ASSOCIATION OF OSTEOPONTIN GENE SINGLE NUCLEOTIDE POLYMORPHISM WITH SYSTEMIC LUPUS ERYTHEMATOSUS

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BONAFIDE CERTIFICATE

This is to certify that this dissertation work entitled "**ASSOCIATION OF OSTEOPONTIN GENE SINGLE NUCLEOTIDE POLYMORPHISM WITH SYSTEMIC LUPUS ERYTHEMATOSUS"** is the original bonafide work done by **Dr.V.YOGESWARI,** Post Graduate Student, Institute of Biochemistry, Madras Medical College, Chennai under our direct supervision and guidance.

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ABBREVIATIONS

AP-1	-	Activator protein-1
OPN	-	Osteopontin
SLE	-	Systemic Lupus Erythematosus
ICs	-	Immune Complexes
Mφs	-	Macrophages
TLR	-	Toll Like Receptor
DCs	-	Dendritic Cells
BAFF	-	B cell Activation Factor
TIM	-	T cell Immunoglobulin Mucin
TBMφs	-	Tingible Body Macrophages
MFG-E8	-	Milk Fat Globule Epidermal Growth Factor 8
HMGB1	-	High Mobility Group Box 1protein
SIBLING	-	Small Integrin-Binding Ligand N-linked Glycoprotein

AER	-	Albumin Excretion rate
ACR	-	Albumin Creatinine Ratio
IL	-	Interleukin
TGF-β	-	Transforming growth factor-β
PCR	-	Polymerase Chain Reaction
PKC	-	Protein Kinase C
EDTA	-	Ethylene Diamine Tetra Acetic Acid
DNA	-	Deoxyribonucleic acid
RFLP	-	Restriction Fragment Length Polymorphism
LN	-	Lupus Nephritis
FcγRIIb	-	Fc Receptor 2 b(CD 32)
IFNα	-	Interferron alpha
TNF	-	Tumour necrosis factor
MLR/lpr	-	Murine model of Murphy Roths Large/lymphoproliferative strain
IRF-5	-	Interferon regulatory factor-5
SHP-1	-	Src homology region 2 domain-containing phosphatase-1

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INTRODUCTION

Systemic lupus erythematosus (SLE) is a prototype autoimmune disease and is characterized by the presence of autoantibodies which target self antigens. SLE is commoner in women than men by nearly ten times, and is typically seen in women of child- bearing age[1].Genome screening studies done in twins have shown the significance of genetic factors and multiple loci of interest [2]. A number of candidate genes susceptibility to SLE have been identified. The occurence of the disease in monozygotic twins is approximately 25–50%[3]. This clearly suggests that genetic factors do play a key role in the pathogenesis of the disease. Environmental factors like sunlight, certain drugs, infections may precipitate the disease in susceptible individuals. Abnormalities in the activation and maturation of T and B lymphocyte leads to the production of autoantibodies, which cause multiple organ damage. Lupus nephritis is the most frequent and potentially serious complication of SLE.

Osteopontin(OPN) or early T lymphocyte activation 1(Eta-1) a member of T helper 1 cytokine plays a key role in the pathogenesis of SLE[4] and Lupus nephritis. Osteopontin gene is located on the long arm of chromosome 4, consisting of 7 exons. OPN mediates its immunoregulatory effects by enhancing the proinflammatory T-helper1 cell response and by inhibiting the T- helper 2 responses [5,6]. It also

stimulates B lymphocytes to express multiclone antibodies. Increased serum levels of Osteopontin is detected in patients with SLE [7]and increased OPN expression is found in SLE patients with lupus nephritis[8,9]. A number of haplotype analysis shows the association of osteopontin gene polymorphisms and SLE. Similarly, Wong et al[10] has found significantly higher plasma OPN levels compared to healthy controls.

In this study, the association of osteopontin gene in exon 7 at position 9250 C→T single nucleotide polymorphism (SNP) with SLE was investigated.

REVIEW OF LITERATURE

Systemic lupus erythematosus (SLE) is a prototype autoimmune disease affecting almost all organs and tissues[11].

The term 'lupus'was derived from Latin meaning 'wolf' which was initially used to describe the classical skin lesions evincive of a 'wolf's bite'. The term 'lupus erythematosus' was coined by a Viennese physician named Ferdinand von Hebra in 1856 [12]. Although the term was coined in the 19th century, nearly a 100 years lapsed to conclude that SLE is a systemic disease of autoimmune etiology. SLE is characterized by hyperactive T and B cells, auto-antibody production, and immune complex(IC) deposition [13] in multiple organs causing end organ damage. Lupus nephritis is one of the most common clinical manifestation affecting nearly 50%[14] of SLE patients.

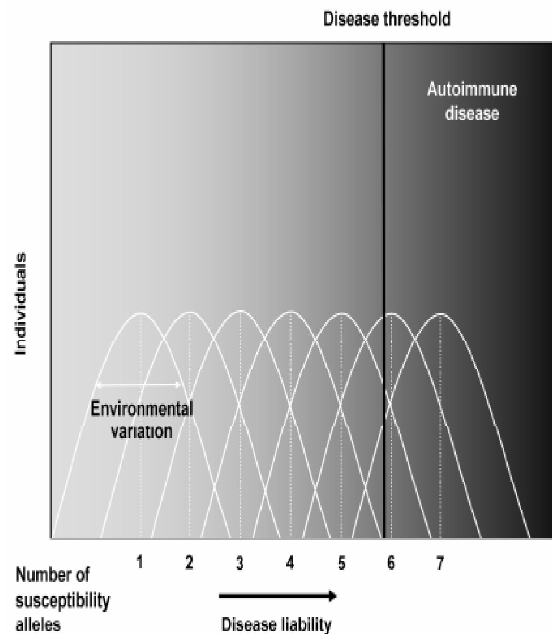
Epidemiology :

SLE is 9 times commoner in women of child-bearing age group(15 to 35) than in men, and more common in non-European descent[15,16]. A study done in Delhi, India showed a point prevalence of 3 per 100,000[17] which is much lower when compared to 12.5 per100,000 adults in England[18], 39 per 100,000 in Finland[19] and 124 per 100,000 in USA[20]. The median age of onset of SLE in India is 24.5 years and sex ratio (F:M) is 11:1[21].

Etiology :

The etiology of SLE is complex and multitude involving genetic factors, environmental factors, female gender, socioeconomic status, ethnicity and immunological factors. Rhodes et al[22] has proposed that number of these factors occurs either simultaneously or step by step over a time period for the disease to develop, which occurs when a threshold of genetic and environmental susceptibility effects is reached.This is shown in Figure 1.

DISEASE MODEL OF A AUTOIMMUNE DISEASE



For people with numerous susceptible genes a minor environmental trigger might predispose to the disease and on the other

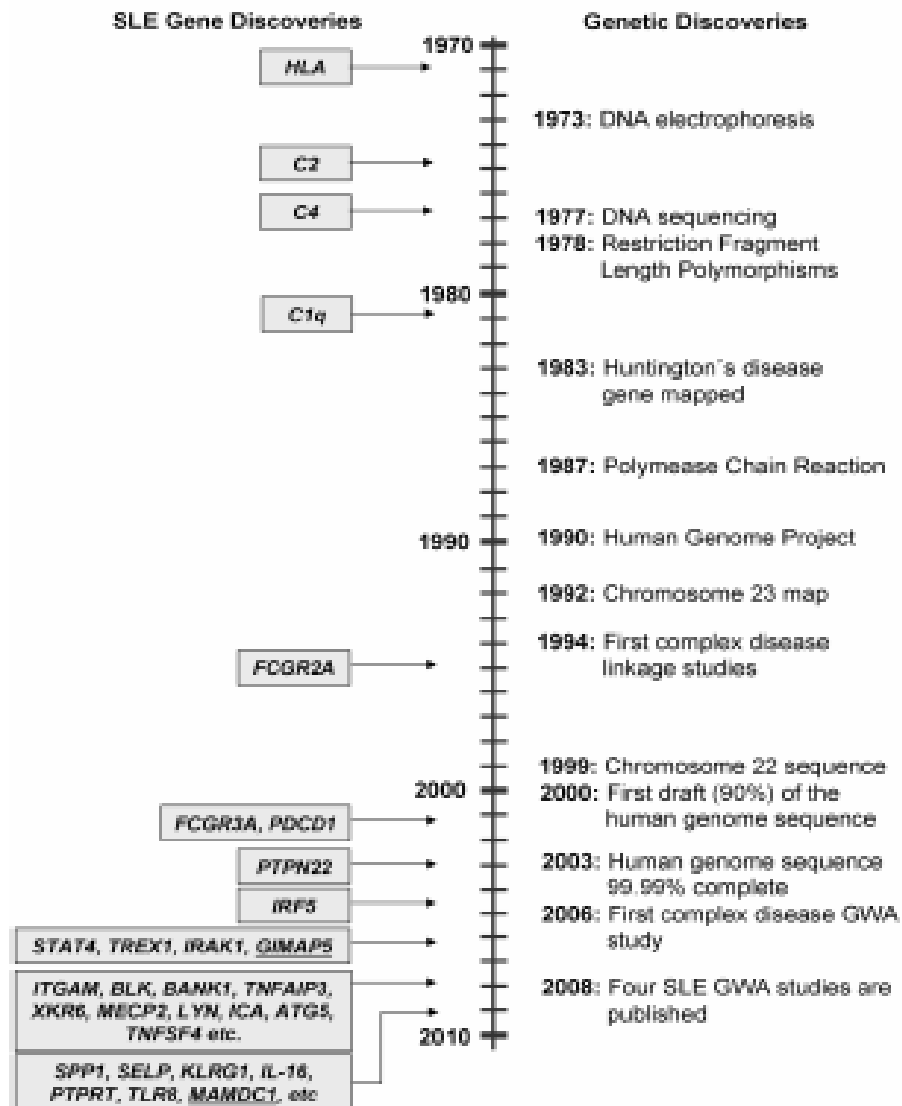
end even strong environmental trigger might not predispose to the disease in people with minimal genetic risk.

Genetic factors:

The fact that SLE has strong familial predisposition is supported by studies done in twins which show that the occurrence of the disease was about 25-50% in monozygotics and nearly 5 % in dizygotics[23]. A number of candidate genes susceptible to the development of SLE have been identified .Figure:2 shows the susceptible genes in SLE. Various studies have shown that Human Leucocyte Antigens -A1, B8, and DR3 and Early Complement Component deficiencies increases the risk of developing SLE. At least 35 candidate genes have been detected in patients with SLE[24] . Single nucleotide polymorphisms (SNP) in the various immune regulatory substances are associated with increased risk of SLE . These include SNPs in the interferon pathway *IRF5, IRF7*, and *IRF8* resulting in increased levels of *IRF5, IRF7*, and *IRF8* transcript and protein expressions[25].Dysregulation in the expression of genes in the IFN pathway is seen in more than 50% of SLE patients[26].Others include polymorphisms in the following genes, *PTPN22*,*FCGR2A, FCGR2B*, and *FCGR3A* ,*ITGAM* ,*STAT4* [27,28].

FIGURE-2

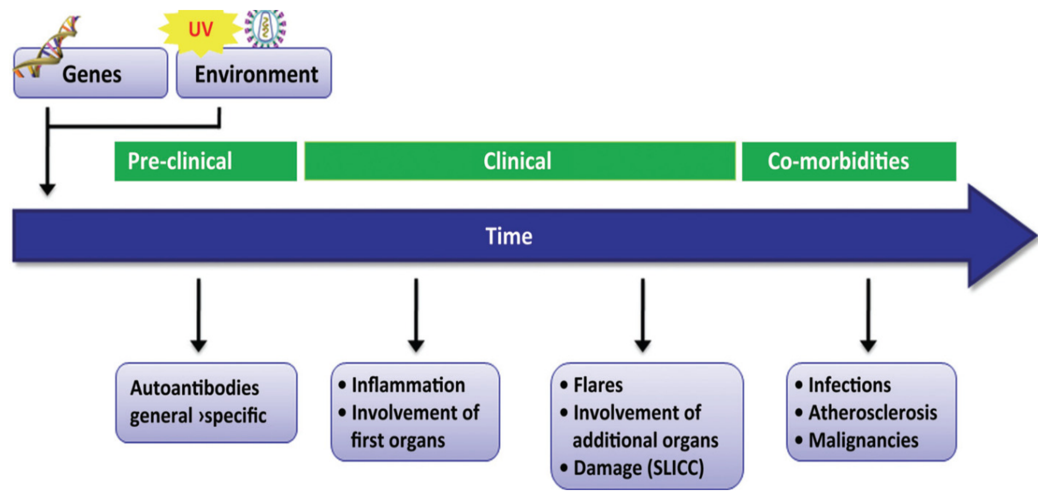
GENE DISCOVERIES IN SLE



Environmental factors:

Smoking and exposure to sun light (UVlight) are important factors implicated in the disease[29] (Figure:3).

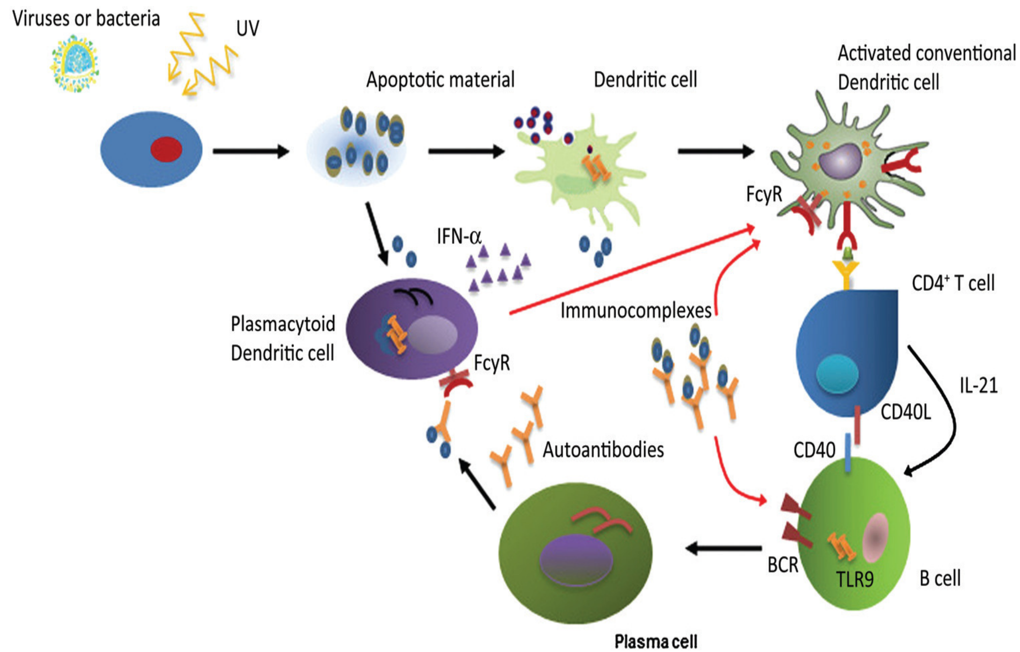
NATURAL HISTORY OF SYSTEMIC LUPUS ERYTHEMATOSUS.



Drug induced SLE occurs due to DNA hypomethylation [30].Some patients with Ebstein Barr virus infection are prone to develop SLE due to molecular mimicry between EBV nuclear antigen 1 and the common lupus autoantigen Ro[31].The Antigen presenting cells take up Environmental and Self antigens, process them into smaller peptides and present them to T cells. This activates the T cells which in turn stimulates the B cells to produce pathogenic autoantibodies (Figure:4).

FIGURE 4

IMMUNE CELLS IN THE PATHOGENESIS OF SLE.



Hormonal factors:

SLE is predominantly a disease affecting women, the reason attributed been the gene CD40 located in X chromosome [32] . Increased serum levels of oestrogen proliferates B cells and hence increase the production of antibodies.[33,34,35,36]Oestrogen is also found to increase the cell surface expression of CD40 ligand in cultured T cells from SLE patients escalating calcineurin mRNA levels [37,38]. These effects appear to

be distinctive to patients with SLE, signifying that T cells in SLE patients are more responsive to oestrogens. On the other hand serum levels of androgens were found to be inversely related to SLE disease activity[39].

Women with SLE usually tolerate pregnancy without disease flares. However, a small proportion develops severe flares requiring aggressive glucocorticoid therapy or early delivery and a positive link between pregnancy outcome and the status of disease at conception has been noted[40]. Treating patients with dehydroepiandrosterone has shown some clinical benefit[41].

Pathogenesis:

The pathogenesis of SLE is linked to autoimmunity directed against native cellular components. The three important causes of pathogenesis include

i) impaired removal of apoptotic bodies &dysregulation of apoptosis.

ii) dysregulation of T and B cells.

iii) dysregulation of expression of certain cytokines.

Multiple susceptible genes have been discovered. The major histocompatibility complexes [42] present antigens to T-cells,which elicits an immune response against self-antigens. This results in deposition of immune complexes and tissue damage.

Autoantibodies are the prime cause of tissue injury in SLE patients. The role of the immune system in the SLE development was studied using mouse strains in the last few years.Based on these findings,dysregulation of immune system has been divided into two events :

(I) systemic production of autoantibodies which leads on to formation of antinuclear antibodies and anti-double stranded DNA antibodies in serum.

(II) local production of autoantibodies against target organs resulting in tissue damage. Defective cellular as well as humoral immunity play an important role in the causation and development of lupus.

