

Haploinsufficiency and acquired loss of *Bcl11b* and *H2AX* induces blast crisis of chronic myelogenous leukemia in a transgenic mouse model

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Chronic myelogenous leukemia (CML) is a hematological malignancy that begins as indolent chronic phase (CP) but inevitably progresses to fatal blast crisis (BC). p210BCR/ABL, a chimeric protein with enhanced kinase activity, initiates CML CP, and additional genetic alterations account for progression to BC, but the precise mechanisms underlying disease evolution are not fully understood. In the present study, we investigated the possible contribution of dysfunction of *Bcl11b*, a zinc-finger protein required for thymocyte differentiation, and of *H2AX*, a histone protein involved in DNA repair, to the transition from CML CP to BC. For this purpose, we crossed CML CP-exhibiting p210BCR/ABL transgenic (*BA*^{tg}) mice with *Bcl11b* heterozygous (*Bcl11b*^{+/-}) mice and *H2AX* heterozygous (*H2AX*^{+/-}) mice. Interestingly, p210BCR/ABL transgenic, *Bcl11b* heterozygous (*BA*^{tg}-*Bcl11b*^{+/-}) mice and p210BCR/ABL transgenic, *H2AX* heterozygous (*BA*^{tg}-*H2AX*^{+/-}) mice frequently developed CML BC with T-cell phenotype and died in a short period. In addition, whereas p210BCR/ABL was expressed in all of the leukemic tissues, the expression of *Bcl11b* and *H2AX* was undetectable in several tumors, which was attributed to the loss of the residual normal allele or the lack of mRNA expression. These results indicate that *Bcl11b* and *H2AX* function as tumor suppressor and that haploinsufficiency and acquired loss of these gene products cooperate with p210BCR/ABL to develop CML BC. (*Cancer Sci* 2009; 100: 1219–1226)

Chronic myelogenous leukemia (CML) is a disorder of hematopoietic stem cells, characterized by excessive and uncontrolled proliferation of differentiated myeloid cells.⁽¹⁻³⁾ Clinically, CML undergoes two different stages.⁽¹⁻³⁾ In the initial stage, chronic phase (CP), the leukemic cells retain the ability to differentiate into mature granulocytes and are sensitive to conventional therapies. However, after several years' duration of CP, the disease inevitably accelerates and ultimately progresses to the terminal stage, blast crisis (BC), which exhibits aggressive proliferation of immature blast cells and is resistant to intensive therapies.⁽¹⁻³⁾

The cytogenetic hallmark of CML CP is t(9;22)(q34;q11) (known as Philadelphia chromosome, Ph), which generates a *BCR-ABL* fusion gene encoding a 210-kDa chimeric protein (p210BCR/ABL).⁽¹⁻³⁾ p210BCR/ABL possesses a constitutively active tyrosine kinase activity, which plays an essential role in the initiation of the disease.⁽¹⁻³⁾ Although Ph is the unique and sole chromosomal abnormality in CP, additional and non-random chromosomal abnormalities are frequently observed in BC, indicating that secondary genetic events account for the disease progression.⁽¹⁻³⁾

To understand the pathogenesis of the disease, it is necessary to establish animal models that express p210BCR/ABL and

recapitulate the clinical course of CML. For this purpose, we generated transgenic mice expressing p210BCR/ABL under the control of the mouse *TEC* promoter.⁽⁴⁾ The p210BCR/ABL transgenic (hereafter, designated as *BA*^{tg}) mice reproducibly exhibited a myeloproliferative disorder closely resembling human CML CP.⁽⁴⁾ In addition, by crossing *BA*^{tg} mice with p53 heterozygous mice and *Dok-1/Dok-2* knockout mice, we showed that the loss of p53 and absence of *Dok-1/Dok-2* accelerated the disease and caused CML BC.^(5,6) Furthermore, by applying retroviral insertional mutagenesis to *BA*^{tg} mice, we demonstrated that overexpression and enhanced kinase activity of p210BCR/ABL and altered expression of *Notch1* contribute to CML BC.⁽⁷⁾ These results demonstrated that the *BA*^{tg} mouse is not only regarded as a model for CML CP, but is also useful for investigating the molecular mechanisms underlying the progression from CP to BC.

Chromosomal and molecular analyses have revealed that several mechanisms are implicated in this process, such as: (i) loss of tumor suppressor; (ii) differentiation arrest; and (iii) chromosomal instability.⁽³⁾ Indeed, as an example of (i), we demonstrated that loss of p53 cooperates with p210BCR/ABL and induces CML BC.^(5,6) In the present report, as candidate genes for (ii) and (iii), we chose *Bcl11b* (also known as *Rit1* and *Ctip2*), encoding a transcription factor required for thymocyte differentiation,⁽⁸⁾ and *H2AX*, encoding a histone protein involved in DNA repair,⁽⁹⁾ and examined the possible contribution that dysfunction of these gene products for the disease progression of CML. For this purpose, we crossed *BA*^{tg} mice with mice heterozygous for *Bcl11b* (*Bcl11b*^{+/-}) or *H2AX* (*H2AX*^{+/-}) and generated *BA*^{tg}-*Bcl11b*^{+/-} mice and *BA*^{tg}-*H2AX*^{+/-} mice. Interestingly, both types of double transgenic mouse frequently developed CML BC and died in a short period. The pathological, flow cytometric, molecular, and chromosomal analyses of the diseased mice are described.

Materials and Methods

Mice. p210BCR/ABL transgenic, *Bcl11b* heterozygous, and *H2AX* heterozygous mice were generated as described previously.^(4,8,10) Crossing and genotyping of the mice were carried out as described previously.⁽⁵⁾ All of the mice were kept according to the guidelines of the Institute of Laboratory Animal Science, Hiroshima University.

Pathological analysis. Autopsies were carried out on dead or moribund animals. Peripheral blood smears were stained with Wright-Giemsa. After gross examination, tissues were fixed in

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10% neutral buffered formaldehyde and representative slices were stained with hematoxylin–eosin (HE).

Western blot analysis. Proteins were extracted from tissues, separated by SDS-PAGE, transferred to a nitrocellulose membrane, and blotted with appropriate antibodies as described previously.^(4,8) The antibodies used in this study were: anti-ABL monoclonal antibody, Ab3 (Oncogene Science, Cambridge, MA, USA); an anti-Bcl11b polyclonal antibody;⁽⁸⁾ and an antihistone H2AX antibody (Millipore, Bedford, MA, USA). Positive signals were detected with the enhanced chemiluminescence system.

