

Frequency of *SMN1* deletion carriers in a Mestizo population of central and northeastern Mexico: A pilot study

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Abstract. Individuals who suffer from spinal muscular atrophy (SMA) exhibit progressive muscle weakness that frequently results in mortality in the most severe forms of the disease. In 98% of cases, there is a homozygous deletion of the survival of motor neuron 1 (*SMN1*) gene, and both parents carry the same heterozygous genetic abnormality in the majority of cases. Various population studies have been conducted to estimate the frequency of carriers and thereby identify the communities or countries in which children are at a high risk of being affected by SMA. However, the prevalence of SMA in Mexican populations has not yet been established. In the present pilot study, the frequency of the heterozygous deletion of the *SMN1* gene was determined in two groups from northeastern (n=287) and central (n=133) Mexican Mestizo populations and compared with other ethnic populations. Amplification refractory mutation system polymerase chain reaction analysis yielded a disease carrier frequency of 11/420 (2.62%) healthy individuals, comprising 9/287 (3.14%) northeastern and 2/133 (1.5%) central Mexican individuals. In summary, no significant differences were identified between the northeastern and central populations of Mexico and other ethnic populations, with the exception of the general worldwide Hispanic population, which exhibited the lowest carrier frequency of 8/1,030.

The results of the present study may be used to improve the evaluation procedure, and appear to justify further studies involving larger sample populations.

Introduction

Spinal muscular atrophy (SMA) is an autosomal recessive disease that is a result of the degeneration and progressive death of α motor neurons in the anterior horns of the spinal cord and brainstem nuclei (1). SMA is characterized by progressive muscle weakness, and is the most common genetic cause of infant mortality (1). SMA presents with an extremely variable phenotype. The clinical spectrum of SMA is continuous; however, variations in the age of onset and symptoms have led to a classification system of the clinical variants of SMA that is based on maximum patient motor skill achievement. This classification system has resulted in an improved guideline design for the clinical management and follow-up of SMA, which is based on the patient's maximum motor skills (2,3). Sixty percent of SMA cases are type I, which is characterized by proximal muscle weakness, affecting the legs most severely, areflexia, and abdominal breathing that produces a bell-shaped chest deformity with breathing complications. SMA type I is also known as Werdnig-Hoffman disease (OMIM, #253,300) and has a prevalence of 4.1/100,000 live births, with patient mortality typically occurring at ~2 years of age (4). SMA type II, the intermediate form (OMIM, #253,550) of the disease, has an onset age of 6-18 months. Patients with SMA type II may be able to sit, but develop hypotonia, hyporeflexia and lingual fasciculation, and 98% of affected individuals survive to 5 years of age, although overall lifespan is reduced (2,3). SMA type III (Kugelberg-Welander type; OMIM, #253,400) has an onset age of 18 months, and patients affected with this SMA type are able to sit and walk (5). SMA type IV presents in adults >21 years; these patients exhibit a normal acquisition of all fine motor skills prior to the onset of neuromuscular symptoms and usually have a normal lifespan (6).

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The overall prevalence of SMA is 1/6,000-1/10,000, with a carrier frequency range of 1/40-1/60 (7,8). Homozygous loss of the survival of motor neuron 1 (*SMN1*) gene accounts for 94-95% of all SMA cases. The remaining 5% of cases consist of compound heterozygotes with the *SMN1* deletion in one allele and an alternative mutation in the remaining allele. This mutational event occurs in <1/4,000 affected individuals and follows the Hardy-Weinberg equilibrium (9-11). The chromosomal region 5q11.2-13.3 is susceptible to non-allelic homologous chromosomal rearrangements. The two variants of the *SMN* gene, a telomeric (*SMN1* or *SMNT*) (OMIM, #600,354) and a centromeric gene (*SMN2* or *SMNc*) (OMIM, #601,627), differ in a single nucleotide (840C>T), which results in the alternative splicing of exon 7 in the *SMN2* gene in 75-90% of the transcripts produced (2,12-16). The severity of SMA is primarily determined by the copy number of the *SMN2* gene. The SMN protein forms a complex of molecules that regulates motoneuron survival by maintaining normal axonal transport and the growth, maturation and formation of axons and neurites (17-19).

