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Growth Hormone Strongly Induces h*SMN2* Promoter Driving Construct Gene Expression in Mammalian Cells

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What is already known on this topic?

Prolactin increases survival motor neuron (SMN) expression and survival in a mouse model of severe spinal muscular atrophy (SMA) via the STAT5 pathway. STAT5 constitutive activation rescues defects in SMA. Human growth hormone (hGH) increases SMN expression and survival in severe SMA mouse model. hGH treatment upregulates SMN protein in NT2 cells (shown total SMN protein in the manuscript, it is not known how much SMN is coming from *SMN1* gene or how much SMN protein is coming from *SMN2*). GH majorly activates STAT5 activation.

What this study adds?

Nobody shows effect of GH on human *SMN2* promoter using luciferase specific gene expression in mammalian cells. We did the first GH-*SMN2* Promoter study in the world and our study shows GH specifically-strongly affect *SMN2* promoter. Results showed that luciferase activity of the GH-treated pGL3-human *SMN2* (h*SMN2*) promoter 1 region increased 191.6-fold, GH-treated pGL3-h*SMN2* promoter 2 region increased 348-fold and GH-treated pGL3-h*SMN2* promoter 3 region increased 133-fold compared to GH-treated plasmid alone. These fold increases are too huge amount. GH may be used to increase *SMN2* gene expression to treat SMA.

ABSTRACT

Objective: Spinal muscular atrophy (SMA) is the most common neurodegenerative disease caused by the absence or insufficiency of the survival motor neuron (SMN) protein. Human *SMN1* (h*SMN1*) produces fully functional SMN protein but h*SMN2* produces only about 10% functional protein. Deletion or mutation in h*SMN1* gene leads to SMA, while the h*SMN2* copy number modifies disease severity. Increasing h*SMN2* expression has emerged as a potential therapeutic approach. In this study, we investigated the effect of growth hormone (GH) on h*SMN2* promoter activity using a reporter in Chinese hamster ovary (CHO) cells.

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Methods: Three different h*SMN2* promoter regions (588 bp, 1036 bp and 1705 bp) were used to show the effect on gene expression of reporter response to GH in this study. Promoters were amplified by polymerase chain reaction (PCR) and cloned into the pGL3 luciferase reporter vector. The ligation reactions were transformed into DH5α cells and positive colonies containing specific h*SMN2* promoter inserts were confirmed by PCR with h*SMN2*-primers. The plasmids carrying h*SMN2* promoters were transfected into CHO cells. After transfection, the cells were treated with GH for 24 hours and luciferase activity was measured to assess promoter activity.

Results: All h*SMN2* promoter constructs responded to GH. The 1036 bp promoter construct showed the highest luciferase expression upon GH treatment. However, the 1705 bp promoter construct exhibited reduced gene expression compared to the control vector treated with GH.

Conclusion: These findings suggest that GH can modulate h*SMN2* expression in h*SMN2* promoter dependent manner. GH may be a candidate hormone for SMA treatment by enhancing h*SMN2* expression.

Keywords: Spinal muscular atrophy, growth hormone, survival motor neuron protein, survival motor neuron 2 promoter, genetic disease

Introduction

Spinal muscular atrophy (SMA) is an autosomal recessive motor neuron disease characterized by degeneration of spinal cord motor neurons associated with proximal muscle weakness and muscular atrophy (1). SMA affects 1 in 6,000 to 1 in 10,000 individuals worldwide (2). Based on the age of onset and severity of the clinical course, childhood-onset SMA can be classified into four types (types I-IV). The most severe and most common (45%) type is type 1, which presents in infancy, and the mildest form is type 4, which is present in adults (3). Muscle weakness and impaired mobility are characteristic features of SMA (4). SMA is caused by a deficiency of the survival motor neuron (SMN) protein (1). The gene responsible for encoding the SMN protein maps to the reverse duplication site on 5q11.2-q13.3 and is called the *SMN* gene (5). In humans, there are two copies of the *SMN* gene, identified as human SMN1 (h*SMN1*) (telomeric) and h*SMN2* (centromeric) and this is unique to Homo sapiens (6). All forms of SMA result from homozygous loss of the hSMN1 gene due to gene deletion, conversion or mutation (7). Consistent with this gene duplication being a very recent evolutionary event, the h*SMN1* and h*SMN2* genes share more than 99.8% sequence homology over a 30 kb segment containing the entire coding region (8). h*SMN1* is composed of nine exons, 1, 2a, 2b, 3, 4, 5, 6, 7 and 8 (untranslated exon 8) encoding a 294 amino acid (aa) protein with a molecular weight of 38 kDa (9). h*SMN1* gene is transcribed into a full-length (FL) messenger RNA (mRNA). However, h*SMN2* is primarily transcribed into alternatively and naturally spliced mRNA lacking exon 7. This alternative splicing is caused by a silent mutation (C to T) in exon 7 of the h*SMN2* gene, which results in the loss of an exon splicing enhancer or the creation of an exon splicing repressor (1). The h*SMN2* gene mainly produces 90% transcripts lacking exon 7 and 5-10% wild type transcripts (10). When the h*SMN1* gene is deleted or mutated, the h*SMN2* gene cannot produce sufficient levels of functional SMN protein, resulting in SMA. All SMA patients have one or more copies of h*SMN2* gene and there is an inverse correlation between SMA severity and h*SMN2* copy number. The relationship between disease severity and copy number for h*SMN2* correlates with an increase in the FL SMN produced by each additional h*SMN2* gene (11). Increasing expression level of the h*SMN2* gene is considered an important strategy in the treatment of SMA.

