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RESEARCH ARTICLE

IRF4 and IRF8 expression are associated with clinical phenotype and clinico-hematological response to hydroxyurea in essential thrombocythemia

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Abstract **The morbidity and mortality of myeloproliferative neoplasms (MPNs) are primarily caused by arterial and venous complications, progression to myelofibrosis, and transformation to acute leukemia. However, identifying molecular-based biomarkers for risk stratification of patients with MPNs remains a challenge. We have previously shown that interferon regulatory factor-8 (IRF8) and IRF4 serve as tumor suppressors in myeloid cells. In this study, we evaluated the expression of IRF4 and IRF8 and the JAK2V617F mutant allele burden in patients with MPNs. Patients with decreased IRF4 expression were correlated with a more developed MPN phenotype in myelofibrosis (MF) and secondary AML (sAML) transformed from MPNs versus essential thrombocythemia (ET). Negative correlations between the JAK2V617F allele burden and the expression of IRF8 ($P < 0.05$) and IRF4 ($P < 0.001$) and between white blood cell (WBC) count and IRF4 expression ($P < 0.05$) were found in ET patients. IRF8 expression was negatively correlated with the JAK2V617F allele burden ($P < 0.05$) in polycythemia vera patients. Complete response (CR), partial response (PR), and no response (NR) were observed in 67.5%, 10%, and 22.5% of ET patients treated with hydroxyurea (HU), respectively, in 12 months. At 3 months, patients in the CR group showed high IRF4 and IRF8 expression compared with patients in the PR and NR groups. In the 12-month therapy period, low IRF4 and IRF8 expression were independently associated with the unfavorable response to HU and high WBC count. Our data indicate that the expression of IRF4 and IRF8 was associated with the MPN phenotype, which may serve as biomarkers for the response to HU in ET.**

Keywords myeloproliferative neoplasms; IRF4; IRF8; hydroxyurea; essential thrombocythemia

Introduction

Myeloproliferative neoplasms (MPNs), including essential thrombocythemia (ET), polycythemia vera (PV), and primary myelofibrosis (PMF), are clonal hematological malignancies characterized by deregulated hematopoietic progenitor proliferation and are associated with myeloproliferation, splenomegaly, and constitutional symptoms [1,2]. As a myeloproliferative disorder (MPD), MPNs are linked to the somatic acquisition of genetic alterations targeting genes involved in the intracellular signaling pathways, the most frequent being the JAK2V617F mutation, found in approximately 95% of PV, 60% of ET, and 50% of PMF patients [3]. Constitutive JAK2 activation triggers several signaling pathways linked to cell survival and proliferation, promoting myeloproliferation and resistance to cell death [4,5]. At present, cytoreductive treatment in PV and ET primarily aims to prevent severe thrombo-hemorrhagic complications [6,7]. However, a proportion of patients experience transformation to secondary myelofibrosis (MF), myelodysplastic syndrome, or acute myeloid leukemia (AML) [8,9]. Recently, a series of studies has addressed the relevance of leukocytosis, spleen size, JAK2V617F mutational allele burden, and degree of fibrosis as factors associated with prognosis, leukemic disease transformation, and the risk of vascular events [10–12]. However, more quantitative clinical

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markers indicative of aggressive phenotype and prognosis still need to be defined.

Essential thrombocythemia (ET) is a relatively benign MPN characterized by increased platelet production and persistently elevated platelet counts [13,14]. This condition is frequently associated with major and minor vascular complications that cause increased morbidity and sometimes fatal complications. The overall estimated risk for major thrombotic and bleeding episodes in ET is 6.6% per patient year, which increases to more than 10% per year if left untreated in patients with risk factors such as age older than 60 years or a history of vascular complications [15,16]. With regard to its efficacy, hydroxyurea (HU) has been reported to provide adequate control of platelet and leukocyte counts in most ET patients and reduce the risk of major thrombosis in ET patients [17,18]. Predictors associated with the clinical outcomes of HU treatment are still lacking.

Interferon regulatory factors (IRFs) are a family of transcription factors that play important roles in the transcriptional regulation of type I IFNs and are also involved in the regulation of tumorigenesis, cell growth, differentiation, and myeloid cell development [19]. IRFs comprise nine members from IRF1 to IRF9. Among them, IRF4 and IRF8 are two structures closely related to hematopoietic-specific IRFs, which play critical roles in the development of multiple lineages of hematopoietic cells [20]. They have both unique and overlapping functions [21]. Mutational profiling shows recurrent IRF8 mutations in childhood ET patients [22], and low expression of IRF4 and IRF8 has been observed in chronic myeloid leukemia (CML) patients with poor responses to IFN-α therapy [23]. Mouse studies also show the importance of IRF4 and IRF8 in myeloid malignancies. Mice lacking Irf8 spontaneously develop a myeloproliferative syndrome resembling CML [24]. We have shown that Irf8 is downregulated in a BCR/ABL-induced murine CML and that forced overexpression of Irf8 in this model represses the resulting MPD and prolongs survival [25]. In addition, Irf4 and Irf8 deficiencies can promote the development of myeloid and lymphoid tumors [26]. However, the relationship of IRF4/IRF8 expression with the clinical phenotypes and treatment outcomes of MPNs remains unknown.

In the current study, we focused on the evaluation of IRF4 and IRF8 expression and their clinical implications in ET, PV, PMF, and sAML. We explored whether or not the burden of the JAK2 mutation was associated with IRF4 and IRF8 expression and investigated the association of the levels of IRF4 and IRF8 with WBC counts in ET and PV patients. Furthermore, we examined the IRF4 and IRF8 expression in JAK2V617F-mutated ET patients treated with HU as first-line therapy to estimate their prognostic value for CR responses according to the ELN criteria [27] and assess their correlations with HU treatment outcomes.

Patients and methods

Patients

A total of 108 samples from patients with newly diagnosed MPNs, including 85 ET patients, 13 PV patients, 5 PMF patients, and 5 sAML patients at Shanghai Ruijin Hospital from September 2015 to April 2017, were collected. The diagnosis and classification of the MPN subtypes and sAML were determined by expert hematologists based on the 2016 World Health Organization criteria [28]. The PV, PMF, and sAML patients were all positive for JAK2V617F. Of the 85 ET patients, 57 (67.1%) harbored JAK2V617F mutation, which had a significantly higher risk of thrombosis than other mutations in ET [29]. We focused on all the JAK2V617F mutated patients. We sequentially excluded ET patients treated with disease-modifying drugs at any time before or on the date of base cohort entry (HU, interferon, ruxolitinib; $n = 17$). The final 40 JAK2V617F-mutated ET patients were enrolled in the HU treatment response study. This study was approved by the Shanghai Ruijin Hospital Ethics Committee (No. 2008-07). Informed consents were obtained from all the enrolled patients.