Southern blot analysis and genomic PCR. For Southern blotting, DNA was digested with restriction enzymes, separated in an agarose gel, blotted to a nylon membrane, and hybridized with a ³²P-dCTP-labeled *TCRβ* probe. Genomic PCR was carried out using the following primers as described previously:⁽¹¹⁾ P1 (5'-TGCAGCTTCCGGGCGATGCCA-3'), P2 (5'-ACTTTCCCAGAACCCACGC-3'), and P3 (5'-CCTGCTTGCCGAATATCATGGTGG-3') for *Bcl11b*; and P1 (5'-TCACATTGTTTCCTTCG-GTGTAC-3'), P2 (5'-AAGTGTGTGATTGGGAAGCGTAG-3'), P3 (5'-AGATCCCGTTGACTGAACACAGG-3'), P4 (5'-TTCAG-GTTTTGTTGTCGCGCCGTAG-3'), and P5 (5'-TCAGCTCTTTCTGTGAGGGAGTGG-3') for *H2AX*.

Northern blot analysis and RT-PCR. Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA), separated in 1.2% formaldehyde gel, blotted to a nylon membrane, and hybridized with a ³²P-dCTP-labeled *H2AX* probe. RT-PCR was carried out using the following primers as described previously:⁽¹¹⁾ 5'-CGAGCTCAGGAAAGTGTCCGAG-3' and 5'-GGAAATTCATGAGCGGG-GACTG-3' for *Bcl11b*; 5'-CCTTCTGGAAGACTTGGCCTTC-3' and 5'-GAGGAAGATGTGCCTGTTACC-3' for *H2AX*; and 5'-TTCAACACCCAGCCATGA-3' and 5'-CTCAGGAGGAGCAATGATCT-3' for *β-actin*.

Flow cytometric analysis. Cells were stained with FITC- or phycoerythrin (PE)-conjugated anti-Thy-1.2, anti-B220, anti-Mac1, and anti-Gr1 monoclonal antibodies (Pharmingen, San Diego, CA, USA), as described previously.⁽⁵⁾

Chromosomal analysis. Chromosomes were prepared by means of standard culture procedures for tumor cells and treated with trypsin-Giemsa as described previously.⁽¹²⁾

Patient samples and normal bone marrow cells. Patient samples were taken after obtaining informed consent and approval from the institutional review board at Hiroshima University.⁽¹³⁾ Diagnosis of CML CP or CML BC (myeloid or B-lymphoid lineage) was carried out based on morphological, cytogenetic, and immunophenotypic analyses. Normal bone marrow cells were obtained from a healthy volunteer.

Results

***BA^{tg/-}Bcl11b^{+/-}* and *BA^{tg/-}H2AX^{+/-}* mice developed acute leukemia and died in a short period.** To investigate the contribution of haploinsufficiency of *Bcl11b* and *H2AX* to the disease progression of CML, we crossed CML-exhibiting *BA^{tg/-}* mice with *Bcl11b^{+/-}* mice and *H2AX^{+/-}* mice. Mice with four different genotypes were generated by each crossing: *BA^{tg/-} × Bcl11b^{+/-}* created *BA^{-/-}Bcl11b^{+/+}* (wild type), *BA^{tg/-}Bcl11b^{+/+}* (*p210BCR/ABL* transgenic), *BA^{-/-}Bcl11b^{+/-}* (*Bcl11b* heterozygous), and *BA^{tg/-}Bcl11b^{+/-}* (*p210BCR/ABL* transgenic, *Bcl11b* heterozygous); and *BA^{tg/-} × H2AX^{+/-}* produced *BA^{-/-}H2AX^{+/+}* (wild type), *BA^{tg/-}H2AX^{+/+}* (*p210BCR/ABL* transgenic), *BA^{-/-}H2AX^{+/-}* (*H2AX* heterozygous), and *BA^{tg/-}H2AX^{+/-}* (*p210BCR/ABL* transgenic, *H2AX* heterozygous). Mice with these genotypes were normally born approximately at the expected Mendelian ratio (see the mouse number shown in parentheses in Fig. 1), indicating that the crossing did not affect the embryonic development of the mice.

All of the mice were observed continuously and peripheral blood parameters were counted routinely. The genotype-based survival curves of the mice in each crossing are shown in