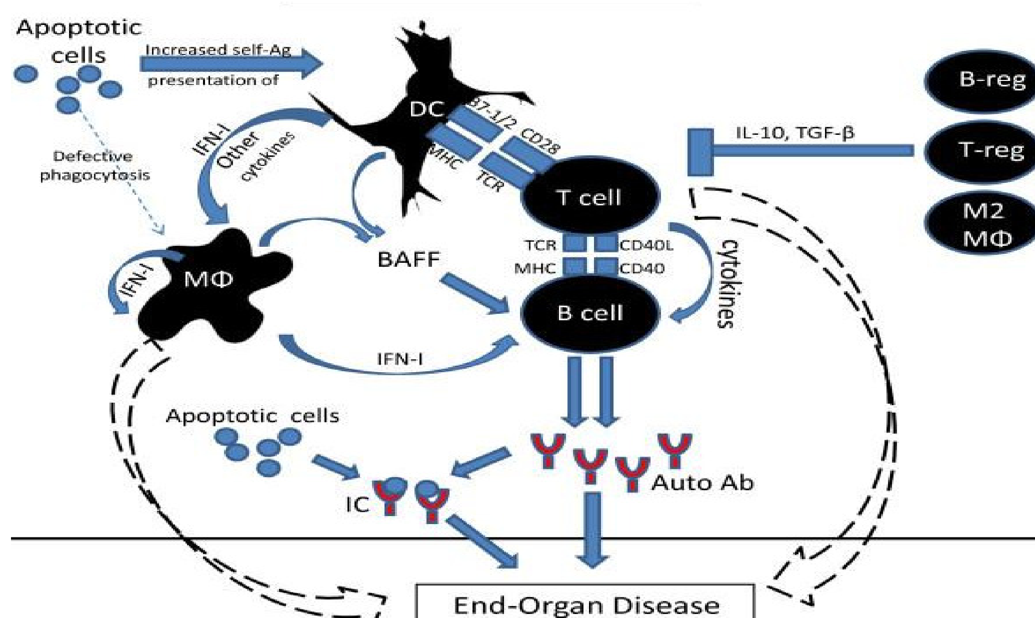
Elicting an immune responses against self antigens is characteristic of SLE. The self antigens present on the surface of apoptotic blebs are engulfed by dendritic cells which present them to T cells resulting in their activation. These T cells secrete interleukin 10 (IL10) and interleukin 23(IL23),which stimulate B cells to produce autoantibodies. There is an another way of production of autoantibodies not depending on T cells through the both B cell antigen receptor (BCR) and TLR signalling.

Defects in the apoptosis leads to persistence of endogenous nucleic acids.This induces the production of IFNα which augment the

autoimmune process by destroying self-tolerance (Figure:5). The immune complexes following their deposition amplify and maintain the inflammatory events. The antibody complexes damage the critical vessels, such as the glomeruli of the kidney resulting in lupus nephritis. Researchers have identified the individual genes and their corresponding inflammatory mediators produced.One such protein is osteopontin (OPN), overexpressed in SLE. Each protein is linked to autoimmune disease and research is being conducted to discover drugs against those links[43,44] .

FIGURE 5

SYSTEMIC PROCESS IN SLE



Autoantibodies:

The core of the disease is immune dysregulation with subsequent formation of autoantibodies. B lymphocytes produce antibodies against self antigens which are present in the nucleus, cytoplasm, and soluble molecules like immunoglobulins and clotting factors. The presence of antinuclear autoantibodies (ANA) is the immunological hallmark of SLE. About 98% of SLE patients show positive for antinuclear antibodies (ANA) which is used as a screening test. Approximately 60% of patients show positive for anti-DNA antibodies [45] which is used as a specific test. The Sm antigen i.e., small nuclear ribonucleoprotein (snRNP) and anti-dsDNA antibodies are specific to SLE[46]. Anti-Sm antibodies directed against core proteins is constantly produced, whereas anti-DNA antibodies produced against DNA found over the basement membrane of glomerulus, antigens like C1q, nucleosomes, heparan sulfate, and laminin[47] varies.

Anti-DNA antibodies reflect disease activity[48], being pathogenic in some people . Hence, these are used as disease marker. These antibodies cause glomerulonephritis both in healthy mice and severe combined immunodeficient mice[49,50]. Not all anti-DNA antibodies cause nephritis as shown by the previous studies where they are found to be negative in active nephritis cases and in some they are positive with no renal

impairment.The differences in anti-DNA antibodies pathogenicity can be due to different isotypes, varying ability of fixing complement. Studies in SLE have shown that certain clinical features in SLE is linked with certain antibodies in serum. For instance the anti-ribosomal P antibodies are associated with psychosis and anti-Ro antibodies with congenital heart block. Deposition of immune complex with subsequent activation of complement system is a postulated mechanism of autoantibodies mediated tissue damage. This is supported by the association finding of decreased complement levels with vasculitis in SLE. The autoantibodies also damage the tissues directly through cytotoxic reactions.

Disturbed apoptotic cell clearance :

Casicala-Rosen *et al*. demonstrated that target autoantigens are found in the blebs of apoptotic cells[51]. Apoptosis may be increased by exposure to UV light, certain infections and drugs. Various studies have revealed that few T cells in SLE patients increase the production of the oncogene bcl-2, thereby increase the survival of the dying cells by attenuating apoptosis. This results in persistence of autoreactive T cells and progression of the autoimmune disease.

Inefficient removal of apoptotic bodies can be one of the postulated mechanism in the causation of SLE.This is supported by the research

work conducted in mice strains in which the genes coding for DNase I, serum amyloid protein P (SAP), serum immunoglobulin M,and tyrosine kinase c-mer,normally involved in the removal of apoptotic bodies are found to be defective and associated with increased production of anti-nuclear antibodies[52].

The C1q-deficient mice was found to have defective engulfment of apoptotic bodies[53] by peritoneal macrophages and they develop proliferative glomerulonephritis. This finding reveals that the complement system and FcR are involved in the clearance of apoptotic substances.

Apoptosis regulators defects:

Apoptotic cells in the earlier stage express signals such as phosphatidylserine (PS), to the outer leaflet. Phosphatidylserine (PS) is the efficient signal to mediate apoptotis by specialized phagocytes called tingible body macrophages[54] (TBMφs). Chemo-attractant factors or "find me signals" such as ribosomal protein S19 are secreted in situ for phagocytosis. "Find me signals" such as Tyro-3, Axl, and Mertk (TAM) receptor tyrosine kinases are capable of binding to apoptotic cells by vitamin K-dependent factors, growth arrest specific protein 6 and protein S which acts like bridging molecule[55,56]. This has been studied in mice

that those lacking Mertk was found to have autoimmune response due to defective removal of apoptotic bodies. Tisch and colleagues found the important of Mertk on T-cell central tolerance where they increase the negative selection of autoreactive T cells.

MFG-E8 (milk fat globule epidermal growth factor 8) [57] acts as the connecting molecule between dying cells and phagocytic cells. Low levels of MFG-E8 has been reported in some pediatric and adult SLE patients [58]. Subsequently the another molecule involved in the clearance was identified which is the T-cell immunoglobulin mucin (TIM) gene. Its function include the regulation of tolerance and removal of apoptotic bodies by binding to phosphatidylserine (PS) found on the surface of dying cells[59]. Mice deficient in TIM was found to have autoantibodies directed against dsDNA with increased activation of T and B cells[60].

High mobility group box 1 (HMGB1) protein was found to be increased in SLE patients[61].It is made of two DNA strapping domains (i.e,) HMG boxes A and B. HMGB1 is formed during late apoptosis and necrosis[62] and function of this protein is to prevent the double stranded DNA, single stranded DNA, distorted DNA from undergoing apoptosis by interfering in the binding of "find me signals" to phosphatidylserine. The exact mechanism of this protein in SLE has yet to be studied.

Microparticles (MPs) are vesicles derived from plasma membrane which engulf cytokines, growth factors, acute phase proteins, DNA and RNA. Plasma MPs was found to be increased in SLE patients [63,64]. Microparticles has the capacity to induce B cells by binding to their immunoglobulin receptors on the surface of B cells and increases the longivity of DNA and RNA-specific autoreactive B cells. Normally these microparticles behave like autoadjuvants in the control of both central tolerance and activation of peripheral B-cells. They express increased level of phosphatidylserine on their outer leaflet and restrain the phagocytosis of normal apoptotic cells resulting in accumulation of apoptotic bodies and aggravate the autoimmune response.

Neutrophil extracellular traps (NETs) consisting of chromatin networks function to detain and exterminate invading microorganisms. SLE patients were found to have antibodies directed against NETs, myeloperoxidase and proteinase-3. Type I interferons [65] in SLE induces the expression of neutrophils and the formation of NETs. Immediately after the function of NETs is completed it is removed to avoid the presentation of self-antigens.

The function of the enzyme DNase I is to break the phosphodiester bonds between nucleotides resulting in breakdown of chromatin during

apoptosis.Some studies have shown significant low level of DNase I in serum and urine of mice with lupus when compared to normal[66].Further the mice lacking the enzyme presented with lupus like syndrome and clinical manifestations like nephritis. The delayed clearance in SLE was found due to lack of this enzyme[67].This leads on to use DNase I as a enzyme replacement therapy in those SLE patients lacking the enzyme[68,69].

Dysregulation of B cell and T cell:

SLE is characterised by a abnormal B cells, T cells and monocytic lineage cells leading to activation of polyclonal B cells, increased antibody producing cells, increased immunoglobulins, autoantibody production, and formation of immune complexes in serum as well as in tissues. These immune cells release diverse cytokines and various inflammatory mediators, thereby causing progression of the disease. This results in increased activation of the leukocytes as well as the autoantibodies production which finally mediate end organ damage,most commonly affected is the kidneys.

Systemic autoimmunity; Adaptive immune cells: The role of B cells in SLE pathogenesis was studied by Shlomchik and colleagues[70].In one of their study they found that the mice deficient in B cells do not have autoantibodies and showed an absence of disease concluding the role of B cell in the production of autoantibodies in the pathogenesis of lupus . In a different study, Shlomchik and his associates[71] showed that B cells produce disease independent of autoantibodies. B cell also reciprocally activate T-cell which in turn stimulate B cell to produce autoantibodies .Activated T cells secrete cytokines which causes inflammatory response and organ damage. The most common cytokine produced by the T helper1 cells is IFN-γ, which augments the self antigen presentation with production of antinuclear autoantibodies.

B cells secrete cytokines which mediate inflammation like interleukin-6 (IL-6), interferon-gamma (IFN-γ) as well as anti-inflammatory cytokine IL-10. Kumar and colleagues[72] found that the defect in the negative selection of autoreactive B cells in their immature stage is due to polymorphism of *Ly108* gene, located at *Sle1* locus, resulting in loss of B-cell tolerance .B cell autoimmunity in SLE is also the result of increased B-cell signaling by CD19 particularly of mature peripheral B cells and lack of inhibitors like SHP-1, Lyn and FcγRIIb[73] which attenuate the B-cell receptor (BCR) signaling.

Toll-like receptors (TLRs) participate in the additive stimulation of B cells by both BCR and TLR signaling. Activation of both BCR and TLR signaling pathways[74] is due to simultaneous involvement of BCRs which are specific for DNA in apoptotic material and TLR9 on B cells . In the study conducted in mouse, binding of a synthetic DNA to TLR9 results in progression of nephritis and elevated levels of antibodies to ds-DNA [75]. TLR9-deficient autoimmune mouse models are associated with low level of antibodies which are directed against DNA and chromatin. B-cell activation factor (BAFF) is essential for the survival of B-cell. Transgenic mice with BAFF was found to contract a disease akin to SLE with increased number of peripheral B-cell producing autoantibodies[76]. B-cell activation factor promote the continued existence of B cells in the germinal center and in the periphery by removing the checkpoint at the level of the T1 transitional stage. These findings leads on to development of a soluble receptor specific for BAFF, TACI-Ig, which can be used in the treatment of lupus in murine[77].

Cell mediated immune cells:

The number of monocytes and macrophages are found to be decreased in the exudates arising from inflammation of SLE patients and associated with reduced capacity in phagocytosis of apoptotic cells . The

defective phagocytosis contribute to defective removal of apoptotic debris, resulting in autoimmunity and also cause elaboration of proinflammatory cytokines and chemokines. Kilmon and colleague[78] studies reveal the presence of regulatory molecules such as IL-6 and CD40L produced by the macrophages which keeps check on the proliferation of B-cell was found to be decreased in mice with lupus. Dentritic cells (DCs) contribute to pathogenesis of SLE by producing proinflammatory cytokine, IFN-α. IFN- α stimulate B cells to produce immunoglobulin G targetting soluble autoantigens [79] and also upregulate production of B cell activation factor leading to increased longivity of autoreactive B cells and plasmablasts[80]. This results in a pathogenic cycle, resulting in increased production of autoantibodies.

Tissue autoimmunity:

This is mediated by (i) ICs and infiltrating cells

(ii) resident cells

Immune complexes (ICs) produced by antibodies against dsDNA get deposited in the kidney, which initiates the inflammatory response in the target organ by Fc gamma receptor and complement dependent mechanisms .There is an antigen similarity between DNA and antigens found in the kidney like laminin, alpha-actinin, and heparan sulfate

which results in cross reactivity of antibodies against DNA to renal antigens. Heymann and colleagues [81] found the role of both cytotoxic T (Tc) cells and T helper cells in the causation of glomerulonephritis. This results in local release of cytokines, IFN-γ and IL-12 [82] and chemokines with recruitment of inflammatory cells depicting type IV hypersensitivity-reaction.

Cytokines:

The function of cytokines is to regulate systemic inflammation and immunomodulation and hence they play an important role in SLE pathogenesis. IL-6, TNFα, IFNα, and BLyS are candidate biomarkers for SLE and are been evaluated for potential target oriented therapies. Serum Interleukin-6 and TNF α levels are found to correlate with the disease activity and response to therapy in SLE [83]. Autoreactive T-cell clones in SLE generate huge amounts of IL-6 and TNF α, and thereby triggering B-cell activation and autoantibody production[84].Increased expression of IL-6 was detected in renal biopsy specimens in lupus nephritis and its urinary levels was also found to be raised[85, 86,87]. About 52% of SLE nephritis patients[88] was found to have upregulated TNFα gene expression with local production of TNFα .

Interferons prevent apoptosis and increase the proliferation of B-cells[89]. The primary gene implicated in IFNα secretion which is to be associated with the propensity to develop SLE was transcription factor IRF5 gene [90]. The risk loci of IRF5 is associated with the generation of autoantibodies to dsDNA and RNA-binding proteins[91]. Increased levels of type I interferon-regulated chemokines are used as markers of disease

activity and also found to be associated with the development of nephritis[92], [93].

B-lymphocyte stimulator (BLyS) levels are high in nearly half of SLE patients and is also found to parallel disease activity. Recently, novel therapeutic drugs targeting BLyS are been developed.

IL-10,a T helper 2 cytokine acts as a powerful activator of B cell production and differentiation and hence polyclonal B cell activation in SLE. IL-10 transcript expression and serum levels were significantly raised in SLE patients compared with controls and further paralleled disease activity[94],[95,96,97] .

Mechanism of tissue injury:

Accumulation of immune complexes and activation of complement pathways cause organ damage through cytokines production. Normally these accumulated complexes are removed by Fc gamma receptors and complement receptors.Defective removal of complexes results in their accumulation in the target organs resulting in recruitment of inflammatory cells, generation of free radicals, secretion of proinflammatory cytokines, and initiation of the coagulation cascade .The outcome of the above events leads to target organ damage.

Neuropsychiatric SLE (NPSLE)was one of the manifestation of autoantibody dependent tissue injury . Mutations in the DNA repair enzyme 3’ exonuclease 1 (TREX1) is associated with the defective breakdown of single stranded DNA which results in augmentation of immune mediated response causing tissue injury.

Lupus nephritis:

Nephritis is one of the common and lethal presentation present in nearly half of the SLE patients. The International Society of Nephrology revised the World Health Organization classification of lupus nephritis[98].

International Society of Nephrology/Renal Pathology Society 2003 classification of systemic lupus erythematosus (SLE) nephritis

Class I

Minimal mesangial

Normal histology with mesangial deposits

Class II

Mesangial proliferation

Mesangial hypercellularity with expansion of the mesangial matrix

Class III

Focal nephritis

Focal endocapillary ± extracapillary proliferation with central subendothelial immune deposits and minimal mesangial expansion

Class IV

Diffuse nephritis

Diffuse endocapillary ± extracapillary proliferation with disseminated subendothelial immune deposits and mesangial alterations

Class V

Membranous nephritis

Thickened basement membranes with diffuse subepithelial immune deposits, may occur with Class III or IV lesions and is otherwise called as mixed membranous and proliferative nephritis

Class VI

Sclerotic nephritis

Global sclerosis of almost all glomerular capillaries

In situ production of inflammatory cytokines like interferon alpha (IFNα) and tumour necrosis factor (TNF) mediate the inflammation and injury in the renal cells. Three established theories that lead to the placement of immune complexes over the basement membrane of glomeruli and result in the heterogeneity of disease [99].These includes,

a) deposition of preformed serum ICs[100]

b) increase affinity of autoantibodies for the native antigens present in the glomerulus.Native antigens include heparin, laminin and annexin II.

c) antibodies to double stranded DNA and to chromatin have affinity towards nucleosomes and the DNA found in the matrix of glomerulus[101].

