DNA-methylation changes have been observed in cancer [11,12]. One is a global hypomethylation associated with increased chromosomal instability, the reactivation of transposable elements, and loss of imprinting. The other is hypermethylation of CpG islands located in promoter regions of tumor suppressor genes that has conventionally been associated with transcriptional silencing in cancer. These aberrant DNA methylations are thought to be closely related to the development of cancer. Therefore, the identification of specific DNA methylation markers would be helpful for understanding the pathogenetic mechanism as well as for developing new therapeutic strategies. In Wilms' tumor, hypermethylation of *HACE1*, *RASSF1A* and *SIM1* and hypomethylation of *GRIPR* were reported [13–16], whereas the DNA methylation analysis in pediatric renal sarcomas including RTK, CCSK has not been reported yet.

In an attempt to investigate the characteristics of DNA methylation of pediatric sarcomas including CCSK, RTK, and ESFT, we performed DNA methylation analysis using Illumina Infinium HumanMethylation27. In this paper, we demonstrated that each sarcoma had a distinct DNA methylation profile and could be classified by the methylation pattern of a set of specific genes. We further proposed a convenient assay for the differential diagnosis of CCSK from other pediatric renal tumor.

Materials and Methods

Ethics Statement

This study was approved by the ethics committee/IRB at the National Center for Child Health and Development, and written informed consent was obtained from parents for samples from JWiTS. Since written informed consent was not obtained in a subset of samples collected before 2001, the identifying information for them was removed before analysis, in accordance with the Ethical Guideline for Clinical Research enacted by the Japanese Government. The ethics committee/IRB approved the waiver of written informed consent for latter samples.

Table 2. The number of hyper- and hypomethylated genes in comparison with non-neoplastic kidney.

	CpG island probe	
	Hypermethylated	**Hypomethylated**
CCSK	490 probes (437 genes)	46 probes (36 genes)
RTK	130 probes (107 genes)	65 probes (62 genes)
ESFT	66 probes (58 genes)	55 probes (46 genes)
	Non-CpG island probe	
	Hypermethylated	**Hypomethylated**
CCSK	184 probes (166 genes)	117probes (112 genes)
RTK	179 probes (160 genes)	320probes (275 genes)
ESFT	113 probes (101 genes)	136probes (124 genes)

Hypermethylation: difference of average β-value >0.3. Hypomethylation: difference of average β-value<−0.3.
doi:10.1371/journal.pone.0062233.t002

Clinical Materials

Clinical specimens from pediatric patients, including 6 each with RTK and Ewing's sarcoma, 21 with CCSK, 9 with CMN, and 41 with Wilms' tumor, used in this study were selected from the files of specimens collected in our laboratory between 1985 and 2001, and the JWiTS (Japan Wilms Tumor Study). In each case, the pathological diagnosis was confirmed by J.H. and H.O. based on morphological observations and the immunophenotypic characteristics. Three non-neoplastic kidney (NK) tissues were obtained from the non-tumorous part of the above specimens.

Sample Preparation

Each disease tissue was evaluated by preparing frozen section and the neoplastic cells accounted for more than 80% of viable

Table 1. Primers used for MassARRAY.

Primer name	5′ - 3′ sequence
ADRA1D_F	aggaagagagTGGTAGGTAATTTGTTTGTTATTTTTTT
ADRA1D_R	cagtaatacgactcactatagggagaaggctCTTCCAACCCAACAAAAACCCTA
ALDOC_F	aggaagagagTTGAATTTGGGTATTTTGAAGATGT
ALDOC_R	cagtaatacgactcactatagggagaaggctCAAATAAAACTACAACCCTAACTCCC
CREG1_F	aggaagagagGTGAGTAATTTGTAGGTGAGTTGGG
CREG1_R	cagtaatacgactcactatagggagaaggctCCACTACACTCCAACCTAAACCA
MGMT_F	aggaagagagTGAGATTGTTAGAGTGTGTTTTTGG
MGMT_R	cagtaatacgactcactatagggagaaggctTCCACTCAAACCAACTAAATTACCTA
PKN1_F	aggaagagagGGTTTTTTTTGGAGAATTAGAAGGG
PKN1_R	cagtaatacgactcactatagggagaaggctCCAACCACCATACAAAAAAATAAAA
PTEN_F	aggaagagagGGGGTTGTAAATAGATTTGATAGGTT
PTEN_R	cagtaatacgactcactatagggagaaggctAAAAAAAATCCCCAAACTAATACCA
THBS1_F	aggaagagagGGAGAGAGGAGTTTAGATTGGTTTT
THBS1_R	cagtaatacgactcactatagggagaaggctACCTTACCCTAAAAAATCCTCCAAC
VHL_F	aggaagagagTTTTGGGGAGATTGATAGATGTAAA
VHL_R	cagtaatacgactcactatagggagaaggctAACCACTTAACCCCAAATAACAAAT

F, forward; R, reverse. Lower case letters indicate tag sequences.
doi:10.1371/journal.pone.0062233.t001

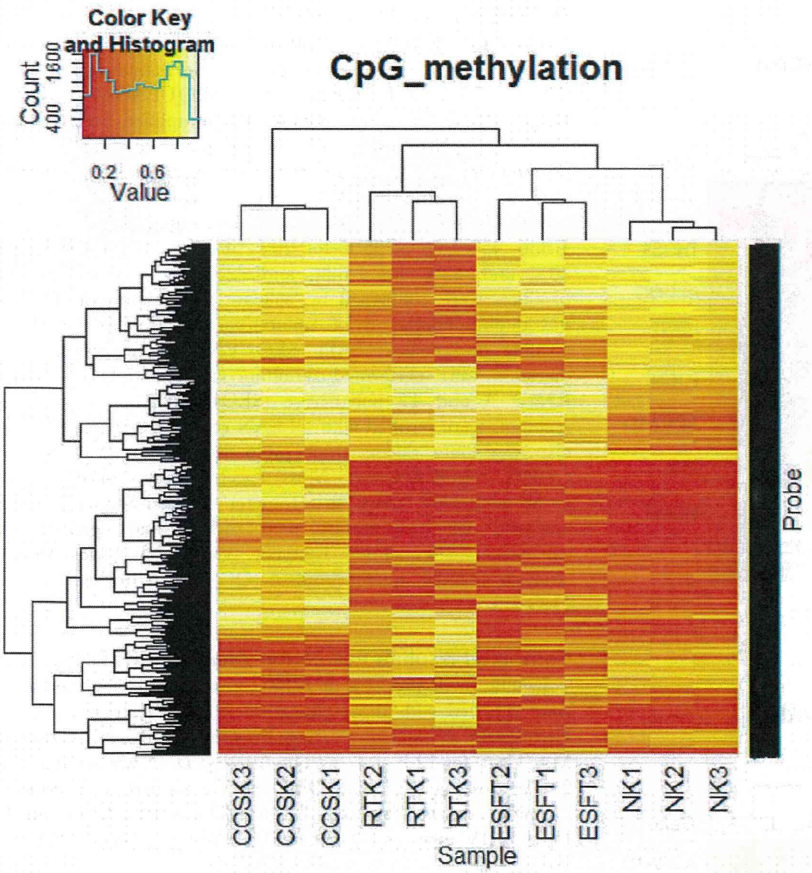

Figure 1. Hierarchical cluster analysis of methylation value (β) in Infinium assay on pediatric tumors. Two-way hierarchical cluster analysis of the methylation level of 1,494 probes (rows, equivalent to hyper- and hypomethylated genes shown in Table 2) and three cases for each of clear cell sarcoma of the kidney (CCSK), rhabdoid tumor of the kidney (RTK), the Ewing's sarcoma family of tumors (ESFT), and non-neoplastic kidney (NK) (columns) were performed using hclust in the R clustering package. The β-values ranged from 0 (unmethylated) to 1 (fully methylated) on a continuous scale.
doi:10.1371/journal.pone.0062233.g001

cells in each sample. The pathologic images of the disease tissues were presented in Figure S1. Genomic DNA was extracted from above evaluated fresh-frozen tissues using the illustra tissue & cells genomicPrep Mini Spin kit (GE Healthcare Bio-Sciences UK Ltd, Chalfont, UK) according to the manufacturer's instructions. The quantity of DNA was assessed by Quant-iT Pico-Green dsDNA Reagent and Kits (Life Technologies Corporation, Carlsbad, CA, USA) and the quality was assessed by agarose gel electrophoresis.

Table 3. The number of specifically methylated genes compared with other each of tumors and non-neoplastic kidney.

	Hypermethylation	Hypomethylation
CCSK	270 genes	28 genes
RTK	65 genes	155 genes
ESFT	7 genes	35 genes

Hypermethylation: difference of average β-value >0.3. Hypomethylation: difference of average β-value<−0.3.
doi:10.1371/journal.pone.0062233.t003

Bisulfite Conversion

Starting from 1 μg of genomic DNA, bisulfite conversion of genomic DNA was performed using the Epitect Bisulfite kit (QIAGEN Inc., Valencia, CA, USA) and EZ DNA Methylation Kit (Zymo Research, Irvine, CA, USA) for the Illumina Infinium Methylation Assay and SEQUENOM MassARRAY, respectively, according to the manufacturer's protocol.

Infinium Methylation Assay

Illumina Infinium HumanMethylation27 (Illumina, Inc., San Diego, CA, USA), containing the 27,578 CpG sites, spanning 14,495 genes, was used for methylation analysis. The bisulfite converted DNA was processed on the chip according to the Illumina protocol. The BeadChips were scanned using iScan system. Our data of Infinium assay have been deposited on Gene Expression Omnibus (GEO) database at the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/geo/, series accession number GSE44847). The β-value and the detection p-value for each locus were calculated using GenomeStudio v2010.1. β-value, a ratio of methylated probe signal intensity to sum of methylated and unmethylated probe signal intensities, was used to estimate the methylation

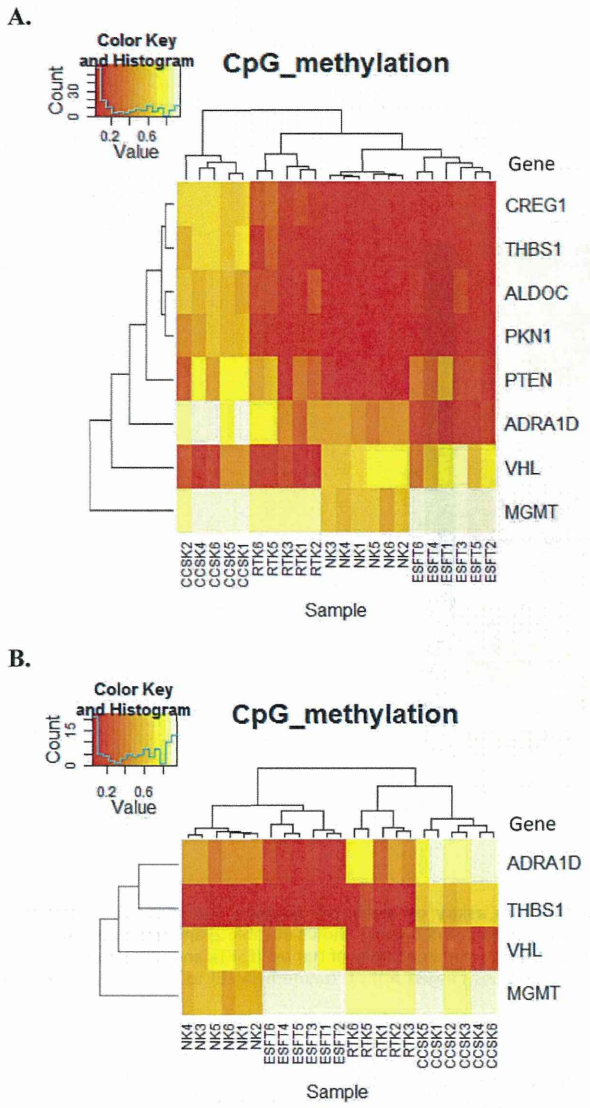

Figure 2. Hierarchical cluster analysis of methylation level of CpG analyzed by MassARRAY. (A) Based on the results indicated in Figure 1, 8 genes were selected as described in the text and analyzed by MassARRAY. Two-way hierarchical cluster analysis was performed using the CpG methylation average of all CpG that have passed QC from each gene. Two cases (RTK4 and CCSK3) showed failed analysis of some CpG sites, and were excluded from hierarchical analysis. (B) Four genes were further selected, and cluster analysis using the methylation average was performed as in (A). CCSK3 was successfully analyzed in all four genes, and included in this analysis.
doi:10.1371/journal.pone.0062233.g002

level of the target locus. Detection p-value is computed from the background model characterizing the chance that the target sequence signal was distinguishable from negative controls. The obtained data were filtered by exclusion of the probes with a detection p-value>0.05 from all probes and SD>0.2 within each entity. The numbers of the detected CpG sites for each sample and filtering process were shown in Table S1. As a result, 23,700 probes (13,385 genes) remained.

