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The beta 1 tubulin R307H single nucleotide polymorphism is associated with treatment failures in immune thrombocytopenia (ITP)

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Summary

Predictive biomarkers are needed in ITP. Single nucleotide polymorphisms (SNPs) in beta 1 tubulin are potential candidates, as beta 1 tubulin is integral for platelet production and function, and SNPs in beta 1 tubulin have been associated with distinct phenotypes in platelets. We investigated the most prevalent beta 1 tubulin SNP (R307H) as a biomarker in patients with ITP via a retrospective chart review. Allelic frequencies between a group of 191 ITP patients and a healthy control group showed no difference, suggesting no direct etiologic role for the SNP in ITP. However, over similar periods of follow-up, both heterozygote and homozygote minor allele ITP patients were treated with significantly more treatment modalities and had significantly higher risk of failure to immune-modulatory therapies (RR=1.5, 95%CI=1.1–2.1;p=0.01); with rituximab, in particular, ITP patients with the SNP experienced a 58% failure rate (RR=1.6, 95%CI=1.03–2.5; p=0.04). Analysis of the absolute immature platelet fraction (A-IPF) as a marker of platelet production showed that SNP patients had significantly higher median A-IPFs compared to non-SNP patients when complete responses were achieved using immune modulatory therapies. The data suggest that the beta 1 tubulin R307H SNP has potential for use as a biomarker in ITP and may affect platelet turnover.

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Author Contributions

P.A.B., J.B.B., and P.G. designed the research; Z.H. collected patient data; P.A.B. performed laboratory experiments; P.A.B., J.B.B., P.J.C., and P.G. analyzed and interpreted the data, and wrote the paper; P.A.B. and P.J.C. performed statistical analysis and made the figures

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Keywords

immune thrombocytopenia; single nucleotide polymorphisms; tubulin; rituximab; immature platelet fraction

Introduction

Immune thrombocytopenia (ITP) is an autoimmune hematologic disorder in which accelerated platelet destruction and decreased platelet production lead to a reduction in peripheral blood platelets (Ballem, *et al* 1987, Chang, *et al* 2003, McMillan 2000, McMillan, *et al* 2004, Nugent, *et al* 2009). The clinical features of ITP are highly variable: a wide range of platelet counts is observed among patients, and serious bleeding events, though rare, are difficult to predict (Neunert, *et al* 2009).

Further complicating the picture are the wide range of treatment options available and the unpredictability of responses to specific treatments (Cohen, *et al* 2000). Immune-modulatory therapies are the mainstay of treatment for ITP. Steroids, IVIG, and anti-D remain the first-line treatments designed to immediately increase the platelet count. Second-line therapy has historically been splenectomy, although rituximab has become increasingly popular, and multiple other agents are options as well (Provan, *et al* 2010). These immune-modulatory therapies are thought to act primarily via reduction of platelet destruction (Cines, *et al* 2003). Thrombopoietin receptor (TPO-R)-agonists have been approved worldwide for use as second- and third-line agents in ITP and work primarily via activation of the TPO-R to stimulate platelet production (Evangelista, *et al* 2007, Kuter 2007).

There is, however, uncertainty regarding the exact therapeutic mechanisms of both TPO-agents and immune-modulatory therapies in ITP. In some instances immune-modulators may stimulate platelet production by reducing the autoimmune anti-megakaryocyte effect (Barsam, *et al* 2011), while TPO-agents may modulate the immune response by increasing—and even normalizing—T-regulatory cell activity (Bao, *et al* 2010).

Due to the limitations of knowledge described above, tailoring therapy for the individual ITP patient is difficult. Studies of genetic variations have been performed to aid in tailoring therapy. Most studies in ITP have focused on the immune system. Evaluations of single nucleotide polymorphisms (SNPs) in cytokines, the Fc γ receptor (Foster, *et al* 2001, Fujimoto, *et al* 2001, Suzuki, *et al* 2008), and methylation enzymes (Chen, *et al* 2008, Zhao, *et al* 2009, Zhao, *et al* 2010) have shown some correlations with disease characteristics and treatment responses. They have yet to be applied to clinical practice.

