Aberrant methylation of protocadherin 17 and its prognostic value in pediatric acute lymphoblastic leukemia

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ABBREVIATION

ALL	Acute lymphoblastic leukemia
BCP	B-cell precursor
BM	Bone marrow
COBRA	Combined bisulfite restriction analysis
CR	Complete remission
DAVID	Database for Annotation, Visualization, and Integrated Discovery
EFS	Event-free survival
HR	Hazard ratio
MNCs	Mononuclear cells
OS	Overall survival
PB	Peripheral blood
RT-PCR	Reverse transcriptase PCR
WBC	White blood cell

ABSTRACT

Background: The outcome of approximately 20% of acute lymphoblastic leukemia (ALL) patients remains poor because of disease recurrence. We examined whether the DNA methylation of cadherin superfamily genes is a useful biomarker for ALL relapse.

Procedure: We used Infinium Methylation 450K Array to assess the genome-wide DNA methylation status. The methylation status of each individual gene was then determined by combine bisulfite restriction analysis and genome bisulfite sequencing. mRNA expression was evaluated by reverse transcriptase PCR (RT-PCR) and quantitative real-time PCR.

Results: Cadherin superfamily genes including *cadherin (CDH)1*, *protocadherin (PCDH)8*, and *PCDH17* were selected for analyzing the methylation status. In 40 B-cell precursor (BCP) ALL samples at onset, the methylation frequencies of *CDH1*, *PCDH8*, and *PCDH17* were 62.5%, 55%, and 30%, respectively. *CDH1* and *PCDH8* methylation was also detected in 80% and 20% of control BM samples, respectively. On the contrary, *PCDH17* was unmethylated in all control BM samples. There was a significant correlation between the methylation status of *PCDH17* (but not *CDH1* and *PCDH8*) and event-free survival or overall survival. By univariate and multivariate analyses, only *PCDH17* methylation was associated with the increased risk for relapse and mortality in patients with BCP ALL.

Conclusion: *PCDH17* methylation at onset was closely related to poor prognosis, and thus it could be used as a new biomarker to predict relapse in BCP ALL.

INTRODUCTION

The cure rate of childhood acute lymphoblastic leukemia (ALL) has improved over the past four decades. However, the prognosis of approximately 20% of patients with ALL remains poor because of disease recurrence. A main cause of refractory ALL is the development of chemotherapeutic drug-resistant clone(s).[1-3] The acquisition of chemoresistance is considered to be associated with either genetic or epigenetic alterations.[4-6] Moreover, recent studies demonstrated that relapse clones already present as a small subset of leukemic cells at diagnosis and eventually reemerge as the major clone at relapse.[7] In recent years, many studies have attempted to identify new clinical biomarkers that can predict high-risk patients and serve as targets for novel therapeutic interventions using gene-expression microarrays, DNA-methylation arrays, and next-generation sequencing.[8-11]

DNA methylation and histone modifications are the two major epigenetic mechanisms regulating gene expression. Hypermethylation of CpG islands in the promoter region of tumor suppressor genes, that results in transcriptional silencing, plays an important role in lymphoid-lineage leukemogenesis and may be an important contributor toward relapse.[4, 5] Moreover, DNA methylation profiling is useful for the subtype classification of newly diagnosed ALL patients and prediction of outcome and relapse risk.[12]

Here we present our data demonstrating that the methylation of *protocadherin (PCDH)17*, a member of non-clustered protocadherins, is related to poor prognosis, and thus proposing *PCDH17* methylation as a novel biomarker to predict relapse in B-cell precursor (BCP) ALL.

MATERIALS AND METHODS

Clinical samples and leukemic cell lines

This study was approved by the institutional review board of the Shinshu University School of Medicine. Bone marrow (BM) cells were aspirated from 40 children with BCP ALL diagnosed between 1995 and 2008 at Shinshu University Hospital after obtaining informed consent from each of them and/or their parents. The patients were treated according to TCCSG ALL protocol

(L95-14) between 1995 and 2001, and according to TCCSG ALL protocol (L99-15 and L04-16) between 2002 and 2008. BM mononuclear cells (MNCs) were separated and stored in liquid nitrogen until the time of experiments. In this study, only the samples with 80% or more leukemic blasts were analyzed. Immunophenotypic diagnosis of BCP ALL was based on either $CD10^{+}$, $CD19^{+}$, and cytoplasmic μ^{-} or $CD10^{+/-}$, $CD19^{+}$, and cytoplasmic μ^{+}. The following leukemic cell lines were used: Kasumi 2, Kasumi 7, and Kasumi 8 (purchased from the Japanese Collection of Research Bioresources Cell Bank, Ibaraki, Japan); MV4-11, REH, and RCH-ACV (purchased from the German Culture Collection, DSMZ, Braunschweig, Germany). Peripheral blood (PB) MNCs obtained from healthy adult volunteers and BM MNCs obtained from ALL patients in complete remission (CR) were used as controls.

Infinium Methylation 450K Array

To examine the methylation status of BM samples at diagnosis, CR, and relapse in a relapsed patient with BCP ALL, a global DNA methylation analysis was performed by Takara Bio Inc. (Kusatsu, Japan) using Infinium Methylation 450K Array (Illumina Inc., Tokyo, Japan). The quantitative scores of DNA methylation levels were obtained as beta values determined by Illumina analysis, ranging from 0 for completely unmethylated to 1 for completely methylated. Data were then analyzed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) bioinformatics software (https://david.ncifcrf.gov) to identify the candidate genes with CpG sites in which their beta values obtained at relapse were higher than those obtained at diagnosis and CR.

