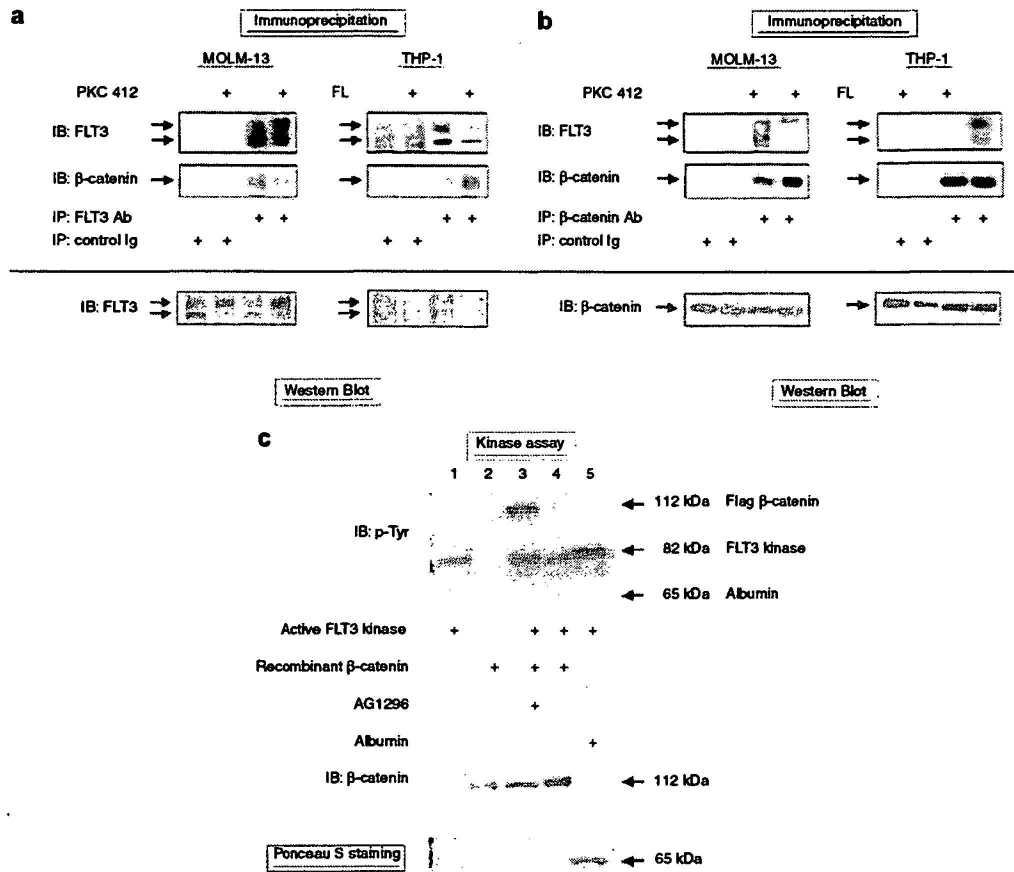

Figure 6 β-Catenin can be directly phosphorylated by FLT3. (a) Physical interaction between FLT3 and β-catenin. (a, b) MOLM-13 and THP-1 cells were treated with or without PKC412 or FL, respectively. After cell lysis, 1 mg of total protein was immunoprecipitated with an antibody recognizing either FLT3 (a) or β-catenin (b). To demonstrate specificity of the reaction, an equal amount of protein lysate was immunoprecipitated with control IgG. After SDS-PAGE and electrotransfer, blots were probed with antibodies to both FLT3 and β-catenin as described. The level of β-catenin and FLT3 protein in whole-cell lysates is shown in the lower panel. (c) *In vitro* kinase assay. Recombinant β-catenin was incubated with recombinant active FLT3 in an *in vitro* kinase reaction buffer containing either active FLT3 kinase alone, or active FLT3 kinase and AG1296. To demonstrate the specificity of the reaction, albumin was substituted for β-catenin. After the reaction, proteins were resolved by SDS-PAGE and immunoblotted for phosphotyrosine (top panel). The membrane was stripped and reprobed with anti-β-catenin antibody (middle panel). The membrane was stained with Ponceau S to identify the location of albumin (bottom panel).

nucleus and the cytoplasm, its retention in one location or the other appears to be mediated by association with other proteins.[42] Tyrosine-phosphorylated β-catenin preferentially associates with the BCL9 homolog BCL9-2, which promotes its nuclear retention and association with TCF,[43] thereby stimulating TCF-driven transcriptional activity.[25,33,38,40] Although it has been reported that several tyrosine kinases phosphorylate β-catenin *in vivo* or *in vitro* (for example, Src kinase,[44] ErbB2[33] and oncogenic mutants of RON and MET[26,45]), there has been no investigation of FLT3 in this regard, nor of other tyrosine kinases in hematologic malignancies.

In the current study, we investigated the relationship between FLT3 activation and the tyrosine phosphorylation status of β-catenin. Tyrosine phosphorylation of β-catenin is markedly increased in activated mutant FLT3 transfectants, FLT3-ITD-32D and FLT3-TKD-32D, compared to the FLT3-WT transfectant FLT3-WT-32D. Moreover, although the β-catenin expression level was lower in the FLT3-ITD human leukemia cell line MOLM-13 than in the FLT3-WT leukemia cell line THP-1, β-catenin was highly tyrosine-phosphorylated in MOLM-13 but not in THP-1. Immunoprecipitation assays showed that endogenous activated FLT3 binds endogenous β-catenin, and *in vitro* kinase assays showed that recombinant active FLT3 kinase can phosphorylate tyrosine residues of β-catenin directly. FLT3-dependent β-catenin tyrosine phosphorylation was confirmed in cells by using pharmacologic FLT3 inhibitors, FLT3 siRNA and the FLT3 activator FL. In MOLM-13 AML cells, treatment with FLT3 inhibitor suppressed both FLT3 and β-catenin tyrosine phosphorylation, concomitant with decreased FLT3 and β-catenin association. Dependence of β-catenin tyrosine phosphorylation on FLT3 was confirmed when FLT3 expression was silenced with siRNA. In contrast, treatment of THP-1 cells with FL increased phosphorylation of FLT3 and β-catenin, and increased the association of FLT3 and β-catenin. β-catenin was located primarily in the nucleus in FLT3-ITD AML cells, while it was located mainly in the cytoplasm in FLT3-WT AML cells. Nuclear localization of β-catenin was decreased by PKC412 and FLT3 siRNA treatment and was increased by FL treatment. Real-time RT-PCR confirmed that nuclear localization of β-catenin was correlated with its enhanced transcriptional activity. Furthermore, the *ex vivo* data obtained from FLT3-ITD-positive AML patient bone marrow mononuclear cells

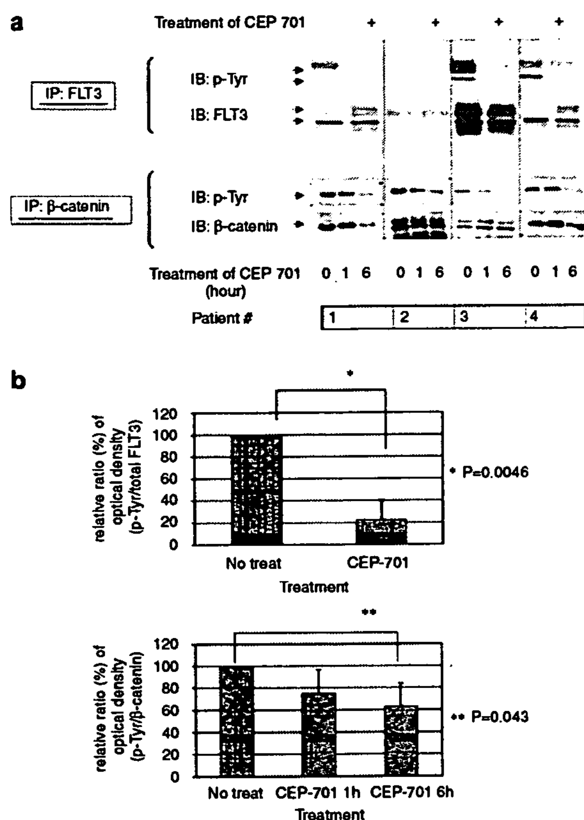

Figure 7 Tyrosine phosphorylation of β-catenin in bone marrow mononuclear cells of FLT3-ITD-positive AML patients is suppressed following FLT3 inhibition. (**a**) A total of 2×10^7 cells from patients with FLT3-ITD-positive AML were incubated with or without CEP-701 (50 nM) for the times indicated and then subjected to immunoprecipitation and western blotting as described in the legend to Figure 1. (**b**) optical density ratios of phosphotyrosine/FLT3 and phosphotyrosine/β-catenin were obtained using a GS-800 densitometer with Quantity One software, and statistical analysis was performed using StatView software.

support the potential clinical relevance of FLT3-dependent β-catenin tyrosine phosphorylation.

Although FLT3 activates PI3K[3,4] and signaling via PI3K/AKT stabilizes β-catenin protein level through inhibition of GSK3β,[22,23,38] use of a GSK3β inhibitor revealed that FLT3-dependent regulation of tyrosine phosphorylation of β-catenin is not mediated by FLT3 activation of the PI3K/AKT axis. These data suggest that two mechanisms might exist to regulate β-catenin in FLT3-AML cells: (1) β-catenin protein stabilization via PI3K/AKT and (2) β-catenin tyrosine phosphorylation mediated directly by FLT3 kinase. Taken together, our data strongly suggest that tyrosine phosphorylation of β-catenin by FLT3 is one of the principal mechanisms by which FLT3 activates β-catenin signaling, and that enhanced nuclear β-catenin signaling promotes malignant transformation associated with activating FLT3 mutations.

Recently, deregulated nuclear β-catenin signaling has been reported in a wide range of hematologic malignancies. It is therefore of interest that over 40 chromosomal translocations leading to deregulation of at least 12 protein tyrosine kinases have been reported in hematologic malignancies, including, for example, the deregulated tyrosine kinase activities associated with the oncogenic fusion proteins BCR/ABL and NPM/ALK.[46] Indeed, BCR/ABL has been shown to promote the tyrosine phosphorylation and enhanced transcriptional activity of β-catenin in chronic myelogenous leukemia, and β-catenin silencing inhibited proliferation of these cells.[47] Further, the efficacy of disrupting the β-catenin/TCF transcriptional complex as a therapeutic approach in treating multiple myeloma has very recently been reported.[48] Thus, enhanced β-catenin nuclear retention and signaling may be a shared downstream event among various hematologic malignancies and β-catenin is suggested to be a significant therapeutic target in these neoplasms.

Acknowledgements

We thank Dr Kazutaka Ozeki, Nagoya University School of Medicine, for his help in preparing MOLM-13 cells and stably transfected 32D cell lines. This work was supported by the Intramural Research Program of the National Institutes of Health, National Cancer Institute, Center for Cancer Research. Support was also provided to TK by the Sumitomo Life Social Welfare Sciences Foundation. *Statement of authorship*: TK, LN, MJL, JBT designed research , TK, EJC, MJL, SL, AS performed research, TK wrote the paper, and HK, MJL, TN contributed vital new reagents.

References

1 Lyman SD, James L, Vanden Bos T, de Vries P, Brasel K, Gliniak B *et al.* Molecular cloning of a ligand for the flt3/flk-2 tyrosine kinase receptor: a proliferative factor for primitive hematopoietic cells. *Cell* 1993; **75**: 1157–1167.
2 Hannum C, Culpepper J, Campbell D, McClanahan T, Zurawski S, Bazan JF *et al.* Ligand for FLT3/FLK2 receptor tyrosine kinase regulates growth of haematopoietic stem cells and is encoded by variant RNAs. *Nature* 1994; **368**: 643–648.
3 Lavagna-Sevenier C, Marchetto S, Birnbaum D, Rosnet O. FLT3 signaling in hematopoietic cells involves CBL, SHC and an unknown P115 as prominent tyrosine-phosphorylated substrates. *Leukemia* 1998; **12**: 301–310.
4 Zhang S, Broxmeyer HE. Flt3 ligand induces tyrosine phosphorylation of gab1 and gab2 and their association with shp-2, grb2, and PI3 kinase. *Biochem Biophys Res Commun* 2000; **277**: 195–199.
5 Nakao M, Yokota S, Iwai T, Kaneko H, Horiike S, Kashima K *et al.* Internal tandem duplication of the flt3 gene found in acute myeloid leukemia. *Leukemia* 1996; **10**: 1911–1918.
6 Yokota S, Kiyoi H, Nakao M, Iwai T, Misawa S, Okuda T *et al.* Internal tandem duplication of the FLT3 gene is preferentially seen in acute myeloid leukemia and myelodysplastic syndrome among various hematological malignancies. A study on a large series of patients and cell lines. *Leukemia* 1997; **11**: 1605–1609.
7 Meshinchi S, Woods WG, Stirewalt DL, Sweetser DA, Buckley JD, Tjoa TK *et al.* Prevalence and prognostic significance of Flt3 internal tandem duplication in pediatric acute myeloid leukemia. *Blood* 2001; **97**: 89–94.
8 Schnittger S, Schoch C, Dugas M, Kern W, Staib P, Wuchter C *et al.* Analysis of FLT3 length mutations in 1003 patients with acute myeloid leukemia: correlation to cytogenetics, FAB subtype, and prognosis in the AMLCG study and usefulness as a marker for the detection of minimal residual disease. *Blood* 2002; **100**: 59–66.
9 Thiede C, Steudel C, Mohr B, Schaich M, Schakel U, Platzbecker U *et al.* Analysis of FLT3-activating mutations in 979 patients with acute myelogenous leukemia: association with FAB subtypes and identification of subgroups with poor prognosis. *Blood* 2002; **99**: 4326–4335.
10 Yamamoto Y, Kiyoi H, Nakano Y, Suzuki R, Kodera Y, Miyawaki S *et al.* Activating mutation of D835 within the activation loop of FLT3 in human hematologic malignancies. *Blood* 2001; **97**: 2434–2439.

