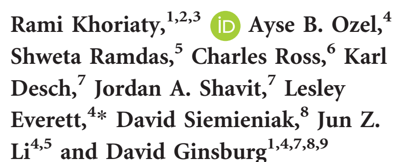
bjh short report

Genome-wide linkage analysis and whole-exome sequencing identifies an *ITGA2B* mutation in a family with thrombocytopenia



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Summary

Hereditary thrombocytopenias can be subclassified based on mode of inheritance and platelet size. Here we report a family with autosomal dominant (AD) thrombocytopenia with normal platelet size. Linkage analysis and whole exome sequencing identified the R1026W substitution in *ITGA2B* as the causative defect. The same mutation has been previously reported in 7 Japanese families/patients with AD thrombocytopenia, but all of these patients had macrothrombocytopenia. This is the first report of a family with AD thrombocytopenia with normal platelet size resulting from mutation in *ITGA2B*. *ITGA2B* mutations should therefore be included in the differential diagnosis of this latter disorder.

Keywords: hereditary thrombocytopenia, linkage analysis, whole exome sequencing, *ITGA2B*, autosomal dominant thrombocytopenia.

Hereditary thrombocytopenias (HT) are a heterogeneous group of bleeding disorders characterized by varying degrees of thrombocytopenia and a wide spectrum of clinical manifestations. Recent advances in molecular genetics have improved our understanding of the pathophysiology of these disorders. HT can be subclassified based on the mode of inheritance as well as platelet size (Balduini & Savoia, 2012). Here we report a family with autosomal dominant (AD) thrombocytopenia characterized by normal platelet size and absence of other phenotypic abnormalities. Linkage analysis and whole exome sequencing identified the genetic defect as mutation in the *ITGA2B* gene resulting in an R1026W substitution.

Methods

Sample collection and DNA preparation

A 4-generation family with 10 affected individuals (Fig 1A) was studied. Following enrollment on a research protocol approved by the University of Michigan Institutional Review

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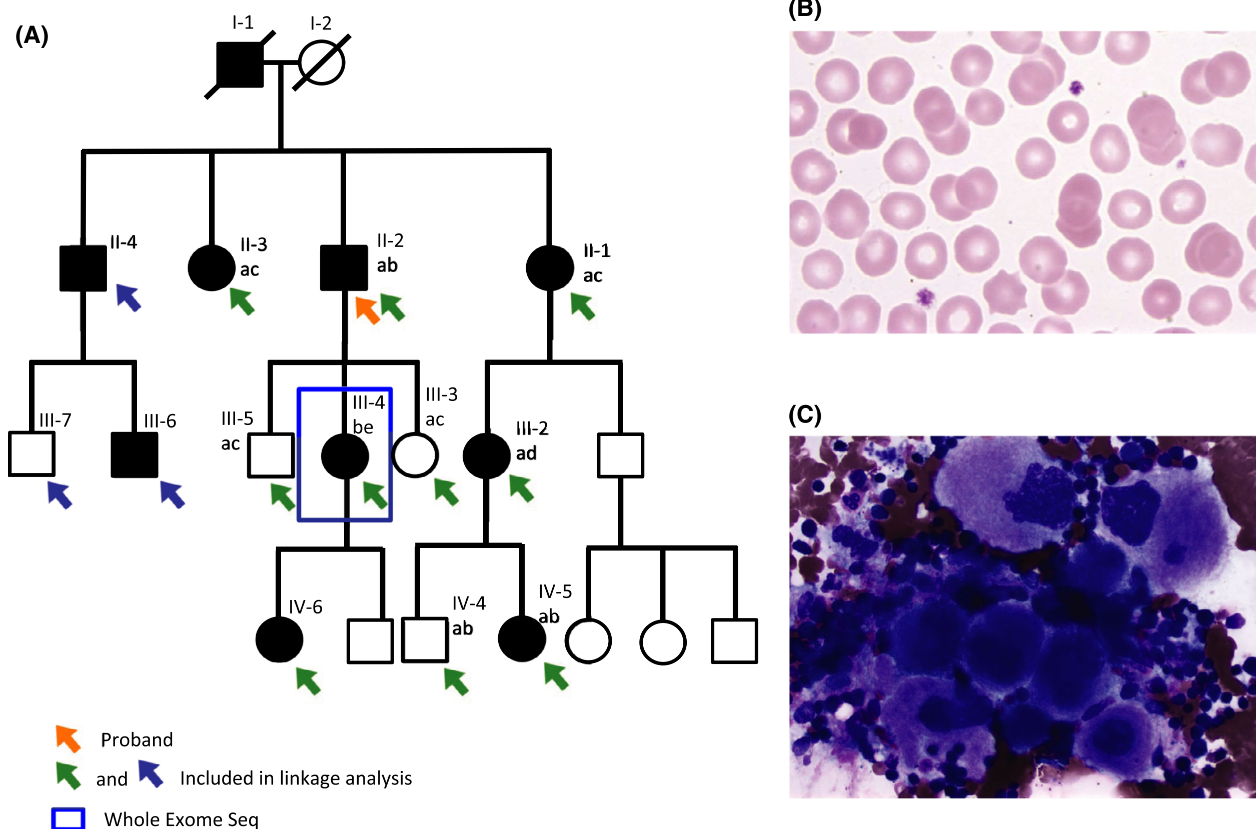

