Discrimination of Polycythemias and Thrombocytoses by Novel, Simple, Accurate Clonality Assays and Comparison with PRV-1 Expression and BFU-E Response to Erythropoietin

Enli Liu¹, Jaroslav Jelinek¹, Yves D. Pastore², Yongli Guan¹, Jaroslav F. Prchal³, and Josef T. Prchal¹,²

¹Hematology/Oncology, ²Pediatric Hematology/Oncology, Baylor College of Medicine, Houston, TX 77030 USA, ³Hematology/Oncology, McGill University, Montreal, Canada

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*Address correspondence to:

Josef T. Prchal, MD
Baylor College of Medicine
One Baylor Plaza, 802E
Houston, TX 77030
Tel: (713) 798-7866
Fax: (713) 798-6132
jprchal@bcm.tmc.edu

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Abstract

Essential thrombocythemia (ET) and polycythemia vera (PV) are clonal myeloproliferative disorders that are often difficult to distinguish from other causes of elevated blood cell counts. Assays that could reliably detect clonal hematopoiesis would therefore be extremely valuable for diagnosis. We previously reported three X-chromosome transcription-based clonality assays (TCA) involving the G6PD, IDS and MPP1 genes, which together were informative in ~65% of female subjects. To increase our ability to detect clonality, we developed simple TCA for detecting the transcripts of two additional X chromosome genes: Bruton Tyrosine Kinase (BTK) and four-and-a-half LIM domain 1 (FHL1). The combination of TCA established the presence or absence of clonal hematopoiesis in ~90% of female subjects. We show that both genes are subject to X-chromosome inactivation and are polymorphic in all major U.S. ethnic groups. The five TCA were used to examine clonality in 46 females along with assays for erythropoietin-independent erythroid colonies (EEC) and granulocyte PRV-1 mRNA levels to discriminate polycythemias and thrombocytoses. Of these, all 19 patients with familial polycythemia or thrombocytosis had polyclonal hematopoiesis, while 22/26 patients with clinical evidence of myeloproliferative disorder and one patient with clinically obscure polycythemia were clonal. Interestingly, Interferon α therapy of two PV patients was associated with reversion of clonal to polyclonal hematopoiesis. EEC were observed in 14/14 PV and 4/12 ET patients; and increased granulocyte PRV-1 mRNA levels were found in 9/13 PV and 2/12 ET. Thus, these novel clonality assays are useful in the diagnosis and follow-up of polycythemic and disorders with increased platelets.
Introduction

The concept of clonality in hematology contributed greatly to our understanding of hematopoiesis and its hierarchy. The myeloproliferative disorders, chronic myelogenous leukemia, polycythemia vera (PV), essential thrombocythemia (ET), and agnogenic myeloid metaplasia, are characterized by clonal hematopoiesis of the myeloid cells. Thus, the demonstration of clonal hematopoiesis has a role in their differential diagnosis, and evaluation of their response to therapy.

Lyon-Beutler's hypothesis of random X-chromosome inactivation provided the basis for assessing clonality of hematopoietic cells. Either the maternal or the paternal X-chromosome is randomly inactivated in each cell at an early stage of embryogenesis. Therefore, clonal tissue will consist of a population of cells all containing the same active X-chromosome, whereas a mixture of paternally and maternally inherited active X-chromosomes is present in non-clonal tissue. Methods for determining X-chromosome inactivation have been devised at the protein, DNA, and RNA levels. The original clonality assay was an analysis of electrophoretic differences among human glucose-6-phosphate dehydrogenase (G6PD) isoenzyme patterns in G6PD heterozygous females of African descent; however, its application was limited to one particular racial group. Subsequent clonality assays exploited the differences in DNA methylation between the active and inactive X-chromosome encoded genes, such as phosphoglycerate kinase (PGK), hypoxanthine phosphoribosyl transferase (HPRT), the DXS255 locus (M27), and the human androgen receptor (HUMARA). Although these assays increased the rate of heterozygosity (informativeness), methylation differences may be also affected by non-genetic factors and carcinogenesis, may vary from gene to gene, and an incomplete DNA digestion may lead to an unreliable clonality result. Finally, these assays cannot be used to...
study non-nucleated cells, such as platelets and reticulocytes. Therefore, we searched for other approaches that could circumvent these shortcomings. The initial assays were based on differentiating the active X chromosome by its transcript (transcriptional clonality assay, TCA) using reverse transcription polymerase chain reaction followed by ligase detection reaction (rtPCR-LDR) to identify the transcribed alleles of the exonic polymorphism of *G6PD* (C/T at coding sequence, cds, #1311, dbSNP: 2230037).\(^{16,22}\) The second TCA exploited an exonic polymorphism of membrane palmitoylated protein 1 (*MPP1*; also known as *P55*) (G/T at cds #358, dbSNP: 1126762).\(^{15,17}\) While these methods were sensitive, reproducible, quantitative, and useful for non-nucleated cells; however, they were time-consuming, laborious and required a large amount of radioactivity and only about 50% of the females were informative for these polymorphisms. To overcome these disadvantages, another method, allele specific polymerase chain reaction (ASPCR) was used to detect three exonic polymorphisms of X-chromosome genes *G6PD*, *MPP1* and another exonic X-chromosome polymorphism studied previously by El-Kassar and colleagues\(^{23}\) iduronate-2-sulfatase (*IDS*, C/T at cds #438, dbSNP:1141608).\(^{18,19,24}\) While these methods were less laborious and technically demanding, they were not quantitative and 35% of females were still not informative;\(^{19}\) further, South-East Asians are not polymorphic at the *IDS* locus.\(^{19}\) Clearly, increased informativeness, and simplification of transcriptional based clonality assays would enhance their general usefulness.

