Genetic Modifiers of the Spinal Muscular Atrophy Phenotype

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Abstract—Proximal spinal muscular atrophy (SMA) is an autosomal recessive neurodegenerative disease caused by homozygous deletion in the seventh exon of the *SMN1* gene. The aim of this work is to analyze the association of the allelic polymorphism of telomeric genes *SMN1* and *NAIP* and the centromeric gene *SMN2* of the 5q13 region with the clinical phenotype of SMA. It was shown that the homozygous genotype, which contains a telomeric deletion, covering both *SMN1* and *NAIP*, is significantly more often observed in patients with the most severe type of SMA. Three or more copies of *SMN2* are associated with a milder phenotype; the number of *SMN2* copies affects the SMA phenotype more heavily than the length of the telomeric deletion. It was shown that one *SMN2* copy is significantly more frequent than three or more copies of this gene in SMA-patients with homozygous deletion of *SMN1* and *NAIP*. This fact may indicate the presence of a large deletion of all the three studied genes in SMA genotypes associated with the most severe type of SMA. It is noted that congenital SMA (type 0) is significantly less common in female patients, which may indicate the presence of SMA modifier genes on the X-chromosome.

Keywords: spinal muscular atrophy, *SMN1*, *NAIP*, *SMN2*, phenotype, gender

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INTRODUCTION

Proximal spinal muscular atrophy (SMA) is a monogenic autosomal recessive disease characterized by degeneration of the anterior horn cells of the spinal cord, less often the motor nerves of the brain stem, which leads to symmetrical muscle weakness and atrophy [1]. In terms of the severity of the disease, childhood SMA is classified into four clinical types according to the age of onset, maximum muscle activity, and patient survival [2, 3]. Type 0, congenital SMA: diagnosed in the prenatal or early perinatal period, children have severe hypotension since birth and need breathing support. Life expectancy in most cases does not exceed 6 months. Type I, childhood SMA, Verdnig-Hoffmann disease: the first symptoms of the disease appear at the age of 1−6 months. Most often, life expectancy is limited to 2 years. Patients have delayed motor development, difficulties with breathing, sucking, and swallowing, children do not hold their heads and cannot sit without support. Type II, intermediate type of SMA: the onset of the disease occurs in the period of 0.5−2 years. Patients with this type of SMA can eat, sit on their own, but can never walk on their own. Life expectancy is reduced, patients live up to 18 years, the prognosis in these cases depends on the degree of pathology of the respiratory muscles. Type III, juvenile SMA, Kugelberg-Welander disease: symptoms

appear in 12−36 months. The most favorable type of disease, life expectancy is reduced slightly. The patient is able to stand and walk, but experiences severe muscle weakness with a tendency to disability (wheelchair mobility). In addition, a very rare type IV, adult SMA, is distinguished: symptoms appear in the second decade or even later, motor function is hardly impaired (frequency 1−9 : 1000000).

The 5q13 chromosomal region contains an inverted duplication of 500 kbp, which is found only in humans and represents a genetically unstable region with "hot spots" of nonallelic homologous recombinations (ectopic recombination, ER) [4]. Due to ER, this region is subject to genomic reorganizations: deletions, duplications, and gene conversions. The genetic determinants of SMA are homozygous deletions in the telomeric copy of duplication. Four genes were identified in this part of the 5q13 locus, including *SMN1* (the survival motor neuron gene 1) and *NAIP* (the neuronal apoptosis inhibitory protein gene). The severity of the SMA phenotype correlates with the length of the deletion in the telomeric copy of the 5q13 locus, which can include both *SMN1* and *NAIP* [5]. The centromeric region contains copies of these genes (pseudogenes and expressed genes), which differ slightly in nucleotide sequence from ancestral telomeric copies of genes. The *SMN2* gene is an expressed

copy of the *SMN1* gene. Genetic factors are known that modify the severity of the SMA phenotype, one of which is the variation in the *SMN2* gene copy number, which is due to ER-mediated genomic reorganizations.

