Clonal analyses reveal associations of JAK2V617F homozygosity with hematologic features, age and gender in polycythemia vera and essential thrombocythemia

Anna L. Godfrey,[1,2,3] Edwin Chen,[1-3] Francesca Pagano,[1] Yvonne Silber,[1,3] Peter J. Campbell,[4] and Anthony R. Green[1-3]

[1]Cambridge Institute for Medical Research and Department of Haematology, University of Cambridge, Cambridge; [2]Department of Haematology, Addenbrooke's Hospital, Cambridge; [3]Wellcome Trust – Medical Research Council Cambridge Stem Cell Institute, University of Cambridge, Cambridge; and [4]Wellcome Trust Sanger Institute, Hinxton, Cambridge, UK

ABSTRACT

Subclones homozygous for *JAK2V617F* are more common and larger in patients with polycythemia vera compared to essential thrombocythemia, but their role in determining phenotype remains unclear. We genotyped 4564 erythroid colonies from 59 patients with polycythemia vera or essential thrombocythemia to investigate whether the proportion of *JAK2V617F* -homozygous precursors, compared to heterozygous precursors, is associated with clinical or demographic features. In polycythemia vera, a higher proportion of homozygous-mutant precursors was associated with more extreme blood counts at diagnosis, consistent with a causal role for homozygosity in polycythemia vera pathogenesis. Larger numbers of homozygous-mutant colonies were associated with older age, and with male gender in polycythemia vera but female gender in essential thrombocythemia. These results suggest that age promotes development or expansion of homozygous-mutant clones and that gender modulates the phenotypic consequences of *JAK2V617F* homozygosity, thus providing a potential explanation for the long-standing observations of a preponderance of men with polycythemia vera but of women with essential thrombocythemia.

Introduction

The *JAK2V617F* mutation is found in over 95% of patients with polycythemia vera (PV) and approximately 60% of those with essential thrombocythemia (ET).[1] but the additional mechanisms which determine their distinct clinical phenotypes remain unclear. Circumstantial evidence suggests that *JAK2V617F* homozygosity may have a role in determining the PV phenotype: i) homozygous-mutant hematopoietic precursors are isolated from a higher proportion of patients and form larger clones in PV compared to ET;[2-4] ii) mouse models have demonstrated PV-like, rather than ET-like, phenotypes associated with higher *JAK2V617F* gene dosage;[5,6] iii) higher *JAK2V617F* allele burdens in granulocyte DNA have been associated with higher hemoglobin levels and lower platelet counts in PV.[7,8]

However, several lines of evidence indicate that the role of *JAK2V617F* homozygosity in PV pathogenesis is not simple. Clonal analyses indicate that, in fact, small homozygous-mutant clones arise frequently and recurrently in both PV and ET, suggesting that the mere presence of homozygosity is insufficient for a PV phenotype, and additional factors are required.[4] Studies of some,[9-11] but not other[12] knock-in mouse models have reported that heterozygosity for *JAK2V617F* is associated with marked erythrocytosis, with no further increase in hemoglobin levels in homozygous mice.[10] Associations of *JAK2V617F* allele burden with blood counts show inconsistencies between studies[13] and have not determined the extent to which allele burdens reflect the relative proportions of heterozygous and homozygous-mutant cells. Moreover, reduced levels of STAT1 activation have been identified in heterozygous-mutant erythroblasts from patients with PV, compared to those with ET, and may contribute causally to the PV phenotype.[14] It is, therefore, unclear whether *JAK2V617F* homozygosity makes a significant contribution to the pathogenesis of human PV.

Here we used a clonal approach to test the hypothesis that *JAK2V617F* homozygosity has a causal role in the PV phenotype, and investigated its associations with demographic features, by utilizing genotype data from 4564 erythroid colonies from 59 patients with PV or ET.

Design and Methods

Patients were recruited from Addenbrooke's Hospital, Cambridge, UK. Demographic and clinical features, together with methods used for colony culture and genotyping, have been previously described.[4] Patients met diagnostic criteria for *JAK2V617F*-positive PV or ET according to the British Committee for Standards in Haematology.[15,16] The study was approved by the Cambridge and Eastern Region Ethics Committee, patients gave written informed consent, and research was carried out in accordance with the Declaration of Helsinki.

