UDC 575.
https://doi.org/10.2298/GENSR2203313M
Original scientific article

C9ORF72 REPEAT EXPANSION IS NOT ASSOCIATED WITH ATYPICAL PARKINSONISM IN THE SERBIAN POPULATION

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Expansion of hexanucleotide repeats (G_4C_2) in the non-coding region of the *C9orf72* gene is the most known genetic cause of amyotrophic lateral sclerosis (ALS), frontotemporal dementia (FTD), and the combined ALS/FTD phenotype. Besides ALS and FTD, G_4C_2 repeat expansions were detected in other neurological disorders with variable frequency. These include, among others, two forms of atypical Parkinsonism, multiple system atrophy (MSA) and progressive supranuclear palsy (PSP). This study aimed to assess the potential role of *C9orf72* repeat expansions among Serbian patients diagnosed with MSA and PSP. Genomic DNA of 44 MSA patients, 73 PSP patients, and 96 controls was extracted from peripheral blood, and normal *C9orf72* alleles were analyzed by standard quantitative fluorescence polymerase chain reaction (QF-PCR) and fragment analysis. Subsequently, for all samples presenting a single allele, repeat-primed PCR was performed with two different sets of primers to avoid a false-negative result. Thirty repeats were used as a pathogenic cut-off and 20-29 repeats for the intermediate alleles. No pathological *C9orf72* expansions were detected in the MSA and PSP patients nor the control subjects. In the MSA group, the most common was the allele with 2 repeats, and the largest repeat number was 14. Among PSP patients, the most common allele also had 2 repeats, while the largest detected repeat size within the normal range was 17. Also, we

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identified one PSP patient that had an intermediate size allele (25 repeats). We did not find correlation between the number of repeats and disease onset, age at the time of examination, or disease duration in MSA or PSP patients. Regarding family history, in PSP the sum of both allele repeats numbers was higher in patients with positive family history than in sporadic cases. The results presented in this study are the first systematic assessment of *C9orf72* allele sizes among patients diagnosed with MSA and PSP in the Serbian population. Although the potential role of intermediate *C9orf72* repeats in neurodegenerative disorders is still to be elucidated, our results support the current knowledge that *C9orf72* repeat expansions are not associated with MSA and PSP.

Key words: atypical Parkinsonism, *C9orf72*, multiple system atrophy (MSA), progressive supranuclear palsy (PSP), repeat expansion

INTRODUCTION

Expansions of GGGGCC repeat in the non-coding region of the *C9orf72* gene were discovered a decade ago, and they are the most common cause of amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) (DEJESUS-HERNANDEZ *et al.*, 2011; RENTON *et al*., 2011). At the same time, they represented a genetic link between these two disorders. The number of G_4C_2 repeats can vary from 2 to above 4000 repeats (DEJESUS-HERNANDEZ *et al*., 2011; BECK *et al*., 2013, GIJSELINCK *et al*., 2016). Although the clear cut-off value that would distinguish the normal and pathological number of repeats is not well defined, it is commonly considered that more than 30 repeats are disease-causing (RENTON *et al*., 2011; MAJOUNIE *et al*., 2012), while alleles carrying 20-30 repeats are considered as intermediate alleles (MAJOUNIE *et al*., 2012; NUYTEMANS *et al*., 2013). Besides ALS and FTD, G_4C_2 repeat expansions were detected in other neurological disorders with variable frequency. These include Alzheimer's disease (AD) (BECK *et al*., 2013; CACACE *et al*., 2013), Huntington's disease-like syndrome (HD-like) (BECK *et al*., 2013; KOSTIC *et al*., 2014), Multiple system atrophy (MSA) (GOLDMAN *et al*., 2014; LINDQUIST *et al*., 2013), and Progressive supranuclear palsy (PSP) (LESAGE *ET AL*., 2013; ORIGONE *ET AL*., 2013; LE BER *et al*., 2013;WILKE *et al*., 2016). Patients whose clinical characteristics are presented with parkinsonian symptoms but also have atypical features including early dementia, falls, problems with eye movement, dysautonomic or ataxia signs are called atypical parkinsonism syndromes and include MSA, PSP, corticobasal degeneration (CBD), and dementia with Lewy bodies (MCFARLAND, 2016).

