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RESEARCH ARTICLE

Analysis of *SMN1*, *NAIP* and *GTF2H2* gene status in correlation with spinal muscular atrophy

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ABSTRACT

Introduction. Spinal Muscular Atrophy (SMA) is a genetic disorder caused by the loss of the survival motor neuron (SMN1) gene in over 95% of cases. Additionally, mutations in genes associated with the SMA chromosomal region can influence disease progression. Aim: To analyze the status of the NAIP and GTF2H2 genes in correlation with SMA.

Material and methods. The study included 105 patients suspected for SMA of which 50 with confirmed with SMA and 55 without causative deletions, and 107 healthy, unrelated individuals. The molecular genetics methods used were mPCR, PCR-RFLP and MLPA.

Results. From 105 patients, 50 were confirmed with SMA. In this group were identified in 8 patients (16%) with a homozygous deletion of exon 5 of the NAIP gene, 4 patients (8%) had a heterozygous status, and 2 (4%) had duplications. In the rest of the patients (55), in which deletions of SMN1 exon 7 were not identified, homozygous deletion of exon 5 of the NAIP gene was established in one patient (2%), 3 patients (5%) had duplications of exon 5 of the NAIP gene, and one patient had 5 copies of the NAIP gene. In the 107 healthy controls, one patient (1%) was identified with a deletion of exon 5 of the NAIP gene. None of the patients with combined deletions of SMN1 and NAIP had deletions in GTF2H2.

Conclusions. The frequency of deletions in the NAIP gene was found to be higher in the SMA patient group compared to the control group. Thus, a significant relationship was identified, the P value being <0.00001. The significance threshold was set at p<0.05. The genetic patterning of genes associated with SMA is an important aspect in the study of molecular pathophysiology and assessment of disease prognosis, especially in the approach to gene therapies.

Keywords: SMA, *NAIP, GTF2H2, SMN1*, deletions, frequency, molecular genetics**.**

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Key messages

What is not yet known on the issue addressed in the submitted manuscript?

The worldwide understanding of Spinal Muscular Atrophy (SMA) genetics remains incomplete, with ongoing research needed to uncover the precise molecular mechanisms, global variations in genetic patterns, and the full spectrum of genotype-phenotype correlations. Additionally, the therapeutic implications of genetic profiles and the long-term outcomes of individuals with SMA, especially in complex cases, are areas that require further investigation to enhance SMA diagnosis and treatment strategies.

The research hypothesis

The research endeavors to explore the relationship between the genetic status of the *NAIP* and *GTF2H2* genes, located in the SMA

chromosomal region, and the diversity of mutations associated with Spinal Muscular Atrophy (SMA).

The novelty added by manuscript to the already published scientific literature

It has been demonstrated that there is a significant correlation between the presence of deletions in the NAIP gene and the occurrence of Spinal Muscular Atrophy (SMA), suggesting that NAIP gene deletions may play a crucial role in SMA susceptibility and severity. Also this research aims to reflect the deletion profile of the patients from Moldavian population in relation to the prevalence of deletions in the genes associated with Spinal Muscular Atrophy (SMA).

Introduction

Spinal muscular atrophy (SMA) is an autosomal recessive disorder that causes muscular atrophy and hypotonia [1, 2]. In over 95% of cases, SMA is caused by homozygous deletion of exon 7 in the *SMN1* gene [3-5]. However, mutations in other genes in the SMA region can contribute to the disease, such as *SMN2*, *NAIP*, and *GTF2H2* genes [6-8].

The *NAIP* gene (OMIM: 600355) [9] is located near the SMN gene and is also duplicated in the 5ql3 region. However, the copy associated with deletions in SMA patients can be distinguished because only this copy contains exon 5 [10-12]. Therefore, in some studies, deletion of exon 5 is present in approximately 50% of patients with SMA type I and ~20% in patients with type II and type III [13]. Experiments demonstrating that the *NAIP* gene is responsible for expressing a protein that suppresses cellular apoptosis support the idea that the protein acts as a negative regulator of motor neuron apoptosis [14]. When this protein is deficient or absent, it contributes to the SMA phenotype [15-17]. Thus, a moderate correlation has been demonstrated between mutations in the *NAIP* gene and the pathophysiology of SMA, especially when considered together with the number of *SMN2* copies, particularly in SMA types I, II, and III [18].

