thought to be caused by compromised Polycomb activities because of *Sf3b1* haploinsufficiency.[12] Polycomb group proteins are epigenetic transcriptional repressors with an important role in the regulation of hematopoiesis, and recent studies have shown that mutations in Polycomb group genes occur in hematological neoplasms (including MDS).[25,26] Furthermore, most *Hox* genes are expressed in HSCs and immature progenitors and are downregulated during differentiation and maturation, and the critical role of Hox gene clusters in normal hematopoiesis has been demonstrated repeatedly in many literatures.[27,28] For example, overexpression of *Hoxb4* leads to expansion of HSCs, and *Hoxb4*-deficient mice exhibit a reduced reconstitution capacity similar to that seen in *Sf3b1*$^{+/-}$ mice.[29] Similarly, *Hoxa 9* and *10* are also reported to be functional regulators of HSCs.[30,31] However, in our gene expression data based on RNA sequencing, expression of *Hoxb4* was increased in HSCs from *Sf3b1*$^{+/-}$ mice compared with that from *Sf3b1*$^{+/+}$ mice, and other clustered *Hox* genes, including *Hoxa9* and *10*, were not significantly changed (Supplementary Figure 3b). Thus, the exact mechanism of action that leads to reduced numbers of HSCs and their compromised function remains unclear.

The other important issue to be discussed regarding the phenotype of *Sf3b1*$^{+/-}$ mice is the impact of *Sf3b1* haploinsufficiency upon the formation of ring sideroblasts because the *SF3B1* mutation is closely associated with MDS with increased numbers of ring sideroblasts. Although a previous study reported an increased frequency of ring sideroblasts in *Sf3b1*$^{+/-}$ mice,[21] no increase in ring sideroblasts was demonstrated in the present study, even though both studies analyzed the identical *Sf3b1*$^{+/-}$ mouse strain.[12] However, we considered that the lack of increased numbers of ring sideroblasts, together with the compromised repopulation capacity of *Sf3b1*$^{+/-}$ stem cells, should be rather expected, because most of the *SF3B1* mutations thus far reported in MDS are clustered in the 5th–9th HEAT domains, mainly involving 5 hot spot amino-acid positions (K700 and, to a lesser extent, K666, H662 and E662), and no nonsense or frameshift changes have been reported, suggesting that these *SF3B1* mutations will not lead to simple loss of function but should be associated with some gain of function.[1,2,9] We could not exclude the possibility that an *SF3B1* mutation acts as a dominant negative mutation that leads to more severe functional deficiency than haploinsufficiency, which would be responsible for tumorigenesis. However, our findings suggest that the simple haploinsufficiency of *SF3B1* may not be responsible for the development of the MDS phenotype with increased formation of ring sideroblasts and other clonal disorders. In this regard, *Sf3b1*$^{+/-}$ mice may not be a suitable animal model for MDS, but further functional experiments using a conditional knock-in *Sf3b1* mutant allele is required to understand the molecular mechanisms of *SF3B1* mutations.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

MM, RY, MS, MO and HN performed mouse experiments; MM, AS-O, Y Shiozawa, KY, Y Shiraishi and SM performed bioinformatics analyses of RNA sequencing data; KI and HK generated *Sf3b1* knockout mice; MM, RY, MS and AS-O analyzed the data and generated figures and table; and MM, MS and SO designed the experiments and wrote the manuscript. All authors participated in the discussion and interpretation of data and results.

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ORIGINAL ARTICLE

Comprehensive analysis of genetic alterations and their prognostic impacts in adult acute myeloid leukemia patients

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To clarify the cooperative roles of recurrently identified mutations and to establish a more precise risk classification system in acute myeloid leukemia (AML), we comprehensively analyzed mutations in 51 genes, as well as cytogenetics and 11 chimeric transcripts, in 197 adult patients with *de novo* AML who were registered in the Japan Adult Leukemia Study Group AML201 study. We identified a total of 505 mutations in 44 genes, while only five genes, *FLT3, NPM1, CEBPA, DNMT3A* and *KIT*, were mutated in more than 10% of the patients. Although several cooperative and exclusive mutation patterns were observed, the accumulated mutation number was higher in cytogenetically normal AML and lower in AML with *RUNX1-RUNX1T1* and *CBFB-MYH11*, indicating a strong potential of these translocations for the initiation of AML. Furthermore, we evaluated the prognostic impacts of each sole mutation and the combinations of mutations and/or cytogenetics, and demonstrated that AML patients could be clearly stratified into five risk groups for overall survival by including the mutation status of *DNMT3A*, *MLL*-PTD and *TP53* genes in the risk classification system of the European LeukemiaNet. These results indicate that the prognosis of AML could be stratified by the major mutation status in combination with cytogenetics.

