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RESEARCH ARTICLE

CLINICAL SIGNIFICANCE OF SOLUBLE SERUM FAS AND APO1/FAS GENE POLYMORPHISM (RS1800682) -670A>G IN SYSTEMIC LUPUS ERYTHEMATOSUS.

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Abstract

Systemic lupus erythematosus (SLE) is a complex genetically autoimmune disease with poorly understood pathogenesis. Lupus nephritis (LN) is one of the most serious complications of SLE. Abnormalities of apoptosis may be involved in the development of autoimmune disorders. Abnormal FAS-mediated apoptosis is one of the susceptibility factors in development of SLE. Promoter variants in the APO-1/Fas gene have been studied in SLE and other autoimmune diseases. **Aim:** The present case control study was conducted to detect the possible association APO1/FAS-670A>G gene polymorphism with susceptibility to SLE and Lupus nephritis (LN) and detect an association between the SNP and disease activity. In addition, investigate the possible relation between the polymorphism and sFAS levels and their possible association with of lupus nephritis. **Patients and Methods:** fifty SLE patients and 44 healthy control subjects were included in the study. sFas levels were detected by ELISA. PCR-RFLP was used to detect APO1/FAS-670A>G gene polymorphism. Lupus nephritis patients had proteinuria higher than 0.5g/24 hours. SLEDAI score was used to assess disease activity status in SLE and LN patients. **Results:** sFas levels were significantly higher in SLE compared to healthy control subjects (P<0.001). SLE patients with moderate SLE disease activity group followed by severe activity had higher sFas levels compared to low activity group (P=0.022). The heterozygous AG genotype of APO1/FAS-670A>G gene polymorphism was significantly higher in SLE patients compared to control group (P=0.029). There was a non-significant association between APO1/FAS-670A>G gene polymorphism and LN patients (P=0.326). There was a statistically significant positive correlation between sFas levels and SLEDAI score in patients carrying the AG genotype. **Conclusion:** Our results suggest a possible genetic association between increased risk of SLE and APO1/FAS-670A>G gene polymorphism. In addition we suggest a possible relation between sFas levels and SLE activity status.

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Introduction:-

Systemic lupus erythematosus (SLE) is a complex autoimmune disease with poorly understood pathogenesis. The genetic heritability of SLE is approximately 66%. Genome wide association studies have mapped of 28 disease susceptibility loci which account for not more than 10% of the genetic heritability (Guerra et al; 2012). Certain major histocompatibility complex class II (MHCII) alleles, such as: HLA-DRB* 0301 and DRB1* 1501, are involved in genetic susceptibility to SLE (Fernando et al; 2007).

Systemic lupus erythematosus is characterized by immune complex formation, auto antibody production and multiple organ damage (Akahoshi et al; 2009). Although the pathogenic mechanisms of SLE are not fully understood, previous studies have indicated that abnormalities of apoptosis may be involved in the development of autoimmune disorders (Kaplan, 2004).

Lupus nephritis is considered as one of the most serious complications of SLE. More than one factor has been proposed in the initiation and progression of LN. Two important factors that are suggested are overproduction of certain cytokines and apoptosis imbalance (Eman et al; 2015).

In lupus patients clearance of apoptotic cells is defective, which may play an important role in disease pathogenesis. During apoptosis, cells from membrane bound blebs containing intracellular proteins, act as a source of auto antigens. Defective clearance of apoptotic blebs, allows cells to undergo secondary necrosis and release nuclear auto antigens (Munoz et al; 2009).

The presence of excess lymphocyte apoptosis and defective clearance of apoptotic cells may promote B-cell lymphocyte hyper activity and subsequent auto antibody over production (Ren et al; 2003).

The APO-1/FAS gene a highly polymorphic gene and it is the longest gene of its family. The human APO-1/FAS gene has been mapped to chromosome 10q24 or 10q23 and spans ~ 25Kb of the chromosome. It has nine exons (25bp to >1.44Kb) and eight introns (152 bp to ~12Kb as well as the promoter responsible for allelic variations in FAS which can alter the rate of transcriptional. For example if the adenine (A) is replaced by guanine (G) at position -670, the resulting polymorphism increases the binding affinity of the transcriptional factor STAT-1 for the interferon gamma-activated sequence (GAS), which in turn modifies the transcriptional rate of the FAS receptor (Huang et al; 1997).

FAS (CD95/AP0-1/TNSF6), as a trans-membrane receptor among cell surface death receptors, are one of the tumor necrosis factor receptor (TNFR) superfamily. The Fas receptor is present in two forms; one is anchored to the plasma membrane, whereas the other is soluble (sFas). The later form is highly regulated at the transcriptional level (Kamihira and Yamada, 2001). It is expressed on many types of immune cells and plays a key role in the homeostasis of immune cells, regulation of T-cells, and removal of malignant and infected cells. Cross linking of the FAS receptor, either by its natural ligand (FASL) or by specific monoclonal antibodies, results in target cells apoptosis (Papo et al; 1998). Abnormal FAS-mediated apoptosis may lead to failure to eliminate auto reactive cells or impaired clearance of apoptotic cells, which is one of the susceptibility factors in development of SLE (Navratil and Aheam, 2000).

