The American Journal of Pathology, Vol. 184, No. 8, August 2014

ELSEVIER

The American Journal of PATHOLOGY

ajp.amjpathol.org

MUSCULOSKELETAL PATHOLOGY

Abnormal Splicing of *NEDD4* in Myotonic Dystrophy Type 2

Possible Link to Statin Adverse Reactions

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Accepted for publication April 8, 2014.

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Myotonic dystrophy type 2 (DM2) is a multisystemic disorder caused by a $(CCTG)_n$ repeat expansion in intron 1 of CNBP. Transcription of the repeats causes a toxic RNA gain of function involving their accumulation in ribonuclear foci. This leads to sequestration of splicing factors and alters pre-mRNA splicing in a range of downstream effector genes, which is thought to contribute to the diverse DM2 clinical features. Hyperlipidemia is frequent in DM2 patients, but the treatment is problematic because of an increased risk of statin-induced adverse reactions. Hypothesizing that shared pathways lead to the increased risk, we compared the skeletal muscle expression profiles of DM2 patients and controls with patients with hyperlipidemia on statin therapy. Neural precursor cell expressed, developmentally downregulated-4 (NEDD4), an ubiquitin ligase, was one of the dysregulated genes identified in DM2 patients and patients with statin-treated hyperlipidemia. In DM2 muscle, NEDD4 mRNA was abnormally spliced, leading to aberrant NEDD4 proteins. NEDD4 was down-regulated in persons taking statins, and simvastatin treatment of C2C12 cells suppressed NEDD4 transcription. Phosphatase and tensin homologue (PTEN), an established NEDD4 target, was increased and accumulated in highly atrophic DM2 muscle fibers. PTEN ubiquitination was reduced in DM2 myofibers, suggesting that the NEDD4-PTEN pathway is dysregulated in DM2 skeletal muscle. Thus, this pathway may contribute to the increased risk of statin-adverse reactions in patients with DM2. *(Am J Pathol 2014, 184: 2322–2332; http://dx.doi.org/10.1016/j.ajpath.2014.04.013)*

Myotonic dystrophy type 2 (DM2; Online Mendelian Inheritance in Man 602668) is an autosomal dominant multisystemic disease with a highly variable phenotype, characterized by adult- or late-onset proximal muscle weakness, myalgia, myotonia, cardiac conduction defects, cataracts, insulin resistance, mild cerebral involvement, and liver enzyme elevation.[1,2] DM2 is caused by an uninterrupted $(CCTG)_n$ expansion of between 75 and 11,000 repeats in a polymorphic $(TG)_n(TCTG)_n(CCTG)_n$ repeat tract in intron 1 of the *CNBP* gene on chromosome 3q21.[3,4] Typical features of DM2 muscle histopathology include extreme atrophy in a subpopulation of type IIA fibers, some of them as nuclear clump fibers, and an increased amount of internal nuclei.[5] DM2 is a common form of muscular dystrophy in adults, at least in some European populations.[6] The mutation frequency is as high as 1 in 1830 in the Finnish population,[6] which suggests a clinical manifestation frequency of 1 in 5000. On the basis of the late

Supported by Folkhälsan Research Foundation, University of Helsinki, National Doctoral Program of Musculoskeletal Disorders and Biomaterials, Liv och Hälsa Foundation, Vasa Central Hospital District Medical Research funds Finnish Foundation of Cardiovascular Research (T.L.), Pirkanmaa Hospital District grant 9N035 (T.L.), Tampere Tuberculosis Foundation (T.L.), Emil Aaltonen Foundation (T.L.), NIH grant AR48171 (R.K.), a Muscular Dystrophy Association grant (R.K.), and a Kleberg Foundation grant (R.K.).

Disclosures: None declared.

http://dx.doi.org/10.1016/j.ajpath.2014.04.013

onset of the symptoms, more than one-half of mutation carriers are asymptomatic at any given time.

In contrast to the more severe myotonic dystrophy type 1 (DM1), there is no congenital form of DM2, and the age of onset and disease severity is not linked to the length of the repeat expansion.[7] DM2 pathogenesis has been shown to result from an RNA gain-of-function pathomechanism that involves sequestration of *trans*-acting nuclear proteins, such as muscleblind-like 1, which co-localizes with mutant RNA repeats in ribonuclear foci.[8] This leads to missplicing of a range of effector genes, including *INSR*,[9] *CLCN1*,[10] *BIN1*,[11] *CACNA1S*,[12] and other genes,[13,14] which likely contribute to the phenotypic features in patients with DM2. However, other mechanisms, such as decreased CCHC-type zinc finger, nucleic acid binding protein (CNBP) expression may also have a role in the DM2 pathology.[15]

Patients who take statins have a dose-related and time-dependent increased risk of adverse muscle reactions and rhabdomyolysis.[16] There is also a clear increase in the frequency of adverse events during combination therapy or if there is an underlying subclinical myopathy.[17] Patients with DM2 frequently have hyperlipidemia and often require statin treatment.[18,19] Among the patients diagnosed with DM2 in Finland, a larger than average proportion had statin-induced adverse muscle reactions, including the occasional rhabdomyolysis.

Certain polymorphisms in *SLCO1B1*[20] have been associated with an increased risk of statin-induced myopathy. Global expression profiling of skeletal muscles of patients without myopathy on statin treatment has shown changes in the calcium regulatory and the membrane repair machinery.[21] In addition, changes in the cholesterol metabolism pathways in muscle cell lines treated with statins have been reported.[22]

Our aim was to identify molecular factors that increase the susceptibility of patients with DM2 to statin-adverse muscle reactions by comparing the expression profiles in three different muscle biopsy sets. Expression profiles of DM2 muscle biopsies were compared with previously published DM2 muscle expression profiles[13] and profiles of patients with hyperlipidemia with no muscle disorders, taking simvastatin.[23] Our hypothesis was that similar molecular pathways would be affected in patients with DM2 and by statin treatment. In this combined analysis we found 21 genes with shared dysregulated expression. In addition, the prevalence and relevance of the risk-associated polymorphism in *SLCO1B1* was analyzed in a large Finnish DM2 cohort.

