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Complications of whole-exome sequencing for causal gene discovery in primary platelet secretion defects

Marcin M. Gorski,[1,2] Anna Lecchi,[1] Eti A. Femia,[1] Silvia La Marca,[1] Andrea Cairo,[1] Emanuela Pappalardo,[1,2] Luca A. Lotta,[1] Andrea Artoni[1] and Flora Peyvandi[1,2]

[1]Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Angelo Bianchi Bonomi Hemophilia and Thrombosis Center, Milan and [2]Università degli Studi di Milano, Department of Pathophysiology and Transplantation and Fondazione Luigi Villa, Milan, Italy

ABSTRACT

Primary platelet secretion defects constitute a heterogeneous group of functional defects characterized by reduced platelet granule secretion upon stimulation by different agonists. The clinical and laboratory heterogeneity of primary platelet secretion defects warrants a tailored approach. We performed a pilot study in order to develop DNA sequence analysis pipelines for gene discovery and to create a list of candidate causal genes for platelet secretion defects. Whole-exome sequencing analysis of 14 unrelated Italian patients with primary secretion defects and 16 controls was performed on Illumina HiSeq. Variant prioritization was carried out using two filtering approaches: identification of rare, potentially damaging variants in platelet candidate genes or by selecting singletons. To corroborate the results, exome sequencing was applied in a family in which platelet secretion defects and a bleeding diathesis were present. Platelet candidate gene analysis revealed gene defects in 10/14 patients, which included *ADRA2A, ARHGAP1, DIAPH1, EXOC1, FCGR2A, ITPR1, LTBP1, PTPN7, PTPN12, PRKACG, PRKCD, RAP1GAP, STXBP5L*, and *VWF*. The analysis of singletons identified additional gene defects in *PLG* and *PHACTR2* in two other patients. The family analysis confirmed a missense variant p.D1144N in the *STXBP5L* gene and p.P83H in the *KCNMB3* gene as potentially causal. In summary, exome sequencing revealed potential causal variants in 12 of 14 patients with primary platelet secretion defects, highlighting the limitations of the genomic approaches for causal gene identification in this heterogeneous clinical and laboratory phenotype.

Correspondence:

FLORA PEYVANDI
flora.peyvandi@unimi.it

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Introduction

Disorders of platelet function are characterized by highly variable mucocutaneous bleeding manifestations and excessive hemorrhage following surgical procedures or trauma.[1-4] Primary platelet secretion defects (PSD) are the most common platelet functional defects[5] and display both clinical and laboratory heterogeneity.[6] From a clinical standpoint, PSD may be associated with a mild to severe bleeding tendency.[7] Thus, given the heterogeneous nature of PSD, laboratory testing is limited to specialized laboratories and accurate mechanistic diagnosis remains challenging.

Platelet aggregation and secretion studies with lumi-aggregometry, in which dense granule secretion is assessed in parallel with traditional light transmission aggregometry, provide evidence for platelet dysfunction.[8,9] PSD is characterized by reduced or absent δ-granule secretion upon stimulation by one or more platelet aggregation agonists either at low or high doses.[8,9] However, lumi-aggregometry, the gold standard technique for platelet function studies, is not always predictive of the molecular mechanisms, rendering the mechanistic differentiation of primary PSD difficult.

Multiple inherited alterations of platelet function have been described, including forms with different patterns of inheritance.[2,4,10] When the laboratory phenotype is

not discriminatory, genotyping using next-generation DNA sequencing (NGS) could be a comprehensive and cost-effective strategy for the diagnosis of platelet function disorders.[11-13] Indeed, NGS-based approaches, based on whole-exome sequencing (WES) or custom gene panels, proved to be successful for the diagnosis of inherited platelet defects.[11,13,14] Leo *et al.* applied WES to study 329 candidate genes involved in platelet function defects and identified gene variants in patients with defects in Gi signaling and with platelet secretion abnormalities.[15] WES was also successful in identifying causal mutations in the *RASGRP2* gene, which encodes a protein required for signaling and platelet activation,[16,17] and in identifying a causal mutation displaying autosomal dominant inheritance located in the *THBD* gene.[18] However, a standardized pipeline or procedure linking the identified gene defects to the specific sub-phenotype of diverse platelet function disorders is still lacking.

