Association of Osteopontin gene single nucleotide polymorphism with Systemic Lupus Erythematosus

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Abstract

Introduction: Osteopontin plays an important role in the pathogenesis of Systemic Lupus Erythematosus[(1)] and lupus nephritis. OPN may influence the autoimmune disease, SLE through its immunoregulatory effects, enhancing the pro-inflammatory Th1 cell response and inhibiting the Th2 responses.[(2,3)] We carried out this study to determine the association of osteopontin gene 9250 C→T polymorphism and increased plasma osteopontin activity with systemic lupus erythematosus.

Aim: The aim of the study was 1) To determine the association of single nucleotide polymorphism at 9250 C→T in exon 7 of OPN gene among the patients with SLE (with and without nephritis) and healthy controls. 2) To assess the plasma OPN activity among study groups and correlate their level with the genotype.

Materials and Method: The study population includes Group 1A: 50 SLE patients with lupus nephritis, Group 1B:50 SLE patients without lupus nephritis, Group 2: 50 age and sex matched healthy individuals. OPN gene 9250 polymorphism was detected by polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP). Plasma Osteopontin level was estimated by ELISA.

Results: A significant difference was observed in the frequencies of OPN gene 9250 T allele between the SLE patients with nephritis and the controls (72% vs 33%, $P<0.05$). 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).

Conclusion: There was a significant association between OPN gene 9250 C→T polymorphism and increased plasma OPN level with SLE.

Keywords: Osteopontin, Eta-1-Early T-lymphocyte activator, Systemic Lupus Erythematosus, Lupus nephritis.

Received: 10th May, 2017 **Accepted:** 18th August, 2017

Introduction

Systemic lupus erythematosus (SLE) is a clinically heterogeneous autoimmune disease characterized by the presence of autoantibodies directed against nuclear antigens. SLE is ten times more common in women than men, and typically has a predilection for women in their child-bearing years.[(4)] Earlier studies in Twin individuals and genomic screening in patients with SLE indicated the role of genetic factors, highlighting a number of potential loci of interest.[(5)] A number of candidate genes susceptible to SLE have been identified. SLE is characterized by hyperactive T and B cells, auto-antibody production, and immune complex(IC) deposition[(6)] in multiple organs causing end organ damage. Lupus nephritis is the most frequent and potentially serious complication of SLE. The major causes of pathogenesis of SLE include dysregulation and impaired clearance of apoptotic bodies, dysregulation of cytokine expression and dysregulation of T and B cells. Cytokines also play a direct role in pathogenesis of SLE. Osteopontin, being a cytokine is evaluated as therapeutic target in SLE. It is a potential biomarker for disease activity and serves as a candidate target of novel biologic agents.

Human plasma osteopontin(OPN), also called as early T lymphocyte activator 1(Eta-1) is a glycoprotein of 34 KDa, expressed upon activation during inflammation in endothelial cells, macrophages, and smooth muscle cells.[(7)] OPN plays an important role in the pathogenesis of SLE[(8)] and lupus nephritis. OPN as a member of Th1 cell play a role in promoting activation of T lymphocyte, regulating balance between T-helper 1 and T-helper 2 cells,[(9,10)] participate in cell-induced immunologic response and stimulate B cell to express multi-clone antibodies.[(11,12,13)] Cell-mediated immunity is necessary for immune protection against most intracellular pathogens, but overexpression can mediate organ-specific autoimmune destruction. This was confirmed by an in-vitro study showing that anti-osteopontin antibodies and drugs targeting OPN have decreased the severity of disease in mice. This provides the basis for the contribution of single nucleotide polymorphism of OPN at position 9250 in exon 7 to the susceptibility of SLE and lupus nephritis. This polymorphism is considered to be a major determinant of variation in plasma OPN activity. Individuals with T allele (TT/TC genotype) have higher activity of OPN than individuals with C allele(CC genotype).

In view of this we have evaluated the distribution of OPN polymorphism by RFLP and the concerned phenotype (OPN activity) was analysed by ELISA. The aim of the study was (a) to determine the association of single nucleotide polymorphism at 9250 C→T in exon 7 of OPN gene among SLE patients, with and without nephritis and healthy controls.(b) To assess the plasma OPN activity among study groups and correlate their levels with the genotype.

