RESEARCH ARTICLE

Molecular and Muscle Pathology in a Series of Caveolinopathy Patients

Luigi Fulizio,[1,2]* Anna Chiara Nascimbeni,[1,2] Marina Fanin,[1,2] Giulio Piluso,[3] Luisa Politano,[4] Vincenzo Nigro,[3] and Corrado Angelini[1,2]

[1]Department of Neurosciences, University of Padova, Padova, Italy; [2]Venetian Institute of Molecular Medicine, Padova, Italy; [3]Department of General Pathology, II University of Naples, Naples, Italy; [4]Department of Internal Medicine and Medical Genetics, II University of Naples, Naples, Italy

Communicated by Jean-Claude Kaplan

Mutations in the caveolin-3 gene (CAV3) cause limb girdle muscular dystrophy (LGMD) type 1C (LGMD1C) and other muscle phenotypes. We screened 663 patients with various phenotypes of unknown etiology, for caveolin-3 protein deficiency, and we identified eight unreported caveolin-deficient patients (from seven families) in whom four CAV3 mutations had been detected (two are unreported). Following our wide screening, we estimated that caveolinopathies are 1% of both unclassified LGMD and other phenotypes, and demonstrated that caveolin-3 protein deficiency is a highly sensitive and specific marker of primary caveolinopathy. This is the largest series of caveolinopathy families in whom the effect of gene mutations has been analyzed for protein level and phenotype. We showed that the same mutation could lead to heterogeneous clinical phenotypes and muscle histopathological changes. To study the role of the Golgi complex in the pathological pathway of misfolded caveolin-3 oligomers, we performed a histopathological study on muscle biopsies from caveolinopathy patients. We documented normal caveolin-3 immunolabeling at the plasmalemma in some regenerating fibers showing a proliferation of the Golgi complex. It is likely that caveolin-3 overexpression occurring in regenerating fibers (compared with caveolin-deficient adult fibers) may lead to an accumulation of misfolded oligomers in the Golgi and to its consequent proliferation. Hum Mutat 25:82–89, 2005.   © 2004 Wiley-Liss, Inc.

INTRODUCTION

Caveolae are 50–100-nm vesicular invaginations of the plasma membrane, which are flat and either detached or fused to form grape-like structures and tubules [Smart et al., 1999; Scherer et al., 1994; Parton et al., 1997]. They are mainly composed of cholesterol and sphingolipids. Caveolins are members of a gene family of 21–25 kDa integral membrane proteins and they are the principle protein components of caveolae [Galbiati et al., 2001; Minetti et al., 2002]. The mammalian caveolin family consists of caveolin-1, -2, and -3, encoded by different genes. Caveolin-1 and -2 are coexpressed in many cell types (particularly in adipocytes), whereas caveolin-3 (or M-caveolin) is specific to striated muscle [Tang et al., 1996]. Like other caveolins, caveolin-3 forms homooligomers in the endoplasmic reticulum [Galbiati et al., 1999], which then interact with each other to form complexes that are thought to function as building blocks in the construction of caveolae membranes. Through the interaction of caveolin with itself, and the caveolin-mediated selection of endogenous lipid components, a caveolae vesicle is generated. It has been proposed that caveolins are directly implicated in interactions with signaling proteins (e.g., G-proteins, conserved kinase domain IX, and many nonkinases) in the caveolin

scaffolding domain [Galbiati et al., 2000; Kubisch et al., 2003; Wyse et al., 2003].

Caveolin-3 is localized to the muscle plasmalemma, where it forms a complex with dystrophin and associated-proteins [Song et al., 1996; Herrmann et al., 2000], suggesting that caveolae might play an important role in muscle membrane physiology. The caveolin-3 gene (CAV3; MIM# 601253), which was mapped to chromosome 3p25, spans 12 kb of genomic DNA and contains two exons [Minetti et al., 1998; McNally et al., 1998]. Caveolin-3 is a 151–amino acid (aa) protein with four separate domains [Minetti et al., 1998]: the N-terminal domain (aa 1–73), which is responsible for the homooligomerization and the interaction with caveolin-associated signaling molecules; the scaffolding domain (aa 54–73), which is a conserved region where caveolin interacts

with signaling molecules localized to the caveolae; the transmembrane domain (aa 74–106), which is believed to form a hairpin loop structure in the plasmalemma; and the C-terminal domain (aa 107–151). The caveolin signature sequence is a stretch of eight amino acids (FEDVIAEP, aa 41–48) within the N-terminal domain, which is highly conserved in all caveolins of different species and is responsible for nNOS inhibition in vitro [Venema et al., 1997; Minetti et al., 1998].

Mutations in *CAV3* gene have been described in association with various phenotypes including dominant limb-girdle muscular dystrophy (LGMD1C; MIM# 607801) [Minetti et al., 1998; Figarella-Branger et al., 2003], idiopathic hyperCKemia (MIM# 123320) [Carbone et al., 2000], rippling muscle disease (RMD; MIM# 606072) [Betz et al., 2001; Vorgerd et al., 2001; Herrmann et al., 2002; Kubisch et al., 2003], distal myopathy [Tateyama et al., 2002], and hypertrophic cardiomyopathy [Hayashi et al., 2004]. Moreover, the same mutation also causes variable phenotypes within family-related patients [Cagliani et al., 2003; Fischer et al., 2003].

A possible pathogenic mechanism is that *CAV3* gene mutations might exert a dominant-negative effect, causing the rapid degradation of both the wild-type and the mutant caveolin-3 proteins [Minetti et al., 1998]. It has been proposed [Minetti et al., 2002] that mutations impair the caveolin-3 homooligomerization, producing unstable, high molecular mass aggregates, which could be retained within the Golgi complex and not targeted to the plasma membrane. Other studies have showed that caveolin-3 mutants of NIH3T3 transfected cells undergo ubiquitination and proteasomal degradation [Galbiati et al., 2000].

