

TARGETED SCREENING OF THE *C9orf72* GENE IN BULGARIAN AMYOTROPHIC LATERAL SCLEROSIS PATIENTS

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Abstract. Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease, characterized by progressive degeneration of the upper and lower motor neurons, leading to muscle weakness, hypotrophy, swallowing and respiratory failure. The cause of ALS is not yet fully elucidated, but there are 35 associated genes and 2 gene loci with an unidentified gene. The most common are *C9orf72*, *SOD1*, *TARDBP* and *FUS* found in approximately 10% of patients. Variants in the *C9orf72* gene are the main cause of fALS – 25-40% of cases (and a small percentage of sALS). The goal of the present study was to evaluate the significance of the *C9orf72* hexanucleotide repeat expansion in Bulgarian patients with ALS, through the means of in house and triplet repeat-primed PCR assay (TP-PCR). From 171 patients diagnosed with ALS and included in the current study, we have identified the repeat expansion with more than 145 GGGGCC repeats in 7 (4,1%). Short expansions or borderline values (24 to 30 repeats) were not detected. Due to absence of sufficient data, we have established an ALS-focused research for the association of the *C9orf72* gene in clinically well-characterized Bulgarian ALS patients. Published data show variable percentage ratios for genetically verified cases (4-40%), which is mainly due to small sample counts and sALS-fALS ratios. Our patients' group also contains sALS and fALS cases, which explains the low percentage of genetic verification. The obtained results enrich the worldwide database and shed light onto genetically characterized Bulgarian ALS patients. Affected patients and their families can receive adequate medical-genetic consultation and prenatal diagnostic testing.

Key words: amyotrophic lateral sclerosis, *C9orf72* gene, hexanucleotide repeat expansion

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INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease, characterized by progressive degeneration of the upper and lower motor neurons, leading to loss of function, followed

by muscle weakness, hypotrophy, swallowing and respiratory failure. ALS can be divided in two forms: sporadic (sALS) and familial (fALS). The most common is the sporadic form (90-95%), and the familial form can be seen in 5-10% of cases, associated with dominant inheritance [1].

The cause of ALS is not yet fully elucidated, but there are more than 30 associated genes. After *SOD1*, the second most frequent genetic variant causing ALS is located in the *C9orf72* gene. The function of this open reading frame is not fully understood, but it is known, that it contains a hexanucleotide repeat GGGGCC (G_4C_2), located between exon 1a and 1b. Normal size of the nucleotide repeats ranges from 2-5 repeats (50% of the population has only 2 repeats). Typically, it does not go over 30 repeats and in the affected patients it usually spans hundreds of repeats [2]. Updated meta-analysis, summarizing all available data, shows 16% occurrence in fALS patients and 6-8% in sALS patients [3].

C9orf72 associated ALS patients show behavioral changes, which tend to mimic those of frontotemporal dementia, Parkinson's disease, ataxia, etc. The penetrance of the dynamic mutation is about 50% at the age of 60 and close to 100% at the age of 80 [4]. Like in other nucleotide expansion-related diseases, the stability of the hexanucleotide repeat is of great significance for the disease onset in subsequent generations (early symptoms can occur at a younger age, up to 7 years earlier) [5].

The open reading frame undergoes alternative splicing, through which at least 3 different transcription variants are formed (V1, V2 and V3). The G_4C_2 expansion is localized either in the intronic (V1 and V3) region or in the promoter region of V2 [6, 7].

There is a lack of data for the association of the *C9orf72* gene with ALS in Bulgarian patients. To the best of our knowledge, the only published article concerning this association is focused on dementia patients and includes 7 FTD-ALS patients [8]. Other ALS-associated articles with Bulgarian patients focus on other genes, such as *SOD1*, *UBQLN2*, *PFN1* and *TBK1* [9, 10, 11]. Due to the lack of data, we have established an ALS-focused research for the association of the *C9orf72* gene in clinically well-characterized Bulgarian ALS patients. Published data show variable percentage ratios for genetically verified cases, which is mainly due to small sample counts, sALS-fALS ratios and difference in ethnic groups.

MATERIALS AND METHODS

A total of 171 patients, clinically diagnosed with ALS were selected, including 98 males (57,3%) and 73 females (42,7%). It was assumed that the cohort of patients contains both fALS and sALS cases, with the possibility that some fALS cases could be misdiagnosed as sALS, due to lack of medical history and/or deceased family members. The patients' age

of onset ranged from 21 to 82 years, the mean age for male patients was 58,6 and 63,2 years for female patients.

DNA was extracted from whole blood using a standard salting-out method. The noncoding intron 1 and intron/exon boundaries of the *C9orf72* gene were amplified through the means of in-house and triplet repeat-primed PCR assay. The in-house method, using designed primers flanking the hexanucleotide region, allows for accurate detection of up to 30 G_4C_2 repeats.

