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Hemizygosity for the Gene Encoding Glycoprotein Ib beta (GPIbβ) Is Not Responsible for Macrothrombocytopenia and Bleeding in Patients with 22q11 Deletion Syndrome

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SUMMARY

Background and Objectives: Macrothrombocytopenia and bleeding are frequently associated with 22q11 deletion syndrome (22q11DS). *GPIBB*, which encodes the glycoprotein Ib beta (GPIbβ) subunit of GPIb-IX-V, is commonly deleted in 22q11DS. Absence of functional GPIb-IX-V causes Bernard Soulier Syndrome, which is a severe bleeding disorder characterized by macrothrombocytopenia. Patients with 22q11DS are often obligate hemizygotes for *GPIBB*, and those with only a pathogenically disrupted copy of *GPIBB* present with Bernard Soulier Syndrome. How *GPIBB* hemizygosity and sequence variation relate to macrothrombocytopenia and bleeding in patients who do not have Bernard Soulier Syndrome, however, is not clear.

Patients/Methods: We thoroughly characterized bleeding severity, mean platelet volume, platelet count, and *GPIBB* copy number and sequence in patients with 22q11DS.

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AUTHORSHIP DETAILS

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Disclosure of Conflict of Interests

R. S. Bercovitz reports other from Velico Medical, outside the submitted work. The other authors state that they have no conflict of interest.

Results and Conclusions: Macrothrombocytopenia and mild bleeding were observed in incompletely overlapping subsets of patients, and *GPIBB* copy number and sequence variation did not correlate with either macrothrombocytopenia or bleeding in patients with 22q11DS. These findings indicate that *GPIBB* hemizygosity does not result in either macrothrombocytopenia or bleeding in these patients. Alternative genetic causes of macrothrombocytopenia, potential causes of acquired thrombocytopenia and bleeding, and ways in which platelet size, platelet count, and *GPIBB* sequence information can be used to aid in the diagnosis and management of patients with 22q11DS are discussed.

Keywords

22q11 Deletion Syndrome; Bernard Soulier Syndrome; Glycoprotein Ib; Giant Platelets; Thrombocytopenia

INTRODUCTION

Chromosomal rearrangements that occur early in embryogenesis are a frequent cause of birth defects. Four low copy repeats (LCR) located within the q11 region of chromosome 22 enable chromosomal rearrangements to occur at a high frequency [1], making chromosome 22q11 deletion syndrome (22q11DS) the most common microdeletion syndrome in humans (reviewed in [2–8]). The segments of DNA that are commonly deleted in 22q11DS encompass genes that are important for development of organs that derive from the pharyngeal apparatus, which include the heart, face, thymus and parathyroid glands (reviewed in [3]). Consequently, 22q11DS is characterized by cardiac defects, abnormal facial features, thymic hypoplasia, cleft palate and hypocalcemia, which exhibit variable expressivity [9–15].

Thrombocytopenia and excessive bleeding have both been reported in patients with 22q11DS; however, the relationship between these conditions is complex and poorly understood. The simplest and best understood relationship, which is also the rarest, occurs in conjunction with Bernard-Soulier Syndrome. Bernard Soulier Syndrome is a rare, autosomal recessive, severe bleeding disorder that is characterized by low numbers of large platelets (i.e., macrothrombocytopenia) that are also dysfunctional (reviewed in [16–19]). Bernard Soulier Syndrome results from failure of platelets to express a functional form of the glycoprotein (GP) Ib-IX-V complex, which is essential for platelets to adhere to and initiate hemostasis at sites of vessel injury [20]. GPIb-IX-V is comprised of four different subunits (GPIbα, GPIbβ, GPIX and GPV), of which three (GPIbα, GPIbβ, GPIX) are required for surface expression of the complex [21] and one (GPV) is not [22]. Bernard Soulier Syndrome is typically the manifestation of homozygous or compound heterozygous pathogenic variants in the genes encoding GPIbα, GPIbβ or GPIX; variants in the gene encoding GPV have thus far not been associated with Bernard Soulier Syndrome [23]. Patients with 22q11DS are at higher than normal risk for development of Bernard Soulier Syndrome because they are hemizygous for *GPIBB*, which is the gene that encodes GPIbβ [24]. Since the first report of Bernard Soulier Syndrome in association with 22q11DS, in which the absence of GPIBβ was confirmed [24] and a pathogenic variant in the *GPIBB* promoter was subsequently identified [25], several additional cases attributable to

pathogenic nonsense [26] or missense [27–29] variants in *GPIBB* have been described. All of these patients had classic phenotypic features of Bernard Soulier Syndrome.

The relationship between macrothrombocytopenia and excessive bleeding in patients with 22q11DS is much more complex and perplexing. Case reports have documented both the absence of any bleeding problems [30–33] and the occurrence of life-threatening and sometimes fatal hemorrhage [34–37] in patients with 22q11DS. In most cases, these patients were found to have had large platelets and platelet counts that were either low or on the low end of normal. Several case series have reported that patients with 22q11DS have, on average, larger platelets and lower platelet counts than normal and either no or mild bleeding symptoms [30, 38–42]. One case series reported increased need for blood products following heart surgery amongst patients with 22q11DS, especially among those with large platelets, relative to patients with other causes of heart defects [43]. In the vast majority of these studies, *GPIBB* copy number and sequence information were not obtained. Consequently, it remains poorly understood how macrothrombocytopenia relates to excessive bleeding, and how *GPIBB* copy number and sequence variation relate to either macrothrombocytopenia or excessive bleeding, in the vast majority of patients with 22q11DS.