Definition of HU responses

ET patients were scheduled an initial HU dose of approximately 15 mg/kg per day (usually 1000 mg/day), which was subsequently adjusted to maintain a platelet count of $< 400 \times 10^9$/L, without significant cytopenias. They were included in this study if HU treatment had lasted at least 12 months. The response to HU treatment was categorized using the recently published ELN criteria [27]. Accordingly, a complete clinico-hematological response (CR) was defined as normalization of the platelet count ($< 400 \times 10^9$/L) in the absence of disease-related symptoms, with a normal spleen size and WBC count of $< 10 \times 10^9$/L. A partial response (PR) was defined as a reduction in the platelet count by 50% from baseline or a platelet count of $< 600 \times 10^9$/ L in patients not fulfilling the criteria for CR. Any response that did not satisfy the PR criteria was classified as a nonresponse (NR).

Methods

Sample processing

Heparinized bone marrow (BM) samples were drawn after informed consent was obtained in accordance with the

Declaration of Helsinki. The initial processing of the samples was performed within 12 h after their collection, in most cases within the first 4 h. Mononuclear cells were separated from the samples by centrifugation on a density gradient medium Lymphoprep™ (Stemcell, Canada). Total RNA (1 mg) used for cDNA synthesis was extracted using TRIzol (Thermo Fisher Scientific, USA). The RT step was adapted from the PrimeScript™ RT Reagent Kit (TaKaRa, Japan) protocol. Samples were incubated for 15 min at 37 °C, 5 min at 85 °C, followed by 10 min at 4 °C. DNA was purified using the QIAamp DNA Blood Kit (Qiagen, Germany) and quantified with NanoDrop technology (Wilmington, USA).

Generation of plasmid standard curves

Real-time quantitative polymerase chain reaction (RQ-PCR) was used to determine the mutant allele burden and gene expression of IRF4 and IRF8 in the BM samples. We cloned the JAK2, JAK2V617F, IRF4, IRF8, and β-actin genes to create a standard curve and calculated the copy numbers of such genes in patients. Mutant DNA was obtained from a PV patient harboring 100% mutant allele as evaluated by direct sequencing. Other DNA was obtained from a healthy volunteer. In constructing JAK2, JAK2V617F, IRF4, IRF8, and β-actin plasmids for the standard curve of the RQ-PCR assay, we added the primers and reagents (Table S1) to reach a final volume of 50 μL and used thermal cycler temperatures and time conditions (Table S1) to amplify the gene RT-PCR products. Then, we cloned the PCR products into the pGEM-T Easy Vector (Promega, USA) according to the manufacturer's instructions. The selected clones were screened for the presence of the insert by PCR and then sequenced for confirmation. The plasmid was extracted using the QIAGEN Plasmid Mini Kit (Qiagen, Germany) and quantified spectrophotometrically. The copy number for 1 μg was estimated on the basis of the molecular weight of the vector plus the insert. Seven 10-fold serial dilution series of a known concentration of each gene were prepared to obtain a standard curve.

Absolute quantification by real-time QPCR

All QPCR reactions were performed on a 7900H ABI platform. We designed specific primers and probes using PrimerExpress 3.0 software for the detection of JAK2V617F, JAK2, IRF8, and β-actin, with reaction conditions recommended by Premix Ex Taq™ (TaKaRa, Japan; Table S2). The detection of IRF4 and β-actin was performed using the QuantiTect SYBR Green RT-PCR Kit (QIAGEN, Germany), and the reaction and incubation conditions are listed in Table S2. Melting curve analysis was frequently performed to exclude nonspecific PCR products. The CT values of each dilution were measured in duplicate using real-time QPCR and were plotted against the logarithm of their initial template copy numbers. Each standard curve was generated by linear regression of the plotted points (Table S3, Fig. S1). Quantitative analysis of gene copies was performed by standard curve analysis (Fig. S1). The mutant allele burden was calculated as the percentage of total JAK2 represented by JAK2V617F (JAK2V617F copies/(JAK2V617F copies + JAK2 wild-type copies)). The expression of IRF4 and IRF8 was normalized against that of the control β-actin gene (copies 10^6 IRF4/β-actin, copies 10^5 IRF8/β-actin).

Analysis of the results and statistical considerations

The ELN response criteria of HU were assessed in the whole cohort of 40 ET patients at 3 and 12 months of treatment. The following clinical characteristics at ET diagnosis were evaluated for their potential relationship with the response to HU: age, sex, history of prior thrombosis, splenomegaly, Hb concentration, white blood cell (WBC) count, platelet count, JAK2 mutational allele burden, and expression of IRF4 and IRF8.

In addition to these baseline characteristics, the ELN response categories after 12 months of HU treatment were evaluated in the Cox models as time-dependent covariates. The optimal cut-off point for IRF4 and IRF8 expression was determined by constructing receiver operating characteristic (ROC) curves generated by calculating the sensitivities and specificities of the data at several predetermined cut-off points. According to the ELN criteria, cut-offs for WBC count, platelet count, age, and JAK2V617F allele burden were selected. In all analyses, Cox regression models were first fitted for each of the parameters studied. The proportional hazard assumption was checked using graphic and analytic methods. Factors reaching a significant level ($P < 0.1$) in univariate analysis were included in a Cox proportional hazard model to assess the independent effect of each covariate controlled for the other covariate. Finally, clinically important factors without statistical significance at the univariate level were individually entered in the final model to ensure that no significant changes in the final estimates were produced.

Statistical analysis was performed using SPSS 19 (SPSS Inc., Chicago, IL, USA). Parametric and nonparametric tests, including the Mann–Whitney test, Student's *t*-test, and ANOVA, were performed on the basis of data distribution, and the interpretations of the results were described in the next section. Associations among subject variables (covariates) were assessed for pairs of numerical variables by Spearman's correlation. In the present study, the data were reported as the median and range, and $P < 0.05$ was considered statistically significant.

