

ARTICLE

Molecular characterization of *SMN* copy number derived from carrier screening and from core families with SMA in a Chinese population

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Screening for carriers of spinal muscular atrophy (SMA) is necessary for effective clinical/prenatal diagnosis and genetic counseling. However, a population-based study of SMA prevalence in mainland China has not yet been conducted. In this study, the copy number of survival motor neuron (*SMN*) genes was determined in 1712 newborn cord blood samples collected from southern China and from 25 core families, which included 26 SMA patients and 44 parents, to identify SMA carriers. The results presented 13 groups with different *SMN1/SMN2* ratios among 1712 newborn individuals, which corresponded to 1535 subjects with two copies of *SMN1*, 119 with three copies of *SMN1*, 17 with four copies of *SMN1*, and 41 with a heterozygous deletion of *SMN1* exon 7. Simultaneously, two '2+0' genotypes and two point mutations were found among the 44 obligate carriers in the core families, including a novel *SMN1* splice-site mutation that was identified in the junction between intron 6 and exon 7 (c. 835–1G > A). These results indicated that the carrier frequency is 1/42 in the general Chinese population and that duplicated *SMN1* alleles and *de novo* deletion mutations are present in a small number of SMA carriers. In addition, we developed and validated a new alternative screening method using a reverse dot blot assay for rapid genotyping of deletional SMA. Our research elucidated the genetic load and *SMN* gene variants that are present in the Chinese population, and could serve as the basis for a nationwide program of genetic counseling and clinical/prenatal diagnosis to prevent SMA in China.

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INTRODUCTION

Spinal muscular atrophy (SMA) is one of the most common autosomal recessive disorders worldwide: the carrier frequency is between 1/40 and 1/60 in diverse populations and it affects 1 in 10 000 live births.^{1,2–4} SMA is caused by the degeneration of anterior horn cells of the spinal cord, which leads to symmetric proximal muscle weakness and atrophy.⁵ On the basis of the age at onset and the clinical course, SMA is classically subdivided into three types: type I is an infantile acute form, and shows the greatest severity; type II is an infantile chronic form with intermediate severity; and type III is a childhood and adolescent form and shows the mildest severity.⁶

All three types of SMA are linked mostly to the survival motor neuron (*SMN*) gene at 5q13, which is found as two nearly identical copies: *SMN1* and *SMN2*.⁷ Mutations in the telomeric copy of the *SMN* gene (*SMN1*) are responsible for SMA, whereas *SMN2* functions as a disease-modifying gene that can ameliorate the clinical severity of SMA in a copy-dependent manner. Each *SMN* gene encompasses 27 kb and comprises 9 exons (exons 1, 2a, 2b, and 3–8). Typically, the DNA sequence of *SMN1* differs from that of *SMN2* by only five nucleotides: one in intron 6, one in exon 7, two in intron 7, and one in exon 8.^{8,9} Of the SMA patients whose disorder is linked to 5q13,

~96% are characterized by a homozygous deletion of *SMN1* exon 7, which results from unequal crossover or *SMN1*-to-*SMN2* conversion events. The remaining 4% of cases are caused either by compound heterozygosity with a point mutation in one allele and a deletion in the other or by compound heterozygous point mutations in the *SMN1* gene.¹⁰ To date, ~40 subtle mutations have been identified in the *SMN1* gene of SMA patients worldwide, with the most common sites of mutation located in exons 3 and 6.^{11,12}

Given that homozygous deletion of exon 7 of *SMN1* is a common genotype and *SMN2* can influence the severity of SMA, it is fundamentally important to directly determine the copy numbers of *SMN1* and *SMN2* for carrier screening and clinical/prenatal diagnosis of SMA. Using quantitative analysis of *SMN1/SMN2* gene dosage, a range of molecular investigations of the incidence of carriers and the genetic burden of SMA in diverse ethnic populations have been carried out.^{2,3,13–19} However, a population-based study of SMA prevalence in mainland China has not yet been executed. Although some data have been reported for subjects from Taiwan and Hong Kong, the analyses included a relatively small number of SMA patients.^{20,21} To estimate the future burden of SMA and the requirements for its appropriate treatment, management, and prevention in

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the Chinese population, accurate population frequency data are essential. Such data could be obtained through the genotyping of *SMN* genes in a large number of samples from mainland China.

In the study reported herein, we determined *SMN* copy number in 1712 samples of newborn cord blood from southern China and from 25 core families that contained individuals with SMA using a modified denaturing high-performance liquid chromatography (DHPLC)-based quantitative assay, which has been proven to be a highly efficient and reliable carrier-screening test.^{20,22,23} We also assessed the genotype contribution of SMA carriers in the Chinese population by comparative genotyping of newborn samples and SMA core families. In addition, during the analysis of SMA patients, we found a novel *SMN1* splice-site mutation at the junction between intron 6 and exon 7 (c.835-1G>A). Simultaneously, we developed and validated a new alternative method that used a reverse dot blot (RDB) assay for rapid genotyping of deletional SMA in our sample cohorts. We anticipate that our research will provide a basis for a nationwide program of clinical/prenatal diagnosis to prevent SMA in China.

