

**Running title:** *β-tubulin VI variability and paclitaxel toxicity*

**Title:** The hematologic-specific β-tubulin VI exhibits genetic variability influencing paclitaxel toxicity

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***Running title:  $\beta$ -tubulin VI variability and paclitaxel toxicity***

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**ABSTRACT**

Cellular microtubules, composed of  $\alpha$ - $\beta$ -tubulin heterodimers, are essential eukaryotic structures for cell shape, intracellular transport and cell division, and are successful targets for anticancer therapy. The highly conserved superfamily of  $\beta$ -tubulin genes has distinct expression patterns. Specifically, previous studies suggest a major hematologic role for  $\beta$ -tubulin VI and functional genetic variants in the gene. In this study we show that  $\beta$ -tubulin VI is highly expressed in blood cells with a substantial inter-individual variability (7-fold variation in mRNA). Sequencing of the gene revealed missense variations leading to Q43P, T274M and R307H, and a rare nonsense variant, Y55X. Variation in the hematologic target of the microtubule binding drugs might alter their myelosuppressive action. To test this, we created cell lines stably expressing the different  $\beta$ -tubulin VI full length variants and found that the T274M change conferred a significantly decreased sensitivity to paclitaxel tubulin polymerization. Furthermore, patients treated with paclitaxel and carrying  $\beta$ -tubulin VI T274M showed a significantly lower thrombocytopenia than wild type homozygous patients (P=0.031). All together, this is the first study showing that  $\beta$ -tubulin VI is a hematologic isotype subjected to relevant genetic variability, which might affect the myelosuppressive action of microtubule binding drugs.

## **INTRODUCCION**

Microtubules are ubiquitous and highly dynamic polymers of  $\alpha$ - $\beta$ -tubulin heterodimers indispensable for a variety of cellular functions such as structure maintenance, intracellular transport, cell signaling, migration and mitosis. Several of the most common chemotherapeutic drugs, such as taxanes, *Vinca* alkaloids and epothilones, base their mechanism of action on binding to microtubules and altering their dynamics, which leads to mitosis arrest and cell death (1, 2). The therapeutic target of these drugs is  $\beta$ -tubulin, which consists of eight isotypes encoded by multiple genes that exhibit a tissue-specific expression. In a previous study we showed that isotypes I, IVb and V are constitutive, isotypes IIa, IIb, III and IVa are neuronal and isotype VI is hematopoietic cell-specific and has been detected in platelets, lymphocytes, bone marrow and spleen(3, 4). The  $\beta$ -tubulin isotypes are highly conserved and have similar sequences, however, the C-terminal region exhibits higher variability and seems to confer differences in microtubule polymerization and stability. The fact that rare mutations in  $\beta$ -tubulin IIb and III lead to a spectrum of severe neuronal disorders, suggests specific functions that cannot be compensated by alternative isotypes (5, 6).  $\beta$ -tubulin VI knock-out mice studies proved a specialized role for this protein in platelet synthesis, structure and function(7, 8).

Due to the crucial role of  $\beta$ -tubulin in cells, genetic variation is not allowed. However, the hematologic isotype VI is an exception, and a common missense polymorphism (Q43P) has been associated with an altered risk of cardiovascular disease by modulating platelet function and structure (9, 10). There is also a report on a rare  $\beta$ -tubulin VI missense variant (R318W) responsible for congenital macrothrombocytopenia (11). Other genetic variations in  $\beta$ -tubulin isotypes correspond to cell lines and lead to acquired resistance to microtubule binding drugs (12-14). Variations in  $\beta$ -tubulin isotype composition in tumors have also been associated with decreased sensitivity to these drugs (15-18). Thus, polymorphisms in  $\beta$ -

***Running title:  $\beta$ -tubulin VI variability and paclitaxel toxicity***

tubulin VI, leading to a variant protein or altered expression, might result in differences in the effect of  $\beta$ -tubulin binding drugs among patients. Because of the hematologic role of  $\beta$ -tubulin VI, these polymorphisms could affect the action of the drugs in these cells, leading to inter-patient differences in myelosuppression. However, at the moment, the expression of  $\beta$ -tubulin VI has not been well established and polymorphisms in this gene have not been investigated.

In this study we characterized  $\beta$ -tubulin VI expression in hematologic cells and screened for genetic variation by sequencing the coding region of the  $\beta$ -tubulin VI gene (*TUBB1*). We generated cell lines stably expressing the variant  $\beta$ -tubulin VI proteins detected and conducted functional assays. Furthermore, the clinical implications of these polymorphisms were studied in patients with hematological malignancies treated with paclitaxel.

## **MATERIALS AND METHODS**

### **Human samples and patients**

cDNAs from 7 different blood fractions were obtained from BD Biosciences (Human Blood Fractions MTC™ Panel), peripheral blood lymphocytes, platelets and neutrophils were isolated from healthy volunteers and CD34- bone marrow cells were isolated from bone marrow aspiration following previously described procedures (19). Frozen samples from peripheral T cell lymphomas were collected through the Spanish National Cancer Center tumor bank network from pathology departments of different hospitals in Spain (20). The main characteristics of this human material and cell lines used for mRNA quantification are shown in Supplementary Table 1. For the estimation of allele and haplotype frequencies we used DNA isolated from unrelated individuals from different ethnic groups, specifically, 481 Caucasians from Spain, 106 Asians from China and 71 Africans from Tanzania.

Forty nine patients with hematological malignancies planned for autologous transplant (20 with lymphoma, 15 with multiple myeloma and 14 with acute leukemia) who underwent mobilization of hematopoietic progenitor cells with paclitaxel 170 mg/m<sup>2</sup> i.v. by continuous infusion for 24 hr followed by 8 µg/kg s.c. rhG-CSF daily, and that had leukapheresis product available, were included in the study (21). In this series, recruited between 1999 to 2008, the time elapsed from the last cytotoxic treatment was at least three weeks, to allow recovery of peripheral blood counts before paclitaxel administration. Peripheral blood counts were assessed on days 5, 7 and daily afterwards. Maximal hematological toxicity was retrospectively assessed, and recorded according to the National Cancer Institute-Common Toxicity Criteria version 3. The characteristics of the patients, including age at treatment, previous treatments, state of disease at stem cell harvest, weeks from last treatment, and baseline platelet counts at paclitaxel treatment initiation, are provided in Supplementary Table 2.

### **RNA isolation and quantitative RT-PCR**

RNA was extracted from blood cells, cell lines and frozen tumoral tissue using TRI-reagent (Molecular Research Center Inc., Cincinnati, USA) and the concentration quantified by using Nanodrop ND-1000 (Wilmington, DE, USA). One µg of total RNA was reverse transcribed using Superscript II (Invitrogen, Carlsbad, USA) and an oligo dT14 primer following the manufacturer's instructions. The mRNA content of the different β-tubulin isoforms was quantified by qRT-PCR with the Sequence Detection System 7900HT (Applied Biosystems Foster City, USA) using conditions, primers and probes previously described (4). Normalization was carried out with the internal standard beta-glucuronidase (GUS). Negative controls were present in all PCR series and assays were carried out in triplicates. The delta-delta Ct method (22) was used for the calculation of mRNA content.

