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ORIGINAL ARTICLE

JAK2 V617F is a rare finding in *de novo* acute myeloid leukemia, but STAT3 activation is common and remains unexplained

DP Steensma¹, RF McClure¹, JE Karp², A Tefferi¹, TL Lasho¹, HL Powell¹, GW DeWald¹ and SH Kaufmann¹

¹Mayo Clinic College of Medicine, Rochester, MN, USA and ²Sidney Kimmel Cancer Center at Johns Hopkins, Baltimore, MD, USA

Signal transducer and activator of transcription (STAT) proteins are phosphorylated and activated by Janus kinases (JAKs). Recently, several groups identified a recurrent somatic point mutation constitutively activating the hematopoietic growth factor receptor-associated JAK2 tyrosine kinase in diverse chronic myeloid disorders – most commonly classic myeloproliferative disorders (MPD), especially polycythemia vera. We hypothesized that the JAK2 V617F mutation might also be present in samples from patients with acute myeloid leukemia (AML), especially erythroleukemia (AML-M6) or megakaryoblastic leukemia (AML-M7), where it might mimic erythropoietin or thrombopoietin signaling. First, we documented STAT3 activation by immunoblotting in AML-M6 and other AML subtypes. Immunoperoxidase staining confirmed phosphorylated STAT3 in malignant myeloblasts (21% of cases, including all AML-M3 samples tested). We then analyzed genomic DNA from 162 AML, 30 B-cell lymphoma, and 10 chronic lymphocytic leukemia (CLL) samples for JAK2 mutations, and assayed a subset for SOCS1 and FLT3 mutations. Janus kinase2 V617F was present in 13/162 AML samples (8%): 10/13 transformed MPD, and three apparent *de novo* AML (one of 12 AML-M6, one of 24 AML-M7, and one AML-M2 – all mixed clonality). FLT3 mutations were present in 5/32 (16%), while SOCS1 mutations were totally absent. Lymphoproliferative disorder samples were both JAK2 and SOCS1 wild type. Thus, while JAK2 V617F is uncommon in *de novo* AML and probably does not occur in lymphoid malignancy, unexplained STAT3 activation is common in AML. Janus kinase2 extrinsic regulators and other proteins in the JAK-STAT pathway should be interrogated to explain frequent STAT activation in AML.

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Introduction

The mammalian Janus kinases (JAK) protein family consists of four cytoplasmic tyrosine kinases (JAK1, JAK2, JAK3, and TYK2) that are recruited to and activated by certain cell surface cytokine receptors, including receptors that are important in normal and disordered hematopoiesis (e.g., erythropoietin, thrombopoietin, granulocyte-monocyte colony-stimulating factor, interleukin-3, and interleukin-6; reviewed in Yamaoka *et al.*¹). Upon activation by an associated receptor, JAKs phosphorylate cytoplasmic signal transducer and activator of transcriptions (STATs), inducing a conformational change that allows STATs to dimerize, translocate to the nucleus, and alter

expression of target genes (reviewed in Yu and Jove²). Janus kinases/signal transducer and activator of transcription-mediated signal transduction is felt to play a critical role in the proliferative and anti-apoptotic effects of multiple cytokines that activate this pathway; and alterations in JAK/STAT pathways have been associated with diverse human diseases (reviewed in Schindler³).

Activating phosphorylation of STAT3 and STAT5a/b has been reported in various malignant cells, including several acute myeloid leukemia (AML) cell lines and a substantial proportion (44–76%) of primary AML patient samples.^{4–8} As a result, it has been postulated that aberrant STAT activation might play a central role in the apoptosis resistance, growth factor independence, and dysregulated cell proliferation that characterize AML.^{4,8–13} The molecular basis for frequent STAT activation in AML is unknown, although leukemogenic fusion protein tyrosine kinases (e.g., TEL (translocation Ets leukemia)-JAK2 and TEL-ABL1 (Abelson oncogene 1)) and mutated tyrosine kinases (e.g., FLT3 (FMS-like tyrosine kinase 3) and c-Kit) can be associated with activation of STAT3, STAT5, or both.^{6,14}

