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A study of *FHL1, BAG3, MATR3, PTRF* and *TCAP* in Australian muscular dystrophy patients

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Abstract

FHL1, BAG3, MATR3 and *PTRF* are recently identified myopathy genes associated with phenotypes that overlap muscular dystrophy. *TCAP* is a rare reported cause of muscular dystrophy not routinely screened in most centres. We hypothesised that these genes may account for patients with undiagnosed forms of muscular dystrophy in Australia. We screened a large cohort of muscular dystrophy patients for abnormalities in *FHL1* ($n = 102$) and *TCAP* ($n = 100$) and selected patients whose clinical features overlapped the phenotypes previously described for *BAG3* ($n = 9$), *MATR3* ($n = 15$) and *PTRF* ($n = 7$). We found one *FHL1* mutation (c.311G>A, p.C104Y) in a boy with rapidly progressive muscle weakness and reducing body myopathy who was initially diagnosed with muscular dystrophy. We identified no pathogenic mutations in *BAG3, MATR3, PTRF* or *TCAP*. In conclusion, we have excluded these five genes as common causes of muscular dystrophy in Australia. Patients with reducing body myopathy may be initially diagnosed as muscular dystrophy.

Keywords

Muscular dystrophy; Diagnosis; *FHL1*; *BAG3*; *MATR3*; *PTRF*; *TCAP*

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1. Introduction

The muscular dystrophies (MDs) are a genetically and phenotypically heterogeneous group of disorders defined by progressive muscle weakness and wasting, genetic aetiology and dystrophic changes on muscle pathology. They are subdivided into the dystrophinopathies, FSHD, myotonic dystrophies, distal dystrophies, congenital (CMD) and limb-girdle muscular dystrophies (LGMDs) based on clinical features. Currently, there are at least 18 genetically defined forms of LGMD identified [1] and distinguishing between the different types can be difficult [2–4]. Diagnosis relies on a combination of clinical information, muscle imaging, protein analysis (immunohistochemistry (IHC) and Western blot) and DNA sequencing. Identifying the primary mutation(s) is essential to provide accurate genetic counselling and aids clinical care. The genetic aetiology of many cases of MD is not known even after extensive investigation [4–7]. One of the likely reasons is that further genetic causes of MD are still to be identified.

There has been a number of muscle disease genes recently described including *FHL1, BAG3, MATR3* and *PTRF*. For each gene, only a handful of cases have been reported to date (especially for *BAG3* and *MATR3*) and as a result, the full spectrum of clinical phenotypes associated with mutation of these genes remains uncertain. The clinical and histological phenotypes described to date overlap with the MDs. We hypothesised that these genes may be responsible for some patients diagnosed with MD who lack a genetic diagnosis. We have screened well-characterised cohorts of CMD and LGMD patients in whom the known genetic causes have been largely excluded [4,8] to investigate whether these genes cause MD, in addition to the myopathies with which they have been associated so far.

Four and a half LIM domain 1 (*FHL1*) is a 32 kDa protein (*FHL1* gene on Xq26.3) that was identified as a cause of myopathy in 2008 [9]. Mutations have been associated with four overlapping clinical phenotypes: reducing body myopathy [9], scapuloperoneal muscular dystrophy [10,11], X-linked myopathy with postural muscle atrophy and generalised hypertrophy [12] and a form of Emery–Dreifuss muscular dystrophy (EDMD) [13]. The age of onset has been highly variable in the families identified to date, ranging from late infancy to adulthood [9], and both sporadic [9] and familial cases have been reported [12]. Early onset disease is usually associated with rapid disease progression. The course in later-onset disease is variable with some patients having relatively rapid progression to quadriplegia and respiratory failure and others showing only very slow decline.

Mutations in *FHL1* have been associated with a range of histological abnormalities on muscle biopsy, including dystrophic features such as increased internal nuclei, increased fibre-size variation, necrotic and regenerating fibres, fibrosis, increased adipose tissue [11,12] and histochemical reactivity for menadione staining [9]. Some patients also have intracytoplasmic inclusions which immunoreact with *FHL1* protein, desmin, ubiquitin, dystrophin, lamp1, Myo-BP-C, GRP78 [9] and phalloidin [11]. *FHL1* protein localises to the sarcomere (I-band/Z-disc) and sarcolemma in skeletal muscle but its precise roles remain uncertain. We considered *FHL1* to be an excellent candidate gene for MD, because the broad

range of clinical and histological phenotypes described to date overlaps significantly with LGMD.

