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The role of Janus Kinase 2 V617F mutation in extramedullary hematopoiesis of the spleen in neoplastic myeloid disorders

Pin-Pen Hsieh[1,2], Randy J Olsen[1], Dennis P O'Malley[3], Sergej N Konoplev[4], Jerry W Hussong[5], Cherie H Dunphy[6], Sherrie L Perkins[7], Liang Cheng[3], Pei Lin[4] and Chung-Che Chang[1,8]

[1]*Department of Pathology and Laboratory Medicine, The Methodist Hospital and the Methodist Hospital Research Institute, Houston, TX, USA;* [2]*Department of Pathology and Laboratory Medicine, Veterans General Hospital-Kaohsiung, Kaohsiung, Taiwan;* [3]*Department of Pathology, Indiana University, Indianapolis, IN, USA;* [4]*Department of Hematopathology, The University of Texas MD Anderson Cancer Center, Houston, TX, USA;* [5]*Laboratory Medicine Consultants and Sunrise Hospital, Las Vegas, NV, USA;* [6]*Department of Pathology, University of North Carolina, Chapel Hill, NC, USA;* [7]*Department of Pathology, University of Utah and ARUP, Salt Lake City, UT, USA and* [8]*Department of Pathology, Weill Medical College of Cornell University, New York, NY, USA*

Extramedullary hematopoiesis (EMH) in the spleen is a characteristic feature of the chronic myeloproliferative disorders (CMPDs) and various other neoplastic or reactive myeloid conditions. However, the origin of these hematopoietic precursor cells and the molecular mechanisms underlying their development in the spleen is uncertain. The V617F mutation in the *Janus Kinase 2* gene (*JAK2*^{V617F}) was recently shown to be frequently and preferentially present in the peripheral blood and bone marrow cells of CMPD patients, and the resulting dysregulation of its downstream targets is important to CMPD pathogenesis. To determine the occurrence and potential role of *JAK2*^{V617F} in splenic EMH cells, we studied splenectomy specimens from 47 patients with significant EMH. *JAK2*^{V617F} was detected by real-time PCR melting curve analysis in 22 specimens, including 11/17 chronic idiopathic myelofibrosis, 7/7 polycythemia vera, 1/1 essential thrombocythemia, 1/3 CMPD unclassifiable, 1/5 chronic myelomonocytic leukemia, 0/5 chronic myelogenous leukemia, 1/3 myelodysplastic syndrome and 0/6 acute myeloblastic leukemia cases, whereas only the *JAK2* wild-type allele was detected in the other 25. Nineteen of 20 cases with adequate bone marrow samples available for molecular examination demonstrated concordant *JAK2* genotypes. Laser-capture microdissection was then used to enrich the EMH and non-EMH splenic cell fractions, confirming that the mutant alleles specifically originated from the EMH cells. Furthermore, megakaryocytes in the *JAK2*^{V617F}-positive splenectomy specimens expressed higher levels of Bcl-xL, an antiapoptotic protein and downstream target of the *JAK2/STAT5* pathway. Thus, *JAK2*^{V617F} is frequently present in splenic EMH cells associated with CMPD, but it is rarely identified in splenic EMH cells associated with other myeloid disorders. Our results indicate that the precursor cells leading to extramedullary hematopoietic expansion in CMPD most likely originate from the transformed bone marrow clone. Also, dysregulation of downstream pathways such as Bcl-xL may be important to CMPD disease pathogenesis in the spleen.
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Extramedullary hematopoiesis (EMH) in the spleen is a characteristic feature of the chronic myeloproliferative disorders (CMPD). EMH may also occur less frequently in patients with reactive conditions or other neoplastic disorders such as myelodysplastic syndrome (MDS) or chronic myelomonocytic leukemia (CMML). The normal adult spleen virtually always contains a very small number of mature hematopoietic elements, but the origin of the precursor cells giving rise to EMH and the molecular mechanisms underlying their

Correspondence: Dr C-C Chang, MD, PhD, Department of Pathology and Laboratory Medicine, The Methodist Hospital, 6565 Fannin Street, M227, Houston, TX 77030, USA.
E-mail: jeffchang@tmh.tmc.edu
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development are uncertain. It has been hypothesized that EMH cells are derived from either circulating neoplastic stem cells, which undergo terminal differentiation within the spleen as part of the neoplastic process or reactivated embryonic remnants of fetal hematopoiesis as compensation for marrow failure.[1–5] The former concept is more strongly favored, as hematopoietic elements in EMH have histomorphological and molecular findings similar to those of the corresponding marrow biopsies.[1–5] However, the clonal relationship between splenic EMH cells and neoplastic bone marrow cells remains unconfirmed.