Multiple laboratory groups recently reported a recurrent, constitutively activating, somatic point mutation in a conserved residue of the auto-inhibitory JH2 pseudo-kinase domain of JAK2 (V617F mutation) in blood and bone marrow samples from patients with various chronic myeloid disorders.^{15–21} This mutation causes excessive myeloid cell proliferation by aberrantly signaling in the absence of ligand binding to JAK2-associated receptors. Janus kinase2 V617F is detectable in almost all patients with PV, as well as 35–50% of patients with either essential thrombocythemia (ET) or myelofibrosis with myeloid metaplasia (MMM).^{15–20} JAK2 V617F is also detectable in a small proportion of samples (<10%) from patients with myelodysplastic syndrome or atypical myeloproliferative/myelodysplastic disorders not already defined by mutations or translocations activating another tyrosine kinase (e.g., platelet-derived growth factor receptor (PDGFR), TEL, c-Kit, or FLT3).^{21,22} To date, JAK2 V617F has been absent in samples obtained from healthy persons, patients with BCR/ABL1-positive chronic myeloid leukemia, and individuals with secondary (reactive, non-clonal) erythrocytosis or reactive thrombocytosis.^{21,23} Thus, the discovery of JAK2 V617F promises to improve diagnostic algorithms for chronic myeloid disorders and suggests novel, attractive therapeutic targets.^{20,24–28}

The role of JAK2 V617F in AML is not clear from the preceding studies. On the one hand, it is known that multiple pathways besides JAKs can lead to STAT activation.^{17,29} On the other hand, the association of the JAK2 V617F mutation with multiple chronic myeloproliferative syndromes that display diverse pathologies and natural histories raises the possibility that this mutation might be associated with additional neoplastic lymphohematopoietic conditions, including AML. Although it

Correspondence: Dr DP Steensma, Mayo Clinic and Mayo Clinic College of Medicine, 200 First Street SW, Rochester, MN 55905, USA. E-mail: Steensma.david@mayo.edu

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was initially reported that four AML samples tested negative for this mutation,²¹ JAK2 V617F is present in at least one AML cell line, the HEL erythroleukemia line.¹⁶

To determine whether JAK2 V617F might be associated with a broader spectrum of neoplasia than classic or atypical chronic myeloid disorders, and whether the mutation might account for the frequent STAT activation observed in AML, we studied a large number of AML patient samples of all French-American-British (FAB) Working Group subtypes,³⁰ both *de novo* and arising in a background of chronic MPD, as well as a samples from a group of patients with B-cell lymphoproliferative diseases. We not only confirmed frequent STAT3 phosphorylation in AML, but also demonstrated that JAK2 V617F is common in leukemic transformation of MPD, rare in *de novo* AML, and absent in lymphoma. We also analyzed the critical JAK2 negative regulator SOCS1 (suppressor of cytokine signaling 1), and found no mutations in AML samples.

Methods

Patient samples

In March 2005, the study was approved by the Institutional Review Board of the Mayo Clinic and by the Johns Hopkins Hospital. State and Federal guidelines regarding patient and medical record privacy were followed. Protein and genomic DNA were obtained from leukemic cells donated by consenting patients to institutional cell banks, and additional DNA was obtained from methanol-preserved cultured waste cells from marrow obtained for clinically-indicated cytogenetic studies at Mayo Clinic. B-cell non-Hodgkin lymphoma (B-NHL) primary patient samples, mostly diffuse large cell and follicular lymphoma collected as part of the Mayo Clinic/University of Iowa Lymphoma SPORE, were a gift of Dr Stephen M Ansell, and chronic lymphocytic leukemia (B-CLL) samples were kindly provided by Dr Neil Kay. Myelodysplastic syndrome and myeloproliferative disorder samples were obtained from Mayo Clinic cell banks. All cases were assigned diagnoses by Mayo Clinic hematopathologists according to standard FAB and World Health Organization (WHO) criteria, and AML samples were additionally reviewed by RFM.³¹ Genomic DNA was extracted using High Pure Template Preparation Kit (Roche Diagnostics, Penzberg, Germany).

Immunoblotting

Whole cell lysates from leukemic blast cells were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and probed with various antibodies as described previously.³² The following immunological reagents were utilized: affinity-purified polyclonal anti-phospho-tyrosine⁷⁰⁵-STAT3, anti-STAT3, anti-phospho-tyrosine⁶⁹⁴-STAT5, anti-JAK2, and anti-phospho-tyrosine^{1007/1008}-JAK2 (no. 9131, no. 9132, no. 9351, no. 3773, and no. 3771 from Cell Signaling Technology, Danvers, MA, USA); affinity-purified polyclonal anti-STAT5 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); monoclonal anti-proliferating cell nuclear antigen (PCNA) (PC10, Sigma-Aldrich, St Louis, MO, USA); and monoclonal antibody to histone H1 (a gift of James Sorace, Veterans Administration Hospital, Baltimore, MD, USA), which served as loading control.

