Identification of novel TUBB1 variants in patients with macrothrombocytopenia

Zihni Onur ÇALIŞKANER1,2, Abdullah Abdul W AHEED1, Merve TUZLAÇOĞLU ÖZTÜRK3, Yeşim OYMAK3, Uygar Halis TAZEBAY4, Nejat AKAR4, Ayten KANDILÇİ4, Didem TORUN ÖZKAN5,*

1Department of Molecular Biology and Genetics, Faculty of Science, Gebze Technical University, Kocaeli, Turkey
2Department of Bioengineering, Faculty of Engineering and Natural Sciences, Üsküdar University, İstanbul, Turkey
3Department of Pediatric Hematology, Dr. Behçet Uz Children's Hospital, İzmir, Turkey
4Department of Pediatrics, Faculty of Medicine, TOBB-ETU University, Ankara, Turkey
5Medical Laboratory, Vocational School of Health Services, İstanbul Okan University, Istanbul, Turkey

* Correspondence: didemtorun@gmail.com

1. Introduction

Although the frequency of rare bleeding disorders ranges from 1 in 500,000 to 1 in 2 million, in countries with a high rate of consanguineous marriages they occur more frequently and constitute an important health problem [1]. Macrothrombocytopenia is a congenital autosomal-dominant blood disorder characterized by increased platelet size and a decreased number of circulating platelets [2]. Macrothrombocytopenia is classified within the genetically heterogeneous group of rare disorders. There are more than 12 genes that are thought to be associated with macrothrombocytopenia including MYH9 (MIM155100), ACTN1 (MIM615193) GP1A (MIM606672), GP9 (MIM173515), FLI (MIM193067), FLNA (MIM3000017), ITG2A/ITGB3 (MIM 607759/173470), GATA1 (MIM305371), NBEAL2 (MIM614169), and VWF (MIM613160). Although the mutations in these genes have been associated with macrothrombocytopenia, an understanding of their role in the development of the disease remains elusive [3,4].

The membrane skeleton, and the link between actin filaments of the skeleton and microtubules, which consist of alpha and beta tubulin [including the tubulin beta-1 chain (TUBB1)] heterodimers, are important for normal platelet morphology, and defects in these systems are associated with macrothrombocytopenia.

Materials and methods: In this study, we sequenced the exons of the TUBB1 gene using DNA isolated from the peripheral blood samples of healthy controls (n = 47) and patients with macrothrombocytopenia (n = 37) from Turkey. The TUBB1 expression levels in fractioned blood samples from patients and healthy controls were analyzed by RT-qPCR and Western blot. Microtubule organization of the platelets in the peripheral blood smears of patients, and in mutant TUBB1-transfected HeLa cells, were analyzed by immunofluorescence staining.

Results: A new TUBB1 c.803G>T (p.T178T) variant was detected in all of the control and patient samples. Importantly, we found 3 new heterozygous TUBB1 variants predicting amino acid substitutions: G146R (in 1 patient), E123Q (in 1 patient), and T274M (in 4 patients); the latter variant was associated with milder thrombocytopenia in cancer patients treated with paclitaxel. Ectopic expression of TUBB1 T274M/R307H variant in HeLa cells resulted in irregular microtubule organization.

Conclusion: Further clinical and functional studies of the newly identified TUBB1 variants may offer important insights into their pathogenicity in macrothrombocytopenia.

Key words: TUBB1, macrothrombocytopenia, MYH9, platelets

1. Introduction

Although the frequency of rare bleeding disorders ranges from 1 in 500,000 to 1 in 2 million, in countries with a high rate of consanguineous marriages they occur more frequently and constitute an important health problem [1]. Macrothrombocytopenia is a congenital autosomal-dominant blood disorder characterized by increased platelet size and a decreased number of circulating platelets [2]. Macrothrombocytopenia is classified within the genetically heterogeneous group of rare disorders. There are more than 12 genes that are thought to be associated with macrothrombocytopenia including MYH9 (MIM155100), ACTN1 (MIM615193) GP1A (MIM606672), GP9 (MIM173515), FLI (MIM193067), FLNA (MIM3000017), ITG2A/ITGB3 (MIM 607759/173470), GATA1 (MIM305371), NBEAL2 (MIM614169), and VWF (MIM613160). Although the mutations in these genes have been associated with macrothrombocytopenia, an understanding of their role in the development of the disease remains elusive [3,4].