Recently, the V617F mutation in the *Janus kinase 2* gene (*JAK2*^{V617F}) was shown to be frequently and preferentially identified in bone marrow and peripheral blood cells of CMPD patients. The *JAK2*^{V617F} allele, present in all hematopoietic cells arising from the neoplastic clone, has been detected in the majority of cases of polycythemia vera (PV), essential thrombocythemia (ET), chronic idiopathic myelofibrosis (CIMF) and acute leukemia transformed from a preexisting CMPD, but it is rarely identified in healthy controls or patients with other myeloid disorders.[6–13] Functioning as a constitutively activated cytoplasmic tyrosine kinase, the mutated *JAK2*^{V617F} protein probably mediates myeloproliferation via dysregulation of various *JAK2*/*STAT5* signaling pathways. Targeted genes, including the antiapoptotic protein Bcl-xL, have been implicated in vital cellular processes such as proliferation and survival.[13–17] Furthermore, retroviral transduction of *JAK2*^{V617F} into murine hematopoietic stem cells was recently shown to result in the development of a polycythemia-like phenotype.[7,18]

The role of *JAK2*^{V617F} in splenic EMH has not been previously studied. We used laser-capture microdissection (LCM), real-time PCR melting curve analysis and immunohistochemistry to characterize the hematopoietic precursor cells found in spleen and bone marrow specimens of patients with significant EMH. These results demonstrate that the *JAK2*^{V617F} mutation is frequently present in splenic EMH cells associated with CMPD. Thus, the precursor cells that lead to extramedullary hematopoietic expansion most likely originate from the transformed bone marrow clone. In addition, the overexpression of Bcl-xL in *JAK2*^{V617F}-positive megakaryocytes suggests that the *JAK2*/*STAT5*/Bcl-xL pathway may be important to the myeloproliferative process, at least partially, in splenic EMH.

Materials and methods

Patient Samples

Retrospective review of pathology and hematology files from our respective institutions identified 47 splenectomy specimens demonstrating significant EMH from patients previously diagnosed with CMPD, MDS, CMPD/MDS, chronic myelogenous leukemia (CML) and acute myeloid leukemia (AML). Biopsies were performed between 1993 and 2005, and patient ages ranged from 37 to 90 years with a median age of 64 years. The morphologic diagnosis of EMH was confirmed, and the pattern of EMH was classified as nodular (macronodular proliferation of EMH), diffuse (diffuse pattern of EMH with trilineage myeloid involvement), granulocyte predominant (immature granulocyte predominance) or scant (few hematopoietic cells present in no distinct pattern).[19] Twenty cases had adequate bone marrow specimens available for correlative molecular studies; the time interval between splenectomy and bone marrow biopsy was less than 12 months for 17 cases and less than 33 months for the remaining three cases. None of the cases had additional biopsies from other anatomical sites that typically demonstrate EMH. Results of a pilot study evaluating *JAK2* mutational status on a small subset of these cases using a different methodology and different criteria is also currently under review (Table 1). This study was approved by the Institutional Review Board.