MATERIALS AND METHODS

Population and patient samples

A total of 1712 cord blood samples obtained from healthy newborns (867 males and 845 females), with no family history of SMA and registered parental origin from the northern and southern districts of Guangdong province, were continuously collected from July 2006 to December 2007. Of these individuals, 95% were ethnically southern Chinese Han. In addition, 25 unrelated SMA core families from across China were enrolled in this study, including 26 SMA patients and 44 parents (2 affected siblings in one family), comprised of 19 parental pairs and 6 single parents. All patients fulfilled the diagnostic criteria of the International SMA Consortium.⁶ Genomic DNA was extracted from the peripheral blood of the core family members or from newborns' cord blood. The local medical research ethics committee reviewed the study, and informed consent was obtained from all participants.

DHPLC analysis of *SMN* copy number in the tested samples

The diagnostic flow chart for determining *SMN1* and *SMN2* gene dosages linked to SMA in this study is illustrated in Figure 1. This protocol includes a single PCR to generate amplified segments containing exon 7 of *SMN1/SMN2*, followed by DHPLC quantitative analysis to display the *SMN1/SMN2* ratio representing genotypic patterns of samples on the WAVE Nucleic Acid Fragment Analysis System (Model 4500 HT, Transgenomic, Omaha, NE, USA). To

test the samples for an equal *SMN1/SMN2* ratio (1:1 or 2:2), multiplex PCRs were performed to amplify *SMN1/SMN2* by simultaneously amplifying two reference genes (X-linked *CYBB* and *KRIT1* on 7q), followed by DHPLC quantitative analysis to distinguish the *SMN1/SMN2* ratio as 1:1 or 2:2. We could clearly differentiate *SMN1* and *SMN2* PCR fragments of the same size by the DHPLC elution pattern, which is dependent on a single-nucleotide polymorphism (SNP) in exon 7 (C→T) present in these two genes. All primers used in this assay were described in detail by Su *et al*²⁰. The reaction mixture components and PCR/DHPLC conditions were similar to the description by Su *et al* with minor revisions. All of the genomic DNA from the tested individuals were subjected to single PCR/DHPLC analysis to determine carriers with an *SMN1/SMN2* ratio not equal to 1 (*SMN1/SMN2*=1:4, 1:3, and 1:2), whereas the normal individuals (accounting for nearly 40% of the total sample) were characterized by an unequal *SMN1/SMN2* ratio (*SMN1/SMN2*=2:1, 2:3, 3:2, 3:1, and 4:1) or by the detection of an *SMN1*-only peak (*SMN1/SMN2*=2:0, 3:0, and 4:0). Samples with an equal *SMN1/SMN2* ratio were then analyzed by the multiplex PCR/DHPLC assay to distinguish carriers with an equal *SMN1/SMN2* ratio (1:1) from normal individuals with an equal *SMN1/SMN2* ratio (2:2). Finally, all samples with abnormal DHPLC peaks were subjected to direct PCR sequencing to detect any novel *SMN1/SMN2* gene mutations, and the SNP functional prediction and alignment analyses were performed according to the programs found at <http://compbio.cs.queensu.ca/F-SNP/>, whereas *SMN1* and *SMN2* gene dosages were determined for the diagnosis of individuals as either carrier or normal individuals. To analyze SMA patients with homozygous deletions from core families, SMA-affected cases were detected efficiently simply by recognizing an *SMN2*-only DHPLC peak.

Development and validation of the RDB assay to genotype the SMA deletion

We established RDB analysis to detect SMA deletions according to a previously described RDB system.²⁴ The two pairs of primers labeled with biotin to selectively amplify the exon 7 and exon 8 regions of the *SMN1* (Genebank NG: 008691) and *SMN2* (Genebank NG: 008728) genes were as follows: biotin-AGACTATCAACTTAATTCTGATCA-3' (from 31844 to 31868 bp) and biotin-TAAGGAATGTGAGCACCTTCCTC-3' (from 32022 to 32045 bp); biotin-GTAATAACCAAATGCAATGTGAA-3' (from 32666 to 32688 bp) and biotin-CTACAACACCCTTCTCACAG-3' (from 32835 to 32855 bp), respectively. The two sets of oligonucleotide probes immobilized on strips were designed to distinguish between the *SMN1* and *SMN2* genes. The four specific hybridization probes, which were linked to the amino group at the 5'-end, were *SMN1*-Ex7: NH₂-TGTCTGAAACCCTGTA-3' (from 31996 to 32011 bp), *SMN2*-Ex7: NH₂-TGTCTAAAACCCTGTAAG-3' (from 31992 to 32009 bp), *SMN1*-Ex8: NH₂-AAAGACTGGGGTGGG-3' (from 32726 to 32740 bp), and