The current case-control study was designed to detect the possible association of sFAS and APO1/FAS-670A>G gene polymorphism with SLE, disease activity. Investigate the possible relation between the polymorphism and sFAS levels with the development of lupus nephritis. In addition, we studied the distribution of renal biopsy classes and their association with APO-1/Fas genotype

Materials and Methods:-**Patients:-**

A total of 94 subjects of Egyptian origin, including 50 SLE patients and 44 healthy control subjects, were included in the study.. The SLE patients (44 females and 6 male) were recruited from outpatient's clinic of the Rheumatology Department of BeniSuef University Hospital, Egypt, in the duration between June 2014 and March 2015. The cases were diagnosed by a rheumatologist according to American College of Rheumatology (ACR) revised criteria (Hochberg , 1997).The healthy control subjects; unrelated Egyptian age- and sex matched individuals had no family

history of autoimmune diseases. The control group lives in the same geographical area and has the same ethnic origin as the patients. All cases and control subjects were informed of the purpose of the study, and their consent was obtained. The local ethics committee approved the study. All procedures performed in studies involving human participants were in accordance with ethical standards of the institutional and with the 1964 Helsinki declaration and its later amendments in 2004.

Clinical and Laboratory assessment:-

All patients were subjected to standard hospital questionnaire and thorough clinical examination. Relevant data on history, and pathological finding of the patients were obtained from the hospital medical records. Laboratory assessment was done in the form of erythrocyte sedimentation rate (ESR), serum complement level (C3 and C4), antinuclear antibody (ANA), and anti-double stranded deoxyribonucleic acid (anti-DNA) antibody, complete urine analysis and kidney function tests. The SLE patients were stratified into two groups; the first group (non-lupus nephritis patients and the second group had LN).

Lupus nephritis was defined as clinical and laboratory manifestations that met the ACR criteria (persistent proteinuria > 0.5g/day or greater than +++ by dip stick, and/or cellular casts including red blood cells, hemoglobin, granular, tubular or mixed) and/or renal biopsy samples demonstrating immune complex mediated glomerulonephritis compatible with LN (Petri et al, 2012). The disease activity was assessed using the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) (Bombardier et al; 1995).

The pathological finding in renal specimens obtained from the patients' medical records of LN cases were routinely stained by hematoxylin-eosin, periodic acid Schiff (PAS) staining and examined by light microscopy. The biopsies were classified according to the International Society of Nephrology/Renal Pathology Society (ISN/RPS) Classification of lupus nephritis (Weening et al; 2004).

Blood samples were collected in vacutainer EDTA tubes and used for DNA extraction and simultaneously, tubes without anticoagulant were used to obtain serum. The EDTA samples were used for determination of erythrocyte sedimentation rate (ESR; Westergreen) the serum samples were used for determination of C-reactive protein (CRP) by semi quantitative latex (AVITEX® CRP, Omega Diagnostics). CRP was considered positive > 6mg/Litre. ANA was detected by immunofluorescence (IF) using HEP-2 cells (Kallestad™, BIO RAD) and tests were considered positive for titers above 1/40. Anti-dsDNA were determined by IF, with Crithidia luciliae (Kallestad™, BIO RAD) and tests were considered positive for titers above 1/10. C3 and C4 were measured by radial immune diffusion (Biocientifica SA).

Soluble Fas levels were measured using a commercial ELISA kit (Quantikine Human sFas/TNFRSF6 R&D System, Abingdon, UK), and the optical density was measured at 450nm using an ELISA plate reader (Stat Fax). The sFas concentration was expressed in pg/mL according to the curves obtained from the standards.

Genotyping:-

Genomic DNA was extracted from EDTA anti-coagulated whole blood using QIAamp DNA Mini Kit (Cat.no. 51104, QIAGEN) according to the manufacturer's protocol. The sequences flanking the APO-1/FAS (-670A/G) single nucleotide polymorphism were amplified by polymerase chain reaction (PCR). Genotyping of the polymorphism was determined by restriction fragment length polymorphism (RFLP) (Avis, 1994).

The 332-bp fragment of the APO-1/Fas (-670) gene, encompassing the polymorphic site, was amplified via PCR. The primer sequences were as follows: the sense (5'-CTACCTAAGAGCTATCTACCGTTC-3') and antisense (5'-GGC TGTCCATGTT GTGGCTGC-3') oligonucleotides (Vandana, 2012). The polymerase reaction was performed using Pfu DNA polymerase (# EP0571) as follows: first, 2.5 µl of DNA was placed in an eppendorf tube with a reaction mixture containing 0.5 µl of each primer; then 2.5 µl of 10x Pfu PCR buffer with MgSO₄, 0.5 µl of dNTP Mix, 10mM each (# R0191) and 0.7 µl Pfu DNA polymerase and adjusted to a final volume of 25 µl. The PCR reaction was performed in (Biometra) Thermal cycler under the following conditions: initial denaturation at 95°C for 1-3 minutes, followed by 35 cycles of amplification; denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, extension at 72°C for 1 minute and followed by a final extension phase for 5 minutes. The PCR products were electrophoresed and separated on 2% agarose gel using 1x TAE buffer for 40 min at 100 volts, followed by ultraviolet visualization. The PCR generated a 332-bp fragment carrying polymorphic site.