Immunohistochemistry

Immunoperoxidase stains were performed on B5-fixed, paraffin-embedded bone marrow biopsy specimens from AML patients

using antibody to phospho-tyrosine⁷⁰⁵-STAT3 clone 3E2 (no. 9138 from Cell Signaling). Sections were deparaffinized with xylene (three changes), rehydrated through an ethanol series (100, 95, and 70% concentrations), rinsed with distilled water, treated for 30 min with preheated 1 mM ethylenediaminetetraacetic acid buffer (EDTA, pH 8.0, Sigma), cooled for 5 min, and rinsed again in distilled water. The slides were then processed in a Dako auto-stainer (Dako A/S, Carpinteria, CA, USA) using the following reagents and staining sequence: 5 min in 3% H₂O₂ in ethanol (Sigma), 30 min in a 1:100 dilution of phospho-tyrosine-STAT3 antibody, rinse in 0.125% Tris-buffered saline with Tween (TBST, Sigma), 10 min in biotin-free polymer mouse MACH3 probe (Biocare Medical, Concord, CA, USA), rinse in TBST, and 5 min in 3,3'-diaminobenzidine (Betazoid DAB, Biocare Medical, USA). The slides were then manually stained with modified Schmidts's hematoxylin for 5 min, rinsed with tap water, dehydrated through an ethanol series (70, 95, and 100% concentrations), cleared in three changes of xylene, and mounted. Samples were reviewed by a hematopathologist blinded to the results of molecular analyses and disease subtype.

Janus kinase2 mutation analysis

DNA was analyzed for the JAK2 mutation by polymerase chain reaction (PCR) and fluorescent dye chemistry sequencing with the BigDye terminator reaction (Applied Biosystems, Foster City, CA, USA) as described previously.²² Sense and anti-sense primers for amplification and sequencing were 5'-TGCTGAAAGTAGGAGAAAGTGCAT-3' and 5'-TCCTACAGTGTTTCAGTTTCAA-3', respectively. PCR cycling parameters were: one cycle of 94°C for 2 min; 35 cycles of 94°C for 30 s, 52°C for 40 s and 72°C for 40 s; followed by one cycle of 72°C for 2 min. Amplicons confirmed by ethidium-impregnated agarose gel electrophoresis were purified with QIAquick PCR Purification Kit (QIAGEN, Valencia, CA, USA), and an ABI PRISM 3700 DNA Analyzer (Applied Biosystems) was used for sequencing. We used Sequencher v4.2 (Gene Codes Corporation, Ann Arbor, MI, USA) for sequence analysis, comparing to GenBank RefSeq NM_004972 (c.1849 G>T mutation).

FLT3 and SOCS1 mutation analysis

FLT3 mutation analysis was performed using a clinically validated multiplex PCR assay with two sets of oligonucleotide primers: one designed to amplify the portion of the *FLT3* gene susceptible to juxtamembrane internal tandem duplication (ITD) mutations, and the second designed to amplify the segment of *FLT3* coding for the D835 mutation, with one primer in each set labeled with a fluorescent dye for fragment analysis. The PCR products were then digested with *EcoRV* restriction enzyme (New England Biolabs, Ipswich, MA, USA) and analyzed by capillary gel electrophoresis using an ABI 3100 device (Applied Biosystems). The ITD-specific primers amplify a 328 bp fragment from normal genomic DNA and a larger fragment if an ITD mutation is present. The D835-specific primers amplify a 222 bp fragment from normal genomic DNA that contains an *EcoRV* cut site, which leaves a 151 bp fragment in wild-type DNA. In the presence of D835 codon mutation, the *EcoRV* site is eliminated and the 222 bp amplicon is detected during analysis.

