The potential role of Serum Level of Growth Arrest–Specific 6 (GAS6) Protein and its Genetic Variations in systemic lupus erythematosus patients

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Received 12 August 2017
Accepted 22 October 2017

ABSTRACT

Objective: To determine whether c.843+7G>A Gas6 polymorphism is related to the development of systemic lupus erythematosus (SLE) and to investigate the association of its plasma level with different clinical manifestations and disease activity in SLE.
Methods: This study was conducted on 50 SLE patients together with 40 age and sex matched apparently healthy volunteers. Disease activity was defined by SLE Disease Activity Index (SLEDAI) score. The Gas6 protein was measured in serum using a commercially available ELISA kit and Gas6 gene polymorphism by polymerase chain reaction-Restriction Fragment Length Polymorphism (PCR-RFLP).
Results: The GAS6 c.834+7 GG genotype was associated with an increased risk for SLE (GG vs. GA (OR; 3.4; 95% CI 1.2- 9.5); and GG vs. AA (OR 3.6, 95% CI 1.1-11.8) with significant differences were observed among the 3 genotypes (P <0.001). Serum Gas6 concentrations were higher among SLE patients (15.9±0.8) compared with healthy control subjects (13.2±0.9) (P<0.001) and correlated with markers of renal inflammation and SLEDAI score. Some statistically significant differences were observed between Gas6 c.843+7G>A polymorphisms (p<0.05) with arthritis and renal disorders.
Conclusion: The GAS6 c.834+7 GG genotype could be a genetic determinant of SLE disease pathogenesis with special attention to lupus nephritis and could be used as a marker of disease activity.

KEY WORDS: systemic lupus erythematosus
Gas6
Single nucleotide polymorphism

Introduction

Systemic Lupus Erythematosus (SLE) is an auto-immune disease which vary widely in clinical appearances and disease course which resulted because of the creation of pathogenic auto-antibodies and a wide range of auto-antigens which results in formation and deposition of immunocomplexes resulting in the development of lupus nephritis (LN) and end-organ damage [1,2]. Apoptosis is the principle pathway for cell death, Inefficient clearance of apoptotic cells and accumulation of those apoptotic cell can produce a chronic inflammatory reaction and may results in self-tolerance failure [3]. It is believed that SLE autoimmunity could be related to impair or delay clearance of apoptotic cells with certain key autoantigens are demonstrable on blebs of apoptotic cells [4,5].

Materials and methods

Subjects

This case control study was conducted on 50 SLE Female patients diagnosed according to the revised criteria for the SLE classification. together with 40 age and sex matched apparently healthy volunteers were enrolled in this study as a control group [9,10]. They were recruited from the outpatients' clinic and inpatients of the Rheumatology, Rehabilitation and Physical Medicine department of Benha University Hospitals during the period from December 2016 to April 2017.

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All patients were subjected to full history taking, thorough clinical examination, Assessment of disease activity was done using the modified SLEDAI-2K. SLEDAI score change > 12 was expected to diagnose severe activity [11]. Laboratory investigations including CBC using a Sysmex 5000 counter; ESR determination using the Westergren method, complete urine analysis and kidney function tests, 24 hs urinary protein and protein to creatinine ratio (P/ C ratio). Antinuclear antibodies (ANA) by indirect immunofluorescent test, anti-dSDNA antibodies with Enzyme-

Linked immunosorbent assay (ELISA) technique and Complement (C3&C4) by immunodiffusion plate method. Blood samples: About 5cc of venous blood was collected by sterile venipuncture, the samples were divided into two parts. 2 cc allowed to clot naturally for 30 minutes. Then centrifuge at 1000 x g for 15 minutes; sera were separated and kept frozen at – 20ºC till used in ELISA and the remaining volume of venous blood was collected into an EDTA tube. The samples were stored at -20°C until further processing.

Measurement of Gas6

The Gas6 protein was measured in serum using a commercially available ELISA kit (Human Gas6 ELISA Kit, Entrez Gene; Swiss-Prot) according to the manufacturer's protocol. This particular immunoassay utilizes the quantitative technique of a "Sandwich" Enzyme-Linked Immunosorbent Assay.

Gas6 gene polymorphism by polymerase chain reaction-Restriction Fragment Length Polymorphism (PCR-RFLP)

DNA was extracted from whole blood using DNA extraction kit (GeneJET Whole Blood Genomic DNA Purification Mini Kit, Fermentas, Germany) according to the manufacturer's instructions.

