduce release of neurotransmitter acetylcholine from the nerve terminals into the synaptic gaps.

Anti-phospholipid syndrome (APS), now accepted as a systemic autoimmune disease, was reported in the setting of SLE as associated with recurrent thrombotic episodes and fetal losses, together with pre-eclampsia (Wilson et al., 1999). The autoantibodies detected in APS are anti-phospholipid antibodies, which are detectable by tests for β-2 glycoprotein or anti-cardiolipin antibodies or lupus anticoagulant. Anti-phospholipid antibodies play a pathogenic role in addition to being a diagnostic marker for APS (Meroni and Riboldi, 2001). These antibodies have pathogenic roles against the normal physiological functions of soluble coagulation factors and coagulation cells (De Groot and Derksen., 2000). Although the pathogenic roles on the obstetric manifestations are not definitively clear, they do interfere in the physiological function of trophroblast cells via reduction in proliferation and differentiation, and impairment of gonadotrophin secretion. These may promote a defective placenta, which results in pre-embryonic loss and utero-placental insufficiency (Di Simone et al., 2000).

Antibodies to the Ro/SS-A were first reported in 1962, as antibodies from sera of patients with Sjogren's syndrome were reactive with RNA protein particles (Anderson et al., 1962). There are two molecular types of the protein, 52-kD and 60-kD iso-forms. Anti-Ro antibodies are also detected in SLE (40-50%), however their significant association with Sjogren's syndrome (60-75%) was well demonstrated (Reichlin, 1994) and used as criteria for the latter condition. The pathogenic roles of

anti-SS-A antibodies in neonatal congenital heart blocks have been shown by detection of these antibodies on affected fetal heart, induction of AV block in rabbit's heart after perfusion with anti-SS-A antibodies and development of congenital heart block in BALB/c mice after immunization of purified SS-A protein. (Reichlin et al., 1994; Garcia et al., 1994; Miranda-Carus et al., 1998). Therefore pregnant women with anti-SS-A autoantibodies are at risk of having infants with congenital heart block. In this autoimmune-mediated congenital heart block, it can be concluded that the autoantibodies that cross the placenta, have altered the normal function of the conduction system of fetal heart by injuring the cardiac conductive tissue.

In SLE, heterogeneous autoantibodies are detectable against different cellular components and most of these autoantibodies' functions are still unknown with only some of them having diagnostic values. This is mainly due to heterogenicity of the disease itself. There is a need to clarify the roles and practical importance of these autoantibodies in SLE.

1.3.1. Prevalence of autoantibodies in SLE

Sherer et al. (2004) reported that more than 100 different autoantibodies (116 in total) have been found in both human and animal lupus studies. Antibodies are directed against nuclear components, cytoplasmic proteins, cell membranes and lipid-related proteins. Most SLE patients have more than one autoantibody, however the vast majority of the listed autoantibodies are not found in lupus patients. In addition to the different autoantibody frequencies, there are great variability regarding the correlation with disease activity and clinical associations. Some autoantibodies correlate with

disease severity and clinical manifestations. For example, increased anti-dsDNA antibodies level preceding disease exacerbations, anti-Ro antibodies associated with congenital heart blocks and anti-ribosomal P proteins antibodies associated with nephritis and central nervous system involvement. However it is not clear how many of these autoantibodies contribute to pathogenesis of SLE.

1.3.2. Usefulness of detection of autoantibodies in SLE

Since autoantibodies in SLE are abundant in nature and can be detected easily by improved methodology, this unique feature of autoantibodies was used in diagnosis, disease activity and disease severity assessments in SLE (**Table 1.4**).

In the 1997 updated ACR criteria for classification of SLE, two criteria based on the detection of autoantibodies are included for the purpose of SLE classification. They are

i) Immunologic disorder: -

a) Anti-DNA: antibody to native DNA in abnormal titer (OR)

b) Anti-Sm: presence of antibody to Sm nuclear antigen (OR)

c) Positive finding of antiphospholipid antibodies based on

- an abnormal serum level of IgG or IgM anticardiolipin antibodies,
- a positive test result for lupus anticoagulant using a standard method, or
- a false-positive serologic test for syphilis known to be positive for at least 6 months and confirmed by *Treponema pallidum* immobilization or fluorescent treponemal antibody absorption test.

ii) Antinuclear antibody

An abnormal titer of antinuclear antibody by immunofluorescence or an equivalent assay at any point in time and in the absence of drugs known to be associated with "drug-induced lupus" syndrome.

Apart from their use in establishing the diagnosis of SLE, prospective serum level of autoantibodies are also useful in management and disease activity monitoring in lupus clinics. Anti-dsDNA antibodies are used as disease activity markers in SLE and useful in assessing lupus nephritis. Anti-histone antibodies are found mainly in drug-induced lupus. Recently reports on the association of ribosomal P protein antibodies with NP lupus have been published.

We decided to explore further the roles of specific autoantibodies in two areas, namely autoantibodies to the kidneys and brain, since their involvement can lead to serious adverse outcomes.

1.3.3. Renal involvement in SLE

Renal involvement in systemic lupus erythematosus (SLE) is one of the commonest disease manifestations and a strong predictor of poor outcome. Most patients with SLE have immunoglobulins deposited in their glomeruli, but only about half have clinical nephritis as defined by proteinuria. According to ACR criteria for the classification of SLE, “Persistent proteinuria greater than 0.5 grams per day or greater than 3+ if quantitation not performed” is considered as one of the criteria for lupus nephritis. However, the clinical spectrum of lupus nephritis ranges from

Table 1.4. Some of the autoantibodies present in SLE with practical value in diagnosis and monitoring SLE patients.

ANTIBODY	ANTIGEN	DISEASE ASSOCIATION
Anti-nuclear antibodies	Nuclear materials	SLE (>95%)
Anti-DNA	Double stranded DNA (native DNA)	SLE (50-70%); Especially with glomerulonephritis
Anti-Histone	Basic proteins bound to DNA	SLE (30%); Drug induced lupus (95-100%)
Anti-Sm	Acidic nucleoprotein	SLE (25-30%)
Anti-SSA (Ro)	Acidic protein	SLE (10-20%); ANA Negative-SLE and other forms of Lupus (30-40%)
Anti-SSB (La)	Acidic protein	SLE (10-20%).
Anti-Cardiolipin	Cardiolipin	SLE (60%)
Lupus Anticoagulant	Phospholipid coagulation activator complex	SLE (5-10%)
Anti-Nucleolar	Nucleolar antigens	SLE
Anti-PCNA	Proliferating Cell Nuclear Antigen	SLE (5-10%)
Anti-RNP	Acidic protein	SLE: (25-40%)

asymptomatic, low-grade proteinuria to a rapidly progressive course with hypertension, edema, leading to severe renal failure within days.

Immune complexes are present at the sites of injury in glomeruli and in tubules. Whether these are derived from circulating immune complexes or from in situ combination of antigen with antibody, is still unclear. The incidence of renal disease in lupus varies with the criteria used to define renal involvement and the selection of patients. In early stages of lupus, renal abnormalities can be detected in 25-50% of patients. It has been shown that up to 2/3 of patients with lupus will develop some renal abnormalities in the course of their disease. However, if kidney tissue from lupus patients (obtained by biopsy) is analyzed using refined histology techniques such as immunofluorescence or electron microscopy, abnormalities can be found in almost all cases, regardless of the presence of clinical signs and symptoms.

Lupus patients develop nephritis early in their disease and it is uncommon to have renal disease onset more than ten years after the appearance of SLE. Studies have suggested a mild increase in the proportion of male lupus patients compared to women with renal disease. Asians, African-Caribbeans and African-Americans may have more nephritis than other ethnic groups (Lee and Spargo., 1985). HLA antigens have been associated with an increased risk of developing nephritis and the HLA-DR2 and HLA-B8 are associated with the development of lupus renal disease (Pistiner et al., 1991; Freedman et al., 1993 ; Klippel, 1995). Polymorphisms of Fc receptors for IgG (FcgammaR) were recently identified as a risk factor, implicating defective handling of circulating immune complexes in the development of renal disease (Salmon et al., 1996). The prevalence of renal disease in eight large cohort studies

consisting of 2649 SLE patients varied from 31% to 65% (Wallace, 1996). A recent study analyzed the annual incidence of nephritis in 384 lupus patients followed at the Johns Hopkins Medical Center between 1992 and 94. The one year incidence of acute renal disease was 10% (Skovron and Petri, 1997).

i. Pathogenesis of renal involvement in SLE

Up to 50% of lupus patients develop clinically relevant nephritis at some time in the course of their illness. The pathogenetic mechanisms involved in lupus nephritis are still unclear. However at least three potentially overlapping, immuno-pathogenic mechanisms are supported by experimental data. Firstly, circulating immune complexes consisting chiefly of DNA and anti-DNA are deposited in the kidney. This results in complement activation and chemotaxis of neutrophils, leading to a local inflammatory process. Secondly, in-situ formation of antigen and antibody complexes may similarly lead to complement activation and leucocyte mediated injury. Thirdly, antibodies against specific cellular targets may produce renal injury. For example, antibodies against kidney tissue such as glomerular basement membrane, may bind to specific antigens, and this antigen-antibody reaction could cause disruption of cellular function.

ii. Autoantibodies in lupus nephritis

The majority of patients with lupus nephritis are positive for anti-dsDNA antibody and rising anti-DNA titers accompanied by hypocomplementemia, especially a low C3 level, are often harbingers of active and deteriorating lupus glomerulonephritis.

Since different substrates are used (i.e. bacterial DNA in Farr assay, mammalian DNA, such as calf thymus, in ELISA, and protozoan DNA within the kinetoplast organelle in Crithidia lucilae assay) patients may be negative in one but have a positive test in another assay. Occasionally lupus patients who are genuinely anti-DNA test-negative develop clinically significant nephritis. This may occur because of antigen excess and inability to detect circulating anti-DNA, which is deposited in the kidney. Alternatively, antibodies such as anti-ribosomal P, anti-Ro, or antibodies to C1q may mediate renal disease. However, the antibodies directly reacting with or specifically targeting against renal tissue, have not been demonstrated in lupus nephritis patients.

iii.Diagnosis and management of lupus nephritis

Testing the urine for abnormalities is important in detecting renal involvement in lupus. Various degrees of proteinuria, red blood cells in the urine and abnormalities of urine sediments are seen. Patients with lupus nephritis have significant proteinuria. Active lupus renal disease can be categorized clinically or histologically. Clinical disease is evaluated by urinalysis, 24 hour urine protein assay and creatinine excretion, serum creatinine, anti-DNA titers, and serum complements. Additionally, serum albumin and cholesterol can be used to help characterize the nature of lupus renal disease.

Lupus nephritis is highly variable in its histological manifestations and presentation. The only way to define the histological type of lupus nephritis is by performing renal biopsies. Renal biopsies are necessary in all patients with lupus who have abnormal

urine and/or reduced renal function. It provides information for prognostication and management. The World Health Organization (WHO) has defined six histological types of lupus nephritis (**Table 1.5**). Exacerbation of lupus nephritis is often preceded by a rise in the titre of serum anti-dsDNA antibodies, a fall in the serum concentrations of complement components, C3 and C4, an increase in the serum concentration of immune complexes and a markedly elevated erythrocyte sedimentation rate.

The five-year survival rate of treated patients with lupus nephritis from 1990 to 1995 was 82%. About 10-15% of patients with lupus nephritis progressed to end-stage renal failure. Renal failure supervenes usually within the first decade of follow-up. A proportion of lupus nephritis patients in endstage renal failure still requires immunosuppression. Recurrence of lupus nephritis is not uncommon, but renal transplant cases are rare.

In some lupus patients in remission, the phenomenon of fixed proteinuria is present. These are patients who do not have active immunologic injury, but who have persistent proteinuria. It is hypothesized that the prior immunologic injury and inflammatory process caused dysfunctional glomeruli incapable of preventing protein leakage. Patients may excrete 1-3 grams per day of proteins even during periods of disease remission. The postulated mechanism is the reaction of an autoantibody against a structural protein, causing proteinuria without damaging the structural protein. From extensive literature search, we found a monoclonal antibody, which causes transient proteinuria in animal models.

Table 1.5. World Health Organization Classification of Lupus Nephritis

Class	Description by light microscopy	Clinical presentation
I	Normal	Normal Urine
II a	Normal	Asymptomatic hematuria & proteinuria
II b	Mild mesangial hypercellular	-do-
III	Focal & segmental proliferative GN Necrosis and proliferation < 50% glomerul	Proteinuria, Hypertension,Hematuria
III a	Focal & segmental proliferative GN with active necrotizing lesions	-do-
III b	Focal & segmental proliferative GN with active necrotizing and sclerosing lesions	-do-
III c	Focal & segmental proliferative GN with sclerosing lesions	-do-
IV	Diffuse proliferative GN; > 50% glomeruli; ±crescents; focal necrosis	Acute nephritis ±nephrotic syndrome
V	Membranous GN	Nephrotic syndrome
VI	Chronic sclerosing GN	-do-

iv. Significance of mAb 5-1-6 in proteinuria

In 1988, Orikasa et al. generated a monoclonal antibody (mAb 5-1-6) which was produced in BALB/c mice immunized with collagenase-treated Winstar rat glomeruli. This monoclonal antibody reacts with the slit diaphragm in immunoelectron microscopy. (Kawachi et al. 1995). Single intravenous injection of mAb 5-1-6 into the rat causes transient but massive proteinuria. Protein excretion started immediately following mAb injection and sustained an average value of 138.5 mg/24 hours on day 5, This was followed by a gradual decline, so that normalization was virtually resumed by day 15. No histological abnormalities could be detected by light microscopy on day 5 when the proteinuria was most severe. Deposition of host (rat) IgG and complement proteins, and the infiltration of inflammatory cells were not detected during the proteinuria phase. Thus it was concluded that mAb 5-1-6 induced the proteinuria in a complement- and inflammatory cells- independent manner and it was postulated that its antigen is important for the filtration function of the slit diaphragm. It was proposed that mAb 5-1-6 affects glomerular permeability by altering the molecular composition of the slit diaphragm.

The characteristics of the protein that mAb 5-1-6 binds remained unknown until 2000 when Topham et al. reported that mAb 5-1-6 was directed against the extracellular domain of nephrin by protein mass spectrometry. The extracellular domain of nephrin is a member of the podocyte proteins, which can alter the configuration of slit diaphragm, thus regulating the glomerular permeable-selectivity. Massive proteinuria is thus observed immediately after intravenous injection of mAb 5-1-6 to normal rats. It is possible that these antibodies are gradually being removed by the host immune

system, normalizing glomerular filtration function in about two weeks (Kawachi et al. 1995). We postulate that anti-nephrin antibodies are associated with proteinuria in SLE patients.

1.3.4. NP involvement in SLE

People with lupus can experience headaches, dizziness, difficulties with concentration, and may be confused, have seizures, strokes, or other signs of central nervous system involvement. The variety of NP manifestations in lupus has been well described in prospective and retrospective studies. (Kaell et al., 1986; Sibley et al., 1995; Futrell et al., 1992; West et al., 1995; Rood et al., 1999). However, it is often difficult to differentiate if these manifestations are disease-related or the result of therapy (Jennekens and Kater., 2002).

Three classifications for neurological and psychiatric disorders of SLE have been published; the first consists of 12 diagnostic criteria proposed by The Ad Hoc NP Lupus Workshop Group (Singer and Denburg., 1990) (**Table 1.6**), the second consists of a list of 29 lupus related CNS descriptors ranked according to diagnostic weightage, (Rood et al., 1999) (**Table 1.7**) and the last is the nomenclature system comprising 19 lupus related CNS syndromes with detailed definitions.(ACR Ad Hoc Committee, 1999) (**Table 1.8**). However all reports indicated that the classification, nomenclature and definitions are designed to facilitate clinical research and multicenter trials in order to generate unique diagnostic criteria for NP lupus. They suggested these classifications and definitions to be used critically. Currently there are no single diagnostic tests or investigations to establish NP lupus. Many investigations

(a series of hematological, biochemical, radiological and psychological tests) must be performed to establish NP lupus.

i. Pathogenesis of NP Lupus

Postulated mechanisms to explain the pathogenesis of NP lupus are many, but no concrete pathogenesis was reported. Inflammatory processes are no longer considered to be the main cause of cerebral damage. Complex processes due to a variety of factors including immunological and vascular factors, are acknowledged to cause cerebral damage (Jennekens and Kater., 2002). Autoantibodies against cerebral components are considered as one of the possible mechanisms for CNS involvement in SLE. SLE is a disease that presents diverse autoantibodies against its own cells and tissues, autoantibodies targeting neuronal cells or vascular tissue in central nervous system, are not rare. Nerve tissues may be damaged when antibodies target nerve cells or blood vessels, resulting in neuronal cell dysfunction. In addition, autoantibodies may bind to neuronal cells or tissues without causing physical damage, but may alter their physiological function. Clinically these altered processes manifest as NP-lupus.

ii. Autoantibodies in NP lupus

Many reports of autoantibodies detected in SLE patients with NP manifestations have been published (Bonfa and Elkon., 1986; Robbins et al., 1988; Denburg and Behmann., 1994; Hanly et al., 1988; Hanson et al., 1992, Hirano et al., 1980). However their diagnostic value remains unclear. There have been a few reports on profiles of autoantibodies used in diagnostic evaluation of NP lupus (Conti et al.,

Table 1.6. The Diagnosis criteria for NP SLE (Singer and Denburg., 1990)

	Criterion
1	Aseptic meningitis
2	Cerebrovascular disease
3	Demyelinating syndrome
4	Headache (including migraine and benign intracranial hypertension)
5	Movement disorder (chorea)
6	Myelopathy
7	Seizure disorders
8	Acute confusional state
9	Anxiety disorder
10	Cognitive dysfunction
11	Mood disorder
12	Psychosis

Table 1.7. The Descriptors of CNS manifestation of SLE (Ranked according to decreasing importance) (Rood et al., 1999)

	Descriptors
1	Seizures, primary generalized
2	Psychosis(brief reactive or atypical)
3	Transverse myelitis
4	Global cognitive dysfunction (dememtia)
5	Seizures focal (motor or sensory)
6	Seizures, complex partial
7	Stroke syndrome
8	Limited cognitive dysfunction (objective)
9	Status epilepticus
10	Optic neuropathy
11	Meningitis, aspetic
12	Movement disorders
13	Attentional cognitive dysfunction
14	EEG abnormality
15	CSF abnormality
16	Schizophreniform disorder
17	Cerebral angiographic abnormality
18	Brain scan abnormality
19	Major affective disorder
20	Absence attacks
21	Transient ischaemic attacks
22	Headache, vascular
23	Headache, intractable
24	Schizophrenia
25	Benign intracranial hypertension
26	Limited cognitive dysfunction (subjective)
27	Other psychiatric syndrome/symptoms
28	Generalized anxiety/pain disorder
29	Headache tension

Table 1.8. Case Definitions for NP Syndromes in Systemic Lupus Erythematosus

(ACR Ad Hoc Committee., 1999)

	Descriptions
1	Acute Confusional State
2	Cognitive Dysfunction
3	Myasthenia Gravis
4	Acute Inflammatory Demyelinating Polyradiculoneuropathy (Guillain-Barré Syndrome)
5	Demyelinating Syndrome
6	Myelopathy
7	Anxiety Disorder
8	Headache
9	Neuropathy, Cranial
10	Aseptic Meningitis
11	Mononeuropathy (single/multiplex)
12	Plexopathy
13	Autonomic Disorder
14	Mood Disorders
15	Polyneuropathy
16	Cerebrovascular Disease
17	Movement Disorder (Chorea)
18	Psychosis
19	Seizures and Seizure Disorders

NB. For detail definitions of NP syndromes, basic laboratory evaluation and diagnostic imaging in NP-SLE and suggested proposed One-Hour neuropsychological Battery for SLE, refer to "ACR Ad Hoc Committee on NP lupus nomenclature. The American College of Rheumatology nomenclature and case definitions for NP lupus syndromes". Arthritis Rheum. 1999; 42: 599-608"

2004; Nakamura., 1997). Autoantibodies associated with NP lupus can be grouped into five categories. They are (1) anti-neuronal antibodies, (2) brain-lymphocyte cross-reactive antibodies, (3) anti-ribosomal P proteins, (4) anti-phospholipid antibodies and (5) anti-gangliosides antibodies (Greenwood et al., 2002).

Bluestein and Zvaifler reported an association of brain-lymphocyte cross-reactive antibodies with NP lupus (Bluestein and Zvaifler 1976). These antibodies were identified with reactive lymphocytes antigen of 32, 52, 56 and 98 kD and significant associations with cognitive impairment in patients is observed with the 52 kD proteins (Denburg et al., 1994). However, brain-lymphocyte cross-reactive antibodies react with 52kD autoantigen from both lymphocytes (CD4+ HUT-78) and neuroblastoma cells (SK-N-SH and IMR-6) (Denburg and Behmann., 1994). Thus it is not clear that if these antibodies should be classified as anti-neuronal antibodies.
An association between anti-ribosomal P protein antibodies and SLE psychosis was reported by western blot analysis. (Bonfa and Elkon., 1986). It was confirmed by some, but also refuted by others. Anti-ribosomal P protein antibodies are reported frequently in patients with lupus psychosis, lupus nephritis and also increased during the active phase of SLE (Teh and Isenberg., 1994), and associated with liver dysfunction in SLE patients (Yoshio et al., 1998). Thus anti-ribosomal P antibodies are not solely associated with NP lupus.

Anti-cardiolipin antibodies, associated with antiphospholipid syndrome, are also found in a variety of disorders, such as SLE, syphilis, Q fever, Acquired Immune Deficiency Syndrome (AIDS) (Mouritsen et al., 1989; Harris et al., 1988; Ordi-Ros et al., 1994; Canoso et al., 1987). However, their association with NP lupus is debatable.

Although anti-cardiolipin antibodies are found, not exclusively, to be in high titers in NP lupus, Alarcon-Segovia et al (1997) did not observe an association of serum anticardiolipin antibodies and NP lupus.

Anti-gangliosides antibodies are antibodies against gangliosides located on neuronal and myelin membranes in the central and peripheral nervous system. An association of anti-gangliosides antibodies in NP lupus was reported by Hirano et al. in 1980. However the incidence of these antibodies in NP lupus is yet to be confirmed. Currently it provides little assistance in the management of NP lupus. Many anti-neuronal antibodies have been described in relationship with NP lupus. However the identification of antigens that these antibodies binds to is still unclear except for neurofilament proteins (Robbins et al., 1988). Currently, many of the autoantibodies detected in NP lupus do not show any diagnostic value because of poor specificity and sensitivity. Greenwood et al., (2002) suggested (1) to standardize the methods to prevent discrepancies in antibody detection and (2) to use the ACR definitions and classification for NP lupus to prevent the subjective definitions of CNS syndromes/symptoms.

Anti-neuronal antibodies were first reported in NP SLE using radioimmunoassay and SK-N-SH, a human neuroblastoma cell line (Bluestei., 1978). Subsequently, a variety of human and animal neuronal substrates have been used to detect autoantibodies in NP lupus using a range of methods, such as immunofluorescence, immunoblotting and ELISA. However the identity of most brain-specific proteins remains unknown except for their associations in NP lupus. Whether the presence of these autoantibodies contributes to the development of NP-lupus is not known.

The neuronal proteins used as antigens in detection of autoantibodies can be divided into following categories; (1) neuronal protein from normals, such as human, rat, mouse or cow, and (2) neuronal protein from tumor cell lines such as human neuroblastoma and mouse neuroblastoma. Using tumor cell line as antigens to detect autoantibodies in NP lupus will pose a question of whether these proteins are expressed under normal conditions in neuronal cells and tissues. However a variety of autoantibodies reacting against tumor cell lines was reported to be associated with human and mouse NP-lupus. (Isshi and Hirohata., 1998; Hanly and Hong., 1993; Hoffman et al., 1988; Danon and Garty., 1986; How et al., 1985).

The autoantibodies from human and mouse NP-lupus sera bind to different proteins from human and other animals. (Hanly and Hong., 1993; Klein et al., 1991; Hanson et al., 1992; Moore et al., 1994). Besides the presence of these anti-neuronal antibodies in lupus sera from both human and murine models, attempts to show the presence of antibodies in murine model of NP lupus was also done. Using laser confocal microscopy, the presence of immunoglobulin in brain section of murine model mice (MRL/lpr and BXSB) was reported by Zameer and Hoffman in 2001. Antibodies eluted from brain parenchyma of NZBxW (F1) mice were shown to react with brain tissue of BALB/c mice. (Moore 1992). Furthermore, Narendran and Hoffman (1989) and Moore (1990) demonstrated that multiple autoantibodies are identified against normal mouse brain tissue in sera of different murine lupus models. Among these anti-neuronal antibodies that have shown associations with NP-lupus, none of them has been identified except for one that is partially cloned. (Moore et al., 1998).

