Title:

Investigation of vitamin D receptor polymorphisms in amyotrophic lateral sclerosis

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Key words: amyotrophic lateral sclerosis, ApaI, neurogenetics, VDR gene

Abbreviated title: VDR gene polymorphisms in ALS

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Abstract

BACKGROUND:

Amyotrophic lateral sclerosis (ALS) patients manifest aberrations in the vitamin D–endocrine system, with a vitamin D deficiency.

Genetic investigations have identified those proteins which link vitamin D to ALS pathology: major histocompatibility complex class II molecules, toll-like receptors, poly(ADP-ribose) polymerase-1, haeme oxygenase-1, the reduced form of nicotinamide adenine dinucleotide phosphate and calcium-binding proteins. Vitamin D additionally impacts ALS through cell-signalling mechanisms: glutamate, matrix metalloproteinases, the Wnt/β-catenin signalling pathway, mitogen-activated protein kinase pathways, prostaglandins, reactive oxygen species and nitric oxide synthase, but its role has been only poorly investigated.

OBJECTIVE:

Our aim was to investigate vitamin D receptor (VDR) gene single nucleotide polymorphisms (SNPs) in an ALS population. This gene encodes the nuclear hormone receptor for vitamin D3.

MATERIALS AND METHODS:

75 consecutive sporadic ALS patients (~ 20% of the Hungarian ALS population) and 97 healthy controls were enrolled to investigate the possible effects of the different VDR alleles. A restriction fragment length polymorphism technique was utilized for allele discrimination.

RESULTS:

One of the four investigated SNPs was associated with the disease, but none of the alleles of these SNPs influenced the age at disease onset. The ApaI A allele was more frequent in the ALS group than in the control group, and may be an ALS risk factor.

CONCLUSIONS:

This is the first verification of the genetic link between ALS and VDR. However, further studies are needed to confirm these findings.

Key words: amyotrophic lateral sclerosis, ApaI, neurogenetics, VDR gene

Abbreviated title: VDR gene polymorphisms in ALS

Abbreviations:

ALS: amyotrophic lateral sclerosis
SNP: single nucleotide polymorphism
VDR: vitamin D receptor
MS: multiple sclerosis
PD: Parkinson's disease
AD: Alzheimer's disease

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a devastating disease in which the upper (cortical) and lower (spinal and ponto-bulbar) motor neurones undergo relentless degeneration resulting in death within several months or years. There is apparently no cure for it; currently the only effective treatment (Riluzole) (1) leads to merely a modest slowing of the disease progression. ALS is a rare disease, with a mean incidence of 2.8/100 000, and a mean prevalence of 5.40/100 000 in Europe (2).

Only 10% of the cases are classified as familial, with the remaining 90% as sporadic (3). A genetic aetiology plays a role in ~65% of the familial cases and about ~11% of the sporadic cases (3). The high-risk causative genes and low-risk susceptibility genes have been well surveyed in two reviews (3, 4).

Genetic investigations have contributed to a clarification of the mechanisms and signalling routes involved in the aetiology of the disease: glutamate excitotoxicity, damage by free radicals, a mitochondrial dysfunction, intracellular protein aggregation, excessive poly(ADP-ribose) polymerase activation, autoimmune inflammatory processes and the accumulation of intracellular calcium.

The rise in the level of intracellular calcium in the motor neurones is a common denominator leading to cell death in ALS and its experimental animal models, as a result of glutamate excitotoxicity or damage by free radicals or autoantibodies. One of the putative causes of the vulnerability of the motor neurones in the disease is their low levels of calcium-binding proteins (parvalbumin and calbindin-D28K), which can be elevated by gene therapy in experimental animals (5) and by vitamin D supplementation (6, 7).