Growth hormone (GH) has long been known to be a regulator of growth and sugar-fat metabolisms, but mechanisms of the transcription regulation by GH for some specific genes, such as h*SMN2*, are not described. GH binds to two GH receptors (GHR) and this ternary complex activates GHR-associated Janus kinase 2 (JAK2), which in turn phosphorylates tyrosines residues in itself, on the GHR and on intracellular proteins. Phosphorylated tyrosines on the receptor form docking sites for a number of signaling proteins, including members of the signal transducers and activators of transcription (STAT) family. Phosphorylated STAT proteins are released from the receptor and then they are dimerized, migrate to the nucleus and play an important role in the regulation of gene transcription (12). The role of the JAK/STAT signaling pathway in the regulation of h*SMN2* expression has also been demonstrated (5). STAT5 transcription factor in the STAT family plays an important role in the JAK2/STAT5 pathway. The PRL JAK2/STAT5 pathway is known to be involved in the regulation of h*SMN2* gene expression (5). GH is plays a major role in activation of STAT5 but there is no information about increased h*SMN2* gene expression by GH through the JAK-STAT5 signaling pathway in humans.

There is currently no cure for SMA. There are only treatments that slow the progression of disease severity and reduce symptoms. Recent studies have indicated that up-regulating h*SMN2* gene expression may be a possible treatment for SMA.

Previous *in vivo* studies have suggested that GH may influence SMN expression through STAT5 pathway activation. In particular, MacKenzie et al. (13) demonstrated that systemic administration of human GH (hGH) in severe SMA mouse models increased SMN protein levels in the brain and spinal cord, improved disease phenotype, and significantly prolonged survival. These results identified hGH as a potential therapeutic compound acting via STAT5 signaling (13). Building upon these findings, our study focused on the direct transcriptional regulation of the h*SMN2*

promoter by GH in a cell culture system, aiming to provide mechanistic evidence for the promoter-level responsiveness of *SMN2* to GH.

In this study, our purpose was to determine whether or not GH specifically increased the expression level of luciferase gene for the reporter vectors containing three different promoter regions of h*SMN2* gene in Chinese hamster ovary (CHO) cells.

Methods

Bioinformatic Analysis

The promoter regions of h*SMN1* and h*SMN2* genes were analyzed using data from the National Center of Biotechnology Information (NCBI) and The Eukaryotic Promoter Database (EPD). The promoter sequence of the two genes was compared and differences were determined using the bioinformatic tool, VectorBuilder (VectorBuilder Inc. 1010 W 35th Street, Suite 515 Chicago, IL 60609, USA Tel: +1 800-517-2189; https://en.vectorbuilder.com/). STAT5 transcription sites in promoter region of h*SMN2* gene were analyzed using EPD. A restriction enzyme map in the h*SMN2* promoter region was analyzed using NEBcutter 3.0 (a tool provided by New England Biolabs, Inc., 240 County Road, Ipswich, MA 01938-2723, USA; Tel: +1 978-927-5054; Email: info@neb.com; Website: https://www.neb.com/). Restriction enzyme cut sites were determined for cloning, based on restriction enzyme analysis of h*SMN2* promoter and the pGL3 vector cloning site. Restriction enzymes used in the study were NheI and XhoI (New England Biolabs, Inc., 240 County Road, Ipswich, MA 01938-2723, USA). Primers for h*SMN2* promoter regions were designed specifically and NheI and XhoI restriction sequences were added to 5' site of the primers. The primers for h*SMN2* promoter regions were analyzed for Tm and GC values using OligoAnalyzer™ Tool (Integrated DNA Technologies, Inc., Coralville, IA 52241, USA). Specificity and size of amplicons were also checked with NCBI Primer Blast Tool.