Recently, 2 other autoantibodies have been described as associated with NP lupus. Antibodies to triosephosphate isomerase (TPI) was reported to be associated with NP lupus (Watanabe et al, 2004). TPI is a highly conserved 29 kD glycolytic enzyme that catalyses the interconversion of dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate. Deficiency of TPI causes hemolytic anemia and neurological disorders (Schneider, 2000). TPI, though quite similar in molecular weight to one of the 2 novel BRAAs identified by us, has recently been found to be associated with osteoarthritis and advocated as a diagnostic marker for OA (Xiang et al, 2004). N-methyl-D-aspartate (NMDA) receptors are responsible for the majority of excitatory synaptic transmission in the central nervous system. Antibodies to NMDA receptor types NR2a or NR2b, have been reported as being associated with NP lupus, specifically to depressed mood, decreased short-time memory and learning (Omdal et al, 2005). They are about 180 kD in molecular weight (Stephenson, 2001).

iii. Diagnosis and management of NP lupus

During the course of SLE, a variety of NP disturbances are reported (Kaell et al., 1986; Sibley et al., 1995; Futrell et al., 1992; West et al., 1995; Rood et al., 1999). The reported prevalence of these disorders varies widely, raging from 17% to 75%. The diagnosis of NP-lupus is difficult and depends on the exclusion of other causes of CNS manifestations such as metabolic, cardiovascular or infectious causes (Jennekens and Kater., 2002). Many CNS manifestations are subjective and difficult to interpret, requiring a careful and complete psychological evaluation. Thus diagnosis depends on a series of tests (series of hematological, biochemical, radiological and psychological tests).

1.4. Aims of the study

Effective management of lupus with immunosuppressive drugs, which lead to increased survival rate of lupus patients, are also associated with higher risk of recurrent infections. Presence of certain MBL variants favoring MBL deficiencies, can aggregate the infectious risks. The presence of autoantibodies, which target specific organ system, may pose additional severity factors in lupus patient undergoing current aggressive lupus management. We therefore aim to study following:

- The role of polymorphic MBL gene in lupus patients and infections
- The role of anti-nephrin antibodies in lupus patients and renal involvement
- The role of BRAA in lupus patients and NP manifestations

We will also determine if specific MBL variant, anti-nephrin antibodies and brain reactive autoantibodies are associated with disease severity in systemic lupus erythematosus.

CHAPTER 2

MATERIALS AND METHODS

2.1 Patient selection and sample collection

One hundred and two healthy volunteer controls were recruited into the study. One hundred unselected lupus patients, fulfilling the 1997-updated ACR criteria for classification of SLE (Hochberg, 1997), were recruited into this study. One hundred and thirty non-lupus rheumatic patients (20 Rheumatoid Arthritis patients, 20 Ankylosing Spondylitis patients, 20 Psoriatic Arthritis patients, 20 Osteoarthritis patients, 50 patients with Primary Anti-phospholipid syndrome) were also recruited into the study. All non-lupus patients were diagnosed by attending rheumatologists using established disease criteria. All lupus patients and non-lupus rheumatic patients were attending the rheumatology clinics at the Singapore General Hospital.

After informed consent, 5 ml each of peripheral venous blood from controls and lupus patients, were taken in both plain tube and Na_2EDTA tube for DNA extraction and sera preparation. 5 ml of the peripheral venous blood from non-lupus patients was collected in plain tubes for sera preparation. The sera were separated from clotted blood using standard protocol and genomic DNA extraction was done using modified salting-out method (Miller et al., 1998). All aliquoted sera and DNA samples were kept in -80°C freezer until further experiments.

A standard protocol was used to collect demographic and clinical data of lupus patients. Briefly the drugs used in lupus treatment (especially steroid and cytotoxic drugs), renal and NP involvements as defined by the 1997-updated ACR criteria for classification of SLE (Hochberg, 1997) and ACR-defined NP lupus nomenclature, detailed renal presentation, and infections (bacterial, viral and fungal) were recorded

together with patients' demographic data. SLE Damage Index of lupus patients was defined for twelve organ systems using SLICC/SCR DI during this study (**Appendix 1**).

2.2 Mannose Binding Lectin

Currently the available methods used in genotyping and haplotyping of MBL gene were PCR-based methods, such as PCR-SSP, PCR-SSO, realtime PCR, UHG-PCR. PCR-RFLP methods were also available to genotype codons 54 and 57 of exon 1 and –221 bp site (X/Y alleles) of promoter region. The lack of readily available restriction endonucleases for codon 52 and –550 bp site (H/L alleles) made PCR-RFLP a partial method to genotype and haplotype the MBL gene. We therefore decided to design mismatched primers where restriction endonucleases sites are incorporated so that genotyping and haplotyping of MBL gene can be completely determined by PCR-RFLP method.

2.2.1. A novel PCR-RFLP for genotyping and haplotyping of MBL gene.

We introduced a restriction endonuclease recognition sites at codon 52 of exon 1 and –550 bp site (H/L alleles) of promoter region of MBL gene, by altering some nucleotides at mismatched primers and validated a novel PCR-RFLP method for complete genotyping and haplotyping of the MBL gene polymorphisms.

i. Design and synthesis of primers

All primers were synthesized locally (Research Biolabs and BST Scientific, Singapore).

Two mismatched primers were manually designed for use in this study. The forward mismatched primer for MBL exon 1 (MBL-E1-FOR; 5'-CATCAACGGCTTCCCAGG**gc**AAGATGGG-3') containing *Mwo* I recognition site was designed at the 5' end of Codon 52. The reversed primer for exon 1 (MBL-E1-REV; 5'-GTCTCCTCATATCCCCAGGC-3') was also synthesized for PCR amplification (**Figure. 2.1**). The forward mismatched primer for promoter region (MBL-PRO-FOR; 5'-GAAAATGCTTACCCAG**a**CAAGCCTGT-3') was also designed manually at the 5' end of the polymorphic site H/L and *Drd* I enzyme cleavage site was added. The reversed primer for promoter PCR (MBL-PRO-REV; 5'-TCATCTGTGCCTAGACACCTGGC-3') was designed away from the X/Y mutation site to allow for differentiable fragments and easy interpretations after RFLP (**Figure. 2.2**).

Sequence specific primers for exon 1 and promoter gene polymorphism were synthesized according to Steffensen et al. (2000). (**Table 2.1**)

ii. Polymerase Chain Reactions

MBL-E1-FOR and MBL-E1-REV primers were used to amplify the polymorphic sites at exon 1 while MBL-PRO-FOR and MBL-PRO-REV were used for polymorphic sites at the promoter gene region.

PCR conditions, using BioMetra T3 thermocycler, were as follows: 1 cycle of 95°C for 5 minutes followed by 35 cycles of denaturing at 94°C for 10 seconds, annealing at 62°C for 30 seconds and extension at 72°C for 30 seconds. The final extension step was done at 72°C for 10 minutes. 30 µl of PCR reaction contained 50ng of genomic DNA, 0.2 µM each of forward and reverse primers; 0.1 mM of dNTPs, 1x reaction buffer (10 mM Tris-HCl pH 8.8 at 25 °C, 1.5 mM $MgCl_2$,, 50 mM KCl and 0.1% Triton X-100) and 0.2 units of DyNAzyme ™ II DNA polymerase (FinnZymes).

4 µl of PCR products were visualized on Ethidium Bromide stained 2% agarose gel in 0.5xTBE buffer before cleavage by different restriction endonucleases.

iii. Restriction Fragment Length Polymorphisms

All restriction endonucleases used in this study were purchased from New England Biolabs.

4 µl each of MBL exon 1 PCR products were incubated with *Mbo* II (1 unit), *Ban* I (0.13 unit) at 37°C, and *Mwo* I (0.13 unit) at 60°C for one hour according to

```
                                        gcnnnnnnngc←I    ggyrcc←II
                    -------------------gc---------
**********catcaacggcttcccaggcaaagatgggcgtgatggcacc      → +234
   ggaga←III
aagggagaaaaggggaaccaggtacgtgttgggctgttctgtctctgca       → +284

attctttaccttccagaggaaactgcctggggatatgaggagac******      → +334
```

Figure. 2.1 Design of forward mis-matched and reversed primers for Exon 1 MBL ; *Mwo* I (I) will cut wild type allele, *Ban* I (II) will cut wild type allele and *Mbo* II (III) will cut mutant allele. r = a or g; y = c or t ; n = a or c or t or g:

```
                                          gacnnnnnngtc←IV
                 ----------------a---------
*****************gaaaatgcttacccaggcaagcctgtgtaaaac      → -544

accaaggggaagcaaactccagttaattctgggctgggttggtgactaag      → -494

gttgaggttgatctgaggttgagaccttcctctttggatcaccagctttc      → -444

agctcagggcctgccaatgagtaaatgatagttaacaggtcctggagggg      → -394

aatcagctgcccagatacaaagatgggattcaggtggcagatggacccga      → -344

agaggacatggagagaaagaggaagctcctacagacacctgggtttccac      → -294

tcattctcattccctaagctaacaggcataagccagctggcaatgcacgg      → -244
                      ccyrgg←V
tcccatttgttctcactgccacggaaagcatgtttatagtcttccagcag      → -194

caacgccaggtgtctaggcacagatga***********************      → -144
```

Figure. 2.2 Design of forward mis-matched and reversed primers for promoter region of MBL; *Drd* I (IV) will cut L allele and *Btg* I (V) will cut Y allele:
r =a or g ; y =c or t ; n = a or c or t or g.

Table 2.1. List of primers (for PCR-RFLP and PCR-SSP) to genotype the exon 1 and promoter region of MBL.

No	Primers	Sequence	Methods	Remark
1	MBP-E1-FOR (mismatched)	5'-CATCAACGGCTTCCCAGG**GC**AAGATGGG-3'	PCR-RFLP	#
2	MBP-E1-REV	5'-GTCTCCTCATATCCCCAGGC-3'	PCR-RFLP	#
3	MBP-PRO-FOR (mismatched)	5'-GAAAATGCTTACCCAG**A**CAAGCCTGT-3'	PCR-RFLP	#
4	MBP-PRO-REV	5'-GCCAGGTGTCTAGGCACAGATGA-3'	PCR-RFLP	#
5	MBLSSP	5'-CTGCACCCAGATTGTAGGACAGAG-3'	PCR-SSP	*
6	52DR	5'-TCTCCCTTGGTGCCATCACA-3'	PCR-SSP	*
7	52AR	5'-TCTCCCTTGGTGCCATCACG-3'	PCR-SSP	*
8	54BR	5'-CCCCCTTTTCTCCCTTGGTGT-3'	PCR-SSP	*
9	54AR	5'-CCCCCTTTTCTCCCTTGGTGC-3'	PCR-SSP	*
10	57CR	5'-ACGTACCTGGTTCCCCCTTTTCTT-3'	PCR-SSP	*
11	57AR	5'-ACGTACCTGGTTCCCCCTTTTCTC-3'	PCR-SSP	*
12	H-s	5'-GCTTACCCAGGCAAGCCTGTG-3' 76-96	PCR-SSP	*
13	Y-as	5'-CTGGAAGACTATAAACATGCTTTCC-3'	PCR-SSP	*
14	L-s	5'-GCTTACCCAGGCAAGCCTGTC-3'	PCR-SSP	*
15	X-as	5'-GGAAGACTATAAACATGCTTTCG-3'	PCR-SSP	*

(NB: # newly designed primer, * R. Steffensen et al.)

manufacturer's instructions. The cleaved DNA fragments were visualized on Ethidium bromide stained 2 % agarose gel (SeaKem LE agarose) in 0.5x TBE buffer.

4 μl each of MBL promoter gene region PCR products were incubated with *Drd* I (0.13 unit) and *Btg* I (0.5 unit) overnight at 37°C. The cleaved DNA fragments were visualized on Ethidium-bromide-stained 3 % agarose gel (1% NuSieve GTG agarose and 2% SeaKem LE agarose) in 0.5x TBE buffer.

iv. Validation of the PCR-RFLP results.

We have tested the robustness and reproducibility of our novel PCR-RFLP method by i.) DNA sequencing, ii.) comparison with PCR-SSPs and iii) independent performance by volunteers.

Samples with homozygous and heterozygous results at exon 1 and samples with different promoter gene haplotypes were randomly selected for sequencing. DNA sequencing reactions were performed with appropriate forward and reversed primers on purified PCR products (spin columns, QIAGEN) using ABI PRISM BigDye Terminator v3.0 Sequencing Kit and ABI 3100 Genetic Analyzer (Applied Biosystem). PCR-SSP typing method was performed using established method of PCR-SSP to genotype and haplotype the MBL gene in selective samples. PCR conditions and compositions are as described elsewhere (Steffensen et al., 2000).

To test reproducibility and simplicity of use, DNA samples containing different genotypes of exon 1 and promoter gene region polymorphisms were distributed to

four different laboratory-personnel volunteers together with the detailed protocol. These volunteers performed the PCR-RFLP using this detailed protocol, the results of which were collected separately and compared.

2.2.2. Genotyping of Exon 1 and Haplotyping of Promoter region of MBL gene.

Upon successful optimization, healthy individuals and lupus patients were genotyped and haplotyped for exon 1 and promoter region of MBL gene using novel PCR-RFLP protocol.

A total of 102 healthy Chinese individuals were used in genotyping and haplotyping of MBL exon 1 and promoter region using our novel PCR-RFLP method.

The genotype of exon 1 and haplotypes of promoters region of MBL gene of 100 lupus patients were also analyzed using PCR-RFLP method.

2.2.3. Prevalence of MBL gene variants in lupus patients

The frequencies of different variants were analyzed in lupus patients compared to control individuals. All significant MBL variants were further correlated with infections among lupus patients. Chi-square test was used to determine the significance of these findings and Fisher's exact test was used when necessary.

2.3 Nephrin

The nephrin gene was responsible for Finish-type of familial nephrotic syndrome. It was well established that mutations in the nephrin gene is associated with proteinuria. Recently it was reported that the monoclonal antibodies (mAb 5-1-6), which caused transient proteinuria in rat when injected intravenously, was targeted against the extracellular domain of nephrin. We decided to express the fragment of extracellular portion of mouse nephrin protein, which has similar amino acid sequence among the mouse, rat and human nephrin genes (**Figure. 2.3**).

2.3.1. Expression of nephrin protein fragment

All animals were purchased from the Animals Holding Unit, National University of Singapore. All procedures and protocols used in this study are in concordance with standard practice. The flowchart for cloning of nephrin protein is presented in **Figure 2.4**.

i. Animal selection and RNA extraction

Two 8 week-old female BALB/c mice were sacrificed by using Chloroform intoxication. Abdominal cavities were opened under standard sterile conditions and the kidneys were removed, weight measured and then snap-frozen in liquid nitrogen. One pair of kidneys was kept using frozen tissue media for immunohistochemistry studies.

```
"DNGVAPAARGLVRLVVRFAPQVDHPTPLTKVAAAGDSTSSATLHCRARGVPNIDFTWTKNGVPLDLQDPRYTEH
KYHQGVVHSSLLTIANVSAAQDYALFKCTATNALGSDHTNIQLVSISRPDPPLGLKVVSVSPHSVGLEWKPGFDG
GLPQRFQIRYEALETPGFLYMDVLPAQATTFTLTGLKPSTRYRIWLLASNALGDSGLTDKGIQVSITTPGLDQAP
EDTDQPLPTEQPPGPPR"
```

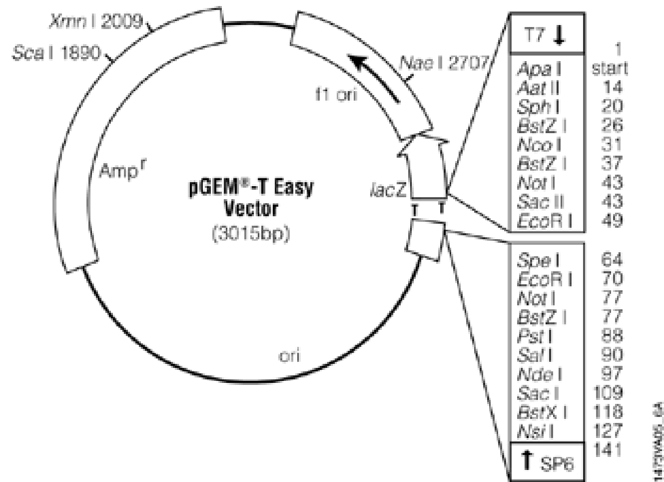
```
Mouse   DNGVAPAARGLVRLVVRFAPQVDHPTPLTKVAAAGDSTSSATLHCRARGVPNIDFTWTKNG
Rat     -----------------------Q-------------------------------------
Human   ------P--R-L----------E------------------------------V-------

Mouse   VPLDLQDPRYTEHKYHQGVVHSSLLTIANVSAAQDYALFKCTATNALGSDHTNIQLVSISR
Rat     -------------R-----------------------------------------------
Human   -------------T----G--------------------T----------Q----------

Mouse   PDPPLGLKVVSVSPHSVGLEWKPGFDGGLPQRFQIRYEALETPGFLYMDVLPAQATTFTLT
Rat     -----------I----------------------------------HV----T--------
Human   ----S------LT--------------------C------G----H-V--V-P--------

Mouse   GLKPSTRYRIWLLASNALGDSGLTDKGIQVSITTPGLDQAPEDTDQPLPTEQPPGPPR***
Rat     -------------------------------V----P--------HQ----L------***
Human   --Q------V-------------A---T-LP------H-PSGEPEDQ----P-S--SG***
```

Figure. 2.3. The fragment of murine nephrin protein for expression and its homology across mouse, rat and human



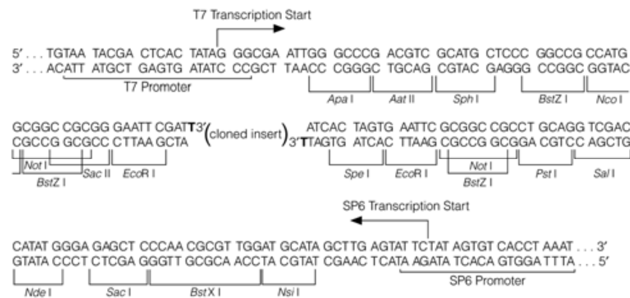


Figure. 2.5. pGEM®-T Easy vector and its multiple cloning site

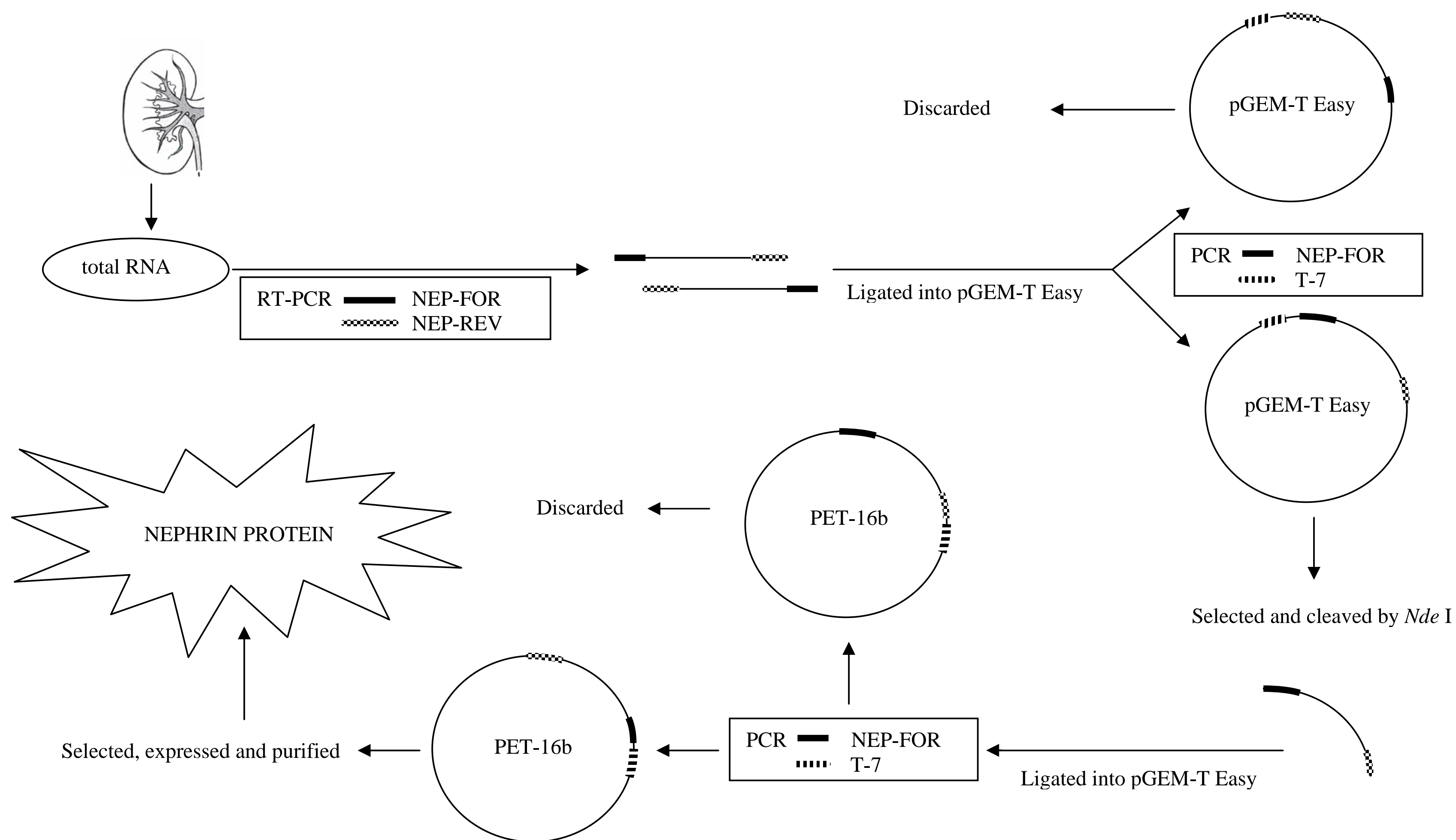


Fig. 2.4. Molecular cloning flowchart for mouse nephrin;
Murine nephrin of BALB/c origin was cloned into pET-16b (His-tagged expression system), with brief construction of expression vector

Frozen kidneys were homogenized by means of mortar and pestle in the presence of continuous flow of liquid nitrogen. Using syringes and needles (from 19G to 25 G sizes) the tissue lysates were further homogenized. Homogenized tissues were transferred to Trizol reagent (Gibco BRL) according to manufacturer's instructions (1 ml of Trizol reagent per 100 mg tissue). The amount of extracted total RNA was measured by spectrophotometer. Reverse transcriptions and polymerase chain reactions (RT-PCR) were then performed.

ii. Design and Synthesis of primers

The primers used in RT-PCR are manually designed and vector construction and selection of correctly orientated clone were available from manufacturer's instruction manuals. All of them were synthesized locally (Research Biolabs and BST Scientific, Singapore) (**Table 2.2**).

iii. Reverse transcription and polymerase chain reaction

The first strand cDNA from murine kidney RNA was synthesized with random primers using RNase H Reverse transcriptase (SuperScript^{TM} II, Gibco-BRL) according to manufacturer's instructions. RNase inhibitors were used when handling RNA samples in the experiments.

The cDNA containing gene segment of interest was amplified in PCR using forward and reversed primers flanking the region of interest by means of standard PCR

Table 2.2. List of primers for RT-PCR, construction of expression vector in cloning of nephrin protein fragment.

No	Primers	Sequence	Used in	Remark
1	NEP-FOR	5'- GGCCCATATGGACAACGGGGTGGCTCCC -3'	RT-PCR	#
2	NEP-REV	5'-GGCCCATATGTCACCTCGGGGGTCCCGGAGG -3'	RT-PCR	#
3	T7- promoter	5'- TAATACGACTCACTATAGGG -3'	pGEM-T easy vector	*
4	SP6- primer	5'- ATTTAGGTGACACTATAG-3'	pGEM-T easy vector	*
5	T7-primer	5'- TAATACGACTCACTATAGGG -3'	pET-16 expression vector	@
6	T7-terminator	5'- GCTAGTTATTGCTCAGCGG -3'	pET-16 expression vector	@

(NB: # manually designed primer, * Promega; @ invitrogen.)

procedure, utilizing DyNAzyme DNA polymerase kit in Thermocycler (MJ Research).

iv. Sub-cloning for PCR product and selection of correct orientation

The PCR product was transferred into the pGEM-T easy vector (**Figure 2.5**) using Promega TA-cloning kit. Briefly, the PCR product was ligated into pGEM-T easy vector using DNA ligase, transformed into chemically competent JM 109 cells using heat-shock method. The E. Coli (JM 109) cells grown in LB media without antibiotics for one hour were then transferred to LB+ampicillin agar-plate and incubated overnight at 37°C.

The single colonies were picked and grown in 1 ml LB media for three hours and the clone containing the corrected orientation were selected by using PCR method. The clones with correct orientation were further grown in 10 ml LB liquid media containing Ampicillin as selection agent. Upon obtaining reasonable culture, the plasmid DNA was extracted using standard alkaline miniprep. The plasmid DNA, containing correct orientation of PCR product, was cleaned by phenol method and measured the amount of plasmid DNA measured by spectrophotometer.

v. Construction and selection of expression vector

The His-tagged protein expression system was used to express the gene of interest in this study.

