Accepted: 3 September 2017

DOI: 10.1111/ejh.12965

ORIGINAL ARTICLE

WILEY European Journal of Haematology

Novel mutations in Thai patients with glanzmann thrombasthenia

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Funding information
The Ratchadapiseksompotch Fund,Faculty of Medicine, Chulalongkorn University, Grant/Award Number: RA58/074; Chulalongkorn Academic Advancement into Its 2nd Century Project and Thailand Research Fund, Grant/Award Number: BRG5980001

Abstract

Objectives: Glanzmann thrombasthenia (GT) is an autosomal recessive platelet disorder, caused by defects of the platelet integrin αIIbβ3 (GPIIb/IIIa) resulting from pathogenic mutations in either *ITGA2B* or *ITGB3*. It is characterized by spontaneous mucocutaneous bleeding. The molecular features of GT in Thailand have not been identified. This study aimed to determine the clinical and molecular features of unrelated Thai patients with GT.

Methods: Four patients with clinically suspected GT were recruited at the Division of Pediatric Hematology/Oncology, King Chulalongkorn Memorial Hospital. The diagnosis was based on clinical and hematological parameters as well as genetic analysis. Whole exome sequencing (WES) was performed in all cases.

Results: Of the four patients studied, the median age at first suspicion of GT was 2.5 years. All presented with severe bleeding symptoms (WHO bleeding scale 3). Flow cytometry to assess the surface GPIIb/IIIa complex showed reduced expression. By WES, we successfully identified seven mutant alleles in *ITGA2B*. One alteration, the c.2915dup (p.Leu973Alafs*63), was detected in two unrelated families. One patient was homozygous for the c.617T>A (p.Val206Asp). Of the five different mutations, three have never been previously described. These include a missense, c.617T>A (p.Val206Asp), a deletion, c.1524_1533del (p.Gln508Hisfs*3), and a nonsense, c.2344C>T (p.Arg782Ter).

Conclusion: This study reported three novel mutations expanding the genotypic spectrum of *ITGA2B* causing GT.

KEYWORDS
glanzmann thrombasthenia, ITGA2B, ITGB3, novel, variants

1 | INTRODUCTION

Glanzmann thrombasthenia (GT; MIM #273800) is an autosomal recessive platelet disorder, characterized by spontaneous mucocutaneous bleeding, prolonged bleeding time, and impaired or absent platelet aggregation in response to multiple physiologic agonists including adenosine diphosphate (ADP), arachidonic acid (ARA), and collagen. It is caused by qualitative and/or quantitative defects of the platelet glycoprotein (GP) IIb/IIIa complex (platelet integrin αIIbβ3).[1] αIIbβ3 functions as a key mediator of platelet aggregation.

Ittiwut and Suchartlikitwong are equally contributed to this study.

Mutations in the integrin αIIbβ3 receptor subunit genes *ITGA2B* and *ITGB3* have been identified in patients with GT. Both genes are located on chromosome 17 and code for the platelet integrin αIIbβ3. The *ITGA2B* contains 30 exons while the *ITGB3* has 15 exons.[2,3] The bleeding phenotype among patients with GT is quite variable. GT has been classified into three types based on the levels of αIIbβ3 on the platelet surface. Type I contains 0%-5% and type II has 5%-20% of normal αIIbβ3. GT type III has near normal levels but non-functional αIIbβ3.[4]

At least 150 and 100 different causative variants have been described in *ITGA2B* and *ITGB3*, respectively (Human Gene Mutation Database (HGMD; http://www.hgmd.cf.ac.uk/ac/index.php), accessed June 4, 2017). The majority of mutations include missense and nonsense mutations, small deletions, and insertions.[5] There have been limited studies of GT in the Thai population. Here, we report on the clinical and molecular characteristics of four unrelated Thai families with GT. Seven causative variants in *ITGA2B* were identified. Three have never been described expanding the genotypic spectrum of *ITGA2B*.

