Aberrant expression of microRNA in polycythemia vera

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The online version of this article contains a supplementary appendix.

ABSTRACT

Background

Polycythemia vera is a clonal hematopoietic stem cell disorder in which the *JAK2 V617F* mutation is observed in >95% of patients, but an as yet unidentified process appears to initiate the clonal expansion of hematopoiesis. Because microRNA regulate hematopoietic differentiation, we hypothesized that dysregulated expression of microRNA may contribute to the pathophysiology of polycythemia vera.

Design and Methods

We performed gene expression profiling in five patients with polycythemia vera and in five controls using CombiMatrix MicroRNA CustomArray. ANOVA identified deregulated microRNA in polycythemia vera, and their expression was studied in a larger set of samples by quantitative reverse transcriptase polymerase chain reaction. The expression of these microRNA was also analyzed in other myeloproliferative disorders.

Results

We observed down-regulation of *let-7a* and up-regulation of *miR-182* in polycythemia vera granulocytes, up-regulation of *miR-143*, *miR-145* and *miR-223* in polycythemia vera mononuclear cells, up-regulation of *miR-26b* in polycythemia vera platelets, and down-regulation of *miR-30b*, *miR-30c* and *miR-150* in polycythemia vera reticulocytes. *JAK2 V617F* frequency was positively correlated with *miR-143* expression and inversely correlated with *let-7a*, *miR-30c*, *miR-342* and *miR-150*. Transcript level of predicted target genes was determined, and overexpression of *IRAK2* was detected in all granulocytes from patients with myeloproliferative disorders and in polycythemia vera reticulocytes. Abnormally high *HMGA2* microRNA was found in myelofibrosis granulocytes.

Conclusions

Our study demonstrates that peripheral blood cells from patients with polycythemia vera have microRNA signatures distinct from those of controls. Our findings of aberrant microRNA expression underline the complexity of the molecular basis of polycythemia vera.

Key words: polycythemia vera, peripheral blood cells, microRNA expression, *JAK2 V617F* correlation.

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Introduction

Polycythemia vera (PV) is a clonal myeloproliferative disorder (MPD) caused by somatic mutation(s) arising in a hematopoietic multipotent cell. Hematopoiesis in PV is characterized by the accumulation of phenotypically normal erythrocytes with variable overproduction of platelets and myeloid leukocytes. A single nucleotide substitution (G1849T) generates the *JAK2 V617F* tyrosine kinase that is present in the bone marrow and peripheral blood myeloid cells of more than 95% of PV patients and in approximately 50% of patients with essential thrombocythemia (ET) and primary myelofibrosis (PMF). *JAK2 V617F* has also been identified in a small proportion of patients with chronic neutrophilic leukemia, acute megakaryocytic leukemia, and in most patients with refractory anemia with ringed sideroblasts and thrombocytosis.[1,2] The non-specificity of the mutation, the absence of *JAK2 V617F* in some PV patients, the existence of familial PV in which affected members can be either *JAK2 V617F*-negative or positive, only a proportion of clonal PV cells are *JAK2 V617F*-positive, and the existence of erythropoietin-independent burst-forming units-erythroid (BFU-E) that are either *JAK2 V617F*-negative or positive demonstrate that somatic mutation of *JAK2* is not the sole pathogenic process in PV.[3-7] Moreover, several mutations in exon 12 of *JAK2* have been described in *JAK2 V617F*-negative PV patients.[8,9]

MicroRNA (miRNA) are non-coding 18-22nt RNA that regulate gene expression either by destabilizing target mRNA or by inhibiting protein translation.[10] Both *in vitro* and *in vivo* data show that miRNA are important regulators of hematopoiesis and that they play a role in the pathogenesis of some acquired hematologic disorders,[11] functioning either as tumor suppressors (*miR-15/16*) or as oncogenes (*miR-17-92* cluster). For example, in chronic lymphocytic leukemia, somatic deletion of 13q14 is observed in more than 50% of clonal lymphocytes, resulting in loss of expression of *miR-15* and *miR-16*.[12] As these miRNA upregulate expression of the anti-apoptotic gene *BCL2*, mutant cells have a survival advantage.[13] Abnormally high expression of *miR-155* has been reported in both Hodgkin's lymphoma and diffuse large B-cell lymphoma.[14,15] The precursor sequence of *miR-155* is located in the non-coding region of the *BIC* locus. *BIC* activation accelerates lymphopoiesis and is associated with over-expression of *MYC* (V-myc myelocytomatosis viral oncogene homolog).[16]

Microarray studies have defined miRNA signatures in some hematopoietic cell lineages and hematologic diseases,[17-22] and comparison of samples from patients and controls samples revealed aberrantly expressed miRNA that correlated with disease phenotype.[23-27] Disease-specific miRNA expression may have diagnostic significance, prognostic significance, or both and may provide new insights into the pathogenesis of disorders such as PV in which the etiology is incompletely understood. To define the miRNA profiles in PV, miRNA expression was analyzed in peripheral blood cells from healthy volunteers and from patients with PV.

