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ZNF384-related fusion genes define a subgroup of childhood B-cell precursor acute lymphoblastic leukemia with a characteristic immunotype

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ABSTRACT

Fusion genes involving *ZNF384* have recently been identified in B-cell precursor acute lymphoblastic leukemia, and 7 fusion partners have been reported. We further characterized this type of fusion gene by whole transcriptome sequencing and/or polymerase chain reaction. In addition to previously reported genes, we identified *BMP2K* as a novel fusion partner for *ZNF384*. Including the *EP300-ZNF384* that we reported recently, the total frequency of *ZNF384*-related fusion genes was 4.1% in 291 B-cell precursor acute lymphoblastic leukemia patients enrolled in a single clinical trial, and *TCF3-ZNF384* was the most recurrent, with a frequency of 2.4%. The characteristic

immunophenotype of weak CD10 and aberrant CD13 and/or CD33 expression was revealed to be a common feature of the leukemic cells harboring *ZNF384*-related fusion genes. The signature gene expression profile in *TCF3-ZNF384*-positive patients was enriched in hematopoietic stem cell features and related to that of *EP300-ZNF384*-positive patients, but was significantly distinct from that of *TCF3-PBX1*-positive and *ZNF384*-fusion-negative patients. However, clinical features of *TCF3-ZNF384*-positive patients are markedly different from those of *EP300-ZNF384*-positive patients, exhibiting higher cell counts and a younger age at presentation. *TCF3-ZNF384*-positive patients revealed a significantly poorer steroid response and a higher frequency of relapse, and the additional activating mutations in RAS signaling pathway genes were detected by whole exome analysis in some of the cases. Our observations indicate that *ZNF384*-related fusion genes consist of a distinct subgroup of B-cell precursor acute lymphoblastic leukemia with a characteristic immunophenotype, while the clinical features depend on the functional properties of individual fusion partners.

Introduction

B-cell precursor acute lymphoblastic leukemia (BCP-ALL) is a heterogeneous disease that can be subdivided according to primary recurrent genetic abnormalities that are strongly associated with characteristic biological and clinical features.¹ The detection of these abnormalities can facilitate diagnosis, risk stratification, and targeted therapy. In approximately two-thirds of pediatric patients with BCP-ALL, well-characterized genetic abnormalities, including t(9;22) *BCR-ABL1*, the rearrangement of *MLL* at 11q23, t(1;19) *TCF3-PBX1*, t(17;19) *TCF3-HLF*, t(12;21) *ETV6-RUNX1*, hyperdiploidy, and hypodiploidy, can be detected by standard genetic analyses.¹ In the remaining BCP-ALL patients, major pathogenic or driver cytogenetic abnormalities have yet to be clarified, and they are called "B-others".

Recent studies using advanced analytical approaches have stratified a variety of subgroups harboring novel genetic abnormalities. For example, a high-risk subtype in B-others with a number of fusion genes involving tyrosine kinases, so-called "Ph-like acute lymphoblastic leukemia (Ph-like ALL)", was recently discovered.²⁻³ Besides Ph-like ALL, other subgroups such as iAMP21, dic(9;20), and patients with *PAX5* abnormalities have also been identified in B-others.⁴⁻⁷ However, a certain portion of B-others still remain genetically unclassified.

The zinc-finger protein 384 (*ZNF384*) gene encodes a transcription factor that regulates promoters of the extracellular matrix genes⁸ and is known to be involved in ALL through fusion with the *TET* family genes, such as the Ewing sarcoma breakpoint region 1 gene [*EWSR1*, t(12;22)], TATA box binding protein-associated factor [*TAF15*, t(12;17)], and transcription factor 3 [*TCF3* or *E2A*, t(12;19)].⁹⁻¹⁰ In addition to those previously reported *ZNF384*-related fusion genes, we identified *EP300-ZNF384*, t(12;22), as a novel recurrent fusion gene with an incidence of approximately 1% in B-others in a Japanese cohort.¹¹ The recurrence of *EP300-ZNF384* in pediatric BCP-ALL patients as well as a higher incidence in adolescent and young adult (AYA) BCP-ALL patients was confirmed by other groups.¹²⁻¹⁵ Furthermore, the CREB binding protein gene [*CREBBP*, t(12;16)(p13;p13)] has been most recently identified as another novel fusion partner for *ZNF384*.¹³ So far, however, patients harboring *ZNF384*-related fusion genes have been considered to be a minor subgroup in pediatric BCP-ALL.⁸⁻¹⁵ The BCP-ALL patients with *EP300-ZNF384* possess a characteristic

immunophenotype of weak CD10 and aberrant CD13 and/or CD33 expression.¹¹ Although certain immunophenotypic features were still observed in some of the remaining B-others patients, cytogenetic abnormalities in these cases have yet to be clarified.

In order to further investigate unknown cytogenetic alterations in the remaining B-others, we employed whole transcriptome sequencing (WTS). Herein, we report the identification of an unexpectedly high incidence of fusion genes involving *ZNF384* genes in BCP-ALL in our cohort. The immunophenotypic and gene-expression characteristics, accompanying genetic abnormalities as well as the clinical features of BCP-ALL harboring the *ZNF384*-related fusion gene are evaluated and discussed.

Methods

Patient selection

Following previous publication,¹¹ 91 ribonucleic acid (RNA) samples obtained from pediatric BCP-ALL patients classified as B-others were selected from the Tokyo Children's Cancer Study Group (TCCSG) biobank as a discovery cohort for WTS to screen for unknown fusion genes. We then extended analysis to another 122 B-others' RNA samples that were available in our biobank as a validation cohort by employing reverse transcription polymerase chain reaction (RT-PCR) (detailed information is presented in Figure 1, *Online Supplementary Information* and *Online Supplementary Table S1*).

Diagnoses were made on the basis of the morphology and routine examinations, including the immunophenotype, cytogenetic analysis, DNA content analysis, and the RT-PCR detection of 7 conventional fusion transcripts, as described previously.¹⁶ The investigations were approved by the institutional review boards of all participating institutions. Informed consent was obtained from parents or guardians, and informed assent was obtained from the patients when appropriate, based on their age and level of understanding. In the TCCSG study, we first assigned patients to standard- (SR), intermediate- (IR) and high-risk (HR) groups based on their age and leukocyte count, and risk assessment was revised on day 8 based on the response of the patients to steroid monotherapy.¹⁷

Whole transcriptome sequencing and RT-PCR

Using total RNA extracted from the bone marrow or peripheral blood samples of the patients, WTS was performed and the fusion transcripts were investigated by employing deFuse, an algorithm for gene fusion discovery, as described in the *Online Supplementary*

Table 1A. Clinical characteristics of acute lymphoblastic leukemia (ALL) with ZNF384-related fusion genes.

