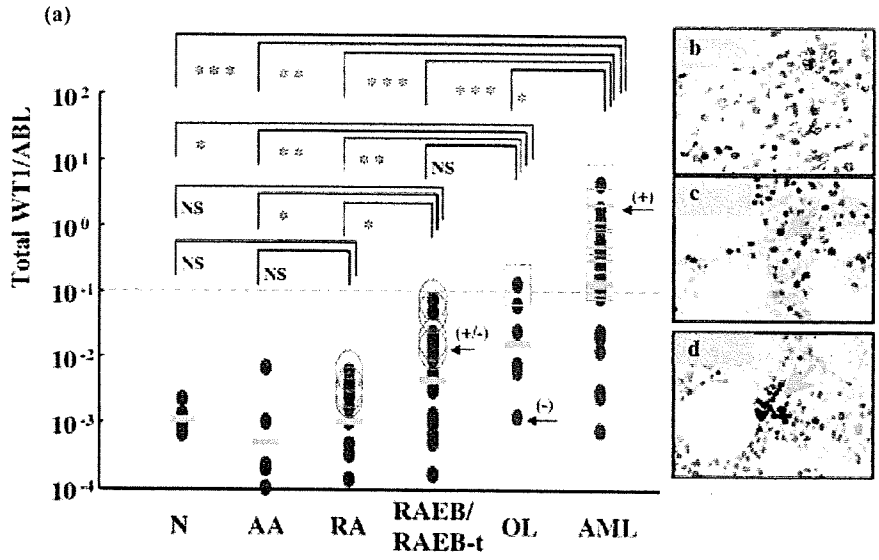

Figure 1 (**a**) Total Wilms' tumor 1 (*WT1*) message level was examined using quantitative (TaqMan) reverse transcription–polymerase chain reaction (RT-PCR). The y axis indicates the ratio of total *WT1/ABL* message (relative *WT1* message level) shown as the log scale. N, normal; AA, aplastic anemia; RA, refractory anemia; RAEB/RAEB-T, refractory anemia with excessive blast/refractory anemia with excessive blast in transformation; OL, overt leukemia transformed from myelodysplastic syndrome; AML, acute myelocytic leukemia. Horizontal bar, mean; broken line, putative threshold for WT1-positive immunostaining; red squares, WT1-positive staining; blue circles, weakly positive staining according to the criteria described in the present study. (**b–d**) Representative staining patterns of WT1. Arrow indicates nuclear staining positive cells. (**b**) Negative staining was from OL (a, black arrow); (**c**) weakly positive staining (+/–) was from RAEB/RAEB-T (a, black arrow); (**d**) strong positive staining (+) was from AML (a, black arrow). NS, not significant. $^{*}P < 0.05$, $^{**}P < 0.001$, $^{***}P < 0.0001$ according to one-way factorial analysis of variance and multiple comparison test (Fisher's method).

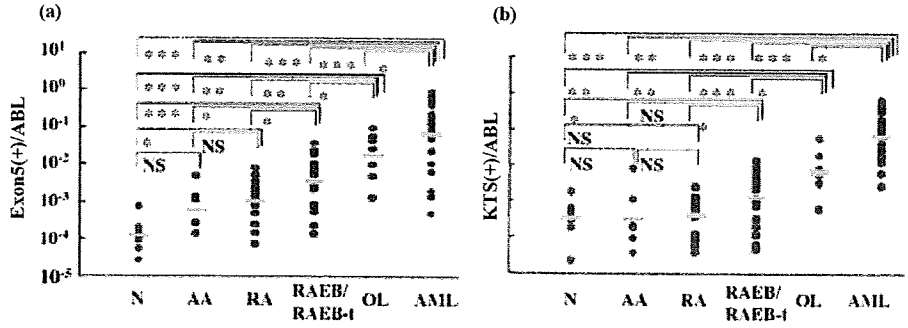

Figure 2 (**a**) Exon 5(+) and (**b**) KTS(+) Wilms' tumor 1 (*WT1*) message analyzed on quantitative reverse transcription–polymerase chain reaction (RT-PCR). Data are expressed as the ratio of the message of each *WT1* isoform/*ABL*. Specific primer sets are as described in Table 2. N, normal; AA, aplastic anemia; RA, refractory anemia; RAEB/RAEB-T, refractory anemia with excessive blast/refractory anemia with excessive blast in transformation; OL, overt leukemia transformed from myelodysplastic syndrome; AML, acute myelocytic leukemia. Horizontal bar, mean. NS, not significant. $^{*}P < 0.05$, $^{**}P < 0.001$, $^{***}P < 0.0001$ according to one-way factorial analysis of variance and multiple comparison test (Fisher's method).

Correlation between total *WT1* and exon 5(+) or between total *WT1* and KTS(+) message

Figure 3 shows a correlation between total *WT1* and exon 5(+) *WT1* and also between the total *WT1* and KTS(+) *WT1* message. Both graphs indicated a good correlation between these two indexes (total and each spliced form), denying the possibility that there is some unique distribution of special splice variant *WT1* message in the diseases analyzed. The results also suggest that the total *WT1* message analysis is sufficient for the clinical diagnosis and treatment of BFS and AML.

DISCUSSION

Because the mutations of the *WT1* gene were the cause of Wilms' tumor, Denys–Drash syndrome and Frasier syndrome, wild-type *WT1* has been regarded as the tumor suppressor gene.[11] After the discovery of WT1 overexpression in

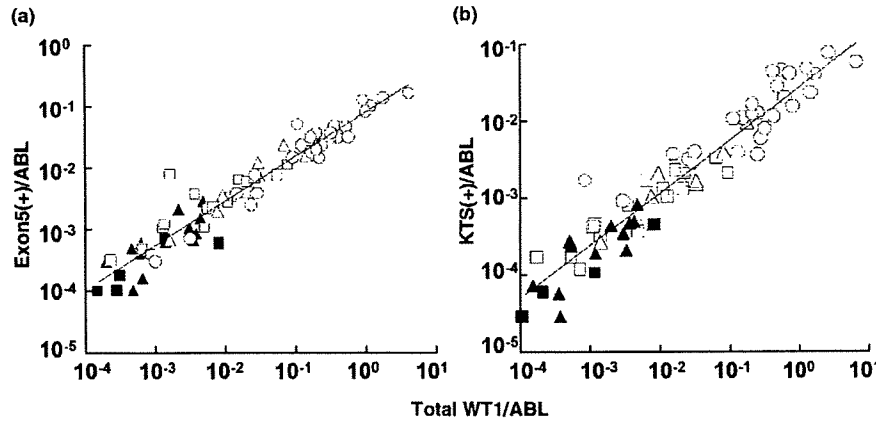

Figure 3 Correlation between total Wilms' tumor 1 (WT1) and (**a**) exon 5(+) *WT1* message and between total *WT1* and (**b**) KTS(+) *WT1* message in MDS, OL and AML, based on data of total *WT1*, exon 5(+) and KTS(+) *WT1* message level. (■) AA, aplastic anemia; (▲) RA, refractory anemia; (□) RAEB/RAEB-T, refractory anemia with excessive blast/refractory anemia with excessive blast in transformation; (△) OL, overt leukemia transformed from myelodysplastic syndrome; (○) AML, acute myelocytic leukemia. Correlation efficiency: a, $R^2 = 0.922$; b, $R^2 = 0.914$.

hematological malignancies,[3,4] WT1 was also taken to be the oncogene in some situations.[19] Importantly, Hosoya *et al.* reported the absence of mutation of *WT1* gene in MDS.[20] WT1 is a zinc finger protein and works as the transcription factor to regulate its target gene expression, but the respective function of four WT1 isoforms as the transcription factor has not been completely clarified. Therefore, the significance of each WT 1 isoform in malignant diseases also remains to be determined.

