MYH9-Related Thrombocytopenia: Four Novel Variants Affecting the Tail Domain of the Non-Muscle Myosin Heavy Chain IIA Associated with a Mild Clinical Evolution of the Disorder

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Abstract

MYH9-related disease (*MYH9*-RD) is an autosomal-dominant thrombocytopenia caused by mutations in the gene for non-muscle myosin heavy chain IIA (NMMHC-IIA). Patients present congenital macrothrombocytopenia and inclusions of NMMHC-IIA in leukocytes, and have a variable risk of developing kidney damage, sensorineural deafness, presenile cataracts and/or liver enzymes abnormalities. The spectrum of mutations found in *MYH9*-RD patients is limited and the incidence and severity of the non-congenital features are predicted by the causative *MYH9* variant. In particular, different alterations of the C-terminal tail domain of NMMHC-IIA associate with remarkably different disease evolution. We report four novel *MYH9* mutations affecting the tail domain of NMMHC-IIA and responsible for *MYH9*-RD in four families. Two variants cause amino acid substitutions in the coiled-coil region of NMMHC-IIA, while the other two are a splicing variant and a single nucleotide deletion both resulting in frameshift alterations of the short non-helical tailpiece. Characterization of phenotypes of affected individuals shows that all of these novel variants are associated with a mild clinical evolution of the disease.

Keywords
► inherited platelet disorders
► inherited thrombocytopenia
► *MYH9*-related disease
► non-muscle myosin IIA

Zusammenfassung

MYH9-assoziierte Erkrankung (MYH9-RD) ist eine autosomal-dominante Thrombozytopenie, die durch Mutationen im Gen für die Nicht-Muskel-Myosin schwere Kette IIA (NMMHC-IIA) verursacht wird. Die Patienten weisen eine kongenitale

Makrothrombozytopenie und Einschlüsse von NMMHC-IIA in Leukozyten auf und haben ein variables Risiko, Nierenschäden, sensorineurale Taubheit, präsenile Katarakte und / oder Leberenzyme zu entwickeln. Das Spektrum der bei MYH9-RD-Patienten gefundenen Mutationen ist begrenzt und die Inzidenz und der Schweregrad der nicht-kongenitalen Merkmale werden durch die verursachende MYH9-Variante prognostiziert. Insbesondere assoziieren verschiedene Veränderungen der C-terminalen Schwanzdomäne von NMMHC-IIA mit einer bemerkenswert unterschiedlichen Krankheitsentwicklung. Wir berichten über vier neue MYH9-Mutationen, die die Schwanzdomäne von NMMHC-IIA betreffen und in vier Familien für MYH9-RD verantwortlich sind. Zwei Varianten verursachen Aminosäuresubstitutionen in der Coiled-Coil-Region von NMMHC-IIA, während die anderen beiden eine Splicing-Variante und eine Einzelnucleotid-Deletion sind, die beide zu Frameshift-Veränderungen des kurzen nicht-helikalen Schwanzstückes führen. Die Charakterisierung der Phänotypen betroffener Individuen zeigt, dass alle diese neuen Varianten mit einer milden klinischen Entwicklung der Erkrankung einhergehen.

Introduction

MYH9-related disease (*MYH9*-RD) is an autosomal-dominant syndromic thrombocytopenia caused by mutations in *MYH9*, the gene encoding for the heavy chain of non-muscle myosin IIA (NMMHC-IIA).[1-3] *MYH9*-RD represents the most prevalent form of inherited thrombocytopenia worldwide. Patients present congenital thrombocytopenia, marked platelet macrocytosis with giant platelets and aggregates of NMMHC-IIA in the cytoplasm of granulocytes, which may be detectable after conventional staining of blood smears as basophilic 'Döhle-like' inclusion bodies.[4,5] Thrombocytopenia can result in a variable degree of bleeding tendency.[6] The low platelet count is often discovered only in adulthood and patients are frequently misdiagnosed as having immune thrombocytopenia (ITP) and inappropriately treated with immunosuppressive therapies and/or splenectomy, which are ineffective in this condition.[5,7] Causative *MYH9* mutations predispose to a series of extrahaematological defects, and most *MYH9*-RD patients develop one or more non-congenital features of the disorder, namely, proteinuric nephropathy often evolving to end-stage kidney failure, sensorineural deafness, presenile cataracts and/or abnormalities of liver enzymes.[6,8]

