Published in final edited form as:

Muscle Nerve. 2018 August ; 58(2): 286–292. doi:10.1002/mus.26135.

Quantification of Lectin Fluorescence in GNE Myopathy Muscle Biopsies

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Abstract

Introduction—GNE myopathy is an adult-onset muscle disorder characterized by impaired sialylation of (muscle) glycans, detectable by lectin histochemistry. We describe a standardized method to quantify (lectin-)fluorescence in muscle sections, applicable for diagnosis and response to therapy for GNE myopathy.

Methods—Muscle sections were fluorescently labeled with the sialic acid-binding *Sambucus nigra* agglutinin (SNA) lectin and antibodies to sarcolemma residence protein Caveolin-3 (CAV-3). Entire tissue sections were imaged in tiles and fluorescence was quantified.

Results—SNA fluorescence co-localizing with CAV-3 was ~50% decreased in GNE myopathy biopsies compared to muscle-matched controls, confirming previous qualitative results.

Discussion—This quantitative fluorescence method can accurately determine sialylation status of GNE myopathy muscle biopsies. This method is adaptable for expression of other membrane-associated muscle proteins, and may be of benefit for disorders in which therapeutic changes in expression are subtle and difficult to assess by other methods.

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Ethical Publication Statement:

The authors confirm that they have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

Conflict of Interest Disclosure:

None of the authors has any conflict of interest to disclose.

Part of the material presented in this manuscript is contained within poster presentations made at the 22nd International Congress of the World Muscle Society (Oct 3–7, 2017; St. Malo, France) and at the Society for Glycobiology 2017 Annual meeting (Nov 5–8, 2017; Portland, OR).

Keywords

Caveolin-3; N-acetylmannosamine (ManNAc); sarcolemma; sialylation; SNA lectin

INTRODUCTION

GNE myopathy is an autosomal recessive disorder characterized by adult-onset, slowly progressive, distal and proximal myopathy that typically leaves patients wheelchair dependent 10–20 years after onset. Histologically, it is associated with muscle fiber degeneration and formation of tubulofilamentous vacuoles in muscle tissue.^{1–5} GNE myopathy is caused by biallelic, mostly missense mutations in the *GNE* gene, encoding the rate-limiting enzyme of sialic acid biosynthesis, UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosamine kinase (GNE).^{6–9} Sialic acids are the most abundant terminal sugar residues on glycans (glycoproteins and glycolipids), where they regulate several biologic functions, including cellular interactions, adhesions and signaling.^{10,11}

The exact pathophysiology of GNE myopathy remains unknown, but the partial dysfunction in GNE enzyme activities due to missense mutations suggests involvement of impaired sialylation of muscle glycans.^{12,13} Such impaired sialylation was identified in a select group of (sialo-) glycans in GNE myopathy patients;^{14–18} some of the glycans may aid in diagnosing GNE myopathy.^{17,18} There are no robust biomarkers developed for diagnosis or for demonstrating intracellular response to therapy.

Lectins are sugar-binding proteins with ligand specificities for defined carbohydrate sequences.¹⁹ Staining of GNE myopathy human and mouse muscle sections with sialic acid binding lectins or lectins binding to desialylated sugar moieties demonstrated hyposialylation of sarcolemmal membranes.^{4,12,15,18,20,21} In particular, use of the lectin SNA (*Sambucus nigra* agglutinin) that predominantly recognizes terminal sialic acid (Neu5Ac) in an $\alpha(2,6)$ -linkage with either galactose or with N-acetylgalactosamine (GalNAc),^{24,25,26} was previously proven informative for GNE myopathy muscle sialylation status.^{4,15,18}

Lectin histochemistry demonstrated that sialic acid residues in a $\alpha(2,6)$ -linkage with either galactose or with N-acetylgalactosamine (GalNAc), present on sarcolemmal glycans appeared to be absent or decreased in human and mouse GNE myopathy muscle sections (Supplemental Figure S1).^{4,15,18} In addition, GNE myopathy mice receiving 12 weeks of oral therapy with the sialic acid precursor N-acetylmannosamine (ManNAc) showed re-sialylation of sarcolemmal membranes by lectin histochemistry (Supplemental Figure S1C),²¹ and ManNAc therapy ameliorated the myopathic phenotype in GNE myopathy mice.²² These encouraging murine results suggest that lectin staining of muscle biopsies not only serves as a biomarker aiding diagnosis of GNE myopathy,^{4,18} but may also to demonstrate intracellular response to sialylation-increasing therapies. Clinical studies of oral ManNAc therapy in GNE myopathy subjects are currently ongoing ([clinicaltrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT02346461) Identifier NCT02346461)^{4,23} and a robust biomarker of intracellular response to therapy of skeletal muscle, the only affected tissue in GNE myopathy, is pivotal for demonstration of biochemical efficacy.

