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RESEARCH ARTICLE

Validity of CD4 enhancer gene polymorphism in susceptibility and severity of some Iraqi rheumatoid arthritis patients.

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Abstract

Rheumatoid arthritis is one of the most common systemic autoimmune diseases. The CD4 enhancer and promoter seem to be the major regulatory regions for CD4 transcription in T cells; therefore, the study aimed to investigate the role of CD4 enhancer gene polymorphism in susceptibility and severity of rheumatoid arthritis (RA). Fifty RA Iraqi patients and 50 apparently healthy controls enrolled in this study during May to September 2015 in rheumatology consultation clinic/Baghdad teaching hospital. Genotyping of CD4-10845 gene polymorphism had determined by PCR – RFLP. Patients with AA genotype of CD4-10845 were significantly ($p<0.01$) more likely to develop RA and having of A allele was significantly more likely to have sever RA ($\chi^2=9.121$, $P<0.01$).

Introduction:-

Rheumatoid arthritis (RA) is an inflammatory disease that affects approximately 1% of the adult population. Although there is no cure, patients may reach a state of remission, which has become an achievable goal with optimal early treatment (1). The 2010 American college of rheumatology (ACR)/ European league Against Rheumatism (EULAR) classification criteria developed in a three-stage process to replace existing criteria, which deemed out of date. The 2010 ACR/EULAR criteria comprise a scoring system that considered the number and distribution of the affected joints, serology, duration of symptoms, and acute phase reactants (2).

Previous studies have shown that T cells play an important role in the development of such autoimmune disease (3). The balance between regulatory T cells and pro-inflammatory effectors T cells has been shown to be of critical importance in the development and persistence of autoimmune diseases.(4)

CD4+T cells that stimulate monocyte, macrophages, synovial fibroblasts, and other cells to produce cytokines such as tumor necrosis factor – α (TNF – α), interleukin – 1 (IL – 1), IL – 6, IL- 15, IL – 17, and metalloproteinases that produce tissue damage, play as the main role in the development of most human autoimmune disease bordering on RA (5).

CD4+ Th cells exist in a variety of epigenetic states that determine their function, phenotype and capacity for persistence, and form long-term immune memory. The CD4 is a glycoprotein expressed on the surface of helper T cells colocalizes with the T cell receptor (TCR) and major histocompatibility complex (MHC) class II molecules. It interacts with the non – antigen-binding regions in the MHC class II molecule in V – shaped binding mode during antigen recognition. The binding of CD4 to the MHC – TCR – CD3 complex during antigen recognition brings the active LCK close to the immunoreceptor tyrosine – based activation motifs (ITAM) located within the TCR – CD3 complex, there by phosphorylation them (8). The Zeta – associated protein of 70KD (ZAP 70) is then recruited to

the complex and activated, allowing further phosphorylation of downstream molecules, eventually leading to gene activation of cellular function. The inhibition of CD4 MHC class II interaction severely impairs the response of T cells to antigen exposure (6). CD4 gene is a candidate gene in autoimmune diseases, including rheumatoid arthritis (RA). Because the CD4 receptor is crucial for appropriate antigen responses of CD4 T cell activity, may influence tolerance or tissue destruction in autoimmune diseases and contribute to their risk. The CD4 gene is located in chromosome 12p – 12pter, and its expression regulated primarily at the transcriptional level. So that, the analysis of the CD4 enhancer polymorphism can provide insights into regulation of its expression the importance of CD4 in the functional performance of T cells, as well as its consequent crucial effect in the immune response of an individual(7,8)

The current study hypothesized genetic variation in the CD4 enhancer may be associated with the risk of development of autoimmune diseases such as RA. To verify this hypothesis, the study compared the allelic and genotyping frequencies of the 10845 A/G polymorphism in the CD4 enhancer of 50 patients with RA and 50 apparently healthy controls living in Iraq. Further, compared the genotypes among RA patients with various clinical variables to investigate whether a relationship exists between CD4 enhancer polymorphisms and the clinical manifestations of RA.

Subjects and methods:-

The study included 50 Iraqi RA patients. These patients diagnosed according to the criteria of American College of Rheumatology (2). Patients included in the study underwent routine biochemical blood investigations and x-rays of the hands and feet. The evaluation of Patients included physical examination, with particular focus on the pattern of joint involvement, presence of nodules , other extra-articular features (such as vasculitis, anemia, subcutaneous nodules, vasculitis manifestation, and organ involvement), and laboratory features such as rheumatoid factor (RF). Disease activity has been determined based on defined parameters (the number of swollen and tender joints, ESR, Health Assessment Questionnaire (HAQ), C reactive protein (CRP) and a global physician's assessment. Disease severity has been determined based on defined parameters (RF and X-ray erosion). Patients deemed to have an erosive arthropathy if one or erosions that are more definitive appear in any of the peripheral joints that have identified to be predictive of disease progression. Fifty apparently healthy Iraqi adult male and female as a healthy control group.