Given the positive experience acquired with the use of WES in identifying potentially pathogenic genetic variants in platelet function defects, the use of NGS-based diagnostics provides a great opportunity to improve causal gene identification and understand the underlying clinical phenotype.[19-22] For this reason, we decided to apply exome sequencing in a well-characterized group of patients with primary PSD and clinically relevant bleeding.[5] The aim of our pilot study was to test whether WES could be an adequate diagnostic tool for causal gene discovery in a heterogeneous group of platelet function defects such as primary PSD.

Methods

Study population

Fourteen unrelated patients with a diagnosis of primary PSD were enrolled from among 360 individuals with suspected platelet function disorders referred to our outpatient clinic at Ospedale Maggiore Policlinico (Milan, Italy).

The patients' inclusion criteria were: (i) European ancestry; (ii) platelet count >120x10^9/L; (iii) impaired platelet ATP secretion after stimulation with two or more agonists measured by lumi-aggregometry; (iv) normal expression of platelet glycoprotein (GP) Ib/IX/V and GPIIb/IIIa to exclude Bernard-Soulier syndrome and Glanzmann thrombasthenia; (v) absence of any other known platelet disorder; and (vi) absence of von Willebrand disease. Four family members of one patient (C740) were also included and studied.

All studied subjects abstained from taking drugs that affect platelet function for 2 weeks before blood sampling. All platelet function results were compared with our internal normal ranges.

The study was approved by the local Ethical Committee of the Ospedale Maggiore Policlinico and carried out according to the Declaration of Helsinki. All participants signed informed consent.

Platelet phenotyping

Personal and family histories and results of blood tests including a complete blood count, prothrombin time and activated partial thromboplastin time determined by standard methods, von Willebrand factor antigen and von Willebrand factor ristocetin cofactor determined by an automated latex enhanced immunoassay (Instrumentation Laboratory, Milan, Italy)[23] were collected (*Online Supplementary Methods*). The bleeding severity score (BSS) was calculated for each patient according to Tosetto *et al.*[24] (normal values: children <2; men <5; women <6).

Blood samples were drawn into trisodium citrate for coagulation, von Willebrand factor measurement, and platelet function studies and into K–EDTA for DNA extraction[25] and blood cell counts.

Platelet aggregation and ATP secretion induced by ADP (4 and 20 μM), collagen (2 μg/mL), thrombin receptor activator peptide-14 (10 μM), and thromboxane A2 analog U46619 (1 μM) were measured in platelet-rich plasma by lumi-aggregometry (Chronolog 560, Mascia Brunelli, Milan, Italy).[26] Platelet-rich plasma was prepared as previously reported.[27] Intraplatelet ADP, ATP, serotonin, and fibrinogen content were measured as previously reported[28,29] (*Online Supplementary Methods*).

Whole-exome sequencing

Individual exomes were enriched using a SeqCap EZ Human Exome Library Kit v2.0 (Roche NimbleGen) and paired-end sequencing was carried out on the HiSeq2000 (Illumina, San Diego, CA, USA) at the Beijing Genomics Institute (www.bgi.com).

The Short Oligonucleotide Analysis Package aligner (soap2.21)[30] was used to align reads to the reference human genome (hg19/GRCh37) and produce individual binary alignment map (BAM) files. The Genome Analysis Tool Kit was used for quality recalibration, duplicate read marking, insertions/deletions (indels) realignment, and BAM sorting to produce a merged, sample-level variant calling file (VCF) (*Online Supplementary Methods*).

Variant filtering and candidate gene discovery

Variant filtering and candidate gene discovery were performed on the project level, merged VCF file containing 14 unrelated Italian PSD patients and 16 healthy controls by using two different filtering strategies: selection of singletons and filtering for the single nucleotide variants (SNV) reported by Leo *et al.*[15] (*Online Supplementary Methods*).

Variant pathogenicity was assigned according to the American College of Medical Genetics and Genomics (ACMG) pathogenicity classification.[31] Platelet gene expression was evaluated using the Human Proteome Map (HPM).[32] (*Online Supplementary Methods*).

