Two Clinical Phenotypes in Polycythemia Vera

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Abstract

BACKGROUND—Polycythemia vera is the ultimate phenotypic consequence of the V617F mutation in Janus kinase 2 (encoded by *JAK2*), but the extent to which this mutation influences the behavior of the involved CD34+ hematopoietic stem cells is unknown.

METHODS—We analyzed gene expression in CD34+ peripheral-blood cells from 19 patients with polycythemia vera, using oligonucleotide microarray technology after correcting for potential confounding by sex, since the phenotypic features of the disease differ between men and women.

RESULTS—Men with polycythemia vera had twice as many up-regulated or down-regulated genes as women with polycythemia vera, in a comparison of gene expression in the patients and in healthy persons of the same sex, but there were 102 genes with differential regulation that was concordant in men and women. When these genes were used for class discovery by means of unsupervised hierarchical clustering, the 19 patients could be divided into two groups that did not differ significantly with respect to age, neutrophil *JAK2* V617F allele burden, white-cell count, platelet count, or clonal dominance. However, they did differ significantly with respect to disease duration; hemoglobin level; frequency of thromboembolic events, palpable splenomegaly, and splenectomy; chemotherapy exposure; leukemic transformation; and survival. The unsupervised clustering was confirmed by a supervised approach with the use of a top-scoring-pair classifier that segregated the 19 patients into the same two phenotypic groups with 100% accuracy.

CONCLUSIONS—Removing sex as a potential confounder, we identified an accurate molecular method for classifying patients with polycythemia vera according to disease behavior, independently of their *JAK2* V617F allele burden, and identified previously unrecognized molecular pathways in polycythemia vera outside the canonical JAK2 pathway that may be amenable to targeted therapy.

Polycythemia vera is a clonal stem-cell disorder characterized by unregulated production of red cells, white cells, and platelets and complicated by extramedullary hematopoiesis, myelofibrosis, and acute leukemia. An explanation for this phenotype was provided by the discovery of an activating mutation (V617F) in Janus kinase 2 (encoded by *JAK2*).[1] However, the same mutation occurs in essential thrombocytosis and primary myelofibrosis, which are diseases with overlapping phenotypes but distinctly different natural histories. Although it is undisputed that *JAK2* V617F can produce a myeloproliferative phenotype, the *JAK2* V617F allele burden cannot be the sole explanation for the pathogenesis of these three different diseases, since it overlaps substantially among them.[2] Several lines of evidence suggest that additional genetic and epi-genetic factors are involved. For example, in polycythemia vera and essential thrombocytosis, gene expression in CD34+ bone marrow cells does not differ between *JAK2* V617F–positive and *JAK2* V617F–negative patients,[3,4] and clonal granulocytes in patients with polycythemia vera do not always express *JAK2* V617F.[5] Furthermore, *JAK2* V617F expression, regardless of the allele burden, has been shown not to influence signal transduction in circulating CD34+ cells in patients with polycythemia vera.[6] Finally, polycythemia vera is more common among women,[7] in whom it appears earlier and is associated with higher frequencies of splenomegaly,[8] masked erythrocytosis,[9] and hepatic-vein thrombosis[10] and a lower neutrophil *JAK2* V617F allele burden than in men.[2]

To further define the molecular abnormalities in polycythemia vera at the stem-cell level, we examined gene expression in circulating CD34+ cells from 19 *JAK2* V617F–positive patients with polycythemia vera, controlling for sex as a possible confounder.