The E. Coli (JM109) containing the expression vector pET-16b (**Figure 2.6**) was grown in LB media without antibiotics, the plasmid DNA was then extracted and cleaned by phenol method. The amount and purity of plasmid DNA was measured.

The expression vector (pET-16b) and pGEM-T easy vector, containing gene of interest were subjected to cleavage by restriction endonuclease *Nde* I (New England Biolabs) according to recommended conditions. The cleaved plasmids (pET-16b) were separated on 0.8% normal agarose gel electrophoresis while the cleaved plasmids (pGEM-T easy) were separated on 1.5% gel electrophoresis. The cleaved fragments were confirmed by enzyme map, then the cleaved expression vector (pET-16b) and the fragments containing gene of interest were excised from the gel. The cleaved expression vector and fragment were spun in glass-wool columns and purified using phenol method. The linearised pET-16b was dephosphorylated, prior to ligation with DNA insert, to prevent self-ligation.

The dephosphorylated expression vector (pET-16b) and DNA insert (fragment of nephrin gene) were ligated using Promega DNA T4 ligase according to manufacturer's instructions. The ligated plasmids were transformed into the chemically competent JM109 cell using heat-shock method. The transformed JM109 cell grown after one hour in LB media without antibiotics were plated onto LB+Ampicillin agar plate and incubated at 37°C overnight. The single colonies were selected and grown in LB media with ampicillin as selection agent for three hours with shaking. The E.Coli clones, containing the pET-16b expression vector with correct orientation, were selected using PCR technique with cell lysate as templates. The pET-16b with correct orientation were further grown in 10 ml of LB media

containing ampicillin at 37°C with constant shaking. Upon reasonable growth of bacterial cells, the plasmid DNA was extracted using standard mini-prep. The plasmid DNA was cleaned by using phenol method and quantity of plasmid DNA was measured by spectrophotometer.

vi. Expression of nephrin protein fragment

The plasmid DNA (Expression pET-16b vector containing gene of interest) was transformed into chemically competent E. Coli BL21 (expression strain) by using heat-shock method. The transformed BL21 cell grown after one hour in LB media without antibiotics were plated onto LB+Ampicillin agar plate and incubated at 37°C overnight. The single colonies were selected and grown on LB media containing ampicillin as selection marker, separated into two eppendorfs tubes after three hours and grown further. One of the eppendorf tubes was induced using 0.1mM IPTG for one more hour while the other eppendorf tube acted as control without IPTG induction. After an hour's induction, *E.coli* pallet was collected and SDS-PAGE was done using both cell lysates (both induced and uninduced) to determine if the expected protein was seen.

An expression experiment was done to find out if the protein was expressed as soluble protein or insoluble protein (inclusion bodies) after confirming the expression of nephrin protein. Briefly, the IPTG-induced E Coli were lysed using different approaches (sonication, homogenization by syringe and needle, proteolytic lysis, free-thaw cycles, combination of these methods). Insoluble fraction was separated by

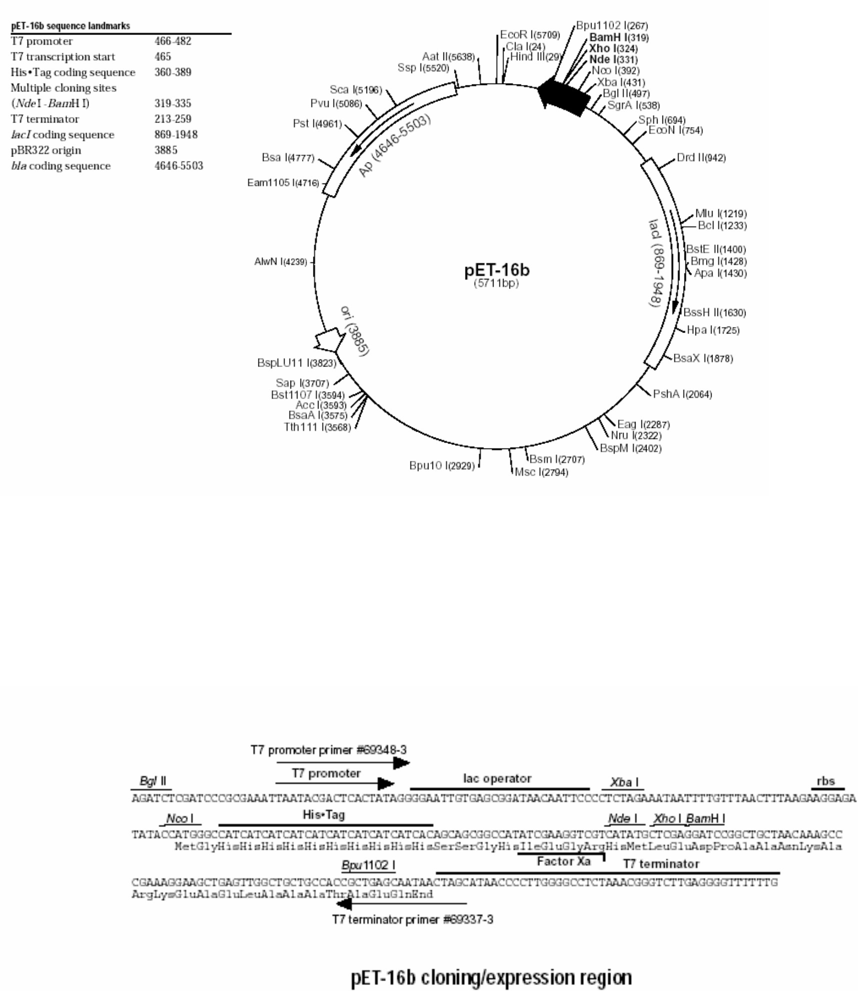


Figure. 2.6. pET-16b expression vector and its multiple cloning site

using centrifugation and solubilised using 8M Urea. Both soluble and insoluble fractions were visualized on SDS-PAGE to find out the nature of expressed protein.

2.3.2.Purification of expressed nephrin protein

Small scale of purification was carried out in 5 ml of LB-media of the expressed protein. E.coli were collected and lysed by means of mechanical and proteolytic approaches (combination of free-thaw cyles and proteolytic approaches). The insoluble fractions (inclusion bodies) was washed completely and solubilised in 8M Urea. The expressed nephrin protein was purified from the mixture of urea-solubilised proteins using Ni-NTA agarose (Qiagen) (**Figure 2.7**). Briefly, the urea-soluble protein was incubated with Ni-NTA agarose and washed completely to remove any unbound proteins. Then the 6xHis-tagged expressed protein was eluted using acidic elution buffer. All proteins from the different fractions (binding step, washing steps and elution steps) were eletcrophoresed on SDS-PAGE.

Large scale expression and purification was carried out using 1 liter LB medium after the confirmation of the small-scale purification result. Briefly, the columns were made using 10 ml syringe with 2 ml of Ni-NTA agarose bed volume. Glass wool plugs were used to prevent agarose leaking from the syringes during all steps of purification. The concentration of protein purified from Ni-NTA was measured using BIORAD protein assay. All proteins were aliquoted and kept in -80°C freezer.

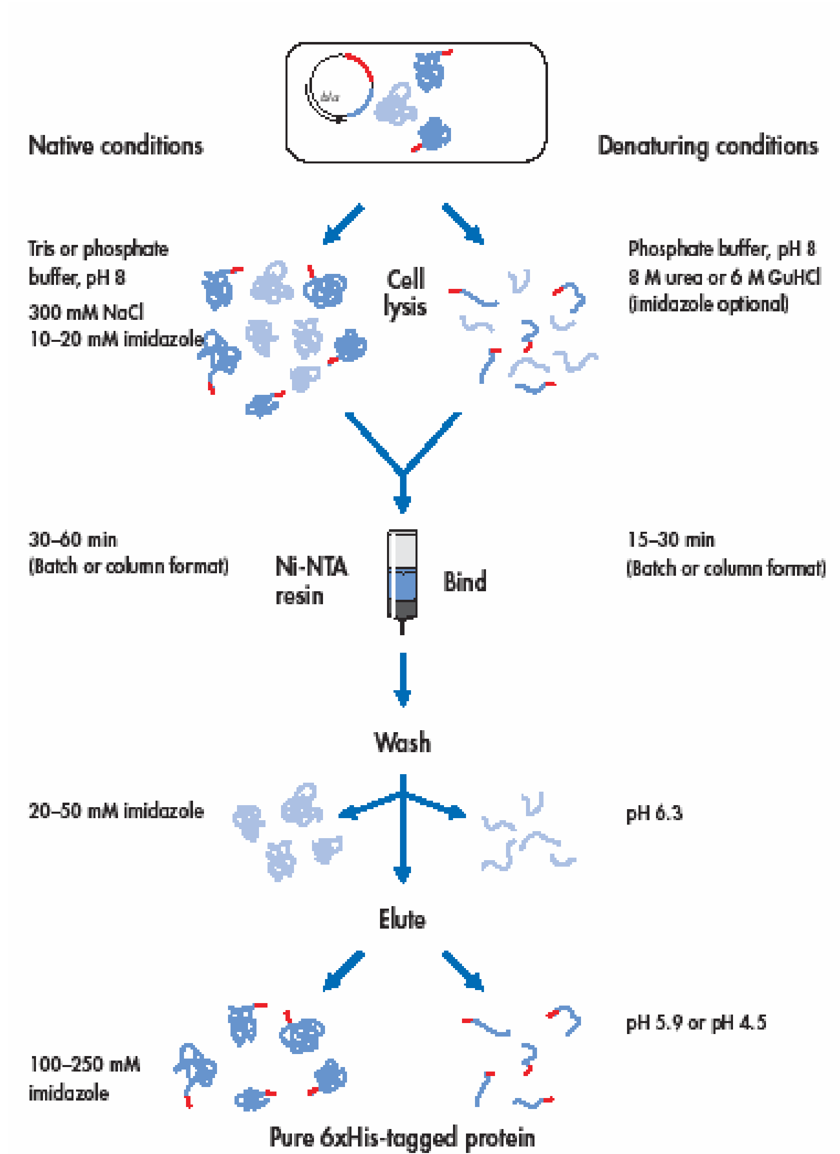


Fig. 2.7. His-tag fusion protein purification system

2.3.3.Production of polyclonal antibodies against expressed protein

All animals were purchased from the Animals Holding Unit, National University of Singapore and maintained in Animals Room, Department of Physiology, National University of Singapore with standard accommodation and food provided during the period of production of polyclonal antibodies. All procedures and protocols used in this study are recognized internationally.

i. Animal selection, bleeding and culling

Two New Zealand white (NZW) female rabbits (about 2.5 kilograms) each obtained from the Animal Holding Unit, National University of Singapore were allowed to get accustomed to their new environment (Animals Room, Department of Physiology, National University of Singapore) for one week before the first pre-bleed and first immunization.

Before the immunization, the pre-bleed (5 ml of whole blood per rabbit) was taken from both rabbits. The subsequent bleedings (25 ml of whole blood per rabbit) were done every two weeks after booster doses. Immunizations and bleedings were done under anesthesia at the Animals Holding Unit, NUS, under standard sterile condition. The rabbits were kept for four months after which they were sent to Animal Holding Unit for culling according to standard procedures.

ii. Rabbit immunization.

500 µg of purified protein per rabbit was used. The antigens were prepared in Complete Freund's adjuvant (containing 0.5 mg/ml microbacterium cell wall components) and the total volume of 1 ml consisted of 500 µl CFA and 500 µl of 1xPBS containing 500 µg of antigen for one rabbit. Upon complete suspension by vigorous mixing, the antigen were injected into four sites, two on the thighs and two on lower back. All sub-cutaneous immunizations were done under sterile conditions. Booster doses with antigen prepared only with incomplete Freund's adjuvant were done every 4 weeks, with 25 ml-bleed per rabbits 14 days after each injection.

The quantity of the antibodies in rabbit's serum is monitored using indirect ELISA. The cut-off point for anti-nephrin antibodies was set at 3 S.D (Standard Deviation) above the mean value (OD) of anti-nephrin antibodies from BALB/c mice sera. The indirect ELISA is used primarily to determine the strength and/or amount of antibody response in a sample, whether it is from the serum of an immunized animal or the cell supernatant from growing hybridoma clones.

iii. Purification of polyclonal antibodies.

A standard ELISA method for the assaying of anti-nephrin antibodies with pre-bleed sera as controls, were used to determine the titers of anti-nephrin antibodies. When significantly high titers of anti-nephrin antibodies were present in the rabbit serum, the polyclonal antibodies were purified by ammonium sulfate precipitation. Two-thirds volume of saturated ammonium sulfate solution was added to pooled rabbit's sera and the pallet was separated by centrifugation. The precipitation was repeated

and precipitated polyclonal antibodies were suspended in 1xPBS. After dialysis against 1xPBS thrice, the protein concentration was determined by BioRad protein assay.

2.3.4.Production of monoclonal antibodies against expressed protein

All animals were purchased from the Animal Holding Unit, National University of Singapore and maintained in Animals Room, Department of Physiology, National University of Singapore with standard accommodation and food provided until scarified. All procedures and protocols used in this study are recognized internationally.

i. Animal selection and bleeding

Two 8-week old female BALB/c mice were used for production of monoclonal antibodies. These mice were allowed to get accustomed to their new environment for one week before the first pre-bleed and first immunization. All standard accommodation and food were provided during the period of production of monoclonal antibodies. Before the immunization, the pre-bleed (50μl) was taken from all mice from tail vein.

ii. Mouse immunization.

100 μg of purified protein per mouse was used. The antigens were prepared in complete Freund's adjuvant (containing 0.5 mg/ml microbacterium cell wall components) and total volume of 200 μl of consisted 100 μl CFA and 100 μl of

1xPBS containing 100 μg of antigen for one mouse. Upon complete suspension by vigorous mixing, antigens were injected into mouse intraperitoneally using standard sterile method. Booster dose with antigen prepared only with incomplete Freund's adjuvant was repeated every 2 weeks, with 50μl-bleed per mouse.

Indirect ELISA monitors the quantity of the antibodies in serum (humoral immune response) of the bleeds. The cut-off point was defined as described.

iii. Preparation of myeloma cells and splenic cells

The myeloma cell line of murine BALB/c origin (P3X63Ag8.653) was used as immortal cell line in fusion because they do not secrete any antibodies. The cell line was maintained for about two weeks at $4x10^5$cells/ml in ClonalCell™-HY pre-fusion Medium (Medium A of ClonaCell™ complete kit) using standard sterile cell culture procedure. This is to ensure that they are well adapted to this medium before fusion Since the recommended cell density for fusion is $2x10^5$ cells/ml, double density of myeloma cells are kept to make sure sufficient numbers are available on fusion day. Cells harvested on the fusion day were washed three times with serum-free ClonalCell™-HY fusion Medium (Medium B of ClonaCell™ complete kit). The viable cells were counted after the third wash, the cell suspension containing $2x10^7$ cells was prepared and kept on ice until the fusion step.

The BALB/c mouse immunized with expressed nephrin protein and had shown high titer of antibodies against immunized protein was sacrificed. The spleen was removed aseptically and splenic cells were prepared for fusion. Briefly, the splenic cells were

collected through smashing against a metal sieve, collected cells washed thrice with serum-free ClonalCell^{TM}-HY fusion Medium (Medium B of ClonaCell^{TM} complete kit). After the last wash, the viable splenic cells were counted using 3% acetic acid where red cells will be lysed. The cell suspension containing $2x10^8$ cells was prepared and kept on ice until fusion step.

iv. Fusion using ClonaCell^{TM} complete kit.

The fusion of the myeloma cells and splenic cell was done using PEG solution according to manufacturer's instructions. After the fusion step, the cell pallet was resuspended in Medium C of ClonaCell^{TM} complete kit and incubated at 37°C in 5% CO_2 atmosphere for one day. On the following day, ClonalCell^{TM}-HY Hybridoma selection Medium (Medium D of ClonaCell^{TM} complete kit) was used to resuspend the fused cells and this resuspended fused cells were plated on ten petri plates which were incubated at 37°C in 5% CO_2 atmosphere for two weeks without any disturbances.

v. Selection of the correct clone from fused myeloma.

After a 2-week incubation, the plates were examined for the presence of colonies visible to the naked eye. Each visible clone was transferred to an individual well of 96-wells tissue culture plates containing 200 µl of ClonalCell^{TM}-HY Growth Medium (Medium E of ClonaCell^{TM} complete kit) and incubated at 37°C in 5% CO_2 atmosphere for 4 days without feeding. The supernatant of each hybridoma was collected for screening to detect anti-nephrin antibodies using ELISA method. The

positive clones were resuspended in 1 ml of ClonalCell^{TM}-HY Growth Medium (Medium E of ClonaCell^{TM} complete kit) and incubated at 37°C in 5% CO_2 atmosphere to grow further so as to reach a suitable density.

2.3.5.Confirmation of expressed protein and its polyclonal antibodies

The expressed protein (fragment of mice nephrin protein) was checked by enzymes map during vector construction before protein expression and by molecular weight in SDS-PAGE gel after protein expression. Further more, the expression protein was confirmed by protein finger printing and its polyclonal antibodies were also confirmed by immunohistochemical methods.

i. Peptide Mass Fingerprinting

The expressed nephrin protein was electrophoresed on acrylamide gel at denatured condition. Upon completion of SDS-PAGE, Coomassie blue was used to detect presence of expressed nephrin protein. The protein was punched using a yellow pipette tip and the punched gel piece was sent for peptide mass fingerprinting using MALDI-TOF-MS (matrix-assisted laser desorption ionization- Time of Flight - Mass Spectrometry) at Agenica Research Pte Ltd, Singapore.

ii. Immunohistochemistry

Two female BALB/c mice were sacrificed, and the kidneys were removed and frozen using freezing media. The kidneys were cut into 5µm thick frozen sections using

cryostat, fixed using acetone for 2 minutes, then used for immunohistochemical staining. Briefly, endogenous peroxidase activity was blocked by incubating the frozen sections with 0.3% H_2O_2 for 10 minutes, followed by incubation with rabbit polyclonal antibodies against expressed and purified nephrin protein fragment. Second antibodies (goat anti-rabbit IgG) conjugated with Horse Raddish Peroxidase and chromogen 3,3'-diaminobenzidine (DAB) were used to visualize the location of antigen where antibodies bind.

2.3.6.Detection of anti-nephrin antibodies (ELISA method)

A standard ELISA was set up for detection of anti-nephrin antibodies in both mouse and human samples. The expressed nephrin protein was coated in standard ELISA plate using carbonate buffer. Samples diluted at 1:100 dilution in TBS-T were then incubated, followed by the second antibodies, which were either goat anti-human or goat anti-mouse immunoglobulins according to sera used. The OPD substrate was used to detect colourimetric changes in the final step as second antibodies were conjugated with horse raddish peroxidase enzymes (HRP).

Using pre-bleed immunized sera of BALB/c mice as negative and positive controls respectively, the BALB/c (normal mice), MRL lpr/lpr mice (lupus model), the experimental lupus mouse model of (PL/JxNZW) F1 x NZB mice were tested for anti-nephrin antibodies. All sera in duplicate were tested in two ELISA tests (one with coated expressed protein and one without any coated protein) and all ELISA tests were repeated twice to ensure validity of results.

The sera of healthy individuals (102 samples) and lupus patients (100 samples) were tested for presence of anti-nephrin antibodies using standard ELISA protocol. However, absorbance values (non-specific binding) were unexpectedly high in both control and lupus sera. Different blocking agents such as bovine serum albumin (BSA), scanned milk powder, fetal calf serum, gelatin, human albumin, were used in optimization of ELISA to reduce the non-specific binding. Among all different blocking agents, the ELISA result using human albumin was best among all blocking agents tried.

2.3.7.Detection of anti-nephrin antibodies (Immunobloting method)

The anti-nephrin antibodies were detected using immunoblotting technique. Briefly, the expressed nephrin protein, electrophoresed in 12% denatured polyacrylamide gel, was transferred onto nitrocellulose membrane. The membrane containing nephrin protein was cut into appropriate strips and incubated in samples sera diluted at 1:100 dilution in TBS-T. Secondary antibody was goat anti-human immunoglobulin conjugated with alkaline phosphatase diluted at 1:10,000 dilution in TBS-T. NBT/BCIP was used to detect presence of antibodies in sample sera.

Using pre-bleed immunized sera of BALB/c mice as negative and positive controls respectively, the BALB/c (normal mice), MRL lpr/lpr mice (lupus model), the experimental lupus mice model of (PL/JxNZW) F1 x NZB mice was tested for anti-nephrin antibodies by immunoblotting method.

The sera of healthy individuals (102 samples) and lupus patients (100 samples) were tested for presence of anti-nephrin antibodies using immunoblotting methods. A total of 100 non-lupus samples (20 Rheumatoid Arthritis patients, 20 Ankylosing Spondylitis patients, 20 Psoriatic Arthritis patients, 20 Osteoarthritis patients, 20 patients with Primary Anti-phospholipid syndrome) were also tested for presence of anti-nephrin antibodies by using immunoblotting method.

2.3.8.Prevalence of anti-nephrin antibodies in lupus patients

The frequencies of anti-nephrin antibodies were analyzed in lupus mouse model (PL/JxNZW F1 x ZNB) compared to BALB/c mice and correlated with presence of proteinuria. Similarly the frequencies of anti-nephrin antibodies of lupus patient were analyzed and correlated with renal involvement as defined by the ACR criteria. Chi-square test was used to determine the significance of these findings and Fisher's exact test was used when necessary.

2.4.Brain reactive autoantibodies

Brain lysate from different species and different cell lines were studied. Since cell membrane is the structure that can readily react with pathogenic antibodies, we decided to focus on neuronal membrane proteins.

2.4.1.Selection of brain tissues for protein lysates

Two 8 weeks-old female BALB/c mice purchased from the Animals Holding Unit, National University of Singapore, were sacrificed by chloroform intoxication method, then perfused with 50 ml of 1xPBS through cardiac ventricles. The brains and other tissues (liver, heart, kidney and spleen) were removed, immediately snap-frozen in liquid nitrogen, and kept in -80°C freezer until protein extraction.

The female Wistar rat's brain was a gift from Ms Irene Kee of Department of Experimental Surgery, Singapore General Hospital. The brains of pig, chicken, duck, quail, frog and fish (Indian Mackerel-*Rastrelliger kanagurta*) were bought from local markets.

Human neuronal membrane proteins (normal adult male) were purchased from BioChain, USA.

2.4.2.Membrane protein preparations

All chemicals were purchased from Sigma Aldrich, unless otherwise mentioned.

The frozen mouse brain was thawed, cut into smaller pieces followed by homogenisation using mortar and pestle in 1xPBS containing a cocktail of proteinase inhibitors (5μg/ml leupeptin, 5μg/ml approtinin and 1mM PMSF). Brain lysates were further homogenised using syringe and needles method (started from 19G and gradually reduced in bore sizes to 27G). The water-insoluble pallet was separated by

centrifugation at 12,000xg for 10 minutes at 4°C. Repeated washings with 1x PBS containing cocktail of protein inhibitors were done until proteins were not detected in wash buffer using BioRad protein assay. The water-insoluble protein was dissolved in 1xPBS containing 6M urea together with 1 mM PMSF and incubated on shaker for 15 minutes at room temperature. The urea-soluble protein was separated using centrifugation at 12,000xg for 30 minutes at room temperature. The protein concentration was measured using BioRad protein assay and aliquoted proteins were kept in -80°C freezer for future use.

Membrane proteins were also extracted from different mouse tissues (kidney, liver, heart and spleen) using the standard protocol described. Concentrations of extracted membrane proteins were determined using BioRad protein assay and aliquoted membrane proteins were kept in -80°C freezer until further experiment.

The described protocol was used to extract brain membrane proteins from different species (rat, pig, duck, chicken, quail, frog and fish). The proteins were kept in –80°C freezer for further experiments after protein concentrations were determined by BioRad protein assay.

2.4.3 Protocol for detection of Brain Reactive Autoantibodies

The protocol for detection of brain reactive antibodies was set up using immunoblotting approach. Briefly brain membrane lysates were separated on denatured polyacrylamide gel electrophoresis, transferred to solid support and hybridized with controls and patients sera.

i. SDS-PAGE electrophoresis and gel staining

The sodium dodecyl sulphate-polyacrylamide gel electrophoreses (SDS-PAGE) were done using analytical comb or preparative comb according to needs of experiments. The membrane proteins (20μg proteins per well for analytical comb or 200 μg proteins per well for preparative comb) were denatured and separated in 12 % or 20% denatured SDS-PAGE according to Laemmli. Upon completion of protein separation, the polyacrylamide gels were either visualized with Coommasie blue or Silver stain or transferred to nitrocellulose membrane (Hybond, Amshersham). Commassie blue stain was done according to established protocol and silver staining was done with SilverPlus (BioRad) which is compatible with MS analysis.

