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Clinical implication of ACE gene polymorphism in Systemic Lupus Erythematosus patients

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ABSTRACT

Systemic Lupus Erythematosus is a complex genetic disease, associated with environmental and genetic factors. Angiotensin-converting enzyme (ACE) plays an important role in the development of SLE, is expressed in a wide range of tissues including lung, vascular endothelium, kidney, cardiac muscles and testis. ACE gene I/D polymorphisms, particularly the DD genotype has been associated with lupus susceptibility and with risk of renal and cardiovascular disease. This study analysed the ACE gene polymorphic variants among the recruited 48 SLE patients (SLE was confirmed by Anti nuclear antibody (ANA) and dsDNA tests) associated with the cardiovascular and renal disease and 48 controls. The present study also analyzed the biochemical parameters (total serum cholesterol HDL, LDL, VLDL, TGL) and C-reactive protein (CRP) in SLE patients and controls. Our study found that, SLE patients had higher levels of cholesterol (40%) than controls and the and CRP was found to be higher in SLE patients in comparison with controls and established normal reference range (<5mg/L). The distribution of genotype in SLE patients was DD 62.50%, ID 20.83% and II 16.66%. A significantly higher frequency of DD genotype ($p<0.0004$) was observed in SLE patients in comparison with controls.

Keywords: Systemic Lupus Erythematosus, ACE, CRP, Anti nuclear antibody, Cholesterol.

INTRODUCTION

Systemic Lupus Erythematosus (SLE) is a complex, chronic, multi-system autoimmune disease which varies in prevalence and incidence depending on ethnicity (Lau *et al.,* 2006; D'Cruz *et al.,* 2007). While the precise etiology of SLE still remains vague, genetic predisposition, environmental and hormonal factors are deemed to play important roles in its pathogenesis. Severity, acquisition risk and clinical manifestations of this disease can vary by ethnicity, geography and sex, with a prevalence

that is higher in women during their childbearing ages and some non-European populations such as African Americans, Hispanics and Asians (Lau *et al.,* 2006; Voskuhl, 2011). Genetic, hormonal, environmental and immunoregulatory factors contribute to the expression of the disease (Kammer *et al.,* 1999).

Genetic studies have demonstrated a strong contribution of many gene variants to SLE incidence and the clinical manifestations of SLE (Wong and Tsao, 2006; Hom *et al.,* 2008; Flesher *et al.,* 2010).

SLE is a complex prototypic autoimmune disease that predominantly affects women; the hallmark of SLE is the generation of auto antibodies that react with self nuclear and cytoplasmic antigens, culminating in immunologic attacks to body organs (Petri, 2006). With very distinct forms of presentation, includes articular and mucocutaneous involvement, renal disease, hematological abnormalities and central nervous systems disease (Cervera *et al.,* 1993). It is a multisystem disease with a variable course and a wide range of clinical manifestations (Gudmundsson and Steinsson, 1984). Among a range of factors that are thought to be contributing to the pathophysiology of SLE, chronic inflammation is thought to play a pivotal role in the pathogenesis of SLE (Alarcon-Segovia *et al*., 2005).

Several studies have shown that the C-reactive protein (CRP) levels in SLE patents are abnormally elevated both in the absence and presence of infection (Royand Tan, 2001). The value of using CRP to monitor SLE disease activity has remained in practice. Serum lipids can be used to identify patients with SLE who are at increased risk of renal dysfunction (Font *et al.,* 2001).

Rheumatoid factor (RF) and anti nuclear antibody (ANA) are associated with cardio vascular disease (CVD) events and overall mortality both in those with and without rheumatic diseases. Inflammation (as studied through elevations in CRP and/or Erythrocyte Sedimentation Rate (ESR)) contributes to increased risk of CVD, both in patients with autoimmune diseases (Danesh *et al*., 2004). ESR develops in 5–20 % of patients with SLE and renal involvement (Cortes-Hernandez *et al.,* 2005).

Patients with SLE also develop arterial stiffness, but it is uncertain if arterial stiffness occurs independently of age, hypertension, renal function, or atherosclerosis (Yildiz *et al*., 2008). Renal injury in SLE is one of the most serious complications and its pathogenesis has not yet been completely clarified (Sprovieri and Sens, 2005). Angiotensin-converting enzyme (ACE) plays an important role in the development of SLE, expressed in a wide range of tissues including lung, vascular endothelium, kidney, cardiovascular and testis (Kaufman *et al*., 2001). ACE catalyses the conversion of Angiotensin I to Angiotensin II by its metalloproteinase enzymatic activity and plays a major role in the renin–angiotensin and kallikrein–kininogen systems. A major component of the renin-angiotensin system, ACE is up regulated in pressure overload–induced cardiac hypertrophy as well as heart failure (Schunkert *et al*., 1993). It is long recognized that autoimmune diseases such as SLE are associated with increased mortality and an increased risk of CVD, which is not explained by traditional CVD risk factors alone (Doria *et al.,* 2005).

