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Plasma levels of *JAK2* mRNA in patients with chronic myeloproliferative diseases with and without V617F mutation: implications for prognosis and disease biology

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SUMMARY

The association of V617F *JAK2* expression levels with disease behavior has not been studied in patients with nonchronic myelogenous leukemia (CML) myeloproliferative disease (MPD). We found plasma levels of total *JAK2* mRNA to be higher in patients with non-CML MPD ($n = 175$) than in CML patients ($n = 45$) and normal controls ($n = 58$) (each $P < 0.001$). Overall survival was studied in 68 patients and showed positive correlation with levels of total and mutant *JAK2* mRNA in patients with the V617F mutation, but not those without the mutation. These findings suggest that total *JAK2* expression levels play a role in the biology of the disease in V617F-positive patients, and a therapy aiming at downmodulating the expression of the total *JAK2* mRNA should be considered. In conclusion, we studied JAK2 total and V6217F mutant mRNA levels in plasma. We show high levels of JAK2 expression in MPD patients and these levels correlate with survival.

Keywords

Polycythemia vera; idiopathic myelofibrosis; essential thrombocythemia; *JAK2*; mutation; plasma; exon 12

INTRODUCTION

The dominant gain-of-function V617F point mutation in the *JAK2* gene is now considered the most important molecular abnormality characterizing patients with nonchronic myeloid leukemia (CML)-myeloproliferative diseases (MPDs). This mutation is found in nearly all patients with polycythemia vera (PV) and approximately 50% of patients with idiopathic myelofibrosis (IMF) or essential thrombocythemia (ET) (Baxter *et al.*, 2005; James *et al.*,

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2005; Jones *et al.*, 2005; Kralovics *et al.*, 2005; Levine *et al.*, 2005; Steensma *et al.*, 2005; Zhao *et al.*, 2005; Nelson *et al.*, 2006).

JAK2 encodes a nonreceptor tyrosine kinase responsible for signal transduction from type 1 cytokine receptors – such as the erythropoietin receptor (EpoR) – that acts by phosphorylation and activation of the STAT5 transcription factor (Schindler, 2002). The V617F mutation results in substitution of phenylalanine for valine at amino acid 617 (V617F), located in the JH2 pseudokinase domain that normally autoinhibits JAK2 kinase activity (Schindler, 2002; Baxter *et al.*, 2005; James *et al.*, 2005; Jones *et al.*, 2005; Kralovics *et al.*, 2005; Levine *et al.*, 2005). The V617F mutant kinase thus displays constitutive activity that is independent of growth factors (James *et al.*, 2005; Levine *et al.*, 2005; Ma *et al.*, 2006). Recently, four somatic gain-of-function mutations in *JAK2* exon 12 were reported in patients with a V617F-negative form of PV. These exon 12 mutations were found mainly in isolated erythroid colonies in heterozygous form (Scott *et al.*, 2007). Although *JAK2* mutation results in constitutive activation, the association of *JAK2* expression levels with disease behavior in patients with non-CML MPDs has not been investigated.

In patients with hematologic diseases, the plasma is enriched with tumor-specific DNA, RNA and proteins (Manshouri *et al.*, 2003; Rogers *et al.*, 2004; Ma *et al.*, 2007). This enrichment results from the high turnover of tumor cells and allows the detection of *JAK2* mutations in plasma samples (Ma *et al.*, 2006). Furthermore, our previous findings demonstrated that use of plasma allows more sensitive detection of *JAK2* mutations than with peripheral blood cells (Ma *et al.*, 2006). In this study, we evaluated the association of plasma levels of *JAK2* mRNA with clinical behavior in patients with non-CML MPDs. Reverse transcription-polymerase chain reaction (RT-PCR)-based assays were used to measure both wild-type and V617F mutant mRNA. To assess potential implications of *JAK2* mRNA expression levels for disease biology, we also compared expression in patients with CML and non-CML MPDs. CML was selected as a MPD with similar increase in white cell count, but a leukemogenic process that depends on the BCR-ABL and not the *JAK2* pathway.