The PCR products were digested using MvaI restriction enzyme (# FD0554 Thermo Scientific) according to the manufacturer's protocol. The digested products were electrophoresed and separated on 4% agarose gel using 1x TAE buffer for 40 min at 100 volts followed by ultraviolet visualization. After digestion, Fas-670 G allele gave rise 188 bp, 99bp and 44-bp fragments, while the A allele gave rise to 233bp and 99 bp fragments.

Statistical Analysis:-

The collected data was reviewed, coding and statistical analysis was done by using SPSS program (statistical package of social science; SPSS Inc., Chicago, IL, USA) version 16 for Microsoft Windows. Mean, median, range and standard deviation, were calculated to measure central tendency and dispersion of quantitative data while frequency of occurrence was calculated to measure qualitative data. Student t test was used to determine the significance in difference between two means. Chi-square-test (χ^2) was done for comparison of qualitative data and fisher's exact test was used instead when the count of cell was less than 5. Odds ratios (ORs) with 95% confidence intervals (CI) were calculated whenever applicable, to test association between genotype and RA. Analysis of variance (ANOVA) test used to determine the difference between more than 2 means. The significance of the OR was calculated by a 2 by 2 contingency table. Genotype distributions were compared with those expected for samples from populations in Hardy-Weinberg equilibrium using a χ^2 test (1df). The level of significance was taken at p-value of <0.05.

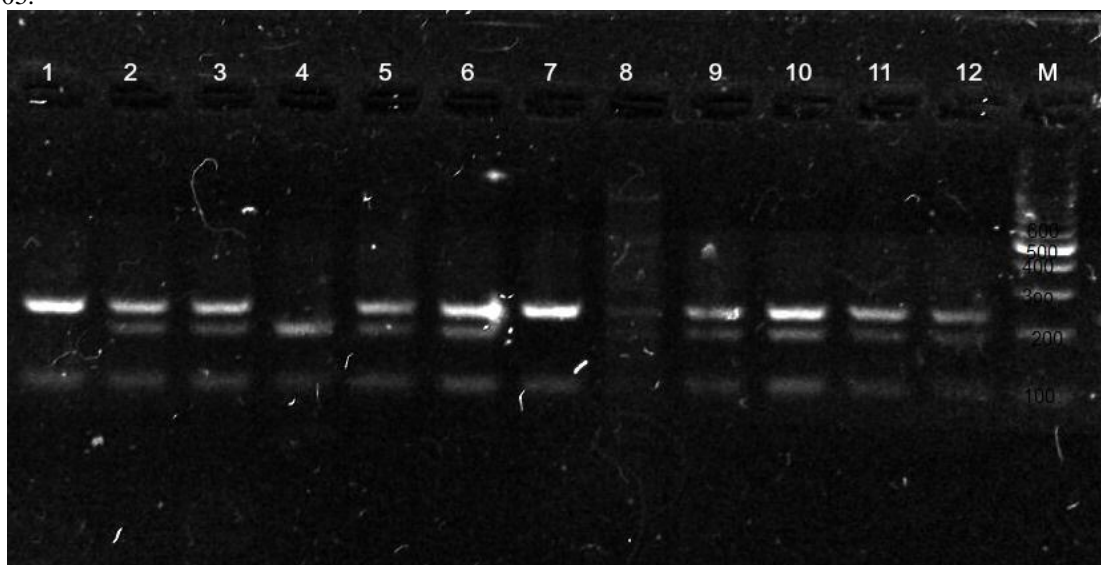


Fig 1:- PCR-RFLP analysis of APO1/FAS Gene Polymorphism 670A>G using MvaI restriction enzyme:

M: DNA molecular weight marker: (100-200-300-400-500-600 bp)

Lanes 1,7: Wild type (AA) genotype :233 and 99 bp

Lanes 2,3,5,6,8,9,10,11,12: Heterzygous (AG) genotype : 233, 188,99 bp

Lanes 4 : Homozygous mutant (GG) genotype: 188, 99 bp

Results:-

In the current case control study, among the 50 SLE patients the percentage of female patients was 88% while the percentage of male patients was 12%. The mean age of patients was 27.48 ± 9.58 years while the mean age of the healthy control group was 25.86 ± 4.47 years. On the other hand out of 50 SLE patients, 33 patients had LN with a mean age of 24.29 ± 20 while the mean age of non-LN patients was 27.38 ± 8088 . The demographic clinical and laboratory data of, control group, SLE, LN and non-LN patients are demonstrated in Table.1.

The distribution of APO-1/FAS -670 (A/G) genotypes in SLE cases showed slight deviation from Hardy-Weinberg Equilibrium (HWE) ($P=0.046$), while the genotype frequencies of the healthy control subjects conformed to HWE ($P=0.835$). In addition, both the genotype frequencies of LN and non-LN patients conformed to HWE ($P=0.428$; $P=0.377$, respectively).