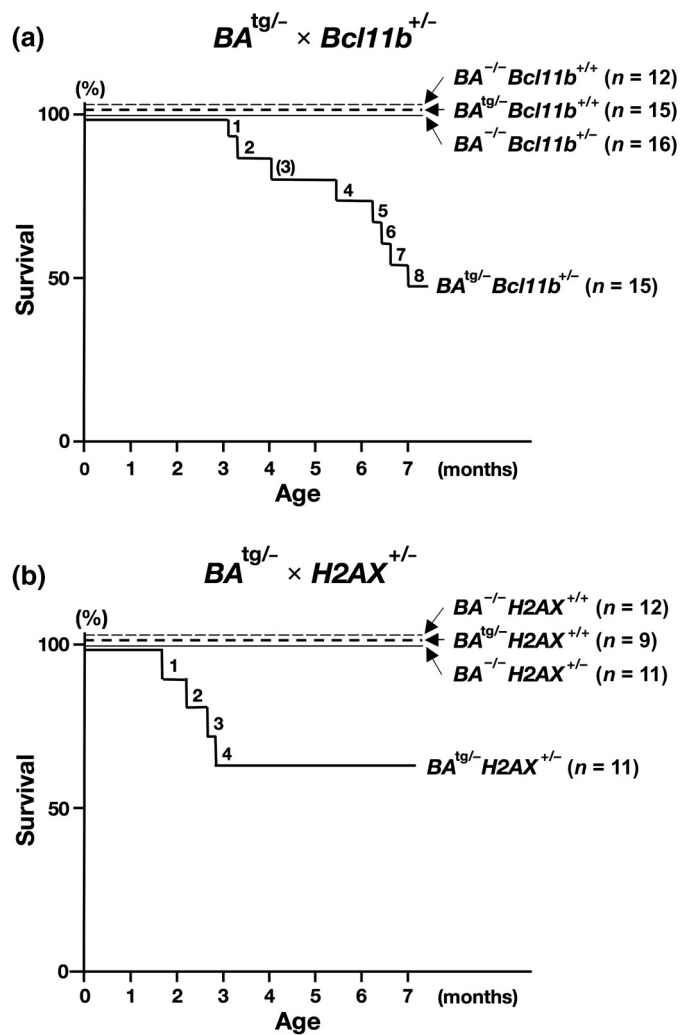


Fig. 1. Survival curves of mice generated by (a) *BA^{tg/-} × Bcl11b^{+/-}* and (b) *BA^{tg/-} × H2AX^{+/-}*. The survival curves of *BA^{-/-}Bcl11b^{+/+}* and *BA^{-/-}H2AX^{+/+}*, *BA^{tg/-}Bcl11b^{+/+}* and *BA^{tg/-}H2AX^{+/+}*, *BA^{-/-}Bcl11b^{+/-}* and *BA^{-/-}H2AX^{+/-}*, and *BA^{tg/-}Bcl11b^{+/-}* and *BA^{tg/-}H2AX^{+/-}* mice are shown as thin dotted, thick dotted, thin continuous, and thick continuous lines respectively. In the *BA^{tg/-} × Bcl11b^{+/-}* group, 8 of 15 *BA^{tg/-}Bcl11b^{+/-}* mice died within 7 months of age and in the *BA^{tg/-} × H2AX^{+/-}* group, 4 of 11 *BA^{tg/-}H2AX^{+/-}* died within 3 months of age. The number of an unanalyzable *BA^{tg/-}Bcl11b^{+/-}* mouse due to death (no. 3) is shown in parentheses.

Figure 1. During a 7-month observation period, in the *BA^{tg/-} × Bcl11b^{+/-}* group, 8 of 15 *BA^{tg/-}Bcl11b^{+/-}* died of acute leukemia, in contrast *BA^{-/-}Bcl11b^{+/+}*, *BA^{tg/-}Bcl11b^{+/+}*, and *BA^{-/-}Bcl11b^{+/-}* littermates did not show any disorders (Fig. 1a). As for the *BA^{tg/-} × H2AX^{+/-}* group (lower panel), 4 of 11 *BA^{tg/-}H2AX^{+/-}* mice exhibited proliferation of blast cells and died within 3 months of birth, whereas no disease was observed in *BA^{-/-}H2AX^{+/+}*, *BA^{tg/-}H2AX^{+/+}*, and *BA^{-/-}H2AX^{+/-}* littermates (Fig. 1b).

The representative results of pathological analysis of *BA^{tg/-}Bcl11b^{+/-}* and *BA^{tg/-}H2AX^{+/-}* leukemic mice are shown in Figure 2. Macroscopically, both leukemic mice exhibited marked thymic enlargement with splenomegaly, which were occasionally associated with lymph node swelling or pleural effusion (data not shown). The peripheral blood smears exhibited proliferation of blast cells morphologically resembling lymphoblasts (upper panels of Fig. 2). Tissue sections showed that the blast cells caused destruction of the basic structure of the thymus (second panels of Fig. 2) and infiltrated in non-hematopoietic tissues, such as liver (third panels of Fig. 2). In contrast, the bone marrow

Table 1. Characteristics of *p210BCR/ABL*^{tg/-} *Bcl11b*^{+/-} leukemic mice

Mouse no.	Age at disease (months)	PB parameters			Macroscopic tumor sites	<i>TCRβ</i> status	<i>p210BCR/ABL</i> expression	<i>Bcl11b</i> expression	<i>Bcl11b</i> status
		WBC (× 10 ³ /μL)	Hb (g/dL)	Plt (× 10 ⁴ /μL)					
1	3.1	35.0	12.5	65.6	Thy, Spl	G/R	+	+	G/T
2	3.3	5.0	10.5	64.8	Thy, Spl	G/loss	+	+	G/T
3	4.0 [†]	ND	ND	ND	Thy	ND	ND	ND	ND
4	5.3	2.3	7.1	44.2	Thy	G/loss	+	+	G/T
5	6.0	12.0	12.3	35.5	Thy, PE	G/loss	+	-	T/loss
6	6.1	6.6	13.9	53.5	Thy	G/R	+	-	T/loss
7	6.4	14.6	15.5	47.1	Thy, Spl	G/R	+	+	G/T
8	6.9	1.5	14.1	74.9	Thy, PE	G/R	+	-	T/loss

[†]Found dead. G, germline; Hb, hemoglobin; ND, not done; PB, peripheral blood; PE, pleural effusion; Plt, platelet; R, rearranged; Spl, spleen; T, targeted; Thy, thymus; WBC, white blood cell.

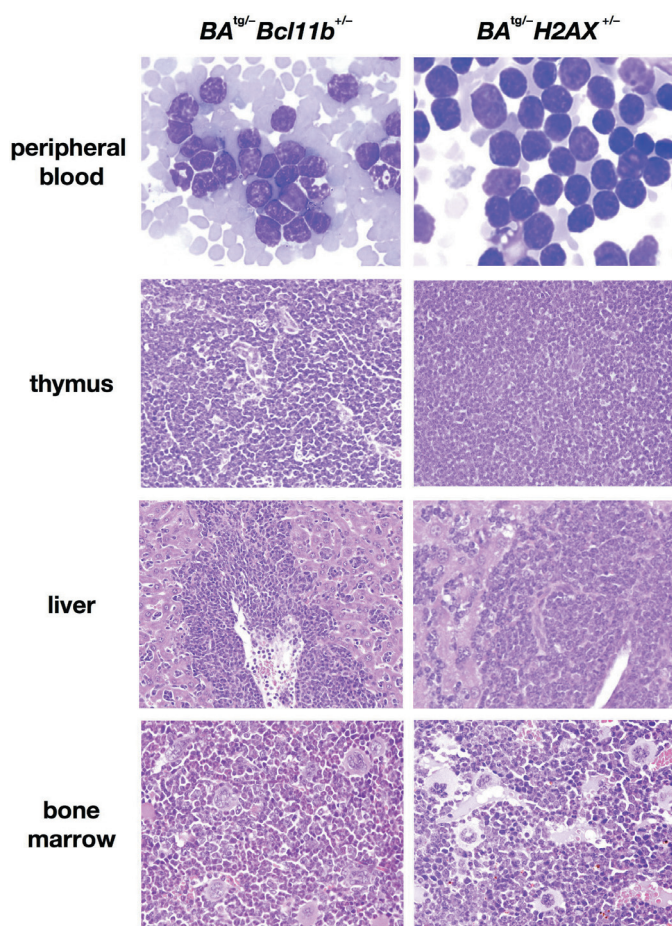


Fig. 2. Representative results of pathological analysis of *BA*^{tg/-} *Bcl11b*^{+/-} (left panels) and *BA*^{tg/-} *H2AX*^{+/-} (right panels) leukemic mice. Wight-Giemsa-stained peripheral blood smears and HE-stained tissue slices are shown. In both leukemic mice, blast cells proliferated in the peripheral blood (upper panels), caused destruction of the basal structure of the thymus (second panels), and infiltrated around the vessel and in the sinusoids in the liver (third panels). In contrast, bone marrow exhibited myeloid cell hyperplasia with differentiation and proliferation of megakaryocytes (bottom panels).