The present study was designed to clarify the relationship between bleeding severity and macrothrombocytopenia in patients with 22q11DS, and to evaluate the contribution of *GPIBB* copy number and sequence variation to both of these phenotypes. To accomplish this goal, we thoroughly characterized a small group of patients with 22q11DS with respect to bleeding severity, mean platelet volume (MPV), platelet count, and copy number and sequence information for *GPIBB* and other genes that encode requisite subunits of the GPIb-IX-V complex and that are associated with Bernard Soulier Syndrome.

METHODS

Patients

This study was approved by the Medical College of Wisconsin (MCW) Institutional Review Board (IRB). A total of 27 patients with a confirmed molecular diagnosis of 22q11.2 DS who were being treated at Children's Hospital of Wisconsin were consented for enrollment in the study. Complete sets of data were obtained for fifteen patients. Subject selection was unbiased relative to bleeding symptoms. Patients enrolled in this study were genetically unrelated subjects. Enrolled patients were administered the International Society of Thrombosis and Haemostasis Bleeding Assessment Tool (ISTH-BAT) to determine severity of bleeding symptoms [44]. In children, scores of 0–2 pre-puberty, <4 post-puberty in males, and <6 post-puberty in females define normal levels of bleeding severity [45]. Demographic information, diagnostic test results, platelet counts and mean platelet volumes (MPV) were extracted from medical records. Platelet function testing, GPIb-IX-V expression levels, gene copy number and sequencing results were obtained using blood (5 mL) drawn into a 3.2% sodium citrate tube (BD Biosciences, San Jose, CA). Control gene copy number results were obtained using blood drawn from healthy adult subjects who were consented for enrollment in the study.

Flow Cytometry

Platelet surface expression levels of the human GPIb-IX-V complex were determined using the GPIbα-specific monoclonal antibody, AP1, and levels of expression of the human GPIIb/IIIa complex were determined using the complex-specific monoclonal antibody, AP2. Both antibodies were purchased from the BloodCenter of Wisconsin Hybridoma Core (Milwaukee, WI). AP1 was conjugated to AlexaFluor-647 and AP2 was conjugated to AlexaFluor-488 using an antibody labeling kit (Thermo Fisher, Waltham, MA). Mouse IgG1/kappa isotype control antibodies conjugated to AlexaFluor-647 or Alexafluor-488 were purchased from Biolegend and used as negative controls.

Flow cytometry was performed using whole blood samples diluted 1:10 in HEPES/Tyrode's buffer (20mM HEPES, 137mM NaCl, 2.7mM KCl, 1mM $MgCl_2$, 5.6mM Glucose, 1 mg/ml bovine serum albumin, pH 7.4). The diluted whole blood samples were incubated in the dark for 20 minutes at room temperature with AP1–647, AP2–488, or isotype control antibodies. To quench the reactions, samples were diluted 10 fold with HEPES/Tyrode's buffer and analyzed immediately using an Accuri C6 flow cytometer (BD Biosciences, San Jose, CA). Median fluorescence intensity of GPIbα and GPIIb/IIIa were determined using FlowJo Ver10.0 software (FlowJo LLC, Ashland, OR).

Whole blood aggregometry

Platelet aggregation was determined in whole blood using a Multiplate aggregometer (Diapharma, West Chester, OH) according to the manufacturer's instructions. Agonists, and the final concentrations at which they were used, included arachidonic acid (ASPItest; 0.5mM), adenosine-5'-diphosphate (ADPtest; 6.5μM), thrombin receptor activating peptide (TRAPtest; 32μM), collagen (3.2 μg/ml), and high dose ristocetin (RISTOtest; 0.77 mg/ml).

Gene copy number and sequencing assays

Genomic DNA was prepared using the Gentra Puregene Blood Core Kit B (Qiagen, Hilden, Germany) from buffy coats that were isolated from whole blood. For gene copy number results, real-time quantitative PCR was performed using kits purchased from Life Technologies (Carlsbad, CA) with proprietary primers and probes for the target genes *GP1BA*, *GP9*, *GP1BB*, *DGCR6L*, *MED15*, and *SERPIND1* or for *RnaseP* as a reference. Extracted DNA, primers, and probes were combined with TaqMan polymerase (Life Technologies) and run on an ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA) under conditions that encompassed denaturation at 95°C for 10 min, followed by 40 amplification cycles that involved denaturation at 95°C for 15 sec and annealing/extension at 60°C for 1 min. Final copy number analysis was performed using Copy Caller Version 2.0 software (Applied Biosystems), using possession of 2 copies of the target genes *GP1BB*, *GP1BA*, and *GP9* in healthy adult controls and possession of 2 copies of the reference gene, *RnaseP* in both 22q11.2 DS patients and healthy adult controls, as calibrators.

Genomic DNA was amplified, in order to be sequenced, in 10% dimethyl sulfoxide (Sigma-Aldrich, Saint Louis, MO) on a BioRad T100 thermal cycler (Hercules, CA) using a HotStarTaq DNA Polymerase Kit (Qiagen), 10mM dNTPs (Life Technologies), and primers

that were designed by and purchased from Integrated DNA Technologies (IDT, Coralville, IA). The primers used for amplification are listed in the Supplementary Table 1. The conditions used for amplification of *GP1BB* and *GP9* involved an initial denaturation step at 95°C for 15 min, 35 amplification cycles that involved denaturation at 94°C for 45 sec, annealing at 60°C for 1 min, and extension at 72°C for 1min, and a final extension step of 10 min at 72°C. The conditions used for amplification of *GP1BA* involved an initial denaturation step at 95°C for 15 min, 45 amplification cycles that involved denaturation at 94°C for 45 sec, annealing at 55°C for 1 min, and extension at 72°C for 3 min, and a final extension step at 72°C for 10 min.