Siehl *et al.* reported that major splice variants could be separately calculated using their RT-PCR strategy.[16] A similar method was also reported using semiquantitative RT-PCR.[21] However, contrary to a previous report,[16] our preliminary analysis found that the combination of total *WT1*, exon 5(+), and KTS(+) *WT1* messages cannot always estimate exon 5(−) and KTS(−) *WT1* messages. Another method is necessary such as the RNase protection assay as reported by Haber *et al.*,[22] which needs a considerable amount of RNA in order to be performed. Therefore we presented and discussed the data of total *WT1*, exon 5(+) *WT1* and KTS(+) message of BFS and AML in the present study.

The relationship between total *WT1* message and International Prognostic Score System (IPSS) of MDS has been reported.[9] The *WT1* message level is considered to be a universal marker for minimal residual disease and quantification of leukemic cells.[23] It was also reported that *WT1* is highly expressed in the CD34(+) population of AML,[24] although some controversy remains as to whether or not the percentage of CD34 expression is related to the *WT1* message level.[25] The prognostic relevance of high *WT1* levels at diagnosis in acute leukemia is controversial.[26–29] Because the gradual increase of total *WT1* message was observed from low-grade MDS to AML, the present analysis is consistent with previous reports suggesting a good correlation between *WT1* message and MDS disease progression to overt leukemia.

Another important issue is the differential diagnosis between RA and non-severe AA when they have no morphological and/or chromosomal abnormality. We observed that total *WT1* as well as any isoform *WT1* messages could not clearly discriminate between normal and RA and between RA and AA, respectively. There was always some overlap of *WT1* message among normal, AA and RA. An expression level $>2 \times 10^{-3}$ *WT1/ABL* (higher than normal) might be regarded mostly as either MDS or AML. Although severe AA patients were excluded from the current analysis (mostly because of low RNA yield), the present results suggest the limitation of *WT1* message analysis in discriminating RA and AA. Several reports comparing the *WT1* level between RA and normal subjects have presented conflicting results.[9,10] Another possible interpretation of the present results is that *WT1* overexpression is not the early cellular event of RA.

Nakatsuka *et al.*[14] reported on *WT1* message level in various solid tumor cell lines, and >80% of them expressed the *WT1* message. These results were presumed to reflect the possible role of WT1 in the oncogenic process of solid tumors. But it was reported that there was no relationship between *WT1* message level and positive immunostaining.[12] Moreover, the problem in WT1 immunostaining of these solid tumors was the localization of WT1 protein in the cytoplasm but not in the nucleus. The present analysis showed clearly its nuclear localization in the bone marrow clot sections of MDS and AML. Cells staining positive for WT1 could be regarded as blast cells or leukemia cells.

The present study demonstrated positive staining only in AML and OL or in samples with $>10^4$ *WT1* copies/μg RNA. The present results suggest a difference in the sensitivity of each assay method (quantitative RT-PCR and immunostaining) for the quantification of *WT1* expression, and that the WT1 staining was not sufficiently sensitive for clinical practice in MDS and AML cases.

Numerous studies have confirmed that the specific effect of WT1 on cell death is critically dependent on the cell type being studied and that different WT1 isoforms can have a distinct effect on gene regulation and cell fate.[30,31] It is of interest to know whether some isoform of WT1 is particularly

important for leukemogenesis or which form of *WT1* is prevalent in hematological malignancy. Previous reports showed that exon 5(+) isoform was the dominant form in AML as well as lung, head and neck squamous carcinomas.[16,21] The present analysis suggested that both exon 5(+) and KTS(+) message was strongly correlated with the total *WT1* message level. It is less likely that other isoforms (exon 5 (–) or KTS (–)) played some unique role in the leukemogenesis. Recently, the anti-apoptotic function of the exon 5(+) *WT1* gene has also been reported.[32]

Taken together, the present analysis indicates a considerable overlap of *WT1* message among normal, AA and RA, and that WT1-positive nuclear immunostaining was clearly observed in patients whose *WT1* message of the bone marrow was higher than 1/10 the *ABL* gene message (or 10^4 copies/μg RNA). These data provide a basic understanding of WT1 expression in BFS and AML.

ACKNOWLEDGMENT

The authors express sincere thanks to Dr M. Suzuki (Nagoya University Graduate School of Medicine) for his valuable assistance in figure preparation.

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Leukemia (2007) 21, 2476–2484

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ORIGINAL ARTICLE

FLT3 regulates β-catenin tyrosine phosphorylation, nuclear localization, and transcriptional activity in acute myeloid leukemia cells

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Deregulated accumulation of nuclear β-catenin enhances transcription of β-catenin target genes and promotes malignant transformation. Recently, acute myeloid leukemia (AML) cells with activating mutations of FMS-like tyrosine kinase-3 (FLT3) were reported to display elevated β-catenin-dependent nuclear signaling. Tyrosine phosphorylation of β-catenin has been shown to promote its nuclear localization. Here, we examined the causal relationship between FLT3 activity and β-catenin nuclear localization. Compared to cells with wild-type FLT3 (FLT3-WT), cells with the FLT3 internal tandem duplication (FLT3-ITD) and tyrosine kinase domain mutation (FLT3-TKD) had elevated levels of tyrosine-phosphorylated β-catenin. Although β-catenin was localized mainly in the cytoplasm in FLT3-WT cells, it was primarily nuclear in FLT3-ITD cells. Treatment with FLT3 kinase inhibitors or FLT3 silencing with RNAi decreased β-catenin tyrosine phosphorylation and nuclear localization. Conversely, treatment of FLT3-WT cells with FLT3 ligand increased tyrosine phosphorylation and nuclear accumulation of β-catenin. Endogenous β-catenin co-immunoprecipitated with endogenous activated FLT3, and recombinant activated FLT3 directly phosphorylated recombinant β-catenin. Finally, FLT3 inhibitor decreased tyrosine phosphorylation of β-catenin in leukemia cells obtained from FLT3-ITD-positive AML patients. These data demonstrate that FLT3 activation induces β-catenin tyrosine phosphorylation and nuclear localization, and thus suggest a mechanism for the association of FLT3 activation and β-catenin oncogeneic signaling in AML.
Leukemia (2007) **21**, 2476–2484; doi:10.1038/sj.leu.2404923; published online 13 September 2007
Keywords: FLT3 AML; β-catenin; tyrosine phosphorylation

