Investigation of carrier frequency for spinal muscular atrophy in Thailand using dried blood spot specimens

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ABSTRACT

Spinal muscular atrophy (SMA) is an autosomal recessive genetic disorder, characterized by the degeneration of motor neurons of the spinal cord, thus leading to the deaths of newborns. More than 95% of SMA patients are caused by the absence of survival motor neuron 1 (*SMN1*) gene exon 7, located on chromosome 5q13. The SMA disease prevalence and SMA mutations in Thailand have never been reported. In this study, we aimed to explore the frequency of SMA carriers (heterozygous, loss of exon 7 in *SMN1* gene) and simultaneously determine the *SMN2*-exon 7 copy number in Thai neonate population. We used a denaturing high-performance liquid chromatography (DHPLC)-based semi-quantitative multiplex-PCR technique to detect the copy number of *SMN* genes by comparing with two reference genes, palmitoyl-protein thioesterase 1 and proteolipid protein 1. DNA samples were extracted from dried blood spot (DBS) specimens (n = 1,000) leftover from the Thai National Neonatal Screening Program. Results indicated that the SMA carrier frequency in the Thai newborns was 2.8% (1/36 cases). According to this data, the prevalence of SMA caused by homozygous deletion of *SMN1* exon 7 in Thai newborns was estimated at 1 in 5,102 cases. In conclusion, we reported a relatively high rate of SMA carriers in Thai newborns. Additionally, the estimated incidence of SMA found in this study was comparable to that reported in other countries. Therefore, SMA could be considered as a national health problem and results from this study may be useful to establish a national program for clinical, prenatal diagnosis and genetic counseling to effectively prevent SMA in Thailand.

Keywords: spinal muscular atrophy (SMA); dried blood spots (DBS); *SMN1*-exon 7 deletion; carrier frequency; Thailand

INTRODUCTION

Spinal muscular atrophy (SMA) is one of the most common fatal autosomal recessive genetic disorders in the world (Kesari *et al*., 2005a; Ogino and Wilson, 2002.). The incidence is high and varies among countries. It is about one in 6,000 to 10,000 live births with a carrier frequency of 1/40-1/60 (Ogino and Wilson 2002; Pearn 1978). The presentation of disease symptoms is due to the degradation of the anterior horn cells of the spinal cord, resulting in hypotonia and muscle weakness and possibly lead to death in severe cases (Lunn and Wang, 2008). SMA is classified into four types according to age of onset, ranging from adolescence into young adulthood, and severity based on motor function test (Kesari *et al*., 2005a). Type I SMA (Wernig–Hoffman) is the most severe form with an onset before 6 months and death within 2 years of life. Type II, intermediate type, is the most common form found in SMA patients with an onset between age six and 12 months. The life span varies from two years to the third decade of life. Type III SMA is the milder

form with onset in childhood after 12 months of age. The last form, type IV, is the very mild form that is usually found in adults. Until now, no medical treatment is implemented to effectively cure the disease.

Studies on SMA patients indicate that the genes responsible for the disease are located in part of a large inverted duplication region on chromosome 5q13 (Melki *et al.*, 1990; 1994), including, survival motor neuron (*SMN*), neuronal apoptosis inhibitory protein (*NAIP*), basal transcription factor subunit (*p44*) and the novel protein of unknown function *H4F5* genes (Kesari *et al.*, 2005a, 2005b). In these gene clusters, only the *SMN1* gene at the telomeric region has been shown to cause the disease (Kesari *et al*., 2005b; Su *et al*., 2005; Ogino and Wilson, 2002). About 95-98% of individuals with SMA are homozygous for the *SMN1* deletion or gene conversion between *SMN1* and *SMN2*. About 2-5% of the mutations are compound heterozygous for the *SMN1* deletion or gene conversion and the *SMN1* point mutation (Mailman *et al*., 2002). These mutations cause a reduction of the SMN protein, which lead to the disease manifestations. However, SMA patients carrying the same mutation usually presented with difference disease progression and severity. This is explained via the presence of an almost identical copy of the *SMN1* gene, a centromeric *SMN* (*SMN2* or *SMNc*) (Lefebvre *et al*., 1995). However, only 10% of the SMN protein expressed from this gene is functional whereas the rest of 90% is abnormal protein and rapidly degraded (Lorson and Androphy, 2000). The *SMN2* exon 7 sequence differs from the *SMN1* at +6 position (C-to-T nucleotide substitution or c.840C>T), but does not cause amino acid change. The previous study has shown that this is a position located in an exonic splicing enhancer sequence (ESE) (Cartegni and Krainer, 2002). This c.840C>T interrupts the binding of splicing enhancer protein to ESE and recruits a nuclear ribonuclear protein (hnRNP) A1, the splicing repressor protein. Eventually, it promotes an alternative splicing causing lack of exon 7 in the mature mRNA (Cartegni and Krainer, 2002). However, the full length protein can be expressed up to 50% in some cases, depending on the splicing efficiency. In summary, homozygous deletion of *SMN1* causes development of SMA whereas heterozygous loss does not cause any SMA phenotypes. The loss of *SMN2* (5% in population) (Lefebvre *et al*., 1995) is not directly responsible for SMA. Moreover, increase in the *SMN2* copy number often alleviates SMA phenotypes (Kesari *et al*., 2005a). The *SMN2* copy number has not yet been investigated in Thai SMA patients and normal population.

