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Protective association of VDR gene polymorphisms and haplotypes with multiple sclerosis patients in Egyptian population

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Abstract

Background: Hypovitaminosis D is one of the hazardous factors for multiple sclerosis (MS) and can be attested by expanding clinical studies. We aimed to study vitamin D receptor (VDR) gene polymorphisms: FokI, BsmI, ApaI, TaqI, and Tru9I genotype; frequency; haplotype structure; and linkage disequilibrium (LD) in MS Egyptian patients. The study was conducted on 50 MS patients and 50 healthy controls of matching age and sex. Alleles and genotype variants of VDR gene SNPs were analyzed by PCR using the restriction fragment length polymorphism (RFLP). Haplotype and linkage disequilibrium analysis based on the five genetic loci was studied on the detected genotypes.

Results: Frequency of FokI genotype (Ff+ff) was significantly higher in healthy controls (50%) compared to MS (28%) ($P = 0.03$), and "f" allele was significantly associated with the control group (OR = 0.42, CI = 0.26–0.85, $P = 0.015$). Frequency of ApaI genotype (Aa+aa) was significantly higher in MS (60%) ($P = 0.002$), and "a" allele was significantly associated with MS cases (OR = 2.47, CI = 1.25–4.88, $P = 0.008$). TaqI allelic distribution showed significant association of "t" allele with control group (OR = 0.55, CI = 0.31–0.98, $P = 0.04$). Statistically significant LD was detected between BsmI and ApaI in controls and MS ($D' = 0.89$ and $P < 0.001$, and $D' = 0.52$ and $P < 0.001$), respectively. Odd ratios of "fAt" and "BAt" were 0.2 (95% CI = 0.06–0.66) and 0.43 (95% CI = 0.19–0.97), respectively, suggesting that MS risk was 5 times and 2.3 times lesser, respectively, for haplotype carriers compared to non-carriers.

Conclusion: The study findings suggest that VDR gene variants "f," "A," and "t" alleles as well as VDR gene haplotypes "BAt" and "fAt" may have a protective effect against MS disease in Egyptian population.

Keywords: Haplotype structure, Linkage disequilibrium, Multiple sclerosis, PCR-RFLP, VDR-SNPs

Background

Multiple sclerosis (MS) is an immunoinflammatory and neurodegenerative disease caused by autoimmune reaction to the central nervous system (CNS) [1]. These inflammatory insults result in MS symptoms such as optic nerve damage and motor difficulty [2]. MS is a multifactorial disease that is reliant on both genetic and environmental susceptibility [2–4]. In the perspective of substantial results from experimental work, reliable epidemiological information, and encouraging clinical findings, the theory that hypovitaminosis D as one of MS risk factors has quickly gained support and can, before long, be affirmed by more broad clinical studies. Vitamin D (VD) role in MS pathogenesis was highlighted in several studies that showed decreased level of active form of VD in initial and serious phases of MS [5–8]. Genetic variations in vitamin D receptor (VDR) gene as single nucleotide polymorphisms (SNPs) might alter VD-VDR pathway causing disturbance of VD immune-regulatory functions which consequently is reflected on MS risk [9–12]. Most of genomic studies in MS pathogenesis suggested that no single gene locus predisposed to MS

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disease and multiple genes may have an impact on MS pathogenesis, risk, and presentation. The currently studied VDR polymorphisms include FokI polymorphism (rs10735810) in the 5′ promoter area and are referred to as start codon polymorphism (SCP) [13]. Presence of FokI restriction enzyme site results in the variant "f" allele and translation of a 3 amino acid longer VDR protein, while the "F" allele, the wild type, produces shorter VDR protein and is associated with an increased transcriptional activity [13]. Other VDR SNPs are located in the 3′UTR and defined by the restriction enzymes as BsmI polymorphism (rs1544410) A/G in intron 8, ApaI polymorphism (rs7975232) G/T in intron 8, TaqI polymorphism (rs731236) T/C in exon 9, and Tru9I polymorphism (rs757343) G/A in intron 8 [6–8, 14–18]. Considering each SNP site independently may not uncover critical impacts or clarify variations of numerous endeavors to associate VDR genotype with MS [7, 19–22]. Hence, our aim was to study the genotype and frequency of VDR gene polymorphisms: FokI, BsmI, ApaI, TaqI, and Tru9I, in MS Egyptian patients. Additionally, we analyzed the haplotype structure and possible genetic linkage disequilibrium (LD) of the studied loci. To our knowledge, no previous studies have covered those genetic aspects in MS Egyptian population, and we believe it may help in establishing clinical registries for future studies.

