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Deletion of introne-4 of Endothelial nitric oxide synthase gene in Behcet's disease-Iraq .Ali alkazzaz*
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Abstract
Behcet's disease (BD) is multi systemic inflammatory disorder of unknown cause, it characterized by recurrent oral and genital ulcer, skin lesion and uveitis. The etiopathogensis of Behcet's Disease had long been postulated, possibility that immunological abnormalities induced by microbial pathogens in genetically susceptible individual are important in the pathogenesis. There was no positive association between eye involvement, arteritis and HLA5 tissue typing and positive pethergy test .The HLA5 was positive in 62% of patients with Behcet's disease in Iraqi patients. Endothelial nitric oxide synthase is synthesized by arginine oxidation, it is one of nitric oxide synthase family, this enzyme has three isoform tow constitutive (neuronal NOS include nNOS and NOS-I)and one inducible NOS (Inos, nos-II), endotheline enzyme responsible of generate small amount of nitrous oxide, The eNOS gene is located on chromosome 7q35-36. 33 Iraqi patients with Behcet's disease compared with the same number of healthy population and it was conducted in Merjan teaching hospital in Babylon –Iraq in rheumatology unit from same ethnic Arabic group. The genotype study was done for both groups in the biological department of Science Collage in Babylon University during 2013. Genomic DNA was extracted from peripheral blood leukocytes using standard protocols. PCR experiment was performed using Forward primer 5-AGGCCCTATGGTAGTGCCTT-3 and revers primer 5- TCTCTTAGTGCTGTGGTCAC-3 in (clever scientific thermo cycler PCR). The study aims to detection deletion in of introne-4 of Endothelial nitric oxide synthase gene in Behcet's disease, the total number of patients was 33 patients, 11[33.3 %] were female and 22 [66.6%]were male, the mean age of the patients was (34.45± 11.51)years. (69.7%) of patients did not had family history of Behcet's disease, (57.6%) of patients were less than 5 years having the disease.57.6%of patients were got no active disease at time of study and 42.3% got active disease and there was significant association with genital involvement , central nervous system and disease duration with disease activity . The genetic study revealed that no significant related of deletion of Endothelial nitric oxide synthase gene in Behcet's disease Iraqi patients.
Keywords: Behcet's disease [BD], Iraq, Endothelial nitric oxide synthase[e NOS], PCR.

Introduction

Behcet'sdisease (BD) is multi systemic inflammatory disorder of unknown cause ,it characterized by recurrent oral and genital ulcer, skin lesion and uveitis ,the other manifestation may include arthritis ,central nerves manifestation and gastrointestinal tract involvement historically BD traces the old silk road , the ancient trade route following the eastern shores of the Mediterranean sea . (1) Onset of (BD) usually occurs in the 3rd decade of live with equal sex predication in most patients' series (2).

A Study done in Iraq in 1985 show the incidence BD was 1.2 per 10.000 popular which was equivalent to near countries except turkey, there was no positive association between Eye involvement, arteritis and HLA5 tissue typing and positive pethergy test .The HLA5 was positive in 62% of patients with BD in Iraqi patients [3] Regional difference in the clinical characteristic may exist between Eastern & Western population in the west the ocular lesions are less common, with less familial & less pethergy test (4). Systemic review has refuted ethnic variation in BD phenotype (5)

The etiopathogensis of BD had long been postulated, possibility that immunological abnormalities induced by microbial pathogens in genetically susceptible individual are important in the pathogenesis (3 , 5) A finding have been supported the significance of genetic factor & better defined the nature of inflammation of the disease (6)

BD usually appear sporadic Disease , but familial aggregation is well known &an increased prevalence has been observed in the siblings & parents of pediatric patients ,the silk rood region had higher frequency of HLA5 in the healthy population [7]

The BD is not a genetic disease with Medellin inheritance model, the majority of patients are sporadic cases with no family history, however, a familial aggregation of BD patients has long been noted and increased risk has been observed among first degree relative [8] .

