The association of Cluster of differentiation 34 gene (CD34) polymorphism with oral ulceration in Systemic Lupus Erythematosus Iraqi patients

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Abstract

Objectives: Systemic lupus erythematosus (SLE) is a prototype autoimmune disease with extreme clinical heterogeneity and significant differences between populations , genetic factors are highly involved in this disease, ulcerations of the oral cavity in the long setting of SLE have been considered as predictors of systemic vasculitis and worse prognosis, the aim of this study was investigate cluster of differentiation 34 (CD34) gene polymorphism among systemic lupus erythematosus (SLE) patients with and without oral ulceration and control in some Iraqi peoples. **Materials and Methods:** Study was took place in Mirjan teaching hospital in Hilla city, Babylon (Rheumatology unit) during the period from May 2018 till January 2019. All the subjects were from the same ethnic group (Arabic) and were from the same geographical region. The American College of Rheumatology (ACR) 1997 criteria of SLE were used for diagnosis by rheumatologist. A total of 53 patients were recruited for the study and 31 control subjects were recruited for this study, the manufacturer protocol (Geneaid / Taiwan) was followed for extraction of the DNA from frozen blood samples, Polymerase Chain Reaction and then single-strand conformational polymorphism was done. **Results**: The PCR product of CD34 gene amplification was 608 bp , and 59.5 º C is the best temperature for target segment amplification. Eight haplotypes were observed in the present study (A,B,C,D,E,F,G and H) for CD34 gene polymorphism ,with significant difference (p value 0.05)between control and SLE patients at all haplotypes except at haplotypes (B and C), the association among haplotypes according to oral ulcer types(single or multiple) was highly significant different ($p \leq 0.001$) at haplotypes (B, D, E,G and H) and the association among haplotypes according to presence of oral ulcer pain was significant in haplotypes (A,C,D,E,G and H). **Conclusion**: this study found that CD34gene polymorphism different between SLE patient and controls. Related to oral ulceration in SLE most common in haplotypes except F, and there were differences between haplotypes according to ulcer type and presence of pain, Future prospective longitudinal studies with adequate sample size are needed in order to ascertain any causation factors or common etiologic pathways and other oral manifestations.

Key words: Oral ulcerations, Systemic lupus erythematosus, Haplotypes, CD34 gene.

Introduction

Systemic lupus erythematosus is a prototype autoimmune disease with extreme clinical heterogeneity and significant differences between populations. East Asian populations are known to have higher prevalence and more severe clinical manifestations for SLE than Europeans [1]. The difference could be the result of genetic and environmental factors, and the interactions between them. Systemic lupus erythematosus (SLE) is a complex and potentially fatal disease characterized by production of a wide-spectrum of autoantibodies and multi-system damage. Genetic factors are highly involved in this disease, as evidenced by a sibling recurrence risk ratio at around 20–40 years, and much higher concordance rate for monozygotic twins compared with dizygotic twins [2-4]. There is also marked differences in genetic susceptibility and disease epidemiology across different populations. African

Americans, Hispanics and Asians, all have higher disease prevalence than Caucasians, and lupus nephritis is also more prevalent in Chinese than in European populations [5-6].

The SLE is a multigenic disease. A combination of genome-wide association studies (GWAS) and candidate gene approaches has led to the identification of > 40 robust genetic associations with SLE [7-10]. These are genes which induce the transcription of proteins involved in key pathogenic pathways, including apoptosis and clearance of apoptotic material or immune complexes, innate and adaptive immunity functions, and the production of cytokines, chemokines, or adhesion molecules [11].

The CD34 is a 115kDa transmembrane protein of unknown function and is expressed in l-4% of human bone marrow cells; this population consists of pluripotential hematopoietic stem cells as well as committed progenitors of each hematopoietic lineage [12-13]. It also expressed in the vascular endothelium of a variety of organs [14,15].

Materials and methods

A case-control study included "52" Iraqi patients from Babylon Province with systemic lupus erythematosus (SLE) and "31" of apparently healthy subjects, the SLE were subdivided into two groups, 24 patients of SLE with oral ulcer(P+OU), 28 SLE patients without oral ulcer (P-OU). Collection of samples was done in Mirjan teaching hospital in Hilla city, Babylon (Rheumatology unit) during the period from 2nd May 2018 till January 2019. All the subjects were from the same ethnic group (Arabic). Both the patient and control groups were from the same geographical region. Ethical approved was obtained from each subjects.