Fig 1. Family with autosomal dominant thrombocytopenia. (A) Affected individuals are indicated by filled symbols. Arrows indicate individuals included in the linkage analysis, with green or blue arrows denoting collection of blood sample or buccal swab, respectively. The blue box indicates the individual whose DNA was subjected to whole exome sequencing. The proband is indicated by an orange arrow. Genotyping for a microsatellite, ~3 Mb upstream of *ANKRD26* was performed on 9 family members (6 affected and 3 unaffected), ruling out *ANKRD26* as the causative gene in this family. Each genotype is indicated by a different letter (a, b, c, d, e). Affected patients did not share a common microsatellite genotype. (B) Peripheral blood smear from family member II-2 demonstrates thrombocytopenia with uniform, normal-sized platelets. Original magnification ×500. (C) Bone marrow aspirate from family member III-4 demonstrates abundant (slightly increased in number) megakaryocytes, 5–10% of which were small with nuclear hypolobation with no other bone marrow abnormalities. Original magnification ×500. [Colour figure can be viewed at wileyonlinelibrary.com].

Board, genomic DNA was prepared from the individuals indicated in Fig 1A as described in the Data S1.

Microsatellite genotyping

Genotyping for a microsatellite located ~3 megabases (Mb) 5′ of *ANKRD26* (Chromosome 10:30 059 574–30 059 640) was performed by polymerase chain reaction (PCR with primers MS F1 and MS R1 (Table SI), as described in the Data S1.

Sanger sequencing

PCR and Sanger Sequencing were performed as described in the Data S1. PCR primers are listed in Table SI.

Linkage analysis

DNA samples from the 13 individuals highlighted with a green or blue arrow in Fig 1A were genotyped on the Infinium HumanCoreExome v24.1 BeadChip (Illumina, San Diego, CA), which yielded ~530K autosomal single nucleotide polymorphisms (SNPs). After applying multiple filters for quality control, parametric linkage analysis was performed using an AD inheritance model on a range of marker sets and parameters (See Data S1).

Whole-exome sequencing and analysis

Genomic DNA from family member III-4 (Fig 1A) was sonicated and subjected to exome capture using a NimbleGen SeqCap EZ Exome Enrichment Kit v3.0 (Roche, Madison, WI, USA) and to 100 base pair paired-end sequencing on an Illumina HiSeq 2000 at the University of Michigan Sequencing Core (UMSC). This sample was one of 733 samples sequenced at the UMSC for which pooled variant calling was performed. The details of the sequencing and downstream analysis are included in the Data S1.