showed a predominance of myeloid cells with differentiation and proliferation of megakaryocytes (bottom panels of Fig. 2). These results demonstrated that haploinsufficiency of *Bcl11b* and *H2AX* cooperated with *p210BCR/ABL*, transformed *p210BCR/ABL*-expressing hematopoietic cells, and caused CML

BC. The characteristics of *BA*^{tg/-} *Bcl11b*^{+/-} and *BA*^{tg/-} *H2AX*^{+/-} leukemic mice are summarized in Table 1 and Table 2, respectively.

Leukemias that developed in *BA*^{tg/-} *Bcl11b*^{+/-} and *BA*^{tg/-} *H2AX*^{+/-} mice were of T-cell lineage and were mostly clonal in origin. To determine the cell lineage and clonality of the leukemias that developed in *BA*^{tg/-} *Bcl11b*^{+/-} and *BA*^{tg/-} *H2AX*^{+/-} mice, blast cells were subjected to flow cytometric and Southern blot analyses.

The representative results of flow cytometric analysis of *BA*^{tg/-} *Bcl11b*^{+/-} and *BA*^{tg/-} *H2AX*^{+/-} leukemic cells are shown in Figure 3(a). In both types of mice, leukemic cells were highly positive for Thy1.2, the antigen specific for T lymphocytes, but were negative for CD19, Gr1, and Mac1, the markers for B lymphocytes, granulocytes, and macrophages respectively.

The clonality of the leukemic cells was examined by gene rearrangement analysis. DNA extracted from a control thymus and tumor tissues of *BA*^{tg/-} *Bcl11b*^{+/-} and *BA*^{tg/-} *H2AX*^{+/-} leukemic mice were digested with a restriction enzyme and blotted with the T-cell receptor β (*TCR-β*) gene. As shown in Figure 3(b), more than half of the samples (no. 1 and no. 6–8 in *BA*^{tg/-} *Bcl11b*^{+/-} and no. 1 and 2 in *BA*^{tg/-} *H2AX*^{+/-}) showed rearranged bands, and in the remaining samples (no. 2, 4, and 5 in *BA*^{tg/-} *Bcl11b*^{+/-} and no. 3 and 4 in *BA*^{tg/-} *H2AX*^{+/-}), loss of the upper germline band was observed (the positions of germline bands are indicated by arrows and shown as ‘G’). These results demonstrated that the blast cells of *BA*^{tg/-} *Bcl11b*^{+/-} and *BA*^{tg/-} *H2AX*^{+/-} leukemic mice were committed to the T-cell lineage and most of the tumors were clonal in origin.

Frequent and acquired loss of *Bcl11b* and *H2AX* protein expression in the tumor tissues of *BA*^{tg/-} *Bcl11b*^{+/-} and *BA*^{tg/-} *H2AX*^{+/-} leukemic mice. We then investigated protein expression in the tumor tissues of *BA*^{tg/-} *Bcl11b*^{+/-} and *BA*^{tg/-} *H2AX*^{+/-} leukemic mice. Proteins extracted from a control thymus and tumor tissues of *BA*^{tg/-} *Bcl11b*^{+/-} and *BA*^{tg/-} *H2AX*^{+/-} leukemic mice were blotted with antibodies against c-ABL, *Bcl11b*, and *H2AX*.

The results of *p210BCR/ABL* expression in these tumors are shown in the upper panels of Figure 4(a,b). As shown in both panels, the 210-kDa band was detected in all of the tumor samples, indicating that the blast cells originated from *p210BCR/ABL*-expressing hematopoietic precursors. We next examined the expression of *Bcl11b* and *H2AX* proteins in *BA*^{tg/-} *Bcl11b*^{+/-} and *BA*^{tg/-} *H2AX*^{+/-} leukemic samples respectively. Interestingly, in the anti-*Bcl11b* western blot, the expression of *Bcl11b* was found to be lost in three of seven samples (no. 5, 6, and 8, middle panel of Fig. 4a). In addition, in the anti-*H2AX* blot, the expression of *H2AX* was undetectable in two of four samples (no. 2 and 3, middle panel of Fig. 4b). These results indicated that the protein expression of *Bcl11b* and *H2AX* was lost in several samples of *BA*^{tg/-} *Bcl11b*^{+/-} and *BA*^{tg/-} *H2AX*^{+/-} leukemic mice.

To investigate the molecular mechanism underlying the loss of *Bcl11b* and *H2AX* expression, DNA extracted from tumor