Methods

Patients and Controls and Study Oversight

The study protocol was approved by the institutional review board of the Johns Hopkins University School of Medicine, and written informed consent was obtained from each patient, in accordance with the Declaration of Helsinki. The diagnosis of polycythemia vera was based on Polycythemia Vera Study Group criteria.[11] Enrollment of patients was predicated solely on obtaining sufficient CD34+ peripheral-blood cells for analysis. Clinical data were extracted from the patients' medical records at the time of study entry and termination. For controls, granulocyte colony-stimulating factor–mobilized CD34+ cells from three healthy men and three healthy women were obtained from commercial sources (AllCell Technologies, Chicago, and STEMCELL Technologies, Vancouver, BC, Canada). The study was initiated in 2003 and terminated in 2012. There was no commercial support for this study. The authors designed and executed the study, analyzed the data, wrote the manuscript and made the decision to submit it for publication, and vouch for the accuracy and completeness of the data and the analysis. No one who is not an author contributed to the writing of this article.

Laboratory Analyses

Neutrophil isolation and DNA preparation were performed as described elsewhere.[12] *JAK2* V617F analysis was performed with the use of an allele-specific, quantitative real-time

polymerase-chain-reaction (PCR) assay with a lower limit of detection of 5% of either the nonmutant or mutant allele.[12] Our methods of CD34+ cell isolation and RNA preparation are described in the Supplementary Appendix (available with the full text of this article at NEJM.org). Oligonucleotide microarray analysis was performed with the use of the Affymetrix U133A chip (for details, see the Supplementary Appendix). The microarray data were deposited in the Gene Expression Omnibus MIAME (minimum information about a microarray experiment)–compliant database (www.ncbi.nlm.nih.gov/geo) under the accession number GSE47018.

Results

Patients

The clinical features of the 19 patients are listed in Table 1, and in Table S1 in the Supplementary Appendix. The median age and disease duration did not differ significantly between the men and women. All patients had *JAK2* V617F expression, and the median neutrophil allele burdens were also similar in the men and women (94% and 100%, respectively); in 13 patients, the median CD34+ cell *JAK2* V617F allele burden was 82% (range, 50 to 100) (data not shown), which indicated clonal dominance at both the progenitor-cell and neutrophil levels.[13] The two groups differed significantly only with respect to their platelet counts, with men having a lower median platelet count than women (421,000 vs. 948,000 per cubic millimeter; P = 0.02).

Gene Expression in Male and Female Patients

Given the differences in disease behavior between men and women with polycythemia vera, we hypothesized that there may be sex-specific differences in gene expression that are independent of *JAK2* V617F expression. Therefore, we compared gene expression in the patients with that in controls of the corresponding sex and found that there was differential gene expression in female patients as compared with male patients, with 235 genes differentially regulated (126 up-regulated and 109 down-regulated) in the women, versus 571 genes differentially regulated (486 up-regulated and 85 down-regulated) in the men (Tables S2A and S2B in the Supplementary Appendix). Despite the fact that female patients had a smaller number of deregulated genes, a Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis revealed that more than three times as many molecular pathways were activated in the female patients, including one — the pentose phosphate pathway — that was not activated in any male patient (Fig. S1A and S1B in the Supplementary Appendix).

Identification of Concordantly Deregulated Genes in Male and Female Patients

The subtraction of genes with sex-specific expression left 102 genes (68 up-regulated and 34 down-regulated) with differential expression that was concordant in men and women (Fig. S2 in the Supplementary Appendix) and that was a potential core set of genes involved in the pathogenesis of polycythemia vera. We validated gene expression for 9 of the 102 genes by means of a quantitative reverse-transcriptase–PCR (RT-PCR) assay (Table S3 in the Supplementary Appendix), using CD34+ cells from a subset of the patients, and found that there was a close correlation between the observed microarray gene-expression changes and the quantitative RT-PCR measurements. (Fig. S3 in the Supplementary Appendix)

Annotation of the Concordantly Deregulated Genes

Annotation of the 102 genes is provided in Table S4 in the Supplementary Appendix. There was differential regulation of the stem-cell maintenance genes *HES1*, *HOXA9*, *PTGER4*, and *NR4A2*; the master transcription factor *SOX4*; and the oncogenes *SETBP1* and *MIR21*. Eight antiapoptotic genes (*LEPR*, *CKAP4*, *RRAS2*, *TIMP1*, *IER3*, *THBS1*, *POSTN*, and *LGALS3*) were up-regulated, and six proapoptotic genes (*EIF5A*, *EMP1*, *ZFP36L2*, *LUC7L3*, *HLF*, and *HOPX*) and three tumor-suppressor genes (*SSBP2*, *TLE4*, and *KLF6*) were down-regulated.