Therefore, we sought to develop SNA lectin staining of skeletal muscle biopsies as a biomarker for GNE myopathy. GNE myopathy patients' muscle biopsies are available, since they are often acquired as part of the diagnostic evaluation.^{1,2,4,5} In previous lectin studies for human GNE myopathy muscle biopsy sections, either paraffin embedded^{4,18} or frozen,^{12,15} were imaged and presented in a qualitative way, with the investigator determining the microscope settings and the muscle region in the biopsy appropriate for imaging. Here we present a standardized, reproducible method to image and quantify fluorescent lectin binding to muscle membranes (marked by sarcolemma residence protein Caveolin-3) in entire muscle biopsy slides.

MATERIALS and METHODS

Subjects and Muscle Biopsies

Frozen human control muscle biopsy slides (n=4) and human GNE myopathy muscle biopsy slides (n=6) (Table 1) were acquired from the Department of Neuromuscular Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry (NCNP), Tokyo, Japan. Muscle tissue was obtained through open biopsies on control individuals and patients participating in clinical studies approved by the NCNP Institutional Review Board; written informed consent was obtained from all muscle biopsy donors. Biopsies were frozen in 2-methylbutane, cooled in liquid nitrogen and cut into 8mm cross sections.

Sectioning and Fluorescence Staining

Muscle sections were double stained with Caveolin-3 (CAV-3) antibody (R&D Systems, Minneapolis, MN) and SNA lectin (Vector Laboratories). The CAV-3 protein is not glycosylated and is found in plasma membranes of most cell types. CAV-3 antibodies are commonly used as sarcolemma markers in muscle imaging studies.^{27,28} The lectin SNA predominantly recognizes terminal sialic acid (Neu5Ac) in an $\alpha(2,6)$ -linkage with either galactose or with N-acetylgalactosamine (GalNAc).^{24,25,26}

Slides containing frozen muscle sections were transferred from -80°C storage into pre-chilled glass dishes and thawed to room temperature for 30 min. Staining for all 9 slides (GNE myopathy and controls) was performed in 1 batch under the same conditions. Thawed muscle sections were fixed in acetone for 20 min, followed by 1 min of air drying and washing with phosphate-buffered saline (PBS) for 10 min. The fixed muscle sections were then blocked at room temperature with 1X Carbo-Free Blocking solution (Vector Laboratories, Burlingame, CA) for 1 h, followed by overnight incubation at 4°C in a mixture of 8 $\mu\text{g}/\text{mL}$ Caveolin-3 (CAV-3) antibody (R&D Systems, Minneapolis, MN) and 5 $\mu\text{g}/\text{mL}$ fluorescein isothiocyanate (FITC)-labeled SNA lectin (Vector Laboratories) in Carbo-Free Blocking solution. Next, the sections were washed at room temperature 3 times 5 min with 0.1% Tween/PBS and 5 min with PBS. The slides were then incubated at room temperature for 1 h with secondary antibodies (to visualize CAV-3 antibody staining) donkey anti-mouse AlexaFluor555 (A-31570, Invitrogen/Thermo Fisher Scientific, Rockville, MD) in Carbo-Free Blocking solution. After washing again 3 times 5 min with 0.1% Tween/PBS and 5 min with 1X PBS, the slides were mounted with Vectashield containing the nuclear dye DAPI (Vector Laboratories).

Image Acquisition Procedure by Inverted Microscopy

Imaging was performed on an Axio Observer Z1 inverted microscope (Zeiss Microimaging Inc, Thornwood, NY) with a 20× DIC objective, using an Axiocam MRm Rev 3 digital camera with Colibri LED illumination (Zeiss Microimaging Inc). Image settings were established on a control stained muscle slide. Entire muscle sections on each slide were digitally imaged using Tile-imaging (with local focus surface support points, 10% Tile Overlap) and Z-stack imaging (5 Z-slices per slide; 8 µm range, 2 µm interval slices) using ZEN2 Pro software (Zeiss Microimaging Inc.). All slides were imaged in one session with the same microscope settings. Image processing was performed with ZEN2 Pro software, the focus planes of the entire Z-Stack image was calculated to one extended depth of focus (EDF) image with Maximum Projection parameters. All tiles were stitched together into one image file by stitching processing.