NMMHC-IIA comprises two distinct domains: the N-terminal globular head domain, responsible for actin binding and generation of mechanical force through hydrolysis of adenosine triphosphate (ATP), and the C-terminal tail domain, which is mainly involved in assembly of the myosin molecule. Two NMMHC-IIAs dimerize through the tail domain to form a long coiled-coil rod, which is also the site for the association of NMMHC-IIA dimers in functional myosin filaments.[9] The coiled-coil region of the tail domain ends at the C-terminus with a 37-residue non-helical tailpiece.[10] The spectrum of mutations responsible for *MYH9*-RD is quite peculiar. Causative variants are mainly missense mutations affecting either the head domain or the coiled-coil region of NMMHC-IIA. Nonsense or frameshift alterations have been

also reported, all affecting the C-terminal non-helical tailpiece. Short in-frame deletions or duplications have been identified in very few families.[6,11,12]

MYH9-RD is a disorder characterized by a very wide variability in clinical evolution. In fact, some patients present a mild and asymptomatic thrombocytopenia as the only manifestation of the disease throughout life, while other individuals develop complex syndromic pictures in which thrombocytopenia associates with early-onset end-stage kidney failure, severe deafness and cataracts. Genotype-phenotype studies indicated that the risk and the severity of non-congenital manifestations are predicted by the causative *MYH9* mutation.[6,13,14] Globally, mutations affecting the head domain of NMMHC-IIA are associated with a more severe clinical evolution than those involving the tail domain. Moreover, recent studies have shown that different alterations of the tail domain have a very different prognostic significance; for instance, the p.Asp1424His substitution in the coiled-coil is associated with high incidence of non-congenital manifestations, whereas patients carrying the p.Asp1424Asn mutation or deletions of the non-helical tailpiece have a very low risk of developing these features.[6]

Here, we report four novel mutations that affect the tail domain of NMMHC-IIA and are responsible for *MYH9*-related thrombocytopenia in four families. Thrombocytopenia came to medical attention only in adulthood in all the index cases. The study of the phenotypes of affected individuals indicates that these novel variants are associated with a mild clinical evolution of the disease.