Subjects and methods

Subjects

One hundred subjects were recruited in this study and divided into 2 groups: group I, 50 MS patients that were diagnosed according to McDonald criteria [23], and group II, 50 healthy subjects of matching age and sex were included as a control group. All patients were subjected to thorough history taking, clinical, and neurological examination. The study has been carried out following the approval of the Ethics Committee of the Faculty of Medicine, Alexandria University, in accordance with Helsinki Declaration (reference number: 0101952). Informed consent to participate in the study was obtained from all participants.

VDR gene polymorphisms genotyping by PCR-RFLP

Genomic DNA was extracted from EDTA-peripheral blood samples obtained from both groups using Thermo Scientific GeneJET Whole Blood Genomic DNA Purification Mini Kit (USA #K0781) following the manufacturer's recommendations. The extracted DNA was stored at − 20 °C till used. The targeted SNP was identified by PCR-RFLP where the targeted SNP was amplified by conventional PCR followed by restriction digestion. SNP primer sequences, PCR thermal profiles, and expected amplicon size following restriction digestion are summarized in Table 1. All VDR polymorphisms were named according to their corresponding restriction enzymes, using the commonly used BAT nomenclature of VDR (*NCBI* official nomenclature, reference sequence AY342401.1, GI: 32891816) [24]. The presence of the restriction site was indicated with small letters, and its absence was indicated with capital letters. Only in Tru9I, its presence was indicated by "u" letter and absence by "U" (Table 2).

PCR-RFLP reactions were performed in a 50-μl final volume; 25 μl Thermo Scientific Dream Taq green PCR master mix, 2 μl of forward and reverse SNP primers (Sigma Aldrich, USA), 10 μl genomic DNA, and 11 μl DNase-free water and PCR protocols designed to the corresponding SNP were applied as in Table 1. The amplicons were examined by 3% agarose gel

Table 1 Primers sequences and PCR thermal profile of VDR gene polymorphisms

SNP	Primers sequences and PCR thermal profiles	Amplicon (bp)
FokI	F 5′-AGC TGG CCC TGG CAC TGA CTC TGC TCT-3′ R 5′-ATG GAA ACA CCT TGC TTC TTC TCC CTC-3′ *Initial denaturation: 95 °C for 10 min; 35 cycles: 94 °C for 30 s, 60 °C for 20 s, and 74 °C for 50 s; and final extension: 74 °C for 5 min	265
BsmI	F 5′-GGG AGA CGT AGC AAA AGG-3′ R 5′-AGA GGT CAA GGG TCA CTG-3′ *Initial denaturation 94 °C for 3 min; 32 cycles:94°C for 45 s, 60 °C for 45 s, and 72 °C for 45 s; and final extension: 72 °C for 5 min	358
ApaI and TaqI	F 5′-CAG AGC ATG GAC AGG GAG CAA-3′ R 5′-GCA ACT CCT CAT GGC TGA GGT CTC-3′ *Initial denaturation: 94 °C for 10 min; 35 cycles: 94 °C for 15 s, 55 °C for 30 s, and 72 °C for 30 sec; and final extension: 72 °C for 10 min	ApaI = 740 TaqI = 495, 245
Tru9I	F 5′-TGA GGT TTC TTG CGG GCA GGG TA-3′ R 5′-CAG GGC CGC CCC TCT TTG GA-3′ *Initial denaturation: 95 °C for 3 min, 35 cycles: 95 °C for 30 s, 72 °C for 60 s, and 72 °C for 60 s; and final extension: 72 °C for 10 mins	212

NCBI official nomenclature, reference sequence AY342401, GI:32891816. *SNP* single nucleotide polymorphisms, *F* forward primer sequence, *R* reverse primer sequence, *bp* base pair

*: PCR amplification conditions for each SNP

Table 2 VDR gene polymorphism genotype and allele frequencies in the two study groups

