Hereditary platelet function disorder from *RASGRP2* gene mutations encoding CalDAG-GEFI identified by whole-exome sequencing in a Korean woman with severe bleeding

Inherited platelet disorders (IPD) are a group of genetically heterogeneous disorders with thrombocytopenia or platelet dysfunction (platelet function disorders, PFD). Mutations in more than 50 genes have been reported to be the underlying genetic defects of IPD.[1,2] PFD typically have normal counts of platelets but with impaired function. Among them, Glanzmann thrombasthenia (GT) is the best recognized PFD and is caused by mutations in the genes *ITGA2B* or *ITGB3*, encoding the integrin molecules glycoprotein IIb-IIIa (GPIIb/IIIa or $\alpha_{IIb}\beta_3$) on the platelet membrane (OMIM 273800). The GPIIb/IIIa complex plays an essential role in platelet aggregation by mediating platelet-platelet interactions, and quantitative or qualitative defects of the complex results in GT. The RAS guanyl-releasing protein-2 gene (*RASGRP2*) encodes for the calcium and diacylglycerol (DAG)-regulated guanine exchange factor-1 (CalDAG-GEFI), the critical protein for normal hemostasis by affinity regulation of GPIIb/IIIa for fibrinogen by inside-out signaling in platelets.[3] PFD caused by mutations of *RASGRP2* is a rare autosomal recessive disorder with severe bleeding, with 20 unique mutations from 24 families reported in the literature.[2,4-11] Recently, high-throughput next-generation sequencing is being increasingly applied to the diagnosis of patients with bleeding diatheses, and uncovers rare bleeding disorders, delineating a better landscape of genetic defects in PFD.[2,12] In this report, we describe a patient with a PFD caused by compound heterozygous mutations of *RASGRP2* identified by whole-exome sequencing (WES).

A 41-year old pregnant woman was referred to the coagulation clinic of Samsung Medical Center with a clinical impression of GT. She had a history of a bleeding tendency including frequent nose bleeding and hematuria since early childhood. Her family members, including mother, father, elder sister, and younger brother, had no history of bleeding. The patient did not have a history of unusual or recurrent infections suggesting immune deficiency. The severity of bleeding in the patient was assessed according to the standardized questionnaire of the International Society on Thrombosis and Haemostasis-Bleeding Assessment Tool (ISTH-BAT) and showed a severe phenotype with a score of 17 (Table 1).[13] The number and morphology, including size, of her platelets were normal. Coagulation screening tests such as prothrombin time and activated partial thromboplastin time were all within reference ranges. However, rapid platelet function tests using a PFA-100 revealed prolonged closure times, indicating platelet dysfunction (>300 s on collagen/epinephrine and 153 s on collagen/ADP). The platelet aggregation test showed markedly decreased aggregation responses to ADP (20 μM) and epinephrine (300 μM), a minimally decreased aggregation response to ristocetin (1500 μg/mL), and a normal aggregation response to collagen (10 μg/mL). The expression of CD41a (GPIIb/IIIa complex) and CD61 (GPIIIa) was normal, as assessed by flow cytometric analysis. Based on these results, a PFD other than GT was suspected.

For molecular genetic diagnosis, we performed WES using DNA extracted from peripheral blood with written informed consent from the patient. Seventy-two genes associated with platelet disorders were screened.[2] The variants detected were classified based on the 2015 American College of Medical Genetics (ACMG) guidelines. ClinVar, Human Gene Mutation Database, and *in silico* analysis including polyphen[2] and SIFT were used to characterize the variants detected, and all potentially pathogenic variants were confirmed by Sanger sequencing. As a result, we found two heterozygous mutations in the *RASGRP2* gene, c.659G>A (R220Q) and c.1142C>T (S381F) (Figure 1A). S381F was a previously reported pathogenic variant in PFD,[2,5] and R220Q was a novel variant. The R220 residue is located in the catalytic domain (CDC25) of CalDAG-GEFI which is important for interaction with the GTPase,[4] and is conserved across mammalian species (Figure 1B,C). *In silico* analyses including polyphen-2 (*http:// genetics.bwh.harvard.edu/pph2*), SIFT (*https://sift.bii.a-star.edu.sg*), MutationAssessor (*http://mutationassessor.org*), MutationTaster (*http://www.mutationtaster.org*) and PROVEAN (*http://provean.jcvi.org*) predicted that R220Q exerts a damaging effect on the protein. Family study by targeted mutation analyses showed that the patient's father carries R220Q and her mother carries S381F, confirming the autosomal recessive inheritance of the disease in the patient. Collectively, R220Q was classified as a likely pathogenic variant according to the ACMG guidelines.[14] No remarkable variants were detected in other candidate genes, including *ITGA2B* and *ITGB3*.

Table 1. The patient's bleeding symptoms and score.

