Inherited Thrombocytopenias with Predisposition the Hematological Malignancies

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Abstract

Inherited thrombocytopenias (IT) are rare, clinically and genetically heterogeneous diseases caused by mutations in more than 30 genes. Mutations of three of these genes, RUNX1, ANKRD26, and ETV6, cause Familial Platelet Disorder with propensity to Acute Myelogenous Leukemia (FPD/AML), ANKRD26-related thrombocytopenia (ANKRD26-RT), and ETV6-related thrombocytopenia (ETV6-RT), respectively. They are similar forms characterized by moderate thrombocytopenia with platelets of normal size. In addition to mild bleeding tendency, individuals affected with these forms of IT have an increased risk of developing hematological neoplasms, including myelodysplastic syndrome, acute myeloid leukemia, and acute lymphoblastic leukemia. The pathogenic mechanisms responsible for these ITs are partially known, likely resulting in defects of a pathway controlling the expression of ANKRD26 in hematopoietic cells. Due to their rarity, FPD/AML, ANKRD26-RT, and ETV6-RT are still poorly defined diseases, requiring extensive genetic analysis and clinical characterization. Considering the increased risk of leukemia, improvement of our knowledge of these ITs would be of fundamental importance in recognizing affected individuals.

Introduction

Inherited thrombocytopenias (IT) are a heterogeneous group of diseases characterized by low platelet count associated or not with hemorrhagic diathesis and/or other clinical manifestations. ITs are genetically heterogeneous diseases, as they are caused by mutations in more than 30 genes. However, these genes can explain only 50% of the cases, suggesting that novel genes have yet to be identified for a comprehensive understanding of platelet biogenesis defects [1,2].

Among the different ITs, there are forms of mild/moderate thrombocytopenia due to maturation defects of megakaryocytes. Indeed, although they are usually produced in normal number, megakaryocytes are small with hypolobulated nuclei and produce proplatelets of short length with few branches. Consistent with the relatively mild defect in platelet production, the bleeding tendency is usually mild or insignificant. The more common bleeding symptoms are petechiae, ecchymoses, gum bleeding, epistaxis, and menorrhagia. Of the ITs with these features, three are characterized by an increased risk of developing hematological malignancies: familial platelet disorder with propensity to acute myelogenous leukemia, ANKRD26-related thrombocytopenia, and ETV6-related thrombocytopenia, autosomal dominant diseases caused by mutations in the ANKRD26, RUNX1, and ETV6 genes, respectively. They are forms recognized as "myeloid neoplasms with germ line predisposition and preexisting platelet disorders" by the 2016 revision of the WHO classification of hematological neoplasms [3]. Considering the importance in their recognition for appropriate management of patients and their families, we provide an update focusing on the molecular basis and potential pathogenic mechanisms of these three ITs (Table 1).

Familial Platelet Disorder with Propensity to Acute Myelogenous Leukemia

Familial platelet disorder with propensity to acute myelogenous leukemia (FPD/AML) is the IT with predisposition to hematological malignances best characterized. It is known since 1999, when heterozygous germline mutations of the RUNX1 gene were identified in the first families [4]. RUNX1 encodes for the alpha subunit (CBFalpha) of the core-binding factor (CBF) transcription complex. The subunit contains a runt-homologous (RHD) and transactivation (TAD) domain at the NH2- and COOH terminus domain, respectively. The RHD mediates interaction with both the

Table 1: Features of ITs with predisposition to hematological malignancies.

Features	FPD/AML	ANKRD26-RT	ETV6-RT
OMIM	601399	188000	n.a.
Relative frequency	0.03	0.18	0.05
Transmission	Autosomal dominant		
Gene	RUNX1	ANKRD26	ETV6
Chromosomal localization	21q22	10p12.1	12p13.2
Thrombocytopenia	Moderate	Moderate/severe	Mild/moderate
Platelet size and volume	Normal		
Platelet function	Aggregation defects (delta-granule deficiency)	No dysfunction reported (alpha-granule deficiency)	Reduced spread of platelet on fibrinogen
Megakaryocytes	Decrease of Mk progenitor number; immature Mks	Increase of Mk number; immature Mks	Immature Mks
Proplatelet formation	Few proplatelets, shorter in length with decreased number of branches		
Bleeding tendency	Absent or mild	Mild/moderate	Absent or mild
Frequent hematological malignancy (incidence of malignancies)	MDS/AML (0.40)	MDS/AML (0.08)	ALL (0.25)

CBF-beta subunit of the CBF complex and the TGT/cGGT consensus sequence of target genes.

