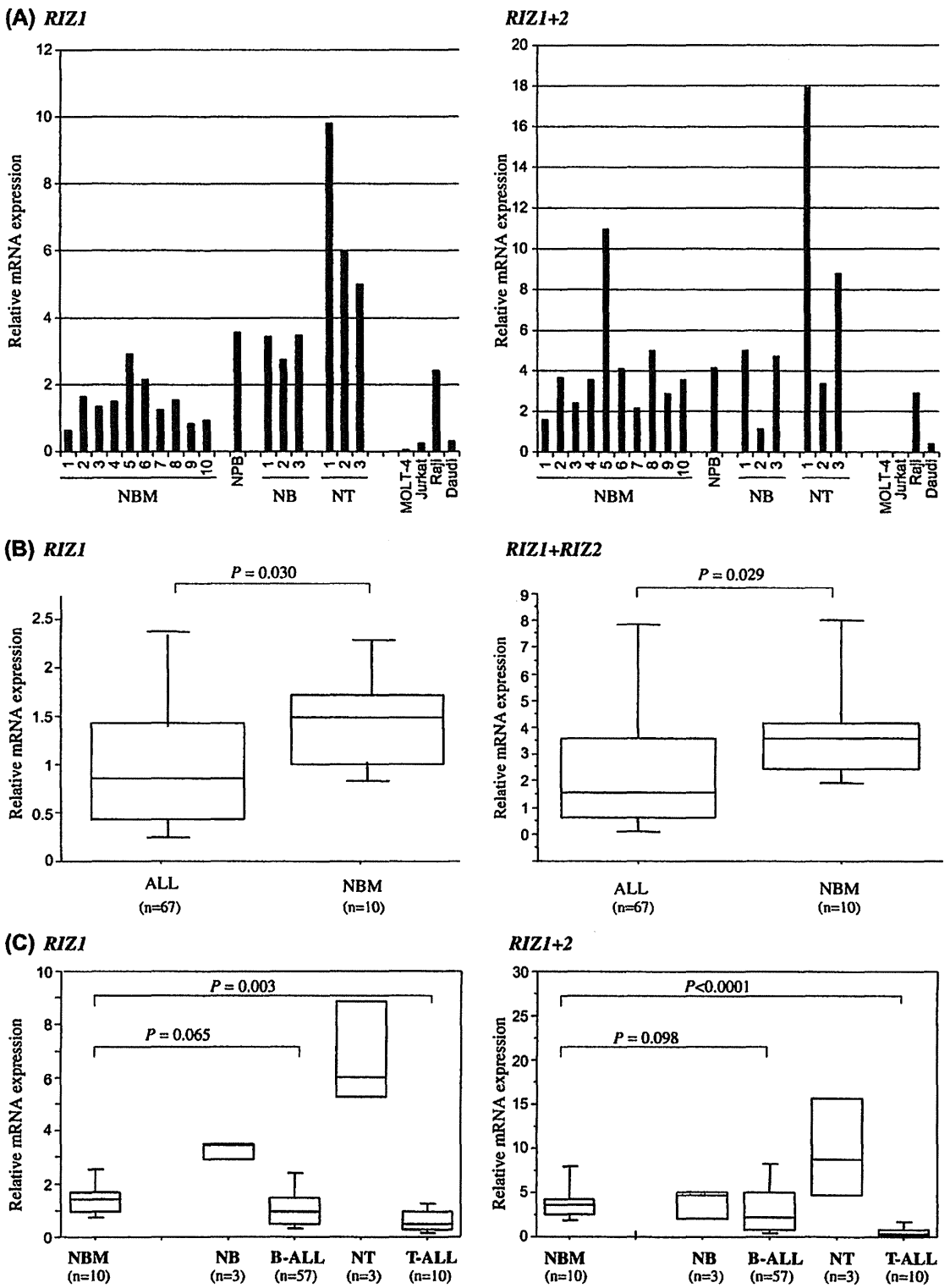

Figure 1. qRT-PCR analysis of the *RIZ* gene. NBM, normal bone marrow cells; NPB, normal peripheral blood; NB, normal peripheral B cells; and NT, normal peripheral T cells. (A) Expression of *RIZ* in normal tissues (NBM $n = 10$, NPB $n = 1$, NB $n = 3$ and NT $n = 3$) and cell lines of lymphoid tumors. Number represents each normal sample. (B) Expression of *RIZ1* and *RIZ1 + 2* in NBM and all samples of ALL. Large boxes represent 25th and 75th percentiles, upper and lower bars represent 10th and 90th percentiles of samples. Lines inside boxes represent median. (C) Expression of *RIZ1* and *RIZ1 + 2* in NBM, NB, B-ALL, NT and T-ALL. Large boxes represent 25th and 75th percentiles, upper and lower bars represent 10th and 90th percentiles of samples. Lines inside boxes represent median. (D) Relationship between expressions of *RIZ1* and *RIZ1 + 2* in each sample.

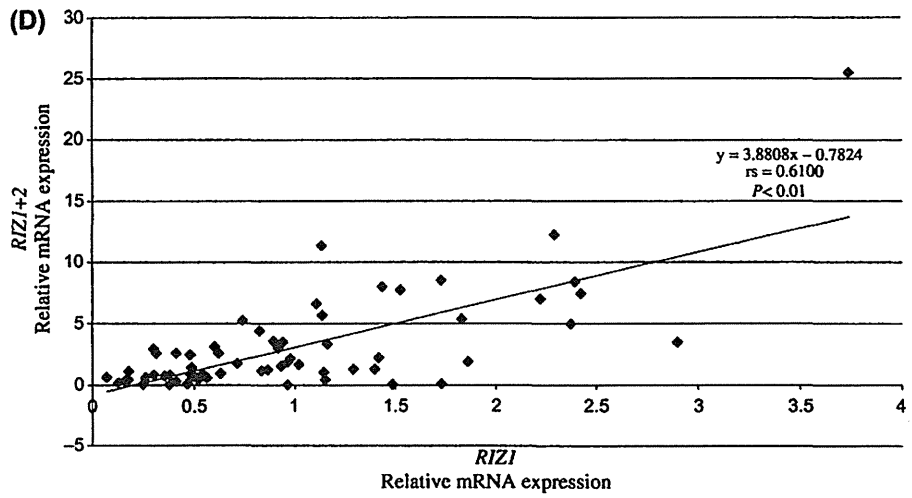

Figure 1. *(Continued).*

MS-PCR analysis of the *RIZ1* gene in normal samples and leukemic cells

To investigate the *RIZ1* promoter methylation status, we performed MS-PCR for 71 of the 73 patients whose DNA was available. Samples that showed only methylated bands were regarded as completely methylated, and samples that had both methylated and unmethylated bands were identified as partially methylated. Methylation-negative samples had unmethylated bands only. Methylation statuses of the *RIZ1* gene in normal samples and the lymphoid cell lines are shown in Figure 2(A). No methylation of the *RIZ1* gene was detected in NBM and NPB cells from healthy controls. Of the four cell lines, only MOLT-4 had methylation of the *RIZ1* gene. Although *RIZ1* mRNA expressions were decreased in Jurkat and Daudi, they were not methylated [Figures 1(A) and 2(A)].

Methylation of the *RIZ1* gene promoter was detected in 11 of the 71 samples of ALL (15.5%). Figure 2(B) shows all the methylation-positive ALLs and examples of methylation-negative ALL. The characteristics of methylation-positive patients are shown in Table II.

Table III shows the *RIZ1* methylation status and characteristics of patients. Concordant with the result of *RIZ1* expression, correlation between *RIZ1* methylation and phenotype of ALL was found. Methylation was more frequent in T-ALL (63.6%) than in B-ALL (6.7%) ($p < 0.0001$). Many of the patients with ALL with *RIZ1* methylation had T-ALL (7/11, 63.6%), although only a small proportion of the cases of ALL (11/71, 15.5%) had T-cell phenotype. Furthermore, *RIZ1* methylation was more frequent in Ph − ALL (23.3%) than in Ph + ALL (3.7%) ($p = 0.027$). No correlations were observed between *RIZ1* methylation and other clinical characteristics of ALL.

To determine whether transcriptional silencing of *RIZ1* is associated with promoter hypermethylation in ALL, we assessed the correlation between methylation status and *RIZ1* mRNA expression. There was no difference in *RIZ1* mRNA levels between methylation-positive (mean 0.818)

Table I. *RIZ* mRNA expression and characteristics of patients with ALL.

Variable		Number of samples*	*RIZ1* mRNA level (mean ± SD)	*p*-Value	*RIZ1 + 2* mRNA level (mean ± SD)	*p*-Value
Age	≤ 35 years	26	0.820 ± 0.610	0.102	1.850 ± 2.331	0.011
	> 35 years	41	1.165 ± 0.866		3.840 ± 4.720	
Sex	Male	35	1.067 ± 0.870	0.821	3.111 ± 4.780	0.706
	Female	32	1.017 ± 0.705		2.977 ± 3.174	
Phenotype	B-ALL	57	1.145 ± 0.817	0.045	3.577 ± 4.252	< 0.001
	T-ALL	10	0.606 ± 0.437		0.566 ± 0.668	
WBC ($\times 10^9$/L)	≤ 30	33	0.920 ± 0.699	0.464	2.180 ± 2.900	0.115
	> 30	30	1.134 ± 0.882		3.934 ± 5.081	
LDH	Normal	3	0.989 ± 0.483	0.807	0.177 ± 0.211	0.004
	> Normal	56	1.070 ± 0.816		3.327 ± 4.306	
Karyotype	Better/ intermediate	20	0.992 ± 0.944	0.399	3.093 ± 5.713	0.340
	Poor	33	1.122 ± 0.669		2.871 ± 2.594	
Ph	Positive	26	1.189 ± 0.857	0.086	4.072 ± 5.009	0.015
	Negative	40	0.941 ± 0.706		2.505 ± 3.293	
Response†	CR	53	1.057 ± 0.811	0.543	2.752 ± 3.909	0.318
	Non-CR	12	1.116 ± 0.732		4.445 ± 4.643	

*67 samples were available for qRT-PCR.
†Response to induction therapy.
ALL, acute lymphoblastic leukemia; WBC, white blood cell count; LDH, lactate dehydrogenase; Ph, Philadelphia chromosome; CR, complete remission; qRT-PCR, quantitative real-time reverse transcription polymerase chain reaction.