SNP	MS ($n = 50$)		Controls ($n = 50$)		Significance	OR (95% CI)
	No.	%	No.	%		
FokI						
FF (wt)	36	72.0	25	50.0	$\chi^2 = 6.33^*$	0.42 (0.26–0.85)
Ff (ht)	14	28.0	22	44.0	$^{MC}p = 0.029^*$	
ff (mut)	0	0.0	3	6.0		
F	86	86.0	72	72.0	$\chi^2 = 5.91^*$	
f	14	14.0	28	28.0	$P = 0.015^*$	
BsmI						
BB (mut)	20	40.0	22	44.0	$\chi^2 = 1.24$	1.33 (0.76–2.32)
Bb (ht)	12	24.0	15	30.0	$P = 0.54$	
bb (wt)	18	36.0	13	26.0		
B	52	52.0	59	59.0	$\chi^2 = 0.99$	
b	48	48.0	41	41.0	$P = 0.32$	
ApaI						
AA (mut)	20	40.0	36	72.0	$\chi^2 = 11.103^*$	2.47 (1.25–4.88)
Aa (ht)	28	56.0	12	24.0	$^{MC}p = 0.002^*$	
aa (wt)	2	4.0	2	4.0		
A	68	68.0	84	84.0	$\chi^2 = 7.02^*$	
a	32	32.0	16	16.0	$P = 0.008^*$	
TaqI						
TT (wt)	24	48.0	18	36.0	$\chi^2 = 4.16$	0.55 (0.31–0.98)
Tt (ht)	20	40.0	18	36.0	$P = 0.13$	
tt (mut)	6	12.0	14	28.0		
T	68	68.0	54	54.0	$\chi^2 = 4.12^*$	
t	32	32.0	46	46.0	$P = 0.04^*$	
Tru9I						
UU (wt)	28	56.0	37	74.0	$\chi^2 = 4.80$	1.66 (0.82–3.35)
Uu (ht)	20	40.0	10	20.0	$^{MC}p = 0.11$	
uu (mut)	2	4.0	3	6.0		
U	76	76.0	84	84.0	$\chi^2 = 2.0$	
u	24	24.0	16	16.0	$P = 0.16$	

wt homozygote wild type, *ht* heterozygote mutated, *mut* homozygote mutated, *SNP* single nucleotide polymorphism, ^{MC}p Monte Carlo test, χ^2 chi-square test, *OR* odds ratio, *CI 95%* 95% confidence interval

*Significant at $P \leq 0.05$

electrophoresis to ensure appropriate amplification. The amplified PCR products were digested using the corresponding restriction enzymes, and the resulted RFLP products were analyzed by 3% agarose gel electrophoresis and visualized by Dolphin-Doc gel documentation system (Wealtec, USA). The restriction enzyme mixture reaction was 30 μl final volume and contained 17 μl nuclease-free water, 2 μl 10X FastDigest green buffer, 10 μl PCR product, and 1 μl FastDigest enzyme. Restriction enzymes were used following the manufacturer's instructions: FokI code number: #FD2144, BsmI code number: #FD0964, Tru9I code number #FD0984, ApaI code number #FD1414, and TaqI code number: #FD0674 (Thermo Scientific FastDigest, USA).

Statistical analysis

Statistical analysis was done using SPSS software package version 18.0 (SPSS, Chicago, IL, USA). Odds ratios (OR) are given with 95% confidence intervals (CI). Qualitative data were analyzed using the chi-square test and Monte Carlo to compare different groups. Normally distributed quantitative data were analyzed using

Student's *t* test. Parametric quantitative data were expressed using mean and standard deviation. *P* value was assumed to be significant at ≤ 0.05. Haplotype frequencies and distribution study were conducted using Haploview 4.2 software, and haplotypes were assembled as different combinations of the five VDR SNPs. Normalized linkage disequilibrium coefficient *D′* (*D′*)[25] values range from − 1 to 1, and values close to − 1 or 1 indicate high polymorphism linkage on one allele and were calculated using SPSS software [26].

