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ORIGINAL ARTICLE

CREB3L1 overexpression as a potential diagnostic marker of Philadelphia chromosome–negative myeloproliferative neoplasms

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Abstract

Discrimination of Philadelphia-negative myeloproliferative neoplasms (Ph-MPNs) from reactive hypercytosis and myelofibrosis requires a constellation of testing including driver mutation analysis and bone marrow biopsies. We searched for a biomarker that can more easily distinguish Ph-MPNs from reactive hypercytosis and myelofibrosis by using RNA-seq analysis utilizing platelet-rich plasma (PRP)-derived RNAs from patients with essential thrombocythemia (ET) and reactive thrombocytosis, and *CREB3L1* was found to have an extremely high impact in discriminating the two disorders. To validate and further explore the result, expression levels of *CREB3L1* in PRP were quantified by reverse-transcription quantitative PCR and compared among patients with ET, other Ph-MPNs, chronic myeloid leukemia (CML), and reactive hypercytosis and myelofibrosis. A *CREB3L1* expression cutoff value determined based on PRP of 18 healthy volunteers accurately discriminated 150 driver mutation–positive Ph-MPNs from other entities (71 reactive hypercytosis and myelofibrosis, 6 CML, and 18 healthy volunteers) and showed both sensitivity and specificity of 1.0000. Importantly, *CREB3L1* expression levels were significantly higher in ET compared with reactive thrombocytosis ($P < .0001$), and polycythemia vera compared with reactive erythrocytosis ($P < .0001$). Pathology-affirmed triple-negative ET (TN-ET) patients were divided into a high– and low–*CREB3L1*-expression group, and some patients in the low-expression group achieved a spontaneous remission during the clinical course. In conclusion, *CREB3L1* analysis has the potential to single-handedly discriminate driver mutation–positive Ph-MPNs from reactive hypercytosis

Abbreviations: AUC, area under the receiver operating characteristic curve; BM, bone marrow; CML, chronic myeloid leukemia; CPM, counts per million mapped reads; CV, coefficient of variation ; DDBJ, DNA Data Bank of Japan; DE, differential expression; DEG, differentially expressed gene; ET, essential thrombocythemia; FC, fold change; FDR, false discovery rate; MF, myelofibrosis; PCA, principal component analysis; Ph-MPNs, Philadelphia-negative myeloproliferative neoplasms; PMF, primary myelofibrosis; PRP, platelet-rich plasma; PV, polycythemia vera; RPKM, reads per kilobase of exon per million mapped reads; RT-qPCR, reverse-transcription quantitative PCR; TN-ET, pathology-affirmed triple-negative ET.

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and myelofibrosis, and also may identify a subgroup within TN-ET showing distinct clinical features including spontaneous remission.

KEYWORDS
biomarker, *CREB3L1*, Philadelphia-negative myeloproliferative neoplasms, platelet RNA, RNA-seq

1 | INTRODUCTION

Philadelphia-negative myeloproliferative neoplasms (Ph-MPNs) including polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF) are a group of clonal hematopoietic stem cell diseases characterized by the overproduction of mature blood cells. *JAK2*, *CALR*, and *MPL* mutations are well-recognized recurrent driver mutations found in Ph-MPNs. In PV, 97% are *JAK2*V617F-mutated, and the remaining 3% are positive for *JAK2* exon12 mutations. In ET and PMF, 50%-60% are *JAK2*V617F-mutated, 20%-30% are *CALR*-mutated, a very small percentage are *MPL*-mutated, and 10%-20% show no driver mutations and are so-called "triple-negative" ET/PMF.[1-4] Diagnosis of Ph-MPNs requires not only driver mutation analysis, but a bone marrow (BM) biopsy is ultimately necessary.[5] However, erythrocytosis, thrombocytosis, and myelofibrosis (MF) can occur due to various reactive causes other than Ph-MPNs, and discrimination of Ph-MPNs from reactive cases is crucial because treatment strategies differ greatly. In addition, reactive cases are not at all rare, and as for thrombocytosis, more than 85% of all cases are reported to be due to a reactive cause.[6] Furthermore, the diagnostic processes, including diver mutation analysis and BM biopsies, that are indispensable for discriminating Ph-MPNs from reactive cases are not only costly, but also impose a physical burden on the patient. Therefore, simple and universally utilizable methods that can discriminate Ph-MPNs from reactive cases are eagerly awaited. Here, we compared gene expression profiles of platelet-rich plasma (PRP)-derived RNA of Ph-MPNs and reactive cases and found that *cyclic AMP-response element-binding protein 3-like 1* (*CREB3L1*) expression levels can accurately discriminate the two disorders.