MSA is a progressive neurodegenerative disease characterized by parkinsonism, autonomic failure, cerebellar ataxia, and pyramidal signs that begin with adult-onset (WENNING *et al*., 2003). Clinically, MSA can be presented in two major subtypes: MSA-P if parkinsonian characteristics prevail and MSA-C if the cerebellar ataxia is the primary clinical sign (GILMAN *et al*., 1998). Although no causative genes were discovered for MSA yet, variants in several genes have been implicated to be associated with MSA. A significant association between single nucleotide polymorphisms at the *SNCA* locus and risk for the development of MSA was reported (SCHOLZ *et al*., 2009), but these findings were not replicated in a subsequent study where the number of MSA cases was doubled (SAILER *et al*., 2016). Other genes were also screened for the possible association with susceptibility to MSA including *COQ2* (MULTIPLE-SYSTEM ATROPHY

RESEARCH, 2013), *PINK1*, *Parkin* (BROOKS *et al*., 2011), *LRRK2* (CHO *et al*., 2009), *GBA* (GOKER-ALPAN *et al*., 2006), *MAPT* (MORRIS *et al*., 2000), *CYP1A1*, *GSTM1*, *NAT2*, *CYP2D6*, *DAT1* (NICHOLL *et al*., 1999) as well as other genes reviewed in (FEDEROFF *et al*., 2015). The given findings were negative or inconclusive, and some needed further verification. The genome-wide association study (GWAS) performed by SAILER *et al*. (2016) was not able to find any significant loci, but loci in *FBXO47*, *MAPT*, *ELOVL7*, and *EDN1* were of potential interest.

PSP is a neurodegenerative disorder characterized by clinical features that include postural instability, vertical supranuclear gaze palsy, akinesia, and cognitive dysfunction (HOGLINGER *et al*., 2017). The pathological feature of PSP is the presence of Tau-positive neurons and glia in the subcortical regions (SPILLANTINI and GOEDERT, 1998; SPILLANTINI *et al*., 1998). The presence of Tau pathology implied the connection of PSP and *MAPT* gene. At the genetic level, this link was supported by the discovery that dinucleotide polymorphic repeat marker in *MAPT* is associated with PSP (CONRAD *et al*., 1997). Later, it was found that association exists also for the extended H1 haplotype that covers the human *MAPT* gene (BAKER *et al*., 1999). Moreover, both heterozygous (POORKAJ *et al*., 2002; ROS *et al*., 2005) and homozygous (NICHOLL *et al*., 2003) mutations in *MAPT* were reported in PSP patients. Besides mutations in *MAPT*, variants in *LRRK2* were shown to be associated with PSP (ZIMPRICH *et al*., 2004; SPANAKI *et al*., 2006). However, studies on neuropathologically proven PSP cases did not confirm these findings (ROSS *et al*., 2006; GAIG *et al*., 2008). Further GWAS studies identified several loci in *MAPT*, *MOBP*, *STX6*, *EIF2AK3*, *SLCO1A2*, *DUSP10* to be associated with PSP (HOGLINGER *ET AL*., 2011; SANCHEZ-CONTRERAS *et al*., 2018; CHEN *et al*., 2019).

Based on the current knowledge about *C9orf72*, several novel therapeutic approaches have been proposed targeting *C9orf72* disease mechanisms (MAYL *et al*., 2021; HAUTBERGUE *et al*., 2021), so uncovering the expansion carriers in other diseases is very important from the perspective of the potential therapies.

In this study, we aimed to assess the potential role of *C9orf72* repeat expansions among Serbian patients diagnosed with MSA and PSP.

MATERIALS AND METHODS

This study was approved by the Ethics Committee of the University Clinical Center of Serbia (UCCS). All participants included in this study were informed about the genetic testing, and they gave written consent.

