Research Article

Clinical SMN1 and SMN2 Gene-Specific Sequencing to Enhance the Clinical Sensitivity of Spinal Muscular Atrophy Diagnostic Testing

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Purpose. Therapeutic advances in the treatment of spinal muscular atrophy (SMA) prompt the need for robust and efficient molecular diagnosis of this disease. Approximately five percent of SMA cases are attributable to one copy of SMN1 with a hypomorphic or inactivating variant in trans with a deleted or converted allele. These intragenic variants are challenging to definitively localize to SMN1 due to its sequence homology with the SMN2 gene. To enhance the clinical sensitivity of SMA diagnostic testing, we present an optimized gene-specific sequencing assay to localize variants to either SMN1 or SMN2.

Methods. SMN1 and SMN2 genes are independently amplified by long-range allele-specific PCR. Long-range products are used in subsequent nested PCR reactions to amplify the coding exons of SMN1 and SMN2. The resulting products are sequenced using standard Sanger-based methodologies and analyzed for disease-associated alterations.

Results. 83 probands suspicious for a clinical diagnosis of SMA with a nondiagnostic SMN dosage result were sequenced for intragenic variants in the SMN1 gene. Gene-specific sequencing revealed likely disease-associated variants in SMN1 in 42 cases (50.6%). Of the 42 variants, 27 are unique including 16 loss-of-function variants, 9 missense variants, 1 in-frame deletion variant, and 1 splice site variant.

Conclusions. Herein, we describe an optimized assay for clinical sequencing of the full coding region of SMN1 and SMN2. This assay uses standard techniques and equipment readily available to most molecular diagnostic laboratories.

1. Introduction

Spinal muscular atrophy (SMA) is an autosomal recessive motor neuron disease characterized by symmetric muscle atrophy and weakness secondary to anterior horn cell degeneration [1]. With an incidence of approximately 1 in 11,000 live births, SMA was considered the most common genetic cause of infant mortality; however, the recent approval of several therapies has drastically altered the natural progression of this disease [2]. Clinically, SMA disease presentations are heterogeneous with profound proximal muscle weakness, hypotonia, and early death observed in type I patients and decreasing severity with later presentations seen in types II, III, and IV [3].

SMA exhibits locus heterogeneity; however, the vast majority of SMA patients (~96%) segregate biallelic loss-of-function variants in the survival motor neuron gene (SMN1), a gene that encodes a protein involved in small nuclear ribonucleoprotein biogenesis (snRNP) and pre-mRNA processing [4, 5]. Pathogenesis may relate to motor neuron dysfunction from abnormal snRNP assembly producing ineffectual splicing or abnormal mRNA trafficking [6]. The human genome encodes
2. Materials and Methods

2.1. Samples. SMN1 and SMN2 gene-specific sequencing was performed at The Ohio State University Molecular Pathology/James Molecular Laboratory on 9 probands from our own institution and 74 from other hospitals (Supplementary Table 1). The primary reason for referral was gene-specific sequencing to amplify and sequence the coding regions of these genes allows for more comprehensive diagnostics thereby enhancing the ability to identify additional therapy-eligible patients.

2.2. SMN Copy Number Analysis. SMN1 and SMN2 copy number determination was carried out using a competitive dosage method. In brief, SMN1 and SMN2 were coamplified with an internal control and a calibrator. Resulting products were analyzed by capillary electrophoresis, and a semiquantitative approach was used for copy number determination as previously described [14].

2.3. SMN Gene Sequencing Strategy. First, the coding regions of SMN1 (GRCh38/hg38; NM_000344.4) and SMN2 (GRCh38/hg38; NM_017411.3) were coamplified and sequenced using standard Sanger-based methodologies (Supplementary Table 2). If a known pathogenic or uncharacterized variant was identified, the case was reflexed to gene-specific sequencing whereby SMN1 and SMN2 were independently amplified using allele-specific long-range PCR (Supplementary Figure 1). Subsequently, long-range products were assessed by nested PCR amplification of exon 8 and Sanger sequencing whereby the ratio of cytosine (C) from SMN1 (GRCh38/hg38 chr 5 g.70951946) to thymine (T) from SMN2 (GRCh38/hg38 chr 5 g.70076526) was used to confirm SMN1/SMN2 specificity. The exon harboring the putative disease-associated variant was also amplified and sequenced in a separate reaction to determine whether the variant originated from the SMN1 or the SMN2 long-range PCR product (Supplementary Methods).

