Table 2. Characteristics of 190 patients with AML, excluding patients with the M3 subtype, treated according to JALSG protocols

	Total N = 190	*NPM1* Mutation, n = 49	*NPM1* Wild type, n = 141	*P*
Age, y	50 (15-85)	58 (15-77)	47 (15-85)	.003
WBC count, × 10^9/L	24.5 (0.9-372)	52.2 (1.0-372)	23.3 (0.9-337.6)	.002
PB blast count, × 10^9/L	14.9 (0.017-357)	29.6 (0.093-357)	12.6 (0.017-324)	.029
FAB subtype				.004
M0	3	1	2	
M1	44	12	32	
M2	78	10	68	
M4	46	16	30	
M5	14	8	6	
M6	4	1	3	
M7	1	1	0	
Cytogenetics				< .001
Favorable	33	1	32	
t(8;21)	27	0	27	
inv(16)	6	1	5	
Intermediate	119	42	77	
Normal	79	37	42	
Others	40	5	35	
Poor	14	1	13	
t(9;22)	3	1	2	
del(5) or del(7)	11	0	11	
Unknown	24	5	19	
Outcome				.025
CR	139	42	97	
Failure	51	7	44	
FLT3				
Mutation, total	51	31	20	< .001
ITD mutation	43	27	16	< .001*
D835 mutation	8	4	4	.018*
Wild type	139	18	121	
P53				NS
Mutation	8	1	7	
Wild type	182	48	134	
NRAS				NS
Mutation	26	8	18	
Wild type	164	41	123	

Clinical characteristics of the 190 patients with AML, excluding those with the M3 subtype, who were treated according to the JALSG protocols, are shown. Median values (with range in parentheses) are indicated for age, WBC count, and PB blast count. Numbers of patients are shown by FAB type, cytogenetics, outcome, and gene mutation. The favorable risk group was defined by t(8;21) or inv(16); the poor risk group by t(9;22), del(5), or del(7); and the intermediate risk group by normal or other karyotypes.

PB indicates peripheral blood; NS, not significant.

*Compared with wild-type variables.

of *NPM1* was not associated with the high relapse rate in patients with FLT3/ITD, whereas it tended to be a worse factor for relapse in those without FLT3/ITD ($P = .084$) (Figure 3).

Comparison of mutational status of the *NPM1* gene at diagnosis and relapse

Mutational status of the *NPM1* and *FLT3* genes was compared between diagnosis and relapse in 39 AML patients. *NRAS* and *TP53*

Table 3. Logistic regression analysis results of the effect of unfavorable factors for CR in 190 patients, excluding those with the M3 subtype, treated according to JALSG protocols

Variable	*P*	Odds ratio	95% CI
NPM1, wild type	< .001	8.434	2.562-27.77
FAB, other than M2	.008	3.247	1.353-7.813
Cytogenetic, other than good risk	.039	5.240	1.091-25.16
NRAS, mutation	.056	2.667	0.978-7.299
TP53, mutation	.058	6.024	0.941-38.46
WBC count, more than 100 × 10^9/L	.090	2.427	0.872-6.757
FLT3, mutation	.279	1.719	0.645-4.583
Age, older than 60 y	.960	2.427	0.446-2.340

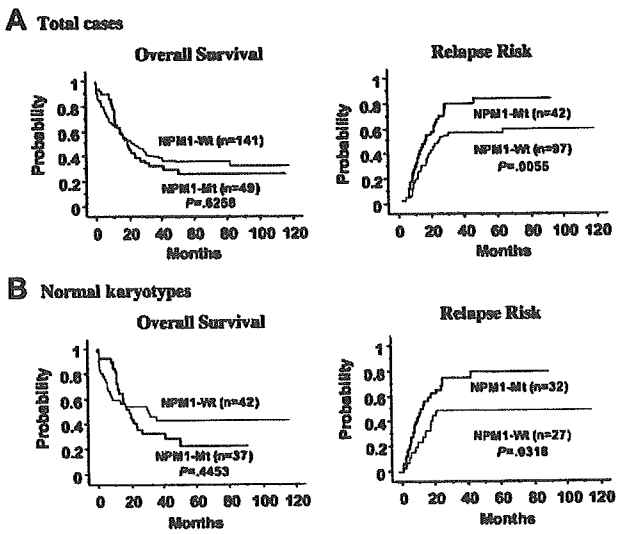

Figure 2. Kaplan-Meier curves according to NPM1 mutation. (A) Overall survival and relapse risk in all patients. (B) Overall survival and relapse risk in patients with normal karyotype. Statistical difference was evaluated with the log-rank test.

Table 4. Unfavorable prognostic factors for overall survival in 190 patients, excluding those with the M3 subtype, treated according to JALSG protocols

Prognostic factors	*P*	Odds ratio	95% CI
TP53 mutation	.003	4.002	1.876-8.538
Age, older than 60 y	.009	1.651	1.131-2.410
FAB, other than M2	.010	1.643	1.127-2.396

mutations were also analyzed in 20 and 29 patients, respectively (Table 6). An *NPM1* mutation was found in 17 of 39 patients at diagnosis. Of the 17 patients with *NPM1* mutations at diagnosis, 15 carried the same mutation at relapse, although 2 patients (unique patient number [UPN] 16 and UPN 17) lost the mutation at relapse. In UPN 16, the karyotype of leukemia cells was normal (46XY) at diagnosis, although it changed to 46XYdel(20)(q1?) at relapse. In contrast, *FLT3, NRAS,* and *TP53* were of the wild type at both stages. In UPN 17, leukemia cells showed normal karyotype (46XX), FLT3/ITD, and wild-type *NRAS* and *TP53* mutations at diagnosis, and these were retained at relapse.

An *FLT3* mutation was found in 11 of 39 patients at diagnosis, and all mutations were FLT3/ITD. The *FLT3* mutation was lost at relapse in 1 patient, but it emerged at relapse in 4 patients who did not have the *FLT3* mutation at diagnosis. An *NRAS* mutation was found in 5 of 20 patients at diagnosis. The *NRAS* mutation was lost at relapse in 2 patients, but it emerged at relapse in 1 patient. A *TP53* mutation was found in 3 of 28 patients at diagnosis. The *TP53* mutation was lost at relapse in 1 patient, but it emerged at relapse in 1 patient. Taken together, in 11 of 39 (28.2%) patients, the gene status of *NPM1, FLT3, NRAS,* and *TP53* was different at diagnosis than it was at relapse (Table 6). However, *NPM1* gene status was relatively stable, in contrast to that of *FLT3*, and was not related to inducing a genotypic change in leukemia cells at relapse. In addition, age and CR duration were not associated with the genotypic change of leukemia cells at relapse. Importantly, the *NPM1* mutation was not associated with achieving second CR after first CR.

Discussion

In this study, we found *NPM1* mutations in 64 of 257 (24.9%) adult patients with de novo AML. Of the 64 *NPM1* mutations, 60 were variants reported previously, and 4 were novel. All the novel variants had a 4-bp insertion at position 960, resulting in the same frameshift as previously reported. The C-terminal of NPM is important to the nuclear localization of NPM,[36] and tryptophan residues at positions 288 and 290 are reportedly associated with the nucleolar localization of NPM.[37] Interestingly, predicted mutant proteins contain a nuclear export signal (NES) motif at the C-terminal, which may be a reason for the cytoplasmic dislocation of mutant NPM.[38,39] All novel variants found in the present study also contained the NES motif. Therefore, we believe that the generation of the NES motif by the insertion mutation is strongly associated with the cytoplasmic dislocation of mutant NPM.

Table 5. Unfavorable prognostic factors for relapse in 139 patients with AML, excluding those with the M3 subtype, who achieved CR

Prognostic factors	*P*	Odds ratio	95% CI
Poor cytogenetics	.001	3.876	1.718-8.772
NPM1 mutation	.002	2.106	1.324-3.350

FLT3, TP53, and *NRAS* mutations are the most frequent genetic alterations involved in the pathogenesis of AML. It was reported that the *NPM1* mutation is closely associated with FLT3/ITD. We found that it is also associated with FLT3/D835Mt, but a large-scale study is necessary to confirm this association because of the small number of patients with FLT3/D835Mt in this study. It is noteworthy that the *NPM1* mutation was not associated with *TP53, NRAS,* and *MLL-TD* mutations. In fact, the *NPM1* mutation was found in only 2 of 16 patients with the *TP53* mutation, although this was not statistically significant. Because the *TP53* mutation is known to be present in patients with karyotypic abnormalities,[40,41] the *NPM1* mutation seems to be infrequent in this group. The *NRAS* mutation is negatively related to FLT3/ITD but is found in all patients with AML, regardless of karyotypic abnormalities.[6,42] In contrast, a possible association between MLL-TD and FLT3/ITD was reported.[43] Therefore, to identify factors associated with *NPM1* mutations, we performed multivariate logistic regression analysis that included the presence or absence of *FLT3, TP53, NRAS,* and *MLL-TD* mutations and karyotypic abnormality in 119 patients in whom all these genetic alterations were determined. The analysis showed that the normal karyotype ($P < .001$; odds ratio, 26.0 [95% CI, 6.231-108.73]) and the *FLT3* mutation ($P < .001$; odds ratio, 15.2 [95% CI, 3.690-62.50]) were independently associated with *NPM1* mutation. These results suggested that the *NPM1* mutation might be involved in the pathogenesis of AML with and without *FLT3* mutations.

The most important finding in the present study is that in patients with AML, excluding those with the M3 subtype, the *NPM1* mutation is a favorable factor for achieving CR after induction chemotherapy, but it implicates a high relapse rate. FLT3/ITD is clinically demonstrated to be a poor prognostic factor in patients with AML, especially those in the intermediate-risk group. FLT3/ITD confers autonomous proliferation to hematopoietic progenitors through its constitutive kinase activity, though alone it is not sufficient for the development of AML. In the murine APL model, the transduction of FLT3/ITD into *PML-RARA* transgenic bone marrow cells resulted in a shortened latency with

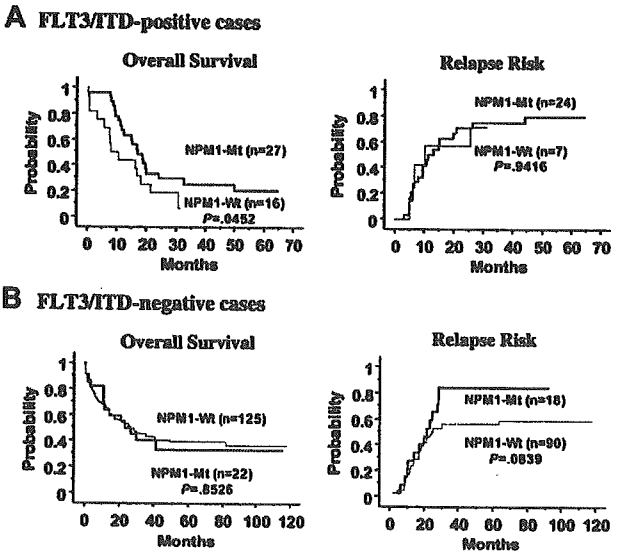

Figure 3. Kaplan-Meier curves according to NPM1 mutation in the FLT3/ITD-positive and -negative patients. (A) In the FLT3/ITD-positive group, the *NPM1* mutation was a favorable prognostic factor for overall survival, but did not affect the relapse risk. (B) In the FLT3/ITD-negative group, the *NPM1* mutation did not affect overall survival. The *NPM1* mutation tended to be a worse factor for relapse in this group, although it was not statistically significant. Statistical difference was evaluated with the log-rank test.