Materials and Methods

Patient Material and Expression Studies

All patients with DM1 and with DM2 were from Finland and were diagnosed by DNA mutation testing.[4] Altogether nine different DM2 patient samples were studied (clinical information is summarized in Table 1). The main clinical symptoms of the patients with DM2 consisted of muscle pain and stiffness, especially after exercise, and proximal muscle weakness that was more pronounced in the lower limbs. Only two patients had clinically detectable myotonia. Half of the patients had hyperlipidemia, but none had diabetes mellitus. Whole genome expression array analysis was performed on six DM2 and four control biopsies from the vastus lateralis muscle with the use of the Illumina expression array platform (Illumina, Inc., San Diego, CA) (Table 1). For the comparison, we included previously published expression studies on DM2 patient biopsies[13] and simvastatin-treated hyperlipidemic persons.[23] Biopsies of the patients with DM2 A and B (Table 1) were used both in the Illumina DM2 array study and in the previously reported Affymetrix DM2 study.[13] The study was approved by the institutional review board of Tampere University Hospital, and all patients gave written informed consent.

Microarray Expression Profiling

RNA Amplification and Labeling

Muscle biopsies were homogenized with ultra-turrax (IKA turrax, S8N-5 G), and total RNA was extracted with Trizol (15596-018; Invitrogen, Carlsbad, CA) and purified with the RNeasy kit (74106; Qiagen, Valencia, CA). The total RNA was treated with DNase (79254; Qiagen) according to the manufacturer's recommendations. One hundred nanograms of total RNA was amplified and biotinylated with the use of the Illumina RNA Total Prep Amplification kit (AMIL1791; Ambion, Austin, TX) for 14 hours.

Gene Expression Analysis

The Illumina gene expression array analysis was performed at the Microarray Center at Turku University by using 1.50 μg of sample RNA with Sentrix Human-6 Expression BeadChips version 2 (BD-25-113; Illumina, Inc.) at 58°C overnight (17 hours) according to Illumina whole genome gene expression with IntelliHyb Seal-protocol, revision B. The hybridization was detected with 1 μg/mL cyanine 3−streptavidine (PA43001; GE Healthcare Bio-Sciences Corp., Piscataway, NJ). The expression arrays were scanned with Illumina BeadArray Reader and analyzed with Bead Studio version 3.

The expression array raw intensity signals were analyzed with Inforsense KDE version 2.0.4 (Inforsense, London, UK) by using quantile normalization. This software was used for single-gene analyses, including fold-change calculations and filtering of the probes. Statistical significance was calculated with an unpaired *t*-test. Probes that did not reach statistical significance ($P \leq 0.05$) were removed from the data.

The microarray data are available from Gene Expression Omnibus (*http://www.ncbi.nlm.nih.gov/geo*; accession number GSE45331).

Pathway Analysis

Pathway changes were identified with the Database for Annotation, Visualization and Integrated Discovery ontology

Table 1 Patient and Control Biopsies Used in Illumina Gene Expression Analysis, RT-PCR, and Western Blot Analysis

Name	Sex/age at biopsy (years)	Method	Muscle symptoms	Clinical findings	Hyper-lipidemia	Diabetes
Myotonic dystrophy type 2						
Patient A	M/49	EA	Exercise-induced myalgia	Normal muscle strength, calf hypertrophy	Yes	No
Patient B	M/38	EA	Exercise-induced myalgia and weakness	Clinical myotonia, mild proximal lower limb atrophy	Yes	No
Patient C (*1)	F/34	EA	Muscle stiffness, clumsiness	Normal muscle strength, myotonia on EMG	No	No
Patient D (*2)	F/41	EA	Difficulties in climbing stairs, muscle stiffness	Proximal lower limb weakness, clinical myotonia	No	No
Patient E (*3)	M/55	EA	Exercise-induced myalgia, muscle stiffness	Proximal weakness and atrophy, calf hypertrophy, myotonia on EMG	No	No
Patient F (*4)	M/37	EA	Muscle stiffness	Mild proximal weakness, myotonia on EMG	Yes	Insulin resistance
DM2-A (*5)	M/44	PCR	Exercise-induced myalgia and weakness, muscle stiffness	Mild proximal weakness	NA	No
DM2-B (*4)	M/37	PCR	See patient F			
DM2-C	F/63	PCR	Mild bent spine syndrome	Axial and proximal weakness	Yes	No
DM2-D	M/51	PCR	Difficulties in climbing stairs, muscle stiffness	Proximal weakness, myotonia on EMG	NA	No
D1 (*3)	M/55	WB	See patient E			
D2 (*1)	F/34	WB	See patient C			
D3 (*2)	F/41	WB	See patient D			
D4 (*5)	M/44	WB	See DM2-A			
Controls						
Ctrl A	M/52	EA				
Ctrl B	M/45	EA				
Ctrl C	M/50	EA				
Ctrl D	M/54	EA				
C-1	M/79	PCR				
C-2	M/80	PCR				
C-3	F/U	PCR				
C1	U/>65	WB				
C2	U/>65	WB				
Myotonic dystrophy type 1						
DM1-A	M/34	PCR				
DM1-B	F/42	PCR				
DM1-C	M/50	PCR				
DM1-D	F/47	PCR				

*Overlapping biopsies indicating the same patient's biopsy was present in different experiments.

F, female; M, male; Ctrl, control; DM1, myotonic dystrophy type 1, DM2, myotonic dystrophy type 2; EA, expression array; EMG, electromyography; NA, not available; U, unknown; WB, Western blot analysis.

database (*http://david.abcc.ncifcrf.gov*, last accessed January 24, 2011) with KEGG (Kyoto Encyclopedia of Genes and Genomes) downstream annotations. Significant pathway enrichment in the DM2 expression data were calculated with EASE score (modified Fisher exact *t*-test) with multiple testing correction (Benjamini–Hochberg). The pathways were ranked with the EASE score *t*-test ($P \leq 0.05$).

SLCOB1 Genotyping

Genomic DNA was extracted from peripheral blood leukocytes by using the QIAamp DNA Blood Mini-kit. The *SLCO1B1* single nucleotide polymorphism (SNP) *rs4149056* was genotyped with TaqMan genotyping assays (Applied Biosystems, Foster City, CA). Random duplicates were used as controls.