2 | MATERIALS AND METHODS

2.1 | Patients and families

A total of four unrelated patients with GT were recruited in the study. Written informed consent was obtained from all patients and their parents according to a protocol approved by the Institutional Review Board, Faculty of Medicine, Chulalongkorn University (Med Chula IRB No.051/58). Both clinical and laboratory data of each patient were collected from their medical records which included age, gender, age of onset, age at diagnosis, bleeding manifestations, family history of hemostatic defects and consanguinity, platelet count number and morphology, bleeding time measurements, the results of clot retraction, and platelet aggregation test. The bleeding severity was graded by WHO bleeding scale.

2.2 | Whole blood platelet aggregation test

Platelet aggregation tests were performed using the Chrono-log Whole Blood Aggregometer (Chrono-log WBA, model 592, Havertown, PA, USA) based on electrical impedance to measure platelet aggregation in whole blood. An aliquot of citrated whole blood (0.5 mL) was diluted with an equivalent volume of isotonic saline in the sample cuvette and incubated for 6 minutes at 37°C in the instrument reaction well. The electrode was inserted in the sample cuvette, and then uniform monolayers of platelets were formed and coated the electrode wires during incubation. A small voltage was applied, and the impedance caused by the platelets coating the electrode wires was measured as a baseline. After a stable baseline was established, the agonist such as ADP 10 μmol/L, arachidonic acid 0.5 mmol/L, ristocetin 1.0 mg/mL, and collagen 2 μg/mL was added to the sample in each reaction well. Platelets in the specimen were activated and began to aggregate. The coating of platelets was thicker on the wires over the next 6 minutes with a corresponding increase in electrical impedance between the electrode wires. This change in impedance was directly proportional to the extent of platelet aggregation and was indicated as amplitude reported in ohms.

2.3 | Flow cytometry analysis of platelets

Briefly, the blood sample from each patient was obtained, using ethylene diamine tetra-acetic acid (EDTA) as anticoagulant. Platelet-rich plasma (PRP) of each patient was prepared by centrifugation at 200 *g* for 5 minutes. The amount of integrin αIIbβ3 on platelet surface was measured by flow cytometry method using monoclonal antibodies (Ab) against glycoprotein IIb (CD41) and glycoprotein IIIa (CD61) (Biolegend, San Diego, CA, USA). Fluorescence intensity was measured with theBD Accuri™ C6 flow cytometer and analyzed with BD Accuri™ C6 Plus software.

2.4 | Whole exome sequencing

After informed consent, genomic DNA was extracted from peripheral blood leukocytes obtained from the patients and their available parents using a Puregene blood kit (Qiagen, Hilden, Germany). As the *ITGA2B* (NM_000419.4) and *ITGB3* (NM_000212.2) genes consisted of 30 exons and 15 exons, respectively, whole exome sequencing (WES) was performed. WES can help speed up the process and reduce the cost. The DNA sample was prepared as an Illumina sequencing library and in the exome capture step. The sequencing libraries were enriched by SureSelect Human All Exon V5 kits. The captured libraries were sequenced using IluminaHiSeq 2000 Sequencer at Macrogen Inc., Seoul, Korea. The raw data per exome were aligned to the human genome reference sequence (UCSC hg19) using Burrows-Wheeler Alignment. Variant calling was performed using GATK with HaplotypeCaller. The variants were subsequently filtered out if they were present in our in-house database of 678 unrelated Thai exomes. The variants would be called novel if they were not listed in the Human Gene Mutation database (www.hgmd.cf.ac.uk/ac/index.php) and the Exome Aggregation Consortium database (exac.broadinstitute.org).