Selected Area Imaging by Confocal Microscopy

After finalizing the Tile-imaging for each slide, 4–5 selected areas in each slide were also digitally imaged as a Z-stack with a Zeiss LSM 510 META confocal laser-scanning microscope (Zeiss Microimaging Inc). Images were acquired using a Plan-Apochromat 20X DIC objective. All confocal images are displayed as 1D projections of confocal Z-stacks (Supplemental Figure S1).

Membrane Fluorescence Quantitation with Image Analysis Software

Image analysis of SNA/CAV-3 fluorescent image files was performed using Definiens Architect Software (Definiens, Cambridge, MA) with specialized algorithms and applications. Entire image files (Figure 1A, 1B) were imported in the software. Nonspecific artifacts/fluorescence, scrambled biopsy edges and non-muscle or non-intact areas (i.e., mostly fibrous tissue areas) (Figure 1C) were manually annotated in each image file to be excluded from analysis (Figure 1D). Images were analyzed automatically in one batch. For image analysis, the CAV-3 fluorescent area was identified by the software and set as a mesh for sarcolemma membrane area (Figure 1E). The software then determined the SNA fluorescent signal co-localization (intensity (pixels), area of fluorescence) on the CAV-3 mesh (Figure 1F). The software provided fluorescence quantitation per image file in the following outputs:

- Area of SNA fluorescence co-localized on CAV-3 mesh (area is calculated in pixels)
- Total intensity of SNA fluorescence co-localized on CAV-3 mesh (intensity is calculated by sum of intensity and # of pixels).

In addition, the number of cells per biopsy was manually determined, using ImageJ software (NIH, Bethesda, MD). The diameter per cell was manually annotated with Image J software (annotated as narrowest distance at/near center of each cell), which was used to determine membrane length per cell and average membrane length per cell for each biopsy (calculated with Image J software).

Statistical Analysis

Statistical analysis of fluorescence quantitation was analyzed with the Mann-Whitney unpaired t-test.

RESULTS

The biopsy donors in both the control and GNE myopathy groups were comparable in male/female distribution, range of age at biopsy and variation in *GNE* gene mutations, and most biopsies were acquired from the *biceps brachii* (Table 1). Each biopsy was of adequate quality with a large number of intact muscle cells per biopsy slide (range 1,223–13,304 total cells) (Table 1, Supplemental Figure S2). The average cell membrane length varied from 11.53–23.81, reflecting the cut of each biopsy (pure cross-section or more longitudinal cut resulting in larger cells).

Each biopsy slide was double-stained with FITC-labeled SNA lectin and CAV-3 antibodies (visualized with AlexaFluor555-labeled secondary antibodies). Initial qualitative imaging on muscle biopsy sections showed that in frozen sections, the SNA fluorescent signal is less distinctive than in paraffin embedded sections (Supplemental Figure S1A, S1C), which called for quantitation of the SNA fluorescent signal.

To standardize quantitative lectin analysis, staining methods and imaging procedures were optimized using control and GNE myopathy muscle slides. All biopsies were stained with SNA and CAV-3 in one batch, followed by imaging entire tissue sections on each slide in one batch by Tile and Z-stack imaging in automated fashion with similar microscope settings by the same investigator. One image file was created for each biopsy, with annotations for regions to exclude from analysis (Figure 1, Supplemental Figure S2), followed by quantitative analysis of fluorescent area and intensity in each image file (Table 1).

Because the size and quality of each biopsy varied, we used the relative measures SNA area and SNA intensity colocalized with CAV-3 per biopsy as outcome parameters, and divided them by the average length of cells per biopsy to correct for a different cut (cross-section cut versus more longitudinal cut) of each biopsy. These values were averaged for each group (control and GNE myopathy) of biopsies. Both the SNA area (299.5×10^3 in control group versus 137.6×10^3 in GNE Myopathy group) and SNA intensity (97.5×10^6 in control group versus 48.1×10^6 in GNE Myopathy group) co-localized with CAV-3 per the average cell length showed a ~ 50% decrease in the GNE myopathy group compared to the control group (Table 1). Although substantial, this difference was not statistically significant (unpaired t-test; $P = 0.0667$, Figure 2).

