Genetic effect of rs2248359 and rs2248137 CYP24A1 with serum level of CYP24A1 enzyme and 25-OHvitD3 on multiple sclerosis disease

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Abstract

Our study attempted to clarify the genetic association between rs2248359 and rs2248137 CYP24A1 polymorphism and risk of MS in Iraq, and to explore their possible interactions with CYP24A1 enzyme and 25-hydroxyvitamin D3 (25-OHvitD3) serum level; as well as, with certain features in terms of age at onset, gender, disease duration, Expanded Disability Status Scale (EDSS) and disease lines.

Keywords: Multiple sclerosis, CYP24A1, 25-OHvitD3, Polymorphism, Genetic, Vitamin D.

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Introduction

Multiple Sclerosis (MS) is a multifactorial autoimmune disease of the Central Nervous System (CNS). Until now, the underlying cause of this disease which remains uncertain MS had attacked about 2.5 million young adults in worldwide. A number of studies had found both genetic variation and numbers of environmental factors particularly (vitamin D, ultraviolet light, Epstein–Barr virus infection, obesity and smoking could be related with disease susceptibility. Several analytical epidemiological studies have recommended an association between low level of vitamin D and MS CYP24A1 is located at chromosome 20q13.2 responsible for expression of 25-hydroxyvitamin D3 24-hydroxylase (CYP24A1). Human CYP24A1 enzyme is one of seven mitochondrial cytochrome that are commonly found in the kidney and other parts of the body. The human CYP24A1 enzyme can catalyze the degradation of 1,25-dihydroxyvitamin D3 (1,25-(OH)2D3) through a series of sequential oxidation reactions that target particular carbon atoms in the side chain through a highly complex regulatory mechanism. Generally, in normal human tissues, expression of CYP24A1 remains at low levels. Functional deletion of the CYP24A1 gene has been found to lead to an increased concentration of 1,25-(OH)2D3 in serum [1-3].

Previously, number of studies were identified significantly related between Single Nucleotide Polymorphism (SNP) in CYP24A1 with low level of vitamin D such as rs6013897 and rs2248137while, rs2248359 and rs2248137 were related with lower 25-OHvit D3 and MS risk. The aim of this study was to identify the relative association between the polymorphisms rs2248359 and rs2248137 of CYP24A1 gene and the risk of MS in Iraq and to explore their possible interactions with CYP24A1 enzyme and 25-hydroxyvitamin D3 (25-OHvitD3) serum level; as well as with certain features in term of age at onset, disease duration, gender, Expanded Disability Status Scale (EDSS) and disease lines [4-6].

Materials and Methods

Study group

In our study, blood samples were obtained from 60 MS patients (33 female and 27 male) and 40 healthy controls (20 female and 20 male) from December 2019 to February 2020 with ages from 18 to 55 years. All patients were diagnosed according to the McDonald criteria at Baghdad teaching hospital/medical-city in Baghdad, Iraq. Patients were divided into three groups. Group I consisted of 14 patients with relapsing-remitting multiple sclerosis who did not receive immune modulatory therapy (N-RRMS), group II included 20 patients with Relapsing-Remitting Multiple Sclerosis (RRMS) who received Betaferon®, and group III included 26 patients with secondary-progressive multiple sclerosis who received Tysabri therapy (SPMS) [7-9].

Biochemical analysis

Enzyme-Linked Immunosorbent Assay (ELISA) kits (Mybiosource, USA) were used to measure the levels of CYP24A1 and 25-OHvitD3 in serum.

Molecular analysis

In the molecular analysis, frozen whole blood samples in EDTA tubes were used. A commercial kit (Geneaid/Taiwan) was used to extract genomic DNA from 200 µl of blood. The content of the DNA was determined using electrophoresis in a 0.8% agarose gel, then quantified using nanodrop 8000 Spectrophotometer from Thermo Scientific/USA. Finally all DNA samples were stored at −20˚C for further use. All samples were genotyped for rs2248359 and rs2248137 of CYP24A1 using Sanger sequencing *via* ABI3730XL, automated DNA sequences, by Macrogen. Corporation/Korea [10-12].

The results were analyzed using geneious software. A conventional PCR protocol was used to amplify the extracted DNA with using Go Tag Green Master Mix kit (Promega/USA)

with thermal cycler (thermo fisher scientific/USA). The PCR reaction, optimized as follows, was: initial denaturation at 95˚C for 5 minute, followed by 30 cycles of amplification (denaturation at 95˚C for 30 second, annealing at 58˚C (rs2248359) and at 65˚C (rs2248137) for 30 second, extension at 72˚C for 30 second). Then, final extension at 72˚C for 7 minute, followed by 10 minute in hold at 10˚C. The PCR primer were designed with Primer3 v4.0 software, the primers sequences in this study were list in Table 1. Electrophoresis on 1.5% agarose gels was used to check the 725 bp and 874 bp for PCR-generated amplicons of rs2248359 and rs2248137 respectively [13-15].

