

Single section Western blot

Improving the molecular diagnosis of the muscular dystrophies

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Abstract—Single section Western blot (SSWB) is an improved methodology for molecular diagnosis of the muscular dystrophies, requiring only a single 8- μ m muscle biopsy cryosection for the simultaneous analysis of multiple disease candidates. The authors demonstrate that SSWB can be used for diagnosis of dystrophinopathies, to identify haploinsufficiency in autosomal dominant laminopathy, and as a tool to distinguish between primary and secondary immunohistochemical abnormalities in limb-girdle muscular dystrophy type 2B.

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The clinical and pathologic phenotype of the limb girdle muscular dystrophies (LGMD) is very similar. Therefore, diagnosis of specific subtypes relies predominantly on immunohistochemistry, and quantitative analysis of proteins by Western blotting is often essential to distinguish between primary and secondary immunohistochemical abnormalities. However, current immunoblot protocols require the solubilization of a significant portion of a muscle biopsy specimen (20 to 100 mg).^{1–3} We have developed an improved methodology for Western blot analysis, requiring only a single 8- μ m biopsy cryosection for the simultaneous analysis of multiple disease candidates for a range of human muscular dystrophies and myopathies. Such a small commitment of patient muscle tissue significantly increases the routine diagnostic potential of Western blot analysis for the molecular diagnosis of muscle disorders.

Materials and methods. *Antibodies.* Antibodies to the following proteins were used: dystrophin (NCL-DYS1), dysferlin (NCL-Hamlet), β -dystroglycan (NCL-b-DG), δ -sarcoglycan (NCL-d-SARC), lamin A/C (NCL-LAM-A/C), calpain-3 (NCL-CALP-2C4, NCL-CALP-12A2), and β -spectrin (NCL-SPEC1) (Novocastra Laboratories, Newcastle, UK); merosin (mAb1922, Chemicon, Temecula, CA); nebulin (NB2, Sigma, St. Louis, MO); caveolin-3 (C38320, Transduction Laboratories, Lexington, KY); dystrophin (Dys 6-10, provided by Dr. Louis Kunkel, Harvard University, Boston); α -actinin2 (4B2, provided by Dr. Alan Beggs, Children's Hospital, Boston); and myotilin (provided by Dr. Olli Carpen, Uni-

versity of Helsinki). The following secondary antibodies were used: horseradish peroxidase (HRP)-conjugated sheep antimouse and donkey antirabbit immunoglobulin G (IgG) (Amersham Biosciences, Uppsala, Sweden) and Cy3-conjugated goat antimouse IgG (Jackson Immunoresearch Laboratories, West Grove, PA).

Single section Western blot (SSWB) sample preparation. We studied muscle from patients with a range of genetically defined muscular dystrophies (see figures 1 through 3), as well as age-matched patient control muscle with normal histology (osteosarcoma amputees). The use of samples for this study was approved by the Children's Hospital at Westmead ethics committee. We optimized protein solubilization from muscle tissue in four lysis buffer mixtures varying both the sodium dodecyl sulfate (SDS) (detergent phase) and glycerol (aqueous phase) concentrations (solubilization studies not shown). Optimal solubilization was achieved using a lysis buffer (SSWB-LB) containing 4% SDS, 125 mM Tris pH 8.8, 40% glycerol, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 100 mM dithiothreitol (DTT), and bromophenol blue (BPB). Forty microliters of SSWB-LB per biopsy cryosection (8 μ m thickness, 10 mm² cross-sectional area) was shown to provide saturating solubilization conditions. Samples were sonicated, heated to 94 °C for 4 minutes, and briefly spun (3 min, 15,000 g) before loading. (In this study, 20 μ L muscle lysate was loaded per lane [5 mm² biopsy area]. However, for routine screening, we recommend ~10 mm² biopsy tissue loaded per lane, normalizing for myosin content.)