There is significant heterogeneity in the clinical presentation of FPD/AML. Thrombocytopenia is usually moderate, though platelet count might be near the lower limit of the normal range. For this reason, thrombocytopenia is diagnosed from early childhood into the sixth decade. The reduced platelet count is associated with decrease in the number of megakaryocytic progenitors and impairment of their maturation. Indeed, megakaryocytes are immature characterized by high nucleo-cytoplasmic ration and poorly lobulated nuclei, which leads to significant reduction of proplatelet formation.

Other numerous platelet abnormalities have been reported in FDF/AML, including reduced alpha-granules, impaired platelet aggregation, decreased platelet spreading and activation of alpha-IIb/beta3. However, the most recurrent platelet dysfunction is associated with delta-granule deficiency and release. As consequences, platelet aggregation might be defective in response to ADP and other agonists [5]. The platelet phenotypic heterogeneity is consistent with the function of RUNX1, which regulates directly or indirectly the expression of many genes. Of interest, two targets are MYH9 and MYH10, both encoding for non-muscle myosins of class II that are important for platelet biogenesis. Expression of MYH9, whose mutations are responsible for another form of IT (MYH9-related disease) [6], is increased during megakaryopoiesis and its product (myosin IIA) is required for proplatelet formation. Instead, MYH10 (myosin IIB) is physiologically down-regulated up to being almost undetectable in mature megakaryocytes and platelets, thus allowing the switch from mitosis to endomitosis and polyploidization in megakaryocytic maturation [7]. Since its expression persists in FPD/AML platelets, detection of MYH10 can be used as a prescreening assay for molecular genetic testing of RUNX1 [8].

As mentioned above, individuals with heterozygous mutations of RUNX1 are at risk of hematological malignancies. This is not unexpected, as the RUNX1 locus is affected by numerous acquired translocations and point mutations in acute lymphoblastic leukemia, acute myeloid leukemia, chronic myelomonocytic leukemia, and myolodysplastic syndrome [9,10]. In FPD/AML, acute myeloid leukemia is the most prevalent neoplasm, though predisposition to T-acute lymphoblastic leukemia or B-lymphoid cell malignancies may also be possible [5,11]. We cannot exclude that germline mutations even predispose to solid tumors [12].

Hematological malignancies have been reported in almost 40% of patients with a median age of onset in the early 30s [13]. However, considering that one of the indications for molecular genetic testing of RUNX1 is the thrombocytopenia associated with occurrence of hematological malignancies among the proband's family membrers, the enrolment criteria might considerably influence the evaluation of the leukemic risk. Indeed, thanks to next generation sequencing approaches, RUNX1 mutations have been identified in families with IT of unknown origin without evidence for hematological neoplasms [12,14]. Therefore, a systematic analysis of the RUNX1 gene in IT families and a strict follow up of carriers will help determining a reliable estimation of the incidence of leukemia among the FPD/AML patients.

At present, almost 50 different, mainly private, alterations of RUNX1 have been reported [15]. They include loss-of-function alterations, such as small deletions and insertions, or nonsense mutations, which are likely to lead to haploinsufficiency of the transcription factor. A syndromic thrombocytopenia associated with numerous congenital features has also been described in individuals with de novo deletions of the 21q22 chromosomal region that includes RUNX1 and several nearby genes [5,16]. Dominant-negative effects have also been reported for missense mutations, which mainly affect the DNA binding domain (Figure 1A) [17,18].

There are no extensive studies on correlation between genotype and the risk of leukemia. However, mutations with dominant-negative effects are likely to correlate with a higher risk [18]. Accordingly, the RUNX1 haploinsufficiency does not affect proliferation of granulomonocytes, which is instead increased in cells with mutations exerting dominant-negative effect, as demonstrated in induced pluripotent stem cells generated from patients [19]. A significant reduction of the megakaryocyte production was instead observed in both models, suggesting that low level of RUNX1 may be critical for development of leukemia but not for thrombocytopenia.