Few studies have so far been conducted to analyze the skeletal muscle from caveolinopathy patients [Minetti et al., 1998; Betz et al., 2001]: caveolin-3 immunolabeling is typically lost, and the protein level on blots ranges from absent to 20% of controls. Other membrane proteins involved in muscular dystrophies (i.e., dystrophin, sarcoglycan, merosin) are expressed at normal levels. Moreover, in mutant patients there is a severe impairment of caveolae formation at the membrane and a disruption of the T tubule at the subsarcolemmal level [Minetti et al., 2002].

In this study, we screened a large population of patients with different phenotypes of unknown etiology for caveolin-3 deficiency, in order to select protein-deficient cases for subsequent *CAV3* mutation analysis. In a series of eight caveolin-deficient patients, we then analyzed the effect of gene mutations on both protein level and clinical phenotype, and performed a muscle morphological and immunohistochemical study to investigate muscle pathology features possibly involved in the pathogenic mechanism of the disorder.

MATERIALS AND METHODS
Patient Selection Criteria

Our muscle biopsy bank contains more than 5,300 specimens that were collected for diagnostic purposes after obtaining the patients' informed consent. We selected 663 muscle biopsies for caveolin-3 protein analysis, as they fulfilled the following criteria:

1. normal muscle expression of dystrophin, alpha-sarcoglycan, dysferlin, calpain-3, and emerin;

2. muscle biopsy histopathology consistent with a dystrophic or myopathic process;

3. increased serum creatine kinase (CK) level (>500 U/l).

Of these 663 patients, 144 had been clinically diagnosed as affected with unclassified LGMD, 273 with proximal myopathy (including cases with dominant family history), 13 with distal myopathy, 11 with scapuloperoneal atrophy, 25 with idiopathic myoglobinuria, 186 with high CK (with or without muscle cramps and myalgia), and 11 had unclassified hypertrophic cardiomyopathy. No patients with overt rippling muscle disease (RMD) have been found in our muscle tissue bank; however, since the reported RMD patients always have elevated CK values as common feature, it is likely that some RMD candidates were included in the group of patients selected for caveolin-3 protein screening.

We excluded those patients diagnosed on a clinical and/or on a molecular basis as being affected with myotonic dystrophy and facioscapulohumeral dystrophy from the screening.

Muscle Biopsy Histopathology

Microscopy inspection of routinely stained sections was used to determine the degree of the dystrophic/myopathic process, on the following graded scale: 1) dystrophic process (marked increase of fiber size variability, increased central nuclei, few degenerating and regenerating fibers, moderate increase of connective tissue); 2) moderate myopathic picture (moderate increase of fiber size variability, increased central nuclei, fiber splitting, slight increase of connective tissue); and 3) mild myopathic picture (slight increase of fiber size variability, increased central nuclei).

Immunohistochemical Analysis

Serial muscle biopsy specimens were cryostat sectioned (8 μm thick), collected onto gelatin-coated slides, air-dried for 30 minutes, blocked for 15 minutes with 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS), and incubated for 1 hr with appropriate primary antibodies diluted 1:100 in 1% BSA-PBS. We used monoclonal antibodies against caveolin-3 (Transduction Laboratories, Lexington, KY), Golgi complex (Chemicon, Temecula, CA), and fetal myosin (Novocastra, Newcastle upon Tyne, UK). After washes, specific labeling was developed by immunofluorescence, using anti-mouse cyanine-3 conjugated immunoglobulin (Caltag, Burlingame, CA) diluted 1:100 and incubated for 30 minutes. Sections were mounted with anti-fading medium and examined with epifluorescence microscopy; the same optical fields were identified in serial sections.

For neuromuscular control of muscle regeneration, we used muscle biopsies from two polymyositis patients and two Duchenne muscular dystrophy patients.

Immunoblot Analysis

Immunoblot analysis was performed as previously described [Fanin et al., 2003], with minor modifications. Briefly, 20 muscle biopsy sections were dissolved in SDS-loading buffer, boiled, and centrifuged. Muscle proteins contained in the supernatant were resolved by SDS-PAGE and electroblotted to nitrocellulose membrane. The blots were blocked with BSA and then incubated for 1 hr with anti-caveolin-3 antibodies diluted 1:500. Immunoreactive bands were visualized using the avidin-biotin system and developed by chemiluminescence. The amount of caveolin-3 protein in the patients sample was normalized to the amount of muscle tissue loaded, as determined by the myosin band in the posttransfer Coomassie blue–stained gels.

Molecular Studies

Genomic DNA extraction. DNA was extracted from peripheral blood or muscle tissue (collected after informed consent), using the GenElute Mammalian Genomic DNA kit and the procedure recommended by the manufacturer (Sigma, St. Louis, MO). A working dilution of 100 ng/μl DNA sample was prepared.

PCR amplification of the DNA sequence and direct sequencing. The complete coding sequence of the *CAV3* gene

was amplified by PCR in three amplicons, using the following primers:

Exon 1 F: 5′-ACACAGCTCGGATCTCCTCCT-3′;

Exon 1 R: 5′-GCAAACCTGACACTCTCCGC-3′;

Exon 2-1 F: 5′-CACACACCCAAAAGCTTGAGA-3′;

Exon 2-1 R: 5′-GCTCTTAATGCATGGCACCA-3′;

Exon 2-2 F: 5′-GGTGGTGCCATGCATTAAGAG-3′;

Exon 2-2 R: 5′-CCTGTGAAGAAGTCCCCCG-3′.

