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MicroRNA deregulation in polycythemia vera and essential thrombocythemia patients

Huichun Zhan[a,b,*], **Christopher Cardozo**[a,c], **Wayne Yu**[d], **Antai Wang**[b], **Alison R. Moliterno**[d], **Chi V. Dang**[e], and **Jerry L. Spivak**[d]

[a]James J. Peters VA Medical Center, Bronx, NY, USA

[b]Columbia University Medical Center, New York, NY, USA

[c]Mount Sinai School of Medicine, New York, NY, USA

[d]Johns Hopkins University School of Medicine, Baltimore, MD, USA

[e]Abramson Cancer Center of the University of Pennsylvania, Philadelphia, PA, USA

Abstract

Polycythemia vera (PV) and essential thrombocythemia (ET) are the two most common myeloproliferative neoplasms. The same JAK2^{V617F} mutation can be found in both disorders and is able to recapitulate many of the phenotypic abnormalities of these diseases in the murine models. The disease phenotype is also influenced by other unknown genetic or epigenetic factors. MicroRNAs (miRNA) are 18–24 nucleotides single-stranded non-protein-coding RNAs that function primarily as gene repressors by binding to their target messenger RNAs. We performed miRNA expression profiling by oligonucleotide microarray analysis in purified peripheral blood CD34+ cells from eight JAK2^{V617F}-positive PV patients and six healthy donors. A quantitative reverse-transcription polymerase chain reaction assay was used to verify differential miRNA expression. Since erythrocytosis is the only feature that distinguishes PV from ET, we also compared specific miRNA expression in the nucleated erythroid cells directly descended from the early erythroid progenitor cells of PV and ET patients. Our data indicate that significant miRNA deregulation occurs in PV CD34+ cells and confirm a genetic basis for the gender-specific differences that characterize PV with respect to miRNA. The results of our study also suggest that deregulated miRNAs may represent an important mechanism by which the PV erythrocytosis and ET thrombocytosis phenotypes are determined.

Keywords

MicroRNA; Myeloproliferative neoplasm; Polycythemia vera; Essential thrombocythemia; Hematopoiesis

[*]Corresponding author at: James J. Peters VA Medical Center, Room 3F-12, 130 West Kingsbridge Rd, Bronx, NY 10468, USA. Fax: +1 718 741 4675. Huichun.Zhan@va.gov (H. Zhan).

Author contribution

HZ, CVD, and JLS designed the research study. HZ performed the research. HZ, CC, WY and AW analyzed the data. ARM provided CD34+ cell JAK2^{V617F} allele burden data. HZ wrote the paper. JLS, CC, and CVD helped edit the paper. All authors approved the submitted version of the manuscript.

Introduction

The chronic myeloproliferative neoplasms (MPN), including polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF), are clonal stem cell disorders characterized by deregulated stem cell expansion and production of red cells, white cells and platelets alone or in combination. The discovery of a somatic mutation (V617F) in the tyrosine kinase, Janus Kinase 2 (JAK2) [1–5], was the most important advance in MPN since the discoveries 30 years ago that hematopoiesis in these disorders was both autonomous and clonal [6–8]. Although the murine models have provided unequivocal evidence that $JAK2^{V617F}$ is able to cause MPNs, there is significant heterogeneity in disease phenotypes between different murine lines and even within the same line, suggesting that disease phenotype is affected by other unknown genetic or epigenetic factors [2,9–12]. In particular, PV is characterized by raised red cell mass and sometimes increased platelet and white cell counts, while ET is defined by an elevated platelet count alone and a normal red cell mass. As the erythroid and megakaryocytic lineages are closely associated during differentiation and are generated from a common progenitor cell, additional mechanisms must exist to regulate erythropoiesis and megakaryopoiesis differently between $JAK2^{V617F}$-positive PV and $JAK2^{V617F}$-positive ET patients [13] and further characterization of these molecular differences in PV and ET should lead to a better understanding of the development of these diseases, their clinical manifestations, and their treatment.

MicroRNAs (miRNA) are 18–24 nucleotides single-stranded non-protein-coding RNAs that function primarily as gene repressors by binding to the target messenger RNAs to regulate gene expression [14–16]. There is growing evidence that miRNAs regulate hematopoiesis in both hematopoietic stem cells and committed progenitor cells [17–25]. Deregulated miRNA profiles have been reported in MPN patients during in vitro erythroid differentiation as well as in peripheral blood granulocytes, platelets, and reticulocytes [26,27]. Since the MPNs are stem cell disorders, comprehensive miRNA analysis in MPN stem cells is needed to further delineate their roles in MPN pathogenesis but so far there is only limited data in this area [26,28–30].