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Keywords: acute myeloid leukemia; gene mutations; prognosis; risk factor

INTRODUCTION

Acute myeloid leukemia (AML) is a clinically and genetically heterogeneous disease.[1,2] Although about 80% of younger adults with AML achieve complete remission (CR) with induction chemotherapy, more than half of the CR patients relapse, even if they receive intensive consolidation therapies. Allogeneic hematopoietic stem cell transplantation (allo-SCT) is applied to the patients who have risk factors for relapse, and it has been demonstrated by meta-analysis that allo-SCT at the first CR improves the long-term prognosis of the cytogenetically intermediate- and adverse-risk groups.[3] Cytogenetic-risk classification for AML is well established and commonly used as criteria for the application of allo-SCT at the first CR, whereas there is clinical heterogeneity in the intermediate-risk group, particularly cytogenetically normal (CN)-AML.[4] Recent advances and the accumulation of information on the prognostic relevance of recurrent genetic alterations have made more detailed risk stratification possible in AML patients.[5–19] The European LeukemiaNet (ELN) has recommended a novel risk classification system on the basis of the cytogenetic and genetic status.[2] In this system, CN-AML is stratified into two risk groups according to the mutation status of *FLT3*, *NPM1* and *CEBPA*: patients with *NPM1* mutation but not *FLT3*-ITD and those with *CEBPA* mutation are included in the favorable-risk (FR) group, and patients with *FLT3*-ITD and those with neither *NPM1* mutation nor *FLT3*-ITD are categorized into the intermediate-I-risk (IR-I) group. Long-term prognosis according to the ELN classification system was retrospectively evaluated in well-established cohorts, and it has

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been demonstrated that the ELN system is useful for further risk stratification of younger adult patients with CN-AML.[20,21] However, it has been reported that another genetic status, such as mutations in epigenetic modifier-encoding genes, could more precisely distinguish the prognosis in each ELN-risk group.[5,22] In addition, the prognostic impacts of recently identified mutations in spliceosome and cohesin complex genes on AML remain unclear.

The Japan Adult Leukemia Study Group (JALSG) conducted six phase III trials for adult *de novo* AML from 1987 (AML87, AML89, AML92, AML95, AML97 and AML201).[23] In the JALSG AML201 study, we prospectively compared a standard dose of idarubicin (IDR) with a higher dose of daunorubicin (HiDNR) in combination with cytarabine (Ara-C) as induction therapy, and three courses of high-dose Ara-C (HiDAC) with four courses of conventional standard-dose multiagents as consolidation therapy in CR patients.[24,25] We demonstrated that HiDNR was equivalent to IDR, as induction therapy and HiDAC was of benefit only to patients with core-binding factor (CBF)-AML as consolidation therapy. Although the CR rate remained at 75–80% during the six JALSG studies, 7-year overall survival (OS) was improved to 48% in the AML201 study from 29% in the AML87 study. Allo-SCT was conducted in only 7.1% of registered patients in the AML87 study, whereas 45.8% of registered patients received allo-SCT not only at the first CR but also after relapse or primary induction failure in the AML201 study, indicating that active application of allo-SCT even after relapse or primary induction failure might contribute to the improvement of OS. These results collectively suggested that a novel risk stratification system for decision making of allo-SCT at the first CR is required.

In this study, we comprehensively analyzed mutations in 51 genes that have been recurrently identified in myeloid neoplasm as well as cytogenetics, and evaluated the association of genetic status with prognostic and clinical features in patients who were registered in the AML201 study.

