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Research Article

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

Yücedal D and Arman A. Growth Hormone Activity on SMN2 Promoter

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What is already known on this topic?
-Prolactin increases SMN expression and survival in a mouse model of severe spinal muscular atrophy via the STAT5 pathway.
-STAT5 constitutive activation rescues defects in SMA.
-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-hSMN2 promoter 1 region increased 191.6-fold, GH-treated pGL3-hSMN2 promoter 2 region increased 348-fold and GH-treated pGL3-hSMN2 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 protein (SMN). h*SMN1* is producing fully functional SMN protein but h*SMN2* is producing 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 *hSMN2* expression has emerged as a potential therapeutic approach. In this study, we investigated the effect of growth hormone (GH) on *hSMN2* promoter activity using a reporter in CHO cells.
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 respond to GH in this study. They were amplified by 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 candidate hormone for SMA treatment by enhancing h*SMN2* expression.
Key Words: *Spinal muscular atrophy, growth hormone, survival motor neuron protein, survival motor neuron 2 promoter, genetic disease*

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1. 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 is presented 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 characterized 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 survival motor neuron (SMN) [5]. In humans, there are two copies of the *SMN* gene, identified as *hSMN1* (telomeric) and *hSMN2* (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 the fact that this duplication is a very recent evolutionary event, the *hSMN1* and *hSMN2* genes share more than 99.8% sequence homology over a 30 kb segment containing the entire coding region [8]. *hSMN1* gene is composed of nine exons, 1, 2a, 2b, 3, 4, 5, 6, 7 and 8 (untranslated exon 8) encoding 294 aa amino acid protein (38 kDa) [9]. *hSMN1* gene is transcribed into a full-length (FL) messenger RNA (mRNA), however, *hSMN2* 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 *hSMN2* gene, which results in the loss of an exon splicing enhancer or the creation of an exon splicing repressor [1]. The *hSMN2* gene mainly produces 90% transcripts lacking exon 7 and 5-10% wild type transcript [10]. When the *hSMN1* gene is deleted or mutated, the *hSMN2* gene cannot produce any SMN protein or sufficient SMN protein, resulting in SMA. All SMA patients have one or more copies of *hSMN2* gene and there is an inverse correlation between SMA severity and *hSMN2* copy number. The relationship between disease severity and number of copy for *hSMN2* correlates with an increase in the full-length SMN produced by the additional *hSMN2* gene [11]. Increasing expression level of the *hSMN2* 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 *hSMN2* are not described. GH binds to the Growth hormone receptor (GHR) and this ternary complex activates GHR-associated JAK2, which in turn phosphorylates tyrosine residues in itself, on the GHR and 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 receptor and then they are dimerized, travel to the nucleus and play an important role in the regulation of gene transcription [12]. The role of the Janus kinase (JAK)/STAT signaling pathway in the regulation of *hSMN2* 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 *hSMN2* gene expression [5]. GH is majorly involved in activation of STAT5 but, there is no information for increasing *hSMN2* gene expression by GH thoroughly 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 regulating *hSMN2* gene expression and increasing its expression level may be a possible treatment for SMA.

Previous *in vivo* studies have suggested that growth hormone may influence *SMN* expression through STAT5 pathway activation. In particular, MacKenzie et al. (2014) demonstrated that systemic administration of human growth hormone (HGH) in severe SMA mouse models increased SMN protein levels in the brain and spinal cord, improved disease phenotype, and significantly prolonged survival. Their results identified HGH as a potential therapeutic compound acting via STAT5 signaling [13]. Building upon these findings, our study focuses 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 increases the expression level of luciferase gene for the reporter vectors containing 3 different promoter regions of *hSMN2* gene in CHO cells. Results showed that all constructs containing *hSMN2* promoters responded to GH and 1036 bp *hSMN2* promoter region in the reporter construct gave highest level of gene expression of luciferase gene induced by GH. Our results show that GH may use to treatment of SMA disease.

2. Methods

2.1. Bioinformatic analysis

The promoter regions of h*SMN1* and h*SMN2* genes were analyzed by 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 bioinformatic tool (VectorBuilder). STAT5 transcription sites in promoter region of h*SMN2* gene were analyzed using EPD. Restriction enzyme map of the h*SMN2* promoter region was analyzed using NEBcutter 3.0. Restriction Enzymes cut sites were determined for cloning based on restriction enzyme analysis of h*SMN2* promoter and pGL3 vector cloning site. Restriction enzymes used in the study are NheI and XhoI. Primers for h*SMN2* promoter region were designed specifically and NheI and XhoI restriction sequence 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 (IDT). Specificity and size of amplicons were also checked with NCBI Primer Blast Tool.