Amplified DNA was purified using Exo-SAP IT (Applied Biosystems) and sequenced using a BigDye Terminator kit (Thermo Fisher, Waltham, MA). Primers used for sequencing were designed and purchased from IDT and are listed in Supplementary Table 1. Sequencing reactions were run on a BioRad T100 thermal cycler with one sequencing primer in each reaction and conditions that involved 25 cycles of denaturation at 96°C for 30 sec, annealing at 50°C for 15 sec, and extension at 60°C for 4 min. Sequencing reactions were cleaned up on Centri-Sep spin columns (Princeton Separations, Freehold, NJ), lyophilized with a Savant DNA120 SpeedVac Concentrator (Thermo Fisher), and analyzed on a 3130xl Genetic Analyzer (Applied Biosystems). Sequences were analyzed for single nucleotide polymorphisms (SNPs) using SnapGene 3.3.3 software (GSL Biotech, Chicago, IL) and reference genomic DNA sequences from the NCBI RefSeq database. Non-consensus SNPs in *GP1BB*, *GP1BA*, and *GP9* that were identified in our patients were verified with the NCBI SNP database.

Variable Number of Tandem Repeat (VNTR) analysis

GP1BA has a variable number of tandem repeats (VNTR), each of which encodes a 13-amino acid long segment, in the region that encodes the macroglycopeptide stalk of GPIBα [46]. Humans possess four *GPIBA* VNTR variants with four (haplotype A), three (B), two (C), and one (D) repeat [46, 47]. For patients who were homozygous for the *GPIBA* VNTR variant, haplotypes were assigned based on *GPIBA* sequence information. For patients who were heterozygous for the *GPIBA* VNTR variant, the *GPIBA* VNTR was amplified by PCR and haplotypes were assigned based on the pattern of migration of PCR products in an agarose gel. Genomic DNA from heterozygous patients was amplified using the HotStarTaq DNA Polymerase Kit, 10mM dNTPs, and primers specific to the VNTR region that were designed by and purchased through IDT (Supplementary Table 1). Amplification involved an initial denaturation step at 95°C for 15 min, 30 amplification cycles that involved denaturation at 94°C for 45 sec, annealing at 60°C for 1 min, and extension at 72°C for 3 min, and a final extension step at 72°C for 10 min. Amplified PCR products were electrophoresed on a prepared 4% agarose gel (Fisher Scientific, Hampton, NH), stained with ethidium bromide, and imaged using an AlphaImager HP system (Protein Simple, San Jose, CA).

Statistics

The Students' t-test was performed using GraphPad Prism 6 software (GraphPad, La Jolla, CA) to determine the statistical significance of differences in the extent of platelet count,

mean platelet volume, and platelet aggregation between patients with high vs. low ISTH-BAT scores.

RESULTS

Patient Characteristics

The ages of the patients ranged from 3 to 17 years, with a median age of 9 years. Half of the patients (8/15, 53.3%) were female. All patients had a confirmed molecular diagnosis of 22q11.2 DS, which was based on results of a fluorescence *in situ* hybridization test for the majority (9/15, 60%), and a chromosomal microarray test for the remainder of the patients.

Self-reported ISTH-BAT scores

The patients enrolled in our study reported ISTH-BAT scores ranging from 0–9, with a median score of 3 (Table 1). The scores of half of the patients (7/15, 46.6%) fell within the normal range. The remainder of the patients (8/15, 53.3%) reported scores reflective of higher than normal levels of bleeding severity. Cutaneous bleeding was the symptom most commonly associated with higher than normal ISTH-BAT scores, with 40% (6/15) of patients (#6, 12, 13, 18, 24, and 27) reporting unexplained and/or frequent bruising, all of whom had higher than normal ISTH-BAT scores. Epistaxis was the next most common symptom, with 25% (4/15) of patients (#1, 6, 12, and 25) reporting spontaneous and frequent nosebleeds, three of whom had higher than normal ISTH-BAT scores. Excessive bleeding from the mouth was reported by two patients (#10 and 27), one of whom had a higher than normal ISTH-BAT score. Prolonged bleeding from minor wounds (patients #13 and 27) and gastrointestinal bleeding (patients #22 and 25) were each reported by two patients, all four of whom had higher than normal ISTH-BAT scores. The patient with the highest ISTH-BAT score (patient #27) also reported excessive post-surgical bleeding following cardiac surgery with cardiopulmonary bypass. Based on these findings, we conclude that the severity of bleeding symptoms was higher than normal in 53% of our cohort of patients with 22q11.2 DS.

Gene copy number analysis

We found that, regardless of their ISTH-BAT scores, all of the patients enrolled in our study had 2 copies of *GP1BA* and *GP9 and* 1 copy of *GPIBB* (Fig. 1). With the exception of one patient who had 2 copies of *SERPIND1*, all patients had 1 copy each of *DGCR6L*, *MED15*, and *SERPIND1*, indicating that their deletion encompassed LCRA-D of chromosome 22 (Fig. 1). These results signify no relationship between ISTH-BAT scores and either *GPIBB* copy number or the extent of the deletion within chromosome 22 in patients with 22q11.2 DS.

Sequencing of GP1BB, GP1BA, and GP9

Our results revealed seven single nucleotide variants (SNVs) and one insertion in the genes encoding GP1bβ, GP1bα, and GPIX in our patients (Table 1). These included 1 SNV in *GPIBB*, 2 SNVs in *GP9*, and 4 SNPs and one insertion in *GPIBA*. With one exception, all of the sequence variants that we identified have been previously reported (Table 1). The one exception was a synonymous G>A substitution at position 336 within *GPIBA*. Also with one

exception, none of the sequence variants that we identified affected the amino acid sequence of the protein it encodes. The one exception was a missense C>T substitution at position 482 of *GPIBA*, which results in substitution of threonine with methionine at position 145 of the mature GPIBα polypeptide and defines the human platelet antigen (HPA)-2b polymorphism (reviewed in [48]). HPA-2b has been previously associated with thrombocytopenia in genome-wide association studies [49, 50]. Interestingly, the two patients enrolled in our study who possessed this variant also had the lowest platelet counts (117 and 120 × 10^3 platelets/μL); however, both of these patients reported ISTH-BAT scores of 0. None of the sequence variants that we identified was more prevalent amongst patients with higher than normal ISTH-BAT scores relative to those with normal scores. These results indicate that variants in *GPIBB*, *GPIBA* or *GP9* are not associated with ISTH-BAT scores in patients with 22q11.2 DS.

