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Research Article

Molecular Analysis of Spinal Muscular Atrophy: A genotyping protocol based on TaqMan® real-time PCR

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Abstract

Spinal muscular atrophy (SMA) is an autosomal recessive inherited disorder caused by alterations in the survival motor neuron I (*SMN1*) gene. SMA patients are classified as type I-IV based on severity of symptoms and age of onset. About 95% of SMA cases are caused by the homozygous absence of *SMN1* due to gene deletion or conversion into *SMN2*. PCR-based methods have been widely used in genetic testing for SMA. In this work, we introduce a new approach based on TaqMan® real-time PCR for research and diagnostic settings. DNA samples from 100 individuals with clinical signs and symptoms suggestive of SMA were analyzed. Mutant DNA samples as well as controls were confirmed by DNA sequencing. We detected 58 SMA cases (58.0%) by showing deletion of *SMN1* exon 7. Considering clinical information available from 56 of them, the patient distribution was 26 (46.4%) SMA type I, 16 (28.6%) SMA type II and 14 (25.0%) SMA type III. Results generated by the new method was confirmed by PCR-RFLP and by DNA sequencing when required. In conclusion, a protocol based on real-time PCR was shown to be effective and specific for molecular analysis of SMA patients.

Keywords: SMA, SMN1 gene, gene conversion, molecular analysis.

Introduction

Spinal muscular atrophy (SMA) is a genetic disorder characterized by symmetric proximal muscle weakness due to degeneration of the anterior horn cells of the spinal cord. Based on the severity of symptoms and age of onset, SMA is divided into four clinical types (Zerres and Rudnik-Schoneborn, 1995). Type I [Werdnig-Hoffman disease, Online Mendelian Inheritance in Man (OMIM) 253300] is the most severe form, characterized by muscle weakness and hypotonia within first days/months of life, resulting in death before the age of two. Type II (OMIM 253550) is characterized by proximal muscle weakness with onset between six to 12 months after birth, inability to walk and variable survival. Type III (Kugelberg-Welander disease, OMIM 253400) is marked by proximal muscle weakness after the age of 18 months, with survival to adulthood. Type IV (OMIM 271150) shows similar findings to those described for SMA III, except that the onset of muscle weakness usually only occurs in the second or third decade of life. SMA is an autosomal recessive disease with a prevalence of 1 in 10,000 and a carrier incidence of 1 in 50 (Ogino and Wilson, 2002).

The genomic locus of the disease is located at 5q11.2-q13.3 within a region characterized by a large inverted duplication of a 500 kb element (Lefebvre *et al.*, 1995). Included in this region is the SMA-determining gene, the survival motor neuron gene (*SMN*), which is also duplicated and both copies expressed (Burglen *et al.*, 1996). Both *SMN* genes are highly homologous and differ by few nucleotides changes within their 3' regions. These differences in exon 7 and exon 8 are used to distinguish the telomeric (*SMN1*, OMIM 600354) from the centromeric *SMN* copy (*SMN2*, OMIM 601627) in DNA analysis

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(Wirth, 2000). Despite the high similarity between these two genes, SMN1 protein is the only one that is required for the survival of motor neurons. In about 95% of the patients, the pathogenic mutation consists of the homozygous functional absence of exon 7 and 8, or only exon 7, of *SMN1* due to deletion or conversion to *SMN2*, respectively (Cobben *et al.*, 1995; Hahnen *et al.*, 1995; Lefebvre *et al.*, 1995; Velasco *et al.*, 1996; Simard *et al.*, 1997). The remaining cases are compound heterozygotes for a deletion/conversion of one *SMN1* allele and a small intragenic mutation of the other allele, or more rarely, intragenic mutations in both alleles (Wirth *et al.*, 1999). *SMN*2 is present in all patients but is unable to fully compensate for loss of *SMN1*.

A transition 840C > T in exon 7 leads to exon skipping (Cartegni *et al.*, 2006). As a result, most transcripts from *SMN2* lack exon 7, and the resultant truncated protein appears biochemically unstable and is rapidly degraded *in vivo* (Chang *et al.*, 2004; Vitte *et al.*, 2007). *SMN2* is unrelated to the disease, and deletion of both *SMN2* genes occurs in about 5%-10% of unaffected individuals (Gerard *et al.*, 2000). Nevertheless, *SMN2* is considered a disease-modifying gene because its copy number is related to disease severity and survival of affected patients (McAndrew *et al.*, 1997; Wirth *et al.*, 1999; Feldkotter *et al.*, 2002).

