ORIGINAL ARTICLE

UDC: 575:61]:[616.8:616.74-007.23
DOI: 10.2298/VSP140328072Z

Association between the SMN2 gene copy number and clinical characteristics of patients with spinal muscular atrophy with homozygous deletion of exon 7 of the SMN1 gene

Povezanost broja kopija SMN2 gena i kliničkih karakteristika bolesnika sa spinalnom mišićnom atrofijom sa homozigotnom delecijom egzona 7 gena SMN1

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Abstract

Background/Aim. Spinal muscular atrophy (SMA) is an autosomal recessive disease characterized by degeneration of alpha motor neurons in the spinal cord and the medulla oblongata, causing progressive muscle weakness and atrophy. The aim of this study was to determine association between the SMN2 gene copy number and disease phenotype in Serbian patients with SMA with homozygous deletion of exon 7 of the SMN1 gene. **Methods.** The patients were identified using regional Serbian hospital databases. Investigated clinical characteristics of the disease were: patients' gender, age at disease onset, achieved and current developmental milestones, disease duration, current age, and the presence of the spinal deformities and joint contractures. The number of SMN1 and SMN2 gene copies was determined using real-time polymerase chain reaction (PCR). **Results.** Among 43 identified patients, 37 (86.0%) showed homozygous deletion of SMN1 exon 7. One (2.7%) of 37 patients had SMA type I with 3 SMN2 copies, 11 (29.7%) patients had SMA type II with 3.1 ± 0.7 copies, 17 (45.9%) patients had SMA type III with 3.7 ± 0.9 copies, while 8 (21.6%) patients had SMA type IV with 4.2 ± 0.9 copies. There was a progressive increase in the SMN2 gene copy number from type II towards type IV ($p < 0.05$). A higher SMN2 gene copy number was associated with better current motor performance ($p < 0.05$). **Conclusion.** In the Serbian patients with SMA, a higher SMN2 gene copy number correlated with less severe disease phenotype. A possible effect of other phenotype modifiers should not be neglected.

Key words:
muscular atrophy, spinal; genetic diseases, inborn; chromosome aberations; serbia.

Apstrakt

Uvod/Cilj. Spinalna mišićna atrofija (SMA) je autosomno recesivno oboljenje koje se karakteriše degeneracijom alfa motornih neurona kičmene moždine i produžene moždine, što uzrokuje progresivnu mišićnu slabost i atrofiju. Cilj rada bio je da se utvrdi povezanost broja kopija gena SMN2 i fenotipa kod srpske populacije bolesnika sa SMA sa homozigotnom delecijom egzona 7 gena SMN1. **Metode.** Podaci o bolesnicima sa SMA preuzeti su iz registara regionalnih bolnica u Srbiji. Ispitivane su sledeće kliničke karakteristike: pol bolesnika, uzrast pri pojavi bolesti, postignuti stepen motornog razvoja na početku bolesti i trenutno stanje, trajanje bolesti, sadašnji uzrast i prisustvo deformiteta kičme i kontrakture zglobova. Broj kopija gena SMN1 i SMN2 utvrđivan je pomoću lančane reakcije polimeraze (*polymerase chain reaction* – PCR) u realnom vremenu. **Rezultati.** Od 43 identifikovana bolesnika, 37 (86.0%) je imalo homozigotnu deleciju egzona 7 gena SMN1. Jedan (2.7%) od 37 bolesnika imao je SMA tipa I sa 3 kopije SMN2, 11 (29.7%) je imalo

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SMA tipa II sa 3.1 ± 0.7 kopija, 17 (45.9%) imalo je SMA tipa III sa 3.7 ± 0.9 kopija, dok je 8 (21.6%) bolesnika imalo SMA tipa IV sa 4.2 ± 0.9 kopija. Zabeležen je progresivan porast broja kopija gena SMN2 od tipa II ka tipu IV ($p < 0.05$). Veći broj kopija gena SMN2 bio je povezan sa boljim trenutnim motornim sposobnostima ($p < 0.05$). **Zaključak.** U srpskoj populaciji bolesnika sa SMA, veći broj kopija gena SMN2 koreliše sa blažim fenotipom bolesti. Pored toga, ne treba isključiti ni mogući uticaj drugih faktora koji mogu modifikovati fenotip.

