MYH10 protein expression in platelets as a biomarker of RUNX1 and FLI1 alterations

Iléana Antony-Debré¹²³, Dominique Bluteau¹²³, Raphael Itzykson¹²³, Véronique Baccini⁴, Aline Renneville⁵, Françoise Boehlen⁶, Margot Morabito¹²³, Nathalie Dron¹²³, Caroline Deswarte⁷⁸, Yunhua Chang¹²³, Guy Leverger⁷⁸⁹, Eric Solary¹²³, William Vainchenker¹²³, Rémi Favier°¹⁹, Hana Raslova°¹²³

* These two authors have equally contributed; ** These two authors have equally contributed

1. Institut National de la Santé et de la Recherche Médicale, UMR 1009, 114 rue Edouard Vaillant, 94805 Villejuif, France
2. University Paris-Sud 11, 114 rue Edouard Vaillant, 94805 Villejuif, France
3. Institut Gustave Roussy, 114 rue Edouard Vaillant, 94805 Villejuif, France
4. Institut National de la Santé et de la Recherche Médicale, UMR 1062, Faculty of Medicine La Timone, Marseille, France
5. Laboratoire d’Hématologie, Centre de Biologie-Pathologie, CHRU de Lille, Lille, France.
6. Division of Angiology and Hemostasis, Geneva University Hospital, 1211 Geneva 14, Switzerland
7. University Paris 6, Faculty of Medicine Saint Antoine, 75012 Paris, France
8. Institut National de la Santé et de la Recherche Médicale, UMR S938, Faculty of Medicine Saint Antoine, 75012 Paris, France
9. Assistance Publique - Hôpitaux de Paris, Hôpital Trousseau, CRPP, Services d’Hématologie biologique et clinique, 26 Avenue du Docteur Netter, 75012 Paris, France

Corresponding author: Hana Raslova, INSERM UMR1009, Institut Gustave Roussy, 114 rue Edouard Vaillant, 94805, Villejuif cedex, France.
Phone number (+33) 1 42 11 46 71
Fax number (+33) 1 42 11 52 40
E-mail: hraslova@igr.fr

Short title: MYH10 biomarker for detection of RUNX1 malignancies
Scientific Heading: Hematopoiesis
Abstract

*RUNXI* gene alterations are associated with acquired and inherited hematological malignancies that include Familial Platelet Disorder/Acute Myeloid Leukemia (FPD/AML), primary or secondary acute myeloid leukemia, and chronic myelomonocytic leukemia (CMML). Recently we reported that *RUNXI*-mediated silencing of non-muscle myosin heavy chain IIB (MYH10) was required for megakaryocyte ploidization and maturation. Here, we demonstrate that *runx1* deletion in mice induces the persistence of MYH10 in platelets and a similar persistence was observed in platelets of patients with constitutional (FPD/AML) or acquired (CMML) *RUNXI* mutations. MYH10 was also detected in platelets of patients with the Paris-Trousseau syndrome, a thrombocytopenia related to the deletion of the transcription factor FLI1 that forms a complex with RUNX1 to regulate megakaryopoiesis, while MYH10 persistence was not observed in other inherited forms of thrombocytopenia. We propose MYH10 detection as a new and simple tool to identify inherited platelet disorders and myeloid neoplasms with abnormalities in *RUNXI* and its associated proteins.
Introduction

Alterations in RUNX1 are associated with several acquired and inherited hematological malignancies that include familial platelet disorder with propensity to develop acute myeloid leukemia (FPD/AML, OMIM 601399), primary or secondary acute myeloid leukemia (AML) and chronic myelomonocytic leukemia (CMML). FPD/AML is an autosomal dominant disorder characterized by a mild to moderate thrombocytopenia, a normal platelet size and morphology, an abnormal platelet aggregation and heterozygous germ-line mutations or deletions in RUNX1. Acquired RUNX1 mutations have been detected in 15-30% of CMML and 12% of AML. The frequency of RUNX1 alterations is underestimated for at least two reasons. First, intragenic deletions, demonstrated to be causal in some FPD/AML pedigrees, are not identified by common gene sequencing that is limited to coding exons. Secondly, in most FPD/AML pedigrees, gene sequencing is performed only when an AML/MDS occurrence is observed, which prevents identification of most FPD/AML pedigrees with thrombocytopenia alone. The improved detection of RUNX1 alterations is necessary for better molecular characterization of AML and CMML, and also for earlier diagnosis of FPD/AML.