Direct DNA testing for detecting homozygous *SMN1* loss is an effective method for molecular diagnosis of SMA patients and has been widely used for confirmation of clinical diagnosis. Almost all genetic tests for SMA are designed to identify the homozygous absence of *SMN1* exon 7 due to the fact that the vast majority of symptomatic SMA patients have homozygous *SMN1* exon 7 deletion/conversion. In order to detect alteration in the *SMN1* gene, the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay remains a commonly used method along with modern approaches (van der Steege *et al.*, 1995). However, a number of other methodologies have been introduced to date, mainly quantitative assays based on different strategies and focusing dosage analysis on hemizygous *SMN1* deletions (Wirth *et al.*, 1999; Scheffer *et al.*, 2000; Xu *et al.*, 2003; Gómez-Curet *et al.*, 2007; Passon *et al.*, 2009; Maranda *et al.*, 2012), considering that gene dosage is essential for carrier detection.

We have previously used the PCR-RFLP test in our laboratory, which is routinely performed to identify SMA cases. In this present work, we describe a protocol using TaqMan® real-time PCR technology for detecting SMA patients that are homozygous for *SMN1* exon 7 deletion. This method was based upon the fact that *SMN1* exon 7 differs from *SMN2* exon 7 by a single nucleotide change, and specific probes will only detect the *SMN1* or *SMN2* sequence. We applied both methods in a cohort of individuals with clinical signs and symptoms of SMA, aiming to identify new cases and the applicability of a new method in research and diagnostic settings.

Material and Methods

The study comprised 100 unrelated individuals that were referred to the Medical Genetics Service of Hospital de Clínicas de Porto Alegre (HCPA) for genetic investigation due to clinical signs and symptoms of SMA. The great majority (80 cases) were from the State of Rio Grande do Sul, Brazil). This study was approved by the local Ethics Committee in Porto Alegre.

Genomic DNA from peripheral blood of individuals was isolated by a standard procedure (Miller *et al.*, 1988). DNA quantity was determined by Quant-iT dsDNA BR Assay kit (Invitrogen, Carlsbad, USA) and samples were diluted to 50 ng/μL and to 10 ng/μL in water. Negative and positive control samples were also included in the analyses.

Presence or deletion of exon 7 and exon 8 *SMN1* and *SMN2* genes were detected primarily by PCR-RFLP analysis as previously described (van der Steege *et al.*, 1995). The PCR amplification of exon 7 and exon 8 of the SMN genes was carried out in a total volume of 25 μL with the following final concentrations: 200 μM of each dNTP, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 800 nM of each primer, 1.5 mM $MgCl_2$, 1.25 U Taq DNA polymerase, 100 ng of genomic DNA, and 4% (v/v) of dimethylsulfoxide (DMSO) for exon 8 only. Amplification was performed as follows: an initial denaturation at 94 °C for 10 min, followed by 32 cycles of denaturation at 94 °C for 1 min, annealing at 57 °C (exon 7)/58 °C (exon 8) for 1 min, and extension at 72 °C for 1 min, followed by a final extension step at 72 °C for 10 min. Exon 7 was amplified with a forward intron 6 primer R111 (Lefebvre *et al.*, 1995) and a mismatched reverse exon 7 primer X7-Dra (van der Steege *et al.*, 1995) that creates a *Dra*I restriction site in the SMN2 product. Exon 8 was amplified with forward primer 541C960 and reverse primer 541C1120 (Lefebvre *et al.*, 1995). Digestion reactions were done using 10 units of restriction enzymes, *Dra*I for exon 7 and *Dde*I for exon 8, and kept at 37 °C overnight. Digested PCR products were analyzed by electrophoresis in a 3% (w/v) agarose gel and visualized by ethidium bromide staining under UV light.

Probes and primers for real-time PCR were designed using the Primer Express® Software v.3.0 (Applied Biosystems, Foster City, USA). Probes were specific for the SMN1 and SMN2 copies at base position 6 in exon 7. SMN1 and SMN2 probes were labeled with 6FAM™ and NED™ fluorescin dye at the 5' end respectively and contained a minor groove binder (MGB) and a nonfluorescent quencher (NFQ) at the 3' end (Table 1).

PCR was carried out in a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, USA) using a 96-well format. PCR amplifications for detecting *SMN1* exon 7 was performed in a total volume of 12 μL, containing 6 μL of TaqMan® Genotyping PCR Master Mix (Applied Biosystems, Foster City, USA), 900 nM of each primer, 250 nM of SMN1ex7 probe (*SMN1* gene) or

Table 1 - Primers and probes for detecting *SMN1* and *SMN2* genes using TaqMan® analysis.

