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RESEARCH ARTICLE

C-REACTIVE PROTEIN GENE POLYMORPHISM 1468G>A (RS 1205) AND THE RISK OF SYSTEMIC LUPUS ERYTHEMATOSUS AND LUPUS NEPHRITIS

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ABSTRACT

Objectives: Low serum C- Reactive Protein (CRP) has been implicated in Systemic lupus Erythrematosus (SLE) pathogenesis, so we studied the CRP rs1205 polymorphism and its role in the risk association of both SLE and Lupus nephritis (LN).
Methods: DNA from 90 patients who met the ACR criteria for SLE and 40 healthy controls was genotyped for CRP 1468G>A rs1205. Genotyping was performed using PCR-RFLP. Serum CRP levels were measured using particle enhanced immunonephelometry.
Results: We Found the genotype distribution of CRP rs1205 G>A polymorphism was In SLE: AA, 6 (12%), GA, 9 (18%) and GG, 35 (70%), in LN: AA, 9 (22.9%), GA, 17 (42.5%) and GG, 14 (35%)(p value=0.041). Carriers of the rs1205 A allele were characterized by low serum CRP levels compared with major homozygotes 2.5(1.55 - 11.35) vs. 9.55(6.45 - 14.83)mg/L; p = 0.029).
Conclusion: Our data indicates that rs1205 variant allele predisposes to SLE and LN, potentially being a genetic risk marker of disease progression.

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INTRODUCTION

Systemic lupus erythematosus (SLE) is a complicated and multi factorial chronic autoimmune inflammatory disease includes genes polymorphisms, hormones and different environmental causes. Family and twin studies lead to believe that genetic factors play an important role in the pathogenesis of SLE (Kyttaris *et al*., 2005; Block *et al*., 1975). SLE is characterized by producing a different types of autoantibodies and immune complex deposition leading to multiple organ failure. It is believed that defective treatment of immune complexes and consequent tissue deposition leading to excretion of inflammatory mediators and a collection of inflammatory cells can develop a wide range of clinical manifestations (Alarcon-Segovia *et al*., 2005). Lupus nephritis (LN) is the most frequent clinical complication of SLE, happen in up to 74% of patients. LN results from immune complex deposition in the renal glomeruli causing chronic inflammation, complement activation, renal insufficiency and the appearance of proteinuria and cellular casts, white blood cells and red blood cells in urine (Cervera *et al*., 2006). 10%-12% of patients with SLE have first or second degree family members with the disease as shown in familial aggregation studies in SLE, in comparing to < 1% of controls (Alarcon-Segovia *et al*., 2005; Hochberg *et al*., 1987). In addition, a genetic susceptibility of LN is supported by an over-manifestation of LN among children with SLE, linkage studies of LN (Quintero-Del-Rio *et al*., 2002) and familial aggregation of end-stage renal disease (ESRD) among African Americans with LN (Freedman *et al*., 1997). C- Reactive Protein (CRP) is a liver derived acute-phase protein that can raise up to 1000-fold in serum as a reaction to different stimuli such as injury and infection (Moser, 1998). Pentameric CRP under particular circumstances irreversibly dissociates into monomers (mCRP) and manifests new epitopes (Moser, 1998; Kushner, 1988). mCRP has been reported to bind histones (Robey, 1984), chromatin (Du Clos, 1988), and apoptotic cells (Gershov, 2000). These particular features of CRP are speculated to support its ability to change the autoimmune disease phenotype by improving the removal of apoptotic and necrotic cells, clearance of immune complexes, recruiting complement through interaction of mCRP with complement factor H and C1q (Du Clos, 1988).