Molecular Biology Studies

DNA Isolation and PCR

Genomic DNA was isolated from blood using a salting out technique (14). DNA concentration and purity were measured with a NanoDrop (Thermo Fisher, USA) and DNA with an A260/A280 ratio between 1.8-2.0 was used for polymerase chain reaction (PCR). The PCR reaction for promoter regions 1 and 2 of h*SMN2* was prepared in a total volume of 25 µL and composed of 1x10X PCR Buffer, 1.5 mM MgCl2, 200 µM dNTP mixture, 0.5 µM h*SMN2* Forward1 or 2 primer, 0.5 µM h*SMN2* reverse primer, 500 ng genomic DNA, 1U Taq polymerase (Promega, USA). PCR amplification was performed using Thermal Cycler (Bio-Rad T100 96-Well, US) under the following conditions: initial denaturation: 94 °C 2 min (1X); first cycle: 94 °C 30s, 61 °C 30s, 72 °C 1 min (5X); following second cycle: 94 °C 30s, 65 °C 30s, 72°C 1 min. (25x); final extension: 72 °C 10 min. The long-range PCR for h*SMN2* promoter region 3 was performed in a total volume of 50 µL and composed of 1x PCR Buffer with MgCl2, 200 µM dNTP mixture, 0.5 µM h*SMN2* Forward3 primer, 0.5 µM h*SMN2* Reverse primer, 500 ng Genomic DNA, 1U Taq polymerase (Takara, Japan). PCR amplification was performed using Thermal Cycler (Bio-Rad T100 96-Well, US) under the following conditions: initial denaturation: 94°C 2 min (1X), 94 °C 30s, 65 °C 30s, 72 °C 2 min (5x); following cycle: 94 °C 30s, 68 °C 30s, 72 °C 2 min, Final extension: 72 °C 10 min. PCR products were analyzed by 1% agarose gel electrophoresis and the ethylene bromide-stained gel was visualized using the Gel Imaging System (Biolab, UK).

PCR Purification

PCR purification was performed using the High Pure PCR Product Purification Kit (Roche, Switzerland). Concentration of the purified PCR products was measured by NanoDrop (Thermo Fisher, USA).

Double Digestion

Double cut was performed for the h*SMN2* promoter PCR products and pGL3 vector using 25 µL PCR product (2 µg) or pGL3 vector (2 µg), 5 µL 10X Buffer (rCutSmart), 1 µL XhoI (20U), 1 µL NheI (20U) with a total volume of 50 µL. The digestion reactions were incubated at 37 °C for 4 hours (Bacterial Incubator, Binder, Germany). Digested pGL3 vectors and PCR products were purified as previously described. Concentrations of double cut PCR products and pGL3 vector were measured and analyzed by agarose gel electrophoresis.

Ligation

The double cut h*SMN2* PCR promoter products were ligated into double cut pGL3 vector using T4 DNA ligase (3U, Promega, USA). The ligation reactions were performed in 1/1, 1/3 and 1/5 ratios and incubated at +4 °C overnight. Restriction enzymes were inactivated 80 °C and 65 °C for 20 min respectively. Then, transformation was performed using heat shock method with 5 µL ligation product and 100 µL DH5α competent cells (Takara, Japan). Transformed products were plated on bacterial plates with ampicillin and incubated at 37 °C overnight (Bacterial Incubator, Binder, Germany).

Colony PCR

Direct colony PCR was performed to determine insertion of promoter regions of h*SMN2* gene in the pGL3 vectors on colonies. The transformed colonies on the plates were transferred into tubes containing 10 µL distilled, DNA-free water (dwater), from which 4 µL bacterial aliquot were taken into sterile tubes. The remaining 6 µL were incubated at 95 °C for 10 minutes. For direct colony PCR content: 2 µL template, 1.5 µL 10X PCR Buffer, 1.5 mM

MgCl2, 200 µM dNTP, 0.4 µM forward primer for vector, 0.4 µM reverse primer for vector, 0.5U Taq DNA Polymerase (Promega), 7.5 µL dwater. PCR amplification was performed using Thermal Cycler (Bio-Rad T100 96-Well, US) under the following conditions: initial denaturation: 94 °C 2 min (1X), 94 °C 30s, 55 °C 30s, 72 °C 90 sec (25X), final extension: 72 °C 5 min. PCR products were analyzed by 1% agarose gel electrophoresis and visualized using the Gel Imaging System (Biolab, UK).

