Mann-Whitney U test, we found that the patients with stage IV showed higher expression of *MET* than those with stage I–III ($P = 0.04$). Furthermore, the expression level of *MET* was significantly higher in the patients who died of cancer than those who were alive ($P = 0.02$). In all samples including cell lines, the group with *PAX3-FOXO1A* or *PAX7-FOXO1A* transcript showed higher expression of *MET* than that without such transcripts ($P = 0.04$). There was no statistical significance between the groups classified by histology ($P = 0.16$) and age ($P = 0.27$), although a tendency of higher expression of *MET* was shown in ARMS as well as in patients over 3 years old (Fig. 2A). Using the median expression level (58.30) as a cutoff, we also compared the difference in proportions among groups by Fisher's exact test. In all samples, overexpression of *MET* (>58.30) was detected in 7 of 9 ARMSs compared with that in 5 of 15 ERMSs ($P = 0.05$), as well as in 7 of 8 samples with *PAX3-FOXO1A* or *PAX7-FOXO1A* transcripts compared with that in 5 of 16 samples without such transcripts ($P = 0.03$). In fresh tumors, overexpression of *MET* was shown in 7 of 9 patients who died of cancer but only in 1 of 8 patients who survived ($P = 0.03$), as well as in 5 of 6 patients with stage IV compared with 3 of 11 patients with stage I–III ($P = 0.05$). The survival curve on Kaplan-Meier analyses showed that higher expression of *MET* correlated with poorer survival ($P = 0.05$, log-rank test) (Fig. 3).

On the other hand, significantly lower expression levels of $p16^{INK4A}$ and $p14^{ARF}$ were found in patients younger than 3 years ($P = 0.0065$ and $P = 0.0009$, respectively); however, the expression levels of $p16^{INK4A}$ and $p14^{ARF}$ showed no significant difference between groups classified by stage, histology, expression of *PAX3-FOXO1A* or *PAX7-FOXO1A* transcript, or prognosis (Mann-Whitney U test; Figs. 2B and 2C).

DISCUSSION

It has been reported that HGF/SF-MET signals induce proliferative and antiapoptotic responses in various cell types (Trusolino and Comoglio, 2002; Birchmeier et al., 2003). Analyses of HGF/SF and Met in mice have shown their essential regulatory role in development, such as the growth and survival of epithelial cells and migration of myogenic precursor cells (Bladt et al., 1995; Schmidt et al., 1995; Uehara et al., 1995). Upregulation of MET and HGF/SF expression is observed in several injured tissues, whereas deregulation of MET and HGF/SF signaling has emerged as a crucial feature

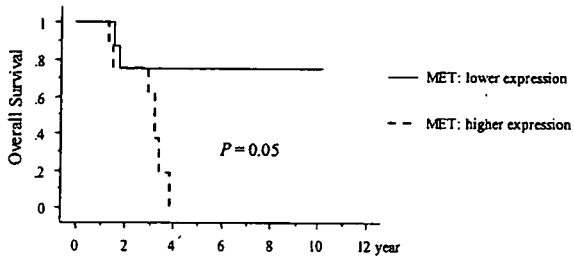

Figure 3. Kaplan-Meier curve for overall survival rates of patients with higher (>median) or lower expression (≤median) of *MET*. *P* value was calculated by log-rank test.

of many human malignancies. For instance, germline or somatic activating mutations in the tyrosine kinase domain of *MET* have been demonstrated in papillary renal carcinomas (Schmidt et al., 1997), in childhood hepatocellular cancer (Park et al., 1999), and in metastases of carcinoma (Lorenzato et al., 2002). Overexpression and/or amplification of *MET* with autocrine or paracrine loop have been noted in carcinomas of breast (Tuck et al., 1996), thyroid (Di Renzo et al., 1992), and pancreas (Di Renzo et al., 1995), as well as in sarcomas, such as osteosarcoma (Ferracini et al., 1995) and RMS (Ferracini et al., 1996). Characterization of MET and HGF/SF activities in proliferation, invasion, angiogenesis, and antiapoptosis delineates the stages at which these molecules participate in tumor progression (Birchmeier et al., 2003). Furthermore, a number of studies have shown that HGF/SF and/or MET over- or misexpression often correlates with poor prognosis in many malignancies (Birchmeier et al., 2003). It is also reported that Pax3 modulates the expression of *Met* during limb muscle development by mediating the migration of myogenic precursor cells into the limb anlage (Bladt et al., 1995; Epstein et al., 1996), while the expression of *MET* is repressed in differentiated myotubes and in adult nondividing muscle cells (Sonnenberg et al., 1993). The *PAX3-FOXO1A* fusion protein upregulates the expression of *MET* in alveolar type RMS (Ginsberg et al., 1998).

In this study, we detected the expression of *MET* in all of the RMS samples by RQ-PCR and the expression levels showed a large variety among samples. This is comparable to an early report that the expression of *MET* was detected in 6 of 6 RMS cell lines and 9 of 14 RMS fresh tumors by Western blot analysis (Ferracini et al., 1996). In their study, cell lines RD and SJRH-30 with a higher expression of MET also showed amplification of *MET* on Southern blot analysis, whereas SJRH-1 and SJRH-4 with a relatively lower expression level showed no amplification. We found that the expression level of

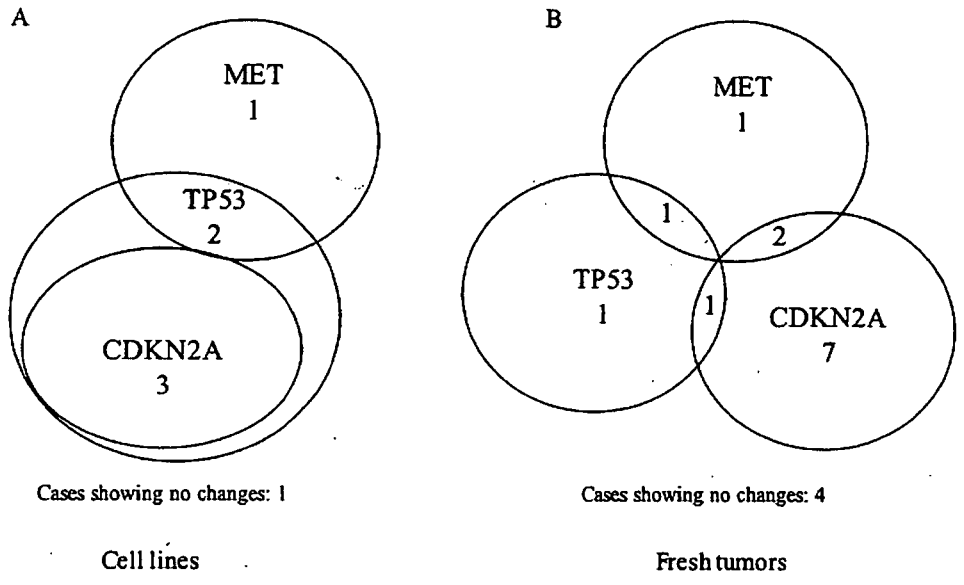

Figure 4. The distributions of overexpression of *MET* (> mean value), reduced or absent expression of $p16^{INK4A}$ and $p14^{ARF}$ (< half the mean value), and the mutations of *TP53* in seven cell lines (A) and 17 fresh tumors (B).

MET is higher in ARMS cell lines SJRH-4, SJRH-18, SCMC-RM2, and SJRH-30 than in ERMS cell lines SJRH-1, RMS, and RD by RQ-PCR (Fig. 1A). These results were also consistent with the results of Western blotting for MET protein expression. Furthermore, immunoprecipitation analysis showed constitutive tyrosine phosphorylation of MET in SJRH-4, SJRH-30, and SCMC-RM2, which indicated an autocrine manner. There was no obvious change of the relative DNA copy number observed by RQ-PCR and no mutations detected by PCR-SSCP in all samples. Thus, our results indicated that overexpression of *MET* in RMS was probably not induced by amplification/hyperploidy and that mutations of *MET* may not be of major pathogenetic importance in RMS.

Our data also showed a significantly higher expression level of *MET* in patients who died of cancer than in patients who were alive. The higher expression level was also observed in patients with stage IV as well as in patients with chimeric transcripts. A tendency toward higher *MET* expression was observed in ARMS as well as in patients over 3 years of age. Similar results were obtained when using the median level as a cutoff for *MET* expression. The survival curve on Kaplan-Meier analyses further confirmed that the expression level of *MET* correlates with the outcome of RMS patients. In RMS, it has previously been shown that older age at diagnosis, advanced stage, alveolar type, and expression of *PAX3-FOXO1A* are associated with poor prognosis (Crist et al., 1990; Newton et al., 1995; Sorensen et al., 2002). Earlier studies have also suggested that HGF/SF stimulates transmigration and invasiveness of RMS cells in vitro (Ferracini et al., 1996). It has also been reported recently that Met is necessary for a *Pax3-Foxo1a*-mediated effect in mice, and that Met has a role in RMS maintenance (Taulli et al., 2006). Taken together, it seems that MET may play an important role in the progression of RMS. A larger panel of samples should be studied to confirm this.

The *CDKN2A* gene encodes two unrelated proteins that function in tumor suppression: $p16^{INK4A}$ binds to and inhibits the activity of CDK4 and CDK6, and $p14^{ARF}$ promotes MDM2 (transformed 3T3 cell double min 2, TP53 binding protein) degradation and arrests the cell cycle in a TP53-dependent manner (Zhang et al., 1998). Thus, deletion of the *CDKN2A* locus simultaneously impairs both INK4A-cyclinD/CDK4-RB and ARF-MDM2-TP53 pathways. Mutations, homozygous deletions and altered expression of *CDKN2A* have been discovered in a wide range of human solid tumors as well as hematological malignancies (Kamb et al., 1994; Okamoto et al., 1994; Ohnishi et al., 1995; Takita et al., 2004). There has been some controversy about the significance of $p16^{INK4A}/p14^{ARF}$ in predicting the prognosis of malignancies such as neuroblastoma (Takita et al., 1998; Omura-Minamisawa et al., 2001) and childhood acute lymphoblastic leukemia (Mekki et al., 1999; Dalle et al., 2002). The association of $p16^{INK4A}$ and $p14^{ARF}$ expression with prognosis in RMS has not been well docu-

mented. In this study, we identified a nonsense mutation at codon 80 of $p16^{INK4A}$ in one cell line, SJRH-18, resulting in reduced expression of both $p16^{INK4A}$ and $p14^{ARF}$. This result is consistent with an early report showing that *CDKN2A* mutations are rare in RMS (Iolascon et al., 1996). No significant correlation was shown between the reduced or absent expression of $p16^{INK4A}$ and $p14^{ARF}$ and the clinicopathological factors, except for patients' age, which showed that the expression levels of $p16^{INK4A}$ and $p14^{ARF}$ were significantly lower in patients younger than 3 years. There were 3 of 7 cell lines (two ERMSs and one ARMS) and 2 of 17 fresh tumors (one ERMS and one ARMS) having less than a half of the mean DNA copy number, which suggested a loss of heterozygosity (LOH) in these samples; however, no homozygous deletion of *CDKN2A* was detected. The mRNA expression level of $p16^{INK4A}$ and $p14^{ARF}$ showed no obvious correlation with the relative DNA copy number.

