Research Article

Osteopontin 9250 C/T Gene Polymorphism in Egyptian Lupus Nephritis Patients

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ABSTRACT

Objective: To investigate the possible association of osteopontin 9250 C/T (rs 1126616) SNP with the development of lupus nephritis among a cohort of Egyptian lupus patients and evaluate its correlation with laboratory and clinical data in addition to both activity and chronicity indices (according to NIH).

Methods: Detection of osteopontin 9250 C/T (rs 1126616) SNP for 100 lupus nephritis patients and 100 controls by PCR-RFLP

Results: TT, CT+TT genotypes, and T allele were significantly higher in Lupus Nephritis (LN) group versus controls(p=0.005, 0.048, <0.001 respectively).No statistically significant increased TT and CT+TT genotypes and T allele frequencies in LN patients with renal failure compared to those without renal failure. A non-significant association was found between rs1126616 genotypes and each of the demographic data, laboratory data, and clinical features in addition to activity and chronicity indices in all studied LN patients. Logistic regression analysis showed that only OPN (CT+TT) genotype could be considered a predictor of LN development in Egyptian patients.

Conclusion: OPN 9250TTand CT+TT genotypes and T alleles are considered risk factors for lupus nephritis development in Egyptian SLE patients. However, these genotypes showed no association to each of the demographic data, laboratory data, and clinical features in addition to activity and chronicity indices in all studied LN patients. OPN 9250(CT+TT)genotype could be used as a predictor of LN development in SLE.

INTRODUCTION

SLE is a multisystem autoimmune disease in which immune dysfunction is affected by environmental, genetic, and hormonal factors [1]. B and T cells activation is disrupted in this disease, leading to abnormal expression of cytokines and autoantibodies production. This expression leads to the formation of antigen-antibody complexes that deposit in different organs leading to organ inflammation and tissue damage [2]. Lupus Nephritis (LN) is a frequent and severe complication of SLE [3]. It affects SLE prognosis regarding the kidney survival rate, patient survival, and quality of life [4]. Osteopontin (OPN) is a glycoprotein of 34 KDa, expressed upon activation during inflammation in macrophages, smooth muscle, and endothelial cells [5]. It is an essential player in the pathogenesis of SLE and lupus nephritis. As a member of the Small Integrin-Binding Ligand N-Linked Glycoproteins (SIBLINGs) family, OPN plays a

role in activating T cells and controlling TH1 & TH2 lymphocytes function through Interleukin (IL)-10 dependency. They also participate in immunologic response and stimulate B cells to produce different antibodies [6]. OPN also promotes the differentiation of T_{FH} and T_{FR} through expression in T_{reg}, thus plays a role in regulating the quantity and quality of humoral immunity [7].

High OPN levels were proved to differentiate SLE patients from healthy subjects [8,9]. Associations between OPN level and SLE disease activity [10], as well as with organ damage [11], have also been observed. Furthermore, high OPN levels have been reported to precede organ damage [12]. In lupus-prone mice, OPN over expression was found to activate B-cell leading to anti-ds DNA antibodies production, a diagnostic marker of SLE [13].

Genome-wide association studies and SNP arrays have identified different genetic variants associated with autoimmune diseases. These polymorphisms can affect susceptibility to disease, clinical presentations, and response to therapy [14,15]. Cytokines production and function are also affected by the polymorphisms in sequences of their genes [16]. These cytokine genetic variants are associated with different autoimmune diseases, including SLE [17]. Different inflammatory mediators, including osteopontin, are now under investigation from the perspective of autoimmune diseases.

AIM OF THE STUDY

To investigate the possible association of osteopontin 9250 C/T (rs 1126616) SNP with the development of lupus nephritis among a group of Egyptian lupus nephritis patients and to evaluate its correlation with laboratory and clinical data in addition to both activity and chronicity indices.

SUBJECT AND METHODS

The Mansoura Faculty of Medicine Institutional Research Board "MFM-IRB" approved this study protocol (Approval no. R/20.06.902). All methods were performed following relevant guidelines and regulations. Informed consent was obtained from all participants.