The *SMN1* and *SMN2* genes encode the SMN protein, which is present both in the cytoplasm and in the nucleus of cells of various tissues in the composition of SMN complexes. This protein is most actively expressed in motor neurons of the spinal cord, muscles, and liver; in patients with SMA, its expression is most significantly reduced in the spinal cord [6, 7]. SMN is necessary to maintain the activity of motor neurons that control muscle movement. Protein products of the *SMN1* and *SMN2* genes are involved in pre-mRNA splicing, transport of mature mRNA, and axon growth [8, 9]. In SMA patients with homozygous deletions of the *SMN1* gene, protein is expressed from *SMN2*, which is able to provide 10−30% of the SMN protein required for motor neurons [10, 11].

Until recently, there was no effective treatment for this disease. In recent years, the FDA (US Food and Drug Administration) and EMA (European Medicines Agency) have approved several drugs for the treatment of SMA using gene therapy [12, 13], and several more are undergoing clinical trials. However, despite the breakthrough in gene therapy for SMA, the price of such treatment ranges from 500 000 to 2 million dollars. In addition, the effectiveness of gene therapy drugs directly depends on the patient's genotype, not only on the determinant gene (*SMN1*) but also all possible genetically determined modifiers of the clinical phenotype. Therefore, the identification of the role of genetic factors in the modification of the clinical phenotype of SMA remains relevant both for basic research and for assessing the economic component of therapy.

This paper aims to analyze the association of allelic polymorphism of the *SMN1*, *SMN2*, and *NAIP* genes of the 5q13 chromosome region and other genetic factors with the clinical phenotype of spinal muscular atrophy.

MATERIALS AND METHODS

Analysis of clinical data and sampling. The object of research is a DNA collection isolated from peripheral blood lymphocytes of patients with clinical signs of childhood spinal muscular atrophy from various regions of Ukraine. In the period of 1990−2018, blood samples and clinical data of patients, subject to informed consent, were provided by regional medical-genetic centers of Ukraine and Crimea and clinics in Kyiv: OKhMATDET, Isida-IVF, and Institute of Pediatrics, Obstetrics, and Gynecology of the National Academy of Sciences of Ukraine.

DNA was isolated by the standard method: hydrolysis of cell lysates with proteinase K (Termo Fisher Scientific, EU) followed by phenolic extraction [14]. The quality and quantity of DNA preparations was determined by spectral characteristics and by electrophoresis in 1.2% agarose gel. The criteria for assessing the quality of DNA samples consisted in the ratios of optical density λ260/λ280 and λ260/λ230 calculated using the ND-1000 Spectrophotometer (NanoDrop, United States).

Determination of the copy number of the SMN2 gene using quantitative real-time PCR. Two variants of quantitative PCR (qPCR) were used in this work: with an intercalating dye (screening method) and TaqMan probes (refinement method). qPCR with TaqMan probes was used to more accurately determine copies of the studied gene in DNA samples with three or more *SMN2* copies, since this method has a higher resolution [15−17].

To amplify exon sequences of the *SMN2* and *ALB* genes, we used specific primers and probes published previously with our modifications [17−19]. The specificity of the modified oligonucleotide sequences was verified using the online resources NCBI BLAST, WASP, and Genome Browser Gateway [20−22]. Considering the fact that the studied nucleotide sequence of the seventh exon of the *SMN2* gene differs from the corresponding sequence of the *SMN1* gene by only one nucleotide, it was critical for primer design to provide the specificity of annealing of primer oligonucleotides close to 100%.

The reaction mixture for qPCR with the intercalating dye EvaGreen® in a volume of 20 μL contained a 1-time commercial mixture HOTFIREPol® EvaGreen® qPCRSuper-mix (SolisBiodyne, EU), 0.7 μm primers, and 50−75 ng (5 μL) of the genomic DNA template.

For qPCR with TaqMan probes, 20 μL of the mixture contained a onefold commercial mixture HOT FIREPol® Probe qPCR MixPlus (SolisBiodyne, EU), 0.3 μm probes (with FAM dye for *SMN* and Cy5 for *ALB* genes, synthesized by Simesta, Odessa), 1.0 μm primers, and 50−75 ng (5 μL) of the genomic DNA template.

