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Association of *CYP27B1* Polymorphism and Vitamin D Levels with Multiple Sclerosis Development in Lebanese Population of Bekaa Region: A Preliminary Study.

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DOI: 10.31383/ga.vol5iss1pp18-25

Abstract

Multiple sclerosis (MS) is a neurodegenerative disease of the central nervous system (CNS). Interaction between genetic and environmental factors guides the development of the disease. Among environmental factors, vitamin D deficiency is shown to increase the risk of MS development. Several single nucleotide polymorphisms (SNPs) in cytochrome P450 family 27 subfamily B (*CYP27B1*) gene that encodes the rate-limiting enzyme involved in vitamin D metabolism, were shown to be correlated with MS. We aimed at investigating the association of *CYP27B1* gene polymorphisms and vitamin D level with MS development in a sample of Lebanese MS patients living in the Bekaa region. Enrolled MS patients and controls were age and gender matched. Genotyping was performed by sequencing the amplified *CYP27B1* PCR products. Vitamin D levels were measured using a VIDAS® 25 OH Vitamin D total assay based on enzyme linked fluorescent assay (ELFA). Chi-square and Mann-Whitney U tests were used for statistical analysis. A significant association was shown between vitamin D deficiency and MS without any association between *CYP27B1* studied SNPs and the disease. We confirmed that vitamin D deficiency was associated with MS with no implication of the studied SNPs of *CYP27B1* gene with disease susceptibility among the Lebanese MS patients living in the Bekaa region.

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Received

January, 2021

Accepted

May, 2021

Published

June, 2021

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Keywords

CYP27B1, multiple sclerosis, polymorphism, vitamin D

Introduction

Multiple sclerosis (MS) is a chronic hostile inflammatory disorder targeting the myelin sheath of nerves (Gonzalez, 2012). It is characterized by plaque formation in the central nervous system (CNS) accompanied with demyelination and axonal injury.

Various studies showed that MS initial event occurs when auto-reactive T cells are activated in the periphery (Domingues et al., 2010; Holman et al., 2011) and this activation increases the immune response by recruiting different pro-inflammatory cells like B cells that adhere to and cross the blood-brain barrier (BBB).

Following their entry to CNS, T cells are reactivated by antigen presenting cells (APCs) and then produce various types of pro-inflammatory cytokines that activate microglial cells, inducing myelin phagocytosis. B cells can also secrete myelin specific antibodies resulting in myelin destruction. Oligodendrocytes, producers of myelin in CNS, are also affected by macrophages (Nikbin et al., 2007; Frischer et al., 2009).

The disease may evolve from relapsing-remitting disease course to much more severe clinical forms (Milo and Kahana, 2010; Mehta, 2010). The main MS symptoms include numbness, fatigue, muscle spasms, walking difficulty, bladder and bowel dysfunction, and many others (Yamout et al., 2008). MS prevalence varies around the world. No recent epidemiological study about MS patients number in Lebanon. However, more than 1000 cases were registered in 2008 (Yamout et al., 2008), and this number is still rising in the absence of a total effective cure.

Although MS etiology is still unknown but it is believed that an interaction between genetic and environmental factors guides the development and the progression of the disease. Among these environmental factors, vitamin D deficiency is shown to be correlated with MS (Brutting et al., 2021). Several single nucleotide polymorphisms (SNPs) in cytochrome P450 family 27 subfamily B (*CYP27B1*) gene, encoding for the rate-limiting enzyme in vitamin D metabolism, were confirmed to be associated with MS in diverse populations (Sundqvist et al., 2010; Cierny et al., 2019; Smagina et al., 2020).

Few works on MS genetic and environmental contribution were performed in the Middle East region despite the disease-accelerating rate (Heidari et al., 2011; Al Jumah et al., 2018).

In the light of this, we aimed in this preliminary study to investigate the association of *CYP27B1* gene polymorphism and vitamin D levels with MS development in a sample of Lebanese population living in the Bekaa region.