SMA is diagnosed using various molecular procedures; the majority are polymerase chain reaction (PCR)-based methods and rely on an analysis of the gene dosage. In the present study, the frequency of *SMN1* gene deletion was determined in healthy carriers and non-carriers from northeastern and central Mexican Mestizo populations. The results may aid in establishing a basis for implementing carrier screening programs across the country following further validation.

Materials and methods

This pilot study (no. GN08-005) was conducted at the Department of Genetics, School of Medicine, Autonomous University of Nuevo León (Monterrey, Mexico). The study was approved by the committee for ethics, research and biosafety of the Autonomous University of Nuevo León.

A total of 420 individuals aged >18 years, with parents and four grandparents of Mexican origin, were recruited from northeastern (n=287) and central Mexico (n=133). Informed consent was obtained from all participants prior to peripheral blood collection by venipuncture in accordance with the ethical guidelines established by the Declaration of Helsinki of the World Medical Association in 1964 and modified in 1989. Genomic DNA was obtained from peripheral blood cells using a QIAamp® Blood Mini kit employing the automated system QIAcube (Qiagen GmbH, Hilden, Germany).

Molecular detection of the *SMN1* deletion. A molecular assay was designed to simultaneously determine the relative gene dosage of the *SMN1* deletion and *SMN2* in relation to the *TP53* gene, which was used as an internal control. This method was based on amplification refractory mutation system PCR (ARMS-PCR) (20) using six primers and capillary electrophoresis (Fig. 1). The four primers (Applied Biosystems Life Technologies, Foster City, CA, USA) used to amplify the *SMN1* and *SMN2* genes were as follows: *SMN1*, forward 5'-TGTGAAACAAAATGCTTTTAAACATCC-3' and reverse 5'-AAAACATTTGTTTTCCTTATTTTCCTTACAGGGTGTC-3' and reverse 5'-FAM-CACCTTCCT

TCTTTTGTGATTGTTGTATA-3'. The primers used to amplify the *TP53* gene were as follows: Forward 5'-GGTCCAGATGAAGCTCCCAGAAT-3' and reverse 5'-FAM-TCACAGACTTGGCTGTCCCAGAAT-3'. Primers labeled 5'-FAM were 5' fluorescently labeled with the fluorophore 6-FAM.

The method was validated by assaying triplicate DNA samples from six individuals with SMA type I that were homozygous for the deletion of the *SMN1* gene, based on a PCR-restriction fragment length polymorphism analysis. In addition, samples from healthy carriers of the deletion were used in the validation assay.

PCR procedure. PCR reactions were conducted in a reaction system containing 20 ng genomic DNA, 0.031 μ M *TP53* primers, 0.038 μ M forward and 0.42 μ M reverse external primers for *SMN1/SMN2*, 0.022 μ M primer specific to the 'C' nucleotide (c.840C of *SMN1*), 0.030 μ M primer specific to the 'T' nucleotide (c.840T of *SMN2*), 0.75 U GoTaq DNA polymerase (Promega Corporation, Madison, WI, USA), 1.5 mM MgCl₂ and 0.3 mM dNTPs (Promega Corporation), with a final volume of 15 μ l. The cycling procedure included one cycle of initial denaturation at 95°C for 1 min, 29 cycles of denaturation at 95°C for 40 sec, annealing at 53°C for 40 sec and extension at 72°C for 1 min; and a final 72°C extension step for 20 min. Immediately, 1 μ l of the amplicon was mixed with 0.3 μ l GeneScan™-500 LIZ® Size Standard (Applied Biosystems Life Technologies) and 8.7 μ l formamide. This mixture was placed in a Hybaid PCR Express HBPX110 thermocycler (Thermo Fisher Scientific, Renfrew, UK) for 5 min at 95°C, then at -20°C for 5 min. Following denaturation, the samples were analyzed using a 3100 Avant Genetic Analyzer (Applied Biosystems Life Technologies).

