Association of Plastin 3 Expression With Disease Severity in Spinal Muscular Atrophy Only in Postpubertal Females

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**Objective:** To investigate the potential association of plastin 3 (PLS3) expression levels in the blood with disease severity in spinal muscular atrophy (SMA).

**Design:** Measurement of PLS3 messenger RNA levels in the blood of patients with types I, II, and III SMA.

**Setting:** Pediatric Neuromuscular Clinical Research Network SMA Natural History study.

**Participants:** A cohort of 88 patients of both sexes who had SMA.

**Main Outcome Measures:** Levels of PLS3 messenger RNA in relation to SMA type and SMN2 copy number.

**Results:** Prepubertal female and younger male (<11 years) patients had approximately 2-fold-higher levels of PLS3 expression than did postpubertal female and older male (≥11 years) patients, respectively (P ≤ .001). Expression of PLS3 in male patients did not correlate with SMA clinical type or SMN2 copy number in either age group (P > .10). In postpubertal female patients, PLS3 expression was greatest in patients with type III SMA, was intermediate in patients with type II SMA, and was lowest in patients with type I SMA. Expression of PLS3 correlated with SMA type, SMN2 copy number, and the gross motor function measure only in postpubertal female patients.

**Conclusion:** The PLS3 gene may be an age- and/or puberty-specific and sex-specific modifier of SMA.

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**Sample Collection**

Subjects were enrolled in the Pediatric Neuromuscular Clinical Research (PNCR) Network SMA Natural History study after approval from the institutional review boards of The Children’s Hospital of Philadelphia, Boston Children’s Hospital, Columbia University, and the University of Rochester. Subject characteristics are given in the Table. In brief, at study entry, patients were clinically evaluated electrophysiologically and by using measures of motor function. The gross motor function measure (GMFM), an 88-item test measuring motor function that has been validated for SMA types II and III, was administered to type II and type III SMA patients. Evaluator training and reliability testing had been performed by the PNCR clinical evaluators before data collection. Electrophysiological measurement of the com-

**METHODS**

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Table. Characteristics of 88 Patients With SMA

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Male Patients</th>
<th>Female Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;11 y (n=29)</td>
<td>≥11 y (n=12)</td>
</tr>
<tr>
<td>Age, mean, y</td>
<td>3</td>
<td>15</td>
</tr>
<tr>
<td>SMA type, No. (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>12 (41)</td>
<td>2 (17)</td>
</tr>
<tr>
<td>II</td>
<td>13 (45)</td>
<td>2 (17)</td>
</tr>
<tr>
<td>III</td>
<td>4 (14)</td>
<td>8 (67)</td>
</tr>
<tr>
<td>SMN2 copy, No. (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>9 (31)</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>18 (62)</td>
<td>8 (67)</td>
</tr>
<tr>
<td>4</td>
<td>2 (7)</td>
<td>4 (33)</td>
</tr>
<tr>
<td>GMFM by SMA type, mean</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>II</td>
<td>21</td>
<td>14</td>
</tr>
<tr>
<td>III</td>
<td>72</td>
<td>46</td>
</tr>
<tr>
<td>Maximum CMAP by SMA type, mean, mV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>358</td>
<td>345</td>
</tr>
<tr>
<td>II</td>
<td>1804</td>
<td>1134</td>
</tr>
<tr>
<td>III</td>
<td>5524</td>
<td>5077</td>
</tr>
<tr>
<td>MUNE by SMA type, mean</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>II</td>
<td>22</td>
<td>13</td>
</tr>
<tr>
<td>III</td>
<td>81</td>
<td>60</td>
</tr>
</tbody>
</table>

Abbreviations: CMAP, compound motor action potential; GMFM, gross motor function measure; MUNE, motor unit number estimate; SMA, spinal muscular atrophy.

a Because of rounding, percentages may not total 100.

pound motor action potential (CMAP) of the ulnar nerve and calculation of the motor unit number estimate (MUNE) followed the procedure described by Swoboda et al. Reliability training of the clinical evaluators was performed before initiating the study. Blood was drawn at the PNCR study sites and shipped by express delivery from the remote sites to the molecular core laboratory. Genomic DNA was isolated from blood using a kit (Puregene DNA Isolation Kit; Qiagen, Germantown, Maryland). The SMA diagnosis was molecularly confirmed by detecting the homozygous exon 7 deletion in SMN1 by a polymerase chain reaction (PCR) restriction digest with DraI. With the use of SMN2-specific primers and primers for the control gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH), the SMN2 copy number was quantified by real-time PCR using a thermal cycler platform (LightCycler; Roche Diagnostics Corp, Indianapolis, Indiana) in which the intensity of fluorescence was detected in real time. The hybridization probes had an annealing temperature of 58°C with fluorescence detection at the annealing phase. Crossing points were determined and compared with GAPDH. Control samples with known SMN2 copy numbers were used to determine the relative SMN2:GAPDH ratio for each genotype. Melting curve analysis was used to ensure the specificity of the PCR product. Each sample was run in triplicate. SMN2 copy number was independently determined using a real-time TaqMan assay with samples run in duplicate. Patients were clinically categorized by type of SMA on the basis of the most advanced motor milestones achieved (sitting for type II and walking for type III).

