Haploinsufficiency of the platelet P2Y12 gene in a family with congenital bleeding diathesis

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ABSTRACT

Two sisters with inherited, severe platelet dysfunction associated with P2Y12 deficiency displayed a single base pair deletion in their P2Y12 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 P2Y12 sequence. We hypothesized that the abnormal platelet phenotype of GL is due to haploinsufficiency of his P2Y12 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 P2Y12 allele, inherited from his father. RT-PCR revealed that GL, his mother and aunt have one single intact P2Y12 allele, while his father has two P2Y12 alleles. The single GL P2Y12 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 P2Y12 haploinsufficiency.

Key words: haploinsufficiency, P2Y(12) gene, congenital bleeding diathesis.

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Introduction

Adenosine diphosphate (ADP) activates platelets through its interaction with two P2 receptors: the Gq-coupled P2Y1 receptor, which mediates a transient rise in cytoplasmic Ca2+, platelet shape change and rapidly reversible aggregation, and the Gi-coupled P2Y12 receptor, which mediates inhibition of adenylyl cyclase and amplifies the platelet aggregation response.1 Concomitant activation of both the Gq and Gi pathways by ADP is necessary to elicit normal aggregation. The platelet P2Y1 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,2 and its inherited defects are associated with abnormal bleeding.1 Six patients with congenital defect of the platelet P2Y12 receptor have been described so far: they have mild to moderate bleeding diathesis, characterized by easy bruising, mucosal bleedings, excessive post-operative hemorrhage.1,3-6 The molecular basis of P2Y12 defects has been analyzed in all these patients.5-9 In the present study, we focused our attention on two sisters with congenital P2Y12 deficiency (IG and MG) and the son of MG (GL). In the present study, we focused our attention on two sisters with congenital P2Y12 deficiency (IG and MG) and the son of MG (GL). Molecular analysis of the P2Y12 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 P2Y12; the mutation results in a frame shift (Thr126 frame shift x34) and premature truncation of the expressed protein.9 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.9 As expected, the 13-year old son GL displayed a phenotype that is compatible with partial defect of the platelet P2Y12 receptor: abnormal aggregation and ATP secretion induced by several agonists, moderate deficiency of platelet-binding sites for [33P]2MeS-ADP, and partial impairment of inhibition of adenylate cyclase by ADP.9 However, PCR analysis of GL's P2Y12 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 P2Y12 gene, and, accordingly, his mother and aunt suffer from P2Y12 deficiency owing to haploinsufficiency and the previously described 378delC mutation in their remaining P2Y12 allele.
**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. Briefly, IG and MG had severely prolonged bleeding time and suffered a lifelong 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. The same amplification primers and two additional internal primers (P2Y12-3 5'-gctaccagaagaccaccagg-3', P2Y12-4 5'-tctcggctgcctgttggtcag-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 BamHI (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<sup>™</sup> Hybridization Solution (Clontech, Cellbio). 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) (P2Y12-2 5'-taaggattcgcctgttgga-3', P2Y12-10 5'-tggctcagggtcatgtagt-3'). Amplification of ZNF80 and GPR15 genes was carried out using the primers described by Hoebeck et al. The PCR reaction mix (25 μL) contained Taq 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 Δ-A-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. 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 (S78delC) that had been previously described by Conley et al. 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; 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.
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 \( P2Y_{12} \) gene, which does not affect \( P2Y_{12} \) expression or function, as demonstrated by the normal platelet phenotype of subject LL.5

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.

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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 3. Results of quantitative real-time PCR. DNA copy numbers for \( P2Y_{12} \) in 7 healthy controls, patients IG and MG, with severe \( P2Y_{12} \) 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 BamHI and carries the already described single bp deletion (378delC) in the open reading frame of exon 2 of P2Y12; and an allele with a genomic deletion spanning either the entire P2Y12 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 BamHI 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 P2Y12 allele, while his father contained two P2Y12 alleles, comparable to normal controls (Figure 3). However, GL’s single allele encodes a normal P2Y12, while his mother and aunt each have a single mutant (378delC) allele, which encodes a truncated P2Y12. This explains the differences in clinical phenotypes between GL and his mother and aunt. Therefore, the results of both quantitative PCR and Southern blotting analysis, support our hypothesis and illustrates the platelet phenotype associated with P2Y12 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.

References