

Heavy chain myosin 9-related disease (*MYH9*-RD): Neutrophil inclusions of myosin-9 as a pathognomonic sign of the disorder

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Summary

MYH9-related disease (*MYH9*-RD) is an autosomal dominant thrombocytopenia with giant platelets variably associated with young-adult onset of progressive sensorineural hearing loss, presenile cataract, and renal damage. *MYH9*-RD is caused by mutations of *MYH9*, the gene encoding for non-muscle heavy-chain myosin-9. Wild-type and mutant myosin-9 aggregate as cytoplasmic inclusions in patients' leukocytes, the identification of which by immunofluorescence has been proposed as a suitable tool for the diagnosis of *MYH9*-RD. Since the predictive value of this assay, in terms of sensitivity and specificity, is unknown, we investigated 118 consecutive unrelated patients with a clinical presentation strongly consistent with *MYH9*-RD. All patients prospectively underwent both the immunofluorescence assay for myosin-9 aggregate detection and molecular genetic analysis of the *MYH9* gene. Myo-

sin-9 aggregates were identified in 82 patients, 80 of which (98%) had also a *MYH9* mutation. In the remaining 36 patients neither myosin-9 aggregates nor *MYH9* mutations were found. Sensitivity and specificity of the immunofluorescence assay was evaluated to be 100% and 95%, respectively. Except for the presence of aggregates, we did not find any other significant difference between patients with or without aggregates, demonstrating that the myosin-9 inclusions in neutrophils are a pathognomonic sign of the disease. However, the identification of the specific *MYH9* mutation is still of importance for prognostic aspects of *MYH9*-RD.

Keywords

MYH9-related diseases, thrombocytopenia, giant platelets, *MYH9* gene, neutrophil inclusions

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Introduction

MYH9-related disease (*MYH9*-RD) is an autosomal dominant disorder characterised by congenital thrombocytopenia with giant platelets. Patients are at risk of developing during youth or adult life progressive sensorineural hearing loss, presenile cataract and/or a proteinuric nephropathy that often leads to end-stage renal disease. *MYH9*-RD includes syndromes previously known as May-Hegglin anomaly and Sebastian, Epstein or Fechtner syndromes, which were thought to be distinct disorders prior to the demonstration that they were all due to mutations of *MYH9*, the gene encoding for the heavy chain of non-muscle myosin-9 (1–3). Myosin-9 assembles into a hexameric complex consisting of two heavy chains and two pairs of light chains. Each heavy chain contains a

N-terminal motor domain, a neck region, and a C-terminal α -helical coiled-coil tail domain. While it has been recently shown that mutations of myosin-9 cause thrombocytopenia by affecting proplatelet formation and platelet release (4), pathogenesis of hearing loss, nephropathy and cataract is completely unknown.

A genotype/phenotype study recently found that most individuals with mutations in the region encoding for the motor domain of myosin-9 have a severe thrombocytopenia (platelet count usually lower than $50 \times 10^9/l$) and a high risk of developing nephropathy and deafness before the age of 40 (5). Affected individuals with mutations affecting the tail domain have instead a mild phenotype characterised by moderate macrothrombocytopenia and a lower risk of later onset manifestations. In particular, patients carrying the R1933X mutation have only a moderate thrombocy-

topenia with no observed risk for non-haematological manifestations.

MYH9-RD was considered a very rare disease, being only a few cases reported in the literature before the cloning of the gene (6–11). Since the identification of the first mutations, the number of patients and families described has greatly been increasing, especially in Italy and Japan. Since *MYH9*-RD has a worldwide distribution, it is likely that many patients are not recognised in many countries and that the overall prevalence of this disorder is highly underestimated. In fact, among *MYH9*-RD patients with an ascertained diagnosis based on genetic testing there are cases presenting with only a macrothrombocytopenia and cases with additional extra haematological manifestations, such as hearing, ocular and/or kidney defects, which might not be recognised as a part of the complex clinical trait of *MYH9*-RD. Moreover, *MYH9*-RD can be caused by *de novo* mutations of *MYH9* and therefore not regarded as a inherited disease (5, 12). As a consequence, patients are often misdiagnosed with idiopathic thrombocytopenic purpura and receive unnecessary therapies when an accurate and prompt diagnosis would instead allow them to benefit from proper therapies and preventive measures (6, 13, 14).