Marked synergism between aberrant Met signaling and *Ink4a/Arf* inactivation has been shown to induce RMS at high frequency in mice (Sharp et al., 2002). Furthermore, *Pax3-Foxo1a* homozygosity with accompanying Ink4a/Arf or Tp53 pathway disruption substantially increases the frequency of alveolar RMS tumor formation in mice (Keller et al., 2004a). As shown in Figure 4A, 2 cell lines with overexpression of *MET* also had mutations of *TP53*; however, no cell lines with overexpression of *MET* showed reduced or absent expression of *CDKN2A*. Interestingly, all of the cell lines with reduced or absent expression of *CDKN2A* had mutations of *TP53*. Of 17 fresh tumors (Fig. 4B), 10 showed reduced or absent expression of *CDKN2A*; 2 of them with overexpression of *MET* and 1 of them having mutation of *TP53* simultaneously. One sample with overexpression of *MET* had mutation of *TP53*. Only 1 of 7 cell lines and 1 of 17 fresh tumors showed overexpression of *MET* alone, and one cell line and four fresh tumors did not show changes in any of the three genes.

In conclusion, our data suggest that *MET*, *CDKN2A* as well as *TP53* are involved in the pathogenesis of RMS, and that *MET* may play an important role in the progression of RMS. Molecules that specifically inhibit MET and HGF/SF are therefore promising in the treatment of RMS patients predicted to have poor prognosis.

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Letter to the Editor

Low Frequency of *KIT* Gene Mutation in Pediatric Acute Myeloid Leukemia with inv(16)(p13q22): A Study of the Japanese Childhood AML Cooperative Study Group

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Acute myeloid leukemia (AML) patients with t(8;21)(q22;q22) or inv(16)(p13q22) are known to have a good prognosis. Recently, mutations of the *KIT* gene have been found in 12.7% to 48.1% of adult AML patients with t(8;21) or inv(16) and in approximately 20% of pediatric AML patients with t(8;21) [1-5]. *KIT* gene mutations in adult and pediatric AML patients with t(8;21) and in adult AML patients with inv(16) have been associated with a poorer prognosis than in those without *KIT* gene mutations [1-5]. However, the frequency and clinical impact of *KIT* gene mutations in pediatric AML patients with inv(16) remain unknown. Pediatric AML patients with inv(16) have been reported to represent 3.4% to 6% of the total number of pediatric AML patients. Thus, the number of patients in this subgroup is very small [6,7].

Three hundred eighteen patients were enrolled in the Japanese Childhood AML Cooperative Study Group Protocol AML 99 from January 2000 to December 2002, and 12 (3.8%) of these AML patients comprised 11 patients with inv(16) and 1 patient with t(16;16)(p13;q22) [5,8]. The 5-year overall survival rate was 100%, and the event-free survival rate was 90.9%. Of these 12 AML patients with inv(16) or t(16;16), 7 patients were available for molecular analysis (age range, 11 months to 14 years; median, 10 years) (Table 1). The 5-year overall survival rate for these 7 patients was 100%, and the event-free survival rate was 85.7% (Table 1). We used the reverse transcriptase–polymerase chain reaction method in a mutational analysis of the extracellular domain (exons 8 and 9), the transmembrane domain (exon 10), the juxtamembrane domain (exon 11), and the second intracellular kinase domain (exons 17 and 18) of the *KIT* gene and then carried out a sequencing analysis [5]. Sequencing was performed directly or, if necessary, after subcloning.

KIT mutation (deletion of D419 in exon 8) was found in an 11-month-old male patient (1 [14.3%] of 7 patients). The initial white blood cell count for this patient (no. 7) was 199,000/μL, and a karyotype analysis revealed 46,XY,inv(16)(p13q22). This patient received a total of 6 consecutive chemotherapies; however, he relapsed 16 months after the initial diagnosis. He then underwent unrelated allogeneic stem cell transplantation during the second complete remission and has been alive for 40 months from the diagnosis. The remaining 6 AML patients with inv(16) have maintained a complete remission without relapse for more than 41 months.

As for *FLT3* and *RAS* gene alterations, we found 2 *FLT3* D835 mutations (28.6%) and 2 *NRAS* mutations (28.6%) in these 7 AML patients with inv(16) (Table 1). No patient had an *FLT3* internal tandem duplication or a *KRAS* gene mutation. The majority of these patients (5 [71.4%] of 7) had one of the chimeric *CBFβ-MYH11* transcripts, which have most frequently been found in AML cases with inv(16) (*CBFβ* at nucleotide 495 fused to

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Table 1.
Correlations of Clinical Features with *KIT*, *FLT3*, and *RAS* Gene Mutations in 7 Acute Myeloid Leukemia Patients with inv(16) or t(16;16)*

Patient No.	Age	Sex	FAB Classification	Karyotype	*CBFβ-MYH11*	*KIT* Mt	*FLT3* D835 Mt	*NRAS* Mt	EFS Time, mo
1	14 y	M	M4Eo	46,XY,inv(16)(p13q22), add(7)(q32)	†	–	–	+‡	63+
2	10 y	M	M4Eo	46,XY,inv(16)(p13q22)	1	–	–	–	67+
3	7 y	M	M1	46,XY,inv(16)(p13q22)	1	–	–	–	63+
4	13 y	F	M1	46,XX,inv(16)(p13q22)	1	–	+	–	59+
5	3 y	F	M5a	47,XX,+8,t(16;16)(p13;q22)	2	–	–	+‡	43+
6	14 y	F	M5b	46,XX,inv(16)(p13q22)	1	–	+	–	41+
7	11 mo	M	M4Eo	46,XY,inv(16)(p13q22)	1	+§	–	–	16

**CBFβ-MYH11* transcripts were detected by reverse transcriptase–polymerase chain reaction analysis and direct sequencing (1, nucleotide 495 of *CBFβ* fused to nucleotide 1921 of *MYH11*; 2, nucleotide 399 of *CBFβ* fused to nucleotide 1201 of *MYH11*). *KRAS* mutations were not found in any of the 7 patients. FAB, French-American-British; Mt, mutation; EFS, event-free survival.

†Chimeric transcripts were not detected.

‡Codon 12.

§Deletion D419 in exon 8.

MYH11 at nucleotide 1921, Table 1) [9,10]. The *FLT3* D835 mutation, *NRAS* mutation, and subtypes of *CBFβ-MYH11* transcripts were not associated with the clinical outcome.

We also looked for *KIT* mutations in 11 pediatric AML patients with inv(16) who were treated with the previous protocol in Japan (age range, 8 months to 15 years; median, 3 years), but we did not identify *KIT* mutations in any of the patients. Interestingly, 3 of the 11 AML patients with inv(16) were infants, and 2 of them died, although all 3 exhibited no mutations in *KIT*, *FLT3*, or *RAS*. These data together with those described in our previous report [11] suggest that infant AML patients with inv(16) have a poor prognosis, regardless of the status of these genes.

A few reports have suggested that adult AML patients who have inv(16) with *KIT* mutations were associated with a poorer prognosis than those without KIT mutations [2,3]. A recent study by the Berlin-Frankfurt-Münster Study Group revealed that 6 (54.5%) of 11 pediatric AML patients with inv(16) had *KIT* mutations but that the clinical impact was limited [12]. The Acute Leukemia French Association (ALFA) and the Leucémies Aiguës Myéloblastiques de l'Enfant (LAME) cooperative study groups also suggested that *KIT* gene mutations were not associated with a poor prognosis in pediatric and adult AML patients with inv(16) [4]. We considered the frequency of *KIT* gene mutations (1 [5.6%] of 18) among the pediatric AML patients with inv(16) in this study to be lower than that of adult AML patients with inv(16) [2-4]. We must await the results of a larger study regarding the correlation between *KIT* gene mutations and prognosis in pediatric AML patients with inv(16).

Acknowledgments

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Fms-like Tyrosine Kinase 3 Ligand Stimulation Induces *MLL*-Rearranged Leukemia Cells into Quiescence Resistant to Antileukemic Agents

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Abstract

Fms-like tyrosine kinase 3 (FLT3) is highly expressed in acute lymphoblastic leukemia with the *mixed-lineage leukemia* (*MLL*) gene rearrangement refractory to chemotherapy. We examined the biological effect of FLT3-ligand (FL) on 18 B-precursor leukemic cell lines with variable karyotypic abnormalities, and found that nine of nine *MLL*-rearranged cell lines with wild-type FLT3, in contrast to other leukemic cell lines, are significantly inhibited in their proliferation in a dose-dependent manner by FL. This inhibition was due to induction of the G_0-G_1 arrest. A marked up-regulation of p27 by suppression of its protein degradation and an abrogation of constitutive signal transducers and activators of transcription 5 phosphorylation were revealed in arrested leukemia cells after FL stimulation. Importantly, FL treatment rendered not only cell lines but also primary leukemia cells with *MLL* rearrangement resistant to chemotherapeutic agents. *MLL*-rearranged leukemia cells adhering to the bone marrow stromal cell line, which expresses FL as the membrane-bound form, were induced to quiescent state resistant to chemotherapeutic agents, but their chemosensitivity was significantly restored in the presence of neutralizing anti-FL antibody. The FL/FLT3 interaction between leukemia cells and bone marrow stromal cells expressing FL at high levels should contribute, at least in part, to persistent minimal-residual disease of *MLL*-rearranged leukemia in bone marrow. [Cancer Res 2007;67(20):9852–61]

Introduction

Fms-like tyrosine kinase 3 (FLT3) belongs to the receptor tyrosine kinase class III family and plays an important role in an early stage of hematopoiesis (1–3). In normal bone marrow, FLT3 is expressed predominantly in hematopoietic stem/progenitor cells (2, 4) and FLT3-ligand (FL) shows a strong synergy with other cytokines in their proliferation (5, 6). FLT3 is also expressed at considerable levels in most clinical samples from acute myelogenous leukemia (AML) and B-precursor acute lymphoblastic leukemia (ALL) patients (7, 8). However, the growth-promoting activity of FL alone in AML and ALL cells is heterogeneous and rather modest in comparison with other effective cytokines (6, 8, 9). Recently, two types of FLT3 mutations were found in leukemic cells, which activate constitutively the FLT3 tyrosine kinase. One is internal tandem duplications (ITD) in the juxtamembrane domain of FLT3 (10), and the other is a point mutation of D835/I836 within the activation loop of the second tyrosine kinase domain of FLT3 (FLT3-TK mutation; refs. 11, 12). Although less frequently, active point mutations other than D835/I836 have been known in the juxtamembrane domain of FLT3. Several studies have shown that FLT3-ITDs confer a poor prognosis in adult AML (13, 14) and in childhood AML (15, 16).

The *mixed-lineage leukemia* (*MLL*) gene rearrangement results from chromosomal translocation at 11q23, and frequently observed in infantile ALL and therapy-related secondary leukemia with a very poor prognosis (17, 18). Armstrong et al. (19) reported that *MLL*-rearranged leukemia has a highly distinct gene expression profile that is consistent with the developmental stage of a very early hematopoietic progenitor, and that the *FLT3* gene is the most differentially expressed gene that distinguishes *MLL*-rearranged leukemia from other subtypes of ALL and AML. We recently showed that a FLT3-TK mutation is also detected in 16% of *MLL*-rearranged ALL in infants (20). Under the hypothesis that constitutive activation of FLT3 might be involved in maintenance and development of *MLL*-rearranged leukemia as a second hit, new therapeutic approaches using FLT3-targeted tyrosine kinase inhibitors are emerging for the treatment of *MLL*-rearranged leukemia overexpressing FLT3 with or without activating mutations (21, 22).

In the present study, we showed that FL stimulation specifically induced *MLL*-rearranged leukemia cells into quiescence resistant to antileukemic agents, and postulated that the FL/FLT3 interaction possibly implicated in the formation of minimal residual disease (MRD) of this leukemia.

