DNMT3A/TET2/ASXL1 mutations are an age-independent thrombotic risk factor in polycythemia vera patients: an observational study

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Abstract

Background: Polycythemia vera (PV) patients are classified as being of high or low thrombotic risk based on age >60 years and prior history of thrombosis. Despite adherence to treatment recommendations, vascular events remain frequent, even in younger patients, leading us to question whether thrombotic risk stratification could be improved. We previously reported an association between thrombotic events and mutations in DTA genes (DNMT3A, TET2 and ASXL1, frequently mutated in clonal hematopoiesis of indeterminate potential, CHIP). The objective of this study was to confirm this observation in a larger series of PV patients.

Methods: Patients with a confirmed diagnosis of PV and a minimum follow-up of 3 years were recruited from 8 European centers. Medical history was searched for thrombotic event recorded at any time and next-generation sequencing carried out with a myeloid panel to detect somatic mutations. Logistic regression multivariable analysis evaluated the impact of age, myeloid mutations and previous event on thrombotic risk. Kaplan–Meier thrombosis-free survival curves were compared by the Log Rank test. Associations in the total cohort were confirmed in a case-control study to exclude selection bias.

Results: Of the 136 patients recruited, 74 (56.1%) had a thrombotic event during a median follow-up of 9.4 years. The incidence density of vascular events was 2.83 per 100 person-years, with cerebral events the most frequent. In multivariable analysis, age ≥ 60 years was not a risk factor for thrombotic event but presence of DTA mutation was. Importantly, presence of DTA mutation was predictive of shorter thrombosis-free survival (p=0.007). A gender- and age-matched case (with event, n=47) control (without thrombotic events, n=47) study was carried out, confirming the association between thrombotic event and DTA mutation, even in a subgroup of patients aged ≤ 60 years.

Conclusions: Our study confirms that the presence of DTA mutations is an age-independent risk factor for a thrombotic event and predicts for shorter thrombosis-free survival in PV patients. In conclusion, our results support the use of molecular testing at diagnosis to help predict which PV patients (including younger patients and those with classical risk factors) are at higher risk of developing thrombosis.

BACKGROUND

The myeloproliferative neoplasms (MPN) are a group of diseases produced by a clonal expansion of an abnormal haematopoietic stem cell and characterized by a propensity for cardiovascular events. Polycythaemia vera (PV) has the highest risk of cardiovascular events, affecting 34–39% of patients [1], including those classified as low-risk according to the current ELN recommendations [2]. Such events are often the cause for MPN diagnosis, but also occur frequently during follow-up despite strict adherence to the prophylactic recommendations set out in international guidelines [3]. Indeed, annual incidence rates of 3.6 events/100 person-years were reported in patients aged 70 years receiving hydroxyurea treatment [4].
Carriers of clonal haematopoiesis of indeterminate potential (CHIP), a natural phenomenon associated with older age, are at risk of progression to haematological disease (approximately 0.5–1% per year) [5, 6]. Moreover, patients with CHIP have a 40% higher mortality due to an increased cardiovascular predisposition, independently of classical risk factors such as diabetes, high cholesterol, etc. [7, 8].

In the last few decades, the diagnosis, treatment and follow-up of PV patients has changed dramatically as a result of our increased understanding of the underlying pathogenic mechanisms. However, the same risk stratification has continued in use for over 20 years [9–11].

In a previous study we reported an association between the presence of mutations in DTA genes (DNMT3A, TET2 and ASXL1, the most frequently mutated genes in CHIP), and vascular events in patients with PV [12]. We aimed to confirm this observation in a larger series of PV patients from various European centers.

**METHODS**

**Patients**

Patients aged ≥ 18 years with a confirmed diagnosis of PV according to WHO criteria and a minimum follow-up of 3 years were recruited from 7 hospitals in Spain and 1 in Poland. Medical records were searched for thrombotic event recorded at any time in the medical history as well as information on cardiovascular risk factors (CVRF), including and hypertension.

For the case-control study, two homogeneous groups of gender- and age-matched PV patients were formed. Patients with thrombotic events in their medical history (case) were identified from eligible patients. Then patients without thrombotic events (control) in their medical history were taken and gender- and age-matched with the cases. Cases that did not age-match with a control were removed from the case-control analysis.

**Next-generation sequencing (NGS)**

NGS (MiSeq, Illumina) was performed on 200 ng genomic DNA extracted from peripheral blood at diagnosis of 136 PV patients with the 30-gene panel Myeloid Solution (SOPHiA Genetics). Only variants with an allelic frequency (VAF) ≥ 2% and annotated as pathogenic or probably pathogenic were considered [5].

**Statistical analysis**

Data normality was determined using the Shapiro-Wilk test. Differences between group means were compared using the Student paired t-test of equal variance for unpaired samples. Incidence density (events/person-year) of thrombotic events was calculated by dividing the total number of thrombotic events by the total time of follow-up from diagnosis of all patients. Chi-squared univariable analyses were used to determine associations with thrombotic event and the level of significance calculated by the
Fisher exact test. Logistic regression multivariable analysis evaluated the impact of the independent variables on thrombotic risk and was only carried out for variables that reached statistical significance in the univariable analysis. Thrombosis-free survival was measured from the time of diagnosis until the thrombotic event or date of last follow up. Survival probabilities were estimated using the Kaplan–Meier method and the Log Rank test was used for statistical comparison. P-values < 0.05 were considered statistically significant. Analyses were performed using the SPSS statistical software, version 22.0.

