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Comparison of Methods for Polycythemia Rubra Vera-1 mRNA Quantification in Whole-Blood Leukocytes and Purified Granulocytes, Lars Palmqvist,1 Philipp Goertler,4 Carina Wasslavig,1 Peter Johansson,2,3 Björn Andreasonn,2,3 Soodabeh Safai-Kutti,2 Jack Kutti,2 Heike L. Pahl,4 and Anne Ricksten1* (1 Department of Clinical Chemistry and Transfusion Medicine, Institute of Laboratory Medicine and 2 Hematology and Coagulation Section, Department of Medicine, Sahlgrenska University Hospital, Göteborg, Sweden; 3 Department of Medicine, Uddevalla Hospital, Uddevalla, Sweden; 4 Department of Experimental Anaesthesiology, University Hospital, Freiburg, Germany; † address correspondence to this author at: Department of Clinical Chemistry and Transfusion Medicine, Sahlgrenska University Hospital, SE-413 45 Göteborg, Sweden; fax 46-31-828458, e-mail anne.ricksten@clinchem.gu.se)

In the absence of pathognomonic markers, the diagnosis of the two chronic myeloproliferative disorders polycythemia vera (PV) and essential thrombocytosis (ET) has relied on a set of clinical and laboratory criteria (1–5). The cloning of the cell surface receptor polycythemia rubra vera-1 (PRV-1) has recently been described (6), and the consistent overexpression of PRV-1 mRNA observed in PV patients indicates that this might constitute a new diagnostic marker for the disease. In the initial cohort examined by Northern blot analysis, PRV-1 expression was increased in all PV patients examined as well as in some ET patients, but not in healthy controls (6). These results have also been verified and extended using a quantitative reverse transcription-PCR (RT-PCR) assay. All PV as well as 50% of ET patients displayed increased PRV-1 expression (7, 8). Patients with secondary erythrocytosis and healthy controls tested showed PRV-1 concentrations within the reference interval. Interestingly, the observed increase in PRV-1 mRNA expression does not lead to a corresponding increase in protein expression on the cell surface (9). Erythropoietin-independent colony growth and PRV-1 overexpression seem to go hand in hand in both PV and ET patients (7), raising the hope that RT-PCR for PRV-1 could replace the need for the technically demanding erythropoietin-independent colony assay, although a recent report suggests that the erythropoietin-independent colony growth assay is a more reliable method (10). The aim of the present work was to develop a quantitative RT-PCR method to measure PRV-1 transcripts in whole-blood leukocytes and to determine the potential usefulness of the method in the differential diagnosis of polycythermas and thrombocythias. The assay was compared with a method using isolated granulocytes (7, 9) to assess whether granulocyte purification is necessary before RNA extraction. Granulocyte fractionation is cumbersome to standardize, and an assay that uses whole-blood leukocytes would simplify the analysis and perhaps make it more suitable for a routine laboratory setting.

Blood samples were collected from 78 patients with
well-characterized myeloproliferative diseases, 25 with PV and 53 with ET (Table 1). At the time of the study, 92% of the PV patients and 66% of the ET patients were receiving therapy with myelosuppressive agents in addition to phlebotomies (Table 1). Duplicate samples were transferred to the two laboratories that participated in the study for analysis of PRV-1 expression by their respective methods. The samples arrived within 24 h from the time of sampling to both laboratories. All patients fulfilled the Polycythemia Vera Study Group criteria as well as the additional criteria proposed for their respective diagnoses (1–5). The study was approved by the Ethics Committee of Göteborg University, and informed written consent was obtained from all participants. Details for the laboratory procedures, assay performance, and establishment of reference intervals for the two methods are given in the Data Supplement that accompanies the online version of this Technical Brief at http://www.clinchem.org/content/vol50/issue3/ as well as in the text and in the report by Klippel et al. (8).

Both RT-PCR assays used in this report are quantitative, which was not the case in some of the earlier reports (6, 11). The cycle threshold (C_T) for PRV-1 was determined by real-time PCR and was divided by the C_T value for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript. Consequently, a lower PRV-1:GAPDH C_T ratio indicates higher PRV-1 mRNA expression. Both methods had approximately the same assay imprecision, as determined by their between-day CV (3%). A PRV-1:GAPDH C_T ratio <0.93 indicated increased expression of PRV-1 in peripheral whole-blood leukocytes, and a C_T ratio <1.16 indicated increased expression of PRV-1 in purified granulocytes.