Results

Clinical characteristics of patients with platelet secretion defects

Of 360 patients with suspected platelet disorders investigated at our center, 14 unrelated patients (12 females and 2 males; median age 23 years) fulfilled the study inclusion criteria (Table 1). The patients' BSS ranged between 0 and 15 and 64% of the cases resulted abnormal (Table 1). Prothrombin time, activated partial thromboplastin time, plasma fibrinogen, and von Willebrand factor levels were within the normal ranges (*data not shown*). Platelet count was normal in all PSD patients (median 258 x10^9/L, minimum-maximum 120-357; normal values 150-450), except for patient C749 who had a slightly low platelet count (120 x10^9/L).

Platelet functions studies

Platelet aggregation was lower than the normal range in the majority of the patients with all agonists tested (Figure 1A) and rapidly reversible in 60% of the cases when induced by ADP (4 μM). Platelet ATP secretion was absent after stimulation by ADP (4 μM) in all patients and lower than the normal range in response to the other agonists in the majority of cases (Figure 1B). In particular, platelet

secretion was impaired with two stimuli in 4/14 patients, with three stimuli in 4/14, and with more than three stimuli in 6/14 (Table 1). These findings confirmed the diagnosis of primary PSD in all patients.

The concentrations of total serotonin, ADP, and ATP were normal in all patients as was the ATP/ADP ratio, which is considered a diagnostic hallmark for δ-storage pool deficiency (*Online Supplementary Table S1*). Similarly, fibrinogen from platelet α-granules was normal. All together, these data excluded that the secretion defect of these patients was attributable to the presence of α- or δ-storage pool deficiency.

Exome sequencing and candidate gene discovery

NGS data analysis revealed 101,562 variants that passed quality control and were sequenced with an average read depth of 51 over each site. Of those, 96,432 were single SNV and 5,130 were indels. The number of singletons, defined as private variants occurring exclusively in a single individual, was 11,430 (mean, 762) in PSD cases and 23,564 (mean, 1,473) in controls. In addition, we identified 30,973 rare variants with a minor allele frequency (MAF) ≤1% and 11,187 of these variants were considered novel, i.e., not listed in the Database of Single Nucleotide Polymorphisms (dbSNP) or any other variant database.

Table 1. Clinical and biological characteristics of 14 unrelated patients with platelet secretion defects.

ID	Sex	Age	BSS	Parents consang.	First-grade family Bleeding history	Platelet secretion stimulus				
						ADP 4 μM	ADP 20 μM	Collagen 2 μg/mL	U46619 1 μM	TRAP 10 μM
C696	F	34	4	no	No	+	-	-	+	-
C708	F	36	5	no	Mother (mild PSD)	+	+	+	+	-
C729	F	3	2	no	No	+	+	-	-	n/p
C732	F	25	15	no	No	+	+	-	+	-
C739	M	5	0	no	Mother	+	+	-	-	n/p
C740	M	19	10	no	Father (mild PSD), Sister (mild bleeding diathesis without PSD)	+	+	+	+	+
C749	F	55	9	no	Mother and sister with thrombocytopenia	+	+	+	-	+
C783	F	63	13	no	Mother	+	+	-	-	-
C797	F	31	5	no	No	+	+	-	+	-
C831	F	20	7	no	Mother	+	-	+	-	+
C847	F	19	7	no	Mother	+	+	-	+	+
C862	F	3	7	no	No	+	+	-	+	n/p
C1075	F	52	15	no	Brother	+	+	+	-	+
C1107	F	60	8	no	No	+	+	-	+	+

ID: patient identity; BSS: bleeding severity score; U46619: a thromboxane A2 analog; TRAP: thrombin receptor activator peptide. F: female; M: male; PSD: platelet secretion defect; (+) indicates defective platelet secretion response to the stimulus; (-) indicates response within the normal range; n/p: data not present.