### **DNA isolation, sequencing and genotyping**

Genomic DNA was isolated from the peripheral blood lymphocytes of the healthy volunteers and from the leukapheresis product of the hematological patients using FlexiGene DNA Kit (QIAGEN, Valencia, USA) and the DNA concentration was determined using PicoGreen dsDNA quantification reagent (Invitrogen). For *TUBB1* sequencing, the exons and proximal promoter region were amplified by PCR using specific primers designed to avoid cross-reactions with other β-tubulin isotype genes/ pseudogenes (Suppl. Table 2). PCR amplification products were purified using the PCR Purification Kit (QIAGEN) and run on an ABI PRISM 3700 DNA Analyzer capillary sequencer (Applied Biosystems). Genotyping for the *TUBB1* coding polymorphisms was performed in duplicates with the KASPar SNP Genotyping System (Kbiosciences, Herts, UK) using 15 ng of genomic DNA. All assays included DNA samples with known genotypes and negative controls. The sequence Detection

**Running title:** *β-tubulin VI variability and paclitaxel toxicity*

System 7900HT (Applied Biosystems) was used for fluorescence detection and allele assignment.

**Platelet activation and aggregation assays**

The effect of  $\beta$ -tubulin VI R307H polymorphism in platelet function was investigated by performing aggregation assays in 6 healthy volunteers of known genotype (3 women homozygous for the wild type allele and 2 women and 1 man homozygous for the variant allele). Platelet aggregation was measured in non adjusted-citrated platelet-rich plasma obtained by centrifuging blood at 140 g for 12 min. Platelets were stimulated with 6-mer thrombin receptor agonist at 0.78-25  $\mu$ M (Sigma-Aldrich, St. Louis, USA), collagen at 0.125-2  $\mu$ g/mL (Menarini Diagnostics, Florence, Italy), ADP at 0.16-0.5  $\mu$ M (Menarini Diagnostics), arachidonic acid at 0.4-1.6 mM (Bio-Rad, Hercules, USA) and ristocetin at 0.75-1.25 mg/mL (Sigma-Aldrich). Changes in light transmission of platelet-rich plasma over baseline were recorded for 5 min using an Aggrecoorder II aggregometer (Menarini Diagnostics).

**Generation of cell lines expressing  $\beta$ -tubulin VI wild type and variant proteins**

We amplified the full coding sequence of *TUBB1* using specific primers that introduced *NheI* and *NotI* cleavage sites (Suppl. Table 2) and cDNA from peripheral blood lymphocytes that carried in homozygosity the wild type *TUBB1* coding region. The PCR product was cloned into pIRESpuro2 vector (Clontech, Palo Alto, CA) to generate pIRESpuro2\_TUBB1 wild type plasmid. By means of the GeneTailor Site-Directed Mutagenesis System (Invitrogen) and following the manufacturer's indications and adequate primer pairs (Suppl. Table 2), the pIRESpuro2\_TUBB1 wild type construct was mutated to generate plasmids which contained *TUBB1* sequence with variants encoding for the following



***Running title:  $\beta$ -tubulin VI variability and paclitaxel toxicity***

protein changes: Q43P, Y55X, T274M, R307H and T274M/R307H. Correct *TUBB1* sequence and lack of artifacts was confirmed by sequencing.

MCF7 breast cancer cells were chosen for heterologous expression of  $\beta$ -tubulin VI based on their lack of endogenous expression of this protein and sensitivity to microtubule binding drugs. MCF7 cells were transiently transfected with the different *TUBB1* constructs and the empty vector by using Lipofectamine-2000 (Invitrogen) following the manufacturer's instructions. To generate stable cell lines, the different *TUBB1* constructs and the empty vector were electroporated using the Gene Pulser XCell Electroporation System (Bio-Rad) into cells and 24 hr later 0.5  $\mu$ g/ ml of puromycin (Sigma-Aldrich) was added for selection. Puromycin resistant clones were analyzed for  $\beta$ -tubulin VI expression by western blot. Control cells were also selected with puromycin and all resistant clones were pooled together. Stable transfectants were maintained in DMEM supplemented with 10% FBS, penicillin, streptomycin and puromycin at a concentration of 0.4  $\mu$ g/ ml.

**$\beta$ -tubulin VI protein detection**

For western blotting, total cell extracts from cell lines were separated by using the Criterion XT Gels Bis-Tris 10% and the Criterion electrophoresis cell (Bio-Rad) and transferred to polyvinylidene fluoride membranes (Immobilon-P Membrane, Millipore, Billerica, USA) using the Trans Blot Semi-Dry Blotter (Bio-Rad). Protein concentration was measured by using Bio-Rad Protein Assay (Bio-Rad) and equal loading of proteins was verified by Ponceau S staining. The membranes were blocked and then incubated with a rabbit polyclonal antibody specific for human  $\beta$ -tubulin VI (from Dr. Paraskevi Giannakakou) (3), a mouse antibody recognizing all  $\beta$ -tubulin isotypes (clone 2.1, Sigma; 1:1,000 dilution) or a rat anti- $\alpha$ -tubulin (clone YL1/2; Chemicon International; 1:1,000 dilution). After washing, the membranes were incubated with the corresponding secondary antibody: goat

***Running title:  $\beta$ -tubulin VI variability and paclitaxel toxicity***

anti-rabbit (Alexa Fluor 680 nm, Molecular Probes; 1:15,000 dilution), goat anti-mouse (DyLight 800, Thermo Scientific; 1:10,000 dilution) or donkey anti-rat (IRDye 800 nm, Rockland Immunochemicals; 1:15,000 dilution). The fluorescence signal was detected using the Odyssey infrared imaging system (LI-COR).

Immunofluorescence microscopy was performed as previously described (23). In brief, cells were cultivated on coverslips overnight and fixed the next day with 4% paraformaldehyde, permeabilized with Triton-X-100 0.5% in TBS and blocked with 10% goat serum. The same primary antibodies as those described for western blotting were used. Specific secondary antibodies Alexa-rabbit-405nm and Alexa-rat-568 were used. Cells were imaged using a Zeiss LSM 5 LIVE confocal microscope using a 40x/1.3 EC Plan Neofluar objective, a 63x/1.4 Plan APOCHROMAT objective and 100x/1.4 Plan APOCHROMAT objectives. All images were acquired and analyzed using Zeiss LSM 5 LIVE software.

**Tubulin polymerization assay**

Quantitative drug-induced tubulin polymerization assays were performed as previously described (12). In brief, cells were grown in 24-well plates overnight and incubated for 6 hrs with paclitaxel at concentrations of 0, 10, 100 and 1000 nM. The cells were washed twice with PBS prior to lysis at 37 °C for 5 min in the dark, with 100  $\mu$ l of hypotonic buffer (20 mM Tris-HCl pH 6.8, 1 mM MgCl<sub>2</sub>, 2 mM EGTA, 0.5% Nonidet P40) containing protease inhibitors (Protease Cocktail inhibitor tablets, Roche). The lysates were transferred to 1.5-ml Eppendorf tubes, and rinsed with 100  $\mu$ l of hypotonic buffer, mixed by vortexing and centrifuged at 14,000 rpm for 10 min at room temperature. The 200  $\mu$ l supernatants containing soluble (cytosolic) tubulin were transferred to another tube and pellets containing polymerized (cytoskeletal) tubulin were resuspended in 200  $\mu$ l of hypotonic buffer. The cytosolic and cytoskeletal fractions were each mixed with electrophoresis sample

***Running title:  $\beta$ -tubulin VI variability and paclitaxel toxicity***

buffer, sonicated, boiled and analyzed by immunoblotting. The percentage of polymerized tubulin was determined by dividing the densitometry value of polymerized tubulin by the total tubulin content (the sum of the densitometry values of soluble and polymerized tubulin), in at least 3 independent experiments.

