Severe bleeding and absent ADP-induced platelet aggregation associated with inherited combined CalDAG-GEFI and $P2Y_{12}$ deficiencies

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Supplementary material

Materials

[^{3}H]PSB-0413 was from General Electric, Healthcare (Buckinghamshire, UK). ADP, collagen, the thromboxane/prostaglandin endoperoxide analogue 9,11-dideoxy-11,9-epoxymethano-prostaglandin F_2 (U46619), thrombin receptor activating peptide 6 (TRAP-6), prostaglandin (PG) E_1, PGI_2, were from Sigma (St. Louis, MO, USA). Apyrase was purified from potatoes.[1] Commercial preparations of luciferin/luciferase reagent were used to measure the platelet ATP and ADP contents (ATP Assay Kit, BioOrbit Oy, Turku, Finland), and platelet secretion concurrently with platelet aggregation (Chronolume, Chrono-log Corp, Havertown, PA, USA). Serum thromboxane (Tx) B_2 was measured by a commercially available enzymatic immunoassay (Thromboxane B_2 EIA kit, Cayman Chemical Co., Ann Arbor, MI, USA).

Preparation of Platelet-Rich Plasma (PRP) and Washed Platelet Suspensions

For studies with PRP, 9 volumes of blood were drawn into 1 volume of 109 mmol/L trisodium citrate, and then centrifuged at 200xg for 10 min. The supernatant PRP was transferred into a clean plastic tube; the platelet counts in PRP samples were not adjusted to a pre-defined value. For the preparation of washed platelet suspensions, 6 volumes of blood were drawn into 1 volume of acid-citrate-dextrose anticoagulant, centrifuged at 200xg for 10 min to obtain PRP, which was used to prepare twice washed platelet suspensions in Tyrode's buffer containing 500 nmol/L PGI_2 during the first and second wash. Platelet counts in washed platelet suspensions were adjusted to $3x10^{11}$/L.

Studies of Platelet Aggregation and Secretion

The first screening of platelet aggregation in the studied patients was done in Florence, suing the platelet aggregometer APACT4004 (LABiTec®, LAbor BioMedical Technologies GmbH, Ahrensburg, Germany). Additional studies of platelet aggregation in patient II-1 were done in Milan, where platelet aggregation and secretion were studied simultaneously by lumi-aggregometry. Samples of PRP (0.45 mL) were incubated with 50 μL luciferine/luciferase reagent at 37 °C for 30 sec and stirred at 1,000 rpm in a lumi-aggregometer (Lumi-aggregometer, Chrono-log Corp., Havertown, PA, USA). After incubation, 10 μL of an aggregating agent was added and the aggregation and ATP secretion tracings were recorded for 3 min.

Binding of [^{3}H]PSB-0413 to Washed Platelets

Binding experiments were performed using [^{3}H]PSB-0413, which is a tritiated derivative of a selective nucleotide antagonist of the $P2Y_{12}R$, AR-C67085MX (2-propylthioadenosine-5'-adenylic acid (1,1-chloro-1-phosphonomethyl-1-phosphonylanhydride), and $9x10^7$ washed platelets. Nonspecific binding was defined in the presence of 1 mM ADP. Washed platelets were incubated with the ligand at 37°C for 5 min; then bound and free radioactivity was separated by filtration through Whatman GF/B glass-fiber filters. Filters were then washed with 5x2 ml of ice cold washing buffer (Tris HCl 50 mM pH 7.5, EDTA 1 mM, $MgCl_2$ 5 mM, NaCl 100 mM). Filter-bound radioactivity was counted in 2 ml liquid scintillation counter.

Measurement of platelet cAMP

Platelet cAMP was measured by a radioisotopic assay, using a commercially available kit (Cyclic AMP [^{3}H] assay system, Amersham International, UK). Duplicate samples of 1 mL citrated PRP containing 1 mM theophylline were incubated with Tyrode's buffer and PGE_1 (1 μM), PGE_1 and ADP or

epinephrine (0.1 and 1.0 μM) or Tyrode's buffer alone in a control mixture. After incubation at 37 °C (2 min), 1 mL of 5% trichloroacetic acid was added, and the samples were snap-frozen in dry ice and methanol, thawed at ambient temperature, and then shaken at 4 °C for 45 min. After centrifugation at 4 °C for 30 min, the supernatant was extracted three times with 5 mL of water-saturated ether, dried under a stream of nitrogen at 60 °C, and stored at –20 °C. Before assay, the samples were reconstituted with 0.05 mol/L Tris buffer containing 4 mmol/L EDTA.