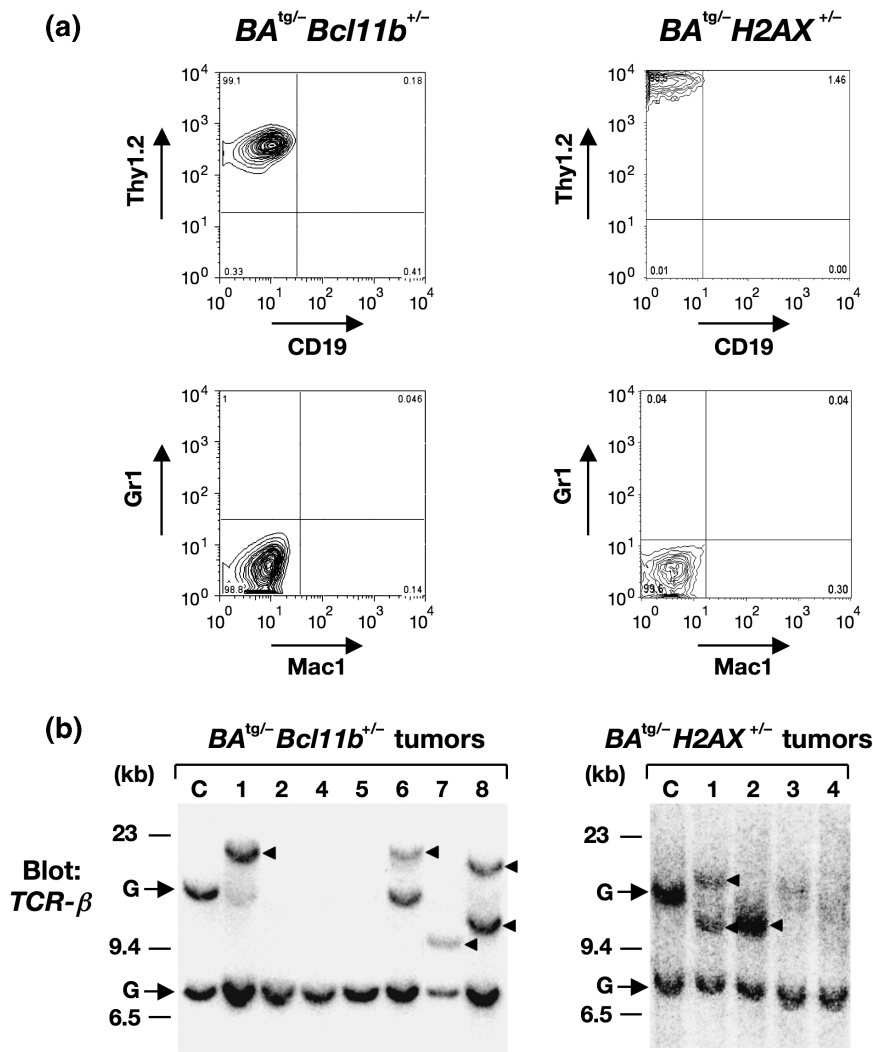


Fig. 3. Results of flow cytometric and Southern blot analyses of $BA^{tg/-} Bcl11b^{+/-}$ and $BA^{tg/-} H2AX^{+/-}$ leukemic mice. (a) Representative results of flow cytometry of leukemic cells that developed in $BA^{tg/-} Bcl11b^{+/-}$ (left panel) and $BA^{tg/-} H2AX^{+/-}$ (right panel) mice. In both samples, blast cells were positive for Thy1.2 but negative for CD19, Gr1, and Mac1, indicating that they were of T-cell phenotype. (b) Results of gene rearrangement analysis in tumors that developed in $BA^{tg/-} Bcl11b^{+/-}$ (left panel) and $BA^{tg/-} H2AX^{+/-}$ (right panel) mice. (c) DNA extracted from control thymus and thymomas that developed in $BA^{tg/-} Bcl11b^{+/-}$ (left panel) and $BA^{tg/-} H2AX^{+/-}$ (right panel) mice were digested with *Bam*HI and blotted with *TCR- β* probe. Germline and rearranged bands are indicated by arrows and arrowheads respectively. Molecular markers are shown on the left.

Table 2. Characteristics of p210BCR/ABL^{tg/-} H2AX^{+/-} leukemic mice

Mouse no.	Age at disease (months)	PB parameters			Macroscopic tumor sites	<i>TCRβ</i> status	p210BCR/ABL expression	H2AX expression	H2AX status
		WBC ($\times 10^3/\mu\text{L}$)	Hb (g/dL)	Plt ($\times 10^4/\mu\text{L}$)					
1	1.8	15.3	16.1	56.4	Thy, Spl	G/R	+	+	G/T
2	2.2	160.8	10.4	53.4	Thy, Spl, LN	G/R	+	-	G/T
3	2.5	128.4	12.0	90.9	Thy, Spl, LN	G/loss	+	-	G/T
4	2.8	84.7	12.4	36.0	Thy, Spl, LN	G/loss	+	+	G/T

G, germline; LN, lymph node; R, rearranged; Spl, spleen; T, targeted; Thy, thymus.

tissues was subjected to genomic PCR that distinguished the PCR product of the wild-type allele from that of the knockout allele (upper panels of Fig. 4c,d). The results showed that the wild-type *Bcl11b* allele-derived band was not amplified in the three samples without *Bcl11b* expression (no. 5, 6, and 8, lower panel of Fig. 4c), indicating that the absence of *Bcl11b* protein was attributed to the loss of the residual wild-type *Bcl11b* allele. In contrast, the PCR product from the wild-type *H2AX* allele was retained in the two samples lacking *H2AX* expression (no. 2 and 3 in the lower panel of Fig. 4d). Because the PCR primer set detecting the wild-type allele (P1 + P2) did not amplify the coding region of the *H2AX* gene (upper panel of Fig. 4d), we designed another primer set encompassing the *H2AX* exon. As

H2AX is a single-exon gene,⁽¹⁰⁾ this primer set (shown as P4 and P5 in the upper panel of Fig. 4d) amplified a part of the promoter and the whole coding region. The results showed that a PCR product of expected size was detected in all of the $BA^{tg/-} H2AX^{+/-}$ tumors (lower panel of Fig. 4d). To examine the possibility that subtle deletion and/or base substitution had occurred in this region, we sequenced the whole PCR product but could not detect any mutation (data not shown). In addition, Southern blotting using a 5' external probe for the *H2AX* gene⁽¹⁰⁾ did not show any gross rearrangement (data not shown). These results indicated that the structure of the *H2AX* gene was largely unaffected. We next examined *H2AX* mRNA expression in the $BA^{tg/-} H2AX^{+/-}$ tumors by northern blotting. Interestingly, as shown