Design and Methods

Blood samples

Peripheral blood from patients and controls was obtained following written informed consent using a protocol approved by the Institutional Review Board of the University of Utah. The diagnosis of PV, ET and PMF was made using the criteria of the Polycythemia Vera Study Group, and these clinical criteria were further supported by the following laboratory studies: low serum erythropoietin, identification of erythropoietin-independent colonies, and clonal hematopoiesis in informative females.[28] The allele frequency of the *JAK2 V617F* mutation was quantified in patients' cells as described elsewhere.[29] The PV group consisted of 17 patients, in whom the *JAK2 V617F* mutation frequency ranged from 0.8% to 100%. The patients were categorized according to *JAK2 V617F* allele frequency into two groups: those with a *low allele burden* (patients with a mutation frequency <50%; n=6) and those with a *high allele burden* (patients with a mutation frequency >50%; n=11). We tested nine patients with PMF (6/9 *JAK2 V617F*-positive) and nine with ET (4/9 *JAK2 V617F*-positive). All patients were negative for *MPLW515L* and *MPLW515K* mutations.[30] The control group was composed of ten volunteer donors.

Platelets were separated from plasma by centrifugation at 500g for 10 min. Mononuclear cells and granulocytes were isolated by Ficoll-Paque (Sigma, St. Louis, MO, USA) density gradient centrifugation.

Gene expression profiling

Total RNA was extracted using Tri-Reagent (MRC, Cincinnati, OH, USA) according to the manufacture's instructions. Reticulocyte RNA was isolated as described previously.[22]

CombiMatrix MicroRNA CustomArrays (#3725) were used for gene expression profiling. These arrays contained 326 probes for human mature miRNA including corresponding mismatch oligonucleotides. We used labeling and hybridization protocols as described elsewhere.[31] Briefly, 6 μg of total RNA were 3'-end labeled with Cy3 dinucleotides (Dharmacon, Lafayette, CO, USA) using T4 RNA ligase (NEB, Ipswich, MA, USA) by incubating on ice. After 2 hours, the labeled RNA was precipitated and resuspended in 12% formamide, 5% sodium dodecyl sulfate (SDS), 0.8% bovine serum albumin (BSA) and 400 mM Na_2HPO_4 (pH 7.0). The sample was hybridized on array slides overnight at 37°C. The slides were washed by incubation (3 minutes) once in 2xSSC, 0.029% SDS at room temperature, three times in 1.6xSSC at 23°C and twice in 0.8xSSC at 4°C. Processed slides were scanned using a GenePix 4000B (Axon, Sunnyvale, CA, USA) scanner at a resolution of 10 μm.

Microarray data analysis

The raw data were extracted by CombiMatrixImager software (*www.combimatrixcorp.com*). The median signal from all mismatch probes was set to background. This value was subtracted from all miRNA probe signals.

Probe signals greater than 1.5 times background were considered *present*. By using Genesis 1.6.0Beta1 software (*http://genome.tugraz.at/*), signal intensity values were normalized to per-chip mean values, and hierarchical clustering analysis was performed using average linkage and Pearson's correlation. Additionally, the data were processed by one-way ANOVA (Welch analysis of variance) to determine differentially expressed miRNA (a p value of <0.05 was considered statistically significant).

Expression analysis by quantitative real time polymerase chain reaction (qRT-PCR)

Expression of selected miRNA was analyzed by qRT-PCR using TaqMan MicroRNA Expression Assays (Applied Biosystems, Foster City, CA, USA) as previously described.[22] Briefly, 10 ng of total RNA were reverse transcribed under the following conditions: 16°C for 30 min, 42°C for 30 min, 85°C for 5 min. The conditions for the PCR reaction were as follows: 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min using an ABI PRISM 7000 thermal cycler. The miRNA expression levels were normalized to *RNU6B* (Applied Biosystems).

