Cellular and Molecular Biology

Tanhapour *et al. Cell. Mol. Biol.*2016, 62 (12): 56-61
ISSN: 1165-158X
doi: 10.14715/cmb/2016.62.12.10

*Original Research*

# Association between the cytotoxic T-lymphocyte antigen-4 mutations and the susceptibility to systemic lupus erythematosus; Contribution markers of inflammation and oxidative stress

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**Abstract:** The cytotoxic T lymphocyte antigen-4 (CTLA-4) also known as CD152 (cluster of differentiation 152) is a crucial negative regulator of the immune system. This protein receptor provides negative signals in order to suppress T-cell activation and immune attack against self-antigens, although its role is unclear. The ability of CTLA-4 to limit T cell-mediated immune response has made it a major target in treatment of tumors and autoimmune diseases such as systemic lupus erythematosus (SLE). In this study, we investigated whether CTLA-4 $G_{-1661}A$ and CTLA-4 $T_{-1722}C$ mutations are associated with SLE. So one hundred nine SLE patients and 101 gender and age-matched unrelated healthy controls were recruited for this case-control study. The promoter mutations were detected by PCR-RFLP, neopterin, malondialdehyde (MDA) and serum lipid concentration were determined by HPLC and enzyme assay, respectively. Result: We found that both codominant (AA vs. GG) and recessive (AA vs. GA+GG) CTLA-4 $G_{-1661}A$ mutation significantly decreased the risk of SLE by 1.7 and 3.7 times, respectively. Interestingly, SLE patients with AA genotypes of CTLA-4 $G_{-1661}A$ have lower neopterin and MDA concentration compared with GA+GG genotypes. The overall distribution of CTLA-4 $T_{-1722}C$ genotypes and alleles in SLE patients were similar to those in control group. In conclusion, our findings showed, that there is an association between systemic inflammatory markers, oxidative stress and the CTLA-4 $G_{-1661}A$ GG+AG genotypes, MDA and neopterin which are the most conventional risk factors for coronary heart disease, therefore these mutations may be consider as a risk factor for susceptibility to heart disease in SLE patients.

***Key words:*** CTLA-4, neopterin, MDA, SLE.

## Introduction

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease in which the body produces autoantibodies against self-antigens (1). The etiology and pathogenesis of SLE are unknown, although various factors such as environmental factors, sibling, hormonal factors and genetics play a role in the onset of the disease (2,3). The gene encoding CTLA-4 on chromosome 2q33 is one of the potential regions associated with an increased risk of developing SLE (2,4). CTLA-4 is a glycoprotein that is expressed by both $CD4^+$ and $CD8^+$ T cells (5). Due to alternative splicing of CTLA-4 gene there are 2 isoforms of protein, a full-length (flCTLA-4) and a soluble (sCTLA-4) form lacking transmembrane domain (4). CTLA-4 is related to T cell receptor, CD28. Both molecules interact with CD80 and CD86 ligands found on antigen presenting cells. Despite structural similarities, the functions of CD28 and CTLA-4 are almost completely opposite. CD28 enhances T cell proliferation, cytokine production, survival and also enables T cells to provide help for B cells, while CTLA-4 acts as an inhibitor of T cell responses (5). CTLA-4 binds to CD80 and CD86 and blocks their interactions with CD28 and down-regulates T-cell activation, which may help to maintain peripheral tolerance and contribute to SLE treatment (4,6). Several CTLA-4 gene mutations have been reported but only few of them are associated with SLE (6). Two of the most important CTLA-4 gene variations are G/A nucleotide transition at position -1661 and T/C change at −1722 within the promoter region (4). T/C change at position −1722 affects binding sites of transcription factors, whereas G/A transition at position -1661 may alter the potential response element for myocyte enhancer factor 2 (MEF2) (7).

Previous studies demonstrated that neopterin is generated following activation of cellular immune system (8). Neopterin is derived from guanosine triphosphate (GTP) in response to interferon-(IFN) γ secreted by activated T cells (9,10). Although other cell types can produce neopterin but its amount is negligible (11). Neopterin elevated in patients with infections, neoplastic and

**Received** May 11, 2016; **Accepted** October 17, 2016; **Published** October 31, 2016

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inflammatory diseases (11).

Shah et al. reported excessive amount of reactive oxygen species (ROS) that may cause abnormal activation of apoptosis, are considered as crucial factor in generation of antibody and pathogenesis of SLE (12).