The membrane skeleton, and the link between actin filaments of the skeleton and microtubules, maintain normal platelet morphology [5], which is critical for macrothrombocytopenia given that patients display defects in the size and structure of platelets. Alpha- and beta-tubulin heterodimers are the key components of these structures [6]. Among the beta-tubulin isoforms, expression of TUBB1 is restricted to the megakaryocytes and platelets, and its expression is induced during the differentiation of megakaryocytes, suggesting a role in proplatelet production and platelet release [7]. TUBB1, a 7401 base-pair gene, is located on human chromosome...
20q13.3 and consists of 4 exons which encode 451 amino acids [6]. It functions in the building of the marginal band in platelets, a unique cytoskeleton structure that is composed of bundles of circumferential microtubules that support the maintenance of the shape and function of platelets [8,9]. Sequencing studies in patients with macrothrombocytopenia described several TUBB1 variants that are mainly found in exon 4, which is the longest exon of the gene. Whether the same variants exist with similar frequency and whether there are additional undescribed TUBB1 variants in Turkey is not known. Here, we sequenced the TUBB1 gene in patients with macrothrombocytopenia and in healthy controls from Turkey using exon-specific PCR. Due to sample limitations, we were only able to analyze TUBB1 mRNA expression and platelet structure in a sample of one patient carrying the missense variants of TUBB1 (p.T274M/p.R307H). We also generated a Flag-tagged TUBB1 variant (p.T274M/p.R307H) by using the cDNA of the same patient. The microtubule structure of HeLa cells expressing this ectopic Flag-tagged mutant TUBB1 was analyzed using confocal microscopy after IC staining with ACTIN and TUBB1 (or Flag) antibodies.

2. Materials and methods

2.1. Blood sampling, DNA isolation, and sequencing

Written, informed consent for genetic analysis was obtained from the patients. Approval was also obtained from the Okan University Clinical Research Ethics Committee (24.09.2014; no: 09.2014.50) in order to comply with the ethical rules of the study. Blood samples of the patients (n = 37) and healthy controls (n = 47) were obtained from various hematology clinics in Turkey. Platelet counts of all patients and healthy controls were evaluated. Patients were asymptomatic at the time of admission, and peripheral blood sampling revealed macrothrombocytopenia. Platelet counts of 150–450 × 10³/μL and MPV values of 7.5 fl–11.5 fl were accepted as control in the study. These were then subjected to molecular genetic analysis. The MYH9-related disorder was previously thought to be four separate disorders; May–Hegglin anomaly, Epstein syndrome, Fechtner syndrome, and Sebastian syndrome. All of these disorders involved thrombocytopenia and enlarged platelets and were distinguished by some combination of hearing loss, renal disease, and cataracts. Nonhematological findings (renal disease, cataracts, hearing loss, etc.) were evaluated by the relevant clinics.

The PCR reactions were performed using 25 ng DNA and GoTaq green master mix reagent in a 20 μL PCR volume (Promega, Wisconsin, USA). Primers were designed using an online tool from the Santa Cruz Genome Browser¹ (Table 1). Sequences were analyzed with the Finch TV program.

¹ Santa Cruz Genomic Institute. Genome Browser. Website https://genome.ucsc.edu/
dilution) at room temperature for 1 h. Slides were mounted with DAPI solution (1 µg/10 mL prepared in ddH₂O) for nuclei staining. Stained cells were visualized under LSM 880 laser scanning confocal microscope (Zeiss, Göttingen, Germany).

2.5. Western blot analysis

Whole protein lysate was isolated using RIPA buffer [150 mMNaCl, 50 mMTris-HCl (pH = 8), 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS] completed with 100X protease/phosphatase inhibitor cocktail (Thermo Fisher, Massachusetts, USA), and protein concentration was measured by BCA protein assay kit (Thermo Fisher). Each lysate from different samples was separated by using Mini-PROTEAN TGX 10% precast gel (Bio-Rad, California, USA) and transferred to nitrocellulose membrane using Trans-Blot Turbo transfer system (Bio-Rad). Membranes were blocked with 5% BSA (Sigma–Aldrich) in TBS-T and then incubated with a mouse anti-human TUBB1 antibody (OriGene, TA506654; 1:3000 dilution) at 4 °C overnight, an anti-Flag HRP antibody (Abcam, ab49763; Cambridge, UK) at room temperature for 1 h, a rabbit antihuman GAPDH antibody (Cell Signaling, 5174; 3:10000 dilution) at room temperature for 1 h, or a rabbit antihuman β-actin antibody (Cell Signaling, 8457; 1:1000 dilution) at room temperature for 1 h. Upon washing with TBS-T, membranes were incubated with compatible HRP-conjugated antimouse IgG (Cell Signaling, 7076; 1:5000 dilution) or antirabbit IgG (Cell Signaling, 7074; 1:5000 dilution) secondary antibodies at room temperature for 1 h. Protein bands were detected using ECL Plus Western blotting substrate (Thermo Fisher) and ChemiDoc XRS+ imaging system (Bio-Rad).