The qPCR reaction was carried out using the CFX96 real-time PCR system (Bio-Rad, United States). The samples were preheated for 12 min at 95°C to denature the DNA and activate the HOT FIREPol® DNA polymerase. Further PCR was carried out in the following temperature regime: 95°C for 15 s, 57°C for 40 s, and 72°C for 20 s, 40 cycles. The fluorescence signal was detected at the end of the elongation stage. The relative copy number of the *SMN2* gene was calculated using the simplified Livak formula ($2^{-\Delta\Delta Ct}$) [23]. Quantitative PCR values were evaluated using the CFX Manager™ Software (Bio-Rad).

Statistical processing of results. The results were evaluated using the OpenEpi software package, version 3.0. The significance of the revealed difference in the frequency of genotypes was evaluated using the χ^2

test. The significance of differences in the frequency distribution of genotypes and phenotypes was evaluated by the OR (Odds Ratio) indicator. The relationship of qualitative characteristics was analyzed using the Spearman correlation analysis.

The threshold value of statistical significance for all the tests was $p < 0.05$.

RESULTS AND DISCUSSION

Homozygous gene deletions of the telomeric copy of the 5q13 region (seventh and eighth exons of *SMN1* and fifth exon of *NAIP*) were studied in 1950 patients clinically diagnosed with SMA. A homozygous deletion of at least the seventh exon of the *SMN1* gene was identified in 571 probands, which confirms their clinical diagnosis. To analyze the copy number of the *SMN2* gene, DNA material was collected from 170 probands with the confirmed diagnosis of SMA; these samples constituted the study group. The quality and quantity of DNA samples was also one of the selection criteria, since these characteristics are critical for qPCR.

The study sample included patients with different ages of disease onset (from birth to 3 years of age) and different degrees of motor neuron damage, which met the criteria for all of the above types of SMA. The minimum age of the patients was 0.01 years and the maximum age was 42.76 years. The following distribution by types of SMA was observed: type 0 was encountered with the lowest frequency, 16%; II and III were more common, 29 and 20%, respectively; the most frequent was type I, 35%.

The most common patients had a homozygous deletion of the seventh and eighth exons of the *SMN1* gene (63.5%). Less common were probands with a longer deletion, which involved the *NAIP* gene (21.8%) in addition to the *SMN1* gene. The rarest was the genotype with a homozygous deletion of only the seventh exon of the *SMN1* gene (14.7%).

Previously, we found that patients with a homozygous deletion of only the seventh exon of the *SMN1* gene are significantly more likely to have type III SMA, while probands with a homozygous deletion of two telomeric genes (*SMN1* and *NAIP*) are significantly more likely to be diagnosed with SMA of types 0 and 1 [18]. Table 1 shows the frequency distribution of genotypes for the *SMN1* and *NAIP* genes in SMA probands with various clinical types of disease in the study sample ($n = 170$).

The obtained results are consistent with the data that we obtained earlier. It was found that patients with a congenital type of SMA (type 0) significantly less frequently had a genotype with a homozygous deletion of only the seventh exon of the *SMN1* than a genotype with an extended telomeric deletion, while patients with type II SMA significantly more often had a deletion of the seventh and eighth exons of the *SMN1* gene

Table 1. Frequency distribution of genotypes for homozygous deletion of *SMN1* and *NAIP* in patients with different types of SMA

Type of SMA	Telomeric deletion genotype		
	del ex7	del ex7, 8	del ex7, 8, 5
0 type ($n = 28$)	0.036*	0.464	0.500*, †
I type ($n = 59$)	0.170	0.559	0.271
II type ($n = 49$)	0.163	0.755*	0.082*, †
III type ($n = 34$)	0.177	0.735	0.088†

del ex7, homozygous deletion of the seventh exon of the *SMN1* gene; del ex7, 8, homozygous deletion of the seventh and eighth exons of the *SMN1* gene; del ex7, 8, 5, homozygous deletion of the seventh and eighth exons of the *SMN1* gene as well as the fifth exon of the *NAIP* gene; *, †, differences in the frequencies of genotypes are significant, $p < 0.05$.

in a homozygous state than a genotype with a more extended homozygous deletion of the telomeric genes. On the other hand, an extended telomeric deletion in a homozygous state is significantly more common in patients with the most severe type 0 SMA than in patients with types II and III.

