When summer season was defined as June to September based on a minimum outside temperature ≥15°C, HR, DP, and SI showed significant inverse associations with the daily minimum outside temperature in summer when compared with the other seasons. Systolic blood pressure and DP were consistently and significantly correlated with daily minimum outside temperature throughout the year (summer: β (SBP) = −0.3055 and β (DP) = −0.2172, and in the other seasons: β (SBP) = −0.1999 and β (DP) = −0.2051, all $P < .0001$). The correlations between HR and daily minimum outside temperature were significant but weak in each season (summer: $\beta = 0.0095$, in the other seasons: $\beta = -0.0560$, all $P < .0001$). Although SI was positively correlated with daily minimum outside temperature in summer ($\beta = 0.2235$), in the remaining seasons weak correlations were observed ($\beta = 0.0811$; $P = <.0001$). The interactions between daily minimum outside temperature and seasonality (summer vs. the other seasons) for SBP, HR, DP, and SI were significant (Table 1, all interactions $P < .0001$).

Hemodynamic Parameters, Gestational Age, and Seasonal Variation

The associations among hemodynamic parameters (SBP, HR, DP, and SI), gestational age, and seasonal variation are shown in Figure 3. Systolic blood pressure increased gradually and achieved its peak values (≥110 mm Hg) at gestational week 40. Heart rate increased gradually and reached its peak (≥75 bpm) at gestational week 32. Double product increased gradually as gestational age increased and reached its peak (≥8000 mm Hg bpm) at gestational week 40. The effect of expected date of birth on HR and DP was smaller than the effect of gestational age. On the other hand, women who gave birth in winter had a high SI (≥0.73 bpm/mm Hg) in their first or second trimester, and women who gave birth in autumn had a high SI (≥0.73 bpm/mm Hg) in their third trimester.

DISCUSSION

This is the first study to describe the relationships of HR, DP, and SI with a combination of gestational age and seasonality in a cohort of normal pregnant women. This study collected daily serial hemodynamic data during pregnancy based on the self-measurement of BP and HR at home.

Heart Rate

As in a previous study that used ambulatory BP measurement (16), home HR showed the highest value in gestational week 32 in this study. Seasonal variations exist in home BP values (17); however, there are few studies that have observed seasonal variation of HR. Some articles reported that there is no significant relationship between seasonality and HR (18,19), whereas Izzo et al. (20) reported wintertime HR increased by 7% ($P < .017$), with larger parallel increases in systemic vascular resistance (+24%, $P < .0017$) and plasma norepinephrine (+26%, $P < .017$). In this study, the association between HR and temperature was weak, but the association was significant.

Double Product

The DP of SBP and HR indicates cardiovascular load. It is a surrogate measure of myocardial oxygen demand and cardiac workload, which has recently become widely used in cardiovascular medicine (5). There is a report using ambulatory BP monitoring that showed that the DP increased from summer to winter (daytime) by 1053 mm Hg bpm for smokers (21). In this study, DP was also higher in winter than in summer. The interaction between seasonality and temperature on the effect on DP was significant.

Rang et al. (4) reported that cardiac output was higher in preeclampsia or gestational hypertension without fetal growth restriction but not in preeclampsia or gestational hypertension with fetal growth restriction. Recently, De Paco et al. (3) reported that cardiac output between 11^{+0} and 13^{+6} weeks of gestation was increased in women who developed preeclampsia. In this study, DP increased gradually as gestational age increased, and the effect of seasonality and expected date of birth on DP was less marked than that of gestational age. Double product might be a good way of evaluating cardiac workload in pregnancy due to its linear association with gestational age.

Shock Index

Shock index, which is calculated from HR divided by SBP, is an effective way to diagnose hemorrhagic shock

Table 1. Associations between hemodynamic parameters and daily minimum outside temperature in summer and in other seasons

		Summer (from June to September)			Other seasons (from October to May)			Interaction[a]
		β	SE	P	β	SE	P	P
SBP	(mm Hg)	−0.3055	0.0049	<.0001	−0.1999	0.0074	<.0001	<.0001
HR	(bpm)	0.0095	0.0052	<.0001	−0.0560	0.0080	<.0001	<.0001
DP	(10^2 mm Hg bpm)	−0.2172	0.0069	<.0001	−0.2051	0.0104	<.0001	<.0001
SI	(10^{-2} bpm/mm Hg)	0.2235	0.0058	<.0001	0.0811	0.0088	<.0001	<.0001

Abbreviations: SBP – systolic blood pressure, HR – heart rate, DP – double product, SI – shock index.
[a]Interaction between daily minimum outside temperature and seasonality and hemodynamic parameters.