RT-PCR and Quantitative Real-Time PCR

cDNA was generated with 1 μg of total RNA from myotube cultures or from muscle tissue by using the Trizol reagent (Invitrogen) according to the manufacturer's instructions. *In vitro* transcription was performed with random hexamers and oligo-(dT) priming according to the manufacturer's instructions (SuperScript III First-strand cDNA Synthesis Kit; Invitrogen). The PCR products were amplified with

DreamTaq master mix (EP0702; Fermentas, Burlington, ON, Canada) and were separated on an agarose gel. PCR products were identified by sequencing of representative DNA bands. Primer sequences are given in Table 2.

Quantification of the cDNA was performed with TaqMan-based quantitative real-time PCR by using *NEDD4* (Hs00406454_m1), *Gapdh (*Mm99999915_g1), and *GAPDH* (4333764F) primers and probes (Life Technologies, Carlsbad, CA). TaqMan master mix (10 mL; Applied Biosystems), 0.5 μL of 1:10 diluted cDNA, and 2 μL of primer and probe sets were used in a 20-μL total reaction volume. Amplification and detection were performed with the ABI 7500 system (Applied Biosystems). The PCR thermal conditions were 50°C for 2 minutes, 95°C for 10 seconds, and 60°C for 1 minute. Each sample was performed in triplicate and normalized to *GAPDH* by using standard curves for each gene on the same plate.

Western Blot Analysis

Muscle biopsies were prepared for SDS-PAGE by homogenization with 19 volumes of sample buffer that contained 4 mol/L urea and 4% SDS at 100°C for 5 minutes. Samples were resolved with 12% SDS-PAGE gels with 4% stacking gels and transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions.

Antibodies

Antibodies used in immunoblotting, immunofluorescence (IF), and immunohistochemistry (IHC) are rabbit monoclonal anti−phosphatase and tensin homologue (PTEN; IF and IHC dilution 1:100; 6H2.1; Millipore, Billerica, MA), rabbit polyclonal anti-ubiquitin (dilution 1:150; Z0458; Dako UK Ltd., Ely, UK), mouse anti-dystrophin (dilution 1:50; Dy4/6D3; Novocastra, Newcastle, UK), polyclonal rabbit anti-NEDD4 (dilution 1:1000; NBP1-03462; Novus Biologicals, Inc., Littleton, CO), mouse anti-GAPDH (dilution 1:20,000; ab8245; Abcam, Cambridge, MA), and rabbit monoclonal anti-MBNL1 (dilution 1:1000; ab108519; Abcam). Primary antibodies were detected with secondary horseradish peroxidase−conjugated antibodies (dilution 1:100; DAKO P260; DakoCytomation, Glostrup, Denmark) and enhanced chemiluminescence with the Immun-Star kit (Bio-Rad Laboratories). Secondary horseradish peroxidase−conjugated antibodies were diluted at 1:1000 in Western blot analysis.

Table 2 A List of Primers Used in RT-PCR Analysis

Oligonucleotide	Sequence
NEDD4 006-1L	5′-CTCCTCCTCCTCCACAGTTG-3′
NEDD4 006-1R	5′-CGGTGCTGCTGAGGATGA-3′
NEDD4	Fwd: 5′-TTGCAGCAACAACAAGAACC-3′
	Rev: 5′-GCAAGTCCAGGCGATTAAAA-3′
Nedd4	Fwd: 5′-CACAGTGGAAAAGGCCAAGC-3′
	Rev: 5′-ACCTGGTGGCAATCCAGATG-3′
GAPDH	Fwd: 5′-TTAGCACCCCTGGCCAAGG-3′
	Rev: 5′-CTTACTCCTTGGAGGCCATG-3′

Fwd, forward; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NEDD4, neural precursor cell expressed, developmentally down-regulated 4, E3 ubiquitin protein ligase; Rev, reverse.

Cell Culture and IP

Cell culture of biopsies was performed as described previously.[15] Immunoprecipitation (IP) assays of PTEN were done according to the manufacturer's instructions (Pierce cross-linking IP kit; Thermo Fisher Scientific, Inc., Waltham, MA). PTEN was captured with the anti-PTEN antibody (dilution 1:100; ab32199; Abcam) and identified by Western blot analysis as described above.

IHC and IF

DM2 muscle biopsies ($n = 6$) and control muscle biopsies ($n = 2$) were snap frozen in liquid nitrogen-cooled isopentane for 6 μm cryosections on SuperFrost. IHC staining was performed with the NovoLink Min Polymer detection system (reference RE7290-K; Leica Microsystems, Milton Keynes, UK) by using the aforementioned antibodies. IF was performed as previously described[15] by using Alexa Fluor 680 donkey anti-mouse (A10038; Invitrogen) and Alexa Fluor 405 goat anti-rabbit (A31556; Invitrogen) secondary antibodies (dilution 1:1000). Fluorescence was detected with an Axioplan Imager 2 microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany) with a high-resolution, cooled camera. AxioVision version 4.6 (Carl Zeiss) was used for image acquisition.

Cell Culture and Simvastatin Treatment

Simvastatin (S6196; Sigma-Aldrich, St. Louis, MO) was converted into the active acid according to the manufacturer's instructions. C2C12 myoblasts were grown in Dulbecco's modified Eagle's medium (Gibco 41965; Invitrogen) with high glucose (4.5 g/L) and supplemented with 10% fetal bovine serum, 50 U/mL penicillin, and 50 μg/mL streptomycin. Myoblasts were seeded in T25 flasks for 2 days until fully confluent and differentiated in Dulbecco's modified Eagle's medium with high glucose that was supplemented with penicillin, streptomycin, and 2% horse serum. After 1 week simvastatin-containing media were added to the myotubes (no treatment, ethanol vehicle, 1 μmol/L, and 10 μmol/L) in quadruplicate. The cells were harvested after 6 hours, and RNA was extracted with an RNeasy kit (Qiagen). cDNA was then produced, and PCR products were amplified with mouse primers. Cells were also harvested after 2 days of simvastatin treatment, and total protein was extracted for Western blot analysis. Band intensities were quantified with ImageJ version 1.46f (NIH, Bethesda, MD). Significance of changes between treatment groups was calculated with a two-tailed equal variance *t*-test.