2.2. Molecular biology studies

DNA Isolation and PCR. Genomic DNA was isolated from blood using 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 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 1x 10X PCR Buffer, 1.5 mM $MgCl_2$, 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 regions 3 was performed in a total volume of 50 µL and composed of 1x 10X PCR Buffer with $MgCl_2$, 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. The EtBr-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), 1µl 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). The ligation reactions were performed by 1/1, 1/3 and 1/5 ratio and incubated at +4°C for overnight. Restriction enzymes were inactivated 80ºC and 65 ºC 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 amp and incubated at 37ºC 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 10 µl dwater containing tubes. 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 $MgCl_2$, 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 1:30 min (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 Genopure Plasmid Isolation Kit (Roche, Switzerland) using its 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 $MgCl_2$, 200 µM dNTP, 0.5 µM forward primer (h*SMN2* Forward 1or 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, 60°C 30s, 72°C 1:30 min (30x), Final extension: 72°C 5 min.

2.3. Cell culture studies

CHO (Chinese hamster ovary) 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 37ºC 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 h*SMN2* gene. Transfected CHO cells were incubated at 37°C with 5% CO_2 for 24 hours.

Growth Hormone 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 GH (Genotropin Goquick, 5.3 mg/mL); (1000 ng/ul to 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, US) was used for the lysis of cells in this study. 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 shaked 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.

3. Results

STAT5 binding sites in the promoter region of h*SMN2* gene are analyzed by EPD tool and binding sites 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 existence in 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 is 588 bp, 1036 bp and 1705 bp fragments respectively shown in Figure 1.

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 are correct 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 are specific h*SMN2* promoter sequences and they are correct sizes.

In order to determine effect of GH 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 GH 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 alone with or without GH treatment. Although the pGL3-h*SMN2* promoter2 construct induced by GH 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 among 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.

4. Discussion

SMA is an inherited autosomal recessive neurodegenerative disease presenting different phenotype 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]. 95% of cases of SMA patients show a homozygous deletion in *hSMN1* gene on chromosome 5q13 and SMA is the second most common inherited disease. However, clinical heterogeneity in disease phenotype depends on h*SMN1* gene (Telomeric) and h*SMN2* (Centromeric) gene with patients carrying varying copy number of h*SMN2*. h*SMN1* gene produces a functionally full-length mRNA encoding functional SMN protein, however, h*SMN2* gene produces 10-15% functionally complete mRNA encoding SMN protein [11]. 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 exogenic splicing enhancer [1]. C>T substitution causes abnormal splicing resulting removal of exon 7. [1] Exon 7 deleted mRNA gives truncated non-functional protein. SMA patients lacking h*SMN1* dependent on a few h*SMN2* functional SMN protein for alpha motor neuron function. Several reports showed that there is a big 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 disease and one of them increases h*SMN2* gene expression. There are numbers of studies that have been shown to increase h*SMN2* expression levels. Andreassi et al. investigated the effect of 4-phenylbutyrate (PBA) treatment on h*SMN2* gene expression in fibroblast cell cultures obtained from SMA patients and PBA increased full-length h*SMN2* transcript levels and SMN protein in cells from patients with all SMA types (Type I, II and III) [7]. Grzeschik et al. 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 full-length h*SMN2* transcript ratio in a dose and time-dependent manner [1]. Addition to this, a significant increase in SMN protein levels and significantly increase nuclear gem structures were shown with treatment of HU [1]. Biondi et al. presented that NMDA receptor activation accelerates motor neuron maturation, reduced apoptosis and increased h*SMN2* gene expression in SMA model mice. [16]. It was reported that GH induces SMN expression in 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 GH strongly increased h*SMN2* promoter driving gene expression of luciferase gene in construct in mammalian cells. We can say that GH can induce expression of h*SMN2* gene expression.

Although it has been reported that GH can regulate motor neuron function through the JAK/STAT pathway [5]. This study fills an important gap by discussing how GH could be used as a potential therapeutic target in the treatment of SMA.

Our results, showing strong and specific activation of the h*SMN2* promoter by GH *in vitro*, are consistent with the findings of MacKenzie et al. 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 for SMA. However, translation from promoter-reporter assays to clinical application requires additional validation in motor neuron-derived cells and patient-based models.

Our h*SMN2* promoter studies showed that h*SMN2* promoter regions exhibited different levels of transcriptional activity in response to GH 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 GH-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 amount h*SMN2* promoters may be existence of enhancers and suppressor sequences located at the promoter regions where binding sites for transcription factors activated by GH. 669 bp h*SMN2* promoter region between residues 1036 and 1705 has suppressor sequence. Addition to *in vitro* studies on CHO cells, GH-induced h*SMN2* expression studies should be done in human motor neurons cells or human fibroblast cells obtained from different type of SMA patients or in vivo models.