The electrophoretic transfer of separated proteins to nitrocellulose membrane was performed at 100 constant Volts for one-hour using buffer transfer tank (BioRad) in cold room. All polyacrylamide gels after protein transfer were stained with Coomassie blue to confirm complete transfer of protein and for documentation.

ii. Immunoblotting experiment

The nitrocellulose membrane, on which the proteins were transferred, was incubated in blocking solution (1xTBS containing 3% scanned milk powder) on a shaker for one hour at room temperature to block non-specific reactions prior to sample incubation. Depending on the purpose of experiment, nitrocellulose membrane with proteins separated using analytical combs were used for sample incubation straight away, while those with proteins separated using preparative combs were cut into strips and

incubated with samples separately. The samples were diluted at 1:200 in blocking solution and incubated with membrane on shaker for overnight at 4°C. Membranes were washed by 1xTBS-T (1xTBS containing 0.1% Tween 20) thrice with 10 minutes soaking while shaking between washes. The membranes were further incubated with goat anti-human Ig conjugated with Alkaline Phosphatase (Chemicon) diluted in blocking buffer 1:10,000 on shaker for one hour at room temperature. The membranes were, after incubation, washed with 1xTBS-T thrice with 10 minutes shaking between washes. The 5-Bromo-4-Chloro-3'-Indolyphosphate p-Toluidine/Nitro-Blue Tetrazolium Chloride (BCIP/NBT) substrate (Zymed Laboratories Inc) was used to visualise the positive protein bands on the membranes. When the optimal colour is visualized, the substrate was removed, membranes were dried and documented.

2.4.4.Detection of BRAA in controls, non-lupus patients and lupus patients

Standard immunoblotting protocol was used for detection of lupus antibodies in sera of lupus, non-lupus and healthy individuals. Western blot analyses were repeated at least twice for confirmation and both positive and negative lupus autoantibodies reacting with neuronal autoantigens were used in every Western blot analysis. The screening of immunoblotting was started with 12% SDS-PAGE and repeated using 20% SDS-PAGE for higher resolution when necessary.

The sera of healthy individuals (102 samples) were used to detect brain reactive autoantibodies against membrane protein lysate prepared from mouse brain. 12% SDS-PAGE was used in protein separation and sera prepared at 1:200 dilution with TBS-T were used. All tests were repeated twice to validate the results. One sample

was chosen to represent the negative control samples in subsequent immunoblotting experiment.

A total of 100 unselected lupus patients were also tested for presence of Brain reactive antibodies in their sera against membrane protein lysate prepared from mouse brain. 12% SDS-PAGE was used and 1:200 diluted samples were used in immnoblotting experiment. After repeating the experiment twice, one positive sample was selected to represent the positive lupus group in subsequent experiment.

130 non-lupus rheumatic patients (20 Rheumatoid Arthritis patients, 20 Ankylosing Spondylitis patients, 20 Psoriatic Arthritis patients, 20 Osteoarthritis patients, 50 patients with Primary Anti-phospholipid syndrome) were also tested for presence of Brain reactive autoantibodies in their sera using immunoblotting experiments. 20% SDS-PAGE was used to separate the membrane protein lysate prepared from mouse brain and sera were diluted at 1:200 in TBS-T. As standard practice, all tests were repeated to validate the result and a positive sample from the lupus group and a negative sample from control group were used as internal controls.

2.4.5.Detection of BRAA in different tissues and different species

The detected brain reactive autoantibodies against membrane protein lysate prepared from mouse brain were further tested for their tissue specificity and species specificity using different membrane lysates prepared from different tissues, different species and tumour cell lines too. In this experiment, a sample representing positive lupus

group and a sample from negative control group were used to detect autoantibodies against different membrane lysates preparations.

i. Detection of cross reactive BRAA in different tissues

The sera were immunoblotted against membrane protein lystae prepared from different mouse tissues; namely heart, liver, spleen and kidney. 12 % SDS-PAGE was used to separate protein lysate and 1:200 diluted sera were used in immunoblotting experiment. All tests were repeated to validate the results. 20% SDS-PAGE was also used to differentiate the same autoantigens binding to autoantibodies of positive samples.

ii. Detection of cross-reactive brain autoantibodies in different species

These two representative samples were again tested in immunoblotting experiment using brain membrane proteins extracted from different animal species (rat, pig, chicken, duck, quail, frog, and fish) together with human brain membrane proteins (BioChain). 12 % and 20% SDS-PAGE were used to separate the proteins well and samples were diluted at 1:200 with TBS-T.

2.4.6. Two-dimensional gel electrophoresis

The membrane proteins from mouse brain were used in two-dimensional gel electrophoresis. The same protocol described was used, except that the water-insoluble pallet was resuspended in 2-DE sample solubilization buffer containing 6M

urea, 50mM DTT, 4% CHAPS, 0.2% carrier amphyolytes (1:2 Biolyte pH 7-9:Biolyte pH 8-10), 0.0002% Bromophenol Blue. Protein concentration was determined by BioRad RC DC protein assay.

100 µg brain membrane proteins each were applied onto a pair of pH 7-10 7cm ReadyStrip IPTG strip (BioRad) during passive rehydration in Protean IEF cell (BioRad). The first dimension (Isoelectric Focusing) was done for 8000 V-hr at 15°C on the same machine. Upon completion of first dimensional gel electrophoresis, the strips were equilibrated in SDS-buffers prior to second dimensional gel electrophoresis. The second dimensional separation was done in 12%SDS-PAGE. The paper wick soaked with mouse brain membrane protein and another paper wick soaked with pre-stained markers were added next to IPG strips when the IPG strips were loaded onto SDS-PAGE gel. The second dimension was carried out at 45 mAmp constant. Upon completion of second dimensional gel electrophoresis, the gel was visualized with silver staining and a separate gel was used for immunoblotting using brain reactive autoantibodies positive serum. Immunoblotting was performed according to protocol described above.

The gel spots from silver stained polyacrylamide gel matching with positive spots of immunoblotting were punched and sent for peptide mass finger-printing (PMF) using MALDI-TOF-MS (matrix-assisted laser desorption ionization- Time of Flight - Mass Spectrometry) at Agenica Research Pte Ltd, Singapore.

2.4.7. Peptide mass finger-printing

Peptide mass finger-printing was done using the MALDI-TOF-MS analysis. Briefly, the silver-stained gel spots were destained with freshly prepared destaining buffer (5 μl of 30mM potassium ferricyanide and 5μl of 100mM sodium thiosulfate). The gel spots were washed properly and incubated with trypsin (Promega) overnight. The trypsin-cleaved peptides released by sonication were desalted by ZipTip μ-C18 (Millipore). The peptides together with 1 μl of matrix mix containing 5mg/ml Alpha-Cyano-4-Hydroxycinnamic Acid (CHCA) and 5mg/ml 2,5-dihydroxybenzoic acid (DHB) were loaded onto MALDI plate. The MS analysis was done by the curved field reflectron instrument Axima CFR-plus (Kratos, Shimadzu). The MALDI mass spectra were searched in Swissport and NCBI protein databases for matches with known proteins.

2.4.8. Calculation of possible molecular weights of brain membrane proteins reactive with lupus autoantibodies

The known molecular weights of prestained protein ladder (MBI, Fermentus) were plotted against corresponding distance from upper edge of the polyacrylamide gel in semi-log (log-linear) graph. Then the approximate expected molecular weights of brain membrane protein were calculated using linear regression. Similar observations were made in 10 available western-blot results. The mean theoretical molecular weights of two mammalian neuronal autoantigens were calculated.

2.4.9.Prevalence of BRAAs in lupus patients

The prevalence of BRAAs was analyzed in lupus patients compared to control individuals and non-lupus patients, and correlated with Psychoses or Seizures as defined by 1997-ACR criteria or NP lupus syndromes as defined by ACR Ad Hoc Committee on NP lupus nomenclature. Chi-square test was used to determine the significance of these finding and Fisher's exact test was used when necessary.

2.5. Correlation of MBL genetic variants, anti-nephrin antibodies and brain reactive antibodies with clinical data and SLE damage index

The SLE disease severity was analyzed in lupus patients using SLICC/ACR Damage Index. The MBL genetic variants were further analyzed in lupus patients with regards to infections and immunosuppressive drugs used in lupus management. The anti-nephrin antibodies and brain reactive antibodies were correlated with renal involvement and NP involvement in unselected lupus patients. All of these findings were further evaluated against SLE Damage Index.

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2.6. Statistical analysis

The demographic data of controls, lupus and non-lupus patients together with clinical manifestations and SLICC/ACR disease damage index were analyzed using SPSS version 10.1 for Windows.

Genetic frequencies (haplotypes and haplotypes) were calculated from PCR-RFLP results and the significant difference between controls and lupus patients were determined by 2x2 Chi-square test with continuity correction or Fisher's exact test. All controls and lupus patients were tested if they are in Hardy-Weinberg equilibrium with 5% significant level at 1 degree of freedom.

In optimization of anti-nephrin ELISA, mean value of control mouse sera was calculated based on average of three runs and the cut-off OD for normal anti-nephrin antibodies was determined as mean with three standard deviations (mean + 3 SD). The ODs showed above the cut-off were considered as positive anti-nephrin antibodies. Significant presence of anti-nephrin antibodies in mouse sera and lupus sera were determined using 3x2 Chi-square test.

Linear regression analysis was used to determine the approximate expected molecular weights of brain membrane proteins reacting with BRAA. BRAA positivity in controls, lupus and non-lupus patients was tested for determination of statistical significance using 3x2 Chi-square test.

In analysis of clinical manifestation with genetic results or autoantibodies, appropriate Chi-square test was used to determine the statistical significance because these data were unpaired categorized data. Similar approach applies to determine the statistical significance between SLICC/ACR damage and genetic results or autoantibodies too.

All given p value are two-sided. The p values of <0.05 were considered significant and those of <0.001 are considered highly significant.

CHAPTER 3

RESULTS

3.1. Demographic data of controls, lupus patients and non-lupus patients

There were 102 healthy control individuals, 100 lupus patients and 130 non-lupus patients (**Table 3.1 and 3.2**). There were 2 deaths within the cohort of lupus patients during the study period. One of the patients perished from severe lupus disease and multiple infections while the second patient died of hepatosplenic T cell lymphoma. The lupus patients, in this study, presented with abnormal titers of antinuclear antibodies (97%), immunological disorder (95%), hematological disorder (81%), arthritis (77%) and renal disorder (53%) followed by the remaining ACR criteria (8-41%) (**Table 3.3**).

NP involvement in SLE patients were found in 10 lupus patients as defined by 1997-updated ACR criteria for classification of SLE and 27 lupus patients according to 1999-proposed NP lupus syndromes by ACR Ad Hoc Committee on NP Lupus Nomenclature (**Table 3.4**). However the number of lupus patients with renal involvement did not change under both inclusion criteria; according to a.) 1997-updated ACR criteria for classification of SLE, and b.) modified inclusion criteria made up of proteinuria, cellular casts, nephrotic syndrome, nephritic syndrome and renal histology. 53 lupus patients were found to have proteinuria or cellular cast as renal involvement according 1997-updated ACR criteria. The number of patients (where all were same patients) still maintained as 53 with modified inclusion criteria as well **(Table 3.5**).

Fifty-three lupus patients (53%) did not have any damage recorded, as indicated by a total SLICC/ACR score of 0. The NP and renal damages were the commonest organ

system damage detected with SLICC/ACR score of 1 or more. The 26 lupus patients having NP and/or renal damage (patients with both NP and renal damages = 2; patients with NP damage = 4 and patients with renal damage = 20) as defined by SLICC/ACR Damage Index have a score of 1 or more (**Table 3.6**).

There were 51 lupus patients treated with corticosteroids and cytotoxic drugs and 45 patients with only corticosteroids. 2 patients were treated only with cytotoxic drugs alone and 2 did not require corticosteroid or cytotoxic drugs. Infections were grouped into bacterial, viral and fungal causes in this study. One case of systemic fungal infection was noted. Bacterial infections were more common than viral infections. A total of 66 lupus patients had infections. 22 patients had bacterial and viral infections, 32 had bacterial infections only and 12 had viral infections only.

3.2. Mannose Binding Lectin

3.2.1. A novel PCR-RFLP protocol for genotyping and haplotyping of MBL

The PCR-RFLP approach was limited in the genotyping of exon 1 and haplotyping of promoter region of MBL gene because restriction endonucleases are not available for codon 52 mutation site at exon 1 and –550 bp site of promoter region. To overcome this limitation, we designed mismatched primers where restriction enzyme recognition sites were carefully inserted. During the process of optimization, DNA samples with rare genotypes at codons 52 and 57 at exon 1 were provided as gifts from overseas researchers. We optimized our novel PCR-RFLP protocol for all possible genotypes

Table 3.1 Demographic data of controls, lupus and non-lupus patient cohort.

Group	Parameter	Value	
Lupus (n=100)			
	Female: Male ratio	9:1	
	Mean Age (range)	39 (19-71 yrs)	
	Race	Chinese	81
		Malay	14
		Indian	5
	Disease duration (range)	11 (1-20 yrs)	
Non-Lupus (n=130)			
	Female: Male ratio	1.4:1	
	Mean Age (range)	52 (21-81 yrs)	
	Race	Chinese	91
		Malay	10
		Indian	29
Control (n=102)			
	Female: Male ratio	2.7:1	
	Mean Age (range)	35 (21-41 yrs)	
	Race	Chinese	82
		Malay	14
		Indian	6

Table 3.2. Demographic data of non-lupus patient cohort.

Group	n	Parameter	Value	
RA	(n=20)	Female: Male ratio	4:1	
		Mean Age (range)	54 (30-75 yrs)	
		Race	Chinese	16
			Malay	1
			Indian	3
PA	(n=20)	Female: Male ratio	1:1.5	
		Mean Age (range)	51 (21-70 yrs)	
		Race	Chinese	15
			Malay	0
			Indian	5
OA	(n=20)	Female: Male ratio	9:1	
		Mean Age (range)	62 (40-81 yrs)	
		Race	Chinese	14
			Malay	1
			Indian	5
AS	(n=20)	Female: Male ratio	1:9	
		Mean Age (range)	44 (27-60 yrs)	
		Race	Chinese	18
			Malay	0
			Indian	2
APS	(n=50)	Female: Male ratio	4:1	
(Primary)		Mean Age (range)	50 (26-67 yrs)	
		Race	Chinese	28
			Malay	8
			Indian	14

Table 3.3. ACR criteria presentation of lupus patients at diagnosis (n=100)

No	ACR Criteria	No of Pts	(%)
1	Antinuclear antibody	97	(97)
2	Immunologic Disorder	95	(95)
3	Hematologic Disorder	81	(81)
4	Arthritis	77	(77)
5	Renal Disorder	53	(53)
6	Malar Rash	41	(41)
7	Photosensitivity	31	(31)
8	Oral Ulcers	29	(29)
9	Serositis	16	(16)
10	Neurologic Disorder	10	(10)
11	Discoid Rash	8	(8)

Table 3.4. NP syndromes recorded in SLE cohort (n=100)

NP syndromes	no of patients (%)
Total	27 (27)
Acute Confusional State	0 -
Acute Inflammatory Demyelinating Polyradiculoneuropathy	0 -
Anxiety Disorder	0 -
Aseptic Meningitis	2 (2)
Autonomic Disorder	0 -
Cerebrovascular Disease	2 (2)
Cognitive Dysfunction	6 (6)
Demyelinating Syndrome	0 -
Headache	12 (12)
Mononeuropathy (single/mutiplex)	0 -
Mood Disorders	0 -
Movement Disorder (Chorea)	0 -
Myasthenia Gravis	0 -
Myelopathy	0 -
Neuropathy, Cranial	0 -
Plexopathy	0 -
Polyneuropathy	0 -
Psychosis*	4 (4)
Seizures and Seizure Disorders*	6 (6)
Psychosis* and/or Seizures*	10 (10)

(* Psychosis and Seizure were neurological disorder defined by 1997-updated ACR criteria for classification of SLE)

NB. Some patients presented more than one NP syndrome.

Table 3.5. Renal involvement in SLE cohort (n=100)

Renal involvement	no of patients (%)
Total	53 (53)
Persistent proteinuria*	46 (46)
Cellular casts*	25 (25)
Nephrotic syndrome	12 (12)
Nephritic syndrome	6 (6)
Rapidly Progressive GN	2 (2)
Persistent proteinuria* and/or Cellular casts*	53 (53)

(* Proteinuria and cellular casts were renal disorders defined by 1997-updated ACR criteria for classification of SLE)

NB. Some patients presented with more than one renal involvement.

Table 3.6. Distribution of damage according to the organ system (n=100)

Organ system or disease	no of patients with score ≥1	(%)
Total	47	(47)
NP and/or Renal	26	(26)
Ocular	12	(12)
NP	6	(6)
Renal	22	(22)
Pulmonary	0	-
Cardiac	4	(4)
Peripheral vascular	2	(2)
Gastrointestinal	4	(4)
Musculoskeletal	6	(6)
Skin	2	(4)
Premature gonadal failure	0	-
Diabetes Mellitus	6	(6)
Malignancy	0	-

of MBL exon 1 using DNA samples from our database and gifts of rare genotypes.

Using Web Cutter (*http://rna.lundberg.gu.se/cutter2/index.html*), the expected different fragments of MBL Exon 1 PCR product (134-bp) were calculated for different restriction enzymes *Mwo* I, *Ban* I and *Mbo* II. The cleaved products were tabulated together with uncleaved PCR products, according to possible homozygous and heterozygous alleles at respective codons (**Table 3.7.A**). PCR product of MBL promoter (410-bp product) was also analysed using Web Cutter for double-digest with *Btg* I and *Drd* I enzymes. The expected and logical different haplotypes were presented (**Table 3.7.B**).

All three polymorphic sites at exon 1 of MBL were tested successfully. Since we introduced an enzyme recognition site (*Mwo* I) at Codon 52 in the mismatched primer (MBL-E1-FOR), all Codon 52 wild type alleles were cleaved by restriction endonuclease *Mwo* I and seen clearly on gel electrophoresis. Codon 54 polymorphic alleles were also cleaved by restriction endonuclease *Ban* I, which has been described elsewhere. Although we did not expect to observe any mutant alleles of codon 57 from our local control group, we used gift-samples with all different alleles in order to optimize the PCR-RFLP protocol **(Figure 3.1.A)**. PCR product containing L allele (-550 bp site) will be cleaved by restriction endonuclease *Drd* I and PCR product containing Y allele (-221 bp site) will be cleaved by *Btg* I restriction endonuclease. Our results showed the presence of all possible haplotypes except HX/HX, HX/HY, HX/LY and HX/LX haplotypes (**Figure 3.1.B**). These four haplotypes were not present in the cohort of individuals studied and these are also not reported elsewhere.

A

Polymorphic sites	Homozygous (Wildtype)	Homozygous (Mutant)	Heterozygous (Wildtype+Mutant)
Codon 52	109 bp	134 bp	109+134 bp
Codon 54	95 bp	134 bp	95+134 bp
Codon 57	134 bp	78 bp	78+134 bp

B

Haplotypes	410 bp	389 bp	354 bp	333 bp
HX/HX	+			
LX/LX		+		
HY/HY			+	
LY/LY				+
HX/LX	+	+		
HX/HY	+		+	
HX/LY	+			+
HY/LX		+	+	
LX/LY		+		+
HY/LY			+	+

Table 3.7. Expected fragments of PCR-RFLP genotyping for MBL gene
A: Exon 1 polymorphism for respective codons
B: Promoter polymorphism for respective haplotypes

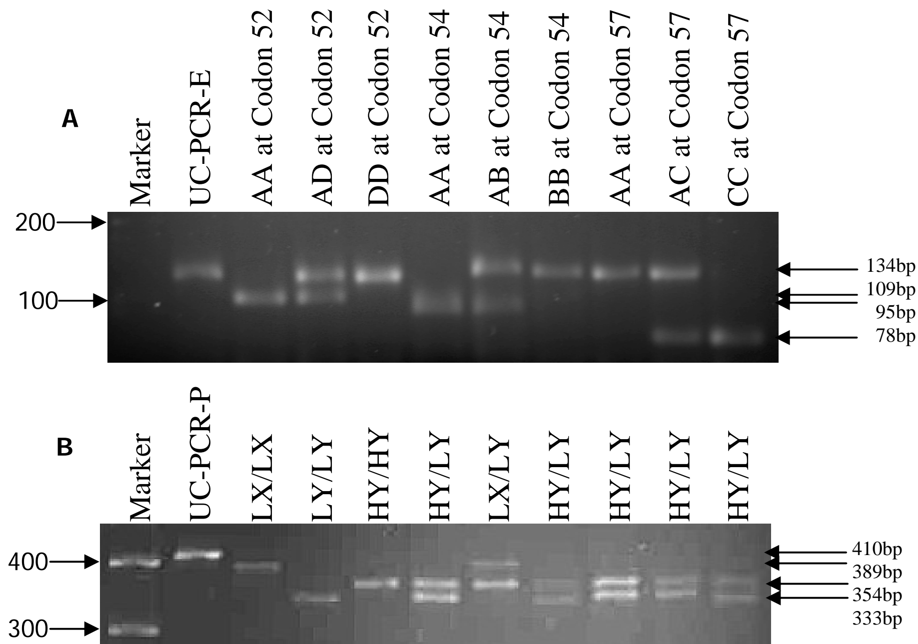

Fig 3.1: Observed fragments of RFLP in MBL genotyping; UC-PCR-E= uncut Exon 1 PCR product; UC-PCR-P= uncut promoter PCR product control.
A: Exon1 polymorphism; B: Promoter haplotypes

We have validated the novel PCR-RFLP method using various approaches and our PCR-RFLP method is found to be able to genotype codons 52, 54 and 57 of exon 1 and haplotype the promoter region of MBL gene.

DNA sequencing

The sequencing results of the different genotypes and haplotypes of MBL gene showed concordance with that of PCR-RFLP (data not shown).

Comparison with PCR-SSP

The PCR-SSP was done according to conditions described (Steffensen et al., 2000). We found (in agreement with previous reports) that optimization of the PCR-SSP is not easy to perform. However it was observed that optimization of PCR-SSP for the promoter gene region was easier than that for PCR-SSP of exon 1. We have tested all possible genotypes and haplotypes using PCR-SSP methods. All results are in concordance with our PCR-RFLP results (Data not shown).

Independent performance by volunteers

The various genotyped and haplotyped DNAs were distributed among five volunteer laboratory personnels to analyze according to our PCR-RFLP protocol. The results were tabulated and found to be in concordance with our PCR-RFLP typing results, DNA sequencing results and PCR-SSP results (Data not shown). There was no discordant result among the 5 volunteers.

3.2.2.Genetic frequencies of control individuals

We have genotyped the exon 1 and haplotyped the promoter gene of MBL in our local control group and compared our results with Chinese (Mainland) and Japanese gene polymorphisms obtained by PCR-based methods (Huang et al., 2003, Wang et al 2001) (**Table 3.8 and Table 3.9**). The genetic variations at codon 54 are observed among three control groups. However haplotype frequencies of promoter regions for local controls (PCR-RFLP), Japanese (PCR-SSP) and China Chinese (PCR-UHG) are almost similar with minor variations in LY/LY, LX/LX, HY/HY, LX/LY. This has to be confirmed by more genotyping on different ethnic groups.

3.2.3.Genetic frequencies of lupus patients

We have carried out genotyping of structural polymorphism of exon 1 and haplotyping of promoter region of MBL gene in 100 lupus patients using our novel PCR-RFLP approach. The gene frequencies of these polymorphisms were presented in **Table 3.10** and **Table 3.11**.

3.2.4. Prevalence of MBL genetic variants in lupus patients

The genetic frequencies of MBL gene (both exon 1 and promoter region) were compared in both control group and lupus patients. It was noted that mutant alleles at codon 52 were significantly increased in lupus patient ($p < 0.0001$) while other polymorphic sites at codons 54 and 57 did not show significant difference (**Table 3.10**). It was also interesting to note the genetic frequencies of MBL high- and

Table 3.8. Genotype frequencies of MBL exon 1 (Singaporean Controls, Mainland Chinese Controls, Japanese controls) * Hung et al. 2003 ; + Wang et al. 2001

Exon 1 MBL Genotypes	Singaporean (n=102)	Chinese (China)* (n=111)	Japanese + (n=143)
Codon 52			
WT/WT	102 (100)	ND	ND
WT/MU	0 (0)	ND	ND
MU/MU	0 (0)	ND	ND
Codon 54			
WT/WT	76 (74.5)	92 (82.9)	77 (53.8)
WT/MU	25 (24.5)	18 (16.2)	58 (40.6)
MU/MU	1 (1.0)	1 (0.9)	8 (5.6)
Codon 57			
WT/WT	102 (100)	ND	ND
WT/MU	0 (0)	ND	ND
MU/MU	0 (0)	ND	ND

Haplotypes frequencies were presented as absolute numbers with percentage in parenthesis; ND = Not done; PCR-RFLP = Singaporean controls, Japanese controls; PCR-UHG = Chinese (China) controls.

