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ORIGINAL ARTICLE

Clinical significance of *ASXL2* and *ZBTB7A* mutations and C-terminally truncated *RUNX1-RUNX1T1* expression in AML patients with t(8;21) enrolled in the JALSG AML201 study

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Abstract

We analyzed the clinical significance and genetic features of *ASXL2* and *ZBTB7A* mutations, and the alternatively spliced isoform of the *RUNX1-RUNX1T1* transcript, which is also called *AML1-ETO*9a (AE9a), in Japanese CBF-AML patients enrolled in the JALSG AML201 study. *ASXL2* and *ZBTB7A* genes were sequenced using bone marrow samples of 41 AML patients with t(8;21) and 14 with inv(16). The relative expression levels of AE9a were quantified using the real-time PCR assay in 23 AML patients with t(8;21). We identified *ASXL2* (34.1%) and *ZBTB7A* (9.8%) mutations in only AML patients with t(8;21). *ASXL2*-mutated patients had a significantly higher WBC count at diagnosis ($P = 0.04$) and a lower frequency of sex chromosome loss than wild-type patients (33 vs. 76%, respectively, $P = 0.01$). *KIT* mutations were the most frequently accompanied with both *ASXL2* (36%) and *ZBTB7A* (75%) mutations. Neither *ASXL2* nor *ZBTB7A* mutations had an impact on overall or event-free survival. Patients harboring cohesin complex gene mutations expressed significantly higher levels of AE9a than unmutated patients ($P = 0.03$). In conclusion, *ASXL2* and *ZBTB7A* mutations were frequently identified in Japanese AML patients with t(8;21), but not in those with inv(16). Further analysis is required to clarify the detailed biological mechanism of AE9a regulation of the cohesin complex.

Keywords Acute myeloid leukemia · Core-binding factor · *ASXL1/2* · *ZBTB7A* · *RUNX1-RUX1T1* transcript

Introduction

Core-binding factor (CBF)-acute myeloid leukemia (AML) accounts for about 25% of Japanese adult AML patients, who are categorized as a favorable risk group; however, relapse occurs in approximately half of the patients [1]. Chromosomal aberrations t(8;21)(q22;q22) and inv(16)(p13.1q22)/t(16;16)(p13.1;q22) result in fusion transcripts encoding subunits of CBF [2]. Their fusion proteins, RUNX1-RUNX1T1, and CBFB-MYH11, reportedly block myeloid maturation, but are not sufficient to induce leukemia [3, 4]. Additional driver mutations are, therefore, required for the clonal expansion of leukemia cells. *KIT* mutation is the most frequently identified driver mutation in CBF-AML, and it has been suggested to be a poor prognostic factor [5]. Recently, recurrent *ASXL1* or *ASXL2* (additional sex combs-like 1 or 2, respectively) and *ZBTB7A* (zinc-finger and BTB domain containing 7A) mutations in AML patients with t(8;21) were identified. *ASXL1* and *ASXL2* are epigenetic regulators involved in the regulation or recruitment of the polycomb-group repressor complex and trithorax-group activator complex, and the loss of *ASXL2* promoted AML in association with t(8;21) [6, 7]. *ASXL1* and *ASXL2* mutations were exclusively identified with an incidence of 12–19% and 17–23% in AML patients with t(8;21), respectively [8–13]. *ZBTB7A* is known as a leukemia/lymphoma-related factor (LRF) that encodes a transcription factor of the POK (poxvirus and zinc finger and

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Krüppel)/ZBTB (zinc finger and broad complex, tramtrack, and bric-a-brac) family [14]. *ZBTB7A* mutations were identified in 10–23% of AML patients with t(8;21), and commonly consisted of missense or truncating mutations, resulting in alteration of the DNA binding ability of the zinc-finger domains [12, 15, 16]. It has been reported that loss of *ZBTB7A* was associated with the upregulation of glycolysis metabolism and tumor cell proliferation [17]. These results indicate that *ASXL1/2* and *ZBTB7A* mutations are closely associated with the development of CBF-AML, whereas their prognostic significance has not been clarified yet.

In addition, it has been reported that an alternatively spliced isoform of the *RUNX1-RUNX1T1* (also called *AML1-ETO*: AE) transcript, *RUNX1-RUNX1T19a* (also called *AML1-ETO*9a: AE9a), which encodes a C-terminally truncated RUNX1-RUNX1T1 protein, might be associated with the development of AML with t(8;21) [18]. Co-expression of AE and AE9a is shown to block myeloid cell differentiation and reduce the latency of AML development in murine models. However, the clinical significance and genetic mechanism of AE9a expression remain unclear.