Results

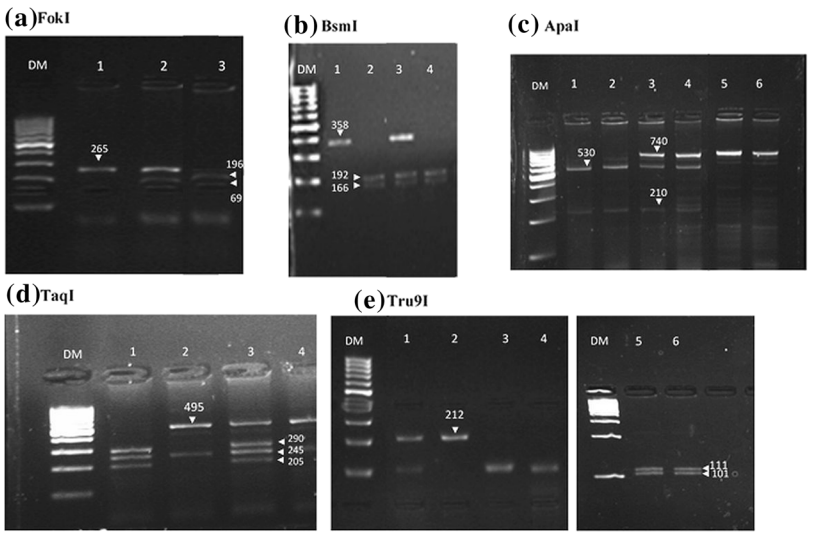
Demographic data

The studied cases were divided into group I (MS patients; *n* = 50, 22 males (44%) and 28 females (56%), age range 18–50 years (mean 30 ± 8 years)) and group II (healthy controls; *n* = 50, 18 males (36%) and females 32 (64%), age range 17–55 years (mean 32 ± 11 years)). Demographically, among MS patients, females were presented more than males with percentage of 56%. Studied MS patients were categorized according to clinical and neurological examination into relapsing/remitting MS (RRMS) which is characterized by clearly discrete attacks with neurological stability between attacks (*n* = 44 (88%)) and secondary progressive MS (SPMS) which usually starts as RRMS with progressive worsening of neurologic function (*n* = 6 (12%)), while primary progressive MS (PPMS) which is characterized by deteriorating neurologic function from the onset of symptoms without acute attacks or progressive relapsing MS (PRMS) which is characterized by steady functional deterioration with occasional attacks had no presentation among studied MS cases.

VDR gene polymorphism genotype and allelic distribution (Fig. 1, Table 2)

VDR gene polymorphisms were genotyped for all studied subjects, and the resulted RFLP products were visualized by 3% agarose gel electrophoresis (Fig. 1). Distribution of FokI, BsmI, ApaI, TaqI, and Tru9I genotypes with the respective allele frequencies and associations was analyzed in MS and controls (Table 2).

1. FokI genotype distribution in MS was as follows: FF (wild) = 72%, Ff (heterozygous) = 28%, and ff (mutant) = 0%, while in controls: FF = 50%, Ff = 44%, and ff = 6%. FokI genotype (Ff+ff) percentage was higher in controls 50% vs MS case 28% (*P* = 0.029) (Table 2). There was a statistically significant association of "f" allele with the control group compared to MS cases (OR = 0.42, 95% CI = 0.26–0.85, *P* = 0.015) (Table 2).
2. BsmI genotype distribution in MS was as follows: BB (mutant) = 40%, Bb (heterozygous) = 24%, and bb (wild) = 36%, while in controls: BB = 44%, Bb = 30%, and bb = 26%. No statistically significant difference was detected between MS cases vs controls with respect to BsmI genotype or allelic distribution (Table 2).
3. ApaI genotype distribution in MS was as follows: AA (mutant) = 40%, Aa (heterozygous) = 56%, and



SNP	genotype	No of bands	Size in bps
FokI	FF	1	265
	Ff	3	265, 196, 69
	ff	2	196,69
BsmI	BB	1	358
	Bb	3	358, 192, 166
	bb	2	192, 166
ApaI	AA	1	740
	Aa	3	740, 530, 210
	aa	2	530, 210
TaqI	TT	2	495, 245
	Tt	4	495, 290, 245, 205
	tt	3	290, 245, 205
Tru9I	UU	1	212
	Uu	3	212, 111, 101
	uu	2	111, 101

Fig. 1 PCR-RFLP based genotyping of VDR gene polymorphisms. VDR SNP was genotyped by PCR-RFLP (described in methodology), and the RFLP bands were visualized by 3% agarose gel electrophoresis. **a** FokI: lane 1: FF, lane 2: Ff, and lane 3: ff. **b** BsmI: lane 1: BB, lane 2, 4: bb, and lane 3: Bb. **c** ApaI: lane 1, 2: aa, lane 3, 4: Aa, and lane 5, 6: AA. **d** TaqI: lane 1: tt, lane 2: TT, and lane 3: Tt. **e** Tru9I: lane 1: Uu, lane 2: UU, lane 3, 4: uu, and lane 5, 6: DNA from lanes 3 and 4 were reloaded on 5% agarose gel for better resolution. DM, 100 bps DNA marker, size of the band in bps is written above the corresponding band with white arrowhead. VDR SNPs genotypes with the expected RFLP bands are summarized in a table (right-hand side)

aa (wild) = 4%, while in controls: AA = 72%, Aa = 24%, and aa = 4%. ApaI genotype (Aa+aa) percentage was higher in MS cases 60% vs control group 28% ($P = 0.002$) (Table 2). Statistically significant association was detected between "a" allele and MS cases (OR = 2.47, 95% CI = 1.25–4.88, $P = 0.008$) Table 2.

