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ORIGINAL ARTICLE

Correlation of *PLS3* expression with disease severity in children with spinal muscular atrophy

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Spinal muscular atrophy (SMA) is a common autosomal recessive neuromuscular disease in children caused by homozygous deletion of the survival motor neuron 1 gene (*SMN1*). Plastin 3 (*PLS3*) has been identified as a protective modifier of SMA. We analyzed the levels of *PLS3* expression in peripheral blood from 65 children with SMA and 59 healthy controls by using real-time PCR. In healthy controls, younger children ($\leqslant$3 years) showed $>$1.75-fold higher levels of *PLS3* expression than did older child cohorts ($\sim$3–6 years, $\sim$6–12 years and $>$12 years). In the older female subjects with SMA ($>$3 years), *PLS3* expression was 56.7% lower in type II subjects than in type III patients ($P=0.011$). When these female subjects carried three copies of *SMN2*, *PLS3* expression was 62.6% lower in the type II subjects than in type III subjects ($P=0.008$). Moreover, there was a trend toward higher *PLS3* expression in older female patients who could walk unaided ($>$3 years and *SMN2* copy number = 3) than those who could not. However, these differences were not observed in male subjects examined by SMA clinical type and *SMN2* copy number ($P>0.05$). We concluded that the *PLS3* gene may have an age- and gender-specific role in the clinical severity of SMA in children afflicted with this condition.
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INTRODUCTION

Spinal muscular atrophy (SMA) is a common autosomal recessive neurodegenerative disorder with a prevalence of $\sim$1/10 000; it primarily presents with progressive muscular weakness and atrophy resulting from degeneration of the anterior horn cells in the spinal cord. Childhood onset SMA are usually classified into three types: SMA type I (MIM no. 253300), SMA type II (MIM no. 253550) and SMA type III (MIM no. 253400), by increasing age at onset and decreasing clinical severity.

The survival motor neuron 1 (*SMN1*) is the primary disease-causing gene. Over 90% of patients with SMA are homozygous for a deletion of *SMN1*, whereas 2–5% of patients are compound heterozygotes for an *SMN1* deletion and an *SMN1* intragenic mutation.[1] The *SMN2* gene, a nearly identical copy to *SMN1*, is considered a phenotypic modifier of SMA. *SMN2* copy number is inversely correlated with disease severity.[2,3] However, this correlation is not absolute, and other genetic factors also appear to modify the SMA phenotype.

Plastin 3 (*PLS3*) has been identified as a gender-specific SMA modifier, first reported by Oprea *et al.*[4] It was found that *PLS3* was expressed at higher levels in asymptomatic female subjects with homozygous deletions in *SMN1* when compared with their SMA-affected male siblings. Moreover, a study involving patients with SMA showed that *PLS3* may ameliorate SMA phenotype in older postpubertal female patients.[5]

We analyzed *PLS3* expression in 65 children with SMA and 59 healthy controls to determine whether the protective effect of *PLS3* was also fulfilled in our cases.

MATERIALS AND METHODS

Subjects

In this study, 65 subjects with SMA and 59 healthy volunteers without neuromuscular symptoms were enrolled in the Department of Medical Genetics in the Capital Institute of Pediatrics (Beijing, China). All subjects with SMA were diagnosed by molecular analysis based on homozygous exon 7 deletion in the *SMN1* gene. Informed consent was obtained from the minors' parents and the study protocol was approved by the Ethical Committee of the Capital Institute of Pediatrics.

DNA extraction and *SMN* copy number

Genomic DNA was isolated from whole blood leukocytes by using the proteinase K phenol/chloroform method. The *SMN1* and *SMN2* copy numbers were determined by multiplex ligation-dependent probe amplification (MRC-Holland, Amsterdam, Netherlands), according to the manufacturer's instruction.

RNA extraction and *PLS3* expression level

Total RNA was extracted from peripheral blood by using RNA simple Total RNA Kit (Tiangen, Beijing, China). Total RNA (500 ng) was subjected to reverse transcription for 60 min at 37 °C using 3.75 mM of random primers (Sangon, Shanghai, China) and 2U of M-MLV reverse transcriptase

