Original Article
Smooth muscle atrophy and colon pathology in SMN deficient mice

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Abstract: Spinal muscular atrophy (SMA) is an autosomal recessive genetic disorder characterized by loss of motor neurons in the ventral horn of the spinal cord. Clinical features such as progressively lethal respiratory weakness and associated muscle wasting have been extensively studied but less attention has been given to gastrointestinal (GI) dysfunction, which is common symptomatology in SMA patients with 43% constipation, 15% abdominal pain, and 14% meteorism. In the current study, the PrP92-SMN mouse model of SMA was utilized, to complement previous studies in which cells of the Enteric Nervous system (ENS) were susceptible to Smn (survival motor neuron) deficiency and could possibly be the basis of the observed GI symptoms. Necropsy of our mouse model showed impairment in feces excretion and smaller bladder mass, compared to Wild-Type (WT) animals. Along with the reduction in bladder mass, we also observed a decrease in the size of smooth muscles, due to reduction in Cross-Sectional Area (CSA). Interstitial cells of Cajal (ICC) provide important regulatory functions in the GI tract. To investigate if ICC are implicated in Smn deficient-induced colonic dysmotility, we assessed ICC distribution and abundance, by c-Kit, a well-established marker. SMA mice exhibited fewer c-Kit positive cells with altered localization, compared to WT. In conclusion, the observed histopathological abnormalities of our mouse model, can be secondary to SMN deficiency and could possibly be the basis of the observed GI symptoms. Necropsy of our mouse model showed impairment in feces excretion and smaller bladder mass, compared to Wild-Type (WT) animals. Along with the reduction in bladder mass, we also observed a decrease in the size of smooth muscles, due to reduction in Cross-Sectional Area (CSA). Interstitial cells of Cajal (ICC) provide important regulatory functions in the GI tract. To investigate if ICC are implicated in Smn deficient-induced colonic dysmotility, we assessed ICC distribution and abundance, by c-Kit, a well-established marker. SMA mice exhibited fewer c-Kit positive cells with altered localization, compared to WT. In conclusion, the observed histopathological abnormalities of our mouse model, can be secondary to SMN deficiency and could possibly underlie the GI symptoms observed in SMA patients. Future therapeutic approaches for SMA, must address not only CNS symptoms, but also non-motor-neuron-related symptoms. The PrP92-SMN mouse model could be a useful model for assessing therapeutic rescue of GI dysfunction in SMA.

Keywords: Spinal muscular atrophy, gastrointestinal dysfunction, smooth muscle atrophy, colon pathology, interstitial cells of Cajal, c-kit

Introduction

Spinal muscular atrophy (SMA) is an autosomal recessive genetic disorder characterized by loss of the anterior horn motor neurons of the spinal cord [1]. This rare neuromuscular disease is caused by mutation in the SMN1 (survival motor neuron) gene that encodes the full-length SMN protein [2, 3]. In humans a second form of the SMN gene exists, SMN2 [4]. SMN1 gene differs from SMN2 gene by only 5 nucleotides (nt); more specifically 1 nt at exons 7 and 8 and intron 6 and 2 in intron 7. The nucleotide exchange in exon 7, results in the creation of a new exonic splice silencer (ESS) and consequently 90% of the pre-mRNAs resulting from SMN2 are alternatively spliced, lacking exon 7. A small percentage (10%) of SMN proteins arising from SMN2 are fully functional [5]. The clinical manifestation of SMA includes pediatric progressive muscle wasting and paralysis, eventual respiratory failure [6] or even death based on the maximal motor function achieved [7, 8]. In SMA, the pathogenic mechanisms of frequently reported clinical features such as progressively lethal respiratory weakness [6] and other skeletal muscle wasting have been exten-
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SMA, which is primarily investigated, however, the gastrointestinal dysfunction enuresis and some other autonomic-nerve-associated symptoms like bradycardia hyperhidrosis and abnormal vasodilation that account for a proportion of symptoms in SMA patients [9-12] are rarely studied. According to the 2007 Consensus Statement for Standard of Care in SMA, patients with spinal muscular atrophy suffer from gastrointestinal dysmotility problems; such as delayed gastric emptying, and potentially life-threatening gastroesophageal reflux (GER) [13]. It has been shown by clinical studies that 4% of patients with SMA type II have been diagnosed with GER and another 7% with GER related symptoms. Furthermore, 43% of patients complain of constipation, 15% had abdominal pain, and 14% had meteorism [14].