Patients and Methods

Patients

Two Italian (►**Fig. 1**, F1 and F2), one Hungarian (F3) and one Finnish (F4) Caucasian pedigrees have been included in this study. The investigation was approved by the Institutional Review Board of the IRCCS Policlinico San Matteo Foundation,
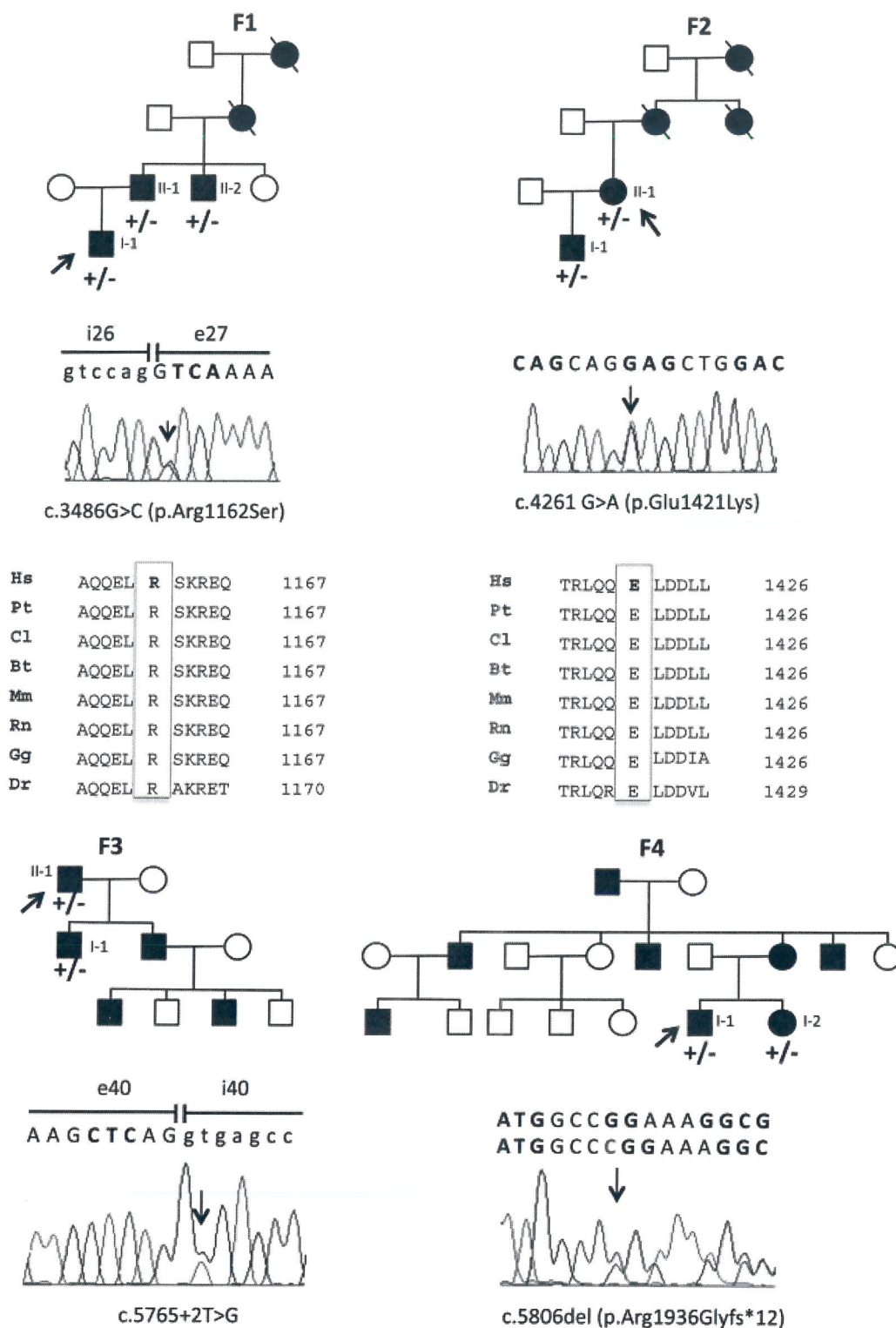
Fig. 1 Pedigrees of families enrolled in the study and direct sequencing of polymerase chain reaction (PCR) products showing the respective mutations. For the p.Arg1162Ser and p.Glu1421Lys alignment of *MYH9*, orthologues from different species is shown. Hs, H. sapiens (NP_002464), Pt, P. troglodytes (XP_016794573), Cl, C. lupus (NP_001104237), Bt, B. taurus (NP_001179691), Mm, M. musculus (NP_071855), Rn, R. norvegicus (NP_001292806), Gg, G. gallus (NP_990808) and Dr, D. rerio (NP_001091647) at http://www.ncbi.nlm.nih.gov/sites/homologene/144. The mutated residues are boxed. Arrows indicate the probands of each family.

Pavia, Italy. All the reported patients or their legal guardians provided written informed consent for the study, which was conducted in accordance with the principles of the Declaration of Helsinki.