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(Invitrogen, Carlsbad, CA, USA) in a 40 μl volume. We performed real-time PCR to evaluate the expression level of *PLS3*. *PLS3* cDNA was amplified using the primer pair: 5′-TGGCTACCACTCAGATTTCCAA-3′ and 5′-GAATC CGTTGCTGTTGAGATCA-3′. For *PPIB* (peptidylprolyl isomerase b) cDNA, an endogenous reference primer pair 5′-ATGATCCAGGGCGGAGACTT-3′ and 5′-GGAAGCGCTCACCGTAGATG-3′ was used. Subsequently, 10-fold dilutions of purified PCR products from a control fibroblast cell line were used to construct an external standard curve for *PLS3* and *PPIB*. The relative standard curve method was used to quantify *PLS3* expression. The 25 μl real-time PCR reaction included 1 × SYBR Green PCR mixture (Kangwei, Beijing, China), 5 μl of *PLS3* cDNA and 0.25 pmol μl^{-1} of each primer. Real-time PCR conditions were 95 °C for 1 min as a denaturation step, and 40 cycles of 95 °C for 15 s and 64 °C for 1 min. The quantities of *PLS3* and *PPIB* were determined by the appropriate standard curve. Each sample was run in triplicate.

Statistical analysis

The levels of *PLS3* expression in different groups were compared using Student's *t*-test or ANOVA when appropriate. Statistical analyses were performed by the software package SPSS 19.0 for Windows (SPSS Inc., Chicago, IL, USA). A value of $P<0.05$ was considered to be statistically significant.

RESULTS

To determine whether *PLS3* expression was associated with SMA phenotypes in children, we examined the level of *PLS3* in 59 healthy controls and 65 subjects with SMA types I, II and III. The characteristics of subjects were summarized in Table 1. Consistent with findings of the previous study, expression of *PLS3* was very low in peripheral blood.[4]

Interestingly, we observed a relationship between age and *PLS3* expression in controls. Stratification by age showed that younger children (⩽3 years) had >1.75-fold higher levels of *PLS3* expression than did older child cohorts. However, *PLS3* expression was comparable among the groups of preschool-aged children (~3–6 years), school-aged children (~6–12 years) and adolescents (>12 years) (Figure 1). It was surmised that children showed higher expression at earlier stages of development, which then decreased with age and gradually stabilized. In addition, the level of *PLS3* was 2-fold and 1.7-fold higher in SMA groups of preschool-aged children (~3–6 years) and school-aged children (~6–12 years) than in controls, respectively ($P=0.001$ and $P=0.01$, respectively) (Table 2).

In the absence of age or gender stratification, no differences in expression of *PLS3* were observed among SMA clinical types ($P>0.05$). A cutoff of 3 years was chosen to avoid bias because of the amount of *PLS3* expression, which significantly decreased after this age. When stratified by age (⩽3 vs >3 years) and gender, differences in *PLS3* expression by SMA type were observed in female subjects alone. However, male subjects in both age groups showed no difference in *PLS3* expression when examined by SMA type ($P>0.05$). In older female subjects (>3 years), *PLS3* expression was 55.6 and 56.7% lower in age-matched controls (mean age ± s.d., year: 6.24 ± 2.31) and type II patients (5.82 ± 0.77) than that in type III subjects (7.17 ± 2.80), respectively (Figure 2; $P=0.001$ and $P=0.011$, respectively), with comparable age distribution ($P=0.338$). Moreover, in older female patients with type III, 10 of them could walk unaided but 3 could not. Although no correlation was found between *PLS3* expression and phenotype in older female SMA type III patients ($P=0.261$), there was a trend that *PLS3* expression of patients who could walk independently (mean ± s.d., 3.75 ± 1.5) was higher than those of patients who could not (2.68 ± 0.4).

Among the 65 subjects with a *SMN1* homozygous deletion, the distributions of *SMN2* copy number were as follows: 50 subjects with three copies (76.9%), 14 subjects with two copies (21.5%) and 1 subject with four copies (1.5%). No difference was observed in *PLS3* expression divided by *SMN2* copy number (Table 1). To eliminate the modification of *SMN2* to SMA phenotypes, we analyzed the *PLS3* expression in the subjects carrying the same *SMN2* copy number. In the 22 female subjects with three copies of *SMN2*, *PLS3* expression was 62.6% lower in the type II subjects than in the type III subjects (Figure 3; $P=0.008$). However, in the six female patients with two copies of *SMN2*, five were type I and only 1 was type II. Thus, stratification analysis could not be applied. Moreover, this difference in *PLS3* expression was not observed in male patients with three or two copies of *SMN2*.