We report on two additional exonic X-chromosome polymorphisms and the use of single-stranded conformation polymorphism (SSCP) analysis for genotyping and TCA of *BTK*, *FHL1* and three previously used polymorphic X-chromosome genes. The gene frequencies of *BTK* and *FHL1* polymorphisms in the common U.S. ethnic groups and the clinical applicability of TCA in studies of the patients diagnosed with clinical thrombocythemic or
polycythemic disorders are shown. These novel TCAs are compared with other laboratory
tests previously utilized for discrimination of PV and ET, i.e. \textit{in vitro} formation of
erthropoietin-independent erythroid colonies (EEC)\textsuperscript{25,26} and an increased expression of
\textit{PRV-1} mRNA in granulocytes.\textsuperscript{27-29} The results of these tests are now reported to demonstrate
their relative value and clinical usefulness for differential diagnosis of polycythemias and
thrombocytoses.

\section*{Materials and Methods}

\subsection*{Subjects}

All patients with clinical diagnosis of PV and ET fulfilled the clinical diagnostic criteria of
Polycythemia Vera as defined in the widely used Hematology text.\textsuperscript{30} Most of the studied
subjects were seen and examined by one of the authors (JTP). Nineteen others were referred
to us by other hematologists, 9/19 were subsequently seen and examined by one of us (JTP).
These patients were referred for the establishment or the confirmation of diagnosis and/or
additional diagnostic and research assays not routinely available. ACD-anticoagulated
peripheral blood samples were obtained from the individuals with proven or suspected MPD.
The study was approved by the Baylor College of Medicine (BCM) IRB; all studied subjects
participated for the studies after signing Informed Consent. DNA samples from unrelated
healthy females of four major US ethnic groups (Caucasian, Hispanic, African-American,
and Southeast Asian) were kindly provided by the BCM Polymorphism Resource Core.

\subsection*{Cell fractionation}

Platelets and granulocytes were separated by differential centrifugation and isopycnic density
gradient separation using standard protocols.\textsuperscript{2} T lymphocytes were isolated from 5 ml of
whole blood using RosetteSep™ Antibody Cocktail (StemCell Technologies Inc. Vancouver, British Columbia, Canada) following the manufacturer’s protocol.

**Preparation of DNA and RNA and PCR for genotype determination**

Genomic DNA was extracted from 200 µl of peripheral blood by using QIAamp DNA Blood Mini Kit (QIAGEN Inc. Valencia, CA). RNA was prepared from platelets, granulocytes and T lymphocytes using TRI REAGENT (Molecular Research Center, Inc. Cincinnati, OH) in combination with RQ1 RNase-free DNase (Promega Corporation, Madison, WI) and RNeasy Mini Kit (QIAGEN) according to the manufacturers’ protocols to avoid DNA contamination. PCR was used for genotype determination. We used a rapid PCR amplification program to amplify the polymorphisms of four genes (*BTK, FHL1, MPP1* and *G6PD*) at the same time, i.e. a touch-down program which consisted of 3 min at 94 °C, 23 cycles of 30 s at 94 °C, 40 s at 65 °C, -0.5 °C per cycle, 40 s at 72 °C, then 25 cycles of 30 s at 94 °C, 40 s at 54 °C and 40 s at 72 °C, and a final 10-min extension at 72 °C in a PTC-200 Peltier thermal cycler (MJ Research, Inc. Waltham, Massachusetts). The PCR products were labeled with ^32^P by 0.1 µl (1 µCi) of [α-^32^P]dCTP (Amersham Pharmacia Biotech, Inc. Piscataway, NJ) in 10 µl of PCR reaction mixture. The sequences of the primers and PCR conditions are shown in Table 1. The *IDS* genotyping was performed by PCR-restriction enzyme digestion method as previously reported.23