Table 3 The Frequency of the *rs4149056* SNP Allele in Finnish Patients with DM2

Genotype	T/T [% (*n*)]	C/T [% (*n*)]	C/C [% (*n*)]
Finnish population	63.9	31.8	4.3
Patients with DM2 ($n = 126$)	57.1 (72)	38.9 (49)	4.0 (5)
Patients with DM2 with statin complications ($n = 6$)	33 (2)	66 (4)	0

The normal Finnish population frequency has been previously published.[1] The *SLCO1B1* c.521 TC and c.521 CC genotypes are associated with a significantly increased risk of statin-induced myopathy compared with the c.521 TT genotype. The c.521 CC genotype confers the highest risk of adverse reactions.

DM2, myotonic dystrophy type 2; SNP, single nucleotide polymorphism.

Plasmids for shRNA expression that target all protein-coding isoforms of MBNL1 and a scramble control were purchased from GeneCopoeia (Rockville, MD). Two combined shRNAs were used that contained the target sequence n7 (5′-CAATTGCAACCGAGGAGAA-3′) or n8 (5′-AGATCAAGGCTGCCCAATA-3′) in the HSH011081-7-mU6 vector. HEK293T cells were plated on 6-well plates at 150,000 cells per well and transfected with 2 μg of combined MBNL1 shRNA plasmids or a scramble control (CSHC TR001-mU6) by using FuGene 6 (Promega, Madison, WI) and OptiMem (Life Technologies) according to the manufacturer's instructions. Cells were harvested 72 hours after transfection for Western blot analysis and RNA extraction.

Results

Clinical Observation of Incidence

Eight patients referred to the Neuromuscular Research Center (Tampere University and University Hospital, Finland) because of simvastatin-induced muscle symptoms (muscle pain/weakness and elevated levels of creatine kinase, including one case of rhabdomyolysis) proved to have DM2 on genetic examination. In the same 2004 to 2007 period, a total of 89 patients were diagnosed with DM2. Twelve of these patients were diagnosed in family studies, 77 were primary referrals of undetermined muscle disease, and among them were the eight patients with simvastatin-adverse reactions. Thus, 10% of the primary referred patients who proved to have DM2 had simvastatin-induced symptoms as the main cause of referral. During the 2008 to 2009 period, 19 further patients were referred for neuromuscular evaluation because of statin-induced muscle symptoms, of which 4 patients (21%) had a subsequent genetic diagnosis of DM2.

Risk-Associated *SLCO1B1* SNP Frequency in Patients with DM2

Genotyping of the known myopathy-associated SNP *rs4149056* in *SLCO1B1*[20] was performed in six of the eight patients with DM2 referred for primary statin-induced muscle reactions, including one patient with rhabdomyolysis. In addition, a second larger cohort of 126 patients with DM2 was also included. No correlation was found between the *C/C* genotype and statin susceptibility in patients with DM2, at least for the highest risk of adverse reaction (Table 3).

Microarray Gene Expression Profiling

Shared expression changes were identified in the expression profiles of DM2 muscle biopsies and in persons who were on statin medication because of hyperlipidemia. The expression

Table 4 Summary of the DAVID Ontology Analysis

KEGG pathway term	Percent (%)*	Fold enrichment	EASE *P* value	BH *P* value
hsa04120: Ubiquitin-mediated proteolysis	1.13	1.65	0.000003	0.0006
hsa04510: Focal adhesion	1.51	1.51	0.000005	0.0005
hsa04310: Wnt signaling pathway	1.16	1.54	0.000036	0.0023
hsa04210: Apoptosis	0.74	1.70	0.000073	0.0036
hsa05200: Pathways in cancer	2.17	1.33	0.000109	0.0043
hsa05016: Huntington's disease	1.28	1.42	0.000319	0.0104
hsa04010: MAPK signaling pathway	1.78	1.34	0.000327	0.0092
hsa04722: Neurotrophin signaling pathway	0.93	1.49	0.000618	0.0151
hsa04520: Adherens junction	0.62	1.62	0.001053	0.0228
hsa03018: RNA degradation	0.49	1.71	0.001464	0.0284
hsa05210: Colorectal cancer	0.66	1.56	0.001662	0.0293
hsa05010: Alzheimer disease	1.13	1.39	0.001750	0.0283
hsa04144: Endocytosis	1.25	1.36	0.001970	0.0294

DAVID ontology analysis with KEGG pathway indicates significantly dysregulated pathways in DM2 muscles expression profile versus controls by using an EASE score of gene enrichment analysis. Multiple testing corrections were applied to the EASE score, and a threshold ($P \leq 0.05$) was applied. A minimum threshold of two genes for a given term was used.

*Number of genes involved in a term/total genes of the pathway.

BH, Benjamini–Hochberg; DAVID, Database for Annotation, Visualization and Integrated Discovery; EASE score, modified one-tailed Fisher exact *t*-test; KEGG, Kyoto Encyclopedia of Genes and Genomes; MAPK, mitogen-activated protein kinase.

array analysis of DM2 muscle biopsies by using the Illumina platform did not provide identical significant differences for some of the genes that were reported in our previous Affymetrix-based microarray study.[13] The differences observed between the two platforms may be due to the different probe sets recognizing different transcript isoforms. Because of these differences, some of the expression profile leads were verified in DM2 muscle samples by RT-PCR, with the aim of identifying aberrantly spliced genes. There were 33 genes that were abnormally expressed in both the DM2 expression profiling and in expression profiling of persons taking statins compared with controls (Supplemental Table S1). Of the 33 genes identified, four (*BIRC3*, *NEDD4*, *UBE2D1*, and *USP6*) belonged to the ubiquitin proteasome pathway.

Pathway Analysis

Pathway analysis indicated that the ubiquitin-mediated proteolysis pathway was the most severely affected, both ranked by significance and by representation in the DM2 expression array pathway analysis (Table 4).