Laser-Capture Microdissection

LCM was performed on 10 unstained splenectomy sections using a PixCell II Laser Capture Microdissection system (Arcturus Engineering, Mountain View, CA, USA) to enrich cell fractions for either EMH or non-EMH cells.[20,21]

DNA Extraction and Real-Time PCR Melting Curve Analysis for *JAK2* Genotype

DNA was extracted from formalin-fixed paraffin-embedded spleen sections, bone marrow aspirates and LCM-enriched cell fractions as described previously.[22] Real-time PCR melting curve analysis for the *JAK2* wild-type and V617F mutant allele was performed as described previously.[22] Homozygous mutant human erythroleukemia (HEL) and homozygous wild-type multiple myeloma (RPMI8226) cell lines were used as positive and negative controls, respectively. As previously reported, the analytical sensitivity of our real-time PCR melting curve assay is capable of detecting one homozygous V617F mutant cell in 20 total cells (5% lower limit of mutant allele frequency detection).[22] Furthermore, the peak height (dF/dT *vs* T) of each *JAK2* allele curve (*JAK2*^{V617F} *vs* wild-type) proportionately increases or decreases with its relative frequency, providing a semiquantitative measurement (Figure 1). The relative proportion of wild-type and mutant *JAK2* alleles was scored as either mutant only (M), mixed clonality (M&W) for cases with similar peak heights of both *JAK2*^{V617F} and wild-type alleles, mutant predominant (M > W) for cases with a large

Table 1 Molecular, histological and clinical results from patients with splenic extramedullary hematopoiesis (EMH)

Case no.	*BM diagnosis*	*JAK2 whole spleen*	*JAK2 EMH cells*	*JAK2 non-EMH cells*	*JAK2 BM*	*Spleen weight (g)*	*Histology pattern*	*Bcl-xL (%)*
1	PV	M>W	—	—	—	1390	D	79
2	PV	M>W	—	—	—	5536	D	—
3	PV	M>W	—	—	—	3943	N	—
4	PV	M>W	—	—	—	1645	N	—
5[a]	PV	M>W	—	—	M>W	2970	D	78
6[a]	PV	M>W	—	—	W>M	3070	N	64
7[a]	PV	W>M	—	—	M&W	4700	D	56
8	ET	W>M	W>M	W>M	—	285	D	35
9	CIMF	M>W	—	—	—	3500	D	81
10[a]	CIMF	M>W	—	—	M>W	1700	D	52
11[a]	CIMF	M>W	—	—	W	3332	D	82
12	CIMF	M&W	—	—	M>W	3000	D	—
13[a]	CIMF-BC	M&W	—	—	M>W	2190	D	77
14	CIMF	M&W	M	M&W	—	2400	D	33
15	CIMF	W>M	M>W	W>M	—	960	D	—
16[a]	CIMF	W>M	—	—	W>M	1400	D	82
17	CIMF	W>M	—	—	—	—	D	—
18	CIMF	W>M	—	—	—	3693	D	—
19	CIMF	W[b]	M	W	—	1470	G	—
20	CIMF	W	—	—	—	3840	D	—
21[a]	CIMF	W	—	—	W	2280	D	22
22	CIMF	W	W	W	—	2320	D	—
23[a]	CIMF	W	—	—	W	820	D	30
24	CIMF	W	—	—	W	1233	N	—
25[a]	CIMF-BC	W	—	—	W	885	D	34
26	CMPDu	M&W	M>W	M&W	—	2300	D	18
27	CMPDu	W	—	—	W	3850	D	—
28[a]	CMPDu-BC	W	—	—	W	3900	D	31
29	CML	W	W	W	—	1405	D	34
30	CML	W	W	W	—	360	S	0
31	CML-BC	W	—	—	—	1880	G	0
32	CML	W	—	—	—	1510	N	—
33	CML	W	W	W	—	550	S	0
34[a]	CMML	W>M	—	—	W>M	1390	D	72
35	CMML & fib	W	—	—	W	1319	—	—
36[a]	CMML-BC	W	—	—	W	1099	D	41
37[a]	CMML	W	—	—	W	2800	N	60
38[a]	CMML	W	—	—	W	2250	D	9
39	MDSu	W>M	—	—	—	822	D	—
40	MDSu	W	—	—	—	1342	D	—
41	RAEB	W	—	—	W	170	D	44
42	AML	W	—	—	—	362	D	—
43	AML-MDS	W	—	—	—	1056	—	—
44	AML-CMML	W	—	—	—	3100	—	—
45	AML-CMPD	W	—	—	—	2040	D	—
46	AML-CMPD	W	—	—	—	3476	D	—
47	AML-CIMF	W	W	W	—	2100	D	47