For SOCS1 molecular analysis, we used two sets of primers to amplify *SOCS1* genomic DNA: 5'-CCCAGCTCACCTTTTGTCT-3' (sense) with 5'-AGGGGCCCCCAGTAGAAT-3' (anti-sense), and 5'-ACTTCCGCACATTCGGTTC-3' (sense) with 5'-AGGGGAAGGAGCTCAGGTAG-3' (antisense). These same primers were used for fluorescent sequencing, which was

performed as for JAK2. The PCR mix included 1 U of Roche Taq polymerase with its buffer (Roche Diagnostics; final MgCl₂ concentration 1.5 mM), dNTPs (final concentration 200 μ M; Roche Diagnostics), 40 pM each of the forward and reverse primers (Integrated DNA Technologies, Coralville, IA, USA), and 100 ng template DNA. Polymerase chain reaction cycling conditions were as follows: 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 60 s; followed by a final extension at 72°C for 2 min. Sequences were compared with GenBank RefSeq NM_003745 and the corresponding region of the chromosome 16p13 contig.

Results

Signal transducer and activator of transcription 3 phosphorylation was confirmed in six of 10 AML-M6 samples tested by Western blotting and in 16 of 21 non-M6 AML specimens (Figure 1 and data not shown), confirming previous observations^{4,9,10} that STAT3 activation is common in AML of all FAB types, including AML-M6.⁹ In contrast, STAT5 phosphorylation was much more rarely detectable (Figure 1, lane 4). Janus kinase2 and phosphoJAK2 were also detectable in many of the samples shown in Figure 1, but the signal was of low intensity, possibly because of the quality of the antibodies or low levels of the JAK2 polypeptide (data not shown).

In order to determine whether STAT3 phosphorylation observed during immunoblotting represented pathway activation in malignant myeloblasts, immunoperoxidase staining was

performed on 98 AML patient samples. Several distinct patterns of phosphorylated STAT3 staining were observed (Figure 2). In some samples, including six of seven from those with AML-M3, malignant myeloblasts, were all strongly positive for phospho-STAT3 (Figure 2a), with a prominent nuclear component to the staining. In other cases, blast staining was focally positive (Figure 2b and c). Overall, 21% of the 98 patients demonstrated strong phospho-STAT3 staining in a uniform or patchy distribution. While the percentage of samples with blasts that were scored positive by immunohistochemistry was somewhat lower than the percentage that were positive by immunoblotting, it is important to emphasize that the samples for immunohistochemistry were fixed with B5 fixative, a mercury containing preparation that is known to damage many epitopes. Accordingly, the immunohistochemical analysis might have underestimated the percentage of samples that contained phosphorylated STAT3. Nonetheless, this analysis demonstrated that blasts rather than contaminating normal myeloid elements contain phosphorylated STAT3 in a subset of AML patients. Among the 11 patients with JAK2 V617F mutations from whom uncut blocks were available for phospho-STAT3 staining, six had phospho-STAT3 staining in malignant blasts.

A previous study demonstrated that phosphorylated STAT3 staining occurs normally in some bone marrow fibroblasts and macrophages.³³ Consistent with this report, we also noted that fibroblasts, endothelial cells and macrophages stained positive for phospho-STAT3 in 92% of patients, including many samples in which the blasts themselves did not demonstrate identifiable staining (Figure 2d and e). As stromal cells represent far fewer than 1% of the cells in the Ficoll-purified blast populations analyzed in Figure 1, it is unlikely that these cells represent the source of the phosphorylated STAT3.

In additional experiments, we sequenced genomic DNA from patients with B-cell malignancies and AML. All 40 B-NHL and B-CLL samples analyzed for JAK2 V617F contained exclusively wild-type JAK2 sequences. Of the 162 AML samples tested (Table 1), 13 (8%) had JAK2 V617F. Of these 13 AML samples, 10 were from patients with known antecedent MPD (seven PV, two MMM, one ET); and only three were believed to have *de novo* AML (one of 23 AML-M6, one of 24 AML-M7, and one AML-M2). Whereas all three *de novo* AML samples and seven of 10 post-MPD AML samples had an intermediate mutated allele burden (i.e., appeared heterozygous by sequencing, with both wild-type and mutant alleles visible on sequencing chromatograms), three of seven post-PV samples contained a very high mutated allele burden (i.e., appeared homozygous for V617F by sequencing analysis). Pre-leukemia transformation samples were available for only two of the seven post-PV AML patients. One of these had an intermediate mutated allele burden before developing AML, then evolved a very high mutated allele burden; the other had an intermediate mutated allele burden at both time points (i.e., chronic-phase PV and post-PV AML), with a slightly greater proportion of the mutated allele in the AML sample. In contrast, 146 of 149 samples of AML occurring in patients lacking a history of an antecedent MPD were negative for the JAK2 V617F mutation, including all of the M6 AML samples that were positive for phospho-STAT3 in Figure 1.