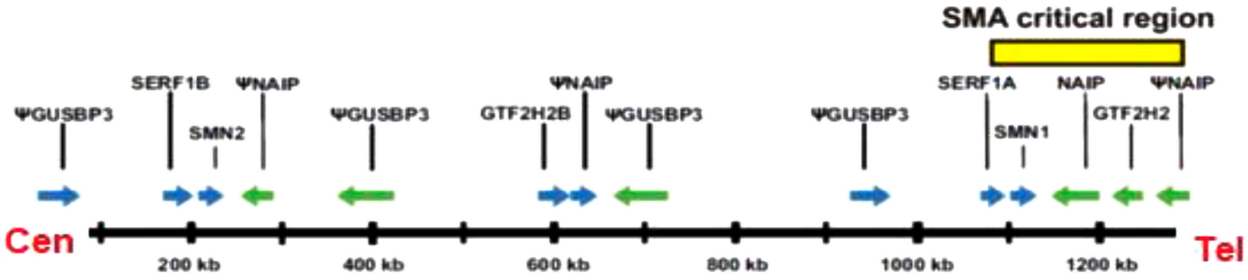

Fig. 1. Map of the genomic localisation of "SMA critical region" showing all genes and their orientation.
SMN1 and *SMN2* are in the same orientation and are approximately 848 kb away from each other. Near *SMN1* are *NAIP and GTF2H2* genes. Also are represented the pseudogenes that are indicated with a (Ψ) [19].
Note: *Cen – centromer; ΨGUSBP3 – Glucuronidase, Beta 3 Pseudogene; SERF1B – Small EDRK-Rich Factor 1B (Centromeric); SMN2 – Survival Of Motor Neuron 2, Centromeric; ΨNAIP – Neuronal Apoptosis Inhibitory Protein pseudogene; GTF2H2 – General Transcription Factor IIH Polypeptide 2; SERF1A – Small EDRK-Rich Factor 1A (Telomeric); SMN1 – Survival Of Motor Neuron 1, Telomeric; NAIP – Neuronal Apoptosis Inhibitory Protein; Tel – Telomeric; kb – kilobases.*

The gene *GTF2H2* (OMIM: 601748) a subunit of the basal transcription factor TFIIH, involved in the transcription process, DNA repair mechanisms and probably in other cellular processes was also characterized and located in the SMA region.

Thus, studies were reported on unrelated patients with SMA that showed that large deletions involving *SMN, NAIP, GTF2H2* gene loci are associated with the most severe SMA phenotype (SMA type I) [20, 21]. The identification of deletions in these genes can be performed by different molecular genetics methods such as PCR, qPCR or MLPA [13, 17, 21].

Objective

The aim of this study was to analyze the profile of modifier genes associated with SMA, such as *NAIP* and *GTF2H2*, in patients with SMA.

Material and methods

The study was conducted at the Institute of Mother and Child, Human Molecular Genetics Laboratory. A total of 105 patients suspected of having SMA were enrolled in the study, including 50 patients with a molecular-genetic diagnosis of SMA, 55 patients with hypotonia but without confirmed molecular-genetic SMA, and 107 unrelated healthy individuals from the Human Molecular Genetics Laboratory's database. All patients have signed the consent for participation (approved by Research Ethics Committee of *Nicolae Testemițanu State University of Medicine and Pharmacy*, Act No. 3, from February 16, 2021). Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and multiplex ligation-dependent probe amplification (MLPA) (The innovation Act "*The method of diagnosing Spinal Muscular Atrophy by the MLPA technique*" No. 483 from 17.03.2022) were used to detect deletions of exons 7

and 8 of the *SMN1* gene for diagnostic purposes. Multiplex polymerase chain reaction (mPCR) was used for exon 5 of the *NAIP* gene and exon 4 of the *GTF2H2* gene. Primers for identifying mutations in exon 5 of *NAIP* (The innovation Act *„ Diagnostic method for the deletion of exon 5 of the NAIP gene based on the PCR genetic molecular technique*" No.517 from 14.03.2023) and exon 4 of *GTF2H2* through mPCR (The innovation Act "*Diagnostic method of deletion of exon 4 of the GTF2H2 gene based on the PCR genetic molecular technique*" No. 516 from 14.03.2023) were custom-designed, along with the parameterization of the amplification program. The innovative diagnostic methods used, as outlined in the acts mentioned above, contain the detailed protocol information, safeguarding that more specific data will not be made public to facilitate the filing of a patent application. Statistical analyzes were calculated via *Social Science Statistics* (Online) [22].