Molecular genetic test for homozygous *SMN1* exon 7 deletions is mainly used for diagnosis of SMA (Lai *et al*., 2005). Patients with clinical phenotypes similar to SMA should be tested for the presence of exon 7 homozygous deletion (Lai *et al*., 2005). Most of molecular genetic tests previously reports are based on enzymatic digestion of PCR products amplified from both the *SMN1* and *SMN2* genes (Su *et al*., 2005). The restriction enzyme digestion method can discriminate *SMN1* from *SMN2* based on the nucleotide difference at the position c.840 (Ogino and Wilson, 2002). This allows detection of the existence or deletion of *SMN1* in the homozygous states. However, this method is unable to detect one allele loss of *SMN1* because of the limitation of detection at an endpoint PCR. Various techniques have thus been developed for *SMN1* copy number analysis that can detect both homozygous and heterozygous losses of the *SMN1* exon 7. DHPLC is a simple, fast and non-gel based method that is very sensitive and specific for detection of variations in DNA including the detection of the homozygous *SMN* gene deletion (Su *et al*., 2005).

Until now, there is no report on the SMA incidence and carrier frequency in Thailand. In this study, we aimed to test if the modified SMA screening technique, named DHPLC-based semi-quantitative multiplex-PCR, was efficient to determine *SMN1/SMN2* gene dosage in genomic DNA samples extracted from dried blood spot (DBS) specimens. The SMA carrier frequency in Thai newborn population was also investigated.

MATERIALS AND METHODS

DNA samples

One thousand DBS specimens of Thai newborns of year 2007 were taken anonymously from the biobank of the National Neonatal Screening Program, Department of Medical Sciences, Ministry of Public Health, Thailand, using systematic random sampling method with the departmental ethical approval. The specimens were from 480 boys (48%) and 520 girls (52%)**.** Fifty DBS samples were taken from each five representative provinces of the four regions of Thailand. The Northern region included Tak, Lumphun, Nan, Sukhuthai and Uttaradit provinces. The Central region included Chachoengsao, Chai-nat, Ratchaburi, Sa-keaw and Singburi provinces. The Northeastern region included Chaiyaphum, Burirum, Nakhon-phanom, Udon-thani and Yasothon provinces. Finally, the Southern region included Nara-thiwat,

Trang, Ranong, Songkha and Surat-thani provinces. Genomic DNA was extracted from three pieces of 3-mm diameter of DBS using QIAamp®96 DNA Blood Kit (QIAGEN, Germany) according to the manufacturer's protocol. The average concentration of DNA obtained was 6.4 ng/µl, with the average OD 260/280 ratio of 1.94. The positive control samples for *SMN1* exon 7 homozygous deletion and carrier (heterozygous deletion) were from anonymous SMA patients and their parents. Negative controls were DNA from healthy individuals of both genders. All control samples were anonymously obtained from DNA bank of Molecular Genetics Laboratory, Faculty of Medicine Siriraj Hospital, Mahidol University with informed consent at the time of blood collection carried out as part of a routine medical examination following the institutional ethical guideline. The genomic DNA was extracted using standard phenol-chloroform extraction procedure**.** The positive control samples for *SMN1* exon 7 homozygous deletions have been previously analyzed in our laboratory using restriction fragment length polymorphism (RFLP) (Lefebvre *et al*., 1995 and Wirth *et al*., 1999).