This study was conducted on two hundred individuals subdivided into two groups. The patient group included one hundred lupus nephritis patients: 5 males and 95 females, with their age 34.6 ± 9.6 years (mean ± SD). The control group included one hundred apparently healthy subjects: 4 males & 96 females, their age 34.6 ± 9.2 years (mean ± SD). Patients and controls gave complete history. They were subjected to clinical examination and laboratory investigations, including ESR, CBC, anti-DNA antibodies, C3, C4 levels, serum creatinine level, and urine analysis for proteinuria & hematuria. All patients were subjected to renal biopsy for lupus nephritis staging and measurement of activity and chronicity indices. 2.5 ml venous blood was withdrawn from all subjects and collected in tubes containing EDTA, stored at -20°C until the time of assay. DNA extraction: was performed using QIAamp DNA Mini Kit (50), Catalog no.51304.

Genotyping of Osteopontin 9250 C→T (rs1126616) was done using the PCR–RFLP technique [3]. In a 25µl reaction mixture, DNA (1µg) was amplified. The mixture contained 0.3µmol/L of each primer and master mix (EmeraldAmp® GT PCR Master Mix (2X) code No. RR310A). Primers used included:

Forward primer– 5'TACCCTGATGCTACAGACGAGG –3' and Reverse primer – 5'-CTGACTATCAATCACATCGGAATG – 3'.

In Arktik Thermal Cycler (Finland), DNA was denatured for 3 min at 95°C. The reaction mixture was subjected to 35 cycles of denaturation (94°C for 30 sec), annealing (60°C for 45 sec), and extension (72°C for 30 sec). The final extension was carried over at 72°C for 10 min. RFLP was done by mixing 1.0 ul of Restriction Enzyme (RE) Thermo Scientific™ FastDigest AluI (FD0014), 10 ul of PCR products, 2.0 ul 10x Fast Digest green buffer, 17ul nuclease-free water, incubating the mixture at 37°C (10 min) then heating at 65°C (10 min). The digested fragments were resolved in 2.5% agarose gel and ethidium bromide staining and visualized under UV trans-illuminator. Digestion of PCR products yielded: for (CC genotype): 147 and 105 bp, while for the (TT genotype): 147, 61, and 44 bp. Heterozygous individuals (TC) included 147, 105, 61, and 44 bp fragments, as shown in (Figure 1).

STATISTICAL ANALYSIS

It was done by using SPSS version 20. Qualitative data were in the form of numbers and percentages. Qualitative data were described as mean and standard deviation. A P-value less than (0.05) means statistical significance. According to the Hardy-Weinberg formula, the genotype frequencies of each group were examined using the Chi-Square test on the observed

versus the expected frequencies. The Chi-Square test was used to describe genotype and allele frequencies between the studied groups. The Mann-Whitney test was used for the difference in disease parameters in lupus nephritis.

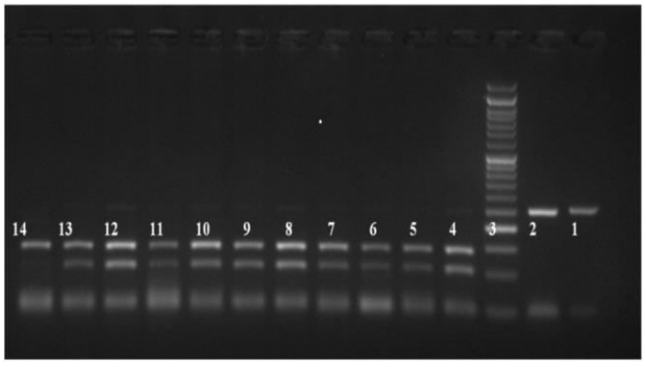

Figure 1: Gel Electrophoresis of Alu-1 Re Products. A gel electrophoretic image showing the bands for the different genotypes.

