INVESTIGATION OF THE APOLIPOPROTEIN E GENE POLYMORPHISM VARIATIONS AND CORRELATION WITH ANTI-CYCLIC CITRULLINATED PEPTIDES ANTIBODIES IN IRAQI PATIENTS WITH RHEUMATOID ARTHRITIS

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Abstract: Objective: To the assessment of Apolipoprotein E genetic polymorphism variations (E2/E3, E3/E4, E3/E2, E2/E4, E2/E2, E4/E4) and correlation with anti-cyclic citrullinated peptides (ACCP) antibodies in Iraqi patients with rheumatoid arthritis. Methods: A case-control study was conducted at the rheumatologic outpatient department at Baghdad teaching hospital and included forty-five patients with rheumatoid arthritis (seven male and 38 female) enrolled in the study. APOE and ACCP levels were estimated by ELISA. RT-PCR was used to investigate APOE SNP (rs7412 and rs429358).
Results: The results of this study reveal the statistical difference in the level of Apo E between the patients and the control group. The mean of the APOE in the patient's group compared to the control group (p-value <0.001). The results also show a significant difference between the level of ACCP in the patient group compare to the control group (p-value <0.001). In the RA and control groups, the results of (rs7412 and rs429358) (T, C) genotyping showed that (17vs.11) carry homozygous genotype (E2/E2), whereas (52 vs. 61) homozygous genotype (E3/E3), and (16vs14) respectively homozygous genotype (E4/E4). Since the frequency in these groups is (5vs4) of heterozygous genotype (E2/E4) in both the RA and control groups. Heterozygous anthers were not present in the sample group (E2/E3, E3/E4). Since our sample had small subgroups of APOE E2/E4 (p-value>0.05).
Conclusion: There were significant differences in APOE and ACCP levels but not significant in genotyping analysis between RA and control groups.

Keywords: Rheumatoid arthritis, APOE, ACCP, SNP

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INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disorder that mainly affects the synovial joints of the hand and feet. It is characterized by a progressive symmetric inflammation of affected joints resulting in cartilage destruction, bone erosion, and disability [1]. The prevalence of RA is estimated at 1% in most developed countries, with a frequency ranging from less than or equal to 0.1% to 1.9% in surveys from different parts of the world [2]. RA is a systemic illness, in addition to articular involvement, extra-articular manifestations like ocular, skin, cardiac, pulmonary, neurological can occur. The actual mechanism causes an invasion of joints by the immune system. These responses to the immune system cause inflammation and spread of the joint capsule and also affect cartilage and bone [3]. Although the etiology of RA has not yet been thoroughly established, a combination of genetic and environmental factors as well as many other autoimmune-initiating agents are thought to have been involved [4]. RA can strike at any age, with the peak incidence usually between the ages of 40 and 60. Moreover, the risk increases with age progression [48]. Women are two to three times likely to get rheumatoid arthritis than men. hormones are believed to play a role. This is evidenced in part by research showing women often develop the disease after major shifts in their hormones [5]. Apolipoprotein E (APOE), a circulating 34 kDa glycoprotein composed of 299 amino acids, mainly synthesized in the liver, is associated with lipoproteins rich in triglycerides to mediate the clearance of their remnants in the circulation following enzymatic lipolysis. Its synthesis in macrophages allows high density-like lipoproteins to be produced to reverse the transport of cholesterol to the liver [6-9]. Four exons and three introns are in it. In the human APOE gene, several individual SNPs have been identified. In particular, the three main alleles are responsible for two SNPs, rs7412 (C/T) and rs429358 (C/T): epsilon-2 (ε2), epsilon-3 (ε3), and epsilon-4 (ε4). Six genotypes of APOE are available: ε2/ε2, ε2/ε3, ε2/ε4, ε3/ε3, ε3/ε4, and ε4/ε4. Three homozygous (ε2/ε2, ε3/ε3, and ε4/ε4) and three heterozygous (ε2/ε3, ε2/ε4, and ε3/ε4) [10]. The aim of this study to assessment of above

six genotypes of APOE and find the correlation with anti-cyclic citrullinated peptides (ACCP) antibodies in Iraqi patients with rheumatoid arthritis.

