

Phenotypic approaches to gene mapping in platelet function disorders

Identification of new variant of P2Y₁₂, TxA₂ and GPVI receptors

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Keywords

Platelet dysfunction

Summary

Platelet number or function disorders cause a range of bleeding symptoms from mild to severe. Patients with platelet dysfunction but normal platelet number are the most prevalent and typically have mild bleeding symptoms. The study of this group of patients is particularly difficult because of the lack of a gold-standard test of platelet function and the variable penetrance of the bleeding phenotype among affected individuals.

The purpose of this short review is to discuss the way in which this group of patients can be investigated through platelet phenotyping in combination with targeted gene sequencing. This approach has been used recently to identify patients with mutations in key platelet activation receptors, namely those for ADP, col-

lagen and thromboxane A₂ (TxA₂). One interesting finding from this work is that for some patients, mild bleeding is associated with heterozygous mutations in platelet proteins that are co-inherited with other genetic disorders of haemostasis such as type 1 von Willebrand's disease. Thus, the phenotype of mild bleeding may be multifactorial in some patients and may be considered to be a complex trait.

Schlüsselwörter

Thrombozytendysfunktion

Zusammenfassung

Störungen der Thrombozytenzahl oder -funktion führen zu einer Reihe von leichten bis schweren Blutungssymptomen. Patienten mit Thrombozytendysfunktion, aber normaler Thrombozytenzahl zeigen die höchste Prävalenz, haben aber üblicherweise nur leichte Blu-

tungen. Die Untersuchung dieser Patientengruppe gestaltet sich besonders schwierig, da kein Goldstandard-Test für die Thrombozytenfunktion existiert und die Penetranz des Blutungsphänotyps bei den Betroffenen individuell variiert.

Mit dieser kurzen Übersicht soll dargestellt werden, wie diese Patientengruppe mittels Thrombozytenphänotypisierung in Kombination mit der Sequenzierung von Zielgenen untersucht werden kann. Dieser Ansatz wurde kürzlich zur Identifizierung von Patienten genutzt, die Mutationen in Schlüsselrezeptoren der Thrombozytenaktivierung aufweisen, nämlich den Rezeptoren für ADP, Kollagen und Thromboxan A₂ (TxA₂). Ein interessantes Ergebnis ist, dass bei einigen Patienten leichte Blutungen mit heterozygoten Mutationen in Thrombozytenproteinen verbunden sind, die gemeinsam mit anderen genetisch bedingten Hämostasestörungen wie dem von-Willebrand-Jürgens-Syndrom Typ 1 vererbt werden. Folglich kann der Phänotyp einer leichten Blutung bei einigen Patienten multifaktoriell sein und als komplexes Merkmal angesehen werden.

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Phänotypische Ansätze zur Genkartierung bei Thrombozytenfunktionsstörungen – Identifizierung neuer Varianten von P2Y₁₂-, TxA₂- und GPVI-Rezeptoren

Hämostaseologie 2010; 30: 29–38

For almost 100 years, the study of bleeding disorders has provided an unprecedented insight into the pathways that regulate platelet function. The recognition and functional characterisation of the major bleeding disorders, Glanzmann's thrombasthenia (GT) and Bernard Soulier syndrome (BSS), was pivotal in the identification of

integrin α IIb β 3 and GPIb-IX-V, respectively. An antiserum from a patient with immune thrombocytopenia and defective collagen aggregation provided the first evidence for GPVI as a collagen receptor (1) and was later used to demonstrate the association between GPVI and the FcR γ -chain (2). Even relatively recently, cloning

of the ADP receptor, P2Y₁₂, was reported alongside a patient with a history of bleeding and a naturally occurring mutation in the P2Y₁₂ gene (3).

Despite these advances, the repertoire of platelet function disorders for which a genetic cause has been identified is surprisingly small. Therefore, patients with this com-

mon group of disorders represent an under-exploited resource that offer to significantly advance understanding of platelet haemostatic function.

This review describes a systematic approach to gene mapping in platelet function disorders that first requires a detailed evaluation of the clinical and laboratory phenotype of affected individuals and then targeted gene sequencing. In some circumstances, clinical data alone can be used to select candidate genes, particularly if platelet dysfunction is part of a wider syndromic disorder. However, when bleeding from platelet dysfunction is the only abnormal clinical feature detailed phenotypic analysis of platelets is first required before selecting candidate genes. Plausible nucleotide variations identified by direct sequencing of these genes are then investigated using *ex vivo* expression studies. We summarise recent successes in mapping gene defects in receptors for collagen, ADP and TxA₂ which have been identified by this phenotype-directed approach (► Tab. 1).

Human genetic variation as a tool to investigate platelet physiology

The fundamental principle that underlies the study of genetic variation in human platelet function disorders is that nucleotide variations identified in platelet genes can be used confidently to predict changes in expression level or structure of encoded proteins. The functional consequences of a predicted change in a platelet protein can then be investigated with specific tests of protein function in platelets or in *ex vivo* models in which the variant protein is expressed in heterologous cells. Naturally occurring nucleotide variations may therefore enable the identification of new proteins not previously known to regulate human platelet function. This approach can also yield insights into which protein domains and amino acid residues are essential for protein function. Genetic platelet function disorders therefore enable correlation of the structure and function of

platelet proteins and compliment techniques such as *ex vivo* mutagenesis and generation of transgenic animal models.