In the present study, we focused on the clinical and genetic features of CBF-AML harboring *ASXL2* and *ZBTB7A* mutations, and the *AE9a* expression level of t(8;21) AML in Japanese patients enrolled in the JALSG AML201 study.

Patients and methods

Patients and treatment

The JALSG AML201 study was a multi-center phase 3 randomized study for newly diagnosed de novo adult AML patients, except for those with acute promyelocytic leukemia (UMIN Clinical Trials Registry C000000157, http://www.umin.ac.jp/ctrj/). The patients were prospectively registered and randomly assigned to receive either idarubicin (IDR) or high-dose daunorubicin (HiDNR) for induction therapy, and those who achieved CR were again randomized to receive either four courses of conventional consolidation therapy or three courses of high-dose cytarabine (HiDAC) therapy. The AML201 study included 247 CBF-AML patients, of whom 55 patients' samples were available for comprehensive genetic analysis, and their clinical and genetic data were used for this study. This study was approved by the institutional review board of each participating institution and all patients provided informed consent for banking and molecular analysis before registration in accordance with the Declaration of Helsinki.

Mutation analysis

In the previous study, comprehensive genetic analysis targeting 51 genes was performed, but it did not include *ASXL2* and *ZBTB7A* genes [19]. We, thus, additionally analyzed their mutations in this study. Exons 12 and 13 of *ASXL2* and exons 2 and 3 of *ZBTB7A* genes were amplified using each forward and reverse primer set (Supplementary Table 1). Amplified products were purified using the MinElute 96 UF PCR Purification kit (Qiagen, Hilden, Germany), and sequenced on an ABI 3100 Avant sequence detection system (Applied Biosystems, Foster City, CA). Cytogenetic G-banding analysis was performed using standard methods. In addition, we also examined *RUNX1-RUNX1T1* and *CBFB-MYH11* chimeric gene transcripts by reverse transcriptase-mediated quantitative PCR (RQ-PCR), as previously reported [20].

Quantification of *RUNX1-RUNX1T1* (*AE*) and *RUNX1-RUNX1T19a* (*AE9a*) *m*RNA expressions

Complementary DNA (cDNA) was generated from total RNA using the SuperScriptII Reverse Transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The *AE* fusion transcript was amplified by nested PCR using the previously described primers (Supplementary Table 2) and AccuTaq LA DNA Polymerase (Sigma-Aldrich, St Louis, MO, USA) for 25 cycles in a volume of 50 μL with an initial denaturation step of 98 °C for 30 s followed by 25 cycles of denaturation at 94 °C for 15 s, annealing at 58 °C for 20 s, extension at 68 °C for 3 min, and with a final extension cycle of 68 °C for 10 min [18]. To quantify the relative expression level of *AE9a*, the real-time quantitative (RQ)-PCR assay was performed in a total reaction mixture (20 μL) containing 2 μL of amplified *AE* fusion transcript, 10 μl of 2 × TaqMan Universal Master Mix II with UNG (Applied Biosystems), forward and reverse primers, and each probe (Supplementary Table 2). *GAPDH* was quantified as the internal control using TaqMan Gene Expression Master Mix (Applied Biosystems). RQ-PCR was performed with Applied Biosystems 7300 Real-time PCR System (Applied Biosystems) using the following protocol: an initial denaturation and polymerase activation step for 2 min at 50 °C and 10 min at 95 °C, followed by 45 cycles of denaturation at 95 °C for 15 s and 60 °C for 1 min. Each RQ-PCR assay contained the dilution series of a standard for the calibration curve, and all samples and standards were run in duplicate. The standards were plasmid controls that contained the PCR products amplified by each primer set.

Statistical analysis

Demographic factors and disease characteristics were compared using the standard χ^2 test for categorical data and the Mann–Whitney *U* test for continuous variables. Probabilities of overall survival (OS) were estimated using the Kaplan-Meier method, and the log-rank test was used for univariate comparisons. The survival time was calculated from the date

of entry into the JALSG AML201 study to death due to any cause or the last follow-up. Event-free survival (EFS) was defined as the time from the day of achieving CR to relapse, death due to any cause, or the last follow-up. Patients undergoing allogeneic stem cell transplantation were not censored at the time of transplantation. A significance level of $P < 0.05$ was considered significant for all analyses. All statistical analyses were performed by Stata version 12 (Stata Corp., College Station, TX).

