Table 1 Patient characteristics

Disease status at auto-SCT	*PTCL*			*DLBL*
	CR1/PR1	*Sensitive relapse*	*Refractory*	*CR1/PR1*
No. of cases	23	9	7	64
Age (range) at transplantation	55 (16–66)	52 (27–68)	45 (21–51)	53 (21–67)
Sex, M/F	18/5	6/3	7/0	36/28
Histology (n)				
Unspecified	7	4	1	
Angioimmunoblastic T-cell lymphoma	6	3	3	
Anaplastic large-cell lymphoma	5	2	2	
Natural killer/T-cell lymphoma	5	0	1	
Ann Arbor stage				
I–II	4	2	1	8
III–VI	19	7	6	55
NA	0	0	0	1
Age-adjusted IPI score at diagnosis				
0–1	10	2	1	38
2–3	13	6	3	24
NA	0	1	2	2
Conditioning regimen				
MCEC	20	5	7	60
TBI-containing regimen	3	2	0	2
Others	0	2	0	2
Months from diagnosis to auto-SCT (range)	6 (2–12)	12 (9–29)	5 (4–7)	7 (2–37)

Abbreviations: CR1/PR1 = first complete or partial response; DLBL = diffuse large B-cell lymphoma; F = female; IPI = International Prognostic Index; M = male; MCEC = pre-transplant regimen, consisting of ranimustine, carboplatin, etoposide and CY; PTCL = peripheral T-cell lymphoma.

had angioimmunoblastic T-cell lymphoma, seven had NK/T-cell lymphoma and twelve had PTCL-U. Clinical characteristics at diagnosis are shown in Table 1. Stage at diagnosis was defined according to the Ann Arbor staging system.[11] The age-adjusted International Prognostic Index (aa-IPI) at diagnosis was retrospectively evaluated in 36 of 39 patients (92%).[12]

Exclusion criteria were involvement of the central nervous system, inadequate major organ functions, concomitant malignancy and active viral infection (for example, hepatitis B, hepatitis C, human immunodeficiency virus).

Treatment

Patients received anthracycline-containing regimens, mainly CHOP regimens (CY, doxorubicin, vincristine and prednisone), as an induction therapy. In addition to chemotherapy, most patients with NK/T-cell lymphoma also received local radiotherapy. Autologous PBSCs were collected during hematological recovery after treatment with a high-dose etoposide (500 mg/sqm for 3 days) or intermediate-dose Ara-C (500 mg/sqm two times daily for 5 days) followed by s.c. injection of G-CSF as previously described.[3] The target cell dose was $>2 \times 10^6$ CD34$^+$ cells/kg in a PBSC harvest. Harvested PBSCs were cryopreserved until use. The median number of infused CD34$^+$ cells was 5.7×10^6 cells/kg (range, 2.4–26.1).

Transplantation procedure

Patient characteristics at transplantation are shown in Table 1. The median age at transplantation was 49 years (range, 16–68 years), and the median time from diagnosis to auto-PBSCT was 7 months (range, 2–29 months). At transplantation, 22 patients were in CR1, 1 in PR1, 9 in chemotherapy-sensitive relapse and 7 in a chemotherapy-resistant state. Thirty-two patients were chemotherapy-sensitive, whereas seven patients were chemotherapy resistant.

The conditioning regimen prepared for auto-PBSCT was referred to as MCEC. It consisted of ranimustine 200 mg/m^2 on days –8 and –3, carboplatin 300 mg/m^2 from days –7 to –4, etoposide 500 mg/m^2 from days –6 to –4 and CY 50 mg/kg on days –3 and –2 in 32 patients (82.1%) as previously described.[3] Five patients were treated with TBI-containing conditioning regimens. On day 0, unpurged PBSCs were reinfused followed by administration of G-CSF. Engraftment was confirmed by granulocyte counts $>0.5 \times 10^9$/l and plt counts $>20 \times 10^9$/l on three consecutive occasions, or independence of plt transfusion.

Statistical methods

OS was defined as days from transplantation to death of any cause. Progression-free survival (PFS) was defined as days from transplantation to disease progression or death of any cause. TRM included all causes of death other than disease progression within 100 days after transplantation. Survival was estimated using the Kaplan–Meier method. Comparisons among those variables of interest at the time of diagnosis were performed by the log-rank test. All *P*-values reported were two-sided and statistical significance was defined at a *P*-value <0.05.

Ethical considerations
This study was conducted in accordance with the ethical guidelines mandated by the Declaration of Helsinki.

Results

Engraftment and treatment-related complications
Engraftment was rapid and documented in all patients. The median days to granulocyte count $>0.5 \times 10^9$/l and a plt count $>20 \times 10^9$/l were 9 (range, 8–11) and 11.5 (range, 7–17), respectively. TRM were not observed. Significant adverse events scored as more than grade 3 according to the National Cancer Institute Common Toxicity Criteria were not seen.

Survival and progression of the disease
With a median follow-up time of 78 months (range, 7–127 months) after auto-PBSCT, CR was achieved and maintained in 23 patients: 17 patients in CR1/PR1, 2 in sensitive relapse and 4 in the chemotherapy-resistant state before transplantation. The 5-year OS and PFS were 62.2% (95% confidence interval (CI), 46.4–77.9%) and 60.6% (95% CI, 45.0–76.2%), respectively (Figure 1a). Progressive disease (PD) was observed in 14 patients: five patients in CR1, three in sensitive relapse and six in the chemotherapy-resistant state before transplantation. Median time to PD was 149 days (range, 28–3815 days) and cumulative incidence of PD at 5 years was 33.8 %. Sixteen patients died. The primary cause of death was PD in 13 patients; the other three patients eventually died of secondary leukemia on day 521, heart failure on day 826 and pneumonia on day 2587, respectively. These three patients died in CR.

To investigate the effect of HDCT with auto-PBSCT in the treatment of PTCL, we compared the outcomes of 23 patients with PTCL, who were in CR/PR1 at transplantation, with those of 64 patients with diffuse large B-cell lymphoma, who were in CR, CR of undetermined significance and PR at transplantation from 1990 to 2005. Patient characteristics were well balanced and not statistically different between the two groups with regard to age, time to transplantation and conditioning regimen. There was no difference in outcomes between these two groups; 5-year OS and PFS were 72.9% vs 75.4% ($P=0.82$) and 73.1% vs 57.1% ($P=0.42$), respectively (Figure 2).

Prognostic factors
Data were evaluated using univariate analysis with the use prognostic factors such as histology, age, disease status at transplantation, stage, aa-IPI and lactate dehydrogenase at diagnosis. Patients having chemotherapy-sensitive disease at the time of transplantation showed significantly better 5-year OS and PFS than those having chemotherapy-resistant disease; OS was 67.2% vs 38.1% ($P=0.014$) and PFS 67.5% vs 28.6% ($P=0.033$). Patients in CR1/PR1 at transplantation showed significantly better 5-year OS and PFS than those with other status; OS was 72.9% vs 45.8% ($P=0.033$) and PFS 73.1% vs 42.2% ($P=0.023$) (Figure 1b). The low score of aa-IPI was also a significant factor influencing survival; OS of patients at low risk by

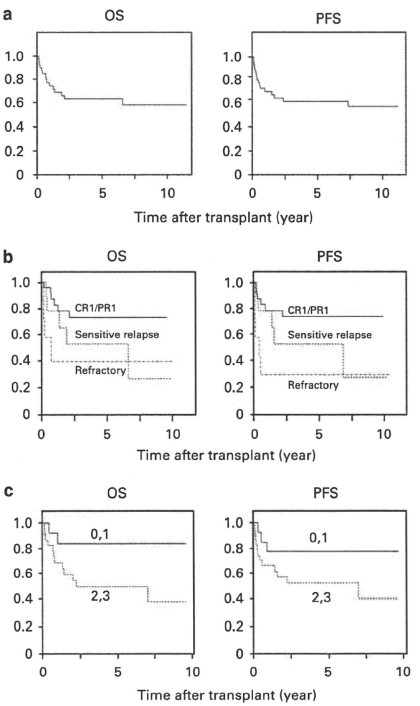

Figure 1 Kaplan–Meier estimates of the OS and progression-free survival (PFS) in all peripheral T-cell lymphoma (PTCL) patients (**a**). Analyzed according to disease status at transplantation (**b**). Analyzed according to age-adjusted International Prognostic Index (aa-IPI) at diagnosis (**c**). Patients who received transplantation as consolidation of initial response and aa-IPI low-risk patients at diagnosis show longer survival by univariate analysis.

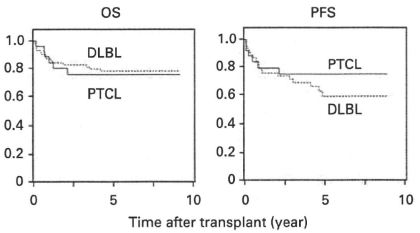

Figure 2 Kaplan–Meier estimates of OS and progression-free survival (PFS) in peripheral T-cell lymphoma (PTCL) and diffuse large B-cell lymphoma (DLBL) transplanted in initial response analyzed according to histology. Histology does not affect survival.

Table 2 Prognostic factors on survival (multivariate analysis)

Parameter	*OS*			*PFS*		
	HR	*95% CI*	P-*value*	*HR*	*95% CI*	P-*value*
Age	1.045	1.002–1.089	0.040	1.043	1.002–1.086	0.040
Histology (ALCL/non-ALCL)	3.973	0.697–0.120	0.120	4.350	0.875–21.62	0.072
aa-IPI score (0, 1/2, 3)	4.691	0.919–23.95	0.063	3.788	0.877–16.36	0.074
Status at transplant (CR/non-CR)	3.117	1.019–9.542	0.046	3.021	1.049–8.700	0.040

Abbreviations: ALCL = anaplastic large-cell lymphoma; CI = confidence interval; HR = hazards ratio; aa-IPI = age-adjusted International Prognostic Index; PFS = progression-free survival.

aa-IPI (score 0 and 1) and at high risk by aa-IPI (score 2 and 3) were 83.9% vs 49.2% ($P = 0.035$) and PFS 76.9% vs 50.2% ($P = 0.070$) (Figure 1c). Lactate dehydrogenase (<normal vs ⩾normal) also affected OS and PFS; OS was 90.0% vs 49.9% ($P = 0.023$) and PFS 90.0% vs 48.4% ($P = 0.018$). Neither histological subtypes (ALCL vs non-ALCL), age (<50 years vs ⩾50 years), nor stage (Ann Arbor I–II vs III–IV) showed a significant difference in survival.