METHODS

Patient samples

The diagnosis of the studied patients (175 non-CML MPD, 45 CML) was confirmed by morphology and cytogenetic studies. A subset of non-CML MPD patients ($n = 68$) had complete clinical, morphological, cytogenetic, laboratory and survival data. Samples were collected and testing according to institutional guidelines and an IRB-approved protocol. Plasma was prepared from ethylenediaminetetraacetic acid anticoagulated peripheral blood samples by centrifugation and stored at −70 °C until assay. No preservative or special treatment was used in collecting plasma. All samples were processed within 48 h of collection. Samples that had complete clinical data were frozen within 24 h of collection. The Hel (92.1.7) cell line used in mixing studies was obtained from the American Type Culture Collection (Manassas, VA, USA) and was maintained in RPMI 1640 medium with 10% fetal calf serum.

Determination of JAK2 mutation status

Total nucleic acid was extracted from plasma and peripheral blood cells using the NucliSens extraction kit (bioMerieux Inc, Durham, NC, USA) according to the manufacturer's instructions. One-step RT-PCR was performed in a 25-μl reaction volume using SuperSript III one-step RT-PCR systems with Platinum Taq (Invitrogen, Carlsbad, CA, USA). Concentrations used for RT-PCR were as follows: 1× reaction buffer, 400 nM of each of the forward and reverse *JAK2* primers, 1 unit of SuperScript III and 5 μl of the RNA template. The thermocycler conditions were 30 min at 55 °C for reverse transcription, followed by 2 min at 94 °C and 40 cycles of 94 °C for 15 s, 60 °C for 30 s, and 68 °C for 1 min, with a final step of 68 °C for 7 min.

The following primers were used (uppercase letters indicate the actual sequences and lower case letters represent the M13 linker used for sequencing): 5′-tgt aaa acg acg gcc agt CTA AAT GCT GTC CCC CAA AG-3′; and 5′-cag gaa aca gct atg acc CCA TGC CAA CTG TTT AGC AA-3′. The amplification products and sequenced fragment were 491 bp. The PCR product was filtration-purified using a Multiscreen PCR plate (Millipore, Billerica, MA, USA) and then sequenced in both forward and reverse directions using the ABI Prism BigDye® Terminator v3.1 Cycle Sequencing Kit and the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequencing data were base-called by sequencing analysis software and assembled and analyzed with ABI Prism® SeqScape software (Applied Biosystems, Foster City, CA, USA) using the *JAK2* sequence in GenBank (accession number NM 004972) as a reference.

Quantification of JAK2 mRNA and DNA in plasma

Total *JAK2* PCR product was amplified and quantified from MPD patient RNA using real-time RT-PCR with a 6-point, 5-log external RNA standard. The PCR product was then filtration-purified and sequenced in forward and reverse directions using an ABI PRISM 3730XL genetic analyzer. Sequencing data were base-called and V617F mutant mRNA was quantified by mutation surveyor software using GenBank accession number NM 004972 as reference. The tumor load (pg *JAK2* V617F/ml plasma) was reported by multiplying the total *JAK2* mRNA (pg/ml) from the real-time result by the % V617F *JAK2* mRNA from sequencing analysis.

The quantitative *JAK2* V617F mutation assay detects the V617F tumor load in absolute terms: pg *JAK2* V617F/ml plasma. It combines the TaqMan real-time and sequencing technology with the application of the Mutation Surveyor's DNA quantification feature. A similar approach was used for quantification of *JAK2* DNA, except the following primers were used: 5′-AGCAAGCTTTCTCACAAGCA-3′ and 5′-CTGACACCTAGCTGTGATCCTG-3′. The probe sequence was 5′-CAGGCTTTCTAATGCCTTTCTCAGAGCA-3′. The Mutation Surveyor V617F quantification uses GenBank accession number NT 008413 as reference. Detected levels from eight samples collected and frozen at 24 h were compared with levels obtained from the same samples frozen at 48 h. The CV between the two groups was <20%.