EPITYPER Assay (MassARRAY)

The SEQUENOM EpiTYPER assay was performed according to the protocol recommended by the manufacturer. Using the Complete PCR Reagent Set (SEQUENOM Inc., San Diego, CA, USA), target regions were amplified from bisulfite-converted DNAs using the primer pairs containing a T7-promoter tag to allow further *in vitro* transcription. The primers used in this study (Table 1) were designed by EpiDesigner (SEQUENOM). The cycle conditions used were: 95°c for 4 min, 45 cycles of 95°C for 20 s, 56°C (65°C for *CREG1*) for 30 s. and, 95°C for 1 min, and 72°C for 3 min. The PCR products were confirmed by agarose gel electrophoresis. After the dephosphorylation of unincorporated dNTPs by shrimp alkaline phosphatase (SAP) (SEQUENOM), transcription and digestion were performed simultaneously at 37°C for 3 h by RNase A and T7 polymerase (SEQUENOM). The cleavage reactants were purified with CLEAN resin (SEQUENOME) and dispensed onto silicon chips preloaded with matrix (Spectro-CHIPS, SEQUENOM). Mass spectra were collected using a MassARRAY mass spectrometer (Bruker-Sequenom) and analyzed using proprietary peak picking and signal-to-noise calculations (Sequenom Epityper v1.0.5). In MassARRAY analysis, initially, quality control (QC) was performed in each CpG site.

Combined Bisulfite Restriction Analysis (COBRA)

Bisulfite PCR products of *THBS1* produced as described above were digested with the methylation-sensitive restriction enzyme HpyCH4IV (5′-ACGT-3′) (New England Biolabs, NEB, Ipswich, MA, USA) for 12 h at 37°C The digested DNA was separated on 2% agarose gels in 0.5× TBE buffer, stained with ethidium bromide, and visualized on a UV transilluminator. As a control of HpyCH4IV digestion, 0, 50, and 100% methylated DNA were used.

Statistical Analysis

Two-way hierarchical cluster analyses of Infinium assay and MassARRAY were performed using hclust in the R clustering package with Euclidean metric and complete linkage for statistical computing.

Results

DNA methylation profiling of 3 each of CCSK, RTK, ESFT and NK, 12 samples in total, were performed using Infinium HumanMethylation27. First, we analyzed the general methylation status of each tumor group by defining hyper- and hypomethylated CpG sites in each tumor group as those with average β-value differences of >0.3 and <−0.3 compared to NK, respectively. The numbers of selected hyper- and hypomethylated CpG probes in each tumor are listed in Table 2. Among them, the number of selected hypermethylated CpG probes mapped on the CpG island in CCSK, RTK, and ESFT were 490, 130, and 66, respectively, while those of hypomethylated non-CpG probes were 117, 320, and 136, respectively (Table 2).

To test whether the tumors can be distinguished by the methylation level, we performed two-way hierarchical cluster analysis of methylation patterns using hyper- and hypomethylated sites (1,494 probes; equivalent to 1,281 genes selected in Table 2). As shown in Figure 1, each case in the same tumor was clustered in the same group, indicating the tumor-type-dependent methylation pattern of the selected probes.

To further select marker genes for methylation-based tumor-type classification, we defined tumor-specific differentially meth-

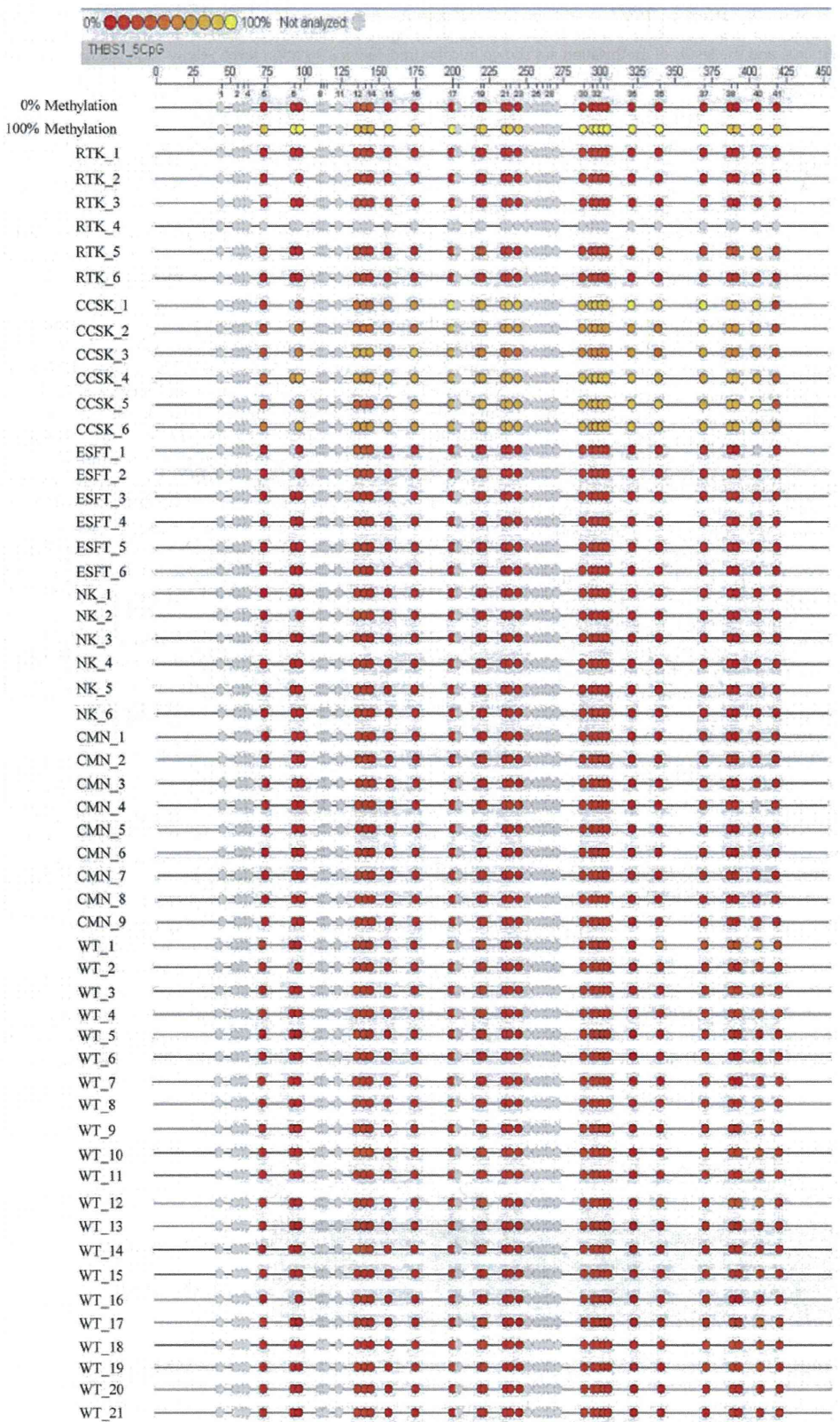
0%
100%
Not analyzed
THBS1_5CpG
0
25
50
75
100
125
150
175
200
225
250
275
300
325
350
375
400
425
450
0% Methylation
100% Methylation
RTK_1
RTK_2
RTK_3
RTK_4
RTK_5
RTK_6
CCSK_1
CCSK_2
CCSK_3
CCSK_4
CCSK_5
CCSK_6
ESFT_1
ESFT_2
ESFT_3
ESFT_4
ESFT_5
ESFT_6
NK_1
NK_2
NK_3
NK_4
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Figure 3. MassARRAY analysis of methylation in *THBS1*. In addition to the 6 cases for each tumor group, 9 and 21 cases of CMN and Wilms' tumor (WT), respectively, were analyzed for DNA methylation of the *THBS1* CpG site, as in Figure 2A. Different colored of circles mark the position of CpG within the sequence (straight line) and the levels of methylation are shown in color (red, low methylation level; yellow, high methylation level). Gray circles represent the unanalyzed CpG sites. WT: Wilms' tumor.
doi:10.1371/journal.pone.0062233.g003

ylated genes as those with average β-value differences of >0.3 (hypermethylated) or <−0.3 (hypomethylated) compared to each of other tumor groups and NK. As shown in Table 3, 270 and 28 genes were identified as CCSK-specific hyper- and hypomethylated genes, respectively, while 65 and 155 genes were selected as RTK-specific hyper- and hypomethylated genes, respectively.

Employing MassARRAY, next we analyzed the surrounding CpG of some specific probes filtered in Table 2, in detail to validate the results of an Infinium assay and further search for candidate genes for a differential diagnostic marker of renal sarcomas. Genes *ALDOC*, *CREG1*, *PKN1*, and *Thrombospondin-1* (*THBS1*) were selected because of their tendency to be hypermethylated in CCSK by the Infinium assay, while *ADRA1D* was selected because of distinct methylation levels among tumors. *MGMT* and *PTEN* were selected because of hypermethylation in 3 tumors, while *VHL* was selected because of hypomethylation in CCSK and RTK. These are known as tumor suppressor genes and these methylation changes are reportedly involved in specific tumors. [17,18].

We analyzed 6 each of RTK, CCSK, ESFT, and NK, the results of MassARRAY were well correlated with those of the Infinium assay, and each type of tumor revealed a specific DNA methylation pattern (Figure S2). In fact, when we performed two-way hierarchic cluster analysis using the CpG methylation average derived from the results, the cases for each tumor type were successfully classified into the same group (Figure 2A). As shown in Figure 2A, *CREG1*, *ALDOC*, *THBS1*, and *PKN1* were hypermethylated in CCSK, whereas RTK, ESFT, and NK were hypomethylated at specific CpG loci, as analyzed by the Infinium assay. *PTEN* was hypomethylated in NK, while variably methylated in sarcoma cases. *ADRA1D* was also hypermethylated in CCSK but hypomethylated in ESFT in comparison with NK, whereas variably methylated in RTK. Although *VHL* was hypomethylated in CCSK and RTK, it was hypermethylated in NK (Figure S3 and Figure S4). *MGMT* was hypermethylated in tumor groups compared to NK.

To distinguish these tumors by the DNA methylation pattern more simply, we further selected four genes characteristically methylated among different tumor groups: *ADRA1D*, *MGMT*, *VHL*, and *THBS1*, and performed cluster analysis using the methylation average of 4 genes. As shown in Figure 2B, CCSK, RTK, and ESFT were successfully classified according to the DNA methylation pattern of these genes, suggesting that the DNA methylation analysis of these 4 genes is sufficient for the differential diagnosis of these tumors.

