Ruxolitinib as potential targeted therapy for patients with *JAK2* rearrangements

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ABSTRACT

JAK2 fusion genes are rare but recurrent abnormalities associated with diverse, clinically heterogeneous hematologic malignancies. Here we assess the JAK1/2 inhibitor ruxolitinib as therapy for patients with *JAK2*-rearrangement associated myeloproliferative neoplasms (MPN). Ruxolitinib-treated Ba/F3 cells transformed to IL3 independence by *ETV6-JAK2* showed reduced proliferation and survival (IC_{50} = 370 nM) compared with KG1A or Ba/F3 cells transformed by *BCR-ABL1*, *SPBN1-FLT3* and *ZMYM2-FGFR1* (IC_{50} > 10 μM for all). Inhibition was associated with reduced phosphorylation of ETV6-JAK2, ERK, STAT5 and AKT. Primary cell growth from 2 patients with JAK2 rearrangement and one patient with *JAK2* amplification was assessed in methylcellulose assays. Reduced colony growth was seen for all patients in ruxolitinib-treated cultures compared with healthy controls (n=7). Fluorescence *in situ* hybridization showed reduced growth of *JAK2*-rearrangement positive colonies compared to *JAK2*-rearrangement negative colonies. Our data, therefore, provide evidence that ruxolitinib is a promising therapy for treatment of patients with *JAK2* fusion genes.

Introduction

Chromosomal translocations targeting *JAK2* are rare but recurrent abnormalities are seen in myeloproliferative neoplasms (MPN), acute myeloid leukemia, acute lymphoblastic leukemia and lymphoma.[1-7] Three fusion variants in MPN, namely *PCM1-JAK2, BCR-JAK2* and *ETV6-JAK2*, are thought to be constitutively activated drivers of the disease process analogous to *BCR-ABL1* in chronic myeloid leukemia. *JAK2*-rearranged malignancies are typically aggressive and, apart from stem cell transplantation, current therapies have limited efficacy.

Ruxolitinib (INC424) (Novartis/Incyte) is an orally available, selective JAK1/2 inhibitor recently approved by the United States Food and Drug Administration for the treatment of intermediate or high-risk myelofibrosis. JAK2 inhibitors are obvious candidates for treatment of patients with *JAK2* fusion genes[8] and, therefore, we have examined the activity of ruxolitinib in primary patient material and cell line models. We present data showing that ruxolitinib is a promising candidate for treatment of patients with MPN and chromosomal abnormalities involving *JAK2*.

Design and Methods

To assess response to ruxolitinib in cell line models, Ba/F3 cells were transfected with pcDNA3.1-*ETV6-JAK2* (ETV6 amino acid 154 fused to JAK2 amino acid 534; kindly provided by Dr Dwayne Barber, Ontario Cancer Institute, Toronto, Canada). Clones were isolated and transformed to IL3 independence. Ba/F3 cells transformed to IL3 independence by *BCR-ABL1* (kindly provided by Dr Junia Melo, University of Adelaide, Australia), *ZMYM2-FGFR1*[9] or *SPTBN1-FLT3*[10] and the KG1A cell line were used as controls. All cell lines were grown in RPMI-1640 plus 10% serum.

To assess proliferative response to ruxolitinib (provided by Novartis, Basel, Switzerland), cell lines were grown in 96-well plates (Ba/F3 lines at $1x10^5$/mL, KG1A at $2\text{-}3x10^5$/mL) with concentrations of 10 nM to 10 μM at half-log intervals in triplicate in 100 μl volumes. CellTiter 96 Aqueous One Solution Cell Proliferation Assay (MTS) (Promega, Madison, WI, USA) was used to measure live cells at 0 and 48 h. Absorbance was measured using an MRX Microplate Reader (Thermo Labsystems, Waltham, MA, USA). Each experiment was performed three times. Cellular IC_{50} values were calculated using GraphPad Prism 4 software (GraphPad Software, San Diego, CA, USA).