PCR reactions were prepared in a 25 µl mixture containing 100 ng of genomic DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl$_2$, 200 µM of each dNTP (Applied Biosystems [AB], Foster City, CA), 2.5 U AmpliTaq Gold polymerase (AB), 0.2 µM of each primer (Sigma Genosys, Woodlands, TX). PCR amplification was performed on a 9700 GeneAmp Thermal Cycler (AB) using the following conditions: denaturation at 95°C for 10 minutes, followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 59°C for 30 seconds, extension at 72°C for 30 seconds, and final extension at 72°C for 10 minutes. PCR products were purified using the ExoSap-It PCR purification kit (Amersham), quantified on agarose gel electrophoresis, and sequenced using the Big Dye dideoxy terminator cycle sequencing kit (AB) and the 3100 ABI-PRISM automated sequencer (AB) at CRIBI Biotechnology Centre at Padova University. Sequence analysis was obtained using the Chromas 1.45 software (Technelysium, Australia).

PCR amplification with allele-specific primers. All mutations identified by direct sequencing have been confirmed by a classic amplification refractory mutation system (ARMS)-PCR test using allele-specific primers designed ad hoc. The same tests were also used to check 100 normal chromosomes from control subjects, to exclude the possibility they could be polymorphic variants, and to analyze the segregation of mutation in the family members of Patient 8.

ARMS-PCR primers were designed using Primer-3 software (http://frodo.wi.mit.edu/primer3/primer3_code.html) with the human CAV3 gene sequence as reference: *Homo sapiens* caveolin 3 (*CAV3*), transcript variant 1, mRNA (GenBank: NM_033337.1); *Homo sapiens* chromosome 3 clone RP11-128A5 map 3p, complete sequence (GenBank: AC068312). Previous studies on *CAV3* gene mutations used different mutation nomenclatures (e.g., c.136G>A, p.A46T mutation was given as p.A45T). To avoid misunderstanding, we used the DNA mutation numbering based on cDNA sequence as reported in the Leiden Muscular Dystrophy database (www.dmd.nl) and OMIM database (www.ncbi.nlm.nih.gov), using the A of ATG start codon as +1. Moreover we used the nomenclature "c." to indicate cDNA or "p." to indicate protein sequence in accordance with journal guidelines (see www.hgvs.org/mutnomen).

PCR reactions were prepared as described for the amplification of genomic sequences. To reduce nonspecific products and optimize the allele-specific reaction, we used a touchdown program: in the first cycle, the temperature was set 5°C higher than the annealing temperature, it was decreased by half a degree per cycle for 10 cycles, and then held constant for a further 25 cycles. PCR products were electrophoresed on a 2% agarose gel stained with ethidium bromide. Each experiment was designed to run the PCR products from patients to be tested and one normal control in the same gel.

RESULTS
Screening for Caveolin-3 Protein Deficiency

Immunohistochemical screening for caveolin-3 deficiency in 663 patients allowed us to identify eight unreported patients with either absent or marked reduction of immunolabeling (Table 1; Fig. 1). Caveolin-3 immunoblot analysis in these eight patients confirmed a severe reduction of protein level, which ranged from absent to 10% of controls (Table 1; Fig. 1).

Caveolinopathy patients accounted for about 1% of cases with various clinical phenotypes of unknown etiology, including proximal and distal myopathy and hyperCKemia. A similar proportion of caveolinopathies (1/144 cases) was found among patients affected with LGMD, which suggests that caveolinopathy is rarely involved in LGMD.

Patients

The eight patients with *CAV3* gene mutations described in this study belong to seven families (one pair of mother-daughter patients is included) (Table 1). Positive family history with autosomal dominant pattern of inheritance was reported in 5 out of 7 families. In the family of Patient 1, the patient's father and two paternal uncles were referred as asymptomatic with only high CK. In the family of Patient 2, the patient's mother suffered from a similar myopathy with high CK and proximal muscle weakness. In the family of Patient 4, the patient's maternal uncle had LGMD with difficulty walking in late adulthood, and the patient's mother was referred to as healthy but no clinical data or CK values were available. In the family of Patients 6 and 7 (mother and daughter, respectively), a maternal cousin of Patient 7 was also affected with myopathy. In the family of Patient 8, the analysis of DNA from both parents and younger brother showed that the mother of the patient was a carrier of the mutation found in her son.

Serum CK level was found to be elevated in all patients, the levels found ranged from a three-fold to an over 40-fold increase.

One patient presented only hyperCKemia at the age when the biopsy was done. In the other patients, the onset of muscle weakness had occurred from 7 to 37 years of age (mean 19.1 years) and involved either proximal or distal limb girdle muscles. Only one patient showed the LGMD phenotype. Muscle cramps, myalgia, or pain was a rather common complaint (Table 1). Cardiomyopathy was not reported in any patient.

Muscle Morphology and Histopathology

In most of the patients in our series, the muscle pathological picture was relatively benign and showed mild or moderate myopathic features. In only three patients was the muscle pathology pattern clearly dystrophic, showing degenerating/regenerating fibers and increased fibrosis (Fig. 2). The degree of muscle pathology appeared unrelated to the age at biopsy and to the extent and distribution of muscle weakness. However, on average, patients with dystrophic muscle biopsy had had the onset of muscle weakness at an earlier age (14.6 years) than the others (22.5 years). In the former group, CK levels were only moderately increased, suggesting long-term muscle damage. The dystrophic muscle pathological pattern was observed in association with different gene mutations, involving the scaffolding domain, the signature sequence, and the N-terminal domain.