A further critical advantage of studying platelet function disorders is that affected individuals only undergo study because they have a clinical bleeding phenotype. This means that when a causative nucleotide variation is identified, it is likely that the protein affected by the variation is essential for normal platelet haemostatic function.

This pre-selection of functionally important platelet proteins avoids unnecessary analysis of platelet proteins in redundant pathways that have less direct relevance in platelet physiology.

Gene mapping of platelet function disorders may be informative if the causative nucleotide variation prevents expression of a platelet protein. In this circumstance, the function of the absent protein can be inferred directly from identifying the functional deficit in platelets in the laboratory.

receptor	description			reference
	heredity	location	functional effect in platelets	
collagen receptor (GPVI)	compound heterozygous	R38C substitution in 1 st Ig domain	reduced ligand binding	(54)
		5 bp insertion in e4	absent expression	
	compound heterozygous	S175N substitution in 2 nd Ig domain	reduced membrane expression	(55)
		16 bp deletion in e3	absent expression	
ADP receptor (P2Y ₁₂)	homozygous	2 bp deletion in e2	absent expression	(47)
	homozygous	M1R substitution in e2	absent expression	(48)
	homozygous	1 bp deletion in e2	absent expression	(40)
	? compound heterozygous	2 bp deletion in e2	absent expression	(3)
		second 'null' mutation not identified		
	compound heterozygous	R256Q substitution in 6 th TM loop, R265W substitution in 3 rd EC loop	normal expression and ligand binding but reduced receptor function	(49)
	heterozygous	P258T substitution in 3 rd EC loop	reduced P2Y ₁₂ receptor function	(50)
	heterozygous	K174E substitution in 2 nd EC loop	reduced ligand binding	(44)
heterozygous	P341A substitution in PDZ binding domain	abnormal receptor trafficking		
TxA ₂ receptor (TP)	homozygous	R60L substitution in 1 st IC loop	reduced receptor coupling	(51)
	heterozygous	D304N substitution in 7 th TM loop	reduced ligand binding	(41)

Ig: immunoglobulin; e: exon; TM: transmembrane; EC: extracellular; IC: intracellular

Tab. 1

Mutations in platelet receptors for collagen (GPVI), ADP (P2Y₁₂) and thromboxane A₂

Alternatively, some nucleotide variations do not prevent expression but instead, alter platelet protein function. These protein variants may be highly informative because they enable fine mapping of individual platelet protein domains or interaction sites. These principles are illustrated by the severe bleeding disorder GT in which platelets show defective fibrinogen-mediated aggregation and activation. GT is associated with failure of expression of functional platelet integrin α IIb β 3 and is caused by nucleotide variations in *ITGA2* and *ITGB3* that encode the α IIb- and β 3-integrin subunits, respectively (4). GT was initially associated with *ITGA2* and *ITGB3* nonsense, frameshift and splice site mutations that cause absent or reduced expression of the α IIb or β 3 subunits. These observations confirmed the essential role of integrin α IIb β 3 in platelet haemostatic function but were not informative about the domains within the integrin that mediate this function. A spectrum of point mutations in *ITGA2* and *ITGB3* have now been identified in patients with a GT phenotype but who express variant α IIb- or β 3-subunits that are dysfunctional. Systematic analyses of these naturally occurring variants have enabled functional mapping of the structural motifs within the integrin α IIb β 3 complex that bind the major integrin ligands such as fibrinogen and those that mediate activation and signalling events (5). More recently, a novel *ITGA2* mutation has been identified in a kindred with large platelets and thrombocytopenia but without significant bleeding (6). This mutation causes expression of a variant α IIb subunit that is constitutively active indicating a previously unsuspected function of the integrin α IIb β 3 in regulating human platelet formation in line with previous studies in mice (7).

The study of patients with platelet function disorders may often be the only valid approach for investigating structure-function relationships in platelet proteins. This is because techniques that enable the study of proteins in nucleated cells using *ex vivo* mutagenesis are not directly applicable to proteins present in anucleate platelets. Although megakaryocytes are genetically tractable and platelets can be generated from these cells *ex vivo*, there are important

limitations in the numbers of platelets that can be produced in this way and the extent to which they are functionally equivalent to their *in vivo* counterparts. For this reason, genetically-modified mice are currently the model of choice for studying structure-function relationships of platelet proteins. This approach has greater validity because the major regulatory events underlying platelet activation are conserved with those in human platelets. However, the functions of specific proteins in mouse and human platelets can be distinct, especially where platelets express multiple protein members within the same family. For example, human platelets express two G protein-coupled receptors for thrombin, PAR1 and PAR4, with PAR1 being considered the predominant mediator of platelet activation. In mouse platelets, PAR3 and PAR4 are expressed, but only PAR4 is able to induce platelet activation. There are also more fundamental differences between the human and mouse genomes of relevance to platelet function. For example, the low affinity immune receptor Fc γ RIIA which is expressed on human platelets, is encoded by the *FCGR2A* gene, which is absent from the mouse genome.