GPIBA VNTR polymorphisms

The majority of the patients enrolled in our study (12/15, 80%) were homozygous for the C haplotype of the *GPIBA* VNTR polymorphism (Table 1). Of the three remaining patients, two were heterozygous for the CD haplotype and one was heterozygous for the BC haplotype. The finding of either homozygosity or heterozygosity for the C haplotype of the *GPIBA* VNTR polymorphism in all of our patients is compatible with the previous report that the C haplotype is the most frequent phenotype observed in humans [51]. None of the *GPIBA* VNTR haplotypes was associated with ISTH-BAT scores in patients with 22q11.2 DS.

Platelet number, size and function

As shown in Table 2, MPVs were within or above the normal range and platelet counts were within or below the normal range for all 22q11DS patients. Platelet responses to agonist stimulation fell within normal reference ranges for all agonists except collagen and Ristocetin. Only one patient in each group exhibited slightly lower than normal responses to Ristocetin; aggregation responses to all other agonists were normal or slightly higher than normal. GPIb-IX-V expression levels ranged from approximately 50%, to slightly more than 100%, that of the platelet-specific integrin, GPIIb, for all patients. Median MPVs, platelet counts, levels of GPIb-IX-V expression and agonist-induced platelet aggregation responses did not correlate with ISTH BAT scores. Collectively, the results shown in Table 2 indicate that in patients with 22q11.2 DS ISTH-BAT scores do not correlate with larger platelets, lower platelet counts, lower levels of GPIb-IX-V expression on platelet surfaces, or platelets that are less responsive to agonist stimulation.

DISCUSSION

This study enabled clarification of the relationship between macrothrombocytopenia and bleeding in patients with 22q11DS by thoroughly characterizing bleeding severity, platelet size and count, and *GPIBB* genetics in a small group of patients. As summarized in Fig. 2, we found that the patients included in our study were all hemizygous for *GPIBB* and none had a deleterious variant in their sole copy of *GPIBB*. Approximately 50% of the patients reported higher than normal ISTH-BAT scores and 25% had MPVs and platelet counts

characteristic of macrothrombocytopenia. One patient had thrombocytopenia but not large platelets and one patient had large platelets but not thrombocytopenia. Compared to previously described patients who had macrothrombocytopenia in association with Bernard Soulier Syndrome, which was caused either by homozygous or compound heterozygous pathogenic variants in *GPIBB* (Supplementary Table 2) or by hemizygosity for a mutated copy of G*PIBB* (Supplementary Table 3), bleeding was generally less severe, platelet counts were generally not as low and platelet volumes did not range as high in patients who had macrothrombocytopenia in association with 22q11DS. The four patients with, and the eleven patients without, macrothrombocytopenia were equally distributed amongst patients with normal and higher than normal ISTH-BAT scores. These findings reveal that mild macrothrombocytopenia and a mild bleeding diathesis are characteristic of incompletely overlapping subsets of patients, and that neither *GPIBB* copy number nor *GPIBB* sequence variation accounts for either macrothrombocytopenia or excessive bleeding in patients with 22q11DS.

Only 25% of the patients with 22q11DS in our study had both large platelets and low platelet counts, suggesting that macrothrombocytopenia may not be as representative a feature of the wide spectrum of 22q11DS as has been previously supposed [32, 36, 39, 41, 52, 53]. This supposition is based, in part, on case reports, all of which described patients with large platelets (where platelet size was reported) and platelet counts that dropped below normal on at least one occasion (Supplementary Table 4). Case series of 22q11DS patients have perpetuated the perception that macrothrombocytopenia is characteristic of 22q11DS by reporting that groups of patients with 22q11DS have, on average, higher MPVs and lower platelet counts than control populations (Supplementary Table 5). However, the incidence of macrothrombocytopenia was not reported in most studies and, where reported, was less than 50% [39, 52]. Furthermore, average MPVs and platelet counts may be susceptible to skewing by a small number of patients with high MPVs and/or low platelet counts. The finding that the existence in our study population of only five patients with MPVs > 10 fL brought the mean MPV for all fifteen patients up to 10.2 fL, which is comparable to the mean MPVs reported for patients with 22q11DS in other case series, exemplifies the latter problem. Based on these findings, we propose that consideration of 22q11.2DS in association with congenital heart disease should not be limited to infants with increased MPV and mild thrombocytopenia [54], but should be extended to infants without platelet abnormalities as well.