Cell membrane lipids are one of the most important target molecules which can be attacked by the free radicals (13). MDA, an oxidation product of lipid peroxidation, contribute to oxidative damage in various diseases such as cancer, atherosclerosis, Alzheimer's disease, diabetes mellitus and autoimmune diseases (14).

In this study, we investigated association between CTLA-4 $G_{-1661}A$ and CTLA-4 $T_{-1722}C$ gene promoter mutations with susceptibility to SLE. Also we examined whether the MDA as a marker of oxidative stress and the neopterin as an activated immune system mediator correlate with developing and severity of disease.

## Materials and Methods

### Study groups

A total of 109 SLE patients (mean age, 37.3 ±11.3 years; range, 15–75 years; 90 females and 19 males) meeting the 2012 revised criteria of the American College of Rheumatology (ACR) for SLE (15) were recruited from Imam Reza Hospital of the Kermanshah University of Medical Sciences. Clinical and laboratory findings were defined using ACR criteria and were collected with respect to age, gender and involved organs.

Renal and cardiovascular diseases were identified by X-ray, electrocardiogram, ultrasonic cardiogram, urinalysis, brain computed tomography scan or magnetic resonance imaging.

Systolic blood pressure of ≥ 140 mmHg or diastolic blood pressure ≥ 90 mmHg was defined as hypertension. Lupus nephritis was diagnosed through urinalysis (proteinuria greater than 500 mg/day), blood tests (high levels of creatinine) and kidney biopsy.

One hundred one control healthy volunteers (mean age, 37.1±11.5 years; range, 15–72 years; 81 females and 20 males) without any history of autoimmune diseases, coronary heart disease, and SLE whom they had no specific diseases at their annual medical checkup were selected from Hospitals of the Kermanshah University of Medical Sciences.

### Chemical analysis

The neopterin and MDA levels of serum samples were determined by High Performance Liquid Chromatography (HPLC) system (Agilent Technologies 1200 Series, Agilent Corp., Germany) using EC 250/4.6 Nucleodur 5 µm C18 column (Macherey-Nagel, Du¨ ren, Germany) as previously described (16).

### Serum lipids

Serum lipid profiles included low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), total cholesterol (TC) and triglyceride (TG), were measured by the standard enzymatic method (Pars Azmon kit, Iran), using an automated Erba XL-600 (Mannheim, Germany).

### DNA Extraction and CTLA-4 Genotyping

DNA was extracted from peripheral blood samples using phenol chloroform extraction method according to the standard protocol (17). The mutations at positions $G_{-1661}A$ and $T_{-1722}C$ of CTLA-4 gene were determined by PCR using forward 5'-CTA AGA GCA TCC GCTTGC ACC T-3' and reverse 5' -TTG GTG TGA TGC ACA GAA GCC TTTT-3' primers. The PCR products were digested with restriction enzyme *Bvb*I for T/C at position −1722 and *Mse*I for G/A at position -1661. The digested products were analyzed on 2.5% agarose gel and visualized by ethidium bromide staining. The CTLA-4 $T_{-1722}C$ mutation generated a single 486 bp fragment for T allele and two fragments of 270 bp and 216 bp for C allele "figure1". CTLA-4 $G_{-1661}A$ mutation generated a 486 bp for G allele and 347bp and 139 bp fragments for A allele "figure 2" (4).

Ethical Considerations

The study protocol was approved by the Ethics Committee of the Kermanshah University of Medical Sciences and was in accordance with the principles of the Declaration of Helsinki II and all subjects provided written informed consent.

### Statistical analyses

The allelic frequencies were calculated by the gene counting method. The CTLA-4 $G_{-1661}A$ and CTLA-4 $T_{-1722}C$ genotypes and allele frequencies in SLE patients were compared to controls using the t-test. Odds ratios

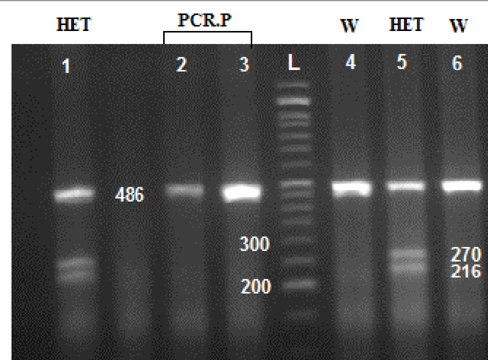


**Figure 1.** Electrophoresis patterns for CTLA-4 T−1722C genotypes analyzed by PCR-RFLP. L: 50 bp DNA ladder, PCR.p: PCR product (486bp), W: homozygous wild-type (TT: 486bp), HET, heterozygous mutant (TC: 486, 270, 216bp). The expected PFLP fragments were detected using 2% agarose gel electrophoresis.