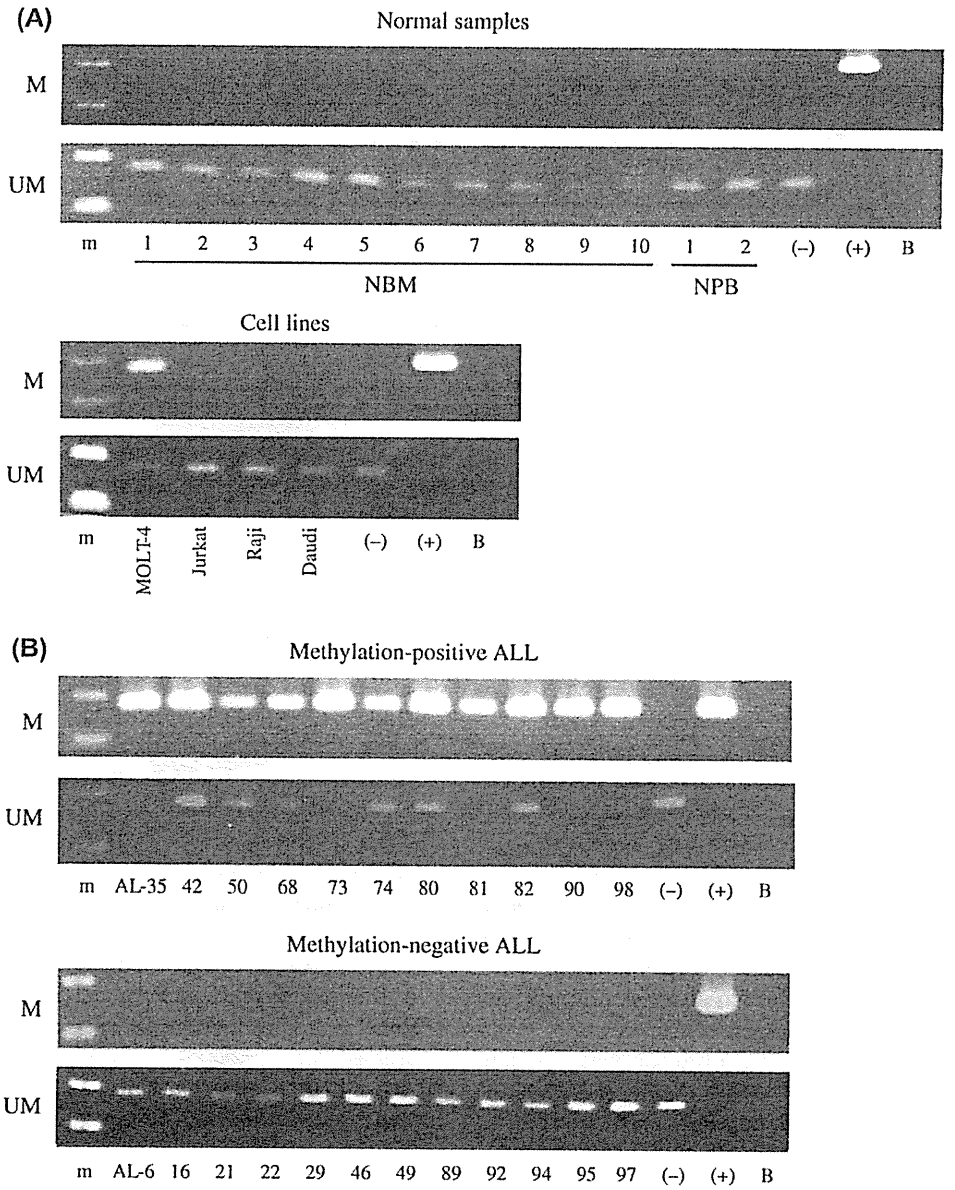

Figure 2. Methylation status of the *RIZ1* gene in adult ALL. Twenty-five μL of PCR products were separated by electrophoresis through 2% agarose gel, stained with ethidium bromide and visualized by ultraviolet illumination. Upper panel indicates methylated bands (M), while lower panel indicates unmethylated bands (UM). m, 100 bp DNA ladder marker; NBM, normal bone marrow cells; NPB, normal peripheral blood cells; (−), negative control; (+), positive control; and B, blank. (A) Methylation of *RIZ1* gene in normal samples and cell lines of lymphoid tumors. Number represents each normal sample. (B) Examples of methylation status of ALL samples. Number represents each patient with ALL. DNA methylation was detected in AL-35, 42, 50, 68, 73, 74, 80, 81, 82, 90 and 98. AL-6, 16, 21, 22, 29, 46, 49, 89, 92, 94, 95 and 97 were methylation-negative ALL.

and -negative patients (mean 1.114) in total ALL samples ($p = 0.151$). A similar observation was also found in *RIZ1* + 2 mRNA levels (mean 2.398 vs. 3.166, $p = 0.111$).

Mutation analysis of the *RIZ1* gene in leukemic cells

A previous study demonstrated that frequent A563G missense mutation in exon 7 was restricted in DLBCL but not in other malignant lymphomas [7]. However, this mutation in other hematological neoplasms has not been analyzed. Therefore, we performed PCR-SSCP analysis on exon 7 of the *RIZ1* gene in the 71 cases of ALL, and no abnormal SSCP band was detected in any of the cases.

Frequent frameshift mutations were also reported in tumors with microsatellite instability (MSI) [8,9]. The mutations are located at two polyadenosine tracks within the coding region of *RIZ*: one $(A)_8$ track at coding nucleotide position 4273–4280 and one $(A)_9$ track at 4462–4471 in exon 8. No frameshift mutations were found in the 71 patients with ALL.

5-Aza-dC treatment of MOLT-4 cells

To determine whether promoter methylation is associated with *RIZ1* silencing in T-ALL, MOLT-4 cells were treated with 5-Aza-dC. Numbers of cells and viabilities were not statistically different between control and DMSO treatment. Dose- and time-dependent inhibitions of cell growth and viability due to 5-Aza-dC were observed [Figures 3(A) and 3(B)]. When the cells were treated with 3 μM 5-Aza-dC, cell growth was completely inhibited [Figure 3(A)]. Cell death

Table II. Characteristics of patients with *RIZ1* methylation.

No.	Age	Sex	Phenotype	WBC ($\times 10^9$/L)	BM blasts (%)	LDH (U/L)	Karyotype	Response*	*RIZ1* mRNA level	*RIZ1* + 2 mRNA level
AL-35	61	M	T	9.1	82.0	434	Hyperdiploid, der(13), add(13), del(13)	CR	0.070	0.650
AL-42	57	M	T	1.6	NE	239	46,XY	CR	1.018	1.691
AL-50	83	F	B	7.8	83.7	1896	Hypertetraploid, add(14), i(17)	CR	0.179	1.138
AL-68	60	F	B	4.9	46.7	NE	46,XX	CR	0.300	2.965
AL-73	54	F	B	50.8	96.0	1169	46,XX	CR	1.432	8.001
AL-74	21	M	T	190.0	85.0	6110	46,XY, ins(17;1) (p13;p11p22.3)	CR	0.357	0.765
AL-80	31	F	T	41.7	98.4	129	Complex, t(10;11), del(12), i(17)	CR	0.963	0.051
AL-81	67	F	B	7.6	97.8	781	t(9;22)(q34;q11.2)	CR	1.829	5.377
AL-82	66	F	T	0.9	95.7	163	Complex, t(10;11), t(10;12), r(12)	CR	1.485	0.058
AL-90	30	F	T	2.6	88.0	276	Complex, del(6), del(10), add(16), add(18)	Non-CR	0.468	0.082
AL-98	34	M	T	34.2	75.1	566	Complex, del(6)	CR	0.248	0.081

*Response to induction therapy.
M, male; F, female; WBC, white blood cell count; BM, bone marrow; NE, not examined; LDH, lactate dehydrogenase; CR, complete remission.

was obviously induced after 72 h of treatment with 0.5 or 3 μM 5-Aza-dC [Figure 3(B)]. Treatment of MOLT-4 cells induced demethylation of the *RIZ1* promoter in a dose- and time-dependent manner [Figures 3(C) and (D)]. Four days of treatment induced demethylation in a concentration-dependent manner [Figure 3(C)] and restoration of expression was induced by 3 μM 5-Aza-dC [Figure 3(D)].

Forced *RIZ1* expression in T-ALL cell lines

To examine whether forced *RIZ1* expression by DNA methyltransferase inhibitor is associated with antiproliferative effects, we transfected p3RIZRH1 plasmid into T-ALL cell lines, whose *RIZ1* expression was decreased before transfection. *RIZ1* expression in MOLT-4 and Jurkat were confirmed by qRT-PCR and Western analysis. *RIZ1* transfections into these cells resulted in sustained mRNA expression level more than that of the *RIZ1* positive control 24–96 h after transfection [Figure 4(A)]. *RIZ1* protein expression in these cells 96 h after transfection is shown in Figure 4(B). In contrast, *RIZ1* mRNA and protein expression levels did not increase in MOLT-4 or Jurkat cells, or empty vector transfected controls [Figures 4(A) and 4(B)]. In comparison with the controls, inhibition of cell growth was observed in both *RIZ1* transfected MOLT-4 and Jurkat cells [Figure 4(C)]. Cell cycle analysis showed that forced *RIZ1* expression in Jurkat led to a higher percentage of cells in G2/M phase ($p = 0.014$) and a lower percentage of cells in G0/G1 phase ($p = 0.010$), 96 h after transfection. A similar trend was found in MOLT-4 cells (G2/M phase [$p = 0.09$], G0/G1 phase [$p = 0.013$]) [Figure 4(D)]. The percentage of apoptotic cells by annexin V assay was significantly higher in MOLT-4 cells, 72 h ($p = 0.036$) and 96 h ($p = 0.020$) after *RIZ1* transfection [Figure 4(E)]. In contrast, *RIZ1* transfection did not lead to apoptosis significantly in Jurkat cells (data not shown).

Table III. Methylation status of *RIZ1* and characteristics of patients.

Variable		Number of samples*	Methylation-positive *n* (%)	*p*-Value
Age	≤35 years	27	4 (14.8)	0.591
	>35 years	44	7 (15.9)	
Sex	Male	37	4 (10.8)	0.210
	Female	34	7 (20.6)	
Phenotype	B-ALL	60	4 (6.7)	<0.0001
	T-ALL	11	7 (63.6)	
WBC ($\times 10^9$/L)	≤30	35	7 (20.0)	0.354
	>30	30	4 (13.3)	
LDH	Normal	3	2 (66.7)	0.065
	>Normal	59	8 (13.6)	
Karyotype	Better/ intermediate	21	6 (28.6)	0.114
	Poor	34	4 (11.8)	
Ph	Positive	27	1 (3.7)	0.027
	Negative	43	10 (23.3)	
Response†	CR	56	10 (17.9)	0.335
	Non-CR	13	1 (7.7)	

*71 samples were available for MS-PCR.
†Response to induction therapy.
WBC, white blood cell count; LDH, lactate dehydrogenase; Ph, Philadelphia chromosome; ALL, acute lymphoblastic lymphoma; CR, complete remission.

Discussion

RIZ1 is a tumor suppressor gene that is mapped to chromosome band 1p36.2 and frequently undergoes deletion, rearrangements and loss of heterozygosity in a broad spectrum of tumors [26]. Its expression is decreased in MDS, AML, CML and DLBCL. However, in adult ALL, the role of *RIZ1* has not been well examined. We examined *RIZ1* expression and evaluated whether hypermethylation was related to *RIZ1* silencing in adult patients with ALL.