The levels of putative target mRNA (*MYB*: Hs00920571_m1, *HMGA2*: Hs00171569_m1, *CCND2*: Hs00922418_g1, *HIC2*: Hs00740546_s1; *IRAK2*: Hs00176394_m1, *KRAS*: Hs00270666_m1) were quantified by TaqMan Expression Assays (Applied Biosystems) according to the manufacturer's instruction. Briefly, 500 ng of total RNA were reverse transcribed using SuperScript III First-Strand Synthesis SuperMix for qRT-PCR kit (Invitrogen, Carlsbad, CA, USA), under the following incubation conditions: 25°C for 10 min, 50°C for 30 min and 85°C for 5 min. Two microliters of cDNA were used for TaqMan qRT-PCR. The PCR reaction was performed under the following conditions: 95°C for 10 min, followed by 40 cycles of 94°C for 15 s and 60°C for 1 min. Expression levels of the genes were normalized to 18S *rRNA* (Hs99999901_s1, Applied Biosystems).

Relative fold changes of gene expression were calculated by the $\Delta\Delta C_T$ method and the values are expressed as $2^{-\Delta\Delta C_T}$.[32]

Prediction of putative miRNA targets

Putative gene targets of miRNA were predicted using the following two algorithm tools: TargetScan 4.0 (*www.targetscan.org*) and Pictar (*http://pictar.bio.nyu.edu/*). The target genes predicted in both databases are reported.

Results

miRNA signatures of peripheral blood cell lineages

For initial gene expression profiling by microarrays, we used peripheral blood cells from five PV patients and five controls. Hierarchical clustering analysis determined miRNA expression clusters for particular peripheral blood cell lineages in both controls and PV patients (e. g., cluster –R: down-regulation of *miR-23a*, *miR-23b*, *miR-342*, *miR-27a*, and *miR-150* in reticulocytes; cluster R: up-regulation of *miR-185*, *miR-195*, *let7-c/b/i*, *miR-92*, *miR-363*, and *miR-182* in reticulocytes; cluster G: up-regulation of *miR-338*, *miR-148a*, *miR-424*, *miR-143*, and *miR-199b* in granulocytes; cluster P: up-regulation of *miR-199a**, *miR-221*, and *miR-130a* in platelets, and down-regulation of *miR-451* in mononuclear cells) (*Online Supplementary Figure S1*).

Validation of miRNA array data by qRT-PCR

The array data of 12 miRNA were validated in the tested cell lineages by qRT-PCR. The relative fold changes of miRNA expression, controls versus PV patients, determined by qRT-PCR were similar to those detected by microarrays (r=0.95, p<0.01) (*Online Supplementary Figure S2*). Relative gene expression was assessed using both quantitation derived from a standard curve and the $\Delta\Delta C_T$ method. A high concordance (r>0.96, p<0.01) was observed when the two methods were compared.

Differentially expressed miRNA in polycythemia vera peripheral blood cells

The ANOVA of array data showed significantly different expression of 40 miRNA in particular PV cells as compared to in control cells (p<0.05) (Figure 1). Of these, we selected 25 miRNA (p<0.03) for further testing in a larger set of PV patients (n=17) and controls (n=10) by qRT-PCR. We confirmed down-regulation of *let-7a* (p<0.05) and up-regulation of *miR-182* (p<0.01) in PV granulocytes; up-regulation of *miR-143* (p<0.01), *miR-145* (p<0.01) and *miR-223* (p<0.01) in PV mononuclear cells; up-regulation of *miR-26b* (p<0.05) in PV platelets; and down-regulation of *miR-30b* (p<0.05), *miR-30c* (p<0.05) and *miR-150* (p<0.05) in PV reticulocytes (Figure 2). *miR-30b* and *miR-30c* belong to the same gene family and their levels of expression were significantly correlated with each other (r=0.96, p<0.01). Array data showed *miR-342* down-regulation in PV cell lineages except reticulocytes but we did not confirm this by qRT-PCR at a p value of <0.05.

miRNA expression in cells from patients with primary myelofibrosis and essential thrombocythemia

To investigate PV specificity of deregulated miRNA, we analyzed miRNA expression in peripheral blood cells of patients with PMF (n=9) and ET (n=9). In granulocytes, the down-regulation of *let-7a* was specific for PV, but abnormally high expression of *miR-182* was also found in all other MPD patients (p<0.05). In addition, we detected significantly decreased levels of *miR-30c* in ET granulocytes (p<0.01). We did not confirm PV specificity of *miR-143*, *miR-145* or *miR-223* over-expression in mononuclear cells, as increased levels of those miRNA were also detected in mononulcear cells from patients with PMF and ET (p<0.01). Because of the limited amount of blood available from non-PV MPD patients, we did not attempt to isolate reticulocyte RNA from patients with PMF or ET.