The effect of ruxolitinib on phosphorylation was assessed by Western blot. Cells were washed of serum then incubated with inhibitor for 4 h. At each inhibitor concentration equal numbers of cells were lysed in SDS-lysis buffer (62.5 mM Tris pH 6.8, 2% SDS, 10% glycerol, 0.1% bromphenol blue) with addition of protease inhibitors. Proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Antibodies were: anti-STAT5 (#9363, Cell Signalling Technology, Beverley, MA, USA), anti-phospho-STAT5 (#9359, Cell Signalling Technology), anti-ERK (sc-94, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-phospho-ERK (sc-7383, Santa Cruz Biotechnology, CA, USA), anti-AKT (ab8805, Abcam, Cambridge, UK), anti-phospho-AKT (ab66138, Abcam), anti-JAK2 (#2863-1, Epitomics, Burlingame, CA, USA) and

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anti-phospho-JAK2 (#TA303559, Origene, Rockville, MD, USA). Signals were detected using enhanced chemiluminescence (ECL) plus Western blotting detection reagent (Amersham Biosciences, Little Chalfont, UK).

Primary peripheral blood mononuclear cells from 2 patients with *JAK2* rearrangements and one patient with a *JAK2* amplification were used in *in vitro* response assays. Peripheral blood samples from 7 healthy individuals and bone marrow samples from 3 chronic myeloid leukemia (CML) patients were used as controls. The study was approved by the relevant internal review boards and/or ethics committees, and informed consent was provided according to the Declaration of Helsinki.

Case #1

A 51-year old male presented with a history of night sweats. Peripheral blood showed left-shifted leukocytosis of 12×10^9/L with a hemoglobin level of 13.7g/dL and a platelet count of 138×10^9/L. Eosinophils and basophils were not elevated. Spleen size was increased (20x10 cm). Bone marrow was hypercellular with pronounced granulopoiesis, prominent eosinophilia, and reduced numbers of megakaryocytes and dysplastic erythropoiesis. Marrow fibrosis was grade 2. Cytogenetic analysis revealed a 46,XY,t(8;9)(p22;p24) in 17 of 20 metaphases and real-time polymerase chain reaction (RT-PCR) amplified a chimeric mRNA with *JAK2* exon 11 fused to *PCM1* exon 36.

Case #2

The peripheral blood of a 68-year old male patient showed left-shifted leukocytosis (39×10^9/L) with 9% eosinophils (absolute numbers 3.5×10^9/L), thrombocytosis (780×10^9/L) and normal hemoglobin (13 g/dL). Liver and spleen were slightly enlarged (13x8 cm). Trephine biopsy revealed a hypercellular marrow with pronounced granulopoiesis predominantly caused by eosinophils and reduced numbers of erythropoietic precursors and megakary-