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Imputation and haplotype analysis

In a previous report (Kunishima *et al*, 2011), the disease haplotype at the *ITGA2B* locus was defined using a set of 11 SNPs. The disease haplotype was defined in this study to determine if the *ITGA2B* mutation occurs on a different haplotype in our family (see Data S1). The candidate risk allele was also examined for segregation with the disease haplotype (see Data S1).

Results and discussion

This family (Fig 1A) is of European ancestry and exhibits AD thrombocytopenia with normal platelet size and appearance on peripheral smear (Fig 1B and Table SII) and normal mean platelet volume of 10·2 fl for the proband (normal range 9–12·2 fl). Bone marrow evaluation of individual III-4 demonstrated a slightly increased number of megakaryocytes, 5–10% of which appeared small with nuclear hypolobation (Fig 1C). The bone marrow was otherwise normal and metaphase cytogenetics revealed a normal karyotype.

Given the lack of acute leukaemia history or phenotypic abnormalities in this AD thrombocytopenia family with normal platelet size, *ANKRD26*-related thrombocytopenia, also known as thrombocytopenia 2, was suspected. Thrombocytopenia 2 results from mutations in the 5′UTR of *ANKRD26* leading to loss of transcription factor binding (Pippucci *et al*, 2011; Bluteau *et al*, 2014). The 5′UTR of *ANKRD26* was Sanger sequenced in family members II-2 and III-4, and no mutation was identified. To rule out a disease-causing mutation in *ANKRD26* outside of the 5′UTR, genotyping for a microsatellite, ~3 Mb upstream of the gene was performed in 9 family members (6 affected and 3 unaffected) (Fig 1A), excluding the *ANKRD26* locus as the cause of thrombocytopenia in this family [logarithm of the odds (LOD) score minus 15·6].

To map the disease-causing gene, we performed linkage analyses on 9 affected and 4 unaffected family members (details described in Methods). All parameter combinations and marker sets highlighted two consensus linkage regions: Chr17:37 222 473–48 110 703 (LOD score 3·31) and Chr13:50 246 074–78 461 133 (LOD score 2·62).

Whole exome sequencing was performed on genomic DNA obtained from family member III-4, with an average coverage of 45·96× (95·95% of the target sequence covered at least 10×). Non-synonymous, nonsense, splice-site, stop-loss and frameshift variants within the identified linkage peaks were subjected to a variety of filters (see Methods and Fig 2A). Three variants (one in each of *AOC3*, *CDC27* and *ITGA2B*) passed all filters (Fig 2B). *AOC3* is not expressed in platelets (Rowley *et al*, 2011), and the *CDC27* variant was predicted to be benign/tolerated (Fig 2B). Therefore, the leading candidate variant was a G to A substitution in *ITGA2B*, resulting in an Arginine (R) to Tryptophan (W) substitution at amino acid 1026 (p.R1026W). This variant was predicted to be damaging and has been reported previously in AD macro-thrombocytopenia (Kunishima *et al*, 2011; Kashiwagi *et al*, 2013), further supporting its identification as the causative mutation in this family. Sanger sequencing showed that the *ITGA2B* variant was present in heterozygous form in all affected family members and was absent in all unaffected family members.

Homozygous or compound heterozygous loss of function mutations in *ITGA2B* result in Glanzmann thrombasthenia, a bleeding disorder characterized by normal platelet count but abnormal platelet function (Nurden *et al*, 2011). In contrast, the *ITGA2B* R1026W mutation (referred to as R995W in some reports) is thought to result in constitutive activation of the αIIbβ3 receptor (Kunishima *et al*, 2011) and has been previously reported in 7 Japanese families with AD thrombocytopenia (Kunishima *et al*, 2011; Kashiwagi *et al*, 2013). The common geographic origin of these latter families suggested the possibility of a single founder allele, although a mutation in another gene tightly linked to *ITGA2B* could not be excluded. There are many examples where the true causative mutation is in linkage disequilibrium with many other variants, some of which reside at a distance or even in another gene. For example, before *ANKRD26* was identified as the causative gene for thrombocytopenia 2, the disease was initially attributed to mutations in one of two nearby genes, *ACBD5* or *MASTL* (Pippucci *et al*, 2011.).