To the best of our knowledge, there have been no published studies of SNPs within factors specific to platelet physiology as biomarkers in ITP. One candidate for this is beta 1 tubulin (Class VI, *TUBB1*), a tubulin isotype that is specific to hematopoietic cells and comprises 90% of beta-tubulin found in the platelet marginal band (Kenney and Linck 1985, Lecine, *et al* 2000), a structure known to be important in platelet production (Patel, *et al* 2005, Richardson, *et al* 2005, Schwer, *et al* 2001) and morphology (White and Rao 1982, White and Rao 1998). Beta 1 tubulin is unique among tubulins in the occurrence of non-

synonymous SNPs. Some SNPs have been shown to have significant impact on platelet physiology, with reports of spherical, hypo-reactive platelets with Q43P (Freson, *et al* 2005, Navarro-Nunez, *et al* 2011) and congenital macrothrombocytopenia with R318W (Kunishima, *et al* 2009).

The associations between beta 1 tubulin SNPs with changes in platelet physiology make it a potential candidate for a biomarker in ITP. We focused our analysis on the R307H SNP, which is the most common beta 1 tubulin SNP, as this would allow for application of any findings to the largest group of ITP patients. Here we report on our findings of an association between the R307H SNP and treatment outcomes and platelet dynamics in ITP.

Methods

Patients and clinical data

Two hundred randomly selected patients with chronic ITP seen at the Platelet Disorders Center at Weill Cornell Medical College were enrolled in the study after providing written, informed consent in accordance with the Declaration of Helsinki and the Weill Cornell Internal Review Board (IRB). Separate consent was obtained for treatment with experimental agents, if used. ITP was diagnosed by international consensus guidelines (Provan, *et al* 2010). Age was not an exclusion criterion. Treatment selection was at the discretion of the patient and provider.

A previously reported population of 361 healthy individuals was used for genotype frequency comparisons. Details of recruitment, collection, and analysis are reported separately (Leandro-Garcia, *et al* 2012). Clinical information was obtained by retrospective chart review by investigators blinded to beta 1 tubulin R307H genotypes. Patient demographics obtained were age at analysis of R307H SNP, sex, and concurrent autoimmune disease. Age of onset of ITP and platelet count at initial presentation of ITP were obtained. Duration of ITP follow-up was defined as the time from initial presentation of ITP to the time of the current analysis.

Treatments were categorized as T P O-agent (romiplostim, eltrombopag, AKR501/E5501, and ligand LGB4665) and immune-modulatory (all others). The total number of different treatments used was counted for each patient; treatments used in combination were counted individually. Treatments evaluated for response in this study were only those for which platelet counts were available for analysis. Treatment response was defined as a platelet count $>50 \times 10^9/l$ and an absolute increase in platelet count $20 \times 10^9/l$ from baseline.

Genetic analysis

Whole blood was collected by phlebotomy and stored at -70°C . Genomic DNA was extracted from whole blood using the GenElute Mammalian DNA MiniPrep Kit (Sigma-Aldrich, St. Louis, MO). Identification of rs6070697G>A (leading to R307H substitution) in *TUBB1* (the gene encoding beta 1 tubulin) was performed by PCR amplification using previously published primers (Freson, *et al* 2005) and Sanger sequencing. Nine patient samples did not yield sufficient DNA for analysis.

Absolute Immature Platelet Fraction

Peripheral blood samples for measuring absolute immature platelet fractions (A-IPF) were obtained from a subset of ITP patients. Blood was collected in ethylenediaminetetraacetic acid tubes which were then processed at the Platelet Disorders Center at Weill Cornell Medical College campus of New York Presbyterian Hospital, on a Sysmex XE-2100 within 8 hours of venesection to obtain IPF. A-IPF was calculated by multiplying the simultaneously measured platelet count by the IPF. Platelet counts were divided into three ranges for A-IPF analysis: no response ($1-50 \times 10^9/l$), partial response ($51-100 \times 10^9/l$), and complete response ($101-450 \times 10^9/l$). Platelet counts $>450 \times 10^9/l$ were excluded from the analysis. A-IPFs were further classified by type of treatment received: immune-modulatory or TPO-agent. For each patient, a median A-IPF was determined for each platelet range by treatment type. The mean of all median A-IPFs for each BETA 1R307H genotype group was then calculated for each platelet range by treatment type.

