Investigation on the Effects of Modifying Genes on the Spinal Muscular Atrophy Phenotype

Drenush¿ Zhuri1, Hakan Gurkan1, Damla Eker1, Yasemin Karal2, Sinem Yalcintepe1, Engin Atli1, Selma Demir1, Emine Ikbal Atli1

1Department of Medical Genetics, Trakya University Faculty of Medicine, Edirne, Turkey
2Department of Pediatric Neurology, Trakya University Faculty of Medicine, Edirne, Turkey

Introduction
Spinal muscular atrophy (SMA) is an autosomal recessive neuromuscular disorder caused by the degeneration of motor neurons, muscle weakness, and atrophy that leads to infant’s death. The duplication of exon 7/8 in the SMN2 gene reduces the clinical severity of disease, and it is defined as modifying effect. In this study, we aim to investigate the expression of modifying genes related to the prognosis of SMA like PLS3, PFN2, ZPR1, CORO1C, GTF2H2, NRN1, SERF1A, NCALD, NAIP, and TIA1.

Methods
Seventeen patients, who came to Trakya University, Faculty of Medicine, Medical Genetics Department, with a preliminary diagnosis of SMA disease, and eight healthy controls were included in this study after multiplex ligation-dependent probe amplification analysis. Gene expression levels were determined by real-time reverse transcription polymerase chain reaction and delta–delta CT method by the isolation of RNA from peripheral blood of patients and controls.

Results
SERF1A and NAIP genes compared between A group and B + C + D groups, and A group of healthy controls, showed statistically significant differences (p = 0.037, p = 0.001).

Discussion
PLS3, NAIP, and NRN1 gene expressions related to SMA disease have been reported before in the literature. In our study, the expression levels of SERF1A, GTF2H2, NCALD, ZPR1, TIA1, PFN2, and CORO1C genes have been studied for the first time in SMA patients.
(SMN) protein. As a result of a pathogenic variation, a
defective or low-level expression of the SMN protein leads
to the loss of function of α neuronal cells in the anterior horn
region of the spinal cord, causing the skeletal muscles to
weaken and shrink. It has been reported that the c.859G > C
(p. Gly287Arg) variation in the seventh exon of the SMN2
gene increases the copy number of the transcript at the
mRNA level. The increase in the copy number of the SMN2
gene contributes to the progression of the disease, and this
has been defined as a modifying effect.1–4 The SMN1 and
SMN2 genes are mapped in the centromeric and telomeric
parts of the chromosome 5q13.2 region. The nucleotide
sequences between these two genes are more than 99%
similar, encoding a 294-amino acid-long, 28-kDa SMN pro-
tein.5 Although the SMN protein produced by the SMN2 gene
is sufficient for all cells, it is not enough for motor neurons.6
As a result of the studies performed with heterogeneous
nuclear ribonucleoproteins (hnRNPs), the SMN protein is
determined to interact with the regulator gene of the glu-
cosyltransferase (RGG) box. After the SMN protein forms a
complex with the hnRNPs and the RGG box, the complex
interacts with pre-mRNA and nuclear mRNA and plays an
important role in the processing and transport of mRNA.7
Owing to the faulty production of the SMN protein, snRNPs
cannot interact with other molecules and the functions of
motor axons are negatively affected because of splicing
errors.8 The faulty production of the SMN protein leads to
defective functions of both molecular biological features and
metabolic activities.9 The clinical severity of SMA disease
may differ among patients. Whereas some patients die in
infancy, some with the same mutation may survive with
many symptoms, such as muscle discomfort, wheelchair
dependency, or milder clinical symptoms. Exon 7/8 homo-
zygous deletion in the SMN1 gene may cause different
phenotypes in patients. Therefore, SMA is divided into five
clinical subtypes according to physical examination findings:
SMA type 0, type I, type II, type III, and type IV.10 An increase
in the number copies of the SMN2 gene increases the clinical
severity of SMA.1 The results of the studies conducted in
recent years have revealed the existence of new modifying
genesis in SMA and the prognosis of the disease in patients
with SMA.

The differences in the gene expression levels of plasmin 3
(PLS3), profilin 2 (PFN2), zinc finger (ZPR1), coronin 1C
(CORO1C), general transcription factor 2H, polypeptide 2
(GTF2H2), neuritin 1 (NRN1), small EDRK-rich factor 1A
(SERF1A), neurocalcin delta (NCALD), neuronal apoptosis in-
hibitory protein (NAIP), and cytotoxic granule-associated
RNA-binding protein (TIA1) genes predicted to have modify-
ing effects on SMA were evaluated statistically in the patient
and control groups.

Materials and Methods

Patients and Control Subjects
Seventeen patients (5 female and 12 male) who applied to
the outpatient clinic of Trakya University Hospital, Medical
Genetics Department, Genetic Diseases Diagnosis Center
with a prediagnosis of SMA were included in the study. Six
patients had SMA type I, eight had SMA type II, and three had
SMA type III according to the clinical prediagnosis. The
patients were aged between 9 months and 15 years, and
the ages of the individuals included in the control group
were determined to be close to the average age of the
patients. The control group was composed of eight individ-
uals who did not have a neurological disease or had no
family history. Among the SMA patients, deletions and
duplications in the SMN1 and SMN2 genes were evaluated
using multiplex ligation-dependent probe amplification
(MLPAs) probes (P460-A1 and P060-B2, MRC-Holland
Salsa) SMA carrier probes through the MLPAs method.
Patients with exon 7/8 homozygous deletion in the SMN1
gene were included in the study. Table 1 presents the
detailed patient information.