The identical disease-associated *ITGA2B* haplotype was previously reported for 4 of the Japanese families (Kunishima *et al*, 2011). We determined the *ITGA2B* haplotype in our Caucasian family, confirmed that it segregates with the disease (Fig 2C), and demonstrated that the *ITGA2B* R1026W mutation arose on a different haplotype compared to the previous reports, strongly supporting at least 2 independent origins for this same point mutation. This R to W substitution represents a C to T transition at a CpG site, a known hot spot for human mutations (Rahbari *et al*, 2016). Taken together, these results suggest that R1026W confers a unique gain of function, consistent with a previous report (Kunishima *et al*, 2011).

In contrast to our family (European ancestry), all previously reported families (Japanese ancestry) with HT due to the *ITGA2B* R1026W substitution exhibit macrothrombocytopenia (thrombocytopenia with large platelet size) (Kunishima *et al*, 2011; Kashiwagi *et al*, 2013). The lack of macrothrombocytopenia in our family could be the result of a modifier gene(s) difference in these disparate genetic backgrounds. Strain differences in mice have been demonstrated to contribute to variations in platelet size in Gray Platelet Syndrome (Tomberg *et al*, 2016), analogous to this observation in humans.

A different substitution at the same position of *ITGA2B* (R1026Q, previously designated R995Q) was reported to result in thrombocytopenia with an mean platelet volume of 10·3 μm^3 (control range of 8·6 ± 1μm^3) in one patient (Hardisty *et al*, 1992; Peyruchaud *et al*, 1998). Point mutations in *ITGB3* have also been reported to result in an

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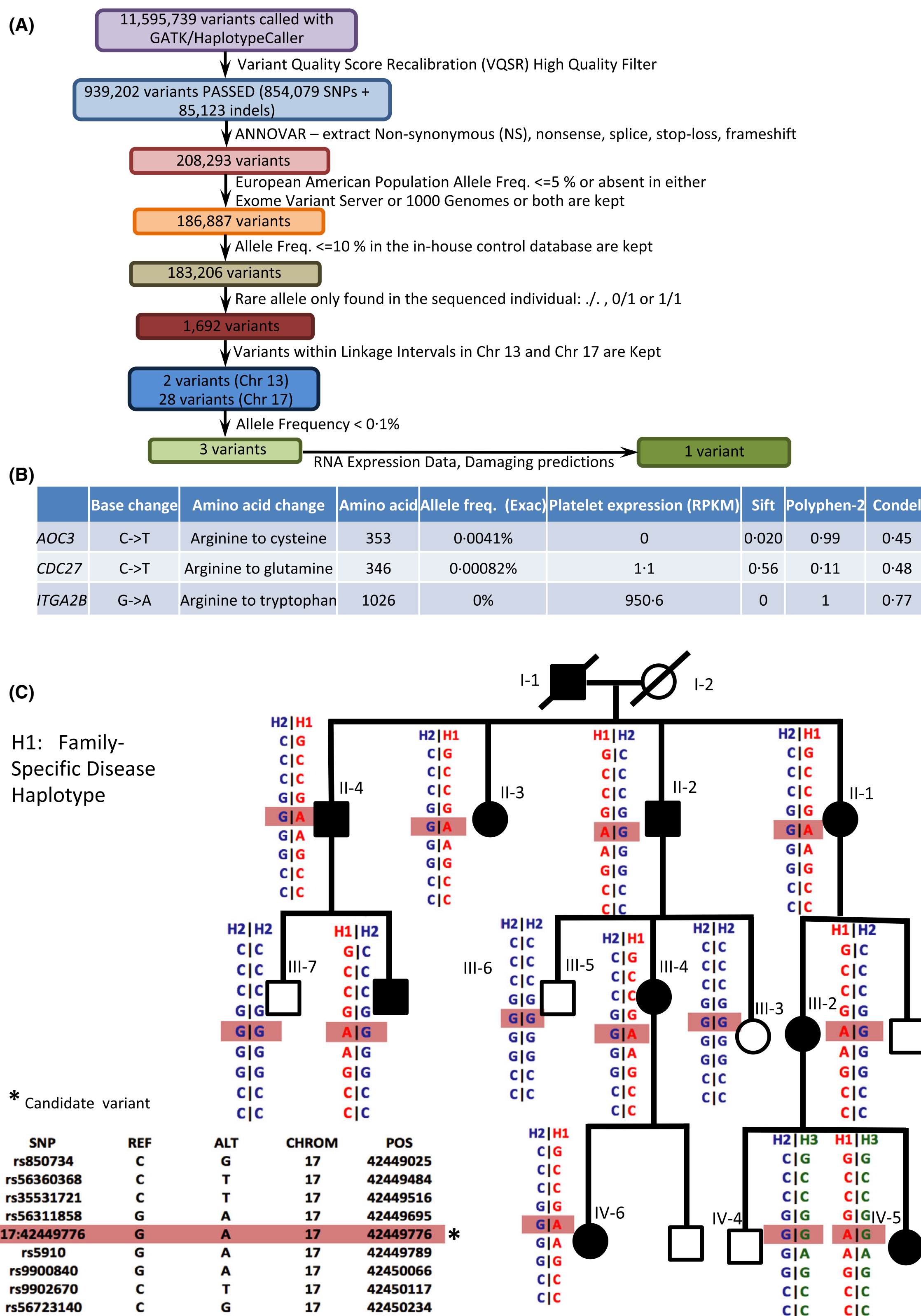