Phenotype as a tool to select candidate genes

Advances in understanding of the transcriptomes of haemopoietic cells reveals surprising complexity in the array of genes expressed within each cell lineage. Recent estimates suggest that more than 10 000 transcripts are expressed in megakaryocytes (MK) from healthy donors including approximately 1000 that are not expressed in other haemopoietic cell lineages other than erythroblasts (8). While much of the MK transcriptome encodes regulators of cell cycle and proliferation, there remains a wide array of receptors, signalling mediators and regulators, synthetic enzymes and cytoskeletal proteins that are expressed in mature platelets and are plausible determinants of platelet haemostatic function. This is a highly significant observation from a functional viewpoint as only a minority of these proteins have been tested comprehensively in human platelets.

The large number of MK genes with protein products expressed in platelets raises significant practical barriers to determining the relationship between variation in individual genes and platelet function.

Correlation is hampered further by the background sequence variation in platelet genes caused by common polymorphisms, some of which themselves affect platelet function. One way of linking genotype and phenotype is through genome-wide association studies in which associations are sought between nucleotide variations at many different loci and platelet function traits. This approach has successfully identified several new proteins that were not previously recognised as regulators of platelet function (9). However, genome-wide association studies are limited by the requirement of large study cohorts to demonstrate associations between uncommon genetic variants and functional assay responses. This need for large cohorts in turn raises significant practical barriers to the range of platelet function tests that can be performed so evaluation of complex platelet phenotypes is not possible.

Our group has followed an alternative approach that enables genetic associations to be identified in individual families with platelet function disorders. This is achieved by first performing a systematic clinical evaluation followed by screening laboratory tests so that only subjects with bleeding disorders likely to originate from platelet dysfunction undergo further study. This 'pre-selection' of patients provides a highly enriched study population with genetic platelet function disorders which then undergo detailed phenotypic study using a standardised panel of validated laboratory reference tests. For many patients, the functional defect can be assigned to a particular platelet pathway (e.g. thromboxane A₂ synthesis defect) that contains small numbers of platelet proteins. This reduces significantly the number of genes in which to search for candidate mutations and may even enable single candidate genes to be sequenced if the laboratory phenotype is sufficiently specific. We now describe in greater detail the approach to clinical evaluation, screening tests and detailed platelet phenotyping that has enabled targeted

gene mapping in our laboratories and others.

Identifying patients with platelet function disorders

Platelet function disorders are associated with bleeding symptoms that range from a mild tendency for mucocutaneous bleeding to a major bleeding diathesis characterised by recurrent spontaneous visceral and central nervous system bleeding. Bleeding symptoms are usually indistinguishable clinically from a number of other disorders of primary haemostasis such as von Willebrand disease (VWD) and separating pathological mild mucocutaneous bleeding from normal variation in bleeding tendency may be difficult. Genetic platelet function disorders should also be distinguished from acquired platelet disorders which are highly prevalent in the general population and are related to a range of co-morbid disorders or therapeutic drugs (10, 11). The accurate identification of patients with genetic platelet function disorders therefore requires a systematic clinical assessment to determine whether there is an abnormality in primary haemostasis. Screening laboratory investigations are then required to exclude other causes of abnormal primary haemostasis, most notably coagulation defects and VWD, and to demonstrate abnormality in platelet function (12).

Clinical history of abnormal primary haemostasis

An abnormal bleeding history is highly predictive of disorders of primary haemostasis, and may be evaluated using a standardised questionnaire that allows enumeration of bleeding symptoms at multiple sites into a single bleeding score (13, 14). Although this approach provides an objective assessment of the likelihood and severity of the primary haemostatic defect, it is important to recognize that an abnormal bleeding score may indicate a defect in platelets, coagulation factors or vessel wall, or a combination of these. Typical symptoms associated with platelet dysfunction include

- unexplained or extensive skin bruising
- prolonged epistaxis
- oral cavity bleeding that requires admission to hospital
- menorrhagia and post-partum bleeding
- surgical and traumatic bleeding.

Prolonged bleeding after invasive procedures or trauma is highly predictive of abnormal primary haemostasis particularly if severe enough to require blood transfusion or re-admission to hospital for medical management of bleeding (13). Abnormal bleeding after minimally invasive procedures has greater significance than after major procedures. It should be recognised that children with mild bleeding disorders may have few abnormal bleeding symptoms because they have not yet experienced the haemostatic challenge of an invasive surgical procedure (15). In contrast, women with bleeding disorders are more readily identified because of heavy menstrual bleeding and obstetric haemorrhage (13). Chronicity of abnormal bleeding or a family history of abnormal bleeding supports a diagnosis of a genetic defect. In contrast, a history of a co-morbid disorder or exposure to some prescription or non-prescription drugs that coincides with the development of bleeding symptoms is suggestive of an acquired disorder.

Severe bleeding disorders, including the platelet function disorders GT and BSS, typically present in the neonatal period with events such as intracranial or subdural bleeding or with prolonged bleeding from the umbilical stump or after circumcision or vaccination. In infancy there may be easy bruising after handling and minor trauma. Epistaxis, post-procedural bleeding, menorrhagia and obstetric bleeding are commonplace in GT and BSS and may be life threatening (16, 17).