Since *THBS1* was found to be characteristically hypermethylated in CCSK (Figure 2B), we next examined whether the hypermethylation of *THBS1* alone can distinguish CCSK from other tumor groups. To confirm the specificity of *THBS1* hypermethylation in CCSK among pediatric renal tumors, we additionally analyzed Wilms' tumor and CMN. As shown in Figure 3, when we analyzed 6 each of RTK, CCSK, ESFT, and NK as well as 21 cases of Wilms' tumor and 9 cases of CMN, the CpG sites of *THBS1* were unmethylated in all of the cases, indicating that the CpG sites of *THBS1* are specifically hypermethylated in CCSK among pediatric renal tumors.

To detect the methylation of *THBS1* more easily, we have developed combined bisulfite restriction analysis (COBRA) and analyzed 21 cases of CCSK and 41 cases of Wilms' tumor, 6 cases of RTK, 9 cases of CMN, and 6 cases of NK. As shown in Figure 4 and Table 4, the digestion of bisulfite PCR products with HpyCH4IV clearly indicated that a CpG site of *THBS1* in all CCSK cases was hypermethylated. However, none of other tumor groups exhibited hypermethylation of the CpG site of *THBS1*. The results strongly indicate that hypermethylation of *THBS1* is a specific characteristic of CCSK among pediatric renal tumors, and could be utilized as diagnostic maker of this tumor.

Discussion

In this study, we carried out a DNA methylation analysis to identify genes differentially methylated in a series of pediatric tumors and we clearly showed that different pediatric sarcomas occurring in the kidney possess a distinct DNA methylation profile. Especially, CCSK is more frequently hypermethylated, but less hypomethylated, at the CpG island, compared with other tumors. Since pediatric renal sarcomas have overlapping morphologic and clinical features, the significant differences in epigenetic characteristics between these tumors are of particular interest and may represent the distinction of their cell origin or developmental mechanism. We also showed that these tumors can definitely be classified based on the DNA methylation profile, indicating the usefulness of epigenetic profiling for the differential diagnosis of

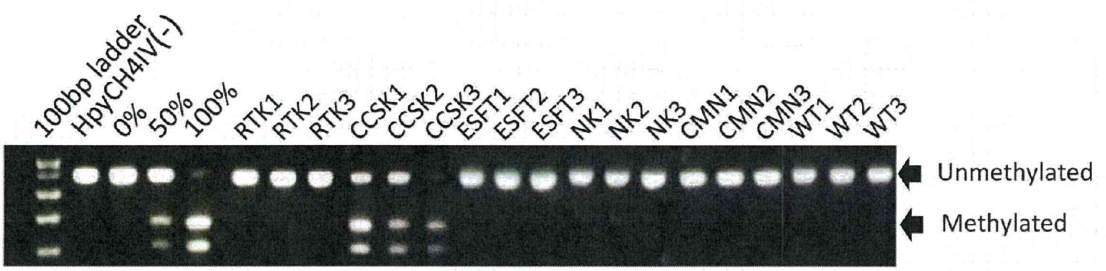

Figure 4. Combined bisulfite restriction analysis (COBRA) of *THBS1* in pediatric renal tumors. Using the same PCR products as in Figure 3, COBRA analysis was performed by digesting with the HpyCH4IV enzyme. The HpyCH4IV site is equivalent to CpG18 (chr15:37,660,642-37,660,645/hg18) in Figure 3. The positions of bands representing methylated and unmethylated DNA are indicated by arrows. As the control of HpyCH4IV digestion, 0, 50, and 100% methylated DNA were loaded on the same gel. WT: Wilms' tumor.
doi:10.1371/journal.pone.0062233.g004

Table 4. Frequency of *THBS1* methylation by COBRA.

	Hypermethylated cases
RTK	0/6
CCSK	21/21
ESFT	0/6
CMN	0/6
Wilms	0/41
NK	0/6

doi:10.1371/journal.pone.0062233.t004

pediatric renal sarcomas, and found that a combination of four genes is sufficient. Furthermore, the DNA methylation status of the *THBS1* CpG site detected by COBRA alone can distinguish CCSK cases from other pediatric renal tumors, including Wilms' tumor and CMN.

The pathological diagnoses of pediatric renal tumors are often supported by immunohistochemical and molecular genetic findings. For example, biallelic inactivation of *SMARCB1* as evidenced by negative immunohistochemical staining has high sensitivity and specificity for the diagnosis of RTK, and *ETV6-NTRK3* fusion is a marker for CMN of the cellular type. In the case of CCSK, however, it has neither immunohistochemical nor molecular genetic markers, while *YWHAE-FAM22* fusion was reported in only a minority [19]. Since *THBS1* hypermethylation is highly specific for CCSK, as we presented in this study, this finding should be useful for a molecular marker of this tumor. Especially, COBRA of the *THBS1* CpG site is highly accurate, reproducible, and can be performed without particular equipment, and, thus, it could be a candidate for routine examination for the differential diagnosis of CCSK from other pediatric renal tumors.

DNA methylation has been proposed as a diagnostic marker for certain cancers. For example, Goto reported that malignant mesothelioma could be differentiated from lung adenocarcinoma by methylation profiles [20], and Mahoney reported that embryonal and alveolar subtypes of rhabdomyosarcoma have a distinct DNA methylation profile [21]. In their cases, however, the methylation status of at least several genes was required for the differentiation of only two entities. In contrast, our findings showed that hypermethylation of a single locus of *THBS1* is sufficient for the differentiation of CCSK from other pediatric tumors and it could serve as a robust diagnostic marker for this tumor.

Hypermethylation of the *THBS1* CpG site has been observed in some tumors. For example, Guo et al. reported that the rate of methylation of *THBS1* was significantly higher in gastric cardia adenocarcinoma than that in the corresponding normal tissues and accompanied by reduction of its mRNA and protein expressions [22], and Guerrero et al. also reported that the hypermethylation of *THBS1* is associated with a poor prognosis in penile squamous cell carcinoma [23]. In both cases, the hypermethylation of *THBS1* has been suggested to be correlated with the pathogenesis.

THBS1 is a member of the thrombospondin family, and is known for putative antiangiogenic factor [24,25]. By employing a knockout mouse model, several studies have shown that the absence of *THBS1* leads to increased vascularization and THBS1 protein inhibits tumor progression in several ways, including direct effects on cellular growth and apoptosis in the stromal compartment [26–29]. Since CCSK is known to be rich in a fine vascular network, it is reasonable to presume that hypermethylation of the *THBS1* CpG site is involved in the pathogenesis of CCSK. However, we observed no significant correlations between the methylation status and expression of THBS1 by realtime RT-PCR and immunohistochemistry (data not shown). This is possibly due to hypermethylated CpG sites of *THBS1* in CCSK that we identified as not being responsible for *THBS1* expression, and other CpG sites are related to the regulation of *THBS1* expression. In fact, other CpG probes (cg19570574: chr15: 37660116–37660117/hg18, cg04051458: chr15: 37660352–37660353/hg18) in the upstream region of the *THBS1* transcription start site were hypomethylated in CCSK based on our assay. Another possibility is that THBS1 is expressed in a limited period during tumorigenesis. Further studies to elucidate the biological significance of CCSK hypermethylation are now underway.

In conclusion, pediatric renal sarcomas possess a distinct DNA methylation profile in a tumor-type-specific manner. The DNA methylation status of the *THBS1* CpG site detected by COBRA alone is sufficient for the distinction of CCSK from other pediatric renal tumors. Although further analysis to elucidate the biological importance of the differential DNA methylation of each tumor is required, our observation should shed light on the significance of the epigenetic diversity of pediatric renal tumors on their biological features and mechanism of pathogenesis.

Supporting Information

Figure S1 Images of frozen tissue sample by H.E. staining. Frozen tumor tissues were embedded in OCT-compound and sectioned in 6 μm, stained with H.E. The proportion of viable tumor tissue was evaluated under light microscope.
(TIF)

Figure S2 Correlation of the methylation values between Infinium assay and MassARRAY. In cases of *THBS1* and *CREG1*, methylation values of same CpG site measured by MassARRAY (average of 6 samples) and Infinium BeadChip Assays (average of 3 samples) were indicated in the scattergrams and values of coefficient of determination (R^2) were calculated. In case of *VHL*, two probe sites were shown. Since each site of *VHL* could not be discriminated from the neighbouring site by MassARRAY, the methylation value was obtained as an average of two sites. Coefficient of determination between the values obtained by two methods was larger than 0.99 in each case. Methylation levels of the equivalent CpG sites were correlated between Infinium assay and MassARRAY.
(TIF)

Figure S3 MassARRAY analysis of *VHL* in pediatric renal tumors. MassARRAY analysis of the *VHL* was carried out in 6 each of RTK, CCSK, ESFT, NK. Different colored of circles mark the position of CpG within the sequence (straight line) and the levels of methylation are shown in color (red, low methylation level; yellow, high methylation level). Gray circles represent the unanalyzed CpG sites. CpG5 and CpG13 are equivalent to Infinium assay probes.
(TIF)

Figure S4 Combined bisulfite restriction analysis (COBRA) of *VHL* in pediatric renal tumors. Bisulfite PCR amplification of *VHL* was carried out by using same primer for MassArray. COBRA analysis was performed by digesting with the TaqI restriction enzyme. The TaqI site is equivalent to CpG3 in MassARRAY analysis. The site of genome sequence is CCGA, which is converted to TaqI sequence (tCGA) by bisulfite reaction. PCR amplification of RTK4 was failed. The digested DNA was

separated on 2% agarose gels in 1×TAE buffer, stained with ethidium bromide, and visualized on a UV transilluminator. (TIF)

Table S1 Numbers of probes filtered with p-value and SD. (DOCX)

Acknowledgments

We thank Keiko Nakasato for technical assistance.

Author Contributions

Conceived and designed the experiments: HU HO NK. Performed the experiments: HU HO SA KN. Analyzed the data: HU HO. Contributed reagents/materials/analysis tools: HO JF JH MF KN KH. Wrote the paper: HU HO KK NK.

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Assessment of Corticosteroid-induced Osteonecrosis in Children Undergoing Chemotherapy for Acute Lymphoblastic Leukemia: A Report From the Japanese Childhood Cancer and Leukemia Study Group

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Summary: Steroid-induced osteonecrosis (ON) is a challenging complication encountered during modern chemotherapy for childhood acute lymphoblastic leukemia (ALL). We retrospectively assessed the incidence of ON and its risk factors in a total of 1095 patients enrolled in 3 consecutive Japanese Children's Cancer and Leukemia Study Group ALL studies (ALL941 [1994 to 2000], n = 464; ALL2000 [2000 to 2004], n = 305; and ALL2004 [2004 to 2010], n = 326). ON was diagnosed in 16 patients, of whom 15 were symptomatic. The cumulative incidence of ON was 0.76% in ALL941, 0.35% in ALL2000, and 3.6% in ALL2004. The incidence of ON in ALL941/2000, in which only prednisolone was administered as a steroid, was significantly lower than that in ALL2004, in which dexamethasone was used as a partial substitute for prednisolone ($P < 0.01$). In ALL2004, sex and age were significantly correlated with the incidence of ON (1.3% in boys vs. 6.7% in girls, $P = 0.0132$; 0.42% for age < 10 y vs. 15.6% for age ≥ 10 y, $P < 0.0001$), suggesting that girls aged 10 years and above are at a greater risk of ON onset. These results indicate that the risk of ON should be considered when administering dexamethasone as part of ALL protocol treatment in girls aged 10 years and above.