**One step RT-PCR**

One step RT-PCR was performed for the TCA. It was carried out with Access RT-PCR System (Promega Corporation, Madison, WI) in one reaction following the manufacturer’s protocol. The reactions were subjected to a PCR condition consisting of 45 min at 47 °C, 2 min at 92 °C, then 35 cycles of 30 s at 94 °C, 30 s at 62 °C and 45 s at 68 °C, and a final 5-min extension at 68 °C according to the manufacturer’s recommendations. The products of
one step RT-PCR were labeled with $^{32}$P in the same manner as for genotype determination. The sequences of the primers for five polymorphic markers (BTK, FHL1, MPP1, IDS, and G6PD) are depicted in Table 1.

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<th>Gene</th>
<th>Primer</th>
<th>Primer Sequence (5'-3')</th>
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<th>DMSO [%]</th>
<th>PCR Product (bp)</th>
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<td>TCTCCATCTGCGCCTCGGC</td>
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</table>

Table 1. Primers and PCR conditions for determination of genotype and TCA of 5 X-chromosome markers. PCR primers for genotyping (D-For, D-Rev), RT-PCR primers for TCA (R-For, R-Rev).

SSCP analysis

SSCP analysis was used for both genotype determination and TCA. $^{32}$P-labeled products of
PCR or one step RT-PCR were used directly for SSCP analysis. Two microliters of $^{32}\text{P}$-labeled product mixed with 2 µl sequencing loading dye were heated for 3 min at 95 ºC to denature and then snap cooled on ice. The samples were then loaded and run on a non-denaturing sequencing 0.5xMDE (mutation detection enhancement) polyacrylamide gel (Biowhittaker Molecular Applications, Rockland, ME) with constant power (6W) at room temperature for 16 hours. After electrophoresis, the gel was dried for 1h at 80 ºC and exposed to an X-ray film at -80 ºC for autoradiography.

**Sequencing analysis**

In order to confirm the different genotypes of $BTK$ and $FHL1$ in SSCP analysis, unlabelled PCR products were purified with QIAquick Gel Extraction Kit (QIAGEN Inc. Valencia, CA). Five microliters of the eluate were used as the template for cycle sequencing with a DNA Sequencing Kit (BigDye™ Terminator Cycle Sequencing Ready Reaction) (Applied Biosystems, Foster City, CA) in the PTC-200 Peltier thermal cycler. Sequences for both the coding and the complementary strands were analyzed on the ABI Prism 377 DNA Sequencer (Applied Biosystems, Foster City, CA) according to the manufacturer’s protocol.

**In vitro assay of erythroid responsiveness to Epo**

*In vitro* assay of erythroid responsiveness to Epo was performed as previously described. Briefly, mononuclear cells from peripheral blood were isolated on Histopaque 1077 (Sigma, St Louis, MO) density gradient and cultured at a final concentration of $3 \times 10^5$ cells/ml in Methocult H-4531 medium (StemCell Technologies Inc., Vancouver, Canada) in 35-mm Petri dishes in the presence of 0, 30, 60, 125, 250 and 3000 mIU/ml of Epo. Cultures were maintained in a humidified atmosphere of 5% carbon dioxide at 37 ºC. Erythroid colonies were scored at 14 days by standard criteria.