3. Results

3.1. Redesign and Optimization of a SMN1 and SMN2 Gene-Specific Sanger Sequencing Assay. Given the lack of exon 1 coverage and inconsistent allele-specific amplification of our prior SMN1/SMN2 sequencing assay, we designed a long-range PCR/sequencing assay that built upon the work of Kubo et al. [15]. Using a common SMN1/SMN2 forward primer (SMN_FL_EX1-654_F) and the same exon 9 variant (SMN1–GRCh38/hg38 chr5 g.70952674; SMN2–GRCh38/hg38 chr5 g.70077254), a series of reverse primers were placed at varying positions and tested to identify the primer combination that yielded the highest degree of allele specificity (Supplementary Table 3). By comparing cytosine (SMN1) to thymine (SMN2) levels at coding position 840 (exon 8), the SMN1-specific reverse primer, SMN1_FL_EX8_R_16, and the SMN2-specific reverse primer, SMN2_FL_EX8_R_3, gave the highest degree of gene-specific amplification.

Using these optimized primer pairs, increased specificity was further achieved by introducing a dimethyl sulfoxide (DMSO) gradient with a concentration of 7% DMSO (range: 6%-15%) being optimal (Supplementary Figure 2). Finally, additional allele specificity was achieved by decreasing the molar concentration of the allele-specific reverse primer compared to the common forward primer (Supplementary Figure 3) [16].

3.2. Accuracy of the SMN1 and SMN2 Gene-Specific Sequencing Assay. To confirm accuracy of the new assay, 13 previously tested positive cases (with variants in each of the 8 coding exons using the standard salting-out method or the EZI DNA Blood Kit (Qiagen, cat # 951054) [13].
of SMN1 including five cases with exon 1 variants that could not be localized by the prior assay) and 12 negative cases were assessed. Using these optimized PCR conditions, all 25 cases showed gene-specific amplification as confirmed by C to T ratio at coding position 840 of exon 8 of each long-range product (Table 1). In the 8 positive cases with variants in exons 2-8, the SMN1 versus SMN2 localization of the sequence variant previously reported was confirmed. For the five exon 1 cases with the c.5C>G (p.Ala2Gly) variant, the new assay showed unequivocal SMN1 localization (Figure 1). Additional studies to assess the reproducibility of this assay were conducted including intra-assay, inter-assay, and inter-technician studies, all of which demonstrated concordant results (data not shown). Although it was not observed, it is perceivable that residual genomic DNA in the long-range PCR reaction could affect the specificity of the assay.

3.3. Summary of Results in Our Entire Patient Cohort. Since SMN1 and SMN2 gene-specific sequence analysis was launched at our institution in 2005, a total of 83 probands have been analyzed, including 81 with our prior SMN1/SMN2 sequencing assay. The median age at the time of referral was 3.72 years (range = 9 days to 76 years) and included 53 males, 27 females, and 3 cases where the sex was not reported. As expected, this cohort was enriched for cases with a single copy of SMN1 (n = 65); however, 10 cases with two copies of SMN1 and 8 cases where SMN1 copy number was not assessed were also tested (Supplementary Table 1).

After resolving the exon 1 cases with our new assay, forty-two cases harbored disease-associated variants in the SMN1 gene (42/83; 50.6%) including 27 unique variants (16 premature stop variants, 9 missense variants, 1 in-frame deletion variant, and 1 splice site variant). The missense variants clustered in the Tudor domain (exon 4) and the YG box (exon 7), with the most frequently occurring missense variant being c.5C>G (p.Ala2Gly) in exon 1 (Figure 2 and Supplementary Table 1). Loss-of-function variants occurred throughout the SMN1 gene with the most frequent being the c.770_780dup (p.Gly261LeufsTer8) variant in exon 7, which was seen in six cases (Figure 2 and Supplementary Table 1). The c.275G>A (p.Trp92*) and c.684dupA (p.Leu229Thrfs*27) variants, to our knowledge, have not been previously described in the literature and therefore represent novel findings. Noticeably absent from our cohort were the two most frequently reported variants in the literature, c.815A>G (p.Tyr272Cys) and c.399_402del (p.Arg133fsTer15) [17].