Key Words: acute lymphoblastic leukemia, osteonecrosis, corticosteroid, dexamethasone

(*J Pediatr Hematol Oncol* 2014;36:22–29)

Recent advances in treatment strategies for childhood acute lymphoblastic leukemia (ALL) have improved the overall survival rate by 80% to 90%.[1–3] Enhanced chemotherapeutic agents, refined risk classification criteria, and improved supportive care have contributed to these high cure rates, but significant toxicity remains a major risk factor that causes long-term morbidity and decreased quality of life. Osteonecrosis (ON) has been increasingly documented in pediatric ALL and presents a challenging complication during modern chemotherapy.[4,7] ON can result in joint dysfunction and subsequent impairments in activities of daily living among long-term survivors.[8,9] Well-known risk factors for ON include age above 10 years, female sex, and use of dexamethasone (DEX).[5,7] Although the precise pathophysiology of ON remains unknown, corticosteroid administration has been shown to induce ischemia, upregulate apoptosis of osteoblasts and osteocytes, and prolong osteoclast lifespans.[10]

Most previous studies regarding ON in children with ALL have been limited to European and North American study groups, as there is little data concerning Japanese or Asian patients. Therefore, the aim of the present study was to assess the incidence, risk factors, and morbidity of corticosteroid-induced ON in ALL studies conducted by the Japanese Childhood Cancer and Leukemia Study Group (JCCLSG). We retrospectively analyzed the data of 1095 patients enrolled in 3 consecutive ALL studies (ALL941, ALL2000, and ALL2004) conducted by the JCCLSG. Prednisolone (PSL) was used as the primary corticosteroid in all studies, with DEX acting as a partial substitute for PSL in ALL2004. ON patients were practically identified by symptoms and ON was confirmed with imaging studies in all patients.

MATERIALS AND METHODS

Patients and Treatment

ALL941, ALL2000, and ALL2004 were conducted between 1994 and 2000, 2000 and 2004, and 2004 and 2010, respectively. The therapies on these studies were risk adjusted but not randomized. Patients enrolled in ALL941 and ALL2000 were aged 1 to 15 years, whereas those enrolled in ALL2004 were aged 1 to 18 years. All participants were newly diagnosed with B-precursor ALL or T-cell ALL, and those with a mature B-cell phenotype and Philadelphia chromosome-positive ALL were excluded. All studies were conducted across 18 hospitals that were members of the JCCLSG. The study protocol was approved by the institutional review board of each study,

Received for publication March 27, 2013; accepted August 26, 2013.
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Supplemental Digital Content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's Website, www.jpho-online.com.

and written informed consent was provided by the patients or their legal guardians before treatment.

The treatment protocols adopted in ALL941 and ALL2000 were reported previously.[11,12] The patients were stratified according to leukocyte count and age at the time of diagnosis into standard-risk (SR), high-risk (HR), and high-high-risk (HHR) groups. The ALL941 and ALL2000 study protocols were almost identical except for the addition of doxorubicin administration to patients with a leukocyte count $<10{,}000/\mu L$ and age below 5 years in ALL2000. Treatment schedules and adopted drugs are briefly described in the supplement (see Supplemental Digital Content 1, Table 1,http://links.lww.com/JPHO/A55).

The ALL2004 treatment protocols are described in Figure 1 and Table 1. Previous risk classification criteria were modified according to the National Cancer Institute criteria,[13] resulting in a shift from HR to SR in 6- to 9-year-old patients with leukocyte counts of 5000 to 10,000 cells/μL. After a 7-day PSL regimen, induction therapy in the SR and HR groups was almost identical to that in previous studies.[11,12] In the HHR group, cyclophosphamide was added on day 8. After achieving complete remission, all risk groups received the same intensification therapy (Int-1). At week 15, SR patients with MRD levels $<10^{-3}$ received further intensification therapy (Int-2) that was followed by maintenance therapy (M-1 and M-2) until week 110. In the HR and HHR groups, patients with MRD levels $<10^{-3}$ at week 15 received 2 cycles of reinduction/intensification therapy (Rc1/Int-2 and Rc1/Int-3 in HR, Rc2/Int-4 and Rc2/Int-5 in HHR group) that was followed by the same maintenance therapy (M-3 and M-4) until week 165. Patients with MRD levels $\geq 10^{-3}$ at week 12 in the SR and HR/HHR groups were assigned to salvage arms 1 and 2, respectively. In the salvage regimen, patients received intensification therapy comprising 2 cycles of Rc-2/etoposide + cytarabine + L-asparaginase. For CNS prophylaxis, SR and HR patients received extended TIT injections beginning from day 1. When IT was combined with a high dose of methotrexate, only cytarabine and hydrocortisone were injected (double intrathecal [DIT] injection). TIT injections were repeated every 6 weeks in the first year, every 8 weeks in the second year, and every 12 weeks in the third year. The HHR group patients included in salvage arm 2 received 18 Gy of CRT in addition to 6 and 7 doses of TIT injections until week 22 and 32 of therapy, respectively.

Cumulative doses of the corticosteroids administered in ALL941/2000 and ALL2004 are listed in Table 2.

Identification of ON Patients

Because the significance of this therapy-related toxicity had not been fully appreciated until the early 2000s, case report form in the 3 studies did not request data regarding ON. Thus, cases with ON were collected by the questionnaire specified for ON to the investigators of JCCLSG. Most of the ON patients were identified based on clinical symptoms such as bone pain and further confirmed with diagnostic imaging studies (x-ray/magnetic resonance imaging [MRI]) by the each institutional radiologists, except one who was asymptomatic and diagnosed by imaging studies at the discretion of primary physician.

Statistical Analysis

Differences in the categorical variables of patient characteristics were analyzed using the χ^2 test. The cumulative incidence of ON during the study period was estimated using Kaplan-Meier analysis. The median follow-up periods were 147.4, 100.3, and 43.6 months for patients enrolled in ALL941, ALL2000, and ALL2004, respectively (median follow-up period, 78.7 mo for all patients). Differences in cumulative incidence between patient subsets were tested using the log-rank test.

RESULTS

A total of 1095 patients were enrolled in the present study (ALL941, n = 464; ALL2000, n = 305; and ALL2004, n = 326). Sixteen of 1095 patients developed ON during or after treatment, 4 (0.86%), 2 (0.65%), and 10 (3.1%) were in ALL941, ALL2000, and ALL2004, respectively. In patients with ON, the median age at diagnosis of ALL was 11.5 years (range, 5 to 16 y) and the male to female ratio was 1:3. When patients were evaluated by risk classification, only 1 patient in the SR group and 15 patients in the HR/HHR groups developed ON.

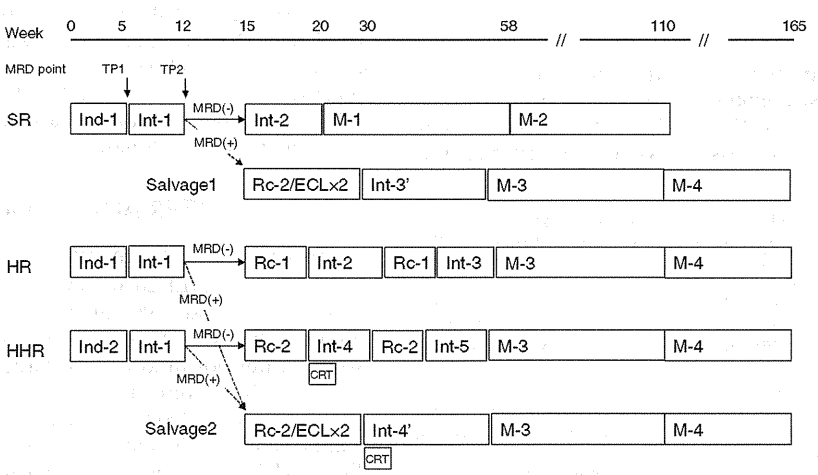

FIGURE 1. Treatment framework and minimal residual disease (MRD) stratification in the ALL2004 study. Patients with MRD levels $\geq 10^{-3}$ at week 12 received salvage therapy (dotted arrows), whereas the remainder continued to receive the initial risk-adapted therapy (solid arrows). Treatment schedules are shown in Table 1. CRT indicates cranial radiotherapy; HR, high-risk group; HHR, high-high-risk group; SR, standard-risk group.

TABLE 1. Drug Dosage and Schedule for ALL2004

	Regimen	Daily Dose	Administration	Days
Induction phase				
Ind-1	VCR	2 mg/m^2	IV	8, 15, 22, 29
	LASP	2000 U/m^2	IM	9-34 (3/wk)
	PSL*	60 mg/m^2	Oral	1-28
	DOX	25 mg/m^2	IV	8, 15, 22
Ind-2	Same as in Ind-1 except for CY (1200 mg/m^2)			8
Intensification phase				
Int-1				
AA	THP	20 mg/m^2	IV	1, 43
	VCR	2 mg/m^2	IV	1, 43
	PSL	120 mg/m^2	Oral	1-5, 43-47
	6MP	250 mg/m^2	Oral	1-5, 43-47
C	CY	400 mg/m^2	IV	15
	CA	50 mg/m^2 × 2	IV	15-18
	6MP	125 mg/m^2	Oral	15-19
BH	MTX	3000 mg/m^2	IV	29
Weekly LASP	LASP	6000 U/m^2	IM	1-50 (1/wk)
Int-2				
C + BH × 3 + A	VCR	2 mg/m^2	IV	43
	PSL	120 mg/m^2	Oral	43-47
	6MP	250 mg/m^2	Oral	43-47
Int-3				
CH + BH × 3	CY	500 mg/m^2	IV	1, 15
	CA	75 mg/m^2 × 2	IV	1-4, 15-18
	6MP	60 mg/m^2	Oral	1-28
AA + C + BH†				63-91
Weekly LASP†				63-98 (1/wk)
Int-4				
CHs	CY	500 mg/m^2	IV	1, 15
	CA	75 mg/m^2 × 2	IV	1-4, 15-18
	6MP	30 mg/m^2	Oral	1-28
AA + C + BI‡	MTX	500 mg/m^2	IV	63
Weekly LASP‡				35-70 (1/wk)
Int-5				
ECL + AA + C + BI	E	100 mg/m^2	IV	1-4
	CA	2000 mg/m^2 × 2	IV	1-4
	LASP	6000 U/m^2	IM	5
Weekly LASP				21-98 (1/wk)
Reinduction phase				
Rc-1	VCR	2 mg/m^2	IV	1, 8, 15, 22
	LASP	2000 U/m^2	IM	2-20 (3/wk)
	DEX*	10 mg/m^2	Oral	1-14
	DNR	25 mg/m^2	IV	1, 8, 15
Rc-2	VCR	2 mg/m^2	IV	1, 8, 15, 22
	LASP	6000 U/m^2	IM	9-20 (3/wk)
	DEX*	10 mg/m^2	Oral	1-14
	DNR	40 mg/m^2	IV	1, 8, 15
	CY	1200 mg/m^2	IV	1
Maintenance phase				
M-1 (C + B + A)	MTX	225 mg/m^2	IV	15
	LASP	2000 U/m^2	IM	15
M-2 (B + As)	VCR	2 mg/m^2	IV	15
	PSL	80 mg/m^2	Oral	15-19
M-3 (AA-C-B)				1-29
M-4 (Bs + As)	MTX	225 mg/m^2	IV	1
CNS prophylaxis				
TIT	MTX	12 mg/m^2	IT	
	CA	30 mg/m^2		
	HDC	50 mg/m^2		
DIT	Same as TIT except for methotrexate			

*PSL and DEX were tapered off (PSL; 30 mg/m^2 for 3 d and 15 mg/m^2 for 4 d, DEX; 5 mg/m^2 for 3 d and 2.5 mg/m^2 for 4 d).
†Repeat 2 cycles in Int-3′ for salvage 1.
‡Repeat 3 cycles in Int-4′ for salvage 2.
6MP indicates 6-mercaptpopurine; CA, cytarabine; CY, cyclophosphamide; DEX, dexamethasone; DNR, daunorubicin; DOX, doxorubicin; E, etoposide; HDC, hydrocortisone; LASP, L-asparaginase; MTX, methotrexate; PSL, prednisolon; THP, pirarubicin; VCR, vincristine.