**Quantification of PRV1 mRNA expression in granulocytes**
Real time RT-PCR was used to quantify PRV-1 mRNA, in total RNA isolated from peripheral blood granulocytes using TaqMan® One-Step RT-PCR Master Mix Reagents Kit and ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). The primers and TaqMan® MGB probes designed by using Primer Express software (Applied Biosystems) were: PRV-1-1158F 5'-CAACCTTCCAGCTTCTTGTA-3', PRV-1-1219R 5'-TTCTCACGCAGCAGAGATC-3', and probe: PRV-1-1182T 5'-CACACCAGACAAATC-3’ labeled with the FAM fluorophore. The PRV-1 amplicon was based on the assay designed by Dr. Pahl (personal information). To normalize the PRV-1 expression level GAPDH mRNA and 18S ribosomal RNA were used as reference standards (the primers were GAPDH-284F 5'-ATGGAAATCCCATCACCATTT-3’, GAPDH-340R 5’-CGCCCCACTTGATTTTGG, h18S-542F 5’-TCGAGGCCCTGAATTGGAA -3’, h18S-602R 5’-CCCTCCAATGGATCCTCGTT-3’, the probes were GAPDH- 307T 5’-CAGGAGCGAGATCC-3’ labeled with the VIC fluorophore and h18S-564T 5’-AGTCCCACCTTTAATCTTT-3’ labeled with the FAM fluorophore). The primers were used at 900 nM, the probes at 100 nM concentration. We analyzed the expression of each gene (PRV-1, GAPDH and 18S) in separate reactions. We used the RNA amount giving the linear range of response for PRV-1,GAPDH and 18S, typically 10 ng of RNA per reaction for PRV-1 and GAPDH, and 0.1 ng for 18S. We used a universal RT-PCR protocol recommended by the manufacturer, i.e. reverse transcription at 48°C for 30 minutes, denaturation and polymerase activation at 95°C for 10 minutes, followed by 45 cycles of denaturation at 92°C for 15 seconds and annealing/extension/plate reading at 60 °C for 1 minute. RT-PCR reactions for each sample and each gene were done in triplicate. PCR without reverse transcriptase was performed for each sample to control for the possible interference from genomic DNA contamination. The threshold amplification cycles (CT) at the normalized
reporter signal minus the baseline signal level of 0.2 for PRV-1, GAPDH and 18S rRNA were
determined and their differences ΔCT(GAPDH-PRV1) and ΔCT(18S-PRV1) were calculated.
The cut-off for normal PRV-1 mRNA level was established as the 95% upper confidence
limit (mean + 1.96 standard deviations) of ΔCT(GAPDH-PRV1) and ΔCT(18S-PRV1)
analyzed in neutrophil RNA samples from 33 healthy volunteer donors. The PRV-1/GAPDH
ratio was calculated as $2^{ΔCT(GAPDH-PRV1)}$.

**Results**

*BTK and FHL1 Exonic Genotypes and TCA.*

To improve the usefulness of TCA, we searched for new X-chromosome exonic genomic
polymorphisms based on the following criteria: 1) exonic polymorphisms of sufficient
frequency which makes them suitable for transcriptional clonality assays; 2) their location on
the X chromosome is at a significant distance from the three polymorphic genes previously
used for TCA (G6PD, IDS and MPP1 located on chromosome Xq28) to be in linkage
equilibrium; 3) they are subject to X-chromosome inactivation; 4) they are expressed in
hematopoietic cells.

We examined two additional exonic X-chromosome polymorphic genes: Bruton tyrosine
kinase (*BTK*: C/T at cds #1899, dbSNP: 1135363) and four-and-a-half LIM domain1 (*FHL1:*
G/A at cDNA #1958, 3’-UTR, dbSNP: 9018). *BTK* is located at Xq21.3-q22, and *FHL1* at
Xq27.2. The genotypes for *BTK* and *FHL1* were first determined on 20 females that were
randomly chosen. Three different SSCP patterns were obtained on both *BTK* and *FHL1*
genes corresponding to the three different genotypes: homozygous T or C, or heterozygous T/C for
*BTK*; homozygous A or G, or heterozygous A/G for *FHL1* (Fig.1A). The genotypes of the six
females (all with a distinct SSCP pattern) were then confirmed by direct sequencing of the
PCR products.
We optimized the PCR-SSCP method for *MPP1* and *G6PD* genes. DNA from 20 females with known genotypes for *MPP1* and *G6PD* were subjected to the PCR-SSCP analysis. Identical results were obtained with PCR-SSCP (Fig.1B) and PCR-restriction enzyme digestion assays of *MPP1* and *G6PD* genes (data not shown).

Subsequently, we developed RT-PCR-SSCP for detecting the allelic transcripts of all five polymorphic genes *FHL1*, *BTK*, *MPP1*, *IDS* and *G6PD* (Fig.1C, D).