| Exons | Forward primer | Reverse primer | Amplicon (bp) |
|-------|----------------|----------------|---------------|
| 1     | CATACCACGGTCACTAGGGC | AAAGCCCAAGGCATTGTC | 587 |
| 2     | GGAACAGGGCTTGGAATG | CATTTCCATCTCCTGGGC | 282 |
| 3     | TTTGGACCAGTATACAAGATTCC | AAAAGAGAACGACGAGGG | 295 |
| 4 – 1 | GCCATGCATCTAGCAGAATTAGG | ATCGAACAATCTGCTGGTG | 766 |
| 4 – 2 | TACCCCTGAAGCTTGACGACAC | GCTGCAGGAGAACACTCATC | 789 |
| 4 – 3 | AACGAATTGGGGAAGCTG | GAAAGCAGATGCCAGTCAG | 608 |
| 4 – 4 | GGAATTCCAGGGAGGCCAC | TTCCTGCATTTGAATGGTG | 623 |
| 4 – 5 | TTTCATTCCAGTCACTACCCCC | TTGATATGTATTTTAGTATTCCTCG | 604 |
| 4 – 6 | GGGTTCTCCTACCTGATTTCG | CGAAAGAGTAGATGAGATG | 620 |

| Table 1. Primers that were used in the PCR reactions and sequencing of TUBB1. |
| Exon-4 specific primer pairs | Alteration type | Amino acid change | Nucleotide change |
|-----------------------------|-----------------|------------------|------------------|
| Exon 4.1 Silent             | T178T           | c. 803G > T      | All Patients (n=37) and controls (n=47) |
| Exon 4.1 Missense           | G146R           | c.705 G > A      | Patient #36 |
| Exon 4.1 Missense           | E123Q           | c.636 C > G      | Patient #17 |
| Exon 4.1 Silent             | H122H           | 635 C > T        | Patient # 30 |
| Exon 4.2 Missense           | T274M           | 821 C > T       | Patients #2,3,7,37 |
| Exon 4.2 Known polymorphism in macrotrombocytopenia | R307H | 920 G > A | 19 out of 37 Patients 13 out of 47 controls |
Table 3. Results of MYH9 and TUBB1 genomic DNA analysis.

