Effective Valproic Acid Treatment in Motor Function Is Caused by Possible Mechanism of Elevated Survival Motor Neuron Protein Related With Splicing Factor Gene Expression in Spinal Muscular Atrophy

TWMUJ 6: 57-66, 2022

Original

Effective Valproic Acid Treatment in Motor Function Is Caused by Possible Mechanism of Elevated Survival Motor Neuron Protein Related With Splicing Factor Gene Expression in Spinal Muscular Atrophy

Kozue Takano,[1,2] Toshitaka Uchiyama,[2] Noriko Otsuki,[2] Hisahide Nishio,[3] Yuji Kubo,[2,4] Reiko Arakawa,[2] Toshio Saito,[5] Yasuhiro Takeshima,[6] Kotaro Yuge,[7] Toshio Ikeda,[8] Zenichiro Kato,[9] Takashi Nakajima,[10] and **Kayoko Saito[2]**

[1]Affiliated Field of Medical Genetics, Division of Biomedical Engineering and Science, Graduate Course of Medicine, Graduate School of Tokyo Women's Medical University, Tokyo, Japan

[2]Institute of Medical Genetics, Tokyo Women's Medical University, Tokyo, Japan

[3]Department of Occupational Therapy, Faculty of Rehabilitation, Kobe Gakuin University, Hyogo, Japan

[4]Technical Research Institute, Toppan Printing Co., Ltd., Saitama, Japan

[5]Division of Child Neurology, Department of Neurology, National Hospital Organization Osaka Toneyama Medical Center, Osaka, Japan

[6]Department of Pediatrics, Hyogo College of Medicine, Hyogo, Japan

[7]Department of Pediatrics and Child Health, Kurume University School of Medicine, Fukuoka, Japan

[8]Department of Community Pediatrics and Support Raising Next-Generation Pediatricians of Miyazaki City, Faculty of Medicine, University of Miyazaki, Miyazaki, Japan

[9]Department of Pediatrics, Graduate School of Medicine, Gifu University, Gifu, Japan

[10]National Hospital Organization Niigata National Hospital, Niigata, Japan

(Accepted February 14, 2022)

(Advance Publication by J-STAGE May 20, 2022)

Background: Spinal muscular atrophy (SMA) is a lower motor neuron disease caused by *SMN1*. Several clinical trials have indicated that valproic acid (VPA) benefits a limited number of SMA patients. To clarify the difference in VPA responsiveness and elucidate the mechanism, we analyzed gene expression changes by VPA treatment in Japanese pediatric patients using data from clinical trials.

Methods: To identify VPA responders, we correlated the changes in motor function and survival motor neuron (SMN) protein levels. To determine the effects of VPA on gene expression profiles, a microarray analysis was performed. The Gene Ontology (GO) analysis evaluated statistically overexpressed GO terms within a group of genes.

Results: The group with significant improvement showed elevated SMN protein levels following VPA administration, whereas that with the highest SMN levels at baseline did not improve immediately. GO analysis suggested that specific factors contributed to the correlation between changes in motor function and the SMN protein levels, including splicing factors *HNRNPC* and *SNRNP70*.

Corresponding Author: Kayoko Saito, Institute of Medical Genetics, Tokyo Women's Medical University, 8-1 Kawada-cho, Shinjuku-ku, Tokyo 162-8666, Japan. saito.kayoko@twmu.ac.jp

doi: 10.24488/twmuj.2021020

Conclusions: This is the first study to indicate that the time for VPA effectiveness varies among individuals and is associated with SMN protein levels at baseline and expression changes in splicing factor genes.
Clinical Trials Registry of the Center for Clinical Trials, Japan Medical Association, a registry of the Japan Primary Registries Network certified by the World Health Organization as a primary registry (registration numbers: SMART01 trial, JMA-II A00190; SMART02 trial, JMA-II A00231; SMART03 trial, JMA-II A00259).

Keywords: spinal muscular atrophy, valproic acid, motor function, SMN protein, splicing

Introduction

Spinal muscular atrophy (SMA) is a progressive lower motor neuron disease induced by degeneration of anterior horn cells of the spinal cord, causing trunk and extremity muscle weakness and atrophy. SMA is ultimately caused by the survival motor neuron 1 gene (*SMN1*) located at chromosome 5q13. The SMN protein is essential for small nuclear ribonucleoprotein (snRNP) assembly;[1] snRNPs are the core elements of spliceosomes. Previous studies have suggested that SMN protein deficiency influences numerous splicing events.[2,3] Therefore, the splicing pattern of transcripts encoding a protein with critical functions in motor neuron biology and development may be altered by changes in snRNP abundance due to SMN protein depletion,[4] contributing to SMA pathology. A highly homologous gene, *SMN2*, differs from *SMN1* by only five nucleotides, which predominantly produces alternatively spliced transcripts with exon 7 skipping that are translated into truncated and unstable SMN protein.[5] Although *SMN2* cannot compensate for loss of *SMN1*, all SMA patients have at least one copy of *SMN2*;[6] SMN protein levels correlate with disease severity.[7]

Increasing the full-length and stable SMN protein levels is the basis of therapeutic strategy. Before developing new drugs, histone deacetylase (HDAC) inhibitors were considered since they may promote *SMN2* transcription and eventually increase functional SMN protein levels. Valproic acid (VPA), used to treat epilepsy, mood disorders, and migraines, has also been assessed as a potential SMA treatment agent. Human fibroblasts derived from VPA-treated SMA patients exhibited increased *SMN* promoter acetylation,[8,9] modulated splicing factor expression,[10,11] and elevated full-length *SMN* messenger RNA (mRNA) and protein levels.[10,12] A pilot trial observed that VPA elevated full-length *SMN2* mRNA levels in the blood in approximately one-third of SMA patients,[13] whereas another study reported improved muscle strength in children rather than adult patients.[14] Moreover, clinical trials results suggest that VPA is most efficacious in younger patients and upon long-term treatment.[15-21] Furthermore, it has been suggested that individual factors besides *SMN* genotype may affect responses to VPA.[22] As VPA affects expression of some genes,[23] it may affect responsiveness; however, there are no reports of the effects of VPA on gene expression profiles in SMA patients.