Mutations in *BAG3* are a newly identified cause of a severe myofibrillar myopathy (MFM), a genetically heterogeneous form of myopathy characterised by desmin-positive protein inclusions on muscle biopsy [14]. The Bcl-associated athanogene-3 (*BAG3*) protein, encoded by *BAG3* is highly expressed in striated muscle, where it localises to the Z-disc [15]. Only three patients have been reported to date, in a cohort study of 85 unrelated MFM patients and all three had a common dominant *de novo* change (c.626C>T; p.Pro209Leu) [14]. These patients had a severe atypical MFM clinical phenotype, characterised by onset within the first decade, severe, rapidly progressive muscle weakness and rigid spine in 2/3 cases [14]. The *BAG3* knock-out (KO) mouse has a severe fulminant myopathy with use-dependent muscle degeneration and death at day 25 from respiratory insufficiency [15]. There is already overlap between LGMD and MFMs as mutations in MYOT (myotilin) are established causes of both disorders [16]. To date, the main focus of *BAG3* research has been in MFM and we hypothesised that mutations in *BAG3* may cause other phenotypes such as MD.

Vocal cord and pharyngeal weakness with distal myopathy (VCPDM) due to mutations in *MATR3* was identified through two separate linkage studies that found the same mutation (c.C254G p.S85C) in unrelated families from Southern Tennessee and Bulgaria [17,18]. *MATR3* is a 14 exon gene on 5q31 [18] that encodes matrin 3, a highly conserved 130 kDa protein [19] that localises to the nuclear matrix in skeletal muscle [20]. VCPDM is a slowly progressive, autosomal dominant, adult-onset disease, with a distinctive clinical pattern of distal limb and bulbar weakness. Clinical presentation includes foot drop, hand weakness, swallowing difficulties, vocal cord changes and mild-moderate elevation in CK (maximum eight times normal) [17,18]. As the disease progresses, muscle involvement can become more asymmetric, and the limb girdle muscles are involved. Histopathological changes in patient muscle biopsies included rimmed vacuoles, variation in fibre size and fibre splitting. While the clinical phenotype described to date is distinctive, as the disease advances, symptoms overlap with MD and we hypothesised that *MATR3* may account for a broader spectrum of clinical and histological phenotypes that overlaps MD.

Recessive mutations in *PTRF* (17q21.2, also known as Cavin-1) have been associated with MD with generalised lipodystrophy in Japanese [21], Omani [22] and UK families [22]. A range of other clinical features have been associated, such as muscle hypertrophy, muscle mounding, metabolic complications, cardiac involvement, spinal involvement and contractures, and creatine kinase levels have ranged between 500 and 2650 μ/l [21,22]. Muscle biopsies from affected patients demonstrate chronic dystrophic changes, mislocalisation of caveolin 1, 2 and 3, [21] and reduced caveolae [21,22]. *PTRF* immunolocalises to the sarcolemmal membrane in normal muscle [21]. Mutations have been associated with absent or barely detectable *PTRF* staining [21,22] together with greatly reduced sarcolemmal and increased cytoplasmic caveolin-3 staining [21].

The *TCAP* gene was first identified as a cause of LGMD2G a decade ago, but to date mutations have only been reported in four Brazilian families, and one European case

[23,24]. *TCAP* (named after titin-cap) has two exons that encode a 19 kDa protein called telethonin [25]. *TCAP* is exclusively expressed in striated muscle where it localises to the Z-disc of the sarcomere [26]. The only study to look at disease frequency estimated the incidence of LGMD2G to be 2.2% in the Brazilian general LGMD population [27]. LGMD2G is a variable autosomal recessive disease that is sometimes associated with atrophy of distal muscles or calf hypertrophy [27]. All mutations identified to date have been frameshift or nonsense mutations that are associated with absence of telethonin immunostaining in patient muscle [23,28,29]. We hypothesised that *TCAP* may be responsible for a greater proportion of LGMD than currently appreciated.