Genetic studies

The *P2RY12* gene (NCBI Ref Seq: NG_016019.1) was genotyped by PCR and direct sequencing of the three exons and splicing junctions, of 1.1Kb in the 5' gene region, 0.64 Kb of intron 1 and the whole intron 2 (1.7Kb) (Figure 2D). Primer sequences and position are shown in the Supplementary Table 1. Known polymorphisms were identified, which permitted the definition of *P2RY12* haplotypes in the family (Figure 2D). The *P2RY1* gene was amplified and sequenced using the forward 5'-CCCTGTTGTGTAAGCTCGGCG-3' and reverse 5'- CTTTTTGAGCCGGCCCAGGG-3' primers, and the forward 5'-CCATGTGTAAACTGCAGAGG-3' and 5'- CAAACAAGCTAAGTGTGGATG-3' primers. Total RNA was extracted from platelets using Tempus Blood RNA Tubes and by Tempus Spin RNA Isolation Kit (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA USA). The *P2RY12*, *ITGB3* and *ITGA2B* mRNAs were reverse transcribed using random primers and the M-MLV Reverse Transcriptase Kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA USA). For *P2RY12*, a 644 bp cDNA fragment, spanning exons 2-3, was amplified with primers 19 and 20 (Supplementary Table 1). The nested-PCR fragment (547 bp, primers 21 and 22) was sequenced (Supplementary Table 1). The cDNA allelic ratio was determined by densitometric analysis of chromatogram peaks. Evaluation of the ratios was obtained by comparison of three independent

nucleotide sequences and normalization for flanking C and T peaks. The 16 primers for *ITGB3* and *ITGA2B* cDNA studies are available on request.

We performed genetic analysis by means of whole-exome sequencing (WES), and direct DNA sequencing. For WES, genomic DNA was isolated from peripheral blood by Wizard® Genomic DNA Purification Kit (Promega Corporation, Madison, WI, USA), and coding regions were enriched by using SureSelectXT Human All Exon V5 kit (50MB; Agilent Technologies). DNA sequencing was undertaken on the HiSeq2000 platform (Illumina, San Diego, CA, USA) with 125-bp paired-end reads. Mean coverage was >95x and 51 Megabases was the target size that required ~4 Gigabases of sequencing per sample. Sequence reads were aligned to the human genome reference sequence (GRCh37/hg19). Exome analysis produced a large number of variants (~65.000 Single Nucleotide Variations and ~12.000 InDels). Variant annotation (*i.e.* exonic, intronic, UTRs; for exonic: synonymous, nonsynonymous, stop gain/loss, frameshift, allele frequency, etc) and prioritization were performed using an open–source software (Variant Studio, Illumina). To minimize the number of potentially deleterious gene defects different approaches, the following strategies were adopted: (*i*) filtering based on quality score>30; (*ii*) excluding variants having a Minor Allele Frequency (MAF) greater than 0.01; (*iii*) removing variants outside coding regions or synonymous coding variants; (*iv*) filtering the data for novelty by comparison to dbSNP (http://www.ncbi.mln.nih.gov/snp), Exome Aggregation Consortium (http://exac.broadinstitute.org/), Exome Variant Server (http://evs.gs.washington.edu/EVS), 1000 Genomes Projects (http://browser.1000genomes.org), published studies; (*v*) selecting variants that segregate according to the presumed pattern of inheritance; (*vi*) querying disease databases, such as ClinVar (http://www.ncbi.nlm.nih.gov/clinvar), OMIM, (http://www.omim.org), HGMD Locus-specific database (http://www.hgvs.org/). After these filtering strategies, the number of variants were reduced to~300-400. To further prioritize the

candidate gene defects, a functional annotation was undertaken based on effect on protein function and *a priori* knowledge of phenotype.

After this initial prioritization, additional strategies were used to find the causative mutation: *i.e.* the linkage strategy, in which multiple affected family members were sequenced to identify shared variation and, in addition, unaffected relatives were sequenced to exclude a benign variation; the overlap strategy, that is the searching for mutation in multiple unrelated patients with similar phenotype. Variants were validated by Sanger sequencing and segregation analysis of the prioritized variants was performed in additional affected family members when constitutive DNA was available.