We performed miRNA expression profiling by microarray analysis in purified peripheral blood CD34+ cells from six healthy donors and eight $JAK2^{V617F}$-positive PV patients. Since erythrocytosis is the only feature that distinguishes PV from ET, we also compared specific miRNA expression in the nucleated erythroid cells directly descended from the burst-forming unit erythroid (BFU-E) progenitor cells of PV and ET patients. Our data indicate that significant miRNA deregulation occurs in the CD34+ cells of PV patients and confirm a genetic basis for the gender-specific differences that characterize PV [31–34]. The results of our study also suggest that deregulated miRNAs may represent an important mechanism by which the PV erythrocytosis and ET thrombocytosis phenotypes are determined.

Materials and methods

Patients

Thirteen PV patients and 4 ET patients from the Johns Hopkins University Hospital and the James J. Peters VA Medical Center Hematology clinics were studied. The diagnosis of PV and ET was based on the Polycythemia Vera Study Group Criteria [35]. All patients gave written consent for venipuncture and allowed their clinical and laboratory data (e.g. cell counts, family history, chemotherapy, and spleen size) to be recorded in a dataset for later analysis. Splenomegaly was considered present if the spleen was palpable by physical exam. The study protocol was approved by the Institutional Review Boards of both institutions.

Mononuclear cell (MNC) and CD34+ cell isolation

Peripheral blood MNCs were isolated using Ficoll-Paque (Sigma, St Louis, MO) gradient centrifugation. Peripheral blood CD34+ cells were isolated from the Ficoll buffy coats using immunomagnetic beads (Miltenyi, Auburn, CA). CD34+ cells were analyzed for expression of CD34, CD38, CD117, and CD41 using commercially available labeled antibodies. Fluorescence of at least 10,000 cells was measured on a FACS Caliber and analyzed with CELLQuest and Paint-a-gate software (BD Biosciences, San Jose, CA, USA). Similar to other published reports of the CD34+ cell phenotype in the MPN, the peripheral blood CD34+ cells were >95% positive both for CD34 and CD38, were dimly positive for CD117, were CD41 negative, and were primarily in G_0/G_1 of the cell cycle [31]. Peripheral blood CD34+ cells from healthy controls were purchased from AllCells™.

$JAK2^{V617F}$ determination

The CD34+ cell $JAK2^{V617F}$ allele burdens were measured using an allele-specific, quantitative real-time polymerase chain reaction assay sensitive to 10% of either the wild-type or mutant JAK2 allele as previously described [31].

Methocult colony formation assay

Peripheral blood MNCs (3×10^5 cells) was cultured using Methocult semisolid media with growth factors (StemCell Technologies, Vancouver, Canada) according to manufacturer's recommendations and incubated in a humidified environment at 37 °C in the presence of 5% carbon dioxide for 14 days. Burst Forming Unit-Erythroid (BFU-E) colonies, identified by visual inspection, were plucked. Up to 30 colonies were pooled for total RNA extraction for specific miRNA expression analysis.

Microarray analysis

Total RNA from the peripheral blood $CD34^+$ cells of healthy controls and PV patients was prepared using the miRNeasy Mini Kit (Qiagen) with on-column DNase I digestion. All microarray samples were processed at the Sidney Kimmel Comprehensive Cancer Center Microarray Core Facility at Johns Hopkins University, Baltimore, USA. RNA was tested for integrity using the Bioanalyzer 2100 (Agilent, Santa Clara, CA) and RNA integrity (RIN) scores above 8.0 were present in all samples. Human miRNA Microarray Version 3 Kit (Agilent Technologies, Santa Clara, CA),which contains probes for 866 human miRNAs

from the Sanger database v12.0, was used for microarray assays to examine the global miRNA expression. Briefly, 150 ng total RNA from each sample was labeled using the miRNA Labeling Reagent & Hybridization Kit (Agilent Technologies) as described in the instruction manual. All arrays were hybridized at 55 °C for 20 hours followed by wash procedure according to the miRNA Microarray System Protocol, Version 1.5, December 2007 (Agilent Technologies). Fluorescent signals were obtained by scanning with an Agilent scanner controlled by Agilent Scan Control 7.0 software. Data were acquired with Agilent Feature Extraction 9.5.3.1 software for miRNA microarray. Data analysis was performed using GeneSpring GX 11 software. The Student's unpaired *t* Test and multiple comparison adjustment were used to select differentially expressed miRNAs between sample groups with a *p* value<0.05 considered statistically significant. The statistical analysis was performed independently by W.Y. and A.W.

Quantitative real-time polymerase chain reaction

The TaqMan MicroRNA Assay (Applied Biosystems, Foster City, CA) was used for quantitative real-time polymerase chain reaction (qRT-PCR) to verify differential miRNA expression on an ABI ViiA™ 7 Real-Time PCR machine (Applied Biosystems). The miRNA expression levels was normalized to *RNU6B* (Applied Biosystems) and relative fold changes of gene expression was calculated by the $2^{\Delta\Delta CT}$ method. All assays were performed in triplicate. The Student's unpaired *t* test was used to test the significance of differences using a *p* value≤0.05 as the threshold for significance.