Introduction

FLT3 is a member of the class III receptor tyrosine kinase family and plays an important role in regulating the proliferation, differentiation and survival of hematopoietic cells.[1,2] Binding of FLT3 ligand (FL) to FLT3 promotes receptor dimerization and activation of FLT3.[1] The activated receptor then activates the phosphotidylinositol 3–kinase (PI3K) and RAS signal-transduction cascades.[3,4] FLT3 mutations are the most frequent genetic lesion seen in acute myeloid leukemia (AML). Of patients with AML, 15–35% have an internal tandem duplication (ITD) of the juxtamembrane domain of FLT3 (FLT3-ITD)[5–8] and 5–10% have mutations in the tyrosine kinase domain (TKD) of FLT3 (FLT3-TKD).[9,10] Both types of FLT3 mutation result in ligand-independent activation of the receptor and activation of downstream signaling pathways.[11–13] The presence of FLT3-ITD is associated with leukocytosis and poor clinical outcome in both pediatric and adult patients with AML.[7,8,14,15]

β-catenin is a multifunctional protein that plays an important role in both cell – cell interactions[16,17] and transcriptional regulation.[18–20] In epithelial cells, β-catenin is localized at the inner surface of the plasma membrane, where it functions in conjunction with E-cadherin as part of the adherens junction, a specialized cytoskeletal complex that regulates cell–cell adhesion.[21] β-catenin is also the critical effector of the canonical Wnt signaling pathway, in which nuclear β-catenin coactivates transcription in association with LEF/TCF family members. In the absence of secreted Wnts, the modular protein axin provides a scaffold for the binding of glycogen synthase kinase-3β (GSK-3β), adenomatous polyposis coli (APC) protein and β-catenin. This facilitates serine/threonine phosphorylation in the N terminus of β-catenin by GSK-3β and subsequent degradation of β-catenin by a proteasome-dependent process.[22,23] Wnt stimulation leads to β-catenin stabilization, nuclear accumulation and interaction with T-cell factor/lymphoid enhancer factor (TCF/LEF) transcription factors to regulate genes important for proliferation and survival.[18,24–26]

Wnt/β-catenin has previously been implicated in the development of several hematologic malignancies.[27–31] Recently, it was reported that TCF-dependent transcriptional activity of FLT3-ITD cells is higher than in FLT3-WT cells, suggesting that aberrant β-catenin signaling may contribute to FLT3-ITD-related myeloid transformation.[32] However, the relationship between FLT3 activity and activation of β-catenin-dependent nuclear signaling has not been elucidated. While serine/threonine phosphorylation facilitates degradation of β-catenin in the proteasome, we and others have demonstrated that tyrosine phosphorylation facilitates β-catenin nuclear localization and enhances β-catenin-associated transcription.[25,33,34] Thus far, tyrosine phosphorylation of β-catenin and a potential role for FLT3 in this regulatory event have not been examined in AML. In this study, we have examined the effects of FLT3 mutation or activation on β-catenin tyrosine phosphorylation, subcellular localization and transcriptional activity.

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Received 10 May 2007; revised 7 July 2007; accepted 23 July 2007; published online 13 September 2007

Materials and methods

Antibodies and reagents

FLT3 inhibitor AG1296 was purchased from Calbiochem (San Diego, CA, USA). The FLT3 inhibitor PKC 412 and GSK3β

inhibitor SB415286 were purchased from LC Laboratories (Woburn, MA, USA). FLT3 siRNA, β-catenin siRNA and control siRNA were purchased from Dharmacon (Lafayette, CO, USA). Recombinant human FLT3 ligand (FL) and recombinant mouse IL-3 were purchased from R&D systems (Minneapolis, MN, USA). Fetal bovine serum (FBS) was purchased from Gibco-BRL (Gaithersburg, MD, USA). Anti-β-catenin monoclonal antibody was purchased from BD Biosciences (San Jose, CA, USA). Anti-FLT3-antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phosphotyrosine monoclonal antibody was purchased from Upstate (Charlottesville, VA, USA). Anti-phospho-β-catenin antibody (Ser33/37/Thr41), anti-phospho-GSK3 antibody (Ser21/9), anti-GSK3β antibody and anti-topoisomerase II antibody were purchased from Cell Signaling Technology (Danvers, MA, USA). Western blotting blocking reagent was purchased from Roche Applied Science (Indianapolis, IN, USA). GammaBind Plus Sepharose beads and horseradish peroxidase (HRP)-labeled goat anti-mouse and goat anti-rabbit antibodies were purchased from Amersham Biosciences (Uppsala, Sweden). BCA Protein Assay reagent, western blot chemiluminescence reagents and Restore Western Blot Stripping Buffer were purchased from Pierce Chemical Co (Rockford, IL, USA). PVDF membrane was purchased from Bio-Rad Laboratories (Hercules, CA, USA).

Cells

The murine IL-3-dependent myeloid progenitor cell line, 32D, was obtained from the RIKEN cell bank (Tsukuba, Japan) and maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum and 1.0 ng ml^{-1} recombinant murine IL-3. WT, ITD and TKD (D835) FLT3-expressing 32D cell lines were reported previously.[10–12,35] MOLM-13, a human AML-M5 cell line with the FLT3-ITD mutation,[6] was kindly provided by Dr Yoshinobu Matsuo (Fujisaki Cell Center, Okayama, Japan). MV 4-11, a human AML cell line expressing FLT3-ITD, and THP-1, a human AML-M5 cell line expressing FLT3-WT,[6] were purchased from ATCC (Manassas, VA, USA). MOLM-13 and THP-1 were maintained in RPMI-1640 medium supplemented with 10% FBS. MV 4-11 was maintained in Iscove's modified Dulbecco's medium (IMDM) with 20% FBS.

Immunoprecipitation and immunoblotting assays. Cells were washed twice with ice-cold PBS, and were lysed in lysis buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 50 mM NaF, 1 mM Na_3VO_4, 10 μg ml^{-1} aprotinin, 10 μg ml^{-1} leupeptin and 1 mM phenylmethylsulfonyl fluoride). After incubation for 1 h at 4 °C, lysates were spun at 12 000 *g* for 25 min and pellets were discarded. Lysates were immunoprecipitated with each primary antibody overnight at 4 °C. GammaBind Plus Sepharose (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) beads were added and the mixture was rocked for 1 h at 4 °C. The beads were subsequently washed three times with lysis buffer and mixed with sodium dodecyl sulfate (SDS) sample buffer. After boiling for 5 min, samples were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted onto PVDF membranes (Millipore, Billerica, MA, USA). The membranes were incubated overnight with primary antibody in 10% blocking reagent in TNE washing buffer: (50 mM NaCl, 10 mM Tris–HCl, pH 7.5, 2.5 mM EDTA, 0.1% Tween 20). Primary antibodies were detected by HRP-labeled secondary antibody (1:2000) and were visualized using chemiluminescence reagents. For reprobing, the membranes were stripped with stripping buffer and reprobed with the indicated antibodies. Band optical densities were measured using a GS-800 densitometer and Quantity One software (Bio-Rad).