Manzi *et al.* (1997) established in the Pittsburgh cohort that certain risk factors were more common in SLE patients with cardiovascular events than in those without events, including older age at diagnosis, longer disease duration, hypercholesterolemia, postmenopausal status (Manzi *et al*., 1999).

ACE gene I/D polymorphisms, particularly DD genotype has been associated with lupus susceptibility and with risk of renal disease and cardiovascular disease (Kennon *et al*., 1999). Inhibition of ACE induces regression of cardiac hypertrophy independent of load (Linz *et al*. 1992) and prevents dilation and remodelling of the ventricle after myocardial infarction (Pfeffer *et al*., 1992). Inflammatory mediators and an array of inflammatory cells can induce a broad spectrum of clinical manifestation (Kyttaris *et al*., 2005).

The purpose of this study was to determine the relationship between the biochemical profile, autoantibodies, and ACE gene polymorphisms in relation to renal and cardiac disease.

MATERIALS AND METHODS

Sample selection

The study group comprised of 48 SLE female patients and equivalent number of healthy normal control subjects, matched for age (±5), gender, and socio-economic conditions. The samples were collected from SLE patients belonged to Coimbatore, Madurai, Erode districts in Tamil Nadu, South India. The work was

followed and carried out in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. After obtaining written informed consent from all participating subjects, information regarding health status, habit and occupation was recorded. Individuals who reported a history of chronic or acute diseases were excluded from the study. About 4 ml of peripheral blood sample was collected from SLE and control subjects in heparinzed and EDTA coated vaccutainers. The blood samples were brought to the laboratory within 24 hours for analysis of immunological, biochemical and genotypic analysis.

The immunological and biochemical parameters (Serum Cholesterol, ANA, RF, ESR and CRP) were analyzed through a commercial laboratory (Religare SRL Ranbaxy Mumbai India) and X-rays (for heart and lungs) and ECG (for the heart, pulmonary functions for the lungs) were done from Sugapriya Hospital Madurai, India.

Genotyping of ACE I/D Polymorphism

Genomic DNA was isolated according to the standard protocol (Miller *et al*., 1988). The specific segment of ACE gene was amplified by using the previously reported primers (Salem and Batzer, 2009). The primer sequences were as follows, Sense primer: 5'- CTGGAGACCACTCCCATCCTTTCT -3' and antisense primer: 5'-GATGTGGCCATCACATTCGTCAGAT-3'. PCR reaction was performed in a final volume of 25 µl with 0.25µl of forward and 0.25µl of reverse primers, followed by 200ng of template DNA, 1.25units of Taq DNA polymerase, 2mM dNTPs in 2mM $MgCl_2$. Thermal conditions consisted of an initial denaturation at 94°C for 5 minutes and 35 cycles of denaturation at 94°C for 1 minute, annealing at 58°C for 30 seconds, extension at 72°C for 1 minute and final extension at 72°C for 8 minutes. A 190bp amplicon was observed in case of homozygous DD genotype, 490bp in case of homozygous II genotype and heterozygous DI genotype.

All samples that were identified initially as a DD genotype were reanalyzed using an insertion-specific primer pair, as reported by Lindpaintner *et al.* (1995). Oligonucleotide sequences specific for I alleles primers were 5'-TGGGACCACAGCGCCCGCCACTAC-3' and 5'-TCGCCAGCCCTCCCATG CCCATAA-3'. PCR was performed with an initial denaturation at 94°C for 1 minute and for 30 cycles of denaturation at 92ºC for 40 seconds, annealing at 63ºC for 40 seconds, extension at 72ºC for 40 seconds and a final extension at 72ºC for 5 minutes. The PCR products were separated by 8% polyacrylamide gel in 1X TBE buffer at 65V/cm for 1:45 hours. A 335 bp band was obtained only in the presence of the I allele and no bands were detected for samples with DD genotype

Statistical Analyses

Statistical analysis was performed with SPSS 16.0 for windows. The distribution of genotypes in all groups was tested for deviation from Hardy–Weinberg equilibrium. The Odds ratio (OR) and 95% Confidence Intervals (95% CI) were calculated with statistical significance at *p*-value <0.05 (J Martin Bland and Douglas G Altman Statistics Notes: The odds ratio BMJ 2000; 320:1468). Chi-square analysis ($\chi 2$ tests) was used to test the association between the polymorphic variants and biochemical parameters among the controls and experimentals. $p < 0.05$ was used as the criterion of significance. The association between clinical manifestation, production of autoantibodies and polymorphism distribution in patients with SLE was determined by Fisher exact test.