To test the associations of the relative proportions of *JAK2V617F*-homozygous and heterozygous colonies with hematologic or demo-

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Correspondence: arg1000@cam.ac.uk

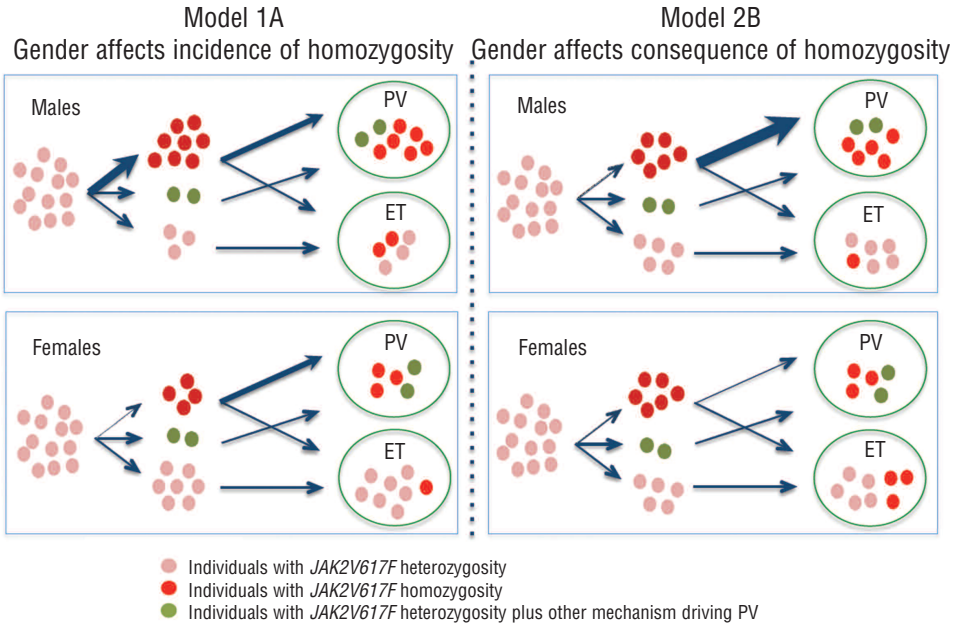

Figure 1. Possible models to explain the associations of *JAK2V617F* homozygosity with gender in PV and ET. Each symbol represents one patient. Starting from a pool of individuals with subclinical *JAK2V617F* heterozygous mutations (pink symbols), there are three possible outcomes: acquisition of a significant *JAK2V617F*-homozygous clone (red symbols); acquisition of other mechanisms that can drive PV (green symbols); or maintenance of *JAK2V617F* heterozygosity alone. (A) *JAK2V617F* homozygosity occurs at a higher rate in males compared to females, but the phenotypic consequences of homozygosity are equivalent between males and females: most individuals develop PV and a minority develop ET. Of those individuals who remain *JAK2V617F*-heterozygous, some develop PV as a consequence of mechanisms other than *JAK2V617F* homozygosity, and others develop ET. (B) *JAK2V617F* homozygosity occurs at an equivalent rate in males and females but the phenotypic consequences are different: homozygosity in males is more likely to lead to PV than homozygosity in females, because of other factors that facilitate erythropoiesis in males and/or constrain erythropoiesis in females. The relative numbers of individuals with each genotype are approximations for illustrative purposes, and are not derived directly from the colony analysis.

graphic features, Poisson's regression analyses were performed for the count of *JAK2V617F*-homozygous colonies, with total *JAK2V617F*-mutant colonies as an offset. $P<0.05$ was considered significant.

Results and Discussion

In order to assess the importance of *JAK2V617F* homozygosity in hematologic phenotype, we first investigated the relationship between relative proportions of *JAK2V617F*-homozygous and heterozygous hematopoietic precursors and hematologic features in PV and ET. We used BFU-E colonies because overactive erythropoiesis is a feature of both PV and *JAK2V617F*-positive ET[1] and is important in distinguishing between the two. Moreover, the proportions of colony genotypes are similar between BFU-E and CFU-G in PV patients.[3] Colony genotypes are summarized in *Online Supplementary Table S1*. Homozygous-mutant erythroid precursors were identified in 24 of 30 patients with PV and 15 of 29 patients with ET. The ratios of homozygous-mutant to heterozygous colonies were generally higher in PV than ET, but varied significantly within each subgroup, and were reproducible for individual patients.[4]