Statistical analysis

The significance of differences in clinical characteristics between groups was analyzed by chi-squared or Kruskal–Wallis test for categorical data and *t*-test for continuous data. Estimates of survival curves were calculated according to the Kaplan–Meier product-limit method and were calculated from the time of referral to MD Anderson Cancer Center. Survival times were compared by means of the log-rank test.

RESULTS

Higher levels of plasma JAK2 mRNA in patients with non-CML MPDs

Plasma levels of *JAK2* mRNA were significantly higher in patients with non-CML MPD ($n = 175$) than in those with Philadelphia-positive CML ($n = 45$) and normal control subjects ($n = 58$) ($P < 0.001$) (Table 1; Figure 1a). Among non-CML MPD patients, however, *JAK2* mRNA levels did not differ significantly between those with and those without the V617F mutation (Table 1; Figure 1a).

To confirm that the difference in *JAK2* mRNA levels between the CML and non-CML MPD patients was not because of variation in the number of cells, we also compared measured plasma levels of *JAK2* DNA. *JAK2* DNA copy numbers did not differ significantly between CML and non-CML MPD patients, irrespective of *JAK2* mutation status, but were significantly lower in control subjects (Figure 1b; Table 1).

Sensitivity of JAK2 V617F detection with mRNA and DNA analysis

Given that plasma mRNA levels are higher than corresponding DNA levels, we hypothesized that mRNA would provide more sensitive detection of *JAK2* mutation than would DNA. Among 68 paired plasma DNA and mRNA samples from non-CML MPD patients (Table 2), the *JAK2* V617 mutation was detected by mRNA testing in 37 and by DNA testing in only 33; thus, four (11%) mutation-positive samples would have been missed with DNA testing alone. Moreover, mRNA analysis yielded a higher relative ratio for the mutant peak (Figure 2).

Clinical correlations

Complete clinical data were available for 68 patients with non-CML MPD; their baseline characteristics are shown in Table 2. *JAK2* mRNA levels did not differ significantly by sex ($P = 0.6$) or diagnosis (myelofibrosis, PV, ET, nonclassified-MPD; $P = 0.09$). Plasma levels of *JAK2* mRNA correlated positively with white blood cell count and hemoglobin level, and negatively with bilirubin and creatinine levels (Table 3). In contrast, plasma levels of V617F *JAK2* mRNA only correlated negatively with percent bone marrow progranulocytes (Table 3), indicating that *JAK2* expression level as a whole plays a more important role in MPDs. As expected patients with PV had significantly ($P = 0.002$) higher levels of V617F mutant *JAK2* mRNA, most likely because of the higher rate of V617F mutation in this group of patients as compared with myelofibrosis, ET or nonclassified-MPD.

Correlation with survival

As a continuous variable, higher total plasma *JAK2* mRNA levels were associated with longer survival in non-CML MPD patients with the *JAK2* V617F mutation ($P = 0.03$, Figure 3), but not in the overall group. Plasma levels of V617F mRNA (as a continuous variable) were also associated with survival in patients with the V617F mutation ($P = 0.05$). Similarly, as a dichotomous variable, plasma levels of *JAK2* mRNA (total or V617F mutant) above the upper quartile were associated with longer survival when patients with V617F mutation were considered alone or when all patients were considered (Figures 3 and 4). In contrast, copy number of *JAK2* genomic DNA in the plasma did not correlate with survival when considered as a continuous variable nor when median, high or low quartile cut-off points were considered. The number of patients is too small for studying survival in each of the subgroup (myelofibrosis, PV, ET, nonclassified-MPD).

DISCUSSION

The STAT-JAK2 pathway is becoming recognized as playing an important role in the pathophysiology of non-CML MPDs. The data presented here show that this involvement is not necessarily only through the *JAK2* V617F mutation. Clearly, the V617F mutation or exon 12 mutations are important and cause constitutive activation. However, our findings suggest that patients with non-CML MPDs have high levels of *JAK2* expression, irrespective of V617F mutation status; *JAK2* mRNA levels were similar in patients with and without the V617F mutation. In addition, *JAK2* mRNA levels were significantly higher in these patients than in those with CML, who have a similar MPD.