In the present study, *RIZ1* expression in the total ALL samples and lymphoid cell lines was decreased compared with that in normal bone marrow mononuclear cells. Expressions

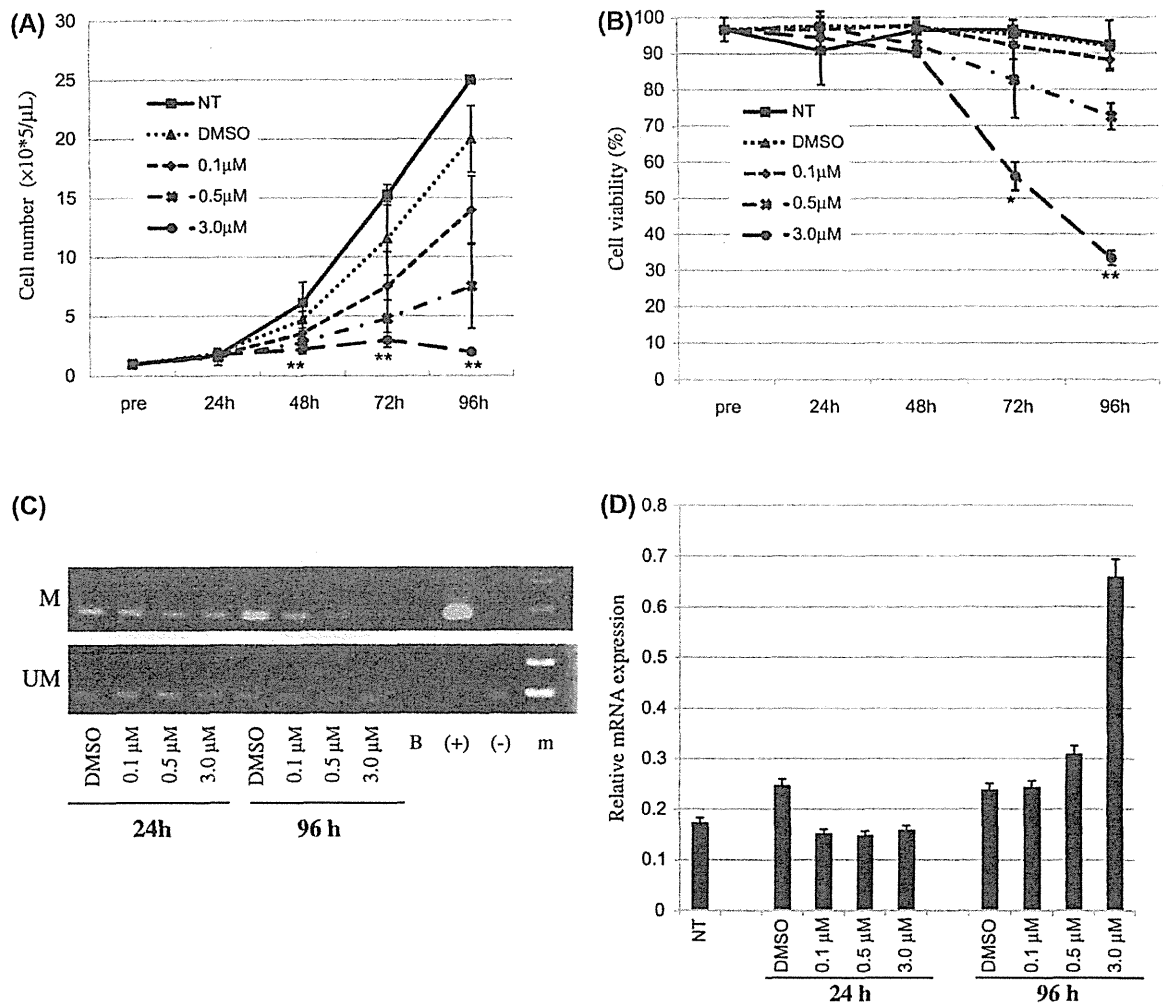

Figure 3. Treatment with 5-Aza-dC in T-ALL cells. Bars represent mean of three independent experiments. NT, no treatment; DMSO, only DMSO added. (A) Number of MOLT-4 cells (mean) is indicated according to treatment (*$p < 0.05$, **$p < 0.005$ vs. control). (B) Cell viability (mean) is indicated according to treatment (*$p < 0.05$, **$p < 0.005$ vs. control). (C) Methylation status of MOLT-4 cells at different times after 5-Aza-dC treatment. Upper panel indicates methylated bands (M), while lower panel indicates unmethylated bands (UM). B, blank; (+), positive control; (−), negative control; and m, 100 bp DNA ladder marker. (D) Expression of *RIZ1* mRNA in MOLT-4 cells at different times after 5-Aza-dC treatment.

of *RIZ1* and *RIZ1* + 2 were correlated in the all samples, and *RIZ1* + 2 in ALL was also significantly decreased compared with that in normal samples. Furthermore, the ratio *RIZ1*/*RIZ1* + 2 varied among patients. Our results revealed that decreased expression of *RIZ1* but not overexpression of *RIZ2* was related to adult ALL. Sasaki *et al.* showed that *RIZ1* expression was decreased in only four of 11 (37%) patients with ALL. They speculated that increased *RIZ2* expression might be related to leukemic transformation in ALL [12]. However, the number of patients was relatively small, and most of the patients in the study had B-cell phenotype.

Correlations between *RIZ1* expression and clinical characteristics were also examined. Significantly decreased *RIZ1* expression was found in T-ALL. *RIZ1* has been independently isolated as GATA-3 binding protein G3B [2]. Since GATA-3 is a transcription factor known to be expressed very specifically in the T-cell lineage at its early developmental stage, it may be important in T-cell development [2], and decreased *RIZ1* expression may induce dysregulation of GATA-3. Therefore, *RIZ1* is potentially important in leukemogenesis of T-ALL.

Although RIZ is a downstream effector of estrogen action [27] and estrogen receptor co-activator [5], no relationship was found between sex and its expression. The expression was not significantly different among cytogenetic risk groups. *RIZ1* + 2 but not *RIZ1* expression was significantly different in other characteristic groups (age, LDH and Ph chromosome). This indicates that the difference in *RIZ2* is prominent for these characteristics. Further investigation may clarify the role of *RIZ2* in ALL.

Methylation of the *RIZ1* gene was detected in 11 of the 71 patients (15.5%), but not in the healthy controls. Decreased *RIZ1* expression in all cases of ALL was not statistically associated with methylation. Several possibilities may account for this. First, some cell lines (Jurkat or Daudi) and samples without methylation had lower levels of *RIZ1* expression. Thus, methylation-independent mechanisms of *RIZ1* silencing may occur in some ALLs. Mutation in promoter sequences, loss of heterozygosity, histone methylation, microRNA (miRNA) and alteration of transcription factors may account for the low *RIZ1* expression. Second, a

few samples with methylation showed higher *RIZ1* expression. This may be related to the high sensitivity of MS-PCR. Third, the above might be related to a substantial proportion of cases of ALL having partial methylation. Another explanation is heterogeneity of ALL regarding methylation. In unknown entities of ALL, hypermethylation of *RIZ1* might be important in silencing.

Similar to expression, we found a strong relationship between *RIZ1* methylation status and ALL phenotype. Although methylation of the *RIZ1* promoter was infrequent in total ALL, it was frequently detected in T-ALL. Many patients with *RIZ1* methylation had T-ALL. As *RIZ1* expression in patients with T-ALL was lower than that in patients with B-ALL, and methylation of the *RIZ1* promoter was more common in T-ALL, it is suggested that *RIZ1* promoter methylation is important for silencing in T-ALL.

MOLT-4 cells were treated with 5-Aza-dC, a known DNA methyltransferase inhibitor. It induced demethylation of the *RIZ1* promoter, and suppression of cell proliferation and viability in a dose- and time-dependent manner. Restoration

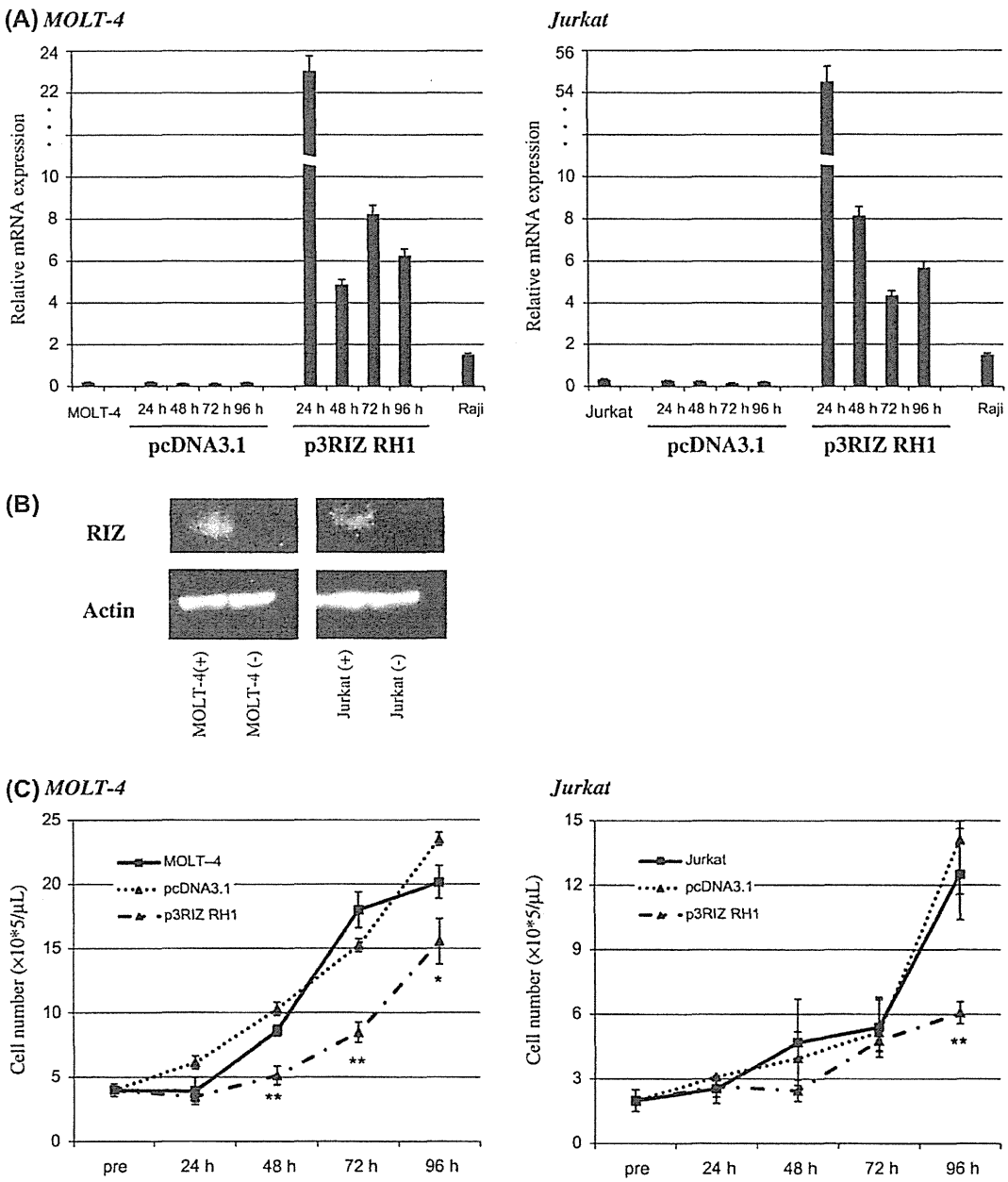

Figure 4. Forced RIZ1 expression in T-ALL cell lines. Bars represent mean of three independent experiments. (A) Expression of *RIZ1* mRNA in MOLT-4 and Jurkat cells after *RIZ1* transfection. p3RIZRH1, transfected with *RIZ1* plasmid; pcDNA3.1, transfected with empty vector for control. (B) RIZ1 protein expression of MOLT-4 and Jurkat cells 96 h after transfection. (+), transfected with *RIZ1* plasmid; (−), transfected with empty vector. (C) Cell proliferation of *RIZ1*-transfected MOLT-4 and Jurkat cells (mean). Inhibition of cell growth was observed in both MOLT-4 and Jurkat ($*p < 0.05$, $**p < 0.005$ vs. control). p3RIZRH1, transfected with *RIZ1* plasmid; pcDNA3.1, transfected with empty vector for control. (D) Cell cycle analysis of *RIZ1*-transfected MOLT-4 and Jurkat cells. Forced *RIZ1* expression led to an increase of cells in G2/M arrest and a decrease of cells in G0/G1 phase, 96 h after transfection ($*p < 0.1$, $**p < 0.05$ vs. control). (+), transfected with *RIZ1* plasmid; (−), transfected with empty vector. (E) Annexin V assay in *RIZ1*-transfected MOLT-4 cells. Percentage of apoptotic cells was significantly increased 72 and 96 h after *RIZ1* transfection ($*p < 0.05$ vs. control). (+), transfected with *RIZ1* plasmid; (−), transfected with empty vector.