We reviewed these univariate analyses by the multivariate method using four parameters: histology, aa-IPI at diagnosis, and age and disease status at transplantation. Despite no significant difference by univariate analysis, we also included histology (ALCL or non-ALCL) as a parameter because it was shown to be a favorable marker in a previous report.[13] Two independent factors (age and disease status at transplantation) were found to significantly influence survival (Table 2).

Discussion

Progress in PTCL therapy is slow partly because of the rarity of the disease, variation in incidence according to geographic location and absence of a common marker for therapy using monoclonal antibodies. Consensus regarding optimal treatment for PTCL is therefore lacking, and the prognosis of PTCL is reported to be inferior to that of aggressive B-cell lymphoma. Despite high and good initial response rates, most patients with PTCL fare poorly and develop progression early during or shortly after sequential conventional chemotherapy.[14] The 5-year survival reported is <30% with CHOP regimen or more intensive regimens, such as those used in LNH programs and Hyper CVAD regimen.[15–17] Recent studies have shown the superiority of HDCT with auto-SCT to conventional sequential chemotherapy and salvage chemotherapy for patients with aggressive NHL.[4,5,18] These studies included in part T-cell lymphomas, and have given encouraging retrospective results of PTCL patients treated by HDCT with auto-SCT; estimated OS was reported to be ~30–50%, which was similar to that of aggressive B-cell NHL despite fewer cases analyzed. Thus, these results indicate that HDCT with auto-SCT can benefit some patient populations of PTCL, and the parameters preferentially indicated for HDCT with auto-SCT should be clarified. To address this issue, we retrospectively analyzed our results of 39 consecutive patients with PTCL who underwent HDCT with auto-PBSCT in our institutions (FBMTG) since 1990.

In our study, 5-year OS and PFS in PTCL patients who were autografted in CR1/PR1 were 72.9 and 73.1%, respectively. These values were comparable with the corresponding results in patients with aggressive B-cell lymphoma who underwent HDCT with auto-PBSCT (Figure 2). Detailed analysis suggests that HDCT with auto-SCT may be effective for PTCL patients who have maintained CR1 or PR1 at transplantation, as well those who were chemotherapy sensitive after relapse. aa-IPI and histology type did not affect OS and PFS by multivariate analysis. Of the 39 patients in our study, 23 patients underwent auto-PBSCT in CR1/PR1, 9 patients were in chemotherapy-sensitive relapse and 7 cases in refractory phase. Approximately, 75% of patients in remission and half of those after chemotherapy-sensitive relapse had a durable response. Only two of seven patients transplanted with refractory disease showed a prolonged response. Our results are comparable with previous studies. For example, 5-year OS was reported to be 80% in patients transplanted in CR1 and 45% in other status by the GEL-TAMO group;[19] 5-year OS was reported to be 76 and 30% in patients with CR1/PR1 and refractory disease by Stanford group;[14] and 5-year OS was 80% in CR1 by EBMT group.[20] Therefore, these data strongly indicate that HDCT with auto-SCT can be one of the treatment choices for patients with PTCL as consolidation after achieving initial response, or as salvage after sensitive relapse. This treatment would be less favorable for chemotherapy-resistant patients, with no long-term survivors after HDCT with auto-SCT.[19]

Previous studies have stated the importance of the histological type of PTCL on auto-SCT outcome. Jagasia *et al.*[21] reported improved survival in ALCL type because these studies included relatively more cases of ALK-positive ALCL, which generally confers good prognosis. Corradini *et al.*[10] reported that up-front auto-SCT could induce a high rate of long-term CR only in patients with ALK-positive ALCL. Although number of patients were limited and information about ALK was not available in any of the patients in our study, there was no significant difference in 3-year OS between ALCL and the other subtypes of PTCL (66.7% vs 59.1%; $P = 0.12$). Similarly, City of Hope[22] and Stanford series[14] also showed no difference in survival after HDCT with auto-SCT based on histological subtypes of PTCL, although these studies also did not afford a sufficient number of cases to stratify by type and disease status. A large Korean multicenter retrospective analysis including 139 patients also reported no survival difference depending on histological subtypes of

PTCL.[23] Discrepancies in survival data from the several groups mentioned above may in part be due to the heterogeneity of histological subtypes of PTCL. Therefore, larger retrospective and prospective studies are required to evaluate prognostic factors influencing survival in each histological subtype of PTCL.

According to our study and previous reports, the patients who seemed to benefit from HDCT were those in first remission or sensitive relapse at the time of transplantation.[8,10] However, some risk factors, such as prognostic index for PTCL (PIT), IPI and β2-microglobulin, identified some proportion of the patients in remission, who did not benefit from HDCT.[8,24] Therefore, allogeneic SCT as consolidation or salvage for the patients with higher prognostic factors at diagnosis in remission should be investigated. Furthermore, chemotherapies with novel agents that aimed at increasing the CR rate have potential to improve the outcomes of auto-SCT.[25]

In conclusion, our retrospective analyses suggest that HDCT with auto-PBSCT is feasible and safe for the treatment of PTCL. It is necessary to elucidate who could be benefited by this treatment modality depending on clinical features, prognostic markers and histological subtypes. HDCT with auto-SCT after improved initial or salvage chemotherapy must be explored for better outcomes. Prospective clinical trials, including a sufficiently large study population for statistical power, are necessary to define the role of HDCT for this rare disease.

Conflict of interest

The authors declare no conflict of interest.

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bjh research paper

JAK2 V617F uses distinct signalling pathways to induce cell proliferation and neutrophil activation

Seido Oku,[1] Katsuto Takenaka,[1] Takuro Kuriyama,[1] Kotaro Shide,[2] Takashi Kumano,[1] Yoshikane Kikushige,[1] Shingo Urata,[1] Takuji Yamauchi,[1] Chika Iwamoto,[1] Haruko K. Shimoda,[2] Toshihiro Miyamoto,[1] Koji Nagafuji,[3] Junji Kishimoto,[4] Kazuya Shimoda[2] and Koichi Akashi[1,5]

[1]Department of Medicine and Biosystemic Science, Kyushu University Graduate School of Medical Sciences, Fukuoka, [2]Faculty of Medicine, Department of Gastroenterology and Haematology, Miyazaki University, Miyazaki, [3]Department of Medicine, Division of Haematology, Kurume University School of Medicine, [4]Digital Medicine Initiative, Kyushu University, and [5]Centre for Cellular and Molecular Medicine, Kyushu University Hospital, Fukuoka, Japan

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Correspondence: Katsuto Takenaka, Department of Medicine and Biosystemic Science, Kyushu University Graduate School of Medical Sciences, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan.
E-mail: takenaka@intmed1.med.kyushu-u.ac.jp

Summary

The acquired *JAK2* V617F mutation is observed in the majority of patients with *BCR-ABL1* negative chronic myeloproliferative neoplasms (MPN). *BCR-ABL1* negative MPN displays myeloproliferation with an elevated leucocyte alkaline phosphatase (LAP) activity, a neutrophil activation marker. We tried to separate the downstream signalling of JAK2 V617F to stimulate myeloproliferation and LAP activity. NB4, a myeloid lineage cell line, was transduced with *Jak2* V617F mutation or wild-type *Jak2*. We found that *Jak2* V617F mutation, but not wild-type *Jak2* enhanced LAP expression in NB4-derived neutrophils and proliferation of NB4 cells. JAK2 V617F induces constitutive phosphorylation of STAT3 and STAT5, and uses signalling targets such as Ras/MEK/ERK and PI3K/Akt pathways. By using MEK1/2 inhibitor U0126, PI3K inhibitor LY294002, and STAT3 or STAT5 siRNAs, JAK2 V617F was found to specifically use the STAT3 pathway to enhance LAP expression, while STAT5, Ras/MEK/ERK and PI3K/Akt, but not STAT3 pathways, were able to stimulate cell proliferation. These data strongly suggest that JAK2 V617F uses distinct signalling pathways to induce typical pathological features of MPN, such as high LAP activity and enhanced cell proliferation.

Keywords: myeloproliferative neoplasm, JAK2 V617F, STAT3, STAT5, leucocyte alkaline phosphatase.

The *BCR-ABL1* negative chronic myeloproliferative neoplasms (MPN) include polycythaemia vera (PV), essential thrombocythaemia (ET) and primary myelofibrosis (PMF) (Dameshek, 1951). The G-to-T point mutation at position 617 (V617F) in Janus kinase 2 (JAK2) that results in substitution of phenylalanine for valine has been reported in patients with *BCR-ABL1* negative MPN. This mutation is seen in approximately 95% of the patients with PV and in about 50% of the patients with ET or PMF (Baxter *et al*, 2005; James *et al*, 2005; Jones *et al*, 2005; Kralovics *et al*, 2005; Levine *et al*, 2005). JAK2 is a member of the non-receptor tyrosine kinase family that signals between type I cytokine receptors and downstream pathways, such as STAT3 (signal transducers and activators of transcription 3) and STAT5 (Witthuhn *et al*, 1993; Parganas *et al*, 1998). These pathways are critical for cell growth and differentiation. The *JAK2* V617F mutation induces constitutive activation of the downstream signalling pathways such as STAT3/STAT5, Ras/MEK/ERK and PI3K/Akt: the expression of JAK2 V617F in cytokine-dependent cell lines confers cytokine-independent growth by constitutively activating STAT3, STAT5, Akt and ERK (James *et al*, 2005; Levine *et al*, 2005; Levine & Wernig, 2006; Shide *et al*, 2007). The enforced expression of JAK2 V617F in murine bone marrow cells *in vivo* resulted in erythrocytosis and subsequent myelofibrosis in recipient mice (James *et al*, 2005; Lacout *et al*, 2006; Wernig *et al*, 2006; Zaleskas *et al*, 2006). Therefore, it is possible that the *JAK2* V617F mutation directly induces pathogenic features of *BCR-ABL1* negative MPN.