Immunocytochemistry

Cells that had been attached to glass slides by cytocentrifugation (Shandon, Pittsburgh, PA, USA) were fixed with 3.7% formaldehyde in PBS for 10 min and permeabilized with 0.2% Triton X-100 for another 10 min at room temperature. The cells were stained with monoclonal anti-β-catenin antibody (BD Biosciences), Alexa Fluor 488 goat anti-mouse immunoglobulin (Molecular Probes, Carlsbad, CA, USA) and 4′,6-diamidino-2-phenylindole (DAPI; Molecular Probes). The cells were viewed using a Leica DM IRB fluorescence microscope (×100 objective) equipped with a *Z*-axis motor (Ludl Electronics, Hawthorne, NY, USA). Stacks of images (13–19 optical sections at a step size of 0.3 μm) were taken with a digital camera (Hamamatsu) and processed using Openlab Volume Deconvolution software (Improvision, Lexington, MA, USA). Nuclear β-catenin was determined using the colocalization feature in Openlab. Briefly, the areas of colocalization, depicted as violet, were generated by calculation of measurement statistics based on intensity information in the whole image obtained from two fluorescence channels detecting β-catenin and DAPI.

Preparation of subcellular fractions

Cells were washed and treated with ice-cold low-salt lysis buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 1 mM Na_3VO_4, 10 μg ml^{-1} aprotinin, 10 μg ml^{-1} leupeptin) for 10 min at 4 °C. Cells were homogenized with 15 strokes of a Dounce homogenizer. Homogenates were centrifuged at 700 *g* for 5 min and supernatant fractions were saved as cytoplasmic extracts. The pellets were resuspended in high-salt lysis buffer (20 mM HEPES, pH 7.9, 25% glycerol, 420 mM NaCl, 1% TritonX-100, 1 mM Na_3VO_4, 10 μg ml^{-1} aprotinin, 10 μg ml^{-1} leupeptin) and rotated for 10 min at 4 °C. Cellular debris was removed by centrifugation and supernatants were saved as nuclear fractions. Equal amounts of protein were subjected to western blot analysis.

Quantitative Reverse Transcriptase-PCR. Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and cDNA was prepared using the TaqMan Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Analysis of mRNA expression was carried out by real-time RT-PCR using the ABI Prism 7700 Sequence Detection System (Applied Biosystems). All sample mRNA levels were normalized to the level of 18S ribosomal RNA. The primer and probe sequences for real-time RT-PCR were as follows: rRNA forward 5′-AGTCCCTGCC CTTTGTACACA-3′, rRNA reverse 5′-CGATCCGAGGGCCTC ACTA-3′, c-*myc* forward 5′-CGTCTCCACACATCAGCACAA-3′, c-*myc* reverse 5′-TCTTGGCAGCAGGATAGTCCTT-3′, cyclin D1 forward 5′-GCATGTTCGTGGCCTCTAAGAT-3′, cyclin D1 reverse 5′-TCGGTGTAGATGCACAGCTTCT-3′.

In vitro *kinase assay*

Recombinant β-catenin (Upstate) was incubated with or without recombinant FLT3 kinase (Cell Signaling Technology) in reaction buffer (60 mM HEPES pH 7.5, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 3 μM Na_3VO_4, 1.25 mM DTT, 200 μM ATP) for 30 min at 25 °C. After the reaction, samples were mixed with SDS-sample buffer and were resolved by SDS-PAGE, electrotransferred and immunoblotted with anti-phospho-tyrosine antibody (Upstate). The membrane was stripped and reprobed with

anti-β-catenin antibody. Since albumin is not phosphorylated by FLT3, the membrane was stained with Ponceau S to identify the location of albumin.

RNAi-mediated silencing of FLT3

MOLM-13 cells (2×10^6 in 100 μl) were transfected with 1 μM FLT3 siRNA, β-catenin siRNA or control siRNA by electroporation (Nucleofector, Amaxa Biosystems, Gaithersburg, MD, USA). Following transfection, the cells were incubated for 48 h and then were subjected to immunoprecipitation and immunoblot assay. Band optical density was measured using a GS-800 densitometer and Quantity One software.

Primary AML samples

Bone marrow and peripheral blood samples were obtained from leukemia patients under an Institutional Review Board-approved