The study of serial muscle sections immunolabeled with caveolin-3, Golgi, and fetal myosin (a marker of muscle regeneration) showed different pathological patterns. One pattern is characterized by scattered and atrophic caveolin-positive fibers, which are clearly reacting for fetal myosin (thus regenerating) and are faintly and diffusely labeled for the Golgi complex (Fig. 2). Other muscle fibers showed subsarcolemmal Golgi accumulation (possibly referring to sarcoplasmic bodies) in association with faint caveolin-3 labeling at the plasma membrane

TABLE 1. Clinical, Histopathological, and Molecular Data*

Patient sex	Family history	CK (U/L)	Age at onset (yrs)	Clinical phenotype	Main clinical features (age at last examination)	Age at biopsy (years)	Muscle biopsy pathology	Caveolin-3 protein on WB	CAV3 gene mutation	Caveolin-3 protein domain
1,M	+	891	10	Myopathy	Myalgia, calf hypertrophy, mild proximal weakness (10)	10	Moderate myopathic	<5%	c.136G>A, p.A46T, exon 2	Signature sequence
2,F	+	↑	37	Myopathy	Muscle pain, mild proximal weakness (37)	37	Mild myopathic	10%	c.136G>A, p.A46T, exon 2	Signature sequence
3,M	–	4000	13	Myopathy	Mild proximal weakness (13)	13	Mild myopathic	<5%	c.136G>A, p.A46T, exon 2	Signature sequence
4,M	+	600	10	LGMD	LGMD, cramps, myoglobinuria (21)	21	Dystrophic	5%	c.136G>A, p.A46T, exon 2	Signature sequence
5,M	–	1272	7	Myopathy	Mild proximal weakness (7)	7	Dystrophic	0	**c.183C>A, p.S61R**, exon 2	Scaffolding
6,F[a]	+	1780	30	Distal myopathy	Distal weakness, calf hypotrophy (50)	50	Moderate myopathic	0	**c.99C>G, p.N33K**, exon 1	N-terminal
7,F[a]	+	674	27	Distal myopathy	Mild distal weakness (27)	27	Dystrophic	0	**c.99C>G, p.N33K**, exon 1	N-terminal
8,M	+	8500	14	Hyperckemia	High CK (14)	14	Moderate myopathic	0	c.80G>A, p.R27Q, exon 1	N-terminal

*Novel mutations are indicated in bold. We used the DNA mutation numbering based on cDNA sequence, using the A of ATG start codon as +1. We used the nomenclature c. to indicate cDNA sequence and p. to indicate protein sequence. Human *CAV3* gene sequence: *Homo sapiens* caveolin 3 (*CAV3*), transcript variant 1, mRNA (GenBank: NM_033337.1); *Homo sapiens* chromosome 3 clone RP11-128A5 map 3p, complete sequence (GenBank: AC068312). CK, creatine kinase; WB, western blot.
[a]Relatives (patient #6 is the mother of patient #7).
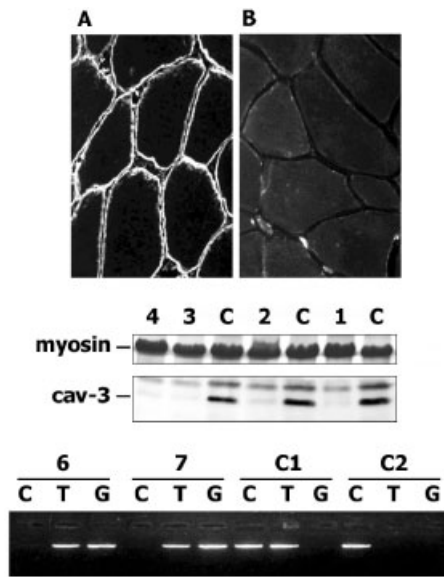
FIGURE 1. **Caveolin-3 biochemical and molecular diagnosis. Caveolin-3 immunolabeling on muscle biopsy sections (upper panel): Example of immunoreaction showing marked reduction in Patient 7 (B) as compared to normal control (A). Caveolin-3 Western blot analysis (middle panel): Normal caveolin-3 bands correspond to a 21-kDa molecular weight protein (caveolin-3). Muscle biopsies from Patients 1–4 show a marked quantitative reduction of caveolin-3 as compared to control (C) following normalization with myosin in the posttransfer Coomassie blue–stained gel. ARMS-PCR test for p.N33K CAV3 gene mutation (lower panel): The agarose gel electrophoresis showed the 339-bp PCR products for the p.N33K mutation. Normal controls (C1 and C2) and Patients 6 and 7 were tested for the wild-type (C nucleotide), the mutant (G nucleotide), and the neutral polymorphic (T nucleotide) alleles. Patients 6 and 7 are heterozygous for both the mutation and the polymorphism. Control C1 is heterozygous for both the polymorphism and the wild-type allele, whereas control C2 is homozygous for the wild-type allele.**

(Fig. 2). Last, some atrophic caveolin-positive fibers were not regenerating, and did not react for Golgi (Fig. 2); it is likely that these fibers are degenerating, and they possibly express caveolin-3 because of the reduced cell surface and consequent concentration of caveolae. To check if Golgi proliferation may also be present in other muscle disease with active regeneration, we used Duchenne muscular dystrophy and myositis as neuromuscular controls. We observed that regenerating fibers from neuromuscular controls showed positive Golgi accumulation, but a reduced intensity of caveolin-3 labeling as compared to nonregenerating fibers (data not shown).