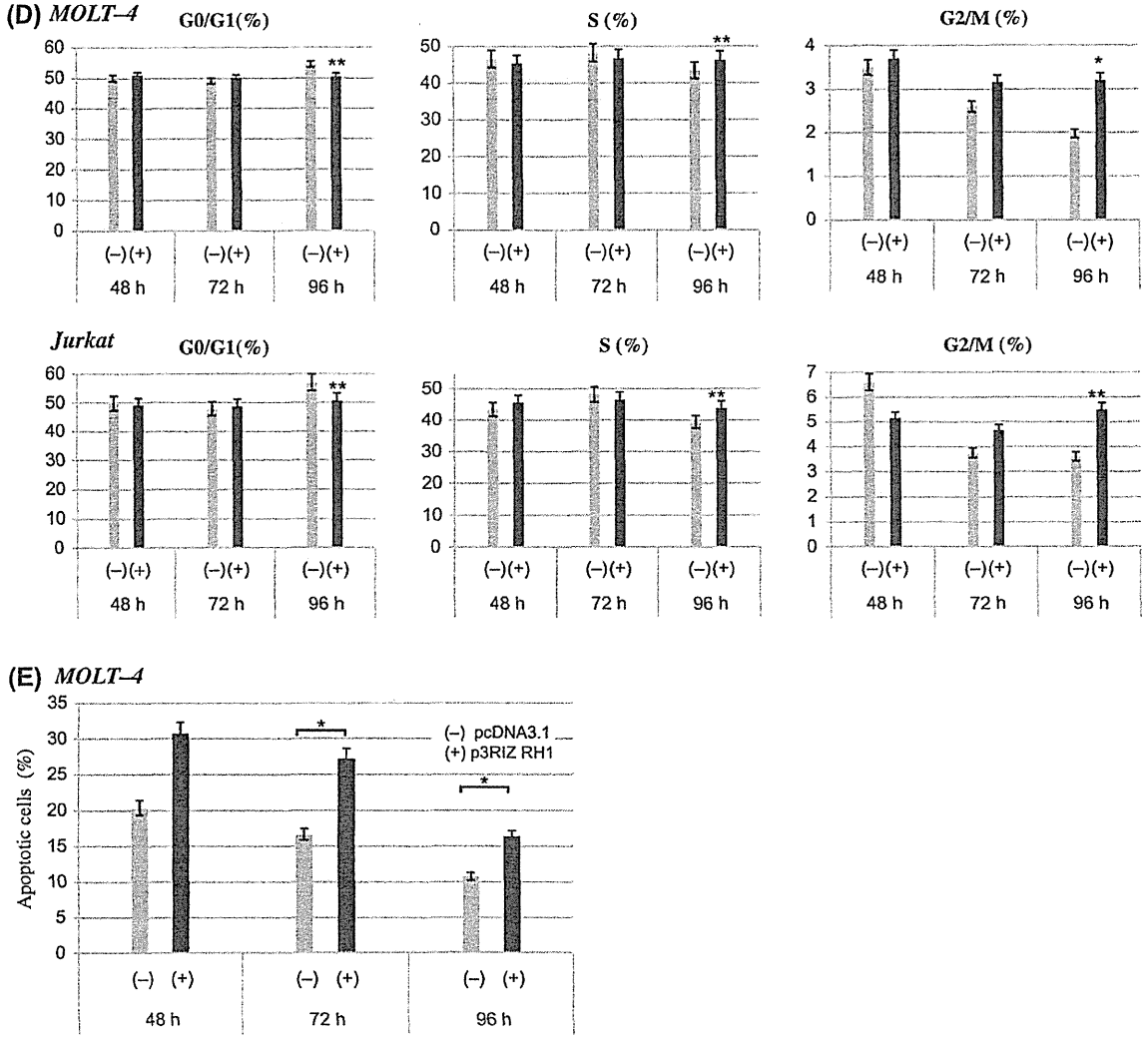

Figure 4. *(Continued).*

of *RIZ1* expression was induced in cells treated with a high concentration of 5-Aza-dC. These results suggest that aberrant DNA methylation affects silencing of *RIZ1* expression in T-ALL. Previous reports revealed that the *p73* [28] and *Lyn* [29] genes are frequently methylated in T-ALL in comparison with B-ALL. One possibility is that expression of DNA methyltransferase enzymes (DNMTs) is elevated in T-ALL and hypermethylation is not restricted to a single gene. Nevertheless, with re-expression of *RIZ1* in T-ALL cell lines, inhibition of cell growth, G2/M arrest and increase of apoptotic cells were observed. These results were consistent with a previous report in breast cancer [6]. Antiproliferative effects by the DNA methyltransferase inhibitor in MOLT-4 could be related to *RIZ1* re-expression. Moreover, although there are methylation-independent mechanisms of *RIZ1* silencing in Jurkat, cell growth inhibition and G2/M arrest were observed with forced *RIZ1* expression. Therefore, *RIZ1* inactivation is assumed to be involved in pathogenesis in T-ALL.

Regarding other clinical characteristics, *RIZ1* methylation was more frequent in Ph− ALL than in Ph+ ALL. Methylation of *RIZ1* might play a role in leukemogenesis in Ph− ALL. A previous study reported that methylation of certain genes was associated with aging [30]. However, we found no correlation between *RIZ1* methylation and patient age.

ALL is a heterogeneous group of lymphoid disorders, and many patients are incurable despite intensified chemotherapy or hematopoietic stem cell transplantation, particularly in adult ALL. An earlier study revealed that aberrant methylations of *p73*, *p15* and *p57KIP2* are common abnormalities in adult ALL, and these methylation patterns predict for prognosis [31]. Thus, the methylation profile may further divide the classification of adult ALL, and recognition of distinct gene expression profiles may identify new patient subgroups with response to therapies and prognosis.

We found no cases showing missense mutations in exon 7 or frameshift mutations at $(A)_8$ and $(A)_9$ tracks in exon 8 by PCR-SSCP analysis. The reasons for the difference in the incidence of the *RIZ1*-exon 7 mutations in DLBCL and other hematological malignancies including ALL are not clear. However, another PR domain-containing protein, PRDM1, is also mutated in DLBCL.

In conclusion, decreased expression of *RIZ1* but not overexpression of *RIZ2* is related to leukemogenesis in adult ALL. Aberrant methylation is associated with decreased

expression of *RIZ1* in T-ALL. Therefore, *RIZ1* is one of the candidate target tumor suppressor genes particularly in T-ALL, and further study to determine the mechanism of *RIZ1* inactivation will help to understand the variety of pathogenesis in ALL.

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Potential conflict of interest: Disclosure forms provided by the authors are available with the full text of this article at www.informahealthcare.com/lal.

References

[1] Buyse I, Takahashi E, Huang S. Physical mapping of the retinoblastoma interacting zinc finger gene RIZ to D1S228 on chromosome 1p36. Genomics 1996;34:119-121.
[2] Shapiro V, Lee P, Winoto A. Identification and cloning of the G3B cDNA encoding a 3' segment of a protein binding to GATA-3. Gene 1995;163:329-330.
[3] Muraosa Y, Takahashi K, Yoshizawa M, et al. cDNA cloning of a novel protein containing two zinc-finger domains that may function as a transcription factor for the human heme-oxygenase-1 gene. Eur J Biochem 1996;235:471-479.
[4] Liu L, Shao G, Steele-Perkins G, et al. The retinoblastoma interacting zinc finger gene RIZ produces a PR domain-lacking product through an internal promoter. J Biol Chem 1997;272:2984-2991.
[5] Carling T, Kim KC, Yang XH, et al. A histone methyltransferase is required for maximal response to female sex hormones. Mol Cell Biol 2004;24:7032-7042.
[6] He L, Yu JX, Liu L, et al. RIZ1, but not the alternative RIZ2 product of the same gene, is underexpressed in breast cancer, and forced RIZ1 expression causes G2-M cell cycle arrest and/or apoptosis. Cancer Res 1998;58:4238-4244.
[7] Steele-Perkins G, Fang W, Yang XH, et al. Tumor formation and inactivation of RIZ1, an Rb-binding member of a nuclear protein-methyltransferase superfamily. Genes Dev 2001;15:2250-2262.
[8] Chadwick RB, Jiang GL, Bennington GA, et al. Candidate tumor suppressor RIZ is frequently involved in colorectal carcinogenesis. Proc Natl Acad Sci USA 2000;97:2662-2667.
[9] Piao Z, Fang W, Malkhosyan S, et al. Frequent frameshift mutations of RIZ in sporadic gastrointestinal and endometrial carcinomas with microsatellite instability. Cancer Res 2000;60:4701-4704.
[10] Oshimo Y, Oue N, Mitani Y, et al. Frequent epigenetic inactivation of RIZ1 by promoter hypermethylation in human gastric carcinoma. Int J Cancer 2004;110:212-218.
[11] Du Y, Carling T, Fang W, e al. Hypermethylation in human cancers of the RIZ1 tumor suppressor gene, a member of a histone/protein methyltransferase superfamily. Cancer Res 2001;61:8094-8099.
[12] Sasaki O, Meguro K, Tohmiya Y, et al. Altered expression of retinoblastoma protein-interacting zinc finger gene, RIZ, in human leukaemia. Br J Haematol 2002;119:940-948.
[13] Lakshmikuttyamma A, Takahashi N, Pastural E, et al. RIZ1 is potential CML tumor suppressor that is down-regulated during disease progression. J Hematol Oncol 2009;2:28.
[14] Xie W, Li X, Chen X, et al. Decreased expression of PRDM2 (RIZ1) and its correlation with risk stratification in patients with myelodysplastic syndrome. Br J Haematol 2010;150:242-244.
[15] Mori N, Yoshinaga K, Tomita K, et al. Aberrant methylation of the RIZ1 gene in myelodysplastic syndrome and acute myeloid leukemia. Leuk Res 2011;35:516-521.
[16] Pui C, Robison L, Look A. Acute lymphoblastic leukaemia. Lancet 2008;371:1030-1043.
[17] Faderl S, O'Brien S, Pui C, et al. Adult acute lymphoblastic leukemia: concepts and strategies. Cancer 2010;116:1165-1176.
[18] Takeuchi J, Kyo T, Naito K, et al. Induction therapy by frequent administration of doxorubicin with four other drugs, followed by intensive consolidation and maintenance therapy for adult acute lymphoblastic leukemia: the JALSG-ALL93 study. Leukemia 2002;16:1259-1266.
[19] Towatari M, Yanada M, Usui N, et al. Combination of intensive chemotherapy and imatinib can rapidly induce high-quality complete remission for a majority of patients with newly diagnosed BCR-ABL-positive acute lymphoblastic leukemia. Blood 2004;104:3507-3512.
[20] Yanada M, Takeuchi J, Sugiura I, et al. High complete remission rate and promising outcome by combination of imatinib and chemotherapy for newly diagnosed BCR-ABL-positive acute lymphoblastic leukemia: a phase II study by the Japan Adult Leukemia Study Group. J Clin Oncol 2006;24:460-466.
[21] Slater D, Mertelsmann R, Koziner B, et al. Lymphoblastic lymphoma in adults. J Clin Oncol 1986;4:57-67.
[22] Rowe J. Prognostic factors in adult acute lymphoblastic leukaemia. Br J Haematol 2010;150:389-405.
[23] Fang W, Piao Z, Simon D, Sheu JC, Huang S. Mapping of a minimal deleted region in human hepatocellular carcinoma to 1p36.13-p36.23 and mutational analysis of the RIZ (PRDM2) gene localized to the region. Genes Chromosomes Cancer 2000;28:269-275.
[24] Mori N, Morosetti R, Mizoguchi H, Koeffler HP. Progression of myelodysplastic syndrome: allelic loss on chromosomal arm 1p. Br J Haematol 2003;122:226-230.
[25] Wang Y, Takanashi M, Tsuji K, et al. Level of DNA topoisomerase IIalpha mRNA predicts the treatment response of relapsed acute leukemic patients. Leuk Res 2009;33:902-907.
[26] Buyse I, Shao G, Huang S. The retinoblastoma protein binds to RIZ, a zinc-finger protein that shares an epitope with the adenovirus E1A protein. Proc Natl Acad Sci USA 1995;92:4467-4471.
[27] Abbondanza C, Medici N, Nigro V, et al. The retinoblastoma-interacting zinc-finger protein RIZ is a downstream effector of estrogen action. Proc Natl Acad Sci USA 2000;97:3130-3135.
[28] Liu M, Taketani T, Li R, et al. Loss of p73 gene expression in lymphoid leukemia cell lines is associated with hypermethylation. Leuk Res 2001;25:441-447.
[29] Hoshino K, Quintás-Cardama A, Yang H, Sanchez-Gonzalez B, Garcia-Manero G. Aberrant DNA methylation of the Src kinase Hck, but not of Lyn, in Philadelphia chromosome negative acute lymphocytic leukemia. Leukemia 2007;21:906-911.
[30] Toyota M, Issa JP. CpG island methylator phenotypes in aging and cancer. Semin Cancer Biol 1999;9:349-357.
[31] Shen L, Toyota M, Kondo Y, et al. Aberrant DNA methylation of p57KIP2 identifies a cell-cycle regulatory pathway with prognostic impact in adult acute lymphocytic leukemia. Blood 2003;101:4131-4136.