We screened an undiagnosed cohort of Australian CMD and LGMD patients for abnormalities in *FHL1, TCAP, BAG3, MATR3* and *PTRF* to investigate whether mutations in these genes are responsible for cases diagnosed as MD, in addition to the phenotypes for which they have been associated to date.

2. Materials and methods

2.1. Patient ascertainment

This study was approved by The Human Ethics Committee of The Children's Hospital at Westmead (Biospecimen bank 2005-042). We selected study participants from patients enrolled in a myopathy biospecimen bank in a single Australian institution and therefore the cohort includes individuals from varied ethnic backgrounds due to Australia's diverse heritage (70% Anglo-Celtic, 18% European, 9% Asian, 1.5% Aboriginal or Torres Strait Islander) [30]. LGMD patients had proximal limb-girdle or generalised muscle weakness, age of onset greater than 2 years and dystrophic histopathological muscle changes. CMD patients had progressive muscle weakness and wasting from birth or within the 1st year of life and dystrophic histopathological changes on muscle biopsy. All study participants had been screened for the common forms of CMD or LGMD depending on clinical presentation and histological findings, through protein and/or genetic-based testing [4,8].

2.2. Patient selection

We investigated *FHL1* in 102 patients in total; 71 with undiagnosed LGMD or EDMD, 12 with undiagnosed CMD and 19 with unspecified myopathies who had one or more of the following features previously associated with mutations in *FHL1*: foot drop, muscle hypertrophy, prominent spinal involvement, scapula winging, scapuloperoneal weakness, early respiratory involvement, cardiomyopathy, vacuoles/inclusions, early onset and progressive weakness, or a family history suggestive of X-linked inheritance. We excluded patients who had congenital onset with slow clinical progression.

For the *BAG3* study we performed mutation analysis on nine undiagnosed LGMD patients who had specific clinical features that have been previously associated with mutation of *BAG3*; six myopathy patients with severe muscle disease and/or respiratory, cardiac and bulbar involvement, and three patients with MFM-like histological changes.

We sequenced the *MATR3* gene in a cohort of 15 LGMD patients with features overlapping VCPDM. Twelve patients had bulbar involvement, four patients had at least one rimmed

vacuole on muscle biopsy and one patient without an available histological report had a first-degree relative with rimmed vacuoles.

We assessed seven patients for primary *PTRF* abnormalities by mutation analysis. Five patients had lipodystrophy or abnormal fat distribution with or without muscular dystrophy and two patients had abnormal caveolin-3 protein levels or localisation and normal *CAV3* gene analysis.

For the *TCAP*/telethonin studies we investigated 100 patients with undiagnosed LGMD, unselected by clinical features, by immunohistochemical analysis.

2.3. Immunohistochemistry

All muscle biopsies were snap frozen in isopentane cooled in liquid nitrogen and stored in liquid nitrogen until required. Indirect immunofluorescence was performed on 8 μm-thick muscle cryosections cut onto glass slides (Thermoscientific Superfrost plus). Sections were blocked in 2% bovine serum albumin (BSA) in 1× PBS solution at room temperature (RT) for 15 min. Sections were incubated in primary antibody (diluted in 2% BSA) at 4 °C overnight, followed by three washes in PBS. Secondary antibodies were applied and sections were incubated at RT for 2 h. After an additional three washes in PBS, coverslips were applied over Immumount (Shandon, Pittsburgh, PA, USA). Fluorescence microscopy imaging was performed (Olympus BX50) and images were captured using SciTech camera and ProgRes® CapturePro 2.6 software (Jenoptik). Antibodies to the following proteins were used: spectrin (NCL-Spec1; Leica Microsystems, Wetzlar, Germany); telethonin (G-11/sc-2537; Santa Cruz Biotechnology, Inc. CA, USA) and Cy3-conjugated mouse anti-human IgG secondary antibody (Jackson Immunoresearch Laboratories, PA, USA).