2.5 | Segregation analysis

Polymerase chain reaction (PCR) and direct sequencing were performed in the DNA samples of the patients and their available parents to verify the presence of the identified mutations. PCR primers are shown in Table 1. The PCR products were sent for Sanger sequencing by Macrogen Inc. (Seoul, South Korea). The sequences were analyzed and compared to those of the unaffected control using the Mutation surveyor and Chromas Lite software (Technelysium Pty Ltd, South Brisbane, Australia). For the description of sequence variants, Mutalyzer 2.0.25 was used according to the guideline of the Human Genome Variation Society (HGVS).[6] Amino acid conservation was analyzed using Ensembl Ortholog alignment (https://doi.org/10.1093/database/bav096).

TABLE 1 Primer sequences for Sanger sequencing of novel mutations

Variant	Primer name	Primer sequence from 5′ to 3′	Tm (°C)
c.617T>A	ITGA2B_E5F ITGA2B_E5R	5′-AGCCCTTGCTTTGGATCTG-3′ 5′-AAGAAATAATAGCCGCCAGGA3′	53
c.1524_1533del	ITGA2B_delF ITGA2B_delR	5′-CCATCACCCTATCCCATCAG-3′ 5′-CAAAGACGTAAGTGGGGCTCA-3	60
c.2344C>T	ITGA2B_E23F ITGA2B_E23R	5′-GTTTCTCCTCATCCCCTCC-3′ 5′-GAGAGAGTGTGATGCCCTG-3′	58

3 | RESULTS

3.1 | Clinical and hematological findings

Of four patients recruited in this study, the median age at first suspicion of GT was 2.5 years. All patients were male and presented with severe bleeding symptoms as measured by WHO bleeding scale of 3. The common presentations included recurrent epistaxis, bleeding in oral cavity, and easy-bruising spontaneously or after trauma. There was one patient with parental consanguinity (Table 2). No history of hemostatic defects was noted in any patients' family members. The platelet count number was between 201 000 and 479 000/mm^3. The platelet size and stain were also normal in all patients. However, all had abnormal clot retraction and prolonged bleeding time. The platelet aggregation test was typically defective with all physiological agonists except ristocetin. Flow cytometry to analyze the platelet surface CD41 and CD61 showed reduced expression in all patients.

3.2 | Genetic findings

Exome sequencing in four unrelated patients identified seven mutations in *ITGA2B* (NM_000419.4). There were five different mutations including one missense, two nonsense, one insertion, and one deletion. Among these five mutations, three have never been previously described (Table 2). The proband of Family 1 was found to be compound heterozygous for two different mutations, c.1750C>T (p.Arg584Ter) and c.2915dup (p.Leu973Alafs*63) in the *ITGA2B* gene. The c.1750C>T mutation in exon 17 was transmitted from the father, and the c.2915dup in exon 28 was transmitted from the mother (Figure 1). Patient 2 was homozygous for the c.617T>A (p.Val206Asp) mutation. Both parents were found to be heterozygous for this mutation. Two different mutations, c.2344C>T (p.Arg782Ter) and c.2915dup (p.Leu973Alafs*63), were identified in the proband of Family 3. The father was heterozygous for the p.Arg782Ter. The maternal DNA sample was unavailable. The patient from Family 4 was found to harbor one novel mutant allele, c.1524_1533del (p.Gln508Hisfs*3) which was inherited from the father. No disease-causing variant could be identified in the other allele (Table 2).

4 | DISCUSSION

This study described four patients with GT from different unrelated families. All cases were classified as being GT type 1 based on the clinical features and flow cytometry findings. Seven mutations in the *ITGA2B* gene were identified in four patients (87.5%). Of the five different variants, three were novel including one missense, one nonsense, and one deletion. A homozygous mutation was found in one patient. The previously known c.1750C>T (p.Arg584Ter) and c.2915dup (p.L973Afs*63) mutations were identified in patient 1.[7,8]A homozygous c.617T>A (p.Val206Asp) mutation was detected in patient 2. Patient 3 was compound heterozygous for two different mutations, c.2344C>T (p.Arg782Ter) and c.2915dup (p.Leu973Alafs*63). Interestingly, the c.2915dup (p.Leu973Alafs*63) was also found in patient 1. A previously undescribed mutation, c.1524_1533del (p.Gln508Hisfs*3), was detected in patient 4.