In the gastrointestinal (GI) tract, one of the major Enteric Nervous System (ENS) components is Auerbach’s plexus (or myenteric plexus), which provides motor innervation to both longitudinal and circular muscular layers of the GI tract, with parasympathetic and sympathetic input [15, 16]. Besides direct innervation of motor neurons to smooth muscle cells, the innervation can also occur through interstitial cells of Cajal (ICC), a type of interstitial cell that interacts with and forms electrical connectivity with neurons and smooth muscle cells [17-20]. ICC provide important regulatory functions in the GI tract including: (a) pacemakers of electrical slow wave activity [21-24], (b) coordination of pacemaker activity and active propagation of slow waves [25-29], (c) neurotransmission by facilitating motor neural innervation from the myenteric ganglion [30], and (d) mechano-sensation to stretch of GI muscles [31-34].

There are different types of ICC: myenteric ICC (MY-ICC) are multipolar shape cells serve as a pacemaker which creates the bioelectrical slow wave potential that leads to contraction of the smooth muscle [35-38]. Intramuscular ICC (IM-ICC) are bipolar shaped cells involved in the stimulation of smooth muscle cells, and that neurotransmitters act through [39-42]. Submucosal interstitial cells of Cajal (SM-ICC) are multipolar shaped cells still thought to be pacemaker cells in the colon [35, 43]. It is reasonable to hypothesize that the ENS and ICC innervating the gastrointestinal smooth muscles may be vulnerable to SMN deficiency, as are motor neurons in the spinal cord innervating skeletal muscles.

In the current study, in order to investigate whether ENS (Auerbach’s plexus) and ICC innervating the GI smooth muscle are vulnerable to SMN deficiency, we utilized the SMN2; Smn; PrP92-SMN mutant mice (which we refer to as “SMA” mice). In this strain of mice, SMN production is localized to the CNS. Homozygous SMN2; Smn; PrP92-SMN mice are rescued from the severe SMA phenotype, have significantly increased lifespan (average of 210 days) and have normal lumbar motor neuron root counts and body weight [44]. They can potentially be utilized for studies evaluating SMN restoration only in the brain and spinal cord, thus modeling some of the current human therapeutic strategies. In these mice, we observed significant alterations in micro-pathohistology of the GI tract, including a decrease of smooth muscle cross sectional area in the SMN-deficient bladder and colon. More specially, c-kit positive ICC volume was decreased in the SMN-deficient colon. Both pathological alterations may cause reduced contractile force and problems with neuromuscular activation and transmission, leading to GI dysmotility, as is observed in SMA patients.

Materials and methods

Animal breeding and genotyping

Animal studies were performed in accordance with the Institutional Animal Care and Use Committee and approved by the University of Florida. (30 weeks) Male and female mice were used in all studies. Age and sex-matched C57BL/6J were used as controls.

SMN1-deficient, SMN2; Smn; PrP92-SMN mutant mice were utilized as the SMA mouse model. When maintaining a colony, mice heterozygous for the Smm1tm1Msd targeted mutation, homozygous for the SMN2 low copy line 89 transgene, and homozygous for the PrP92-SMN transgene were bred together. Unlike the delta7 mouse, which is homozygous for the Smm1tm1Msd targeted mutation (Smn null allele) and human SMN2 transgene (SMN2 low copy line 89) and exhibits symptoms, neuropathology, and early lethality similar to human type I proximal spinal muscular atrophy (SMA) patients, this strain carries the additional

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PrP92-SMN transgene (Figure S1). With the mouse prion protein (PrP92) promoter directing full-length human SMN, SMN is expressed at high levels in neurons, but low levels in cardiac muscle, skeletal muscle, kidney, and lower GI tract, with no expression in upper GI tract (Figure S1), liver and spleen [44-46]. With this approach, homozygous SMN2; Smn; Prp92-SMN mice are CNS rescued, have normal lumbar motor neuron root counts and much increased lifespan [44], which should be a good model to investigate the impact of SMN deficiency on organs that have low or no SMN expression.

The genotyping of the mice was completed according to the protocol of The Jackson Laboratory (JAX) [47]. Genomic DNA was extracted from tail clips and amplified with PCR using the following primers: Common primer, 5’-CTC CGG GAT ATT GGG ATT G-3’; Mutant reverse primer, 5’-GGT AAC GCC AGG GTT TTC C-3’; Wild type reverse primer, 5’-TTT CTT CTG GCT GTG CCT TT-3.

Colon tissue processing

Mice were sacrificed at 30-weeks of age, which is the average reported lifespan (210 days) of these animals [44] and weighed. Various tissues were dissected, weighed and processed. The entire colon was collected, its length was measured and dissected along the mesenteric border. The colon was divided into three pieces: one piece was fixed in ice cold acetone at 4°C for whole mount as previously described [48], another was embedded in optimal cutting temperature (OCT, Fisher HealthCare, US) and frozen in liquid nitrogen-cooled iso-pentane, the rest was snap frozen and stored at -80°C for western blotting.