Results

Distinct miRNA expression in PV patients' peripheral blood CD34+ cells in comparison to healthy controls

Eight $JAK2^{V617F}$-positive PV patients (four females and four males, median CD34+ cell $JAK2^{V617F}$ allele burden = 70%, range 16–100%) and six healthy controls (three females and three males) were included in the miRNA expression profiling by microarray analysis. Table 1 shows the clinical and laboratory features of the eight $JAK2^{V617F}$-positive PV patients. Overall, in the eight PV patients as a group, there was differential regulation of 71 miRNAs compared to healthy controls ($p<0.05$) (36 up-regulated and 35 down-regulated) (Table 2). All the 71 deregulated miRNAs were concordantly up or down-regulated in both the male and female patients. There was no correlation between the miRNA microarray expression and the CD34+ cell $JAK2^{V617F}$ allele burden (not shown).

Given the phenotypic and genotypic differences between male and female PV patients [31–34], we analyzed differential miRNA expression separately by gender to identify gender-specific effects. Consistent with their different phenotypes, 79 miRNAs were differently regulated in the male PV patients (42 up and 37 down-regulated, $p<0.05$) compared to healthy male controls, while only 25 miRNAs were differently regulated in the female PV patients (9 up and 16 down-regulated, $p<0.05$) compared to healthy female controls. Only four miRNAs (miR-575 and miR-887, up; miR-196b and miR-551b, down) were significantly deregulated in both the male and female patients ($p<0.05$) suggesting that they may have a key role in disease pathogenesis (Fig. 1A). In a second group of five $JAK2^{V617F}$-

positive PV patients and two normal donors, quantitative RT-PCR confirmed the up-regulation of miR-575 (mean 9.3-fold) and miR-887 (mean 2.4-fold) and down-regulation of miR-196b (mean 9.1-fold) andmiR-551b (mean 3.3-fold) in PV patients (Fig. 1B). Using the PicTar, TargetScan, MicroCosm, and miRDB algorithms, we predicted the potential targets of these four core miRNAs (Table 3).

A unique miRNA expression signature for increased erythropoiesis in PV patients

We looked for miRNAs that can possibly contribute to increased erythroid proliferation and differentiation, which is the principal feature of PV. As previously reported, miR-145 promotes erythrocyte differentiation of the megakaryocyte-erythroid progenitor (MEP) cell, the common progenitor of the erythroid and megakaryocytic lineages [36,37]. MiR-451 is an erythroid-specific miRNA that is significantly up-regulated during erythroid differentiation [26,38–40] (Fig. 2A). Among the differentially regulated miRNAs in our eight PV patient microarray analysis, there was up-regulation of miR-451 and miR-145 in PV patients compared to healthy controls, consistent with their roles in increased erythropoiesis (Fig. 2B). Quantitative RT-PCR confirmed the up-regulation of miR-451 (~900-fold, range 1–8003) and miR-145 (23-fold, range 1–206), although these changes were not statistically significant likely due to the small sample size and large sample-to-sample variation (Fig. 2C). A similar result was obtained in the second group of five JAK2^{V617F}-positive PV patients (not shown).

Since erythrocytosis is the only feature that distinguishes PV from ET, we then compared miR-145 and miR-451 expression in the nucleated erythroid cells directly descended from the BFU-E progenitor cells of three JAK2^{V617F}-positive PV, two JAK2^{V617F}-positive ET, and two JAK2^{V617F}-negative ET patients. Briefly, patient peripheral blood MNCs were cultured using Methocult semisolid media with growth factors (Stem Cell Technologies, Vancouver, Canada) for 14 days and BFU-E colonies were plucked and pooled for total RNA extraction. Quantitative RT-PCR confirmed the up-regulation of miR-145 (mean 1.8-fold) and miR-451 (mean 10.6-fold) in PV BFU-E colony cells than in ET (both JAK2^{V617F}-positive and JAK2^{V617F}-negative) BFU-E colony cells (Fig. 3). These findings suggest that up-regulation of both miR-451 and miR-145 might be a potential “erythroid miRNA signature” of the PV phenotype.

Discussion

We report for the first time a unique CD34+ cell miRNA expression signature in JAK2^{V617F}-positive PV patients compared to healthy controls. Consistent with the phenotypic and genotypic differences between male and female PV patients [31–34], there are different miRNA deregulations between male and female patients (Fig. 1). 79 miRNAs were differently regulated in the male PV patients compared to healthy male donors, while only 25 miRNAs were differently regulated in the female PV patients compared to healthy female donors. Only four miRNAs (“core miRNAs”) were significantly up- (miR-575 and miR-887) or down-regulated (miR-196b andmiR-551b) by both the male and female patients and they may have a key role in disease pathogenesis.

