Platelets, 2009; 1–5, Early Online

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CASE REPORT

MYH9 related disease: A novel missense Ala95Asp mutation of the *MYH9* gene

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(Received 26 August 2009; accepted 18 September 2009)

Abstract
MYH9-related disease (*MYH9*-RD) is a rare autosomal dominant disorder caused by mutations in *MYH9*, the gene encoding the heavy chain of non-muscle myosin IIA. Patients present with congenital macrothrombocytopenia and inclusion bodies in neutrophils and might develop sensorineural deafness, presenile cataract, and/or progressive nephritis leading to end-stage renal failure. In a family with eight individuals suffering from macrothrombocytopenia and hearing impairment we identified a novel c.Ala95Asp mutation. Affecting the motor domain of the protein, the mutation is likely to be associated with a severe phenotype. Therefore, this family should be carefully monitored to follow-up the renal status even though the affected members do not seem to be at risk of early kidney disease.

Keywords: MYH9-*related disease, macrothrombocytopenia, neutrophil aggregate,* MYH9 *gene, mutational screening*

Introduction

MYH9-related disease (*MYH9*-RD) is an autosomal dominant disorder characterized by congenital macrothrombocytopenia and inclusions in neutrophils due to aggregation of mutant and wild type proteins [1, 2]. During their lives patients may develop sensorineural hearing loss, presenile cataract and proteinuric nephropathy that often leads to end-stage renal disease. *MYH9*-RD is caused by mutations of *MYH9*, the gene encoding for the heavy chain of the non-muscle myosin A of class II (myosin-9) [3, 4]. Myosin-9, as well as the other myosins of class II, is a hexameric complex consisting of two heavy chains and two pairs of light chains. Each heavy chain contains a N-terminal globular head or motor domain attached to a long tail coiled-coil domain [5].

At least 36 different mutations of the *MYH9* gene have been reported [6–10]. Most of them are missense mutations hitting a few residues, such as 96 and 702 localized in the head or 1165, 1424 and 1841 within the tail, which together account for *MYH9*-RD in approximately 65% of families. Moreover, there is a correlation between genotype and phenotype, as it was demonstrated that mutations of the motor domain are associated with a severe thrombocytopenia and early onset of sensorineural hearing loss and proteinuric nephropathy whereas those within the coiled-coil domain correlate with a mild form of the disease, characterized by slight thrombocytopenia and a much lower risk of non-hematological manifestations [10].

In this paper we report a novel missense variation, p.Ala95Asp, of the motor domain in a family affected with a severe thrombocytopenia and hearing impairment. Due to the limited number of patients with mutations at this residue, we cannot predict their risk for proteinuric nephropathy that should be defined after a careful follow-up.

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ISSN 0953–7104 print/ISSN 1369–1635 online © 2009 Informa Healthcare Ltd.
DOI: 10.3109/09537100903349620

Patients and methods

Case report

A 29-year-old woman (III-7) was referred for evaluation of thrombocytopenia, which was detected during early childhood (Figure 1a). She had a lifelong history of excessive bleeding, including easy bruising, gums bleeding, sporadic epistaxis, prolonged bleeding after tooth extraction and menorrhagia, which required oral contraceptives administration. She developed bilateral sensorineural hearing loss (B-SNHL) at age 15, which became progressively worse during follow-up, and required hearing aids. Her family history was remarkable because seven other members suffered from thrombocytopenia, bleeding diathesis and hearing loss (Figure 1a), one of them (individual II-2) presenting also end-stage renal failure disclosed at the age of 60. A cochlear implant was performed in individual II-4 because of severe hearing impairment.

Blood samples and complete clinical and laboratory evaluation were available for the propositus and her 32-year-old sister (III-4), who had easy bruising, gums bleeding and sporadic epistaxis since childhood, and developed B-SNHL at age 30.