| ID | Sex | Clinical diagnosis      | MYH9 Variants | TUBB1 Variants | ACMG classification |
|----|-----|-------------------------|---------------|----------------|---------------------|
|    |     |                         | Found only in patients | Found in controls and patients |                        |
|    |     |                         |                |                | Found only in patients | Found in controls and patients |
| 1  | M   | Macrothrombocytopenia   | -             | -              | -                   | c.803 G>T, c.920 G>A       |
| 2  | M   | Macrothrombocytopenia   | c.3756 C>A,   | -              | c.821 C>T           | c.803 G>T, c.920 G>A       |
|    |     |                         | c.3762 G>A    |                | PM2/PS3             |
| 3  | F   | Macrothrombocytopenia   | -             | -              | c.821 C>T           | c.803 G>T                   |
| 4  | M   | Macrothrombocytopenia   | -             | c.3660 T>G     | -                   | c.803 G>T                   |
| 5  | M   | Macrothrombocytopenia   | -             | -              | -                   | c.803 G>T, c.920 G>A       |
| 6  | M   | Thrombocytopenia        | c.197 G>C     | -              | -                   | c.803 G>T, c.920 G>A       |
| 7  | F   | Macrothrombocytopenia   | -             | c.3660 T>G     | c.821 C>T           | c.803 G>T, c.920 G>A       |
| 8  | F   | May Hegglin Anomaly     | -             | -              | -                   | c.803 G>T                   |
| 9  | M   | Macrothrombocytopenia   | -             | -              | -                   | c.803 G>T                   |
| 10 | M   | Thrombocytopenia        | -             | -              | -                   | c.803 G>T                   |
| 11 | M   | Macrothrombocytopenia   | -             | -              | -                   | c.803 G>T, c.920 G>A       |
| 12 | F   | Thrombocytopenia        | -             | -              | -                   | c.803 G>T, c.920 G>A       |
| 13 | M   | Macrothrombocytopenia   | -             | -              | -                   | c.803 G>T, c.920 G>A       |
| 14 | M   | Macrothrombocytopenia   | -             | -              | -                   | c.803 G>T, c.920 G>A       |
| 15 | M   | Thrombocytopenia        | -             | -              | -                   | c.803 G>T                   |
| 16 | M   | Macrothrombocytopenia   | c.3814 T>G    | -              | -                   | c.803 G>T                   |
| 17 | M   | Macrothrombocytopenia   | -             | -              | c.636 C>G           | c.803 G>T, c.920 G>A       |
| 18 | M   | May Hegglin Anomaly     | -             | -              | -                   | c.803 G>T, c.920 G>A       |
| 19 | M   | Macrothrombocytopenia   | -             | -              | -                   | c.803 G>T                   |
| 20 | M   | Macrothrombocytopenia   | -             | -              | -                   | c.803 G>T                   |
| 21 | F   | Thrombocytopenia        | -             | -              | -                   | c.803 G>T                   |
| 22 | M   | Gray Platelet Syndrome  | -             | -              | -                   | c.803 G>T, c.920 G>A       |
| 23 | M   | Thrombocytopenia        | -             | -              | -                   | c.803 G>T                   |
| 24 | M   | Thrombocytopenia        | -             | -              | -                   | c.803 G>T, c.920 G>A       |
| 25 | M   | Macrothrombocytopenia   | -             | -              | -                   | c.803 G>T                   |
| 26 | M   | Macrothrombocytopenia   | -             | -              | -                   | c.803 G>T                   |
| 27 | F   | Macrothrombocytopenia   | -             | -              | -                   | c.803 G>T, c.920 G>A       |
| 28 | M   | Macrothrombocytopenia   | -             | c.3660 T>G     | -                   | c.803 G>T                   |
| 29 | M   | Macrothrombocytopenia   | -             | -              | -                   | c.803 G>T, c.920 G>A       |
| 30 | F   | Macrothrombocytopenia   | -             | -              | c.635 C>T           | c.803 G>T, c.920 G>A       |
| 31 | F   | Thrombocytopenia        | -             | -              | -                   | c.803 G>T                   |
| 32 | M   | Macrothrombocytopenia   | -             | -              | -                   | c.803 G>T                   |
| 33 | F   | Thrombocytopenia        | -             | -              | -                   | c.803 G>T, c.920 G>A       |
| 34 | M   | May Hegglin Anomaly     | -             | -              | -                   | c.803 G>T                   |
| 35 | M   | Thrombocytopenia        | -             | -              | -                   | c.803 G>T                   |
| 36 | M   | Thrombocytopenia        | -             | c.705 G>A      | -                   | c.803 G>T, c.920 G>A       |
| 37 | M   | Thrombocytopenia        | -             | c.821 C>T      | c.803 G>T           | c.803 G>T, c.920 G>A       |
2.6. Bioinformatic analysis
Computational mutation analysis was performed using online prediction tools such as PROVEAN\(^2\), MutationTaster\(^2\), and PolyPhen-2\(^4\). MutationTaster\(^2\) was also used for the multiple amino acid sequence alignment of TUBB1 homologues.

3. Results
3.1. Sequencing of TUBB1 exons
Initially, we sequenced all of the exons (1 to 4) of TUBB1 in patient samples (n = 37) to determine any possible TUBB1 variant. Healthy controls (n = 47) were subsequently sequenced to screen for TUBB1 variants that we found in patient samples. All of the determined variants were confirmed by repeating the corresponding PCR reactions and using forward and reverse primers in the sequencing analysis. Sequencing results of the patient samples did not show any new variant in exons 1, 2, or 3 of TUBB1; however, in exon-2 we found heterozygous TUBB1 variant c.130-131 AG>CC (p.Q43P) in 4 patients (11%) and 7 healthy controls (15%) (Figure 1A), which had been previously reported as a functional polymorphism in congenital

