Multiplex ligation-dependent probe amplification improves diagnostics in spinal muscular atrophy

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Abstract

Spinal muscular atrophy (SMA) is an autosomal recessive disease caused by decreased levels of survival motor neuron protein (SMN). In the majority of cases, this decrease is due to absence of the SMN1 gene. Multiplex ligation-dependent probe amplification (MLPA) is a modern quantitative molecular method. Applied in SMA cases, it improves diagnostics by simultaneously identifying the number of copies of several target sequences in the SMN1 gene and in nearby genes.

Using MLPA in clinical diagnostics, we have identified a previously unreported, partial deletion of SMN1 (exons 1–6) in two apparently unrelated Swedish families. This mutation would not have been detected by conventional diagnostic methods.

This paper illustrates the broad clinical and genetic spectrum of SMA and includes reports of MLPA results and clinical descriptions of a patient with homozygous absence of SMN1 and only one SMN2 (prenatal onset SMA type 1), an asymptomatic woman with five SMN2 (lacking SMN1) and representative patients with SMA types 1, 2 and 3.

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1. Introduction

Spinal muscular atrophy (SMA) is characterized by degeneration of the anterior horn cells in the spinal cord, leading to progressive symmetrical muscle weakness and atrophy with very variable age at onset and clinical severity [1]. It is estimated to be the second most common autosomal recessive disease with an overall incidence of around 1 in 10,000 live births and a carrier frequency that may be as high as 1 in 35 [2,3]. The inconsistency between the incidence and the carrier frequency is at least partly caused by the presence of certain alleles that, in specific combinations, results in asymptomatic individuals and of other allele combinations that are assumed never to result in live births. SMA is divided into types 1, 2 and 3, based on age at onset and the maximum attained motor function. Children with SMA 1 (MIM 253300) usually manifest before 6 months of age and are never able to sit unaided. Children with SMA 2 (MIM 253550) usually manifest before 18 months of age and are never able to walk unaided. Patients with SMA 3 (MIM 253400) achieve the ability to walk unaided.

The vast majority of all SMA cases are caused by mutations in the survival motor neuron gene (SMN)
on chromosome 5q13. The mutational mechanism behind SMA, in around 94% of typical cases, is lack of SMN1 due to either SMN1 deletion or SMN1-to-SMN2 conversion. Furthermore, around 30 different small intragenic mutations have been reported [1]. 

SMN1 is located in a highly complex genetic region containing a variety of pseudogenes and repetitive elements due to an inverted duplication of approximately 500 kilobases. SMN is present in two versions, SMN1 (MIM 600354; GenBank: NM_000344) and SMN2 (MIM 601627; GenBank NM_022875), which differ by only one nucleotide in the coding region. This transition of a cytosine to a thymine in SMN2 exon 7 alters the splicing pattern, creating a truncated transcript (∆7 SMN) which encodes a non-functional protein lacking the last 16 residues at the C-terminal end. Nevertheless, SMN2 also creates a small proportion (10–20%) of full-length SMN transcripts and it has been shown that the number of SMN2 copies modulates the severity of the disease. The SMN protein is located in cytoplasmic and nuclear sub-structures, called gems, and appears to play an important role in spliceosomal snRNP biogenesis [4].

The purpose of this report is to show the strength of the multiplex ligation-dependent probe amplification technique (MLPA) as a clinical method for SMA diagnosis and to describe a previously unreported mutation of SMN1 that would not have been identified using conventional methods.

2. Materials and methods

2.1. Patients and samples

The study was approved by the Ethics Committee of Göteborg University and informed consent was obtained from all families. Genomic DNA was extracted from blood using the Puregene™ Kit from Gentra systems (USA). In one case, RNA was extracted from blood using the RNeasy™ Kit from Qiagen (Germany).