METHODS

Specimens collection

A case-control study was conducted at the rheumatologic outpatient department at Baghdad teaching hospital and department of biochemistry/college of medicine/Babylon university from Oct 2020 to Mar 2021. A total of forty-five patients with rheumatoid arthritis (seven male and 38 female) were enrolled in the study. Forty–five apparently healthy persons were taken as a control group consisted of (8 males and 37 females) were matched with the patients group in case of sex and age status to increase the accuracy of the results. About 5 ml of blood were collected from each individual by vein puncture, 1 ml was collected in EDTA tubes for genetic analysis, the remaining 4 ml pushed into a disposable gel tube for serum separation for biochemical analysis.

Determination of APOE and ACCP by ELISA

The Sandwich-ELISA method is used for the estimation of APOE and ACCP levels depending on manufacturing instructions. Figure 1 showing the standard curves of APOE and ACCP.

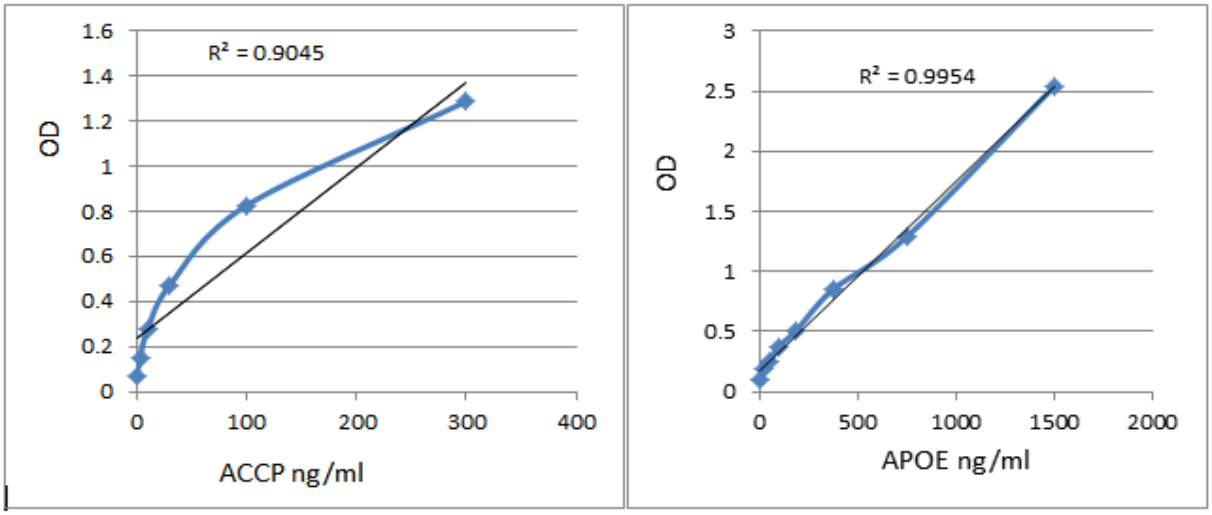

Fig.1: APOE and ACCP ELISA standard curves

Total DNA extraction

Genomic DNA was extracted from blood using human DNA extraction and purification kit (INTRON). A volume of 200µl of whole blood or body fluid is pipetted into a 1.5 micro centrifuge tube and DNA extracted depending on manufacture instructions provided by the kit. Genomic DNA was detected by agarose gel electrophoresis to showing the bands in all cases as showing in figure 2. Genotyping was carried out by the Real-Time Polymerase Chain Reaction polymorphism (RT- PCT) for human APOE SNPs (rs7412 and rs429358).