Table 6. Comparisons of mutations between diagnosis and relapse

					NPM1		*FLT3*		*NRAS*		*TP53*		
UPN	Age, y	Sex	FAB	CR, d	D	R	D	R	D	R	D	R	2nd CR
1	56	M	M4	255	Mt	Mt	Mt	Mt	WT	WT	WT	WT	No
2	38	F	M2	574	Mt	Mt	Mt	Mt	WT	WT	WT	WT	Yes
3	62	M	M1	328	Mt	Mt	Mt	Mt	ND	ND	WT	WT	Yes
4	55	M	M5	408	Mt	Mt	Mt	Mt	ND	ND	WT	WT	No
5	15	F	M2	181	Mt	Mt	Mt	Mt	ND	ND	WT	WT	No
6	62	F	M1	521	Mt	Mt	Mt	Mt	ND	ND	WT	WT	No
7	50	M	M2	264	Mt	Mt	Mt	Mt	ND	ND	ND	ND	Yes
8	66	M	M1	608	Mt	Mt	*Mt*	*WT*	WT	WT	WT	WT	Yes
9	67	F	M4	483	Mt	Mt	*WT*	*Mt*	WT	WT	WT	WT	Yes
10	57	F	M1	603	Mt	Mt	WT	WT	*Mt*	*WT*	WT	WT	Yes
11	54	M	M1	175	Mt	Mt	WT	WT	WT	WT	WT	WT	No
12	53	F	M1	214	Mt	Mt	WT	WT	WT	WT	WT	WT	No
13	54	M	M4	1026	Mt	Mt	WT	WT	ND	ND	ND	ND	Yes
14	53	M	M4	542	Mt	Mt	WT	WT	ND	ND	ND	ND	No
15	70	M	M1	773	Mt	Mt	WT	WT	ND	ND	WT	WT	No
16	64	M	M4	489	*Mt*	*WT*	WT	WT	WT	WT	WT	WT	No
17	44	F	M0	147	*Mt*	*WT*	Mt	Mt	WT	WT	Mt	Mt	No
18	59	M	M1	222	WT	WT	Mt	Mt	ND	ND	ND	ND	No
19	55	M	M5	128	WT	WT	Mt	Mt	ND	ND	ND	ND	Yes
20	16	M	M4	308	WT	WT	*WT*	*Mt*	Mt	Mt	WT	WT	No
21	34	F	M4	370	WT	WT	*WT*	*Mt*	Mt	Mt	WT	WT	No
22	56	F	M4	161	WT	WT	*WT*	*Mt*	WT	WT	*Mt*	*WT*	No
23	29	M	M0	361	WT	WT	WT	WT	Mt	Mt	WT	WT	No
24	26	F	M4	430	WT	WT	WT	WT	*Mt*	*WT*	WT	WT	Yes
25	48	M	M2	525	WT	WT	WT	WT	*WT*	*Mt*	WT	WT	Yes
26	74	F	M1	147	WT	WT	WT	WT	WT	WT	*WT*	*Mt*	Yes
27	44	F	M1	137	WT	WT	WT	WT	WT	WT	WT	WT	No
28	39	F	M2	200	WT	WT	WT	WT	WT	WT	WT	WT	Yes
29	22	M	M1	168	WT	WT	WT	WT	WT	WT	WT	WT	Yes
30	68	M	M2	208	WT	WT	WT	WT	WT	WT	WT	WT	No
31	23	M	M5	133	WT	WT	WT	WT	ND	ND	Mt	Mt	No
32	62	M	M2	271	WT	WT	WT	WT	ND	ND	WT	WT	No
33	55	F	M2	281	WT	WT	WT	WT	ND	ND	WT	WT	Yes
34	32	M	M1	257	WT	WT	WT	WT	ND	ND	ND	ND	No
35	60	F	M5	429	WT	WT	WT	WT	ND	ND	ND	ND	Yes
36	47	M	M2	435	WT	WT	WT	WT	ND	ND	ND	ND	Yes
37	19	M	M2	389	WT	WT	WT	WT	ND	ND	ND	ND	Yes
38	58	M	M3	412	WT	WT	WT	WT	ND	ND	ND	ND	Yes
39	44	F	M4	266	WT	WT	WT	WT	ND	ND	ND	ND	Yes

Mutational status of *NPM1, FLT3, NRAS,* and *TP53* mutations was analyzed in 39 paired samples obtained at diagnosis and relapse. Italics in data field indicate a change in mutational status.

D indicates diagnosis; R, relapse; Mt, mutation; WT, wild type; ND not done.

increasing penetration of APL-like disease.[44] The *NPM1* mutation is essentially absent in patients with APL or CBF leukemia, in contrast to the high frequency of FLT3/ITD and *KIT* mutations in patients with APL and CBF leukemia, respectively. Only the mutation of *NPM1* was an independent favorable prognostic factor for achieving CR and a marginally favorable prognostic factor for overall survival in patients with FLT3/ITD. However, it did not affect the CR rate, and its prognostic value for relapse was not significant in patients without FLT3/ITD. Mutant NPM, therefore, might be involved in the pathogenesis of AML by its conferring a differentiation block or properties of self-renewal, or both, to the hematopoietic progenitors in concert with mutant FLT3, and it might be associated with sensitivity to chemotherapy. More recently, it was reported that several genes putatively involved in the maintenance of a stem cell phenotype, such as *HOX* and *JAG1* genes, were up-regulated in NPMc+ AML cells,[45] supporting this hypothesis. However, the exact role of mutant NPM in the pathogenesis of AML, especially without FLT3/ITD, has not been fully resolved. Our sequential analysis using the paired samples obtained at diagnosis and relapse demonstrated that the *NPM1* mutation is not related to acquiring additional genetic changes in *FLT3, TP53,* and *NRAS* genes. In addition, the *NPM1* mutation was not a favorable factor for achieving second CR after relapse, suggesting that unknown genetic or epigenetic changes might additionally occur in AML cells with the *NPM1* mutation during disease progression. It is notable that *NPM1* mutations were lost at relapse in 2 of the 17 patients who had *NPM1* mutations at initial diagnosis. Loss of the mutation at relapse has been repeatedly observed in the mutations of *FLT3* and *NRAS*, and these mutations are thought of as late or secondary events in leukemogenesis. Loss of the *NPM1* mutation at relapse suggests that it is not necessarily an earlier event and that it is not needed for continuance of the disease. However, because NPM is involved in ribosome assembly/transport, cytoplasmic/nuclear trafficking, regulation of DNA polymerase alpha activity, centrosome duplication, acute response of mammalian cells to environmental stress, and stabilization of the p53 pathway,[25,26,46-48] it is possible that mutant NPM also has a multifunctional role in the pathogenesis of AML. Alternatively,

mutant NPM might use its multiple functions according to the genetic state of leukemia progenitors. Further biologic studies are necessary to clarify how mutant NPM is involved in the pathogenesis of AML and what kinds of genetic or epigenetic alterations cooperate with mutant NPM for the development of AML.

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SPOTLIGHT

Efficacy of gemtuzumab ozogamicin on ATRA- and arsenic-resistant acute promyelocytic leukemia (APL) cells

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Acute promyelocytic leukemia (APL) cells express a considerable level of CD33, which is a target of gemtuzumab ozogamicin (GO), and a significantly lower level of P-glycoprotein (P-gp). In this study, we examined whether GO was effective on all-*trans* retinoic acid (ATRA)- or arsenic trioxide (ATO)-resistant APL cells. Cells used were an APL cell line in which P-gp was undetectable (NB4), ATRA-resistant NB4 (NB4/RA), NB4 and NB4/RA that had been transfected with MDR-1 cDNA (NB4/MDR and NB4/RA/MDR, respectively), ATO-resistant NB4 (NB4/As) and blast cells from eight patients with clinically ATRA-resistant APL including two patients with ATRA- and ATO-resistant APL. The efficacy of GO was analyzed by ^{3}H-thymidine incorporation, the dye exclusion test and cell cycle distribution. GO suppressed the growth of NB4, NB4/RA and NB4/As cells in a dose-dependent manner. GO increased the percentage of hypodiploid cells significantly in NB4, NB4/RA and NB4/As cells, and by a limited degree in NB4/MDR and NB4/RA/MDR cells. Similar results were obtained using blast cells from the patients with APL. GO is effective against ATRA- or ATO-resistant APL cells that do not express P-gp, and the mechanism of resistance to GO is not related to the mechanism of resistance to ATRA or ATO in APL cells.
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Introduction

Recently, gemtuzumab ozogamicin (GO, Mylotarg™), a calicheamicin-conjugated humanized anti-CD33 monoclonal antibody (mAb), has been introduced for the treatment of acute myeloid leukemia (AML).[1] However, the clinical outcome after the treatment with GO was negatively associated with P-glycoprotein (P-gp) function in AML.[2] In our previous studies, we found that resistance to GO was mainly mediated by P-gp.[3,4] Acute promyelocytic leukemia (APL) cells express a considerable level of CD33 antigen and a significantly lower level of P-gp compared with other types of AML.[5] Therefore, GO may become established as a successful treatment for APL. In fact, GO has been introduced with the clinical efficacy in the treatment of APL.[6,7] However, *in vitro* efficacy of GO on APL cells as well as all-*trans* retinoic acid (ATRA)- and arsenic trioxide (ATO)-resistant ones has not been studied well. Moreover, drug interaction among ATRA, ATO and GO, and the mechanisms of resistance of APL cells to them remain unclear.[8]

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Materials and methods

Cells

The cell lines used were a human APL cell line, NB4, which was kindly provided by Dr M Lanotte (Hospital Saint-Louis, Paris, France);[9] ATRA-resistant NB4 (NB4/RA) cells; NB4 and NB4/RA cells transfected with MDR-1 cDNA (NB4/MDR and NB4/RA/MDR, respectively); and ATO-resistant NB4 (NB4/As) cells. The NB4/RA and NB4/As cells were obtained by culturing NB4 cells with gradually increasing concentrations of ATRA and ATO, respectively.[10] mdr-1 messenger RNA (mRNA) was not detectable in NB4, NB4/RA or NB4/As cells by RT-PCR.[11,12] NB4/MDR and NB4/RA/MDR cells had detectable mdr-1 mRNA, but did not have detectable MDR-related protein (MRP) mRNA or lung-resistant protein (LRP) mRNA. Blasts were collected from four patients with APL at diagnosis, six patients with clinically ATRA-resistant but ATO-sensitive APL and two patients with clinically ATRA- and ATO-resistant APL.

Flow cytometric analysis for CD33 and Pgp expression

For evaluation of CD33 expression, cells were stained with phycoerythrin (PE)-conjugated anti-CD33 mAb (Becton Dickinson Immuno-cytometry Systems, San Jose, CA, USA), according to the manufacturer's instructions. For P-gp analysis, cells were incubated with biotinylated MRK16 (Fab') mouse mAb or a subclass-matched control mAb, and stained with streptavidine-Per CP (Becton Dickinson Immuno-cytometry Systems) as previously described.[11] Over 10 000 events were analyzed with the Epics XL flow cytometer (Beckman Coulter, Fullerton, CA, USA). APL cells obtained from the patients were also gated by CD-45 staining.[4]

Humanized anti-CD33 mAb and GO

GO consists of three essential parts: an antibody a cytotoxic agent, and a linker. The antibody, humanized IgG4 (hP67.6), targets the CD33 antigen. The cytotoxic agent is *N*-acetyl (NAc)-γ calicheamicin dimethyl hydrazide (DMH), a derivative of calicheamicin antitumor antibiotics.[1,2] GO, humanized non-conjugated anti-CD33 mAb (hP67.6) and free NAc-γ calicheamicin DMH were kindly provided by the Wyeth Research Division of Wyeth Pharmaceuticals Inc. (Philadelphia, PA, USA). The amount of GO used in an experiment was determined based on the concentration of NAc-γ calicheamicin DMH bound to the antibody. One microgram of GO contains 27.1 ng of NAc-γ calicheamicin DMH, and approximately 97% of a GO molecule is composed of the linker and antibody.