Material and methods

Participants

Fifty-six participants (28 patients with clinically definite MS of the relapsing-remitting disease course

(RRMS) and 28 controls) were enrolled in this preliminary study. All patients and controls were of Lebanese ancestry from the Lebanese Bekaa region. Each participant signed a consent form and filled a well-designed questionnaire pertaining to the study including information about age, age onset, disease duration, treatment, family neurological history and clinical complications. Expanded disability status scale (EDSS), generally used to quantify MS disability, EDSS progression index, that is obtained by dividing EDSS score by the disease duration since the patient's clinical diagnosis, and multiple sclerosis status scale (MSSS), which is derived from the global MSSS table, were also calculated (Kurtzke, 1983; Roxburgh et al., 2005). This work was approved by the Institutional Review Board at Beirut Arab University (code #: 2019A-0042-S-P-0337) and from Al-Abdallah hospital in Bekaa, Lebanon.

DNA extraction and PCR amplification

Venous blood sample (5 ml) was collected in EDTA tube from each participant. Following centrifugation at 2,000 rpm for 20 minutes, the lower fraction containing blood cells was used for DNA extraction using DNA purification kit (Sigma-Aldrich, NA2010, Germany). DNA quantification and purity check were performed by measuring absorbance at 260 nm and 280 nm using a UV-Spectrophotometer (Thermo Fisher Scientific, 840-189900, USA).

DNA integrity was validated by agarose gel electrophoresis. DNA primer sequences were designed using Primer-BLAST and manufactured by Macrogen company (Korea). The following shows the primers used to amplify the region covering exon 3 and the region covering intron 6 and exon 7 containing the studied SNPs of *CYP27B1*:

For exon 3:

F: 5'- ACGTAGTCTGCGACCTTGTG-3' and R: 5'- CGACACGGAGACCTTCATCC-3'.

For intron 6 and exon 7:

F: 5'- AAGTGCTAAGGTGAGGGGGA- 3' and R: 5'- AAGGGGAGTGTTTGAAGGGC- 3'.

Table 1 shows the different *CYP27B1* SNPs investigated and the amino-acid change in the specified exons.

Amplicons were purified using PCR clean-up kit (Sigma-Aldrich, NA1020, Germany) and sequenced at MacroGen company- Korea. PCR reaction was performed in a total volume of 25 µl consisting of 1 µl DNA template (10 ng), 1.5 µl of forward and reverse primers (10 µM), 12.5 µl of PCR master mix (2x) (Sigma-Aldrich, R2523, Germany) and nuclease free water to make up to volume. PCR conditions were as followed: one cycle of denaturation (95 °C, 5 min), followed by 30 cycles consisting of denaturation (95 °C, 30 sec), annealing (55 °C, 30 sec) and extension (72°C, 30 sec) and one cycle of final extension (72°C, 5 min). PCR products size was checked using 2% agarose gel electrophoresis and visualized under UV light (Bio-Rad, ChemiDoc-It® 2515 Imager P/N 95-0441-04, USA).

Serum 25(OH)D₃ assay

Serum 25(OH)D₃ levels of all participants were measured, according to manufacturer instructions', using an immunoassay (VIDAS® 25 OH Vitamin D total assay, cat # 9304004, France) that is based on enzyme linked fluorescent assay (ELFA). The assay principle combines an enzyme immunoassay competition method with a final fluorescent detection.

Statistical analysis

Data entry and statistical analysis were performed using SPSS version 20. Chi-square and Fischer's exact tests were used in order to evaluate SNPs contribution to MS. Mann-Whitney U test was used to assess 25(OH)D₃ level differences between groups.

The risk of outcome, taking a confidence interval of 95%, was calculated as a measure of association and statistical significance was defined for a P-value ≤ 0.05.

Results and Discussion

Characteristics of participants

This preliminary study was conducted on 56 participants (28 patients with clinically definite relapsing-remitting multiple sclerosis (RRMS) according to McDonald's criteria and 28 controls) from the same Lebanese Bekaa region.