ISOLATION OF TOTAL MESSENGER RNA AND COMPLEMENTARY DNA SYNTHESIS

Total messenger RNA (mRNA) was extracted from whole blood and treated with deoxyribonuclease using the PAXgene Blood RNA System (PreAnalytiX GmbH, Franklin Lakes, New Jersey) according to the manufacturer’s instructions. Complementary DNA (cDNA) synthesis from 1 µg of total mRNA was performed at 50°C for 50 minutes using a synthesis system (SuperScript III First-Strand Synthesis System; Invitrogen, Carlsbad, California) with oligo (dT)18 primers. Subsequently, the reverse transcriptase was inactivated at 85°C for 5 minutes, and the sample was treated with ribonuclease H for 20 minutes at 37°C.

QUANTITATIVE PCR

Transcript quantification was performed on the thermal cycler platform using a kit (LightCycler FastStart DNA Master SYBR Green I; Roche Diagnostics Corp) according to the manufacturer’s instructions. Samples were heated at 95°C for 10 minutes, followed by 40 cycles of 93°C for 15 seconds, 62°C for 10 seconds, and 72°C for 12 seconds. For the amplification of the endogenous control genes GAPDH and beta actin (ACTB), the annealing temperature was 60°C. Each 20-µL reaction contained 2.5 µL of cDNA previously diluted to a 1:4 ratio, 1.6mM magnesium chloride, 3µM of each primer, and the recommended amount of the SYBR Green I mix (Roche Diagnostics Corp). Primers for the endogenous control genes used were as follows: for GAPDH, 5’-GCAGTGGAAGTAATTGTGC and 5’-CCCGTTGATGACAAGCTTCCCATTC, and for ACTB, 5’-TCTGGTGATCCACCATCTGCCAG and 5’-TGGAAGGTTGACAGTGAGGCCAG. The PLS3 primers amplified part of exon 13 (5’-CTCTGAATGATGGGAACCAAACCCTG), exon 14, and part of exon 13 (5’-CCACAACCTGCCAAACTTGAGCTG).

Transcript quantification was performed using the automated absolute quantification mode of the thermal cycler software (LightCycler Software; Roche Diagnostics Corp). Standard curves were calculated for each set of primers using cDNA from patient mRNA. The PLS3-specific primer set had an amplification efficiency of approximately 1.8. The crossing point was defined as the first maximum of the second derivative of the fluorescence curve.

Transcript levels of GAPDH and ACTB showed only modest variation (1%-5%) among patients studied. We used the geo-
metric mean of GAPDH and ACTB to normalize PLS3 expression in each sample.

STATISTICAL ANALYSIS

Levels of PLS3 expression were compared among groups using Mann-Whitney nonparametric tests (StatView 5.0; SAS Institute Inc, Cary, North Carolina). Correlation analysis was performed using Statistica 6.0 software (StatSoft Inc, Tulsa, Oklahoma). All analyses were performed using a 5% significance level (2-tailed).

RESULTS

Expression of PLS3 was assessed in 88 male and female SMA types I, II, and III patients. Without age stratiﬁcation, PLS3 expression levels were comparable across SMA
type or SMN2 copy number for male and female patients \( (P > .10) \). However, stratification by age and sex demonstrated differences in PLS3 expression by SMA type or SMN2 copy number but only in female patients. Similarly, stratification by pubertal status defined by age at menarche showed that postpubertal female patients had 64% lower levels of PLS3 expression than did prepubertal female patients \( (P < .001) \) (Figure, A). In the younger prepubertal female patients, PLS3 expression was 45% lower in the prepubertal type II patients compared with prepubertal type III patients (Figure, B) \( (P = .04) \). There was no difference in PLS3 expression in prepubertal female patients by SMN2 copy number. There were significant differences in PLS3 expression in older postpubertal female patients by SMA type and SMN2 copy number. Expression of PLS3 in postpubertal type II female patients was 56% lower than that in postpubertal type III female patients \( (P = .03) \) (Figure, B). In addition, PLS3 expression in postpubertal type I female patients was 62% \( (P = .02) \) and 83% \( (P < .001) \) lower than those in postpubertal type II and III female patients, respectively (Figure, B). Similarly, PLS3 expression in 14 postpubertal female patients with 3 SMN2 copies was 54% \( (P = .02) \) lower than that in 2 female patients with 4 SMN2 copies. There was a trend toward lower PLS3 expression levels in postpubertal female patients with 2 SMN2 copies compared with those with 3 SMN2 copies.