Blood Samples: About 2 ml of venous blood were taken from anti-cubital vein from each subject in this study for genetic analysis.

Extraction of DNA (from frozen blood)

The manufacturer protocol (Geneaid / Taiwan) was followed for extraction of the DNA from frozen blood samples. The DNA then stored at -20 °C purified DNA. The Estimation of DNA Concentration and Purity using a Nanodrop.

Reconstituting and diluting primers : Primer was constructed by using Bioneer kit in a clean room under strict ISO 9001:2000 to confirm DNase/RNase and DNA free environment. Macro gene primers were commonly shipped in a lyophilized state. The units of a lyophilized

primer were given as a mass, in picomoles to create a stock of primers. The primer was reconstituted in sterile nuclease-free H_2O to obtain a master stock that would be used again to obtain a working stock.

Polymerase Chain Reaction: PCR optimization was done as a first step using a gradient temperature ranging from 55 ^{0}C to 66 ^{0}C. After the determination of optimum annealing temperature target sequences were amplification by 35 cycles consist of 95^{0}C for 30 sec, 58.9 ^{0}C for 20 sec then 72 ^{0}C for 30 sec also in addition to per denaturation 95 ^{0}C for 5 min and final extension for 5min at 72 ^{0}C, using GTC series thermo cycler (Cleaver Scientific /UK) apparatus. Primer of CD 34 were F:5'-CGGCTGTTAAGACTTGCAGTG-3', R: 5'-TGAGGTGTGAGAATCGCTTG-3, Amplified DNA fragments were electrophoresed on 1% agarose, (0.5x) TBE buffer for (40 min at 75 V).

Polyacrylamide-Bis-acrylamide stock solution preparation

Stock solution was prepared as follows[16] : A 40 % w/v of 29:1 of acrylamide-bisacrylamide is prepared by weighing 38.66gm of acrylamide and 1.33gm of biscrylamide then dissolved in ultrapure distil water and complete the volume to 100ml.

While dissolving the acrylamide-bisacrylamide in ultra-pure water the reaction temperature drops down to extremely chilled. This is because the reaction is energy absorbance and this could be a good indicator for the purity of the materials used [17]. The stock solution is wrapped with aluminum foil and stored in 4 ^{o}C.

A 50µl (10%) freshly prepared ammonium persulfate and 8µl TEMED were added to 3 ml of the acrylamide – bisacrylamide gel stock solution (30%) and completed to 10ml with TBE 0.5X . The mixture was mixed briefly. Take 8ml of 0.5x TBE buffer and mixed it with 2.8 of glycerol (100%), then add 20.8 deionized distill water, 40 µl of TEMED and 400 µl of freshly prepared ammonium persulfate (10%) and mix with the acrylamide – bisacrylamide gel stock solution (40%) . The mixture was mixed briefly (that depends on the quality of ammonium persulfate and TEMED used).

Steps for single-strand conformational polymorphism (SSCP)

A10 µl of PCR product and 10 µl of 2x SSCP gel loading dye were added to a micro centrifuge tube and the contents were mixed gently, then the tubes were placed into a 95^0C water bath for 10 minutes and then on ice for about 5 minutes. A 10 µl of the samples were loaded into wells of an 8% acrylamide /bis-acrylamide gel by specialized gel loading tips. Electrophoresis was took place in mini-slab gel provided by Cleaver Scientific –UK and performed at room temperature using pre-cold electrophoresis buffer(stored in refrigerator) and the whole tank was placed in a big container that was loaded with ice pack and cooled water, finally the power supply was set up at 125V for about 3.5 hours. Then polyacrylamide gel was visualized in a UV translluminator provided with gel documentation unit, after the gel was stained with Ethidium Bromide (www.thermofisher.com).

All statistical analysis was performed by using SPSS 21 version. Data were expressed as (mean ± SD) by using T-test. The normality of the distribution of all variables was assessed by the student′s ANOVA test and Pearson correlation analyses that have been used to determine the significant difference between the groups. Genetic analysis was performed using Chi-square ($\chi 2$) test. P values less than (0.05) is considered significant and less than (0.01) is considered highly significant.

RESULTS

The CD34 gene polymorphism is shows in figure (1), the gradient PCR showed that 59.5 º C is the best temperature for target segment amplification (figure 1).