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Leucocyte alkaline phosphatase (LAP) is a monophosphoesterase that is the product of the gene encoding the liver/bone/kidney-type alkaline phosphatase (ALP) isoforms (Hayhoe & Quaglino, 1958; Weiss *et al*, 1988; Gianni *et al*, 1994; Sato *et al*, 1994). In neutrophils, LAP is stored in unique secretory vesicles, and is exported to the plasma membrane by chemotactic agents (Borregaard *et al*, 1987, 1992). Neutrophils play a critical role in host defence by phagocytozing and digesting microorganisms. This task is accomplished by mobilization of several types of neutrophil granules, including azurophilic (primary) granules, specific (secondary) granules, gelatinase (tertiary) granules and secretory vesicles. These granules contain potent antimicrobial compounds, proteolytic enzymes and also many membrane proteins. The azurophilic granules are formed during the promyelocytic stage, while specific granules and gelatinase granules are formed in more matured cells. Secretory vesicles appear in segmented neutrophils, and constitute a reservoir of membrane protein needed for neutrophil-mediated inflammatory response. Alkaline phosphatase is located at the luminal face of the membrane in secretory vesicles. Acquisition of LAP enzymatic activity is a very late event during myeloid maturation, and indicates that mature neutrophils acquire secretory vesicles. Thus, LAP is an indispensable marker of functionally mature neutrophils (Stewart, 1974; Garattini & Gianni, 1996; Borregaard & Cowland, 1997; Falanga *et al*, 2000; Faurschou & Borregaard, 2003). In clinics, the level of LAP in neutrophils is scored to evaluate neutrophil activation, which was classically designated as the neutrophil alkaline phosphatase (NAP) score (Kaplow, 1955, 1963; Shibata *et al*, 1985). Evaluation of the LAP score has been useful to distinguish chronic myeloid leukaemia (CML) from *BCR-ABL1* negative MPN, or from infection- or inflammation-associated leucocytosis: untreated CML patients usually have a low LAP score, whereas it is elevated in patients with PV, PMF and reactive leucocytosis.

In CML patients, the release of premature granulocytes from the bone marrow into the peripheral blood might result in low LAP scores (Dotti *et al*, 1999). It is, however, unclear why the LAP scores in patients with PV or PMF are elevated. Recent reports have shown that patients with *BCR-ABL1* negative MPN carrying the *JAK2* V617F mutation display higher LAP scores as compared to those carrying the wild-type *JAK2* alleles (Passamonti *et al*, 2006; Basquiera *et al*, 2007; Kondo *et al*, 2008). This finding suggests that an elevated LAP score in these diseases could be due to constitutive activation of the JAK2 signalling via the *JAK2* V617F mutation.

Neutrophil activation is the key feature of MPN as well as myeloproliferation. To understand the developmental mechanisms of MPN, we tried to separate the downstream signalling of JAK2 V617F in each pathway, and we used LAP expression as a neutrophil maturation/activation marker. The present study showed that the JAK2 V617F signalling stimulates LAP expression in neutrophils through specifically the activation of STAT3-dependent signalling pathway, whereas its stimulation of cell proliferation is dependent upon STAT5 activation. Thus, the acquisition of *JAK2* V617F at the haematopoietic stem cell (HSC) stage might induce at least two independent signals to alter myelopoiesis, including myeloproliferation and in elevation of the LAP score at the neutrophil stage, both of which are the pathological features specific to MPN.

Materials and methods

Cell lines and culture conditions

A human acute promyelocytic leukaemia (APL) cell line NB4, which retains t(15;17), was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). NB4 cells were cultured in RPMI 1640 medium (Wako, Osaka, Japan) containing 10% fetal bovine serum (ICN, Osaka, Japan). NB4 cells were differentiated into neutrophil-like cells after they were cultured with *all-trans* retinoic acid (ATRA) (10 μmol/l) (Sigma-Aldrich, St. Louis, MO, USA) and granulocyte colony-stimulating factor (G-CSF) (10 ng/ml) (Sigma-Aldrich) for 4 d (Gianni *et al*, 1994). Cells were counted by trypan blue dye exclusion staining.

Preparation of neutrophils from patients and healthy donors

This study was approved by Institutional Review Board of Kyushu University and all patients gave informed consent. Heparinized peripheral blood samples were collected from 15 healthy volunteer donors, 15 patients with PV, 21 patients with ET, and nine patients with PMF according to the World Health Organization criteria. Neutrophils were isolated by dextran sedimentation, hypotonic lysis of contaminating erythrocytes, and centrifugation with lymphocyte separation medium (LSM) (MP Biomedicals, Irvine, CA, USA) as described previously (Sullivan *et al*, 1984).

Mutation analysis and DNA sequencing of JAK2

DNA from patient's neutrophils was extracted using the Blood and Cell Culture DNA Mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. To examine the putative *JAK2* V617F mutation, exon 14 of JAK2 was amplified using 5′-TATAGTCATGCTGAAAGTAGG-3′ and 5′-TAACTGAATAGTCCTACAGTG-3′ primers as described previously (Shide *et al*, 2007). The polymerase chain reaction (PCR) products were sequenced directly using an ABI3130XL DNA sequencer (Applied Biosystems, Foster City, CA, USA). Furthermore, when the *JAK2* V617F mutation was not detected by direct sequencing, we used the three-primer allele-specific PCR assay as described previously (Baxter *et al*, 2005).

Evaluation of LAP expression

Leucocyte alkaline phosphatase expression was evaluated by LAP score, flow cytometric analysis, or ALP enzyme assay.

Leucocyte alkaline phosphatase scores were measured by the LAP staining kit (Muto, Tokyo, Japan) according to the manufacturer's instructions (Sakamoto, 1966; Shibata *et al*, 1985).

Flow cytometric analysis was used for the analysis of cell surface expression of LAP (sLAP). Isolated neutrophils were washed once in phosphate-buffered saline (PBS) (Wako) and stained with phycoerythrin (PE)-conjugated anti-human LAP (BD PharMingen, San Diego, CA, USA) or PE-conjugated mouse $IgG_1\kappa$ (isotype-matched negative control) (BD PharMingen). The mean fluorescent intensity (MFI) of sLAP was used as the LAP expression value as described previously (Shibano *et al*, 1999).

In the ALP enzyme assay, LAP expression in 5×10^5 NB4-derived neutrophils or neutrophils was measured by the SensoLyte™ p-Nitrophenyl phosphate (pNPP) alkaline phosphatase assay kit (Ana Spec, San Jose, CA, USA) according to the manufacturer's instructions.

Jak2 lentiviral infection

Murine *Jak2* cDNA was kindly provided by Dr J. Ihle (St. Jude Children's Research Hospital, Memphis, TN, USA). Oligonucleotide-directed mutagenesis was performed to substitute phenylalanine for valine at residue 617 of *Jak2* as described previously (Shide *et al*, 2007). For the wild-type *Jak2* or the *Jak2* V617F construct, each of the cDNAs were cloned downstream of an E1Fα promoter in a third-generation lentiviral vector backbone consisting of a reporter gene encoding GFP driven by the promoter of the human gene that encodes phosphoglycerate kinase. The control vector CEP contained only the gene encoding GFP. Vector construction was confirmed by sequencing. Viruses 'pseudotyped' with the vesicular stomatitis virus G protein were produced by transient transfection of 293T cells as described previously (Takenaka *et al*, 2007) and were concentrated by ultracentrifugation. Functional titres of virus producing wild-type JAK2 or JAK2 V617F, as determined by infection of HeLa cells, were 1×10^7 or 2×10^7 infectious particles/ml, respectively. Virus, at a multiplicity of infection of 40–50, was added to 24-well tissue culture plates of NB4 cells. Infected cells were collected on day 3. The efficiency of gene transfer into cells was 20–45%, as assessed by GFP fluorescence performed using a FACSCalibur flow cytometer (Becton Dickinson [BD] Biosciences, San Jose, CA, USA). GFP^+ cells were sorted by a FACS Aria cell sorter (BD Biosciences) and then the GFP^+ NB4 cells were cloned at one cell per well into 96-well tissue culture plates.

Inhibitions of JAK2, Ras/MEK/ERK and PI3K/Akt pathways by protein kinase inhibitors

We investigated the effects of protein kinase inhibitors on LAP expression in neutrophils/NB4-derived neutrophils, and on proliferation of NB4 cells. Protein kinase inhibitors used to suppress JAK2 and its downstream pathways were as follows: AG490 (Calbiochem, San Diego, CA, USA) was used as a JAK2 inhibitor at a concentration of 5–50 μmol/l, U0126 (Calbiochem) as a MEK1/2 inhibitor at a concentration of 1–20 μmol/l, and LY294002 (Calbiochem) as a PI3K inhibitor at a concentration of 5–50 μmol/l.

Leucocyte alkaline phosphatase expression was evaluated by flow cytometric analysis after neutrophils were cultured with/without protein kinase inhibitors in the presence of G-CSF (50 ng/ml) for 24 h.

Leucocyte alkaline phosphatase expression was evaluated by ALP enzyme assay after NB4 cells were cultured with ATRA (10 μmol/l), G-CSF (10 ng/ml), and protein kinase inhibitors for 4 d.

The proliferation of NB4 cells with medium alone or protein kinase inhibitors was evaluated for 4 d.

Inhibitions of STAT3/STAT5 pathways by small interfering RNAs (siRNAs)

To investigate the role of the STAT3 and STAT5 pathways in LAP expression in NB4-derived neutrophils and in proliferation of NB4 cells, STAT3 or STAT5 expression in NB4 cells was knocked down using double-stranded siRNAs. The siRNAs to knock down human STAT3, STAT5a and STAT5b were obtained from Invitrogen (Carlsbad, CA, USA). NB4 cells were transiently transfected with siRNAs using Nucleofector technology (Amaxa Inc., Gaithersburg, MD, USA) according to the manufacturer's instructions. Non-specific siRNA (Invitrogen) was used as a negative control.

Leucocyte alkaline phosphatase expression was evaluated by ALP enzyme assay after NB4 cells were cultured with ATRA (10 μmol/l) and G-CSF (10 ng/ml) for 4 d.

The proliferation of NB4 cells with medium alone was evaluated for 5 d.