We recently observed that the expression of non-muscle myosin heavy chain IIB (NMMHC-IIB, MYH10) was almost completely silenced during the ploidization process and terminal maturation of megakaryocytes due to RUNX1-mediated negative regulation of MYH10 gene. RUNX1 knock-down in human/mice was associated with a decreased ploidy of megakaryocytes and an increased expression of MYH10, while other myosins were rather downregulated. We show here that the biochemical detection of MYH10 in platelets can be used as a marker to screen for RUNX1 gene inactivation in inherited as well as acquired myeloid diseases.
Material and Methods

Patients

The study was approved by the Local Research Ethics Committee from AP-HP and INSERM. Blood samples from patients and healthy subjects were collected with informed consent in accordance with the Declaration of Helsinki.

Samples

Granulocytes, red blood cells (RBC), and mononuclear cells were separated by standard techniques. CD14 (Monocytes), CD3 (T Lymphocytes) and CD19 (B Lymphocytes) cells were separated by double-positive selection using a magnetic cell-sorting system (AutoMACS, Miltenyi Biotec SAS). The platelet-rich plasma was prepared by centrifugation at 900 rpm for 10 minutes. Platelets were pelleted by centrifugation at 3,000 rpm for 10 minutes. Remaining RBC (GPA⁺) were depleted using immunomagnetic beads (Miltenyi Biotec SAS).

Immunoblotting

Thirty μg of proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Blots were incubated with a rabbit anti-MYH10 antibody (Cell Signaling) and reprobed with an antibody against HSC70 (Sigma) followed by HRP-linked secondary antibodies and, respectively, Super Signal West Femto Maximum Sensitivity Substrate (Thermo Scientific) for MYH10 detection and Immobilon Western Chemiluminescent HRP Substrate (Millipore Corporation) for HSC70 detection.

Sequence and CGH array analysis

Gene sequencing was performed using BigDye® Terminator Cycle Sequencing Kit (Applied Biosystems) and analyzed on the Applied Biosystems 3130xl Genetic Analyzer. The genomic profile was assessed by CGH arrays (Agilent AMADID 021850). All data and protocols have
been submitted to ArrayExpress at the European Bioinformatics Institute with the accession number E-MTAB-1121.

Results and discussion

RUNX1 mediated silencing of MYH10 during normal megakaryopoiesis\(^5\) suggested that, contrary to normal platelets, MYH10 should be expressed in platelets of \(\text{runx1} \text{ KO mice}\) as well as in platelets of patients with \(\text{RUNXI} \text{ gene alterations}\). Therefore we induced \(\text{runx1} \text{ knock-out in } \text{runx1}^{0/0(\text{Mxe-Cre}+)} \text{ mice by pIpC injections}\)\(^5\). As expected, contrary to control mice (\(\text{runx1}^{0/0(\text{Mxe-Cre}^-)}\)), MYH10 protein was detected in the platelets of KO (\(\text{runx1}^{0/0(\text{Mxe-Cre}+)}\)) animals (Supplementary figure 1).

MYH10 expression was then studied in human platelets from patients and healthy subjects. Immunoblotting experiments failed to detect any MYH10 protein in healthy donor platelets. We also tested the expression of MYH10 in other blood cells as platelets can be contaminated upon purification, especially in pathological samples. MYH10 protein was not detected in red blood cells, granulocytes, monocytes, and T and B cells sorted from healthy donor peripheral blood (Figure 1A). In contrast, MYH10 expression was easily detected in platelets of five patients from three FPD/AML pedigrees (A, B, D, supplementary table 1) compare to three control samples (Co1-3) (Figure 1B). MYH10 was easily detected in a dividing immature megakaryocytic/erythroid cell line, HEL, used as a positive control.

To determine whether MYH10 strong expression in platelets was specific for FPD/AML, we collected the platelets of eight patients with well identified inherited platelet disorders (Supplementary table 1) including Bernard-Soulier syndrome (BS); Paris Trousseau syndrome (PTS)\(^6\); gray platelet syndrome (GPS)\(^7\); mild thrombocytopenia associated with a GPlV (CD36) defect (GPIVD) and MYH9 syndrome (MYH9) (Figure 1C,
Supplementary table 1). In these series, MYH10 was detected exclusively in platelets from patients with a Paris Trousseau syndrome, which is characterized by FLI1 haploinsufficiency. Interestingly, FLI1 and RUNX1 transcription factors bind distinct regions on the MYH10 promoter and cooperate during megakaryopoiesis as components of a large transcriptional complex.