Symptom	Score
Epistaxis	4
Cutaneous	2
Bleeding from minor wounds	0
Oral cavity	2
Gastrointestinal bleeding	0
Hematuria	2
Tooth extraction	4
Surgery	0
Menorrhagia	0
Post-partum hemorrhage	3
Muscle hematoma	0
Hemarthrosis	0
Central nervous system bleeding	0
Other bleedings	0
Sum	**17**

At 39 weeks of pregnancy, the patient delivered a healthy baby by Cesarean section with a Pfannenstiel skin incision. During the operation, two units of single-donor platelets were prophylactically transfused, and 400 mL of blood loss occurred. Her pre-operative hemoglobin was 11.3 g/dL, which decreased to 6.4 g/dL on post-operative day 2 and to 5.4 g/dL on post-operative day 3. Since there was no evidence of clinically recognizable uterine atony or subfascial hematoma, the relatively severe drop in hemoglobin was considered to be partially due to the small maternal blood volume due to the patient's short stature (155 cm in height). Two units of leukocyte-reduced red blood cells were transfused, and her hemoglobin concentration was 8.0 g/dL on day 4. She started oral iron replacement therapy. At the 1-month follow-up visit, both the patient and her baby were doing well without any medical issues.

The *RASGRP2* gene encodes CalDAG-GEFI, a major

Table 2. Summary and American College of Medical Genetics classification of previously reported variants in the *RASGRP2* gene.

cDNA change	AA change	Exon	ACMG classification	References
c.199_200delAA	p.N67Lfs*24	4	PV	(7)
c.337C>T	p.R113*	5	PV	(5)
c.372-3C>G		IVS5	PV	(7)
c.542T>C	p.F181S	7	LPV	(7)
c.659G>A	p.R220Q	7	LPV	This report
c.706C>T	p.Q236*	8	PV	(8)
c.742G>T	p.G248W	8	PV	(10)
c.778G>T	p.E260*	8	PV	(7)
c.1033G>C	p.A345P	9	LPV	(7)
c.1081_1083delCTG	p.L361del	9	LPV	(6)
c.866A>G	p.Y289C	9	LPV	(7)
c.886T>C	p.C296R	9	LPV	(7)
c.887G>A	p.C296Y	9	LPV	(8)
c.914G>A	p.G305D	9	PV	(9)
c.925A>T	p.K309*	9	PV	(6)
c.990C>G	p.N330K	9	LPV	(7)
c.1142C>T	p.S381F	10	PV	(5)
c.1159C>G	p.R387G	10	VUS	(15)
c.1479dupG	p.R494Afs*54	13	PV	(11)
c.1490delT	p.F497Sfs*22	13	PV	(7)
c.1777C>T	p.R593C	16	VUS	(15)

AA, amino acid; ACMG, American College of Medical Genetics; PV, pathogenic variant; LPV, likely pathogenic variant; VUS, variant of uncertain significance; IVS, intervening sequence.

signaling molecule in platelets.[3] CalDAG-GEFI is critical for Ras-like GTPase activation whose main target is Rap1. Rap1 is one of the predominant small GTPases in platelets and is the key signaling protein that directly regulates integrin-mediated platelet aggregation and granule secretion. In our patient, we detected compound heterozygous mutations in *RASGRP2* by WES. One mutation, R220Q in exon 7, was a novel missense mutation (likely pathogenic variant) in the CDC25 catalytic domain based on the ACMG variant classification guidelines. The other mutation, S381F in exon 10, is also located in the CDC25 domain and was previously described in a Chinese boy with severe bleeding who had homozygous S381F from consanguinity.[2,5] The expression of CalDAG-GEFI was severely impaired in the boy. Interestingly, all the pathogenic missense mutations were located in the CDC25 domain except two variants of uncertain significance[15] (Figure 1C and Table 2) according to the ACMG guidelines.[14] PFD due to *RASGRP2* mutations was first described in three siblings with severe bleeding born to consanguineous parents.[4] The authors detected a homozygous missense mutation (G248W) in the siblings by WES and demonstrated that the mutation impaired CalDAG-GEFI's ability to activate Rap1. Westbury *et al.* screened 2,042 cases in pedigrees with unexplained bleeding by high-throughput sequencing and found 11 cases (0.5%) with biallelic *RASGRP2* mutations. Very recently, Bastida *et al.* developed a high-throughput sequencing panel for IPD to cover 72 genes including *RASGRP2*.[2] Among 82 patients with suspected IPD (34 suspected to have a particular disease and 48 with uncertain etiology), they found three patients with homozygous *RASGRP2* mutations, which accounted for 3.6% (3/82) of the total cohort and 6.3% (3/48) of cases with uncertain etiology. The authors suggested that IPD/PFD due to *RASGRP2* mutations could be more frequent than had been thought. The lower frequency of *RASGRP2* mutations in the study by Westbury *et al.* could be partly due to the fact that the ThromboGenomics panel used for the second set of patients did not include the *RASGRP2* gene.[12]