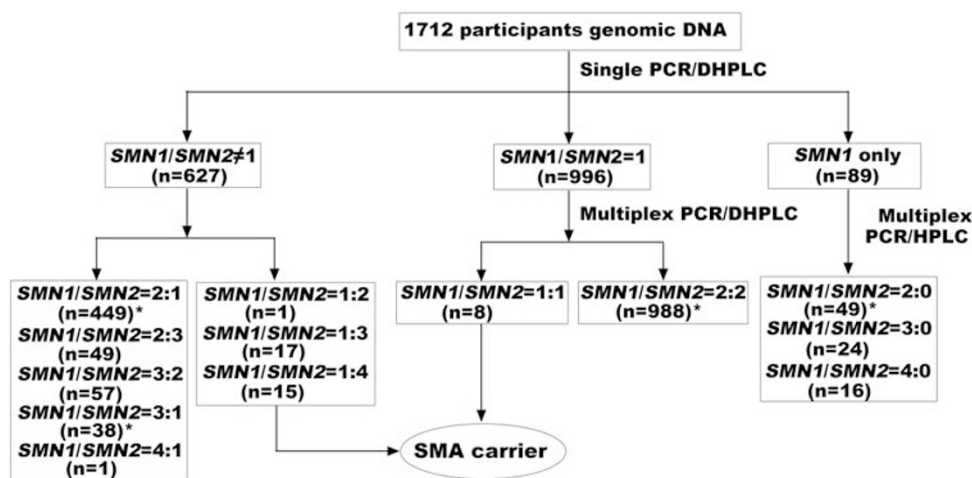


Figure 1 Diagnostic flow chart for targeted detection of *SMN1* and *SMN2* gene dosages linked to SMA in a population-based study. The sample numbers detected at each step are indicated in brackets. *Some samples with abnormal peaks screened by DHPLC were sequenced to distinguish point mutations or SNPs.

SMN2-Ex8: NH₂-AAAGACTGAGGTGGG-3' (from 32724 to 32738 bp). Multiplex PCR amplification was performed, and the 50 µl total reaction mixture contained 100 ng of gDNA, 0.5 µM of each primer for exon 7, 0.4 µM of each primer for exon 8, 400 µM of each dNTP, 4 Units of Taq polymerase (Takara, Dalian, China), and 5 µl of 10×buffer I. The cycle conditions used for PCR were 5 min at 94°C for denaturation, followed by 35 cycles of 45 s at 94°C, 30 s at 55°C, 30 s at 72°C, and a final extension of 10 min at 72°C. The conditions used for hybridization, washing, and color development in this RDB assay were as described in our previously published paper²⁴. The RDB assay was validated using 183 samples (which were blinded with regard to the previous diagnosis) by DHPLC/DNA sequencing, including 25 samples with homozygous deletions of exon 7 and/or exon 8 of the *SMN1* gene and 158 samples with an *SMN1*/*SMN2* ratio that derived from normal or SMA carrier individuals. The DHPLC/DNA sequencing results were unknown until the genotypes obtained from each of the given RDB results were scored.

Molecular identification of a novel mutation in a Chinese family

The proband was obtained from an 8-month-old male infant from 1 of the 25 SMA families who was clinically diagnosed as SMA type I, without the homozygosity typical of *SMN1* gene deletion. The patient was referred to us for further molecular analysis. Blood samples were collected from the patient, his elder sister, and his parents for identification with the new mutation and functional study with *SMN1* RNA expression and abnormal RNA spliceosome.

Table 1 Different *SMN1*/*SMN2* copy numbers identified by DHPLC in newborn screening

SMN gene copy number (SMN1/SMN2)	Expected total copy number of the SMN gene	Measure (SMN1/SMN2) ratio ^a	Number of subjects	Frequency (%)	Status
1:4	5	0.26	1	0.06	Carrier
1:3	4	0.34 ± 0.02	17	0.99	Carrier
1:2	3	0.50 ± 0.04	15	0.88	Carrier
1:1	2	0.99 ± 0.05	8	0.47	Carrier
2:3	5	0.65 ± 0.01	49	2.86	Normal ^b
2:2	4	0.99 ± 0.05	988	57.71	Normal ^b
2:1	3	1.96 ± 0.08	449	26.23	Normal ^b
3:2	5	1.54 ± 0.08	57	3.33	Normal
3:1	4	2.98 ± 0.05	38	2.22	Normal
4:1	5	4.02	1	0.06	Normal
2:0	2	No <i>SMN2</i> peak	49	2.86	Normal ^b
3:0	3	No <i>SMN2</i> peak	24	1.40	Normal
4:0	4	No <i>SMN2</i> peak	16	0.93	Normal
Total			1712		

^aData were expressed as means ± SD, and the ratio with 'mean ± SD' is based on one mean of a single determination in all tested individuals for a given genotype.

^bVery few carriers with two *SMN1* genes on one chromosome ('2+0') may be involved in them as this genotype could not be detected by *SMN1* copy-number analysis.