In the present study, we compared the sFas serum levels in the healthy control (559.82 ± 232.86 pg/ml) group and the SLE (1284.00 ± 577.64) patients and we detected a significant difference between the levels sFas in these groups

($P < 0.001$). However, there was no statistically significant difference detected on comparing the level of sFas in both LN (1307.79 ± 644.95) and non-LN (1237.82 ± 431.88) patients ($P = 0.0689$) (Table.2).

Analysis of the distribution of APO-1/Fas -670(A/G) alleles, revealed that the allele frequency for healthy control subjects was 47.73% and 52.7% for the A and G alleles respectively. On the other, the allele frequency for the SLE group of patients was 48% and 52% for the A and G alleles respectively, ($\chi^2 = 0.34$; $P = 0.558$) (Table.4).

Regarding the distribution of APO-1/Fas -670(A/G) genotypes in SLE patients and healthy control groups, the heterozygous mutant (AG) genotype was significantly higher in SLE patients compared to the control group ($OR = 3.11$; $\chi^2 = 4.73$; $P = 0.029$) (Table.4).

Further on, analysis of the allele and genotype frequencies of APO-1/Fas -670(A/G) gene polymorphism in LN and non-LN patients, revealed a non-significant association between allelic and genotypic distribution between both LN and non-LN patients (for allele frequency $\chi^2 = 0.96$; $P = 0.326$ and for genotype frequencies $\chi^2 = 0.12$; $P = 0.730$; $\chi^2 = 1.17^b$; $P = 0.279$; $\chi^2 = 0.34$; $P = 0.557^c$, respectively). (Table.4)

For the next analysis, we attempted to detect an association between the status of disease activity and APO-1/Fas -670 (A/G) gene polymorphism. The patients carrying the heterozygous (AG) genotype revealed higher disease activity (58.3%) compared to patients carrying the wild type and the mutant homozygous genotypes (41.7% and 0%, respectively). We also detected that the percentage of patients having moderate disease activity was highest among patients carrying the AG genotype (64.7%) compared to patients carrying the GG (23.5%) and AA (11.8%) genotypes. Further on, the percentage of patients showing mild disease activity was highest among SLE patients carrying the AG genotype compared to the GG and AA genotypes (66.6%; 28.6% and 4.8%, respectively). The difference between the three groups was statistically significant ($\chi^2 = 10.15$; $P = 0.038$). (Table.5)

However, on investigating the association between disease activity represented by SLEDAI scoring system and APO-1/Fas -670 (A/G) gene polymorphism in LN and non LN patients, there was no statistically significant difference regarding diseases activity in both patients groups ($\chi^2 = 9.09$; $P = 0.059$). (Table.5)

In the current case control study, we stratified SLE patients into three groups according to APO-1/Fas -670 (A/G) gene polymorphism genotypes and we compared the levels of sFas in the three groups. The sFas level was slightly higher in the SLE patients carrying the AG genotype (1354.66 ± 621.44) compared to patients carrying the GG and AA genotypes (1163.10 ± 397.58 ; 1115.33 ± 579.382), however the difference in sFas level between the three groups was statistically non-significant (Table. 6). In addition, we stratified the patients into three groups according to the APO-1/Fas -670 (A/G) gene polymorphism genotypes, and we correlated the level of sFas and disease activity status represented by SLEDAI score. There was a statistically significant correlation between sFas level and SLEDAI score in patients carrying the AG genotype ($r = 0.47$; $P = 0.004$) (Figure 2).

In addition, we divided both LN and non LN patients into three groups according to APO-1/Fas -670 (A/G) gene polymorphism genotypes, and we compared the sFAS level in LN and non-LN patients in the three groups. In the lupus nephritis patients' group the serum Fas level was higher in patients having the AG genotype compared to patients having the GG and AA genotypes (1425.86 ± 681.86 ; 1024.40 ± 402.27 ; 1111.83 ± 625.84 , respectively). However, the in sFas level between the three groups was statistically non-significant ($f = 1.14$; $P = 0.333$). Similarly, there was a statistically non-significant difference ($f = 0.15$; $P = 0.86$) in sFas level in non-lupus nephritis patients carrying AG, GG or GG genotypes (1240.22 ± 459.76 , 1302.80 ± 380.84 , 1122.33 ± 570.97 ; respectively) (Table.6).

Among the 33 LN patients included within this study, we observed that out of 4 patients with type I LN, 3 patients had the AG genotype and 1 patient had the GG genotype. Out of 4 patients with type II LN, 2 patients had AG genotype and 2 patients had the GG genotype. Among the 12 patients with type III LN, 8 patients had the AG genotype and 4 patients had the GG genotype. In addition, the 6 patients with type IV LN, 4 patients had AG genotype, 1 patient had GG genotype and 1 patient had AA genotype. There were also 7 patients with type VI LN, 5 of them had the AG genotype, 1 patient had GG genotype and 1 patient had AA genotype.

Finally, we divided the SLE patients into three groups according to the genotype distribution (AA, AG and GG genotypes groups) among patients, and we correlated SLEDAI score and the sFas levels within each group. There

was a statistically significant positive correlation between sFas levels and SLEDAI score in patients carrying the AG genotype ($r= 0.47$; $P=0.004$) (Figure.2).

