

Brief report

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

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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 6 families with PV. The familial predisposition to PV appears to follow an autosomal dominant inheritance pattern with incomplete pen-

etrance. 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 healthy 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. (Blood. 2003;102:3793-3796)

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Introduction

Sporadic myeloproliferative disorders (MPDs) are due to an acquired mutation of a single hematopoietic cell resulting in clonal circulating myeloid progeny.¹ 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 (EECs) in clonogenic cultures in the absence of exogenous erythropoietin (Epo).² This unique feature permits the distinction of PV from other primary polycythemias with or without a family history.^{3,4} 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.⁵ Familial clustering of polycythemia is seen in congenital polycythemic states including primary familial and congenital polycythemia, Chuvash polycythemia, high oxygen-affinity globin mutants, and biphospho-glycerate mutase deficiency.⁶ However, few case reports of familial occurrence of PV have been reported.⁷⁻¹⁰

elsewhere.^{12,13} Microsatellite polymerase chain reaction for the *EPOR* and *c-mpl* genes was done as previously described.^{14,15}

Results and discussion

We studied 6 white families of heterogeneous ethnic background, each with multiple members with PV (Figure 1A). We detected EECs 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 preselected. In addition to the affected members with the full PV phenotype, we tested family members without PV for the presence of EECs in their peripheral blood. We identified subjects in families A and B who had no clinical signs or symptoms of PV but EECs were present in their peripheral blood (subjects A04, A06, A09, A14, A17, B01). Clonality was demonstrated in the informative females with full PV phenotype (Table 1; Figure 1B). The members with partial phenotype (EECs 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 EECs 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 blast-forming units-erythroid [BFUEs] harvested from 10 methylcellulose plates followed by magnetic-activated cell sorting for glycophorin A. Each BFUE consists of approximately 800-2000 proerythroblasts). We detected clonal EECs in one informative female who had otherwise polyclonal

Study design

All affected family members had classical diagnosis of PV based on the PV Study Group criteria.¹¹ All studies were performed under approved institutional review board protocols (Baylor College of Medicine and University of 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, loss of heterozygosity (LOH) detection, and linkage analysis are listed

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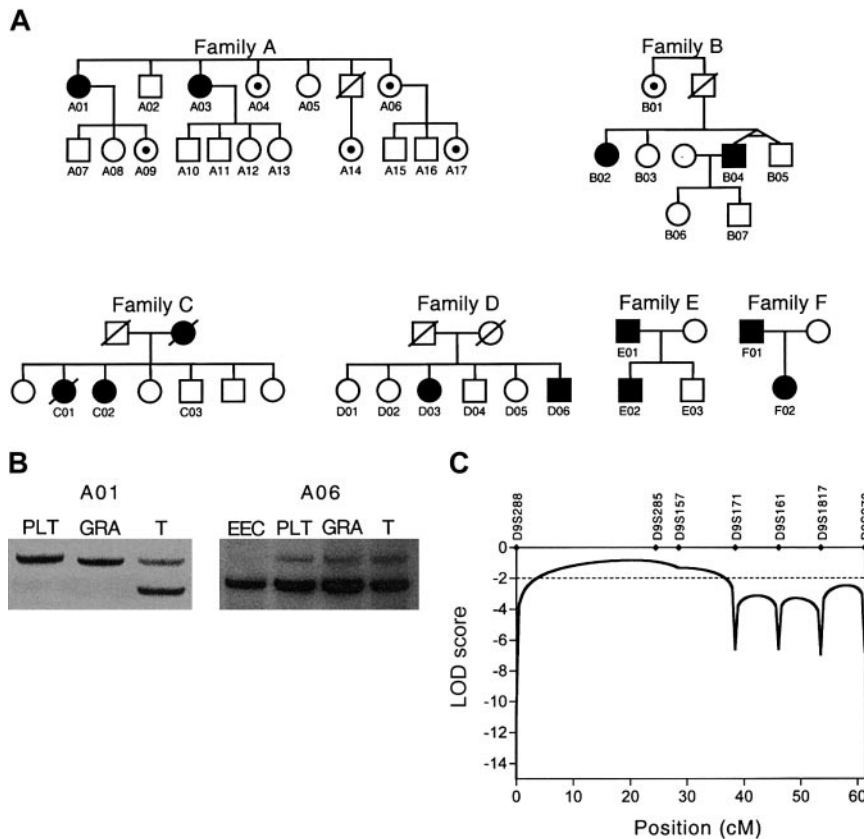
Submitted April 7, 2003; accepted May 9, 2003. Prepublished online as *Blood* First Edition Paper, June 26, 2003; DOI 10.1182/blood-2003-03-0885.

