NCAM is hyposialylated in hereditary inclusion body myopathy due to *GNE* mutations

Abstract—The authors found that the neural cell adhesion molecule (NCAM) is hyposialylated in hereditary inclusion body myopathy (HIBM) muscle, as suggested by its decreased molecular weight by Western blot. This abnormality represented the only pathologic feature differentiating HIBM due to *GNE* mutations from other myopathies with similar clinical and pathologic characteristics. If further confirmed in larger series of patients, this may be a useful diagnostic marker of *GNE*-related HIBM.

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Autosomal recessive hereditary inclusion body myopathy (HIBM, MIM# 600737) is associated with mutations in the UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosamine kinase gene (*GNE*) that codes for a bifunctional enzyme with a critical role in sialic acid biosynthesis.¹ Whether *GNE* mutations always result in abnormal sialylation of glycoproteins in HIBM muscle has not been fully elucidated.^{2–4}

The neural cell adhesion molecule (NCAM) is a member of the immunoglobulin superfamily of adhesion molecules. NCAM binds long linear homopolymers of α 2,8-linked sialic acid residues, thus forming polysialic acid (PSA)-NCAM. In skeletal muscle PSA-NCAM plays a role during muscle fiber development and regeneration and in the organization and function of the neuromuscular junction (NMJ).^{5,6}

In the present study we investigated the expression and the molecular characteristics of NCAM in HIBM muscle.

Methods. *Patients.* Five patients from four unrelated families were diagnosed as having HIBM based on clinical findings, muscle pathology, and genetic study.^{1,7} The mean age at onset of symptoms was 28.2 ± 7.2 years. *GNE* mutations of these patients have been reported elsewhere³ (table).

Six unrelated patients with a quadriceps sparing myopathy but without *GNE* mutations were included in this study. The mean age at onset of symptoms was 37.5 ± 12.5 years, with weakness and atrophy initially involving the distal compartments of lower limbs, later proximal progression and sparing of the quadriceps. Three patients had a family history of myopathy with a possible autosomal recessive inheritance.

The other three were apparently sporadic cases although an autosomal recessive transmission could not be excluded. In all these patients, clinical assessment also included an MRI scan of lower limb muscles (figure 1A). Muscle biopsies from patients of both groups showed very similar pathology, including absence of inflammation and necrosis, fibers with rimmed vacuoles, and variable amount of angulated atrophic fibers. Diagnostic workup on muscle biopsies included immunohistochemistry with the SMI-31 antibody recognizing phosphorylated tau-containing filaments.⁷ This showed the presence of cytoplasmic immunoreactive inclusions in four of the five *GNE*-related HIBMs, and in three of the five non-*GNE* quadriceps sparing myopathies analyzed (figure 1B, table). For simplicity, this latter group of patients will be identified as having non-*GNE* hereditary inclusion-body myopathy (NG-HIBM). Additional control muscle biopsies were as follows: polymyositis ($n = 4$), dermatomyositis ($n = 4$), amyotrophic lateral sclerosis ($n = 4$), Duchenne muscular dystrophy (DMD, $n = 3$), and normal muscles ($n = 6$). All biopsies were obtained from the quadriceps after informed consent. This research was approved by the ethical committee of our institution.

Immunohistochemistry. Immunohistochemistry was performed as previously described.³ The following primary antibodies were used: monoclonal anti-NCAM (Santa Cruz Biotechnology, Santa Cruz, CA), diluted 1/100, monoclonal anti-myosin heavy chain developmental type (MHCd, Novocastra, Newcastle upon Tyne, UK), diluted 1/20, and monoclonal anti-SMI-31 (Sternberger Monoclonals, Lutherville, MD), diluted 1/1000. Controls for staining specificity were the omission of the primary antibody or its replacement with non-immune serum. The relative amount of immunopositive fibers was evaluated in at least 500 muscle fibers from four representative low-power microscopic fields of each section.

Western blot. Western blot was performed as previously described.³ The following primary antibodies were used: monoclonal anti-NCAM (Santa Cruz Biotechnology), diluted 1/100, and monoclonal anti-actin (Novocastra), diluted 1/2000.

Enzyme treatment. Muscle homogenates were incubated with *Vibrio cholerae* neuraminidase (VCN, SIGMA, St. Louis, MO), at the concentration of 1 mU/ μ g of proteins in Tris buffer pH 5.5 for 20 minutes at 37°C, and then analyzed by Western blot. Control experiments consisted of the omission of VCN from the mixture.