Using the PicTar, TargetScan, MicroCosm, and miRDB algorithms, we predicted the potential targets of these four core miRNAs (Table 3). MiR196b plays an important role in the control of hematopoietic stem cells (HSC) by regulating HSC self-renewal, proliferation and differentiation. MiR-196b is nearly undetectable in long-term HSCs (LT-HSCs) and is up-regulated during the transition from LT-HSCs to short-term HSCs (ST-HSCs); it is most abundant in ST-HSCs and is down-regulated in more differentiated cells. Overexpression of miR-196b causes both a differentiation block and a reduced HSC capacity in a bone marrow reconstitution assay [19,41,42]. The stem cell compartment in MPN is heterogeneous with the presence of both JAK2 wild-type clones and $JAK2^{V617F}$ mutant clones in most MPN patients. In PV, there is usually a gradual increase in the $JAK2^{V617F}$ mutant stem/progenitor cell population over time with a decrease in the normal stem cell population [32,43]. The effects of miR-196b down-regulation in $JAK2^{V617F}$-positive clonal expansion and increased erythroid proliferation in PV are worth further exploration (Fig. 4). Potential targets of miR-575 are the serine/arginine-rich splicing factors SFRS2 (also known as SRSF2) and SFRS1 (also known as SRSF1), which are members of pre-mRNA splicing factors and constitute part of the spliceosome. An SRSF2 mutation is present in ~12% of patients with myelodysplastic syndromes [44–46] and frequently mutated in MDS/MPN patients (24%), particularly in CMML (28%) [47]. Although not common in the classic MPNs, SRSF2 mutations are more common in acute myeloid leukemia transformed from MPNs (18.9%) compared with leukemia transformation after myelodysplasia (4.8%) or de novo AML (5.6%) [48].

As previously reported, miR-145 promotes erythrocyte differentiation of the megakaryocyte-erythroid progenitor cell while miR-451 is an erythroid-specific miRNA that is significantly up-regulated during erythroid differentiation [26,36–40]. Up-regulation of miR-451 and miR-145 was previously reported during in vitro erythroid differentiation from PV patient peripheral blood mononuclear cells and in PV patient peripheral blood mononuclear cells, respectively [26,27]. Enforced expression of miR-451 promoted erythroid differentiation of the K562 cells [49]. Our study shows for the first time that there is indeed up-regulation of miR-451 and miR-145 in PV CD34+ cells. This is consistent with a role for these miRNAs in erythropoiesis, which is the principal feature of PV. This is not surprising as PV CD34+ cells have been shown to be more lineage-committed than the normal CD34+ cells [50,51]. Our data provide the first microRNA basis for this lineage preference of PV hematopoietic stem/progenitor cells. We show further that in the nucleated erythroid cells of the BFU-E colonies there appears to be greater miR-145 and miR-451 expression in PV than in ET (both $JAK2^{V617F}$-positive and $JAK2^{V617F}$-negative) although our sample size is too small for statistical significance (Fig. 3). This suggests that an erythroid miRNA signature (i.e. up-regulation of miR-451 and miR-145) in PV may serve in part as the mechanism for promoting the differential phenotypes between PV and ET (Fig. 4). Further verification with larger sample size is needed.

