Clonal hematopoiesis in familial polycythemia vera suggests the involvement of multiple mutational events in the early pathogenesis of the disease

Robert Kralovics,1 David W. Stockton,2,3,4 and Josef T. Prchal3

1Department of Research, Experimental Hematology, Basel University Hospital, 4031 Basel, Switzerland and the Departments of 2Molecular and Human Genetics, 3Medicine, and 4Ophthalmology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030.

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Corresponding author: Josef T. Prchal, M.D., Department of Medicine, Baylor College of Medicine, One Baylor Plaza 802E, Houston, TX 77030, tel. 1-713-798-7520, fax. 1-713-798-6132, e-mail: jprchal@bcm.tmc.edu. The work was supported by the MPD Foundation.
ABSTRACT

Familial clustering of malignancies provides a unique opportunity to identify molecular causes of cancer. Polycythemia vera (PV) is a myeloproliferative disorder due to an unknown somatic stem cell defect that leads to clonal myeloid hyperproliferation. We studied six families with PV. The familial predisposition to PV appears to follow an autosomal dominant inheritance pattern with incomplete penetrance. All examined females informative for a transcriptional clonality assay had clonal hematopoiesis. We excluded linkage between PV and a number of previously proposed candidate disease loci (c-mpl, EPOR, 20q, 13q, 5q, 9p). Therefore, mutations at these loci are unlikely primary causes of familial PV. The finding of erythropoietin-independent erythroid progenitors in normal family members indicated the presence of the PV stem cell clone in their hematopoiesis. This finding, together with clonal hematopoiesis in the affected individuals, supports the hypothesis of multiple genetic defects involved in the early pathogenesis of PV.

jprchal@bcm.tmc.edu
Introduction

Sporadic myeloproliferative disorders (MPD) are due to an acquired mutation of a single hematopoietic cell resulting in clonal circulating myeloid progeny. Accumulation of erythrocytes is a hallmark of polycythemia vera (PV) while the accumulation of platelets, neutrophils, basophils, and eosinophils is variable. The molecular lesion responsible for PV is unknown. The erythroid progenitors in PV form erythropoietin-independent erythroid colonies (EEC) in clonogenic cultures in the absence of exogenous erythropoietin (Epo). This unique feature permits the distinction of PV from other primary polycythemias with or without a family history. The EEC assay also identifies an early PV stage that lacks the full PV phenotype and allows differentiation of PV in those patients initially presenting with thrombocytosis from those individuals having essential thrombocythemia. Familial clustering of polycythemia is seen in congenital polycythemic states including primary familial and congenital polycythemia, Chuvash polycythemia, high oxygen affinity globin mutants, and BPG mutase deficiency. However, few case reports of familial occurrence of PV have been reported.

Patients, Materials and Methods

All affected family members had classical diagnosis of PV based on the PV Study Group criteria. All studies were performed under approved Institutional Review Board protocols (Baylor College of Medicine and Univ. Alabama at Birmingham) and all subjects included in this study provided written consent to perform DNA and cell culture studies on their blood samples.
Detailed description of the EEC assay, cell isolations, clonality analysis, LOH detection, and linkage analysis are listed elsewhere. Microsatellite PCR for the EPOR and c-mpl genes were done as previously described.