Results

In the pursuit of unraveling the intricate genetic landscape underlying Spinal Muscular Atrophy (SMA), our study meticulously examined the distribution and implications of deletions within the *SMN1, NAIP* and *GTF2H2* genes, elucidating potential links to SMA. The following results present a comprehensive analysis, detailing the frequency of deletions in the *NAIP, GTF2H2* and SMN1 genes across distinct patient cohorts, providing numerical insights crucial for advancing our understanding of SMA pathogenesis. The distribution of patient groups was predicated on clinical manifestations and the analysis of exon 7 status in the *SMN1* gene. After carrying out molecular genetics analyzes by PCR and MLPA technique, within the SMA group, 8 out of 50 patients (16%) exhibited a homozygous deletion of exon 5 of the *NAIP* gene, while 4 patients (8%) showed a heterozygous status, and 2 patients (4%) displayed duplications. In the cohort of 55 patients with hypotonia suspected of SMA but lacking deletions in *SMN1* exon 7, one patient (2%) had a homozygous deletion of exon 5 of the *NAIP* gene, 3 patients (5%) exhibited a duplication of exon 5 of the *NAIP* gene, and one patient showcased 5 copies of the *NAIP* gene. Among the 107 unrelated healthy controls, one patient (1%) manifested a deletion of exon 5 of the *NAIP* gene (Figure 2).

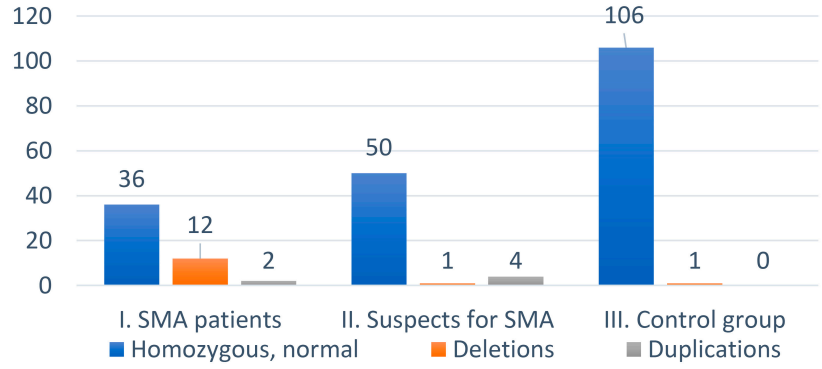

Fig.2 The deletion profile of the *NAIP* gene identified in the patients included in the study.
Representation of the number and type of mutations: Blue-homozygous, normal, Orange - deletions, Gray -Duplications, in each subgroup: I- SMA patients, II- Suspects for SMA, III- Control group; SMA - Spinal Muscular Atrophy.

Notably, the frequency of exon 5 deletions of the *NAIP* gene was calculated for the entire SMA patient group without categorization by SMA types.

Furthermore, molecular genetic analysis of exon 4 of the *GTF2H2* gene was exclusively performed for patients with combined deletions of *SMN1* and *NAIP*, revealing no deletions of exon 4 of the *GTF2H2* gene in any patient.

Statistical analysis, employing the Chi-squared test to explore the relationship between mutations in the *SMN1* gene and those in the *NAIP* gene, yielded compelling results. The Chi-squared statistic was X^2=24.97, with an associated p-value of < 0.00001, indicating a statistically significant association between deletions in the *SMN1* gene and deletions in the *NAIP* gene.

These findings, backed by numerical data and percentages, strongly support the hypothesis that genetic alterations in the *NAIP* gene are intricately linked with susceptibility to SMA. This underscores the paramount importance of delving into these genetic patterns for a nuanced understanding of SMA pathophysiology and a more accurate prognosis.

Discussions

SMA is an inherited disease that leads to progressive hypotonia and damage to the lower motor neurons in the ventral horn of the spinal cord [23]. According to previous studies, changes in genes located near the SMN gene locus have been correlated with the severity of SMA (Jiang et al., 2019) [24], and an important factor influencing the clinical severity of SMA is the duplication of exon 7/8 in the *SMN2* gene, which exerts a modifying effect on the disease. Additionally, prior research in various populations has linked severity to alterations in the *NAIP* and *GTF2H2* genes (Liu et al., 2016) [21]. Additionally, it has been demonstrated that *NAIP* plays a role in preventing motor neuron apoptosis and is homozygously deleted in approximately 50% of SMA type 1 cases [25], while the *GTF2H2* gene is important in transcription and DNA repair [8].