Results

Characteristics, JAK2V617F allele burden, and IRF4 and IRF8 expression of the patients

We studied 63 subjects who were diagnosed on the basis of the WHO criteria; their clinical characteristics and hematological parameters at diagnosis, as well as JAK2V617F mutational burden and IRF4 and IRF8 expression normalized against β-actin, are reported in Table 1. Consequently, ET patients had significantly higher platelet (PLT) counts than PV (median 956 $\times$ 10^9/L vs. 660 $\times$ 10^9/L, $P = 0.006$), and sAML patients had lower WBC counts (median 956 $\times$ 10^9/L vs. 231 $\times$ 10^9/L, $P < 0.001$) than PV (median 10.72 $\times$ 10^9/L vs. 26 $\times$ 10^9/L, $P < 0.001$) patients. PV patients had higher hemoglobin (Hb) levels than ET (188 g/L vs. 145.5 g/L, $P < 0.001$), PMF (188 g/L vs. 81 g/L, $P < 0.001$), and sAML (188 g/L vs. 118 g/L, $P < 0.001$) patients (Table 1). All investigated patients were positive for JAK2V617F. The JAK2V617F mutational burden showed a varied pattern in each group: ET (median 0.408; range 12%–97%), PV (median 0.587; range 40%–97%), PMF (median 0.524; range 35%–77%), and sAML (median 0.704; range 21%–98%; Table 1, Fig. 1A). IRF8 expression was higher in ET (median 69.82 copies 10^5 IRF8/β-actin) and PV (median 68.21 copies 10^5 IRF8/β-actin) patients than in PMF (median 51.89 copies 10^5 IRF8/β-actin) and sAML (median 42.62 copies 10^5 IRF8/β-actin) patients, although the difference did not reach statistical significance (Table 1, Fig. 1C). IRF4 expression was significantly higher in ET patients than in PMF (176.04 vs. 55.51 copies 10^6 IRF4/β-actin, $P = 0.022$) and sAML (176.04 vs. 30.06 copies 10^6 IRF4/β-actin, $P = 0.008$) patients (Table 1, Fig. 1B).

Comparison and correlation of IRF4 and IRF8 expression with clinical factors and parameters

Considering the sample size and standard error of IRF expression measurement in the PMF and sAML group, we studied the association of IRF expression with clinical factors in the whole and separate group of ET and PV patients. In the whole study group, median IRF8 expression was 69.72 copies 10^5 IRF8/β-actin (range 15.28 to 121.29 copies 10^5 IRF8/β-actin), and high IRF8 expression was found in patients who had a low WBC count ($< 15 \times 10^9$/L: median 74.36 vs. 59.32 copies 10^5 IRF8/β-actin; $P = 0.041$) and low JAK2V617F allele burden ($< 50\%$: median 78.04 vs. 60.4 copies 10^5 IRF8/β-actin; $P = 0.006$). Median IRF4 expression was 154.77 copies 10^6 IRF4/β-actin (range 33.84 to 465.1 copies 10^6 IRF4/β-actin), and significantly high IRF4 expression was recorded in patients

Table 1 Laboratory and clinical characteristics of MPNs and sAML with JAK2V617F mutation at diagnosis

	ET (A)	PV (B)	PMF (C)	sAML (D)	*P* (A) vs. (B)	*P* (A) vs. (C)	*P* (A) vs. (D)	*P* (B) vs. (C)	*P* (B) vs. (D)	*P* (C) vs. (D)
No. of patients (male:female)	40 (27:13)	13 (4:9)	5 (1:4)	5 (3:2)	0.026	0.06	1	1	0.326	0.524
Age (year)	55.5 (25–84)	57 (47–71)	62 (48–68)	58 (51–80)	0.315	0.233	0.107	0.64	0.394	0.748
WBC ($\times$ 10^9/L)	10.72 (5.2–39)	12.2 (7.39–21.48)	5.05 (0.82–18.52)	26 (17.33–55.8)	0.86	0.336	<0.001	0.331	<0.001	<0.001
Hb (g/L)	145.5 (54–170)	188 (160–207)	81 (50–123)	118 (74–152)	<0.001	<0.001	<0.001	<0.001	<0.001	0.128
Hematocrit (%)	45.45 (27.0–49.1)	60 (48.2–70.8)	37.3 (18.7–61.7)	25.5 (22.9–46.9)	<0.001	0.394	0.137	<0.001	<0.001	0.375
Erythrocyte count ($\times$ 10^9/L)	5.02 (2.18–5.79)	6.93 (5.78–9.91)	2.99 (1.80–4.65)	2.52 (2.20–5.65)	<0.001	0.001	0.179	<0.001	<0.001	0.765
PLT ($\times$ 10^9/L)	956 (634–2070)	660 (146–1167)	248 (29–2609)	231 (146–272)	0.006	0.055	<0.001	0.977	0.048	0.102
JAK2V617F allele burden (%)	40.8 (12–97)	58.7 (40–97)	52.4 (35–77)	70.4 (21–98)	0.124	0.832	0.137	0.453	0.683	0.335
IRF8 expression (copies 10^5 IRF8/β-actin)	69.82 (15.28–121.29)	68.21 (46.26–98.81)	51.89 (43.75–80.31)	42.62 (32.28–111.84)	0.784	0.29	0.531	0.42	0.697	0.698
IRF4 expression (copies 10^6 IRF4/β-actin)	176.04 (33.84–465.1)	111.6 (51.06–268)	55.51 (21.38–84.88)	34.06 (29.35–74.65)	0.207	0.022	0.008	0.165	0.101	0.91

All values are expressed as the median and range.