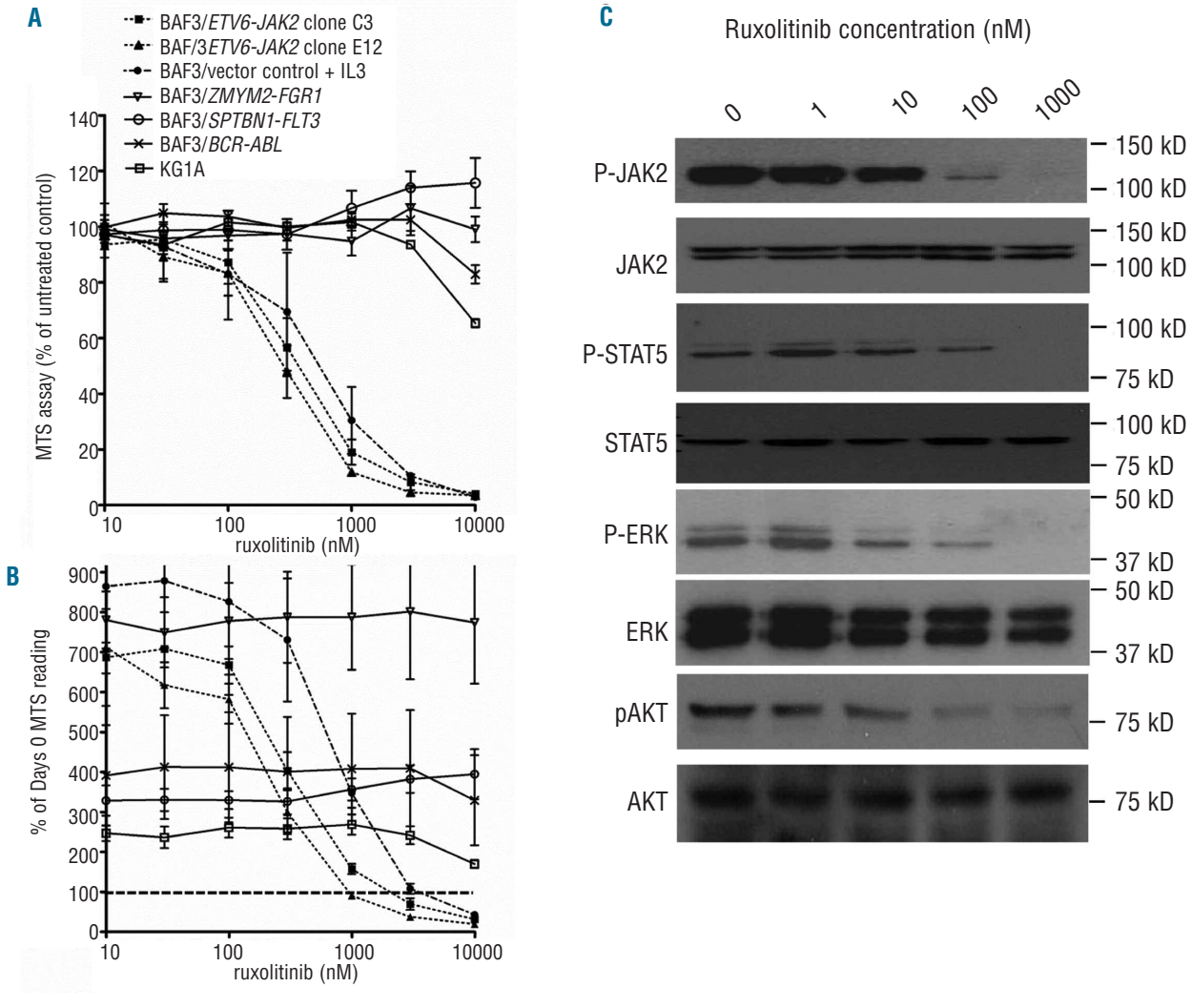

Figure 1. Ruxolitinib is active against Ba/F3 cells transformed by ETV6-JAK2. (A) KG1A cells (which contain *FGFR1OP2-FGFR1*) and Ba/F3 cells transformed to IL3-independence by the fusion genes *ETV6-JAK2*, *SPTBN1-FLT3*, *ZMYM2-FGFR1* or *BCR-ABL1* were exposed to ruxolitinib for 48 h. Values are means +/- SE of 3 independent experiments. (B) Expression as a percentage of Day 0 MTS reading (where line crosses y-axis at 100%) indicates a net reduction in numbers of Ba/F3-*ETV6-JAK2* cells over 48 h. Values are means +/- SE of 2 independent experiments. (C) Treatment of cells with ruxolitinib for 4 h resulted in a dose-dependent reduction in phosphorylation of ETV6-JAK2, ERK, STAT5 and AKT. Translation from an internal methionine results in two isoforms of ETV6-JAK2;[2] we found only the larger to be phosphorylated.

