Development of SMA mouse model plasma protein biomarker assays

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Abstract

Several putative SMA plasma protein biomarkers have been reported, based on association with motor function. These proteins have been identified as potential markers of disease state and progression, and may serve as pharmacodynamic (PD) markers that respond to SMN changes in target tissues. With data from SMA clinical trials pending, SMA mouse models provide a useful screening tool for PD markers.

Using human SMA plasma protein markers as a starting point, 10 mouse biomarker immunoassays were developed and optimized. This mouse biomarker panel provides a useful tool for screening genotypic differences in SMA mice as well as potentially identifying which markers, if any, respond to SMN upregulating drug candidates.

Plasma protein biomarker candidates identified as hits from SMA biomarker analyses were selected for development of mouse immunoassays. Using commercially available antibodies and protein standards, singleplex immunoassays were developed with Meso Scale Discovery (MSD) technology. Quantitative immunoassays were developed for the following biomarker proteins: AXL, COMP, DPP4, Fetuin A, IGF1, Osteopontin, Tetranectin, Vitronectin, Cadherin 13 (heart) and YKL-40. The dynamic range of the resultant assays spanned 3-5 units on the log scale using standards in assay buffer. Similar results were observed when measuring standards in normal mouse plasma. Detected concentrations of endogenous protein in the plasma ranged from pg/mL to µg/mL levels using different plasma dilutions. Genotypic differences were also observed among biomarker proteins when plasma from wild type and C/C mild-model mice was evaluated; several analytes were significantly related to SMN dosage. Assays that performed in a sensitive and quantitative manner using purified standards and which detected the protein of interest in normal mouse plasma were converted to MSD multiplex format.

Using SMA biomarker data from human studies, we have produced sensitive and dose responsive immunoassays for use in studies employing mouse SMA models. These assays provide a tool for monitoring biochemical changes resulting from changes in SMN levels. The assays may also serve to identify analytes responsive to pharmacodynamic SMN changes in disease-relevant target tissues. Our next steps will be to evaluate these biomarkers against SMN protein levels in target tissues in C/C mice, to examine plasma from the more severe phenotype Delta7 mice to determine if analytes can be robustly detected in cerebrospinal fluid, and to measure plasma responses in C/C mice treated with SMN upregulating small molecules and other therapeutic approaches.

Materials and methods

Biomarker selection

The plasma protein biomarkers were selected as a result of the Biomarkers for SMA (BforSMA) clinical study (Finkel et al., 2012 & Crawford et al., 2012) that identified 97 potential biomarkers as strong predictors of disease severity in SMA patient plasma samples. The 10 plasma protein biomarkers (**Table 1**) that were ultimately selected for this study are included in a human biomarker assay panel, SMA-MAP, of 27 markers co-developed by the SMA Foundation and Myriad-RBM.

Assay Protocol

Protein standards and antibody pairs for each protein were previously identified for use in the Meso Scale Discovery assay format. A description of the biomarker characteristics are provided in **Table 2**.

Antibodies were labeled using protocols provided by Meso Scale Discovery (MSD). Yield of antibody protein was estimated via BCA protein assay.

Pre-wetted MSD standard plates were solution coated with 30 µl of diluted capture antibody solution and incubated overnight at 4 °C. All washing steps were 3X using 200 µL of Tris buffered saline, 0.05% Tween 20 pH 7.5 (TTBS). Prior to addition of sample plates were blocked using 5% BSA, 1X PBS, 0.05% Tween 20 (5% Blocker A).

Protein standards, plasma and detection antibodies were diluted into 1% BSA, 1X PBS, 0.05% Tween 20, pH 7.4 (1% Blocker A) except in the case of IGF1 where plasma was diluted into 1% Blocker A, 5% Tween 20. Standards and diluted plasma samples (25 µl) were incubated for 2h at room temperature with shaking at 650 rpm. Following incubation and wash steps 25 µL of biotinylated detection antibody + sulfotagged streptavidin or sulfotagged detection antibody (where applicable) were added to each well and incubated for 1 hour at room temperature with shaking (650 rpm). Following incubation and wash steps 150 µL of 1X Read Buffer-T was added and plates were read on an MSD 2400 instrument.

STUDY 1: Optimization of biomarker singleplex immunoassays

The goal of Study 1 was to develop a new set of assays to investigate levels of proteins.