Correlation of JAK2 V617F allele frequency with

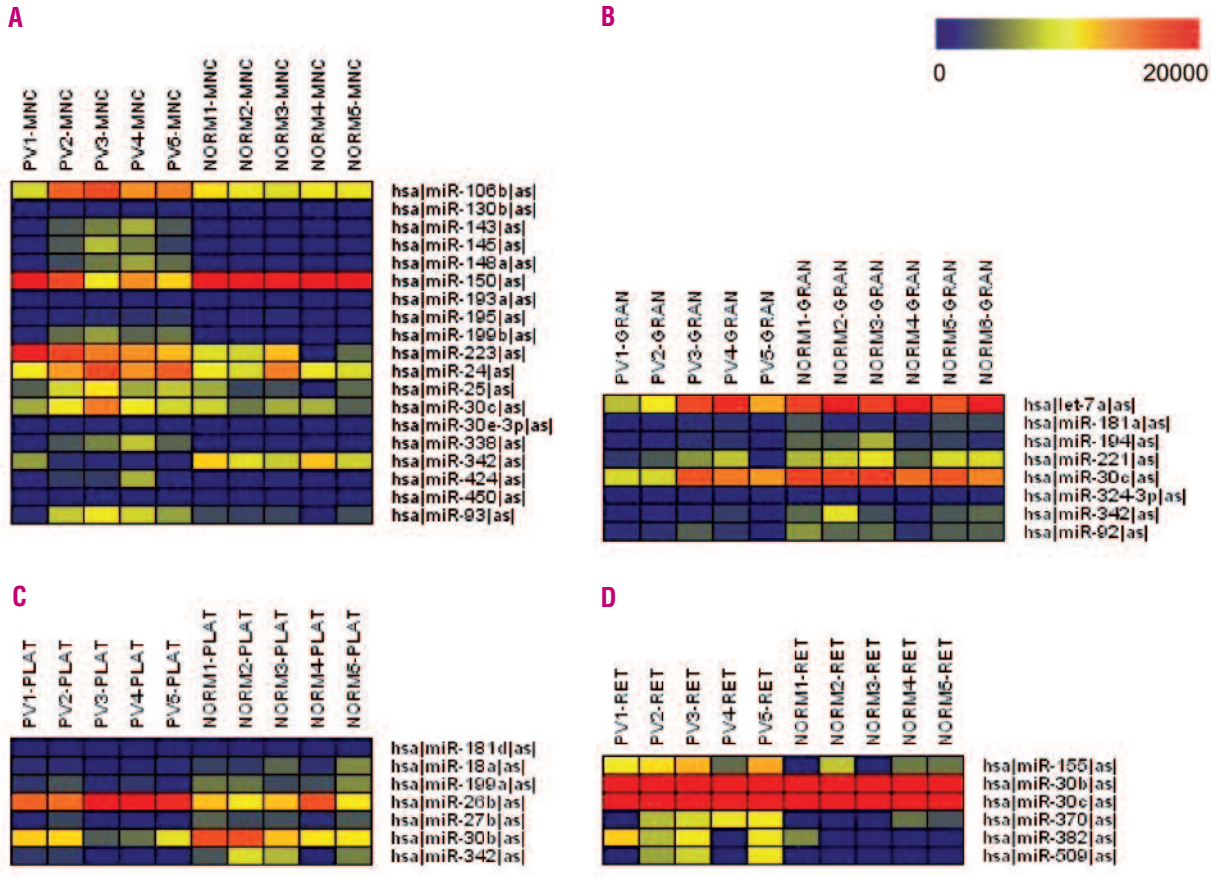

Figure 1. Differently expressed miRNA in PV peripheral blood cells detected by microarrays. The one-way ANOVA of array data revealed significantly deregulated miRNA ($p<0.05$) in PV mononuclear cells (A), granulocytes (B), platelets (C) and reticulocytes (D). The PV patients are listed according to their *JAK2 V617F* level, from the lowest level in PV1 to the highest level in PV5. The relative gene expressions are expressed by a gradient intensity of color, as shown in the color scale at the right upper corner of this Figure. The dark blue color indicates low expression and the lightest red color indicates maximal expression. NORM: control; PV: polycythemia vera patient; MNC: mononuclear cells; GRAN: granulocytes; PLAT:platelets; RET: reticulocytes.

