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Platelet glycoprotein VI genetic quantitative and qualitative defects

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Abstract

Platelet membrane glycoprotein VI (GPVI) is increasingly recognized as an important receptor for thrombus formation and growth. Numerous arguments have been published indicating that GPVI plays a major role in thrombosis without being essential for physiological hemostasis. In humans, GPVI deficiencies are rarely reported. These are most often deficiencies occurring in the context of autoimmunity and, more rarely, genetic deficits. The purpose of this review is to compile data on the quantitative and qualitative genetic abnormalities of GPVI.

Keywords

Glycoprotein VI, genetic deficiency, platelet disorder

History

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Human platelets express three major immune and immune-like receptors: the low-affinity IgG receptor FcγRIIA [1], platelet glycoprotein VI (GPVI) and C-type lectin-like receptor (CLEC-2) which signal through immunotyrosine-based activation motifs [2]. GPVI and CLEC-2 have been identified relatively recently as compared to other major platelet glycoproteins such as the GPIb-V-IX complex and integrin γIIbγ3. This could be due to the absence or scarcity of clinical manifestations related to quantitative or qualitative modifications of these receptors.

GPVI was initially described as the major receptor for platelet activation by collagen [3] and more recently GPVI was also identified as a receptor for fibrin [4,5]. Moreover, upon platelet binding to fibrinogen, GPVI was shown to trigger outside-in signals contributing to platelet spreading and to platelet aggregation under flow [6]. Importantly, collagen or fibrin-mediated activation of GPVI promotes the procoagulant activity of platelets that supports thrombin generation [4,7]. GPVI is thus recognized as (i) being critical for the initiation of thrombus formation at sites of vascular injury and in particular on eroded atherosclerotic lesions enriched in collagen, (ii) contributing to the growth of the thrombus thanks to the recruitment of additional platelet by the fibrin-rich thrombus and (iii) favoring the stabilization of the thrombus.

GPVI belongs to the immunoglobulin receptor superfamily 89. It is co-expressed at the platelet surface with the γ chain common to Fc receptors (FcRγ) that bears the ITAM motif responsible for intracellular signaling [10]. The FcRγ gene (*FcER1G*) is located on chromosome 1q23 and to date, no mutations have been described that lead to pathogenicity.

The GPVI gene (*GP6*) maps to chromosome 19 within the leukocyte receptor cluster (LCR) together with leukocyte Ig-like receptors (LIRs), killer-cell Ig-like receptors (KIRs), leukocyte-associated Ig-like receptors (LAIRs) and the receptor for IgA, FcγRI [9]. The GPVI gene is composed of 8 exons: exons 1–7 code for the extracellular domain with exon 3 coding for the first Ig-like domain D1, exon 4 for the second Ig-like domain D2, exons 5–7 for the stem domain; exon 8 codes for the transmembrane and intracellular domain [11]. GPVI expression is restricted to platelets and megakaryocytes [9,12]. GPVI is type I transmembrane protein with a 58–60 kDa molecular mass, 45% of which is carbohydrate. The human GPVI contains 339 amino acids residues. The extracellular portion of 247 amino acid presents the two immunoglobulin-like domains (D1 and D2) linked by a hinge and orientated at ~90° [13]. Several amino-acid residues at the surface of the D1 domain have been identified as participating to GPVI binding to collagen [14]: a collagen-binding domain (CBD) composed of a basic groove formed of K41, K59, R60, and R166 [15,16], a more distal site including hydrophobic residues L36, V34 [17], and the N-glycan chain at N72 [18]. It is yet unknown whether the collagen-binding-domain and the fibrin-binding-domain share common structures or are different. The D2 domain is also involved GPVI binding to collagen [14] because it contributes to the formation of GPVI dimers [19] that considerably increase the affinity of GPVI for collagen [20,21]. The D1-D2 domain is followed by a highly O-glycosylated sequence that forms a mucin-like stalk domain. Proximal to the membrane, GPVI presents cleavage sites for metalloproteases of the ADAM family whose action leads to the release of the extracellular domain [22]. The transmembrane domain of GPVI is characterized by the presence of a positively charged residue (R252) that forms a salt bridge with a negatively charged residue (D11) of the FcRγ-chain required for the expression of functional GPVI. The cytoplasmic domain of human GPVI contains 51 amino acid residues without apparent homology with other receptors of this family but that contains several functional motifs: a basic sequence that stabilizes the interaction with FcRγ, a calmodulin binding motif and a proline-rich domain (PRD) to which the Src-family kinases, Lyn and Fyn are constitutively bound [23,24].