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The enhanced clearance of apoptotic cells and their nuclear contents by phagocytosis via CRP opsonization may arrest the development of probable nuclear antigen autoimmune reactions (Du Clos, 1988; Gershov *et al*., 2000). CRP's autoimmunity prevention capability may drive from its ability to stop activation of autoreactive B cells by enhancing clearance of immune complexes (Kravitz *et al*., 2005). While other acute phase protein reactants increase in active SLE, levels of CRP generally remain low (Gershov, 2005). This might because of a suppression of interleukin (IL)-6-mediated CRP production in liver cells by overproduction of inter-feron alpha (IFNα), CRP gene polymorphisms (Szalai, 2004) and an accelerated conversion of CRP into mCRP (Kravitz *et al*., 2005). CRP has a unique ability to change SLE phenotypes, and its status as an important candidate gene, CRP aids as a promising SLE susceptibility gene. Russell *et al*. (2004) reported that levels of CRP were affected independently by 2 CRP polymorphisms (+838 & +2043), and the latter was also associated with antinuclear autoantibody production and SLE. They hypothesized that defective removal of immunogenic substances, caused by low CRP levels, may be a complementary factor in lupus pathogenesis. In the present study, we analyzed CRP rs 1205 polymorphism and its association with serum CRP level, SLE risk and LN risk in SLE patients.

SUBJECT AND METHODS

Subjects: Ninety SLE cases and 50 healthy controls were included in this work. All cases were admitted to Rheumatology and Rehabilitation Department, Assiut University, Egypt. All subjects met the 1982 and 1997 American College of Rheumatology (ACR) criteria for SLE (Tan, 1982; Hochberg, 1997) at the time of recruitment, and were divided into two groups: 40 patients with LN and 50 patients with SLE according to the laboratory data. Controls were sex and age matched, and had no apparent history of SLE. The patients' written consent was obtained according to the declaration of Helsinki and the study has been approved by local ethics committee prior to their inclusion in the study.

SLE Clinical and Laboratory Characteristics

SLE disease activity was measured by the same physician (SM) in all patients, using the Systemic Lupus Disease Activity Index (SLEDAI)(20). Active LN was described as active urinary sediment and cellular casts, and/or worsened glomerular filtration rate (GFR) >25 % above baseline/normal range caused by active LN and/or proteinuria ≥0.5 g/day, and/or C3 hypocomplementemia. At least two of the previous criteria had to be found, and renal biopsy showing lupus nephritis. Sixty five patients (72.2%) had arthritis, 40 (44.4%) patients had a diagnosis of SLE-renal disease, 4 (4.4%) patients had Seizures, 5 (5.5%) patients had psychosis 31(34.4%) patients had Malar rash, 16(17.7%) patients had oral ulcers, 9(10%) patients had alopecia. Patients' median SLICC score at the time of recruitment was 4 and mean SLAM score was 7.1. Additional measurements included complete blood picture, urine analysis, urinary 24 hour protein, urea, creatinine, creatinine clearance, glomerular filteration rate, liver function test, lipogram, antinuclear antibodies, anti-double stranded DNA, serum C3, and C4. high sensitivity CRP: Serum samples were withdrawn from each patient and control stored at –20ºC until tested.

Serum hsCRP concentrations were measured using BN Prospec from Dade Behring is based on particle enhanced immunonephelometry (N Hs CRP, cat. no. OQIY 13, supplement reagent OUMU194E0003V; Dade Behring, Liederbach, Germany). None of the SLE cases with CRP measurement had any evidence of infection at the time of the study CRP level.

Molecular Analysis

DNA extraction

Genomic DNA was extracted from peripheral blood samples at diagnosis using an QIA amp® DNA Blood Mini Kit, Germany, by QIA cube Extractor.

Restriction Fragment Length Polymorphism (RFLP)

For CRP 1846G>A (rs 1205) mutation analysis, we added 200ng of DNA, 50mM KCL, 10mM Tris-HCL, pH8.3, 1.5mM MgCL2, 200 µM dNTPs, 0.4µM of forward primer 5'-CTTATAGACCTGGGCAGT-3' and 0.4µM reverse primer 5'-GGAGTGAGACATCTTCTTG-3', and 1U of polymerase, in a volume of 50µl. The PCR consisted of an initial incubation step at 95°C for 15 minutes, followed by 35 cycles of reaction at 95°C for 30 seconds, 56°C for 30 seconds, and 72°C for 30 seconds. The final extension step at 72°C for 5 minutes. HpyCH4III (Taal) was added to the PCR amplification product acquired from the operation, and after incubation at 65 °C for 2 hours, RFLP was performed by electrophoresis on standard 3% agarose gels. Inhibitory allele (A) has a fragment of 227 bp, while Permissive allele (G) has two fragments of 97 and 130 (Figure 1).