11 Hayakawa F, Towatari M, Kiyoi H, Tanimoto M, Kitamura T, Saito H et al. Tandem-duplicated Flt3 constitutively activates STAT5 and MAP kinase and introduces autonomous cell growth in IL-3-dependent cell lines. Oncogene 2000; 19: 624–631.
12 Kiyoi H, Towatari M, Yokota S, Hamaguchi M, Ohno R, Saito H et al. Internal tandem duplication of the FLT3 gene is a novel modality of elongation mutation which causes constitutive activation of the product. Leukemia 1998; 12: 1333–1337.
13 Kiyoi H, Ohno R, Ueda R, Saito H, Naoe T. Mechanism of constitutive activation of FLT3 with internal tandem duplication in the juxtamembrane domain. Oncogene 2002; 21: 2555–2563.
14 Kiyoi H, Naoe T, Nakano Y, Yokota S, Minami S, Miyawaki S et al. Prognostic implication of FLT3 and N-RAS gene mutations in acute myeloid leukemia. Blood 1999; 93: 3074–3080.
15 Nakano Y, Kiyoi H, Miyawaki S, Asou N, Ohno R, Saito H et al. Molecular evolution of acute myeloid leukaemia in relapse: unstable-and FLT3 genes compared with p53 gene. Br J Haematol 1999; 104: 659–664.
16 Ozawa M, Baribault H, Kemler R. The cytoplasmic domain of the cell adhesion molecule uvomorulin associates with three independent proteins structurally related in different species. EMBO J 1989; 8: 1711–1717.
17 Peifer M, McCrea PD, Green KJ, Wieschaus E, Gumbiner BM. The vertebrate adhesive junction proteins beta-catenin and plakoglobin and the Drosophila segment polarity gene armadillo form a multigene family with similar properties. J Cell Biol 1992; 118: 681–691.
18 Shtutman M, Zhurinsky J, Simcha I, Albanese C, D'Amico M, Pestell R et al. The cyclin D1 gene is a target of the beta-catenin/LEF-1 pathway. Proc Natl Acad Sci USA 1999; 96: 5522–5527.
19 Behrens J, von Kries JP, Kuhl M, Bruhn L, Wedlich D, Grosschedl R et al. Functional interaction of beta-catenin with the transcription factor LEF-1. Nature 1996; 382: 638–642.
20 Bienz M, Clevers H. Linking colorectal cancer to Wnt signaling. Cell 2000; 103: 311–320.
21 Barker N, Clevers H. Catenins, Wnt signaling and cancer. Bioessays 2000; 22: 961–965.
22 Easwaran V, Song V, Polakis P, Byers S. The ubiquitin-proteasome pathway and serine kinase activity modulate adenomatous polyposis coli protein-mediated regulation of beta-catenin-lymphocyte enhancer-binding factor signaling. J Biol Chem 1999; 274: 16641–16645.
23 Aberle H, Bauer A, Stappert J, Kispert A, Kemler R. beta-catenin is a target for the ubiquitin-proteasome pathway. EMBO J 1997; 16: 3797–3804.
24 Tetsu O, McCormick F. Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. Nature 1999; 398: 422–426.
25 Piedra J, Martinez D, Castano J, Miravet S, Dunach M, de Herreros AG. Regulation of beta-catenin structure and activity by tyrosine phosphorylation. J Biol Chem 2001; 276: 20436–20443.
26 Danilkovitch-Miagkova A, Miagkov A, Skeel A, Nakaigawa N, Zbar B, Leonard EJ. Oncogenic mutants of RON and MET receptor tyrosine kinases cause activation of the beta-catenin pathway. Mol Cell Biol 2001; 21: 5857–5868.
27 McWhirter JR, Neuteboom ST, Wancewicz EV, Monia BP, Downing JR, Murre C. Oncogenic homeodomain transcription factor E2A-Pbx1 activates a novel WNT gene in pre-B acute lymphoblastoid leukemia. Proc Natl Acad Sci USA 1999; 96: 11464–11469.
28 Chung EJ, Hwang SG, Nguyen P, Lee S, Kim JS, Kim JW et al. Regulation of leukemic cell adhesion, proliferation, and survival by beta-catenin. Blood 2002; 100: 982–990.
29 Derksen PW, Tjin E, Meijer HP, Klok MD, MacGillavry HD, van Oers MH et al. Illegitimate WNT signaling promotes proliferation of multiple myeloma cells. Proc Natl Acad Sci USA 2004; 101: 6122–6127.
30 Lu D, Zhao Y, Tawatao R, Cottam HB, Sen M, Leoni LM et al. Activation of the Wnt signaling pathway in chronic lymphocytic leukemia. Proc Natl Acad Sci USA 2004; 101: 3118–3123.
31 Simon M, Grandage VL, Linch DC, Khwaja A. Constitutive activation of the Wnt/beta-catenin signalling pathway in acute myeloid leukaemia. Oncogene 2005; 24: 2410–2420.
32 Tickenbrock L, Schwable J, Wiedehage M, Steffen B, Sargin B, Choudhary C et al. Flt3 tandem duplication mutations cooperate with Wnt signaling in leukemic signal transduction. Blood 2005; 105: 3699–3706.
33 Bonvini P, An WG, Rosolen A, Nguyen P, Trepel J, Garcia de Herreros A et al. Geldanamycin abrogates ErbB2 association with proteasome-resistant beta-catenin in melanoma cells, increases beta-catenin-E-cadherin association, and decreases beta-catenin-sensitive transcription. Cancer Res 2001; 61: 1671–1677.
34 Ilan N, Tucker A, Madri JA. Vascular endothelial growth factor expression, beta-catenin tyrosine phosphorylation, and endothelial proliferative behavior: a pathway for transformation? Lab Invest 2003; 83: 1105–1115.
35 Zhao M, Kiyoi H, Yamamoto Y, Ito M, Towatari M, Omura S et al. In vivo treatment of mutant FLT3-transformed murine leukemia with a tyrosine kinase inhibitor. Leukemia 2000; 14: 374–378.
36 Grundler R, Thiede C, Miething C, Steudel C, Peschel C, Duyster J. Sensitivity toward tyrosine kinase inhibitors varies between different activating mutations of the FLT3 receptor. Blood 2003; 102: 646–651.
37 Stone RM, DeAngelo DJ, Klimek V, Galinsky I, Estey E, Nimer SD et al. Patients with acute myeloid leukemia and an activating mutation in FLT3 respond to a small-molecule FLT3 tyrosine kinase inhibitor, PKC412. Blood 2005; 105: 54–60.
38 Harris TJ, Peifer M. Decisions, decisions: beta-catenin chooses between adhesion and transcription. Trends Cell Biol 2005; 15: 234–237.
39 Polakis P. Wnt signaling and cancer. Genes Dev 2000; 14: 1837–1851.
40 Aberle H, Schwartz H, Hoschuetzky H, Kemler R. Single amino acid substitutions in proteins of the armadillo gene family abolish their binding to alpha-catenin. J Biol Chem 1996; 271: 1520–1526.
41 Aono S, Nakagawa S, Reynolds AB, Takeichi M. p120(ctn) acts as an inhibitory regulator of cadherin function in colon carcinoma cells. J Cell Biol 1999; 145: 551–562.
42 Krieghoff E, Behrens J, Mayr B. Nucleo-cytoplasmic distribution of \{beta\}-catenin is regulated by retention. J Cell Sci 2006; 119: 1453–1463.
43 Brembeck FH, Schwarz-Romond T, Bakkers J, Wilhelm S, Hammerschmidt M, Birchmeier W. Essential role of BCL9-2 in the switch between beta-catenin's adhesive and transcriptional functions. Genes Dev 2004; 18: 2225–2230.
44 Roura S, Miravet S, Piedra J, Garcia de Herreros A, Dunach M. Regulation of E-cadherin/catenin association by tyrosine phosphorylation. J Biol Chem 1999; 274: 36734–36740.
45 Zinser GM, Leonis MA, Toney K, Pathrose P, Thobe M, Kader SA et al. Mammary-specific Ron receptor overexpression induces highly metastatic mammary tumors associated with beta-catenin activation. Cancer Res 2006; 66: 11967–11974.
46 Chalandon Y, Schwaller J. Targeting mutated protein tyrosine kinases and their signaling pathways in hematologic malignancies. Haematologica 2005; 90: 949–968.
47 Coluccia AM, Vacca A, Dunach M, Mologni L, Redaelli S, Bustos VH, Benati D, Pinna LA, Gambacorti-Passerini C. Bcr-Abl stabilizes beta-catenin in chronic myeloid leukemia through its tyrosine phosphorylation. EMBO J 2007; 26: 1456–1466.
48 Sukhdeo K, Mani M, Zhang Y, Dutta J, Yasui H, Rooney MD et al. Targeting the beta-catenin/TCF transcriptional complex in the treatment of multiple myeloma. Proc Natl Acad Sci USA 2007; 104: 7516–7521.

achieved morphologic and cytogenetic remission after 2 months of treatment with lenalidomide therapy and a second patient who achieved a platelet response after only 1 month of therapy before receiving an unrelated allogeneic stem cell transplant. Both patients remain without any evidence of relapse with a maximum follow-up of 8 months.

Both radiation therapy and traditional DNA-interactive antineoplastics, such as alkylating agents and topoisomerase II inhibitors, are known genotoxins with the potential to induce MDS or acute myeloid leukemia (AML) that commonly harbors a chromosome 5q deletion with high frequency of evolution to AML and short overall survival.[4–6] Our findings indicate that lenalidomide has therapeutic potential in patients with secondary MDS with complex karyotype accompanied by chromosome 5q deletion.

M Melchert[1], C Williams[2] and A List[1]
[1]*Malignant Hematology, Moffitt Cancer Center and Research Institute, Magnolia Drive, Tampa, FL, USA and*
[2]*Kansas City Blood and Marrow Transplant Program, Suite, Kansas, MO, USA*
E-mail: melcheme@moffitt.usf.edu

References

1 Shali W, Helias C, Fohrer C, Struskei S, Gervais C, Falkenrodt A *et al.* Cytogenetic studies of a series of 43 consecutive secondary myelodysplastic syndromes/acute myeloid leukemias: conventional cytogenetics, FISH, and multiplex FISH. *Cancer Genet Cytogenet* 2006; **168**: 133–145.
2 List AF, Kurtin S, Roe D, Buresh A, Mahadevan D, Fuchs D *et al.* Efficacy of lenalidomide in myelodypslastic syndromes. *N Engl J Med* 2005; **352**: 549–557.
3 List AF, Dewald G, Bennett J, Giagounidis A, Raza A, Feldman E *et al.* Lenalidomide in the myelodysplastic syndrome with chromosome 5q deletion. *N Eng J Med* 2006; **355**: 1456–1465.
4 Pedersen-Bjergaard J, Andersen MK, Chrstiansen DH. Therapy-related acute myeloid leukemia and myelodysplasia after high-dose chemotherapy and autologous stem cell transplantation. *Blood* 2000; **95**: 3273–3279.
5 Darrington DL, Vose JM, Anderson JR, Bierman PJ, Bishop MR, Chan WC *et al.* Incidence and characterization of secondary myelodysplastic syndrome and acute myelogenous leukemia following high-dose chemoradiotherapy and autologous stem-cell transplantation for lymphoid malignancies. *J Clin Oncol* 1994; **12**: 2527–2534.
6 Hake CR, Graubert TA, Fenske TS. Does Autologus transplantation directly increase the risk of secondary leukemia in lymphoma patients? *Bone Marrow Transplant* 2006 [E-pub ahead of print].

Recurrent chromosomal aberration at 12q15 in chronic idiopathic myelofibrosis with or without JAK2^{V617F} mutation

Leukemia (2007) **21,** 1578–1580; doi:10.1038/sj.leu.2404700; published online 19 April 2007

Mutation of JAK2^{V617F} is currently known to play a potential role in the development of chronic myeloproliferative disorders (CMPD);[1] 78% (393/506) of polycythemia vera (PV) cases have JAK2^{V617F}, while only 43% (55/127) of reported chronic idiopathic myelofibrosis (CIMF) cases have JAK2^{V617F}.[2] Thus the question naturally arises whether the CIMF patients without JAK2^{V617F} might have another pathway towards myelofibrosis or a common pathogenic factor may exist with or without the

Table 1 JAK2-V617F status and cytogenetic results at the time of myelofibrosis

Case no.	*Age at diagnosis/sex*	*Cytogenetics at the time of myelofibrosis*	*JAK2 V617F*
Idiopathic myelofibrosis			
JAK2_0048	41/male	46,XY,**t(1;12)(p34;q15)**[10]	G/G
JAK2_0039	56/male	46,XY,del(11)(q13)[18]/46,XY[3]	G/G
JAK2_0057	78/female	46,XX[21]	G/G
JAK2_0098	67/male	46,XY,del(20)(q11)[5]/46,XY[3]	G/G
JAK2 0112	33/male	46,XY[23]	G/G
JAK2_0163	63/female	46,XX,**t(12;20)(q15;q11)**[7]/47,XX,+9[10]	G/T
JAK2_0036	54/male	46,XY,del(20)(q11)[2]/ 46,XY,idem,t(2;17)(q24;q22)[13]/ 46,XY,idem,i(17q)[5]	G/T
JAK2_0105	70/male	46,XY,add(9)(p21)[16]	G/T
JAK2_0148	56/male	46,XY[21]	T/T
Myelofibrosis with prior history of myelodysplastic syndrome			
JAK2_0021	70/male	46,XY,**t(4;12)(q27;q15)**[22][a]	G/T
Polycythemia vera developing myelofibrosis			
JAK2_0042	55/female	46,XX,del(7)(q22)[9]/45,X,add(X)(p22),−18[6]/ 46,XX[5]	G/T
JAK2_0061	60/female	46,XX,tan(1q12−1qter)[8]/46,XX[1]	G/T
JAK2_0065	43/female	43,XX,−1,−3,−7,−9,−10,−12,−13, −16,+5m[13]/46,XX[8]	T/T
JAK2_0118	46/female	46,XX[20]	T/T
JAK2_0141	62/male	46,XY[20]	T/T

Table 1 *Continued*

Case no.	*Age at diagnosis/sex*	*Cytogenetics at the time of myelofibrosis*	*JAK2 V617F*
Essential thrombocythemia developing myelofibrosis			
JAK2_0013	58/female	46,XX[13]	G/G
JAK2_0035[b]	49/male	46,XY,t(2;5)(p16;q14),add(11)(q23)[23]	G/G
JAK2_0005[b]	57/male	46,XY,+1,der(1;7)(q10;p10),del(20)(q11)[20]/ 46,XY,idem,add(18)(p11)[2]/46,XY[4]	G/T
JAK2_0034	67/male	46,XY,i(7q)[6]/48,XY,+8,+21[9]/46,XY[6]	G/T
JAK2_0054[b]	59/male	46,XY,+1,der(1;7)(q10;p10)[9]/46,XY[3]	G/T
JAK2_0055	76/male	46,XY,add(18)(p11)[20]	G/T
JAK2_0158	46/female	46,XX[17]	G/T

[a]This case was reported as myelodysplastic syndrome developing myelofibrosis with a 6-year interval;[5] however, the continuity of the disease is uncertain.[3]
[b]JAK2_0035 was UPN-12, JAK2_0005 was UPN-6 and JAK2_0054 was UPN-8 in Hsiao *et al.*[6]
Bold signifies chromosomal translocations involving 12 of 15 region.

presence of JAK2^{V617F}. During the series of mutational assay of JAK2^{V617F} in CMPD using the sequence-specific primer-single molecule fluorescence detection assay,[3] we studied cytogenetic changes in nine patients with CIMF, 51 with essential thrombocythemia (ET) and 34 with PV. Myelofibrosis developed in seven of 51 patients (13.7%) with ET and five of 34 patients (14.7%) with PV, and we compared clinical and cytogenetic changes between patients with CIMF and those with myelofibrosis developing in PV/ET.