For comparison, muscle biopsy tissue was also solubilized according to currently used protocols.^{2,3} Briefly, 15 mg of muscle tissue was cryosectioned (~80 to 100, 10 mm², 8 μ m sections) and solubilized in 19 volumes (w/v; 285 μ L) of lysis buffer (125 mM Tris pH 8.8, 4% SDS, 4 M urea, 10% glycerol, 100 mM DTT, 0.5 mM PMSF, BPB).

SDS-polyacrylamide gel electrophoresis (PAGE) and Western blot. Samples were electrophoresed on 2 to 9% or 2 to 12% gradient SDS-PAGE gels (0.75 mm thickness) at 30 to 35 mA constant current with recirculating water cooled to 18 °C. A total of 10 mM 2-mercaptoethanol was added to the upper buffer chamber. Transfer efficiency was influenced significantly by current and temperature, and optimal results were achieved using 35 V constant voltage (~300 mA) overnight onto 0.45 μ m PVDF membranes

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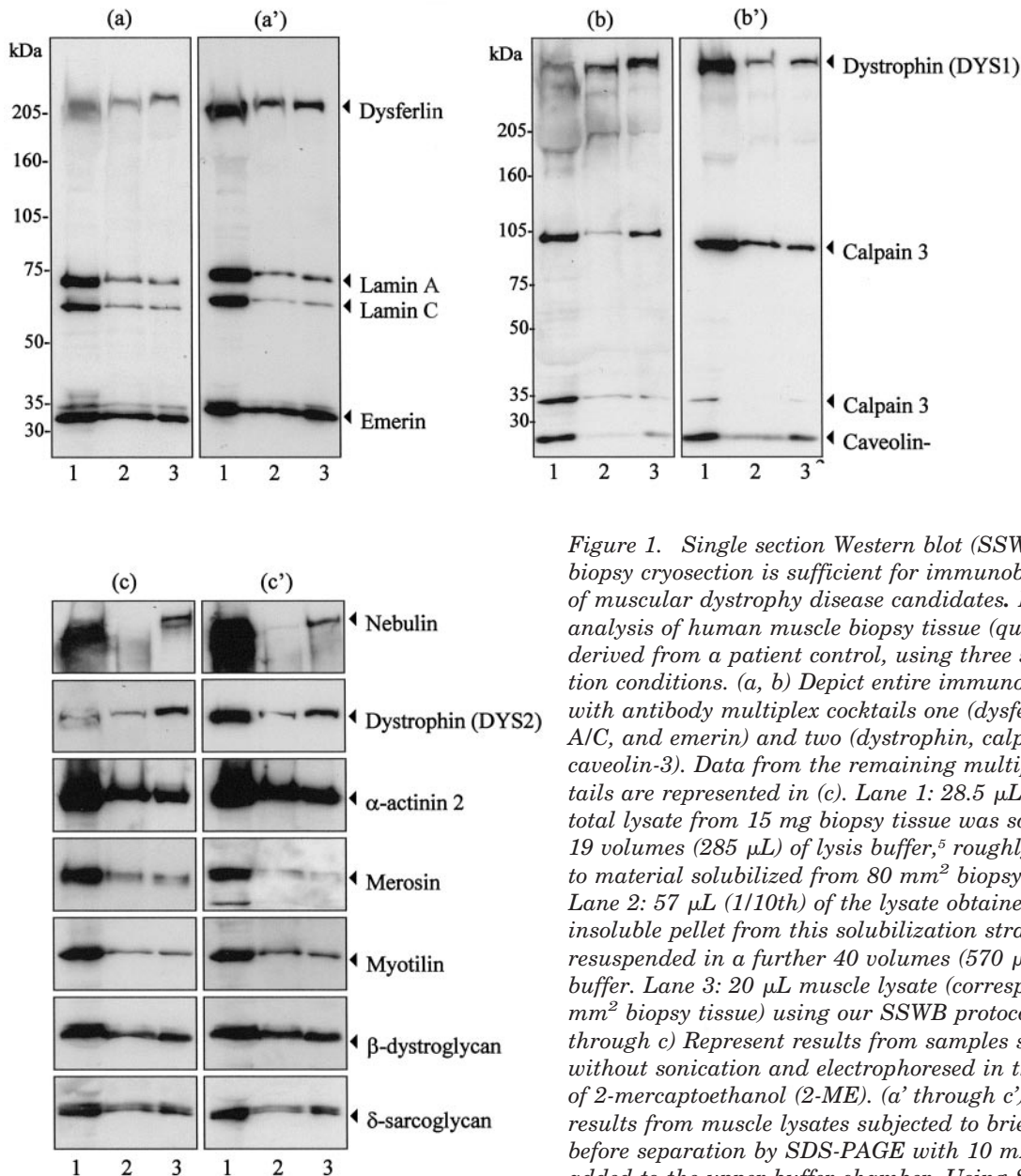