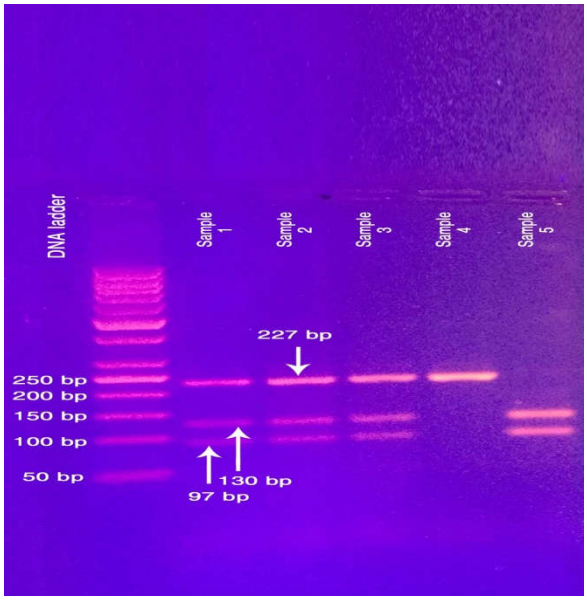

Figure (1). Agarose gel electrophoresis of CRP rs 1205 of five samples. Lane 1: 50 bp molecular marker, lane 2,3,4: (GA) heterozygote genotype, lane 5: (AA) minor homozygote genotype, lane 6: (GG) major homozygote genotype

Statistics

The data were tested for normality using the Anderson-Darling test and for homogeneity variances prior to further statistical analysis. Categorical variables were described by number and percent (N, %), where continuous variables described by mean and standard deviation (Mean, SD).

Chi-square test and fisher exact test used to compare between categorical variables where compare between continuous variables by t-test and Independent-Samples T test, Mann-whitney U, kruskal wallis test. A two-tailed $p < 0.05$ was considered statistically significant. All analyses were performed with the IBM SPSS 20.0 software.

RESULTS

The age of 90 subjects with SLE and LN (50 patients with SLE and 40 patients with LN) included in the study ranged from 22.5-43 years with a median of 28years and 25.75-43.5 with a median 30.5 years respectively. All of them are females (100%) for SLE patients, while 18(81.8%) are females and only 4 (18.2%) patients are males in N patients. The genotype distribution of CRP rs1205 G>A polymorphism was as follows:

In SLE: AA, 6 (12%), GA, 9 (18%) and GG, 35 (70%), in LN: AA, 9 (22.9%), GA, 17 (42.5%) and GG, 14 (35%)(p value=0.041) showing no deviation from Hardy-Weinberg Equilibrium (HWE; $p = 0.43$). However, to approve the genotyping method, it was also tested in a group of 50 healthy subjects aged between 19 and 48 years (median of 30.5 years) and containing 52 females (84%), in whom the following genotype distribution was obtained: GG, 26 (52%), GA, 17 (34%) and AA, 7 (14%), also being in HWE ($p = 0.35$). The rs1205 polymorphism minor allele was in frequency of 34**%.** The genotype distribution and allele count were compared between the groups and showing no significant differences ($p = 0.53$ and $p = 0.48$, respectively).

Association of CRP rs 1205 with SLE and LN Risk: We genotyped CRP rs 1205 in ((90 cases (50 SLE, 40 LN patients) and 50 controls)).