The close association between HLA5 allele and BD represent the clearest evidence of genetic contribution to the disease, there are considerable effort had been made to understand if HLA51 loci play a role in pathogenesis of BD or it represent a marker for other predisposing gene in linkage disequilibrium with it [9] Although BD

does not have the features of a classical autoimmune disorder, an Antigen-driven immune response is seen in BD that possibly develops on the background of enhanced innate Immune reactivity. Familial aggregation of BD patients, association of HLA-B51 with BD, and peculiar geographical distribution of this disease along the old silk route, running from the Mediterranean, through the Middle East, and to Asia including countries such as Turkey and Iran [10, 11.12]

Endothelial nitric oxide synthase is synthesized by arginine oxidation, it is one of nitric oxide synthase family, this enzyme has three isoform tow constitutive (neuronal NOS include nNOS and NOS-I) and one inducible NOS (Inos, nos-II), endotheline enzyme responsible of generate small amount of NO result from receptor stimulation or shear stress which contribute to regulate vascular tone[13].

The eNOS gene is located on chromosome 7q35-36 and comprise of 26 exon spanning 21 kb when it variant it causes deficient NOS may be causes disease process [14].

Many studies was carried out to determinate the relationship of this gene with different disease such as myocardial infraction, hypertension, renal disease and stroke all this disease showed related with Endothelial nitric oxide synthase gene polymorphism in different sites of gene in some population but no relation in others, thus it's polymorphism was restricted by population[15].

The histological hallmark of BD is known to be a vasculitis of any size or type blood vessels which characteristic of disease ,the endothelial dysfunction is thought to paly important role in pathogenesis of these lesions ,a decreased endothelial NO activity may contribute to vascular lesions in BD[16]

Aim of the study:

The aim of this study was to detect the deletion in of introne-4 of endothelial nitric oxide synthase gene in BD patients in Babylon province in Iraq in comparison with normal healthy persons

Materials and methods:

This was comparison study done during 2013 and it included 33 Iraqi patients from Babylon province with BD and compared with the same number of healthy population and it was conducted in Merjan teaching hospital in Babylon –Iraq in rheumatology unit, all patients and control were from the same ethnic group [Arabic], all patients were diagnosed according to the diagnostic criteria proposed by international study of BD [17].

The pethrgy test was done for all patients with optholmlgical examination and if patient had neurological feature of the disease MRI of brain was done .The family history of same illness was obtained and if the father and mother of the patients were relative. Each participant was informed about the study and consent was obtained from them, the ethical approval was obtained from both Babylon collage of medicine and health department in Babylon.

The activity of BD was measure by Behcet's disease current activity form[BDCAF] for all patients at time blood aspiration for the test in Merjan teaching hospital by researcher [18]

The genotype study was done for both groups in the biological department of Science Collage in Babylon University; Genomic DNA was extracted from peripheral blood leukocytes using standard protocols [19].

The PCR experiment was performed using Forward primer 5-AGGCCCTATGGTAGTGCCTT-3 and revers primer 5- TCTCTTAGTGCTGTGGTCAC-3 in (clever scientific thermo cycler PCR) using tow program the first was gradient program to determinate optimum annealing temperature as following: pre-denaturation tm was 95 for 5 min, denaturation tm was 95 30 sec, annealing tm was (58-65 c) for 30 sec, extension tm 72 and final extension 72 for 10 min, this program for 30 cycle. The second program was performed in annealing tm 62 for detection deletion in introne-4 of endothelial nitric oxide synthase.

PCR product was electrophoresis by 30% acrylamide in 1/5 x of TBE buffer for 90 min at 100 v and 20 mAm , gel was stained by ethudium bromide in shaker with 1/2 X staining buffer then it visualized in UV transliminator to calculate deletion and wild type of intron-4. 420 bp was wild type which contain five 27 bp repeats (b allele) and 393 of mutant type contain four 27 repeats (a allele).

Result:

The total number of patients was 33 patients, 22[66.6 %] were male and 11 [33.4 %] were female patient's .the age range between 62 years and 14 years. And the mean age 34.45-+11.5 years, while the mean age of control was[33.2-+12.3] as shown in fig[1] .25[75.7%] patients live in urban area and the other in rural area,8[24.3%],table[1]

Mean duration of the disease at the time of the test was 4 years while 19 patients the disease duration less than 5 years[75.6%],5 patient more than10 years[15,1%] and only 9 patients duration between both [between 5-10 years][27.2%].tab[1] The average duration for all patients was [7.54] years. All patients has mouth ulcer as part for criteria for diagnosis of BD except one patients [96.9%],25[75.75%] of them got positive pethergy test, 20 of them done at time of the study while the other done before the study start

Majority (63.6%) of patients aged 20-40 year .There was no significant difference between the mean age of male (36.43± 11.74) years old and female (31.00± 10.68) years old (t=1.318, df= 31, p=0.197).