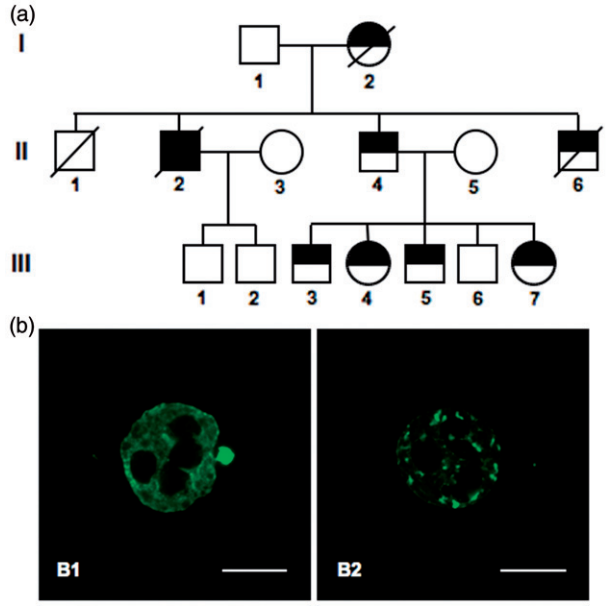

Figure 1. Pedigree and morphological features of myosin-9 aggregates. (a) Pedigree of the *MYH9*-RD family enrolled in the study. Open symbols indicate unaffected disease status. Half solid symbols indicate family members with macrothrombocytopenia and hearing impairment. Solid symbols indicate affected disease status characterized macrothrombocytopenia, hearing impairment, and renal failure. (b) Immunofluorescence localization of myosin-9 in neutrophil granulocytes of a healthy individual (B1) and propositus III-7 (B2) disclosing cytoplasmic aggregates of the protein typical of the *MYH9*-RD. The staining pattern was characterized by numerous aggregates per cell with small size (less than 0.5 μm) scattered throughout the cytoplasm. Scale bars correspond to 10 μm.

In both patients, examination of May-Grünwald-Giemsa-stained peripheral blood smears revealed platelet macrocytosis with giant platelets and basophilic ''Döhle-like'' inclusion bodies in granulocytes. Platelet counts determined by phase contrast microscopy in a Neubauer chamber were 22×10^9/L and 12×10^9/L in the propositus and her sister, respectively. The platelet count was lower (2 and 3×10^9/L) when measured by an electronic cell counter, as frequently occurs in patients with very large platelets, reflecting the lack of accuracy of automated counters which fail to recognize the large platelets. Likewise, mean platelet volume could not be determined by an electronic counter. Platelet aggregation could not be evaluated because of severe thrombocytopenia. A defective platelet surface expression of the GPIb/IX/V glycoprotein complex relative to GPIIIa was revealed by flow cytometry in both patients. When compared to control platelets, the GP Ib/IIIa ratio was 20% and 52% in the propositus and her sister, respectively, whereas the GPIX/IIIa ratio was 47% and 64%. Plasma thrombopoietin levels in patients III-7 and III-4, measured by ELISA, were 45 pg/mL and 51 pg/mL, respectively (reference values 0–22 pg/mL).

Audiometric evaluation showed bilateral sensorineural hearing loss in both patients, while ophtalmological evaluation was performed in the propositus and did not reveal cataracts. Urinanalysis, 24-hour proteinuria and serum creatinine were normal in both the propositus and her sister.

Immunofluorescence and mutational analyses

Immunofluorescence localization of myosin-9 was performed on peripheral blood smears using the NMG2 mouse monoclonal antibody; the staining procedures were previously described [10, 11]. The coding exons 1 and 16 and the respective exon-intron boundaries of the *MYH9* gene were amplified by PCR and the 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) as previously described [10].

Bioinformatics

The structure with the closest sequence homology with MYH9 was identified by a Blast search of the PDB database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The query sequence was aligned to that of the template by the clustalx software [12]. The model was built using the expasy Swiss-Model repository used in automated mode (http://swissmodel.expasy.org/repository/).

Results and discussion

On the basis of the clinical picture, a diagnostic suspicion of *MYH9*-RD was raised for this family. Therefore, immunofluorescence localization of myosin-9 was performed on peripheral blood smears of the propositus and her sister. In both cases, the analysis revealed the presence of typical myosin-9 aggregates in neutrophil granulocyte cytoplasm. The distribution pattern was characterized by numerous aggregates per cell with small size (less than 0.5 μm) scattered throughout the cytoplasm (Figure 1b).

Since we observed the presence of small and numerous aggregates, which are typical mutations in exons 1 and 16, we performed the mutational screening of these two exons. In fact, direct sequence analysis using genomic DNA of individual III-7 revealed a novel c.284C>A nucleotide substitution within exon 1, which would result in an amino acid substitution p.Ala95Asp (Figure 2a).