Table 1. Genotype and allele frequencies of CRP rs 1205

Genotype	SLE cases n(%)	LN cases n(%)	P value	Allele	SLE cases n(%)	LN cases n(%)	P value
GG	35 (70)	14 (35)	0.041	G	79(79)	45(56.25)	0.048
GA	9 (18)	17(42.5)					
AA	6 (12)	9(22.5)		A	21(21)	35(43.75)	

Table 2. Laboratory characteristics of SLE patients with regard to the C-reactive protein (CRP) gene rs1205 polymorphism

	rs1205 G>A			
	Major (AA) Homozygotes Median (Range)	Heterozygotes (CT) Median (Range)	Minor (GG) Homozygotes Median (Range)	P.value
Age(years)	31.5(27.25 - 45)	36(32 - 42)	27(20 - 44)	0.257
Duration (years)	6(4 - 6)	2(1.5 - 11.5)	4.5(3 - 7)	0.549
ESR(1^{st}hr)(mm/hr)	50.5(30.75 - 67.5)	64(21.5 - 90)	28.5(24 - 60)	0.419
ESR (2^{nd}hr)(mm/hr)	71(49.75 - 98.5)	83(42.5 - 115)	51(40 - 88.75)	0.234
CRP(mg/L)	2.5(1.55 - 11.35)	4(1.05 - 13.3)	9.55(6.45 - 14.83)	0.029
ANA(AI)	12(0.78 - 52.78)	37(33.85 - 52.55)	27.9(8.88 - 45.6)	0.285
Anti-ds DNA(IU/ml)	83.5(18.5 - 182.5)	110(15 - 200)	18(12 - 30.75)	0.038
Hb(g/dL)	11.5(8.55 - 12.53)	10.5(9.35 - 12.05)	10.7(10.05 - 12.38)	0.828
WBCs(10^3/µl)	5.78(2.95 - 9.5)	5.47(5 - 7.16)	5.4(3.58 - 7.47)	0.878
PLTs (10^3/µl)	242.5(177.25 - 329)	204(166 - 259)	214(163 - 338.25)	0.721
AST(U/L)	25(19 - 29.5)	25(14 - 35.5)	22.5(16.25 - 29.75)	0.852
ALT(U/L)	18(14 - 29)	17(7 - 31)	19(11.25 - 20)	0.689
S. Cholesterol(mg/dl)	181.5(122.5 - 197.75)	202(149.5 - 223)	187(144.25 - 211.25)	0.444
Triglycerides(mg/dl)	111(63 - 172.75)	141(124 - 208)	120(94.5 - 183.5)	0.285
HDL(mg/dl)	57.5(50.5 - 69.75)	48(37 - 51)	54.75(45.25 - 72.75)	0.141
LDL(mg/dl)	121.15(72.75 - 133)	127(99.3 - 148.65)	118.9(91.68 - 139.15)	0.543
C3(g/L)	0.85(0.65 - 1.12)	0.71(0.65 - 0.97)	0.94(0.76 - 1.28)	0.181
C4(g/L)	0.14(0.08 - 0.2)	0.06(0.06 - 0.1)	0.13(0.09 - 0.3)	0.032
Urine WBCs(hpf)	10(0 - 28.75)	0(0 - 32.5)	0(0 - 11.75)	0.633
Urine RBCs(hpf)	1(0 - 3.75)	5(0 - 31)	0(0 - 5.5)	0.438
Urine 24hr Protein(mg/24hr)	191.5(28.5 - 730.5)	200(98.8 - 1655.5)	203(38.55 - 827)	0.960
Createnine Clearance(ml/min)	98.15(67.5 - 129.5)	98.5(62 - 143.3)	107(98.25 - 128)	0.496
S. creatinine(mg/dL)	64.5(33.13 - 88.5)	58(47.5 - 74.52)	64(51 - 82.93)	0.894
eGFR (ml/min/1.73m²)	99.5(65.25 - 118.25)	108(81.5 - 141.5)	113.5(87.5 - 132.75)	0.515
SLEDAI Score	6(0.75 - 15.25)	4(0 - 18)	6(0.25 - 16)	0.932

Table (3). The effect of C-reactive protein (CRP) gene rs1205 G>A polymorphism on the risk of SLE and LN analyzed by logistic regression

Genotype Groups	Crude OR (95% CI)	p- value	Adjusted OR (95% CI)	p- value
GG(reference/GA/AA				
GA vs. GG	3.1(1.68-6.2)	0.026	2.9(1.44-5.9)	0.03
AA vs. GG	0.019 4.3(1.88-8.1)		4.56(1.95-8.9)	0.01
GG(reference)/GA+AA				
GA+AA vs. GG	2.4(1.5-5.91	0.021	3.1(1.49-6.3)	0.02

OR denotes odds ratio; CI, confidence interval; Adjusted for laboratory characteristics of SLE (ANA, Anti ds ANA, C3 and C4).