Statistical Analysis

Descriptive statistics (including mean, standard deviation, standard error of mean, median, range, frequency, percent) were calculated to characterize the study population and were stratified by genotype and allele frequency groupings. Fisher's exact test was used to evaluate the association between categorical variables of interest and genotype grouping (i.e., G/G, G/A, and A/A, as well as SNP (G/A+A/A) and non-SNP (G/G)). The Mann-Whitney U test and Kruskal-Wallis test were used, as appropriate, to compare 1) median platelet count at initial ITP diagnosis, 2) median age, 3) median duration of ITP follow-up, and 4) the median number of ITP treatment modalities used, between the three genotype categories. The two-sample t-test was used to compare mean values of median A-IPFs between the genotype categories. Linear regression analysis was used to evaluate the relationship between platelet count and A-IPF, performed separately for SNP and non-SNP patients. Pearson correlation coefficients for each regression model are presented to assess the degree of the observed linear correlation between platelet count and A-IPF. P-values for the correlation coefficient were adjusted for clustering by patient (i.e., robust standard errors were calculated to correct for clustering within patient groups). All p-values are two-sided with statistical significance evaluated at the 0.05 alpha level. Ninety-five percent confidence intervals (for the relative risk) were calculated to assess the precision of the obtained estimates. All analyses were performed in SPSS Version 19.0 (SPSS Inc., Chicago, IL) and STATA Version 12.0 (StataCorp, College Station, TX).

Results

Genotype and allele frequencies for BETA 1R307H (rs6070697G>A)

We initiated an investigation of the role of the beta 1 tubulin R307H SNP as a biomarker in ITP. We genotyped 191 randomly selected ITP patients and compared the frequency of the R307H SNP with that reported from genotyping of 361 individuals from a healthy control population. Neither allele frequency nor genotype frequency of the R307H SNP differed significantly between the two groups (Supplementary Table I). Heterozygote SNP (G/A) genotype frequency was 24.7% in the control group and 20.9% in the ITP group;

homozygote minor allele (A/A) genotype frequency was 3.9% in the control group and 5.2% in the ITP group ($p=0.5$ for both comparisons).

Beta 1 tubulin R307H SNP and ITP Treatments and Outcomes

The similar allele frequencies between healthy controls and ITP patients did not suggest a role for the SNP in the direct pathogenesis of ITP, but this did not exclude a role as a predictive biomarker. Among the ITP patients, demographics and baseline disease characteristics did not differ among the beta 1 tubulin genotypes (Table I), though there was a trend toward more severe thrombocytopenia at initial presentation in the homozygote minor allele group. We investigated the number of treatment modalities that ITP patients received and correlated this with the R307H genotype. With similar periods of follow-up after diagnosis, homozygote major allele (G/G) ITP patients received a median of 4 different treatment modalities as compared with 5 in heterozygote (G/A) patients and 8 in homozygote minor allele (A/A) patients ($p<0.0001$ for overall comparison; $p=0.0008$, G/G v A/A; $p=0.02$, G/A v A/A; Figure 1).

We next analyzed response to various treatment types, and found a significant association between the initial responses to immune modulatory treatments and the presence of the R307H SNP allele. Analysis of patients with a SNP allele (the composite group of heterozygote and homozygote minor allele patients) compared to non-SNP (homozygote major allele) patients revealed a failure rate of 31% in the SNP allele patients, which was significantly higher than that seen in the group without a SNP allele (20%; $p=0.01$). The relative risk of initial failure to immune modulatory treatments for the SNP group compared to the non-SNP group was 1.5 (95%CI=1.1–2.1; $p=0.01$; Table II). On further analysis of individual genotypes, heterozygote (G/A) patients had a 32% initial failure rate to immune modulatory treatments, which was significantly higher than the 20% observed in homozygote major allele (G/G) patients ($p=0.02$). Homozygote minor allele (A/A) patients had a similar initial failure rate (28%) to heterozygote patients, but the difference compared to homozygote major allele patients did not reach significance ($p=0.3$), likely because of the small numbers of homozygotes.

The initial response to each individual immune-modulatory treatment was analyzed next. The composite SNP group had a significantly higher risk of failure to both steroids and rituximab compared to the non-SNP patients (RR=2.1, 95% CI 1.01–4.3, $p=0.05$ for steroids; RR=1.6, 95%CI 1.03–2.5, $p=0.04$ for rituximab). On further analysis of each genotype, heterozygote patients had a significantly increased risk of initial failure to steroids (35% v 15% for homozygote major allele; RR=2.3, 95%CI=1.1–4.9, $p=0.04$) and rituximab (70% v 36% for homozygote major allele; RR=1.9, 95%CI=1.3–3.0; $p=0.003$). The differences in the homozygote minor allele group did not reach statistical significance (Table II).

Homozygote minor allele (A/A) patients did have a significantly increased risk of failure to anti-D (50%) compared to both heterozygote and homozygote major allele patients (13% and 0%, respectively; RR versus homozygote major allele patients=3.8; 95% CI=1.5–9.90; $p=0.007$; Table II).