^{3}H-Thymidine (^{3}H-TdR) incorporation analysis for assessment of cell proliferation

Cells were plated in a 96-well microplate (BD Biosciences, Billerica, MA, USA) at 2×10^5 cells per well in the presence or absence of GO containing 5, 10 or 100 ng/ml NAc-γ calicheamicin DMH or the respective concentration of hP67.6, in 100 μl of RPMI 1640 medium containing 10% fetal calf serum (FCS) and 1 μCi of ^{3}H-TdR. The detailed method was described in our previous papers.[3] The level of ^{3}H-TdR incorporation upon incubation with GO was compared with that upon incubation with hP67.6. The analysis was repeated five times.

Dye exclusion test with propidium iodide staining

After incubation of cells with GO or hP67.6. for the indicated period of time, cells were stained with 0.2 μg/ml propidium iodide (PI) (Sigma, Saint Louis, MO, USA) solution and counted. The numbers of dye-stained (dead) and unstained (living) cells both decreased and the amount of debris rapidly increased, making it difficult to evaluate the cell viability properly. Therefore, viable cells were evaluated. The viable cell count (/ml) after incubation with GO was compared with that after incubation with hP67.6. The analysis was repeated five times.

Cell cycle distributions

The cell cycle distribution was analyzed by flow cytometry with PI staining. The detailed method was described in our previous papers.[3,4] Cell cycle distribution could be analyzed after incubation with 10 or 100 ng/ml of GO for 24 or 48 h. GO temporarily arrests NB4 cells at the G2/M phase, and increases the percentage of hypodiploid cells, by which we evaluated the effect of GO.[3,4] Then, the GO-sensitive cells rapidly change to debris. The analysis was performed in triplicate.

Results

Flow cytometric analysis of CD33 and P-gp expression on NB4 cells and its sublines

The amount of CD33 expressed on the cells did not significantly differ among NB4, NB4/RA, NB4/MDR, NB4/RA/MDR and NB4/As cells. P-gp was not expressed on NB4, NB4/RA or NB4/As cells, in agreement with previous reports.[11,12] Equivalent levels of P-gp were expressed on NB4/MDR and NB4/RA/MDR cells.

^{3}H-TdR incorporation into NB4 cells and its sublines

Upon 72-h incubation with GO containing 5, 10 or 100 ng/ml of NAc-γ calicheamicin DMH, the level of ^{3}H-TdR incorporation into NB4 cells and its sublines decreased in a dose-dependent manner (Figure 1a). In each cell line, the level of ^{3}H-TdR incorporation was lower than that in the same cell line that had been incubated with the corresponding concentration of hP67.6. Upon incubation with GO containing 100 ng/ml NAc-γ calicheamicin DMH, there were significant differences in the level of ^{3}H-TdR incorporation between NB4 and NB4/MDR cells at 48 and 72 h ($P<0.01$ each), and between NB4/RA and NB4/RA/MDR cells at 48 and 72 h ($P<0.01$ each) (Figure 1b). There were no significant differences in the level of ^{3}H-TdR incorporation between NB4 and NB4/RA cells, or between NB4/MDR and NB4/RA/MDR cells at any time point. ^{3}H-TdR incorporation in NB4 cells upon 72-h incubation with GO containing 10 ng/ml NAc-γ calicheamicin DMH corresponded with that with 1000 ng/ml free NAc-γ calicheamicin DMH, which corresponded to a concentration of NAc-γ calicheamicin DMH that was approximately 100 times greater than that in GO. The rate did not change between P-gp-negative and positive NB4 cells.

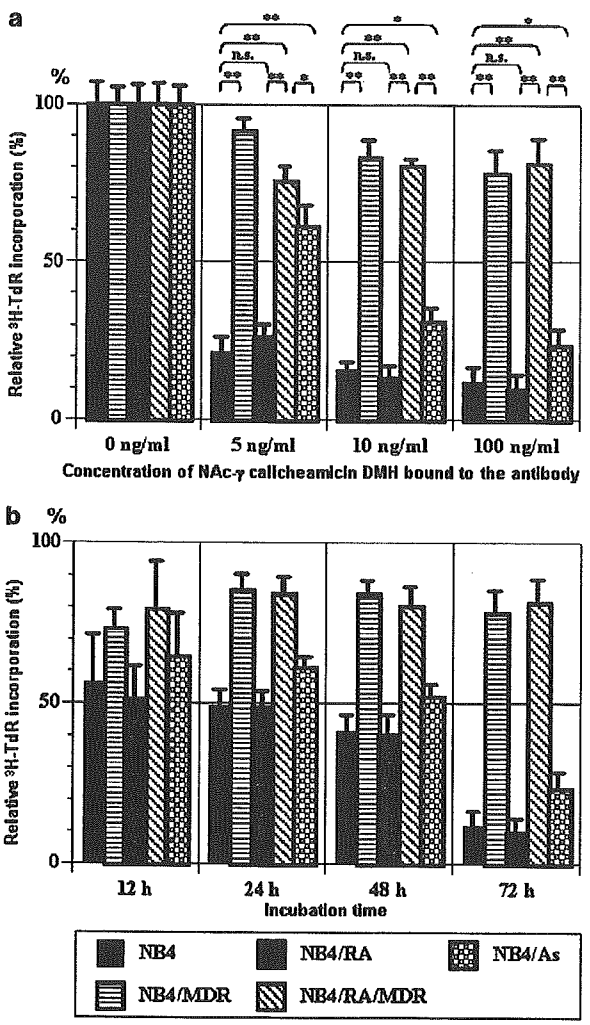

Figure 1 (a) ^{3}H-TdR incorporation by NB4 cells and its sublines after incubation with GO containing 5, 10 or 100 ng/ml NAc-γ calicheamicin DMH or with the respective concentrations of hP67.6 for 72 h. At 72 h, the amount of incorporated ^{3}H-TdR (cpm) was determined by liquid scintillation counting. In (a, b) the amount of incorporated ^{3}H-TdR are expressed as the ratio (%) of incorporated ^{3}H-TdR after incubation with GO to that after incubation with the respective concentration of hP67.6, normalized to 100%. The data from five independent experiments are expressed as mean values of the % response±standard deviation (s.d.). At 72 h incubation with hP67.6, the mean level of ^{3}H-TdR incorporation by NB4 cells was 57 000 (53 200–62 300). Statistical significance was calculated by Student's *t*-test. $^*P<0.05$, $^{**}P<0.01$. (b) Time course of ^{3}H-TdR incorporation by NB4 cells and its sublines. Cells were incubated in medium with GO containing 100 ng/ml NAc-γ calicheamicin DMH or the respective concentration of hP67.6 for 12, 24, 48 or 72 h.

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Viable cell count analysis by flow cytometry

By the incubation with GO containing 5, 10 or 100 ng/ml of NAc-γ calicheamicin DMH for 72 h, the viable cell counts of NB4 cells and its sublines decreased in a dose-dependent manner (Figure 2a). Figure 2c shows their cell counts upon 24-, 48- or 72-h incubation of these cell lines with GO containing 100 ng/ml NAc-γ calicheamicin DMH or the respective concentration of hP67.6. GO similarly decreased the number of NB4 and NB4/RA cells. GO also decreased the count of NB4/As cells. GO slightly reduced the counts of NB4/MDR and NB4/RA/MDR cells. The counts of NB4 cells and its sublines upon 72-h incubation with GO containing 10 ng/ml NAc-γ calicheamicin DMH corresponded with those with 1000 ng/ml free NAc-γ calicheamicin DMH, respectively.

The combination of ATRA and GO reduced the count of NB4 cells by a greater degree than GO or ATRA alone ($P=0.019$ and $P<0.01$, respectively), but did not reduce the count of NB4/RA cells by a significantly greater degree than GO or ATRA alone (Figure 2b). Upon incubation with GO and ATO, the counts of NB4 and NB4/RA cells were less than those upon incubation with GO ($P<0.01$ each) or ATO alone ($P<0.01$ each).

Cell cycle distribution

The increase percentage in the number of hypodiploid cells on cell cycle distribution upon incubation with GO containing 10 or 100 ng/ml of NAc-γ calicheamicin DMH for 48 h is summarized in Figure 3. The percentage of hypodiploid cells in the NB4, NB4/RA and NB4/As cells was increased upon 12-h incubation with GO, and it was the highest upon 48-h incubation. Beyond 48 h, it was difficult to evaluate the proportion of hypodiploid cells accurately because these GO-sensitive cells transformed into debris, as reported previously (Figure 4).[3] Upon incubation with GO, the percentage of hypodiploid cells in the NB4/MDR and NB4/RA/MDR cells were significantly less than those in the NB4 and NB4/RA cells, respectively ($P<0.01$ each). The addition of ATRA to GO further increased the percentage of hypodiploid cells in NB4 cells ($P=0.043$), but not in NB4/RA cells ($P=0.97$). Upon incubation with GO and ATO, the percentage of hypodiploid cells in NB4 and NB4/RA cells were slightly higher than those upon incubation with GO alone ($P=0.091$ and 0.082, respectively).

Similar results were obtained using blast cells derived from the patients with APL (Table 1). Upon incubation with GO for 48 or 60 h, the hypodiploid portion considerably increased in APL cells that had been obtained from not only the four cases at diagnosis, but also five ATRA-resistant and two ATRA- and ATO-resistant cases. Two patients, whose APL relapsed after achieving complete remission (CR) by ATRA and receiving postremission chemotherapy, were treated according to the Japanese phase 1 and 2 study of GO. They were resistant to re-induction therapy by ATRA, but achieved CR and CR without platelets recovery (CRp) after treatment with GO, respectively.

Discussion

Recently, GO with or without ATRA was introduced for the treatment of APL, and the clinical efficacy of these therapies has been reported in newly diagnosed or relapsed patients with APL.[6,7] There are two basic reasons that support the clinical

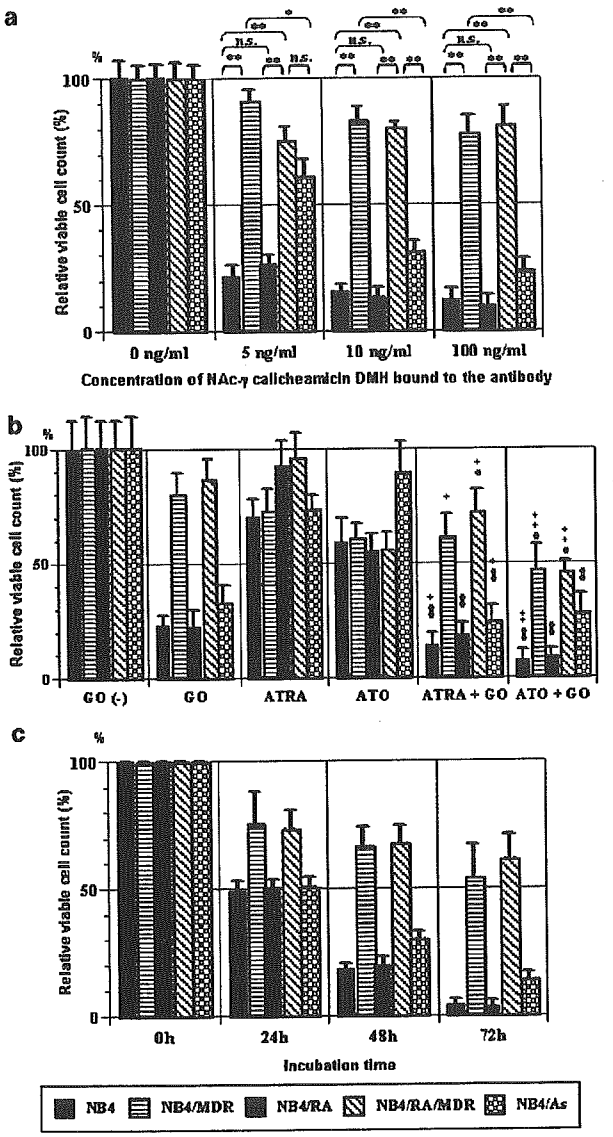

Figure 2 (a) Viable cell counts of NB4 cells and its sublines after incubation with GO containing 5, 10 or 100 ng/ml NAc-γ calicheamicin DMH or with the respective concentrations of hP67.6 for 72 h. The cell counts are expressed as the ratio of the cell counts after incubation with GO to that after incubation with the respective concentration of hP67.6, normalized to 100%. The data from five independent experiments are expressed as mean values of the % count ± s.d. Statistical significance was calculated by Student's *t*-test. $^{*}P<0.05$, $^{**}P<0.01$. (b) Viable cell counts of the five cell lines upon incubation with GO containing 5 ng/ml NAc-γ calicheamicin DMH with or without ATRA or ATO for 72 h. The ratio of the cell count after incubation with the agents to that after incubation in the absence of both agents was calculated. Statistical significance was calculated by Student's *t*-test. $^{*}P<0.05$, $^{**}P<0.01$ comparing GO + ATRA or GO + ATO *vs* ATRA alone or ATO alone, respectively. $^{+}P<0.05$, $^{++}P<0.01$ comparing GO + ATRA or GO + ATO *vs* GO alone. (c) Viable cell counts of the five cell lines upon incubation with GO containing 100 ng/ml NAc-γ calicheamicin DMH, or with the respective concentration of hP67.6 for up to 72 h. The ratio (%) of the cell count after incubation with GO to that after incubation with the respective concentration of hP67.6 was calculated.

