Introduction

Background: Biomarkers are measures of biological states that are useful in assessing disease severity, designing proof-of-concept trials, tracking therapeutic responses, and generating hypotheses about disease pathophysiology. Biomarker discovery in Spinal Muscular Atrophy (SMA) is related to muscle and nerve function and also assesses neuropathological changes. The goal is to identify new potential protein biomarkers that could aid in prognosis or monitoring treatment efficacy in SMA patients.

Objective: Our objective was to use a multiplex immunoassay panel (MAP) technology to expand on the plasma protein biomarker discovery approach of the prospective Biomarker for SMA (BforSMA) clinical study conducted in 2009 and create a custom biomarker panel for SMA for use in the research and clinical communities.

Strategy: Using two existing Rule-Based Medicine Multi-analyte Profile (MAP) panels comprising 257 markers (DiscoveryMAP and OncologyMAP), SMA and control plasma samples collected from the BforSMA study were analyzed to identify novel plasma protein biomarker candidates that correlate to the Modified Hammersmith Functional Motor Scale (MHFSM) measure of disease severity in SMA patients. New immunomarkers for the best hits identified by LC/MS but not in existing MAPs were also developed.

Results: Testing in the existing MAPs produced 72 new candidate markers. Another 9 markers identified separately in the BforSMA study were reproduced in the MAPs. Working assays for the top 9 BforSMA hits have been produced in singleplex and pilot multiplex formats, and a final panel of the top 35 combined hits will be tested with new SMA plasma samples to verify the candidate markers.

Methods

Data and Samples

The data herein was generated in an effort to identify a marker or panel of markers in plasma from a wide range of SMA patients. The study utilized multiple plasma samples from subjects of varying age, gender, and disease status. The subjects were identified as having SMA based on their association with disease severity as determined by motor function or other functional outcome measures. Data was generated across three different discovery studies or campaigns. The platform was a LC/MS-ITRAQ analysis done by RSI Genomics. The second and third were the DiscoveryMAP and OncologyMAP multiplex immunomarkers developed by Rule-Based Medicine. Samples used were plasma samples collected in the Biomarkers for SMA for the research and clinical communities. This study was a multi-center, pilot study enrolling 150 subjects, age 2 to 50 years, SMA type 2 or 3 (both autosomal dominant and autosomal recessive), and nerve pathology. Each subject was analyzed as a single, time point, during which assessment of functional ability, psychometric status, and nutritional status was performed. No therapeutic intervention occurred.

Three groups of SMA patients and one cohort of control children were enrolled according to the classifications below:

- Children with type I SMA (n=17)
- Children with type II SMA (n=46)
- Children with type III SMA (n=42)
- Control children (n=22)

125 plasma samples were collected from the SMA patients and matched control subjects. These samples were used in ongoing LC/MS and MAP platform biomarker analyses.

Other samples from the Pediatric-Nervous System Clinical Research Network (PNCSR) SMA-Natural History Study (NHS) from Columbia University, Boston Children’s Hospital, and Children’s Hospital of Philadelphia were used for assay development. This was a multi-site, longitudinal, prospective study enrolling 103 patients 4 months to 45 years from three academic pediatric neuromuscular clinics. Subjects were seen at 5, 6, 12, 18, 24, 36 and 39 months, during which assessments using multiple motor scales and tests were made; psychometric status, functional ability, and nutritional status was assessed. Sustained intervention occurred. Three SMA groups from the PNCSR NHS were enrolled according to the classifications below:

- Subjects with type I SMA (n=27)
- Subjects with type II SMA (n=46)
- Subjects with type III SMA (n=43)

Statistical Analysis

The LC/MS analysis data was analyzed using univariate and multivariate regression methods. Age and gender were controlled for in the analysis. Hits were identified by significant p-values whereby the lowest p-value was the trimmed hit. The MAPs were analyzed using ANOVA, t-test, and Fisher’s Exact test, and Pearson’s correlation and multivariate regression analysis (linear, basic, random forest). Analyses that had a high number of missing values (greater than 40%) below limit of detection were excluded from the analysis. Analyses whose values were driven by a few outliers generally fell out of the following analyses. The false discovery rate (FDR) was controlled by the Benjamin-Hochberg method and the FDR q-value cutoff was set at q ≤ 0.1.

Further multiplex analyses were performed on both the LC/MS and MAP data to identify the best hits from all three discovery campaigns (Figure 1). The ability of top hits to predict SMA function scores is represented in Figure 2.