Lane1&2: PCR product at 252bp.
Lane3: 50 bp DNA ladder
Lane4-13: TC genotype (147, 105, 61 bp).
Lane14: TT genotype (147, 61 bp).

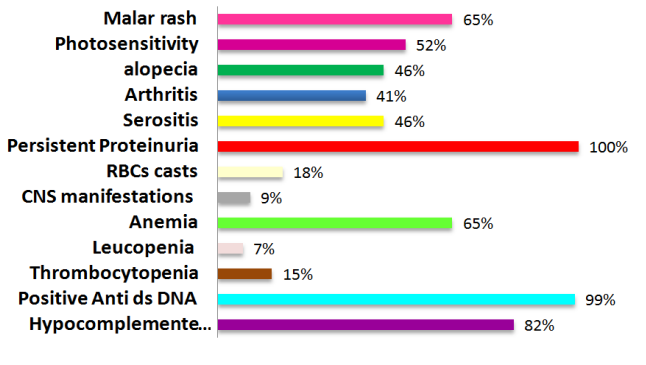

Figure 2: Clinical and laboratory data of studied patients. Shows the different clinical manifestations and laboratory parameters of the studied patients.

RESULTS

As shown in (Figure 2); clinical criteria of LN patients in the present study, the most common presentations were Malar rash (65%), photosensitivity (52%) followed by alopecia (46%) and arthritis (41%), while the least manifestations were thrombotic formation (13%), CNS affection (9%) as complications of the disease. Regarding laboratory data, 100% of patients show proteinuria, 99% show positive anti-ds-DNA, 82% show hypocomplementemia, and 65% show anemia. (Figure 2) shows the clinical and laboratory data of lupus nephritis patients.

Comparison of laboratory data between patients and control shows a statistically significant decrease in C3 and C4 concentrations in lupus nephritis patients while ESR at 1st and 2nd hours and creatinine are significantly higher in patients versus controls. Data not shown. Renal biopsies were performed for all patients, and classification was done according to ISN/RPS lupus nephritis classification. Class I & II were found in 6% of patients, class III in 28% of patients, class IV in 44% patients, class V in 14% of patients, and class VI was found in 2% of patients. Data not shown Taking rs1126616 CC genotype as the reference genotype and C as the reference allele; TT, CT + TT genotypes and T allele showed higher frequency in LN versus control groups (p= 0.005, 0.048, <0.001 respectively) with risk to develop lupus nephritis in SLE patients (OR = 2.131, 1.656, 1.617 respectively). (Table 1). There are increased TT and CT + TT genotypes and T allele frequencies in LN patients who were complicated with renal failure compared to those without renal failure. However, this increase shows non-statistical significance (Table 2).

Table 1: Comparison of OPN rs1126616 genotypes and alleles between LN patients and controls.

	Controls N=100		LN patients N=100		*P*	Odds Ratio (OR)	95% Confidence Interval. (CI)
	N	%	N	%			
CC	20	20	10	10	-	1	Reference
CT	45	45	31	31	0.476	1.219	0.707-2.100
TT	35	35	59	59	0.005*	2.131	1.253-3.623
CT+TT	80	80	90	90	0.048*	1.656	1.004-2.733
C	85	42.5	51	25.5	-	1	Reference
T	115	57.5	149	74.5	<0.001*	1.617	1.243-2.104

*Significant p<0.05

Table 2: Comparison of OPN rs1126616 genotypes and alleles frequencies between LN cases with and without Renal Failure (RF).

	No RF N=88		RF N=12		*P*	OR	95% CI
	N	%	N	%			
CC	9	10.2	1	8.3	-	1	Reference
CT	30	34.1	1	8.3	0.416	0.567	0.145-2.221
TT	49	55.7	10	83.3	0.571	1.385	0.449-4.266
CT+TT	79	89.8	11	91.7	0.836	1.125	0.370-3.416
C	48	27.3	3	12.5	-	1	Reference
T	128	72.7	21	87.5	0.113	1.630	0.890-2.984

No significant association was found between rs1126616 genotypes and any demographic data, laboratory data, or SLE features in all studied LN patients. Data not shown. No significant association was found in histopathological findings between rs1126616 genotypes and both activity and chronicity indices in all studied LN patients (Table 3). Logistic regression analysis was conducted using age, gender, and OPN genotype as covariates. Only OPN (CT + TT) genotype is considered a predictor of LN development in Egyptian patients- (Table 4).