4. TaqI genotype distribution in MS was as follows: TT (wild) = 48%, Tt (heterozygous) = 40%, and tt (mutant) = 12%, while in controls: TT = 36%, Tt = 36%, and tt = 28%. No statistically significant difference was detected between MS cases vs controls with respect to TaqI genotype distribution (Table 2). However, there was a statistically significant association of "t" allele with the control group (OR = 0.55, CI = 0.31–0.98, $P = 0.042$) (Table 2).
5. Tru9I genotype distribution in MS was as follows: UU (wild) = 56%, Uu (heterozygous) = 40%, and uu (mutant) = 4%, while in controls: UU = 74%, Uu = 20%, and uu = 6%. No statistically significant difference was detected between MS cases and controls with respect to Tru9I genotype or allelic distribution (Table 2).

Haplotype analysis

In controls; haplotype analysis revealed statistically significant pairwise LD between FokI/ApaI ($D' = 0.39$, $P = 0.012$), FokI/TaqI ($D' = 0.47$, $P = 0.002$), BsmI/ApaI ($D' = 0.89$, $P < 0.001$), and BsmI/TaqI ($D' = -0.26$, $P = 0.017$) (Table 3). While in MS cases, statistically significant pairwise LD was detected between BsmI/ApaI($D' = 0.52$, $P < 0.001$) and Tru9I/ApaI ($D' = 0.26$, $P = 0.03$) (Table 3). Haplotype frequencies for the linked di- or tri-allelic SNPs were listed in Tables 4 and 5, respectively. We classified haplotypes into *non-protective haplotypes*, where when detected, suggested that carriers of the corresponding haplotypes would be at greater MS risk than non-carriers, and *protective haplotypes*, where when detected, suggested that carriers of the corresponding haplotypes would be at lower MS risk than non-carriers. Studying bi-allelic haplotypes (Table 4) showed that non-protective haplotypes were "*Ba*," "*ua*," *and* "*Fa*" with the ORs 10.55 (95% CI = 1.27–87.46), 5.07 (95% CI = 1.36–18.85), and 3.59 (95% CI = 1.45–8.94), respectively. This means that MS risk was 10.5 times, 5 times, and 3.6 times, respectively, greater for individuals carrying this haplotype compared to non-carriers. Protective haplotypes were "*fA*" and "*ft*" where ORs were 0.331 (95% CI = 0.124–0.884) and 0.23 (95% CI = 0.09–0.62), respectively, which means that MS risk was 3 times and 4.3 times lesser for these haplotype carriers compared to non-carriers. Structuring tri-allelic haplotypes (Table 5) showed two protective haplotypes: "*fAt*" and "*BAt*" haplotype with ORs 0.2 (95% CI = 0.06–0.66) and 0.43 (95% CI = 0.19–0.97), respectively, with MS risk 5 times and 2.3 times lesser for these haplotype carriers compared to non-carriers. No statistically significant associations were detected for haplotypes compiled as a combination of four or five SNPs (tables not included).

Table 3 Pairwise linkage disequilibrium of VDR gene polymorphisms

	Variant 1	Variant 2	*D'*	*P*
Controls	FokI	BsmI	0.213	0.11
	FokI	Tru9I	0.045	0.76
	FokI	ApaI	0.392*	0.012*
	FokI	TaqI	0.471*	0.002*
	BsmI	Tru9I	0.258	0.18
	BsmI	ApaI	0.894*	< 0.001*
	BsmI	TaqI	− 0.265*	0.017*
	Tru9I	ApaI	0.033	0.74
	Tru9I	TaqI	0.074	0.73
	ApaI	TaqI	0.389	0.07
MS	FokI	BsmI	− 0.099	0.68
	FokI	Tru9I	0.060	0.74
	FokI	ApaI	0.370	0.06
	FokI	TaqI	0.160	0.37
	BsmI	Tru9I	0.199	0.25
	BsmI	ApaI	0.519*	< 0.001*
	BsmI	TaqI	− 0.202	0.15
	Tru9I	ApaI	0.265*	0.03*
	Tru9I	TaqI	0.044	0.87
	ApaI	TaqI	0.081	0.42