In this study, we analyzed the correlation between changes in motor function and SMN protein levels following long-term VPA treatment using data from pediatric SMA patients. Further, to elucidate the underlying mechanism and associated factors of VPA responsiveness, we analyzed changes in gene expression profiles before and after VPA treatment using microarrays and performed Gene Ontology (GO) analyses, which is a method for analyzing the relationship between genes of a gene set by annotating and categorizing a corresponding molecular function and biological process, to investigate whether specific genes affect VPA responsiveness.

Materials and Methods

Clinical trials

The SMA Research and Treatment (SMART) clinical trial comprised three parallel studies. SMART01 was an open-label, uncontrolled, exploratory phase II study. SMART 02 was a placebo-controlled, double-blind, parallel-group comparison, confirmatory phase III study. The participants were randomly assigned to either the treatment or placebo arm. Either VPA and L-carnitine or a matched placebo was administered once daily after supper for 32 weeks. SMART03, with continuous administration following SMART02, was an open-label, uncontrolled phase III trial. The standard protocol for measure-

Table 1. Baseline demographic characteristics of 26 participants.

Characteristic	VPA group (N = 13)	Placebo group (N = 13)
Age (years)		
Mean	3.7	3.7
SD	1.7	1.7
Median	3.7	3.3
Range	1.1-6.8	1.3-6.9
Sex		
Male	9	8
Female	4	5
SMA type		
I	3	0
II	10	13
Disease Duration (years)		
Mean	2.2	1.9
SD	1.9	1.8
Median	1.9	1.6
Range	0-5.7	0.2-6.2

SD, standard deviation; SMA, spinal muscular atrophy; VPA, valproic acid.

ments is shown in **Supplementary Figure 1**.

These trials were performed following the instructions presented by the Pharmaceutical and Medical Devices Agency in Japan and are registered with the Clinical Trials Registry of the Center for Clinical Trials, Japan Medical Association, a registry of the Japan Primary Registries Network certified by the World Health Organization as a primary registry (registration numbers: SMART01 trial, JMA-II A00190; SMART02 trial, JMA-II A00231; SMART03 trial, JMA-II A00259).

Herein, we report an exploratory analysis using the results of SMART02 and SMART03, with a focus on whether VPA improved motor function, increased SMN protein levels, and influenced gene expression profiles.

Study participants

Twenty-nine Japanese children aged < 7 years with SMA types I-II treated at six hospitals in Japan were included. All diagnoses for SMA type I or type II with a homozygous deletion of exon 7 in *SMN1* were clinically and genetically confirmed. Based on the classification system of Kaneko et al.,[24] the SMA types were further subtyped (**Supplementary Table 1**). The progression of all participants is represented in **Supplementary Figure 2**. **Table 1** lists the baseline demographic properties of the 26 participants who completed SMART02.

Motor function evaluation

To evaluate gross motor function, the Hammersmith Functional Motor Scale-Expanded (HFMSE) was used; the HFMSE comprises 33 items scored 0-66 and has been specifically validated to evaluate children with SMA.[25,26] Measurements were obtained before week 4 and at weeks 0, 12, 24, 28, 32, 52, 64, 76, and 88 (**Supplementary Figure 1**).

Classification of participants according to change in HFMSE score

Participants were classified to define VPA treatment efficacy by subtracting the best HFMSE score before treatment from the best HFMSE score at weeks 24, 28, and 32. Groups A, B, and C were defined as participants with score difference of over 3, 1-2, and < 0, respectively (**Supplementary Table 1**).

Evaluation of SMN protein levels

Otsuki et al.[27] developed a semi-quantitative analysis for SMN protein levels. Peripheral blood cells stained with Alexa Fluor 488-conjugated 2B1 (Novus Biologicals, Littleton, CO, USA) against human SMN protein expressed in the classified cell population were detected using imaging flow cytometry (ImageStreamX Mark II, Merck, Darmstadt, Germany). SMN spots implied the presence of functional SMN protein in the cell nucleus. The percentage of SMN-spot$^+$ cells was regarded as the SMN protein levels. The SMN spot analysis was performed at weeks 0, 8, 24, 32, 36, 52, 64, 76, and 88 as described (**Supplementary Figure 1**).[27]

RNA extraction and microarray

Blood samples were collected using PAXgene blood RNA tubes before and at week 32 of the trial (**Supplementary Figure 1**). Total RNA was isolated using the PAXgene Blood RNA Kit (Qiagen, Hilden, Germany). Microarray analysis was performed using the Applied Biosystems™ GeneChip™ Human Genome U133 Plus 2.0 Array (Thermo Fisher Scientific, Waltham, MA, USA) comprising 54,675 probe sets representing 38,500 human genes.

Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR)

To validate the results of microarray experiments, quantitative real-time RT-PCR analysis of *HNRNPD* (MIM #601324), *U2AF2* (MIM #191318), *HNRNPC* (MIM #164020), *HNRNPH1* (MIM #601035), and *SNPNP70* (MIM #180740) was performed. Complementary DNA was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). The reverse-transcribed sample was used for RT-PCR using the StepOnePlus Real-Time PCR System and TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific). The assay IDs are *HNRNPD*: Hs01086912_m1, *U2AF2*: Hs00200737_m1, *HNRNPC*: Hs01028910_g1, *HNRNPH1*: Hs04979572_g1, and *SNPNP70*: Hs05041646_g1. Each RT-PCR was performed in triplicate, and mRNA levels were quantified based on the Ct value, normalized to *GAPDH,* and expressed as relative amounts.

Statistical analyses

Differential HFMSE score and protein levels changes between groups during SMART02, SMART03, and SMART02-03 were analyzed using a two-sample *t*-test. SMN protein levels were compared between pre-treatment and each week using a one-sample *t*-test. Differential changes in gene expression between Groups A and C were examined using the Wilcoxon exact test. To evaluate statistically overrepresented GO terms within a group of genes, Database for Annotation, Visualization, and Integrated Discovery (DAVID) version 6.7 was used.[28] To assess differential splicing factor gene expression between Groups A and C, a two-sample *t*-test was used.

Ethics statement

Participants were recruited with the approval of the Tokyo Women's Medical University Ethics Committee (Approved protocols No. 2709-R). SMART trials were approved by the Tokyo Women's Medical University Institutional Review Board [examination Nos. N2015040 (SMART02) and N2016024 (SMART03)] and by each participating institution (Toneyama National Hospital; Hyogo College of Medicine; Tokyo Women's Medical University Yachiyo Medical Center; Kurume University Hospital; Faculty of Medicine, University of Miyazaki Hospital). All procedures were conducted according to the principles described in the Declaration of Helsinki. Written informed parental consent and written child assent were obtained from all participants.

Results

Table 1 outlines participant baseline characteristics. Mean patient age in both groups was 3.7 ± 1.7 years; disease duration was 2.2 ± 1.9 years and 1.9 ± 1.8 years in the treatment and placebo groups, respectively.

Effects of VPA therapy on HFMSE score

Changes in motor function during each period of SMART02 and SMART03 were monitored using the HFMSE score (**Figure 1**). HFMSE scores are summarized in **Supplementary Table 2**. While Group A had a markedly improved HFMSE score compared with Group C ($p = 0.0177$) and the placebo group ($p = 0.0463$), it was not significant (but trending) compared with Group B ($p = 0.0629$) during SMART02 (**Figure 1A**) but changed marginally during SMART03 (**Figure 1B**). Although Group C had a rather reduced HFMSE score during SMART02 compared with that before VPA administration (**Figure 1A**), they showed improvements during SMART03. In SMART03, there were no significant differences in score changes between Groups C and A, despite significant differences during SMART02; there was a significant increase compared with the scores of Groups B and C ($p = 0.0270$, **Figure 1B**). The HFMSE score changes differed between Group C and the placebo group ($p = 0.0509$, **Figure 1B**); however, they were not significant. Overall changes from week 0 in SMART02 to the end of SMART03 were significantly greater for Group A than for the placebo group ($p = 0.0442$, data not shown). In Group B, some participants exhibited increased scores and others exhibited decreased scores; thus, there was no overall trend in motor function over time (**Supplementary Table 2**). Long-term treatment increased the HFMSE score of Group C (**Figure 1B**, **Supplementary Table 2**). The specific clinical course of participants in Groups A and C are shown in **Supplementary Figure 3**.