miRNA expression

To analyze the relationship between miRNA expression and *JAK2 V617F* allele frequency, PV patients were grouped into two categories according to their mutational status and Spearman's correlation analysis was applied. The patients with an allelic burden of <50% were categorized into a *low allele burden* group (n=6) and those with an allele frequency of >50% were categorized into a *high allele burden* group (n=11). We determined a positive correlation of *miR-143* (r=0.62) ($p<0.05$) in PV mononuclear cells; an inverse correlation of *let-7a* (r=-0.67)($p<0.01$), *miR-30c* (r=-0.63)($p<0.01$), *miR-150* (r=-0.74)($p<0.001$) and *miR-342* (r=-0.63) ($p<0.01$) in PV granulocytes; and an inverse correlation of *miR-150* (r=-0.75)($p<0.01$) in PV platelets. In PV reticulocytes, only *miR-150* showed a slight trend towards an inverse correlation (r=-0.42)($p>0.05$). We also correlated expression levels of these miRNA in PMF and ET. Although we had a limited number of *JAK2 V617F*-positive PMF and ET patients, we found an inverse correlation of *miR-199a* (r=-0.77)($p<0.05$) and *miR-342* (r=-0.82)($p<0.05$) in PMF platelets. Generally, we observed the same correlation trend (either positive or negative) in PV and PMF, but in ET, the correlation trend was in the opposite direction for some miRNA (e.g., *miR-143*, *miR-342*, *miR-27b*).

Transcript levels of putative target genes

Putative miRNA targets were predicted by TargetScan 4.0 and PicTar software (Table 1), and transcript levels were measured by qRT-PCR (Figure 3). The highest scored target of *miR-150* is *MYB* (V-myb myeloblastosis viral oncogene), but we did not detect a significant difference in *MYB* mRNA levels between control and MPD granulocytes or between control and PV reticulocytes. Another high scored target of *miR-150* is *IRAK2* (interleukin-1 receptor-associated kinase 2), and we detected its significant over-expression in all MPD granulocytes ($p<0.01$) and in PV reticulocytes ($p<0.05$). The highest scored target of *let-7a* is *HMGA2* (high mobility group AT-hook 2), and we observed significantly increased expression of *HMGA2* mRNA in PMF granulocytes ($p<0.05$) but not in PV and ET granulocytes. *CCND2* (cyclin D2) may be regulated by *let-*

7a, *miR-182* or *miR-145*, and we found decreased expression of *CCND2* in all MPD granulocytes; however, in PMF and ET, the *p* values were of borderline significance (0.06 and 0.05, respectively). *HIC2* (hypermethylated in cancer 2 gene), a potential target of *let-7a*, *181a* or *miR-145*, was not expressed aberrantly in MPD granulocytes. As *let-7a*, *miR-143* or *miR-150* may target *KRAS* (v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog), we found a significantly decreased level of *KRAS* in PMF granulocytes ($p<0.05$).

Discussion

We determined the miRNA expression profiles of peripheral blood cells from patients with PV and compared these data to those of control samples and samples from patients with PMF and ET (the other two Philadelphia chromosome negative MPD). We identified aberrant expression of eight miRNA (*let-7a*, *miR-182*, *miR-143*, *miR-145*, *miR-223*, *miR-26b*, *miR-30b*, *miR-30c* and *miR-150*) in either granulocytes, mononuclear cells, platelets, or reticulocytes of PV patients. We observed correlations between aberrant expression of *miR-143*, *let-7a*, *miR-30c*, *miR-342* and *miR-150* and *JAK2 V617F* mutation frequency. In some cases (*miR-30c*, *miR-150* and *miR-342*), a statistically significant difference in granulocyte expression was observed only after samples were categorized into *low allele burden* (<50% *JAK2 V617F* allelic frequency) and *high allele burden* (>50% *JAK2 V617F* allelic frequency) groups. Granulocytes from patients with a *high allele burden* had significantly lower expression of these miRNA com-

Table 1. Putative target genes of deregulated miRNA in polycythemia vera peripheral blood cells. Target genes of the miRNA were predicted by TargetScan 4.0 and PicTar software and the genes predicted in both databases are reported.