Vitamin D is synthesized from its prohormone in the skin in response to exposure to sunlight. Its active form, 1α-25-(OH)2D is bound to the vitamin D nuclear receptor (VDR) and modulates the transcription of several genes and mineral ion homeostasis. Vitamin D

regulates the serum calcium level, which may modify immune functions (8). It has been localized to neurones and glial cells. Genes which encode the enzymes in the metabolization of this hormone are also found in the brain. Vitamin D is additionally responsible, among others, for the biosynthesis of neurotrophic factors, inducible nitric oxide synthase and increased glutathione levels, all of which play a part in the pathomechanisms of ALS and other neurodegenerative diseases. In general, vitamin D can reduce the inflammatory reaction, which is a significant contributor in neurodegeneration. The roles of the possible genetic and cellular signalling mechanisms of vitamin D in ALS, and particularly in its G93A transgenic mouse model, have been extensively reviewed by Long and Nguyen (9).

The gene is located on the 12q13.11 chromosome region encoding the nuclear hormone receptor for vitamin D3. Associations have been published between VDR polymorphism and various neurological disorders, but only limited data are available as concerns ALS.

In light of the above, we set out to investigate single nucleotide polymorphisms (SNPs) of the VDR gene in ALS in a Hungarian cohort.

2. Patients and Methods

2.1. ALS patients and controls

All the study participants provided their written informed consent. 75 unrelated ALS patients and 97 healthy controls were enrolled for analysis of the possible effects of four SNPs of the VDR gene: rs1544410 (BsmI), rs7975232 (ApaI), rs731236 (TaqI) and rs2228570 (FokI). The ALS and control groups did not differ from each other in sex ratio (p=0.976) or mean age (p=0.935). The ALS group consisted of 47 females and 28 males; their average age was 60.3±11.0 years, and their average age at the onset of the disease was 58.9±11.8 years. The age at onset was determined from the medical records and the cases were categorized as early-onset (diagnosed ≤60 years) or late-onset (diagnosed >60 years) ALS (the median of the age at onset was 60 years). The diagnosis was established according to the El-Escorial criteria (10). The control group comprised 97 healthy volunteers, 61 of whom were females and 36 were males, with an average age of 60.1±11.3 years. The sociodemographic data are summarized in Table 1. The study protocol was approved by the Medical Research Council Scientific and Research Ethics Committee (47066-3/2013/EKU (556/2013)) and was in full accordance with the Helsinki Declaration. Blood samples were collected at the Department of Neurology.

2.2. Methods

2.2.1. DNA isolation

Genomic DNA was isolated from peripheral blood by the standard desalting method developed by Miller et al. (11). The purified genomic DNA was stored at -20 °C at the biobank of the Department of Neurology until further use (biobank licence: Regional Human Biomedical Research Ethics Committee: 135/2008).

2.2.2. Genotyping

Three of the four investigated SNP are intron variants and one is a missense change in the genome.

rs1544410 (BsmI)

For amplification of the DNA region near the intronic rs1544410 SNP, the following primers were designed: forward primer: 5'- CAA CCA AGA CTA CAA GTA CCG CGT CAG TGA -3', and reverse primer: 5'- AAC CAG CGG GAA GAG GTC AAG GG -3'. For allele discrimination, 10 U/μl of the enzyme Mva1269I (BsmI) (Thermo Scientific) was applied according to the manufacturer's recommendation.

PCR amplification was carried out at 95 °C for 5 min, followed by 44 cycles of 95 °C for 30 s, and then 60 °C for 30 s, 72 °C for 1 min; 72 °C for 5 min.

rs731236 (TaqI)

For amplification of the intronic rs731236 SNP, the following primers were used: forward primer: 5'- CAG AGC ATG GAC AGG GAG CAA -3', and reverse primer: 5'- CAC TTC GAG CAC AAG GGG CGT TAG C -3'. For separation of the alleles, 10 U/μl of the enzyme TaqI (Thermo Scientific) was applied according to the manufacturer's recommendation.

PCR amplification was carried out at 95 °C for 5 min, followed by 44 cycles of 95 °C for 30 s, and then 60 °C for 30 s, 72 °C for 1 min; 72 °C for 5 min.

rs2228570 (FokI)

For discrimination of the missense SNP at rs2228570 from the VDR gene, the following primers were consumed: forward primer: 5'- AGC TGG CCC TGG CAC TGA CTC TGC TCT -3', and reverse primer: 5'- ATG GAA ACA CCT TGC TTC TTC TCC CTC -3'. For

allele discrimination, 10 U/µl of the enzyme BseGI (BtsCI) (Thermo Scientific) was used according to the manufacturer's recommendation.

