Accepted Article

Mutations of *RUNX1* in families with inherited thrombocytopenia

Daniela De Rocco[1], Federica Melazzini[2], Caterina Marconi[3], Alessandro Pecci[2], Roberta Bottega[4], Chiara Gnan[1], Flavia Palombo[3], Paola Giordano[5], Maria Susanna Coccioli[6], Ana C. Glembotsky[7], Paula G. Heller[7], Marco Seri[3], Anna Savoia[1,4] and Patrizia Noris[2]

[1]Institute for Maternal and Child Health – IRCCS Burlo Garofolo, Trieste, Italy;

[2]Department of Internal Medicine, IRCCS Policlinico San Matteo Foundation and University of Pavia, Pavia, Italy;

[3]Department of Medical and Surgical Science, Policlinico Sant'Orsola Malpighi and University of Bologna, Bologna, Italy;

[4]Department of Medical Sciences, University of Trieste, Trieste, Italy;

[5]Department of Biomedical Science and Human Oncology, Clinical Pediatrics "B. Trambusti", University of Bari, Bari, Italy;

[6]U.O.C of Pediatrics, Hospital "D. Camberlingo", Francavilla Fontana, Brindisi, Italy;

[7]Instituto de Investigaciones Médicas Alfredo Lanari, Universidad de Buenos Aires, IDIM-CONICET, Buenos Aires, Argentina

Running heads: Novel RUNX1 mutations.

Corresponding author:

Dr. Anna Savoia

Department of Medical Sciences, University of Trieste

IRCCS Burlo Garofolo

Via dell'Istria 65/1 - 34137 Trieste, Italy

Tel +39 040 3785527 - Fax +39 040 3785540

Email: anna.savoia@burlo.trieste.it

Main test words count: 972

No of tables: 1

No of references: 6

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as an 'Accepted Article', doi: 10.1002/ajh.24703

This article is protected by copyright. All rights reserved.

Accepted Article

Familial platelet disorder with propensity to myeloid malignancy (FPD/AML) is a rare autosomal dominant disease characterized by thrombocytopenia and platelet functional defects [1]. Although bleeding tendency is usually mild to moderate, an important hallmark of FPD/AML is the increased risk of myeloid neoplasms, such as myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML).

FPD/AML is caused by different, usually private, mutations of *RUNX1*, a gene encoding the DNA binding subunit (also known as CBF-alpha) of the core binding factor (CBF) transcription complex [2]. The N-terminal domain of RUNX1 (runt-homologous domain) mediates DNA binding and heterodimerization to CBF-beta, the other subunit of the CBF complex. At the C-terminus, RUNX1 is constituted of domains involved in transcription activation and repression.

We report three families with pathogenic variants of *RUNX1* identified in our cohort of 274 consecutive unrelated probands with inherited thrombocytopenia (IT) (Supporting information in materials and methods). They carry three different heterozygous mutations, all segregating with thrombocytopenia in the respective families: one missense (c.578T>A/p.Ile193Asn) variant affecting a well conserved residue of the runt-homologous domain, and two nucleotide substitutions of the canonical "gt" dinucleotide in the donor splice sites of intron 4 (c.351+1G>A) and intron 8 (c.967+2_5del) (Supporting information Fig. S1, S2, S3). The effect of c.351+1G>A on splicing mechanisms was not determined because RNA from members of family 2 was not available. In family 3, RT-PCR analysis revealed two alternative spliced products, sp1 and sp2 that led to frameshift/truncation and in-frame deletion alterations at the transactivation domain of RUNX1, respectively. The sp1 RNA was expressed at higher level than the sp2 form.

The 13 individuals from the three families carrying the *RUNX1* mutations had mild thrombocytopenia (platelet count ranging from 70 to $130X10^9/L$) and mild bleeding tendency (Table 1). The platelet size was normal in all the six cases analyzed. Consistent with the mild reduction in platelet count, patients' serum thrombopoietin levels were normal or moderately increased (Table 1). No specific morphological alteration of platelets was detected at the May-Grunwald-Giemsa staining, except for moderate reduction in the alpha-granule content in family 1, as confirmed by immunofluorescence analysis (data not shown).