A causal relationship between *JAK2V617F* homozygosity and PV would predict an association between a higher proportion of *JAK2V617F*-homozygous colonies and more extreme PV-like clinical features, even within a cohort of PV patients. We used Poisson's regression analyses to ask whether the relative proportion of homozygous-mutant precursors, as a proportion of all mutant precursors, was associated with hematologic features at diagnosis. This was a retrospective analysis since blood counts at diagnosis were being compared with the proportions of homozygous- and heterozygous-mutant precursors at the time of colony assays. In univariable analyses of the combined PV and ET cohort, a larger proportion of homozygous precursors were associated with higher hemoglobin, higher white cell count and lower platelet count at diagnosis (all $P<0.0001$). In a multivariable analysis, controling for diagnosis, age, gender, disease duration, presence of cytoreductive therapy and erythropoietin concentration in colony assays, an increased proportion of homozygous colonies remained significantly associated with higher hemoglobin, higher white cell count and lower platelet count at diagnosis in the whole patient group and the PV subgroup, but not in the ET subgroup (Table 1). A larger proportion of homozygous colonies were, therefore, independently associated with more extreme PV-like features at diagnosis in the whole *JAK2V617F*-positive cohort and in the PV subgroup.

We considered the possibility that expansion of homozygous clones is secondary to the abnormal blood counts in PV, for example, through erythrocytosis suppressing erythropoietin levels and selecting for *JAK2V617F*-homozygous progenitors. However, multi-

Table 1. Associations between the number of homozygous-mutant colonies (as a proportion of all mutant colonies) and full blood count parameters at diagnosis, in *JAK2V617F*-positive patients.

	Fold change in homozygous colonies (as a proportion of all mutant colonies)					
	All *JAK2V617F* patients		*JAK2V617F* PV		*JAK2V617F* ET	
Parameter	Fold change (95% CI)	*P*	Fold change (95% CI)	*P*	Fold change (95% CI)	*P*
Hb at presentation (per g/dL)	1.054 (1.011-1.100)	0.014	1.053 (1.007-1.102)	0.022	0.784 (0.576-1.066)	NS
White cell count at presentation (per 10^9/L)	1.023 (1.003-1.042)	0.023	1.021 (1.001-1.041)	0.043	0.899 (0.731-1.105)	NS
Platelet count at presentation (per 100x10^9/L)	0.932 (0.884-0.982)	0.009	0.913 (0.860-0.970)	0.003	1.090 (0.931-1.278)	NS

Multivariable Poisson's regression analysis was performed for the number of homozygous-mutant colonies, with total JAK2-mutant colonies as an offset. Fold changes refer to the increase in occurrence of homozygous colonies for each specified parameter. The analysis also controlled for diagnosis (PV or ET), age, gender, disease duration (i.e. time from diagnosis), erythropoietin concentration in the colony assay and the use of cytoreductive therapy at time of assay. NS: not significant.

Table 2. Associations between the number of homozygous-mutant colonies (as a proportion of all mutant colonies) and demographic features.

	Fold change in homozygous colonies (as a proportion of all mutant colonies)					
	All *JAK2V617F* patients		*JAK2V617F* PV		*JAK2V617F* ET	
Parameter	Fold change (95% CI)	*P*	Fold change (95% CI)	*P*	Fold change (95% CI)	*P*
Diagnosis of PV (*vs.* ET)	22.8 (11.7-44.5)	<0.0001				
Age at colony assay (per additional decade)	1.18 (1.12-1.24)	<0.0001	1.19 (1.13-1.26)	<0.0001	1.57 (1.16-2.12)	0.004
Male gender (*vs.* female)	Interaction with diagnosis[1]	0.003	1.39 (1.16-1.66)	0.0003	0.366 (0.176-0.761)	0.007
	PV: 1.37 (1.15-1.65)	0.0006				
	ET: 0.464 (0.230-0.938)	0.033				
Time from diagnosis to assay (per additional year)	Interaction with diagnosis[1]	<0.0001	0.775 (0.776-0.822)	<0.0001	1.30 (1.16-1.46)	<0.0001
	PV: 0.804 (0.781-0.828)	<0.0001				
	ET: 1.25 (1.13-1.38)	<0.0001				

Multivariable Poisson's regression analysis was performed for the number of homozygous-mutant colonies, with total JAK2-mutant colonies as an offset. Fold changes refer to the increase in occurrence of homozygous colonies for each specified parameter. The analysis also controlled for erythropoietin concentration in the colony assay and the use of cytoreductive therapy at time of assay. 1In the analysis of all JAK2V617F-positive patients, significant interactions were identified between diagnosis and gender (P=0.003) and between diagnosis and disease duration (P<0.0001). Fold changes are, therefore, shown for gender and disease duration according to diagnosis.

variable analyses showed that a higher proportion of homozygous colonies was not associated with higher hemoglobin, higher white cell count or lower platelet count at the time of colony assays (*data not shown*). A higher proportion of homozygous-mutant colonies were, therefore, associated with more extreme PV-like blood counts at diagnosis but not at the time of colony assay, consistent with the concept that *JAK2V617F* homozygosity contributes causally to a PV phenotype.