Table 2 Distribution of the *SMN1* allele and genotype frequency observed in 1712 newborn individuals

Allele	Number (%)	Genotype	Designation	Observed no. (%)	Expected no. (%)	χ ²	P-value
0-copy	41 (1.20)	0+0	p ²	0 (0)	0.25 (0.02)		
1-copy	3230 (94.34)	1+0	2pq	41 (2.39)	38.76 (2.26)	9.792	0.084
		1+1	q ²	1535 (89.66)	1523.36 (88.98)		
2-copy	153 (4.46)	2+0	2pr	0 (0) ^a	1.84 (0.11)		
		2+1	2qr	119 (6.95)	144.37 (8.43)		
		2+2	r ²	17 (0.99)	3.42 (0.20)		
Total	3424 (100)			1712 (100)	1712 (100)		

No significant differences were detected between the observed number and the expected number ($P > 0.05$).

^aThe '2+0' genotype could not be detected by the DHPLC assay. It is very rare in the general population, and therefore the observed number is referred to as '0'.

DHPLC, followed by direct DNA sequencing, was used to identify the unknown mutation in the *SMN1* gene. Total RNA was isolated from peripheral blood lymphocytes using TRIZOL Reagent (Invitrogen, Carlsbad, CA, USA). First-strand cDNA synthesis and reverse transcription were performed according to the manufacturer's instructions (Fermentas Fermentas, Hanover, MD, USA) using 30 ng each of the forward primer (5'-ACCACCACCCCACTTA CTA-3', specific for exon 5 of *SMN1*/*SMN2*) and the reverse primer (5'-TT TTTCTTTCCCAACTTGAT-3', specific for exon 8 of *SMN1*/*SMN2*). The reaction mixture components and PCR conditions were identical to those described for the above single PCR reaction used before the DHPLC assay.

Statistical analyses

Current and predicted vital statistics were obtained from the 'National Population and State Family Planning Commission of China'.²⁵ Statistical analyses were conducted using SPSS 17.0 statistical software (SPSS Inc., Chicago, IL, USA). Differences in the allele/genotype frequency of *SMN1* genes were calculated from the modified Hardy-Weinberg formula.

RESULTS

SMN1/*SMN2* copy number and population prevalence

Characterization of the *SMN1*/*SMN2* ratio or copy numbers in 1712 newborn individuals by DHPLC analysis resulted in the identification of 13 different groups. We identified 41 subjects with a heterozygous deletion of *SMN1* exon 7, which indicated that the carrier prevalence was 2.39% in the general Chinese population. The number and percentage of subjects with each different *SMN1*/*SMN2* ratio among our samples are listed in Table 1, and the frequencies of the *SMN1* alleles and genotypes defined by this study are listed in Table 2. Representative DHPLC profiles for different *SMN1*/*SMN2* peak ratios are shown in Figure 2. From the results of the DHPLC analysis, we identified a group of subjects who seemed to carry novel *SMN1*/*SMN2* gene mutations. Seven SNPs in the introns or exons of the *SMN1* or *SMN2* gene were identified in 31 subjects by direct sequencing; this included 20 subjects with mutation IVS6-50A>G, 6 with IVS6-24dupT, 4 with IVS7+287dupC, 2 with IVS7+137G>T, 2 with IVS7+183C>T, 1 with c.875C>G, and 1 with EX8+204del2. These seven variants of the *SMN1* and *SMN2* genes could not be associated with causative factors of SMA in the Chinese population on the basis of data obtained from SNP functional prediction and alignment analysis. No deviation from Hardy-Weinberg and genotypic equilibrium was observed by testing the allele and genotype distributions for the *SMN1*/*SMN2* loci.

Characterization of the SMA genotypes of the core families

Among the 26 SMA patients from the 25 unrelated core families, 13 cases of type I, 5 cases of type II, and 8 cases of type III SMA were detected (Table 3). In all cases, at least 1 copy of the *SMN1* gene was deleted: 24 were homozygous deletions of *SMN1*, and 2 were