Amplification Gas6

The SNP selection and primers using intron 8c.834+7G>A Gas6 were described by Hurtado et al. Gas6 E8-F primer 5'-TTC CCT CAA GAA AGA GCC CG-3' Gas6 E8-R Primer 5'-TCT CAT CCC AAA CCT CCA CA-3' (Biosearch technologies, USA).Genotyping was performed using Thermal Cycler (Biometra) and DreamTaq Green PCR Master Mix (2X) (Thermo Scientific,Germany) containing DreamTaq™ DNA polymerase, optimized DreamTaq Green buffer, MgCl2 and dNTPs. The reaction mixture contained 25 µl DreamTaq Green PCR Master Mix (2X) , 10 µl of tested DNA, 0.5µM of each primer, and water, nuclease-free was added to a PCR mixture to give a final reaction volume of 50 µl. Negative control reaction was confirmed by inserting a tube containing all components needed for amplification except DNA template in each amplification run.

Genotyping of c.843+7G>A Gas6 using restriction fragment length polymorphism analysis (RFLP)

RFLP analysis was done using Fast Digestion restriction enzyme AL WNI (Fermentas, Germany).5µl of purified PCR product were mixed with 1 µl AL WNI at 37°C for 15 minutes, according to the manufacturer's protocol. The Gas6 A allele digested into fragments (345 bp and 136 bp) but G allele remains uncut. The digested products were analyzed by 2% agarose gel electrophoresis stained by ethidium bromide and visualized by UV [13].

Statistical Analysis

Statistical analysis was undertaken using SPSS computer software (SPSS Version 16 for Microsoft Windows). Appropriate statistical tests were used (Student's t-test, ANOVA (F) with post hoc analysis, chi square test (X2), correlation co-efficient "r" test and risk association was assessed by Odds Ratio (OR) and the corresponding 95% Confidence Interval (CI)). Results were considered to be statistically significant at $P<0.05$, and highly significant at $P < 0.001$.

Results

The study included 50 SLE female patients (ages from 19 to 46 years with a mean of 27.9±6.6 years and 40 healthy control females of a matched age (age range, 21 to 46 years with a mean of 27.7±8.2 years). The main features of the studied groups are summarized in Table 1.

Table 2 shows: Allelic and genotypic frequencies of gas 6 gene polymorphisms at position c.834+7G>A in SLE patients and controls. GG frequencies in the SLE patients were 70 %, while GA frequencies were 18 % and AA genotype frequencies were12%. There was a significant difference ($P<0.05$) between G and A allelic frequency in SLE patients and normal controls. The GAS6 c.834+7 GG genotype was associated with an increased risk for SLE (GG vs. GA (OR; 3.4; 95% CI 1.2- 9.5); and GG vs. AA (OR 3.6, 95% CI 1.1-11.8).

Table 1. Characteristics of SLE patients and controls.

Features	Groups SLE patients (n = 50)	Controls (n = 40)
Age (years) Mean ± SD(Range)	27.9±6.6 (22-46)	27.7±8.2 (21-45)
Disease duration(years)		
Mean ± SD(Range)	8± 3.6 (3-11)	NA
Mucocutaneous features, no(%)	50(100%)	NA
Serositis, no (%)	12 (24%)	NA
Arthritis/arthralgia, no (%)	35 (70%)	NA
Renal features, no (%)	28 (56%)	NA
CNS features, no (%)	14 (28%)	NA
Hematologic features, no (%)	27 (54%)	NA
SLEDAI		
Mean ± SD(Range)	16.7 ± 5.9 (6-26)	NA
ANA, +ve no (%)	50 (100%)	NA
Anti-dsDNA, +ve no (%)	41 (82%)	NA
↓C3, no (%)	25 (50%)	NA
↓C4, n (%)	13 (26%)	NA
*NA: not available		

Table 2. Allelic and Genotypic Frequencies of Gas6 c.834+7G>A genotype among SLE subjects and controls.