The c.1750C>T (p.Arg584Ter) is a mutation previously located in several studies and occurs across continents.[7] The previously reported c.2915dup (p.Leu973Alafs*63) was identified in two families (Family 1 and 3). This frameshift mutation which affected the transmembrane

TABLE 2 Molecular characteristics of Thai patients with Glanzmann thrombasthenia (GT)

Case no.	Consanguinity	Exon	Mutation[a]	Status[b]	Amino acid change[a]	Father	Mother
1	N	17 28	c.1750C>T/ c.2915dup	Het.	p.Arg584Ter/ p.Leu973Alafs*63	p.Arg584Ter/WT	p.Leu973Alafs*63/WT
2	Y	5	c.617T>A	Hom.	p.Val206Asp	p.Val206Asp/WT	p.Val206Asp/WT
3	N	24 28	c.2344C>T/ c.2915dup	Het.	p.Arg782Ter/ p.Leu973Alafs*63	p.Arg782Ter/WT	Unavailable
4	N	15	c.1524_1533del/ND	Het.	p.Gln508Hisfs*3	p.Gln508Hisfs*3/WT	No mutations in the coding region

[a]According to the standard nomenclature recommendations of the HGVS (Human Genome Variation Society).
[b]Het.: heterozygous; Hom.: homozygous.
ND, not detected; WT, wild type.

domain and the cytoplasmic tail was reported in a Japanese patient with GT.[5,8] The effect of the insertion of an additional C was revealed by transfection experiments using COS-7 cells. The protein containing the insertion C was expressed and able to form a complex; however, it was not transported to Golgi apparatus.

The newly identified variant c.617T>A (p.Val206Asp) in patient 2 was inherited from both parents. Several lines of evidence suggest this variant as a disease-causing mutation. It was not identified in the 678 in-house exomes. The valine residue at codon 206 was highly conserved (Figure 1). PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) predicted the c.617T>A (p.Val206Asp) to be probably damaging with a score of 0.999. In addition, SIFT (http://sift.jcvi.org/) predicted the c.617T>A to be damaging with a score of 0.002. The p.Val206Asp is located within the αIIb β-propeller domain. The Val to Asp substitution at position 206 of the αIIb β-propeller resulted in changing from a hydrophobic to a negatively charged residue. There was a report describing the p.Val286Asp, a similar substitution in this region. It is located within the αIIb β-propeller and introduces a negatively charged residue. It was shown to disturb the structure of the αIIb β-propeller.[5] In addition, it has been demonstrated that the previously reported p.Leu214 Pro, an adjacent causative variant, disrupts the ligand-binding conformation of the integrin αIIbβ3 receptor.[9] The novel p.Val206Asp might have similar effects on the structure of the αIIb β-propeller and blocked ligand binding as shown in the previous studies.[5,9,10] Most of the missense mutations in the *ITGA2B* are located in the β-propeller region of αIIb.[5,11-13] Several studies have shown that they could prevent complex formation[5,9,14,15] as well as prevent processing of pro-αIIbβ3 within the endoplasmic reticulum and across the Golgi apparatus.[11,16,17]

The c.2344C>T (p.R782X) and c.1524_1533del (p.Gln508Hisfs*3) alterations have never been previously described. The nonsense c.2344C>T (p.Arg782Ter) mutation located in exon 24 is expected to be degraded through nonsense-mediated mRNA decay. The c.1524_1533del (p.Gln508Hisfs*3), located in exon 15, is predicted to result in a frameshift leading to premature termination. It is located