Figure 1. Single section Western blot (SSWB): a single biopsy cryosection is sufficient for immunoblot analysis of muscular dystrophy disease candidates. Immunoblot analysis of human muscle biopsy tissue (quadriceps) derived from a patient control, using three solubilization conditions. (a, b) Depict entire immunoblots probed with antibody multiplex cocktails one (dysferlin, lamin A/C, and emerin) and two (dystrophin, calpain-3, and caveolin-3). Data from the remaining multiplex cocktails are represented in (c). Lane 1: 28.5 μL (1/10th) the total lysate from 15 mg biopsy tissue was solubilized in 19 volumes (285 μL) of lysis buffer,⁵ roughly equivalent to material solubilized from 80 mm^2 biopsy tissue. Lane 2: 57 μL (1/10th) of the lysate obtained when the insoluble pellet from this solubilization strategy was resuspended in a further 40 volumes (570 μL) of lysis buffer. Lane 3: 20 μL muscle lysate (corresponding to 5 mm^2 biopsy tissue) using our SSWB protocol. (a through c) Represent results from samples solubilized without sonication and electrophoresed in the absence of 2-mercaptoethanol (2-ME). (a' through c') Represent results from muscle lysates subjected to brief sonication before separation by SDS-PAGE with 10 mM 2-ME added to the upper buffer chamber. Using SSWB gel

conditions, the band resolution of larger molecular weight proteins in samples derived using traditional solubilization protocols (lane 1) was poor, but was improved significantly by sonication of the sample (contrast a through c with a' through c'). The addition of 2-ME to the upper buffer chamber slightly improved the resolution of higher molecular weight proteins (a' through c'), in accordance with previous reports.⁴

(Millipore) in Towbin transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, 0.075% SDS), with stirring and recirculating water cooled to 18 $^{\circ}\text{C}$.

Blocked membranes were probed with antibody cocktails (see below) followed by HRP-conjugated secondary antibodies (α -rabbit^{HRP}@1:3000; α -mouse^{HRP}@1:2000), also diluted in blocking solution, for 2 hours at room temperature (RT). Membranes were developed with ECL chemiluminescent reagents using Hyperfilm ECL (Amersham Biosciences).

The following antibody cocktails were optimized, based on size of the protein and time for incubation, to allow simultaneous analysis of multiple proteins:

Cocktail 1: NCL-Hamlet@1:1000, NCL-Lam A/C@1:100, and NCL-Emerin@1:200 diluted in phosphate-buffered saline (PBS)-block (PBS, 5% skim milk powder, 0.1% Tween20); RT for 2 hours.

Cocktail 2: NCL-DYS1@1:300, NCL-CALP-2C4@1:200, and Cav3 C38320@1:1000 diluted in TBS-block (15 mM Tris pH8.0, 150 mM NaCl, 5% skim milk powder, 0.1% Tween20); 37 $^{\circ}\text{C}$ for 1 hour.