To search for another potential explanation for the presence of phospho-STAT3 detected in Figure 1 and in previous studies,⁴⁻⁸ a subset of 32 JAK2 wild-type AML patients were tested for SOCS1 mutations; none were detected. In addition, all 40 B-NHL and CLL patients were SOCS1 wild type, as were 32 JAK2 wild-type MDS/MPD samples. FLT3 mutations were present in 5/32 AML patient samples assayed (16%; two AML-M1, one AML-M3, one AML-M5, and one post-PV AML), and included

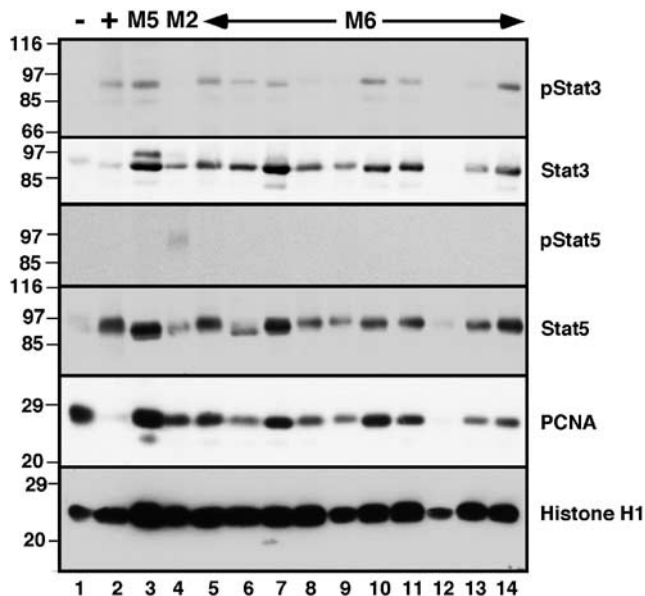


Figure 1 Immunoblot demonstrating frequent activating phosphorylation of STAT3 in acute myeloid leukemia (AML). Aliquots containing protein from 5×10^6 cells ($>80\%$ blasts) were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis and probed with reagents that recognize (from top to bottom) phospho-Tyr⁷⁰⁵-STAT3, STAT3, phospho-Tyr⁶⁹⁴-STAT5, STAT5, proliferating cell nuclear antigen (PCNA), and, as a loading control, histone H1. Leftmost axis: molecular weight in kilodaltons. Lanes 1 and 2: negative and positive controls (lysates from patients with myeloid neoplasia with known STAT3 activation status). Lanes 3–14: 12 AML samples. The 10 AML-M6 samples, including all of those with phospho-Tyr⁷⁰⁵-STAT3 bands, were analyzed for Janus kinase2 (JAK2) V617F and were negative for this mutation. JAK2 and phospho-JAK2 immunoblot are included in Supplementary Information.