Study patients and subjects

In the time period from 2008 to 2021 from all examined patients with MSA and PSP, DNA for the genetic analysis was available only for 44 MSA and 73 PSP patients. Also, in this study we included 96 healthy controls without neurological and cognitive disturbances. All the patients were diagnosed at the Neurology Clinic UCCS, Faculty of Medicine, University of Belgrade. All MSA patients were diagnosed as probable MSA based on Gilman criteria (GILMAN *et al*., 2008), and diagnosis of PSP patients was made based on Hoglinger criteria (HOGLINGER *et al*., 2017).

DNA extraction, fragment analysis QF-PCR, repeat sizing, and data interpretation

Genomic DNA was extracted from peripheral blood using PureLink™ Genomic DNA Mini Kit (*Life Technologies*, USA) following the manufacturer's instructions.

PCR amplification

Alleles in the normal range were determined by standard fluorescent PCR amplification of the region containing repeats and subsequent fragment analysis by capillary electrophoresis. PCR reaction mix in the total volume of 10 µl included 1x Long buffer with 25mM $MgCl_2$ (*Thermo Scientific*, USA), 0.2 mM of each deoxyribonucleotides mix (dNTPs, *Thermo Scientific*, USA), 0.4 pmol/µl of each forward (FAM-5' GAAACAACCGCAGCCTGTAG 3') and reverse primer (5' GCCTCCTCACTCACCCACT 3'), DMSO (*Thermo Scientific*, USA) in 10% of the total reaction volume and 0.5 U of Long PCR enzyme Mix (*Thermo Scientific*, USA), DNA sample, and nuclease-free water. Thermal cycling profile included: 5 min 94°C, 10 cycles (30s 94°C, 30s 60°C, 45s 72°C), 20 cycles (30s 94°C, 30s 60°C delta 0.5°C, 60s 72°C), 10 cycles (30s 94°C, 30s 50°C, 45s 72°C), final extension 60 min 60°C. All homozygous samples were further analyzed by repeat-primed PCR (RP-PCR) using previously published primers (RENTON *et al*., 2011). PCR reaction was performed in the total volume of 10 µl including 1x Long buffer with 25mM $MgCl_2$ (*Thermo Scientific*, USA), 0.75mM of additional 25mM $MgCl_2$ (*Thermo Scientific*, USA), 0.2 mM of each deoxyribonucleotides mix (dNTPs, *Thermo Scientific*, USA), 1.4 pmol/µl of forward and 0.7 pmol/µl of reverse and anchor primer, DMSO (*Thermo Scientific*, USA) in 10% of the total reaction volume, 0.5M of 5M Betaine solution (*Serva*, Germany) and 2.5 U of Long PCR enzyme Mix (*Thermo Scientific*, USA), DNA sample, and nuclease-free water on thermal cycling profile: 5 min 95°C, 14 cycles (60s 95°C, 30s 70°C delta 1°C, 4 min 72°C), 22 cycles (60s 95°C, 30s 56°C, 4 min 72°C), final extension 15 min 60°C. To avoid potential false-negative results, all homozygous samples confirmed with RP-PCR were re-tested with another set of primers (DEJESUS-HERNANDEZ *et al*., 2011), where PCR reaction mix in the total volume of 10 µl included 1X Xtreme buffer (*Toyobo Novagen*, Japan), dNTPs (0.4 mM each) (*Toyobo Novagen*, Japan), 0.3 pmol/µl of each forward and anchor primer and 0.15 pmol/µl of reverse primer, DMSO (*Thermo Scientific*, USA) in 10% of the total reaction volume, 0.2 U KOD Xtreme Hot Start DNA Polymerase (*Toyobo Novagen*, Japan), DNA sample and nuclease-free water. Thermal cycling profile was: 2 min 94°C, 14 cycles (30s 98°C, 60s 67°C delta 1°C, 3 min 72°C), 22 cycles (15s 98°C, 60s 53°C, 3 min +10s 72°C), final extension 60s 72°C.