NEDD4 Analysis

The DM2 muscle expression analysis indicated an increase in ubiquitin ligase NEDD4 mRNA at the probe level (Affymetrix 213012_at; fold change, 1.72; $P = 0.00007$). This increase was also found by quantitative real-time PCR analysis of the main NEDD4 isoform in DM2 muscles (Figure 1A). The total protein level of NEDD4 did not appear to change in IHC (data not shown) or by Western blot analysis (Figure 2). However, two previously unreported splice isoforms of NEDD4 were identified by RT-PCR analysis. A smaller isoform of NEDD4 was shown to be present in DM2 and DM1 biopsies but not in controls (Figure 1B). The total level of cDNA in samples was measured by GAPDH levels (Figure 1B). This splice isoform was found to be in frame but lacking 16 full exons and parts of exons 5 and 22 (002, ENST00000508342) (Figure 1C). By Western blot analysis, a NEDD4-positive band of approximately 75 kDa was identified only in the DM2 samples (Figure 2), which could result from alternative splicing of transcripts NEDD4-002 (ENST00000508342) or NEDD4-003 (ENST00000506154) with a theoretical size of 80 kDa and 78 kDa, respectively. In addition, a second novel short isoform was present in DM2 biopsies but not in controls or DM1 biopsies (Figure 1B). This isoform was missing all of exon 3 and 4 of the NEDD4-006 (ENST00000435532) transcript, causing a frameshift that lead to 18 new amino acids and a premature stop codon (all accessed from Ensemble, *http://www.ensembl.org/index.html*). The level of the full-length NEDD4-006 isoform in DM2 biopsies was decreased compared with controls (Figure 1B).

MBNL1 Silencing

To further validate the abnormal expression of the two novel isoforms of NEDD4 we knocked down MBNL1 with the

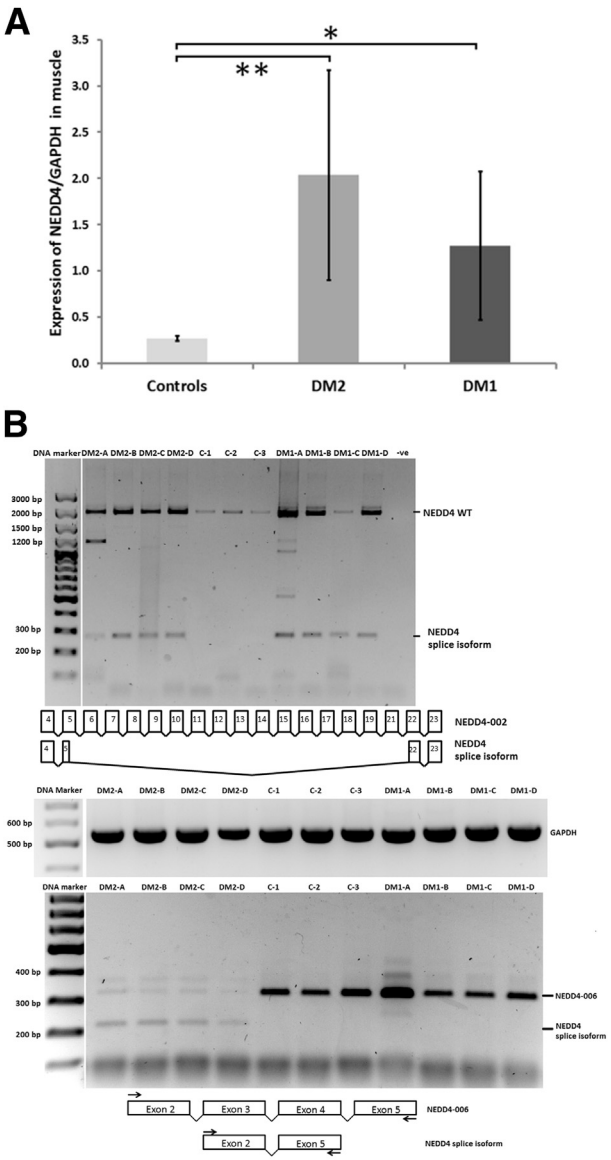

Figure 1 **A:** Quantitative PCR analysis of NEDD4 expression in DM2, control, and DM1 muscle cDNAs. The primers annealed to a region present in all of the protein coding NEDD4 isoforms (001 to 006; Ensembl). **B:** The NEDD4 forward × reverse primers set bind and amplify the native full-length NEDD4 isoforms (2033-bp bands) and another novel NEDD4 splicing form (274-bp band) that was confirmed by sequencing. GAPDH shows similar levels of expression between samples. RT-PCR analysis of the NEDD4-006 (ENST00000435532) transcript in DM2, control, and DM1 muscle biopsies. The NEDD4 006-1L and 006-1R primer set bind and amplify the native NEDD4-006 transcript (327 bp) and a novel (207 bp) extra band. The band was confirmed by sequencing to be a novel splice isoform that was missing all of exon 3 and 4, leading to a change in reading frame, 18 new amino acids, and a premature stop codon. **C:** The short isoform-specific splice site locations in the NEDD4-002 (ENST00000508342) transcript in **B**. Expressed sequence is in bold. Data are expressed as means ± SEM. $*P \leq 0.05$, $**P \leq 0.01$, *t*-test. WT, wild type.

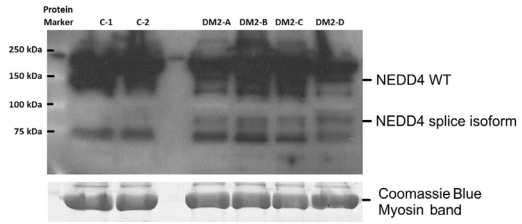

Figure 2 Western blot of DM2 (DM2, 1 to 4) and control (C1 to C2) muscle biopsy extracts stained with NEDD4 and PTEN antibodies. WT NEDD4 isoforms are predicted from Ensembl to be 149, 147, 141, 105, and 104 kDa in size. A DM2-specific NEDD4 band (approximately 75 kDa) corresponding to the aberrant DM2 transcript was present in all four patient biopsies. Protein loading is shown by the myosin band in Coomassie Blue staining. WT, wild-type.

use of shRNAs in HEK293T cell cultures. Silencing of MBNL1 (Figure 3A) led to a significant increase in the level of the novel NEDD4-006 isoform missing exon 3 and 4 compared with the scramble controls (Figure 3B). The small NEDD4 isoform missing 16 full exons (Figure 1B) was not significantly different from the controls after MBNL1 silencing (not shown).