Estimation of gene copy number. Three peaks corresponding to the *SMN2* (135 bp), *SMN1* (149 bp) and *TP53* (194 bp) genes were obtained using electropherograms generated by ABI Prism 3100 AVANT genetic analyzer (Applied Biosystems Life Technologies) (Fig. 2). The area of each peak was determined based on the GeneMapper software, version 3.1 with a peak detection algorithm (Applied Biosystems Life Technologies) and following a previously described protocol (21) with modifications (allele size detection adjusted for *SMN1*, *SMN2* and *TP53* amplicons). The ratio of the peak area of the *SMN1* gene to the *TP53* gene represented the relative copy number of the target gene *SMN1*. For this analysis, a *TP53* gene dosage of 100% (two gene copies of *TP53*) was assumed to be present in all subjects, as none presented with Li-Fraumeni syndrome (22). Based on this analysis, subjects with at least two copies of *SMN1* were considered to be healthy non-carriers, those with one copy of *SMN1* were considered true carriers, and those with no copies of the *SMN1* gene were considered to be affected with SMA. A limitation of this detection method is that it did not distinguish whether the two copies of the *SMN1* gene were in *cis* or *trans*.

Statistical analysis. Fisher's exact test was applied to compare the northeastern and central Mexican Mestizo populations. The χ^2 test was applied to compare the carrier frequency among the populations in the present study, worldwide populations and the Hispanic population.

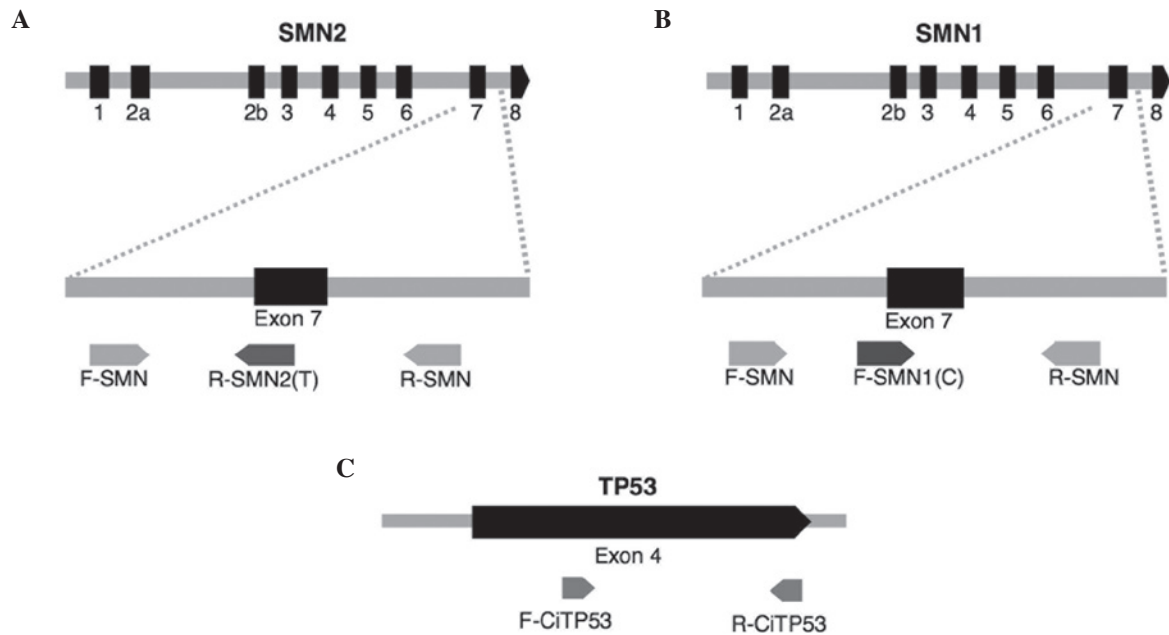


Figure 1. Primer binding in the *SMN1*, *SMN2* and *TP53* genes. The primer (A) R-*SMN2* binds specifically to exon 7 in *SMN2* and (B) F-*SMN1* binds specifically to exon 7 in *SMN1*. (C) Binding region of primers in the *TP53* gene for copy number reference control. F, forward; R, reverse; *SMN*, survival of motor neuron.