Given the reported frequency of exon 12 mutations in patients with V617F-negative non-CML MPD (Martínez-Avilés *et al.*, 2007; Pardanani *et al.*, 2007; Scott *et al.*, 2007), we tested for exon 12 mutations in all 68 patients with follow-up data and found none. However, the quantitation of *JAK2* mRNA was performed using plasma rather than cells. This decision was based on our previous data showing that plasma RNA and DNA are less diluted by nucleic acids from normal tissue, and therefore not significantly influenced by interindividual variation in the levels of disease, whether bone marrow or peripheral blood cells are used. We believe there is no reason for the plasma levels to be biased in any way or different from one group to the other.

The high level of *JAK2* mRNA in patients with only wild-type *JAK2* is most likely driven by cofactors that activate the STAT-JAK2 pathway, such that the *JAK2* mutation is not required for leukemogenesis. However, our data show that higher levels of mutant *JAK2* mRNA are associated with *less* aggressive disease – i.e. longer survival (Figure 4). This finding suggests that disease driven by a mechanism other than *JAK2* mutation tends to be more aggressive. The demonstration that *JAK2* expression levels (both mutant and wild-type) are clinically relevant only when patients with the mutation are considered (Figure 3) supports this conclusion. In addition, the finding of relatively high levels of *JAK2* mRNA in plasma indicates that mRNA may be preferable to DNA for detecting *JAK2* mutations in plasma; the high levels of mRNA in patients with an MPD should facilitate detection of the mutation at an earlier stage. The comparison between DNA and mRNA showed that 11% of positive cases could potentially be missed if DNA rather than RNA is used. In addition, we have

reported in recent paper that even when RNA is analyzed, using plasma is more sensitive in detecting JAK2 mutations than using cells (Ma *et al.*, 2008).

In conclusion, our findings suggest that plasma levels of *JAK2* mRNA may play a role in the biology of disease in V617F-positive patients. Therapeutic approaches targeting the STAT-JAK2 pathway may be more likely to be effective in patients with *JAK2* mutation. However, such therapies might still be useful in patients without the mutation, because the STAT-JAK2 pathway appears to be deregulated regardless of mutation status.

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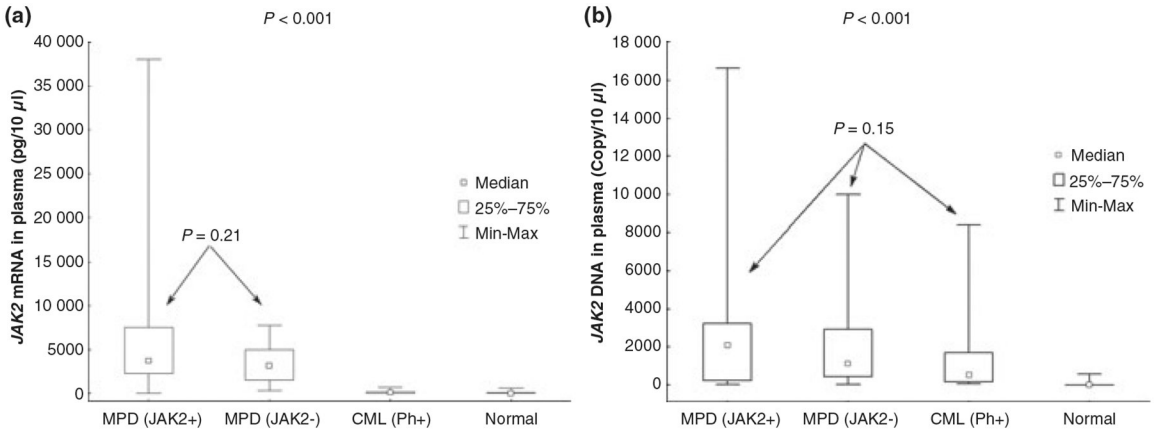

Figure 1.