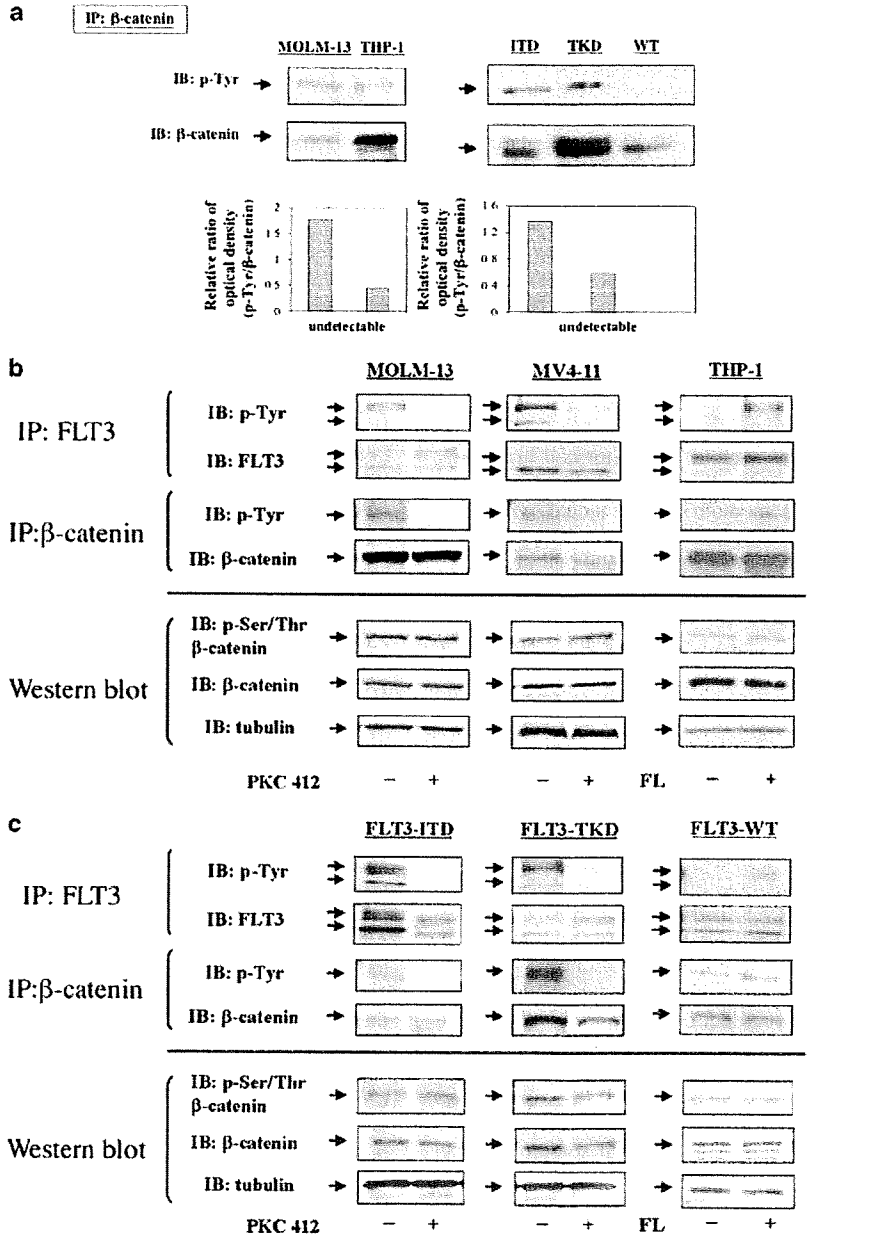

Figure 1 Tyrosine phosphorylation of β-catenin in FLT-3-expressing cell lines. (**a**) Cell lysates were obtained as described in 'Materials and methods'. To detect tyrosine phosphorylation of β-catenin, 1 mg of proteins from cell lysates was precipitated using 2 μg of anti-β-catenin antibody. Proteins were resolved by reducing 10% SDS-PAGE, electrotransferred to PVDF membrane and western blotted for anti-phosphotyrosine (upper panel). Membranes were stripped and reprobed with anti-β-catenin antibody (middle panel). Optical density ratio of phosphotyrosine/total β-catenin was measured using a GS-800 densitometer with Quantity One software (lower panel). (**b**, **c**) Effect of FLT3 inhibition or activation on β-catenin tyrosine phosphorylation. Cells were treated with or without PKC412 (20 nM for 2 h) or FLT3 ligand (FL; 50 ng ml^{-1} for 1 h) and then lysed. Protein (1 mg) from cell lysates was precipitated with 2 μg of anti-β-catenin antibody or 1.5 μg of anti-FLT3 antibody. Whole-cell lysates and immunoprecipitated samples were resolved by reducing 7.5% SDS-PAGE, electrotransferred to a PVDF membrane and western blotted with corresponding antibodies.

protocol. All patients gave informed consent according to the Declaration of Helsinki. Four individual cryopreserved AML samples, each harboring an FLT3-ITD mutation, were thawed in warm RPMI medium supplemented with 10% FBS and penicillin/streptomycin. After overnight incubation at 37 °C and 5% CO_2, samples were treated for 5 min with DNAse I (150 U ml^{-1}, Amersham) and subjected to centrifugation on Ficoll–Hypaque (Amersham) to remove necrotic cells. Eighty million cells from each sample were split into two aliquots. To one aliquot was added CEP-701 to a final concentration of 50 nM (FLT3 inhibited), while the other aliquot contained dimethylsulfoxide (DMSO) (control). After incubation at 37 °C, cells were divided into two aliquots, pelleted, lysed and immunoprecipitated for either FLT3 or β-catenin. Statistical analysis was performed using StatView software (SAS, Cary, NC, USA).

Results

Tyrosine phosphorylation of β-catenin is increased in activated FLT3-expressing AML cells

Tyrosine phosphorylation of β-catenin in FLT3-expressing cell lines was investigated by immunoprecipitation assay. As shown in Figure 1a (left panel), β-catenin in the FLT3-ITD cell line, MOLM-13, was more tyrosine-phosphorylated compared to a FLT3-WT cell line, THP-1. The phosphotyrosine/total β-catenin ratio was 3.75-fold greater in the FLT3-ITD-expressing cells than in the FLT3-WT-expressing cells. Similarly, using 32D transfectants (Figure 1a, right panel), β-catenin was more tyrosine-phosphorylated in cells expressing the activated FLT3 mutants FLT3-ITD and FLT3-TKD-32D, than in FLT3-WT transfectants.

To confirm the importance of FLT3 activation for tyrosine phosphorylation of β-catenin, we treated the cell lines with the FLT3 kinase inhibitor PKC412, which effectively inhibits the constitutive kinase activity of FLT3-ITD and FLT3-TKD[36,37] and the kinase activity stimulated by FLT ligand (FL). In the FLT3-ITD cell lines MOLM-13 and MV 4-11, treatment with PKC412 (20 nM for 2 h) decreased tyrosine phosphorylation of both FLT3 and β-catenin. Similar results were obtained with the FLT3 inhibitor AG1296 (data not shown). Conversely, FL (50 ng ml^{-1} for 1 h) induced tyrosine phosphorylation of FLT3 and β-catenin in THP-1 cells (Figure 1b, upper panel). In these settings, little change was observed in the total protein level of β-catenin and in its serine/threonine-phosphorylation status (Figure 1b, lower panel). In addition, PKC412 decreased tyrosine phosphorylation of FLT3 and β-catenin in FLT3-ITD-32D and FLT3-TKD-32D and FL increased phosphorylation of FLT3 and β-catenin in FLT3-WT-32D cells (Figure 1c, upper panel). As in the AML cell lines,

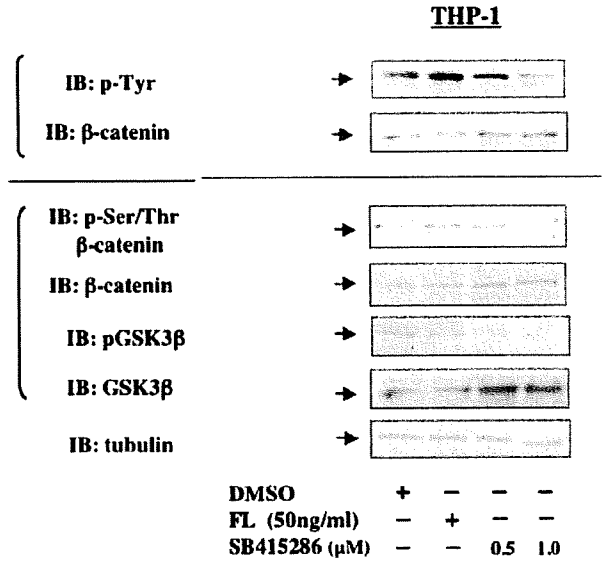

Figure 2 FLT3 regulates tyrosine phosphorylation of β-catenin. Effect of GSK3β inhibitor on total and tyrosine phosphorylation level of β-catenin. Cells were pretreated with or without SB415286 (0.5 or 1.0 μM for 3 h), then treated with FL (50 ng ml^{-1}) for 2 h. Because SB415286 was dissolved in DMSO, the same amount (0.1% final concentration) of DMSO was added to control cells. Preparation of cell lysates, immunoprecipitation and western blot are described in Materials and methods.