Supplementary Results

The intron less *P2RY1* gene (coding sequence, 1122bp) sequencing detected only previously reported synonymous codons (Ala19Ala, rs1065776 C/T and Val262Val, rs701265 A/G), in the homozygous condition (CC and AA, respectively) in the patient II-5 and in the heterozygous form in her mother (I-4) and brother (II-4).

The P2RY12 mRNA extracted from platelets of the patient II-5, heterozygous for the c.318C/T polymorphism and the c.678delC frameshift mutation, was studied. The cDNA allelic ratio was estimated at the c.618C/T position through the peak area in the chromatograms derived from two independent RT-PCR and sequencing. The cDNA corresponding to the alleles 318T and 318C, which marks the c.678delC and thus the translational frameshift, were similarly represented (T/C ratio 0.77-1.27, Supplementary Figure 1). The nonsense mediated decay (NMD) mechanism is not expected to alter this ratio because of the introduction of a premature nonsense triplet in the last exon. Sequencing of the coding portion of exon 3 (1 Kb) in the cDNA failed to detect any additional mutation.

The *ITGB3* and *ITGA2B* mRNA were characterized by sequencing of the *ITGB3* and *ITGA2B* platelet cDNAs in the patient II-5. Only previously described *ITGB3* synonymous polymorphisms (Val381Val, rs15908 A/C, Glu511Glu rs4642 A/G and Arg515Arg rs4634 G/A) were detected in the heterozygous condition.

References

1. Cazenave JP, Ohlmann P, Cassel D, Eckly A, Hechler B, Gachet C. Preparation of washed platelet suspensions from human and rodent blood. Methods in Mol Biol (Clifton NJ). 2004;272:13–28

Supplementary Table 1. *P2RY12* primer sequence and position.

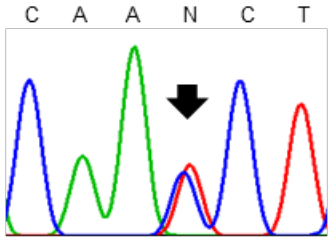
Primer	**Primer sequence (5'- 3')**	**Position** [a]
1	ACTTTCTGATCGCTTGTCTCC	4698
2	AACTCTATGCTTGGACTGGC	5244
3	TTCTCAGCCATCCTCATCCC	48839
4	TGAGGCAAAGTAACTAAGACCA	49416
5	GTGCTTTAAGAGGCAAACATTCA	50622
6	TGCCAGACTAGACCGAACTC	51471
7	TCTCTGTTGTCATCTGGGCA	51342
8	TGTCGTTTGTTTTGCTGCTAA	52170
9	GAATGTCGGTGGTTGCTTACTG	3842
10	AGGCATATGCTTGTCTTCTAAG	4452
11	TTCAGGGAAACATTTTAAGTCC	4339
12	ATTGTGATCACTACCCTGGA	5008
13	GGAAGCTGTTTCACCTACAAAG	48473
14	TCAGTAAAGTCTTGAGTGCTC	49016
15	AATACCAGATGCCACTCTGC	49081
16	ATTGGCCTCACGGAGATTCA	50249
17	GGAATGCCAACTCATGACCA	50056
18	CGCCAGGCCATTTGTGATAA	51049
19	CCACTCTGCAGGTTGCAATAAC	49092
20	TGCCAGACTAGACCGAACTC	51471
21	GATACATTCAAACCCTCCAGAATC	49126
22	TGCCTGTTGGTCAGAATCATGT	51408

Grey lines, forward primers; white lines, reverse primers. Primers 1-8, primers used to amplify and sequence exons and splicing junctions of the *P2RY12* gene; primers 9-18, primers used for the 5'

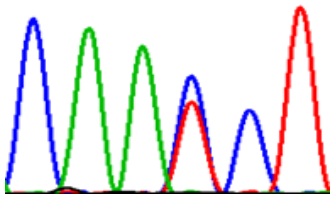
and intronic regions; primers 19-22, primers for the cDNA. [a] Corresponding to the position of the 5' end in the sequence database.

Supplementary Figure 1 - Chromatograms of the exonic nucleotide change c.318C/T (patient II-5) in the genomic DNA (upper panel) and platelet cDNA (lower panel).

P2RY12 c.318C/T



II-5
genomic DNA



II-5
platelet cDNA