---

\(^2\) http://provean.jcvi.org
\(^3\) http://www.mutationtaster.org
\(^4\) http://genetics.bwh.harvard.edu/pph2/
macrothrombocytopenia patients [10]. Given that the fourth exon of \textit{TUBB1} is the largest one (2950 base pairs) and contains most of the published genetic variations [6], we designed 6 different primer pairs to sequence this exon (Table 1). Primer pairs, named exon 4.1 and 4.2 (Table 1), cover the coding region of the 4th exon. The \textit{TUBB1} variants described below were all covered within the PCR products of the 4.1 and 4.2 primer pairs. In this region of the 4th exon, we found a novel synonymous \textit{TUBB1} c.803G>T (p.T178T) variant in all of the healthy control and patient samples (Figure 1B) (Table 3). In addition, we showed previously-described \textit{TUBB1} variant c.920 G>A (p.R307H) [11,12] for the first time in patients (19 out of 37; 51%) and healthy controls (13 out of 47; 28%) from Turkey (Figure 1C) (Table 3). Importantly, we identified one new silent (\textit{TUBB1} c.635C>T; p.H122H in patient #30) (Figure 2A) and 3 new heterozygous missense \textit{TUBB1} variants in patient samples only (c.636C>G, p.E123Q in patient #17; c.705G>A p.G146R in patient #36; and c.821C>T, p.T274M in patients #2, #3, #7, and #37) (Tables 2 and 3) (Figures 2B–2D). In the literature, the \textit{TUBB1} variant T274M was associated with milder thrombocytopenia in cancer patients treated with paclitaxel [13]; however, to our knowledge, it was not previously described in macrothrombocytopenia patients. The patients carrying p.G146R, p.E123Q, and T274M were also positive for the R307H variant. Two of the T274M carriers (Table 3; patients #2 and #3) were relatives (a son and his mother, respectively). We also showed that patients #2 and #7 are carriers of \textit{TUBB1} variant c.920G>A (p.R307H) and \textit{MYH9} variants c.3756C>A (p.L1176M)/c.3762G>A (p.E1182K) and c.3660T>G (p.A1144L), respectively (Table 3); this is the first presentation of the coexistence of the \textit{TUBB1} and \textit{MYH9} variants in macrothrombocytopenia patients. Since we did not find \textit{TUBB1} variation in the remaining portions of exon 4 in the patient samples (screened with exon 4.3, 4.4, 4.5, and 4.6 primers), we did not screen these regions of exon 4 in healthy control samples.

3.2. \textit{TUBB1} variant p. T274M/R307H changes the microtubule structure

Due to sample limitations and the unavailability of other patients carrying heterozygous missense mutations (p.G146R and p.E123Q), we were able to clone and analyze only the effect of \textit{TUBB1} variant p.T274M/ R307H (rs35565630/rs6070697) on the morphology of the platelets of patient #7 and on the microtubule organization of HeLa cells. The clinical data of patient #7 is summarized in Table 4. Both variants exist in the ExAC database with high MAF values (p.T274M MAF value: 0.018 and p.R307H MAF value: 0.173). Computational mutation analysis tools PROVEAN, MutationTaster2, and PolyPhen-2 also predicted deleterious effects of p.T274M variation on \textit{TUBB1} protein function (data not shown). Endogenous \textit{TUBB1} mRNA was detectable in both the peripheral blood mononuclear cells (PBMC) and platelet-rich plasma samples of patient #7 (Figures 3A and 3B),

![Figure 2. Sequencing also revealed heterozygous TUBB1 variations identified for the first time in macrothrombocytopenia patients in Turkish population. (A) A silent TUBB1 variation c.635C>T (p.H122H), and 3 missense variants (B) c.636C>G (p.E123Q), (C) c.705G>A (p.G146R), and (D) c.821C>T (p.T274M) were detected in patient #30, patient #17, patient #36 and patients #2, #3, #7, and #37, respectively.](image-url)
Figure 3. TUBB1 expression was detected in both mRNA and protein levels by using blood samples from healthy control (n=4, pooled) and patient #7. RT-qPCR analysis showed relative TUBB1 gene expression in both (A) PBMC and (B) PRP fractions. All Taqman assays were carried out in triplicates and relative fold change was calculated with the ΔΔCt method. (C) TUBB1 protein band was not detectable in the whole lysate isolated from patient #7 contrary to healthy control.

Table 4. Clinical data of all patients carrying newly identified TUBB1 variants and patient #7 which is proceeded for functional analysis.