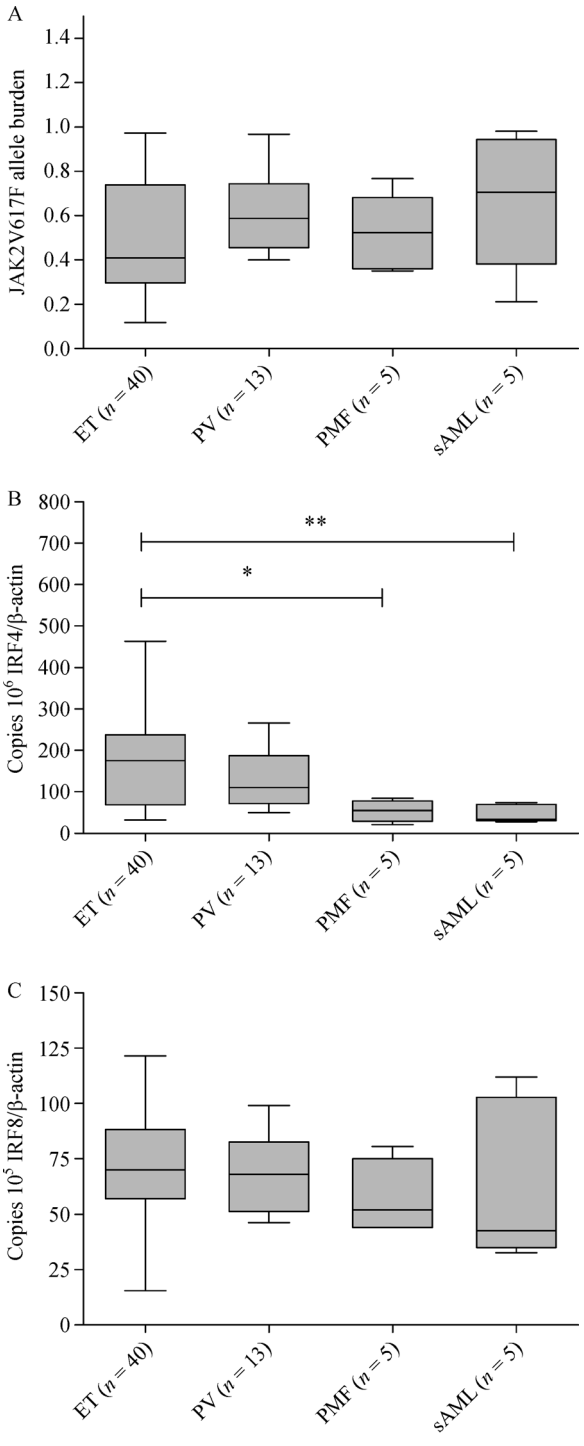

Fig. 1 JAK2V617F allele burden (A) and IRF4 (B) and IRF8 (C) expression in the 63 patients enlisted in the study. Boxes represent the interquartile range that contains 50% of the subjects; the horizontal line inside marks the median, and the bars show the upper and lower ranges of values.

who were younger (< 60 years; median 193.21 vs. 108.59 copies 10^6 IRF4/β-actin; $P = 0.002$) and had a low JAK2V617F allele burden (< 50%: median 214.06 vs. 88.75 copies 10^6 IRF4/β-actin; $P < 0.001$). Detailed data are presented in Table 2. We performed Spearman's correlation to analyze IRF4 and IRF8 expression data and clinical parameters. Detailed data are presented in Table S4. In the whole study group and ET group, we observed a negative correlation between IRF4 expression and age (r = –0.361; $P = 0.008$; r = –0.398; $P = 0.011$). The relationship between the JAK2V617F allele burden and WBC count at diagnosis (the two principal risk factors for MPNs) and IRF4 and IRF8 expression was studied. In ET patients, JAK2V617F mutation and baseline leukocytosis seemed to be associated with a high thrombotic risk, particularly in the high-risk category, and poor survival [29–31]. In ET patients, the leukocyte count > 15 × 10^9/L was identified as an independent predictor of poor survival [30]. In the entire ET patient cohort, we observed a negative correlation between JAK2V617F allele burden and IRF8 (r = –0.354; $P = 0.025$) and IRF4 expression (r = –0.651; $P < 0.001$), a negative correlation between WBC count and IRF4 expression (r = –0.381; $P = 0.015$), and a positive correlation between JAK2V617F allele burden and WBC count (r = 0.601; $P < 0.001$; Fig. 2A–2C, 2E). In PV patients, the JAK2V617F allele burden had strong effects on thrombosis and prognosis [29,32]. In the entire PV patient cohort, we observed that IRF8 expression was negatively correlated with JAK2V617F allele burden (r = –0.564; $P = 0.045$), whereas WBC count was positively correlated with JAK2V617F allele burden (r = 0.651; $P = 0.016$; Fig. 2F–2H).

IRF4 and IRF8 expression in different HU response groups of ET patients

The response to HU treatment in ET patients was categorized using the recently published ELN criteria. Fig. 3 reports the rates of responses in 40 patients at 3 and 12 months of treatment. Response was assessed at 3 months, being categorized as CR (n = 16, 40%), PR (n = 12, 30%), and no response (n = 12, 30%; Fig. 3). The highest rates of CR and PR were obtained after 12 months and were 67.5% and 10%, respectively, and no responses were found in 22.5% of the patients (Fig. 3). The CR and PR groups of patients were significantly younger ($P < 0.05$), and they had lower WBC counts ($P < 0.001$) and JAK2V617F allele burden ($P < 0.001$) than the NR group of patients at 3 months (Table S5, Fig. 4A–4C). IRF8 expression was significantly higher in the CR group of patients (median 80.65 copies 10^5 IRF8/β-actin; range 64.38–116.46 copies 10^5 IRF8/β-actin) than in the PR (median 65.68 copies 10^5 IRF8/β-actin; range 44.07–121.29 copies 10^5 IRF8/β-actin, $P = 0.03$) and NR (median 45.69 copies 10^5 IRF8/β-actin; range 15.28–92.59 copies 10^5 IRF8/β-actin, $P < 0.001$; Table S5, Fig. 4E) group of patients. For the PR and NR groups, a difference in IRF8 expression was identified ($P = 0.03$). The median

Table 2 Baseline characteristic of the study group and comparison of IRF4 and IRF8 expression depending on clinical factors

Factors	*n* (%)	IRF8 expression (copies 10^5 IRF8/β-actin)	*P* value	IRF4 expression (copies 10^6 IRF4/β-actin)	*P* value
Age					
<60 years	32 (60.38%)	71.96	0.557	193.21	0.002
⩾60 years	21 (39.62%)	67.99		108.59	
Gender					
Male	31 (58.49%)	71.85	0.599	150.71	0.445
Female	22 (41.51%)	68.32		172.31	
Diagnosis					
ET	40 (75.47%)	70.94	0.769	168.69	0.255
PV	13 (24.53%)	68.68		131.97	
WBC count					
$<15 \times 10^9$/L	39 (73.58%)	74.36	0.041	171.66	0.148
$\geqslant 15 \times 10^9$/L	14 (26.42%)	59.32		126.32	
JAK2V617F allele burden (%)					
<50%	30 (56.60%)	78.04	0.006	214.06	<0.001
⩾50%	23 (43.40%)	60.40		88.75	

IRF4 expression in the CR group was 220.77 copies 10^6 IRF4/β-actin, which was higher than 179.1 copies 10^6 IRF4/β-actin in the PR group ($P = 0.02$) and 55.27 copies 10^6 IRF4/β-actin in the NR group ($P < 0.001$), and the difference between the PR group and NR group was also significant ($P < 0.001$; Table S5, Fig. 4D).