RESULTS AND DISCUSSION

The mean age of SLE patients and controls was 45.11 ± 11.50 and 44.18±11.50 respectively. In this study 40% of the SLE patients had increased total cholesterol levels and were classified into two groups based on cholesterol level such as SLE high (>200 mg/dL) and SLE low (<200 mg/dL) (Table 1). In Patients with high and low cholesterol levels, high TGL levels showed corresponding high levels of LDL and VLDL suggesting a correlation between these levels but HDL levels in both high and low level patients remained unaffected by total cholesterol level.

RF and ESR level were analysed in the SLE and control subjects and found that, in SLE subjects ESR 53.45±10.32 and RF 119±199.19 were significantly (P<0.05) increased as compared to control subjects (Table 1 and 2). CRP levels as expected was higher in all the SLE patients than the normal reference range (<5 mg/L) (Table 2). ANA score was 4+ in severe SLE patients and 1+ in low severity patients (Table 2). The patients in SLE high and SLE low groups had insignificant difference in the levels of CRP and ESR, but had high and low levels of RF in 4+ and 1+ patient respectively which points towards a positive correlation of ANA and RF.

The antinuclear antibody test is positive in almost all individuals with SLE (97%) and in the present study it was found to be elevated in all the SLE patients as compared to controls (Table 2).

Chi square analysis revealed that there was significant difference in the distribution of DD genotype between controls and SLE patients (Table 3). The patients with DD genotype had high total cholesterol levels suggesting a relationship between the two. Gel electrophoresis of amplified PCR products showed 490 bp and 190 bp products, corresponding to the PCR amplification of I and D alleles, respectively (Figure 2). The distribution of ACE genotypes in the study population was as follows 62% DD, 20.83% ID and 16.66% II. The frequency of allele D was 0.72 and I allele was 0.27.

Table 1: Immunological and biochemical profile in control and SLE subjects

Particulars	Age	ESR (mm at I hr)	HDL 35-60 mg/dL	LDL <100 mg/dL	VLDL 7.0-35 mg/dL	TGL 30-200 mg/dL	Total Cholesterol 30-200 mg/dL	CRP<5 mg/dL
Controls	44.18 ±11.50	15.95 ±5.55	44.23 ±11.00	85.10 ±18.57	24.73 ±7.75b	135.75 ±50.49	146.90 ±40.45	6.039 ±5.55
SLE High	46.77 ±11.25	53.45 ±10.32	41.04 ±16.50	129.49 ±28.14[bc]	40.67 ±22.55[abc]	184.09 ±83.35[abc]	233.95 ±33.99[abc]	10.66 ±7.24[bc]
SLE Low	43.46 ±12.58	62.38 ±42[abc]	43.57 ±27.12	88.95 ±24.10	24.13 ±6.08	127.31 ±33.48	151.15 ±129.02	6.59 ±4.40

[a] Homogeneity Variances are significant compared to the controls and low level of cholesterol in the experimental subjects (ANOVA)

[b] Bonferroni test were used an the value are compare to the controls and low level of the experimental subjects

[c] post hoc analysis (Dunnett analysis) were used and values are significant ($p<0.05$) to the one way ANOVA.

Table 2: Immunological and biochemical profiles in control and SLE subjects

Particulars	RF (IU/ml) <40	ANA,AB-IFA,HEP2	Anti CCP antibodies (U/ml) <15.0	DsDNA Test IU/ml 0.0-4.2	CRP
Controls	34.52 ±20.62	N	14.65 ±9.4	2.37±2.12	5.26 ±3.98
SLE High	119.35 ±199.19	4+	32.48 ±63.73	46.29±25.44	21.78 ±2097
SLE Low	191.21 ±234	2+	26.98 ±51.89	15.25±12.15	45.52 ±47.78