D' normalized linkage disequilibrium coefficient *D* values normally range from − 1 to 1, where values close to − 1 or 1 indicate high polymorphism linkage on one allele

*Significant at $P \leq 0.05$

Discussion

Studying VDR gene polymorphisms in association with MS has provoked a large body of research with special interest to the studied race and population. It was concluded from our study that VDR gene variants "f," "A," and "t" alleles as well as VDR haplotypes "BAt" and "fAt" may have a protective effect against MS disease in Egyptian population. In our study, females represented most of the studied MS cases which agrees with general MS epidemiology [27, 28] as well as in a recent published survey conducted by Egyptian team on Egyptian MS patients [29]. In the recent years, females outnumbered men with MS which was related to increased levels of sphingosine-1-phosphate receptor 2 (S1PR2), a

Table 4 Association of biallelic haplotypes BsmI/ApaI, Tru9I/ApaI, FokI/ApaI, FokI/TaqI, and BsmI/TaqI in the two study groups

Haplotype	Haplotype frequencies (%)		OR (95% CI)
	MS ($n = 50$)	Controls ($n = 50$)	
BsmI/ApaI			
BA	0.44	0.58	1
Ba	0.08	0.01	10.55* (1.27–87.46)
bA	0.24	0.26	1.22 (0.62–2.4)
ba	0.24	0.15	2.11 (0.99–4.49)
Tru9I/ApaI			
UA	0.56	0.71	1
Ua	0.20	0.13	1.95 (0.89–4.26)
uA	0.12	0.13	1.17 (0.50–2.76)
ua	0.12	0.03	5.07* (1.36–18.85)
FokI/ApaI			
FA	0.62	0.65	1
Fa	0.24	0.07	3.59* (1.45–8.94)
fA	0.06	0.19	0.331* (0.124–0.884)
fa	0.08	0.09	0.932 (0.338–2.57)
FokI/TaqI			
FT	0.60	0.46	1
Ft	0.26	0.26	0.77 (0.39–1.492)
fT	0.08	0.08	0.77 (0.27–2.20)
ft	0.06	0.20	0.23* (0.09–0.62)
BsmI/TaqI			
BT	0.32	0.26	1
Bt	0.20	0.33	0.49 (0.23–1.05)
bT	0.36	0.28	1.05 (0.51–2.14)
bt	0.12	0.13	0.75 (0.29–1.92)

OR odds ratio, *95% CI* 95% confidence interval, *NA* not applicable

Table 5 Association of tri-allelic haplotypes in the two study groups

Haplotype	Haplotype frequencies (%)		OR (95% CI)
	MS ($n = 50$)	Controls ($n = 50$)	
BsmI/Tru9I/ApaI			
BUA	0.38	0.52	1
BUa	0.04	0	NA (NA)
BuA	0.06	0.06	1.37 (0.41–4.57)
Bua	0.04	0.01	5.47 (0.59–50.95)
bUA	0.18	0.19	1.30 (0.60–2.80)
bUa	0.16	0.13	1.68 (0.72–3.91)
buA	0.06	0.07	1.17 (0.36–3.77)
bua	0.08	0.02	5.47 (1.10–27.25)
FokI/ApaI/TaqI			
FAT	0.46	0.40	1
FAt	0.16	0.25	0.56 (0.26–1.19)
FaT	0.14	0.06	2.03 (0.71–5.78)
Fat	0.10	0.01	8.70 (1.07–70.93)
fAT	0.02	0.02	0.87 (0.117–6.46)
fAt	0.04	0.17	0.20 (0.06–0.66)
faT	0.06	0.06	0.87 (0.26–2.91)
fat	0.02	0.03	0.58 (0.09–3.65)
BsmI/ApaI/TaqI			
BAT	0.28	0.25	1
BAt	0.16	0.33	0.43 (0.19–0.97)
BaT	0.04	0.01	3.57 (0.37–34.11)
Bat	0.04	0	NA (NA)
bAT	0.20	0.17	1.05 (0.45–2.44)
bAt	0.04	0.09	0.40 (0.11–1.45)
baT	0.16	0.11	1.30 (0.51–3.32)
bat	0.08	0.04	1.79 (0.48–6.66)

OR odds ratio, *95% CI* 95% confidence interval, *NA* not applicable

blood vessel receptor protein that is expressed at higher levels by female brain tissue and controls the immune cells passage across brain blood vessels causing the inflammation that promotes MS [30].