Ključne reči:
mišići, atrofija, spinalna; genetičke bolesti, urođene; hromosomi, aberacije; srbija.

Introduction

Spinal muscular atrophy (SMA) is an autosomal recessive disease characterized by degeneration of alpha motor neurons in the spinal cord and the medulla oblongata, causing progressive muscle weakness and atrophy. The incidence of the disease is 1 per 10,000 live births, with a carrier frequency of approximately 1 in 50 [1, 2]. In 95% of cases, the cause is deletion of both copies of exon 7 on the SMN1 gene in chromosome 5, whereas point mutations are present in the rest of the cases [3].

The SMN1 gene normally provides production of fully functional survival of the motor neuron protein (SMN), unlike the SMN2 gene transcript, which results in 80–90% of the cases with dysfunctional protein chains [4]. Insufficient production of SMN1 results in apoptosis of alpha motor neurons [5]. Several studies have demonstrated a correlation between the number of SMN2 gene copies and the type of SMA. The lowest number of SMN2 gene copies is seen in SMA type 1, while in other types this number is higher [6–8]. However, the correlation between symptom severity and SMN2 copy number is not linear and there seem to exist other factors impacting on the disease phenotype, such as gender, other genes, epigenetics and variant point mutations [9].

The aim of this study was to determine the association between SMN2 gene copy number and disease phenotype of Serbian SMA patients with homozygous deletion of exon 7 of the SMN1 gene.

Methods

The study included 43 patients with SMA identified through hospital databases of the Neurology Clinics in Novi Sad and Belgrade in 2011. It was approved by the Ethical Committee of the Clinical Center of Vojvodina, Novi Sad. All the patients or the adult guardians of the children gave written informed consent to take part in the study.

SMA was uniformly classified according to the International SMA Consortium Meeting Report recommendations [10]. Disease onset before 6 months of age, i.e. before the ability to sit independently, was classified as SMA type I. Disease onset between 6 and 15 months of age, with the absence of the ability to walk independently, was classified as SMA type II. Disease onset after the patient developed the ability to walk, between 15 months of age and 20 years, was classified as SMA type III. Disease onset after the age of 20 years was classified as SMA type IV.

Medical documentation and clinical examination were used to collect the following data: patients' gender, age at disease onset, achieved and current developmental milestones (graded as follows: not able to sit, sitting with support, sitting independently, walking with support, walking independently), disease duration, current age, and the presence of the spinal deformities and joint contractures.

Molecular diagnosis

The genomic DNA isolation from single samples was performed by the silica membrane-based DNA extraction using the QIAamp DNA Micro Kit (Qiagen, Hilden, Germany) and followed by the spectrophotometric measurement of DNA concentration in each isolate. Isolated nuclear DNAs were amplified using the Taqman Universal PCR Master Mix. For SMN1 and SMN2 exon 7 amplification, we used the sequences of primers and probes described by Passon et al. [11]. The same authors described the conditions for performance of PCR reactions. PCR conditions were 2 min 50°C, 10 min 95°C, 40 cycles consisting of 15 s 95°C, and 1 min 60°C. Serum albumin was employed as a reference gene. The PCR reaction of amplification of SMN1, SMN2 and the reference gene was performed using the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The DNA sample of the patient with a known SMN1 copy number and the DNA sample of the patient with a known SMN2 copy number were used as calibrators in all SMN1 and SMN2 exon 7 amplification reactions. DNA of each sample, including calibrators, was used for amplification of SMN1, SMN2 and the reference gene. SMN1 and SMN2 copy number was determined using $\Delta\Delta C_t$ method.

