PROGENITORS HOMOZYGOUS FOR THE V617F JAK2 MUTATION OCCUR IN MOST PATIENTS WITH POLYCYTHEMIA VERA, BUT NOT ESSENTIAL THROMBOCYTHEMIA.

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ARG co-wrote the manuscript.
ABSTRACT

An acquired V617F JAK2 mutation occurs in patients with polycythemia vera (PV) or essential thrombocytemia (ET). In a proportion of V617F-positive patients, mitotic recombination produces mutation-homozygous cells that come to predominate with time. However, the prevalence of homozygosity is unclear, as previous reports studied mixed populations of wildtype, V617F-heterozygous and V617F-homozygous mutant cells. We therefore analysed 1766 individual hematopoietic colonies from 34 patients with PV or ET in whom granulocyte sequencing demonstrated that the mutant peak did not predominate. V617F-positive BFU-e were more frequent in PV patients compared to ET patients (p=0.022) and, strikingly, V617F-homozygous BFU-e were detected in all 17 PV patients, but in none of the ET patients (p<0.0001). Moreover, mutation-homozygous cells were present in two ET patients after polycythemic transformation. These results demonstrate that V617F-homozygous erythroid progenitors are present in the vast majority of patients with PV but occur rarely in those with ET.
INTRODUCTION

An acquired V617F mutation in the tyrosine kinase JAK2 occurs in most patients with polycythemia vera (PV) and in half of those with essential thrombocythemia (ET) or idiopathic myelofibrosis\(^1-5\). This mutation increases JAK2 kinase activity\(^2-5\), confers cytokine independence on cell-lines\(^2-5\), is required for erythropoietin (Epo)-independent erythroid progenitor growth in affected patients\(^1,3\), and produces erythrocytosis in a retroviral bone marrow transplant model\(^3\). JAK2 mutation status divides ET patients into two distinct subgroups, with V617F-positive patients resembling a *forme fruste* of PV\(^6\). These data have led to the suggestion that V617F-positive ET and PV form a disease continuum, with the extent of erythrocytosis influenced by a combination of genetic and physiological modifiers\(^6\).

Homozygosity for the V617F mutation occurs in 30% of patients with PV\(^1,5,7\), but is rare in patients with ET, suggesting that it may favor a polycythemic phenotype. These and other studies\(^8-10\) have inferred the presence of V617F-homozygosity from sequence analysis of leukocyte DNA showing predominance of the mutant peak. However, patients with PV or ET in whom the mutant peak does not predominate may nonetheless harbor a subpopulation of V617F-homozygous cells. To investigate this possibility, we assessed the JAK2 mutation status of individual hematopoietic colonies from V617F-positive patients with PV or ET.
MATERIALS & METHODS

Mutation Screening. Studies were approved by the Addenbrooke’s NHS Trust Local Research Ethics Committee. Approved by MREC (Multi-Regional Ethics Committee), UK. All patients gave verbal and written informed consent, and research was carried out according to the principles of the Declaration of Helsinki. Patient diagnoses were according to the modified PVSG criteria for PV\textsuperscript{11} and ET\textsuperscript{12}. The clinical and biological features of all patients have been listed in Supplemental Table 1. Patient WBC preparation and JAK2 sequence analysis were performed as described\textsuperscript{1}. Sequence patterns were scored as V617F-heterozygous if the mutant peak was $\leq 50\%$ total peak height. Peripheral blood burst forming unit-erythroid (BFU-e), Epo-independent erythroid colonies (EECs) and colony forming unit-granulocyte/macrophage (CFU-GM) were genotyped by Bsa\textsubscript{XI} digestion\textsuperscript{1,13}. Bsa\textsubscript{XI} digestion of JAK2 exon 14 was confirmed by digestion of a control (SCL) fragment and/or by sequencing of colony DNA in all 700 colonies so analyzed.