DISCUSSION

Fluorescent stained muscle biopsy slides often show varied fluorescent intensity in different areas of the tissue; this is related to differences in size, cut, and quality (fibrous tissue vs muscle cells) of each biopsy. It is often at the discretion of the investigator performing the imaging to capture representative fields of the tissue, determine microscope settings and

qualitatively analyze the results, i.e., score the fields by eye. While this is an accepted practice,^{12,14,15,17,18} quantitation of the fluorescence signal in the entire biopsy may be more suitable if diagnosis/outcome or clinical trial decisions are based on such results. A few other studies have explored quantitation of fluorescence signals in muscle biopsy slides, mostly for dystrophin-expression related studies.^{29,30,31}

Here we assessed quantitative analysis of SNA fluorescent signal in entire muscle biopsy slides as a biomarker for GNE myopathy, which is associated with impaired sarcolemma sialylation. The quantitative analysis method described in this manuscript includes standardized fluorescent staining and imaging of entire cross-section biopsy slides, with unbiased software analysis of the image files. This software program has previously been used in muscle image analysis for other purposes.³¹ Previous imaging demonstrated that SNA-reactive glycans are expressed at muscle membranes and all muscle fibers appear to express SNA-reactive glycans equally.^{4,15,18,21} Therefore, we imaged entire muscle sections and included each intact muscle cell (stained by caveolin) in our analysis.

Even with the small number of biopsies per group, we demonstrated that average areas and intensities of SNA fluorescence per CAV-3 area was ~50% ($p = 0.0667$) reduced in GNE myopathy biopsies compared to controls. Statistical significance was absent likely to the limited number of biopsies in each group. These findings are in line with previously published, sometimes subtle, qualitative GNE myopathy lectin imaging results.^{12,15,18}

The use of the non-glycosylated protein, CAV-3, as sarcolemma marker proved appropriate, because CAV-3 is evenly distributed among sarcolemma membranes and muscle fiber types. The use of a software program for muscle membrane analysis using CAV-3 as a mesh, as established in this manuscript, is easily adaptable for any other purpose involving expression analysis of muscle membrane components, and may be of benefit for disorders in which therapeutic changes in expression are subtle and difficult to assess by other methods.

For future use of this method for diagnosing GNE myopathy, ranges of normal and disease fluorescence intensities/areas need to be established in a larger group of control and GNE myopathy muscle biopsies. A suspected GNE myopathy diagnosis based on muscle biopsy lectin analysis needs to be confirmed by genetic analysis identifying biallelic mutations in the *GNE* gene, because it is possible that other muscle disorders (e.g., dystroglycanopathies) show decreased sialylation/ glycosylation of sarcolemma membranes.³²

Our established quantitation method is well-suited to demonstrate response to therapy, particularly in muscle biopsies of the same muscles in the same individuals acquired before and after therapy. We have now established and validated this method for GNE myopathy muscle biopsies, and intend to apply it to muscle biopsies acquired in our ongoing Phase 2 clinical trial of ManNAc for GNE myopathy ([clinicaltrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT02346461) Identifier NCT02346461).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank the participating muscle biopsy donors. We thank Megumu Ogawa (NIN, NCNP, Japan) for her technical expertise in preparing the muscle biopsy specimen, Carla Ciccone (NHGRI, NIH, USA), Heidi Dorward (NHGRI, NIH, USA) and Steven Titus (NCATS, NIH, USA) for expert laboratory assistance and training, Andrzej Cholewinski (Definiens Inc., USA) for assistance with image parameters and analysis using the Definiens software, and the UDP Translational Laboratory (UDP, NIH, USA) for microscope use.

This study was supported by the Intramural Research Programs of the National Human Genome Research Institute and the National Center for Advancing Translational Sciences, National Institutes of Health, Bethesda, Maryland, USA. This study was partially funded by Escala Therapeutics (New York, USA) through a Cooperative Research and Development Agreement.