Fragment analysis by capillary electrophoresis and data interpretation

PCR products were separated by size with capillary electrophoresis on ABI 3500 Genetic analyzer (*Applied Biosystems*, Foster City, CA, USA) using GeneScan 500 LIZ (*Applied Biosystems*, USA) as a size standard and genotyping software package GeneMapper software version 4.1 (*Applied Biosystems*, Foster City, CA, USA) for allele calling. Alleles with less than 20 repeats are considered as a "wild type", those 20-29 repeats as "intermediate" alleles (CANNAS *et al*., 2015), and those with more than 30 repeats as "expanded" alleles (RENTON *et al.*, 2011).

Statistical analysis

The members of pair of repeats on both alleles for each patient were labeled as smaller and larger. Two additional variables calculated were the difference and sum of repeats on both alleles. The total of 4 variables (smaller, larger, difference, and sum) was treated as numerical normally distributed data and compared between groups with t-test and with other numerical variables using Pearson correlation. One observation of 25 repeats for the larger allele was treated as missing for the purpose of these analyses. Comparison between multiple groups was performed using a one-way ANOVA.

RESULTS

In a total number of 44 MSA patients, 73 PSP patients, and 96 controls, we performed *C9orf72* repeat sizing to determine the distribution of normal alleles and to detect the possible presence of *C9orf72* repeat expansion. Clinical and demographic characteristics of MSA, PSP, and control group are shown in Table 1.

Table 1. Clinical and demographic characteristics of patients diagnosed with MSA, PSP, and the control group.

	MSA			PSP			Controls		
	Mean	SD	Range (min-max)	Mean	SD	Range (min-max)	Mean	SD	Range (min-max)
Disease onset (years)	55.64 (95% CI:53.75-57.53)	6.22	37.0-69.0	63.05 (95% CI:61.50-64.61)	6.69	49.0-79.0	/	/	/
Age at the time of examination (blood draw)	59.32 (95% CI:57.49-61.15)	6.02	42.0-73.0	66.70 (95% CI:65.16-68.24)	6.60	52.0-81.0	56.37 (95% CI:53.81-58.94)	12.67	36.0-88.0
Disease duration* (years)	3.72 (95% CI:2.98-4.46)	2.43	0.6-14.0	3.66 (95% CI:3.12-4.20)	2.32	0.1-10.0	/	/	/

SD-standard deviation; CI-confidence interval

*Disease duration is defined as a period from disease onset to the time of the examination when the blood is drawn for genetic analysis

Our MSA group included 21 men (47.73%) and 23women (52.27%), with an average age of (59.32±6.02 years) and an average disease duration at the time of the examination of (3.72±2.43) years. Negative family history was reported in 88.64% of the patients, 4.55% had a positive family history for parkinsonism or Parkinson's disease, while for 6.82% family history was unknown. The most frequent allele in the MSA group was the allele with 2 repeats (55.68% of the total number of alleles), followed by the allele with 5 repeats (17.05%). The largest number of detected repeats was 14 (1.14%). Thirteen patients (29.55%) were homozygous and confirmed with another set of primers. None of the patients carried the repeat expansion in *C9orf72*. We did not find any significant correlation of the disease onset, age at the time of

examination (blood draw), and disease duration with the number of repeats. Also, there was no statistical difference in repeats sizes in relation to family history (Table 2).

Table 2. Results of statistical analysis of the size of the repeats and the observed parameters in MSA patients

		Larger allele	Smaler allele	Difference	Sum
Disease onset	r	0.093	-0.157	0.151	0.038
	p	0.550	0.308	0.329	0.809
Age at the time of examination (blood draw)	r	0.093	-0.143	0.146	0.042
	p	0.549	0.353	0.344	0.789
Disease duration	r	-0.028	0.053	-0.047	-0.010
	p	0.856	0.732	0.760	0.949
Family history	t	-0.921	-0.792	-0.288	-1.372
	p	0.363	0.572	0.775	0.178

r – Pearson correlation coefficient; p – statistical significance; t- t-test value