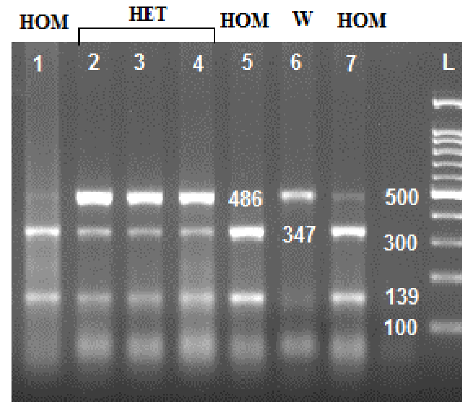


**Figure 2.** Electrophoresis patterns for CTLA-4 G-1661A genotypes analyzed by PCR-RFLP. L: 100 bp DNA ladder, PCR.p: PCR product (486bp), W: homozygous wild-type (GG: (486bp), HOM, homozygous mutant (AA: 347, 139bp), HET, heterozygous mutant (GA: 486, 347, 139bp). The expected RLFP fragments were detected using 2% agarose gel electrophoresis.

**Table 1.** The demographic characteristics and distribution of serum lipids and other risk factors in SLE patients and control group in population from Kermanshah province.

| | **SLE patient (n=109)** | **Control subjects (n=101)** | **p values** |
|---|---|---|---|
| Age (years) | 35.6±16.3 | 37.1±11.5 | #NS |
| Sex (M/F) | 19/90 | 20/81 | #NS |
| Neopterin (nmol/L) | 25.8±38.1 | 6.5±2.9 | <0.001 |
| MDA (µmol/L) | 1.84±1.27 | 1.41±0.6 | 0.003 |
| HDL-cholesterol (mg/dL) | 42.1±21.1 | 42.7±11 | 0.067* |
| Total cholesterol (mg/dL) | 196±37 | 188±61 | 0.019* |
| Triacylglycerols (mg/dL) | 179±141 | 173±117 | 0.76 |
| SLE activity | 21±12.2 | - | - |

*p>0.05; #NS not significant: MAD: malondialdehyde, HDL: high-density lipoprotein, LDL: low-density lipoprotein.

(OR) were calculated as estimates of relative risk for disease and 95% confidence intervals (CI) obtained by SPSS logistic regression. The interaction between CTLA-4 G-$_{1661}$A and CTLA-4 $T_{-1722}C$ mutations was determined using a logistic regression model. The correlation of serum levels of neopterin, MDA, HDL-C, LDL-C, TC and TG with the CTLA-4 $G_{-1661}A$ and CTLA-4 $T_{-1722}C$ mutations between studied groups were calculated using linear regression and unpaired t test (Pearson and Spearman). A two-tailed Student's t test, analysis of variance (ANOVA) and nonparametric independent-sample Mann-Whitney analyses were used to compare quantitative data. Statistical significance was assumed at $p<0.05$. The SPSS statistical software (SPSS version 16.0; SPSS Inc, Chicago, IL, USA) was used for the statistical analysis.

## Results

The demographic features and laboratory test results for the SLE patients and control group are shown in table 1. Details of the clinical and laboratory features of SLE patients and control group have been described previously (16).

The frequency of CTLA-4 $G_{-1661}A$ and $T_{-1722}C$ genotypes and alleles in SLE patients and control group are demonstrated in Tables 2 and 3. As shown in Table 2, the overall distribution of the CTLA4 $G_{-1661}A$ genotypes in SLE patients were significantly different from that the control group ($\chi2=28.9$, df=2, p=0.001). The frequency of the CTLA4 $G_{-1661}A$, G allele was increased in SLE patients: 72.5% versus 68.4% in control subjects, although the difference was not significant (Table 2). The overall distribution of CTLA-4 $T_{-1722}C$ genotypes and alleles in SLE patients were not significantly different from that of control group (Table 3). The age and sex adjusted OR indicated that both codominant (AA vs. GG), and recessive (AA vs. G/A+G/G) CTLA-4 $G_{-1661}A$ gene mutations significantly decreased the risk of SLE by 1.7 and 3.7 times, respectively (Table 4). However, CTLA-4 $G_{-1661}A$ codominant (G/A vs. GG) gene mutation significantly increased the risk of SLE by 2.45 (1.4–4.7, p=0.001) times (Table 4).

