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CORRESPONDENCE

Correlation of mutation status and morphological changes in essential thrombocythaemia and myelofibrosis

Sir,

The identification of a myeloproliferative neoplasm (MPN) driver mutation [Janus Kinase 2 V617F (*JAK2*), Calreticulin exon 9 (*CALR*) or Myeloproliferative Leukaemia Virus Oncogene exon 10 (*MPL*)] has become a major criterion in the 2016 updated World Health Organization (WHO) diagnosis and classification of tumours.[1] Despite the common activation of the JAK-STAT pathway, these mutations are increasingly found to define different clinical courses and outcomes.[2] For instance, JAK2-mutant essential thrombocythaemia (ET) has a higher risk of thrombosis, whereas CALR-mutant ET has a lower thrombosis risk but a higher risk of progression to myelofibrosis.[3] On the other hand, JAK2-and MPL-mutant primary myelofibrosis (MF) have a worse outcome compared to CALR-mutant primary MF. Therefore, it is important to test for these mutations at the diagnosis of MPN as they have prognostic and treatment implications. In this study, we aim to investigate whether the different driver mutations in ET and MF (both primary and post-ET) result in differing characteristic peripheral blood and bone marrow trephine morphological features.

We retrospectively reviewed 59 cases with a new diagnosis of untreated ET and primary MF between 1 July 2007 and 31 July 2017 across two tertiary hospitals in Melbourne, Victoria. Eight cases of newly diagnosed post-ET MF were also included for analysis. Cases were identified and retrieved from each hospital's bone marrow databases based on the final diagnosis assigned. All cases underwent molecular testing for *JAK2 V617F, CALR* and *MPL* mutations. If all three mutations were not detected the case was classified as triple negative MPN. Ethics approval was obtained from the Human Research Ethics Committees from both sites (HREC/14/Austin/490; SSA/15/MH/53).

Two haematopathologists co-evaluated the blood films (May–Grünwald Giemsa stain) and bone marrow trephines (formalin fixed, paraffin embedded, and stained with haematoxylin and eosin, reticulin) in a blinded manner [only age and full blood examination (FBE) parameters provided] using a multi-headed microscope to overcome the limitations of inter-observer reproducibility. FBE parameters were obtained from the laboratory information systems; however, a manual differential cell count was performed. Peripheral blood (PB) film features were graded as present/absent for polychromasia, nucleated red blood cells, teardrop cells, left shift, platelet anisocytosis, anisochromia, giant platelets and leukoerythroblastic change. Trephines were graded according to predetermined criteria adapted from Wilkins *et al.*;[4] cell lineage cellularity was graded taking into account patient age. Megakaryocyte morphology was graded as absent (<10%), present (10–50%) or predominant (>50% of cells) for the following features: multilobated/staghorn, cloud-like, pyknotic, dysplastic (monolobated/separated lobes) and bare megakaryocyte nuclei. Megakaryocyte cluster characteristics were assessed: >6 versus ≤6 megakaryocytes per cluster, tight/loose and whether clusters were occasional or predominant in the trephine section. Reticulin was scored according to the bone marrow fibrosis grading system by Thiele *et al.*[5] A consensus diagnosis was allocated to each case according to WHO classification without reference to the original diagnosis.[1] Clinical history was subsequently assessed for antecedent haematological conditions to correctly classify the post-ET MF category. Patients with post-polycythaemia vera MF were excluded due to the exclusive *JAK2* mutation in this group.

Molecular analysis was performed on DNA extracted from PB. Molecular methods used during 2007–2016 were: *JAK2* V617F by high resolution melting analysis;[6] *CALR* exon 9 mutations by Sanger sequencing; and *MPL* mutations first screened by high resolution melting analysis and then confirmed by Sanger sequencing.[7] From 2016–2017, *JAK2* V617F analysis was performed by either reverse transcriptase polymerase chain reaction or next generation sequencing; both *CALR* and *MPL* mutations were analysed by next generation sequencing.

Descriptive statistics (counts, percentage frequencies, mean or median, range or interquartile ranges, 95% confidence intervals) were provided as appropriate. Categorical variables were evaluated using the chi-squared test, or Fisher's exact test when frequencies were <5. Mann–Whitney (rank-sum for comparison of two groups) or Kruskal–Wallis (for ≥3 groups) test was applied to assess non-normally distributed continuous variables. Statistical significance was defined as a two-tailed p value of <0.05. Statistical analysis was performed with GraphPad Prism 6 software (GraphPad Software, USA). Morphological features were mapped using principal component analysis with MATLAB-2017b (MathWorks, USA). This analysis assessed whether morphological features cluster with mutation status.

Of the 59 cases evaluated, 36 (61%) had ET, 8 (14%) post-ET MF and 15 (25%) primary MF. *JAK2* V617F was detected in 21 (35%) cases, *CALR* in 26 (43%), *MPL* in six (10%) and triple negative in seven (12%). There were no disagreements between the consensus diagnosis and the original diagnosis. Table 1 shows a summary of morphological characteristics.

Cases with ET had a median age of 56 years (range 21–89 years) with a slight female predominance (22/36, 61%). *CALR* positive ET, when compared with *CALR* negative ET, had significantly higher platelet count (p = 0.02), a predominance of megakaryocyte clustering [12/16 (75%) vs 8/20 (40%), p = 0.04] and large (≥6) cluster sizes [9/16 (56%) vs 3/20 (15%), p = 0.01]. A higher proportion of *CALR* positive ET was observed to have pyknotic nuclei [7/16 (44%) vs 2/21 (10%), p = 0.11] but the difference was not statistically significant. *JAK2* positive ET had a higher number of cases with a predominance of megakaryocyte staghorn nuclei [6/13 (46%) vs 2/21 (10%), p = 0.03]. There were no characteristic features observed in the small number of *MPL* or triple negative groups. There were no differences in PB morphology in all mutation groups in ET.