Combined bisulfite restriction analysis (COBRA) and bisulfite sequencing

Genomic DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen, Chuo-ku, Tokyo, Japan), followed by sodium bisulfite treatment using the EZ DNA Methylation Kit (Zymo Research, Irvine, CA, USA) as reported previously.[13, 14] PCR reaction was performed as described previously.[15]

For combined bisulfite restriction analysis (COBRA), PCR products were digested with *BstU*I enzyme (New England BioLabs Japan Inc., Tokyo, Japan), electrophoresed on agarose gels stained with ethidium bromide. The minimal amount of methylated DNA detectable in an unmethylated DNA pool was 20%.

For bisulfite sequencing of the *PCDH17* CpG island, 10μl of each PCR product was electrophoresed onto a 3% agarose gel. The bands were then excised and purified using the Geneclean 2 Kit (Bio 101 Inc., La Jolla, CA, USA). Purified PCR products were cloned into the pGEM-T Easy vector (Promega Corp., Madison, WI, USA). The plasmids were extracted using the Wizard Plus Minipreps DNA Purification System (Promega). Individual clones were sequenced on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) using a DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Biosciences, Piscataway, NJ, USA).

Reverse transcriptase PCR (RT-PCR) and quantitative real-time PCR

Total RNA was isolated using the RNeasy Mini Kit (Qiagen), and cDNA was prepared using PrimeScript II first strand cDNA Synthesis Kit (Takara Bio Inc.). The cDNA was used as a template for RT-PCR, and quantitative real-time PCR with primers and probes listed in Supplemental Table S2. RT-PCR was performed as reported previously.[15] Real-time PCR was carried out in an ABI sequence detection system using the Master Mix (Applied Biosystems). Each assay was performed in triplicates for each sample.

Multiplex ligation-dependent probe amplification (MLPA)

IKZF1 deletion was identified by MLPA. The MLPA assays, using SALSA MLPA P202 *IKZF1* kit (MRC-Holland, Amsterdam, The Netherlands), were performed according to the manufacturer's protocol. The data were analyzed by Coffalyser.Net software (free download at https://www.mlpa.com, MRC-Holland).

Statistical analyses

The chi-square or Fisher's exact tests was used to determine the association of the methylation of *PCDH17* with that of other cadherin superfamily genes, and to compare their methylation

status at diagnosis with that at relapse. Spearman's rank correlation test was performed to determine the relationship between two variables.

Actuarial estimates of event-free survival (EFS) and overall survival (OS) were calculated using the Kaplan–Meier method. Patients were censored for EFS if they were alive and in remission at last follow-up, and were censored for OS if they were alive at last follow-up. Follow-up data were available through December 31, 2014. Risk factors for relapse or mortality were applied to the univariate and multivariate Cox proportional hazards regression analysis. All *P* values were two-sided and *P* values 0.05 or less were considered statistically significant. All statistical analyses were performed with EZR (Saitama Medical Center, Jichi Medical University; http://www.jichi.ac.jp/saitama-sct/SaitamaHP.files/statmedEN.html;Kanda, 2012).

RESULTS

The methylation status of cadherin superfamily genes in BCP ALL

The genome-wide DNA methylation status of BM samples (at diagnosis, CR, and relapse) of a 2-year-old male diagnosed with BCP ALL was examined, followed by functional analysis of large gene lists using bioinformatics DAVID program which identified cadherin superfamily genes as the top candidate genes with aberrant methylation at relapse (Supplemental Appendix S1). Among these cadherin superfamily genes, *Cadherin (CDH)1*, *PCDH8*, and *PCDH17* were previously demonstrated as tumor suppressor genes and frequently detected as hypermethylated in various cancers such as gastric, esophageal squamous cell, hepatocellular, and breast cancers. [16-19] Therefore, we selected these 3 genes for further analysis. First, we examined the methylation status of *CDH1*, *PCDH8*, and *PCDH17* in 6 ALL cell lines, in 40 newly diagnosed Ph1-negative BCP ALL samples obtained at diagnosis, and in control PB and BM cells using COBRA method. The results are shown in Fig 1. *CDH1* and *PCDH8* methylation was observed in all cell lines, whereas *PCDH17* methylation was detected in 4 of them. *CDH1*, *PCDH8*, and *PCDH17* were methylated in 62.5%, 55%, and 30% of ALL cases, respectively. However,

104 methylation of *PCDH8* and *CDH1* was also detected in 80% and 20% of control BM samples,
105 respectively. These findings were partly due to the methylation of CD71-positive BM cells. In
106 contrast, *PCDH17* was unmethylated in control BM cells. There was a significant association
107 between *CDH1* methylation and *PCDH8* methylation ($P < 0.001$). Furthermore, *PCDH17*
108 methylation was associated with both *CDH1* and *PCDH8* methylation ($P = 0.018$ and $P = 0.013$,
109 respectively).
110 Thereafter, we compared the methylation status of 31 *PCDH17* CpG sites at diagnosis and at
111 relapse in 3 patients using bisulfite sequencing. As presented in Supplemental Figure S2, total
112 methylated *PCDH17* sites obtained from 8 clones increased significantly at relapse, as compared
113 with those obtained from clones at diagnosis in 2 of 3 patients ($P < 0.0001$).
114 We examined the relationship between the methylation status and the mRNA expression of
115 *PCDH17* in various cell types. *PCDH17* methylation was almost negligible in control PB cells,
116 whereas a total number of methylated sites varied among ALL samples and ALL cell lines, as
117 determined by bisulfite sequencing (Supplemental Figure S3). Control PB cells expressed
118 *PCDH17* mRNA due to the present of PB CD33+ cells, whereas the mRNA was variable in ALL
119 samples and cell lines (Supplemental Figure S4). There was no correlation between *PCDH17*
120 methylation and mRNA expression levels (Supplemental Figure S5).
121 ***The relationship between methylation status of cadherin superfamily genes at diagnosis and***
122 ***clinical characteristics of patients with BCP ALL***
123 We then determined whether the methylation status of *CDH1*, *PCDH8*, and *PCDH17* at
124 diagnosis examined by COBRA was associated with the clinical characteristics of BCP ALL. As
125 shown in Table 1, *PCDH17* methylation was significantly correlated with high white blood cell
126 (WBC) counts ($P = 0.047$), however, no correlation was found with age, gender, genetic
127 abnormalities including *IKZF1* deletion, t(4;11) and t(12;21), or steroid response. There were
128 substantial relationships between *CDH1* methylation and male sex, as well as between *PCDH8*
129 methylation and both male sex and high WBC. The EFS and OS of 14 patients treated between