Family 1. The proband (►Fig. 1, F1, subject I-1) was a 17-year-old male referred for investigation of persistent thrombocytopenia (platelet count between 40 and 50 × 10⁹/L), which was found incidentally about 2 years earlier on a routine blood cell count. He was initially diagnosed as having ITP and treated with some courses of prednisone, without any benefit. Bleeding tendency was limited to occasional episodes of mild epistaxis. Examination of peripheral blood smears after conventional staining showed prominent platelet macrocytosis with giant platelets (platelets larger than erythrocytes); platelet morphology was otherwise normal. Moreover, basophilic Döhle-like inclusions could be observed in a proportion of neutrophil granulocytes (►Fig. 2A). Investigation of family members disclosed that the proband's father (II-1), as well as the paternal uncle (II-2), grandmother and great grandmother, had a history of asymptomatic thrombocytopenia and adult-onset bilateral hearing loss. Examination of blood smears of subjects II-1 and II-2 showed a picture similar to that of the proband.

Family 2. The index case (F2, II-1) was a 64-year-old female who started a diagnostic work-up for a long-standing thrombocytopenia (platelet count 30–45 × 10⁹/L). The low platelet count was discovered after a tooth extraction complicated by prolonged bleeding, while the patient had no spontaneous bleeding. She previously received a diagnosis of ITP and was treated with steroids without any benefit. Physical examination revealed a moderate bilateral hearing defect. Collection of family history disclosed that her 45-year-old son (I-1) and several members of the maternal bough of the family (►Fig. 1) also had a history of thrombocytopenia. Because of the ab-

sence of relevant bleeding symptoms, none of these relatives had undergone specific diagnostic investigations. Examination of peripheral blood smears showed platelet macrocytosis, giant platelets and basophilic inclusions in granulocytes.

Family 3. The propositus (F3, II-1) was a 66-year-old male referred for thrombocytopenia (platelet count 35–55 × 10⁹/L) without spontaneous bleeding tendency. He referred also hearing loss and the recent finding of bilateral cataract at ophthalmologic examination. Assessment of family history revealed that other family members also had thrombocytopenia (►Fig. 1). At our evaluation, May–Grünwald Giemsa stained blood smears showed macrothrombocytopenia with giant platelets and basophilic inclusions in neutrophils in both subjects II-1 and I-1. The other family members refused to undergo further examinations.

Family 4. The probands (F4, I-1 and I-2) were a 19-year-old male and his 17-year-old sister, who were referred to haematologist for the investigation of chronic thrombocytopenia (platelet count 35–60 × 10⁹/L for both). They did not suffer of spontaneous bleeding and their medical history was unremarkable. Examination of peripheral blood smears showed platelet macrocytosis and giant platelets in both subjects. Dominant inheritance of thrombocytopenia was suggested by the discovery of low platelet count in several subjects of the maternal bough of the family (►Fig. 1). Of note is that three of them (the probands' mother and two of her brothers) also complained moderate hearing loss.

Immunofluorescence Assay for NMMHC-IIA

Immunofluorescence analysis of NMMHC-IIA distribution in neutrophils was performed on peripheral blood smears using the anti-NMMHC-IIA mouse monoclonal antibody NMG2.[15] For the secondary detection, a goat anti-mouse
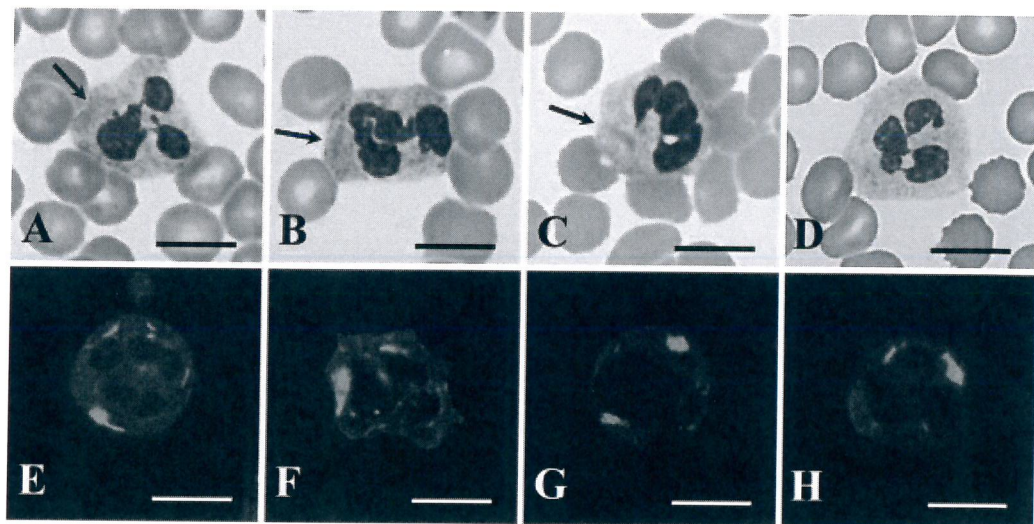