PATIENTS AND METHODS

Patients and samples

The study population included 197 newly diagnosed *de novo* AML patients, except for those with acute promyelocytic leukemia, who were registered in the JALSG AML201 study (UMIN Clinical Trials Registry C000000157, http://www.umin.ac.jp/ctrj/). The diagnosis of AML was on the basis of the French–American–British (FAB) classification.[26] Median follow-up time was 32.5 months. The age distribution is presented in Table 1. In the AML201 study, patients were randomly assigned to receive either IDR or 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 HiDAC therapy.[24,25] Of the 197 patients, 98 and 99 patients were assigned to IDR and HiDNR arms for induction therapy, respectively. CR was achieved in 161 of 197 (81.7%) patients, and 80 and 77 patients were assigned to HiDAC and conventional consolidation therapies, respectively (Table 1).

High molecular weight DNA and total RNA were extracted from bone marrow samples using standard methods.[27–29]

Cytogenetic G-banding analysis was performed by standard methods. We also examined 11 chimeric gene transcripts (Major: *BCR-ABL1*, Minor: *BCR-ABL1*, *PML-RARA*, *RUNX1-RUNX1T1*, *CBFB-MYH11*, *DEK-NUP214*, *NUP98-HOXA9*, *MLL-MLLT1*, *MLL-MLLT2*, *MLL-MLLT3*and *MLL-MLLT4*) by reverse transcriptase-mediated quantitative PCR as previously reported.[30]

Morphological diagnosis, the FAB classification and karyotypes were reviewed and confirmed by the central review committees of the JALSG using the BM samples obtained at diagnosis.

We obtained informed consent from all patients to use their samples for banking and molecular analysis, and approval was obtained from the ethics committees of the participating institutes.

Screening for mutations in 51 genes

A custom-made oligonucleotide probe library was designed to capture the exons of 51 genes that have been recurrently identified in myeloid neoplasm (Supplementary Table 1). Captured and enriched exons were subjected to sequencing on an Illumina HiSeq (Illumina, San Diego, CA, USA).[31–33] Sequence variation annotation was performed using known polymorphism databases, followed by mutation characterization. Each predicted variant sequence was confirmed by Sanger sequencing. Internal tandem duplication of the *FLT3* gene (*FLT3*-ITD) and partial tandem duplication of the *MLL* gene (*MLL*-PTD) were examined as previously reported.[28,34]

Table 1. Characteristics of the 197 patients

Characteristics	*Number*	*(%)*
Age (year)		
15–19	6	3.0
20–29	32	16.2
30–39	35	17.8
40–49	33	16.8
50–59	69	35.0
60–64	22	11.2
FAB subtype		
M0	7	3.6
M1	36	18.3
M2	89	45.2
M4	34	17.3
M4Eo	9	4.6
M5	21	10.7
M6	1	0.51
Cytogenetic-risk group		
Favorable	55	27.9
RUNX1-RUNX1T1	41	20.8
CBFB-MYH11	14	7.1
Intermediate	100	50.8
Normal cytogenetics	72	36.5
Unfavorable	23	11.7
Complex karyotype	16	8.1
t(11q23) excluding t(9;11) and t(11;19)	3	1.5
t(9;22)	2	1.0
−7	1	0.5
Not determined	19	9.6
Induction therapy		
IDR + Ara-C	98	49.7
DNR + Ara-C	99	50.3
Achieving CR	161	81.7
Consolidation therapy		
High-dose Ara-C	80	51.0
Multiagent CT	77	49.0

Abbreviation: IDR, idarubicin. The study population included 197 newly diagnosed *de novo* AML patients except for acute promyelocytic leukemia, and equally assigned to induction and consolidation arms. Nine patients showed the M4Eo FAB type, and all of them harbored the *CBFB-MYH11* transcript.

Statistical analysis

Differences in continuous variables were analyzed by the unpaired *t*-test or the Mann–Whitney *U*-test for distribution between two groups. Analysis of frequencies was performed using Fisher's exact test for 2×2 tables or Pearson's χ^2 test for larger tables. A multivariate analysis to identify risk factors for achieving CR was performed by the logistic regression model. Survival probabilities were estimated by the Kaplan–Meier method, and differences in the survival distributions were evaluated using the log-rank test. OS was defined as the time from the date of entry into the AML201 study to death due to any cause or last follow-up. Disease-free survival (DFS) was defined as the time from the day of achieving CR to relapse, death due to any cause or last follow-up. Patients undergoing SCT were not censored at the time of transplantation. The prognostic significance of the clinical variables was assessed using the Cox proportional hazards model. These statistical analyses were performed with Prism 5 (GraphPad Software, La Jolla, CA, USA) and JMP Pro10 (SAS Institute Japan,

Tokyo, Japan). For all analyses, the *P*-values were two-tailed, and a *P*-value of less than 0.05 was considered statistically significant.