Statistical Methods. Frequencies of V617F-positive and V617F-homozygous colonies in ET and PV patients were compared using an unpaired Student’s t-test. Frequencies of V617F-homozygous colonies were compared in V617F-positive ET patients that had not transformed and those that had transformed to PV using the Fisher’s exact test. To assess the relationship between clinical/laboratory characteristics and the proportion of either V617F-homozygous BFU-e or V617F-positive BFU-e, logistic regression models were fitted, and significance tested using likelihood ratio tests.
RESULTS & DISCUSSION

We identified five patients with PV and a V617F-homozygous sequence pattern in their granulocytes (mutant peak >50% total peak height) for whom an earlier granulocyte DNA sample was available. In 3 cases, the earlier samples (taken 2, 5 or 6 years previously) had a heterozygous pattern (Figure 1A). These results demonstrate that V617F-homozygous clones may predominate with time, and are consistent with the concept that patients with an apparently V617F-heterozygous sequence pattern may harbor a subpopulation of V617F-homozygous cells.

We therefore analysed 1082 hematopoietic colonies from seventeen PV patients with a heterozygous sequence pattern recorded in their peripheral blood granulocytes in the preceding six months. These were assessed by BsaXI digestion\(^1,6,13\), which distinguishes between wildtype, V617F-heterozygous and V617F-homozygous genotypes (Figure 1B). As expected, BFU-e from a patient with a homozygous granulocyte sequence pattern were predominantly V617F-homozygous (Figure 1C, patient 19). However, V617F-homozygous BFU-e were also present in all patients with a heterozygous granulocyte sequence (patients 1-17). To exclude the possibility that the BsaXI site was destroyed by mutations other than the 1849G>T (V617F) mutation, exon 14 was sequenced in colonies with a restriction pattern consistent with a V617F-homozygous genotype. In 48 colonies from 8 PV patients, the 1849G>T mutation was detected in both JAK2 alleles. The frequencies of V617F-homozygous or total V617F-positive BFU-e did not correlate significantly with age, gender, disease duration or treatment, or with hemoglobin levels, white cell counts or platelet counts either at diagnosis or the time of analysis (p>0.5 for all variables). All patients analysed within three months of diagnosis had V617F-homozygous BFU-e (Figure 1C; patients 2-4, 9, 11), suggesting
that homozygosity occurs at an early stage of the clinically overt disease. As the V617F mutation is detectable as a homozygous or heterozygous granulocyte sequence trace in 80% of PV patients\textsuperscript{1-5}, our demonstration that PV patients with a heterozygous sequence trace do harbor V617F-homozygous progenitors suggests that these cells are present in the vast majority of all PV patients. However, a small minority of patients have a normal granulocyte JAK2 sequence but are V617F-positive by PCR-based methods\textsuperscript{1}. In one such patient, 3% of BFU-e were V617F-positive (Figure 1C, patient 18); the frequency of V617F-homozygous colonies may have therefore been below the level of detection, although it is possible that occasional PV patients lack V617F-homozygous cells.

All 316 erythropoietin-independent erythroid colonies (EECs) from fifteen of the PV patients with a V617F-heterozygous granulocyte sequence pattern had at least one mutant JAK2 allele, in agreement with previous observations\textsuperscript{1}. There was no consistent relationship between the proportion of mutation-positive colonies that were homozygous when cultured in the presence or absence of saturating Epo concentrations (116/289 versus 130/270 colonies, p=0.4; Supplemental Figure 1A). Ninety-six CFU-GM colonies from six PV patients were also analysed (Supplemental Figure 1B). Mutation-homozygous colonies were identified in five cases, demonstrating that the mutational event responsible for V617F homozygosity occurs at or before the common myeloid progenitor stage. In each patient, the proportions of CFU-GM and BFU-e that were V617F-positive were comparable, as were the proportions of CFU-GM and BFU-e that were V617F-homozygous, suggesting that this mutation does not strongly bias commitment towards the erythroid lineage.
To investigate whether ET patients also have a subpopulation of V617F-homozygous progenitors, we analysed 684 BFU-e from V617F-positive ET patients (Figure 2A, patients 20-36). V617F-heterozygous BFU-e were identified in all patients. The proportion of V617F-positive progenitors did not correlate significantly with age, gender or disease duration (p>0.5 for all variables). Eleven patients treated with hydroxyurea had a trend towards reduced numbers of V617F-positive BFU-e (p=0.09), consistent with the observation that, compared to V617F-negative ET patients, blood counts of V617F-positive ET patients are particularly sensitive to hydroxyurea\(^6\). The proportion of V617F-positive BFU-e was significantly lower in ET patients than in PV patients (30.6±27.1% versus 52.5±26.6% respectively; p=0.02), an observation that did not reflect a shorter time from diagnosis (median disease durations of 59 months and 18 months, respectively). Moreover, whereas homozygous-mutant BFU-e were found in all 17 PV patients, none were detected in any of the 17 ET patients (p<0.0001). In PV patients, 129/335 V617F-positive BFU-e were mutation-homozygous compared to 0/248 in ET patients (p<0.0001). Furthermore, 0/76 EECs in 3 ET cases (patients 30, 33 and 34) were V617F-homozygous, compared to 130/270 EECs from the PV patients (p<0.0001). These data demonstrate that, in contrast to PV patients, ET patients rarely harbor V617F-homozygous erythroid progenitors, although the existence of rare ET patients with mutation-homozygous progenitors cannot be excluded.