Fig. 2 Non-muscle myosin heavy chain IIA (NMMHC-IIA) inclusions in leukocytes after conventional staining and immunofluorescence assay for the *MYH9* protein. (A–D) Peripheral blood smear, May–Grünwald Giemsa staining, probands of family 1 (A), 2 (B), 3 (C) and 4 (D). NMMHC-IIA inclusions are detectable as faint basophilic (sky-blue) inclusion bodies (Döhle-like bodies) in the cytoplasm of neutrophil granulocytes (arrows). Döhle-like bodies were evident upon conventional staining in all the investigated probands, with the exception of the proband of family 4. (E–H) Peripheral blood smear, immunofluorescence staining for NMMHC-IIA, probands of family 1 (E), 2 (F), 3 (G) and 4 (H). Immunofluorescence assay evidences typical aggregates of the MYH9 protein in granulocytes of all the investigated subjects. The finding of one to three large (2–5 μm) aggregates per cell together with further small aggregates suggests that the causative mutation involves the tail domain of the protein. Scale bars correspond to 10 μm.

antibody conjugated with Alexa Fluor 488 (Invitrogen, Milan, Italy) was used.

Mutational Screening of *MYH9* and Bioinformatic Analysis

The *MYH9* (GenBank Accession Number RefSeq NM_002473.3) coding exons and the respective exon–intron boundaries were amplified by polymerase chain reaction (PCR) as described. Amplification reactions were performed in a final volume of 35 µL containing 100 ng of deoxyribonucleic acid (DNA), 17.5 µL of KAPA2G Fast Hot Start Ready Mix (Kapa Biosystems, Resnova, Cape Town, South Africa) and 1.75 µL of a 10 µM solution of each primer. 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, California, United States). The variant identified in probands was then tested in all the available relatives.

The missense variants were evaluated using the bioinformatic programs Combined Annotation Dependent Depletion (CADD, http://cadd.gs.washington.edu), Mutation Taster (http://www.mutationtaster.org), Sorting Intolerant From Tolerant (SIFT) (http://sift.jvci.org/) and Mutation Assessor (http://mutationassessor.org). For the CADD algorithm, we considered probably pathogenetic only the variants with score ≥ 15. The in silico analyses of splicing mutation were performed using Human Splicing Finder (HSF) Version 3 (http://www.umd.be/HSF3/HSF.html).

Characterization of *MYH9*-RD Phenotype

Whenever possible, platelet count was measured using both automated cell counters and phase-contrast microscopy.[3] History of bleeding symptoms, based on the patients' entire lifetime, was standardized by means of the ISTH Bleeding Assessment Tool questionnaire.[16,17] Extra-haematological features of the *MYH9*-RD were searched in all patients independently of the presence of a symptomatic disease.[6,18] Kidney impairment was investigated by measurement of proteinuria in a 24-hour urine sample or protein/creatinine ratio in a spot urine sample, and assessment of serum creatinine levels. Protein loss was considered as significant for values higher than 0.5 g/24 hour or for protein/creatinine ratio higher than 100 mg/g on two consecutive measurements. Hearing function was investigated by pure-tone audiometry: hearing loss was recorded for a bone threshold average greater than 25 dB at 1,000, 2,000 and 4,000 Hz. Cataract was searched for by ophthalmological evaluation. Measurements of the following plasma liver markers were performed: alanine aminotransferase, aspartate aminotransferase and gamma-glutamyl transferase. Elevation of liver enzymes was considered as related to the *MYH9* mutation in the absence of any other appreciable causes of liver damage.[8]