| Case     | #2  | #3  | #7  | #17 | #30 | #36 |
|----------|-----|-----|-----|-----|-----|-----|
| Age      | 21  | 13  | 13  | 12  | 4   | 14  |
| Sex      | Male| Male| Female| Male| Male| Female|
| WBC (10^3 uL) | 14.47 | 3.9 | 11.4 | 6.68 | 10.57 | 7.9 |
| RBC (uL) | 4.88 | 4.49 | 4.38 | 5.23 | 4.9 | 4.57 |
| HB (g/dL) | 14.3 | 11.9 | 11.4 | 13.6 | 11.6 | 12 |
| HCT (%) | 42.3 | 35 | 38.1 | 40.3 | 36.2 | 36.1 |
| MCV (fL) | 86.7 | 78.1 | 78.3 | 77.1 | 73.9 | 79.1 |
| MCH (pg) | 29.3 | 26.6 | 26.2 | 26 | 23.7 | 26.3 |
| MCHC (g/dL) | 33.8 | 34.1 | 32.8 | 33.7 | 32 | 33.3 |
| PLT (10^3 uL) | 52 | 89 | 32 | 29 | 87 | 69 |
| MPV (fL) | 9.9 | 8.4 | 11.5 | nd | 13 | 9.6 |
| PDW (fL) | 12.2 | 17.1 | 13.6 | nd | 18.1 | 16 |
| PCT (%) | 0.05 | 0.074 | 0.04 | nd | 0.11 | 0.31 |
| Consanguineous marriages | - | + | + | - | - | - |
| Bleeding problem | + | - | - | - | - | - |
| Renal disease | - | - | - | + | - | - |
| Cataracts | - | - | - | - | - | - |
| Hearing loss | - | - | - | - | - | - |
who is heterozygous for the variant p.T274M/R307H. Although the endogenous TUBB1 protein was detectable in the platelet samples of a healthy control, we could not detect the TUBB1 protein in the platelets of patient #7 using Western blot analysis (Figure 3C). The IC analysis of platelets by confocal microscopy showed a normal marginal band structure (ring shaped) in a healthy control sample; however, the marginal band was disrupted and a diffuse staining with both α-TUBULIN and TUBB1 antibodies was observed in the majority of platelets from the same patient (Figure 4). We also transiently expressed the Flag-tagged TUBB1 variant p.T274M/R307H in the HeLa cell line to determine its effect on microtubule distribution under steady-state conditions. The IC analysis of HeLa cells transfected with wild type Flag-tagged TUBB1 showed a more organized and clear microtubule net. These results suggested that TUBB1-p.T274M/R307H variant causes a disorganized microtubule structure under steady-state conditions (Figure 5).

Interestingly, we also found the MYH9 (p.K64N) and TUBB1 (p.R307H) variants in the same patient (patient #7). While the variant in MYH9 causes a defect in proplatelet formation and platelet secretion [14,15], an understanding of the effects of the coexistence of these two variants remains elusive.

4. Discussion
In the current study we screened all exons of the TUBB1 gene using DNA from the peripheral blood samples of patients with macrothrombocytopenia from Turkey (n = 37) (Table 3). The patients were selected based on symptoms affecting platelet morphology, such as large

Figure 4. Peripheral blood smears were stained for marginal band detection in healthy or patient’s platelets. Endogenous TUBB1 and α-Tubulin which are subunits of marginal band were marked with specific primary antibodies in combination with Alexa-555 (red) or Alexa-488-conjugated (green) secondary antibodies, respectively. Marginal band was determined as a hollow-ring structure in healthy platelets but circular microtubule arrangement was disappeared in patient #7’s platelets carrying TUBB1 variant p.T274M/R307H. Merged images (yellowish) were captured in oiled 63X magnification by using LSM 880 (Zeiss) confocal microscope (upper panel). Platelets labeled with an asterisk were zoomed in while image processing (bottom panel).
abnormal platelet structure, and the platelet count. We also included DNA samples from healthy controls (n = 47).

We found 2 types of previously described TUBB1 polymorphisms (Q43P and R307H) for the first time in Turkey. We also showed 3 new missense and 1 new silent variant in the fourth exon of TUBB1 in the macrothrombocytopenia patients but not in healthy controls (Table 2), which suggests that these are not common polymorphisms. The findings are of great importance, as they represent single nucleic acid base changes which affect the amino acid sequences in the protein. These changes can end up providing an abnormal biochemical property, such as charge, hydrophobicity, or size, within the wild type protein. To check this theory, different function prediction programs such as PROVEAN and Poly Phen-2 were utilized. Both analyses suggest that the variants G146R and T274M have potentially deleterious effects on TUBB1 (data not shown). The p.G146R (the glycine in position 146 of β1-tubulin) is highly conserved, and the variation is located in the amino-terminal guanosine triphosphate domain of β1-tubulin. In silico modeling confirmed its strong influence on the structure of this domain, and the gene where this mutation occurs is related to macrothrombocytopenia.

