Original Article

Gene Dosage Analysis of Proximal Spinal Muscular Atrophy Carriers using Real-Time PCR

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Abstract

Background: Autosomal recessive spinal muscular atrophy is a disease resulting from homozygous absence of SMN1 gene in approximately 94% of SMA patients. To identify patients who retained a single SMN1 copy, SMN1 dosage analysis was performed by quantitative Real-time PCR using SYBR green dye. SMN1 dosage analysis results were utilized to identify carriers before offering prenatal diagnosis.

Method: Carrier testing was performed for 150 individuals. Copy number of the SMN1 gene was determined by the comparative threshold cycle (Ct) method and human serum albumin gene was used as a reference.

Result: Analysis of 150 DNA samples with quantitative PCR determined the number of SMN1 gene copies. Of these, 50 (33.33%) cases had one SMN1 gene copy, 87 (58%) had two copies and 13 (8.66%) did not have any copies of SMN1. The homozygous SMN1 deletion ratio was 0.00 and deletion of one copy of SMN1 gene ratio ranged from 0.3 to 0.58.

Conclusion: This report demonstrates modification of risk estimation for the diagnosis and detection of SMA carriers by accurate determination of SMN1 copy number. SMN1 copy number analysis is an important parameter for identification of couples at risk of having children affected with SMA. It also reduces unwarranted prenatal diagnosis for SMA. Furthermore, the dosage analysis might be useful for the counseling of clinically suspected SMA patients with negative diagnostic SMA tests.

Keywords: gene dosage, Real-Time PCR, SMN1, spinal muscular atrophy

Introduction

Proximal spinal muscular atrophy (SMA) is an autosomal recessive disorder resulting in the loss of motor neurons in the spinal cord. SMA has an estimated incidence of 1 in 10,000 with a carrier frequency of 1 in 40 to 60.[1,2] Recessive proximal childhood SMA is clinically classified into three groups. Type I (Werding-Hoffmann) is the most severe form with onset at <6 months and death typically at <2 years. Type II SMA displays an intermediate severity, with onset at <18 months and an inability to walk. Type III (Kugelburg-Welander) individuals are able to walk independently and have a relatively mild phenotype with onset at >18 months. The SMA chromosomal region on 5q13 includes the disease locus for SMA types I, II, and III. This is a complex region with a 500-kb inverted duplication that contains several repeated genes including the survival motor neuron gene (SMN),[3] the neuronal apoptosis inhibitory protein gene (NAIP),[4] the gene encoding the p44 subunit of the transcription factor TFIIH,[5,6] and the H4F5 gene.[7] Large deletions removing all of these genes occur frequently in SMA patients. The telomeric copy of SMN gene (SMN1) is homozygously lost in over 94% of patients in all three types of SMA.[3,8,9,10,11] Identification of several intragenic SMN1 mutations in some rare cases proves that SMN1 is the primary SMA-determining gene.[12] The centromeric copy of SMN gene (SMN2) is almost identical to SMN1. The only critical difference in the genomic sequence is a base change in exon 7 (840C→T) that affects the splicing pattern.[13] Both SMN genes code for a full-length RNA with nine exons, but SMN2 mainly produces a transcript without exon 7 that encodes a truncated protein.[3,14] The SMN1 gene is detectable in 94% of SMA patients, either because of conversion of sequences in the SMN1 gene to those in the SMN2 gene or as a result of SMN1 gene deletions.[3,8,9,10,11,15] The absence of a homozygous SMN1 deletion does not rule out a diagnosis of SMA since approximately 6% of cases do not have a homozygous SMN1 deletion. The presence of a hemizygous SMN1 exon 7 deletion is identified by a quantitative determination of SMN1 exon 7 copy number. Several small mutations in the SMN1 gene (2%) are reported in patients without a deleted or sequence converted SMN1 allele. These mutations include disrupted splicing of exon 7,[3] a 4,[16] or a 5[17] bp deletion in exon 3, an 11-bp duplication in exon 6,[2] and a clustering of missense mutations in exon 6.[3,8,18]

In this report, we have described a Real-Time PCR strategy to determine the SMN1 gene copy number in SMA patients. SMN1 gene specific primers were used in Q-PCR with serum albumin gene as a reference for normalization. SMA carrier frequency in the Iranian population is high.[19] Therefore, it is necessary to design a specific and sensitive quantitative Real-Time PCR method reproducible for determination of copy number of SMN1 and gene dosage analysis.