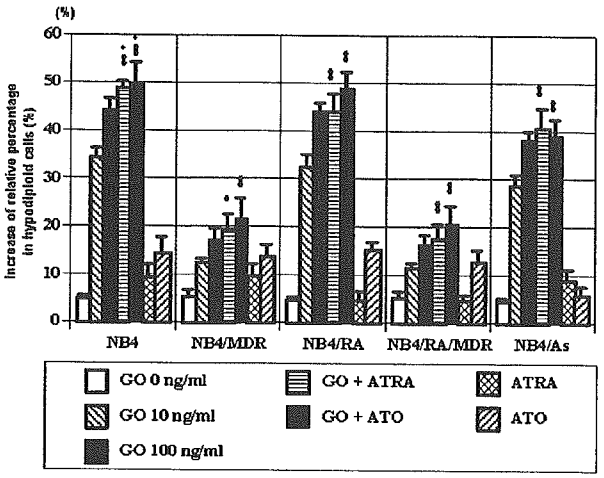

Figure 3 Percentage of hypodiploid cells in cell cycle distribution patterns upon 48-h incubation with GO containing 10 or 100 ng/ml of NAc-γ calicheamicin DMH or with the respective concentrations of hP67.6. Cells were also incubated with GO containing 100 ng/ml of NAc-γ calicheamicin DMH in combination with 10^{-6} M ATRA or 10^{-6} M ATO. Each bar represents the mean ± s.d. of three experiments. $^{*}P<0.05$, $^{**}P<0.01$ comparing GO + ATRA or GO + ATO *vs* ATRA alone or ATO alone, respectively. $^{+}P<0.05$, $^{++}P<0.01$ comparing GO + ATRA or GO + ATO *vs* GO alone.

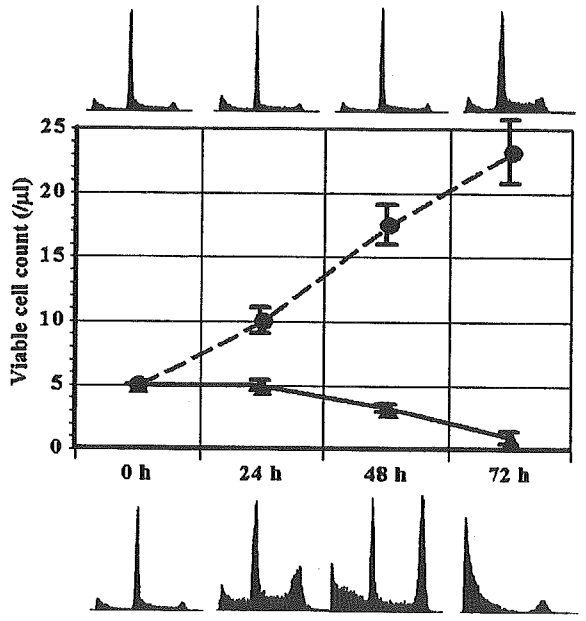

Figure 4 The cell growth curve and cell cycle distributions of NB4 cells upon 24-, 48- and 72-h incubation with GO containing 10 ng/ml NAc-γ calicheamicin DMH (continuous line and lower distribution patterns, respectively) or with the respective concentrations of hP67.6 (dotted line and upper distribution patterns, respectively).

application of GO on APL. One is that large amounts of CD33 are commonly expressed on the surface of APL cells. Therefore, several different anti-CD33 mAbs have been used for the treatment of APL, and notable results have been reported, especially in the use of GO.[6,7] Another reason is that a low level of P-gp is expressed on APL cells.[5] GO is sometimes not effective in some other subtypes of AML because the detached calicheamicin derivative is pumped out by P-gp.[3,4] Therefore, this mechanism of resistance to GO is not theoretically applicable to APL cells.

In this study, GO showed antiproliferative and cytocidal effects on ATRA-resistant NB4 cells as well as NB4 cells. We previously demonstrated that MDR modifiers, PSC833 and MS209, had no effect on ATRA-resistance in APL, which indicated that P-gp has a limited role in ATRA-resistance.[11] Intracellular ATRA concentration was not influenced by P-gp.[11] Clinical evidence, including our reports, also supported the independence of P-gp and ATRA-resistance.[13] Taking these data into consideration, GO is predictably effective on ATRA-resistant APL unless P-gp is expressed.

In the previous report, the combination of GO and ATRA was given to some patients with APL. In a study conducted in the US, GO was administered with ATRA to 19 patients with untreated APL.[6] The CR rate was 16/19 (84%), and 14 became PCR-negative. In relapsed APL, Lo-Coco *et al*[7] reported 14 cases of patients who achieved molecular remission after treatment with GO among 16 relapsed APL cases. However, there has been no *in vitro* study to explain these clinical efficacies. We performed the present study using NB4 and its drug-resistant sublines in an attempt to elucidate the mechanisms of GO. In APL, the drug resistance, which has been studied and discussed previously, might be built up by multiple causes and procedures.[8,13] Further studies on APL should be performed from many directions. It is also important to determine the optimal dosage of these drugs as well as the optimal timing of their administration.

GO also showed efficacy on ATO-resistant NB4 cells, which do not express P-gp. The cellular glutathione and MRP levels are reported with their relationship to ATO-resistance.[10,14] Walter *et al*[15] reported that MRP1 might attenuate the susceptibility to GO, although by a smaller degree than P-gp. We could not find an obvious relationship between MRP1 and GO-resistance.[16] Our data suggest that the MRP and the cellular glutathione levels play limited roles, while P-gp plays a major role in GO-resistance.

GO showed antiproliferative and cytocidal effects on APLs that do not express P-gp (Figure 4). GO increased the percentage of hypodiploid cells (Figure 3) while it inhibited cell proliferation in the early phase (Figures 1 and 2). After undergoing these changes, GO-sensitive cells rapidly collapsed into debris. The time-lag and variation of the effect of GO on APL cells might be explained by differences in the level of CD33 expression on the cells, and the length of time required for binding, and internalization of GO and detachment of calicheamicin from GO. Alternatively, GO could have various different actions against cells. Apoptosis, which is one of the main mechanisms of GO, did not explain all of the observed morphological changes of GO-treated cells in our previous study using videomicroscopy.[3] However, analysis of the changes in cell cycle distribution could be a valuable test for analyzing the susceptibility of AML cells to GO. It has a high degree of usability for samples derived from cases that contain different phenotypes.

We confirmed the antileukemia effect of GO on APL in an *in vitro* study using an APL cell line and its ATRA- and ATO-resistant sublines. GO seems to be promising for the treatment of not only untreated but also relapsed APL. A larger clinical study of GO for the treatment of relapsed and refractory APL is needed. The results of such study may suggest how GO should be integrated into the management of APL.

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Table 1 Background of the patients and *in vitro* effect of GO on the cell cycle

Case no.	Sex	Age (year)	t(15;17)[a]	PML/RARα[b]	Status	Blast (%) in bone marrow	CD33 (%)	P-gp (%)	% increase in hypodiploid cells by GO[c]		Clinical response to ATRA	Clinical response to ATO	Clinical response to GO
									48 h	60 h			
DIAG-1	M	63	(+)	(+)	At diagnosis	92.2	99	3.7	13.2	22.7	CR	NT	NT
DIAG-2	F	21	(+)	(+)	At diagnosis	88.0	97.9	1.8	17.5	25.3	CR	NT	NT
DIAG-3	M	55	(+)	(+)	At diagnosis	67.6	97.8	3.5	12.1	17.9	CR	NT	NT
DIAG-4	M	51	(+)	(+)	At diagnosis	68.1	88.3	4.1	18.3	35.8	CR	NT	NT
ATRA-1	F	36	(+)	(+)	2nd relapse	20.2	98.7	1.8	10.7	18.4	NR	CR	NT
ATRA-2	M	57	(+)	(+)	2nd relapse	83.2	89.8	9.1	6.6	8.1	NR	CR	NT
ATRA-3	F	38	(+)	(+)	2nd relapse	77.2	94.6	1.4	16.9	27.9	NR	CR	NT
ATRA-4	F	21	(+)	(+)	2nd relapse	94.8	97.8	0.1	32.4	43.5	NR	CR	NT
ATRA-5	M	51	(−)	(+)	3rd relapse	30.8	97.9	4.4	9.6	12.8	NR	NT	CR
ATRA-6	M	48	(+)	(+)	3rd relapse	86.0	97.1	4.2	13.8	25.6	NR	NT	CRp
ATO-1	M	50	(+)	(+)	3rd relapse	78.0	83.6	11.6	10.8	13.4	NR	NR	NT
ATO-2	F	38	(+)	(+)	2nd relapse	38.7	85.4	4.5	9.5	15.3	NR	NR	NT

[a]Karyotype was analyzed by G-banding.
[b]PML/RARα was analyzed by RT-PCR or FISH.
[c]Defined as the difference in the percentage of hypodiploid cells between samples that had been incubated with nonconjugated anti-CD33 mAb or GO.
NT, not treated by ATO or GO *in vivo*; NR, no response; CR, complete remission; CRp, CR without platelets recovery.
After treatment with GO, the percentage of hypodiploid cells considerably increased in APL cells that had been obtained not only from the four patients at diagnosis but also from several ATRA-resistant patients and ATRA-and-ATO-resistant patients. Two patients, whose APL relapsed after achieving CR by ATRA and receiving postremission chemotherapy, were treated according to the Japanese phase 1 or 2 study of GO. They were resistant to reinduction therapy by ATRA, but achieved CR and CRp by treatment with GO, respectively. The coefficient of correlation (*r*) between the percentage of P-gp expression and the increase in hypodiploid portion was 0.60 and 0.65 upon 48- and 60-h incubation with GO, respectively.