ID	Fusion partner	Age (years)	Gender	Initial WBC count	Karyotype	Initial risk	Day 8 blasts (/ μ L)	CNS involvement	Current status	Relapse (date, site)	Salvage therapy after relapse	FUP (years)	Status
Case-1	<i>TCF3</i>	2	F	36,600	46,XX	IR	0	CNS1	1 st CR			3.5	Alive
Case-2	<i>TCF3</i>	9	M	3,200	46,XY	IR	14	CNS1	Relapse	4.7y, BM	SCT	7.0	Alive
Case-3	<i>TCF3</i>	3	M	137,260	46,XY	HR	639	CNS1	1 st CR			6.1	Alive
Case-4	<i>TCF3</i>	2	M	4,000	46,XY	SR	0	CNS1	1 st CR			6.4	Alive
Case-5	<i>TCF3</i>	1	F	21,100	46,XX	IR	1,617	CNS1	1 st CR			4.5	Alive
Case-6	<i>TCF3</i>	9	F	2,750	ND	IR	1,824	CNS1	1 st CR			2.0	Alive
Case-7	<i>TCF3</i>	1	M	49,970	46,XY	IR	11,941	CNS1	Relapse	1.6y, BM	SCT	2.5	Death
Case-8	<i>TCF3</i>	11	F	1,220	46,XX	IR	10	CNS1	1 st CR			6.7	Alive
Case-9	<i>TCF3</i>	2	F	150,200	46,XX	HR	44,156	CNS1	1 st CR			3.6	Alive
Case-10	<i>TCF3</i>	10	F	4,800	46,XX	IR	0	CNS1	1 st CR			0.7	Alive
Case-11	<i>TCF3</i>	3	F	130,900	46,XX	HR	16,816	CNS1	Relapse	3.4y, BM	SCT	4.3	Alive
Case-12	<i>TCF3</i>	2	M	49,160	46,XY	IR	7,443	CNS1	Relapse	2.9y, BM	SCT	3.6	Death
Case-13	<i>TCF3</i>	2	M	27,450	46,XY	IR	5,425	CNS1	Relapse	1.6y, BM	SCT	2.4	Death
Case-14	<i>TCF3</i>	3	M	105,900	46,XY	HR	17,797	CNS1	1 st CR			2.4	Alive
Case-15	<i>TCF3</i>	8	M	76,500	46,XY	IR	52	CNS2	1 st CR			2.3	Alive
Case-16	<i>TAF15</i>	5	M	12,200	46,XY	SR	224	CNS1	1 st CR			4.7	Alive
Case-17	<i>TAF15</i>	3	M	22,000	46XY	IR	537	CNS1	Relapse	5.7y, BM	Chemotherapy	6.3	Alive
Case-18	<i>TAF15</i>	8	M	34,100	46,XY	IR	2,704	CNS1	1 st CR			1.5	Alive
Case-19	<i>CREBBP</i>	3	F	19,270	47,X,add(X)(p22), t(12;12)(p13;q13),+21	SR	3,076	CNS1	1 st CR			5.7	Alive
Case-20	<i>CREBBP</i>	12	F	58,000	46,XX	HR	574	CNS1	1 st CR			6.4	Alive
Case-21	<i>CREBBP</i>	2	M	26,500	46,XY	IR	538	CNS1	Relapse	3.5y, BM	Chemotherapy	6.5	Alive
Case-22	<i>BMP2K</i>	5	F	4,100	46,XX	SR	17	CNS1	1 st CR			10.1	Alive

F: female; M: male; WBC: white blood cell; CNS: central nervous system; FUP: follow up; SR, standard-risk; IR: intermediate-risk; HR: high-risk; CR: complete remission; SCT: stem cell transplantation; BM: bone marrow.

Table 1B. Additional genetic characteristics of acute lymphoblastic leukemia (ALL) with ZNF384-related fusion genes.

ID	Fusion partner	MLPA	Whole transcriptome sequencing	Methods		Additional genetic mutation				
				Whole exome sequencing	Samples obtained at	MLPA	Signaling/adhesion	Epigenetic regulator	Fusion points	
Case-1	<i>TCF3</i>	○			Newly diagnosed	NP		NT		Ex11-Ex3
Case-2	<i>TCF3</i>	○			Newly diagnosed	NP		NT		Ex13-Ex3
Case-3	<i>TCF3</i>	○			Newly diagnosed	NP		NT		Ex17-Ex7
Case-4	<i>TCF3</i>	○	○		Newly diagnosed	<i>RBI</i> del		NP		Ex13-Ex3
Case-5	<i>TCF3</i>	○	○	○	Newly diagnosed	NP			<i>ASHIL</i>	Ex13-Ex3
Case-6	<i>TCF3</i>	○			Newly diagnosed	NP		NP		Ex13-Ex3
Case-7	<i>TCF3</i>	○	○	○	Newly diagnosed	NP	<i>PTPN11</i>			Ex13-Ex3
Case-8	<i>TCF3</i>				Newly diagnosed	NT		NT		Ex17-Ex7
Case-9	<i>TCF3</i>	○			Newly diagnosed	NP		NT		Ex11-Ex3
Case-10	<i>TCF3</i>				Newly diagnosed	NT		NT		Ex16-Ex2
Case-11	<i>TCF3</i>	○			Newly diagnosed	<i>CDKN2A/2B</i> del		NT		Ex17-Ex7
Case-12	<i>TCF3</i>	○			Newly diagnosed	NP		NT		Ex13-Ex3
Case-13	<i>TCF3</i>	○	○	○	1 st relapse	NP	<i>NRAS</i>		<i>EZH2</i>	Ex11-Ex3
Case-14	<i>TCF3</i>		○	○	Newly diagnosed	NP	<i>KRAS, PTPN11</i>		<i>MLL2</i>	Ex13-Ex3
Case-15	<i>TCF3</i>		○		Newly diagnosed	NP			<i>ASHIL</i>	Ex13-Ex2
Case-16	<i>TAF15</i>				Newly diagnosed	NT		NT		Ex6-Ex3
Case-17	<i>TAF15</i>	○	○	○	Newly diagnosed, 1 st relapse	NP		NP		Ex6-Ex3
Case-18	<i>TAF15</i>		○		Newly diagnosed	NP	<i>NRAS</i>		<i>MLL2</i>	Ex6-Ex3
Case-19	<i>CREBBP</i>	○		○	Newly diagnosed	<i>CDKN2A/2B</i> del		NP		Ex6-Ex2, Ex5-Ex3
Case-20	<i>CREBBP</i>	○			Newly diagnosed	NP		NT		Ex6-Ex3, Ex5-Ex3, Ex4-Ex3
Case-21	<i>CREBBP</i>				Newly diagnosed	NT		NT		Ex6-Ex3, Ex4-Ex3, Ex7-Ex3
Case-22	<i>BMP2K</i>			○	Newly diagnosed	NP		NP		Ex14-Ex3, Ex15-Ex3

MLPA: Multiplex Ligation-dependent Probe Amplification; NP: not particular; NT: not tested; del: deletion; Ex: exon.

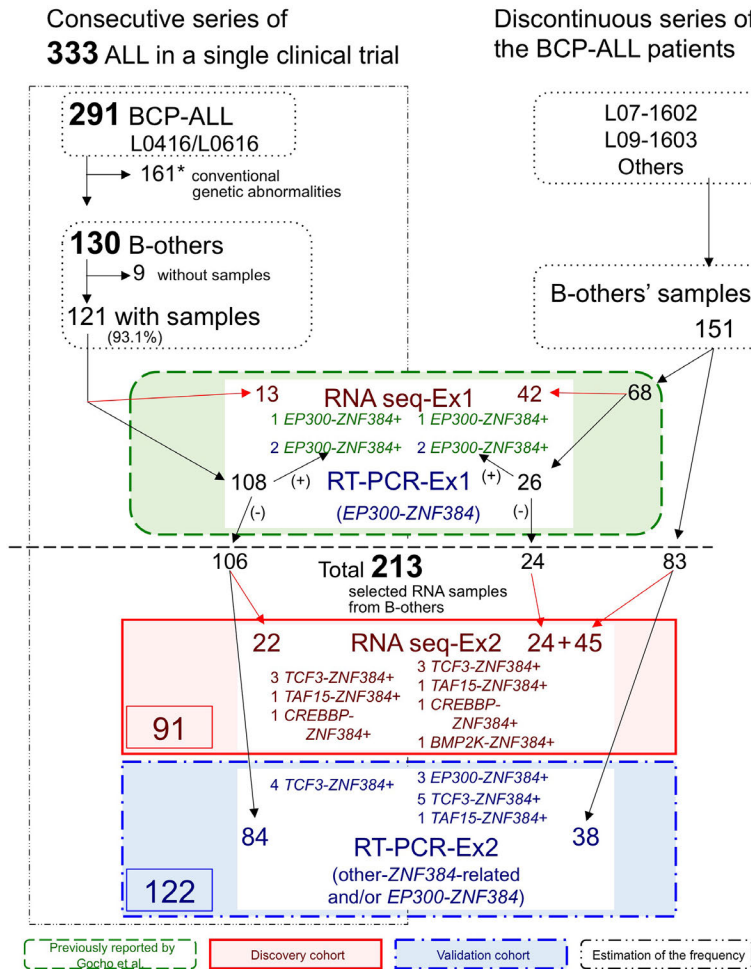


Figure 1. Flow chart of the analysis of patients. The respective numbers of patients and cohorts that were investigated are presented in a hierarchical fashion. See also the *Online Supplementary Information* and *Online Supplementary Table S1*. ALL: acute lymphoblastic leukemia; BCP-ALL: B-cell precursor ALL; B-others: BCP-ALL without conventional genetic abnormalities; RNA: ribonucleic acid; RNA-seq-Ex1 and Ex2: RNA-sequencing experiment 1 and 2; RT-PCR-Ex1 and Ex2: reverse transcription polymerase chain reaction experiment 1 and 2; Ex: experiment.