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Materials and Methods

Subjects

Patient selection for this study was according to clinical findings that included muscle biopsy, electromyography (EMG) and laboratory results. We performed quantitative analysis of the SMN1 gene in 13 affected patients, 20 carrier couples with at least one child with a homozygous deletion or with a child with clinical findings for SMA. Additional families of diseased affected children and CVS samples from carrier couples that referred to our laboratory for prenatal diagnosis were also analyzed for SMN1 copy number. We analyzed a total of 150 individuals for SMA. Prior to any genetic testing all individuals signed a consent form approved by the Division of Genetics, Ministry of Health and Human Services, Tehran, Iran.

DNA extraction

Genomic DNA was extracted from peripheral blood samples by the salting out method. Samples with a purity of 1.5 – 1.8 (260/280 nm ratio) and a final concentration of 5 ng/μL were used for highly efficient amplifications.

PCR-RFLP

All subjects were assessed for deletions of exons 7 and 8 in the SMN1 gene, using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method described previously.[20] DNA from parents of an affected newborn with the homozygous deleted SMN1 gene was used as a positive control.

Comparative Real-Time PCR

Homozygous absence of SMN1 exons 7 and 8 in affected individuals was confirmed using PCR-RFLP. The quantitative Real-Time PCR assay was based on primers that specifically amplified segments of exons 7 and 8 of the SMN1 gene.[8] Exon 12 of human serum albumin gene was used as a reference gene.[21] PCR was performed in a total volume of 25 μL containing 12.5 μL of SYBR Green PCR master mix (Fermentas, Lithuania), 20 ng of genomic DNA (4 μL), and 0.1 μM and 0.5 μM of SMN1, and albumin gene primers, respectively. Each sample was run in duplicate in separate tubes to allow an accurate and precise quantification of the SMN1 gene. The PCR condition consisted of an initial denaturation of 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Data calculations were performed by the comparative Ct (threshold cycle, ΔΔCt) method. Ct value of each sample was calculated for SMN1 and albumin gene. The gene copy numbers of the samples were determined by the following formula: ΔΔCt = [ΔCt albumin (calibrator sample) - ΔCt SMN1 (calibrator sample)] - [ΔCt albumin (unknown sample) - ΔCt SMN1 (unknown sample)].[21] The range of gene dosage or relative copy number was determined from $2^{-\Delta\Delta Ct}$.

Result

Accurate dosage analysis is necessary in order to identify SMA carriers and to distinguish between patients with or without homozygous deletions of the SMN1 gene. SMA heterozygotes can be distinguished by a method that can differentiate between individuals with one or more copies of the SMN1 gene.[22] In this report we applied a quantitative PCR assay that uses serum albumin gene as a reference to determine the copy number of the SMN1 gene. The relative gene copy numbers were calculated by the formula $2^{-\Delta\Delta Ct}$. The calculated ranges of $2^{-\Delta\Delta Ct}$ were expected to be approximately 0.5 – 2 in normal controls, 0.3 – 0.58 in carriers and 0 in patients with SMA. Ct values of SMN1 and albumin gene for normal control (calibrator) and Ct values of albumin gene for normal, carrier and patient individuals showed almost identical values at the amplification plots. In carriers with only one copy of the SMN1 gene, the Ct values of SMN1 increased to around 0.8 – 1.6 compared to albumin (Figure 1). In SMA patients with homozygous absence of the SMN1 gene, the albumin gene was amplified as in normal controls but the SMN1 gene was not amplified as expected and Ct ratios of these samples were 0.00. The results of our optimized gene dosage assays were verified by RFLP-PCR (Figures 2A and 2B). Following optimization of the competitive PCR assay, we quantitated the SMN1 copy number in patients without homozygous deletions of SMN1 gene who had clinical features consistent with SMA and their parents, individuals referred for carrier testing, and couples who requested prenatal diagnosis. The majority of individuals, 50 out of 150 (33.33%) were carriers, 87 out of 150 (58%) were normal and had two copies of SMN1, and 13 out of 150 (8.66%) were homozygous mutants in which the SMN1 gene did not amplify. The SMN1 gene dosage ($2^{-\Delta\Delta Ct}$) range is given in Table 1. In this study, from fetuses of 14 families who went through prenatal diagnosis, 4 were heterozygous, 3 were homozygous for SMN1 deletion and 7 were normal. A total of 58% of fetuses or children suspected of SMA were normal with two copies of this gene. These fetuses or children might have an intragenic point mutation or they might have been misdiagnosed with similar clinical symptoms as SMA.