The characteristics of both MS patients and controls are shown in Table 2. Thirteen MS patients were male and 15 were female with a mean age of 40.6. As for the controls, 9 were male and 19 were female with a mean age of 37.8. The mean age onset and disease duration were 28 and 14.9 respectively. Serum level mean of 25(OH)D₃ in MS patients was 14.7 ng/ml which is significantly lower to that in controls which is 25.8 ng/ml (P < 0.05). The EDSS, EDSS progression index and MSSS mean scores were 4.6, 0.3 and 5.5, respectively.

Clinical profile of MS patients

Bladder dysfunction was observed in around 50% of MS patients. Constipation was a common symptom observed in 50% of patients and that may be the result of spinal cord impairment, reduced motility and diet composition.

Low physical disability was noticed in 46.4% of patients and 28.6% of the patients were self-dependent and able to perform daily activities. These symptoms were accompanied with the partially unaffected cognitive functions of the patient group.

Table 1. CYP27B1 considered regions along with the studied SNPs and corresponding amino-acid change

CYP27B1 coding/non-coding region	SNP	Amino-acid change
Exon 3	Rs118204012 (A>G)	Glutamic acid → Glycine
Intron 6	Rs4646536 (T>C)	-----
Exon 7	Rs28934607 (C>T)	Proline → Serine
Exon 7	Rs118204010 (C>G/T)	Arginine → Glycine/Cysteine
Exon 7	Rs118204009 (G>A)	Arginine → Histidine

Table 2. Summary of studied parameters in MS patients and controls

	Cases (n=28)	Controls (n=28)	P-value
Age (mean $\pm$ SD)	40.6 $\pm$ 12.27	37.8 $\pm$ 16.55	0.544
Gender (F/M)	15/13	19/9	0.322
F:M ratio	1.15:1	2.1:1	-----
Disease course	Relapsing-remitting	-----	-----
Age onset (years) (mean $\pm$ SD)	28 $\pm$ 16.1	-----	-----
Disease duration (years) (mean $\pm$ SD)	14.9 $\pm$ 4.9	-----	-----
25(OH)D ₃ (ng/ml) (mean $\pm$ SD)	14.7 $\pm$ 4.6	25.8 $\pm$ 9.1	0.000*
EDSS mean	4.6	-----	-----
EDSS progression index mean	0.3	-----	-----
MSSS	5.5	-----	-----

* Statistically significant

Allelic and genotypic frequencies of CYP27B1 studied SNPs

The amplified polymerase chain reaction (PCR) products of different *CYP27B1* regions were run on 2% agarose gel, with 100 bp ladder to confirm the size of amplicons. Figure 1 shows representative gels of the amplified fragments of exon 3 of *CYP27B1* gene yielding a fragment of 274 bp (Figure 1A), and that of intron 6 - exon 7 amplicon of 394 bp (Figure 1B). Table 3 shows the allele and genotype frequencies of *CYP27B1* (exon 3, intron 6, and exon 7) SNPs in MS patients and controls.

For all the studied SNPs of exon 3 (rs118204012) and exon 7 (rs118204009, rs118204010, and rs28934607) of *CYP27B1*, all MS patients and controls carried the wild type allele and genotype showing by this a non-significant difference in terms of allelic and genotypic frequencies between MS patients and controls ($P=1.000$).

However, for rs4646536 SNP of intron 6, 67.9% of MS patients and 50% of controls were homozygous for T allele, 32.1% of MS patients and 7.1% of controls were heterozygous and none of the MS patients and 50% of controls were homozygous for C allele. On the other hand, 32.1% of MS patients and 50% of controls carried the wild type allele T and 67.9% of MS patients and 50% of controls carried the mutant allele C. Based on this, no significant difference was found in terms of allelic

and genotypic frequencies between both groups ($P=0.174$).