The contribution of deletions of the *SMN1* and *NAIP* genes to the modification of the disease phenotype was estimated using correlation analysis. The Spearman correlation coefficients between the type of SMA and the length of the homozygous deletion of the telomeric region 5q13 ($r = -0.30$; $p = 8 \times 10^{-5}$) were calculated. According to the data obtained, the revealed correlation is statistically significant and confirms the previously obtained data. However, the correlation is rather weak since it explains no more than a third of the SMA phenotypes in our patient sample. The data obtained indicate that this disease has more significant modifying factors.

One of the SMA modifier genes is the centromeric gene *SMN2*. In our study, we analyzed the number of copies of this gene in the DNA samples of SMA patients and the associations of the number of *SMN2* copies with the clinical type of SMA. According to the data obtained (Fig. 1), it was found that the majority of patients in the study sample had a genotype with two copies of *SMN2* (56%), less often genotypes with 1, 3, or 4 copies. In isolated cases, patients were identified to have five copies of this gene.

We analyzed the distribution of genotype frequencies by the number of *SMN2* gene copies in each subgroup of SMA patients depending on their clinical phenotype (Table 2).

It should be noted that the number of *SMN2* gene copies, except for one patient, did not exceed two in patients with severe congenital form of the disease (type 0, average age 0.18 years, SD = 0.16). Moreover, in almost half of the cases these patients had only one copy of the *SMN2* gene, while patients with a mild
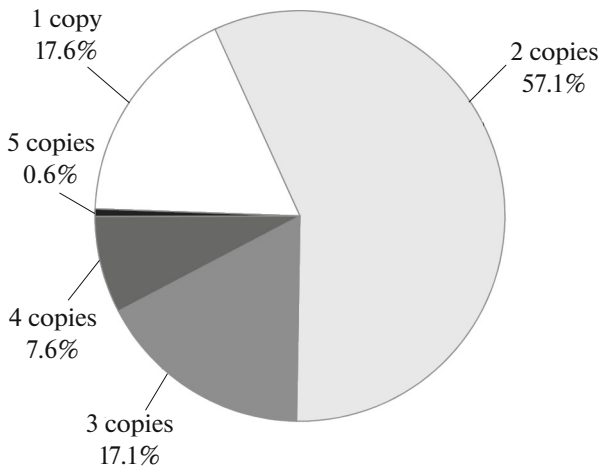
Fig. 1. Distribution of genotype frequencies for the copy numbers of the *SMN2* gene in SMA patients with homozygous deletions in the *SMN1* gene (*n* = 170).

type of SMA (type III, average age 18.74 years, SD = 12.50) had no genotypes with one copy of this gene. Based on these results, we can assume that the genotype with one copy of the *SMN2* gene is the most unfavorable for predicting the course of SMA and the life expectancy of patients. It was found that the frequency of genotypes with one and two copies of the *SMN2* gene (84.4%) is significantly higher (*p* < 0.0001) in patients with types 0–II SMA compared with patients with type III (33.3%). Genotypes with 3–5 copies were significantly more often (*p* < 0.0001) observed in the subgroup of patients with type III SMA (66.7%) than in patients with severe types of SMA (15.6%). Genotypes with four and five copies of the *SMN2* gene were significantly more common in patients with type III SMA (*p* < 1 × 10⁻⁵). It is significant that the majority of patients with type III SMA (23 out of 33 patients, 69.7%) at the time of the last examination were older than 10 years, and the average age in this group was 18.56 years (SD = 12.65), which is well above the average age of patients with more severe types of SMA.