Information. For the confirmation of the whole transcriptome screening results and screening for additional cases in the validation cohort, RT-PCR was performed as described previously,¹⁸ using the primers listed in the *Online Supplementary Table S2*, followed by Sanger sequencing. The details of the data analyses of WTS to identify genetic mutations and deletions are also described in the *Online Supplementary Information*.

Whole exome sequencing (WES)

Exome libraries were prepared from 100 ng of genomic DNA using the Nextera Rapid Capture Exome Kit (Illumina, Inc., San Diego, CA, USA), and sequenced using SBS v4 reagents with the HiSeq2500 sequencing system (Illumina). More than 10 Gb of sequence was obtained for each library by paired-end sequencing (126 bp x2). The details of the whole exome data analyses are described in the *Online Supplementary Information*.

Microarray and data analyses

The complementary DNAs (cDNAs) were amplified and labeled using the Ovation® Pico WTA System and Encore™ Biotin Module (NuGEN Technologies Inc., San Carlos, CA, USA), as instructed by the manufacturer. The labeled probes were hybridized to Human Genome U133 Plus 2.0 Arrays (Affymetrix, Santa Clara, CA, USA). The arrays were analyzed using GeneChip Operating Software 1.2 (Affymetrix, Santa Clara, CA, USA) and GeneSpring GX 13.1 software (Agilent Technologies, Santa Clara,

CA, USA), and the details of data analyses, including gene set enrichment analysis (GSEA), are described in the *Online Supplementary Information*.

Statistical analysis

An unpaired *t*-test with Welch's correction was performed for the positivity of cell surface antigens.

Mutual univariable analysis of characteristics were conducted using the Fisher's exact test or χ^2 test for qualitative variables. Overall survival (OS) and event-free survival (EFS) were estimated by the Kaplan-Meier method and compared with the log-rank test. Analyses were performed with the use of Prism software, version 6.0 (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Detection of the fusion genes involving ZNF384 in pediatric BCP-ALL patients

The 213 selected RNA samples from B-others patients were analyzed by WTS and/or RT-PCR followed by Sanger sequencing (Figure 1 and *Online Supplementary Tables S1* and *S3*). We identified a total of 15, 3 and 3 patients with *TCF3-ZNF384*, *TAF15-ZNF384* and *CREBBP-ZNF384*, respectively. Three fusion genes were reported previously.^{9,10,12-15,19-22} In addition, we identified a

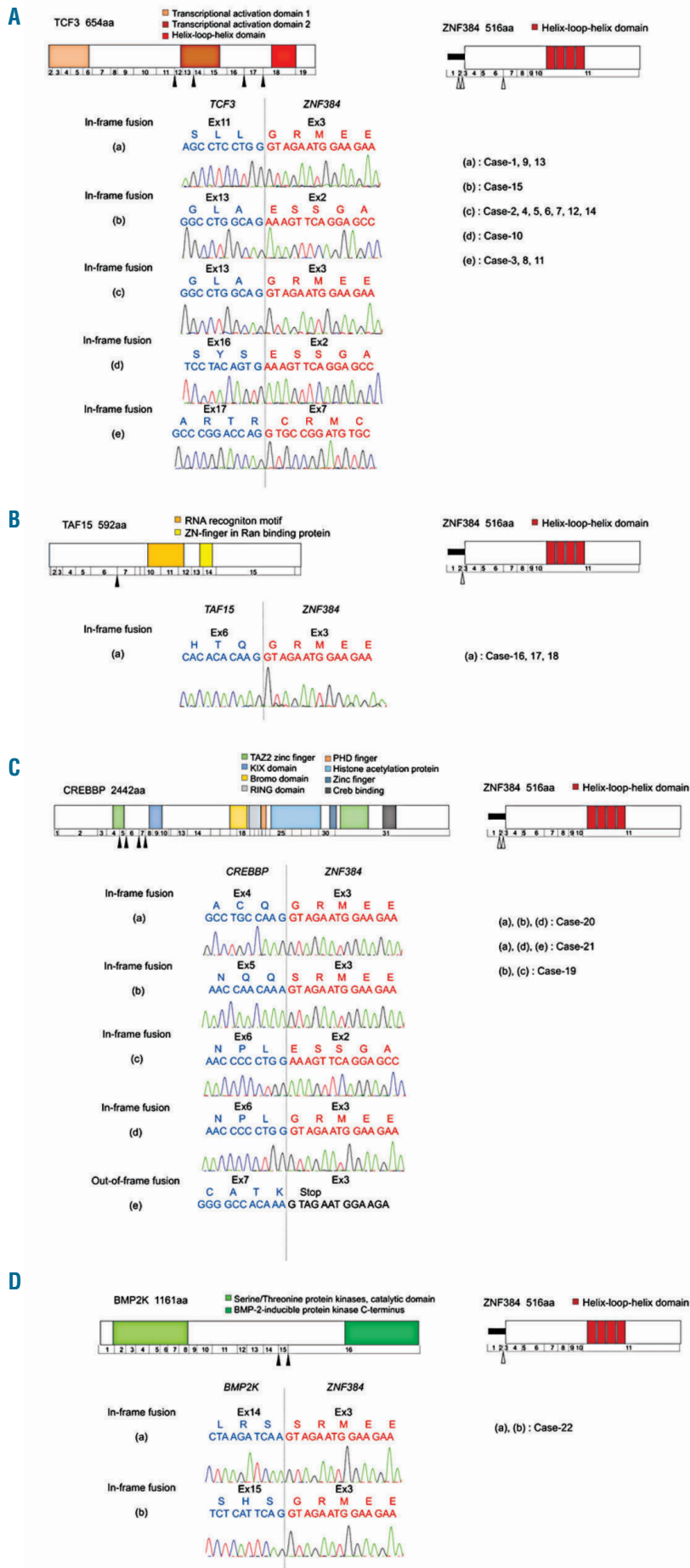


Figure 2. Structure of the ZNF384-related fusions. Structures of fusion proteins and nucleotide sequences of fusion points of (A) *TCF3-ZNF384*, (B) *TAF15-ZNF384*, (C) *CREBBP-ZNF384*, and (D) *BMP2K-ZNF384* are presented. Exon numbers and boundaries are marked below the protein structures. The black arrowhead shows the donor site fusion point. The white arrowhead shows the acceptor site fusion point. Five and four different in-frame fusions for *TCF3-ZNF384* and *CREBBP-ZNF384*, respectively, are depicted with the nucleotide sequence, predicted amino acids, and chromatogram. The patients in whom a particular fusion isoform was found are indicated on the right. Ex: exon; TAZ2: transcription adaptor putative zinc finger; KIX: kinase-inducible domain interacting; RING: really interesting new gene; PHD: plant homeodomain; CREB: cyclic adenosine monophosphate (AMP) response element binding protein; RNA: ribonucleic acid; ZN: zinc finger; RAN: Ras-related nuclear proteins; BMP-2: bone morphogenetic protein 2.

