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Association between Vitamin D Receptor Gene Polymorphism and Alzheimer's Disease

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Gezen-Ak, D., Dursun, E., Ertan, T., Hanağası, H., Gürvit, H., Emre, M., Eker, E., Öztürk, M., Engin, F. and Yilmazer, S. *Association between Vitamin D Receptor Gene Polymorphism and Alzheimer's Disease*. Tohoku J. Exp. Med., 2007, **212** (3), 275-282 —— Vitamin D_3 is known to be involved in neuroprotection and exert its neuroprotective effects by modulating neuronal calcium homeostasis and production of neurotrophins. The single nucleotide polymorphisms (SNP) in vitamin D receptor (VDR) gene which can influence the affinity of vitamin D_3 to its receptor may be related to neurodegenerative diseases and neuronal damage by altering the vitamin D-mediated pathways. In this study, our aim was to determine whether there is an association between VDR gene and late-onset Alzheimer's disease (AD) in order to see if vitamin D contributes to AD or not. One hundred and four cases of dementia of Alzheimer type and 109 age-matched controls were genotyped according to ApaI (a: + restriction site and A: no restriction site) and TaqI (t: + restriction site and T: no restriction site) sites in intron 8 and exon 9 of the ligand-binding site of VDR gene. When the controls and patients were compared for their ApaI genotypes, the frequency of the patients with Aa genotype was significantly higher than the frequency of the healthy individuals with the same genotype ($p = 0.008$, $\chi^2 = 9.577$, OR = 2.30). Thus, the "Aa" genotype may increase the risk of developing AD 2.3 times when compared with the "AA" genotype. On the other hand, the "AT" haplotype was significantly higher in controls ($p = 0.006$) indicating a protective role of the "AT" haplotype in AD. Consequently, this study provides evidence for a possible link between AD and vitamin D. ——— vitamin D; VDR; Alzheimer's disease; SNP; haplotype
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The most common form of degenerative dementia is Alzheimer's disease (AD) constituting approximately 60-70% of cases (Emilien et al. 2004). It is a chronic, degenerative, dementing illness with typically insidious onset (Rocca et al. 1991). The key aims in therapeutic strategies of AD are to decrease the neuronal damage, provide maintenance or regeneration of neurons

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(Tuszynski et al. 2002). Therefore, the candidate molecule capable of treating AD should have access to the mechanisms of survival and detoxification of neurons.

The active form of vitamin D, 1,25-dihydroxyvitamin D_3 (1,25 $[OH]_2 D_3$) is a steroid hormone and a nuclear transcription regulator acting via a nuclear hormone receptor (VDR). In addition to its role in the regulation of calcium and phosphate homeostasis and bone formation, vitamin D is also found to be involved in brain function and neuroprotection (Evans 1988; Ferrari et al. 1998; Garcion et al. 2002). Vitamin D is synthesized from 7-dehydrocholesterol by ultraviolet B radiation, in the skin (Bouillon et al. 1998). However, recent data showed that the central nervous system can locally perform bioactivation of the vitamin D prohormone (Garcion et al. 2002). The relation between vitamin D associated cellular pathways and neurodegeneration should be considered under three titles: 1. Oxidative stress prevention and 2. Neurotrophic factor regulation 3. Ca^{+2} homeostasis. Vitamin D is an antioxidant which up-regulates γ glutamyl transpeptidase activity and thus controls brain detoxification processes. It also regulates the synthesis of neurotrophic factors such as nerve growth factor (NGF), neurotrophin (NT) 3, NT 4, and glial cell-line derived neurotrophic factor (GDNF), which are important for neuron fate and neuronal survival (Wion et al. 1991; Neveu et al. 1994a; Jehan et al. 1996). On the other hand, calcium homeostasis affects many cellular mechanisms, especially neurotransmission. Vitamin D was found to alter calcium uptake in some excitable cells by modulating the L-type voltage sensitive calcium channels in hippocampus and by inducing the synthesis of calcium binding proteins (Cai et al. 1993; Landfield et al. 1998; Garcion et al. 2002).