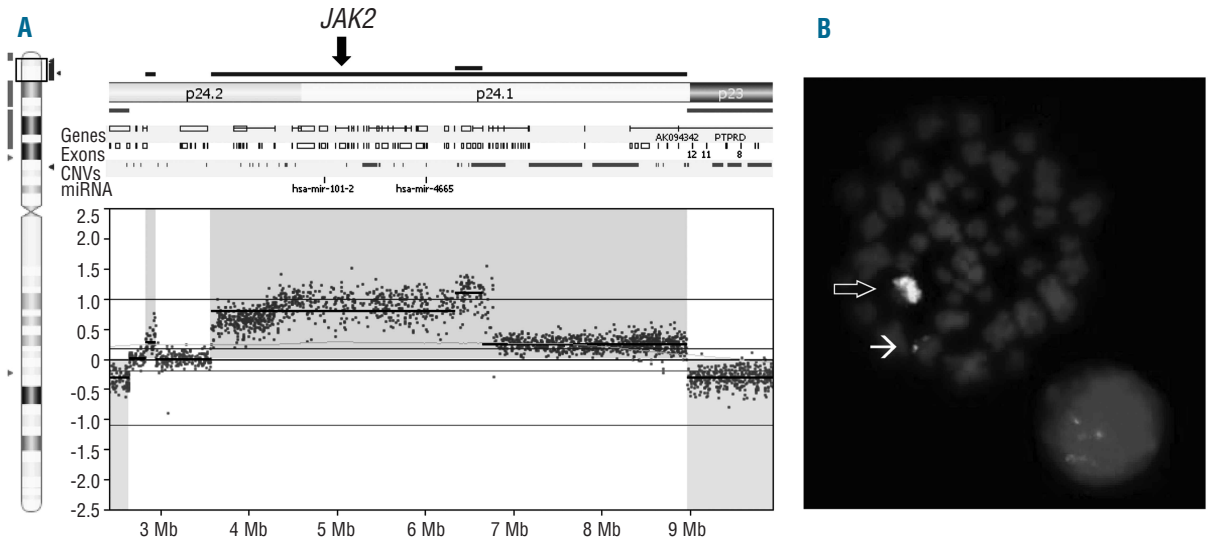

Figure 2. Amplification of *JAK2* in case 3 with myelodysplastic syndrome. Comparative genomic hybridization using a NimbleGen CGH 6x630K Whole-Genome Tiling Array (NimbleGen Inc., Madison, WI, USA) (A) and fluorescence *in situ* hybridization (FISH) with probes flanking the *JAK2* locus (Kreatech, Amsterdam, The Netherlands) (B) revealed a *JAK2* amplification contained within a single chromosome (⇨) and a non-amplified JAK2 allele (→).