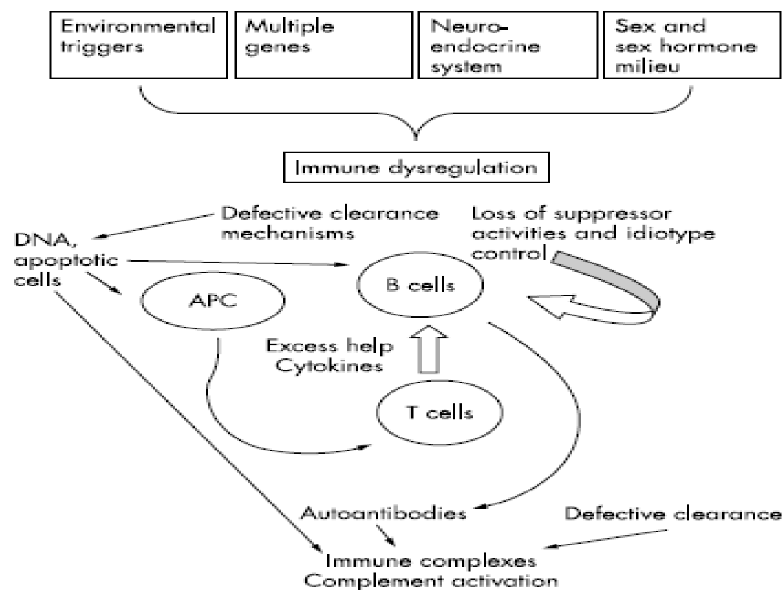
The nucleosomes present in the injury site can arise both from the plasma and from necrotic debris of the glomerular cells. The nucleosomes and DNA present in the plasma gets attracted towards the glomerular basement membrane because of charge to charge interactions and act as source of antigen for autoantibodies. All these leads to repression of the gene for the enzyme, DNAse I which normally degrades DNA with accumulation of nucleosomes in the glomerulus[102]. These accumulated materials further activate immune mediated response with simultaneous induction of FcγRs and Toll-like receptors (TLRs) and complement cascade [103].

Figure 6:

Shows an overview of multiple factors involved in the causation of SLE and their probable effects on the immune system. Defective clearance of

apoptotic materials leads on to presentation to B cells which produces autoantibodies against self-antigens such as heparin ,laminin in the kidney. This leads to accumulation of immune complexes (ICs) in the glomerulus which induces the activation of complement cascade and proliferation of native mesangial cells (MC) and endothelial cells (EC). Activated renal cells (MC and podocytes), and inflammatory cells such as macrophages and dendritic cells (DCs) form free radicals resulting in additive tissue damage affecting both the tubular and the glomerular cells. **FIGURE 6**

SCHEMATIC VIEW OF AUTOANTIBODY FORMATION



Clinical features:

SLE may involve one or several organ systems; over time, additional manifestations may occur. Systemic symptoms, particularly fatigue and myalgias/arthralgias, are most common and are present most of the time. Most patients experience relapses and remissions, but permanent complete remissions are rare. The clinical features of SLE are diverse involving the following systems,

1.Mucocutaneous features

Lupus dermatitis can be classified as discoid lupus erythematosus (DLE), systemic rash, subacute cutaneous lupus erythematosus (SCLE). The classical SLE rash is a photosensitive, slightly raised erythema, which is sometimes scaly,mostly over the face .It predominantly involves the cheeks and nose depicting the "butterfly" rash. It may also involve the ears, chin, V region of the neck, upper back, and extensor surfaces of the arms. Clinical exacerbation of the rashes might be associated with flares of the disease.

2.Musculoskeletal involvement

Most SLE patients have discontinuous polyarthritis, varying from mild to severe, characterized by soft tissue swelling and tenderness in

joints, commonly involving the hands, wrists, and knees. Joint deformities (hands and feet) develop in only 10% of patients

3. Nephritis

Nephritis is the most grave manifestation of SLE,since it is one of the leading causes of death in SLE. Since nephritis is asymptomatic in most lupus patients, urinalysis should be ordered in any person suspected of having SLE. Lupus nephritis is currently defined as the presence of more than 3+ or 500mg/24 hrs proteinuria or presence of cellular casts of any type. The classification of lupus nephritis is primarily histologic and renal biopsy is the Gold Standard investigation.[104].

4. Neurological

The most common manifestation of diffuse CNS lupus is cognitive dysfunction. Headache is also a common symptom among patients, but when severe, it is considered to indicate a flare in SLE. Any type of seizure may be caused by lupus. Rarely the principal clinical manifestation of SLE can be psychotic behaviour [105]. The ACR recommends the term neuropsychiatric SLE (NPSLE).

5. Haematological abnormalities

Red blood cells

The most frequent hematologic manifestation of SLE is anemia, generally normochromic and normocytic. Sometimes intravascular hemolysis can occur, which might be acute and life threatening.

Platelets

Two forms of thrombocytopenia (platelet count $<100\times109$/l) are found in SLE: (1) a chronic form generally associated with mild disease and (2) an acute form similar to idiopathic autoimmune thrombocytopenic purpura.

Platelet destruction appears to be mediated by antiplatelet antibodies and antiphospholipid antibodies.

White blood cells

Leukopenia is also common and almost always consists of a decrease in lymphocytes, thus rendering patients susceptible to infections.

6. Gastrointestinal problems

Oral ulcers, dyspepsia and peptic ulcers are common. Vasculitis involving the intestine may be grave occasionally by causing bowel perforations, ischemic necrosis of bowel, bleeding and sepsis. The incidence of hepatomegaly is 12–25%.

7. **Cardiovascular features**

Pericarditis is the most frequent cardiac manifestation; usually cured with anti-inflammatory drugs and infrequently causes cardiac tamponade. Other serious cardiac manifestations are myocarditis and Libman-Sacks endocarditis. The endocardial involvement can lead to valvular insufficiencies, most commonly of the mitral or aortic valves, or to embolic events[106]. Patients with SLE are at increased risk for myocardial infarction, usually due to accelerated atherosclerosis, which probably results from chronic inflammation [107].

8. **Lungs**

SLE patients most frequently develop pleuritis with or without pleural effusion as a pulmonary disease. Rarely grave manifestations include interstitial pneumonitis leading to fibrosis, shrinking lung syndrome, and intraalveolar hemorrhage.

9. **Eye**

The commonest ocular involvement is Sicca syndrome and nonspecific conjunctivitis. Infrequently retinal vasculitis and optic neuritis are serious manifestations blindness can develop over days to weeks.

Diagnosis of SLE:

The American College of Rheumatology has a criteria for the classification of patients as having SLE. Criteria for SLE classification was developed in 1971, revised in 1982, and revised again in 1997[108]. If a patient has, at any time in his or her medical history, 4 of the 11 criteria documented, the diagnosis of SLE can be made with about 95% specificity and 85% sensitivity.

Criterias,

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. Nonerosive Arthritis	Involving 2 or more peripheral joints, characterized by tenderness, swelling, or effusion
6. Pleuritis or Pericarditis	1. Pleuritis--convincing history of pleuritic pain or rubbing heard by a physician or evidence of pleural effusion 1. OR 2. Pericarditis--documented by electrocardigram or rub or evidence of pericardial effusion
7. Renal Disorder	1. Persistent proteinuria > 0.5 grams per day or > than 3+ if quantitation not performed 1. OR 2. Cellular casts--may be red cell, hemoglobin, granular, tubular, or mixed
8. Neurologic Disorder	1. Seizures--in the absence of offending drugs or known metabolic derangements; e.g., uremia, ketoacidosis, or electrolyte imbalance 1. OR 2. Psychosis--in the absence of offending drugs or known metabolic derangements, e.g., uremia, ketoacidosis, or electrolyte imbalance

9. Hematologic Disorder	1. Hemolytic anemia with reticulocytosis OR 2. Leukopenia< 4,000/mm3 on ≥ 2 occasions OR 3. Lyphopenia--< 1,500/ mm3 on ≥ 2 occasions OR 4. Thrombocytopenia--<100,000/ mm3 in the absence of offending drugs
10. Immunologic Disorder	1. Anti-DNA: antibody to native DNA in abnormal titer *OR* 2. Anti-Sm: presence of antibody to Sm nuclear antigen *OR* 3. Positive finding of antiphospholipid antibodies on: a) an abnormal serum level of IgG or IgM anticardiolipin antibodies, b) a positive test result for lupus icoagulant using a standard method, OR c) a false-positive test result for at least 6 months confirmed by Treponema pallidum immobilization or fluorescent treponemal antibody absorption test

11. Positive 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 .

Serology in SLE[109] :

1. Antinuclear antibody (ANA)

ANAs are antibodies which are produced against any one of the following auto-nuclear antigens:

1. Double stranded-DNA
2. Extractable nuclear antigens (ENA)
3. Histones
4. Nuclear RNA

ANA testing by direct immunofluorescence method is the gold standard modality. ANA testing is highly sensitive as nearly 95% patients show a high titre and hence a negative result commends re-testing or re-evaluation.

Anti-double stranded DNA antibody (anti-dsDNA)

This antibody is highly specific for SLE. The positivity of anti-dsDNA by radioimmunoassay and Crithidia lucilae method in SLE is in around 60% in SLE cases, moreover the collective positivity during the

entire period of the disease is nearly 90%. Thus it is not a good screening test. The anti-dsDNA titres correlate with the severity of the disease.

Antibodies to extractable nuclear antigens (anti-ENA)

These comprise anti-Sm, anti-UIRNP, anti-Ro and anti-La antibodies.They are detected in only a half of the patients tested positive for ANA. The most specific antibody for SLE is Anti-Sm antibody but it is detected in only one-third of SLE patients. Anti-Ro is antibody is found in patients with ANA negative SLE, neonatal SLE and subacute cutaneous lupus erythematosus. Anti-La is positive in cases with SLE and Sjogren's syndrome. Antihistone antibodies are coupled with drug-induced SLE.

Complement levels (C3 and C4)

Serum levels of C3 and C4 are useful to follow up patients with SLE as they are negatively correlated with lupus activity.

Management[189]:

Patient Education

Avoidance of sun-exposure – by using protective clothing and sun screen lotions.

Pharmacotherapy in SLE

Mild SLE

The drugs prescribed in order are NSAIDs and analgesics, anti-malarials (chloroquine, hydroxychloroquine). These are predominantly useful for the dermatological lesions in SLE.

Moderate SLE

Steroids are the mainstay of treatment, prednisolone 1 mg/kg per day, taken orally, is the drug of choice. Along with steroids anti-malarials are also prescribed.

Calcium supplements and vitamin D are prescribed along with steroids to retard osteoporosis.

Severe SLE

Both steroids and cytotoxics/ immunosuppressants are used to treat severe flares. The various immunosuppressants used are cyclophosphamide, mycophenolate mofetil, azathioprine, chlorambucil, methotrexate and leflunomide.The pulse therapies are given once a month for 6 months and then a maintenance pulse is administered every 3 months for a total of 2 years of cytotoxic therapy.

Osteopontin (OPN)

Osteopontin (Eta-1), secreted phosphoprotein[110] is a glycoprotein secreted in bone, inflamed renal tissues, and T cells. Osteopontin (OPN) is an important cytokine found to have key roles in inflammation and immunity. OPN carry out a variety of functions in the body which includes T lymphocyte activation, increases T-helper 1 cell population and decreases T-helper 2 cells, contribute to cell-mediated immunologic response and induce B lymphocyte to produce multi-clone antibodies[111]. Increased production of OPN was found to be associated with the progression of the autoimmune diseases .

OPN is constitutively expressed in bone and epithelial tissues mediating bone remodeling, repair of tissue and migration[112] of cells. OPN is expressed upon activation during inflammation in endothelial cells, macrophages, and smooth muscle cells[113]. OPN is pleiotropic cytokine secreted by natural killer (NK) cells and activated T cells. OPN is also called as Eta-1 (early T-cell activation-1) as it is produced soon after the activation of cells and enhance the T-cell helper 1 (TH1) and inhibit the T helper 2 responses[114,115] .The function of the innate immunity is to protect the organs from various intracellular pathogenic microbes. Autoimmunity against specific organs results due to increase response of the innate immunity causing organ damage.These findings were

confirmed by a study conducted in mice with absence of Eta-1 gene expression. Mice shows defective cell mediated(innate) immunity against certain viral , bacterial infections and failure to develop sarcoid like granulomas. Deficiency of osteopontin leads to decreased production of Interleukin-12 (IL-12), interferon-gamma and increases the IL-10 production.OPN acts over the target cells through a phosphorylation-dependent interaction between the amino-terminal portion of Eta-1 and its integrin receptor and increases IL-12 production.In other way OPN interacts with CD44 receptor through phosphorylation-independent mechanism where it decreases IL-10 production .These findings led to hypothesis that Eta-1 as a T helper1cytokine is essential for mounting type-1 immune responses through its variable control of production of macrophages ,IL-12 and IL-10 cytokines.

Osteopontin receptors and receptor-binding motifs:

The osteopontin mediates its function by binding to the receptors on the cell surface.This binding is carried out by the presence of specific receptor-binding motifs in the osteopontin gene sequence.Most commonly OPN interacts with integrins and CD44.

Integrins are transmembrane, heterodimeric proteins made of α and β subunits.There are number of combinations of α and β subunits so that

they can bind to a different type of ligands. OPN binding will cause clustering and activation of the focal adhesion complex, consisting of a number of regulatory and structural proteins such as like focal adhesion kinase, Src, cytoskeletal proteins. This results in activation of a number of diverse signal transduction pathways ultimately affecting the proliferation and survival of cells[116].

Osteopontin was found to interact with a number of heterodimeric integrins $\alpha_v\beta_3$, $\alpha_v\beta_1$ and $\alpha_v\beta_5$ [117,118] through the RGD sequence of arginine,glycine,aspartate aminoacids. Additional integrins by which osteopontin interacts are $\alpha_4\beta_1$, $\alpha_9\beta_1$, and $\alpha_8\beta_1$.

Through the RGD sequence of arginine,glycine,aspartate aminoacids osteopontin interacts with $\alpha_v\beta_3$ integrin receptor in osteoclasts , smooth muscle cells and tumor cells. Thrombin cleavage site is present close to the RGD sequence.So the osteopontin is liable to cleavage by thrombin which is formed during the blood coagulation cascade .The osteopontin cleaved by thrombin was found to exist in side-by-side with the full-length of protein(OPN-FL) [119].Thrombin cleaved osteopontin shows an increase effects in the cell survival when compared to uncleaved osteopontin.

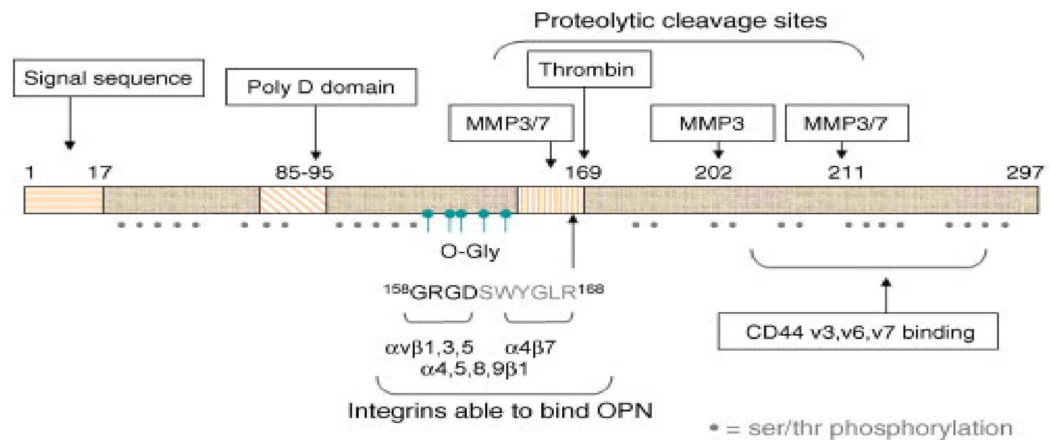
Thrombin-cleaved osteopontin binds mostly to a active $\alpha_v\beta_3$ integrin receptor. The cleavage of osteopontin induce the conformation change of the molecule, around the RGD motif, and thereby promote the binding to the $\alpha_v\beta_3$ integrin.The RGD motif is present in the amino terminal region of thrombin-cleaved osteopontin which induces an amplified response [120].

Figure 7:

Thrombin cleavage of human osteopontin. Thrombin cleave the osteopontin between Arg^{152} and Ser^{153} aminoacids producing two fragments an amino terminal fragment consisting of RGD sequence and carboxyl-terminal fragment consisting of SVVYGLR sequences (OPN-R). The carboxyl-terminal fragment can be further acted upon by Carboxypeptidase B (CPB) which removes C-terminal arginine converting OPN-R to OPN-L. Both OPN-R and OPN-L are able to bind integrin receptors. Thrombin-cleaved OPN (OPN-R) exposes an epitope for the integrins $\alpha4\beta1$,$\alpha9\beta1$,and $\alpha9\beta4$.These integrin receptors are also found on the surface of immune cells such as mast cells,neutrophils,and T cells which use diverse signal transduction pathways to elicit immune responses .

FIGURE 7

THROMBIN CLEAVAGE OF HUMAN OSTEOPONTIN



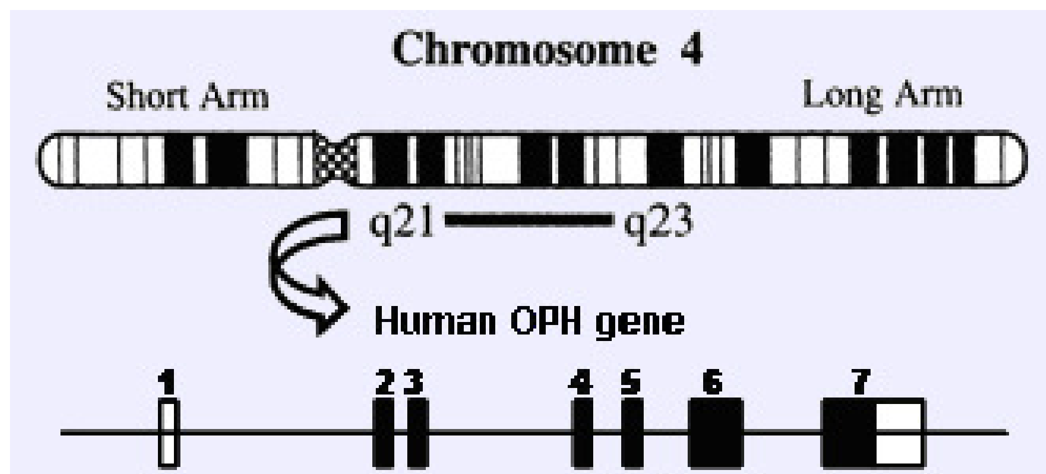
The CD44 family encoded by a single gene produces different protein isoforms, because of alternative splicing of sequences.Osteopontin interaction with CD44 on the surface of macrophages results in decrease production of interleukin-10 by macrophages and are implicated in the development of metastases [121]. Osteopontin mediate the chemotaxis and attachment of cells mostly by binding to CD 44 receptor.