**FHL1 and BTK genes are subjects to X chromosome inactivation.**

In order to demonstrate that *FHL1* and *BTK* genes are X-chromosome inactivated, we tested the RNAs from platelets, granulocytes and T lymphocytes of four subjects with PV or ET who were also heterozygous for both *IDS* and *BTK/FHL1* exonic polymorphisms and were previously shown to be clonal using the *IDS* assay (RT-PCR-restriction enzyme digestion method) (Fig.1C). RT-PCR-SSCP analyses of *BTK* or *FHL1* genes on the same patients demonstrated that only one allele of the *FHL1* gene (Fig.1C) and the *BTK* gene (Fig.1C) were expressed in their platelets and granulocytes, while both alleles were expressed in T lymphocytes. This confirmed that *BTK* and *FHL1* genes are subject to X-chromosome inactivation.
Figure 1. Genotyping and expression of five X-chromosome exonic polymorphic genes. (A). Genotype determination and sequence analysis of BTK and FHL1. Sequencing using reverse primers is shown. (B). Genotypes of MPP1 and G6PD. (C). FHL1 and BTK are subject to X-chromosome inactivation. Clonal expression of IDS, FHL1 and BTK (shown here patient #38 and #37) was detected in platelets (PLT) and granulocytes (GNC) (the weak upper band of IDS expression in GNC was 3.35% of the major band which is clearly outside of the normal range), while T lymphocytes were polyclonal. (D). IDS, MPP1, and G6PD expression patterns using SSCP analysis (the RNA mixture of PLT and GNC was used). Lane 1 and 2: expression patterns of homozygosity in IDS, MPP1 and G6PD; lane 3: expression pattern of heterozygosity.

Polymorphic frequency of BTK and FHL1 genes in different U.S. ethnic groups.

The applicability of X-chromosome polymorphisms for TCA depends on their informativeness. Therefore, we studied 174 unrelated healthy females’ DNA. We found that BTK and FHL1 genes are highly polymorphic among all of the common U.S. ethnic groups (Caucasian, Hispanic, African-American and Southeast Asian). The frequency of
heterozygosity as depicted in Figure 2 was found to be 26-50% for \textit{BTK} and 41-54% for \textit{FHL1}. These two new polymorphisms in combination with previous markers-\textit{MPP1}, \textit{IDS} and \textit{G6PD} rendered \(~90\)% of the female subjects informative for clonality studies.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{Distribution of heterozygosity for X-chromosome exonic polymorphisms in common US ethnic groups. Caucasian (n=47), African-American (n=42), Asian (n=40), Hispanic (n=45). Overall: percentage of females heterozygous for at least one marker.}
\end{figure}

\textbf{Sensitivity, linearity, and reproducibility of the \textit{BTK} and \textit{FHL1} RT-PCR-SSCP assays.}

The sensitivity and linearity of the methods were tested by using serial dilutions of RNA from a healthy homozygous female. As shown in Figure 3, the assay was linear for the \textit{FHL1} RT-PCR-SSCP assayed in the range of 2-36 ng of total RNA per sample (\(r^2=0.98\)).
Similar data were generated for BTK assay (data not shown). The results were highly reproducible with inter test variation of 4%.

![Graph showing sensitivity and linearity of FHL1 TCA](image)

**Figure 3. Sensitivity and linearity of FHL1 TCA.** The RT-PCR-SSCP analysis of FHL1 showed a linear response in the range of 2 to 36 ng of total RNA per reaction ($r^2=0.98$). As little as 2 ng of total RNA can be detected.

**Studies of females with polycythemic and thrombocytemic disorders.**

We used five exonic X chromosome polymorphic markers (BTK, FHL1, MPP1, IDS and G6PD) to analyze the clonality of hematopoiesis on 46 informative patients. The T-lymphocyte fraction was used as a control non-clonal tissue for the X-chromosome inactivation patterns, since previous studies had shown that T-lymphocytes are not involved in myeloproliferative disorders;\(^{35,36}\) the data are shown in Table 2.
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Table 2: Clonality analysis, PRV-1 mRNA expression and the *in vitro* response of erythroid progenitors to erythropoietin in patients with polycythemias and thrombocytoses. Hb: hemoglobin; Plt: platelet. H or N indicates a high or normal value at diagnosis, respectively. Clonality: poly – polyclonal. PRV-1/GAPDH ratio of PRV-1 to GAPDH mRNA in granulocytes, the values in bold are increased above 95% confidence interval based on 33 healthy controls (mean PRV-1/GAPDH and 95% confidence interval for the 33 normal controls: 0.33, 0.023-3.45, respectively). BFU-E response to Epo: EEC – endogenous erythroid colonies growing in the absence of added Epo, HS – hypersensitivity of erythroid progenitors manifesting as the growth of erythroid colonies in presence of 30 and 60 mIU of Epo per ml of culture. Diagnosis: indicate the presumed clinical diagnosis. PV – polycythemia vera, UP – unexplained polycythemia, CFP – congenital or familial polycythemia, ET – essential thrombocytemia, FT – familial thrombocytosis. N.D. – not done. A – anagrelide, HU – hydroxyurea, P – phlebotomy, B – busulfan, IFN – interferon, No – no treatment.