In the present study, we analyzed VDR gene SNPs, FokI in the 5′ promoter area and BsmI, ApaI, TaqI, and Tru9I in the 3′UTR (three prime untranslated region), and their relation to MS in Egyptian patients for a better understanding of MS susceptibility. According to our data, statistically significant higher percent of FokI genotype (Ff+ff) was detected in the healthy controls compared to MS cases with statistically significant association of "f" allele with the control group indicating the possible association of "f" allele with decreased MS risk.

Our result is in accordance with other studies that demonstrated the association between "ff" genotype with reduced MS risk suggesting a role for FokI SNP in MS pathogenesis [3, 15]. Mamutse et al. reported an association between ff genotype and less MS disability outcome in patients diagnosed with MS for over 10 years [15]. However, Bettencourt et al.'s study detected ff genotype more in MS patients with no difference in disease course or disability progression, even though they could not rule out FokI role in MS [19]. Simon et al. showed no significant association between genotypes of FokI and MS disease [31]. Nevertheless, they observed an interaction between dietary intake of vitamin D and FokI polymorphism on MS risk, where the protective effect of increasing vitamin D supplement was apparent in women with the "ff" genotype with significant 80% reduced risk of MS [31]. They explained their findings in the context of variation in VDR protein functionality resulted from FokI polymorphism effect, where among women with increased target cell activity, any slight exposure to vitamin D might be enough to maintain

a healthy immunologic environment. Meanwhile, there were studies that could not detect any association between FokI and MS diseases [18, 20]. The study conducted by Smolders et al. showed an association between "F" allele and low 25(OH) D in patients and controls which highlighted FokI role in vitamin D metabolism in MS [18].

Changes in VDR protein sequence result in major functional impacts, as changes in DNA binding, transcriptional activation or heterodimerization, and hormonal ligand affinity [13, 32]. FokI SNP results in frameshift mutations and introduces premature methionine start codon which creates shorter/wild/F-VDR or a long/f-VDR proteins resulting in different VDR protein structure and function [13]. The short "F" isoform has been associated with a higher transcriptional activity and more efficient protein interaction [13, 33, 34]. van Etten et al. showed that VDR FokI polymorphism affected immune cell behavior as monocytes, dendritic cells, and lymphocytes with a more active immune system for the short F-VDR, highlighting its role in immune-mediated diseases [35, 36].

We expanded our study and included 3′UTR region VDR polymorphisms BsmI, ApaI, TaqI, and Tru9I and studied their frequencies and association with MS. 3′ UTR regulatory region affects mRNA stability and translational activity, which is important in understanding the functionality of sequence variations in the 3′UTR [37]. Our study showed statistically significant higher percent of ApaI genotype (Aa+aa) in MS compared to controls with statistically significant association of "a" allele with MS cases than with control group. For TaqI, although no statistically significant difference was detected between TaqI genotype in MS cases and controls, TaqI allelic distribution showed a statistically significant association of "t" allele with control group than with MS cases. No statistically significant differences were detected between MS cases or controls with respect to BsmI and Tru9I genotype or allelic distribution. Most of the published work agreed with part of our data and disagreed with others, keeping in mind these variations mainly correlated to the studied population. The study published by Cierny et al. on Slovaks showed an association between BsmI and MS risk, yet no association between ApaI or TaqI and MS was detected [7]. Another study conducted by a Turkish group concluded an association between FokI and MS, yet no association was detected between TaqI or ApaI and MS risk [22]. Studying the Greek population, being epidemiologically comparable to the Middle East and North Africa where Egypt belongs, showed no association between BsmI and MS [38]. The effect of allele variation between populations can be attributed to differences in environmental factors affecting these populations, e.g., exposure to ultraviolet irradiation and diet [12]. This may explain the significant association between some VDR polymorphisms and MS in certain populations, e.g., BsmI and ApaI polymorphisms in Japanese population [39], TaqI and ApaI polymorphisms in Australian population [20], and FokI, ApaI, and TaqI in Egyptian population according to our study.