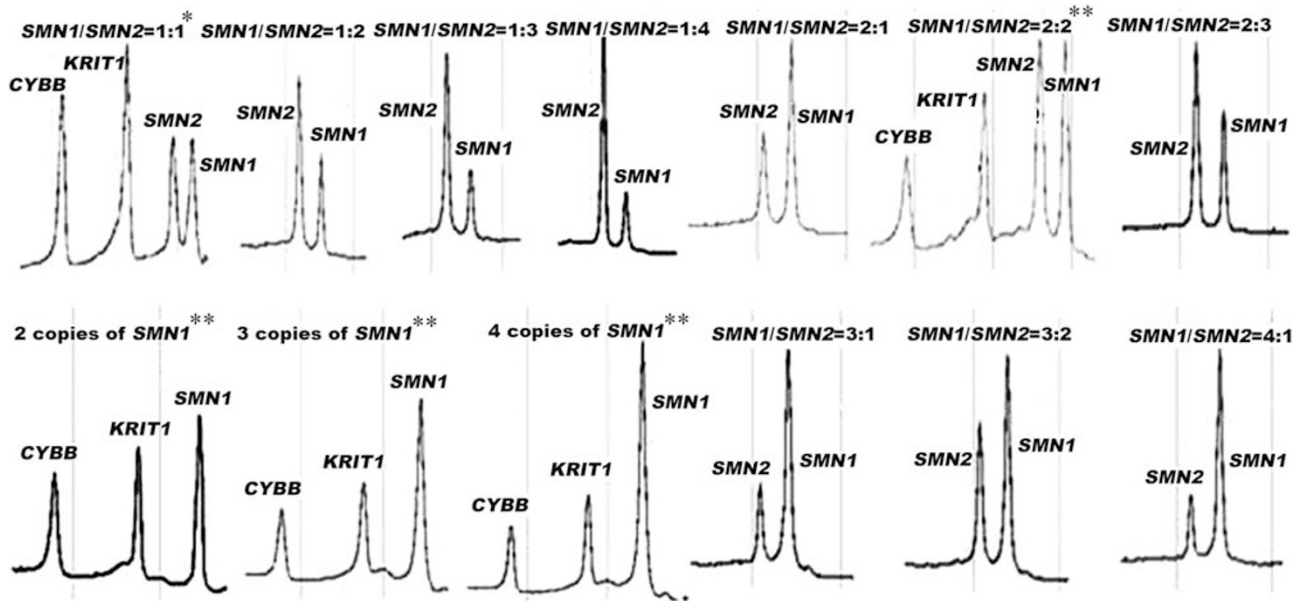


Figure 2 Representative DHPLC profiles of single/multiplex PCR analysis for different *SMN1/SMN2* ratios. *Female individuals with *SMN1/SMN2*=1:1; **male individuals with various *SMN1/SMN2* ratios.

Table 3 *SMN* gene copy numbers observed in 25 core families

Case no.	Clinical type	<i>SMN1/SMN2</i> ratio			
		Patient	Father	Mother	Sib
1	I	0:2(M)	1:1	1:1 or 1:2 ^a	
2	I	0:2(F)	1:1	1:2	
3	I	0:2(M)	1:2	1:1	
4	I	0:2(F)	1:3	2:0 ^a	
5	I	0:3(M)	2:1 ^b	1:3	
6	I	0:2(M)	1:2	1:1 or 1:2 ^a	
7	I	0:2(F)	1:1	1:2	
8	I	0:3(M)	1:2	1:3	
9	I	1 ^D :3(M)	1+1 ^D :2	1:3	2:2(F)
10	I	0:2(M)	1:1	1:2	
11	I	0:2(M)	1:2	1:1	
12	I	0:2(M)	1:2	1:3	
13	I	0:2(F)	1:2	1:3	
14	II	0:3(M)	1:2	1:3	
15	II	0:3(M)	1:3	1:1	
16	II	0:3(F)	2:1 ^b	1:3	
17	II	0:3(M)	1:1	1:2 or 1:3 ^a	
18	II	0:3(F)	1:2	1:3	
19	III	0:4(F)	1:2	1:3	0:2(F)
20	III	0:3(M)	1:2	1:3	
21	III	0:3(F)	1:3	1:2 or 1:3 ^a	
22	III	0:3(F)	1:1	1:3	
23	III	0:4(M)	1:3	1:2 or 1:3 ^a	
24	III	0:4(F)	1:3	1:3	
25	III	1 ^D :3(F)	1+1 ^D :1	1:3	

M, male; F, female.

^aBlood samples were not available from these individuals, and therefore their *SMN* genotypes were assumed based on the genotypes determined for their patients and fathers from the family study.

^bThese two fathers were found to be the 'carriers with the '2+0' genotype by the further family testing.

Table 4 Comparison of the genotype frequency of SMA carriers between two groups: newborns vs the SMA core family

<i>SMN1/SMN2</i>	Newborn		SMA core family		χ^2	P-value
	No.	%	No.	%		
1:1	8	19.51	9	20.45		
1:2	15	31.58	12	27.27		
1:3	17	44.74	19	43.18		
1:4	1	2.63	0	0	2.749	0.432
2:1	0	0	3 ^a	6.82		
2:2	0	0	1 ^b	2.27		
Total	41	100	44	100		

^aOne case with 1+1^D: 1 and two cases with the '2+0' genotype.

^bOne case with the 1+1^D: 2 genotype.

heterozygous deletions. The *SMN1/SMN2* copy numbers in the 44 carriers from these SMA families were determined by DHPLC analysis and compared with the copy numbers obtained for the 41 carriers in the newborn population (Table 4). No significant differences in the genotype frequencies of SMA carriers were observed between these two groups ($P > 0.05$). As expected, the '1+0' genotype was predominant, and accounted for 90.9% (40/44) of carriers in the SMA core families and 100% (41/41) in the newborn population. Among three carriers from the core families with an *SMN1/SMN2* ratio of 2:1, we found that the fathers of two unrelated patients had the '2+0' genotype and we also identified two '1+1^D' genotypes in individuals with an *SMN1/SMN2* ratio of 2:2 or 2:1.