Table 2. Statistical comparison of clinical characteristics between TCF3-ZNF384-, EP300-ZNF384-, TCF3-PBX1-positive, and ZNF384-related fusion gene-negative B-cell precursor acute lymphoblastic leukemia (BCP-ALL).

	TCF3-ZNF384 (N=15)	EP300-ZNF384 (N=9)	TCF3-PBX1* (N=17)	ZNF384 wild-type* (N=115)	
Sex					
Male	8 (53%)	6 (67%)	10 (59%)	63 (55%)	
Female	7 (47%)	3 (33%)	7 (41%)	50 (43%)	
Age (years)					
1-6	10 (67%)	1 (11%)	10 (59%)	71 (62%)	#1
7-9	3 (20%)	2 (22%)	3 (18%)	14 (12%)	
>10	2 (13%)	6 (67%)	4 (24%)	26 (23%)	
WBC (x10 ⁹ /L)					
<20	5 (33%)	7 (78%)	7 (41%)	80 (70%)	#2
20-50	5 (33%)	0 (0%)	6 (35%)	17 (15%)	
50-100	1 (7%)	1 (11%)	2 (12%)	5 (4%)	
>=100	4 (27%)	1 (11%)	2 (12%)	4 (3%)	
Initial risk					
SR	1 (7%)	1 (11%)	3 (18%)	42 (37%)	#3
IR	10 (67%)	6 (67%)	12 (71%)	53 (46%)	
HR	4 (27%)	2 (14%)	2 (12%)	4 (3%)	
Treatment response					
PGR	7 (47%)	8 (89%)	14 (82%)	99 (86%)	#4
PPR	8 (53%)	1 (11%)	3 (18%)	5 (4%)	

Wild-type ZNF384; BCP-ALL without conventional genetic abnormalities as well as ZNF384-related fusions; SR: standard-risk; IR: intermediate-risk; HR: high-risk; WBC: white blood cells; PGR: predomine good responder; PPR: predomine poor responder. *L0416/0616-registered cases. #P<0.01 EP300-ZNF384 vs. wild-type ZNF384; P<0.05 EP300-ZNF384 vs. TCF3-ZNF384. #P<0.05 TCF3-ZNF384 vs. wild-type ZNF384, TCF3-PBX1 vs. wild-type ZNF384. #P<0.01 TCF3-ZNF384 vs. wild-type ZNF384; P<0.05 EP300-ZNF384 vs. wild-type ZNF384. #P<0.01 TCF3-ZNF384 vs. wild-type ZNF384.

novel ZNF384-related fusion gene, namely BMP2K-ZNF384 (1 patient). Although we recently reported 6 patients with EP300-ZNF384,¹¹ another 3 patients with this fusion gene were further identified in this study (Figure 1 and Online Supplementary Tables S1 and S4).

Structure of the fusion genes involving ZNF384

The structure and sequences of the fusion points of each fusion gene as well as a schematic representation of the predicted fusion proteins are depicted in Figure 2. In 3 out of 15 TCF3-ZNF384 patients, the fusion point of ZNF384 was the same as those previously reported, and exon 11 of TCF3 was fused to exon 3 of ZNF384 (presented in Figure 2A as Type (a)).¹⁰ In addition to this known fusion point, we identified several novel fusion points of this fusion gene. Most frequently, for example, exon 13 of TCF3 was fused to exon 3 of ZNF384 with in-frame joining in 7 patients (Figure 2A Type (c)). The predicted protein from all of the fusions does not have the principal functional domains of wild-type TCF3, whereas it retains almost all of the part of the ZNF384 protein.

The structure and sequences of the fusion point of TAF15-ZNF384 are depicted in Figure 2B. Although several isoforms have been reported in this fusion gene,¹⁶ only one isoform joining exon 6 of TAF15 to exon 3 of ZNF384 was observed in 3 of our patients.

In the case of CREBBP-ZNF384, we identified 5 isoforms in 3 patients (Figure 2C). The exons 4, 5, and 6 of CREBBP fused in-frame to exon 3 or 2 of ZNF384 (Figure 2C Type (a-d)). Among all of the fusion points, most functional domains of CREBBP were lacking in the resulting fusion proteins, while the complete ZNF384 protein was retained. In contrast, exon 7 of CREBBP was fused out-of-frame to exon 3 of ZNF384 in 1 patient (Figure 2C Type (e)).

In the case of BMP2K-ZNF384, 2 isoforms were identi-

fied in one patient and nucleotide 2228 (exon 15) and nucleotide 2117 (exon 14) of BMP2K were fused in-frame to nucleotide 266 (exon 3) of ZNF384 (Figure 2D, Type (a, b)), and the predicted protein retained the kinase domain of BMP2K but lacked the c-terminus portion. The 3 patients with EP300-ZNF384 exhibited identical split sequences, as we reported previously (*data not shown*).¹¹

Frequency of ZNF384-related fusion gene-positive patients

A consecutive series of 333 ALL patients enrolled in the TCCSG L0416/0616 study¹¹ included 130 B-others patients and 161 BCP-ALL patients with conventional genetic abnormalities (291 BCP-ALL patients, Figure 1 and Online Supplementary Table S1). Since the majority of these B-others patients of the L0416/0616 cohort (121 patients, 93.1% of B-others) were studied in the present analysis, we estimated the frequency of ZNF384-related fusion genes in childhood ALL based on this cohort. As we reported previously, 3 patients with EP300-ZNF384 were included in the L0416/0616 cohort.¹¹ In the study herein, we identified another 9 patients with ZNF384-related fusion genes in this cohort. Therefore, the frequency of the total of 12 patients with ZNF384-related fusion genes in B-others was estimated as 9.2% (4.1% in BCP-ALL, Figure 1 and Online Supplementary Table S1). Of those fusion genes, TCF3-ZNF384 was the most recurrent (7 patients), with a frequency of 5.4% in B-others (2.4% in BCP-ALL).

Immunophenotypic characteristics of BCP-ALL patients with ZNF384-related fusion genes

As we reported previously, all BCP-ALL patients with EP300-ZNF384 showed a dull or negative expression of CD10 and aberrant expression of one or more myeloid antigens based on immunophenotypic examination.^{11,13}

Similar to *EP300-ZNF384*-positive patients, all of the other BCP-ALL patients with *ZNF384*-related fusion genes were found to have a weak or negative expression of CD10, ranging from 0.39% to 67.34% (mean: $19.44 \pm 18.23\%$, Figure 3 and *Online Supplementary Table S5*), and there was a significant difference compared with *TCF3-PBX1*-positive patients and B-others in which *ZNF384*-related fusion genes were not identified (wild-type *ZNF384*), for the most part strongly expressing CD10 (mean: $98.66 \pm 0.89\%$, $P < 0.001$ and mean: $77.71 \pm 25.79\%$, $P < 0.001$, respectively).

In addition, 7 and 17 patients out of 22 patients with *ZNF384*-related fusion genes (31.82% and 77.27%, respectively) exhibited more than 20% expression of CD13 (mean: $10.70 \pm 10.37\%$) and CD33 (mean: $59.86 \pm 34.01\%$), significantly distinct from BCP-ALL patients with *TCF3-PBX1* and wild-type *ZNF384* expressing neither CD13 (mean: $0.26 \pm 0.33\%$, $P < 0.001$ and mean: $2.76 \pm 7.9\%$, $P < 0.0015$, respectively), nor CD33 (mean: $0.59 \pm 0.76\%$, $P < 0.001$ and mean: $6.63 \pm 1.72\%$, $P < 0.0001$, respectively).