The patients signed an informed consent form prior to
participating in the study. Ethics approval was granted by the
Scientific Research Ethics Committee of Trakya University
Faculty of Medicine (No. 16/30 dated January 10, 2018).

Methods

A total of 2-mL peripheral venous blood taken from the
patients and healthy controls was placed into ethylenedia-
minetetraacetic acid for RNA isolation, which was performed
in accordance with the protocol of the kit (QIAGEN QIAamp
RNA Isolation Kit, Germany). The concentration and purity
(260/280 nm) of the isolated RNA samples were measured
using a nanodrop device. RNA samples with the proper
concentration and purity were stored at −80°C. Each RNA
sample was transformed into complementary DNA (cDNA) in
accordance with the protocol of the kit (Thermo Fisher
Scientific High Capacity cDNA Reverse Transcription Kit,
Lithuania). Subsequently, PLS3, PFN2, ZPR1, CORO1C,
GTF2H2, NRN1, SERF1A-H4F5, NCALD, NAIP, and TIA1
gene expression studies were performed by the TaqMAN Gene
Expression Assay Kit (Thermo Fisher Scientific, Wilmington,
MA, United States) in Applied Biosystems Step One Plus
(Thermo Fisher Scientific, Wilmington, MA, United States)
using appropriate primers and assays created specifically
for each gene. The procedure was repeated three times for
each patient on a 96-well plate. As an endogenous control,
the β-actin housekeeping gene was used as an internal control.

The delta CT (ΔCt) values were calculated from the data
obtained using the following formula: ΔCt = gene Ct-house-
keeping Ct.12 The values were calculated using ΔΔCt and
2−ΔΔCt, and the gene expression levels were determined.
|   | SMA type | Age | Gender | SMN1                                | SMN2                                | Relative marriage | Mother                        | Father                        |
|---|----------|-----|--------|-------------------------------------|-------------------------------------|-------------------|-------------------------------|-------------------------------|
| P1| Type II  | 14  y | M      | Exon 7–8 homozygous deletion         | Exon 7 (1.5-fold) increased         | –                 | SMN1–7–8 Heterozygous deletion | SMN1–7–8 Heterozygous deletion |
| P2| Type I   | 4   y | M      | Exon 7–8 homozygous deletion         | Normal                              | –                 | –                            | –                            |
| P3| Type II  | 9   y | M      | Exon 7–8 homozygous deletion         | Exon 7 (1.5-fold) increased         | –                 | SMN1–7–8 Heterozygous deletion | SMN1–7–8 Heterozygous deletion |
| P4| Type II  | 15  y | M      | Exon 7 homozygous deletion Exon 8 heterozygous deletion | Exon 7 (1.5-fold) increased | –                 | –                            | –                            |
| P5| Type III | 6   y | F      | Exon 7–8 homozygous deletion         | Exon 7 (1.5-fold) increased         | –                 | –                            | –                            |
| P6| Type II  | 9   y | M      | Exon 7 homozygous deletion Exon 8 heterozygous deletion | Exon 7 (1.5-fold) increased | –                 | SMN1–7–8 Heterozygous deletion | –                            |
| P7| Type I   | 11  y | M      | Exon 7–8 homozygous deletion         | Normal                              | –                 | –                            | –                            |
| P8| Type II  | 15  y | M      | Exon 7–8 homozygous deletion         | Exon 7 (1.5 fold) increased         | –                 | SMN1–7–8 Heterozygous deletion | SMN2-7. Heterozygous deletion |
| P9| Type I   | 3   y | M      | Exon 7–8 homozygous deletion         | Normal                              | –                 | –                            | –                            |
| P10| Type II | 2   y | F      | Exon 7–8 homozygous deletion         | Normal                              | Yes (uncle’s children) | –                            | –                            |
| P11| Type I  | 2   y | F      | Exon 7–8 homozygous deletion         | Normal                              | –                 | SMN1–7–8 Heterozygous deletion | SMN1–7–8 Heterozygous deletion |
| P12| Type III | 3   y | F      | Exon 7–8 homozygous deletion         | Normal                              | –                 | –                            | –                            |
| P13| Type II  | 5   y | M      | Exon 7–8 homozygous deletion         | Exon 7–8 (1.5-fold) increased       | –                 | –                            | –                            |
| P14| Type II  | 2   y | M      | Exon 7–8 homozygous deletion         | Exon 7–8 (1.5-fold) increased       | –                 | SMN1–7–8 Heterozygous deletion | SMN1–7–8 Heterozygous deletion |
Table 1 (Continued)

| SMA type | Age | Gender | SMN1                  | SMN2                  | Relative marriage | Mother                  | Father                  |
|----------|-----|--------|-----------------------|-----------------------|--------------------|-------------------------|-------------------------|
| P15      | Type III | 15 y | M | Exon 7–8 homozygous deletion | Exon 7–8 (1.5-fold) increased | – | – | – |
| P16      | Type I | 9 mo | F | Exon 7–8 homozygous deletion | Normal | – | SMN1–7–8 Heterozygous deletion | SMN1–7–8 Heterozygous deletion |
| P17      | Type I | 10 mo | M | Exon 7–8 homozygous deletion | Normal | Yes (aunt and uncle children) | SMN1–7–8 Heterozygous deletion | SMN1–7–8 Heterozygous deletion |

Abbreviations: F, female; M, male; MLPA, multiplex ligation-dependent probe amplification; SMA, spinal muscular atrophy.