Overexpression of lung resistance-related protein and P-glycoprotein and response to induction chemotherapy in acute myelogenous leukemia

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Abstract

Lung resistance-related protein (LRP) and P-glycoprotein (P-gp) are associated with multidrug resistance. P-gp overexpression reduces intracellular anticancer drug concentrations and is correlated with low remission rates. However, whether the presence of LRP influences the response to induction chemotherapy remains controversial. Therefore, we investigated the relationship of LRP and P-gp overexpression with the response to induction chemotherapy. Univariate analysis revealed that there was a significant difference between complete remission rates for acute myelogenous leukemia patients depending on their blast cell expressions, between LRP positive versus negative, P-gp positive versus negative, and LRP/P-gp double positive versus other groups. Crude odds ratios (ORs) for complete remission were 0.390, 0.360, and 0.307 for LRP positive, for P-gp positive, and LRP/P-gp double positive patients, respectively. After controlling the confounding variables by stepwise multivariate logistical regression analysis, the presence of LRP/P-gp double positivity and P-gp positivity were found to be independent prognostic factors; adjusted ORs were 0.233 and 0.393, respectively. Furthermore, the monoclonal antibody against LRP significantly increased daunorubicin acumulation (P=0.004) in the nuclei of leukemic blast cells with LRP positivity in more than 10% of the cells. An LRP reversing agent, PAK-104P, was found to increase the daunorubicin content with marginal significance (P=0.060). The present results suggest that not only the presence of P-gp, but also LRP in leukemic blast cells is a risk factor for resistance to induction chemotherapy. Inhibiting LRP function, similar to the inhibition of P-gp function, will be necessary to improve the effectiveness of induction chemotherapy.

Introduction

Although the clinical outcome of acute myelogenous leukemia (AML) has improved with advancements in chemotherapy, treatment is still problematic. One of the major challenges for treatment is the resistance of leukemia blast cells to anticancer drugs. The most well-known proteins associated with multidrug resistance to anticancer drugs are P-glycoprotein (P-gp), lung resistance-related protein (LRP), and multidrug resistance-associated protein. P-gp overproduction reduces intracellular drug concentrations by binding to the drugs and acting as an adenosine triphosphate-dependent efflux pump.[1] Several studies, including ours, found that P-gp overexpression in AML is correlated with a low remission rate.[2-7] However, whether the presence of LRP influences the response to induction chemotherapy remains controversial.[8-11] Our previous study showed that the co-existence of LRP and P-gp significantly decreases the effectiveness of induction chemotherapy whereas the isolated presence of LRP or P-gp does not.[7] Scheper *et al.*[12] identified LRP as a drug resistance-associated protein in a P-gp-negative multidrug-resistant lung carcinoma cell line. LRP is the human major vault protein; vaults are localized in nuclear pore complexes and are involved in nucleocytoplasmic transport. Nuclear pore complexes are multicomponent structures that allow bidirectional nucleocytoplasmic exchanges of molecules and particles.[13] A decrease in the nucleus/cytoplasm ratio of doxorubicin content has been demonstrated in an LRP positive non-small cell lung cancer cell line.[14] No pharmacological study for LRP has been reported. Therefore, in the present study, we investigated the effects that the presence of LRP has on daunorubicin (DNR) content in the nuclei of leukemic blast cells. We also assessed whether LRP and P-gp affect the response to induction chemotherapy in AML patients.

Materials and Methods

Patients

The study cohort was made up of 151 previously untreated AML patients, including 138 patients with *de novo* AML and 13 patients with overt leukemia transformed from myelodysplastic syndrome (MDS). All patients provided written informed consent for induction chemotherapy. The leukemic subtypes of *de novo* AML were determined according to the French-American-British classification as follows:[15] 2, 34, 57, 19, 23, and 3 cases of M0, M1, M2, M4, M5, and M6, respectively. Patient age ranged from 16 to 84 years (mean 49 years). The immunophenotype (CD34 and CD7) of blast cells was examined with a flow cytometer and defined as positive when more than 20% of the blasts were stained. Cytogenetic analysis was performed using the Q-banding method and assessed according to International system for human cytogenetic nomenclature guidelines.[16] Chromosomal abnormalities were classified into three categories according to the report by Slovak *et al.*[17] The favorable risk category included patients with abnormalities of inv(16)/t(16;16)/del(16q) or (15;17) with any additional abnormalities, or t(8;21) without either a del(9q) or a complex karyotype. The intermediate risk category included patients characterized by +8, -Y, +6, del (12p), or a normal karyotype. The unfavorable risk category was defined by the presence of one or more of -5/del(5q), -7/del(7q), inv(3q), abnormalities 11q, 20q or 21q, del(9q), t(6;9), t(9;22), abnormalities 17p, and a complex karyotype defined as 3 or more abnormalities.

Chemotherapy

For *de novo* AML patients and patients with overt leukemia transformed from MDS, three regimens were used. Thirty-nine *de novo* AML patients and 2 patients with overt leukemia

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transformed from MDS were treated with behenoylcytarabine, DNR, and 6-mercaptopurine according to the Japan Adult Leukemia Study Group (JALSG) AML92 protocol.[18] Ninety-six *de novo* AML patients and 8 patients with overt leukemia transformed from MDS were treated with idarubicin and arabinosylcytosine according to the JALSG AML95 and MDS protocols.[19-20] The remaining 3 *de novo* AML patients and 3 patients with overt leukemia transformed from MDS were treated according to the CAG protocol.[21] The response to treatment was assessed after one course of induction therapy. Complete remission (CR) was defined as the achievement of a morphologically normal marrow, a granulocyte count of 1.5×10^3/L or over, and a platelet count of 100×10^9/L or over. Remission failure was defined according to the classification proposed by Preisler.[22] Relapse was defined as the presence of more than 5% blasts in marrow aspirates or development of extra-medullary leukemia in patients with previously documented CR after one course of induction chemotherapy according to National Cancer Institute criteria.[23]

Separation of leukemic blast cells

Mononuclear cells were separated through Ficoll-Conray (specific gravity: 1.077) density gradient centrifugation from bone marrow or peripheral blood taken at the initial diagnosis. The leukemic blast cells thus prepared were either used immediately or cryopreserved in liquid nitrogen with 10% dimethylsulphoxide and 50% fetal calf serum (FCS; Intergen, Purchase, NY, USA) until used as previously described.[7] The presence of more than 80% leukemic blast cells in each sample was confirmed by cytospin preparation.

Detection of lung resistance-related protein and P-glycoprotein expression on leukemic blast cells

The avidin-biotin-glucose oxidase method was carried out on the cytospin preparations. The slides were fixed with paraformaldehyde and incubated with 10% normal rabbit serum (Nichirei, Tokyo, Japan) in order to block nonspecific reactions, and stained with a monoclonal antibody (mAb) against LRP (LRP56, Nichirei) as previously described.[7] Positivity was classified according to the positive percentage as reported previously: negative (-); less than 10%, positive (+); and more than 10% positive (++).

P-gp expression was detected by indirect immunofluorescence staining by using MRK16 mAb (Kyowa Medix, Tokyo, Japan) as previously reported.[6] Positivity was classified according to our previous report as follows: less than 20% (-) and more than 20% (+). No difference was observed in the P-gp positivity between fresh and cryopreserved samples.

Isolation of nuclei

Nuclei were isolated as described by Newmeyer *et al.*[24] Leukemic blast cells were suspended in reticulocyte standard buffer (RSB; NaCl 0.01 M, $MgCl_2$ 0.0015 M, Tris-HCl 0.01 M, pH 7.4) and centrifuged for 5 min at 2000 rpm. The cells were incubated in RSB with 10% Nonidet P40 (Sigma, St. Louis, MO, USA), and then centrifuged for 1 h at 40,000 g. The nuclei that formed a sediment were resuspended in solution A (sucrose 250 mM, Dithiothreitol 1 mM, 1× buffer A salts, spermidine 0.5 mM, spermine 0.2 mM, and phenylmethylsulfonyl fluoride 1 mM).

Accumulation of [^{3}H] daunorubicin in isolated nuclei

The accumulation of 1 M [^{3}H] DNR (18.5 Ci/mmol; Perkin Elmer Life Sciences, Boston, MA, USA) in isolated nuclei was studied as described by Kitazono *et al.*[25] The isolated nuclei suspended in solution A were incubated with 1 M DNR for 10 min at 37°C in the presence or absence of 100 g/mL mAb LRP56 (Kamiyama Biomedical, Seattle, WA, USA) or 3 M PAK-104P{2-[4-(diphenylmethyl)-1-piperazinyl]ethyl-5-(trans-4,6-dimethyl-1,3,2-dioxaphosphorinan-2-yl)-2,6-dimethyl-4-(3-nitrophenyl)-3-pyridinecarboxylate P-oxide} as an LRP reversing reagent.[26] The PAK-104P was a gift from Dr S. Akiyama (Cancer Research, Kagoshima University, Japan). The nuclei were suspended in aqueous counting scintillant (Amersham Biosciences, Buckinghamshire, UK), and the radioactivity in the nuclei of 1×10^4 cells was determined by a liquid scintillation counter system (LSC-700; Aloka, Tokyo, Japan).

Statistical analysis

The relationships of LRP and P-gp expression with the response to induction chemotherapy, and with the clinical parameters such as age, subtype (*i.e.* AML and MDS), WBC count, immunophenotype (*i.e.* CD34 and CD7), LD, chromosome abnormalities were assessed by the χ^2 *test*. The relationship between LRP expression and DNR accumulation in isolated nuclei was evaluated by a two-sided t-test. Univariate logistic regression analysis was conducted to calculate crude odds ratios (ORs) and 95% confidence intervals (CIs). Furthermore, we used stepwise multivariate logistical regression analysis to calculate adjusted ORs and 95% CIs and to account for other confounding factors. All statistical analyses were performed by SAS software (Version 9.1.3 (TS1M3), SAS Institute Inc., Cary, NC, USA).