Statistical analysis

Biochemical and genetic data were evaluated by SPSS (statistical package for social sciences) version 23. For biochemical analysis all data were represented as mean ± Standard Division (mean ± SD). Multiple comparisons analysis was performed by One-Way Variance Analysis (ANOVA). Chi-square ($\chi 2$) test was used to determine if all genotypes were in Hardy-Weinberg Equilibrium (HWE). The Differences in the genotypic and allelic distribution between study groups were determined using Fisher's exact test, Odds Ratio (OR) with 95% confidence intervals (95% CI).

	Primer name	Primer sequences	Annealing Tm (°C)	Amplicon size (bp)
CYP24A1	rs2248359- F	5`-CCCTCCAGACAAATCAGAAAT-3`	58	725
	rs2248359- R	5`-ACTGAAATGCACCAGAAGAG-3`		
	rs2248137- F	5`-TCTCATCTACCCTCCTTGAC-3`	65	874
	rs2248137- R	5`-GCCATACTTCTTGTGGTACTC-3`		

Table 1. *Primers used to amplify the specific SNPs in CYP24A1. F: Forward primer, R: Reverse primer.*

Comparison of genotypes distribution of SNPs according to CYP24A1 and 25-OHvitD3 levels, age of onset, disease duration and EDSS was performed by (ANOVA). While, ($\chi 2$) test was performed to compare the genotypes distribution of SNPs according to gender and disease line. The results of statistical analysis were deemed significant if the level of significance was $p \leq 0.05$ [16-20].

Results

Table 2 was summarized the basic characteristics for both MS patient and control groups. There were no statically significant differences in age and BMI between research groups (P>0.05). In MS patients both CYP24A1 and 25-OHvitD3 levels were significantly lower than in control.

Parameters	Patients (N=60)	Controls (N=40)	P-value
Age (years)[a]	34.1 ± 8.41	35.2 ± 7.965	0.509
Gender[b]			
Female	33 (54.55)	20 (52.38)	
Male	27 (45.45)	20 (47.62)	
BMI (kg/m2)[a]	25.031 ± 3.583	24.529 ± 3.305	0.48
Disease duration, (years)[a]	5.412 ± 4.207	-	-
Age at onset, (years)[a]	28.683 ± 8.081	-	-
Disease line[b]			
N-RRMS	14 (23.33%)		
RRMS	20 (33.33%)		
SPMS	26 (43.33%)		
EDSS level[a]	1.266 ± 1.404	-	-
MS Treatment [b]			

Betaferone	20 (33.33%)		
Tysabri	26 (43.33%)		
No treatment	14 (23.33%)		

Table 2. *Basic characteristics of the patients and control groups. N: Sample size, a mean ± SD, b number and percentage N (%) P>0.05 indicates a non-significant difference.*

(1.443 ± 0.671 ng/mL vs. 4.050 ± 1.586 ng/mL, P ≤ 0.01 and 55.702 ± 13.703 ng/mL vs. 70.394 ± 16.751 ng/mL, P ≤ 0.01, respectively; Table 3).

In patients, a non- significantly higher levels of CYP24A1 enzyme were found in advance stage patients (SPMS and RRMS) than N-RRMS (1.554 ± 0.628 ng/mL, 1.304 ± 0.629 ng/mL *vs.* 1.192 ± 0.671 ng/mL, respectively; Figure 1).

While, the level of CYP24A1 in control group was 4.114 ± 1.498. On other hand, regarding the levels of 25-OHvitD3 in patients, the opposite manner were found. They were significantly higher in level of 25-OHvitD3 in N-RRMS than other patients (60.473 ± 12.877 ng/mL *vs* 46.327 ± 20.722 ng/mL and 43.584 ± 17.081 ng/mL, respectively; Figure 1). Whereas, in control group the level of 25-OHvitD3 was 72.170 ± 14.699 ng/mL. As well as that, in all patients group the level of CYP24A1 was significantly difference (P ≤ 0.01) with control. The levels for 25-OHvitD3 were significantly decreased (P ≤ 0.05) in N-RRMS than control group. The serum levels for 25-OHvitD3 were significantly decreased (P ≤ 0.01) in SPMS and RRMS than control, and also, significantly decreased in level of 25-OHvitD3 in both SPMS and RRMS than N-RRMS Figure 1 [22,23].