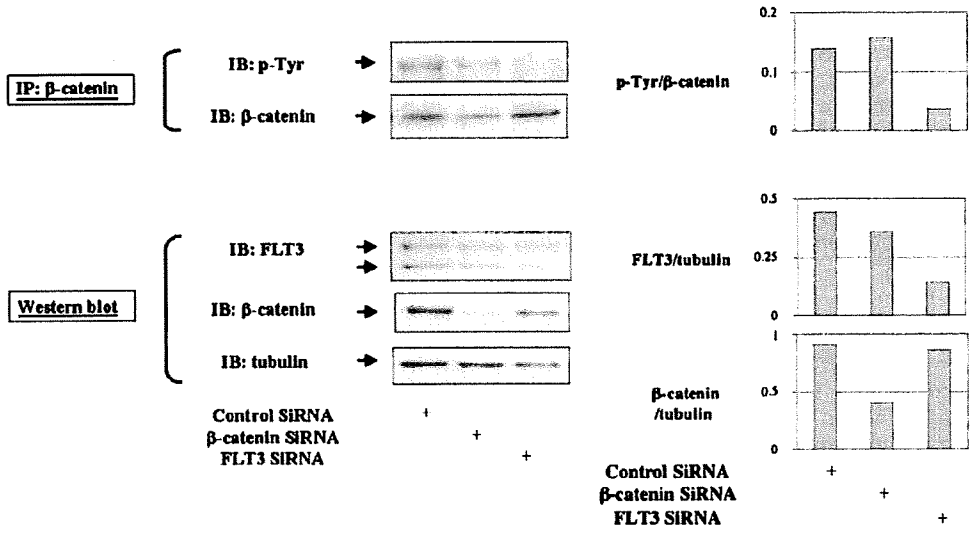

Figure 3 Silencing of FLT3 leads to reduced tyrosine phosphorylation of β-catenin. MOLM-13 cells were transfected with FLT3 siRNA, β-catenin siRNA or control siRNA. Following transfection, cells were incubated for 48 h. After incubation, cells were lysed and subjected to immunoprecipitation and immunoblotting assay. Optical density ratios were obtained using a GS-800 densitometer with Quantity One software.

no change was found in either the total level or phosphoserine/threonine status of β-catenin in 32D transfectants (Figure 1c, lower panel). These results suggest that tyrosine phosphorylation of β-catenin, not its serine/threonine phosphorylation or total level, closely parallels FLT3 activation status.

Stabilization of β-catenin following GSK3β inhibition does not promote enhanced tyrosine phosphorylation of β-catenin

To study the relationship between the tyrosine phosphorylation and the total level of β-catenin further, we investigated the effect of GSK3β inhibition on β-catenin tyrosine phosphorylation status. As shown in Figure 2, treatment with the GSK3β inhibitor SB415286 increased the total β-catenin level in THP-1 cell line and decreased β-catenin serine/threonine phosphorylation. However, SB415286 did not increase tyrosine phosphorylation of β-catenin in these cells, rather it decreased the ratio of tyrosine-phosphorylated/total β-catenin.

Silencing of FLT3 gene expression leads to reduced phosphorylation of β-catenin in FLT3-ITD AML cells

We next investigated whether RNAi-mediated knockdown of FLT3 expression affected β-catenin tyrosine phosphorylation similarly to small-molecule FLT3 kinase inhibitors. As shown in Figure 3, when the expression of FLT3 in MOLM-13 cells was suppressed with FLT3 siRNA, tyrosine phosphorylation of β-catenin was concomitantly reduced, while the total protein level of β-catenin was not changed. In contrast, while the protein level of β-catenin was suppressed by β-catenin siRNA, the ratio of tyrosine-phosphorylated/total β-catenin was not significantly affected.

Nuclear localization of β-catenin in activated FLT3-expressing cell lines

Because tyrosine phosphorylation of β-catenin has been reported to be associated with increased nuclear accumulation,[25,33,38] we investigated the subcellular localization of β-catenin by immunocytochemistry and western blot. Although β-catenin was located mainly in the cytoplasm in THP-1, it was primarily nuclear in MOLM-13 (Figures 4a and b). After treatment with PKC412, the amount of nuclear β-catenin was markedly decreased in MOLM-13 cells. Similar to PKC412 treatment, FLT3 siRNA decreased nuclear β-catenin while control siRNA had no effect. In contrast, after treatment of THP-1 cells with FL, the amount of nuclear β-catenin was increased. Western blotting of subcellular fractions confirmed β-catenin to be enriched in the nuclear fraction of cells with active FLT3 signaling (Figure 4c). These findings suggest that activated FLT3 is associated with increased nuclear accumulation of β-catenin and that nuclear β-catenin is preferentially tyrosine-phosphorylated.

Activation status of FLT3 affects β-catenin target gene expression in AML and 32D cell lines

Because enhanced nuclear localization of β-catenin was correlated with the activation status of FLT3, we wished to determine whether β-catenin-dependent transcription could be affected by FLT3 activity in MOLM-13, THP-1 and 32D transfectants. To examine this question, the mRNA levels of two β-catenin target genes, cyclin D1 and c-*myc*, were measured using real-time RT-PCR. In cells with FLT3 gain-of-function mutations, PKC412 treatment markedly decreased RNA expression of both c-*myc* and cyclin D1 (Figure 5a). In cells with