Table 3.9. Haplotype frequencies of MBL promoter gene region (Singaporean Controls, Mainland Chinese controls, Japanese controls) * Hung et al. 2003 ; + Wang et al. 2001

MBL Promoter region Haplotypes	Singaporean (n=102)	Chinese (China)* (n=111)	Japanese + (n=143)
HX/HX	0	0	0
LX/LX	8 (7.8)	1 (0.9)	2 (1.4)
HY/HY	28 (27.5)	15 (13.5)	28 (19.6)
LY/LY	15 (14.7)	30 (27.0)	39 (27.3)
HX/LX	0	0	0
HX/HY	0	0	0
HX/LY	0	0	0
HY/LX	1 (1.0)	9 (8.1)	10 (7.0)
LX/LY	14 (13.7)	8 (7.2)	10 (7.0)
HY/LY	36 (35.3)	48 (43.2)	54 (37.8)

Haplotypes frequencies were presented as absolute numbers with percentage in parenthesis; PCR-RFLP = Chinese (Singaporean); PCR-SSP = Japanese; PCR-UHG = Chinese (China) controls

Table 3.10. Genotypic frequencies of exon 1 of MBL in controls and lupus patients.

Exon 1 MBL Genotypes	Controls (n=102)	Lupus patients (n=100)	Chi-square *p*
Codon 52			
WT/WT	102 (100)	74 (74)	
WT/MU	0 (0)	26 (26)	<0.0001
MU/MU	0 (0)	0 (0)	
Codon 54			
WT/WT	76 (74.5)	63 (63)	
WT/MU	25 (24.5)	37 (37)	0.0764
MU/MU	1 (1.0)	0 (0)	
Codon 57			
WT/WT	102 (100)	100 (100)	
WT/MU	0 (0)	0 (0)	
MU/MU	0 (0)	0 (0)	

Gene frequencies were presented as absolute numbers with percentage in parenthesis
WT, Wild-type; MU, mutant

Table 3.11. Haplotype frequencies of MBL promoter gene region in controls and lupus patients

MBL Promoter region Haplotypes	Controls (n=102)	Lupus patients (n=100)	Chi-square *p*
HX/HX	0 (0)	0 (0)	
LX/LX	8 (7.8)	14 (14)	0.2386
HY/HY	28 (27.5)	14 (14)	0.0291
LY/LY	15 (14.7)	6 (6)	0.0724
HX/LX	0 (0)	0 (0)	
HX/HY	0 (0)	0 (0)	
HX/LY	0 (0)	0 (0)	
HY/LX	1 (1.0)	0 (0)	nd
LX/LY	14 (13.7)	9 (9)	0.4034
HY/LY	36 (35.3)	57 (57)	0.0031

Haplotypes frequencies were presented as absolute numbers with percentage in parenthesis; nd = not done

intermediate-producing promoter haplotypes (HY/HY and LY/LY) were lower. The LX/LX haplotype associated with low-production of MBL showed a higher frequency in lupus patients, though it did not reach statistical significance (**Table 3.11**).

3.3.Nephrin

3.3.1.Expression and purification of nephrin protein fragments.

The theoretical properties of expressed nephrin protein fragment were calculated from Proteomics and sequence analysis tools of ExPASy Proteomics Server (*http://au.expasy.org/tools/pi_tool.html*) (**Figure. 3.2**).

Expression of protein was done using expression strain BL21 competent cells. The expected protein of 28.5 kD was seen in SDS-PAGE after inducing with 1mM IPTG for one hour (**Figure. 3.3**). The expressed fragment of mouse nephrin protein, separated on SDS-PAGE, was visualised using Coommaie blue stain and it was punched out from polyacrylamide gel. The punched gel was sent to Agenica Protein Services, Singapore, for peptide mass fingerprinting (PMF) using matrix-assisted laser desorption ionization- Time of Flight - Mass Spectrometry (MALDI-TOF-MS).

The trypsin-cleaved peptide masses were analyzed in available protein databases (NCBI and Swissport) and it was reported as murine nephrin protein. The expressed protein is thus confirmed as nephrin protein of mouse origin, and this protein was used to raise polyclonal antibodies and monoclonal antibodies. It was also used as antigen in anti-nephrin antibodies ELISA experiments.

“MGHHHHHHHHHHSSGHIEGRHMDNGVAPAARGLVRLVVRFAPQVDHPTPLTKVAAA
GDSTSSATLHCRARGVPNIDFTWTKNGVPLDLQDPRYTEHKYHQGVVHSSLLTIANV
SAAQDYALFKCTATNALGSDHTNIQLVSISRPDPPLGLKVVSVSPHSVGLEWKPGFD
GGLPQRFQIRYEALETPGFLYMDVLPAQATTFTLTGLKPSTRYRIWLLASNALGDSG
LTDKGIQVSITTPGLDQAPEDTDQPLPTEQPPGPPR”

Theoretical pI = 6.83

Molecular weight = 28544 Daltons

Fig 3.2: Physical properties of eexpressed protein with His-tag using web base software (*http://au.expasy.org/tools/pi_tool.html*)

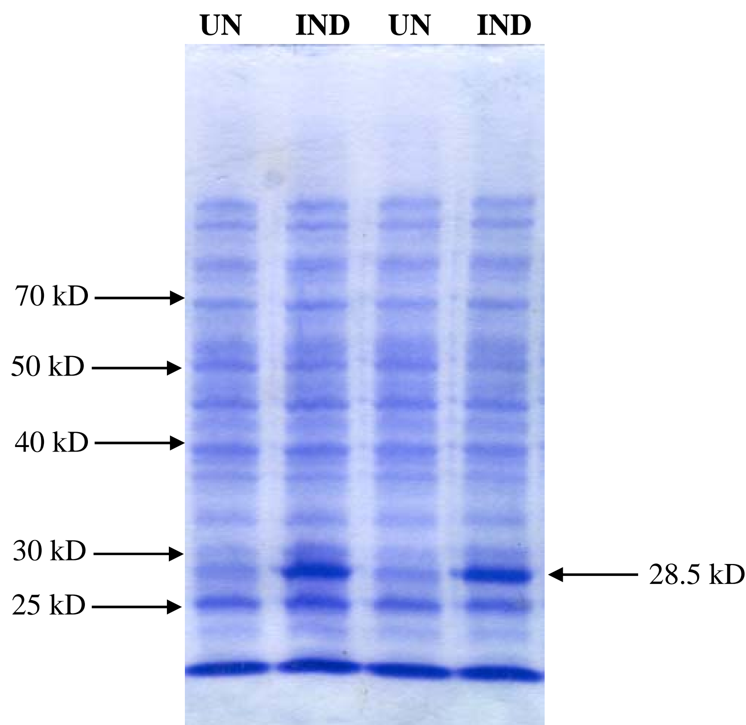

Fig 3.3: Induced and Uninduced *E. Coli* proteins electrophoresed on SDS-PAGE and stained with Coomassie Blue stain; UN= uninduced; IND=induced
The nephrin protein fragment (28.5 kD) was seen on IPTG-induced expression.

3.3.2.Polyclonal antibodies against nephrin protein fragment

The polyclonal antibodies were raised in Rabbits using purified nephrin protein fragment of mouse origin. During the production of polyclonal antibodies, we have noted that one of the rabbits died after one week of immunization, however no infection or abscess was seen at the site of immunization. The definitive cause of death was not known. The production of polyclonal antibodies was continued in remaining rabbit. The polyclonal antibodies were crudely purified using ammonium sulfate precipitation.

3.3.3.Monoclonal antibodies against nephrin protein fragment

The monoclonal antibodies were raised using myeloma cell line of murine BALB/c origin (P3X63Ag8.653) and splenic cells of BALB/c mice immunized with the aid of ClonaCell^{TM} complete kit. The splenic cells were harvested upon observation of the increased titer of anti-nephrin antibodies in the immunized mice. The fusion steps were satisfactory and we have noted that supernatant of three wells (fused cells) were showing significantly increased anti-nephrin antibodies. These cells were further expanded, but unfortunately these cells did not survive in the expansion media. Therefore, we failed to obtain monoclonal antibodies against nephrin protein fragment in this study.

3.3.4.Characterization of nephrin protein fragment and its polyclonal antibodies

The nephrin protein fragment of mouse origin expressed in *E. Coli* system was separated on SDS-PAGE and sent for peptide mass fingerprinting. The results had shown the protein expressed was nephrin of mouse origin (**Figure. 3.4**).

Furthermore, the polyclonal antibodies that raised in rabbits were tested in frozen sections of mouse kidney using immunohistochemical approach (**Figure. 3.5**). It was noted that these antibodies bound to glomerulus of mouse kidney. We conclude that the proteins expressed were nephrin proteins and polyclonal antibodies raised were targeting against the nephrin protein.

The nephrin protein was used in ELISA and Immunoblotting experiment to detect anti-nephrin antibodies in controls, lupus and non-lupus patients in our study.

3.3.5.Detection of anti-nephrin antibodies

The anti-nephrin antibodies in lupus mouse model (PL/JxNZW) F1xNZB) were detected by ELISA and 19 (95%) out of 20 lupus mouse models showed positive anti-nephrin antibodies in their serum (**Table 3.12**).

The 102 control individuals, 100 unselected lupus patients and 130 non-lupus patients were screened for presence of anti-nephrin antibodies (**Table 3.13**). The ELISA approach could not be optimized for human samples, hence an immunoblotting approach was used for human samples. We observed that the antibodies against

expressed fragments of nephrin protein were negative in all control samples (n=102) and non-lupus samples (n=130) while there were 14 lupus patients (14%) showed positive anti-nephrin antibodies (n=100) (**Figure. 3.6**).

3.3.6. Prevalence of anti-nephrin antibodies in lupus nephritis

The anti-nephrin antibodies were found in sera of lupus mouse models and human lupus patients. None of the BALB/c mice, controls and non-lupus patients showed positive anti-nephrin antibodies. This finding was significant for mice samples ($p<0.0037$) and human samples ($p<0.0001$) (**Table 3.12** and **Table 3.13**).

3.4.Brain reactive autoantibodies

All membrane protein preparations were carried out according to protein solubility.

3.4.1.Observation of BRAA in lupus patients by standard protocol

W started immunoblotting assay using mouse neuronal membrane protein lysates in unselected lupus patients. Antibodies reactive with two brain antigens (between 24 kD and 35 kD) were detected and used for our study. These autoantibodies were collectively named as Brain Reactive AutoAntibodies (BRAA). The calculated molecular weights of these proteins were 27.5 kD and 29.5 kD.

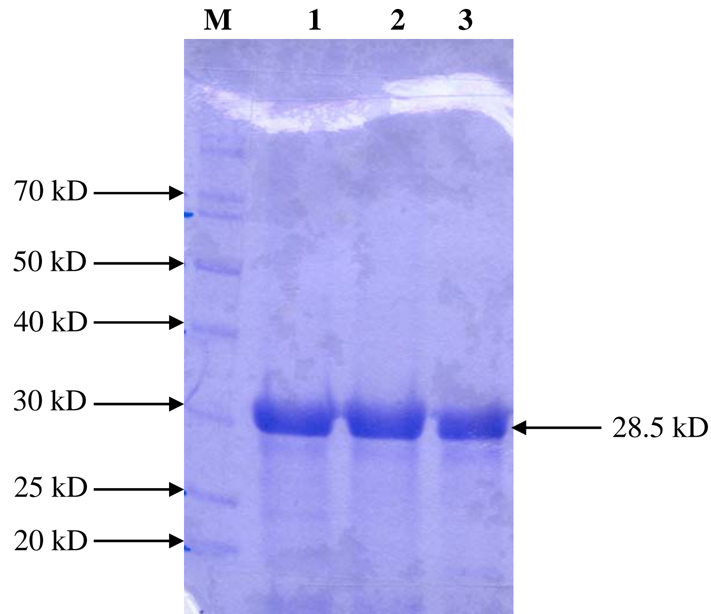

Fig 3.4: purified nephrin proteins (M.W 28.5 kD) electrophoresed on SDS-PAGE and stained with Coomassie Blue stain; the protein was punched and sent for protein finger-printing by MALDI-TOF-MS method.

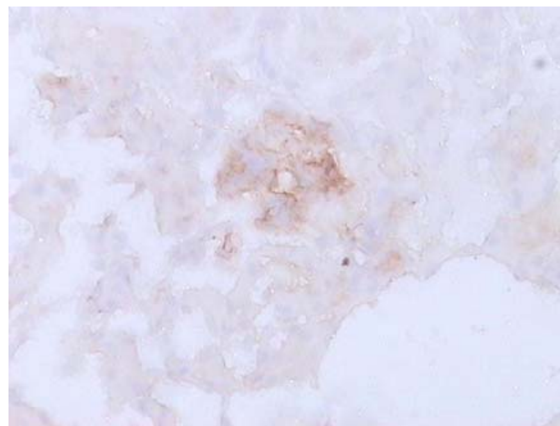

Fig 3.5: Immunohistochemically stained glomerulus of mouse kidney frozen section; using polyclonal antibodies against expressed nephrin protein and DAB stains.

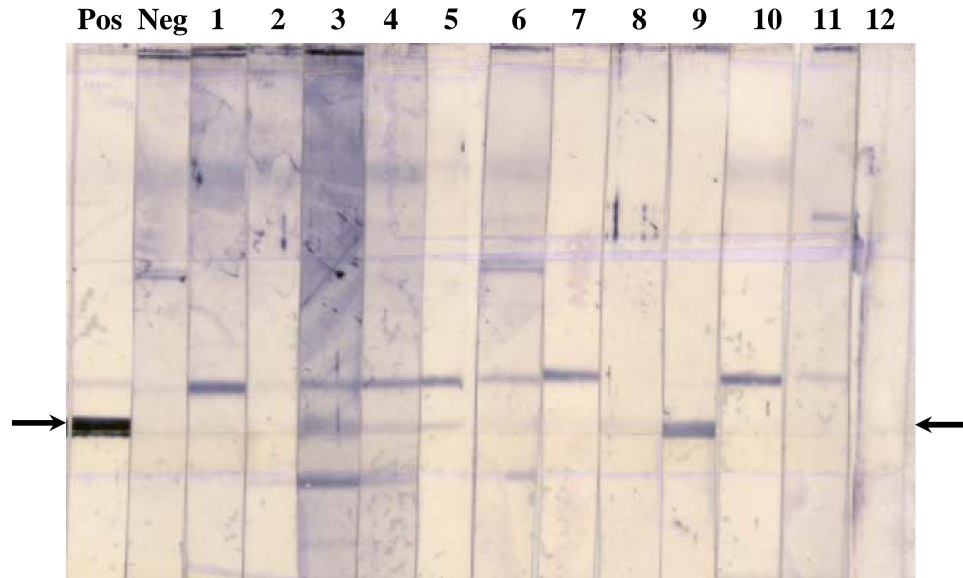

Fig 3.6: Immunoblotting result of anti-nephrin antibodies in control, non-lupus and lupus samples;
Pos: Anti-nephrin positive serum; Neg: Anti-nephrin negative serum;
1-4: representative control samples; 5-8: representative non-lupus sample;
9-12: representative lupus samples

NB: Sample 9 showed anti-nephrin positive results

Table 3.12. Anti-nephrin antibodies in control and lupus mouse model (ELISA)

		Positive	Negative
BALB/c mice	n = 10	-	10
MRL/lpr mice	n = 5	4	1
PL/J F1xNZB	n = 20	19*	1

*p=0.0037; The cut-off point for both anti-nephrin was mean OD value of BALB/c mice + 3 SD

Table 3.13. Anti-nephrin antibodies in controls, lupus and non-lupus groups

		Positive	Negative
Control group	n = 102	-	102
Lupus patients	n = 100	14	86
Non-lupus patients	n = 130	-	130

*p = <0.0001

3.4.2.Detection of BRAAs in controls, non-lupus and lupus patients

We found BRAAs present in 10 (10%) out of 100 unselected lupus patients and none (0%) of the 100 healthy individuals. One BRAA-positive serum (from unselected lupus patients) and one BRAA-negative serum (from healthy controls) were used as positive and negative samples in subsequent experiments (**Figure. 3.7**).

To detect these antibodies in other rheumatic diseases, we further tested 130 non-lupus rheumatic patients. Positive and negative controls were added in all immunoblotting experiments. The 130 non-lupus patients comprised of 20 RA, 20 OA, 20 AS, 20 PsA and 50APS. They did not show presence of BRAAs. Thus BRAAs reacting with mice neuronal membrane proteins were found only in lupus patients, not in the healthy individuals and non-lupus rheumatic patients ($p < 0.0007$) (**Table 3.14**).

3.4.3.Tissue and Species specificity of BRAAs

The immunoblotting experiment was carried out using membrane protein lysates from different mouse tissues (heart, liver, spleen and kidney). It was noted that BRAAs react with only membrane protein lysates from mouse brain tissue (**Figure 3.8**).

The same experiment was carried out using neuronal membrane protein lysates of different animals. It is interesting to note that BRAAs target neuronal membrane protein lysates of mammalian origin and did not react with those of other species such as avian, fish and amphibian. (**Figure 3.9**).

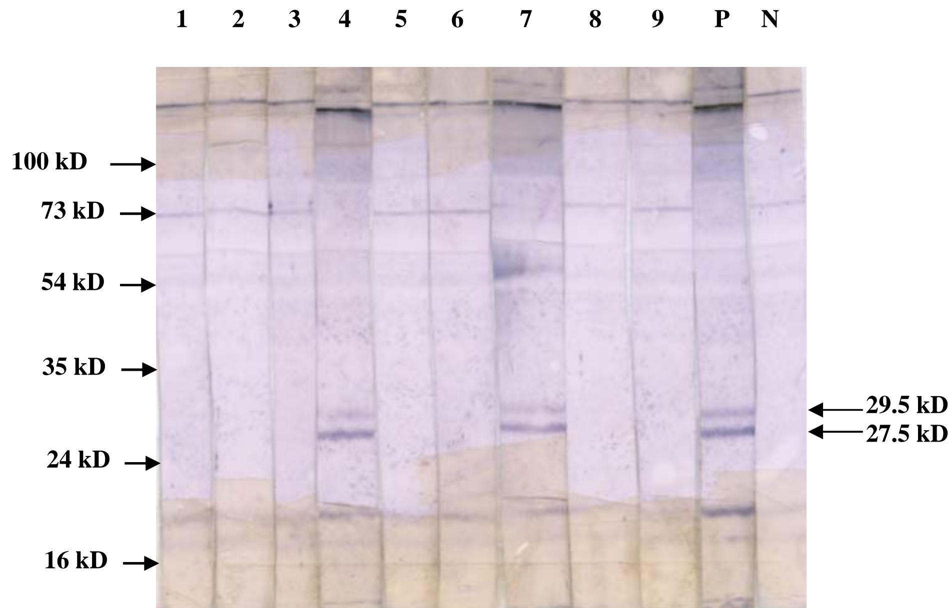

Fig. 3.7. Immunoblotting results of BRAAs in sera of lupus, non-lupus and healthy controls; 1-3 ➔ non-lupus patients; 4-7➔ lupus patients; 8-9➔ controls lupus patients; P ➔ positive serum; N ➔ negative serum; this is the representative immunoblotting results of our study. The positive lupus sample (P) and negative controls sample (N) were selected to represent the positive and negative groups and used in subsequent immunoblotting experiments. All serum samples were tested at least twice in immunoblotting experiments,

Table 3.14. BRAA in controls, lupus and non-lupus groups

		Positive	Negative
Control group	n = 102	-	102
Lupus patients	n = 100	10	90
Non-lupus patients	n = 130	-	130

$p < 0.0007$

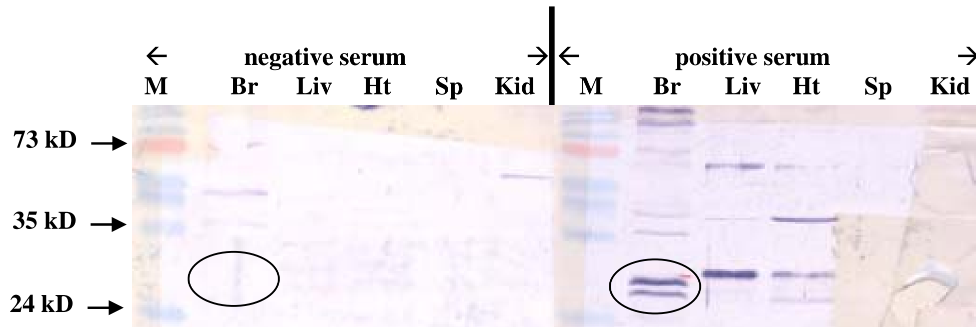

Fig.3.8. Immunoblotting results of BRAA against membrane proteins extracted from different mouse tissues; M ➔ protein marker; Br ➔ membrane proteins of mouse brain; Liv ➔ membrane proteins of mouse liver; Ht ➔ membrane proteins of mouse heart; Sp ➔ membrane proteins of mouse spleen; Kid ➔ membrane proteins of mouse kidney; Positive serum➔ serum representing positive samples; Negative serum ➔ serum representing negative samples.

N.B: The immunoblotting experiment done using 12% SDS-PAGE was not able to differentiate proteins reacting with BRAA. Therefore, we repeated immunoblotting experiments using 20% SDS-PAGE and it was clearly observed the brain proteins reacted with the BRAA were not expressed in other tissues.

NB: 20% SDS-PAGE was used in this experiment.

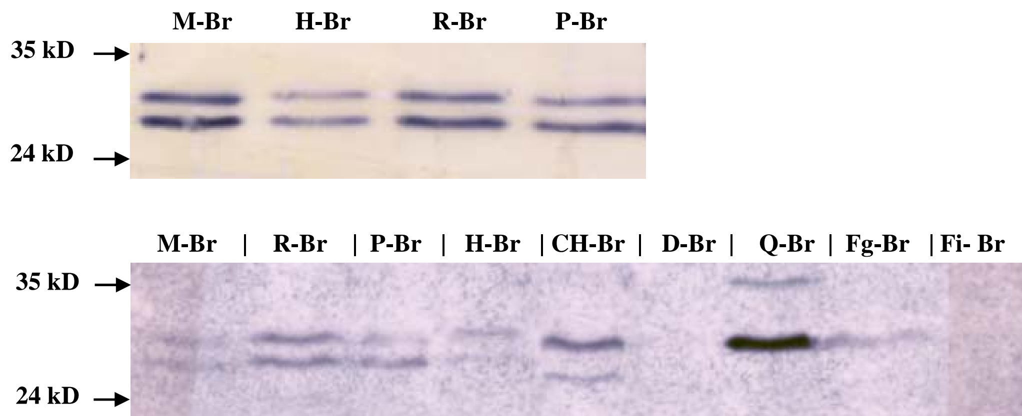

Fig.3.9. Immunoblotting results of BRAA against brain membrane proteins extracted from different animal species;
M-Br ➔ mouse brain membrane proteins; R-Br ➔ rat brain membrane proteins; P-Br ➔ pig brain membrane proteins; H-Br ➔ human brain membrane proteins; CH-Br ➔ chicken brain membrane proteins; D-Br ➔ duck brain membrane proteins; Q-Br ➔ quail brain membrane proteins; Fg-Br ➔ Frog brain membrane proteins; Fi-Br ➔ Fish brain membrane proteins.

3.4.4. Peptide Mass Fingerprinting of brain antigens reacting with BRAA

We used 2-D gel electrophoresis to separate the BRAAs. The protein spots of silver stained SDS-PAGE, matching with BRAAs result of immunoblotting results were sent for peptide mass fingerprinting using MALDI-TOF-MS analysis (**Figure 3.10**). The trypsin-cleaved peptides mass spectra were matched against public protein databases (NCBI and Swissport). It was noted that these membrane proteins did not match to any published protein sequences.

