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Published in:
Platelets

DOI:
10.3109/09537104.2015.1064882

2015

Link to publication

Citation for published version (APA):
Zetterberg, E., Carlsson Alle, M. S., Najm, J., & Greinacher, A. (2015). Thrombin generation in two families with MYH9-related platelet disorder. *Platelets*, (Aug 6), 1-4. https://doi.org/10.3109/09537104.2015.1064882

Total number of authors:
4

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Title Page

Thrombin generation in two families with MYH9-related platelet disorder

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Sort title: Thrombin generation in MYH-9-related disorder

Key words: MYH-9; platelet; thrombosis; thrombin generation

Abstract

MYH9-related platelet disorders are inherited macrothrombocytopenias with additional clinical manifestations including renal failure, hearing loss, pre senile cataract and inclusion bodies in leucocytes that are present in different combinations. The *MYH9* gene codes for the cytoplasmic contractile protein non-muscular myosin heavy chain IIA, present in several tissues. The bleeding tendency is usually mild to moderate but rarely, thrombotic complications are also seen. We report on the thrombin generation potential (ETP) in patients with MYH9-related disease with and without arterial thrombosis.

In family A, four affected members (c.5521G>A mutation causing p.(Glu1841Lys)) were evaluated. Three of them had a moderate bleeding tendency and in two renal insufficiency and hearing loss were already present. These two patients had an arterial thrombosis (myocardial infarction and pons infarction, respectively) before 50 years of age. In family B two members were affected (c.4679T>G, resulting in p.(Val1560Gly)). Their bleeding tendency was mild (bleeding scores 4 and 3, respectively). Thrombelastography (ROTEM) was normal in all six individuals. ETP was below the normal range in family B. However, in family A, the two members affected by thrombosis had a normal ETP, indicating that other factors compensated for the low platelet count and might have contributed to the arterial thrombosis.

Introduction

MYH9-related disorders (MYH9-RD; MIM 160775) are autosomal dominantly inherited platelet disorders caused by mutations in the *MYH9* gene. The gene encodes the non-muscular myosin heavy chain IIA (NMMHC-IIA), a contractile protein that is part of the cytoskeleton. Mutations give rise to a defective protein, which results in impaired shedding of platelets from megakaryocytes resulting in macrothrombocytopenia. NMMHC-IIA is also present in granulocytes, the inner ear, podocytes of the kidney and the eye lens, explaining the other clinical manifestations of MYH9-related disorders namely presence of inclusion bodies in granulocytes, sensorineural hearing loss, nephritis and presenile cataract. Historically, different combinations of these manifestations have been described as May-Hegglin anomaly (MHA; MIM 155100 - only large inclusion bodies), Fechtner syndrome (FTNS; MIM 153640 - small inclusion bodies, hearing loss, nephritis and cataract), Epstein syndrome (EPTS; MIM 153650 - hearing loss and nephritis) and Sebastian platelet syndrome (SBS; MIM 605249 - small inclusion bodies and sometimes hearing loss and cataract)[1, 2]. The bleeding tendency is mild to moderate and easy bruising, epistaxis and menorrhagia are the most prevalent symptoms [3].

Despite thrombocytopenia, episodes of thrombosis have been reported in a minor subset of patients [3, 4]. In one systematic review seven patients with thrombotic events are described, four with myocardial infarctions, one with stroke and two with venous thrombosis (recurrent deep venous thrombosis and portal venous thrombosis respectively) [4]. Another review described two members of the same family with MYH9-RD who developed coronary artery disease and myocardial infarction requiring coronary artery bypass surgery [3].

No particular *MYH9* mutation has been associated with an increased risk of thrombosis and it has been speculated that the increased platelet volume could to some extent compensate for thrombocytopenia and cause thrombosis in patients with a high risk profile [5].

Thrombin generation (TGA) and thrombelastography are assays testing the global coagulation capacity, which has been applied in hemophilia patients [6, 7], patients at risk for thrombosis [8, 9], as well as in the transfusion setting [10]. We found higher values of TGA parameters in MHY9-RD patients who developed thrombosis compared to MYH9-RD patients without thrombosis.

Methods

Patient population

Patients referred to the Coagulation Unit, Skane University Hospital, Malmö with MYH9-RD were asked to participate in the study. Six patients belonging to two different families (A and B) agreed. With informed consent blood samples were drawn (citrated blood as well as EDTA) with atraumatic technique.

Bleeding score

The bleeding tendency was assessed by the BAT/ISTH score [11]. A value <4 was considered normal.

