

## Haploinsufficiency of the platelet P2Y<sub>12</sub> gene in a family with congenital bleeding diathesis

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### ABSTRACT

Two sisters with inherited, severe platelet dysfunction associated with P2Y<sub>12</sub> deficiency displayed a single base pair deletion in their P2Y<sub>12</sub> genes (378delC), resulting in a frame-shift and premature truncation of the protein. GL, the son of one of them, displayed mild platelet dysfunction and normal P2Y<sub>12</sub> sequence. We hypothesized that the abnormal platelet phenotype of GL is due to haploinsufficiency of his P2Y<sub>12</sub> gene. We analyzed genomic DNA from the family by Southern Blotting and real-time (RT) PCR. Southern Blotting results demonstrated that GL has a single P2Y<sub>12</sub> allele, inherited from his father. RT-PCR revealed that GL, his mother and aunt have one single intact P2Y<sub>12</sub> allele, while his father has two P2Y<sub>12</sub> alleles. The single GL P2Y<sub>12</sub> allele contains normal sequence, while his mother

and aunt have the 378delC allele. The results of this study support our hypothesis and illustrate the platelet phenotype associated with P2Y<sub>12</sub> haploinsufficiency.

Key words: haploinsufficiency, P2Y<sub>12</sub> gene, congenital bleeding diathesis.

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### Introduction

Adenosine diphosphate (ADP) activates platelets through its interaction with two P2 receptors: the G<sub>q</sub>-coupled P2Y<sub>1</sub> receptor, which mediates a transient rise in cytoplasmic Ca<sup>2+</sup>, platelet shape change and rapidly reversible aggregation, and the G<sub>i</sub>-coupled P2Y<sub>12</sub> receptor, which mediates inhibition of adenylyl cyclase and amplifies the platelet aggregation response.<sup>1</sup> Concomitant activation of both the G<sub>q</sub> and G<sub>i</sub> pathways by ADP is necessary to elicit normal aggregation. The platelet P2Y<sub>12</sub> receptor plays a very important role both in thrombosis and hemostasis, because its pharmacological inhibition with the thienopyridine drugs ticlopidine, clopidogrel and prasugrel reduces the risk of atherothrombotic events,<sup>2</sup> and its inherited defects are associated with abnormal bleeding.<sup>1</sup> Six patients with congenital defect of the platelet P2Y<sub>12</sub> receptor have been described so far: they have mild to moderate bleeding diathesis, characterized by easy bruising, mucosal bleedings, excessive post-operative hemorrhage.<sup>1,3-6</sup> The molecular basis of P2Y<sub>12</sub> defects has been analyzed in all these patients.<sup>5,9</sup> In the present study, we focused our attention on two sisters with congenital P2Y<sub>12</sub> deficiency (IG and MG) and the son of MG (GL).<sup>5</sup> Molecular

analysis of the P2Y<sub>12</sub> gene of each sister revealed an identical single base pair (bp) deletion (378delC) occurring just beyond the coding sequence for the third transmembrane domain in P2Y<sub>12</sub>; the mutation results in a frame shift (Thr126 frame shift x34) and premature truncation of the expressed protein.<sup>9</sup> Only alleles encoding the mutated DNA sequence were found by PCR analysis. Our first hypothesis was that patients IG and MG were homozygous for the 378delC mutation.<sup>9</sup> As expected, the 13-year old son GL displayed a phenotype that is compatible with partial defect of the platelet P2Y<sub>12</sub> receptor: abnormal aggregation and ATP secretion induced by several agonists, moderate deficiency of platelet-binding sites for [<sup>35</sup>S]2MeS-ADP, and partial impairment of inhibition of adenylyl cyclase by ADP.<sup>5</sup> However, PCR analysis of GL's P2Y<sub>12</sub> gene revealed normal DNA sequence only (Conley P and Cattaneo M, unpublished observations, 2001).

The present study was undertaken in order to test the hypothesis that the abnormal platelet phenotype of GL is due to haploinsufficiency of his P2Y<sub>12</sub> gene, and, accordingly, his mother and aunt suffer from P2Y<sub>12</sub> deficiency owing to haploinsufficiency and the previously described 378delC mutation in their remaining P2Y<sub>12</sub> allele.

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## Design and Methods

### Patients

The clinical characteristics of IG and her sister MG, and of LL and GL, husband and son of MG (Figure 1), have been described in a previous report.<sup>5</sup> Briefly, IG and MG had severely prolonged bleeding time and suffered a life-long history of easy bruising, epistaxis, menorrhagia, and bleeding complications after dental extractions or major surgery. GL had mildly prolonged bleeding time, never suffered spontaneous bleeding episodes and never underwent surgical interventions. LL had normal bleeding time and never suffered abnormal bleeding episodes. Informed consent was obtained from all the study subjects. The study was approved by the Ethical Committee of the Ospedale San Paolo, Milano, Italy.