3.4.5. Prevalence of BRAAs in lupus patients

The prevalence of BRAAs was calculated in control, lupus and non-lupus patients. BRAAs were detected in 10 out of 100 lupus patients (10%) while none of healthy individuals (n=100) and non-lupus (n=130) present their presence in their sera (p=0.0035) (**Table 3.14**)

3.5. Correlation of MBL genetic variants, anti-nephrin antibodies positivity and BRAAs positivity with clinical data and SLE Damage Index

Based on the results we have, we focused on the mutant alleles of codon 52 and MBL low-producing haplotypes (LX/LX) of promoter region, we postulated that this combination will be associated with infections in lupus patients, hence leading to more severe disease in SLE. **Table 3.15** shows the correlation between infections and MBL polymorphisms. Though the codon 52 variants and MBL (LX/LX) haplotypes did not reach statically significance, there was a trend showing predisposition to

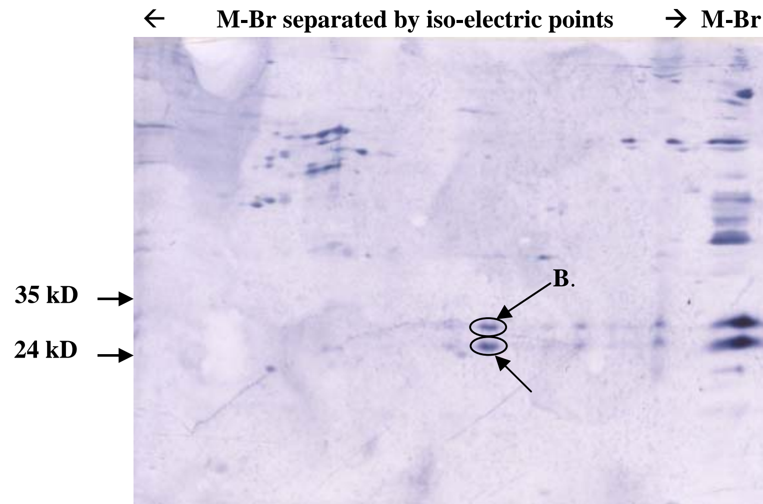

Fig.3.10. Immunoblotting results of BRAA against mouse brain membrane proteins separated by 2-D gel electrophoresis.
This experiment was done for a pair of 2-D gel strips with a guide lane using mouse brain membrane proteins (M-Br) was used. One gel was processed to immunoblotting using BRAA positive serum. Another gel was visualised using silver plus stain. The protein spots of silver-stained similar gel matching to A and B were punched, processed for trypsin-cleaved peptide mass finger printing using MALDI-TOF-MS.

Table 3.15. Correlation of infection with MBL polymorphism in lupus patients

Correlation of MBL polymorphism with infections

Lupus patients with infections (Bacterial and/or viral)		
n =66		
with mutant codon 52	20	(30%)
with wildtype codon 52	46	
Lupus patients without infections		
n = 34		
with mutant codon 52	6	(18%)
with wildtype codon 52	28	

p = ns

Correlation of MBL promoters polymorphism with infections

Lupus patients with infections (Bacterial and/or viral)		
n =66		
with LX/LX	10	(15%)
without LX/LX	56	
Lupus patients without infections		
n = 34		
with LX/LX	4	(12%)
without LX/LX	30	

p = ns

infections – codon 52 mutant variant (30% vs 18%) and LX/LX haplotype (15% vs 12%) .

To evaluate the relationship of MBL variants and infection under the influence of immunosuppressive drugs, the lupus patients were divided into four groups according to immunosuppressive drugs used and analyzed the infections together with MBL genetic variants (**Table 3.16**). The patients treated with corticosteroids and cytotoxic drugs did not differ between mutant codon 52 variant frequencies, but patients with LX/LX haplotypes were more prone to infections. For lupus patients on corticosteroids alone, mutant MBL variant may predispose to increased frequency of infections compared to those with normal MBL alleles. The small numbers in the other group do not allow us to detect any trends.

The lupus mouse models with positive anti-nephrin antibodies were found to be significantly associated with proteinuria ($p<0.0001$), however we did not detect an association in human lupus patients (**Table 3.17** and **Table 3.18**).

The BRAAs were found to be highly associated with psychoses and seizures. 6 (60%) out of 10 BRAA-positive lupus patients had psychoses and/or seizures while only 4 (2%) of 90 BRAA-negative lupus patients had psychoses and/or seizures ($p<0.0001$) (**Table 3.19**). This strong association was still present when BRAA-positive patients were analyzed with NP lupus syndromes as described by ACR Ad Hoc Committee for NP lupus nomenclature ($p<0.0186$) (**Table 3.20**).

Table 3.16. MBL influence on infection in lupus patients under immunosuppressive drugs

Correlation of MBL polymorphism with infection

	No	Infection
Lupus patients with steroid and cytotoxic drugs		
n =50		
with mutant codon 52	14	10 (71%)
with wildtype codon 52	36	26 (72%)
Lupus patients with steroid		
n = 46		
with mutant codon 52	12	10 (83%)
with wildtype codon 52	34	19 (56%)
Lupus patients with cytotoxic drugs only		
n = 2		
with mutant codon 52	0	
with wildtype codon 52	2	0 (0%)
Lupus patients without steroid and cytotoxic drugs		
n = 2		
with mutant codon 52	0	
with wildtype codon 52	2	1 (50%)

Correlation of MBL promoters polymorphism with infections

	No	Infection	
Lupus patients with steroid and cytotoxic drugs			
n =50			
with LX/LX	8	8	(100%)
without LX/LX	42	28	(66%)
Lupus patients with steroid			
n = 46			
with LX/LX	2	1	(50%)
without LX/LX	44	27	(61%)
Lupus patients with cytotoxic drugs			
n = 2			
with LX/LX	2	1	(50%)
without LX/LX	0	-	
Lupus patients without steroid cytotoxic drugs			
n = 2			
with LX/LX	2	1	(50%)
without LX/LX	0	-	

Table 3.17. Correlation of Anti-nephrin antibodies with renal involvement (proteinuria) in control and lupus mice model (ELISA)

	Positive	Negative	Proteinuria	anti-dsDNA
BALB/c mice n = 10	-	10	ND	ND
MRL/lpr mice n = 5	4	1	ND	ND
PL/J F1xNZB n = 20	19*	1	17	16

*p=0.0037; The cut-off point for both anti-nephrin and anti-dsDNA antibodies were mean OD value of BALB/c mice + 3 SD; Proteinuria was defined as > 100 mg/dL of protein in urine; ND = not done

Table 3.18. Correlation of Anti-nephrin antibodies with renal involvement (proteinuria/cellular cast) in controls, lupus and non-lupus groups (Immunoblotting method)

	Proteinuria/cast	No proteinuria/cast
Lupus patients + anti-nephrin abs n=14	6 (42%)	8 (58%)
Lupus patients - anti-nephrin abs n=86	47 (54%)	39 (46%)

p=n.s

Table 3.19. Correlation of BRAA in lupus patients with Psychosis and Seizure

	Psychosis/seizure	No Psychosis/seizure
Lupus patients + BRAA (n=10)	6 (60%)	4 (40%)
Lupus patients – BRAA (n=90)	4 (2%)	86 (98%)

p<0.0001

Table 3.20. Correlation of BRAA in lupus patients with NP-lupus syndromes (ACR suggested guideline for NP-lupus)

	NP lupus	No NP-lupus
Lupus patients + BRAA (n=10)	6 (60%)	4 (40%)
Lupus patients – BRAA (n=90)	21 (23%)	69 (77%)

p<0.0186

The SLE disease Damage Index was analyzed with regards to immunosuppressive drugs in lupus management. Not surprisingly, we noted that the patients treated with corticosteroids and cytotoxic drugs were found to be associated with the Disease Damage Index and combined NP and renal damage index ($p<0.0001$). The index was also associated with patients on corticosteroids alone ($p <0.0001$). The number was small for those with only cytotoxic and those without any of steroid or cytotoxic drugs, it was noted the association with disease Damage Index was low.

The MBL genetic variants were also analyzed with the SLE Damage Index in order to find out the if they predispose or worsen the SLE disease. First of all, the drugs used in lupus management were analyzed with SLE Damage Index. Disease damage was divided into two groups, i.e. NP and renal damage and overall damage based on the SLICC/ACR damage index. The lupus patients under both steroids and cytotoxic drug were found to have more damage compared to those with single drug therapy (**Table 3.21**). For the influence of MBL genetic variant on SLE damage Index, both MBL codon 52 mutant alleles and LX/LX haplotypes were analyzed. It was found lupus patients with mutant codon 52 variant were more prone to have damage in NP and renal organ system damage and in overall SLE damage (**Table 3.23**). However we did not such finding in lupus patients with LX/LX alleles (**Table 3.24**). Therefore we decided to analyze this finding in lupus patients with mutant codon 52 variants with regards to drugs used in lupus management. It was interesting to know MBL codon 52 variant was associated with NP and renal organ system damage and in overall SLE damage compared to those with wild-type alleles.

Table 3.21. Correlation of drug used in lupus management with SLICC/ACR damage index (NP and renal damage vs all systems)

	NP/Renal	All
Lupus patients on corticosteroids and cytotoxic drugs		
n = 50	22 (44%)[1]	36 (72%)[2]
Lupus patients on corticosteroids alone		
n = 46	4 (9%)[3]	10 (22%)[4]
Lupus patients on cytotoxic drugs alone		
n = 2	0	2 (100%)
Lupus patients not on corticosteroids and cytotoxic drugs		
n = 2	0	0

p = 0.0003 (1 vs.3); p < 0.0001 (2 vs.4); p = 0.0084 (1 vs.2); p = n.s (3 vs.4)

Table 3.22. Correlation of MBL exon 1 polymorphism with SLICC/ACR damage index (NP and renal damage vs all systems)

		NP/Renal	All
Lupus patients on corticosteroids and cytotoxic drugs			
n =50			
MBL 52 mutant	n = 14	8 (57%)*	10 (71%)
MBL 52 wildtype	n = 36	14 (39%)	26 (72%)
Lupus patients on corticosteroids alone			
n = 46			
MBL 52 mutant	n = 12	4 (33%)*	4 (33%)
MBL 52 wildtype	n = 34	0 (0%)	6 (18%)
Lupus patients on cytotoxic drugs alone			
n = 2			
MBL 52 mutant	n = 0	-	-
MBL 52 wildtype	n = 2	0 (0%)	2 (100%)
Lupus patients not on corticosteroids and cytotoxic drugs			
n = 2			
MBL 52 mutant	n = 0	-	-
MBL 52 wildtype	n = 2	0 (0%)	0

Table 3.23. Correlation of MBL exon 1 polymorphisms with SLICC/ACR damage index (NP and renal damage vs all systems)

	NP/Renal	All
Lupus patients with MBL 52 mutant		
n =26	12 (46%)[1]	14 (54%)[2]
Lupus patients without MBL 52 mutant		
n =74	14 (19%)[3]	34 (46%)[4]

p = 0.0138 (1 vs.3); p = n.s (2 vs.4);

Table 3.24. Correlation of MBL promoters polymorphism with SLICC/ACR damage index (NP and renal damage vs all systems)

	NP/Renal	All
Lupus patients with LX/LX n = 14	2 (14%)[1]	8 (57%)[2]
Lupus patients without LX/LX n = 86	24 (28%)[3]	40 (47%)[4]

p = n.s (1 vs.3); *p* = n.s (2 vs.4)

Table 3.25.Correlation of anti-nephrin autoantibodies with renal damage score

	Renal D-Score >0	Renal D-Score =0
Lupus patients + anti-nephrin antibodies n=14	2 (14%)	10 (86%)
Lupus patients - anti-nephrin antibodies n=86	20 (23%)	68 (77%)

p=n.s

Table 3.26. Correlation of BRAA in lupus patients with NP damage score

	NP D-Score >0	NP D-Score =0
Lupus patients + BRAA (n=10)	0 (0%)	10
Lupus patients – BRAA (n=90)	0 (0%)	81

$p<0.0186$

For anti-nephrin antibodies and BRAA, we could not find any significant association of those antibodies with Damage Index (**Table 3.25** and **Table3.26**).

CHAPTER 4

DISCUSSION

4.1.Mannose Binding Lectin

Mannose Binding Lectin (MBL) is an acute phase protein secreted by the hepatocytes into the blood circulation. MBL recognises, binds and enhances phagocytosis of mannose-rich microorganisms, i.e. bacteria, viruses, protozoas and yeasts without the need for specific antibodies. Thus MBL plays an important role in innate immunity.

Low levels of MBL cause defective opsonization and phagocytosis, resulting in increased susceptibility to bacterial, viral and fungal infections in humans, especially in children. Patients with low plasma MBL are reported to have association with prolonged and recurrent infections. Significantly, immunosuppressed patients with low level of plasma MBL are more prone to having infections than those with normal plasma level.

Affinity purified MBL from pooled fresh human plasma (pMBL) has been used to treat patients with frequent and chronic infections and initial results were encouraging (Valdimarsson et al, 2003). Now recombinant MBL (rMBL) have been used as replacement protein in patients with low level of plasma MBL in clinical trials. MBL replacement therapy is potentially available soon for patients with uncontrolled infections, pending the results of clinical trials.

Currently, plasma levels of MBL are measured by ELISA methods. However, serum MBL level may not represent actual normal levels because it is an acute phase protein. Circulating MBL in human varies from <0.01 to >5μg/ml and MBL concentration in the umbilical cord blood was reported to be lower than that in the

systemic circulation (Thiel et al.,1995). Furthermore, MBL being an acute phase protein, its concentration can show an increase of up to three-fold following infections or surgical trauma (Thiel et al., 1992). Dysfunctional proteins resulting from mutant structural MBL gene can give normal levels of serum MBL, but poor functionality.

Its gene is assigned at chromosome 10q11.2-q21 (Sastry et al 1989). Genetic polymorphisms of MBL gene have been recorded in the promoter region as well as in part of exon 1. Although the frequency of the different genotypes varies among different population groups, mutant genotypes of exon 1 and different haplotypes of MBL are associated with low levels of plasma MBL. Thus patient with mutant allotypes are more prone to having infections, especially in immunocompramised state.

Since low-MBL producing mutant alleles and haplotypes has been identified, genotyping of MBL gene will be able to provide an alternative way of predicting serum MBL level. Two studies have shown association of infections with different MBL alleles and haplotypes in children. Summerfield et al. (1997) reported a retrospective study of children attending a pediatric hospital and concluded that mutations in the MBL gene are an important risk factor for infections in children. In a prospective population-based study on young children, Koch et al. (2001) suggested that MBL insufficiency play an important role in host defense, particularly during childhood from age 6 through 17 months, when the adaptive immune system is immature. These two findings suggest that the MBL pathway is important in defense against infection during the interval between the loss of passively acquired maternal

antibodies and beginning of a mature immunologic repertoire.

Current methods for assessing MBL gene polymorphisms include PCR-SSP, PCR-SSO, PCR-RFLP (partially) and multiplex PCR using UHG. The disadvantage of the PCR-SSP method is the need for many PCR cycles, which requires many primers. Difficulties with optimization compound this problem. In the case of MBL gene polymorphisms, at least 10 PCRs using sequence specific primers will be needed (Steffensen et al., 2000). In other words, more genomic DNA, more reagents and longer processing time will be required. Furthermore, Steffensen et al. (2000) highlighted that difficulties in optimization of PCR-SSP may require re-optimization whenever a new set of reagents or a new PCR machine is used. These difficulties have been confirmed in our experience with optimization of PCR-SSP for detection of MBL gene polymorphisms.

PCR-SSO needs an experienced operator to perform, longer time to complete and stringent conditions for clear and good results. Two PCRs and 10 hybridization steps were necessary in the radioactive detections of MBL gene polymorphisms. (Madsen et al., 1994, 1995) The PCR-SSO method is more complex and more costly than PCR-SSP and PCR-RFLP to perform.

The genotyping method based on the generation of DNA heteroduplexes using a PCR-amplifiable synthesized DNA (Universal Heteroduplexes Generator – UHG) has been successfully employed to detect all three mutant alleles in exon 1 of MBL gene (Jack et al., 1997). However, the nature of the test requires an experienced person to

perform, use of more resources, time and at increased cost as compared to other methods.

Steffensen et al. (2003) reported rapid genotyping of MBL gene polymorphism by using real-time PCR with fluorescent hybridization probes. The genotyping was done in a relatively short time. However, the costs of real-time PCR machine and fluorescent hybridization probes are significant.

The PCR-RFLP method is simple, easy to perform, does not require special training and gives unambiguous results. PCR-RFLP approach is available mainly for Codons 54 and 57 at exon 1 and for X/Y polymorphic site at promoter gene region. No enzyme-recognition sites are readily available at Codon 52 and H/L polymorphic sites. Since there are only partial PCR-RFLP methods (Lipscombe et al., 1992 and Roelofs et al., 2003) available for genotyping of MBL gene polymorphisms, we, therefore, decided to design mismatched primers to introduce enzyme-recognition sites in the PCR product.

The ideal method to detect genetic polymorphisms should be simple, inexpensive, reproducible, reliable, with a rapid turnaround time, and should be able to be performed in laboratories with basic facilities. Though the focus of our study is not to design new molecular biological techniques, we felt the need to do so for our study as well as the benefits that arise from it when the technique is published. We have demonstrated a simple and reproducible PCR-RFLP method to genotype the MBL gene polymorphisms using manually-designed mismatched primers which possess recognition sites for restriction endonucleases. We have optimized the conditions with

regards to simplicity and reproducibility. Our method is suitable for population studies targeting large sample numbers. Our method can be used in routine diagnostic laboratories where limited manpower is available and rapid turn-around time is required.

In our PCR-RFLP, only 2 PCR reactions (one for exon 1 and one for promoter gene) will be required for subsequent cleavage by the respective restriction endonucleases. We need to do only one PCR for genotyping of MBL exon 1 which is followed by a one-hour digestion with respective restriction endonucleases. We therefore can genotype all three polymorphisms at exon 1 within a day. We tested different incubation times for enzyme cleavage and it was noted total cleavage by enzymes occurs after as short a period of time as 30 minutes. The amount of restriction endonucleaes required for RFLP was optimized (2 units, 1unit, 0.5unit, 0.25unit and 0.13unit of corresponding enzymes tested for over night incubation). Based on the minimum amount of enzymes required for overnight digestion, the time required for enzyme cleavage were also tested (one hour, two hours, four hours and over-night incubation). We found that *Mwo* I (0.13 unit) and *Ban* I (0.13 unit) for 4μl of exon 1 MBL PCR product and *Mbo* II (1 unit) for 1 μg of plasmid DNA are sufficient for complete cleavage at their respective optimum temperature with one hour incubation.

For promoter gene region polymorphisms, only one PCR product will be required for double-digestions by *Drd* I and *Btg* I with overnight incubation at 37°C. We also optimized for time and amount of enzymes required for double digestions. However we settled on the double digest with *Drd* I (0.13 unit) and *Btg* I (0.5 unit) overnight

because we wanted to use minimum enzyme amount in double digestion and overnight incubation showed clearer results.

One of the advantages of our PCR-RFLP is the use of routine gel electrophoresis to visualize the PCR-RFLP products. We do not require polyacrylamide gel to visualize the cleaved fragment, as agarose gel electrophoresis is sufficient to separate the fragments after enzyme cleavages. We successfully showed that these PCR products are accurately cleaved by appropriate restriction endonucleases to easily identifiable fragments. Furthermore, one agarose gel will be enough for visualization of the three different exon 1 enzyme-digests because the fragments will not overlap in the gel electrophoresis. In our laboratory, one enzyme-digest is loaded every 30 minutes so that the time required for separation and the cost of agarose gel are significantly reduced.

Since we have introduced an enzyme recognition site at Codon 52 in the mismatched primer, all Codon 52 wild type alleles were cleaved by restriction endonuclease *Mwo* I and seen clearly on gel electrophoresis. The heterozygous sample was also seen in our control group. These samples (both wild type homozygous and heterozygous samples) were sent for sequencing to validate our PCR-RFLP method. For Codon 54, mutant alleles were cleaved by restriction endonuclease *Ban* I, which has been described elsewhere. We did not find any mutant allele at Codon 57 where restriction endonuclease *Mbo* II would cleave, however we managed to receive such DNA samples from overseas. The optimization of PCR-RFLP was done using all possible MBL variants.

We have successfully genotyped the exon 1 and haplotyped the promoter gene of MBL in our local healthy controls using novel PCR-RFLP method. We have presented those gene frequencies of our control group (**Table 3.8** and **Table 3.9**). We also compared our results with those of Chinese (Mainland) and Japanese gene polymorphisms obtained by PCR-based methods (Huang et al., 2003, Wang et al 2001). It is understandable to note that they did not report codons 52 and 57 results in their local population because firstly there are no PCR-RFLP methods available at that time and secondly mutant alleles of codon 57 are not expected in Oriental population. The comparison of codon 54 polymorphisms at MBL exon 1 results was carried out and the frequencies of codon 54 are comparable between our cohort and their populations. PCR-RFLP method was used for our local and Japanese populations while PCR-UHG approach was employed for Chinese (China). The frequencies of promoter gene haplotypes among local population (PCR-RFLP), Japanese (PCR-SSP) and China Chinese (PCR-UHG) are almost similar with minor variations in LY/LY, LX/LX, HY/HY, LX/LY. This has to be confirmed by more genotyping on different ethnic groups.

We genotyped the lupus patients using our novel PCR-RFLP protocol and gene frequencies results were presented (**Table 3.10**). We found no mutant alleles in codon 57 in both control and lupus groups. Similar polymorphic patterns were observed between codons 54 and 57 of control and lupus patients groups. However the mutant allele of codon 52 is significantly increased in lupus patients group compared to control group. For promoter regions, the gene frequencies of control and lupus patients groups were presented (**Table 3.11**). It is widely accepted that HX haplotypes of MBL gene are not reported so far and their association with MBL

production is as follow; HY as high-promoting haplotype, LY as medium-promoting haplotype and LX as low-promoting haplotype. We did not find any of HX haplotypes, which is in accordance with other reports. There were decreased high-promoting haplotypes (HY/HY), decreased medium-promoting haplotypes and increased low-promoting haplotypes in lupus patients compared to control group. And it was noted that lupus patients had decreased LX/LY haplotypes and increased HY/LY haplotypes. This finding will provide important information on the MBL gene as a disease predisposing factor in pathogenesis or aggravating factor in SLE, especially for those under immunosuppressive drugs. Low-promoting haplotypes (LX) will cause "MBL Insufficiency State" which eventually lead to an inefficient MBL complement system compared to those with high-promoting haplotypes (HY) and medium-promoting haplotypes (LY). Complement system plays an active role in innate immunity and disposal of immune complexes. Hence, genetic variation (codon 52 mutant alleles or low-promoting haplotypes) may confer disease susceptibility and severity predisposing factor in lupus patients. The severity state may be the result of predisposition to recurrent infections. We further explore if these genetic variants are associated with infections while treated with immunosuppressive agents in lupus patients.

Among 100 unselected lupus patients, 98 patients were treated with immunosuppressive agents (50 with corticosteroids and cytotoxic agents; 46 with corticosteroids alone; 2 with cytotoxic agents alone). Only 2 patients did not have corticosteroids or cytotoxic agents. We found that 66 lupus patients (66%) had significant infections during their disease course. 20 (30%) of 66 lupus patients with infections had codon 52 mutant alleles while 6 (18%) of 34 lupus patients without

infections. 10 (15%) of 66 patients presented with low-promoting LX/LX haplotypes and 4 (12%) of 34 lupus patients without infection also had low-promoting LX/LX haplotypes. However we did not find anyone having codon 52 mutant alleles and low-promoting LX/LX haplotypes.

The importance of MBL variants with MBL insufficiency with regards to infection was studied in lupus patients with different immunosuppressive drugs used. It is interesting to note that a higher percentage of lupus patients with codon 52 mutant allele (71%) or low-promoting haplotype (100%) presented with infections while being treated by immunosuppressive drugs. However this needs to be studied further if this is because of MBL variant or due to extensive use immunosuppressive drug. It is noted that those without codon 52 mutant alleles (72%) and those without LX/LX haplotypes (66%) do also have infections too. However those with codon 52 mutant alleles (83%) presented with more infections compared to those without codon 52 mutant alleles (56%) when corticosteroid is used.

The relationship of SLE Damage Index with drugs used in lupus treatment was significant in those treated with steroids and immunosuppressive drugs in both overall damage score and NP and renal damage scores. The damage score was reduced in those with steroids alone or cytoxic drugs. This finding support that survival rate of lupus patients are increasing with effective lupus management with steroid and cytotoxic drug, lupus patients are presenting with organ damage. In such situation, MBL, complement protein, may have a role in predisposing or worsening the situation. We found that lupus patients with MBL 52 mutant and LX/LX haplotypes did not show any significant effect on overall SLE disease damage. However lupus

patients with MBL codon 52 mutant alleles are associated with renal and NP damages.

4.2.Anti-nephrin antibodies

The kidney is one of the most commonly affected organ systems in SLE. With the use of sensitive light, electron, and immunofluorescence microscopy, at least one abnormality is seen in kidney biopsy specimens from almost all patients with lupus. Approximately 75% of renal biopsy specimens reported in several series have been classified as focal proliferative, diffuse, or membranous glomerulonephritis (Golbus and McCune., 1994). In unselected group of lupus patients, abnormalities in urine or renal functions occur in about 25%-50% early in the course of the disease (Cameron., 1999). In the study of Vlachoyiannopoulos et al (1993), renal diseases manifested as proteinuria, microscopic hemuturia, decreased clearance of creatinine, increased serum creatinine level or the presence of casts was found in about 50% of lupus cases. In other studies, using similar definitions, the prevalence of renal diseases ranged from 29% to 75% (Wallace et al., 1980; Cervera et al., 1993).