PCR amplification was carried out at 95 °C for 5 min, followed by 44 cycles of 95 °C for 30 s, and then 60 °C for 30 s, 72 °C for 1 min; 72 °C for 5 min.

rs7975232 (ApaI)

For the allele discrimination of the intronic SNP at rs7975232 on chromosome 12, the following primers were applied: forward primer: 5'- CAG AGC ATG GAC AGG GAG CAA -3', and reverse primer: 5'- CAC TTC GAG CAC AAG GGG CGT TAG C -3'. For the allele discrimination, 10 U/µl of the enzyme ApaI (Thermo Scientific) was used according to the manufacturer's recommendation.

PCR amplification was carried out at 95 °C for 5 min, followed by 44 cycles of 95 °C for 30 s, and then 60 °C for 30 s, 72 °C for 1 min; 72 °C for 5 min.

The PCR experiments were performed with a BioRad CFX96 C1000 real-time thermal cycler machine. After the PCR, the samples were digested with the appropriate enzymes and the resulting restriction fragments were separated according to their lengths by gel electrophoresis (Figure 1). The agarose gels were 2% (3 g agarose (SeaKem LE agarose) and 150 ml 1x TBE buffer (diluted from TBE buffer 10x from Applichem) with 15 µl ethidium-bromide (Sigma, (500 µg/ml)), the applied voltage was 120 V. We used Gene Ruler 50 bp (left side of the gels) and the 100 bp (right side of the gels) DNA ladders from the Thermo Scientific. The separated alleles of the TaqI SNP were C allele (293 bp, 201 bp, 7 bp) and T allele (494 bp, 7 bp). In case of ApaI SNP the separated alleles were C allele (284 bp, 217 bp) and A allele (501 bp). The alleles of the FokI SNP were C allele (267 bp) and T allele (169 bp, 96 bp). The last investigated VDR SNP was BsmI with A allele (822 bp) and G allele (646 bp and 176 bp).

2.2.3. Statistical methods

SPSS software version 20.0 was used for evaluation of the data. The chi-square test was utilized for comparisons of the distribution of alleles and genotypes, and the t-test for comparisons of the averages of the two groups. Odds ratio (OR) and 95% confidence intervals (CI) were calculated to detect the association between the VDR and the risk of ALS. A p value of < 0.05 was considered significant.

The observed genotype frequencies in the control and ALS groups were in accordance with the Hardy-Weinberg equilibrium.

3. Results

rs1544410 (BsmI) SNP

This SNP, localized in the intronic segment of the VDR gene, is an A/G change. The genotype distribution in the ALS group was 9 AA, 35 AG and 31 GG, and that in the control group was 19 AA, 37 AG and 41 GG. The allele frequencies in the two groups were similar (Table 2). This SNP variant is not associated with the disease (genotype: p=0.327, allele frequency: p=0.527) or with the age at disease onset (genotype p=0.244). Moreover, no significant difference was found between this SNP and gender in the ALS group (p= 0.074).

rs731236 (TaqI) SNP

This intronic variant of the VDR gene is a C/T change. The genotype distribution was 9 CC, 34 CT and 32 homozygote TT in the patient group and 18 CC, 38 heterozygote and 41 TT in the control group. The allele frequency results: 35% C allele in the ALS group vs 38% C allele in the control group, and 65% T allele in the patient vs 62% T allele in the control group. This SNP variant is not associated with the disease (genotype: p=0.462, allele frequency p=0.507) or with the age at disease onset (genotype p=0.328) and no significant difference was observed in the male to female ratio (p=0.134) (Table 2).