Results

Patients were divided into four groups based on the MLPA results:

Group A: This group comprised eight patients with SMN1 exon 7/8 homozygous gene deletion and normal SMN2 copy number (no increase in copy number) (P2, P7, P9, P10, P11, P12, P16, and P17). The most common clinical findings in patients were unable to sit without support, tongue fasciculation, decreased or absent reflexes, muscle weakness and atrophy, and hypotonia.

Group B: This group comprised four patients with SMN1 exon 7/8 homozygous gene deletion and an increased copy number of exon 7 in the SMN2 gene (P1, P3, P5, and P8). The patients could sit unsupported but never ambulated, and they had problems while walking or could not walk.

Group C: This group comprised two patients with SMN1 exon 7 homozygous deletion/exon 8 heterozygous deletion and an increased copy number of SMN2 exon 7 (P4 and P6). Patients had muscle weakness, they could sit by themselves, and they had late muscle development.

Group D: This group comprised three patients with SMN1 exon 7/8 homozygous deletion and an increase in the copy number of SMN2 exon 7/8 (P13, P14, and P15). Patients had problems while walking and muscle soreness.

The patients included in this study were divided into three clinical subtypes based on the clinical classification criteria of the 1991 SMA International Consortium:

SMN type I: Six patients (P2, P7, P9, P11, P16, and P17)

SMN type II: Eight patients (P1, P3, P4, P6, P8, P10, P14, and P15)

SMN type III: Three patients (P5, P12, and P13)

2^(-ΔΔCt) Values of the Patient and Control Groups

For the expression levels of the relevant genes in the patient and control groups, each sample was analyzed three times based on the Ct values obtained at the end of each analysis. The expression of each gene was calculated as 2^(-ΔΔCt) (Table 2).

The Ct value of the NAIP gene could not be calculated in two patients (P2 and P10). This was interpreted as a deletion of the NAIP gene, and RNA primers were designed for confirmation. The expression of each gene in the control group was calculated as 2^(-ΔΔCt) based on the Ct values obtained at the end of each study. Statistical analysis was performed (Table 3).

The results were presented as the mean ± standard deviation. The suitability of the quantitative data to normal distribution was determined using the Shapiro–Wilk test. Comparison of the gene expression levels (PLS3, PFN2, ZPR1, CORO1C, GTF2H2, NRN1, SERF1A, NCALD, NAIP, and TIA1) between the groups (control, group A and group B+C+D) and SMA types (type I, II, and III) was performed using the Kruskal–Wallis test.

The SPSS version 20.0 (License No: 10240642) program was used for statistical analysis by the Department of Biostatistics and Medical Informatics at Trakya University. A (p) value of less than 0.05 was considered statistically significant.

Evaluation of Group A, group B + C + D, and the Control Group Using the Nonparametric Kruskal–Wallis Test

A statistically significant difference was determined using the Kruskal–Wallis test in the SERF1A and NAIP gene expression levels among group A, group B+C+D, and the control group (p = 0.001) (Fig. 1).

SERF1A Gene Expression

A statistically significant difference was found in the SERF1A gene expression between group A and group (B+C+D) (p = 0.037) and between group A and the control group (p = 0.001). No statistically significant difference was observed in the SERF1A gene expression between group (B+C+D) and the control group (p = 0.090) (Fig. 2a).
A statistically significant difference was found in the NAIP gene expression between group A and group (B + C + D) \((p = 0.001)\) and between group A and the control group \((p = 0.001)\). No statistically significant difference was observed in the NAIP gene expression between group (B + C + D) and the control group \((p = 0.873)\) (\(\text{Fig. 2b}\)).

NN2 gene copy number, and a clinical diagnosis of SMA type II was associated with a higher NRN1 gene expression level (4.29 times) compared with other patients and the control group (\(\text{Fig. 3}\)). The result of the real-time polymerase chain reaction (PCR) analysis showed that the Ct value associated with the NAIP gene expression level could not be detected in our patient. Anticipating that this could be related to a possible deletion in the NAIP gene, we designed an RNA primer specific to the NAIP gene. Exon regions with no band patterns were detected by PCR, and agarose gel electrophoresis confirmed the deletion (\(\text{Fig. 4}\)).