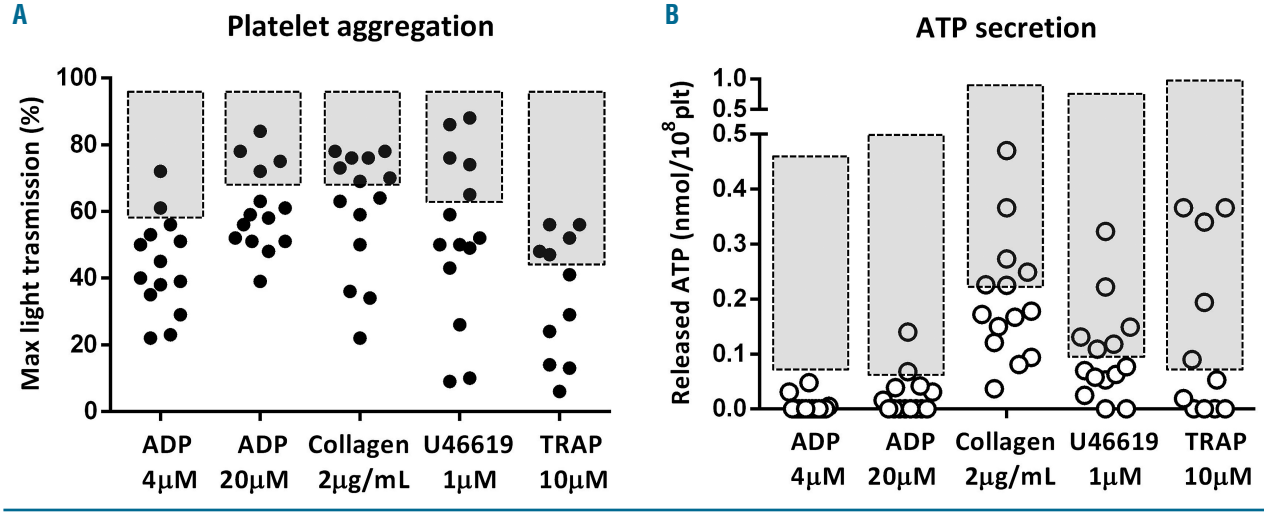

Figure 1. Platelet aggregation and secretion of 14 unrelated patients with platelet secretion defects. Dot plots of platelet (A) aggregation and (B) secretion. Boxes indicate our internal range of normality (5th-95th percentiles). U46619: thromboxane A2 analog; TRAP: thrombin receptor activator peptide.

Platelet candidate gene filtering approaches

Candidate gene discovery was carried out by two independent filtering approaches: by identification of variants in platelet candidate genes and by selecting singletons (*Online Supplementary Figure S1*). In the former approach, we selected from PSD patients all rare, potentially deleterious variants located in the coding regions of 329 candidate platelet genes listed by Leo *et al.*[15] This prioritizing strategy revealed 37 gene defects, of which six were novel (*Online Supplementary Table S2*). Since this variant prioritizing strategy yielded multiple SNV for the following patients, C729 (5 SNV), C732 (4 SNV), C739 (4 SNV), C740 (7 SNV), and C831 (4 SNV), we used the ACMG variant pathogenicity classification,[31] which revealed 14 gene defects classified as variants of uncertain significance (VUS) in eight patients. To provide functional analysis of these genes, we assessed their expression patterns in platelets using the HPM, which integrates mass spectrometry analysis of different human tissues and cell types as part of the human proteome project.[32] This evaluation identified potential gene defects in seven PSD patients, with the genes involved being: *EXOC1* (C732), *DIAPH1* (C739), *STXBP5L* and *PRKACG* (C740), *PTPN12* (C749), *VWF* (C831), *PRKCD* (C1075), *PTPN7* and *PRKCD* (C1107).

Singleton filtering approach

Given that the first approach failed to identify gene defects in six patients, we decided to apply another filtering strategy based on the isolation of singletons. To this end, we selected from all 14 patients private variants, which were rare and possibly deleterious and we obtained 2,875 SNV in 2,162 genes. To prioritize these SNV for their putative role in PSD, we performed functional annotation using the Database for Annotation, Visualization and Integrated Discovery (DAVID).[33] Significantly associated Gene Ontology (GO) annotations were found for gene clusters in the following functional categories: biological process - extracellular matrix organization for 48 genes (P=2.1x10^{-7}, Bonferroni P=9.9x10^{-4}); cellular component - basal lamina containing 10 genes (P=5.7x10^{-6}, Bonferroni P=4.4x10^{-3}); molecular function - extracellular matrix structural constituent comprising 22 genes (P=5.6x10^{-6}, Bonferroni P=8.3x10^{-3}). In addition, Kyoto Encylopedia of Genes and Genomes (KEGG) pathway analysis (*www.genome.jp/kegg/pathway.html*) revealed once again a cluster of 26 genes with functional annotation associated with extracellular matrix-receptor interactions (P=2.9x10^{-6}, Bonferroni P=7.9x10^{-4}). The extracellular matrix functional category can be defined as any material produced by cells and secreted into the surrounding medium, includiing collagen, laminin, fibronectin proteins and glycosaminoglycans (*http://www.uniprot.org/keywords/?query=Extracellular%20matrix*), indicating that our prioritizing method had indeed identified genes potentially affected in PSD.