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Table I.

Case	Genomic variation	Protein effect	Inheritance	Variation type	Collagen-induced platelet aggregation	Bleeding	Reference
A	c.172 C > T	p.R58C^{het}	heterozygous(mother)	Missense	Normal	No	29
A	c.356_dupAGCCC	p.G121SfsX12^{het}	heterozygous (father)	Insertion	Normal	No	29
A		p.R58C^{het}	Compound heterozygous (proband)		Absent	Ecchymoses during childhood	29
		p.G121SfsX12^{het}					
B	c.524 G > A	p.S175N ^{het}	(mother?)	Missense	ND	ND	30
B	c.145_159del	p.Gln49_Gly53del ^{het}	heterozygous (father)	Deletion	ND	ND	30
B		p.S175N^{het}	Compound heterozygous (proband)		Absent	Ecchymoses, epistaxis, menorrhagia Post surgical and traumatic	30
		p.Gln49_Gly53del ^{het}					
C	c.711_712insA	p.(Val238Serfs*5)	heterozygous (parents)	Insertion	Normal	No	31
C		p.(Val238Serfs*5)	homozygous/recessive (probands)		Absent	Minor in 4 sujects No in one	31

GPVI Polymorphisms

GP6 locus is highly polymorphic. Eighteen SNPs have been identified, six of which encode amino acid substitutions in the mature form of the protein. The 18 SNPs segregate into 34 predicted sequence haplotypes in three linkage disequilibrium blocks across the gene [25].

Only one nonsynonymous SNP has been identified in the exons encoding the first Ig-like domain, encoding for a 103Leu > Val with a very low frequencies of the Val allele (0.005) [25]. This substitution results in reduced ligand binding. The five other nsSNPs encode for five common amino acid substitutions resulting in 14 GP6 protein isoforms. These five nsSNPs are restricted to the stem and cytoplasmic tail, with three replacements in the glycosylated stem (Ser199Pro, Lys217Glu, Thr229Ala) and two in the cytoplasmic domain (Gln297Leu and His302Asn). In the Caucasoid population, the observed genotypes indicate the presence of a major and minor haplotype encoding protein isoforms allele "a" encoding amino acids SKTQH and allele "b" encoding PEALN, with frequencies of 0.82 and 0.15 [26]. Individuals homozygous for the minor haplotype have significantly reduced GP6 expression. Platelets homozygous for the low-frequency allele tend to be less able to form a thrombus on a collagen surface in flowing whole blood and have a reduced procoagulant response to collagen-related peptide [27,28].

GPVI Genetic Deficiencies

Only three reports describing subjects with an inheritable GPVI deficiency have so far been published (Table 1). Two describe compound heterozygous mutations responsible for the GPVI deficiency in two individuals [29,30], while in the third report a homozygous mutation causing the deficiency in five subjects from four unrelated families [31]. These studies are referenced as A, B and C, respectively, in the following of this manuscript.

Characterization of the GPVI Deficiency

In the three studies, the GPVI deficiency was evidenced by flow cytometry and confirmed by immunoblot. Flow cytometry experiments were performed with different antibodies [3J24 (A); HY101 (B); 6B12 and 11A12 (C)] and a quantitative analysis obtained once (A). GPVI was almost absent on the platelet surface from probands of studies B and C and decreased to less than 15% of the control for proband of A. Of note, most of the flow cytometry GPVI expression studies have up to now been performed using different monoclonal antibodies without these having been compared with each other; the now available quantification assay using the monoclonal antibody 1G5 (GP Screen Biocytex) should establish international standards for the number of GPVI sites at the platelet surface.

The GPVI deficiency was confirmed by immunoblot that also provided interesting information regarding the structure of GPVI. In A, the GPVI band was of decreased intensity and migrated as a smear when the blot was realized with a polyclonal antibody but no band was detected using two monoclonal antibodies directed to the D2 loop suggesting that a structurally abnormal GPVI was synthesized in low amounts without being expressed on the platelet surface; no low molecular weight GPVI remnant was detected using an anti-GPVI-tail antibody arguing against shedding of the GPVI extracellular domain. The expression of the FcRγ chain was normal. In B, the immunoblot was realized with a polyclonal antibody and showed a decrease in intensity of the GPVI band as compared to a control. In C, the immunoblot realized with a polyclonal as well as a monoclonal antibody revealed a band of lower molecular mass than normal GPVI suggestive of a truncated form that appeared sequestered in platelets as indicated by immunofluorescence studies.