Vitamin D deficiency and/or nuclear VDR polymorphisms, which affect the affinity of VDR to its ligand vitamin D, may be relevant to neuronal damage and neurodegenerative diseases by means of affecting oxidative stress, levels of neurotrophic factors and calcium homeostasis. Several linkage studies have reported that the chromosome 12, including VDR gene, has susceptibility loci for developing AD (Podulso et al. 2001). To our knowledge, only one study examined FokI polymorphism in the DNA-binding site of VDR gene and reported no association with the disease (Luedecking-Zimmer et al. 2003). Based on these biological and genetic backgrounds, we have focused on the ligand-binding site of the VDR gene in order to see if vitamin D contributes to AD or not and aimed at determining whether there is an association between VDR gene ApaI and TaqI single nucleotide polymorphisms (SNPs) and late onset AD.

Subjects and Methods

One hundred and four late-onset AD patients and 109 age-matched controls free from any neurodegenerative disorders (mean ages 75.1 ± 5.7, with age ranging from 65 to 94 and 73.6 ± 7.3 years, with age ranging from 65 to 90, respectively) were included in this study. Patients were diagnosed at Istanbul University, Cerrahpasa Faculty of Medicine, Department of Geropsychiatry and Istanbul Faculty of Medicine, Department of Neurology, Behavioral and Movement Disorders Unit according to DSM-IV criteria.

The subjects of this study were treated according to the World Medical Association Declaration of Helsinki ethical principles for medical research involving human subjects and the study was approved by Ethics Committee of Istanbul University, thus appropriate approval and procedures were used concerning human subjects.

DNA was extracted from 10 ml of K_3EDTA (Ethyle nediaminetetraacetic acid) treated peripheral blood samples by salting-out method. A 740-base pair (bp) fragment which includes intron 8 and exon 9 of the vitamin D receptor gene on chromosome 12 was amplified by polymerase chain reaction (PCR) with forward (5′-CAGAGCATGGACAGGGAGCAAG-3′) and reverse (5′-GCAACTCCTCATGG-CTGAGGTCTCA-3′) (Kohama et al. 2000) primers to detect ApaI and TaqI restriction sites (Fig. 1). PCR products were generated in a 25-μl reaction volume containing 50-100 ng of genomic DNA, 1 x PCR buffer, 1.8 mM $MgCl_2$, 200 μM of dNTP, 10 pmol/μl of each primer and 0.5 U of *Taq* DNA polymerase. PCR was performed as follows: incubation for 5 min at 94°C, 10 cycles of incubation for 20 sec at 94°C, 40 sec at 64°C, and 1 min at 72°C, 25 cycles of incubation for 20 sec at 94°C, 40 sec at 62°C, and 1 min at 72°C, followed by an extension step of 6 min at

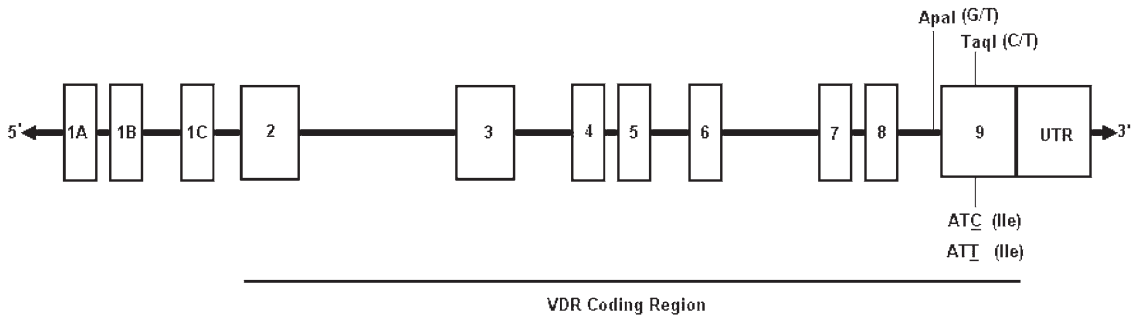

Fig. 1. ApaI and TaqI polymorphic sites on VDR gene. Boxes represent the exonic sites. Exon 2 to exon 9 is the VDR coding region which includes DNA binding site (exon 2 and 3) and ligand binding site (exon 4 – 9). ApaI restriction site is located in intron 8 and determines a G/T change (chromosome position 46525024). TaqI restriction site is located in exon 9 and determines a C/T change (chromosome position 46525104) (modified from Zmuda et al. 2000).