Plasmid Isolation

Positive colonies were cultured overnight at 37 °C and the plasmid isolation was performed using a Genopure Plasmid Isolation Kit (Roche, Switzerland) following manufacturer protocol and DNA concentrations were measured.

Specific PCR for plasmids obtained from positive colonies. PCR was performed using 100 ng/µL plasmids, 1.5 µL 10X PCR Buffer, 1.5 mM MgCl2, 200 µM dNTP, 0.5 µM forward primer (h*SMN2* forward 1 or h*SMN2* forward 2 or h*SMN2* forward 3), 0.5 µM reverse primer (h*SMN2* reverse), 1U Taq DNA Polymerase and 8.5 µL dwater. PCR amplification was performed using Thermal Cycler (Bio-Rad T100 96-Well, US) under the following conditions: Initial denaturation: 94 °C 2 min, 94 °C 30s, 67 °C 30s, 72 °C 90 sec (30x), Final extension: 72 °C 5 min.

Cell Culture Studies

CHO cells were cultured in T25 flask containing High Glucose with L-Glutamine (500 mL, PAN-Biotech, Germany), 10% Fetal Bovine Serum, heat inactivated (500 mL, Wisent Inc, Canada) and 500 µL penicillin + streptomycin and incubated at 37 °C with 5% CO_2 (Mammalian cell culture incubator, Binder CB150. Germany).

Transfection

CHO cells were plated into 24 well plates containing $0.05x10^6$ cells. After 24 hours incubation, transfection was done using TransIT®-2020 Transfection Reagent (Mirus, USA) based on company protocol. Transfection was performed by 400 ng GHR, 400 ng STAT5 and 300 ng different reporter constructs containing different promoter regions of the h*SMN2* gene. Transfected CHO cells were incubated at 37 °C with 5% CO_2 for 24 hours.

GH Treatment

After 24 hours of transfection, the medium of the transfected CHO cells was removed and the cells were washed 3 times with DMEM. The transfected cells were starved with 0.5 mL DMEM for 1 hour at 37 °C in a 5% CO_2 incubator. The transfected cells were treated with GH (Genotropin Goquick, 5.3 mg/mL); (1000 ng/mL for each well) for 24 hours at 37 °C with 5% CO_2.

Cell Culture Lysis and Luciferase Assay

Cell Culture Lysis Buffer 5X Reagent (Promega, USA) was used for the lysis of cells. Medium in 24-well plate was removed and the cells were washed 3 times with cold 1X PBS. 100 µL Luciferase Cell Culture Lysis Reagent (1X) was added to each well and shaken for 15 min. The cell lysates were transferred into sterile centrifuge tubes and luciferase activity was performed using Promega Luciferase Assay System protocol. 100 µL of luciferase substrate and 20 µL of cell lysate were added into each well of a 96-well plate and luciferase activities were measured on EnSpire Multimode Plate Reader (PerkinElmer Inc., USA).

Statistical Analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) for Windows, version 30.0.0.0 (IBM Inc., Armonk, NY, USA). Normality of the data was assessed using the Shapiro-Wilk tests. A one-way ANOVA was performed to compare the luciferase activity among the four experimental groups. A post-hoc test (Tukey) was conducted to identify specific group differences. A p-value<0.05 was considered statistically significant.

Results

STAT5 binding sites in the promoter region of h*SMN2* gene were analyzed using the EPD tool and binding sites were located at -26, -334, -523, -750, -1631, -1686 from transcription start site. NheI and XhoI restriction sites were chosen for cloning based on pGL3 vector cloning sites and no presence in the h*SMN2* promoter. Three h*SMN2* promoter regions from transcription start site were amplified successfully by specific h*SMN2* promoter primers shown in Table 1. Amplified PCR products were analyzed by agarose gel electrophoresis and size of PCR products was 588 bp, 1036 bp and 1705 bp fragments, respectively (Figure 1).

Table 1. PCR primers used for h*SMN2* promoter amplification and for determination of insert in the pGL3 vector in this study

Name	Sequence (5'-3')
h*SMN2* reverse	TTAACTCGAGCGTCCCTTCTTAAGAGTGACGACTTC
h*SMN2* forward 1	ATTGCTAGCTAAGGATCTGCCTTCCTTCCTGC
h*SMN2* forward 2	ATTGCTAGCGGGCTGAGGCAGAATTGCTTG
h*SMN2* forward 3	ATTGCTAGCCCCGAGTTCAAGTGATTCTCCTGG
RV3 forward	CTAGCAAAATAGGCTGTCCC
GL2 reverse	CTTTATGTTTTTGGCGTCTTCCA