Plasma levels of *JAK2* mRNA and DNA in patients with MPD. (a) Plasma levels of *JAK2* mRNA were significantly higher in non-CML MPD patients than in CML patients and healthy control subjects; levels did not differ significantly between non-CML MPD patients with and without the V617F mutation. (b) Similarly, *JAK2* DNA levels in plasma did not differ significantly among patients with V617F+ non-CML MPD, V617F− non-CML MPD, and Philadelphia chromosome-positive CML.

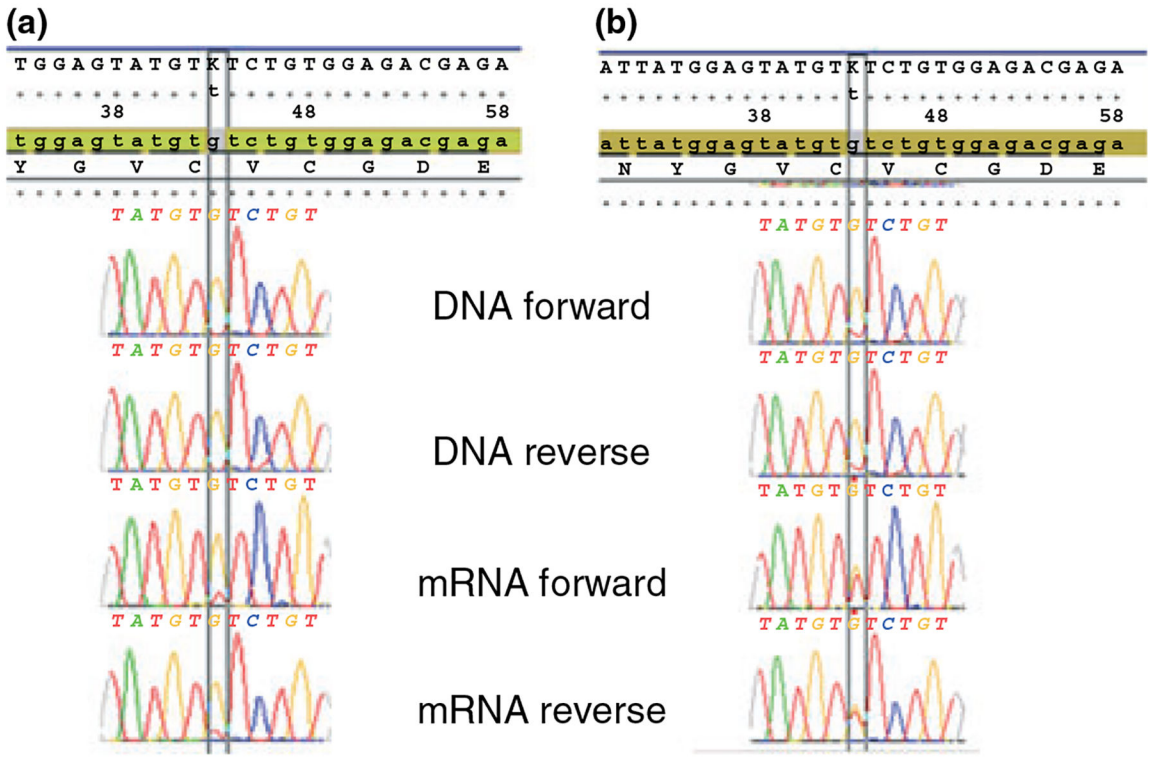

Figure 2.

Higher sensitivity for detecting *JAK2* V617F (1849G→T) mutation with plasma mRNA than with plasma DNA. (a) Representative patient plasma sample in which the V617F mutation was detected by mRNA but not DNA analysis. (b) A second representative sample shows weak detection of the V617F mutation with DNA analysis, while mRNA analysis clearly identifies this sample as having heterozygous V617F mutation. Note the relative intensity of orange peak (G) and the red peak (T). The intensity of the mutant peak is significantly higher when the RNA is used.