Results from the 47 cases are shown. Diagnoses included PV (polycythaemia vera); ET (essential thrombocythaemia); CIMF (chronic idiopathic myelofibrosis); CIMF-BC (CIMF in blast crisis); CMPDu (chronic myeloproliferative disease, unclassified); CMPDu-BC (CMPDu in blast crisis); CML (chronic myeloid leukemia); CML-BC (CML in blast crisis); CMML (chronic myelomonocytic leukemia); MDSu (myelodysplastic syndrome, unclassified); RAEB (refractory anemia with excess blasts); AML (acute myeloid leukemia) and AML- (AML transformed from a preexisting condition). *JAK2* genotype by real-time PCR melting curve analysis of EMH cells extracted from the whole spleen, splenic EMH cells enriched by laser capture microdissection, and splenic non-EMH cells enriched by laser capture microdissection is shown. Genotype is reported as M>W (predominately mutant allele detected via a dominant $JAK2^{V617F}$ melting curve peak), M&W (equal amounts of mutant and wild-type allele detected via approximately equivalent peaks), and W>M (predominately wild-type allele detected via a dominant *JAK2* wild-type peak) (Figure 1). Spleen weight is reported in grams (g). Histology pattern of EMH cells is reported as N (nodular), D (diffuse), G (granulocyte predominant) or S (scant). — indicates that an adequate specimen was not available for further analysis.

[a]The 15 cases also reviewed by different methodologies and different criteria as described in the Materials and methods section.

[b]That case 19 was considered to be $JAK2^{V617F}$ positive for subsequent statistical analyses.

peak of mutant alleles and a smaller peak of wild-type alleles, wild-type predominant (W>M) for cases with a large peak of wild-type alleles and a smaller peak of mutant alleles, or wild-type only (W).

Bcl-xL Immunohistochemical Staining

Bcl-xL expression in splenectomy specimens was studied by immunohistochemical staining using standard methods.[23,24] Briefly, antigen retrieval was

performed at 100°C in a steamer for 30 min (Tris, pH9 buffer), BCL-xL antibody (2H12, Zymed Laboratories Inc., San Francisco, CA, USA) was applied at 1:100, and DAB was used for signal detection. Bcl-xL expression was scored as percentage of positive megakaryocytes.

Results

The *JAK2* V617F mutant allele was detected in 22 of the 47 splenectomy specimens, including 11/17 CIMF (65%), 7/7 PV (100%), 1/1 ET (100%), 1/3 CMPD unclassifiable (33%), 1/5 CMML (20%), 0/5 CML (0%), 1/3 MDS (33%) and 0/6 AML (0%) cases, whereas only the *JAK2* wild-type allele was detected in the other 25 specimens (Table 1). Twenty cases had corresponding bone marrow biopsies available for *JAK2* genotyping. Nineteen (95%) demonstrated a concordant genotype with the spleen, including eight showing both mutant and wild-type alleles and 12 showing only wild-type alleles (Table 1). The single case (case 11) with a discordant *JAK2* genotype showed both mutant and wild-type alleles in the spleen but only wild-type alleles in the bone marrow. Of note, another case (case 6) showed a mutant allele predominance in the spleen and a wild-type allele predominance in the bone marrow.