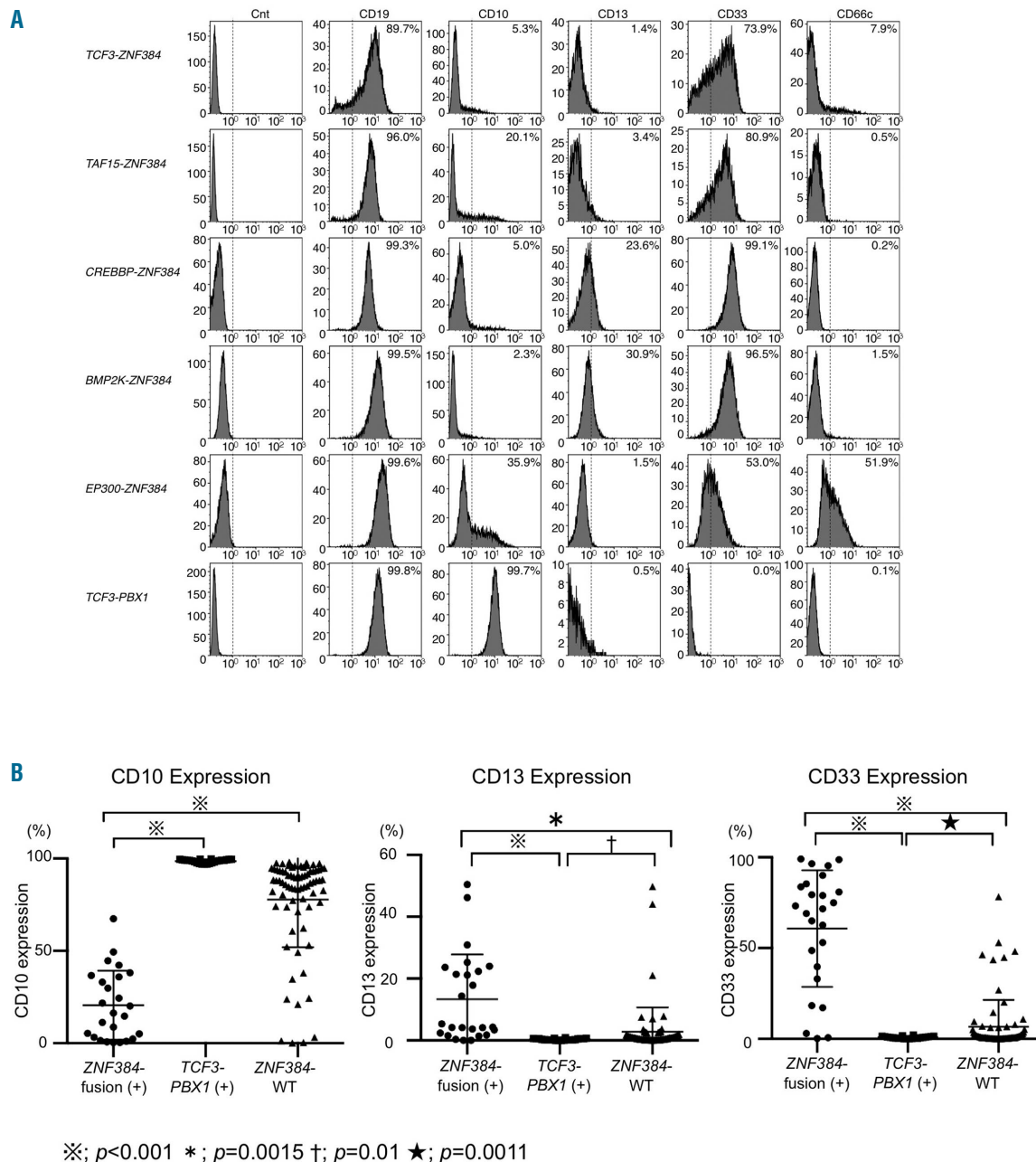


Figure 3. Immunophenotypic characteristics of B-cell precursor acute lymphoblastic leukemia (BCP-ALL) patients with *ZNF384*-related fusion genes. (A) Typical histograms of CD19, CD10, aberrant myeloid antigens (CD13 and CD33), and CD66c of *ZNF384*-related fusion gene-positive patients and *TCF3-PBX1* patients are indicated with a positive rate (%). X-axis, fluorescence intensity; Y-axis, relative cell number. (B) The positivity (percentage) of CD10, 13, and 33 of *ZNF384*-related fusion gene-positive BCP-ALL patients (22 cases, excepting *EP300-ZNF384*) and *TCF3-PBX1*-positive or wild-type *ZNF384* BCP-ALL patients was plotted on a scattergram. The detailed list of positivity for each immunophenotypic marker of the patients is presented in the *Online Supplementary Table S5*. *ZNF384-WT* indicates cases without *ZNF384* rearrangement.