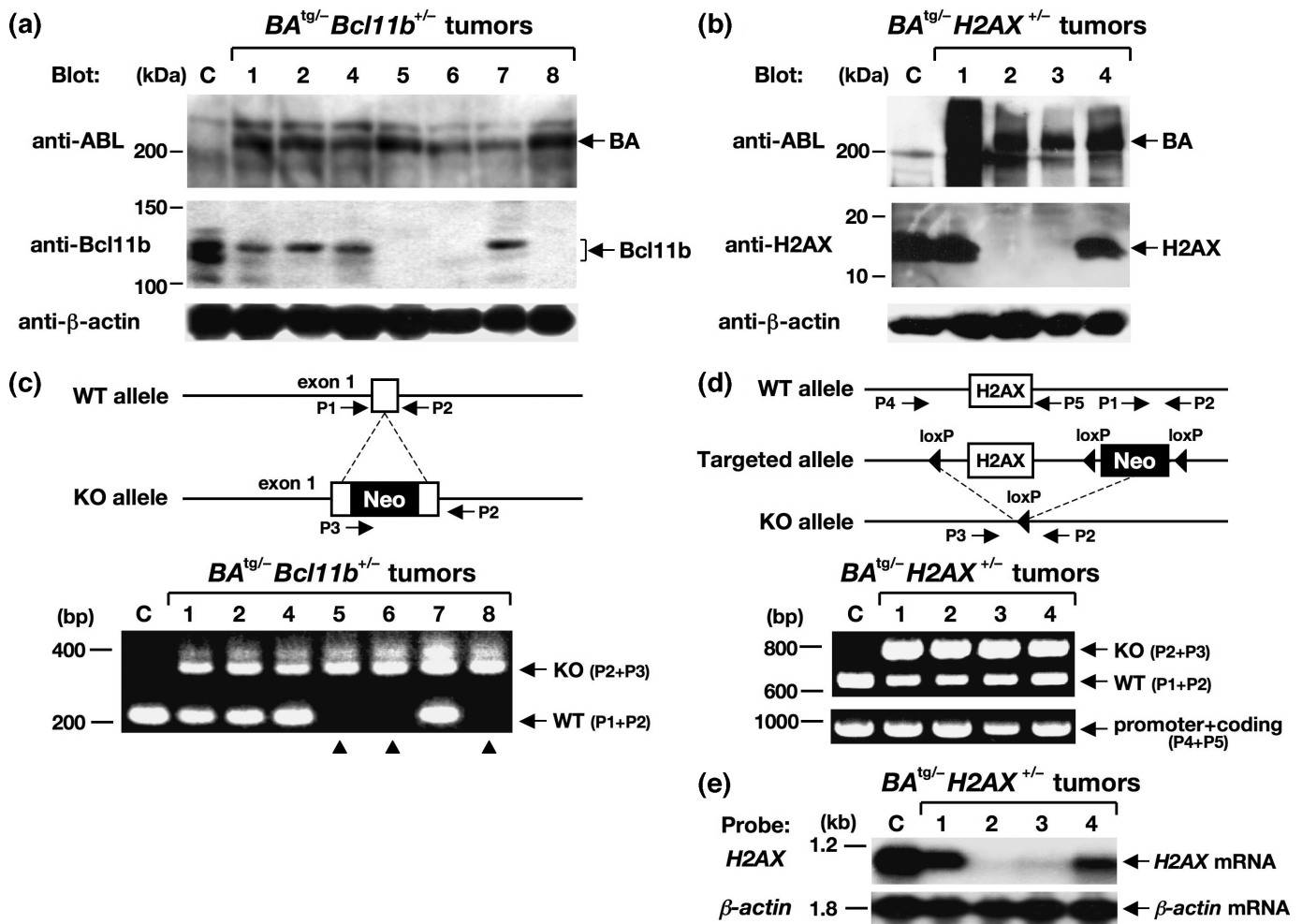


Fig. 4. Gene expression and PCR analyses of the tumors that developed in *BA^{tg/-}Bcl11b^{+/-}* (left panels) and *BA^{tg/-}H2AX^{+/-}* (right panels) mice. (a,b) Western blot analysis for the expression of p210BCR/ABL, Bcl11b, and H2AX proteins. Proteins extracted from a control (C) thymus and tumor tissues of *BA^{tg/-}Bcl11b^{+/-}* (no. 1, 2, and 4–8) and *BA^{tg/-}H2AX^{+/-}* mice (no. 1–4) were blotted with an anti-ABL antibody (upper panels) and anti-Bcl11b or anti-H2AX antibody (middle panels). The positions of p210BCR/ABL (BA), Bcl11b, and H2AX proteins are indicated by arrows. An anti-β-actin blot was carried out as an internal control (bottom panels). Protein markers are shown on the left. (c,d) Schematic illustrations of wild-type and targeted alleles for *Bcl11b* and *H2AX* genes (upper panels) and the resultant genomic PCR products (lower panels). DNA extracted from a control (C) thymus and tumor tissues of *BA^{tg/-}Bcl11b^{+/-}* (no. 1, 2 and 4–8) and *BA^{tg/-}H2AX^{+/-}* mice (no. 1–4) were amplified with sets of primers (P1 and P2 for wild-type [WT] alleles, P2 and P3 for knockout [KO] alleles, and P4 and P5 for a part of the promoter and the whole coding region of *H2AX*). The positions of primers are shown in the upper panels and WT- and KO-derived PCR products are indicated by arrows in the lower panels. Molecular markers are shown on the left. Samples without *Bcl11b* expression are indicated by arrowheads. *Neo*, neomycin resistance gene. (e) Expression of *H2AX* mRNA in *BA^{tg/-}H2AX^{+/-}* tumors. RNA extracted from a control thymus (C) and tumor tissues of *BA^{tg/-}H2AX^{+/-}* mice (no. 1–4) were hybridized with *H2AX* cDNA probe. *β-Actin* hybridization was carried out as an internal control. Molecular markers are shown on the left.

in Figure 4(e), no H2AX mRNA was detected in tumors lacking H2AX protein expression (no. 2 and 3). These results indicated that the absence of H2AX protein was not due to deletion or mutation in the *H2AX* gene but to a lack of mRNA expression.

Chromosomal abnormalities in the leukemic cells developed in *BA^{tg/-}H2AX^{+/-}* mice. We finally examined the chromosomal status of the leukemic cells developed in *BA^{tg/-}H2AX^{+/-}* mice, as previous reports demonstrated that haploinsufficiency and absence of H2AX led to increased incidence of chromosomal abnormalities.^(14,15) In the four tumors that arose from *BA^{tg/-}H2AX^{+/-}* mice, although two samples showed a normal karyotype (no. 1 and 4, data not shown), the other two samples (no. 2 and 3) that did not express H2AX protein (Fig. 4b) exhibited chromosomal aberrations. As shown in the left panel of Figure 5, sample no. 2 contained an additional chromosome (indicated by an arrowhead). In addition, as shown in the right panel of Figure 5, sample no. 3 exhibited deletions in the long arm of chromosome 6 and in the short arm of chromosome 13,

and a breakage in chromosome 11 (indicated by arrows). These results suggested the possibility that the acquired loss of H2AX induced chromosomal instability and resulted in the chromosomal abnormalities observed in samples no. 2 and 3.