Results

Frequency and clinical features of *ASXL2* mutations in t(8;21) AML

We identified *ASXL2* mutations in 14 of 41 (34.1%) AML patients with t(8;21), but in no AML patients with inv(16). *ASXL2* mutations were preferentially identified in the 3′ region of the gene with 13 frame-shift mutations and 1 single nucleotide variant mutation. Eight of 41 patients (19.5%) had *ASXL2* mutations in exon 12, whereas 6 of 41 (14.6%) had them in exon 13 (Fig. 1). The characteristics of the AML patients with t(8;21) are summarized in Table 1. *ASXL2*-mutated patients had a significantly higher WBC count at the time of diagnosis than those with the wild-type (median: 12,400 vs. 7100 cells/μL, respectively, $P = 0.04$). In cytogenetic analysis, the loss of sex chromosomes was observed in 4 (33%) *ASXL2*-mutated patients, with the rate being significantly lower than in wild-type patients (76%, $P = 0.01$). There was no difference in the frequency of del(9q) and no patient carried the trisomy 8 chromosome in our cohort.

The median follow-up period of survivors was 1547 days (range, 1002–2355). *ASXL2* mutations had no impact on the CR rate ($P = 0.48$), OS ($P = 0.64$, Fig. 2a), or EFS ($P = 0.57$, Fig. 2b) compared with those with the wild-type, with a 3-year OS rate of 81.5% (95% confidence interval [CI], 61.1–91.8)

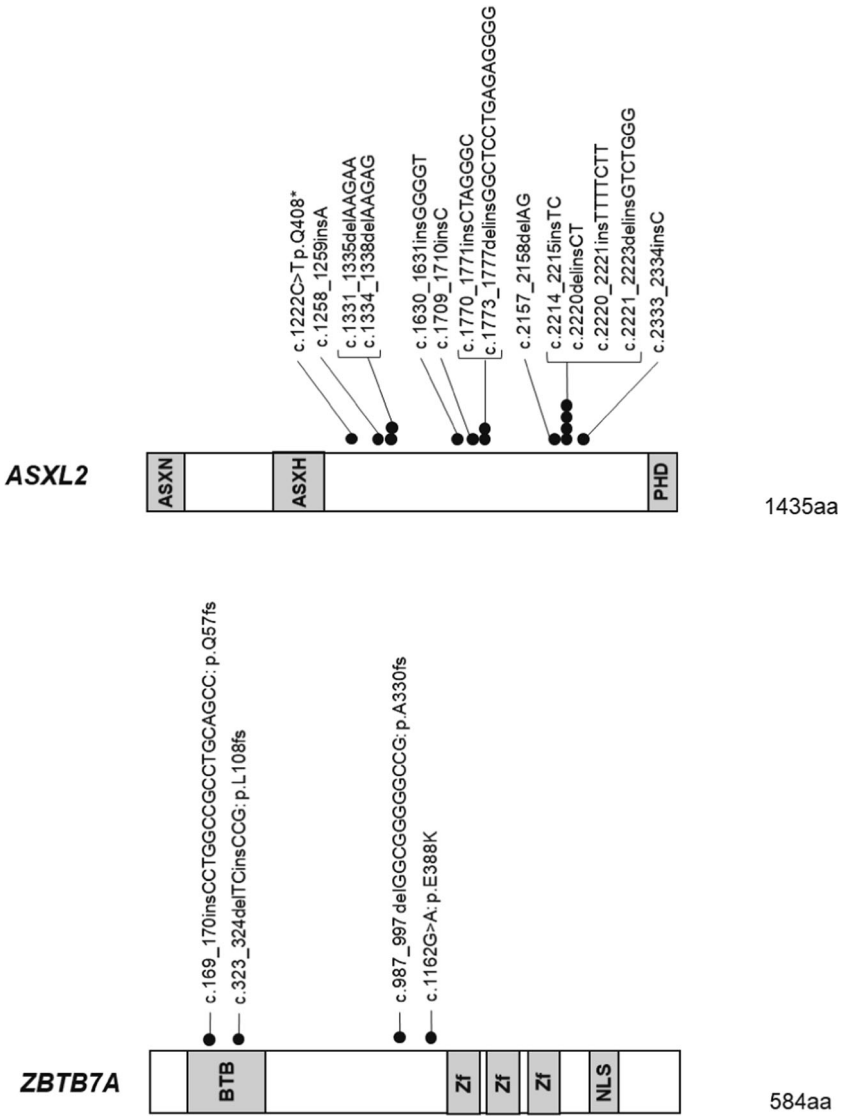

Fig. 1 Schematic representation of *ASXL2* and *ZBTB7A* mutations. ASXN, Asx homology N; PHD, plant homeodomain; BTB, BR-C ttk and bab; NLS, nuclear localization sequence; Zf, zincfinger