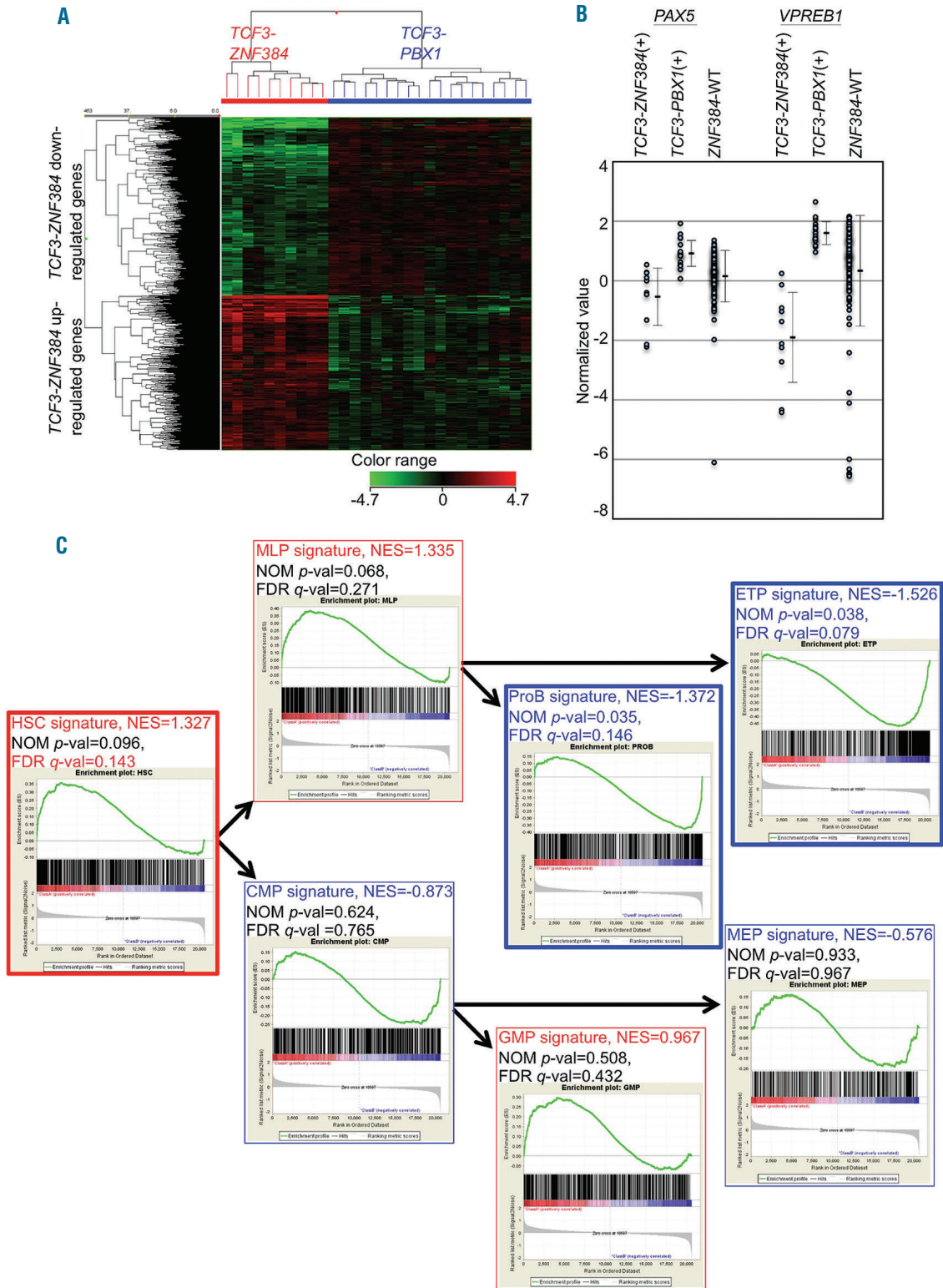


Figure 4. Characteristics of gene expression profile in TCF3-ZNF384-positive acute lymphoblastic leukemia (ALL). (A) Two-way hierarchical clustering was performed on filtered microarray probes, those up- or downregulated by 2-fold or more in TCF3-ZNF384- (red, n=10), in comparison with TCF3-PBX1-positive (blue, n=19) ALL. The results are displayed using a heat map as a dendrogram. (B) Among the genes that exhibited a 2 times higher or lower expression in either TCF3-ZNF384- and TCF3-PBX1-positive ALL (listed in the *Online Supplementary Table S6*), the expressions of PAX5 and VPREB genes measured by microarray analysis were plotted on a scatter diagram, and bars representing the mean±SD are presented. The data of wild-type ZNF384 B-cell precursor acute lymphoblastic leukemia (BCP-ALL) patients are also presented. (C) Gene set enrichment analysis (GSEA) for curated gene sets of hematopoietic precursors was performed on the differentially expressed genes between TCF3-ZNF384- (red) and TCF3-PBX1-positive (blue) ALL. Enrichment plots for the hematopoietic stem cells (HSCs), multi-lymphoid progenitor (MLP), pro-B cell (Pro-B), early T-cell precursors (ETP), common myeloid progenitors (CMP), granulocyte-monocyte progenitor (GMP), and megakaryocyte-erythroid progenitor cell (MEP) signatures are presented. Bold lines represent significant enrichments (false discovery rate (FDR) q-value<0.25 and/or nominal (NOM) P-value<0.05)). NES: normalized enrichment score.

Additional genetic abnormalities in ZNF384-related fusion gene-positive patients

To detect the additional genetic abnormalities in BCP-ALL with *ZNF384*-related fusion genes, we performed multiplex ligation-dependent probe amplification (MLPA) analysis. However, MLPA analysis did not reveal that *ZNF384*-translocated cases had deleted or amplified regions in *IKZF1*, *PAX5*, *ETV6*, *BTG1*, *EBF1*, *CRLF2*, and *ERG*, and only 2 patients with *CDKN2A/B* deletion and 1 patient with *RB1* deletion out of 15 were detected (Table 1 and *Online Supplementary Figure S1*).

We preliminarily identified 126 genes as candidates for recurrent genetic abnormalities occurring in BCP-ALL by performing somatic point mutation calling from matched leukemia-normal patient samples based on the results from WES data from 100 patients with pediatric BCP-ALL. We therefore subjected 7 DNA samples of patients with *ZNF384*-related fusion genes to WES to filtrate the 126 genes by multi-sample calling for single-nucleotide variants and short insertions/deletions (*Online Supplementary Information*). Together with the data of WTS performed for 9 patients (6 patients were subjected to both WES and WTS), we analyzed them to identify somatic mutations impacting coding sequences. As a consequence, we identified 7 recurrently mutated genes which had been recognized earlier as abnormalities involved in leukemia, including *NRAS*, *KRAS*, *PTPN11*, *EZH2*, *MLL2*, and *ASH1L* (Table 1 and *Online Supplementary Figure S1*). Principally, activating mutations in RAS signaling pathway genes, including *NRAS*, *KRAS*, and *PTPN11*, were detected in 4 out of 10 *ZNF384*-translocated patients subjected to WES and/or WTS. On the other hand, mutations in B-cell developmental genes, such as *PAX5*, *VPREB1*, *BTG1*, and *IKZF1*, were not detected at all in DNA samples from BCP-ALL with *ZNF384*-related fusion genes.

Gene expression profile of TCF3-ZNF384-positive patients

To assess the functional aspects of *TCF3-ZNF384*, we performed DNA microarray-based expression profiling. Hierarchical clustering analysis using the selected gene probe sets²³ showed that the major subtypes of BCP-ALL patients with conventional genetic abnormalities separated into distinct clusters. Remarkably, *ZNF384*-related fusion gene positive patients were located in the same cluster and clearly separated from those of other conventional genetic subtypes (*Online Supplementary Figure S2A*) as well as *TCF3-PBX1*-positive patients (*Online Supplementary Figure S2B*). The data indicate that the gene expression profile of *TCF3-ZNF384*-positive ALL is related to *EP300-ZNF384*- and *CREBBP-ZNF384*-positive ALL, but not to other conventional genetic subtypes.

Therefore, we proceeded to directly compare the gene expression of the most frequent *TCF3-ZNF384*-positive ALL to that of *TCF3-PBX1*-positive ALL possessing the same fusion partner, and with both translocations disrupting one allele of *TCF3* that drives the B-cell differentiation program. As shown in the *Online Supplementary Figure S2C* and Figure 4A, *TCF3-ZNF384*-positive patients were clearly separated in a distinct cluster from *TCF3-PBX1*-positive patients by hierarchical clustering, and the differential expression of 3,698 genes (up: 1,984, down: 1,714, >2.0) were identified (*Online Supplementary Table S6*), indicating that two *TCF3*-translocated subtypes were signifi-

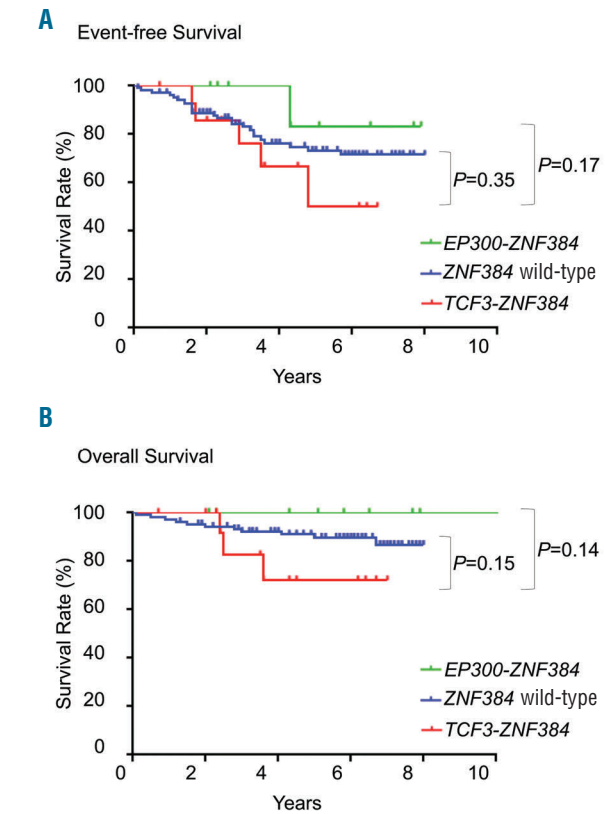


Figure 5. Outcomes of patients with *ZNF384*-related fusion genes. (A) Kaplan-Meier estimates of event-free survival (EFS) for patients with *TCF3-ZNF384*, *EP300-ZNF384*, and wild-type *ZNF384* (log-rank $P=0.35$ in wild-type *ZNF384* vs. *TCF3-ZNF384*, log-rank $P=0.17$ in *TCF3-ZNF384* vs. *EP300-ZNF384*). (B) Overall survival (OS) for the same as above (log-rank $P=0.15$ in wild-type *ZNF384* vs. *TCF3-ZNF384*, log-rank $P=0.14$ in *TCF3-ZNF384* vs. *EP300-ZNF384*).