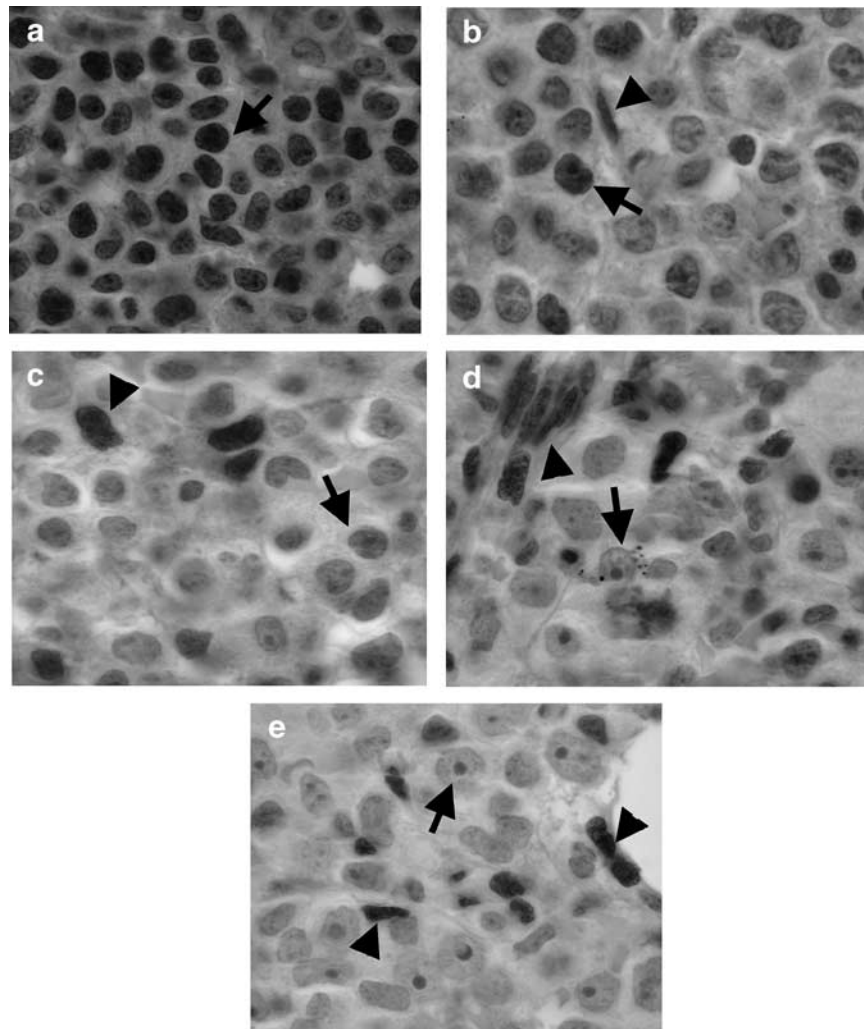


Figure 2 Immunoperoxidase staining of phosphorylated STAT3 demonstrating cellular distribution of STAT3 activation in B5-fixed marrow from primary AML patient samples (peroxidase and Schmidt's hematoxylin, $\times 1000$ magnification). Representative samples of 3 distinct staining patterns are shown. (a) Strong staining of malignant myeloblasts (arrow) with accompanying staining of stromal elements. This sample is from a patient with AML-M3 and the *PML-RARA* gene rearrangement. (b and c) Weak phospho-tyrosine-STAT3 staining of malignant blasts (arrows) and strong stromal staining (arrowheads), from patients with AML-M5 and AML-M6. (d and e) Absent staining of malignant blasts (arrows), with strong staining of some stromal cells (arrowheads), from patients with AML-M2 and AML-M4.

Table 1 JAK2 mutation testing results for AML samples by FAB subtype

<i>Leukemia subtype includes both de novo and post-MPD</i>	<i>Number tested</i>	<i>Number with JAK2 V617F (proportion)</i>
AML-M0 and unclassified	24	5 (21%)
AML-M1	18	0 (0%)
AML-M2	24	2 (8%)
AML-M3	11	0 (0%)
AML-M4	20	0 (0%)
AML-M4Eo	2	0 (0%)
AML-M5 (M5a and M5b)	13	1 (8%)
AML-M6	23	2 (10%)
AML-M7	27	3 (11%)
Total	<i>N</i> = 162	13 (8%)

three ITD mutations and two D835V mutations, consistent with published mutation rates for FLT3 in AML (including AML-M3) that generally range from 15–30%.^{34,35} Only one of these five FLT3-mutation positive samples, from a post-MPD AML patient

who also had JAK2 V617F, exhibited phospho-STAT3 staining (focally) in myeloblasts (Table 2).

Discussion

The present study demonstrates that the JAK2 V617F is frequently observed when MPD transforms to AML. In contrast, in *de novo* AML, JAK2 V617F is a very rare finding, despite the relatively frequent occurrence of STAT3 phosphorylation in myeloblasts. As most patients with AML have near-total marrow replacement by neoplastic cells, unlike MDS³⁶ or early MPD where an admixture of normal and abnormal hematopoietic clones is more typical, it seems unlikely that our failure to detect widespread JAK2 V617F in AML samples was due to insensitivity of sequencing methods.