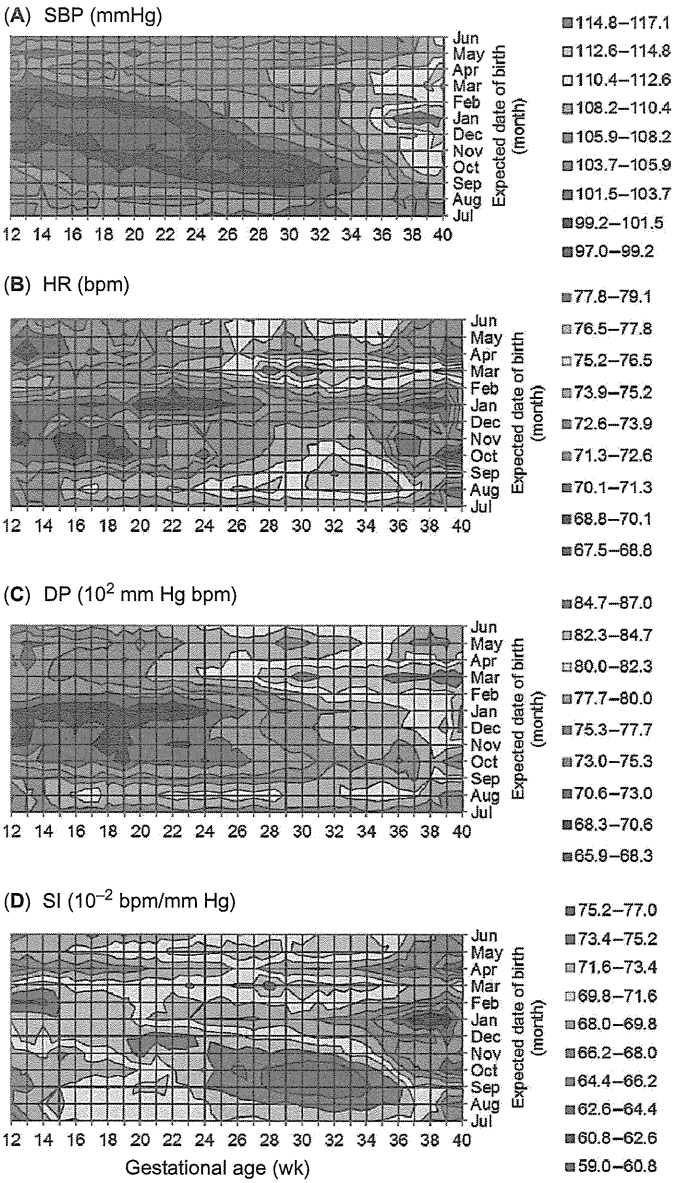

Figure 3. (A) Systolic blood pressure (SBP), (B) heart rate (HR), (C) double product (DP), and (D) shock index (SI) values for the combination of gestational age and expected date of birth, calculated on the basis of a mixed linear model. The horizontal axis shows gestational age, and the vertical axis shows the expected date of birth.

following injury, which is accompanied with hypotension and tachycardia (6,7). In this study, women who gave birth in winter had a high SI in their first or second trimester. Women who gave birth in winter spent their first trimester or second trimester during the summer season. The first trimester SI of the women who gave birth in winter was 0.72–0.77 bpm/mm Hg, while the first trimester SI of the women who were to give birth in other seasons was 0.66–0.70 bpm/mm Hg. Birkhahn et al. reported that acute blood loss of 450 mL significantly increased the SI from 0.61 to 0.65 bpm/mm Hg (7). Shock index might also represent hypovolemia. Women who are to give birth in winter might have hypovolemia in their first to second trimester. Similarly, women who are to give birth in autumn might have hypovolemia in their last trimester.

Plasma volume in pregnancies complicated by preeclampsia is reported to be significantly lower than in normal pregnancies in the first trimester (2). In this study, plasma volume is reported to be the first parameter to show significant intergroup difference among the parameters of progesterone, aldosterone, estradiol, and their combination. Although further studies are necessary to investigate which factors changed before and after hypovolemia, there might be some association between hypovolemia in the first trimester in summer and the

incidence of preeclampsia in winter. Shock index might be a good marker of dehydration in summer, since a similar trend was observed for HR (Figure 2).

LIMITATIONS

There are some limitations in this study. First, SI seems to be a good way to identify hypovolemia within one subject; however, it is impossible to compare SIs among individuals because the SI is low with high BP. Another method may be necessary to identify hypovolemia. There are no previous reports showing that SI reflects chronic hypovolemia, in the same way as acute hypovolemia. Further study is needed to evaluate the amplitude of hypovolemia in chronic conditions. Second, in our study, we did not perform echocardiography or electrocardiography; therefore, we cannot evaluate the real clinical meaning of the DP using such physiological examinations. Another approach might be necessary to evaluate the real clinical meaning of DP. Third, these data are limited to normotensive pregnant women, because we did not perform a similar analysis in preeclamptic women, since few subjects developed preeclampsia. Serial changes of indirect indices might be modified by hospitalization, medication, and termination in subjects with preeclampsia.

CONCLUSION

This study collected daily serial hemodynamic data during pregnancy using home BP monitoring. Double product increased gradually as gestational age increased, and the effect of seasonality and expected date of birth on Double product was less marked than that of gestational age. Shock index might be useful for identifying hypovolemia within individuals. Such data might be useful for examining hemodynamic changes during normal pregnancy, as well as identifying hemodynamic changes during abnormal pregnancy.

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Declaration of interest: H.M. is conducting a collaborative research with Omron Healthcare Ltd.

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Casitas B-cell lymphoma mutation in childhood T-cell acute lymphoblastic leukemia

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ABSTRACT

Somatic *CBL* mutations have been reported in a variety of myeloid neoplasms but are rare in acute lymphoblastic leukemia (ALL). We analyzed 77 samples from hematologic malignancies, identifying a somatic mutation in *CBL* (p.C381R) in one patient with T-ALL that was associated with a uniparental disomy at the *CBL* locus and a germline heterozygous mutation in one patient with JMML. Two *NOTCH1* mutations and homozygous deletions in *LEF1* and *CDKN2A* were identified in T-ALL cells. The activation of the RAS pathway was enhanced, and activation of the NOTCH1 pathway was inhibited in NIH 3T3 cells that expressed p.C381R. This study appears to be the first to identify a *CBL* mutation in T-ALL.