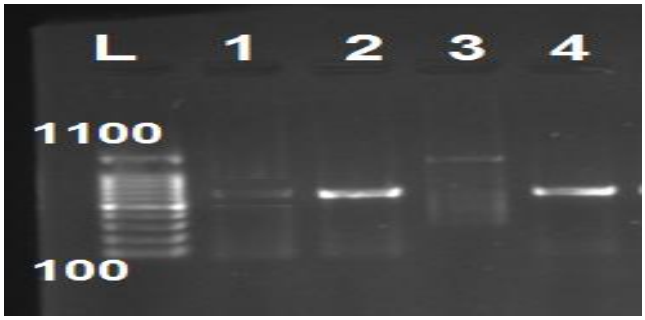

Figure (1) : The electrophoresis pattern of gradient PCR products for the CD34 gene ,this amplification product one band 608 bp,1% agarose ,75V,20 mA for 1h.Lane L: DNA ladder, lane 1 :55 ºC, lane 2: 57.2 ºC , lane 3 :58.4 ºC and lane4: 59.5 º C.

The PCR product of CD34 gene amplification was 608 bp , with 59.5 º C, as showed in figure (2).

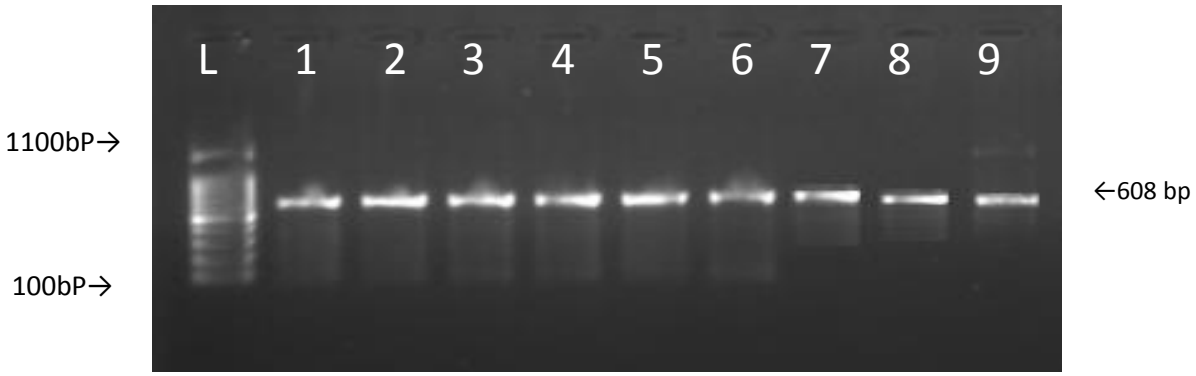

Figure (2) : The electrophoresis pattern of PCR product for CD34, this amplification product one band 608 bp for both patients and control ,.Lane DNA marker, lane 1-6 PCR product of patient, lane 7-9 PCR products of control.

The PCR product of electrophoresis pattern of CD 34 single-strand conformational polymorphism (SSCP) as in figure (3)

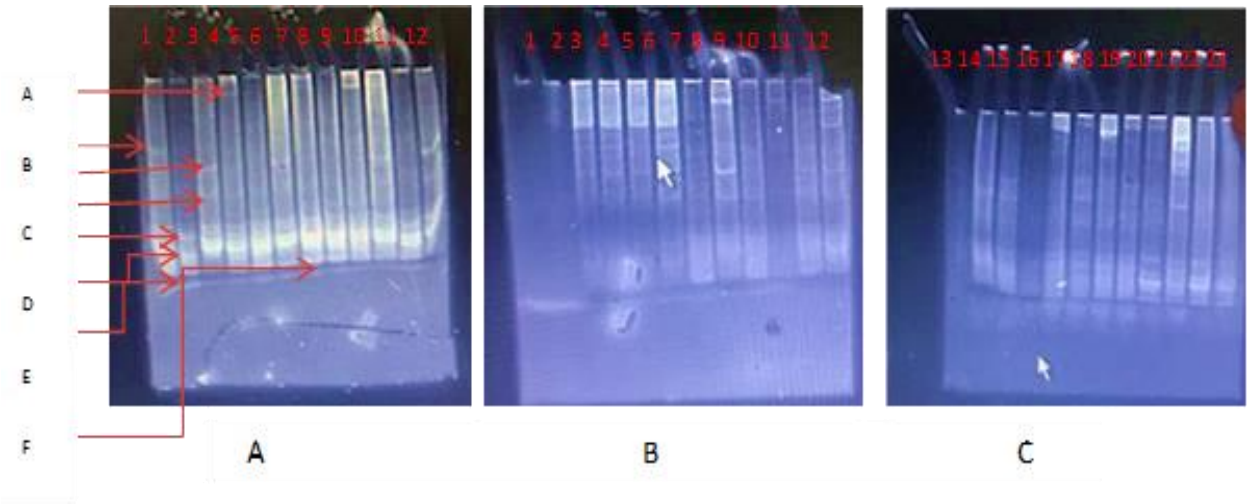

Figure (3): Electrophoresis of SSCP pattern of CD 34; A: control, B (P+OU) and C (P-OU) SLE patients.