Materials and Methods

Human cell lines and primary leukemia cells. Twenty human lymphoid leukemic cell lines were used in this study. Eighteen were B-lineage including 10 with *MLL* rearrangement, 4 with Philadelphia chromosome (Ph1), and 4 with other karyotypes. Two T-lineage cell lines were also used as controls. All cell lines have been established in our laboratory and are maintained in RPMI 1640 supplemented with 10% FCS as reported previously (23, 24). Their characteristics are summarized in

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Table 1. Characteristics of B-precursor leukemic cell lines and their FLT3 expression

	Surface antigens			Karyotype	FLT3 expression	
	CD 10	19	13		%	MFI
MLL rearrangement						
KOCL-33	−	+	−	t(11;19)(q23;p13)	99	204.1
KOCL-44	−	+	−	t(11;19)(q23;p13)	99	340.1
KOCL-45	−	+	+	t(4;11)(q21;q23)	95	115.0
KOCL-50	−	+	−	t(11;19)(q23;p13)	77	80.3
KOCL-51	−	+	+	t(11;19)(q23;p13)	98	162.1
KOCL-58	−	+	+	t(4;11)(q21;q23)	79	41.9
KOCL-69	−	+	+	t(4;11)(q21;q23)	57	63.6
KOPN-1	−	+	−	t(11;19)(q23;p13)	94	169.1
KOPB-26	−	+	−	t(9;11)(p22;q23)	99	1046.8
YACL-95	+	+	−	t(9;11)(p22;q23)	98	381.3
MLL germ line						
KOPN-30bi	+	+	+	t(9;22)(q34;p11.2)*	88	75.1
KOPN-55bi	+	+	+	t(9;22)(q34;p11.2)†	90	367.0
KOPN-57bi	+	+	+	t(9;22)(q34;p11.2)*	99	581.7
KOPN-72bi	+	+	+	t(9;22)(q34;p11.2)*	99	266.3
KOPN-32	−	+	−	t(5;11)(q31;q21)	73	54.0
KOPN-36	+	+	−	t(1;19)(q23;p13.3)	22	53.3
YAMN-92	+	+	−	t(1;19)(q23;p13.3)	28	43.8
KOPN-70	+	+	−	-X,-4,-17,+3mar	98	329.5

Abbreviation: MFI, mean fluorescence intensity.
*Minor bcr-abl.
† Major bcr-abl.

Table 1. KOCL-33 with t(11;19) is a special cell line with a D835 point mutation of FLT3 (20), whereas other cell lines with or without *MLL* gene rearrangement had neither FLT3-TK mutation nor ITDs. Human bone marrow stromal cell line KM-104 was used as the feeder expressing FL as the membrane-bound form (25). Fourteen primary leukemia samples with (n = 9) and without (n = 5) *MLL* rearrangement were also used after obtaining written informed consents.

Antibodies and reagents. FL was purchased from Peprotech. Biotinylated FL and neutralizing anti-FL monoclonal antibody (mAb) were purchased from R&D Systems. Cycloheximide, bromodeoxyuridine (BrdUrd), and propidium iodide were purchased from Sigma. Daunorubicin and 1-β-D-arabinofuranosylcytosine (AraC) were obtained from Meiji Seika and Nippon Shinyaku, respectively. PKC412 (*N*-benzoylstaurosporine, a FLT3 kinase inhibitor) was kindly provided by Novartis. Polyclonal antibodies against phosphorylated signal transducers and activators of transcription 5 (STAT5; Tyr^{694}), p44/42 mitogen-activated protein kinase (MAPK), phosphorylated p44/42MAPK (Thr^{202}/Tyr^{204}), Akt, and phosphorylated Akt (Ser^{473}) were purchased from Cell Signaling Technology; polyclonal antibodies against cyclin-dependent kinase 4 (CDK4), cyclin E, and p27 were from Upstate Biotechnology, Inc.; CDK2 was from PharMingen; and cyclin A was from Santa Cruz Biotechnology. mAbs against cyclin B, cyclin D3, and STAT5 were purchased from Transduction Laboratories; phosphotyrosine, CDK6, p21, c-Myc, and α-tubulin were from UBI, Lab Vision Corporation, PharMingen, Santa Cruz Biotechnology, and Sanbio, respectively.

Flow cytometric analysis. For FLT3 expression, 5×10^5 leukemia cells were incubated with biotinylated FL for 1 h at 4°C followed by incubation with avidin-FITC for 30 min at 4°C. As a negative staining control, anti-FL blocking antibody was mixed with FL-biotin. For FL expression, cells were incubated with biotinylated anti-FL antibody followed by incubation with avidin-FITC. IgG-biotin was used for a negative staining control. These cells were washed and analyzed using a flow cytometer (FACSCalibur, Becton Dickinson).

[^{3}H]thymidine uptake analysis. Leukemia cells (2.5×10^4 to 5×10^4 per well) were cultured in RPMI 1640 supplemented with 10% FCS in a 96-well flat-bottomed culture plate in triplicate in the presence or absence of various concentrations of FL at 37°C for the indicated periods. Subsequently, wells were pulsed with [^{3}H]thymidine (1 μCi/well) for 4 h, after which the cells were harvested onto glass-fiber filters. In some experiments, a FLT3 kinase inhibitor, PKC412, was included at various concentrations. [^{3}H]thymidine incorporated to DNA was measured using a liquid scintillation counter. Percentage stimulation was calculated as follows: {[(cpm of treated) / (cpm of untreated)] − 1} × 100. Percentage thymidine uptake was calculated as follows: {(cpm of treated) / (cpm of untreated)} × 100.

Cell cycle analysis. Leukemia cells (5×10^5/mL) were cultured in the presence or absence of FL (20 ng/mL) for 3 days. These cells were pulsed with BrdUrd for 30 min at 37°C and harvested. After fixation in 70% ethanol on ice, cells were treated with RNase (Funakoshi) for 15 min at 37°C, and subsequently washed with 4 N HCl, 0.1 mol/L $Na_2B_4O_7$, and 0.5% Tween 20. Cells were stained with FITC-conjugated anti-BrdUrd antibody for 20 min at 37°C and then treated with propidium iodide (50 μg/mL) for 20 min on ice. The signals generated by FITC and propidium iodide were analyzed using a flow cytometer.

Apoptosis analysis. Leukemia cells (5×10^5/mL) were incubated for 24 h in the presence or absence of FL (20 ng/mL) and then irradiated (4 Gy) followed by culture for 48 h, or exposed to daunorubicin (10 ng/mL) or AraC (200 nmol/L) for 48 h. Cells were then harvested and stained doubly with FITC-conjugated Annexin V and propidium iodide (MEBCYTO Apoptosis Detection Kit; MBL) at 37°C for 15 min in dark. Ten thousand events were analyzed using a flow cytometer.

Western blot analysis. Leukemia cells were solubilized in lysis buffer [50 mmol/L Tris-HCl (pH 7.5), containing 150 mmol/L NaCl, 1% NP40, 5 mmol/L EDTA, 0.05% NaN_3, 0.2 TIU/mL aprotinin, 1 μg/mL pepstatin A, 10 mmol/L iodoacetamide, 1 mmol/L phenylmethylsulfonyl fluoride, and 1 mmol/L sodium vanadate]. SDS was added to the lysis buffer

at a final concentration of 0.1% for analysis of nuclear proteins. The lysates were separated on a 6% to 15% SDS-polyacrylamide gel under reducing conditions and transferred to nitrocellulose membranes, which were incubated with various primary antibodies at 4°C overnight, and then with horseradish peroxidase-conjugated second antibody for 1 h at room temperature. The detection of the bands was done using an enhanced chemiluminescence kit (Amersham Japan).

RNase protection assays. Leukemia cells (5×10^5/mL) were cultured in the presence or absence of FL (20 ng/mL) for 24, 48, and 72 h, and their total RNAs were extracted. The RNase protection assay was done using [^{32}P]UTP-labeled multiprobe template hCC-1 and the RiboQuant Multi-Probe RNase Protection Assay system (PharMingen).

Dye-exclusion test. Leukemic cell lines (4×10^4/well) were precultured in the presence or absence of FL (20 ng/mL) for 24 h, and then exposed to daunorubicin (10–20 ng/mL) or AraC (200–400 nmol/L) for the indicated hours. In some experiments, the human bone marrow–derived stromal cell line KM-104 was used as the feeder. In analysis of primary samples, leukemia cells (1×10^5/well) were precultured in the presence or absence of FL (40 ng/mL) or KM-104 cells with or without anti-FL antibody for 24 h, and then exposed to AraC (100 nmol/L) for 24 h. The numbers of living and dead cells were counted by dye-exclusion test in triplicate after each of culture conditions, and viability (%) and Δviability (treated viability − control viability) were calculated.

Statistics. The Mann-Whitney's test was used for the comparison of the differences in FLT3 expression among leukemic cell lines and fresh leukemia cells, and unpaired *t* test for the comparison of the differences in [^{3}H]thymidine uptake and dye-exclusion analyses. Differences in the Δviabilities of primary leukemia cells between culture conditions were analyzed using the matched paired Wilcoxon's test. A P value of <0.05 was considered significant.

Results

Surface expression of FLT3 in leukemic cell lines. Surface expression of FLT3 in leukemic cell lines used in this study was first checked. B-precursor cell lines (n = 18) expressed a considerable amount of FLT3 on their surfaces (median 94.5%, range 22–99%) as shown in Table 1, whereas T-lineage cell lines (n = 2) did not (<10%; data not shown). There were no significant differences in percentage of FLT3-positive cells between B-precursor cell lines with (n = 10) and without (n = 8) *MLL* rearrangement (median of positive population; 96.5% versus 89.0%). There were also no significant differences in the FLT3 expression levels between B-precursor cell lines with and without *MLL* rearrangement (median of mean fluorescence intensity; 165.6 versus 170.7).

FL stimulation inhibits proliferation of *MLL*-rearranged leukemia cells by induction of cell cycle arrest. To investigate the biological effect of FL, 18 B-precursor leukemic cell lines were incubated in the presence or absence of various concentrations of FL for 72 h, and their [^{3}H]thymidine uptakes were assayed in the final 4-h incubation. As shown in Fig. 1*A*, three of four Ph1-positive leukemic cell lines showed stimulative responses to FL in a dose-dependent manner. Two of other four B-precursor cell lines without *MLL* rearrangement also showed a marked stimulative response to FL. Unexpectedly, however, all of the *MLL*-rearranged cell lines with wild-type FLT3 (n = 9), irrespective of the types of translocation, showed inhibitory responses to FL in a dose-dependent manner as shown in Fig. 1*B*. The *MLL*-rearranged cell line with a D835 mutation, KOCL-33, was not affected by the addition of FL. The kinetics of [^{3}H]thymidine uptakes after FL stimulation (20 ng/mL) was next analyzed using representative cell lines. The FL-induced inhibition in *MLL*-rearranged KOCL-51 and KOCL-58 reached maximal levels between 48 and 96 h of culture, whereas the FL-induced stimulation in KOPN-55bi (Ph1), KOPN-36 with t(1;19), and KOPN-70 (other B-precursor) peaked between 96 and 144 h of culture (data not shown). These results indicate that,