**Statistical Analysis**

The myelotoxicity of the patients was analyzed taking into account gender, age at paclitaxel treatment, type of disease, previous treatments, state of disease at stem cell harvest, weeks from last treatment, and baseline platelet counts at initiation of paclitaxel and genotypes. For the analysis quantitative variables were dichotomized according to the median. To include previous chemotherapy as a covariable, we used a bone marrow toxicity scoring system which takes into account drugs and number of chemotherapy cycles used (24). This chemotherapy score correlates with damage to the stem cell pool caused by previous chemotherapy and with the ability to mobilize hematopoietic cells (25, 26). Acute leukemias were excluded from this analysis because, although treatments only include low/medium myelotoxic drugs and they have low chemotherapy score, the intense dosages in these protocols cause prolonged aplasias (Supplementary Table 3). The  $\chi^2$  test and Fisher Exact test were used to compare the hematologic toxicity grade of patients with clinical variables and genotype. To correct for the possible effect of previous treatments on the thrombocytopenia, the Mantel-Haenszel test was used. Nominal two-sided P-values <0.05 were considered statistically significant. All statistical analyses were carried out using SPSS software package version 17.0 (SPSS, Chicago, IL, USA).

## RESULTS

### **β-tubulin isotype VI has a broad and variable expression in blood cells**

We previously showed that β-tubulin VI expression was exclusively found in hematopoietic lineage cells, specifically, in platelets, lymphocytes, bone marrow and spleen, however, a detailed characterization of β-tubulin VI expression has not been performed. Here we show that β-tubulin VI mRNA was detected in all blood samples tested, with the highest expression corresponding to erythrocytes, platelets and monocytes (cells of myeloid origin) (Fig. 1A). To further investigate the expression of β-tubulin VI, we also determined β-tubulin VI expression in hematologic malignancies by studying tumoral cell lines and samples. Again, cells of myeloid origin showed higher expression than those of lymphoid origin, with the megakaryocytic MC-3 and CHRF288-11 cell lines showing the highest expression levels (Fig. 1B).

To confirm the relevance of β-tubulin VI in blood cells, we studied its expression in peripheral blood lymphocytes from 100 healthy volunteers. Figure 2A shows that isotype VI was the major β-tubulin component of lymphocyte microtubules, followed by β-tubulins IVb and I, accounting for 82, 10 and 7.5 % of the total β-tubulin content, respectively. Interestingly, β-tubulin VI mRNA content was subjected to a substantial inter-individual variability (7.2-fold variation in expression). To substantiate this variability, we also measured the expression of the ubiquitous and highly conserved β-tubulins IVb and I in the same samples, finding a 2.5 and 2.2-fold variation in mRNA content, respectively (data not shown).

### ***TUBB1* gene has common missense polymorphisms**

To determine whether *TUBB1* gene could differentiate from the other β-tubulin isotypes and be subjected to common genetic variability, we sequenced the four exons of

**Running title:** *β-tubulin VI variability and paclitaxel toxicity*

*TUBB1* and part of the 5' and 3' UTR in genomic DNA from 20 unrelated Caucasians. In addition to the already studied missense polymorphisms, rs463312 (Q43P) and rs415064 (Q43H), we found two other nucleotide changes leading to amino acid changes, rs35565630 (T274M) and rs6070697 (R307H), and one resulting in a premature stop codon (c.585G>A; Y55X). To determine the allelic frequency of these genetic variations, we performed genotyping in a series of unrelated Caucasians, Asians and Africans (Table 1). The nucleotide change leading to an early stop codon was only detected in the initial case used for sequencing. Regarding the other variants, the polymorphism leading to R307H was the most frequent, followed by those corresponding to Q43P, Q43H, and T274M, which was not detected in Asians. When we established the haplotypes formed by these missense variants (Table 1) we found that, as previously observed, rs463312 and rs415064 were in total linkage disequilibrium resulting exclusively in Q43P. rs35565630 was found only in the presence of rs6070697, thus, encoding a protein with two amino acid changes (T274M and R307H), while a relatively high number of cases carried only rs6070697 (R307H).

Concerning the putative effect of the *TUBB1* coding variants, previous results have shown that Q43P affects cardiovascular risk through an alteration of platelet aggregation. To determine whether the newly described missense polymorphisms could also alter platelets function, we isolated DNA from 100 healthy volunteers and identified individuals carrying the variants in homozygosity. For T274M we could not find homozygous variant individuals, thus, we performed platelet activation and aggregation assays only for rs6070697 (R307H), however, we did not detect significant differences (data not shown). Additionally, we tested whether the missense polymorphisms could be associated with an altered *TUBB1* mRNA content and thus explain the inter-individual variation found in the expression of this gene (Fig. 2A), however, none of the polymorphisms was significantly associated with an altered mRNA content (data not shown). Sequencing of *TUBB1* proximal promoter region in

individuals with maximal and minimal mRNA content did not identify polymorphisms associated with the expression of the gene.

**β-tubulin VI T274M alters paclitaxel effect on tubulin polymerization and is associated with decreased thrombocytopenia in patients treated with paclitaxel**

To test the effect of the *TUBB1* missense polymorphisms, we carried out transient transfections using appropriated expression vectors in MCF7 cells. These cells are derived from solid tumors and do not express β-tubulin VI protein. As shown in Figure 3A-B, the exogenously expressed β-tubulin VI proteins were correctly produced and colocalized with α-tubulin, indicating a correct incorporation into the cytoskeleton of the transfected cells. Cells stably expressing the different β-tubulin VI proteins were used to study tubulin polymerization after treatment with increasing amounts of paclitaxel. We found that in control cells, transfected with the empty vector, most of the cellular β-tubulin shifted to a polymerized form at 100 nM paclitaxel. This same effect was also appreciated in cells expressing wild type, Q43P and R307H β-tubulin VI proteins. However, cells expressing T274M and T274M/R307H variant proteins had significantly higher amounts of soluble β-tubulin at 100 nM paclitaxel (P=0.011) (see Fig. 3C and 3D). These results suggest that β-tubulin VI T274M variant protein is less sensitive to the polymerization effect of paclitaxel.