<table>
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<th>Table 1. Antibodies utilized for immunostaining</th>
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<td>Antibodies</td>
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<tr>
<td>Primary antibodies</td>
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<tr>
<td>PGP 9.5</td>
</tr>
<tr>
<td>C-kit</td>
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<tr>
<td>Dystrophin</td>
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<tr>
<td>Secondary antibodies</td>
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<tr>
<td>Goat anti-Rabbit IgG, Alexa Fluor 594</td>
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<td>Goat anti-Rat IgG, Alexa Fluor 488</td>
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Histopathology

Transverse and longitudinal cryosections (10 μm) of distal regions of the colon were prepared using a cryostat (Leica, Germany) and were stained with haematoxylin and eosin (H&E). Images were captured on a light microscope (Leica DM4 B, Leica, Germany).

Immunohistochemistry

The preparation and immunostaining for whole mount samples and cryosections were performed according to the protocol from JOVE [48]. All sections were analyzed by immunohistochemistry using the primary and secondary antibodies listed in Table 1. Smooth muscle and bladder CSA was determined on 10 μm cryosections stained with dystrophin. For PGP9.5 primary antibody staining, sections were subjected to antigen retrieval by boiling slides in 10 mM citrate buffer (pH 6.0) for 20 minutes.

Imaging and quantification

Images were captured on a confocal laser scanning microscope (TCS SP8, Leica, German). All the images were quantified with ImageJ (Fiji 1.0) software. For the smooth muscle, the cellular membrane outline was identified by dystrophin positive pixels (green), and the cross-sectional area (CSA) was determined and qualified by combined use of ‘Trainable weka segmentation 3D’, ‘Binary’ and ‘Particles’ analysis. Similarly, the PGP9.5 positive pixels and DAPI stained nuclei were used to identify Auerbach’s plexuses (enteric nervous ganglia included). The ganglia cells and ganglionic cross section areas were counted and measured. The numbers of ICC, the c-kit positive (green) labeled...
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Table 2. Antibodies utilized for Western blot

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Host</th>
<th>Dilution</th>
<th>Company</th>
<th>Catalog number</th>
</tr>
</thead>
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<td>SMN</td>
<td>mouse</td>
<td>1:1000</td>
<td>Cell Signaling Technology</td>
<td>12976S</td>
</tr>
<tr>
<td>Goat anti-Mouse IgG, Alexa Fluor 488</td>
<td>Goat</td>
<td>1:1000</td>
<td>Thermo Scientific</td>
<td>A-11001</td>
</tr>
<tr>
<td>C-kit</td>
<td>Goat</td>
<td>1:2000</td>
<td>Bio-Techne Corporation</td>
<td>AF1356</td>
</tr>
<tr>
<td>Rabbit anti-Goat IgG, Alexa Fluor 790</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>Thermo Scientific</td>
<td>A27019</td>
</tr>
<tr>
<td>Anti-Actin Antibody, Alexa Fluor 488</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>Merck millipore</td>
<td>ABT1485-AF488</td>
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Figure 1. Anatomical features of the SMN2; Smn; PrP92-SMN mouse (SMA mouse) strain. (A) No difference was observed in Esophagus, Small Intestine and Colorectum lengths or (B) Body weight between SMA and WT mice. (C) The colon was dissected from SMA and WT mice. The arrows point to well-pelleted feces. SMA mice displayed increased numbers of pelleted-feces, indicating constipation. (D) H&E staining of SMA and WT colon. Secondary lesions (arrow) were present in SMA mice. (E) Representative images of colon smooth muscles stained for dystrophin. (F) Quantification of colon smooth muscle CSA. SMA mice have approximately 35% smaller smooth muscle CSA, compared to WT (8.476 ± 1.052 µm² N=7 vs 13.82 ± 1.735 µm² N=7, P=0.0218), exhibiting a significant shift in distribution towards smaller CSA fibers (Kolmogorov-Smirnov D=0.2584, P < 0.0001).