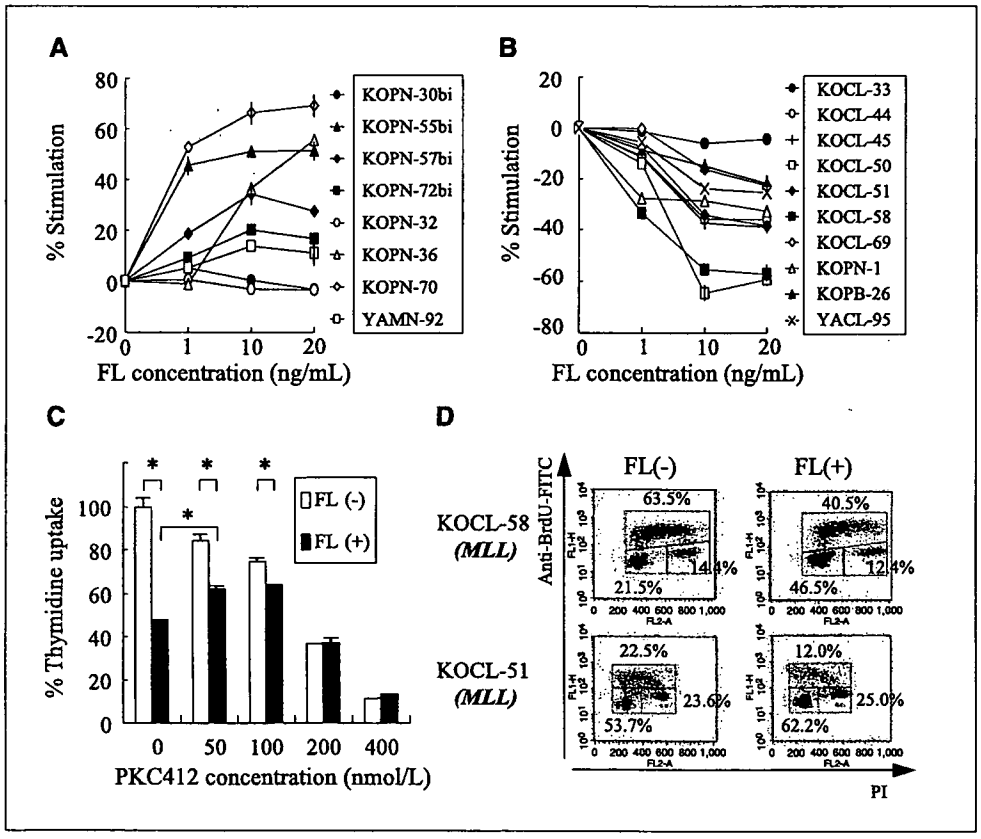

Figure 1. Effect of FL on [^{3}H]thymidine uptakes and cell cycle progression in B-precursor leukemic cell lines. In [^{3}H]thymidine uptake analysis, leukemic cell lines were cultured in triplicate in the presence or absence of FL for 72 h, pulsed, and harvested. Data are representative of three separate experiments and are shown as the mean. In cell cycle analysis, *MLL*-rearranged KOCL-58 and KOCL-51 cells were cultured in the presence or absence of FL (20 ng/mL) for 72 h, and stained with FITC-conjugated BrdUrd and propidium iodide. *A*, % stimulation of [^{3}H]thymidine uptakes by various concentrations of FL in Ph1-positive (n = 4, *closed symbols*) and other B-precursor cell lines without *MLL* rearrangement (n = 4, *open symbols*). *Bars*, SE >3%. *B*, % stimulation of [^{3}H]thymidine uptakes by various concentrations of FL in *MLL*-rearranged leukemic cell lines (n = 10). KOCL-33 is a special cell line with a D835 mutation of FLT3. *Bars*, SE >3%. *C*, effect of the FLT3 inhibitor (PKC412) on % thymidine uptake. *MLL*-rearranged KOCL-58 cells were cultured in the presence or absence of FL (20 ng/mL) with or without the addition of various concentrations of PKC412 (50–400 nmol/L), and % thymidine uptake was determined in each of culture conditions. *, P < 0.01, significant difference by *t* test. *D*, flow cytometric analysis of cell cycle progression. *Vertical* and *horizontal axes*, log fluorescence intensities of FITC and propidium iodide (*PI*), respectively.

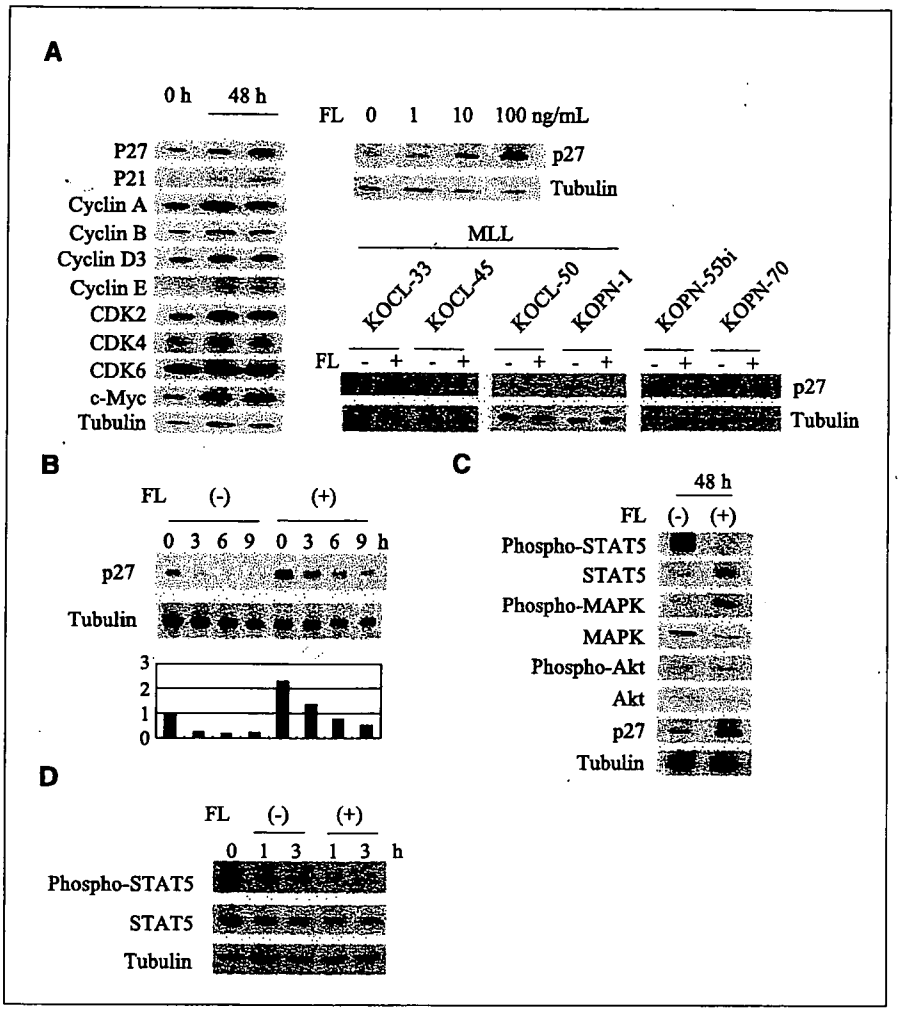

Figure 2. Changes in expression of cell cycle–related proteins and phosphorylation of FLT3-mediating signal transducing molecules after FL stimulation in *MLL*-rearranged leukemia cells. *A*, Western blot analysis of changes in expression of cell cycle–related proteins at 48 h after culture in the presence or absence of FL (20 ng/mL). *Left*, changes in expression of p27, p21; cyclins A, B, D3, and E; CDK2, CDK4, and CDK6; and c-Myc were examined in KOCL-58 cells. *Right, top*, changes in expression of p27 were examined in KOCL-58 cells after culture with increasing concentrations of FL; *bottom*, changes in expression of p27 were examined in leukemic cell lines with ($n = 4$) or without ($n = 2$) *MLL* rearrangement. *B*, half-life of p27 after FL stimulation. KOCL-58 cells were incubated for 24 h in the presence or absence of FL (20 ng/mL), further incubated in the presence of cycloheximide, and harvested at indicated time points. Changes in the p27 expression were measured by densitometry and half-life of p27 was calculated. Data are representative of three separate experiments. *C*, Western blot analysis of changes in phosphorylation of STAT5, MAPK, and Akt after FL stimulation. KOCL-58 cells were incubated for 48 h in the presence or absence of FL (20 ng/mL). *D*, Western blot analysis of changes in STAT5 phosphorylation after FL stimulation. KOCL-58 cells were incubated for 1 or 3 h in the presence or absence of FL (20 ng/mL).

in contrast to other types of leukemia cells, proliferation of *MLL*-rearranged leukemia cells with wild-type FLT3 is specifically suppressed by ligand activation of FLT3.

To determine whether the inhibitory effect of FL is dependent on the kinase activity of FLT3, *MLL*-rearranged KOCL-58 cells were cultured with or without FL (20 ng/mL) for 72 h in the presence of various concentrations of the FLT3 kinase inhibitor PKC412. As shown in Fig. 1*C*, PKC412 alone inhibited [^{3}H]thymidine uptake of KOCL-58 cells, as previously reported (21). Of note, thymidine uptake, which was inhibited by FL to 47.8% of the control level, was restored to 62.3% in the presence of 50 nmol/L PKC412, a concentration that can markedly suppress FLT3-mediating signal transduction molecules such as STAT5, MAPK, and Akt (Supplementary Fig. S1). This result suggests that the kinase activity of FLT3 is required for the FL-induced growth inhibition exhibited by *MLL*-rearranged leukemia cells.

To investigate the mechanism of the FL-induced growth inhibition seen in *MLL*-rearranged cell lines, cell cycle analysis was done at 72 h after FL treatment (20 ng/mL) using the BrdUrd/propidium iodide double staining method (Fig. 1*D*). In both KOCL-58 and KOCL-51 cell lines, the population in S phase after FL stimulation significantly decreased compared with that measured without FL stimulation with a concomitant increase in the population in G_0-G_1 phase. The population of cells in G_2-M phase remained unchanged after FL stimulation. Importantly, the apoptotic population, which appears in the hypodiploid region, did not increase in either cell line. These results were consistently observed in three separate experiments, indicating that the FL-induced growth inhibition seen in *MLL*-rearranged cell lines results from induction of G_0-G_1 arrest, but not from apoptosis.

Phosphorylation of FLT3, STAT5, MAPK, and Akt is transiently up-regulated after FL stimulation in *MLL*-rearranged leukemia cells. It is known that FLT3 is dimerized after FL stimulation, and this evokes biological effects through the signaling pathways acting via STAT5, RAS/p44/42 MAPK, and phosphatidylinositol 3-kinase/Akt (26–28). To investigate activation patterns of signaling pathways after FL stimulation in *MLL*-rearranged cell lines, changes in phosphorylation of STAT5, MAPK, and Akt in KOCL-58 cells were pursued by Western blot using antibodies against the phosphorylated (active) form of these molecules. FLT3 phosphorylation was examined using anti-phosphotyrosine antibody after FLT3 immunoprecipitation. FLT3, STAT5, MAPK, and Akt were constitutively phosphorylated before FL stimulation as we reported previously (20), and their phosphorylation was further up-regulated within 1 min after FL stimulation (100 ng/mL) and returned to the prestimulated level at 15 min (Supplementary Fig. S2). A similar pattern of changes in phosphorylation of these molecules after FL stimulation was observed in KOPN-70, which showed a stimulative response to FL in the [^{3}H]thymidine uptake assay (data not shown), suggesting that an opposite biological

effect of FL, that is, stimulation or suppression of cell growth, is not simply due to differences in an early event of signaling after the FL/FLT3 interaction.

FL stimulation markedly up-regulates p27 expression in *MLL*-rearranged leukemia cells. It is known that cell cycle progression is controlled primarily by activities of CDKs that are up-regulated or down-regulated by cyclins and CDK inhibitors (CDKI), including p16, p21, and p27, respectively. To examine the expression of these cell cycle-associated proteins after FL stimulation, KOCL-58 cells (5×10^5/mL) were cultured 48 h in the presence or absence of FL (20 ng/mL). As shown in Fig. 2*A* (*left*), expression levels of cyclins A, B, D3, and E, and CDK2, CDK4, and CDK6, were completely unchanged. Expression of p16 was not detected as reported previously (23). Although expression of p21 was modestly up-regulated by FL, this was not seen consistently in repeated experiments. Of interest, expression of p27 was consistently and markedly up-regulated in the presence of FL. This p27 up-regulation was observed in a dose-dependent manner in response to FL, and reached a maximum level at 100 ng/mL (Fig. 2*A*, *top right*). In other experiments, it was shown that p27 was maximally up-regulated at FL concentrations between 20 and 40 ng/mL. Moreover, the FL-induced p27 up-regulation 48 h after FL stimulation was also observed to varying degrees in other *MLL*-rearranged cell lines with wild-type FLT3, but not in KOCL-33 with a D835 mutation (Fig. 2*A*, *bottom right*). Importantly, the FL-induced up-regulation of p27 was more profoundly observed in KOCL-58 and KOCL-50, which showed a marked suppression of [^{3}H]thymidine uptake by FL than in KOCL-45 and KOPN-1, which showed a modest suppression of [^{3}H]thymidine uptake by FL. In KOPN-55bi, KOPN-70 without *MLL* rearrangement that showed stimulative responses to FL, the p27 expression was somewhat down-regulated after FL stimulation.