Proteinuria is considered a hallmark of renal involvement in lupus and it was reported to occur in 100% of lupus patients, sometime during the course of their disease (Golbus and McCune., 1994). Proteinuria in SLE is due to deposition of immune-complexes in the filtration unit, the glomerulus. The glomerular filtration barrier to proteinuria composed of: -(1) innermost endothelial cells, (2) middle glomerular basement membrane and (3) outermost slit diaphragm (Tryggvason and Wartiovaara. 2001). The plasma filtrate first passes through the vascular endothelium, where the

blood cells were retained. The glomerular basement membrane then serves as a pre-filter of larger plasma proteins and finally the slit diaphragm is thought to function as the decisive ultrafilter that prevents proteins larger than albumin from leaving the blood circulation (Tryggvason K and Wartiovaara. 2001). Of the three components of glomerular filtration barrier, the crucial barriers for plasma proteins larger than 70 kDa are the glomerular basement membrane (GBM) and the glomerular slit diaphragm (Farquhar et al., 1999; Schneeberger., 1974; Rodewald and Karnovsky., 1974). Thus, Karnovsky and Ainsworth (1972) proposed that the glomerulus functions as two molecular filters acting in series:- the GBM (the coarse filter) and the slit diaphragm (the ultimate fine filter).

Slit diaphragm, the ultimate molecular size filter, is a specialized cell adhesion structure between the interdigitating glomerular podocyte foot processes. Based on the electron microscopic studies on mouse and rat kidney tissues, Rodewald and Karnovsky (1974) proposed that the slit diaphragm is an orderly isoporous filter. According to them, the slit diaphragm is a three dimensional protein structure with highly ordered periodic cross bridges forming zipper-like appearance. While the filtration barrier is freely permeable by water and small solutes, the slit diaphragms of podocytes will decide the permeability of molecules at larger extent. Several proteins have been shown to localize in the slit diaphragm, however, it may require more studies to understand how they interact or how they contribute to maintain the functional integrity of the slit diaphragm. Within a recent few years, several molecules such as, ZO-1 (Schnabel et al. 1990), nephrin (Ruotsalainen et al. 1999), CD2AP (Shih et al.1999), FAT (Inoue et al. 2001) and P-Cadherin (Reiser et al. 2000)

have been shown to be expressed with in slit diaphragm. This suggests that some/all of these proteins may play a major role in maintaining integrity of slit diaphragm.

Nephrin is a member of an immunoglobulin superfamily (IgCAM) and transmembrane protein expressed in podocytes and suggested to play an important role in maintaining selective permeability in slit diaphragm. Extracellular domain has eight IgG motifs and one type III-fibronectin domain. Topham et al. (1999) reported the relationship between the antigen recognized by mAb 5-1-6 and nephrin. In their study, rat glomeruli were incubated in vitro with mAb 5-1-6, then cross-linked the antibody to its antigen with chemical cross-linker, solubilised glomeruli, extracted the immune complexes, separated the immunoglobulin (IgG) and antigen on a gel by electrophoresis, and then identified the isolated antigen by mass spectrometry. The antigen that was recognized by mAb 5-1-6 turned out to be part of the extracellular domain of nephrin.

Currently pathogenesis of proteinuria in SLE is not a well-understood process. Some proposed the circulating immune complexes composed of antibodies are inappropriately deposited in the kidney of lupus patients, which in turn cause altered permeability for serum protein by damaging the glomerular basement membranes (Mavragani and Moutsopoulos., 2003). Autoantibodies may have a role in pathogenensis of renal involvement in SLE (Termaat RM et al., 1993). Autoantibodies, which are characteristics of SLE, may play a role in pathogenesis of renal involvement and proteinuria. Glomerulonephritis mediated by antibodies to glomerular basement membrane (GBM) is the prototype of human glomerular disease produced by pathogenic antibodies to intrinsic glomerular components. Anti-

glomerular basement membrane (anti-GBM) antibody disease is a rare autoimmune disorder in which circulating antibodies are directed against an antigen normally present in the GBM and alveolar basement membrane. The target antigen is the α-3 type IV collagen and the resultant clinical syndrome due to antiGBM antibodies provides a spectrum from mild renal involvement to a rapidly progressive glomerulonephritis.

The disease is more common in whites than in African Americans. The disease can manifest in persons of any age with equal distribution on both sexes. In adults, anti-GBM disease is responsible for approximately 5% of glomerulonephritis and is diagnosed in 1-2% of renal biopsy specimens. Anti-GBM glomerulonephritis accounts for 10-20% of rapidly progressive glomerulonephritis in both adults and children. In untreated patients, the disease usually progresses to renal failure or death. Treated patients have a significant risk of morbidity and mortality from renal failure, pulmonary hemorrhage, and complications of treatment.

The combination of glomerulonephritis and pulmonary hemorrhage has been commonly referred to as Goodpasture syndrome. Pulmonary and/or renal manifestations can be encountered in a variety of conditions such as antineutrophilic cytoplasmic antibody (ANCA)– positive vasculitis and other autoimmune disorders. Consequently, the identification of anti-GBM antibodies in the patient's serum or tissues is of paramount importance in the diagnosis of Goodpasture disease. Anti-GBM antibodies are uncommon in lupus patients. The prevalence of renal involvement is high in local Chinese lupus patients (Fong et al. 1997). Consequently anti-nephrin antibodies may explain proteinuria in lupus patients and their presence

may represent a novel severity factor in SLE. Thus we decided to express the most homologous area of extra-cellular domain among mouse and human nephrin proteins.

We have expressed this fragment of nephrin protein using His-Tag expression vector (pET-16b) in *E.Coli* expression system. The expressed protein was confirmed as “nephrin” protein fragment by protein fingerprinting (MALDI-TOF-MS) after identifying the trypsin-cleaved fragments in available protein databases. We raised the monoclonal antibodies and polyclonal antibodies against expressed protein, however the monoclonal antibody production was unsuccessful because of technical problems. The purified polyclonal antibodies were found to be reacting with glomeruli of frozen section of mouse kidney.

An ELISA was set up to detect antibodies against nephrin protein in both human and mice sera. It was noted the antibodies against nephrin protein were raised in 19 (95%) out of 20 lupus mouse models (PL/J F1xNZB) and 4 (80%) out of 5 MPR/lpr mice and none (0%) of 10 BALB/c mice. These anti-nephrin antibodies were associated with proteinuria (89%) and anti-dsDNA antibodies (84%) in these lupus mouse models.

However, optimization of ELISA for use with human sera was unsuccessful despite repeated attempts using different blocking reagents. Among gelatin, fetal bovine sera, bovine sera albumin, nonfat dry milk, casein, human albumin, none worked well enough to yeild clear and consistent results. We therefore switched to immunoblotting to detect anti-nephrin antibodies in human sera for both controls and patients.

The 102 control individuals, 100 unselected lupus patients and 130 non-lupus patients were screened for presence of anti-nephrin antibodies. The results were presented in **Table 3.13**. Briefly, we observed that the antibodies against expressed fragments of nephrin protein were not seen in all control samples and non-lupus samples. 14 (14%) out of 100 unselected lupus patients have shown positive results against anti-nephrin antibodies.

These antibodies were further correlated with proteinuria and renal organ-system Damage score of unselected lupus group. Even though we have significant association of anti-nephrin antibodies in lupus patients, we did not find any significant association of these antibodies with proteinuria or renal damage in lupus patients. Firstly it was shown that resultant clinical syndrome due to antiGBM antibodies which are found to be associated with Goodpasture disease, provides a spectrum from mild or no renal involvement to a rapidly progressive glomerulonephritis. Secondly, autoantibodies can predate the clinical presentation in autoimmune disease. Arbuckle et al. presented a retrospective study on development of autoantibodies before clinical onset of SLE in 2003. They found that at least one SLE autoantibodies (anti-nuclear antibodies, anti ds-DNA antibodies, anti-phospholipid antibodies, anti-Ro antibodies, anti-La antibodies and anti nuclear ribonucleoprotein antibodies) was present in 88 percentage of lupus patients before the clinical diagnosis of SLE (the mean duration is 3.3 year with 0- 9.4 year range). Therefore, those with positive anti-nephrin antibodies may not present with proteinuria or renal damage yet, but may present in the later course of disease. This phenomenon can be seen in myasthenia gravis where anti-acetylcholine receptor antibody can be detected several weeks before clinical presentation. This

would explain the nature of autoantibodies where they can be detected without a symptom.

The pathogenesis of renal involvement is rather diverse with many possible explanations at the moment. Although proteinuria and cellular cast are presented in about half of lupus patients, biopsy studies demonstrate some degree of renal involvement in almost all patients. It may not be able to conclude a single pathogenic mechanism for all renal involvements. We may need to recruit more lupus patients in the study to be able to correlate anti-nephrin antibodies with renal involvement. It will be better if sera from same cohort of lupus patients are serially collected and analyzed together with renal involvement.

Furthermore, expressed nephrin protein was from mouse origin, which may not antigenic to human nephrin. Thus we will plan to express complete extracellular domain of nephrin protein so that it will be able to match most antigenic epitopes in lupus patients. The expressed nephrin protein will be used in determination of anti-nephrin antibodies in sera serial collected from larger cohort of lupus patients.

4.3.Brain Reactive Autoantibodies (BRAAs)

It was reported that 31%-70% of SLE patients has significant neuro-psychiatric (NP) manifestations in both retrospective and prospective studies (West et al., 1995; Kaell et al., 1986; Futrell et al., 1992; Sibley et al., 1992; Rood et al., 1999). The spectrum of NP manifestation in SLE includes both neurologic and psychiatric features, many of which may be subjective in nature. They vary from overt neurologic dysfunctions

due to psychoses, seizures, to subtle abnormalities in neurocognitive functions such as memory, intellect etc. (Hanly and Hong., 1993). They are not easily identifiable because lupus itself or drugs used in lupus management or other associated pathological condition may contribute to pathogenesis of NP-lupus.

The unclear pathogenesis of NP-lupus contributes to difficulties in diagnosis and management. Clinicians depend on exclusion of other causes to diagnose NP-lupus (Hanson et al., 1992). The pathogenic roles of many autoantibodies are not well defined in autoimmune disorders and also in SLE, especially in NP-lupus. Prevalence of brain reactive autoantibodies reacting against different neuronal proteins was reported. They were detected in sera and cerebrospinal fluid (CSF) in both human and animal studies (Hanson et al., 1992; Bluestein and Zvaifler 1976; Wilson et al., 1979; Toh and Mackay 1981; Hoffman et al., 1987; Zameer and Hoffman 2001; Moore PM; 1992). However, not all brain reactive autoantibodies are responsible for NP-lupus, i.e, they are not necessarily of pathogenic importance (Khin and Hoffman., 1993). Of these autoantibodies, some pathogenic autoantibodies might also attack neuronal tissue and lead to NP manifestations. These autoantibodies can bind to neuronal surface membranes (Koren et al., 1992) and cause injury via a direct toxic effect, or alternatively it disrupts physiological function of neuronal tissue when bound, but without causing cell death.

In our immunoblotting experiments using neuronal membrane proteins extracted from the mouse brains to detect any antibodies in unselected-lupus patients, we found, not surprisingly, that many lupus antibodies were reactive with mouse neuronal membrane proteins. Among them, two lupus antibodies reacting against mouse brain

membrane proteins between 24 and 35 kD molecular weights were particularly prominent. We found their presence in 10 (10%) out of 100 unselected lupus patients and in none (0%) of the 100 healthy individuals. Calculated molecular weights of the membrane proteins are 27.5 and 29.5 kD and we used the term "Brain Reactive Autoantibodies" as both seem to be present in concordance. We tested whether these antibodies are specific to lupus sera, we immunoblotted the sera of 130 non-lupus rheumatic patients comprising 20 RA, 20 OA, 20AS, 20 PsA and 50 APS patients. We were particularly interested in the primary APS patients with history of CNS manifestation e.g. stroke. Hence we are confident that the BRAAs represent independent factors that are associated with NP-lupus and not due to antiphospholipid antibodies. However these antibodies (BRAAs) were not found in rheumatic patients with non-lupus disease.

The membrane proteins extracted from different mouse tissues (liver, heart, spleen and kidney) were used as antigen source in immunoblotting experiments and no similar bands were seen. BRAAs were specifically reacting with membrane proteins of mouse brain neuronal cells and they did not cross react with membrane proteins from other cells.

To explore if similar brain membrane proteins extracted from the different species react with lupus antibodies, immunoblotting experiment was set up using brain membrane proteins extracted from rat, pig, chicken, duck, quail, frog and fish. The results showed the lupus antibodies still react with similar membrane proteins extracted from mammalian brains such as rat and pig. However lupus antibodies failed to show binding with such proteins from neuronal membrane proteins of other

species such as avian, amphibian and fish species. Our main interest is whether these BRAAs react with human neuronal membrane proteins. Immunoblots using human neuronal lysate produce the 2 bands as predicted. We therefore conclude that these BRAAs react with human and mammalian neuronal membrane proteins.

With 2-dimensional gel approach, we attempt to identify the autoantigens by peptide mass fingerprinting using MALDI-TOF-MS analysis. Matching the results against public protein databases, NCBI and Swissport, it was noted that they may be previously undescribed proteins. We did not attempt to expand further time and effort in further identifications at present as this is not the focus of our current project.

We concluded that these two membrane antigens of mammalian brain origin are reacting with antibodies present in the sera of lupus patients. We found these BRAAs in 10 out of 100 lupus patients (10%) while none of the healthy individuals (n=102) and non-lupus (n=130) patients had these antibodies. We then proceeded to determine the association of these antibodies with neuro-psychiatric manifestations (psychosis and seizure) and whether they represent a disease severity factor in SLE. According to chart review, of the 10 patients with positive BRAAs, 6 patients presented with NP manifestations (3 with psychosis and 3 with seizures) according to 1997-updated ACR criteria. However, only 4 patients out of 90 lupus patients without BRAAs had NP-lupus (seizures). This association is statistically significant ($p<0.001$). We extended our analysis to include the NP-lupus syndromes, which is more diverse. The significant association of BRAA with NP lupus is still present. The pathogenesis of NP lupus is yet unclear. There are probably many sub-sets of NP-lupus in existence.

BRAAs could be antibodies that are associated with the sub-set presenting with psychosis or seizure in NP-lupus.

NP manifestations may be related to targets of autoantibodies. Khin and Hoffman (1993) reported that monoclonal antibody prepared against brain membrane proteins was seen not binding to membranes of all neuronal cells. Thus autoantibodies may selectively bind to different area of brain (perhaps different expression of protein in different anatomical location) and NP-manifestations may differ from one patient to another depending on anatomical locations. Sakic et al., (1998) reported that cognitive deficits are associated with hippocampal lesions in the mice. The BRAAs target area in mouse brain will need further studies to be defined.

Presence of different autoantibodies is hallmark of lupus disease, antibodies alone will not be significant. It is important to correlate presence of autoantibodies with clinical manifestations. We have identified the BRAA, which are highly associated with psychosis and/or seizure. We hypothesized that over-expressed BRAA which can cross the blood-brain-barrier in certain clinical conditions, bind membrane proteins of brain and alter physiological functions causing psychosis and/or seizure in lupus patients. We plan to identify and characterize the membrane proteins reacting with BRAA. It is necessary to prepare animal studies to see if our hypothesis is correct.

CHAPTER 5

CONCLUSION

Conclusions

1. We have successfully optimized the PCR-RFLP approach to genotype the Exon 1 and haplotype promoter region of MBL gene and applied this method to genotype and haplotype the MBL gene in controls and lupus patients. We found that codon 52 mutant alleles are highly associated with lupus patients and they can be considered as predisposing factor in pathogenesis of SLE as a result of impaired immune-complex clearing function by the MBL protein. More MBL low-producing haplotypes are seen in lupus patients. They may also worsen the disease severity by failing to protect against invading pathogenic microorganism when intensive immunosuppressive lupus treatment is being carried out.

2. We have identified anti-nephrin antibodies, which are highly associated with proteinuria in lupus mouse models. However, we did not find this association with proteinuria in human samples. Firstly, the difference in origin of proteins might be an important factor to determine the specificity between mouse and human. Secondly the different epitopes may play an important role in their antigen-antibody reaction. Moreover its action on mouse models indicates a potential role in disease severity.

3. We have identified BRAAs in lupus patients, which are highly associated with psychosis, or seizure in NP-lupus. These BRAAs are highly tissue, disease and species-specific and do not correlate with other NP syndromes in lupus patients.

4. Among these three findings, codon 52 mutant MBL alleles confer increased disease severity by increasing disease Damage Index (SLICC/ACR score) both in overall and NP and renal damages.

Future work

1. MBL gene has three mutation sites at exon 1 and two polymorphic sites at promoter. We plan to design a method to haplotype the all these polymorphic site so that we can further study its functional correlation. We also plan to administer purified MBL into the lupus mouse model to study whether it attenuates severity of lupus manifestation.

2. The nephrin protein will be expressed in full length or nephrin protein of human origin will be expressed, and further studied as to their relationship with renal involvement in lupus patients.

3. Brain autoantigens reacting with BRAA will be isolated and identified so that an ELISA method will be employed to detect BRAA in lupus patients prospectively in a large scale. It may be a useful diagnostic and monitoring marker in NP-lupus management.

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APPENDICES

APPENDX 1

"Patients Data Entry Form"

Code ______________________
Sex ______________________
Ethnic Group ______________________
Date of Birth ______________________
Date of Disease Onset ______________________

ACR criteria

		Tick Y or N Y	N
ACR1	**Malar rash** Fixed erythema, flat or raised, over the malar eminences, tending to spare the nasolabial folds.		
ACR2	**Discoid rash** Erythematous raised patches with adherent keratotic scaling and follicular plugging, atrophic scarring may occur in older lesions		
ACR3	**Photosensitivity** Skin rash as a result of unusual reaction to sunlight, documented by patient history or physician observation.		
ACR4	**Oral ulcers** Oral or nasopharyneal ulceration, usually painless, observed by a physician.		
ACR5	**Arthritis** Nonerosive arthritis involving two or more peripheral joints characterized by tenderness, swelling, or effusion		
ACR6	**Serositis** a) Pleuritis - convincing history of pleuritic pain or rub heard by a physician or evidence of pleural effusion OR b) Pericarditis – documented by ECG or rub or evidence of pericardical effusion.		
ACR7	**Renal disorder** a) Persistent proteinuria greater than 0.5 grams per day or greater than 3+ if quantitation not performed OR b) Cellular casts – may be red cell, hemoglobin, grandular, tubular, or mixed		
ACR8	**Neurologic disorder** a) Seizures – in the absence of offending drugs or known metabolic derangements, eg uremia, ketoacidosis, or electrolyte imbalance OR b) Psychosis – in the absence of offending drugs or known metabolic derangements, eg uremia, ketoacidosis, or electrolyte imbalance		
ACR9	**Hematological disorder:** a) Hemolytic anemia – with reticulocytosis OR b) Leukopenia – less than 4,000/mm3 total on two or more occasions OR c) Lymphopenia – less than 1,500/mm3 on two or more occasions OR d) Thrombocytopenia – less than 100,000/mm3 in the absence of offending drugs.		
ACR10	**Immunological disorder**: a) Anti-DNA antibody to native DNA in abnormal titer OR b) Anti-Sm: presence of antibody to Sm nuclear antigen OR c) Positive finding of antiphospholipid antibodies based on i) an abnormal serum level of IgG or IgM anticardiolipin antibodies, OR ii) a positive test result for lupus anticoagulant using a standard method, OR iii) a false-positive serologic tests for syphilis known to be positive for at least 6 months and confirmed by Treponema pallidum immobilization or fluorescent treponemal antibody absorption test.		
ACR11	**Antinuclear antibody** An abnormal titer of antinuclear by immunofluorescence or an equivalent assay at any point in time and in the absence of drugs known to be associated with "drug-induced lupus" syndrome.		

MODIFEID RENAL INVOLVEMENT

Enter Y or N

		Y	N
R 1	Persistent Proteinuria: proteinuria > 0.5 gm/day or > 3+ on lab-stix on > 1 occasion		
R 2	Cellular Casts: presence of red cell, hemoglobin, granular, tubular, mixed casts.		
R 3	Nephrotic Syndrome: proteinuria > 3.0 gm/day with peripheral edema and serum Albumin < 30 gm/dL.		
R 4	Nephritic Syndrome: microscopic haematuria (> 5 RBC/hpf), proteinuria, elevated serum creatinine with or without hypertension.		
R 5	Rapidly Progressive Glomerulonephritis: deterioration of renal function over days to weeks in the absence of any other cause		

NEUROPSYCHIATRIC SYNDROMES

Enter Y or N

		Y	N
NP 1	Acute Confusional State		
NP 2	Acute Inflammatory DemyelinatingPolyradiculoneuropathy (Guillain-Barre Syndrome)		
NP 3	Anxiety Disorder		
NP 4	Aseptic Meningitis		
NP 5	Autonomic Disorder		
NP 6	Cerebrovascular Disease		
NP 7	Cognitive Dysfunction		
NP 8	Demyelinating Syndrome		
NP 9	Headache		
NP 10	Mononeuropathy (single / multiplex)		
NP 11	Mood Disorders		
NP 12	Movement Disorder (Chorea		
NP 13	Myasthenia Gravis		
NP 14	Myelopathy		
NP 15	Neuropathy, Cranial		
NP 16	Plexopathy		
NP 17	Polyneuropathy		
NP 18	Psychosis		
NP 19	Seizures and Seizure Disorder		

MBL, anti-nephrin antibodies and BRAA result

Enter appropriate result..

1	MBL	
	MBL Codon 52 polymorphism (wildtype homo=0; mutant homp=1; hetro=2)	
	MBL Codon 52 polymorphism (wildtype homo=0; mutant homp=1; hetro=2)	
	MBL Codon 52 polymorphism (wildtype homo=0; mutant homp=1; hetro=2)	
	MBL promoter (Refer to PCR-RFLP chart of MBL promoter)	
2	Anti-nephrin antibodies (Y or N)	
3	BRAA (Y or N)	

THERAPEUTIC MODALITIES

Indicate yes if patient has at any time been on the modality since the last entry:

Enter Y or N

		Y	N
DRG1	Prednisolone		
DRG2	Anti-malarial		
DRG3	Azathioprine		
DRG4	Methotrexate		
DRG5	Cyclophosphamide (p.o.)		
DRG6	Cyclophosphamide (i.v.)		
DRG7	Methylprednisolone		
DRG8	Vincritstine (i.v.)		
DRG9	Immunoglobulin (i.v.)		
DRG10	NSAIDS		
DRG11	Danazol		
DRG12	Plasmapheresis		
DRG13	Warfarin/Heparin		
DRG14	Aspirin		
DRG 15	Alternative medication: Traditional medication (Chinese, Malay and Indian) or herbal medication, either oral or topical, or acupuncture.		

DEFINITIONS FOR INFECTIONS

Enter Y or N

		Y	N
Bacterial			
INF1	Pneumonia ➔air space shadowing on X-rays.		
INF2	Urinary ➔ Pyuria (> 10 WBC/high power field) and/or positive urine cultures		
INF3	CNS ➔cerebrospinal fluid results or imaging suggestive of a cerebral infection or EEG/CSF features suggestive of a viral encephalitis.		
INF4	Hepatobiliary ➔ serum alkaline phosphatase more than 2 times normal range with suggestive imaging (thickened gall bladder, biliary sludge, cholelithiasis demonstrated).		
INF5	Bacteraemia➔ organism isolated on blood cultures with no source identified.		
INF6	Skin➔ cellulitis, necrotising fascitis, etc		
INF7	Joints➔ purulent joint aspirate or positive joint fluid cultures or suggestive operative findings		
INF8	Others➔ infections at other sites and confirmed by positive cultures or histology		
Viral			
INF9	Herpes Zoster➔ grouped vesicles as on an erythematous base in a dermatomal distribution involving 1 dermatome.		
INF10	Disseminated Varicella ➔ grouped vesicles as on an erythematous base involving > 1 dermatome.		
INF11	Disseminated Herpes Simplex ➔ grouped vesicles as on an erythematous base distributed extensively (may not be possible to distinguish this clinically from disseminated varicella).		
INF12	Cytomegalovirus➔ any of the following: (a) 4 fold rise in antibody titres in 2 weeks (b) strongly positive CMV IgM assay (c) cytopathic effect on histological specimen.		
Fungal			
INF13	Cryptoccocal Meningitis ➔ infection involving the meninges and has any of the following: (a) positive cryptococcal antigen assay (b) torula seen on fungal smears or isolated on cultures		
INF14	Other Cryptoccocal Infections ➔ infection involving sites other than the meninges and has any of the following: (a) positive cryptococcal antigen assay (b) torula seen on histology or isolated on cultures.		
INF	Other Infections ➔ specific infections not mentioned above.		

Systemic Lupus International Collaborating Clinics / American Collegue of Rheumatology (SLICC / ACR) Damage InNdex for Systemic Lupus Erythematosus*

(* Damage: nonreversible change not related to active inflammation occurring since onset of lupus, ascertained by clinical assessment and present for at least 6 months unless otherwise stated. Repeat episodes must occur at least 6 months apart to score 2. The same lesion cannot be scored twice.)