CAV3 Gene Mutations

In the eight caveolin-deficient patients, the search for mutations in the CAV3 gene allowed us to identify four different missense mutations (Table 1). The substitution c.136G>A in exon 2 causing an amino acid change of alanine to threonine at position 46 (c.136G>A, p.A46T) was found in the heterozygote state in four unrelated patients. The latter patients came from different geographic areas of Italy (i.e., northeast, northwest, center). The substitution c.80G>A in exon 1 causing an amino acid change of arginine to glutamine at position 27 (c.80G>A, p.R27Q), and the substitution c.183C>A in exon 2 causing an amino acid change of serine to arginine at position 61 in exon 2 (c.183C>A, p.S61R), were each found in the heterozygote state in one patient. In a pair
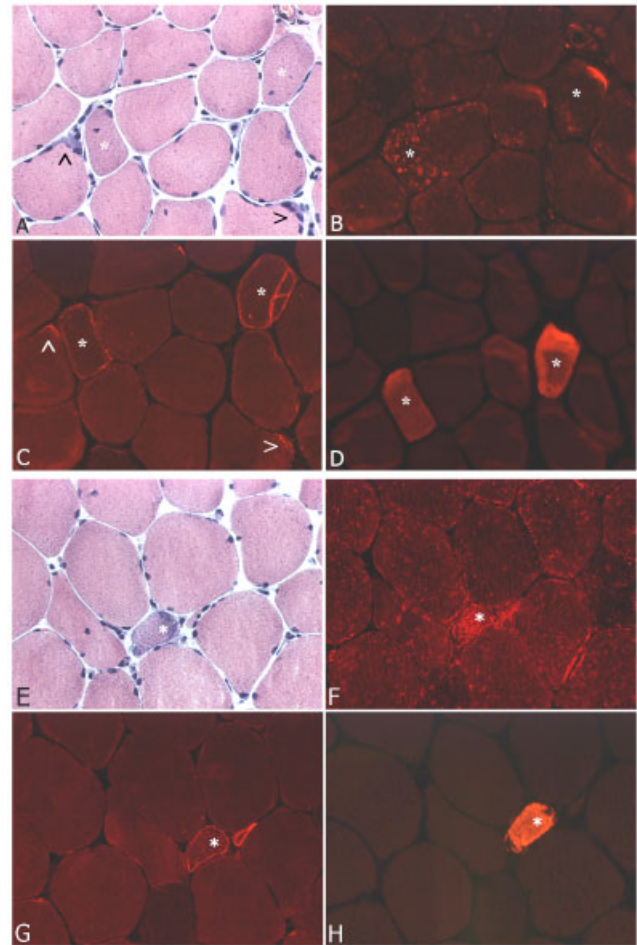
FIGURE 2. **Two sets of serial muscle biopsy sections (Patient 8) show muscle morphology and immunolabeling with different antibodies: hematoxylin-eosin (H&E) staining (A,E), anti-Golgi zone (B,F), anti caveolin-3 (C,G), and anti-fetal myosin (D,H). The asterisks indicate the same fiber in different serial sections. The upper set (A–D) shows two centrally nucleated regenerating muscle fibers that positively reacted for caveolin-3, and positive Golgi labeling either in scattered cytoplasmic or in subsarcolemmal regions. Additional fibers show subsarcolemmal purple bodies at H&E staining that are positive for caveolin-3 (arrowheads). The lower set (E–H) shows two adjacent atrophic fibers which are immunolabeled for caveolin-3: one of these fibers is undergoing regeneration and shows positive Golgi reaction in scattered cytoplasmic granules. Original microscope magnification × 200. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]**

of mother-daughter patients, we identified the same substitution c.99C>G in exon 1, causing an amino acid change of asparagine to lysine at position 33 (c.99C>G, p.N33K). In the family of Patient 8, the mother showed the same mutation in her affected son (c.80G>A, p.R27Q). Two of the mutations reported here (c.99C>G, p.N33K and c.183C>A, p.S61R) have not been reported before, according to the Leiden Muscular Dystrophy database (www.dmd.nl) and the OMIM database (www.ncbi.nl.nih.gov). We did not find these new mutations in 50 control subjects when using allele-specific tests, thus these variations were not polymorphisms.