Figure 1: Biomarker protein standard curves

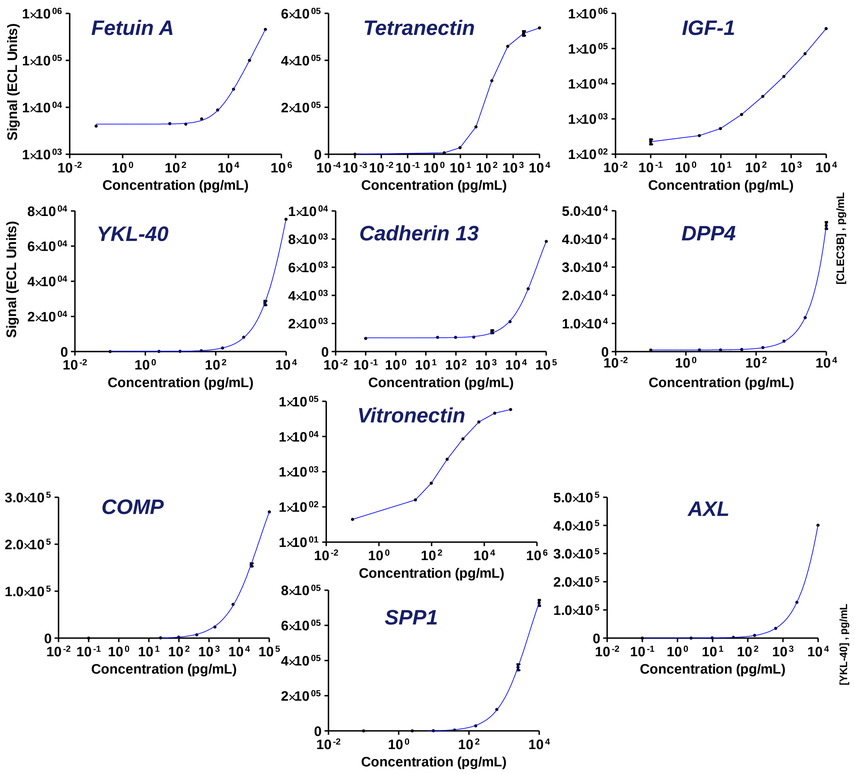

Table 1: Analyte Description

Analyte	Expression pattern	Function
AXL Receptor Tyrosine Kinase, AXL	Ubiquitous, non-nerve	Neuronal migration, control neuronal apoptosis
Cadherin 13, CDH13	Ubiquitous, enriched in connective tissue	Neuronal projection
Cartilage Oligomeric Matrix Protein, COMP	Ubiquitous, enriched in connective tissue	Cartilage damage marker due to acute exercise
Dipeptidyl peptidase 4, DPP4	Ubiquitous, enriched in parathyroid	T-cell activation, glucose homeostasis
FetuinA, AHSG	Somewhat restricted, enriched in liver	Bone resorption, negative bone mineralization
Insulin-like Growth Factor 1, IGF1	Ubiquitous, enriched in uterus, liver, adipocyte, ovary, fetal tissues	CNS development, muscle generation/regeneration
Osteopontin, SPP1	Ubiquitous	Neurite outgrowth, axonal sprouting post-injury
Tetranectin, CLEC3B	Somewhat restricted expression, not in nerve	Skeletal system development
Vitronectin, VTN	Ubiquitous, enriched in liver tissue	promotes cell adhesion and spreading, hemostatis; tumor malignancy
Chitinase 1 Like 3, CHI3L1 or YKL40	Ubiquitous, enriched in connective tissue	Choroid expression

STUDY 2: Evaluation of biomarker protein levels in Delta7, Taiwanese, Burgheron SMA mice plasma

The goal of Study 2 was to evaluate plasma protein levels from wild type (WT), heterozygous (HET) and homozygous (HOM) in 3 SMA model mice in a singleplex assay using the Meso Scale Discovery platform.

Plasma samples (36 total) from three SMA mouse model systems were evaluated for levels of ten SMA biomarkers in singleplex assays **(Figure 2)**. The Delta7 (hSMN2+/+, hSMN2Δ7+/+, Smn-/-) represents a severe type SMA phenotype and the Taiwanese SMA Type 3 (Smn-/-,SMN2) and Burgheron models (Smn-/- SMN2+/+ mice and C-allele) represent mild SMA phenotypes.