The PRV-1:GAPDH C_T ratios did not follow a gaussian distribution for the patients; therefore, the Kruskal–Wallis nonparametric method was used to determine the significance of the difference between the groups. All individual results can be found in Table 1 of the online Data Supplement. With either assay, the differences between PV and ET patients vs healthy controls were significant (P <0.0001), as was the difference between the PV and ET patients (P <0.0001). In 23 of the 25 PV patients, PRV-1 was overexpressed in whole-blood leukocytes. PRV-1 was overexpressed in the same number of patients when purified granulocytes were used for the assay. One patient was found to express normal amounts of PRV-1 by both methods. One additional PV patient had normal PRV-1 expression when measured with one or the other method, but the PV patient with normal PRV-1 expression in granulocytes but overexpression in leukocytes was one of two outliers in the method comparison (see below) and was excluded because we suspected that this sample was a mix up with the other outlier, an ET patient. This gave an assay sensitivity of 92% (23 of 25) for PV patients when we used whole-blood leukocytes and 96% (23 of 24) for PV patients when we used purified granulocytes. Thus, the assay sensitivity for PV does not seem to be severely decreased by omitting granulocyte purification from whole-blood leukocytes. The fact that two PV patients did not show increased PRV-1 expression in one or both assays suggests that treatment could have some effect on PRV-1 expression.

It has been shown in a recent study that two PV patients reverted from clonal to polyclonal hematopoiesis after interferon-α treatment (12), indicating that treatment could affect PRV-1 expression. This study also reported several PRV-1-negative PV patients (4 of 13). A treatment effect is also supported by the finding that interferon-α might normalize PRV-1 expression (13). In contrast, the PV patient who was normal in both of the RT-PCR assays used in the present study has never been treated with interferon-α, but with hydroxyurea instead. It is unclear whether this patient, who fulfilled all of the criteria for PV, has ever been PRV-1-positive. Both patients who were PRV-1-negative in the whole-blood leukocyte assay have been reanalyzed with concordant results. Furthermore,

### Table 1. Clinical characteristics of the patients with PV and ET.

<table>
<thead>
<tr>
<th></th>
<th>PV</th>
<th>ET</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>25</td>
<td>53</td>
</tr>
<tr>
<td>M/F</td>
<td>15/10</td>
<td>20/33</td>
</tr>
<tr>
<td>Mean (SD) [range] age, years</td>
<td>65 (11) [26–78]</td>
<td>61 (16) [27–89]</td>
</tr>
<tr>
<td>Median (range) duration of the disease, years</td>
<td>5.0 (0.5–33)</td>
<td>4.0 (0.3–21)</td>
</tr>
<tr>
<td>Current treatment, n</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydroxyurea</td>
<td>18</td>
<td>19</td>
</tr>
<tr>
<td>Radiophosphorus</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>Interferon α</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>Anagrelide</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>No myelosuppressive treatment</td>
<td>2</td>
<td>18</td>
</tr>
<tr>
<td>PRV-1:GAPDH C_T ratio*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole-blood leukocytes</td>
<td>0.80 (0.08) [0.77–0.83]</td>
<td>0.99 (0.10) [0.96–1.02]</td>
</tr>
<tr>
<td>Positive results/number tested</td>
<td>23/25</td>
<td>14/53</td>
</tr>
<tr>
<td>Purified granulocytes</td>
<td>1.01 (0.08) [0.97–1.04]</td>
<td>1.17 (0.08) [1.14–1.19]</td>
</tr>
<tr>
<td>Positive results/number tested</td>
<td>23/24</td>
<td>24/52</td>
</tr>
</tbody>
</table>

* A PRV-1:GAPDH C_T ratio <0.93 is considered to indicate PRV-1 overexpression in whole-blood leukocytes, and a ratio <1.16 is considered to indicate overexpression in isolated granulocytes. Values are the mean (SD) [95% confidence interval].
we found no significant difference in PRV-1 expression between treated (n = 35) and untreated (n = 18) ET patients (whole-blood leukocytes, \( P = 0.32 \); isolated granulocytes, \( P = 0.88 \), Kendall rank correlation) that would indicate any inhibitory or stimulatory effect on PRV-1 expression by myelosuppressive treatment. PRV-1 was overexpressed in 14 of 53 (25%) whole-blood leukocyte samples from ET patients. In contrast, 24 of the 52 ET patients (46%) displayed overexpression when purified granulocytes were analyzed. Ten patients who were PRV-1-negative in the whole-blood leukocyte assay showed PRV-1 overexpression in the granulocyte assay. No patient was found to be positive in the whole-blood leukocyte assay but negative in isolated granulocyte assay. The difference between the two methods in detecting PRV-1 overexpression in ET patients can to some extent be explained by the fact that nongranulocytes (e.g., lymphocytes) contribute GAPDH transcripts but no PRV-1 transcripts when whole blood is used for the RNA preparation (6). We also observed a significant correlation between PRV-1 expression in whole-blood leukocytes and lymphocyte count (\( P = 0.014 \)). However, regression analysis demonstrated a poor fit (\( r^2 = 0.10 \); data not shown), which indicates that additional factors other than lymphocyte number contribute to the difference between the two methods. When whole-blood leukocytes were used, a correlation between PRV-1 overexpression and increased risk for vascular complications was detected in ET patients (14) as well as an association between PRV-1 overexpression and low plasma erythropoietin concentrations (15). These findings indicate that the whole-blood leukocyte assay is a good predictor for patients with a more aggressive ET.