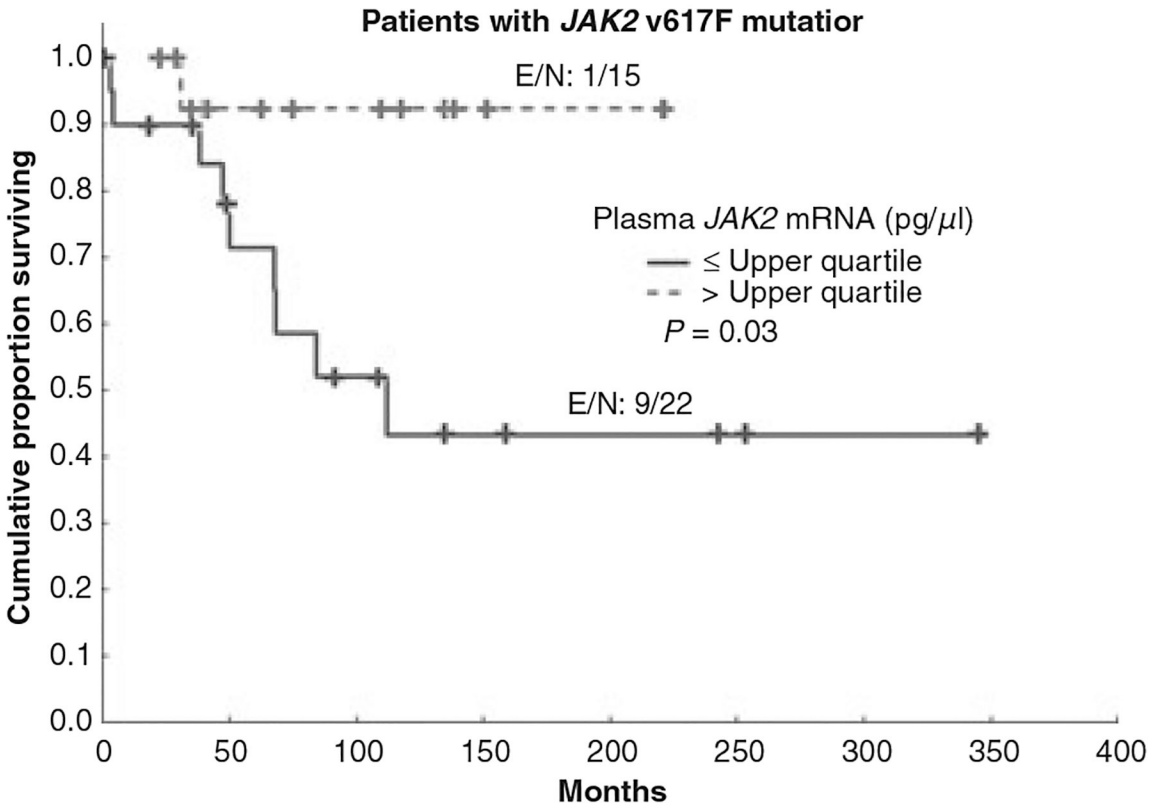

Figure 3.

Association of plasma *JAK2* mRNA levels with survival in non-CML MPD patients with *JAK2* V617F mutation. Patients with elevated plasma levels of *JAK2* mRNA (upper quartile) had significantly longer survival than those with lower levels. N, total number; E, event (death).