We introduced the MLPA technique into clinical practice in 2003. A total of 181 individuals, referred to the Department of Clinical Genetics for molecular genetic investigation due to suspected SMA or carriership, have been analysed using MLPA. The diagnosis of SMA has been confirmed in 43 patients and 54 carriers have been identified using this method; 84 analyses were negative. Cases 2 and 3 were included in this report with the aim of describing the previously unreported mutation consisting of a deletion of exons 1–6 in SMN1. To illustrate the strength of the MLPA technique, we chose representative cases (from those for which we have the clinical data) possessing 1–5 SMN2 copies and one case with a point mutation in SMN1. All chosen cases were of Swedish origin.

Clinical data concerning cases 1–9 are summarized in Table 1. Dynamic muscle strength was measured by manual muscle testing, according to a protocol developed by Scott et al. [5].

2.2. Multiplex ligation-dependent probe amplification

The MLPA technique is a gene-dosage method that can be used to detect deletions, duplications and certain point mutations [6]. The MLPA was performed in a thermal cycler with a heated lid, using the SALSA SMN region test kit, P021, from MRC-Holland (NL). This kit contains a probe mixture for SMN1 exons 7 and 8, SMN2 exons 7 and 8, SMN (1 and 2) exons 1, 4, 6 and 8, BIRC1 (and NAIPψ), GTF2H2 (BTFFp44) and several control fragments on different chromosomes, as well as the standard MLPA control probes used to determine ligation efficiency and DNA concentration. All reactions were carried out according to the manufacturer’s recommendations (www.mlpa.com). The quality of the genomic DNA was estimated using standard agarose electrophoresis. Approximately 400 ng genomic DNA was used for the MLPA reaction. The MLPA method is highly sensitive to the quality and concentration of the DNA. The MLPA products were analysed using an ABI 3100 genetic analyser from Applied Biosystems (USA), with ROX 500 as the internal size standard. Data analysis was performed using an Excel file. The peak height (and area) of each specific probe was normalised by dividing it with the combined heights (or areas) of the control probes. The relative peak height (and area) of each probe was compared with the relative peak height (or area) of the same probe in the control samples. Control samples were chosen from blood donors for those that have two similar alleles, each with one copy of SMN1, SMN2, BIRC1, NAIPψ and two copies of GTF2H2. All samples were analysed at least twice.

2.3. Sequencing of the SMN genes

The exons of the SMN genes were amplified and sequenced using primers 1–18 (Table 2) [7,8], ABI Prism™ BigDye ™ Terminator Cycle Sequencing v3.1 and the ABI 3100 genetic analyser. Analyses of heterozygote positions were aided by the knowledge of the number of SMN1 and SMN2 copies present.

2.4. Transcript analysis

Total RNA was extracted from blood using the Qia- gen RNeasy kit. cDNA was synthesised using random hexamer primers and Superscript™ II Reverse Transcrip- tase from Invitrogen (USA). Full-length and ∆7 SMN transcripts were amplified using primers in exon 6 and exon 8 (primers 37/38 in Table 2, full-length
<table>
<thead>
<tr>
<th>Case</th>
<th>Type of SMA</th>
<th>Age at onset</th>
<th>Age at last follow-up or death</th>
<th>Maximum motor functions achieved</th>
<th>Vital capacity on last exam (%)</th>
<th>Dynamic muscle strength MRC range</th>
<th>Number of SMN1 copies</th>
<th>Number of SMN2 copies</th>
<th>Haplotype</th>
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<tbody>
<tr>
<td>1</td>
<td>SMA 1</td>
<td>Birth</td>
<td>1 week*</td>
<td>None</td>
<td>0</td>
<td>0 (1 partial SMN1, exons 7–8)*</td>
<td>1</td>
<td>1</td>
<td>C212 maternal: 237*</td>
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<td></td>
<td>SMA 1</td>
<td>Birth</td>
<td>9 months*</td>
<td>None</td>
<td>0</td>
<td>0 (1 partial SMN1, exons 7–8)*</td>
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<td>2</td>
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<tr>
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<td>7 months*</td>
<td>None</td>
<td>0</td>
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<td>2</td>
<td>2</td>
<td>C212 maternal: 237*</td>
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<tr>
<td>5</td>
<td>SMA 2</td>
<td>1 year</td>
<td>10 years</td>
<td>Stand/walk with support</td>
<td>32</td>
<td>1–3</td>
<td>0</td>
<td>2</td>
<td>C212 maternal: 237*</td>
</tr>
<tr>
<td>6</td>
<td>SMA 2</td>
<td>1 year</td>
<td>61 years</td>
<td>Stand/walk with support</td>
<td>38</td>
<td>0–2</td>
<td>1 (SMN1 c.815A &gt; G, p.Y272C)</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>SMA 3</td>
<td>1.5 years</td>
<td>7 years</td>
<td>Walks with waddling gait.</td>
<td>90</td>
<td>2–4</td>
<td>0</td>
<td>4</td>
<td>Ag1-CA: 96, 104, 108</td>
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<tr>
<td>8</td>
<td>SMA 3</td>
<td>3 years</td>
<td>18 years</td>
<td>Walks, difficulties with stairs</td>
<td>81</td>
<td>3–4</td>
<td>0</td>
<td>4</td>
<td>No phase could be determined</td>
</tr>
<tr>
<td>9</td>
<td>Asymptomatic</td>
<td></td>
<td>42 years</td>
<td></td>
<td>4–5</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>100</td>
</tr>
</tbody>
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* The exon 1–6 deletion.
* Age at death.
SMN fragment of 497 bp and Δ7 SMN fragment of 441 bp). Only full-length SMN transcripts were amplified using primers in exon 7 and exon 8 (primers 39/40 in Table 2). The fragments were analysed using standard agarose gel electrophoresis and sequenced.