A total of 16 extracellular-matrix genes, including 6 collagen genes (*COL1A1*, *COL1A2*, *COL3A1*, *COL4A1*, *COL4A2*, and *COL6A3*) and the matricellular genes *SPARC*, *POSTN*, *TIMP1*, *THBS1*, *HPSE*, *FN1*, *S100A9*, *EFEMP1*, *LGALS3*, and *LTBP3*, were up-regulated, essentially constituting a "stromal gene signature." This finding is consistent with the propensity for myelofibrosis to develop in patients with polycythemia vera. Furthermore, there was increased expression of 10 cytokine and inflammatory mediator genes — *CCL3* (*MIP1A*), *CCL5* (*RANTES*), *CXCL5*, *SERPINE1* (*PAI1*), *S100A9*, *LCN2*, *PTX3*, *PF4V1*, *FCN1*, and *CFD* — which similarly constituted a "cytokine gene signature," a finding that is consistent with the inflammatory milieu that characterizes the myeloproliferative disorders.[14]

Unsupervised Hierarchical Clustering with the Concordantly Deregulated Genes

Unsupervised hierarchical clustering was used to determine whether the set of 102 core genes segregated the patients with polycythemia vera from the controls. As shown in Figure 1, the patients with polycythemia vera were clustered in two distinct groups: one group clustered independently from the controls and was heterogeneous with respect to core-gene expression, whereas the other was more homogeneous and overlapped with the controls. Table 2 lists the clinical features of the two patient groups, which did not differ significantly with respect to age, neutrophil *JAK2* V617F allele burden, white-cell and platelet counts, or clonal dominance. However, they did differ significantly with respect to disease duration; hemoglobin level; frequency of thromboembolic events, palpable splenomegaly, and splenectomy; chemotherapy exposure; leukemic transformation; and survival; this indicated that disease behavior was aggressive in one group and indolent in the other.

To determine whether the disease duration could drive the observed changes in gene expression, we constructed a multiple regression model with disease duration as the dependent variable. Only two genes were weakly associated with disease duration, an association that became non-significant after correction for multiple testing, which indicated that the observed differential gene expression was not a consequence of disease duration.

Clustering of Extracellular-Matrix Gene Expression According to Clinical Phenotype

Because of the striking deregulation of genes encoding extracellular-matrix proteins, we used unsupervised hierarchical clustering to determine whether stromal gene expression segregated with a particular clinical phenotype. As shown in Figure 2, the stromal gene set also separated the groups of patients with aggressive and indolent disease, with the latter

group characterized by the same heterogeneity seen when the 102-core-gene classifier was used (Fig. 1).

Gene Expression According to Clinical Phenotype

Analysis of differential gene expression in the groups with aggressive or indolent disease reinforced the importance of *JAK2* V617F–independent expression of disease phenotype–modifying genes. For example, although there was no difference in the *JAK2* V617F allele burden between the two groups (Table 2), gene expression differed markedly, with 707 genes differentially regulated (248 up-regulated and 459 down-regulated) in the group with indolent disease, as compared with the controls (Table S5A in the Supplementary Appendix), whereas only 149 genes were differentially regulated (68 up-regulated and 81 down-regulated) in the group with aggressive disease (Table S5B in the Supplementary Appendix). Furthermore, the two groups differed markedly with respect to expression of the 102 core genes (Tables S6A and S6B in the Supplementary Appendix). The results of KEGG analysis (Fig. S4A and S4B in the Supplementary Appendix) underscored the predominance of deregulated molecular pathways involving DNA and RNA metabolism and function in both groups, but histone gene deregulation predominated in the group with aggressive disease.