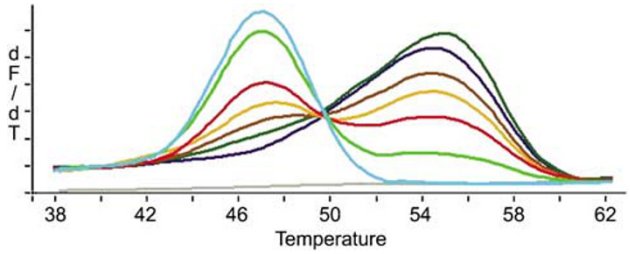

Figure 1 *JAK2* real-time PCR melting curve. A representative *JAK2* real-time PCR melting curve is shown. Serial dilution of a homozygous $JAK2^{V617F}$ human erythroid leukemia cell line (HEL) with a homozygous *JAK2* wild-type multiple myeloma cell line (RPMI8226) demonstrates the semiquantitative measurement of allele frequency by comparison of relative peak heights (47°C for $JAK2^{V617F}$ compared with 55°C for wild-type). $JAK2^{V617F}$ to wild-type ratio (HEL:RPMI8226) is indicated by light blue = 1:0, light green = 1:1, red = 1:4, orange = 1:9, brown = 1:19, dark green = 1:39 and dark blue = 0:1. The negative control (water only) is shown in gray. As demonstrated in the brown curve with a barely observable $JAK2^{V617F}$ peak, the lower limit of detection for this assay is one homozygous mutant cell in total 20 cells. The relative peak height of the $JAK2^{V617F}$ allele decreases as the ratio of mutant to wild-type cells decreases, providing a semiquantitative measurement of allele frequency.

To enhance the sensitivity of $JAK2^{V617F}$ detection and determine whether $JAK2^{V617F}$ alleles specifically originated from the splenic EMH cells, 10 cases were further analyzed by LCM of either EMH or non-EMH cells. *JAK2* genotyping results from the EMH-enriched cell fractions were concordant with the whole-spleen studies, and they demonstrated that LCM significantly increases the mutant allele proportion (Table 1). For example, cases 14, 15, 19 and 26 demonstrated a significantly increased mutant allele proportion in the LCM EMH-enriched splenic cell fraction compared with the whole-spleen samples. In comparison, the non-EMH cell fractions showed enrichment for the wild-type allele. Only case 8 revealed similar amounts of mutant and wild-type *JAK2* alleles in the EMH and non-EMH cell fractions. This finding suggests that the $JAK2^{V617F}$ alleles were predominantly possessed by the hematopoietic elements of splenic EMH rather than being derived from contaminating bone marrow hematopoietic cells in circulation.

The *JAK2* genotype in splenic EMH cells was then correlated with other clinicopathologic parameters. Among all cases, the weight of spleens with $JAK2^{V617F}$ alleles tended to be higher than those with only wild-type alleles (2461 ± 1348 g for $JAK2^{V617F}$-positive compared with 1837 ± 1142 g for *JAK2* wild-type only, student's *t*-test, $P = 0.096$) (Table 2). Similarly, spleen weights among only the non-CML CMPD cases (PV, ET and CIMF) were slightly higher in $JAK2^{V617F}$-positive cases (2627 ± 1371 g for $JAK2^{V617F}$-positive compared with 2390 ± 1269 g for *JAK2* wild-type only, student's *t*-test, $P = 0.713$). There were no statistically significant differences between the hemoglobin concentration, platelet count or white blood cell count in cases with or without $JAK2^{V617F}$ alleles in splenic EMH cells at the time of splenectomy among all cases studied or among the non-CML CMPD cases (Table 3). There was also no correlation between presence of *JAK2* mutant alleles and histological pattern of splenic EMH cells (Table 1).

Expression of Bcl-xL, a well-described antiapoptotic protein and downstream target of *JAK2*, was studied in splenic EMH cells by immunohistochemistry. In the 26 cases available for further morphologic evaluation, the percentage of megakaryocytes expressing Bcl-xL was significantly higher in spleens with the $JAK2^{V617F}$ allele ($62 \pm 22\%$ for $JAK2^{V617F}$-positive compared with $27 \pm 20\%$ for *JAK2* wild-type only, student's *t*-test, $P = 0.0002$) (Table 1 and Figure 2). Furthermore, expression of

Table 2 Mean and standard deviation of spleen weight, hemoglobin concentration, platelet count and white blood cell count in all cases

JAK2 genotype	*Spleen weight (g)*	*Hemoglobin (g/dl)*	*Platelet ($\times 10^9$/l)*	*WBC (10^9/l)*
V617F mutant alleles detected	2461 ± 1348 ($n = 21$)	10.2 ± 2.5 ($n = 12$)	192.2 ± 174.6 ($n = 12$)	19.6 ± 13.3 ($n = 12$)
Wild-type alleles only	1837 ± 1142 ($n = 25$)	9.1 ± 1.4 ($n = 16$)	108.6 ± 130.5 ($n = 16$)	26.4 ± 33.8 ($n = 16$)
P-value, Student's *t*-test	0.096	0.423	0.159	0.514