| Table 2 Gene expression values of patients |
|------------------------------------------|
| **PLS3** | **PFN2** | **ZPR1** | **CORO1C** | **GTF2H2** | **NRN1** | **SERF1A** | **NCALD** | **NAIP** | **TIA1** |
| P1 | 1.19 | 2.96 | 0.83 | 1.67 | 1.09 | 2.78 | 1.18 | 1.18 | 2.32 | 2.29 |
| P2 | 1.48 | 0.14 | 0.05 | 0.78 | 0.10 | 0.41 | 0.52 | 0.21 | 0.0 | 0.46 |
| P3 | 1.23 | 1.21 | 0.63 | 0.52 | 0.33 | 1.19 | 0.67 | 0.45 | 0.32 | 1.10 |
| P4 | 1.22 | 1.79 | 0.41 | 1.33 | 0.45 | 0.36 | 0.41 | 0.32 | 0.46 | 0.70 |
| P5 | 1.69 | 1.31 | 1.01 | 0.95 | 0.78 | 1.09 | 0.95 | 0.68 | 0.16 | 1.11 |
| P6 | 0.93 | 0.55 | 0.43 | 0.65 | 0.44 | 2.78 | 0.65 | 0.34 | 0.93 | 1.06 |
| P7 | 1.11 | 1.49 | 0.56 | 1.19 | 0.57 | 1.56 | 0.47 | 0.77 | 0.13 | 0.73 |
| P8 | 1.44 | 2.17 | 1.16 | 1.44 | 0.63 | 1.73 | 0.77 | 0.61 | 0.63 | 0.77 |
| P9 | 1.51 | 0.73 | 0.69 | 1.21 | 0.63 | 0.67 | 0.21 | 0.19 | 0.05 | 0.41 |
| P10 | 2.83 | 0.87 | 1.43 | 1.03 | 0.55 | 4.29 | 0.56 | 0.60 | 0.0 | 0.95 |
| P11 | 1.70 | 1.22 | 1.68 | 0.93 | 0.89 | 0.83 | 0.69 | 0.87 | 0.14 | 0.89 |
| P12 | 9.08 | 0.92 | 1.61 | 1.39 | 0.65 | 1.11 | 0.63 | 0.97 | 0.08 | 1.28 |
| P13 | 1.98 | 1.11 | 0.79 | 0.49 | 0.47 | 0.50 | 0.75 | 0.48 | 1.15 | 0.56 |
| P14 | 2.96 | 0.84 | 1.56 | 0.83 | 0.57 | 3.39 | 0.81 | 0.46 | 0.18 | 1.56 |
| P15 | 0.82 | 1.11 | 0.61 | 1.40 | 0.67 | 0.24 | 0.75 | 0.35 | 1.63 | 0.88 |
| P16 | 2.46 | 0.90 | 0.92 | 1.34 | 0.62 | 1.29 | 0.59 | 0.72 | 0.18 | 1.67 |
| P17 | 2.46 | 2.00 | 2.02 | 0.76 | 0.93 | 1.31 | 0.73 | 0.798 | 0.20 | 1.59 |

**NAIP Expression**

A statistically significant difference was found in the NAIP gene expression between group A and group (B + C + D) \((p = 0.001)\) and between group A and the control group \((p = 0.001)\). No statistically significant difference was observed in the NAIP gene expression between group (B + C + D) and the control group \((p = 0.873)\) (\(\text{Fig. 2b}\)).

**P12**

**SMA type III, group A.** We predicted that the patient with exon 7/8 homozygous deletion in the SMN1 gene, normal SMN2 gene copy number, and a clinical diagnosis of SMA type III clinical diagnosis was associated with a higher PLS3 gene expression level (9.8 times) compared with other patients and the control group (\(\text{Fig. 3}\)).

**P10**

**SMA type II, group A.** We predicted that the patient with exon 7/8 homozygous deletion in the SMN1 gene, normal SMN2 gene copy number, and a clinical diagnosis of SMA type II was associated with a higher NRN1 gene expression level (4.29 times) compared with other patients and the control group (\(\text{Fig. 3}\)). The result of the real-time polymerase chain reaction (PCR) analysis showed that the Ct value associated with the NAIP gene expression level could not be detected in our patient. Anticipating that this could be related to a possible deletion in the NAIP gene, we designed an RNA primer specific to the NAIP gene. Exon regions with no band patterns were detected by PCR, and agarose gel electrophoresis confirmed the deletion (\(\text{Fig. 4}\)).

**P2**

**SMA type I, group A.** The Ct value associated with the NAIP gene expression level was not detected (\(\text{Fig. 3}\)) as a result of the real-time PCR analysis in the patient with exon 7/8 homozygous SMN1 gene deletion and normal SMN2 gene copy number. Anticipating that this could be related to a

| Table 3 Gene expression values of controls |
|------------------------------------------|
| **PLS3** | **PFN2** | **ZPR1** | **CORO1C** | **GTF2H2** | **NRN1** | **SERF1A** | **NCALD** | **NAIP** | **TIA1** |
| C1 | 2.27 | 1.89 | 0.85 | 0.54 | 0.42 | 0.33 | 1.03 | 1.13 | 0.29 | 0.94 |
| C2 | 1.33 | 0.84 | 4.28 | 0.85 | 0.26 | 0.94 | 0.77 | 0.29 | 2.12 | 1.47 |
| C3 | 2.96 | 2.01 | 1.04 | 0.61 | 0.65 | 0.45 | 0.99 | 0.63 | 0.43 | 0.84 |
| C4 | 3.92 | 3.87 | 1.40 | 0.73 | 1.09 | 1.33 | 1.18 | 0.95 | 0.31 | 1.18 |
| C5 | 2.14 | 0.80 | 1.00 | 0.64 | 0.40 | 0.48 | 0.70 | 0.49 | 1.11 | 0.99 |
| C6 | 1.55 | 2.13 | 1.62 | 1.27 | 2.11 | 1.26 | 2.82 | 1.43 | 0.36 | 1.02 |
| C7 | 2.65 | 1.46 | 1.14 | 0.86 | 0.53 | 1.79 | 1.58 | 0.69 | 0.74 | 1.15 |
| C8 | 1.29 | 0.67 | 0.93 | 0.87 | 0.45 | 3.50 | 0.84 | 0.51 | 1.74 | 0.87 |
possible deletion in the \textit{NAIP} gene, we designed an RNA primer specific to the \textit{NAIP} gene. Exon regions with no band patterns were detected by PCR, and agarose gel electrophoresis confirmed the deletion (Fig. 4).