The group of patients with myelofibrosis associated with PV/ET had high incidences of history of thrombosis (4/12 versus 0/9; $P=0.0542$), requirement of cytoreductive chemotherapy (12/12 versus 5/9; $P=0.0103$) and acute leukemia development (7/12 versus 1/9; $P=0.0274$) compared with the CIMF group. Depending on the status of JAK2^{V617F}, the group of patients with myelofibrosis associated with PV/ET with GT/TT mutation of JAK2 had a high incidence of chemotherapy requirement (10/10 versus 2/4; $P=0.0157$) and tended to have a frequent thrombosis history (4/10 versus 0/4; $P=0.1345$) (Supplementary Table 1). However, there was no particular difference in the percentage of abnormal karyotypes at the time of myelofibrosis according to CIMF diagnosis or the mutational status of JAK2^{V617F}. We also noted a high frequency of myelofibrosis development in patients with JAK2^{V617F} in PV (wild-type JAK2/heterozygous JAK2^{V617F}/homozygous JAK2^{V617F}: 0/9 versus 2/18 versus 3/7: $P=0.0460$), but not in ET (2/18 versus 5/30 versus 0/1: $P=0.7970$), in agreement with the report by Kralovics *et al.*[4]

In myelofibrosis patients, we noticed that two of the nine patients with CIMF had chromosome abnormalities at the 12q15 region; one had t(1;12)(p34;q15), while the other had t(12;20)(q15;q11?) (Table 1 and Figure 1). Another patient (JAK2_0021) reported as showing t(4;12)(q31;q21) had a prior history of myelodysplastic syndrome (MDS)-refractory anemia with a normal karyotype 6 years before myelofibrosis.[5] Re-assessment by the spectral karyotypic analysis revealed that this anomaly was t(4;12)(q27;q15) (Supplementary Figure 1). In contrast to the results of CIMF, no patients with myelofibrosis developing from PV/ET had 12q15 anomaly. Of the 12 patients with myelofibrosis associated with PV/ET, four had −7/7q−, including two with der(1;7)(q10;p10); both patients with der(1;7)(q10;p10) had a prior ET diagnosis[6] with heterozygous JAK2^{V617F} (Table 1). Of the nine patients with CIMF and one myelofibrosis associated with MDS, four had heterozygous and

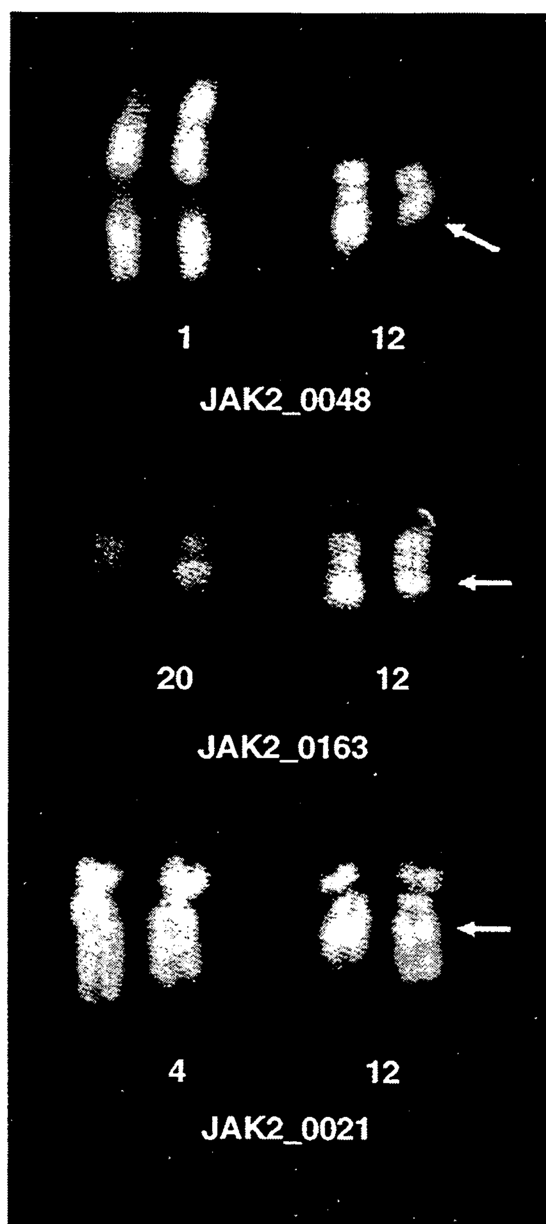

Figure 1 Partial quinacrine-banded karyotypes obtained from two patients with chronic idiopathic myelofibrosis (JAK2_0048 and JAK2_0163) and one patient with myelofibrosis with prior history of myelodysplastic syndrome (JAK2_0021) showing 12q15 anomalies, that is, t(1;12)(p34;q15), t(12;20)(q15;q11?) and t(4;12)(q27;q15). Arrows indicate possible breakpoint of 12q15.

one had homozygous JAK2^{V617F}; the 12q15 anomaly was detected in one patient with wild-type JAK2, while two had heterozygous JAK2^{V617F}.

Cytogenetic changes in CIMF are well documented: +8, del(20q), −7/7q−, del(11q) and del(13q) are known to be recurring nonspecific cytogenetic abnormalities, and some of them are also detectable in PV or ET patients. In the literature, Andrieux *et al.*[7] reported a possible role in the association between *HMGA2* and translocation involving 12q15 in CIMF. In the current study, we found that 12q15 anomaly does not depend on the JAK2 mutational status; thus genetic anomaly, independent to JAK2^{V617F}, may exist in CIMF, and molecular study on the 12q15 region, including *HMGA2*,[8] may disclose another pathogenetic pathway in CIMF. The 12q15 chromosomal abnormality was recurrently detected in patients with CIMF, while der(1;7)(q10;p10) was only noted in ET patients who had myelofibrosis with JAK2^{V617F}.[6] These findings clearly indicate that myelofibrosis among CMPD might be cytogenetically heterogeneous.

Acknowledgements

We thank Professor J Patrick Barron for his review of this manuscript. This work was supported in part by the 'High-Tech Research Center' Project from the Ministry of Education, Culture, Sports and Technology (MEXT) and by the 'University-Industry Joint Research Project' from MEXT.

K Ohyashiki[1], T Tauchi[1], M Kuroda[2], A Kodama[3] and JH Ohyashiki[4]

[1]First Department of Internal Medicine (Hematology Division), Tokyo Medical University, Tokyo, Japan;
[2]Department of Pathology, Tokyo Medical University, Tokyo, Japan;
[3]Laboratory Medicine (Chromosome Unit), Tokyo Medical University, Tokyo, Japan and
[4]Intractable Immune System Diseases Research Center, Tokyo Medical University, Tokyo, Japan
E-mail: ohyashik@rr.iij4u.or.jp

References

1 De Keermaecker K, Cools J. Chronic myeloproliferative disorders: a tyrosine kinase tale. *Leukemia* 2006; **20**: 200–205.
2 Schafer AI. Molecular basis of the diagnosis and treatment of polycythemia vera and essential thrombocythemia. *Blood* 2006; **107**: 4214–4222.
3 Ohyashiki K, Aota Y, Akahane D, Gotoh A, Miyazawa K, Kimura Y *et al.* The JAK2 V617F tyrosine kinase mutation in myelodysplastic syndromes (MDS) developing myelofibrosis indicates the myeloproliferative nature in a subset of MDS patients. *Leukemia* 2005; **19**: 2359–2360.
4 Kralovics R, Passamonti F, Buser AS, Teo SS, Tiedt R, Passweg JR *et al.* A gain-of-function mutation of JAK2 in myeloproliferative disorders. *N Engl J Med* 2005; **352**: 1779–1790.
5 Nunoda K, Sashida G, Ohyashiki K, Kodama A, Fukutake K. The translocation (4;12)(q31;q21) in myelofibrosis associated with myelodysplastic syndrome: impact of the 12q21 breakpoint. *Cancer Genet Cytogenet* 2006; **164**: 90–91.
6 Hsiao HH, Ito Y, Sashida G, Ohyashiki JH, Ohyashiki K. *De novo* appearance of der(1;7)(q10;p10) is associated with leukemic transformation and unfavorable prognosis in essential thrombocythemia. *Leukemia Res* 2005; **29**: 1247–1252.
7 Andrieux J, Demory JI, Dupriez B, Quief S, Plantier I, Roumier C *et al.* Dysregulation and overexpression of HMGA2 in myelofibrosis with myeloid metaplasia. *Genes Chromosomes Cancer* 2004; **39**: 82–87.
8 Odero MD, Grand FH, Iqbal S, Ross F, Roman JP, Vizmanos JL *et al.* Disruption and aberrant expression of *HMGA2* as a consequence of diverse chromosomal translocations in myeloid malignancies. *Leukemia* 2005; **19**: 245–252.

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

Neighboring adipocytes participate in the bone marrow microenvironment of multiple myeloma cells

Leukemia (2007) **21**, 1580–1584; doi:10.1038/sj.leu.2404658; published online 22 March 2007

Multiple myeloma (MM) is a hematological malignancy, characterized by the accumulation of monoclonal plasma cells in the bone marrow (BM). MM disease progression has been recently recognized as the result of an evolving crosstalk between different cell types within the BM. Although genetically abnormal plasma cells define the tumor compartment itself, the surrounding and interwoven stroma provides the supporting framework of the tumor. This framework includes extracellular matrix proteins, secreted growth factors and cellular interactions with fibroblasts, macrophages, endothelial cells, osteoblasts and osteoclasts.[1] Little attention has been given to another cell type present in the BM cavity: namely the adipocyte. These are absent in the BM of a new-born individual; however, their number increases with advancing age, resulting in adipocytic deposits occupying up to 70% of the BM cavity in elderly persons. MM is typically a disease of the elderly with a median age of diagnosis of 65 years and the incidence increases with age. Knowing that with advancing age, the BM cavity is filled with adipocytes and that MM cells closely interact with their neighboring cells, we assumed functional interactions between BM adipocytes and MM cells. We studied these interactions using the 5T33MM model and the human MM5.1 cell line. In this study, we further tried to characterize the secreted cytokines and explored the potential role of leptin in mediating the effects of adipocytes. We finally evaluated the expression of leptin receptor on both murine and human MM cells and tried to correlate this with different clinical parameters.

From the observation that MM cells, at interstitial disease stages, can be found in close contact with adipocytes, functional interactions between these cells are reasonable and prompted us to start *in vitro* tests. The murine BM adipocytic cell line 14F1.1 (obtained from Professor Zipori D, Department of Molecular Cell Biology, The Weizmann Institute of Science, Rehovot, Israel) and primary isolated human adipocytes were used. The 14F1.1 cells initially have a

International Journal of
HEMATOLOGY

The Prophylactic Effect of Itraconazole Capsules and Fluconazole Capsules for Systemic Fungal Infections in Patients with Acute Myeloid Leukemia and Myelodysplastic Syndromes: A Japanese Multicenter Randomized, Controlled Study

Yoshikazu Ito,[a] Kazuma Ohyashiki,[a] Isao Yoshida,[b] Makoto Takeuchi,[b] Yasutaka Aoyama,[c] Atsuko Mugitani,[c] Yasuhiro Matsuura,[d] Hisashi Wakita,[d] Mitsuhiro Matsuda,[e] Erina Sakamoto,[f] Toru Kiguchi,[g] Akio Urabe,[h] Kazuo Tamura,[i] Akihisa Kanamaru,[e] Toru Masaoka,[j]
The Japan Febrile Neutropenia Study Group

[a]First Department of Internal Medicine (Hematology Division), Tokyo Medical University, Tokyo, Japan; [b]Department of Internal Medicine, National Hospital Organization Minami-Okayama Medical Center, Okayama, Japan; [c]Department of Hematology, Fuchu Hospital, Izumi, Japan; [d]Department of Internal Medicine, Narita Red Cross Hospital, Narita, Japan; [e]Department of Hematology, Kinki University School of Medicine, Osaka-Sayama, Japan; [f]Department of Clinical Hematology and Clinical Diagnostics, Graduate School of Medicine, Osaka City University, Osaka, Japan; [g]Department of Internal Medicine, Kagawa Rosai Hospital, Marugame, Japan; [h]Division of Hematology, NTT Kanto Medical Center, Tokyo, Japan; [i]First Department of Internal Medicine, School of Medicine, Fukuoka University, Fukuoka, Japan; [j]Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka, Japan

Received March 31, 2006; received in revised form October 14, 2006; accepted November 9, 2006

Abstract

We performed a randomized, controlled study comparing the prophylactic effects of capsule forms of fluconazole (n = 110) and itraconazole (n = 108) in patients with acute myeloid leukemia (AML) or myelodysplastic syndromes (MDS) during and after chemotherapy. There were 4 cases with possible systemic fungal infection in the itraconazole group, and there were 8 possible and 3 probable cases in the fluconazole group. Adverse events did not significantly differ in the 2 groups. In patients with MDS or in the remission-induction phase of chemotherapy, the numbers of cases with probable or possible infections were lower in the itraconazole group than in the fluconazole group, whereas no difference was seen in patients with AML or in the consolidation phase of therapy. In patients with neutrophil counts of $<0.1 \times 10^9$/L lasting for more than 4 weeks, the frequency of infection in the fluconazole group (5 of 9 patients) was significantly higher than in the itraconazole group (0 of 7 patients; $P = .03$). Our results suggest that both drugs were well tolerated in patients with AML or MDS who received chemotherapy and that the efficacy of itraconazole for prophylaxis against systemic fungal disease is not inferior to that of fluconazole.
Int J Hematol. 2007;85:121-127. doi: 10.1532/IJH97.06079
© 2007 The Japanese Society of Hematology

Key words: Acute myeloid leukemia; Myelodysplastic syndromes; Chemotherapy; Fungal infection; Prophylaxis

1. Introduction

Systemic fungal infection is the major cause of death in patients with severe neutropenia. Prophylaxis of systemic fungal infections during neutropenia after anticancer chemotherapy has been studied for many years. A previous meta-analysis showed that antifungal prophylaxis resulted in significant reductions in fungal infection–related mortality and invasive fungal infection compared with the control group [1]. In patients undergoing hematopoietic stem cell transplantation or with prolonged neutropenia, overall mortality was improved by antifungal prophylaxis [1].