Because β-tubulin VI is a hematologic isotype, we investigated whether β-tubulin VI T274M patients could be less sensitive to the myelosuppressive effect of paclitaxel. To determine this, we genotyped a series of 49 patients with non-solid tumors scheduled for autologous transplant treated for mobilization of hematopoietic progenitor cells with paclitaxel 170 mg/m<sup>2</sup> i.v. by continuous infusion for 24 hr followed by 8 μg/kg s.c. rhG-CSF daily. With this highly myelosuppressive protocol, grades 3-4 leukocytopenia and

***Running title:  $\beta$ -tubulin VI variability and paclitaxel toxicity***

thrombocytopenia occurred in 55% and 43% of patients, respectively. We did not find a statistically significant association between clinical variables (e.g. type of disease, previous treatments, state of disease at stem cell harvest, weeks from last treatment, and platelet counts at baseline before initiation of treatment with paclitaxel) and the hematological toxicity of the patients, however, we found a statistically significant effect for T274M polymorphism. Heterozygous T274M patients showed significantly lower thrombocytopenia than homozygous wild type subjects. Thus, the two T274M patients exhibited grade 1 toxicity, while 85% of homozygous wild type patients developed higher thrombocytopenia grades (2, 3 or 4) (P= 0.031; Fig.4). The association was also significant (Mantel-Haenszel test, P=0.028) when the chemotherapy previous treatments were taken into account. No significant association was found between T274M and neutropenia. We also genotyped the patients for Q43P and R307H variants and, as expected, no differences in thrombocytopenia and neutropenia were found (data not shown).

## **DISCUSSION**

In this study we show that β-tubulin VI is a hematologic-specific isotype subjected to a substantial genetic variability that is likely contributing to the inter-individual variability observed in the myelosuppressive effect of paclitaxel. A thorough characterization of β-tubulin VI expression in cells of hematopoietic lineage revealed a high β-tubulin VI expression not only in lymphocytes and platelets, as previously described (3, 4), but also in most blood cells and several hematologic malignancies (Fig. 1). These data and the lack of β-tubulin VI in non-hematopoietic tissues (4), indicate that this is a hematologic specific isotype, and hence a major target mediating the hematologic toxicity of the β-tubulin binding drugs. In contrast, the low expression of this isotype in hematologic malignancies of lymphoid origin does not support a major role of β-tubulin VI in the physiopathology of these diseases. Furthermore, we found substantial inter-individual variability in β-tubulin VI mRNA content and common amino acid changes affecting *TUBB1* gene (Fig. 2 and Table 1).

So far, β-tubulin isotypes genes have been considered highly conserved (27); however, here we prove that β-tubulin VI differentiates in this respect from the rest of isotypes and exhibits common variation in the coding region. β-tubulin VI Q43P was previously described and associated with a reduced risk of arterial thrombosis and an increased risk of intracerebral hemorrhage by modulating platelet function and structure (9, 10). Additionally, a rare β-tubulin VI variant, R318W, was described in a case of congenital macrothrombocytopenia (11). In this study a nonsense variant (Y55X) was detected in heterozygosity, however, no deviations from normal ranges were found in the hemograms of this individual. So far, all the β-tubulin pathogenic variants are missense mutations (5, 6, 11), suggesting that early truncated proteins, that do not integrate into microtubules might not be as damaging as variant proteins that lead to altered dynamic instability of microtubules (5, 6). This is in agreement with the lack of Y55X phenotype. The R307H polymorphism is located in a conserved region



**Running title:** *β-tubulin VI variability and paclitaxel toxicity*

encoding β-tubulin VI M-loop, however, computational algorithms (PolyPhen and SIFT) predicted a benign variant and we found no significant effects on platelet activation and aggregation. T274M change also affects a conserved amino acid in the M-loop and was predicted as probably damaging by PolyPhen and SIFT, however, we detect this variant in the general population suggesting it will not substantially alter its function. In regards to microtubule targeted drugs, residue 274 is located near the taxanes binding site (28).

We found that cells stably expressing β-tubulin VI 274M variant, alone or in combination with 307H, were less sensitive to the polymerizing effect of paclitaxel compared to control cells transfected with the empty vector and those expressing β-tubulin VI wild type, 43P and 307H variant proteins (Fig. 3). Human β-tubulin isotypes are essentially invariant within paclitaxel binding site and, specifically, residue 274 is evolutionarily conserved in all vertebrate β-tubulins and all known β-tubulin isotypes in these organisms (29). Interestingly, residue 274 clusters in a space that has been identified as essential for paclitaxel's interaction with tubulin and an acquired mutation in residue 274 of β-tubulin I (T274I) renders cell lines resistant to paclitaxel and epothilone A (28, 30). These data suggest that variants affecting threonine 274 can lead to impaired drug binding and, thus, patients carrying the T274M variant could be resistant to the myelosuppressive effects of these microtubule binding drugs. To investigate the impact of this polymorphism in patients and its potential as a biomarker of hematological toxicity, we worked with a series of patients treated with paclitaxel. Since paclitaxel hematologic toxicity greatly depends on the time used for drug infusion (31), we decided to work with an outstanding set of 49 patients treated with a highly myelosuppressive protocol used for mobilization of hematopoietic progenitor cells. Although only two patients were T274M carriers, they showed a statistically significant lower grade of thrombocytopenia (P=0.031; Fig. 4), suggesting that *TUBB1* T274M conferred protection against paclitaxel thrombocytopenia. It is important to highlight that the T274M carriers had similar

***Running title:  $\beta$ -tubulin VI variability and paclitaxel toxicity***

characteristics than the non carriers in terms of age, disease state, base line platelets and chemotherapy score (Supplementary table 3). With respect to the type of disease, both were myeloma patients with treatments that have an important effect on platelet production. Concerning the interval from last chemotherapy, carriers were among the patients with shorter times. No significant associations were found for leukocytopenia but the results are inconclusive, because patients were treated with G-CSF to stimulate the production of neutrophils, and this is an important confounding factor. The effect found for T274M in thrombocytopenia risk is supported by the critical role that  $\beta$ -tubulin VI plays in platelets, demonstrated both in knock-out mice (7) and in patients carrying a mutation in this gene (11). Although in these previous studies no pathogenic phenotypes were reported in neutrophils, we detected high contents of  $\beta$ -tubulin VI in these cells (Fig. 1A). This, together with the fact that the microtubule binding agent 2-methoxyestradiol does not have an effect on  $\beta$ -tubulin VI (3) and does not result in any type of myelosuppression (32), supports a role for this isotype also in neutrophils and consequently in drug induced neutropenia. The similar structure of paclitaxel and docetaxel, which share the binding domain with epothilones, and the fact that cell lines expressing  $\beta$ -tubulin I T274I are resistant to all of these agents (28), warrants further studies to investigate the role of T274M in the hematologic toxicity of these drugs. It can be estimated that approximately 7% of Caucasians carry T274M and might show differences in myelosuppression.

In summary, this is the first study demonstrating that  $\beta$ -tubulin VI is a hematologic specific isotype that differentiates from other  $\beta$ -tubulin genes by relevant genetic and expression variability. In addition, we show that  $\beta$ -tubulin VI T274M decreases the effect of paclitaxel on tubulin polymerization and, although validation in independent patient series is required, we provide data supporting that it might constitute a marker of hematological toxicity induced by paclitaxel.