72°C (Gezen-Ak et al. 2005). To determine the presence of ApaI and TaqI restriction sites, Restriction Fragment Length Polymorphism (RFLP) was performed. Five μl PCR products were digested with 2 μl of DNase, RNase free water and 2 U of ApaI enzyme at 37°C and 2 U of TaqI enzyme at 66°C separately. Digestion products were analyzed in 1.5% agarose gel stained with ethidium bromide (Applichem, Darmstadt, Germany). All samples were tested twice by independent researchers. DNA fragments were visualized by ultraviolet illumination and fragment size was estimated by comparing with a ladder of 50 bp. The presence of ApaI restriction site causes splitting of the PCR product into two bands, 529 bp and 211 bp, was designated as *a*. When ApaI restriction site is not found in the corresponding sequence the PCR product remains as a 740 bp single band, was designated as *A*. The presence of TaqI restriction site causes splitting of the PCR product into three bands, 291 bp, 247 bp and 202 bp, respectively, was designated as *t*. If RFLP-associated TaqI restriction site is not found in the corresponding sequence, the PCR product splits into two bands, 493 bp and 247 bp respectively, was designated as *T*.

Statistical analysis were performed by UNISTAT 5,0® and SPSS 11® software. The distributions of alleles, genotypes and combined genotypes were statistically analyzed with Chi square test. All data were given as mean ± S.E. The data were considered significant at α = 0.05. Bonferroni correction which lowers the significance level (α_c value) in order to avoid a type I error performed for the p values of allele and haplotype assignments and the data were considered significant at $p < \alpha_c$ value. While determining risk (odds ratio estimates) of alleles and genotypes as independent variables and patients/controls as dependent variables, logistic regression was performed as well as Wald's confidence intervals (CI). An exact test for Hardy-Weinberg Equilibrium (HWE) was also performed. Haplotype analysis was performed by "Haploview 3.32" software with chromosome position of TaqI 46525024 and ApaI 46525104 taken as reference.

Results

The alleles of the controls for ApaI and TaqI polymorphisms were in HWE. The distribution of the ApaI genotypes was 33.7% for AA, 60.6% for Aa, 5.8% for aa in the patients and 50.5% for AA, 39.4% for Aa, 10.1% for aa in the healthy controls. There was a highly significant difference in the frequency of the Aa genotype in patients vs controls ($p = 0.008$, $\chi^2 = 9.577$, OR = 2.30) (Table 1).

TaqI genotype distribution was 36.5% for TT, 48.1% for Tt, 15.4% for tt in the patients and 48.6% for TT, 35.8% for Tt, 15.6% for tt in the healthy controls. Though it was not statistically significant, there was a slight increase in the frequency of the "Tt" genotype in patients vs controls ($p = 0.15$, $\chi^2 = 3.75$) (Table 2).

No significant difference was found for the distribution of both TaqI (T, t) and ApaI (A, a) alleles (p = 0.17, p = 0.20, respectively; α_c = 0.025).

The AATT combined genotype was observed with the highest frequency in healthy controls

Table 1. The distribution of VDR gene ApaI genotypes.

Group	Genotype		
	AA *n* (%)	Aa *n* (%)	aa *n* (%)
Patients	35 (33.7)	*63 (60.6)	6 (5.8)
Controls	55 (50.5)	43 (39.4)	11 (10.1)

*$p = 0.008$, $\chi^2 = 9.577$, OR = 2.30.

Table 2. The distribution of VDR gene TaqI genotypes.

Group	Genotype		
	TT *n* (%)	Tt *n* (%)	tt *n* (%)
Patients	38 (36.5)	50 (48.1)	16 (15.4)
Controls	53 (48.6)	39 (35.8)	17 (15.6)

$p = 0.15$, $\chi^2 = 3.75$.

($p = 0.0064, \chi^2 = 17.92; \alpha_c = 0.0071$) (Table 3).