### PCR and sequencing

Genomic DNA was isolated from peripheral blood using standard protocols. To screen the *P2Y<sub>12</sub>* gene for mutations, we performed direct sequencing of the entire coding region in the genomic DNA. Exon 2 of the *P2Y<sub>12</sub>* gene, which encodes the entire 342-amino acid protein, and flanking sequences were amplified by PCR as described.<sup>6</sup> The same amplification primers and two additional internal primers (P2Y12-3 5'-gctaccagaagaccaccagg-3', P2Y12-4 5'-tctcggctgctgttgctag-3') were used in the sequencing reactions. Cycle sequencing was performed using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready reaction Kit (Applied Biosystems, Foster City, CA, USA). The fragments were sequenced by automated sequencing analysis on an ABI Prism 310 sequencer (Applied Biosystems).

### Southern blot analysis

DNA was digested with *Bam*HI (Figure 2B). After agarose gel electrophoresis and transfer onto nitrocellulose membranes (BioRad), the genomic DNA was hybridized with a DNA probe that had been labeled with <sup>32</sup>P using the Primer-It® II Random Primer Labeling kit (Stratagene), according to the manufacturer's instructions. The radiolabeled probe was generated by PCR amplification of exon 2 of the *P2Y<sub>12</sub>* gene (Figure 2A). Hybridization was performed in ExpressHyb™ Hybridization Solution (Clontech, Celbio). Membranes were visualized by autoradiography by using Kodak Biomax MS imaging film (Fisher Scientific).

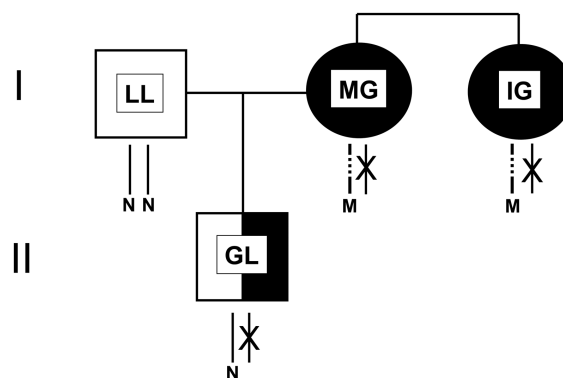
### Real-time quantitative PCR

DNA copy numbers for *P2Y<sub>12</sub>* were determined in a 7900HT Sequence Detection System (Applied Biosystems) using the DNA-binding dye SYBR Green. To account for possible variations related to DNA input amounts or the presence of PCR inhibitors, two reference genes (both located on chromosome 3, like *P2Y<sub>12</sub>* gene), *ZNF80* (3q13.3) and *GPR15* (3q11.2-q13.1), were simultaneously quantified in separate tubes for each patient sample. Amplification of exon 2 of *P2Y<sub>12</sub>* was done using primers that had been designed using Primer Express 2.0 software (Applied Biosystems) (P2Y12-9 5'-caaaagaactgtaccggtcatcag-3', P2Y12-10 5'-tggtcagggtg-