This article is protected by copyright. All rights reserved.

Accepted Article

Flow cytometry of platelets showed normal expression of the major surface glycoprotein (GP) complexes GPIIb-IIIa and GPIb-IX-V (Table 1). In family 1, we found moderate reduction (25-45%) of GPIa-IIa, whose alteration was independent of genotypes at the *ITGA2* locus [4]. In this family, platelet aggregation was defective after stimulation with low doses of collagen (4 μg/mL), a defect that could not be explained by the reduction of GPIa-IIa, as the specific reagent used for the platelet aggregation assay does not bind this receptor. No second wave aggregation was observed in response to low doses of ADP (2 μM) in the five individuals investigated, suggesting alterations of the platelet delta-granules (Table 1). Although we did not support this hypothesis through specific assays, deficiency or defective release of the dense-granules is one prevalent defect in individuals with FPD/AML [3]. Increasing the concentration of ADP (5 μM), we found defective response only in family 1. Normal responses were observed using higher concentrations of both the agonists (collagen 20 μg/mL and ADP 20 μM) and with ristocetin (1.5 mg/mL), suggesting that the platelet dysfunction was mild.

Consistent with data from literature [3], our findings indicate that multiple aspects of platelet structure and function are compromised in FPD/AML, which is expected considering that RUNX1 regulates expression of multiple genes and that their expression is modulated by many genetic factors.

Individuals with heterozygous mutations of *RUNX1* are at risk of hematological malignancies, which have been reported in almost 40% of patients with a median age of onset in the early 30s [1]. In our cohort of 14 carriers (individual II-3 of family 3 is an obligate carrier), only three individuals (one from family 2 and two from family 3) had developed AML (Supporting information in materials and methods). This low prevalence (20%) could depend on the relatively young age of some of the individuals studied, as well as on the fact that the probands were not all selected on the basis of their personal or family history of hematological malignancies. Indeed, families 1 and 2 were identified by diagnostic work-up of an IT of unknown origin of IT, which was not associated with MDS or AML. Therefore, only systematic molecular genetic testing will provide the actual risk of malignancies in individuals with *RUNX1* mutations. Although AML remains the most prevalent neoplasm, we cannot exclude that mutations of *RUNX1* predispose also to solid tumors, as those (lung and uterine cancer) diagnosed in two members of family 3.

This article is protected by copyright. All rights reserved.

Accepted Article

Although mutations of *RUNX1* are relatively rare (1% in our cohort), their identification in families with IT is important because of the associated risk of malignancies. However, it is difficult to discriminate FPD/AML from other IT due to lack of pathognomonic signs. The most promising features could be the defects of the alpha- and delta-granules, or persistence of MYH10 protein expression in platelets [3]. However, these investigations cannot be performed on a routine basis, as they require specialized laboratories.

Since mutations not only of *RUNX1* but also of the *ANKRD26* and *ETV6* genes are responsible for thrombocytopenia (*ANKRD26*-related thrombocytopenia, *ANKRD26*-RT; *ETV6*-related thrombocytopenia, *ETV6*-RT) and predisposition to hematological malignancies [5], the recognition of these disorders would provide patients with appropriate genetic counseling, clinical follow-up, and treatment, especially in case of selection of donors for hematopoietic stem cell transplantation in patients who develop AML or MDS. Like FPD/AML, *ANKRD26*-RT and *ETV6*-RT are rare autosomal dominant forms of IT that are hard to diagnose because they lack of specific clinical or laboratory features. However, they are all characterized by normal platelet size, which is a relatively uncommon finding in IT [6]. Therefore, we suggest that all the individuals with autosomal dominant thrombocytopenia and normal platelet size should be tested for mutations in *RUNX1*, *ANKRD26*, and *ETV6*.

Authorship and Disclosure

DDR, CG, CM, FP performed whole exome, Sanger sequencing and bioinformatics analysis. RB carried out RT-PCR analysis. FM, AP, PG, MSC, ACG, PH, MS, PN enrolled patients for the study, collected patients' clinical and laboratory data. DDR, FM, MS and AS were responsible for writing the manuscript; DDR, AS, PN participated in the study design. All authors revised and approved the manuscript.