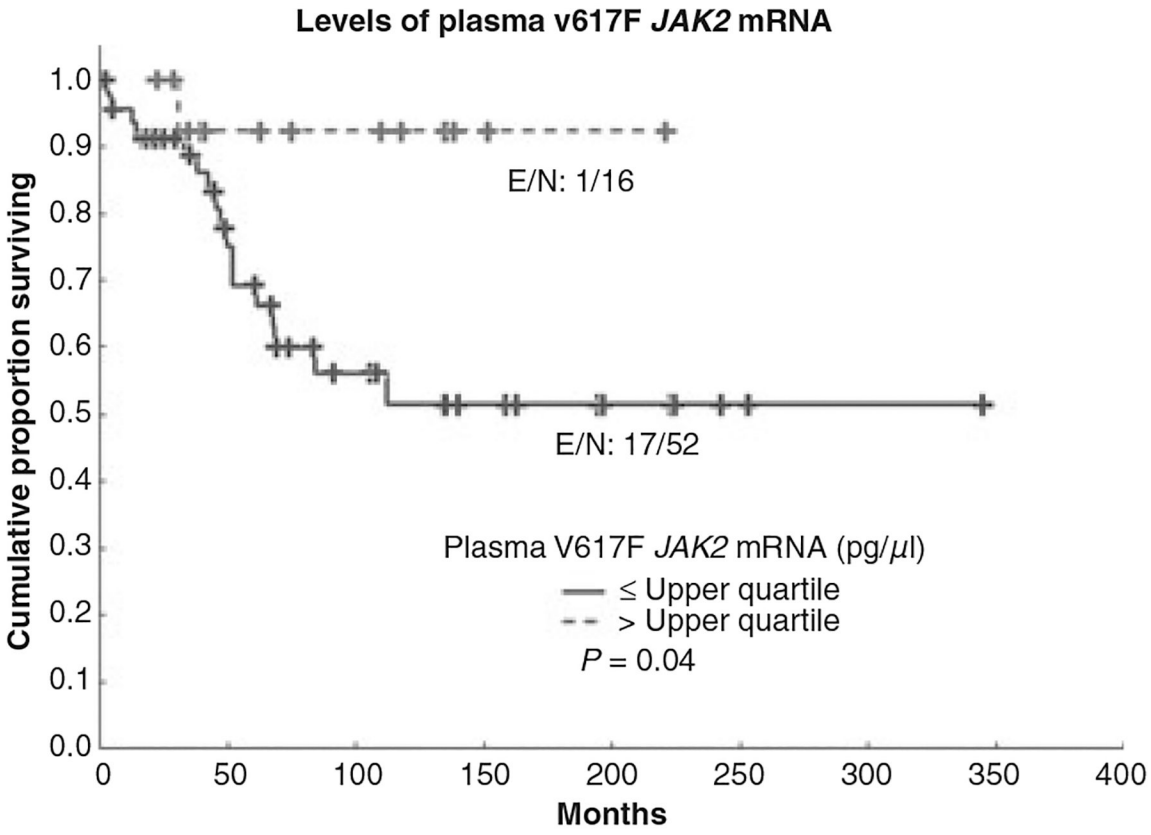

Figure 4.

Association of plasma *JAK2* V617F mRNA levels with survival in all patients with non-CML MPDs, irrespective of the mutation status of *JAK2*. Patients with elevated plasma levels of *JAK2* V617F mRNA (upper quartile) had significantly longer survival than those with lower levels. N, total number; E, event (death).

Table 1.

Plasma levels of *JAK2* mRNA and DNA in patients with non-CML MPDs, CML and normal control subjects

	Plasma *JAK2* mRNA (pg/μl)			**Plasma *JAK2* DNA (copies/10 μl)**		
	Median	**Range**		**Median**	**Range**	
Non-CML MPD						
V617F+	3704	15.87	38 072	1107	52.0	9984
V617F−	3202	347.9	7747	2106	39.38	16 644
CML	65.03	3.78	667.1	509.7	55.7	8399
Control subjects	34.34	1.55	653.1	7.35	0.55	587.6

CML, chronic myelogenous leukemia; MPD, myeloproliferative disease.

Table 2.