Platelet dysfunction in syndromic genetic disorders

In some circumstances, platelet dysfunction may be part of a more complex phenotype as part of multi-system genetic disease in which the main clinical manifestations are in other tissues. This is unsurprising since many proteins that regulate platelet

function are expressed widely in haemopoietic cells and in other tissues. This means that a single gene defect may have multiple manifestations in any of the tissues in which the gene product serves an essential function. This is illustrated by the autosomal recessive disorder Hermansky Pudlak Syndrome (HPS) which is caused by defective trafficking of a group of related organelles that include platelet dense granules, melanosomes and cytotoxic T cell and natural killer cells granules. These organelles share many common structural components which are defective in the eight human and fifteen mouse forms of HPS that have so far been identified (18). Accordingly, patients with HPS consistently exhibit platelet dysfunction arising from dense granule deficiency and various degrees of oculocutaneous albinism because of failure of melanocyte synthesis. In addition, there may be additional immunodeficiency, respiratory and gastro-intestinal manifestations depending on which HPS gene product is defective.

The association of a platelet function disorder with characteristic patterns of syndromic features can sometimes be used to refine the selection of candidate genes. This is illustrated by the identification of the eighth form of HPS in a kindred from the Birmingham area in the UK, who presented with the cutaneous and ocular manifestations of albinism (19). Interestingly, abnormal bleeding had only been recognized in two of the six family members with oculocutaneous albinism. However, the absence of any other clinical features or gene defects associated with known HPS variants suggested that this was a novel form of the disorder. Subsequent autozygosity mapping of affected family members demonstrated a defect in the *BLOC1S3*/reduced pigmentation gene not previously associated with HPS in humans. Another disorder in which platelet dysfunction is part of a wider phenotype is leukocyte adhesion deficiency III (LAD-III) in which mutations in *kindlin-3* underlie defective integrin activation in both leukocytes and platelets (20–23). Further examples include the Ca^{2+} entry channel, *Orai1*, which was first identified in a patient with an immunodeficiency syndrome (24) and was later shown to be the major Ca^{2+} entry channel

in platelets, albeit through use of transgenic mice (25). Our study of subjects with the immunodeficiency syndrome, X-linked agammaglobulinaemia, demonstrated a role for the tyrosine kinase Btk in the regulation of PLC γ 2 by collagen in platelets (26).

Screening for platelet function disorders

The initial laboratory investigation of patients with symptoms of abnormal primary haemostasis is aimed at identifying numerical platelet disorders, VWD and coagulation factor disorders which may also cause abnormal mucocutaneous bleeding (12, 27). These initial screening investigations are often performed at the same time as simple tests to evaluate platelet function such as the template bleeding time (BT) or measurement of closure times with adrenaline/collagen and ADP/collagen using the Platelet Function Analyser (PFA) 100.

However, the BT and PFA100 tests have low sensitivity for mild platelet function disorders. They may also give abnormal results in patients with VWD and thrombocytopenia and therefore have low specificity (28).

The BT test is now performed rarely to investigate abnormal bleeding and the role of the PFA100 as a screening tool for platelet function disorders remains controversial. The attributes of these tests have recently been reviewed elsewhere (29) and are not discussed further in this review.

The most widely used clinical assay to screen for platelet function disorders is Born aggregometry (30, 31) in which changes in light transmission are measured in platelet rich plasma (PRP) in response to different agonists which induce platelet activation and aggregation. This enables a simple quantification of platelet activation, usually expressed as the maximum amplitude (MA) of aggregation relative to light transmission of platelet poor plasma (designated as 100% transmission) and PRP (0% transmission). Born aggregometry also provides other parameters of platelet activation including

- lag phase before onset of aggregation
- platelet shape change
- aggregation that is
 - monophasic or biphasic and
 - sustained or transient.

In most clinical laboratories, a diagnosis of a platelet function disorder is made if there are sufficient qualitative differences in these features between PRP obtained from the patient and that obtained from a healthy donor control. Higher diagnostic accuracy may be achieved if laboratory reference intervals for the MA of the responses to each agonist concentration are first determined from a panel of healthy donors (32, 33). A platelet function disorder is highly likely if there is an abnormal degree of aggregation with two or more standard agonists (34, 35) although the optimum panel of agonists for this screening application of Born aggregometry remains controversial.

Although Born aggregometry continues to be regarded as the gold-standard assay to identify patients with platelet function disorders, there are still significant constraints to this approach. For example, the assay may be markedly affected by pre-analytical variables such as exposure of patients to non-prescription drugs and dietary factors that affect platelet function. The assay may be highly unreliable with some agonists if the test subject is thrombocytopenic and there is poor standardization in the practice of normalising platelet counts in PRP before analysis and in the choice of agonists and their concentrations (31). Finally, the objective comparison of aggregation parameters such as the MA to healthy donor reference ranges is not practiced widely and diagnostic criteria for most mild platelet function disorders are not established. There is also now evidence that Born aggregometry performed with 'standard' agonist concentration ranges may be insensitive to some defects in platelet secretion which can only be detected using tests of dense granule release such as luminometric assays of ATP release or Born aggregometry using an increased range of agonist concentrations (32–34).