We analyzed association of CTLA-4 $G_{-1661}A$ genotypes (G/G+A/G, A/A) with serum level of LDL-C, HDL-C, TC, TG, neopterin and MDA. As observed in Table 5, the presence of CTLA-4 $G_{-1661}A$ genotypes had a considerable effect on neopterin, MDA and LDL-C serum levels in both SLE patients and control subjects.

**Table 2**. Odds ratio and distribution of cytotoxic T lymphocyte CTLA-4 $G_{-1661}A$ genotypes and alleles in SLE patients and control subjects.

| **CTLA-4 $G_{-1661}A$** | **SLE patients (n=109)** | **Control subjects (n=101)** |
|---|---|---|
| genotypes | | |
| G/G | 57 (53%) | 60 (59%) |
| G/A | 44 (40%) | 18(18.3%) |
| A/A | 8(7%) | 23(22.7%) |
| | ($\chi2=28.9$, df=2, p=0.001) | |
| alleles | | |
| G | n=158 | n=138 |
| A | 0.892 (0.7-1.05, p=0.1, n=60) | n=64 |
| | ($\chi2=1.8$, df=1, p=0.1) | |

SLE: systemic lupus erythematosus, *CTLA- 4:* cytotoxic T lymphocyte associated antigen 4, odd ratio is an estimate relative risk for disease that was calculated and 95% confidence interval was obtained by using $\chi^2$ regression binary logistic analysis.

**Table 3**. Distribution of cytotoxic T lymphocyte CTLA4 $T_{-1722}C$ genotypes and alleles in patients with SLE and control subjects.

| **Promoter CTLA-4 $T_{-1722}C$** | **SLE patients (n=109)** | **Control subjects (n=101)** |
|---|---|---|
| genotypes | | |
| T/T | 88(80.7%) | 82 (81%) |
| T/C | 21 (19.3%) | 19 (19%) |
| C/C | - | - |
| | ($\chi^2$=0/01, df=1, p=0.9) | |
| alleles | | |
| T | 197 (81.2%) | 183 (81%) |
| C | 21 (18.8%) ($\chi^2$=0.02, df=1, p=0.8) | 19 (19%) |

SLE: systemic lupus erythematosus, CTLA4: cytotoxic T lymphocyte associated antigen 4;, df: degree of freedom. The distribution of CTLA4 alleles and genotypes in SLE group were compared with those in control group using the $\chi^2$ test analysis.

**Tables 4.** Odds ratio of CTLA-4 $G_{-1661}A$ genotypes and alleles with respect to codominant, dominant and recessive model in SLE patients after adjusted sex and age.

| Promoter CTLA-4 $G_{-1661}A$ | SLE patients reference group OR (95%confidential interval) | Control reference group |
|---|---|---|
| **Codominant** | | |
| A/A vs. G/G | 0.589 (0.4–0.8, p=0.003) (n=8 vs. n=57) | (n=23 vs. n=60) |
| A/G vs. G/G | 2.45 (1.4–4.7, p=0.001) (n=44 vs. n=57) | (n=18 vs. n=60) |
| **Dominant** | | |
| A/A+ A/G vs. G/G | 1.28 (0.83–1.96, p=0.2) (n=52 vs. n=57) | (n=41 vs. n=60) |
| **Recessive** | | |
| A/A vs. G/G+ A/G | 0.27 (0.13–0.53, p=<0.001) (n=8 vs. n=101) | (n=23 vs. n=78) |
| CTLA4 alleles | | |
| G | n=158 | n=138 |
| A | 0.892 (0.7-1.05, p=0.1, n=60) | n=64 |

SLE: systemic lupus erythematosus, *CTLA- 4:* cytotoxic T lymphocyte associated antigen 4, odds ratios is an estimate relative risk for disease that were calculated and 95% confidence intervals were obtained by using $\chi^2$ regression binary logistic analysis.

Also we observed in Table 5, SLE patients with one or two copies of G allele (G/A+G/G) of CTLA-4 $G_{-1661}A$, had significantly higher neopterin (p<0.001), MDA (p=0.002), and LDL-C (p<0.001) concentrations compared with control subjects.

In addition, we compared distribution and odds ratios of the functional CTLA-4 $G_{-1661}A$ promoter genotypes in SLE patients with and without HBP, HD and LN in Table 6. Recessive model of CTLA-4 $G_{-1661}A$ genotypes decreased the risk of HD in SLE patients (OR=0.32), but the correlation had a trend to become significant (p=0.08) (Table 6).