Table 3 Mean and standard deviation of spleen weight, hemoglobin concentration, platelet count and white blood cell count in non-CML CMPD cases

JAK2 genotype	*Spleen weight (g)*	*Hemoglobin (g/dl)*	*Platelet ($\times 10^9$/l)*	*WBC (10^9/l)*
V617F mutant alleles detected	2627±1371 ($n=18$)	10.2±2.5 ($n=10$)	207.1±187.4 ($n=10$)	23.1±11.6 ($n=10$)
Wild-type alleles only	2390±1269 ($n=6$)	8.4±1.1 ($n=4$)	66.3±80.9 ($n=4$)	48.4±47.9 ($n=4$)
P-value, Student's *t*-test	0.713	0.197	0.180	0.126

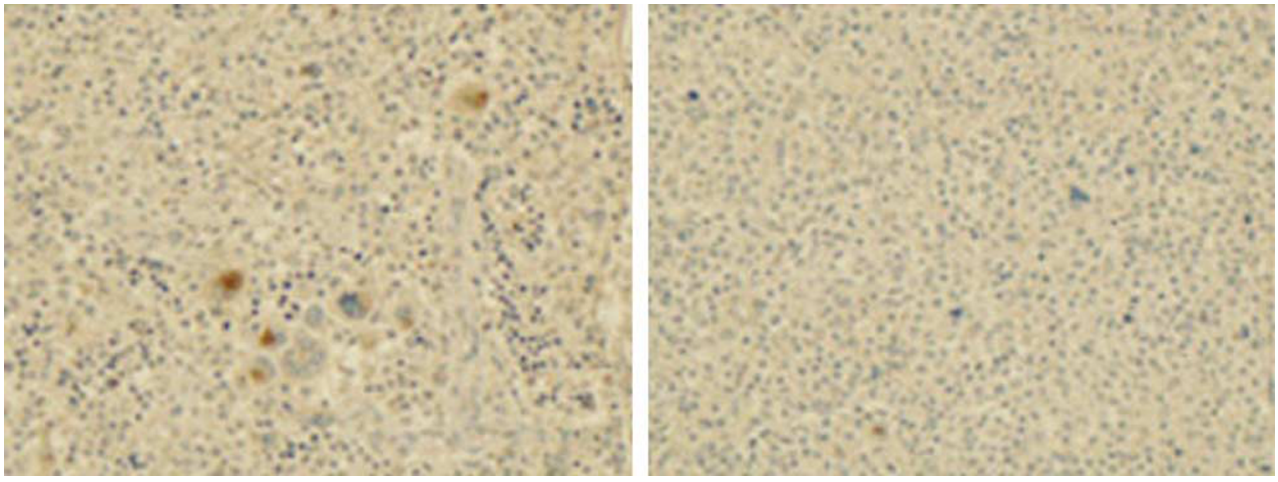

Figure 2 Bcl-xL immunohistochemistry. Representative micrographs of positive cytoplasmic Bcl-xL immunohistochemical staining of EMH megakaryocytes in a *JAK2*^{V617F}-positive spleen (left panel, case 11, ×200) and negative Bcl-xL immunohistochemical staining in a *JAK2*^{V617F}-negative spleen (right panel, case 23, ×200).

Bcl-xL in more than 50% of megakaryocytes was observed in 10/13 *JAK2*^{V617F}-positive cases (77%) but only 1/13 *JAK2*^{V617F}-negative cases (8%) (Fisher's exact test, $P=0.001$). This finding suggests that *JAK2*^{V617F} upregulates Bcl-xL expression and may contribute to the extramedullary expansion of EMH clones with mutant alleles.

Discussion

In this study, we demonstrated that the *JAK2*^{V617F} allele is frequently present in the splenic EMH cells of CMPD patients but rarely present in the splenic EMH cells of patients with other myeloid disorders. This frequency of *JAK2*^{V617F} positivity in splenic EMH cases is similar to those previously reported using peripheral blood and bone marrow samples from patients with PV, CIMF and ET.[6–9,12,13,25–27] These findings confirm that *JAK2*^{V617F} is predominantly associated with the hematopoiesis of non-CML CMPD clones, even in the setting of splenic EMH. Of interest, although our sample size was relatively small, the frequency of *JAK2*^{V617F} in splenic EMH cases associated with MDS and CMML appears to be slightly higher than those previously described in peripheral blood or bone marrow studies.[26–29] This suggests that patients with MDS or CMML carrying the *JAK2* V617F mutation may be more likely to develop splenic EMH, a feature more commonly observed in CMPD than MDS or CMML. Further studies are needed to confirm this observation.