Statistical tests were performed using the commercial software SPSS (IBM, USA), with the level of statistical significance of $p < 0.05$ and the high statistical significance of $p < 0.01$. The following methods of descriptive statistics were used: proportion, mean and standard deviation. For comparisons between non-continuous variables, χ^2 test was used. For continuous non-normal distributions, we used Mann-Whitney *U*-test for analysis between the two groups, and Kruskal-Wallis test for analysis of more than two groups. For normal distributions, data were analyzed using Student *t*-test for comparison between the two groups and ANOVA one way breakdown test for comparison between three or more groups. We also calculated the odds ratio (OR) with 95% confidence interval (CI) describing the risk of patients with 4 or 5 copy numbers of SMN2 gene compared to patients with 3 copies to develop mild form SMA (type 3 or 4 SMA *vs* type 2). Comparison of survival in patients with different SMN2 gene copy numbers was analyzed by the Kaplan–

Meier log-rank test, with SMA onset as zero time and inability to walk as the end-point event.

Results

A total of 43 patients with SMA were identified. Among them, 37 (86.0%) patients showed homozygous deletion of SMN1 exon 7. The additional four (9.3%) cases showed deletion of the exon 7 of only one allele of SMN1 gene, while two (4.7%) patients had both copies of the SMN1 exon 7. Only the homozygous cases were further analyzed in the study.

Only one of 37 (2.7%) patients with homozygous deletion had SMA type I. SMA type II was registered in 11 (29.7%) cases, SMA type III in 17 (45.9%) and SMA IV in 8 (21.6%) patients.

Sociodemographic and clinical features of investigated patients with different SMA types are presented in Table 1. There was no association between disease type and gender ($p > 0.05$). Significant association between better current motor performance and less severe SMA types was observed ($p < 0.01$). Also, the increased incidence of cases with spine and extremity deformities was observed in more severe SMA types ($p < 0.05$).

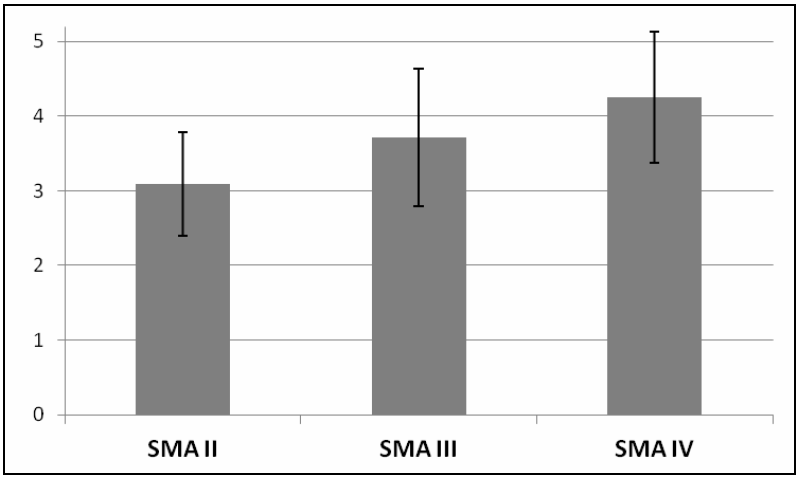
Regarding all SMA types, mean number of SMN2 copies was 3.6 ± 0.9; 2 copies were present in 1 (2.8%) patient, 3 in 20 (55.6%), 4 in 7 (19.4%), 5 in 7 (19.4%) patients and 6 in 1 (2.8%) patient. A significant increase in SMN2 copy number was observed from SMA type II towards types IV (3.1 ± 0.7 copies in SMA II, 3.7 ± 0.9 in SMA III, and 4.2 ± 0.9 in SMA IV, $p < 0.05$) (Figure 1). The patients with 4 copies of SMN2 gene had 1.6 times higher risk to develop mild form of SMA (OR 1.6, 95% CI 1.1–2.4; $p < 0.05$), while patients with 5 copies had 4.9 times higher risk but this was not

Table 1

Sociodemographic and clinical features of the investigated patients with spinal muscular atrophy (SMA) types II, III and IV (n = 36)