rs2228570 (FokI) SNP

This SNP of the VDR gene is a missense mutation (C/T), which affects the structure and function of the encoded protein (12). The two variants of this exonic SNP code for structurally different receptor proteins. The wild-type, C allele codes a 424-amino acid protein, while the T allele codes a 427-amino acid receptor. These two receptors exhibit different efficiencies of VDR binding with transcription factor II B (12), and hence have different abilities to induce the transcription of vitamin D-dependent genes. Our results indicate the lack of an association between rs2228570 and ALS (genotype: p=0.801, allele

frequency p=0.542) and the mutation does not affect the age at disease onset (p=0.341) or the gender distribution in the ALS group (genotype p=0.982) (Table 2).

rs7975232 (ApaI) SNP

This intronic variant of the VDR gene is an A/C change. The genotype distribution in the ALS patient group was 25 AA, 43 AC and 7 CC and in the control group 28 AA, 40 AC and 29 CC. The allele frequencies were 62% A allele and 38% C allele in the patient group and 49.5% A and 50.5% C in the control group (Table 2).

There was a significant difference in genotype distribution between the patient and control groups (χ^2=11.09; p=0.004). The frequency of the genotype with A (AA+AC) vs CC was significantly higher among the ALS patients as compared with the controls ($\chi2$=10.807, df=1, p=0.001, OR:4.143 and 95% CI=1.699-10.100) (Figure 2). Additionally, the A allele proved to be significantly associated with the ALS group ($\chi2$=5.352, df=1, p=0.021, OR=0.600, 95% CI=1.080-2.569).

The investigated two alleles had no effect on the age at disease onset (genotype p=0.289) and there was no sifnificant difference between this SNP and gender (p=0.327) in the ALS group (Table 2).

To summarize these results, one of the four investigated SNPs was associated with ALS (ApaI), but none of them affected the age at the onset of the disease and there were no statistical differences in the gender ratio (Table 2).

4. Discussion

Earlier reports revealed that the 25-hydroxyvitamin D levels are decreased in various neurological disorders, including multiple sclerosis (MS) (13, 14), Alzheimer's disease (AD) (15, 16) and Parkinson's disease (PD) (17, 18). However, there have been only a few studies of the possible role of vitamin D in ALS (6, 7, 19-21).

A subnormal serum level of 25-hydroxyvitamin D was measured in a group of ALS patients (22). Supplementation which ensured at least a low level in the normal range resulted in only a slight slowing-down in disease progression as measured by the ALSFRS-R score decline during a 9-month period (6). Another report demonstrated that chronic vitamin D treatment upregulates the VDR mRNA level in cultured rat cortical neurones during neurotoxicity by glutamate (23). ALS shares pathophysiological similarities with neurodegenerative diseases such as MS, AD and PD, e.g. oxidative stress, neurodegeneration, inflammation, apoptosis and a mitochondrial dysfunction. Recent studies have shown that vitamin D ameliorates these pathophysiologies in animal models of these diseases and in human trials (21, 24-27). Moreover, genetic studies on VDR SNPs have suggested the involvement of VDR in these diseases (28-33), but only limited data are available on VDR SNPs in ALS. We therefore set out to examine the possible associations between four SNPs of VDR gene and ALS.

In our study, the ApaI A allele proved to be more frequent in the ALS group than in the healthy controls, suggesting that it may have a risk effect in ALS. This work led to the first genetic evidence that the VDR gene may have a role in ALS.

Kamel et al. earlier investigated the BsmI SNP of the VDR gene, but this was not associated with the causative lead levels or ALS risk (19). We verified this observation in our study, because we did not identify a significant association with the BsmI SNP. Moreover, no significant differences were found between the other two investigated SNPs in the ALS group.

Several studies have recently been conducted on the impact of VDR SNPs in PD. As an example, the protective or harmful roles of different SNPs of the VDR gene were examined in the Faroe Islands (where the prevalence of PD is double that in other European countries). It emerged that there were no statistical differences in genotype frequencies in the PD patients relative to the controls in the three examined SNPs (ApaI, BsmI and TaqI) (34). However, in a Chinese study Taq I seemed to be a risk factor for males with PD (31), while in a Hungarian report the Fokl C allele frequency was significantly higher in PD patients than in controls (32).