1. Introduction

Casitas B-cell lymphoma (CBL) is the cellular homologue of the v-Cbl transforming gene of the Cas NS-1 murine leukemia virus [1]. CBL primarily functions as an E3 ubiquitin ligase and is responsible for the intracellular transport and degradation of a large number of receptor tyrosine kinases. CBL also retains important adaptor functions; approximately 150 proteins associate with or are regulated by CBL [2]. The majority of *CBL* somatic mutations have been reported in myelodysplastic syndrome/myeloproliferative disorder (MDS/MPD), including chronic myelomonocytic leukemia (CMML; approximately 15%), juvenile myelomonocytic leukemia (JMML; approximately 17%) and atypical chronic myeloid leukemia (approximately 5%) [3–9]. *CBL* mutations are primarily associated with an 11q-acquired uniparental disomy (aUPD) that involves the *CBL* locus and converts *CBL* mutations into a homozygous state [3]. However, *CBL* mutations have been rarely reported in acute lymphoblastic leukemia (ALL).

Germline mutations in *CBL* have been identified in three JMML patients who displayed a variable combination of dysmorphic features reminiscent of the facial gestalt of Noonan syndrome [10], as well as in 17 children with JMML [11] and two patients with sporadic Noonan syndrome [12]. Noonan syndrome and related disorders are autosomal dominant congenital anomaly syndromes, and patients with these disorders have distinctive faces, heart defects, mental retardation and tumor predisposition [13]. *CBL* mutations have been shown to activate the downstream RAS pathway, and patients with germline *CBL* mutations have been grouped with those with Noonan syndrome and related disorders, i.e., RAS/mitogen-activated protein kinase (MAPK) pathway syndromes or RASopathies [13,14].

In this study, we analyzed somatic and germline *CBL* mutations in leukemia cells from 77 patients with hematopoietic malignancies and identified a somatic *CBL* mutation in a T-ALL sample. The functional properties of the mutant CBL protein were further analyzed.

2. Materials and methods

2.1. Patients with hematopoietic malignancies

A total of 77 children with hematopoietic malignancies (40 ALL, including 29 B cell ALL, 6 T-ALL, 1 mixed lineage ALL and 4 unknown; 28

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acute myeloid leukemia (AML); 3 malignant lymphoma; 2 transient abnormal myelopoiesis (TAM) associated with Down syndrome; 2 MDS; 1 JMML; and 1 CML) were studied (Supplementary Table 1). The AML subtypes, according to the French–American–British (FAB) classification, were as follows: M0 ($n = 6$), M1 ($n = 3$), M2 ($n = 8$), M4 ($n = 3$), M5 ($n = 4$), M7 ($n = 3$) and unknown subtype ($n = 1$). Bone marrow (BM) and/or peripheral blood (PB) cells were obtained from these patients at the time of diagnosis, and pleural effusions were obtained from the malignant lymphoma patients. Using a standard protocol, genomic DNA was prepared from the BM, PB and pleural effusion samples that contained tumor cells. The Ethics Committee of the Tohoku University School of Medicine approved this study.

2.2. Mutation analysis

Sequencing was conducted for exons 8 and 9 of *CBL*, exons 4–12 of *FBW7* and exons 26, 27 and 34 of *NOTCH1*, which correspond to the heterodimerization [HD] and proline-, glutamic acid-, serine- and threonine-rich [PEST] domains of NOTCH1. If a *CBL* mutation was detected in a sample, then the remainder of the coding exons of *CBL* were also sequenced (Supplementary Table 2). The PCR products were purified using a MultiScreen PCR plate (Millipore, Billerica, MA, USA) and sequenced on an Applied Biosystems 3500xL genetic analyzer (Applied Biosystems, Foster City, CA, USA).

2.3. SNP array karyotyping analysis

DNA from the T-ALL sample and the paired DNA from remission leukocytes were analyzed on a high-density Affymetrix single-nucleotide polymorphism array (SNP-A; 250 K) to identify loss of heterozygosity (LOH), microamplification and microdeletion, as described previously [15].

2.4. Construction of expression vectors

The expression construct pCMV6-CBL, which included the *CBL* cDNA, was purchased from OriGene (Rockville, MD, USA). One of two single-base substitutions, either c.1141T>C, resulting in p.C381R, or c.1259G>A, resulting in p.R420Q, was introduced using a QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA). All of the mutant constructs were verified by sequencing. An HES-Luc expression construct in the pGV-B vector [16] and a mouse intracellular NOTCH1 (ICN1) region expression construct in the pEF-BOSneo vector [17] were obtained from Riken BRC DNA Bank (Tsukuba, Ibaraki, Japan).

2.5. Reporter assay for ELK and c-Jun

NIH 3T3 cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). The NIH 3T3 cells were maintained in DMEM containing 10% newborn calf serum (NCS), 100 U/ml penicillin and 100 μg/ml streptomycin. The NIH 3T3 cells were plated in 24-well plates at a density of 5×10^4 cells per well one day prior to the transfection. The cells were transiently transfected using Lipofectamine and PLUS Reagents with 350 ng pFR-luc, 25 ng pFA2-ELK1 or pFA2 c-Jun, 3.5 ng phRLnull-luc and 200 ng wild-type (WT) or mutant expression constructs of CBL for ELK or c-Jun transactivation. The luciferase activity was assayed using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). Renilla luciferase, expressed by phRLnull-luc, was used to normalize the transfection efficiency. All of the experiments were performed in triplicate.