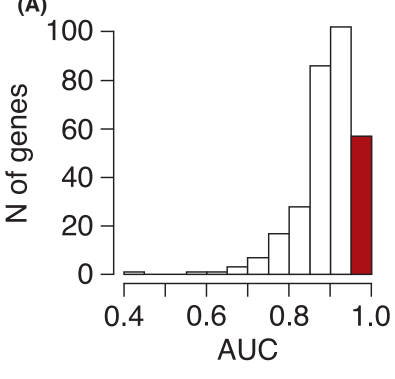
2 | MATERIALS AND METHODS

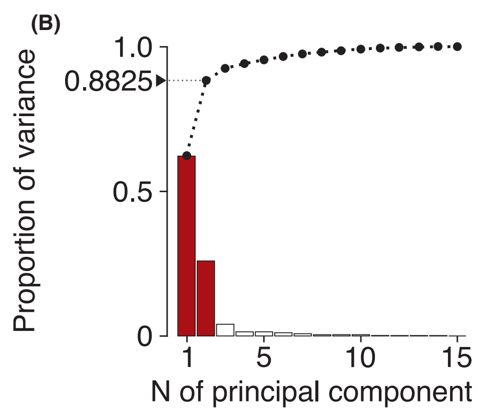
2.1 | Sample collection

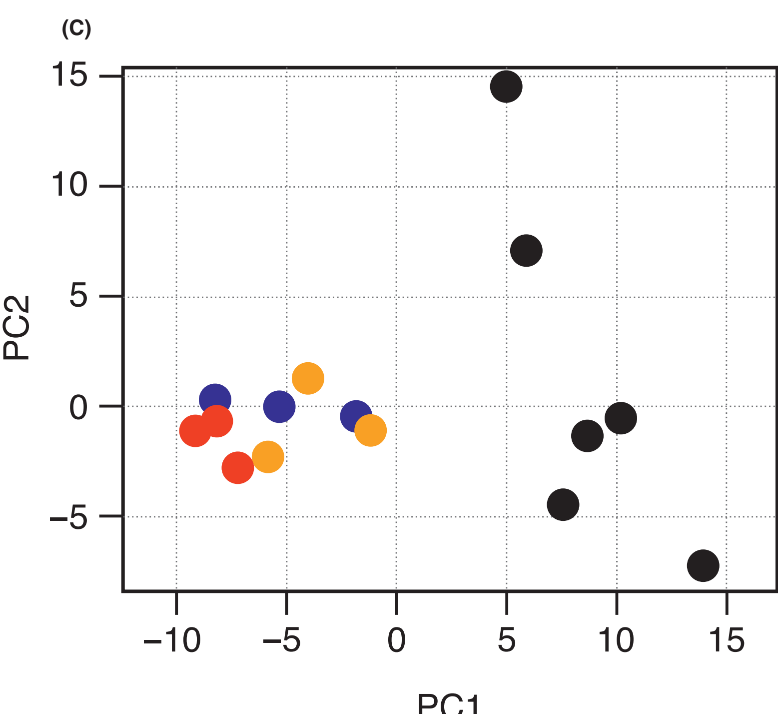
This study was conducted in accordance with the Declaration of Helsinki and was approved by the ethics committee of Juntendo University School of Medicine (IRB#2016119). Written informed consent was obtained from all participants prior to sample collection. All data collected were from the time of study entry. *JAK2*, *CALR*, and *MPL* mutations were analyzed by methods previously published.[7-9] All of our Ph-MPN patients were strictly diagnosed according to the 2016 WHO criteria, and thus all had undergone BM biopsies. Chronic myeloid leukemia (CML) patients were also diagnosed according to the 2016 WHO criteria, and *CREB3L1* analyses concerning CML patients were done prior to treatment. In order to set equal terms with the 2016 WHO requirements for ET, PV, and PMF, all reactive thrombocytosis patients had platelet counts ≥ 450 × 10^9/L, reactive erythrocytosis patients had either elevated hemoglobin levels (>165 g/L for men, >160 g/L for women) or elevated hematocrit (>0.49 L/L for men, >0.48 L/L for women), and reactive MF patients had MF grade > 1.

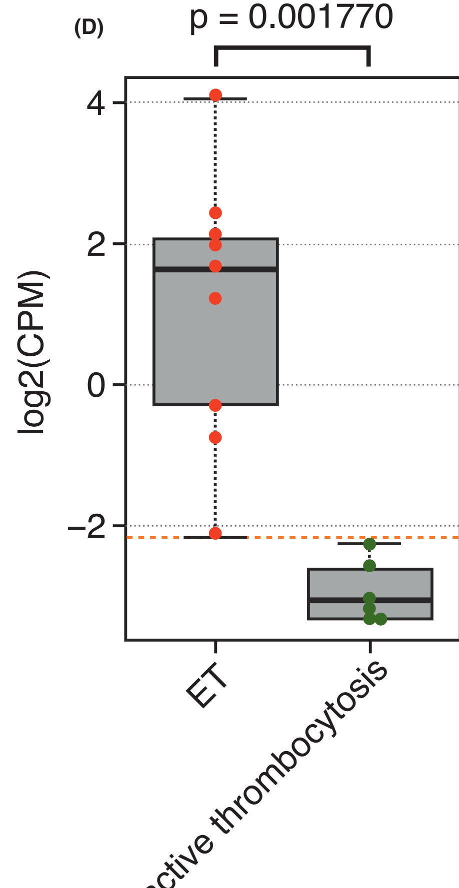
Peripheral blood samples (10 mL) were collected using 0.38% w/v sodium citric acid as an anticoagulant, and PRP was isolated by using Allegra® X-12 centrifuge (Beckman Coulter) for 5 minutes at 233 *g* (1000 rpm) without use of the brake. PRP was prepared by collecting the plasma layer using a disposable dropper. The PRP was dissolved in TRIzol LS reagent (Thermo Fisher Scientific). PRP-derived RNA was extracted from the lysate using an isopropanol precipitation and stored at −80°C until use. We collected PRP from 9 ET patients (3 patients with *JAK2*V617F, 3 patients with *MPL*W515L/K, and 3 patients with *CALR* exon 9 frameshift mutation) and 6 patients with reactive thrombocytosis (3 cases due to chronic inflammation and 3 cases due to rebound thrombocytosis) for RNA-seq as a derivation cohort, and, as a validation cohort, we further collected 150 driver mutation–positive Ph-MPN patients (78 ET, 30 PV, 16 overt PMF, 7 prefibrotic PMF, and 19 post-PV/ET MF patients, Table S1), 6 patients with CML, 38 patients with reactive thrombocytosis (15 chemotherapy patients with rebound thrombocytosis following myelosuppression, 12 patients with iron deficiency anemia, 8 patients with chronic inflammation, 2 patient with bleeding, and 1 patient undergoing splenectomy), 30 patients with reactive erythrocytosis (24 patients with an unknown cause, 5 patients with sleep apnea syndrome, and 1 patient with hypoxia due to emphysema), and 3 patients with reactive MF (1 patient with multiple myeloma, 1 patient