Statistical analyses (Newman-Keuls Multiple Comparison Test) were performed on the resultant data comparing SMA mouse model groups. Statistically significant differences were observed among and between strains dependent on the biomarker and the strain comparator. Statistical analysis of Cadherin plasma levels was not attempted due to the highly variable nature of the assay results.

Figure 2: Plasma protein levels in Delta7, Taiwanese & Burgheron mice

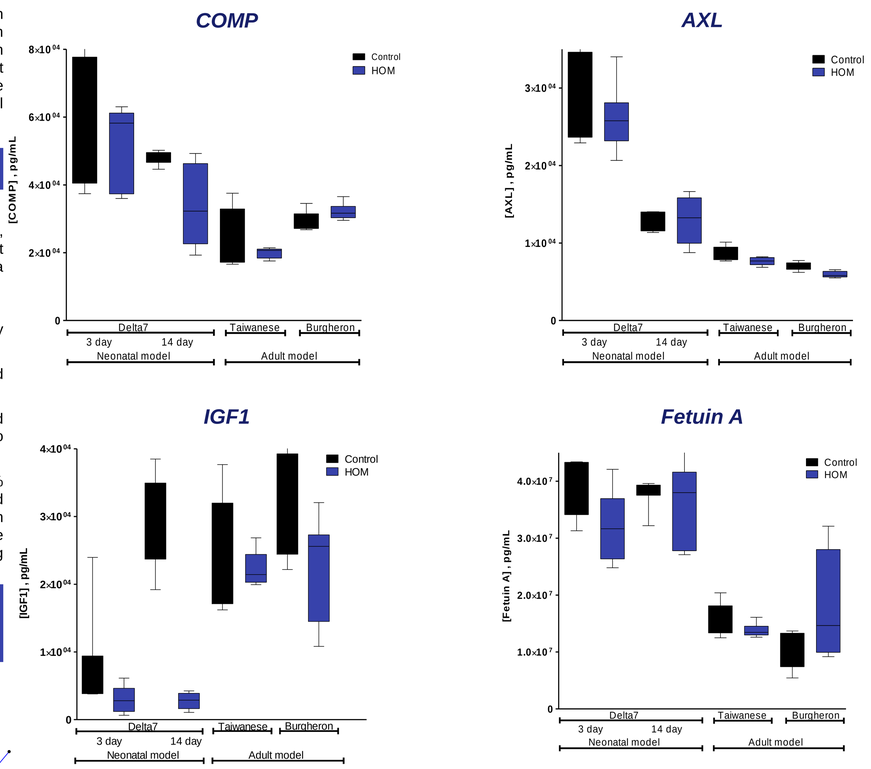

Table 2: Biomarker Characteristics

Plasma protein	% Amino acid identity mouse:human	% Amino acid length mouse/hum	Est. levels in mouse plasma
Fetuin A	58.8	345/367	10-50 µg/mL
Tetranectin	79.2	202/202	1-20 µg/mL
IGF1	65.6	153/195	100-400 ng/mL
Cadherin	93.4	714/713	1-20 ng/mL
Vitronectin	74.2	478/478	10-70 µg/mL
YKL-40	72.9	381/383	100-400 ng/mL
DPP4	84.5	760/766	10-100ng/mL
COMP	90.6	755/757	10-100 ng/mL
AXL	86.8	879/894	70-270 ng/mL
SPP1	63.3	294/314	1-5 µg/mL

STUDY 3: Comparison of biomarker protein levels in wild type and homozygous C/C SMA mouse plasma

The goal of Study 3 was to evaluate plasma protein levels from wild type (WT) and C/C homozygous (HOM) mice using singleplex immunoassays developed and optimized by PharmOptima and Meso Scale Discovery.

Plasma samples from 5 wild type and 5 homozygous C/C SMA adult mice were evaluated for levels of 10 biomarker proteins using the optimized singleplex immunoassays (**Figure 3**). Blood samples were centrifuged at 10,000 rpm and clarified plasma was separated and frozen and maintained at -20 °C until assayed.