In this study, we stratified patients according to gender, and non-significantly decreased was found in CYP24A1 and 25-OHvitD3 levels in women with respect to men (1.316 ± 0.606 ng/mL vs. 1.473 ± 0.694 ng/mL and 46.12 ± 18.397 ng/mL vs. 51.788 ± 18.992 ng/mL respectively; P>0.05).

Parameters	Group	N	Mean	± SD	P-value
CYP24A1 (ng/ mL)	Control	40	4.05	1.586	0
	Patient	60	1.443	0.671	
25-OHvitD3 (ng/ mL)	Control	40	70.394	16.751	0
	Patient	60	55.702	13.703	

Table 3. *The serum level of CYP24A1 and 25-OHvitD3 in study groups. P ≤ 0.05 indicates a significant difference.*

Genotypic distribution of the CYP24A1 SNPs rs2248359 and rs2248137 in both MS patients and controls was shown to be in Hardy-Weinberg equilibrium (H.W.E).

The (χ2) of genotype distributions for rs2248359 were 0.042 in patients vs. 2.736 in control P>0.05.

While, the (χ2) of genotype distributions for rs2248137 were 0.066 in patients vs. 2.736 in control P>0.05.

Furthermore, the accession numbers for GG, GA and AA in CYP24A1 SNPs rs2248359 were LC632059, LC626469 and LC626459 respectively.

The accession numbers for GG, GC and CC in CYP24A1 rs2248137 were LC632061, LC626464 and LC626458 respectively.

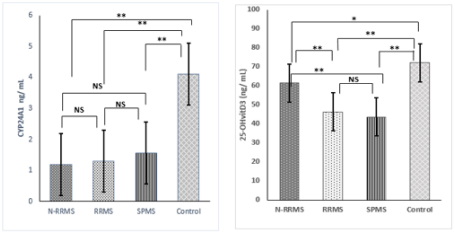

Figure 1. *The serum level of CYP24A1 and 25-OHvitD3 of patients within different disease lines and control group. **: P ≤ 0.01, *: P ≤ 0.05, NS: Non-significant at P>0.05.*

When, the genotype distributions and allele frequencies of rs2248359 and rs2248137 were compared in study groups, significant differences were found Table 4. Besides that, in patients there was significantly higher in minor allele (A) frequency of the rs2248359 than in control (54.17 % vs. 18.75%, P ≤ 0.01; Table 4).

CYP24A1 SNP	Genotype/allele	Patient		Control	OR	95% CI	P- value	
		N	(%)	N				(%)
rs2248359	GG	13	21.67	28	70	0.119	0.0475 to 0.2955	0.0001
	GA	29	48.33	9	22.5	2.9	1.2097 to 6.9522	0.017
	AA	18	30	3	7.5	5.286	1.4409 to 19.3893	0.012
	G	55	45.83	65	81.25	0.195	0.1003 to 0.3802	0.0001
	A	65	54.17	15	18.75	5.121	2.6299 to 9.9726	0.0001
rs2248137	GG	15	25	28	70	0.143	0.0584 to 0.3492	0.0001
	GC	26	43.33	9	22.5	2.634	1.0700 to 6.4840	0.0351
	CC	19	31.67	3	7.5	4.171	1.1242 to 15.4728	0.0328
	G	56	46.67	65	81.25	0.201	0.1037 to 0.3931	0.0001
	C	64	53.33	15	18.75	4.952	2.5438 to 9.6415	0.0001

Table 4. *The SNPs of CYP24A1 gene polymorphisms association in MS patients and control group. P ≤ 0.05 indicates a significant difference.*

SNPs	Genotype	CYP24A1 (ng/ mL)	P-value	25-OHvitD3 (ng/ mL)	P-value
rs2248359		(Means ± SD)		(Means ± SD)	
	GG (N=13)	0.913 ± 0.273	0.021a	66.879 ± 11.211	0.000a
	GA (N=29)	1.468 ± 0.664	0.008b	42.339 ± 16.907	0.002b
	AA(N=18)	1.597 ± 0.665	0.759c	45.728 ± 18.060	0.767c
rs2248137	CC(N=18)	1.457 ± 0.748	0.840a1	61.913 ± 13.486	0.006a1
	CT (N=26)	1.337 ± 0.555	0.964b1	43.960 ± 18.627	0.015b1
	TT (N=16)	1.398 ± 0.705	0.948c1	44.669 ± 18.188	0.989c1

Table 5. Comparison of CYP24A1 and 25-OHvitD3 between the diffrent genotypes distribution of the rs2248359 and rs2248137 in MS patients. P ≤ 0.05 indicates a significant difference. a: GG genotype vs. GA genotype; b: GG genotype vs. AA genotype; c: GA genotype vs. AA genotype; a1: GG genotype vs. GC genotype; b1: GG genotype vs. CC genotype; c1: GC genotype vs. CC genotype.