Following the study of VDR polymorphisms, we thought it is important to understand how they relate to each other. Polymorphisms can be related genetically where the alleles are linked to each other and can be studied by linkage disequilibrium (LD), or biologically (functionally) and studied by analyzing alleles interaction that can enhance or reduce gene functional effects. Additionally, analyzing multiple polymorphisms and haplotypes may help to understand contradictory results from other studies.

In controls, our study demonstrated a statistically significant LD between FokI and both ApaI and TaqI, with nearly comparable *D*' strength. FokI is often considered an independent marker in the VDR gene showing no LD with any of the other VDR SNPs [40, 41]; however, Sweeney et al.'s study on colorectal cancer showed LD between FokI-BsmI and FokI-Poly (A) polymorphisms in Asians [42].

In the current study, haplotype structure analysis revealed that both BAt and fAt haplotypes were associated with controls more than MS cases, which means that MS risk was 2.3 and 5 times lesser, respectively, for individuals carrying any of these haplotypes compared with non-carrier. The identified haplotypes matched our detected LD and were distributed sensibly between the study group. Bsm-Apa-Taq haplotype can be considered the commonest studied haplotype in respect to VDR function [42–44]. Researches who studied bone mineral density demonstrated that BAt haplotype expressed better response than baT haplotype in improving bone mineral density in response to several treatments [42–44]. This was explained in terms of better mRNA stability and half-life, which would hypothetically result in higher quantities of VDR protein being translated in the target cells with preferred response to vitamin D effect. Thakkinstian et al. pointed in their study that baT was the most common haplotype for the VDR gene, regardless of ethnicity, followed by BAt and bAT in Caucasians, and bAT and BaT in Asians [43]. We identified BAt and fAt haplotypes as protective haplotypes from MS in Egyptians. Studying the four SNPs haplotype together, FokI, BsmI, ApaI, and TaqI showed that there was a trend of increased frequency of "fBAt" haplotype in controls than in MS cases, suggesting that this haplotype could be a protective haplotype, and on a bigger scale study, more definitive results can be concluded. Similar study investigated FokI, BsmI, ApaI, and TaqI haplotype in Korean population and found no significant association for BsmI and TaqI but observed that the haplotype "fbaT" was associated with a reduced colorectal cancer risk [45].

Conclusion

A corollary of this work is that studying several VDR gene polymorphic site variations is imperative to comprehend VDR functionality and increase the likelihood for distinguishing alleles contributing to common diseases risk. LD pattern and degree at each genomic region contrasted broadly, and the discrepancy seen between populations strengthens the requirement for characterizing haplotypes and LD of studied population. Establishing registries in Egypt to study the clinical course of the MS disease, safety and benefit of including vitamin D supplements in treatment protocols, and the epidemiology of MS in Egypt are highly recommended [29]. Further investigation is needed with the inclusion of more cases to avoid results that can be inflated by small samples or low frequencies of minor alleles.

Abbreviations

3′UTR: Three prime untranslated region; bp: Base pair; CI: Confidence intervals; CNS: Central nervous system; *D′*: Linkage disequilibrium coefficient *D′*; LD: Linkage disequilibrium; MS: Multiple sclerosis; OR: Odds ratios; PPMS: Primary progressive MS; PRMS: Progressive relapsing MS; RFLP: Restriction fragment length polymorphism; RPMS: Relapsing/remitting MS; S1PR2: Sphingosine-1-phosphate receptor 2; SCP: Start codon polymorphism; SNPs: Single nucleotide polymorphisms; SPMS: Secondary progressive MS; VD: Vitamin D; VDR: Vitamin D receptor

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Authors' contributions

AH conceived of the study and revised the manuscript draft. AD participated in conceiving the study and supervised the selection of MS cases and controls. DE supervised the molecular genetic studies and the statistical analysis and interpretation, and helped in drafting the manuscript. IT carried out the molecular genetic work and the statistical study and interpretation, and helped in drafting the manuscript. AF participated in the design and coordination of the study and the statistical analysis and interpretation, and drafted the manuscript. All authors read and approved the final manuscript and accepted the publication.

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Availability of data and materials

The data used or analyzed during the study are available from the corresponding author on reasonable request

Ethics approval and consent to participate

The study has been carried out following the approval of the Ethics Committee of the Faculty of Medicine, Alexandria University, in accordance with Helsinki Declaration (reference serial number: 0101952). Informed written consent to participate in the study was obtained from all participants.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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