We also collected the platelets of eleven patients with unexplained inherited thrombocytopenia (Supplementary table 1). Of six who showed an increase in their mean platelet volume, none expressed MYH10 (Figure 1D) and none presented RUNX1 mutations. The five other patients, including three isolated cases (I-1, I-2, I-3) and two cases from the same pedigree (PG3-1 and PG3-2), had a normal mean platelet volume. MYH10 was detected in the platelets of only one of these five patients (Figure 1E, I-2) who was the only one in which RUNX1 gene sequencing identified a mutation (R174Q). In three out of the four other patients, we identified a mutation in ANKRD26 gene, which has also been associated with inherited thrombocytopenia with potential predisposition to acute leukemia (THC2) (Figure 1E, I-1, PGS-1, PGS-2, Supplementary table 1). We confirmed these results at the mRNA level using Q-RT-PCR by showing that the MYH10 transcript was 4 to 7-fold higher in platelets from FPD/AML patients than from controls or TCH2 patients (Supplementary figure 2). According to these results, we established a diagnostic algorithm of un-explained thrombocytopenia (Figure 1F) based on the detection of MYH10 in platelets of thrombocytopenic patients with normal platelet volume, allowing to separate patients with mutations in RUNX1 repressor complex from those with mutations in ANKRD26 or other non identified genes. This first screening will drive to diagnose the main platelet pathologies: FPD/AML, PTS and THC2. In a given pedigree with RUNX1 mutation, the test may be useful to detect rapidly the family members without thrombocytopenia but with the RUNX1 mutation and more importantly family members without RUNX1 mutations to select the best donor for
bone marrow transplantation.

MYH10 protein expression was also examined in the platelets of twenty-four patients suspected of CMML. The firm diagnosis of CMML was made according to the WHO criteria only in nineteen of them (Supplementary table 2). MYH10 protein expression was detected in the platelets of eight patients and RUNXI mutations were detected in five of these eight patients. All patients negative for platelet MYH10 were negative for RUNXI mutation (Figure 2A, Supplementary table 2). Interestingly, the platelet number was lower in eight CMML patients expressing MYH10 in their platelets compared to other patients (Figure 2B, Supplementary table 2). No significant correlation was found between the presence of MYH10 and the status of other genes frequently mutated in CMML including TET2, ASXL1, KRAS, NRAS, CBL and SRSF2 (Supplementary table 2). To investigate whether the patients expressing MYH10 in platelets without RUNXI mutation harbor RUNXI or FLI1 deletion, CGH arrays were performed in two of them (N°15 and N°16, Supplementary table 2). No deletion in these two transcription factors and in other hematopoietic regulators was found suggesting either the presence of mutation(s) in non explored sequences of the gene, or mutation(s) in another member of the RUNXI repressor complex, or in epigenetic regulators.

All together, the detection of MYH10 expression in platelets indicates a defective silencing of MYH10 gene during the terminal differentiation of megakaryocytes, due to a germ-line or a somatic alteration in either RUNXI gene or a gene encoding a member of the repressor complex regulating MYH10 gene expression such as FLI1. MYH10 protein detection in platelets may be used as a biomarker of these gene alterations, e.g. as a first-line assay to screen for RUNXI and FLI1 alterations in autosomal forms of inherited thrombocytopenia, as well as RUNXI in acquired myeloid malignancies.
Acknowledgments: We thank the patients and their families for participation in this study. We are grateful to Dr. A. Auvrignon, Pr. P. Fenaux, Pr. B. Quesnel, Pr. F. Dreyfus, Dr. C. Berthon, Dr. A. Toma, Pr. O. Beyne-Rauzy, Pr. O. Hermine, Dr. S. de Botton, and Pr. M. Fontenay for patients samples, to Dr. D.G. Gilliland and Dr. T. Mercher for kindly providing of \textit{runx1}^{0/0/(Mse-Cre+)} mice and to Dr. G. Meurice for analysis of CGH array data.

