Development of Digital Tissue Image Analysis Solutions for Muscle Biopsies in Support of Disease-Mediating Therapies for Duchenne Muscular Dystrophy

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Abstract

The continual expression of utrophin protein by pharmacological maintenance of utrophin transcription in dystrophin-deficient muscle fibres is potentially a disease-modifying treatment for Duchenne muscular dystrophy (DMD) regardless of the specific dystrophin mutation. The evaluation of molecular biomarkers of muscle regeneration and structural protein complexes, such as developmental myosin and dystroglycans respectively, may be important endpoints in future clinical trials of utrophin modulators. Building on a recently published manual quantification approach, which demonstrated a positive correlation between utrophin levels and the degree of muscle fiber regeneration in DMD and Becker muscular dystrophy (BMD) muscle biopsies, the development of fully automated processes is ongoing.

Here, we report the development of multiplex immunohistochemical (ICH) assays and digital tissue image analysis tools to gauge for robustly quantifying utrophin, developmental myosin heavy chain, and beta-dystroglycan expression in DMD, BMD, and control muscle biopsies. The ICH approach enabled detection of biomarker signal features (e.g., cumulative intensities) and tissue morphometrics (e.g., fiber area and minimum diameter) in whole-slide images of muscle cross-sections at an individual muscle fiber resolution.

The ICH approaches reproducibly demonstrated quantifiable differences in levels of utrophin, and regeneration between DMD, BMD, and control biopsies. These biomarkers may be informative endpoints for evaluating pharmacologic benefit in dystrophin-deficient muscle in future clinical trials of utrophin modulators and potentially other DMD therapeutic approaches.

Introduction & Objectives

Muscular dystrophies are a collection of progressive, muscle wasting diseases. The most prevalent and severe of these diseases is Duchenne muscular dystrophy (DMD). Those afflicted with DMD suffer through the fragility of life impairments and reduced lifespan. Patients are typically wheelchair-bound during their teenage years, require mechanical ventilation assistance in their twenties, and due to cardiomyopathy or respiratory failure before they reach their early thirties.

DMD, and the related, but less severe, Becker muscular dystrophy (BMD), are caused by mutations in the dystrophin gene. These mutations result in loss or reduction of dystrophin expression, function, or both. In healthy muscle, dystrophin serves as a structural protein: its loss of function compromises the structural integrity of the muscle, resulting in increased muscle damage and myosynaptic dysfunction during the normal process of contraction and relaxation (see Barres 2011; Skeletal Muscle 1; Tinsley 2019). Myofibers are histologically distinct from the classification (blue, 1; yellow, 2; orange, 3; red). The algorithms are undergoing final review which may result in methodological modifications in subsequent studies. Zoomed images are at 10x magnification; scale bar represents 100 μm.

Methods

Samples

Frozen skeletal muscle biopsies from DMD, BMD, and non-DMD/BMD control patients (CTRLs) were used. The control muscle biopsies originated from patients presenting with a clinical neuromuscular phenotype that warranted a muscle biopsy; however, these patients were ultimately not diagnosed with either DMD or BMD, or control muscle biopsies had no histologic abnormalities. Samples were previously stained with monoplex ICH assays for utrophin, dystrophin, and developmental myosin heavy chain. Whole-slide images were semi-quantitatively evaluated by a board-certified, MD pathologist to approximate percentages of biomarker-positive myofibers. Samples were obtained from the University of Iowa, and Paul D. Wilkenson Muscular Dystrophy Cooperative Research Center. Sample details are listed in Table 1.

Immunohistochemistry

Duplex ICH assays were developed and optimized for lamin A/C (clone 4H8-2, Enzo Life Sciences ALB-A-001, Cat No. 405379), and for dystrophin (clone 43G4/1-105, Leica Biosystems), or developmental myosin heavy chain (HC-MHC Clone RNM20/920, Leica Biosystems). In each duplex assay, lamin A/C expression is visualized by a yellow chromogen and utrophin expression by a blue chromogen. All staining was performed using the Leica Biosystems Bondmax autostainer.

Image Analysis

ICH-stained slides were scanned on an Aperio ScanScope CS2 brightfield scanner at 20x magnification to generate whole slide images. Aperio's proprietary software analyses the color information, generating new images that distinguish yellow and blue staining. MuscleMap™ algorithms then quantify the blue signal intensities in the regions defined by the yellow stain (myofiber membrane or cytoplasm).