Multiplex PCR

Multiplex PCRs were performed to amplify exon 7 and flanking sequences of both *SMN1* and *SMN2* genes together with 2 reference genes including palmitoyl-protein thioesterase 1 (*PPT1*; chromosome 1p32) and proteolipid protein 1(*PLP1*; X-linked). Three pairs of primers used in this study are shown in Table 1. The PCR reaction was conducted in a final volume 25 µl, containing 4 µl of genomic DNA extracted from DBSs (about 25 ng), 1x ImmoBuffer, 3.5 mM MgCl2, 0.28 mM of dNTPs, 0.23 µM of each SMNint6-7F and SMNint6-7R primers, 0.15 µM of each PLP-4F and PLP-4R primers, 0.16 µM of each NCL1-9F and NCL1-9R primers, and 0.5 U of Immolase® (Bioline Ltd., UK). The PCR amplification was performed in a MJ Research PTC-100 thermal cycler (MJ Research, Inc., Canada) with an initial denaturation step at 94ºC for 7 min, followed by 35 cycles consisting of denaturation at 94ºC for 30 sec, annealing at 58ºC for30 sec, extension at 72ºC for 30 sec and then a final extension step at 72ºC for 10 minutes. The reference genes were used to determine the relative gene copy number of *SMN1* and *SMN2*. Prior to testing, the PCR primer concentration and ratio were optimized based on the DHPLC peak height corresponding to known *SMN1* copy number sample, including obligate carriers of both sexes. For female carriers, the *SMN1* peak height was adjusted equal to half of those *PPT1* and *PLP1*, whereas that for male carriers was adjusted equal to *PLP1* and a half of *PPT1* (see Figure 1, males and females in the carrier group). Amplification of the peripheral blood DNA of the control samples was performed as described above, but the PCR cycle was reduced to 26 cycles. Control samples with known *SMN1* copy number including DNA from SMA patients (0-copy of *SMN1* exon 7) and SMA carrier (1-copy of *SMN1* exon 7) were used to amplify in parallel with other unknown samples in each batch to show the reproducibility of the technique and to ensure that the copy number of other samples could be correctly interpreted.

Table 1 The primers used in the multiplex PCR.

Primer Name	Sequence (5´ to 3´)	Target amplified	Product size (bp.)	References
SMNint6-7F	TGTCTTGTGAAACAAAATGCTT	*SMN1*&*SMN2* exon 7	459	Su *et al*., 2005
SMNint6-7R	AAAAGTCTGCTGGTCTGCCTA			
NCL1-9F	ACTCAGGACAAACTGCATTT	*PPT1* exon 9 (autosome)	236	in this study
NCL1-9R	TTGCAAGCTGGATCTGAGCT			
PLP-4F	ACTCCAGGATCTCCCAGTTT	*PLP1* exon 4 (x-linked)	374	Mimault *et al*., 1999
PLP-4R	AGACCCAATCATTCATCATC			in this study