Component	Sequence (5' > 3')
Primers	
SMNex7-F	AAT GCT TTT TAA CAT CCA TAT AAA GC
SMNex7-R	CTT AAT TTA AGG AAT GTG AGC ACC
Probes	
SMN1ex7	6FAM-CAG GGT TT**C*** AGA CAA A-MGB-NFQ
SMN2ex7-anti	NED-ATT TTG TCT **A***AA ACC C-MGB-NFQ

*Asterisks denote specific nucleotides for *SMN1* or *SMN2* gene copies.

SMN2ex7-anti probe (*SMN2* gene), 0.3 µL of eukaryotic 18S rRNA TaqMan® Endogenous Control (VIC®/MGB Probe, Primer Limited; Applied Biosystems, Foster City, USA) and 20 ng of genomic DNA. The following amplification protocol was used: activation of AmpErase® UNG function at 50 °C for 2 min, AmpliTaq Gold® activation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s, and annealing/extension at 62 °C (*SMN1* exon 7) or 60 °C (*SMN2* exon 7) for 1 min.

To estimate the influence of cross hybridizations between the *SMN1* and *SMN2* genes and respective probes, DNA samples from patients homozygous for *SMN1* or *SMN2* deletions were analyzed using both probes. PCR efficiency was monitored by adding specific primers for amplification of the eukaryotic 18S rRNA gene in each well as endogenous control. Data generated were analyzed using the Sequence Detection Software v.1.4.0 (Applied Biosystems, Foster City, USA).

Direct DNA sequencing was performed to confirm the genotype of three DNA samples as follows: one sample carrying both genes, one sample lacking the *SMN2* gene and a sample lacking the *SMN1* gene (indicative of SMA). Direct DNA sequencing was also used to confirm cases of gene conversion.

For DNA sequencing, exon 7 and exon 8 of the *SMN* genes were amplified using the same primers employed in PCR-RFLP analysis. Amplification conditions were similar except for primer concentration (600 nM of each primer) and amount of DNA (50 ng). After PCR amplification, samples were purified using 2.5 U of Exonuclease I (USB Corporation, Cleveland, USA) and 0.25 U of Shrimp Alkaline Phosphatase (USB Corporation, Cleveland, USA). The reaction mixtures were kept at 37 °C for 30 min followed by 15 min at 80 °C. Purified amplicons were sequenced using the BigDye® Terminator v.3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, USA), according to the manufacturer's recommendations. Sequencing products were purified by ethanol/ethylenediamine tetraacetic acid (EDTA) precipitation, and resuspended in formamide solution. Fragments were then resolved by capillary electrophoresis in the ABI Prism 3130xl Genetic Analyzer (Applied Biosystems, Foster City, USA). Sequencing data were analyzed using Sequencing Analysis Software v.5.3.1 (Applied Biosystems, Foster City, USA).

Results

Within the population of 100 individuals we detected 58 SMA cases (58.0%) through both methodologies, confirming the lack of *SMN1* exon 7 in these patients. We also evaluated exon 8 of the *SMN* genes by PCR-RFLP analysis, and the presence of *SMN1* exon 8 was detected in 8 out of these 58 confirmed SMA patients (13.8%), indicating the occurrence of gene conversion events in these patients. One of these patients showed a homozygous deletion of *SMN2* exon 8, probably due to the fact that only one allele with this region of *SMN2* was originally present, which was converted to *SMN1* exon 8, as stated above. Additionally, we found 5 individuals with a complete deletion of the *SMN2* gene within the remaining 42 individuals of the studied population. Figure 1 represents results obtained with this assay.

Clinical information available from SMA confirmed patients permitted to define the clinical type for 56 of them, according to the revised diagnostic criteria for SMA suggested by the European Neuro Muscular Centre (ENMC) (Zerres and Rudnik-Schoneborn, 1995). This is based on the age of onset and achievable motor milestones. Patient distribution was 26 (46.4%) with SMA type I, 16 (28.6%) with SMA type II and 14 (25.0%) with SMA type III.