Fluconazole is widely used to prevent systemic fungal infection during the treatment of hematologic malignancies [2]. Fluconazole is not fully effective against *Candida* other than *C albicans* and is not effective against *Aspergillus*, whereas itraconazole reportedly exhibits activity against

Correspondence and reprint requests: Yoshikazu Ito, MD, First Department of Internal Medicine, Tokyo Medical University, 6-7-1-Nishishinjuku, Shinjuku-ku, Tokyo 160-0023, Japan; 81-3-3342-6111 ext 5895; fax: 81-3-5381-6651 (e-mail: yito@tokyo-med.ac.jp).

these fungi [3,4]. Another meta-analysis showed a significant reduction in invasive fungal infection by the prophylactic use of itraconazole [5]. A recent meta-analysis of multiple randomized trials of fluconazole and itraconazole failed to demonstrate any significant difference in mortality or the frequency of invasive fungal infection [6]. An additional randomized study comparing itraconazole and fluconazole may be needed.

In Japan, no randomized multicenter study comparing itraconazole and fluconazole has been published. It is also important to determine whether there is any difference between Japan and Western countries in the effects of antifungal drugs, because the colonizing fungi may be different. Although the oral-solution form of itraconazole has been recommended for prophylactic use to prevent systemic fungal infection [5], only the capsule form is available in Japan; however, the capsule form of itraconazole has an inherent problem with aberrant absorption through the intestine [7]. The prophylactic effect of the capsule form must also be studied. We tried to perform a randomized, controlled study to investigate the noninferiority of itraconazole capsules versus fluconazole capsules for systemic fungal infection prophylaxis in patients receiving intensive chemotherapy for acute myeloid leukemia (AML) or high-risk myelodysplastic syndromes (MDS).

2. Patients and Methods

2.1. Patients

Each institution was required to obtain approval by the local institutional review board for the treatment protocol and the consent forms. Adult patients with AML or MDS who were to receive conventional chemotherapy as a remission-induction or consolidation therapy were eligible for the study, because their neutrophil counts were expected to fall to less than 1.0×10^9/L. Both untreated and relapsed patients were enrolled in the study. The use of laminar airflow was not included in the eligibility criteria on the assumption that the patients in each group would be treated the same in a single institution. Considering the interaction of other concomitantly used drugs with itraconazole or fluconazole, patients taking triazolam, pimozide, quinidine sulfate, simvastatin, azelnidipine, ergotamine, or dihydroergotamine were excluded. Pregnant women, patients with hepatic dysfunction, and those with a history of sensitive reaction to itraconazole or fluconazole were also excluded. Informed consent was obtained in writing prior to enrollment.

2.2. Study Protocol

The eligibility of patients was evaluated in each institution. The diagnosis, according to French-American-British classification criteria and the type of chemotherapy, was described in the registration form that was sent to the registration center by fax when a course of chemotherapy was to be administered. Patients were randomized by computer to receive either itraconazole or fluconazole. Reregistration and individual rerandomization were permitted for patients who had not developed systemic fungal infection in the previous course of chemotherapy.

To prevent Gram-negative bacterial infection during and after chemotherapy, we administered 300 mg levofloxacin (100 mg, 3 times a day) orally to all patients. Each group was orally administered 200 mg of itraconazole capsules (once a day) or 200 mg of fluconazole capsules (once a day). Although serum itraconazole or fluconazole concentrations were not measured in this study, the itraconazole dosage was based on a previous study in Japan, which suggested that 200 mg a day was more effective than 100 mg [8]. In cases of febrile neutropenia during or after chemotherapy, antibacterial treatment was given according to the Japanese guidelines for treating febrile neutropenia [9]. All prophylactic drugs were started together with chemotherapy and were continued until the improvement of neutrophil counts to 1×10^9/L, the improvement of leukocyte counts to 2×10^9/L, or the replacement of antifungal drugs. Other antifungal agents were prohibited during the prophylactic period. Oral administration of the prophylactic antifungal drugs in the study was stopped when patients required intravenous antifungal therapy. Vinca alkaloids were avoided because of their interactive effect with itraconazole. Patients who wished to discontinue the study treatment could do so at any time.

A previous nationwide survey in Japan showed that most patients received a 200-mg dose of fluconazole, the maximum dose permitted in Japan [10]. Every week we monitored symptoms, signs, and such laboratory data as the leukocyte count, neutrophil count, and serum C-reactive protein concentration. Serum β-D-glucan, now widely used in Japan as a diagnostic marker of fungal infection, was also measured at least every month. Conducting additional frequent measurements was also encouraged at each institution. Serum galactomannan was measured if invasive aspergillosis was suspected. Furthermore, bacteriologic culturing was performed when necessary. In addition to the monitoring of subjective and objective symptoms, a radiologic examination, including chest computed tomography scanning, was performed when fungal infection was suspected.

The frequency of systemic fungal infection in the 2 groups was the primary end point of this study. Fluconazole prophylaxis was expected to cause fungal infection in 2.8% to 7% of patients, based on data reported in previous studies [11,12]. Thus, the number of patients to be registered was calculated to obtain a result similar to these 2 studies [11,12] with a power of 0.6, considering the general difficulty in recruiting participants into a prospective study in Japan.

Our diagnostic criteria for systemic fungal infection were based on the criteria of the European Organisation for Research and Treatment of Cancer (EORTC) [13], and the guidelines of the Japanese Deep-Seated Mycosis Guidelines Editorial Committee were used [14]. We divided patients into 3 groups with respect to systemic fungal infection (ie, proven, probable, and possible fungal infection). Proven infection was diagnosed when fungus was identified pathologically or microbiologically and its presence was accompanied by symptoms compatible with systemic fungal infection. Probable infection was defined as findings

Table 1.
Characteristics of Patients*

	Itraconazole Group	Fluconazole Group
Cases enrolled, n	108	110
Evaluable	103	106
Not evaluable	5	4
Age, y†	58 (16-80)	53 (16-80)
Male/female sex, n	57/46	64/42
Therapy, n		
Induction	41	44
Consolidation	62	62
AML, n	88	93
M0	4	1
M1	13	16
M2	36	41
M3	8	14
M4	11	4
M5	14	15
M6	2	1
Unclassifiable	0	1
MDS, n	15	13
RAEB	4	2
RAEBt	1	0
CMML	1	2
Post-MDS AML	9	9
Body temperature, °C†	36.7 (35.3-39.4)	36.7 (35.7-38.0)
Neutrophil count, $\times 10^9$/L†	1.580 (0-26.576)	1.591 (0-13.832)
Leukocyte count, $\times 10^9$/L†	3.445 (0.360-91.200)	3.250 (0.600-64.200)

*AML indicates acute myeloid leukemia; MDS, myelodysplastic syndrome; RAEB, refractory anemia with excess of blasts; RAEBt, RAEB in transformation; CMML, chronic myelomonocytic leukemia.
†Data are presented as the median (range).

compatible with systemic fungal infection according to computed tomography or radiography results and accompanied by positive results of mycologic examinations. Intraocular candidiasis was also considered as probable infection according to the criteria of the Japanese guidelines. Possible infection was diagnosed in cases with at least 1 positive finding in a computed tomographic, radiographic, or serologic examination that suggested a systemic fungal infection. The diagnosis of systemic fungal infection was made at each institution. Because the EORTC criteria are heavily dependent on the suspicion and positive action of responsible doctors and on the activity of institutions, additional evaluation of the diagnostic process was conducted in each case at the headquarters of our study group, in an effort to reduce interinstitution bias.

2.3. Statistical Analysis

Data were statistically analyzed with the χ^2 test, the Fisher exact probability test, or the Student t test, and a P value of less than .05 was considered to indicate statistical significance. Data from initial and repeated prophylactic treatments were analyzed separately, because the primary end point was set to be a breakthrough for systemic fungal infection.

3. Results

A total of 218 episodes were recorded in the 142 patients from 33 institutions throughout Japan enrolled between June 2003 and March 2005 (Table 1). Patients were randomized into either itraconazole (108 episodes) or fluconazole (110 episodes) groups. Nine episodes (5 in the itraconazole group and 4 in the fluconazole group) were not evaluable because of the inappropriate administration of antifungal drugs. Among the remaining 209 episodes (103 in the itraconazole and 106 in the fluconazole group), the median age was 58 years (range, 16-80 years) in the itraconazole group and 53 years (range, 16-80 years) in the fluconazole group. Background characteristics, including subcategories of leukemia or MDS, types of therapy, and the male-female ratio were similar in the 2 groups. Median body temperatures and neutrophil and leukocyte counts were also similar in the 2 groups. Among the evaluable patients, 64 (62.1%) of 103 episodes in the itraconazole group developed febrile neutropenia, compared with 73 (68.9%) of 106 episodes in the fluconazole group (Table 2). In 21 (20.4%) of 103 episodes in the itraconazole group and 20 (18.9%) of 106 episodes in the fluconazole group, intravenous antifungal drugs were empirically used instead of discontinuing the prophylactic use of oral antifungals. According to our diagnostic criteria, 4 possible and no probable cases of systemic fungal infection were noted in the itraconazole group, and 8 possible and 3 probable cases were seen in the fluconazole group (Table 3). There were no cases of proven systemic fungal infection in either group.

We also analyzed serologic test results for the presence of fungi (Table 4). Serum β-D-glucan was measured in 59 episodes in the itraconazole group and in 70 episodes in the fluconazole group. Among these episodes, 4 patients in the itraconazole group and 2 in the fluconazole group were positive for β-D-glucan before the start of chemotherapy. In 4 episodes in the fluconazole group, the serum β-D-glucan concentration increased from undetectable to the positive range, whereas no increase was found in the itraconazole group. Serum galactomannan was also tested in 6 episodes in the itraconazole group and 11 episodes in the fluconazole

Table 2.
Infection-Related Events during and after Chemotherapy*

	Itraconazole, n	Fluconazole, n	P†
Systemic fungal infection	4	11	.12
Proven	0	0	
Probable	0	3	.26
Possible	4	8	
Febrile neutropenia	64	73	.31
Empiric use of intravenous antifungals	21	20	.78
Micafungin	18	18	
Fluconazole	0	1	
Fosfluconazole	2	0	
Amphotericin B	1	1	

*Systemic fungal infection was diagnosed with the criteria described in the text. The difference between the 2 groups was not statistically significant at any stage.
†χ^2 test.

Table 3.
Details of 3 Probable Cases of Systemic Fungal Infection in the Fluconazole Group*

Age, y	Sex	Diagnosis	Chemotherapy	Fungal Infection
73	M	AML: M2	Induction	Invasive pulmonary aspergillosis
56	F	AML: M2	Induction	Invasive pulmonary aspergillosis
50	M	AML: M2	Consolidation	Intraocular candidiasis

*AML indicates acute myeloid leukemia.

group. With a cutoff of 0.5, 4 patients with 4 episodes in the fluconazole group tested positive, compared with only 1 patient (1 episode) in the itraconazole group.

Adverse events are shown in Table 5. Four episodes in the itraconazole group and 2 in the fluconazole group were associated with adverse events. Among them, discontinuation of the study was necessary following 4 episodes in 4 patients of the itraconazole group and 1 episode in the fluconazole group. In the itraconazole group, 3 episodes of erythema and 1 of liver dysfunction were seen, and 1 episode with renal dysfunction and 1 with gingivitis were noted in the fluconazole group.

Possible risk factors for systemic fungal infection were established to divide patients into subgroups (Table 6). Risks associated with induction therapy are usually higher than those of consolidation therapy. In patients receiving remission-induction therapy, probable and possible systemic fungal infections were found in 2 (4.9%) of 41 episodes in the itraconazole group, and 7 (15.9%) of 44 episodes were found in the fluconazole group. The numbers of patients who received consolidation therapy were similar in the 2 groups. In patients with MDS, a susceptibility to infection or a delay of recovery from bone marrow suppression is occasionally seen after chemotherapy. MDS is considered to involve a greater risk of developing severe infection compared with de novo AML. Among patients with MDS, there was no episode (0%) of probable or possible systemic fungal infection among 15 episodes in the itraconazole group, whereas 3 episodes (23.1%) of possible infection were noted among 13 episodes in the fluconazole group. In patients with AML, no difference between the 2 groups in the development fungal disease was found.