**PRV-1 mRNA expression in granulocytes.**

*PRV-1* expression in granulocytes was assessed to test its diagnostic value in PV. Linear response between 100 ng and 0.1 ng of RNA per 20 µl reaction was observed for *PRV-1*, *GAPDH* and *18S* rRNA by real time RT-PCR analysis of RNA isolated from granulocytes of a typical PV patient. The RT-PCR results were highly reproducible, the mean inter test standard deviation calculated from 105 repeated assays was 0.8 amplification cycle (data not shown). *GAPDH* RNA as a normalizer gave a lower variance in the *PRV-1* expression values.
in 33 control samples (the standard deviation was 2.3 amplification cycles for ΔCT (18S-PRV1) and 1.7 cycles for ΔCT (GAPDH-PRV1). Therefore, we used the *GAPDH* normalizer to calculate the *PRV-1* expression level in the patients (Fig. 4).

![Figure 4](image)

**Figure 4.** *PRV-1* mRNA expression in granulocytes. *PV* – polycythemia vera, *CFP* – congenital or familial polycythemia, *ET* – essential thrombocythemia. The boxes represent the interquartile range which contains 50% of values. The bars are lines that extend from the box to the highest and lowest values. A line across the box indicates the median. *PRV-1/GAPDH* expression ratio (calculated as $2^{\Delta CT(GAPDH-PRV1)}$) was significantly increased in patients with polycythemia vera (**P**<0.0001) when compared with Mann-Whitney nonparametric test to 33 healthy controls or CFP patients.

Surprisingly, 4/13 *PV* patients did not have elevated *PRV-1* mRNA level in their granulocytes. Clinical and laboratory characteristics of these patients are shown in the Table 3.
Table 3: PV patients with low PRV-1 mRNA level in granulocytes and the diagnostic criteria for polycythemia vera.\textsuperscript{30}#The presence of elevated red cell mass and any three additional criteria are diagnostic of polycythemia vera.

Correlation of the TCA data with the \textit{in vitro} assay of erythroid progenitor responsiveness to Epo and \textit{PRV-1} mRNA expression in granulocytes.

The TCA data were correlated with \textit{PRV-1} mRNA neutrophil expression and the \textit{in vitro} assay of erythroid progenitor responsiveness to Epo. The results are summarized in Table 2. All patients with polycythemia vera had clonal hematopoiesis and endogenous erythroid colonies (EEC) growing without added Epo. \textit{PRV-1} expression in granulocytes was significantly increased it the group of PV patients (P<0.0001, Fig. 4), however, not all PV patients had increased \textit{PRV-1}. Normal level of \textit{PRV-1} expression in granulocytes was detected in 4/13 (31\%) of PV patients. Clonal hematopoiesis was detected in 8/12 essential thrombocythemia patients, EEC were observed in 4/12, and increased \textit{PRV-1} expression in 2/12 ET patients. All 15 patients with congenital or familial polycythemia had polyclonal hematopoiesis, 12/15 had erythroid progenitors hypersensitive to Epo (responding to low concentrations of Epo in vitro), while 1/15 of these patients had a slightly increased
expression of PRV-1. Polyclonal hematopoiesis was found in 2/2 patients with familial thrombocytosis, both patients had erythroid progenitors hypersensitive to Epo.