Table 1 Patient characteristics

	All patients ($n = 41$)	*ASXL2* wild ($n = 27$)	*ASXL2* mutation ($n = 14$)	*P* value*	*ZBTB7A* wild ($n = 37$)	*ZBTB7A* mutation ($n = 4$)	*P* value†
Age (years)				0.25			0.92
10–19	3	2	1		3	0	
20–29	5	2	3		4	1	
30–39	10	10	0		9	1	
40–49	7	4	3		6	1	
50–59	14	8	6		13	1	
60–65	2	1	1		2	0	0.74
WBC ($\times 10^3/\mu L$), median [range]	9.5 [0.2–67.6]	7.1 [0.2–51.4]	12.4 [1.2–67.6]	0.04	8.8 [0.2–57.6]	16.4 [8.3–51.4]	0.18
Additional cytogenetic abnormalities‡							
-X or -Y	23 (62%)	19 (76%)	4 (33%)	0.01	20 (54%)	3 (75%)	0.56
del(9q)	4 (10%)	3 (11%)	1 (7%)	0.73	4 (11%)	0 (0%)	0.46
Gene mutations							
KIT	19 (46%)	14 (52%)	5 (36%)	0.33	16 (43%)	3 (75%)	0.55
exon 8	4 (10%)	2 (7%)	2 (14%)	0.48	3 (8%)	1 (25%)	0.28
exon 10–11	1 (2%)	1 (4%)	0 (0%)	0.47	1 (3%)	0 (0%)	0.74
exon 17	14 (34%)	11 (41%)	3 (21%)	0.22	12 (32%)	2 (50%)	0.48
FLT3	3 (7%)	2 (7%)	1 (7%)	0.98	4 (11%)	0 (0%)	0.49
ITD	2 (5%)	2 (7%)	0 (0%)	0.30	3 (8%)	0 (0%)	0.55
KDM	1 (2%)	0 (0%)	1 (7%)	0.26	1 (3%)	0 (0%)	0.74
NRAS	2 (5%)	1 (4%)	1 (7%)	0.63	1 (3%)	1 (25%)	0.05
Outcomes							
CR (%)	37 (90%)	25 (93%)	12 (86%)	0.48	33 (89%)	4 (100%)	0.49
3-year OS, % [95% CI]	78.1 [62.1–87.9]	81.5 [61.1–91.8]	71.4 [40.6–88.2]	0.64	81.1 [64.4–90.5]	50.0 [5.8–84.5]	0.15
3-year EFS, % [95% CI]	56.8 [39.4–70.8]	52.0 [31.3–69.2]	66.7 [33.7–86.0]	0.57	60.6 [42.0–74.9]	25.0 [0.9–66.5]	0.14

*P value comparing *ASXL2* mutation versus wild patients.

† P value comparing *ZBTB7A* mutation versus wild patients

‡ Data only available in 37 patients

vs. 71.4% (95%CI, 40.6–88.2) and with a 3-year EFS rate of 52.0% (95%CI, 31.3–69.2) vs. 66.7% (95%CI, 33.7–86.0), respectively (Table 1). Among 14 *ASXL2*-mutated patients, 12 achieved CR after induction therapy and received subsequent consolidation therapies (HiDAC group: 6, multi-agent chemotherapy group: 6). According to the consolidation treatment, there was no significant difference in OS ($P = 0.14$) or EFS ($P = 0.15$) in *ASXL2*-mutated patients.

Frequency and clinical features of *ZBTB7A* mutations in t(8;21) AML

ZBTB7A mutations were identified in 4 of 41 (9.8%) AML patients with t(8;21), but none in AML patients with inv(16). Two of the 4 (50%) *ZBTB7A*-mutated patients had *ASXL2* mutations. Two had a frame-shift insertion resulting in the loss of the N-terminal BTB domain, one had a missense mutation resulting in an amino acid change, and the final one had a frame-shift deletion resulting in alternation or loss of the C-terminal zinc-finger domain (Fig. 1).

There was no significant difference in patients' age, sex, or additional cytogenetic abnormality according to *ZBTB7A* mutations (Table 1). The 3-year OS rates of *ZBTB7A*-mutated and -unmutated patients were 50.0% (95%CI, 5.8–84.5) and 81.1% (95%CI, 64.3–90.5), respectively, and 3-year EFS rates were 25.0% (95%CI, 8.9–66.5) and 60.6% (95%CI, 42.0–74.9), respectively; however, there was no significance in either OS ($P = 0.15$, Fig. 2c) or EFS ($P = 0.14$, Fig. 2d).