, meanwhile, (57.6%) of the patients was non-employer, (69.7%) of patients did not had family history of BD. (54.5%) of patients were overweight. Table [1]

The distribution of patients with BD by disease signs and symptoms. (96.9%%) of the patients had mouth involvement; meanwhile, (90.0%) of the patients had skin involvement. (51.5%) of the patients had genital involvement, as well as, (84.4%) of the patients had eye involvement. (51.5%) of the patients had CNS involvement, meanwhile, (66.7%) had joint involvement, and (57.6%) had good respond to their therapy in control the disease symptom.as shown in table[2]

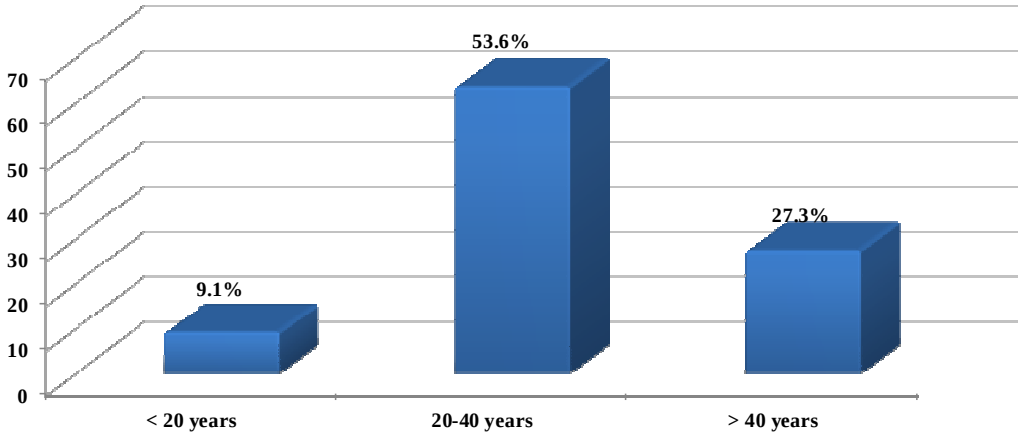


Figure [1]: Distribution of patients with BD by age groups

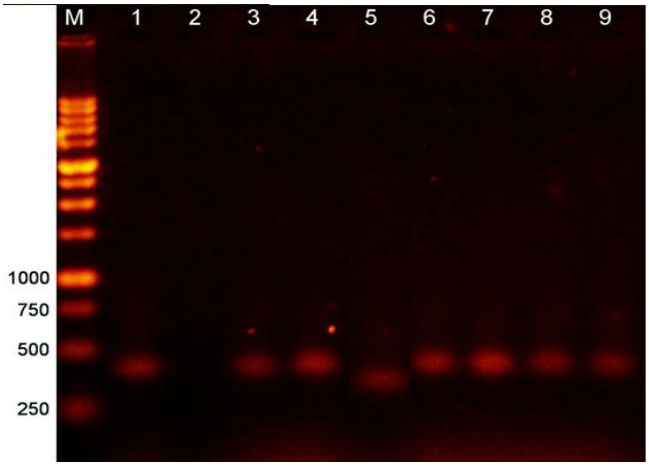
There was no significant difference between the mean age of male (36.43± 11.74) years old and female (31.00± 10.68) years old (t=1.318, df= 31, p=0.197).
There were 57.6% of patients have non active disease at the time of this study while 42.4% had active disease at the same time by measurement of Behcet's disease current activity form][BDCAF]
There was significant association between disease activity and duration of disease; meanwhile there were no significant associations between activity of disease with age groups, sex, residence, and family history and BMI .tab [3]

There were significant associations between disease activity with genital and CNS involvement as well as responding to the therapy; meanwhile there were no significant associations between activity of disease and other disease signs and symptoms as shown in table [4]

The genetic study revealed that no significant related of deletion gene with BD as show in table (5). And figure (2,3) . Polyacrylamide more persist than agarose in electrophoresis pattern of PCR product.

Table(5) genotype of introne-4 of Endothelial nitric oxide synthase gene in Behcet's disease and normal control.

Allele type	Patient	Normal	Odd ratio	CI (95%)
BB	30%	70%	5.360	1.1472-25.1058
AA	12.5%	87.5%		



igure (2). 1% agarose gel electrophoresis of PCR amplified fragments using specific primers for introne-4 of Endothelial nitric oxide synthase gene taken from BD patients.
M; refers to 1 kb DNA size marker (Geneaid – USA, Cat # DL005), lanes 1-9 show PCR product line 5 refer to deletion and others lines show wild type .