Two of the mutations found are localized in the N-terminal domain of the protein, one in the signature sequence and one in the scaffolding domain (Table 1; Fig. 3).
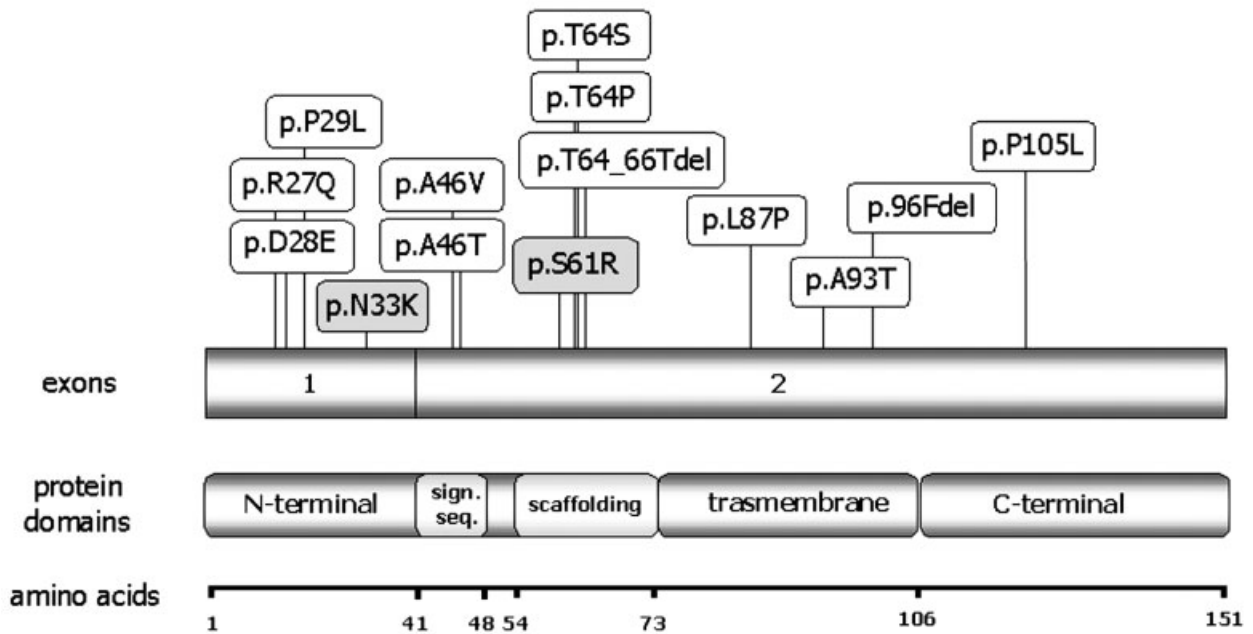
FIGURE 3. **Localization and distribution of the CAV3 gene mutations identified so far. The relative size of exons is represented. Gray boxes indicate new mutations found in this study. The lower panel shows a schematic representation of the corresponding caveolin-3 protein domains: the N-terminal domain, which includes the signaling sequence (sign. seq.) and the scaffolding domain, the trans-membrane domain, and the C-terminal domain.**

During the analysis of gene mutations, we also identified a polymorphism, c.99C>T in exon 1, resulting in a synonymous codon variation at position 33 (p.N33N) (dbSNP rs1008642), which accounted for about 30% of control alleles in our Italian population.

Genotype–Protein–Phenotype Correlations

The clinical phenotype was variable both within the whole series of cases and between patients with the same mutation. In particular, four unrelated patients shared the same c.136G>A (p.A46T) gene mutation, which always resulted in a low, but still detectable, caveolin-3 protein level on immunoblot.

Interestingly, the patient showing the highest level of residual protein (10% of normal) had onset of muscle weakness at the latest age in our series (37 years), while the patient carrying the c.183C>A (p.S61R) mutation in the scaffolding domain had onset of weakness at the earliest age in our series (7 years) and had dystrophic muscle pathology. The other three mutations were invariably associated with absent protein but patients showed various clinical phenotypes, ranging from hyperCKemia to distal muscular dystrophy.

DISCUSSION

Several familial or isolated caveolinopathy patients, found because of new *CAV3* gene mutations or their clinical phenotype different from LGMD, have been reported in the literature [McNally et al., 1998; Minetti et al., 1998; Carbone et al., 2000; Herrmann et al., 2000, 2002; Betz et al., 2001; Vorgerd et al., 2001; Tateyama et al., 2002; Cagliani et al., 2003; Figarella-Branger et al., 2003; Fischer et al., 2003; Kubisch et al., 2003; Hayashi et al., 2004]. However, a systematic search for caveolinopathies has never been performed, due to the rarity of the disease and the wide spectrum of clinical phenotypes of

candidate patients. The availability of a collection of more than 5,300 muscle biopsies in our tissue bank allowed us to carry out the first screening for caveolin-3 protein deficiency on a large population of patients with different phenotypes.

We demonstrated that the immunohistochemical analysis of caveolin-3 is a highly specific method of identifying primary caveolinopathies; therefore, caveolin-3 protein and molecular analysis should be considered as part of diagnostic work in patients with unclassified LGMD, hyperCKemia, and myopathy.

With regard to the sensitivity of the immunohistochemical analysis of caveolin-3, all patients with *CAV3* gene mutations and caveolin-3 protein analysis showed severe protein deficiency [Minetti et al., 1998; Carbone et al., 2000; Herrmann et al., 2000, 2002; Betz et al., 2001; Vorgerd et al., 2001; Tateyama et al., 2002; Cagliani et al., 2003; Kubisch et al., 2003], whereas nonpathogenetic *CAV3* gene nucleotide variations were associated with normal protein expression [McNally et al., 1998; De Paula et al., 2001]. Actually, the demonstration of the pathogenic effect (dominant-negative) of mutations in this gene [Minetti et al., 2002] is indeed the detection of protein defect. Therefore, it is likely that the sensitivity of protein testing in detecting primary caveolinopathies is high, and screening for protein defect is more convenient to identify primary caveolinopathies than the more laborious screening of *CAV3* gene mutations.

The observation that only a limited number of caveolinopathy families have been reported so far (approximately 25) would suggest that this disease is somewhat rare; however, no estimate of its frequency has been made so far. Though the results from our screening approach are likely an underestimate the real situation, we found that *CAV3* gene mutations accounted for only 1% of patients with LGMD of unknown etiology. Although one could expect to find a higher frequency of caveolinopathies among patients with various phenotypes other than LGMD (e.g., idiopathic hyper-CKemia including possible RMD, and proximal

and distal myopathy), we found that in our series there was a similar frequency in this latter group of patients.

The availability of immunochemical data showing the specific muscle protein deficiency is crucial for demonstrating the pathogenic role of gene mutations, but this proof has not been always provided in previous studies.