		SLE patients (n=50) N (%)	Controls group (n=40) N (%)	OR	95% CI	*P value
Gas6 allele	G	79 (79)	46 (57.5)	2.8	1.4-5.3	< 0.05
	A	21 (21)	34 (42.5)			
Gas6 Genotypes (rs8191974)	GG	35 (70)	16 (40)	3.4	1.2-9.5	<0.05*
	GA	9 (18)	14 (35)	3.6	1.1-11.8	< 0.05†
	AA	6 (12)	10 (25)	1.1	0.3-3.9	>0.05€
	GG vs AA/GA			3.5	1.5-8.4	<0.05

* between GG & GA † between GG & AA € between GA & AA

Serum Gas6 concentrations were higher among SLE patients (15.9±0.8) compared with healthy control subjects (13.2±0.9) (P<0.001) (Table 3).

The concentrations of serum Gas 6 for SLE patients who had GG genotype was 13.9±0.8, 13.2±0.6 for GA genotype and 12.7±0.3 for AA genotype. Significant differences were observed among the 3 genotypes (P <0.001); Table (4). Table 5 shows Correlation coefficients results of serum Gas 6 levels with laboratory and disease activity markers in SLE patients. Serum Gas6 levels showed significant positive correlations with the ESR 1sthr (r= 0.89, P <0.05), protein /creatinine ratios (r = 0.88, P <0.05), 24hs urinary proteins (r=0.38, p<0.05), anti- dsDNA titers (r =0.44, P <0.05), and SLEDAI scores (r = 0.91, P <0.05).

Table 3. Serum Concentrations of Gas6 among SLE patients and healthy Controls.

Groups	Serum Gas6 Mean ±SD (ng/mL)	P value
SLE Patients	15.9±0.8	<0.001
Healthy Controls	13.2±0.9	

Table 4. Serum concentrations of Gas6 among different .genotypes

Gas6 843+7G>A	Serum Gas6 Mean (ng/mL)	*P value	Post-hoc test
GG	13.9±0.8		P1>0.05
GA	13.2±0.6	<0.01	P2<0.001
AA	12.7±0.3		P3>0.05

* P1 between GG and GA, P2 between GG and AA, P3 between GA and AA

Table 5. Correlation coefficients of serum Gas6 levels with laboratory and disease activity markers in SLE patient

Parameter	serum Gas6 R	*P value
HB (g/dl)	- 0.67	< 0.05
ESR mm / 1st hour	0.89	< 0.05
Platelet/HPF	0.02	NS
WBCs/HPF	- 0.12	NS
S. creatinine (mg/dl)	0.2	NS
S. urea (mg/dl)	0.18	NS
Creatinine clearance (ml/min)	-0.89	< 0.05
Protein /creatinine ratio	0.89	< 0.05
24hs urinary proteins	0.38	<0.05
C3 (mg/dl)	-0.9	< 0.05
C4 (mg/dl)	-0.82	< 0.05
Anti-ds DNA (u/ml)	0.45	< 0.05
ANA (u/ml)	0.2	NS
SLEDAI score	0.92	< 0.05

* P> 0.05 is not significant (NS).

But it had significant negative correlations with HB% (r = - 0.68, P <0.05), creatinine clearance (r = -0.87, P <0.05) C4 (r=-0.82, p<0.05) and C3 (r =-0.81, P <0.05). As regard association of Gas6 c.843+7G>A polymorphisms with clinical manifestations in patients with SLE, there were statistically significant differences (p<0.05) with arthritis and renal disorders and insignificant differences as regard other clinical manifestations (p>0.05) table (6).

Table 6. Association of Gas6 c.843+7G>A polymorphisms with clinical manifestations in patients with SLE.

Clinical features	Genotype SNP c.843+7G>A Gas6 genotypes (n=50)			
	GG n=34	GA n=10	AA n=6	P-value
ANA	34(100%)	10(100%)	6(100%)	--
Discoid rash	4(12%)	2(10%)	1(16%)	NS
Alopecia	14(41%)	3(30%)	1(16%)	NS
Malar rash	32(94%)	9(90%)	3(50%)	NS
Oral ulcers	17(50%)	6(60%)	2(33.3%)	NS
Arthritis	28(82%)	4(40%)	2(34%)	< 0.05
Photosensitivity	6(18%)	1(10%)	1(16%)	NS
Renal disorder	26(76%)	3(50%)	2(34%)	< 0.05
Serositis	11(32%)	4(40%)	1(16%)	NS
CNS disorder	10(29%)	3(30%)	0	NS
Hematological disorder	23(68%)	4(40%)	2(33.3%)	NS

* P> 0.05 is not significant (NS).