The amino acid alignment of the MYH9 orthologs (Canis lupus familiaris, Bos Taurus, Mus musculus, Rattus norvegicus, Gallus gallus, and Danio rerio; at http://www.ncbi.nlm.nih.gov/homologene) demonstrates that residue 95 is a highly conserved alanine (data not shown). Moreover, the alignment of the 13 human myosins of class II showed also conservation of alanine 95 among the non muscle (MYH9, MYH10 and MYH14) and smooth muscle (MYH11) myosins, suggesting that a substitution at this position should have deleterious effects on the function of myosin-9 (Figure 2b).

To have a better understanding of the effects of the mutation on the structure of MYH9, we built a three-dimensional model using the coordinates of the chicken smooth muscle myosin motor domain (PDB accession number 1BR2) as a template (Figure 3). Visual inspection of the model shows that alanine 95 (corresponding to alanine 98 in 1BR2) is at the N-terminus of helix α1 and that this residue is completely buried (exposed surface area 0 Å^2). It packs directly against the aromatic ring of tryptophan 33 and is surrounded by phenylalanine 41 and histidine 99 (Figure 3a). We expect that substitution of this residue with a bulkier hydrophilic group, as is that of an aspartic acid, would destabilize the fold. Additionally, the Ala95Asp mutation would imply burying a charged group in the protein interior, which is energetically costly. These observations strongly suggest that the mutation has a structural role that will affect the fold stability. A different disease-causing mutation of amino acid 95 (p.Ala75Thr) was previously described in a family from Korea [9]. Similarly, mutation of alanine 95 in threonine would cause local strain due to a clash with tryptophan 33.

In order to better characterize platelet abnormalities deriving from MYH9 mutations, we studied surface glycoproteins in platelets from two of the eight patients and showed a marked defect of the

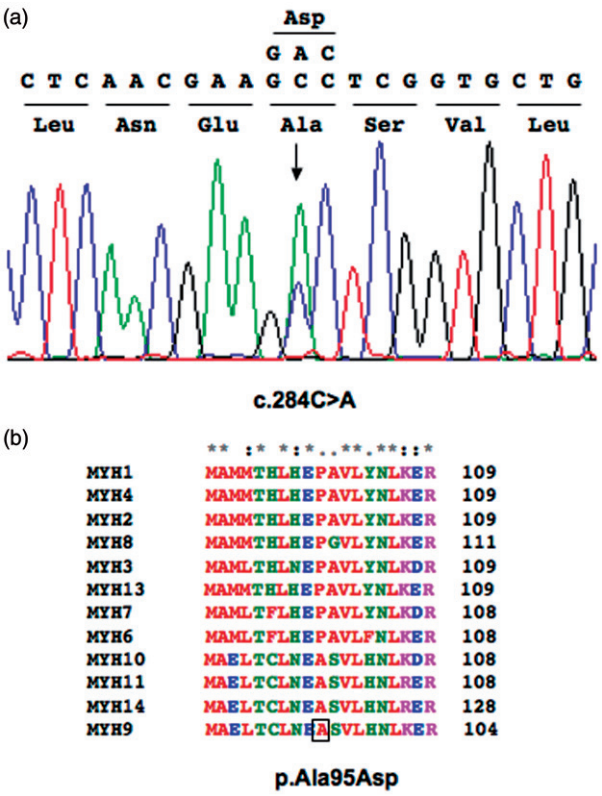

Figure 2. Molecular analysis of the *MYH9* gene. (a) Direct sequencing analysis of PCR product of *MYH9* exon 1 showing the heterozygous nucletotide substitution leading to the c.284C>A (p.Ala95Asp) mutation. Nucleotide A of the ATG translation initiation start site of the *MYH9* cDNA in GenBank sequence NM_002473.3 is indicated as nucleotide +1. (b) Alignment of all human muscle and non muscle myosins of class II. MYH1 (NM_005963), MYH4 (NM_017533), MYH2 (NM_017534), MYH8 (NM_002472), MYH3 (NM_002470), MYH13 (NM_003802), MYH7 (MN_000257), MYH6 (NM_002471), MYH9 (AB191263), MYH10 (NM_005964), MYH11 (NM_002474), and MYH14 (AY165122). The mutated residue is boxed.