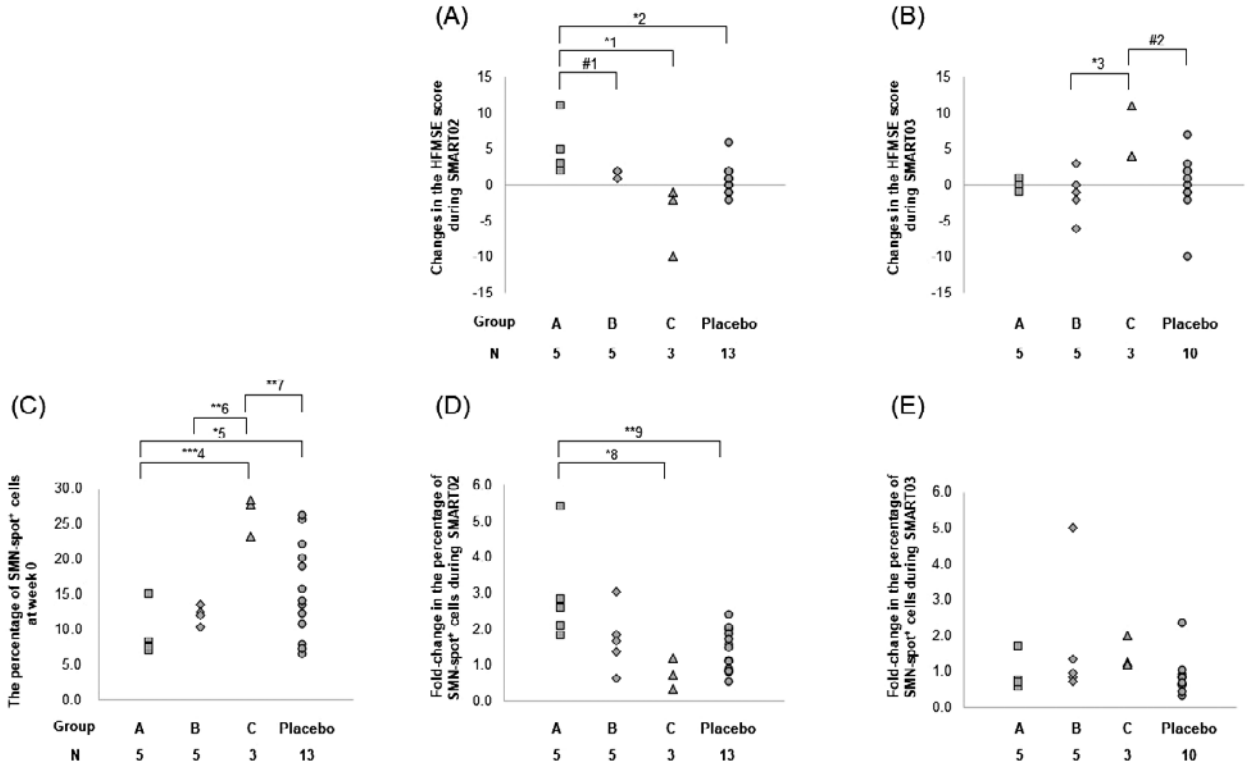

Figure 1. Changes in HFMSE score and SMN protein level in the VPA and placebo groups. (Top) Changes in the HFMSE score during SMART02–SMART03. *$p < 0.05$; *1: $p = 0.0177$, *2: $p = 0.0463$, *3: $p = 0.0270$, #1 = 0.0629, #2 = 0.0509. (Bottom) Fold-changes in the percent of SMN-spot$^+$ cells during SMART02–SMART03. Fold-changes were calculated as the ratio of the final value to the initial value, for each period. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; ***4: $p = 0.0005$, *5: $p = 0.017$, **6: $p = 0.008$, **7: $p = 0.002$, *8: $p = 0.045$, **9: $p = 0.0046$.
HFMSE, Hammersmith Functional Motor Scale-Expanded; VPA, valproic acid; SMART, SMA Research and Treatment.

Quantitative evaluation of SMN protein levels

Biomarkers for assessing the phenotype of SMA patients and therapeutic efficacy of drugs are controversial.[29,30] In this study, SMA protein levels were measured to evaluate the effect of VPA. Changes in SMN protein levels during each period of SMART02 and SMART03 were examined (**Figure 1**). SMN protein analysis results are summarized in **Supplementary Table 3**. While SMN protein levels of Group C were significantly higher than Groups A ($p = 0.0005$) and B ($p = 0.008$) before VPA treatment (**Figure 1C**), it was not statistically significant post-VPA treatment (**Supplementary Table 3**). SMN protein levels in Group A were significantly elevated compared with that in Group C ($p = 0.045$) and the placebo group ($p = 0.0046$) during SMART02 (**Figure 1D**), although there was no between-group difference during SMART03 (**Figure 1E**). Overall changes from week 0 in SMART02 to the end of SMART03 were significantly greater for Group A than for the placebo group ($p = 0.030$, data not shown).

Effects of VPA treatment on gene expression profiles

Based on the Wilcoxon exact test, expression changes from 1,262 probe sets were significantly different between Groups A and C ($p < 0.05$). To investigate whether these probe sets were clustered according to specific functions, we analyzed the frequency of GO annotations using DAVID.[28] The probe sets with different expression changes between Group A and C displayed significantly enriched GO terms, including "alternative splicing" (44.8%, $p = 6.45E-13$) and "splice variant" (44.7%, $p = 1.23E-12$; **Supplementary Table 4**).

Table 2. Microarray and RT-PCR results for differences in splicing factor gene expression changes between Groups A and C after VPA treatment.

Gene	Probe ID	Expression change	Group A			Group C			p-value
			Mean	SD	Median	Mean	SD	Median	
HNRNPD	221480_at	U133	0.99	0.10	1.00	0.77	0.06	0.78	0.0143*
		PCR	0.98	0.19	1.05	0.94	0.21	0.91	0.7566
U2AF2	218382_s_at	U133	0.87	0.15	0.92	1.32	0.37	1.12	0.0493*
		PCR	0.35	0.11	0.37	1.13	1.12	0.70	0.1529
HNRNPC	1568941_a_at	U133	1.06	0.36	1.02	2.92	0.8	2.55	0.0036**
		PCR	0.69	0.08	0.72	1.08	0.38	1.05	0.0607
HNRNPH1	201031_s_at	U133	0.99	0.11	0.99	0.78	0.09	0.83	0.0307*
		PCR	0.79	0.18	0.81	1.01	0.35	1.08	0.2857
SNRNP70	213121_at	U133	0.76	0.22	0.84	2.11	0.95	2.55	0.0183*
		PCR	0.21	0.10	0.18	1.81	1.72	1.56	0.0718

*$p < 0.05$, **$p < 0.01$; RT-PCR, reverse transcription-polymerase chain reaction; SD, standard deviation; VPA, valproic acid.

VPA modulates *HNRNPC* and *SNRNP70* expression

Previous studies have indicated that *SMN* transcript splicing is influenced by multiple splicing factors,[31] and our microarray analysis indicated the expression change of some splicing-related genes in response to VPA treatment. Thus, we analyzed 83 splicing factor genes using a microarray approach (**Supplementary Table 5**). According to the two-sample *t*-test, 5 out of 54,675 probe sets were mapped to 5 genes-*HNRNPD*, *U2AF2*, *HNRNPC*, *HNRNPH1*, and *SNRNP70*-that were differentially expressed between Groups A and C (**Table 2**). Our microarray results were confirmed using real-time RT-PCR. Among the five genes, changes in the expression of *HNRNPC* and *SNRNP70* differed between Groups A and C, although no significant difference was observed (**Table 2**), indicating that VPA tends to decrease the expression of *HNRNPC* and *SNRNP70* in Group A compared to that in Group C.

