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A new multiplex PCR and conformation-sensitive gel electrophoresis strategy for mutation detection in the platelet glycoprotein α_{IIb} and β_3 genes

G. JAYANDHARAN, E. J. R. NELSON, S. BAIDYA, M. CHANDY and A. SRIVASTAVA
Department of Haematology, Christian Medical College, Vellore, India

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Glanzmann thrombasthenia (GT) (OMIM 273800) is a rare, autosomal recessive bleeding disorder, caused by a quantitative and/or qualitative defect of the platelet glycoprotein (GP) $\alpha_{IIb}\beta_3$ (IIb–IIIa or integrin [ITG]A2B–ITGB3) complex [1]. Several genetic defects (~160) associated with GT have been identified in either the α_{IIb} or β_3 genes (http://sinaicentral.mssm.edu/intranet/research/glanzmann/menu; accessed 13 November 2006). A majority (75%) of them are point mutations, the detection of which has been difficult because of the combined size of 67 kb and the presence of 45 exons in α_{IIb} and β_3. The currently available mutation-screening methods for α_{IIb} and β_3 are Southern blotting, denaturing gradient gel electrophoresis (DGGE) and single-strand conformation polymorphism analysis (SSCPA) [2–4]. These methods require ~35–50 amplification reactions, may involve radiolabeling or laborious optimization procedures, and tend to have less than 90% sensitivity. We report here a simplified method for mutation screening in α_{IIb} and β_3 by multiplex polymerase chain reaction (PCR) amplification (MPCR) and conformation-sensitive gel electrophoresis (CSGE).

Sixteen DNA samples from GT patients with mutations previously identified ($n = 17$) by PCR–SSCPA [5,6] were used as positive controls for validating the MPCR–CSGE strategy. This method was then used to evaluate samples from four unrelated patients with hematologically confirmed GT but in whom candidate disease-causing mutations had not been identified by the PCR–SSCPA technique (Table 1) [5]. Analysis of surface expression of α_{IIb} and β_3 was performed by flow cytometry with antibodies 10E5 (anti-α_{IIb}/β_3, CD41/61), 7E3 (anti-α_{IIb}/β_3 + anti-vitronectin, CD41/61 and CD51/61 respectively), and 6D1 (anti-α_{Ib}, CD42b). Western blot analysis for platelet $\alpha II_b\beta_3$ was performed as described previously [5]. Ten MPCR groups were designed to coamplify the α_{IIb} and β_3 coding and flanking intronic sequences and the β_3 promoter region (Table 2) using 37 primer pairs described elsewhere [4,7]. The β_3 promoter + exon 1 region was amplified as a 299-bp fragment using forward primer 5′-TAGAGAAGCGCGAGGGGATCT-3′ and reverse primer 5′-AGATCCTGGGGCAGCTGCGA-3′. MPCR for groups 1–10 (except for groups 5 and 8) was performed in a 25-μL volume containing 250–500 ng of genomic DNA, 250 μM each dNTP, and 1.05 units of Expand High Fidelity PCR system, in the supplied buffer-2 (Roche Diagnostics, GmbH, Mannheim, Germany). Dimethylsulfoxide (7.5%) was included for the multiplex amplification of groups 1 and 7. MPCR for groups 5 and 8 was performed in a 25-μL reaction volume containing 250–500 ng of genomic DNA, 750 μM each dNTP, and 2 units of DyNAzyme II™ DNA polymerase (Finnzymes, Espoo, Finland), in the supplied buffer at a concentration of 2× [20 mM Tris–HCl (pH 8.8), 100 mM KCl and 0.2% Triton X-100]. Following an initial denaturation at 94 °C for 5 min, 30 cycles of PCR amplification were performed, as follows: denaturing at 94 °C for 1 min, annealing at the specific temperatures for 1 min, and extension at 72 °C for 1 min. The final extension was performed at 72 °C for 5 min. MPCR products were screened by CSGE as described previously [8]. Abnormal CSGE profiles were

Correspondence: Alok Srivastava, Department of Haematology, Christian Medical College, Vellore 632 004, TN, India.
Tel.: +91 416 2282352; fax: +91 416 2232035; e-mail: aloks@cmcvellore.ac.in

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Table 1 Clinical, hematologic, biochemical and molecular genetic data of the four Glanzmann thrombasthenia (GT) patients who were evaluated with the validated multiplex PCR (MPCR)–conformation-sensitive gel electrophoresis strategy