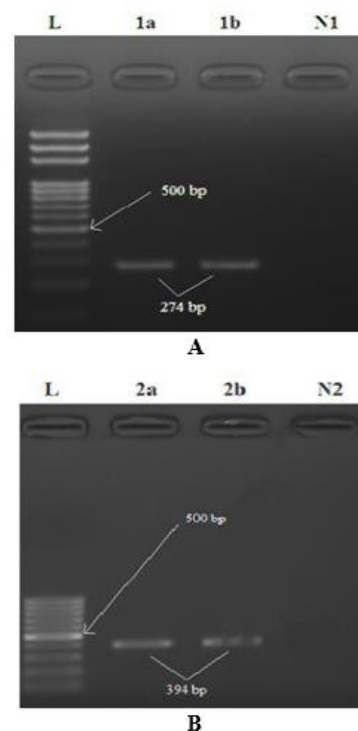


Figure 1. Agarose gel electrophoresis of amplified PCR products. Panels A and B show amplified products of exon 3 and intron 6-exon 7 respectively. (L): 100 bp DNA Ladder; (N1): Negative control for exon 3 primers; (N2): Negative control for intron 6-exon 7 primers; (1): PCR product of *CYP27B1* (exon 3) of 274 bp; (2): PCR product of *CYP27B1* (intron 6 with exon 7) of 394 bp; (a) control; (b) MS patient.

MS is an autoimmune neurodegenerative disease targeting the myelin sheath of axons (Goldenberg, 2012). Although its etiology is not well established, it is thought to be the result of gene-environment interactions. This study assessed the association between 5 different SNPs in *CYP27B1* and MS risk, and investigated whether low vitamin D level in serum is associated with either increased MS susceptibility or worsen disability progression among a sample of Lebanese MS patients living in the Bekaa region.

The heterogeneous clinical profile of the enrolled MS participants in this preliminary study resembles to certain extent to that of Indian and Kashmiri MS patients engaged in Bhatia and Zahoor studies (Zahoor et al., 2017; Bhatia et al., 2015) where the female to male ratio was 1.5:1 and 3.1:1 respectively and the age of the majority of MS participants ranged between 31 and 40 years.

Vitamin D, known as the modulator of calcium homeostasis, is believed to play a role in osteoporosis, resulting in decreased bone mineral density through the reduction of dietary calcium absorption (Howe and Dellavalle, 2007). It was also suggested that inadequate levels of 25(OH)D₃ play a role in the pathogenesis of several autoimmune diseases such as rheumatoid arthritis, autoimmune thyroid disease and MS (Gonzalez, 2010).

Worldwide, various studies tackled the association between vitamin D deficiency and MS. In this study, the mean level of 25(OH)D₃ in serum of MS patients was 14.7 ± 4.6 ng/ml which is significantly lower compared to control group that was 25.8 ± 9.1 ng/ml, indicating an association between vitamin D and MS development in the enrolled Lebanese MS patients living in the Bekaa region.

This result was in accordance with a case-control study suggesting that an increased level of vitamin D is associated with lower MS risk (Munger et al., 2012). Another systemic meta-analysis review consisting of 11 studies also verified that lower serum vitamin D level is associated with increased MS risk (Duan et al., 2014). Furthermore, a Mendelian randomized study showed a significantly strong correlation between genetically lowered vitamin D level and the increased susceptibility to MS (Mokry et al., 2015). However, a case-control