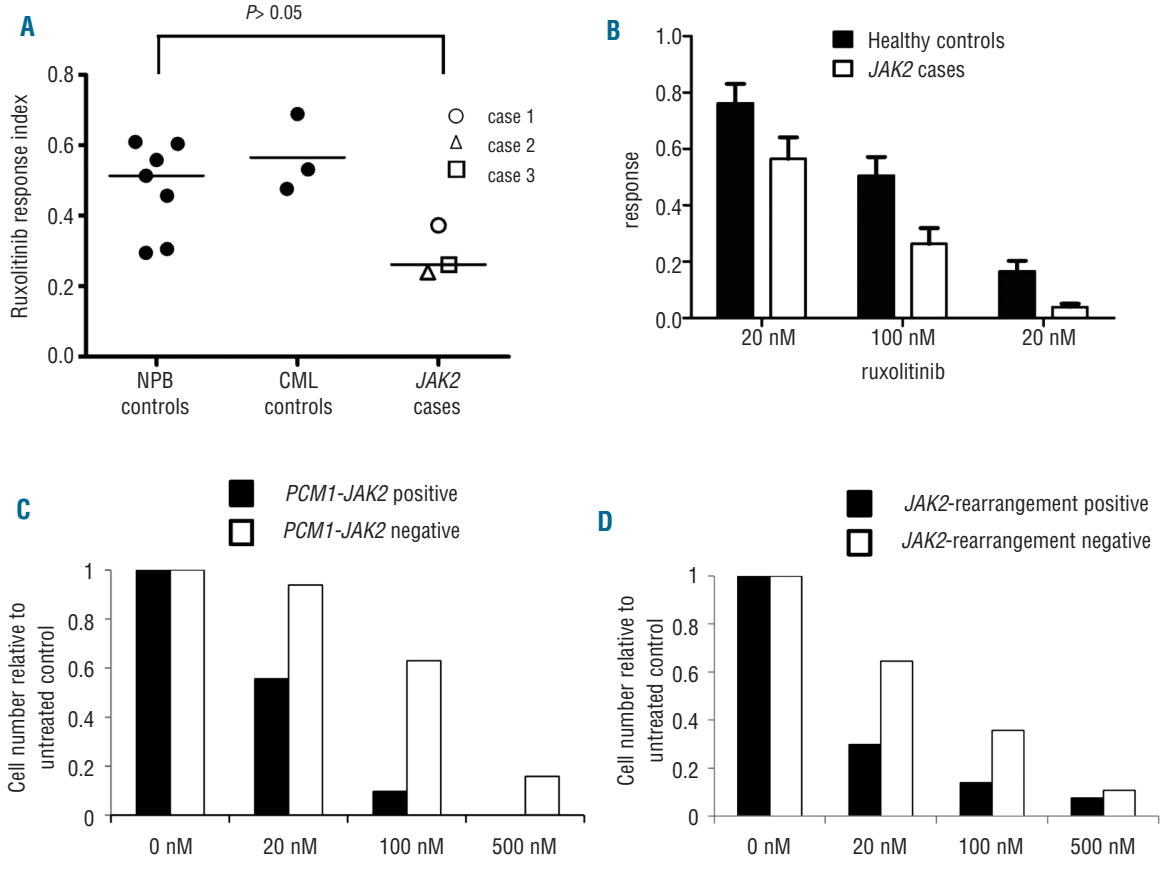

Figure 3. Ruxolitinib inhibits colony growth of *JAK2* rearranged or amplified primary cells. (A) Colony numbers of samples from 3 patients with *JAK2* rearrangements or amplification, 7 healthy controls and 3 CML controls were counted at Days 7 and 14. The y-axis response index is the mean reduction in Days 7 and 14 colony counts in ruxolitinib-treated methylcellulose cultures compared to untreated cultures for all ruxolitinib concentrations. (B) Comparison of JAK2 cases and healthy controls showed a progressive reduction in colony numbers with increasing ruxolitinib concentrations. In cases 1 (C) and 2 (D) a subset of colonies were plucked on Day 14 for *JAK2* FISH analysis allowing the size of the *JAK2*-rearrangement positive and negative fractions to be estimated. In both cases, the number of *JAK2*-rearrangement positive colonies fell at lower ruxolitinib concentrations than *JAK2*-rearrangement negative colonies.

ocytes. Blasts were slightly elevated.

Marrow fibrosis was grade 1. Cytogenetic analysis revealed 46,XY,t(9;18)(p24;q12),t(14;18)(q21;q23)[24]. A rearrangement of *JAK2* was confirmed by fluorescence *in situ* hybridization (FISH) with probes flanking the *JAK2* gene (Kreatech, Amsterdam, The Netherlands). The putative *JAK2* fusion partner remains to be identified.

Case #3

A 71-year old male patient presented with moderate leukocytopenia ($3.4x10^9$/L), transfusion-dependent anemia (Hb 7.6 g/dl) and thrombocytopenia ($53x10^9$/L). The spleen was slightly enlarged. Bone marrow biopsy showed approximately 50% ringed sideroblasts with dysplastic granulopoiesis and megakaryopoesis leading to refractory cytopenia with multilineage dysplasia. No marrow fibrosis could be detected.

Cytogenetics revealed a complex karyotype (46,XY,der(5)t(3;5)(q26;q14)[1]/46,XY,der(5)t(3;5)(q26;q14),der(7)t(7;9)(q21;p24),r(9)(p13q34),der(12)t(12;18)(p12;p11),r(18)(p11q21)[19). FISH and comparative genomic hybridization (CGH) analysis revealed overamplification of *JAK2* and genetic analysis showed the patient to be negative for *JAK2* V617F.

Primary cells were set up at $2x10^5$/mL in methylcellulose with cytokines without erythropoietin (Stem Cell Technologies, Vancouver, BC, Canada) in triplicate for each inhibitor dose. Colonies greater than 50 cells were counted on Day 7 and colonies greater than 100 cells were counted on Day 14. An index of growth response was calculated as described previously.[11] FISH was used to assess any differential effect of ruxolitinib upon colonies with or without rearranged or amplified *JAK2*. Colonies were plucked into 3:1 methanol/acetic acid, stored at -20°C until required, and then pipetted onto slides. Split-apart FISH with differentially labeled bacmid probes RP11-3H3 (5´ *JAK2*) and RP11-28A9 (3´ *JAK2*) was performed according to established techniques[12] with the addition of a 70% acetic acid wash immediately after slide making and a refix in 1% paraformaldehyde in PBS for 10 min before hybridization.

