J Korean Med Sci 2004; 19: 870-3
ISSN 1011-8934

Quantitative Analysis of *SMN1* Gene and Estimation of *SMN1* Deletion Carrier Frequency in Korean Population based on Real-Time PCR

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Received : 26 March 2004
Accepted : 25 June 2004

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Spinal muscular atrophy (SMA) is an autosomal recessive disorder, caused by homozygous absence of the survival motor neuron gene (*SMN1*) in approximately 94% of patients. Since most carriers have only one *SMN1* gene copy, several *SMN1* quantitative analyses have been used for the SMA carrier detection. We developed a reliable quantitative real-time PCR with SYBR Green I dye and studied 13 patients with SMA and their 24 parents, as well as 326 healthy normal individuals. The copy number of the *SMN1* gene was determined by the comparative threshold cycle (Ct) method and albumin was used as a reference gene. The homozygous *SMN1* deletion ratio of patients was 0.00 and the hemizygous *SMN1* deletion ratio of parents ranged from 0.39 to 0.59. The △△Ct ratios of 7 persons among 326 normal individuals were within the carrier range, 0.41-0.57. According to these data, we estimated the carrier and disease prevalence of SMA at 1/47 and 1/8,496 in Korean population, respectively. These data indicated that there would be no much difference in disease prevalence of SMA compared with western countries. Since the prevalence of SMA is higher than other autosomal recessive disorders, the carrier detection method using real-time PCR could be a useful tool for genetic counseling.

Key Words : *Muscular Atrophy, Spinal; SMN Protein; Gene Deletion; Heterozygote; Polymerase Chain Reaction; PCR, Real-time*

INTRODUCTION

Spinal muscular atrophy (SMA) is one of the most common autosomal recessive disorders characterized by progressive muscle weakness caused by degeneration of the spinal anterior horn cells. SMA has a prevalence of 1/6,000 to 1/10,000 births and a carrier frequency of 1/40 to 1/50 (1-3). According to age of onset and severity of symptoms, patients with SMA are subdivided into three clinical types (4). Type I (Werdnig-Hoffmann disease; OMIM 253300) is the most severe form with the onset at birth to the first few month and death by 2 yr of life. Type II (OMIM 253550), intermediate form, is characterized by the onset before 18 months of age and survival beyond 4 yr-old age. Type III (Kugelberg-Welander disease; OMIM 253400) is the mildest form with the onset after the age of 2 yr and survival into adulthood.

All types of SMA are associated with homozygous deletion in the survival motor neuron 1 gene (*SMN1*; OMIM 600354) (5). *SMN1* and its homologous gene, *SMN2* (OMIM 601627), are located on 5q13. The two SMN genes are different at only five nucleotide exchanges within their 3′ ends (5, 6). In contrast to *SMN1*, the expression of SMN2 protein is truncated from skipping of exon 7 by a single nucleotide exchange (840C→T) (7, 8). Approximately 94% of SMA patients lack both copies of *SMN1* exon 7 (9), which can be detected by the commonly used molecular analyses (2, 10).

As the carrier frequency in population is high, the carrier test for SMA is important issue in genetic counseling. Until now, several quantitative PCR methods for *SMN1* analysis have been developed for carrier detection (11-14). However, these methods were intricate and easily submitted to errors. Recently reported quantitative real-time PCR assays (15, 16) allow the specific amplification of only *SMN1* gene, and become powerful tools to avoid these problems. However, these tests include still several limitations in relation to the specificity, and cost-effectiveness.

In consideration of these defects, we developed a reliable quantitative real-time PCR method using *SMN1* gene specific primers and SYBR Green I dye for the carrier detection of SMA. The comparative Ct (threshold cycle) method was used to quantify the copy numbers of *SMN1* gene. This test allowed us to analyze a large number of samples efficiently. Here, we report the results of quantitative analysis of *SMN1* gene in SMA patients and parents, and the estimated carrier frequency in Korean population.

MATERIALS AND METHODS

Subjects

Quantitative analysis of *SMN1* gene was done in 13 patients

with SMA and their 24 parents. Clinical diagnosis was fulfilled on all patients and homozygous absence of *SMN1* exon 7 was confirmed with polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) (2, 10).