2.4. Genetic analysis

Genomic DNA was isolated from either frozen muscle using the QIAamp DNA Mini Kit (Qiagen Pty Limited, Hilden, Germany), or from whole blood using a modified salting out protocol [31]. Primers were designed to amplify the coding exons of *FHL1, BAG3, MATR3, PTRF* & *TCAP* and adjacent intronic regions. The amplicons for *FHL1* (all exons), *PTRF* (all exons), *TCAP* (all exons) and *BAG3* (exons 2–4) were generated using Taq DNA Polymerase (Invitrogen, CA, USA). For *BAG3* exon 1, Expand Long Template PCR System enzyme (Roche Diagnostics, Basel, Switzerland) and for *MATR3*, amplitaq gold (Applied Biosystems by life technologies, CA, USA) was used. PCR fragments were resolved on a 1% agarose gel and visualised with ethidium bromide. The PCR products were either gel purified (Jetquick gel extraction spin kit, Astral Scientific, Caringbah, Australia) or treated with 1 U Shrimp Alkaline Phosphatase (USB Corporation, Cleveland, Ohio, USA) and 6 U Exonuclease I (New England Biolabs, Ipswich, MA, USA). All DNA sequencing was performed on a 3730*xl* capillary sequencer (Applied Biosystems by life technologies, CA, USA).

3. Results

3.1. FHL1

We found a likely disease-causing sequence change in *FHL1* in one out of 102 undiagnosed MD and myopathy patients. A hemizygous missense change c.311G>A (p.C104Y) was found in exon 3, in a boy (Patient 1) with rapidly progressive muscle weakness and a dystrophic muscle biopsy. Parental samples were not available. This change was not observed in the latest 1000 genomes data release which contains data from 629 control individuals [32].

3.2. Case report

Patient 1 is a male with no reported family history of muscle weakness, except for a maternal uncle and great uncle who were reported to have waddling gait in adulthood. This boy was well in the neonatal period and achieved early gross motor milestones at normal ages until the onset of symptoms at age 2.5 years. On examination at age 4 years he had repeated falls, a waddling gait, difficulty sitting upright, positive Gowers' manoeuvre, wasting of the shoulder girdle and quadriceps muscles, shoulder weakness, a lumbar lordosis and respiratory muscle weakness. At age 5 years he had normal nerve conduction studies and echocardiogram, an elevated serum CK level (700 μ/l) and myopathic changes on biceps muscle electromyography. His condition was rapidly progressive and he died at age 7 years from respiratory failure.

3.3. Histopathology

Quadriceps muscle biopsy of Patient 1 at age 5 years was reported as showing marked dystrophic changes with necrotic and regenerating fibres, variation in fibre size and mild endomysial and perimysial fibrosis (Fig. 1A and B). There was increased internalised nuclei, particularly in regenerating fibres. Numerous fibres contained rimmed vacuoles (Fig. 1A and B), and occasional fibres contained rod-like bodies. Menadione-NBT stains, performed after *FHL1* genetic analysis, revealed positive staining for reducing bodies (Fig. 1C). Irregular granular foci stained positive for *FHL1* (Fig. 1D) and desmin. Immunohistochemistry for dystrophin, α-sarcoglycan, γ-sarcoglycan, laminin-α2, α-dystroglycan, dysferlin, caveolin-3, sarcospan, α7-integrinB, α7-integrinA, dystrobrevin, α-dystrobrevin2, β1-syntrophin, α-actinin-3 and myosin heavy chain, fast were normal and β2-syntrophin IHC was moderately reduced. Western blot analysis of dystrophin, dysferlin, calpain-3, caveolin-3, lamin A/C and emerin was also normal.

3.4. BAG3, MATR3 and PTRF gene sequencing

We found several heterozygous previously reported single nucleotide polymorphisms (SNPs) in *BAG3* and *MATR3* in addition to unreported single base changes in intronic regions and 5′ & 3′ UTR regions of *BAG3* that are unlikely to alter splicing or gene expression. We did not find the previously reported *BAG3* mutation or any novel changes in *BAG3, MATR3* or *PTRF* that were likely to be pathogenic.