Results

Impaired feces excretion and reduced colorectal smooth muscle fiber size in SMA mice

It has been reported that patients with SMA type II have constipation and GI symptoms [14]. To determine the state of the GI system in our animal model, we measured the length of “major” parts of the gastrointestinal system (esophagus, small intestine and colorectum) (Figure 1A) and body weight (Figure 1B). No significant changes were observed in either part of the system.
the GI system in our SMA mice (esophagus: 28.52 ± 2.72 mm, small intestine: 382.6 ± 26.7 mm, colorectum: 105.2 ± 5.56 mm; N=2) compared to their controls (esophagus: 31.18 ± 2.58 mm, small intestine: 399.1 ± 24.13 mm, colorectum: 107.9 ± 6.24 mm; N=2). Also, no significance difference was observed in body weight in SMA mice (38.38 ± 6.74 g, N=8), compared to control (31.43 ± 12.01 g, N=4), neither is weight in specific muscles, because the skeletal muscles have similar SMN expression (Figure S1B and S1C). However, increased fecal loading was observed in SMA mice (Figure 1C). To determine the origin of the impaired fecal excretion, transverse sections of SMA mice colon were stained with Hematoxylin and Eosin (H&E) and examined for morphological alterations and possible inflammation. Evaluation of colon sections show the presence of secondary lesions in SMA mice and infiltration by inflammatory cells (Figure 1D). Another possible etiology, of the gastrointestinal problems observed in SMA patients is the involvement of smooth muscles of the GI system. We found that the CSA of smooth muscle in the colon was approximately 40% lower in SMA compared to control mice (Figure 1E, 1F). From the above we conclude, while the PrP92-SMN transgene rescues the SMA motor neuron and CNS phenotypes, dysfunctions of the GI system are still present.

SMA mice exhibit reduction in bladder and smooth muscle size

Another area that the role of SMA has been overlooked is enuresis and urinary incontinence. To the best of our knowledge there is just one clinical study that has examined that issue and reported that a third of all children diagnosed with SMA have signs of urinary incontinence [12]. To investigate whether a similar phenomenon is present in our animal model, we measure the bladder weight (mg)/body weight (g) (BD/BW), as a possible indication of the phenomenon. We found that BD/BW of our SMA mice was nearly 50% lower compared to WT mice (Figure 2A). Furthermore, based on immunohistological analysis, SMA mice bladder smooth muscle fibers were by 20% smaller than WT mice (Figure 2B, 2C). The smaller sized smooth muscle fibers may cause poor contraction and can be the etiology for urinary incontinence.

SMA mice have a lower volume of c-kit positive ICC in colon

The GI tract is innervated by extrinsic (sympathetic, parasympathetic and visceral afferent) axons and intrinsic neurons of the enteric nervous systems (ENS). Defects of these innervations or ICC facilitation may cause GI dysmotility [50-55]. In the current study, we examined enteric neurons and ICC in our animal model. Neuron density in the colon was identified and quantified by PGP9.5 staining. ICC abundance and distribution were determined by immunoblot and staining, respectively [48]. Examining the cryosections from serosa to mucosa, we found that no significant difference was observed in the mesh formed by PGP9.5-positive nerve cells, between SMA and WT mice. However, SMA mice exhibited 50% fewer c-Kit positive stained ICCs compared to WT mice (Figure 3A-J). The reduction of c-Kit pro-
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Protein levels in the SMA mice was confirmed by immunoblot (Figure 3K, 3L). Whole-mount immunofluorescence at three different tract layers (Myenteric (MY), Intramuscular (IM) and submucosa (SM)) (Figure 4), showed that there is a lower abundance (approximately 50%) of c-kit positive ICC in SMA mice compared to controls. These findings could be an indication that ICC may play a role in facilitating motor neural innervation from the myenteric plexus, and SMN deficiency might cause c-kit positive ICC maldevelopment and reduced abundance impacting neurotransmission.

Discussion

Spinal Muscular Atrophy patients present with GI-related symptomatology, which include gastroesophageal reflux, constipation and delayed gastric emptying. These phenotypes have received limited attention in SMA animal model studies. In the current study, 30-week old Prnp92-SMN mice (SMA mice) were used for investigation, allowing organs to be larger and better developed for histological analysis than in Delta 7 mice, which have an average survival of only 13 days [56]. Using the PrP92-SMN mice model of SMA, that exhibits a mild phenotype due to CNS and motor neuron rescue, we were able to examine the phenotypical and morphological alterations in the GI tract associated with the loss of SMN.