2.6. HES1 reporter assay

The NIH 3T3 cells were plated in 24-well plates at a density of 5×10^4 cells per well one day prior to the transfection. The cells were transiently transfected using Lipofectamine and PLUS Reagents with 100 ng HES-Luc, 5 ng phRLnull-luc, 120 ng ICN region expression construct and 60 ng, 120 ng or 240 ng WT or mutant expression constructs of CBL. The luciferase assays were performed as described above.

3. Results

3.1. Mutation analysis

We sequenced exons 8 and 9 in *CBL* in 77 children with hematopoietic malignancies. *CBL* mutations were detected in 2 patients. A T-to-C substitution at nucleotide 1141 (c.1141T>C) in *CBL*, which resulted in a p.C381R homozygous mutation, was detected in Patient PL1, who was diagnosed with T-ALL (Fig. 1A). DNA isolated from the buccal mucosa and peripheral blood during complete remission revealed no mutation of *CBL*, suggesting that the p.C381R mutation occurred somatically. Additionally, c.1222T>C, which resulted in a p.W408R homozygous mutation, was identified in JMML cells from Patient PL52 (Fig. 1B). An analysis of a DNA sample from the buccal mucosa revealed a heterozygous mutation in c.1222T>C, suggesting a heterozygous germline mutation. No mutations were identified in any of the coding exons in *PTPN11*, *HRAS*, *KRAS* or *SOS1*, exons 6, 11–16 in *BRAF*, exons 7, 14 or 17 in *RAF1* or exon 1 in *SHOC2* [13,18] in Patient PL52.

3.2. Clinical course of PL1 and PL52

Patient PL1 was the first son of unrelated healthy parents. He developed a swelling of the cervical lymph glands at 10 years of age, and he was admitted to our hospital following a laboratory finding of leukocytosis and thrombocytopenia. The laboratory findings were hemoglobin 12.3 g/dl, white blood cells 403.4×10^9/l and platelets 83×10^9/l. Bone marrow aspiration revealed a hypercellular marrow with 93.4% lymphoblasts with a T-cell phenotype: the cells were positive for CD2, CD3, CD5, CD7, CD4, CD8, cytoplasmic CD3 and TdT and negative for CD10, CD13, CD19, CD20 and CD33 according to immunophenotyping using flow cytometry. Chromosomal testing demonstrated 46, XY. T-ALL was diagnosed, and the cerebrospinal fluid was negative for leukemia. Induction therapy, which consisted of vincristine, prednisolone, tetrahydropyranyl adriamycin, cyclophosphamide and *Escherichia coli* asparaginase, was performed. Although this patient underwent leukapheresis before induction therapy, he developed tumor lysis syndrome that required dialysis therapy. Complete remission was achieved at Day 15, and he has remained in complete remission.

Patient PL52 was a three-month-old girl. She developed a fever and was hospitalized for leukocytosis and thrombocytopenia. The laboratory data were hemoglobin 8.8 g/dl, white blood cells 32.5×10^9/l (2.0% myelocytes, 4.0% stab neutrophils, 16% segment neutrophils, 11% monocytes and 67% lymphocytes) and platelets 23×10^9/l. Bone marrow aspiration revealed hypercellular marrow. Spontaneous growth and hypersensitivity to granulocyte/macrophage colony-stimulating factor (GM-CSF) were observed in the colony assay. This patient was diagnosed with JMML. Her brain CT was normal at 3 months of age. She was developmentally normal with no obvious dysmorphic features. At 1 year and 3 months of age, her stature was 79.1 cm (+0.9 SD), body weight was 10.6 kg (+1.3 SD) and no heart murmur was observed. The laboratory data were hemoglobin 8.8 g/dl, white blood cells 17×10^9/l (2.0% myelocytes, 4.0% stab neutrophils, 16% segment neutrophils, 10.3% monocytes and 67% lymphocytes) and platelets 23×10^9/l. She has been observed in outpatient care and will obtain hematopoietic stem cell transplantation if her blood features deteriorate.

3.3. The analysis of the NOTCH1 and FBXW7 genes and of the copy number in the T-ALL sample

Activating mutations of the *NOTCH1* gene that involve the extracellular HD domain and/or the C-terminal PEST domain have been identified in more than half of all T-ALL cases [19]. FBXW7 is a ubiquitin ligase of NOTCH1, and mutations in *FBXW7* are observed in almost 10% of T-ALL cases [20–22]. Exons 26, 27 and 34 in *NOTCH1* and exons 4–12 in *FBXW7* were analyzed in a sample from Patient PL1 to confirm that the leukemia cells had the properties of T-ALL. *NOTCH1* sequencing revealed two mutations in the HD and PEST domains. One mutation, a missense mutation (c.4724T>C) that results in a p.L1575P in the HD domain, has previously been identified in a sample from T-ALL patients [19]. Another mutation, a novel c.7416-7417insGA that causes a frame shift in the amino acid in Position 2478 (p L2473fs(2478*)), has been predicted to result in a partial deletion of the PEST domain. No mutations in *FBXW7* were identified. These results and the analysis of T cell markers confirmed that the sample from Patient PL1 had properties of T cell leukemia.