PTEN Analysis

To check for possible downstream effects of NEDD4 abnormality in DM2 muscle, we studied the NEDD4 ligand PTEN. The original DM2 muscle expression analysis indicated an increase in PTEN mRNA levels (Affymetrix 225363_at; fold change, 1.5; $P = 0.01$). IF staining of DM2 biopsies showed clear accumulation of PTEN in the highly atrophic type IIA fibers (Figure 4, A–D), including the nuclear clump fibers. IHC confirmed that the increased PTEN signal was not due to multichannel-fluorescent lipofuscin (Figure 4, E and F). The total PTEN level showed a trend toward being increased in DM2 myotube cultures compared with controls in Western blot analysis (Figure 5A), but it did not reach significance.

PTEN Ubiquitination

We performed IP of PTEN from DM2 patient and control myotube culture extracts. The level of ubiquitinated PTEN was decreased in the DM2 pull-downs compared with the control samples when total PTEN was normalized (Figure 5B).

Simvastatin Treatment of Skeletal Muscle Cells

The expression profiling of hyperlipidemic persons on statin medication showed a decrease in the NEDD4 transcript at the probe level (Illumina 3060017; fold change, −1.59; $P = 0.03$). C2C12 myotube cultures showed a significant dose-dependent reduction of NEDD4 mRNA expression compared with the vehicle controls after 6 hours of simvastatin treatment (Figure 6A). After 2 days of simvastatin treatment, C2C12 myotube cultures showed a significant dose-dependent increase in the PTEN protein levels compared with vehicle controls (Figure 6B). Decreased NEDD4 levels have been shown to cause decreased PTEN ubiquitination and thereby increase PTEN levels.[24] We therefore performed IP of PTEN from the C2C12 cell extracts to enable measurements of its ubiquitination. Once total PTEN was normalized, the simvastatin-treated cells showed reduced ubiquitination of PTEN compared with untreated cells (Figure 6C).

Discussion

Patients with DM2 are frequently diagnosed with hyperlipidemia, and many are therefore prescribed statin medication. The observed increase of statin-adverse muscle reactions that cause medical attention among patients with DM2 did, however, not appear to be linked to the co-segregation of the reported statin-adverse reaction risk *C*-allele of SNP *rs4149056*[20] in Finnish patients with DM2. Therefore, some other molecular factor(s) apparently underlie the increased susceptibility of statin-provoked muscle

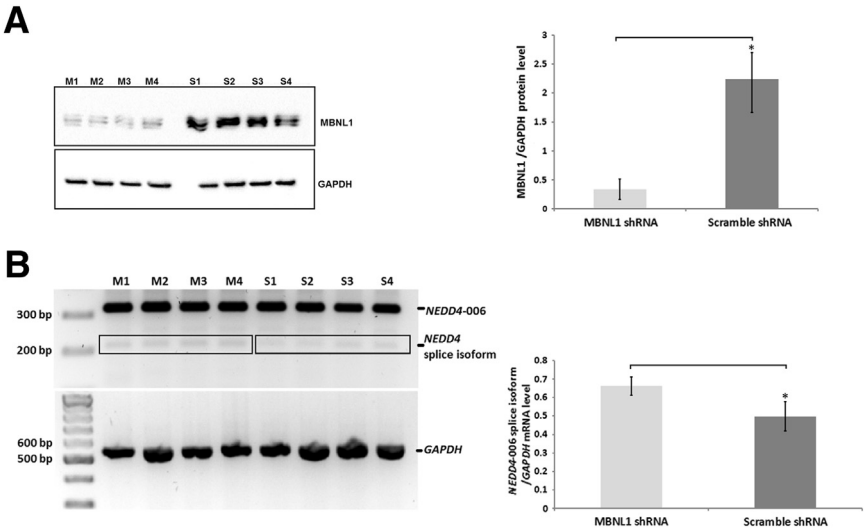

Figure 3 HEK293T cell cultures were transfected with two combined vectors that express shRNA against a region of MBNL1 that is present in all protein coding isoforms (M1 to M4) or a scramble control (S1 to S4). **A:** Western blot analysis of MBNL1 shRNA– and scramble shRNA–treated protein extracts, normalized to GAPDH, shows an 80% decrease in MBNL1 protein levels. cDNA was produced from RNA extracted from the same transfected HEK293T cell cultures and amplified with the NEDD4 006-1L and 006-1R primer set. **B:** MBNL1 shRNA–transfected cell culture shows a significant increase in the level of the novel NEDD4-006 splice isoform (missing exon 3 and 4 are shown in the **box**) after normalization to GAPDH. Data are expressed as means ± SEM. $^*P \leq 0.05$, *t*-test.