GPIb/IX/V complex. These data are consistent with those we previously obtained in eight patients carrying four different *MYH9* mutations, which indicate that the cytoskeletal alteration induced by *MYH9* mutations can result in a reduced expression of GPIb/IX/V on platelet surface [13]. Since the GPIb/IX/V complex mediates the *in vivo* platelet adhesion to subendothelium at the sites of vascular injury, this defect could contribute to the bleeding tendency of the propositus and her sister.

As emerged from a genotype/phenotype study, a few mutations are regarded as risk factors for non-hematological manifestations. In particular, individuals with amino acid substitutions in the motor domain (exons 1-18) are likely to have severe thrombocytopenia, nephropathy and deafness before the age of 40 [10, 14]. However, this correlation is not clearly ascertained for the mutations of the first exon because of the limited number of patients reported. In this regard, patients of our family have a marked thrombocytopenia and severe

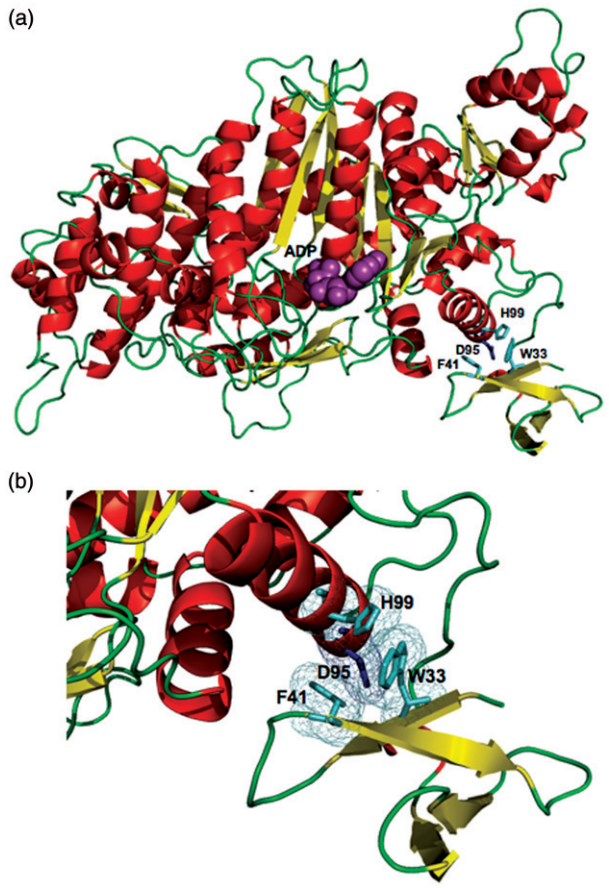

Figure 3. Effect of mutation on the structure of MYH9. (a) Ribbon representation of the Ala95Asp mutant of MYH9 as modeled from the coordinates of chicken smooth muscle myosin motor domain (1BR2). Helices and strands are indicated in red and yellow respectively. The side chains of Asp95 (in blue) and of Trp33, Phe41 and His99 (in cyan) are shown explicitly. In purple is indicated the position of ADP which does not interfere with the mutated position. (b) Close up of the structure of the MYH9 model zoomed around the mutation. The van der Waals radii of Asp95 and of the surrounding residues are indicated to show potential clashes, which are expected to destabilize the protein fold.

hearing impairment developed at a young age but not kidney disease, having only one member end-stage renal failure with a relatively late onset. Consistent with a potential lack of nephropathy associated with mutations at position 95, the Korean family with the p.Ala75Thr mutation was reported to have no extra-hematological characteristics [9]. However, the affected individuals of our family should be carefully monitored to promptly recognize proteinuria, which is the first sign of kidney involvement of the *MYH9*-RD. In fact, recent evidence suggests that treatment of the kidney impairment at its early stage with renin-angiotensin inhibitors could effectively slow down the progression to end-stage renal disease in *MYH9*-RD patients [15].

Acknowledgments

We are grateful to Silvina Bonaccorso for referring the patients. This work was supported by grants from the Telethon Foundation (GGP06177), the Italian Ministry of Health, Italian ISS (Istituto Superiore di Sanità), Grant 34/07 of IRCCS Burlo Garofolo and the Agencia Nacional de Promoción Científica y Tecnológica, Argentina (PICT 01931).

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