No statistically significant deviations from Hardy– Weinberg equilibrium were found. Of the total 140 subjects (90 cases and 50 controls) genotyped for CRP rs1205, we repeated genotyping on 50% of the subjects for the SNP a second time and had higher than 99% concordance rate. (Table 1) presents the genotype and allele frequencies in our cases and controls for CRP rs1205. We detected that there is significant difference between SLE and LN regarding minor allele frequency (43.75% in LN cases in opposite to 21% in SLE cases).

Association of CRP rs 1205 with SLE Clinical Characteristics

Because we reported a significant association between CRP rs 1205 and SLE, we do follow-up analyses to detect the association between CRP rs 1205 and specific SLE characteristics. Patients with rs 1205 AA and GA genotypes exhibited significantly higher Anti ds ANA levels compared to GG individuals {83.5(18.5 - 182.5), 110(15 - 200) and 18(12 - 30.75) respectively, (p = 0.038)}. Additionally, we found significant association between rs 1205 AA and GA genotypes and C4 level {0.14(0.08 - 0.2), 0.13(0.09 - 0.3) and 0.06(0.06 - 0.1) respectively, (p= 0.032)}. However, no significant associations were observed between SNP rs 1205 and SLEDAI Score, ESR, complete blood picture, Lipogram, C3, creatinine, oral ulcers, arthritis, vasculitis, pericarditis, pleurisy and psychosis (Table 2).

CRP rs 1205 Association with Serum C-Reactive Protein Level

In our study cohort, CRP showed a significant association with CRP rs1205 polymorphism in a genotype model; CRP levels were the lowest in minor homozygotes and the highest in patients having two major alleles 2.5(1.55 - 11.35) vs. 4(1.05 - 13.3)vs. 9.55(6.45 - 14.83)mg/L; p = 0.029) (Table 2, Figure 2). Furthermore, linear regression stated that rs1205 polymorphism is associated with CRP concentrations also in an additive model, which remained strongly significant after adjustment for accompanying characteristics (Table 3). Similar results were obtained when the effect of the polymorphism on CRP levels was analyzed in a dominant model (CRP concentration in patients carrying the minor allele, 2.11 (1.51–11.06) mg/L; p < 0.025) (Table 2).

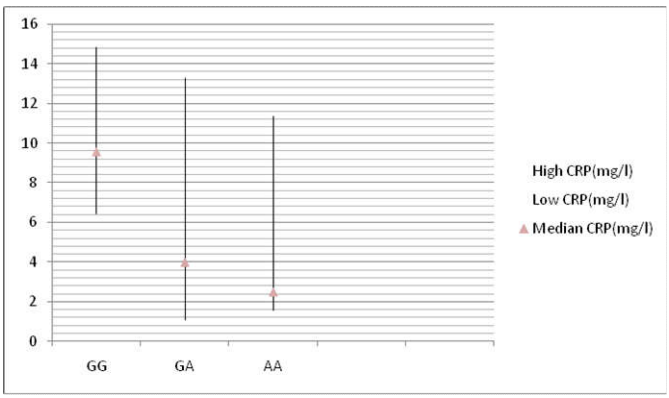

Figure 2: Describing the relationship of Serum C-reactive protein (CRP) gene rs1205 G>A polymorphism with the CRP levels. A triangle indicates the median of serum CRP level, upper and lower end of the line indicate the high and low levels of serum CRP, respectively