Other platelet function testing platforms include flow cytometry which is used

widely to quantify platelet surface expression of the integrin α IIb β 3 and GPIb-IX-V to enable diagnosis of GT and BSS in children where the volume of blood may be limiting (36). Other flow cytometry assays (e. g. measurement of the α -granule protein, P-selectin) and techniques such as electrical impedance aggregometry, shear-dependent assays of platelet adhesion and visualisation of platelet ultrastructure using electron microscopy have an established role in platelet research laboratories (37, 38). However, these assays are not available widely in clinical platelet laboratories and it is uncertain whether they have any value in screening for platelet function disorders in patients presenting with abnormal primary haemostasis.

Gene mapping in mild platelet function disorders

We developed a systematic approach to gene mapping in platelet function disorders that utilized two different strategies to pre-select patients according to phenotype. In both groups, patients were first evaluated clinically by expert haemostasis clinicians and were evaluated further only if there was a clear history of abnormal primary haemostasis. One group of study subjects also underwent screening for platelet function disorders at their referring haemostasis centre and then underwent detailed laboratory evaluation in a single reference laboratory before sequencing of candidate genes. For the second group, who were recruited as part of the EU MCMDM-1VWD study, we sequenced platelet genes with a high likelihood of abnormality without prior examination of the platelet phenotype. If candidate mutations were identified in this group then the platelet phenotype was determined after a call-back of the affected patient. Both of these approaches yielded nucleotide variations that were highly informative about platelet proteins. The detailed methodology of these studies is described below.

Patients with a prior diagnosis of a platelet function disorder

We chose lumiaggregometry in PRP using the Chronolog lumiaggregometer as the platelet function test to determine the detailed platelet phenotype in our study subjects since this assay combines aggregometry and on-line monitoring of ATP release. Measurement of ATP secretion is an essential adjunct to measuring aggregation since it enables greater sensitivity for platelet dense granule disorders. We firstly established reference curves for platelet aggregation and ATP secretion from a range of healthy controls from the ethnically-rich population of 5.7 million covered by the Birmingham Hospitals and from control blood transported from other UK Haemophilia Centres including major collaborating sites in Bristol and Sheffield. These preliminary studies revealed a high level of reproducibility in platelet aggregation and secretion for up to six hours following preparation of PRP (33). We are aware that this time interval is longer than the 4 hours limit to test platelet reactivity recommended by the ISTH SSC Working Party on Platelet Aggregometry. However, in our laboratory this approach is highly reliable and enabled transport and processing of PRP in a central reference laboratory to ensure standardization of the assay. We also showed that it was not necessary to adjust the platelet concentration in PRP if the whole blood platelet count was within the normal population range of $150\text{--}500 \times 10^9$ platelets/l and that there are no major differences with age (range: 18–55 yrs) or gender of the donors. If the whole blood platelet count was below 150×10^9 /l, we noted diminished functional responses. This therefore defined the lower threshold limit at which we could guarantee reliability of the assay.

Working within these parameters, we generated a series of standard curves for aggregation and ATP secretion to nine platelet agonists both in the absence and the presence of antagonists of the $P2Y_1$ and $P2Y_{12}$ ADP receptors, and of indomethacin, which prevents TxA_2 formation. These were chosen because ADP and TxA_2 mediate major feedback pathways of platelet

activation (33). Several agonists in our panel are not used routinely in clinical platelet laboratories. These include PAR1 and PAR4 peptides, which in contrast to thrombin can be used to test citrated PRP, and collagen-related-peptide (CRP) which has greater specificity for defects in GPVI than collagen reagents which also binds integrin $\alpha 2\beta 1$ (39). These published curves and representative aggregation traces have been invaluable in the assessment of patient samples (33).

To date we have monitored aggregation and ATP secretion in PRP from over 90 patients with platelet function defects and have used the results of these assays to identify the likely defective pathway underlying platelet dysfunction in each case. We have also established a bio-bank of genomic DNA from this group thus providing an invaluable resource of linked functional and genetic data. For many patients, we have also performed additional functional tests such as flow cytometry or measurement of cyclic nucleotides to document in greater detail the platelet phenotype. The most common defects in our study cohort were

- abnormal dense granule secretion (~25%)
- impaired signalling by Gi-coupled receptors (~25%)
- defective activation by arachidonic acid (~20%).

Within this group, initial analysis has identified one patient who was homozygous for a frameshift mutation in the $P2Y_{12}$ receptor gene that prevented receptor expression (40) and a second with a heterozygous missense mutation in the TxA_2 receptor that abolished ligand binding (41). These abnormalities are discussed in further detail in the final section of this review.

EU MCMDM-1VWD study

We also investigated whether the variable penetrance of abnormal bleeding in patients with mild type 1 VWD was due to co-inheritance of platelet function disorders. The rationale for this hypothesis is that the symptoms of mild type 1 VWD and mild platelet function disorders are clinically in-

distinguishable and both disorders are prevalent in the general population. Co-inheritance of type 1 VWD and platelet function disorders is therefore unlikely to be a rare event.