The "Aa" genotype was found significantly higher in patients, in the logistic regression analysis of genotypes ($p = 0.006$, OR = 1.908, 95% CI 1.200-3.034). Additionally, "TT" genotype was also found to be associated with the disease. ($p = 0.012$, OR = 0.552, 95% CI 0.347-0.878) (Table 4).

When patient and control groups were compared for the frequency of VDR gene haplotypes, "AT" haplotype was found to be significantly increased in the controls ($p = 0.006, \chi^2 = 7.55; \alpha_c = 0.013$) (Table 5) and the LD between the two polymorphisms was 14.05 (D' = 0.911, r-squared = 0.233).

Table 3. The distribution of combined genotypes.

Group	Genotype						
	AATT *n* (%)	AATt *n* (%)	AAtt *n* (%)	AaTT *n* (%)	AaTt *n* (%)	aaTT *n* (%)	aaTt *n* (%)
Patients	3 (2.9)	16 (15.4)	16 (15.4)	30 (28.8)	33 (31.7)	5 (4.8)	1 (1.0)
Controls	*19 (17.4)	19 (17.4)	17 (15.6)	24 (22.0)	19 (17.4)	10 (9.2)	1 (0.9)

*$p = 0.0064$, $\chi^2 = 17.92$, $\alpha_c = 0.0071$ (Bonferroni adjustment), $p < \alpha_c$.

Aatt and aatt combined genotypes were excluded in the table as none observed in the subjects. The difference resulting from AATT was determined by proceeding Chi-square test. Reanalyzing the data after excluding AATT genotype values became $p = 0.33$, $\chi^2 = 5.78$.

Table 4. Logistic regression analysis of VDR genotypes.

VDR	Odds ratio estimates	95% Wald CI	χ^2	*p*
Genotypes				
ApaI				
AA	0.578	0.337-0.994	3.928	0.047
Aa	1.908	1.200-3.034	7.450	0.006
aa	1*			
TaqI				
TT	0.552	0.347-0.878	6.309	0.012
Tt	1.111	0.739-1.672	0.256	0.613
tt	1*			

1*, Referent estimate.

TABLE 5. The comparison of VDR haplotypes.

VDR haplotypes	Patients *n* (%)	Controls *n* (%)	Total *n* (%)	χ^2	*p* value
AT	53 (25.5)	83 (37.9)	136 (31.8)	7.55	*0.006
At	79 (38.0)	70 (32.3)	149 (35.1)	1.51	0.219
aT	73 (35.1)	63 (29.1)	136 (32.0)	1.77	0.183
at	3 (1.4)	2 (0.7)	5 (1.1)	0.51	0.473
Total	208 (100)	218 (100)	426 (100)		

*$\alpha_c = 0.013$ (Bonferroni adjustment), $p < \alpha_c$.

DISCUSSION

The genetics of the most common, late onset form of AD is more complex and less understood when compared with early onset AD. Apolipoprotein E (ApoE) is the only confirmed susceptibility gene for late onset AD. However, strong evidence indicates that additional risk genes exist on chromosome 12. Several studies supported this relation. (Blacker et al. 1998; Hollenbach et al. 1998; Luedecking-Zimmer et al. 2003; Bertram and Tanzi 2005). Poduslo et al. (2001) showed that placing the disease between the markers D12S364 and D12S78 including the VDR gene (12q12-q14) gave the highest Lod scores. Luedecking-Zimmer et al. (2003) focused on five candidate genes on chromosome 12 including VDR gene and found no relation with FokI polymorphism in DNA-binding site of VDR gene.

This present study, examined the association between ApaI and TaqI polymorphisms in the ligand binding site of VDR gene and late onset AD, for the first time. We found a highly significant association between ApaI polymorphism but no association between TaqI polymorphism and the AD. Thus, we suggest that the polymorphism in the ligand binding site of the VDR gene increases the risk for AD development. Our findings are also in correlation with studies that show the wider biological role for vitamin D in brain (Chatterjee 2001; Langbug et al. 2001; Garcion et al. 2002; Eyles et al. 2003). Those studies demonstrated that vitamin D can act on nervous system by modulating the expression of neurotrophins (Neveu et al. 1994a, b; Naveilhan et al. 1996; Garcion et al. 2002).