cantly distinct. Interestingly, the B-cell developmental genes *VPREB1*, *PAX5*, and *IKZF1-3* were listed as more highly expressed genes in *TCF3-PBX1*-positive ALL than in *TCF3-ZNF384*-positive ALL (*Online Supplementary Table S6* and Figure 4B). The transcriptional regulators, such as *BACH2*, *HIST1H3A*, and *LEF1*, were also upregulated in *TCF3-PBX1*-positive ALL, while *GATA3*, *ERG*, *NCOR1*, and *TOX* were upregulated in *TCF3-ZNF384*-positive ALL. It is noteworthy that gene set enrichment analysis revealed a significant enrichment for hematopoietic stem cell (HSC) signatures in *TCF3-ZNF384*-positive ALL while lymphoid features, such as Pro-B cells and early T-cell precursors (ETP), were more prominent in *TCF3-PBX1*-positive ALL (Figure 4C and *Online Supplementary Table S7*). The signatures of granulocyte-macrophage progenitor (GMP)/multi-lymphoid progenitor (MLP) and megakaryocyte-erythroid progenitor (MEP) were enriched in *TCF3-ZNF384*- and *TCF3-PBX1*-positive ALL, respectively, whereas statistical significance was not observed.

The above analyses could just as well detect the lack of specific *TCF3-PBX1* features rather than the common features among cases with *TCF3-ZNF384*. Thus we subsequently compared the gene expression of *TCF3-ZNF384*-positive ALL to that of wild-type *ZNF384*. There is the possibility that the wild-type *ZNF384* patients still include a variety of subgroups harboring novel genetic abnormali-

ties, and some of them might be related to ZNF384-related fusion genes by their gene expression. Indeed, hierarchical clustering analysis showed that wild-type ZNF384 patients were not necessarily located in the same cluster, and some of the cases were dispersed in the clusters of the major subtypes of BCP-ALL with conventional genetic abnormalities (Online Supplementary Figure S2D), indicating the heterogeneity of wild-type ZNF384 patients. Furthermore, hierarchical clustering of ZNF384-related fusion gene positive patients and wild-type ZNF384 patients indicated that 21 out of 104 wild-type ZNF384 patients located in the same cluster as ZNF384-related fusion gene positive patients (Online Supplementary Figure S2E). However, when we compared TCF3-ZNF384-positive patients and wild-type ZNF384 patients; excepting those located in the ZNF384-related fusion gene positive cluster; they were clearly separated into a distinct cluster by hierarchical clustering (Online Supplementary Figure S2F), and the differential expression of 4,515 genes (up: 2,485, down: 2,030, >2.0) were identified (Online Supplementary Table S8). The B-cell developmental genes *VPREB1* and *IKZF1*, but not *IKZF2* or *IKZF3*, were listed as more highly expressed genes in wild-type ZNF384 ALL than in TCF3-ZNF384-positive ALL, and *PAX5* also listed as a relatively highly expressed gene in wild-type ZNF384 ALL (1.83-fold). The transcriptional regulators such as *BACH2*, *HIST1H3A*, and *LEF1* were also upregulated in wild-type ZNF384 ALL, while *GATA3* and *NCOR1*, but not *ERG* and *TOX*, were upregulated in TCF3-ZNF384-positive ALL. The gene set enrichment analysis also revealed an enrichment for HSC, GMP and MLP signatures in TCF3-ZNF384-positive ALL while Pro-B, ETP and MEP were more prominent in wild-type ZNF384 ALL, whereas statistical significance was not obvious (Online Supplementary Figure S2G and Online Supplementary Table S7).

Clinical characteristics and outcomes of ZNF384-related fusion gene positive patients

The clinical findings and outcomes of BCP-ALL patients with ZNF384-related fusion genes, other than EP300-ZNF384, are summarized in Table 1. They were aged between 1 and 12 years (median: 3) at presentation and comprised of 12 males and 10 females. Their initial white blood cell (WBC) counts at presentation ranged from 1,220 to 150,200 (median: 26,975). The analysis of fluids obtained by lumbar puncture revealed no indication of central nervous system involvement, with the exception of one patient with TCF3-ZNF384 (Case-15). Thirteen out of 22 patients (59.1%) were classified into an IR at the initial diagnosis, based on an advanced age (4 patients), elevated WBC counts (7 patients), or both (2 patients). In addition, 5 patients were classified as HR (22.7%) based on markedly elevated WBC counts, but not an advanced age, and thus the SR included only 4 patients (18.2%). Five out of 15 (33.3%) patients with TCF3-ZNF384 had a relapse in the bone marrow, all of the patients received stem-cell transplantation, and 3 patients died. One patient each of TAF15- and CREBBP-ZNF384-positive patients had a relapse in the bone marrow, but both patients were salvaged by chemotherapy. It should be emphasized that no patient died in those with ZNF384-related fusion genes, other than TCF3-ZNF384.

Importantly, 21 out of 22 patients showed a normal karyotype on cytogenetic analysis by conventional G-banding, but the predicted chromosomal translocations,

including t(12;19)(p13;p13) for TCF3-ZNF384, t(12;17)(p13;q11) for TAF15-ZNF384, t(12;16)(p13;p13) for CREBBP-ZNF384, and t(4;12)(q21;p13) for BMP2K-ZNF384 were not detected at all (Table 1). The remaining patient with CREBBP-ZNF384 (Case-19) showed 47,X,add(X)(p22),t(12;12)(p13;q13),+21 at the initial diagnosis, but this was not consistent with the predicted chromosomal translocation.

Since TCF3-ZNF384 was the most recurrent among ZNF384-related fusion genes detected in the pediatric BCP-ALL patients, we next compared the characteristics of TCF3-ZNF384-positive patients with those of wild-type ZNF384, as well as EP300-ZNF384- and TCF3-PBX1-positive patients (Table 2). When compared with wild-type ZNF384, initial WBC counts and a trend to be classified in higher risk groups (IR and HR) were significantly higher in TCF3-ZNF384-positive patients ($P < 0.05$ and $P < 0.01$, respectively). In contrast, the age at diagnosis was not significantly different between TCF3-ZNF384-positive patients and those with wild-type ZNF384. The response to steroid monotherapy using the cut-off of 1,000/ μ L for the blast count in peripheral blood on day 8 was significantly poorer in TCF3-ZNF384-positive patients than in those with wild-type ZNF384 ($P < 0.01$).

As we reported previously, BCP-ALL patients with EP300-ZNF384 exhibit an advanced age but not significantly elevated WBC counts.¹¹ Indeed, compared to the patients with wild-type ZNF384, those with EP300-ZNF384 exhibit a significantly older age at presentation ($P < 0.01$) but not significantly elevated WBC counts ($P = 0.37$), as presented in Table 2. Therefore, the clinical features of TCF3-ZNF384-positive patients were clearly distinct from those of EP300-ZNF384-positive patients. In the case of TCF3-PBX1-positive ALL, initial WBC counts were significantly higher than in wild-type ZNF384, whereas other features were not significantly different.

The 5-year EFS among TCF3-ZNF384-positive patients and those with wild-type ZNF384 were 50.0% and 73.3% ($P = 0.35$) and the 5-year OS rates were 72.2% and 89.8% ($P = 0.15$), respectively (Figure 5). Although there was no statistic significance, the clinical outcomes of TCF3-ZNF384-positive patients were more unfavorable than in wild-type ZNF384 patients. In contrast, the 5-year EFS and the 5-year OS rates of EP300-ZNF384-positive patients were 83.3% and 100%, respectively, thus being more favorable than in TCF3-ZNF384-positive and wild-type ZNF384 patients.