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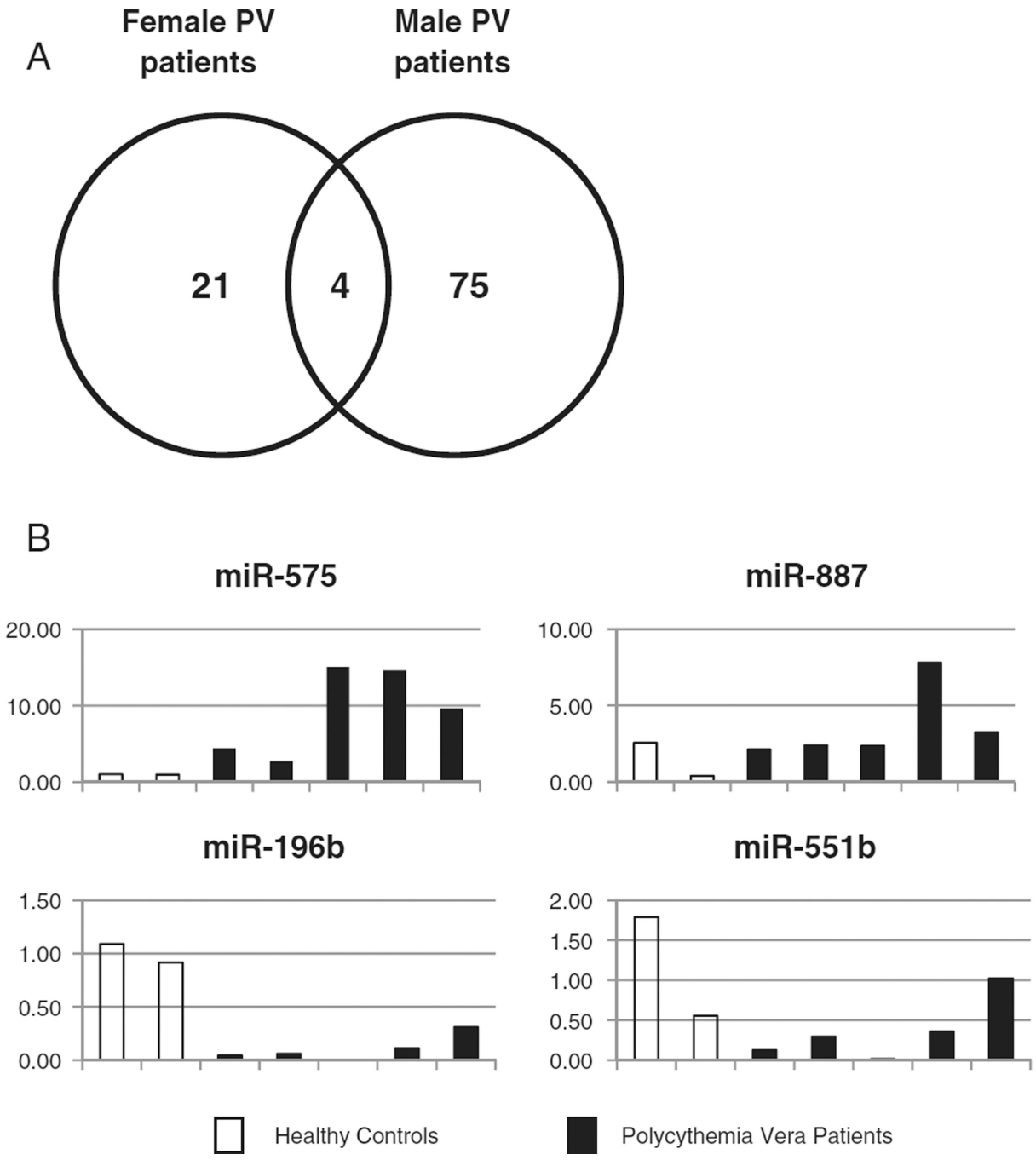

Fig. 1.

MicroRNA deregulation in both male and female polycythemia vera patients. (A) The Venn diagram shows the gender-specific differential miRNA expression in polycythemia vera (PV) patients. 79 miRNAs were differently regulated in the male PV patients compared to healthy male donors, while only 25 miRNAs were differently regulated in the female PV patients compared to healthy female donors. Only four miRNAs were significantly up- (miR-575 and miR-887) or down- (miR-196b and miR-551b) regulated in both the male and female patients ($p<0.05$). (B) Quantitative real-time polymerase chain reaction of

miRNA-575, miR-887, miR-196b and miR-551b expression in healthy control ($n = 2$) and PV patient ($n = 5$) peripheral blood CD34+ cells. The individual sample miRNA expression was shown as the fold-change compared to the average healthy control expression which was set as “1”.