To estimate the carrier frequency in Korean population, we selected 326 unrelated healthy individuals aged 19-81 yr who participated in a comprehensive health screening in Ansan Health Center (17). Informed consents for DNA analysis were obtained from the patients and normal individuals.

DNA extraction

Genomic DNA was extracted from peripheral blood samples by the QIAamp DNA blood kit (Qiagen, Hilden, Germany) and was measured by an Ultrospec®2100 (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.). Samples that had purity in the range of 1.5-1.8 (260 nm/280 nm ratio) were used for highly efficient amplification. DNAs were diluted to a final concentration of 2.5 ng/μL.

Quantitative real-time PCR of *SMN1*

The quantitative real-time PCR assay was based on primers that specifically amplify *SMN1* gene. To distinguish *SMN1* from *SMN2* (GenBank AH006635) in the amplication reactions, 3′ ends of the primers were designed to end on the *SMN1* specific sequence in exon 7 at position 6 and intron 7 at position +2: forward primer is 5′-CCTTTTATTTTCCTTACAGGGTTT*C*-3′; reverse primer is 5′-GATTGTTTTACATTAACCTTTCAACTTTT*-*3′. Specificity of the *SMN1* primers was confirmed by multiplex PCR with *COL1A1* gene (forward primer, 5′-GGGGGAACAAGGCTGTCT-3′; reverse primer, 5′-TCCTGGGGTTCAGACCAA-3′) in patient samples and a normal individual's. As a reference gene, we used exon 12 of human serum albumin: forward primer is 5′-AGCTATCCGTGGTCCTGAAC-3; reverse primer is 5′-TTCTCAGAAAGTGTGCATATATCTG-3′. Using the ABI PRISM®7700 Sequence Detection System (PE Applied Biosystems, Foster City, CA, U.S.A.), the PCR was performed in a total volume of 20 μL in each well,