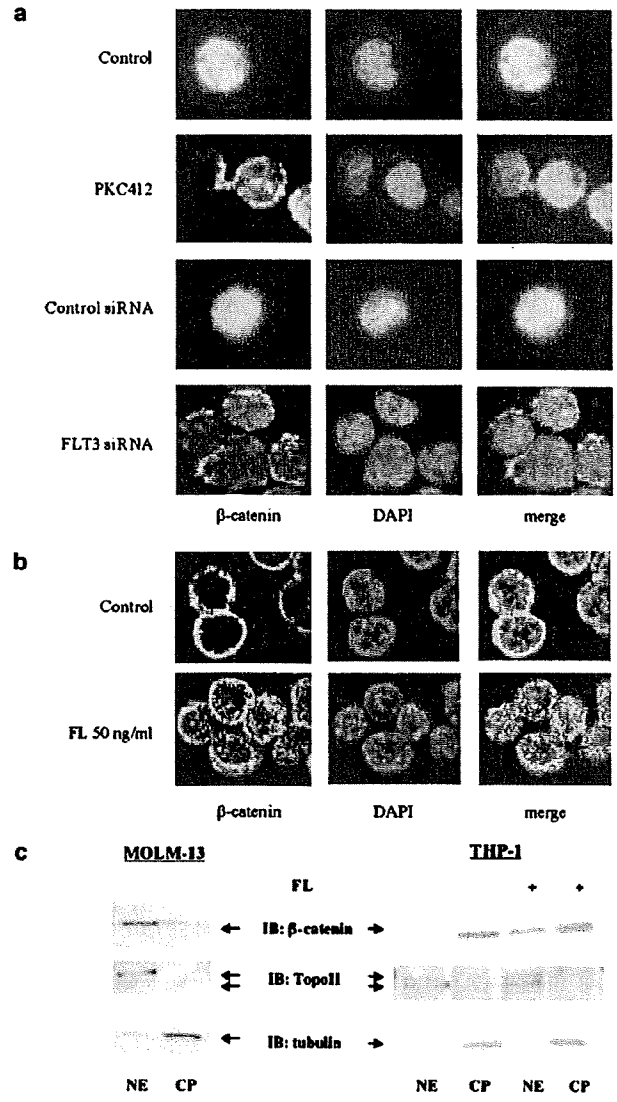

Figure 4 Nuclear localization of β-catenin in activated FLT3 cell lines. (**a**, **b**) MOLM-13 (**a**) and THP-1 (**b**) cells were treated with or without PKC412 (20 nM for 2 h), control siRNA (48 h), FLT3 siRNA (48 h) or FL (50 ng ml^{-1} for 30 min) and then were cytocentrifuged. Cells were fixed in 3.7% formaldehyde/PBS and permeabilized with 0.2% Triton X-100. β-Catenin was visualized by immunofluorescence (green, left panel). The DNA-intercalating dye DAPI was used to identify cell nuclei (blue, center panel). The right panel presents a merged image to highlight the nuclear pool of β-catenin. To facilitate visualization of the nuclear localization of β-catenin in THP-1, the green/blue merged signal was converted to violet by the image analysis software. Images were collected using a ×100 objective. (**c**) Western blot analysis of β-catenin in subcellular fractions. Preparation of subcellular fractions is described in Materials and methods. To verify the purity of subcellular fractions and to control for protein loading, blots were reprobed with antibodies to topoisomerase II (Topo II) for nuclear fractions and α-tubulin (tubulin) for cytoplasmic fractions.

FLT3-WT, expression of c-*myc* and cyclin D1 was increased after treatment with FL (suggesting functional nuclear localization of β-catenin), while SB415286 had very little effect (Figure 5b). Similar to PKC412 treatment, FLT3 siRNA decreased expression of cyclin D1, as did β-catenin siRNA (Figure 5c).

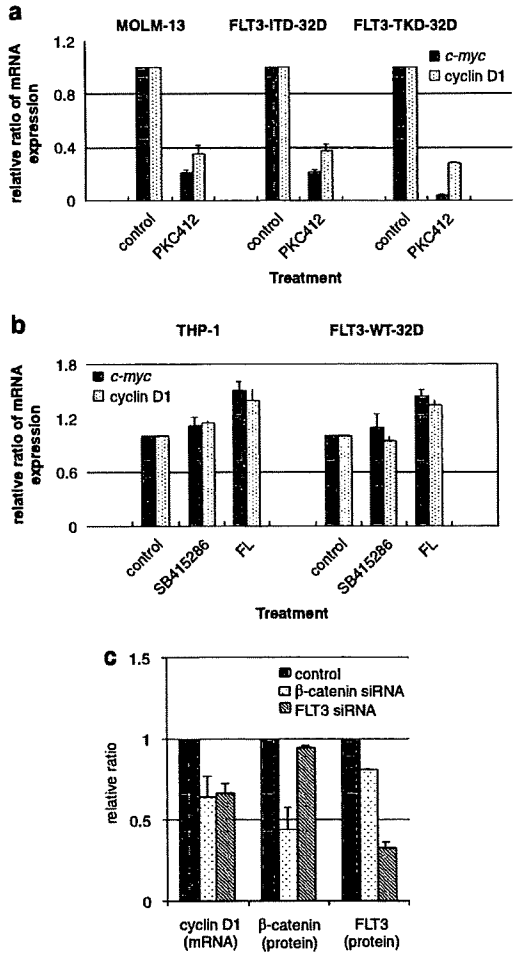

Figure 5 Effect of FLT3 activation on β-catenin target gene expression in AML cell lines. (**a**, **b**) Cells were treated as follows: 0.1% DMSO (control), 20 nM PKC412 for 4 h (PKC412) or 50 ng ml^{-1} FL for 4 h (FL). Total RNA was isolated from the cells and quantitative RT-PCR was performed in duplicate. All samples were normalized to the level of 18S ribosomal RNA. The mean of two individual experiments and standard deviations are shown. (**c**) MOLM-13 cells were transfected with indicated siRNA. After 48 h, total RNA was isolated and quantitative RT-PCR was performed as described.

Active FLT3 binds to β-catenin

We used co-immunoprecipitation techniques to investigate the possible physical interaction between FLT3 and β-catenin. In MOLM-13 cells, endogenous β-catenin co-immunoprecipitated with endogenous FLT3. This association was markedly reduced in cells treated with PKC412 (Figure 6a). In the reciprocal experiment, FLT3 was co-immunoprecipitated by anti-β-catenin antibody in untreated cells, but this association was reduced in PKC412-treated cells (Figure 6b). In THP-1 cells, although a small amount of β-catenin was co-immunoprecipitated with anti-FLT3 antibody in the absence of FL, the proportion of β-catenin bound to FLT3 was increased after FL treatment (Figure 6a). This observation was confirmed by co-immunoprecipitation with anti-β-catenin antibody (Figure 6b). These results demonstrate that active FLT3 preferentially binds to β-catenin, whether the kinase is mutated or not, and that inhibition of FLT3 activation decreases interaction between FLT3 and β-catenin.

Active FLT3 kinase phosphorylates β-catenin directly

To determine whether active FLT3 can directly phosphorylate tyrosine residues of β-catenin, we performed an *in vitro* kinase assay using recombinant active FLT3 as enzyme source and recombinant β-catenin as substrate. As shown in Figure 6c, no tyrosine phosphorylation of β-catenin was detected in the absence of FLT3 protein (upper panel, lane 2). Addition of active FLT3 kinase induced tyrosine phosphorylation of β-catenin, while inclusion with the FLT3 inhibitor AG1296 decreased tyrosine phosphorylation of both FLT3 and β-catenin (upper panel, lanes 3 and 4). These results suggest that active FLT3 kinase can directly phosphorylate tyrosine residues of β-catenin.

Tyrosine phosphorylation of β-catenin in mononuclear cells obtained from FLT3-ITD-positive AML patients is suppressed by the FLT3 inhibitor CEP-701

To determine whether these observations are of clinical relevance, we investigated the effect of FLT3 inhibition on the tyrosine phosphorylation status of β-catenin in mononuclear cells of AML patients. Four individual isolates of bone marrow mononuclear cells obtained from patients harboring the FLT3-ITD mutation were treated *ex vivo* with the FLT3 inhibitor CEP-701 (50 nM). As shown in Figure 7, this treatment markedly reduced the level of FLT3 phosphorylation ($P=0.0046$). In each case, tyrosine phosphorylation of β-catenin was concomitantly reduced in a time-dependent manner ($P=0.043$).