TABLE 2. Cumulative Dose of Corticosteroid in Trials ALL941/2000 and ALL2004

	ALL941/2000		ALL2004	
	PSL (mg/m²)	DEX (mg/m²)	PSL (mg/m²)	DEX (mg/m²)
Induction	1830	—	1830	—
Reinduction				
SR	—	—	—	—
HR/HHR	1830	—	—	330
Intensification				
SR	600	—	1200	—
HR/HHR	1200	—	2400	—
Maintenance				
SR	18,000	—	9200	—
HR/HHR	15,000	—	12,200	—
Total*				
SR	20,430		12,230	
HR/HHR	19,860		18,575	

*Calculated in PSL equivalents (1 mg of DEX = 6.5 mg of PSL).
DEX indicates dexamethasone; HHR, high-high risk; HR, high risk; PSL, prednisolone; SR, standard risk.

Comparisons of the characteristics of patients with and without ON are presented in Table 3, which shows a predominance of females aged 10 years and above, treatment with ALL2004, and high risk ($P < 0.01$) in patients with ON. Notably, 9 of the 12 female and 3 of the 4 male patients with ON were aged 10 years and above, the latter was marginally significant ($P = 0.044$). ON was diagnosed at median treatment weeks 56.5 (range, 32 to 264) and 66 (range, 37 to 120) in ALL941/2000 and ALL2004, respectively. The median cumulative corticosteroid doses at the time of ON onset were as follows: PSL, 5700 mg/m² (range, 3480 to 13,880 mg/m²) in ALL941/2000 and PSL, 6030 mg/m² (range, 3480 to 13,800 mg/m²) and DEX, 330 mg/m² (range, 240 to 330 mg/m²) in ALL2004. As described in Table 2, SR patients in ALL2004 originally did not receive DEX, and despite the cumulative dose of PSL far exceeded the median doses for patients with ON at onset, none of them eventually developed ON. To obtain total PSL equivalents, DEX was multiplied by a conversion factor of 6.5[14]; therefore, a relatively higher steroid dose

TABLE 3. Comparison of Patient Characteristics Between With and Without ON

	Patients With ON (%)	Patients Without ON (%)	P (χ^2)
All	16	1079	
Sex			< 0.01
Male	4 (25)	606 (56)	
Female	12 (75)	473 (44)	
Age (y)			< 0.01
Male			0.044
1-5	1 (6)	345 (32)	
6-9	0	124 (11)	
>10	3 (19)	137 (13)	
Female			< 0.01
1-5	0	271 (25)	
6-9	3 (19)	102 (10)	
>10	9 (56)	100 (9)	
WBC			
< 10,000	6 (38)	571 (53)	
10,000-100,000	9 (56)	398 (37)	
> 100,000	1 (6)	110 (10)	
Immunophenotype			
BCP	9 (56)	739 (68)	
T	3 (19)	99 (9)	
Others	3 (19)	158 (15)	
NK	1 (6)	90 (8)	
Treatment			0.015
ALL941	4 (25)	460 (43)	
ALL2000	2 (12)	303 (28)	
ALL2004	10 (63)	316 (29)	
Risk			< 0.01
SR	1 (6)	629 (58)	
HR/HHR	15 (94)	450 (42)	

BCP indicates B-cell precursor; HHR, high-high risk; HR, high risk; NK, not known; ON, osteonecrosis; SR, standard risk; WBC, white blood cell count.

was used in ALL2004 compared with that used in ALL941/2000.

The cumulative 5-year incidence of ON was 0.76% (SE, 0.43%), 0.35% (SE, 0.35%), and 3.6% (SE, 1.1%) in ALL941, ALL2000, and ALL2004, respectively (Fig. 2), with a significant difference between ALL2004 and ALL941/2000 ($P < 0.01$). To assess the contribution of sex and age to ON incidence in patients receiving DEX-containing protocols, the cumulative incidence of ON was estimated in ALL2004 (Figs. 3A, B). Both sex and age were significantly associated with the 5-year ON incidence rate ($P < 0.01$), whereas female sex and age 10 years and above were HR factors for ON. The cumulative 5-year incidence of ON for girls over 10 years of age was 25.6% (SE, 8.4%), which was extremely higher than the rest of patients in ALL2004 ($P < 0.0001$) (Fig. 3C).

The characteristics of the 16 patients who eventually developed ON are listed in Table 4. All patients showed typical imaging findings on MRI except 1 (case 941-3) who underwent only x-ray that showed bilateral flattened femoral head. The most commonly affected joints and bones were the hip joint (44%), the knee joint (25%), and the femur (13%). Three patients (19%) exhibited multiple lesions. Nine (56%) continued to receive the planned steroid therapy despite the diagnosis of ON, whereas the doses were decreased or withdrawn in 7 (44%). ON management varied for each patient depending on the physician discretion. Most patients (75%) received supportive care only and were advised to avoid lifting heavy weights (grade 2 according to Common Terminology Criteria for Adverse Event version 4.0). Three patients (19%) underwent surgical intervention (grade 3) and 1 was treated with oral bisphosphonates (grade 2). With the median follow-up times of 33 months (range, 4 to 194), the clinical outcomes of ON were as follows: 12 with amelioration of ON and 3 with stable disease, except 1 who suffered a relapse of leukemia.

DISCUSSION

In the 3 most recent JCCLSG ALL studies, we found that a significant number of patients developed ON during or after treatment. ALL2004 study was conducted to evaluate the efficacy of DEX usage as a corticosteroid in the context of intensification of reinduction phase, comparing with the preceding 2 studies wherein PSL was the only corticosteroid adopted. This strategy also enabled us to compare the DEX toxicity with that of PSL. The results clearly demonstrated the higher incidence of ON in

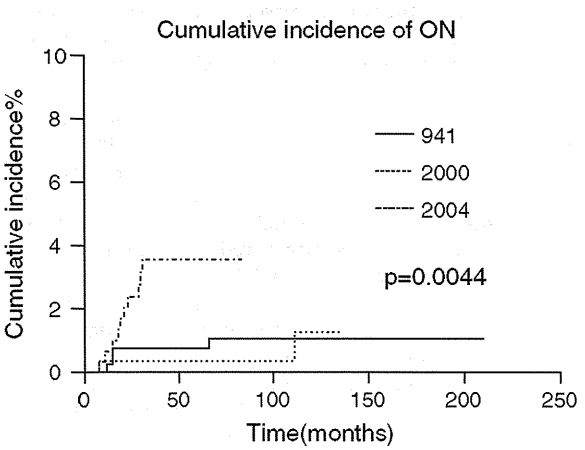

FIGURE 2. The cumulative incidence of osteonecrosis (ON) in the 3 Japanese Childhood Cancer and Leukemia Study Group studies on acute lymphoblastic leukemia (ALL). ALL941: 0.76%, SE, 0.43%; ALL2000: 0.35%, SE, 0.35%; ALL2004: 3.6%, SE, 1.1%.

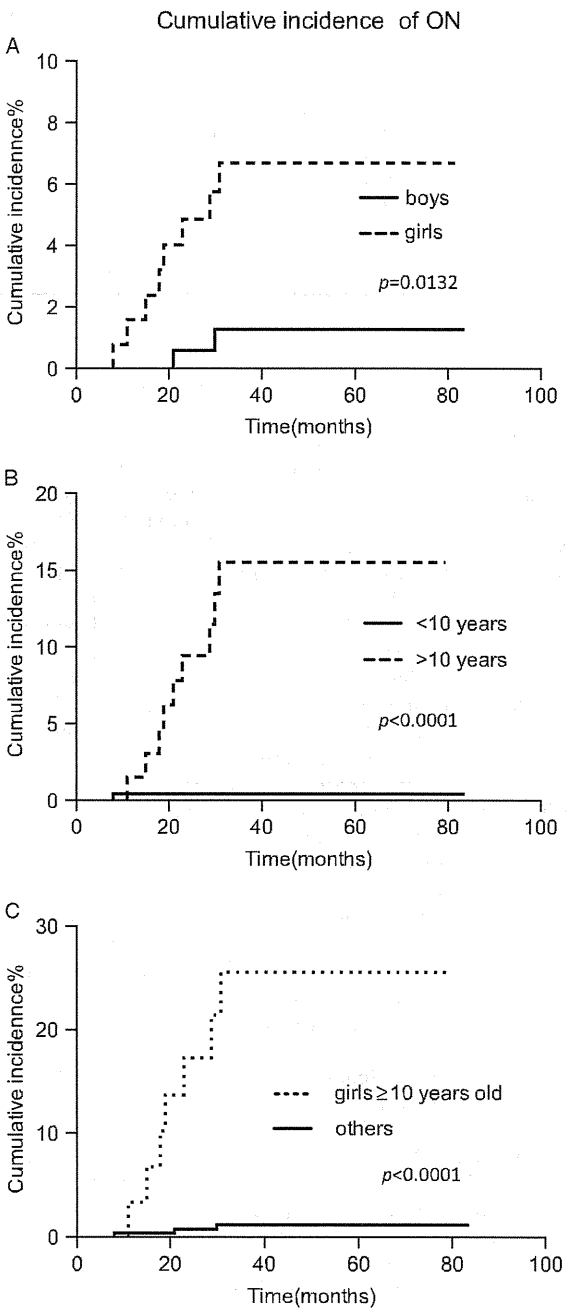

FIGURE 3. The cumulative incidence of osteonecrosis (ON) in ALL2004 according to sex (a), age (b), and combined (c). A, Boys (n=2/190): 1.3%, SE, 0.9%; girls (n=8/136): 6.7%, SE, 2.3%. B, Age below 10 years (n=1/249): 0.42%, SE, 0.42%; age 10 years and above (n=9/77): 15.6%, SE, 4.8%. C, Girls 10 years and above (n=7/33): 25.6%, SE, 8.42%; others (n=3/293): 1.19%, SE, 0.68%.

TABLE 4. Affected Joints and Clinical Course of Patients With ON

Cases	Affected Lesion	Corticosteroids	Management	Outcome
941-1	Right hip	Reduced	Avoidance of weight-bearing	Improve
2	Bilateral hips	Continue	Prohibit hard exercise	Stable
3	Bilateral hips	Continue	Avoidance of weight-bearing	Improve
4	Bilateral hips	Reduced	Avoidance of weight-bearing	Improve
2000-1	Left hip	Continue	Bracing	Improve
2	Bilateral knees, right talus	Withhold	Observation	Improve
2004-1	Bilateral hips	Withhold	Avoidance of weight-bearing	Stable
2	Bilateral hips	Withhold	Observation	Improve
3	Bilateral knees	Continue	Surgery	Improve
4	Left femur	Continue	Avoidance of weight-bearing	Improve
5	Right femur	Continue	Avoidance of weight-bearing	Improve
6	Bilateral hips and knees	Continue	Observation	ALL relapse
7	Right knee	Continue	Observation	Improve
8	Right knee	Withhold	Surgery	Improve
9	Bilateral hips and knees	Reduced	Bisphosphonate	Stable
10	Bilateral knees	Continue	Surgery	Improve

ALL indicates acute lymphoblastic leukemia; ON, osteonecrosis.

ALL2004, indicating DEX exposure was the risk for ON in ALL chemotherapy.