\textbf{P5}

\textit{SMA type III, group B}. The patient with exon 7/8 homozygous deletion in the \textit{SMN1} gene and an increase in the \textit{SMN2} exon 7 copy number had a clinical diagnosis of \textit{SMA type III}, unlike the other patients in group B (P1, P3, and P8). Thus, this could be related to another gene expression level investigated previously in our study (Fig. 3).

\textbf{P4, P6}

\textit{SMA type II, group C}. In these two patients, we suggested that the heterozygous deletion in the exon 8 \textit{SMN1} gene did not contribute to the progression of the patients’ clinic, as P4 and P6 had the same clinical diagnosis (\textit{SMA type II}) and P14 and P15, which are in group D, had exon 8 homozygous deletion in the \textit{SMN1} gene (Fig. 3).

\textbf{P13}

\textit{SMA type III, group D}. The patient with exon 7/8 homozygous deletion in the \textit{SMN1} gene and an increased copy number in exon 7/8 in the \textit{SMN2} gene had an SMA type III clinical
diagnosis, unlike the other patients in group D (P14, P15). Thus, this could be related to another gene expression level other than those investigated in our study (►Fig. 3).

The comparison of the expression levels according to SMA clinical type showed that the PLS3 expression level increased in SMA type III and that the NAIP expression level decreased in SMA type I (►Fig. 5).

The mean PLS3 gene expression level was found to be 1.8, 1.6, and 4.25 in SMA type I, type II, and type III, respectively. Although the PLS3 gene expression level increased in SMA type III, no statistically significant difference was found between SMA type I and type II \((p = 0.197)\) (►Fig. 5). This result could be related to the low number of patients included in the SMA type III patient group (P5, P12, and P13). A statistically significant result could be obtained if the study were repeated with an increased number of patients.

The mean NAIP gene expression level was found to be 0.12, 0.81, and 0.46 in SMA type I, type II and type III, respectively. Although the NAIP gene expression level was lower in SMA type I than in types II and III, no statistically significant difference was found \((p = 0.081)\) (►Fig. 5). The low average NAIP expression level in SMA type I could be related to the failure to obtain the NAIP gene-specific Ct value in our two patients (P2 and P10). Statistically significant results could be obtained if the study were repeated with an increased number of patients.

**Discussion**

SMA is an autosomal recessive neuromuscular disease characterized by weakness and atrophy in the proximal muscles. Homozygous deletion in the 7th and 8th exons of the SMN1 gene or only in the seventh exon was found in 95% of the patients. As a result of the deletions in these exons, weakness and atrophy in skeletal muscles can be observed, as the SMN protein cannot be produced or is damaged.\(^1^3\)

Although the protein expressed by the SMN2 gene compensates for the deficiency of the protein that the SMN1 gene cannot express, some patients do not achieve the expected level of recovery. The better clinical course in SMA patients with an increased number of SMN2 gene copies was interpreted as the modifying effect of the SMN2 gene.\(^1^4\)

In some single-gene disorders, the effect of the mutation in a particular gene on the phenotype may differ among individuals carrying the same mutation. This can be explained by the change in expressivity and/or penetrance. Similar phenotypic differences can also be detected in SMA patients. The SMA consortium has clinically divided SMA...
into five subtypes, namely SMA type 0, SMA type I, SMA type II, SMA type III, and SMA type IV, taking into account the physical examination findings of the patients. In his study on humans and mice, Nadeau reported that modifying genes could be a factor affecting the clinical course of a disease (age and penetrance). The detection of modifying genes will contribute to a better understanding of the pathogenesis of SMA and to the development of drug studies. Riordan et al also emphasized the importance of modifying genes in their studies. In the present study, to explain the prognostic differences in patients diagnosed with SMA, we investigated the expression levels of \( \text{PLS3}, \text{PFN2}, \text{ZPR1}, \text{CORO1C}, \text{GTH2H2}, \text{NRN1}, \text{SERF1A}, \text{NCALD}, \text{NAIP}, \text{and TIA1} \) genes, which we predicted to have modifying effects on the patient and healthy control groups. We found a statistically significant difference in the SERF1A and NAIP gene expression levels \( (p = 0.001) \). The SERF1A gene expression level being low and the prognosis being worse in the patients in group A could be explained by the modifying effect of the SERF1A gene. Considering the results on the NAIP gene expression level, we found that the NAIP gene also had a modifying effect.

Arkblad et al investigated the relationship between SMA disease and the SERF1A gene using the MLPA method. They reported that a deletion in an allele of the SERF1A gene was detected in all SMA type I patients included in the study, 50% of SMA type II patients, and 31% of SMA type III patients. However, no significant relationship was observed between the number of copies of the SERF1A gene and the clinical severity of SMA.

In their study performed on 26 SMA patients, Amara et al found that the copy number of the SERF1A gene was observed as one copy in exon 1 in 60% of the mild SMA type I cases and as two copies in the mild clinical ones of the SMA type II and type III cases. Medrano et al reported that a deletion in the NAIP and SERF1A genes was observed in approximately 73 and 35% of SMA type I patients, respectively. As a result, deletions in the NAIP and SERF1A genes were detected in 90% of SMA type II and type III patients and 21% of SMA type I patients, as these two genes could have modifying effects.