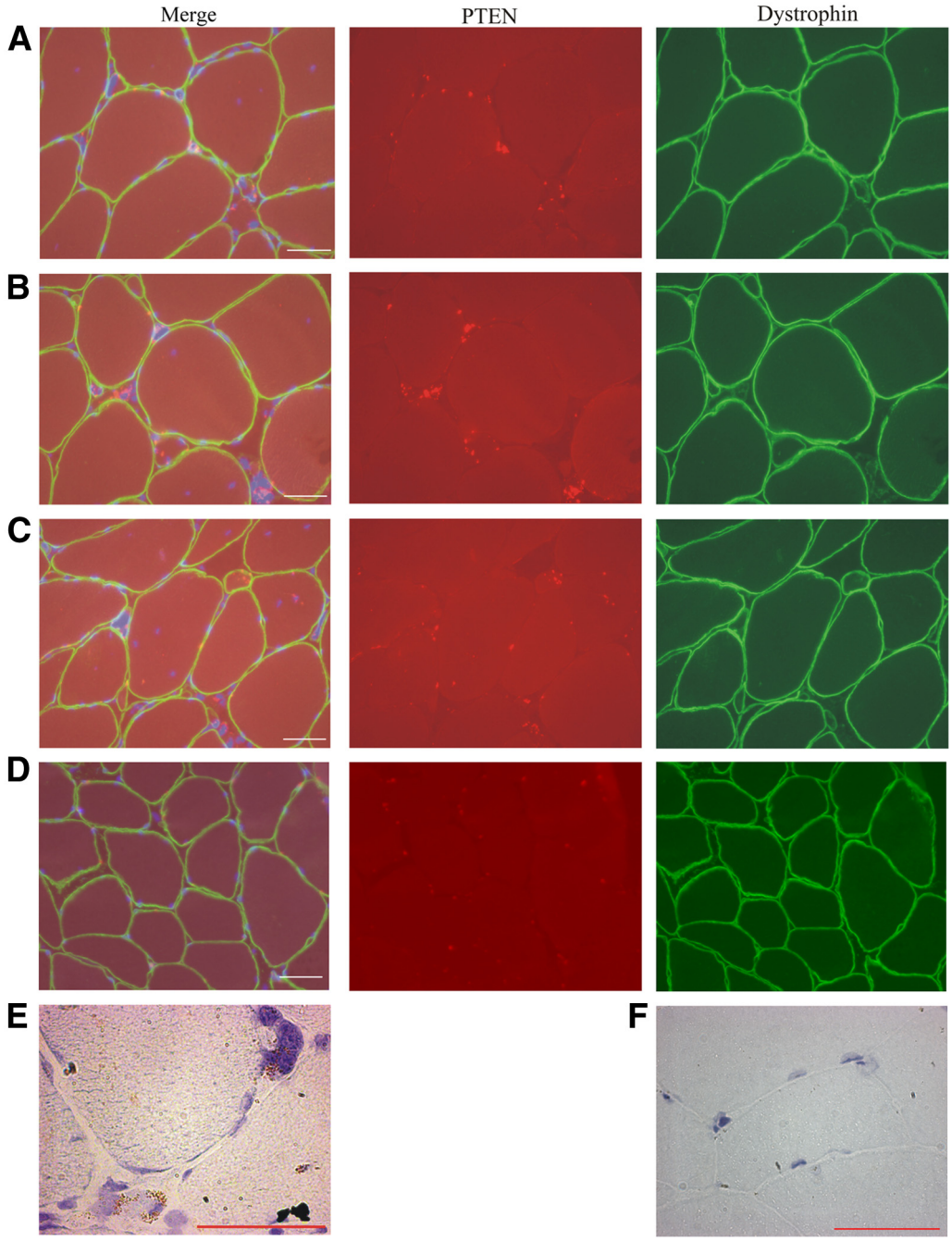

Figure 4 IF staining for PTEN (red), dystrophin (green), and DAPI (blue) in three different DM2 muscle biopsies (**A**–**C**) and a control (**D**). IHC of PTEN and hematoxylin counterstain in a patient with DM2 (**E**) and a control (**F**). Both techniques show accumulation of PTEN in nuclear clump fibers and highly atrophic fibers in DM2 samples. Scale bars: 50 μm (**A**–**F**).

reactions in patients with DM2. Statin-induced myopathy in the general population has been proposed to have an immune-mediated mechanism.[25] However, all our patients with DM2 with statin-adverse reactions underwent muscle biopsy, and no evidence of immune reactivity was identified by major histocompatibility class 1 IHC (not shown).

By expression profiling analysis, we found dysregulation in ubiquitination pathway components in the DM2 biopsies. Increases in expression of components in the ubiquitin-proteasome pathway have previously been described in muscle of persons who are exercising and taking statins[26] and in persons with statin-induced adverse reactions.[27] The up-regulation of genes involved in the ubiquitin-proteasome pathway have been suggested to be a response to myofibrillar damage,[28] inducing ubiquitination of proteins targeted for proteasome degradation.

The expression profiling of persons treated with statins, but with no muscle disorders, indicated that statin treatment

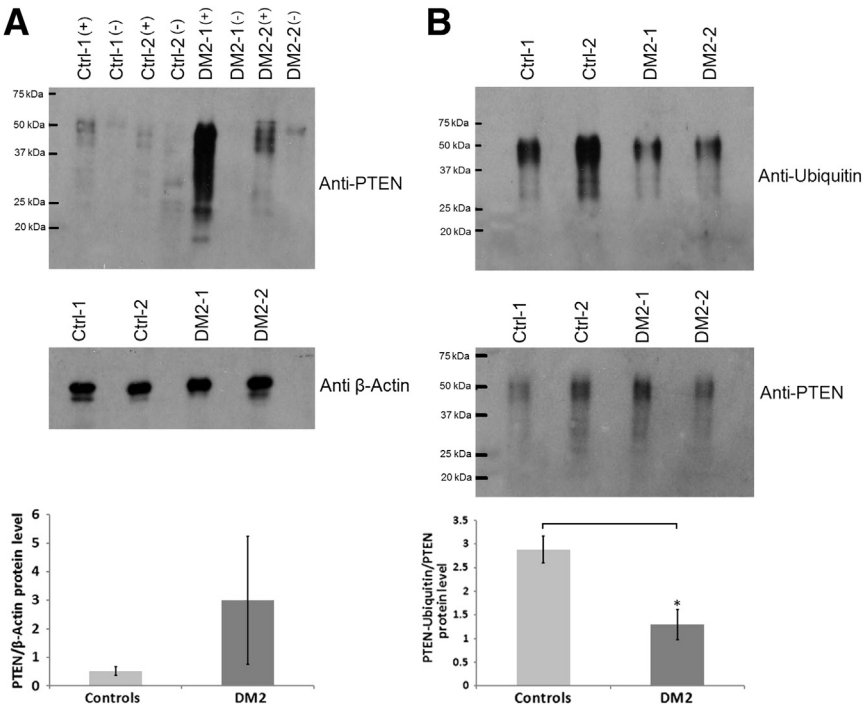

Figure 5 PTEN IP assay from DM2 and control myotube culture extracts. **A:** Background IP levels are shown by the (−) lanes. The (+) lanes indicate the presence of the primary antibody. Total protein levels were measured from β-actin immunostaining of myotube protein extracts, and the same amount of IP extract was then PTEN stained. **B:** PTEN levels were determined by staining of IP extracts, and the same amount of PTEN IP extract was then ubiquitin stained. Data are expressed as means ± SEM. $*P \leq 0.05$, *t*-test. Ctrl, control.

represses transcription of NEDD4 in human muscle tissue. Here, we confirm that simvastatin treatment causes repression of NEDD4 transcription in mouse myotube cultures. Because NEDD4 is a negative regulator of PTEN,[24,29] this result is in agreement with our results showing that simvastatin increases PTEN protein levels in C2C12 myotubes. NEDD4 is an E3 ubiquitin-protein ligase that transfers ubiquitin from an E2 ubiquitin-conjugating enzyme to target substrates. Ubiquitinated substrates are then transferred to the proteasome for degradation.[30] Alterations in the expression of NEDD4 may therefore directly affect the cellular level of its substrates, such as PTEN.[24,29] The importance of NEDD4 in muscle is supported by *Nedd4*$^{+/-}$ knockout mice, which have muscle weakness and histologically show a marked reduction in skeletal muscle fiber size and increased amount of internal nuclei.[31]