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Expression and methylation status of the *FHIT* gene in acute myeloid leukemia and myelodysplastic syndrome

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To clarify the role of fragile histidine triad (FHIT) in hematological malignancies, we examined the methylation status and the expression level of the *FHIT* gene in myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) cells in comparison with the methylation of the *p15*^{INK4B} gene. The *FHIT* methylation was found in 13 of 94 (13.8%) AML and 22 of 40 (55.0%) MDS cases, but not in normal mononuclear cells (MNCs). Both the frequency and density of methylation increased in the advanced-stages MDS and the relapsed AML cases. Although *FHIT* and *p15*^{INK4B} methylations were not correlated in MDS and AML, increased *FHIT* methylation at the relapse in AML was associated with *p15*^{INK4B} methylation. The median expression level in AML was significantly higher than in normal MNCs, although the median expression level in those with methylation was significantly lower than in those without methylation. Furthermore, the methylation level at relapse was significantly higher than at diagnosis in AML. These results suggested that *FHIT* methylation was accumulated through the disease progression of MDS and AML, and the role of the *FHIT* gene as a tumor suppressor seemed different in AML and MDS.
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Introduction

The fragile histidine triad (*FHIT*) gene was isolated from the chromosome band 3p14.2, which includes the FRA3B common fragile region.[1] Alterations of the *FHIT* gene, including loss of heterozygosity, homozygous deletions and the expression of aberrant transcripts, are frequently found in a variety of solid tumors and cancer cell lines, and the loss or reduced expression of FHIT is reportedly associated with tumor progression and worse prognosis.[2–5] The *FHIT* gene has, therefore, been considered a tumor suppressor gene, while the exact molecular pathway of its tumor suppressor function remains unclear.[6]

In hematological malignancies, aberrant FHIT transcripts have been reported in acute and chronic leukemia, although it was demonstrated that those aberrant transcripts were observed in both normal and malignant hematopoietic cells.[7–10] In contrast, the loss or reduced expression of FHIT protein was restricted to malignant hematopoietic cells, while neither loss nor reduction was associated with progression, response to therapy or prognosis in chronic myeloid leukemia (CML) and prognosis in acute lymphocytic leukemia (ALL).[11,12] These results indicated that inactivating alterations of the *FHIT* gene, which result in the loss or reduced expression of its product, may be involved in the pathogenesis of hematological malignancies, but the clinical significance was not well documented. Since genomic alterations of the *FHIT* gene, such as loss of heterozygosity and somatic mutations, are rare in hematological malignancies, we focused on promoter methylation as an inactivating mechanism. To date, it has been shown that many tumor suppressor genes were inactivated by methylation within their promoter regions in various kinds of malignant cells. Among them, it was demonstrated that the *p15*^{INK4B} gene, but not the *p14*^{ARF} and *p16*^{INK4A} genes, was frequently inactivated by promoter methylation in myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML).[13] In particular, *p15*^{INK4B} was shown to be associated with the high blast percentage in BM and transformation to AML in MDS.[14–16] Methylation of the *FHIT* gene was found in several solid tumors, including the esophagus, colon, lung and breast cancers, and was shown to be associated with loss of expression.[17–19] In addition, it was reported that the loss or reduced expression of FHIT protein resulting from gene methylation was associated with disease progression in solid tumors.[20–22] However, it was also suggested that FHIT inactivation seems to occur at different stages of cancer development among the cancer types and is necessary, but not sufficient, for conferring a growth advantage to cancer cells.[23]

In this study, to clarify how FHIT is involved in the development and progression of hematological malignancies, we examined the methylation status and expression level of the *FHIT* gene in MDS and AML cells in comparison with *p15*^{INK4B} methylation.

Methods

Patients and samples

The diagnosis of MDS and AML was based on morphology, histopathology, the expression of leukocyte differentiation antigens and/or the French–American–British (FAB) classification. The study population included 40 MDS cases consisting of 10 refractory anemia (RA), 13 refractory anemia with excess of blasts (RAEB), five RAEB in transformation (RAEBt), seven overt leukemia from MDS (MDS-overt) and five chronic myelomonocytic leukemia (CMMoL) and 94 newly diagnosed *de novo* AML cases consisting of 1 M0, 26 M1, 31 M2, 6 M3, 20 M4, 9 M5 and 1 M6 FAB types. In 21 of the 94 AML cases, samples at both diagnosis and at the first relapse were available. In three of the 21 cases, sequential samples at diagnosis, complete remission (CR) and the first relapse were available. Furthermore, in six cases, samples at both diagnosis and CR were available. Bone marrow (BM) samples from patients with MDS or AML were subjected to Ficoll-Hypaque (Pharmacia LKB, Uppsala, Sweden) density gradient centrifugation and cryopreserved in liquid nitrogen before use. For the normal control, five BM, four peripheral blood (PB) and five cord blood (CB) mononuclear cells (MNCs) were used. We obtained informed consent from all patients and volunteers to use their samples in this study. CB was collected after full-term deliveries with informed consent

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approved by the Review Board of Tokai Cord Blood Bank.[24] CD34$^+$ and CD34$^-$ fractions of MDS, CB and BM MNCs were separated by using Dynabeads M-450 conjugated with an anti-CD34 monoclonal antibody and DETACHaBEAD (Dynal, Oslo, Norway), according to the manufacturer's instructions. Separated samples were subjected to flow cytometry for analyzing the purity. We also examined three human myeloid leukemia cell lines consisting of THP1, MOLM13 and U937. All cell lines were maintained in RPMI1640 medium (Gibco BRL, Gaithersburg, ND, USA) supplemented with 10% fetal calf serum (Gibco BRL).

Analysis of FHIT *and* p15^{INK4B} *gene methylation*

In this study, we analyzed the methylation status of 30 CpG sites in the *FHIT* gene promoter and tentatively numbered them from 5′ to 3′ (Figure 1). High molecular weight DNA was prepared from cell samples and cell lines by standard methods, and bisulfite modification of genomic DNA was performed as previously reported.[25] Methylation-specific PCR (MSP) was performed by using primer pairs for the methylated *FHIT* gene (FHIT-MF, 5′-TTGGGGCGCGGGTTTGGGTTTTTACGC-3′ and FHIT-MR, 5′-CGTAAACGACGCCGACCCCACTA-3′) and for the unmethylated *FHIT* gene (FHIT-UF, 5′-TTGGGGTGTGGGTT TGGGTTTTTATG-3′ and FHIT-UR, 5′-CATAAACAACACCAA CCCCACTA-3′), according to the previous report.[18] For bisulfite genomic sequencing (BGS) of the *FHIT* 5′ CpG island, bisulfite-treated DNA was amplified by seminested PCR using primer pairs for the first round PCR (FHIT-1F, 5′-GAAAAAGTTAAAGAT TGTGCGA-3′ and FHIT-1R, 5′-ATCCCACCCTAAAACCTCG TAAAAC-3′) and for the second round PCR (FHIT-2F, 5′-AGT TGTGTTTTGTGGTTAGTGTTTTT-3′ and FHIT-1R) (Figure 1a).[17] Amplified products were separated through agarose gel and purified using a QIAquick gel extraction kit (Qiagen Inc., Chatsworth, CA, USA). The fragments were cloned into a pGEM-T-vector (Promega, Madison, WI, USA), then transfected into the *Escherichia coli* strain, DH5α. In total, 10 recombinant colonies were randomly selected from PCR-amplified libraries, and plasmid DNA was prepared using a QIAprep Spin Miniprep Kit (Qiagen Inc.). DNA sequencing was performed on a DNA sequencer (310; Applied Biosystems, Foster City, CA, USA) using a BigDye terminator cycle sequencing kit (Applied Biosystems).

The methylation status within the exon 1 of the *p15^{INK4B}* gene was analyzed by MSP using the primer pairs for the methylated (p15-MF, 5′-GCGTTCGTATTTTGCGGTT-3′ and p15-MR, 5′-CGTACAATAACCGAACGACCGA-3′) and for the unmethylated genes (p15-UF, 5′-GTTTTGAGTTTGGTTGTGTTTGT-3′ and p15-UR, 5′-ATTAACTCCAAACTTTTCCTAACA-3′), as previously described.[26] BGS of the *p15^{INK4B}* 5′ CpG island was performed as described above, using primer pairs p15-F, 5′-TGGGGATTAGGAGTTGAGGG-3′ and p15-R, 5′-CCTTCCTA AAAAACCTAAACTCAA-3′ (Figure 1b).

Quantitation of FHIT transcript expression

Total RNA was extracted from the samples using a QIAamp RNA Blood Mini Kit (Qiagen Inc.). cDNA was synthesized from each RNA using a random primer and Moloney murine leukemia virus reverse transcriptase (Super-Script II; Gibco BRL, Gaithersburg, ND, USA), according to the manufacturer's recommendations. The expression level of the FHIT transcript was quantitated using a real-time fluorescence detection method on an ABI Prism 7000 sequence detection system (Applied Biosystems).[27] The primer and probe sequences for real-time PCR of FHIT were sense primer, 5′-AAACATTTCCA TGGGACCTCTCT-3′, antisense primer, 5′-GAGCTCCTCATAG ATGCTGTCATTC-3′, and TaqMan probe, 5′-FAM-CCGGACA GACTGTGAAGCACGTTCAC-TAMRA-3′. The housekeeping gene, *GAPDH*, served as a control for cDNA quality. Relative gene expression levels were calculated using standard curves

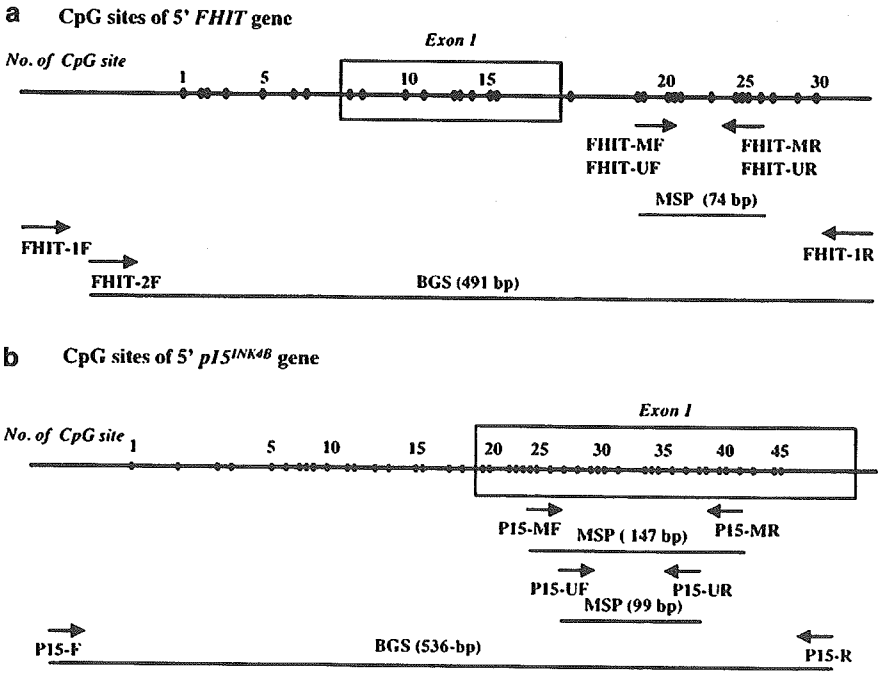

Figure 1 Schematic representation of the 30 and 45 CpG sites in the *FHIT* (a) and *p15^{INK4B}* (b) gene promoters. The positions of the PCR primers for MSP and BGS are indicated by arrows.

and adjusted based on the expression level of the *GAPDH* gene. Each gene expression level was analyzed in triplicate and the mean was subjected to analysis.

5-Aza-2′-deoxycytidine treatment

5-Aza-2′-deoxycytidine (5-Aza-dC) was purchased from Sigma Chemical Co. (St Louis, MO, USA), and dissolved in phosphate-buffered saline at appropriate concentrations. Primary AML and MDS cells or cell lines were plated at a density of 1×10^6 cells/ml on a six-well tissue plate (Becton Dickinson, Franklin Lakes, NJ, USA) in RPMI1640 medium (Gibco BRL) containing 10% FCS with or without 1 μM 5-Aza-dC. In this culture condition, we did not add any growth factors. After 48 h treatment in a humidified incubator containing 5% CO_2 in air at 37°C, DNA and RNA were extracted from the treated cells and subjected to analysis for methylation status and expression of the *FHIT* gene as described above.

