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Quick MLPA test for quantification of *SMN1* and *SMN2* copy numbers

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ABSTRACT

Spinal muscular atrophy (SMA) is an autosomal recessive disease caused in about 95% of SMA patients by homozygous deletion of the survival motor neuron 1 (*SMN1*) gene or its conversion to the highly homologous *SMN2* gene. In the majority of cases, disease severity correlates inversely with increased *SMN2* copy number. Because of the comparatively high incidence of healthy carriers and severity of the disease, detection of sequence alterations and quantification of *SMN1* and *SMN2* copy numbers are essential for exact diagnosis and genetic counselling. Several assays have been developed for this purpose. Multiplex ligation-dependent probe amplification (MLPA) is a versatile technique for relative quantification of different nucleic acid sequences in a single reaction. Here, we establish a quick MLPA-based assay for the detection of *SMN1* and *SMN2* copy numbers with high specificity and low complexity.

1. Introduction

Spinal muscular atrophy (SMA) is one of the most common autosomal recessive diseases, affecting approximately 1 in 10,000 live births [1–3]. About 95% of SMA cases are caused by homozygous deletion of the *SMN1* gene (OMIM 600354; Genbank NM_000344) or its conversion to the homologue *SMN2* gene (OMIM 601627; GenBank NM_022875). The remaining cases include compound heterozygotes for a deletion/conversion of one *SMN1* allele and intragenic mutations of the other allele [4]. The two closely related genes located on chromosome 5q13 differ in only 5 positions. The C-to-T substitution in *SMN2* exon 7 decreases the activity of an exonic splice enhancer and alters the splicing pattern so that the *SMN2* mRNA excludes exon 7 sequence. As a consequence, *SMN2* produces insufficient amounts of full-length *SMN* transcript and protein to rescue the SMA phenotype [5–7]. The frequency of healthy *SMN1* deletion carriers in the general population is at least 1 in 50, thus identifying these subjects may be relevant in genetic counselling [4,8–10]. The *SMN2* copy number can vary between 1 and 6, potentially modifying severity of the disease [4,7,11,12]. Therefore, the assessment of *SMN2* copy numbers could have prognostic relevance for affected subjects. *SMN* duplication renders simultaneous and reliable *SMN1* and *SMN2* quantification rather difficult. Recently, several different quantitative tests for analyzing *SMN* gene dosage have been developed [13–16]. Multiplex ligation-dependent probe amplification (MLPA) is a versatile technique for relative quantification of different nucleic acid sequences in a single reaction [17].

We developed a simple, sensitive and quick MLPA-based assay for quantification of *SMN* dosage in patients and carriers.

2. Material and methods

2.1. Subjects

We genotyped the DNA samples of 185 subjects. All DNAs were characterized for *SMN1*/*SMN2* copy numbers by Multiplex Real-Time PCR [18] and by TaqMan technology [19].

Twenty-five subjects out of 185 carried a homozygous deletion of the *SMN1* gene; 22 out of 160 healthy subjects were parents of SMA patients; 92 were healthy relatives at reproductive risk for SMA, and the remaining 46 subjects were from the general population without a family history for SMA. Six healthy subjects (3 females and 3 males having two copies of the *SMN1* and *SMN2* genes each) were used as sex-matched controls. Written informed consent was obtained from all subjects.

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2.2. DNA extraction

Genomic DNA was isolated from peripheral blood leukocytes by either the phenol–chloroform method [20] and resuspended in Tris-EDTA (Tris–HCl 20 mM, EDTA 0.5 mM, pH 8.0) or the PurageneTM DNA purification kit (Gentra Systems, Milan, Italy), according to the manufacturer's protocol. DNA concentration was determined by NanoDrop 1000 Spectrophotometer (Thermo Scientific, Milano, Italy).

2.3. Probes and primer design

Ten gene-specific oligonucleotides were synthesised (Eurofins MWG Operon, Milan, Italy) so that, after the ligation reaction, 6 different probes were amplified by PCR and detected as 6 different gene-specific signals. Briefly, each 5′ synthetic oligonucleotide contained a target-specific sequence at the 3′ end, a common 19 base sequence at the 5′ end (forward universal PCR primer) and a stuffer sequence of variable length in between where indicated (Table 1). Each 3′ synthetic oligonucleotide contained a target-specific sequence at the 5′ phosphorylated end, a common 19 base sequence at the 3′ end (reverse universal PCR primer) and a stuffer sequence of variable length in between where indicated (Table 1). Gene-specific sequences were derived from NCBI RefSeqGene: *SMN1* (NG_008691.1), *SMN2* (NG_008728.1), *ALB* (Albumin gene, NG_009291.1) and *F8* (Coagulation Factor VIII, NG_005114.1). The gene-specific oligonucleotides were selected for a Tm $>$ 60 °C at 100 mM NaCl (http://www.promega.com/biomath/calc11.htm). Discrimination between *SMN1* and *SMN2* genes was performed utilizing the SNPs at position +6 (C $>$ T) in exon 7 and at position +254 (G $>$ A) in exon 8. The entire sequences of each probe were checked for hairpins formation at 60 °C (http://dinamelt.bioinfo.rpi.edu/). Each probe gave rise to an amplification product of unique size between 98 and 128 bp (Table 1).

2.4. Probe specificity

Each probe was tested in single on appropriate genomic DNA samples. Different annealing and ligase working temperatures (54 °C or 62 °C), as well as different concentrations of the thermostable Ligase-65 enzyme (1 U or 0.2 U) were tested. Five microliters of each amplification product were analyzed by polyacrylamide gel electrophoresis (geneGelTM Clean SSCP Gel, GE Healthcare, Milano, Italy), utilizing GenePhore electrophoresis unit (Pharmacia Biotech, GE Healthcare, Milano, Italy) at 4 °C. The gel was stained by a DNA Silver Staining Kit (Amersham Biosciences, GE Healthcare, Milano, Italy) in accordance with the manufacturer's protocol.