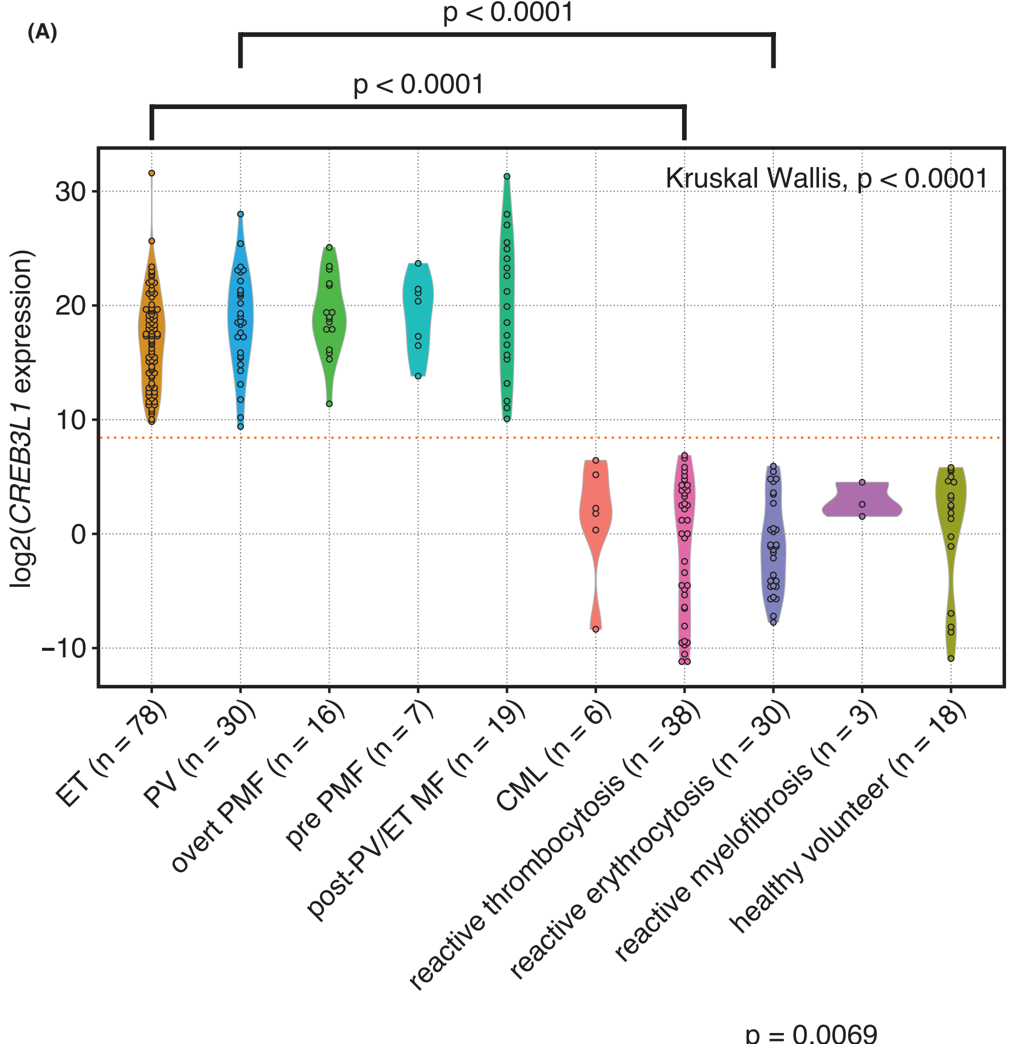
FIGURE 1 RNA-seq analysis. A, Histogram showing area under the receiver operating characteristic curve (AUC) of the 303 genes. Red bar expresses AUC > 0.95. B, Pareto plot showing the proportion of variances among principal components (PC) based on the reads per kilobase of exon per million mapped reads (RPKM) of the 57 differentially expressed genes (DEGs) in 9 essential thrombocythemia (ET) patients and 6 reactive thrombocytosis patients. PC1 and PC2 cover 88.25% of all variances (red bars). C, Primary component analysis (PCA) plot using the RPKM of the 57 DEGs. Color-coded dots represent patients with the following entities: *JAK2*V617F ET (red), *MPL*W515L/K ET (yellow), *CALR* exon 9 frameshift ET (blue), and reactive thrombocytosis (black). D, Box plot expressing the logarithm of counts per million mapped reads (logCPM) of ET and reactive thrombocytosis. Dotted pink line shows the cutoff value discriminating ET from reactive thrombocytosis.


(A)
100
80
60
40
20
0
N of genes
0.4
0.6
0.8
1.0
AUC


(B)
1.0
0.8825
0.5
0
Proportion of variance
1
5
10
15
N of principal component


(C)
15
10
5
0
−5
PC2
−10
−5
0
5
10
15
PC1


(D)
p = 0.001770
4
2
0
−2
log2(CPM)
ET
reactive thrombocytosis


(A)
p < 0.0001
p < 0.0001
Kruskal Wallis, p < 0.0001
log2(CREB3L1 expression)
30
20
10
0
−10
ET (n = 78)
PV (n = 30)
overt PMF (n = 16)
pre PMF (n = 7)
post-PV/ET MF (n = 19)
CML (n = 6)
reactive thrombocytosis (n = 38)
reactive erythrocytosis (n = 30)
reactive myelofibrosis (n = 3)
healthy volunteer (n = 18)