Routine analyses

Hemoglobin (Hgb), white cell blood count (WBC), platelet count and mean platelet volume (MPV) were measured on an automated cell counter (Sysmex XN-10,Sysmex, Osaka japan). Since automated cell counters sometime have difficulties to distinguish enlarged platelets from red blood cells and thus, might underestimate platelet counts, platelets counts were also determined manually on a blood smear. In case of divergent results, results from the manual count were used. Antithrombin, protein C, protein S, FV Leiden mutation, prothrombin mutation, lupus anticoagulant, and homocysteine were determined by routine procedures at the Department of Clinical Chemistry, Skane University Hospital, Malmö. Born platelet aggregation was performed on a Chrono-Log-aggregometer, Triolab, Mölndal Sweden using ADP, epinephrine, collagen, ristocetin and arachidonic as agonists.

Genetic analyses

Genomic DNA was isolated from peripheral blood using standard techniques. The 40 coding exons (1-40) of the *MYH9* gene including their flanking splice sites were analyzed by direct sequencing starting with the hot spot exons 1, 10, 16, 24, 25, 26, 30, 38 and 40. Primer pairs for genomic amplification were generated according to the GenBank Refseq NM_002473.5 (ATG=1 in coding exon 1). PCR products were purified using ExoSAP- (Affymetrix UK Ltd High Wycombe, United Kingdom). DNA sequencing of both strands was performed, using a commercial kit (BigDye Terminator Cycle Sequencing v3.1 kit, Applied Biosystems,

Foster City, CA, USA) and an automated DNA sequencer (model ABI 3130XL, Applied Biosystems). SeqPilot analysis software (JSI medical systems GmbH, Kippenheim, Germany) was used for final sequence readout and mutation documentation. The amino acid numbering and nomenclature is used according to international recommendations for the description of sequence variants of the Human Genome Variation Society (HGVS; http://www.HGVS.org; update March 2015) with exon 1 being the first coding exon of the transcript and A of the ATG translate initiation start site as nucleotide +1.

Light microscopy and immunoflourescence

Blood smears were either stained by May-Grünwald-Giemsa stain to assess platelets and granulocytes and by immunofluorescence using a polyclonal rabbit anti-non-muscular myosin IIA (Sigma-Aldrich Chemie GmbH, Steinheim, Germany).

Rotational thrombelastography (ROTEM)

ROTEM analyses were performed on a ROTEM Delta (Triolab, Mölndal, Sweden), according to the instructions of the manufacturer and using EXTEM and INTEM as starting agents. Clotting time (CT), angle, clot formation time (CFT) and maximum clot firmness (MCF) were recorded.

Thrombin Generation

Preparation of platelet rich and platelet poor plasma

Blood samples were collected into tubes containing 0.129 m citrate. “Platelet rich plasma” (PRP) was prepared by centrifuging the tubes once for 10 min at 200 g. Platelet poor plasma (PPP) was prepared by centrifuging the tubes repeatedly two times for 20 min at 1830g. Platelet count should be adjusted to 150 by measuring the platelet count in a cell counter (Coulter Ac T diff 2, Beckham Coulter) and dilution with PPP. However, due to the low platelet count of all included patients, the platelet count could not be adjusted and was below 50 in all patients. PRP was then frozen at 80ºC and thawed for 10 minutes at 37ºC just before the assay.

Thrombin generation assay, CAT

Thrombin generation assay (CAT) was carried out using calibrated automated thrombin generation method (CAT) on frozen/thawed PRP and PPP.

All materials needed for the test including the calibrator (640 nM human thrombin), PRP reagent (final concentration 0.5 pM TF and minimal with phospholipids), PPP-Reagent LOW (final

concentration 1.0 pM TF and a mixture of phospholipids at 84μM), the fluorogenic substrate and fluorogenic buffer (FluCa-kit) were supplied by Thrombinoscope BV® (Maastricht, the Netherlands). The CAT method was carried out according to instructions of the manufacturer. Analyses were performed in triplicates 96-well round bottom microplates (Immulon 2 HB plate, Fisher Scientific, Roskilde, Danmark). The samples were first preincubated for 10 min in an incubator and analysis performed at 37ºC in a Microplate Fluoroscence Reader (Fluoroskan Ascent, Thermo Scientific, Thermo Fisher Scientific Instruments Co. Ltd., Shanghai, China) equipped with 390/460 filter where the fluorescence intensity is monitored for 90 min. The fluorescence intensity (measured in relative fluorescence units (RFU) is proportional to the amount thrombin generated in the assay. Results are delivered in a thrombogram where TGA parameters lagtime, endogenous thrombin potential (ETP), peak, and time to peak (ttpeak/second) arepresented.