2.5. Haplotype analysis

Multiallelic microsatellite markers, Ag1-CA (D5S1556) [9] and C212 (D5F149S1/S2) [10], located in the duplicated region of 5q13, were analysed with dye-labeled primers on an ABI 3100 genetic analyser giving multiallelic haplotypes as described [3,11,12].

3. Results

The MLPA results for cases 1–3, 6, 8–9 and two control individuals are shown in Fig. 1 and a summary of clinical and genetic data for all cases is presented in Table 1. For comparison purposes, the inferred deletions in cases 1–5 and 7–9 and their parents are shown in Fig. 2. No samples were available from the parents of case 6 and no inferred genotypes could thus be constructed in this case.

All cases except cases 2, 3 and 6 had homozygous deletions of SMN1.

In case 2, with SMA type 1, MLPA revealed one copy each of SMN1 exon 7 and SMN1 exon 8, indicating that the boy had one deletion and was at least a carrier of SMA (Fig. 1). The analysis also showed two copies each of SMN2 exon 7 and SMN2 exon 8. The probe indicating the total number of SMN exon 8 showed three copies, which concurs with the above. In contrast, the probes indicating the total number of SMN exons 1, 4 and 6 detected only two copies of each exon. MLPA analysis of the mother showed that she had two copies each of SMN1 exons 7 and 8, the normal number, as well as two copies each of SMN2 exon 7 and SMN2 exon 8, a total of four SMN copies, the same result as that detected with the SMN exon 8 probe. As in the son, the probes indicating the total number of SMN exons 1, 4 and 6 each detected one fewer copy than the total number of exons 7 and 8. Analyses showed that the father had one SMN1 (Fig. 1). PCR with primers in exon 6 and exon 8 in the SMN genes (37/38 in Table 2), we were able to PCR-amplify the full-length product. Sequencing of this PCR product showed that it had the SMN2 sequence in exon 8 (data not shown) and hence that the full-length cDNA originated from SMN2. In summary these analyses indicate that there is a mutation in the remaining SMN1 copy, consisting of a deletion of exons 1 through 6. The exact breakpoints have not been identified. The 5' breakpoint is assumed to be located approximately in the centre of the inverted repeat, between SMN1 and the corresponding SMN2. This mutation has been inherited from the