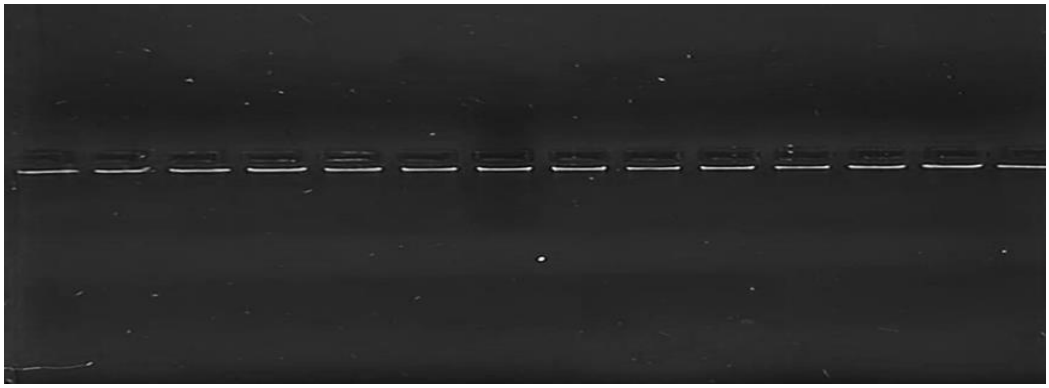

Fig.2: Gel electrophoresis of genomic DNA extraction from a blood cases in 2% agarose gel

Real Time RT-PCR Analysis

For detection the genetic variations of APOE SNPs (rs7412 and rs429358), the RT-PCR were used. The following briefly protocol of this analysis: Dilute the 40X Custom SNP Genotyping Assay to a 20X working stock solution. Vortex and centrifuge the 20X Assay Working Stock. Thoroughly mix TaqMan® Genotyping Master Mix by swirling the bottle. Suspended the thawed frozen samples by vortexing, and then centrifuge the tubes briefly. Calculate the number of reactions to be performed for each assay. Calculate the total volume of each component needed for each assay, as showing in table 1.

Table 1: The volumes of reagents that used in RT-PCR procedures

Component	20 µL (Final volume)
2X TaqMan® Master	5 µL
20X Assay Working	0.25 µL
Nuclease-free water	2.75 µL
DNA Sample Volume	12 µL

Prepare the reaction mixed for each assay before transferring it to the optical reaction plate for thermal cycling. Pipette the required volumes of 2X TaqMan® Genotyping Master Mix and 20X Genotyping Assay mix into a sterile tube. Cap the tube and vortex briefly to mix the components. Centrifuge the tube briefly to spin down the contents and to eliminate air bubbles from the solution. The thermal cycling conditions showing in table 2:

Table 2: PCR conditions for APOE SNP analysis

Cycle Type	Temperature℃	Time	Number of cycles
Enzyme activation	95	10min.	HOLD
Denaturation	95	15 sec.	40 cycle
Annealing	63	1min.	

Statistical analysis

The statistical analysis of this prospective study performed with the statistical package for social sciences (SPSS) 20.0 and Microsoft Excel 2013. Numerical data were tested for normality testing using Shapiro-Wilk test found that the data were normally distributes. The data described as mean and standard deviation and independent sample t-test used for comparison between two groups while, analysis of variance (ANOVA) test used for comparison among more than 2 groups. Categorical data were described as count and percentage. Chi-square test used to estimate the association between variables. The lower level of accepted statistically significant difference is bellow or equal to 0.05.Odd ratio was calculated to estimate the potential risk of allele among their carriers.

Ethical issue

This study was performed depending on ethical instructions of the department of community medicine /college of medicine/ Babylon university. The approval was taken from all subjects whom participate in this study.