Results

Lung resistance-related protein and P-glycoprotein expression in acute myelogenous leukemia patients

LRP and P-gp expression of in AML patients is summarized in Table 1. The frequency of LRP positivity was 47.7% (72 of 151). There were no significant differences in the expressions of these proteins between *de novo* AML patients (47.1%, 65 of 138) and patients with overt leukemia transformed from MDS (53.8%, 7 of 13). The overall proportion of P-gp positive samples was 42.4% (64 of 151); this was found to be significantly higher in patients with overt leukemia transformed from MDS (69.2%, 9 of 13) than that in *de novo* AML patients (39.9%, 55 of 138) (P=0.041). While there were no correlations between LRP and clinical parameters, the expression of P-gp was found to be frequently positive when expression of CD7 or CD34 was positive (CD7, P=0.016; CD34, P=0.006). In addition, P-gp expression was found to be frequently negative when the

Table 1. Lung resistance-related protein and P-glycoprotein expression in acute myelogenous leukemia patients.

AML subtype	LRP positive		P-gp positive	
	n	%	n	%
M0	0/2	0	1/2	50
M1	16/34	47.1	13/34	38.2
M2	27/57	47.4	29/57	50.9
M4	9/19	47.4	4/19	21.2
M5	12/23	52.2	8/23	34.8
M6	1/3	33.3	0/3	0
Subtotal	65/138	47.1	55/138	39.9
Overt leukemia transformed from MDS	7/13	53.8	9/13	69.2
Total	72/151	47.7	64/151	42.4

AML, acute myelogenous leukemia; LRP, lung resistance-related protein; P-gp, P-glycoprotein; MDS, myelodysplastic syndrome.

white blood cell (WBC) count exceeded 50,000 (P=0.014). There was a negative correlation between percentage of P-gp cells and WBC count (r=-0.207, P=0.011).

Lung resistance-related protein and P-glycoprotein overexpression, clinical parameters, and the response to induction chemotherapy

The overall CR rate was 67.5% (102 of 151 patients); the failure rate of induction chemotherapy was 32.5% (49 of 151 patients). Patients were classified according to Preisler's classification:[22] 24 patients had hypocellular marrow at any time during chemotherapy (type I), 10 achieved hypocellular marrow but leukemic cells regrew within four weeks (type II), one survived for more than four weeks with hypocellular marrow (type III), 2 died with hypocellular marrow without evidence of residual leukemia (type IV), and 12 patients could not be typed. Of these 49 patients, the leukemic blast cells of 22 (44.9%) were LRP/P-gp double positive. As shown in Table 2, the CR rate of LRP positive patients (56.9%, 41 of 72) was significantly lower than that of LRP negative patients (77.2%, 61 of 79) (P=0.0079). The CR rate of P-gp positive patients (54.7%, 35 of 64) was also significantly lower than that of P-gp negative patients (77.0%, 67 of 87) (P=0.0038). The CR rate of LRP/P-gp double positive patients (38.9%, 14 of 36) was significantly lower than that of other groups (P=0.0005). Univariate logistical regression analysis (Table 2) revealed that the crude ORs for CR were 0.390 (95% CI: 0.193-0.788) for LRP positivity, 0.360 (95% CI: 0.179-0.726) for P-gp positivity, and 0.307 (95% CI: 0.131-0.720) for LRP/P-gp double positivity. After adjusting for the confounding factors using the stepwise multivariate logistical regression analysis, the presence of LRP/P-gp double positivity and P-gp positivity were found to be independent prognostic factors; ORs were 0.233 (95% CI: 0.103-0.529) and 0.393 (95% CI: 0.188-0.820), respectively.

Regarding the relationships between the CR rate and various clinical parameters, the CR rate of patients with unfavorable chromosomal abnormalities was significantly lower than that of patients with favorable and intermediate chromosomal abnormalities (33.3% *vs* 68.8%, P=0.0296). The OR of CR was 0.227 (95% CI: 0.054-0.954) by univariate logistical regression analysis. There were no significant differences with respect to CR regarding other clinical parameters.

Table 2. Risk factors which affect inferior complete remission rates.

	Univariate analysis		Logistic regression analysis	
	CR rates	χ^2	Odds ratio	95% CI
LRP+ vs LRP-	56.7% *vs* 77.2%	0.0079	0.390	0.193-0.788
Pgp+ vs P-gp-	54.5% *vs* 77.0%	0.0038	0.360	0.179-0.726
LRP+/Pgp+ vs others	38.0% *vs* 76.5%	0.0005	0.307	0.131-0.72
Chromosomal abn unfavourable *vs* good + intermediate	33.3% *vs* 68.8%	0.0296	0.227	0.054-0.954

CI, confidence interval; CR, complete remission; LRP, lung resistance-related protein; P-gp, P-glycoprotein.

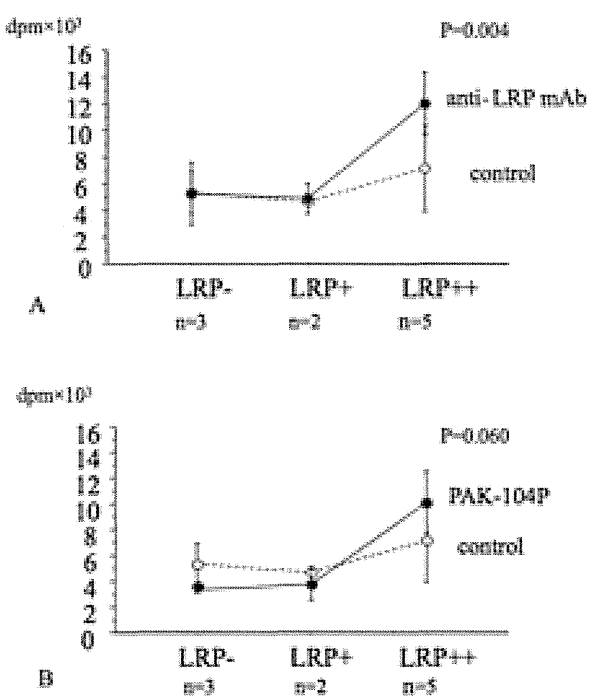

Figure 1. DNR accumulation in isolated nuclei and effects of the addition of (A) anti-LRP mAb and (B) PAK-104P. A) DNR accumulation with the addition of anti-LRP mAb increased significantly when the leukemic blast cells were LRP++ (P=0.004); B) PAK-104P increased DNR accumulation when the samples were LRP++, with marginal significance (P=0.06). Open circles (○) indicate the untreated controls; closed circles (●) indicate the presence of anti-LRP mAb or PAK-104P. Data are expressed as the mean±SD of triplicate results.

Functional assay of lung resistance-related protein expression

Figure 1 shows the differences in DNR accumulation with respect to LRP positivity with and without anti-LRP mAb or PAK-104P. Anti-LRP mAb significantly increased DNR accumulation when the samples were LRP++ (P=0.004). PAK-104P also increased DNR accumulation when the samples were LRP++, with marginal significance (P=0.060). These results indicate that anti-LRP mAb and PAK-104P increase DNR accumulation in leukemic cells that highly express LRP.

Discussion

The *LRP* gene is located on chromosome 16, proximal to the multidrug resistance-associated protein gene. It encodes a nuclear major vault protein homolog that may disrupt the transport of drugs from the cytoplasm to the nucleus.[13] In this study, we demonstrated that the addition of an mAb against LRP or PAK-104P increases DNR accumulation in isolated nuclei. DNR accumulation in the nuclei was higher when the cells highly expressed LRP. Our results agree with those of a previous report demonstrating that a polyclonal antibody against LRP in adult T-cell leukemia enhances doxorubicin efflux in isolated nuclei.[27] Furthermore, the addition of verapamil is reported to partially restore the doxorubicin nucleus/cytoplasm ratio in non-small cell lung cancer cell line.[14] This indicates that the inhi-

bition of LRP function may lead to an increase in DNR accumulation in nuclei, thus increasing the effectiveness of the treatment. In the present study, we evaluated whether the presence of LRP, P-gp alone or the co-existence of LRP and P-gp in leukemic blast cells influences the response of AML patients to induction chemotherapy. The CR rate was not only significantly lower in LRP/P-gp double positive patients, but also lower in only LRP or P-gp positive patients. Furthermore, multivariate analysis confirmed a significantly lower CR rate in LRP/P-gp double positive patients. Therefore, the presence of both LRP and P-gp may be a sufficiently strong factor for predicting the response to induction chemotherapy. In our previous report, the presence of LRP or P-gp alone was not powerful enough to predict the effectiveness of induction chemotherapy.[7] Compared to our previous research, a greater number of AML patients were examined in the present study, and analyzed cases were limited to AML, except M3. These different conclusions may be due to differences in patient populations. As for clinical parameters, only chromosomal abnormalities influenced the CR rate.

Conclusions

The present results indicate that the presence of LRP and/or P-gp in leukemic blast cells may be risk factors for resistance to induction chemotherapy. As our data demonstrate that high LRP levels obstruct the transport of DNR into the nucleus, trials inhibiting LRP function, similar to the inhibition of P-gp, are necessary to potentially improve the clinical response.

References

1. See YP, Carlsen SA, Till JE, et al. Increased drug permeability in Chinese hamster ovary cells in the presence of cyanide. Biochim Biophys Acta 1974;373:242-52.
2. Campos L, Guyotat D, Archimbaud E, et al. Clinical significance of multidrug resistance P-glycoprotein expression on acute non-lymphoblastic leukemia cells at diagnosis. Blood 1992;79:473-6.
3. Del Poeta G, Stasi R, Aronica G, et al. Clinical relevance of P-glycoprotein expression in de novo acute leukemia. Blood 1996;87:1997-2004.
4. Michieli M, Damiani D, Germin A, et al. Overexpression of multidrug resistance - associated p-170- glycoprotein in acute non-lymphocytic leukemia. Eur J Haematol 1992;48:87-92.
5. Hunault M, Zhou D, Delmer, A, et al. Multidrug resistance gene expression in acute myeloid leukemia: major prognosis significance for in vivo drug resistance to induction treatment. Ann Hematol 1997;74:65-71.
6. Wang YH, Motoji T, Motomura S, et al. Recovery of drug resistance-reversing agent on acute myelogenous leukemic blasts and K562 cells resistant to adriamycin cell line. Eur J Haematol 1997;58: 186-94.
7. Tsuji K, Motoji T, Sugawara I, et al. Significance of lung resistance-related protein in the clinical outcome of acute leukaemic patients with reference to P-glycoprotein. Br J Haematol 2000;110:370-8.
8. Legrand O, Simonin G, Zittoun R, et al. Lung resistance protein (LRP) gene expression in adult acute myeloid leukemia: a critical evaluation by three techniques. Leukemia 1998;12:1367-74.
9. Michieli M, Damiani D, Ermacora A, et al. P-glycoprotein, lung resistance-related and multidrug resistance associated protein in de novo acute non-lymphocytic leukaemias: biological and clinical implications. Br J Haematol 1999;104:328-35.
10. Leith CP, Kopecky KJ, Chen IM, et al. Frequency and clinical significance of the expression of multidrug resistance proteins MDR1/P-glycoprotein, MRP1, and LRP in AML. A southwest oncology group study. Blood 1999;94:1086-99.
11. Tsimberidou AM, Paterakis G, Androutsos G, et al. Evaluation of the clinical relevance of the expression and function of P-glycoprotein, multidrug resistance protein and lung resistance protein in patients with primary acute myelogenous leukemia. Leuk Res 2002;26:143-54.
12. Scheper RJ, Broxterman HJ, Scheffer GL, et al. Overexpression of a M(r)110,000 vesicular protein in non-P-glycoprotein-mediated multidrug resistance. Cancer Res 1993;53:1475-9.
13. Scheffer GL, Wijngaard PLJ, Flens MJ, et al. The drug resistance-related protein LRP in the human major vault protein. Nat Med 1995;1:578-82.
14. Schuurhuis GJ, Broxterman HJ, de Lange JHM, et al. Early multidrug-resistance, defined by changes in intracellular doxorubicin distribution, independent of P-glycoprotein. Br J Cancer 1991;64:857-61.
15. Bennett JM, Catovsky D, Daniel MT, et al. Proposals for the classification of acute myeloid leukemia. A report of the French-American-British cooperative group. Ann Int Med 1985;103:626-9.
16. ISCN. Guidelines for Cancer Cytogenetics. In: An international system for human cytogenetic nomenclature. Basel, Switzerland: Karger; 1995.
17. Slovak ML, Kopecky KJ, Cassileth PA, et al. Karyotypic analysis predicts outcome of preremission and postremission therapy in adult acute myeloid leukemia: a southwest oncology group/eastern cooperative oncology group study. Blood 2000;96:4075-83.
18. Miyawaki S, Tanitomo M, Kobayashi T, et al. No beneficial effect from addition of etoposide to daunorubicin, cytarabin, and 6-mercaptopurine in individualized induction therapy of adult acute myeloid leukemia: the JALSG-AML92 study. Int J Hematol 1999;70:97-104.
19. Ohtake S, Miyawaki S, Kiyoi H, et al. Randomised trial of response-oriented individualized versus fixed-schedule induction chemotherapy with idarubicin and cytarabine in adult acute myeloid leukemia: the JALSG AML95 study. Int J Hematol 2010;91:276-83.
20. Okamoto T, Kanamaru A, Shimazaki C, et al. Combination chemotherapy with risk factor-adjusted dose attenuation for high-risk myelodysplastic syndrome and resulting leukemia in the multicenter study of the Japan Adult Leukemia Study Group (JALSG): results of an interim Analysis. Int J Hematol 2000;72:200-5.
21. Yamada K, Furusawa S, Saito K, et al. Concurrent use of granulocyte colony-stimulating factor with low-dose cytosine arabinoside and aclarubicin for previously treated acute myelogenous leukemia: a pilot study. Leukemia 1995;9:10-4.
22. Preisler HD. Failure of remission induction in acute myelocytic leukemia. Med Ped Oncol 1978;4:275-6.
23. Chenson BD, Cassileth PA, Head DR, et al. Report of the National Cancer Institute-sponsored workshop on definitions of diagnosis and response in acute myeloid leukemia. J Clin Oncol 1990;8:813-9.
24. Newmeyer DD, Wilson KL. Egg extracts for nuclear import and nuclear assembly reactions. Method Cell Biol 1991;36:607-34.
25. Kitazono M, Sumizawa T, Takebayashi Y, et al. Multidrug resistance and the lung resistance-related protein in human colon carcinoma SW-620 cells. J Natl Cancer Inst 1999;91:1647-53.
26. Shudo N, Mizoguchi T, Kiyosue T, et al. Two pyridine analogues with more effective ability to reverse multidrug resistance and with lower calcium channel blocking activity than their dihydropyridine counterparts. Cancer Res 1990;50:3055-61.
27. Ohno N, Tani A, Uozumi K, et al. Expression of functional lung resistance-related protein predicts poor outcome in adult T-cell leukemia. Blood 2001;98:1160-5.