Platelet Functions

In none of the cases was the platelet count changed (280 and 208 G/L in A and B, ranging from 242 to 304 G/L in C) indicating that platelet production was not impaired by the GPVI deficiency.

Platelet aggregation was measured by light transmission aggregometry in PRP (A, B, C) and washed platelets (A). It was comparable to controls in response to ADP (B, C), arachidonic acid (B, C), U46617 (A, B), TRAP1 (A) and ristocetin (A, C). In contrast, platelets from all subjects failed to aggregate in response to collagen (Horm collagen). The specificity of the functional defect was confirmed using the GPVI specific agonists convulxin (A, B, C) or CRP (B, C). Platelet dense granule secretion was assessed by measuring ATP (A, B) or serotonin (C) and was null in response to collagen (A, B, C), convulxin (A, C) and CRP (C) but normal in response to other agonists. Moreover, in A, P-selectin exposure was almost null in response to collagen. These results confirmed the critical role played by GPVI in the activation of platelets by collagen *in vitro*.

Platelet interaction with collagen was additionally reduced when perfusing whole blood in collagen-coated flow chambers at a moderate 600 s^{-1} (B) or high 1500 s^{-1} (A) shear rate. In both cases, the adhesion of single platelets was observed but the formation of platelet aggregates was dramatically reduced. Flow chamber experiments are recognized to better reflect the events leading to the formation of a platelet thrombus in a vessel than platelet aggregation does because of the use of whole blood and of the shear stress applied [32]. Despite platelets from GPVI-deficient patients being unable to form aggregates on collagen in these conditions, their capacity to adhere onto collagen is preserved suggesting these platelets are still able to ensure some hemostatic functions thanks to compensatory mechanisms.

Thrombin generation was measured in one case (A) and found to be severely decreased upon platelet stimulation with collagen. This was in agreement with the well-documented inhibition of thrombin generation and platelet procoagulant activity by GPVI-blocking antibodies.

GPVI-deficient platelets obtained from families described in C have been very useful to establish the role of GPVI in platelet interaction with fibrinogen and fibrin. Indeed, GPVI-deficient platelets were found to have a reduced adhesion and failed to spread on immobilized fibrinogen and fibrin and were unresponsive to fibrin [6,33].

GPVI Mutations

In A, sequence analysis of *GP6* indicated that the proband was homozygous for the common SKTQH allele. It was reported that one allele presented a single nucleotide substitution in exon 3 resulting in a missense mutation pArg38 Cys (R39C in [29]), and was inherited from the mother (Figure 1); the other allele presented a 5 bp duplication in exon 4 resulting in a frameshift and an early stop codon and was inherited from the father. Analysis of platelet mRNA showed that all transcripts contained the single point mutation in exon 3 and none the duplication found in exon 4 due to the premature arrest of the transcription. Since GPVI function was abolished from the proband platelets despite one allele was transcribed, the effect of the Arg38Cys mutation on GPVI expression and function was assessed by introducing the mutation in recombinant GPVI (GPVI-Fc). The mutant protein exhibited an abnormal migration in electrophoresis, a decreased binding to collagen and an impaired capacity to inhibit collagen-induced platelet aggregation as compared to wild-type GPVI-Fc. R38 is localized in the D1 domain of GPVI and its interactions with Y66 and K41 stabilize the loop which contains residues of the CBD such as K41 and K59 [15,16]. Furthermore, a cysteine in this position could result in the formation of aberrant disulfide bridges responsible for the abnormal migration of the protein.

In B, sequence analysis of *GP6* cDNA obtained from platelet mRNA also revealed heterozygous compound mutations confirmed by gDNA sequencing. One allele bearing the rare (b) haplotype (PEALN) presented an out-of-frame 16 bp deletion in exon 3 resulting in an early stop codon and was inherited from the father. The presence of the corresponding potentially synthesized