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h*SMN2*: human survival motor neuron 2, PCR: polymerase chain reaction

The amplified h*SMN2* PCR promoter products and pGL3 vector were digested by NheI and XhoI restriction enzymes. The digested PCR products were ligated into NheI and XhoI sites located in front of luciferase gene in pGL3 vector producing three pGL3-h*SMN2* promoter1, pGL3-h*SMN2* promoter2 and pGL3-h*SMN2* promoter3 constructs. In order to determine h*SMN2* promoter insert in the transformed colonies on amp plates, colony PCR was performed successfully using pGL3 vector primers shown in Table 1 and Figure 2 shows the agarose gel electrophoresis results of colony PCR. The size of PCR products including vector sequence were 788 bp, 1236 bp and 1905 bp respectively and these were the expected sizes for h*SMN2* promoters plus part of vector.

In order to confirm specific h*SMN2* promoter inserts in positive colonies, plasmids were isolated from cultured bacteria and they were amplified by specific h*SMN2* primers and PCR-agarose gel electrophoresis analysis were performed (data not shown). Results showed that inserts in plasmids were specific h*SMN2* promoter sequences and the sizes were correct.

In order to determine the effect of hGH on h*SMN2* promoters driving luciferase gene expression, transfected CHO cells expressing pGL3 alone, pGL3-h*SMN2* promoter1, pGL3-h*SMN2* promoter2 and pGL3-h*SMN2* promoter3 were treated with hGH and then their luciferase activities were measured. Luciferase results showed that GH strongly induced luciferase reporter gene expression for all reporter constructs driven by h*SMN2* promoters compared to luciferase expression of pGL3 vector

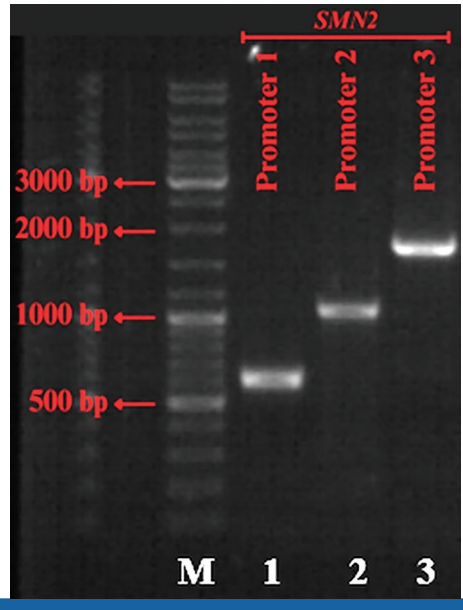

Figure 1. Agarose gel electrophoresis of purified h*SMN2* promoter PCR products. h*SMN2* promoter regions 1 and 2 were amplified by normal PCR and h*SMN2* promoter region 3 was amplified with long-range PCR. Purified h*SMN2* promoter PCR products were run on a 1% agarose gel. M shows GeneRuler DNA Ladder Mixture, line 1 shows h*SMN2* promoter 1 region corresponding to 588 bp, line 2 shows h*SMN2* promoter 2 region corresponding to 1036 bp and line 3 shows h*SMN2* promoter region 3 corresponding to 1705 bp DNA fragments, respectively