Statistical analysis. All data were expressed as mean $\pm$ SD. Comparison between groups was assessed by Student *t* test. Significance was set at $p \leq 0.05$.

Results. In all myopathies, increased NCAM immunoreactivity was found in regenerating muscle fibers, identified by strong MHCd expression. In HIBM biopsies, NCAM expression was increased in $12 \pm 4.6\%$ ($n = 5$) of non-regenerating muscle fibers (both vacuolated and non-vacuolated) mainly in the form of a diffuse cytoplasmic staining, presence of cytoplasmic granular deposits, or both (see figure 1B). In the NG-HIBMs ($n = 5$), NCAM expression was found increased in $12.2 \pm 7.5\%$ of non-

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Table Summary of clinical, genetic, and pathologic data

Patient/sex/ age at onset, y	Family history	EMG features	CK	Clinical involvement			<i>GNE</i> mutation	SMI-31 positivity	NCAM positive fibers, %
				Lower limbs	Quadriceps	Upper limbs			
HIBM									
1/M/25	+	Mixed	↑ 2–3 fold	Distal and proximal	Spared	Distal and proximal	p.N519S + p.N519S	+	11
2/F/37	+	Myopathic	↑ 2–3 fold	Distal and proximal	Spared	Distal and proximal	p.N519S + p.N519S	+	10
3/F/35	–	Myopathic	Normal	Distal and proximal	Spared	Proximal	p.P27S + p.P27S	+	20
4/F/23	+	Mixed	↑ 2 fold	Distal and proximal	Spared	Distal and proximal	p.M171V + p.M712T	+	11
5/F/21	–	Myopathic	Normal	Distal and proximal	Spared	Distal and proximal	p.R246Q + p.Q355_C357del/ p.I377fsX16	–	8
NG-HIBM									
1/F/50	–	Myopathic	Normal	Distal and proximal	Spared	Distal and proximal	NF	+	11
2/M/20	+	Myopathic	↑ 2–3 fold	Distal and proximal	Spared	Distal and proximal	NF	–	8
3/F/25	+	Myopathic	↑ 2–3 fold	Distal and proximal	Spared	Distal and proximal	NF	+	14
4/M/40	–	Myopathic	↑ 2–3 fold	Distal and proximal	Spared	Distal and proximal	NF	+	24
5/M/50	–	Myopathic	Normal	Distal and proximal	Spared	Distal and proximal	NF	–	4
6/M/40	+	Mixed	↑ 2–3 fold	Distal and proximal	Spared	Distal and proximal	NF	NA*	NA*

*For this patient no muscle sample was available for morphologic analysis.

Mixed = mixed myopathic and neurogenic EMG features; NF = not found; NA = not available.

regenerating fibers ($p = 0.48$ when HIBMs and NG-HIBMs were compared), with a pattern of immunoreactivity identical to that of HIBM muscle (see figure 1B).

By Western blot analysis, in all normal control muscles NCAM was almost undetectable. In all HIBMs, NCAM was identified as a discrete band of 130 kDa, whereas in all other myopathies (NG-HIBMs, polymyositis, dermatomyositis, and DMD) NCAM migrated as a broad band of 150 to 200 kDa.

To verify whether the reduced molecular weight of NCAM in HIBM reflected a markedly reduced sialylation, muscle homogenates of representative control myopathies, characterized by the presence of numerous regenerating fibers and showing normally sialylated NCAM (two DMD, two polymyositis, and two dermatomyositis), were digested with VCN, to remove sialic acid residues from glycoproteins, and then subjected to SDS-PAGE. Indeed, after VCN treatment, NCAM migrated as a discrete band of 130 kDa, as observed in HIBM muscles (figure 2B).

Discussion. Previous studies attempting to elucidate whether mutations in the *GNE* gene result in hyposialylation of muscle glycoproteins have provided differing results.^{2–4} In the present study we have found that in the muscle biopsies of all our

HIBM patients NCAM is hyposialylated, as suggested by its decreased molecular weight by SDS-PAGE.⁵ Such abnormality was not connected with the impairment of a specific domain of the enzyme, as it was detected in patients harboring mutations in different portions of the *GNE* gene. Although other studies have shown that specific mutations do not result in reduction of membrane-bound sialic acid,⁴ it is possible that highly sialylated proteins, such as PSA-NCAM, may still be affected even if the overall level of sialic acid remains within normal range.