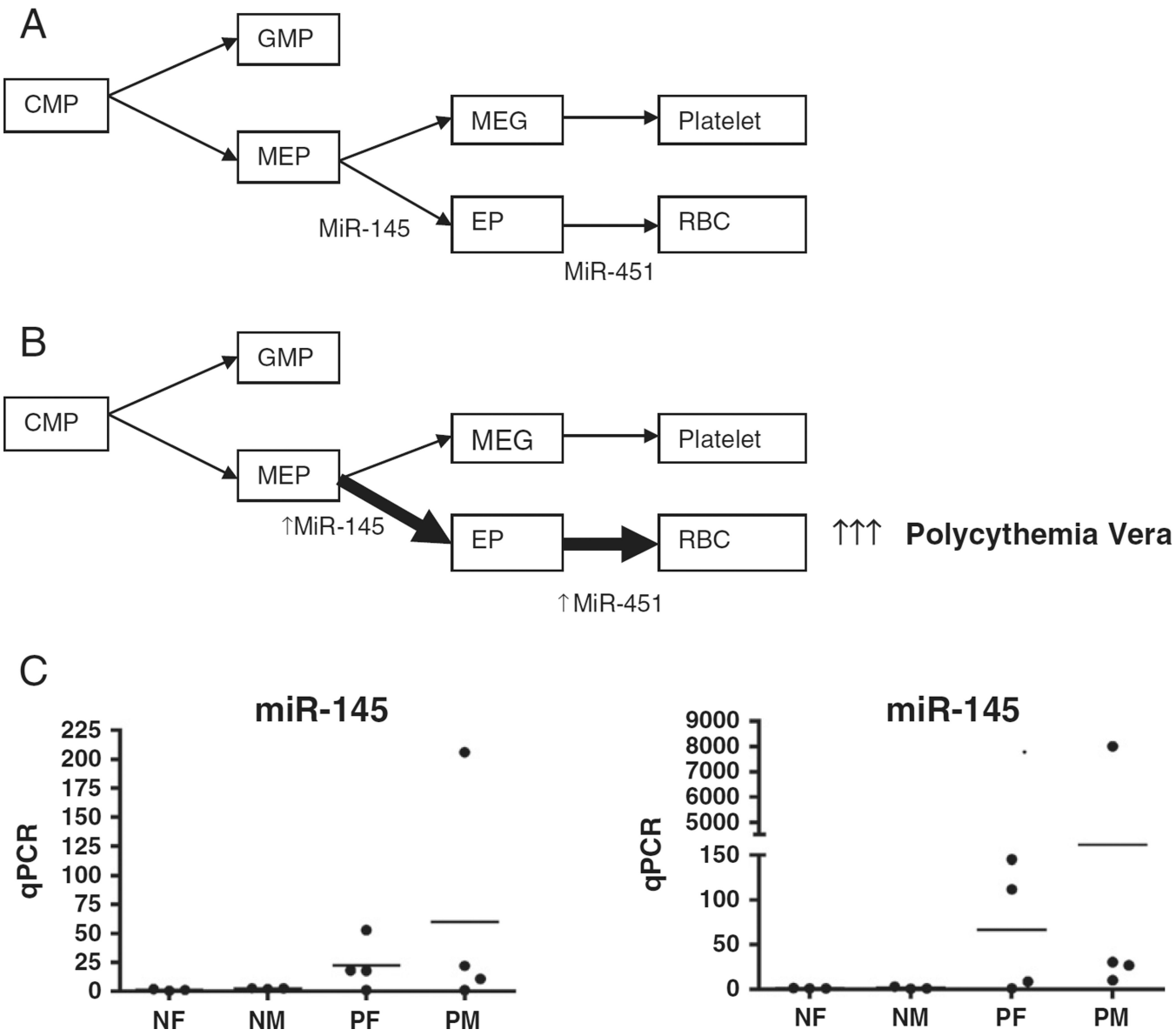

Fig. 2.

A unique microRNA expression signature for increased erythropoiesis in polycythemia vera (PV). (A) In normal hematopoiesis, the developmental fate of MEP is regulated in part by miR-145, while miR-451 is associated with erythroid differentiation. (B) In microRNA microarray analysis of PV patients' peripheral blood CD34+ cells, we observed up-regulation of miR-145 and miR-451, which will promote increased erythropoiesis. (C) Point plot illustration of peripheral blood CD34+ cell expression of miRNA-145 and miRNA-451 in healthy controls ($n = 6$) and PV patients ($n = 8$) by quantitative real-time polymerase chain reaction. The individual sample miRNA expression is shown as the fold-change compared to the average normal female expression which was set as “1”. CMP, common myeloid progenitor; GMP, granulocyte macrophage progenitor; MEP, megakaryocyte-erythrocyte progenitor; EP, erythroid precursor; MEG, megakaryocyte precursor; RBC, red

blood cell; NF, healthy female control; NM, healthy male control; PF, PV female patient; PM, PV male patient.

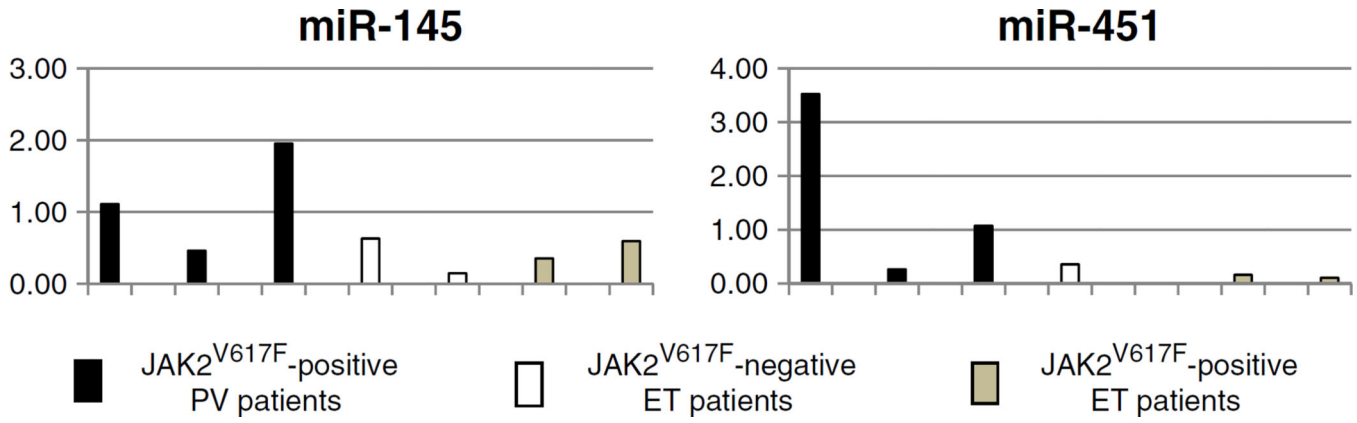

Fig. 3.