In order to assess whether other clinical features predispose to or interact with *JAK2V617F*-homozygous clones, we next used Poisson's regression analysis to test whether the number of homozygous-mutant precursors, as a proportion of all mutant precursors, was associated with demographic features or disease duration. In univariable analyses of the whole cohort, a larger proportion of homozygous colonies were associated with a diagnosis of PV (rather than ET), older age at assay, male sex and shorter time from diagnosis to assay (all $P<0.0001$). In a multivariable analysis, a larger proportion of homozygous colonies were associated with a diagnosis of PV and older age at time of assay (both $P<0.0001$, Table 2). A surprising interaction was identified between gender and diagnosis ($P=0.003$): an increased proportion of homozygous colonies were associated with male gender in PV ($P=0.0006$) but with female gender in ET ($P=0.033$). A significant interaction was also found between disease duration and diagnosis ($P<0.0001$), with an increased proportion of homozygous colonies associated with shorter disease duration in PV but with longer disease duration in ET (both $P<0.0001$). These associations were confirmed to be significant in multivariable analyses within the PV and ET subgroups (Table 2).

The association between an increased proportion of homozygous progenitors and increasing age, identified in both PV and ET, was also found in a cohort of 18 patients with JAK2 exon 12-mutated PV[4] ($P=0.024$ in multivariable analysis) and was independent of disease duration. These data raise the possibility that age-dependent changes in DNA damage[17] and/or DNA repair mechanisms[18,19] contribute to an increased rate of mitotic recombination, and thus acquisition of *JAK2* mutation homozygosity, in older patients. Alternatively, expansion of *JAK2V617F*-homozygous subclones may require additional mutations[4] which accumulate with age.

The associations of an increased proportion of homozygous-mutant colonies with shorter disease duration in PV, but with increasing disease duration in ET, are likely to reflect distinct mechanisms in the two diseases. In ET, homozygous-mutant clones are present in approximately 50% of patients and are small.[4] Our results indicate that, over time, mitotic recombination is more likely to occur and/or that an increasing proportion of ET patients develop clones above the detection threshold. By contrast, most PV patients have large homozygous-mutant clones.[4] The association of an increased proportion of homozygous-mutant colonies with shorter time from diagnosis suggests that the size of homozygous-mutant clones, relative to heterozygous clones, decreases with time. This observation does not merely reflect suppressed erythropoietin lev-

els and selection for homozygous-mutant precursors soon after diagnosis, since the association remained significant ($P<0.0001$) when we removed 3 patients with hemoglobin levels or hematocrit above the normal range at the time of colony assay, or when hemoglobin at time of assay was incorporated as a variable. Instead, we favor the possibility that hydroxycarbamide suppresses *JAK2V617F*-homozygous progenitors more than their heterozygous counterparts. This effect would be more evident in PV given the large number of homozygous-mutant colonies in most PV patients, and is consistent with evidence that hydroxycarbamide reduces granulocyte *JAK2V617F* allele burden especially in patients with PV and higher initial allele burdens.[20,21]

An association between male gender and increasing granulocyte *JAK2V617F* allele burden has been reported in PV, and was suggested to reflect gender-related differences in the frequency of mitotic recombination.[22] Our data, however, indicate that the proportion of homozygous-mutant colonies was higher in males with PV than in females with ET, and do not favor this interpretation (Figure 1A). An alternative model is that *JAK2V617F* homozygosity occurs in both sexes, but that the phenotypic consequences of homozygosity are modulated by gender, so that the occurrence of a dominant *JAK2V617F*-homozygous clone is more likely to lead to development of PV in a male than in a female (Figure 1B). This would predict an excess of males with homozygosity in PV but an excess of females with homozygosity in ET, consistent with our data. The effect of *JAK2V617F* homozygosity on erythropoiesis could be modulated by gender-specific factors including a permissive effect of androgens in men and a constraining effect of iron depletion in pre-menopausal women. Importantly, this model provides an explanation for the long-standing observations that PV is more common in men[23] whereas ET is more common in women.[24]

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