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Table 2

<table>
<thead>
<tr>
<th>No</th>
<th>Position</th>
<th>Sequence</th>
<th>Reference</th>
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<tr>
<td>1</td>
<td>ex1−32</td>
<td>5'-CGTCACTTAAAGAAGGAGAC-3'</td>
<td>[8]</td>
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<tr>
<td>2</td>
<td>ex1+34</td>
<td>5'-GGAGAAGGAGGTGGCTGAGGAC-3'</td>
<td>[8]</td>
</tr>
<tr>
<td>3</td>
<td>ex2a−63</td>
<td>5'-GGATAAAGATGACTCTTGGTAC-3'</td>
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<tr>
<td>4</td>
<td>ex2a+35</td>
<td>5'-CCTTTCCAATGAAATAAAGCA-3'</td>
<td>[8]</td>
</tr>
<tr>
<td>5</td>
<td>ex2b−25</td>
<td>5'-CTTGCACACCCCTGTAACA-3'</td>
<td>[8]</td>
</tr>
<tr>
<td>6</td>
<td>ex2b+31</td>
<td>5'-TAAAGTAAATGACATCC-3'</td>
<td>[8]</td>
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<td>7</td>
<td>ex3−45</td>
<td>5'-CGAGATGATAGTTGGCCTC-3'</td>
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<td>5'-CATCTATGTCTCGTTCGCCA-3'</td>
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<td>9</td>
<td>ex4−19</td>
<td>5'-ACCTTTAATAACAAAACTGC-3'</td>
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<td>[7]</td>
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<td>[7]</td>
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<td>c.SMN 1223</td>
<td>5'-CTACAACCAATGTCCTGAG-3'</td>
<td>[7]</td>
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<td>5'-GATTCTCTTGATGATGCTAT-3'</td>
<td>[7]</td>
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<td>5'-TTTAGATACTACAAACAGGCTTCT-3'</td>
<td>[7]</td>
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<td>39</td>
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<td>5'-GACAAAAATCAGAAGAAGGAA-3'</td>
<td>[7]</td>
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<td>40</td>
<td>c.SMN 1235</td>
<td>5'-GACAGTCTTTTATAAACTACAACA-3'</td>
<td>[7]</td>
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Fig. 1. MLPA results shown as number of gene copies versus probes: a, SMN-exon 1; b, SMN-exon 4; c, SMN-exon 6; d, SMN-exon 8; e, SMN1-exon 7; f, SMN1-exon 8; g, BIRC1; h, SMN2-exon 7 and i, SMN2-exon 8. Case 1 had no SMN1 and only 1 SMN2. Her mother and father had one SMN1, respectively, as do the majority of SMA carriers. Case 2 had only one copy of SMN1 exon 7 and fewer copies of SMN exons 1–6 (2 copies) than the total number of SMN exons 7–8 (3 copies). His mother had 2 copies of SMN1 exon 7 and fewer copies of SMN exons 1–6 (3 copies) than the total number of SMN exons 7–8 (4 copies), while his father had one SMN1 and two SMN2. Case 3 is very similar to case 2 having only one copy of SMN1 exon 7 and fewer copies of SMN exons 1–6 (2 copies) than the total number of SMN exons 7–8 (3 copies). His mother had 2 copies of SMN1 exon 7 and fewer copies of SMN exons 1–6 (3 copies) than the total number of SMN exons 7–8 (4 copies), while his father had one SMN1 and two SMN2. Case 8 had no SMN1 and 4 SMN2 copies. Her mother, case 9, had no SMN1 and 5 SMN2 copies, while her father had one SMN1. C1, control DNA (2 SMN1 and 2 SMN2). C2, control DNA (2 SMN1 and 0 SMN2). Case 6 had only one SMN1 and 3 SMN2 copies. The mutation SMN c.815A > G (Y272C) was later identified. (All probes are not shown.)
subject’s mother and, in combination with the zero copy SMN1 allele from the father, it results in SMA. Haplotype analyses of multi-copy micro-satellites were performed (Table 1).