Study Limitations

One of the limitations of our study is that we did not perform electrophoretic mobility shift assay (EMSA) 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.

5. 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 large animals. This study opens the door to a new therapeutic approach 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 provide a basis for further studies.

Ethics

Ethics Committee Approval: This research was approved by Marmara University's ethics committee in Turkey. All human subjects' rights in this research were protected and any necessary approval was secured from the ethics committee. (Approval number: 09.2024.640).

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

Footnotes

Authorship Contribution: Concept: Prof. Dr. Ahmet ARMAN, Design: Prof. Dr. Ahmet ARMAN, Experimental Processing: MSc Dilara YÜCEDAL, Analysis: Prof. Dr. Ahmet ARMAN, Literature Research: MSc Dilara YÜCEDAL, Writing: MSc Dilara YÜCEDAL and Prof. Dr. Ahmet ARMAN.

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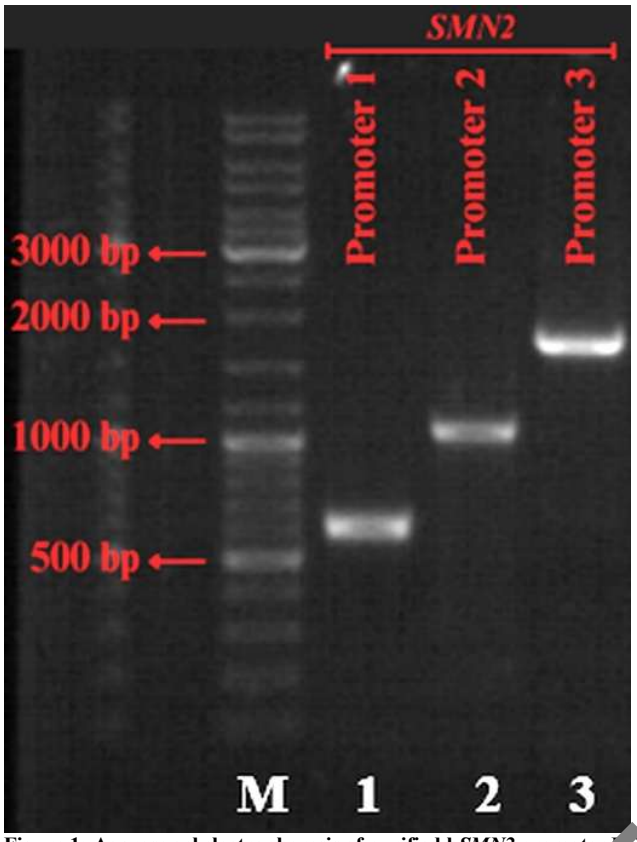

Figure 1: Agarose gel electrophoresis of purified h*SMN2* promoter PCR products
h*SMN2* promoter regions 1 and 2 were amplified by normal PCR and h*SMN2* promoter region 3 was amplified with long-range PCR. Purified h*SMN2* promoter PCR products were run on a 1% agarose gel. M shows GeneRuler DNA Ladder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

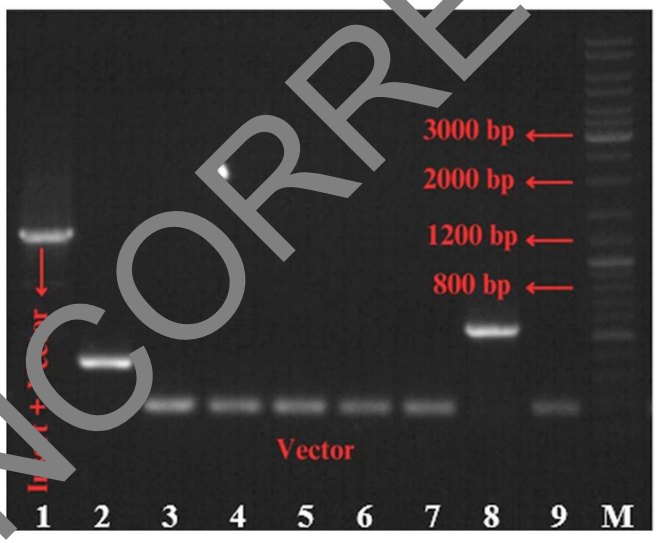

Figure 2a:

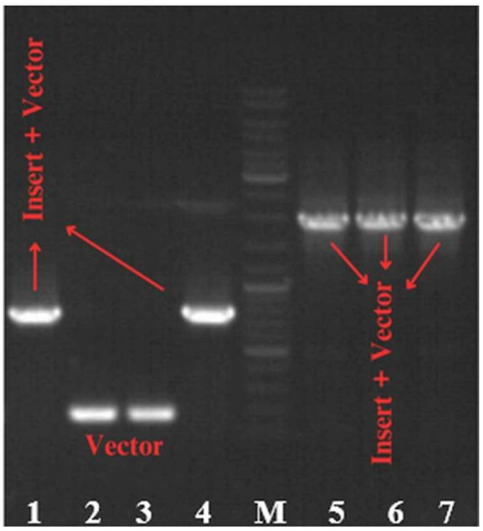

Figure 2b:

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. **In Figure 2a**: 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. **In Figure 2b**: 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 1905 bp DNA fragment (vector + insert). M shows GeneRuler DNA Ladder Mixture.

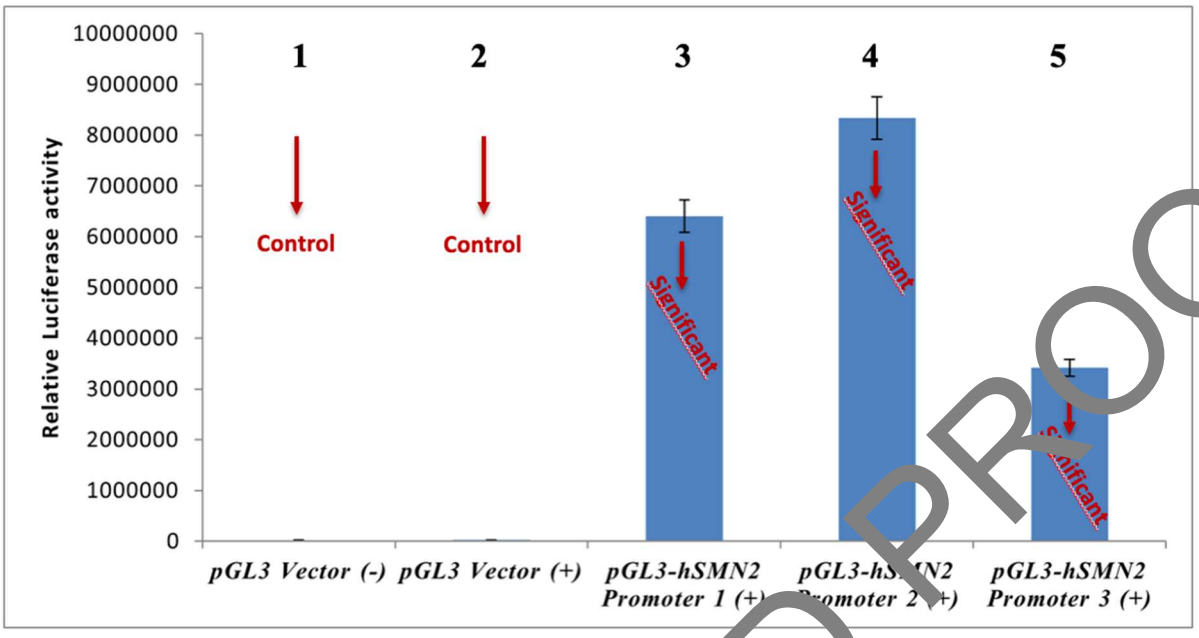

Figure 3: Relative luciferase activity of different pGL3-h*SMN2* promoter constructs in response to growth hormone stimulation (1000 ng/ml).

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's luciferase activities respectively. Data are presented as mean ± standard deviation.

Table 1: PCR primers used for hSMN2 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
Oligomer Biotechnology Inc.	

Table 2: Tukey post-hoc test			
Group 1	**Group 2**	**p-value**	**Significant**
pGL3 Vector (-)	pGL3-h*SMN2* Promoter 1 (+)	**0.007**	**Yes**
pGL3 Vector (+)	pGL3-h*SMN2* Promoter 2 (+)	**0.001**	**Yes**
pGL3 Vector (-)	pGL3-h*SMN2* Promoter 3 (+)	0.141	No
pGL3-h*SMN2* Promoter 1 (+)	pGL3-h*SMN2* Promoter 2 (+)	0.531	No
pGL3-h*SMN2* Promoter 1 (+)	pGL3-h*SMN2* Promoter 3 (+)	0.210	No
pGL3-h*SMN2* Promoter 2 (+)	pGL3-h*SMN2* Promoter 3 (+)	**0.030**	**Yes**
Note: A p-value < 0.05 indicates a statistically significant difference			

Table 3: Luciferase activity results for pGL3 vector with or without GH treatment and pGL3-h*SMN2* promoter constructs with GH treatment		
Promoter	**Mean of 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 (+).		