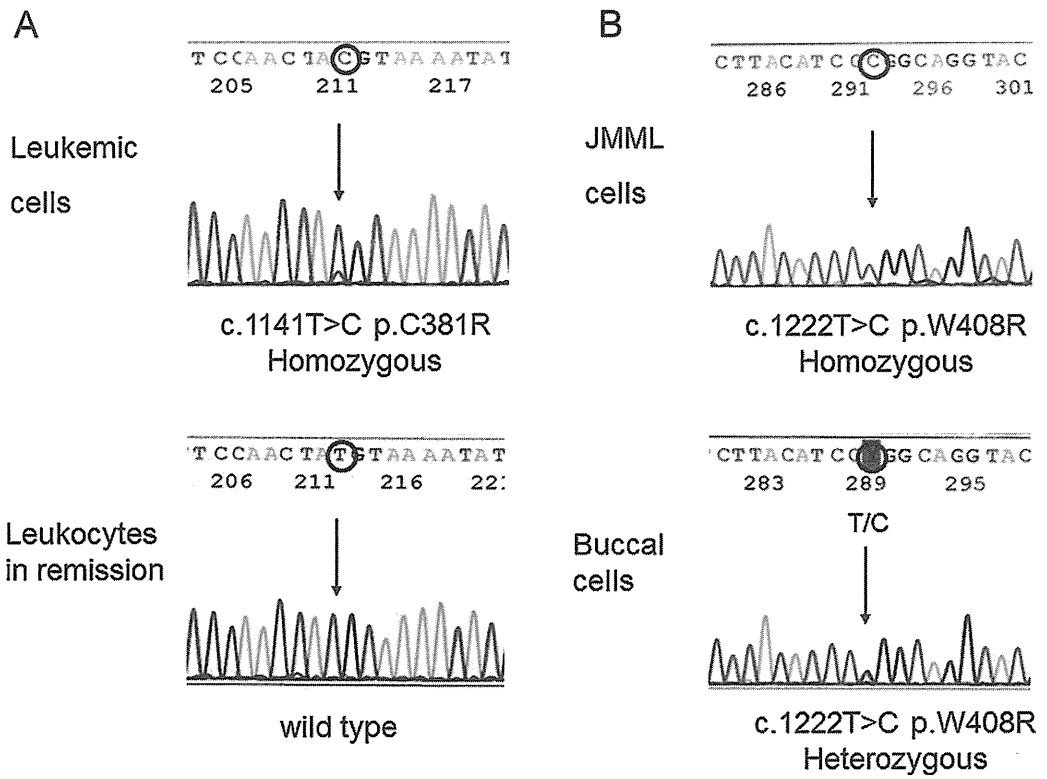

Fig. 1. *CBL* mutations identified in patients. (A) Sequencing charts of leukemic cells and peripheral blood at complete remission from Patient PL1. (B) Sequencing charts of JMML cells and buccal mucosa from Patient PL52.

CBL mutations are associated with an 11q-acquired uniparental disomy (aUPD) involving the *CBL* locus, which converts these mutations into a homozygous state [3]. An SNP array analyzed the difference in DNA between PB samples at disease onset (leukemia cells) and leukocytes in remission. The analysis revealed a UPD 11q13.1qter that contained the *CBL* locus only in the T-ALL sample (Table 1). In addition, homozygous deletions of 4q25, which encodes the *LEF1* gene, and of 9p21.3, including the region that encodes *CDKN2A*, were detected. A UPD at 9pter-p13.3 was also observed. The deletion of 14q11.2, which encodes *TCRA*, and the one-copy deletion in 7q34 (including *TCRB*) that were observed in the DNA from the T-ALL cells may be due to a TCR rearrangement. The effects of the gain of the immunoglobulin light chain at 2p11.2 and the gain at 17q12, which contains *CCL3L3*, *CCL4L2* and *TBC1D3*, are unknown.

3.4. ELK and c-Jun transactivation in cells expressing mutant CBL proteins

The *CBL* p.C381R mutation that was identified in one T-ALL patient has also been identified in a JMML patient and a single patient with MDS [5,23]. However, a functional analysis of p.C381R has not been performed.

A WT allele and the two *CBL* mutants, p.C381R and p.R420Q, were introduced in NIH 3T3 cells, and ELK transactivation was examined to elucidate the activation of the ERK pathway. The allele p.R420Q was used as a positive control because this mutant activates ERK [12]. ELK is a transcription factor that is phosphorylated by activated ERK and that binds the serum response element in the promoters of the immediate early genes, including c-FOS [24]. ELK transactivation was remarkably enhanced in cells expressing

Table 1
Genetic abnormalities of T-ALL at diagnosis.

Chromosomal sites	Copy number state (leukemia)	Copy number state (germline)	Loss/gain	Size (kb)	[a]Start_Linear_Position	[a]End_Linear_Position	Genes included in the region
11q13.1qter		2	UPD	69575.00	64877380	134452384	*CBL* and others
14q11.2	1	2	Loss	369.94	21660717	22030660	*TCRA*, *TCRD*, *TCR*
17q12	3	2	Gain	192.98	31460821	31653797	*CCL3L3*, *CCL4L2*, *TBC1D3*
2p11.2	4	3	Gain	460.63	88914227	89374858	*IGK@*
2p11.2	3	2	Gain	109.16	89753412	89862571	*IGK@*
4q25	0	2	Loss	104.82	109199454	109304271	*LEF1*
7q34	0	2	Loss	491.97	141711730	142203700	*TCRB*
9p11.2	3	2	Gain	127.88	44667843	44795721	
9p21.3	0	2	Loss	117.67	21864256	21981923	*CDKN2A*
9pterp13.3		2	UPD	33701.54	1	33701540	*CDKN2A* and others

Abbreviations: CBL, Cas-Br-M (murine) ecotropic retroviral transforming sequence; TCRA, T cell receptor alpha; TCRD, T cell receptor delta; CCL3L3, chemokine ligand 3-like 3; CCL4L2, chemokine ligand 4-like 2; TBC1D3, TBC1 domain family, member 3; IGK@, immunoglobulin kappa locus; LEF1, lymphoid enhancer binding factor 1; TCRB, T cell receptor beta; CDKN2A, cyclin-dependent kinase inhibitor 2A.