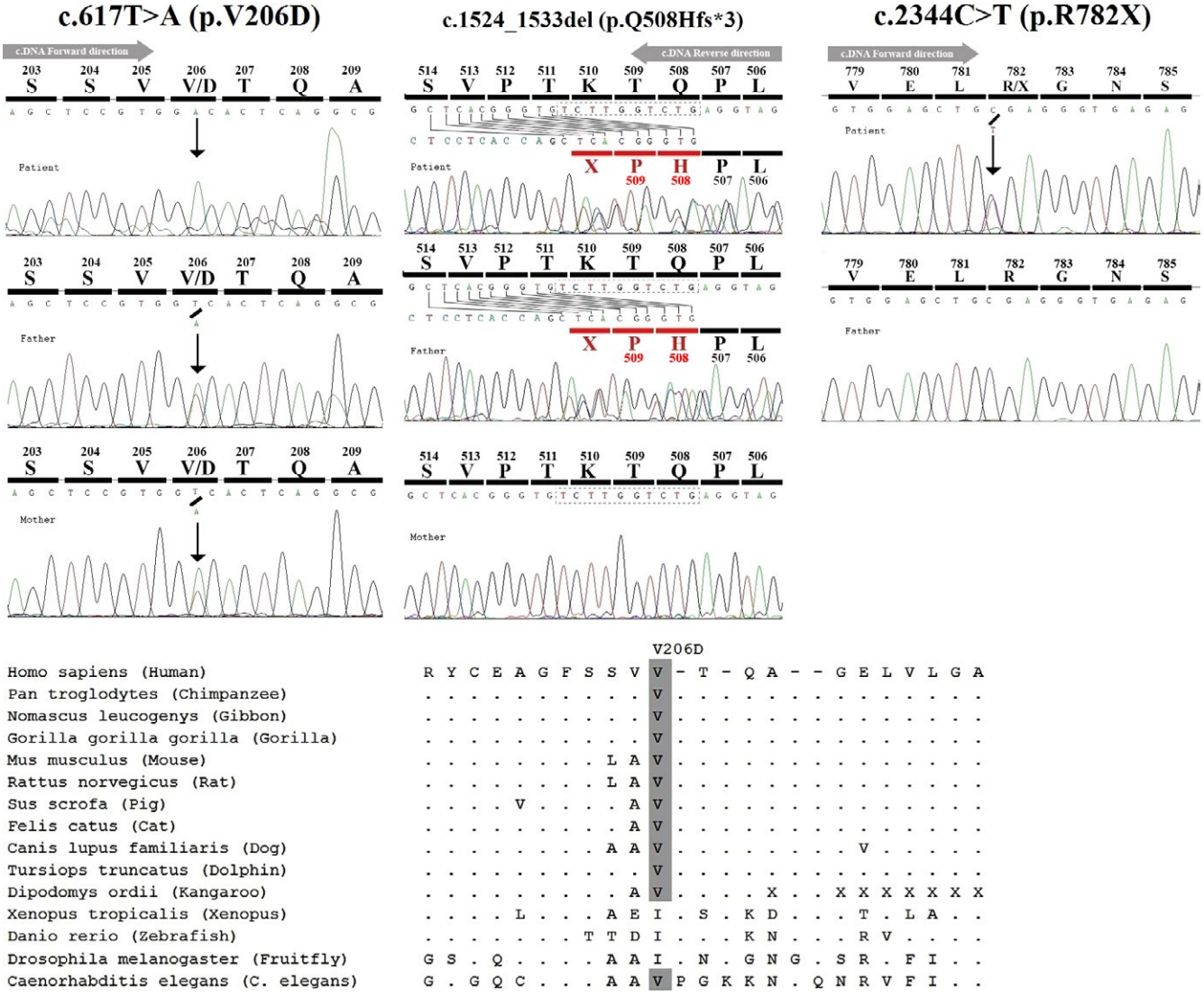

FIGURE 1 Mutation analysis of the novel *ITGA2B* mutations. A, Electropherograms showing c.617T>A (p.Val206Asp), c.1524_1533del (p.Gln508Hisfs*3), and c.2344C>T (p.Arg782Ter). The site of each mutation is indicated by an arrow. B, Sequence alignment of partial amino acid sequence of ITGA2B from various species. The valine residue at codon 206 is indicated by a gray bar