Table 3: Association between rs1126616 genotypesand both Activity Index (AI) and Chronicity Index (CI) in all studied LN cases.

	CC N=10		CT N=31		TT N=59		CT+TT N=90		P^1	P^2
	Median	range	median	range	Median	range	median	range		
AI	6	0-21	5	0-21	6	0-22	6	0-22	0.754	0.600
CI	0.5	0-5	1	0-9	2	0-16	2	0-16	0.275	0.141

P1: Comparison between CC, CT, TT; p2, comparison between CT+TT versus CC

Table 4: Regression analysis for prediction of development of LN in Egyptian patients.

	P	OR	95% CI
Age	0.964	1.001	0.971-1.031
Gender	0.734	0.792	0.206-3.039
OPN (CT+TT)	0.048*	1.656	1.004-2.733

*Significant p<0.05

DISCUSSION

Osteopontin (OPN) is a member of the SIBLINGS family that plays a role in promoting T lymphocyte activation and regulating the balance between T-helper 1 and T-helper 2 cells [18]. It participates in cell-induced immunologic response and stimulates B cells to express multi-clone antibodies [19-21]. Several studies reported that OPN is an essential component in the immunopathogenesis of SLE and lupus nephritis. Others reported that OPN gene polymorphism caused an increase in its level, which is associated with SLE susceptibility and clinical manifestations of the disease in humans [22]. The polymorphic OPN alleles were implicated in the mouse model of lupus [23]. The authors assumed that OPN gene polymorphism induces enhanced immunoglobulins expression: IgG3, Ig2a, and IgM and cytokines: IFN-γ, TNF- α, IL-1b, which play an essential role in lupus mice models and human SLE development. The association between different OPN gene polymorphisms and SLE susceptibility in different human populations has also been investigated [24-28].

D'Alfonso, Barizzone [24] suggested that OPN genetic variants have a crucial role in creating a background favoring lymphocyte accumulation and leading to autoimmunity development. They assumed that OPN might exert its effect through either its capacity to stimulate proliferation and to inhibit apoptosis of lymphocytes or through its ability to modulate the immune response by inducing Th1 responses and potentiating polyclonal activation of B cells.

Few studies regarding the association of the OPN rs1126616 (9250 C/T) polymorphism and lupus nephritis are available, and to our knowledge, this is the first study in the Egyptian population. We observed a higher frequency of TT genotype of OPN (rs1126616 SNP) in lupus nephritis patients (59%) when compared to controls (35%). This was statistically significant ($p<0.005$, OR = 2.131). The frequency of T-containing genotype (CT+TT) also was significantly higher (90% vs. 80%) in the LN patients than controls ($P<0.048$, OR= 1.656). Furthermore, a significant increase in the frequencies of T allele in the SLE patients with nephritis was observed when compared to the controls (74.5% vs. 57.5%, $P<0.001$, OR = 1.617), indicating that TT genotype, combined (CT+TT) genotype and T allele could be considered risk factors for susceptibility to LN in Egyptian patients.

In a study among the Korean population, Kim and Song [29] reported that SNP at position 9250 (C to T) in exon 7 of the osteopontin gene was highly associated with the susceptibility to SLE. Han, Guthridge [30] also reported that minor alleles of rs1126616 and rs9138 (T and C, respectively) were correlated with a higher risk of SLE in European-American and African-American male populations. Like our results, Yogeswari, Nirmaladevi [3] observed that the TT genotype frequency of the OPN gene 9250 was significantly higher (58% vs. 6.0%) in SLE patients with nephritis than in the controls. A significant difference was observed in the T allele frequencies between the SLE patients with nephritis and the control group (72% vs. 33%, $P<0.05$), indicating the association of OPN gene polymorphism with the development of LN in the South Indian

population. They also proved that the OPN TT genotype is associated with increased circulating OPN levels.