1995 and 2001 were similar to those of 26 patients treated between 2002 and 2008 [10-year EFS, 64.3% (95% CI: 34.3–83.3) vs. 61.5% (95% CI: 40.3–77.1)]; 10-year OS, 70.7% (95% CI: 39.4–87.9) vs. 72.5% (95% CI: 59.5–91.4), although ALL protocols were different between the two groups. Thus, we analyzed the impact of the methylation of cadherin superfamily genes on the outcomes of all 40 patients. EFS of *PCDH17* methylation-positive group was profoundly inferior to that of *PCDH17* methylation-negative group: 33% (95% CI, 10–59) vs. 75% (95% CI, 55–87); $P = 0.005$ (Fig 2). A significant difference in OS was also found between the two groups: 50% (95% CI, 21–73) vs. 82% (95% CI, 62–92); $P = 0.016$. Conversely, there were no substantial correlations between the methylation status of *CDH1* and EFS or OS, and between the methylation status of *PCDH8* and EFS or OS.

As shown in Table 2, univariate analysis revealed that *PCDH17* methylation was associated with increased risk for relapse and mortality in patients with BCP ALL [hazards ratio (HR), 3.85; $P = 0.001$ and HR, 5.05; $P = 0.017$, respectively]. According to multivariate analysis, only *PCDH17* methylation was related to poor outcome [HR, 5.23; $P = 0.016$ for relapse and HR, 8.22; $P = 0.016$ for mortality]. However, *CDH1* and *PCDH8* methylation did not influence ALL prognosis.

DISSCUSSION

DNA hypermethylation plays a significant role in the leukemogenesis and hematologic relapse in ALL.[4, 5] For a comprehensive evaluation of the DNA methylation in a pediatric patient with ALL, we performed a genome-wide DNA methylation analysis. The patient already harbored the relapse clone as a minor population at diagnosis. Therefore, to identify the candidate genes associated with relapse, we selected genes that were methylated at higher levels at relapse than at onset. As a result, cadherin superfamily genes, including *CDH1*, *PCDH8*, and *PCDH17,* were the top gene-family in this patient. Among 40 ALL samples at onset, the methylation frequencies of *CDH1*, *PCDH8*, and *PCDH17* were 62.5%, 55%, and 30%, respectively. The methylation of *CDH1* and *PCDH8* was also detected in 80% and 20% of control BM samples, respectively. On

the contrary, *PCDH17* was unmethylated in all 10 control BM samples. These results suggest that *PCDH17* methylation is a specific phenomenon for ALL blasts at onset.

In our study, methylation of *PCDH8* and *CDH1* in BM cells was detected in CD33+/CD71+ cells. CD71 was expressed on proliferating cells. Myeloid BM precursors from dividing blast cells to myelocytes were variably CD71 positive, whereas non-dividing late precursors were CD71 negative.[20-22] Therefore, the methylation of *PCDH8* and *CDH1* in CD33+/CD71+ cells may be related to the proliferative status of myeloid precursors.

Interestingly, the Kaplan–Meier estimates showed that EFS and OS of the *PCDH17* methylation-positive group were significantly lower than those of the *PCDH17* methylation-negative group. Conversely, there were no significant correlations between the methylation status of either *CDH1* or *PCDH8* and EFS or OS. According to univariate and multivariate analysis, *PCDH17* methylation, but not *CDH1* or *PCDH8* methylation, was associated with an increased risk for relapse or mortality in BCP ALL patients. These results suggest that *PCDH17* methylation at onset is related to poor prognosis and thus it could be considered as a new biomarker to predict relapse in such subset of patient. However, a larger study is necessary to clarify this issue. *PCDH17* is a member of non-clustered protocadherin, a subfamily of cadherin superfamily genes.[23] *PCDH17* is frequently downregulated through the promoter hypermethylation in a number of cancers.[24-26] The restoration of *PCDH17* expression reduces cell proliferation and migration in esophageal squamous cell carcinoma, [17] and induces tumor cell apoptosis and autophagy in gastric and colorectal cancers.[18] However, there was no correlation between the methylation status and the mRNA expression level of *PCDH17* in patients with BCP ALL in this study, implying the involvement of alternative mechanisms in regulation of *PCDH17* gene expression. Accordingly, the association of *PCDH17* methylation with poor prognosis may occur through a mechanism that are independent of methylation-mediated transcriptional silencing.

In our genome-wide DNA methylation analysis of one relapsed case with BCP ALL, the relapse-mediated increases in beta values of *PCDH10*, *CDH11*, and *CDH13* methylation were less than 0.3. Therefore, we excluded these 3 genes from the subsequent candidate gene analyses in this study. However, Narayan et al. identified *PCDH10* hypermethylation in approximately 80% of patients with B-ALL.[27] Hogan et al. found that *CDH1*, *CDH11*, and *CDH13* were hypermethylated and downregulated in relapsed pediatric ALL cases.[5] Considering one single sample with a clonal expansion of a minor clone at relapse, we cannot completely rule out the impact of methylation of cadherin superfamily genes other than *PCDH17* and the other family genes on ALL relapse.