Collection of Blood Samples:-

Eight ml of venous blood drawn from each individual of the two groups under complete aspect condition after an overnight fasting. Three ml of blood was collected in EDTA containing tube for separation of peripheral blood mononuclear cells (PBMCs) for determination of CD4 enhancer genotypes. The other 3 ml of blood collected in anticoagulant-free tubes used for separation of serum to detect RA and CRP, ACCP levels. Two mL was collected in ESR tube.

Methods:-

Biochemical analysis:-

Blood samples drawn from all subjects after an overnight fasting. Sera separated immediately and stored at -20°C. CRP assayed by high-sensitivity enzyme-linked immunosorbent assay (ELISA). RF and ACCP assayed by enzyme linked immunoassay.

Isolation of DNA:-

Genomic DNA extracted from EDTA whole-blood sample using a spin column method according to the protocol (ReliaPrep Blood g DNA Miniprep System, Promega).

Detection of CD4 polymorphic site:-

The patients were genotyped for CD4-10845 A/G polymorphism by polymerase chain reaction restricted fragment length polymorphism (PCR – RFLP). PCR reaction carried out in a 25ul reaction containing 12.5 ul of Green Master Mix, 1ul of 10pmol/ul of each primer, 5ul of DNA template and the volume completed to 25ul using nuclease-free water. Master Mix consist of dNTPs, Mgcl2, Taq polymerase.

For CD4-10845 A/G polymorphism, the region surrounding the polymorphism amplified with following forward primer

5 GAAATGAGAAGTAGCACACAGT 3 and reverse primer 5 AAAAGTTAAGCAGAATCAGGC 3. PCR was performed at initial step at 95°cfor 4min, followed by denaturation step at 95°cfor 30 sec, annealing step at 58°cfor 30 sec, extension step at 72c°for 1 min and repeat step 2 – 4 for 34 cycles. After that extension at 72°cfor 10 min. holding at 4°c.

PCR products were resolved on 1% Agarose gel. The gel was prepared by dissolving 1g of Agarose in 100ml of 1x TAE buffer using a microwave oven. The mixture was left to cool to about 55-60C before a 1ul of 10mg/ml of Ethidium bromide was added. It then poured into the electrophoresis tank, secure the combs in place, and left to cool and solidify for about 30min. After the gel was set, the combs were removed carefully and the tank was placed in the electrophoresis system containing running buffer consisting of 1x TAE. The buffer was poured until it covered the gel for about 1-2 mm. Five µl of each PCR product along with the negative control and a 100 bp DNA ladder were loaded into the wells, the system cover was then put into place and the system was turned on. The gel left to run for 90min with a 100 volt/50 mAmp current. Following electrophoresis, visualization conducted with a UV Tran illuminator and the image captured by digital camera (Canon, US). This camera has the appropriate filter and a suitable program for illumination of EtBr-stained gels (8).

Twenty-microliter PCR products digested with 0.5ul of HaeII enzyme and incubated in a thermal cycler at 37°C for 3h. Digested products were resolved on 1.5% agarose gel.

Statistical Analysis:-
The Statistical Analysis System- SAS (2012) program was used to effect of difference factors in study parameters. Chi-square test used for significant compare between percentage and least significant difference –LSD test, which also used to significant compare between means of the study.

Result:-

Study parameters such as RF, ESR, CRP, ACPA in patients compared with control group are shown in table (1), which explained the significant differences between patients and control group (P <0.01).

Genotype and allele frequencies of the CD4-10845 A/G polymorphisms of patients with RA and controls shown in table (2). The frequencies of AA genotype of CD4-10845 A/G significantly increased in patients with RA compared with control group. patients with genotype AA were significantly more likely to develop RA (OR =1.355), also there were significant difference increased in patients with RA compared with healthy control in GG genotype but patients are more likely to develop RA with AA genotype. However there were no significant difference (P>0.01) in AG genotype between patients and control.

Allele frequency of CD4-10845 A/G gene in patients and control group are shown in table (3) that was showed A alleles are higher frequency in patients than apparently healthy control and are more likely to develop RA.

Relationship of CD4 genotypes frequencies with study parameters (ESR, CRP, RF, and ACPA) in RA patients shown in table (4). These parameters of disease activity and severity, there was high significant difference increase in ESR levels in patients with RA with genotype AA than AG and GG. Also high significant difference (p<0.01) increase in CRP, RF, and ACPA in RA patients with AA genotype than AG, GG.

There was a significant relationship between genotype of CD4 10845 A/G gene and study parameters, but highly significant in RA patients carry AA compared those carry AG and GG (P<0.05) (P<0.01).

In addition, there was no relationship (P>0.01) between genotype of CD4 10845 A/G gene and study parameters of healthy control group (Table 5).

Table 1. Compare between patients and control in study parameters

Group	No.	Mean ± SE			
		ESR	CRP	RF	ACPA
Patients	50	52.96 ± 3.68	38.39 ± 4.31	168.87 ± 31.62	114.85 ± 21.06
Control	50	10.44 ± 0.74	16.49 ± 2.51	4.96 ± 0.71	1.71 ± 0.13
LSD value	---	7.459 **	9.918 **	62.766 **	41.801 **
** (P<0.01).					