Cooperative gene mutations with *ASXL2* and *ZBTB7A*

Cooperative gene mutations accompanied by *ASXL2* and *ZBTB7A* mutations are shown in Tables 1 and 2, and Fig. 3 [21]. *KIT* mutations were the most frequently observed in both *ASXL2* and *ZBTB7A* mutations with frequencies of 36 and 75%, respectively. There was no significant correlation of

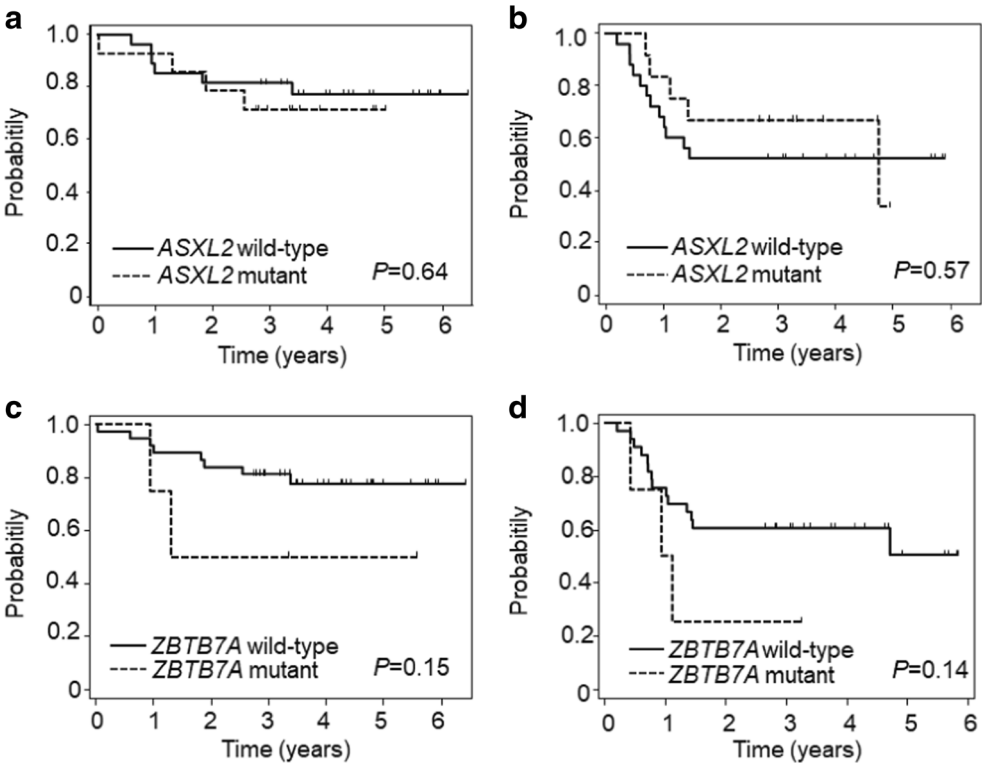

Fig. 2 Outcomes according to *ASXL2* and *ZBTB7A* mutations in t(8;21) AML. **a** Overall survival and **b** event-free survival according to *ASXL2* mutations. **c** Overall survival and **d** event-free survival according to *ZBTB7A* mutations

ASXL2 and *ZBTB7A* mutations with *KIT*, *FLT3*, and *NRAS*, which are frequently observed driver mutations in CBF-AML (Table 1). Within the 51 gene variants screened by target sequencing, chromatin modifier genes were significantly accumulated in *ASXL2*-mutated patients compared with those with wild-type ($P = 0.02$). *ASXL2* mutations were exclusive with *ASXL1* mutations. There was no significant correlation between other evaluated gene mutation families (Table 2). On the other hand, there was no significantly accumulated gene mutation accompanied by *ZBTB7A* mutation.

Clinical features and outcomes according to the expression level of *RUNX1-RUNX1T19a (AE9a)*

Quantitative analysis of *AE9a* expression was performed in 23 AML patients with t(8;21). The *AE9a* transcript was

Table 2 Cooperative gene mutations

Gene	All patients	*ASXL2*		*P* value*	*ZBTB7A*		*P* value†
		wild	mutation		wild	mutation	
	($n = 41$)	($n = 27$)	($n = 14$)		($n = 37$)	($n = 4$)	
Activating kinase	26	18	8	0.55	22	4	0.11
FLT3, *KIT*, *NRAS*, *KRAS*, *CBL*, *JAK1*, *JAK3*, *PTPN11*	(63%)	(67%)	(57%)		(59%)	(100%)	
Chromatin modifier	11	4	7	0.02	9	2	0.27
ASXL1, *ATRX*, *BCOR*, *BCORL1*, *EZH2*, *KDM6A*	(27%)	(15%)	(50%)		(24%)	(50%)	
Transcription factor	6	3	3	0.38	6	(0%)	0.38
ETV6, *GATA2*, *MLL*, *NCOR1*, *NCOR2*, *RUNX1*	(15%)	(11%)	(21%)		(16%)		
Cohesin	7	4	3	0.60	7	0	0.34
RAD21, *SMC1A*, *SMC3*, *STAG2*	(17%)	(15%)	(21%)		(19%)	(0%)	
DNA methylation	5	3	2	0.77	5	0	0.43
DNMT3A, *DOT1L*, *IDH1*, *IDH2*, *TET2*	(12%)	(11%)	(14%)		(14%)	(0%)	
Tumor suppressor	9	4	5	0.13	7	2	0.15
WT1, *TP53*, *NOTCH1*, *NOTCH2*, *PBRM1*, *PHF6*, *EPB41L5*	(22%)	(15%)	(36%)		(19%)	(50%)	
Spliceosome	1	1	0	0.47	1	0	0.74
SF3B1, *SRSF2*, *ZRSR2*, *U2AF1*	(2.4%)	(4%)	(0%)		(3%)	(0%)	