Supervised Clustering Based on Top-Scoring Pairs

We used a supervised approach based on top-scoring pairs to validate the unsupervised clustering results (see the Supplementary Appendix). We identified 30 gene pairs, none of which were in the set of 102 core genes, which segregated the patients into the same groups with aggressive or indolent disease with 100% accuracy (Fig. S5 in the Supplementary Appendix). For external validation (Tables S7 through S15 and Fig. S6, S7, and S8 in the Supplementary Appendix), we used the 6 highest-scoring gene pairs from the original 30 gene pairs, 8 of the original 19 patients as the training set, and a quantitative RT-PCR assay (which obviated the previous need for large numbers of CD34+ cells), and we were able to segregate a test group of 30 patients with polycythemia vera, selected without knowledge of their disease severity, into groups with the same aggressive and indolent clinical phenotypes.

Expression of Polycythemia Vera Core Genes in Chronic Myeloid Leukemia

Finally, to determine whether differential expression of the 102 core genes was unique to polycythemia vera or a nonspecific consequence of constitutive tyrosine kinase activation, we compared the expression of these genes in the chronic and blast-crisis phases of chronic myeloid leukemia (CML), a disease characterized by constitutive tyrosine kinase signaling in which STAT5 phosphorylation has a role in pathogenesis (see the Additional Reading section in the Supplementary Appendix).[15] As shown in Table 3, there was concordant up-regulation of 16 polycythemia vera core genes in the chronic phase of CML and concordant down-regulation of 9 genes. This pattern was reversed in the blast-crisis phase of CML, with up-regulation of 6 core genes that were down-regulated in polycythemia vera, including the oncogene *SETBP1*, and down-regulation of 11 core genes that were up-regulated in polycythemia vera.

Discussion

The cause of polycythemia vera remains an enigma. To address this issue, we examined gene expression in circulating CD34+ cells from patients with polycythemia vera, with the analyses performed separately for male and female patients order to eliminate sex as a potential confounder. This led to the identification of 102 genes; the importance of these genes in the disease process was substantiated by the fact that they could be used to segregate the patients with polycythemia vera into two groups with distinctly different clinical features that were independent of disease duration, the *JAK2* V617F allele burden, and white-cell and platelet counts. The group with aggressive disease fit the phenotype of patients with polycythemia vera in whom myelofibrosis develops[16] and who are at risk for spontaneous leukemic transformation. In this regard, it was possible with the use of gene expression to identify patients with chronic-phase CML who, despite their clinical phenotype, had disease that had already progressed to the accelerated phase at the molecular level.[17] Thus, given the low frequency of cytogenetic abnormalities in polycythemia vera before disease transformation,[18] gene-expression profiling could have prognostic relevance for patients with polycythemia vera.

With respect to disease specificity, it was informative to examine the expression of the 102 polycythemia vera core genes in CD34+ cells from patients with CML in the chronic or blast-crisis phase (see the Additional Reading section in the Supplementary Appendix). Fifty-five of the 102 polycythemia vera core genes were deregulated in CML, with concordant deregulation of 25 core genes in the chronic phase. However, in the blast-crisis phase, there was a reversal of the up-regulation or down-regulation of 17 polycythemia vera core genes; this provided a window into the genetic mechanisms that govern the clinical behavior of these two disorders and the potential relevance of specific genes or pathways in maintaining normal differentiation or promoting leukemic transformation. The gene-expression profile of our group with aggressive disease, as in CML in the blast-crisis phase, was closest to that of normal CD34+ cells.[17]

The mechanisms driving gene deregulation in the groups of patients with indolent or aggressive disease are unknown, but gene expression appeared to be cell autonomous (i.e., intrinsic to the cell rather than caused by external influences), a contention supported by the comparison with CD34+ cell gene expression in CML (Table 3). The extent to which *JAK2* V617F contributed to gene expression remains undefined, but no significant differences in gene expression were observed in a study of CD34+ bone marrow cells from *JAK2* V617F–positive and *JAK2* V617F–negative patients with polycythemia vera.[3] In our study, the *JAK2* V617F allele burdens did not differ between the group with aggressive disease and the group with indolent disease, even though their gene-expression patterns differed overall (Tables S5A and S5B in the Supplementary Appendix) and with respect to the set of 102 core genes (Table S6A and S6B in the Supplementary Appendix), suggesting an important role of other signaling pathways in the pathogenesis of poly-cythemia vera.