VWD may be diagnosed if abnormal bleeding symptoms are accompanied by low plasma von Willebrand factor (VWF) activity. However, definitive diagnosis of mild type 1 VWD may be difficult in some patients because plasma VWF activity varies as much as 2–3 fold in the background population and may vary significantly within individuals in blood samples taken at different times. Plasma VWF activity is also modified by other genetically determined traits such as ABO blood group.

There is a considerable overlap in VWF activity between patients with mild VWD and unaffected controls and identification of patterns of inheritance within affected families is often complex.

As a proof-of-principle, we chose to sequence the *P2RY12* gene that encodes the $P2Y_{12}$ ADP receptor in patients with type 1 VWD. For this work, we studied a genomic DNA bio-bank from 148 index cases recruited through the EU MCMDM-1VWD study led by Drs Ian Peake and Anne Goodeve at the University of Sheffield and Professor Francesco Rodeghiero at the University of Vicenza. This detailed phenotype and genotype study of index cases with type 1 VWD, and at least one other affected and one unaffected family member, recruited patients from 14 Centres throughout Europe (42). DNA was also isolated from approximately 100 unrelated control subjects from the same geographical regions as the index cases. Sequencing of the *P2RY12* gene in this cohort identified two heterozygous missense mutations, one of which causes the variant $P2Y_{12}$ receptor to have reduced ligand binding and the other, abnormal receptor trafficking (43, 44).

Genetic defects in $P2Y_{12}$, TxA_2 and GPVI receptors

A number of new kindreds have been recently characterised with variant receptors for the two major platelet feedback agonists,

ADP and TxA₂, and with variant GPVI which is the major signalling receptor for collagen. For the majority of these kindreds, the only clinical manifestation was abnormal mucocutaneous bleeding and identification of the genetic defect relied entirely on detailed phenotypic analysis of platelets. A description of the current repertoire of naturally occurring nucleotide variations in the genes encoding the ADP P2Y₁₂ receptor, the TxA₂ receptor and the collagen GPVI receptor is presented below.

ADP P2Y₁₂ receptor

Ten different P2Y₁₂ receptor defects have been reported in nine index cases with mild bleeding disorders, five resulting in a failure to express the functional receptor. Homozygous deletions of either 1 or 2 bp in the coding sequence caused frameshifts leading to introduction of premature stop codons and a lack of demonstrable P2Y₁₂ expression in patients from two families (45–47). We have recently identified a third kindred in Birmingham with a small *P2RY12* deletion and frameshift who has a similar laboratory platelet phenotype (40). A further patient was homozygous for a missense mutation in the *P2RY12* translation initiation codon which also resulted in a failure to express functional P2Y₁₂ (48). All patients predicted to have absent P2Y₁₂ expression presented with similar clinical histories of lifelong spontaneous mucocutaneous bleeding, excessive bleeding in response to haemostatic challenges and prolonged bleeding times. Platelets from these patients displayed severely impaired ADP-induced aggregation, normal shape change (reflecting normal P2Y₁ function), a failure of ADP to inhibit PGE-stimulated adenylyl cyclase and a reduction in the number of binding sites for radiolabelled ADP (45, 46, 48). A heterozygous carrier of one of the null alleles described above had a mildly prolonged bleeding time and platelets which showed only moderate reductions in platelet ADP binding sites and in adenylyl cyclase inhibition by ADP (46). Moreover, platelets in this heterozygous subject showed a normal aggregation response to a high concentration of ADP (20 µmol/l), but reduced and reversible aggregation to a

lower concentration (4 µmol/l). The ATP secretion response to a range of agonists was also impaired consistent with the positive feedback role of ADP in platelet activation initiated by a range of stimuli (46). A fifth patient with a complete absence of P2Y₁₂ receptor expression was found to be heterozygous for a 2 bp deletion in *P2RY12* causing frameshift and introduction of a premature stop codon (3). Analysis of platelet mRNA revealed the presence of *P2RY12* transcripts derived only from the mutated allele although no coding sequence abnormality could be identified in the second allele in genomic DNA to account for absent transcription. Platelets from the daughter of this patient, who was also heterozygous for the 2bp *P2RY12* deletion displayed a moderate reduction in ADP binding sites, and impaired ADP-dependent aggregation at low concentrations of ADP (3).

A further patient with a lifelong history of easy bruising and excessive bleeding after surgery and trauma was found to have compound heterozygous R256Q and R265W point mutations in *P2RY12* (49). Platelets underwent normal shape change but demonstrated reduced aggregation in response to a high concentration of ADP (20 µmol/l). This observation of a partial reduction in the aggregation response to this concentration of ADP indicates that either one or both mutations cause impairment rather than abolition of function. The R256Q and R265W variants both supported normal binding to (³H)2MeS-ADP suggesting a failure in receptor function downstream of agonist interaction (49).

The other three patients with naturally occurring *P2RY12* nucleotide variations all had heterozygous mutations. This group includes a patient with abnormal bleeding predicted to have a heterozygous P258T P2Y₁₂ substitution. Platelets from this patient failed to aggregate in response to low concentrations of ADP (≤4 µmol/l), but showed reduced and reversible aggregation at higher ADP concentrations (20 µmol/l) (50).