Consistent with the "second-hit" hypothesis, the leukemic transformation has been associated with biallelic alterations of RUNX1 [20]. Somatic mutations of CDC25C are recurrent in the early phase of leukemic progression [21]. Throughout the cellular interphase, CDC25C is inactive, sequestered into the cytoplasm by protein 14-3-3 l, preventing cells from entering mitosis. When
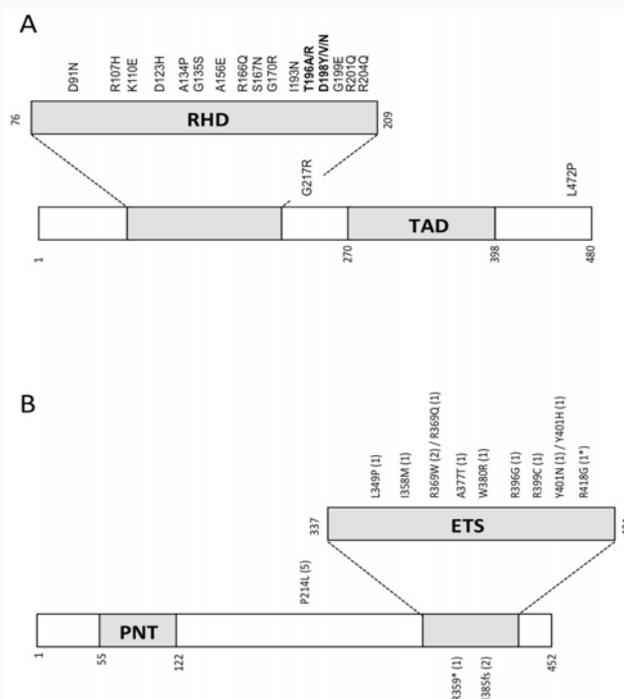
Figure 1: Mutations affecting transcription factors RUNX1 and ETV6. A) Schematic representation of the RUNX1 domains [56], including the Runt homology DNA-binding domain (RHD) spanning amino acids 76 to 209, the transactivation domain (TAD) from residue 270 to 398. Among the different alteration identified in FPD/AML individuals, only the missense mutations are indicated. Of note, the amino acid substitutions mainly affect RHD. The other alterations, including small inframe deletions, nonsense and frame shift mutations, are spread through the entire gene [15]. Residues affected by more than one mutation are in bold. Alterations are numbered according to positions in NM_001754.4. B) Domain structure of ETV6 based on Pfam annotation (http://www.ncbi.nlm.nih.gov/gene/2120), including the N-terminal pointed domain (PNT) and the C-terminal DNA binding domain (EST). The number of families carrying each mutation is reported in brackets. *Mutation c.1252A>G, which determines the p.R418G amino acid substitution, also leads to the frameshift skipping of exon 7 (p.N385Vfs*7) [46]. The skipping of exon 7 is also caused by the c.1153-1_1165del and c.1157_1161del mutations identified in two families [47,50].

mutated, CDC25C binds to 14-3-3 more efficiently and persists in the nucleus promoting cell cycle progression. Cells with this phenotype are regarded as pre-leukemic clones, which require somatic mutations in other genes, including GATA2, for neoplastic evolution [21]. However, the molecular events leading to malignant transformation remain unknown. Systematic collection of clinical and molecular data and extensive follow-up will be fundamental to define the risk of any neoplasia in individuals carrying mutations of RUNX1.

ANKRD26-Related Thrombocytopenia

ANKRD26-related thrombocytopenia (ANKRD26-RT) is another rare autosomal dominant form of thrombocytopenia, described for the first time in two large families from Italy and North America, when the disease locus was mapped on chromosome 10p11.1-p12 [22,23]. However, the ANKRD26 gene was identified only more than ten years later, thanks to the study of additional families [24].