hSMN2: human survival motor neuron 2, PCR: polymerase chain reaction

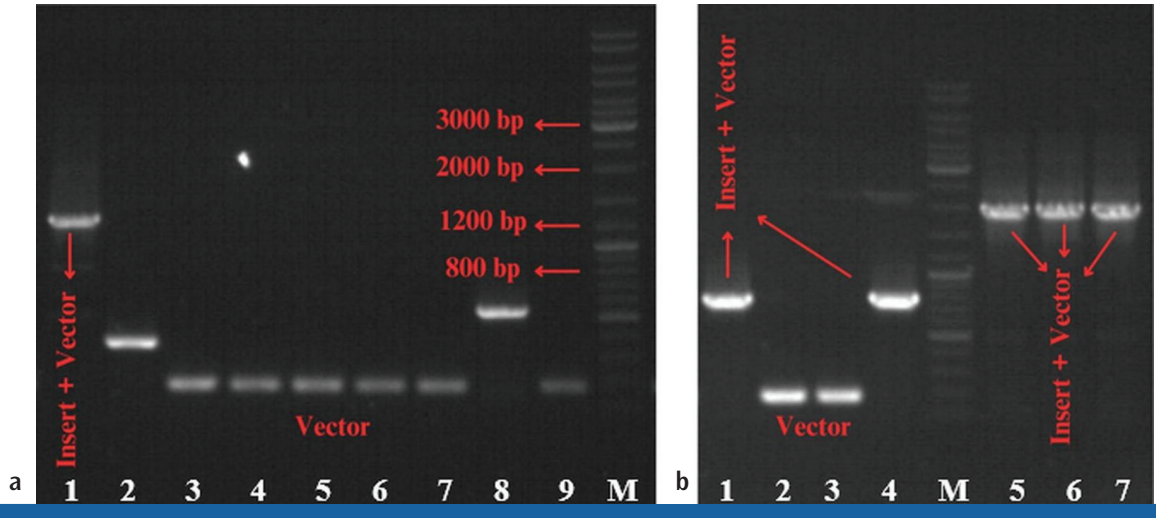

Figure 2. Agarose gel electrophoresis of colony PCR for h*SMN2* promoter regions. Transformed colonies on the bacterial plate were screened by colony PCR using pGL3 vector primers. The colony PCR products were analyzed on 1% agarose gel. **a)** Line 1 shows h*SMN2* promoter 2 region corresponding to 1236 bp (vector + insert), lines 3-7 and 9 show vector alone and line 2 and 8 show different h*SMN2* promoter insert sizes into the vector. **b)** line 1 and 4 show h*SMN2* promoter 1 region corresponding 788 bp (vector + insert), line 2-3 show 200 bp vector alone, line 5-7 show h*SMN2* promoter region 3 corresponding to 1905 bp DNA fragment (vector + insert). M shows GeneRuler DNA ladder mixture

hSMN2: human survival motor neuron 2, PCR: polymerase chain reaction

alone, with or without GH treatment. Although the pGL3-h*SMN2* promoter2 construct induced by hGH produced the highest level of luciferase gene expression, the pGL3-h*SMN2* promoter3 construct suppressed luciferase gene transcription, as shown in Figure 3.

Statistical analysis was performed by assessing the normality of the data using the Shapiro-Wilk test. To assess the normality of each group's data, the Shapiro-Wilk test was conducted. All groups showed p-values greater than 0.05 [pGL3 vector (+): p=0.824, pGL3-h*SMN2* Promoter 1 (+): p=0.567, pGL3-h*SMN2* Promoter 2 (+): p=0.943, pGL3-h*SMN2* Promoter 3 (+): p=0.846], indicating that the data were normally distributed. However, due to the small sample size (n=3 per group), the results of the normality tests should be interpreted with caution. A one-way ANOVA was performed to compare the luciferase activity among the four experimental groups [(1) pGL3 Vector (+), (2) pGL3-h*SMN2* Promoter 1 (+), (3) pGL3-h*SMN2* Promoter 2 (+), (4) pGL3-h*SMN2* Promoter 3 (+)]. The analysis revealed a statistically significant difference between the groups (p=0.002). Post-hoc analyses using Tukey's HSD test revealed significant differences between Group 1 and Group 2 (p=0.007), Group 1 and Group 3 (p=0.001), and Group 3 and Group 4 (p=0.030). No significant differences were found between the other group pairs. These results are presented in Table 2.

Discussion

SMA is an inherited autosomal recessive neurodegenerative disease presenting with variable phenotype and is characterized by the loss of motor neurons from the anterior horn cells of the spinal cord, resulting in progressive muscle loss and respiratory failure (7). Most cases of SMA (95%) have a homozygous deletion in the h*SMN1* gene on chromosome 5q13.

SMA is one of the most common autosomal recessive neuromuscular disorders. However, clinical heterogeneity in disease phenotype depends on h*SMN1* gene (Telomeric) and

Table 2. Tukey post-hoc test results

Group 1	Group 2	p value
pGL3 vector (+)	pGL3-h*SMN2* promoter 1 (+)	**0.007**
pGL3 vector (+)	pGL3-h*SMN2* promoter 2 (+)	**0.001**
pGL3 vector (+)	pGL3-h*SMN2* promoter 3 (+)	0.141
pGL3-h*SMN2* promoter 1 (+)	pGL3-h*SMN2* promoter 2 (+)	0.531
pGL3-h*SMN2* promoter 1 (+)	pGL3-h*SMN2* promoter 3 (+)	0.210
pGL3-h*SMN2* promoter 2 (+)	pGL3-h*SMN2* promoter 3 (+)	**0.030**