Contrary to our results, Yogeswari, Nirmaladevi [3] found that TC genotype frequency was significantly lower in SLE patients with nephritis than in the control group. On the other hand, Salimi, Noora [31] genotyped the rs1126616 SNP in SLE patients and age, gender, and ethnically matched controls. They found no association between the polymorphism and SLE susceptibility. However, CT and TT genotypes' frequency was higher in SLE patients with LN than in those without LN. Also, no correlation between OPN serum levels and rs1126616 polymorphism was found. Additionally, they observed higher serum OPN levels in SLE patients and SLE patients with LN and joint symptoms. They suggested that OPN production was correlated with the inflammatory process.

In a study in the Chinese Han ethnic population, Xu, Bai [26] demonstrated that SNP in exon 7 of the OPN gene (9250C/T) was associated with SLE susceptibility. However, the TT genotype was lower in SLE patients with lupus nephritis compared to controls. The contradiction among results of different studies might be due to genetic variability within different ethnic groups and the influence of other factors that may affect the disease pathogenesis's inflammatory reactions. The current study results revealed increased frequencies of TT and CT+TT genotypes and T allele in LN patients who were complicated with renal failure compared to those without renal failure. However, this increase showed non-statistical significance due to the small sample size and low statistical power.

On comparing the distribution of OPN (9250T/C) genotypes to activity and chronicity indices in addition to demographic data, laboratory data, and SLE features of studied LN patients, no significant associations were found. In agreement with our results, Salimi, Noora [31] showed no correlation between the rs1126616 polymorphism and other SLE manifestations. A study by Forton, Petri [25] showed that the polymorphic T allele of the polymorphism at position 707 in exon 7 (707C/ T, rs1126616) is associated with opportunistic infections and renal insufficiency but is protective for a vascular necrosis in Caucasian SLE patients. Regarding gender, in a study of the Chinese Han ethnic population, Xu, Bai [26] demonstrated that SNP in exon 7 of the OPN gene (9250C/T) showed significant differences of frequencies in TT genotype and TC genotype and allele in women, but not in men. Han, Guthridge [30] reported that the frequency of the 750T and 1239C minor alleles of the OPN gene were associated with a higher risk of SLE in males only in European American and African American populations. Regarding the association between OPN SNPs and clinical features of the disease, D'Alfonso, Barizzone [24] scanned the coding 5' and 3' flanking regions of the OPN gene in SLE patients and revealed the correlation between the 156G allele in the promoter and +1239C allele in the 3'untranslated region of the OPN gene and SLE susceptibility. Besides, an association between lymphadenopathy and 156 genotypes was observed.

Trivedi, Franek [28] also showed a significant association between the 1239C allele and SLE patients' photosensitivity. Additionally, an association between the C allele with thrombocytopenia and hemolytic anemia in patients was identified. In the current study, logistic regression analysis was conducted using age, gender, and OPN genotype as covariates. Only OPN (CT+TT) genotype is proved to be a predictor of the development of LN in Egyptian patients.

CONCLUSION

From the present study, we can conclude that OPN 9250 TT, combined (CT+TT) genotypes and T allele, are considered risk factors for developing lupus nephritis in Egyptian patients. However, these genotypes are not associated with each of the demographic data, laboratory data, and clinical features in addition to activity and chronicity indices in all studied LN patients. OPN 9250 (CT+TT) genotype could be used to predict lupus nephritis development in Egyptian SLE patients. This study can be considered the basis for coming projects to identify the possible polymorphisms associated with different SLE patients' complications in the Egyptian population to recognize the potential genetic susceptibility and hopefully lower the morbidity risks for these patients with an adequately planned prevention and early intervention.

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