The analysis of PSA-NCAM could potentially prove to be a useful diagnostic marker of HIBM due to *GNE* mutations. In our study the presence of 130 kDa NCAM represented the only pathologic feature differentiating all the HIBMs from the NG-HIBMs. In fact, both NG-HIBM and HIBM patients had a very similar clinical presentation. One distinguishing aspect could be the mean age at onset (higher in the NG-HIBM patients) but HIBM patients with a late onset have also been reported.⁸ Muscle biopsies showed identical pathologic features and SMI-31 immunohistochemistry did not provide a definitive diagnostic clue to differentiate the HIBMs from the NG-HIBMs.

Figure 1. (A) MRI scans of lower limb muscles of two representative patients, one with hereditary inclusion-body myopathy (HIBM) and one with non-GNE HIBM (NG-HIBM). In both patients the quadriceps appear to be relatively spared compared to other muscle compartments. (B) Both HIBMs and NG-HIBMs showed remarkably similar pathologic features at the muscle biopsy by hematoxylin and eosin (H-E) staining, including vacuolated muscle fibers, absence of inflammation and necrosis and presence of angulated atrophic fibers. SMI-31 immunohistochemistry showed the presence of cytoplasmic inclusions in muscle fibers of HIBM and NG-HIBM. Neural cell adhesion molecule (NCAM) expression was found increased in comparable amounts of non-regenerating fibers (arrowheads) with an identical pattern of immunoreactivity in both HIBMs and NG-HIBMs. Arrows identify regenerating muscle fibers (immunopositive for MHCd) that also show increased expression of NCAM. Data shown are representative of all muscle biopsies studied. Scale bar corresponds to 20 μ m in all micrographs except for those of SMI-31 immunohistochemistry where the scale bar corresponds to 5 μ m.

Figure 2. (A) By Western blot, in all hereditary inclusion-body myopathy (HIBM), neural cell adhesion molecule (NCAM) was identified as a discrete band of 130 kDa, whereas in non-GNE HIBMs (NG-HIBMs) (as well as in all other myopathies, not shown) NCAM migrated as a broad band of 150 to 200 kDa. Patients shown are indicated as in the table. (B) Muscle homogenate from a patient with Duchenne muscular dystrophy (DMD) was digested with *Vibrio Cholerae* neuraminidase (VCN). After VCN treatment (VCN+), NCAM migrated as a discrete band of 130 kDa, as observed in HIBM muscles. In control experiment, consisting of the omission of the VCN from the mixture (VCN-), NCAM showed its normal molecular mass. Similar results were obtained when muscle homogenates of polymyositis and dermatomyositis were used for digestion studies with VCN (not shown).

An abnormal expression of NCAM has been previously demonstrated in vacuolated fibers of HIBM muscle.⁹ Whether a hyposialylated NCAM has a pathogenic role in HIBM remains to be determined. PSA-NCAM has been shown to have a role in NMJ physiology, as mice lacking NCAM show structural and functional abnormalities of the NMJ.⁶ In vitro it has been shown that cultured HIBM myotubes cannot be properly innervated by neurites of rat spinal cord explants and a mechanism of “myogenous dysreception to innervation” has been proposed.¹⁰ Therefore, it is possible that in HIBM muscle fibers, the underlying metabolic defect results in impairment of NMJ stabilization, as these fibers are initially properly innervated but later probably lose their contact with the nerve terminal. Indeed, HIBM muscle biopsies are often characterized by a variable amount of panesterase-positive angulated atrophic fibers, thus suggesting an ongoing process of denervation.⁷ Further studies are necessary to clarify whether the hyposialylation of NCAM, as demonstrated in our study, plays a role in such abnormalities.

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Figure. (A) CT showing middle cerebral infarction on the right, a massive calcified chronic subdural hematoma on the left producing a mass effect with falx herniation, and signs of cerebral atrophy. (B) Anteroposterior and (C) lateral view of the skull X-ray showing a calcified mass in the left hemisphere.

Asymptomatic huge calcified subdural hematoma in a patient on oral anticoagulant therapy

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A 69-year-old right-handed retired locksmith with atrial fibrillation and cardiomyopathy but without other health problems had

an acute left hemiparesis after being on warfarin for 8 years. An infarction in the right hemisphere and a calcified subdural hematoma in the left hemisphere were demonstrated (figure). On further questioning, previous alcoholism and a trivial cycling accident 10 years ago were reported.

The natural history of calcified subdural hematoma is still poorly understood.¹ It is estimated that calcification occurs in 0.3–10% of subdural hematomas.^{1,2} The interval between hemorrhage and visible calcification varies from 3 months to several years.¹

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