Table 1:- Demographic, clinical and laboratory Data of patients and control group

Clinical and lab. Data	Statistical method	SLE Cases (50)	controls (44)	LN (33)	Non-LN(17)	Significance test and P-value
Age	Mean \pm SD	27.48 \pm 9.58	25.86 \pm 4.47	24.29 \pm 9.20	27.34 \pm 8.88	t=1.02 P=0.308 ^a t=1.12 P=0.267 ^b
Sex	Female Male	44 (88%) 6 (12%)	34 (77.3%) 10 (22.7%)			X ² =1.90 P=0.167 ^a
Disease Duration (month)	Mean \pm SD Range	25.76 \pm 17.98 1-72	27.52 \pm 19.21		22.35 \pm 15.27	t=0.96 P=0.342 ^b
SLEDAI Score	Mean \pm SD Range	12.18 \pm 8.03 0-33		15.48 \pm 7.84	5.76 \pm 4.42	P<0.001 ^b
SLICC Score	Mean \pm SD Range	1.68 \pm 1.73 1-10				
ESR	Mean \pm SD Range	67.98 \pm 41.66 8-180				
CRP(mg/L)	Mean \pm SD Range	25.48 \pm 28.119 3-96				
HB (gm/dl)	Mean \pm SD Range	10.30 \pm 2.06 6-15				
ANA	Positive Negative	50 (100%) 0 (0%)				
Anti-ds DNA	Positive Negative	31(62%) 19 (38%)				
C3 and C4	Consumed Normal	23 (46%) 27 (54%)				
24hrs Urinary proteins(mg/dl)	Mean \pm SD Range	1097.40 \pm 1038.17 85-3500				

LN: lupus nephritis; SLE: systemic lupus erythematosus;^aSLE versus controls; ^bL N versus non LN. P-Value<0.05

Table 2:- Comparison between sFas levels in SLE, LN and non-LN patient

Groups	Controls	SLE	Lupus Nephritis	Non Lupus Nephritis
Serum Fas levels (pg/dl)	559.82 \pm 232.86	1284.00 \pm 577.64	1307.79 \pm 644.95	1237.82 \pm 431.88
-mean \pm SD	Student t test=3.98		Student t test=0.40	
-Significance Test	P value<0.001*		P value=0.689	

* Significant difference (p value<0.05)

Table 3:- Comparison of S.FAS levels among SLE,,LN and non-LN patients according to SLEDAI score

SLEDAI Score** Serum FAS Level (mean \pm SD)	Mild (≤ 8)	Moderate (9-18)	Severe (>18)	ANOVA Test	P value
Among SLE Patients:	1023.90 \pm 386.51	1452.75 \pm 864.95	1486.18 \pm 408.91	F statistics =4.15	0.022*
Among Lupus nephritis Patients	918.33 \pm 305.19	1452.75 \pm 864.96	1454.92 \pm 472.81	F statistics =2.46	0.102
Among Non-Lupus Nephritis Patients	1103.08 \pm 433.51	1237.82 \pm 431.89	1561.20 \pm 210.05	F statistics =4.95	0.042*

*significant difference (p value<0.05). ** patients were grouped arbitrary according to the rheumatologist clinical opinion

Table 4:- genotype and allele frequencies of the -670A/G Fas polymorphism in SLE, LN and non- LN patients

	Controls (44)	SLE Patients (50)	OR,(CI)	Significance Test	Lupus Nephritis (33)	Non- Lupus Nephritis (17)	OR,(CI)	Significance test
Genotype	14 (31.8%)	8(16.0%)	Reference	$\chi^2=4.73$	6(18.2%)	(11.8%)	Reference	$\chi^2=0.12^a$
A/A	18(40.9%)	32(64.0%)	3.11	P=0.029*	22(66.6%)	10(58.8%)	1.364	P=0.730
A/G	12(27.3%)	10(20.0%)	(0.98 ^a)	$\chi^2=0.38$	5(15.2%)	(29.4%)	(0.18-	$\chi^2=1.17^b$
G/G	30(68.2%)	42(84.0%)	10.12 ^a)	P=0.539	27(81.8%)	15 (88.2%)	11.88)	P=0.279
A/G+GG			1.46 (0.37 ^b - 5.83 ^b)				3.00 (0.27- 37.81)	$\chi^2=0.34^c$
			2.45 (0.83 ^c - 7.39 ^c)				1.667 (0.250- 13.75) ^c	P=0.557
Allele	42(47.73%)	52(%52)	1.19	$\chi^2=0.34$	32(48.48%)	20 (58.82%)	0.66	$\chi^2=0.96$
G	46(52.7%)	48(48%)	0.64-2.95	0.558	34(51.52%)	14 (41.18%)	0.26-1.65	0.326
A								

*Significance difference (P value<0.05)

- a- comparison between heterozygous mutant AG genotype versus wild AA genotypes among cases versus controls
- b- - comparison between homozygous mutant GG genotype versus wild AA genotypes among cases versus controls
- c- comparison between AG+GG genotypes versus wild AA genotypes among cases versus controls