PCR was performed by using 80 ng of DNA in a reaction containing 5mM dNTPs mixture, 10x Prime Taq buffer (22.5 mM $MgCl_2$), 5 U/ μ l Thermostable Prime Taq polymerase, and 10 pmol/ μ l reverse and FAM-labeled forward primers (5'-AGCAAGCTCTG-GAACTCAGGAGT CG-3' forward and 5'CCTCACT-CACCCACTCGCCAC-3' reverse primers). Cycle conditions were 5 min at 94°C for the initial denaturation, followed by 35 cycles of 94°C for 45 sec, 56°C for 45 sec, 72°C for 1 min and 5 min at 72°C for the final extension. Fragment analysis was conducted on a genetic analyzer ABI Prism 3130 (Applied Biosystems, Foster City, CA, USA), using standard electrophoresis conditions on a 36 cm capillary array with POP7 polymer (Applied Biosystems, Foster City, CA, USA). 20 μ l Hi-Di formamide and 0.2 μ l ROX500 Size Standard (Applied Biosystem, Foster City, USA) were added to 0,2 μ l of each PCR product for fragment analysis.

This method is mainly applicable for detection of heterozygous profiles within the specified range. If the result shows one allele, a more precise method, such as TP-PCR is needed.

The TP-PCR method uses up to three primers: two gene specific primers (GS forward/reverse) that flank the repeat region, and one forward repeat primer, complementary to the *C9orf72* hexanucleotide repeat region.

TP-PCR was performed by using 80 ng of DNA and the reagents provided with the AmpliX PCR/CE *C9orf72* Kit (Asuragen) – *C9orf72* Gene Specific Primer Mix, *C9orf72* Repeat Primer, GC-Rich Amp Buffer, GC-Rich Polymerase Mix, and Diluent. Cycle conditions were 5 min at 98°C for the initial denaturation, followed by 37 cycles of 97°C for 35 sec, 62°C for 35 sec, 72°C for 3 min and 10 min at 72°C for the final extension. 11 μ l Hi-Di formamide and 2 μ l ROX1000 Size Ladder (Asuragen) were added to 2 μ l of each PCR product for fragment analysis. Fragment analysis conditions from the kit protocol were the same as the in-house method.

Using this method for long expansions leads to a "sawtooth" pattern when analyzing data. PCR-based

screening for variations in the *C9orf72* gene is used as part of the clinical diagnosis of affected patents, as well as a prognostic test for healthy patients under risk of manifesting ALS or frontotemporal dementia. Raw data from fragment analysis was analyzed using the Gene Mapper v.4.0 software.

CLINICAL ASSESSMENT

We studied 171 patients with ALS (according to the revised El Escorial and Awaji criteria), who underwent detailed neurological examination, including manual muscle testing (MRC scale). Nerve conduction studies (NCS) and needle electromyography (EMG) were performed in all of the affected individuals. Brain CT scan was done in all patients. Neuropsychological examination was performed in order to evaluate cognitive function and the presence of dementia. To assess the presence of bulbar and pseudobulbar palsy,

an otorhinolaryngological examination was conducted in all patients.

RESULTS

We have identified that the most common allele among Bulgarian ALS patients is 2 G4C2 (Fig. 1).

We have found an expansion in intron 1 of the *C9orf72* gene greater than 145 G4C2 in 7 out of 171 patients (4,1%) (Fig. 2). The longest normal allele for heterozygotes was 19 G4C2 and short expansions or borderline values (24 to 30 repeats) were not detected. The patients' age of onset ranged from 21 to 82 years, and the mean age of G4C2 expansion carriers was 53,6 years (ranging from 36 to 68 years).

Only 1 out of the 7 affected patients was diagnosed with having familial ALS (14,3%), as for the rest was assumed that they carry the sporadic form (85,7%).

Table 1. Clinical characteristics of the affected patients

№	Sex	Age of onset	First symptoms	Cognitive impairment	Family history (fALS/sALS)	Genotype	Brain CT scan
1	F	56	Dysarthria, weakness in both shoulders	No	No	7/ > 145	Without pathological changes
2	M	36	Dysarthria, weakness in the right thumb	No	Uncle with ALS and bedridden mother with unexplained myasthenia	8/ >145	Without pathological changes
3	M	68	Single fall, pain in the right shoulder	No	No	8/ > 145	Without pathological changes
4	M	52	Clumsiness and heaviness in the feet	No	No	2/ > 145	Without pathological changes
5	M	54	Dysarthria, increased salivation, dysphagia	No	No	7/ > 145	Without pathological changes
6	F	54	Dysarthria, weakness in the right foot	No	No	7/ > 145	Without pathological changes
7	F	55	Dysarthria, weakness in the right shoulder	No	No	4/ > 145	Without pathological changes