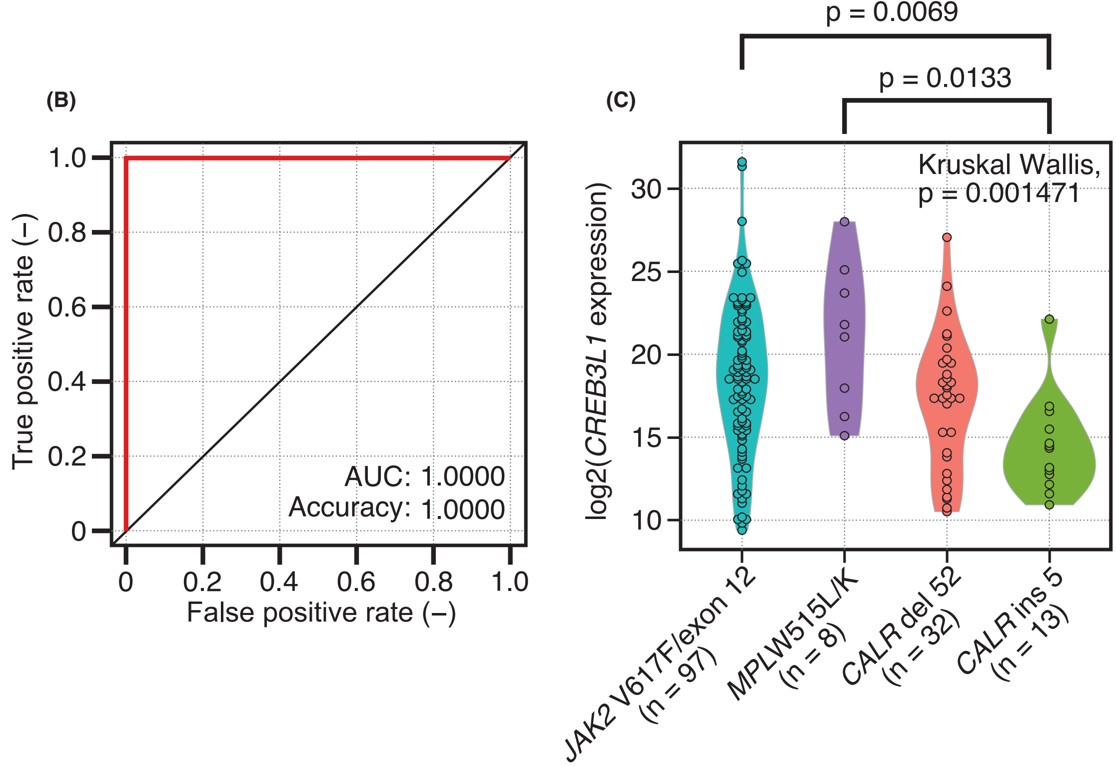

(B)
True positive rate (–)
1.0
0.8
0.6
0.4
0.2
0
AUC: 1.0000
Accuracy: 1.0000
0
0.2
0.4
0.6
0.8
1.0
False positive rate (–)
(C)
p = 0.0069
p = 0.0133
Kruskal Wallis,
p = 0.001471
log2(CREB3L1 expression)
30
25
20
15
10
JAK2 V617F/exon 12 (n = 97)
MPLW515L/K (n = 8)
CALR del 52 (n = 32)
CALR ins 5 (n = 13)

FIGURE 2 Reverse-transcription quantitative PCR (RT-qPCR) analysis. A, Violin plot comparing *CREB3L1* expression levels between different entities. Dotted orange line shows the cutoff value discriminating driver mutation–positive Philadelphia-negative myeloproliferative neoplasms (Ph-MPNs) from chronic myeloid leukemia (CML), reactive hypercytosis, reactive myelofibrosis (MF), and healthy controls. ET, essential thrombocythemia; PMF, primary myelofibrosis; PV, polycythemia vera. B, Receiver operating characteristic (ROC) curve analysis of *CREB3L1* expression for discriminating driver mutation–positive Ph-MPNs from other disorders. C, Violin plot comparing *CREB3L1* expression levels between Ph-MPN patients with different driver mutations.

with splenic marginal zone lymphoma, and 1 patient with primary autoimmune MF) for reverse-transcription quantitative PCR (RT-qPCR) analysis.

2.2 | RNA-seq analysis

The RNA-seq library was prepared from 300 ng of PRP-derived RNAs by using TruSeq Stranded Total RNA Library Prep Kit with Ribo-Zero Human/Mouse/Rat (Illumina) and then applied to HiSeq2500 apparatus (Illumina) for RNA-seq. RNA-seq was performed at the following conditions: sequence mode, High Output Mode; sequence read, 50 Single-End; Real-Time Analysis Software version, 1.17.20; Basecaller "bcl2fastq" software version, 1.8.4; and HiSeq Flow Cell, v3. The fastq data were aligned using hg19 human reference and the STAR program (version 2.4.1d),[10] and the read-count data were obtained using the FeatureCount program (version 1.5.0-p1).[11] For both alignment and read-counting, Gencode V19 reference was used.

2.3 | RT-qPCR analysis

In order to analyze expression levels of *CREB3L1* in PRP-derived RNA through RT-qPCR, we first explored for an internal control gene. To do so, two sets of read-count data generated from fastq targeting platelet RNA were prepared: One set was derived from the abovementioned PRP-derived RNA of 9 ET patients and 6 patients with reactive thrombocytosis in our study, and the other set was derived from fastq reported by Best et al,[12] which was based on 6 patients with various solid cancers and 11 healthy controls and was downloaded from DNA Data Bank of Japan (DDBJ) Sequence Read Archive (SRA accession: SRA260904, http://trace.ddbj.nig.ac.jp/dra/index.html). Next, the two sets of read-count data were compared to select commonly expressed genes regardless of the type of disease. The genes satisfying both logarithm of counts per million mapped reads (logCPM) > 10 and coefficient of variation (CV) <0.3 were screened, and *B2M* was defined as the internal control gene.

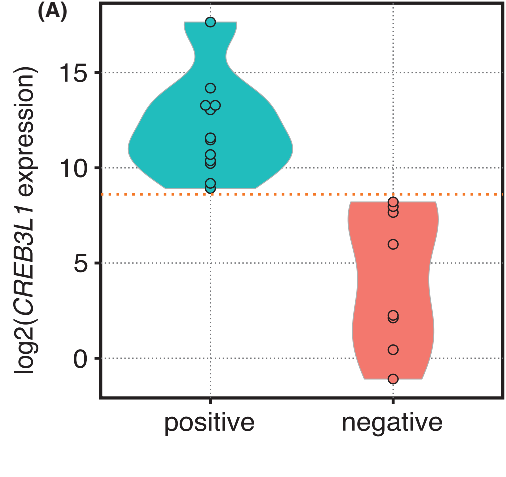
RT-qPCR was performed with a set of primers (forward primer: GGA GAA TGC CAA CAG GAC, reverse primer: ACC AGA ACA AAG CAC AAG G for *CREB3L1*; and forward: CTA TCC AGC GTA CTC CAA AG, reverse: ACA AGT CTG AAT GCT CCA CT for *B2M*) and THUNDERBIRD® SYBR qPCR Mix (Toyobo). The cDNA synthesized with the random hexamers and oligo-dT primers used for RT-qPCR was equivalent to 100 ng of PRP-derived RNA. The thermal conditions of the RT-qPCR were as follows: an initial denaturation at 95°C for 1 minute, 45 cycles of denaturation at 95°C for 30 seconds, annealing at 65°C for 30 seconds, and extension at 72°C for 30 seconds. The relative expression levels of *CREB3L1* in each sample compared with the mean expression level of the 18 healthy controls were calculated by the ΔΔCt method.