Table 1 Characteristics of subjects and *PLS3* expression

Characteristic	*Male*	*Female*
Group (%)		
Control	31 (52.5)	28 (47.5)
SMA I	12 (63.2)	7 (36.8)
SMA II	12 (57.1)	9 (42.9)
SMA III	12 (48)	13 (52)
Age (mean ± s.d., year)		
Control	6.97 ± 5.49	8.45 ± 4.04
SMA I	1.69 ± 2.09	1.34 ± 1.94
SMA II	4.27 ± 3.05	4.21 ± 2.03
SMA III	5.36 ± 3.50	6.21 ± 3.03
SMN2 copy (%)		
2	8 (57.1)	6 (42.9)
3	28 (56)	22 (44)
4	0	1
PLS3 expression[a] *(mean ± s.d.)*		
Controls	2.30 ± 1.49	2.11 ± 1.03
SMA I	3.51 ± 1.56	2.63 ± 0.79
SMA II	3.21 ± 1.87	2.45 ± 0.73
SMA III	3.59 ± 1.80	3.50 ± 1.39
SMN2 copy		
2	3.69 ± 1.52	2.79 ± 0.95
3	3.36 ± 1.77	3.05 ± 1.25

[a]The amount of *PLS3* expression is calculated by *PLS3*/*PPIB* × 1000.

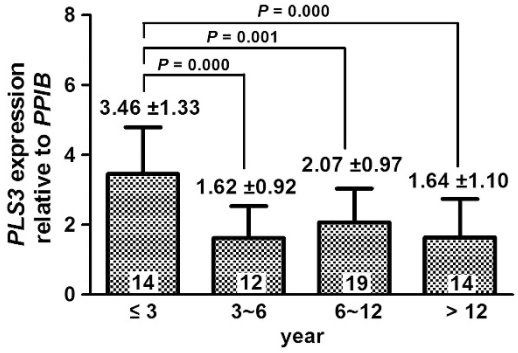

Figure 1 *PLS3* expression in controls. The younger children (⩽3 years) had higher levels of *PLS3* expression than did in ~3–6-year group ($P=0.000$), ~6–12-year group ($P=0.001$) and >12 year ($P=0.000$), respectively. Date are plotted as (mean ± s.d.) × 1000, and the number of each group (*n*) is shown within each bar.

Table 2 ***PLS3*** **expression in controls and SMA patients**

Group	*⩽3 years (n, mean ± s.d.)*	*~3–6 years (n, mean ± s.d.)*	*~6–12 years (n, mean ± s.d.)*	*12 years (n, mean ± s.d.)*
Controls	(14) 3.46 ± 1.33	(12) 1.62 ± 0.92	(19) 2.07 ± 0.97	(14) 1.64 ± 1.10
SMA	(30) 3.14 ± 1.50	(20) 3.17 ± 1.34	(15) 3.49 ± 1.74	—
P-value	0.489	0.001	0.01	—

The amount of *PLS3* expression is calculated by *PLS3*/*PPIB* × 1000, and analyzed for significance by Student's *t*-test.

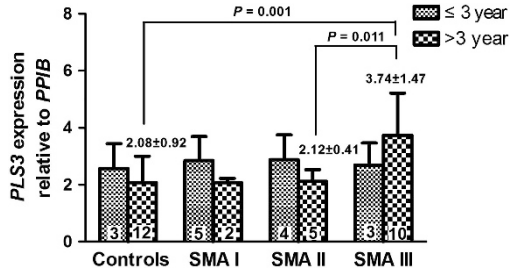

Figure 2 *PLS3* expression in female controls and patients. In older female subjects (>3 year), *PLS3* expression was higher in type III patients than that in type II patients ($P=0.011$) and controls ($P=0.001$), respectively. In older female controls, the age distribution were from >3 year to ⩽10 year in order to match the age of the control group and SMA groups. Date are plotted as (mean ± s.d.) × 1000, and the number of each group (*n*) is shown within each bar.

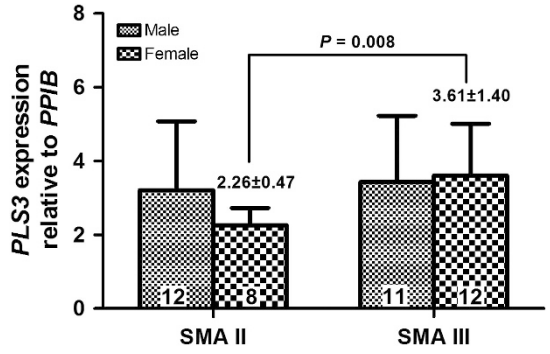

Figure 3 *PLS3* expression in SMA patients with three *SMN2* copies. In female patients with three *SMN2* copies, *PLS3* expression was lower in the type II than in the type III ($P=0.008$). The age distribution (mean ± s.d., year) of the female groups were comparable (4.54 ± 1.87 in type II, 6.47 ± 3.00 in type III, $P=0.125$). Date are plotted as (mean ± s.d.) × 1000, and the number of each group (*n*) is shown within each bar.