Since *MYH9* is a large gene with 40 coding exons, molecular genetic analysis of the entire gene is expensive, time consuming, and not feasible for a large series of patients. We and others have reported an altered localisation of myosin-9 in the peripheral blood neutrophils of *MYH9*-RD patients, where it forms cytoplasmic aggregates instead of being diffusely distributed in cytoplasm (3, 15–17). These aggregates correspond to the basophilic leukocyte inclusions that can be observed in some but not all patients using conventional May-Grünwald-Giemsa (MGG) staining. Therefore, immunofluorescence analysis of myosin-9 distribution on blood smears was proposed as a useful tool for the diagnosis of *MYH9*-RD. However, the predictive value of this assay, in terms of sensitivity and specificity for diagnosis of the disease, has not been defined. In fact, although in the last years the myosin-9 aggregates have been described in several patients with *MYH9* mutations (5, 16, 18), no prospective studies have been performed to measure its accuracy in the diagnosis of the disease in case of clinical suspicion.

In this paper, we report data from 118 consecutive unrelated patients with a strong clinical suspicion of *MYH9*-RD, who all prospectively underwent both the immunofluorescence assay for the detection of myosin-9 aggregates and molecular genetic testing of *MYH9*. Granulocyte aggregates of myosin-9 were identified in about 70% of patients. Whereas in patients with aggregates we identified mutations in 98% of the cases, no mutation was detected when inclusions were absent, indicating that the analysis of myosin-9 in neutrophils is a powerful discriminating tool for the diagnosis of *MYH9*-RD.

Patients and methods

Patients

A series of consecutive unrelated patients with a diagnostic suspicion of *MYH9*-RD was enrolled by the Italian Registry for

MYH9-RD from January 2002 to January 2008. This study included exclusively one patient from each investigated family, that is the first member referred to the Registry and evaluated at the time of the initial referral. The activity of the Registry, as well as that of present study, was approved by the Institutional Review Board of the IRCCS Policlinico San Matteo Foundation, Pavia, Italy. All the investigated subjects or their parents or legal guardian gave written informed consent.

The inclusion criteria for this study were defined as follows: patients with an autosomal-dominant or sporadic form of congenital thrombocytopenia associated with at least one of the following features of the *MYH9*-RD disease: giant platelets in the peripheral blood, early-onset sensorineural deafness, unexplained proteinuric nephropathy, and/or presenile cataract (3, 5, 19). Since it has been shown that automated cell counters often fail to recognise very large platelets of patients with congenital macrothrombocytopenias, underestimating thus platelet volume (20), platelet size was evaluated by peripheral blood smears examination. In each subject, platelet diameters were measured on at least 200 platelets by optical microscopy on MGG stained smears using a software-assisted image analysis method (Axiovision 4.5; Carl Zeiss, Goettingen, Germany). Measurement of platelet size was centralised at the Department of Internal Medicine, Pavia, Italy. The presence of giant platelets was defined by platelets with a major diameter larger than 8 μm , which are never detected in healthy subjects (6, 21). Platelet macrocytosis was instead defined if more than 4% of platelets were larger than 4 μm (3). The presence of sensorineural hearing loss was based on audiometric examination. Deafness was recorded for a bone threshold average greater than 25 dB at 1,000 Hz, 2,000 Hz, and 4,000 Hz (5). For infants the hearing function was examined by sensory evoked potentials. Only sensorineural deafness detected earlier than the age of 60 was considered for this study. Proteinuric nephropathy was recorded when 24-hour proteinuria was more than 0.5 g in at least two consecutive examinations during a period of at least six months, in the absence of any other possible causes of proteinuria (5). Proteinuria could be associated with chronic renal failure and/or nephrotic syndrome. Presenile cataract was searched for by ophthalmological evaluation in the early or middle life (age <60 years) (5).

Patients with an heterozygous p.Ala156Val mutation of the GPIBA gene (monoallelic form of Bernard-Soulier syndrome) were excluded *a priori* from the present study as this mutation is relatively frequent, at least in the Italian population (22). Indeed, the immunofluorescence analysis revealed a normal distribution of myosin-9 in neutrophils of all these patients (data not shown).

