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Biomarker Assay Development: Urinary Titin in Becker Muscular Dystrophy

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Introduction

Mutations in a structural muscle protein called dystrophin result in muscle fiber degradation and diseases such as Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD). Such muscle fiber degradation can result in large titin fragments being released into the serum and excreted in the urine. Urinary titin can be a key indicator for disease progression and a valuable biomarker compared to current invasive methods that use muscle biopsy or serum collection. ***Objective:*** *To develop a laboratory-based high-throughput assay utilizing liposomal technology to track the progression of BMD and DMD via urinary titin. Our liposomes offer ease of use, lower limits of detection, and better signal to noise over enzymes traditionally used for immunoassay detection.*

Methods

Liposome Conjugation with Streptavidin (StAv) for Detection

- Liposomes were prepared encapsulating high concentrations of fluorescent dye sulforhodamine B (Fig.1).
- EDC was utilized to propagate the bond between the liposomes and StAv, forming StAvasomes.

Titin Immunoassay (ELISA)

- Anti-Titin (Abnova™ clone 2F12) and biotin-conjugated Anti-Titin (Abnova™ clone 6H5) antibodies were used for capture and detection forming a sandwich complex with titin.
- StAvosomes were added to recognize the bound biotinylated detection antibody and were quantitatively measured using fluorescence following via surfactant-induced lysis using n-octyl-β-D-glucopyranoside (OG).
- The concentration of titin in BMD urine samples was determined using a standard curve of titin in artificial urine (AFU) (Fig.1 & 4).

Normalization: Micro-BCA Assay, Creatinine Assay, Urine Analyzer

- BMD urine samples were prepared through the ThermoScientific Compat-Able Protein Assay™ and standard ThermoScientific Micro-BCA™ assay practices were used to determine total protein concentrations via absorbance (Fig.2 & 4).
- Standard practices for Cayman Chemical Creatinine (urinary) Colorimetric™ assays were performed for comparison of previous literature on DMD to BMD (Fig.2).
- A clinical urine analyzer provided a readout of data that is automatic and accurate. Specific gravity (SG) was used to normalize the concentration of particles in and compares the density of urine with water between samples (Fig.3)

Western Blot

- Standard Western blot practices were utilized to visualize and compare urinary titin between differing severity of BMD patients; control (no disease), mild (ambulatory), and severe (non-ambulatory) (Fig.5).

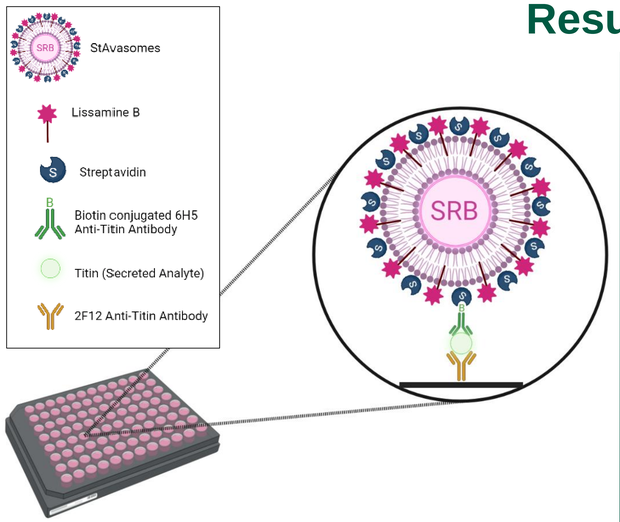


Figure 1. Streptavidin labeled fluorescent dye-entrapping liposomes for titin detection using a sandwich immunoassay. This technology has a lower limit of detection (LOD) and higher signal-to-noise ratio (S:N) compared to standard enzymatic detection practices.

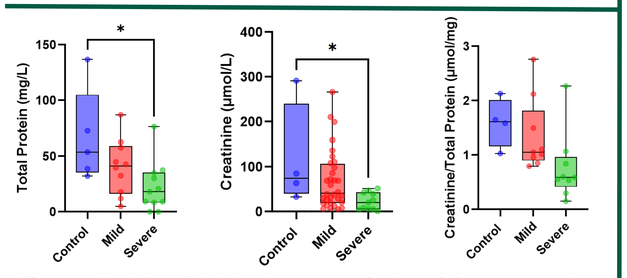


Figure 2. MicroBCA™ Assay and Creatinine assay for Normalization: A significant difference between control and severe BMD patients in both total protein and creatinine concentration was observed. However, when creatinine was normalized to total protein concentration, no significant difference was found between disease severities (p-value ≤ 0.05).

