Evaluation of a panel of polymorphisms in vitamin D-related genes, vitamin D status and Multiple Sclerosis

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ABSTRACT

Introduction: the role of hypovitaminosis D as risk factor for Multiple Sclerosis (MS) is well known. Vitamin D status is the result of the interaction between environmental and genetic factors. Several single nucleotide polymorphisms (SNPs) in genes codifying for molecules involved in vitamin D pathway have been associated with an increased risk of MS. However, few studies evaluated the association of these SNPs with MS severity. The aim of this study was to investigate the association among a panel of vitamin D-related SNPs, vitamin D *status*, and MS severity.
Methods: one hundred MS patients were enrolled in the study. Serum 25(OH)D_3 levels and genotyping of SNPs in vitamin D-related genes were evaluated in all patients by high-performance liquid chromatography or real-time polymerase chain reaction. MS severity was assessed by Multiple Sclerosis Severity Score (MSSS).
Results: three SNPs of the *NADSYN1* gene, namely rs3829251, rs7944926 and rs12785878, and the rs2248137 SNP of the *CYP24A1* gene were significantly associated with 25(OH)D_3 levels. However, neither serum 25(OH)D_3 levels nor the SNPs of the *NADSYN1* or of the *CYP24A1* genes were associated with disease severity.
Discussion: in this study, we assessed the hypothesis that the presence of SNPs in vitamin D-related genes could influence MS severity. However, the statistical analysis indicates that there is no correlation between the severity of the disease and the polymorphisms considered.

Keywords: *gene, multiple sclerosis, vitamin D*

INTRODUCTION

Multiple sclerosis (MS) is a chronic autoimmune neurodegenerative disease of the central nervous system (CNS), characterised by demyelination and axonal degeneration, which lead to progressive irreversible disability (1). It is a multifactorial disease resulting, from the interaction among environmental, genetic, and epigenetic factors. The role of hypovitaminosis D as risk factor for MS is well known and supported by several literature evidence (2-5).

Noteworthy, vitamin D status is influenced by both environment, such as sun exposure, and genetic. Genome-wide association studies and case control studies found several single nucleotide polymorphisms (SNPs) in vitamin D-related genes associated with an increased risk of MS (3,6). However, only a few studies investigated the potential role of such SNPs on the MS severity.

The aim of this study was to assess the association among SNPs in vitamin D-related genes, vitamin D *status* and MS severity.

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METHODS

This observational study was performed at the University Hospital of Palermo, Italy. We enrolled one hundred patients with MS from Neurology Unit the Unit of Neurology. The MS diagnosis was made by an experienced neurologist based on a previous the history of the disease, physical examination, cerebrospinal fluid analysis, and magnetic resonance imaging findings, according to the revised McDonald criteria (7). The neurological status of patients was assessed using Kurtzke's Expanded Disability Status Scale (EDSS). The progression of disability was assessed using the Multiple Sclerosis Severity Score (MSSS) (8). The annualized relapse rate (ARR) was calculated in the year prior to the genotyping.

The study protocol was approved by the Ethics Committee of the University Hospital of Palermo (nr 07/2016) and was performed in accordance with the current revision of the Helsinki Declaration. Informed consent was obtained from all patients included in the study.

All laboratory analyses were performed at the Institute of Clinical Biochemistry, Clinical Molecular Medicine and Clinical Laboratory Medicine, Department of Biomedicine, Neurosciences, and Advanced Diagnostics.

25-hydroxy-vitamin D_3 [$25(OH)D_3$] levels were measured by high-performance liquid chromatography (HPLC) using the Chromosystem reagent kit (Chromsystems Instruments & Chemicals GmbH; Grafelfing, Munich, Germany) and a chromatographic system equipped with a Waters 1525 Binary HPLC pump connected to a photodiode array detector; detection was carried out at 265 nm. Chromatographic separation was performed as follows: C18 analytical column, column temperature 25 "C, flow rate 0.7 mL min^{-1}, wavelength 265 nm and sample injection volume 50 mL. Chromatographic separation was performed with isocratic elution with a retention time of 4.2 min. In accordance with the manufacturer's instruction, a serum $25(OH)D_3$ concentration of 30 µg/L was considered the threshold value for identifying low levels of vitamin D.

