Comparison of molecular markers in a cohort of patients with chronic myeloproliferative disorders

Robert Kralovics, Andreas S. Buser, Soon-Siong Teo, Jörn Coers, Andre Tichelli, Anthonie P. C. van der Maas, and Radek C. Skoda

Decreased expression of c-MPL protein in platelets, increased expression of polycythemia rubra vera 1 (PRV-1) and nuclear factor I-B (NFIB) mRNA in granulocytes, and loss of heterozygosity on chromosome 9p (9pLOH) were described as molecular markers for myeloproliferative disorders (MPDs). To assess whether these markers are clustered in subgroups of MPDs or represent independent phenotypic variations, we simultaneously determined the c-MPL, PRV-1, NFIB, and 9pLOH status in a cohort of 44 MPD patients and 18 healthy control individuals.

Study design

The study was approved by the Ethics Committee of Basel. Blood was obtained with written consent. The diagnosis of MPD subtypes was assigned using the World Health Organization (WHO) criteria. To prevent influence from genomic DNA amplification, the primers for RPL19, PRV-1, and NFIB were designed across exon-intron junctions. The primers for RPL19 were GA TGCCGGAAAAACACCTTG, TGGCTGTAC CCTTCCGCTT, CCTATGCCCATGTCGCTGCTT (probe); for PRV-1: CCCAGACAGACAGGAA, TTGTCCTCTCCAGACAGCC, CAT AGACAAGACAGACTTGGCAACTCAA (probe); and for NFIB: CAG TCCAAACACACAGCAGTC, GCCGGTTAAGATGGGTCTCCTA, GAAAGGAACCAAGCTAGCCCAGGTACCA (probe). The probes were dual-labeled with 5’-6-carboxytetramethylrhodamine (FAM) and 3’-carboxyfluorescein (TAMRA). The primers for MPL used for SYBR-PCR were AGCCCTGAGCCCGCC and TCCACTTCCACAGGATCTGAGA. The ΔCt values were derived by subtracting the threshold cycle (Ct) values for PRV-1, NFIB, and c-MPL from the Ct value for ribosomal protein L19 (RPL19), which served as an internal control. All reactions were run in duplicate using the ABI 7000 Sequence Detection System (Applied Biosystems, Foster City, CA).

Real-time polymerase chain reaction

Total RNA (2 µg) was reverse transcribed after random hexamer priming. To prevent influence from genomic DNA amplification, the primers for MPL, PRV-1, and NFIB were designed across exon-intron junctions. The primers for RPL19 were GATGCCGA'AACACCTTG, TGGCTGTAC CCTTCCGCTT, CCTATGCCCATGTCGCTGCTT (probe); for PRV-1: CCCAGACAGACAGGAA, TTGTCCTCTCCAGACAGCC, CAT AGACAAGACAGACTTGGCAACTCAA (probe); and for NFIB: CAG TCCAAACACACAGCAGTC, GCCGGTTAAGATGGGTCTCCTA, GAAAGGAACCAAGCTAGCCCAGGTACCA (probe). The probes were dual-labeled with 5’-6-carboxytetramethylrhodamine (FAM) and 3’-carboxyfluorescein (TAMRA). The primers for MPL used for SYBR-PCR were AGCCCTGAGCCCGCC and TCCACTTCCACAGGATCTGAGA. The ΔCt values were derived by subtracting the threshold cycle (Ct) values for PRV-1, NFIB, and c-MPL from the Ct value for ribosomal protein L19 (RPL19), which served as an internal control. All reactions were run in duplicate using the ABI 7000 Sequence Detection System (Applied Biosystems, Foster City, CA).

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**EEC assay**

The clonogenic cultures for erythropoietin-independent colony formation (EEC assay) were performed using commercial reagents (Stem Cell Laboratories, Vancouver, BC, Canada).