In case 3, with SMA type 1, MLPA yielded results identical to those in case 2, indicating that the boy had at least one deletion of the entire SMN1 and one deletion of exons 1–6 in SMN1 (Fig. 1). MLPA analysis of the mother showed that she was a carrier of the exons 1–6 deletion in SMN1. The father had one normal SMN1 and, in the other allele, a large deletion covering both BIRC1 and GTF2H2, in addition to SMN1 (Fig. 2). No RNA was available. The partial deletion of SMN1 (exons 1–6) has been inherited from the mother and, in combination with the zero copy SMN1 allele from the father, it results in SMA. Haplotype analyses were performed (Table 1). These haplotypes were identical to the ones in case 2.

In case 6, with SMA 2, MLPA showed heterozygous absence of SMN1 and the presence of three SMN2 copies (Fig. 1). Sequencing of all the exons of SMN1 and SMN2 identified the mutation SMN c.815A > G (Y272C) (data not shown), confirming the diagnosis of SMA. The primers used amplify both the single SMN1 and all three SMN2, showing approximately 25% heterozygosity of the altered position in the sequencing. Haplotype analyses were per-
formed (Table 1). The results were not compatible with the previously identified founder haplotype for the c.815A > G mutation [12,13].

The MLPA probe for SMN2 exon 7 cross-reacts with the SMN1 exon 7 (see Fig. 1, in which C2 is a control lacking SMN2 and the SMN2 exon 7 probe still emits a significant signal). This cross-reaction is not apparent in analysis of individuals with two SMN1 copies and two SMN2 copies, since this is the genotype used as a reference (compare with C1 in Fig. 1). In cases with no SMN1 copies, there will be a trend towards a comparably lower signal from this probe. The difference between apparent SMN2 dose and real SMN2 copy number will increase the more SMN2 copies an individual has (compare, for example, with cases 8 and 9 in Fig. 1). Case 8 has apparently 3.5 copies of SMN2 exon 7, compared to four copies of SMN2 exon 8. This is due to the lack of cross-signals from SMN1, which are present in the normal control to which it is compared. The effect is even greater in case 9, in which there are apparently four copies of SMN2 exon 7, compared to five copies of SMN2 exon 8. This effect would disappear if all analyses were compared to a DNA lacking SMN1 instead of to a normal DNA reference.

4. Discussion

We describe a novel SMN1 deletion mutation and demonstrate how the introduction of MLPA contributes to improving diagnostics in SMA.

Two apparently unrelated cases with a previously unreported mutation, consisting of a deletion of exons 1–6 in SMN1, have been identified. The families of both cases originate from northern Sweden. Haplotype analysis suggests that this might be the same mutation and raises the question of whether this is a founder effect and of whether this allele might contribute to SMA cases in Sweden previously considered to be unrelated to SMN1.

The deletion of exons 1–6 mutation would neither have been detected by standard DNA analysis, by quantitative assays generally directed only at exons 7 and 8 (see, for example, Anhuf et al. [14]), nor by the commonly used qualitative analyses, restriction enzyme digestions or SSCP analysis [15]. Any quantitative method would have identified these patients as at least carriers of SMA and they would therefore probably have been singled out for further studies, e.g. sequencing. Normal exon-by-exon sequencing would not have detected this mutation. RNA-based analysis for SMA is usually not included in standard clinical practices. The MLPA method can detect partial deletions of SMN1. The current version of the MLPA kit for SMA includes probes for exons 1, 4 and 6 in addition to exons 7 and 8. It is technically possible to add probes for the other exons to the kit, which would then be able to detect copy number changes in all exons. Three different patients have previously been reported to carry internal multi-exon deletions and all three had a deletion spanning exons 5–6 [12,13]. These mutations are detectable both by MLPA and by long-range PCR, while the deletion of exons 1–6 is detectable by MLPA only, although it can probably also be detected by FISH if a probe directed at the deletion is used. However, long-range PCR is not applicable for carrier detection. The haplotypes determined for these patients with deletion of exons 5–6 are not identical with the haplotypes in cases 2 and 3 [13].