Genomic DNA was extracted from 200 µL of whole peripheral blood using a commercial kit (Qiagen, Valencia, CA, USA), according to the manufacturer. The DNA quality was evaluated by electrophoresis in a 0.8% agarose gel, quantified by using absorbance spectrophotometric analysis and stored at -20 °C for subsequent analysis. We selected the following 18 SNPs in genes codifying molecules involved in the vitamin D pathway: *NADSYN1* (rs3829251, rs7944926, and rs12785878), *CYP2R1* (rs10766197 and rs10741657), *VDBP* (rs7041 and rs4588), *VDR* (rs1544410, rs7975232, rs731236, rs2228570), *RXR-α* (rs9409929 and rs12004589), *KLOTHO* (rs9536314 and rs1207568), *CYP24A1* (rs2762939 and rs2248137), and *CYP27A1* (rs17470271) (Table 1).

Table 1
Characteristics of vitamin D-related Single Nucleotide Polymorphisms

Gene	Chromosome	SNP	Ancestral allele	Substitution allele
NADSYN1	11	rs3829251	G	A
		rs7944926	G	A
		rs12785878	G	T
CYP2R1	11	rs10766197	G	A
		rs10741657	G	A
VDBP	4	rs7041	G	T
		rs4588	C	A
VDR	12	rs1544410 (Bms-I)	B	b
		rs7975232 (Apa-I)	A	a
		rs731236 (Taq-I)	T	t
		rs2228570 (Fok-I)	F	f
RXR-α	9	rs9409929	G	A
		rs12004589	G	A
KLOTHO	13	rs9536314	T	G
		rs1207568	G	A
CYP24A1	20	rs2762939	G	C
		rs2248137	G	C
CYP27A1	2	rs17470271	T	A

NADSYN1, NAD Synthetase 1; CYP, cytochrome P450; VDBP, Vitamin D Binding Protein; VDR, Vitamin D Receptor; RXR- α, Retinoid X Receptor-α; SNP, single nucleotide polymorphisms.

SNPs in *NADSYN1*, *CYP2R1*, *CYP24A1*, *CYP27A1*, *RXR-α*, and *KLOTHO* genes were analysed by real-time allelic discrimination TaqMan assay on a 7500 real-time polymerase chain reaction (PCR) system (Applied Biosystems), as previously described (9-12). SNPs in VDR and VDBP genes were assessed by PCR followed by restriction fragment length polymorphism (RFLP) analysis, as previously described (13-14).

Statistical analysis was performed by SPSS version 17.0 (SPSS Inc., Chicago, IL, USA) and R Language v.3.6.1 (R Foundation for Statistical Computing, Vienna, Austria). Quantitative variables were expressed by the median, interquartile range (IQR) and min-max range, while categorical variables by relative frequencies. Differences between groups were evaluated by nonparametric Kruskal-Wallis or Mann-Whitney test, with Bonferroni's correction when needed. Association was evaluated by nonparametric Spearman's rank correlation coefficient and the Fisher's exact test.

RESULTS

One hundred MS patients (F:M 75:25%), with a median (IQR) age of 39 (32-47) years, were investigated. 82% and 14% displayed, respectively, a Relapsing-Remitting (RRMS) or a Secondary Progressive Multiple Sclerosis (SPMS) form of the disease. EDSS ranged between 0 and 7.5 with median (IQR) of 2.25 (1.4-5.0). Median (IQR) MSSS and median (IQR) ARR were, respectively, 3.34 (1.45-5.48) and 1 (1-2). Median (IQR, min-max) levels of vitamin D were 20 (16-25, 13-44) µg/L, with up to 87% of the subjects investigated displaying a vitamin D concentration <30 µg/L.