Results

Immunofluorescence Assay

MYH9-RD was suspected in the probands of the four families, as thrombocytopenia was apparently transmitted as an autosomal-dominant trait and associated with giant platelets. We also observed leukocyte basophilic Döhle-like inclusions by examination of conventionally stained blood smears of patients from the families 1 to 3. Moreover, thrombocytopenia was associated with hearing loss in the families 1, 2 and 4. The diagnostic suspicion was confirmed in all the probands through immunofluorescence assay for NMMHC-IIA on blood smears, which demonstrated typical aggregates of the MYH9 protein in granulocytes.[15,19,20] In all the subjects, NMMHC-IIA staining pattern was characterized by one to three large aggregates (2–5 µm) per cell, often together with further small aggregates (►**Fig. 2E–H**). Based on previous genotype–phenotype studies, this pattern suggested that the causative *MYH9* mutations affect the tail domain of NMMHC-IIA.[15,19,21]

Mutational Screening

In the affected members of family 1, we identified the c.3486G > C variant in exon 27 leading to substitution of arginine 1,162 with a serine (p.Arg1162Ser) in the coiled-coil of NMMHC-IIA. Of note, since the 'G' at position 3,486 is the first nucleotide of exon 27, the variant could potentially affect the 3′ splice site of intron 26. Ribonucleic acid (RNA) samples of these patients were not available to test this hypothesis. However, the substitution of the same nucleotide with a 'T' (c.3486G > T) was previously reported in one Japanese *MYH9*-RD patient.[22] In this subject, the analysis of the messenger RNA (mRNA) from platelets detected mainly the normally spliced product and only a small amount of RNA with skipping of exon 27; no alternative splicing forms were instead identified in the other blood cells, suggesting that the majority of the c.3486G > T mRNA resulted in synthesis of the p.Arg1162Ser protein. Based on this finding and considering that the score of the natural acceptor splice site of intron 26 is 95.39 and that those predicted by c.3486G > C and c.3486G > T are equivalent (91.23 and 91.09, respectively), it is likely that even the c.3486G > C variant leads to the same amino acid substitution in the MYH9 protein. Family 2 carries the c.4261G > A variant in exon 31 leading to another amino acid substitution (p. Glu1421Lys) in the coiled-coil of NMMHC-IIA. In family 3, we identified the c.5765 + 2T > G variant: the substitution of this 'T' nucleotide destroys the 5′ splice site of exon 40, as it was previously reported in one *MYH9*-RD individual carrying the c.5765 + 2T > A mutation.[11] Since in that case the mutation led to a frameshift alteration (p.Arg1922Argfs*43) due to recognition of a cryptic 'GT' splice site 50 nucleotides (nts) downstream of intron 40, it is likely that even the c.5765 + 2T > G has the same effect. However, we could not confirm this hypothesis, as the RNA samples were not available from the affected subjects. Finally, in family 4 we identified the deletion c.5806del of exon 41, resulting in a frameshift alteration (p. Arg1936Glyfs*12) of the non-helical tailpiece of NMMHC-IIA.