Materials and Method

The case-control study was conducted after obtaining ethical committee clearance. The study was carried out at Madras medical college and Rajiv Gandhi government general hospital.

Study Population

Cases: 100 SLE cases according to the ACR 1997 criteria[14] (American College of Rheumatology) attending rheumatology department of our hospital were included in the study and were categorised into **Group 1A:** 50 SLE patients with lupus nephritis, **Group 1B:** 50 SLE patients without lupus nephritis. Nephritis cases were included based on renal biopsy findings. Cases with clinical or laboratory evidence suggestive of mixed connective tissue disorders were excluded from the study. Written informed consent according to the declaration of Helsinki was obtained from all participants.

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

Sample collection: About 5 mL of blood was drawn from the antecubital vein of the subjects and collected in EDTA tube. The samples were centrifuged, plasma was separated and transferred into 2 mL eppendorf. Plasma was stored at -20°C for estimation of osteopontin. Early morning urine samples were collected in sterile plastic containers and Albumin Creatinine ratio was estimated. Blood Urea was estimated by GLDH method. Serum Creatinine was estimated by Modified Jaffe's Method. Urine Microalbumin was measured by Immunoturbidimetry method.

OPN Polymorphism Screening: DNA was extracted from buffy coat by high salt method and the 252bp target region in the OPN gene was amplified by PCR. Fig. 1 shows the 252bp osteopontin gene pcr product (lane 1 to 4) on 2.5% agarose gel with 50bp DNA ladder. Primers Used: Forward primer– 5'TACCCTGATGCTACAGACGAGG–3' and Reverse primer – 5'-CTGACTATCAATCACATCGGAATG – 3'. Genomic DNA (1µg) was amplified in 25µl reaction mixture containing 0.3µmol/L of each primer and red dye master mix (Bangalore Genei) containing 100µmol/L of each dNTP, 2.5µL of 10x reaction buffer and 0.6 unit of Taq DNA polymerase. After the DNA was denatured for 3 minutes at 95ºC, the reaction mixture was subjected to 35 cycles of denaturation for 30seconds at 94ºC, 45 seconds of annealing at 60ºC and 30 seconds of extension at 72ºC. Final extension was carried over at 72ºC for 10 minutes. OPN polymorphism was detected by digestion of the PCR amplified product with the Alu 1 restriction enzyme (10 units for 4 hours) followed by size fractionation in 3% Agarose Gel Electrophoresis. The 252-base pair (bp) PCR fragment was digested with the AluI restriction enzyme. The C allele had one AluI cleavage site and was digested to 147 and 105 bp fragments, whereas for the T allele, the 105 bp fragment was cleaved to 61 and 44 bp fragments. Heterozygous individuals (TC) cut with Alu1 generated fragments of 147 bp, 105 bp, 61 bp and 44 bp. Analysis was done using 25 bp DNA ladder from Bangalore genei. In Fig. 2 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.

Phenotype analysis: OPN activity in plasma was measured by using Solid phase sandwich ELISA. The OPN activity was expressed in ng/mL.

Statistical analysis: Age, blood urea, serum creatinine, urine microalbumin, urine creatinine and urine albumin creatinine ratio were compared between three study groups by ANOVA.OPN genotype frequency distribution and OPN activity was compared between the study groups by ANOVA. Statistical analysis was done using SPSS software.

Results

Patients had significantly higher frequency of T-allele than control subjects (0.72 versus 0.33; p value=0.001). The odds ratio between T-allele (TT+TC genotypes) and C-allele (CC genotype), for developing SLE with nephritis was 5.22(95% CI, 2.86 to 9.55; p=0.001) and SLE without nephritis was 1.47(95% CI, 0.83 to 2.62; p=0.189) as shown in Table 2. Significantly high plasma OPN activity was observed in SLE with nephritis (283.78± 88.27ng/ml; p=0.001) as compared to SLE without nephritis (188.76± 56.71 ng/ml; p=0.001) and healthy controls (107.78± 67.06 ng/ml; p=0.001). Age, blood urea, serum creatinine, urine microalbumin, urine creatinine and urine albumin creatinine ratio was compared between cases and controls by ANOVA. There was a highly significant difference in the values of blood urea, serum creatinine, urine microalbumin, and urine albumin creatinine ratio between the groups (p value=0.001) as shown in Table 1, 3 & Fig. 4 Shows the genotype distribution of OPN in Group 1A (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 highly significant difference in the distribution of TT genotype between Lupus nephritis group and the other two groups (p value =0.001). TC and CC genotypes were distributed more in SLE without nephritis than in patients with lupus

