Analysis of LGMD2i Patient Biopsies for O-glycosylation Content of Alpha Dystroglycan Using a Ratio-Metric Western Blot Method

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Abstract

Limb Girdle Muscular Dystrophy type 2i (LGMD2i) is an autosomal recessive disease caused by mutations in the fukutin-related protein (FKRP) gene. The Majority of FKRP mutations lead to LGMD2i which is the third most common form of Limb Girdle Muscular Dystrophy (LGMD). All FKRP related diseases result in the absence or reduction of O-glycosylation of alpha dystroglycan (a-DG). This results in the disruption of sarcolemma integrity and leads to contraction induced damage, inflammation and loss of muscle mass and function. Currently there are no directed therapies for the treatment of LGMD2i. We have established a multiplexed western blot-based method that reports on the relative O-glycosylation of alpha dystroglycan from muscle biopsies. Here we report the results on 13 LGMD2i patients representing homozygous and compound heterozygous mutations in FKRP. In all patients there is decreased matriglycan expression as compared to healthy human donor muscle. Four of these patients have longitudinal biopsies and there is little change in matriglycan expression from the initial baseline biopsy measurement but still significantly reduced as compared to donor tissue. The semi-quantitative nature of this method may be of use in understanding the longitudinal changes in patients with LGMD2i and in assessing the impact of therapeutics directed at increasing the O-glycosylation of alpha dystroglycan.

Materials & Methods

Muscle Biopsy

Muscle biopsy samples were collected from tibialis anterior using a fine needle aspirate. In total three passes were collected. Biopsy samples were snap-frozen immediately after collection and stored at -80 C at Virginia Commonwealth University until analysis.

Healthy Human Tissue

Control healthy human muscle tissue (male and female) was obtained from BioIVT and stored at -80 C until analysis.

Murine Mouse Tissue

Murine muscle tissue from healthy, P448L and L276I FRKP mutant mice was provided by the McColl Lockwood lab at Atrium Health. Samples were stored at -80 C until analysis.

Tissue Lysis

Tissue samples were processed using a TissueLyzer II (Qiagen). Approximately 250 mgs of tissue was place in a 2 mL microtube with a steel bead and 1 mL of RIPA buffer supplemented with protease and phosphatase inhibitors. The microtubes were subjected to three cycles of disruption (2 min at 25 Hz) in the TissueLyzer II. Lysates were clarified by centrifugation and the supernatant was transferred to fresh tubes and lysate concentration was determined using a BCA assay (Pierce).

SDS-PAGE

Polyacrylamide gel electrophoresis was carried out on 4-20% StainFree gels (BioRad). Tissue lysates were diluted to 20 ug/ 15 uL with RIPA buffer in LiCor loading buffer. A total of 20 ug of lysate was loaded per well. Pre-stained protein markers were also loaded onto gel. After electrophoretic separation the gel was washed in water and total protein was imaged using the ChemiDoc imaging system (BioRad).

Western Blotting

Proteins were transferred to a PVDF membrane using an iBlot Gel Transfer Device (Invitrogen). The membrane was dried and activated with a methanol wash and then rinsed with water. The membrane was blocked with Intercept blocking buffer (LiCor) and probed with AF6868 alpha dystroglycan antibody (R&D Systems) and IIH6C4 alpha dystroglycan antibody (Millipore). The primary antibody solution was removed and the washed four times with TBST. The blot was then probed with IR790 Mouse Anti-Sheep and IR680 Goat Anti-Mouse secondary antibodies (LiCor). The secondary antibody solution was removes and the membrane was wash four times with TBST. The membrane was visualized on an Odyssey CLx imager (LiCor) and intensities of the 700 and 800 nm channels were recorded.

Data Analysis

Analysis was carried on GraphPad Prism (version 8). Signal intensities were normalized to total protein and the ratio of 700 nm normalized signal/800 nm normalized signal was determined. Linear fits were assessed using the linear regression function of Prism. Statistical significance was determined using an unpaired t-test using Prism.