Mutational screening of the family members demonstrated the segregation of the mutations with the phenotype within the respective families. All the four variants are not reported in public databases, including the SNP (www.ncbi. nlm.nih.gov/SNP), the 1000 genomes (www.1000genomes. org) or the Exome Aggregation Consortium (www.exac. broadinstitute.org) and GnomAD (http://gnomad.broadinstitute.org/) databases. Regarding the two missense variants,

Table 1 Main clinical features of the investigated patients

Family/patient	MYH9 mutation[a]/NMMHCIIA mutation	Gender/age (y)	Basophilic Döhle-like inclusions[b]	Platelet count ($\times 10^9$/L) automated/microscopic	ISTH BAT Score[c]	Proteinuria/renal failure [age at onset]	Sensorineural hearing loss[d] [age at onset]	Cataract[e] [age at onset]	Liver enz alteratio... [age at o...
1/I-1	c.3486G > C/p.Arg1162Ser	M/17	Yes	41/68	1	No/No	No	No	Yes [15]
1/II-1		M/52	Yes	49/67	2	No/No	Bilateral [48]	No	Yes [52]
1/II-2		M/45	Yes	70/79	0	No/No	Bilateral [40]	No	Yes [45]
2/II-1	c.4261G > A/p.Glu1421Lys	F/64	Yes	18/50	1	No/No	Bilateral [64]	No	No
2/I-1		M/45	Yes	15/40	0	No/No	No	No	No
3/II-1	c.5765 + 2T > G/p.Arg1922Argfs*43[f]	M/66	Yes	42/na	0	Yes [66]/No	Bilateral [66]	Bilateral [66]	No
3/I-1		M/41	Yes	30/na	0	No/No	No	No	No
4/I-1	c.5806del/p.Arg1936Glyfs*12	M/19	No	35/na	0	No/No	Bilateral [18]	No	No
4/I-2		F/17	No	69/na	0	No/No	No	No	No

Abbreviations: cDNA, complementary deoxyribonucleic acid; ISTH BAT score, International Society on Thrombosis and Haemostasis Bleeding Assessment Tool score; na, not available; NMMHCIIA, non-myosin heavy chain IIA.

[a]Nucleotide A of the ATG translation initiation start site of the MYH9 cDNA in GenBank sequence NM_002473.5 is indicated as nucleotide +1.

[b]As evaluated by examination of blood smears after conventional staining (May-Grünwald Giemsa).

[c]The International Society on Thrombosis and Haemostasis (ISTH) Bleeding Assessment Tool (BAT) score was assessed as reported.[16,17]

[d]As determined by pure-tone audiometric examination.

[e]As determined by ophthalmologic evaluation.

[f]Predicted on the basis of data from the literature.[11]

multiple sequence alignment showed that Arg1162 and Glu1421 are highly conserved between different species (►Fig. 1). The CADD for p.Arg1162Ser and p.Glu1421Lys is 24.2 and 34.0, respectively. Other bioinformatic predictive tools, such as Mutation Taster and SIFT, or Mutation Assessor, classify both the missense variants as disease-causing and deleterious variants, or variants with medium/high impact on protein function. Taken together, these data suggest that the MYH9 variants identified in the four families are likely to be pathogenic.

Phenotype Investigation

Results of the study of the MYH9-RD phenotype in nine affected individuals from the four investigated families are summarized in ►Table 1. The degree of thrombocytopenia was moderate in all the subjects; only three individuals had a history of very mild spontaneous bleeding episodes (easy bruising and/or epistaxis). Five patients presented sensorineural deafness. In four cases, the age at onset of symptomatic hearing loss was above 40 years. In these patients, audiometric examination showed a moderate, bilateral and symmetric hearing defect involving the middle and high tones. Audiometric studies showed that one subject (4/I-1) had a mild bilateral hearing impairment at the age of 18 years; in this case, the defect affected only the high tones and was not self-reported by the patient. One patient (3/II-1) had proteinuric nephropathy, disclosed at the age of 66, in the absence of alteration of kidney function; kidney examinations returned normal results in the other eight patients. The same individual 3/II-1 had bilateral cataracts found at the age of 66, while ophthalmological findings were normal in the other investigated patients. All the three patients of family 1 had persistent, moderated elevation of alanine aminotransferase. Alternative causes of liver damage were excluded in these subjects[8] and liver ultrasound scan showed normal findings, suggesting that the abnormality can be attributed to the MYH9-RD.