The proposed protocol based on TaqMan® real-time PCR analysis was introduced and amplification conditions were able to eliminate cross hybridizations. We have also

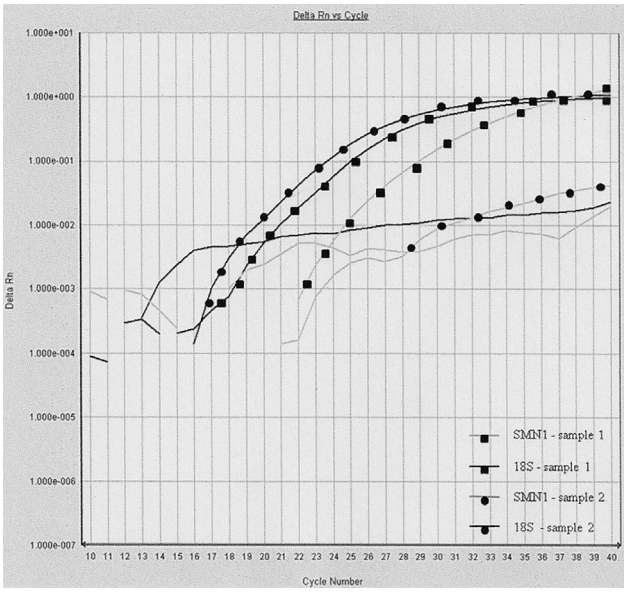

Figure 1 - TaqMan® assay for the *SMN1* gene. Sample 1 is DNA from an individual with the *SMN1* gene, showing both *SMN1* gene and eukaryotic 18S rRNA gene amplification. Sample 2 is DNA from a patient with a deletion of *SMN1* (indicative of SMA) showing only amplification of the eukaryotic 18S rRNA gene. Remaining lines with no amplification represent the negative control.

ruled out false positive results by simultaneously amplifying an endogenous control gene. Results from this new approach were all confirmed by the well established genotyping methodology (PCR-RFLP analysis). All negative and positive controls were submitted to direct DNA sequencing, the gold standard in sequence validation, to confirm genotypes (data not shown).

Both methodologies were accurate and reproducible for detecting the homozygous loss of the *SMN1* gene, corroborating molecular detection for a great majority of SMA patients. Once optimized, the TaqMan® real-time PCR analysis was faster due to fewer manipulation steps. In addition, we were also able to reduce the amount of DNA required for analysis due to the higher sensitivity of this technology.

Discussion

Molecular analysis to detect the lack of *SMN1* has been proven to be a specific tool for the diagnosis of SMA patients. Besides PCR-RFLP analysis, more sophisticated approaches, such as multiplex ligation-dependent probe amplification (MLPA) and methodologies based on capillary electrophoresis, have also been applied in recent years to determine *SMN1* and *SMN2* copy number (Passon *et al.*, 2010; Wang *et al.*, 2010). These methodologies are very powerful and generate highly informative results. However, they require sophisticated equipment and highly skilled personnel, therefore the need for an alternative for use in a routine diagnostic laboratory.

We present herein a protocol designed to improve molecular analysis of SMA. When compared to classic PCR-RFLP analysis, we found that the TaqMan® real-time PCR-based assay produced results within a considerably shorter period of time, with fewer manipulation steps and requiring smaller amounts of DNA than the PCR-RFLP method. These aspects become particularly relevant in the diagnosis of patients with SMA type I, for which onset is at an early age and disease progression is fast and devastating. These severe cases are in general presented in newborns where sample collection can be difficult and a result is essential for taking appropriate therapeutic measures. The protocol can be also adapted for the quantification of *SMN1* and *SMN2* copy number. This adapted protocol should be also useful for the detection of heterozygotes.

In the study cohort, 42.0% of the cases were not homozygous for *SMN1* exon 7 deletion. These results were confirmed by both methodologies. It remains possible that some of these patients may carry intragenic mutations, as previously reported to occur in around 4% of the cases in other studies (Wirth, 2000; Zapletalova *et al.*, 2007). Available clinical data showed a high frequency of SMA type I patients (46.4%), which is similar to other studies with SMA patients (Velasco *et al.*, 1996; Wirth, 2000). Gene conversion was also identified and presented in 13.8% of the confirmed patients. This frequency is comparable to data from Hahnen *et al.* (1995) who found a frequency of 12% for conversion events in SMA patients. In our cohort, such conversion events occurred in patients with milder SMA (SMA type II and type III cases). They were present in 28.6% of the SMA type III patients and in 18.8% of the SMA type II ones. These results corroborate the hypothesis that an increase in *SMN2* gene copy number would be responsible for the less severe SMA phenotypes (Wirth *et al.*, 2006; Watihayati *et al.*, 2009). Gene conversion was only detected in a single SMA type I patient, but this patient was also homozygous for a *SMN2* exon 8 deletion, which may be the primary cause of a more severe phenotype. Another interesting finding was that five individuals were found to be homozygous for a *SMN2* gene deletion. It remains to be shown whether the phenotype presented by these individuals was caused by a different clinical condition or by mutations in *SMN1* other than the exon 7 deletion.

In conclusion, a protocol based on real-time PCR was shown to be effective and specific for detecting SMA patients. Therefore, this method should become an additional option for SMA genotyping for research and/or diagnosis.

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