Other studies, regarding the gene expressions of *GTF2H2, NAIP*, and others related to SMA, have been reported in the literature. For example, a study conducted in the

Turkish population examined the expression levels of *SERF1A, GTF2H2, NCALD, ZPR1, TIA1, PFN2*, and *CORO1C* genes for the first time in SMA patients (Zhuri et al., 2022) showed statistically significant differences ($p = 0.037$, $p = 0.001$) between *SERF1A* and *NAIP* genes compared between control group and patients groups [26]. Another study conducted between 2018 and 2021 included 58 SMA patients and 40 healthy individuals as a control group also in Turkish population (Karasu et al., 2022) showed that the genes *NAIP* (p = 0.0095) and *GTF2H2* (p = 0.0049) exhibited a significant difference between healthy subjects and those with SMA [8].

In a study involving the Egyptian population, Hassan et al. in 2020 determined that *SMN2* and *NAIP* are the primary modifier genes, and alterations in their copy numbers can impact the severity of SMA. They found that homozygous deletion of exon 5 of *NAIP* was observed in 60% to 73% of SMA cases, depending on the SMA type [2].

In this study, our aim was to present the mutational profile of the *NAIP* and *GTF2H2* genes and examine the relationship between the *SMN1, NAIP*, and *GTF2H2* genes to determine the frequency of *NAIP* and *GTF2H2* deletions in patients with SMA. According to our findings, combined homozygous deletion in both the *SMN1* and *NAIP* genes was found in 16% of SMA patients. Therefore, a significant association between deletions in the *SMN1* gene and deletions in the *NAIP* gene was established. The chi-square statistic was $X^2 = 24.97$, and the p-value was <0.00001. These data once again underscore the presence of deletions in other genes in the immediate vicinity of the SMA-causing genes in the case of patients from the Republic of Moldova.

However, in our study, no relationship was demonstrated between the *GTF2H2* gene and patients with deletions in *SMN1* and *NAIP*. This phenomenon was described in a study by Arkblad et al. in 2009 [27], although the presence of mutations in the *GTF2H2* gene has been reported to be closely associated with a severe form of SMA (type I) by He et al. in 2013 [28].

Due to the fact that the frequency of deletions in genes associated with SMA was calculated for the entire group of SMA patients, without categorizing them by SMA types, the percentage of deletions is different compared to other populations. However, this still demonstrates that such genetic profiles are characteristic of this disease in the Moldovan population, especially given that the p-value (<0.00001) and the chi-square test statistic (X^2=24.97) showed a highly significant correlation between these two gene mutations. Regarding patients with duplication of exon 5 of *NAIP*, both in the SMA patient group and the hypotonia group, Tomoko Akutsu et al. in 2002 reported in their work that approximately 2 to 5 copies of intact or truncated *NAIP* gene have been identified in the general population, suggesting that duplications in the *NAIP* gene do not have clinical significance [29].

These findings suggest a complex link between *SMN1, NAIP* and *GTF2H2* genes in the pathogenesis of SMA and highlight the importance of molecular genetic studies for understanding and characterizing the disease in different populations. Our study contributes to the knowledge of the mutational profile of the *NAIP* and *GTF2H2* genes in the context of SMA in Moldova, while underlining the need for continued research to develop more effective therapies and to increase the understanding of this complex condition.

Some aspects that are not yet known or require investigation can be categorized as limitations but also ideas for further research:

- *Perspectives on Molecular Mechanisms*: Further investigations are warranted to elucidate the molecular pathways involved in establishing causality and risk factors.

- *Genetic Variability:* Examination of a broader spectrum of possible genetic factors implicated is essential.

- *Clinical Implications*: Subsequent research could shed light on how this genetic information can be practically applied in a clinical framework, with long-term monitoring of individuals with associated mutations.

Conclusions

The present study revealed a higher prevalence of *NAIP* gene deletions within the SMA patient group as compared to the control group, establishing a significant relationship with a *p*-value of $p < 0.00001$. This suggests that the likelihood of this relationship occurring by chance is exceedingly low. Consequently, these alterations merit consideration in the assessment of molecular pathophysiology and disease prognosis. This observation is particularly pertinent in the context of genetic therapies, as it signifies that the genetic profile characterized by modifications in genes within the SMA region is also representative of the population in the Republic of Moldova.

Competing interests

None declared.

Authors' contribution

IC conceived conceptualization, methodology, data collection, analysis and interpretation, writing - original draft preparation. VS conceived writing review and editing, supervision, funding acquisition, validation. The authors read and approved the final version of the manuscript.

Patient consent

Obtained.

Ethics approval

This study was approved by the Research Ethics Committee of *Nicolae Testemițanu* State University of Medicine and Pharmacy (Act No. 3, from February 16, 2021).

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