We have found further two subjects with heterozygous mutations in *P2RY12* within the EU MCMDM-1VWD study cohort. One of these predicts a K174E substitution in the P2Y₁₂ receptor second extracellular

loop which is a region that is critical for the receptor interaction with ADP. This patient and several family members live close to our laboratory in Birmingham enabling us to perform detailed functional studies on three heterozygous individuals alongside control subjects from the same family but who lacked the mutation. Importantly, platelets from all three heterozygous individuals had impaired aggregation to low and intermediate concentrations of ADP, but showed normal aggregation responses with a higher ADP concentration (44). In a stably transfected cell line, variant P2Y₁₂ containing the K174E substitution was expressed normally at the cell surface but showed a marked reduction in ligand binding confirming the importance of this residue in the interaction with ADP (44). This is the first published association of a platelet defect in a family with type 1 VWD.

The second *P2RY12* missense mutation to be identified in our laboratory predicted a P341A substitution in the PDZ binding domain at the C-terminus of the P2Y₁₂ receptor. PDZ binding domains regulate critical aspects of receptor signalling and endocytic sorting, although no other clinical defect linked to a mutation in a PDZ binding domain has been reported to date. Stable expression of the variant P341A P2Y₁₂ receptor revealed that it is expressed at the cell surface and able to inhibit formation of cAMP. However the ability of the variant receptor to internalize, recycle and resensitize in the stably transfected cells was significantly impaired (43). This has led us to hypothesise that platelets continually internalise the P2Y₁₂ receptor in response to exposure to low levels of ADP in the circulation in order to prevent unwanted activation. The P2Y₁₂ receptor is then returned to the surface to maintain platelet sensitivity. We speculate that defective recycling of the variant P341A P2Y₁₂ causes the receptor to become trapped within the platelet, thus causing a net reduction in surface expression.

Thromboxane A₂ receptor

The first of two reported naturally occurring *TXA2R* nucleotide variations reported to date is a missense point mutation ident-

ified in multiple unrelated Japanese patients that predicts an R60L substitution in the first cytoplasmic loop of the TxA₂ receptor. One of the first affected patients to be described in detail was a 53-year-old man with a lifelong tendency for abnormal bleeding after surgery and trauma and recurrent oral cavity bleeding but with no other demonstrable haemostatic defect. Platelets from this index patient showed an absent aggregation response to the stable TxA₂ mimetic STA2 and *TXA2R* gene sequencing showed homozygosity for the predicted R60L substitution (51). A number of relatives of the index patient were heterozygous for the R60L substitution and these subjects showed reduced, but not absent platelet aggregation responses to STA2. Consequently, this platelet function disorder was described as showing autosomal dominant inheritance even though heterozygous relatives did not have significant a clinical bleeding phenotype (51). When the variant R60L TxA₂ receptor was co-expressed with the wild type receptor in CHO cells there was decreased agonist-induced second messenger formation despite normal ligand binding affinity. This led the authors to suggest a dominant negative effect of the substitution consistent with the apparent autosomal dominant inheritance in the affected index family (51). Unfortunately, no functional analyses were performed on heterologous cells expressing only the variant receptor so this conclusion may be premature. Later studies in a number other Japanese kindreds with an identical R60L variant TxA₂ receptor confirmed quantitative differences in the aggregation responses to STA2 between heterozygotes and homozygotes but also differences in the extent to which second messenger activation was impaired (52, 53). These data argue against a simple dominant negative effect of the R60L substitution in heterozygotes but, instead point to a more complex defect in TxA₂ receptor coupling.

We have recently identified a second naturally occurring nucleotide variation in the TxA₂R in a teenage boy with a history of severe nose bleeds and whose platelets showed a selective loss of response to the stable TxA₂ mimetic, U46619 (41). The patient was heterozygous for a D304N substi-

tution in the 7th transmembrane region of the TxA₂ receptor. The heterozygous mutation was associated with an approximate 50% reduction in ligand binding to the patient's platelets and abolition of binding to the variant receptor and functional responses in stably transfected CHO cells despite expression of the variant receptor at the cell surface. Thus, this work identifies a structural element within the TxA₂ receptor not previously implicated as a determinant of ligand binding. This kindred is also informative in that the index case is first patient in which abnormal bleeding has been associated with a heterozygous TxA₂ receptor defect in the receptor. However, the father of the index case who possesses the same *TXA2R* mutation and has a similar defect in platelet activation and ligand binding, does not have a history of abnormal bleeding. This suggests that the symptomatic index case had a further unidentified haemostatic defect that contributed to the abnormal clinical phenotype.