As our results were being prepared for submission, pre-publication electronic manuscripts of two other manuscripts describing analyses of the frequency of JAK2 V617F in AML became available, while two additional studies appeared during manuscript revision.^{37–40} The first of these analyses used a novel

Table 2 Correlations between AML FAB subtype, immunohistochemical STAT3 phosphorylation pattern, and FLT3/JAK2 mutation analysis

<i>Leukemia subtype</i>	<i>Cases examined for pSTAT3</i>	<i>pSTAT3 staining in stromal elements</i>	<i>pSTAT3 staining in blasts</i>	<i>FLT3 mutation status (proportion demonstrating mutation)</i>	<i>JAK2 mutation status (proportion demonstrating mutation)</i>
AML-M0 and classified	13	7 of 13	1 of 13	0 of 3	0 of 10
AML-M1	10	10 of 10	2 of 10	2 of 2, each pSTAT3+ stromal elements only	0 of 10
AML-M2	13	11 of 13	0 of 13	0 of 4	1 of 11, pSTAT3+in stromal elements only
AML-M3	7	7 of 7	6 of 7	1 of 3, pSTAT3+in stromal elements only	0 of 7
AML-M4 and M4Eo	14	14 of 14	0 of 14	0 of 2	0 of 12
AML-M5	9	9 of 9	2 of 9	1 of 1, pSTAT3+in stromal elements only	1 of 8, pSTAT3+in stromal elements only
AML-M6	12	12 of 12	1 of 12	0 of 3	1 of 11, pSTAT3+in stromal elements only
AML-M7	11	11 of 11	3 of 11	0 of 2	1 of 8, pSTAT3+in both blasts and stroma
Previous MPD, all types	9	9 of 9	6 of 9	1 of 7, pSTAT3+in both blasts and stroma	7 of 7, all patterns seen
Total	N=98	90 of 98 (92%)	21 of 98 (21%)	5 of 27	11 of 84

pyrosequencing technique for mutation detection, instead of fluorescent dye chemistry sequencing^{16–19,22} or allele-specific PCR^{15,21} as used by other groups. These investigators found JAK2 V617F in two of 11 (18%) of patients with AML-M7, 0 of 28 patients with other FAB AML subtypes, and 0 of eight non-HEL AML cell lines.³⁷ The second AML JAK2 V617F analysis included 222 patients with AML (subtypes unspecified) and identified mutations in only four cases (2%), three of whom had a preceding MPD.³⁸ Other domains of JAK2 were studied in a subset of patients, and were wild type. The third study analyzed 90 AML samples, and found that JAK2 V617F was present in five elderly men (5.6%), a subset of whom had MPD. Among 25 AML cell lines in the same analysis, only two demonstrated the mutation.³⁹ None of the five AML patients with JAK2 V617F had FLT3-ITD mutations, or the AML-associated t(8;21), inv(16), or t(15;17) chromosomal rearrangements. In the other patients, the entire coding regions of *STAT5A*, *STAT5B*, *JAK1*, *JAK2*, *JAK3*, and *TYK2* genes were free of mutations.³⁹ The final recent study analyzed 113 primary AML samples, and detected two JAK2 V617F mutations, as well as a novel mutation, K607N, that was not detected in our series or any of the other analyses (overall mutation rate in study 4, 3%).⁴⁰

The present results compliment and extend these other studies by showing that JAK2 V617F mutations are common in AML that arises in the setting of prior MPD, and suggesting that JAK2 mutations detected in *de novo* AML may be preferentially observed in patients with the rare M6 or M7 AML subtypes (one of 23 M6 AML and one of 24 M7 AML, but only one of 102 *de novo* specimens of other FAB subtypes were JAK2 mutant). Combining the present study with the other published analyses that included AML patient samples, 630 patients with AML have now been analyzed for JAK2 V617F, with 26 mutations detected (4.1%).

Distinction between AML-M7,⁴¹ megakaryoblastoid transformation of MPD including MMM,^{42–44} advanced or atypical MMM, 'acute myelofibrosis',^{45,46} panmyelosis with fibrosis,^{47,48} and MDS with fibrosis⁴⁹ continues to prove challenging, and is of uncertain clinical relevance. Janus kinase2 V617F was detected in two *de novo* cases morphologically consistent with

AML-M7 in the pyrosequencing analysis³⁷ and one in this study, further obscuring this issue.

Despite the absence of the JAK2 V617F mutation in the vast majority of AML specimens, STAT3 phosphorylation was frequently observed by immunoblotting (six of 10 samples of M6 AML and 16 of 21 non-M6 AML). In the present study, immunohistochemistry demonstrated strong staining of blasts in a uniform or patchy distribution in 21% of cases. Importantly, the staining in these cases was predominately or exclusively nuclear (Figure 2a and b), suggesting that the detected phospho-STAT3 had translocated to the nucleus and was active. Because the bank providing samples for immunoblotting did not contain unstained blocks, we were unable to directly compare the two assays in the same specimens and cannot rule out the possibility that the incidence of STAT3 positivity varied between the two sample sets. Alternatively, it is possible that the immunohistochemical assay was somewhat less sensitive, either because of the deleterious effects of the fixative on the epitope or the lower sensitivity of the monoclonal antibody used for immunohistochemistry vs the polyclonal antibody used for immunoblotting.