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Author contributions

L.T.N.U performed the research, analyzed the data and wrote the paper. K.S. contributed to data management and statistical analysis and wrote the paper. K.K. revised the manuscript and contributed to paper writing. Y.N., T.K., and A.L.F.Y assisted in doing experiments.

Conflict of interest

The authors declare no potential conflicts of interest

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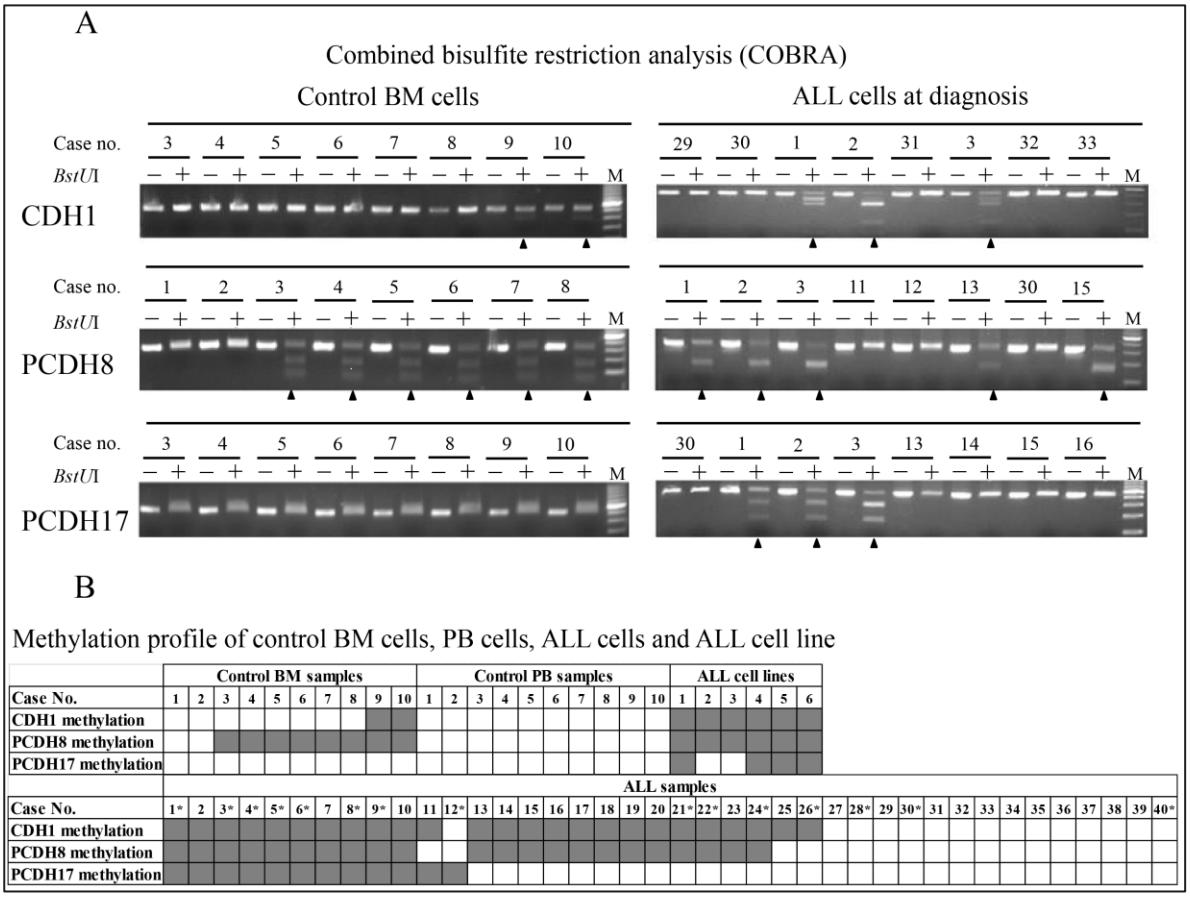
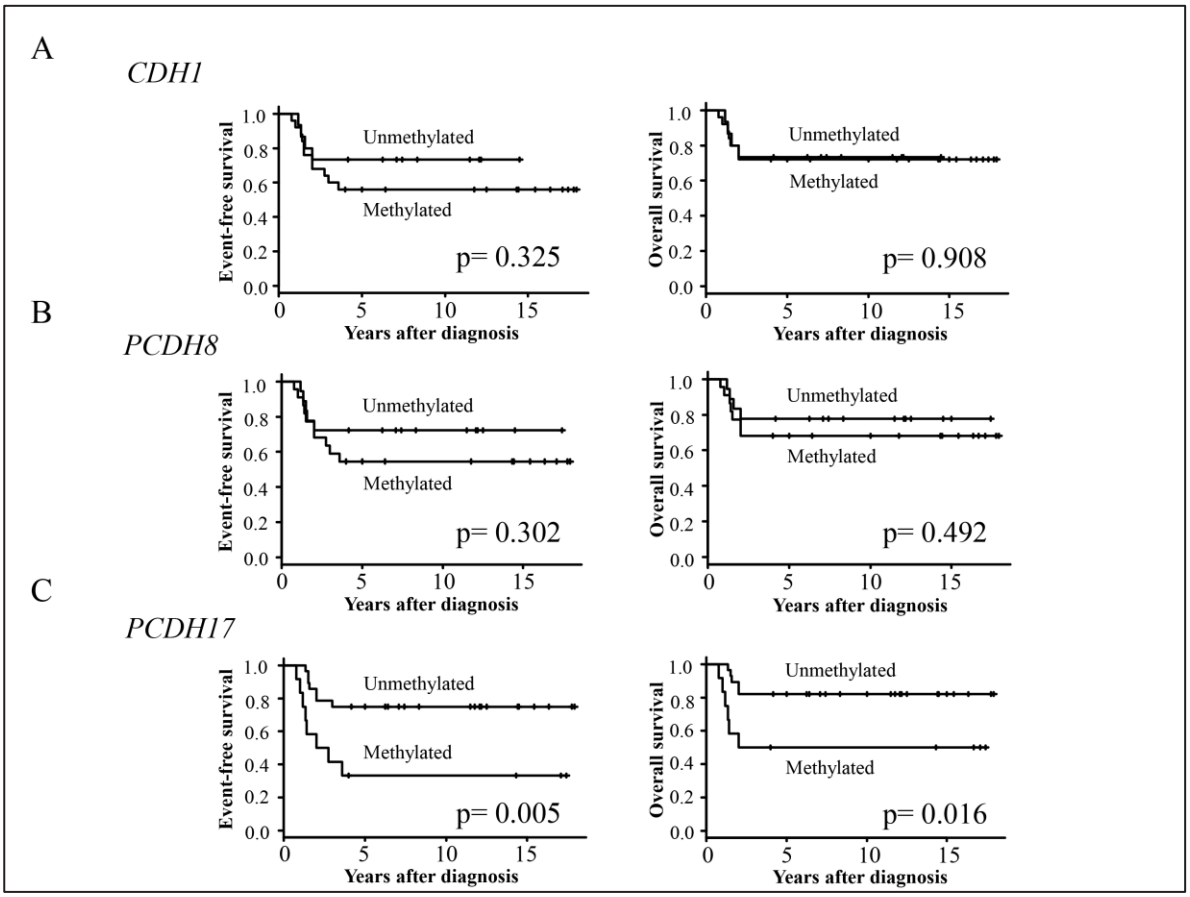
Figure legends