Table 5:- Comparison of disease activity (SLEDAI Score) among SLE, LN and non -LN patients according to genotype

SLEDAI Score** Genotype	Mild (≤8)	Moderate (9-18)	Severe (>18)	Chi Square Test	P value
Among SLE Patients :					
AA	1(4.8%)	2(11.8%)	5(41.7%)	$\chi^2=10.15$	0.038
AG	14(66.6%)	11(64.7%)	7(58.3%)		
GG	6(28.6%)	4(23.5%)	0(0.0%)		
Among Lupus nephritis Patients:					
AA	0(0.0%)	1(8.3%)	5(41.7%)	$\chi^2=9.09$	0.059
AG	7(77.8%)	8(66.7%)	7(58.3%)		
GG	2(22.2%)	3(25.0%)	0(0.0%)		
Among Non-Lupus Nephritis Patients :					
AA	1(8.3%)	1(20.0%)	0(0.0%)	$\chi^2=0.62$	0.732
AG	7(58.3%)	3(60.0%)	0(0.0%)		
GG	4(33.4%)	1(20.0%)	0(0.0%)		

*Significant difference (P<0.05). ** Mild disease activity(≤8) , Moderate disease activity(9-18) , Severe disease activity (>18)

Table 6:- comparison of sFas level in according to genotype in LN, non-LN and all SLE patients

	Cases of AG Genotype	Cases of GG Genotype	Cases of AA Genotype	ANOVA	P value
Serum FAS Level(mean ±SD) among Lupus Nephritis Cases	1425.86± 681.86	1023.40 ±402.27	1111.83±625.84	f= 1.14	0.333
Serum FAS Level(mean ±SD) among non- Lupus Nephritis Cases	1240.22±459.76	1302.80±380.84	1122.33±570.97	F=0.15	0.865
Serum FAS Level(mean ±SD) among all SLE patients	1354.66±621.45	1163.10±397.58	1152.50±598.95	F=0.66	0.523

-Significance difference (P<0.05)

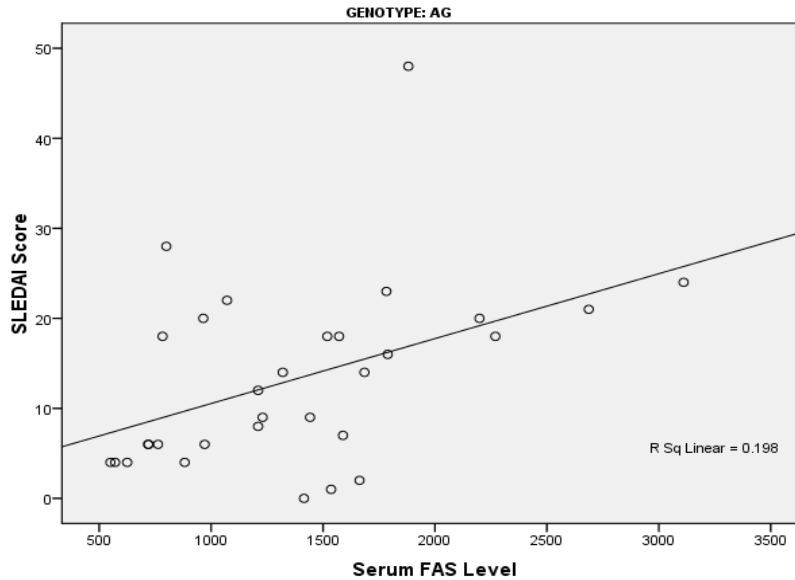


Figure 2:- Correlation OFSLEDAI score and sFas levels in SLE patients with AG genotype.

Discussion:-

Serum soluble Fas (sFas) is a variant of the receptor molecule which lacks the trans membrane segment (Jia et al; 2006). The role of serum FAS mediated apoptosis in immunity and removal of auto reactive lymphocytes is quite clear, but the function of sFas as one of the markers of apoptosis in autoimmune diseases, especially SLE is currently under investigation (Sahin; 2006) .

In the current case control study, there was highly statistically significant difference on comparing sFas levels in SLE patients and healthy control subjects. The mean serum FAS level was 1284.00 ± 577.64 pg/ml in patients group and it was 559.82 ± 232.86 in healthy controls ($P > 0.001$).

In accordance with the results of the current study, Maryam et al (2010) indicated that in SLE patients the mean sFas levels 372 ± 20 pg/ml against a mean of 190.38 ± 127.77 in healthy controls ($P = 0.001$). In addition, our results regarding the significant difference in sFas level detected between SLE patients and healthy controls ,was in agreement with the results of the studies conducted by Jodo et al(1997) , courteny et al(1999), Al-Mainietal(2000) and Vandana(2012).

The results of the current study regarding the high levels of sFas in SLE patients could be explained by the hypothesis proposing that during active apoptosis, released sFas may interact with fasL, leading to inhibition of cellular Fas/FasL interaction and hence inhibition of apoptosis(Maryam et al; 2010). Al Maini et al. (2000) proposed that sFas may contribute to the pathogenesis of SLE by protecting the autoreactive and Fas receptor-bearing lymphocytes from apoptosis.