LC/MS ITRAQ

Abundant proteins were depleted in order to facilitate a good dynamic range of protein measurements. In this project a dual affinity depletion strategy was implemented. In the first stage, 14 highly abundant proteins (serum albumin, IgG, fibrinogen, transferrin, IgA, IgG2, haptoglobin, α-1 acid glycoprotein, α-1 antitrypsin, Apo-A, Apo-A1, complement C3, and Apo B-100) were depleted by tPF antibody column. The remaining proteins were extracted from non-proteocomplexes processes by isoelectric focus chromatography. The proteins were reduced, alkylated, tryptic digestion and purified using tPF resin. The resulting peptide pool was then labeled with the isotope specific (ITRAQ) reagent. Eight samples labeled with different isotopes were combined into eight iTRAQ mixtures and analyzed by a single sample using mass spectrometry. Each ITRAQ mix is analyzed by two dimensional LC MS/MS. ITRAQ ratios were pre-fractionated by strong cation exchange into six fractions that are further separated by HPLC. The mass-to-charge ratio is the primary data used to determine whether individual analysis are hits (Table 1).

Immunomass Multi-Analyte Profile (MAP)

Multiplexing is accomplished by assigning each analyte specific assay a microscope slide set labeled with a unique fluorescence signature. To obtain distinct microscope signatures, two fluorescent dyes, red and far red, are mixed in various combinations using different intensities of each dye. Each set of microscope is encoded with a fluorescence signature by imprinting the microscope with a unique dye signature. After encoding the process, an assay-specific capture reagent is conjugated covalently to a unique set of microspheres, creating an ELISA-like assay on each fixed surface. After optimizing the parameters of each assay separately, Multi-Analyte Profiles (MAPs) are formed by mixing up to 100 different sets of the microspheres in a single well of a 96- or 384-well format microplate. A small volume of sample (75 μL or 25 μL) is added to the well and allowed to react with the microspheres. The assay-specific capture reagent on each individual microsphere binds the analyte of interest. A cocktail of assay-specific, biotinylated-detected reagents (e.g., antigens, antibodies, ligands, etc.) is reacted with the microsphere mixture, followed by a streptavidin-labeled fluorescent “reporter” molecule. Finally, the microscope is washed to remove unbound detected reagents. After washing, the mixture of microspheres is analyzed using the Luminescent 100™ instrument similar to a flow cytometer, the instrument uses hydrodynamic focusing to pass the microsphere analyte mixture directly into light guides where the fluorescence emitted by the reporter reagents, is analyzed for size, encoded fluorescence signature and the amount of fluorescence generated in proportion to the analyte. Each microscope is encoded with a unique signature, the classification identifies the analyte being measured on that individual microscope. As the microsphere passes through a green diode pumped solid state laser (532 nm), a fluorescent “reporter” signal (580 nm) is generated in proportion to the analyte concentration. Analyte concentrations from the DiscoveryMAP and OncologyMAP panels were analyzed to identify hit proteins (Table 2).

SMA Panel

The top hits were identified and selected for their statistical value and assay performance for use in a new SMA MAP panel with both LC/MS and DiscoveryMAP and OncologyMAP analytes. New immunomarkers were created for the top 9 LC/MS plasma hits: Tenascin-C, Cartilage Intermediate Layer Protein 2, C3OS, Cartilage-Oligomeric Matrix Protein, Lumican, Claudin13, Diphosphatase 6, Peptidase D, and Thrombospondin-4 (Table 4). LC/MS Plasma Protein Hit List

Table 1: LC/MS Plasma Protein Hit List

Table 3: OncologyMAP Plasma Protein Hit List

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Discussion and Next Steps

- Hits from the LC/MS and MAP biomarker discovery studies have been combined into a new custom multiplexed immunomarker panel for SMA.
- The SMA panel is being tested with other SMA sample collections to verify the initial Biomarker hits.
- Results will be available by end of July.
- Analysis will include regression against several other SMA motor scales (e.g. GMFM, MHFSM) and outcome measures (e.g. PIV, CMAP/MUNE).
- This SMA panel will be available in August 2011 for further community testing as a diagnostic and possibly pharmacodynamic biomarker tool.
- A streamlined version of the panel will be available in Fall 2011.
- Please see BioStar database portal at.

Acknowledgments

Our thanks are given to the following groups and people for their contributions to this work. The Biomarkers for SMA Study Group and the PNCSR SMA Biopsy Committee provided the plasma samples and SMA clinical data. Sarah Providakis worked on poster design, Jason Scoll of Rule-Based Medicine developed new panel assays. All work was funded by the SMA Foundation.