Western blotting

Total cell lysates were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride nitrocellulose membranes (Amersham, Uppsala, Sweden) as described previously (Shide *et al*, 2008). Membranes were probed using the appropriate antibodies and visualized by enhanced chemiluminescence (Amersham). Anti-phospho Tyr^{705} STAT3, anti-phospho Ser^{727} STAT3, anti-phospho Tyr^{694} STAT5, anti-phospho Thr^{202}/Tyr^{204} ERK1/2, anti-ERK1/2, anti-phospho-Ser^{473} Akt, anti-Akt and anti-β-actin antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-JAK2, anti-STAT3, anti-STAT5a and anti-STAT5b antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Real-time quantitative PCR

Total RNA was extracted from NB4 cells. Reverse transcription was performed using a high-capacity cDNA

reverse transcription kit (Applied Biosystems) according to the manufacturer's instructions. Power SYBR Green Master Mix (Applied Biosystems) and subsequent primers were used for real-time quantitative PCR amplification of the murine *Jak2* cDNA, using 5′-AAGTGGAGCTTCGGGAC-CACTCT-3′ and 5′-GCTTATCTTCATAGAACTGCAGC-3′ primers on an ABI 7000 sequence detection system (Applied Biosystems).

Statistical analysis

Statistical analysis was performed using SAS or JMP software (SAS Institute Inc., Cary, NC, USA). LAP expressions among the groups were compared by Tukey's test or Dunnett's test. For inhibitor experiments, LAP expressions were compared using the least-squares mean test or paired *t*-test. For siRNAs experiments, LAP expressions were compared by paired *t*-test. For cell proliferation experiments, data were plotted on a single logarithmic graph and approximated by the straight line from the origin for each group. The gradients of the straight lines among the groups were compared by Tukey's test. Differences were considered statistically significant when the *P*-value was <0·05.

Results

Neutrophils with JAK2 V617F mutation express high levels of LAP in all types of MPN

The presence of the *JAK2* V617F mutation was documented by direct sequencing, or by allele-specific PCR of isolated neutrophils' DNA from *BCR-ABL1* negative MPN patients ($n = 45$). Patients were judged to be homozygous for the *JAK2* V617F mutation if their mutational load exceeded 50%. *JAK2* V617F mutation was identified in 13 of the 15 PV patients, 11 of the 21 ET patients and five of the nine PMF patients. Among them, five PV patients, eight ET patients and five PMF patients carried heterozygous mutations.

We analysed LAP expression of isolated neutrophils in *BCR-ABL1* negative MPN patients ($n = 45$) and healthy volunteer donors ($n = 15$) by flow cytometric analysis (Fig 1). *JAK2* V617F positive MPN patients ($n = 29$) had a higher LAP expression than *JAK2* V617F negative MPN patients ($n = 16$) or than healthy controls ($n = 15$) ($P < 0·01$). Among *JAK2* V617F positive MPN patients, those carrying homozygous mutation ($n = 11$) had higher levels of neutrophil LAP than those carrying heterozygous mutation ($n = 18$) ($P < 0·01$). Patients carrying heterozygous mutation also displayed higher LAP expression than healthy controls ($P < 0·05$), although the difference between *JAK2* V617F negative patients and patients carrying heterozygous mutation was not statistically significant. These data suggest that the expression level of JAK2 V617F correlates with the LAP levels in MPN neutrophils.

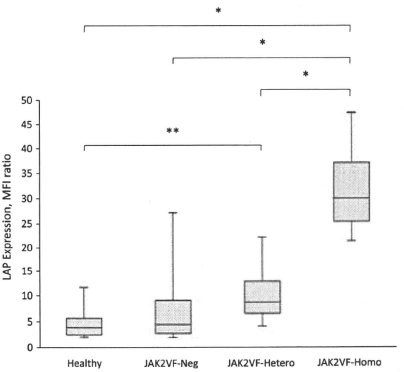

Fig 1. LAP expression of neutrophils in healthy volunteer donors ($n = 15$), *JAK2* V617F negative MPN (JAK2VF-neg, $n = 16$), *JAK2* V617F heterozygous MPN (JAK2VF-hetero, $n = 18$), and *JAK2* V617F homozygous MPN (JAK2VF-homo, $n = 11$). LAP levels were evaluated by flow cytometric analysis. LAP expression (ratio of mean fluorescence intensity, MFI) is shown in a box plot. JAK2VF-homo patients had a significantly higher level of LAP than those in the other three groups. *$P < 0·01$; **$P < 0·05$.

AG490, a JAK2 inhibitor, suppresses LAP expression in neutrophils with JAK2 V617F

To test whether the high level of LAP in *JAK2* V617F positive neutrophils is dependent upon JAK2 activation, we evaluated the effect of AG490, a JAK2 inhibitor, on LAP activity in *JAK2* V617F positive neutrophils. Neutrophils were purified from MPN patients with homozygous *JAK2* V617F, and were cultured for 24 h in the presence of AG490. AG490 significantly suppressed the LAP expression (Fig 2), confirming that the expression of LAP is dependent upon JAK2 activation in *JAK2* V617 positive neutrophils.

The enforced expression of JAK2 V617F stimulates the expression of LAP

To verify that JAK2 V617F signalling activates the expression of LAP in myeloid cells, we used NB4, a myeloid lineage cell line. NB4 was infected with lentiviral vectors harbouring wild-type *Jak2* or *Jak2* V617F mutation with GFP reporters. Infected cells were purified by positive expression of GFP by FACS. NB4 cells transfected with wild-type *Jak2* and *Jak2* V617F mutation showed an increased expression of JAK2 protein as compared to control by Western blotting, but their levels did not differ between these *Jak2*- or *Jak2* V617F-transduced NB4 clones (Fig 3A).

NB4 cells differentiated into neutrophil-like cells in the presence of ATRA and G-CSF for 4 d (Gianni *et al*, 1994). The NB4-derived neutrophils were then evaluated for LAP

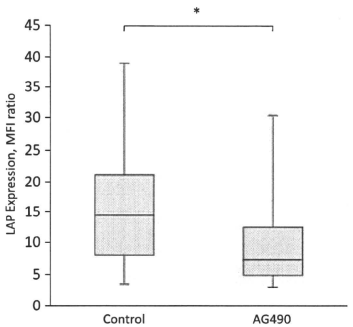

Fig 2. Effect of the AG490, a JAK2 inhibitor, on LAP expression in *JAK2* V617F positive neutrophils. Neutrophils from *JAK2* V617F positive MPN patients ($n = 16$) were cultured in medium in the presence of G-CSF (50 ng/ml), and the effect of addition of AG490 (50 μmol/l) on LAP expression was evaluated 24 h after culture on a flow cytometry. LAP expression (ratio of MFI) is shown in a box plot. AG490 suppressed LAP expression. *$P < 0{\cdot}01$.

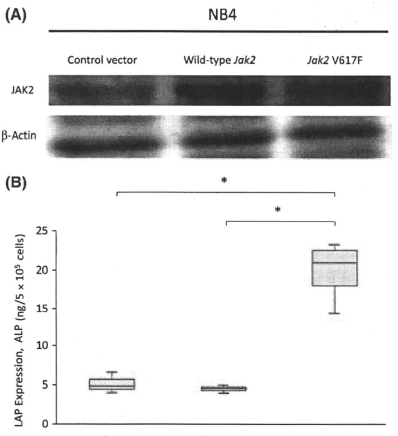

Fig 3. (A) JAK2 protein expression in NB4 cells by Western blotting. NB4 cells expressing control vector, wild-type JAK2 or JAK2 V617F were lysed and analysed by Western blotting with the indicated antibodies. Expression of JAK2 protein in wild-type *Jak2* and *Jak2* V617F-transduced NB4 cells was equivalent, but higher than that in the control vector-transduced cells. (B) Effect of the *Jak2* V617F mutation on LAP expression in NB4-derived neutrophils. NB4 cells expressing control vector, wild-type JAK2 or JAK2 V617F were cultured with ATRA (10 μmol/l) and G-CSF (10 ng/ml) for 4 d and differentiated into neutrophil-like cells. LAP expression was evaluated by ALP enzyme assay. LAP expression (ALP level) obtained from three independent experiments is shown in a box plot. *Jak2* V617F-transduced NB4-derived neutrophils had a higher LAP expression than the other two groups. *$P < 0{\cdot}01$.

expression. We measured LAP levels in neutrophils differentiated from *Jak2* V617F clones, from wild-type *Jak2* clones, and from control clones with an empty vector by ALP enzyme assays. Representative data are shown in Fig 3A, B. Neutrophils from *Jak2* V617F clones expressed LAP at a much higher level, as compared to those from wild-type *Jak2* clones, whereas the expression levels of JAK2 in these clones were equivalent ($P < 0{\cdot}01$) (Fig 3A, B). Thus, JAK2 V617F signalling is able to stimulate LAP expression in neutrophils.

STAT3 but not STAT5 signalling is required for JAK2 V617F-dependent LAP expression

JAK2 V617F mutation constitutively activates multiple JAK2 downstream signalling molecules, including STAT3/STAT5, Ras/MEK/ERK and PI3K/AKt (James *et al*, 2005; Levine *et al*, 2005; Levine & Wernig, 2006; Shide *et al*, 2007). We therefore tried to identify the signalling pathway responsible for LAP expression. We first tested the requirement of STAT3 or STAT5 for LAP expression stimulated by JAK2 V617F in *Jak2* V617F-transduced NB4 cells after inducing neutrophil lineage differentiation by ATRA and G-CSF. STAT3 and STAT5 expression were inhibited by STAT3 and STAT5 siRNAs respectively, in NB4-derived neutrophils: *Jak2* V617F-transduced NB4 cells were transduced with STAT3 or STAT5 siRNA, and cultured with ATRA and G-CSF for 4 d. As shown in Fig 4A, C, STAT3 siRNA, but not STAT5 siRNA, inhibited LAP expression ($P < 0{\cdot}05$). Western blot analysis showed that STAT3 and STAT5 siRNAs specifically inhibited the expression of STAT3 and STAT5 respectively, but did not inhibit the signalling molecules on Ras/MEK/ERK and PI3K/Akt pathways (Fig 4B, D). These data suggest that STAT3 is indispensable for LAP expression in JAK2 V617F signalling.

The LAP expression is dependent upon the MEK, but not the PI3K signalling pathway

We then tested the effects of MEK1/2 inhibitor (U0126) and PI3K inhibitor (LY294002) on LAP expression in *Jak2* V617F-transduced NB4 cells. LAP expression was evaluated by ALP enzyme assay. U0126 but not LY294002 significantly suppressed LAP expression in *Jak2* V617F-transduced NB4-derived neutrophils (Fig 5A, C). Further analysis of signalling molecules, such as ERK and Akt, downstream of MEK and PI3K respectively, revealed that U0126 and LY294002 efficiently inhibited phosphorylation of ERK and Akt respectively (Fig 5B, D).