3.5. TCAP

We performed IHC on 100 LGMD patients to screen for abnormalities in telethonin, as all previously reported patients have had absent telethonin expression. Most patient biopsies showed normal levels of homogenous cytoplasmic staining, identical to control biopsies. Several patient biopsies had mildly abnormal staining patterns, sometimes only in isolated fibres (fibres with absent staining or prominent sarcolemmal staining). Four representative patients with minor abnormalities in telethonin IHC were further investigated by *TCAP* gene sequencing and we found no abnormal sequence changes.

4. Discussion

Currently, even the most experienced laboratories cannot identify a genetic cause in around a third of patients with limb-girdle or congenital muscular dystrophy [6,7]. One of the likely reasons is that further muscular dystrophy genes are still to be identified. We considered that several recently identified myopathy genes were good candidates for causing MD because they share clinical and/or histological features in common with MD. We screened an Australian cohort of patients for abnormalities in *FHL1, BAG3, MATR3* and *PTRF*, as well as *TCAP* for which there is limited data on disease frequency. The full clinical spectrums associated with mutation of these genes remain uncertain and the frequency of mutations in Australia is unknown.

We chose a direct gene sequencing approach to screen patients for primary abnormalities in *FHL1* since *FHL1* protein expression has varied widely in patients with *FHL1* mutations to date [9,12]. We identified one patient with a likely disease-causing mutation in *FHL1* who had been diagnosed with a muscular dystrophy, albeit with additional features such as rimmed vacuoles and protein inclusions. In retrospect this patient's muscle biopsy and clinical phenotype is consistent with reducing body myopathy. The p.C104Y change we identified is highly likely to be pathogenic for several reasons. This change affects a highly conserved cysteine residue in the second LIM domain that is implicated in coordinating zinc molecules and is important for the protein's tertiary structure [9,12]. All *FHL1* mutations associated with reducing body myopathy identified so far have involved zinc-binding residues in the second LIM domain and the p.C104Y mutation we found continues this strong association [33]. In addition, a different change affecting this same residue (p.C104R) has recently been reported in association with reducing body myopathy [34]. Since we identified only one case of *FHL1* myopathy out of a large cohort of undiagnosed LGMD and CMD patient, we are confident that mutations in *FHL1* are not a common cause of muscular dystrophy in Australia.

We screened LGMD patients for primary telethonin abnormalities by IHC and found only minor variations in telethonin staining that were most likely non-specific changes. We identified no mutations on sequencing representative patients with those changes for *TCAP*. Therefore, in Australia, we found telethonin to be an uncommon cause of LGMD. It is possible that we missed patients with *TCAP* mutations that do not alter telethonin staining significantly, but we think this is unlikely as all *TCAP* mutations to date have been recessive changes that lead to loss of protein expression [23,28,29].

We performed focused studies for *BAG3, MATR3* or *PTRF* as to date, all have been associated with distinctive phenotypes and we considered the most likely MD patients to have mutations in those genes were patients with clinical features that overlapped the disease patterns currently described. Therefore we restricted gene analysis to patients who shared key clinical features with the most distinctive clinical features associated each disease gene. We identified no patients with mutations in *BAG3, MATR3* or *PTRF*. If other quite different phenotypes are caused by mutations in these genes we may have missed them with this approach. It is difficult to draw firm conclusions from the relatively small cohorts screened for each gene but they do not appear to be common causes of myopathy in Australia.

In this study we have excluded *FHL1* and *TCAP* as common causes of MD in Australia and we found no evidence that *BAG3, MATR3* and *PTRF* are causes of MD. This study highlights that patients with reducing body myopathy share many clinical and histological features with muscular dystrophies and the diagnosis may be missed unless the more specific histological features, such as rimmed vacuoles are noted and specific stains for reducing bodies or *FHL1*-rich inclusions are performed. These findings can be used to prioritise genetic testing in Australian populations and have relevance for other populations who share similar ethnic backgrounds such as northern Europe and North America.