In 34 SMA patients, Tran et al investigated the SMN2 and NAIP genes, which are considered to have modifying effects. The SMN2 gene copy number was detected as three copies in only one of the 13 patients with SMA type I and two copies in the remaining patients; three copies in 9 of the 11 patients with SMA type II and two copies in the remaining patients; and two copies in 2 of the 10 patients with SMA type III, three copies in five patients, and four copies in the remaining three patients. This result was reported as a modifying effect of the
SMN2 gene. The NAIP gene was observed as a homozygous deletion in five SMA patients, a single copy in 20 patients, and a normal copy number in nine patients. Therefore, it was interpreted as the modifying effect of the NAIP gene.²⁰

No studies have yet examined the relationship between SMA prognosis and the expression levels of SERF1A and NAIP genes. SERF1A gene function is a general regulator of protein aggregations, and NAIP gene function is related to the negative regulator of motor-neuron apoptosis.²¹ According to their functions, NAIP gene is directly related to IAP (Inhibitor of Apoptosis) apoptosis family and has direct effect on motor neurons which are related to SMA prognosis, and SERF1A gene may regulate protein aggregation of SMN proteins; the location of SERF1A is near to SMN1 and SMN2 gene but there is not yet any functional study performed about the contribution of SMA prognosis for these two genes.

So, the expression levels of the SERF1A and NAIP genes being lower in SMA type I patients and the statistical comparisons between the groups in our study support the idea that the SERF1A and NAIP genes have modifying effects.²⁰³⁹

As a result of the comparison of the expression levels of the genes with SMA (SMA type I, SMA type II, and SMA type III) types, the PLS3 gene expression increased in SMA type III and the NAIP gene expression decreased in SMA type I. However, we did not find a statistically significant difference between the SMA clinical types and the expression levels of the PLS3 and NAIP genes. We considered this result to be related to the low number of patients included in our study. The result can become statistically significant if the number of patients is increased.

The NRNI gene synthesizes the protein required for the growth of neurite. In the only study in the literature examining the relationship between the SMA and the NRNI gene, the expression levels of the PLS3 and NRNI genes were analyzed in nine patients with SMA diagnosis in four different families. Both siblings, P1 (21 months old, unable to walk) and P2 (14 years old, able to walk), in the first family were diagnosed with SMA type III, and the expression level of the NRNI gene was reported to be 0.9 and 1.4 times higher in P1 and P2, respectively. This result was interpreted as a modifying effect of the NRNI gene. No statistically significant difference was found between the PLS3 gene expression level and the clinical course of SMA. In the second family, three siblings (P3, P4, and P5) with SMA type III diagnosis were included in the study, and the clinical course of H4 was more severe in P3 and P5. Contrary to the result for the first family, the PLS3 gene had a modifying effect but the NRNI gene had no modifying effect. In the third family, two siblings were diagnosed with SMA type III, and the expression level of the PLS3 gene was found to be associated with the clinical course. Moreover, the NRNI gene expression level was found to be unrelated to the clinical course in the third family, and the gene had no modifying effect. In the fourth family, two siblings were diagnosed with SMA type II (P9) and type III (P8) and included in the study. The PLS3 gene expression level was determined to be 1.7 in the sibling diagnosed with SMA type II (P9) and 0.8 in the sibling diagnosed with SMA type III (P8). Thus, the NRNI gene was reported to have no modifying effect.²¹²²

In this study, the NRNI gene expression was higher (4.29 times) in our patient with SMA type II (P10) than in the other patients and the healthy control group. This result supports the literature reporting the modifying effect of the NRNI gene.

In the study in which the PLS3 gene expression and SMA relationship were investigated in 88 SMA patients (29 males less than 11 years old, 12 males older than 11 years old, 29 prepubertal females, and 18 postpubertal females), the highest PLS3 gene expression was found in SMA type III post-pubertal females. PLS3 gene expression was reported as a modifier gene in females, as it varied according to age and puberty stage.²³

Analyzing the PLS3 gene expression levels in 19 SMA type I patients, 21 SMA type II patients, 25 SMA type III patients, and 59 healthy controls, Yanyan et al evaluated the SMN2 copy number using the MLPA method and found three copies of the SMN2 gene in 76.9% of the patients, two copies in 21.5% of the patients and four copies in 1.5% of the patients. The PLS3 gene expression levels were found to be 56.7% lower in SMA type II patients (i.e., those with one and two copies of the SMN2 gene) than in SMA type III patients. The PLS3 gene expression was 62.6% less in SMA type II patients (i.e., those with three copies of the SMN2 gene) than in SMA type III patients.²⁴

This study showed that PLS3 gene expression was increased 9.8 times in our SMA type III patient (P12 with SMN1 gene exon 7/8 homozygous deletion and SMN2 gene exon 7/8 without an increased copy number) compared with other patients and the healthy controls. Although the SMN2 copy number of our patient was normal, the good clinical course could be related to the increased PLS3 gene expression. This result can be interpreted as the modifying effect of the PLS3 gene.