Plasma exchange experiment

PPPof the two members with arterial thrombosis (A1 and A3) was added to PRP of a healthy donor and diluted with PPP from the patients to obtain a final concentration of $40x10^9$platelets/mL; the same platelet count as was present in the PRP of these two patients thereafter peak thrombin and ETP were determined. Addition of normal PPP to the same platelets served as control.

Results and Discussion

Patient characteristics

In family A, four members were evaluated: a 51 year old woman (platelet count 36; A1), the brother of A1 (57 years; platelet count 39; A2), the 24 year old daughter of A1 (platelet count 46; A3), and the 30 year old son of A2 (platelet count 44; A4). Their clinical and laboratory features are summarized in Table I. All four had macrothrombocytopenia, inclusion bodies consisting of NMMHCIIA in the leucocytes and Born aggregometry showed loss of shape change

when platelets were stimulated with collagen. The underlying mutation in the *MYH9* gene c.5521G>A caused the amino acid change p.(Glu1841Lys). Three of the family members (A1, A2 and A3) had a moderate bleeding tendency (ISTH /SSC bleeding scores 9, 13, 4). In A1 and A2, renal insufficiency and hearing loss were already present. A1 had a myocardial infarction and A2 a pons infarction before the age of 50 and both were treated for hyperlipidemia. The present laboratory evaluation revealed mild hyperhomocysteinemia in both siblings, all other thrombophilia markers were normal.

In family B, macrothrombocytopenia and small to medium size inclusion bodies of NMMHCIIA were found in the leucocytes of the 38-year old proposita (platelet count 36; B1) and her 15-year old daughter (platelet count 46; B2). Platelet aggregation studies showed loss of the shape change after stimulation with collagen in both mother and daughter. The underlying mutation in the *MYH9* gene c.4679T>G resulted in the amino acid change p.(Val1560Gly). Thrombophilia testing was normal in both members of the family and bleeding tendency was mild (bleeding scores 4 and 3 respectively).

Results from Global Coagulation tests

Thrombelastography (ROTEM) was normal in all six individuals of the two families, while thrombin generation showed interesting differences. Peak thrombin concentration in PPP was elevated in all four members of the family A while it was normal in family B. The ETP in PPP was normal in both families. In PRP, ETP was found to be below the normal range in family B, which should be expected since the platelet count was just one third of that of control PRP. However, in family A, the two members affected by thrombosis had a normal ETP despite the low platelet count (Fig 2). Since ETP in PRP reflects both the contribution of coagulation factors as well as the

contribution of platelets to thrombin generation, these findings could be explained either by an increased availability of platelet phospholipids, due to the larger size of the platelets, or by a plasma factor present in the patients that presented with thrombosis. To address this question, plasma from these two patients was added to platelets of normal donors. With the plasma of both patients, the ETP (+7±4%) and peak thrombin concentration (+13.6± 1.2%) were higher as compared to the control experiment in which normal plasma was added to the same platelets. These findings indicate that the bleeding tendency, which was more pronounced in family A than in family B, does not predict the risk for arterial thrombosis in MYH9-RD patients. Indeed the normal ETP of the individuals of family A and the results of the plasma exchange experiment indicate that a plasma factor rather than increased availability of platelet phospholipids, due to the larger platelet size determine the peak thrombin and ETP in PRP of MYH9-RD patients. This is further underscored by the observation that the plasma of these patients increased the ETP when added to PRP of normal donors in comparison to the autologous plasma of the platelet donor. These index families suggest that thrombin generation studies could be an interesting tool to assess the risk of thrombosis or bleeding in patients with this rare disorder.

Limitations of the study: One limitation of the study is that TGA was performed on frozen samples. The freeze-thaw cycle will cause platelet lysis and any functional aspect of platelets in thrombin generation have not been evaluated. The other major limitation is of course the small size of the study, including only two families. We therefore, suggest that other centers also assess the ETP in their MYH9-RD patients according to our protocol (www.med.lu.se/klinvetmalmo/koagulationsforskning/forskningsprojekt) to gather data on the potential association of the ETP with the phenotype.

Acknowledgements

Marcus Ljungqvist is acknowledged for expert technical assistance in performing the thrombin generation assays and research nurse Eva Lindén for her administrative efforts.

Declaration of Interest statement

The authors report no declarations of interest.

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Tables and Figures

Table I. Clinical and laboratory features of two families with MYH9 related disorder

Figure 1. Endogenous Thrombin potential in platelet rich plasma in two families with MYH9 related disorder

The endogenous thrombin potential was measured by thrombin generation assay in frozen/thawed "PRP". ETP was seen to be below the normal range in the family not affected by thrombosis (family 2) as expected considering the low platelet counts. In the two family members affected by thrombosis (marked by a star) ETP was normal