* *P* value comparing *ASXL2* mutation versus wild patients

† *P* value comparing *ZBTB7A* mutation versus wild patients

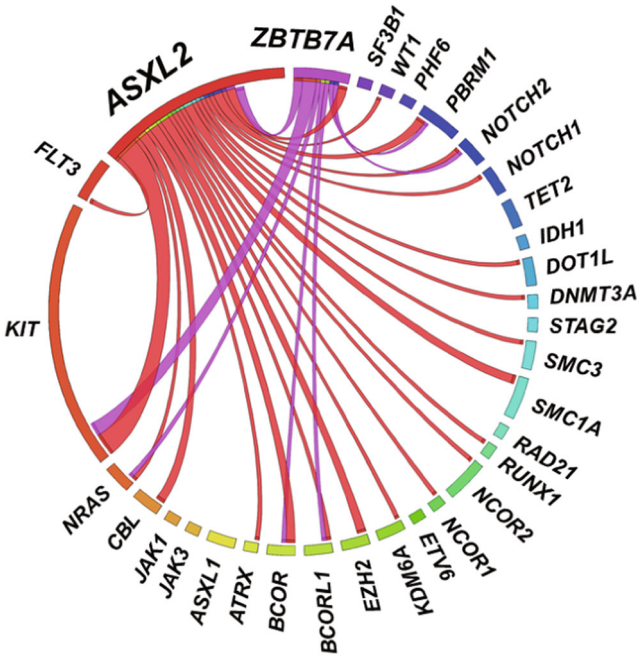

Fig. 3 Corporative mutations with *ASXL2* and *ZBTB7A*. Circos plot of mutated genes according to the gene mutation families are shown

detected in 18 of 23 patients (85.7%). The median relative expression level of *AE9a* to *GAPDH* was 0.15 (range, 0–31.6). The patients were divided into two groups on the basis of a nearly bimodal distribution of relative *AE9a* expression to *GAPDH* ($P = 0.0001$, Fig. 4a). Therefore, a relative *AE9a* expression level of 0.5 was defined as the cut-off value. Nine patients who presented with higher relative amounts of *AE9a* to *GAPDH* than 0.5 (0.88 ± 10.15) were referred to as a High-*AE9a* group, whereas 14 who expressed lower relative amounts of *AE9a* to *GAPDH* than 0.5 (0.05 ± 0.07) were referred to as a Low-*AE9a* group. There was no difference in the patient age ($P = 0.30$) or WBC count ($P = 0.28$) between these groups; however, more patients in High-*AE9a* group carried additional cytogenetic abnormalities, including the loss of sex chromosomes and del(9q) (Supplementary Table 3). The median follow-up period of survivors was 1008 days (range, 274–2150). The relative expression level of *AE9a* had no impact on the CR rate ($P = 0.24$), cycles of induction therapy ($P = 0.25$), OS ($P = 0.26$), or EFS ($P = 0.12$).