In the PSP group, we have analyzed 41men (56.16%) and 32 women (43.84%) with an average age of (66.70±6.60 years) and the disease duration at the time of the examination of (3.66±2.32) years. The family history was negative for 71.23% of the patients. Positive family history, which also included other neurological disorders such as dementia, parkinsonism, or psychiatric disturbances like suicide attempts, was present in 15.07% of the patients. For 13.70% of the patients, data on family history was not available. The most frequent allele in PSP patients was allele with 2 repeats (52.74%) followed by the allele with 5 repeats (13.70%). In the normal range (up to 20 repeats) the largest number of detected repeats was 17 (0.68%). Thirty patients (41.10%) were confirmed as homozygous. We also detected one heterozygous patient (1.37%) carrying one allele in the normal size range (8 repeats) and one intermediate allele with 25 repeats (Figure 1). At the time of the examination, this patient was 75 years old, and the disease onset was at the age of 69 years. Family history was negative. His clinical picture was typical PSP with slow and difficult walking more right-sided, resting tremor right-sided, common falls, and vertical gaze palsy with limited response to levodopa. His CT scan showed signs of

frontotemporal cortico-reductive changes. We did not detect the presence of the pathological *C9orf72* repeat expansions among PSP patients. There was no significant correlation between the observed parameters and the number of repeats. In relation to family history, the sum of both allele repeats numbers was higher in the group with positive family history using t-test (t=-2.231, p=0.029), when patients with an unknown family history together with the patient with 25 repeats were excluded from the analysis (Table 3).

Table 3. Results of statistical analysis of the size of the repeats and the observed parameters in PSP patients

		Larger allele	Smaller allele	Difference	Sum
Disease onset	r	0.209	0.100	0.195	0.186
	p	0.078	0.399	0.100	0.118
Age at the time of examination (blood draw)	r	0.196	0.107	0.184	0.174
	p	0.099	0.368	0.123	0.143
Disease duration	r	-0.041	0.003	-0.029	-0.043
	p	0.731	0.980	0.809	0.720
Family history	t	-1.855	-1.932	-0.805	**-2.231**
	p	0.068	0.058	0.424	**0.029**

r – Pearson correlation coefficient; p – statistical significance (bold- statisticly significant); t- t-test value

The control group included 27 male (28.12%) and 69 female (71.87%) subjects with an average age at the time of the genetic testing of 56.37±12.67 years. The largest allele detected among control subjects had 17 repeats (0.50%), while the most frequent allele was the allele with 2 repeats (47.40%) followed by the allele with 8 repeats (19.30%). None of the control subjects carried the pathological repeat expansion nor intermediate repeat number in *C9orf72*. The distribution of allele sizes for MSA, PSP, and control group is shown in Figure 2. Repeat size did not correlate with the age at the blood draw.

In all three groups, more than 90% of alleles had up to 10 repeats (92.05%, 90.36%, and 95.8% in MSA, PSP, and controls, respectively).

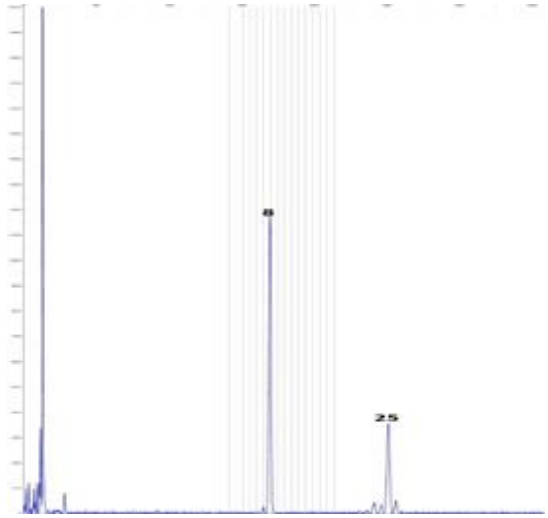

Figure 1. Electropherogram of the *C9orf72* fluorescent sizing PCR for the PSP patient carrying one normal allele (8 repeats) and one intermediate allele (25 repeats)