Reviewing TUBB1 and its genetic variations in macrothrombocytopenia to date, we found that reported mutations in mammals included 2 missense mutations, p.R318W and p.F260S in exon 4 in humans [8,16], and a missense mutation of p.D249N in canines [17]. All of these variations are believed to be located in or near the α- and β-tubulin intradimer interface, reportedly causing macrothrombocytopenia in humans and dogs, respectively. The intradimer interface of the platelet structure is believed to be disrupted by these mutations, causing frayed cytoskeleton formation and affecting platelet morphology. With the exception of p.R307H and p.Q43P polymorphisms, the variants discovered in macrothrombocytopenia patients in our study are distinct and do not overlap with earlier studies performed in different populations, which suggests an evolutionary difference in the Turkish population. Previously it was shown that the ectopic expression of R307H variant in

![Figure 5. IF analysis of HeLa cells transiently transfected with C-terminus Flag-tagged wild-type TUBB1 (WT) or T274M/R307H variant constructs. Exogenous TUBB1 was labeled with anti-Flag primary antibody (Sigma-Aldrich, F7425; 1:100 dilution) and α-Tubulin was labeled with anti-alpha Tubulin primary antibody (Santa Cruze, sc-5286; 1:100 dilution) in combination with Alexa-555 (red) or Alexa-488-conjugated (green) secondary antibodies, respectively. Cells expressing WT protein have a branched microtubule organization but transfection of T274M/R307H variant resulted in a disrupted and diffuse microtubule staining compared with WT and untransfected cells. Successfully transfected and exogenous Flag-tagged TUBB1 expressing HeLa cells were labeled with an asterisk. Images were captured in oiled 63X magnification by using LSM 880 (Zeiss) confocal microscope.](image-url)
human MCF7 cells causes defects in microtubule regrowth after treatment of the cells with nocodazole, a reagent that depolymerizes the microtubules [18]. Given that it is observed at high frequency in healthy individuals [27% in our study (Tables 2 and 3) and 30% in Basciano et al.] [18], it is likely that its effect on platelets is not disastrous. On the other hand, although the p.T274M/R307H variant was seen at a low frequency (0.023) in Caucasian populations without a known history of macrothrombocytopenia, we describe for the first time a tendency towards this variant in macrothrombocytopenia patients (4 out of 37; 10%), but not in healthy individuals from Turkey (n = 47); this suggests a potential role for variant T274M/R307H in the disturbed platelet structure that we observed in the platelets of the patient sample (Figure 4). In support of our hypothesis, Western blot analysis demonstrated that MCF7 cells express ectopic p.T274M or p.T274M/R307H, and cancer patients carrying these variants are less sensitive to the polymerization effect of paclitaxel on microtubules [13]. Supporting these findings, we showed for the first time that cytoplasmic distribution of the microtubule net was disturbed under steady-state conditions in HeLa cells.

Although we were not able to functionally link the variants p.G146R and p.E123Q to macrothrombocytopenia, our data involving the T274M/R307H variant suggest that it may be useful as a biomarker in immune thrombocytopenia given its potential effect on platelet turnover (which leads to lower platelet counts). Finally, given that the synonymous TUBB1 p.T178T variant was found in all of our healthy control and patient samples, it is unlikely that this variant is associated with macrothrombocytopenia.

The American College of Medical Genetics and Genomics (ACMG) has recommended a five-tier classification system [19]. According to this classification system, a sequence change can be categorized as pathogenic, likely pathogenic, uncertain significance, likely benign, and benign. In our study, the p.705G>A, p.636C>G, and p.821C>T variants were found only in patients, and they had a small minor allele frequency (MAF) value, suggesting their pathogenicity [nsv2778234 (ClinVar)]. Additionally, the variants that we found in 6 patients (#2, #3, #7, #17, #30, and #36) could be categorized as PM2/PS3 (pathogenic modified/pathogenic strong criteria). Here, PM2 indicates that the mutation is found only in the patient group and is absent in the controls; PS3 shows the functional effect of mutation (Table 4).

In summary, our findings suggest that further functional and clinical analyses of the new TUBB1 variants that we identified in macrothrombocytopenia patients may provide new insights into the molecular mechanisms of normal and abnormal platelet production and morphology.

Author contributions
DTÖ and AK designed and guided the study, analyzed the data, and wrote the manuscript. ZOÇ and AAW performed the experiments and analyzed the data. MTÖ and YO provided the patient material and clinical data. UHT guided the experiments and reviewed the data and manuscript.

Acknowledgments
This research was financially supported by the Starting R&D Projects Funding Program (3001) of the Scientific and Technological Research Council of Turkey (TÜBİTAK) (project no.: 214S601).