All the authors report no potential no conflicts of interest.

Acknowledgements

This study was supported by the ERA-Net for Research Program on Rare Diseases (EUPLANE), Telethon Foundation (Grant GGP13082), Cariplo Foundation (2012-0529), Italian Ministry of Health (RF-2010-2309222). C.G. receives a fellowship

This article is protected by copyright. All rights reserved.

(RF13679 2013-2014 and RF16440 2015) from AIRC (Italian Association of Cancer Research).

References

1. Liew E, Owen C. Familial myelodysplastic syndromes: a review of the literature. Haematologica 2011;96:1536-1542.

2. Song WJ, Sullivan MG, Legare RD, et al. Haploinsufficiency of CBFA2 causes familial thrombocytopenia with propensity to develop acute myelogenous leukaemia. Nat Genet 1999;23:166-175.

3. Latger-Cannard V, Philippe C, Bouquet A, et al. Haematological spectrum and genotype-phenotype correlations in nine unrelated families with RUNX1 mutations from the French network on inherited platelet disorders. Orphanet J Rare Dis 2016;11:49.

4. Noris P, Guidetti GF, Conti V, et al. Autosomal dominant thrombocytopenias with reduced expression of glycoprotein Ia. Thromb Haemost 2006;95:483-489.

5. Noris P, Biino G, Pecci A, et al. Platelet diameters in inherited thrombocytopenias: analysis of 376 patients with all known disorders. Blood 2014;124:e4-e10.

6. Savoia A. Molecular basis of inherited thrombocytopenias. Clin Genet 2016;89:154-16

Accepted Article

This article is protected by copyright. All rights reserved.

Table 1. Clinical and laboratory finding of individuals carrying *RUNX1* heterozygous germline mutation.

Affected individuals (probands in bold)	Age (years)[1]	Platelet count ($x10^9$/L)[2]	MPV (fL)[3]	Serum THPO level (pg/mL)[4]	MGG-stained blood smear examination	Flow cytometry of platelet surface GPs	Platelet aggregation by collagen and ADP	Bleeding symptoms	Hematological malignancies (age of onset)
F1/I-2	54	87	7.8	30.33	Normal platelet size; reduced alpha-granule content	Normal GPIIb-IIIa and GPIb-IX-V; reduction of GPIa-IIa	Collagen (4 µg/ml): reduced collagen; ADP (2 µM): no second wave	Menorrhagia, mild bleeding after dental extraction	No
F1/II-1	25	86	11.1	14.57	Normal platelet size; reduced alpha-granule content		Collagen (4 µg/ml): reduced collagen; ADP (5 µM): no second wave	No	No
F1/II-2	24	70-120	11.2	14.73	Normal platelet size; reduced alpha-granule content		Collagen (4 µg/ml): reduced collagen; ADP (5 µM): no second wave	Easy bruising, ecchymosis	No
F2/I-2	52	80-110	8.9	nd	Normal platelet size and morphology	Normal GPIIb-IIIa and GPIb-IX-V	ADP (2 µM): no second wave	Easy bruising	AML (53y)
F2/II-1	12	80-140	8.2	nd	Normal platelet size and morphology			Easy bruising	No
F3/IV-1[5]	3	110 - 130	8.4	110	Normal platelet size and morphology	nd	nd	Epistaxis, easy bruising, ecchymosis	No

Notes: [1]Age at the last evaluation. [2]When more than one platelet count is available, the lowest and highest counts are reported. [3]Data have been acquired by automated counters using different normal range for MPV; all the MPV described were within the normal range. [4] Normal range of serum THPO: 6.9 - 18 pg/mL. [5]Except for platelet count and cancer development, no additional information is available for other family members. **Abbreviations:** AML: acute myeloid leukemia; GPs = glycoproteins. nd = not determined; MGG = May-Grünwald-Giemsa; MPV = mean platelet volume; THPO = thrombopoietin.

This article is protected by copyright. All rights reserved.