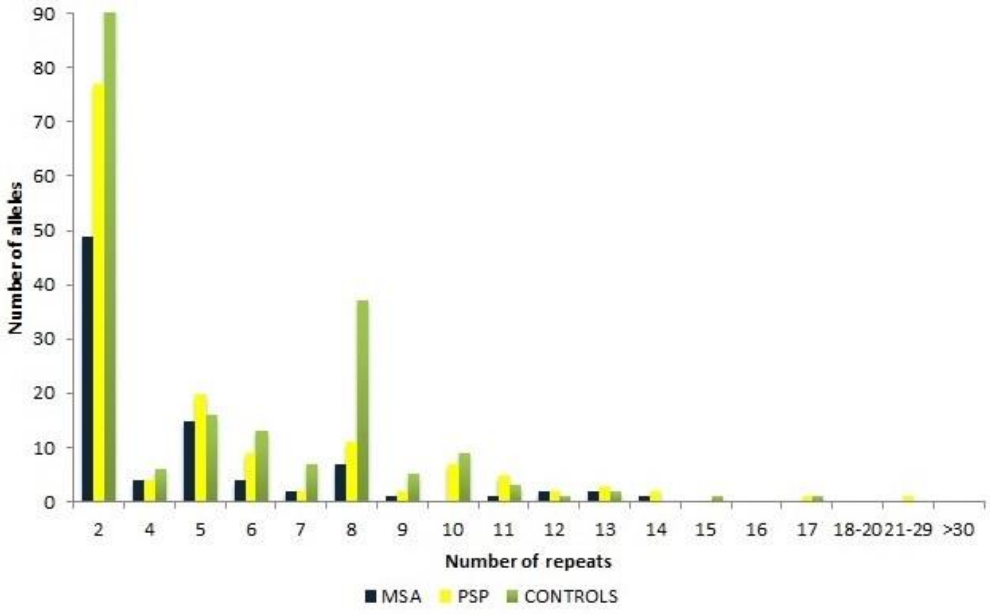

Figure 2. Distribution of the *C9orf72* allele sizes among MSA, PSP, and the control group.

DISCUSSION

In the present study we have investigated the possible presence of the *C9orf72* repeat expansions among patients clinically diagnosed with MSA and PSP, as well as we wanted to gain insight into allele sizes distribution. Overall, the results we obtained are in line with the data available in the literature.

The number of repeats within normal alleles did not exceed 14 among the MSA patients and 17 in the PSP group. One intermediate size allele with 25 repeats was detected in a single PSP patient. These results are in accordance with published data where the distribution of the normal repeats ranged 2-14 in the MSA patients (AKIMOTO *et al*., 2013; CHEN *et al*., 2016) and 2-18 in the PSP patients (AKIMOTO *et al*., 2013; TICOZZI *et al*., 2014). Furthermore, for the alleles in the normal range up to 20 repeats, literature data show that 92.3% of patients have less than 10 repeats (AKIMOTO *et al*., 2013). Similarly, in our MSA and PSP group, 91.45% of the alleles had up to 10 repeats.

Since the discovery of the hexanucleotide repeat expansion in the *C9orf72* gene and its relevance to the genetic basis of ALS and FTD, many studies focused on the elucidation of the potential role of the G_4C_2 repeat expansions in other neurodegenerative disorders. Of particular interest were disorders presenting with parkinsonian symptoms due to the observation that the prevalence of parkinsonian symptoms among *C9orf72* expansion carriers is increased (BOEVE *et al*., 2012; SIMON-SANCHEZ *et al*., 2012). Bradykinesia or hypokinesia is present in 94% of the cases followed by rigidity and resting tremor in 86 % and 39% of the cases with parkinsonian features respectively (WILKE *et al*., 2016). Although a few positive cases were reported in PD (LESAGE *et al*., 2013), corticobasal syndrome (SCHOTTLAENDER *et al*., 2015), PSP (LE BER *et al*., 2013), and dementia with Lewy bodies (SNOWDEN *et al*., 2012) cohorts thus far, generally, there was not enough evidence to pinpoint *C9orf72* expansions as the cause of PD or atypical PD (reviewed in (BOURINARIS and HOULDEN, 2018)).