Immunofluorescence assay for detection myosin-9 aggregates

Distribution of myosin-9 was investigated on peripheral blood smears using the mouse NMG2 monoclonal antibody (kindly provided by Dr. Saverio Sartore, University of Padua, Italy (3, 23). Eighty out of the 118 patients were also investigated using the

BTI561 (Biomedical Technologies Inc., Stoughton, MA, USA) and the PRB-440P (Covance Research Products, Berkeley, CA, USA) rabbit polyclonal antibodies confirming the results obtained with NMG2. Goat anti-mouse or anti-rabbit conjugated with Alexa Fluor 488 (Invitrogen, Milan, Italy) was used for secondary detection. General staining procedures have been previously described (24). The immunofluorescence assay was performed either on freshly prepared blood smears or on smears shipped at room temperature and processed up to 15 days after preparation. Preliminary experiments had shown that the assay gives identical results when performed on freshly prepared smears or on smears stored at room temperature up to 30 days after preparation. As control, the immunofluorescence assay was performed in 50 healthy donors, whose neutrophils never showed myosin-9 aggregates. Fluorescence microscopy was performed as blind analysis by two different observers (V.B. and A.P.) using an Axioscope 2 Plus microscope (Carl Zeiss) with a 100x/1.30 Plan Neofluar oil-immersion objective. No disagreement in the interpretation of the specimens was recorded. The size of myosin-9 aggregates was measured on digital images acquired by an AxioCam MRC camera and the Axiovision 4.5 software (Carl Zeiss). The software module Axiovision Auto-measure was used for image analysis.

Mutational screening of the MYH9 gene

All patients were screened for mutations of the MYH9 gene. The coding exons and the respective exon-intron boundaries were amplified by PCR as previously described (5). The PCR products were sequenced using an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit and an ABI 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The analysis was performed with a tiered approach that focuses initially on “hot” exons (exons 16, 30, 38, and 40; the first coding exon is referred as exon 1), where mutations have been found most commonly, then on the other six exons (exons 1, 10, 25, 26, 31, and 37) where mutations have also been identified, and finally on the remainder of the gene. PCR products of the so-called “hot” exons were obtained using two different sets of primers in order to prevent allelic drop out due to the presence of single nucleotide polymorphisms. Since a genomic deletion of 1220 leading to an in-frame deletion of exon 25 was recently detected in one MYH9-RD Japanese family (25), a long PCR was also carried out to detect this potential alteration of the gene.

Results

Enrolment of patients

One hundred eighteen consecutive patients with a clinical suspicion of MYH9-RD entered the study (► Table 1). They were 62 males and 56 females, with a median age of 27 years (range, 2–87). The inclusion criteria are detailed in the Patients and Methods sec-

tion. Sixty-three patients had congenital thrombocytopenia and giant platelets without extra-haematological manifestations (non-syndromic thrombocytopenia). In the remaining 55 patients, congenital thrombocytopenia was associated with one or more extra-haematological features of MYH9-RD (syndromic thrombocytopenia). Fifty-one of the syndromic patients presented giant platelets. The mean platelet count of the 118 enrolled patients was $64 \times 10^9/l$ (range, 3–148).

Myosin-9 aggregates strongly associated with MYH9 mutations

Of the 118 enrolled individuals, 82 showed myosin-9 aggregates in neutrophils (Table 1). Molecular genetic test identified 27 different pathogenetic MYH9 mutations in 80 of the 82 cases (98%). The clinical features of the affected members of 50 of these families were previously reported in a genotype/phenotype correlation study (3). The percentage of patients with MYH9 mutations was not different between the two groups classified as non-syndromic or syndromic thrombocytopenia (65% vs. 71%, respectively).

Of note, 35% of all cases were *de novo* mutations. Mutations in the motor domain were more frequently *de novo* (17 out of 24 cases, 71%) than those in the tail domain (11 out of 56, 20%) (► Table 2). The nucleotide that most frequently was hit by a *de novo* mutation was at position 2104, leading to the p.R702C mutation.