Development and validation of the RDB assay

The RDB assay was developed to genotype deletions of *SMN1* exon 7 and exon 8 using a panel of samples from SMA patients, carriers,

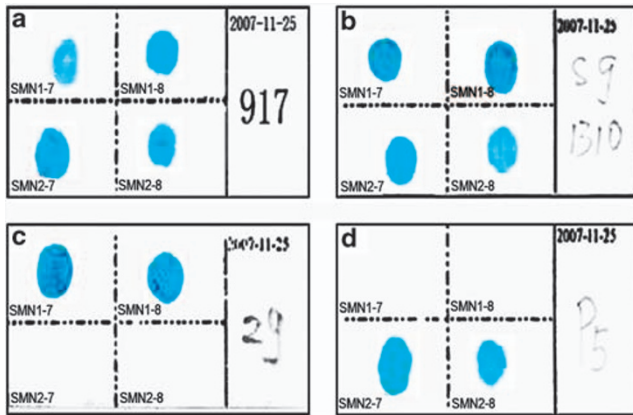


Figure 3 Representative results for RDB detection of different *SMN1/SMN2* ratios. (a) Typing of a normal individual with *SMN1/SMN2*=2:N; (b) typing of an SMA carrier with *SMN1/SMN2*=1:N; (c) typing of a normal individual with *SMN1* only; (d) typing of SMA patients with *SMN1/SMN2*=0:N.

or normal individuals who had been characterized previously by molecular analysis. Figure 3 shows representative RDB profiles for four tested samples. Results indicate that the assay could clearly distinguish SMA patients who carried a homozygous deletion (only *SMN2* could be detected) from normal individuals or carriers with only *SMN1* genes or with different ratios of *SMN1/SMN2*. The assay could not discriminate a normal individual from a carrier with different ratios of *SMN1/SMN2* genes. In a blinded experiment, the results for 182 of the 183 samples (99.5%) tested by the RDB assay were in agreement with the results obtained by DHPLC/DNA sequencing; the exception was the misdiagnosis of one sample that contained one of the below-mentioned novel mutations.

A novel *SMN1* splice-site mutation

A novel acceptor splice-site mutation (c.835-1G>A) within intron 6 of the *SMN1* gene was discovered in 1 of the 25 unrelated core families using DHPLC, RDB, and DNA sequence analysis (Figure 4). The proband had inherited a typical deletion of *SMN1* exon 7 from his mother and a novel mutation from his father. DNA sequencing revealed unique peak heights at the c.835-1G>A mutation (Figure 4e) in the genomic DNA of both the proband and his father and confirmed the results obtained by the DHPLC quantitative assay, which showed that the ratio of *SMN1/SMN2* was 2:2 in the father and 1^D:3 in the proband. In addition, the sequences of *SMN1* and *SMN2* vary at nucleotide position +6 in exon 7 (C in *SMN1*, T in *SMN2*). Figure 4e shows that the peak size of C was equal to that of T for the father, whereas the peak size of C was one-third that of T for the proband, which further confirmed that the ratio of *SMN1/SMN2* was 2:2 in the father and 1^D:3 in the proband. RT-PCR analysis of RNA from the father demonstrated the expected pattern of *SMN1/SMN2* expression: the abundance of the 294-bp product (*SMN1* Δexon7 allele and *SMN2*) was much greater than that of the 348-bp product (*SMN1* wild-type allele). The 294-bp product represents amplification of both the *SMN2* gene transcript, from which exon 7 is normally deleted, and the *SMN1* transcript with the splice-site mutation (c.835-1G>A), which indicates that the mutation inhibits splicing of the *SMN1* gene.

DISCUSSION

We studied the incidence of carriers and genetic background of SMA in mainland China by quantifying the copy numbers of *SMN1* and

SMN2 genes in 1712 newborn individuals using a DHPLC-based assay. This is the first large-scale population-based genetic investigation of SMA in the general population of mainland China. We selected random individuals from the southern Han Chinese population as representative of the population of mainland China, because the two groups of Chinese, the southern Han Chinese and the northern Han Chinese, who represent the largest population in mainland China share a common origin and similar genetic structure.^{26,27} Nearly all of the possible different ratios of *SMN1/SMN2*, as determined by quantitative DHPLC-based assay, could be observed clearly in the Chinese population. This confirmed the complex structural organization of the human *SMN* gene and is similar to previous observations in Caucasian, Korean, Australian, and African populations.^{2,3,13-19} Simultaneously, we compared *SMN1* alleles and genotype frequencies among different races. There were no significant differences among Chinese, Caucasian, Korean, Australian, American, and African populations, which showed that there were no racial or regional differences with respect to SMA in general populations. As expected, the '1+0' genotype was predominant among carriers, and accounted for 90.9% (40/44) of the carriers in the SMA core families and 100% (41/41) in the newborn population, but it should be noted that a few distinct, rare genotypes (2+0 and 1+1^D) were neglected in our newborn cohort because they could not be detected by the present assay. In addition, among three carriers from the core families with an *SMN1/SMN2* ratio of 2:1, we found that the fathers of two unrelated patients had the '2+0' genotype and we also identified two '1+1^D' genotypes in individuals with an *SMN1/SMN2* ratio of 2:2 or 2:1. Given that the number of copies of the *SMN2* gene is undoubtedly the major modifier of the SMA phenotype, the elucidation of the *SMN2* gene patterns presented herein will provide fundamental knowledge of this disorder in the Chinese population. These data demonstrate a carrier prevalence of 1/42 (2.39%) in the general Chinese population with respect to heterozygosity for deletion and intragenic mutations of *SMN1* exons 7 and 8; the infrequent *SMN* gene defects that consist of point mutations outside of exons 7 and 8 were not detected. In addition, the '2+0' genotype could not be identified in this study. The data provide a reliable representation of the incidence of SMA carriers in the general Chinese population because analysis of the allele and genotype distributions for the *SMN1/SMN2* loci showed no deviation from Hardy-Weinberg equilibrium. In addition, the '1+0' genotype was proven to be predominant in the Chinese population on the basis of data obtained from the SMA core family study (Table 4). Therefore, our results could provide a basis for a nationwide program of genetic counseling, clinical screening, and prenatal diagnosis to prevent SMA in China. According to the expected frequency of homozygous deletion of *SMN1* in newborns (Table 2), 14.34 individuals in 100 000 (95% CI, 13.66/100 000 ~ 15.03/100 000) will have the '0+0' genotype. With a current annual birth rate of ~ 16 080 000,²⁵ we can estimate that the number of newborns in mainland China who will be affected by SMA is 2306 (95% CI, 2196 ~ 2417) per year.