Results

Figure 1: Duplex ICH Assays for Assessment of Utophin, Beta-Dystroglycan, and MHC in Skeletal Muscle. DMD, BMD, and CTRL skeletal muscle sections were stained with duplex ICH assays for lamin A/C (yellow), to outline myofibers, and either a marker of muscle regeneration (utrophin) or MHC, that is a marker of the dystrophin associated protein complex (beta-dystroglycan, blue). Zoomed images are at 10x magnification; scale bar represents 100 μm. Insets are 10x whole-slide images of the tissue sections.

Figure 2: Digital Tissue Image Analysis of Whole-Slide Images. Whole slide images were annotated prior to analysis to remove artifacts (tissue fields, tears, etc.). Panel A shows an annotated, fit image of a section from BMD 2 stained with the lamin A/C and MHC duplex assay. The annotated image is analyzed by MuscleMap™ to identify and classify myofibers according to their biomarker expression (B). This allows for precise biomarker quantification in thousands of myofibers. Staining intensity thresholds were then determined by a board-certified, DMD pathologist to classify myofibers by biomarker expression (C). Myofibers were artificially colored according to their classification (blue, 1; yellow, 2; orange, 3; red). The algorithms are undergoing final review which may result in methodological modifications in subsequent studies. Zoomed images are at 10x magnification; scale bar represents 100 μm.

Figure 3: ICH Duplex IHC Assay Distinguishes Biomarkers within the same Cellular Compartment. Immunofluorescence images from DMD 2 stained with the duplex ICH assays were digi- tized and processed to separate the yellow and blue signals (A-C, and E-G). MuscleMap™ algorithms were developed to identify myofibers through detection of lamin A/C and MHC expression. This approach, and MuscleMap’s R and D division, have developed a small molecular therapeutic called ezutifumbistatin, which is currently in DMD clinical trials, to assess the effectiveness of utrophin modulation.

In order to assess the efficacy of utrophin upregulation therapy, Flagship Biosciences has developed ICH assays and digital tissue image analysis tools (IAA) to measure biomarkers of muscle health. Specifically, we have developed three duplex ICH assays combining lamin A/C with utrophin, beta-dystroglycan or developmental myosin heavy chain respectively. The ICH tools allow quantification of biomarker expression and tissue morphometrics across whole-slide images of DMD, BMD, and control patient biopsies, allowing for a robust assessment of muscle health.

Immunohistochemistry

Duplex ICH assays were developed and optimized for lamin A/C (clone 4H8-2, Enzo Life Sciences ALB-A-001, Cat No. 405379), and for dystrophin (clone 43G4/1-105, Leica Biosystems), or developmental myosin heavy chain (HC-MHC Clone RNM20/920, Leica Biosystems). In each duplex assay, lamin A/C expression is visualized by a yellow chromogen and utrophin expression by a blue chromogen. All staining was performed using the Leica Biosystems Bondmax autostainer.

Figure 4: MuscleMap™ Quantifies Differential Expression of Utophin in DMD, BMD, and Control Skeletal Muscle

The MuscleMap™ algorithms collected biomarker expression data from each analyzed image. Signal intensity histograms (A) showed that the dynamic range of biomarker signal fell within the detectable limits for each disease state; signal was neither saturated nor too faint to quantify differences from the control samples. With utrophin (Al), we observed a right shift in signal intensity from CTRL to DMD as a more myofibers expressed higher levels of this biomarker. Average signal intensity also grossly showed this trend (B, C: utrophin signal intensity for DMD 1; D: DMD 2; 0.05; CTRL 1, 0.04; CTRL 2, 0.05). MuscleMap™ sampled a subtle right shift with increased disease severity and beta-dystroglycan showed no difference in signal intensity between samples (data not shown). For utrophin, both signal intensity and the percentage of membrane expression of the biomarker (membrane completeness) varied both within sample and between sample types (B, D). To achieve a more comprehensive understanding of such complex expression patterns, Flagship has developed the 4CAHx analysis method. The 4CAHx method accounts for both membrane completeness and signal intensity for each myofiber to derive a summary score for individual images. These methods can clearly differentiate samples by taking into account multiple aspects of biomarker expression, as demonstrated for utrophin in C.

Conclusions

- Here, we have shown three duplex, ICH assays and IAA™ solutions that enable quantification of utrophin, beta-dystroglycan, and MHC in whole-slide images to robustly assess muscle health in DMD, BMD, and control biopsies.

- We have demonstrated that MuscleMap™ can measure biomarker expression and muscle fiber morphometrics in all muscle fibers in a biopsy in a quantitative fashion. This tool can aid pathologists in evaluating the disease-status of skeletal muscle biopsies and the efficacy of novel neuromuscular therapeutics.

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