The most important result from our biochemical and molecular screening is the identification and description of the largest group of unrelated primary caveolinopathy patients identified so far, which has allowed us to try to correlate the genotype with the protein and the clinical phenotype. We described four different missense mutations (two unreported) in the CAV3 gene in eight patients. The pattern of clinical phenotype was variable in the whole series of cases and even between the four unrelated patients sharing the same mutation: they had widely varying age at onset (from 10 to 37 years), and variable degree of muscle involvement, both at the clinical and at the muscle histopathological level. Moreover, they came from different regions of Italy, suggesting that there is no relationship between them; however, the existence of a founder effect could not be verified (the DNA from patients' relatives was not available for the study).

The p.N33K mutation was found in a couple of relatives who had similar age at onset of distal myopathy, but who showed different degrees of muscle involvement and histopathological patterns. These data indicate that clear genotype–phenotype correlations do not exist and that as yet unidentified modulating factors must play an important role in the individual genetic background in determining both clinical phenotype and progress of the disease.

Regarding the correlation between the genotype and the protein expression, we observed that the p.A46T mutation always resulted in low but still detectable caveolin-3 level on immunoblot, whereas the other mutations were invariably associated with absent protein. Interestingly, the patient showing the highest level of residual protein (10% of normal) had onset of muscle weakness at the latest age in our series (37 years), suggesting a possible prognostic value of residual protein. On the other hand, the patient carrying the p.S61R mutation, which is localized in the scaffolding domain, had absent protein, had onset of weakness at the earliest age in our series (7 years), and had a dystrophic muscle pathology pattern. We can speculate that mutations in this latter domain might have more serious consequences at the protein and clinical level, possibly affecting homooligomerization and the interaction of caveolin-3 with associated signaling molecules [Minetti et al., 1998; Wyse et al., 2003].

Genotype–protein correlations are important when studying the effects of different mutations, but they are not useful when explaining the pathogenic mechanism underlying the disease. CAV3 gene mutations might be exerting a dominant-negative effect, causing unstable and high molecular mass aggregates of caveolin-3, which are retained within the Golgi complex and are not targeted to the plasma membrane [Minetti et al., 2002]. Impaired caveolin-3 homooligomerization would result in a defective protein [Minetti et al., 1998], and misfolded oligomers would be targeted for degradation [Galbiati et al., 1999].

To examine the potential caveolin-3 accumulation within the Golgi complex, we performed an immunohistochemical study in caveolinopathies patients and documented a proliferation of the Golgi complex in some regenerating fibers, which also expressed normal caveolin-3 staining at the plasmalemma. The same Golgi accumulation was observed in regenerating fibers from neuromuscular controls, suggesting that this feature may be a physiological and general reaction. The expression of caveolin-3 has been demonstrated to be induced during the differentiation of skeletal myoblasts to myotubes, suggesting that its role may be crucial in the early stages of muscle development [Song et al., 1996].

The study of neuromuscular controls showed a reduced caveolin-3 expression in regenerating fibers as compared to nonregenerating fibers, while in some regenerating fibers from caveolinopathy patients we observed an increased caveolin-3 labeling.

One possible explanation of the preserved caveolin-3 immuno-labeling in regenerating fibers is that it might derive from rescued wild-type caveolin-3 that is not degraded and reaches the plasma membrane. We suggest that in caveolinopathy muscle an over-expression of caveolin-3 protein during regeneration could be responsible for the accumulation of misfolded oligomers in the Golgi complex and for its consequent proliferation. The increased expression of both mutant and wild-type protein might account for a residual amount of nonmisfolded oligomers, resulting in caveolin-3 staining at the plasmalemma of regenerating fibers. This hypothesis could also explain the caveolin-3 and Golgi immunolabeling observed in the aberrant regeneration associated with sarcoplasmic bodies and fibers undergoing splitting.

ACKNOWLEDGMENTS

REFERENCES

Betz RC, Schoser BGH, Kasper D, Ricker K, Ramirez A, Valentin S, Torbergsen T, Lee YA, Nothen MM, Wienker TF, Malin JP, Propping P, Reis A, Mortier W, Jentsch TJ, Vorgerd M, Kubisch C. 2001. Mutations in CAV3 cause mechanical hyperirritability of skeletal muscle in rippling muscle disease. Nat Genet 28: 218–219.

Cagliani R, Bresolin N, Prelle A, Gallanti A, Fortunato F, Sironi M, Ciscato P, Fagiolari G, Bonato S, Galbiati S, Corti S, Lamperti C, Moggio M, Comi GP. 2003. A CAV3 microdeletion differentially affects skeletal muscle and myocardium. Neurology 61: 1513–1519.

Carbone I, Bruno C, Sotgia F, Bado M, Broda P, Masetti E, Panella A, Zara F, Dagna Bricarelli F, Cordone G, Lisanti MP, Minetti C. 2000. Mutation in the CAV3 gene causes partial caveolin-3 deficiency and hyperCKemia. Neurology 54:1373–1376.

De Paula F, Vainzof M, Bernardino ALF, McNally E, Kunkel LM, Zatz M. 2001. Mutations in the caveolin-3 gene: when are they pathogenic? Am J Med Genet 99:303–307.

Fanin M, Nascimbeni AC, Fulizio L, Trevisan CP, Meznaric-Petrusa M, Angelini C. 2003. Loss of calpain-3 autocatalytic activity in LGMD2A patients with normal protein expression. Am J Pathol 163:1929–1936.

Figarella-Branger D, Pouget J, Bernard R, Krahn M, Fernandez C, Levy N, Pellissier JF. 2003. Limb-girdle muscular dystrophy in a 71-year-old woman with an R27Q mutation in the CAV3 gene. Neurology 61:562–564.

Fischer D, Schroers A, Blumcke I, Urbach H, Zerres K, Mortier W, Vorgerd M, Schroder R. 2003. Consequences of a novel caveolin-3 mutation in a large German family. Ann Neurol 53:233–241.