The list of 102 core genes provides ample evidence for this contention, as well as for synergistic interactions among these pathways. For example, *HES1* up-regulation suggests activation of either the Notch or Hedgehog pathway and couples these pathways with

JAK2–STAT3 signaling and activation of nuclear factor B and hypoxia-inducible factor 1a.[19–22] LEPR, LGALS3, LTBP3, and THBS1 activate transforming growth factor β1,[23–26] whose target genes include *SOX4*, *SPARC*, *SERPINE1* (*PAI1*), and *MIR21*.[27–29] SOX4 is also associated with activation of the Wnt, Notch, and Hedgehog pathways[27] and up-regulation of *HOXA9*,[30] which in turn enhances the transcriptional activity of SOX4 and STAT5.[31] LGALS3 also enhances Wnt pathway activity,[32] TIMP1 and SOX4 activate the PI3K–AKT pathway,[27,33] and RRAS2 activates the RAF–MAPK–ERK and PI3K–AKT pathways.[34,35]

Other striking abnormalities were the up-regulation of 16 genes encoding important matricellular proteins, essentially constituting a stromal signature similar to that described in lymphomas,[36] and 10 inflammatory cytokine genes, constituting a cytokine signature. The stromal signature represents genes probably involved in polycythemia vera myelofibrosis[37] and confirms the importance of malignant CD34+ cells in bone marrow stem-cell niche maintenance and remodeling.[38] The cytokine signature not only adds new members to those previously identified as involved in polycythemia vera[14] but also represents genes linking inflammation with coagulation, such as the complement-activating genes, *CFD*, *FCN1*, and *PTX3*. Finally, the data suggest a potential prothrombotic role of PF4V1, SERPINE1 (PAI1), HSPE, the prothrombinase FGL2,[39] and THSB1, which antagonizes both nitric oxide and ADAMTS13.