***Running title:  $\beta$ -tubulin VI variability and paclitaxel toxicity***

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## REFERENCES

1. Jordan MA, Wilson L. Microtubules as a target for anticancer drugs. *Nat Rev Cancer* 2004; 4: 253-65.
2. Risinger AL, Giles FJ, Mooberry SL. Microtubule dynamics as a target in oncology. *Cancer Treat Rev* 2009; 35: 255-61.
3. Escuin D, Burke PA, McMahon-Tobin G, et al. The hematopoietic-specific beta1-tubulin is naturally resistant to 2-methoxyestradiol and protects patients from drug-induced myelosuppression. *Cell Cycle* 2009; 8: 3914-24.
4. Leandro-Garcia LJ, Leskela S, Landa I, et al. Tumoral and tissue-specific expression of the major human beta-tubulin isotypes. *Cytoskeleton (Hoboken)* 2010; 67: 214-23.
5. Jaglin XH, Poirier K, Saillour Y, et al. Mutations in the beta-tubulin gene TUBB2B result in asymmetrical polymicrogyria. *Nat Genet* 2009; 41: 746-52.
6. Tischfield MA, Baris HN, Wu C, et al. Human TUBB3 mutations perturb microtubule dynamics, kinesin interactions, and axon guidance. *Cell* 2010; 140: 74-87.
7. Italiano JE, Jr., Bergmeier W, Tiwari S, et al. Mechanisms and implications of platelet discoid shape. *Blood* 2003; 101: 4789-96.
8. Schwer HD, Lecine P, Tiwari S, Italiano JE, Jr., Hartwig JH, Shivdasani RA. A lineage-restricted and divergent beta-tubulin isoform is essential for the biogenesis, structure and function of blood platelets. *Curr Biol* 2001; 11: 579-86.
9. Freson K, De Vos R, Wittevrongel C, et al. The TUBB1 Q43P functional polymorphism reduces the risk of cardiovascular disease in men by modulating platelet function and structure. *Blood* 2005; 106: 2356-62.
10. Navarro-Nunez L, Lozano ML, Rivera J, et al. The association of the beta1-tubulin Q43P polymorphism with intracerebral hemorrhage in men. *Haematologica* 2007; 92: 513-8.
11. 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: 458-61.
12. Giannakakou P, Sackett DL, Kang YK, et al. Paclitaxel-resistant human ovarian cancer cells have mutant beta-tubulins that exhibit impaired paclitaxel-driven polymerization. *J Biol Chem* 1997; 272: 17118-25.
13. Kavallaris M, Tait AS, Walsh BJ, et al. Multiple microtubule alterations are associated with Vinca alkaloid resistance in human leukemia cells. *Cancer Res* 2001; 61: 5803-9.
14. Verrills NM, Flemming CL, Liu M, et al. Microtubule alterations and mutations induced by desoxyepothilone B: implications for drug-target interactions. *Chem Biol* 2003; 10: 597-607.
15. Ferrandina G, Zannoni GF, Martinelli E, et al. Class III beta-tubulin overexpression is a marker of poor clinical outcome in advanced ovarian cancer patients. *Clin Cancer Res* 2006; 12: 2774-9.
16. Rosell R, Scagliotti G, Danenberg KD, et al. Transcripts in pretreatment biopsies from a three-arm randomized trial in metastatic non-small-cell lung cancer. *Oncogene* 2003; 22: 3548-53.
17. Seve P, Dumontet C. Is class III beta-tubulin a predictive factor in patients receiving tubulin-binding agents? *Lancet Oncol* 2008; 9: 168-75.
18. Seve P, Mackey J, Isaac S, et al. Class III beta-tubulin expression in tumor cells predicts response and outcome in patients with non-small cell lung cancer receiving paclitaxel. *Mol Cancer Ther* 2005; 4: 2001-7.
19. McCoy JP, Jr. Handling, storage, and preparation of human blood cells. *Curr Protoc Cytom* 2001; Chapter 5: Unit 5 1.
20. Rodriguez-Antona C, Leskela S, Zajac M, et al. Expression of CYP3A4 as a predictor of response to chemotherapy in peripheral T-cell lymphomas. *Blood* 2007; 110: 3345-51.
21. Fernandez A, De Arriba F, Rivera J, Heras I, Vicente V, Lozano ML. Successful mobilization of hematopoietic peripheral blood progenitor cells with paclitaxel-based chemotherapy as initial or salvage regimen in patients with hematologic malignancies. *Haematologica* 2008; 93: 1436-8.

**Running title:** *β-tubulin VI variability and paclitaxel toxicity*

22. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2<sup>-</sup>(-Delta Delta C(T)) Method. *Methods* 2001; 25: 402-8.
23. Mabeesh NJ, Post DE, Willard MT, et al. Geldanamycin induces degradation of hypoxia-inducible factor 1alpha protein via the proteasome pathway in prostate cancer cells. *Cancer Res* 2002; 62: 2478-82.
24. Aksentijevich I, Flinn I. Chemotherapy and bone marrow reserve: lessons learned from autologous stem cell transplantation. *Cancer Biother Radiopharm* 2002; 17: 399-403.
25. Clark RE, Brammer CG. Previous treatment predicts the efficiency of blood progenitor cell mobilisation: validation of a chemotherapy scoring system. *Bone Marrow Transplant* 1998; 22: 859-63.
26. Drake M, Ranaghan L, Morris TC, et al. Analysis of the effect of prior therapy on progenitor cell yield: use of a chemotherapy scoring system. *Br J Haematol* 1997; 98: 745-9.
27. Sullivan KF, Cleveland DW. Identification of conserved isotype-defining variable region sequences for four vertebrate beta tubulin polypeptide classes. *Proc Natl Acad Sci U S A* 1986; 83: 4327-31.
28. Giannakakou P, Gussio R, Nogales E, et al. A common pharmacophore for epothilone and taxanes: molecular basis for drug resistance conferred by tubulin mutations in human cancer cells. *Proc Natl Acad Sci U S A* 2000; 97: 2904-9.
29. Torin Huzil J, Luduena RF, Tuszynski J. Comparative modelling of human beta tubulin isotypes and implications for drug binding. *Nanotechnology* 2006; 17: S90-S100.
30. Huzil JT, Chen K, Kurgan L, Tuszynski JA. The roles of beta-tubulin mutations and isotype expression in acquired drug resistance. *Cancer Inform* 2007; 3: 159-81.
31. Eisenhauer EA, ten Bokkel Huinink WW, Swenerton KD, et al. European-Canadian randomized trial of paclitaxel in relapsed ovarian cancer: high-dose versus low-dose and long versus short infusion. *J Clin Oncol* 1994; 12: 2654-66.
32. Sweeney C, Liu G, Yiannoutsos C, et al. A phase II multicenter, randomized, double-blind, safety trial assessing the pharmacokinetics, pharmacodynamics, and efficacy of oral 2-methoxyestradiol capsules in hormone-refractory prostate cancer. *Clin Cancer Res* 2005; 11: 6625-33.

## FIGURE LEGENDS

**Figure 1. β-tubulin VI mRNA content in hematologic cells.** qRT-PCR was used to determine β-tubulin VI (*TUBB1*) mRNA quantities in different cells. *TUBB1* mRNA content in **A)** different non-tumoral human hematologic cells and **B)** in different cell lines and tumors of myeloid and lymphoid origin. mRNA content is compared to platelets expression (100%). Relative units, ru.