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Table 1 Peripheral blood and bone marrow trephine megakaryocyte characteristics separated by diagnosis and mutation status

	JAK2	*CALR*	*MPL*	Triple negative
Essential thrombocythaemia (n = 36)				
No. cases, n (%)	13 (36)	16 (44)	3 (8)	4 (11)
Platelet count, $\times 10^9$/L, median (IQR)	563 (459−630)	835 (684.25−1017.25)	622 (600.5−741)	661 (539−1016)
Megakaryocyte cellularity, n (%)				
Normal	0	0	0	0
Mildly increased	5 (38)	6 (38)	1 (33)	2 (50)
Moderately increased	7 (54)	7 (43)	1 (33)	1 (25)
Severely increased	1 (8)	3 (19)	1 (33)	1 (25)
Cluster size, n (%)				
<6 megakaryocytes	11 (85)	7 (44)	3 (100)	3 (75)
≥6 megakaryocytes	2 (15)	9 (56)	0	1 (25)
No. of clusters, n (%)				
Occasional (<10%)	9 (69)	4 (25)	1 (33)	2 (50)
Predominant (≥10%)	4 (31)	12 (75)	2 (66)	2 (50)
Staghorn nuclei, n (%)				
Absent	2 (15)	5 (31)	1 (33)	3 (75)
10−50%	5 (38)	9 (56)	2 (66)	1 (25)
>50%	6 (46)	2 (12)	0	0
Cloud-like nuclei, n (%)				
Absent	11 (85)	12 (75)	1 (33)	2 (50)
10−50%	2 (15)	4 (25)	2 (67)	2 (50)
Dysplastic nuclei, n (%)				
Absent	12 (92)	13 (81)	3 (100)	3 (75)
10−50%	1 (8)	3 (19)	0	1 (25)
>50%	0	0	0	0
Pyknotic nuclei, n (%)				
Absent	11 (85)	9 (56)	3 (100)	4 (100)
10−50%	2 (15)	7 (44)	0	0
Myelofibrosis (n = 23)				
No. cases, n (%)	7 (30)	10 (43)	3 (13)	3 (13)
Platelet count, $\times 10^9$/L, median (IQR)	746 (494.5−814.5)	582.5 (286.25−912.25)	181 (129.5−232.5)	58 (56−827)
Cluster size, n (%)				
Absent	0	1 (10)	0	1 (33)
<6 megakaryocytes	4 (57)	2 (20)	2 (67)	1 (33)
≥6 megakaryocytes	3 (43)	7 (70)	1 (33)	1 (33)
No. of clusters, n (%)				
Absent	0	1 (10)	0	1 (33)
Occasional (<10%)	1 (14)	2 (20)	1 (33)	1 (33)
Predominant (≥10%)	6 (86)	7 (70)	2 (67)	1 (33)
Staghorn nuclei, n (%)				
Absent	4 (57)	6 (60)	2 (67)	3 (100)
10−50%	2 (29)	4 (40)	1 (33)	0
>50%	1 (14)	0	0	0
Cloud-like nuclei, n (%)				
Absent	5 (71)	2 (20)	3 (100)	3 (100)
10−50%	2 (29)	8 (80)	0	0
>50%	0	0	0	0
Dysplastic nuclei, n (%)				
Absent	7 (100)	9 (90)	2 (67)	1 (33)
10−50%	0	1 (10)	0	2 (67)
>50%	0	0	1 (33)	0
Pyknotic nuclei, n (%)				
Absent	0	5 (50)	0	2 (67)
10−50%	6 (86)	4 (40)	3 (100)	1 (33)
>50%	1 (14)	1 (10)	0	0

Of the 16 *CALR* positive ET cases, eight had type 1 mutations (52 base pair deletion, p.L67fs*46), seven had type 2 mutations (5 base pair insertion, p.K385fs*47) and one had a combination of 14 base pair insertion and 7 base pair deletion. Comparison of type 1 and type 2 mutated *CALR* revealed that type 2 cases had a lower median age [52 years (range 30−74) vs 74 years (range 48−82), p = 0.016] and predominance of megakaryocyte clusters (7/7 vs 4/8, p = 0.03). In addition, there was a non-significant trend to higher platelet count (median platelet count 992 $\times$ 10^9/L vs 723 $\times$ 10^9/L, p = 0.12), and more megakaryocyte proliferation (3/7 vs 0/8, p = 0.12) in the type 2 mutation group. The remaining parameters were similar.

MF cases had a median age of 65 years (range 30−84 years) with male predominance (17/23, 89%). Fifteen cases had primary MF and eight cases had post-ET MF. Of the post-ET MF cases, four had *JAK2* mutation, three had *CALR* mutation and one had *MPL* mutation. *CALR* positive cases had more cloud-like nuclei observed [8/10 (80%) vs 2/13 (15%), p = 0.01]. The remaining morphological findings of the PB and trephines were similar between all the mutation groups. No significant differences were found between primary MF and post-ET MF cases.

Principal component analysis was used to evaluate the relevant morphological features responsible for the highest variation in our dataset and to determine whether these

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