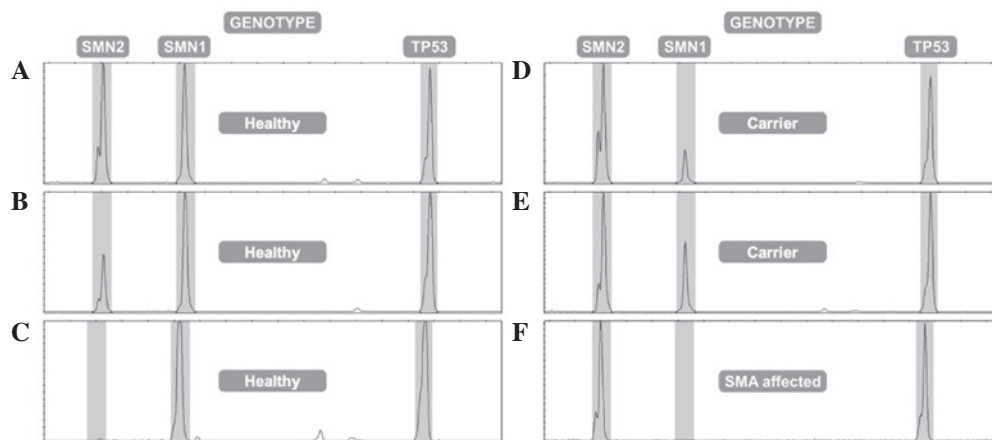


Figure 2. Electropherogram profiles of the affected, heterozygote and healthy individuals. (A, B and C) The presence of a *SMN1* gene peak equal to *TP53* indicates a healthy individual even in the presence of the *SMN2* gene deletion. (D and E) The peak for *SMN1* is half the size of the peak for the *TP53* gene, which indicates the presence of a single copy of the *SMN1* gene. (F) The absence of a peak indicates a homozygous deletion of *SMN1* affected with spinal muscular atrophy. *SMN1*, survival of motor neuron 1.

Data were analyzed using SPSS statistical software, version 22.0 (IBM SPSS, Armonk, NY, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

A total of 420 DNA samples were obtained from male and female participants aged 18-71 years, with four grandparents of Mexican descent. Among this population, 68.33% ($n=287$) were from northeastern and 31.67% ($n=133$) from central Mexico. The *SMN1* copy number was standardized using DNA from affected and healthy carrier individuals. The peak electropherogram area of the *SMN1* gene in the affected patients was 0.00-0.05, indicating a mean loss of the *SMN1* gene among homozygous carriers. In heterozygous carriers, the range of the peak area was 0.3-0.52, representing half

of the subjects. The range was >0.6 for healthy individuals, which indicated complete gene dosage (Fig. 3). If it was not possible to determine whether a sample corresponded to a carrier or a non-carrier (*SMN1/TP53* ratio between 0.52-0.6), the sample was reanalyzed in triplicate, and the mean of the three measurements was used.

Among the study population, 11 subjects (2.62%) carried one copy of *SMN1* and 409 subjects (97.38%) carried two copies (Table I). Nine of the single-copy carriers (3.14%) were from northeastern and two (1.50%) were from central Mexico. No significant difference was observed between these groups using two-tailed Fisher's exact test ($P=0.5143$; Table I). The overall frequency of carriers among the two Mexican population groups was 11/420 (or 1/38). Thus, the carrier frequency detected in the present pilot study was 2.62%.

Table I. Frequencies of *SMN1* gene deletion carriers.