At present, collection of clinical and laboratory data from approximately 60 unrelated families accounting for almost 200 individuals has allowed us to define this form of IT [25,26]. Like PFD/AML, ANKRD26-RT is characterized by moderate thrombocytopenia with normal-sized platelets and alpha-granule deficiency. A small subset of individuals can have elevated hemoglobin levels and leukocyte counts, without a clear explanation. Though increased in number, megakaryocytes are small with reduced cytoplasm and hypolobulated nuclei, all features indicating maturation defects of these cells. Consistently, the serum TPO level is higher in ANKRD26-RT patients than in controls, indicating that the platelet-megakaryocyte mass is reduced. Moreover, platelets

and megakaryocytes have specific inclusions called "particulate cytoplasmic structures" [27]. These formations are characterized by cylindrical structures with selective immunoreactivity for proteasome and polyubiquitinated proteins. They have been observed even in solid cancers, preneoplastic gastric lesions, and neutrophils of patients with Schwachman–Diamond syndrome, a genetic disease with neutropenia and increased leukemia risk [27].

In addition to mild bleeding tendency, individuals with mutations of ANKRD26 are at risk of hematological malignancies, including myelodiplastic syndrome and acute myeloid leukemia. The incidence of these neoplasms is approximately of 8% [28-31]. A few cases of chronic myelomonocytic leukemia have also been reported [32].

The 17 different heterozygous mutations identified so far are all clustered in a short specific sequence from nucleotides c.-134G to c.-113A of the 5′-untranslated region (5′-UTR) of ANKRD26 [24-26,28,30,33,34]. Nucleotide substitutions at position c.-118C (c.-118C>T or c.-118C>A), c.-127A (c.-127A>T), c.-128G (c.-128G>A, c.-128G>C, or c.-128G>T), and c.-134G (c.-134G>A) represent 75% of the mutant alleles (Figure 2). Although it has been reported as a disease-causing mutation [26,30], the c.-140G>C variant is likely to be a neutral polymorphism (rs41299222). At this locus, the minor allele frequency is up to 11% in different populations, and the C allele has been detected in affected individuals carrying one of the 5'-UTR mutations [30].

The pathogenic mechanisms responsible for ANKRD26-RD are not completely clear. ANKRD26 encodes a protein containing N-terminal ankyrin repeats, which are required for protein-protein
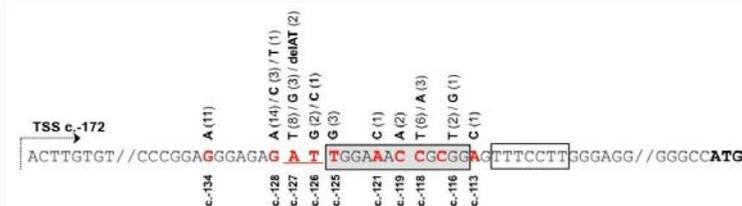
Figure 2: Mutations identified in the 5'-UTR of ANKRD26-RT patients. The 5'UTR spans from position c.-172 indicated as TSS (Transcription Start Site) to the first codon ATG codon (in bold). Mutations are located in a short stretch of 22 nucleotides that can be replaced by more than one alteration. Except for one dinucleotide deletion (c.-127_-126delAT), they are nucleotide substitutions. The number of families in which the single mutation has been detected is in brackets. Consensus sequences for the binding of transcription factors RUNX1 and FLI1 are shown by gray and open boxes, respectively.

interactions. In mice, partial inactivation of the ortholog Ankrd26 gene leads to marked hyperphagia, severe obesity, and increased body size, but not to thrombocytopenia [35,36]. Furthermore, partial or complete hemizygosity at the ANKRD26 locus is not associated with low platelet count or other phenotypes in humans, suggesting that haploinsufficiency or loss-of-function effects are not responsible for platelet defects [37,38].

Functional studies led to hypothesize that mutations prevent ANKRD26 from being downregulated during megakaryoppiesis [24,33]. Indeed, the RUNX1 and FLI1 transcription factors bind the 5′-UTR of ANKRD26, inhibiting its transcription progressively along the megakaryocyte differentiation up to almost the complete absence of mRNA in mature cells [33]. When the 5'UTR is mutated, the ANKRD26 expression is not inhibited, suggesting that mutations interfere with the correct binding of the regulatory factors. As consequence, the MAPK/ERK1/2 pathway is maintained active severely impairing proplatelet formation [33]. Consistent with this hypothesis, an increased expression level of ANKRD26 is also observed in individuals with germline mutations of RUNX1 [33].