Our results support the hypothesis that hematopoietic precursors giving rise to splenic EMH in patients with neoplastic myeloid disorders may be derived from the transformed bone marrow clone. In virtually all cases of this series, the genotype of the splenic EMH cells was concordant with the corresponding bone marrow specimen. LCM-enriched EMH fractions had more *JAK2* V617F mutant alleles than the non-EMH fractions, and one case had *JAK2*^{V617F} only in spleen, suggesting that the mutant allele is exclusive to the splenic EMH cells. The LCM procedure eliminates the possibility that *JAK2*^{V617F} alleles were contributed by contaminating neoplastic bone marrow cells in circulation. We hypothesize that the clonal myeloid cells may enter the bone marrow sinuses via intrasinusoidal hematopoiesis, a morphological finding commonly observed in CIMF.[30] These precursor cells may then circulate to and become trapped within the spleen, leading to a clonal extramedullary hematopoietic expansion. O'Malley *et al*[3,4] recently reported that EMH cases associated with neoplastic myeloid disorders showed concordant loss-of-heterozygosity (LOH) lesions and X-chromosome inactivation patterns in the respective bone marrow and spleen specimens. Furthermore, additional LOH abnormalities were identified in the spleen, suggesting evolution of the original clone. In addition, the

spleen is a common site of blast transformation for patients with neoplastic myeloid disorders.[19] It would be of great interest to further study our cohort of cases once assays using paraffin-embedded tissues become commercially available to detect other mutations associated with CMPD or acute leukemia, such as MPL-W515L, MPL-W515K, *JAK2*-L611S, *JAK2*-K607N and *JAK2*-T875N.[31–36]

The finding of increased Bcl-xL expression in megakaryocytes of *JAK2*^{V617F}-positive spleen specimens suggests that the *JAK2* V617F mutation may induce myeloproliferation via the *STAT5*/Bcl-xL pathway, at least partially, in splenic EMH. In support, sustained high levels of Bcl-xL were recently demonstrated to promote erythropoietin independent colony formation *in vitro*.[14] However, regardless of *JAK2* genotype, Bcl-xL expression was not observed in the erythroid or myeloid lineages of the splenic EMH cells in our series. This is most likely due to the limited sensitivity of immunohistochemistry for detecting low levels of Bcl-xL expression. Furthermore, the observed tendency for larger spleens to occur in *JAK2*^{V617F}-positive cases agrees with a previous report of 166 CMPD patients (43 PV, 111 ET, 12 CIMF) demonstrating the *JAK2* V617F mutation to confer a higher probability of splenomegaly.[37] Thus, the various *JAK2*/*STAT5* signal transduction pathways, including activated expression of Bcl-xL, may be important to the extramedullary expansion of neoplastic myeloid cells.

CMPD patients with the *JAK2* V617F mutation were previously shown to have higher hemoglobulin/hemocrit levels and leukocyte counts at the time of diagnosis.[38–40] However, we did not observe such differences in this series of patients with established disease at the time of splenectomy. This may be due to the effect of splenomegaly and/or EMH in modifying these hematologic parameters. Alternatively, although less likely, it could be secondary to a relatively smaller sample size.

In summary, our results suggest that the *JAK2*^{V617F}-positive cells in splenic EMH originate from the transformed bone marrow clone and the mutation plays a significant role in the initial development or subsequent progression of splenic EMH in *JAK2*^{V617F}-positive patients with CMPD, CMML and MDS. Thus, the *JAK2*/*STAT5* pathway inhibitors currently undergoing clinical trials may have therapeutic efficacy as a pharmaceutical alternative to surgical intervention for *JAK2*^{V617F}-positive CMPD patients with significant splenomegaly.

Disclosure and conflict of interest

The authors disclose no conflicts of interest.

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