The overall incidence of ON was 1.5% (16/1095), which was comparable with that in a previous study by the Japan Association of Childhood Leukemia Study (JACLS) (2.4%, Hiroki H, Yasushi I, Teruaki H, Makoto Y, Megumi O, Tooru K, Shinichiro N, Junichi H, Keizo H, Keiko Y, and Tatsutoshi N; unpublished data). In studies from Europe and the United states, the ON incidence was highly variable (1% to 2% up to 9%) and dependent on patient characteristics and treatment intensity.[5 7] Furthermore, the detection methods of ON have significantly affected the incidence. Recent report from St Jude Total XV study showed that 17.6% of patients had the symptomatic ON, whereas the asymptomatic ON was detected in > 50% of patients by the prospective screening with MRI test.[15] With regard to the effects of race, the incidence of ON is reportedly higher in whites than in patients of African descent.[7] Although it remains unclear whether the Asian race is related to an increased risk of ON, our results showed that the incidence of ON in Japanese children seemed to be comparable with that in European and American children. However, it should be taken into account the limitation of the present assessment: the possible missing of asymptomatic cases and the diagnosis partly depending on physician's discretion.

In this study, female sex, age 10 years and above, and the use of DEX as a corticosteroid were significant risk factors for ON. Of the 33 female patients aged over 10 years who received DEX, 7 developed ON (cumulative incidence, 25.6%). This was the extremely higher incidence of ON comparing with the rest of patients. Although females were found to be at a higher risk of developing ON in the Children's Cancer Study Group (CCG) and Italian studies,[5,7] there was no such correlation in studies performed in the UK and Germany and at the Dana Farber Cancer Institute (Boston, MA).[6,16,17] In addition, a Japanese study conducted by the JACLS failed to show a significant female predominance (male to female ratio, 7:9). Therefore, the effects of sex on ON pathogenesis remain unclear.

A significant contribution of age to ON onset has been robustly documented by most retrospective and prospective studies.[5 7,9,16,18 21] Among children aged 10 years and above, those aged 16 to 20 years were at the highest risk of ON. The eligible patient age was 1 to 15 years in ALL941/2000 and 1 to 18 years in ALL2004; therefore, we may have underestimated the incidence of ON. Further monitoring is necessary when ALL treatment protocols designed for children are extended to adolescence and young adulthood.

The potential effect of DEX on ALL is 6.5 times that of PSL, resulting in an increase in the use of DEX for ALL treatment. Because DEX is more toxic to bone tissues,[14,22] a higher incidence of ON has been a major concern in the design of treatment protocols. In ALL2004, DEX was incorporated only in the reinduction phase because an increased incidence of ON and mortality was reported with the use of DEX in the induction phase.[23] Nonetheless, our data revealed a higher cumulative incidence of ON associated with DEX administration; this finding was comparable with the results of the Dana Farber Consortium study DFCI 00-01, wherein DEX was used in postremission intensification therapy and/or in the maintenance phase.[24] Although the total corticosteroid dose (analyzed as PSL equivalents) at therapy completion were slightly lower in ALL2004 than in ALL941/2000 (Table 2), ON was most frequent in patients who had received only DEX in the HR group in ALL2004. These results suggest that DEX administration at any dose (as PSL equivalents) and in any treatment phase affects the incidence of ON. A recent report from the CCG found that DEX administration could influence the risk of ON[21] and that alternate-week DEX administration during delayed intensification therapy decreased ON incidence compared with continuous DEX. In our ALL2004 protocol, DEX was administered continuously for 2 weeks, and it would have been beneficial to modify the DEX schedule from continuous administration to alternate-week administration.

Recently, biological and genetical basis for ON development has been extensively investigated. Children's Oncology Group tested 12 polymorphisms of candidate genes and identified children with *PAI-1* GA/AA genotypes were significantly associated with ON.[25] Another study from St Jude Children's Research Hospital showed polymorphisms of *ACP1* were associated with risk of

symptomatic ON as well as with lower serum albumin and higher cholesterol levels.[15] These results suggest that some patients are prone to develop ON and individualized therapy should be needed in the future ALL studies.

In the present report, cases with ON were retrospectively collected by the questionnaire, and most of the ON patients were identified by symptoms and confirmed with imaging studies (x-ray/MRI) without central review. Despite such limitations, the clinical features of all 16 ON patients in our study were virtually comparable with those of patients in previous studies.[6,7,16] Weight-bearing joints were commonly affected, whereas asymptomatic lesions might have been overlooked.[15] Once ON is confirmed, the physician must decide whether steroids should be withheld or continued, considering that no consensus guideline is available thus far. Most of our patients were prescribed a planned dose of steroids without compromising functional outcomes after ON development. We believe that it may not be necessary to withhold steroids at the risk of leukemia relapse.

Bisphosphonates, which are structurally similar to pyrophosphates, inhibit osteoclast activity and bone turnover, thus exerting beneficial effects on bone mineralization.[26] Alendronate, a third-generation bisphosphonate, is reportedly effective in the prevention of femoral head collapse in ON patients.[27] Wiernikowski et al[28] showed that alendronate-induced changes in bone mineral metabolism/homeostasis benefited bone mineralization in children with ALL or non-Hodgkin lymphoma with steroid-induced osteopenia. Another bisphosphonate, pamidronate, was shown to be effective in the management of pain and motor function recovery in symptomatic ON occurring in children with ALL.[29] In the present study, alendronate was administered to 1 patient with symptomatic ON of the bilateral hip and knee joints; this resulted in no further deterioration of functional outcome and no treatment-induced side effects. However, further studies are required to clarify the potential benefits of concomitant bisphosphonate and steroid use for ON treatment.

In summary, the overall incidence of ON was 1.5% in the JCCLSG ALL studies, which was comparable with that reported in previous studies conducted in the Unites States and Europe. The known risk factors of age above 10 years, female sex, and DEX use were all significantly associated with an increase in the cumulative incidence of ON. In our future studies, we are intending to routinely screen for ON development with MRI test, especially those incorporating DEX in the treatment protocol. Although an ON management regimen remains to be established, steroids should not be withheld at the risk of ALL relapse.

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Pediatr Blood Cancer 2013;60:1587–1592

IKZF1 and *CRLF2* Gene Alterations Correlate With Poor Prognosis in Japanese *BCR-ABL1*-Negative High-Risk B-Cell Precursor Acute Lymphoblastic Leukemia

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Background: Genome-wide analysis studies have demonstrated that *IKZF1*, *CRLF2*, and *JAK2* gene alterations correlate with poor prognosis in pediatric B-cell precursor acute lymphoblastic leukemia (BCP-ALL). However, the prognostic significance for these gene alterations has not been clarified in Japanese patients. ***Procedure:*** A total of 194 patients with BCP-ALL enrolled in the Japanese Children's Cancer & Leukemia Study Group ALL 2004 clinical trial were assessed for the presence of three different gene alterations: *IKZF1* deletions, *CRLF2* expression and *JAK2* mutation. ***Results:*** *IKZF1* deletions and *CRLF2*-high expression were identified in 22 of 177 (12%) patients and in 15 of 141 (11%) patients, respectively. However, *JAK2* R683 mutation was detected only one of 177 patients. The 4-year event-free survival (4y-EFS) was different when comparing patients with or without *IKZF1* deletions (68.2% vs. 85.2%; $P=0.04$) and was also different when comparing patients with different *CRLF2* expression levels (high, 66.7% vs. low, 88.1%; $P=0.03$). The differences in 4y-EFS were statistically significant in patients with ALL in the National Cancer Institute (NCI)-high risk group (HR-ALL) (*IKZF1* deletions: yes, 58.3% vs. no, 87.0%, $P=0.02$; *CRLF2* expression: high, 55.6% vs. low, 85.3%, $P=0.04$) but not in patients with ALL in the NCI-standard risk group (SR-ALL; *IKZF1* deletions: yes, 80.0% vs. no, 84.4%, $P=0.75$; *CRLF2* expression: high, 83.3% vs. low, 89.2%, $P=0.77$). Coexistence of *IKZF1* deletions and *CRLF2*-high expression associated with poor outcomes. ***Conclusions:*** *IKZF1* deletions and *CRLF2*-high expression predicted poor outcomes in patients with HR-ALL but not in patients with SR-ALL in our Japanese cohort. Pediatr Blood Cancer 2013;60:1587–1592. © 2013 Wiley Periodicals, Inc.

Key words: acute lymphoblastic leukemia; *CRLF2*; *IKZF1*; *JAK2*

INTRODUCTION

Improvements in overall survival in patients with pediatric B-cell precursor acute lymphoblastic leukemia (BCP-ALL) have been achieved via the institution of risk-adapted multi-agent chemotherapy [1]. However, about 20% of patients still show persistent disease or experience relapse. This observation underscores the need for a better understanding of the pathophysiology of the disease as well as the identification of factors that predict outcomes or predict the response to specific therapies.

Findings from genome-wide analysis have demonstrated that alterations in *IKZF1*, which encodes the lymphoid transcription factor, IKAROS, are prevalent in patients with *BCR-ABL1*-positive ALL [2,3]. *IKZF1* alterations have also been demonstrated in patients with high-risk *BCR-ABL1*-negative ALL and are associated with poor prognosis [4–10]. Further, Mullighan et al. [11] identified Janus kinases (*JAK*s) mutations in approximately 10% of the *BCR-ABL1*-negative subgroup and reported that these mutations were associated with *IKZF1* alterations. Recent studies have also revealed that increased expression of *CRLF2*, which is predominantly caused by fusion of *P2RY8-CRLF2* or *IGH-CRLF2*, was found in approximately 5–10% of patients with high-risk ALL and in 50–60% of patients with Down syndrome-associated ALL [12–14]. Alterations in *CRLF2* often coexist with alterations in *IKZF1* and/or *JAK2*, and these gene alterations are associated with poor outcomes [15–17]. However, the prognostic significance for these gene alterations has not been clarified in Japanese patients. Therefore, the incidence and clinical significance of *IKZF1* deletions, *CRLF2* expression and *JAK2* mutations were assessed in Japanese pediatric patients with *BCR-ABL1*-negative BCP-ALL in this study.

DOI 10.1002/pbc.24571
Published online 27 June 2013 in Wiley Online Library (wileyonlinelibrary.com).

MATERIALS AND METHODS

Patients and Samples

A total of 194 patients were selected from 264 pediatric *BCR-ABL1*-negative BCP-ALL patients who were enrolled in the Japanese Children's Cancer & Leukemia Study Group (JCCLSG) ALL 2004 clinical trial from 2004 to 2008. One hundred seventy-seven DNA and 141 RNA samples were available and extracted from total bone marrow (BM) or peripheral blood (PB) at the time of diagnosis. These samples contained over 50% (median, 95%; range, 53.3–100%) blasts. The analyzed cohort included 131 patients with ALL classified as NCI-SR and 63 patients classified as NCI-HR. Treatment stratification in this clinical trial

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Grant sponsor: Clinical Cancer Research; Grant sponsor: Grant-in-Aid for Cancer Research; Grant sponsor: The Ministry of Health, Labour and Welfare of Japan

Conflict of interest: Nothing to declare.

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Received 10 September 2012; Accepted 25 March 2013

was based on age and white blood cell (WBC) count; patients with HR-ALL were treated with a HR- or very-high-risk regimen, and patients with SR-ALL were treated with a SR-regimen, except for 10 patients who were treated with intensified chemotherapy due to positivity for minimal residual disease. There were no statistical differences in the clinical characteristics (e.g., age, initial WBC, gender, and cytogenetic abnormalities) when comparing the analyzed cohort and original cohort (Supplementary Table I). The median (range) follow-up period from diagnosis was 6.0 (0.1–8.2) years. DNA samples from three healthy donors and from seven patients with solid tumor that were free from BM invasion were used as controls. This study was approved by the institutional review board at Nagoya Medical Center. Informed consent to participate in this study was obtained from patients and their guardians.