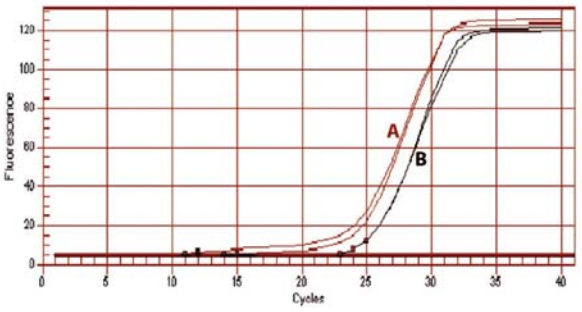

Figure 1. Representative Real-Time amplification plots of SMN1gene, normal individual **(A)**, carrier individual **(B)** and the affected patient which did not amplify.

Table 1. Real-time quantitative analysis of SMN1 gene copy number using a comparative Ct method (Ct ratio) in patients, carriers, and individuals with two copies of SMN1.

Subjects	Mean	$2^{-\Delta\Delta Ct}$ range	No. of patients	Frequency (%)
Patients	0.00	0.00	13	8.7
Carriers	0.4	0.3 – 0.58	50	33.3
Normals	1.25	0. 5 – 2	87	58
Total			150	100

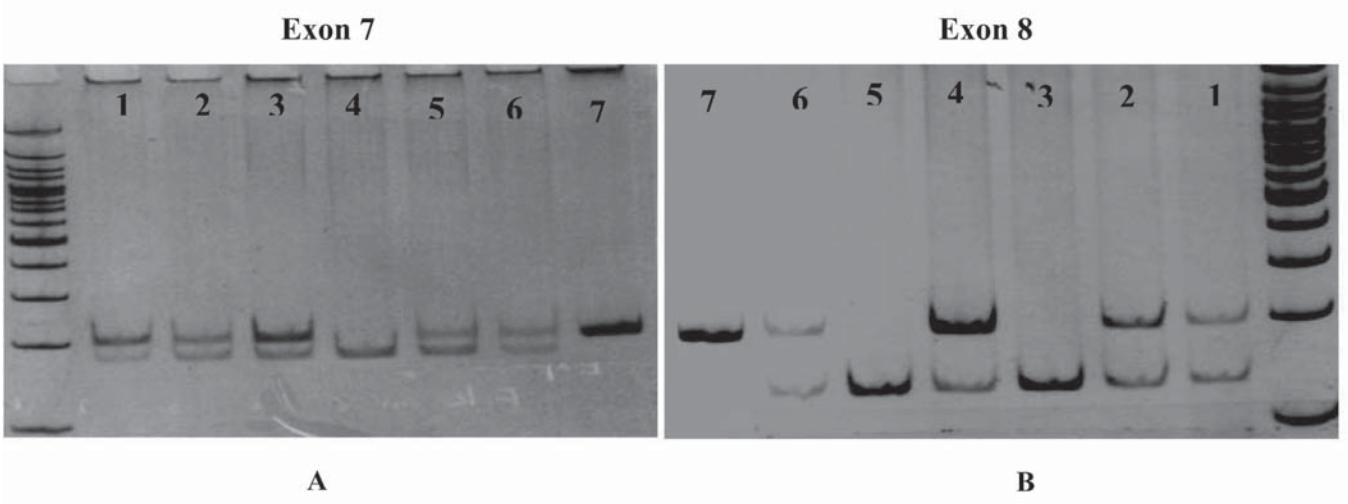

Figure 2. Deletion of exons 7 and 8 in the SMN1 gene using the polymerase chain reaction- restriction fragment length polymorphism (PCR-RFLP) method: **(A)** Analysis of exon 7 deletion in SMN1 gene: Lanes 1 and 3 are normal; 2, 5 and 6 are heterozygous; 4 is homozygous and 7 is uncut. **(B)** Analysis of exon 8 deletion in SMN1 gene: Lanes 1 and 6 are heterozygous; 2 and 4 are normal; 3 and 5 are homozygous and 7 is uncut.