Cocktail 3: Nebulin NB2@1:1000, NCL-CALP-12A2@1:100, and NCL-d-SARC@1:50 diluted in PBS-block; 4 $^{\circ}\text{C}$ overnight.

Cocktail 4: NCL-DYS2@1:300, merosin mAb1922@1:800, and NCL-b-DG@1:400 diluted in PBS-block; RT for 2 hours. Improved results with Cocktail 4 may be achieved by incubation at 4 $^{\circ}\text{C}$ overnight, also incorporating β -spectrin NCL-SPEC1@1:200.

Cocktail 5: Dys6-10@1:50,000, α -actinin2 (4B2) @1:50,000, and myotilin@1:3000 diluted in PBS-block; RT for 2 hours.

Results. SSWB: a single biopsy cryosection is sufficient for immunoblot analysis of muscular dystrophy disease candi-

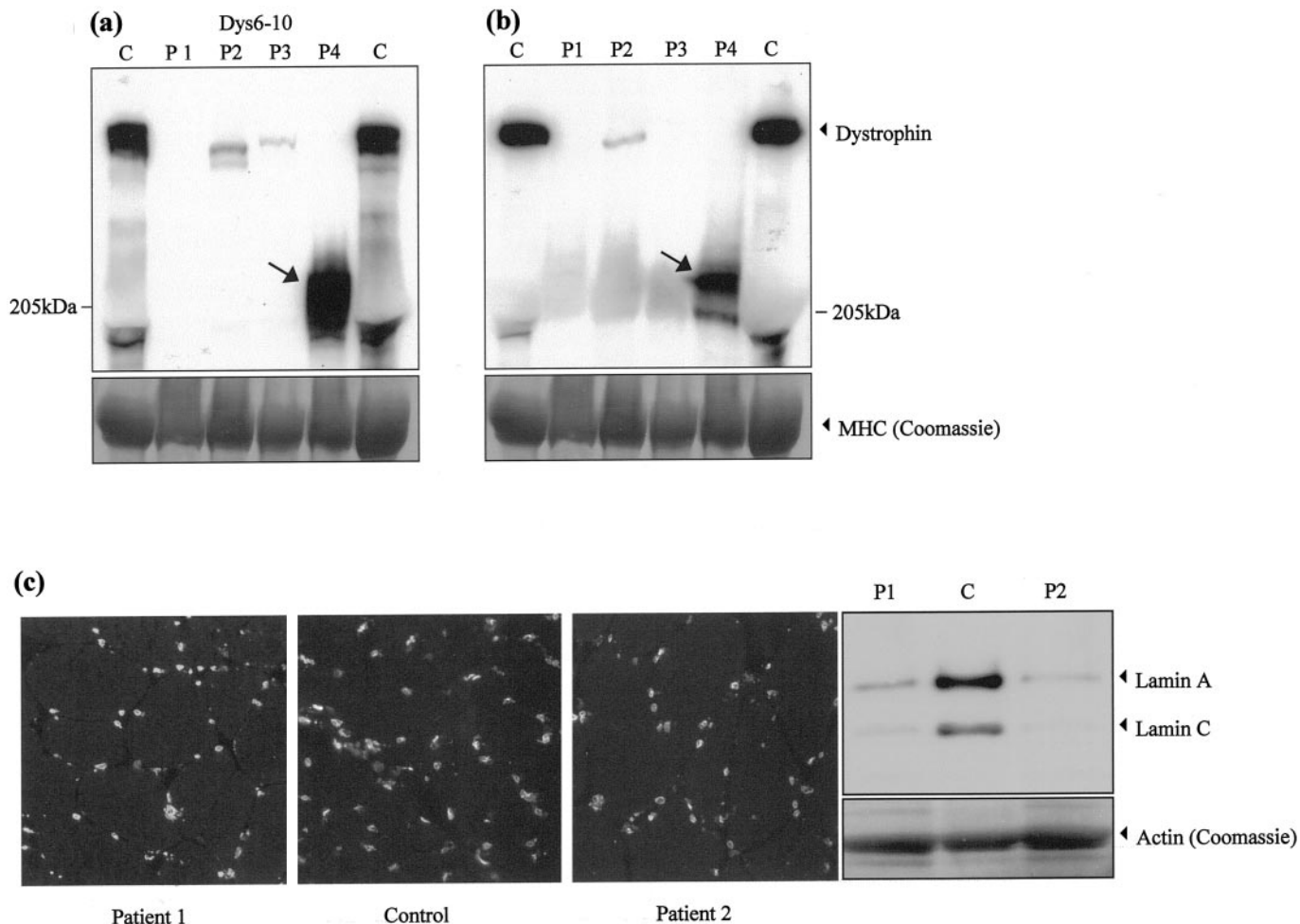


Figure 2. Single section Western blot (SSWB) can identify protein deficiencies, truncations, and reduced expression levels. Based on myosin content, equivalent amounts of solubilized patient biopsy samples (quadriceps) were loaded onto 2 to 9%, 0.75 mm, 16 cm acrylamide gels and transferred to PVDF membranes. The blot was sectioned and the two halves probed with either (a) Dys6–10 (rod domain) or (b) DYS2 (C-terminal dystrophin antibodies). Some nonspecific cross-reactivity with the large myosin band can be detected with DYS2. The gel is flanked by two samples from unaffected age-matched patient controls (c). Lanes 2 through 5 contain samples from dystrophinopathy patients (P1–4): Patient 1, Duchenne muscular dystrophy; Patients 2 through 4, Becker muscular dystrophy. Arrowhead: a dramatically truncated dystrophin protein product. Lower panels: bands corresponding to myosin heavy chain on the Coomassie-stained membrane demonstrate equal protein loading. (c) Immunohistochemical staining of lamin A/C in biopsy samples (quadriceps) from two siblings; Patient 1 (left), Patient 2 (right), and an unaffected patient control (middle) show normal labeling for lamin A/C at the periphery of the nuclear membrane. SSWB