Other mechanisms, independent of the RUNX1/FLI1 interaction with the 5'UTR, could be involved in the pathogenesis of ANKRD26-RT, as it could occur with mutations affecting the ANKRD26 coding region. Among these variants, only one (p.Asp158Gly) has been associated with thrombocytopenia, as reported in a large Saudi Arabian family [39]. The pathogenic mechanisms leading to low platelet count are unknown, though we cannot exclude that the mutation makes the ANKRD26 protein more stable. A few others mutations of the coding region (p.Met1?, p.Tyr35*, and p.Lys1300Asn) has been detected in four of the numerous cases of apparently sporadic myeloid neoplasms without evidence for platelet defects [29,40], suggesting that alterations of ANKRD26 are not recurrent in these cancers.

ETV6-Related Thrombocytopenia

Like for RUNX1, several somatic alterations of ETV6 have been described in leukemia and other haematological malignancies, since its cloning as a fusion gene resulting from a t(5;12) translocation, occurring in the malignant cells of a chronic myelomonocytic leukemia [41-43]. ETV6, a transcription factor belonging to the ETS family, is nuclear phosphoprotein with a strong repression activity. As the other family members, it has two conserved regions, the N-terminal pointed (PNT) and C-terminal DNA-binding (ETS) domains. The PNT domain is responsible for homodimerization or oligodimerization of ETV6. The ETS domain is the positively charged domain that recognizes purine rich stretch of DNA characterized by the GGAA/T core. Between PNT and ETS there is a less conserved central domain that binds several repressors, further controlling the

transcription of the target genes [44].

Despite the numerous acquired alterations of ETV6 reported since its original description, the first time germline mutations of this gene have been identified only in 2015 [45,46]. Two independent studies identified four different variants in six unrelated thrombocytopenic families, defining a new IT called ETV6-related thrombocytopenia (ETV6-RT). In the last two years, 11 additional ETV6 variants have been reported [47-50]. Of the 15 different, mainly private, mutations, 13 are amino acid substitutions affecting EST, which is likely to be severely destabilized, precluding the ETV6 binding to the DNA consensus sequences (Figure 1B). The presence of one relatively frequent mutation (p.P214L) indicates a potential mutational hot spot at this residue. This is the only mutation that does not alter EST but it affects the less conserved central region. Considering that this domain interacts with several transcription repressors, the effect of p.P214L on protein function could be different from those of the other ETV6 mutations.

ETV6-RT is a rare disease accounting for approximately 5% of the families with known forms of IT [50]. However, thanks to the 20 unrelated ETV6-RT families identified so far, this novel form of IT is being characterized. Trombocytopenia is mild with some fluctuations in the patients' platelet counts, showing no evidence for improvement or worsening of thrombocytopenia over time [48,50]. Platelets are normal in size without any evident morphological defect. However, their ability to spread is impaired due to a reduced capacity to form filopodia and lamellipodia. Consistent with defects in reorganization of the cytoskeleton, megakaryocytes elongate few proplatelets, which have shorter length and decreased number of branches, explaining thrombocytopenia in patients [48,50].

The number of circulating CD34+ progenitor cells is increased in affected individuals, perhaps predisposing to hematological malignancies [48]. Moreover, considering that somatic alterations of the ETV6 locus are associated with hematological neoplasms [42,49], it is not surprising that in the ETV6-RT patients are at risk of hematological malignancies, mainly acute lymphoblastic leukemia in childhood but also myelodysplastic syndromes, acute lyeloid leukemia, chronic myeolomonocytic leukemia, and multiple myeloma. The frequency varies in the different cohorts, being 39% in the first reports, where the recruitment criteria of families were mainly based on occurrence of hematological neoplasms [45-47,49]. Instead, when the molecular genetic testing was carried out in families with IT of unknown origin, the incidence of hematological neoplasms ranges from 13 to 20% [48,50].