**Figure 2. Inter-individual variability in β-tubulin VI mRNA expression and β-tubulin isotypes relative expression in lymphocytes.** **A)** *TUBB1* mRNA content was quantified by qRT-PCR in peripheral blood lymphocytes isolated from 100 healthy volunteers. **B)** β-tubulin isotypes mean relative mRNA expression in peripheral blood lymphocytes from 100 healthy volunteers (*TUBB1*: 82%; *TUBB2C*: 10%; *TUBB*: 7.1%; *TUBB6*: 0.35% and *TUBB2A*: 0.27%. mRNA from *TUBB3*, *TUBB2B* and *TUBB4* below detection limits). Relative units, ru.

**Figure 3. Cells expressing β-tubulin VI T274M variant protein are less sensitive to the effect of paclitaxel on tubulin polymerization.** **A)** Confocal microscopy images of β-tubulin VI transient transfection in MCF7 cells. α-tubulin and β-tubulin VI staining are visualized, in red (568 nm) and blue (405 nm), respectively. **B)** β-tubulin VI protein detection by western blot in MCF7 cells stably expressing the different variants and those transfected with the empty vector (EV). **C)** MCF7 cells stably expressing the different β-tubulin VI variants were treated with paclitaxel (PTX) for 6 hrs. Control and paclitaxel treated samples were lysed and the polymerized (P) and the soluble (S) tubulin fractions were separated by centrifugation, loaded on adjacent lanes in SDS-PAGE and immunoblotted with an antibody against total β-

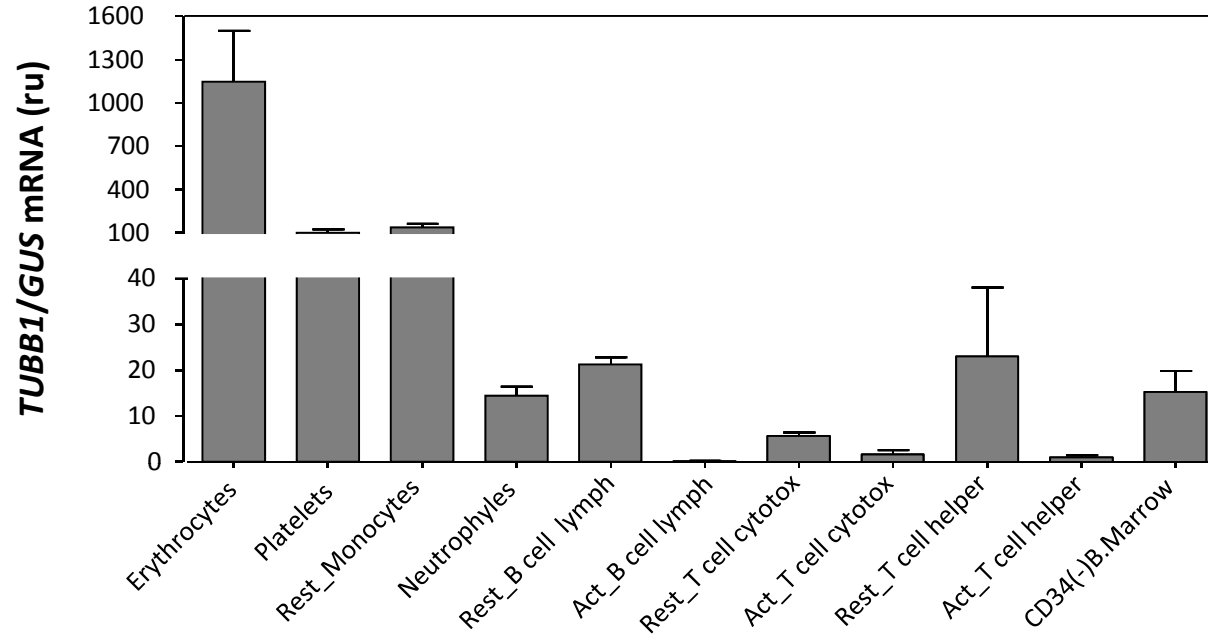
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tubulin. No statistically significant differences were found for any of the variant proteins, except for those with T274M, arrows indicate the lanes with significant differences. **D)** The percentage of polymerized (%P) and soluble (%S) tubulin was determined by dividing the densitometric value of polymerized (P) and soluble (S) tubulin by the total tubulin content (P + S). The bars in the graph represent the mean values of 3 different experiments.

**Figure 4. Patients carriers of  $\beta$ -tubulin VI T274M polymorphism develop less thrombocytopenia after paclitaxel treatment.** Patients treated with paclitaxel 170 mg/m<sup>2</sup> i.v. by continuous infusion for 24 hrs were genotyped and the thrombocytopenia grade developed after paclitaxel treatment (grade 1 *versus* grades 2, 3 or 4) was compared to the homozygous wild type patients using Fisher exact test.

Figure 1

A



B

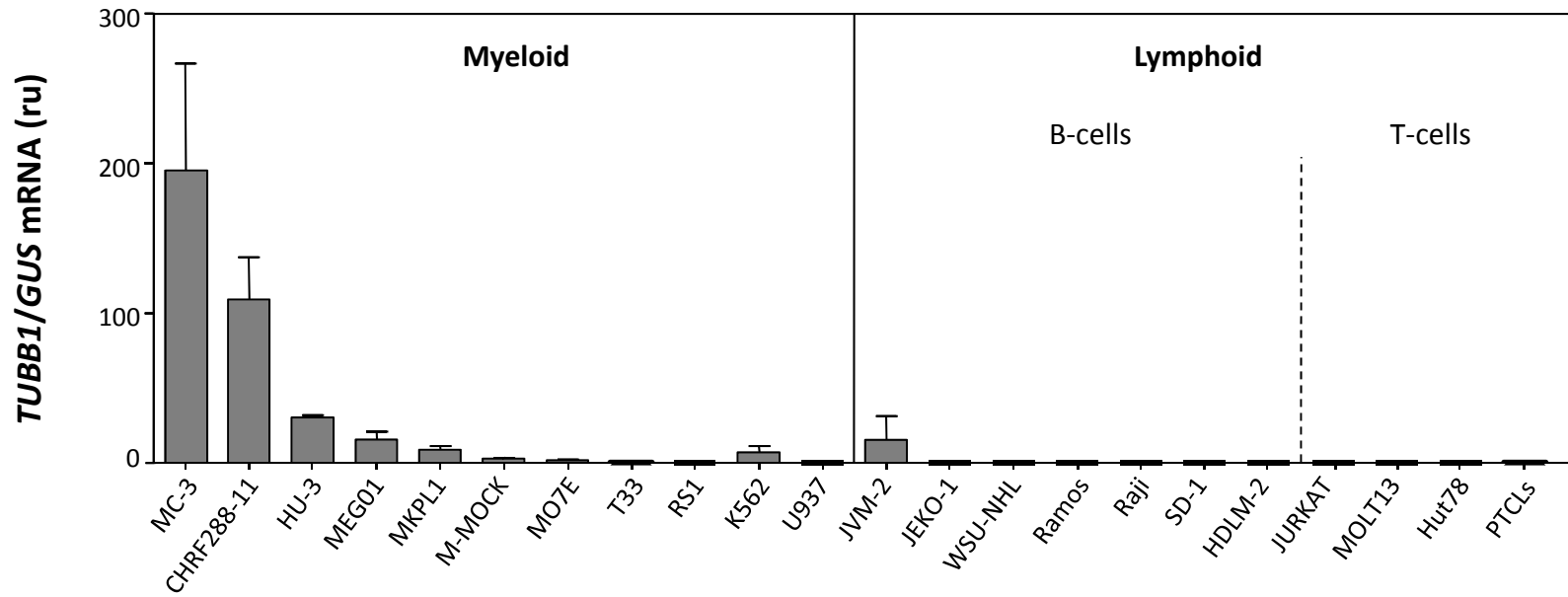
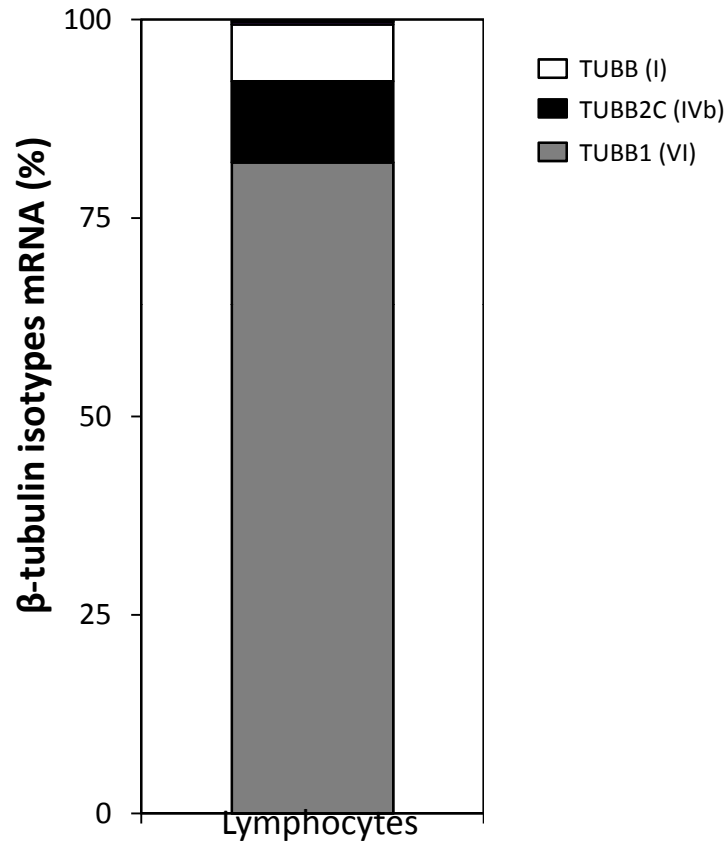