Discussion

MYH9-RD is a complex syndromic disorder characterized by congenital macrothrombocytopenia and the variable risk of developing non-congenital extra-haematological manifestations that have a major impact on prognosis. Since the cloning of MYH9 as the gene responsible for the disease, the spectrum of its mutations has appeared to be peculiar. Although the number of causative variants is being increased in the last years, most of the MYH9 mutations result in single amino acid substitutions affecting only 35 of the 1,960 residues of NMMHC-IIA.[6,11,12] Both domains can rarely be hit by small deletions and duplications, which are however all in frame. Frameshift and nonsense mutations of MYH9 have been identified in patients but only in the last exon of the gene (exon 41): these alterations all result in the deletion of a variable portion of the C-terminal non-helical tailpiece of NMMHC-IIA. The four novel mutations reported in this article appear consistent with this scenario. Indeed, two mutations result in amino acid substitutions in the long coiled-coil region of the tail domain. The other two variants are one splicing mutation and one

single nucleotide deletion, both leading to frameshift alterations of the short non-helical tailpiece of NMMHC-IIA. Of note is that this specific spectrum is plausible with a dominant negative effect of the MYH9 variants. In fact, it is likely that only the mutant molecules stably expressed could exert pathogenic role, thus limiting the spectrum of alterations of MYH9 associated with MYH9-RD.

Another feature of MYH9-RD is the strong association between the genotype and the evolution of the disease. In particular, recent investigations showed that different mutations affecting the tail domain of NMMHC-IIA correlate with very different clinical pictures. For instance, the p.Asp1424His change in the coiled-coil is associated with remarkably high risk of developing evolutive kidney damage, deafness and cataract. Among the other frequent mutations, the substitutions of the arginine 1165 are associated with early-onset and progressive deafness, whereas patients carrying the p. Asp1424Asn, the p.Glu1841Lys or alterations of the non-helical tailpiece, usually have no extra-haematological abnormalities or develop a mild to moderate hearing impairment at advanced age.[6,14] Because of this variability in prognostic significance, when identifying novel variants in the tail domain, it is interesting to study the associated phenotypes. Clinical characterization of our patients suggests that the four novel mutations reported here are associated with a mild disease evolution. In fact, only the oldest patient (3/II-1, ►Table 1) presented the association of hearing loss, bilateral cataracts and non-nephrotic range proteinuria without kidney failure at the age of 66 years. The other individuals did not show any extra-haematological features or had only a mild to moderate hearing loss presenting in most cases after the fourth decade. In the patient 4/I-1 carrying the p.Arg1922Argfs*43, we disclosed a very mild, clinically unnoticed hearing loss at the age of 18 years. However, his 17-year-old sister showed a normal audiogram, and the older members of family 4 reported hearing loss occurring in the middle or advanced age, suggesting that other genetic and/or environmental factors may have co-operated with the MYH9 mutation in determining the early-onset hearing impairment in this individual.[3,4,6] Of note is that a previous study suggested an association between the mutations affecting the N-terminal part of the coiled-coil, such as substitutions of threonine 1155 or arginine 1162, and the elevation of liver transaminases.[8] The finding that all the three individuals from family 1, who carry the p.Arg1162Ser substitution, present a persistent elevation of alanine aminotransferase, appears to support this association.

In conclusion, we identified four novel variants that enlarge the spectrum of mutations responsible for MYH9-RD. These novel mutations affecting the tail domain of NMMHC-IIA appear to be associated with a mild form of the disorder.

Authors' Contributions

C.Z., A.P. and A.S. conceived and designed the study, analysed and interpreted data and wrote the manuscript. D.D.R., T.G., V.B., S.B., J.D., P.L., P.N. and S.R. performed research, collected, analysed and interpreted the data. All the authors critically revised the manuscript and accepted the final version.

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