Table 3: ACE Gene polymorphisms identified in controls and SLE subjects

Subjects	**No**	**Genotypes**			***P*<0.05**	**I allele n (%)**	**D allele n (%)**	**P(χ2)**	**OR (95%CI)**
		II n (%)	ID n (%)	DD n (%)					
SLE patients Controls	48 48	8(16.66) 14(29.16)	10(20.83) 23(47.91)	30(62.50) 11(22.91)	χ2=15.56 (0.0004)	26(27.08) 51(53.12)	70(72.91) 45(46.87)	13.55 (0.0002)	3.05 (1.67-5.57) P<0.0001
SLE and controls Genotypic association and the risk ratio	II/DD RR 1.79 (1.19-2.87) **Odds ratio** 4.77 (1.57-14.48) (χ2)P<0.004 (F test)P<0.005		II/ID RR 0.89(0.55-1.44) **Odds ratio** 0.76 (0.24-2.38) Pearson P=0.63 Log odds -0.27	ID/DD RR 2.31(1.38-3.89) **Odds ratio** 6.27(2.27-17.29) (χ2) (F test, Pearson test)*P*<0.0002	*P*<0.0001 (F test and Pearson)	Risk ratio 1.55(1.21-1.98) Odds ratio 3.05 (1.67-5.57) χ2 *p*<0.0004, Pearson *p*<0.0002 F test *p*<0.0001			

Genotype and allele frequencies were compared with controls and Experimental subjects via χ2 test

Chi-square (χ2) is calculated and statistically significance compare the controls $p<0.0001$.

Statistically significance level <0.0001, OR (CI) = Odd Ratio and Confidential Interval.

Subjects with DD (OR) are at greater risk for SLE when compared to ID and II genotypes. The ID genotype was most prevalent genotype in control.

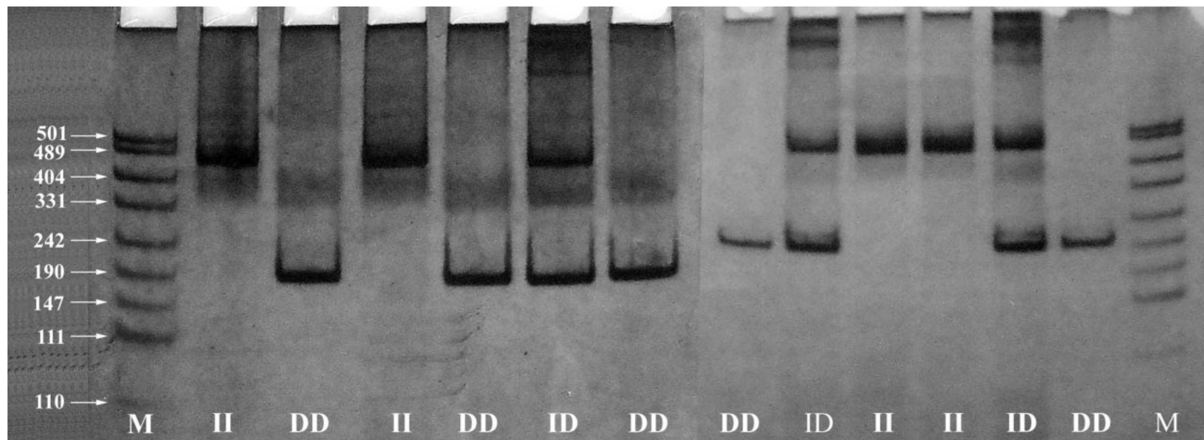

Figure 1: Homozygous DD, Homozygous II and Hetrozygous ID genotypes

From left to right A: Lane 1 PUC 19 Marker. Lane 2-II, Lane 3-DD, Lane 4-II, Lane 5-DD, Lane 6-ID, Lane 7-DD genotypes.
B: From right o left - Lane 1 PUC 19 Markers. Lane 2-DD, Lane 3-ID, Lane 4-II, Lane 5-II, Lane 6-ID, Lane 7-DD genotypes