Eight haplotypes were observed in the present study for CD34 gene polymorphism (A, B, C, D, E, F, G and H). All the Haplotypes showed significant differences at ($p \leq 0.001$) according to their distributions between patients and controls except at haplotypes (B and C) were non significant, as in table (1) and figure (3).

Table (1): Haplotype distribution of CD34gene between SLE patients and control subjects.

Haplotype	Control subjects (31)	SLE patients (52)	Odd ratio (95%CI)	P value
A	4(12.90%)	19(36.53%)	3.88* 1.17 to 12.80	0.02
B	3(9.67%)	11(21.15%)	2.33 (NS) 0.59 to 9.10	0.22
C	10(32.25%)	24(46.15%)	1.80(NS) 0.71 to 4.56	0.21
D	13(41.93%)	11(21.15%)	0.37* 0.14 to 0.98	0.04
E	31(100%)	17(32.69%)	0.007** 0.0005 to 0.13	0.0009
F	31(100%)	8(15.38%)	0.003** 0.0002 to 0.054	0.0001
G	31(100%)	8(15.38%)	0.003** 0.0002 to 0.054	0.0001
H	31(100%)	26(50%)	0.001* 0.0009 to 0.27	0.004

**Highly significant p≤0.001; *Significant p≤0.05.

Regarding the subgroup of SLE patients, it has been showed that significant differences was observed in the haplotypes (C and F) only between P+OU and P-OU, while all others haplotypes (A,B,D,E,G and H) showed no significant differences in their distributions (table 2).

Table (2): Haplotype distribution of CD34gene between P+OU and P-OU

Haplotype	P+OU (24)	P- OU (28)	Odd ratio	P value
A	8(33.3%)	11(39.2%)	0.77(NS) 95 % CI 0.24 to 2.41	0.44
B	6(25%)	5(17.8)%	1.54(NS) 95 % CI 0.41 to 5.85	0.54
C	15(62.5%)	9(32.1%)	4.07* 95 % CI 1.31 to 12.65	0.01
D	5(20.8%)	6(21.4%)	1.09(NS) 95 % CI 0.29 to 4.13	0.89
E	8(33.3%)	9(32.1%)	1.22(NS) 95 % CI 0.38 to 3.85	0.73
F	0(0%)	8(28.5%)	0.03* 95 % CI 0.0021 to 0.73	0.03
G	4(16.66%)	4(14.28%)	1.35(NS) 95 % CI 0.30 to 6.06	0.06
H	12(50%)	14(50%)	1.00(NS) 95 % CI0.37 to 2.98	1.00

*Significant p≤0.05.

Regarding presence of single oral ulcer or multiple oral ulcers, the haplotypes CD34 gene distributions showed a significant differences ($p \leq 0.05$) at (E) while, no differences found in other haplotypes (table 3).

Table (3): Haplotype distribution of CD34gene between patients with single oral ulcer or multiple..

Haplotypes	Single oral ulcer (11)	Multiple oral ulcer (13)	χ^2	P value
A	2(18.16%)	6(46.15%)	1.31 (NS)	0.25
B	2(18.16%)	4(30.7%)	0.50(NS)	0.47
C	7(63.6%)	8(61.6%)	0.01(NS)	0.91
D	1(9.09%)	4(30.7%)	1.69	0.19
E	6(54.5%)	2(15.3%)	4.11*	0.04
F	0	0		
G	2(18.1%)	2(15.3%)	0.03(NS)	0.85
H	4(36.3%)	8(61.5%)	1.51(NS)	0.21

* Significant $p \leq 0.05$; NS: Non significant

Regarding the presence of pain associated with oral ulcer, the haplotypes of CD34 gene showed no differences found in all haplotypes (table 4).

Table (4): Haplotype distribution of CD34gene associated presence of oral ulcer pain.