RESULTS

Cytogenetic analysis

Cytogenetic analysis revealed a normal karyotype in 72 (36.5%) patients and an abnormal karyotype in 106 (53.8%) patients, including 41 AML with t(8;21) (q22;q22); *RUNX1-RUNX1T1* and 14 AML with inv(16) (p13q22); *CBFB-MYH11*. However, karyotypes could not be determined in 19 (9.6%) patients because we could not obtain sufficient mitotic cells. On the basis of the G-banding karyotype and chimeric transcript analyses, patients were assigned to favorable- ($n=55$, 27.9%), intermediate- ($n=100$, 50.8%) and adverse-risk ($n=23$, 11.7%) groups according to the refined MRC criteria (Table 1).[4]

Frequencies of mutations

We identified mutations in 44 of 51 genes analyzed in the 197 AML patients. However, there were only five genes (*FLT3, NPM1, CEBPA, DNMT3A* and *KIT*) that were mutated in more than 10% of the patients (Figure 1a and Supplementary Table 1). Each position and type of mutation is presented in Supplementary Figure 1. As germ-line controls were available in a limited number of patients, we could not completely confirm that all identified mutations were somatic mutations. Therefore, there is a possibility that a part of identified mutations might be rare SNPs. *FLT3* mutation was the most frequently identified (50 patients, 25.4%), followed by *NPM1* (38 patients, 19.2%), *DNMT3A* (32 patients, 16.2%), *CEBPA* (31 patients, 15.7%) and *KIT* mutations (28 patients, 14.2%). Of the 50 patients with *FLT3* mutations, 36 (18.3%) and 17 (8.6%) patients harbored *FLT3*-ITD and *FLT3*-KDM, respectively, and three patients harbored both mutations. Of the 31 patients with *CEBPA* mutations, 19 (9.6%) and 12 (6.1%) patients harbored double *CEBPA* (*CEBPA*-D) mutations and a single *CEBPA* (*CEBPA*-S) mutation, respectively. Of the 28 patients with *KIT* mutations, 4, 2 and 23 patients harbored mutations in exon 8, exons 10–11 and exon 17 of the *KIT* gene, respectively, and one patient harbored mutations in both exons 10–11 and exon 17.

Although mutations in the 51 analyzed genes were not identified in 14 (7.1%) patients, 183 (92.9%) patients harbored one or more mutations; one mutation in 36, two mutations in 56, three mutations in 40, four mutations in 27, five mutations in 17, six mutations in five and seven mutations in two patients. The mean mutation number per patient was 2.56 ± 0.11 in all patients, whereas it was significantly higher in patients with a normal karyotype (3.18 ± 0.16) than in those with an aberrant karyotype (2.10 ± 0.15) ($P<0.0001$). Furthermore, mean mutation numbers per patient in AML with *RUNX1-RUNX1T1* (1.68 ± 0.17) and *CBFB-MYH11* (1.57 ± 0.20) were significantly lower than that in all samples ($P=0.0008$ and 0.0123, respectively) (Figure 1b).

The mean mutation number per patient aged 60–64 years (3.18 ± 0.41) tended to be higher, although there was no significant difference between the mean mutation number and age (Supplementary Figure 2).