Sustained neutropenia is also a risk factor for invasive fungal infection. We analyzed the frequency of systemic fungal infection, including probable and possible cases, for 2 subgroups of patients with neutrophil counts of less than 0.5×10^9/L and less than 0.1×10^9/L (Table 7). These subgroups were further subclassified by the duration of neutropenia in each patient. In patients with neutrophil counts of less than 0.1×10^9/L lasting more than 4 weeks, the frequency of systemic fungal infection in the fluconazole group (5 of 9 patients) was significantly higher than in the itraconazole group (0 of 7 patients; $P = .03$). In other subgroups, the frequency of systemic fungal infection was relatively higher in the fluconazole group compared with itraconazole group, but the differences were not statistically significant.

4. Discussion

In patients with hematologic malignancies, neutropenia is commonly observed after chemotherapy. The most common cause of fever during neutropenia is considered to be infection, although the rate of positive bacterial cultures is low. Klastersky proposed the concept of febrile neutropenia, a persistent fever of unknown origin but strongly suggesting infectious disease during neutropenia [15]. The empiric use of antibiotics is recommended in cases with febrile neutropenia before the results of microbiological studies are obtained. Bacteria are a major pathogen in such cases, but fungi should also be considered when febrile neutropenia persists after the administration of antibacterial agents. Once systemic fungal infection has occurred during neutropenia,

Table 4.
Results of Serologic Examination*

	Itraconazole, n	Fluconazole, n	P†
β-D-glucan			
Tested	59	70	
Initially positive	4	2	
Evaluable	55	68	
Increased to positive range	0 (0%)	4 (5.9%)	.19
Galactomannan			
Tested	6	11	
Increased to positive range			
1.5 or greater	0	1	
1.0 or greater	0	2	
0.5 or greater	1	4	.77

*Cutoff values provided by each manufacturer were used to determine the positivity of serum β-D-glucan tests. Galactomannan values are expressed as the absorbance of the patient serum sample divided by the average of the absorbances of the control samples from the assay kit. Differences between the 2 groups were not statistically significant at any stage.
†χ^2 test.

Table 5.
Adverse Events*

	Itraconazole, n	Fluconazole, n	P†
Evaluable cases	103	106	
Adverse events	4	2	.65
Discontinuation of the study	4	1	.35
Erythema	3	0	
Liver dysfunction	1	0	
Renal dysfunction	0	1	
Gingivitis	0	1	

*Events of grade 2 or higher were described.
†χ^2 test.

Table 6.
Numbers of Patients in Each Subcategory*

	Itraconazole, n	Fluconazole, n	P†
Induction therapy	41	44	
Systemic fungal infection	2 (4.9%)	7 (15.9%)	.19
Probable	0	2	
Possible	2	5	
Consolidation therapy	62	62	
Systemic fungal infection	2 (3.2%)	4 (6.5%)	.68
Probable	0	1	
Possible	2	3	
AML	88	93	
Systemic fungal infection	4 (4.5%)	8 (8.6%)	.43
Probable	0	3	
Possible	4	5	
MDS	15	13	
Systemic fungal infection	0 (0%)	3 (23.1%)	.17
Probable	0	0	
Possible	0	3	

*AML indicates acute myeloid leukemia; MDS, myelodysplastic syndrome.
†χ^2 test.

it is difficult to achieve success with antifungal therapy. Systemic fungal infection in hematologic malignancies is generally linked to a poor prognosis; therefore, the empiric use of antifungal agents is also necessary in neutropenic patients when fever persists despite the use of antibiotics [16]. In addition to empiric therapy for neutropenic patients with persistent fever, the prophylactic use of antifungal drugs during and after chemotherapy has been studied [1,2,5,6].

The routine use of antifungal prophylaxis for all neutropenic patients is currently controversial [1,2]. A meta-analysis by Bow et al showed that antifungal prophylaxis is effective for reducing the frequency of invasive or superficial fungal infection and fungal infection–related mortality [1]. However, the overall mortality did not improve significantly. In a subset analysis, patients with prolonged neutropenia or who had undergone allogeneic stem cell transplantation showed a significant decrease in overall mortality after receiving antifungal drugs, compared with patients who received no prophylactic treatment with antifungals [1]. Among antifungal drugs, fluconazole has been widely used as prophylaxis and is significantly effective in improving overall mortality for patients who undergo allogeneic stem cell transplantation. Another meta-analysis was performed by Kanda et al to clarify the prophylactic effect of fluconazole [2]. They reported that it decreased the overall mortality in patients with a risk of greater than 15% for developing systemic fungal infection, whereas no significant effect was seen in low-risk patients [2]. This result suggests that antifungal prophylaxis with fluconazole should be considered for patients with a high risk for fungal infection.

Fluconazole is very active against *C albicans*, but some non-*albicans Candida* species and *Aspergillus* are resistant to fluconazole. These fungal infections are not rare in patients with hematologic disease. Itraconazole has been reported to inhibit the growth of non-*albicans Candida* species and *Aspergillus* in vitro [3,4]. Itraconazole was also reportedly used for prophylaxis in patients with hematologic malignancies [5]. A meta-analysis showed a significant reduction in the frequency of systemic fungal infection with prophylactic itraconazole use compared with the control [5]. The effect of itraconazole on overall mortality is still unclear. Some studies have been performed to evaluate the difference in prophylactic effect between itraconazole and fluconazole [17-21]. A recent meta-analysis comparing itraconazole and fluconazole showed no significant difference in mortality but did demonstrate a significant increase in adverse effects in the itraconazole group [6]. In the analysis, only 5 randomized studies were included, and the superiority of itraconazole was also questioned after reanalysis of that study [22,23]. Although the prophylactic use of itraconazole is expected to be equal to or more effective than fluconazole, there are not enough data so far to support this expectation.

What category of patients should receive antifungal prophylaxis is still controversial. In patients with hematologic malignancies, the immune system condition apart from neutropenia is quite complicated. Many factors that compromise immunocompetence have to be considered in patients who undergo allo-

Table 7.
Numbers of Patients in Subcategories According to Length of Neutropenia Periods*

	Itraconazole, n		Fluconazole, n		
	SFI Cases	Total Cases	SFI Cases	Total Cases	P
Neutrophils <0.5 × 10^9/L, n					
Any period	4	101	11	104	.13†
≥2 wk	4	74	10	77	.19†
≥3 wk	3	51	7	45	.22†
≥4 wk	1	24	6	26	.10‡
Neutrophils <0.1 × 10^9/L, n					
Any period	3	70	9	75	.17†
≥2 wk	2	47	7	43	.12†
≥3 wk	1	17	6	22	.11‡
≥4 wk	0	7	5	9	.03‡

*SFI indicates systemic fungal infection.
†χ^2 test.
‡Fisher exact probability test.

geneic stem cell transplantation. Hypogammaglobulinemia in B-cell malignancies and poor cellular immunity in T-cell tumors are problematic. Cellular dysfunction has also been reported in patients with MDS [24]. The intensity of chemotherapy varies among leukemias and lymphomas. Remission-induction chemotherapy for leukemias is one risk factor for fungal infection, compared with other conditions in which chemotherapy is employed [25]. Mucosal damage induced by chemotherapy is another important factor in the occurrence of invasive mycotic infection. Corticosteroid use also compromises the immune system. Many other possible factors for invasive fungal infection have been discussed [26]. We have to clarify the indications for the prophylactic use of antifungals, because the routine use of drugs increases the risk of resistance to multiple antifungal drugs, and their high costs and risks of adverse events must also be considered.

In studies to verify prophylactic effects, enrolling heterogeneous categories of patients sometimes leads to a misinterpretation such that the routine use of prophylactic drugs is encouraged. Such a misinterpretation was avoided in this study by enrolling patients with AML or MDS with the expectation that similar types of chemotherapy may be given. To make the study group as uniform as possible, we did not enroll patients who were to undergo allogeneic stem cell transplantation. We also excluded lymphoid malignancies, considering the interaction between itraconazole and the vinca alkaloids that are often used in combination chemotherapy for such diseases [27]. Drug-drug interaction increases the rate and degree of adverse events that lead to early termination of the treatment or, conversely, decreases the effect by lowering the blood levels of the drug. Although overall mortality is a useful primary end point for a prophylaxis study, the patients in the present study were expected to have a low mortality. Because a relatively high frequency of adverse events has been reported with itraconazole, we were afraid that many patients would discontinue participation in the study because of adverse events if we included high-risk patients. Including large numbers of patients who discontinue drugs makes the interpretation of a study difficult. Taking all of these background aspects together, we decided to use the success rate of prophylaxis as a primary end point in this study.

In this study, we administered 200 mg/day of itraconazole or 200 mg/day of fluconazole. The dosages of both drugs were set according to domestic regulations. A previous report indicated that 400 mg of fluconazole was appropriate for high-risk patients, such as those undergoing allogeneic stem cell transplantation [2]. Significant effects have also reportedly been obtained with itraconazole when a dose of 400 mg was given orally as a solution [5]. It is still unclear, however, what dosage is appropriate for patients with hematologic disease but not undergoing stem cell transplantation. A retrospective multicenter analysis in Japan reported that the majority of domestic institutions were using 200 mg of fluconazole or itraconazole for prophylaxis [10]. The plasma concentration of itraconazole has been reported to reach an effective level in more than 50% of the patients who receive the 200-mg capsule [28].

This study was primarily designed to establish the noninferiority of itraconazole capsules versus fluconazole capsules. Fluconazole was reported to have a prophylactic effect in patients undergoing allogeneic stem cell transplantation. According to the retrospective analysis of data from Japanese patients, antifungal prophylactic therapy is widely administered during and after chemotherapy [10]. The analysis recommended that antifungal prophylaxis be administered in patients with prolonged neutropenia [1]; predicting the length of neutropenia was a challenge in the design of this prospective study. Although there is currently limited evidence to support the use of fluconazole during or after chemotherapy, it is the only standard antifungal drug available for this use.

Another challenge in designing a reliable study protocol was the difficulty of recruiting suitable participants in Japan; however, we recognized the importance of conducting a randomized, multicenter study in Japan, because no such study had previously been conducted in Japan in this field. Thus, this study was designed to verify, with a statistical power of 0.6, results from previous studies that reported the efficacy of fluconazole prophylaxis for reducing fungal-infection frequency [11,12].

Itraconazole is known to be liposoluble and to have a high protein-binding affinity. There are significant problems with capsule formulation because absorption of the drug is highly variable [29]. Despite having a wider range of antifungal activity than fluconazole, the serum concentration of itraconazole is estimated to be low in capsule formulation. Antifungal prophylaxis is thought to be accomplished mainly by eradication of *Candida* species colonized in the intestinal tract. The degree of eradication of colonized fungus with itraconazole capsules is not clear.

The prophylactic effect on aspergillosis depends on the absorption of antifungal drugs. Previous reports suggested that a higher dose of itraconazole is necessary to prevent pulmonary aspergillosis [5]. In the present study, the frequency of systemic fungal infection was relatively low, but despite using a low dose, we obtained results that suggested that prophylaxis with the itraconazole capsule was effective in higher-risk patients, such as those with severe neutropenia for more than 4 weeks.

It is of importance to distinguish patients who need a higher dose from those requiring less extensive prophylactic therapy. In addition, the appropriate dose for Japanese patients has to be clarified. The present study suggested that itraconazole had a prophylactic effect for systemic fungal infection at least equal to that of fluconazole in patients with AML or MDS who plan to undergo systemic chemotherapy. Both drugs were well tolerated at a dosage of 200 mg. Further study is warranted to clarify the relative efficacy of prophylactic itraconazole compared with fluconazole and to establish better management of fungal infection during treatment of hematologic malignancies.

Acknowledgments

We thank all physicians and data managers involved in this trial. The authors are indebted to Prof. J. Patrick Barron of the International Medical Communication Center of Tokyo Medical University for his review of this manuscript.

Appendix

Participating staff and institutions: Hirofumi Hasegawa (Kinki University); Masayuki Hino (Osaka City University);

Manabu Hirai (Osaka City General Hospital); Yasunori Ueda (Kurashiki Central Hospital); Kazuyuki Shigeno, Kazunori Ohnishi (Hamamatsu University School of Medicine); Hiromichi Iwasaki (University of Fukui Faculty of Medical Sciences); Tetsuya Goto, Keiji Ozaki (Tokushima Red Cross Hospital); Kimiko Iijima (NTT Kanto Medical Center); Chikara Sakai (Chiba Cancer Center); Koichi Miyamura (Japanese Red Cross Nagoya First Hospital); Tomomi Toubai, Masahiro Imamura (Hokkaido University); Yasunori Nakagawa, Kenshi Suzuki (Japanese Red Cross Medical Center); Nobu Akiyama (Tokyo Metropolitan Bokutoh Hospital); Takaki Shimada, Fumi Mizorogi (Jikei University School of Medicine); Hideho Wada (Kawasaki Medical School); Hidetsugu Mihara (Aichi Medical University); Nobuhiko Uoshima (Matsushita Memorial Hospital); Masarou Tashima (Takatsuki Red Cross Hospital); Masafumi Taniwaki (Kyoto Prefectural University of Medicine); Akira Miyata (Chugoku Central Hospital); Makoto Hirokawa, Kenichi Sawada (Akita University); Kentaro Watanabe (Tokyo Saiseikai Central Hospital); Hiroyuki Sugawara, Yuzuru Kanakura (Osaka University); Toshiharu Tamaki (Izumisano Municipal Hospital); Mahito Misawa, Hiroshi Hara (Hyogo College of Medicine); Hiromi Karasawa (Kanazawa Medical University); Hiroatsu Agou (Shimane Prefectural Central Hospital).