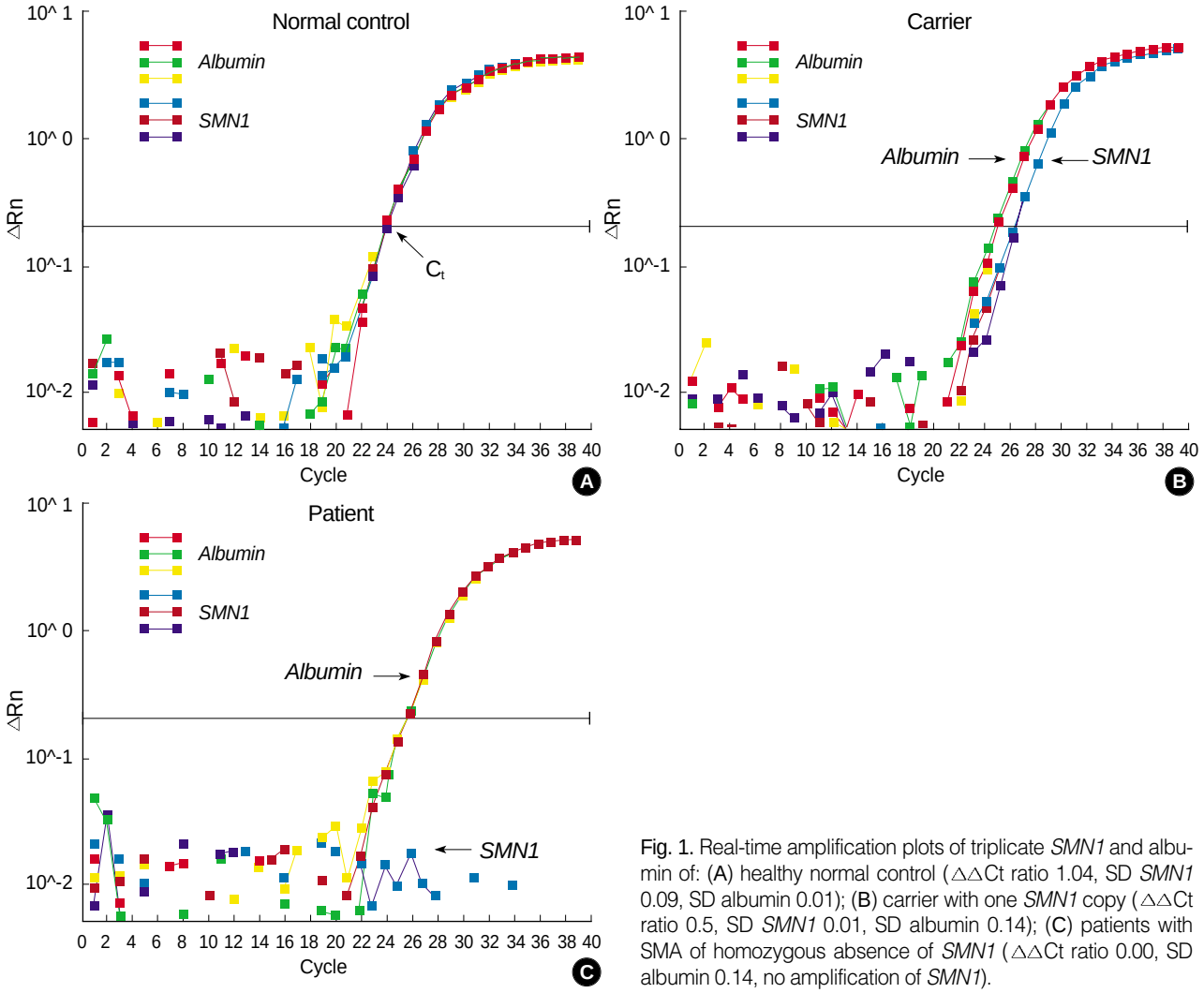

Fig. 1. Real-time amplification plots of triplicate *SMN1* and albumin of: (A) healthy normal control (△△Ct ratio 1.04, SD *SMN1* 0.09, SD albumin 0.01); (B) carrier with one *SMN1* copy (△△Ct ratio 0.5, SD *SMN1* 0.01, SD albumin 0.14); (C) patients with SMA of homozygous absence of *SMN1* (△△Ct ratio 0.00, SD albumin 0.14, no amplification of *SMN1*).

containing 10 μL of SYBR Green® PCR Master Mix (PE Applied Biosystems), 20 ng of genomic DNA (8 μL), and 0.1 μM and 0.5 μM of *SMN1* and albumin primers, which was determined after the analysis of the optimal concentrations of each primer. Each sample was run in triplicates in separate tubes to permit quantification of the *SMN1* gene normalized to albumin. The PCR condition consisted of initial denaturation step of 95℃ for 10 min, followed by 40 cycles of 95℃ for 15 sec and 60℃ for 1 min.

Data analysis

All data were calculated by the comparative Ct method, to detect the relative gene copy numbers (18). By using a calibrator sample of a normal control DNA, the gene copy numbers of unknown samples were estimated. Patients, carrier, and unrelated normal samples were analyzed together with the calibrator sample on every assay plate. △Ct represents the mean Ct value of each sample and was calculated for *SMN1* and albumin. The gene copy numbers of the samples were determined by the following formula: △△Ct=[△Ct albumin (calibrator sample)-△Ct SMN1 (calibrator sample)]-[△Ct albumin (unknown sample)-△Ct *SMN1* (unknown sample)]. The relative gene copy numbers were calculated by the expression $2^{-\triangle\triangle Ct}$. Using this method, a △△Ct ratio, [$2^{-\triangle\triangle Ct}$], was expected to be about 1 in normal controls, about 0.5 in carriers and 0 in patients with SMA.

RESULTS

The Ct values of *SMN1* and albumin triplicates showed almost identical at the amplification plots of a normal control (Fig. 1A). The standard deviations of triplicate Ct values for all samples were very low (mean, 0.10; range, 0.00-0.32). In parents of SMA patients, carrying only one *SMN1* gene copy, the Ct values of *SMN1* increased about 1.32 compared with albumin (Fig. 1B). In the SMA patients, the albumin gene was amplified as in normal controls, but the *SMN1* gene was not amplified as expected (Fig. 1C). Their △△Ct ratios were 0.00 showing homozygous absence of *SMN1* gene. In accordance with this result, there were no visible products of the *SMN1* genes in SMA patients by multiplex PCR with *COL1A1* gene (Fig. 2). The range of measured △△Ct ratios in 24 parents was between 0.39 and 0.59 (Table 1). To estimate the frequency of carrier in general population, we tested 326 healthy individuals. Among them, △△Ct ratios of 7 persons were within the carrier range, 0.41-0.57, indicating that the carrier prevalence was about 1/47 in Korean population (Table 2). The others were detected between 0.84 and 2.19. This result was classified into three groups according to the expected copy numbers of *SMN1* (Table 2).