analysis of lamin A/C expression detects two bands of 70 kDa and 60 kDa corresponding to lamin A and C isoforms. A significant reduction in lamin A/C expression levels was detected in Patient 1 (P1, left lane) and Patient 2 (P2, right lane), compared with control (C, middle lane). Coomassie staining of actin on the PVDF membrane demonstrates equal protein loading.

dates. Solubilization of a single muscle cryosection is sufficient for detection of all muscle-specific proteins examined in this study, including many muscular dystrophy disease candidates (lane 3, figure 1, a through c). We also demonstrate that currently used protocols (lane 1, figure 1, a through c) result in inefficient solubilization of large or membrane-associated proteins, with significant quantities found in the insoluble pellet (lane 2, figure 1, a through c). This is likely due to saturation of the lysis buffer because of the extremely high protein content of muscle tissue.

SSWB can identify protein deficiencies, truncations, and reduced expression levels. Figure 2, a and b, illustrates the use of SSWB for diagnosis of the dystrophinopathies. Patients for this study were identified on the basis of ab-

normal dystrophin immunohistochemistry and dystrophin deletion analysis. SSWB confirms the diagnosis of Duchenne muscular dystrophy for Patient 1, owing to the absence of detectable dystrophin. Each of the patients with Becker muscular dystrophy (Patients 2 through 4) express reduced levels or truncated dystrophin isoforms. Deletion analysis confirmed a small deletion (Δ exons 3 through 6) for Patient 2 and a large deletion (Δ exons 3 through 34) for Patient 4. There was no detectable deletion for Patient 3, although our results suggest a C-terminal truncation (based on observations of a slightly smaller dystrophin product and the absence of immunoreactivity to DYS2, a C-terminal antibody).