To assess the significance of the associations of the copy number of *SMN2* with the SMA phenotype, a correlation analysis was carried out similar to the analysis carried out for telomeric deletions. It was found

Table 2. Frequencies of genotypes for the *SMN2* gene in patients with different clinical types of SMA (*n* = 170)

Type of SMA	Number of copies *SMN2*				
	1	2	3	4	5
0 type (*n* = 28)	0.429	0.500	0.071	0	0
I type (*n* = 59)	0.220	0.644	0.119	0.017	0
II type (*n* = 49)	0.102	0.674	0.204	0.02	0
III type (*n* = 34)	0	0.353	0.294	0.324	0.029

that the copy number of *SMN2* correlates with the SMA phenotype (*r* = 0.50; *p* = 4.35 × 10⁻¹²): the more copies of *SMN2*, the less severe the SMA phenotype. Let us note that the copy number of *SMN2* correlates much more with the SMA type than the extent of telomeric deletion.

Considering that genomic reorganizations at the 5q13 locus, which result in deletions and duplications of the studied genes, are of recurrent origin, we assumed that one of the reorganizations that led to the formation of a mutant allele with deletion of both the *SMN1* and *NAIP* genes can also involve the centromeric region; as a result, the *SMN2* gene is deleted as well. In this case, the association of the *NAIP* homozygous deletion with the severe SMA phenotype may be not so much a consequence of the role of *NAIP* in the survival of motor neurons but primarily due to the lower copy number of *SMN2*. On the other hand, patients with a homozygous deletion of only the seventh exon of the *SMN1* gene, the cause of which in most cases is a gene conversion with the formation of the hybrid gene *SMN2/SMN1*, have a less aggressive course of SMA [24]. According to the hypothesis of the decisive role of the *SMN2* gene in modifications of the SMA phenotype, we can assume that the gene conversion with the formation of the *SMN2/SMN1* gene, the seventh exon of which has the nucleotide sequence of the *SMN2* gene and the eighth exon has that of the *SMN1* gene, leads to the formation of a gene with the exon sequence not different from *SMN2*. Correspondingly, when the centromeric part of 5q13 has no deletions, the copy number of the seventh exon of the *SMN2* gene in the hybrid gene increases to three or more copies. In this case, an increase in the production of protein SMN2 plays a crucial role in alleviating the SMA phenotype in patients with a hybrid gene. To confirm our assumptions about the complex nature of the reorganization of the 5q13 locus, we analyzed the association of the length of telomeric deletions with the copy number of the centromeric gene *SMN2* (Table 3).

According to the data obtained, a relationship was found between the type of telomeric deletion and the copy number of the *SMN2* gene. Patients with a homozygous deletion of the two studied telomeric genes (*SMN1* and *NAIP*) significantly more often than carriers of a homozygous deletion of only the seventh exon of the *SMN1* gene had one copy of *SMN2* (OR = 7.7; CI: 1.44–63.07; *p* = 0.01). Accordingly, genotypes of patients of the second group (the alleged heterozygous carriers of the hybrid gene *SMN2/SMN1*), in comparison with the first group, significantly more often had three or more copies of *SMN2*. These results indicate that an extended deletion that involves the *NAIP* gene adjacent to *SMN1* can also contain centromeric genes, including the *SMN2* gene. Other types of telomeric deletions are much less likely to be accompanied by *SMN2* deletions, presumably due to the fact that the probability of two independent reorganiza-

Table 3. Frequency distribution of genotypes for the *SMN2* gene in patients with different types of telomeric reorganizations of the 5q13 locus (*n* = 170)

Type of telomeric deletion	Number of *SMN2* copies		
	1	2	≥3
del ex7 (*n* = 25)	0.08*	0.56	0.36*
del ex7, 8 (*n* = 107)	0.18	0.54	0.28
del ex7, 8, 5 (*n* = 38)	0.24*	0.66	0.10*

del ex7, homozygous deletion of the seventh exon of the *SMN1* gene; del ex7, 8, homozygous deletion of the seventh and eighth exons of the *SMN1* gene; del ex7, 8, 5, homozygous deletion of the seventh and eighth exons of the *SMN1* gene as well as the fifth exon of the *NAIP* gene; *, differences in the frequencies of genotypes are significant, *p* < 0.05.

tions (in the telomeric and centromeric copy of the 5q13 locus) is lower than the probability of one extended deletion involving both the telomeric and centomeric copies of the region. The hypothesis that the formation of the hybrid gene *SMN2/SMN1* is associated with the higher copy number of *SMN2* is supported by data from population studies: the frequency of genotypes with three or more copies of *SMN2* is significantly higher in patients with SMA and their families than in population samples [25, 26]. Data on the presence of molecular-genetic mechanisms leading to the formation of complex reorganizations were also obtained in studies of other authors [27, 28].