Figure 1. Methylation status of *CDH1*, *PCDH8*, and *PCDH17* by combined bisulfite restriction analysis (COBRA)



(A) Representative results of COBRA analysis: PCR products of promoter *CDH1*, *PCDH8*, and *PCDH17* of control BM cells and ALL samples were digested with *BstU*I enzyme, subjected to 2 % agarose gel stained with ethidium bromide. The black triangle symbols indicate methylated samples with "CGCG" *BstU*I enzyme cut site (digested in smaller fragments). The "minus" symbols represent no *BstU*I treatment, and the "plus" symbols represent *BstU*I treatment. The letter "M" represents DNA size marker. (B) The summary of the methylation profile of control BM, PB samples, ALL cell lines and 40 newly diagnosed ALL samples. Gray squares: cases with methylated genes. *: relapsed case.

Figure 2. Kaplan–Meier survival analysis of ALL children with or without methylation of the three cadherin superfamily genes

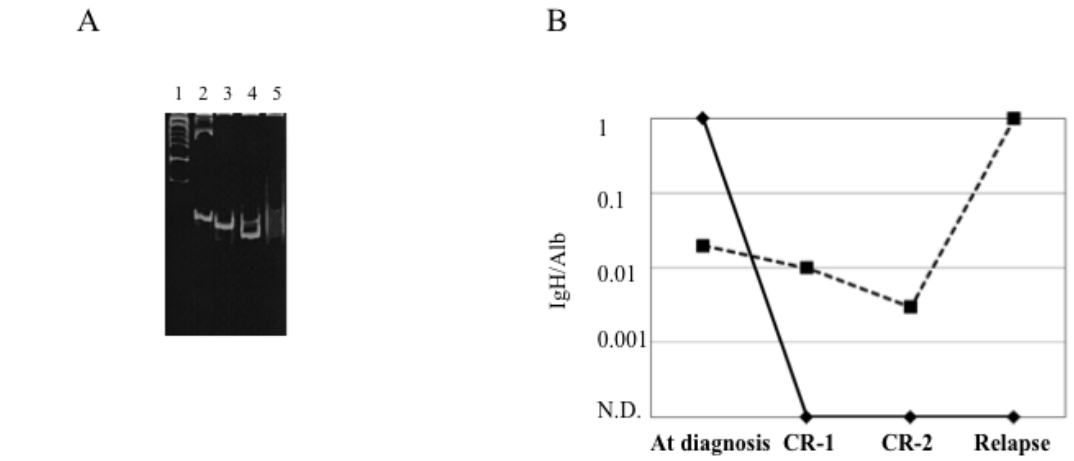


Kaplan–Meier estimates of EFS and OS were compared between methylated and unmethylated *CDH1* (A), *PCDH8* (B), and *PCDH17* (C) determined by COBRA.