[a] Denoted by NCBI 36 reference human genome (hg18).

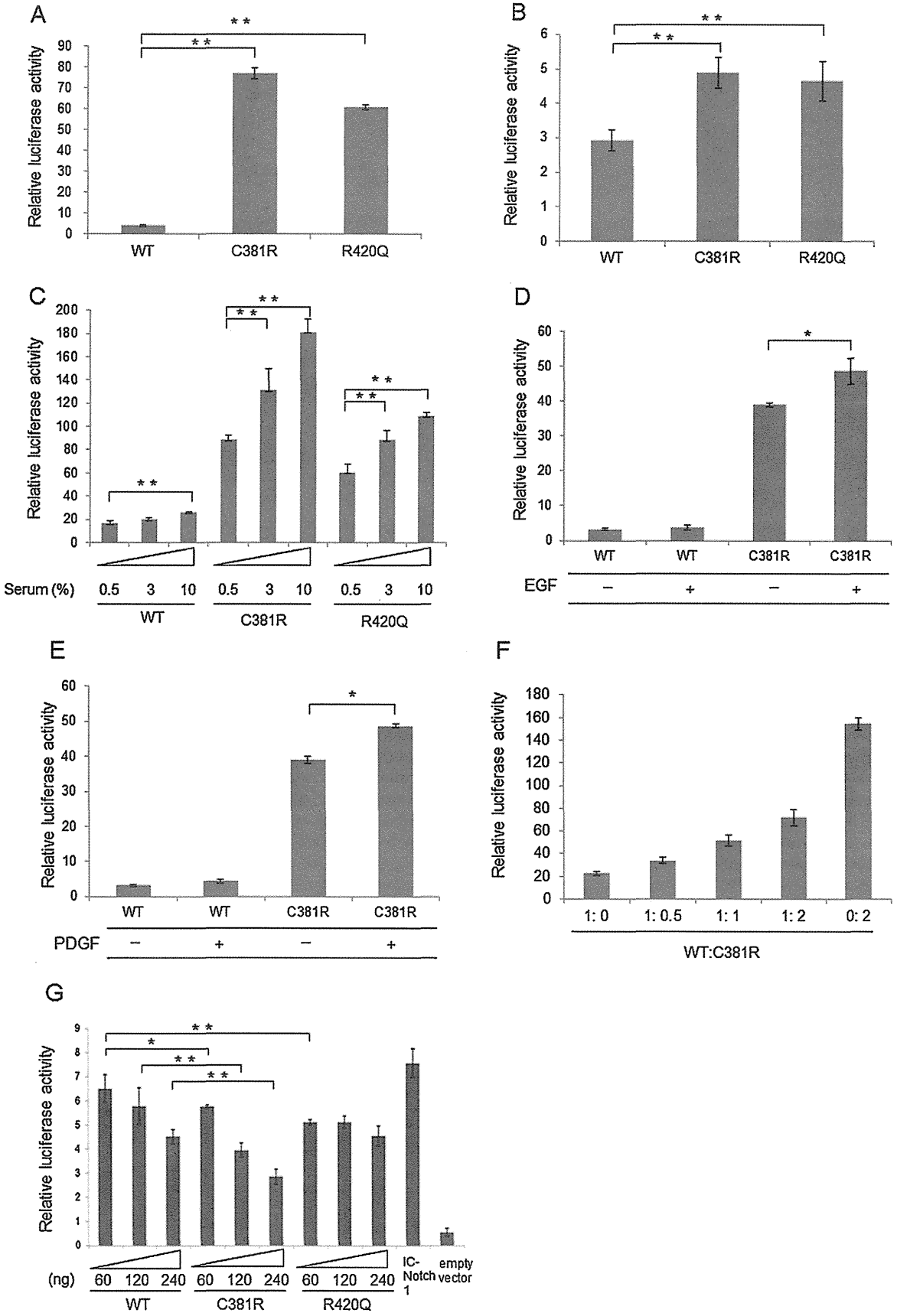

Fig. 2. ELK, c-Jun, and HES1 transactivation in cells expressing mutant CBL proteins. The results are expressed as the mean and standard deviation of mean values from triplicate samples. $^{**}P < 0.01$ and $^{*}P < 0.05$ determined with Student's *t*-test. (A) ELK transactivation in cells with WT CBL and mutant CBL. (B) c-Jun transactivation in cells with WT CBL and mutant CBL. (C) ELK transactivation in NIH 3T3 cells transiently expressing WT CBL and C381R CBL in DMEM that contained the indicated concentrations of newborn calf serum (NCS). (D) For EGF stimulation, the ELK transactivation level in cells expressing p.C381R stimulated with EGF was significantly enhanced compared

p.C381R and p.R420Q in DMEM containing 10% NCS compared with WT *CBL*-transfected cells (Fig. 2A). The transactivation of the transcription factor c-Jun was examined in NIH 3T3 cells. Studies have shown that c-Jun activity is upregulated by the phosphorylation of c-Jun NH2-terminal kinases (JNK) [25]. In this case, c-Jun transactivation was significantly enhanced in cells expressing p.C381R and p.R420Q in DMEM containing 10% NCS (Fig. 2B). These results demonstrate that *CBL* mutants activate the ERK and JNK pathways, possibly via the upstream activation of RAS in the presence of serum.