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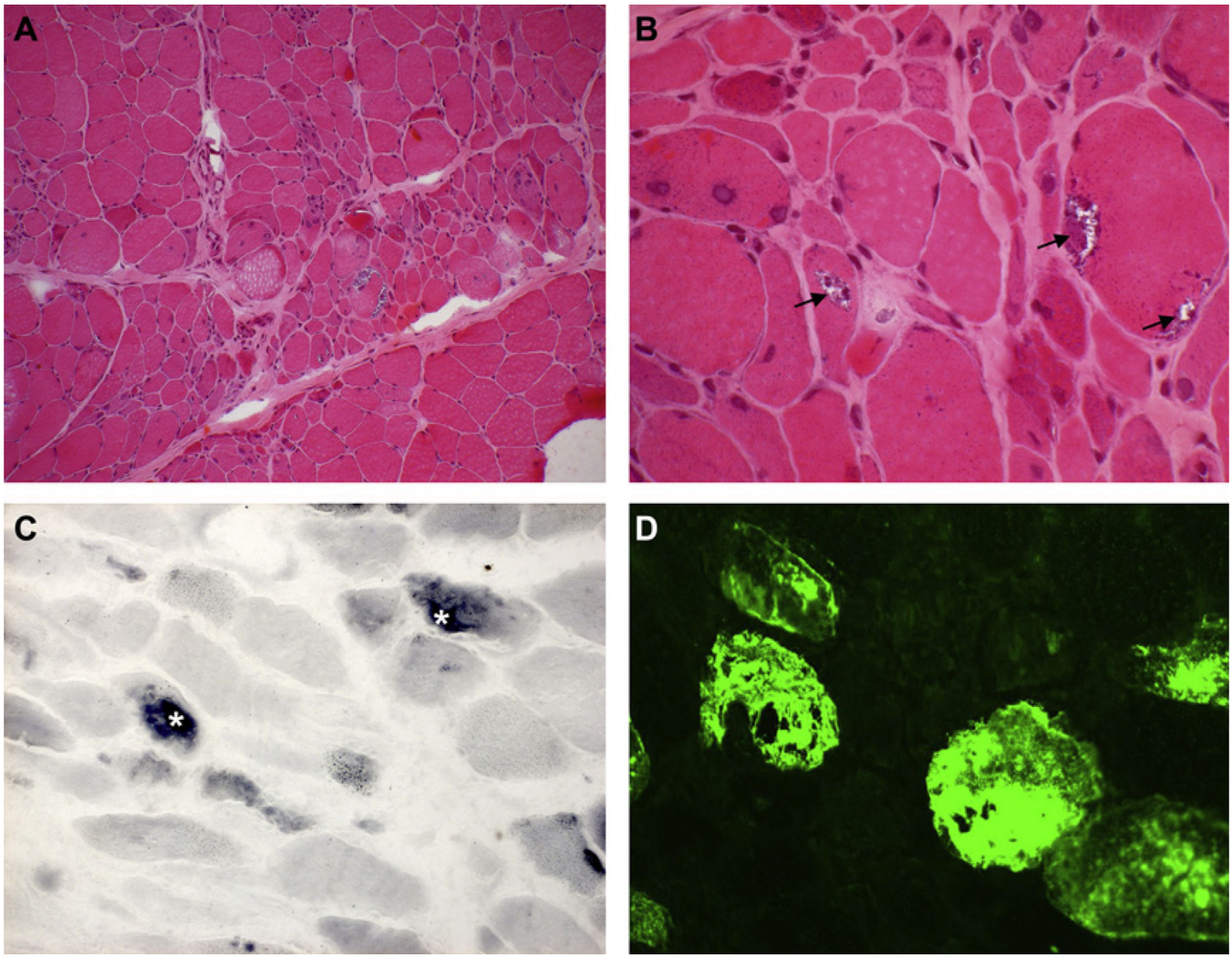

Fig. 1.

Haematoxylin and Eosin, Menadione-NBT and *FHL1* stains of Patient 1. Muscle biopsy findings from Patient 1. Haematoxylin and eosin stains at 100× (A) and 400× magnificaion (B) showing widespread dystrophic changes and rimmed vacuoles (B; arrows). Menadione-NBT stain at 200× magnification (C), demonstrating positive staining of reducing bodies (stars). *FHL1* IHC at 400× magnification (D), demonstrating fibres highly immunoreactive for *FHL1* protein.