Supplemental Figure legends

Supplemental Figure S1. Comparison of *IgH* rearrangements at diagnosis and relapse in a BCP ALL patient

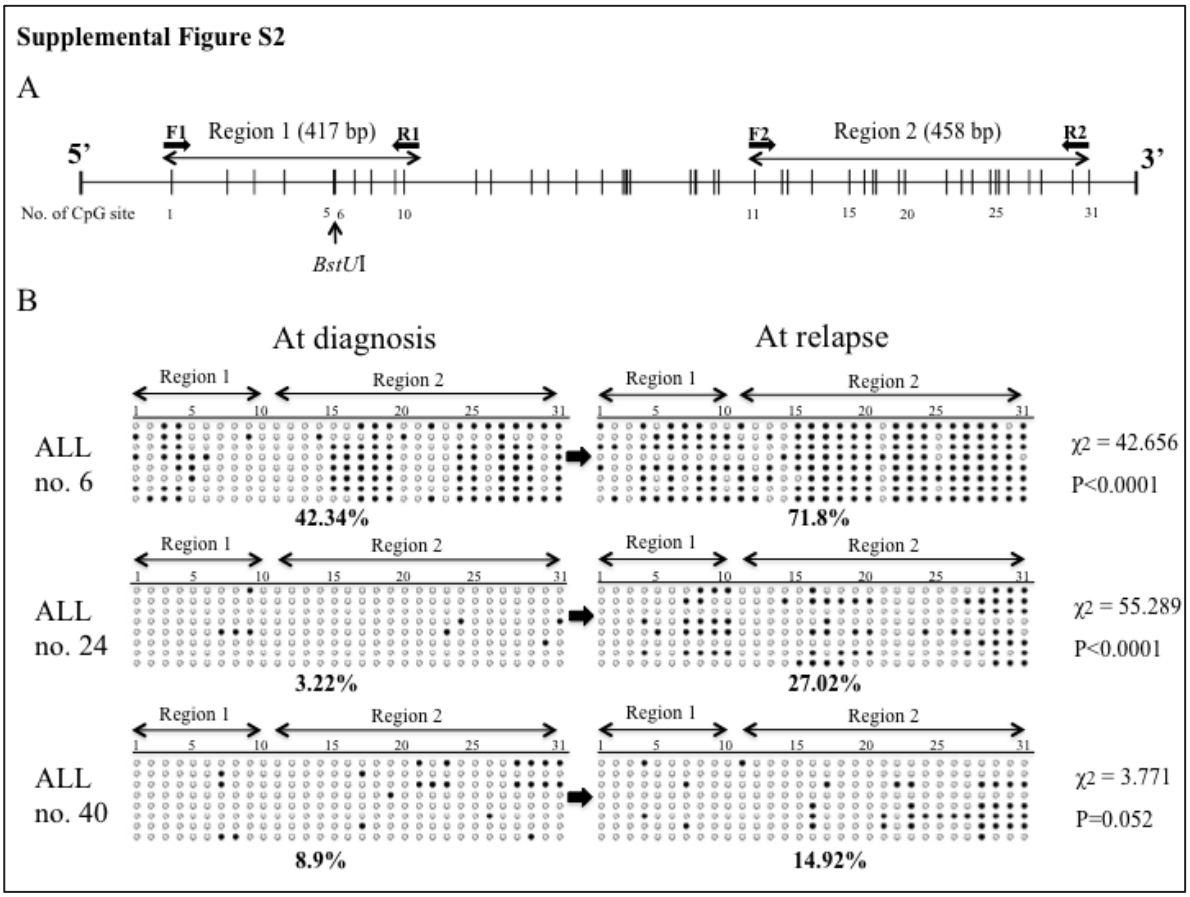
Supplemental Figure S1



Comparison of *IgH* rearrangements at diagnosis and relapse in a BCP ALL patient
(A) Electrophoresis of *IgH* (CDR-III region) gene rearrangements. Lane 1: DNA size marker, lane 2: Raji cells, lane 3: one band indicating the presence of a clonal *IgH* gene rearrangement at diagnosis, lane 4: two bands indicating the presence of two clonal rearrangements at relapse, and lane 5: negative control (normal PB). (B) Real-time PCR-based detection of two leukemic clones with different *IgH* gene rearrangements at diagnosis, CR, and relapse. Albumin (*ALB)* gene was used for normalization, and the values were expressed as relative quantities compared with the value at diagnosis or relapse.

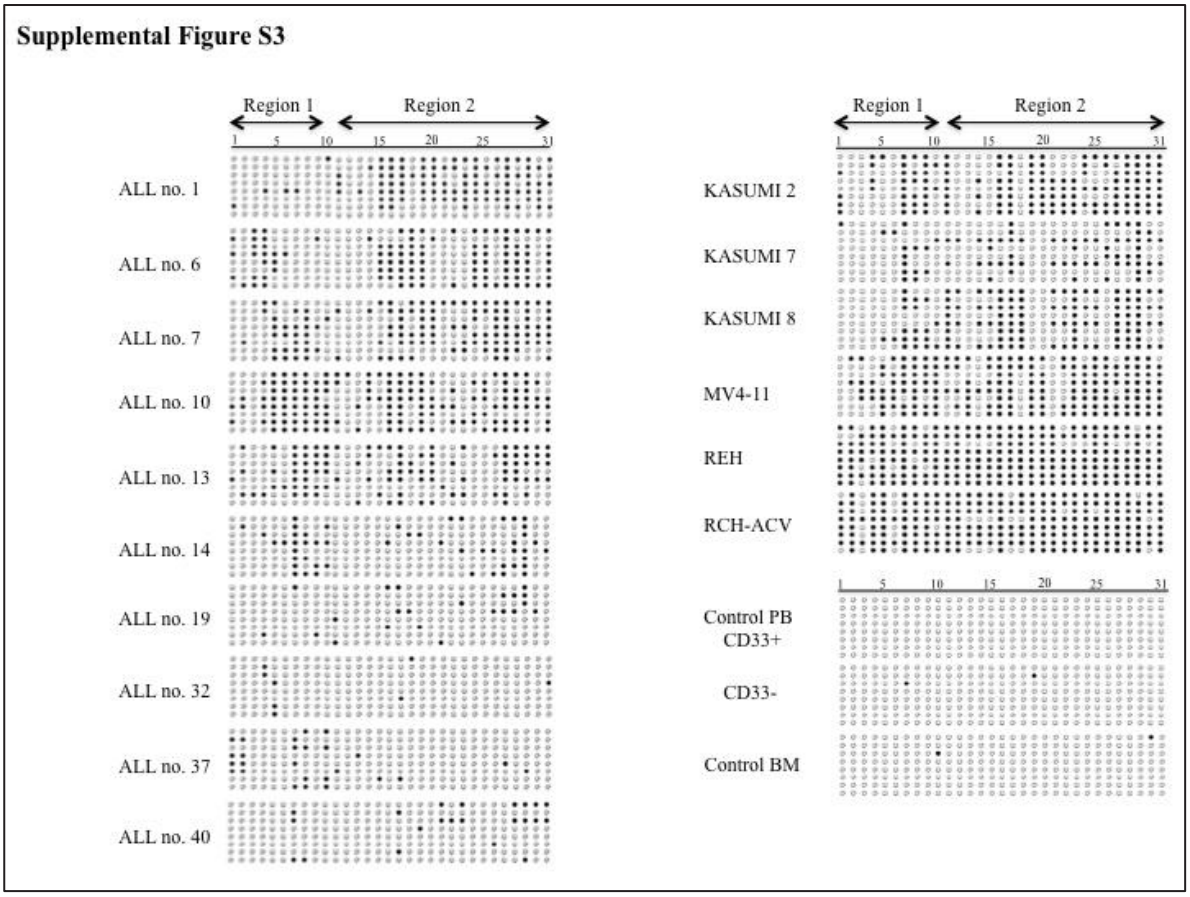
(A) Electrophoresis of *IgH* (CDR-III region) gene rearrangements. (B) Real-time PCR-based detection of two leukemic clones with different *IgH* gene rearrangements at diagnosis, CR, and relapse.