Further on, we compared the sFas levels in SLE patients according to the disease activity status based on the SLEDAI scoring system. SFas level was significantly higher ($P = 0.022$) in thesevere andmoderatedisease activity groups respectively (mean 1486.18 ± 408.91 pg/ml; 1452.75 ± 864.95 pg/ml, respectively), compared to the low activity group (mean: 1023.90 ± 386.51 pg/ml).

In agreement with the results of the current study, Jia et al,(2006) ,indicated that serum sFas levels were significantly higher in the more active SLE patients compared to the less active SLE patients($P = 0.04$). They also, indicated that there is a positive correlation between sFas levels and SLEDAI, however, it was not statistically significant($r = 0.114$; $P = 0.2$).

Similarly, Vandana, 2012 indicated that sFas levels were higher in patients with higher SLEDAI scores (SLEDAI > 18). In addition, several research groups have reported that sFas levels were elevated in serum samples obtained from patients with SLE with active SLE than those with inactive SLE (Al-Maini et al, 2000; van der Linden et al, 2001 and Silvestris et al, 2003).

Maryam et al, (2010), indicated that the mean sFas levels differed significantly between low, medium and high activity groups ($P=0.001$). However, contrary to our results, on evaluating the correlation between the two variables, they detected a significant positive correlation ($r=0.494$; $P=0.001$). These results were contradicted by Al-Maini et al (2000) and Aleuc et al (2001). In a study by Courtney et al (1999), the correlation between sFas and SLEDAI was reported significant only during the relapse phase but not during the entire course of the disease.

The SLE disease activity score system could be affected by many different factors. These scores evaluate the patient's status over a limited time and thus, these controversies might have originated from the low number of patients, duration of activity prior to sampling and medications (Maryam et al; 2010).

Renal involvement is one of the serious complications of SLE due to its involvement in the occurrence of high morbidity and mortality rates (Iwata et al; 2011). The diagnosis of glomerulonephritis is suspected when proteinuria and urinary sediments are accompanied by arterial hypertension. These data may predict kidney involvement, however, the gold standard for diagnosis and classification of LN, remains to be renal biopsy (Bollain et al; 2014).

To investigate the possible association and correlation of sFas levels with LN, SLE patients were stratified into two groups, the first group represented the LN patients (33 patients), while the second group represented the non-lupus nephritis patients (17 patients). There was no statistically significant difference detected between the level of sFas in LN patients and non-lupus nephritis patients (mean 1307.79 ± 664.95 and 1237 ± 431.88 respectively, $P=0.689$).

Similarly, Vandana (2012), indicated that among 35 LN patients, 17 had strong sFas positivity, 6 had weak positivity and 12 were sFas negative. On the other hand, among 35 non-lupus nephritis patients, 13 had strong positivity, 4 patients had weak positivity and 18 patients were sFas negatives. In contrast, Hao et al; 2006 and Eman et al; 2015, reported an association between sFas levels and LN.

The results of the current study, suggest that renal involvement in SLE patients is not associated with changes in sFas levels. This could be explained by the fact that renal tissue cells express Fas antigen in plenty, supporting the view that this tissue may well be involved in the production of sFas. sFas, produced locally by the kidney, may then act protectively by blocking FasL: Fas receptor interaction that leads to the damage sustained by the kidney due to autoimmune disease (Jarad et al; 2004).

The repeated investigations concerning the association of Fas polymorphisms with SLE, in different populations, supported the involvement of this gene in SLE susceptibility (Iu et al; 2012). It has been proposed that polymorphisms in the promoter region regulate transcription or alternate splicing of Fas in particular conditions or cell populations (Vandana, 2012). The human APO-1/Fas was mapped to chromosome 10q24 or 10q23 and spans ~25 kb of the chromosome. It has nine exons (25 bp to >1.44 kb) and eight introns (152bp to ~12 kb) (Richa et al; 2009).

Regarding the association APO-1/Fas (-670 A/G) polymorphism in the occurrence of SLE, we detected that the frequency distribution of the A and the G alleles was not significantly different when compared in SLE patients and the healthy control group ($P=0.558$; $\chi^2=0.34$). As, the frequency of the A allele in the SLE patients was 48% while, in the healthy control group it represented 46%. On the other hand, the frequency of the G allele represented 52% in the SLE patients and 42% in the control group.

The studies on the association between Fas -679 A/G polymorphism and SLE produced conflicting results. Several case control studies were in accordance with the results of the current study regarding the non-significant difference in frequency distribution A and G alleles among SLE cases and healthy controls (Arasteh et al; 2010), (Man-Man et al; 2012), (Vandana, 2012). However, a case control study conducted by Bollain et al, (2014) reported that the -670 Fas polymorphism revealed an association of the G allele with increased SLE susceptibility ($P=0.03$). On the other hand, on evaluation of the Japanese, Kanemitsu et al; (2002), detected that the -670 A allele to be of higher frequency in SLE patients compared to healthy control subjects.

Regarding the frequency distribution of genotypes in APO-1/Fas (-670 A/G) gene polymorphism, the current study revealed a possible association of the heterozygous (AG) genotype with SLE, as the frequency of the AG genotype was significantly higher ($P= 0.029$; $\chi^2 =4.73$) in SLE patients when compared to the wild (AA) genotype among cases versus control group.