The recruitment of inflammatory cells by OPN is carried out by interaction of arginine-glycine-aspartate (RGD) sequence with integrin receptors. OPN is associated with a number of pathologic conditions such as autoimmune disease, cardiovascular disease, cancer, aging, diabetes, obesity and metabolic syndrome.

OPN GENE STRUCTURE:

The human OPN gene (*OPN*) is located on chromosome 4q22.1- figure 8.

CHROMOSOMAL LOCALISATION OF OPN



Osteopontin belongs to the group of SIBLING proteins which consists of five structurally similar proteins. The human protein consists of 314 amino acid residues[122]. OPN undergo posttranslational *modifications* like glycosylation,phosphorylation with a molecular mass of 44 kiloDalton[123]. The OPN gene consists of seven exons. Of the seven exons, six code for the expression of osteopontin.The 5' untranslated region (5' UTR) consists of the first two exons . Exon 2 codes for 17 aminoacids,exon 3 codes for 13 aminoacids,exon 4 codes for 27 aminoacids,exon 5 codes for 14 aminoacids, exon 6 codes for 108 aminoacids and exon 7 codes for 134 aminoacids. Casein kinase II as well as cAMP-dependent protein kinases catalyses the self -phosphorylation[124] of osteopontin. Figure:9

shows the secondary structure of OPN consisting of eight α-helices and six β-sheets[125].Correlation studies have shown the association of certain *OPN* genotypes and the increased production of OPN level[126].

SECONDARY STRUCTURE OF OSTEOPONTIN(OPN).

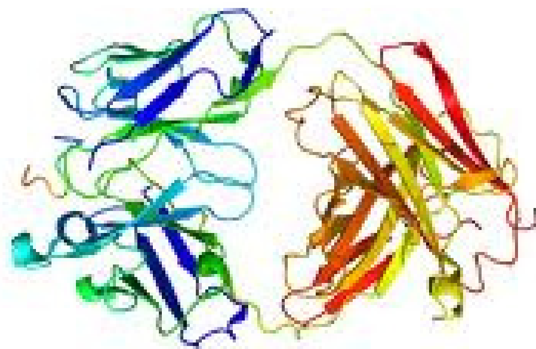


Figure:8-Shows the coding regions of osteopontin. Exon 1 is present in the 5'-untranslated region consisting of a transcription initiation site (AGC).

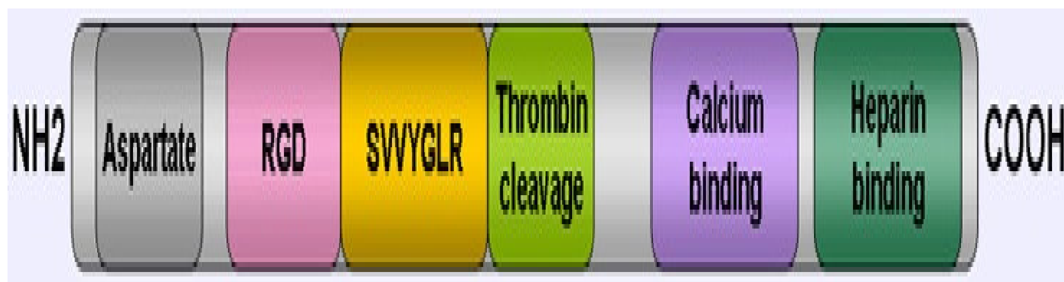
- Half of the exon 7 is present in the 3'-untranslated region having three possible polyadenylation attachment signals (AATAA).

- The leader Sequence that directs the protein to the endoplasmic reticulum and the first two amino acids in the protein is coded by exon 2
- Exon 3 and 5, the two typical Ser-Ser-Glu-Glu phosphorylation sequences.
- Exon 4, the two transglutaminase-reactive glutamine residues.
- Exon 6, the aspartic-rich sequence.
- Exon 7 is the major exon encoding roughly half of the protein together with the RGD motif and the central thrombin cleavage site.

The activity of osteopontin is regulated by the presence of the numerous cell interacting domains in addition to multiple protease cleavage sites.

Figure:10 Shows a synopsis of structural domains

OSTEOPONTIN DOMAINS



* RGD sequence comprising Arg159-Asp159 amino acid sequence is important for binding to multiple forms of integrins such as alphaVbeta3, alphaVbeta1, alphaVbeta5 and alpha5beta1.

* Aspartate domain consisting of amino acid sequence Asp86-Asp89 which is essential for binding hydroxyapatite in the bone.

* Amino acid sequence Ser162-Arg168 present in the SVVYGLR sequence binds alpha9beta1 and alpha1beta1 integrins.

* Calcium binding domain consisting of amino acid sequence Asp216-Ser228 is for binding calcium.

* Amino acid sequence Arg168-Ser169 – with RGD sequence is the thrombin cleavage site.

* Heparin binding domain - amino acid sequence Asp290-Ile305- mediates CD44binding.

OPN undergoes post-translational modifications including

* Phosphorylation of serine residues,

* O- and N-glycosylation

Isoforms:

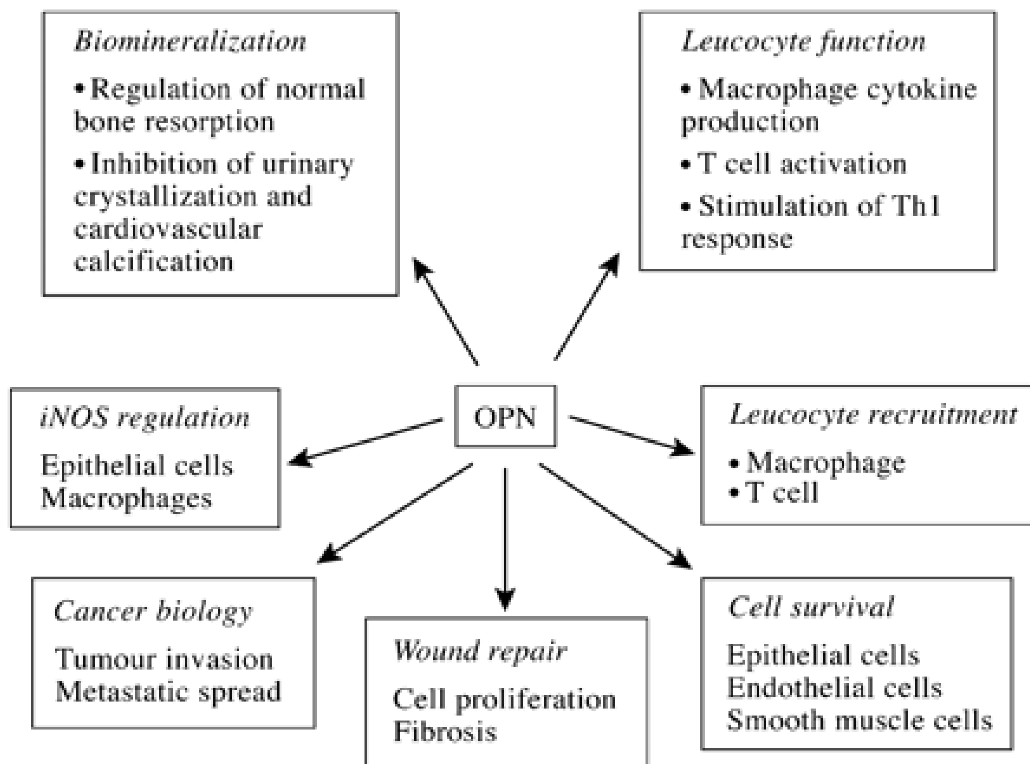
The three splice variants of osteopontin transcripts are OPN-a, OPN-b and OPN-c. These varients are due to alternative splicing of OPN gene.Exon 5 is absent in the OPN-b varient and OPN-c is lacking exon 4.The regulation of transcription of OPN is composite and it includes different pathways of Wnt/b-catenin/APC/GSK-3b/Tcf-4,AP-1, Myc, v-Src, RunX/CBF and TGF-B/BMPS/Smad/Hox .

Intracellular OPN (i-OPN) is produced with an different translation initiation site on the similar mRNA species that codes for the extracellular isoform [127].This different translation initiation site is present in the lower part of the amino-terminal leader sequence.The leader sequence directs the mRNA to lumen of the endoplasmic reticulum thereby permitting the translation of OPN in the cytoplasm.

The secretion of osteopontin is stimulated by many factors and is controlled at the level of transcription [128].It acts through multiple signaling pathways on specific cell types.Analyses of the promoter region of osteopontin revealed the possible sites for interactions [129,130] of transcription factors such as progesterone, glucocorticoids, vitamin D3 and DNA binding motifs basic helix-loop-helix proteins like activator

protein-1. Activator protein-1 binds with a highly preserved enhancer-like element found in many viral and cellular genes, such as osteopontin gene.

Biological functions of Osteopontin,Figure-11:



OPN in bone remodeling

Osteopontin (OPN) is a major noncollagenous bone matrix protein. The GRGDS sequence consisting of glycine-arginine-glycine-aspartic acid-serine amino acids mediate cell attachment through cellular αvb3 integrin[131].Normally osteoclasts expresses OPN during the course of bone remodelling. It is produced by osteoblasts as well as osteoclasts,

and is considered to play important roles in bone formation, resorption, and remodeling.OPN increases the segregation and production of osteoblast cells thereby increases ALP activity[132]. OPN also cause bone resorption by acting on osteoclasts and change them into ruffled borders.OPN present in the urine inhibits formation of the kidney_stone.

Immunological functions

Osteopontin is necessary for the development of cell mediated immunity. It acts by increasing the expression of interferon-gamma and interleukin-12 and decreases the production of interleukin-10. Osteopontin (OPN) is produced by a number of immune cells such as macrophages, neutrophils, dendritic cells,T cells and B cells in different concentrations. OPN interacts with multiple integrin receptors α4β1, α9β1, and α9β4 present on the surface of leukocytes. OPN is an immune modulator[133]. It has multiple functions including recruitment of immune cells to inflammatory sites, an adhesion_protein participating in cell attachment ,wound_healing and augments the survival of cells by regulation of apoptosis.

Chemotaxis

The chemotactic function of OPN was shown by a number of studies.OPN plays an significant role in recruitment of neutrophils in alcoholic liver disease.Another study reveal the function of recruitment of inflammatory cells to arthritic joints in the development of rheumatoid arthritis[134] .In vitro study conducted in 2008[135] inferred that OPN knock-out mast cells showed a reduced level of chemotaxis when compared to normal mast cells.

Activation of cells

Osteopontin induces the secretion of cytokine IL12 which causes T cells activation and make them to differentiate towards the T helper 1cells.The T helper1cells expresses cytokines such as IL-12 and IFN-γ. At the same time OPN attenuate the production of the T helper2 cytokine IL-10,ultimately leading to increased T helper 1cells response. OPN induces cell-mediated immunity and T helper 1cells comprises cytokine functions. OPN stimulate B cells to produce multiple clone of immunoglobulins and their proliferation. The researchers found that IgE-mediated anaphylaxis was considerably low in mice without OPN when compared to mice with OPN. Activation of macrophages by osteopontin was found in a cancer study, where OPN-deficient tumors[136] cells were

unable to cause activation of macrophages compared to tumors cells producing osteopontin.

OPN in Apoptosis

OPN is an significant anti-apoptotic cytokine.OPN prevents the activation-induced apoptosis of macrophages,T cells ,fibroblasts and endothelial cells when exposed to detrimental stimulating factors[137]. OPN was found to inhibit the non-programmed loss of cells in inflammatory colitis. Charles describes research findings that apoptotic dysfunction in the development of autoimmunity[138]. Defective removal of apoptotic bodies was one of the mechanisms that leads to the occurance of SLE.

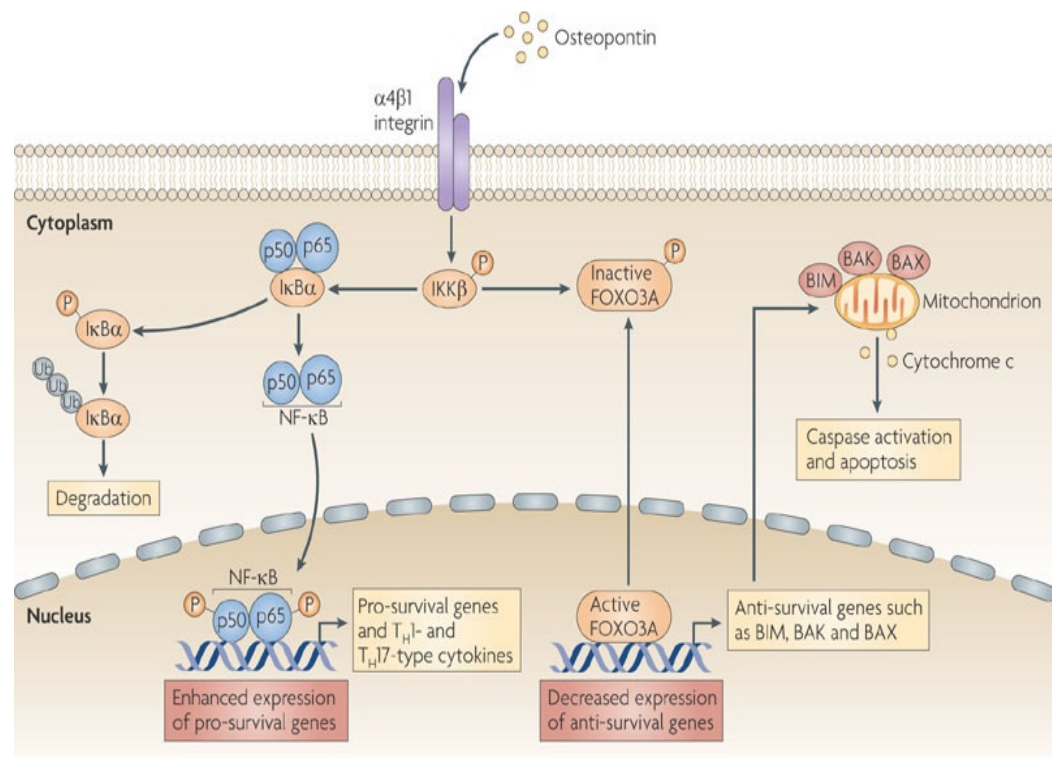
OPN in autoimmune diseases

OPN was found to be involved in the development of *rheumatoid* arthritis.Study workers have found the increased level of OPN-R, the thrombin-cleaved form of OPN in the synovial fluid of rheumatoid arthritis joint.But the exact pathogenic events in rheumatoid arthritis is not studied in detail.One study group found that OPN knock-out mice do not develop arthritis. OPN has also been involved in the pathogenesis of other autoimmune diseases such as autoimmune hepatitis, allergic airway disease, and multiple sclerosis.

OPN in Malignancy and inflammatory diseases

The expression of osteopontin in several human carcinomas was shown by Brown and co-workers [139]. High level of osteopontin mRNA was seen in the screening of tumors of colon, breast, lung, stomach, endometrium and thyroid when compared to normal tissues. Osteopontin promote the development of cancer through numerous and composite mechanisms such as binding with cell surface receptors, regulation of growth factor and receptor pathways and proteases.Proto-oncogene *ras* ,a GTPase protein increases the transcription of osteopontin which is implicated in the transformation, metastasis and progression [140]of neoplastic cells. Normally osteopontin is expressed at low concentrations in tissues but in case of premalignant and malignant cells it is increased. Osteopontin increases the survival of endothelial cells by increasing the expression of pro-survival genes and decreasing the expression of anti-survival genes through the activation of nuclear factor κB [141]Figure;12.Further by interacting with integrin $\alpha_v\beta_3$, it induces the expression of osteoprotegerin, a tumor necrosis factor receptor which has been found to protect endothelial cells from programmed cell death. Antiosteopontin therapeutic strategies are being in research to target OPN in malignancy.

ROLE OF OSTEOPONTIN IN APOPTOSIS



OPN in allergy and asthma

Osteopontin was found recently to be associated with asthma and diseases. From the study conducted in mice it was established that the secreted form of OPN (OPN-s) exhibit opposing effects by increasing the T helper2 responses when compared to other forms of OPN which increases the T helper 1 cells.This results in allergic disease like asthma with primary systemic sensitization through pro-inflammation and anti-inflammatory effects during subsequent exposure to similar pulmonary

antigens through the regulation of different dendritic cell population. The absence of OPN was seen to protect from remodeling and asthma[142].

OPN in muscle disorders

Osteopontin is involved in a number of pathways that lead to development of skeletal_muscle diseases, such as Duchenne_muscular dystrophy. Osteopontin mediates inflammation of dystrophic and injured muscles and cause high scarring of diaphragm muscles in the aged dystrophic mice.A latest study revealed that a mutation in the promoter region of osteopontin gene causes decreased levels of osteopontin expression.This seems to be associated with a decrease in the severity of clinical manifestations in patients with Duchenne muscular dystrophy[143].