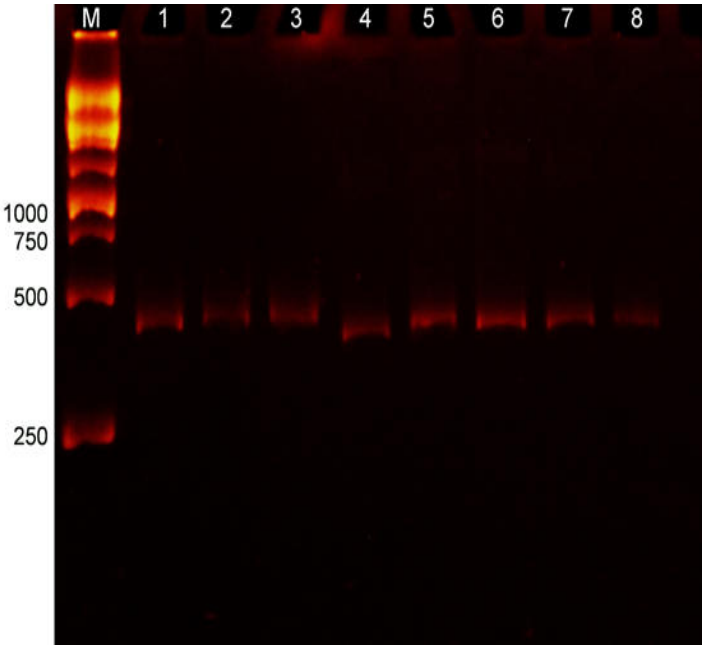


Figure (3). 8% polyacrylamide gel electrophoresis of PCR amplified fragments using specific primers for introne-4 of endothelial nitric oxide synthase gene taken from BD patients.
M; refers to 1 kb DNA size marker (Geneaid – USA, Cat # DL005), lanes 1 into 8; refer to PCR products, lane 4 refer deletion and others refer to wild type.

Discussion

BD was found in different place in world and difference studying was carried out on it such as immune study, hormones, enzyme and genetic analysis, in Iraqi population genetic study was less than other studies, thus it choose in present study, polymorphism of gene may be occur in different loci of genome which is causes less functional protein that may lead to change in e NOS expression and enzymatic activity that related with disease (18),while NOS gene polymorphism does not constitute a risk factor for developing BD in Japan[19]
A number of polymorphism have been identified in the eNOS gene regions, exon , intron, and promoter of gene but most study were performed in exon 7 and intron 4 because its related with development vascular disease such as Myocardial infraction , Hypertension, renal disease and stroke.(20) . present study show that's no related deletion in VNTR of intron 4 with BD in Iraqi patient, because this deletion was occurring in normal control also, thus other polymorphisms must be performed in this gene to estimate genetic polymorphisms which occur in other population such as Korean population , kim et al found that Glu298Asp polymorphism in exone7 and

VNTR polymorphism in intron 4 of eNOS gene(21) Salvarani et al showed that the Glu298Asp polymorphism of the eNOS gene was another susceptibility gene for BD that was independent of HLA-B51 in Italian populations (22).

The previous study record that more than 25% of patients with BD may have systemic venous thrombosis.(23) Endothelial dysfunction is thought to play an important part in the development of thrombosis in these patients (23,24),also the incidence of myocardial infraction has been estimated to be at least 50-fold higher in young patients with systemic lupus erythematous (SLE) than in age matched controls,(25) and occlusive coronary disease in these patients may result from atherosclerosis, thrombosis, or vasculitis (26).

Recently Tappuni et al was performed a comparative study of the genetics of Behcet's disease in Iraq using Micro SSP HLA Class I B locus kit (B locus, generic). Results analysis revealed that samples positive for HLA-B51 were 7.4 times more likely to have BD than the healthy control subjects (27).

Conclusion:

This study showed that no difference between normal population and patients with BD whatever the disease was active or not and also the sex showed no difference in the detection of introne -4Endothelial nitric oxide synthase gene ,the association of e NOS gene polymorphism and vascular disease varies according to the race and ethnic group so further studies to done for detection of other genetic predisposition in the same ethnic group and to compare with other ethnic group live in Iraq .