Heterozygous mutations in lamin A/C, a nuclear enve-

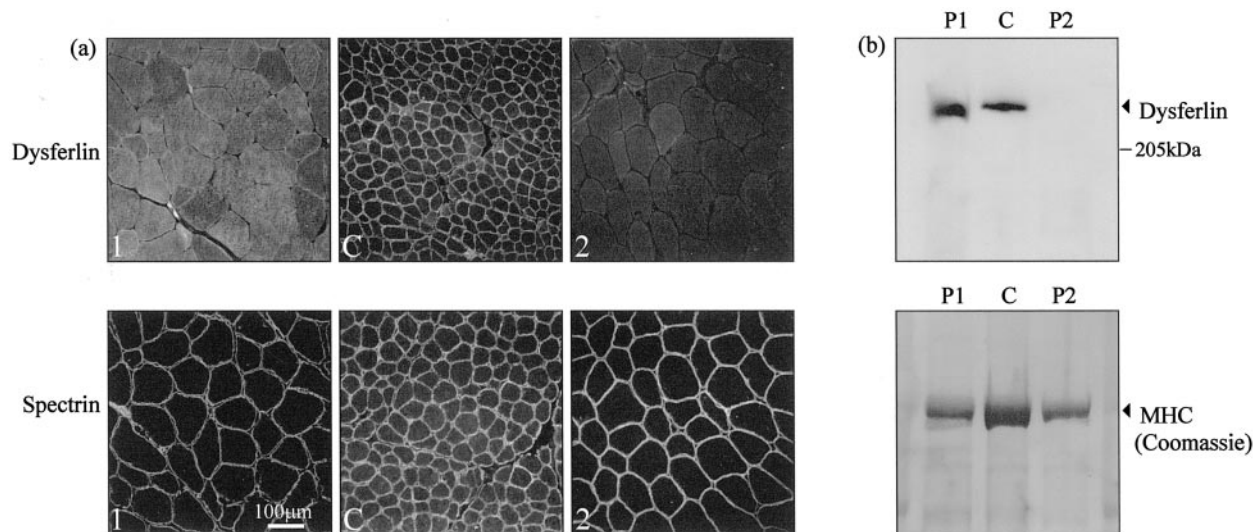


Figure 3. Single section Western blot (SSWB) helps distinguish between primary and secondary immunohistochemical abnormalities. (a) Immunohistochemical analysis of dysferlin expression in two patients with undiagnosed muscular dystrophy and an unaffected patient control. Muscle biopsy sections (deltoid) were stained with an antibody that recognizes dysferlin (NCL-Hamlet, upper panels) or spectrin (NCL-SPEC1, lower panels) followed by a Cy3-conjugated secondary antibody. Patient 1 (upper panel; left) shows faint cytoplasmic or negative staining in most fibers, with specific sarcolemmal staining detected only in occasional fibers. Patient 2 exhibits an absence of staining for dysferlin (upper panel; right). The control sample (middle) shows normal labeling for dysferlin at the sarcolemma of muscle fibers. Identical results were obtained using a second antibody to dysferlin (NCL-Hamlet-2, not shown). (b) SSWB analysis of dysferlin expression. Upper panel: the membrane was probed with an antibody that recognizes dysferlin (NCL-Hamlet), followed by a horseradish peroxidase-conjugated secondary antibody. A normal dysferlin band (230 kDa) was detected in Patient 1 (P1, left lane) and the control sample (C, middle lane). However, no dysferlin was detected in Patient 2 (P2, right lane). Lower panel: Coomassie staining of the PVDF membrane demonstrates equal protein loading for myosin.

lope protein, cause LGMD1B, dilated cardiomyopathy, and familial partial lipodystrophy.⁵⁻⁷ Figure 2c demonstrates that SSWB is sufficiently sensitive to detect lamin A/C haploinsufficiency in two female siblings with dilated cardiomyopathy and a confirmed lamin A/C mutation. Whereas lamin A/C immunohistochemical analysis results were normal, SSWB demonstrates a marked reduction in the levels of lamin A/C protein in the two patients compared with control.