Galbiati F, Razani B, Lisanti MP. 2001. Caveolae and caveolin-3 in muscular dystrophy. Trends Mol Med 7:435–441.

Galbiati F, Volontè D, Minetti C, Chu JB, Lisanti MP. 1999. Phenotypic behaviour of caveolin-3 mutations that cause autosomal dominant limb girdle muscular dystrophy (LGMD1C). J Biol Chem 274:25632–25641.

Galbiati F, Volontè D, Minetti C, Bregman DB, Lisanti MP. 2000. Limb girdle muscular dystrophy (LGMD1C) mutants of caveolin-3 undergo ubiquitination and proteasomal degradation. J Biol Chem 275:37702–37711.

Hayashi T, Arimura T, Ueda K, Shibat H, Hohda S, Takahasi M, Hori H, Koga Y, Oka N, Imaizumi T, Yasunami M, Kimura A. 2004. Identification and functional analysis of a caveolin-3 mutation associated with familial hypertrophic cardiomyopathy. Biochem Biophys Res Comm 313:178–184.

Herrmann R, Straub V, Blank M, Kutzick C, Franke N, Neuen Jacob E, Lenard HG, Kroger S, Voit T. 2000. Dissociation of the dystroglycan complex in caveolin-3-deficient limb girdle muscular dystrophy. Hum Mol Genet 9:2335–2340.

Herrmann RH, Hertel S, Kutzick C, Straub V, Voit T. 2002. A novel caveolin-3 mutation in autosomal dominant rippling muscle disease. Neuromuscul Disord 12:723.

Kubisch C, Schoser BG, von During M, Betz RC, Goebel HH, Zahn S, Ehrbrecht A, Aasly J, Schroers A, Popovic N, Lochmuller H, Schroder JM, Bruning T, Malin JP, Fricke B, Meinck HM, Torbergsen T, Engels H, Voss B, Vorgerd M. 2003. Homozygous mutations in caveolin-3 cause a severe form of rippling muscle disease. Ann Neurol 53:512–520.

McNally EM, de Sa Moreira E, Duggan DJ, Bonnemann CG, Lisanti MP, Lidov HGW, Vainzof M, Passos-Bueno MR, Hoffman EP, Zatz M, Kunkel LM. 1998. Caveolin-3 in muscular dystrophy. Hum Mol Genet 7:871–877.

Minetti C, Sotgia F, Bruno C, Scartezzini P, Broda P, Bado M, Masetti E, Mazzocco M, Egeo A, Donati MA, Volontè D, Galbiati F, Cordone G, Dagna Bricarelli F, Lisanti MP, Zara F. 1998. Mutations in the caveolin-3 gene cause autosomal dominant limb-girdle muscular dystrophy. Nat Genet 18:365–368.

Minetti C, Bado M, Broda P, Sotgia F, Bruno C, Galbiati F, Volontè D, Lucania G, Pavan A, Bonilla E, Lisanti MP, Cordone G. 2002. Impairment of caveolae formation and T-system disorganization in human muscular dystrophy with caveolin-3 deficiency. Am J Pathol 160:265–270.

Parton RG, Way M, Zorzi N, Stang E. 1997. Caveolin-3 associates with developing T-tubules during muscle differentiation. J Cell Biol 136:137–154.

Scherer PE, Lisanti P, Baldini G, Sargiacomo M, Mastick CC, Lodish HF. 1994. Introduction of caveolin during adipogenesis and association of GLUT-4 with caveolin rich vesicles. J Cell Biol 127:1233–1243.

Smart EJ, Graf G, McNiven M, Sessa W, Engelman J, Scherer PE, Okamoto T, Lisanti M. 1999. Caveolins, liquid-ordered domains and signal transduction. Mol Cell Biol 19:7289–7304.

Song KS, Scherer PE, Tang Z, Okamoto T, Li S, Chafel M, Chu C, Kohtz DS, Lisanti MP. 1996. Expression of caveolin-3 in skeletal, cardiac, and smooth muscle cells. J Biol Chem 271:15160–15165.

Tang Z, Scherer PE, Okamoto T, Song K, Chu C, Kohtz DS, Nishimoto I, Lodish HF, Lisanti MP. 1996. Molecular cloning of caveolin-3, a novel member of the caveolin gene family expressed predominantly in muscle. J Biol Chem 271:2255–2261.

Tateyama M, Aoki M, Nishino I, Hayashi YK, Sekiguchi S, Shiga Y, Takahashi T, Onodera Y, Haginoya K, Kobayashi K, Iinuma K, Nonaka I, Arahata K, Itoyoma Y. 2002. Mutation in the caveolin-3 gene causes a peculiar form of distal myopathy. Neurology 58:323–325.

Venema VJ, Ju H, Zou R, Venema RC. 1997. Interaction of neuronal nitric oxide synthetase with caveolin-3 in skeletal muscle. Identification of a novel caveolin scaffolding/inhibitory domain. J Biol Chem 63:28187–28190.

Vorgerd M, Ricker K, Ziemssen F, Kress W, Goebel HH, Nix WA, Kubisch C, Schoser BGH, Mortier W. 2001. A sporadic case of rippling muscle disease caused by a de-novo caveolin-3 mutation. Neurology 57:2273–2277.

Wyse BD, Prior IA, Quian H, Morrow IC, Nixon S, Muncke C, Kurzchalia TV, Thomas WG, Parton RG, Hancock JF. 2003. Caveolin interacts with the angiotensin II type 1 receptor during exocytic transport but not at the plasma membrane. J Biol Chem 278:23738–23746.