**Discussion**

We previously reported TCA for the X-chromosome genes *G6PD, MPP1* and *IDS* detected either by rtPCR-LDR or ASPCR.\(^{15-19}\) Although valuable, these methods have limitations. They were laborious, time consuming and their informativeness was limited by their lower degree of heterozygosity. In order to increase the percentage of informative females, we searched for new X-chromosome polymorphic markers. *BTK* and *FHL1* were two candidates since they both have exonic polymorphisms which make them suitable for clonality assays and they both are at a distant location on the X-chromosome compared to the three previously used X chromosome markers—*G6PD, IDS* and *MPP1*, thus these polymorphisms would be expected to be at the linkage equilibrium. Importantly, *BTK* and *FHL1* are expressed in hematopoietic cells.\(^{34,37}\) We chose to circumvent the technical difficulties encountered by the previous tests and after experimenting with various alternate approaches developed SSCP based analysis for genotype determination and evaluation of their allelic transcripts. SSCP analysis is a simple method,\(^{38,39}\) which has the potential to discriminate single base pair differences between DNA fragments. This method is based on the principle that the electrophoretic mobility of a single stranded DNA molecule in a non-denaturing gel is dependent not only on its length and molecular weight but also on its structure (i.e. conformation). A point mutation or polymorphism at a particular site in the primary sequence can change the conformation of the molecule, which alters its mobility during electrophoresis.\(^{40}\) Moreover, the employment of a touch-down program PCR in the genotype determination and the same one step RT-PCR conditions allowed simultaneous
discrimination of the genotypes and determination of their transcribed alleles. We report the accuracy, sensitivity (as little as 2 ng of total RNA can be detected), and simplicity of these improved TCA methods; the addition of the assays for detection of BTK and FHL1 exonic polymorphisms greatly increased the proportion of females informative for clonality studies. A possible shortcoming of these new TCA assays might be the limitation of their polymorphism to only some of the US ethnic groups. For example, the IDS exonic polymorphism has been the most informative transcriptionally based clonality assay for Caucasian and African females but was entirely uninformative for Southeast Asian females. 19 We report here the results of an analysis of 174 unrelated healthy females for BTK #1899 (at cds) and FHL1 #1958 (at cDNA, 3’-UTR) polymorphisms and show the high frequencies of heterozygosity in all four common US ethnic groups (see Figure 2), combining with three previously established TCA–G6PD, MPP1 and IDS, the percentage of informative female subjects is increased from ~65% to ~90%.

To be useful for clonality studies, the BTK and FHL1 genes must be subject to X chromosome inactivation, i.e. only one allele of the gene is detected in a clonal cell population; for BTK gene this was already suggested by previous studies of mothers of boys with X-linked agammaglobulinemia.41,42 We confirmed that BTK and FHL1 genes are subject to X-chromosome inactivation. Thus, these two exonic polymorphisms of BTK and FHL1 genes are suitable for clonality studies.

The diagnosis of myeloproliferative disorder such as polycythemia vera and essential thrombocythemia can be difficult. Polycythemic disorders include a large variety of disorders, of variable prognostic and therapeutic implications. While PV is characterized by a clonal hematopoiesis, other polycythemic disorders such as familial and congenital polycythemias (PFCP) or secondary polycythemias are polyclonal.43 We have used the assays for five
exonic X chromosome polymorphisms to determine the clonality of easily separated hematopoietic progenies – platelets and granulocytes in 46 informative females that were referred to us for evaluation of polycythemia and thrombocytosis. The demonstration of clonal hematopoiesis supported a diagnosis of myeloproliferative disorder in 22 patients with acquired polycythemia or thrombocytosis.

**Evaluation of subjects with polycythemic disorders.** Of 14 patients with PV, all had had EEC and were clonal, 9/13 patients had elevated PRV1 expression in granulocytes. One subject (#15), a heavy smoker, was found polycythemic at the hospitalization for GI bleeding with Hct of 53, WBC of 16,000/mm³, platelet counts of 580,000/mm³ and mild hepatosplenomegaly at the age of 32, and was diagnosed with PV. Now 15 years later she has normal liver and spleen size and normal blood count without any therapy. She has no EEC, normal PRV-1 expression, but her granulocytes and platelets are clonal. It is speculative whether the clonality data represent a) an acquired clonal hematopoietic disorder with a transient polycythemic phenotype, b) “pseudoclonality”;⁴⁴ or c) “somatic selection after X inactivation” such as seen in female carriers of some X-linked congenital disorders.⁴⁵,⁴⁶ Nineteen patients with congenital or familial polycythemic or thrombocytemic disorder had all polyclonal hematopoiesis. Of 17 subjects with congenital or familial polycythemia, 7 had elevated or inappropriately normal Epo level for their Hct, 5/7 were analyzed for mutation of von Hippel Lindau (VHL) gene; 2/5 had VHL mutations.⁴⁷ Remaining 8 subjects had low Epo level, hypersensitive erythroid progenitors to Epo and most had familial history fulfilling the criteria for the diagnosis of PFCP.⁴³ Interestingly, some of these polycythemic subjects with polyclonal hematopoiesis were previously diagnosed as PV while they had transient (presumably reactive) thrombocytosis or mild splenomegaly, and other minor laboratory criteria of clinical diagnosis of PV.⁴⁰
Although our study was not designed to prospectively follow patients, two patients with PV and clonal hematopoiesis were re-evaluated after treatment with interferon-α (IFNα), an agent successfully used in some patients with MPD. Although clinical remission after IFNα therapy has been reported (decrease in the size of the spleen, stabilization of hemoglobin with decreased need for phlebotomies), the reversal of a clonal hematopoiesis has not. Two of our studied patients (#6 and #14) paralleled their clinical response with reversion of clonal to polyclonal hematopoiesis.