Discussion

Although SMA patients with two identical, non-deleted SMN1 alleles have been identified in Iranian families, it is possible that these alleles are on a single chromosome. It should be noted that this quantitative assay cannot distinguish between a single copy of SMN1 on each chromosome (trans) or two copies of SMN1 on a single chromosome (cis). Ninety-five percent of SMA patients can be detected with this Q-PCR assay while 5% of all SMA patients with clinical symptoms who show two copies of the SMN1 gene with this assay have intragenic point mutations in other exons of the SMN1 gene.[18,23] Vallian et al. have studied defects in genes for survival motor neuron (SMN) and neural apoptosis inhibitory proteins (NAIP) associated with spinal muscular atrophy. By PCR-RFLP, their results showed that a homozygous deletion in exons 7 and 8 of the SMN gene was found with high frequency in types I and II, and with less frequency in type III SMA.[24]

Hasanzad et al. performed detection of exon 7 deletion of the SMN1 gene for patients and fetuses suspected of having SMA and reported a homozygosity frequency for the deletion of SMN1 exon 7 at 94%.[25]

The homozygous deletion frequency rate of the telomeric copy of exons 7 and 8 in the SMN gene for all types of SMA was 97%. Deletions in exons 5 and 6 of NAIP in SMA type I was 87%, and SMA type II was 33% as reported by Derakhshandeh-Peykar et al.[22] These results confirmed previous studies.

Hasanzad et al. found the range of carrier gene dosage in the Iranian population to be 0.29 – 0.55 as compared to the gene dosage found in our study, 0.3 – 0.58. Hasanzad et al.[19] determined the carrier frequency to be 1 in 20. The SMA carrier frequency varies amongst populations from the United Kingdom (1/60), Korea (1/47), Australia (1/40) and Germany (1/50). Considering the higher SMA carrier frequency in Iran relative to many populations worldwide, prenatal diagnosis of SMA for the Iranian population should be implemented.

Conclusion

SMN1 copy number analysis is an important parameter for identification of carrier couples at risk of having children affected with SMA. It also reduces unwarranted prenatal diagnosis for SMA. Furthermore, the dosage analysis might be useful for the counseling of clinically suspected SMA patients with negative diagnostic SMA tests.

References

1. Melki J, Lefebvre S, Burglen L, Burlet P, Clermont O, Millasseau P, et al. De novo and inherited deletions of the 5q13 region in spinal muscular atrophies. *Science.* 1994; **264:** 1474 – 1477.
2. Pearn J. Classification of spinal muscular atrophies. *Lancet.* 1980; **1:** 919 – 922.
3. Lefebvre S, Burglen L, Reboullet S, Clermont O, Burlet P, Viollet L, et al. Identification and characterization of a spinal muscular atrophy-determining gene. *Cell.* 1995; **80:** 155 – 165.
4. Roy N, Mahadevan MS, McLean M, Shutler G, Yaraghi Z, Farahani R, et al. The gene for neuronal apoptosis inhibitory protein is partially deleted in individuals with spinal muscular atrophy. *Cell.* 1995; **80:** 167 – 178.
5. Bürglen L, Seroz T, Miniou P, Lefebvre S, Burlet P, Munnich A, et al. The gene encoding p44, a subunit of the TFIIH, is involved in large-scale deletions associated in Werdning Hoffman disease. *J Hum Genet.* 1997; **60:** 72 – 79.
6. Carter TA, Bonnemann CG, WangCH, Obici S, Parano E, DeFatima Bonaldo M, et al. A multicopy transcription repair gene, BTF2p44, maps to the SMA region and demonstrates SMA associated deletions. *Hum Mol Genet.* 1997; **6:** 229 – 236.
7. Scharf JM, Endrizzi MG, Wetter A, Huang S, Thompson TG, Zerres K, et al. Identification of a candidate modifying gene for SMA by comparative genomics. *Nat Genet.* 1998; **20:** 83 – 86.
8. Hahnen E, Forkert R, Marke C, Rudnik-Schöreborn S, Schönling J, Zerres K, et al. Molecular analysis of candidate genes on chromosome 5q13 in autosomal recessive spinal muscular atrophy: Evidence of homozygous deletion of SMN gene in unaffected individuals. *Hum Mol Genet.* 1995; **4:** 1927 – 1933.
9. Rodrigues NR, Owen N, Talbot K, Ignatius J, Dubowitz V, Davies KE. Deletions in the survival motor neuron gene on 5q13 in autosomal recessive spinal muscular atrophy. *Hum Mol Genet.* 1995; **4:** 631 – 634.
10. Vander Steege G, Grootscholten PM, Vander Vlies P, Draaijers TG, Osinga J, Cobben JM, et al. PCR-based DNA test to confirm clinical diagnosis of autosomal recessive spinal muscular atrophy. *Lancet.* 1995; **345:** 985 – 986.
11. Velasco E, Valero C, Valero A, Moreno F, Hernández-Chico C. Molecular analysis of the SMN and NAIP genes in Spanish families and correlation between number of copies of cBCD541 and SMA phenotype. *Hum Mol Genet.* 1996; **5:** 257 – 263.
12. Wirth B. An update of the mutation spectrum of the survival motor neuron gene (SMN1) in autosomal recessive spinal muscular atrophy