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HLA-C Matching Status Does Not Affect Rituximab-Mediated Antibody-Dependent Cellular Cytotoxicity by Allogeneic Natural Killer Cells

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Risk of leukemia relapse after T cell-depleted hematopoietic stem cell transplantation is lower in the "HLA-C mismatched" recipient-donor combinations. This might be attributable to increased natural killing by allogeneic NK cells carrying a KIR that does not bind to HLA-C on target cells (HLA-C-uncoupled KIR). Considering a new strategy of allogeneic NK cell transfer with rituximab to treat B-cell lymphomas, however, it is unknown whether the HLA-C matching status also affects rituximab-mediated antibody-dependent cellular cytotoxicity (ADCC). To address this issue, we investigated the levels of ADCC by purified NK cells carrying an HLA-C-uncoupled KIR, where the NK cell donors had either matched or mismatched HLA-C combination with target cells. Purified NK cells carrying an HLA-C-uncoupled KIR consistently showed enhanced ADCC against target cells when NK cell donors had an HLA-C-mismatch. When NK cell donors did not have an HLA-C mismatch, it was inconsistent whether HLA-C-uncoupled KIR caused ADCC enhancement. When the levels of ADCC by whole NK cells were compared, there were substantial differences among the donors regardless of the HLA-C matching status. Subjects with HLA-C mismatch may not have an advantage when cytoimmunotherapy using allogeneic NK cells is considered in combination with rituximab.

Keywords HLA-C, Rituximab, ADCC, Allogeneic, NK cells.

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INTRODUCTION

As a treatment modality against various malignancies, monoclonal antibody-based therapy is expanding its indications. Monoclonal antibodies attack tumor cells mainly through complement-dependent cytotoxicity and antibody-dependent cellular cytotoxicity (ADCC). ADCC requires Fc receptor-bearing effector cells such as natural killer (NK) cells. After the Fc receptor engagement by an antibody, NK cells kill antibody-bound target cells by secretion of cytokines such as IFN-γ, and by discharge of the contents of their granules, such as perforin and granzyme (Cartron et al., 2004). NK cells also have a device that prevent themselves from attacking normal autologous cells by recognizing "self" with receptors that transduce inhibitory signals, many of which have specificity for HLA class I or HLA class I-related molecules.

Among such inhibitory receptors, a family of killer immunoglobulin-like receptors (KIRs) has been well-characterized (Lanier, 1998; Parham and McQueen, 2003). HLA-C is the main ligand for most inhibitory KIRs. It has been shown that HLA-C is classified into two groups, C1 and C2, by the amino acid substitutions at positions 77 and 80 in the α-1 helix structure (Boyington and Sun, 2002; Farag et al., 2002; Gumperz et al., 1995).

When NK cells face allogeneic target cells that cannot be recognized by inhibitory KIRs, they sense the missing expression of "self" class I alleles and mediates alloreactions (Ruggeri et al., 1999). Allogeneic cells missing expression of KIR ligands can therefore trigger NK cell alloreactivity (Ruggeri et al., 2002). Based on this theory, a simple algorithm known as the KIR ligand mismatch model was developed, in which comparison between donor and recipient HLA class I genotype allows prediction of NK alloreactivity (Baron et al., 2009).

The benefit of KIR ligand mismatch is well analyzed in allogeneic transplantation, especially T-cell-depleted HLA-mismatched transplantation (Giebel et al., 2003; Ruggeri et al., 1999; Ruggeri et al., 2002). Although the contribution of KIR ligand incompatibility to NK cell cytotoxic activity was reported, there is only a single study that documents the role for KIR ligand incompatibility in ADCC thus far (Stein et al., 2006).

In the current study, we focused on ADCC via a chimeric mAb, rituximab (Reff et al., 1994) that binds specifically to CD20 and induces apoptosis in a subset of CD20-expressing lymphoma cells through ADCC. We hypothesized that ADCC against lymphoma cells expressing HLA class I would be suppressed by KIRs on autologous NK cells. In contrast, ADCC would be enhanced and expected to be effective to refractory B-cell lymphomas if we use allogeneic NK cells with KIR ligand mismatch.

MATERIAL AND METHODS

Study Approval

This study was approved by the institutional review board in University of Tsukuba and all subjects were given written informed consent.

Cell Lines

The CD20-positive human Burkitt lymphoma cell line RAJI was provided by RIKEN BRC CELL BANK (Ibaraki, Japan). Epstein-Barr virus-transformed lymphoblastoid cell line, 103-LCL was provided by Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). HLA-C*0304 and HLA-C*0401 bearing B-lymphoblastoid cell lines (LBL), which were produced by retroviral transduction of HLA class I-deficient 721.221 LBL with each HLA-C molecule, were described previously (Akatsuka et al., 2002). All cell lines were cultured in RPMI1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 μg/ml L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin and incubated at 37°C with 5% CO_2.

Antibodies

For flow cytometric analysis, NK cell sorting, and ADCC assay, the following mouse monoclonal antibodies were used: FITC-conjugated anti-human CD20 (B-Ly1; DAKO), PE-conjugated anti-human HLA-A, -B, and -C (W6/32; BioLegend, San Diego, CA), FITC-conjugated anti-human CD56 (MEM188; eBioscience), PE-conjugated anti-human CD158b1/b2,j (GL183; Beckman Coulter), and APC-conjugated anti-human CD158a,h (EB6.B; Beckman Coulter). Anti-human CD20 mAb rituximab was kindly provided by Zenyaku Kogyo Co. Ltd (Tokyo, Japan). Human polyclonal immunoglobulin and human IgG1γ isotype control antibody were purchased from Baxter Japan (Tokyo, Japan) and Beckman Coulter (Fullerton, CA, USA), respectively.

HLA Typing

HLA-C typing of target cells and NK donors were performed at Human Leukocyte Antigen (HLA) Laboratory, Nonprofit Organization (Kyoto, Japan) by a reverse sequence-specific oligonucleotide-probe method using the Luminex (Austin, TX, USA) platform.

NK Cells

Heparinized blood was obtained from normal healthy subjects, and NK cells were isolated from whole blood by density gradient centrifugation using

RosetteSep NK cell enrichment cocktail (Stemcell Technologies, BC, Canada) according to the manufacturer's instructions. Isolated NK cells were stained with anti-CD158a (KIR2DL1) and anti-CD158b1/b2 (KIR2DL2/3) antibodies, and then sorted by BD FACSAria (BD Biosciences) into populations positive for either KIR. We used NK cells or the sorted NK subgroup populations only when the purity was >90% (data not shown).

RNA Interference

Double-stranded, short (21-mer) interfering RNA (siRNA) corresponding to enhanced green fluorescent protein (EGFP) [sense: 5'-CGUAAACGGCCACAAG UUCTT-3', antisense: 5'-GAACUUGUGGCCGUUUACGTT-3', starting from nucleotide 66 of the EGFP coding sequence (Matin et al., 2004)] and β_2-microglobulin were synthesized by Invitrogen. Two types of siRNA for β_2-microglobulin (accession number AB021288) were used: β_2-microglobulin A, 5'-GAUUCAGGUU UACUCACGUTT-3' (sense) and 5'-ACGUGAGUAAACCUGAAUCTT-3' (antisense), starting from nucleotide 91 of β_2-microglobulin sequence (Matin et al., 2004); and β_2-microglobulin B, 5'-GGUUUCAUCCAUCCGACAUTT-3' (sense) and 5'-AUGUCGGAUGGAUGAAACCTT-3' (antisense), starting from nucleotide 76. siRNA for EGFP was used as a control siRNA for those of β_2-microglobulin. We transferred these siRNAs by electroporation using the amaXa Nucleofector system (Koeln, Germany) according to the manufacturer's instructions.

Briefly, 2×10^6 cells, 150 pmol siRNA, and the 100 μl Nucleofector Solution V were combined, and then transferred to a cuvette. The cells were electroporated using the cell-type specific program. Cells were rinsed with medium and cultured. The cells were used as the targets in ADCC assay after 36 hours, at the time of maximal decrease of HLA class I expression determined by serial flow cytometry analyses (data not shown).

Flow Cytometry Assays for ADCC

NK cell ADCC against RAJI, 721.221LCL HLA-C*0304, 721.221LCL HLA-C*0401, and 103-LCL cells was measured using the LIVE/DEAD®. Cell-Mediated Cytotoxicity Kit (Invitrogen) based on procedures described previously (Kroesen et al., 1992). In brief, target B-cell lines (5×10^4 cells) were incubated for 20 min at 37°C, 5% CO_2 with the green fluorescent dye 3,3'-dioctadecyloxacarbocyanine (DiO) according to the manufacturer's instructions. Then the cells were washed twice with phosphate buffered saline.