Enter appropriate score

Organ system	Descriptions	Score Guide	Score
1	**Ocular (either eye, by clinical assessment)**		
O1	Any cataract ever	1	
O2	Retinal change or optic atrophy	1	
2	**Neuropsychiatric**	1	
N1	Cognitive impairment (eg. Memory deficit, difficulty with calculation, Poor concentration, difficulty in spoken or written language, impaired performance level) or major psychosis	1	
N2	Seizures requiring therapy for 6 months	1	
N3	Cerebrovascular accident ever	Score 2 if > 1	
N4	Cranial or peripheral neuropathy (excluding optic)	1	
N5	Transverse myelitis	1	
3	**Renal**		
R1	Estimated or measured glomerular filtration rate < 50%	1	
R2	Proteinuria >= 3.5 gm/24 hours	1	
R3	End-stage renal disease (regardless of dialysis or transplantation)	3	
4	**Pulmonary**	1	
P1	Pulmonary hypertension (right ventricular prominence, or loud P2)	1	
P2	Pulmonary fibrosis (physical and radiograph)	1	
P3	Shrinking lung (radiograph)	1	
P4	Pleural fibrosis (radiograph)	1	
P5	Pulmonary infarction (radiograph)	1	
5	**Cardiovascular**		
CV1	Angina or coronary artery bypass	1	
CV2	Myocardial infarction ever	score 2 if >1	
CV3	Cardiomyopathy (ventricular dysfunction	1	
CV4	Valvular disease (diastolic, murmur, or systolic murmur > 3/6)	1	
CV5	Pericarditis for 6 months, or pericardiectomy	1	
6	**Peripheral vascular**		
PV1	Claudication for 6 months	1	
PV2	Minor tissue loss (pulp space)	1	
PV3	Significant tissue loss ever (eg loss of digit or limb	1	
PV4	Venous thrombosis with swelling, ulceration, or venous stasis	Score 2 if >1site	
7	**Gastrointestinal**		
GI1	Infarction or resection of bowel below duodenum, spleen, liver, or gall bladder ever, for cause any	Score 2 if >1 site	
GI2	Mesenteric insufficiency	1	
GI3	Chronic peritonitis	1	
GI4	Stricture or upper gastrointestinal tract surgery ever	1	
8	**Musculoskeletal**		
MS1	Muscle atrophy or weakness	1	
MS2	Deforming or erosive arthritis (including reducible deformities, excluding avascular necrosis)	1	
MS3	Osteoporosis with fracture or vertebral collapse (excluding avascular necrosis	1	
MS4	Avascular necrosis	Score 2 if >1	
MS5	Osteomyelitis	1	
9	**Skin**		
SK1	Scarring chronic alopecia	1	
SK2	Extensive scarring or panniculum other than scalp and pulp space	1	
SK3	Skin ulceration (excluding thrombosis) for > 6 months	1	
10	**Premature gonadal failure**	1	
11	**Diabetes (regarding of treatment)**	1	
12	**Malignancy (exclude dysplasia)**	Score 2 if >1 site	
	Total score		

APPENDX 2.1

DNA and RNA protocols

A. Genomic DNA extraction from peripheral blood (salting-out method)

- 5 ml of peripheral blood was withdrawn in Na_2EDTA tube.
- Centrifuge the anti-coagulated blood at 2400 rpm for 15 minutes at RT.
- Remove the plasma and carefully transfer the white cells (buffer coat) to 15ml tube.
- Add Red Cell Lysis Buffer (RCLB; 0.144M NH_4Cl, 10mM $NaHCO_3$) to brim of plain tube and incubate about 5-10 minutes at RT to allow complete lysis of red blood cells. Centrifuge at 2400 rpm for 15 minutes at RT.
- Remove the supernatant and wash the white cell pallet gently once with RCLB.
- Add 3 ml of Nuclei Lysis Buffer (NLB; 10mM Tris-HCl pH 8.2, 0.4M NaCl, 2mM Na_2EDTA), 100 µl of Proteinase K 10mg/ml (dissolved in PK Buffer; 2mM Na_2EDTA, 1% w/v SDS) and 100 µl of 10% SDS. Vortex and incubate for 3 hours/overnight at 56°C.
- Add 1 ml of 5M NaCl and vortex vigorously for 15 seconds.
- Centrifuge at 3000 rpm for 30 minutes at RT. Transfer the supernatant into clean 15-ml centrifuge tube without disturbing the pallet.
- Add 2 volumes of ice-cold absolute ethanol and mix well by gentle inversion.
- Thread-like DNA strands will be removed and re-suspended in 200 µl of TE buffer and incubate at 37°C for 10 minutes. DNA will be further diluted at 1:100 for PCR amplification.

NB: Standard PCR will require 1µl of diluted DNA as template. No spectrometry will be needed for standard PCR.

B. Total RNA extraction

- 50-100mg tissue was crushed on liquid nitrogen. Add approximately 1ml Trizol and used syringe and needle to further homogenize. Incubate the homogenized samples for 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes.
- Add 0.2 ml of Chloroform per 1 ml of Trizol reagent used. And shake vigorously by hand for 15 seconds.
- Incubate again for 3 minutes at room temperature.
- Centrifuge the samples at 12,000xg for 15 minutes at 4°C.
- Transfer the aqueous layer into fresh tube. Add 0.5 ml of isoamyl alcohol per 1 ml of Trizol reagent used.
- Incubate again for 10 minutes at room temperature.
- Centrifuge the samples at 12,000xg for 10 minutes at 4°C.
- Remove the supernatant and wash the RNA pallet once with 75% ethanol (1 ml of 75% ethanol per 1 ml of Trizol reagents used). Mix the sample by vortexing and centrifuge at 7,500xg for 5 minutes at 4°C.
- Remove the supernatant and briefly dry the RNA pallet. Dissolve the RNA pallet in 20 µl RNAse-free water.
- Measure the amount of RNA by spectrophotometer,

NB: 1.0 OD_{260nm} contains 40ng/µl RNA

Clean RNA has ratio of OD_{260nm}/OD_{260nm} about 1.8-2.0.

C. Polymerase Chain Reaction (PCR)

- All PCRs are performed as following standard protocol and optimization are done as and when necessary.
- PCR composition

	Working [C]	Final [C]	Volume [µl]
H20	-	-	15.7
10x Buffer	10x	1x	2.0
dNTP	2.5mM	.1mM	0.8
Forward primer	20µM	.2µM	0.2
Reversed primer	20µM	.2µM	0.2
DNA polymerase	2units/µl	.2 unit	0.1
DNA diluted			1.0
			20.0

- PCR conditions

Initial Denaturing	1 cycle	at 95°C for 5 minutes
Three-step amplification	35 cycles	at 95°C for 10 seconds
		60°C for 30 seconds
		72°C for 30 minutes
Final extension	1 cycle	at 72°C for 10 minutes

D. Reverse transcription and PCR (RT-PCR)

- Reversed transcription-polymerase chain reaction (RT-PCR) was done using QIAGEN OneStep RT-PCR kit.

- RT-PCR composition

	Working [C]	Final [C]	Volume [µl]
RNase-free water	-	-	Variable
5x RT-PCR Buffer	5x	1x	10.0
DNTP	10mM	1.6mM	2.0
5x Q-solution	5x	1x	10.0
Forward primer	20µM	.2µM	1.5
Reversed primer	20µM	.2µM	1.5
RT-PCR enzyme mix	-	-	2.0
RNA template (1pg-2µg)	-	-	Variable
			50.0

- RT-PCR conditions

Reverse transcription	1 cycle	at 50°C for 30 minutes
Initial Denaturing	1 cycle	at 95°C for 15 minutes
Three-step amplification	35 cycles	at 95°C for 30 seconds
		60°C for 30 seconds
		72°C for 60 seconds
Final extension	1 cycle	at 72°C for 10 minutes

- Thaw all reagents and keep them on ice.
- Prepare the reaction-mix according to RT-PCR composition
- Add template RNA not more than 2 µg per reaction was added
- Start the RT-PCR while keeping reaction tube on ice.
- Place the reaction tube in thermocycler only when it has reached 50°C.

NB: Generally RNase inhibitor is not necessary. RNase-free environment was maintained during isolation and RT-PCR setup.

APPENDX 2.2

Molecular Cloning Protocol

A. Preparation of chemically competent cells

- Grow *E.Coli* of interest overnight in LB agar plate without antibiotics
- Pick one colony and grow in 5 ml of LB without antibiotics for 2-3 hours
- Transfer to 50 ml of LB without antibiotics and grow until OD_{600} becomes ≈ 5.0
- Centrifuge at 5000 rpm for 15 minutes at 4°C.
- Discard the supernatant and resuspend the cells in 10 ml (0.2 x original volume) of ice-cold 0.1M $MgCl_2$.
- Centrifuge at 5000 rpm for 15 minutes at 4°C.
- Discard the supernatant and resuspend the cells in 2.5 ml (0.05 x original volume) of ice-cold 0.1M $MgCl_2$ containing 75% of Glycerol.
- Incubate on ice for exactly one hour.
- Keep away from any disturbance to increase transformability.
- Freeze the cells in 50 µl of aliquots at -70°C.

B. Plasmid DNA extraction by Alkaline Prep.

- Streak a ice flake of stock bacteria (empty vector or vector with insert) on LB agar-plate with appropriate selection antibiotics and incubate the agar-plate in 37°C incubator.

- Pickup single colony and grow in 1.5 ml LB/antibiotic on shaker for four hours at 37°C.
- Collect the supernatant by quick spinning for 30 seconds and discard the supernatant.
- Resuspend pellet in 100 µl lysis solution
- Add 200 µl alkaline-SDS solution. Vortex well and incubate for 5 min on ice
- Add 150 µl high salt solution. Vortex well and incubate for 10 min on ice
- Separate the supernatant by quick spinning for 10 min
- Remove 400 µl supernatant and transfer to new tube prefilled with 400 ml isopropanole. Vortex well and incubate tube on ice for 10 min
- Remove the supernatant by quick spinning for 10 minutes
- Wash pellet with 70% ethanol.
- Vacuum dry pellet for 5 min and suspend in 100 µl 1xTE with 20 mg/ml RNAse A.

NB:- Usually 1-2 µl is enough for digest (high-copy plasmid). keep DNA frozen at -20°C.

Lysis solution:(freshly prepared)	***Alkaline SDS solution:(stable for 1 week)***
7.55 ml H_2O	7.6 ml H_2O
2 ml 50% glucose	2 ml 5% SDS
0.2 ml 0.5 M EDTA	0.4 ml 5 N NaOH
0.25 ml 1 M Tris-HCl pH 8	------
10 ml total	10 ml total

C. Dephosphorylation of expression vector.

Dephosphorylation was normally performed prior to ligation process to prevent the self-ligation of expression vector. It was commonly done immediately after the enzymatic cleavage of plasmid vector.

- 20 pmoles of expression vector (pET-16) was cleaved by Nde I enzyme

- The status of plasmid DNA cleavage by enzyme was monitored by 0.8% agarose gel electrophoresis.
- The Nde I enzyme was heat inactivated at incubation for 20 minutes at 60°C.
- 1 unit of the calf intestine alkaline phosphatase (Fermentus) was added
- The reaction was continued incubating for 30 minutes at 37°C.
- Stop the reaction by heating for 15 minutes at 85°C
- Precipitate the dephosphorylated expression vector by ethanol precipitation method.

D. Ligation of dephosphorylated vector and gene of interest.

Ligation was performed to insert "gene of interest" or "gene fragment of interest" into the cloning vector. Promega T4 DNA ligase was used to ligate the gene fragment of interest in our study. Even though molar ratio of vector:insert varies from 1:3 to 3:1 when cloning the fragment into a plasmid vector, we used molar ratio of vector:insert 1:3 in our experiment.

<u>Calculation of amount of vector and insert</u>

pET-16 size ➔ 5.711 kb

insert size ➔ .732 kb

ng of insert = (ng of vector x kb size of insert)/ kb size of vector

= (200x.732)/5.711

= 25 ng

<u>Composition of ligation reaction</u>

- Set up the following reaction in a sterile microfuge.

Vector DNA - 200ng

Insert DNA	-	25ng
10x Ligase buffer	-	1µl
T4 DNA ligase (weiss units)	-	1 unit
Nuclease free water to final volume of		10 µl

Condition of ligation reaction

Incubate the reaction at room temperature for 3 hours or overnight at 4°C.

E. Heat-shock transformation and selection of correct clone by PCR.

- Thaw *E. Coli* competent cells prepared chemically by $CaCl_2$ method on ice.
- Add 20 ng of pDNA into the competent cells, and mix with gentle flicking and swirling.
- Incubate on ice for 30 minutes. Heat shock at 42°C for exactly 90 seconds. Incubate on ice again for two minutes.
- Add 1 ml of room-temperatured LB without antibiotics. Incubate at 37°C, shaking at 250 rpm for one hour.
- Place two LB agar plates with ampicillin at 37°C.
- Spin at 13,000 rpm for 90 seconds in table-top microfuge. Pour off supernatant and add 100 µl room-temperatured LB, and resuspend pellet with glass rod.
- Plate 90 µl of the resuspended cells on LB/Ampicillin plate pre-incubated at 37°C, and the remaining resuspended cells (10µl) on another plate.
- Incubate the plates overnight at 37°C.

- Pick single colony and grow in 1.5 ml microcentrifuge tube containing 1 ml of LB/ampicillin. (grow for as many as possible)
- Incubate at 37°C, shaking at 250 rpm for 4 hours.
- Transfer 100 µl each of bacteria into 8 wells 200µl PCR strip and centrifuge at 2400 rpm for 10 minutes at room temperature.
- Discard the supernatant and add 50 µl of sterile water to each well and mixed well by vortexing.
- Incubate the resuspended bacteria at 100°C for 15 minutes.
- Proceed standard PCR using these bacterial lysates for checking of correct expression vector.

NB:- LB/Ampicillin plates will have 1:1000 Ampicillin at 100 mg/ml.
Normally 2µl of baterial lysate is enough for PCR amplification.

F. Expression and purification of expressed protein.

Small-scale expression is normally carried out to test if expressed proteins are soluble. If expressed proteins are not soluble, purification under denatured condition will be applied for purification. Large-scale, about one-liter culture, is carried out for more protein, and purification process is done in column type instead of batch approach.

- Two 15 ml centrifuge-tubes are used to grow 1:200 dilution of *E. Coli* with expression vector in LB media where appropriate antibiotics are added.
- Incubate at 37°C, shaking at 250 rpm for four hours.
- Read the OD_{600} of the cultures every 30 minutes after four hours.
- When the OD600 reaches 0.5 – 0.6, one of tubes will be induced with 0.1mM IPTG.

- The induction will be carried out for another two hours.
- The pallet collected after centrifugation will be lysed without using Urea or Guanidine-HCl (Lysis buffer;50mM Na2HPO4 pH 8.0, 0.3M NaCl, 1mM PMSF; or mechanically or freeze-thaw cycles)
- The resultant supernatant and pallet of induced and uninduced pallets will be analyzed on SDS-PAGE.

In this experiment, we found that the expressed protein is in insoluble form so that strong denaturant (8M Urea) was used to denature the expressed protein and Ni-NTA purification will proceed.

- Resuspend pellet of 10ml bacterial culture in 1ml lysis buffer (8M Urea, 50mM Na2HPO4 pH 8.0, 0.3M NaCl, 1mM PMSF)
- Sonicate the resuspended pallet on ice 3 x 20 seconds and centrifuge at maximum speed for 15 minutes at 4°C.
- Transfer supernatant into clean tube containing 50µl Ni-NTA beads (QIAGENS) which was equilibrated with equilibration buffer (8M Urea, 50mM Na_2HPO_4 pH 8.0, 0.5M NaCl)
- Add the crude extract to the beads and incubate for 1 hour at 4°C while shaking
- Centrifuge at 3500 rpm for 3min and remove unbound material.
- Wash thrice with wash buffer (8M Urea, 50mM Na_2HPO_4 pH 8.0, 0.5M NaCl)
- Wash thrice with wash buffer containing 10mM Imidazole.
- Elute thrice 100µl of elution buffer (8M Urea, 20mM Tris pH 7.5, 100mM NaCl , 250mM Imidazole)
- Run PAGE-SDS gel for crude extract, wash and elution fractions.

APPENDX 2.3

Protein Protocols

A. Protein extraction from tissues

The membrane proteins are extracted using the simple concept of water-insolubility. This concept is used for extraction of membrane proteins from different tissues of mouse and brains of different species.

- The frozen tissue is cut into small pieces by scalpel.
- Homogenization is carried out by using “motor and pastel method” followed by syringe and needle (started from 19G and gradually reduced in different bore sizes until 27G).
- Homogenized tissues are resuspended in 1xPBS containing a cocktail of proteinase inhibitors (5µg/ml leupeptin, 5µg/ml approtinin and 1mM PMSF).
- The water-insoluble pallet was separated by centrifugation at 12,000xg for 10 minutes at 4°C.
- Repeated washings with 1x PBS containing cocktail of protein inhibitors were done until proteins were not detected in wash buffer using BioRad protein assay.
- The water-insoluble protein was dissolved in 1xPBS containing 6M urea together with 1 mM PMSF and incubated on shaker for 15 minutes at room temperature.
- The urea-soluble protein was separated using centrifugation at 12,000xg for 30 minutes at room temperature.

- The protein concentration was measured using BioRad protein assay and aliquoted proteins were kept in -80°C freezer for future use.

B. Denatured Protein Gel Electrophoresis (SDS-PAGE)

- SDS-PAGEs (denature protein gel electrophoresis) were used to separate protein according to protein molecular weights. It was widely used in protein separation, immunoblotting and 2-dimentional gel electrophoresis. Laemmli's method was applied in SDS-PAGE experiment.
- Gel composition.

	SEPARATING GEL				STACKING GEL
	10%	12%	15%	20%	4%
H_20	4.845	4.345	3.595	2.345	2.278
1.5M Tris 8.8	2.500	2.500	2.500	2.500	-
0.5M Tris pH6.8	-	-	-	-	0.375
40% acrylamide	2.500	3.000	3.750	5.000	0.300
10% SDS	0.100	0.100	0.100	0.100	0.030
10% APS	0.050	0.050	0.050	0.050	0.015
TEMED	0.005	0.005	0.005	0.005	0.002
	10 ml	10 ml	10 ml	10 ml	3 ml

- BioRad Proteon III was used for SDS-PAGE casting. Except for SDS-PAGE gel in 2-dimensional gel electrophoresis with separating gel polymerized overnight, all other SDS-PAGEs were polymerized in three hours.
- Gel electrophoresis was run at constant current (30mA for single gel ad 45mA for double gels).
- Gels were proceeded for comassie staining, silver staining, immunoblotting accordingly upon completion of SDS-PAGE.

APPENDX 2.4

Animal Handling Protocols (Immunization and Bleeding)

A. Bleeding

Both mice and rabbit bleeding were carried out under sterile conditions.

- For mice bleeding
  - Mice were warmed using electric light bulbs for 1 hour before bleeds.
  - Put the mouse in strainer, rub with ethanol swab at tail where a small slit at tail vein was done.
  - The blood was collected in 1.5 ml centrifuge tubes immediately after blood is oozing out from the slit. Collect about 0.1 ml of blood for pre-bleed, 0.3 ml for subsequent bleeds.
  - Apply pressure to the wound. When the bleeding has stopped (~10 seconds), sterilize the slit with ethanol.
  - Remove the mouse from the restrainer and place it in its cage.
  - Leave the collected blood at RT for 2 hours.
  - Centrifuge at 4°C 10,000g for 10 minutes. Remove the serum, label accordingly and keep in -20°C for future uses.

- Rabbit bleeding
  - Rabbit was given a mixture of Ketamine 50 mg/kg and Xylazine 10mg/kg intramuscularly and wait for 10 minutes.
  - Rub with ethanol on the top middle of the ear where the large vein can be seen.

- Insert the butterfly needle in the vein and collect the about 5 ml of blood for pre-bleed, 20 ml for subsequent bleed and 40 ml for final bleed.
- Remove the needle and apply pressure to the wound. When the bleeding has stopped (~10 seconds), sterilize the ear with ethanol.
- Leave the collected blood at RT for 2 hours.
- Centrifuge at 4°C 10,000g for 10 minutes.Remove the serum, label accordingly and keep in -20°C for future uses.

B. Immunization

Both mice and rabbit immunizations were carried out under standard sterile conditions.

- For mice immunization
  - Rub the abdominal wall with ethanol and pre-prepared antigen preparation (Complete Freund's Adjuvant) was given to mouse intraperitoneally.
  - Sterilize again with ethanol.
  - The subsequent immunizations were done with antigens prepared with Imcomplete Freund's Adjuvant and given at 2-week intervals

- Rabbit immunization
  - Rabbit was given a mixture of Ketamine 50 mg/kg and Xylazine 10mg/kg intramuscularly and wait for 10 minutes.
  - Rub with ethanol on both sides of thigh muscles and pre-prepared antigen preparation (Complete Freund's Adjuvant) was given intramuscularly on both sides.

- The injection sites were sterilized again with ethanol.
- The subsequent immunizations were done with antigens prepared with Imcomplete Freund's Adjuvant and given at 4-week intervals.

APPENDX 2.5

Immunological Protocols

A. Enzyme Linked Immunosorbent Assay (ELISA)

The indirect ELISA is used primarily to determine the strength and/or amount of antibody response in a sample, whether it is from the serum of an immunized animal or the cell supernatant from growing hybridoma clones or patients' sera.

- Prepare the antigen solution at 2μg/ml using PBS.
- Add 50μl (0.1μg) in each well of a 96-well ELISA plate, cover with parafilm and incubate overnight at 4°C.
- Remove antigen solution and wash thrice with PBS-T
- Add 100 μl of blocking buffer (1% BSA in PBS-T). Incubate for 1 hour at RT.
- Remove the blocking buffer.
- Add 100 μl of test samples prepared in 1:100 dilution using blocking buffer and incubate for 1 hour at room temperature (RT).
- Remove the first antibody solution and wash 3 times with PBS-T.
- Add 100 μl/well of HRP-conjugated antibodies directed against first species prepared in PBS-T. Incubate for 1 hour at RT.
- Remove the antibody enzyme conjugate and wash 3 times as before.
- Add 100 μl/well of chromogenic peroxidase substrate OPD (o-Phenylenediamine) and incubate for 15 minutes at RT.

- Add 100 µl/well stop solution (2N H_2SO4) and read absorbance at 490 nm in an ELISA reader.

NB: Positive, negative and blank controls were used in every ELISA procedure. Checker board titration for first antibodies and second antibody conjugate were done in initial optimization. If the OD of test samples were beyond the range of 0.2-1.5, repeat the ELISA with necessary dilutions.

B. Immunoblotting protocol

- The proteins separated by SDS-PAGE gel were transferred onto Nitrocellulose (Amercham) using BioRad Transfer Unit at constant 100 Volts for one hour in cold room.
- The nitrocellulose membranes with transferred proteins are blocked by blocking buffer (3% BSA in TBS-T) for 1 hour at RT with constant shaking.
- Remove the blocking solution, add the primary antibodies diluted in blocking solution (3%BSA in TBS-T), incubate overnight in 4°C with constant shaking.
- Remove the first antibodies and wash thrice with TBS-T
- Add second antibodies conjugated with Alkaline Phosphatase diluted in TBS-T, incubate for 1 hour at RT with constant shaking.
- Remove the second antibodies and wash thrice with TBS-T.
- Add pre-prepared NBT/BCIP (ZyMed Laboratories Inc) and incubate for 30 minutes at RT with constant shaking.
- The reaction was stopped by washing the Nitrocellulose membrane in sterile water

C. Immunohistochemistry

- The frozen mouse kidney was prepared and 5 μm-thick section were cut using Cryostat. Fix the slides with cold acetone for 2 minutes, air dry at RT and proceed for immunostaining.
- Rinse slides 3x in PBS, to remove the tissue-freezing matrix.
- Block endogenous peroxidase activity by incubating the slides in 0.3% H_2O_2 solution in PBS for 10 minutes.
- Rinse slides 3x in PBS, 2 minutes each time.
- Block non-specific binding by incubating with blocking buffer (3% BSA in PBS) for 1 hour at RT in a humidified chamber.
- Dilute the polyclonal antibodies in blocking buffer and apply the diluted antibody to the tissue sections on the slide. Incubate for 1 hour at RT in a humidified chamber.
- Rinse slides 3x in PBS, 2 minutes each time.
- Dilute the Horseradish Peroxidase-conjugated second antibody and apply to the tissue sections on the slide and incubate for 30 minutes at RT in a humidified chamber.
- Rinse slides 3x in PBS, 2 minutes each time.
- Prepare DAB substrate solution by adding 1 drop of DAB chromagen to every 1 ml of DAB buffer.
- Drain PBS from slides and apply the DAB substrate solution. Allow slides to incubate for 5 minutes or until the desired color intensity is reached.
- Wash 3X in water, 2 minutes each time.
- Counterstain slides: (Dip twice in Hematoxylin; Rinse thoroughly in water; Dip twice in Bluing Reagent or dilute ammonia water; Rinse thoroughly in water.)

- Dehydrate through 4 changes of alcohol (95%, 95%, 100% and 100%). Clear in 3 changes of xylene and coverslip.