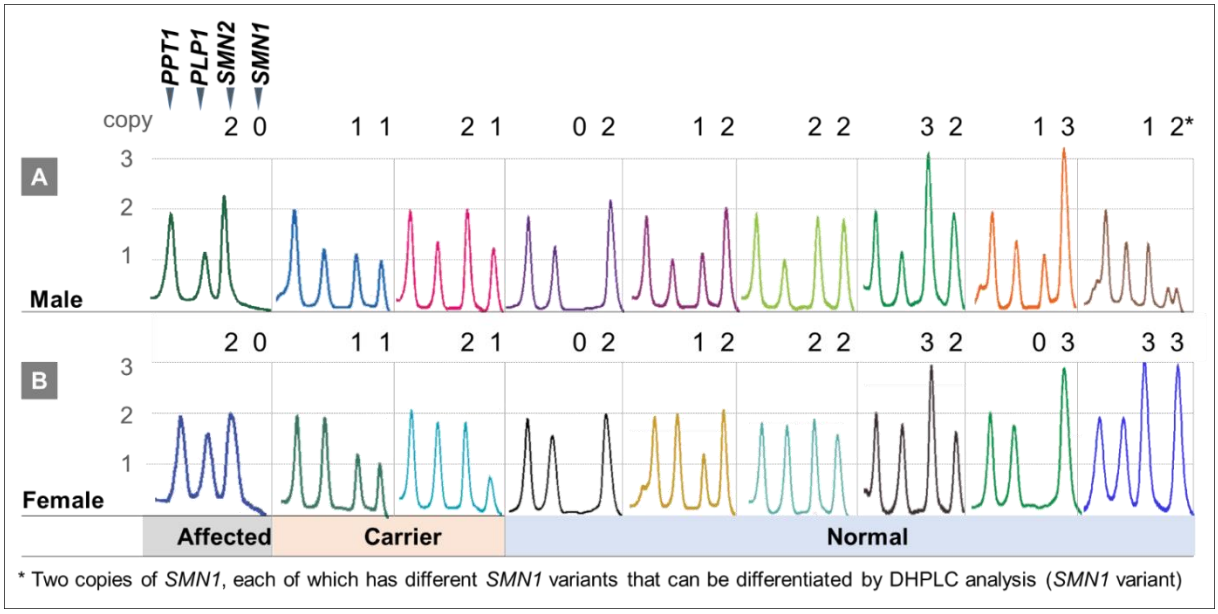

Figure 1 The DHPLC profiles representing copy number ratio of *SMN* genes compared to the reference genes, *PPT1* (autosome) and *PLP1* (X chromosome) in affected SMA patients, SMA carriers and a variety of normal controls in male (panel A) and female (panel B).

DHPLC conditions for the *SMN1* gene copy number analysis

DHPLC analysis of multiplex PCR product was carried out using a non-denaturing mode of the WAVE™ Nucleic Acid Fragment Analysis System (Transgenomic Inc., CA, USA), an automated HPLC instrument equipped with DNASep®HT cartridge (cat. no. DNA-99-3710, Transgenomic). Five microliters of triplex PCR products were eluted with a linear acetonitrile gradient using 45% to 80% of buffer B (0.1 M TEAA, 25% acetonitrile) by mixing with buffer A (0.1 M triethylammonium acetate, TEAA) within a separation time of 3.5 min. Each injection was cleaned with 100% solution D (75% acetonitrile in water) for 0.7 min and equilibrated with 40% B for 0.7 min. The flow rate was set at 0.9 mL/min and at an oven temperature of 52.5 °C. The eluted DNA samples were automatically detected by 260 nm UV absorbance. Generally, the analysis for each sample took about 7.5 min. Reproducibility of the technique was also evaluated from the parallel DHPLC analysis of unknown DBS samples together with the known SMA patient and known-carrier samples in each run.

RESULTS & DISCUSSION

Determination of the *SMN1* gene deletion using DHPLC

Heterozygous loss of the *SMN1* gene in SMA carriers was detected by the DHPLC-based semi-quantitative multiplex-PCR technique. DNA extracted from whole blood samples of the SMA patients' parents (obligate carrier status) and normal individuals were used as controls. The order of peaks from DHPLC chromatogram is based on the size and sequence of the PCR products. The peak of *PPT1* (autosome) comes out first, followed by *PLP1* (X chromosome), *SMN2* and *SMN1*, respectively. The copy numbers of the *SMN* genes were interpreted according to the copy number of the reference genes. *PPT1* has two copy number in both male and female, whereas *PLP1* has one copy number in male but two copy number in female. The SMA carriers have one *SMN1* copy as interpreted from the peak height ratio whereas normal individuals have at least two *SMN1* copies (Figure 1). The ratio of *SMN2*:*SMN1* copy number of normal, affected and carrier of the disease are shown in Figure 1.