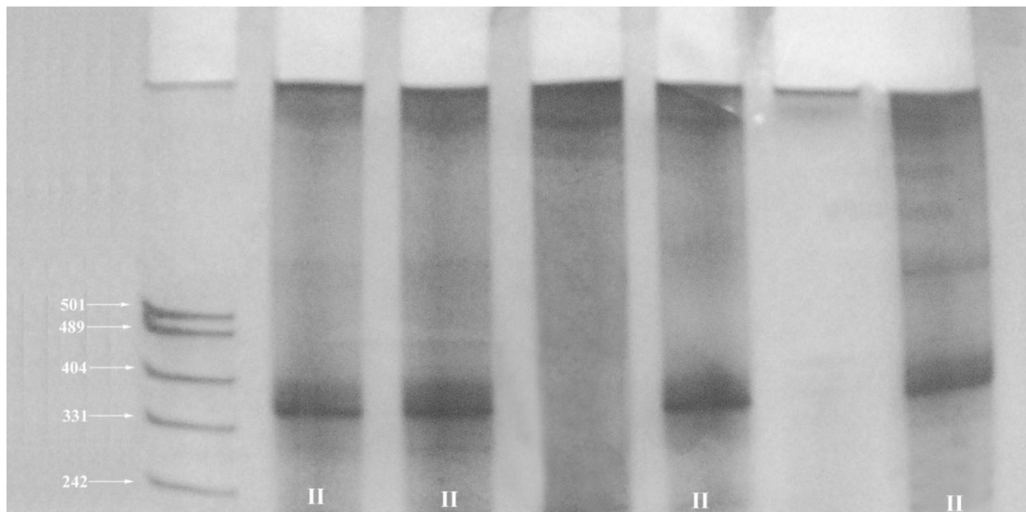

Figure 2: Determination of ACE II genotype by PCR using insertion specific ACE primers

In our study, high cholesterol levels observed in SLE patients were accompanied by an increase in CRP and ESR levels. These results are consistent with a study by Font et *al*. (2001) where SLE patients had higher total cholesterol levels which was associated with renal deterioration. RF is an antibody that is not usually present in normal individual. Out study found that, SLE patients had RF present in their body which is in total agreement with a previous study (Steiner and Smolen, 2002) which reported presence of RF in SLE disease condition. SLE is often accompanied by a number of biochemical, hematological and immunological abnormalities, though several of these abnormalities are non-specific (Dubois *et al.,* 1987).

SLE is associated with high prevalence of dyslipidemia, endothelial dysfunction, hypertension and vascular stiffness (Avalos *et al.,* 2007). The true prevalence of vascular disease in women with SLE is unknown, but could certainly be higher than that defined by cardiovascular events alone (Manzi *et al*., 1997). With improved corticosteroid and immunosuppressive SLE therapy, there is a growing pool of women at increased risk of developing cardiovascular disease, which is now one of the leading causes of death. In a large series, Gladman and Urowitz reported a 9% incidence of *angina* pectoris and/or myocardial infarction that occurred on average 89 months after the onset of SLE (Gladmann and Urowitz, 1987).

Jonsson *et al.* (1989) reported that patients with SLE who developed myocardial infarction had significantly longer duration of the disease. Previous data has suggested that ESR levels in SLE patients can be used to asses disease activity as ESR is an inexpensive diagnostic tool (Luis *et al*., 2005). SLE is an immune

complex mediated disease, which is more common in women. LDL (especially in its oxidized form) may be an important mediator of nephrosclerosis. A high triglyceride level in women and a low HDL cholesterol level in men predicted the decline of renal function (Ettinger *et al.,* 1987).

Font *et al.* (2001) showed link between hyper cholesterolemia with renal failure in a smaller cohort of patients with SLE. In our study, SLE patients had significantly raised triglyceride and LDL cholesterol levels, and significantly lower HDL cholesterol levels in comparison with controls. As women comprise as much as 90% of most SLE cohorts, it would be interesting to investigate whether the triglyceride level is also predictive of renal deterioration in patients with lupus.
The present study found DD genotype overrepresented in SLE patients than controls. In a previous study, the D allele was found to be significantly higher in the SLE patients versus non-related controls (Mattei *et al*., 1989). In another study DD allele frequency was higher in the SLE patients compared to the controls (Kaufman *et al*., 2001). Angiotensin-converting enzyme is an attractive candidate to play a role in the develop-ment of vascular pathological states. Evidence suggests that the presence of the DD genotype increases susceptibility for coronary heart disease, myocardial infarction and both diabetic and non-diabetic renal disease (Kennon *et al*., 1999). Recently two studies have identified an association with the insertion /deletion in the ACE gene with systemic lupus Erythematosus (Pullmann *et al.,* 2002). In addition to several of the variables used in this study, previously reported prognosticators of poor renal function in patients with SLE include the presence of antibodies. Renal function can be evaluated effectively in patients with SLE, by measuring serum total cholesterol levels patients with SLE, who commonly suffer from both kidney dysfunctions.

CONCLUSION

The abnormal elevation pattern of CRP in SLE patients provided the first clinical clue that variation in the CRP may contribute to the pathogenesis of SLE. The study again reemphasizes that cholesterol levels may play a role in SLE patients and may increase risk of cardiovascular and renal diseases. Also DD genotype of ACE may increase the risk of renal and CVD in such patients. Altogether taking into account an array of investigations, SLE patients may be more susceptible to CVD and renal disease risk with ACE polymorphisms having a significant role.

Conflicts of interest: The authors stated that no conflicts of interest.

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