To determine the mechanism of the FL-induced p27 up-regulation, changes in p27 mRNA expression after FL stimulation were examined in KOCL-58 by RNase protection assay. The level of p27 mRNA was completely unchanged after FL stimulation (Supplementary Fig. S3), suggesting that p27 up-regulation might be mediated by a posttranscriptional mechanism. To determine p27 stability, KOCL-58 cells were cultured for 48 h in the presence or absence of FL (20 ng/mL), further cultured after the addition of cycloheximide (150 μg/mL), and harvested 3, 6, and 9 h later. As shown in Fig. 2*B*, the estimated half-life of p27 was elongated from 2.6 h in the absence of FL to 3.9 h in the presence of FL. These results suggest that up-regulation of p27 induced by FL is due to suppression of its protein degradation.

Phosphorylation of STAT5 is specifically abrogated after FL stimulation in *MLL*-rearranged leukemia cells. To determine the activation status of FLT3-mediating signaling pathways at the time point where p27 is up-regulated by FL stimulation, KOCL-58 cells were cultured in the presence or absence of FL (20 ng/mL) for 48 h, and phosphorylation of STAT5, MAPK, and Akt was examined. As shown in Fig. 2*C*, in contrast to marked phosphorylation of STAT5 seen after 48 h of culture without FL, the addition of FL to the culture completely abrogated its phosphorylation. In contrast, phosphorylation of MAPK was up-regulated in the presence of FL at this time point, whereas phosphorylation of Akt showed no difference in the two culture conditions. This STAT5 dephosphorylation was observed in KOCL-58 within 1 h after FL stimulation (Fig. 2*D*). These results suggest that, among FLT3-mediating signaling pathways, the STAT5 pathway is specifically suppressed after FL stimulation.

FL stimulation renders *MLL*-rearranged leukemic cells resistant to anti-leukemic agent–induced apoptosis. It is thought that sensitivity of leukemia cells to irradiation and chemotherapeutic agents is reduced in "dormant" cells whose cell cycle progression is kept in sustained suppression. To assess whether the FL-induced cell cycle arrest affects sensitivity to irradiation-induced apoptosis, *MLL*-rearranged KOCL-58 and KOCL-51 cells (both possessing wild-type p53) were precultured for 24 h in the presence or absence of FL (20 ng/mL) and then irradiated (4 Gy). Induction of apoptosis was examined after 48 h of culture in the presence or absence of FL using FITC-conjugated Annexin V and propidium iodide. As shown in Fig. 3*A* (*left*), the Annexin V–positive apoptotic population decreased by FL from 45.3% to 24.5% in KOCL-58 and from 43.0% to 26.0% in KOCL-51. The propidium iodide–positive late apoptotic population also decreased in the presence of FL in both cell lines, suggesting that irradiation-induced apoptosis is effectively suppressed by pretreatment with FL followed by subsequent stimulation with FL. Similarly, to assess whether the FL-induced cell cycle arrest affects sensitivity to chemotherapeutic agent–induced apoptosis, KOCL-58 cells were precultured in the presence or absence of FL (20 ng/mL) for 24 h, and then exposed to daunorubicin (10 ng/mL) or AraC (200 nmol/L) for 48 h in the presence or absence of FL. As shown in Fig. 3*A* (*right*), the Annexin V–positive population decreased by FL from 38.1% to 20.5% in daunorubicin-treated cells and from 64.3% to 47.3% in AraC-treated cells. These results suggest that ligand activation of FLT3 in *MLL*-rearranged leukemia cells renders them resistant to irradiation- and chemotherapeutic agent–induced apoptosis.

To further evaluate the antiapoptotic activity of FL against chemotherapeutic agents in *MLL*-rearranged leukemia cells, KOCL-58 cells (4×10^4/well) were precultured for 24 h in the presence or absence of FL (20 ng/mL) and then cultured for 3 days with or without the addition of daunorubicin (10 ng/mL) or AraC (200 nmol/L). Numbers of living and dead cells were determined by the dye-exclusion method at days 2, 3, and 4 after the start of preculture (Fig. 3*B*). In the culture without the addition of daunorubicin or AraC (*top*), cell proliferation was gradually inhibited (~50% inhibition at day 4) in the presence of FL. Of note, in the culture to which either daunorubicin (*middle*) or AraC (*bottom*) was added, an increase in the dead cell population was markedly suppressed in the presence of FL (~50% suppression at days 3 and 4). The FL-mediating suppression of cell death was similarly observed at a higher concentration of daunorubicin or AraC (Fig. 3*C*). Thus, it was largely estimated that IC_{50} of daunorubicin and AraC was shifted by FL from 10 to 20 ng/mL and from 200 to 400 nmol/L, respectively. Of importance, the FL-induced inhibition of proliferation and resistance to AraC were specifically canceled by the addition of neutralizing anti-FL antibody in the culture medium (Fig. 3*D*, *left*). This FL effect was not observed in KOCL-33 with a D835 mutation (data not shown). These results indicate that the chemotherapeutic agent–induced apoptosis is suppressed *in vitro* via the interaction of FL with wild-type FLT3 in *MLL*-rearranged leukemia cells.

Coculture with bone marrow stromal cells renders *MLL*-rearranged leukemia cells chemoresistant, which is canceled by anti-FL antibody. Because FL is reported to be expressed at high levels as a soluble or membrane-bound form by bone marrow stromal cells (29), *MLL*-rearranged leukemia cells adhering to bone marrow stromal cells might be induced to cell cycle arrest via the FL/FLT3 interaction, resulting in acquisition of resistance to

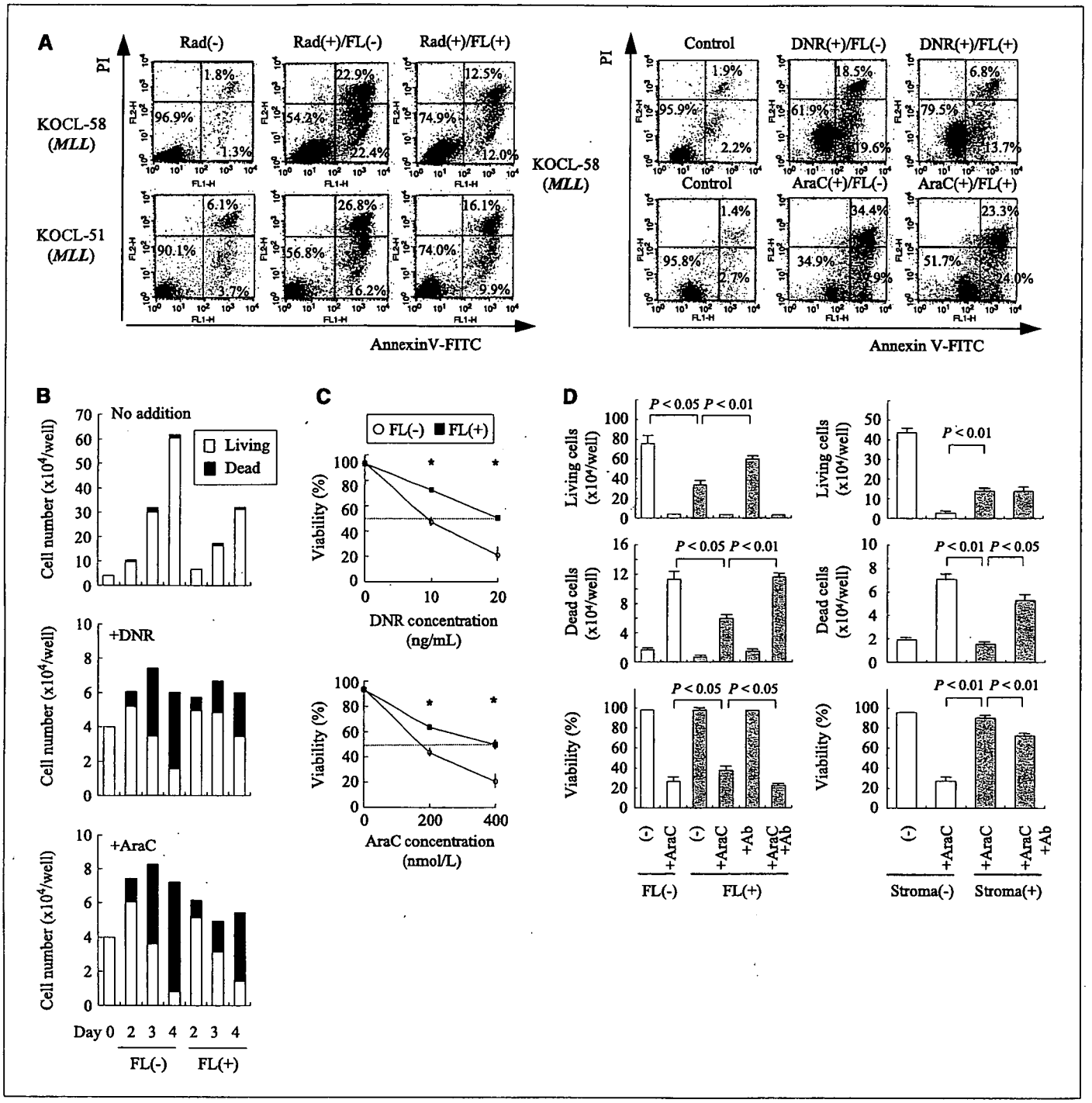

Figure 3. FL-induced resistance to antileukemic agents in *MLL*-rearranged leukemia cells. *A,* flow cytometric analysis of the FL effect on irradiation- and chemotherapeutic agent–induced apoptosis. KOCL-58 and KOCL51 cells were precultured for 24 h in the presence or absence of FL (20 ng/mL), and then irradiated (4 Gy) or exposed to daunorubicin (10 ng/mL) or AraC (200 nmol/L). Flow cytometric analysis was done 48 h later using FITC-conjugated Annexin V and propidium iodide. *Vertical* and *horizontal axes,* log fluorescence intensities of propidium iodide and FITC, respectively. Data are representative of three separate experiments. *B,* analysis of FL-induced resistance to chemotherapeutic agents in *MLL*-rearranged leukemia cells by dye-exclusion test. KOCL-58 cells (4×10^4/well) were precultured in the presence or absence of FL (20 ng/mL) for 24 h, and then cultured with or without daunorubicin (10 ng/mL) or AraC (200 nmol/L) for 3 d. *Columns,* mean numbers of living (*open columns*) and dead (*filled columns*) cells at days 2, 3, and 4 after the start of preculture. Data are representative of three separate experiments. *C,* FL-induced resistance to different concentrations of chemotherapeutic agents. KOCL-58 cells (4×10^4/well) were precultured in the presence or absence of FL (20 ng/mL) for 24 h, and then cultured with or without different concentrations of daunorubicin (10 and 20 ng/mL) or AraC (200 and 400 nmol/L) for 48 h. Viability was calculated by dye-exclusion test. *Points,* mean; *bars,* SE. *, $P < 0.05$, significant difference by *t* test. *D,* effect of anti-FL antibody on soluble FL or bone marrow stromal cell–induced resistance to chemotherapeutic agents. *Left,* KOCL-58 cells (4×10^4/well) were precultured in the presence (*filled columns*) or absence (*open columns*) of FL (20 ng/mL) for 48 h with or without the addition of anti-FL antibody (2 μg/mL), and then cultured with or without AraC (200 nmol/L) for 48 h. *Right,* KOCL-58 cells (4×10^4/well) were precultured with (*filled columns*) or without (*open columns*) KM-104 cells for 48 h in the presence or absence of anti-FL antibody (2 μg/mL), and then cultured with or without AraC (200 nmol/L) for 48 h. Living and dead cell numbers were counted in triplicate by dye-exclusion tests. *Columns,* representative mean from three separate experiments; *bars,* SE.