SSWB can distinguish between primary and secondary immunohistochemical abnormalities. Autosomal recessive LGMD2B and its allelic variant, Miyoshi myopathy, are caused by mutations in the gene encoding dysferlin.^{8,9} Figure 3a demonstrates markedly abnormal dysferlin staining in muscle sections from two patients with a clinical picture suggestive of Miyoshi myopathy. SSWB of biopsy samples from each patient demonstrate normal levels of full-length dysferlin in Patient 1, and complete dysferlin deficiency in Patient 2 (figure 3b). Sequencing of the dysferlin gene identified a disease-causing mutation only in Patient 2. These results highlight that immunohistochemical abnormalities of dysferlin are frequently secondary,¹⁰ and that SSWB analysis is the diagnostic tool of choice for these patients.

Discussion. Definitive diagnoses of patients with inherited muscle disorders are essential for the provision of accurate prognostic and genetic counseling. We have rigorously optimized all parameters of protein solubilization, electrotransfer, and immunodetection for analysis of muscular dystrophy disease

candidates using a single biopsy cryosection (~10 mm²). The simple gradient gels employed in this study are well within the scope of most diagnostic and research laboratories. We currently use high throughput SSWB analysis to screen patients with genetically undefined LGMD, simultaneously screening for dystrophin, dysferlin, calpain-3, lamin A/C, emerin, and caveolin-3 on a single gel (not shown). To date, we have successfully analyzed samples from over 200 patients with muscle disease and identified Duchenne muscular dystrophy, Becker muscular dystrophy, dysferlinopathy, laminopathy, sarcoglycanopathy, merosin deficiency, and caveolin-3 abnormalities, with reproducible results. We have also used SSWB for the analysis of other muscle-specific proteins implicated in the congenital myopathies including nebulin, actin, tropomyosin, troponin, and desmin (not shown). Using this protocol, Western blot analysis is now a realistic and, in many cases, the preferred option for routine diagnosis of the muscular dystrophies, and potentially many muscle disorders where the disease gene is known.

References

- Hoffman EP, Fischbeck KH, Brown RH, et al. Characterization of dystrophin in muscle-biopsy specimens from patients with Duchenne's or Becker's muscular dystrophy. *N Engl J Med* 1988;318:1363-1368.
- Bushby KMD, Anderson LVB. Multiplex Western blot analysis of muscular dystrophy proteins. In: Bushby KMD, Anderson LVB, eds. *Methods in molecular medicine*, vol. 43: Muscular dystrophy: methods and protocols. Totowa, NJ: Humana, 2001;369-386.

3. Anderson LV, Davison K. Multiplex Western blotting system for the analysis of muscular dystrophy proteins. *Am J Pathol* 1999;154:1017–1022.
4. Blough ER, Rennie ER, Zhang F, Reiser PJ. Enhanced electrophoretic separation and resolution of myosin heavy chains in mammalian and avian skeletal muscles. *Anal Biochem* 1996;233:31–35.
5. Bonne G, Di Barletta MR, Varnous S, et al. Mutations in the gene encoding lamin A/C cause autosomal dominant Emery-Dreifuss muscular dystrophy. *Nat Genet* 1999;21:285–288.
6. Fatkin D, MacRae C, Sasaki T, et al. Missense mutations in the rod domain of the lamin A/C gene as causes of dilated cardiomyopathy and conduction-system disease. *N Engl J Med* 1999;341:1715–1724.
7. Shackleton S, Lloyd DJ, Jackson SN, et al. LMNA, encoding lamin A/C, is mutated in partial lipodystrophy. *Nat Genet* 2000;24:153–156.
8. Bashir R, Britton S, Strachan T, et al. A gene related to *Caenorhabditis elegans* spermatogenesis factor Fer-1 is mutated in limb-girdle muscular dystrophy type 2B. *Nat Genet* 1998;20:37–42.
9. Matsuda C, Aoki M, Hayashi YK, et al. Dysferlin is a surface membrane-associated protein that is absent in Miyoshi myopathy. *Neurology* 1999;53:1119–1122.
10. Piccolo F, Moore SA, Ford GC, Campbell KP. Intracellular accumulation and reduced sarcolemmal expression of dysferlin in limb-girdle muscular dystrophies. *Ann Neurol* 2000;48:902–912.