**Results and discussion**

We studied six Caucasian families of heterogeneous ethnic background, each with multiple members with PV (Figure 1a). We detected EEC in peripheral blood cultures in all the affected family members in all families. The clinical findings are summarized in Table 1; these results of clinical findings represent all of the available patients and these were not pre-selected. In addition to the affected members with the full PV phenotype, we tested family members without PV for the presence of EEC in their peripheral blood. We identified subjects in families A and B who had no clinical signs or symptoms of PV but EEC were present in their peripheral blood (subjects A-4, A-6, A-9, A-14, A17, B-1). Clonality was demonstrated in the informative females with full PV phenotype (Table 1, Figure 1b). The members with partial phenotype (EEC only) were polyclonal indicating the contribution of normal stem cells to productive hematopoiesis, and therefore, lack of clinical symptoms of PV. To examine whether the presence of EEC is due to a somatic mutation or an inherited mutant gene, we isolated the Epo-independent erythroid cell population and analyzed its clonality (this sample consisted of approximately 500-1000 BFUE's harvested from 10 methylcellulose plates followed by MACS sorting for glycophorin A. Each BFUE consists of approximately 800-2000 proerythroblasts). We detected clonal EEC in one informative female who had otherwise polyclonal hematopoiesis; please note that the T-cells X-chromosome allelic usage appears skewed; however, the observed skewing is within the range which we reported in normal hematopoietic progeny (Figure 1b).
The inheritance pattern of familial PV is compatible with an autosomal dominant trait with decreased penetrance. If the finding of EEC is considered as an early sign of the PV phenotype, the penetrance increases. Four out of the six families (A-D) were used for linkage analysis since these consisted of at least two affected siblings. It is possible that the clustering of PV in family E and F could be by chance only and that we could have a selection bias to recruit these families since families of unusual polycythemic disorders have been referred to us for over a decade. For these pedigrees, we calculated the power to detect linkage using the SIMLINK software\textsuperscript{17} which predicted a maximum LOD score of 4.4 for the given pedigree structures. The simulated LOD scores further increased to 5.2 when the subjects positive for EEC were considered as affected (data not shown). Using these families, we could examine a number of candidate loci that were previously proposed to play a role in MPD, or PV in particular. We analyzed the linkage between the PV phenotype and the commonly deleted regions on chromosomes 20q, 13q, 5q that were found as genetic aberrations in PV.\textsuperscript{18,19} In addition, the thrombopoietin receptor (\textit{c-mpl}) and Epo receptor (\textit{EPOR}) genes have been proposed in the pathogenesis of myeloproliferative disorders including PV.\textsuperscript{20,21} Recently, we reported the presence of loss of heterozygosity (LOH) involving chromosome 9p as the most common clonal defect in sporadic PV.\textsuperscript{12} We detected LOH on chromosome 9p in subjects A01 and B02 (Table 1). We performed linkage analysis using microsatellite markers mapping to these loci. Only the affected subjects with the full PV phenotype were considered “affected” in the linkage analysis. Since LOD scores below \textasciitilde2.0 are considered exclusion of linkage, we could exclude linkage between the PV phenotype and all the tested loci. LOD scores for the \textit{EPOR} and \textit{c-mpl} genes were \textasciitilde3.16 and \textasciitilde2.24, respectively. The commonly deleted regions found in sporadic MPD were also convincingly excluded with LOD scores \textasciitilde4.40 for 20q, \textasciitilde4.79 for 13q, and \textasciitilde6.27 for 5q. We also fully excluded chromosome 9p region of LOH. These results suggest that the frequently observed somatic mutations in MPD
involving the chromosomal regions on 20q, 13q, 5q, and 9p are secondary genetic changes and do not target the primary PV locus.