(SMA). *Hum Mutat.* 2000; **15:** 220 – 237.
13. Monani UR, Lorson CL, Parsons DW, Prior TW, Androphy EJ, Burghes AH, et al. A single nucleotide difference that alters splicing patterns distinguishes the SMA gene SMN1 from the copy gene SMN2. *Hum Mol Genet*. 1999a; **8:** 1177 – 1183.
14. Gennarelli M, Lucarelli M, Capon F, Dizzuti A, Mertini L, Angelini C, et al. Survival motor neuron gene transcript analysis in muscles from SMA patients. *Biochem Biophys Res Commun.* 1995; **213:** 342 – 348.
15. DiDonato CJ, Ingraham SE, Mendell JR, Prior TW, Lenard S, Moxley R, et al. Deletions and conversion in spinal muscular atrophy patients: Is there a relationship to severity? *Ann Neurol.* 1997b; **41:** 230 – 237.
16. Bussaglia E, Clermont O, Tizzano E, Lefebvr S, Burglen L, Cruaud C, et al. A frame-shift deletion in the survival motor neuron gene in Spanish spinal muscular atrophy patients. *Nat Genet.* 1995; **11:** 335 – 337.
17. Brahe C, Clermont O, Zappata S, Tiziano F, Melki J, Neri G. Frameshift mutation in the survival motor neuron gene in a severe case of SMA type I. *Hum Mol. Genet.* 1996; **5:** 1971 – 1976.
18. Talbot K, Ponting CP, Theodosiou AM, Rodriques NR, Surtees R, Mountford R. Missense mutation clustering in the survival motor neuron gene: A role for a conserved trysine and glycine rich region of the protein in RNA metabolism. *Hum Mol Genet.* 1997; **6:** 497 – 450.
19. Hasanzad M, Azad M, Kahrizi K, Saffar BS, Nafisi S, Keyhanidoust Z, et al. Carrier frequency of SMA by quantitative analysis of the SMN1 deletion in the Iranian population. *Eur J Neurol.* **17:** 160 – 162.
20. Scheffer H, Cobben JM, Matthijs G, Wirth B. Best practice guidelines for molecular analysis in spinal muscular atrophy. *Eur J Hum Genet*. 2001; **9:** 484 – 491.
21. Lee TM, Kim SW, Lee KS, Ji HS, Koo SK, Jo I, et al. Quantitative analysis of SMN1 gene and estimation of SMN1 deletion carrier frequency in Korean population based on real-time PCR. *J Korean Med Sci.* 2004; **19:** 870 – 873.
22. McAndrew PE, Parsons DW, Simard LR, Rochette C, Ray PN, Mendell JR, et al. Identification of proximal spinal muscular atrophy carriers and patients by analysis of SMNt and SMNc copy number. *Am J Hum Genet.* 1997; **60:** 1411-1422.
23. Derakhshandeh-Peykar P, Esmaili M, Ousati-Ashtiani Z, Rahmani M, Babrzadeh F, Farshidi S, et al. Molecular analysis of the SMN1 and NAIP genes in Iranian patients with spinal muscular atrophy. *Ann Acad Med Singapore.* 2007; **36**: 937- 941.
24. Vallian S, Noori N. Molecular analysis and prenatal diagnosis of spinal muscular atrophy in Iranian population: Association of neural apoptosis inhibitory protein (NAIP) deletion with severity of the disease [Abstract]. The 2nd Iranian Annual Neurogenetic Congress. Genetics in the third millennium.
25. Hasanzad M, Golkar Z, Kariminejad R, Hadavi V, Almadani N, Afroozan F, et al. Deletions in the survival motor neuron gene in Iranian patients with spinal muscular atrophy. *Ann Acad Med Singapore*. 2009; **38:** 139 – 141.