Expanded *C9orf72* alleles in MSA patients, thus far, were detected in the presence of positive family history for *C9orf72* associated ALS. Goldman et al. reported a *C9orf72* positive patient clinically diagnosed with possible MSA. This patient had a clinical picture of progressive gait ataxia, autonomic dysfunction without motor neuron signs, and her father and brother were diagnosed with ALS. Southern blot analysis detected more than 1000 repeats in both the patient and her brother (GOLDMAN *et al*., 2014). Another study reported a patient with olivopontocerebellar degeneration, a form of MSA, harboring *C9orf72* repeat expansion with a positive family history of ALS (LINDQUIST *et al*., 2013). On the other hand, several cohort studies on American, European (SCHOLZ *et al*., 2015; SCHOTTLAENDER *et al*., 2015), Chinese (CHEN *et al*., 2016; SUN *et al*., 2015), and Taiwanese (HSIAO *et al*., 2014) MSA patients, showed no association between *C9orf72* expansion and MSA.

In PSP cohorts, several studies reported *C9orf72* repeat sizes in the pathological range. The percent of the expansion carriers ranged from 0.8-8,33% (LESAGE *et al*., 2013; ORIGONE *et al*., 2013) in the total number of tested patients and 5.9-7 % among the patients with positive family history (LESAGE *et al*., 2013; LE BER *et al*., 2013). However, it should be noted that the number of PSP patients included in respective studies was variable and the study with the highest reported frequency of expansion carriers (8.33%) involved only 12 individuals, meaning that only one patient was positive for *C9orf72* expansion (ORIGONE *et al*., 2013). Several other

studies did not identify any *C9orf72* expansion among PSP patients (OGAKI *et al*., 2013; SCHOTTLAENDER *et al*., 2015; GALIMBERTI *et al*., 2013; YEH *et al*., 2013). Additional cases where G_4C_2 repeat expansions were reported in relation to PSP were in ALS-plus, i.e., ALS-PSP syndrome (TICOZZI *et al*., 2014). In line with these findings, the *C9orf72* repeat expansions were not found in Serbian MSA or PSP patients.

At present, repeat lengths of 20-30 are considered intermediate allele sizes (MAJOUNIE *et al*., 2012; NUYTEMANS *et al*., 2013). Their role in ALS and other neurodegenerative disorders is not well understood. In the literature, data are indicating that intermediate repeats could confer risk of neuropsychiatric symptoms but are not directly associated with different neurodegenerative diseases, including ALS (NG and TAN, 2017). On the other side, some studies and meta-analyses reported that intermediate alleles could be disease-causing in ALS (BYRNE *et al*., 2014; IACOANGELI *et al*., 2019). Also, intermediate repeats in patients with the clinical picture of FTD were reported (GOMEZ-TORTOSA *et al*., 2013). For PD and atypical PD cohorts, some studies did hypothesize that intermediate *C9orf72* repeats could be associated with the disease (NUYTEMANS *et al*., 2013; CANNAS *et al*., 2015). However, a large meta-analysis on PD cases (THEUNS *et al*., 2014) and a study in autopsy-confirmed PD cases failed to support that association (NUYTEMANS *et al*., 2014). There are not much data about the role of the intermediate repeats in PD-like disorders, but an interesting finding in autopsy-proven corticobasal degeneration cases showed that intermediate-length repeat expansions (where the minimum size cutoff of 17 repeats was applied) may be a genetic risk factor for CBD and obtained data based on cell models with intermediate repeats revealed that gene expression pathways related to vesicle trafficking and autophagy are affected (CALI *et al*., 2019). One study in the Chinese population detected a single patient clinically diagnosed with MSA to carry an intermediate allele of 20 repeats but with no further clinical data (CHEN *et al*., 2016). In this study, we detected one male patient with an intermediate number of repeats with the clinical picture of PSP and negative family history. According to the published data, one case of clinically diagnosed typical PSP with 27 G_4C_2 repeats with uncertain significance and positive family history for dementia and Parkinson's disease was reported (SCHOTTLAENDER *et al*., 2015). Also, patients with 26 and 30 repeats were reported having parkinsonism, supranuclear gaze-palsy, postural instability, dysarthria, and mild frontal dementia (LESAGE *et al*., 2013). Altogether, these data are not sufficient to draw more concrete conclusions on whether *C9orf72* intermediate alleles contribute to the PSP susceptibility.