Haplotypes	With pain (9)	Without pain (15)	χ^2	P value
A	2(22.2%)	6(40.0%)	0.8(NS)	0.37
B	2(22.2%)	4(26.6%)	1.08(NS)	0.29
C	5(55.5%)	10(66.6%)	1.11(NS)	0.29
D	1(11.1%)	4(60.0%)	0.82(NS)	0.36
E	2 (22.2%)	6(26.6%)	0.08 (NS)	0.37
F	0	0		
G	2 (22.2%)	2(13.3%)	0.32 (NS)	0.57
H	4(44.4%)	8(53.3%)	0.17(NS)	0.67

NS: Non significant

The distributions of CD34 gene polymorphisms haplotypes associated with the presence of oral ulceration according to ulcer site in the oral cavity showed that the haplotypes was more frequents in P+OU at buccal site than others sites (figure 4).

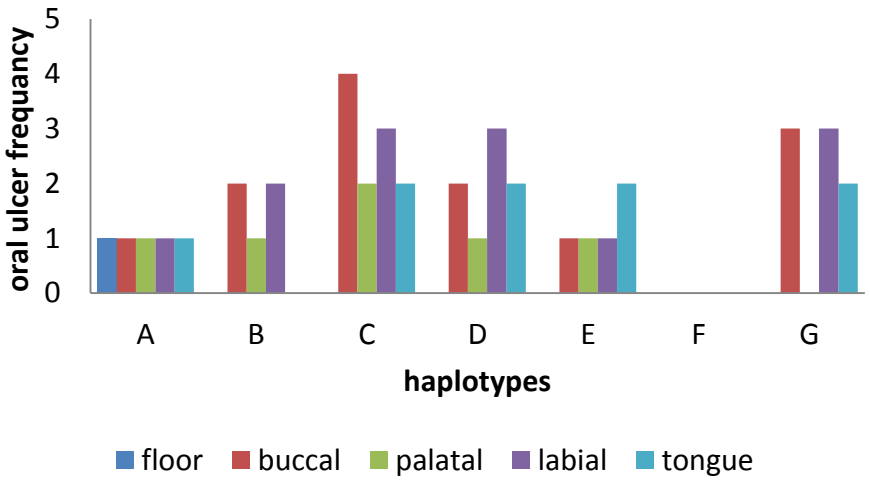

Figure (4): Haplotypes distribution according to ulcer sites

Discussion

Cluster of differentiation 34 (CD34) is an antigen on immune cells, as a results of immune system role in SLE etiology CD34 gene polymorphism was studied using SSCP technique, the results of present study showed that eight haplotypes had been observed in the present study for CD34 gene polymorphism (A, B, C, D, E, F, G and H) and the haplotypes (A, D, E, F, G and H) showed highly significant differences at ($p \leq 0.000$) among patients and controls (table 1) .

Haplotype C was more association with oral ulcer 15(62.5%) than other haplotypes, while, haplotype F showed no relations to oral ulcers (0%) (table 2).

Although CD34 is known to have an alternative splicing variant [18], there was poor information's about CD34 gene polymorphism in the previous studies, and polymorphisms in CD34 has not previously been described. By screening a series of bone marrow samples from donor– recipient pairs we found that polymorphisms are readily detectable in the gene sequence of two proteins of central importance in haemopoiesis. Rhee, [19] found one polymorphism in CD34 gene in hematopiotec stem cells that didn't deal with the results of this study which pointed that more than one haplotypes (E, F, G and H) found in all control subjects, while other haplotypes (A,B,C and D) found in high percentage in SLE patients, these differences could be considered as a genetic marker for early diagnoses of SLE.

The association between CD34 gene polymorphisms and oral ulcer were studied it shows that there was significant association between some haplotypes and oral ulcer appearances; single or multiple, painful and non-painful. This the first study deal with immune genic gene (CD34) with oral ulcer features in autoimmune disease, this relation can be explained that the polymorphism of CD34 can be affect in immune response that contributed in oral ulcer incidence and development, experimental model used *Aloe vera* polysaccharides for treatment of oral ulcer in animal model improved that the enhancement of immune system contributed in oral ulcer healing [20]. Finally Cohen [21] found Genome-wide association analysis of CD34 frequency identified suggestive associations at several loci, including OR4C12 and ENO1 and RERE. Up to the best of our knowledge this is first study conducted in this issue, overall this polymorphism interact with different factors that may be affect the pathogenesis of SLE disease which contributed directly or in direct with oral ulcer features, these theory need more investigations deal with immune marker levels and immune genetic polymorphisms.

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