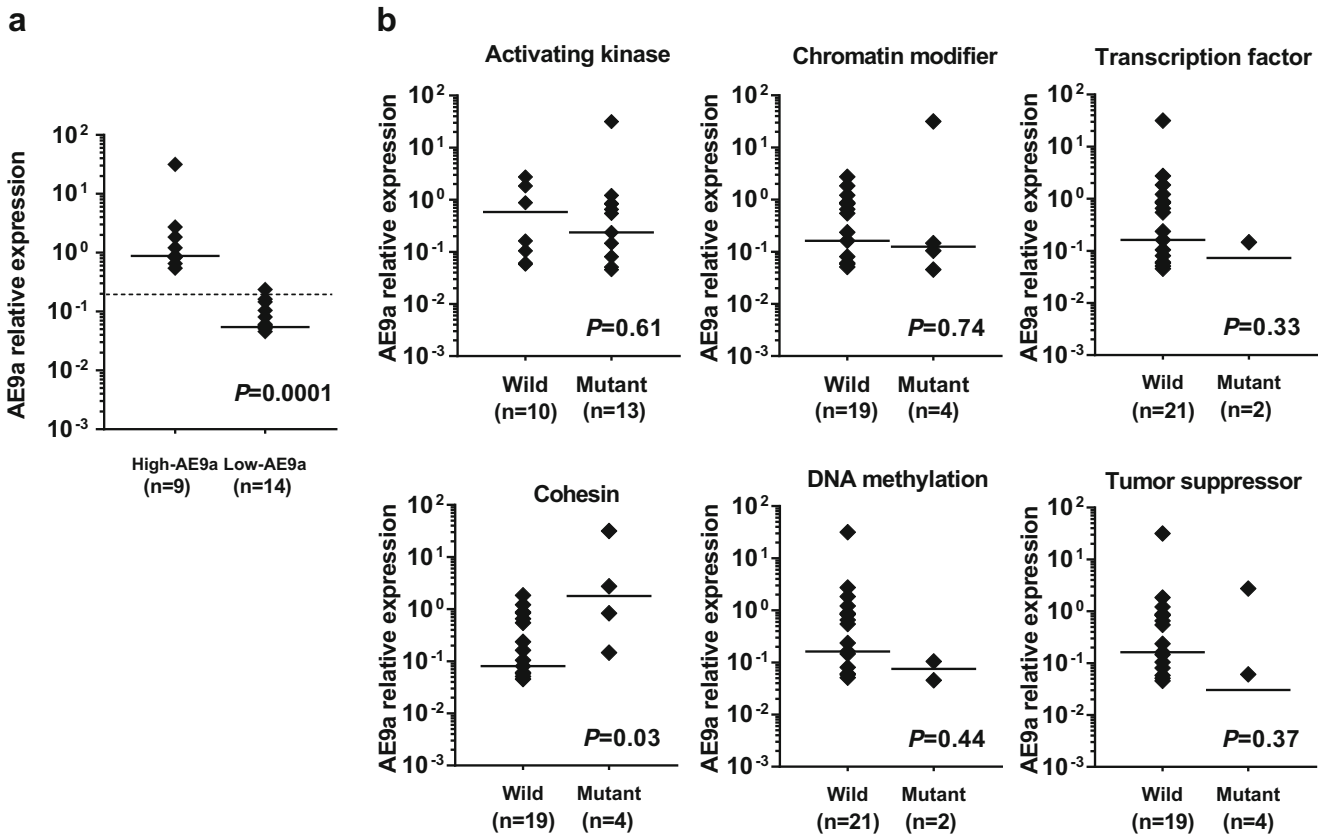

Fig. 4 Relative quantification of *AE9a* expression. **a** The relative expression level of *AE9a* in each patient and median values are shown. The cut-off value (0.5) of High- and Low-*AE9a* groups is shown in a dashed line. **b** The relative expression level of *AE9a* according to gene mutation families and median values is shown

Correlation between *RUNX1-RUNX1T19a* (*AE9a*) expression level and gene mutations

The relative expression level of *AE9a* was analyzed in 23 patients according to the gene mutation families listed in Table 2. *AE9a* expression levels of the patients harboring mutations in cohesin complex genes (*RAD21*, *SMC1A*, *SMC3*, and *STAG2*) were significantly higher than those in unmutated patients (0.08 vs. 1.78, respectively, $P = 0.03$). However, median relative *AE9a* expression levels were similar between wild-type and mutated patients in activating kinase (0.08 vs. 0.24, respectively, $P = 0.61$), chromatin modifiers (0.16 vs. 0.13, respectively, $P = 0.74$), transcription factors (0.16 vs. 0.07, respectively, $P = 0.33$), DNA methylation (0.16 vs. 0.08, respectively, $P = 0.44$), tumor suppressor families (0.16 vs. 0.03, respectively, $P = 0.37$), *KIT* (0.10 vs. 0.60, respectively, $P = 0.33$), and *ASXL2* (0.39 vs. 0.06, respectively, $P = 0.18$) genes (Fig. 4b).