ELK transactivation was examined in different NCS concentrations to evaluate the effect of serum concentration. ELK transactivation in cells expressing p.C381R and p.R420Q was enhanced in an NCS concentration-dependent manner (Fig. 2C). Significant ELK activation was observed in cells expressing p.C381R and p.R420Q in DMEM with 0.5% NCS. The effects of EGF and PDGF on ELK transactivation were examined in cells expressing WT *CBL* or p.C381R *CBL*. The ELK transactivation levels in cells expressing p.C381R that were stimulated with 100 ng/ml EGF (Fig. 2D) or 100 ng/ml PDGF (Fig. 2E) were significantly enhanced compared with those of unstimulated cells. However, EGF and PDGF stimulation did not significantly alter the ELK transactivation levels in cells expressing WT *CBL*. These results suggest that the p.C381R mutation constitutively activates the RAS pathway.

CBL mutations affect endogenous WT CBL in a dominant-negative manner [7]. NIH 3T3 cells were co-transfected with WT CBL and C381R to evaluate the effect of p.C381R on WT CBL. The hypertransactivation response that was induced by the CBL mutant was abolished by the co-transfection of WT CBL (Fig. 2F), suggesting the pathogenic importance of the WT *CBL* allele loss.

3.5. HES transactivation in cells expressing mutant CBL

HES1 is a target gene for NOTCH1. WT or mutant CBL constructs were transiently transfected in NIH 3T3 cells with the HES-Luc reporter and a constitutively active intracellular domain of NOTCH1 (ICN1) construct. ICN1 expression significantly increased the transactivation of HES (Fig. 2G, IC-NOTCH1 lane). The introduction of CBL WT or mutants significantly reduced the HES transactivation levels compared with cells expressing ICN1 (Fig. 2G). The HES1 transactivation levels in cells expressing p.C381R were significantly decreased compared with CBL WT-expressing cells.

4. Discussion

In this study, a homozygous p.C381R mutation and a UPD of the region that included *CBL* were identified in T-ALL cells, and a heterozygous germline p.W408R mutation was identified in one patient with JMML. An additional mutation analysis identified two *NOTCH1* mutations and homozygous deletions of *LEF1* and *CDKN2A* in T-ALL cells. A functional analysis revealed that cells expressing the p.C381R mutant constitutively transactivated ELK and c-Jun. Co-transfection of WT and the p.C381R mutation in NIH 3T3 cells revealed that WT inhibited the ELK-activating effects of p.C381R. The HES1 transactivation levels in cells expressing p.C381R were significantly decreased compared with CBL WT-expressing cells, suggesting that this *CBL* mutation plays a role in NOTCH signaling pathway.

CBL mutations are rare in ALL patients. Recently, mutations in *CBL* have been identified in 2 infant ALL patients with *MLL* gene rearrangements [26]. Nicholson et al. analyzed the linker-RING domains of CBL in a cohort of 180 diagnostic and 46 relapsed ALL patients and identified deletions/insertions of *CBL*, including the splicing acceptor or donor site of exon 8 in three ALL samples [27]. *CBL* mutations in ALL may promote the proliferation of leukemia cells by activating the RAS pathway ([27] and our study). Alternatively, our HES-reporter assay in cells that expressed the *NOTCH1* constitutive active mutant showed that *CBL* p.C381R downregulated the NOTCH1 signaling pathway, suggesting that the *CBL* p.C381R mutation may contribute to leukemogenesis through interaction with NOTCH1. The relationship between CBL and NOTCH1 has not been elucidated, but one report has demonstrated that CBL promotes the ubiquitin-dependent lysosomal degradation of membrane-associated NOTCH1 [28]. In the case of NOTCH3, its interactions with pre-TCR lead to the recruitment and persistence of the CBL to the lipid rafts in thymocytes from mice expressing the constitutively active intracellular domain of NOTCH3, which suggests that CBL may regulate the NOTCH3 and pre-TCR relationship during T-cell leukemogenesis [29]. Further analysis will elucidate the role of the *CBL* mutation in T-ALL leukemogenesis.

Somatic and germline *CBL* mutations have been clustered in either the linker domain or the RING finger domain (Fig. 3). The loss of the ubiquitination of activated receptor tyrosine kinases is thought to contribute to the transforming potential of leukemia-associated mutant CBL proteins. The distributions of somatic and germline mutations were almost similar. However, Y371, which is a hot spot for *CBL* mutations in JMML, is rarely mutated in other myeloid malignancies [5]. The germline p.W408R mutation has been identified in a patient with JMML [5]. Individuals with germline *CBL* mutations display a variable combination of dysmorphic features, including mild hypertelorism, a short upturned nose, a deeply grooved philtrums and thick lips, which are reminiscent of the facial gestalt of NS [10]. Patient PL52, who had a germline p.W408R mutation, had normal development and no dysmorphic features at 15 months of age. However, her young age may have precluded any firm conclusions. Long-term follow-up examinations and an analysis of wider cohorts is necessary to further characterize the phenotypic spectrum that is associated with germline mutations in *CBL*.

The effect of mutant CBLs on ERK activation depends on the level of endogenous WT CBL [7,30]. Therefore, we examined ELK transactivation in NIH 3T3 cells, which have low endogenous CBL protein expression [31]. Our study demonstrated that ELK transactivation in cells expressing p.C381R decreased with increasing WT CBL expression. These results suggest that the p.C381R mutation functions in a dominant-negative manner or as a gain-of-function mutation.