Supplemental Figure S2. Allelic methylation status of the *PCDH17* CpG islands by bisulfite sequencing



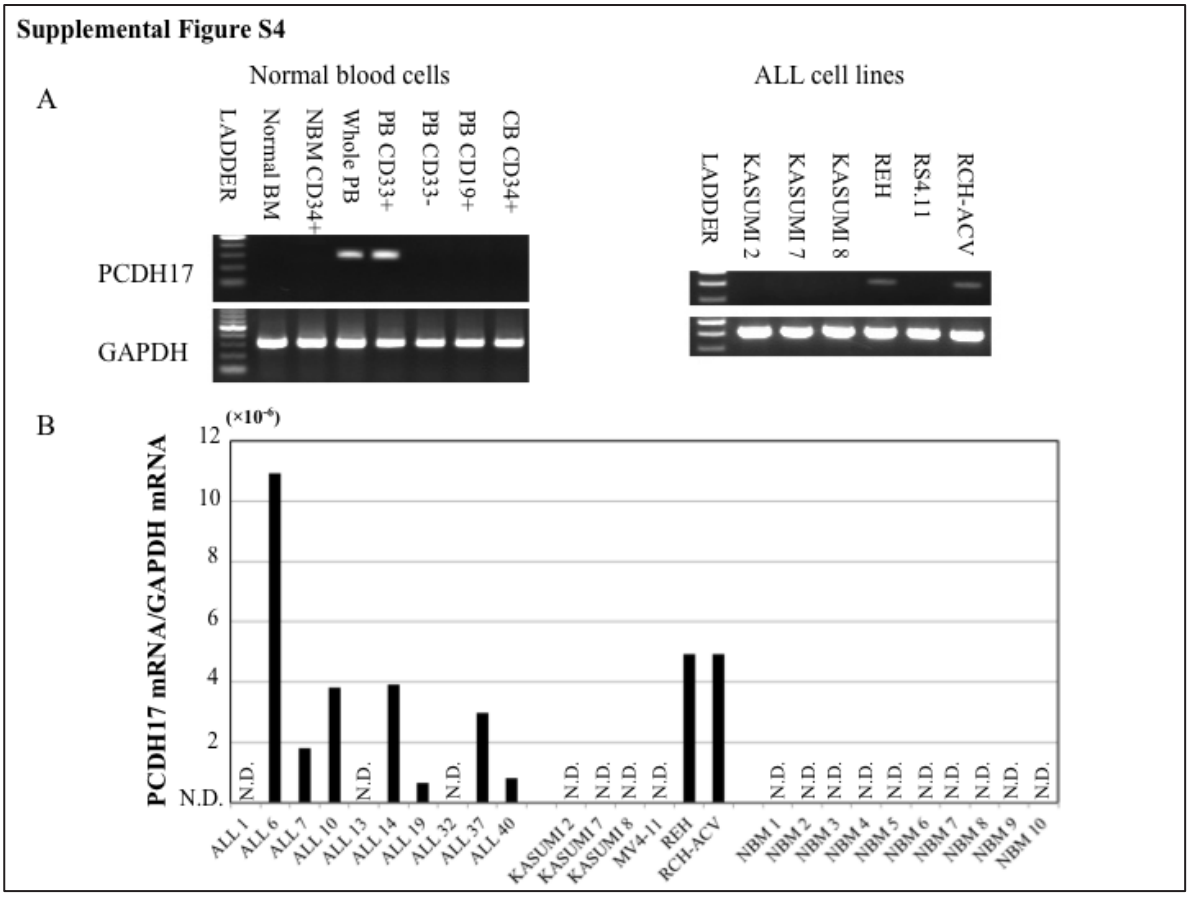
(A) A schematic map of the CpG island region around exon 1 of *PCDH17*. Vertical black bars indicate the location of individual CpG sites. The horizontal arrows indicate the position of two examined fragments (region 1 and region 2). The vertical arrow indicates a restriction site for the *BstU*I enzyme. The binding sites of the primers used in this experiment are also shown (F1 and R1, F2 and R2). (B) The allelic methylation status of the *PCDH17* CpG island at diagnosis and at relapse among 3 ALL cases. Each horizontal row represents an individual cloned and sequenced allele following bisulfite treatment. Methylated CpG sites are marked as closed circles and unmethylated sites as open circles. Numbers above horizontal rows correspond to those of the CpG sites of *PCDH17* shown in (A).