Discussion

Growth arrest specific 6 (Gas6) is a multi-modular circulating protein, which has an inhibitory effect on TNF-α, IL-6 and IL-1 secretion in lipopolysaccharide-stimulated macrophages thus it can stimulate an anti-inflammatory response [12]. The aim of this study was to determine whether c.843+7G>A Gas6 polymorphism is associated with the development of SLE and to investigate the association of its plasma level with different clinical manifestations and disease activity in SLE.

This study revealed that there was a significant difference (P<0.05) between G and A allelic frequency in SLE patients and normal controls. The GAS6 c.834+7 GG genotype was associated with an increased risk for SLE (GG vs. GA (OR; 3.4; 95% CI 1.2- 9.5); and GG vs. AA (OR 3.6, 95% CI 1.1-11.8). In 2012 WU et al. (13) studied two SNPs of the GAS6 gene, the intronic GAS6 834+7G/A and GAS6 +1332C/T, and investigated whether the genetic variation at these positions of Gas6 are associated with the development of SLE or specific clinical manifestations of SLE.for 834+7G/Genotype the study reported that in SLE patients the GG genotype was more prevalent followed by GA then AA genotypes. GG frequencies were 61.5 %, GA frequencies were 33.7 %, and AA frequencies were 4.8%. also the study reported that G allele was more prevalent in SLE patient compared with control groups but the differences in the genotype or allele frequency distribution comparing SLE patients and controls were no statistical significant. A possible explanation for this observation is that apoptotic clearance is dependent on multiple molecules and pathways. Like protein S which has a similar structure to Gas6, is more important than Gas6 in Mer-mediated phagocytosis of apoptotic cells [14]

In humans, a GAS6 polymorphism, the GAS6 834+7AA genotype, may represent a novel independent risk factor of type 2 diabetes [15]. In the current study, serum Gas6 concentrations were higher among SLE patients (15.9±0.8) compared with healthy control subjects (13.2±0.9) (P<0.001). These results were in agreement with those of Kim et al. and Recarte-Pelz et al. where serum levels of Gas6 were higher in SLE patients compared with healthy controls (p<0.001) and (p=0.0067) respectively [16,17]. However Suh et al. reported that plasma concentrations of Gas6 were not different between SLE patients and healthy controls. Gheita et al. reported that serum levels of Gas6 was altered in SLE and behcet;s disease (BD) patients than healthy controls (p 0.001 and 0.04 respectively) [18,19]. These discrepancies may be related to different treatment protocols received by SLE patients. This study showed that significant positive correlations were present between serum Gas6 levels with inflammatory markers such as the ESR 1sthr (r= 0.89, P <0.05), and markers of kidney affection such as protein /creatinine ratios (r = 0.88, P <0.05), 24hs urinary proteins (r=0.38, p<0.05), anti- dsDNA titers (r =0.44, P <0.05), and SLEDAI scores (r = 0.91, P <0.05). But it had significant negative correlations with HB% (r = - 0.68, P <0.05), creatinine clearance (r = -0.87, P <0.05) C4 (r=-0.82, p<0.05) and C3 (r =-0.81, P <0.05) which also indicate renal involvement. Ekman et al.2011 (20) reported Gas6 concentrations correlated with SLEDAI (r = 0.48, P < 0.001), ESR, CRP, anti-DNA antibodies, leucopenia and glomerulonephritis (GN) and inversely correlated with haemoglobin levels. Three previous studies demonstrated significant associations between Gas6 and SLEDAI score [16,17,20]. On the contrary, others did not evidenced the correlation between Gas6 and disease activity in SLE patients [18].

The present study demonstrated that Gas6 c.843+7G>A polymorphisms was found to be statistically ($p<0.05$) associated with arthritis and renal disorders. Wu et al., reported higher Gas6 serum level in patients with nephritis and in patients with cutaneous vasculitis [21]. While others reported an association between Gas6 and neurologic manifestations [18,19]. Others reported elevated Growth arrest-specific gene 6 (Gas6) levels in patients suffering from chronic renal failure [21].

In conclusion, the GAS6 c.834+7 GG genotype could be a genetic determinant of SLE disease pathogenesis with special attention to lupus nephritis and could be used as a marker of disease activity.

Conflict of Interest

I declare that we have no conflict of interest.

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