There was a specific correlation between the morphological aspects of myosin-9 clumps and the respective pathogenetic mutations. Patients with mutations in the motor domain had a “speckled” immunofluorescence pattern characterised by numerous aggregates (> 10) of small size (< 0.5 μm) (pattern A, ► Fig. 1A). Instead, patients with mutations in the rod-tail domain showed one to four large inclusions (2–7 μm) of different shapes (round, oval, spindle-shaped, or rod-like) often together with further small aggregates (pattern B, Fig. 1B). The only exception to this correlation was a patient with the p.V1930CfsX18 mutation (rod-tail domain), which was associated with a pattern A distribution of myosin-9 in neutrophils. The aggregates of pattern B were

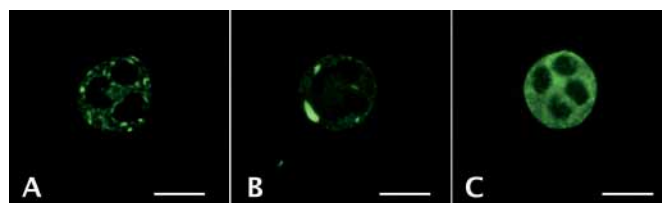


Figure 1: Morphological features of myosin-9 aggregates in neutrophils of patients with different MYH9 mutations. A) Patients with mutations in the motor domain of myosin-9 present a “speckled” pattern characterised by numerous aggregates of small size (<0.5 μm). B) Patients with mutations in the rod-tail domain always show one to four main, large aggregates (2–7 μm) of different shapes, often together with further small aggregates. C) Normal myosin-9 distribution pattern is shown for comparison. Scale bars correspond to 10 μm .

Table 1: Distribution of patients with a suspected diagnosis of MYH9-RD on the basis of their clinical aspects, presence of myosin-9 aggregates in neutrophils and MYH9 mutations.

No. of patients (%)	Non-syndromic thrombocytopenia		Syndromic thrombocytopenia		Total	
	63 (53%)		55 (47%)		118	
Myosin-9 aggregates	MYH9 mutations					
	Yes	No	Yes	No	Yes	No
Yes	41 (65%)	2 (3%)	39 (71%)	0	80 (68%)	2 (2%)
No	0	20 (32%)	0	16 (29%)	0	36 (30%)

Table 2: Mutations of the MYH9 gene identified in 80 families with myosin-9 aggregates in neutrophils.

Nucleotide change ^a	Exon ^b	Aminoacid change	Number of unrelated patients	Patients with de novo mutations
c.279C>G	1	p.N93K	1 (1) ^c	1
c.284C>A		p.A95D	1 (1) ^d	0
c.287C>T		p.S96L	3 (2) ^c	2
c.2104C>T	16	p.R702C	14 (8) ^c	11
c.2105G>A		p.R702H	4 (4) ^c	2
c.2152C>T		p.R718W	1 (1) ^c	1
c.3195_3215del	24	p.E1066_A1072del	1 (1) ^c	1
c.3195_3215dup		p.E1066_A1072dup	1 (1) ^e	0
c.3463A>G	25	p.T1155A	1 (1) ^c	1
c.3464C>T		p.T1155I	1 (1) ^c	0
c.3485G>C		p.R1162T	1 (0)	0
c.3493C>T	26	p.R1165C	3 (3) ^c	1
c.3494G>T		p.R1165L	1 (0) ^c	0
c.4270G>C	30	p.D1424H	5 (4) ^c	2
c.4270G>A		p.D1424N	6 (4) ^c	3
c.4270G>T		p.D1424Y	2 (1) ^c	0
c.4340A>T		p.D1447V	1 (1) ^c	0
c.4546G>A	31	p.V1516M	1 (1) ^f	0
c.4670G>T	32	p.R1557L	1 (1) ^f	0
c.5521G>A	38	p.E1841K	11 (7) ^c	2
c.5770_5779del	40	p.G1924RfsX21	1 (1) ^f	0
c.5773delG		p.D1925TfsX23	1 (1) ^c	0
c.5788delG		p.V1930CfsX18	1 (1) ^f	0
c.5797C>T		p.R1933X	13 (6) ^c	1
c.5797delC		p.R1933EfsX15	1 (1) ^c	0
c.5821delG		p.D1941MfsX7	2 (2) ^c	0
c.5833G>T		p.E1945X	1 (1) ^c	0
Total	80 (56)	28 (35%)		