						Hematologic evaluation						Platelet proteins					
								Aggregation				FCM, 10E5 (%)	Western blot				
UPN	Sex/age (years)	Site of bleeding	Consanguinity	Type of GT†	No. of Tx	BT (min)	Clot retraction	ADP	EPI	COL	RIS	$\alpha_{IIb}\beta_3$	$\alpha_{IIb}\beta_3$	β_3	Fibrinogen	Mutation*	Polymorphisms
12	F/15	Skin, oral, menstrual, dental	Yes	II	0	> 15	A	A	A	A	W	32.7	0	+	0	β_3 c.646T > C, p.Tyr190His (Homozygous)‡	Nil
13	F/41	Skin, oral, menstrual	No	I	15	> 15	A	A	A	A	N	0.5	0	0	0	α_{IIb} c.1750C > T, p.Arg553 (583)stop (homozygous)‡	β_3 c.1533G > A, p.Glu485Glu (heterozygous) β_3 c.1545A > G, p.Arg489Arg (heterozygous) β_3 c.186T > C, p.Leu33Pro (heterozygous) β_3 c.2301 + 9T > C (heterozygous)‡
14	M/4	Skin, oral, gastrointestinal	Yes	III	0	> 15	Poor	A	A	A	N	95.5	4 +	4 +	4 +	Not detected	α_{IIb} c.2188-7C > G (homozygous) α_{IIb} c.2621T > G, p.Ile843Ser (homozygous) α_{IIb} c.3063C > T, p.Val990Val (homozygous)
15	F/13	Skin, oral	Yes	II	0	> 15	A	A	A	A	W	14.4	0	+	0	Not detected	β_3 c.186T > C, p.Leu33Pro (heterozygous) β_3 c.1690 + 23C > G (heterozygous) β_3 c.1533G > A, p.Glu485Glu (heterozygous) β_3 c.1545A > G, p.Arg489Arg (heterozygous)

No. of Tx, number of units of platelet transfusion.

UPN, unique patient identification number; BT, bleeding time; ADP, adenosine diphosphate; EPI, epinephrine; COL, collagen; RIS, ristocetin; A, absent; W, weak; N, normal; FCM, flow cytometry.

*For α_{IIb}, the exon numbering is according to [12], and for β_3, the exon numbering has been modified to include the nucleotides encoding the amino acid residues of the leader sequence as exon 1 [13] and to increase by one the exon numbering according to [14]. The nucleotide numbering of the 'A' nucleotide of the ATG start codon was designated + 1 (GenBank accession number for α_{IIb} cDNA, NM_00419.2; β_3 cDNA nucleotide sequence NM_000212.2).

†Classification according to George *et al.* [1].

‡Nucleotide changes detected only by CSGE and not by single-strand conformation polymorphism analysis.

Table 2 Organization of the exons of α_{IIb} and β_3 into 10 multiplex groups (GT mpx 1–10) and the conditions for their multiplex PCR (MPCR) amplification

MPCR group	Annealing temperature (°C)	Primers	Size (bp)	Forward and reverse primers (pmol)	Final concentration of $MgCl_2$ (mM)	dNTPs (μ L^{-1})	DMSO (%)	DNA polymerase (units)
GTmpx-1	60	GpIIb19 + 20	402	7.5	2.5	300	7.5	1.16
		GpIIb12	284	7.5				
		GpIIb5 + 6	229	7.5				
		GpIIb18	193	7.5				
GTmpx-2	60	GpIIIa11	293	7.5	2.5	250	–	1.16
		GpIIb4	237	7.5				
		GpIIb29	214	7.5				
		GpIIb22	143	7.5				
GTmpx-3	57	GpIIb23 + 24	361	7.5	2.5	250	–	1.16
		GpIIb10 + 11	322	7.5				
		GpIIIa14	284	7.5				
		GpIIb7	190	7.5				
GTmpx-4	57	GpIIb16 + 17	360	7.5	1.5	250	–	1.16
		GpIIIa10B	289	7.5				
		GpIIIa5	240	7.5				
GTmpx-5	55	GpIIb2 + 3	364	7.5	3.5	750	–	2
		GpIIb14 + 15	329	7.5				
		GpIIb13	248	7.5				
		GpIIb21	192	7.5				
GTmpx-6	54	GpIIb8 + 9	338	7.5	1.5	250	–	1.16
		GpIIb1	281	7.5				
		GpIIIa7	220	7.5				
		GpIIb30	130	7.5				
GTmpx-7	62	GpIIIa promoter + 1	299	7.5	1.5	300	7.5	1.16
		GpIIb25	255	7.5				
		GpIIb26	208	7.5				
		GpIIb27	189	30				
GTmpx-8	50	GpIIIa3	267	7.5	4	750	–	2
		GpIIIa6	238	7.5				
		GpIIIa13	207	7.5				
		GpIIIa12	171	7.5				
		GpIIIa2	157	12.5				
GTmpx-9	52	GpIIIa4	319	7.5	3.5	250	–	1.16
		GpIIIa9	209	7.5				
		GpIIIa8	165	7.5				
GTmpx-10	52	GpIIIa10A	302	7.5	2.5	250	–	1.16
		GpIIb28	203	7.5				
		GpIIIa15	144	7.5				