Considering their effect on protein function, the ETV6 mutations prevent the protein from localizing in the nucleus with a significant

reduction of the repression activity of the transcriptional factor [45-48]. Although a dominant negative effect has been hypothesizes, the pathogenic mechanisms involved in ETV6-RT have yet to be characterized. ETV6 plays a fundamental role in hematopoiesis as investigated more accurately in mice. Consistent with a dominant negative effect of the ETV6 mutations, heterozygous Etv6+/- animals are phenotypically normal [51]. Loss of Etv6 function (Etv6-/-) results in early embryonic lethality, which is not associated with defective differentiation and maturation of the myelo/erythroid cell lineages [52]. Etv6 is instead essential during transition of hematopoiesis from the fetal liver to the bone marrow and in definitive hematopoiesis for megakaryocytes maturation [53]. This role is confirmed in human hematopoiesis, where ETV6 is likely to control transcription of genes critical for this process [46,48,50].

How to Recognize Patients with IT and Predisposition to Leukemia

Considering that mutations of RUNX1, ANKRD26, and ETV6 are responsible for thrombocytopenia associated with predisposition to hematological malignancies, recognition of these disorders is of fundamental importance for genetic counseling, clinical follow-up, and appropriate treatment. However, they are not easily recognizable due to lack of pathognomonic signs. For this reason, like other forms of ITs, ANKRD26-RT are often initially misdiagnosed with immune thrombocytopenia, and affected individuals received steroids and underwent splenectomy without producing any increase in platelet count.

In FPD/AML, ANKRD26-RT, and ETV6-RT, thrombocytopenia is not always present in patients, and there is no specific feature of platelets and megakaryocytes that can help in differential diagnosis. There are a few exceptions, that is the presence of particulate cytoplasmic structures in ANKRD26-RT, persistence of MYH10 expression in FPD/AML platelets, increased expression of ANKRD26 in ANKRD26-RT and FPD/AML patients [8,27,33]. Although these biomarkers could be useful for diagnosis, their clinical relevance has not been ascertained in a larger scale and their investigations can be performed only in specialized laboratories.

However, FPD/AML, ANKRD26-RT, and ETV6-RT are characterized by normal platelet size, which is a relatively uncommon finding in ITs considering that the majority of the diseases are characterized by large or giants platelets [54]. 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. This task can be easily addressed by targeted next generation sequencing approaches, which allow the screening of different genes in a single reaction. Despite these innovative techniques, identification of genetic variants does not necessarily confirm a diagnosis, as when affected individuals harbor novel amino acid substitutions of uncertain pathogenic significance. In these cases, the biomarkers mentioned above, as well as functional studies aimed at determining the effect of variants on protein function, would be useful in the diagnostic process.

Considering the high risk of developing malignancy, identification of RUNX1, ANKRD26, and ETV6 mutations is essential when a family member is considered as a donor for hematopoietic stem cell transplantation (HSCT). Indeed, if a disorder predisposing to hematological malignancies is not recognized, family members carrying mutations could be chosen for HSCT [12], leading to insufficient engraftment, poor immune reconstitution, and development of donor-derived malignancies in recipients, as well as hematological malignancies in the donor after hematopoietic stem cell mobilization [55,56].

Conclusions

FPD/AML, ANKRD26-RT, and ETV6-RT are three rare diseases recently recognized as myeloid neoplasms with germ line predisposition and preexisting platelet disorders [3]. They are poorly characterized ITs for different aspects. Due to their rarity, they are not well characterized at the clinical and molecular levels. For this reason, extensive genetic screening of RUNX1, ANKRD26 and ETV6 in individuals with thrombocytopenia and/or hematological malignancies combined with accurate collection of clinical data at diagnosis and during follow-up would be useful to improve our knowledge of these forms of ITs. Moreover, the pathogenic mechanisms responsible for thrombocytopenia are not completely clear and those involved in neoplastic evolution remain obscure. However, considering that RUNX1 controls the expression level of AKKRD26, FPD/AML and ANKRD26-RT are interconnected in a pathway. It would be interesting to determine whether even ETV6 belongs to the same pathway controlling directly or indirectly transcription of the ANKRD26 gene in hematopoietic cells.

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