within the thigh domain of the αIIbβ3 receptor. This alteration resulted in substitution of histidine for glutamine at codon 508, creating a new reading frame and inserting a premature stop codon at codon 511. The abnormal mRNA could be a target for nonsense-mediated mRNA decay. Both alterations would lead to loss of αIIbβ3 expression.

Using exome sequencing, seven mutant alleles were identified in four patients. Even though patient 4 had only one deletion in *ITGA2B* in the heterozygous state, the platelet aggregation test and flow cytometric analysis of platelets strongly suggested the diagnosis of GT. There have been some studies describing patients with GT harboring a single heterozygous mutation or no causative mutations in either *ITGA2B* or *ITGB3*.[5,13,18-20] It remains possible that the causative variants could be located in the promoter, conserved intronic regions or in the non-coding parts of the genes including regulatory elements of *ITGA2B* and *ITGB3*.

Genetic testing for GT has been demonstrated to be useful in several aspects including diagnostic confirmation, carrier detection, and prediction of antiplatelet antibody formation in some cases.[21]

In conclusion, we successfully identified pathogenic variants in four unrelated Thai patients with GT. Seven mutant alleles in *ITGA2B* were detected including three novel ones, expanding the mutational spectrum of *ITGA2B*.

ACKNOWLEDGEMENTS

We would like to thank the patients and their families for participating in this study. This work was supported by the Ratchadapiseksompotch Fund (RA58/074), Faculty of Medicine, Chulalongkorn University, Chulalongkorn Academic Advancement into its 2nd Century Project and Thailand Research Fund (BRG5980001).