miRNA ID[a]	*Chromosomal location*	*Deregulation in PV*	*Putative targets*[b]
hsa-let-7a	9q22.32	down/GR	***HMGA2***,*ARID3B*,***HIC2***,***CCND2***,*DTX2*,***RAS***
hsa-miR-182	7q32.2	up/GR	*ADCY6,RGS17, HAS2,DTR,FRS2*
hsa-miR-143	5q32	up/MNC	***KRAS***, *SSH2,TRPS1,BRD2,AKAP6,ERK5*
hsa-miR-145	5q32	up/MNC	*FLI1*,***HIC2***,*DDR1,TRIM2,ADD3*
hsa-miR-223	Xq32.2	up/MNC	*PRDM1,MEF2C,HLF,SFRS12,DUSP10*
hsa-miR-30b	8q24.22	down/RET	*CELSR3,MKRN3,TNRC6,EED,LHX8*
hsa-miR-30c	1p34.2	down/RET	*CELSR3,MKRN3,TNRC6,EED,LHX8*
hsa-miR-150	19q13.33	down/RET	***MYB***,***IRAK2***,*MLL*,***HMGA2***,*ZNF403*
hsa-miR-26b	2q35	up/PLT	*ATP11C,CHORDC1,ALS2CR2,NAB1,NHS*
hsa-miR-27b	9q22.32	up/PLT	*PLK2,SLC6A1,GPAM,SNAP25,NRK,TXN2*

PV: polycythemia vera; GR: granulocytes; MNC: mononuclear cells; PLT: platelets; RET: reticulocytes, down: down-regulation, up: up-regulation, bold genes-tested genes. [a]The miRNA sequences are available at http://microrna.sanger.ac.uk/sequences/. [b]The gene names are available at http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene.