^aA of the ATG translation initiation start site of the MYH9 cDNA in GenBank reference sequence NM_002473.3 is indicated as nucleotide +1. ^bExon 1 is referred as the first coding exon; exons 1 and 16 code for the motor domain whereas exons 24 to 40 code for the tail domain. ^{c-f}Numbers in brackets indicate the number of MYH9-RD families reported in other studies, such as ^cPecci et al. (5), ^dDe Rocco et al. (31), ^eDe Rocco et al. (32), and ^fPecci et al. (33). The only new mutation reported in this paper is p.R1162T.

phenotypically more heterogeneous than those of pattern A, differing in size and/or shape even among patients carrying the same mutation.

In two out of the 82 patients with myosin-9 aggregates, we were not able to identify any mutations despite an extensive screening of the *MYH9* gene (Table 1). Both subjects presented with a non-syndromic thrombocytopenia, giant platelets, and typical basophilic “Döhle-like” inclusions at MGG staining of peripheral blood smears. Even the immunofluorescence analysis showed a pattern indistinguishable from that of cases with a *MYH9* mutation.

No *MYH9* mutations in patients without myosin-9 aggregates in neutrophils

In 36 of the 118 patients enrolled in the study, myosin-9 was uniformly distributed in the cytoplasm of their neutrophils (Fig. 1C). In these patients, the analysis of all *MYH9* coding exons did not reveal any pathogenetic mutation (Table 1). The 20 patients with a non-syndromic thrombocytopenia had a median age of 18 years (range, 3–52), a mean platelet count of $62.5 \times 10^9/l$ (range, 20–113),

and a percentage of giant platelets ranging from 2% to 36%. These findings were consistent with those of non-syndromic patients with *MYH9* mutations (data not shown). Whereas eight probands were sporadic cases, 12 belonged to families with other affected members (from 2 to 7), where the platelet defect was transmitted as an autosomal dominant trait.

The clinical aspects of the 16 patients with a syndromic thrombocytopenia are summarised in ► Table 3. Even in this group the disorder was sporadic (8 probands) or familial (8 probands). In patient 1, congenital macrothrombocytopenia associated with proteinuric nephropathy, sensorineural hearing loss, and bilateral cataracts notably at the age of 11. Another three patients presented with two additional non-haematological manifestations, such as nephropathy and deafness (patient 2) or deafness and presenile cataracts (patients 3 and 4). Of the remaining 12 patients, congenital thrombocytopenia was associated with either sensorineural deafness (6 cases) or proteinuric nephropathy (6 cases). The onset of nephropathy, hearing loss, and cataracts was at the mean age of 18, 15, and 15 years, respectively, similar to that observed in patients with *MYH9* mutations (data not shown). At a microscopic evaluation of the peripheral blood smears, giant platelets were present in 12 cases; platelet macrocytosis without giant elements was evident in three subjects; in one case (patient 13) the platelet

Table 3: Clinical and laboratory features of 16 syndromic patients without myosin-9 aggregates in neutrophils and *MYH9* mutations.

Patient	Age/ gender	Platelet count ($\times 10^9/L$)	Platelet macro- cytosis ^a	Giant platelets ^a	Nephropathy ^b (age at onset)	Hearing defect ^c (age at onset)	Cataract (age at onset)	Model of transmission ^d (No. of affected members)
1	15/M	29	yes	yes	yes (8)	BSNHL (11)	Bilateral (1)	AD (2)
2	16/M	85	yes	yes	yes (15)	BSNHL (15)	no	AD (3)
3	5/M	72	yes	no	no	BSNHL (5)	Bilateral (4)	Sporadic
4	66/M	97	yes	yes	no	BSNHL (40)	Monolateral (40)	AD (3)
5	24/M	65	yes	yes	no	BSNHL (24)	no	Sporadic
6	52/F	90	yes	yes	no	BSNHL (32)	no	AD (3)
7	23/M	95	yes	yes	no	BSNHL (8)	no	Sporadic
8	15/M	80	yes	yes	no	BSNHL (2)	no	Sporadic
9	2/F	20	yes	yes	no	BSNHL (1)	no	AD (2)
10	8/F	70	yes	yes	no	MSNHL (8)	no	Sporadic
11 ^e	70/M	100	yes	no	yes (nd)	nd	no	AD (4)
12 ^e	25/M	40	yes	yes	yes (25)	no	no	Sporadic
13	4/M	90	no	no	yes (2)	no	no	AD (4)
14	42/F	43	yes	no	yes (35)	no	no	Sporadic
15	27/F	68	yes	yes	yes (15)	no	no	AD (2)
16	35/M	72	yes	yes	yes (28)	no	no	Sporadic