The associations of VDR gene SNPs with AD have been observed by three independent groups. The frequency of the Apal genotype Aa (AC genotype) was significantly higher in AD patients than in healthy individuals (29). In a comparatively large study, ApaI T and TaqI G were associated with the risk of AD, particularly in patients older than 75 years (30). Preliminary evidence of interactions between the VDR SNPs (ApaI and TaqI) and the genes of interleukin 10 and dopamine-β-hydroxylase was also found. Both genes are involved in inflammation too (30).

Moreover, several SNPs were observed to be associated with the age at onset in AD or PD patients (29, 35). Our study did not reveal an association between the age at onset and the VDR SNPs in the ALS group.

The role of the VDR gene in MS has been only poorly investigated to date, but there are several lines of evidence which indicate that certain VDR SNPs are risk factors in MS (33).

Overall, therefore, it appeared worthwhile to examine whether SNPs in the VDR gene may influence the affinity of vitamin D for its receptor, which can be related to the susceptibility to PD, AD, MS or ALS. Of course, our report has the limitation of the low sample size, but this is the first evidence that the VDR gene may have a role in ALS. Further investigations are needed to confirm our findings.

5. Acknowledgements

6. Conflict of interest

The authors declare no conflicts of interest.

7. Author contributions

Conceived and designed the experiments: NT, RT, and PK. Performed the experiments: NT. Selected the patients and controls, established the diagnosis, followed up the patients, and collected the samples: NT and JE. Analysed the data: NT. Wrote the paper: NT, JE, PK and LV. Study supervision and coordination: JE, PK and LV.

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Tables

Table 1. Summary of the sociodemographic data

Groups	Males	Females	Mean age (SD) (years)	Median	Min	Max	Age at onset (SD) (years)	Median of the age at onset
ALS patients (74)	28	47	60.3±11.0	61	33	86	58.9±11.8	60
Controls (97)	36	61	60.1±11.3	62	33	84	-	-

Min: minimum age in the group, Max: maximum age in the group, SD: standard deviation

Table 2. Genotype distributions of the VDR gene in the ALS and control groups

Groups	Genotype				Allele frequency		
rs1544410 (BsmI)	AA (%)	AG (%)	GG (%)	p	A (%)	G (%)	p
ALS	9 (12%)	35 (46.7%)	31 (41.3%)	0.327	53 (35.3%)	97 (64.7%)	0.527
Controls	19 (19.6%)	37 (38.1%)	41 (42.3%)		75 (38.7%)	119 (61.3%)	
Age at onset ≤60 years	3 (7.9%)	16 (42.1%)	19 (50%)	0.244			
Age at onset >60 years	6 (16.2%)	19 (51.4%)	12 (32.4%)				
Male	5 (17.9%)	16 (57.1%)	7 (25%)	0.074			
Female	4 (8.6%)	19 (40.4%)	24 (51%)				
rs7975232 (ApaI)	AA (%)	AC (%)	CC (%)	p	A (%)	C (%)	p
ALS	25 (33.3%)	43 (57.3%)	7 (9.3%)	0.004	93 (62%)	57 (38%)	0.021
Controls	28 (28.9%)	40 (41.2 %)	29 (29.9%)		96 (49.5%)	98 (50.5%)	
Age at onset ≤60 years	10 (26.3%)	23 (60.5%)	5 (13.2%)	0.289			
Age at onset >60 years	15 (41%)	20 (54%)	2 (5%)				
Male	12 (42.9%)	13 (46.4%)	3 (10.7%)	0.327			
Female	13 (27.7%)	30 (63.8%)	4 (8.5%)				
rs731236 (TaqI)	CC (%)	CT (%)	TT (%)	p	C (%)	T (%)	p
ALS	9 (12%)	34 (45%)	32 (43%)	0.462	52 (34.7%)	98 (65.3%)	0.507
Controls	18 (18.6%)	38 (39.2%)	41 (42.3%)		74 (38.1%)	120 (61.9%)	
Age at onset ≤60 years	3 (7.9%)	16 (42.1%)	19 (50%)	0.328			
Age at onset >60 years	6 (16.2%)	18 (48.7%)	13 (35.1%)				
Male	5 (17.9%)	15 (53.6%)	8 (28.6%)	0.134			
Female	4 (8.5%)	19 (40.4%)	24 (51.1%)				