Figure 2

**A**



**B**

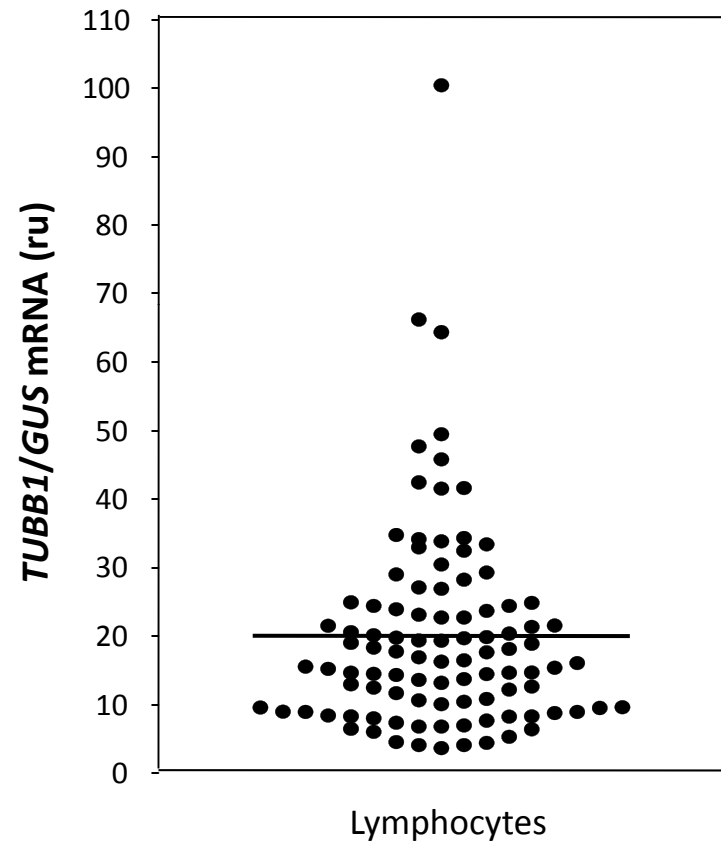
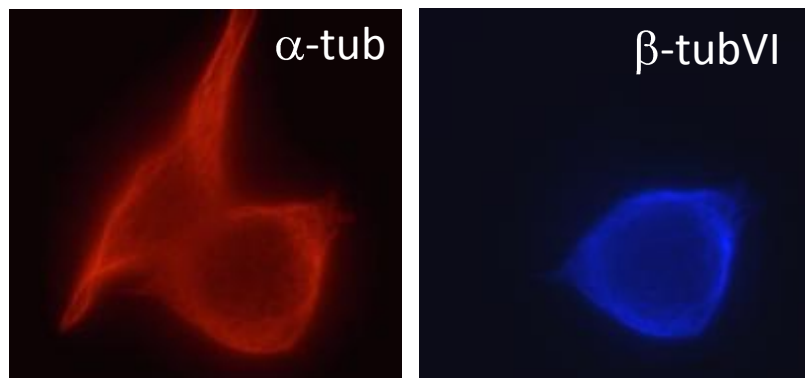
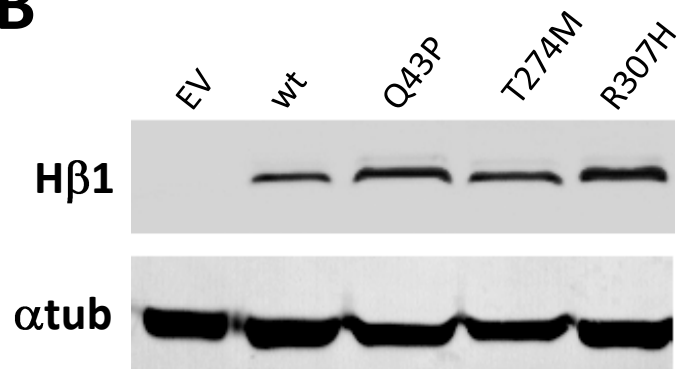