Further analysis demonstrated that STAT3 phosphorylation was detected much more frequently than STAT5 phosphorylation in the same samples (Figure 1). While a formal analysis of the abundance of STAT3 vs STAT5 species in these samples was not performed, immunoblotting demonstrated that unphosphorylated STAT5 species were readily detectable in these same samples and were actually more abundant in the samples that lacked detectable phospho-STAT5 than in samples that contained detectable phospho-STAT5 (e.g., lane 4 vs other lanes in Figure 1). We cannot, however, rule out the possibility that the difference in frequency of detectable STAT3 vs STAT5 phosphorylation in the present study represents a difference in sensitivity of the antibodies.

In any case, it appears that STAT3 phosphorylation is common in AML cases lacking a history of antecedent CMD. As JAK2 V617F mutations do not routinely account for STAT3 activation these cases, the present observations strongly suggest that additional mutations resulting in STAT activation remain to be discovered in AML. FLT3 mutations are present in only 15–

30% of AML cases^{50,51} and were present in only 16% of cases here; FLT3 primarily signals through STAT5,³⁴ but an association between FLT3 ITD mutations and STAT3 activation, perhaps indirect, has been described.⁵²

Promyelocytic leukemia (PML) protein normally suppresses STAT3, but the aberrant PML-RARA fusion dissociates PML from STAT3 and restores its activity.⁵³ and this likely accounts for the strong phospho-STAT3 staining we observed in blasts from AML-M3 patients. None of the patients in this series had other chromosomal translocations (e.g., TEL-ABL1) known to be associated with activation of STAT3.

As sequencing of other JAK2 exons and other members of the JAK family has failed to reveal widespread activating mutations in AML, JAK2 regulators and other tyrosine kinases that signal through STATs are the next logical target for analysis. To this end, we sequenced SOCS1, the major negative regulator of JAK2-STAT signaling. Even though point mutations and deletions of SOCS1 have recently been linked to primary mediastinal B-cell NHL, and hypermethylation of the SOCS1 promoter element has been described in multiple myeloma,^{54–56} the present data suggest that SOCS1 point mutations are not prevalent in AML or in other forms of B-NHL besides primary mediastinal lymphoma.

Other mechanisms might also lead to STAT3 phosphorylation. Decreased internalization and degradation^{57,58} of erythropoietin and its JAK2-bound cell-surface receptor after receptor-ligand binding could lead to prolonged receptor activation, mimicking JAK2 constitutive activation.⁵⁹ Conversely, diminished activity of phosphatases such as SH2-containing tyrosine phosphatase (SHP-1), which ordinarily remove phosphate from activated receptor tyrosine kinases including JAK2, would result in enhanced pathway activation and myeloproliferation, as has been observed in mouse models.⁶⁰ As the full repertoire of JAK-STAT regulators is not yet characterized, complete mutational analysis of this pathway is not possible at present. Moreover, a point mutation, translocation, or deletion of one of these regulators may not be necessary to explain the STAT activation: expression changes due to epigenetic silencing by promoter hypermethylation could be equally effective in silencing phosphatases or SOCS family proteins, as recently described for SOCS1 and SOCS3 in human neoplasia.^{61–63}

In contrast to the AML samples, all of the B-NHL and B-CLL samples in this study lacked JAK2 V617F. These results, again, complement and extend other recently released studies, which excluded widespread JAK2 mutations in NHL, acute lymphoid leukemia, and other lymphoplasmaclastic disorders.^{37–40}

Conclusion

JAK2 V617F is very uncommon in *de novo* AML and is absent in lymphoid malignancy, despite frequent STAT3 activation in the former. JAK2 mutant clones may persist or evolve as MPD transforms to AML. Examination of JAK2 extrinsic regulators and other proteins altering JAK-STAT pathway signaling should be a high priority in AML and other molecularly unexplained myeloid disorders.

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Supplementary Information accompanies the paper on the Leukemia website (<http://www.nature.com/leu>)