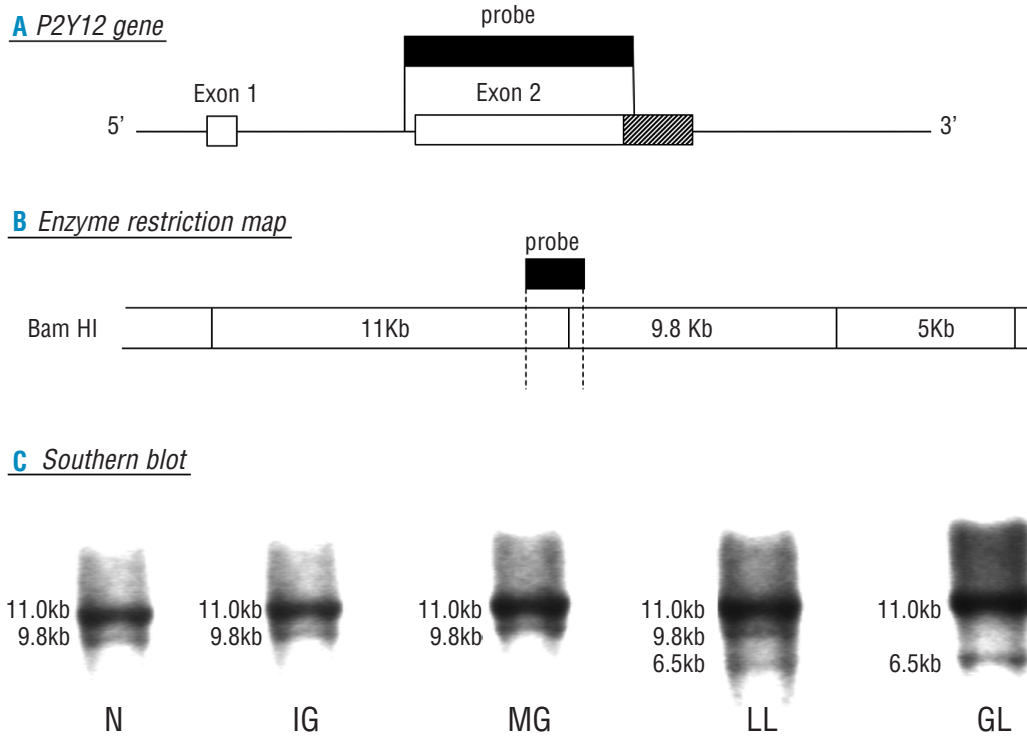
taaggaattc-3'). Amplification of *ZNF80* and *GPR15* genes was carried out using the primers described by Hoebeeck *et al.*<sup>10</sup> The PCR reaction mix (25  $\mu$ L) contained iTaq SYBR Green Supermix with ROX (Bio Rad), 200 nM of each forward and reverse primer and 10 ng template DNA. The cycling conditions were as follows: 10 min at 95°C, 40 cycles at 95°C for 15 s and 60°C for 60 s. After PCR amplification, a melting curve was generated for every PCR product to check for the specificity of the PCR reaction (absence of primer-dimers or other non-specific amplification products). Each assay included: a no-template control, 10 ng of calibrator human genomic DNA (Roche Applied Science, Basel, Switzerland, DNA mixture from healthy individuals) and about 10 ng of test DNA, all in duplex. Calculation of the gene copy number was performed using the  $\Delta\Delta$ -Ct method, which transforms the threshold cycle (Ct) values into normalized relative target amounts, by relating the Ct value of the target gene in the sample to a calibrator sample and the Ct value of a reference gene measured in both samples.<sup>11</sup> Using this method, a haploid copy number of 1 is expected for a normal sample and a haploid copy number of 0.5 for a sample with deletion of the PCR target gene in one allele. We established with preliminary experiments that, for each *P2Y<sub>12</sub>* exon, a haploid copy number <0.7 was considered indicative of hemizygous deletion and a haploid copy number >0.8 was considered normal. Values between <0.7 and >0.8 were considered non-informative.

## Results and Discussion

Sequence analysis of exon 2 of the *P2Y<sub>12</sub>* gene revealed that patients IG and MG had the single bp deletion (378delC) that had been previously described by Conley *et al.*<sup>9</sup> In contrast, normal *P2Y<sub>12</sub>* coding sequence was identified using DNA obtained from GL (son of MG) and LL (father of GL).



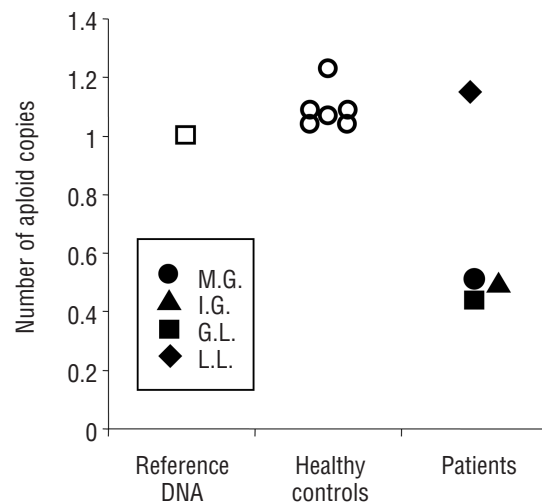
**Figure 1.** Pedigree of the index family. Black symbols: subjects whose platelet phenotype is suggestive of severe *P2Y<sub>12</sub>* deficiency, based on the results of studies that included [<sup>32</sup>P]-2Me-S-ADP binding to platelets, platelet aggregation and inhibition of PGE<sub>1</sub>-induced cyclic AMP formation by ADP;<sup>5</sup> black and white symbol: subject whose platelet phenotype is suggestive of partial *P2Y<sub>12</sub>* deficiency;<sup>5</sup> white symbol: subject whose platelet phenotype is suggestive of normal expression of *P2Y<sub>12</sub>*. A schematic representation of alleles is shown below each individual. (N, normal *P2Y<sub>12</sub>* allele; M, mutant 378delC allele; X, deleted *P2Y<sub>12</sub>* allele).