antileukemic agents. Using the bone marrow stromal cell line KM-104 expressing FL at high levels as the membrane form (Supplementary Fig. S4), we thus did the *in vitro* model study. KOCL-58 cells (4×10^4/well) were precultured for 2 days with or without KM-104 cells growing confluent on the bottom of the plate in the presence or absence of neutralizing anti-FL antibody (4 μg/mL), and then cultured in the presence or absence of AraC (200 nmol/L) for 2 days. As shown in Fig. 3*D* (*right*), the AraC-induced cell death was markedly ($P < 0.01$) suppressed when cocultured with stromal cells. Of importance, this stromal cell effect was partially but significantly canceled by anti-FL antibody in the culture medium, indicating that the FL/FLT3 interaction between *MLL*-rearranged leukemia cells and bone marrow stromal cells contributes, at least in part, to induction of cell cycle arrest of leukemia cells showing resistance to chemotherapeutic agents.

FL stimulation renders primary *MLL*-rearranged leukemic cells resistant to chemotherapeutic agent–induced cell death. To examine whether FL effect is also observed in primary *MLL*-rearranged leukemia cells, peripheral or bone marrow mononuclear cells (blasts >90%) stored in liquid nitrogen were thawed and used for experiments. Characteristics of primary leukemia samples with ($n = 9$) or without ($n = 5$) *MLL* rearrangement are summarized in Table 2. *MLL*-rearranged primary leukemia cells expressed FLT3 at significantly ($P < 0.01$) higher levels than did those without *MLL* rearrangement (median of positive population; 76% versus 25%; median of mean fluorescence intensity; 64.5 versus 30.4). Primary leukemia cells (1×10^5/well) were precultured for 24 h in the presence or absence of FL (40 ng/mL), further cultured for 24 h with AraC (100 nmol/L) in the presence or absence of anti-FL antibody (4 μg/mL), and harvested. As representatively depicted in Fig. 4*A* (case 1), the addition of FL rendered *MLL*-rearranged primary leukemia cells resistant to AraC, which was partially but significantly ($P < 0.05$) canceled by anti-FL antibody. The viabilities (%) after 48 h culture in 14 cases with or without *MLL* rearrangement are summarized in Table 2. Of note, the viabilities after AraC exposure significantly ($P < 0.05$) increased by the addition of FL in five of seven primary leukemia cells with *MLL* rearrangement, but not in five of five primary leukemia cells without *MLL* rearrangement. This FL effect was specifically canceled by anti-FL antibody in all of the cases tested. Statistically, the Δviabilities (treated viabilities − control viabilities) after AraC exposure significantly ($P < 0.05$) increased by the addition of FL, which was canceled by the addition of anti-FL antibody (Fig. 4*B*).

In six cases with *MLL* rearrangement, leukemia cells (1×10^5/well) were precultured for 24 h in the presence or absence of KM-104 cells with or without anti-FL antibody and further cultured for 24 h with AraC (100 nmol/L). As representatively depicted in Fig. 4*A* (case 8), coculture with bone marrow stromal cells rendered *MLL*-rearranged primary leukemia cells resistant to AraC, which was partially but significantly canceled by anti-FL antibody. The Δviabilities after AraC exposure significantly ($P < 0.05$) increased in the presence of stromal cells, which was canceled by the addition of anti-FL antibody (Fig. 4*C*). These results suggest that stimulation by FL, irrespective of its soluble or membrane form, specifically renders primary *MLL*-rearranged leukemia cells resistant to chemotherapeutic agent–induced cell death.

Discussion

MLL-rearranged infant ALL is known to have an especially poor prognosis (30), although its prognosis has gradually improved by intensified chemotherapy and hematopoietic stem cell transplantation (31–33). Nowadays, the complete remission rate in *MLL*-rearranged ALL after the induction chemotherapy has improved to

Table 2. Characteristics of B-precursor primary leukemia cells and their sensitivity to AraC and FLT3 expression

Case no.	Age (mo)/sex	WBC ($\times 10^3/\mu L$)	Sample		Karyotype/fusion gene	Viability (%) after 48 h culture*				FLT3 expression	
			Onset/relapse	BM/PB		Control	AraC	AraC + FL	AraC + FL + Ab	%	MFI
MLL rearrangement											
1	4/M	271	Onset	PB	t(4;11)	65 ± 4	46 ± 4	64 ± 2†	53 ± 2‡	91	64.5
2	1/M	42	Onset	PB	t(11;19)	57 ± 1	38 ± 1	50 ± 1†	40 ± 2‡	91	139.3
3	0/M	182	Relapse	BM	t(4;11)	61 ± 2	53 ± 2	59 ± 3	51 ± 3	70	76.5
4	5/M	440	Onset	PB	t(4;11)	55 ± 5	32 ± 3	45 ± 1†	34 ± 2‡	93	38.5
5	6/M	1,059	Onset	PB	t(4;11)	61 ± 1	54 ± 2	64 ± 2†	53 ± 3‡	92	74.8
6	3/F	40	Onset	PB	t(11;19)	57 ± 3	54 ± 1	58 ± 5	56 ± 2	43	43.3
7	0/M	350	Onset	PB	t(4;11)	51 ± 2	41 ± 2	56 ± 2†	47 ± 2‡	68	94.9
8	2/M	327	Relapse	PB	t(9;11)	66 ± 2	46 ± 3	NT	NT	70	33.4
9	5/F	225	Onset	PB	t(4;11)	63 ± 1	39 ± 4	NT	NT	76	41.3
MLL germ line											
10	56/M	88	Onset	PB	t(1;19)/*E2A-PBX1*	40 ± 2	26 ± 6	36 ± 6	33 ± 4	13	39.2
11	14/M	90	Onset	BM	t(1;19)/*E2A-PBX1*	46 ± 1	33 ± 4	33 ± 4	NT	40	20.3
12	28/M	59	Onset	PB	46,XX/*TEL-AML1*	66 ± 3	58 ± 6	53 ± 3	51 ± 3	7	19.4
13	34/F	9	Onset	PB	Hyperdiploid	66 ± 5	43 ± 1	51 ± 4	52 ± 6	39	32.2
14	18/F	51	Onset	PB	Hyperdiploid	55 ± 5	49 ± 2	47 ± 3	NT	25	30.4

Abbreviations: F, female; M, male; PB, peripheral blood; BM, bone marrow; Ab, neutralizing anti-FL antibody; NT, not tested.
*Data are shown as mean ± SE of triplicate wells.
† Significant increase ($P < 0.05$, t test) compared with AraC+.
‡ Significant decrease ($P < 0.05$, t test) compared with AraC+FL+.

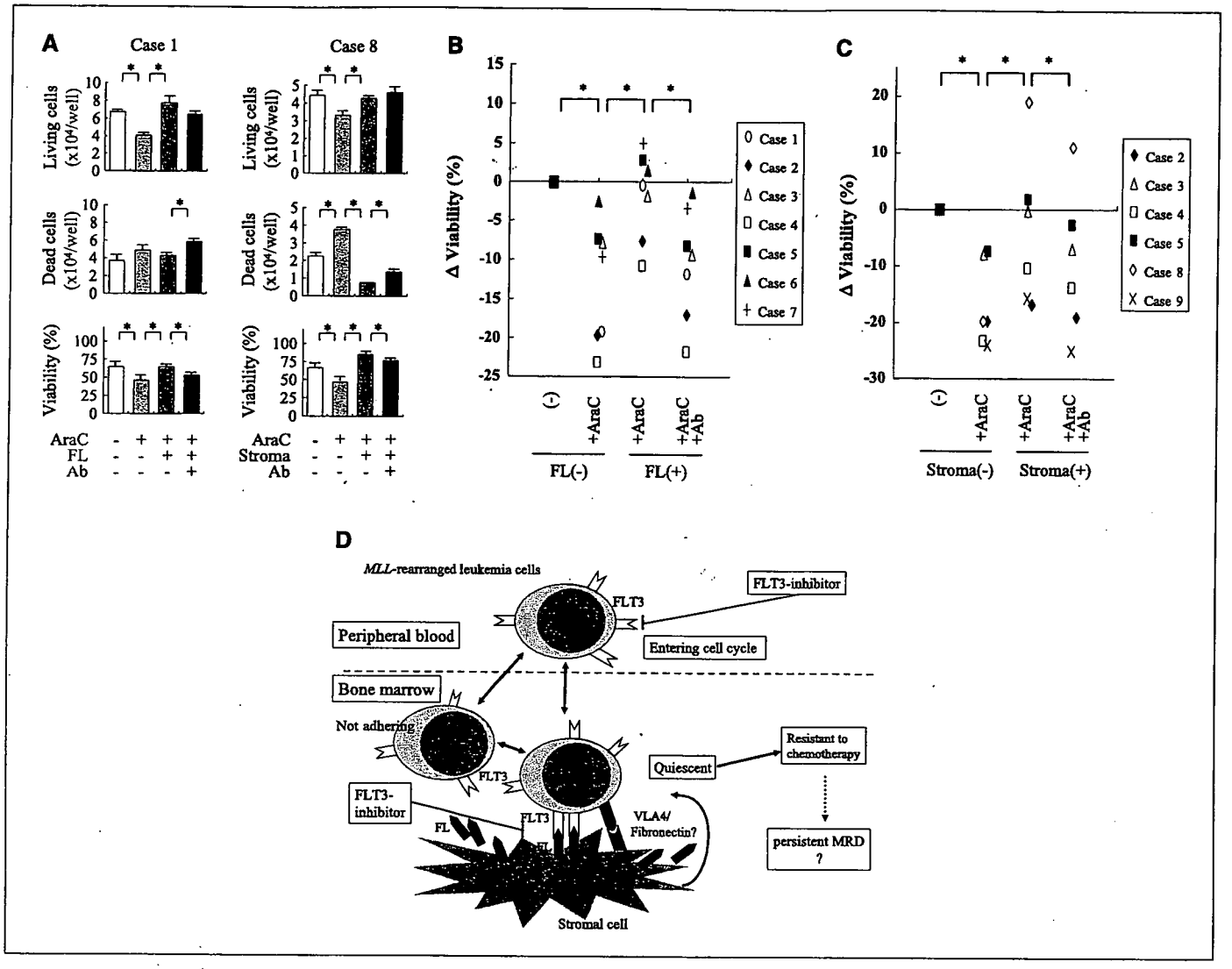

Figure 4. Effect of FL as a soluble or membrane form on chemotherapeutic agent–induced cell death of *MLL*-rearranged primary leukemia cells. *MLL*-rearranged primary leukemia cells (1×10^5/well) were precultured for 24 h in the presence or absence of FL (40 ng/mL) or bone marrow stromal (KM-104) cells with or without the addition of anti-FL antibody (4 μg/mL), and then cultured with or without AraC (100 nmol/L) for 24 h. Numbers of living and dead cells were counted in triplicate by dye-exclusion test and viabilities were calculated. The Δ viability (treated viability − control viability) was also calculated in each culture condition. *A*, representative data in cases 1 and 8. *Columns*, mean; *bars*, SE. *, $P < 0.05$, significant difference by *t* test. *B*, FL-mediating changes in the Δ viabilities in seven primary samples. *, $P < 0.05$, significant difference by the Wilcoxon's test. *C*, bone marrow stromal cell–mediating changes in the Δ viabilities in six primary samples. *, $P < 0.05$, significant difference by the Wilcoxon's test. *D*, schema of the hypothesis illustrating a possible role of the FL/FLT3 interaction for persistent MRD in *MLL*-rearranged leukemia. *MLL*-rearranged leukemia cells not adhering to bone marrow stromal cells are entering cell cycle and considerably sensitive to chemotherapy, but those adhering bone marrow cells are quiescent and resistant to chemotherapy, at least in part via the interaction of FL/FLT3, which results in persistent MRD. The FLT3 inhibitor might be effective by two mechanisms *in vivo*: one directly induces apoptosis of leukemia cells and another awakens quiescent leukemia cells adhering to bone marrow stromal cells to enter cell cycle.

a greater than 90%; the most dismal clinical issue in this disease is an early relapse that frequently occurs during the first 6 months of chemotherapy and before hematopoietic stem cell transplantation (34), resulting in shorter event-free survival and overall survival. Recently, the prognostic value of MRD after induction chemotherapy has been emphasized in childhood ALL (35, 36). Although the importance of MRD is not fully characterized yet in *MLL*-rearranged ALL, persistence of high levels of MRD in bone marrow should be associated with a high and early relapse rate in this disease.