This work was supported by grants from Agence Nationale de la Recherche (ANR-GIS Institut des maladies rares and ANR jeunes chercheurs). IAD was supported by a doctoral fellowship from ARC. DB was supported by a postdoctoral fellowship from ANR. RF, HR and WV are recipients of a research fellowship from AP-HP-INSERM (contrats d’interface 2009-2012 for RF and 2008-2013 for HR) and from IGR-INSERM. WV and ES teams are supported by the Ligue Nationale Contre le Cancer.
Authorship Contributions

WV, RF and HR designed the work, IAD, DB, VB, AR, FB, MM, ND, CD, YC, RF, HR performed the experiments, VB, RI, FB, GL, ES, RF collected the samples, IAD, DB, RI, ND, GL, ES, WV, RF, HR discussed the results, RI, ES, WV, RF and HR wrote the manuscript. IAD* and DB* equally contributed to this work, RF** and HR** have equally contributed to this work.

Disclosure of Conflicts of Interest

The authors declare no competing financial interest.
References

Figure legends

Figure 1

Immunoblot analysis of MYH10 expression in platelets from patients with inherited thrombocytopenia. In all panels, HEL cells were used as positive controls and HSC70 as loading control. (A) Indicated peripheral blood cell populations from a healthy donor (RBC: red blood cells, Platelets-RBC: platelets after depletion of red blood cells; Platelets +RBC: platelets without red blood cell depletion); one representative of two experiments is shown; (B) Platelets of FPD/AML patients (AII-1, AII-2, with R174Q RUNX1 mutation; BII-2, BIII-1 with R139X RUNX1 mutation and D with RUNX1 monoallelic deletion) and healthy donors as controls (Co 1 to 3); (C) Platelets of patients with well-identified inherited thrombocytopenia (BS: Bernard Soulier; PTS: Paris Trousseau Syndrome, GPS: Gray Platelet Syndrome, GPIVD: thrombocytopenia associated with GPIV defect, MYH9: thrombocytopenia associated with MYH9 mutations); (D) Platelets of patients from two pedigrees (PG1 and PG2, three patients in each) with unexplained thrombocytopenia with increased mean platelet volume (MPV); (E) Platelets of patients with unexplained thrombocytopenia with normal MPV. Two patients from the same pedigree are included (PG3); (F) Flowchart of familial thrombocytopenia diagnosis based on MYH10 detection in platelets.

Figure 2

(A) Immunoblot analysis of MYH10 expression in platelets from patients with CMML. In all panels, HEL cells were used as positive controls, Co 1 platelets as negative control, and HSC70 as loading control. (B) Statistical evaluation of correlation between MYH10 presence in platelets of patients with CMML, RCMD, AML-M4 and reactive monocytosis and thrombopenia degree. MYH10+: patients with the presence of MYH10 in
platelets; MYH10-: patients with the absence of MYH10 in platelets. Mann-Whitney’s U test, retaining P<0.05 as statistically significant was used. Statistical analyses were carried out with Prism version 5.0 (Graphpad Software, Inc.).
Figure 1

For personal use only on November 12, 2017 by guest

www.bloodjournal.org

Thrombocytopenia

MPV evaluation

Normal

Increased

MYH10 expression detection in platelets

+ -

RUNX1 DNA alterations screening

FPD/AML

FLI1 DNA alterations screening

PTS

DNA alterations screening of others candidates genes (such as GATA1, ABCD5, MASTL) or High Throughput Sequencing

ANKRD26 DNA alterations screening

THC2
Figure 2

A

CMML

Co 1 HEL 1 2 3 4 5 6 7 8 9 10 11

MYH10

HSC70

B

Platelet number (G/L)

**

MYH10+

MYH10−
MYH10 protein expression in platelets as a biomarker of RUNX1 and FLI1 alterations

Iléana Antony-Debré, Dominique Bluteau, Raphael Itzykson, Véronique Baccini, Aline Renneville, Françoise Boehlen, Margot Morabito, Nathalie Druin, Caroline Deswarte, Yunhua Chang, Guy Leverger, Eric Solary, William Vainchenker, Rémi Favier and Hana Raslova

Information about reproducing this article in parts or in its entirety may be found online at: http://www.bloodjournal.org/site/misc/rights.xhtml#repub_requests

Information about ordering reprints may be found online at: http://www.bloodjournal.org/site/misc/rights.xhtml#reprints

Information about subscriptions and ASH membership may be found online at: http://www.bloodjournal.org/site/subscriptions/index.xhtml

Advance online articles have been peer reviewed and accepted for publication but have not yet appeared in the paper journal (edited, typeset versions may be posted when available prior to final publication). Advance online articles are citable and establish publication priority; they are indexed by PubMed from initial publication. Citations to Advance online articles must include digital object identifier (DOIs) and date of initial publication.