References

1. Bow EJ, Laverdière M, Lussier N, Rotstein C, Cheang MS, Ioannou S. Antifungal prophylaxis for severely neutropenic chemotherapy recipients. *Cancer*. 2002;94:3230-3246.
2. Kanda Y, Yamamoto R, Chizuka A, et al. Prophylactic action of oral fluconazole against fungal infection in neutropenic patients. British Society for Antimicrobial Chemotherapy Working Party. *Cancer*. 2000;89:1611-1625.
3. Shivji A, Noble MA, Bernstein M. Laboratory monitoring of antifungal chemotherapy. British Society for Antimicrobial Chemotherapy Working Party. *Lancet*. 1991;337:1577-1580.
4. Johnson EM, Szekely A, Warnock DW. In-vitro activity of voriconazole, itraconazole and amphotericin B against filamentous fungi. *J Antimicrob Chemother*. 1998;42:741-745.
5. Glasmacher A, Prentice A, Gorschlüter M, et al. Itraconazole prevents invasive fungal infections in neutropenic patients treated for hematologic malignancies: evidence from a meta-analysis of 3,597 patients. *J Clin Oncol*. 2003;21:4615-4626.
6. Vardakas KZ, Michalopoulos A, Falagas ME. Fluconazole versus itraconazole for antifungal prophylaxis in neutropenic patients with haematological malignancies: a meta-analysis of randomised-controlled trials. *Br J Haematol*. 2005;131:22-28.
7. Boogaerts MA, Verhoef GE, Zachee P, Demuynck H, Verbist L, de Beule K. Antifungal prophylaxis with itraconazole in prolonged neutropenia: correlation with plasma levels. *Mycoses*. 1989;32:103-108.
8. Toubai T, Tanaka J, Fujisawa F, Kondo Y, Imamura M. Effect of prophylaxis against mycosis in patients with hematological malignancy disease: efficacy of dosage of itraconazole [in Japanese]. *Jpn J Antibiot*. 2003;56:61-65.
9. Tamura K, Imajo K, Akiyama N, et al. Randomized trial of cefepime monotherapy or cefepime in combination with amikacin as empirical therapy for febrile neutropenia. *Clin Infect Dis*. 2004;39:S15-S24.
10. Yoshida M, Akiyama N, Takahashi M, et al. Management of infecti is complications in patients with acute leukemia during chemotherapy: a questionnaire analysis by the Japan Adult Leukemia Study Group [in Japanese]. *Jpn J Chemother*. 2003;51:703-710.
11. Goodman JL, Winston DJ, Greenfield RA, et al. A controlled trial of fluconazole to prevent fungal infection in patients undergoing bone marrow transplantation. *N Engl J Med*. 1992; 326:845-851.
12. Slavin MA, Osborne B, Adams R, et al. Efficacy and safety of fluconazole prophylaxis for fungal infections after marrow transplantation: a prospective randomized doubleblind study. *J Infect Dis*. 1995;171:1545-1552.
13. Ascioglu S, Rex JH, de Pauw B, et al. Defining opportunistic invasive fungal infections in immunocompromised patients with cancer and hematopoietic stem cell transplants: an international consensus. *Clin Infect Dis*. 2002;34:7-14.
14. Deep-seated Mycosis Guidelines Editorial Committee. *Guidelines for the Diagnosis and the Treatment of Deep-Seated Mycosis* [in Japanese]. Tokyo, Japan: Ishiyaku Publishers; 2003.
15. Klastersky J. Febrile neutropenia. *Curr Opin Oncol*. 1993;5:625-632.
16. Klastersky J. Anti-fungal therapy in patients with fever and neutropenia: more rational and less empirical? *N Engl J Med*. 2004;351:1445-1447.
17. Annaloro C, Oriana A, Tagliaferri E, et al. Efficacy of different prophylactic antifungal regimens in bone marrow transplantation. *Haematologica*. 1995;80:512-517.
18. Morgenstern GR, Prentice AG, Prentice HG, Ropner JE, Schey SA, Warnock DW, on behalf of the U.K. Multicentre Antifungal Prophylaxis Study Group. A randomized controlled trial of itraconazole versus fluconazole for the prevention of fungal infections in patients with haematological malignancies. *Br J Haematol*. 1999;105:901-911.
19. Hüijgens PG, Simoons-Smit AM, van Loenen AC, et al. Fluconazole versus itraconazole for the prevention of fungal infections in haemato-oncology. *J Clin Pathol*. 1999;52:376-380.
20. Winston DJ, Maziarz RT, Chandrasekar PH, et al. Intravenous and oral itraconazole versus intravenous and oral fluconazole for long-term anti-fungal prophylaxis in allogeneic hematopoietic stem-cell transplant recipients. *Ann Intern Med*. 2003; 138:705-713.
21. Marr KA, Crippa F, Leisenring W, et al. Itraconazole versus fluconazole for prevention of fungal infections in patients receiving allogeneic stem cell transplants. *Blood*. 2004;103:1527-1533.
22. Falagas ME, Vardakas KZ, Michalopoulos A. In meta-analysis itraconazole is superior to fluconazole for prophylaxis of systemic fungal infection in the treatment of haematological malignancies: response to Prentice et al. *Br J Haematol*. 2006; 132:658-659.
23. Prentice AG, Glasmacher A, Djulbegvic B. In meta-analysis itraconazole is superior to fluconazole for prophylaxis of systemic fungal infection in the treatment of haematological malignancies. *Br J Haematol*. 2006;132:656-658.
24. Yamaguchi N, Ito Y, Ohyashiki K. Increased intracellular activity of matrix metalloproteinases in neutrophils may be associated with delayed healing of infection without neutropenia in myelodysplastic syndromes. *Ann Hematol*. 2005;84:383-388.
25. Mühlemann K, Wenger C, Zenhäesern R, Täuber MG. Risk factors for invasive aspergillosis in neutropenic patients with hematologic malignancies. *Leukemia*. 2005;19:545-550.
26. Maschmeyer G, Ruhnke M. Update on antifungal treatment of invasive *Candida* and *Aspergillus* infections. *Mycoses*. 2003; 47:263-276.
27. Kamaluddin M, McNally P, Breatnach F, et al. Potentiation of vincristine toxicity by itraconazole in children with lymphoid malignancies. *Acta Paediatr*. 2001;90:1204-1207.
28. Kageyama S, Masuya M, Tanaka I, et al. Plasma concentration of itraconazole and prophylactic efficacy in patients with neutropenia after chemotherapy for acute leukemia. *J Infect Chemother* 1999;5:213-216.
29. Prentice AG, Glasmacher A. Making sense of itraconazole pharmacokinetics. *J Antimicrob Chemother*. 2005;56(suppl 1):i17-i22.

ELSEVIER

Cancer Genetics and Cytogenetics 179 (2007) 19–24

CANCER GENETICS AND CYTOGENETICS

Automated JAK2^{V617F} quantification using a magnetic filtration system and sequence-specific primer-single molecule fluorescence detection

Kazuma Ohyashiki[a], Kunio Hori[b], Tohru Makino[b], Junko H. Ohyashiki[c,*]

[a]First Department of Internal Medicine, Tokyo Medical University, 6-7-1 Nishi-shinjuku, Shinjuku-ku, Tokyo 169-0023, Japan
[b]NovusGene, 2-3 Kuboyama-cho, Hachioji-shi, Tokyo 192-8512, Japan
[c]Intractable Immune System Diseases Research Center, Tokyo Medical University, 6-7-1 Nishi-shinjuku, Shinjuku-ku, Tokyo 169-0023, Japan

Received 1 June 2007; accepted 13 July 2007

Abstract We established an automated mutational analysis detection system using magnetic filtration and the sequence-specific primer-single molecule fluorescence detection (SSP-SMFD) assay to identify the janus activating kinase-2 (JAK2)^{V617F}. DNA was extracted from 100 μL of whole blood automatically by a magnetic filtration system. The JAK2 1849G→T mutation occurs in chronic myeloproliferative disorder (CMPD), and the detection of this change has diagnostic potential. To detect and semiquantitate this mutation, we used two artificial oligonucleotides (wild-type specific and mutated-type specific) and performed the SSP-SMFD assay using an automated fluorescence cell sorter measuring device. The SSP-SMFD assay can detect the presence of a minimum of 5% of the mutated artificial oligonucleotide, thus indicating that this technique is available in detecting contamination of at least 5% cells with the homozygous JAK2^{V617F} mutation. Based on this technique, we analyzed 94 patients with CMPD and compared with the results obtained by the polymerase chain reaction (PCR)-direct sequence. Two homozygous JAK2^{V617F} patients were identified as heterozygous JAK2^{V617F} by the PCR-direct sequence, and four patients judged as wild-type JAK2 by the PCR-direct sequence were identified as heterozygous JAK2^{V617F} by the SSP-SMFD method. Our automated system is simple and suitable for high-throughput analysis in detecting JAK2^{V617F} with a threshold detection limit of 5%. © 2007 Elsevier Inc. All rights reserved.

1. Introduction

The janus kinase-2 (JAK2) 1849G→T mutation results in valine to phenylalanine substitution at amino acid 617 (JAK2^{V617F}), and this molecular change induces constitute signaling of JAK2 in chronic myeloproliferative disorder (CMPD) [1–5]. The JAK2 mutation occurs in most patients with polycythemia vera (PV) and approximately 50% of patients with essential thrombocythemia (ET) or chronic idiopathic myelofibrosis [6,7]. The detection of JAK2 mutational status is important because the presence of this mutation and mutational status (i.e., heterozygous or homozygous JAK^{V617F}) is linked to hematologic and clinical complication in CMPD patients [8–13]. However, the reported incidence of JAK2^{V617F} ranged from 63–91%, and the frequency of heterozygous JAK2^{V617F} was approximately double that of homozygous JAK2^{V617F} in PV patients, ranging from 1.4 to 3.5 [6,7]. The differences among reports may result from technical differences or diagnostic criteria of CMPD, or both.

Methods for detecting the JAK2^{V617F} mutation included direct sequencing with a detection limit around 25% [1,3,4], as well as allele-specific polymerase chain reaction (PCR) [2] or amplification-refractory mutational sequencing (ARMS) PCR with a detection limit of 3% [13]. Another report dealing with AMRS-PCR and capillary electrophoresis showed a detection limit of 1% [14]. Since the detection of JAK2^{V617F} is essential for the diagnosis of PV, the simple and reliable assay system for clinical practice with acceptable detectability would be useful. We set out to develop and evaluate an automated assay system for mass-screening of the JAK2 mutational status.

2. Materials and methods

2.1. Extraction of DNA

DNA was extracted from 100 μL of whole blood by a Magtraction system (SX-6GCN; Precision System

* Corresponding author. Tel.: +81-3-3342-1510; fax: +81-3-5382-6651.
E-mail address: ohyashik@rr.iij4u.or.jp (J.H. Ohyashiki).

0165-4608/07/$ – see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.cancergencyto.2007.07.008

Science, Chiba, Japan) [15]. Magtraction is a magnetic filtration system for automated magnetic particle-based reaction systems. The automation of nucleic acid extraction mediated by magnetic particles has been done in a robot as follows. A sample is first drawn up into a pipette tip, which is loaded into a nozzle. The sample is then dispensed into a well containing reagent and mixed by repeated aspiration and dispensing. Keeping the apical end of the tip soaked in liquid prevents air bubbles or splashes from coming up. The magnetic particles can be effectively separated and captured on the inner wall of the pipette tip by a magnet that comes into contact with the tip at the part with optimal diameter during aspiration or dispensing. Moving the separated particles to the next well and repeating the separating /dispensing operation allows effective resuspending or washing of the magnetic particles (Fig. 1).

2.2. Detection of $JAK2^{V617F}$ mutation by sequence-specific primer-single molecule fluorescence detection (SSP-SMFD)

For the SSP-SMFD study, the 10 ng of extracted DNA was amplified by first-round PCR, and then extended by the fluorescence-labeled single-nucleotide polymorphism (SNP)-specific primer. In this SSP-SMFD method [16,17], DNA containing mutation was amplified by the first-round PCR, and then mutation allele–specific and wild-type allele–specific oligonucleotide labeled by fluorescence was reacted as primers, as described elsewhere [16]. If the sample DNA contains the $JAK2^{V617F}$ mutation (T), the mutation allele–specific oligonucleotide can bind to it and start to extend (Fig. 2, A and B). Fluorescence of PCR products was measured by an automated fluorescence cell sorter measuring device (MF 20; Olympus, Tokyo) (Fig. 1).

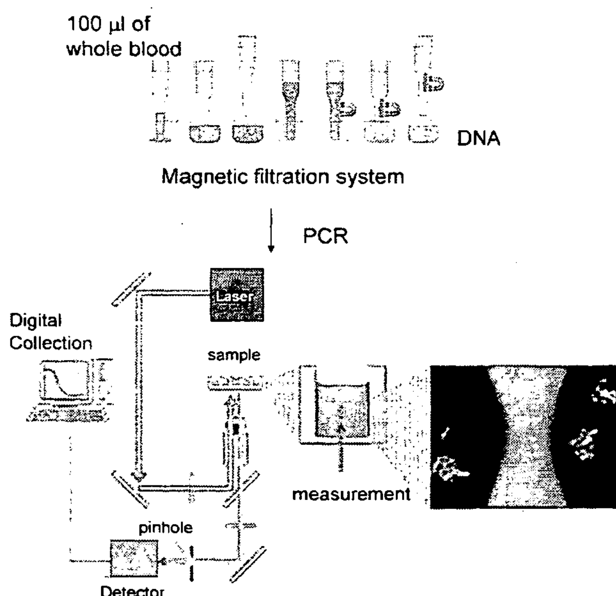

Fig. 1. Workstation of the automated $JAK2^{V617F}$ quantification using a magnetic filtration system and the SSP-SMFD technique.

The first-round forward primer was 5′-TCC TTC TTT GAA GCA GCA AGT ATG ATG AGC AAG CTT TCT CACA.

The first reverse primer was 3′-TAG AAG AGT CCT ACA GTG TTT TCA GTT TCA AAA ATA CTT AAC.

The allele-specific forward primer was 5′-AGC ATT TGG TTT TAA AAT TAT GGA GTATATT.

The allele-specific reverse primer was 3′-TGA AAC TGA AAA CTG TAGGACTATTCAG.

To quantitate the T allele–specific extension, we conducted a plasmid containing mutation allele–specific oligonucleotide (puc18_T: inserted 366-bp mutation-allele-specific-oligonucleotide into puc18) or wild-type allele–specific oligonucleotide (puc18_G), and then used that as a reference. A serial dilution of reference (puc18_T and puc18_G) was used as a standard curve. To confirm the results by SSP-SMFD, the PCR sequence technique was performed with BigDye Termination ver3.1 (Perkin-Elmer Cetus).

2.3. Clinical samples

We studied $JAK2^{V617F}$ in 94 patients with CMPD, including 34 patients with PV, 51 with ET, and 9 patients with idiopathic myelofibrosis, as well as 15 samples obtained from healthy volunteers. We collected frozen marrow cells or peripheral blood (100 µL) from patients after obtaining their written informed consent [17–19], using a SSP-SMFD assay. For detection of $JAK2^{V617F}$ mutation, we used the JAK2 muation kit (NovusGene, Tokyo, Japan).