Functional overlap between the above-mentioned gene clusters was achieved by enriching for variants present in genes exhibiting GO terms such as platelets and secretion, platelets and granules, platelets and signaling.

In this way, we identified 70 potential gene defects, of which 68 were missense variants. We also found a STOP gain variant in the *PHF14* gene (c.G298T, p.E100X) in patient C749 and a frameshift deletion in the *TBXAS1* gene (c.151_152delGT, p.V51fs) present in patient C831. Importantly, all 37 missense variants identified by filtering for gene defects in platelet candidate genes were also found in the list of singletons, which together produced a list of 107 candidate gene defects presented in *Online Supplementary Table S2*.

Similar to the previous filtering strategy, the singleton approach revealed an excess of potential gene defects in several patients (*Online Supplementary Table S2*). To be able to assign causality, a further reduction in the number of SNV was necessary. To this end, we once again used the ACMG variant pathogenicity classification,[31] which resulted in the identification of 22 putative gene defects classified as VUS in ten patients with primary PSD. However, only 13 of these variants were located in genes expressed in human platelets according to the HPM[32] (Table 2). In summary, this variant prioritization approach provided candidate gene defects for four patients, C696, C708, C797 and C847, for whom the previous strategy was ineffective. It is interesting to note that several of these gene defects were missing from the list of Leo *et al.*,[15] indicating that these genomic loci could potentially become novel candidate genes associated with PSD.

Family analysis of patient C740

Only one notable pedigree, case C740, was investigated. The distribution of the PSD phenotype and BSS in his relatives are reported in Figure 2 (father C1300, mother C1301, and two sisters C1302 and 1304). WES was performed in all four individuals and the variant filtering steps were based on MAF ≤1%, selecting SNV with potentially damaging consequences and assuming disease transmission present in affected and absent in unaffected family members (*Online Supplementary Figure S2*). Upon classification according to the ACMG,[31] four SNV were confirmed in a heterozygous state in PSD-affected C740 and father C1300, suggesting an autosomal dominant transmission of the disease. Two of the SNV, p.D1144N in the *STXBP5L* gene and p.P83H in the *KCNMB3* gene, classified as VUS (Table 3) may be involved in the secretion process, thus being the most probable gene defects responsible for the PSD phenotype in this family.

Discussion

In this pilot study, we performed WES in 14 unrelated Italian patients diagnosed with primary PSD and 16 healthy controls. We selected a group with a common phenotype characterized by impaired platelet aggregation and secretion with two or more stimuli as assessed with lumi-aggregometer and a normal platelet content of the granules, confirming the diagnosis of PSD. In our previous study, we demonstrated that a PSD was present in almost one fifth of patients with a mild bleeding diathesis.[5]

To identify causal genes underlying these defects, we carried out two prioritizing approaches, which were based on the identification of rare, potentially deleterious variants present in 329 platelet candidate genes listed by Leo *et al.*[15] or by selecting singletons (*Online Supplementary Figure S1*). These strategies revealed a number of plausible candidate gene defects explaining the phenotypic defects of primary PSD. For instance, patient C740 carries a missense variant p.D1144N in the *STXBP5L* gene (Table 2). In a recent report, another missense variant was identified in this gene as being potentially causal in platelet secretion

abnormalities.[15] Since STXBP5, a paralog of STXBP5L, promotes platelet secretion,[34,35] perhaps STXBP5L may also play a role in this process. Another interesting candidate is the *KCNMB3* gene that carries the p.P83H missense variant. This gene encodes the Calcium-Activated Potassium Channel Subunit Beta-3 protein involved in a pathway activated in response to elevated platelet cytosolic Ca^{2+}.