Baseline characteristics of non-CML MPD patients with available survival data (n = 68)

Variable	Non-CML MPD Patients
Median age, years (range)	61 (25–85)
Gender, No. (%)	
Male	42 (62)
Race/Ethnicity, No. (%)	
Caucasian	63 (93)
African American	4 (6)
Hispanic	1 (1)
Prior malignancy, No. (%)	18 (21)
Prior therapy, No. (%)	3 (4)
Performance status, No. (%)	
0–1	60 (88)
2	4 (6)
Missing	4 (6)
Enlarged liver, No. (%)	10 (15)
Enlarged spleen, No. (%)	32 (47)
Diagnosis, No. (%)	
Myelofibrosis	33
Polycythemia vera	10
Essential thrombocytopenia	5
Not otherwise classified-MPD	20
Bone marrow	
Cellularity, % (range)	60.33 (5–100)
Blasts, % (range)	3.52 (0–61)
Monocytes, % (range)	2.76 (0–17)
Eosinophils, % (range)	2.12 (0–24)
Basophils, % (range)	1.51 (0–30)
Erythroid cells, % (range)	17.12 (0–67)
Peripheral blood	
Hemoglobin, g/dl (range)	11.02 (6.6–19)
Platelets, $\times 10^9$/l (range)	299.67 (8–1181)
White blood cells, $\times 10^9$/l (range)	22.35 (1.7–182)
Blasts, % (range)	2.79 (0–73)
Lymphocytes, % (range)	16.3 (0–59)
Monocytes, % (range)	5.31 (0–35)
Eosinophils, % (range)	2.65 (0–42)
Basophils, % (range)	1.35 (0–10)
Blood urea nitrogen, mg/dl (range)	16.25 (7–39)
Creatinine, mg/dl (range)	0.99 (0.6–3.0)
Bilirubin, mg/dl (range)	0.77 (0.1–5.0)

Variable	Non-CML MPD Patients
Lactate dehydrogenase, U/l (range)	1476.58 (503–4610)
Alanine aminotransferase, U/l (range)	28.9 (11–115)

CML, chronic myelogenous leukemia; MPD, myeloproliferative disease.

Table 3.

Correlation of *JAK2* and V617F *JAK2* mRNA with clinical characteristics of 68 patients with non-CML MPD*

	***JAK2* mRNA (total)**		**V617F+ *JAK2* mRNA**	
Variable	r_s	***P***	r_s	***P***
Age	0.07	0.56	0.16	0.19
Enlarged liver	−0.14	0.25	−0.07	0.59
Enlarged spleen	−0.10	0.41	0.12	0.35
% bone marrow cellularity	0.04	0.78	0.10	0.43
% bone marrow blasts	−0.03	0.78	−0.11	0.36
% bone marrow progranulocytes	0.01	0.96	−0.32	0.01
% bone marrow myelocytes	0.13	0.27	−0.13	0.30
% bone marrow metamyelocytes	0.22	0.07	−0.02	0.84
% bone marrow polymorphoneuclear cells	−0.12	0.35	−0.02	0.85
% bone marrow lymphocytes	−0.17	0.16	−0.07	0.58
% bone marrow monocytes	−0.12	0.32	−0.15	0.22
% bone marrow eosinophils	−0.08	0.53	−0.09	0.47
% bone marrow basophils	0.21	0.09	0.02	0.86
% bone marrow erythroid cells	0.09	0.49	0.21	0.09
Hemoglobin	0.28	0.02	0.20	0.10
Platelets	0.08	0.51	−0.03	0.80
White blood cells	0.32	0.01	0.03	0.81
% peripheral blood blasts	0.14	0.25	0.00	0.98
% peripheral blood polymorphonuclear cells	0.12	0.33	0.20	0.10
% peripheral blood lymphocytes	−0.23	0.06	−0.06	0.60
% peripheral blood monocytes	0.02	0.86	−0.11	0.38
% peripheral blood eosinophils	0.04	0.79	0.10	0.46
% peripheral blood basophils	−0.03	0.81	0.06	0.67
Creatinine	−0.26	0.04	−0.13	0.29
Bilirubin	−0.31	0.01	0.08	0.50

*Spearman correlation.