There was no difference among the genotypes when comparing responses to IVIG and splenectomy, to TPO-agent treatments as a group (Table II) or individually (data not shown). Other treatment modalities had too few observations to analyze.

Absolute Immature Platelet Fractions

We examined platelet production among the R307H genotypes as a possible explanation for the difference observed in failure to immune modulatory but not TPO-agents in SNP patients. There were no significant differences in median A-IPFs between the genotype groups when no response or a partial response was achieved with immune-modulatory treatments; however, when a complete platelet response ($101\text{--}450 \times 10^9/\text{l}$) was obtained with the use of immune modulatory treatments, the median A-IPFs of the heterozygote (G/A) and homozygote minor allele (A/A) patients were significantly greater than those observed in the homozygote major allele (G/G) patients ($p=0.006$; Figure 2). In contrast, when A-IPFs were analyzed for treatment with TPO-agents, no significant differences were observed among the R307H genotypes, regardless of the extent of platelet response (Figure 3).

Linear regression analysis was performed comparing A-IPFs and platelet counts in the complete response range ($101\text{--}450 \times 10^9/\text{l}$) and stratified by genotype. A significant correlation was found between A-IPF and platelet counts for heterozygote and homozygote minor allele (A/A) patients ($r = 0.532$, $p < 0.0001$), but not for homozygote major allele (G/G) patients ($r = 0.111$, $p = 0.23$; Figure 3).

Discussion

In this study, we identified the beta 1 tubulin R307H SNP as a potential predictive biomarker in ITP. This is the first report of a beta 1 tubulin SNP has affecting treatment outcomes for any thrombocytopenic disorder, and the first biomarker for differential responses to immune-modulatory versus TPO-agent treatment in ITP. For rituximab in particular, the finding of an initial failure rate of nearly 60% in patients with the SNP suggests this SNP has the potential to be used in combination with other clinical factors to guide therapeutic choices in ITP; future studies will be needed to validate its clinical utility and define its role in treatment selection. As not all patients who respond well to rituximab initially will experience a long-term remission (Patel, *et al* 2012), the utility of the R307H SNP in predicting long-term response can also be addressed in future studies as well to add to its clinical utility.

Our data suggest that the R307H SNP may lead to increased platelet turnover in ITP patients. This is evidenced by a significantly higher A-IPF during periods of complete response to immune-modulatory treatments. We hypothesize that to maintain platelets in a complete response range after treatment with immune-modulatory agents, a higher rate of platelet production is needed in SNP patients to compensate for platelet turnover associated with the SNP. In this way, the thrombocytopenia in SNP allele patients with ITP would result from both ITP-related destruction and SNP-related platelet turnover (destruction, shortened lifespan, etc). TPO-agents may overcome any deleterious effects the SNP imparts on platelet responses by stimulating supra-physiologic levels of platelet production. Whether

accelerated platelet loss in SNP patients explains increased risk of initial failure to immune-modulatory therapies is unclear.

The trend toward more severe thrombocytopenia at presentation in homozygote minor allele patients may also be explained by SNP-associated platelet loss, as more rapid platelet clearance associated with homozygosity for the minor allele may worsen the thrombocytopenia associated with ITP-related destruction.

Evidence of beta 1 tubulin expression has been reported in immunologic cells at the RNA level (Leandro-Garcia, *et al* 2010); therefore, another possibility is that the SNP may be affecting platelet counts via increased immune destruction. The role of beta 1 tubulin in the immune system has not been characterized, however. Further studies will be needed to explore its role there as well as the potential implications for outcomes in ITP.

Beta 1 tubulin SNPs previously have been shown to affect platelet physiology in other diseases and healthy individuals outside of ITP, and therefore our findings of increased platelet turnover associated with the R307H SNP may extend to these other situations as well. The clinical manifestations of this may not be apparent until normal platelet physiology is disturbed in the setting of a thrombocytopenic disorder, such as was observed here in ITP.

Our study population of 191 patients is relatively large for an ITP study, but due to its relative infrequency, only 10 patients homozygous for the minor allele could be identified. This limits the number of conclusions that can be drawn within that genotype until more subjects can be observed. The retrospective nature of our study also limits the ability to draw conclusions about SNP phenotypes and clinical outcomes.