There are at least three possible explanations for these striking results. Firstly, these PV patients are likely to have lower serum erythropoietin levels than the ET patients, and V617F-homozygous BFU-e may have a selective advantage in a low erythropoietin environment. However, for individual PV patients, the fractions of V617F-homozygous BFU-e and CFU-GM were similar, suggesting that selection for homozygosity occurred...
in multipotent progenitors and was therefore unlikely to be dependent on the erythropoietin receptor, which is first expressed at the BFU-e stage of differentiation\textsuperscript{14}. Secondly, compared to ET patients, PV patients may have a longer pre-diagnosis phase of their disease, allowing more time for homozygosity to occur. Thirdly, mitotic recombination involving the \textit{JAK2} locus may occur more frequently in patients with PV.

It is recognised that patients with ET may infrequently transform to PV\textsuperscript{15,16}. We therefore studied two patients who presented with V617F-positive ET and no evidence of erythrocytosis, but who developed PV 36 or 147 months after their initial diagnosis. V617F-homozygous BFU-e and EECs were present in both patients after polycytemic transformation (\textit{p}=0.0058; Figure 2B). Colony data from the time of their presentation with ET are not available, so we cannot exclude the possibility that these patients had V617F-homozygous colonies prior to transformation. However, taken together with the absence of detectable V617F-homozygous BFU-e in all ET patients, our results raise the possibility that, in some patients, duplication of the mutant \textit{JAK2} allele with loss of the normal allele may be associated with a phenotypic change from thrombocytemia to polycytemia.

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REFERENCES


Figure 1. Frequency of V617F homozygosity in patients with PV.
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(A) JAK2 sequence analysis of granulocyte DNA samples from a patient with PV. Note the V617F-heterozygous sequence pattern in a sample from 1992, and a V617F-homozygous pattern in a sample from 1998. (B) BsaXI digestion of JAK2 exon 14 and SCL intron 1 was used to genotype DNA from BFU-e, EEC and CFU-GM cultured from PV patient 13. Lanes 1-4, BFU-e; lanes 5-8, EEC; lanes 9-12, CFU-GM; lane 13, undigested granulocyte DNA from a V617F-negative control; lane 14, BsaXI-digested granulocyte DNA from a V617F-negative control; lane 15: water. V617F-homozygous colonies are seen in lanes 1, 6, 7 and 11, heterozygous colonies in lanes 3, 5, 8, 9 and 12. (C) Frequencies of wildtype, V617F-heterozygous and V617F-homozygous peripheral blood BFU-e in 17 PV patients with a heterozygous JAK2 granulocyte sequence trace were determined using PCR and BsaXI digestion. The absolute number of colonies genotyped from each patient is at the top of each column. Column 18, V617F-positive PV patient with a wildtype sequence trace; column 19, PV patient with a V617F-homozygous sequence trace.
Figure 2. Frequency of V617F homozygosity in patients with ET.
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(A) Frequencies of wildtype, V617F-heterozygous and V617F-homozygous BFU-e in the peripheral blood of 17 ET patients were determined using PCR and BsaXI digestion. The number of colonies genotyped from each patient is at the top of each column. (B) BsaXI digestion of JAK2 exon 14 and SCL intron 1 was used to genotype DNA from peripheral blood BFU-e cultured from two ET patients following transformation to PV (patients 37 and 38). Lanes 1-10, individual colonies; lane 11, undigested granulocyte DNA from a V617F-negative control; lane 14, BsaXI-digested granulocyte DNA from a V617F-negative control; lane 15: water.
Progenitors homozygous for the V617F JAK2 mutation occur in most patients with polycythemia vera, but not essential thrombocytemia

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