When divided by age \( (<11 \text{ vs } \geq 11 \text{ years}) \), older male patients had 51% lower levels of PLS3 expression (Figure, A). Male patients in both age groups demonstrated no difference in PLS3 expression when analyzed by SMA clinical type or SMN2 copy number \( (P > .10) \); data not shown).

Expression of PLS3 in postpubertal female patients was positively correlated with the GMFM \( (r = 0.83) \) (Figure, C), but this correlation was not observed in prepubertal female or male patients of any age. Expression of PLS3 was not strongly correlated with electrophysiological measures, the maximal CMAP, or the MUNE calculation for male or female patients regardless of age, SMA type, or SMN2 copy number \( (r < 0.4) \).

Understanding the modifiers of SMN2 genotype may suggest therapeutic targets for the treatment of SMA. An example is seen in the recent finding that an SMN2 variant, c.859G>C, promotes exon 7 inclusion, suggesting that not all SMN2 copies are comparable as well as revealing a new therapeutic target to increase SMN2 expression in SMA patients. In addition, the development of informative biomarkers for SMA is important for future clinical trials. We studied PLS3 transcript levels in whole blood of a large series of 88 types I, II, and III SMA patients characterized for SMN1 and SMN2 genotype, GMFM, CMAP, and MUNE to determine whether there were any correlations between PLS3 expression and the clinical phenotype.

Oprea et al reported that asymptomatic female individuals with homozygous mutations in SMN1 had higher PLS3 expression levels than did symptomatic male first-degree relatives matched on SMN2 copy number. Notably, all these individuals were adults aged 26 to 55 years. In a group of 101 symptomatic SMA patients, Oprea et al found that the 3 female patients for whom clinical data were available and in whom PLS3 was highly expressed were all adults aged 22 to 53 years and had a mild phenotype or slowly progressing SMA despite SMN2 copy numbers of 2 and 3. In contrast, all male patients in whom PLS3 was highly expressed (ages not reported) displayed the expected phenotype for the SMN2 copy number. We found that PLS3 transcript levels were higher in younger patients regardless of sex. Transcript levels of PLS3 in types I, II, and III older, postpubertal female SMA patients were inversely correlated with disease severity and positively correlated with SMN2 copy number and GMFM. The failure to observe an association of PLS3 expression with maximal CMAP or MUNE may reflect the decreased reliability of these measures, especially across multiple clinical sites. In younger prepubertal female patients, PLS3 transcript levels were higher in type III compared to type II female SMA patients only. Our findings support those of Oprea et al and further suggest that PLS3 may be an age- and/or puberty-specific and sex-specific modifier of SMA because PLS3 expression levels were associated across all SMA types and SMN2 copy numbers only in older postpubertal female patients and could be related to hormonal interactions. Replication of the association of increased PLS3 expression in more mildly affected postpubertal female patients in addition to data demonstrating that PLS3 colocalizes with SMN in granules of motor neuron axons and can increase neurite length in SMN-deficient cells suggests that it could be a target for modifying SMA disease severity. The fact that PLS3 maps to Xq23 may account in part for the sex-specific effect. Additional studies will be necessary to further elucidate the mechanism for these associations. However, because the association of PLS3 expression with SMA type, SMN2 copy number, and GMFM was apparent in only a subset of the total SMA population, it is unlikely that PLS3 expression alone will be a generalizable biomarker for SMA in most clinical trials.

**COMMENT**

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**Author Contributions:** Dr Chung had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. **Study concept and design:** DeVivo and Chung. **Acquisition of data:** Stratigopoulos, Lanzano, Deng, Guo, Kaufmann, Darras, Tawil, Martens, and Chung. **Analysis and interpretation of data:** Stratigopoulos, Kaufmann, Finkel, Tawil, McDermott, Martens, DeVivo, and Chung. **Drafting of the manuscript:** Stratigopoulos, Guo, DeVivo, and Chung. **Critical revision of the manuscript for important intellectual content:** Stratigopoulos, Lanzano, Deng, Kaufmann, Darras, Finkel, Tawil, McDermott, Martens, DeVivo, and Chung. **Statistical analysis:** Stratigopoulos and McDermott. **Obtained funding:** Kaufmann, Darras, DeVivo, and Chung. **Administrative, technical, and mate-
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REFERENCES


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We plan to utilize videos as part of published papers that highlight and provide convincing information about the observational and visual features of a patient’s neurologic findings. Please refer to Instructions for Authors for instructions on submitting video presentations.