GPVI-FcR γ -chain receptor

A series of patients with defective platelet aggregation responses to collagen have been reported over the last two decades predominantly comprising individuals with autoimmune thrombocytopenia in whom circulating antibodies to the collagen receptor GPVI caused internalisation or shedding of the receptor from the platelet surface as exemplified by the first patient to be described (1). Selective loss of GPVI expression has also been associated as an acquired defect in patients with myeloproliferative disorders and in association with the grey platelet syndrome. In 2009 two patients were independently reported with abnormal platelet aggregation responses to collagen caused by genetic defects in the GPVI receptor (54, 55). One patient was a 10 years old girl with abnormal ecchymoses since infancy and a prolonged bleeding time. Sequencing of the *GP6* gene that encodes the GPVI receptor showed compound heterozygous nucleotide variations comprising a missense mutation in exon 3 of one allele predicting an R38C substitution in the first Ig domain of GPVI, and an insertion of 5 nucleotides in exon 4

on the other allele causing frameshift and loss of GPVI expression (54). Expression of the variant R38C GPVI in heterologous cells confirmed that the naturally occurring variant protein did not bind collagen.

The second patient with a naturally occurring variant GPVI was a 31 year old female with lifelong abnormal bleeding problems associated with a predicted S175N substitution arising from one variant GPVI allele. This substitution affected the highly conserved S175 residue in the second Ig-like domain in GPVI and was shown to cause loss of receptor function when expressed in heterologous cells (55). In common with the other patient with a genetic GPVI defect, this individual also had absent expression from the remaining GPVI allele, in this case caused by frameshift associated with a 16 bp *GP6* deletion.

Is mild platelet bleeding frequently multifactorial?

The study of patients with platelet function disorders is severely hampered by the variable penetrance of bleeding symptoms and by the absence of a gold-standard clinical assay to test for platelet dysfunction. Investigation is further complicated by the knowledge that many bleeding symptoms such as easy bruising or menorrhagia that are reported by patients with platelet function disorders are also prevalent in the general population. For genetic platelet function disorders, which only manifest clinically with mild bleeding, identification of affected individuals is therefore difficult without large-scale systematic laboratory evaluation of patient groups with arrays of platelet function tests that are sensitive to mild disorders.

Subjects with heterozygous platelet protein defects may be prevalent in the general population, but the difficulties in identifying this group on clinical grounds alone mean that these mild defects are likely to be heavily under-recognised. This is illustrated by the paucity of literature reports of families in which the genetic cause of mild platelet dysfunction has been identified. Indeed, amongst the twelve index cases worldwide with reported defects identified in either the P2Y₁₂ ADP receptor, TxA₂ re-

ceptor or the GP-VI collagen receptor, seven had either homozygous or compound heterozygous mutations causing complete loss of expression or function of the receptor. Even these patients displayed a comparatively mild phenotype of mild mucocutaneous or post-surgical bleeding that is comparable with patients receiving aspirin or thienopyridine anti-platelet agents.

The remaining five reported index cases were heterozygous for defects in platelet proteins (four index cases with a P2Y₁₂ defect and one with a TxA₂ receptor defect) yet were identified because they presented with bleeding symptoms. At first site, this seems remarkable given the mild bleeding phenotype associated with homozygosity for loss of function mutations in these receptors. However, two of the three heterozygous P2Y₁₂ defects were found in association with type 1 VWD, while in our analysis of a heterozygous TxA₂ receptor defect, bleeding in the index case was probably caused by co-inheritance of a second, albeit unidentified haemostatic defect (41). We speculate that although there may be a large pool of heterozygous defects in key platelet genes in the background population, clinical presentation is only likely in the very rare homozygotes or compound heterozygotes or in those individuals who have a heterozygous platelet defect and who have co-incidentally co-inherited or acquired other haemostatic disorders. Thus, the phenotype of mild bleeding is likely to be multifactorial in many patients and may be considered to be a complex genetic trait. The careful laboratory evaluation of the platelet phenotype in this group of patients is required before we can properly ascertain the contribution made by defective platelet genes.

Future directions

Gene mapping in patients with mild platelet function disorders is of immediate significance to affected patients since identification of a causative nucleotide variation provides a robust marker to identify other affected individuals within families, including family members such as children in whom phenotypic testing may be prac-

tically difficult. Gene mapping also has wider significance since vital information can be gained about the function of known and novel platelet proteins which ultimately could inform drug development for human disease. Detailed analysis of the platelet laboratory phenotype has demonstrably improved the efficiency of gene mapping by allowing refinement of the choice of candidate genes. It follows from this that there is considerable benefit in refining diagnostic procedures during patient testing that increase the accuracy and scope of phenotypic profiling. Our own experience of this approach allows us to highlight the following considerations:

- Detailed analysis of the platelet phenotype requires the use of low concentrations and a wide range of platelet agonists.
- It should be recognised that mild bleeding is a complex trait and may represent the cumulative defect of multiple defects in platelet function and other pathways in haemostasis, particularly type 1 VWD.
- Heterozygous nucleotide variations in platelet genes may contribute significantly to the bleeding phenotype so apparent autosomal dominant transmission of platelet dysfunction disorders is likely.

Recent advances in high throughput gene sequencing will enable the identification of many more naturally occurring nucleotide variations in genes encoding platelet proteins. The combination of detailed phenotypic analysis and targeted gene sequencing will rapidly advance and facilitate this work.

Acknowledgements

SPW holds a British Heart Foundation (BHF) Chair (CH/03/003). SJM is a BHF lecturer. This work was supported by a BHF grants (PG/06/038; RG/09/007).

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