References
1. Peyvandi F, Spreafico M. National and international registries of rare bleeding disorders. Blood Transfusion 2008; 6 (2): s45-48. doi: 10.2450/2008.0037-08
2. Kunishima S, Okuno Y, Yoshida K, Shiraishi Y, Sanada M et al. ACTN1 mutations cause congenital macrothrombocytopenia. The American Journal of Human Genetics 2013; 92 (3): 431-438. doi: 10.1016/j.ajhg.2013.01.015
3. Bolton-Maggs PH, Chalmers EA, Collins PW, Harrison P, Kitchen S et al. A review of inherited platelet disorders with guidelines for their management on behalf of the UKHCDO. British Journal of Haematology 2006; 135 (5): 603-633. doi: 10.1111/j.1365-2141.2006.06343.x
4. Rao AK, Songdej N. Inherited thrombocytopenias: the beat goes on. Blood 2015; 125 (5): 748-750. doi: 10.1182/blood-2014-11-610675
5. Italiano JE Jr., Bergmeier W, Tiwari S, Falet H, Hartwig JH et al. Mechanisms and implications of platelet discoid shape. Blood 2003; 101 (12): 4789-4796. doi: 10.1182/blood-2002-11-3491
6. Burley K, Westbury SK, Mumford AD. TUBB1 variants and human platelet traits. Platelets 2018; 29 (2): 209-211. doi: 10.1080/09537104.2017.1411587
7. Schwer HD, Lecine P, Tiwari S, et al. A lineage-restricted and divergent beta-tubulin isoform is essential for the biogenesis, structure and function of blood platelets. Curr Biol 2001; 11 (8): 579-586. doi: 10.1016/S0960-9822(01)00153-1
8. Kunishima S, Nishimura S, Suzuki H, Imaizumi M, Saito H. TUBB1 mutation disrupting microtubule assembly impairs proplatelet formation and results in congenital macrothrombocytopenia. European Journal of Haematology 2014; 92 (4): 276-282. doi: 10.1111/ejh.12252
9. Ravid K. Platelet marginal bands: not so marginal. Blood 2008; 111 (9): 4423-4423. doi: 10.1182/blood-2008-02-137786
10. Freson K, De Vos R, Wittevrongel C, Thys C, Defoor J et al. The TUBB1 Q43P functional polymorphism reduces the risk of cardiovascular disease in men by modulating platelet function and structure. Blood 2005; 106 (7): 2356-2362. doi: 10.1182/blood-2005-02-0723
11. Poster Communications. Thrombosis Research 2016; 141: S67-68. doi: 10.1016/S0049-3848(16)30387-5
12. Basciano PA, Bussel J, Hafeez Z, Christos PJ, Giannakakou P. The beta-1 tubulin R307H single nucleotide polymorphism is associated with treatment failures in immune thrombocytopenia. British Journal of Haematology 2012; 160 (2): 237-243. doi: 10.1111/bjh.12124
13. Leandro-Garcia LJ, Leskela S, Inglada-Perez L, Landa I, de Cubbas AA et al. Hematologic beta-tubulin VI isoform exhibits genetic variability that influences paclitaxel toxicity. Cancer Research 2012; 72 (18): 4744-4752. doi: 10.1158/0008-5472.CAN-11-2861
14. D’Andrea G, Chetta M, Margaglione M. Inherited platelet disorders: thrombocytopenias and thrombocytopathies. Blood Transfusion 2009; 7 (4): 278-292. doi: 10.2450/2009.0078-08
15. Mhawech P, Saleem A. Inherited giant platelet disorders. Classification and literature review. American Journal of Clinical Pathology 2000; 113 (2): 176-190. doi: 10.1309/FC4H- LMSV-VCW8-DNJU
16. Kunishima S, Kobayashi R, Itoh TJ, Hamaguchi M, Saito H. Mutation of the beta1-tubulin gene associated with congenital macrothrombocytopenia affecting microtubule assembly. Blood 2009; 113 (2): 458-461. doi: 10.1182/blood-2008-06-162610
17. Davis B, Toivio-Kinnucan M, Schuller S, Boudreaux MK. Mutation in beta1-tubulin correlates with macrothrombocytopenia in Cavalier King Charles Spaniels. Journal of Veterinary Internal Medicine 2008; 22 (3): 540-545. doi: 10.1111/j.1939-1676.2008.0085.x
18. Basciano PA, Matakas J, Pecci A, Civaschi E, Cagioni C et al. Beta-1 tubulin R307H SNP alters microtubule dynamics and affects severity of a hereditary thrombocytopenia. Journal of Thrombosis and Haemostasis 2015; 13 (4): 651-659. doi: 10.1111/jth.12824
19. Qu H, Wang X, Tian L, Hakonarson H. Application of ACMG criteria to classify variants in the human gene mutation database. Journal of Human Genetics 2019; 64: 1091-1095. doi: 10.1038/s10038-019-0663-8