Figure 3

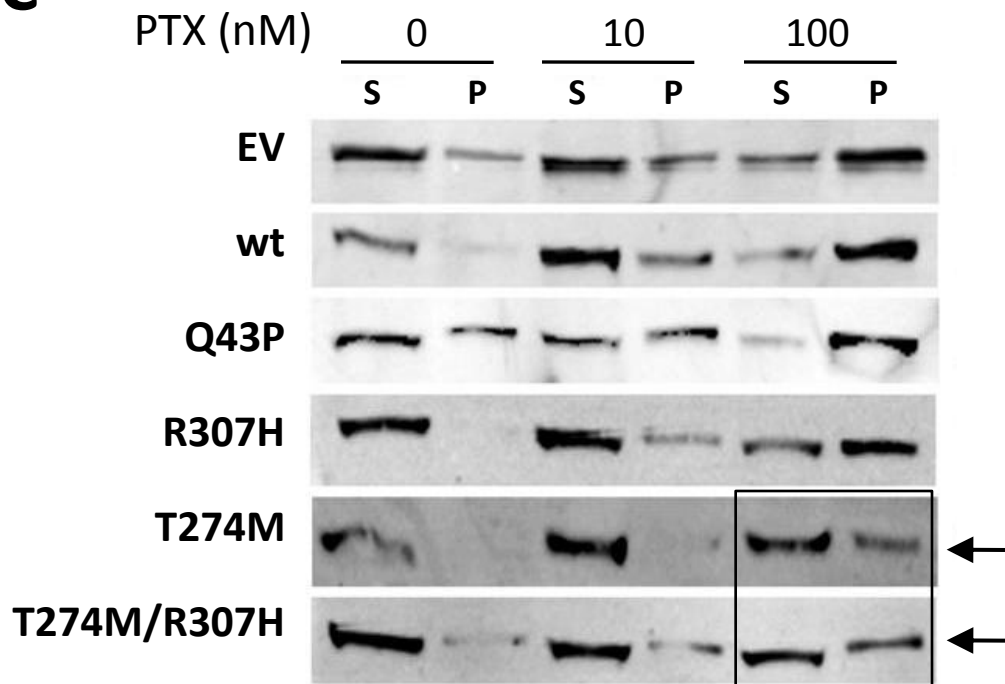
**A**



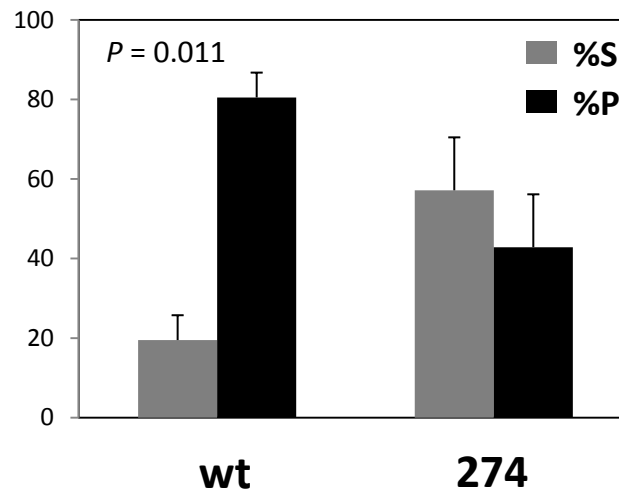
**B**



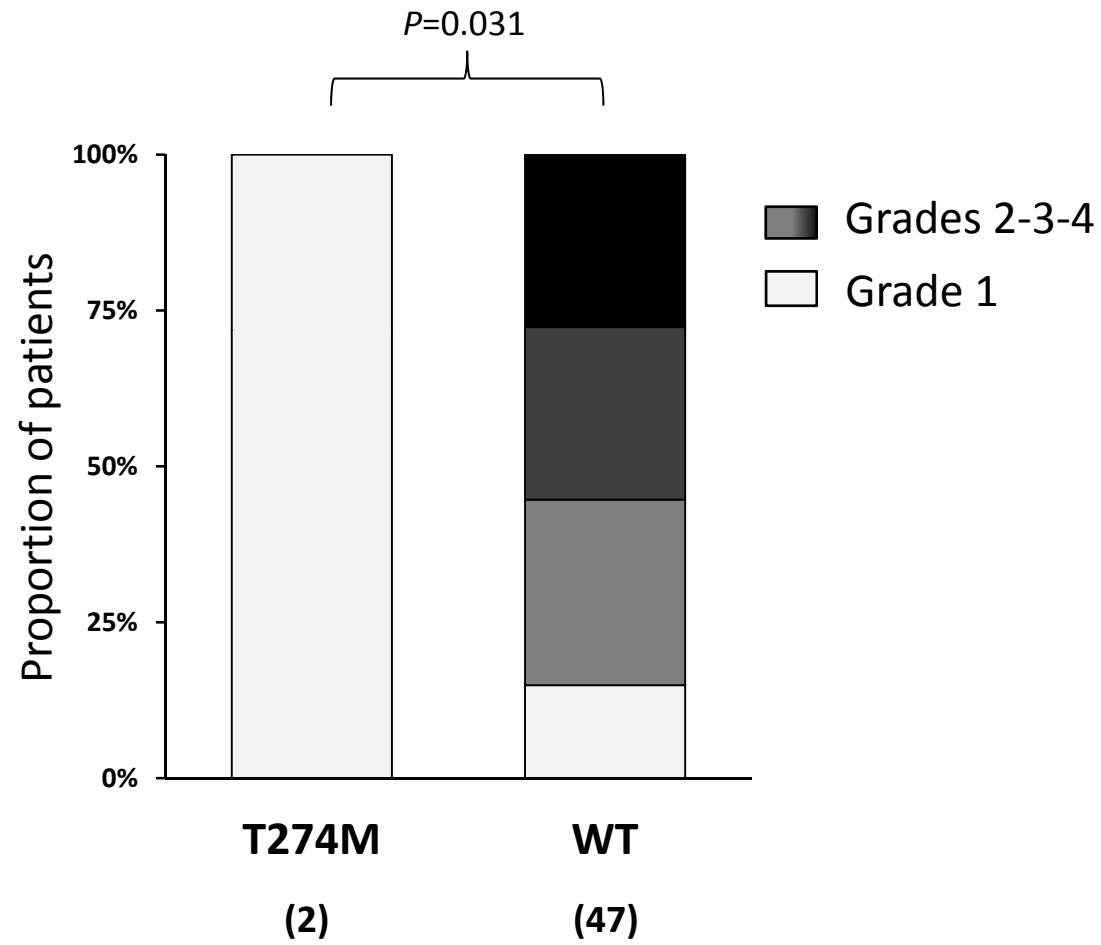
**C**



**D**



**Figure 4**



**Table 1. Genetic variants in *TUBB1* coding region and common haplotypes.**

Variants in coding region	Amino acid change	Caucasians (n=481)		Asians (n=106)		Africans (n=71)	
		Freq <sup>a</sup>	n <sup>b</sup>	Freq	n	Freq	n
rs463312	Q43P	0.042	130	0.086	105	0.029	68
rs415064	Q43H	0.042	130	0.086	105	0.029	68
-	Y55X	0.001	451	0.000	106	0.000	71
rs35565630	T274M	0.036	481	0.000	105	0.014	71
rs6070697	R307H	0.192	453	0.107	98	0.123	69
Haplotypes <sup>c</sup>	Amino acid changes	Freq		Freq		Freq	
Hap_AGCCG	none	0.791		0.823		0.846	
Hap_AGCCA	R307H	0.146		0.107		0.110	
Hap_CCCCG	Q43P	0.037		0.069		0.029	
Hap_AGCTA	T274M/ R307H	0.023		0.000		0.013	

<sup>a</sup> Frequency for the different alleles and haplotypes.

<sup>b</sup> Number of individuals used in each determination.

<sup>c</sup> Haplotypes estimated with frequencies >1%. The nucleotides correspond to rs463312, rs415064, the nucleotide change leading to Y55X, rs35565630 and rs6070697.