Discussion

In this study, we analyzed the clinical characteristics and overlap mutations of the recently identified *ASXL2* and *ZBTB7A* mutations in Japanese CBF-AML patients. Consistent with previous reports, both mutations were identified in AML patients with t(8;21), but not in AML patients with inv(16). *ASXL2* mutations were more frequently observed in Japanese patients (34.1%) than in previous reports (16.7–22.7%) [11–13]. *ASXL2* mutation was significantly correlated with higher WBC counts at diagnosis, as previously reported [11]. In cytogenetics, it has been reported that *ASXL1* and *ASXL2* mutations were significantly correlated with del(9q) [13], whereas such a correlation was not observed in our patients; however, those mutations were negatively associated with the loss of sex chromosomes in our patients. Cooperative driver gene mutations including *KIT*, *FLT3*, and *NRAS* are frequently identified in AML patients with t(8;21). However, they did not show an association with either *ASXL2* or *ZBTB7A* mutations in this study. Although other mutations in chromatin modifier genes were frequently accompanied with *ASXL2* mutations in our cohort, *ASXL1* and *ASXL2* mutations were mutually exclusive, as previously reported, indicating a shared mechanism of transformation for mutations in these genes [11].

Consistent with a previous report, *ASXL2* mutations did not influence OS or EFS [13]. In the present study, we could not find clinical impacts of *ZBTB7A* mutations on OS and EFS. Further study in a large cohort is required to evaluate the prognostic impact of *ZBTB7A* mutation on the long-term prognosis.

We also analyzed the correlation between *AE9a* expression and genetic alterations. Although a previous report suggested that stimulated cell cycle progression under high *AE9a* expression increased the WBC count, WBC counts at diagnosis were similar between groups with high and low expression levels of *AE9a* [22]. Furthermore, regardless of any threshold setting, there were no correlations between the expression level of *AE9a* and prognosis or *KIT* mutation, as previously described [22]. Interestingly, high expression of *AE9a* was observed in patients harboring mutations in cohesin complex genes. The cohesin complex regulates chromosome segregation during meiosis and mitosis, which is essential for cell division [23]. Furthermore, it has been reported that cohesin complex gene mutations were rarely identified in founding clones, and likely represent secondary events in the clonal hierarchy and contribute to clonal transformation [24]. Therefore, C-terminally truncated RUNX1-RUNX1T1 (AE9a) might contribute to AML development by cooperating with the loss of function of cohesin complexes, resulting in rapid disease progression. Further analysis is required to clarify the detailed biological mechanism in AE9a regulation of the cohesin complex.

As our analysis included a limited number of patients, these findings need to be further evaluated in a larger scale cohort to further our understanding of the pathogenesis of AML with t(8;21).

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Compliance with ethical standards

Conflict of interest H. Kiyoi received research funding from Chugai Pharmaceutical Co., Ltd., Bristol-Myers Squibb, Kyowa Hakko Kirin Co., Ltd., Zenyaku Kogyo Co., Ltd., FUJIFILM Corporation, Nippon Boehringer Ingelheim Co., Ltd., Astellas Pharma Inc. and Celgene Corporation, consulting fees from Astellas Pharma Inc. and Daiichi Sankyo Co., Ltd., and honoraria from Bristol-Myers Squibb and Pfizer Japan Inc., N.A. received research funding from Chugai Pharmaceutical Co., Ltd. and Astellas Pharma Inc., S.M. received honoraria from Astellas Pharma Inc. and Otsuka Pharmaceutical Co., Ltd., N.U. received research funding from Nippon Shinyaku Pharmaceutical Co., Ltd., Novartis Pharma, Bristol-Myers Squibb, Celgene Corporation, Fujimoto Pharmaceutical Co., Ltd., Otsuka Pharmaceutical Co., Ltd., Pfizer Japan Inc. and Sysmex Co., Ltd., consulting fees from Celgene Corporation, Fujimoto Pharmaceutical Co., Ltd., Otsuka Pharmaceutical Co., Ltd., Pfizer Japan Inc. and Sysmex Co., Ltd., Astellas Pharma Inc., CIMIC Co., Ltd., Eli Lilly Japan, Huya Bioscience International, Janssen Pharmaceutical Co., Ltd., Kyowa Hakko Kirin Co., Ltd., Nippon Boehiringer Ingleheim Co., Ltd., SymBio Pharmaceuticals Co., Ltd., Takeda Bio Development Center Ltd. and Zenyaku Kogyo Co., Ltd., and honoraria from Bristol-Myers Squibb, Celgene Co., Ltd., Pfizer Japan Inc., Sysmex Co., Ltd., Chugai Pharmaceutical Co., Ltd. and Kyowa Hakko Kirin Co., Ltd. AT received research funding from Chugai Pharmaceutical Co., Ltd., Astellas Pharma Inc., Pfizer Japan Inc. and Takeda Pharmaceutical Co Ltd. T.N. received research funding from FUJIFILM Corporation, Nippon Boehringer Ingelheim Co., Ltd., Astellas Pharma Inc., Dainippon Sumitomo Pharma Co., Ltd., Otsuka Pharmaceutical Co., Ltd. and Toyama Chemical Co., Ltd., patents and

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Ethical approval This study was approved by the institutional review board of each participating institution. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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