We found that *MLL*-rearranged leukemia cells with wild-type FLT3 showed an inhibitory response to FL. This FL-induced inhibition was due to the induction of cell cycle arrest, in the process of which up-regulation of p27 and dephosphorylation of STAT5 might be implicated profoundly. Importantly, these arrested leukemia cells, not only established lines but also primary samples, showed resistance to apoptosis after exposure to irradiation or chemotherapeutic drugs. Because FL is reported to be expressed at high levels as a soluble or membrane-bound form by bone marrow stromal cells (29), it is postulated that *MLL*-rearranged leukemia cells tightly adhering to bone marrow stromal cells are induced to cell cycle arrest via the FL/FLT3 interaction, which might lead leukemia cells to become "dormant cells" that are resistant to antileukemic agents. In addition, the serum level of FL is reported to increase dramatically in patients who experience chemotherapy-induced bone marrow suppression (37). Therefore, leukemia cells

in patients after intensified chemotherapy are speculated to be exposed to high level of FL not only in bone marrow but also in the periphery. We showed in the *in vitro* model study that *MLL*-rearranged leukemia cells adhering to the stroma cell line partially restored sensitivity to antileukemic agents in the presence of anti-FL antibody. In AML, it has been reported that leukemia cells acquire resistance to AraC and daunorubicin via the interaction of VLA4 expressed on AML cells with fibronectin expressed on bone marrow stromal cells (38). This VLA4/fibronectin interaction was confirmed to play a pivotal role in MRD of AML in both the animal model and the clinical study showing a poor prognosis of VLA4-positive AML compared with a good prognosis of VLA4-negative AML (38). We thus present the hypothesis as illustrated in Fig. 4*D*, postulating that *MLL*-rearranged leukemia cells not adhering to bone marrow stromal cells are sensitive to chemotherapy, but those adhering to bone marrow stromal cells are rendered resistant to chemotherapy, at least in part via the interaction of FL/FLT3, which results in persistent MRD that is closely associated with the high relapse rate of this disease. According to this scenario, the FLT3 kinase inhibitors, such as PKC412, should be effective *in vivo* in the treatment of *MLL*-rearranged leukemia because they can exert their inhibitory action through two mechanisms: one directly induces apoptosis of leukemia cells via blockade of the kinase activity required for their survival as recently reported (21, 22, 39) and another awakens "dormant" leukemia cells and induces them to enter a chemosensitive cell cycling state via blockage of the signal through the FL/FLT3 interaction that occurs on the surfaces of leukemia cells and bone marrow stromal cells. The cell surface adhesion molecule VCAM-1 or asparagine synthetase, expressed on or secreted from bone marrow stromal cells, respectively, have also been reported to be involved in resistance to chemotherapy in ALL cells (40, 41).

The precise molecular mechanism of the FL-induced cell cycle arrest in *MLL*-rearranged leukemia cells remains elusive. We found that p27 is markedly up-regulated after FL stimulation, and this was presumably due to prevention of degradation of this protein in *MLL*-rearranged leukemia. As a key member of the KIP/CIP family of CDKIs, p27 blocks cell cycle progression at G_1 phase, primarily by inhibiting the cyclin E/CDK2 complex (42, 43). It is known that p27 is degraded by the ubiquitin-proteasome pathway and that quiescent cells exhibit a smaller amount of ubiquitinating activity (44), which might account for prolongation of the p27 half-life in FL-treated *MLL*-rearranged leukemia cells. Therefore, it is not clear at present whether up-regulation of p27 is the primary molecular event in FL-induced cell cycle arrest, or it is a secondary event in quiescent ("dormant") cells occurring after cell cycle arrest has been induced by other molecular mechanism(s). We also found that phosphorylation of STAT5, but not p44/42 MAPK and Akt, was almost abolished in arrested *MLL*-rearranged leukemia cells after FL stimulation. Because selective activation of STAT5 is shown to play a pivotal role in the self-renewal of leukemic cells as well as in normal hematopoiesis (45), the specific inactivation of STAT5 after FL stimulation might be critical to the induction of cell cycle arrest. The molecular mechanism of FL-induced STAT5 inactivation is still elusive and will be the subject for the future study.

The most important issue to be addressed is why *MLL*-rearranged ALL cells, unlike other B-precursor ALL cells, show a inhibitory response in proliferation after FL stimulation. Analyses of gene expression in leukemia have provided direct insights into the pathogenesis of leukemias and their responses to therapy. Armstrong et al. (19) reported that *MLL*-rearranged ALL has a distinct gene expression profile, including high FLT3 expression compared with other types of ALL. Thus, specific genes and their products that are uniquely activated in *MLL*-rearranged leukemia might be associated with a unique inhibitory response to FL.

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AML1 Mutation and *FLT3*-internal Tandem Duplication in Leukemia Transformed From Myelodysplastic Syndrome

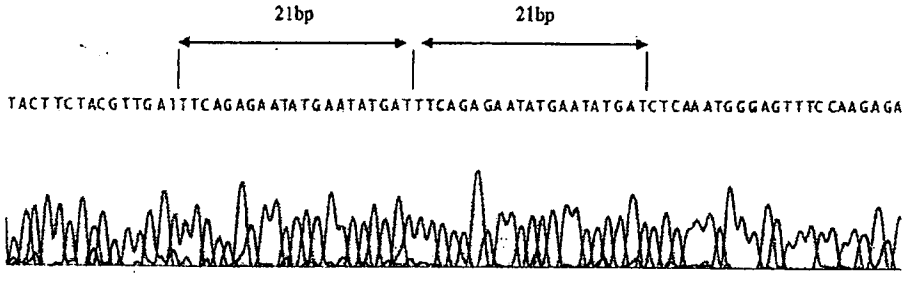

FIGURE 2. Sequence of *FLT3* gene. ITD of 21 bp in exon 11 was observed. The wild type of *FLT3* was not found.

To the Editor:

Myelodysplastic syndrome (MDS) is a clonal disorder of hematopoietic stem cells characterized by ineffective and inadequate hematopoiesis. Recently, gene alterations including *AML1/RUNX1* had been demonstrated to contribute to the development from MDS to secondary acute myeloid leukemia (AML) in adult patients, particular in AML-M0 or AML with acquired trisomy 21.[1,2] Moreover, *FLT3*-internal tandem duplication (ITD) predicts a high risk of progression of MDS to AML in adult patients,[3] but these gene alterations were rarely reported in pediatric MDS.[4]

We reported here a 6-year-old girl with leukocytosis, which consisted of monocytosis and immature myeloid cells. She did not show hepatomegaly, splenomegaly, café-au-lait spots, or other abnormal physical findings. A bone marrow aspirate showed a hypercellular marrow with greatly increased myeloid cells and megakaryocytes, but blasts were less than 1%. Only minimal dysplastic change was seen in her bone marrow cells with normal female karyotype. A provisional diagnosis was adult-type chronic myelomonocytic leukemia. She developed acute mixed-lineage leukemia carrying trisomy 21 after 4 years from the initial diagnosis. The disease progressed rapidly, and she died after allogeneic stem cell transplantation.

We analyzed mutations in the runt domain of *AML1*gene by polymerase chain reaction or reverse-transcription polymerase chain reaction followed direct sequencing using the primers previously described.[5] The patient had a mutation within intron 3 of *AML1* gene (T to A; − 10 from exon 4, Fig. 1). The mutation led to an 8-bp insertion on 1 mRNA allele resulting from change in a splicing acceptor site in intron 3; this induced a frameshift that produced a stop codon. Both normal and mutant *AML1* sequences were found in this patient (Fig. 1).

Moreover, we analyzed the juxtermembrane domain of the *FLT3* gene using primer pairs R5 and R6[6] and found an ITD of 21 bp in exon 11 (Fig. 2), but we could not find the wild-type product of *FLT3* gene. Moreover, mutations of *RAS* and *PTPN11* genes were not found in this patient.

Interestingly, dual mutations in the *AML1* and *FLT3* genes were found in AML-M0 subtype in adult MDS patients.[7] We considered that both *AML1* mutation and *FLT3*-ITD may have a role in disease progression. However, we could not examine the *AML1* mutation and *FLT3*-ITD at the time of chronic phase because the sample was not available. Larger studies regarding gene alterations in pediatric MDS will be needed to clarify these associations.

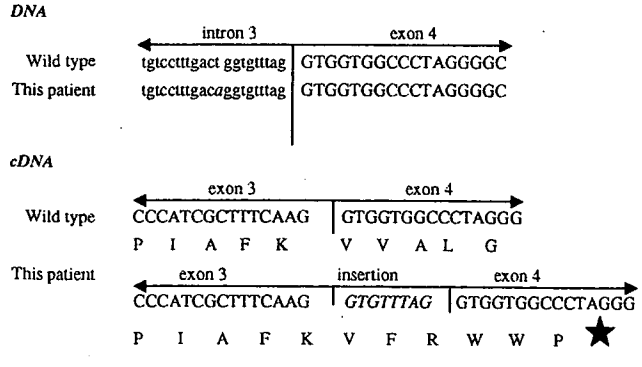

FIGURE 1. The schema of mutation in the runt domain of the *AML1/RUNX1* gene. A point mutation at intron 3 (t→a; italic letter -10 from exon 4), led to an 8-bp insertion (italic letters) on 1 cDNA allele because of the change in the splicing acceptor site in intron 3, inducing a frameshift resulting in a stop codon (pentagram). The wild type of *AML1* was also found.

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Routine Use of PET Scans After Completion of Therapy in Pediatric Hodgkin Disease Results in a High False Positive Rate

In Response:

Drs Mardis and Wong raise important points in response to our published report regarding the routine use of positron emission tomography (PET) scans for surveillance of disease recurrence after completion of therapy in children and adolescents with Hodgkin lymphoma.[1] Our study was designed to evaluate PET scans as they are commonly used in the patient population. Hence, our data included all PET scans, as they are used for surveillance even when PET scans were not used in the diagnostic period. Similarly, in practice, multiple physicians read PET scans and variability in interpretation is itself a limitation of the use of PET scans. Drs Mardis and Wong note that perhaps some of false positives in the early stages were due to inexperience, however, we presented information that the false positive rate remained the same throughout the study period suggesting that a learning curve did not account for the findings of the study.