We found that the body mass of WT and SMA (PrP92-SMN) mice had no significant difference (Figure 1B), in contrast to two other stud-
Figure 4. Whole mount immunofluorescence of colon. Whole mount of colon with PGP9.5 (Red) staining for neurons, c-Kit (Green) staining for ICC and with DAPI (Blue) staining for cell nuclei in three focal planes: (Myenteric (MY), Intramuscular (IM) and submucosa (SM)): Compared to the SMA mice, the WT mice present stronger green intensity
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In a previous study [58], SMN deficiency was found to lead to disrupted ENS signaling to the smooth muscle of colon with a 10-fold greater contractile response with high-frequency electrical field stimulation. However, no neurodegeneration was found to be associated with abnormal ENS signaling in that study. This is consistent with our observation of no significant differences in ganglionic nucleus number and CSA were in our study. But this observation is contrary to a study in Taiwanese SMA mice that exhibited an increase of enteric neuron numbers in the small intestine [57]. Limited clinic data from 8 infants with Werdnig-Hoffmann disease (SMA type I) showed low values of neural tissue in the myenteric plexus in the small intestine and colon [59].

ICC [60] connects motor neurons to local effector cells, such as smooth muscle cells. The current study examines the impact of SMN deficiency on smooth muscle and ICC in SMA. A reduction of c-Kit positive ICC was observed in SMA mice, suggesting that SMN deficiency could lead to loss of ICC that resembles loss of motor neurons in spinal cord. ICC was previously found to integrate excitatory and inhibitory neurotransmission with slow-wave activity to orchestrate peristaltic motor activity [61], therefore ICC loss could be a potential reason for the abnormal ENS signaling in SMA mice. In addition, decreased smooth muscle CSA was seen in colon smooth muscle in our SMA mice. This muscular alteration could be directly caused by SMN-deficiency, as is the case in skeletal muscle, where it has been reported that SMN-deficiency impairs myotube formation by altering myogenic gene expression and focal adhesion dynamics that affect muscle cell migration and organization of the actin cytoskeleton [62]. The muscular alterations might also be the result of denervation resulting from the c-kit positive ICC loss.

In a previous study, macrophage infiltration was observed in the small intestine from the Taiwanese SMA mouse model [57]. Another animal study [63] reported that under the pro-inflammatory conditions of the muscularis-associated GI obstruction, the release of bioactive substances from activated resident macrophages may affect smooth muscle contractility. In addition, under these conditions, both the number and the function of neighboring ICC may also be affected [63, 64]. Thus, the macrophage infiltration in SMA colon tract could be a potential contributor to the ICC volume reduction and smooth muscle atrophy of the colon from SMA mice. Therefore, for the SMN deficiency-associated observed GI symptoms (smooth muscle atrophy, ICC loss, and macrophage infiltration), we cannot distinguish which are the primary pathological alterations due to SMN loss and which are secondary consequences. But in the future, cellular level studies could help determine if these defects are primary or secondary in SMN-depleted smooth muscle cells, ICC and macrophage cells.

In addition to GI symptoms, children with SMA types I and II have a high rate of urinary incontinence [12]. This has been identified as “stress incontinence” due to striated muscles weakness and atrophy in the pelvic floor muscles and the external urethral sphincter, but not the attributed to smooth muscles of the detrusor [12]. However, in our study, we observed a 50% lower bladder weight/body weight and 20% smaller bladder smooth muscle fibers in the SMA-deficient mice, suggesting that SMN deficiency may cause poor contraction of the smooth muscle may contribute to overflow incontinence.

In SMA, besides the well-known motor-neuron-related symptoms, symptoms like GI dysmotilit-
ty, bradycardia, and hyperhidrosis make up a non-motor-neuron-related symptomatic group. The histopathological abnormalities we found in colon and bladder of SMA mice, ICC loss and smooth muscle atrophy secondary to SMN deficiency, could be responsible for the pathological changes in SMA patients. In the future, the therapies for SMA will need to not only target the CNS and motor neurons, but also address such non-motor-neuron-related symptoms. The mice we described herein will be a useful model for testing the ability of therapies to rescue GI dysfunction at different post-natal stages.

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Disclosure of conflict of interest
None.

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Figure S1. A. In genotype, the functional SMN is 800 bp length DNA, the mutated Smn is 500 bp length. B. The weights in specific muscles of tibialis (TA) (52.10 ± 5.738 mg N=4 vs 55.625 ± 2.616 mg N=8, P=0.997) gastrocnemius (Gas) (134.15 ± 20.935 mg N=4 vs 138.9725 ± 5.459268 mg 4=N, P=0.995) and quadriceps femoris (Quad) (217.33 ± 34.139 mg N=4 vs 224.38 ± 5.933 mg N=4, P=0.984) had no difference between WT and SMA. C. SMA mice had lower or even deleted expression level of SMN protein in GI tissues, such as colon, stomach and small intestine, compared to WT. But SMA mice were found having leaky expression from the PrP92-SMN transgene, outside of the neuronal compartment, especially in gastrocnemius (Gas) quadriceps femoris (Quad) and etc.