Discussion

Chronic myelogenous leukemia presents a paradigm for cancers that evolve through accumulation of genetic alterations. Generation of p210BCR/ABL initiates CML CP and additional genetic events progress the disease and develop CML BC.^(1–3) Although chromosomal and molecular analyses revealed that various mechanisms are involved in the transition from CP to BC,^(1–3) genes responsible for the evolution to BC have not fully been identified.

To elucidate the mechanisms underlying the disease evolution of CML, we have developed an *in vivo* model for CML in which expression of *p210BCR/ABL* induces CML CP, and additional

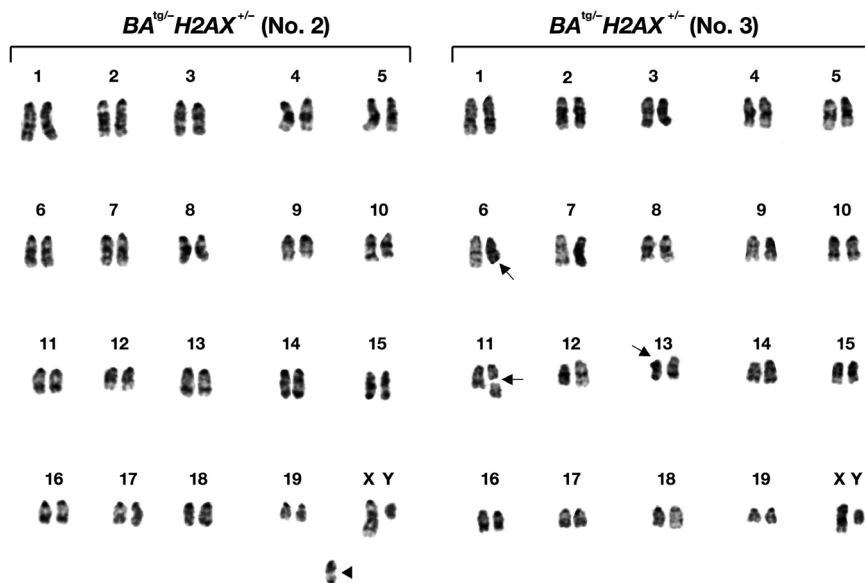


Fig. 5. Chromosomal abnormalities observed in two tumors (no. 2 and 3) that developed in $BA^{tg/-}H2AX^{+/-}$ mice. The additional chromosome in tumor no. 2 is indicated by an arrowhead, and deletion and breakage of the chromosomes in tumor no. 3 are indicated by arrows.

genetic alterations cooperate with *p210BCR/ABL* to progress the disease to CML BC.⁽⁴⁻⁷⁾ Using this as a model system, we examined the possible contribution of haploinsufficiency of *Bcl11b* and *H2AX* to CML BC, by crossing *p210BCR/ABL* transgenic mice ($BA^{tg/-}$) with *Bcl11b* heterozygotes ($Bcl11b^{+/-}$) and *H2AX* heterozygotes ($H2AX^{+/-}$).

Bcl11b encodes a zinc finger protein involved in thymocyte development and differentiation.⁽⁸⁾ *Bcl11b* was originally identified as a gene homologous to *Bcl11a*, that was cloned from t(2;14)(p13;q32.3)-carrying malignant lymphomas,⁽¹⁶⁾ and subsequently shown to be frequently deleted or mutated in radiation-induced thymoma in mice.⁽¹⁷⁾ Conditional knockout analysis showed that acquired ablation of *Bcl11b* in thymocytes resulted in impaired positive selection, altered T-cell receptor signaling, and reduced survival.⁽¹⁸⁾ In addition, a recent study revealed that *Bcl11b* is involved in human leukemia carrying inv(14)(q11.2q32.31), which resulted in generation of the *Bcl11b-TRDC* fusion transcript.⁽¹⁹⁾ On the other hand, *H2AX* is a member of the histone H2A family and a constituent of the nucleosome, the basic subunit of chromatin.^(9,20,21) In response to the DNA double-strand break, *H2AX* rapidly becomes phosphorylated on the serine residue located at the C-terminus to form γ H2AX at the DNA double-strand break sites.^(9,20,21) This event creates a focus in the nucleus, where DNA repair and chromatin remodeling proteins are recruited.^(9,20,21) In human hematopoietic malignancies, a single nucleotide polymorphism upstream of the *H2AX* gene was found to be tightly associated with susceptibility to non-Hodgkin lymphoma.⁽²²⁾ These results indicated that *Bcl11b* and *H2AX* are functionally implicated in cell differentiation and chromosomal stability, respectively, and are involved in subsets of hematopoietic malignancies.

We found that 8 of 15 $BA^{tg/-}Bcl11b^{+/-}$ mice and 4 of 11 $BA^{tg/-}H2AX^{+/-}$ mice developed acute leukemia and died in a short period (Fig. 1). These results indicated that haploinsufficiency of *Bcl11b* and *H2AX* conferred a growth advantage to *p210BCR/ABL*-expressing hematopoietic cells and consequently induced acute leukemia. The blast cells were highly malignant, as evidenced by massive proliferation in the peripheral blood, destruction of the basic structure of the thymus, and marked infiltration in non-hematopoietic tissues (upper 3 panels of Fig. 2). Surface marker analysis showed that the leukemic cells were of T-cell phenotype and Southern blot analysis demonstrated that most of the tumors were clonal in origin (Fig. 3). As the bone marrow showed the typical picture of CML CP (bottom panels

of Fig. 2), the leukemias that developed in $BA^{tg/-}Bcl11b^{+/-}$ and $BA^{tg/-}H2AX^{+/-}$ mice were considered to be CML T-cell BC rather than *de novo* T-cell malignancy.