Quantitative real-time polymerase chain reaction of miRNA-145 and miRNA-451 expression in burst forming unit-erythroid (BFU-e) colony cells derived from polycythemia vera (PV) and essential thrombocythemia (ET) patient peripheral blood mononuclear cells. The individual sample miRNA expression of 3 JAK2^{V617F}(+) PV patients, 2 JAK2^{V617F}(−) ET patients and 2 JAK2^{V617F}(+) ET patients are shown here as the fold-change compared to the average PV BFU-e cell expression which was set as “1”.

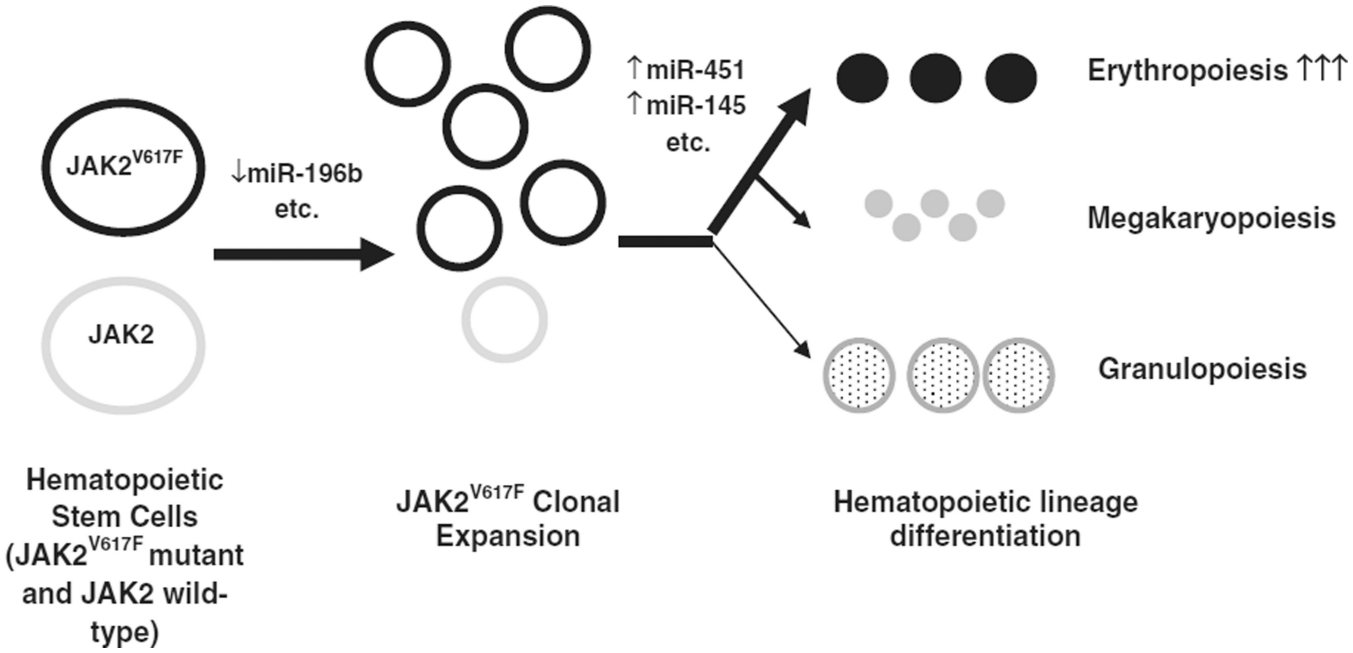

Fig. 4.

Hypothesis: deregulated miRNAs may contribute to JAK2^{V617F}-positive clone expansion and erythroid lineage commitment in polycythemia vera.

Table 1

Clinical and laboratory characteristics of the eight polycythemia vera patients in microRNA microarray expression analysis.

	PV
N	8
% Female	50%
% Antecedent MPN[a]	25%
Disease duration (years), median (range)	5.5 (1–12)
Age (years) at diagnosis, median (range)	
Female	56 (30–59)
Male	66 (60–68)
% with palpable splenomegaly	50%
Therapy at the time of sample collection	
Phlebotomy, *n* (%)	7 (88)
Aspirin, *n* (%)	4 (50)
Hydroxyurea, *n* (%)	4 (50)
Mean CD34 + cell $JAK2^{V617F}$ allele burden (%)	
Female (range)	55 (16–100)
Male (range)	80 (68–100)

PV = Polycythemia vera, MPN = Myeloproliferative neoplasm.