^aEvaluated by examination of peripheral blood smears prepared without anticoagulation and stained by May-Grünwald-Giemsa. Platelet macrocytosis: more than 4% of platelets larger than $4 \mu\text{m}$ (healthy subjects: $\leq 4\%$). Giant platelets: presence of platelets larger than $8 \mu\text{m}$, which are absent in healthy subjects. ^bIdiopathic chronic proteinuria $>1 \text{ g/day}$, with or without nephrotic syndrome (present in 2/8 patients) and/or chronic renal failure (5/8 patients). Any possible cause of acquired proteinuric nephropathy has been carefully excluded in all the reported patients. ^cDetermined by audiometric examination. BSNHL, bilateral sensorineural hearing loss; MSNHL, monolateral sensorineural hearing loss; nd, not determined. ^dAD, autosomal dominant. ^eReported by Seri et al. (3).

size was normal (Table 3). Of note, in all of the four cases without giant platelets myosin-9 aggregates or *MYH9* mutations were not detected.

Discussion

This paper reports a study on 118 affected individuals enrolled because of a diagnostic suspicion of *MYH9*-RD (5), being affected by congenital thrombocytopenia associated with one or more of the features of the disease, such as giant platelets, sensorineural deafness, proteinuric nephropathy, and presenile cataract. All patients prospectively underwent both immunofluorescence analysis for the detection of myosin-9 aggregates in neutrophils and molecular genetic testing of the *MYH9* gene, allowing us to demonstrate that the immunofluorescence test is a valuable tool to select patients suitable for mutational analysis.

When inclusions were present, mutations were detected with a high rate, being identified in 98% of cases. On the contrary, *MYH9* mutations were not identified in patients without aggregates. Evaluated in relation to the presence of mutations, the specificity and sensitivity of the immunofluorescence test is 95% and 100%, respectively. Of note, even in our highly selected population, the predictive value of a negative test result was 100%, while the positive predictive value was 98%. Therefore, while the presence of the myosin-9 aggregates addresses towards the molecular testing and a *MYH9*-RD diagnosis, their absence allows the exclusion of the diagnosis without any risk of missing patients with *MYH9*-RD. Except for the presence of leukocyte inclusions, there was no significant difference in clinical and laboratory features between patients with and without the *MYH9* mutations. However, the four patients enrolled with a syndromic thrombocytopenia but without giant platelets did not carry any *MYH9* mutations, suggesting that platelet size should be regarded as an important feature in the differential diagnosis of *MYH9*-RD.

Whenever the aggregates are present, their morphological characteristics can orient the mutational screening. Indeed, there is a good correlation between the pattern of myosin-9 distribution in neutrophils and the protein domain affected by the causative mutation. The presence of numerous and small aggregates should lead to first investigate exons of the motor domain, such as exons 1 and 16, whereas that of a few large inclusions should induce to analyse the coding region of the tail domain, such as exons 30, 38, or 40. The small aggregates typical of pattern A are rarely detectable as basophilic "Döhle-like" inclusions using a conventional MGG-stained blood smear. Indeed, in patients with mutation at codon 702 the aggregates are rarely recognisable as basophilic inclusions because of their low content in poly(A)⁺ RNA (26). These observations further emphasise how a sensitive test such as the immunofluorescence assay is suitable in the diagnosis of the *MYH9*-RD.