Despite the fact that the copy number of the *SMN2* gene significantly affects the SMA phenotype, the revealed correlation does not explain all disease modifications in the studied sample of patients. It follows that the search for SMA modifier genes is not complete. When analyzing the clinical base of the sample of patients with SMA, it was noted that the most severe clinical type 0 SMA was significantly more often (*p* = 0.02) observed in boys. For other types of SMA, no statistically significant differences were found between the frequencies in boys and girls (Fig. 2).
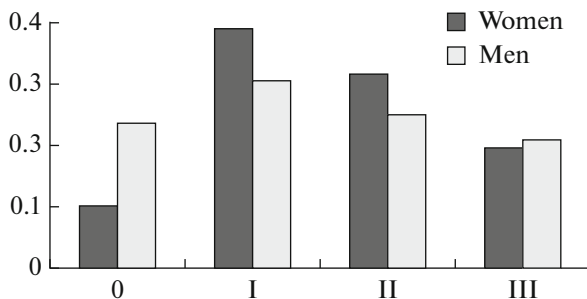

Fig. 2. Distribution of frequencies (vertical) of SMA phenotypes (horizontal) in men and women (*n* = 170).

Let us note that the frequency distributions of genotypes for telomeric deletions and copies of the *SMN2* gene in boys and girls in our sample of patients do not differ significantly (*p* = 0.113 and *p* = 0.534, respectively).

Since gender differences were revealed only for the congenital type of SMA, which is characterized by an onset in the perinatal period, the obtained data may indicate that women with SMA may have specific neuroprotective mechanisms in the early period of development as well as a lesser role of these factors in the postnatal period. Considering the hemizygous state of alleles of X-chromosome genes in men, as well as the presence of epigenetic regulation of the expression of such genes in women (especially in the prenatal period), it can be assumed that SMA modifier genes can, among other things, be X-chromosome genes. Therefore, it seems promising to us to further investigate X-linked SMA modifiers.

CONCLUSIONS

The homozygous genotype with an extended deletion that involves, in addition to the seventh and eighth exons of the *SMN1* gene, the fifth exon of the *NAIP* gene is significantly more often observed in patients with SMA types 0 and Ib, in contrast to patients with less severe types of disease, which more often have a genotype with a homozygous deletion of only the seventh or seventh and eighth exons of the *SMN1* gene (OR = 5.39). It was found that the copy number of *SMN2* correlates with the type of SMA: the more copies, the less severe the SMA phenotype. The copy number of *SMN2* has a stronger effect on the SMA phenotype ($r = 0.50$; $p = 4.35 \times 10^{-12}$) than the length of the telomeric deletion ($r = -0.30$; $p = 8.00 \times 10^{-5}$). SMA patients with a homozygous deletion involving the *NAIP* gene significantly more often than carriers of a homozygous deletion of only the seventh exon of *SMN1* have one copy of *SMN2* (OR = 7.7; CI: 1.44–63.07; $p = 0.01$). It is likely that an extended telomeric deletion can also involve centromeric genes, including the *SMN2* gene, and be a risk factor for the most severe type of SMA. The congenital type 0 SMA is significantly more often ($p = 0.02$) observed in boys. This may indicate the presence of SMA modifier genes on the X chromosome.

A.A. Solov'ev, and S.S. Podlesnaya, who actively participated in the formation of the collection of DNA samples from patients with SMA and in the genotyping of patients by *SMN1* and *NAIP* in the period of 1995−2016.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest. The authors declare that they have no conflict of interest.

Statement of compliance with standards of research involving humans as subjects. All procedures carried out in the studies involving people were consistent with international and national standards, the Helsinki Declaration of 1964, and its later amendments, and approved by the Bioethics Commission of the IMBG NAS of Ukraine.

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