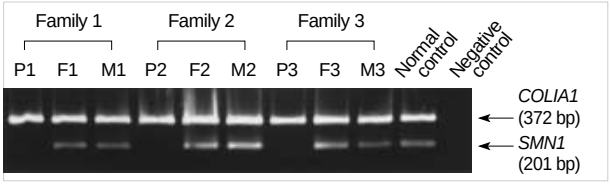

Fig. 2. Identification of specificity of *SMN1* primers in three SMA families and a normal individual by multiplex PCR with *COL1A1*. P, patient; F, father; M, Mother.

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

Subjects	△△Ct ratio of *SMN1* gene			
	Mean	Range	SD	CV (%)
Patient (n=13)	0.00	0.00-0.00	-	-
Parent (n=24)	0.53	0.39-0.59	0.12	6.1
Normal (n=326)	1.12	0.41-2.19	0.25	18.5

SD, standard deviation; CV, coefficient of variation.

DISCUSSION

SMA carrier test based on direct quantitative analysis of *SMN1* gene is one of the most important issues, since homozygous deletion of *SMN1* exon 7 is a common variant. However, the reports of SMA carrier frequency have been concentrated in western countries (3, 11). Therefore, we tried to calculate the SMA carrier frequency in Korea and developed the reliable quantitative test of *SMN1* based on real-time PCR with the SYBR Green I dye using the comparative Ct method. We used primers ending on to the nucleotide exchanges between the *SMN1* and *SMN2* genes in exon 7 to distinguish the *SMN1* and *SMN2* genes. The specificity and sensitivity of the test are 100%, although the presence of two *SMN1* copies per chromosome or intragenic point mutations in some of the SMA chromosomes (approximately 5%) slightly decreases the sensitivity of the test.

We determined the range of carrier gene dosage in 24 parents (0.39-0.59) of SMA patients, in which homozygous deletion of *SMN1* gene were identified previously by PCR-RFLP (Table 1), and then screened 326 healthy normal individuals. As shown in Tables 1 and 2, there is no overlap between the range of △△Ct ratio measured *SMN1* copy numbers in

Table 2. *SMN1* gene copy numbers in Korean population

Expected copy number of	△△Ct ratio range	No. in general population	Frequency (%)
1	0.41-0.57	7	2.2
2	0.84-1.25	254	77.9
3	1.41-1.64	62	19.0
4	1.90-2.19	3	0.9
Total		326	100.0

carriers and normal individuals. The prevalence of carrier was 1/47 in Korean population (95% confidence interval: 1/175-1/27), within the similar ranges reported in western countries (1, 3, 11). In 65 individuals, the *SMN1* copy numbers were three or four copies like as in other countries (11-13).

Since SMA is a fatal disease, coefficient of selection of *SMN1* deleted homozygotes is almost one. Therefore, extrapolation of Hardy-Weinberg equation to calculate the prevalence of *SMN1* deleted homozygotes in a population seems to be unreasonable. Nevertheless, if ignoring this potential bias, the disease prevalence of *SMN1* deleted homozygotes calculated by Hardy-Weinberg equation was 1/8,496 in Koreans. The estimated prevalence was also not much different from those of other European countries. Since there was no available information of SMA prevalence in East Asian countries including Korea, estimated prevalence presented in this study would be valuable for pursuing SMA pathogenesis and genetic counseling.

The *SMN2*, highly homologous gene of *SMN1*, is a well-established disease-modifying gene for SMA and has been shown to decrease the severity of type I-III SMA in gene dosage analyses (11, 15, 19). The copy number of *SMN2* also correlates with longer survival of individuals affected with type I SMA (15). Thus, the quantitative analysis of *SMN2* gene enables it possible to predict that an infant with homozygous deletion of *SMN1* develop type I, II or III SMA. Although we did not carry out the assay of *SMN2* gene in the present study, our real-time quantitative PCR analysis could produce sufficient results.

In conclusion, we detected *SMN1* deletion carriers in a general population and calculated the prevalence as the advantages of real-time PCR with SYBR Green I. With relatively high frequency of SMA compared with other autosomal recessive disorders, the carrier detection method using real-time PCR could be a useful tool for genetic counseling.

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