Since our publication Meany et al[2] published a study, evaluating 23 consecutive pediatric patients with Hodgkin disease and compared PET scan results with clinical status and computed tomography (CT) scans. Their results included a strong negative predictive value of 100% and a positive predictive value of 18.2%, findings that are almost identical to our results. They conclude, as we did, that positive PET scans must be interpreted conservatively and that treatment decisions should not be made on the findings of a positive study.

Ultimately, Dr Mardis and Wong's conclusion that CT/PET scanners will solve many of the issues that commonly occur with the use of PET scans supports the conclusion that PET scans are not an ideal imaging modality for off therapy patients. Our concern, and the rationale for publishing our data, is that PET scans are still used in many centers where combined CT/PET scans are not available and also continue to be used in research studies. Thus, the issues that arise with the use of PET scans, including the presence of false positive results, are likely to continue to present management dilemmas to our colleagues and need to be acknowledged. The International Harmonization Project recently evaluated the existing data on the use of PET scans in lymphoma therapy.[3] Consistent with our recommendations, the report concluded that the current data is inadequate to recommend routine surveillance PET scans after completion of therapy.

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The Accuracy of PET(CT) in Evaluating Pediatric Lymphoma

To the Editor:

We read with great interest the recent article by Levine et al[1] in the November 2006 issue of *Journal of Pediatric Hematology/Oncology*. We have some concerns about the results of this study.

First, only a portion of the cohort had a positron emission tomography (PET) scan at initial diagnosis to serve as a reference for disease response to therapy. Correcting this deficit in surveillance might have minimized false positive results in their patient population. Besides, there was no quantification of metabolic activity, which is important in defining the underlying biologic behavior of the lymphoma. As described by Wong et al,[2] quantitative examination of the glucose metabolic rate by the

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ORIGINAL ARTICLE

Outcome of risk-based therapy for infant acute lymphoblastic leukemia with or without an *MLL* gene rearrangement, with emphasis on late effects: a final report of two consecutive studies, MLL96 and MLL98, of the Japan Infant Leukemia Study Group

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We evaluated the efficacy of a treatment strategy in which infants with acute lymphoblastic leukemia (ALL) were stratified by their *MLL* gene status and then assigned to different risk-based therapies. A total of 102 patients were registered on two consecutive multicenter trials, designated MLL96 and MLL98, between 1995 and 2001. Those with a rearranged *MLL* gene (MLL-R, $n=80$) were assigned to receive intensive chemotherapy followed by hematopoietic stem cell transplantation (HSCT), while those with germline *MLL* (MLL-G, $n=22$) were treated with chemotherapy alone. The 5-year event-free survival (EFS) rate for all 102 infants was 50.9% (95% confidence interval, 41.0–60.8%). The most prominent late effect was growth impairment, observed in 58.9% of all evaluable patients in the MLL-R group. This plan of risk-based therapy appears to have improved the overall prognosis for infants with ALL, compared with previously reported results. However, over half the events in patients with *MLL* rearrangement occurred before the instigation of HSCT, and that HSCT-related toxic events comprised 36.3% (8/22) of post-transplantation events, suggesting that further stratification within the MLL-R group and the development of more effective early-phase intensification chemotherapy will be needed before the full potential of this strategy is realized.

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Keywords: infant acute lymphoblastic leukemia; *MLL* gene; hematopoietic stem cell transplantation; late effects

Introduction

The outcome of therapy for children with acute lymphoblastic leukemia (ALL) has markedly improved over the last four decades, to the extent that approximately 80% of affected patients are now cured.[1] However, infants with ALL, who represent 2.5–5% of all childhood ALL cases, continue to have high relapse rates and a dismal prognosis, as illustrated by recently published event-free survival (EFS) rates of 22–54%.[2–8] ALL in infancy has several distinctive clinical characteristics compared with common childhood ALL, including hyperleukocytosis, hepatosplenomegaly, central nervous system (CNS) disease and a high frequency of molecularly evident rearrangement of the *MLL* gene at chromosome band 11q23.[9–12] Among these features, *MLL* gene rearrangement, found in 70–80% of infant ALL cases studied with molecular techniques, is an independent risk factor most predictive of recurrent leukemia.[13–15]

The high failure rate in infants with ALL, especially those with *MLL* gene rearrangement, can be attributed to early relapse after remission rather than toxicity, and warrants consideration of intensified therapy. We therefore segregated infants with ALL into two subgroups according to their *MLL* gene status in two consecutive nationwide multicenter studies, designated MLL96 and MLL98. Infants with a rearranged *MLL* (MLL-R) gene received intensive chemotherapy followed by hematopoietic stem cell transplantation (HSCT), while those with a germline *MLL* gene were treated with standard intensive chemotherapy for ALL alone. This report updates findings published earlier[16–18] and extends the analysis to long-term side effects. By combining data from two studies with similar treatment strategies, we were able to analyze results for a relatively large cohort with this rare subtype of ALL. The additional detail on prognostic features should facilitate further risk stratification among *MLL*-rearranged cases, while outcome data on the increased number of patients undergoing HSCT should stimulate critical discussion of the role of this procedure in future treatment strategies for infants with ALL.

Materials and methods

Patients

Between December 1995 and December 2001, 102 consecutive infants with ALL, younger than 12 months, were registered and treated on two protocols, designated MLL96 (55 patients) and MLL98 (47 patients). These studies accrued more than 80% of all Japanese infants with ALL over the 6-year enrollment period, based on results of a nationwide surveillance study.[17] The

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diagnosis of ALL was based on bone marrow morphology and cytochemical staining results. Each patient was evaluated with respect to the characteristics of the leukemic cells, including immunophenotype, cytogenetics and *MLL* gene status. Written informed consent, provided according to the Declaration of Helsinki, was obtained from the guardians of the patients, with institutional review board approval obtained for all aspects of this investigation.

Detection of MLL *gene rearrangement and cytogenetic analysis*

The *MLL* gene status of each patient was determined by Southern blot analysis and/or split-signal fluorescence *in situ* hybridization (FISH) as previously described.[16] Leukemic cell karyotypes were determined by cytogenetic analysis with a G-banding technique.[16]

Treatment

The details of the therapeutic regimens used in the MLL96 and MLL98 studies are described in Supplementary Tables.[16–18] Briefly, patients with rearranged *MLL* (MLL-R) received induction therapy and three courses of postremission intensification therapy followed by HSCT if a suitable donor was available. Patients with germline *MLL* (MLL-G) were assigned to a chemotherapy arm consisting of induction, consolidation and CNS prophylaxis, intensification, reinduction and maintenance phases, administered over 83–85 weeks. Except for vincristine, drug dosages on the MLL98 protocol were calculated on the basis of body surface area, while dosages on the MLL96 protocol were based on body weight. This modification increased the dosages of all antileukemic drugs used in the MLL98 study by 1.2- to 2-fold over those in the MLL96 study.

Patients in the MLL-R group underwent HSCT in first remission (CR1), if a 5 to 6/6 human leukocyte antigen-matched related donor, 6/6-matched unrelated donor or 4 to 6/6-matched unrelated cord blood donor was available. The protocol-specified conditioning regimen comprised either a total-body irradiation (TBI; 12 Gy in six fractions, twice a day on days −7 to −5) or busulfan (BU; 35 mg/m^2/dose orally, 4 times a day on days −8 to −5) with a combination of etoposide (60 mg/kg intravenously on day −4) and cyclophosphamide (60 mg/kg intravenously on days −3 and −2). Prophylaxis for graft-vs-host disease (GVHD) consisted of either cyclosporine or tacrolimus combined with short-term methotrexate.

Evaluation of the late effects

Late effects studied included cardiac, pulmonary, renal, endocrine, dental, orthopedic, dermatologic, ophthalmologic, auditory, psychological, growth and development and occurrence of secondary malignancies. Medical records regarding these issues were reviewed by each physician of the participating centers, and these data were collected by questionnaire which was sent to each participating center.

Statistical considerations

The analysis of treatment outcome was updated on 30 September 2006, combining data from both the MLL96 and MLL98 studies because of their similar 5-year survival estimates (see Results). EFS and overall survival (OS) rates were estimated by the method of Kaplan–Meier and standard errors (s.e.) with the Greenwood formula, and then were compared with the log-rank test. Confidence intervals (CIs) were computed with a 95% confidence level. The clinical, demographic and biologic features of patients were compared with χ^2 tests for homogeneity. A Cox regression model was used for the multivariate analysis. *P*-values, when cited, are two-sided, with a value of 0.05 or less taken to indicate statistical significance.

Table 1 Characteristics of infants with ALL treated in the MLL96 and MLL98 studies

	Overall, n *(%)*	*MLL-R,* n *(%)*	*MLL-G,* n *(%)*	*P-value**
Total no. of patients	102	80	22	
Age (months)				<0.001
<3	19 (18.6)	19 (23.8)	0 (0.0)	
3 to <6	31 (30.4)	27 (33.7)	4 (18.2)	
⩾6	52 (51.0)	34 (42.5)	18 (81.8)	
Gender				<0.001
Male	52 (51.0)	32 (40.0)	20 (90.9)	
Female	50 (49.0)	48 (60.0)	2 (9.1)	
WBC count (× 10^9/l)				<0.001
<100	44 (43.2)	25 (31.3)	19 (86.4)	
100 to <300	34 (33.3)	32 (40.0)	2 (9.1)	
⩾300	24 (23.5)	23 (28.7)	1 (4.5)	
CNS disease[a]				0.05
Positive	15 (14.7)	15 (18.8)	0 (0.0)	
Negative	81 (79.4)	59 (73.8)	22 (100.0)	
Unknown	6 (5.9)	6 (7.4)	0 (0.0)	
CD10				<0.001
Positive	24 (23.5)	3 (3.8)	21 (95.5)	
Negative	78 (76.5)	77 (96.2)	1 (4.5)	
Karyotype[b]				<0.001
t(4;11)(q21;q23)	41 (40.1)	41 (51.2)	0 (0.0)	
t(11;19)(q23;p13)	7 (6.9)	7 (8.8)	0 (0.0)	
t(9;11)(p22;q23)	6 (5.9)	6 (7.5)	0 (0.0)	
Other 11q23	6 (5.9)	6 (7.5)	0 (0.0)	
No 11q23 rearrangement	35 (34.1)	13 (16.2)	22 (100.0)	
Unknown	7 (6.9)	7 (8.8)	0 (0.0)	

Abbreviations: ALL, acute lymphoblastic leukemia; CNS, central nervous system; FISH, fluorescence *in situ* hybridization; MLL-G, patients with germline *MLL*; MLL-R, patients with MLL gene rearrangement; WBC, white blood cell count.
*Comparison between the MLL-G and MLL-R subgroups.
[a]CNS disease was diagnosed if more than 5 cells/mm^3 with recognizable blasts were found in cerebrospinal fluid.
[b]All 80 patients in the MLL-R group, including the 13 cases with "No 11q23 rearrangement" and the 7 "Unknown" cases by normal karyotypic analysis, were confirmed as "*MLL* rearranged" by Southern blotting and/or split-signal FISH.

Results

Patient characteristics

The characteristics of the patients at diagnosis are reported in Table 1. We identified 80 patients with MLL-R and 22 with MLL-G by Southern blot analysis and/or FISH. Patients in the MLL-R group were significantly younger (median age, 4 vs 9 months, $P<0.001$), and had higher leukocyte counts (median, 168.4×10^9/l vs 21.8×10^9/l, $P<0.001$). The frequency of CNS disease (defined as more than 5 cells/mm^3 with recognizable blasts in cerebrospinal fluid) was also significantly higher in the MLL-R group. Expression of the CD10 antigen was closely