The GA and AA frequency in MS patients were 48.33% and 30% vs. 22.50% and 7.50 in control (OR=2.900, 95% Cl 1.2097 to 6.9522, P ≤ 0.05 and OR=5.286, 95% Cl 1.4409 to 19.3893, P ≤ 0.01, respectively), demonstrating a mild correlation between allele A and MS.

Furthermore, the minor allele (C) frequency of the rs2248137 in patients was significantly higher than in control (53.33% vs. 18.75%, P=0.0001; Table 4). The genotype frequency of GC and CC in MS patients were (OR=2.634, 95% Cl 1.0700 to 6.4840, P ≤ 0.01 and OR=4.1707, 95% Cl 1.1242 to 15.4728, P ≤ 0.01, respectively), indicting an association between minor allele C and MS Table 4 [24,25].

The analysis of the rs2248359 and rs2248137 distributions in MS patients, is stratified according to the serum levels for both CYP24A1 and 25-OHvitD3 Table 5.

Our results were revealed that patients with genotype AA and GA had statistically significantly higher CYP24A1 than those with genotype GG (1.597 ± 0.665 ng/ mL and 1.468 ± 0.664 ng/mL vs. 0.913 ± 0.273 ng/mL, P ≤ 0.01 and P ≤ 0.05 respectively); while, a trend of statistically significant lower in the levels of 25-OHvitD3 was presented with patients who carried GA and AA in comparison to those with GG genotypes (42.339 ± *16.90*

ng/mL and 45.728 ± 18.060 ng/mL vs. 66.879 ± 11.211 ng/mL, P ≤ 0.01).

When comparing between three genotype distributions of rs2248137, significant differences were observed only in the level of 25-OHvitD3. Significantly lower levels of 25-OHvitD3 are in GC and CC in comparison to those with genotype GG (43.960 ± 18.627 ng/ mL and 44.669 ± 18.188 ng/ mL vs 61.913 ± 13.486 ng/mL P ≤ 0.01 and P ≤ 0.05 respectively). The genotype distributions of the rs2248359 and rs2248137 in MS patients were stratified according to the basic characteristics. As shown in Table 6 rs2248359 had any association with any basic characteristics, simillary rs2248137 as shown in Table 7 [26].

Basic characteristics	Genotype rs2248359			P-value
	GG (N=13)	GA (N=29)	AA(N=18)	
Age at onset ,(years)a	27.154 ± 7.744	28.966 ± 7.844	29.333 ± 8.977	0.509a
				0.467b
				0.881c
EDSSa	1.154 ± 1.049	1.069 ± 1.368	1.667 ± 1.654	0.856a
				0.319b
				0.160c
Disease duration, (years)a	6.077 ± 6.157	5.276 ± 3.731	5.167 ± 3.365	0.575a
				0.560b
				0.932c
Disease lineb				
N-RRMS	4	5	5	0.242
RRMS	7	12	1	
SPMS	7	12	7	
Genderb				
Female	9	13	11	0.279
Male	4	16	7	

Table 6. *Comparison of genotypes distribution of rs2248359 in MS patients according to basic characteristics. a: mean ± SD, b: number N. a: GG genotype vs. GA genotype, b: GG genotype vs. AA genotype, c: GA genotype vs. AA genotype. P>0.05 indicates a non-significant difference.*

Discussion

However, there are limit studies about the CYP24A1 serum level and CYP24A1 gene polymorphism association with MS. In our study we have selected two SNPs in CYP24A1 gene based on their previously identified association with 25-OHvitD3 levels and MS risk in populations of various ethnic origins. When exploring the association among CYP24A1 and 25-OHvitD3 serum levels, SNPs in CYP24A1 gene and group of MS Iraqi patients and control, we found lower serum levels for both CYP24A1 and 25-OHvitD3 in MS patients than in controls, a not statistically significant higher level, of CYP24A1 enzyme in advance stage patients (SPMS and RRMS) than N-RRMS with significantly lower level of 25-OHvitD3. We also, found an association of minor allele (A) of rs2248359, and minor allele (C) frequency of the rs2248137 with MS susceptibility [27-30].