Determination of SMA and SMA carriers in Thai neonates

Of 1000 DBS specimens, none was affected with SMA. Most of the samples were normal (97.2%) having the two most predominant SMN2:SMN1 ratios of 2:2 (49.8%) and 1:2 (35.1%). The SMA carriers had varied *SMN2*:*SMN1* ratios, including 1:1 (1.1%), 2:1 (1.3%) and 3:1 (0.4%) (Table 2). Additionally, in eight DBS samples, the DHPLC profiles of the *SMN1* gene (the 4th peak in the DHPLC chromatogram) exhibited two well separated peaks of equal height of one *SMN1* copy. This indicated 2 copies of *SMN1* (as

shown in the rightmost case on panel A of Figure 1). These eight samples (0.8%, 8/1,000) were classified as normal individuals without *SMN1* exon 7 deletion. However, it was possible that they had existence of *SMN1* point mutations or *SMN1* polymorphisms in exon 7 (we designated as "*SMN1* variant" in Table 2).

Table 2. *SMN2*:*SMN1* ratio analyzed by the multiplex DHPLC-based semi-quantitative multiplex-PCR in 1,000 DBSs of Thai newborns, by regions. *SMN2*:*SMN1* ratios of 1:1, 2:1 and 3:1 indicate SMA carriers.

SMN2*:*SMN1	**Central**	**Northern**	**Northeastern**	**Southern**	**Total (number)**	**Total (%)**
0:2	13	10	8	3	34	3.4
0:3	5	8	5	4	22	2.2
0:4	0	1	1	0	2	0.2
1:1	**3**	**2**	**2**	**4**	**11**	**1.1**
1:2	71	102	99	79	351	35.1
1:3	11	8	4	9	32	3.2
1:4	1	0	0	0	1	0.1
2:1	**4**	**3**	**5**	**1**	**13**	**1.3**
2:2	132	107	117	142	498	49.8
2:3	0	2	1	2	5	0.5
2:4	0	1	0	0	1	0.1
3:1	**1**	**1**	**2**	**0**	**4**	**0.4**
3:2	8	5	4	6	23	2.3
3:3	1	0	1	0	2	0.2
4:3	0	0	1	0	1	0.1
Total	**250**	**250**	**250**	**250**	**1,000**	**100**
Female	**127**	**132**	**136**	**125**	**520**	**52**
Male	**123**	**118**	**114**	**125**	**480**	**48**
***SMN1* variant**	**3**	**2**	**2**	**1**	**8**	**0.8**

Twenty-eight SMA carriers (2.8%) consisting of sixteen males (1.6%) and twelve females (1.2%) were found as summarized in Table 3. It was surprisingly found that most of the carriers were male although the number of the male samples was less than that of the female. Based on the current finding, the carrier frequency rate of SMA in Thai neonates was found at 2.8% or 1:36. Data analysis based on the geographic regions showed that the SMA carrier rate of the Central, the North, the Northeast and the South was 0.8%, 0.6%, 0.9% and 0.5%, respectively. According to the Hardy-Weinberg equation, the calculated frequency of the affected SMA caused by the homozygous deletion of the *SMN1* gene is approximately 1 in 5,102 individuals. The SMA carrier rate found in this study is higher than that reported in Taiwan, of which the carrier rate was about 1:48 (2.1%) and the prevalence of SMA was 1 in 8,968 (Su *et al*., 2011).

Table 3. Frequency of the SMA carriers in Thai neonates regarding to regions.

Sex	**Central**	**Northern**	**Northeastern**	**Southern**	**Total number**	**Percentage (% in total)**	**Carrier Rate**
Female (520)	5	2	3	2	12	1.2	1:43
Male (480)	3	4	6	3	16	1.6	1:30
Total (1,000)	8	6	9	5	28	2.8	1:36

Our finding has made aware of the high prevalence and carrier frequency of SMA disease that is caused by the homozygous deletion of the *SMN1* gene in Thai neonate population. This frequency is corresponding with the data indicated by the World Health Organization that has identified the SMA in 1:6,000 to 10,000 of newborns (Zhu *et al*., 2006; Lai *et al.*, 2005; Frugier *et al*, 2002). Statistical analysis indicated that prevalence of the SMA carriers in the newborns of each region of Thailand was not significantly different. Theoretically, both females and males have an equal chance of being carriers, but in this study the SMA carrier appeared in males more than females. Further study with a larger number of study population is needed to verify this finding.

In conclusion, SMA was relatively prevalent in Thai population and it could be considered as a national health problem. The results of this study may be helpful to initiate a national program for clinical, prenatal diagnosis and genetic counseling to effectively prevent SMA in Thailand.

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