Discussion

We identified a novel fusion gene, BMP2K-ZNF384, in addition to the previously reported ZNF384-related fusion genes, including TCF3-, TAF15-, EWSR-, EP300-, CREBBP-, SYNRG-, and ARID1B-ZNF384, and thus 8 fusion partners for the ZNF384 gene have been identified so far.^{9,15,19,22} Considering the current condition, whereby the frequency of B-others in which specific cytogenetic abnormalities are not present are markedly decreasing, the incidence of ZNF384-related fusion genes in our cohort was unexpectedly high. In the literature, 8 ALL patients with TAF15-ZNF384 have been reported.^{9,19,21} Similarly, 2 and 3 ALL patients with EWSR1- and TCF3-ZNF384, respectively, have also been described previously.^{9,10,20,22} Because 6 out of 13 patients in the literature are adults

(>21 years), this is the first report on the high frequency of the recurrence of *ZNF384*-related fusion genes in childhood BCP-ALL in a single nation. It is possible that the differences in the outbreak frequency of *ZNF384*-related fusion genes in BCP-ALL depending on racial differences are present, and this type of fusion might be common in Asians but not in Caucasians. Alternatively, since *ZNF384*-related fusion genes are difficult to detect with conventional G-banding, as we presented in this report, *ZNF384*-related fusion genes may be found in Caucasians at a similar frequency to that in our cohort upon using more sensitive methods such as PCR or fluorescence *in situ* hybridization (FISH).

As we presented herein, BCP-ALL patients with *ZNF384*-related fusion genes possess a characteristic immunophenotype. All of the BCP-ALL patients with *ZNF384*-related fusion genes, including *EP300-ZNF384* that we reported recently, exhibit the dull or negative expression of CD10 and for the most part express CD13 and/or CD33. Our observations are consistent with previous reports because at least 8 among 13 patients with *ZNF384*-related fusion genes were CD10-negative and expressed CD13 and/or CD33 based on the literature.^{10,20-}

²² Since *ZNF384* is a commonality in all of these fusion genes, the aberrant function of the *ZNF384* protein may be responsible for the characteristics of the immunophenotype. The fact that BCP-ALL patients with *TCF3-PBX1* are clearly positive for CD10 and have no aberrant myeloid antigens should support the above notion. A correlation between the high expression of the *GATA3* gene and CD13/CD33 expression on primary BCP-ALL blasts with *ZNF384*-related fusion genes was suggested,¹⁵ whereas the role of *ZNF384*-fusion proteins in the expression characteristics of the antigen remains largely unknown. BCP-ALL harboring *MLL* rearrangement also reveals the negative expression of CD10 and aberrant expression of myeloid antigens, while such patients frequently express CD65 and CD15 as aberrant myeloid antigens and are mainly accompanied by NG2 (7.1) expression.²³ Thus, the presence of *ZNF384*-related fusion genes should be predictable, based on the characteristic immunophenotype of dull or negative CD10 expression accompanied by the aberrant expression of CD13 and/or CD33.

In contrast to the similarity of the immunophenotypic characteristics, the clinical features of BCP-ALL with the *ZNF384*-related fusion gene are not uniform, and are thought to depend on the fusion partner gene of *ZNF384*. In the *TCF3-ZNF384* fusion gene-positive ALL patients, more than half of the patients were classified into an IR or HR group because of an elevated WBC count at presentation, while their onset age was not advanced and there was no significant difference compared with wild-type *ZNF384* patients. In addition, approximately half of the patients exhibited a poor response to prednisolone, and one-third relapsed, indicating that BCP-ALL patients with the *TCF3-ZNF384* fusion gene include a certain proportion of patients with a poor response to conventional chemotherapy. It is noteworthy that stem cell transplantation is effective as salvage therapy for a part of the relapsed *TCF3-ZNF384*-positive patients. On the other hand, we previously reported that *EP300-ZNF384* fusion gene-positive patients exhibited a relatively advanced age but no significant elevation of the WBC count at presentation, and showed a good response to prednisolone as well as a favorable response to conventional chemotherapy.¹¹

Moreover, although one patient has relapsed, the OS rate of 9 patients with *EP300-ZNF384* is currently 100%. Therefore, the clinical features are significantly different between *TCF3-ZNF384*-positive and *EP300-ZNF384*-positive patients.

As the fusion genes involving the *TCF3* gene, *TCF3-PBX1*, *TCF3-ZNF384*, and *TCF3-HLF* are known, recent reports have indicated that the genomic and transcriptomic landscape of *TCF3-HLF*-positive ALL differs markedly from *TCF3-PBX1*-positive ALL, and that *TCF3-HLF*-positive ALL exhibited enriched stem cell and myeloid features, while lymphoid features were more prominent in *TCF3-PBX1*-positive ALL.²⁴ Moreover, the recurrent intragenic deletions of *PAX5* or *VPREB1* as well as activating mutations in the RAS signaling pathway genes (*NRAS*, *KRAS*, and *PTPN11*) were common in *TCF3-HLF*-positive ALL but rare in *TCF3-PBX1*-positive ALL. In the case of *TCF3-ZNF384*-positive ALL, abnormalities in such B-cell developmental genes were also revealed to be rare. However, the gene expression analysis of *TCF3-ZNF384*-positive ALL revealed that *PAX5* as well as *VPREB1* expressions were lower compared with *TCF3-PBX1*-positive and wild-type *ZNF384* ALL, and the gene expression profile of *TCF3-ZNF384*-positive ALL was markedly distinct from that of *TCF3-PBX1*-positive and wild-type *ZNF384* ALL. In addition, the signature of *TCF3-ZNF384*-positive ALL exhibited enrichment of the stem cell signature, similar to that of *TCF3-HLF*-positive ALL. The low expression of the gene regulating the pro- to pre-B-cell transition and expression of a HSC/myeloid-signature could be features of *TCF3-ZNF384*-positive ALL. Our data also indicate the activating mutations in RAS signaling pathway genes and *CDKN2A/B*-deletion in a part of the patients with *TCF3-ZNF384*. Further studies involving a large series of patients would be required to elucidate the prognostic impact of these gene abnormalities in a part of *TCF3-ZNF384*-positive patients.

It has been shown that *TAF15*-, *EWSR1*-, and *TCF3-ZNF384* induce 3T3 fibroblast transformation.^{9,25} Since *ZNF384* is a commonality in 8 related fusion genes identified in BCP-ALL, the aberrant function of the *ZNF384* protein may participate in the development of BCP-ALL, whereas the precise function of *ZNF384* in cellular transformation has yet to be clarified. Furthermore, the role of the defects in the function of TET family partner molecules in the development of ALL is still controversial. On the other hand, the loss of functions of both EP300 and CREBBP has been reported to participate in tumorigenesis by means of epigenetic alterations mediated by reduced histone acetylation activities.²⁶⁻²⁹ Recent reports also described the marked involvement of *CREBBP* mutations in the relapse of high hyperdiploid BCP-ALL.²⁷⁻²⁸ Therefore, both the loss of function in CREBBP or EP300 and the gain of aberrant *ZNF384* function could cooperatively affect leukemogenesis in ALL harboring these two fusion genes. In contrast, the role of BMP2K abnormality in leukemogenesis is unclear. Further studies to investigate the molecular basis of *ZNF384*-related fusion proteins are required to clarify the precise role of the abnormalities of *ZNF384* as well as its fusion partner genes in leukemogenesis.

In conclusion, we clarified that ALL patients harboring *ZNF384*-related fusion genes constitute a subgroup with a characteristic immunophenotype in B-others of a Japanese cohort with an unexpectedly high incidence, and that the

TCF3-ZNF384 fusion gene is the most recurrent. The clinical features of BCP-ALL with ZNF384-related fusion genes, however, depend on the functional defect of the fusion partner gene of ZNF384. Additional studies to elucidate the biological function of the ZNF384-related fusion protein should shed light on the molecular basis of the development of BCP-ALL.

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