Individuals with the rare '2+0' genotype have two copies of *SMN1* in *cis* on one chromosome and no *SMN1* gene on the other chromosome, which results in a compound SMA heterozygous deletion with a carrier frequency that ranges from 1.7 to 3.3% in different ethnic populations.^{1,3,13} In the family study, we detected two cases of the '2+0' genotype among the 44 parents of the SMA patients; these parents had an *SMN1/SMN2* ratio of 2:1 (Table 3 and 4). This finding suggests that the '2+0' genotype is a considerable SMA determinant and should be tested during carrier screening and clinical molecular diagnosis in the Chinese population. Population estimations suggest that approximately two (1.87) '2+0' carriers should be present among

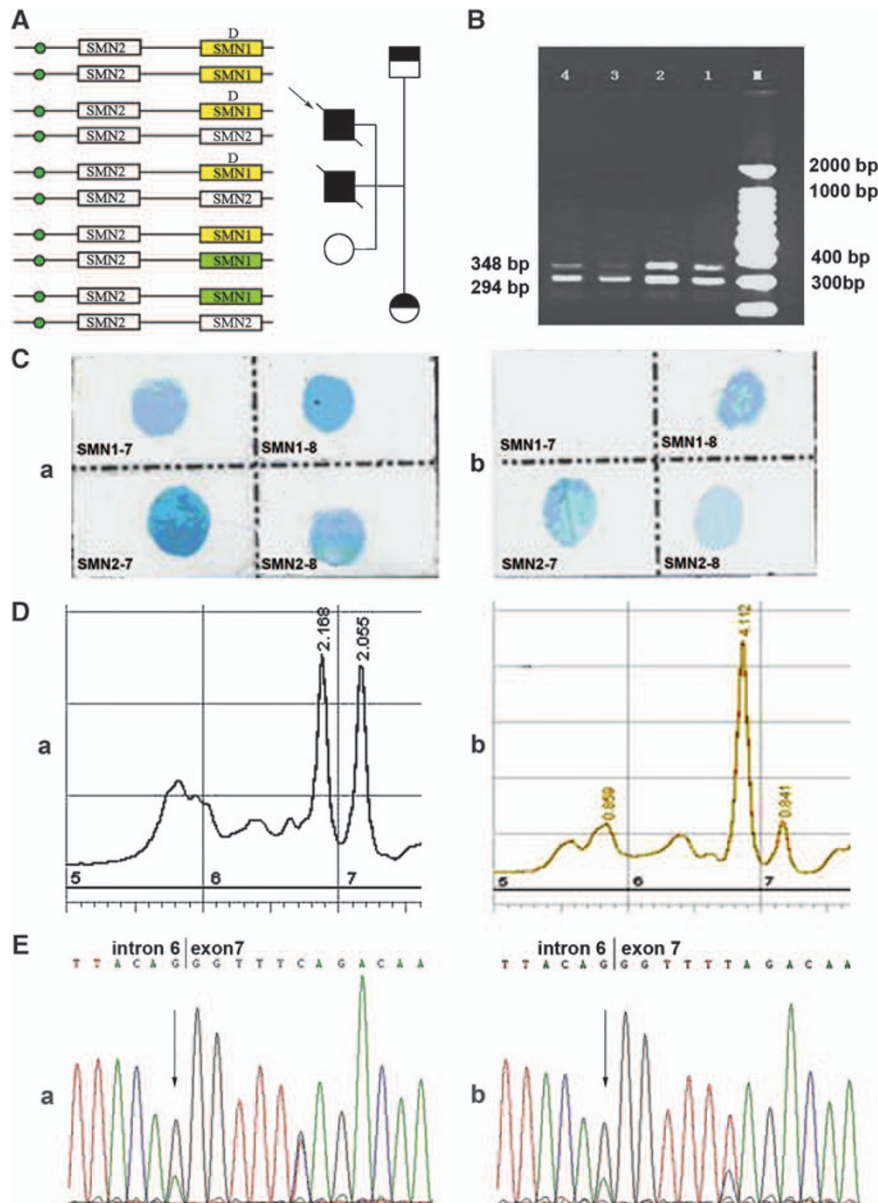


Figure 4 *SMN* analysis for a family carrying the novel *SMN1* splice-site mutation c.835-1G>A. (a) Pedigree and *SMN* copy numbers for the patient's family. (b) RT-PCR amplification of *SMN* gene exons 5-8 to determine *SMN1/SMN2* expression in the RNA sample obtained from the proband's father. The transcript product for Δ exon7 was 294bp and the normal transcript product including *SMN1* exon 7 was 348bp in length. M: DNA molecular weight marker; lanes 1 and 2: normal individuals; lanes 3 and 4: the father carried the *SMN1* splice-site mutation at the junction between intron 6 and exon 7 (c.835-1G>A). (c) RDB analysis of the *SMN* gene in the proband and his father. The DNA fragment hybridized with the probe for *SMN1* exon 7 contained the site with the splice-site mutation c.835G>A, which provided a negative result by the RDB assay for the proband (panel b). However, the father was an SMA carrier with one normal *SMN1* gene, which provided a positive result by the RDB assay (panel a). (d) Quantitative DHPLC analysis of the *SMN1/SMN2* gene dosage in the proband and his father. (Panel a) The father exhibited a gene ratio of *SMN1/SMN2*=2:2; (panel b) the proband exhibited a gene ratio of *SMN1/SMN2*=1:3. (panel e) DNA sequencing of the *SMN1* gene carried by the proband and his father. (Panel a) Sequence of genomic DNA containing *SMN* gene intron 6 and exon 7 from the father; (panel b) sequence of the genomic DNA containing *SMN* gene intron 6 and exon 7 from the proband. The downward arrow indicates a G→A mutation at the acceptor splice site of intron 6.

the 1712 newborns analyzed but presumably they were not detected because of false-negative results obtained in our DHPLC assay. Direct detection of the '2+0' SMA carriers still remains a great challenge because of the time-consuming and laborious detection methods required. Therefore, the finding of two normal dosages of the *SMN1* gene may significantly reduce the risk of being a carrier, but there is still a small risk of recurrence among future offspring for individuals

with two *SMN1* gene copies. Given that it is a rare event and there will be a relatively high number of samples with an *SMN1/SMN2* ratio of 2:1, we suggest that it would be more economical and efficient to focus molecular analysis of the '2+0' genotype on index patients and their family members rather than implementing a population-wide screening. On the other hand, the fact that 2% of individuals with SMA have one *de novo* mutation, and a few individuals have a rare point