Our findings also challenge the prevailing view that *GPIBB* hemizygosity is the cause of macrothrombocytopenia and a bleeding tendency in patients with 22q11DS [32, 39–41, 55]. This view is not based on proof of a causal relationship but is instead founded on overlap of features observed in 22q11DS with the phenotype observed in individuals with Bernard Soulier Syndrome due to hemizygosity or homozygosity for pathogenic variants of *GPIBB* (Supplementary Tables 2 and 3). By determining *GPIBB* copy number and sequence, platelet size and volume, and bleeding severity in individual patients with 22q11DS, we demonstrate definitively that *GPIBB* hemizygosity alone is not correlated with either macrothrombocytopenia or a bleeding tendency as measured by ISTH-BAT scores in these patients. Instead, *GPIBB* hemizygosity exhibits incomplete penetrance with respect to these traits, which reflects the influence of other genetic loci or environmental factors on

macrothrombocytopenia and bleeding in patients with 22q11DS. Other genetic causes of these phenotypes include variations in genes other than *GPIBB* that are located within the deleted region. One such gene is *SEPT5*, which resides 250 nucleotides 5' of *GPIBB* and is important for granule exocytosis in platelets and neurons [56–59]. Homozygous deletion of both *GPIBB* and *SEPT5* was associated with macrothombocytopenia and severe bleeding in a young boy [60]; however, independent contributions of *GPIBB* vs. *SEPT5* deficiency to these phenotypes were not ascertained in this patient. Variants in genes located outside of the deleted region may also cause macrothrombocytopenia and bleeding. Meta-analysis of genome wide association studies recently identified sixty-eight independent loci associated with MPV and/or platelet count in humans [50], among which only one (*ARVCF*) is located within the commonly deleted region in 22q11DS. Whether variants in any of the loci located outside the deleted region cause macrothrombocytopenia and bleeding in patients with 22q11DS remains to be determined. In addition, environmental factors, including surgery or trauma, autoimmune- or alloimmune-mediated platelet destruction, infection- or drug-induced bone marrow suppression, or bone marrow failure (reviewed in [61]), could also cause thrombocytopenia and bleeding in patients with 22q11DS.

Based on our understanding of the complex relationship between *GPIBB*, macrothrombocytopenia and bleeding in patients with 22q11DS, we have developed a decision tree to guide management of bleeding problems in these patients (Fig. 3). The tree uses MPV and platelet count data, obtained from a complete blood count, to diagnose (macro)thrombocytopenia and, if present, its likely cause. MPVs > 10 fL and platelet counts < 150,000/μL would justify sequencing of *GPIBB* to molecularly assess for Bernard Soulier Syndrome; if no pathogenic variant of *GPIBB* is identified, another genetic cause of congenital macrothrombocytopenia should be considered. The degree of thrombocytopenia is the biggest predictor for the risk of bleeding in patients with 22q11DS [30, 32, 34]; therefore, the types of bleeding expected in patients with 22q11DS would depend on the patient's platelet count. Patients with platelet counts above 50,000/μL might exhibit mild to moderate bleeding symptoms, whereas those with platelet counts below 50,000/μL might exhibit severe bleeding. Management of severe bleeding should be dictated by the cause of the low platelet count and should be compatible with published guidelines [62–65].

Our findings have potential relevance to the finding of rare variants of *GPIBB* that, in heterozygous form, are associated with macrothrombocytopenia [66]. Individuals who are heterozygous for such variants are similar to patients with 22q11DS in that they possess only one copy of a normal *GPIBB* allele but different in that they also possess a variant *GPIBB* allele. Whether macrothrombocytopenia in these individuals is due to autosomal dominance of the variant *GPIBB* allele or to variants in other genetic loci depends on how each *GPIBB* variant affects expression and function of GPIb-IX-V. Variants that encode a form of GPIBβ that either doesn't allow trafficking of GPIb-IX-V to the platelet surface or allows trafficking to the surface but causes GPIb-IX-V to be dysfunctional would be autosomal dominant and cause macrothrombocytopenia. In contrast, variants that abrogate expression of GPIBβ would behave like missing alleles, and macrothrombocytopenia that occurs in individuals heterozygous for such alleles would likely be caused by variants in other genetic loci, as they are in patients with 22q11DS. Evaluation of platelet count and size in family members who are homozygous for the wild-type *GPIBB* allele should help to deduce whether the

GPIBB or another genetic variant is responsible for macrothrombocytopenia in individuals who are heterozygous for a given variant *GPIBB* allele. An interesting case-in-point is a previously described family [67], in which members who were homozygous for a variant allele of *GP9* (N45S) had Bernard Soulier Syndrome, whereas those who were either heterozygous for the variant allele or homozygous for the wild-type allele generally had large platelets and, in some cases, also had low platelet counts (Supplementary Figure). It is likely that a variant in a genetic locus other than *GP9* is responsible for the macrothrombocytopenia associated with heterozygosity for the variant *GP9* allele or homozygosity for the wild-type allele in this family. The extent to which variants of *GPIBB* (Supplementary Table 6) or *GPIBA* [68] that, in heterozygous form, are associated with macrothrombocytopenia remain to be determined.

The limitations of our study are that bleeding severity was assessed using only one bleeding assessment tool, that the study included only a small number of patients, and that the causes of large platelets and/or low platelet counts were not determined for the subset of patients who exhibited these platelet defects. Future studies should be done to determine whether the findings are reproducible with other bleeding assessment tools [69, 70] and whether they generalize to a larger, but equally well-characterized, group of patients with 22q11DS. The genetic basis of macrothrombocytopenia in patients with 22q11DS, when it occurs, remains to be conclusively determined. With respect to the latter, patients with 22q11DS may represent a unique and valuable resource for determining how genes other than *GPIBB* that reside in the deleted region contribute to formation of platelets of appropriate size, number and function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ESSENTIALS

- How thrombocytopenia relates to bleeding in 22q11 deletion syndrome (22q11DS) is not clear
- Bleeding severity, platelet count and volume, and *GPIBB* were examined in patients with 22q11DS
- Macrothrombocytopenia and bleeding typified imperfectly overlapping subsets of 22q11DS patients
- *GPIBB* hemizygosity does not cause macrothrombocytopenia or bleeding in patients with 22q11DS