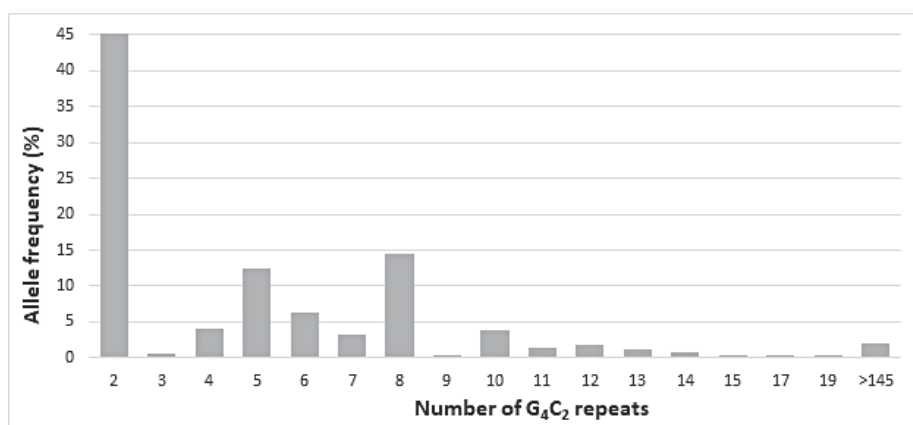


Fig. 1. G4C2 repeats and their allele frequency in all 171 patients

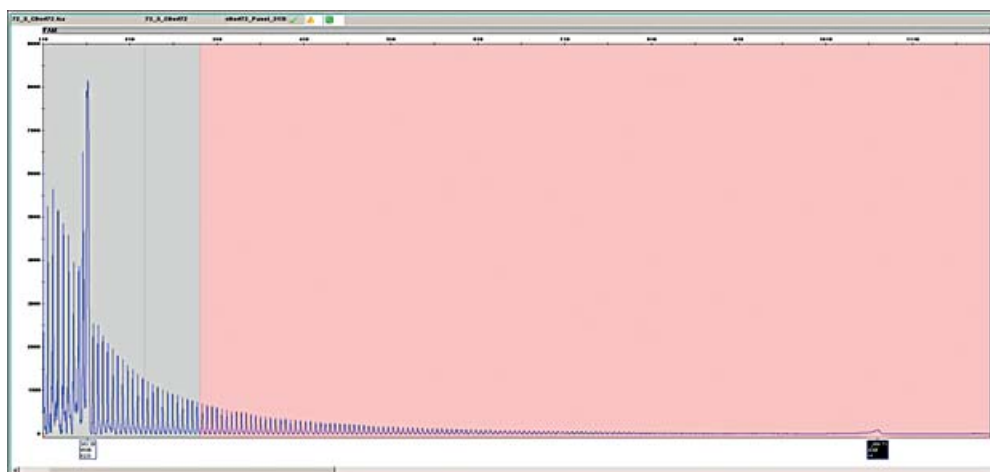


Fig. 2. Fragment analysis profile, using the AmpliX® PCR/CE *C9orf72* Kit (Asuragen). This result shows an expansion of over 145 G4C2 repeats (short allele – 8 G4C2). This kit allows for precise determination of up to 145 G4C2 repeat expansions. Larger expansions are written as > 145 G4C2

The clinical features of the Bulgarian patients with repeat expansion in *C9orf72* are presented in table 1.

DISCUSSION

To the best of our knowledge, this research that aimed at finding out the association of the *C9orf72* gene with ALS is the first of its kind, due to the patients' cohort being comprised of only clinically well-characterized Bulgarian ALS patients. According to previous reports variants in the *C9orf72* gene are the most common cause for ALS in the European population [4, 12, 13], which has led us to focus on this gene for the present study. Published data show variable percentage ratios for genetically verified cases (4-40%), which is mainly due to small sample counts and sALS-fALS ratios. Our patients' group also contains sALS and fALS cases, which explains the low percentage of genetic verification. The low percentage of expansion carriers with fALS (14,3%), compared to sALS (85,7%) could be due to lack of family medical history. Overall occurrence of the expansion (not differentiating between sALS and fALS) was 4,1%, which correlates well with results for the Russian population (6,8%) [13]. The small sample size as well as the relatively wide range of age of initial symptoms of the affected with the *C9orf72* expansion ALS patients (36 to 68 years) makes it difficult to correlate genotype with phenotype. The difference in the disease penetrance must be addressed. Considering this phenomenon, the mean age at onset of 56,5 years (with the exception of one 39-year-old case) proposes an incomplete penetrance. Onset at a later stage of life and the es-

tablished genetic cause of the disease in the family provides an opportunity for medical-genetic consultation. Prenatal testing is an option for the patient's relatives in cases with early disease manifestation (around 30 years of age). This allows to determine if the patient has sALS or fALS and to offer carrier status determination for relatives at risk. The detected asymptomatic carriers may benefit from early specialized clinical follow-up and participation in future clinical trials.

The obtained results from this study enrich the worldwide database and shed light onto genetically characterized Bulgarian ALS patients. Affected patients and their families can receive adequate medical-genetic consultation, prophylactics and prenatal testing.

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