Carrier status	Northeastern		Central		Total	
	n	%	n	%	n	%
Carriers	9	3.14	2	1.50	11	2.62
Non-carriers	278	96.86	131	98.50	409	97.38
Total	287		133		420	

Fisher's exact test, two-tailed $P=0.5143$. *SMN1*, survival of motor neuron 1.

Table II. Distribution of carrier frequencies with an *SMN1* deletion among nine populations worldwide and in the present study.

Population	Carriers		Total	Method	Reference
	Yes (%)	No (%)			
Mexican	11 (2.62)	409 (97.38)	420	PCR-ARMS	Present study
Hispanic	8 (0.8)	1,022 (99.2)	1,030	RT-qPCR	Hendrickson <i>et al</i> , 2009 (4)
African-American	18 (1.8)	997 (98.2)	1,015	RT-qPCR	Hendrickson <i>et al</i> , 2009 (4)
Asian	18 (1.8)	1,009 (98.2)	1,027	RT-qPCR	Hendrickson <i>et al</i> , 2009 (4)
Korean	2 (2.0)	98 (98.0)	100	MLPA	Yoon <i>et al</i> , 2010 (19)
Jewish	22 (2.2)	980 (97.8)	1,002	RT-qPCR	Hendrickson <i>et al</i> , 2009 (4)
Israeli	159 (2.5)	6,235 (97.5)	6,394	MLPA	Ben-Shachar <i>et al</i> , 2011 (23)
Caucasian	28 (2.7)	1,000 (97.3)	1,028	RT-qPCR	Hendrickson <i>et al</i> , 2009 (4)
Arabian	2 (4.0)	48 (96.0)	50	Multiplex-PCR	Majumdar <i>et al</i> , 2005 (8)
Iranian	10 (5.0)	190 (95.0)	200	qPCR	Hasanzad <i>et al</i> , 2009 (9)
Total	278	12,080	12,358		

$\chi^2=22.74$, $df=9$, $P=0.007$ (among 10 populations). $\chi^2=10.84$, $df=8$, $P=0.211$ (Hispanic population was not included). PCR-ARMS, polymerase chain reaction-amplification refractory mutation system; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; MLPA, multiplex ligation-dependent probe amplification; *SMN1*, survival of motor neuron 1.

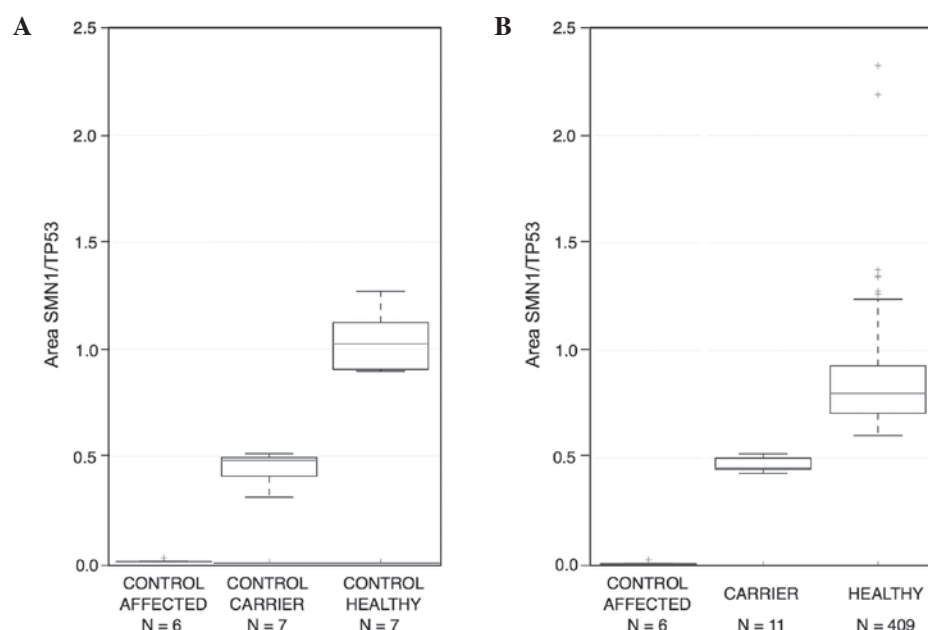


Figure 3. (A) Copy number validation of the *SMN1* gene using the polymerase chain reaction-amplification refractory mutation system method. (B) Copy number determination for the 420 screened individuals presenting the ratio of peak area values of *SMN1/TP53*. Individuals are distributed around ratios of 0.0-0.05, 0.3-0.52 and >0.6 , which represent 0, 1, and ≥ 2 copies, respectively, of *SMN1* exon 7. *SMN1*, survival of motor neuron 1.