Our results indicate that the expression of NEDD4 is increased and abnormally spliced in DM2 muscle tissue. The increased level of NEDD4 mRNA in DM2 muscles may be due to its role in the ubiquitin/proteasome pathway

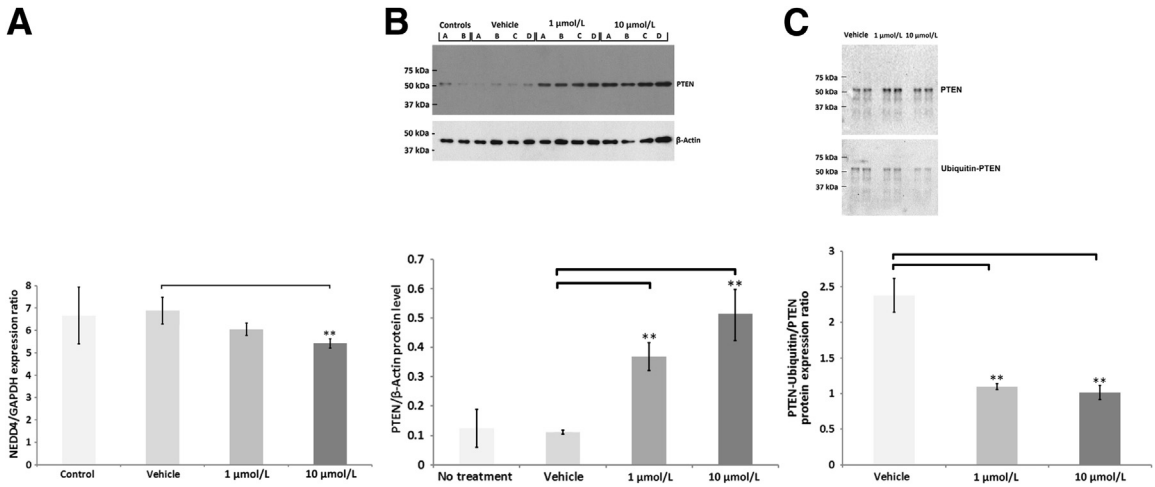

Figure 6 C2C12 myotube cultures were treated with simvastatin (no treatment, vehicle, 1 μmol/L, and 10 μmol/L) for 6 hours. **A:** The NEDD4/GAPDH expression was determined by quantitative real-time PCR. **B:** C2C12 myotube cultures were treated for 2 days and analyzed by Western blot analysis and PTEN immunoblotting. **C:** PTEN IP assay was performed on the same C2C12 myotube protein extracts. The C2C12 myotube extracts were normalized by total PTEN levels and ubiquitin immunoblotted. PTEN, ubiquitin, and β-actin protein levels were determined by Western blot analysis and quantified with ImageJ analysis. Data are expressed as means ± SD according to four replicates (**A**) or as means ± SEM (**B** and **C**). $**P \leq 0.01$, *t*-test for both the quantitative PCR and Western blot analyses.

response to myofibrillar damage. A novel splice isoform that is missing exon 3 and 4 from the NEDD4-006 transcript was present in DM2 muscle but not in controls or DM1 muscle. The loss of the exons causes a frameshift, leading to 18 new amino acids and a premature stop codon. Even though there was an overall NEDD4 increase in DM2 samples (Figure 1A), we observed a reduced amount of the NEDD4-006 isoform in DM2 samples compared with controls. These changes may affect the ability of NEDD4 to function in the ubiquitin-proteasome pathway. The MBNL1 protein has been previously linked to both the DM2 pathology and the DM2 splicing changes.[7] Silencing MBNL1 in cell culture causes the mRNA level of the new 006 isoform to be increased. This suggests MBNL1 regulates NEDD4 splicing in muscle and that it may have a role in the DM2 pathology. Silencing MBNL1 did not affect the mRNA level of the other short NEDD4 splice isoform (Figure 1B). This suggests that other molecules are important for the final regulation of NEDD4 splicing and that these unknown factors are also affected by the DM2 mutation. In Western blot analysis a band that was consistent with the smaller NEDD4 splice isoform (missing 16 full exons) was observed in DM2 samples alongside bands that corresponded to full-length NEDD4 isoforms. The missing region encodes critical domains, such as large parts of the HECT (homologous to the E6AP carboxyl terminus) and WW1 domains, and all of the WW2 to WW4 domains, while still containing the C2 domain. This most likely causes loss of function in the DM2-specific abnormal splice isoform, while still maintaining some of the binding interactions. Although the abnormal isoform is not predominant, on the basis of the amount on Western blots, a dominant negative effect cannot be excluded. Interestingly, the *in vivo* expression of inactivated *Nedd4* in mouse skeletal muscle causes a dominant negative effect.[32]

NEDD4 has been shown to be an important regulator of PTEN turnover. Disruption of NEDD4 in muscle tissue reduces the ubiquitination of PTEN, thus increasing its protein level through a decrease of its degradation turnover. Our results indicate a decrease in the level of PTEN ubiquitination in DM2 myotube cultures compared with controls. PTEN accumulates in a subpopulation of highly atrophic IIA fibers, including the typical nuclear clump IIA fibers in DM2 muscle. Together, our findings suggest defects in NEDD4 activity in DM2. Statin medication reduces the overall level of NEDD4 expression, which in turn increases the level of PTEN and reduces PTEN ubiquitination. The identified NEDD4 dysregulation, including the abnormally spliced NEDD4 isoforms in DM2, could therefore increase susceptibility for the adverse reactions of statin by reducing the level of functionally active NEDD4 even further. This could explain why previously subclinical patients with DM2 more frequently present first symptoms when they are on statin medication and would favor hyperlipidemia therapies that do not have negative effects on the NEDD4 pathway.

Acknowledgments

We thank all participating patients for their cooperation and Linda L. Bachinski for critical review of the manuscript.

Supplemental Data

Supplemental material for this article can be found at *http://dx.doi.org/10.1016/j.ajpath.2014.04.013*.

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