Activated JAK2 transduces the signal cascade through phosphorylation of both the receptors and the major substrates, STATs. STAT3 is phosphorylated by JAK2 on a single tyrosine at position 705. This phosphorylated STAT3 is

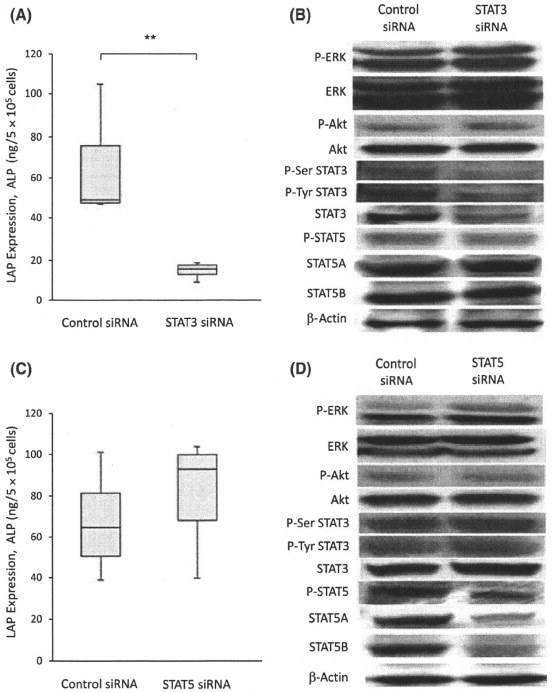

Fig 4. Effect of STAT3 and STAT5 siRNAs on LAP expression in NB4-derived neutrophils. *Jak2* V617F-transduced NB4 cells were transfected with control, STAT3 (A), or STAT5 (C) siRNA, and cultured with ATRA (10 μmol/l) and G-CSF (10 ng/ml) for 4 d and differentiated into neutrophil-like cells. LAP expression in NB4-derived neutrophils was evaluated by ALP enzyme assay. LAP expression (ALP level) obtained from three independent experiments is shown in a box plot. STAT3 siRNA decreased LAP expression, but STAT5 siRNA did not affect LAP expression ($P > 0{\cdot}05$) $^{**}P < 0{\cdot}05$. Phosphorylation and expression of ERK, Akt, STAT3 and STAT5 proteins by Western blotting. NB4 cells expressing JAK2 V617F were transfected with control, STAT3 (B) or STAT5 (D) siRNA, and then cultured with ATRA (10 μmol/l) and G-CSF (10 ng/ml) for 2 d. Cells were then lysed and analysed by Western blotting with the indicated antibodies. STAT3 and STAT5 siRNAs inhibited only phosphorylation and expression of STAT3 and STAT5 proteins, respectively.

dimerized, migrates to the nucleus, and subsequently binds specific regulatory sequences to activate or repress transcription of target genes (Zhong *et al*, 1994; Ihle, 1995). Another phosphorylation event in a single serine at position 727 has been described that modulates transcriptional activity of STAT3. Serine phosphorylation is necessary to potentiate STAT3 transcriptional activity or negatively regulates STAT3 (Wen *et al*, 1995; Zhang *et al*, 1995; Jain *et al*, 1998; Sengupta *et al*, 1998). It has been reported that MEK/ERK mediates signalling to activate the JAK/STAT pathway through phosphorylation of STAT3 at Ser^{727} but not at Tyr^{705} (Chung *et al*, 1997). In fact, interestingly, U0126 inhibited STAT3 Ser^{727} but not Tyr^{705} phosphorylation (Fig 5B). Among the MEK/ERK signalling cascades, signals to induce phosphorylation of STAT3 at Ser^{727} are especially required for LAP expression because STAT3 siRNA inhibited LAP expression but not ERK phosphorylation in our hands (Fig 4A, B). As STAT3 is indispensable for LAP expression (Fig 4A, B), the suppression of LAP expression by U0126 might be due to the inhibition of phosphorylation of STAT3 Ser^{727}. Importantly, LAP expression was inhibited only by U0126 also in neutrophils isolated from *JAK2* V617F positive MPN patients ($n = 10$) (Fig 5E, F). These data collectively suggest that JAK2 V617F stimulates the LAP expression also via MEK/ERK-dependent signalling pathway that phosphorylates STAT3 Ser^{727}.

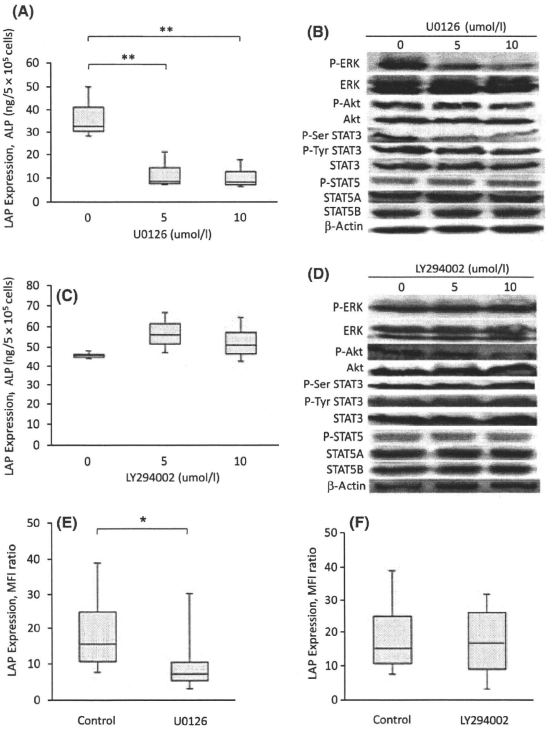

Fig 5. The effects of U0126 (MEK1/2 inhibitor) and LY294002 (PI3K inhibitor) on LAP expression in NB4-derived neutrophils. *Jak2* V617F-transduced NB4 cells were cultured with ATRA (10 μmol/l), G-CSF (10 ng/ml) and U0126 (0–10 μmol/l) (A) or LY294002 (0–10 μmol/l) (C) for 4 d and differentiated into neutrophil-like cells. LAP expression was evaluated by ALP enzyme assay. LAP expression (ALP level) obtained from three independent experiments is shown in a box plot. U0126 suppressed LAP expression in NB4-derived neutrophils, but LY294002 did not affect LAP expression ($P > 0{\cdot}05$). $^{**}P < 0{\cdot}05$. Phosphorylation and expression of ERK, Akt, STAT3 and STAT5 proteins by Western blotting. *Jak2* V617F-transduced NB4 cells were cultured with ATRA (10 μmol/l), G-CSF (10 ng/ml) and U0126 (0–10 μmol/l) (B) or LY294002 (0–10 μmol/l) (D) for 24 h. Cells were then lysed and analysed by Western blotting with the indicated antibodies. The inhibitory effects of MAPK and PI3K pathways by U0126 and LY294002 were confirmed by decreased phosphorylation of ERK and Akt proteins, respectively. U0126 not only inhibited ERK phosphorylation but also STAT3 Ser^{727} phosphorylation. However, LY294002 inhibited only phosphorylation of Akt and did not show any inhibition of phosphorylation of STAT3/STAT5. Neutrophils from *JAK2* V617F positive MPN patients ($n = 10$) were cultured in medium in the presence of G-CSF (50 ng/ml), and the effect of addition of U0126 (10 μmol/l) or LY294002 (50 μmol/l) on LAP expression was evaluated 24 h after culture on a flow cytometry. LAP expression (ratio of MFI) is shown in a box plot. LAP expression was inhibited only by U0126 (E) in neutrophils from *JAK2* V617F positive MPN patients, but LY294002 (F) ($P > 0{\cdot}05$) $^{*}P < 0{\cdot}01$.

Jak2 V617F-induced cell proliferation requires STAT5 but not STAT3 signalling

Enforced expression of JAK2 V617F into NB4 cells significantly enhanced proliferation of NB4 cells *in vitro* (Fig 6A). To test whether stimulation for LAP expression and cell proliferation shared the signalling pathway, we tested the effect of inhibitors (U0126 and LY294002) and STAT3/STAT5 siRNAs on proliferation of NB4 cells. As shown in Fig 6B, C, both inhibitors significantly suppressed proliferation of *Jak2* V617F-transduced NB4 cells. Furthermore, in marked contrast to the LAP expression (Fig 4A, C), STAT5 siRNA but not STAT3 siRNA was able to suppress proliferation of *Jak2* V617F-transduced NB4 cells (Fig 6D, E). These data strongly suggest

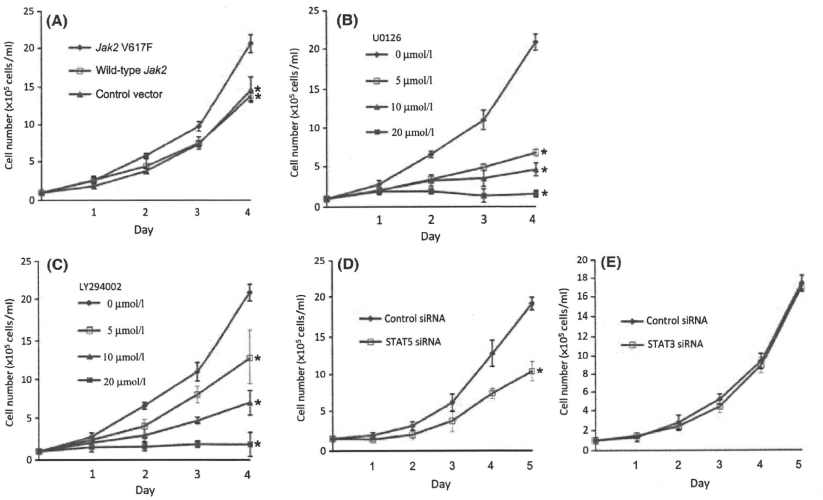

Fig 6. Effect of U0126 (MEK 1/2 inhibitor), LY294002 (PI3K inhibitor), STAT3 siRNA and STAT5 siRNA on proliferation of NB4 cells. (A) Effect of the *Jak2* V617F mutation on proliferation of NB4 cells. NB4 cells expressing wild-type JAK2 or JAK2 V617F were cultured for 4 d. The *Jak2* V617F mutation enhanced cell proliferation. *Jak2* V617F-transduced NB4 cells were cultured with U0126 (0–20 μmol/l) (B) or LY294002 (0–20 μmol/l) (C) for 4 d or cells were transfected with control, STAT5 (D) or STAT3 (E) siRNA, and then cultured with medium alone for 5 d. U0126, LY294002, STAT5 siRNA, but not STAT3 siRNA, inhibited cell proliferation. Cells were cultured at a density of 1×10^5 cells/ml and counted on the indicated days. The mean (±SD) cell numbers obtained from three independent experiments are shown in a line plot. *$P < 0{\cdot}01$ compared with *Jak2* V617F (A), 0 μmol/l (B, C) or control siRNA (D).

that JAK2 V617F uses distinct pathways for stimulation of cell proliferation and neutrophil maturation represented by LAP expression; the former is dependent upon STAT5, Ras/MEK/ERK and PI3K pathways, whereas the latter is dependent upon STAT3.