IRF4 and IRF8 expression are independent factors in predicting HU response

After completing 12 months of therapy, 27 patients attained CR, resulting in a cumulative CR probability of 67.5% from the start of HU (Table 3, Fig. 4). ROC curve analysis revealed a significant cut-off point of IRF4 expression ($P < 0.001$) at 107.11 copies 10^6 IRF4/β-actin, with a sensitivity of 100.0% and specificity of 92.6% (Fig. 5A), and IRF8 expression at 71.83 copies 10^5 IRF8/β-actin ($P = 0.002$), with a sensitivity of 92.3% and specificity of 55.6% (Fig. 5B), which could predict CR of HU therapy in ET patients. CR rates were increased by 2.1-fold (95% CI 1.10–3.99, $P = 0.006$) and 6.73-fold (95% CI 1.86–24.3, $P < 0.001$) in patients aged < 60 years and those who had JAK2V617F allele burden < 50% in the univariable analysis, respectively. Given the correlation among these factors, a multivariate analysis was necessary. In a multivariate cox regression model, IRF4 expression < 107.11 copies 10^6 IRF4/β-actin (HR, 22.18; 95% CI 3.65–134.772), IRF8 expression < 71.83 copies 10^5 IRF8/β-actin (HR, 2.51; 95% CI 1.10–5.77), and leukocyte count > 15×10^9/L (HR, 8.14; 95% CI 1.38–48.05) at diagnosis were independent factors of response to HU (Fig. 6A–6C). The results of the univariate and multivariate analyses of predictors associated with the response to HU in ET patients who attained CR are shown in Table 3. Univariate and multivariable analyses of parameters at diagnosis identified high leukocyte count and low IRF4 and IRF8 expression as independent risk factors for a poor response to HU in ET patients (Table 3). Of note, platelet count ($P = 0.171$) and Hb level ($P = 0.307$) did not seem important to the definition of ELN response (Table 3).

Discussion

We established a reliable assay for IRF4 and IRF8 detection to elucidate the IRF4 and IRF8 expression patterns and their clinical implications in MPNs. Using the method that enables sensitive detection with precise, preferably absolute quantification, we detected IRF4 and IRF8 expression in a representative group of ET, PV, PMF, and sAML patients. Furthermore, we assessed their correlations with HU treatment outcomes in ET patients.

Increasing evidence has implied IRF4 and IRF8 deregulation in diverse hematological malignancies [30–34]. IRF4 and IRF8 function as myeloid tumor suppressors and mediators of IFN treatment in CML [35,36]. We hypothesized that low expression levels of IRF4 and IRF8 may contribute to the clinical phenotype in MPNs patients and serve as biomarkers of drug treatment response in ET patients.

Therefore, we showed that decreased IRF4 levels were associated with a phenotype of a progressive disease. The clinical manifestation of MPN diseases ranges from symptomless to severe constitutional symptoms and thromboembolic events [37,38]. Furthermore, the

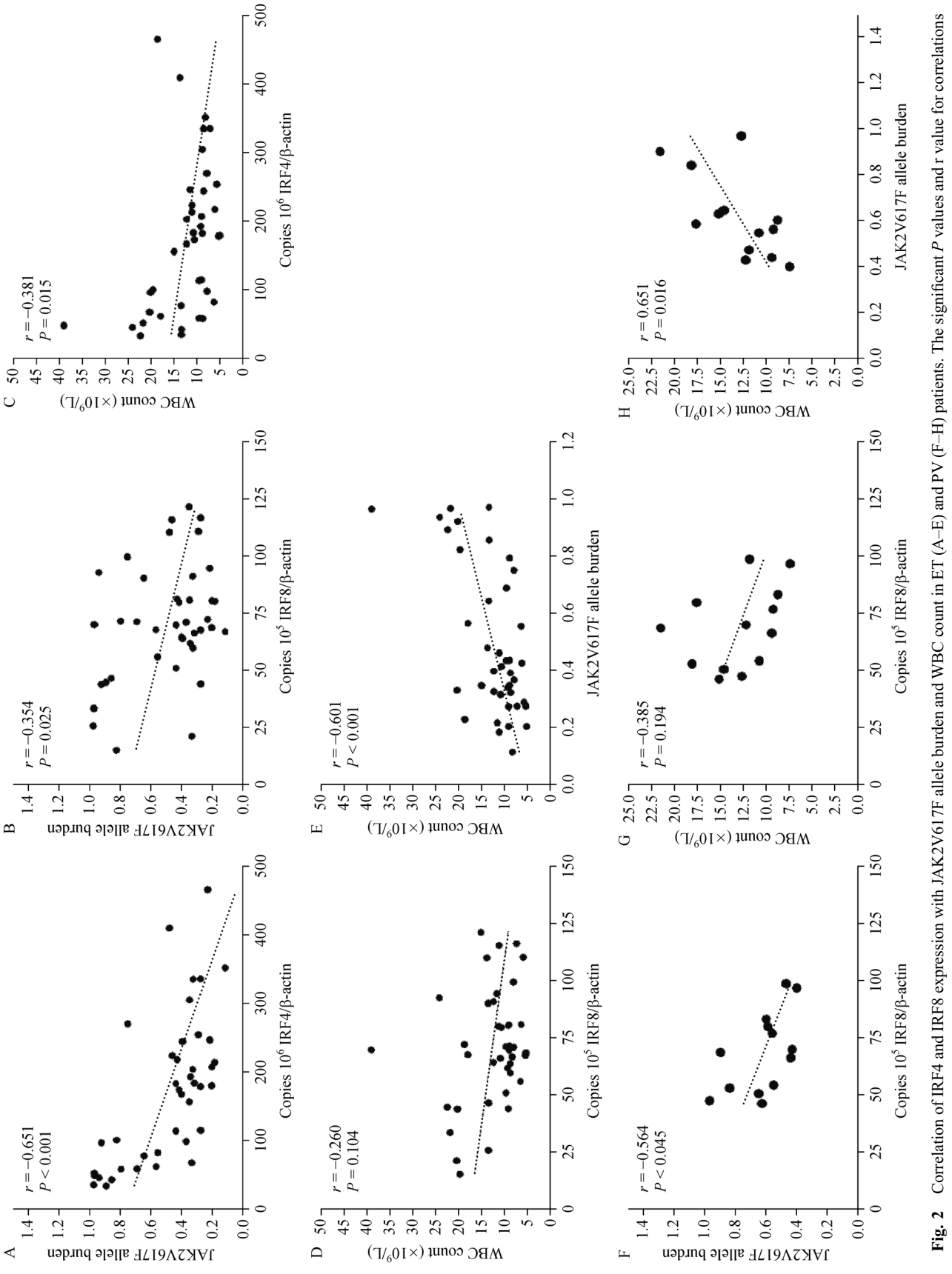

Fig. 2 Correlation of IRF4 and IRF8 expression with JAK2V617F allele burden and WBC count in ET (A–E) and PV (F–H) patients. The significant *P* values and r value for correlations are shown in the figure.