**Detection of 9pLOH**

Three highly polymorphic microsatellite markers D9S1779, D9S157, and D9S161 were used to detect LOH in granulocyte DNA samples as described.9

**Results and discussion**

To determine whether alterations in platelet c-MPL protein expression, granulocyte PRV-1, and NFIB mRNA levels, growth of EECs, and the presence of 9pLOH represent independent phenotypic variations, we examined 44 patients with MPD (23 PV, 15 ET, and 6 IMF) (Figure 1A). As controls, 18 healthy individuals were included (Figure 1B). Decreased expression of c-MPL protein was found in 30% of patients with PV (7 of 23), 40% of ET (6 of 15) and 67% of IMF (4 of 6). Thus, c-MPL cannot be used as a diagnostic test for PV. To assess whether lower expression of c-MPL is specific for MPD, we examined a family with hereditary thrombocythemia (HT). In this family, thrombocytosis is caused by elevated TPO serum levels due to a splice donor mutation in the TPO gene.13 We found lower expression of c-MPL protein in 7 of 8 affected individuals (88%), despite normal c-MPL mRNA levels (Figure 1C). Hence, decrease of c-MPL protein also can occur in patients who display sustained thrombocytosis caused by a molecular mechanism different from sporadic MPD.

PRV-1 mRNA was elevated in 91% of patients with PV (21 of 23), 67% of ET (10 of 15), and 67% of IMF (4 of 6), whereas growth of EEC was present in 100% of patients with PV (20 of 20), 69% of patients with ET (9 of 13), and 60% of patients with IMF (3 of 5). Our results show a higher detection rate of PV than a recently published study, which reported that only 69% of PV patients (7 of 13) had elevated PRV-1.14 This discrepancy might be due to the smaller number of patients examined in the previous report. Using the Agilent 2100 Bioanalyzer, we excluded degradation of the RNA as a potential explanation for low PRV-1 in EEC-positive individuals. Another difference between the 2 studies is that our PRV-1 assay is based on primers that span exon-intron boundaries and should therefore be less influenced by DNA contamination in the patient RNA samples. Irrespective of the MPD subtype, we observed a strong correlation between PRV-1 and EEC: 84% of patients with EECs had elevated PRV-1 (27 of 32) and, conversely, 94% of patients with elevated PRV-1 were EEC positive (29 of 31). Interestingly, we also detected increased expression of PRV-1 in 3 of 4 affected HT family members who were available for analysis (Figure 1C). PRV-1 expression in these individuals did not reach the very high levels observed in most patients with sporadic MPD. Cytokines can influence PRV-1 expression, for example, treatment of normal granulocytes with granulocyte colony-stimulating factor increased PRV-1 mRNA.2 It remains to be examined whether elevated TPO levels in HT patients might indirectly influence PRV-1. Importantly, EECs were negative in all affected family members (Figure 1C). Thus, EECs remain the most reliable auxiliary diagnostic assay for PV.

Loss of heterozygosity on the short arm of chromosome 9 (9pLOH) was recently reported to be the most frequent genetic lesion in PV, affecting approximately 30% of PV patients.9 In comparison, deletion of 20q and various chromosome 9 aberrations were previously reported in 15% and 21% of PV patients, respectively.15,16 In our present study, 7 of 23 PV patients (30%)...
displayed 9pLOH, confirming the previously observed 9pLOH frequency. The 9pLOH also was present in 1 patient with ET. Interestingly, all 8 individuals with 9pLOH were EEC positive and also displayed elevated PRV-1. Expression of NFIB was hypothesized to be a consequence of 9pLOH.9 Our study detected increased NFIB expression in 8 MPD patients with EEC (5 PV, 1 ET, and 2 IMF), but none of these individuals had 9pLOH, indicating that increased expression of the NFIB gene is independent of the presence of 9pLOH. The correlation between 9pLOH and increased NFIB in the original report was most likely a coincidence within a small cohort of 3 patients. No increase in NFIB mRNA was noted in HT family members (Figure 1C).

The concordance between EEC and PRV-1 markers in MPD patients across all diagnostic subclasses suggests that they might be caused by a common molecular mechanism. In contrast, decreased expression of c-MPL appears to be an independent phenotypic variant. Interestingly, low c-MPL and high PRV-1 also can occur secondary to elevated TPO in patients with HT. Increased NFIB expression and presence of 9pLOH are independent of each other but were detected only in patients with EEC and/or high PRV-1. Larger cohort studies will be necessary to determine whether MPD subgroups defined by these molecular markers differ in respect to clinical outcome, for example, leukemic transformation or hemorrhagic and thrombotic complications.

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