DMSO, dimethylsulfoxide.

sequenced (ABI 310 Genetic Analyzer; Applied Biosystems, Foster City, CA, USA). PCR and restriction enzyme analysis for α_{IIb} exons 16 + 17 and β_3 exon 5 was carried out with *Taq*I and *Hae*III (Amersham BioSciences, Buckinghamshire, UK) to confirm the candidate disease-causing mutations identified. Reverse transcription-PCR amplification of a 608-bp fragment corresponding to exons 13–18 of α_{IIb} mRNA of patient 13 and a normal control was performed using exonic primers (forward, 5′-GGCCAAGTGCTGGTGTTC-3′; reverse, 5′-CTCCATGCAGCACGACAG-3′) specific for this region.

All 17 PCR–SSCPA-identified mutations (eight missense, five nonsense and four frameshifts) could be detected with the MPCR–CSGE strategy. We were also able to detect three additional nucleotide changes that were not previously detected by PCR–SSCPA [5]. Two of these were candidate disease-causing homozygous mutations. A c.1750C > T transition in exon 17 of α_{IIb} leading to a premature stop codon, p.Arg553-stop, in the mature α_{IIb} protein (patient 13) and a novel c.646T > C transition in exon 5 of β_3 causing a p.Tyr190His substitution in β_3 (patient 12) were identified. A c.2301 + 9T > C polymorphism (heterozygous) was also detected in β_3 in patient 13. The c.1750C > T transition abolishes a *Taq*I restriction site, and the c.646T > C transition creates an *Hae*III restriction site. Both of these mutations were confirmed in the probands and their parents. RT-PCR amplification of the region spanning exons 13–18 of α_{IIb} in patient 13 revealed two cDNA products of ~530 bp and ~608 bp relative to a normal control (608 bp). Sequencing revealed a deletion of 75 bp (1675–1750del) in the 530-bp cDNA product and a c.1750C > T transition in the 608-bp cDNA product. The relative amounts of these two cDNA fragments were 62% for the transcript containing the 75-bp deletion and 38% for the

transcript containing the 1750C > T transition (QUANTITY ONE software, BioRad, Hercules, CA, USA).

A simplified method for mutation screening of α_{IIb} and β_3 has been developed by multiplexing PCR amplifications and using CSGE for detection of mobility shifts of the amplicons. It is possible to perform a mutation screen in 4 days using this method. The sensitivity of this method also seems to be good, as we were able to detect three nucleotide changes that were not previously detected by PCR–SSCPA [5]. Apart from being easier to perform, the other advantage of this method is the simultaneous detection of multiple mutations. Screening for multiple nucleotide changes is especially important in GT, as ~40% of all α_{IIb} and β_3 mutations reported are compound heterozygous (http://sinaicentral.mssm.edu/intranet/research/glanzmann/menu; accessed 13 November 2006). The overall detection rate with MPCR–CSGE can be estimated to be about 90% ($n = 19/21$), as it detected 17 known and two unknown GT causative mutations. This compares well with the sensitivity (75–82%) of the other techniques for mutation detection in α_{IIb} and β_3 genes, such as PCR–SSCPA [7], DGGE [3] and direct sequencing [9]. There may be several reasons for not identifying mutations in patients 14 and 15. The design of the primers does not permit the identification of intronic changes that might affect transcription. Previous studies [3,9] have also reported an inability to find α_{IIb} and β_3 mutations in ~20% of GT patients, where the defect can be caused by regulatory elements affecting the transcription or by impairment of integrin signaling [10,11].

The p.Arg553stop mutation identified in α_{IIb} has been reported three times in the GT database (http://sinaicentral.mssm.edu/intranet/research/glanzmann/menu; accessed 13 November 2006). This patient had no active α_{IIb} mRNA, because of the loss of amino acid residues 529(560)–553(584) in the mRNA expressed from one allele, and a spliced transcript that carried a premature stop codon, Arg553stop, in the mature α_{IIb} protein in the other, leading to a type I GT phenotype. The β_3 Tyr190His novel mutation is also possibly causative of type II GT. This mutation was absent in 100 normal control alleles screened and occurs at a residue conserved in five different species (NCBI Accession: human AH002711, canine AF116270, rat NM_016780, rabbit AF170529, pig AF170527). Several other examples of thrombasthenic mutations around codon Tyr190, such as Val193Met, Leu196Pro, and Arg214Gln, have been reported to cause type II GT by possibly affecting platelet aggregation through a defect in the binding of soluble fibrinogen (http://sinaicentral.mssm.edu/intranet/research/glanzmann/menu; accessed 13 November 2006).

We conclude that MPCR–CSGE is a simple and sensitive method for mutation screening in α_{IIb} and β_3.

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Disclosure of Conflict of Interests

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