Target cells were seeded in triplicates in 96-well microplates. Either whole or sorted NK cells were used as an effector, which were mixed with target cells at desired effector:target (E:T) ratios in the presence of rituximab or control antibody (human polyclonal immunoglobulin or human IgG1γ isotype control). The ideal concentration of rituximab for each target was determined by measuring ADCC

with various concentrations of rituximab (data not shown). After incubation at 37°C for 2 hours, propidium iodide (PI) was added and the cultures were analyzed by flow cytometry (BD FACSCalibur and CellQuestPro software, BD Bioscience). Specific cytotoxicity against target cells was calculated as: Dead target cells (DiO^+, PI^+)/Total (live and dead) target cells (DiO^+, PI^+ or $^-$) (Reff et al., 1994)

Statistical Analysis

Differences between variables were evaluated using the Student *t*-test using the Statcel software (OMS, Saitama, Japan). In this study, $P < 0.05$ was considered significant.

RESULTS

Knockdown of HLA Class I on Target Cells Enhances ADCC

NK cells prepared from peripheral blood of healthy subjects contained large granular lymphocytes and were >95% CD56-positive and CD3-negative (data not shown). Rituximab-dependent ADCC against the Burkitt lymphoma cell line, RAJI, was observed only when NK cells were present (Fig. 1). Cytotoxicity was saturated at approximately 60% with 1 μg/ml rituximab. As expected, rituximab alone induced almost no cytotoxicity (Fig. 1). Of note, RAJI cells express both HLA-C groups (C1/C2) (Table 1), indicating that NK cells from any donors are not alloreactive to RAJI cells.

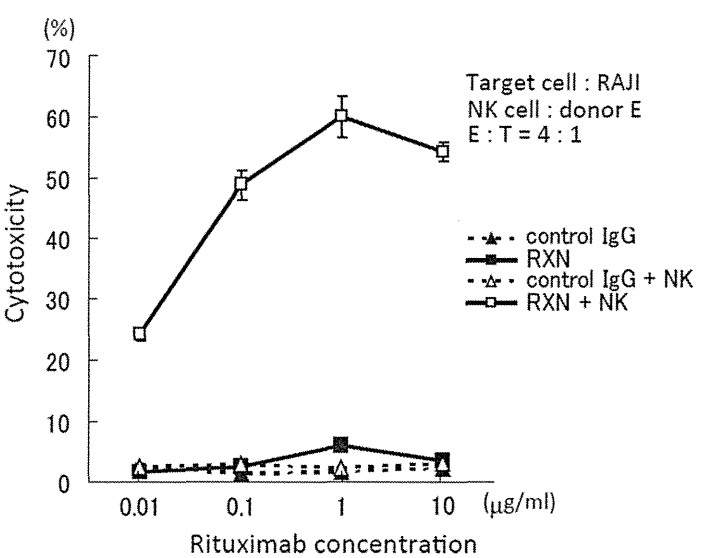

Figure 1: NK cell ADCC on lymphoma cells in the presence of rituximab.A CD20-positive human Burkitt lymphoma cell line RAJI was incubated with or without peripheral blood NK cells from a healthy volunteer donor with various concentration of control polyclonal IgG or rituximab (RXN). Control IgG alone (▲), control IgG with NK cells (Δ), RXN alone (■), RXN with NK cells (□). After 2-hour incubation, RAJI cells were stained by propidium iodide (PI) and analyzed by flow cytometry. Cytotoxicity was calculated by percentage of PI-positive RAJI cells in the total RAJI cell population. Mean cytotoxicity ± standard deviation (SD) of triplicate samples are shown at various antibody concentrations. The results were reproducible in three independent experiments.

Table 1: HLA-C typing and corresponding KIRs of cell lines and donors.

Cells	HLA-C	HLA-C group	Corresponding KIRs	License status	
				KIR2DL2,3$^+$ NK (C1-specific NK)	KIR2DL1$^+$ NK (C2-specific NK)
Target cells					
RAJI	Cw3 / Cw10	C1 / C2	KIR2DL2, 3 / KIR2DL1		
721.221LCL C*0304	Cw3 / -	C1 / -	KIR2DL2,3		
721.221LCL C*0401	Cw4 / -	C2 / -	KIR2DL1		
103-LCL	Cw8 / -	C1 / -	KIR2DL2, 3		
NK cell Donor					
A	Cw14 / Cw15	C1 / C2	KIR2DL2, 3 / KIR2DL1	Licensed	Licensed
B	Cw1 / Cw15	C1 / C2	KIR2DL2, 3 / KIR2DL1	Licensed	Licensed
C	Cw12 / Cw2	C1 / C2	KIR2DL2, 3 / KIR2DL1	Licensed	Licensed
D	Cw1 / Cw8	C1 / C1	KIR2DL2, 3	Licensed	*Unlicensed*
E	Cw1 / Cw7	C1 / C1	KIR2DL2, 3	Licensed	*Unlicensed*
F	Cw12 / Cw14	C1 / C1	KIR2DL2,3	Licensed	*Unlicensed*

Thus, to mimic the effects of allogeneic recognition by NK cells, we down-regulated the surface expression of HLA class I with siRNA targeting β_2-microglobulin (β2MG). Serial flow cytometric analyses demonstrated maximal downregulation of HLA class I at 36 hours after electroporation of the siRNA (data not shown). The mean fluorescence intensity of HLA class I expression was decreased to approximately 20% and 50% with β2MG-KO-A and β2MG-KO-B relative to that of untreated Raji cells, respectively, while the control siRNA (siRNA for EGFP, which is not relevant to this cytotoxicity assay system) had minimal effect (Fig. 2a). In contrast, these siRNA treatments did not affect the expression levels of CD20 (Fig. 2b). Allogeneic NK activity was readily enhanced in inverse correlation with the decreased expression level of HLA class I with the individual siRNAs when NK cells from two donors were tested in the presence of control IgG (Fig. 2c).

Finally, we evaluated the effect of HLA class I down-regulation with siRNAs in combination with rituximab-mediated ADCC. The target cell lysis was significantly enhanced according to the level of HLA class I downregulation. This phenomenon is explained as the increase of natural killing as a result of "missing-self." However, the effect became less clear when the concentration of rituximab was higher (Fig. 2d), suggesting that rituximab-dependent ADCC might be a major component of cytotoxic activity even in the NK cell-mediated allogeneic settings. In this experiment, both donor D and E have homozygous in HLA-C group (C1/C1) but the level of cytotoxicity was different between the donors. ADCC variation among individuals or FcγRIII polymorphisms might have resulted in this difference (see Discussion).

HLA-C-uncoupled KIR Enhances ADCC by Allogeneic NK Cells

We next examined whether ADCC is enhanced by NK cells carrying HLA-C-uncoupled KIR. HLA-C phenotypes were examined in six healthy subjects (Table 1). Three were heterozygous (C1/C2) and the other three were homozygous (C1/C1) in HLA-C groups. We sorted NK cells from these subjects into either KIR2DL2/3-positive (C1-specific) or KIR2DL1-positive (C2-specific) populations (Fig. 3a).

First, we used 721.221LCL HLA-C*0304 and 721.221LCL HLA-C*0401 as the target cells, which express exclusively C1 and C2, respectively. These cells do not express any other HLA class I molecules and expression levels of HLA-C were similar to each other (Fig. 3b). In this setting, KIR2DL1+ NK cells showed higher natural killing than KIR2DL2/3+ NK cells to the 721.221LCL HLA-C*0304 (C1) target, and conversely, KIR2DL2/3+ NK cells showed higher natural killing than KIR2DL1+ NK cells to 721.221LCL HLA-C*0401 (C2), regardless of whether NK cells were prepared from donors having C1/C1 or C1/C2 (Figs. 4a and b).

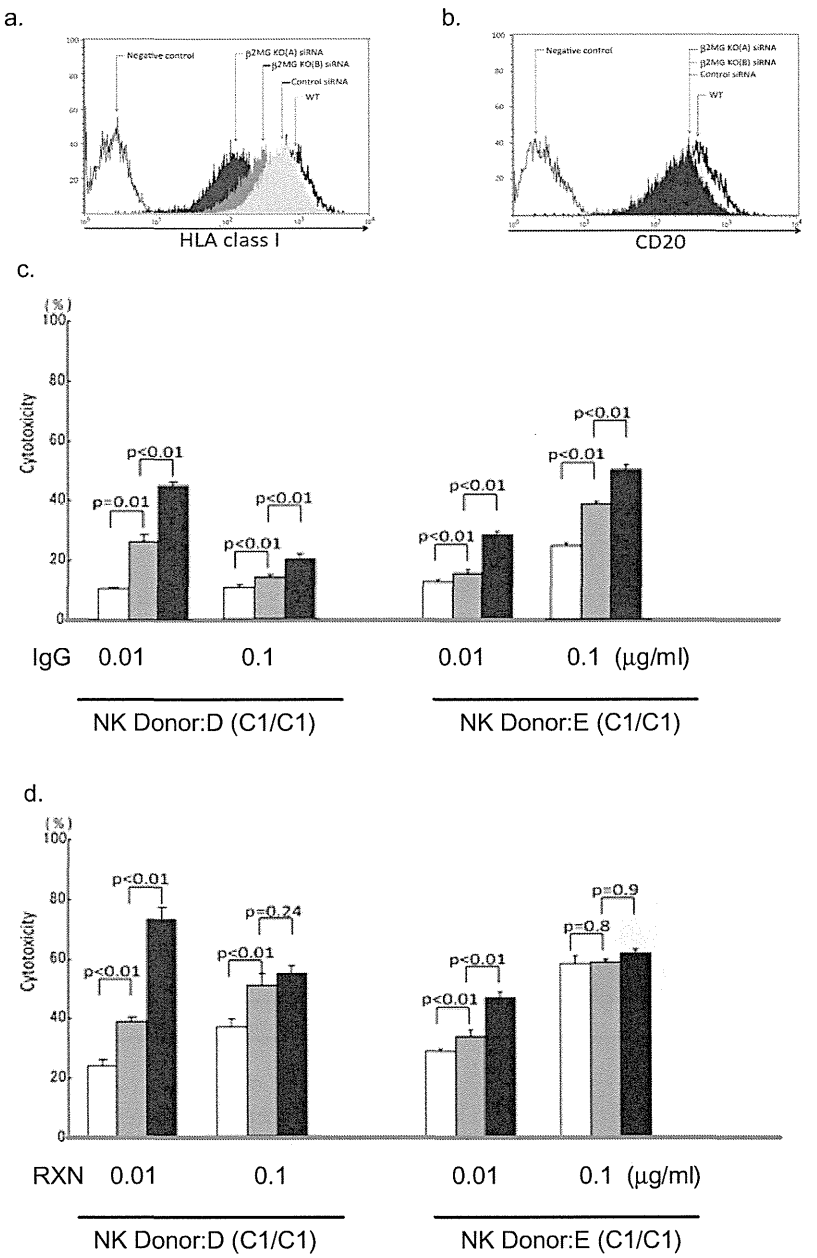

Figure 2: HLA class I knockdown enhances ADCC. (a) Flow cytometric analysis of HLA class I expression in cells treated with two different β2MG siRNAs, A (filled with black) and B (filled with dark gray), or control siRNA (filled with light gray). Isotype control, thin line; HLA class I expression of untreated cells, thick line. Analysis was performed 36 hours after siRNA treatment. (b) CD20 expression in the same cells as in (a). (c, d) RAJI cells, 36 hours after treatment with siRNAs (β2MG-KO-A in black, β2MG-KO-B in gray and control siRNA in white) incubated in the presence of peripheral blood NK cells from healthy volunteer donor D and E with 0.01 μg/ml or 0.1 μg/ml of control IgG (c) or RXN (d) for 5 hours at 37°C, 5% CO_2. E:T ratio was 4:1. Mean cytotoxicity ± SD of triplicate samples was measured. Data are representative of at least three independent experiments.