Genetic Analysis

The multiplex ligation-dependent probe amplification (MLPA) method (IKZF1 P-335, MRC-Holland, Amsterdam, NL) was used to detect *IKZF1* deletion, according to the manufacturer's instructions [18]. A total of 60 ng of DNA was used per reaction. Fragment analysis was performed using GeneScan v.3.5 (ABI310, Applied Biosystems, Foster City, CA). A probe ratio below 1.3 was considered indicative of deletion, as per the manufacturer's instructions [19]. For detection of *Ik6* and *Ik10* of *IKZF1* and the *P2RY8-CRLF2* fusion gene transcript, cDNA synthesis was performed using SuperScript II (Invitrogen Corporation, Carlsbad, CA) with 1 μg of RNA per 20 μl reaction with random primer (Invitrogen), and reverse transcription polymerase chain reaction (RT-PCR) was performed with following primers; *IKZF1* fwd, 5'-CTCCGAGGTTGCTCTT; *IKZF1* rev.: 5'-AGGTAGTT-GATGGCGTTGTTGATG; *P2RY8-CRLF2* primers were as previously reported [12]. To measure *CRLF2* mRNA levels, real-time quantitative (RQ)-PCR was performed using the TaqMan Gene Expression Assay (*CRLF2*, Hs00845692_m1; *GAPDH*, #4310884E, Applied Biosystems). RQ-PCR was performed in duplicate, using 1 μl of cDNA per reaction. The comparative C_t method was used to quantify relative mRNA levels using the endogenous control gene, *GAPDH*. To detect *JAK2* mutations, exon 12, 16, 20, and 21 were amplified and directly sequenced by Sanger sequencing with the ABI310 sequencing system, as previously reported [20].

Statistical Analysis

Descriptive statistical analyses to assess baseline characteristics of patients diagnosed with *BCR-ABL1*-negative BCP-ALL were performed. Event-free survival (EFS) and relapse-free interval (RFI) were analyzed by the Kaplan–Meier method [21], and log-rank tests [22] were used for group comparisons. EFS was defined as the time from the diagnosis to induction failure, relapse, or death from any cause, whichever occurred first. RFI was estimated for patients who achieved complete remission (CR). Cox proportional hazards regression models [23] were used to investigate factors associated with survival in univariate and multivariate analysis. A two-sided *P*-value of more than 0.05 should be interpreted with care. All data analysis was performed using SAS statistical software (version 9.1.3; SAS Institute, Inc., Cary, NC).

Results

Frequencies of *IKZF1, CRLF2,* and *JAK2* Alterations in BCP-ALL

IKZF1 deletions were detected in 22 (12%) of 177 DNA samples, and various deletion patterns were detected by MLPA (Supplementary Table II). Homozygous deletion was not detected. To confirm the results of MLPA, RT-PCR was performed for six patients whose RNA was available. The isoform type in the cases with the deletion of *IKZF1* exon 4–7 and 2–7 was confirmed to be the *Ik6* and *Ik10* isoform variants, respectively (Supplementary Fig. S1). *IKZF1* deletions were significantly associated with older age ($P<0.01$) and NCI-HR ($P=0.02$; Supplementary Table SIII). However, no association was determined between *IKZF1* deletions and any known chromosomal abnormalities.

CRLF2 expression was measured by RQ-PCR in 141 RNA samples. The median expression value was 13.8 copies (range: 0.07–35,100). Fifteen (10%) samples showed *CRLF2* expression that was ≥10-fold of the median value (Fig. 1A). The clinical features of patients with high *CRLF2* expression are shown in Supplementary Table SIII. High *CRLF2* expression was more prevalent in patients with HR-ALL (9/43, 21%) than in patients with SR-ALL (6/98, 6%; $P<0.01$). *P2RY8-CRLF2* fusion was detected in five of 141 patients. Two of these five patients had high *CRLF2* expression, while the other three patients had low *CRLF2* expression (Fig. 1B). Sequencing of predominant fusion transcripts demonstrated that the non-coding exon 1 of *P2RY8* bound to the start of *CRLF2* exon 1 in all five patients (Fig. 1C) [15]. In addition, transcript variants were demonstrated in three patients. One of the clones of unique patient number (UPN) 035, 099, and 219 showed *P2RY8* exon 1 fused to *CRLF2* exon 2. Sequencing of another clone of UPN219 demonstrated that *P2RY8* exon 1 bound to the 34 bp upstream sequence of *CRLF2* exon 1 (Fig. 1C).

In contrast, a *JAK2* R683 mutation was demonstrated in only one of the 177 patients. In this case, *P2RY8-CRLF2* fusion transcript, high *CRLF2* expression and *IKZF1* deletion were also recognized. Furthermore, the patient failed to achieve remission after induction therapy. We further analyzed *JAK2* exons 12, 20, and 21 in 15 patients with high *CRLF2* expression, but no mutation was detected except for a single nucleotide polymorphism (rs10974955) in two patients.

IKZF1 and *CRLF2* Alterations Are Associated With Poor Outcomes in Patients With HR-ALL

In survival analysis, the 4-year EFS was significantly lower for patients with *IKZF1* deletions than for patients without *IKZF1* deletions ($68.2\pm9.9\%$ vs. $85.2\pm2.9\%$; $P=0.04$; Fig. 2A). Interestingly, the difference in this parameter was statistically significant in patients with HR-ALL ($58.3\pm14.2\%$ vs. $87.0\pm5.0\%$; $P=0.02$) and not in patients with SR-ALL ($80.0\pm12.7\%$ vs. $84.4\pm3.5\%$; $P=0.75$; Fig. 2B). Similarly, 4-year EFS for the patients with high *CRLF2* expression was also significantly worse than that for those with low *CRLF2* expression ($62.7\pm12.1\%$ vs. $88.1\pm2.9\%$; $P=0.03$, Fig. 2C), and a statistical difference between these groups was recognized only in patients with HR-ALL ($55.6\pm16.6\%$ vs. $85.3\pm6.1\%$; $P=0.04$ for HR; $83.3\pm15.2\%$ vs. $89.2\pm3.3\%$; $P=0.77$ for SR, Fig. 2D). Similar findings for *IKZF1* and *CRLF2* were noted in the analysis for relapse-free interval (RFI).

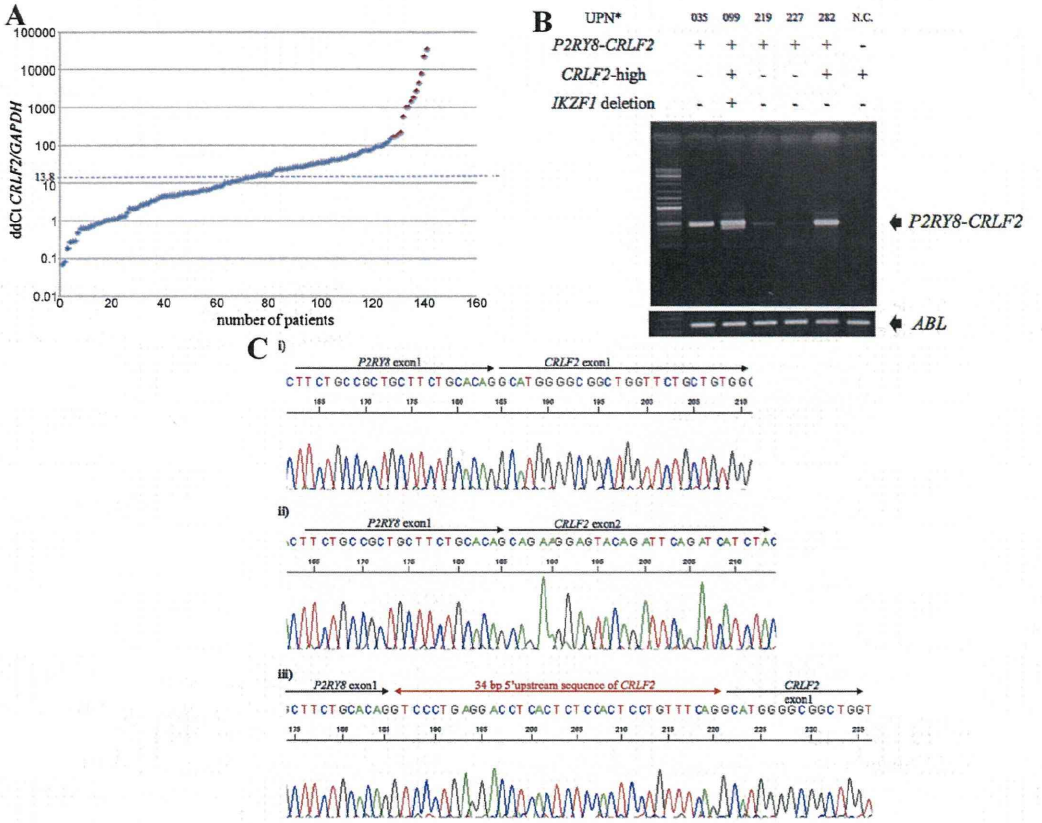

Fig. 1. Assessment of *CRLF2* expression and alterations in *CRLF2*. **A**: Measurement of *CRLF2* expression by RQ-PCR. The median *CRLF2* expression value (normalized to *GAPDH* expression) was 13.8. Red diamonds represent high *CRLF2* expression, defined as *CRLF2* expression that was ≥10-fold higher than the median *CRLF2* expression. Blue diamonds represent low *CRLF2* expression. **B**: *P2RY8-CRLF2* rearrangement detected by RT-PCR. The level of *ABL* transcription served as control. NC indicates negative control. Samples from five patients showed *P2RY8-CRLF2* fusion by RT-PCR. Two of the five samples also showed high *CRLF2* expression, and the remaining three samples showed low *CRLF2* expression. Interestingly, only one patient showed *P2RY8-CRLF2* fusion, *CRLF2* high expression and *IKZF1* deletion. **C**: Sequence results of *P2RY8-CRLF2* fusion. Representative sequences are shown. (i) The major sequence of all samples shows that the 3' end of non-coding *P2RY8* exon 1 bound to the 5' end of *CRLF2* exon 1. Sequence of No. 035 is shown as a representative patient. (ii) One of the No. 035 clones showed that the 3' end of non-coding *P2RY8* exon 1 bound to the 5' end of *CRLF2* exon 2 (skipped *CRLF2* exon 1). This variant was also found in No. 099 and No. 219. (iii) Thirty-four base pair upstream sequence of *CRLF2* exon 1 fused to *P2RY8* exon 1. This fusion was found in No. 219. *UPN indicated unique patient number.

Among the 124 patients whose samples were analyzed for both the *IKZF1* and *CRLF2* genes, five patients had ALL with *IKZF1* deletions and high *CRLF2* expression, simultaneously. All five patients were classified as NCI-HR, and four of the five patients experienced induction failure or relapse. In Kaplan–Meier analysis, EFS was the lowest among patients with ALL and coexisting *IKZF1* deletions and high *CRLF2* expression when compared with other categories of patients (Supplementary Fig. S3).

In comparison with other known prognostic factors in the full cohort and in the NCI-HR cohort, *IKZF1* deletions and high *CRLF2* expression were significant predictors of outcomes in univariate analysis (Table I). However, no variables retained independent prognostic significance in multivariate analysis.

DISCUSSION

Despite recent improvement in outcomes for patients with pediatric BCP-ALL, the genetic pathophysiology of the failure to respond to therapy or the occurrence of relapse remains unclear. *IKZF1*, *CRLF2*, and *JAK2* gene alterations are prognostic factors in patients with pediatric BCP-ALL [4–10,15–17,25]; therefore, we assessed for the presence of these genetic alterations in Japanese patients with *BCR-ABL1*-negative BCP-ALL.