	Base change	Amino acid change	Amino acid	Allele freq. (Exac)	Platelet expression (RPKM)	Sift	Polyphen-2	Condel
AOC3	C->T	Arginine to cysteine	353	0·0041%	0	0·020	0·99	0·45
CDC27	C->T	Arginine to glutamine	346	0·00082%	1·1	0·56	0·11	0·48
ITGA2B	G->A	Arginine to tryptophan	1026	0%	950·6	0	1	0·77

SNP	REF	ALT	CHROM	POS
rs850734	C	G	17	42449025
rs56360368	C	T	17	42449484
rs35531721	C	T	17	42449516
rs56311858	G	A	17	42449695
17:42449776	G	A	17	42449776 *
rs5910	G	A	17	42449789
rs9900840	G	A	17	42450066
rs9902670	C	T	17	42450117
rs56723140	C	G	17	42450234

Fig 2. Sequence analysis. (A) Sequence analysis pipeline. (B) Three variants passed all the applied filters. RPKM refers to reads per kilobase of transcript per million mapped reads and indicates the expression level of the genes in human platelets (Rowley *et al*, 2011). Multiple tools (SIFT, Polyphen2, and CONDEL) were utilized to predict the pathogenicity of the variants. (C) The *ITGA2B* haplotype segregates with the disease. [Colour figure can be viewed at wileyonlinelibrary.com].

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identical phenotype (Ghevaert *et al*, 2008; Gresele *et al*, 2009). All of these mutations cluster on both sides of the transmembrane domains of αIIbβ3 (Rao & Coller, 2014). Recently, rare variants in *GP1BB* were also found to be associated with AD macrothrombocytopenia (Sivapalaratnam *et al*, 2017).

To our knowledge, this is the first report of a family with AD thrombocytopenia with normal platelet size, resulting from a mutation in *ITGA2B*. Mutations in *ITGA2B* should therefore be included in the differential diagnosis of patients with this disorder.

Acknowledgements

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Authorship contributions

RK, ABO, JZL and DG designed the experiments. RK, ABO and SR performed most of the experiments and analyses. KD, JAS, LE and DS contributed to performing the experiments and analysing the data. CR reviewed the peripheral smear and bone marrow. RK, ABO, JZL and DG wrote the paper. All authors contributed to the integration and discussion of the results.

Disclosure of conflicts of interest

The authors declare no competing conflicts of interest.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Data S1. Supplemental methods.
Table SI. Primers used in this study.
Table SII. Platelet counts and Mean platelet volumes (MPV) from available family members.

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