Features	SMA type II	SMA type III	SMA type IV
Number of patients	11	17	8
Gender (males), %	36.4	47.1	25.0
Age at disease onset (years), mean ± SD**	1.00 ± 0.00	5.24 ± 6.02	33.62 ± 16.69
Achieved milestones, % **			
not able to sit	0	0	0
sitting with support	27.3	0	0
sitting independently	27.3	0	0
walking with support	45.4	0	0
walking independently	0	100	100
Disease duration (years), mean ± SD	11.81 ± 6.64	22.05 ± 16.38	12.37 ± 11.24
Current age (years), mean ± SD**	12.36 ± 6.74	27.29 ± 17.02	46.00 ± 15.38
Current motor performance, % **			
not able to sit	0	0	0
sitting with support	36.4	5.9	0
sitting independently	36.4	17.7	0
walking with support	27.2	11.8	37.5
walking independently	0	64.6	62.5
Spinal deformities and joint contractures, % **	72.7	41.2	37.5

**$p < 0.01$



$p < 0.05$

Fig. 1 – SMN2 gene copy number in the patients with different types of spinal muscularatrophy(SMA) with homozygous deletion of exon 7 in SMN1 gene (n = 36).
SMA II – disease onset between 6 and 15 years of age with the absence of the ability to walk independantly; SMA III – disease onset developed the ability to walk, between 15 months of age and 20 years; SMA IV – disease onset after 20 years.

of statistical significance due to the small number of observed cases (OR 4.9; 95% CI 0.5–48.6; $p > 0.05$).

The SMN2 gene copy number did not depend on gender ($p > 0.05$). Also, it did not show any correlation with achieved motor milestones ($p > 0.05$) and with the presence of the spine deformities and limb contractures ($p > 0.05$). A higher SMN2 gene copy number was associated with a better current motor performance ($p < 0.05$) (Table 2). Log-rank test showed similar survival until inability to walk in patients with 4 and 5 SMN2 gene copy numbers, thus we finally compared survival in the patients with 3 *vs* 4 or 5 copies (Figure 2). In the patients with 3 copies inability to walk developed after 23.7 (95% CI 12.2–35.2) years, and in those with 4 or 5 copies after 37.4 (95% CI 31.5–43.2) years ($p < 0.05$).

Table 2

Association between SMN2 gene copy number and current motor performance in the patients with spinal muscular atrophy (SMA) (n = 36)

Achieved milestones	SMN2 gene copy number
Not able to sit	2.8 ± 0.5
Sitting with support	3.0 ± 0.0
Sitting independently	3.5 ± 0.8
Walking with support	3.6 ± 0.9
Walking independently	3.8 ± 1.0

***p* < 0.05**

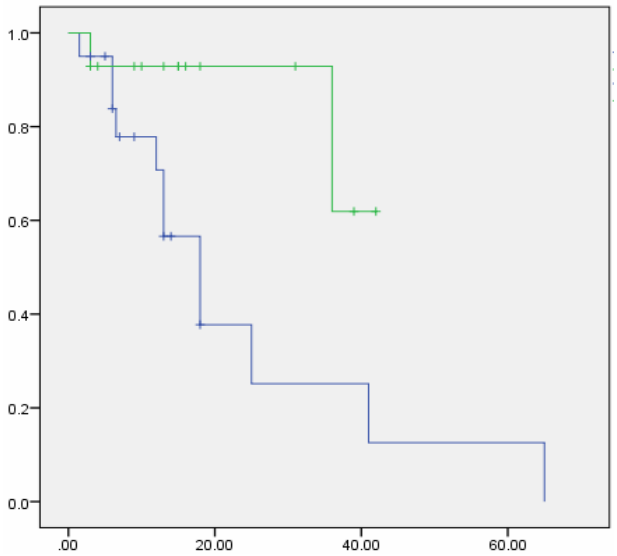


Fig. 2 – Survival analysis until inability to walk in the patients with 3 *vs* 4 or 5 SMN2 gene copy numbers.
x-axis – duration of disease until inability to walk; y-axis – percentage of patients who are able to walk; $p < 0.05$ when compare the patients with 3 SMN2 gene copies (in blue) with 4 or 5 SMN2 gene copies (in green).