h*SMN2*: human survival motor neuron 2

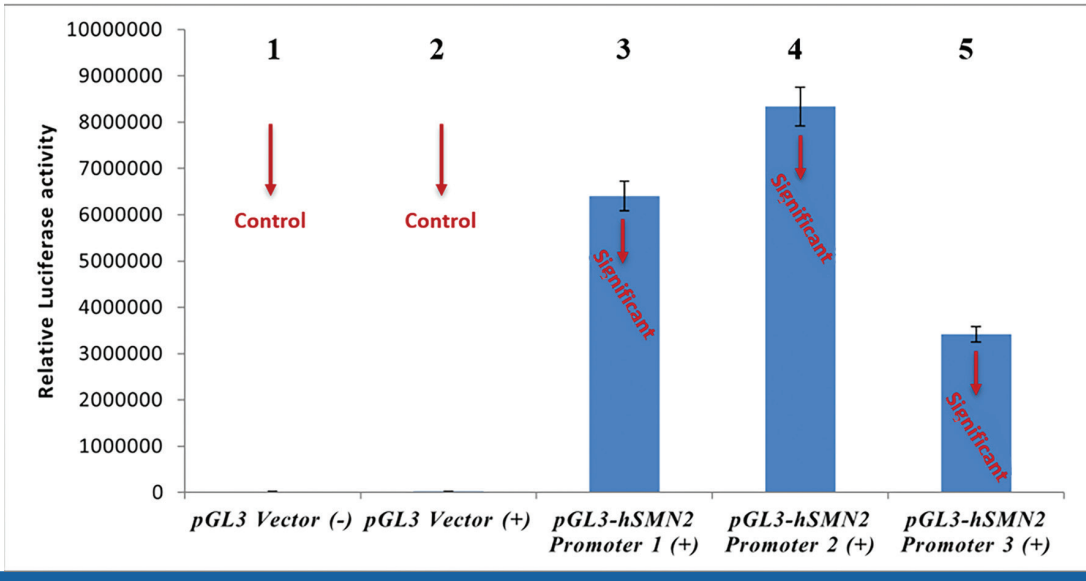

Figure 3. Relative luciferase activity of different pGL3-h*SMN2* promoter constructs in response to growth hormone stimulation (1000 ng/mL). Line 1 shows pGL3 vector alone without GH treatment, line 2 shows pGL3 vector alone with GH treatment, line 3 shows pGL3-h*SMN2* promoter 1 construct with GH treatment, line 4 shows pGL3-h*SMN2* promoter 2 construct with GH treatment, and line 5 shows pGL3- h*SMN2* promoter construct 3 with GH treatment luciferase activities respectively. Data are presented as mean±standard deviation

hSMN2: human survival motor neuron 2, PCR: polymerase chain reaction, GH: growth hormone

Table 3. Luciferase activity results for pGL3 vector with or without GH treatment and pGL3-h*SMN2* promoter constructs with GH treatment

Promoter	Mean relative light units (RLU)	
pGL3 vector (-)	19,169	
pGL3 vector (+)	27,923	
h*SMN2* promoter 1 (+)	5,350.000	191.6 fold
h*SMN2* promoter 2 (+)	9,715.000	348 fold
h*SMN2* promoter 3 (+)	3,715.000	133 fold

Compared to the pGL3 vector (+), luciferase activity increased 191.6-fold in the pGL3-h*SMN2* promoter 1 (+), 348-fold in the pGL3-h*SMN2* promoter 2 (+) and 133-fold in the pGL3-h*SMN2* promoter 3 (+)

h*SMN2*: human survival motor neuron 2, GH: growth hormone

h*SMN2* (Centromeric) genes, specifically the varying copy number of h*SMN2*. There is only one base difference between h*SMN1* and h*SMN2* genes and C residue at exon 7 in h*SMN1* is converted to T residue in *SMN2* gene (c.840C>T) disrupting the exogenic splicing enhancer (1). C->T substitution causes abnormal splicing resulting in removal of exon 7 (1). Exon 7 deleted mRNA gives truncated non-functional protein. SMA patients lacking h*SMN1* are dependent on the amount of residual h*SMN2* functional SMN protein for alpha motor neuron function. Several reports have shown that there is a strong positive correlation between SMA phenotype severity and the number of copies of h*SMN2* gene. Patients carrying high copy number of h*SMN2* show milder SMA (11).