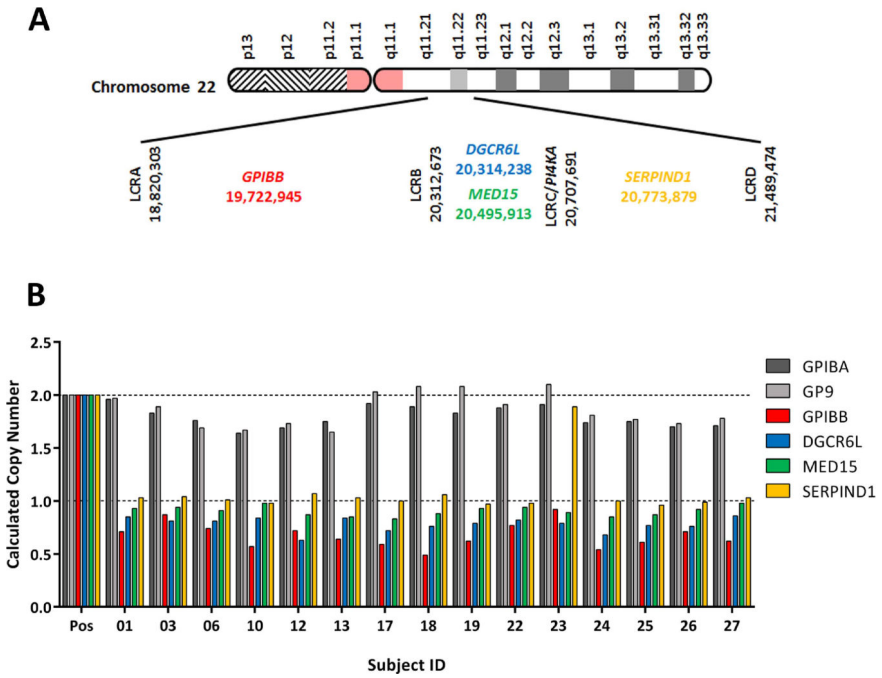

FIGURE 1.

Gene copy number analysis of patients with 22q11.2 DS. (A) Schematic diagram of chromosome 22. The banding pattern is as depicted atghr.nlm.nih.gov/chromosome/22. The locations of LCRA, LCRB, and LCRD are as described in [71]. The locations of *PI4KA* as a reference point for LCRC, and of *GPIBB*, *DGCR6L*, *MED15* and *SERPIND1* are as depicted at www.genecards.org. (B) Reference (*RnaseP*) and target (*GP1BA*, *GP9, GP1BB, DGCR6L, MED15, and SERPIND1*) genes were amplified from genomic DNA extracted from pediatric patients diagnosed with 22q11.2 deletion syndrome. Copy numbers were calculated for each patient and compared to genomic DNA extracted from a healthy adult (Pos) as a 2-copy control calibrator for each target gene. Note that, with the exception of patient 23, who had 2 copies of *SERPIND1*, all of the 22q11.2 DS patients included in this study had 1 copy each of *GPIBB*, *DGCR6L*, *MED15*, and *SERPIND1* and 2 copies each of *GPIBA* and *GP9*.

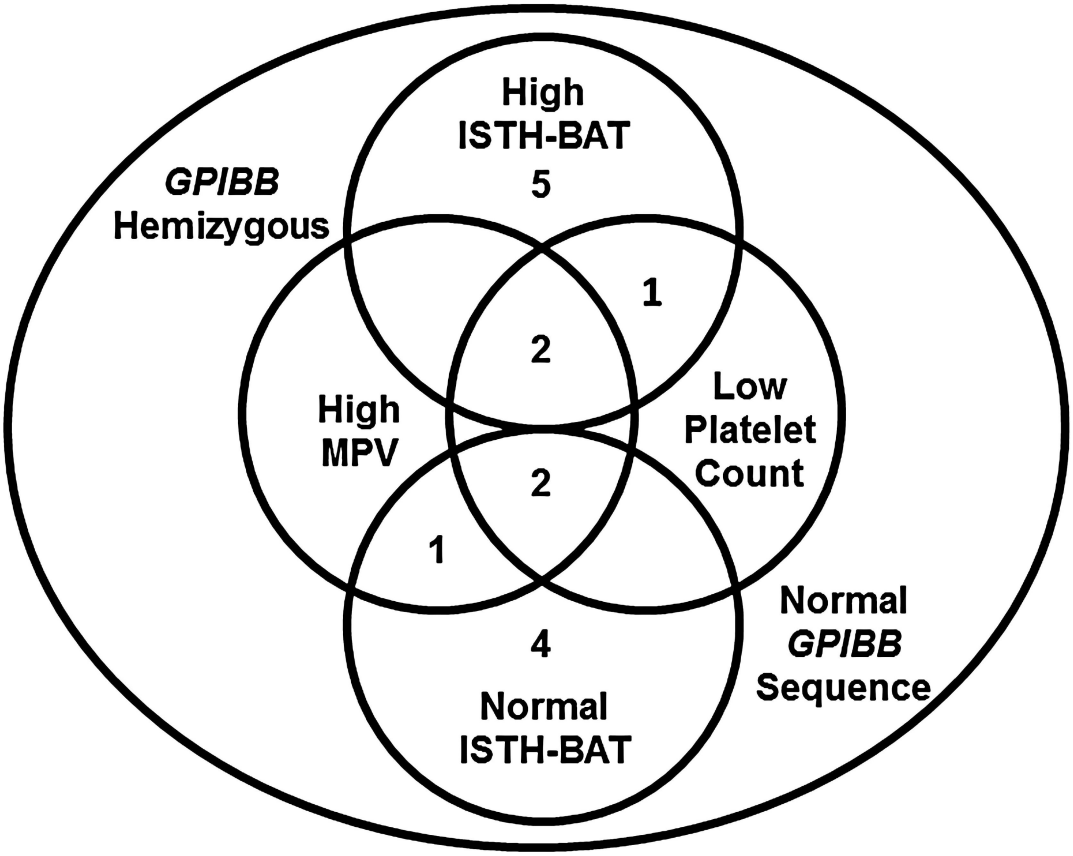

FIGURE 2.