Discussion

The results of the present pilot study indicate that the carrier frequency of *SMN1* in northeastern and central Mexico is 1/38, which is comparable to the reported frequencies in other ethnic populations, including African-American, Asian, Korean, Jewish and Israeli populations (Table II). However, the SMA carrier frequency in this Mexican population is significantly higher compared with that in a Hispanic population from the USA previously examined by Hendrickson *et al* (4). In the northeastern Mexican population, the frequency detected was 1/32 (3.14%).

In Hispanic populations in the USA, the SMA carrier frequency is comparatively low, at 1/125 (24,25). However, the term Hispanic is used as a general term to refer to all persons of Hispanic descent and encompasses a diverse group of individuals (26). A previous study of 7,655 Hispanic individuals in the USA, in which family history and ethnicity was considered (27), observed that *SMN1* one-copy frequency did not differ significantly from that observed in an existing study of 1,030 individuals ($P=0.1869$) (4). A recent study demonstrated that the Mexican population is genetically diverse. Therefore, detailed studies of population structure, including geographical data, may be required in order to assess the frequency and prevalence of genetic diseases in native and Mestizo Mexican populations (28).

The universal implementation of expanded newborn screenings for metabolic inherited diseases has been tailored to specific populations and countries. If the carrier status of an individual for a genetic disease is known, several options are available: i) Choice of a partner who is a non-carrier; ii) pre-implantation diagnosis; iii) prenatal diagnosis; or iv) acceptance of the 25% probability of a child being affected. In cases of infants born affected by the disease, early recognition allows for more effective treatment, which justifies the use of SMA as a target disease for population screening (6,29,30). The identification of SMA carriers may facilitate a reduction in the prevalence of SMA.

To the best of our knowledge, the frequency of SMA carriers in the Mexican populations investigated in the present study has not been reported previously. A carrier frequency of 1/38 (2.62%) was determined in the present study. No significant differences in carrier frequency were identified between populations from the northeastern and central regions of Mexico. Therefore, the frequency of the deletion of the *SMN1* gene remains homogeneous across the Mexican populations examined. Further studies with larger sample sizes may provide an improved understanding of whether this frequency distribution is representative of the entire Mexican population; however, this pilot study provides an initial approximation of the carrier frequency for Mexican individuals. Furthermore, these results may be used to improve evaluation procedures, and thus justify future studies on a larger scale to validate neonatal screening in the Mexican population. It is hypothesized that carrier testing may be a useful technique to prevent SMA, which frequently results in mortality and is currently untreatable. Similar studies have been conducted in other populations, including Israeli, Caucasian, Ashkenazi Jewish, Asian and African-American populations (Table II).

The present study is relevant to the study of carrier and patient frequencies in mixed populations of complex ethnic components. The Mexican population is a convenient model for genetic studies due to its notable ethnic diversity in native and Mestizo populations. A previous study reported significantly different ancestry between individuals from separate geographic regions in Mexico (28). In order to avoid errors such as stratification, false negatives and irreproducibility, the results of the present study may be used as a reference in genetic studies of gene frequency, prevalence and association in characterized populations. In the present study of SMA carrier frequency in the northeastern and central Mexico populations, no statistically significant differences were observed; however, further studies are required to evaluate the remaining regions of Mexico.

The technique applied in the present study is an alternative to existing molecular tests to determine the copy number of the *SMN1* gene in order to detect SMA carriers and affected individuals.

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