Results and Discussion

The IL3-dependent Ba/F3 cell line can be transformed to IL3 independence by expression of activated oncogenes such as *ETV6-JAK2* and thereby provides a model system for assessing tyrosine kinase inhibitors.[1] In initial experiments, Ba/F3-*ETV6-JAK2* clones and untransformed Ba/F3 cells (grown with IL3) showed similar responses to ruxolitinib consistent with the requirement of JAK2 for IL3 signaling.[13] We therefore used the *FGFR1OP2-FGFR1* positive KG1A cell line[14] plus Ba/F3 cells transformed to IL3 independence by *BCR-ABL1, SPTBN1-FLT3* and *ZMYM2-FGFR1* as negative controls since FGFR1, ABL1 and FLT3 are ruxolitinib insensitive. The mean cellular IC50 value for the two Ba/F3-*ETV6-JAK2* clones was 370 nM whilst for all other cell lines the IC50 was over 10,000 nM (Figure 1A). A net loss of cells over the 48-h experimental period was seen with Ba/F3-*ETV6-JAK2* at higher ruxolitinib concentrations, i.e. where the MTS reading at 48 h falls below 100% of the Day 0 MTS reading (Figure 1B). In contrast, almost no reduction was seen in survival of control cell lines. Increasing inhibitor concentration was accompanied by reduced phosphorylation of the JAK2 fusion protein, ERK, STAT5 and AKT (Figure 1C). Together with previous findings on ruxolitinib pharmacology (25 mg dose gives a C_{max} of 934 nM),[15] our data therefore, suggest that effective inhibition of JAK2 fusion proteins would be readily obtainable *in vivo*.

Ruxolitinib was then assessed in *in vitro* assays using primary material from 2 patients with *JAK2* rearrangements, one patient with *JAK2* amplification, 7 healthy controls and 3 CML controls. *JAK2* amplification is very rare in hematologic malignancies and has not previously been described in myelodysplastic syndrome. CGH and FISH showed amplification of a region on chromosome 9 containing *JAK2* (Figure 2A and B). The total number of copies of *JAK2* was estimated at 6-7 per cell suggesting increased JAK2 signaling and a potential response to ruxolitinib. Cells from all 3 patients were grown for two weeks in methylcellulose in the presence of ruxolitinib. An overall reduction in colony growth was seen in patients compared with healthy controls (t-test, $P<0.05$) and CML controls (Figure 3A) which was progressive with increasing ruxolitinib concentration (Figure 3B and *Online Supplementary Table S1*). Colonies were plucked into fixative followed by FISH analysis using split-apart *JAK2* probes to assess the proportions of *JAK2*-rearrangement positive and negative cells at each ruxolitinib concentration and these data were combined with colony counts to show the relative dynamics of the *JAK2*-rearrangement positive and negative fractions (Figure 3C and D; calculations are provided in *Online Supplementary Table S2*). In both cases 1 and 2, the reduction in JAK2-rearrangement positive cells occurs at lower ruxolitinib concentrations and is greater than that seen in *JAK2*-rearrangement negative cells at all concentrations. Both patients with *JAK2* rearrangements showed an overall reduction in *JAK2* rearranged colonies (χ^2 test; case 1, $P<0.02$; case 2, $P<0.05$). In case 1, complete eradication of PCM1-JAK2 positive colonies at 500 nM ruxolitinib was seen. For case 3, only *JAK2*-amplified colonies were seen (65 and 11 colonies analyzed by FISH from 100 nM and 500 nM ruxolitinib-treated cultures, respectively) suggesting that the level of cells negative for the *JAK2*-amplification may be too low to allow detection of a differential effect using this assay.

In summary, we have shown by *in vitro* assays using both cell line models and primary patient material that ruxolitinib has significant activity against JAK2 activated by gene rearrangement and present evidence for potential activity against cells with *JAK2* amplification. Since aberrant activation of JAK2 has also been demonstrated in lymphoid disorders, e.g. by *JAK2* rearrangement or *SOCS1* mutation in lymphoma[7,16] and *CRLF2*, *IL7R* and *JAK* family mutations in acute lymphoblastic leukemia,[17,18] it is possible that treatment with ruxolitinib will have wider potential applicability in addition to the treatment of patients with *JAK2* rearrangement-positive MPN described here.