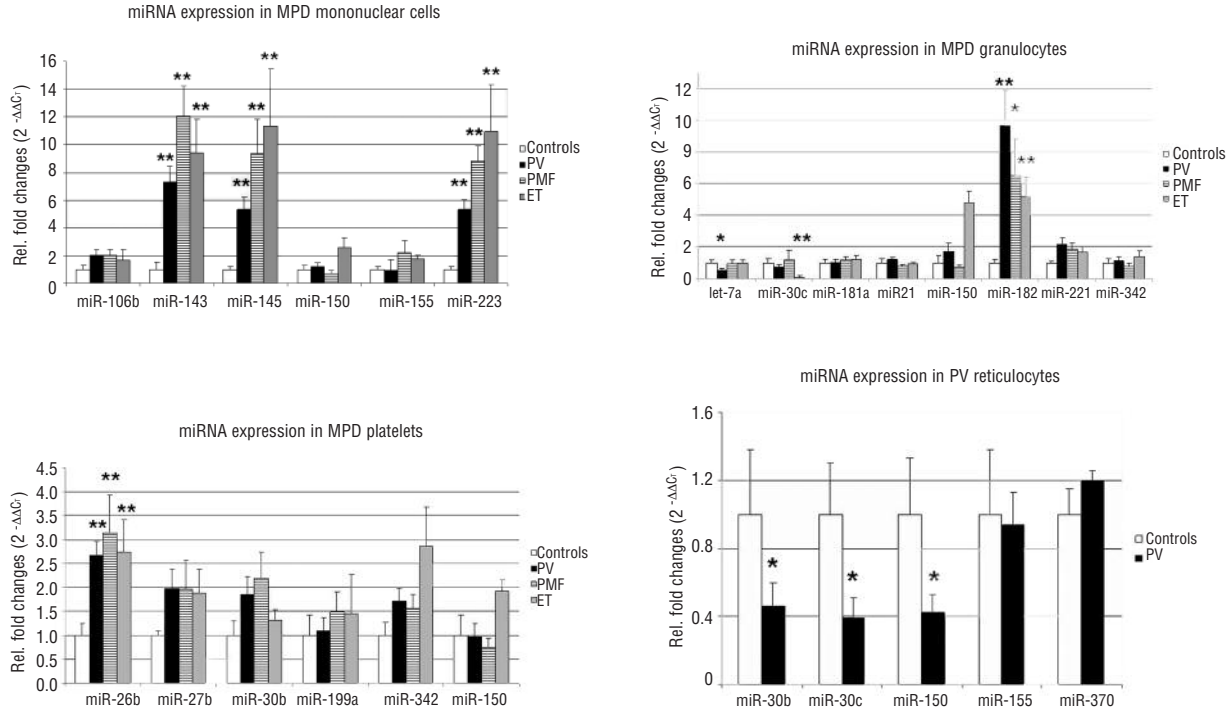

Figure 2. Gene expression of selected miRNA in myeloproliferative disorder peripheral blood cells detected by qRT-PCR. Gene expression of the miRNA was determined by qRT-PCR in peripheral blood mononuclear cells (A), granulocytes (B), platelets (C) and reticulocytes (D) from polycythemia vera, primary myelofibrosis and essential thrombocythemia patients. Relative fold changes of expression were calculated by the $\Delta\Delta C_T$ method and the values are expressed as $2^{-\Delta\Delta Ct}$. Data are presented as the mean plus standard error. The statistical significance between miRNA expression in controls and patients was calculated by Student's t-test. Reticulocyte RNA was available only from polycythemia vera patients. PV: polycythemia vera, PMF: primary myelofibrosis, ET: essential thrombocythemia, *$p<0.05$, **$p<0.01$.

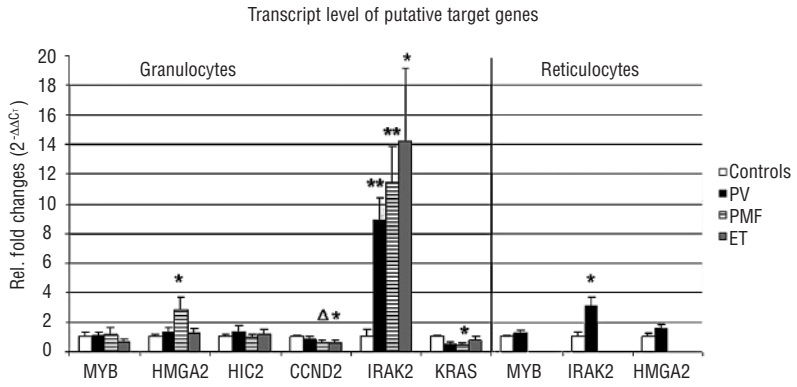

Figure 3. Transcript levels of putative targets of deregulated miRNA in myeloproliferative disorder peripheral blood cells. Putative miRNA targets were predicted by TargetScan 4.0 and PicTar software and transcript levels of selected genes were tested by qRT-PCR in myeloproliferative disorder granulocytes and polycythemia vera reticulocytes. Relative fold changes of expression were calculated by the $\Delta\Delta C_T$ method and the values are expressed as $2^{-\Delta\Delta Ct}$. Data are presented as the mean plus standard error. The statistical significance between miRNA expression of controls and patients was calculated by Student's t-test. PV: polycythemia vera, PMF: primary myelofibrosis, ET: essential thrombocythemia, Δp=0.06, *p<0.05, **p<0.01.

pared to controls, and patients with a *low allele burden* expressed the miRNA at significantly higher levels than the patients with a *high allele burden*. For example, a comparison of granulocytes from *high allele burden* PV patients and those from controls showed a significant difference in *miR-150* expression (p=0.02), but the expression was not significantly different between *low allele burden* PV patients and controls. This demonstrates that *JAK2 V617F* allelic heterogeneity must be considered in comparative analyses.

In our previous study, we tested miRNA expression in *in vitro* expanded erythroid progenitors and found that *miR-150* was reduced in PV erythroid cells.[22] In concordance with these *in vitro* data, we also found significant down-regulation of *miR-150* in native PV reticulocytes. Together, these results suggest that the down-regulation of *miR-150* expression plays a role in the pathophysiology of PV. In contrast, up-regulation of *miR-150* was reported in chronic lymphocytic leukemia.[33]

Transcript levels of some miRNA targets were analyzed to determine their potential dysregulation at the mRNA stability level. High scored targets of *miR-150* are the *MYB* and *IRAK2* genes. We did not detect aberrant expression of *MYB* transcript in MPD granulocytes or in PV reticulocytes; however, the level of mRNA *IRAK2* was significantly up-regulated in these cells. *IRAK2* plays a key role in innate immunity through Toll-like receptor signaling pathways that interface with nuclear factor κB.[34] Its role in MPD is unclear and thus represents an interesting target for further studies.