To investigate interaction between CTLA-4 $G_{-1661}A$ and CTLA-4 $T_{-1722}C$ alleles in SLE patient's logistic regression analysis was used and results were demonstrated in Table 7. We detected no significant association between CTLA-4 $G_{-1661}A$ and CTLA-4 $T_{-1722}C$ alleles in this study.

## Discussion

SLE is a chronic autoimmune disease that is caused

**Tables 5.** Comparison of LDL-C, HDL-C, TC, TG, neopterin and MDA serum levels between CTLA-4 $G_{-1661}A$ genotypes (G/G+A/G, A/A) in SLE patients with control subjects.

| AA | SLE patients | Control subjects | p values |
|---|---|---|---|
| LDL-C (mg/dL) | 113±30 | 75.6±39 | <0.001 |
| HDL-C (mg/dL) | 43.5±18 | 44.4±11 | 0.8 |
| TC (mg/dL) | 186±18 | 193±38 | 0.6 |
| TG (mg/dL) | 134±72 | 161±114 | 0.4 |
| Neoptrin (nmol/L) | 21.1±18.7 | 5.9±3 | <0.001 |
| MDA (mmol/l) | 1.7 + 0.6 | 1.1+0.4 | 0.002 |
| **GG+AG** | | | |
| LDL-C (mg/dL) | 118±27 | 84.3±36 | <0.001 |
| HDL-C (mg/dL) | 43.6±23 | 41.8±10 | 0.4 |
| TC (mg/dL) | 184±57 | 191±35 | 0.2 |
| TG (mg/dL) | 181±152 | 169±1.8 | 0.4 |
| Neoptrin (nmol/L) | 25.5±38 | 6.5±3 | <0.001 |
| MDA (mmol/l) | 2.1±1.2 | 1.05±.3 | <0.001 |

MAD: malondialdehyde, HDL: high-density lipoprotein, LDL: low-density lipoprotein, TC: total serum cholesterol. TG:triacylglycerol.

**Table 6.** The Genotypic distribution and odd ratio of CTLA-4 $G_{-1661}A$ (A/A vs. A/G+G/G) in SLE patients with and without HBP, heart disease (HD) and lupus nephropathy (LN).

| Promoter CTLA-4 $G_{-1661}A$ | HBP | | HD | | LN | |
|---|---|---|---|---|---|---|
| | No (73) | Yes (36) | No (92) | Yes (17) | No (74) | Yes (35) |
| Recessive | | | | | | |
| A/A vs. G/G+A/G | (5 vs. 68) | (2 vs. 34) | (5 vs. 87) | (3 vs. 14) | (6 vs. 68) | (3 vs. 32) |
| | OR=0.8 (0.3- 7.5, p=0.5) | | OR=0.32(0.1- .1, p=0.08) | | OR=0.92(0.2- 3.2, p=0.9) | |
| | ($\chi^2$=0.32, df=1, p=0.51) | | ($\chi^2$=3.2, df=1, p=0.07) | | ($\chi^2$=0.01, df=1, p=0.93) | |

CTLA-4: cytotoxic T lymphocyte associated antigen 4, SLE: systemic lupus erythematosus, HBP: high blood pressure, df: degree of freedom, odds ratios is an estimate relative risk for disease that were calculated and 95% confidence intervals were obtained by using $\chi^2$ regression binary logistic analysis.

**Tables 7.** Carrier odds ratios interaction between -1661 CTLA-4 G allele and -1722 CTLA-4 C allele in SLE patients compared with control group.

| CTLA-4 $G_{-1661}A$ A | CTLA-4 $T_{-1722}C$ C | SLE patients | Control group | OR (95%CI) |
|---|---|---|---|---|
| - | - | n=47.5 (43.5%) | n=49 (48.5%) | References |
| + | - | n=40 (36.6%) | n=37 (36.6%) | 1.28 (0.8- 2.1, p=0.32) ($\chi^2$ =1,df=1,p=0.3) |
| - | + | n=9 (8.2 %) | n=10 (9.9%) | 0.97 (0.7- 1.4, p=0.97) ($\chi^2$ =0.1,df=1,p=0.98) |
| + | + | n=13 (11.9%) | n=6 (5.9%) | 1.12 (0.9- 1.5, p=0.39) ($\chi^2$ =0.8,df=1,p=0.38) |

*CTLA 4:* cytotoxic T lymphocyte associated antigen 4, SLE: systemic lupus erythematosus, CI: confidence interval, df: degree of freedom.