Results

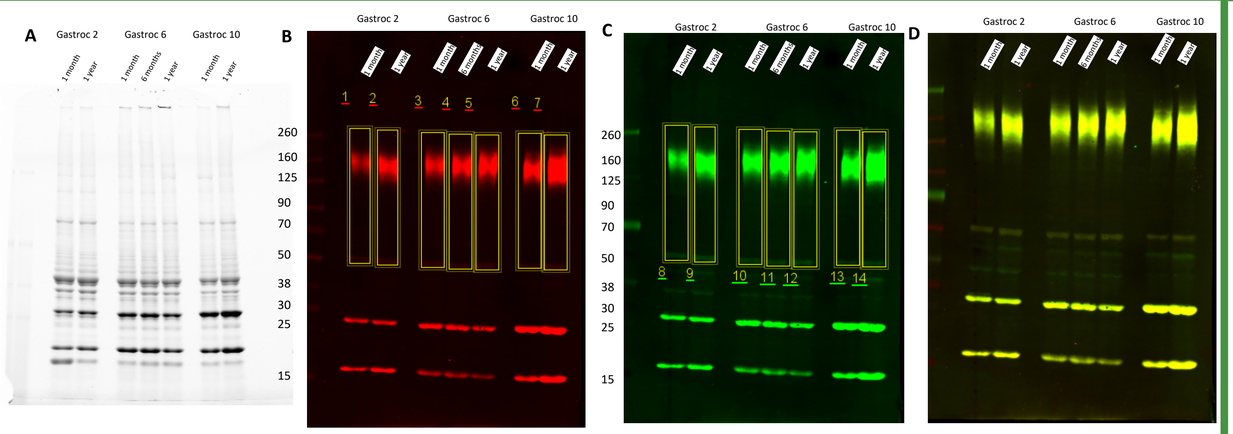


Figure 1. Polyacrilamide gel and western blot of three gastrocnemius healthy human donors. (A) Gel image of gastrocnemius samples tested, each lane was loaded with 20 ug of total lysate and imaged on a ChemiDoc imaging system.. (B) Image of western blot membrane at 700 nm channel showing staining for the glycosylated form of a-DG. (C) Image of western blot membrane at 800 nm channel showing staining for the total a-DG. (D) Image of western blot membrane showing the merge of both detection channels. .

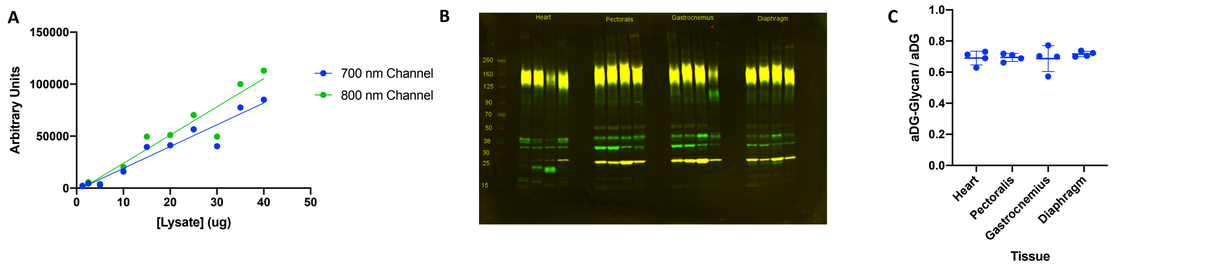
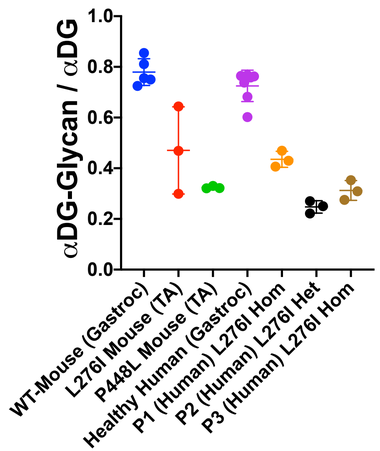