rs2228570 (FokI)	CC (%)	CT (%)	TT (%)	p	C (%)	T (%)	p
ALS	30 (40%)	35 (46.7%)	10 (13.3%)	0.801	95 (63.3%)	55 (36.7%)	0.542
Controls	42 (43.3%)	45 (46.4%)	10 (10.3%)		129 (66.5%)	65 (33.5%)	
Age at onset ≤60 years	17 (44.7%)	18 (47.4%)	3 (7.9%)	0.341			
Age at onset >60 years	13 (35.1%)	17 (46%)	7 (18.9%)				
Male	11 (39.3%)	13 (46.4%)	4 (14.3%)	0.982			
Female	19 (40.4%)	22 (46.8%)	6 (12.8%)				

Figures

Figure 1. Gel electrophoresis images of the four investigated SNPs
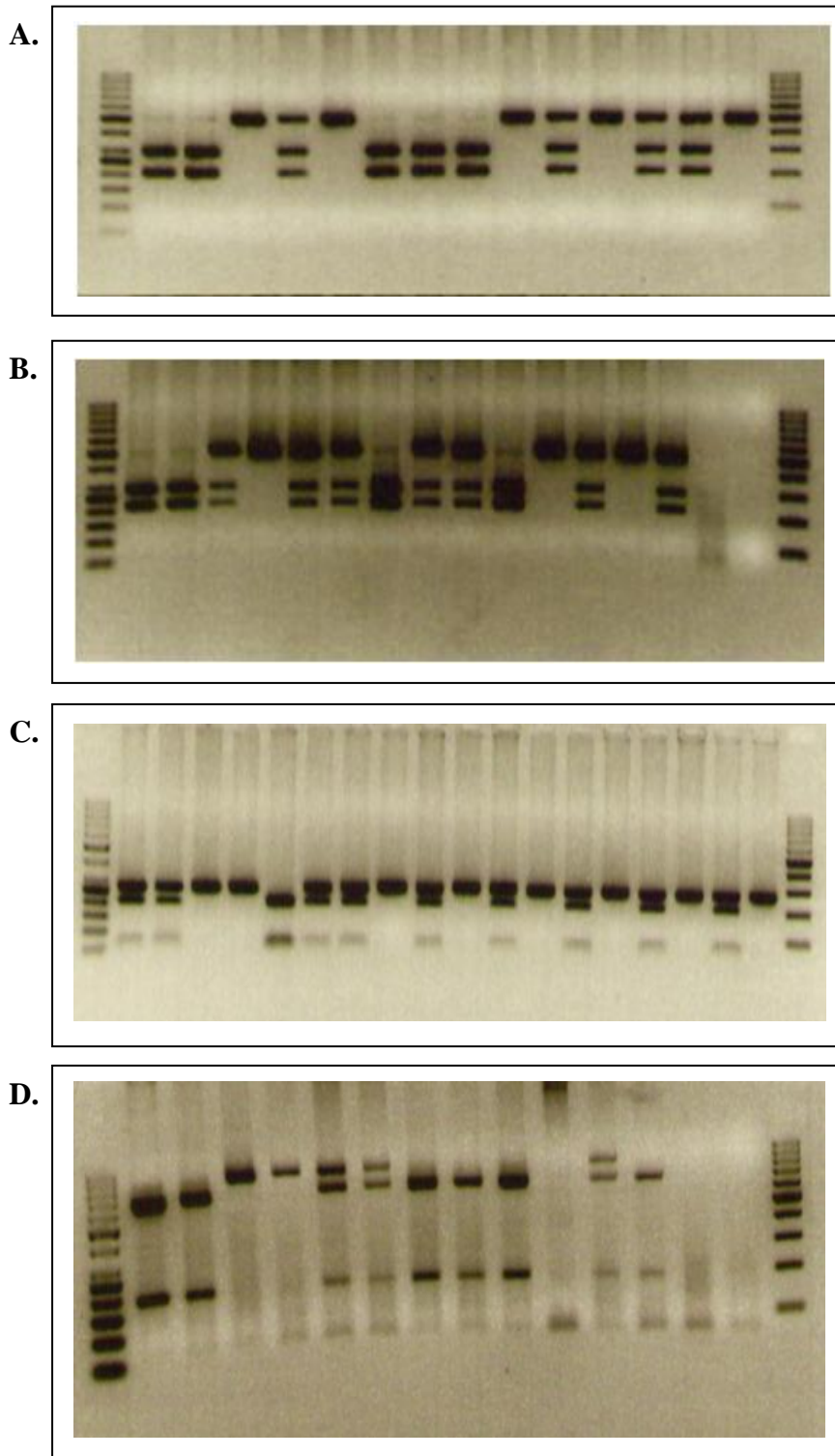
A.