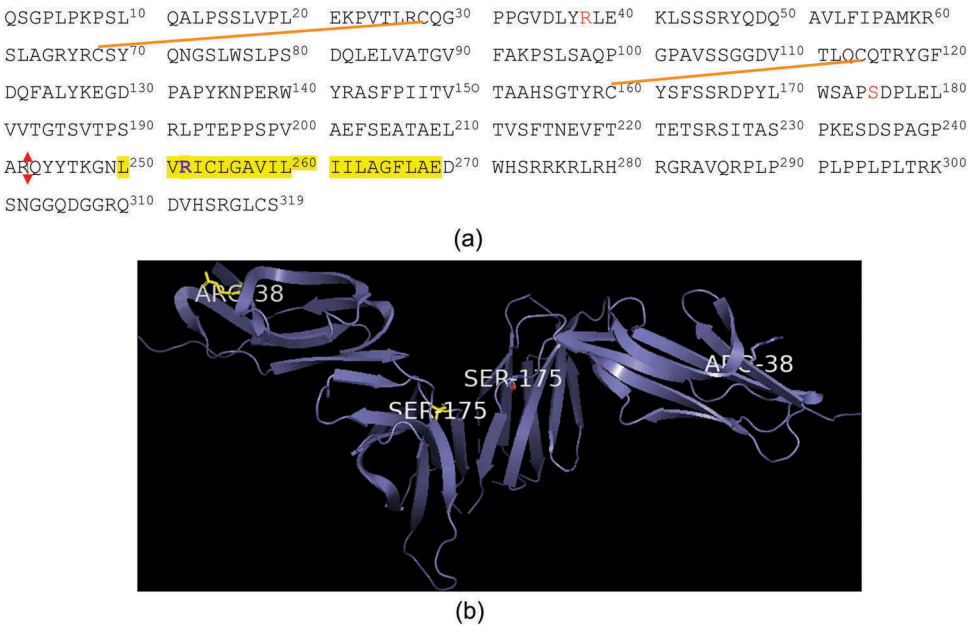

Figure 1. A. GPVI sequence. The transmembrane domain is highlighted in yellow and R252 that forms the salt bridge with the FcRγ chain is in purple. The two disulfide bridges in the extracellular domain are indicated by an orange line. The position of the mutant residues in subjects A and B (R38 and S175) are in red and the site of truncation of the protein in subjects C is indicated by the red arrow. B. The localization of R38 and S175 on the three-dimensional structure of the GPVI dimer is shown using the crystal structure solved by Horii et al. (ref; access number **MMDB ID**: 44970 **PDB ID**: 2GI7).

short (56 amino acid residues) product was not detected in the platelets. The second allele presented one missense mutation pSer175Asn and was inherited from the mother (Figure 1). As in the previous case, the functionality of the Asn175 GPVI was examined by producing recombinant GPVI and found to have a significantly reduced binding to convulxin and CRP. Interestingly, Ser175 is highly conserved in the GPVI D2 domain across species and other LRC proteins. It is part of 5-aa motif, WSXPS found in KIR Ig-like domains one, a similar motif, WSXWS being the signature of class I hematopoietic receptors These motifs have been shown to contribute to tertiary folding.

In C, genomic GP6 sequencing in the first two identified subjects indicated both alleles were of the frequent 'a' allele (SKTQH) and exhibited a homozygous adenine insertion between positions 711 and 712 of exon 6 ('c.711_712insA'), resulting in a frame shift and a premature 'stop codon' five codons ahead (position 242 of the protein) of the transmembrane domain resulting in the synthesis of a truncated protein missing the transmembrane and intracellular domains. In the other subjects, the sequencing directed to exon 6 revealed the same homozygous mutation. Analysis of platelet mRNA indicated that mRNA with the mutation responsible for the transduction of the truncated protein was present in the platelets of all patients but in decreased amount. The truncated protein was synthesized and detected in permeabilized platelets but not at the plasma membrane. In mice, the presence of the FcRγ chain is necessary for GPVI expression at the platelet surface and FcRγ deficient mice are also deficient in GPVI [34]. In fact, R252 within the transmembrane domain is required for GPVI association to the FcRγ chain and signaling downstream of GPVI. However, the expression of recombinant GPVI at the surface of CHO cells [9] and of an R252A GPVI mutant on RBL (Jandrot-Perrus unpublished observation) cells suggest that GPVI can be expressed at the plasma membrane in the absence of association to the FcRγ chain. The combined absence of the transmembrane and cytoplasmic domains thus prevents the truncated GPVI from inserting into the membrane. Whether the truncated protein could be secreted remains an opened question.

Heterozygous Subjects

The father and the mother of the proband have been explored in A, the father in B and several members of the four families from the C study.