Overlapping Venn diagram describing the complex relationship between *GPIBB* hemizygosity and sequence variation, macrothrombocytopenia, and bleeding tendency in patients with 22q11DS. All of the patients included in our study were hemizygous for *GPIBB* and the sequences of their remaining copies of *GPIBB* were all normal. Approximately 50% (8/15) of the patients reported higher than normal ISTH-BAT scores of ≥ 3, and an equivalent percentage (7/15) reported ISTH-BAT scores within the normal range of 0–2. Approximately 25% (4/15) of the patients had high MPVs (> 10 fL) and low platelet counts (< 150,000/μL), which is characteristic of macrothrombocytopenia. One platelet had a high MPV but normal platelet count and another patient had a low platelet count but normal MPV. The four patients with, and the eleven patients without, macrothrombocytopenia were equally distributed amongst patients with normal and higher than normal ISTH-BAT scores. These findings reveal that mild macrothrombocytopenia and a mild bleeding diathesis are characteristic of incompletely overlapping subsets of patients, and that neither *GPIBB* copy number nor *GPIBB* sequence variation accounts for either macrothrombocytopenia or excessive bleeding in patients with 22q11DS. *Abbreviations: ISTH-BAT, International Society of Thrombosis and Haemostasis Bleeding Assessment Tool;* GPIBB*, gene encoding glycoprotein Ibβ.*

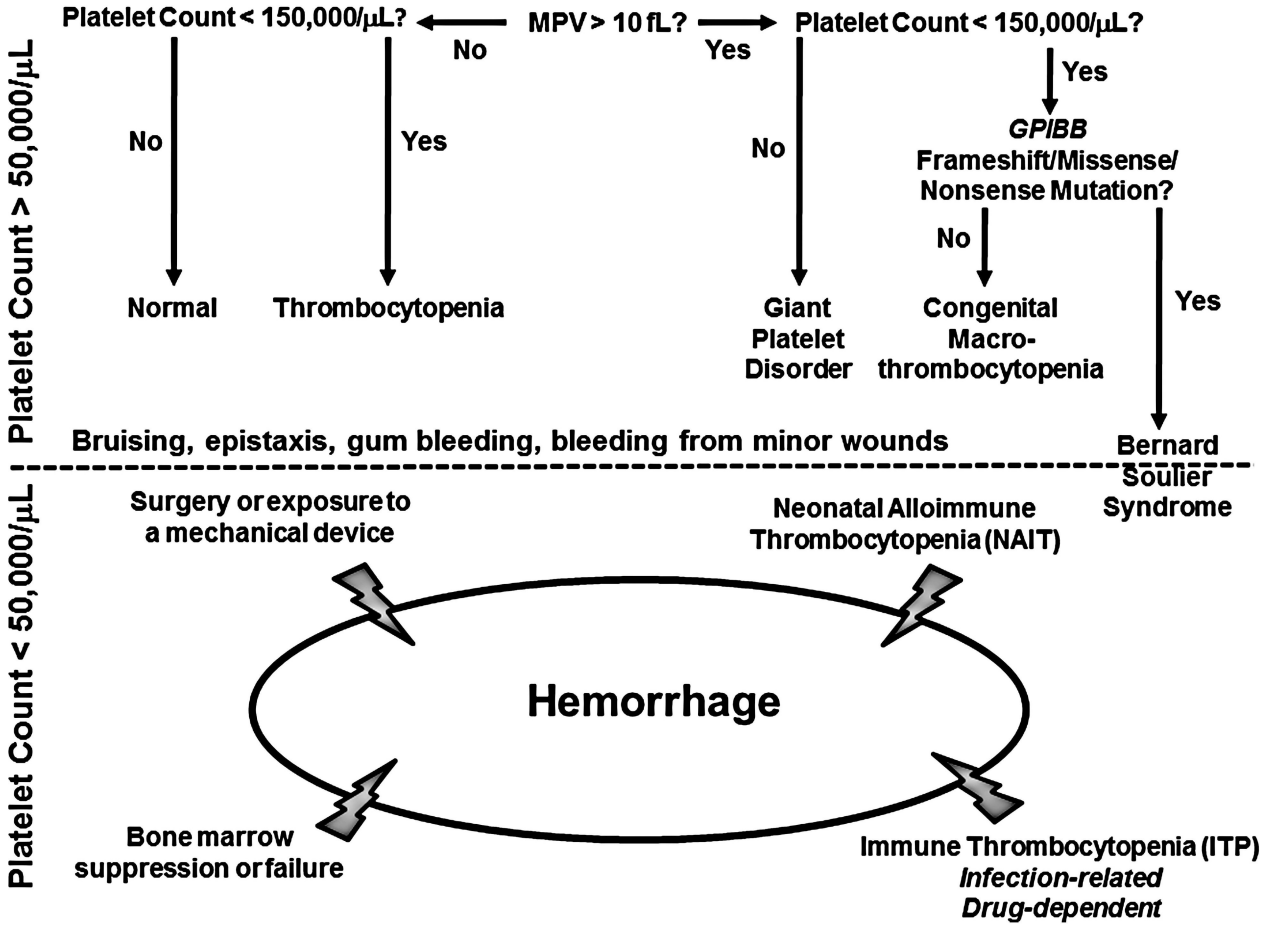

FIGURE 3.