RESULTS

A total of 136 adult PV patients were recruited with an average age of 61.1 years (52.9% were aged 60 or above) and 51.5% were males (Supplementary Table 1). Of these, 74 patients had a thrombotic event recorded at any time in their medical history (56.1%) and 58 had no thrombotic event (43.9%, information on vascular events was not available from the medical history of 4 patients).

Seventy-seven patients (77/136, 56.1%) presented ≥1 additional mutation (i.e. pathogenic non-driver mutation). The most frequently mutated genes were TET2 27.9%, ASXL1 11.8%, and DNMT3A 11% (termed “DTA”; Fig. 1). As expected, patients with DTA mutations were older than those without DTA mutations (65.4 vs. 57.8 years, p = 0.002; Student t-test).

With a median follow-up of 9.4 years, the incidence density of vascular events was 2.83 per 100 person-years. Patients classically stratified as high-risk had shorter thrombosis-free survival as expected, although statistical significance was not reached (Fig. 2A, p = 0.142).

Thirty-seven events (52.9%) were pre-diagnostic (including the cause for diagnosis) and 44 (62.9%) were post-diagnostic; 11 patients had both a pre- and post-diagnostic event. Of the post-diagnostic events with information available (n = 37), 25.7% were venous and 50.0% arterial. The most frequent events were cerebral (30.9%), acute myocardial infarction (25.5%), and portal thrombosis (7.3%).

Comparing the group of PV patients with a thrombotic event at any time in their medical history (n = 74) to those without (n = 58), there was no significant difference in patient age or the VAF of JAK2 mutation, but the group with an event had a higher leukocyte count at diagnosis and more additional mutations (mean 1.1 vs. 0.7, p = 0.004) and DTA mutations (0.53 vs. 0.31, p = 0.001; Supplementary Table 2). Patients with a larger JAK2-mutated clone size (≥ 50% VAF) were not more likely to harbor a DTA mutation (p = 0.858). Patients with a pre-diagnostic event were more likely to have a post-diagnostic event (p = 0.036) and also more likely to have a DTA mutation (p = 0.038).

In univariable analysis, thrombotic event was not associated with age ≥ 60 years or VAF of JAK2 mutation but was associated with the presence of any additional mutation (OR 3.4, p = 0.001) and with any DTA mutation (OR 2.5, p = 0.014). Statistical significance was lost when DTA mutations were not included (non-DTA additional mutation, Supplementary Table 3). The association with any “adverse mutation” (SRSF2, IDH2 or ASXL1, as defined by Tefferi et al. based on impact on overall survival [13]) was marginal (p = 0.064).
DTA mutations were significantly associated with arterial events (OR 4.6, p < 0.001) and marginally with venous events (OR 2.7, p = 0.06). Notably, DTA mutation was predictive of thrombosis-free survival (p = 0.007, Fig. 2B).

Data on cardiovascular risk factors (CVRF) and hypertension was available for 115 patients. Seventy-three of 115 patients (63.5%) were hypertensive. The DTA and CVRF variables are closely related (OR 6.8, p = 0.009), in particular the TET2 mutation with hypertension (p = 0.025). Nevertheless, DTA mutation and additional mutation remained as risk factors for a thrombotic event in hypertensive patients (OR 4.4, p = 0.052; OR 5.8, p = 0.026, respectively; Fisher exact test), with additional mutations losing statistical significance when the DTA genes were removed from the analysis (p = 1.0). The association between thrombotic event and CVRF or hypertension was confirmed in multivariable analysis (OR 3.8, p = 0.030; OR 3.85, p = 0.002, respectively) while the association with DTA mutation was marginal (OR 2.1, p = 0.075; Supplementary Table 4).

To confirm these observations, two homogeneous groups of 47 gender- and age-matched PV patients were formed with (case, n = 47) and without thrombotic events (control, n = 47) in their medical history to exclude selection bias.

In the case-control study, both CVRF and hypertension lost significance in multivariable analysis while DTA mutation was confirmed as a risk factor for thrombotic event (OR 2.9, p = 0.027; Supplementary Table 4), observing a higher number of additional mutations in the group of cases (cases 1.0 vs. controls 0.68, p = 0.026). Importantly, the association between thrombotic event and DTA mutation remained in the group of PV patients aged < 60 years (n = 44; OR 6.67, p = 0.033; Supplementary Table 5) from the case-control study and was predictive for thrombosis-free survival in younger patients (p = 0.024, Supplementary Fig. 1). When all patients in the cohort were dichotomized according to age at diagnosis, the association between thrombosis and DTA mutation was of borderline significance in both age groups (≥ 60 years OR 2.44, p = 0.064; <60 years OR 2.89, p = 0.065). Moreover, when data were dichotomized according to the VAF of mutated JAK2, the association between DTA mutations and thrombosis was significant for the smaller JAK2-mutated clones (VAF < 50%: OR 3.34, CI 1.11–13.67, p = 0.034) and borderline for the larger JAK2-mutated clones (VAF ≥ 50%: OR 3.50, CI 0.82–14.91, p = 0.091).