Discussion

The proportion of our SMA patients with homozygous deletion of exon 7 of the SMN1 gene was 86% which is similar to the findings of other authors, i.e. 94–95% [12]. Deletion of the exon 7 of only one allele of SMN1 gene was found in 9% of our cases and it was probably in association with the point mutation on the other allele which we were not able to test. It is also possible that 5% of patients with both copies of the SMN1 exon 7 had two point mutations, one in each SMN1 copy. However, one should not rule out the possibility that disease in these patients might be caused by mutations in some other gene.

Almost half of our patients had SMA type III, while SMA type I was registered in only one patient. Short survival of patients with SMA type I, and a cross-sectional design of the study resulted in an almost complete lack of patients with the most severe form of SMA.

Less severe SMA types were in association with later age at onset and better milestones achievement, which was expected by SMA classification [10]. Also, patients with less severe SMA types were elder and had better current motor performance which is in line with previous findings that patients with later onset of SMA have normal or almost normal life expectancy [13]. Furthermore, later disease onset is accompanied by a milder disease course and longer independent ambulation [14]. We also observed the increased incidence of cases with spine and extremity deformities in more severe SMA types. It is well-known that muscle weakness in SMA patients leads to the limited joint motion and consequent contractures [15]. Muscle weakness is considered the main factor in the etiology of these changes [16, 17].

In the present study, the number of SMN2 gene copies was statistically significantly higher in the patients with less severe SMA types which correspond with the published data. Patients with SMA type I usually have 1–3 SMN2 gene copies, most frequently 2 [8, 18, 19], patients with type II have 2–4 copies, usually 3 [8, 18, 20]. In type III, more than 90% of cases have 3–4 copies [8, 17, 19]. In type IV, 4–6 SMN2 gene copies have been found [21]. Furthermore, in our patients with the SMA number of SMN2 gene copies correlated with better current motor performance. Survival until inability to walk was almost 14 years longer in SMA patients with 4 or 5 copies of SMN2 gene compared to patients with only 2 copies.

According to our and previous findings, it is generally possible to predict SMA severity on the basis of the SMN2 gene copy number [6–8]. However, the absence of the direct correlation of the SMN2 gene copy number with achieved motor milestones and the presence of the spine deformities and limb contractures, suggest that clear prognosis of SMA phenotype cannot be based only on SMN2 copies. This is in line with previous data [18]. The prognosis might be more precise with determination of the status of another gene responsible for synthesis of the neuronal apoptosis inhibitory protein (NAIP). In SMA patients, homozygous deletion of the NAIP gene is present in 44.8% of patients, and the highest number of patients with homozygous deletions of the NAIP gene and the SMN1 gene belongs to the SMA type I [8, 9]. Other disease modifiers are suggested in the literature, such as genes for plastin 3, chondrolectin, NAIP, p44, H4F5, occludin etc. [22–24]. Also, a larger deletion of the SMN1 gene might be in association with more severe phenotype since neighbor genes (SERF1A, GTF2H2, RAD17) may also be affected. Studies are contradictory in regard to differences in the severity of SMA between males and females. While one study found a greater decline in female pulmonary function [25], another one showed that feminine gender has a mitigating effect on disease severity [17]. In our study patients gender

was not associated with SMA type, the presence of spine and joint contracture, and current motor status. Furthermore, the number of SMN2 gene copies does not differ between sexes. This finding is in accordance with previous studies [9, 25].

Conclusion

In Serbian patients with spinal muscular atrophy, a higher SMN2 gene copy number correlated with the less severe disease phenotype. A possible effect of other phenotype modifiers should not be neglected.

Acknowledgements

This work was funded by the Hungary-Serbia IPA Cross-Border Co-Operation Program: Research Cooperation to Improve Symptoms in Neurological Disorders, and Quality of Life of Patients. 2010-2012, Project ID: HU-SRB/0901/214/052.

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Received on March 28, 2014.
Revised on August 8 , 2014.
Accepted on August 13, 2014.
Online First August, 2015.