Table 2. Distribution of sample study according to genotype of CD4 P10845 A/G

Genotype	Patients		Control		Chi-square (χ^2)
	No.	%	No.	%	
AA	18	36.00	4	8.00	9.121 **
AG	21	42.00	22	44.00	0.694 NS
GG	11	22.00	24	48.00	9.563 **
Total no.	50	100 %	50	100 %	---
Chi-square (χ^2)	----	9.563 **	----	11.814 **	---
** (P<0.01), NS: Non-significant.					

Table 3. Allele frequency of CD4 P10845 A/G gene in patients and Control

Genotype	Patients	Control
A	0.57	0.30
G	0.43	0.70
Total	1 (100%)	1 (100%)

Table 4. Relationship between genotype of CD4 10845 A/G gene and Study parameters of patients

Genotype of CD4	No.	Mean ± SE			
		ESR	CRP	RF	ACPA
AA	18	40.18 ± 5.44	44.26 ± 6.51	186.68 ± 54.36	124.32 ± 34.42
AG	21	32.67 ± 4.74	25.51 ± 4.10	62.51± 19.18	59.99 ± 18.33
GG	11	25.17 ± 4.32	19.25 ± 3.48	54.19 ± 26.09	14.65 ± 8.69
LSD value	---	14.213 *	13.029 **	86.494 **	57.248 **
* (P<0.05), ** (P<0.01).					

Table 5. Relationship between genotype of CD4 10845 A/G gene and study parameters of healthy control

Genotype of CD4	No.	Mean ± SE			
		ESR	CRP	RF	ACPA
AA	4	8.50 ± 1.19	26.37 ± 7.06	4.58 ± 0.32	1.64 ± 0.13
AG	22	10.81 ± 1.15	15.88 ± 4.23	3.30 ± 0.17	1.82 ± 0.28
GG	24	10.41 ± 1.14	15.41 ± 3.35	6.54 ± 1.43	1.62 ± 0.08
LSD value	---	4.975 NS	17.007 NS	4.788 NS	0.906 NS
NS: Non-significant.					

Discussion:-

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by persistent inflammation affecting the musculoskeletal system that results in joints deformation and severe pain. The precise pathogenesis of RA has not been completely elucidated, but $CD4^+$ T cells are thought to play a crucial role, as it stimulate proliferation and differentiation of B-lymphocytes and participate in the induction and propagation of inflammatory responses by secreting pro-inflammatory cytokines, growth factors, and interferon (9).

Trafficking of $CD4^+$ T cells, and consequently their inflammatory mediators into the synovial fluid contribute to initiation, propagation, and maintenance of chronic inflammation of synoviocytes. The migration of $CD4^+$ T cells as well as other immune cells to lymphoid or rheumatoid synovium is dependent on several factors including their chemokine receptors expression profile (10).

Rheumatoid arthritis (RA) associated with the presence of auto reactive $CD4^+$ T cells that produce pro-inflammatory cytokines (11).

The majority of autoimmune disease risk variants are located in non-coding regions of the genome. It is reasonable to hypothesize that a subset of them causes disease by altering gene regulatory mechanisms as expression quantitative trait loci (eQTL) (12,13). So far, studies of gene regulation have largely carried out in cell lines and primary resting blood cells including undifferentiated $CD4^+$ T cells, B cells, monocytes, and dendritic cells. However, to understand the pathogenic mechanisms of risk variants, especially when studying the immune system where cells are highly diverse and functionally specialized, it is essential to focus on relevant cell types and stimulated cellular state (14, 15).

CD4 is a co receptor that assists the T cell receptor (TCR) to activate its T cell following an interaction with an antigen-presenting cell. The genetic regulation of the $CD4^+$ T cell activity might influence tolerance or tissue destruction in RA. T cells indirectly mediate autoantibody production, joint inflammation and bone resorption (16, 17).

Results if present study, established that the frequencies of AA genotype of CD4-10845 were significantly increased in patients with RA compared with healthy control.

In accordance with recent data, Sui-Foon *et al*, created that the patients with RA had a significantly higher frequency of the CD4-10845 AA genotype compared with healthy controls. Similarly, further comparison of the allele frequencies between patients with RA and healthy controls were made and they revealed that the former had a higher frequency of A allele and a lower frequency of G allele. Interestingly, they added that CD4-10845 AA genotype and CD4-10845 A allele increased the risk of RA development.

On the contrary, Hussein *et al.*, found in his study on RA Saudi female patients, there were no significant differences ($p<0.01$)between CD4-10845 polymorphisms through RA patients and healthy control group.

On the other study in RA Egyptian female patients Hussein (8) *et al.*, 2014 detected the association of CD4 enhancer gene polymorphisms with RA, carrier of A allele od CD-10845, which was significantly more likely to develop sever RA, in accordance with present study on RA Iraqi patients.

The study was originated association of CD4-10845 enhancer gene polymorphism with RA. The process in which the enhancer initiates gene transcription via interaction with the promoter is complex. Genetic polymorphisms at the CD4 enhancer locus have shown to be associated with risk of development of RA and the molecular mechanisms are controlling very complex, which need further studies.

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