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REFERENCES

1. George JN, Caen JP, Nurden AT. Glanzmann's thrombasthenia: the spectrum of clinical disease. *Blood*. 1990;75:1383-1395.
2. Heidenreich R, Eisman R, Surrey S, et al. Organization of the gene for platelet glycoprotein IIb. *Biochemistry*. 1990;29:1232-1244.
3. Wilhide CC, Jin Y, Guo Q, et al. The human integrin beta3 gene is 63 kb and contains a 5'-UTR sequence regulating expression. *Blood*. 1997;90:3951-3961.
4. Nurden AT, Fiore M, Nurden P, Pillois X. Glanzmann thrombasthenia: a review of ITGA2B and ITGB3 defects with emphasis on variants, phenotypic variability, and mouse models. *Blood*. 2011;118:5996-6005.
5. Nurden AT, Pillois X, Fiore M, et al. Expanding the mutation spectrum affecting alphaIIbbeta3 Integrin in glanzmann thrombasthenia: screening of the ITGA2B and ITGB3 genes in a large international cohort. *Hum Mutat*. 2015;36:548-561.
6. den Dunnen JT, Dalgleish R, Maglott DR, et al. HGVS recommendations for the description of sequence variants: 2016 update. *Hum Mutat*. 2016;37:564-569.
7. Kato A, Yamamoto K, Miyazaki S, Jung SM, Moroi M, Aoki N. Molecular basis for glanzmann's thrombasthenia (GT) in a compound heterozygote with glycoprotein IIb gene: a proposal for the classification of GT based on the biosynthetic pathway of glycoprotein IIb-IIIa complex. *Blood*. 1992;79:3212-3218.
8. Hayashi T, Tanaka S, Hori Y, Terada C, Ueda Y, Tani Y. Insertion of a C in the exon 28 of integrin alphaIIb gene leading to a frameshift mutation is responsible for glanzmann thrombasthenia in a Japanese case. *J Thromb Haemost*. 2005;3:489-496.
9. Grimaldi CM, Chen F, Wu C, Weiss HJ, Coller BS, French DL. Glycoprotein IIb Leu214Pro mutation produces glanzmann thrombasthenia with both quantitative and qualitative abnormalities in GPIIb/IIIa. *Blood*. 1998;91:1562-1571.
10. Kamata T, Tieu KK, Irie A, Springer TA, Takada Y. Amino acid residues in the alpha IIb subunit that are critical for ligand binding to integrin alpha IIbbeta 3 are clustered in the beta-propeller model. *J Biol Chem*. 2001;276:44275-44283.
11. Nelson EJ, Li J, Mitchell WB, Chandy M, Srivastava A, Coller BS. Three novel beta-propeller mutations causing glanzmann thrombasthenia result in production of normally stable pro-alphaIIb, but variably impaired progression of pro-alphaIIbbeta3 from endoplasmic reticulum to Golgi. *J Thromb Haemost*. 2005;3:2773-2783.
12. Jallu V, Dusseaux M, Panzer S, et al. AlphaIIbbeta3 integrin: new allelic variants in glanzmann thrombasthenia, effects on ITGA2B and ITGB3 mRNA splicing, expression, and structure-function. *Hum Mutat*. 2010;31:237-246.
13. Sandrock-Lang K, Oldenburg J, Wiegering V, et al. Characterisation of patients with glanzmann thrombasthenia and identification of 17 novel mutations. *Thromb Haemost*. 2015;113:782-791.
14. Rosenberg N, Landau M, Luboshitz J, Rechavi G, Seligsohn U. A novel Phe171Cys mutation in integrin alpha causes glanzmann thrombasthenia by abrogating alphabeta complex formation. *J Thromb Haemost*. 2004;2:1167-1175.
15. Rosenberg N, Hauschner H, Peretz H, et al. A 13-bp deletion in alpha(IIb) gene is a founder mutation that predominates in Palestinian-Arab patients with glanzmann thrombasthenia. *J Thromb Haemost*. 2005;3:2764-2772.
16. Afrasiabi A, Gelain F, Artoni A, Mannucci PM. AlphaIIbG236E causes glanzmann thrombasthenia by impairing association with beta3. *Platelets*. 2008;19:322-327.
17. Shen WZ, Ding QL, Jin PP, et al. A novel Pro126His beta propeller mutation in integrin alphaIIb causes glanzmann thrombasthenia by impairing progression of pro-alphaIIbbeta3 from endoplasmic reticulum to Golgi. *Blood Cells Mol Dis*. 2009;42:44-50.
18. D'Andrea G, Colaizzo D, Vecchione G, Grandone E, Di Minno G, Margaglione M. Team GLsTI. Glanzmann's thrombasthenia: identification of 19 new mutations in 30 patients. *Thromb Haemost*. 2002;87:1034-1042.
19. Nelson EJ, Nair SC, Peretz H, et al. Diversity of glanzmann thrombasthenia in southern India: 10 novel mutations identified among 15 unrelated patients. *J Thromb Haemost*. 2006;4:1730-1737.
20. Kannan M, Ahmad F, Yadav BK, Kumar R, Choudhry VP, Saxena R. Molecular defects in ITGA2B and ITGB3 genes in patients with glanzmann thrombasthenia. *J Thromb Haemost*. 2009;7:1878-1885.
21. Fiore M, Firah N, Pillois X, Nurden P, Heilig R, Nurden AT. Natural history of platelet antibody formation against alphaIIbbeta3 in a French cohort of glanzmann thrombasthenia patients. *Haemophilia*. 2012;18:e201-e209.

How to cite this article: Ittiwut R, Suchartlikitwong P, Kittikalayawong Y, et al. Novel mutations in Thai patients with glanzmann thrombasthenia. *Eur J Haematol*. 2017;00:1–5. https://doi.org/10.1111/ejh.12965