Acknowledgement:

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Table[1]Distribution of patients by residence, family history, BMI and duration of disease

Variable	Frequency (%)
Residence	
Urban area	24 (72.7%)
Rural area	9 (27.3%)
Occupational status	
Governmental employer	2 (6.1%)
Non-Governmental employer	12 (36.4%)
Non-Employer	19 (57.6)
Family history	
Yes	10 (69.7%)
No	23 (30.3%)
Duration of disease	
< 5 years	19 (57.6%)
5-10 years	9 (27.3%)
> 10 years	5 (15.1%)
BMI	
< 25 kg/m2	11 (33.3%)
25-29.9 kg/m2	18 (54.5%)
30 kg/m2 and more	4 (12.1)

Table 2: Distribution of patients by disease signs and symptoms

Variable	Frequency (%)
Mouth involvement	
Yes	32 (97.0%)
No	1 (3.0%)
Skin involvement	
Yes	30 (90.9%)
No	3 (9.1%)
Genital involvement	
Yes	17 (51.5%)
No	16 (48.5%)
Eye involvement	
Yes	28 (84.8%)
No	5 (15.2%)
CNS involvement	
Yes	17 (51.5%)
No	16 (48.5%)
Joint involvement	
Yes	22 (66.7%)
No	11 (33.3%)
Pethergy	
Yes	24 (72.7%)
No	9 (27.3%)
Respiratory function	
Good	19 (57.6%)
Poor	14 (42.4%)
Father& mother realtive	
Yes	14 (42.4%)
No	19 (57.6%)
Color	
White	17 (51.5%)
Dark	16 (48.5%)
Vessels involvement	
Yes	13 (39.4%)
No	20 (60.6%)

Table 3: Association of disease activity with age groups, sex, residence, family history, BMI and duration of disease

Variable	Disease Activity		$\chi 2$	P values
	active (%)	Non-active (%)		
Age Groups (years)				
<20 years	2 (10.5)	1 (7.1)		
20-40 years	12 (63.2)	9 (64.3)	0.294a	1.000
≥ 40 years	5 (26.3)	4 (28.6)		
Sex				
Male	12 (63.2)	9 (64.3)	0.004a	0.947
Female	7 (36.8)	5 (35.7)		
Residence				
Urban area	14 (73.7)	10 (71.4)	0.021a	1.000
Rural area	5 (26.3)	4 (28.6)		
Family history				
Yes	6 (31.6)	4 (28.6)	0.035a	1.000
No	13(68.4)	10 (71.4)		
Duration of disease				
< 5 years	15 (78.9)	4 (28.6)		
5-10 years	3 (15.8)	6 (42.9)	8.330a	0.011*
> 10 years	1 (5.3)	4 (28.6)		
BMI				
< 25 years	5 (26.3)	6 (42.9)		
25-29.9 years	11 (57.9)	7 (50.0)	1.232a	0.609
30-34.9 years	3 (15.8)	1 (7.1)		

Table 4 Association of disease activity with disease signs and symptoms

Variable	Disease Activity		χ^2	P values
	active (%)	Non-active (%)		
Mouth involvement				
Yes	19 (100.0)	13 (92.9)	1.400a	0.424
No	0 (0.0)	1 (7.1)		
Skin involvement				
Yes	17 (89.5)	13 (92.9)	0.112a	1.000
No	2 (10.5)	1 (7.1)		
Genital involvement				
Yes	7 (36.8)	10 (71.4)	3.860	0.049*
No	12 (63.2)	4 (28.6)		
Eye involvement				
Yes	16 (84.2)	12 (85.7)	0.014a	1.000
No	3 (15.8)	2 (14.3)		
CNS involvement				
Yes	6 (31.6)	11 (78.6)	7.127	0.008*
No	13 (68.4)	3 (21.4)		
Joint involvement				
Yes	11 (57.9)	11 (78.6)	1.551	0.213
No	8 (42.1)	3 (21.4)		
Pethergy				
Yes	14 (73.7)	10 (71.4)	0.021a	1.000
No	5 (26.3)	4 (28.6)		
Respond to therapy				
Good	16 (84.2)	3 (21.4)	13.007	<0.001*
Poor	3 (15.8)	11 (78.6)		
Father&mother relative				
Yes	9 (47.4)	5 (35.7)	0.448	0.503
No	10 (52.6)	9 (64.3)		
Color				
White	11 (57.9)	6 (42.9)	0.730	0.393
Dark	8 (42.1)	8 (57.1)		
Vessel involvement				
Yes	5 (26.3)	8 (57.1)	3.208	0.073
No	14 (73.7)	6 (42.9)		

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