by complex interactions between genetic, environmental and hormonal factors (6). In addition, regulation of T-cell function by CTLA-4 receptor and deficiency of this receptor have a potential role on the pathogenesis of SLE (18). Two possible causes of progression of SLE disease are inflammation and oxidative stress. MDA and neopterin serum levels are considered as an indicator of oxidation and inflammation (16). Present case-control study, for the first time, demonstrated that distribution of A allele (co-dominant AA vs. GG and recessive AA vs. G/A+G/G) of CTLA-4 $G_{-1661}A$ was significantly higher in the control group. This findings suggest that presence of A allele plays a protective role in the development of SLE; reduces the risk of SLE by 1.69 times. On the other hand, presence of GA and GG genotypes strongly increased the risk of SLE by 2.45 folds. This results show that AA genotype may be induce the expression of CTLA-4 gene and this findings may be compatible with the role of mutation on alterations in the potential response element for myocyte enhancer factor 2 (MEF2). The increase in activity of gene promoter leads to the enhancement of CTLA-4 gene expression and the increase of negative signals to the T lymphocyte and ultimately leads to suppression of immune system. A significant positive association of the G allele with SLE in African-American older than 35 years of age has been reported by Parks' et al. (19). However results of present study were inconsistent with the results has been reported by Shojaa et al. and Farivar et al. from Iran and Taha et al. from china, which there were no association between CTLA-4 G-1661A polymorphism and risk of SLE in Iranian and Chinese population (6,20,21).

Genotypes and alleles frequencies of the CTLA-4 $T_{-1722}C$ gene in lupus patients and controls were not significantly different. Therefore, it seems that there is an absence of a direct and even positive association between mutations at $T_{-1722}C$ position of CTLA-4 gene with SLE disease in Iran′s western population. The lack of $T_{-1722}C$ mutation effect on the susceptibility to SLE was compatible with the researches performed by Shojaa et al. in Iranian population and Aguilar et al in Spanish Autochthonous population (22,23). Fernandez reported that the C allele was significantly associated with increased risk of SLE in Spanish population whereas, Hudson et al. research detected the T allele was more frequent in the Korean patients (24,25).

There are many other mutations on the CTLA-4 locus, but a few of them have been investigated in relation with SLE. A significant association between G allele of CTLA-4 at $A_{+45}G$ position exon 1 and increased risk of SLE has been reported from Japanese and South Indian SLE population, while Barreto et al. failed to indicate such association in Portugal population (26,27,28). These controversial results may be relevant to genetic differences in the pattern of haplotypes on the CTLA-4 position between different ethnic races, inadequate sample size and gene-environmental interactions.

This study for the first time investigated the CTLA-4 $G_{-1661}A$ mutation effect on neopterin, MDA, and lipid serum concentration in SLE involvement and progression. The serum levels of LDL, cholesterol, neopterin and MDA in lupus patients with GG+AG genotypes of CTLA-4 $G_{-1661}A$ were significantly higher than control group. Several studies have indicated that high blood neopterin is correlated with inflammatory disorders and is contributed to heart disease (29) and also there is a significant association of SLE with high levels of MDA (12,30,31). Rho et al. demonstrated macrophage activation, reflected by increased serum neopterin concentrations, which was increased in both SLE and rheumatoid arthritis (RA). Furthermore, they reported neopterin was strongly related with atherogenic mediators of inflammation in SLE and RA, but absence of a direct association with coronary atherosclerosis in either disease (32). Although with respect to research performed by Tewthanom et al. lack of association between plasma MDA concentrations with the degree of severity of SLE has been reported in Thailand SLE patients (33).

We reported, for the first time, that the AA genotype of CTLA-4 $A_{-1661}G$ plays a protective role in susceptibility to lupus, while G allele significantly increases the risk of SLE. SLE patients with AA genotype of CTLA-4 $G_{-1661}A$ have significantly lower neopterin, MDA, and LDL-C concentrations compared with patients who carry GG and AG genotypes. Our findings suggest that A allele may increase the expression of CTLA-4 gene that is a negative regulator of the immune system.

**Source of Funding**

This study was funded by Kermanshah University of Medical Sciences, Kermanshah, Iran; Grant #91304.

**Acknowledgments**

This work was performed in partial fulfillment of requirements for an M.Sc degree in Clinical Biochemistry, Kermanshah University of Medical Sciences, Kermanshah, Iran (Maryam Tanhapour). All authors contributed equally to this study.

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