In this study, *let-7a* down-regulation was specific for PV granulocytes and inversely correlated with the *JAK2 V617* allele burden. Previously, the down-regulation of *let-7a* had been shown in etiologically heterogeneous disorders such as chronic lymphocytic leukemia[35] and gastric carcinoma,[36] demonstrating its relevance in clonal disorders. The target of *let-7a* is *HMGA2*,[37] an architectural transcription factor, whose aberrant expression contributes to clonal hematopoiesis in some cases of paroxysmal nocturnal hemoglobinuria.[38] Compared to levels in normal controls, we detected a significantly higher *HMGA2* mRNA level in PMF granulocytes but not in granulocytes from patients with PV and ET. Similar findings have been reported by others.[39] However, expression of *HMGA2* mRNA in PMF granulocytes did not correlate with *let-7a* expression in either PV (r=-0.46) or PMF (r=0.28) granulocytes, suggesting an alternative mechanism for controlling *HMGA2* expression. For example, in head and neck squamous cell carcinoma, *HMGA2* expression was reportedly controlled by *miR-98*,[40] whereas in Burkitt's lymphoma, *let-7a* down-regulated *MYC*.[41]

RAS oncogenes can be regulated by *let-7*.[42] We observed decreased expression of *KRAS* in all MPD granulocytes; however, only in PMF granulocytes did this decrease reach statistical significance. In the PV group, the p value was 0.10, but when the patient with the lowest *JAK2 V617F* allele frequency (0.8%) was excluded from the analysis, the p value was <0.01, suggesting apparent down-regulation of *KRAS* in patients with a *high allele burden*. Deregulation of Ras signaling has been reported in myeloid malignancies through alternative genetic mechanisms that include somatic mutations in *NRAS* and *KRAS*.[43] Mutations of *RAS* genes are found occasionally in MPD with *NRAS* being affected most commonly and *KRAS* rarely.[44] Interestingly, somatic activation of *KRAS* blocks erythroid differentiation and causes anemia in a mouse model.[45] We may speculate that decreased expression of *KRAS* in PV patients is associated with exaggerated erythropoiesis.

We tested two other genes (*HIC2* and *CCND2*) that are putative targets of deregulated miRNA. *CCND2*, a positive regulator of G_1 phase promotion of the cell cycle, has been shown to be involved in *JAK2 V617F*-mediated signaling.[46] A borderline low *CCND2* transcript level was observed in PMF and ET (p=0.06 and 0.05, respectively).

Although most of the mRNA targets did not show differential expression in the tested MPD cells, this does not rule out their suppression at the translational level, which represents a major mechanism of miRNA-mediated regulation in animals. The functional relevance of the dysregulated targets in the pathogenesis of PV will be studied in detail. These data will need to be compared to those of other heterogeneous acquired and congenital, as well as primary, secondary and Chuvash polycythemias; these studies are in progress.

We analyzed expression of miRNA in ET and PMF

cells to determine whether aberrant miRNA expression was specific to PV. This comparison showed that some miRNA are aberrantly expressed in other MPD (e.g., we demonstrated over-expression of *miR-143*, *miR-145* and *miR-223* in all MPD mononuclear cells and abnormally high expression of *miR-182* in granulocytes of all MPD). In contrast, decreased levels of *miR-143* and *miR-145* were shown in B-cell malignancies.[47] Up-regulated expression of *miR-182* has already been reported in PMF granulocytes.[48] Overlapping dysregulation of some miRNA is not surprising because miRNA may regulate multiple targets or may co-operate to regulate a particular gene. Our study underscores the complexity of aberrant miRNA expression in clonal premalignant and malignant processes.

To our knowledge, this study is the first comparison of the miRNA signatures of PV peripheral blood cells and normal peripheral blood cells. We show that the expression of some dysregulated miRNA correlates with *JAK2 V617F* mutation frequency, suggesting a possible effect of *JAK2 V617F* on miRNA expression. However, we also identified aberrantly expressed miRNA without a correlation with *JAK2 V617F* mutation frequency. As expected from mRNA profiles,[39,49] some miRNA (*miR-143*, *miR-145*, and *miR-150*) are deregulated with expression-specific patterns also in other clonal hematopoietic disorders. Nonetheless, our findings of aberrant miRNA expression support the concept that factors other than constitutive activation of *JAK2* may contribute to the pathogenesis of PV. Additional studies will be needed to determine the molecular consequences of aberrant expression of miRNA in the pathophysiology of PV.

Authorship and Disclosures

HB performed the research and wrote the manuscript; MM analyzed microarray data; JTP designed the research and critically revised the manuscript. The authors reported no potential conflicts of interest.

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