Our study provides only a snapshot of gene expression in a heterogeneous stem-cell population in a chronic disorder characterized by phenotypic variability over time. Repeat studies in patients at more than one time point will be necessary to validate the predictive value of CD34+ cell gene expression for prognostic classification. Other limitations of our study include the small number of patients involved, with selection biased toward those from whom the most CD34+ cells could be obtained. The CD34+ cell population is also diverse, and, although for technical reasons the cells could not be further fractionated, our study confirms that analysis of unfractionated circulating CD34+ cells has clinical usefulness.[17] Gene expression, of course, represents only one component of the complex process from gene transcription to protein product and is subject to epigenetic as well as genetic influences not controlled for in our study. Nevertheless, the data provide new insights into the genetic abnormalities of polycythemia vera, establish a molecular basis for disease heterogeneity, and identify genes and pathways that may have value for targeted therapy outside the canonical JAK2 signaling pathway, as well as previously unrecognized genes potentially involved in promoting myelofibrosis, inflammation, and thrombosis. The possibility that controlling for sex as a potential confounder is applicable to gene-expression analysis in other hematologic cancers warrants evaluation, given the insights described here.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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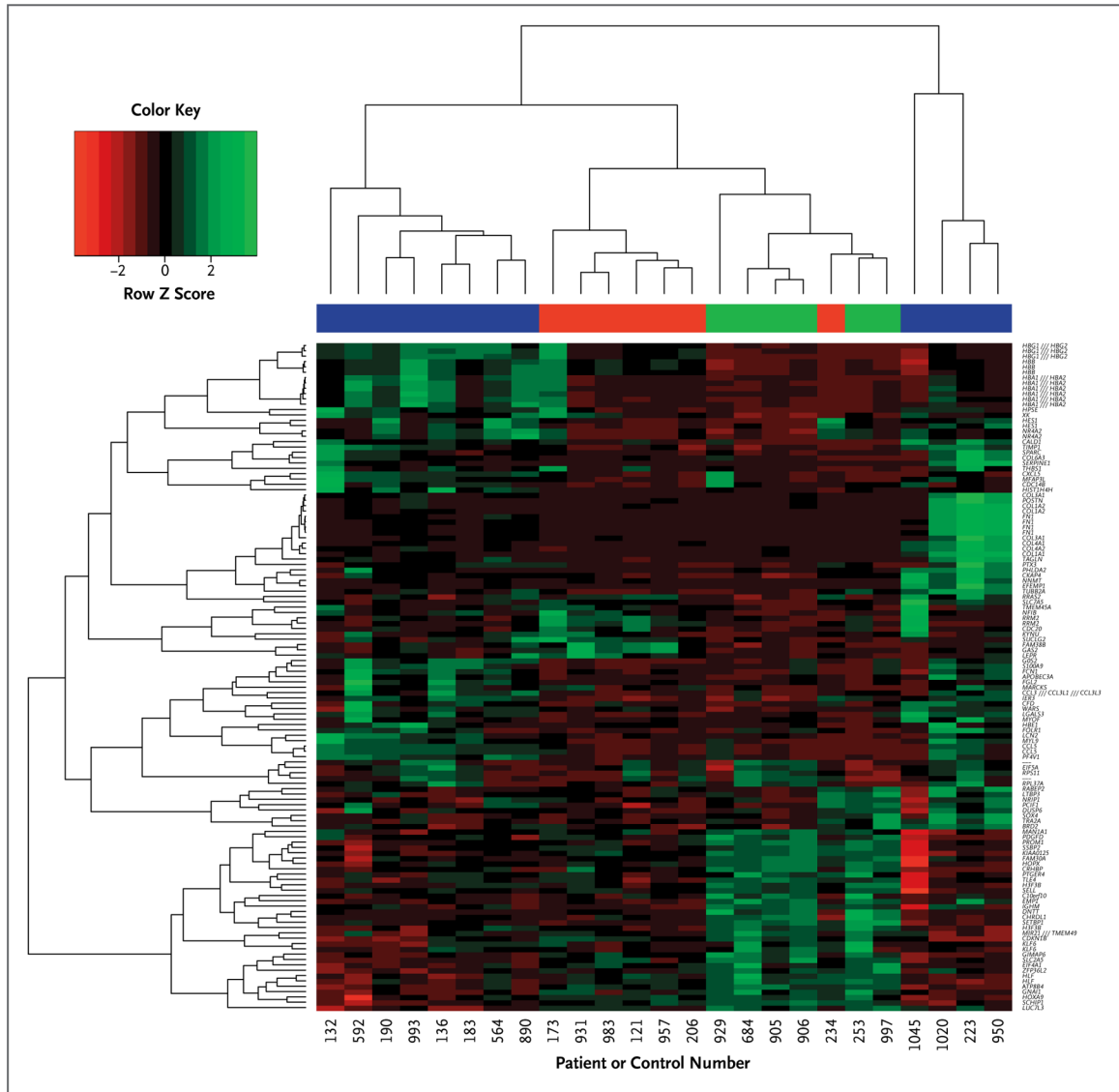
Figure 1. Dendrogram and Heat Map for Unsupervised Hierarchical Clustering in 19 Patients with Polycythemia Vera and 6 Controls, Based on the 102 Core Genes Concordantly Deregulated in Both Sexes