**Figure 2.** Design of the experiments of Southern blot analysis. (A) A radiolabeled probe was generated that spans exon 2 of the P2Y<sub>12</sub> gene. (B) An expanded region of the P2Y<sub>12</sub> gene is shown, with the relevant BamHI restriction sites (see text for further details). (C) Southern blot analysis of genomic DNA from one healthy control (N), patients IG and MG, two sisters with severe deficiency of the platelet ADP receptor P2Y<sub>12</sub>, patient GL (son of MG) and his father, LL (husband of MG). DNA was digested with BamHI and, after agarose gel electrophoresis and transfer onto nitrocellulose membrane, hybridized with a <sup>32</sup>P-labeled DNA probe, which was generated by PCR amplification of exon 2 of the P2Y<sub>12</sub> gene (see Methods for further details). As expected, digestion with BamHI of genomic DNA of the healthy volunteer revealed two autoradiographic signals of 11 kb and 9.8 kb. Identical results were obtained with the DNA of patients IG and MG, while digestion of DNA from LL resulted in the generation of a 6.5 kb fragment, in addition to the 11 and 9.8 kb fragments: the generation of the 6.5 Kb band is likely due to a mutation in the P2Y<sub>12</sub> gene, which does not affect P2Y<sub>12</sub> expression or function, as demonstrated by the normal platelet phenotype of subject LL.<sup>5</sup> Digestion of DNA from GL generated one normal band (11 Kb), but no 9.8 Kb band, and the same 6.5 Kb band that was observed after digestion of his father's DNA.

As expected, Southern blot analysis of normal DNA, after digestion with BamHI and hybridization with the radiolabeled probe including exon 2, revealed two bands of 11 kilobase pairs (kb) and 9.8 kb (Figure 2, C, lane 1). Identical results were obtained with the DNA of patients IG and MG (Figure 2C, lanes 2 and 3). In contrast, BamHI digestion of DNA from subject LL, father of GL and husband of MG, resulted in the generation of an abnormal band of 6.5 Kb, in addition to the two normal bands (Figure 2C, lane 4); the generation of the 6.5 Kb band is likely due to a mutation in the P2Y<sub>12</sub> gene, which does not affect P2Y<sub>12</sub> expression or function, as demonstrated by the normal platelet phenotype of subject LL.<sup>5</sup>

Digestion of DNA from GL with BamHI generated one normal band (11 kb), but no 9.8 kb band, and the same 6.5 Kb band that was observed after digestion of his father's DNA (Figure 2C, lane 5). Confirming our working hypothesis, these data suggest that: (i) subject LL has a normal DNA sequence on one chromosome, giving rise to the 11 kb and 9.8 kb fragments upon digestion with BamHI, and a mutation associated with his second P2Y<sub>12</sub> allele, which is responsible for the creation of a new Bam HI restriction site and the genera-



**Figure 3.** Results of quantitative real-time PCR. DNA copy numbers for P2Y<sub>12</sub> in 7 healthy controls, patients IG and MG, with severe P2Y<sub>12</sub> deficiency, patient GL, son of MG, and LL, father of GL. In addition to 10 ng of test DNA, each assay included 10 ng of calibrator human genomic reference DNA (see Design and Methods for further details).

tion of an abnormal 6.5 Kb fragment, instead of the normal 9.8 Kb band; (ii) MG (mother of GL and wife of LL), and her sister IG, have an allele on one chromosome that is normally digested by *Bam*HI and carries the already described single bp deletion (378delC) in the open reading frame of exon 2 of P2Y<sub>12</sub>, and an allele with a genomic deletion spanning either the entire P2Y<sub>12</sub> gene or a large portion of it, on the other chromosome; (iii) GL (son of LL and MG) inherited the allele identified by the presence of a 6.5 kb *Bam*HI fragment, and the allele with a genomic deletion from his mother (Figure 1).

Our working hypothesis was further supported by the results of quantitative, real-time PCR, which revealed that GL, his mother and aunt each contained a single intact P2Y<sub>12</sub> allele, while his father contained two P2Y<sub>12</sub> alleles, comparable to normal controls (Figure 3). However, GL's single allele encodes a normal P2Y<sub>12</sub>,

while his mother and aunt each have a single mutant (378delC) allele, which encodes a truncated P2Y<sub>12</sub>. This explains the differences in clinical phenotypes between GL and his mother and aunt.<sup>5</sup>

Therefore, the results of both quantitative PCR and Southern blotting analysis, support our hypothesis and illustrates the platelet phenotype associated with P2Y<sub>12</sub> haploinsufficiency.

## Authorship and Disclosures

GF: performance of the laboratory tests, revision of the manuscript; JW: design of the study, revision of the manuscript; MC: design of the study, writing of the manuscript.

The authors reported no potential conflicts of interest.

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