Data about the correlation between the number of repeats and some clinical parameters in MSA and PSP are limited. Two studies on Chinese patients reported no significant association between the number of repeats and the age of onset in MSA (SUN *et al*., 2015; CHEN *et al*., 2016). The lack of association between the age of onset and the repeats below 30 was also reported in studies that included patients with PD, essential tremor, restless leg syndrome (DEJESUS-HERNANDEZ *et al*., 2013), FTD, ALS, and ALS/FTD altogether, concluding that there is no effect of unexpanded alleles on disease onset or disease phenotype (RUTHERFORD *et al*., 2012). On the other side, one study reported a positive correlation between disease onset and the size of the unexpanded alleles in PD patients (CHEN *et al*., 2016). In our MSA and PSP cohorts, the size of normal repeats does not correlate with disease onset, age at the time of the examination, nor disease duration. In the PSP group, the sum of both allele repeats number was bigger in patients

with positive family history, but considering the relatively small sample size of our group these results should be interpreted with caution. Further analysis on larger sample sizes should be performed.

This is the first result of *C9orf72* gene testing among MSA and PSP patients in the Serbian population. In conclusion, herein we report one Serbian PSP patient harboring intermediate-size *C9orf72* allele. Our PSP and MSA cohorts were of modest sample size and we cannot make general conclusions about the role of expanded or intermediate *C9orf72* alleles in PSP and MSA, further studies and meta-analyses might help in resolving this issue.

ACKNOWLEDGEMENTS

This study was supported by the Ministry of Education, Science and Technological Development, the Republic of Serbia (project ON175090 and ON175091, now contract no. 200110).

Received, January 10th, 2022
Accepted November 28th, 2022

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C9ORF72 EKSPANZIJA PONOVAKA NIJE UZROČNIK ATIPIČNOG PARKINSONIZMA U POPULACIJI SRBIJE

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Izvod

Ekspanzija ponovaka (G_4C_2) u nekodirajućem regionu gena *C9orf72* je najčešći genetički uzročnik ALS, FTD i ALS/FTD a prijavljena je i u drugim neurološkim bolestima sa različitom učestalošću. Između ostalog obuhvataju dva oblika atipičnog parkinsonizma, multiplu sistemsku atrofiju (MSA) i progresivnu nuklearnu paralizu (PSP). Genomska DNK 44 MSA bolesnika, 73 PSP bolesnika i 96 kontrola izolovana je iz periferne krvi i veličina normalnih *C9orf72* alela je određena standardnom fluorescentnom PCR amplifikacijom regiona sa ponovcima i fragmentnom analizom. Naknadno, za sve uzorke kod kojih je bio prisutan jedan alel rađen je repeat primed PCR sa dva različita seta prajmera u cilju isključenja lažno negativnih rezultata. Trideset ponovaka je korišćeno kao granica patogenosti. Nisu detektovane *C9orf72* patološke ekspanzije kod bolesnika ni kod kontrola. U MSA grupi najčešći alel je bio sa 2 ponovka a najveći broj detektovanih ponovaka je bio 14. Među PSP bolesnicima najčešći alel je takođe imao 2 ponovka dok je najveći broj detektovanih ponovaka u okviru normalne veličine bio 17. Identifikovan je jedan PSP bolesnik sa 25 ponovaka. Nisu utvrđene korelacije godina početka bolesti, starosti u trenutku uzorkovanja krvi, trajanja bolesti sa brojem ponovaka kod MSA ili PSP bolesnika. Kod PSP bolesnika zbir ponovaka na oba alela bio je veći u grupi sa pozitivnom porodičnom istorijom. Ovo istraživanje predstavlja prvu sistemsku procenu veličine *C9orf72* alela kod MSA i PSP bolesnika u populaciji Srbije. Iako uloga *C9orf72* intermedijarnih ponovaka u neurodegenerativnim bolestima tek treba da bude razjašnjena, naši rezultati podržavaju trenutna saznanja da ekspanzije *C9orf72* ponovaka nisu uzročnik MSA i PSP.

Primljeno 22.I.2022.
Odobreno 28. XI. 2022.