The association between vitamin D levels and polymorphisms was evaluated. Heterozygous carriers (n=25) of the substitution A allele for the rs3829251 SNPs of the *NADSYN1* gene displayed significantly lower vitamin D levels than homozygous carriers (n=75) of the ancestral G allele (19 *versus* 22 µg/L, p=0.024). Homozygous A carriers were not present in the population investigated. Similarly, heterozygous carriers (n=52) of the substitution A alleles for the rs7944926 SNPs of the *NADSYN1* gene displayed significantly lower vitamin D levels than homozygous carriers (n=43) of the ancestral G allele (19 *versus* 24 µg/L, Bonferroni's correction p=0.024). No significant difference was instead evident between A homozygouses (n=5) and G homozygouses (n=43) (24 *versus* 24 µg/L, p=1.000) or between A homozygouses and heterozygouses (24 *versus* 19 µg/L, p=0.822). An association was also found between the rs12785878 SNP of the *NADSYN1* gene and vitamin D levels (KW test, p=0.047): T homozygouses (n=44) displayed marginally significant higher vitamin D levels than heterozygouses (n=51) (23 *versus* 19 µg/L, Bonferroni's correction p=0.051), whereas no other significant *post-hoc* comparison was found. Moreover, G homozygouses (n=5) displayed similar vitamin D levels than T homozygouses (24 *versus* 23 µg/L). Among the other SNPs, the substitution allele C of the rs2248137 SNP of the *CYP24A1* gene was found to be associated with vitamin D levels (KW, p=0.026). In particular, C homozygouses (n=38) displayed significant lower vitamin D levels than heterozygouses (n=45) (19 *versus* 24 µg/L, Bonferroni's correction p=0.036). No other difference resulted to be statistically significant, with C homozygouses showing similar vitamin D levels than G homozygouses (n=17) (19 *versus* 20 µg/L). No other SNPs were found significantly associated to vitamin D. The rs3829251, rs7944926 and rs12785878 SNPs of the *NADSYN1* gene were significantly associated (p<0.001), while no SNP of the *NADSYN1* gene was associated to the rs2248137 SNP of the *CYP24A1* gene. Interestingly, no significant correlation was found between vitamin D and EDSS (rho=-0.091; p=0.403), MSSS (rho=-0.159; p=0.146) or ARR (rho=-0.097; p=0.373). Accordingly, no association was found between the rs3829251, the rs7944926, the rs12785878 SNPs SNPs of the *NADSYN1* gene or the rs2248137 SNP of the *CYP24A1* gene and EDSS, MSSS or ARR.

DISCUSSION

Multiple sclerosis is a complex disease and genetic greatly contributes to its pathogenesis. It is well known that MS is not a monogenic disorder, but several SNPs contribute to the risk of developing it. Noteworthy, each individual SNP contributes little to the MS risk but their combination significantly influences it. Among all investigated SNPs, some Authors found a role for genetic variants related to vitamin D metabolism. The latter is characterised by several steps and, consequently, several enzymes and proteins are involved (15-17). Briefly, vitamin D_3 is mainly synthesised in the skin from 7-dehydrocholesterol (7-DHC), which represents a common substrate between cholesterol and vitamin D. The enzyme NADSYN1 is involved in the regulation of the availability of 7-DHC for vitamin D_3 synthesis. The activation of Vitamin D3 depends on two hydroxylation reactions. The first occurs in the liver and lead to the conversion of vitamin D_3 in $25(OH)D_3$, which represents the main circulating form of vitamin D_3. It is biologically inactive and it has a long half-life. The second hydroxylation catalyses the conversion of $25(OH)D_3$ into $1,25(OH)_2D_3$, which represents the active form of vitamin D_3. This hydroxylation takes place in different tissues, organs, and cells, including kidneys, CNS, and immune cells. CYP2R1 has a key role in the first step of hydroxylation. Also, CYP27A1 contributes to the hydroxylation of vitamin D_3 but to a lesser extends than CYP2R1. $1,25(OH)_2D_3$ exerts its biological function through the interaction with a cytoplasmic complex, consisting of VDR and RXR-α. Such trimeric complex migrates to the nucleus and regulates positively or negatively the expression of genes harbouring vitamin D responsive element (VDRE) in their promoter region. Finally, Klotho is an important regulator of vitamin D homeostasis. The inactivation of $1,25(OH)_2D_3$ is catalysed by CYP24A1, which converts it into 24-hydroxylated products, inactive metabolites.

SNPs in vitamin D-related genes were previously

associated with MS susceptibility. In this study, we investigated the potential relationship among vitamin D status, vitamin D-related SNPs and MS severity. We did not find an association between vitamin D levels, NADSYN1 or CYP24A1, and MS severity.

Our findings are in accordance with other Authors that also failed to find an association between polymorphisms in several genes, including vitamin D-related ones, and MS severity (18-20). Moreover, despite our data have shown significant association between some SNPs and vitamin D, the finding of similar vitamin D levels displayed by homozygouses for the ancestral allele and homozygouses for the substitution allele, seems to suggest the presence of spurious associations. To date, the contribution of genetic to MS disability progression is far to be clarified. It can be hypothesised that different factors can guide the susceptibility and severity of the disease.

This study has some limitations, including the small sample size and the study design. Indeed, we used a candidate gene approach and chose previously-identified common genetic variants. The strength is that we evaluated several vitamin D-related SNPs for each patient.

CONCLUSION

In this study, we first evaluate the possible influence of several SNPs in vitamin D-related genes on MS severity. Although we did not find any association between vitamin D levels, SNPs and MS progression, further studies are required to confirm such findings.

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