Using the MSD singleplex optimized assays, samples were evaluated in quadruplicate from two dilutions. The concentrations of the individual biomarkers of wild type mice were compared with those of homozygous C/C SMN mice using an unpaired t test or Mann Whitney test depending on the variance of the data. Significant differences were observed for the following proteins in plasma samples: AXL, IGF1, COMP, DPPIV, and CHI3L1 (**Table 3**).

Figure 3: Plasma protein levels in wild type and homozygous C/C mouse plasma

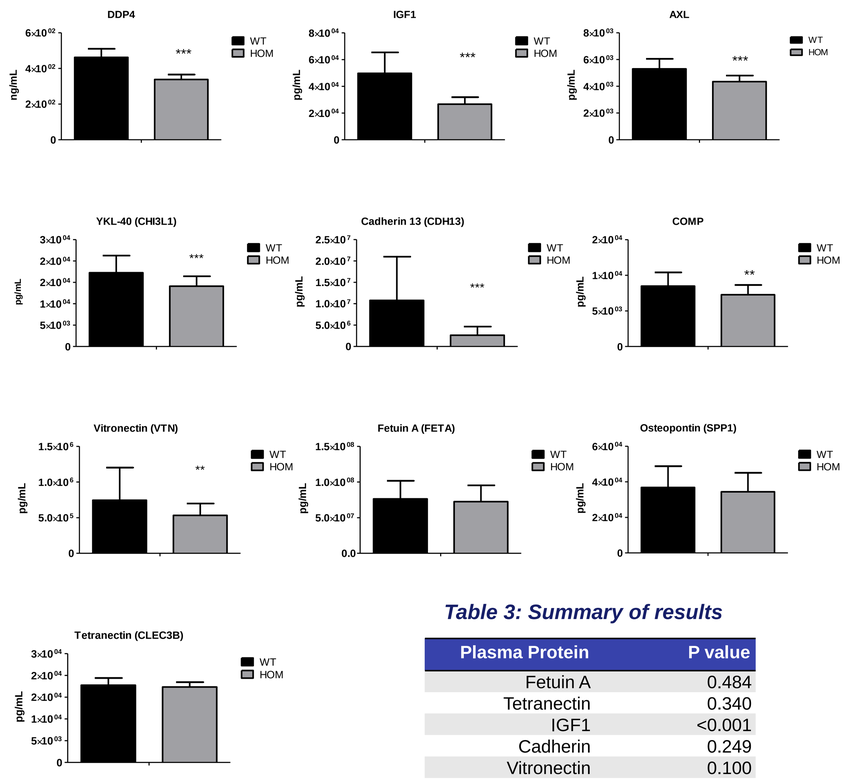

Table 3: Summary of results

Plasma Protein	P value
Fetuin A	0.484
Tetranectin	0.340
IGF1	<0.001
Cadherin	0.249
Vitronectin	0.100
YKL-40	0.0002
DPP4	<0.001
COMP	0.002
AXL	<0.001
SPP1	0.342

STUDY 4: Development and optimization of a multiplex immunoassay biomarker panel using the MSD platform

In Study 4, biomarkers were optimized to run in four-plex and five-plex on seven spot plates. Multiplex plates are being printed and optimization of the multiplex assay is in progress. Preliminary results will be available in July 2012.

Conclusions

Ten biomarker singleplex MSD assays derived from the biomarkers discovered in the BforSMA study were developed for mice. These ten assays were shown to perform in a quantitative and sensitive manner (Study 1), with dynamic ranges spanning 3-5 log units. The assays were able to distinguish between the wild type and homozygous genotypes in the C/C SMA mild mouse model (Study 3), with significant differences in AXL, IGF1, COMP, DPPIV, and YKL-40 levels in wild type and homozygous C/C animals. Several marker plasma levels were different in mouse models that represent severe and mild SMA phenotypes (Study 2).

These assays provide a tool for monitoring biochemical signatures in SMA model mice, and will be explored for responsiveness and relationship to pharmacodynamic SMN changes in disease-relevant target tissues like the spinal cord and muscle.

Next Steps:

- Evaluate plasma biomarkers against SMN protein levels in target tissues in C/C mice
- Conduct a longitudinal study of analyte levels in C/C mice at various timepoints
- Measure plasma responses in C/C mice treated with SMN upregulating therapeutic approaches
- Complete optimization of a more cost-effective multiplex assay format and make it available to the broader research community

Acknowledgments

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