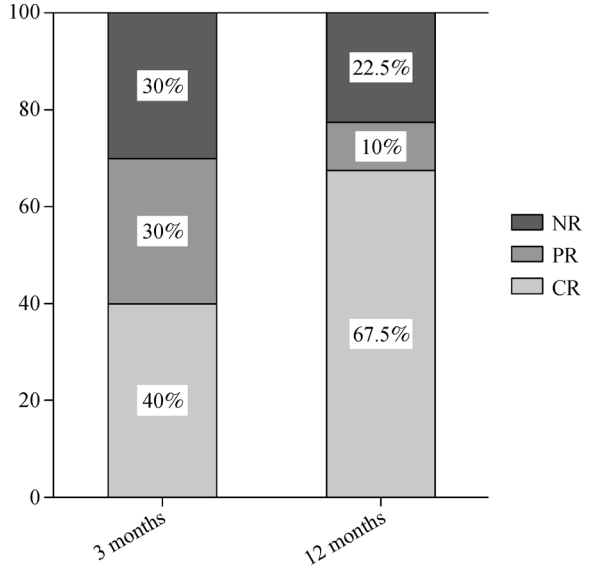

Fig. 3 Rate of responses to HU according to the ELN criteria at 3 and 12 months in ET patients.

transformation of ET to PV, myelofibrosis, and AML is observed in a number of patients [39]. The overall prognosis for PMF is poor, with expected survival ranging from months to several years [40]. In addition, patients with PMF exhibit a propensity for transformation to secondary acute myeloid leukemia (sAML), for which the prognosis is dismal [41]. We found that patients with PMF and sAML had significantly lower IRF4 levels than those with ET. PV patients showed lower IRF4 levels than ET patients and higher IRF4 levels than PMF and sAML patients, but this difference did not reach significance probably because the numbers were too small to draw firm conclusions. However, in our disease model, we did not find that the JAK2V617F allele burden likely has a major impact on disease phenotype, which is in contrast to the findings of previous reports [42,43]. We hypothesized that this difference might be due to the ET patients who came across to Ruijin Hospital for medical advice were seriously ill. It was because of uncontrolled reasons and not because of the selection process.

The JAK2V617F allele burden is associated with granulocyte activation. As the JAK2V617F allele burden increases, the phenotype becomes more proliferative with increasing WBC counts [44,45]. Leukocytosis is a common marker of aggressive disease biology in myeloid malignancies [46,47]. IRF4 and IRF8 serve as notable effectors in the development, growth, and apoptosis of myeloid cells [48]. Studies with myeloid progenitor cells have shown that IRF8 drives their differentiation toward macrophages; however, it inhibits granulocyte differentiation [49,50]. Furthermore, myeloid cells from Irf8$^{-/-}$ mice

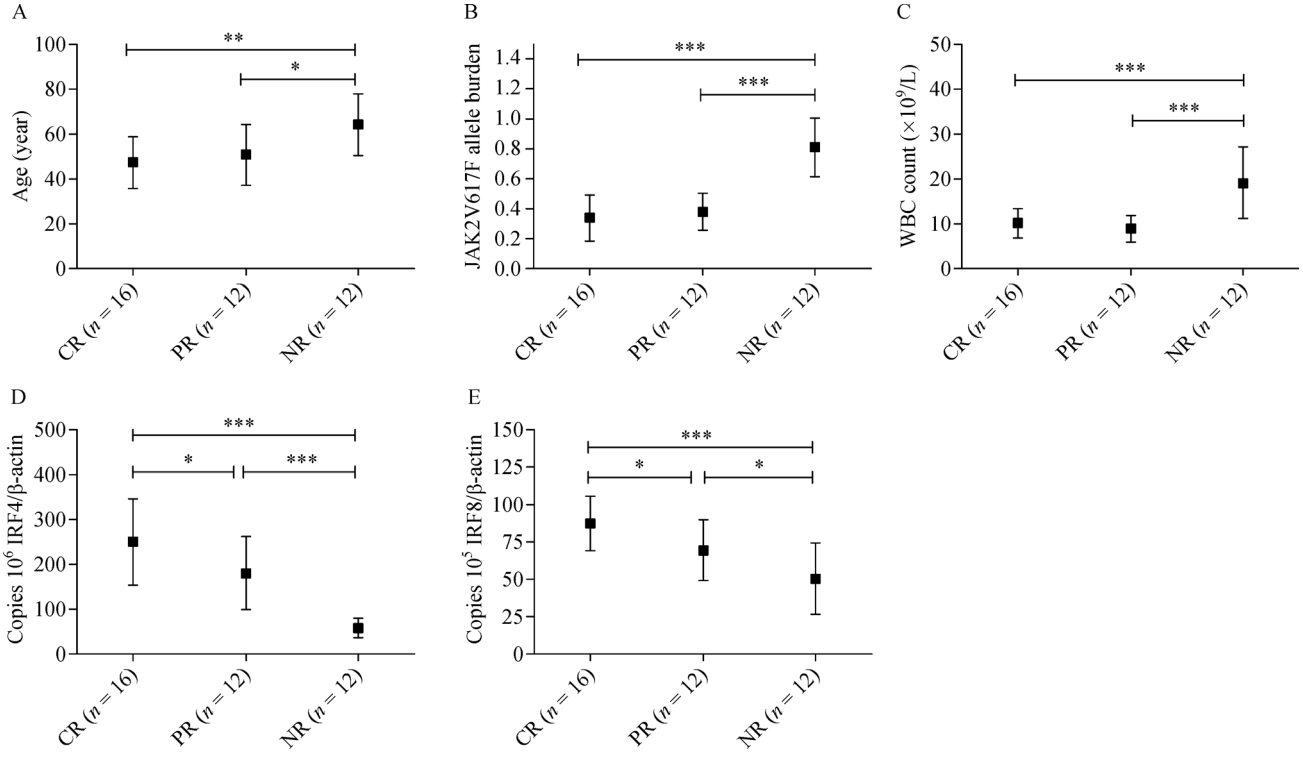

Fig. 4 Clinical characteristics and IRF4 and IRF8 expression in the CR, PR, and NR groups evaluated at 3 months in ET patients. (A) Age; (B) JAK2V617F allele burden; (C) WBC count; (D) IRF4 expression; (E) IRF8 expression. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