For patient C732, a gene defect was found in *EXOC1*, which is another candidate gene that influences platelet granule exocytosis. This gene encodes the Exocyst Complex Component 1 protein that functions as part of the exocyst complex and is required for targeting exocytic vesicles to specific docking sites on the plasma membrane.[36]

We also found a missense variant, p.A464P, in the *RAP1GAP* gene in patient C831. This variant has been classified as likely benign and for this reason, it was excluded from Table 2. Importantly, the Rap1GAP protein plays a regulatory role in platelet aggregation,[37] suggesting that this missense variant may actually have a functional role.

As previously reported, PSD can be associated with proteins acting at different levels: signal transduction, platelet activation, degranulation, or exocytosis.[4] Indeed, we found potential gene defects in proteins involved in all of these processes (Table 2). Importantly, several patients in our study had multiple defects in the above-mentioned genes and gene pathways, which may explain the com-

Table 2. Putative causal variants identified by whole-exome sequencing in 12/14 patients with primary platelet secretion defects according to the classification of Leo *et al.*[15] or by selecting singletons (*Online Supplementary Figure S1*). All variants were heterozygous.

ID	Gene	Nucleotide change	dbSNP	Amino acid change	MAF 1000G	MAF ESP	MAF ExAC	SIFT	Poly phen2	Mutation Taster	CADD C score	Platelet expression (*)	Assess. (**)
C696	*COL24A1*	c.G4673A	-	p.G1558E	-	-	-	D	D	D	25	-	VUS
C708	*TTN*	c.G106955A	rs200497615	p.R35652Q	-	0.0007	0.0003	B	B	D	25	+	VUS
	CSRNP1	c.C673T	rs142034027	p.R225W	-	0.0007	0.0001	D	D	D	32	-	VUS
	NRP1	c.G620A	rs148308681	p.R207H	-	0.0001	0.0001	D	D	D	33	-	VUS
C729	*TTN*	c.C104564A	-	p.S34855Y	-	-	-	D	D	D	20	+	VUS
	ITGA2	c.G305A	rs41392746	p.S102N	-	-	3.01E-05	B	B	B	20	+	VUS
	MYO3A	c.T1525C	rs150793986	p.Y509H	-	0.0003	0.0002	D	D	D	27	-	VUS
	MUC2	c.G6931A	rs200823008	p.V2311I	-	0.0001	0.0008	-	-	-	-	-	VUS
C732	*EXOC1*	c.G2009A	rs35001804	p.G670E	0.003	0.0086	0.009	D	D	D	32	+	VUS
C739	*DIAPH1*	c.T3227G	rs143763573	p.F1076C	-	-	0.0001	D	D	D	26	+	VUS
	ITPR3	c.C5720T	-	p.T1907M	-	-	-	D	D	D	33	+	VUS
C740	*TTN*	c.C72358T	rs372309164	p.L24120F	-	0.0002	0	B	D	D	18	+	VUS
	TTN	c.G1895A	rs150231219	p.G632D	-	0.0002	0	D	B	B	19	+	VUS
	SLC2A7	c.C670T	rs35776221	p.R224C	0.006	0.01	0.008	D	D	D	27	-	VUS
	STXBP5L	c.G3430A	rs139176240	p.D1120N	-	0.0001	0.0001	B	D	D	25	+	VUS
	KCNMB3	c.C248A	rs61734056	p.P83H	-	1.50E-05	0.0001	D	D	D	27	-	VUS
	LCN1	c.G298C	rs117638349	p.G100R	0.006	0.008	0.004	D	D	B	23	-	VUS
	PRKACG	c.C280T	-	p.R94C	-	-	-	D	D	B	23	+	VUS
	MUC2	c.G2594A	-	p.S865N	-	-	-	-	-	-	-	-	VUS
	MUC2	c.A5038G	rs371137719	p.T1680A	0.01	0.0024	0	-	-	-	-	-	VUS
C749	*LYST*	c.G8806A	rs2753327	p.V2936I	0.001	0.0009	0.0009	B	B	D	22	-	VUS
	TTN	c.G49413T	rs202094100	p.W16471C	-	0.0008	0.0006	D	D	D	24	+	VUS
	PHF14	c.G298T	-	p.E100X	-	-	-	-	-	D	38	-	VUS
	PTPN12	c.C1066T	rs752211731	p.P356S	-	-	0	D	D	D	27	+	VUS
C797	*TTN*	c.C17T	rs201490999	p.P6L	-	-	-	D	D	D	24	+	VUS
	EGF	c.G3073A	-	p.A1025T	-	-	-	B	B	D	15	+	VUS
C831	*TTN*	c.T15768A	rs138826545	p.H5256Q	-	0.0002	0.0002	B	B	D	12	+	VUS
	EGF	c.G1723A	rs115396821	p.G575R	0.008	0.0024	0.0027	D	D	D	26	+	VUS
	TBXAS1	c.151_152del	-	p.V51fs	-	-	-	-	-	-	-	+	VUS
	VWF	c.G8171A	-	p.C2724Y	-	-	-	D	D	D	26	+	VUS
C847	*TTN*	c.C91384T	rs373623340	p.R30462W	-	-	3.01E-05	D	D	D	26	+	VUS
	PHACTR2	c.G1360C	-	p.D454H	-	-	-	D	D	D	26	+	VUS
	NOS3	c.C3385T	rs774447524	p.R1129C	-	-	2.31E-05	D	D	D	34	-	VUS
C1075	*PRKCD*	c.A1043G	rs33911937	p.N348S	-	0.0015	0.0016	B	B	D	15	+	VUS
C1107	*PTPN7*	c.G425A	rs115136927	p.R142Q	0.003	0.0072	0.0062	B	D	D	27	+	VUS
	PRKCD	c.G868T	-	p.A290S	-	-	-	B	D	D	25	+	VUS
	MMRN1	c.G3680T	rs147451161	p.R1227L	0.003	0.0031	0.0036	D	D	D	28	+	VUS