Discussion

Wnt signaling is required for normal hematopoiesis, and deregulated Wnt signaling has been implicated in the etiology and progression of several hematologic malignancies.[27–31] Wnt effector β-catenin is expressed in both lymphoid and myeloid leukemia cell lines and primary cells. Inhibition of Wnt/β-catenin signaling in Jurkat T-lymphoblastic cells impaired proliferation and increased susceptibility to apoptosis in response to Fas ligation.[28] In multiple myeloma (MM), β-catenin protein was found to be overexpressed, and stimulation by various Wnts led to its accumulation and nuclear translocation accompanied by cell proliferation.[29] In colorectal cancer, truncation or loss of the APC protein, or mutation of the GSK-3β phosphorylation sites in β-catenin, is thought to be critical mechanism underlying β-catenin cytoplasmic and nuclear accumulation, promoting the expression of β-catenin-regulated pro-proliferative and survival genes.[20,39] However, Wnt signaling was reported to be increased in AML and MM without mutation of APC or β-catenin,[29,31] suggesting that alternative mechanisms might contribute to β-catenin deregulation in hematologic malignancies. Recently, Wnt-independent signaling has been suggested to cooperate with FLT3 in myeloid transformation. Protein expression and activity of β-catenin in fostering TCF/LEF-dependent transcription were increased in FLT3-ITD-32D cells independent of extracellular Wnt ligand.[32] However, the mechanism underlying this phenomenon has remained unclear.

Tyrosine phosphorylation of β-catenin has been studied not only for its impact on the physical association between β-catenin and E-cadherin but also for its relationship to β-catenin-dependent TCF transcriptional activity. Phosphorylation of Tyr-142 and Tyr-654 on β-catenin promotes its release from E-cadherin and its accumulation in the cytoplasm.[25,40,41] Since β-catenin has been shown to freely equilibrate between the

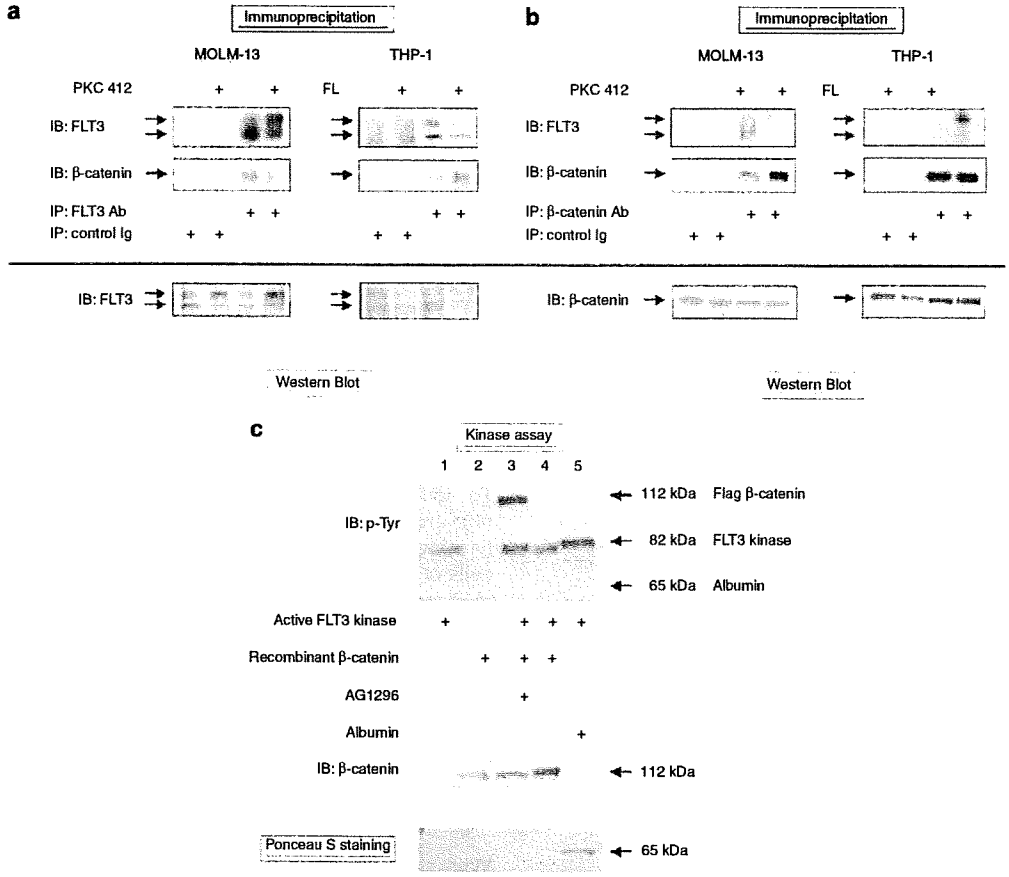

Figure 6 β-Catenin can be directly phosphorylated by FLT3. (**a**) Physical interaction between FLT3 and β-catenin. (**a**, **b**) MOLM-13 and THP-1 cells were treated with or without PKC412 or FL, respectively. After cell lysis, 1 mg of total protein was immunoprecipitated with an antibody recognizing 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

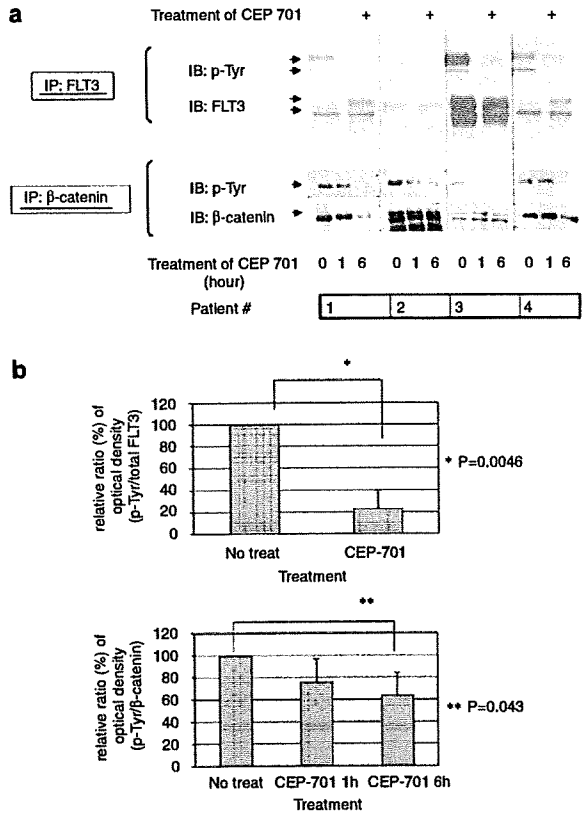

Figure 7 Tyrosine phosphorylation of β-catenin in bone marrow mononuclear cells of FLT3-ITD-positive AML patients is suppressed following FLT3 inhibition. (**a**) A total of 2×10^7 cells from patients with FLT3-ITD-positive AML were incubated with or without CEP-701 (50 nM) for the times indicated and 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.

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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

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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

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

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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.

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.

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 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

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