Challenges and Variability in Dystrophin Detection: Immunofluorescence, Antibody Staining and Imaging Methodologies

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Existence and variability of trace dystrophin & revertants

Dystrophin intensity by a reproducible automated image analysis method

Limitations: variability, linearity, control, biopsy quality

Clinical study perspective on biopsy dystrophin analysis, next steps
Counting positive fibers that look like revertants is complicated

Revertant fibers: bright muscle fibers with high levels of dystrophin

DMD Exon 45-50

Ab15277 (C-terminal) MANDYS106 (exon 43)

detection of revertant fibers

Revertants without exon 43

Revertants with exon 43

Multiple types of revertant fibers present in a biopsy

Counting fibers that look like revertant fibers is difficult to interpret
Variability in trace dystrophin and revertant fibers in DMD

Dystrophin traces: low levels of expression in many fibers throughout a biopsy
  • Patches of low dystrophin-positive areas at the sarcolemma (Nicholson et al., 1990, 1993)

• Trace dystrophin observed in all patients
  (> 500 biopsies; 16 deletion mutations)

Essential
  • representative images capturing many fibers
  • measure dystrophin intensity in the entire membrane
The low trace signal detected in DMD is dystrophin specific a genetic control analysis

MANDYS106 (exon 43)  Isotype control

DMD del 45 (epitope present)

DMD del 43 (epitope absent)

Beekman et al 2014
Method captures the variation in dystrophin intensity between fibers in DMD

Staining & imaging
- revertants
- trace
- Dystrophin
- Spectrin

Automated image analysis
Definiens customised software

Dystrophin measurement
- Trace dystrophin
- dystrophin mean of population
- Revertants

- confocal microscopy (25x magnification)
- identification of individual fibers
  - minimum 90% fibers
  - generally 250 - 600 fibers / section
- measure per fiber dystrophin intensity
  - entire membrane for every fiber
  - operator independent, objective
  - high reproducibility

Beekman et al 2014 (PLoS One)
Reproducibility in dystrophin measurement in different DMD biopsies

- 4 DMD biopsy samples
- 2-3 experiments (over a 1.5 year period) providing inter-assay precision
- 2-4 sections analysed providing intra-assay precision
- 8 operators randomly involved in staining, imaging and image processing

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dystrophin membrane intensity MANDYS106 (a.u)</th>
<th>Ranking</th>
<th>Precision</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMD 3</td>
<td>255</td>
<td>241</td>
<td>208</td>
</tr>
<tr>
<td>DMD 1</td>
<td>286</td>
<td>298</td>
<td>228</td>
</tr>
<tr>
<td>DMD 4</td>
<td>306</td>
<td>427</td>
<td>354</td>
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<tr>
<td>DMD 5</td>
<td>406</td>
<td>-</td>
<td>397</td>
</tr>
</tbody>
</table>

Beekman et al 2014
Immunofluorescence can detect differences but is not quantitative

- Different control muscles and donors vary by 30%
- Negative control differentiates from DMD and is required
- Reproducible differences over a wide range of dystrophin intensity
- Linearity between intensity signal and dystrophin concentration cannot be established
- Relative comparison of dystrophin differences between biopsies is informative
Assay detects differences accurately in DMD

Dystrophin varies between DMD patients thus important to compare with pre-treatment biopsy per patient

Beekman et al 2014
Arechavala-Gomeza et al.
10 circles across membranes; measure min & max; divide by spectrin

Taylor et al.
mean membrane intensity per image; threshold approach based on spectrin; divide by spectrin

Beekman et al.
mean membrane intensity per fiber, not divided by spectrin
Analysis Methods are in agreement on the relative comparison between biopsies.

- Negative control (PBS) can be 50% of DMD trace values.
- Positive control dystrophin intensity vary for different muscles.
- Linearity cannot be established due to lack of dystrophin standards.

Relative comparison between biopsies is most informative.
Biopsies and DMD biomarkers in clinical programmes

Biopsies are valuable for assessment of disease progression and drug effect

- Dystrophin protein detection
- Exon skip
- Drug concentration in muscle
- Explore other biomarkers of disease

~500 biopsies from multicenter studies (80 sites) covering 16 different DMD deletions

- analysis of biopsies under Good Clinical Laboratory Practices for reliability and traceability

Potential for surrogate endpoints, but issues to consider:

- Biopsy sampling: which muscle, biopsy size, disease progression
- Quality of handling, shipping and storage (procedures and training)
Disease progression and heterogeneous sampling can affect analysis

**Healthy control**

**DMD**

**DMD - Extensive disease progression:**

- Sufficient number of muscle fibers required
- Small biopsies and low muscle content are prone to experimental artifacts
Freeze artefacts influence staining and ability to compare biopsies

<table>
<thead>
<tr>
<th>Quality</th>
<th>H&amp;E Staining</th>
<th>Spectrin Staining</th>
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</thead>
<tbody>
<tr>
<td>poor</td>
<td><img src="image" alt="H&amp;E poor" /></td>
<td><img src="image" alt="Spectrin poor" /></td>
</tr>
<tr>
<td>intermediate</td>
<td><img src="image" alt="H&amp;E intermediate" /></td>
<td><img src="image" alt="Spectrin intermediate" /></td>
</tr>
<tr>
<td>good</td>
<td><img src="image" alt="H&amp;E good" /></td>
<td><img src="image" alt="Spectrin good" /></td>
</tr>
</tbody>
</table>

No reliable comparison can be made between biopsies that differ in quality.

% of subjects with evaluable biopsies:
- phase II DMD114117 (13 sites) 88%
- phase II DMD114876 (13 sites) 66%
- phase III DMD114044 (46 sites) 50%
CONCLUSIONS

• Dystrophin immunofluorescence analysis using intensity measurements
  • dystrophin signal localisation at entire membrane
  • variable presence of trace dystrophin and revertant fibers
  • requires good quality biopsies
  • reproducibly and accurately detects differences between DMD biopsies
    - intra-assay precision typically <10%; inter-assay precision <25%

• Advancing immunofluorescence methods for quantification requires development and purified dystrophin protein control with a signal dilution relevant to DMD patients

• Further effort and development to correlate dystrophin to clinical outcome in randomized placebo controlled clinical trials is required
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- **DMD patients and families**