ABBREVIATIONS

CAV-3	caveolin-3
DAPI	4',6-diamidine-2'-phenylindole dihydrochloride
EDF	extended depth of focus
FITC	fluorescein isothiocyanate
GalNAc	N-acetylgalactosamine
GNE	UDP-N-acetylglucosamine-2-epimerase/N-acetylmannosamine kinase
HPA	<i>Helix pomatia</i> agglutinin (lectin)
ManNAc	N-acetylmannosamine
MMA	<i>Maackia amurensis</i> agglutinin (lectin)
NIH	National Institutes of Health
PBS	phosphate-buffered saline
PNA	<i>Peanut</i> agglutinin (lectin)
SBA	<i>Soybean</i> agglutinin (lectin)
SNA	<i>Sambucus nigra</i> agglutinin (lectin)
VVA	<i>Vicia villosa</i> agglutinin (lectin)
WGA	<i>Wheat germ</i> agglutinin (lectin)

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FIGURE 1. Representative Projections of Entire Muscle Section Image Files

Projections of entire image files of biopsy GNE-M3 are shown to illustrate the fluorescence quantitation process. Biopsy slides were co-stained with membrane marker CAV-3 (secondary antibody AlexaFluor555-labeled) (A) and sialic-acid binding lectin SNA (FITC-labeled) (B). CAV-3/SNA fluorescence overlay image (C). Manual annotation to exclude non-specific areas (green) from analysis (D). Caveolin fluorescence set as a mesh for muscle membrane area (E). SNA fluorescence co-localizing on CAV-3 mesh (F). Fluorescence overlay images of all 9 biopsies are displayed in Supplemental Figure S1.

FIGURE 2. Dot Plots of Fluorescence Quantitation Results

Dot plots of SNA/Cav-3 area (A) and SNA/CAV-3 intensity (B) results, comparing control muscle biopsies (n=4) with GNE myopathy muscle biopsies (n=6). A difference in fluorescence expression between groups is present with each output parameter, although not statistically significant (Mann-Whitney unpaired t-test P value 0.0667 for both parameters).

Table 1

Demographics, GNE mutations, muscle type and fluorescence quantitation per biopsy

Donor	Age (y)	Sex	GNE Mutations *	Muscle Biopsied †	No. Cells in Biopsy ‡	Avg cell length §	SNA Area/Cav-3 Area ¶ (per avg cell length)	SNA/Cav-3 Total Intensity ¶¶ (per avg cell length)
Control Group:								
C1	27	Male	Normal	<i>biceps branchii (L)</i>	5,338	23.26	4,988,767 (214,505)	2,141,539,604 (92,080,962)
C2	42	Female	Normal	<i>biceps branchii (L)</i>	6,146	18.44	4,585,638 (248,657)	1,298,005,973 (70,384,594)
C3	32	Female	Normal	<i>biceps branchii (L)</i>	3,137	16.80	2,791,129 (166,133)	882,982,901 (52,556,721)
C4	31	Male	Normal	<i>biceps branchii (L)</i>	13,304	17.94	10,202,823 (568,718)	3,138,608,250 (174,949,970)
Average:					6,981	19.11	5,642,089 (299,503)	1,865,284,182 (97,493,062)
GNE Myopathy Group:								
GENE-M1	27	Male	p.V603L/p.V603L	<i>biceps branchii (L)</i>	1,393	11.53	816,286 (70,798)	367,144,808 (31,843,163)
GENE-M2	29	Male	p.V603L/p.V603L	<i>biceps branchii (L)</i>	7,332	17.46	5,328,311 (305,148)	1,391,871,694 (79,711,274)
GENE-M3	24	Male	p.V603L/p.A622T	<i>biceps branchii (L)</i>	3,114	16.14	1,936,055 (119,976)	816,679,955 (50,608,913)
GENE-M4	55	Male	p.D207V/p.V603L	<i>triceps branchii (L)</i>	1,223	23.14	1,404,210 (60,683)	540,816,086 (23,371,399)
GENE-M5	40	Female	p.D207V/p.K458N #	<i>hamstring (R)</i>	3,318	23.81	3,831,255 (160,939)	1,529,534,462 (64,251,094)
GENE-M6	39	Female	p.D207V/p.V603L	<i>tibialis anterior (R)</i>	2,600	21.06	2,279,575 (108,224)	819,170,403 (38,890,517)
Average:					3,163	18.86	2,599,282 (137,628)	910,869,568 (48,112,727)

* Mutation nomenclature according to GNE2 isoform GenBank NP_001121699.

† L, left; R, right.

‡ Manually determined, using ImageJ software.

§ Avg = average. As calculated by Image J software, see text for details.

¶ Area or Intensity of fluorescence (in pixels) determined by Definiens Architect software.

¶¶ Plotted in Figure 2.

p.K458N is a novel GNE variant (disease causing by MutationTaster, deleterious by SIFT, benign by PolyPhen, CADD_phred score of 25).