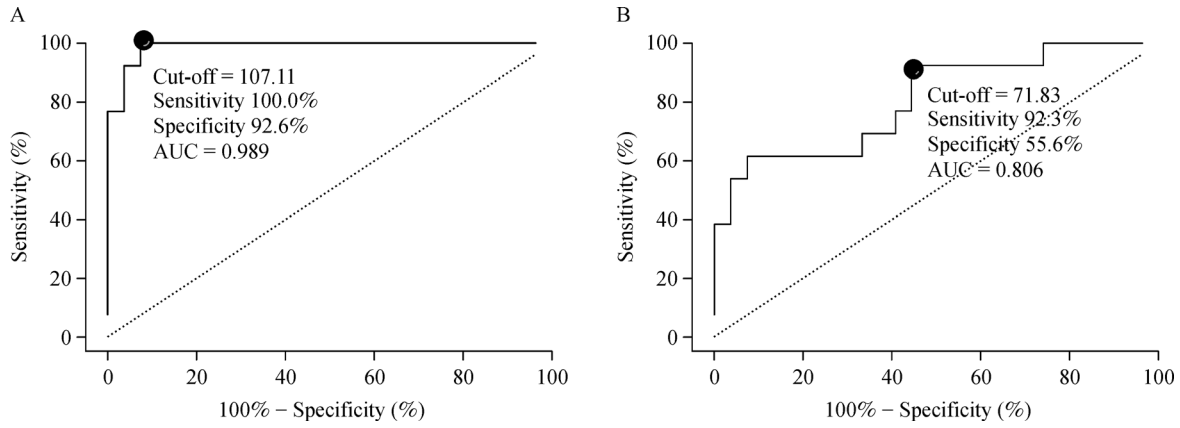

Fig. 5 Receiver operating characteristic (ROC) curve analyses to explore the cut-off expression level of IRF4 and IRF8 in the cohort of ET patients: (A) IRF4 expression; (B) IRF8 expression. Note: The cut-off level for expression was determined using CR as a binary end point within 12 months. AUC, area under the curve.

are resistant to apoptosis [51,52]. The ectopic expression of IRF4 in myeloid progenitor cells *in vitro* inhibits cell growth and promotes macrophage differentiation, but it hinders granulocytic cell differentiation [48]. $Irf8^{-/-}Irf4^{-/-}$ mice exhibit more severe CML-like disease than $Irf8^{-/-}$ mice, involving a disproportionate expansion of granulocytes at the expense of monocytes/macrophages [26]. Our previous study has found that the cooperation among deficiencies of IRF4 and IRF8 promotes myeloid and lymphoid tumorigenesis, and IRF4 and IRF8 function as tumor suppressors in lymphopoiesis and myelopoiesis lineages [26,27,35]. In this study, our data demonstrated a negative correlation between JAK2V617F allele burden and the expression of IRF8 and IRF4 and a negative correlation between WBC count and IRF4 expression, which was in line with previous research showing that IRF4 and IRF8 could inhibit granulocyte differentiation and function as tumor suppressors in the myeloid lineage. JAK2V617F mutation and leukocytosis were considered as an independent prognostic factor for survival [53]. Furthermore, in several studies, the presence of JAK2V617F mutation, allele burden, and leukocytosis was considered as significant predictors of HU response in multivariate analysis in MPN patients [54–56]. Thus, we hypothesized that IRF4 and IRF8 expression may be associated with the clinical response and prognosis of MPN patients.

We applied the proposed criteria to all patients treated with HU in our institutions, focusing on the potential correlations between the ELN response categories and IRF4 and IRF8 expression and clinical characteristics. In our series, most ET patients could achieve CR with HU therapy (67.5% at 12 months), which is consistent with previously reported studies applying the same criteria to a large cohort of ET patients. These data support the well-recognized effectiveness of HU in controlling the platelet count in ET. The minority (10%) of patients receiving HU achieved PR, as defined by a platelet count in the range of 400×10^9/L to 600×10^9/L or a decrease of greater than or equal to 50% from baseline (6%). Nonresponders accounted for 9 of 40 (22.5%) patients, representing the proportion of HU-resistant patients in whom second-line therapy is indicated. The factors statistically associated with NR were the persistence of leukocytosis, high JAK2V617F allele burden, and low IRF4 and IRF8 expression compared with that in the CR and PR groups after HU therapy in 3 months.

After dividing the patients into two groups with low and normal IRF4 and IRF8 levels by ROC curves, which generated an optimal cut-off point, Kaplan–Meier curves showed that patients with low IRF4 and IRF8 levels had a significantly higher risk of not achieving CR in 12 months of HU therapy. This result was supported by univariate analysis of low IRF4 and IRF8 levels showing a significantly increased risk of not achieving CR, with age < 60 years, JAK2V617F allele burden $< 50\%$, and leukocyte count $> 15 \times 10^9$/L. In multivariate analysis, leukocyte count $> 15 \times 10^9$/L and low IRF4 and IRF8 levels were proven to be independent risk factors for not achieving CR. These results confirm that low IRF4 and IRF8 levels were prognostic factors for HU response, thereby showing the clinical significance of the abnormally low IRF4 and IRF8 levels detected in MPNs patients. These results should be considered in the design of future studies addressing the value of new treatment modalities for ET.