Anterior cervical spine fusion and sleep disordered breathing

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Abstract—The authors reviewed 12 patients who developed obstructive sleep apnea (OSA) syndrome in association with anterior cervical spine fusion. Four subsequent patients were studied prospectively before C2 to C4 anterior fusion and documented to have OSA by questionnaire, visual analogue scales, polysomnography, and multiple sleep latency tests. The authors found that placement of the anterior cervical plates reduced the size of the upper airway. Symptoms and objective findings were controlled with nasal continuous positive airway pressure.

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We report on the development of obstructive sleep apnea (OSA) after anterior upper spine fusion for discopathies. Twelve patients whose sleep-related breathing symptoms were associated with the operation alerted us to the problem. We prospectively documented the relationship between the surgery and the onset of OSA in four subjects followed for periodic limb movement syndrome (PLMS).

Methods. *Examinations and record review.* Patients received general and neurologic clinical evaluations. Medical records, x-rays, and MRI obtained before surgery and at follow-ups were reviewed.

Interviews and questionnaires. Subjects and bed partners received separate clinical sleep interviews. Both were asked to mark visual analog scales (VAS) (from 0 [nonexistent] to 100 mm [very severe]) on sleepiness, snoring, and fatigue. Each subject completed the Sleep Disorders Questionnaire¹ and the Epworth Sleepiness Scale (ESS).² For the retrospective study, subjects completed the VAS for time when subject was reported to be normal, now, and during nasal continuous positive airway pressure (CPAP) treatment. For the prospective subjects, these data were obtained at the initial visit, before and after surgery, and during CPAP treatment.

Polysomnography. Polysomnograms were performed three times in the retrospective study and five times in the prospective study with the same protocol, in an 8-hour nocturnal recording, with EEG (C3/A2, C4/A1, O1-O2), chin and leg EMG, electro-oculography, and electrocardiogram (modified V2 lead). Respiration was monitored using nasal-oral flow, initially with thermistors (n = 5) and later with nasal cannula pressure transducer³ and oral thermistor (n = 11); abdominal and thoracic movements, with inductive respiratory plethysmography bands; respiratory effort, with calibrated esophageal manometry⁴; breathing noise and snoring, with a neck microphone; intercostal muscle activity, with surface EMG; and oxygen saturation (SaO₂), with pulse oximeter (Nellcor, Alameda, CA). A five-nap multiple sleep latency test (MSLT)⁵ evaluated daytime sleepiness, twice or three times depending on retrospective or prospective status.

Follow-up for treatment trials. Patients received clinical CPAP follow-up at 1 month, 6 months, and upon request. Patients with PLMS were treated with dopamine agonists: initially pramipexole (0.25 mg), and pergolide (0.10 to 0.15 mg) when reports of fatigue were made.⁶

Data analysis. Subjective results from clinical interviews, questionnaires, VAS, and evaluations were tabulated. The international⁷ and American Sleep Disorders Association (ASDA) atlas criteria were used⁸ to determine sleep/wake distribution, short (>3-second) arousals, and PLM. Apnea and hypopnea were subdivided into central, obstructive, and mixed types. MSLT were scored based on ASDA recommendations.⁵

Statistical analysis. For the retrospective group, univariate repeated measures analysis was used to compare VAS scores for reconstructed (before surgery), baseline, and after 6 months of CPAP treatment. Wilcoxon signed rank test was used to compare 1) reconstructed VAS before surgery and baseline VAS and 2)

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