study on Moroccan MS patients illustrates a contradictory finding by showing that low vitamin D level is neither associated with increased MS risk nor with increased MS severity (Skalli et al., 2018). One study conducted on African Americans showed a lower level of vitamin D in serum of MS patients, with no correlation between vitamin D level and MS severity reflected through EDSS and MSSS scores (Gelfand et al., 2011). These results were partially in contradiction to our findings that showed a negative correlation between vitamin D level and each of EDSS, EDSS progression index and MSSS scores and that lower vitamin D may predict an increase in MSSS. A similar negative correlation was also established in Tasmanian (Van Der Mei et al., 2007) and Australian populations (Sundqvist et al., 2010). On the other hand, *CYP27B1* is considered as one of the vitamin D metabolizing gene (Zhuang et al., 2015) encoding 1-alpha-hydroxylase that catalyzes the hydroxylation of 25(OH)D₃ into 1,25(OH)₂D₃, the bioactive form of vitamin D (Ramagopalan et al., 2011). The association of several genetic variants within *CYP27B1* and the risk of MS development are currently under investigation. *CYP27B1* mutations are thought to reduce circulating levels of calcitriol indicating that a decrease level of bioactive vitamin D due to *CYP27B1* genetic variation can disrupt the genetic-environmental interactions needed for immune and nervous system development, resulting in a potential predisposition to MS development (Reinthal et al., 2014). Our preliminary study provides additional information on the consequence of genetic mutations within *CYP27B1* on MS risk development where five SNPs were analyzed: 1 in exon 3 (rs118204012, A>G, E189G), 1 in intron 6 (rs4646536, T>C, intronic variant) and 3 in exon 7 (rs118204009, G>A, R398H; rs118204010, C>G/T, R389G/C; rs28934607, C>T, P382S). However, no association between the studied SNPs and MS risk was observed among patients. Similarly, rs118204009 was not recorded as a genetic risk factor for MS development among Australian (Reinthal et al., 2014), Han Chinese (Zhuang et al., 2015), Italian and Belgian (Barizzzone et al., 2013) populations. This finding was also confirmed by a genome wide association study (Ban et al., 2013).

Table 3. Allele and genotype frequencies of *CYP27B1* studied SNPs in MS patients and controls

<i>CYP27B1</i> gene	SNP/Risk allele	Genotype and allele	MS patients	Controls	Risk vs. normal genotype or allele	P-value
Exon 3	Rs118204012/G	AA	28 (100%)	28 (100%)	(GG+GA) vs. AA	1.000
		A	28 (100%)	28(100%)	G vs. A	
Intron 6	Rs4646536/C	TT	19 (67.9%)	14 (50%)	(CC+CT) vs. TT	0.174
		TC	9 (32.1%)	12 (42.9%)		
		CC	0 (0%)	2 (7.1%)		0.174
		T	9 (32.1%)	14 (50%)		
		C	19 (67.9%)	14 (50%)	C vs. T	
Exon 7	Rs118204009/A	GG	28 (100%)	28 (100%)	(AA+AG) vs. GG	1.000
		G	28 (100%)	28 (100%)	A vs. G	
	Rs118204010/G/T	CC	28 (100%)	28 (100%)	(GG+GC) vs. CC	1.000
		C	28 (100%)	28 (100%)	G/T vs. C	
	Rs28934607/T	CC	28 (100%)	28 (100%)	(TT+TC) vs. CC	1.000
		C	28 (100%)	28 (100%)	T vs. C	

$P \leq 0.05$ was considered statistically significant. MS: Multiple sclerosis

The contribution of *CYP27B1* in MS is not well established since published works provide contradictory result. Rs118204009 genetic variant was shown to significantly increase MS risk among populations in Canada and the United Kingdom (Traboulsee and Vilariño-Güell, 2016; Ramagopalan et al., 2011). Another study performed on Australian and New Zealand populations confirmed the role of *CYP27B1* in MS confirming the association of 3 SNPs including rs4646536 with increased MS risk (Sundqvist et al., 2010).

Conclusion

As a conclusion, our study revealed an association between vitamin D deficiency and MS by boosting its risk and severity. It provided additional evidence that the studied SNPs of *CYP27B1* have no role in MS development in Lebanese MS patients.

Acknowledgments

The authors like to thank the laboratory medical staff in Al-Abdallah hospital-Riyak, especially Dr. Hassan Al-Abdallah, Mrs. Diala Shalhoub and

Siham Mazbough for facilitating blood samples collection.

Conflict of Interest

The authors state that there is no conflict of interest.

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