Statistical analysis

Differences in continuous variables were analyzed with the Mann–Whitney *U*-test for distribution between two groups, or the Kruskal–Wallis test and the Bonferroni test for distribution among more than three groups. Analysis of frequency was performed using Fisher's exact test for 2×2 tables or Pearson's χ^2 test for larger tables. Analyses of the change of methylation density and the expression level of the transcript were performed using the paired sign test and the Wilcoxon signed rank test, respectively. These statistical analyses were performed with StatView-J 5.0 (Abacus Concepts Inc., Berkeley, CA, USA). For all analyses, the *P*-values were two-tailed, and a *P*-value of less than 0.05 was considered statistically significant.

Results

Methylation status of the FHIT *gene promoter*

Since the MSP used here detected the methylation status only in the 3′ region of the promoter, we first examined whether MSP represented the methylation density of 30 CpG sites in the *FHIT* gene promoter of three leukemia cell lines and 14 normal MNCs. To determine the methylation density, we performed BGS in 10 randomly selected clones from the PCR-amplified libraries of each sample and the methylation density was determined by the number of methylated CpG/total number of CpG sites. In all normal MNCs, no visible products amplified with methylation-specific primers were observed, in agreement with the results obtained by BGS where there was only one methylated CpG site among all the analyzed clones. Furthermore, no methylated CpG site was observed by BGS in both $CD34^+$ and $CD34^-$ fractions separated from each two CB and BM cells. In THP1, MSP revealed unmethylated and weak methylated forms, and the BGS demonstrated that 9/10 clones had at least one methylated site and the methylation density was 13.3%. In U937, MSP revealed both unmethylated and methylated forms, and BGS demonstrated that 8/10 clones had methylated sites and the methylation density was 29.7%. In MOLM13, MSP revealed faint unmethylated and strong methylated forms, and the BGS demonstrated that all clones had methylated sites and the methylation density was 91.0% (Figure 2a). Since the BGS demonstrated that all clones had methylated sites in MOLM13, the DNA of MOLM13 was serially diluted with that of normal PB MNCs to determine the sensitivity of MSP. As shown in Figure 2b, MSP could detect the methylated DNA at the 10^{-2} level. These results indicated that MSP was sensitive and the methylation status obtained by MSP essentially reflected the methylation density of 30 CpG sites. We therefore used MSP to examine the methylation status of the clinical samples. In addition, we classified the methylation status into four levels according to MSP: no methylation, only the unmethylated form was detected; weak methylation, the unmethylated form was stronger than the methylated form; moderate methylation, unmethylated and methylated forms were visible at the same level; strong methylation, the methylated form was stronger than the unmethylated form.

Methylation status of the FHIT *gene in* de novo *AML and MDS*

Using MSP, we found methylation of the *FHIT* gene in 13 of 94 (13.8%) *de novo* AML and 22 of 40 (55.0%) MDS cases, but in none of the normal MNCs from five BM, four PBL and five CB (Table 1). To confirm that the results by MSP reflected the methylation density of 30 CpG sites in AML and MDS samples, we performed BGS in 13 AML and eight MDS cases. Median methylation densities of the clinical samples, which revealed no, weak, moderate or strong methylation by MSP, were 0.2% (range, 0–0.7), 3.3% (range, 0.3–13.1), 34.5% (range, 29.7–43.0) and 51.1% (range, 38.6–75.3), respectively. The distribution of the methylation density well reflected the methylation status obtained by MSP.

In *de novo* AML, there was no different distribution of the methylation status among the FAB types. Furthermore, it was notable that eight of 13 methylated AML cases revealed weak methylation, and two and three cases revealed moderate and strong methylation, respectively (Table 1). Since the BM cells of these AML cases with *FHIT* methylation contained over 80% blast cells, the weak methylation status did not result from the lower percentage of blast cells in BM.

In MDS, the *FHIT* methylation frequency was significantly higher in cases with advanced-stage RAEB (8/13, 61.5%), RAEBt (4/5, 80%) or MDS-overt (6/7, 85.7%) than in those with early-stage RA (1/10, 10%). It was particularly interesting that the methylation level became moderate or strong in advanced-stage MDS in contrast to *de novo* AML (Table 1). In addition, three of five cases with CMMoL showed *FHIT* methylation, all of which had strong levels (Table 1). Since the BM cells from MDS contains heterogeneous cell populations, we analyzed the methylation status of $CD34^+$ and $CD34^-$ fractions separated from 10 MDS samples and compared them with the results obtained by using a total MNCs. Each of the separated $CD34^+$ and $CD34^-$ fractions contained over 85% and less than 10% $CD34^+$ cells, respectively. In four MDS samples, the methylation levels of $CD34^+$ fractions were the same as those of $CD34^-$ fractions. In three samples, the methylation levels of $CD34^+$ fractions were stronger than those of $CD34^-$ fractions, while those of $CD34^+$ fractions were weaker than $CD34^-$ fractions in three samples (Figure 3).

Association of methylation status between FHIT *and* $p15^{INK4B}$ *genes*

Although MSP has been well established to detect the methylation status of the $p15^{INK4B}$ gene by the previous report,[26] we compared the results by MSP with those by BGS in each of the five AML and MDS cases. We performed BGS to determine the methylation density of 45 CpG sites of the 5′ $p15^{INK4B}$ gene

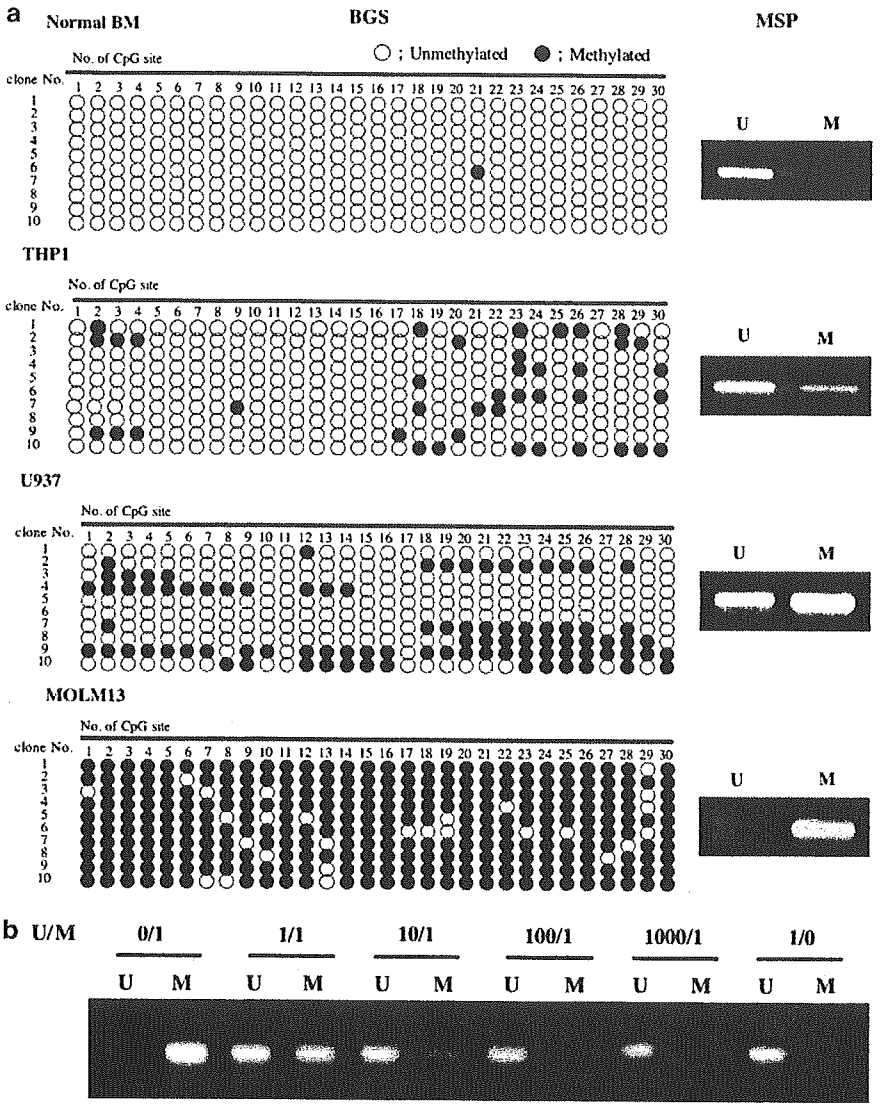

Figure 2 (a) Representative results of *FHIT* methylation by BGS and MSP from normal MNCs and leukemia cell lines. Methylated and unmethylated CpG sites are indicated by closed and open circles, respectively. The methylation density obtained from MSP well reflected the results from BGS. BGS and MSP indicate bisulfate genomic sequence and methylation-specific PCR, respectively. (b) The sensitivity of MSP for *FHIT* methylation. MOLM13 DNA was serially diluted with normal BM DNA. This reaction can detect the methylated DNA at the 10^{-2} level. U and M indicate unmethylated and methylated PCR products, respectively.

and found that the results by MSP and BGS were well correlated. In addition, $p15^{INK4B}$ methylation status was not different in $CD34^+$ and $CD34^-$ fractions separated from MDS samples (Figure 3). $p15^{INK4B}$ methylation was found in 54/94 (57.4%) AML cases and 12/40 (30.0%) MDS cases, but not in normal MNCs by MSP (Table 1). It was notable that *FHIT* methylation, which included all methylation levels, was not associated with $p15^{INK4B}$ methylation in either AML or MDS (Table 2). However, $p15^{INK4B}$ methylation was frequently observed in advanced-stage MDS in agreement with the previous reports (Table 1).[14–16]

Quantitation of the expression level of the FHIT transcript

To analyze the effect of FHIT methylation on its transcript expression, we quantitated the expression level of the FHIT transcript in AML and MDS cells as well as normal MNCs using a real-time fluorescence detection method. We evaluated the cDNA quality from each GAPDH expression, then the ratio of the FHIT Ct value to GAPDH Ct value (FHIT/GAPDH) was determined as the FHIT expression level. In normal MNCs, the median expression level of the FHIT transcript was 0.134 (range, 0.028–1.654). The median expression levels in *de novo* AML and MDS were 1.445 (range, 0–81.7) and 0.6 (range, 0.040–15.3), respectively, and the Bonferroni test revealed that distributions were significantly higher in *de novo* AML than those in normal MNCs ($P<0.0001$) (Figure 4a).

According to the methylation status, the median expression level in *de novo* AML without methylation (1.669; range, 0–81.7) was significantly higher than those with methylation (0.474; range, 0–1.320) ($P=0.0008$) and in normal MNCs ($P<0.0001$). In contrast, the median expression level in MDS was not related to the methylation status (0.637, range, 0.086–15.3 in MDS without methylation; 0.444, range, 0.040–2.775 in MDS with methylation) (Figure 4b).