Meanwhile, given the small sample size of our study, large-scale multicenter studies are needed for the verification of our conclusion and its potential application in clinical practice. Furthermore, prospective studies are needed to identify IRF4 and IRF8 values, which served as reliable cut-offs to define a significant stratification

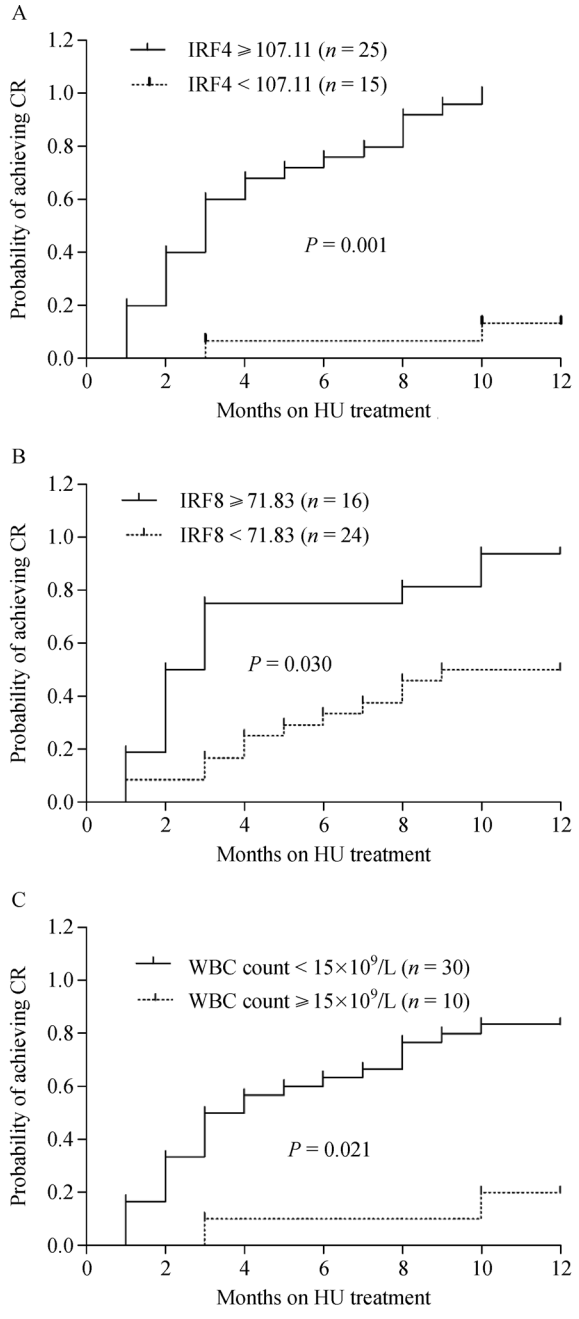

Fig. 6 Probability of achieving a complete clinico-hematological response (CR) to HU in 40 ET patients according to WBC count and IRF4 and IRF8 expression at baseline (A–C).

model for the diagnosis, drug efficacy, and prognosis of MPNs.

IRF8 and IRF4 expression correlates with the cytogenetic response to IFN-α in CML [36,35]. Our data showed that in ET patients, IRF4 and IRF8 expression correlates with HU response, which is a first-line therapy for MPNs. However, HU has no sustained impact upon JAK2V617F mutation with decreasing allelic burden over time [57]. It decreases the JAK2V617F allelic burden to a certain extent in most patients; afterward, the mutation continues to be detectable at levels of 10%–20% in the large proportion of patients having initial levels $>$ 50% [58]. After a few days or weeks after discontinuation of HU, leukocyte and platelet counts will steadily increase to levels before the HU treatment was initiated in all patients [59]. Previous research showed that increasing the expression of IRF4 and IRF8 inhibited tumor-induced myeloid imbalance and enhanced immunotherapy in an *in vitro* study [60,61]. Thus, seeking a way to restore the expression and function of these IRFs could be a new approach to improve MPN therapy.

Conclusions

Our data indicate that the lack of IRF4 and IRF8 may be related to a phenotype of progressive diseases in MPNs. Associations are found between IRF4 and IRF8 expression and the JAK2V617 mutational burden and leukocytes in ET and PV patients. Our study supports the potential use of IRF4 and IRF8 as HU response biomarkers in ET patients. In addition, before these novel biomarkers are introduced in clinical practice, prospective studies in larger cohorts of patients are needed to validate our results.

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Compliance with ethics guidelines

Xiao Huang, Tingting Ma, Yongmei Zhu, Bo Jiao, Shanhe Yu, Kankan Wang, Jian-Qing Mi, and Ruibao Ren declare that they have no conflict of interest. This manuscript does not involve a research protocol requiring approval by the relevant institutional review board or ethics committee. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the *Helsinki Declaration* of 1975, as revised in 2000 (5). Informed consent was obtained from all patients or guardians of patients for being included in the study.

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Table 3 Analysis of factors associated with the clinico-hematological response to HU in 40 patients with ET

Factor	Percentage of CR	Univariate		Multivariate	
		P value	Hazard ratio (95% CI)	*P* value	Hazard ratio (95% CI)
Age					
<60 years	84% (21/25)	0.006	2.1 (1.10–3.99)	0.717	
⩾60 years	40% (6/15)				
Sex					
Male	70.4% (19/27)	0.722	1.14 (0.70–1.88)	0.599	
Female	61.5% (8/13)				
Splenomegaly					
No	67.6% (25/37)	1	1.01 (0.44–2.33)	0.325	
Yes	66.7% (2/3)				
Thrombotic event					
No	65.7% (23/35)	1	0.82 (0.50–1.35)	0.851	
Yes	80% (4/5)				
Hb level					
⩾120 g/L	71.4% (25/35)	0.307	1.79 (0.60–5.33)	0.457	
<120 g/L	40% (2/5)				
WBC count					
$<15 \times 10^9$/L	83.3% (25/30)	0.001	4.17 (1.19–14.54)	0.021	8.14 (1.38–48.05)
$\geqslant 15 \times 10^9$/L	20% (2/10)				
Platelet count					
$<1000 \times 10^9$/L	78.3% (18/23)	0.171	1.48 (0.90–2.43)	0.657	
$\geqslant 1000 \times 10^9$/L	52.9% (9/17)				
JAK2V617F allele burden (%)					
<50%	96.2% (25/26)	<0.001	6.73 (1.86–24.3)	0.223	
⩾50%	14.3% (2/14)				
IRF8 expression (copies 10^5 IRF8/β-actin)					
⩾71.83	93.8% (15/16)	0.004	1.88 (1.23–2.85)	0.030	2.51 (1.10–5.77)
<71.83	50% (12/24)				
IRF4 expression (copies 10^6 IRF4/β-actin)					
⩾107.11	100% (25/25)	<0.001	7.5 (2.06–27.3)	0.001	22.18 (3.65–134.77)
<107.11	13.3% (2/15)				

CI, confidence interval; HR, hazard ratio.

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