**DISCUSSION**

In this observational multicenter study, we confirmed our previous observation that the presence of additional myeloid mutations at diagnosis, particularly DTA mutations, is a risk factor for thrombotic event in PV patients. We further show that the presence of DTA mutations predicts for thrombosis-free survival, even in patients aged < 60 years [12]. The association was lost when the DTA genes were considered individually, probably due to low frequencies. This association was age-independent and, despite the intimate relationship between DTA mutations and CVRF, was an additional risk factor for hypertensive patients [14].
The most frequent site of thrombosis in our cohort was cerebral, with a high incidence of ischemic stroke, in accordance with the recent observation of an age-independent association between DTA mutation and a history of stroke [15]. Since the cardiovascular risk imparted by mutations in CHIP genes has been convincingly demonstrated in healthy individuals [6], its effect in PV patients is not surprising given the additional thrombotic risk imparted by the JAK2 V617F mutation [16]. Nevertheless, not all studies have observed an association between DTA mutations and thrombosis in PV patients [17].

One limitation of our study was the high proportion of PV patients with a post-diagnostic thrombotic event (44 events, 2.83 per 100 person-years), although the incidence of our cohort was in accordance with previous studies [4]. Two groups of a similar number of PV patients were initially included: with and without a thrombotic event recorded in their medical history. However, in a number of cases, records were found of thrombotic events upon closer scrutiny of their medical history, some of which were reported in unusual sites, such as the retina. For this reason, a case-control analysis of two equal groups of sex- and age-matched patients was also performed to exclude potential selection bias.

It is intriguing that some patients present with a thrombotic event leading to their PV diagnosis while others are diagnosed due to blood count abnormalities. It thus appears that a group of PV patients are prone to thrombotic events, including both “low-risk” patients and high-risk PV patients receiving prophylaxis, consequently increasing their comorbidities and reducing their life expectancy. Perhaps there are fundamental differences between these two groups of patients that result in one group being more prone to thrombosis. One such difference could be the presence of DTA mutations, or the order of mutation acquisition, associated with differences in MPN phenotype [18]. However, to demonstrate this, serial sampling and/or phylogenetic methods would be required [19].

**CONCLUSIONS**

Our results support the use of molecular testing at diagnosis to help predict which PV patients are at higher risk of developing thrombosis [12, 13, 20]. Such patients with additional myeloid mutations, particularly in the DTA genes, could perhaps benefit from a more aggressive therapy although randomized controlled trials would be required to confirm optimal prophylaxis management.

In conclusion, patients with a DTA mutation at diagnosis (including younger patients and those with classical risk factors) should be considered to be at higher risk of thrombosis. We question whether the current risk stratification and recommendations of cytoreduction and antiplatelet therapy are effective enough. The incorporation of biomarkers into current algorithms to predict thrombotic events is an unmet need that needs to be addressed.

**Declarations**

**Ethics approval and consent to participate:** This retrospective non-interventional study was approved by our institutional review board (Comité Ético de Investigación Clínica, ref. 2019-230-1) on 28 March 2019.
and conducted in accordance with the Declaration of Helsinki.

All patient data was dissociated and anonymized; informed consent was not required due to the retrospective nature of the study and because the results did not affect the clinical management of patients.

**Availability of data and materials:** The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

**Competing interests:** The authors declare that they have no competing interests.

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**Author contributions:** ASD contributed data, curated data, analysed the data, and wrote the paper; RS coordinated the study, curated data, analysed the data, performed statistical analysis, and wrote the paper; YF performed NGS; MS, AAL, FFM, MPE, GCT, MLF, BTV, and BC contributed data, JFLLP and NFS curated data; JMGM performed statistical analysis; MTGC designed the research study; CBS designed the research study, performed statistical analysis and wrote the paper. All authors read and approved the final version of the manuscript.

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**References**


Figures
Figure 1

Mutations detected in the series of 136 PV patients. The variant allele frequency (VAF) of JAK2 is represented in the top panel. Asterisks (*) mean that a DTA (DNMT3A, TET2 or ASXL1) mutation was detected. Variants detected in genes covered by the targeted myeloid panel (listed on the left) are represented in the bottom panel. Gray square: a pathogenic (or probably pathogenic) variant was detected in the gene; white square: no variant was detected.
Figure 2

Thrombosis-free survival for the total cohort of PV patients. (A) Kaplan–Meier curve with data stratified as high- or low-risk according to conventional thrombotic risk algorithms, and (B) Kaplan–Meier curve with data stratified according to presence or absence of any DTA (DNMT3A, TET2 or ASXL1) mutation. Time calculated from date of diagnosis to date of thrombotic event or date of last follow-up. Significance determined using the Log Rank test.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementarydata.pdf