Discussion

Determination of the LAP score has been a useful tool for differential diagnosis of CML and other MPN. However, the underlying mechanisms for elevation of LAP scores in *BCR-ABL1* negative MPN patients but not in CML has been unclear. We observed that patients with *JAK2* V617F homozygous mutations had a higher LAP expression than other patients (Fig 1). Similar results were reported previously (Passamonti *et al*, 2006; Basquiera *et al*, 2007; Kondo *et al*, 2008), thus suggesting that the LAP levels in neutrophils are dependent upon the level of JAK2 V617F expression. The AG490, a JAK2 inhibitor, significantly decreased LAP expression in neutrophils of *JAK2* V617F positive patients (Fig 2). In addition, the enforced expression of JAK2 V617F but not wild-type JAK2 induced a high level of LAP expression in NB4-derived neutrophils (Fig 3A, B). These data show that signalling from the JAK2 V617F can directly induce LAP activation.

It has been shown that the *JAK2* V617F mutation induces constitutive activation of downstream targets, such as STAT3, STAT5 and other signalling pathways including Ras/MEK/ERK and PI3K/Akt to alter cell proliferation, differentiation and apoptosis (James *et al*, 2005; Levine *et al*, 2005; Levine & Wernig, 2006; Shide *et al*, 2007). We investigated as to which pathway is responsible for inducing LAP expression and cell proliferation. The JAK2 V617F signalling pathways on LAP and cell proliferation based on our results is schematized in Fig 7.

Jak2 V617F mutation enhanced not only LAP expression but also cell proliferation of NB4, a myeloid lineage cell line (Fig 6A). The STAT3 pathway was specifically used when enhancing LAP expression (Fig 4A). On the other hand, STAT5, MEK/ERK and PI3K/Akt, but not STAT3 were used for cell proliferation (Fig 6B–E). The activated JAK2 kinase induces STAT3/STAT5 tyrosine phosphorylation, and then phosphorylated STATs are dimerized, enter the nucleus, and bind specific sequences to regulate transcription of target genes. This pathway is a major downstream signalling cascade of JAK/STAT, and has been considered to be critical in enhancing cell proliferation (Zhong *et al*, 1994; Ihle, 1995). On the other hand, JAK2 also phosphorylates signalling molecules in the Ras/MEK/ERK and PI3K/Akt pathways. We

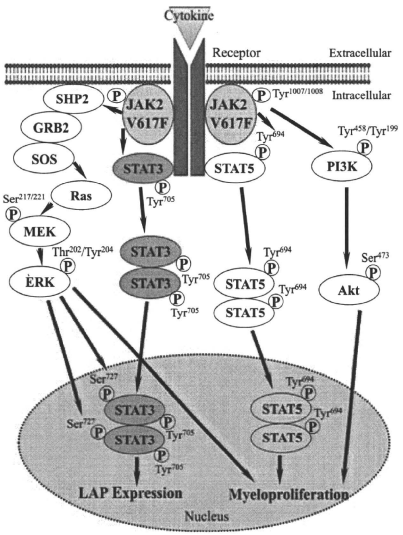

Fig 7. Schematic presentation of JAK2 V617F signalling to activate myeloproliferation and LAP expression. The activated JAK2 kinase induces STAT3/STAT5 tyrosine phosphorylation, and then phosphorylated STATs are dimerized, enter the nucleus, and bind specific regulatory sequences to regulate transcription of target genes. This pathway is a major downstream signalling cascade of JAK/STAT. Tyrosine phosphorylation of STAT5 mediates enhancing myeloproliferation. On the other hand, JAK2 also phosphorylates signalling molecules in Ras/MEK/ERK and PI3K/Akt pathways that are also responsible for cell proliferation. STAT3 has two phosphorylation sites, Tyr^{705} and Ser^{727}. Tyrosine phosphorylation is transduced by JAK2, whereas serine phosphorylation is transduced by MEK/ERK pathway. Serine phosphorylation of STAT3 probably affects the STAT3 transcriptional activation. JAK2 V617F stimulates the LAP expression also via MEK/ERK-dependent signalling pathway that phosphorylates STAT3 Ser^{727}. Thus, JAK2 V617F uses STAT3 pathway to induce LAP expression, and STAT5, Ras/MEK/ERK and PI3K/Akt pathways to stimulate myeloproliferation.

showed that both pathways are responsible for cell proliferation. STAT3 siRNA and the MEK1/2 inhibitor U0126 significantly reduced LAP expression (Figs 4A, 5A, E). Furthermore, U0126 not only inhibited ERK phosphorylation but also STAT3 Ser^{727} phosphorylation (Fig 5B). STAT3 has two phosphorylation sites, which are Tyr^{705} and Ser^{727} sites. Tyrosine phosphorylation of STAT3 is the major signalling cascade of JAK/STAT pathway described above. On the other hand, serine phosphorylation of STAT3 probably affects the STAT3 transcriptional activation, which is mainly transduced by the MEK/ERK pathway. In fact, a STAT3 S727A mutant, where Ser^{727} was replaced with an alanine, exhibited marked reduction in transcriptional activation, indicating that STAT3 Ser^{727} phosphorylation is essential for STAT3 transcriptional activation (Wen *et al*, 1995; Zhang *et al*, 1995). Consistent with this, LAP expression was suppressed significantly by STAT3 siRNA despite ERK phosphorylation (Fig 4A, B). The MEK/ERK signalling unrelated to STAT3 serine phosphorylation was not required for LAP expression. These findings show that STAT3 serine phosphorylation is mainly involved in enhancing LAP expression in Jak2 V617F signalling pathways.

JAK2 V617F mutation occurs at the HSC level (Jamieson *et al*, 2006; Kota *et al*, 2008). It is still unclear how this common mutation can induce three distinct MPN. Our data shows that the JAK2 V617F uses at least two distinct signalling pathways for enhancing LAP expression in neutrophil and cell proliferation of the myeloid lineage cell line (Fig 7). We and others have reported that the balance between constitutively activated STAT3/STAT5 or the expression level of JAK2 V617F could be a determinant for the type of MPN (Mesa *et al*, 2006; Teofili *et al*, 2007; Shide *et al*, 2008; Tiedt *et al*, 2008; Xing *et al*, 2008). To understand the developmental mechanisms of MPN, it is critical to understand the contribution of each signalling pathway toward the proliferation and lineage fate decision of HSCs in different types of MPN.

In conclusion, we obtained direct evidence that JAK2 V617F mainly induces elevation of LAP scores via the STAT3 pathway, whereas it stimulates cell proliferation via the STAT5, Ras/MEK/ERK and PI3K pathways. Thus, JAK2 V617F uses distinct signalling pathways to enhance LAP expression and myeloproliferation, typical characteristics in *BCR-ABL1* negative MPN.

Acknowledgements

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High incidence of false-positive *Aspergillus* galactomannan test in multiple myeloma

Yasuo Mori,[1,2] Yoji Nagasaki,[1,2] Kenjiro Kamezaki,[1] Katsuto Takenaka,[1] Hiromi Iwasaki,[2] Naoki Harada,[1] Toshihiro Miyamoto,[1] Yasunobu Abe,[3] Nobuyuki Shimono,[1] Koichi Akashi,[1,2] and Takanori Teshima[2]

Invasive aspergillosis (IA) remains one of the most significant causes of morbidity and mortality in patients with hematological malignancies undergoing chemotherapy and hematopoietic stem cell transplantation (HSCT), mainly due to the difficulty in its early diagnosis. Monitoring of galactomannan (GM) antigen, an exoantigen of Aspergillus, in the blood by sandwich ELISA is a useful and noninvasive method for early diagnosis of IA. The GM test has a sensitivity of 67–100% with a specificity of 81–99% in neutropenic patients and allogeneic transplant recipients [1–3]. Although it has been widely used as a diagnostic criterion for IA [4,5], one of the major limitations of this assay is false-positivity, particularly in pediatric patients [1], patients with graft-versus-host disease (GVHD) [6,7], and those taking dietary GM [8,9] or fungus-derived antibiotics, such as piperacillin-tazobactam (PIPC/TAZ) [10–12].

Multiple myeloma results from malignant proliferation of a single clone of plasma cells, which produces a monoclonal immunoglobulin. Opportunistic infection is a major cause of death in patients with myeloma [13,14]. The risk for infection primarily resides during periods of chemotherapy-induced neutropenia or in the terminal stages of the disease. Therefore, monitoring of Aspergillus is recommended during chemotherapy-induced neutropenia.

One hundred twenty-four patients with hematological disorders hospitalized in our institution from April 2007 to September 2009 were analyzed retrospectively. The clinical characteristics of these patients are summarized in Supporting Information Table 1. Twenty-seven patients had plasma cell associated disorders (IgG type myeloma: 14, IgA type myeloma: 1, IgD type myeloma: 1, Bence-Jones type myeloma: 3, lymphoplasmacytic lymphoma/Waldenstrom macroglobulinemia: 3, plasma cell leukemia: 1, primary AL amyloidosis: 1, and POEMS syndrome: 2). The remaining 97 patients were diagnosed as acute leukemia (MDS/AML: 32, ALL: 15) or malignant lymphoma (n = 50). Out of the 124 patients, those receiving cytotoxic chemotherapy, autologous peripheral blood stem cell transplantation (PBSCT), and allogeneic HSCT were 81, 10, and 28, respectively. One hundred eleven patients received antifungal prophylaxis, mostly with FLCZ or ITCZ. Eight patients were administered PIPC/TAZ at sampling time points. Seventy patients were low risk for the development of IA, whereas the remaining 54 patients were high risk.