In summary, the beta 1 tubulin R307H SNP is a potential new biomarker in ITP as evidenced by the differential responses to immune-modulatory therapies and particularly to rituximab. As 30% of the ITP population carries this SNP allele, any differences observed in association with the SNP may impact a relatively large number of patients. Also, the association of changes in platelet physiology with changes in this tubulin isotype, which is a major component of the platelet marginal band, suggests the role of beta 1 tubulin and the marginal band warrant further investigation regarding their roles in platelet dynamics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Median number of different ITP treatments received, by beta 1 tubulin R307H genotype

Both heterozygous (G/A) and homozygote minor allele (A/A) SNP patients received significantly more treatment modality types compared to homozygote major allele (G/G) patients. Boxes show interquartile distance. Outliers represented by filled circles.

$p < 0.0001$ by Kruskal-Wallis test for overall comparison; $*p < 0.05$ and $***p < 0.001$ by Mann-Whitney test.

Figure 2. Median A-IPFs in ITP patients by beta 1 tubulin R307H genotype and type of treatment

Data are displayed as Mean $\pm$ SEM of median A-IPFs for responses to immune-modulatory (top) or TPO-agent (bottom) therapies, by beta 1 tubulin R307H genotype. Both heterozygous (light gray) and homozygote minor allele (white) patients had significantly higher mean A-IPFs compared to homozygote major allele (dark gray) when a complete response (platelets 101–405 $\times 10^9/l$) was obtained with immune-modulatory treatments. **p=0.005 by Student t-test

Figure 3. Linear regression analysis of A-IPF versus platelet counts in ITP patients during complete responses to immune modulatory therapies
SNP patients are shown at left; non-SNP patients at right.

Table I

ITP patient demographics and disease characteristics, by beta 1 tubulin R307H genotype

	Beta 1 tubulin Genotype		
	G/G	G/A	A/A
	N (%)	N (%)	N (%)
Female	81 (59)	19 (53)	7 (70)
Other autoimmune disease	30 (23)	8 (33)	4 (40)
	Median (Range)	Median (Range)	Median (Range)
Age at analysis, y	50 (2–87)	50 (8–84)	40 (4–72)
Age at ITP diagnosis, y	26 (1–88)	24 (2–76)	31 (1–76)
ITP duration at analysis, y	7 (1–39)	8.4 (1–33)	9.1 (3–41)
Platelet count at presentation, $\times 10^9/l$	65 (1–100)	21 (1–100)	8 (3–22)

p >0.05 for all statistical comparisons

Table II

ITP patients with the beta 1 tubulin R307H SNP had significantly higher rates of initial failure to immune-modulatory treatments

Immune-Modulatory	Beta 1 tubulin Genotype			
	G/G n (%)	G/A n (%)	A/A n (%)	G/A+A/A n (%)
Overall Response	285 (80)	67 (68)	29 (72)	96 (69)
Overall Failure	73 (20)	32 (32)*	11 (28)	43 (31)
RR (G/G v G/A+A/A): 1.5 (95%CI 1.1–2.1), p=0.01				
<i>Steroids</i>				
Response	66 (85)	15 (65)	6 (75)	21 (68)
Failure	12 (15)	8 (35)	2 (25)	10 (22)
RR (G/G v G/A+A/A): 2.1 (95%CI 1.01–4.3), p=0.05				
RR (G/G v G/A): 2.3 (95%CI 1.1–4.9), p=0.04				
<i>Rituximab</i>				
Response	41 (60)	6 (30)	8 (60)	14 (42)
Failure	23 (36)	14 (70)**	5 (40)	19 (58)*
RR (G/G vs G/A+A/A): 1.6 (1.03–2.5), p=0.04				
RR (G/G vs G/A): 1.9 (1.3–3.0), p=0.003				
<i>Anti-D</i>				
Response	63 (87)	11 (100)	3 (50)	14 (82)
Failure	9 (13)	0 (0)	3 (50)*	3 (18)
RR (G/G v A/A): 3.8 (95% CI 1.5–9.9), p=0.007				
<i>IVIG</i>				
Response	95 (87)	24 (80)	9 (100)	33 (85)
Failure	14 (13)	6 (20)	0 (0)	6 (15)
<i>Splenectomy</i>				
Response	16 (53)	7 (70)	2 (100)	9 (75)
Failure	14 (47)	3 (30)	0 (0)	3 (25)
TPO-Agent				
Overall Response	75 (88)	24 (86)	14(100)	38 (90)
Overall Failure	10 (12)	4 (14)	0 (0)	4 (10)

Patients may have received more than one modality within each class of treatment (immune modulatory and TPO-mimetic).

P values calculated by Fisher exact test versus G/G.

*
p<0.05

**
p<0.01