Discussion

Clinical trials were conducted to assess VPA as a therapeutic candidate for SMA (**Table 3**). A consensus on its therapeutic effect has not been established, but VPA improves motor function upon long-term administration in young patients when treatment is initiated shortly after the onset of symptoms. Therefore, the age of our target patients was less than that of patients in previous clinical trials,[15-20] and the duration of the VPA treatment is the longest to date. From the perspective of SMA pathogenesis, changes in SMN protein levels are thought to affect muscle strength and motor function. We analyzed the correlation between changes in motor function and SMN protein levels. Further, we analyzed gene expression changes before and after VPA treatment using microarrays and performed GO analysis.

Superior efficacies can be achieved by starting treatment immediately after or before onset;[32] however, Group A patients, who had a longer disease duration than Group C patients, exhibited early improvement in motor function and the efficacy was maintained (**Figure 1A, B**). The motor function in Group C patients improved with continuous VPA treatment. Moreover, some participants regained motor activity during VPA treatment, despite the progressive nature of SMA (**Supplementary Figure 3**). These observations indicate a time lag in the effect of VPA on motor function, i.e., these effects were not only "effective or ineffective" but also "rapid or delayed." Thus, Group A could be defined as "rapid responders," Group C as "delayed responders," and Group B, which showed heterogeneous results, as "intermediate-responders." Therefore, factors other than patient age and the disease duration may affect the time lag before the effects of VPA appear.

Changes in the protein levels of Group A suggested that lower baseline levels enabled a quick increase in response to VPA treatment (**Figure 1C, D**), resulting in the early improvement in motor function. Conversely, Group C, which had the highest SMN protein levels before VPA treatment, demonstrated no change in SMN protein levels

Table 3. Summary of previous clinical trials of VPA targeting SMA patients and the study highlights.

Author	Clinical Trial	Participants			VPA Administration	Results
		SMA type	N	Age (Years)		
Swoboda (2009) [15]	open-label trial	I I III	2 29 11	2-3 2-14 2-31	12 months (15-50 mg/kg/day)	Mean MHFMS scores increased in 27 participants with type II; significant improvement was especially observed in participants < 5 years of age. There were no significant changes in FL-*SMN* levels, while Δ*SMN* levels were significantly reduced at 6 and 12 months in type II participants.
Swoboda (2010) [16]	randomized, placebo-controlled, double-blinded clinical trial	II, III "sitter"	61	2-8	Participants were randomized 1:1 to VPA treatment group or placebo group for the first 6 months and all received VPA for the subsequent 6 months. (TL: 50-100 mg/dL)	Post hoc analysis indicated significant improvement in MHFMS in the youngest participants (ages 2-3 years) that received the VPA treatment over a full year. There was no significant change from baseline between both groups at 6 months.
Kissel (2011) [17]	open-label trial	II, III "standers and walkers"	33	3-17	12 months (TL: 50-100 mg/dL)	There was no significant change in MHFMS-Extend and *SMN* transcript levels at either 6 or 12 months.
Darbar (2011) [18]	open-label trial	II, III	22	2-18	12 months (20 mg/kg/day)	Participants younger than 6 years had a better mean HFMS score than participants older than 6 years. There was an improvement in the Barthel Index for evaluating the daily activities at the end of the VPA treatment.
Kissel (2014) [19]	randomized, placebo-controlled, double-blind crossover trial	III "ambulatory"	33	20-55	Participants were randomized 1:1 to VPA treatment group or placebo group for the first 6 months; switched to the other group for the subsequent 6 months. (10-20 mg /kg/day)	There was no significant change in MVICT in adults.
Saito (2015) [20]	open-label trial	II III	6 1	2-34 42	6 months (TL: 50-100 mg/dL)	A significant improvement in MHFMS was observed in 2-year-old participants, but no significant changes were observed in the older participants.
Krosschell (2018) [21]	open-label trial	I	37	0.9-10.6 (months)	6 months (10-30 mg/kg/day)	No significant impact on either survival and respiratory function.
Our study	randomized, placebo-controlled, double-blinded clinical trial	I II	3 23	1.1-6.9	Participants were randomized 1:1 to VPA treatment group or placebo group for the first 32 weeks (SMART02) and all received VPA for the subsequent 52 weeks (SMART03). (25.0 mg/kg/day)	Time of VPA effectiveness for motor function varied among individuals and is correlated with the SMN protein level at the baseline and expression changes in splicing-related genes.

SMA, spinal muscular atrophy; VPA, valproic acid; MHFMS, Modified Hammersmith Functional Motor Scale; HFMS, Hammersmith Functional Motor Scale; MVICT, maximum voluntary isometric contraction testing; TL, trough level.

during VPA treatment (**Supplementary Table 3**). The association between disease duration and SMN protein levels remains to be determined, but it is estimated that the shorter the disease duration, the more the SMN protein levels is conserved, and high baseline SMN protein levels in Group C were maintained during VPA administration, perhaps leading to gradually improved motor function.