In 21 of the 124 (16.9%) patients, GM antigenemia was positive at least two consecutive times and their characteristics are shown in Table I. However, only seven of the 21 patients showed clinical features of IA and were diagnosed with probable IA in the lung (Cases 1–7). Clinical features were relieved with treatment with VRCZ in these patients, confirming a diagnosis of IA. All of the seven patients had received antifungal prophylaxis, but not antibiotics known to cause false-positive results, such as PIPC/TAZ [10–12], amoxicillin/clavulanic acid, or amoxicillin [15]. Four of the 7 patients were high risk for the developing IA and three were low risk. Possible IA was diagnosed in four patients in the absence of positive GM results. On the other hand, no proven or probable IA was detected in 103 patients with negative GM antigenemia.

Fourteen of the 21 (66.7%) patients with positive GM antigenemia did not satisfy the diagnostic criteria of proven or probable IA (Cases 8–21 in Table I); thus, their episodes were considered to be false-positive. None of the 14 patients were treated with antibiotics potentially causing false-positivity of GM test. Antifungal prophylaxis had been given in all 14 episodes (FLCZ in 5, ITCZ in 5, and MCFG in 4). These patients did not show any clinical features suggestive of IA. Chest CT scans did not show any abnormal findings in 10 patients, whereas four patients showed abnormalities in the lung which were not suspicious of IA. Diagnosis of these lung lesions were history of pneumoconiosis in one patient, proven bacterial pneumonia in one, and idiopathic interstitial pneumonia in the remaining two patients. These lesions were not deteriorated without antifungal treatment. (1→3)-β-D-glucan was negative in all patients showing false-positive GM test. With a median follow-up of 10 months (range: 1–19 months), these patients did not develop fungal infection without treatment. On the other hand, false-negative GM results were obtained in four patients (3.2 %). In this study, the sensitivity, specificity, PPV, and NPV of the GM ELISA test were 63.6% (7/11), 87.6% (99/113), 33.3% (7/21), and 96.1% (99/103), respectively.

Surprisingly, 11 out of the 14 patients showing the false-positive results had diagnosis of multiple myeloma. The false-positivity of GM antigenemia was significantly higher in myeloma patients (11/22, 50%) than those with other hematological malignancies (3/102, 2.9%) ($P < 0.001$). Moreover, in myeloma patients false-positive results were exclusively detected in those with IgG subtype. Thus, rate of false-positivity was extremely high in patients with IgG myeloma (11/14, 78.6%). We could not find any difference in the characteristics of IgG myeloma patients with or without GM false-positivity, including serum levels of IgG (5083 ± 2077 mg/dl versus 4713 ± 3729 mg/dl). In addition, the GM test remained to be false-positive even after normalization of IgG levels by chemotherapy in eight of the 11 patients. We also evaluated GM antigenemia in frozen serum samples collected before chemotherapy in three myeloma patients who showed false-positivity after chemotherapy to rule out the possibility that administration of myeloma-specific chemotherapy is associated with the false-positivity, and confirmed GM positivity before chemotherapy in these samples.

In a univariate logistic regression analysis, IgG myeloma and low-risk category were strongly associated with false-positive GM antigenemia. Sex, type of treatment, antibiotics, corticosteroid usage, and serum levels of immunoglobulins were also significant or marginally associated with false-positivity. Multivariate analysis confirmed diagnosis of IgG myeloma as the only independent risk factor for false-positivity (odds ratio, 59.41; 95% confidence interval, 8.19–431.0; $P < 0.001$) (Supporting Information, Table 2). In patients with other diseases, the GM assay showed a high sensitivity (7/11, 63.6%), specificity (96/99, 97.0%), PPV (7/10, 70%), and NPV (96/100, 96%). In contrast, for patients with IgG myeloma, specificity and PPV of the assay were very low (3/14, 21.4%, and 0/11, 0%), whereas NPV was 100% even in this cohort.

A recent meta-analysis addressing the accuracy of a GM assay for diagnosing IA confirmed the clinical usefulness of this test with a sensitivity of 71% and a specificity of 89% [16]. Although our study demonstrated similar sensitivity (7/11:64%) and specificity (103/117:88%) of the GM test, PPV (33%) was lower, compared with previous studies that demonstrate 40–60% PPV [7,17–19]. This difference is due to an unexpectedly high incidence of GM antigen false-positivity (11.3%) in our study. It should be noted, however, that screening of GM antigen was performed less frequently in this study compared with previous studies, where GM antigenemia was evaluated two to three times per week [1,2,20], and such a frequent monitoring is ideal to assure the optimal PPV and NPV.

Diagnosis of multiple myeloma is a major risk factor for GM false-positivity. In particular, the false-positivity was exclusively observed in patients with IgG myeloma and was not observed in patients with other types of plasma cell disorders. These observations should be confirmed in a larger study because some studies previously reported the usefulness of GM antigen assay as a diagnostic tool for IA among patients with hematological malignancies including IgG myeloma [21,22], and only small numbers of patients with plasma cell disorders other than IgG myeloma were included in this study. Low-risk category of developing IA was a risk for false-positive results in a univariate, but not multivariate analysis. IgG myeloma remained a strong risk for false-positivity even after the compensation by the risk categorization.

Mechanisms of high frequency of GM false-positivity in myeloma patients remain to be investigated. (1→3)-β-D-glucan, which is released from the fungal cell wall, is also widely used to support diagnosis of fungal infections

TABLE I. Characteristics of 21 Patients with Positive GM Test

Case	Age/Sex	Disease	Treatment	GM (C.O.I)	Radiological/Clinical	Diagnosis	Times/duration of false-positivity	Antibiotics[a]	Antifungal agents[a]	Steroids	Neutrophil ($\times 10^9$/L)s	IgG (mg/dL)	IgA (mg/dL)	IgM (mg/dL)	Risk of IA
1	49/F	MDS/AML	CTx	1.1	nodules with halo	probable IA	–	CFPM	FLCZ	none	0.01	1419	227	50	High
2	63/M	MDS/AML	CTx	1.2	nodules	probable IA	–	MEPM, AMK	FLCZ	none	0	1253	238	62	High
3	50/F	AML	CTx	1.2	nodules	probable IA	–	MEPM, AMK	ITCZ	none	0	1301	293	235	High
4	57/F	MDS	allo-SCT	2.3	nodules	probable IA	–	CAZ, VCM	VRCZ	none	0.009	1198	256	120	High
5	63/M	ML	CTx	1.3	consolidation with pleural pain	probable IA	–	none	MCFG	DEXA	2.034	689	126	92	Low
6	57/F	ML	CTx	1.5	nodules	probable IA	–	CFPM	FLCZ	PSL	0.081	675	117	67	Low
7	59/F	ML	CTx	2.2	consolidation with pleural pain	probable IA	–	CZOP	MCFG	PSL	0.036	719	95	20	Low
8	70/M	ML	CTx	0.8	pneumoconiosis	F-P	7/7 months	none	FLCZ	PSL	5.096	1870	315	60	Low
9	52/M	ML	allo-SCT	2.5	bacterial pneumonia	F-P	2/1 month	CZOP, CPFX	ITCZ	mPSL	0.806	562	49	19	High
10	54/M	ML	CTx	0.9	negative	F-P	5/3 months	CFPM	FLCZ	none	0.395	691	46	10	Low
11	63/F	IgGλ MM	CTx	0.8	negative	F-P	2/1 month	none	ITCZ	DEXA	1.497	4770	25	27	Low
12	56/F	IgGλ MM	none	1.2	negative	F-P	3/2 months	CAZ	FLCZ	none	3.449	7196	5	38	Low
13	69/M	IgGλ MM	CTx	1.5	negative	F-P	4/4 months	none	MCFG	DEXA	1.72	3344	13	<10	Low
14	69/M	IgGλ MM	CTx	1.2	negative	F-P	5/3 months	none	FLCZ	DEXA	1.777	2385	6	<10	Low
15	64/M	IgGλ MM	CTx	1.6	old inflammatory change	F-P	5/2 months	none	MCFG	PSL	1.919	3263	39	30	Low
16	51/F	IgGλ MM	CTx	2.1	IP	F-P	23/15 months	none	ITCZ	PSL	3.791	8006	98	30	Low
17	54/M	IgGλ MM	CTx	1.6	negative	F-P	15/8 months	none	ITCZ	DEXA	0.899	3956	6	<10	High
18	75/M	IgGλ MM	CTx	0.6	negative	F-P	2/1 month	MEPM	FLCZ	DEXA	0.823	8986	<5	<10	High
19	49/F	IgGλ MM	CTx	1.1	negative	F-P	8/3 months	none	MCFG	DEXA	0.724	4563	37	15	Low
20	74/F	IgGλ MM	CTx	0.7	negative	F-P	6/2 months	none	MCFG	none	1.451	3924	25	18	Low
21	56/M	IgGλ MM	CTx	1.1	negative	F-P	10/3 months	none	ITCZ	DEXA	1.605	4917	9	10	Low

MDS, myelodysplastic syndrome; AML, acute myelogenous leukemia; ML, malignant lymphoma; MM, multiple myeloma; CTx, chemotherapy; allo-SCT, allogeneic stem cell transplantation; C.O.I, cut-off index; IP, interstitial pneumonia; F-P, false-positive; CFPM, cefepime; MEPM, meropenem; AMK, amikacin; CAZ, ceftazidime; VCM, vancomycin; CZOP, cefozopran; PIPC/TAZ, piperacillin/tazobactam; CPFX, ciprofloxacin; PSL, prednisolone; mPSL, methylprednisolone; DEXA, dexamethasone. [a]Administrated at initial positive sampling.

and adopted as one of the microbiological criterion for probable IA in the revised EORTC/MSG definition [5]. A previous study reported that high levels of immunoglobulins interfere with the measurement of (1→3)-β-D-glucan by causing precipitation of insolubilized proteins and increase the nonspecific optical density levels of reaction fluid [23], although (1→3)-β-D-glucan was negative in patients showing false-positive GM test in this study. This phenomenon has not been reported in the GM assay. However, serum levels of IgG were not directly associated with the false-positivity; IgG levels did not differ between IgG myeloma patients with and without false-positivity. In addition, the GM test remained to be false-positive even after normalization of IgG levels by chemotherapy in eight of the 11 patients.