Subjects heterozygous for the missense mutation pArg38 Cys, 5 bp duplication in exon 4 and 'c.711_712insA' had normal collagen-induced platelet aggregation and secretion indicating that the expression of a 50% GPVI level at the platelet surface is sufficient to ensure platelet activation by collagen. This is in agreement with the previous report that platelets with a 50% reduction in GPVI levels did not significantly differ from controls with respect to thrombus formation [27]. Yet, a moderate decreased thrombin generation in collagen challenged PRP was observed in the subject heterozygous for the 5 bp duplication in exon 4. Further studies of subjects heterozygous for GPVI deficiency would be of interest in determining the level of GPVI expression required for the completion of different platelet functional responses.

Bleeding

In A, the Ivy bleeding time was prolonged (>15 min) as well as the PFA closure time on collagen/epinephrine cartridges (210 s for a control <165 s) but the closure time on collagen/ADP cartridges was normal. In B, the PFA closure time on collagen/epinephrine cartridge was in the normal range (113 s vs 93–193 s) and short on collagen/ADP cartridge (62–64 s vs 71–118 s for the reference value). In C, the Ivy bleeding time was of 13 min and 9.5 min in two of the five subjects. It should be noted that an increase in the PFA100 closure time on collagen/epinephrine cartridges has been reported in patients with an acquired GPVI deficiency [35–37], an observation to consider with precaution due to the insufficient sensitivity of the PFA100 assay [38]. None of the heterozygous subjects presented a prolonged bleeding time whatever the type of the mutation and all were free of bleeding tendency.

The clinical manifestations of the GPVI deficiency in the subjects with a genetic GPVI deficiency are heterogeneous and range from zero to severe. In only one case (B) severe bleeding is reported in a 32-year-old woman exhibiting mild spontaneous bleeding episodes (ecchymoses, epistaxis) but also menorrhagia and more severe provoked posttraumatic and post-surgery bleeding. In C, one from each of the four families, two women 22- and 11-year-old, two men 23- and 12-year-old presented a mild to moderate bleeding phenotype since childhood with easy bruising, epistaxis, prolonged bleeding after minor injuries and gum bleeding. The 22-year-old women had heavy menstrual bleeding, requiring platelet and red blood cell transfusions and iron therapy. Two additional women recently diagnosed with the same mutation (D. Mezzano, unpublished observations) presented also with hypermenorrhea that normalized with contraceptive therapy. Interestingly, the 5-year-old sister of one of the patients had no abnormal bleeding despite being homozygous for 'c.711_712insA'.

In contrast, in A, the propositus, an 8-year- old female at the discovery presented only easy ecchymoses during infancy, symptoms that regressed with age. Since now 15 years no bleeding episodes have been reported in this subject: no epistaxis, no gum bleeding, no menorrhagia, no bruising tendency, no surgery apart from a carefree tooth extraction.

From these three reports, it is therefore difficult to establish a standard clinical picture for patients with a GPVI genetic deficiency. It appears, however, that minor to moderate bleeding is the most common phenotype, and especially since post-surgical bleeding encountered by subject B may have been at least partly due to the surgical procedure itself. It is also noteworthy that the 5-year-old child homozygous for the 'c.711_712insA' mutation has remained asymptomatic six years after the diagnosis. It is difficult to say that causal mutations are responsible for the heterogeneous symptomatology because of the small number of observations, and the fact that subjects with the same mutation do not exhibit the same symptoms is not in favor of this hypothesis.

All these observations make it possible to propose two hypotheses: (i) the deficits in GPVI would not be extremely rare, but would remain undiagnosed because of mild or absent bleeding symptoms, (ii) the bleeding when occurring could be due to the association of GPVI deficiency and an unknown intercurrent disorder, possibly additional SNVs in other genes. Indeed, in cases of acquired GPVI deficiency caused by autoimmune antibodies, even a relatively moderate thrombocytopenia has been associated with easy bruising [39]. Inflammation may also trigger bleeding, as shown in GPVI deficient mice. A possible explanation is the role-played by GPVI in sealing the vascular damage inflicted by neutrophils [40]. A more systematic study of the expression and function of GPVI in the population may determine the actual incidence of the quantitative and/or qualitative deficits of GPVI and the associated risk for bleeding.

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Declaration of interest

Martine Jandrot-Perrus is founder and scientific consultant for Acticor-Biotech Diego Mezzano and Cedric Hermans have no conflicts of interest to disclose.

Contribution of each author

M.J-P wrote, C.H and D.M contributed to the manuscript.

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