Additionally, we investigated factors that influenced the changes in SMN protein levels, which may be associated with the time lag that occurs before motor function improvement. According to the microarray analysis, 1,262 probe sets exhibited significant differences in expression changes between Group A and C and were characterized by overexpression of splicing-related genes. Splicing of *SMN* exon 7 is controlled by numerous splicing factors,[31] especially a C to T transition in *SMN2* exon 7 is identified as the causative exon 7 skipping.[33,34] As splicing of *SMN* exon 7 is strongly related to production of a functional SMN protein, expression changes in genes that significantly differ between Groups A and C involved in splicing are especially interesting. Therefore, we verified whether gene expression changes in splicing factors differed between Groups A and C. Microarray

and PCR analyses showed differential expression changes in *HNRNPC* and *SNRNP70* between Groups A and C (**Table 2**). *HNRNPC* encodes heterogeneous nuclear ribonucleoprotein (hnRNP) C1/C2 and is a member of the hnRNP family. In HeLa cells, hnRNP C has no significant effect on *SMN2* splicing,[35] which is consistent with *in vitro* splicing assay results.[36] *SNRNP70* encodes U1-70K, a component of U1 snRNP that is essential to recognize the pre-mRNA 5´ splice site.[37] Downregulation of U1-70K significantly decreases *SMN2* exon 7 inclusion in HEK-293T cells.[38] Expression of splicing factors changes depending on the environment and stress, and pre-mRNA splicing is performed to adapt to the situation.[39, 40] In this study, the expression levels of *HNRNPC* and *SNRNP70* decreased in Group A and increased in Group C at 32 weeks post-VPA administration (**Table 2**). While some splicing factors either enhance or silence pre-mRNA splicing in various genes,[41, 42] it remains unclear how both factors affect the splicing of *SMN2* transcripts. However, *HNRNPC* and *SNRNP70* were affected by VPA and may have directly or indirectly affected the rate and extent of their effect on splicing of the *SMN2* transcript.

Therapeutic agents against SMA are being constantly developed, and novel drugs are being administered to patients.[43-45] However, not all the novel drugs are available worldwide;[46] besides, their cost-effectiveness and long-term safety are still being discussed and determined. VPA can be safely administered for a long duration with monitoring and combined with L-carnitine. A recent systematic review and meta-analysis of clinical trials have reported that VPA treatment significantly improved gross motor function irrespective of carnitine co-administration and study design; however, the lack of significant improvement with the co-administration of carnitine[47] necessitates further evaluation of the effects of the concomitant use of carnitine and VPA. Nusinersen is the first approved drug for SMA targeting an intronic-splicing silencer, and not all SMA patients treated have improved phenotype;[48] therefore, novel therapeutic approaches are being explored. VPA and splice-switching oligonucleotide (SSO) for fibroblasts derived from SMA type I patients showed restoration of full-length *SMN2* mRNA and SMN protein levels when compared with those under monotherapy with each compound.[49] The analysis using LBH589, another HDAC inhibitor, indicated that LBH 589 promoted *SMN2* transcription, leading to augmentation of the target template pre-mRNA for SSO and resulting in elevated exon 7 inclusion and SMN protein levels.[50] These findings suggest that the HDAC inhibitors enhance the function of *SMN2* splicing modifier, and the synergistic effect of combination therapy by the same mechanism is expected for VPA, indicating the possibility of combination therapy.

The study has some limitations. The gene expression was compared only between before and 32 weeks after VPA treatment, in the final stage of SMART02. In addition, evaluating the changes in the expression of genes, such as *HNRNPC* and *SNRNP70*, over a longer period during SMART02 and SMART03, could have helped validate our speculation on the effects of these factors on *SMN2* splicing.

Conclusions

Our findings suggested that some SMA patients respond quickly and effectively to VPA, but the timing of improvement in motor function may be affected by the baseline SMN protein levels. Furthermore, changes in SMN protein levels following VPA treatment were affected by splicing factors such as *HNRNPC* and *SNRNP 70*.

Sources of Funding: This work was supported by the Practical Research Project for Rare/Intractable Diseases of the Japan Agency for Medical Research and Development [grant numbers 17ek0109086h0003, 20ek0109472h0001] and Grants-in-Aid from the Research Committee of CNS Degenerative Diseases, Research on Policy Planning and Evaluation for Rare and Intractable Diseases, Health, Labour and Welfare Sciences Research Grants, the Ministry of Health, Labour and Welfare, Japan.

Conflicts of Interest: The authors declare that there are no conflicts of interest.

Author Contributions: Kozue Takano: Analysis of data, Interpretation of data, Drafting the work, Revising the work.

Toshitaka Uchiyama: Analysis of data, Interpretation of data.

Noriko Otsuki: Analysis of data, Interpretation of data.

Hisahide Nishio: Analysis of data, Interpretation of data.

Yuji Kubo: Analysis of data, Interpretation of data.

Reiko Arakawa: Acquisition of data.

Toshio Saito: Acquisition of data.

Yasuhiro Takeshima: Acquisition of data.

Kotaro Yuge: Acquisition of data.

Toshio Ikeda: Acquisition of data.

Zenichiro Kato: Acquisition of data.

Takashi Nakajima: Acquisition of data.

Kayoko Saito: Design of the work, Acquisition of data, Analysis of data, Interpretation of data, Drafting the work, Revising the work.

Acknowledgments: We are grateful to Dr. Toshiyuki Yamamoto (Tokyo Women's Medical University) for his valuable contributions to this study; Dr. Kitami Hayashi (Department of Pediatric Neurology, Tokyo Women's Medical University Yachiyo Medical Center) for providing the samples and participant information; Dr. Masanori Fukushima (Translational Research Center for Medical Innovation, Foundation for Biomedical Research and Innovation at Kobe) for providing valuable input; Mr. Fumiaki Kobayashi and Mr. Kazuo Watanabe (CTD Co., Ltd.) for their support in our clinical trials; and Ms. Yukari Tateno and Mr. Takahiro Sasaki (Tokyo Women's Medical University) for supporting the clinical trial office. Further, we thank all the children and families who participated in the trials.