mutation in the *SMN1* gene,^{28,29} might provide an explanation of possible false-negative results when interpreting the *SMN1* gene dosage-based test for individuals undergoing genetic counseling. Moreover, Bayesian analysis is essential for the risk assessment calculations that are required for the proper genetic counseling of families with a history of SMA.

By using two groups of samples, the genotype of which had been characterized by the DHPLC assay, we have established and validated a new, simple, and reliable RDB method that is capable of directly detecting the most common homozygous deletion of *SMN1* in exons 7 and 8. This methodology has desirable features that include its ease of operation and the lack of a requirement for expensive equipment or strict experimental conditions, which makes it suitable for use in different laboratories and is especially beneficial for developing countries. According to our experience, it can be used as an alternative method to genotype the SMA homozygous deletion directly in routine clinical diagnosis. The current assay was limited to the detection of this common deletion mutation. We intend to extend the assay to include other relatively rare point mutations so that the assay would be capable of detecting all types of SMA mutations in the Chinese population in the future.

In conclusion, our research has determined the genetic load and the *SMN* gene variants that are present in the Chinese population, and these results should serve as the basis for a nationwide program of genetic counseling, population screening, and prenatal diagnosis to prevent SMA in China. We have also proven that the DHPLC-based dosage analysis is a suitable method to determine the carrier status of individuals with a family history of SMA or to investigate cases of clinically suspected SMA with a negative diagnostic SMA test. However, it should be noted that reasonable counseling modalities must be adapted to different individuals, as described by Smith *et al*.³ Given that the DHPLC-based dosage analysis does not detect all carriers, especially those with the '2+0' genotype or intragenic *SMN* mutations, a risk assessment is an essential component for clinical screening of individuals who have two or more copies of *SMN1*. Genetic risk assessments can be estimated principally on the basis of our reported carrier frequency for the '1+0' genotype. However, the '2+0' genotype, *de novo* mutations, and rates of intragenic mutations in the *SMN1* gene are additional factors that need to be considered. Furthermore, as demonstrated for the Australian population, a multimodal approach that involves *SMN* dosage analysis, linkage analysis, and genetic risk assessment facilitates the resolution of SMA carrier status in individuals with a family history, as well as among individuals in the general population.³ Such an approach should provide Chinese couples who are undergoing genetic counseling with improved choices for their family planning. In addition, we have successfully validated the RDB assay as an alternative method for the rapid genotyping of deletional SMA in routine clinical diagnoses.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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