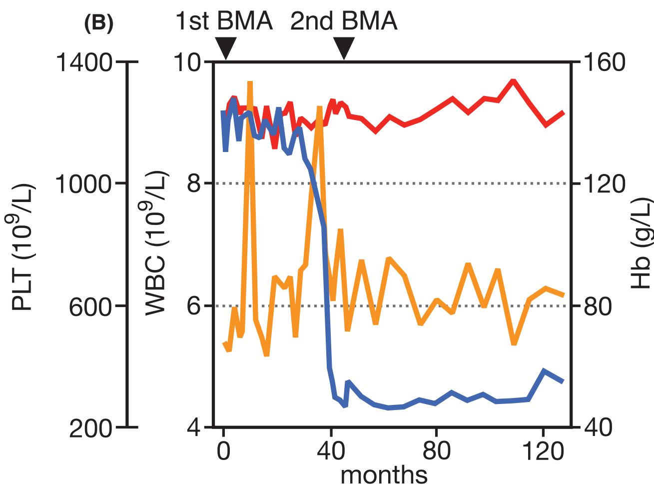
The cutoff value discriminating driver mutation–positive Ph-MPNs from other entities was defined as the mean expression level of the healthy controls +20σ. Statistical analyses were carried out using the R-software (version 3.6.1). The Kruskal-Wallis test was applied for comparison of *CREB3L1* expression levels between different entity groups. For continuous variables, the Mann-Whitney *U* test and Holm's adjustment were applied as post hoc test, and *P*-values less than .05 were considered significant.

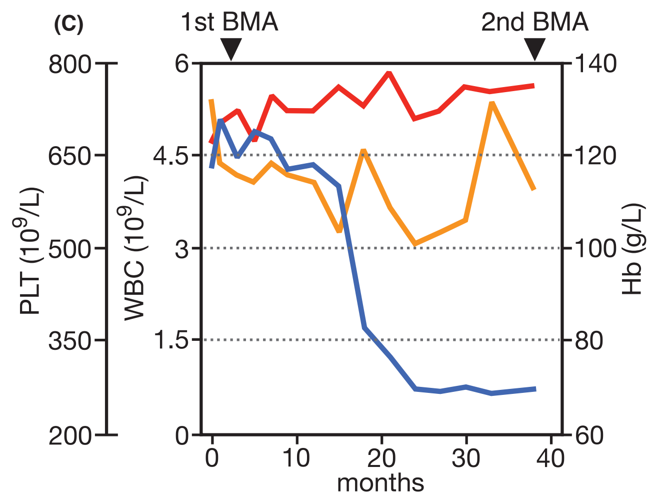
3 | RESULTS

3.1 | *CREB3L1* expression levels can accurately distinguish driver mutation–positive ET from reactive thrombocytosis

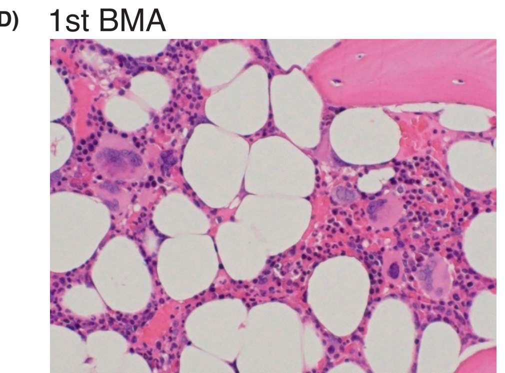
The procedures of how we identified *CREB3L1* through RNA-seq are shown in Figure S1. We performed RNA-seq analysis followed by differential expression (DE) analysis using the edgeR package of R (version 3.6.1)[13] to identify differentially expressed genes (DEGs) of ET and reactive thrombocytosis. 303 genes (67 and 236 genes were highly expressed in ET and reactive thrombocytosis, respectively) satisfying false discovery rate (FDR) ≤ 0.05, logarithm of fold change (logFC) > 2.0 or <−2.0, and logCPM > 1.0 were first filtered through DE analysis. The histogram of the area under the receiver operating characteristic curve (AUC) of the 303 genes led us to define the AUC cutoff as 0.95 (Figure 1A), and as a result, 57 genes (9 and 48 genes were highly expressed in ET and reactive thrombocytosis, respectively) were further filtered as DEGs (Table S2). Principal component analysis (PCA) was performed in order to confirm the expression profile of the DEGs in ET and reactive thrombocytosis utilizing reads per kilobase of exon per million mapped reads (RPKM). PCA was performed using the prcomp package of R. The cumulative frequency of the principal components (PC) calculated by PCA indicated that 88.25% of all variances were attributable to the sum of PC1 and PC2 (Figure 1B). As expected, PCA revealed that the RNA expression profile of the 57 DEGs was clearly different between ET and reactive thrombocytosis (Figure 1C). Furthermore, PCA also demonstrated that the expression profiles within ETs were similar regardless of the driver mutation type, suggesting that the signaling pathways used among ETs are common. On the other hand, the expression profiles


(A)
log2(CREB3L1 expression)
15
10
5
0
positive
negative

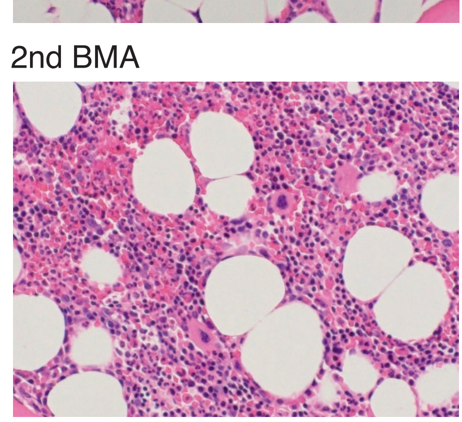

(B)
1st BMA
2nd BMA
1400
1000
600
200
PLT (10^9/L)
10
8
6
4
WBC (10^9/L)
160
120
80
40
Hb (g/L)
0
40
80
120
months