### Table 1: Accuracy results for SMN1 and SMN2 gene-specific validation cohort.

<table>
<thead>
<tr>
<th>Case ID</th>
<th>Copy number (SMN1, SMN2)</th>
<th>Exon</th>
<th>Gene</th>
<th>Previous clinical sequencing result</th>
<th>This study</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1, 1</td>
<td>1</td>
<td>SMN1</td>
<td>c.5C&gt;G</td>
<td>c.5C&gt;G</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>SMN2</td>
<td>c.5C&gt;G</td>
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<tr>
<td>2</td>
<td>n/d</td>
<td>1</td>
<td>SMN1</td>
<td>c.5C&gt;G</td>
<td>c.5C&gt;G</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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<td>c.5C&gt;G</td>
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</tr>
<tr>
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<td>1, 1</td>
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<td>c.5C&gt;G</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
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<td>1, 1</td>
<td>7</td>
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<td>c.770_780dupCTGATGCTTTG</td>
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<td></td>
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<td></td>
<td>SMN2</td>
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<td>SMN2</td>
<td>No variant detected</td>
<td>No variant detected</td>
</tr>
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</table>

NM_000344.4 transcript was used to annotate SMN1 variants. n/d: specimen was referred with no copy number data. Data not shown for negative cases (case ID: 84-95).
In forty of the remaining cases, a causative variant was not detected. Because this assay does not analyze the entirety of SMN1 (i.e., noncoding regions), this result further reduces the possibility of a diagnosis of SMA. The final sample of our cohort tested positive for the c.223G>A (p.Ala75Thr) by an external laboratory; however, they were unable to determine the gene of origin, SMN1 or SMN2. Using the methodology presented here, the c.223G>A (p.Ala75Thr) variant was localized to SMN2.

4. Discussion

We present the validation of an improved gene-specific sequencing assay for the detection of variants across the coding regions of SMN1 and SMN2. Given advancements in SMA therapeutics, expanding molecular diagnostic testing for symptomatic individuals with a single SMN1 allele is imperative for detecting therapy-eligible patients. Further, overcoming the technical hurdles associated with sequencing these paralogous genes contributes to more comprehensive testing for SMA.

The homology between SMN1 and its paralog, SMN2, presents a significant challenge for independently sequencing these loci. While long-range allele-specific PCR methods have been used to successfully localize variants to the SMN1 gene, these approaches have not necessarily offered highly reliable, full gene coverage. For example, one of the first sequencing assays failed to achieve coverage of exon 1 [18], which may have been due to the fidelity of the polymerase and its inability to accurately and efficiently copy across the entire SMN1 and SMN2 loci (~28.5 kB). More recently, an assay that achieved full gene coverage was described; however, in our experience, the specificity was variable [15]. It is important to note that the exact experimental parameters were not able to be replicated due to the unavailability of the polymerase. Due to these challenges (i.e., reagent reliability/availability, reproducibility, and limited coverage), the vast majority of clinical laboratories have elected not to perform gene-specific sequencing.

To achieve a high degree of specificity and reproducibility, long-range gene-specific amplification of the SMN1 and SMN2 loci required significant optimization including primer design, annealing temperature, DMSO concentration, and asymmetric primer concentrations. Numerous allele-specific primer pairs were designed to account for variables affecting allele specificity such as local sequence content and primer stability/thermodynamics. Additional measures including the presence of 7% DMSO and asymmetric primer concentration further contributed to achieving a high degree of gene-specific amplification. These variables, in addition to using commercially available products that have undergone rigorous quality control processes/certification, have contributed to the success of this gene-specific sequencing assay.
Our approach is advantageous because it uses established laboratory technologies that allow for expeditious and cost-efficient testing. Secondly, detection of variants by this DNA-based sequencing approach is not complicated by variants impacting transcript stability that have been seen with RNA-based approaches and it provides an opportunity to pursue family studies. Lastly, long-read sequencing technologies may also be used for sequencing the SMN paralogs, whereas loss-of-function variants localize throughout the coding region of SMN1 with a single copy of SMN1, challenges remain in variant interpretation. The missense variants identified here cluster in exons 4 and 7 whereas loss-of-function variants localize throughout the coding region of SMN1 (Figure 2), as has been noted in other studies [20]. Given the mechanism of disease, loss-of-function variants in symptomatic individual with a single copy of SMN1 typically meet likely pathogenic criteria. Missense variants, on the other hand, remain challenging due to limited functional studies and a lack of SMN1- and SMN2-specific population-level sequence data. Albeit less common, missense variants may elicit splicing defects that result in human disease [21]. While this mechanism has not been associated with SMA, it cannot be discounted. In any case, novel SMN1 missense variants could only achieve a classification of “variants of uncertain significance” using the existing guidelines [22].