DISCUSSION

Despite serum levels of the acute-phase reactant CRP generally coextending disease activity in inflammatory cases, it is extensively accepted that SLE is an exclusion. It was noticed that many patients with active SLE manifest only mildly increased or even normal CRP levels during intervals of severe disease activity (Arbuckle *et al*., 2003; Baumann, 1990; Barnes *et al*., 2005), especially when correlated with patients with rheumatoid arthritis (RA) (Mok, 2013). Genome-wide linkage studies have recognized a locus associated with SLE at chromosome 1q23 (Shai *et al*., 1999; Moser, 1998). CRP is enclosed by the genes that have been mapped to this locus (Floyd-Smith, 1986), and a CRP gene polymorphism, accompanied with low basal serum CRP levels, has been settled to predispose to SLE, production of antinuclear antibodies, and lupus nephritis (Russell, 2004; Jonsen, 2007; Russell *et al*., 2004). Polymorphisms in the promoter of the CRP gene has been associated with SLE or SLE nephritis in African and Caucasian ethnicities (Edberg, 2008; Jonsen, 2007). CRP is a presumable candidate for SLE susceptibility: it is an important innate immune modulator that promotes the clearance of apoptotic bodies and cellular debris, and defects in clearance of apoptotic debris is believed to be important in the advancement and development of autoantibodies in patients with SLE (Edberg *et al*., 2008). Additionally, the low CRP levels noticed in SLE patients are influenced by genetic variation in the CRP gene promoter, and might lead to altered management of self antigens (Edberg *et al*., 2008). Studies on mouse models of SLE theorize that CRP may meliorate SLE (Marnell, 2005). In MRL/*lpr* mice, treatment with CRP delayed onset and progress of nephritis, decreased levels of auto antibodies to DNA and prolonged survival.

This effect was mediated by regulatory T cells. Likewise, CRP administration had beneficial effects in another type of SLE: NZB × NZW mice (Marnell, 2005). In this strain of mice, transgene expression of the CRP led to late onset of glomerulonephritis and prolonged survival Szalai *et al*., 2003; Szalai, 2004). We analyzed the association of CRP rs 1205 in relation to SLE risk and CRP levels in SLE and LN patients. We found significant association of CRP rs 1205 with SLE and LN risk. For the first time, we show that rs1205A allele has significant association with LN than in SLE (17 LN cases opposite to 9 SLE have heterozygote GA and 9 LN cases opposite to 6 SLE have Homozygote AA form, p= 0.41). We also show here that the occurrence of CRP rs1205 G>A polymorphism minor allele is associated with low CRP levels in SLE and LN patients. Some authors stated a colocalization of CRP with IgG and other factors such as C1q and anti-dsDNA-Ab in the renal sub endothelial space and glomerular basement membrane in LN (Becker *et al*., 1980; Morrow, 1981). Genetic variation at the CRP locus could influence SLE risk via its effect on CRP levels. As previously Russell *et al*. (2004) stated a significant association between SNPs +838, +2043 and decreased CRP level in a British SLE study. In addition, a more recent study by Miller *et al*. (2005) reported the same finding of these two SNPs in three large cohorts of healthy populace. However, P. Betty Shih did not notice the same association between SNPs +838, +2043 and decreased CRP in his 273 SLE patients, the lack of association in his SLE women may be referenced to the small sample size of the minor allele carriers in the study, or may be confused by the sequel of anti-inflammatory drugs SLE patients take on the habitual base (e.g corticosteroids) (Betty, 2008).

Gene promoters polymorphisms of CRP may have a role in gene function by changing transcription factor recognition and binding, which in order can affect gene expression and biological pathways. furthermore, SNPs at the intron/exon bounds may result in alternate splicing and influence gene function. Indeed, these association between CRP levels at this SNP has also been founded in healthy, non-SLE population. Russell *et al*. 's family-based study considered that low levels of CRP may stimulate antinuclear autoantibody assembly, which in turn afford to the occurrence of SLE (Russell *et al*., 2004). In summary, our study is constant with some previous studies that genetic variation in CRP affects risk of SLE and levels of CRP in SLE patients, and we suggest that the minor allele of the rs1205 CRP polymorphism could serve as a potential marker identifying subjects prone to develop SLE and LN. This genetic variant might possibly be a risk marker of SLE and LN progression.

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