Therefore, handling of plasma OPN levels can be helpful in the treatment of autoimmune diseases, malignancies, osteoporosis and allergic diseases.

OSTEOPONTIN IN SLE

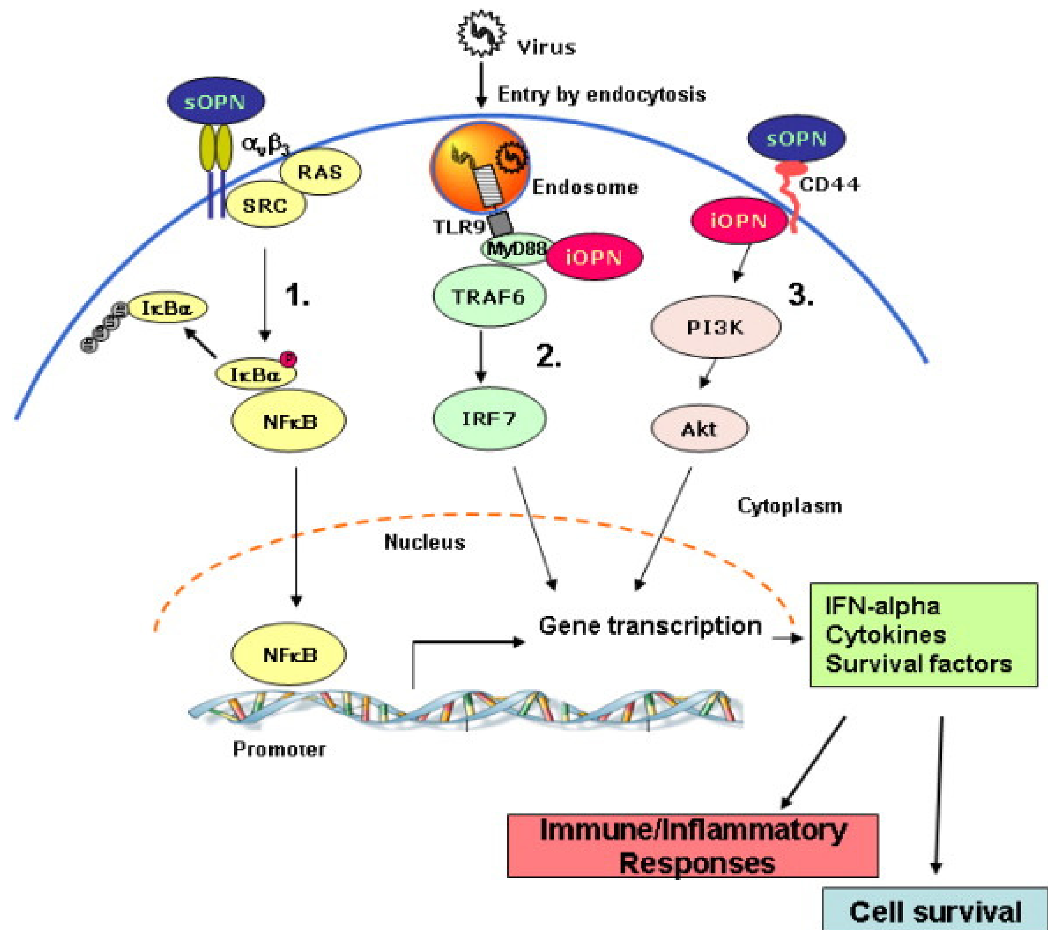
Systemic lupus erythematosus (SLE) is a typical autoimmune disease characterized by abnormal immunologic response.T lymphocyte and B lymphocyte activation results in production of multifarious autoantibodies with several tissue damage.Lupus nephritis (LN) is the most common and lethal manifestation of SLE. Lupus nephritis is frequently associated with abnormal production of cytokines. OPN induces B lymphocyte to produce polyclonal antibodies [144]. OPN carry out a variety of functions in the body which includes early T lymphocyte activation that's why it is called as early T lymphocyte activation 1 (Eta-1), increases T-helper 1 cell population and decreases T-helper 2 cells and thus contribute to cell-mediated immunologic response [145,146].Several studies have found that OPN is an essential component in the autoimmune mediated pathogenesis of SLE[147,148].There is a promising association of OPN gene polymorphism with systemic lupus erythematosus. A single nucleotide polymorphism (SNP) at position 9250 with replacement of C by T in exon 7 of the OPN gene (OPN gene 9250) is newly detected in humans[149].

Humans with SLE overexpress osteopontin suggesting a role of OPN[150] in the pathogenesis of the disease . Enhanced expression of osteopontin is particularly associated with degree of renal damage.This is

confirmed by the study in MRL/lpr –murine model of Murphy roths large/lymphocyte proliferation which shows increased production of osteopontin compared to controls and increased production in the proximal tubule appears to mediate infiltration of macrophage.Osteopontin is upregulated in different types of renal damage[151].Particularly in humans, crescentic [152]glomerulonephritis is associated with enhanced production of osteopontin .Increased production of osteopontin is found to produce certain clinical features of lupus in mice and humans with SLE show overexpression of osteopontin in plasma and at confined sites of renal inflammation. This provoked the present study of an osteopontin gene single nucleotide polymorphism in the region of exon 7 in SLE patients.Multiple polymorphisms in the coding gene of the human OPN was identified in diverse populations,that in Japanese population some polymorphisms have been located in the 5′ flanking region, Chinese population was found to have polymorphisms in the region of exons, introns and 3′ untranslated region [153]. Figure:13- Shows the interaction of OPN with integrin and CD44 receptors.

FIGURE:13

OSTEOPONTIN SIGNAL PATHWAYS



By binding to these receptors OPN stimulate multiple signaling pathways to initiate immune responses[154]. (a) The circular form of osteopontin binds to integrin αvβ3 receptor and the intracellular signals are carried out through the Src and FAK tyrosine kinases activating transcription factor NFκB. OPN causes phosphorylation of the inhibitor of NFκB called IκBα leading to the separation of IκBα from the nuclear

factor and nuclear translocation of NFκB which mediates the transcription of a number of pro-inflammatory cytokines. (b) In dendritic cellsDCs,intracellular OPN (iOPN in red) interacts with MyD88 during TLR9 engagement with viral DNA of endosome, causing TLR9 signaling in the direction of IRF7 instead of IRF-5/NFkB leading to vigorous production of IFN-γ.(c) Interaction of OPN with CD44 increases the intracellular concentration of a second messenger phosphatidyl inositol 3phosphate which is acted upon by kinase cascade of PI3k /Akt signaling mediating cell survival. OPN promoted the survival of activated T cells by decreasing the transcription factor Foxo3a through the translocation of the NF-κB and by varying expression of the pro-apoptotic proteins Bim, Bak and Bax. These events together decrease the death of lupus-reactive T cells, connecting OPN to the development of SLE.

The susceptible genes in SLE which secrete cytokines are of important topic in research. Increased concentration of OPN have been documented in biopsies of injured tissues in SLE and also in other autoimmune diseases .Numerous studies have found that raised plasma OPN level is associated with progression of disease activity in SLE.

In mouse models,OPN is important for the expression of interferon-alpha (IFN-α) . IFN-α levels are found to be raised in more number of SLE patients and elevated IFN-α is a genetic risk factor for

SLE .SLE risk-related allele of OPN rs9138C was linked with elevated levels of serum OPN and IFN-α in adolescent female and men with SLE . This observable fact in which a number of SLE-risk loci associated with the cytokine profiles has been confirmed as well .

Also identified the association of SLE-risk loci with the specific clinical manifestations of SLE .For example a study has found a relationship between rs7687316 in the promoter region and lymphadenopathy in European descent individuals . Another study comprising of 81 SLE patients of European American descent established an connection between a identical change in exon 7 with avascular necrosis and renal damage. The rs11730582 C and rs9138 C alleles of the osteopontin (OPN) gene was found to be separately related with increased possibility of lupus. The cytokine actions of OPN include the activation of macrophage and T-cell migration.OPN is found to protect against herpes viruses and bacterial infections through the activation of the T helper1 activity and stimulation of T helper1 - cell-mediated autoimmunity .

AIMS AND OBJECTIVES

The aim of the study is

1. to determine the association of single nucleotide polymorphism at 9250 C→T in exon 7 of Osteopontin(OPN) gene among systemic lupus erythematosus patients, with and without nephritis and in healthy controls.
2. to assess the plasma OPN activity among study groups and correlate their level with the genotype.

MATERIALS AND METHODS

This is a case-control study and was conducted after obtaining ethical committee clearance. The study was carried out during the period April 2011- September 2012 at Madras medical college and Rajiv Gandhi government general hospital.

Study population:

CASES:

100 SLE cases attending rheumatology outpatient department of our hospital were included in the study after obtaining consent and were categorised into

Group 1A: 50 SLE patients with lupus nephritis

Group 1B: 50 SLE patients without lupus nephritis

Nephritis cases are included based on renal biopsy findings.Cases with clinical or laboratory evidence suggestive of mixed connective tissue disorders were excluded from the study.

CONTROLS:

Group 2: 50 age and sex matched healthy individuals attending master health check- up were selected as controls.

Sample collection:

Blood samples:

About 5 mL of blood was drawn from the cubital vein of the subjects and collected in EDTA tube.The samples were centrifuged and plasma was separated and transferred into 2 mL eppendorf. Plasma was stored at -20°C for estimation of osteopontin.

Urine samples:

Early morning urine samples were collected in sterile plastic containers and Albumin Creatinine ratio was estimated.

BUFFY COAT SEPARATION

Buffy coat was obtained by centrifugation of EDTA tubes at 2000 revolutions for 20 minutes.Buffy coat was transferred to 2 ml eppendorf and was used for DNA extraction. DNA extraction was done on the same day and extracted DNA stored at -20°C.

DNA EXTRACTION BY MODIFIED HIGH SALT METHOD[155]

RBC Lysis:

- 400µL of buffy coat in a 2mL eppendorf is mixed with 1.6mL of 0.17M ammonium chloride and mixed by inversion until red cells are lysed for about 10 minutes

- The cells are centrifuged at 4000rpm for 10minutes.
- The white cell pellet is washed with 800µL of 0.17M ammonium chloride solution. The procedure is repeated till a clear white cell pellet is obtained.

WBC Lysis

- To the pellet 500 µL of TKM I solution is added. It is centrifuged at 10,000rpm for 10minutes.

Nuclear Lysis

- Discard the supernatant. To the pellet add 500 µL of TKM II solution. To that add 300 µL of 6M NaCl and 50 µL of 10% SDS.
- Mix well (vortex), Centrifuge at 10,000 rpm for 10 minutes.
- Save the supernatant. Transfer it to 1.5mL eppendorf.

DNA Precipitation

- To the supernatant double the volume of 100% ethanol is added.
- The sample is stored at -20◦C for 1 hour.
- Then it is centrifuged at 10,000 rpm for 20minutes at 4◦C in a refrigerated centrifuge.

- The supernatant is discarded. To this 500 µL of 70% ethanol is added. The pellet is mixed and centrifuged at 10,000 rpm for 10minutes at 4◦C and later air dried.

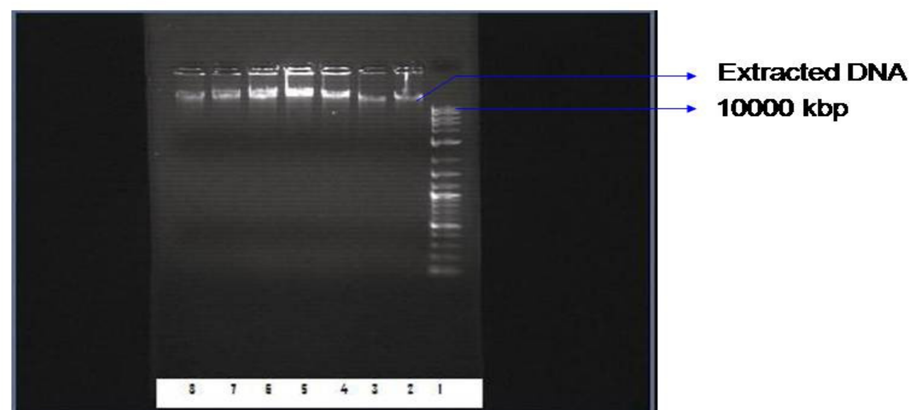
Storage

- To the pellet 30 µL of LTE buffer is added and the extracted DNA is stored at -20◦C for future use.

Identification

- Extracted DNA was identified by 0.8% agarose gel electrophoresis with a constant voltage of 7V/ cm and comparison with a known molecular weight 1kb DNA ladder.Figure:14

DNA EXTRACTION BY HIGH SALT METHOD



•Extracted DNA (lane 2 to 8) was tested on 1% agarose gel using 1kb ladder (lane 1)
•Ladder shows 10000, 8000, 7000,6000, 5000, 4000, 3000, 2000 1000 kbp fragments

Concentration of extracted DNA:

- Concentration of extracted DNA was estimated using UV spectrophotometer at 260 nm.
- Concentration was calculated using the formula :

  1 OD is equivalent to 50 µg/mL.

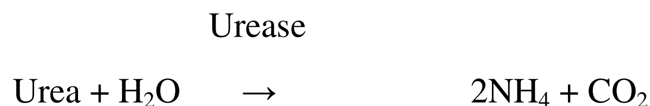
  Conc. of DNA = absorbance X 50 µg/mL X dilution factor

  = y X 50 X 100 ng/µL
- Purity of extracted DNA was assessed by 260nm/280nm ratio. .

Estimation of Blood Urea:

- **Method : GLDH method, Enzymatic method**
- **Principle:**

  The test is performed as a kinetic assay in which the initial rate of the reaction is linear for a limited period of time.



$$Urea + H_2O \xrightarrow{Urease} 2NH_4 + CO_2$$

$$NH_4 + NADH + H^+ + 2\text{-Oxoglutarate} \xrightarrow{GLDH} Glutamate + NAD^+$$

The initial rate of decrease in absorbance at 340 nm is proportional to the concentration of urea in the sample.

- **Procedure:**

To 1 mL of working reagent 10 µL of sample or standard is added. Absorbance is measured after 30 sec (A1) and 90 sec (A2).

- **Calculation:**

$$\frac{\Delta A \text{ Sample}}{\Delta A \text{ Standard}} \times 50 \text{ (Standard Conc)} = \text{mg/dL urea in the sample}$$

- **Reference range:**

Normal blood urea = 15 to 40 mg/dL

Estimation of Serum Creatinine:

- **Method :** Modified Jaffe's Method
- **Principle**:

Creatinine picrate- an orange yellow coloured complex is formed when creatinine in the sample reacts with alkaline picrate.The absorbance of orange-yellow colour developed is directly proportional to concentration of creatinine and is measured in spectrophotometer at 520 nm.

- **Procedure:**

To 500 µL of working reagent 50 µL of serum sample or standard is added and initial absorbance (A1) is measured at 20 sec and final absorbance (A2) is measured at 80 sec after mixing.

- **Calculation:**

$\Delta A = A_2 - A_1$

$$\text{Creatinine} = \frac{\Delta A \text{ of Test}}{\Delta A \text{ of Standard}} \times \text{Concentration of Standard (2 mg/dL)}$$

- **Normal range :** 0.6-1.2 mg/dL

Estimation of Urine Creatinine:

Estimation of urine creatinine is done by modified Jaffe's method after diluting the urine sample ten times and multiplying the derived result with dilution factor i.e.,10.

- Normal Urine creatinine excretion is 1-2 g/day

Estimation of Urine Microalbumin :

- **Method:** Immunoturbidimetry method.
- **Principle :**

The antibodies specific to human albumin are layered over the latex particles.Latex particles are agglutinated with microalbumin, present in the sample. The agglutination causes a change in absorbance which is proportional to the concentration of microalbumin in the patient

sample and estimated by comparison with a calibrator of known microalbumin concentration.

- **Procedure :**

To 1 mL of the working reagent 7µL of calibrator or sample is added and absorbance measured immediately(A_1) and after 2 min (A_2) of sample addition. The temperature of the reagent and the reaction mixture are maintained at 37°C.

- **Calculations:**

$$\frac{(A_2 - A_1)_{sample}}{(A_2 - A_1)_{calibrator}} \times \text{Calibrator concentration} = \text{mg/L albumin}$$

- Calibrator of concentration 64 mg /L was used.
- Normal : 30 mg/24 hrs urine specimen

  20 mg/L in early morning sample

- **Estimation of Urine Albumin Creatinine Ratio (ACR) :**

  Urine microalbumin per gram of Creatinine excreted was calculated for each patient sample.

- ACR ≥ 23 mg/g of creatinine for males and

  ≥ 32 mg/g of creatinine for females is considered as nephropathy range.

Estimation of Plasma Osteopontin:

- **Method:** ELISA

- **Principle:**

Solid phase sandwich ELISA with 2 kinds of high specific antibodies. The coloring agent(chromogen) is a Tetra Methyl Benzidine (TMB).The intensity of colour is proportional to the concentration of plasma osteopontin(OPN) in the sample.

Coating antibody :Anti-Human OPN, Rabbit IgG:The antibody react with the N-terminal region of human OPN.

Labelled antibody : Anti-Human OPN, Mouse IgG- monoclonal antibody. The antibody reacts with carboxy terminal region of thrombin cleavage site of human OPN.

Measurement range: 5 to 320 ng/mL.

Procedure:

The reagents should bring to room temperature 30minutes before use. Then mix it gently and completely. Standard curve can be drawn simultaneously with the measurement of test samples.