There are some functional data that would support pathogenic classification in a subset of missense variants. In particular, missense variants localizing to the Tudor domain, a protein interaction domain that binds core spliceosomal (Sm) proteins, have the most obvious structural correlates. Disruption of the charge distribution within this domain, as is the case for the c.400G>A (p.Glu134Lys) variant, has been shown to abolish interaction with Sm proteins [23]. Supporting this case, we identified a symptomatic patient with a single copy of SMN1 and an in-frame deletion, c.418_432del (p.Asp140_Pro144), that would produce a similar charge distribution shift in this domain. In this disease-specific context, we argue that such missense variants affecting evolutionarily conserved residues within well-characterized functional domains can be regarded as likely pathogenic variants in patients with a single copy of SMN1. It is especially prudent to factor clinical data into the assessment in these cases, with cases presenting with SMA phenotypes providing additional evidence for disease association.

Further, classification of disease-associated variants will require additional resources such as more extensive population-based SMN1- and SMN2-specific sequencing to understand the frequency of variants in various populations. Variant interpretation would also benefit from the development of in vitro screens to rigorously test the functional consequence...
of novel missense variants. Establishing “disease-specific” pathogenicity criteria will also require cross-laboratory collaborations given the relative rarity of missense variants in SMA.

Lastly, the utility of this assay may also be used to further rule out a diagnosis of SMA. In this context, an apparently asymptomatic newborn with a single copy of SMN1 tested positive for a c.223G>A (p.Ala75Thr) variant by an external laboratory; however, the laboratory could not localize the variant to SMN1 or SMN2. Using the assay described here, our laboratory definitively localized the c.223G>A (p.Ala75Thr) variant to SMN2. This result further illustrates the utility of this clinical assay in that the possibility of a SMA diagnosis was drastically reduced for this patient.

5. Conclusions

Given the need for a definitive SMA diagnosis prior to initiating costly therapeutic interventions, we present an optimized assay for the molecular diagnosis of SMA patients with compound heterozygous variants in the SMN1 gene. This assay is cost-effective and may be adapted by nearly any clinical molecular diagnostic laboratory. While the methodology presented here overcomes the gene-specific localization of variants to SMN1 or SMN2, unique challenges for interpreting sequence variants due to the lack of population data remains; however, recent long-read SMN1 and SMN2 locus-specific sequencing approaches may help overcome this gap. Working towards comprehensive detection and a better understanding of SMN1 variants is essential for reliably diagnosing and identifying therapy-eligible patients.

Data Availability

The authors acknowledge that all data supporting the conclusions made by this study are available in the manuscript or as supplementary material.

Ethical Approval

This study was submitted and approved by The Ohio State University Biomedical Sciences IRB (study no. 2021H0153).

Consent

Consent was waived based on minimal risk to participants, and all data was coded and deidentified to study personnel.

Disclosure

The study was part of the employment of the authors at The Ohio State University, Columbus, OH.

Conflicts of Interest

The authors do not acknowledge any conflicts of interest in association with the information presented in this manuscript.

Authors’ Contributions

CRM, MRA, and TWP were responsible for the conceptualization. CRM, JF, PS, and MRA were responsible for the data curation. CRM, JF, PS, SL, TWP, DJ, and MRA were responsible for the formal analysis. MRA and TWP were responsible for the methodology. MRA and JF were responsible for the validation. CRM, DJ, and MRA were responsible for the writing—original draft. MRA, CRM, DJ, and TWP were responsible for the writing—reviewing and editing.

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Supplementary Materials

Supplementary material includes three figures (experimental overview, DMSO optimization, and primer concentration optimization), one section dedicated to description of gene-specific methodology, and three supplementary tables (all gene-specific sequencing results and SMN1 and SMN2 copy number, SMN PCR primers, and SMN1/SMN2 gene-specific PCR primers). (Supplementary Materials)

References