dbSNP: Database of Single Nucleotide Polymorphisms v.138; MAF: minor allele frequency (MAF from European populations is shown); 1000G: the 1000 Genomes Project; ExAC: the Exome Aggregation Consortium; ESP: the Exome Sequencing Project; SIFT: Sorting Intolerant From Tolerant; PolyPhen2: Polymorphism Phenotyping v.2; Mutation Taster, prediction scores, D: damaging; B: benign; CADD C score: Combined Annotation Dependent Depletion score;[41] VUS: variant of uncertain significance. (*) Platelet gene expression evaluated by the Human Proteome Map (HPM) (*http://www.humanproteomemap.org*);[32] (**) Assess. – Assessment of variant pathogenicity assigned according to the American College of Medical Genetics and Genomics pathogenicity classification.[31]

Table 3. Putative causal variants identified by whole-exome sequencing in the family of patient C740 (*Online Supplementary Figure S2*).

Gene	dbSNP	Nucl. change	Amino acid change	C740	C1300	C1301	C1302	C1304	MAF 1000G	MAF ExAC	MAF ESP	SIFT	Poly phen2	Mutation Taster	CADD C score	Plt Exp. (*)	Assess (**)
SLC2A7	rs35776221	c.C670T	p.R224C	het	het	-	-	-	0.006	0.01	0.008	D	D	D	27	-	VUS
STXBP5L	rs139176240	c.G3430A	p.D1144N	het	het	-	-	-	-	0.0004	0.0001	B	D	D	25	+	VUS
KCNMB3	rs61734056	c.C248A	p.P83H	het	het	-	-	-	-	1.50E-05	0.0001	D	D	D	27	-	VUS
LCN1	rs117638349	c.G298C	p.G100R	het	het	-	-	-	0.006	0.008	0.004	D	D	B	23	-	VUS

dbSNP: Database of Single Nucleotide Polymorphisms v.138; MAF: minor allele frequency (MAF from European populations is shown); 1000G: the 1000 Genomes Project; ExAC: the Exome Aggregation Consortium; ESP: the Exome Sequencing Project; SIFT: Sorting Intolerant From Tolerant; PolyPhen2: Polymorphism Phenotyping v.2; Mutation Taster, prediction scores, D: damaging; B: benign; CADD C score: Combined Annotation Dependent Depletion score;[41] VUS: variant of uncertain significance. (*) Platelet gene expression evaluated by the Human Proteome Map (HPM) (*http://www.humanproteomemap.org*);[32] (**) Assess. – Assessment of variant pathogenicity assigned according to the American College of Medical Genetics and Genomics pathogenicity classification.[31]

plex and heterogeneous nature of primary PSD. This indicates that an in-depth functional analysis of platelet receptor and signaling pathways will be necessary to discriminate differences in clinical and laboratory phenotypes of affected individuals.