Supported by the Myeloproliferative Disease Foundation (MPD Foundation).

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hematopoiesis; please note that the T cells' X-chromosome allelic usage appears skewed; however, the observed skewing is within the range that we reported in normal hematopoietic progeny (Figure 1B).¹⁶

The inheritance pattern of familial PV is compatible with an autosomal dominant trait with decreased penetrance. If the finding of EECs is considered as an early sign of the PV phenotype, the penetrance increases. Of the 6 families, 4 (A-D) were used for linkage analysis since these consisted of at least 2 affected siblings. It is possible that the clustering of PV in families E and F could be by chance only and that we could have had a selection bias to recruit these families since families of unusual polycythemic

disorders have been referred to us for more than a decade. For these pedigrees, we calculated the power to detect linkage using the SIMLINK software (<http://www.sph.umich.edu/group/statgen/software>),¹⁷ which predicted a maximum logarithm of odds ratio (LOD) score of 4.4 for the given pedigree structures. The simulated LOD score further increased to 5.2 when the subjects positive for EECs 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, and 5q that were found as genetic aberrations in PV.^{18,19} In addition, the thrombopoietin

Table 1. Summary of clinical findings

Subject	Diagnosis	Sex	Age at diagnosis, y	Hct	Hgb, g/L	PLT, K/ μ L	Splenomegaly	Karyotype	Clonality assay	EEC
A01	PV	F	58	.55	180	443	+	9pLOH	CL	Present
A03	PV	F	62	.64	197	190	+	Normal	CL	Present
A04	Normal	F	—	.422	147	310	—	ND	ND	Present
A06	Normal	F	—	.467	162	204	—	ND	PO, EEC-CL	Present
B02	PV	F	31	.457	127	438	—	9pLOH	CL	Present
B04	PV	M	33	.484	162	284	—	Normal	ND	Present
C01	PV	F	80	.542	177	710	ND	ND	ND	ND
C02	PV	F	72	.409	136	2430	—	ND	CL	Present
C03	PV	F	66	.359	120	1995	+	Normal	ND	Present
D03	PV	F	29	.46	162	1190	—	ND	ND	ND
D06	PV	M	48	.55	195	910	—	Normal	ND	ND
E01	PV	M	25	.573	197	729	+	Normal	ND	Present
E02	PV	M	5	.61	196	614	+	ND	ND	Present
F01	PV	M	52	.566	182	65	—	ND	ND	Present
F02	PV	F	30	.50	164	168	—	Normal	CL	Present

Hct indicates hematocrit; Hgb, hemoglobin; CL, clonal; —, not present; ND, not determined; PO, polyclonal; and EEC, Epo-independent colonies.

receptor (*c-mpl*) and Epo receptor (*EPOR*) genes have been proposed in the pathogenesis of myeloproliferative disorders including PV.^{20,21} Recently, we reported the presence of LOH involving chromosome 9p as the most common clonal defect in sporadic PV.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 less than -2.0 are considered exclusion of linkage, we could exclude linkage between the PV phenotype and all the tested loci. LOD scores for the *EPOR* and *c-mpl* genes were -3.16 and -2.24 , respectively. The commonly deleted regions found in sporadic MPD were also convincingly excluded with LOD scores of -4.40 for 20q, -4.79 for 13q, and -6.27 for 5q. We also fully excluded the chromosome 9p region of LOH (Figure 1C). 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 identified only when symptomatic. Thrombocytopenia was shown to be the first abnormality seen in some PV subjects,⁵ and interestingly in families C and D, thrombocytopenia 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 (1) patients were genotyped for exonic polymorphisms of active X-chromosome genes used for the clonality assays and were not informative, (2) there was failure to get informed consent for this follow-up study, or (3) patients were no longer available or willing to participate in our study.⁴ Clonal hematopoiesis is a marker of fully developed PV. In families A and B, we observed hematologically normal subjects with EECs 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 presymptomatic 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 thrombocytopenia prior to elevation of hematocrit, thrombocytopenia 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 nonfunctional 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 an earlier age than that seen in sporadic PV. This has indeed been the 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 2 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.

Acknowledgments

We thank Dr Hanneke Kluijn-Nelemans and Dr Rangaswamy Govindarajan for providing blood samples and Valerie Irvin and Yongli Guan for their technical assistance.

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