2.4. Statistical analysis

Continuous data were compared by the two-sample t-test and one-way ANOVA for approximately normally distributed data, and the nonparametric Wilcoxon rank-sum test for other distributions. $P<0.05$ was considered to indicate a statistically significant difference.

3. Results

3.1. Validation of SSP-SMFD assay using puc18-T and puc18-G

We first validated the SSP-SMFD assay using two plasmids, puc18_T (Cy-5 labeled) and puc18_G (TAMRA labeled), which contained sequences with mutated or wild-type JAK2. To obtain reproducible results and to clarify the quantitative relationship between the mutated allele and the wild-type allele in the sample, we calculated the ratio of K2(T)%/K2(G)% × 100 [K2(T)%/K2(G)%]. We mixed two references with various ratios (percentages of puc18_T: 0–100%), and measured K2(T)% or K2(G)% (Table 1 and Fig. 3A). In the presence of 5% Cy-5–labeled puc 18_T mutated allele, the K2(T)% increased to 4.6%, while the K2(G)% was 55.2%, then the K2(T)%/K2(G)% ratio [K2(T)%/K2(G)% × 100] was 27.717. Without the puc

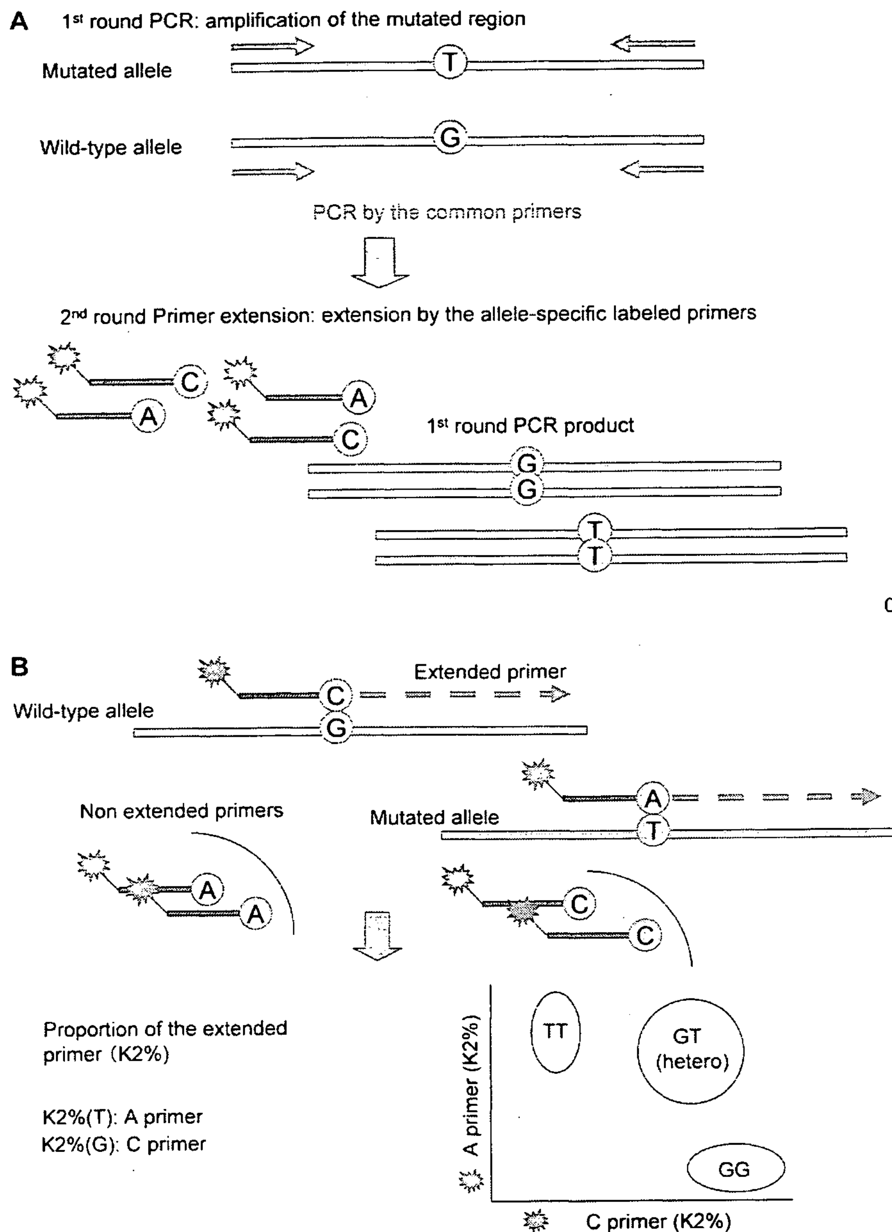

Fig. 2. Schematic presentation of the SSP-SMFD assay. The extracted DNA was amplified by first-round PCR (A), and then extended by the mutation allele–specific and wild-type allele–specific oligonucleotide labeled by fluorescence was reacted as primers (B) [16].

18_T mutated allele, the K2(T)%/K2(G)% ratio was 8.288, therefore we determined that the presence of the 5% mutated allele in the sample could be separated by the K2(T)%/K2(G)% ratio of 30. A K2(T)%/K2(G)% ratio less than 30 was thus considered to be wild-type JAK2. In the presence of 80% Cy-5-labeled puc 18_T mutated allele, the K2(T)%/K2(G)% ratio was 169.431, while the K2(T)%/K2(G)% ratio of 90% of mutated allele was 230.519. Therefore, we tentatively determined that the homozygous JAK2^{V617F} mutation was a K2(T)%/K2(G)% ratio of 230 or more because it was difficult to separate the mutated allele between 40% [K2(T)%/K2(G)% ratio was 128.235] and 80% [K2(T)%/K2(G)% ratio was 169.431] mixture (Table 1 and Fig. 3A). Based on this mixture experiment, we thus identified the JAK2^{V617F} mutational status as follows:

- Wild-type JAK2: K2(T)%/K2(G)% ratio < 30
- Heterozygous JAK2^{V617F} mutation: K2(T)%/K2(G)% ratio 30–230
- Homozygous JAK2^{V617F} mutation: K2(T)%/K2(G)% ratio > 230

Of this mixture condition (5% mutated allele and 95% wild-type allele), the K2(T)%/K2(G)% ratio was 27.717, thus we considered that this method could detect at least

Table 1
Mixture experiment using Cy-5-labeled puc 18_T mutated allele and TAMRA-labeled puc 18_G wild-type allele

Percentage of mutated	Measure d T%	Log K2(T)%	Measure d G%	Log K2(G)%	K2(T)%/K2(G)% × 100
0%	4.60%	0.663	55.50%	1.744	8.288
5%	15.30%	1.185	55.20%	1.742	27.717
10%	33.90%	1.53	57.50%	1.76	58.957
20%	49.30%	1.693	57.80%	1.762	85.294
40%	65.40%	1.816	51.00%	1.708	128.235
80%	71.50%	1.854	42.20%	1.625	169.431
90%	71%	1.851	31%	1.489	230.519
100%	70.90%	1.851	3.70%	0.568	1961.216

5% mixture of the T-specific population [i.e., at least 10% of the population with heterozygous $JAK2^{V617F}$ (Fig. 3B).

3.2. $JAK2^{V617F}$ mutation in clinical specimens

By the SSP-SMFD assay, 9/34 PV patients diagnosed by the Polycythemia Vera Study Group (PVSG) had wild-type JAK2, 18 had heterozygous $JAK2^{V617F}$, and 7 homozygous $JAK2^{V617F}$ (Fig. 4; A and B). There was no particular difference in the K2(T)%/K2(G)% ratio among patients with secondary erythrocytosis (n = 8: 10.9 ± 5.8), those with PV without the World Health Organization (WHO) criteria (n = 7: 8.3 ± 4.9), and those with WHO-PV with wild-type JAK2 (n = 2: 10.4 ± 8). Of the seven patients with homozygous $JAK2^{V617F}$ (TT) by the SSP-SMFD assay, two were identified as heterozygous $JAK2^{V617F}$ (GT) by the PCR-direct sequence. Thirty-two ET patients (62.7%) had $JAK2^{V617F}$ with 31 heterozygous and 1 homozygous $JAK2^{V617F}$ by the SSP-SMFD, and 4 ET patients judged as wild-type JAK2 by the PCR-direct sequence were identified as heterozygous $JAK2^{V617F}$ by the SSP-SMFD method. All the specimens obtained from healthy volunteers showed wild-type JAK2 [n = 15; K2(T)%/K2(G)% ratio = 10 ± 3.6].

4. Discussion

The detection of $JAK2^{V617F}$ is important in diagnosing PV patients because the current PV criteria included the presence of this mutation [20]. To detect this mutation, the PCR-direct sequence method was first applied, but the limitation of detectability was about 25% of mutated alleles. Vannucchi et al. [14] reported a quantitative assay for JAK2 using ARMS-PCR with capillary electrophoresis with a detection limit of around 1%. Capillary electrophoresis is powerful tool to resolve different amplicons and therefore to detect a minor population of JAK2 mutated allele (around 1%), but it might be a complex problem in clinical practice. Poodt et al. [20] developed a semi-quantitative real-time PCR test to detect $JAK2^{V617F}$. With this assay, quantities down to 0.8% JAK2 ^{V617F} among wild-type DNA could be detected reliably, and they concluded that the $JAK2^{V617F}$ qPCR assay is quick, effective, simple,

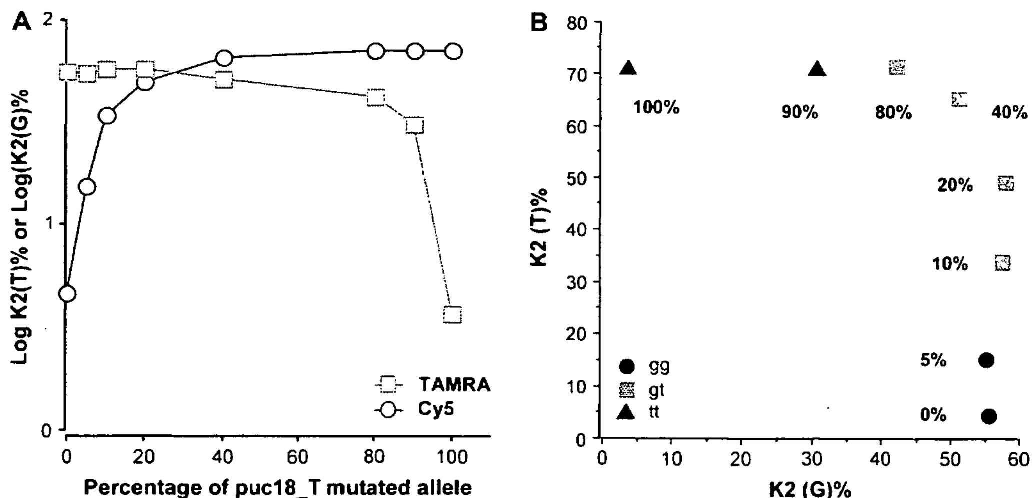

Fig. 3. (A) Quantitative relationship between percentage of mutated alleles and genotypes. The mixture experiment used puc18_T and puc18_G: puc18_T was labeled with Cy5 and puc18_G was labeled with TAMRA. Each fluorescence signal was expressed as K2%. The mixed percentage of puc18_T is indicated in the horizontal axis and log K2(T)% or log K2(G)% are shown in the vertical axis. (B) Scattergram of K2(T)% (vertical axis) and K2(G)% (horizontal axis) of the SSP-SMFD assay in a mixture experiment reference for mutation allele–specific (Cy5) and wild-type allele–specific (Cy3). Percentages in the figure indicate the mixed percentage of mutation-allele-specific reference. Separation is based on the K2(T)%/K2(G)% ratio [=K2(T)%/K2(G)% × 100]. K2(T)%/K2(G)% ratio below 30 are designated as wild-type JAK2 (solid dots), K2(T)%/K2(G)% ratio between 30 and 230 are as heterozygous $JAK2^{V617F}$ (solid squares), and those with 230 or more are considered as homozygous $JAK2^{V617F}$ (solid triangles).