It has been demonstrated that the absence of vitamin D results in decreased NGF expression in the brains of neonatal rats (Eyles et al. 2003). Some studies also showed the role of vitamin D as a potent differentiation agent which reduces mitosis and induces the neurite outgrowth by upregulating NGF synthesis (Neveu et al. 1994a, b; Naveilhan et al. 1996; Comet et al. 1998; Musiol et al. 1997; Brown et al. 2003).

Some neurotrophins such as NGF show their neuroprotective effect by maintaining the calcium homeostasis (Mattson and Cheng 1993). In addition, this regulation can be upgraded by vitamin D via regulating NGF synthesis and expression of calcium channels and calcium binding proteins. While vitamin D shows its neuroprotective effect by reducing the expression of L-type voltage-sensitive calcium channels (L-VSCCs) in brain, an increase in L-VSCCs is demonstrated to be associated with aging and neurodegeneration (Brewer et al. 2001). The effect of vitamin D on the calcium homeostasis depends not only on L-VSCCs but also on calcium binding proteins such as calbindin, parvalbumin and calrectin (Feldman and Christakos 1983; Lee et al. 1987; Alexianu et al. 1998; Brewer et al. 2001; Bastianelli 2003).

Vitamin D might show its neuroprotective effect also by controlling brain detoxification processes (Wiseman 1993; Garcion et al. 1997, 1999; Wang et al. 2001; Garcion et al. 2002).

Expression of 1α-hydroxylase (1α-OHase) which catalyses conversion of 25-dihydroxyvita-

min D_3 to 1,25-dihydroxyvitamin D_3 and VDR was shown in both neuronal and glial cells of most brain regions of human and rat. Moreover it is important to see the expression of VDR and 1α-OHase located particularly in regions CA1 and CA2 of hippocampus, which is more vulnerable in Alzheimer's disease and substantia nigra in Parkinson's disease (Prufer et al. 1999; Ebadi and Pfeiffer 2005; Eyles et al. 2005). Supporting results implying a reduction in the VDR mRNA levels in the CA1 and CA2 pyramidal cells of hippocampus in AD patients have been shown (Sutherland et al. 1992).

Vitamin D deficiency and/or VDR polymorphisms, which may decrease the affinity of VDR to vitamin D (Cai et al. 1993; Bouillon et al. 1998), may affect detoxification mechanisms, calcium homeostasis and expression of neurotrophins. This may lead to neuronal aging, neuronal damage and neurodegeneration via other genomic and environmental factors.

Our data indicate that Aa genotype increases the risk factor 2.3 times for developing AD, when compared with the AA genotype. This intronic polymorphism of ApaI site might be relevant to the disease under three conditions. The polymorphism might be biologically relevant to the disease or it might be in linkage disequilibrium with biological relevant variability elsewhere in the VDR gene or it might be in disequilibrium with genetic variability in another adjacent gene (Johnson et al. 2005). On the other hand VDR acts as a homodimer or heterodimer, and the regions within DNA and ligand binding domains are also important dimerization interfaces (Issa et al. 1998). When two different alleles of ApaI cause an alteration in the splicing process, the VDR products of different alleles (A and a) may create disequilibrium in the homodimerization. Another supporting result for these suggestions is the distribution of combined genotypes in our study. AATT combined genotype was found to be the most frequent genotype in healthy controls indicating a significant difference from the patient group, suggestive of a protective effect on AD. Although it was statistically insignificant, the frequency of AaTt genotype was higher in the patients than the controls. On the other hand, comparison of VDR gene haplotypes showed that the frequency of "AT" haplotype was significantly increased in the controls indicating a protective role of "AT" haplotype of VDR gene in AD.

The reason for this kind of association might be the decrease in the effect of vitamin D resulted from the polymorphisms on its receptor, and this may lead to susceptibility to neurodegenerative diseases together with the other environmental and genetic factors.

The present study shows an association between the VDR gene polymorphisms in the ligand-binding site and late onset Alzheimer's disease. Consequently, this study provides evidence for a probable link between AD and vitamin D.

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