**Evaluation of subjects with thrombocytosis.** Fourteen patients were referred for clarification of thrombocytosis, all but 2 with familial thrombocytosis were considered to have ET, 8 were clonal. The fact that some patients with clinical diagnosis of ET are polyclonal was first reported by El Kassar and later also by others. In our view this fact underlies the difficulties in diagnosing ET and limitations of the clinical phenomenological criteria as the sole diagnostic means. The development of specific laboratory tests for ET diagnosis are sorely needed, albeit some are now being tested. Four clonal subjects with clinical diagnosis of ET (out of 8) had EEC. It should be noted that YL Shi and co-workers had studied a number of subjects with thrombocytosis presenting with normal hematocrit and EEC; most of these during several year follow-up had eventually developed full PV phenotype. We have had a similar experience; at least 4 individuals with a similar clinical course had been followed in our clinic (JTP, unpublished). Six subjects with diagnosis of ET by their hematologist were polyclonal, of these 4 has had significant thrombotic complications, 2 had familial history of thrombocytosis, and 4/6 subjects had hypersensitive erythroid progenitors to Epo. Another patient (#42) in our series with a presumed diagnosis of ET was on hydroxyurea (HU) for more than five years and was found to have polyclonal hematopoiesis. Although there is a possibility that this patient underwent a complete
remission on HU treatment, we know of no other patients where a reversal of clonal hematopoiesis occurred after HU therapy. However, this conclusion is speculative since her peripheral blood was not assayed for clonality prior to the HU therapy.

**PRV-1 mRNA expression.** We confirmed the observations that the increased *PRV-1* mRNA expression level in granulocytes is a useful diagnostic marker for polycythemia vera,27-29 however, it was not entirely specific for PV and some of our PV patients were found to have normal levels of *PRV-1* expression. We compared two different internal standards for quantification of *PRV-1* mRNA: *GAPDH* mRNA used in the *PRV-1* original studies28,29 and *18S* rRNA. The usefulness of *GAPDH* mRNA as a normalizer for gene expression studies has been challenged since its expression can be affected by many factors52 including hypoxia53 a disturbed response to which is a pathophysiological basis of some polycythemic disorders.43,54 On the other hand, the 100- to 1000-fold variation of the *18S* rRNA content has been reported in early hematopoietic progenitors CD34+CD38- cells where *GAPDH* mRNA levels in these cells provided a better reference.55 We show that both *18S* rRNA and the *GAPDH* mRNA levels can be used as a reliable reference for normalization of *PRV-1* expression; the relative advantage of *GAPDH* mRNA as normalizer is that its level is close to the level of *PRV-1* mRNA and the same dilution of RNA can be used for determining the expression of both genes.

We have carefully considered reasons for finding in our studied samples *PRV-1* assay to be less specific for PV than reported by Dr. Pahl’s group.28 It is possible that some of our patients did not represent a typical PV picture, since a significant number of our subjects are the patients who have been referred to us by other hematologists; admittedly because of they were considered a diagnostic challenge. Our laboratory has had extensive experience with erythroid progenitor assays for over two decades and the availability of standardized
commercial reagents in last decade made this assay when rigorously performed very reproducible and reliable. In addition, we have had three decades experience with various clonality assays, many developed by our group. Other patients were referred for additional studies including erythropoietin receptor analyses, clonality studies, searching for VHL gene mutations etc. However, as shown in Table 3 the clinical and routine laboratory characteristics of the 4 PV subjects with normal \( PRV-1 \) mRNA level fulfilled PV diagnostic criteria.

The comparison of various diagnostic assays reported to be useful for discrimination of polycythemic disorders and thrombocytoses demonstrated that the clonality assays significantly contribute to the clarification of the diagnostic dilemma posed on practicing hematologist. Our concomitant performance of \( PRV-1 \) assay, erythroid progenitor assay, clonality and \( PRV-1 \) analyses for differential diagnoses of difficult to classify polycythemic and thrombocytosis conditions augments the specificity and diagnostic usefulness of these diagnostic tests.
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Discrimination of polycythemias and thrombocytoses by novel, simple, accurate clonality assays and comparison with PRV-1 expression and BFU-E response to erythropoietin

Enli Liu, Jaroslav Jelinek, Yves D Pastore, Yongli Guan, Jaroslav F Prchal and Josef T Prchal