Table 1 Methylation status of *FHIT* and *p15*^{INK4B} genes in AML and MDS

	FAB type	Number of analyzed	Methylated number				
			FHIT				*p15*^{INK4B}
			Total	Weak	Moderate	Strong	
AML	M0	1	0	0	0	0	1
	M1	26	3	1	1	1	17
	M2	31	7	5	1	1	15
	M3	6	1	1	0	0	4
	M4	20	0	0	0	0	11
	M5	9	2	1	0	1	5
	M6	1	0	0	0	0	1
	Total	94	13	8	2	3	54
MDS	RA	10	1	1	0	0	0
	RAEB	13	8	6	2	0	2
	RAEBt	5	4	1	1	2	4
	MDS-overt	7	6	4	2	0	4
	CMMoL	5	3	0	0	3	2
	Total	40	22	12	5	5	12
Normal MNCs		14	0	0	0	0	0

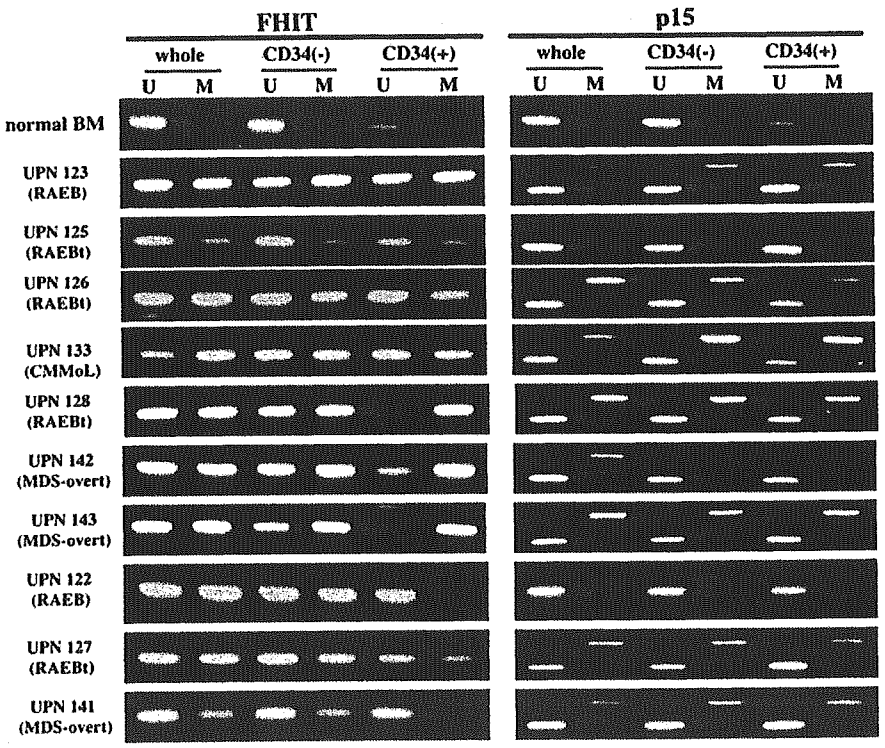

Figure 3 Comparison of the methylation status of *FHIT* and *p15*^{INK4B} genes in CD34$^+$ and CD34$^-$ fractions from MDS samples. In four MDS samples (UPN 123, 125, 126 and 133), *FHIT* methylation levels were the same in CD34$^+$ and CD34$^-$ fractions. In three samples (UPN 128, 142 and 143), the methylation levels were stronger in CD34$^+$ fractions than in CD34$^-$ fractions. However, in three samples (UPN 122, 127 and 141), the methylation levels were stronger in CD34$^-$ fractions than in CD34$^+$ fractions. In contrast, there was no difference of *p15*^{INK4B} methylation levels between CD34$^+$ and CD34$^-$ fractions. U and M indicate unmethylated and methylated PCR products, respectively.

Sequential analysis of FHIT methylation in AML

In 21 AML cases, the methylation status was analyzed at both initial diagnosis and the first relapse (Table 3). *FHIT* methylation was observed in four cases at initial diagnosis, but not in the remaining 17 cases. Among four cases harboring *FHIT* methylation at initial diagnosis, three cases also showed methylation at the first relapse, although one case lost the methylation. Furthermore, increase of the methylation level from weak to strong was observed in one case during the first relapse. Among 17 cases without *FHIT* methylation at initial diagnosis, the *FHIT* gene was still not methylated at the first

Table 2 Association of the methylation status between *FHIT* and *p15*^{INK4B} genes

		AML			MDS		
		p15^{INK4B}			*p15*^{INK4B}		
		M	*UM*	*Total*	*M*	*UM*	*Total*
FHIT	M	7	6	13	9	13	22
	UM	47	34	81	3	15	18
	Total	54	40	94	12	28	40

M and UM indicate methylation and unmethylation, respectively. Methylation category includes all methylation levels from weak to strong.

Table 3 Change of *FHIT* methylation status at relapse

	Change of methylation status			
	Diagnosis		*Relapse*	*Number*
No change	None	→	None	9
	Strong	→	Strong	2
	None	→	Weak	7
Increase	None	→	Strong	1
	Weak	→	Strong	1
Decrease	Moderate	→	None	1

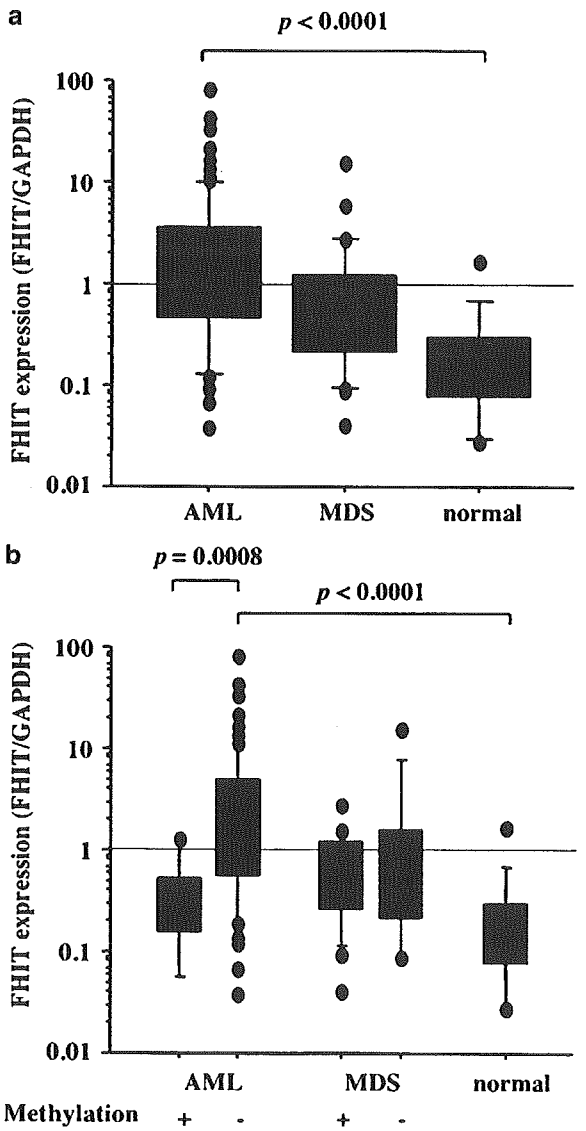

Figure 4 Distribution of the expression level of the FHIT transcript. (a) There was a significant difference among AML, MDS and normal MNCs ($P<0.0001$ by the Kruskal–Wallis test). The Bonferroni test revealed that distributions were significantly higher in AML than in normal MNCs ($P<0.0001$). (b) According to the methylation status, the distributions were significantly higher in *de novo* AML without methylation than in those with methylation ($P=0.0008$) and in normal MNCs ($P<0.0001$). Normal MNCs included five BM, four PBL and five CB.

relapse in nine cases, although eight cases revealed methylation at the first relapse. The paired sign test revealed that the methylation level at the first relapse was significantly higher than that at diagnosis ($P=0.0215$). However, the expression level of the FHIT transcript at the first relapse was not affected by the increased methylation status ($P=0.1823$ by the Wilcoxon signed rank test). Although *FHIT* methylation was not associated with *p15*^{INK4B} methylation at diagnosis, it was particularly interesting that all nine cases, which had increased *FHIT* methylation level at the first relapse, revealed *p15*^{INK4B} methylation at the first relapse.

In addition, we sequentially analyzed the methylation and expression level of the *FHIT* gene at diagnosis, CR and the first relapse in three AML cases. Although we found no methylation at any point in two cases, one case had a dramatic change of methylation status and the expression level of the *FHIT* gene. In this case, *FHIT* methylation was found both at diagnosis and the first relapse, but not at CR. In agreement with the methylation status, the FHIT expression level was five times higher at CR than at diagnosis and first relapse. These results provided clinical correlation that the FHIT expression was downregulated by its promoter methylation (data not shown).

In six cases, both samples at diagnosis and CR were available. Furthermore, CD34$^+$ and CD34$^-$ fractions separated from the BM samples at CR were analyzed. At diagnosis, *FHIT* methylation was found in three cases, but not in both CD34$^+$ and CD34$^-$ fractions from all cases at CR by MSP. These results were confirmed by BGS (Figure 5). Taken together, we found no *FHIT* methylation in nine BM samples at CR, suggesting that the conventional chemotherapeutic agents for AML did not induce *FHIT* methylation.

Effect of 5-Aza-dC treatment on FHIT *methylation and expression*

Previously, it was demonstrated that 1 μM 5-Aza-dC treatment for 6 days demethylated the *FHIT* gene in lung cancer cell lines, resulting in re-expression of the FHIT transcript.[18] However, since our preliminary experiment revealed that most primary AML cells resulted in apoptosis after 5-Aza-dC treatment for 6 days, we evaluated the *FHIT* methylation status and expression level in eight primary samples and three human myeloid leukemia cell lines (THP1, MOLM13 and U937) 2 days after 1 μM 5-Aza-dC treatment (Figure 6). In THP1 and MOLM13, 5-Aza-dC treatment decreased each *FHIT* methylation level and increased each expression level of the FHIT transcript. In U937, the methylation level was not changed by 5-Aza-dC treatment, while the expression level was increased. In four of the eight primary samples, each methylation level was decreased and

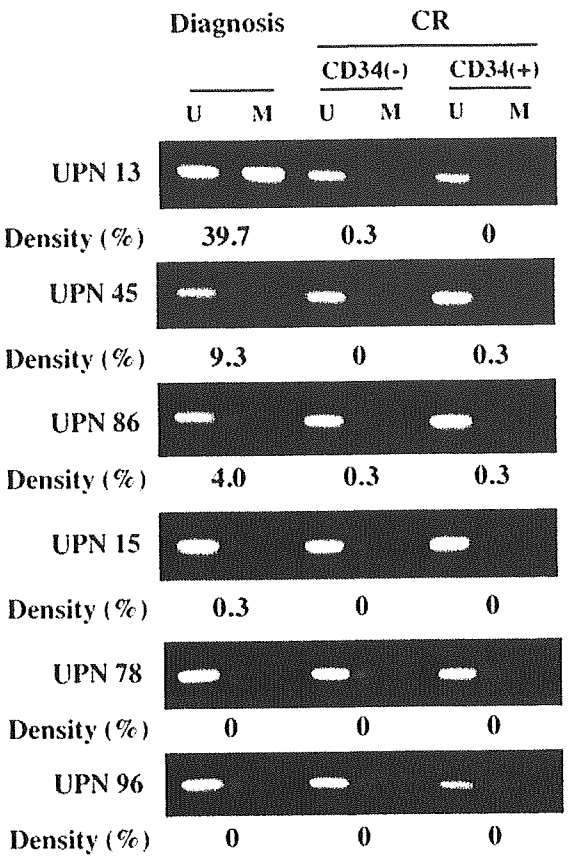

Figure 5 Comparison of the FHIT methylation status at diagnosis and at CR in six AML cases. The methylation was found by MSP in three cases (UPN 13, 45 and 86) at diagnosis, but not in both $CD34^+$ and $CD34^-$ fractions from all CR samples. Each methylation density obtained by BGS is also indicated. U and M indicate unmethylated and methylated PCR products, respectively.

each expression level was increased. Although each methylation level was not changed in the remaining four samples, an increased expression level was observed in one sample. Loss of FHIT expression was observed after treatment in one sample, but this may have been due to the absence of viable cells by 5-aza-dC. Taken together, 5-Aza-dC treatment significantly decreased the *FHIT* methylation level ($P=0.0312$ by the paired sign test) and increased the expression level ($P=0.0208$ by the Wilcoxon signed rank test).