Basic characteristics	Genotype rs2248137			P-value
	GG (N=15)	GC (N=26)	CC(N=19)	
Age at onset, (years)a	27.267 ± 7.421	29.500 ± 7.824	28.684 ± 9.141	0.678a
				0.871b
				0.942c
EDSSa	1.433 ± 1.223	0.923 ± 1.332	1.605 ± 1.587	0.500a
				0.932b

				0.244c
Disease duration, (years)a	6.333 ± 5.753	4.846 ± 3.574	5.473 ± 3.657	0.529a
				0.827a
				0.876c
Disease lineb				
N-RRMS	5	5	4	0.144
RRMS	1	12	7	
SPMS	9	9	8	
Genderb				
Female	9	12	12	0.477
Male	6	14	7	

Table 7. *Comparison of genotypes distribution of rs2248137 in MS patients according to basic characteristics. a: Mean ± SD; b: number N. P>0.05 indicates a non-significant difference. a: GG genotype vs. GA genotype; b: GG genotype vs. AA genotype; c: GA genotype vs. AA genotype.*

But, neither rs2248359 nor rs2248137 had any association with age at onset, disease duration, gender, EDSS and disease lines. As previously mentioned MS was multifactorial, autoimmune disease of the CNS A number of studies has recommended the role of vitamin D in MS susceptibility. Thus, 25-OHvitD3 plays a critical role in the functions of CNS, immune cell maturation, homeostasis of an epithelial barrier, induces differentiation, and inhibits proliferation different type of cell. But, vitamin D level in human can significantly affected by numbers of variation such as genetic, environmental, and lifestyle factors.

As a result, study the enzymes were involved in metabolic path way of vitamin D was fundamental. Particularly, CYP24A1 catalyze vitamin D catabolism by conversion of 25-OHvitD3 into 25,24-(OH)2vitD3 and 1,25-(OH)2vitD3 into calcitroic acid and 1,25-(OH)2D-26-23-lactone that are finally excreted in the bile and urine. In a previous study, we reported in MS patients a significant association between lower levels of CYP24A1 and 25-OHvitD3. It is only in female groups that both of these parameters were positively correlated and decreased significantly with EDSS, which, indicated the association between CYP24A1 level and MS susceptibility, as a result of presented Vitamin D Response Elements (VDRE) in CYP24A1 promoter region, CYP24A1 which respond rapidly and strongly at certain levels of 1,25-(OH)2vitD3. Thus, the rising or falling of 25-OHvitD3 level was controlled the expression of CYP24A1. Therefore, CYP24A1 expression acts as a barrier to vitamin D hormone production. We can speculate that the lower level of 25-OHvitD3 in MS patients than in control group may be related to an increased expression or activity of CYP24A1 gene. Although, not statistically significant, a trend of a higher level of CYP24A1 enzyme is found in advance stage patients (SPMS and RRMS) than N-RRMS and significantly lower level of 25-OHvitD3 may be related to the overexpression of CYP24A1 gene in advance stage vs. early diagnosis. Nonetheless, studies with a larger sample size are needed to support our hypothesis [31].

Thus, risk allele (A) of rs2248359 that located in the 5’ region of the CYP24A1 could change the level of this gene's expression, and thus contribute to the availability of active 25-OHvitD3 in the tissues. Consequently, it could have an effect on immune function which in turn may affect MS susceptibility. While, other research identified that there is no convincing evidence that rs2248359 was related with MS risk. Furthermore, risk allele (C) of rs2248137 that located in intron of CYP24A1 gene increasing the probability that it may alter the expression of the corresponding gene in MS patients. Similarly, rs2248137 polymorphism is associated with lower levels of vitamin D in autoimmune disorders including diabetes 1 and asthma that have pathogenesis similar to multiple sclerosis. Up To the present date, this is the first study that presents evidence of a potential impact of the CYP24A1 and 25-OH vitD3 serum levels with rs2248359 and rs2248137 SNPs in CYP24A1 gene in Iraqi MS patients. We found an association between rs2248359 and rs2248137 of CYP24A1 gene, CYP24A1 and 25-OHvitD3 serum level with MS. However, since this is a study was performed with a limited sample size, our results should be confirmed by a larger sample size study [32-34].

Conclusion

Our study identifies a possible association of CYP24A1 polymorphism with Iraqi multiple sclerosis patients.

Ethics

Written permission was approved by the department of chemistry, college of science, Mustansiriyah university, and Baghdad teaching hospital/medical-city, with approval number (49024), in 19/12/2019, written consent was obtained from all MS patients who volunteered to participate in the study.

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Conflict of Interest

No conflict of interest is associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Shemaa A Soud as first and corresponding author, methodology design, data analysis, writing-original draft. Salwa Hameed Naser Al-Rubae'I, study conceptualization, supervision, writing-review editing. All authors read and approved the final manuscript.

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