In this study, SNP array analyses of samples from leukemia cells and leukocytes obtained from patients in remission revealed a copy number imbalance that was specific for leukemia cells. The homozygous deletion of the entire *LEF1* gene was identified in the T-ALL sample with the *CBL* mutation. LEF1 is a member of the lymphoid enhancer factor/T-cell factor family of DNA-binding transcription factors that interact with nuclear β-catenin in the WNT signaling pathway [32]. Monoallelic or biallelic *LEF1* microdeletions have been identified in 11% (5 of 47) of primary samples from the diagnostic specimens of 47 children with T-ALL, using high-resolution array comparative genomic hybridization [33]. The homozygous deletion of *CDKN2A* and

with unstimulated cells. (E) For PDGF stimulation, the ELK transactivation level in cells expressing p.C381R stimulated with 100 ng/ml PDGF was enhanced compared with unstimulated cells. (F) Co-transfection of WT CBL and C381R CBL. The hypertransactivation response induced by CBL p.C381R was abolished by the co-transfection of WT CBL. (G) Mutant CBL constructs in pCMV6 were transiently transfected in NIH 3T3 cells with the HES-Luc reporter and the intracellular NOTCH1 (ICN1) construct where appropriate.

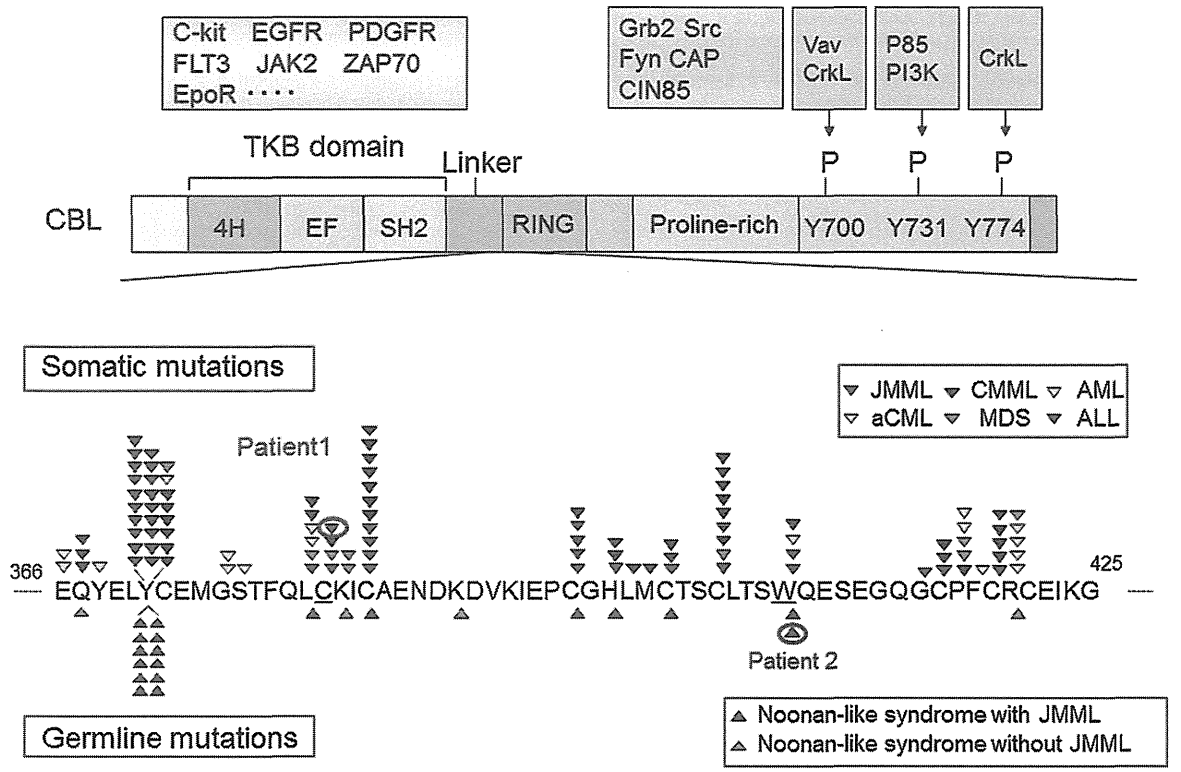

Fig. 3. The CBL structure and mutation spectrum. CBL comprises an N-terminal tyrosine kinase binding domain (TKB) connected by a linker to the RING-finger domain implicated in E2 enzyme binding. These domains are followed by a proline-rich region and a C-terminal portion containing tyrosine phosphorylation sites. The molecular interaction of CBL with cytokine receptors and other signaling molecules are also shown on top. The *CBL* mutations identified in hematologic malignancies partially overlap with those identified in the germline.

CDKN2B, which are frequently inactivated in various hematological malignancies [34], was also identified in the T-ALL sample. A comparison of the copy numbers of DNA samples from leukemia cells and germline DNA will help to highlight the abnormalities in leukemia.

In conclusion, we identified a *CBL* p.C381R mutation in leukemia cells from one patient with T-ALL. A functional analysis demonstrated that the mutation constitutively activated the RAS-MAPK pathway and inhibited the constitutive activation of the NOTCH signaling pathway. Further studies will be needed to determine the relationship between CBL and leukemogenesis.

Conflict of interest statement

All authors declare no competing financial interests.

Acknowledgments

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Contributions. YS, YA and SK designed the research study; YS, HM, HM and TN performed the research; MI, TR, YS, ST and SK provided patients samples; YS, HM, HM and JPM analyzed the data; YS, YA and YM wrote the paper.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.leukres.2012.04.018.

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