(C)
1st BMA
2nd BMA
800
650
500
350
200
PLT (10^9/L)
6
4.5
3
1.5
0
WBC (10^9/L)
140
120
100
80
60
Hb (g/L)
0
10
20
30
40
months

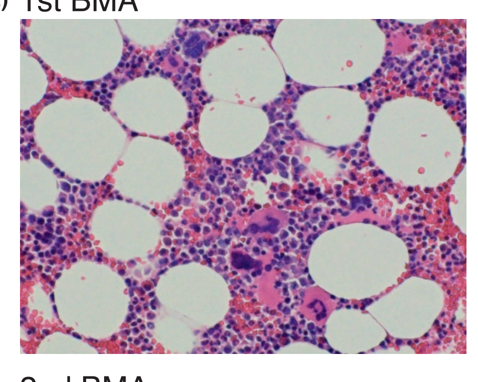
(D) 1st BMA



2nd BMA



(E) 1st BMA



2nd BMA

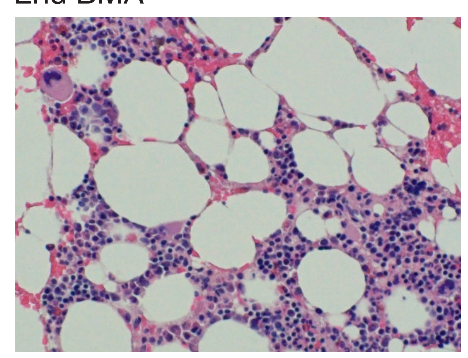


FIGURE 3 *CREB3L1* expressions in pathology-affirmed triple-negative essential thrombocythemia (TN-ET). A, Violin plot comparing *CREB3L1* expression levels among TN-ET patients. Dotted orange line shows the cutoff defined in the present study. B and C, Transition of platelet (PLT, blue) counts, hemoglobin (Hb, red), and white blood cell (WBC, yellow) counts of two TN-ET patients without *CREB3L1* overexpression and showing spontaneous remission. B, A 25-y-old man presenting with an increased PLT count of 1216×10^9/L. Bone marrow (BM) biopsy showed normocellular BM with marked increase in megakaryocytes. After 38 mo of follow-up, the PLT count spontaneously decreased to normal range and was sustained at normal levels for 85 mo. C, A 50-y-old woman presenting with an increased PLT count of 631×10^9/L and a normocellular BM with an increase in mature megakaryocytes. PLT counts spontaneously decreased to 371×10^9/L after 18 mo of follow-up and was maintained within normal range for 15 mo. D and E, Histopathological BM images of (B) and (C) at the first and second bone marrow analysis (BMA), respectively. All BM images were obtained from the time points indicated as arrowheads in (B) and (C).

of reactive thrombocytosis cases were diverse, which reflects the heterogenous nature of this group. Because discriminating ET from reactive thrombocytosis was more effective utilizing PC1 compared with PC2 (Figure 1C), factor loadings of the 57 DEGs in PC1 were calculated, and *CREB3L1* was found to have the highest impact in discriminating ET from reactive thrombocytosis (Table S2). When the logCPM of patients with ET and reactive thrombocytosis were compared, the box plot showed that the expression level of *CREB3L1* was able to completely distinguish ET from reactive thrombocytosis (P = .001770, Figure 1D).

Next, in a validation cohort including 78 ET patients and 38 patients with reactive thrombocytosis, we found that *CREB3L1* expression levels in PRP-derived RNA analyzed through RT-qPCR were able to accurately discriminate ET from reactive thrombocytosis in all patients ($P < .0001$, Figure 2A).

3.2 | *CREB3L1* is a distinct biomarker of driver mutation–positive Ph-MPNs

Because other entities within Ph-MPNs such as PV and PMF also often present with thrombocytosis, and also because PV, PMF, and post-PV/ET MF are considered related disorders of ET, we next extended our validation cohort to patients with Ph-MPNs other than ET. We included 30 PV, 16 overt PMF, 7 pre PMF, 19 post-PV/ET MF, 6 CML, 30 reactive erythrocytosis, 3 reactive MF, and 18 healthy volunteers. A clear cutoff line discriminating 150 Ph-MPNs from other 89 entities was establishable based on the *CREB3L1* expression levels of 18 healthy volunteers (Figure 2A), and sensitivity and specificity for this testing were both 1.0000 (Figure 2B). Furthermore, *CREB3L1* expression levels were significantly higher in 30 PV compared with 30 reactive erythrocytosis ($P < .0001$, Figure 2A), whereas no significant differences in *CREB3L1* expression levels were observed among CML, reactive cases, and healthy volunteers.

In order to confirm that high expression of *CREB3L1* is specific to platelets, *CREB3L1* mRNA levels in purified granulocytes and platelet-deprived plasma were quantified by RT-qPCR and were found to be less than 1/1000 compared with PRP of Ph-MPN patients (data not shown). *CREB3L1* expression was found to be significantly higher in Ph-MPN patients harboring *JAK2* and *MPL* mutations compared with patients with the *CALR* insertion 5 mutation (P = .0069 and P = .0133, respectively, Figure 2C), and when limited to patients with ET, *CREB3L1* expressions among *JAK2*-mutated patients were still significantly higher compared with patients with the *CALR* insertion 5 mutation (P = .03, data not shown).

Spearman rank correlation method revealed a weak positive correlation between *CREB3L1* expression levels and lactase dehydrogenase values (Spearman's correlation coefficient [rs] = .2959, P = .000632). No significant correlations were observed between *CREB3L1* expression levels and other clinical parameters including *JAK2*V617F allele burdens (Figure S2).