2.5. MLPA procedure

Each genomic DNA was diluted with Tris-EDTA (Tris–HCl 20 mM, EDTA 0.5 mM, pH8) to a working solution of 40 ng/μl; 5 μl of this solution was added with 1.5 μl of salt solution (1.5 M KCl, 300 mM Tris–HCl pH 8.5, 1 mM EDTA) and 1.5 μl of probe mix (1 fmole of *ALB* probes, 1.5 fmoles of *F8* probes, 2 fmoles of *SMN1* and *SMN2* ex7 probes, 4 fmoles of *SMN1* and *SMN2* ex8 probes). Samples were denatured at 98 °C for 5 min and incubated for 2 h at 62 °C in a thermalcycler with a heated lid (MastercyclerRep, Eppendorf, Milan, Italy). Ligation of annealed probes was performed in a final volume of 40 μl obtained by adding 32 μl of pre-warmed (62 °C) ligation buffer containing 2, 6 mM MgCl2, 5 mM Tris–HCl pH 8.5, 0.013% Triton X-100 (Sigma–Aldrich, Milan, Italy), 0.2 mM NAD+ (BioLabs, Milan, Italy) and 0.2 U of the enzyme Ligase-65 (MRC-Holland, Milan, Italy). The ligation mix was incubated for 15 min at 62 °C. The Ligase-65 was inactivated by heating at 98 °C for 5 min.

PCR amplification was performed in a final volume of 25 μl containing 5 μl of ligation products, 200 nM of forward and reverse universal primers, 200 μM of dNTPs, 1U of AmpliTaq DNA Polymerase (Applied Biosystems, Milan, Italy) and 2.5 μl of GeneAmp 10×PCR buffer (Applied Biosystems, Milan, Italy). PCR was carried out in a MastercyclerRep (Eppendorf, Milan, Italy) under the following conditions: 95 °C for 1 min, followed by 35 cycles of 30 s at 95 °C, 30 s at 60 °C and 1 min at 72 °C. A final extension step was programmed for 10 min at 72 °C. One microliter of the PCR products was mixed with 1 μl of prediluited (1:10) GeneScanTM −500LIZTM Size Standard (Applied Biosystem, Milan, Italy) and with 13 μl of deionized formamide (Hi-DiTM Formamide, Applied Biosystem, Milan, Italy), denaturated for 2 min at 92 °C and then electrophoresed on a ABI PRISMTM 310 Genetic Analyzer (Applied Biosystem, Milan, Italy). Each electropherogram was analyzed by GeneMapper Software Version 4.0 (Applied Biosystem, Milan, Italy).

2.6. Data analysis

The relative quantification of *SMN1* and *SMN2* copies was obtained by dividing the height of each gene-specific peak by the

Table 1
Probes and primers.

Probes	Size[a]	Oligonucleotide Sequences[b]
F8-ex3	103 bp	5′-agg tgc aac gag gac gga cCC TTT GGC GGA CAT CTC ATT CTT ACA G-3′
		5′-PHO- GTC TGC TAG GTC CTA CCATCC *AGG ATCCAA CTTCAA GCa* gga cga acg ace caa cca-3′
ALB-ex12	109 bp	5′-agg tgc aac gag gac gga cC ACAGAATCCTTG GTG AACAGG CGA-3′
		5′-PHO- CCA TGC TTT TCA GCT CTG GAA GTC GAT *ATTGTA CTTCGGATG GGCCaz* gac gaa cga ccc aac ca-3′
SMN1-ex7	115 bp	5′-agg tgc aac gag gac gga cGC TAT TTT TTT TAA CTT CCTTTA TTT TCC TTA CAG GGTTTC*-3′
		5′-PHO-AGACAAAATCAAAAAGAAGGAAGGTGCTCACATTCC agg acg aac gac cca ace a-3′
SMN2-ex7	124 bp	5′-agg tgc aac gag gac gga *cGG CCA GCG CGC* TAT TTT TTT TAA CTT CCT TTA TTT TCC TTA CAG GGTTTT*-3′
		5′ -PHO- AGA CA A A AT CAA A AA GAA GGA AGG TGC TCA CAT TCC agg acg aac gac cca ace a-3′
SMN1-ex8[c]	119 bp	5′-agg tgc aac gag gac gga c *CT TTAACT CAC CTT* TGCTGG CCT CCCACC CCCACC C*-3′
		5′-PHO-CAG TCTTTT ACA GAT GGTTTT TCA AAATAG AGT CCA *GCCCAC TTC* agg acg aac gac cca ace a-3′
SMN2-ex8[c]	128 bp	5′-agg tgc aac gag gac gga *cCTTTAACTCACCAAA CC TCA CTC* TGCTGG CCT CCCACC CCCACC T*-3′
		5′-PHO-CAG TCTTTT ACA GAT GGTTTT TCA AAATAG AGT CCA G*CCCAC TTC* agg acg aac gac cca ace a-3′
Universal PCR Primers		
Forward		5′-Fam-AGGTGCAAC GAG GAC GGAC-3′
Reverse		5′-TGG TTG GGT CGTTCG TCC T-3′

*Nucleotide difference between *SMN1* and *SMN2* genes in exon 7 and exon 8 respectively.
[a] Amplification products length.
[b] Universal primer sequences are in small letters; gene target sequences are in capital letters; stuffer sequences are in italics underlined letters.
[c] Gene-specific sequences for *SMN1/SMN2* exon 8 are on the opposite strend.