1. Decide wells for reagent blank. Take 100 μL of EIA buffer into the wells.
2. Decide wells for test sample blank, test sample and for standard. Then put 100 μL each of test sample blank, test sample and dilutions of standard into the appropriate wells.
3. Incubate the precoated plate for 1 hour at 37°C after covering it with plate lid.
4. Wash each well with wash buffer and remove buffer completely.This is repeated for 7 times.
5. Pipette 100 μL of labeled antibody into the wells of diluted standard ,test sample blank and test samples.
6. Incubate the precoated plate for 30 minutes at 4°C after covering it with plate lid.
7. Wash the precoated plate 9 times .
8. Chromogen is taken into a disposable test tube.Then pipette 100 μL from the test tube into the wells.

9. Incubate the precoated plate for 30 minutes at room temperature in the dark. The color of the liquid changes to blue after addition of chromogen.
10. Pipette 100 μL of stop solution into the wells. Mix the liquid by tapping the side of precoated plate. The liquid will become yellow. Then run the plate leader and take measurement at 450nm within 30 minutes.

POLYMERASE CHAIN REACTION

- OPN gene was amplified using,

Sense primer (forward) – 5'TACCCTGATGCTACAGACGAGG– 3' and

Antisence primer (reverse) – 5'-CTGACTATCAATCACATCGGAATG – 3'

Primer Reconstitution

Primers are supplied in lyophilized form.

Autoclaved distilled water is used to prepare 100 × concentrations i.e. 10times the molecular weight of primer is the volume of water required to prepare 100 × concentrations which is 100µmolar solution.

- From this stock solution 10 × concentration is prepared as the working solution for PCR.

MASTER MIX:

- Genei Red Dye master mix in the following composition was used.

- Master Mix consists of a unique inert red dye in addition to basic components necessary for PCR.

    - Reaction buffer consisted of Tris Hcl -10mM at pH 8.3

      KCl - 50mM
    - $MgCl_2$ - 1.5mM acts as catalyst.
    - dNTP's were used in a concentration of 2.5mM each.
    - Taq polymerase in a concentration of 1.5 U.

- Primers were used in a concentration of 5 picomole and DNA was used in a concentration of 200ng.

- PCR was carried out in a reaction volume of 12.5 μL with the following components;

    - PCR master mix – 6.5 μL
    - Sence primer – 0.5 μL
    - Antisence primer – 0.5 μL

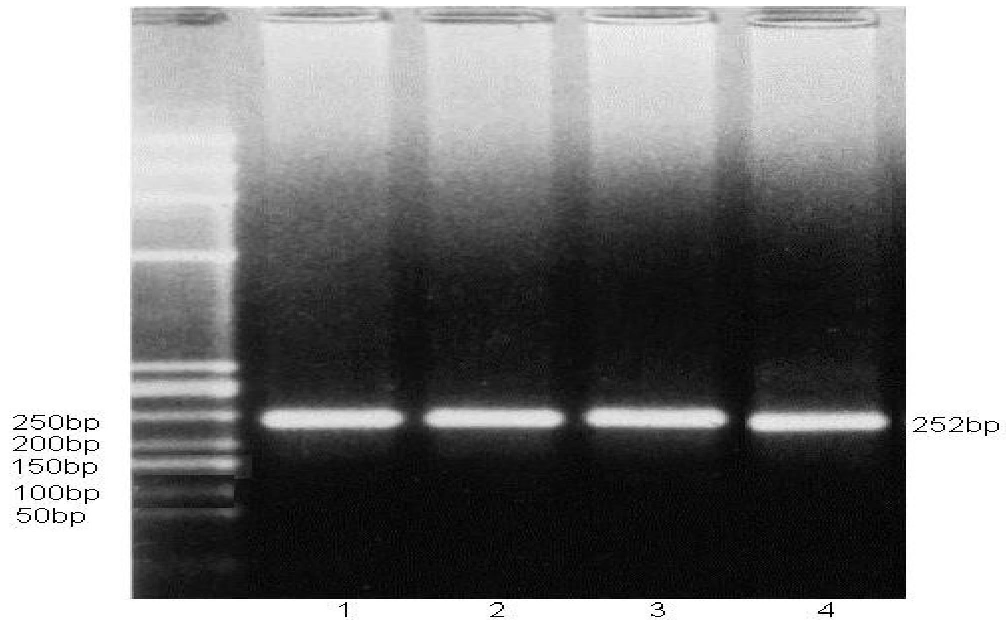
- DNA – 1.0µL
- Distilled water – 4.0 µL
- Total – 12.5 µL

- Amplification was carried out in an Mc Genei thermal cycler with the following cycling conditions.
    - Initial denaturation – 95^{0} C -3min

      35 cycles of

        - Denaturation – 94^{0}C – 30sec
        - Annealing - 60^{0}C – 45sec
        - Extension -72^{0}C – 30sec
    - Final extension at 72^{0}C - 10 min.
- Amplified product is 252 base pairs in length,which is identified by 2.5% agarose gel electrophoresis by comparison with a known 100 base pair DNA ladder, Figure;15.

POLYMERASE CHAIN REACTION



Agarose gel electrophoresis of pcr products. shows the 252bp osteopontin gene pcr product (lane 1 to 4) on 2.5% agarose gel. with 50bp DNA ladder

AGAROSE GEL ELECTROPHORESIS

- PCR product is run on agarose gel in a 25 mL agarose cast as follows: 625mg of agarose is weighed and dissolved in 25mL of TAE buffer with a pH of 8.0.

- It is subjected to microwave for 60 secs, cooled and 2.5 µL of ethidium bromide (10mg/mL) is added. Then poured into a cast and allowed to solidify for 15 mins before placing it in the electrophoresis tank.

- 8 μL of PCR product is loaded onto wells and 4 μL of 100bp DNA ladder is loaded onto single well as a marker. It is electrophoresed at 8V/cm for 45min and visualized under UV illumination.

- **RESTRICTION DIGESTION OF PCR PRODUCTS**

  OPN gene polymorphism was detected by digestion of the PCR amplified product with the Alu1 restriction enzyme.

Principle of Alu1 enzyme digestion

Alu I (Arthrobactor luteus) restriction enzyme cleave the normal C allele into two fragments of length of 147 base pairs and 105 base pairs.

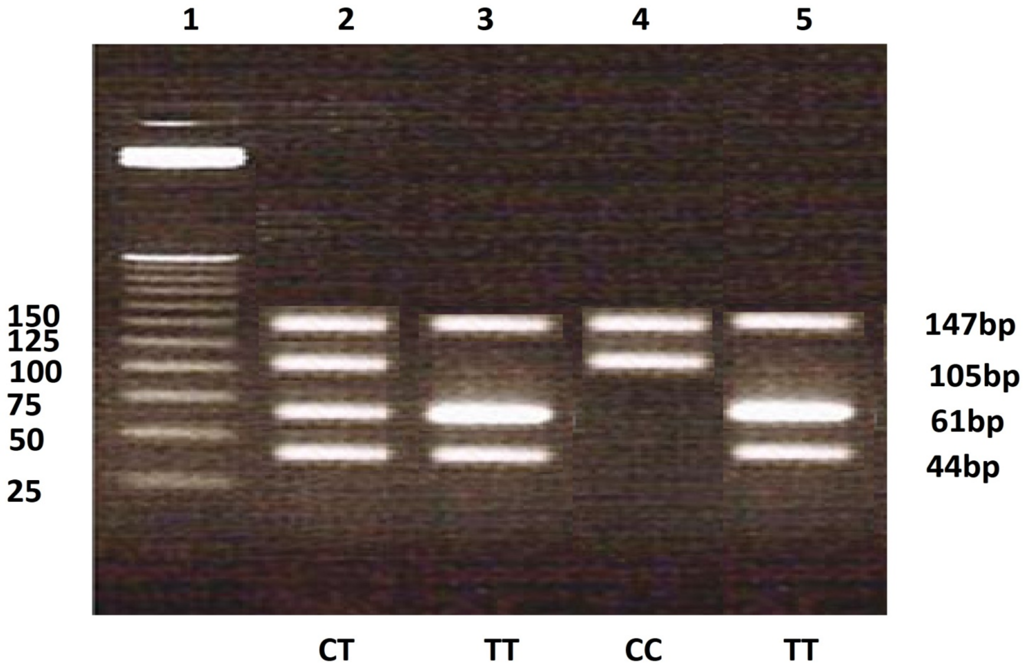
Alu I cleaves the polymorphic variant T allele having two restriction sites to make fragments of 147 base pairs , 61 base pairs and 44 base pairs .Heterozygous genotype (TC) cleaved by Alu1 produce fragments of 147 base pairs,105, base pairs 61 bp and 44 bp.

Analysis was done using 25 bp DNA ladder.

Procedure

10μL of PCR product is aliquoted in an eppendorf , to that added 1μL(10U) of Alu1 restriction enzyme,2.5μL of NE buffer and 11.5μL of distilled water.The entire procedure is carried out in a tube placed over the ice.The contents are mixed thoroughly.

The eppendorf is then placed in a 37°C water bath for 4 hours and the reaction is stopped is by adding 5μL of gel loading dye and mixed thoroughly. After digestion with Alu1 restriction enzyme, products were separated in 3% agarose gel, stained with Ethidium bromide and the gel is visualized in UV illumination & the genotypes are identified by comparison with known molecular weight DNA ladder(25bp) and identifying the various fragments. The separation of fragments by agarose gel electrophoresis shown two bands of length147 base pairs and 105 base pairs for the CC genotype, three bands of length147 base pairs, 61 base pairs ,44 base pairs for the TT genotype and four bands of length 147 base pairs,105 base pairs, 61 base pairs and 44 base pairs for the TC genotype,Figure;16.



Agarose gel electrophoresis of restriction digestion products.lane 1 shows ladder(25,50,75,100,125,150),lane 2 shows 147bp,105bp,61bp,44bp indicating ct genotype, lane 3&5 shows 147bp,61bp,44bp indicating tt genotype, lane 4 shows 147bp,105bp indicating cc genotype.

STATISTICAL ANALYSIS

1. Age, blood urea, serum creatinine, urine microalbumin, urine creatinine and urine albumin creatinine ratio were compared between three study groups by ANOVA.
2. OPN genotype frequency distribution between cases and controls were compared by ANOVA.
3. Odds ratio was calculated for OPN genotype distribution in the study population.
4. Allele frequencies were calculated by allele counting.
5. OPN activity was compared between the study groups by ANOVA.
6. OPN activity for the OPN genotypes were compared by ANOVA.

Statistical analysis was done using SPSS software.

RESULTS

TABLE- 1

COMPARISON OF PARAMETERS BETWEEN STUDY GROUPS BY ANOVA

Variables	Cases		Controls	P value
	Group1A SLE with nephritis	Group1B SLE without nephritis		
Age	**30.7±9.83**	30.04±9.07	30.62±9.89	**0.932–NS**
Blood Urea mg/dL	**71.56±8.53**	37.18±19.57	34.72±19.17	0.001 –S
Serum Creatinine mg/dL	**2.25±0.84**	1.27±1.03	1.23±1.02	**0.001 – S**
Urine Microalbumin mg/L	**85.90±72.93**	13.44±5.96	10.08±5.99	**0.001-S**
Urine Creatinine g/L	**1.02±0.24**	1.08±0.31	1.12±0.41	0.297 -NS
Urine Albumin Creatinine ratio mg/g of creatinine	**75.06±30.81**	12.78±6.07	8.71±4.13	**0.001 – S**

Table 1:

Shows age, blood urea, serum creatinine, urine microalbumin, urine creatinine and urine albumin creatinine ratio comparison between cases and controls by ANOVA.

Blood urea:

The mean blood urea for group 1A was 71.56±8.53mg/dL while that of group 1B was 37.18±19.57mg/dL and that of controls was 34.72±19.17mg/dL. The blood urea values are high in group1A than group1B and controls. There was a statistically significant difference between the groups (p value=0.001).

Serum creatinine:

The mean serum creatinine value for group 1A was 2.25±0.84mg/dL while that of group 1B was 1.27±1.03mg/dL and that of controls was 1.23±1.02mg/dL. There was a statistically significant difference between the groups (p value=0.001).

Urine microalbumin:

Urine microalbumin – mean value for group 1A was 85.90±72.93mg/L while that for group 1B was 13.44±5.96mg/L and that of controls was 10.08±5.99mg/L . It is found that urine microalbumin

levels are elevated in lupus nephritis group and the difference was found to be statistically significant (P value=0.001).

Urine creatinine:

The mean value for group 1A was 1.02±0.24g/L while that of group 1B was 1.08±0.31g/L and that of controls was 1.12±0.41g/L and there was no statistical difference between the groups (p value=0.297).

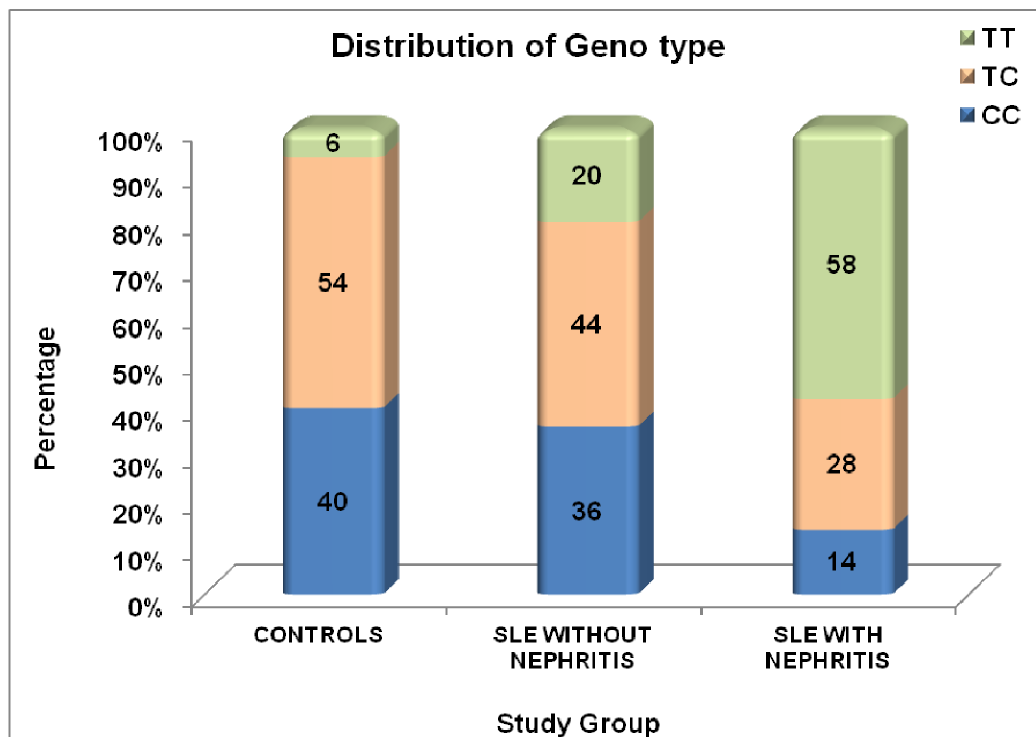
Urine Albumin Creatinine ratio:

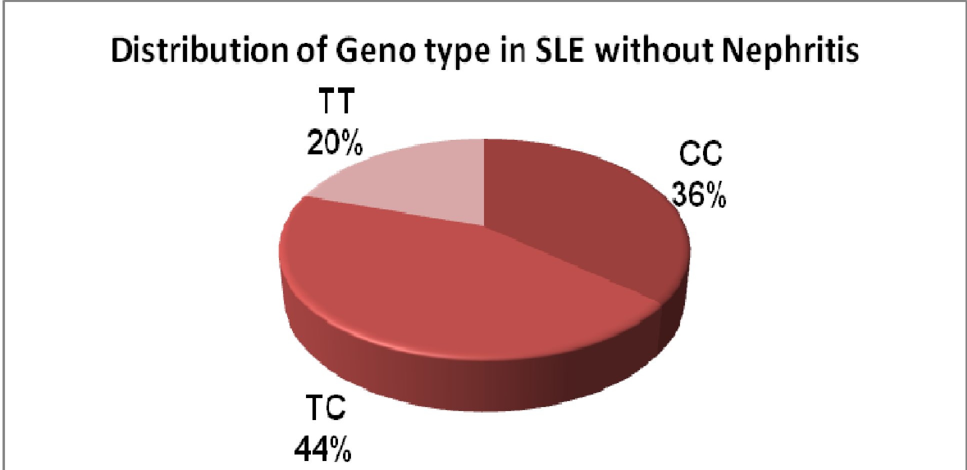
Urine Albumin Creatinine ratio – mean value of group 1A was 75.06±30.81mg/g of creatinine while that of group 1B was 12.78±6.07 mg/g of creatinine and that of controls was 8.71±4.13mg/g of creatinine and statistically the difference was found to be highly significant(p value=0.001). The results show that urine albumin creatinine ratio is highly elevated in lupus nephritis group.