RESULTS

The results of the current study reveal to the statistically difference in the level of the Apo E in between the patients and control group. The mean of the APOE in the patients group was 1498.8± 324.6 than in the control group 1641.9±260.8 and the P-value was <0.001. The results also showing a significant difference between the level of the ACCP in the patient's group than the control group, where the level of the ACCP the patient's group was 232 ± 254 u/ml and in the control group was 4.6 ± 2.1 u/ml, high statically significant p-value (<0.001) as showing in figure 3.

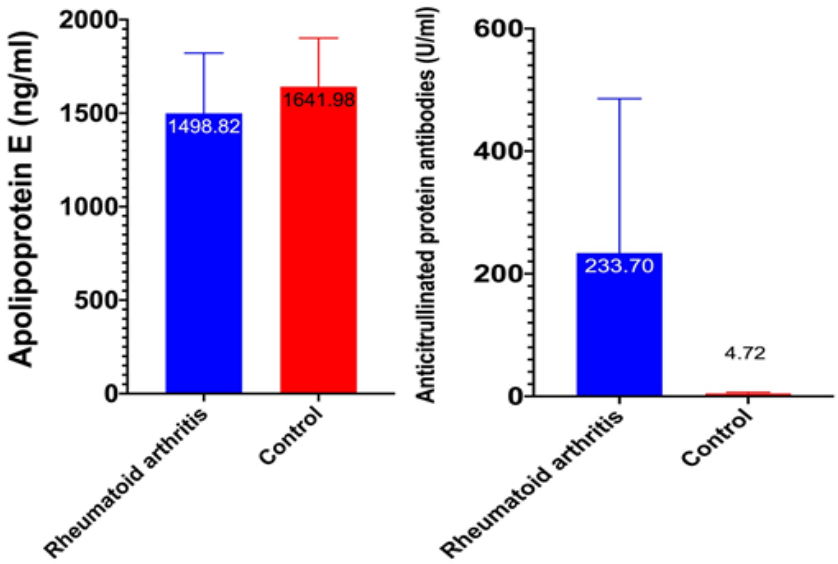

Fig.3: Comparison between APOE (ng/ml) and ACCP (U/ml) levels in patients and control groups

The genetic analysis of APOE showing three alleles (E2,E3,E4), each of which codes for a different protein (APOE2,E3,E4 isoform). These mutations, however, are uncommon, occurring in less than 1% of the human population. Nucleotide substitutions in two SNPs on exon 4 of the APOE gene, rs429358 and rs7412, trigger allele differences. Alleles differ depending on the nucleotide sequence between thymine (T) and cysteine (C) in each SNP. Changes in nucleotide bases take the form of (T rs429358/T-rs7412) in E2, (T-rs429358/C-rs7412) in E3, and (C-rs429358/C-rs7412)in E4 as showing in table 3.

Table (3): APOE SNPs(T rs429358/T-rs7412) in E2, (T-rs429358/C-rs7412)in E3 and(C-rs429358/C-rs7412)in E4.

HAP	Nucleotide base		isoform	Amino acid	Genotypes		Variations	
	Rs429358	rs7412	112		158		HAP1	HAP2
E2	T	T	APOE2	Cys	Cys	E2/E2	TT	TT
						E2/E3	TT	TC
						E2/E4	TT	CC
E3	T	C	APOE3	Cys	Arg	E3/E3	TC	TC
						E3/E4	TC	CC
E4	C	C	APOE4	Arg	Arg	E2/E2	CC	CC

The results of rs7412 and rs429358 shows the amplification fluorescent signal of each sample. The reading of fluorescent signal occurs using two channels (Fam and Hex) depending on the specific probe. the sample which possesses the T allele showed the amplification curve in the Fam channel while the amplification curve of the sample which possesses the C allele appeared in the Hex channel, heterozygotes samples appeared in the two-channel as showing in figures 4 and 5.