Our classification of aggregates is slightly discordant with those reported on 24 Japanese patients (16), who were separated into three groups, I, II, and III according to the number, size, and shape of the

fluorescence-labeled myosin-9 aggregates. On one hand, we can recognise our "speckled" pattern A as localisation type III, which was originally described in two patients with motor-domain mutations (N93K or S96L) or a rare tail-domain missense (I1816V) mutation (16). Moreover, we can confirm and extend these observations, suggesting that a "speckled" distribution of myosin-9 is typical of all the subjects with amino acid substitutions in the motor domain. On the other hand, we observed an extensive heterogeneity in size and/or shape of the large aggregates in patients with rod-tail mutations preventing us from categorising any other specific patterns and further correlate mutations with the morphological aspects of inclusions.

In our large cohort, the *de novo* mutations affected 35% of patients, a frequency higher than that of 20% previously reported (19, 27). Of note, the majority of these mutations affect codon 702, which is associated with the most severe prognosis (5). This observation emphasises that the diagnostic suspicion of *MYH9*-RD

What is known about this topic?

- *MYH9*-related disease (*MYH9*-RD) is an autosomal-dominant thrombocytopenia caused by mutations of *MYH9*, the gene encoding for myosin-9. Patients present since birth with thrombocytopenia, giant platelets, and leukocyte inclusions due to a cytoplasmic aggregation of wild type and mutant myosin-9. Patients can also develop sensorineural deafness, nephropathy, and/or presenile cataract during life.
- Diagnosis of *MYH9*-RD is difficult and most of affected patients have received a previous diagnosis of idiopathic thrombocytopenic purpura and/or undue therapies.
- *MYH9* is a large gene, making its analysis expensive, time consuming, and not feasible for a large series of patients.
- Immunofluorescence search for myosin-9 aggregates on peripheral blood smear has been proposed as a tool to identify patients with *MYH9* mutations. However, sensitivity and specificity of this assay is unknown.

What does this paper add?

- In a large series of patients (n=118) with a clinical picture consistent with *MYH9*-RD myosin-9 immunofluorescence assay predicted the presence of a *MYH9* mutation with a sensitivity and specificity of 100% and 95%, respectively.
- Identification of myosin-9 aggregates in leukocytes is highly predictive for a *MYH9* defect. Their absence allows exclusion of the diagnosis without any risk to miss patients with *MYH9*-RD.
- There is a specific correlation between pattern of the myosin-9 distribution in leukocytes and localisation of the mutations in *MYH9* exons. Thus, the immunofluorescence assay can orient the molecular genetic strategy avoiding useless exon analysis.
- The association of congenital macrothrombocytopenia with sensorineural deafness, nephropathy, and/or presenile cataract should be regarded as a genetic heterogeneous disease, since 30% of patients with such a syndromic presentation had no *MYH9* mutations, suggesting that at least another gene is involved.

must be raised even without a family history of thrombocytopenia or other manifestations of the disease.

In only two patients with aggregates, the *MYH9* mutations were not identified in spite of an accurate screening that covered all the coding exons. Mutations can be localised in any part of the gene, including regulatory regions and introns even though mutations localised outside of the *MYH9* coding regions have not been identified except for a small genomic deletion, leading, however, to an in-frame deletion (25). In fact, since the *MYH9* mutations are likely to act through a dominant negative effect, as hypothesized by functional studies and knock-out mouse models (28–30), most of them are missense and small in-frame deletions or duplication of the coding region (5, 27). We hypothesise that in these two patients mutations have escaped the screening for allelic drop out events or presence of small intragenic rearrangements (25). However, we cannot exclude that another gene could be responsible for a disease indistinguishable from *MYH9*-RD, including the presence of myosin-9 aggregates in neutrophils.

In conclusion, familial or sporadic thrombocytopenia with giant platelets associated or not with nephropathy, hearing impairment, and/or cataract is a genetic heterogeneous disease. Approximately 70% of cases have mutations in the *MYH9* gene and therefore, they are diagnosed as affected with *MYH9*-RD. Moreover, the myosin-9 aggregates in neutrophils should be regarded as a pathognomonic sign of the disease. Therefore, the diagnosis of *MYH9*-RD can be excluded in patients with a normal immunofluorescence pattern even in the presence of a clinical picture suggestive for *MYH9*-RD. On the contrary, the presence of inclusions can alone confirm the diagnosis. However, the identification of the causative mutation is still fundamental for the definition of the prognosis of the individual *MYH9*-RD patient.

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