Causative role of PIPC/TAZ, amoxicillin/clavulanic acid, and amoxicillin in GM false-positivity has been well documented [10–12,15]; therefore, collection of samples before infusion of these antibiotics and the use of a relatively higher cut-off level (>0.7) are recommended in patients receiving these agents [12]. In this study, no patients with GM false-positivity received these antibiotics at the time of sampling. It has been hypothesized that dietary contamination by GM causes GM false-positivity by the translocation of dietary GM into the systemic circulation through the disrupted intestinal mucosal barrier, especially in patients with gastrointestinal GVHD after allogeneic HSCT [6,7,9]. In our cohort, 1(3.6%) of the 28 patients who underwent allogeneic HSCT showed false-positivity. This patient with lymphoma had acute GVHD involving in the skin and intestine. A previous study demonstrated that false-positive results were preferentially observed in patients with febrile neutropenic sepsis [24], although subsequent study was unable to replicate this result [20]. A recent study revealed that serum GM antigen levels was significantly higher in severely neutropenic patients ($< 0.1 \times 10^9$/L) than in the other patients [25]. However, we did not find such an association in this study.

In conclusion, the incidence of false-positive GM antigenemia was high in patients with IgG myeloma. Although the results should be confirmed in a prospective study including larger numbers of patients, positive results of GM antigenemia may be interpreted with caution, and intimate survey including CT scan or other microbiological markers will be recommended in myeloma patients.

[1]*Medicine and Biosystemic Science, Kyushu University Graduate School of Medical Sciences, Fukuoka Japan;* [2]*Center for Cellular and Molecular Medicine, Kyushu University Graduate School of Medical Sciences, Fukuoka Japan;* [3]*Medicine and Bioregulatory Science, Kyushu University Graduate School of Medical Sciences, Fukuoka Japan*

Additional Supporting Information may be found in the online version of this article.

**Correspondence to: Takanori Teshima, Center for Cellular and Molecular Medicine, Kyushu University Hospital, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan*
E-mail: tteshima@cancer.med.kyushu-u.ac.jp

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Mixed phenotype acute leukemia with t(11;19)(q23;p13.3)/MLL-MLLT1(ENL), B/T-lymphoid type: A first case report

Mojdeh Naghashpour,[1]* Jeffrey Lancet,[2] Lynn Moscinski,[1] and Ling Zhang[1]

The majority of cases of acute leukemia belong to a specific lineage origin, either lymphoid or myeloid, and therefore are classified as acute lymphoblastic leukemia (ALL) or acute myelogenous leukemia (AML), based on morphologic features and cytochemical and immunophenotypic profile of the blast cells. A minority of acute leukemias however, show no clear evidence of differentiation along a single lineage. These are now classified under acute leukemias of ambiguous lineage by the most recent WHO classification and account for <4% of all cases of acute leukemia [1]. They include leukemias with no lineage specific antigens (acute undifferentiated leukemias) and those with blasts that express antigens of more than one lineage to such degree that it is not possible to assign the leukemia to any one particular lineage with certainty (mixed phenotype acute leukemias). The latter can either be leukemias with two distinct populations of blasts, each expressing antigens of a different lineage (historically referred to as "bilineal" leukemias) or a single blast population expressing antigens of multiple lineages (historically referred to as "biphenotypic" acute leukemias) [2]. Acute leukemias of ambiguous lineage may harbor a variety of genetic lesions. Those with t(9;22)(q34;q11) or translocations associated with mixed lineage leukemias (MLL) gene, i.e., t(11;V)(q23;V), occur frequently enough and are associated with distinct features, that are considered as separate entities according to the recent WHO classification. Co-expression of myeloid and B-lymphoid antigens is most common in mixed phenotype acute leukemia (MPAL), followed by co-expression of myeloid and T-lymphoid antigens, accounting for 66–70% and 23–24% of MLLs, respectively. Co-expression of B- and T-lineage associated antigens or antigens of all three lineages is exceedingly rare, accounting for <5% of MLLs [3,4]. The requirements for assigning more than one lineage to a single blast population has been established by current WHO classification [1].

Chromosomal rearrangements of mixed lineage leukemias (MLL) gene on chromosomal segment 11q23 occur in a subset of leukemias with poor prognosis and are seen in pediatric, adult and therapy-related acute leukemias [5]. *MLL* translocations can be found in de novo AML and lymphoid lineages (ALL), secondary myelodysplastic syndrome, as well as mixed phenotype acute leukemias [6–8]. Among them, mixed phenotypic acute leukemia has a high incidence of chromosomal abnormalities involving MLL gene [2,3]. Wild-type MLL is a transcriptional regulatory factor involved in the maintenance of clustered homeobox (Hox) gene expression, particularly during hematopoietic development [9]. *MLL* translocations generate an in-frame fusion protein, in which the N-terminal portion of the MLL protein is fused to the C-terminal region of a partner protein, resulting in a chimeric protein with oncogenic properties [10]. Leukemias that harbor *MLL* translocations are a unique biologic subgroup that co-express myeloid and lymphoid associated genes and an overall gene expression profile that characterize those of the precursor cells [11,12]. To date, more than 51 partner genes have been characterized at the molecular level and an additional 35 genetic loci have been identified by cytogenetic analysis [13]. The most frequent translocations, t(4;11)(q21;23) involving *MLLT2* (*AF4*), t(9;11)(p22;23) involving *MLLT3* (*AF9*), and t(11;19)(q23;p13.3) involving *MLLT1* (*ENL*) account for 80% of investigated leukemia samples [13]. The characteristic of the fusion partner seems to determine a bias for generation of myeloid versus lymphoid or mixed lineage type leukemia. While *MLL-MLLT3* and *MLL-MLLT2* are predominantly associated with a myeloid or a lymphoid/mixed lineage phenotype, *MLL-MLLT1* occurs equally likely in both leukemia subclasses [14]. To our knowledge however, B/T-lymphoid MLL harboring t(11;19)(q23;p13.3) has not been reported.

Case Report

A previously healthy 37-year-old male, presented to a local primary care physician with flu-like symptoms. A complete blood count (CBC) was within normal limits, with a normal differential count, at initial visit. He was treated conservatively without relief of his symptoms. A CBC, repeated 1 month later revealed a white blood cell count 10.6×10^9/L, including 3% circulating blasts, hemoglobin 8.6 g/dL, and a platelet count of 179×10^9/L. His absolute neutrophil count was 0.4×10^6/L. With an uncertain diagnosis, the patient was referred to our institution for further consulta-

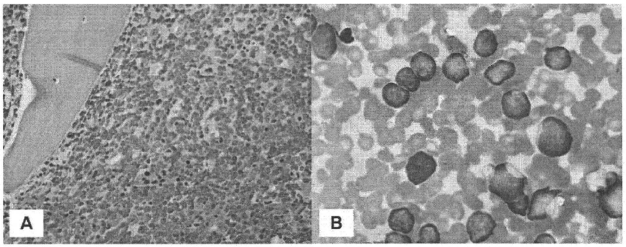

Figure 1. Bone marrow (BM) biopsy and aspirate. (A) Trephine core biopsy showing a hypercellular marrow with diffuse replacement by immature precursors/blasts, comprising over 90% of the marrow cellularity (H&E stain, 400×). (B) Bone marrow aspirate smear showing variably sized blasts with high nuclear to cytoplasmic ratio, immature lacy chromatin, visible nucleoli and scant cytoplasm without obvious vacuoles or granules (H&E stain, 1,000×).

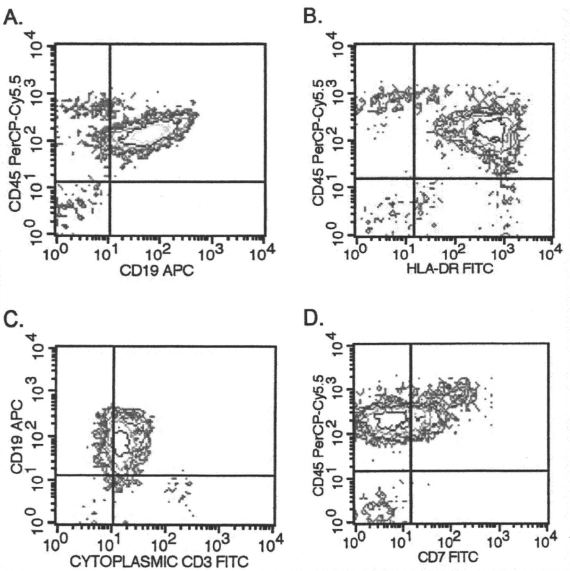

Figure 2. Immunophenotype of blasts as revealed by flow cytometry. (A–D) Positive for CD19 (93%), HLA-DR (95%), cytoplasmic CD3 (84%), and partial CD7 (29%).

tion and management. Several days later, a CBC revealed a white blood cell count of 31.3 × 10^9/L with 95% circulating blasts, hemoglobin 8.1 g/dL, and a platelet count of 67 × 10^9/L. A bone marrow aspirate and biopsy was performed and the samples were submitted for morphologic, cytochemical, flow cytometric, immunohistochemical, cytogenetic, and molecular analysis.

A diagnosis of mixed phenotype acute leukemia B/T-lymphoid was established. He was started on an intensive chemotherapy regimen as for ALL, receiving three cycles of induction chemotherapy with hyper-Cytoxan, Vincristin, Doxorubicin (Adriamycin), Dexamethasone (CVAD) arm A and arm B. His post-treatment course was complicated by nausea, vomiting, fever in an immunocompromised patient, a left axillary abscess and nadir in blood counts. He subsequently recovered and a follow up bone marrow biopsy and aspirate revealed no evidence of leukemia, 6 weeks later. He was placed on maintenance chemotherapy with 6MP and central nervous system prophylaxis, which he tolerated well. Four months later, a repeat bone marrow biopsy revealed a slightly hypocellular marrow with essentially trilieage hematopoiesis and in complete morphologic and cytomolecular remission. He then received high-dose chemotherapeutic conditioning with busulfan, fludarabine, and antithymocyte globulin followed by an eight of 10 mismatched allogeneic hematopoietic stem cell transplantation. He is in stable condition and transfusion/growth factor independent, +150 days post-transplant.

Materials and Methods

Histomorphology, immunohistochemistry, and flow cytometry. Standard Wright-Giemsa stained blood film and bone marrow smears were prepared for morphologic examination. The bone marrow histology was assessed by examining Hematoxylin-eosin stained sections. Immunohistochemical staining on paraffin-embedded bone marrow sections included CD3, CD7, CD10, CD20, CD34, CD79a, myeloperoxidase (MPO), Pax-5, and TdT (Dako, Carpeinteria, CA) were carried out according to the optimized protocols at Moffitt Cancer Center immunohistochemistry laboratory.