He et al investigated the copy number changes in the SMN2, NAIP, GTF2H2, and H4F5 genes in 157 SMA patients and found that the SMN2 gene copy number was 6.72% single copy, 73.83% two copies, 15.43% three copies, and 2.01% four copies. They showed that all patients with a single SMN2 gene copy number having a diagnosis of SMA type I support the findings about the modifying properties of the SMN2 gene. In the same study, the NAIP gene copy number changes were evaluated in 149 patients and homozygous deletion was found in 15 patients, heterozygous deletion in 126 patients, and normal in eight patients.²⁵

Liu et al examined the NAIP and GTF2H2 genes in 75 patients consisting of 41 SMA type I patients, 29 SMA type II patients, and five SMA type III patients. The SMN2 gene was found as two copies in 28 patients, three copies in 29 patients and four copies in 18 patients. They reported that NAIP and GTF2H2 gene deletions were detected in five patients (fourth exon in four patients and fifth exon deletion in one patient) and 10 patients, respectively.²⁶ In our study, we did not find any statistically significant results supporting the modifying effect of the GTF2H2 gene expression level.
In their study conducted on mice, Torres-Benito et al. found that antisense oligonucleotide therapy targeting the NCALD gene was effective for SMA disease.27

No study investigating the NCALD gene expression level in SMA patients has been reported in the literature. Our study is the first to examine the relationship between SMA and the NCALD gene expression level. We found no statistically significant difference between the SMA disease phenotype and the NCALD gene expression level.

More than one study has examined the modifying effect of the ZPR1 gene on SMA patients, and most of these studies were conducted by Gangwani et al. In their study on mice, Gangwani et al. reported that the deficiency of the ZPR1 protein synthesized by the ZPR1 gene caused neurodegeneration.28

Ahmad et al. analyzed the changes made by increasing and decreasing the expression level of the ZPR1 gene in mice with SMA and found that the low expression level of the ZPR1 gene caused loss of motor neurons, hypermyelination of the phrenic nerves, respiratory distress, and a more severe clinical course. They suggested that the high expression level of the ZPR1 gene stimulates neurite growth and repairs axonal growth defects.29 Genabai et al. also reported that the ZPR1 gene had a positive effect on SMN2 gene expression.30

Note that most of the studies that have investigated the relationship between the ZPR1 gene and SMA are mice studies. Unlike the literature, our study is the first to analyze the relationship between the ZPR1 gene expression level and SMA in humans. No statistically significant relationship was found between the ZPR1 gene expression level and the SMA phenotype.

The TIA1 gene regulates alternative splicing at the seventh exon of the SMN2 gene. Owing to this feature, it has been defined as a positive regulator in SMA disease.31 In the literature, there is no study investigating the relationship among the TIA1 gene copy number, the TIA1 gene expression level, and SMA. In this respect, our study is the first to analyze the association between the TIA1 gene expression level and SMA. No statistically significant difference was found between the TIA1 gene expression level and the SMA phenotype in this study.32

Wadman et al. investigated the PFN2 gene variations in SMA patients using the DNA sequence analysis method but could not find any significant relationship.33 No statistically significant difference was found in our study, which is the first to examine the relationship between the PFN2 gene expression level and SMA.

In their study on the functions of PLS3 and CORO1C genes in SMA patients, McCabe et al.34 reported that PLS3 and CORO1C genes could interact with F-actin and SMN1 protein.33 However, no study has yet investigated the expression level of the CORO1C gene in SMA disease. In this respect, our study is the first to analyze the relationship between the CORO1C gene expression level and SMA, and we found no statistically significant difference.

There are some limitations of this study. The number of patients in this study is low. Besides, this study does not include or exclude the effect of studied modifier genes depending on the treatments. Conducting similar studies in different populations with an increased number of patients can provide important insights into SMA disease and make significant contributions to the literature.

Conclusion

The results of our study, in which we investigated the relationship between the expression levels of PLS3, NAIP, and NRN1 genes and SMA, were previously reported in the literature. However, no study has yet investigated the relationship between the expression levels of SERF1A, GIT2H2, NCALD, ZPR1, TIA1, PFN2, and CORO1C genes and SMA. Therefore, this study is the first of its kind in the literature.

Although the results of the study support the modifying effects of SERF1A, NAIP, NRN1, and PLS3 genes in SMA, we did not find a statistically significant difference in the modifying effects of GIT2H2, NCALD, ZPR1, TIA1, PFN2, and CORO1C genes on SMA.

Funding

This work was supported by the Trakya Scientific Research Projects Unit of Trakya University Faculty of Medicine (2018/326).

Conflict of Interest

None declared.

Ethical Approval

The patients signed an informed consent form prior to participating in the study. Ethics approval was granted by the Scientific Research Ethics Committee of Trakya University Faculty of Medicine (No. 16/30 dated January 10, 2018).

References

1. Hoffmann J. Ueber chronische spinale Muskelschäden im Kindesalter auf familiärer Basis. Deutsche Zeitschrift für Nervenheilkunde 1893;3:427–470
2. Paine M, Lapenta L, Abiusi E, et al. Longitudinal assessments in discordant twins with SMA. Neuromuscul Disord 2017;27(10):890–893
3. Prior Th, Leach ME, Finanger E, et al. Spinal Muscle Atrophy. Gene review. Seattle: University of Washington; 1993–2020
4. Ogino S, Gao S, Leonard DG, Paessler M, Wilson RB. Inverse correlation between SMN1 and SMN2 copy numbers: evidence for gene conversion from SMN2 to SMN1. Eur J Hum Genet 2003;11(03):275–277
5. Kashiwa T, Rao N, David CJ, Manley JL. hnRNP A1 functions with specificity in repression of SMN2 exon 7 splicing. Hum Mol Genet 2007;16(24):3149–3159
6. Mourelatos Z, Abel L, Yong J, Kataoka N, Dreyfuss G. SMN interacts with a novel family of hnRNP and spliceosomal proteins. EMBO J 2001;20(19):5443–5452
7. Liu Q, Dreyfuss G. A novel nuclear structure containing the survival of motor neurons protein. EMBO J 1996;15(14):3555–3565
8. Morse R, Shaw D, Todd AG, Young PJ. Targeting of SMN to Cajal bodies is mediated by self-association. Hum Mol Genet 2007;16(19):2349–2358
9. Nash LA, Burns JK, Chardon JW, Kothary R, Parks RJ. Spinal muscular Atrophy more than a disease of motor neurons. Ben- tham science. Curr Mol Med 2016;16(09):779–792
Génin E, Feingold J, Clerget-Darpoux F. Identifying modifier genes of monogenic disease: strategies and difficulties. Hum Genet 2008;124(04):357–368