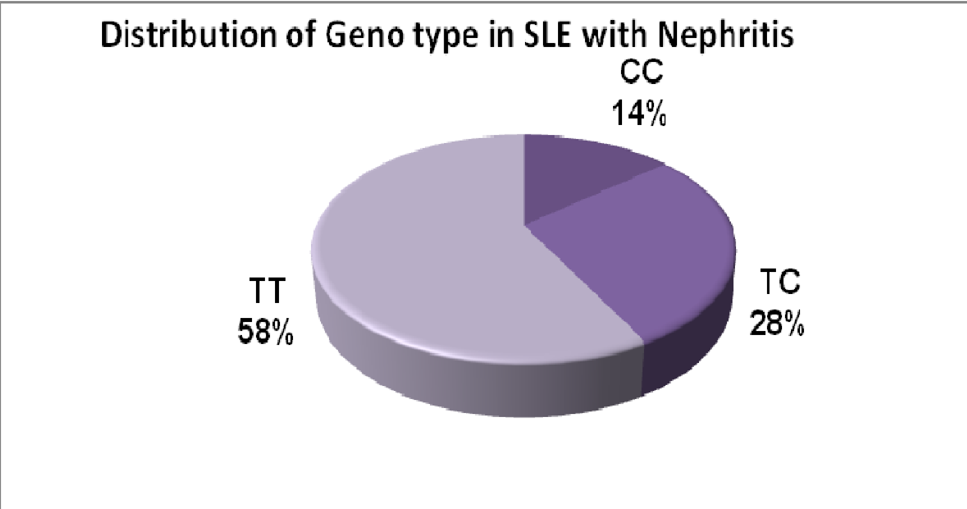
TABLE-2

OPN GENOTYPE DISTRIBUTION IN STUDY GROUPS

Genotype	Group1A- SLE with nephritis 50	Group1B- SLEwithout nephritis 50	Controls 50	P value
TT	29(58%)	10(20.0%)	3(6%)	**<0.001- S**
CT	14(28.0%)	22(44%)	27(54%)	
CC	7(14.0%)	18(36.0%)	20(40.0%)	




Distribution of Geno type in SLE without Nephritis
TT
20%
CC
36%
TC
44%


Distribution of Geno type in SLE with Nephritis
CC
14%
TT
58%
TC
28%

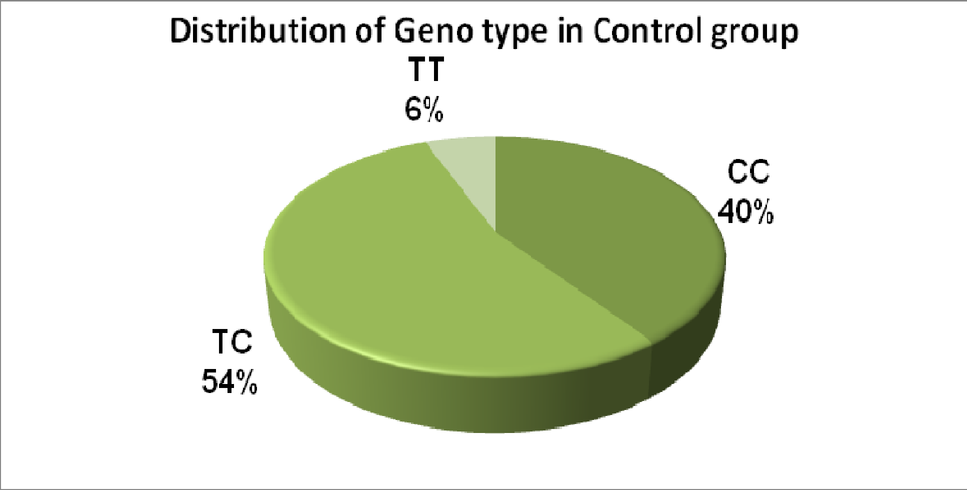

Distribution of Geno type in Control group
TT
6%
CC
40%
TC
54%

Table 2:

Shows the genotype distribution of OPN gene in Group1A (Lupus nephritis), Group 1B (SLE without nephritis) and controls (Group 2).

TT genotype was more frequently distributed among lupus nephritis patients 29(58%) compared to SLE without nephritis 10(20.0%) and controls 3(6%). There was a significant difference in the distribution of TT genotype between Lupus nephritis group and the other two groups as indicated by the P value (0.001).While TC and CC genotypes were distributed more in the SLE without nephritis and in controls when compared to lupus nephritis population.OPN genotype distribution was in agreement with the Hardy-Weinberg expectations.

TABLE -3:

ODDS RATIO FOR OPN GENOTYPES BETWEEN THE STUDY GROUPS

Genotype	Group 1A(SLE with nephritis) Vs Group 1B(SLE without nephritis)	Group1B(SLE without nephritis) Vs controls	Group 1A(SLE with nephritis) Vs controls
TT	7.46(2.41-23.10)	3.7(.88 -15.61)	27.62(6.37-119.8)
CT	1.64(0.54-4.92)	0.9(0.39 -2.12)	1.48(0.51-4.34)
CC	1.0	1.0	1.0

Table- 3:

Shows the odds ratio for OPN genotypes among the study groups. The odds ratio for TT genotype between group 1A and group 1B was found as 7.46 (CI 2.41-23.10) and Odds ratio of 3.7 (CI 0.88-15.61) between group 1B and controls and odds ratio of 27.62 (CI 6.37-119.8) for group 1A and controls.

The results show that TT genotype has 3 times higher association with SLE patients compared to controls.

Other genotypes do not show significant association.

TABLE 4:

ALLELE DISTRIBUTION IN SLE NEPHRITIS CASES AND CONTROLS

Allele	Group 1A-SLE with nephritis	Controls	P Value
T+	43(86%)	30(60%)	0.001 Odds ratio= 5
T-	7(14%)	20(40%)	

Table -4:

Shows the allele distribution of OPN gene between Lupus nephritis cases and controls.

It is found that the T+ allele was distributed more commonly in cases(86%) in lupus nephritis patients than controls(60%).

T+ allele occurs more frequently in cases than controls.

TABLE 5:

PLASMA OPN ACTIVITY IN STUDY GROUPS.

	Group 1A - SLE with nephritis	Group 1B- SLE without nephritis	Control	P value
Plasma OPN activity(ng/mL)	283.78±88.27	188.76± 56.71	107.78±67.06	<0.001-S

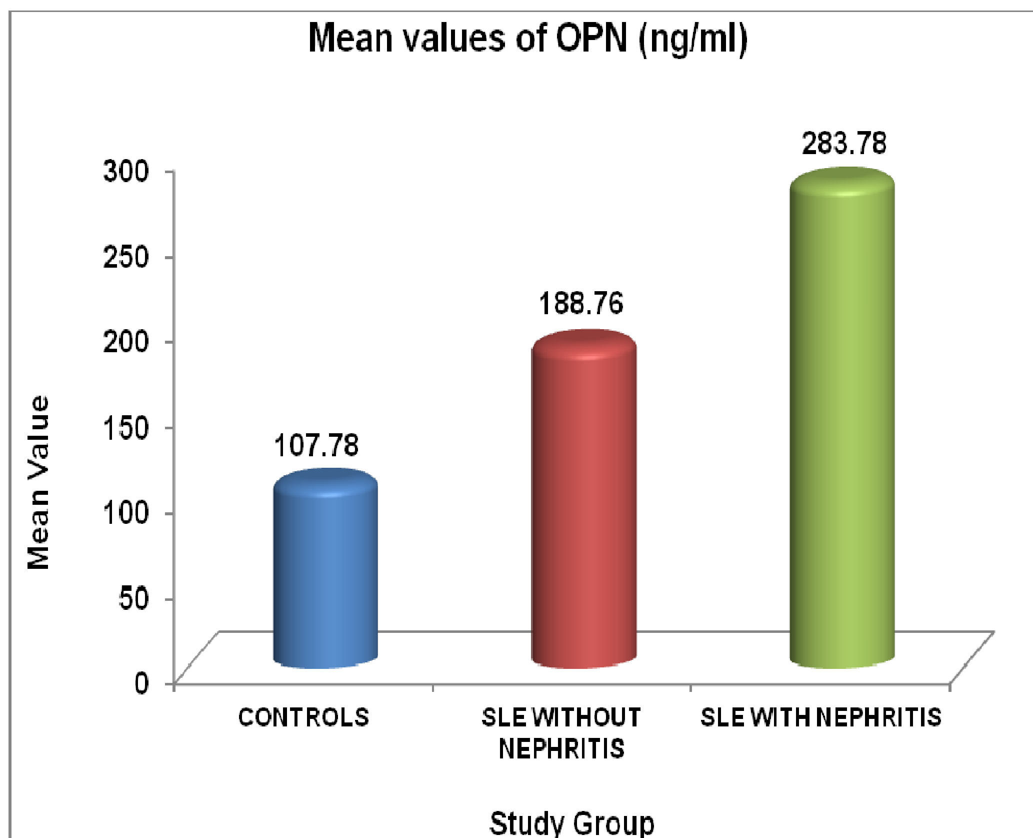


Table- 5:

Shows plasma OPN activity among the three groups. It is found that plasma OPN activity for group 1A(Lupus nephritis) was 283.78ng/mL while that of group 1B was 188.76ng/mL and that of controls was107.78ng/mL, P value of 0.001 indicates that the difference is statistically significant.

Our study results indicate that plasma OPN activity is elevated in lupus nephritis group compared to other groups.

Table- 6 :

Shows the association of OPN genotype with the phenotype(OPN activity)

OPN genotype and its phenotype(OPN activity) were compared.

It is observed that plasma OPN activity is elevated in TT genotype.

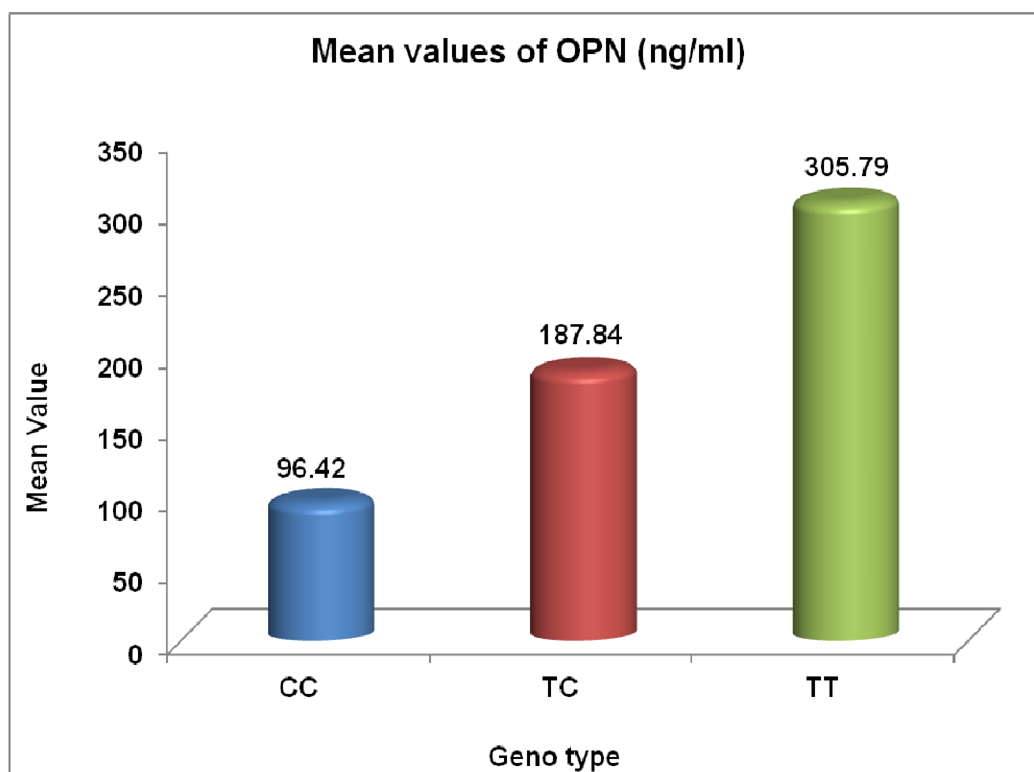
Highest (305.79ng/mL) level of OPN activity in TT genotype, lowest (96.42ng/mL) in CC genotype and intermediate(187.84ng/mL) in TC genotype .

P value =0.001 which is highly significant statistically.

TABLE 6 :

OPN ACTIVITY BETWEEN GENOTYPES

Genotype	OPN activity (ng/mL)	P value
TT	305.79±65.04	**<0.001-S**
CT	187.84±69.09	
CC	96.42±50.15	



DISCUSSION

SLE is an autoimmune disease in which organs and cells undergo damage mediated by tissue-binding autoantibodies and immune complexes. Along with a number of environmental threat factors, genetic factors are implicated in the occurance of SLE. OPN also called as Eta-1 is a competent protein in the extra-cellular matrix.The physiological role of OPN in our body includes adhesion of cells,production of cytokines and chemokines, migration of cell,signal transduction,regulation of immunologic activity,inhibition of cellular apoptosis . Osteopontin (OPN) is an important cytokine found to have key roles in inflammation and immunity.The role of OPN in the pathogenesis of SLE and lupus nephritis has been recognized in recent studies. We investigated a single nucleotide polymorphism of osteopontin gene with replacement of T for C nucleotide at 9250 position.This study is done to find the association of 9250C→T polymorphism with lupus in humans and to establish whether there is any facts that a genetic tendency to altered osteopontin expression could increase the plasma OPN in human SLE patients.

From our study we observed a more frequent association of TT genotype of OPN gene with lupus nephritis group patients (58%) when compared to controls and SLE patients without nephritis. The odds ratio between lupus nephritis and SLE patients without nephritis was found to

be 7 in our study. This is similar to a study done in Korea by Hye Ryoun Kim et al who found that a single nucleotide polymorphism (SNP) at position 9250 (C to T) in exon 7 of the osteopontin (OPN) gene is highly associated with the susceptibility to systemic lupus erythematosus (SLE) among Korean population. Katagiri et al[156] established that serum OPN level is elevated in SLE patients. Wong et al found that plasma concentration of OPN was strongly raised in SLE individuals than normal controls and that increased OPN concentration is related significantly with all SLE patients. Studies by Li et al[157]also revealed that OPN and its corresponding mRNA expression is augmented in peripheral blood mononuclear cells of SLE patients, while more apparent findings exist in lymphocyte too. A Study by Iizuka et al[158] observed that increased expression of OPN leads to enhanced B cell which causes increased anti-ds-DNA antibodies. The above mentioned studies suggest that the production of OPN is associated with SLE development and may serve as a potential disease marker of SLE.

The data of our study showed that the OPN gene 9250 polymorphism exists in the South Indian population. The odds ratio noted in our study for the association of T allele with lupus nephritis was found to be 3.55 . In our study we found that OPN activity is elevated in the lupus nephritis group with the mean value of 283.78ng/mL, while that of

SLE patients without nephritis it was188.76ng/mL and that of controls was 107.78ng/mL. The difference was found to be statistically significant (P value<0.001) between lupus nephritis cases(Group 1A) and controls(Group 2).

The frequency of TT genotype of the OPN gene 9250 was significantly higher (58% vs 6.0%,) in the SLE patients with nephritis than in the controls, and the frequency of TC genotype of the OPN gene 9250 was significantly lower (28% vs 54%, $P<0.001$) in the SLE patients with nephritis than controls. A significant difference was observed in the frequencies of OPN gene 9250 T allele between the SLE patients with nephritis and the controls (72% vs33% ,$P<0.05$), indicating the association of OPN gene polymorphism with SLE in South Indian population.

The OPN TT genotype is associated with increased circulating OPN levels, which are generally two times as high as those found for CC genotypes. CT heterozygotes are associated with intermediate OPN levels. Highest level(305.79ng/mL) of OPN activity in TT genotype, lowest level (96.42ng/mL) in CC genotype and intermediate level(187.84ng/mL) in CT genotype was observed in our study with significant statistical difference(p value<0.001).These findings are in concordance with other studies. Our study indicates that OPN gene

polymorphism is associated with the susceptibility of SLE in South Indian population. In summary, OPN gene 9250C→T polymorphism exists in the South Indian population which is apparently associated with SLE, indicating that the OPN gene might be the receptive gene of SLE.

CONCLUSION

This study was conducted to find out the association of Single nucleotide polymorphism at 9250 C→T in exon 7 of Osteopontin(OPN) gene among systemic lupus erythematosus patients with nephritis and without nephritis.50 cases of systemic lupus erythematosus patients with nephritis were compared with 50 systemic lupus erythematosus patients without nephritis and 50 healthy controls.

From our study we conclude that:

1. SLE patients with nephritis had a higher frequency of OPN TT genotype compared to SLE patients without nephritis and controls.
2. Plasma OPN activity is significantly elevated in SLE patients with nephritis which is responsible for the nephritis changes.
3. The level of Plasma OPN activity was highest in TT genotype, lowest in CC genotype and intermediate in CT genotype and hence TT genotype is strongly associated with nephritis.

4. TT genotype is an independant risk factor for the development of nephritis in SLE patients.

5. Osteopontin activity can be used as a parameter for assessing SLE risk and disease activity.

SCOPE FOR FURTHER STUDY

It is observed that the overexpression of OPN in SLE patients implies the possibility that OPN could participate in the pathogenesis of SLE. Along with OPN estimation in SLE,the cytokines produced by OPN such as interferon alpha,interleukin-12 could be estimated to confirm its action over them.

Future prospects:

1. OPN gene polymorphism screening can be done in families with SLE for risk of development of nephritis.
2. OPN gene polymorphism screening can be done to monitor progression of severity of disease.
3. OPN can be used as a potential disease marker of SLE.

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INSTITUTIONAL ETHICS COMMITTEE
MADRAS MEDICAL COLLEGE, CHENNAI -3

Telephone No: 04425305301
Fax : 044 25363970

CERTIFICATE OF APPROVAL

To
Dr. V. Yogeswari
PG in MD Biochemistry
Madras Medical College, Chennai -3.

Dear Dr. V. Yogeswari

The Institutional Ethics Committee of Madras Medical College reviewed and discussed your application for approval of the proposal entitled "Association of osteopontin gene polymorphism in systemic lupus erythematosus" No. 11072011.

The following members of Ethics Committee were present in the meeting held on 21.07.2011 conducted at Madras Medical College, Chennai -3.