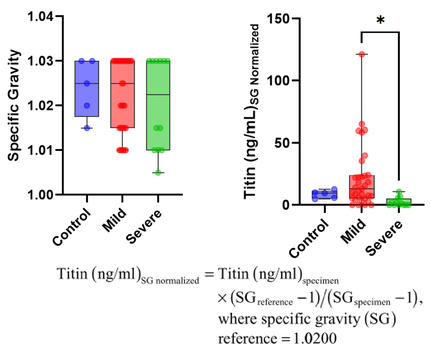


Figure 3. Urine Chemistry Analyzer McKesson Consult™ for SG Normalization: A significant difference between mild and severe BMD patients was observed when normalized to SG using the equation above 2 (p-value ≤ 0.05).

Results

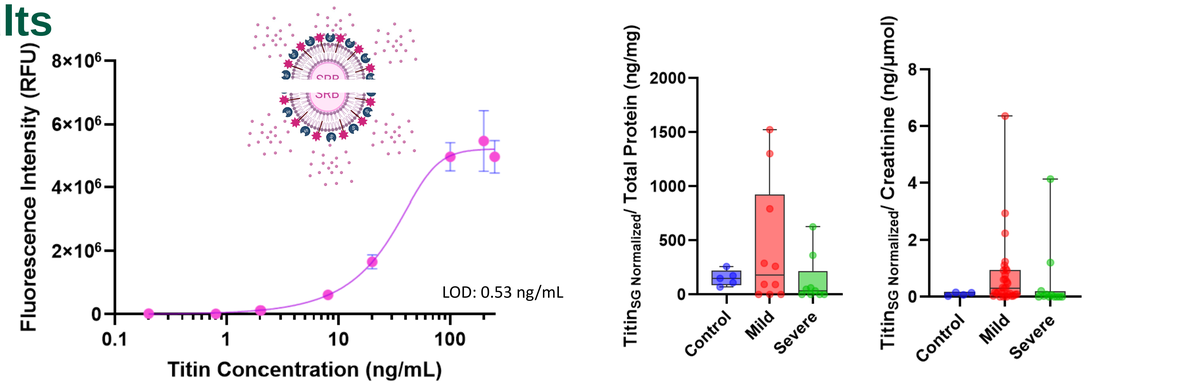
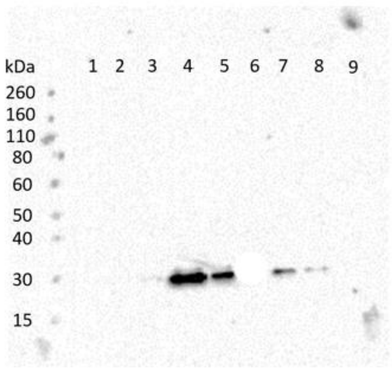


Figure 4. Titin Immunoassay: Following normalization of Titin-SG to Total Protein or Creatinine, no significant difference was observed between disease severities (p-value ≤ 0.05). Total Protein and Creatinine were used for normalization to adjust concentration of titin protein content to total protein content and creatinine content to monitor muscle wasting activity used to observe kidney function.

Label	Sample
L	Ladder
1	C1
2	C2
3	C3
4	BM1
5	BM2
6	BM3
7	BS1
8	BS2
9	BS3

PVDF membrane stained with Ponceau Stain for detection of protein bands in membrane.



PVDF membrane probed with Anti-Titin antibody

Figure 5. Western Blot Analysis: Patients were separated based on ambulatory status, (C) – no disease, (BM) – ambulatory, (BS) – non-ambulatory. Through WB, urinary titin was detected at a greater amount in mild compared to severe BMD patients. Optimization of extraction methods needs to be further explored for consistency of sample loading and downstream mass spectrometry for proteomics.

Conclusion

- DMD being more established in literature in comparison BMD, detecting urinary titin as a noninvasive biomarker could be an effective route in monitoring disease progression.
- The liposome-based high-throughput immunoassay can quantitatively detection titin and could potentially be used for monitoring disease progression if there is a significant difference found between disease severities: mild (ambulatory) and severe (non-ambulatory).
- Our current results indicate that titin fragments are not a promising biomarker for monitoring BMD disease progression.
- However, the development of these universal technologies can benefit other muscular dystrophies and cardiomyopathies impacted by titin, such as DMD, Emery-Dreifuss, limb-girdle, and tibial muscular dystrophy.
- Future work involves further analysis of more samples and mass spectrometry proteomics for titin fragment validation.

References

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