In contrast to our results, Vandana in 2012, indicated that on analysis of association of APO-1/ Fas -670 A/G polymorphism with SLE, the AA (mutant) genotype was significantly more frequently represented among SLE patients as compared to the normal population ($P<0.001$). Similarly, Eman et al; (2015) reported the significant association of AA genotype and SLE. On the other hand, other studies reported the lack of association between the -670 gene polymorphism genotypes and SLE. (Arasteh et al; 2010 and Man-Man et al; 2012).

Thus the studies on the association between Fas -670 A/G polymorphism and SLE revealed controversial results; it may be because of the different ethnicities, clinical heterogeneity, and real genetic heterogeneity. Another possible explanation is the small sample size (Bollain et al; 2014). In addition, SLE is a multi-factorial disease; individual exposure to various environmental factors combined with genetic susceptibility may have contributed to the conflicting results (Man-Man et al; 2012).

Regarding the association of APO-1/Fas (-670 A/G) polymorphism and severity of clinical manifestation, the SLE patients were grouped into three groups based on disease activity status represented by SLEDAI scores. The mild group (score ≤ 8), moderate manifestation group (score 9-18) and severe manifestation group (score >18) (Vandana, 2012). The AG heterozygous genotype was highest (58.3 %) among the severe manifestation group, followed by the AA genotype (41%). The difference between groups was statistically significant ($P=0.038$).

On the other hand, Vandana in 2012, indicated that the AA genotype was highest (47%) among the patients with severe clinical manifestation indicating the possibility of the A allele contribution towards the severe activity status of SLE.

Further on, we detected a statistically significant correlation, on correlating SLEDAI disease activity score with the levels of sFas in patients carrying the AG genotype ($P=0.004$, $r=0.21$). Thus, we might suggest that AG genotype in SLE patients might increase the level of transcription of sFas which might be implicated in increasing the disease activity in patients carrying the AG genotype.

Regarding the association between the -670 polymorphism and sFas levels in SLE patients, on comparing the sFas levels we detected that on comparing AG and GG genotypes, respectively, versus the AA wild genotype, there was no statistically significant difference detected between groups ($P=0.413$ and $P=0.956$, respectively).

In accordance to the results of the current study, Vandana in 2012, after stratifying the patients into sFas negatives, weak positives and strong positives revealed that 50% of SLE patients with the AG genotype, 33% with the AA genotype and 16.7% with GG genotype were sFas negatives. On the other hand, 57.5% patients with AG genotype, 27.5% with AA genotype and 15% with GG genotype had elevated sFas levels.

In addition, we investigated the possible role of APO-1/Fas (-670 A/G) polymorphism in the development of lupus nephritis. The results of the current study indicated that there was no statistically significant association between the developments of lupus nephritis as the frequency distribution of G allele was 48.48% in LN patients and it was 52.82% in non LN patients. Similarly, the frequency of the A allele was 51.52% in LN patients and 41.18% in non LN patients ($\chi^2 = 0.96$, $P=0.326$). Also, no statistically significant association was detected, on comparing the patients carrying the AG genotype in lupus and non-lupus patients versus wild AA genotype ($\chi^2 = 0.12$; $P=0.730$). Further on, no statistically significant association was detected between LN and non LN patients with the GG genotype versus the wild AA genotype ($\chi^2 = 1.17$; $P=0.279$).

In contrast, Huang et al in 1997 reported a higher incidence of AA genotype in LN than in non-LN patients. Similarly, Eman et al, (2015) revealed that, AA genotype was associated with 4.08 times higher risk of lupus nephritis than controls and 1.91 times than SLE group ($P=0.002$). While A allele frequency was higher in LN group and it was associated with 4.16 times higher risk of LN than controls and 1.78 times than SLE group ($P<0.001$).

In Conclusion, this study suggests a possible genetic association behind the susceptibility between SLE and APO-1/Fas (-670 A/G) gene polymorphism in the Egyptian population. The AG heterozygous genotype is more frequently represented in SLE patients than healthy population. SFAS levels are higher in SLE patients in the study

group compared to healthy controls. SFAS levels are significantly higher in SLE and LN patients with severe activity status. According to SLEDAI scoring for disease activity the AG genotype patients had a more severe disease compared to GG and AA genotype patients.

Further on, we detected a significant positive correlation between sFas levels and SLEDAI score for disease activity, in patients carrying the AG genotype. SFas levels are higher in SLE patients than healthy subjects and are associated with severe disease activity status activity.

However, the present work should be regarded as a hypothesis- testing with its limitations, and further studies using larger samples are needed to establish the genetic association. Additionally, the results of this study may have future prognostic value for the future clinical observation of SLE patients.

Compliance with Ethical Standards:-

All procedures performed in studies involving human participants were in accordance with ethical standards of the institutional and with the 1964 Helsinki declaration and its later amendments in 2004.

Informed consent:-

Informed consent was obtained from all individual participants included in the study.

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Conflict of Interest:-

The authors declare that they have no conflict of interest.

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