In the heat map, each column represents an individual patient or control, and each row represents a single gene. Red denotes decreased gene expression, and green increased gene expression. In the color bar above the heat map, green indicates the control group, and the blue and red color bars indicate the 19 patients with polycythemia vera segregated according to expression of the 102 core genes. Virgules separating two gene symbols (e.g., *HBG1 /// HBG2*) indicate that the Affymetrix probe did not distinguish between the genes; a dash indicates that the RNA identified by the probe has not yet been annotated.
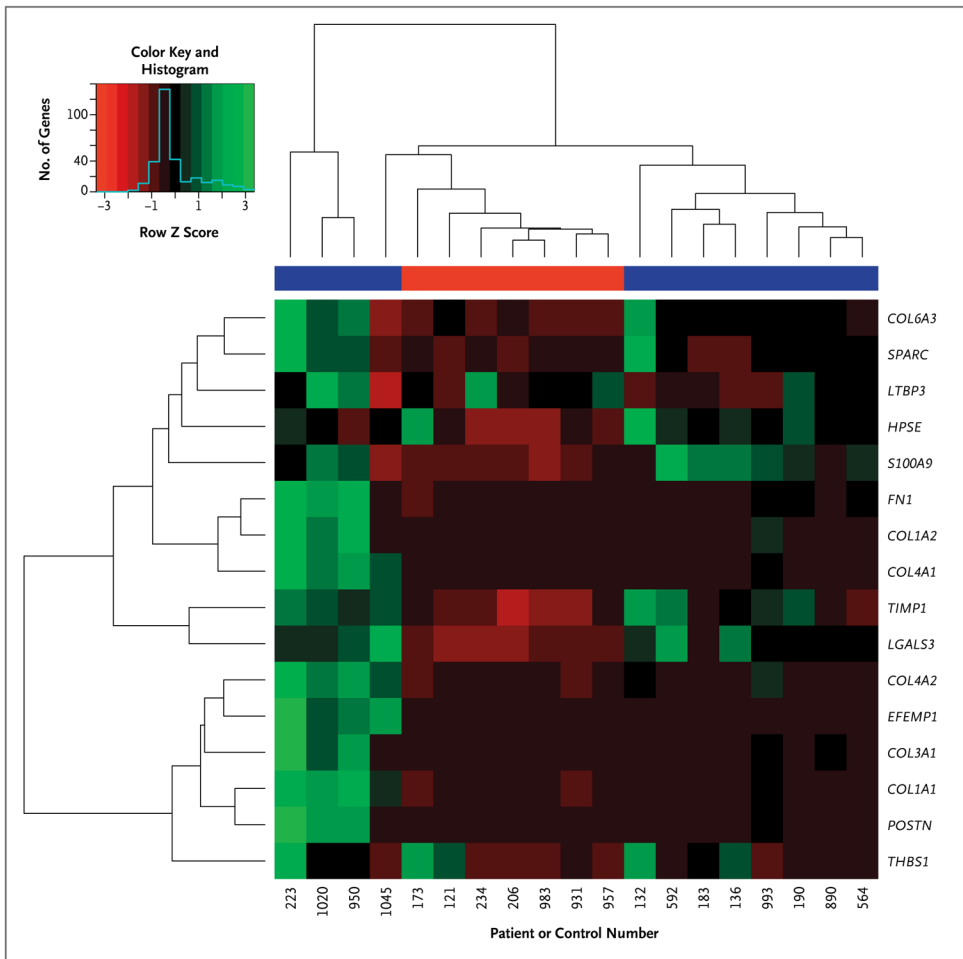
Figure 2. Dendrogram and Heat Map for Unsupervised Hierarchical Clustering in the 19 Patients with Polycythemia Vera, Based on the 16-Gene "Stromal Signature"

In the heat map, each column represents an individual patient, and each row represents a single gene; red denotes decreased gene expression, and green increased gene expression. The blue color bar indicates the patient group with indolent disease, and the red color bar indicates the patient group with aggressive disease.