Discussion

The initiation and development of cancer involves several molecular changes, including epigenetic alterations as well as genetic alterations.[28,29] The methylation of the promoter region of the gene is an important mechanism of epigenetic regulation resulting in gene inactivation. In this study, we demonstrated that *FHIT* methylation is significantly associated with AML and MDS, and that both the frequency and density of *FHIT* methylation increased with the disease progression of MDS. In contrast, *FHIT* methylation was not observed in normal CB, BM and CR samples. Since BM cells from MDS patients contained variously differentiated cell populations, we examined the methylation status of $CD34^+$ fractions in 10 MDS samples. When compared with $CD34^-$ fraction, the *FHIT* methylation level of $CD34^+$ fraction was increased, decreased and not changed in three (UPN 128, 142 and 143), three (UPN 122, 127 and 141) and four (UPN 123, 125, 126 and 133) samples, respectively. On the other hand, there was no significant difference of $p15^{INK4B}$ methylation levels between $CD34^+$ and $CD34^-$ fractions. In $CD34^+$ fractions of UPN 128, 142 and 143, *FHIT* methylation may have preceded $p15^{INK4B}$ methylation. Unmethylated *FHIT* in $CD34^-$ fractions might be derived from non-MDS clones such as lymphocytes. Further analysis using the precisely fractionated samples according to the cell lineage will be required. It remains unclear why in samples of UPN 122, 127 and 141, *FHIT* methylation was dominant in $CD34^-$ fractions. It should be considered that the unmethylated cell population is not necessarily derived from a normal clone. In $CD34^+$ fraction of UPN141, $p15^{INK4B}$ was partially methylated in contrast to *FHIT*. Methylation status may be changed during differentiation of MDS cells. In the future, clonogenic assay distinguishing between normal and MDS clones will clarify the significance of the methylation status of the progenitor cells.

The *FHIT* methylation was not found so frequently and its density was weak in *de novo* AML at diagnosis, while both the frequency and density significantly increased at the first relapse. These results indicated that the *FHIT* methylation was accumulated through the disease progression of AML as well as MDS. However, FHIT seemed to be differently involved in the disease progression between MDS and AML. Both *FHIT* and $p15^{INK4B}$ methylation increased according to the advanced stages of MDS, although these were not correlated with each other, suggesting the independent involvement of FHIT and $p15^{INK4B}$. In AML, these methylations were not correlated at diagnosis, while the *FHIT* methylation level significantly increased at the first relapse in cases where $p15^{INK4B}$ methylation was observed at the first relapse, suggesting that the tumor suppressor function of FHIT may be effective in collaboration with $p15^{INK4B}$. Alternatively, increased methyltransferase activity during disease progression may induce *FHIT* methylation.

It was reported that *FHIT* methylation was closely associated with the loss or reduction of FHIT expression in several kinds of solid tumors, and that the loss of FHIT expression was also observed in hematological malignancies. However, when the median expression level of the FHIT transcript in the methylated and unmethylated cases was compared with *de novo* AML, the former level was indeed lower than the latter, but was the same as that in normal MNCs. At present, it is not clear why the expression level of the FHIT transcript is higher in *de novo* AML and MDS cells than that in normal MNCs. It is possible that FHIT expression is not involved in the pathogenesis of AML and/or MDS at the early stage. On the other hand, previous reports demonstrated that aberrant FHIT transcripts were frequently found rather than a loss of expression in hematological malignancies, while our quantitative conditions detected a total of expressed FHIT transcript regardless of aberrant transcripts.[30,31] Therefore, it is necessary to determine which transcripts are dominantly expressed in AML and MDS cells. In addition, since the reduced expression level of FHIT protein is reportedly restricted to malignant hematopoietic cells, the relationship of expression levels between the transcript and protein should be clarified. It was shown that replacement or overexpression of the wild-type *FHIT* gene in human cancer cell lines reduced tumorigenicity and growth rate.[32,33] In contrast, introduction of the wild-type *FHIT* gene did not alter the tumorigenicity in HeLa cells.[34] Furthermore, it was reported that only half the renal-cell-carcinoma cell lines were suppressed by exogenous FHIT expression.[35] Therefore, there seems to be

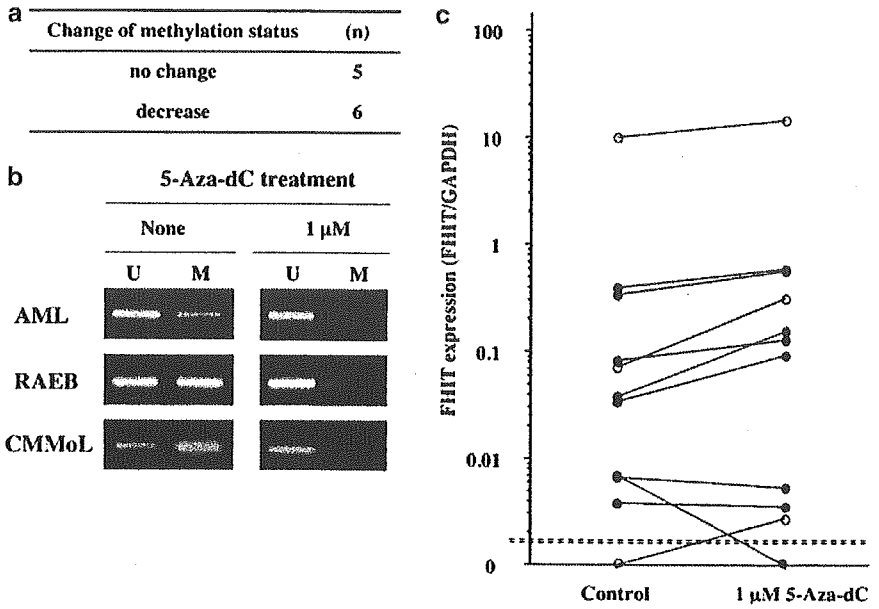

Figure 6 Effect of 5-Aza-dC treatment on the methylation and expression of the *FHIT* gene. The *FHIT* methylation status and expression level in eight primary samples and three human myeloid leukemia cell lines were analyzed 2 days after 1 μM 5-Aza-dC treatment. (a) After the 5-Aza-dC treatment, the FHIT methylation level was decreased in two cell lines and four primary samples. The paired sign test demonstrated that 5-Aza-dC treatment significantly decreased the FHIT methylation level ($P=0.0312$). (b) Representative results of the demethylated effect in clinical samples. (c) The 5-Aza-dC treatment increased the expression level of the FHIT transcript in all three cell lines (open circle) and four clinical samples (closed circle), and the increase was significant ($P=0.0208$ by the Wilcoxon signed rank test).

some different effects of FHIT expression on the pathogenesis among cancer cell types. In this study, the expression level of the FHIT transcript was not related to any clinical variables (data not shown). However, since the expression level decreased in association with methylation at the first relapse of *de novo* AML and at advanced stages of MDS, and the expression level increased by treatment with 5-Aza-dC, the reduced expression of the FHIT transcript caused by methylation might be involved in the pathogenesis of disease progression in AML and MDS.

In conclusion, frequent methylation of the *FHIT* gene was associated with disease progression, and the role of the *FHIT* gene as a tumor suppressor seemed to be important at a later phase during the development of AML and MDS. In addition, a tumor suppressor function of FHIT may be more effective on the pathophysiology of MDS and AML when additional genes alterations would be accumulated. Further analysis is required to clarify the cooperative function of FHIT with other tumor suppressor genes as well as oncogenes.

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SPOTLIGHT

Efficacy of gemtuzumab ozogamicin on ATRA- and arsenic-resistant acute promyelocytic leukemia (APL) cells

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Acute promyelocytic leukemia (APL) cells express a considerable level of CD33, which is a target of gemtuzumab ozogamicin (GO), and a significantly lower level of P-glycoprotein (P-gp). In this study, we examined whether GO was effective on all-*trans* retinoic acid (ATRA)- or arsenic trioxide (ATO)-resistant APL cells. Cells used were an APL cell line in which P-gp was undetectable (NB4), ATRA-resistant NB4 (NB4/RA), NB4 and NB4/RA that had been transfected with MDR-1 cDNA (NB4/MDR and NB4/RA/MDR, respectively), ATO-resistant NB4 (NB4/As) and blast cells from eight patients with clinically ATRA-resistant APL including two patients with ATRA- and ATO-resistant APL. The efficacy of GO was analyzed by ^{3}H-thymidine incorporation, the dye exclusion test and cell cycle distribution. GO suppressed the growth of NB4, NB4/RA and NB4/As cells in a dose-dependent manner. GO increased the percentage of hypodiploid cells significantly in NB4, NB4/RA and NB4/As cells, and by a limited degree in NB4/MDR and NB4/RA/MDR cells. Similar results were obtained using blast cells from the patients with APL. GO is effective against ATRA- or ATO-resistant APL cells that do not express P-gp, and the mechanism of resistance to GO is not related to the mechanism of resistance to ATRA or ATO in APL cells.
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Introduction

Recently, gemtuzumab ozogamicin (GO, Mylotarg™), a calicheamicin-conjugated humanized anti-CD33 monoclonal antibody (mAb), has been introduced for the treatment of acute myeloid leukemia (AML).[1] However, the clinical outcome after the treatment with GO was negatively associated with P-glycoprotein (P-gp) function in AML.[2] In our previous studies, we found that resistance to GO was mainly mediated by P-gp.[3,4] Acute promyelocytic leukemia (APL) cells express a considerable level of CD33 antigen and a significantly lower level of P-gp compared with other types of AML.[5] Therefore, GO may become established as a successful treatment for APL. In fact, GO has been introduced with the clinical efficacy in the treatment of APL.[6,7] However, *in vitro* efficacy of GO on APL cells as well as all-*trans* retinoic acid (ATRA)- and arsenic trioxide (ATO)-resistant ones has not been studied well. Moreover, drug interaction among ATRA, ATO and GO, and the mechanisms of resistance of APL cells to them remain unclear.[8]

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Materials and methods

Cells

The cell lines used were a human APL cell line, NB4, which was kindly provided by Dr M Lanotte (Hospital Saint-Louis, Paris, France);[9] ATRA-resistant NB4 (NB4/RA) cells; NB4 and NB4/RA cells transfected with MDR-1 cDNA (NB4/MDR and NB4/RA/MDR, respectively); and ATO-resistant NB4 (NB4/As) cells. The NB4/RA and NB4/As cells were obtained by culturing NB4 cells with gradually increasing concentrations of ATRA and ATO, respectively.[10] mdr-1 messenger RNA (mRNA) was not detectable in NB4, NB4/RA or NB4/As cells by RT-PCR.[11,12] NB4/MDR and NB4/RA/MDR cells had detectable mdr-1 mRNA, but did not have detectable MDR-related protein (MRP) mRNA or lung-resistant protein (LRP) mRNA. Blasts were collected from four patients with APL at diagnosis, six patients with clinically ATRA-resistant but ATO-sensitive APL and two patients with clinically ATRA- and ATO-resistant APL.

Flow cytometric analysis for CD33 and Pgp expression

For evaluation of CD33 expression, cells were stained with phycoerythrin (PE)-conjugated anti-CD33 mAb (Becton Dickinson Immuno-cytometry Systems, San Jose, CA, USA), according to the manufacturer's instructions. For P-gp analysis, cells were incubated with biotinylated MRK16 (Fab') mouse mAb or a subclass-matched control mAb, and stained with streptavidine-Per CP (Becton Dickinson Immuno-cytometry Systems) as previously described.[11] Over 10 000 events were analyzed with the Epics XL flow cytometer (Beckman Coulter, Fullerton, CA, USA). APL cells obtained from the patients were also gated by CD-45 staining.[4]

Humanized anti-CD33 mAb and GO

GO consists of three essential parts: an antibody a cytotoxic agent, and a linker. The antibody, humanized IgG4 (hP67.6), targets the CD33 antigen. The cytotoxic agent is *N*-acetyl (NAc)-γ calicheamicin dimethyl hydrazide (DMH), a derivative of calicheamicin antitumor antibiotics.[1,2] GO, humanized non-conjugated anti-CD33 mAb (hP67.6) and free NAc-γ calicheamicin DMH were kindly provided by the Wyeth Research Division of Wyeth Pharmaceuticals Inc. (Philadelphia, PA, USA). The amount of GO used in an experiment was determined based on the concentration of NAc-γ calicheamicin DMH bound to the antibody. One microgram of GO contains 27.1 ng of NAc-γ calicheamicin DMH, and approximately 97% of a GO molecule is composed of the linker and antibody.