There is still a problem with the complexity of the region. We have identified a relatively common polymorphism that is the opposite of the exons 1–6 deletion. In approximately 8% of our MLPA analyses (a total of 14 of 181 analysed individuals, 5 of 54 carriers and 9 of 84 with negative results), we identified an extra SMN1 exons 1–6 fragment, compared to the total number of SMN1 and SMN2 exons 7 and 8, illustrated in the mother of case 7 (see Fig. 2). This polymorphism has the potential to hide a SMN1 exons 1–6 deletion in a compound heterozygote but the polymorphism has not yet been identified in any patient with homozygous absence of SMN1 (43 patients). In the previous report on the use of MLPA in children with SMA and their parents, it is not possible to ascertain if the authors noticed any polymorphisms since they only report the results from the probes for SMN1 (1 and 2) exons 7 and 8, which they showed correlate well with the results of other methods [16]. Until further knowledge has been accumulated, deletions that do not include exon 7 should, if possible, be confirmed with a second independent method, i.e. a RNA-based method or long-range PCR.

No individuals have ever been reported to have a complete absence of both SMN1 and SMN2, a condition assumed to be lethal in analogy with the knockout mouse model of SMA [17]. The very severe phenotype of case 1 is explained by the paternal chromosome 5 entirely lacking both SMN1 copies and SMN2 copies, probably due to a large deletion including most of the SMA region. If there are enough SMN2 copies present in an individual, he or she may become asymptomatic. This is represented by case 9 who has an allele with at least three SMN2 copies, resulting in a total of five SMN2 copies, enough to keep her asymptomatic as assessed clinically and by quantitative needle EMG in proximal muscles in upper and lower extremities. In her daughter, this allele is combined with a different allele, resulting in a total of four SMN2 copies and a mild SMA type 3. An allele containing no SMN1 copies and three or four SMN2 copies should be considered a disease allele even though it may, in combination with certain
other zero copy \(SMN1\) alleles, result in asymptomatic/sub-clinical individuals (all combinations resulting in five or more \(SMN2\) copies). Five \(SMN2\) copies are not always enough to keep a person healthy if lacking \(SMN1\), instead they may develop a mild SMA. Even in a sibling-pair, having no \(SMN1\) copies and five \(SMN2\) copies each, one was healthy and the other had a mild SMA type 3 [18].

The majority of patients with SMA have homozygous absence of \(SMN1\) exon 7. Relatively common disease alleles range from deletions spanning only \(SMN1\) and \(BIRC1\) (see Fig. 2: mother of case 1, mother and father of case 4, mother of case 7), and deletions including \(SMN1, BIRC1\) and \(GTF2H2\) (see Fig. 2: fathers of cases 2, 3, 5 and 7). Rarer disease alleles include those with small or intragenic deletions, affecting only \(SMN1\) (see Fig. 2: mothers of cases 2 and 5) and those with larger deletions possibly spanning most of the inverted repeat, including both \(SMN1\) and \(SMN2\) (see Fig. 2: father of case 1). Most carriers have one \(SMN1\) copy and 2–4 \(SMN2\) copies. The number of \(GTF2H2\) gene copies seems to be highly polymorphic and there is no apparent linkage to any pathogenic deletion.

MLPA is a rapid diagnostic method which in addition to detecting homozygous absence of \(SMN1\), both estimates the number of \(SMN2\) and identifies individuals with heterozygous absence of \(SMN1\) who, may potentially have SMA due to an intragenic mutation in the remaining \(SMN1\) copy. These possible compound heterozygotes can therefore immediately be identified for further analyses such as sequencing of the coding regions of \(SMN1\) or analysis of the mRNA for \(SMN1\) and \(SMN2\). This is also true for other quantitative methods e.g. real-time PCR [14]. MLPA can also immediately detect some intragenic deletions such as the one described in this report. SMA patients with two intragenic mutations are, based on the Hardy-Weinberg equilibrium, assumed to be virtually non-existent in non-consanguineous families [2]. In patients with 2 (or more) copies of the \(SMN1\), the diagnosis of 5q-linked SMA can practically be ruled out, unless there is consanguinity in the family [19].

Although MLPA is a robust method, at least double tests should always be performed in the clinical setting. These tests should always be compared to double tests of a well-characterised, known normal reference sample and of a known carrier reference sample. The reference samples should always be included in clinical diagnostics of SMA using MLPA.

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