Clinically, the early-onset, severe bleeding symptoms (score 17) (Table 1) in our patient were similar to those of the previously reported cases with *RASGRP2* mutations. The test results for hemostasis were also largely in line with the previous findings. Particularly regarding the results of platelet aggregation tests, our patient showed a normal or minimally decreased aggregation response to collagen and ristocetin, while aggregation responses were markedly decreased to ADP and epinephrine. According to the study by Westbury *et al.*, reduced aggregation responses to ADP and epinephrine were consistent findings in patients with *RASGRP2* mutations, while aggregation defects to other agonists such as collagen were variable. According to the study by Kato *et al.*, the aggregation response was impaired even at high concentrations of ADP, while the impaired response to collagen was restored to comparable to that of controls by increasing the concentration.[6] Thus, the platelet aggregation responses in PFD due to *RASGRP2* mutations could overlap with those of GT. Indeed, our patient had been on follow-up with an impression of GT based on the platelet aggregation test results until referral to the coagulation clinic. Both PFD from *RASGRP2* mutations and GT are autosomal recessive PFD belonging to the subgroup with defective signal transduction pathways. However, while the bleeding severity is variable in GT, it is consistently moderate to severe in PFD due to *RASGRP2* mutations.[3] Flow cytometry analyses to determine the expression of

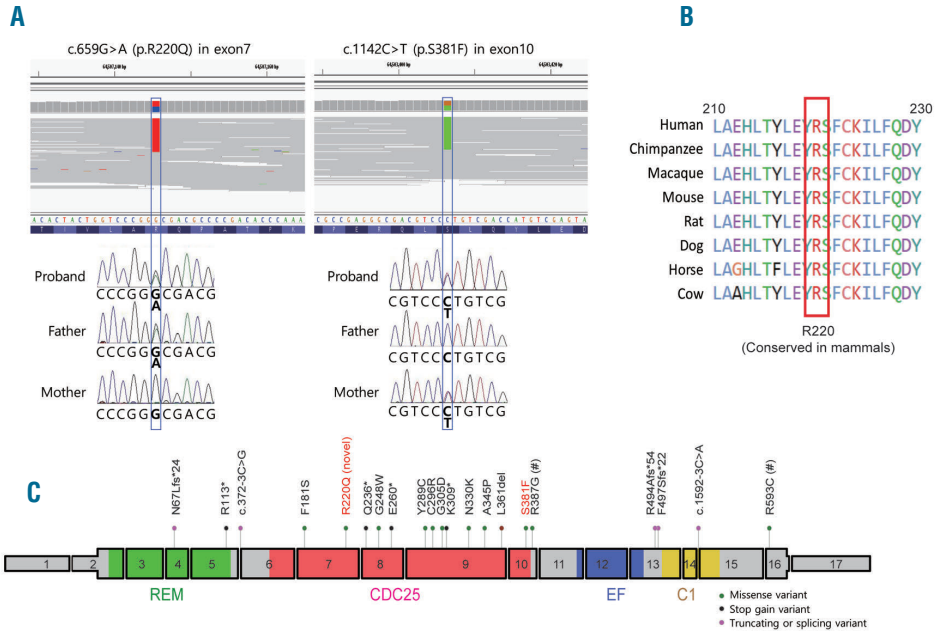

Figure 1. The identified mutations and characteristics. (A) Compound heterozygous mutations (R220Q and S381F) in the *RASGRP2* gene in the proband identified by whole-exome sequencing (Integrative Genomics Viewer). Targeted mutation analyses by Sanger sequencing confirmed the mutations in the proband and her parents. (B) The R220 residue of CalDAG-GEFI, the protein encoded by *RASGRP2*, is conserved across eight mammalian species. (C) The location of R220Q and S381F in the proband (red) and other previously reported disease-causing variants. Note that most missense variants are located in the CDC25 domain. The numbers in each box indicate exons, and the major protein domains are color-coded: green, ras exchange motif (REM); red, catalytic domain (CDC25); blue, calcium-binding EF hands (EF); and yellow, (C1-like domain (C1).

GPIIb/IIIa on platelets and molecular genetic tests, especially high-throughput sequencing, could help the differential diagnosis.

The diagnosis of IPD including PFD has recently been facilitated by the advent of high-throughput sequencing with a growing list of candidate genes. We speculate that inherited CalDAG-GEFI deficiency from *RASGRP2* mutations is a recurrent platelet signal transduction pathway disease and needs to be included in the next-generation sequencing panel or candidate gene lists of WES in the diagnosis of IPD/PFD.

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