Ethical Approval: The SMART trials were approved by the Tokyo Women's Medical University Institutional Review Board [examination Nos. N2015040 (SMART02) and N2016024 (SMART03)] and by each participating institution (Toneyama National Hospital; Hyogo College of Medicine; Tokyo Women's Medical University Yachiyo Medical Center; Kurume University Hospital; Faculty of Medicine, University of Miyazaki Hospital).

References

1. Massenet S, Pellizzoni L, Paushkin S, et al. The SMN complex is associated with snRNPs throughout their cytoplasmic assembly pathway. Mol Cell Biol. 2002;22(18):6533−41.
2. Zhang Z, Lotti F, Dittmar K, et al. SMN deficiency causes tissue-specific perturbations in the repertoire of snRNAs and widespread defects in splicing. Cell. 2008;133(4):585−600.
3. Gabanella F, Butchbach MER, Saieva L, et al. Ribonucleoprotein assembly defects correlate with spinal muscular atrophy severity and preferentially affect a subset of spliceosomal snRNPs. PLoS One. 2007;2(9):e921.
4. Beattie CE, Kolb SJ. Spinal muscular atrophy: Selective motor neuron loss and global defect in the assembly of ribonucleoproteins. Brain Res. 2018;1693(Pt A):92−7.
5. Monani UR, Lorson CL, Parsons DW, et al. A single nucleotide difference that alters splicing patterns distinguishes the SMA gene SMN1 from the copy gene SMN2. Hum Mol Genet. 1999;8(7):1177−83.
6. Calucho M, Bernal S, Alías L, et al. Correlation between SMA type and SMN2 copy number revisited: An analysis of 625 unrelated Spanish patients and a compilation of 2834 reported cases. Neuromuscul Disord. 2018;28(3):208−15.
7. Lefebvre S, Burlet P, Liu Q, et al. Correlation between severity and SMN protein level in spinal muscular atrophy. Nat Genet. 1997;16(3):265−9.
8. Kernochan LE, Russo ML, Woodling NS, et al. The role of histone acetylation in SMN gene expression. Hum Mol Genet. 2005;14(9):1171−82.
9. Garbes L, Heesen L, Hölker I, et al. VPA response in SMA is suppressed by the fatty acid translocase CD36. Hum Mol Genet. 2013;22(2):398−407.
10. Brichta L, Hofmann Y, Hahnen E, et al. Valproic acid increases the SMN2 protein level: a well-known drug as a potential therapy for spinal muscular atrophy. Hum Mol Genet. 2003;12(19):2481−9.
11. Harahap ISK, Saito T, San LP, et al. Valproic acid increases SMN2 expression and modulates SF2/ASF and hnRNPA1 expression in SMA fibroblast cell lines. Brain Dev. 2012;34(3):213−22.
12. Sumner CJ, Huynh TN, Markowitz JA, et al. Valproic acid increases SMN levels in spinal muscular atrophy patient cells. Ann Neurol. 2003;54(5):647−54.
13. Brichta L, Holker I, Haug K, et al. In vivo activation of SMN in spinal muscular atrophy carriers and patients treated with valproate. Ann Neurol. 2006;59(6):970−5.
14. Tsai LK, Yang CC, Hwu WL, et al. Valproic acid treatment in six patients with spinal muscular atrophy. Eur J Neurol. 2007;14(12):e8−9.
15. Swoboda KJ, Scott CB, Reyna SP, et al. Phase II open label study of valproic acid in spinal muscular atrophy. PLoS One. 2009;4(5):e5268.
16. Swoboda KJ, Scott CB, Crawford TO, et al. SMA CARNI-VAL trial part I: double-blind, randomized, placebo-controlled trial of L-carnitine and valproic acid in spinal muscular atrophy. PLoS One. 2010;5(8):e12140.
17. Kissel JT, Scott CB, Reyna SP, et al. SMA CARNIVAL TRIAL PART II: a prospective, single-armed trial of L-carnitine and valproic acid in ambulatory children with spinal muscular atrophy. PLoS One. 2011;6(7):e21296.
18. Darbar IA, Plaggert PG, Resende MBD, et al. Evaluation of muscle strength and motor abilities in children with type II and III spinal muscle atrophy treated with valproic acid. BMC Neurol. 2011;11:36.
19. Kissel JT, Elsheikh B, King WM, et al. SMA valiant trial: a prospective, double-blind, placebo-controlled trial of valproic acid in ambulatory adults with spinal muscular atrophy. Muscle Nerve. 2014;49(2):187−92.
20. Saito T, Nurputra DK, Harahap NIF, et al. A Study of valproic acid for patients with spinal muscular atrophy. Neurol Clin Neurosci. 2015;3(2):49−57.
21. Krosschell KJ, Kissel JT, Townsend EL, et al. Clinical trial of L-Carnitine and valproic acid in spinal muscular atrophy type I. Muscle Nerve. 2018;57(2):193−9.