DISCUSSION

SMA is a debilitating fatal neuromuscular disease in children, characterized by the degeneration of the anterior horn cells of the spinal cord due to reduced expression of the SMN protein. *PLS3* is an isoform of a family of actin-binding proteins involved in the stability and organization of F-actin filaments. Two studies in humans have also shown a correlation between the amount of *PLS3* and SMA severity in female subjects.[4,5] Further studies in SMA cell and zebrafish models showed that high *PLS3* levels can increase the amount of F-actin in motor neurons, resulting in improved axon outgrowth.[4] Therefore, *PLS3* should be considered as a protective SMA modifier.

To determine whether *PLS3* caused a phenotype modification in children with SMA, we analyzed the level of *PLS3* expression in peripheral blood among 65 subjects with SMA type I, II and III and 59 healthy controls. We found that *PLS3* was expressed at very low levels in blood cells, which was consistent with other studies.[4,5] However, *PLS3* was expressed at higher levels in preschool-aged and school-aged subjects with SMA than in age-matched healthy controls, suggesting that a relatively higher expression of *PLS3* in subjects with SMA may act as a compensatory mechanism in humans. Moreover, levels of *PLS3* expression in blood may be associated with age, because we found that children showed higher expression levels at early stages of development (⩽3 years), and these levels decreased with age and gradually stabilized.

The modification of *PLS3* in subjects with SMA was first reported by Oprea *et al*.[4] This study showed that asymptomatic female subjects with *SMN1* deletion exhibited significantly higher expression levels of *PLS3* than did their SMA-affected counterparts. Moreover, female subjects with higher *PLS3* expression sporadically presented with milder phenotypes, which indicated that *PLS3* is a gender-specific modifier of SMA. Another study performed by Stratigopoulos *et al*.[5] showed an inverse correlation between *PLS3* expression in blood and SMA severity in older postpubertal female patients (mean age, 20 years). In younger prepubertal female patients (mean age, 5 years), *PLS3* expression levels were higher in type III than in type II female subjects with SMA only. In our study, we found that in the group of children older than 3 years (mean ± s.d., 6.40 ± 2.41 years), type III female subjects showed higher expression levels of *PLS3* than did type II female subjects. However, no difference was observed in the group of children below 3 years of age. Notably, the SMA subjects that we focused on were all children and the overall age was relatively lower than that of subjects examined by Stratigopoulos *et al*.[5] In our study, only one postpubertal female subject was included. However, in the study by Stratigopoulos *et al*.,[5] postpubertal female subjects accounted for 38% of all female subjects. Moreover, they found that *PLS3* expression levels were higher in younger patients, regardless of gender. Because expression of *PLS3* may be associated with age, this expressed difference among clinical types in younger subjects may be covered, especially in earlier-onset type I patients.

Further, we analyzed the amount of *PLS3* in the subjects with the same number of *SMN2* copies in order to rule out the potential impact of *SMN2* dosage. The data showed that in the presence of three copies of *SMN2*, expression of *PLS3* was higher in type III female subjects than in type II female subjects. Notably, in our study, there was a trend that *PLS3* expression of patients who were able to walk unaided was higher than those of patients who were unable to walk unaided, although no significant difference of *PLS3* expression was observed between these two groups. It will be necessary to verify the correlation between *PLS3* expression and phenotype in a lager sample. In a word, our data suggested that *PLS3* may function as an age and/or gender-specific phenotype modifier in SMA.

A potential association between *PLS3* and SMN was observed in a zebrafish SMA model described by Hao le *et al*.[6] However, more recent data showed that increased *PLS3* expression did not affect SMN levels, as was previously shown in humans, mice and zebrafish,

suggesting that *PLS3* exerts its modifying effects independently of SMN.[7] *PLS3* plays an important role in neuromuscular junction development, maturation, integrity and function by delaying axon pruning, resulting in amelioration of the SMA phenotype.

In view of the gender-specific protection of *PLS3* in humans and in combination with the data demonstrating that over-expressed *PLS3* in both zebrafish and mice could rescue the neuromuscular defects and corresponding movement phenotypes caused by low SMN levels, *PLS3* is suggested as a putative target for SMA therapy.[6,7] Nevertheless, other modified factors, such as zinc finger protein,[8] methylation status[9] and experimental variables, should also be considered in any final analysis, for their potential to function cooperatively to affect the final phenotype in a patient. An investigation of these modifiers will help in understanding the mechanism underlying SMA pathology.

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