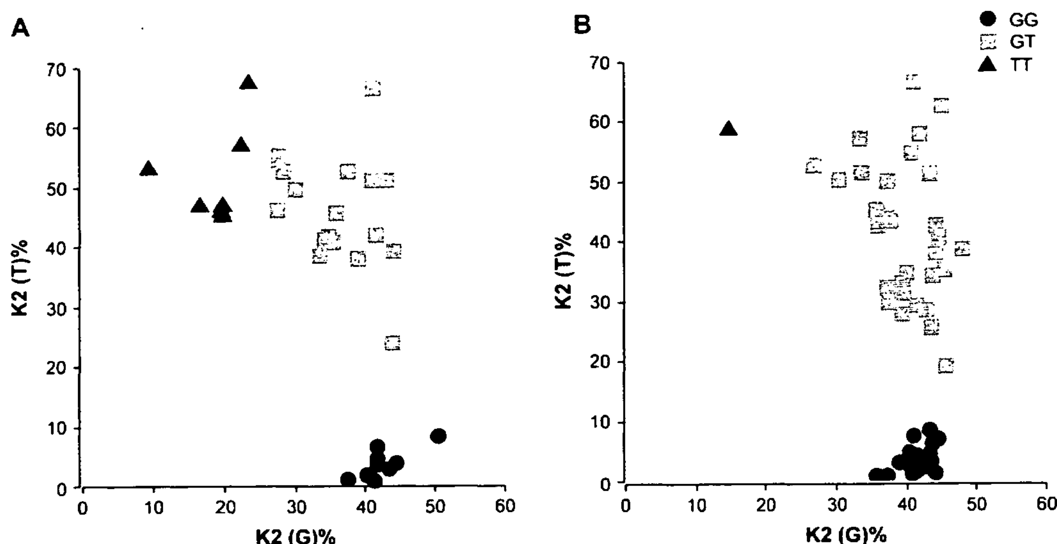

Fig. 4. Scattergram of K2(T)% (vertical axis) and K2(G)% (horizontal axis) of the SSP-SMFD assay in patients with PV diagnosed by PVSG criteria (A) or those with ET (B). Separation of the JAK2 mutational status was based on the K2(T)%/K2(G)% ratio [=K2(T)%/K2(G)% × 100]. Patients with a K2(T)%/K2(G)% ratio less than 30 are designated as wild-type JAK2 (solid dots), those with a K2(T)%/K2(G)% ratio between 30 and 230 are designated as heterozygous $JAK2^{V617F}$ (solid squares), and those with 230 or more as homozygous $JAK2^{V617F}$ (solid triangles).

and more sensitive than direct sequencing, restriction fragment length polymorphism (RFLP), ARMS assay, and other methods published so far to detect JAK2 ^{V617F} [20]. Lay et al. also reported a simple and sensitive method to detect this mutation via PCR and probe dissociation analysis using the LightCycler platform, and they concluded that the LightCycler method offered advantages of speed, reliability, and more straightforward interpretation over the RFLP and sequencing approaches [21]. The detection of a minor population of around 1% of mutated JAK2 allele might be necessary in only a limited number of patients who actually had low levels of JAK2-mutated RNA in the range of 1–4% [14,20,21]. Based on this background, we developed the SSP-SMFD. This technique was originally applied for detection of SNP [16] and used the automated FCS measuring device to obtain the results automatically and process large quantities of materials simultaneously. Therefore, the current SSP-SMFD is suitable for $JAK2^{V617F}$ screening. Our SSP-SMFD is effective to detect $JAK2^{V617F}$ for screening, with a detection threshold of 5%. This is an acceptable level for clinical practice. Indeed, we noticed some discrepancy between the results obtained from the SSP-SMFD assay and those from PCR-direct sequences; 2 (2/25 JAK2 mutated cases) PV patients with heterozygous $JAK2^{V617F}$ and 4 (4/19 wild-type JAK2 cases) ET patients with wild-type JAK2 by direct sequence were found to be homozygous $JAK2^{V617F}$ or heterozygous $JAK2^{V617F}$ by SSP-SMFD, respectively [18]. We therefore concluded that the SSP-SMFD assay may be more sensitive for detection of mutated alleles than the PCR-direct sequence assay. The frequency of $JAK2^{V617F}$ in PV patients due to technical differences is also currently closed up, but we must be cautious in understanding the biologic significance of this mutation with a very low percentage of cells with $JAK2^{V617F}$ in CMPD [22].

In the current study, we tentatively assigned patients with homozygous $JAK2^{V617F}$ mutation as those with a K2(T)%/K2(G)% ratio of more than 230. Based on the mixture experiments using artificial oligonulcotides, this level represents more than 90% of cells with homozygous $JAK2^{V617F}$ (TT). The SSP-SMFD assay detects only a rough percentage of $JAK2^{V617F}$ mutated cells, therefore it is difficult to separate cells with 100% heterozygous $JAK2^{V617F}$ (GT) from those with a 50% population of homozygous $JAK2^{V617F}$ (TT). Further technical developments are required to distinguish patients with a low percentage of homozygous $JAK2^{V617F}$ (TT) from those with a high percentage of heterozygous $JAK2^{V617F}$ (GT). Nevertheless, the current high-throughput SSP-SMFD assay is suitable to identify the $JAK2^{V617F}$ mutation at the clinical level and is more sensitive than the PCR-direct sequence.

Acknowledgments

We thank Professor J. Patrick Barron of the International Medical Communication Center of Tokyo Medical University for his review of this manuscript. This work was supported in part by the "High-Tech Research Center" Project from the Ministry of Education, Culture, Sports and Technology (MEXT) and by the "University-Industry Joint Research Project" from MEXT.

References

[1] James C, Ugo V, Le Couédic J-P, Staerk J, Delhommeau F, Lacout C, Garcon L, Raslova H, Berger R, Bennaceur-Griscelli A, Villeval JL,

Constantinescu SN, Casadevall N, Vainchenker W. A unique clonal JAK2 mutation leading to constitutive signaling causes polycythemia vera. Nature 2005;434:1144–5.

[2] Baxter EJ, Scott LM, Campbell PJ, East C, Fourouclas N, Swanton S, Vassiliou GS, Bench AJ, Boyd EM, Curtin N, Scott MA, Erber WN, Green AR. Cancer Genome Project. Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative disorders. Lancet 2005;365:1054–61.

[3] Kralovics R, Passamonti F, Buser AS, Teo SS, Tiedt R, Passweg JR, Tichelli A, Cazzola M, Skoda RC. A gain-of-function mutation of *JAK2* in myeloproliferative disorders. N Engl J Med 2005;352:779–90.

[4] Levine RL, Wadleigh M, Cools J, Ebert BL, Wernig G, Huntly BJ, Boggon TJ, Wlodarska I, Clark JJ, Moore S, Adelsperger J, Koo S, Lee JC, Gabriel S, Mercher T, D'Andrea A, Frohling S, Dohner K, Marynen P, Vandenberghe P, Mesa RA, Tefferi A, Griffin JD, Eck MJ, Sellers WR, Meyerson M, Golub TR, Lee SJ, Gilliland DG. Activating mutation in the tyrosine kinase JAK2 in polycythemia vera, essential thrombocythemia, and myeloid metaplasia with myelofibrosis. Cancer Cell 2005;7:387–97.

[5] Zhao R, Xing S, Li Z, Fu X, Li Q, Krantz SB, Zhao ZJ. Identification of an acquired JAK2 mutation in polycythemia vera. J Biol Chem 2005;280:22788–92.

[6] Schafer AI. Molecular basis of the diagnosis and treatment of polycythemia vera and essential thrombocythemia. Blood 2006;107:4214–22.

[7] De Keersmaecker K, Cools J. Chronic myeloproliferative disorders: a tyrosine kinase tale. Leukemia 2006;20:200–5.

[8] James C, Delhommeau F, Marzac C, Teyssandier I, Couedic JP, Giraudier S, Roy L, Saulnier P, Lacroix L, Maury S, Tulliez M, Vainchenker W, Ugo V, Casadevall N. Detection of JAK2 V617F as a first intention diagnostic test for erythrocytosis. Leukemia 2006;20:350–3.

[9] Vizmanos JL, Ormazábal C, Larráyoz MJ, Cross NCP, Calasanz MJ. *JAK2* V617F mutation in classic chronic myeloproliferative diseases: a report on a series of 349 patients. Leukemia 2006;20:534–5.

[10] Campbell PJ, Scott LM, Buck G, Wheatley K, East CL, Marsden JT, Duffy A, Boyd EM, Bench AJ, Scott MA, Vassiliou GS, Milligan DW, Smith SR, Erber WN, Bareford D, Wilkins BS, Reilly JT, Harrison CN, Green AR; United Kingdom Myeloproliferative Disorders Study Group; Medical Research Council Adult Leukaemia Working Party, Australasian Leukaemia and Lymphoma Group. Definition of subtypes of essential thrombocythaemia and relation to polycythaemia vera based on *JAK2* V617F mutation status: a prospective study. Lancet 2005;366:1945–53.

[11] Wolanskyj AP, Lasho TL, Schwager SM, McClure RF, Wadleigh M, Lee SJ, Gilliland DG, Tefferi A. JAK2 mutation in essential thrombocythemia: clinical associations and long-term prognostic relevance. Br J Haematol 2005;131:208–13.

[12] Antonioli E, Guglielmelli P, Pancrazzi A, Bogani C, Verrucci M, Ponziani V, Longo G, Bosi A, Vannucchi AM. Clinical implications of the JAK2 V617F mutation in essential thrombocythemia. Leukemia 2005;19:1847–9.

[13] Jones AV, Kreil S, Zoi K, Waghorn K, Curtis C, Zhang L, Score J, Seear R, Chase AJ, Grand FH, White H, Zoi C, Loukopoulos D, Terpos E, Vervessou EC, Schultheis B, Emig M, Ernst T, Lengfelder E, Hehlmann R, Hochhaus A, Oscier D, Silver RT, Reiter A, Cross NC. Widespread occurrence of the JAK2 V617F mutation in chronic myeloproliferative disorders. Blood 2005;106:2162–8.

[14] Vannucchi AM, Pancrazzi A, Bogani C, Antonioli E, Gugliemlli P. A quantitative assay for JAK2V617F mutation in myeloproliferative disorders by ARMS-PCR and capillary electrophoresis. Leukemia 2006;20:1055–60.

[15] Obata K, Segawa O, Yakabe M, Ishida Y, Kuroita T, Ikeda K, Kawakami B, Kawamura Y, Yohda M, Matsunaga T, Tajima H. Development of a novel method for operation magnetic particles, migration technology, and its use for automating nucleic acid purification. J Biosci Bioengineer 2001;91:500–3.

[16] Bannai M, Higuchi K, Akesaki T. Single-nucleotide-polymorphism genotyping for whole-genome-amplified samples using automated fluorescence correlation spectroscopy. Ann Biochem 2004;327:215–21.

[17] Ohyashiki K, Aota Y, Akahane D, Gotoh A, Miyazawa K, Kimura Y, Ohyashiki JH. The JAK2 V617F tyrosine kinase mutation in myelodysplastic syndrome (MDS) developing myelofibrosis indicates the myeloproliferative nature in a subset of MDS patients. Leukemia 2005;19:2359–60.

[18] Ohyashiki K, Aota Y, Akahane D, Gotoh A, Ohyashiki JH. Clinical implication of $JAK2^{V617F}$-positive chronic myeloproliferative disorders: semi-quantitative analysis of mutated allele by sequence-specific primer-single molecule fluorescence detection assay. Leukemia 2007;21:1097–9.

[19] Ohyashiki K, Ito Y, Hori K, Sato K, Makino T, Ohyashiki JH. Thrombosis can occur at any phase of essential thrombocythemia with $JAK2^{V617F}$mutation: a single institutional study in Japan. Leukemia 2007;21:1570–1.

[20] Poodt J, Fijnheer R, Walsh I, Hermans M. A sensitive and reliable semi-quantitative real-time PCR assay to detect JAK2 V617F in blood. Hematol Oncol 2006;24:227–33.

[21] Lay M, Mariappan R, Gotlib J, Dietz L, Sebastian S, Schrijver I, Zehnder JL. Detection of the JAK2 V617F mutation by LightCycler PCR and probe dissociation analysis. J Mol Diagn 2006;8:330–4.

[22] Verstovsek S, Silver RT, Cross CP, Tefferi A. *JAK2*V617F mutational frequency in polycythemia vera: 100%, >90%, less? Leukemia 2006;20:2067.

Leukemia & Lymphoma, November 2007; 48(11): 2141–2144

informa
healthcare

ORIGINAL ARTICLE: CLINICAL

Lack of nucleophosmin mutation in patients with myelodysplastic syndrome and acute myeloid leukemia with chromosome 5 abnormalities

MASAYUKI SHISEKI[1], YUKIKO KITAGAWA[1], YAN-HUA WANG[1], KENTARO YOSHINAGA[1], TOSHIAKI KONDO[1], HANAE KUROIWA[1], MICHIKO OKADA[2], NAOKI MORI[1], & TOSHIKO MOTOJI[1]

[1]*Department of Hematology, Tokyo Women's Medical University, Tokyo, Japan, and* [2]*Chromosome Laboratory, Shiseikai Dai-ni Hospital, Tokyo, Japan*

(Received 4 May 2007; revised 3 August 2007; accepted 4 August 2007)

Abstract

Nucleophosmin (*NPM1*) gene exon 12 mutations are frequently present in patients with acute myeloid leukemia (AML) with normal karyotype. The *NPM1* gene is located on chromosome 5q35, which is often affected in myeloid malignancies including myelodysplastic syndrome (MDS). This suggests that the *NPM1* gene is a one of the target genes affected by chromosome 5 abnormalities and play a role in the development of MDS. It has not been clarified whether *MPM1* mutations are present in patients with MDS and AML with chromosome 5 abnormalities. Therefore, we carried out a mutational analysis on the *NPM1* gene exon 12. *NPM1* mutations were not detected in the 28 patients with MDS and AML with chromosome 5 abnormalities.

Keywords: *Nucleophosmin, MDS, AML, chromosome 5*

Introduction

Nucleophosmin (NPM) is a nuclear phosphoprotein, which functions as a molecular chaperon, and shuttles between the nucleus and cytoplasm [1–3]. Although the biological function of NPM has not been fully elucidated, it is thought to be involved in cell growth and differentiation. Several studies indicate that NPM plays a role in a p53-mediated cellular process, and it may function as a tumor suppressor gene product [4–6]. Recently, it has been reported that mutations in exon 12 of the nucleophosmin (*NPM1*) gene are frequently present in the patients with acute myeloid leukemia (AML) with a normal karyotype [7]. Since the initial report, other groups have reported similar research results, confirming implication of *NPM1* exon 12 mutations in human leukemogenesis [8–13]. The *NPM1* gene is located on chromosome 5q35, which is often affected in myeloid disorders including myelodysplastic syndrome (MDS) and AML [14–17], and the *NPM1* gene is lost in some MDS and AML cases with chromosome 5 rearrangement [18]. In addition, *NPM1* heterozygous ($NPM1^{+/-}$) mice develop MDS-like disorder at 6–10 months of age [19]. These facts suggest implication of *NPM1* abnormality in the development of human MDS. Two recent studies demonstrated that the *NPM1* gene mutations are uncommon in MDS [20,21]. Interpretation of the rare cases of MDS with *NPM1* mutations [20,21] is still a subject of controversy, since multilineage involvement is a distinguishing feature of AML with *NPM1* mutations [22]. Thus, these cases could well represent examples of de novo *NPM1*-mutated AML exhibiting multilineage involvement and dysplastic features [23]. Moreover, it has not been clarified whether the *NPM1* gene mutations are present in MDS and other myeloid malignancies

Correspondence: Masayuki Shiseki, 8-1 Kawada-cho, Shinjuku-Ku, Tokyo 162-8666, Japan. Tel: +81-3-3353-8111. Fax: +81-3-5269-7329. E-mail: shisekim@dh.twmu.ac.jp

ISSN 1042-8194 print/ISSN 1029-2403 online © 2007 Informa UK Ltd.
DOI: 10.1080/10428190701615900