Figure 2. (A) Gastrocnemius tissue lysate was serially diluted, and the linearity of both detection channels was plotted as a function of lysate concentration. The solid line represent the linear regression. (B) Four different muscle types from healthy donors (2 male and 2 female) were analyzed to assess the variability of the a-DG glycan ratio. Shown is the image of the western blot membrane showing the merge of both detection channels. (C) The ratio of the glycosylated form of a-DG to total protein was determined using intensities from the detection channels and plotted for each muscle. The plot shows the mean and the standard deviation of the ratios.



Sample	Mean Ratio	SD	P Unpaired t-test
WT-Mouse (Gastroc)	0.78	0.03	
L276I Mouse (TA)	0.47	0.17	0.10
P448L Mouse (TA)	0.32	0.005	< 0.0001
Healthy Human (Gastroc)	0.72	0.06	
P1 L276I Hom	0.43	0.03	< 0.0001
P2 L276I Het	0.25	0.02	< 0.0001
P3 L276I Hom	0.31	0.04	< 0.0001

Figure 3. The ratio of glycosylated a-DG to total a-DG was assessed from muscle tissue from wild type mouse, an L276I FKRP mutant mouse model, a P448L mutant mouse model, a healthy human donor, and three LGMD2i patients containing an L276I FRKP mutation (two homozygous and one compound heterozygous). The table to the right summarizes the mean and standard deviation of the measures and statistical significance.

Results Cont.

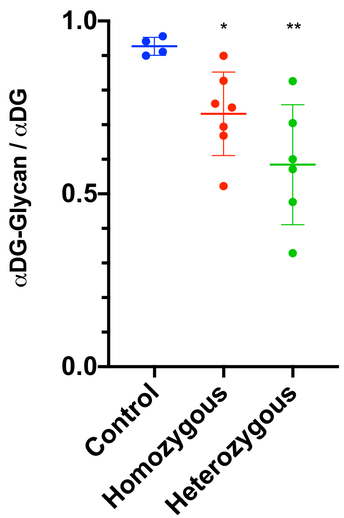


Figure 4. Patient samples for a LGMD2i natural history study were analyzed for a-DG glycosylation ratio. Sample were obtained from VCU and processed as described in the materials and methods. The points on the plot represent the average of the triplicate analysis for each patient. Shown are the mean and standard deviation for the healthy controls, L276I FKRP homozygous and the compound heterozygous patients. The asterisks denote statistical significance. (HOM p=0.0125, HET p=0.0049 relative to control)

Conclusions

1. We have developed a multiplexed western blot method to detect the ratio-metric extend of a-DG glycosylation relative to total a-DG from muscle lysates that can be applied to human biopsy samples.
2. The detection signals show good linearity with lysate concentration and the amount of material required for conducting muscle analysis is compatible with quantities obtain from fine needle aspirate biopsies.
3. Analysis of several muscles from human donors demonstrates that the a-DG ratio is similar across different muscle types.
4. Tibialis anterior from two murine models of LGMD2i were analyzed using this method and a decrease in the glycosylation of a-DG was decreased in both FKRP mutant mouse models with the largest reduction being with the more severe P448L FKRP mutation.
5. The method was initially used to assess three biopsies from LGMD2i patients harboring an L276I mutation and statistically significant reductions in a-DG glycation were observed.
6. Several LGMD2i patients from an ongoing longitudinal study we analyzed and statistically significant reduction in a-DG glycation were observed. The compound heterozygous patients show a great loss in glycosylation content.
7. This method may prove useful in further longitudinal LGMD2i studies and therapies that seek to increase the glycosylation of a-DG.

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