nephritis and in controls. OPN genotype distribution was in agreement with the Hardy-Weinberg expectations.

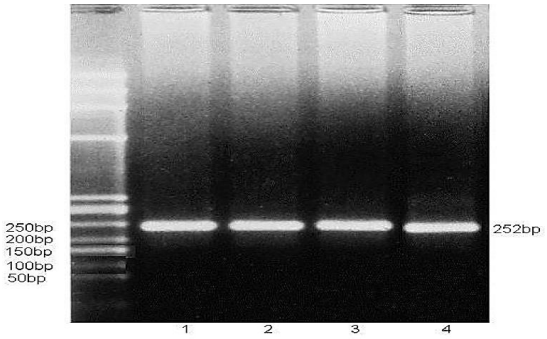

Fig. 1: Agarose gel electrophoresis of per products

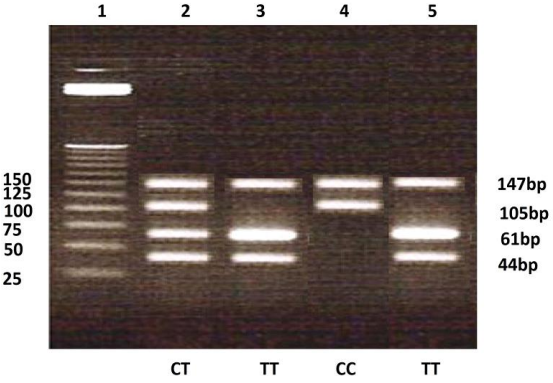

Fig. 2: Agarose gel electrophoresis of Alu-1 restriction digestion products

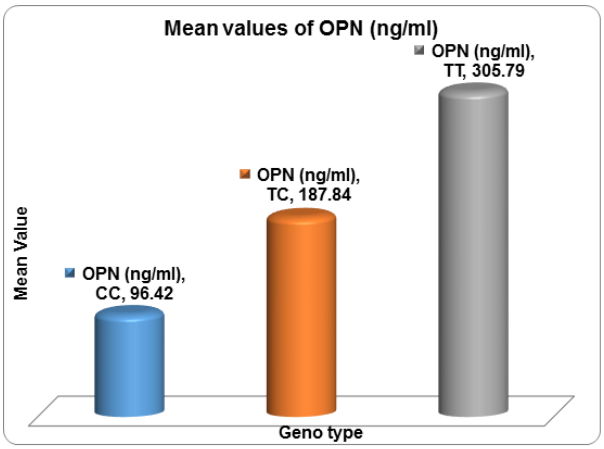

Fig. 3: Comparison of plasma OPN values among OPN genotypes

Table 5 & Fig. 3 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 highly significant. Our study results indicate that plasma OPN activity is elevated in lupus nephritis group compared to other groups.

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Table 1: Comparison of parameters between study groups

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
Blood Urea mg/dL	71.56±8.53	37.18±19.57	34.72±19.17	0.001**
Serum Creatinine mg/dL	2.25±0.84	1.27±1.03	1.23±1.02	0.001**
Urine Microalbumin mg/L	85.90±72.93	13.44±5.96	10.08±5.99	0.001**
Urine Creatinine g/L	1.02±0.24	1.08±0.31	1.12±0.41	0.297

Urine Albumin Creatinine ratio mg/g of creatinine	75.06±30.81	12.78±6.07	8.71±4.13	0.001**

** Highly significant
* Significant

Table 2: Odds ratio for OPN genotype 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: OPN Genotype distribution in study groups