IKZF1 deletions were found in 12% of our cohort (8% of NCI-SR, and 21% of NCI-HR), which is consistent with observations from previous reports (approximately 10–20%) [6–10,15]. Previous studies have reported that *IKZF1* deletions significantly correlated with poor relapse-free survival (RFS). Chen et al. reported that *IKZF1* deletions/mutations retained independent prognostic significance in multivariate analysis in their full cohort and were associated with poor RFS only in NCI-HR patients [9]. In our study, *IKZF1* deletions were significantly associated with outcome in univariate analysis, but not in multivariate analysis within either our full cohort or the NCI-HR cohort. Mi et al. [10] reported that the *Ik6* variant correlated with poor prognosis. In our study, *Ik6* variant was detected in only one-third of *IKZF1*-deletion patients

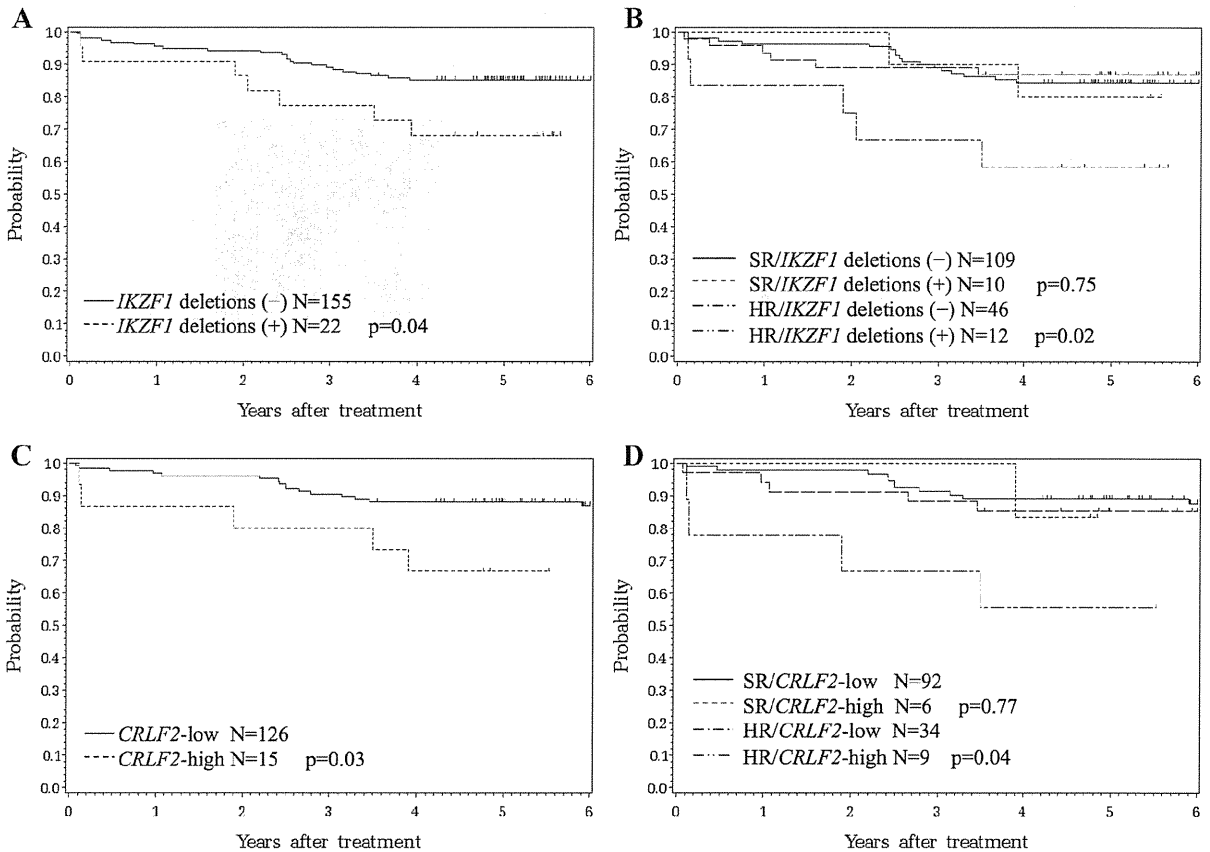

Fig. 2. Probability of EFS according to *IKZF1* deletions, *CRLF2* expression and NCI risk classification. **A**: Probability of EFS for patients with or without *IKZF1* deletions. **B**: Probability of EFS for patients with or without *IKZF1* deletions according to NCI-risk classification. **C**: Probability of EFS for patients with high *CRLF2* expression or low *CRLF2* expression. **D**: Probability of EFS for patients with high *CRLF2* expression or low *CRLF2* expression according to NCI-risk classification.

(Supplementary Table SI), and none of these patients experienced relapsed. The relationship between *Ik6* variant status and patient outcomes remains to be determined.

High *CRLF2* expression was detected in 10% of the patient in this study (6% of SR-ALL, and 21% of HR-ALL), which is consistent with observations from previous reports (5–20% of BCP-ALL) [10–16,24]. Hervey et al. [15] reported that *P2RY8-CRLF2* or *IgH-CRLF2* was highly associated with high *CRLF2* expression. On the other hand, Chen et al. [9] reported that approximately a half of patients with high *CRLF2* expression had these *CRLF2* gene alterations and that these gene alterations were detected only in the patients with high *CRLF2* expression. Palmi et al. [25] reported that *P2RY8-CRLF2* fusion was detected in 45% of patients with high *CRLF2* expression and that it was found in patients with high *CRLF2* expression as well as in patients with low *CRLF2* expression. They also demonstrated that the *P2RY8-CRLF2* fusion was associated with a high incidence of relapse (5-year cumulative incidence of relapse with or without the *P2RY8-CRLF2* fusion: 42.8% vs. 14.5%; $P = 0.001$). In the present study, the *P2RY8-CRLF2* fusion was found in 13% (2 of 15 patients) of ALL samples with high *CRLF2* expression. In addition, the *P2RY8-CRLF2* fusion was also found in 2% (3 of 126 patients) of ALL samples with low *CRLF2* expression, suggesting that a minor population clone had this fusion transcript. Furthermore, five patients with ALL positive for the *P2RY8-CRLF2* fusion are alive in first remission, except for one patient with ALL who had both the *IKZF1* deletion and high *CRLF2* expression. The prognostic impact of the *P2RY8-CRLF2* fusion remains to be clarified in a large-scale study.

Chen et al. [9] also reported that high *CRLF2* expression was associated with poor RFS in a multivariate analysis in HR-ALL patients but not in SR-ALL patients. The present study demonstrated that high *CRLF2* expression was significantly associated with poor outcomes, according to Kaplan–Meier analysis. However, high *CRLF2* expression was associated with only marginal significance for poor EFS, according to univariate and multivariate analysis in the Cox regression model. This discrepancy may be due to the relatively small number of patients analyzed in this study. Some investigators have proposed that ALL patients with high *CRLF2* expression were assigned to the intermediate-risk group because high *CRLF2* expression had no prognostic significance within multivariate analyses [10,17]. The relationship between outcomes and *CRLF2* expression may also be dependent on the specific regimen employed for treatment.

TABLE I. Prognostic Impact of *IKZF1* Deletions and High *CRLF2* Expression in Univariate and Multivariate Analyses

	Full cohort				NCI-HR			
	Univariate analysis		Multivariate analysis		Univariate analysis		Multivariate analysis	
Factors	HR[a] (95% CI[b])	*P*-value	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Age, years								
≥10 versus 10	1.74 (0.81–3.75)	0.16			2.13 (0.58–7.89)	0.26		
Gender								
Male versus female	1.31 (0.66–2.59)	0.44			1.33 (0.43–4.13)	0.62		
WBC[c], $\times 10^9$/L								
≥10 versus 10	0.95 (0.37–2.47)	0.92			0.71 (0.23–2.23)	0.56		
NCI risk classification								
HR[d] versus SR[e]	1.27 (0.62–2.58)	0.51	1.39 (0.50–3.82)	0.53	—	—	—	—
PSL[f] response								
PPR[g] versus PGR[h]	2.63 (0.80–8.60)	0.11	1.56 (0.20–12.08)	0.67	2.70 (0.59–12.34)	0.20	2.03 (0.24–17.49)	0.52
Cytogenetic abnormalities								
Hyperdiploid versus normal	1.48 (0.58–3.76)	0.41			0.49 (0.06–4.23)	0.52		
Other versus normal	1.39 (0.62–3.09)	0.42			0.80 (0.23–2.75)	0.72		
N.D.[i] versus normal	0.83 (0.19–3.69)	0.80			0.74 (0.09–6.29)	0.78		
Fusion genes								
ETV6-RUNX1 versus none	0.74 (0.24–2.34)	0.61			1.71 (0.29–10.21)	0.56		
E2A-PBX1 versus none	1.44 (0.50–4.14)	0.50			1.22 (0.20–7.28)	0.83		
N.D. versus none	1.38 (0.62–3.09)	0.43			2.04 (0.49–8.55)	0.33		
IKZF1 deletions								
Yes versus no	2.38 (1.02–5.55)	0.04	2.78 (0.94–8.27)	0.07	3.61 (1.10–11.84)	0.03	3.93 (0.75–20.75)	0.11
CRLF2 expression								
High versus low	2.97 (1.09–8.11)	0.04	2.24 (0.72–6.95)	0.16	3.56 (0.95–13.27)	0.06	1.97 (0.37–10.37)	0.43

HR[a], hazard ratio; CI[b], confidential interval; WBC[c], white blood cell count; NCI risk classification HR[d], 1–9y. and WBC $<50 \times 10^9$/L; SR[e], ≥10y. or WBC $\geq 50 \times 10^9$/L; PSL[f], prednisolone; PGR[g], prednisolone good responder; PPR[h], prednisolone poor responder; N.D.[i], not determined.

Some studies have reported that high *CRLF2* expression was highly associated with *JAK2* mutation and *IKZF1* deletions [15,16]. In the report by Chen et al. [9], *JAK* mutations were found in 21.8% of patients with high *CRLF2* expression and in 4.4% of their full cohort. Mullighan et al. reported that *JAK* mutations were detected in 20 of 187 patients with high-risk childhood BCP-ALL. A total of 16 cases had *JAK2* mutations, with 13 located in exon 16, and three located within exon 20 or 21. Another four cases had *JAK1* or *JAK3* mutations [11]. In our study, *JAK2* mutation in exon 16 was detected in only 1 of 177 cases. In addition, no mutations in *JAK2* exons 12, 20, and 21 were found in any cases of ALL with high *CRLF2* expression. These results suggest that *JAK2* mutations might be rare in Japanese patients. Further analysis of screening mutations of *JAK1* and *JAK3* as well as other sites of *JAK2* should be performed to confirm this finding.

In the Kaplan–Meier analysis from the present study, the coexistence of *IKZF1* deletions and high *CRLF2* expression, which was found only in patients with HR-ALL and not in patients with SR-ALL, was related to poor outcomes. One of the five patients with ALL and coexisting of *IKZF1* deletions and high *CRLF2* expression was positive for *JAK2* mutation, but the others were not. Therefore, they might have additional genetic alterations similar to those seen in patients with Ph-like ALL [26].

In conclusion, the present study suggests that *IKZF1* deletions and high *CRLF2* expression (and particularly, the combination of these two variables) predicted poor outcome in patients with HR-ALL but not in patients with SR-ALL in our Japanese cohort. However, the small sample size might have limited the statistical power of this study. A large-scale nationwide cohort study is planned to clarify the prognostic significance of these genetic abnormalities in Japan.

ACKNOWLEDGMENTS

We thank all participants of JCCLSG ALL 2004, and we thank Ms. Miho Yamada for her excellent support in sequencing analysis.

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