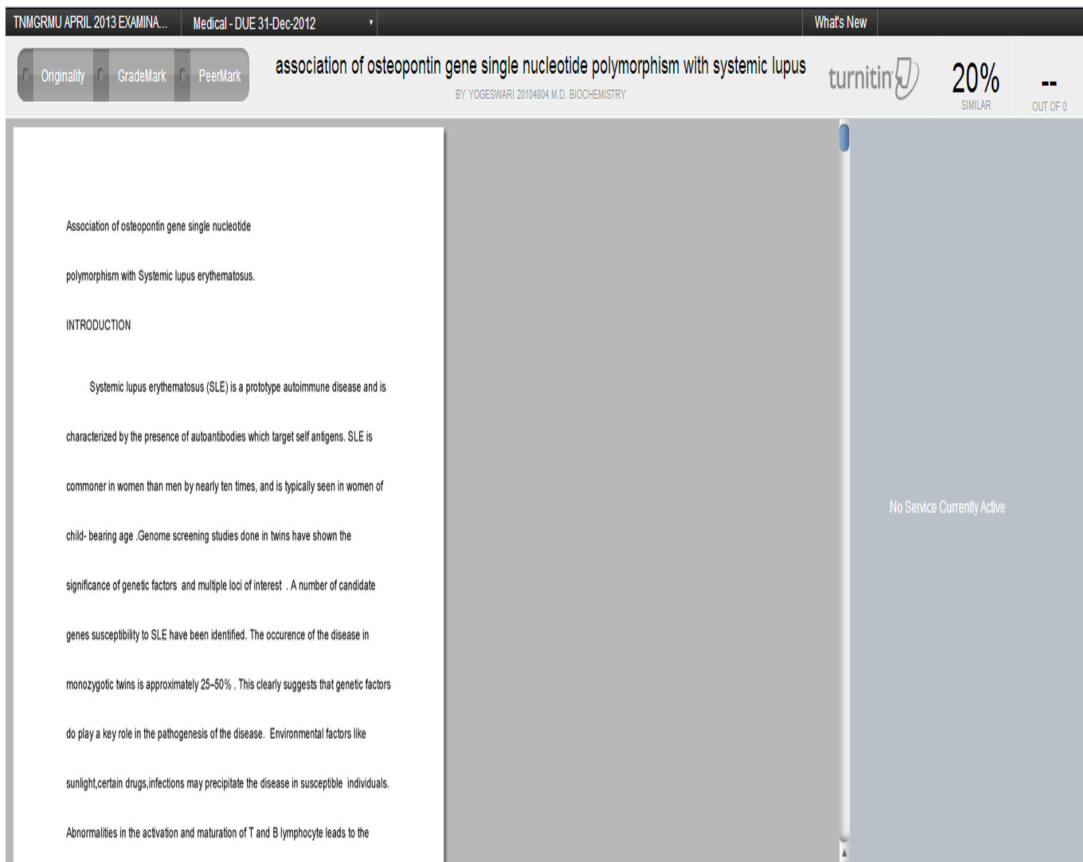
1. Prof. S.K. Rajan, MD -- Chairperson
2. Prof. V. Kanagasabai, MD -- Deputy Chairman
   Dean, Madras Medical College, Chennai-3,
3. Prof. A. Sundaram, MD -- Member Secretary
   Vice Principal, Madras Medical College, Chennai -3
4. Prof R. Sathianathan, MD -- Member
5. Prof R. Nandhini, MD -- Member
   Director, Institute of Pharmacology, MMC, Ch-3
6. Prof. Geetha Subramanian MD, DM -- Member
   Prof & Head, Dept. of Cardiology, MMC, Ch-3
7. Prof. Pregna B. Dolia, MD -- Member
   Director, Institute of Biochemistry, MMC, Ch-3
8. Prof. C. Rajendiran, MD -- Member
   Director, Institute of Internal Medicine, MMC, Ch-3
9. Thiru. A. Ulaganathan -- Layperson
   Administrative Officer, MMC, Chennai -3
10. Thiru. S. Govindasamy. BA.BL -- Lawyer
11. Tmt. Arnold Soulina MA -- Social Scientist

We approve the proposal to be conducted in its presented form

Sd /. Chairman & Other Members

The Institutional Ethics Committee expects to be informed about the progress of the study, any SAE occurring in the course of the study, any changes in the protocol and patient information / informed consent and asks to be provided a copy of the final report

Member Secretary, Ethics Committee


TNMGRMU APRIL 2013 EXAMINA...
Medical - DUE 31-Dec-2012
What's New
Originality
GradeMark
PeerMark
association of osteopontin gene single nucleotide polymorphism with systemic lupus
BY YOGESWARI 20104804 M.D. BIOCHEMISTRY
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SIMILAR
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OUT OF 0
Association of osteopontin gene single nucleotide
polymorphism with Systemic lupus erythematosus.
INTRODUCTION
Systemic lupus erythematosus (SLE) is a prototype autoimmune disease and is
characterized by the presence of autoantibodies which target self antigens. SLE is
commoner in women than men by nearly ten times, and is typically seen in women of
child- bearing age .Genome screening studies done in twins have shown the
significance of genetic factors and multiple loci of interest . A number of candidate
genes susceptibility to SLE have been identified. The occurence of the disease in
monozygotic twins is approximately 25–50% . This clearly suggests that genetic factors
do play a key role in the pathogenesis of the disease. Environmental factors like
sunlight,certain drugs,infections may precipitate the disease in susceptible individuals.
Abnormalities in the activation and maturation of T and B lymphocyte leads to the
No Service Currently Active

PROFORMA

NAME: DATE:

AGE: SEX: M/F

HOSPITAL ID:

ADDRESS:
CASE/CONTROL NO:

DIAGNOSIS:

PRESENTING COMPLAINTS:

PAST H/O:

DM:
HT:
THYROID DISORDER:
RENAL DISORDER:

FAMILY H/O: SLE : YES/NO

DRUG H/O :

MENSTRUAL H/O :

GENERAL EXAMINATION

Ht: Wt:

BUILT: THIN /MODERATE /OBESE

PALLOR/ICTERUS/PEDAL EDEMA

VITAL SIGNS:

B.P :

P.R :

R.R :

SYSTEMIC EXAMINATION:

CVS:

RS:

ABD:

CNS:

DIAGNOSIS:

INVESTIGATIONS:

BLOOD UREA:

SERUM CREATININE:

URINE CREATININE:

URINE MICROALBUMIN:

URINE ALBUNIN CREATININE RATIO:

RENAL BIOPSY FINDING:

OPN GENE POLYMORPHISM:

GENOTYPE:

Renal parameters and genotype of SLE cases with nephritis

S.No	Age	Sex	Blood Urea (mg/dl)	Serum Creatinine (mg/dl)	U. creatinine (g/dL)	U.Micro Albumin (mg/L)	Albumin Creatinine Ratio (mg/g of creatinine)	OPN Polymorphism	OPN (ng/ml)
1	19	F	74	0.70	1.00	60.00	60.00	TT	396
2	17	F	62	1.20	0.80	41.00	51.00	TT	342
3	30	M	78	3.00	0.90	118.00	131.00	TT	354
4	29	F	69	1.80	1.20	108.00	90.00	TC	337
5	22	F	79	3.60	0.90	59.00	66.00	TT	301
6	27	F	82	3.20	0.80	45.00	56.00	TT	400
7	23	F	70	3.70	1.10	79.00	72.00	TT	385
8	29	F	69	3.60	1.00	54.00	54.00	TT	349
9	40	F	65	2.30	1.10	132.00	120.00	TT	406
10	37	M	70	2.10	0.80	57.00	71.00	TC	370
11	32	F	80	3.00	1.10	43.00	38.00	CC	132
12	28	F	58	1.00	1.00	125.00	125.00	TT	198
13	29	M	62	1.20	1.40	115.00	82.00	TC	300
14	22	F	72	1.00	0.80	34.00	42.00	TT	398
15	20	F	70	3.20	0.90	78.00	87.00	TT	206
16	30	F	84	3.10	0.90	85.00	94.00	TT	391
17	27	F	72	2.50	1.90	86.00	43.00	CC	145
18	28	F	64	2.00	1.00	52.00	52.00	TT	383
19	22	F	54	1.60	0.90	43.00	48.00	TC	324
20	28	F	60	1.20	0.90	77.00	86.00	TT	244
21	30	F	70	3.00	1.30	109.00	84.00	TT	315
22	34	M	84	3.10	1.00	54.00	54.00	TC	296
23	19	F	96	3.40	1.00	100.00	100.00	TT	344
24	21	F	74	2.20	1.00	39.00	39.00	CC	106
25	30	F	78	1.20	0.80	40.00	50.00	TC	245
26	27	F	76	2.40	0.90	39.00	43.00	TC	340
27	24	F	80	3.00	0.90	83.00	92.00	TT	378

28	23	F	60	1.80	1.00	76.00	76.00	TC	255
29	40	F	66	1.00	1.10	73.00	66.00	TC	258
30	52	M	68	2.00	0.80	149.00	186.00	TT	305
31	46	F	70	3.10	0.90	118.00	131.00	TT	258
32	22	F	80	3.20	1.20	108.00	90.00	TC	279
33	48	F	76	2.40	0.90	59.00	66.00	TT	368
34	50	F	70	2.10	0.80	45.00	56.00	TC	265
35	46	F	68	1.20	1.10	79.00	72.00	TC	231
36	48	F	66	1.00	1.00	54.00	54.00	TT	264
37	25	F	54	1.80	1.10	132.00	120.00	CC	124
38	24	F	70	3.10	0.80	57.00	71.00	TT	189
39	26	M	74	3.20	1.10	43.00	38.00	CC	167
40	29	F	80	3.20	1.00	125.00	125.00	TT	215
41	23	F	76	2.40	1.40	115.00	82.00	TC	155
42	22	F	60	2.10	0.80	34.00	42.00	CC	169
43	18	F	64	2.00	0.90	78.00	87.00	TT	263
44	28	F	68	1.00	0.90	85.00	94.00	TT	238
45	27	M	76	2.00	1.90	86.00	43.00	CC	100
46	32	F	78	1.80	1.00	52.00	52.00	TT	348
47	46	F	80	2.80	0.90	43.00	48.00	TC	234
48	48	F	70	2.00	0.90	77.00	86.00	TT	345
49	51	F	86	2.10	1.30	109.00	84.00	TT	390
50	37	F	66	2.00	1.00	543.00	54.00	TT	384

Renal parameters and genotype of SLE cases without nephritis

S.No	Age	Sex	Blood Urea (mg/dl)	Serum Creatinine (mg/dl)	U. creatinine (g/dL)	U.Micro Albumin (mg/L)	Albumin Creatinine Ratio (mg/g of creatinine)	OPN Polymorphism	OPN (ng/ml)
1	29	F	30	1.10	0.80	10.00	13.00	TT	255
2	24	F	47	0.80	0.90	4.80	9.00	TC	201
3	42	F	34	0.90	1.00	17.00	17.00	TC	141
4	22	F	20	0.70	0.80	14.00	18.00	CC	140
5	32	M	27	0.90	0.60	4.30	8.00	TT	289
6	30	F	3	0.90	1.00	22.00	22.00	TT	300
7	27	F	32	1.00	1.10	21.00	19.00	CC	112
8	15	F	20	0.80	1.50	15.00	10.00	TC	198
9	30	F	28	0.80	1.40	18.00	24.00	TC	200
10	28	M	30	0.80	0.80	7.00	8.00	CC	134
11	20	F	24	0.70	0.80	9.00	1.00	TC	210
12	30	F	24	0.60	1.70	17.00	10.00	TT	260
13	24	F	42	0.90	1.20	13.00	11.00	CC	150
14	40	F	30	3.80	0.80	16.00	20.00	TC	180
15	37	F	20	4.00	0.90	10.00	11.00	CC	120
16	43	M	26	0.70	0.90	8.40	10.00	TC	213
17	34	F	77	0.90	1.30	18.00	14.00	CC	123
18	31	F	78	1.00	1.70	10.00	5.00	TC	245
19	25	F	22	0.60	1.40	22.00	15.00	CC	154
20	26	F	40	1.00	0.80	2.40	3.00	TC	211
21	50	F	19	0.90	0.90	12.00	13.00	CC	168
22	24	F	30	1.00	1.30	26.00	20.00	TT	264
23	32	F	60	1.50	1.00	5.00	5.00	CC	130

24	47	F	88	4.30	0.80	12.00	15.00	TT	311
25	24	F	60	1.00	1.00	19.00	19.00	CC	112
26	19	F	78	3.80	1.40	17.00	12.00	TC	154
27	35	M	30	0.90	0.90	20.00	22.00	TC	165
28	50	F	32	1.00	1.00	20.00	20.00	TC	149
29	25	F	30	0.90	1.10	10.00	9.50	TC	187
30	45	F	21	1.00	1.70	13.00	7.60	TC	255
31	21	F	20	0.60	0.80	10.00	13.00	TT	234
32	17	F	40	0.70	0.90	4.80	9.00	TC	198
33	20	F	36	1.10	1.00	17.00	17.00	TC	213
34	21	F	24	1.00	0.80	14.00	18.00	CC	134
35	41	F	26	0.70	0.60	4.30	8.00	TT	268
36	36	F	24	0.80	1.00	22.00	22.00	TT	300
37	25	F	89	4.10	1.10	21.00	19.00	CC	115
38	35	F	65	3.80	1.50	15.00	10.00	CC	154
39	21	F	56	1.20	1.40	18.00	24.00	TC	184
40	16	F	65	1.80	0.80	7.00	8.00	CC	140
41	30	F	32	1.00	0.80	9.00	1.00	TC	215
42	21	F	30	1.00	1.70	17.00	10.00	TT	285
43	24	F	47	0.80	1.20	13.00	11.00	CC	138
44	42	M	28	0.70	0.80	16.00	20.00	TC	144
45	41	F	30	1.00	0.90	10.00	11.00	CC	156
46	22	F	22	0.90	0.90	8.40	10.00	TC	214
47	28	F	26	0.70	1.30	18.00	14.00	CC	178
48	32	F	40	0.80	1.70	10.00	5.00	TC	145
49	29	F	30	1.00	1.40	22.00	15.00	CC	116
50	40	F	27	0.80	0.80	2.40	3.00	TC	176

Renal parameters and genotype of controls

S.No	Age	Sex	Blood Urea (mg/dl)	Serum Creatinine (mg/dl)	U. creatinine (g/dL)	U.Micro Albumin (mg/L)	Albumin Creatinine Ratio (mg/g of creatinine)	OPN Polymorphism	OPN (ng/ml)
1	29	F	34	0.90	1.00	3.00	3.00	TC	154
2	22	F	20	0.70	0.90	13.00	14.00	TC	164
3	27	M	27	0.90	1.30	17.00	13.00	CC	62
4	23	F	3	0.90	0.80	4.00	5.00	TC	140
5	29	F	32	1.00	2.20	18.00	8.00	CC	57
6	40	F	20	0.80	0.80	4.00	5.00	TC	32
7	37	M	28	0.80	2.00	10.00	5.00	CC	56
8	32	F	30	0.80	0.70	6.00	8.60	TC	167
9	28	F	24	0.70	1.20	20.00	16.00	TC	156
10	29	M	24	0.60	0.80	5.00	6.00	CC	45
11	22	F	42	0.90	1.10	7.00	6.00	CC	57
12	20	F	30	3.80	0.90	13.00	14.00	TC	41
13	30	F	20	4.00	1.00	6.00	6.00	TC	141
14	27	M	26	0.70	1.50	21.00	14.00	TC	245
15	28	F	77	0.90	2.00	16.80	8.40	CC	53
16	22	F	78	1.00	0.80	2.40	3.00	CC	29
17	28	F	26	0.70	1.00	9.00	9.00	TC	200
18	30	F	24	0.80	0.90	3.60	4.00	TC	170
19	34	M	89	4.10	1.00	7.40	7.40	TC	187
20	19	F	65	3.80	0.80	9.00	11.00	CC	90
21	21	F	56	1.20	0.80	2.00	2.50	CC	68
22	30	M	65	1.80	1.00	15.00	15.00	TT	268
23	27	F	32	1.00	1.20	19.00	16.00	CC	26
24	24	F	30	1.00	0.80	10.00	12.50	TC	134

25	23	F	47	0.80	1.60	16.00	10.00	CC	68
26	40	F	28	0.70	1.00	12.00	12.00	TC	167
27	52	M	30	1.00	1.40	18.00	13.00	TT	230
28	46	F	22	0.90	1.10	7.00	6.00	TC	140
29	22	F	26	0.70	1.00	4.50	4.50	CC	9
30	48	F	40	0.80	0.90	10.00	11.00	TC	119
31	50	M	30	1.00	1.00	3.00	3.00	TC	134
32	46	F	27	0.80	0.90	13.00	14.00	TC	145
33	48	F	47	0.80	1.30	17.00	13.00	CC	78
34	25	F	34	0.90	0.80	4.00	5.00	TC	124
35	24	F	20	0.70	2.20	18.00	8.00	CC	19
36	26	M	27	0.90	0.80	4.00	5.00	TC	134
37	17	F	3	0.90	2.00	10.00	5.00	CC	37
38	23	F	32	1.00	0.70	6.00	8.60	TC	117
39	22	F	20	0.80	1.20	20.00	16.00	TC	156
40	18	F	28	0.80	0.80	5.00	6.00	CC	33
41	28	F	30	0.80	1.10	7.00	6.00	CC	24
42	27	M	24	0.70	0.90	13.00	14.00	TC	137
43	32	F	24	0.60	1.00	6.00	6.00	TC	144
44	46	F	42	0.90	1.50	21.00	14.00	TT	222
45	48	F	30	3.80	2.00	16.80	8.40	CC	34
46	51	F	20	4.00	0.80	2.40	3.00	CC	47
47	37	F	26	0.70	1.00	9.00	9.00	TC	130
48	32	F	77	0.90	0.90	3.60	4.00	TC	27
49	19	F	78	1.00	1.00	7.40	7.40	TC	142
50	23	M	22	0.60	0.80	9.00	11.00	CC	30