Genotype	Group1A- SLE with nephritis n=50	Group1B- SLE without nephritis n=50	Controls n=50	p value
TT	29(58%)	10(20.0%)	3(6%)	<0.001**
CT	14(28.0%)	22(44%)	27(54%)	
CC	7(14.0%)	18(36.0%)	20 (40.0%)	

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**
T-	7(14%)	20(40%)	Odds ratio= 5

Table 5: Plasma OPN activity among study groups

OPN activity	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**

Table 6: OPN activity among the genotypes

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

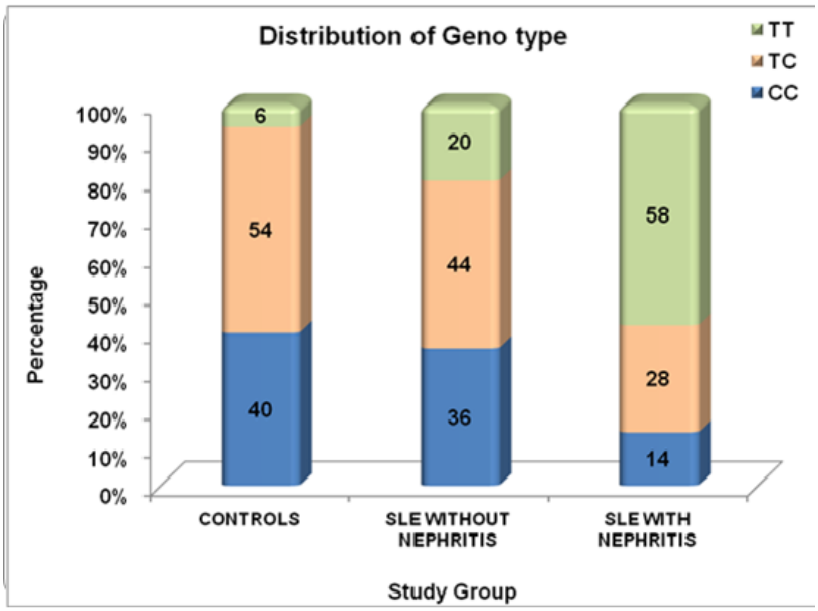

Fig. 4

Discussion

Systemic lupus erythematosus (SLE) is a typical autoimmune disease characterized by abnormal immunologic response. Activation of T and B lymphocytes leads to production of multifarious autoantibodies resulting in tissue damage. Lupus nephritis (LN) is the most common lethal manifestation of SLE, associated with abnormal production of cytokines. OPN induces B lymphocyte to produce polyclonal antibodies.[16] OPN carries out a variety of function in the body which includes early T lymphocyte activation, increases T-helper 1 cell population and decreases T-helper 2 cells contributing to cell-mediated immunologic response.[17,18]

Several studies[19,20] have found that OPN is an essential component in the pathogenesis of SLE. 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.[21]

Humans with SLE overexpress OPN gene suggesting its role[22] in the pathogenesis of the disease. Enhanced expression of OPN is particularly associated with varying degrees of renal damage. OPN is upregulated in different types of renal damage.[23] Crescentic glomerulonephritis[24] is associated with enhanced production of osteopontin. Humans and mice with SLE show overexpression of OPN in plasma at confined sites of renal inflammation and is responsible for the clinical features of lupus in SLE. This provoked the present study of OPN gene single nucleotide polymorphism in the region of exon 7 in SLE patients. Multiple polymorphisms in the coding gene of the human OPN were identified in diverse populations, 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.[25]

Conclusion

From our study we conclude that in the South Indian population, SLE patients with nephritis had a higher frequency of TT genotype compared to SLE patients without nephritis and controls. Plasma OPN activity is significantly elevated in SLE patients with nephritis which is responsible for the nephritic changes. 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.TT genotype is an independent risk factor for the development of nephritis in SLE patients. OPN activity can be used as a parameter for assessing SLE risk and disease activity.

Acknowledgement

I sincerely expresses my gratitude to the Professor, Assistant professor, Statistician, the patients and the persons from whom blood samples were collected and family members for the moral support and encouragement extended by them which gave fulfillment to the study work.

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