Study limitations

Following a positive experience with the application of WES to identify gene defects underlying inherited platelet function disorders,[19-22] we chose to investigate primary PSD using the same technique, hoping that a genomic approach could be effective in identifying causal variants in a heterogeneous clinical and phenotype such as primary PSD. However, exome sequencing followed by two independent variant prioritization approaches yielded inconclusive results. The primary reason for this is undoubtedly the heterogeneous clinical and laboratory phenotype of primary PSD, which may have led to the identification of genes not necessarily associated with the disease. For instance, 20 missense variants were detected in the *TTN* gene in 11 PSD patients, of which eight are VUS. However, *TTN* is one of the most frequently mutated genes in the human genome,[38] implying that the variations found in this gene are probably due to the size of its coding regions (363 exons).

Another limitation of this study was perhaps the choice of the variant prioritization strategy. We applied a generally accepted filtering method based on the selection of rare (MAF ≤1%), potentially damaging variants. This approach revealed a great abundance of variants for most patients, which required further selection based on the ACMG pathogenic classification of SNV (Table 2). This revealed 34 putative gene defects classified as VUS in 12 patients with primary PSD, of which 24 were located in genes expressed in human platelets according to the HPM (Table 2). However, it is possible that many potentially causal SNV, which were classified as likely benign or benign, were excluded due to lack of supporting evidence or because the gene defects may only manifest at the level of megakaryocyte development or platelet maturation.

In addition, some of the functional defects might have been located in the non-coding parts of the genome such as promoters, intronic sequences or enhancers, which were not covered by exome sequencing. Finally, since the identification of gross chromosomal aberration such as copy number variations from the WES data remains a technical challenge, it is likely that these structural variants would not have been detected. Although several bioinformatics methods have been developed for copy number variation analysis from WES data, they require uniform coverage and high resolution of the sequencing data across all exons/coding regions as well as a specialized bioinformatics pipeline of data analysis validated against the whole-genome data.[39] For this reason, whole-genome sequencing is the only sure means for identifying the copy number variations alongside SNV and small indels.

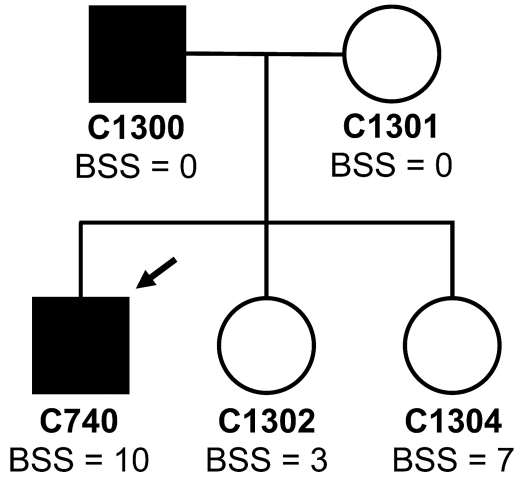

Figure 2. Pedigree of patient C740. Black and white symbols indicate subjects affected by platelet secretion defects and unaffected family members, respectively. The arrow indicates the proband C740. BSS: bleeding severity score.

In conclusion, we carried out exome sequencing in 14 patients with primary PSD and 16 healthy controls, followed by two variant prioritization strategies. Our analysis identified potential gene defects in 12 patients, implying that the NGS-based diagnostic strategies for causal gene identification in such a heterogeneous clinical and laboratory phenotype as primary PSD may be ineffective. In this case, a well-defined, common disease phenotyping and properly established pipeline for variant analysis are necessary. The difficulty in assigning causality can be overcome by genetic screening of affected and unaffected family members, which allows the identification of gene defects that segregate with the clinical phenotype, or by functional studies.

The perils of genetic data sharing with patients may involve ethical concerns, lack of confidence in assessing the causality of identified variants, and the implication of some inherited platelet pathologies with other risks.[40] For these reasons, sharing genetic data with patients is still an open issue that requires further discussion.

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