3.3 | Triple-negative ET is divided into two groups by *CREB3L1* expression levels

We also examined *CREB3L1* expression levels in 20 pathology-affirmed triple-negative ET (TN-ET) patients. All TN-ET patients were confirmed not to have *JAK2*, *MPL*, and *CALR* mutations of atypical genetic loci through amplicon-based target resequencing utilizing the MiniSeq system (Illumina) (data not shown). Within 20 TN-ET patients, *CREB3L1* overexpression was observed in 12 patients and absent in 8 patients (Figure 3A). Platelet and white blood cell counts were significantly higher in patients with *CREB3L1* overexpression than in patients without *CREB3L1* overexpression (Table S3). Also, a trend towards higher *CREB3L1* overexpression levels were seen in females (P = .06233). Importantly, among TN-ET patients, two patients without *CREB3L1* overexpression were initially diagnosed with BM pathology–affirmed ET, but their platelet counts spontaneously decreased to normal levels during the disease course (Figure 3B,C), and disappearance of morphological characteristics of ET upon second BM examinations performed after a decrease in platelet counts was also confirmed (Figure 3D,E).

4 | DISCUSSION

Through comparison of gene expression profiles of PRP-derived RNA, we found that *CREB3L1* was highly expressed not only in ET but also in PV, PMF, and post-PV/ET MF, and expression levels could accurately discriminate driver-mutation–positive Ph-MPNs from reactive hypercytosis, reactive MF, and CML.

CREB3L1, also known as Old Astrocyte Specifically Induced Substance (OASIS), was originally identified in astrocytes as an

endoplasmic reticulin (ER) stress transducer involved in the unfolded protein response signaling pathway.[14] CREB3L1 resides as a transmembrane molecule of the ER at steady state. Responding to ER stress, CREB3L1 is transported from the ER to the Golgi apparatus and then cleaved by S1P and S2P proteases to release the N-terminal fragment. This fragment translocates to the nucleus and functions as a transcription factor.[15] CREB3L1 is known to contribute to osteogenesis and bone angiogenesis by regulating Collagen Type 1 α 1 chain (COL1A1) and Hypoxia Inducible Factor 1 subunit α (HIF1α), respectively.[16,17] Also, CREB3L1 expression is reported to be suppressed in certain cancers[18,19] and is associated with metastatic risk and treatment response in breast cancer.[20,21] Although the pathophysiological role of CREB3L1 concerning Ph-MPNs is still veiled, this is the first report demonstrating its potential of becoming the cornerstone of Ph-MPN diagnosis.

The 2016 WHO criteria ultimately requires a BM biopsy in order to discriminate Ph-MPNs from reactive cases, but this may not necessarily be easy in the real-world setting because previous reports have pointed out that pathological diagnosis of Ph-MPNs is not always reproducible even within expert hematopathologists.[22-24] Early utilization of *CREB3L1* testing in the diagnostic process can guide patients to an efficient diagnosis and free many patients with disorders other than Ph-MPNs from unnecessary BM biopsies. In addition, PRP-derived RNA could be stably stored at 4°C for at least 24 hours in our preliminary observations (data not shown), implying that *CREB3L1* testing has a potential for practical use in diagnosis of Ph-MPNs.

In the current study, patients with a pathology-affirmed diagnosis of PV who were positive for either *JAK2*V617F or *JAK2* exon12 mutations were all found to have overexpression of *CREB3L1*. On the other hand, patients without *CREB3L1* overexpression and negative for both *JAK2*V617F and *JAK2* exon12 mutations who underwent BM biopsies were all pathologically excluded from a diagnosis of PV (data not shown). Therefore, *CREB3L1* analysis alone can reliably discriminate PV and non-PV in patients with erythrocytosis. Under the current algorithm for patients suspected of PV, *JAK2*V617F analysis would be carried out first, and in *JAK2*V617F-negative cases, subsequent *JAK2* exon12 analysis would be required. However, confirmation of low *CREB3L1* expression levels can single-handedly exclude a diagnosis of PV, and early utilization of *CREB3L1* analysis in the diagnostic process can omit many patients from both *JAK2*V617F and *JAK2* exon12 analysis.

Patients with thrombocytosis but no erythrocytosis who were positive for *JAK2*V617F, *CALR*, or *MPL* mutations along with a pathology-affirmed diagnosis of ET or PMF (including prefibrotic PMF) showed *CREB3L1* overexpression. Conversely, triple-negative patients with *CREB3L1* overexpression were all pathologically diagnosed as ET. Therefore, it can be concluded that patients with thrombocytosis but no erythrocytosis who show *CREB3L1* overexpression have either ET or PMF. Although at this point a diagnosis of ET cannot be ruled out in triple-negative patients with no *CREB3L1* overexpression, we encountered two patients who achieved spontaneous remissions within such patients. Moreover, clinical characteristics differed between TN-ET patients with and without *CREB3L1* overexpression such as platelet and white blood cell counts, suggestive that the two groups may be different entities. Therefore, even if a patient is rendered a diagnosis of TN-ET, a careful monitoring of the clinical course without upfront cytoreductive therapy should be considered in patients lacking *CREB3L1* overexpression. TN-ET is a heterogenous disorder, and a large-scale analysis including massively parallel sequencing is awaited, especially in patients lacking *CREB3L1* overexpression.

CREB3L1 expression levels distinguished not only Ph-MPNs from reactive cases but also Ph-MPNs from CML. Although distinct entities, CML and Ph-MPNs are similar in that both are clonal myeloid malignancies and both can present with hypercytosis. This finding shows that *CREB3L1* testing is not a mere discriminator of malignant and nonmalignant disease but is most probably a more specific indicator of Ph-MPNs. Furthermore, we analyzed *CREB3L1* expression levels before and after successful allogeneic stem cell transplantation in one post-ET MF and two overt PMF patients and found that *CREB3L1* expression levels decreased from above to below the cutoff level along with a negative conversion of driver mutations in all three cases (Figure S3), which is further evidence of Ph-MPN-specificity.

The *polycythemia rubra vera-1* (*PRV-1*) gene was also reported to be overexpressed in Ph-MPNs, but the prevalence was inconsistent and ranged from 69% to 100% for PV and 16% to 100% for ET. Furthermore, a major disadvantage was that *PRV-1* overexpression was also found in a proportion of reactive erythrocytosis, reactive thrombocytosis, and in healthy controls.[25] Compared with this, *CREB3L1* overexpression showed both a sensitivity and specificity of 1.0000 for diagnosing driver mutation–positive Ph-MPNs, and practical application of this method is much more realistic. Guo et al have also studied the transcript signature of platelets from Ph-MPN patients and listed *CREB3L1* along with hundreds of other DEGs of Ph-MPNs and controls. However, the importance of this gene concerning Ph-MPN diagnosis has not been addressed until now.[26]

In conclusion, although the etiological significance of *CREB3L1* overexpression in Ph-MPNs has yet to be elucidated, *CREB3L1* testing is a promising method for discriminating Ph-MPNs from other disorders in patients with hypercytosis or MF and has the potential of becoming the hallmark of Ph-MPN diagnosis. Also, early utilization of this method in the diagnostic process will free many patients from time-consuming and costly testing.