Implications of the findings for management of patients with 22q11DS. MPV and platelet count data can be acquired by performing a complete blood count and used to diagnose thrombocytopenia and, if present, its likely cause. MPVs > 10 fL and platelet counts < 150,000/μL would support diagnosis of macrothrombocytopenia; subsequent identification of pathogenic variants in *GPIBB* would support diagnosis of Bernard Soulier Syndrome, whereas their absence would support diagnosis of congenital macrothrombocytopenia caused by another gene defect. MPVs > 10 fL and platelet counts ≥ 150,000/μL would justify diagnosis of a giant platelet disorder. MPVs ≤ 10 fL and platelet counts < 150,000/μL would support diagnosis of thrombocytopenia, and MPVs ≤ 10 fL and platelet counts ≥ 150,000/μL would be indicative of normal platelet parameters. The dashed line divides patients on the basis of whether platelet counts are above or below 50,000/μL, which may determine the type of bleeding that might be expected. Mild to moderate bleeding symptoms would be expected of patients with platelet counts above 50,000/μL, whereas those with platelet counts below 50,000/μL might exhibit severe bleeding. Different factors could cause platelet counts to drop below 50,000/μL, including genetic causes (Bernard Soulier Syndrome) or environmental factors (surgery or exposure to a mechanical device, bone marrow suppression or failure, or platelet clearance by allo- or auto-antibodies. Strategies for management of severe bleeding would be determined by the suspected cause of the low

platelet count. *Abbreviations: MPV, mean platelet volume;* GPIBB*, gene encoding glycoprotein Ibβ; ICH, intracranial hemorrhage; PPH, post-partum hemorrhage.*

TABLE 1

Bleeding severity, platelet parameters, and *GP1BB*, *GP1BA*, and *GP9* sequence information in patients with 22q11 deletion syndrome

Patient ID	ISTH-BAT score	MPV (fL)	Platelet Count(x 10^3/μL)	*GPIb-IX-V Expression(% of GPIIb/IIIa)*	*GP1BB*Sequence	*GP9*Sequence	*GPIBA*Sequence	*GP1BA*VNTR
27	9	9.3	148	91	107 C > T[†]	459 G > A[‡]	336 G > A[¶¶]	CC
25	6	13.7	138	135	Consensus	Consensus	94_95ins G[¶]	CC
							1161 C > T[††]	
13	5	9.1	170	65	Consensus	Consensus	Consensus	CC
12	5	9.2	178	118	Consensus	Consensus	-1207 A > G[‡‡]	CC
18	3	12.5	143	82	107 C > T[†]	Consensus	Consensus	CC
6	3	8.7	157	78	Consensus	Consensus	-1207 A > G[‡‡]	CC
24	3	9.2	226	92	107 C > T[†]	Consensus	-1207 A > G[‡‡]	CC
22	3	8.3	235	120	Consensus	Consensus	Consensus	CD
1	1	8.5	176	72	Consensus	Consensus	Consensus	CC
10	1	9.9	191	91	Consensus	Consensus	Consensus	CD
19	0	14.9	117	68	107 C > T[†]	132 G > A[§]	482 C > T[§§]	BC
23	0	12.1	120	51	107 C > T[†]	Consensus	-1207 A > G[‡‡]	CC
							482 C > T[§§]	
3	0	11.1	176	24	Consensus	Consensus	Consensus	CC
26	0	8.9	279	91	Consensus	Consensus	Consensus	CC
17	0	8.0	299	109	Consensus	Consensus	94_95ins G[¶]	CC
							1161 C > T[††]	

Abbreviations: ISTH BAT, International Society of Thrombosis and Haemostasis Bleeding Assessment Tool; MPV, mean platelet volume; VNTR, variable number of tandem repeats

GP1BA VNTR polymorphism haplotypes: B = 3 repeats, C = 2 repeats, D = 1 repeat

NCBI dbSNP ID, Function Class

[†] rs1059196, 3' UTR

[‡] rs753215807, synonymous codon

§ rs6069, synonymous codon

¶ rs199616811, 3' UTR

†† rs762865345, synonymous codon

rs145319139, upstream variant 2KB

§§ rs6065, missense (T161M)

¶¶ new polymorphism, synonymous codon

TABLE 2

Platelet phenotypic and functional characteristics in pediatric 22q11.2 DS patients

		ISTH-BAT score group		
	Reference	**0–2**	**≥ 3**	
Mean Platelet Volume in fL, median (range)	7.0–9.5†	9.9 (8.0–14.9)	9.2 (8.3–13.7)	$p = 0.6855$
Platelet Count x 10^3/μL, median (range)	150–450†	176 (117–299)	163.5 (138–235)	$p = 0.7080$
GPIb-IX-V expression (GPIbα as % of GPIIb/IIIa MFI), median (range)	N/A	91 (51–124)	101 (65–120)	$p = 0.4507$
Multiplate Aggregation in AUC, median (range)	-	-	-	-
ASPItest	35–102‡	72 (62–99)	74 (49–91)	$p = 0.8570$
ADPtest	33–112‡	64 (51–79)	64 (54–107)	$p = 0.6808$
Collagen	30–86‡	78 (55–100)	83 (55–129)	$p = 0.5676$
TRAPtest	73–145‡	90 (74–122)	108 (88–117)	$p = 0.1910$
RISTOtest	25–127‡	61 (20–71)	75 (24–111)	$p = 0.3463$

Abbreviations: GP, glycoprotein; AUC, area under curve; ISTH-BAT, International Society of Thrombosis and Haemostasis Bleeding Assessment Tool; MFI, median fluorescence intensity

†Normal reference ranges are those indicated by the clinical diagnostic laboratory at Children's Hospital of Wisconsin

‡Healthy adult reference ranges for each platelet agonist as previously described [72]

p-values were derived using a Students' t-test