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Authorship and Disclosures

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

References

1. Ho JM, Beattie BK, Squire JA, Frank DA, Barber DL. Fusion of the ets transcription factor TEL to Jak2 results in constitutive Jak-Stat signaling. Blood. 1999;93(12):4354-64.
2. Ho JM, Nguyen MH, Dierov JK, Badger KM, Beattie BK, Tartaro P, et al. TEL-JAK2 constitutively activates the extracellular signal-regulated kinase (ERK), stress-activated protein/Jun kinase (SAPK/JNK), and p38 signaling pathways. Blood. 2002; 100(4):1438-48.
3. Reiter A, Walz C, Watmore A, Schoch C, Blau I, Schlegelberger B, et al. The t(8;9)(p22;p24) is a recurrent abnormality in chronic and acute leukemia that fuses PCM1 to JAK2. Cancer Res. 2005; 65(7):2662-7.
4. Griesinger F, Hennig H, Hillmer F, Podleschny M, Steffens R, Pies A, et al. A BCR-JAK2 fusion gene as the result of a t(9;22)(p24;q11.2) translocation in a patient with a clinically typical chronic myeloid leukemia. Genes Chromosome Cancer. 2005;44(3):329-33.
5. Peeters P, Raynaud SD, Cools J, Wlodarska I, Grosgeorge J, Philip P, et al. Fusion of TEL, the ETS-variant gene 6 (ETV6), to the receptor-associated kinase JAK2 as a result of t(9;12) in a lymphoid and t(9;15;12) in a myeloid leukemia. Blood. 1997;90(7):2535-40.
6. Najfeld V, Cozza A, Berkofsy-Fessler W, Prchal J, Scalise A. Numerical gain and structural rearrangements of JAK2, identified by FISH, characterize both JAK2617V>F-positive and -negative patients with Ph-negative MPD, myelodysplasia, and B-lymphoid neoplasms. Exp Hematol. 2007;35(11):1668-76.
7. Van Roosbroeck K, Cox L, Tousseyn T, Lahortiga I, Gielen O, Cauwelier B, et al. JAK2 rearrangements, including the novel SEC31A-JAK2 fusion, are recurrent in classical Hodgkin lymphoma. Blood. 2011; 117(15):4056-64.
8. Walz C, Cross NC, Van Etten RA, Reiter A. Comparison of mutated ABL1 and JAK2 as oncogenes and drug targets in myeloproliferative disorders. Leukemia. 2008; 22(7):1320-34.
9. Smedley D, Demiroglu A, Abdul-Rauf M, Heath C, Cooper C, Shipley J, et al. ZNF198-FGFR1 transforms Ba/F3 cells to growth factor independence and results in high level tyrosine phosphorylation of STATS 1 and 5. Neoplasia. 1999;1(4):349-55.
10. Grand FH, Iqbal S, Zhang L, Russell NH, Chase A, Cross NC. A constitutively active SPTBN1-FLT3 fusion in atypical chronic myeloid leukemia is sensitive to tyrosine kinase inhibitors and immunotherapy. Exp Hematol. 2007;35(11):1723-7.
11. Chase A, Grand FH, Cross NC. Activity of TKI258 against primary cells and cell lines with FGFR1 fusion genes associated with the 8p11 myeloproliferative syndrome. Blood. 2007;110(10):3729-34.
12. Sohal J, Chase A, Goldman JM, Cross NC. Assignment of ZNF262 to human chromosome band 1p34-->p32 by in situ hybridization. Cytogenet Cell Genet. 1999; 85(3-4):306-7.
13. Parganas E, Wang D, Stravopodis D, Topham DJ, Marine JC, Teglund S, et al. Jak2 is essential for signaling through a variety of cytokine receptors. Cell. 1998; 93(3):385-95.
14. Gu TL, Goss VL, Reeves C, Popova L, Nardone J, Macneill J, et al. Phosphotyrosine profiling identifies the KG-1 cell line as a model for the study of FGFR1 fusions in acute myeloid leukemia. Blood. 2006;108(13):4202-4.
15. Shi JG, Chen X, McGee RF, Landman RR, Emm T, Lo Y, et al. The pharmacokinetics, pharmacodynamics, and safety of orally dosed INCB018424 phosphate in healthy volunteers. J Clin Pharmacol. 2011; 51(12):1644-54.
16. Weniger MA, Melzner I, Menz CK, Wegener S, Bucur AJ, Dorsch K, et al. Mutations of the tumor suppressor gene SOCS-1 in classical Hodgkin lymphoma are frequent and associated with nuclear phospho-STAT5 accumulation. Oncogene. 2006;25(18):2679-84.
17. Tasian SK, Loh ML. Understanding the biology of CRLF2-overexpressing acute lymphoblastic leukemia. Critical Reviews in Oncogenesis. 2011;16(1-2):13-24.
18. Zhang J, Ding L, Holmfeldt L, Wu G, Heatley SL, Payne-Turner D, et al. The genetic basis of early T-cell precursor acute lymphoblastic leukaemia. Nature. 2012; 481(7380):157-63.