22. Also-Rallo E, Alías L, Martínez-Hernández R, et al. Treatment of spinal muscular atrophy cells with drugs that upregulate SMN expression reveals inter- and intra-patient variability. Eur J Hum Genet. 2011;19(10):1059−65.
23. Rakitin A, Kõks S, Reimann E, et al. Changes in the peripheral blood gene expression profile induced by 3 months of valproate treatment in patients with newly diagnosed epilepsy. Front Neurol. 2015;6:188.
24. Kaneko K, Arakawa R, Urano M, et al. Relationships between long-term observations of motor milestones and genotype analysis results in childhood-onset Japanese spinal muscular atrophy patients. Brain Dev. 2017;39(9):763−73.
25. O'Hagen JM, Glanzman AM, McDermott MP, et al. An expanded version of the Hammersmith Functional Motor Scale for SMA II and III patients. Neuromuscul Disord. 2007;17(9-10):693−7.
26. Pera MC, Coratti G, Forcina N, et al. Content validity and clinical meaningfulness of the HFMSE in spinal muscular atrophy. BMC Neurol. 2017;17(1):39.
27. Otsuki N, Arakawa R, Kaneko K, et al. A new biomarker candidate for spinal muscular atrophy: Identification of a peripheral blood cell population capable of monitoring the level of survival motor neuron protein. PLoS One. 2018;13(8):e0201764.
28. Huang DW, Sherman BT, Tan Q, et al. DAVID Bioinformatics Resources: expanded annotation database and novel algorithms to better extract biology from large gene lists. Nucleic Acids Res. 2007;35:W169−75.
29. Crawford TO, Paushkin SV, Kobayashi DT, et al. Evaluation of SMN protein, transcript, and copy number in the biomarkers for spinal muscular atrophy (BforSMA) clinical study. PLoS One. 2012;7(4):e33572.
30. Sumner CJ, Kolb SJ, Harmison GG, et al. SMN mRNA and protein levels in peripheral blood: biomarkers for SMA clinical trials. Neurology. 2006;66(7):1067−73.
31. Bebee TW, Gladman JT, Chandler DS. Splicing of the Survival Motor Neuron genes and implications for treatment of SMA. Front Biosci. 2010;15(3):1191−204.
32. Dangouloff T, Servais L. Clinical evidence supporting early treatment of patients with spinal muscular atrophy: Current perspectives. Ther Clin Risk Manag. 2019;15:1153−61.
33. Cartegni L, Krainer AR. Disruption of an SF2/ASF-dependent exonic splicing enhancer in SMN2 causes spinal muscular atrophy in the absence of SMN1. Nat Genet. 2002;30(4):377−84.
34. Kashima T, Manley JL. A negative element in SMN2 exon 7 inhibits splicing in spinal muscular atrophy. Nat Genet. 2003;34(4):460−3.
35. Wee CD, Havens MA, Jodelka FM, et al. Targeting SR proteins improves SMN expression in spinal muscular atrophy cells. PLoS One. 2014;9(12):e115205.
36. Irimura S, Kitamura K, Kato N, et al. HnRNP C1/C2 may regulate exon 7 splicing in the spinal muscular atrophy gene SMN1. Kobe J Med Sci. 2009;54(5):E227−36.
37. Nelson KK, Green MR. Mechanism for cryptic splice site activation during pre-mRNA splicing. Proc Natl Acad Sci U S A. 1990;87(16):6253−7.
38. Jodelka FM, Ebert AD, Duelli DM, et al. A feedback loop regulates splicing of the spinal muscular atrophy-modifying gene, SMN2. Hum Mol Genet. 2010;19(24):4906−17.
39. Feng J, Li L, Tong L, et al. The Involvement of Splicing Factor hnRNP A1 in UVB-induced Alternative Splicing of hdm2. Photochem Photobiol. 2016;92(2):318−24.
40. Nakayama K, Kataoka N. Regulation of gene expression under hypoxic conditions. Int J Mol Sci. 2019;20(13):3278.
41. Llorian M, Schwartz S, Clark TA, et al. Position-dependent alternative splicing activity revealed by global profiling of alternative splicing events regulated by PTB. Nat Struct Mol Biol. 2010;17(9):1114−23.
42. Hung LH, Heiner M, Hui J, et al. Diverse roles of hnRNP L in mammalian mRNA processing: a combined microarray and RNAi analysis. RNA. 2008;14(2):284−96.
43. Ottesen EW. ISS-N1 makes the first FDA-approved drug for spinal muscular atrophy. Transl Neurosci. 2017;8:1−6.
44. Mendell JR, Al-Zaidy S, Shell R, et al. Single-dose gene-replacement therapy for spinal muscular atrophy. N Engl J Med. 2017;377(18):1713−22.
45. Dhillon S. Risdiplam: First Approval. Drugs. 2020;80(17):1853−8.
46. Dangouloff T, Vrščaj E, Servais L, et al. Newborn screening programs for spinal muscular atrophy worldwide: Where we stand and where to go. Neuromuscul Disord. 2021;31(6):574−82.
47. Elshafay A, Hieu TH, Doheim MF, et al. Efficacy and safety of valproic acid for spinal muscular atrophy: a systematic review and meta-analysis. CNS Drugs. 2019;33(3):239−50.
48. Finkel RS, Mercuri E, Darras BT, et al. Nusinersen versus sham control in infantile-onset spinal muscular atrophy. N Engl J Med. 2017;377(18):1723−32.
49. Farrelly-Rosch A, Lau CL, Patil N, et al. Combination of valproic acid and morpholino splice-switching oligonucleotide produces improved outcomes in spinal muscular atrophy patient-derived fibroblasts. Neurochem Int. 2017;108:213−21.
50. Pagliarini V, Guerra M, Di Rosa V, et al. Combined treatment with the histone deacetylase inhibitor LBH589 and a splice-switch antisense oligonucleotide enhances SMN2 splicing and SMN expression in Spinal Muscular Atrophy cells. J Neurochem. 2020;153(2):264−75.