There are several approaches to treatment of SMA, one of which increases h*SMN2* gene expression. There are numbers of studies that have been shown to increase h*SMN2* expression levels (1,5,7,13,15,16). Andreassi et al. (7) investigated the effect of 4-phenylbutyrate (PBA) treatment on h*SMN2* gene expression in fibroblast cell cultures obtained from SMA patients and PBA increased FL h*SMN2* transcript levels and SMN protein in cells from patients with all SMA types (Type I, II and III). Grzeschik et al. (1) showed the effect of hydroxyurea (HU) treatment on h*SMN2* gene expression in lymphoblastoid cell lines derived from SMA patients and HU was shown to increase the FL h*SMN2* transcript ratio in a dose and time-dependent manner. In addition to this, a significant increase in SMN protein levels and significantly increased nuclear gem (Gemini of Cajal bodies), which are SMN-containing nuclear structures were shown with treatment using HU (1). Biondi et al. (15) reported that NMDA receptor activation accelerated motor neuron maturation, reduced apoptosis and increased h*SMN2* gene expression in SMA model mice. It was reported that GH induced SMN expression in an SMA animal model (16). Previous studies have shown that GH activates the JAK/STAT pathway (15). However, the direct effect of GH on h*SMN2* promoter activity has not been previously reported in mammalian cells. Our study is first study to show that transfected cells incubated in the presence of hGH strongly increased h*SMN2* promoter driving gene expression of luciferase gene of construct in mammalian cells.

Although it has been reported that GH can regulate motor neuron function through the JAK/STAT pathway (5), the present study fills an important evidence gap by demonstrating the potential for GH to be used as a therapeutic target in the treatment of SMA.

Our results, showing a strong and specific activation of the h*SMN2* promoter by GH *in vitro*, are consistent with the findings of MacKenzie et al. (13) who reported that GH treatment increased SMN protein levels and extended survival in severe SMA mouse models. While their work demonstrated the therapeutic relevance of GH *in vivo*, our data provides mechanistic support at the transcriptional level by confirming that GH-linked signaling pathways can directly activate the h*SMN2* promoter. Together, these complementary studies strengthen the rationale for further investigation of GH or GH-related STAT5 activators as candidate therapeutic agents in SMA. However, translation from promoter-reporter assays to clinical application requires additional validation in motor neuron-derived cells, *in vivo* studies and eventually patient-based models.

Our h*SMN2* promoter studies showed that h*SMN2* promoter regions exhibited different levels of transcriptional activity in response to hGH treatment. pGL3-h*SMN2* Promoter 2 driving construct exhibited the highest luciferase activity among the h*SMN2* promoters, as shown in Figure 3. However, pGL3-h*SMN2* Promoter 3 driving reporter constructed lowered promoter activity, indicating a potentially suppressor regulatory role in promoter region. Luciferase activity of the hGH-treated pGL3-h*SMN2* promoter 1 region increased 191.6-fold, GH-treated pGL3-h*SMN2* promoter 2 region increased 348-fold and GH-treated pGL3-h*SMN2* promoter 3 region increased 133-fold compared to GH-treated plasmid alone, as shown in Table 3. The different transcriptional activities of the three h*SMN2* promoters may indicate the existence of enhancers and suppressor sequences located at the promoter regions for binding sites for transcription factors activated by GH. The 669 bp hSMN2 promoter region between residues 1036 and 1705 contains a

suppressor sequence. Addition to *in vitro* studies on CHO cells, GH-induced hSMN2 expression studies should be performed *in vitro* in human motor neurons cell or human fibroblast cell cultures obtained from patients with different type of SMA or in *in vivo* models.

Study Limitations

One of the limitations of our study was that we did not perform an electrophoretic mobility shift assay to demonstrate the binding of GH-induced STAT5 or other transcription factors to the *SMN2* promoter. Due to limited funding, we were unable to utilize radioactive labeling of the *SMN2* promoter, and we also lacked the necessary equipment to carry out this analysis. Therefore, we could not directly assess the transcription factors involved in the regulation of SMN2 expression.

Conclusion

These findings suggest that GH may be a potential therapeutic target in the treatment of SMA, but this needs to be confirmed *in vivo* in large animals. It is hoped that this study will stimulate investigation of a new therapeutic approach for SMA by demonstrating the effects of GH on h*SMN2* expressions. Our findings are among the first to identify the effects of GH on h*SMN2* promoter regions and this may provide a basis for further studies.

Ethics

Ethics Committee Approval: This research was approved by the Marmara University Faculty of Medicine Non-Drug and Non-Medical Device Research Ethics Committee (approval number: 09.2024.640, date: 10.07.2024). All human subjects' rights in this research were protected and any necessary approval was secured from the ethics committee.

Informed Consent: Informed consent was obtained from the volunteer included in the study.

Footnotes

Authorship Contributions: Concept: Ahmet Arman, Design: Ahmet Arman, Data Collection and Processing: Dilara Yücedal, Analysis or Interpretation: Ahmet Arman, Literature Research: Dilara Yücedal, Writing: Dilara Yücedal, Ahmet Arman.

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