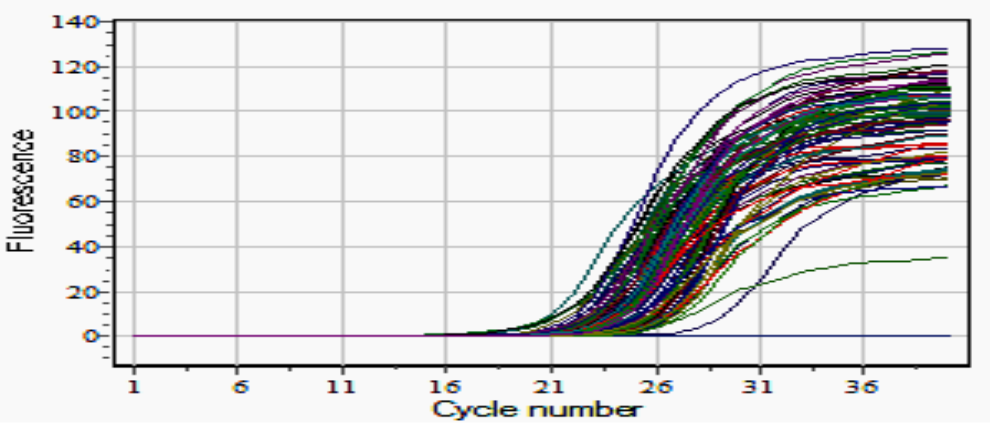

Figure (4): The RT-PCR amplification curve for rs7412 polymorphism of the APOE gene.

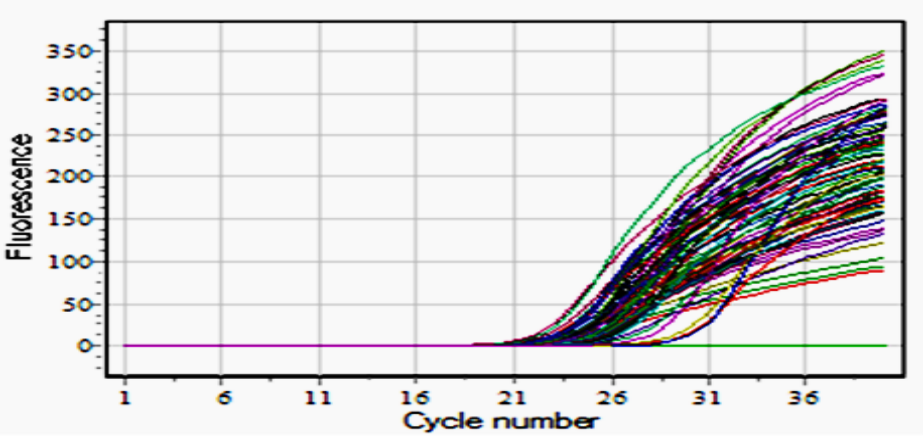

Figure (5): The RT-PCR amplification curve for rs429358 polymorphism of the APOE gene

In RA and control groups, the results of (rs7412 and rs429358) (T, C) genotyping showing that (17vs.11) carry homozygous genotype (E2/E2), whereas (52 vs. 61) homozygous genotype (E3/E3), and (16vs14) respectively homozygous genotype (E4/E4). Since the frequency in these groups is (5vs4) of heterozygous genotype (E2/E4) in both the RA and control groups. Heterozygous anthers were not present in the sample group (E2/E3, E3/E4). Since our sample had small subgroups of APOE E2/E4, APOE genotypes were grouped into three larger subgroups for statistical comparison: E3 carriers (including E3/3) re the reference groups, E2 carriers (including E2/2, E2/4), and E4 carriers (including all 4 carriers: E4/4) as showing in table 5.

Table (5): Distribution of APOE genotypes between RA patients group and control group associated with carriage of (E2,E3,E4) Polymorphism for statistical significance.