The clinical analysis of the affected family members confirmed that they are phenotypically identical to sporadic PV. Familial occurrence of PV provides a unique insight into the stages of PV since we could identify affected members in an early stage of the disease using the EEC assay. This is not possible in sporadic PV since individuals are only identified when symptomatic. Thrombocythemia was shown to be the first abnormality seen in some PV subjects and interestingly in families C and D, thrombocythemia occurred prior to elevation of hematocrit. In all the affected informative females, we observed clonal circulating myeloid cells as seen in sporadic PV; in some clonality could not be determined because a) patients were genotyped for exonic polymorphisms of active X-chromosome genes used for the clonality assays and were not informative, b) failure to get informed consent for this follow-up study, or c) patients were no longer available or willing to participate in our study. Clonal hematopoiesis is a marker of fully developed PV. In families A and B, we observed hematologically normal subjects with EEC present in their peripheral blood. In one of these cases we proved the clonal origin of the Epo-independent cells but the rest of the myeloid cells were polyclonal. Thus it is possible that in pre-symptomatic PV, within polyclonal hematopoiesis the PV stem cell clone may be present but its contribution to blood production is limited. At this stage, the progeny of the PV clone can be detectable by the EEC assay. In the symptomatic stage, the PV clone loses regulation and expands and clonal hematopoiesis appears. It remains to be established if in the individuals with PV who present with thrombocytosis prior to elevation of hematocrit, thrombocytosis precedes (such as seen in subject C02 and in one female with sporadic PV we studied previously) or follows establishment of clonal circulating hematopoietic progeny.
The finding of clonal hematopoiesis suggests that acquired mutations are part of the disease etiology of familial PV. The presence of both inherited and acquired mutations in familial PV allows several interpretations of the disease etiology. As seen in other familial predispositions to cancer (such as retinoblastoma), a mutant non-functional copy of the gene is inherited in the families followed by an acquired mutation of the remaining wild type allele. The disease initiates after both alleles of the gene are mutated (mutation of one allele being inherited and the mutation of the second allele being acquired). Thus in families with PV predisposition, PV phenotype will be expected to develop at the earlier age than that seen in sporadic PV. This has indeed been a case in families B, E, and F. It is possible that the stem cell clone established in this initial stage undergoes further mutagenesis resulting in acceleration of clonal expansion. An alternative interpretation assumes mutations in two or more genes, mutation of one gene being inherited and mutation(s) of other gene(s) being acquired. However, if more than one gene can contribute to the development of a final PV phenotype this will make PV a genetically heterogeneous disorder and some of the positional cloning data interpretation would not be valid. Loss of gene function is a necessary component of the first model, whereas in the second model, gain of function mutations may also be present together with loss of function mutations. In both models, the acquired mutations are responsible for the presence of clonal hematopoiesis. The presence of incomplete penetrance, observed in the families, is compatible with both models.

The chromosomal localization of the “primary PV mutation” remains unknown. The PV phenotype did not show linkage to any of the loci implicated in PV to date. Families with multiple members with PV should prove fundamental in identification of the PV predisposition gene as they offer the possibility for genome-wide linkage analysis and positional cloning.
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References


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Figure legends

Figure 1

A. Pedigrees of six families with polycythemia vera (PV). Affected individuals are indicated with black. In families A and B, black points indicate hematologically normal family members with Epo-independent erythroid cell detectable in their peripheral blood cultures. B. Clonality analysis of individuals A01 and A06 using the *IDS* gene exonic polymorphism. RNA isolated from T lymphocytes (T), granulocytes (GRA), platelets (PLT), and Epo-independent erythroid cells (EEC) were used for the analysis. The presence of only one expressed allele of the IDS gene in platelets, granulocytes, and Epo-independent erythroid cells is consistent with clonal origin of cells. C. Exclusion of linkage between chromosome 9p microsatellite markers and PV. LOD scores below –2.0 satisfy the criteria of exclusion of linkage.
Table 1. Summary of clinical findings.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Diagnosis</th>
<th>Sex</th>
<th>Age at diagnosis</th>
<th>Hct (%)</th>
<th>Hgb (g/dl)</th>
<th>PLT (K/μl)</th>
<th>Spleno-megaly</th>
<th>Clonality</th>
<th>EEC</th>
<th>Karyotype assay</th>
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<td>58</td>
<td>55</td>
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<td>64</td>
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<tr>
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<td>F</td>
<td>-</td>
<td>42.2</td>
<td>14.7</td>
<td>310</td>
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<td>F</td>
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<td>46.7</td>
<td>16.2</td>
<td>204</td>
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<tr>
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<td>168</td>
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(CL – clonal, PO – polyclonal, EEC – Epo-independent colonies, nd – not determined)
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