Table 1

Clinical Features of the Study Population, According to Sex.[*]

Characteristic	Men (N = 8)	Women (N = 11)
Median age (range) — yr	71 (57–82)	60 (46–79)
Median disease duration (range) — yr	12 (1–25)	9 (1–14)
Median *JAK2* V617F neutrophil allele burden (range) — %	94 (55–100)	100 (60–100)
Median hemoglobin level (range) — g/dl	13.2 (8.3–15.9)	11.7 (10.4–14.7)
Median white-cell count per mm^3 (range)	16,690 (4430–177,190)	19,970 (5080–50,070)
Median platelet count per mm^3 (range)	421,000 (151,000–810,000)	948,000 (191,100–1,480,000)
Median spleen size (range) — cm below costal margin	10 (0–32)	5 (0–20)

[*] Characteristics did not differ significantly between the sexes, with the exception of platelet count (P = 0.02).

Table 2

Clinical Features Segregated with the Use of Unsupervised Hierarchical Clustering.

Characteristic	Patients with Aggressive Disease (N = 7)	Patients with Indolent Disease (N = 12)	P Value[*]
Sex — no.			
Male	4	4	
Female	3	8	
Median age (range) — yr	66 (48–74)	68 (46–82)	NS
Median disease duration (range) — yr	14 (7–24)	6 (1–25)	0.05[†]
Median *JAK2* V617F neutrophil allele burden (range) — %	100 (64–100)	85 (55–100)	NS
Median hemoglobin level (range) — g/dl	11.1 (8.3–12.9)	13.3 (10.7–15.9)	0.007[†]
Median white-cell count per mm^3 (range)	17,620 (10,020–171,190)	17,870 (4430–27,270)	NS
Median platelet count per mm^3 (range)	454,000 (171,000–1,017,000)	837,000 (151,000–1,480,000)	NS
Thrombosis — no. of patients	4	1	0.04[‡]
Palpable splenomegaly — no. of patients	7	6	0.03[‡]
Median spleen size (range) — cm below costal margin	20 (5–32)	2 (0–14)	0.005[‡]
Splenectomy — no. of patients	4	0	0.007[‡]
Chemotherapy — no. of patients	5	2	0.03[‡]
Transformation to acute leukemia — no. of patients	4	1	0.04[‡]
Surviving — no. of patients	1	11	0.001[‡]

[*] NS denotes not significant.

[†] The P value was calculated with the use of Student's t-test.

[‡] The P value was calculated with the use of Fisher's exact probability test (two-sided).

Table 3

Regulation of the Core Gene Set in Chronic Myeloid Leukemia (CML) and Genotype Correlations in Polycythemia Vera.[*]

CML, Chronic Phase

Up-regulated genes with concordant regulation in polycythemia vera

   HBA2, HBB, HBG1, COL1A1, RRM2, THBS1, TIMP1, MARCKS, XK, CDC20, MYOF, LEPR, CCL5, IER3, KYNU, LGALS3

Up-regulated genes with discordant regulation in polycythemia vera

   EIF5A, HOXA9

Down-regulated genes with concordant regulation in polycythemia vera

   SCHIP1, HLF, PROM1, MAN1A1, CRHBP, KIAA0125, DNTT, IGHM, SELL

Down-regulated genes with discordant regulation in polycythemia vera

   FGL2, NR4A2, CDC14B, HES1, NRIP1, SOX4

CML, Blast-Crisis Phase

Up-regulated genes with concordant regulation in polycythemia vera

   RRAS2, GAS2, HES1

Up-regulated genes with discordant regulation in polycythemia vera

   EMP1, SSBP2, LUC7L3, GIMAP6, EIF5A, SETBP1

Down-regulated genes with concordant regulation in polycythemia vera

   IGHM, SELL

Down-regulated genes with discordant regulation in polycythemia vera

   EFEMP1, HBA1, FCN1, LCN2, CKAP4, S100A9, APOBEC3A, FGL2, IER3, G0S2, LGALS3

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[*] Listed are 55 genes from the set of 102 core genes, according to their regulation in the chronic and blast-crisis phases of CML.[15]