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DISCLOSURE

Morishita, Yamawaki, Kawaji, Itoh, Tsuneda, and Komatsu applied for a patent concerning *CREB3L1* testing for diagnosis of Ph-MPNs. No other conflict of interest exists.

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REFERENCES

1. Kralovics R, Passamonti F, Buser AS, et al. A gain-of-function mutation of JAK2 in myeloproliferative disorders. *N Engl J Med*. 2005;352(17):1779-1790.
2. Pardanani AD, Levine RL, Lasho T, et al. MPL515 mutations in myeloproliferative and other myeloid disorders: a study of 1182 patients. *Blood*. 2006;108(10):3472-3476.
3. Klampfl T, Gisslinger H, Harutyunyan AS, et al. Somatic mutations of calreticulin in myeloproliferative neoplasms. *N Engl J Med*. 2013;369(25):2379-2390.
4. Misawa K, Yasuda H, Araki M, et al. Mutational subtypes of JAK2 and CALR correlate with different clinical features in Japanese patients with myeloproliferative neoplasms. *Int J Hematol*. 2018;107(6):673-680.
5. Arber DA, Orazi A, Hasserjian R, et al. The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. *Blood*. 2016;127(20):2391-2405.
6. Kvasnicka HM, Orazi A, Thiele J, et al. European LeukemiaNet study on the reproducibility of bone marrow features in masked polycythemia vera and differentiation from essential thrombocythemia. *Am J Hematol*. 2017;92(10):1062-1067.
7. Morishita S, Komatsu N, Kirito K, et al. Alternately binding probe competitive PCR as a simple, cost-effective, and accurate quantification method for JAK2V617F allele burden in myeloproliferative neoplasms. *Leuk Res*. 2011;35(12):1632-1636.
8. Takei H, Morishita S, Araki M, et al. Detection of MPLW515L/K mutations and determination of allele frequencies with a single-tube PCR assay. *PLoS One*. 2014;9(8):e104958.
9. Shirane S, Araki M, Morishita S, et al. JAK2, CALR, and MPL mutation spectrum in Japanese patients with myeloproliferative neoplasms. *Haematologica*. 2015;100(2):e46-e48.
10. Dobin A, Gingeras TR. Mapping RNA-seq reads with STAR. *Curr Protoc Bioinformatics*. 2015;51:1141-1149.
11. Liao Y, Smyth GK, Shi W. featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics*. 2014;30(7):923-930.
12. Best M, Sol N, Kooi I, et al. RNA-Seq of tumor-educated platelets enables blood-based pan-cancer, multiclass, and molecular pathway cancer diagnostics. *Cancer Cell*. 2015;28(5):666-676.
13. Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics*. 2010;26(1):139-140.
14. Kondo S, Murakami T, Tatsumi K, et al. OASIS, a CREB/ATF-family member, modulates UPR signalling in astrocytes. *Nat Cell Biol*. 2005;7(2):186-U12.
15. Sampieri L, Di Giusto P, Alvarez C. CREB3 transcription factors: ER-Golgi stress transducers as hubs for cellular homeostasis. *Front Cell Dev Biol*. 2019;7:123.
16. Cui M, Kanemoto S, Cui X, et al. OASIS modulates hypoxia pathway activity to regulate bone angiogenesis. *Sci Rep*. 2015;5:16455.
17. Murakami T, Saito A, Hino S-I, et al. Signalling mediated by the endoplasmic reticulum stress transducer OASIS is involved in bone formation. *Nat Cell Biol*. 2009;11(10):1205-1211.
18. Rose M, Schubert C, Dierichs L, et al. OASIS/CREB3L1 is epigenetically silenced in human bladder cancer facilitating tumor cell spreading and migration in vitro. *Epigenetics*. 2014;9(12):1626-1640.
19. Ward AK, Mellor P, Smith SE, et al. Epigenetic silencing of CREB3L1 by DNA methylation is associated with high-grade metastatic breast cancers with poor prognosis and is prevalent in triple negative breast cancers. *Breast Cancer Res*. 2016;18(1):12.
20. Feng YX, Jin DX, Sokol ES, Reinhardt F, Miller DH, Gupta PB. Cancer-specific PERK signaling drives invasion and metastasis through CREB3L1. *Nat Commun*. 2017;8(1):1079.
21. Denard B, Jiang S, Peng Y, Ye J. CREB3L1 as a potential biomarker predicting response of triple negative breast cancer to doxorubicin-based chemotherapy. *BMC Cancer*. 2018;18(1):813.
22. Guglielmelli P, Pacilli A, Rotunno G, et al. Presentation and outcome of patients with 2016 WHO diagnosis of prefibrotic and overt primary myelofibrosis. *Blood*. 2017;129(24):3227-3236.
23. Pozdnyakova O, Wu K, Patki A, Rodig SJ, Thiele J, Hasserjian RP. High concordance in grading reticulin fibrosis and cellularity in patients with myeloproliferative neoplasms. *Mod Pathol*. 2014;27(11):1447-1454.
24. Sulai NH, Tefferi A. Why does my patient have thrombocytosis? *Hematol Oncol Clin North Am*. 2012;26(2):285-301.
25. Steimle C, Lehmann U, Temerinac S, et al. Biomarker analysis in polycythemia vera under interferon-alpha treatment: clonality, EEC, PRV-1, and JAK2 V617F. *Ann Hematol*. 2007;86(4):239-244.
26. Guo BB, Linden MD, Fuller KA, et al. Platelets in myeloproliferative neoplasms have a distinct transcript signature in the presence of marrow fibrosis. *Br J Haematol*. 2020;188(2):272-282.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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