	Study groups		P value	Odd ratio	CI , 95%
	RA patients	Control			
E2 carrier	22	15	0.168NS	1.72	0.83-3.61
%	24.4%	16.7%			
E3 carrier	52	61	Reference	-	-
%	57.8%	67.8%			
E4 carrier	16	14	0.540NS	1.34	0.62-2.97
%	17.8%	15.6%			
Total	90	90			

DISCUSSION

APOE is protein that play a key role in the metabolism and clearance of the lipoprotein particles, there are different isoform from the APOE which is APOE2, APO E3 and APOE4. The APOE3 isoform is the predominate normal isoform which play important in the uptake of the lipoprotein particles in the liver and different tissue [11].The main function of the APOE3 is maintaining the structural integrity of the cholesterol carrier molecules and increase its solubility in the blood which making it has an important role in the regulation the level of the lipid in the body by increasing its internalization into the cell, some Apo E particles are originate from the macrophage and these macrophage derived Apo E has an important role in the facilitating the efflux of the cholesterol from the endothelial cell to the HDL molecules which making it help in the facilitating the reverse cholesterol transport [12]. These different isoform of the APOE resulted from the genetic variation in the sequence of the amino acid which making it has different abilities in promoting the cholesterol efflux from the cell and has different function in depending on the type of the isoform, APOE2 has ability to decrease the level of cholesterol while the opposite effect can occur when the APOE4 are presented [13].The important function of the Apo E4 allele it can increase the hepatic production of the cholesterol by increasing the production of the LDL and decreasing the production of the HDL which making it has an increase possibility for developing the cardiovascular disease[14]. In some cases, the APO E has a stimulator effect in the production and elevation the level of both TG and VLDL because it has ability to assembling and secretion the VLDL from the cell in addition to its decreased ability for the clearance of the particles, which making the patients that has an homozygote E4 allele more prone to the hypertriglyceridemia and hypercholesterolemia and making it more prone to the developing the of cardiovascular disease [15]. From the genetic variation can resulted in the lower of the APOE level which resulting in the impairment the metabolism of the Cholesterol and TG carrier molecules which also can resulted in the abnormal level of the cholesterol and TG and these was significantly shown in the RA patients that has higher level of the APOE2 in the blood in comparing to the patients that has predominant APOE4 which has lower level of cholesterol and TG, all these result can conclude in the one conclusion in the depending on the result of the current study which is the APOE4 has an atherogeic role and its higher level can making the patients has higher risk for the developing the different cardiac disease in comparing the Apo E2 or E3 carrier patient which has lower cholesterol level which indicate that these isoform has an cardio protective function [16-18]. The level of the total APOE in the APOE4 carrier patients are lower than the patient carrier for the homozygote Apo E3 and these can occur due to increase the rate of the APOE4 catabolism because it higher affinity for the Apo receptor than the APOE3. the association with VLDL and the ability to faster convert VLDL remnants to LDL [19]. Our findings show that E2 carriers have high levels of APOE with low levels of TC and LDL are statistically significant, Since E4 carriers have high TC and LDL levels but low APOE levels, this suggests that E4 is a more common cause of dyslipidemia in RA patients because lower APOE levels lead to decreased efflux cholesterol to HDL and metabolism of these particles, potentially increasing the risk of CVD. According to the findings, the E4 homozygous allele raises plasma TC and LDL cholesterol thus lowering APOE levels, making it an independent risk factor for the development of atherosclerosis and CVD and this result agrees with the constituent all study of Chen et al [20], Toms et al [21], and Maehlen et al [22].

CONCLUSION

No statistically differences in genotypes or allelic frequency of APOE SNPs (rs7412 and rs429358) were found between patients with rheumatoid arthritis and controls in the Iraqi population.

Ethical Concedration

Ethical issues (including plagiarism, consent to publication, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy) were verified by all authors.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

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Conflect Of Interest

No potential conflict of interest relevant to this article was reported.

Author Contributions

MKS designed the experiments, data analysis, and the manuscript writing. MEA supervising on the experiments. SJA participated in conducting some experiments. All authors read and approved the final manuscript.

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