Supplemental Figure S3.



Allelic methylation status of *PCDH17* individual CpG site by bisulfite sequencing in ALL cases, ALL cell lines and normal blood cells.

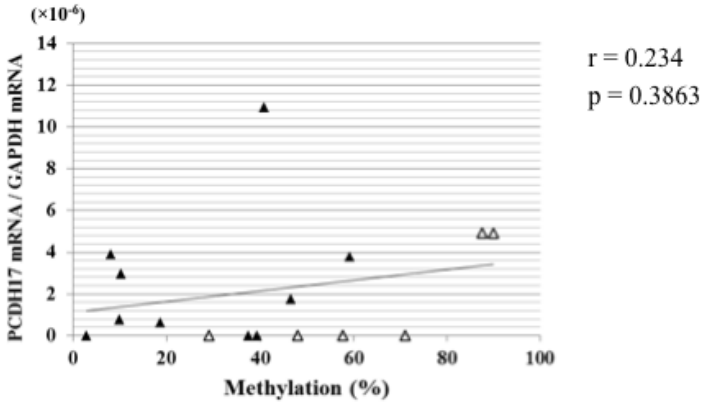
Supplemental Figure S4.



PCDH17 mRNA expression examined by RT-PCR (A) and realtime PCR (B) in normal blood cells, ALL cell lines and ALL samples. The expression levels are displayed as ratios of *PCDH17* and *GAPDH*. N.D., not detectable.

Supplemental Figure S5.

Supplemental Figure S5



No correlation between methylation status and mRNA expression of *PCDH17* among 10 ALL cases and 6 ALL cell lines. Closed triangles, ALL patient samples; open triangles, ALL cell line samples.

Relationship between *PCDH17* methylation and expression levels. Closed triangles: ALL patient samples; open triangles: ALL cell line samples

Tables

TABLE 1. Relationship between methylation status of cadherin superfamily genes at diagnosis and clinical characteristics of patients with BCP ALL

		CDH1 methylation		p	*PCDH8* methylation		p	*PCDH17* methylation		p
		positive	negative		positive	negative		positive	negative	
Methylation status		25	15		22	18		12	28	
Age (year)	10<	21	14	0.369	18	17	0.24	10	25	0.851
	>10	4	1		4	1		2	3	
Gender	male	17	4	0.011	15	6	0.028	7	14	0.628
	female	8	11		7	12		5	14	
WBC ($x10^9$/L)	>50	9	2	0.116	9	2	0.038	6	5	0.047
	<50	16	13		13	16		6	23	
t(4;11)	yes	3	0	0.233	3	0	0.156	2	1	0.209
	no	22	15		19	18		10	27	
t(12;21)	yes	3	1	0.516	2	2	0.617	2	2	0.346
	no	22	14		20	16		10	26	
Steroid response	good	21	14	0.369	18	17	0.24	9	26	0.149
	poor	4	1		4	1		3	2	
IKZF1 mutation	yes	3	0	0.232	3	0	0.156	1	2	0.668
	no	22	15		19	18		11	26	
Relapse	no	14	11	0.273	12	13	0.25	4	21	0.017
	yes	11	4		10	5		8	7	
Outcome	alive	18	11	0.613	15	14	0.377	6	23	0.047
	death	7	4		7	4		6	5	

TABLE 2. Univariate and multivariate analyses of risk factors for outcomes

	Relapse				Overall survival			
	Univariate analysis		Multivariate analysis		Univariate analysis		Multivariate analysis	
	HR (95% CI)	p	HR (95% CI)	p	HR (95% CI)	p	HR (95% CI)	p
WBC (<50 × 10^9 /L / ≥50 × 10^9 /L)	1.09 (0.346-3.43)	0.885	–	> 0.1	1.75 (0.511-5.974)	0.373	–	> 0.1
Age (<10 years / ≥10 years)	1.04 (0.917-1.17)	0.57	–	> 0.1	2.01 (0.434-9.329)	0.372	–	> 0.1
Steroid response (good / poor)	1.36 (0.306-6.016)	0.69	–	> 0.1	1.69 (0.364-7.81)	0.505	–	> 0.1
t(4;11) (negative / positive)	1.04 (0.136-7.902)	0.971	–	> 0.1	1.57 (0.20-12.27)	0.669	–	> 0.1
t(12;21) (negative / positive)	0.51 (0.067-3.908	0.52	–	> 0.1	–*	–	–*	–
IKZF1 mutation (negative /posotive)	2.14 (0.482-9.511)	0.317	–	> 0.1	1.03 (0.132-8.082)	0.975	–	> 0.1
PCDH17 methylation (negative / positive)	3.85 (1.39-10.64)	0.001	5.23 (1.355-20.19)	0.016	5.05 (1.334-19.090)	0.017	8.22 (1.484-45.53)	0.016
PCDH8 methylation (negative / positive)	1.74 (0.593-5.085)	0.313	–	> 0.1	1.53 (0.447-5.213)	0.5	–	> 0.1
CDH1 methylation (negative / positive)	1.75 (0.558-5.507)	0.337	–	> 0.1	1.07 (0.314-3.67)	0.901	–	> 0.1

*: This analysis was excluded because all patients with t(12;21) were alive.