A comparison of PRV-1 expression in whole-blood leukocytes and purified granulocytes is shown in Fig. 1. Deming regression analysis was used to compare the two methods. A regression residual plot between the two methods identified two possible outliers that were outside \( \pm 4 \) SD, and the samples were excluded from the comparison (Fig. 1A). Deming analysis detected both a constant difference and a proportional difference. The presence of a constant difference between the two assays (-0.38; 95% confidence interval, -0.49 to -0.26) was expected because non-PRV-1-expressing cells were included in one of the methods. The proportional difference (slope, 1.17; 95% confidence interval, 1.07–1.27) might reflect the differences in sample preparation and analysis procedures (e.g., RNA preparation method and PCR primer choice) for the two methods. However, the numeric results for the samples analyzed in the two laboratories are clearly linear, and the regression analysis showed a high correlation coefficient (\( r = 0.94 \); 95% confidence interval, 0.90–0.96; Fig. 1B).

In conclusion, our study shows that a quantitative RT-PCR assay of PRV-1 expression in whole-blood leukocytes is a potentially valuable diagnostic tool in the differential diagnosis of polycythemia, especially if the granulocyte purification procedure is unfeasible. The potential usefulness of this method in differential diagnosis and prognosis prediction of thrombocytosis was indicated in two previous studies (14,15), but it needs further investigation. Furthermore, the finding of normal PRV-1 expression in PV patients as well as the possibility that treatment might affect PRV-1 expression needs further investigation. These questions can only be answered in prospective studies involving newly diagnosed patients whose PRV-1 expression is followed consecutively after initiation of different therapeutic regimes.

![Fig. 1. Results of Deming analysis comparing the two quantitative RT-PCR methods for PRV-1 expression analysis in whole-blood leukocytes and purified granulocytes, respectively.](image-url)
This study was supported by grants from “FoU Västra Götaland”, “Stiftelsen Jubileumsklinikens Forskningsfond mot Cancer”, and “Assar Gabriellsions Foundation” as well as the Alfried-Krupp-Förderpreis and the SFB 364 (TP A12).

References
5. Kralovics R, Buser AS, Teo SS, Coers J, Tichelli A, van der Maas AP, et al. The presence of a significant association between IL-1 and the inheritance of obesity does not, however, follow the classic Mendelian patterns that are characteristic of single-gene disorders. Multiple regions of the genome are likely to contain susceptibility genes for obesity and associated phenotypes (3, 4).

Cytokines appear to be major regulators of adipose tissue metabolism. The effects of cytokines within adipose tissue include actions that might be characterized as metabolic. The cytokine tumor necrosis factor-α (TNF-α) is expressed primarily in adipocytes and modulates the expression of several genes in adipose tissue (5, 6). In obese individuals, increased TNF-α correlates strongly with hyperinsulinemia (6). A polymorphism upstream of the transcription start site of TNF-α (G to A at position −308) has been associated with obesity in several European populations (7, 8). In Sweden, the polymorphism influences body fat content only in homozygous individuals (9). Interleukin-1β (IL-1β), like TNF-α, suppresses adipose differentiation and lipoprotein lipase expression and activity (10). IL-1β stimulates lipolysis and inhibits lipogenesis by inhibiting the expression of fatty acid transport protein and fatty acid translocase in adipose tissue (11). IL-1β is significantly more potent than TNF-α and other cytokines in inducing anorexia when administered into the brain (12, 13). These studies suggest that IL-1β may be protectively involved in the onset and progression of weight gain.

Most genes coding for the IL-1 family of proteins and clustered on the 2q12-q21 locus (IL-1α, IL-1β, and the IL-1 receptor antagonist) are polymorphic in multiple loci (14). Three diallelic polymorphisms in IL-1β, all representing C-to-T base transitions, have been reported at positions −511, −31, and +3953 bp from the transcriptional start site (15–17). For the IL-1β +3953 C/T polymorphism, individuals homozygous for the T allele have a fourfold increase in the production of IL-1β compared with individuals homozygous for the C allele (17, 18). We hypothesized that the increased expression of IL-1β as a consequence of the polymorphism can be protective against obesity. We therefore designed this study to investigate whether the IL-1β +3953 C/T polymorphism is associated with obesity in Korean women and to examine the impact of this variant on changes in body mass index (BMI).

The participants were recruited consecutively from the hospital’s obesity clinic to an ongoing project to investigate candidate genes for obesity among the Korean population. All were nonsmokers and had no evidence of cancer, liver, renal, or hematologic diseases or metabolic disorders other than obesity. A total of 257 women met all study criteria and were enrolled into the study. Ages were limited to those between 18 and 47 years, and postmenopausal women were excluded. BMI ranged from 19.2 to 39.1 kg/m². To obtain a better separation among phenotypes, the participants were divided into three BMI groups according to the WHO definitions, with minor modifications: lean (BMI <25.0 kg/m²), overweight (BMI, 25.0–29.9 kg/m²), and obese (BMI, 30.0–40.0 kg/m²). All participants (Korean) gave informed consent before

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Although environmental factors are clearly important determinants of obesity, heredity may contribute to the etiology of obesity (1, 2). The inheritance of obesity does...