B.

C.

D.

C. FokI: CC: 267 bp; CT: 267 bp, 207 bp, 60 bp; TT: 207 bp, 60 bp
D. BsmI: AA: 822 bp; AG: 822 bp, 646 bp, 176 bp; GG: 646 bp, 176 bp
We used Gene Ruler 50 bp (left side) and the 100 bp (right side) DNA ladders from the Thermo Scientific.

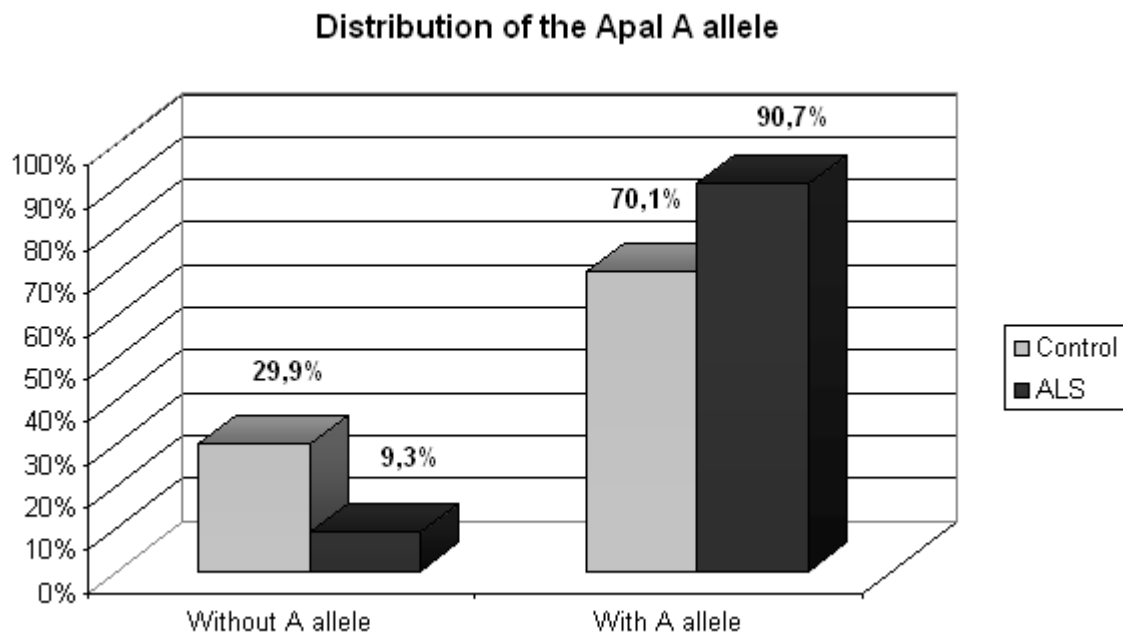Figure 2. Distribution of the ApaI A allele in the ALS patient and control groups

Distribution of the Apal A allele