Maretina MA, Zheleznyakova GY, Lanko KM, Egorova AA, Baranov VS, Kiselev AV. Molecular factors involved in spinal muscular atrophy pathways as possible disease-modifying candidates. Curr Genomics 2018;19(05):339–355

Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta C(T)) method. Methods 2001;25(04):402–408

D'Amico A, Mercuri E, Tiziano FD, Bertini E. Spinal muscular atrophy. Orphanet J Rare Dis 2011;6:71

Arnold WD, Kassar D, Kissel JT. Spinal muscular atrophy: diagnosis and management in a new therapeutic era. Muscle Nerve 2015;51(02):157–167

Nadeau JH. Modifier genes and protective alleles in humans and mice. Curr Opin Genet Dev 2003;13(03):290–295 (03)00061–3

Riordan JD, Nadeau JH, Nadeau JH. From peas to disease: modifier genes, network resilience, and the genetics of health. Am J Hum Genet 2017;101(02):177–191

Arkblad E, Tulinius M, Kroksmark AK, Henricsson M, Darin N. A population-based study of genotypic and phenotypic variability in children with spinal muscular atrophy. Acta Paediatr 2009;98(05):865–872

Amara A, Adala L, Ben Charfeddine I, et al. Correlation of SMN2, NAIP, p44, H4F5 and occludin genes copy number with spinal muscular atrophy phenotype in Tunisian patients. Eur J Paediatr Neurol 2012;16(02):167–174

Medrano S, Monges S, Gravina LP, et al. Genotype-phenotype correlation of SMN locus genes in spinal muscular atrophy children from Argentina. Eur J Paediatr Neurol 2016;20(06):910–917. 2016.07.017

Tran VK, Sasongko TH, Hong DD, et al. SMN2 and NAIP gene dosages in Vietnamese patients with spinal muscular atrophy. Pediatr Int 2008;50(03):346–351

Bikušam M, Kosač A, Jovanović V, et al. Joint effect of the SMN2 and SERF1A genes on childhood-onset types of spinal muscular atrophy in Serbian patients. J Hum Genet 2015;60(11):723–728

Yener IH, Topaloglu H, Erdem-Özdamar S, Dayangac-Erderen D. Transcript levels of plastin 3 and neuritin 1 modifier genes in spinal muscular atrophy siblings. Pediatr Int (Roma) 2017;59(01):53–56

Stratigopoulos G, Lanzano P, Deng L, et al. Association of plastin 3 expression with disease severity in spinal muscular atrophy only in postpubertal females. Arch Neurol 2010;67(10):1252–1256

Yanyan C, Yujin Q, Jinli B, Yuwei J, Hong W, Fang S. Correlation of PLS3 expression with disease severity in children with spinal muscular atrophy. J Hum Genet 2014;59(01):24–27

He J, Zhang QJ, Lin QF, et al. Molecular analysis of SMN1, SMN2, NAIP, GTF2H2, and H4F5 genes in 157 Chinese patients with spinal muscular atrophy. Gene 2013;518(02):325–329

Liu Z, Zhang P, He X, et al. New multiplex real-time PCR approach to detect gene mutations for spinal muscular atrophy. BMC Neurol 2016;16(01):141

Torres-Benito L, Schneider S, Romro R, et al. NCA LD antisense oligonucleotide therapy in addition to nusinersen further ameliorates spinal muscular atrophy in mice. Am J Hum Genet 2019;105(01):221–230

Gangwani L, Mikrut M, Theroux S, Sharma M, Davis RJ. Spinal muscular atrophy disrupts the interaction of ZPR1 with the SMN protein. Nat Cell Biol 2001;3(04):376–383

Ahmad S, Wang Y, Shaik GM, Burghes AH, Gangwani L. The zinc finger protein ZPR1 is a potential modifier of spinal muscular atrophy. Hum Mol Genet 2012;21(12):2745–2758

Genabai NK, Kannan A, Ahmad S, Jiang X, Bhatia K, Gangwani L. Deregulation of ZPR1 causes respiratory failure in spinal muscular atrophy. Sci Rep 2017;7(01):8295

Singh NN, Seo J, Ottesen EW, Shishimorova M, Bhattacharya D, Singh RN. TIA1 prevents skipping of a critical exon associated with spinal muscular atrophy. Mol Cell Biol 2011;31(05):935–954

McCabe ERB. Modifier genes: moving from pathogenesis to therapy. Mol Genet Metab 2017;122(1-2):1–3

Wadman RI, Jansen MD, Curial CAD, et al. Analysis of FUS, PPN2, TDP-43, and PLS3 as potential disease severity modifiers in spinal muscular atrophy. Neurol Genet 2019;5(01):e386