[a]Two patients (one female and one male) had a prior history of ET.

Table 2

Microarray analysis identified 71 significantly deregulated microRNAs in polycythemia vera patients' peripheral blood CD34+ cells.

Up-regulated miRNAs in PV	Fold change (patient/ normal)	*p* Value	Down-regulated miRNAs in PV	Fold change (patient/ normal)	*p* Value
hsa-miR-575	7.69	0.0001	hsa-miR-769-5p	0.51	0.0003
hsa-miR-887	3.70	0.0003	hsa-miR-551b	0.21	0.0014
hsa-miR-630	10.00	0.0003	hsa-miR-181c*	0.29	0.0023
hsa-miR-370	3.23	0.0006	hsa-miR-128	0.67	0.0036
hsa-miR-1207-5p	2.78	0.0013	hsa-miR-181d	0.37	0.0038
hsa-miR-135a*	2.63	0.0014	hsa-miR-874	0.62	0.0042
hsa-miR-1246	2.94	0.0020	hsa-miR-648	0.37	0.0043
hsa-miR-198	3.57	0.0024	hsa-miR-342-5p	0.27	0.0046
hsa-miR-486-5p	5.88	0.0030	hsa-miR-196b	0.19	0.0058
hsa-miR-134	2.17	0.0032	hsa-miR-181c	0.33	0.0071
hsa-miR-188-5p	2.33	0.0034	hsa-miR-181a-2*	0.44	0.0074
hsa-miR-1268	2.08	0.0034	hsa-miR-223*	0.53	0.0075
hsa-miR-298	2.44	0.0045	hsa-miR-361-5p	0.70	0.0136
hsa-miR-422a	1.79	0.0055	hsa-miR-361-3p	0.52	0.0148
hsa-miR-516a-5p	2.27	0.0058	hsa-miR-10b	0.32	0.0152
hsa-miR-1225-5p	2.33	0.0068	hsa-miR-512-3p	0.70	0.0167
hsa-miR-490-5p	2.17	0.0079	hsa-miR-222	0.54	0.0170
hsa-miR-451	10.99	0.0081	hsa-miR-25	0.68	0.0229
kshv-miR-K12-3	2.50	0.0091	hsa-miR-181a	0.51	0.0267
hcmv-miR-US33-5p	1.92	0.0092	hsa-miR-454	0.51	0.0282
hsa-miR-145	2.63	0.0117	hsa-miR-133b	0.31	0.0314
hsv1-miR-H1	2.63	0.0129	hsa-miR-150	0.20	0.0315
hsa-miR-654-5p	2.56	0.0153	hsa-let-7f-1*	0.54	0.0324
ebv-miR-BART7	1.96	0.0160	hsa-miR-374b	0.69	0.0346
hsa-miR-659	2.63	0.0170	hsa-miR-19b-1*	0.50	0.0346
hsa-miR-483-5p	2.78	0.0258	hsa-miR-221*	0.55	0.0351
hiv1-miR-H1	1.59	0.0261	hsa-miR-489	0.43	0.0377

Up-regulated miRNAs in PV	Fold change (patient/ normal)	*p* Value	Down-regulated miRNAs in PV	Fold change (patient/ normal)	*p* Value
hsa-miR-520b	1.54	0.0275	hsa-miR-935	0.42	0.0383
hsa-miR-125a-3p	2.22	0.0283	hsa-miR-363	0.45	0.0393
hsa-miR-1182	2.22	0.0284	hsa-let-7 g	0.68	0.0412
kshv-miR-K12-10b	2.44	0.0350	hsa-miR-149	0.63	0.0421
hsa-miR-490-3p	2.78	0.0387	hsa-miR-30e*	0.63	0.0428
hsa-miR-1290	2.08	0.0457	hsa-miR-501-3p	0.39	0.0475
hsa-miR-1471	2.44	0.0462	hsa-miR-770-5p	0.55	0.0485
hsa-miR-425	1.28	0.0463	hsa-miR-614	0.47	0.0499
hsa-miR-409-3p	4.55	0.0494			

Table 3

Putative target genes of the core deregulated miRNAs predicted using PicTar, TargetScan, MicroCosm, and miRDB prediction algorithms.

miRNA ID	Chromosomal location	Deregulation in PV CD34+ cells	Putative targets[a]
hsa-miR-575	4q21.22	Up	*SFRS2, SFRS1, EPOR, HMGA2, TFPI*
hsa-miR-887	5p15.1	Up	*GSK3A, BIM*
hsa-miR-196b	7p15.2	Down	*HOXA5, HOXA7, HOXA9, HOXA10, HOXB6, HOXB7, HOXC8, HMGA2, ERG*
hsa-miR-551b	3q26.2	Down	*ERBB4*

[a]The gene names are available at http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene.