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RESEARCH ARTICLE

MTHFR gene rs1801133 +677C>T Exon4 Polymorphism in Susceptibility with Rheumatoid Arthritis in Najaf Population

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Abstract

Background: The prevalence of Rheumatoid Arthritis (RA) has been raised in Iraq. The medical costs of such increased prevalence are high. Global studies have revealed polymorphisms of *MTHFR* (Methylenetetrahydrofolate Reductase) gene to be associated with Rheumatoid Arthritis (RA). This polymorphism can cause changes in various metabolic variables. Methods: The current study consists of 100 RA patients and 100 healthy control individuals. Variable parameters data included Anti-Cyclic cytrulinated peptide expression (ACCP), Rheumatoid factor (RF), C - reactive protein (CRP) in serum and Erythrocytes sedimentation rate (ESR) levels. Genotyping of *MTHFR* gene polymorphism is carried out by RFLP-PCR. Various statistical analyses were applied to analyze the data. Results: The estimation of immunological and biochemical data pointed out significant differences in Gender, RF, ACCP, CRP, ESR and age in RA when compared with those of the control group. The genotyping results were found to be consistent with Hardy-Weinberg equilibrium. The analysis of the genotype distribution under various inheritance models highlighted significant differences in SNP of *MTHFR* gene polymorphism among RA patients when compared with the control group under the dominant homozygote, heterozygote and recessive homozygote. The genotype and allele frequencies of the *MTHFR* SNP rs1801133; highlighted significant rises of the risk of RA CT (P =0.0335), (OR=1.9409), (95%CI 1.0532-3.5766) when compared with control subjects. We found that the CT genotype is associated with the risk of RA comparing with CC and TT genotypes. Clinical characteristics were observed to change significantly with respect to the genotype distribution (risk factors) of the investigated SNP. Conclusion: *MTHFR* gene polymorphism (rs1801133) is implicated in the pathogenesis of Rheumatoid arthritis in Najaf population.

Keywords: *Rheumatoid arthritis, MTHFR, Rs1801133, RFLP-PCR, ACCP, CRP, RF.*

Introduction

Rheumatoid arthritis (RA)" is a common "chronic, autoimmune and systemic inflammatory" joint disease .It affects about 1% of the world people; however, prevalence differs between 3-8% depending on the inherited and environmental risk factors [1]. Even with these declines, "RA occurs at twice the rate in women if we compare with men", with a prevalence of 1.06% in women as a percentage of the total population compared with 0.61% in men [2].

Although the RA can occur at any age of human, its incidence increases with age and most cases have an onset between 40 and 70 years [3]. The disease being the common inflammatory affects multiple joints causing "poly-arthritis". This condition, if not cured at earlier stages, results in major joint disability [2, 4, 5].The diagnosis of disease especially in the early time is quite impossible, as the clinical criteria are insufficient at the early stage of the RA. In last 5-6 years, many researches have focused on the value of the diagnostic probability and clinical application of anti-CCP antibody in RA and other rheumatic diseases [6]. RA is characterized by a proliferative disorder of synovial tissue associated with Th1-predominant immune dysregulation [7].

RA pathogenesis is the come from a "complex interaction between genetic and environmental factors", auto-antigen presence with "antigen specific" T and B cells activation and aberrant inflammatory cytokines production [8]. Bartok, et al confirm that much of the effort to define the RA epigenome has focused on fibroblast-like synoviocytes (FLS) of the synovial intimal

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lining, which attack the cartilage and assume a unique aggressive phenotype in patients with Rheumatoid arthritis [9]. Sex hormones can control the immune response via circadian rhythm. Many hormones such as cortisol, that is clear to regulate T cell "mediated inflammation", have a circadian rhythm with a maximum mount at 8:00 a.m. and progressively lower values as the day advances [10].

Methods

The study has been carried out on 100 Rheumatoid arthritis patients (36 male and 64 female). The ages of patients ranged between 15-75 year with a mean ± SD of 44.4300 ± 14.4182 year. Patients have been examined by specialist physicians. They were selected from AL-Sader Teaching Hospital, Al-Hakeem Hospital and AlForat Al-Awsat Hospital in al Najaf al Ashraf Province. Diagnosis of RA was confirmed by specialist physicians for the inclusion of the patients. The control group consisted of 100 obviously healthy subjects (40 male and 60 female). The ages of the control individuals ranged between 19-76 year with a mean ± SD of 42.630± 13.8277 year. The collection of samples is done from April 2018 till January 2019.The biochemical methods, immunological methods and genetic methods were carried out in the laboratories of Biology Department of the Faculty of Science/University of Kufa. All patients were fulfilled the ACR/EULAR 2010 criteria for the classification of RA [11].

Table (1.1).The biochemical parameters were accomplished including estimation sedimentation rate ESR, C - reactive protein CRP, Rheumatoid Factor RF, Anti-cyclic citrulinated protein ACCP, see table (1.2). DNA has been extracted from blood using DNA purification kit (Gene aid). Genotyping has been carried out by using PCR-restriction fragment length polymorphism (RFLP) for gene of MTHFR. Amplification for SNP was carried out with the use of suitable primers and a 2X Master Mix with standard Buffer kit (Bio labs). Products of PCR have been digested with restriction enzymes (Takara, Japan). The digested products were separated on a 2.2% and 2.5% agarose gel.

Table 1.1: Characteristics of study subjects

Parameter	Control subject	RA subject	P-value
No. (M/F)	100(40/60)	100 (36/64)	"P> 0.05"
Age (year)	42.630± 13.8277	44.4300 ± 14.4182	**"P = 0.0181"**
ESR (mm/h)	10.7300± 3.9667	55.1900± 20.3478	**"P < 0.0001"**

Table 1.2: Characteristics RF, ACCP and CRP of study subjects for patients RA

Parameter	RA subject n(100)		P-value Comparing With Control
	Positive (%)	**Negative (%)**	
RF	76 (76%)	24(24%)	P<0.05
ACCP	81(81%)	19(19%)	P<0.05
CRP	67(67%)	33(33%)	P<0.05

Extraction of DNA, Determination of Concentration and Purity

The extraction containing 2ml of blood put in EDTA tube for genotype analysis. From patient and control subjects, collected peripheral blood samples in EDTA tubes were subjected to DNA extraction from whole blood specimens applying g DNA Mini prep System of g SYNC tm DNA Extraction Kit (100 preps)(Gene aid). The concentration and purity of DNA were assessed by measurement of the A260/A280 ratio. Purity of DNA samples (Assessment of Nucleic Acid Purity) was established and the range of concentration of the extracted DNA was 32-240µg/ml of patient groups. Results were clarified in table (1.3). Quantified DNA samples were stored at -20ºC until used for genetic analysis.

Table 1.3: Concentration and purity of DNA of patient groups

DNA	Mean ±SD
Concentration of DNA (µg/ml)	92.35±12.65
Purity of DNA	1.85±0.04

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Amplification Reactions Results

The product of *MTHFR* gene polymorphism analyzed for amplicon size for rs1801133 was 198 bp. Result of amplification of gene polymorphism was analyzed and confirmed by electrophoresis on agarose gel as illustrated in Figure (1.1).

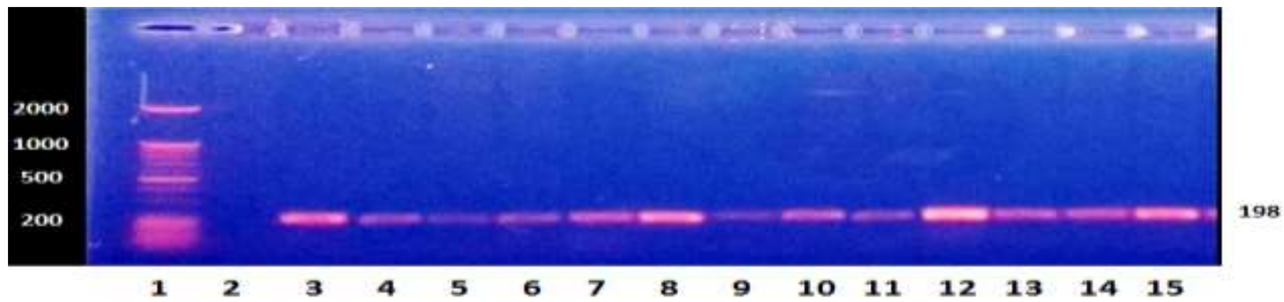

Figure 1.1: PCR product rs1801133 SNP of *MTHFR* gene polymorphism analyzed by agarose gel electrophoresis. Lane 1: Marker of DNA (25bp –2kb). 2: control, Lines 3-15: Ampilcon size of PCR product, 198bp

Detection of *Mthfr* Polymorphism

The *MTHFR* polymorphism on located in +677C>T was genotyped by PCR-RFLP method. was amplified by PCR from genomic DNA followed by digestion with restriction enzyme *HinfI* (Takara, Japan), The primer sequences employed to amplify Methylenetetrahydrofolate Reductase (MTHFR) gene analyzed for polymorphisms (rs1801133) as follows, 5'TG AA GGAGAAGGTGTCTGCGGGA-3' (Forward) and 5'-AG GA CG GT GC GG TG AGAGTG-3' (Reverse) IDT Integrated DNA Technologies (HK). One *Taq*® Quick-Load® 2X Master Mix with Standard Buffer is a premixed ready-to-use solution containing Taq Polymerase, dNTPs, MgCl2 and reaction buffers at optimal concentrations for efficient amplification of DNA templates by PCR. Briefly, each 25µl PCR reaction contained One *Taq*® Quick-Load® 2X Master Mix with Standard Buffer 12.5 µl, Forward-primer 1.5 µl, Reverse-primer 1.5 µl, Genomic DNA 7 µl and Nuclease free water 2.5 µl. Reactions were carried out in a gradient thermocycler (Agilent Technologies, USA) under the following conditions; initial denaturation at 95°C for 5 min, 40 cycles of denaturation at 95°C for 50sec, annealing at 57°C for 60sec, extension at 72°C for 25sec and final extension cycle at 72°C for 9 min.

The product of MTHFR 198bp was then digested with HinfI restriction enzyme (overnight) under the following conditions; 15µl of the reaction were incubated with 1.5U of *HinfI* (Takara, Japan) at 37°C for overnight along with its corresponding buffer. Digested products were electrophoresed on a 2.2-2.5% agarose gel in TBE buffer along with 25bp ladder. Products of digestion: TT 175, 23 bp. CC uncut band of 198 bp. CT 198,175, and 23 bp as shown in Figure (1.2).

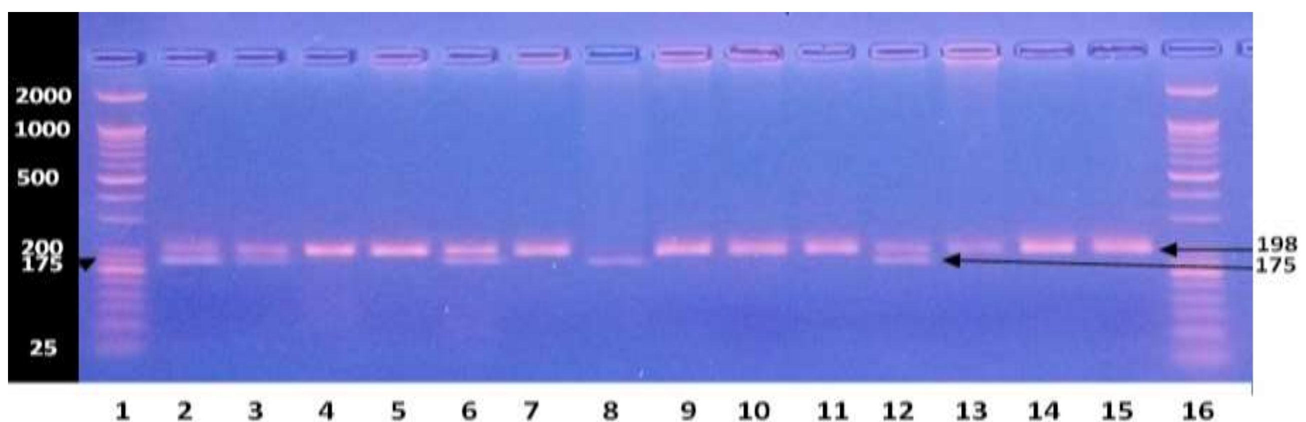

Figure 1.2: Product of PCR of rs1801133 C↔T SNP of *MTHFR* gene digested by restriction enzyme and electrophoresedon 2.5% agaros gel. Lane 1 and 16 Marker of DNA 25 bp. Lanes 4, 5, 7,9,10,11,13,14 and 15: CC genotypes 198 bp. Lanes 2, 3, 6 and 12: CT genotype 198, 175 bp. Lanes : "TT genotype 175 bp, Both the CT and TT alleles were predicted to produce a 23-bp band; however this band was not visible on the agarose gel"

Statistical Analysis

The mean ±SD term is used to express the continuous variables. The differences in means between RA Patients and group of control were determined by Student's t-test. By using Med Calc software for comparing

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level means of continuous parameters across genotypes and multi nominal logistic regression analysis was achieved to assess the association of genotype and allele frequencies with RA under different inheritance models, The ANOVA test is applied to determine the relation between genotype distribution and bio-parameters. Genotype distribution and allele frequency are expressed as non- numerical variables. Differences of these variables are assessed by the chi-squared test.

Changes are considered significant when the p value was<0.05. The mathematical relationship that relates genotypes to allele frequencies is the Hardy–Weinberg equilibrium (HWE) test [12].

The genotype and allele frequencies of rs1801133 SNP *MTHFR* gene of RA and control, persons were also analyzed with use of several approaches. "Our data indicated that, there are significant differences between RA patients and healthy controls in heterozygote CT (P =0.0335), (OR=1.9409), (95%CI 1.0532-3.5766), while genotype CC+TT frequencies represented significant difference without risk of Rheumatoid arthritis (P = 0.033), (OR = 0.5152), (95%CI 0.2796-0.9495) "."We found that the CT genotype is associated with the risk of RA comparing with CC and TT genotypes"."All genotypes, patients and healthy controls had been genotyped successfully, as described in Table"(1.4).The distribution of allelic frequencies, as shown in Figure (1.3).

Results

Table 1.4: Frequency estimation of genotype and allele for *MTHFR* gene polymorphism (rs1801133) in RA and control subjects

rs1801133 (C/T)	RA patients (n=100)	Control (n=100)	Total (n=200)	Odds Ratio	95%CI	(p-value)
CC Wild Type	51(51%)	69(69%)	120	0.4676	0.2625-0.8330	**"P = 0.0099"**
CT	38(38%)	24(24%)	62	1.9409	1.0532-3.5766	**"P = 0.0335"**
TT	11(11%)	7(7%)	18	1.6421	0.6094-4.4245	"P = 0.3268"
CC + TT	49	31	80	0.5152	0.2796-0.9495	**"P = 0.033**
TT versus others	11	7	18	1.701	(0.678 - 4.267)	(0.253)
C	140	162	0.55	1	-	-
T	60	38	0.45	1.857	(1.213 - 2.843)	-

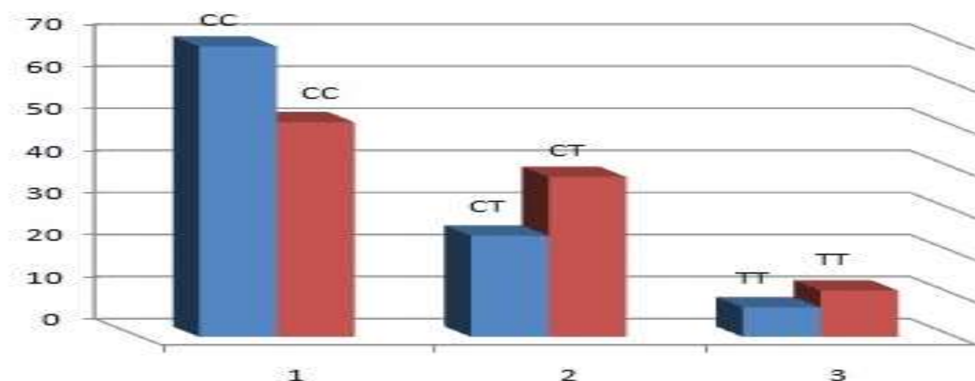

Figure1.3: "Distribution of the *MTHFR* gene rs1801133 SNP genotypes among RA cases compared with healthy control .In each graph columns show wild type CC, heterozygote CT, homozygote TT and total mutant allele frequencies respectively

Clinical characteristics of study subjects with respect to genotyping, Biochemical parameters (ESR; erythrocyte sedimentation rate) as well as serological parameters; C-Reactive Protein (CRP), Rheumatoid Factor (RF) and Anti-Cyclic Cittrulinated Protein (ACCP) values were analyzed in relevance to the genotype of the studied SNP of *MTHFR* gene in RA patients under inheritance models by ANOVA test, as illustrated in table (1.5), demonstrated no significant elevations in ESR (Erythrocyte sedimentation rate) (P=0.1217), statistical difference present in CRP (C-Reactive Protein) , Rheumatoid Factor RF and Anti-Cittrulinated protein ACCP (P < 0.0001, P = 0.0005 and P < 0.0001 respectively).

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Table 1.5: Clinical characteristics of RA patients with genotypes of rs1801133 SNP in *MTHFR* gene

Clinical characteristic	CC (n=51)	CT (n=38)	TT (n=11)	P-value
ESR (mm/h)	54.4±25.8	47.0±13.9	39.0±6.6	0.1217
CRP+ (n = 67)	27 (53%)	**35 (92%)**	5 (45.45%)	**P < 0.0001***
RF+ (n = 76)	37 (72.54%)	**29(76.31%)**	**10(82.64%)**	**P = 0.0005***
ACCP+ (n = 74)	40(78.4)	**33(86.8%)**	1(9%)	**P < 0.0001***

*P<0.05, significant

Discussion

Although the etiology of RA remains unclear."The genetic factors constitute about 50% of these factors" [13].The current study tested 200 individuals distributed between Rheumatoid arthritis patients versus healthy controls . There are no significant differences between the age, gender and variant of allelic distributions, "genotyping of the C677T polymorphism, PCR products were analyzed by using restriction enzyme *HinfI* to verify the presence of the polymorphism or not.

The percentage of genotypes was 35% CC, 38% CT, and 11% TT in RA patients, while 69% CC, 24% CT, and 7% TT in control group. Statistical analysis indicated a significant association between genotype CT, as follows (P = 0.0335), (OR=1.9409), 95%CI 1.053-3.576). Here the Odd Ratio near to two folds of RA risks comparing with controls."The *MTHFR* C677T polymorphism is associated with reduced activity of methylen tetrahydrofolate reductase" [14].

This enzyme is involved in the remethylation of homocysteine (Hcy) to methionine (Met) in the homocysteine–methionine pathway; therefore, reduced activity of the enzyme leads to hyperhomocysteinemia or the elevation of homocysteine in serum or blood" [15]. Szodoray et al have recently shown that an increased serum level of Hcy is correlated with ocular disease [16]. In addition, the association of the C677T polymorphism with preeclampsia, “congenital heart diseases (CHDs) and neural tube defects (NTDs) have been demonstrated [17].

The results of previous studies were heterogeneous; The agreements with our results, Brambila-Tapia et al suggested that the *MTHFR* (C677T) and (A1298C) polymorphisms in "Mexican population" are significantly associated with RA risk [18].

In addition, Egyptian study agreed with our investigation, "that The CT genotype and T allele of *MTHFR* C677 T are associated with RA, C polymorphic forms were not associated with RA" [19]. Another study explains, "racial variations in allele and genotype frequencies as reported by” [20] who diagnosed significant increasing in T allele in *MTHFR* C677 T and C allele of *MTHFR* A1298 C "independent on disease status" in Caucasians, when compared to African Americans. Also, a significant interaction between MTHFR polymorphisms and nutrient/environmental factors (i.e. folate status, age, smoking and alcohol intake) was reported [21], "relation study performed by Kurzawski et al, suggested the relation between *MTHFR* SNP (C677T) and RA patients who treated with Methotrexate (MTX) drug" [22], also in Asians patients [23].The investigations which disagreed with our findings. The heterogeneity between the results comes from difference in Ethnicity and environmental factors.

Inanir et al" suggested that there is no significant association between genotypic frequencies of the *MTHFR* C677T polymorphism with risk of RA [13], but allele frequencies showed statistically significant association (P = 0.01) between patients and controls, in a series of Spanish patients with RA" [24], another one; comparison between African-Americans and Caucasians with rheumatoid arthritis and controls [20], other research performed in West Algerian population, that the *MTHFR* A1298C (rs1801131) polymorphism can be associated with rheumatoid arthritis [25].

As indicated in the previous discussion about the risk factor of Rheumatoid arthritis represented with existing T allele in form CT genotype and TT genotype of MTHFR gene Exon 4 (+677) rs1801133 SNP, matching results of RA risk factor with the clinical parameters CRP, RF, and ACCP. There are significant differences; C - reactive protein for CT genotype (P < 0.0001). (P = 0.0005) of Rheumatoid Factor (RF) represented statistical significant difference in RA patients have MAF (Minor Allele Frequency) in genotype CT and TT (76.31%), (82.64%) respectively)].

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According to the findings of ACCP and its distribution between genotypes, there is a significant difference (P < 0.0001) for individuals who have CT genotype (RA risk factor) 86.8% compared with those without CC and TT (78.4), (9%) respectively. All these observations in CRP, RF and ACCP values suggested the participation of the rs1801133 SNP of *MTHFR* gene in directing metabolic changes of Rheumatoid arthritis. No significant difference about ESR value in all RA patients (p= 0.1217) GG, GA and AA 54.4±25.8, 47.0±13.9 and 39.0±6.6 respectively.

Conclusion

Our results show that *MTHFR* gene polymorphism (rs1801133) specially T allele is implicated as risk factor in the pathogenesis of Rheumatoid arthritis in Najaf population.

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