Interestingly, protein analysis revealed that the expression of *Bcl11b* and *H2AX* was lost in several tumors that developed in the $BA^{tg/-}Bcl11b^{+/-}$ and $BA^{tg/-}H2AX^{+/-}$ mice (Fig. 4a,b, middle panels). These results strongly suggested that the expression of *p210BCR/ABL* rendered genetic instability in the hematopoietic cells and consequently lost the normal residual allele of *Bcl11b* and *H2AX*, as reported in our previous study.⁽⁵⁾ Indeed, in $BA^{tg/-}Bcl11b^{+/-}$ tumors, genomic PCR analysis revealed that the wild-type *Bcl11b*-derived band was not amplified in tumors lacking *Bcl11b* expression (no. 5, 6, and 8 in the lower panel of Fig. 4c), indicating that loss of the normal *Bcl11b* allele was responsible for the lack of the protein product. In contrast, the tumor tissues with no *H2AX* expression in $BA^{tg/-}H2AX^{+/-}$ mice retained the normal *H2AX* allele, including the 3' region, a part of the promoter region, and the whole coding region (no. 2 and 3 in the lower panels of Fig. 4d). Instead, we found that no *H2AX* mRNA was expressed in tumors lacking *H2AX* protein (no. 2 and 3 in the upper panel of Fig. 4e), which indicated that the absence of *H2AX* protein was due to the lack of *H2AX* mRNA expression. Although the mechanism underlying loss of the *H2AX* message in these tumors remains unclear, one possibility is that *p210BCR/ABL*-induced genetic alterations might have occurred in the other regions regulating *H2AX* transcription, such as the enhancer, which led to the loss of mRNA expression. Alternatively, *p210BCR/ABL* might have impaired the transcriptional machinery for *H2AX* mRNA in these tumors by an unknown mechanism. Taken together, our findings demonstrated that *p210BCR/ABL* induces loss of protein expression through several different mechanisms, including genomic instability and transcriptional inhibition.

It is to be noted that four $BA^{tg/-}Bcl11b^{+/-}$ and two $BA^{tg/-}H2AX^{+/-}$ leukemic mice retained *Bcl11b* and *H2AX* protein expression (no. 1, 2, 4, and 7 in the middle panel of Fig. 4a and no. 1 and 4 in the middle panel of Fig. 4b). Thus, the mechanism of how haploinsufficiency of these genes caused disease evolution is to be clarified. Although no obvious phenotypic abnormalities were found in $Bcl11b^{+/-}$ or $H2AX^{+/-}$ mice, previous studies demonstrated that both types of heterozygotes exhibit enhanced susceptibility to hematological malignancies on $p53^{+/-}$ and $p53^{-/-}$ backgrounds.^(14,15,23) These results indicated that both genes function as a dosage-dependent tumor suppressor and their

haploinsufficiency predisposes to cancer development in certain genetic backgrounds. Thus, it is possible that haploinsufficiency of *Bcl11b* and *H2AX* exerted its oncogenic potential in cooperation with *p210BCR/ABL*, conferred a growth advantage to *p210BCR/ABL*-expressing hematopoietic cells, and consequently developed CML BC. An alternative possibility is that because *p210BCR/ABL* is known to promote genetic instability,^(3,5) altered expression of unknown genes synergized with haploinsufficient *Bcl11b* or *H2AX* in *p210BCR/ABL*-expressing hematopoietic cells, accelerated progression of CML, and eventually caused CML BC.

We finally examined the possible chromosomal abnormalities in the leukemic cells of *BA^{tg/-}H2AX^{+/-}* mice, as previous reports demonstrated that haploinsufficiency or deficiency of *H2AX* induced various chromosomal aberrations, especially on a *p53^{-/-}* genetic background.^(14,15) The results showed that two of four tumors exhibited chromosomal abnormalities, which were the presence of an additional chromosome, deletion in part of the long and short arms, and breakage in the body of several chromosomes (Fig. 5). Interestingly, *BA^{tg/-}H2AX^{+/-}* mice with these chromosomal abnormalities exhibited very high white blood cell (WBC) counts ($>1 \times 10^5/\mu\text{L}$, see the right top panel of Fig. 2 and Table 2), suggesting that these events conferred a marked proliferative ability to *p210BCR/ABL*-expressing hematopoietic cells and exhibited a very aggressive phenotype. We also examined the possible contribution of dysfunction of genes involved in error-prone non-homologous end joining, such as *DNA ligase IV* and *XRCC4*, by crossing *BA^{tg/-}* with *DNA ligase IV* heterozygous mice and *XRCC4* heterozygous mice. However, we did not observe disease acceleration or CML BC in *BA^{tg/-}DNA ligase IV^{+/-}* or *BA^{tg/-}XRCC4^{+/-}* double transgenic mice (data not shown), suggesting the possibility that among DNA repair-associated genes, *H2AX* might play a unique role in the disease evolution of CML.

The CML BC observed in *BA^{tg/-}Bcl11b^{+/-}* and *BA^{tg/-}H2AX^{+/-}* mice were of T-cell phenotype. Although T-cell BC is frequently observed in mouse models for CML,^(5,24) it is rarely detected in

human clinical samples. The reason for this discrepancy is not clear but one possibility is that human CML originates from the acquisition of *p210BCR/ABL*-transformed hematopoietic stem cells and the T-cell lineage is rarely involved probably due to its prolonged life span, whereas every cell in transgenic (or knockout) mice inherently contains (or lacks) the target gene and T cells might be more susceptible to the target gene-induced oncogenic transformation than other types of hematopoietic cells.

It is intriguing to examine whether acquired expressional loss of *Bcl11b* and *H2AX* contributes to human CML BC. We examined *Bcl11b* and *H2AX* expression in several CML BC samples by RT-PCR but did not detect the absence of mRNA expression in either gene (Supporting Information Fig. S1), probably due to the limited number of samples available and a lack of T-cell crisis cases. Thus, an expanded study is required to clarify the clinical significance of dysfunction of these genes in the development of CML BC.

In the present report, we demonstrated that haploinsufficiency and acquired loss of protein expression of *Bcl11b* and *H2AX* cooperate with *p210BCR/ABL* and induce CML BC. Our findings demonstrated that altered expression of genes involved in cell differentiation or chromosomal integrity contributes to the development of CML BC, which provides insights into the molecular mechanisms underlying the disease evolution of CML.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Expression of *Bcl11b* and *H2AX* in chronic myelogenous leukemia (CML) chronic phase (CP), CML blast crisis (BC), and normal bone marrow (BM). RNA extracted from one CML CP sample, four CML BC samples (two myeloid and two B-lymphoid), and one normal BM sample were subjected to RT-PCR for the expression of *Bcl11b* and *H2AX*. *β-Actin* RT-PCR was carried out as an internal control.

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