Genetic alterations found in AML have been conceptually grouped into class I mutation, which causes constitutive activation of intracellular signals that contribute to the growth and survival, and class II mutation that blocks differentiation and/or enhance self-renewal by altered transcription factors.[35–37] Recently, it has been suggested that mutations that modify the epigenetic status generate a new class because of their overlap mutations both with class I and class II mutations.[13,38] In this study, Class II mutations (*NPM1, CEBPA, RUNX1* and *GATA2* mutations, and *RUNX1-RUNX1T1* and *CBFB-MYH11*) were the most frequently identified (138/197; 70.1%), followed by Class I mutations (*FLT3, KIT, N/KRAS, PTPN11, JAK1/3* and *TP53* mutations) (116/197; 58.9%) and mutations that modify the epigenetic status (*ASXL1, ATRX, EZH2, TET2, PBRM1, DNMT3A, IDH1/2, KDM6A, MLL* and *DOT1L* mutations) (91/197; 46.2%). Furthermore, mutations of NOTCH family genes (*NOTCH1 and NOTCH2*), cohesin complex genes (*STAG2, SMC1A, SMC3* and *RAD21*), BCOR family genes (*BCOR* and *BCORL1*), NCOR family genes (*NCOR1, NCOR2* and *DIS3*) and spliceosome genes (*SF3B1, U2AF1, SRSF2* and *ZRSR2*) were identified in 19 (9.6%), 22 (11.2%), 17 (8.6%), 22 (11.2%) and 9 patients (4.6%), respectively (Figure 1c).

Association between gene mutations and cytogenetics

The prevalence of each gene mutation differed among the cytogenetic-risk groups. *KIT* mutations were preferentially identified in the favorable cytogenetic-risk group. *FLT3*-ITD, *NPM1, CEBPA* and *DNMT3A* mutations were preferentially identified in the intermediate-risk group, particularly in patients with a normal karyotype. *BCORL1* and *TP53* mutations were preferentially identified in the poor-risk group; in particular, *TP53* mutations were frequent in patients with a complex karyotype. In addition, *PHF6* mutations were also frequently identified in patients with a complex karyotype (Figure 2 and Supplementary Table 2).

Overlap mutations

Several patterns of overlap mutations were identified in this comprehensive mutation analysis (Supplementary Figures 3 and 4). Significantly overlapped mutations were observed between *FLT3* mutations and *NPM1, DNMT3A* and *MLL*-PTD mutations; *NPM1* mutations and *DNMT3A, IDH1* and *IDH2* mutations; *CEBPA* mutations and *TET2* mutations; *ASXL1* mutations and spliceosome gene mutations; *DIS3* mutations and *MLL* mutations; *DNMT3A* mutations and *PTPN11* mutations; *GATA2* mutations and *CEBPA*-D mutations; *K/NRAS* mutations and *WT1* mutations and *BCOR/BCORL1* mutations; *RUNX1* mutations and *U2AF1, MLL-PTD, BCOR/BCORL1* and *PHF6* mutations; *SF3B1* mutations and *NRAS* mutations; and *TET2* mutations and *STAG2* mutations. In contrast, mutually exclusive mutations were observed between *FLT3* mutations and *KIT, K/NRAS* and *CEBPA*-D mutations; *NPM1* mutations and *CEBPA*-D and *RUNX1* mutations; and *CEBPA* mutations and *IDH1/2* mutations.

According to the conceptual classification of the mutated genes, overlap mutations between Class I, Class II and epigenetic modifying gene mutations were frequently observed. However, these major mutations widely coexisted with other family gene mutations, such as the cohesin complex, BCOR family and spliceosome gene mutations (Figure 1d). Although biological functions of mutated genes have not been fully clarified, we also present frequencies and associations of mutated genes according to the provisional gene function in the Supplementary Figure 5.

Association of gene mutations with clinical characteristics

Several associations between mutations and clinical characteristics were observed. *DNMT3A* mutations and *MLL*-PTD were more frequently identified in patients over 50 years old than in those less than 50 years old ($P=0.0064$ and $P=0.0121$, respectively), whereas the other mutations were not significantly associated with age (Supplementary Table 3).

Several mutations were associated with the white blood cell count at diagnosis. *FLT3-ITD, NPM1, DNMT3A* and *NOTCH1* mutations were significantly associated with the high white blood cell count. In contrast, *ASXL1* and *IDH1* mutations were associated with a lower white blood cell count (Supplementary Table 4).

Association of gene mutations with the CR rate

We analyzed the association of mutations with the CR rate. By Fisher's exact test, *RUNX1-RUNX1T1* or *CBFB-MYH11, KIT, NPM1* and *CEBPA*-D mutations were identified as favorable factors for

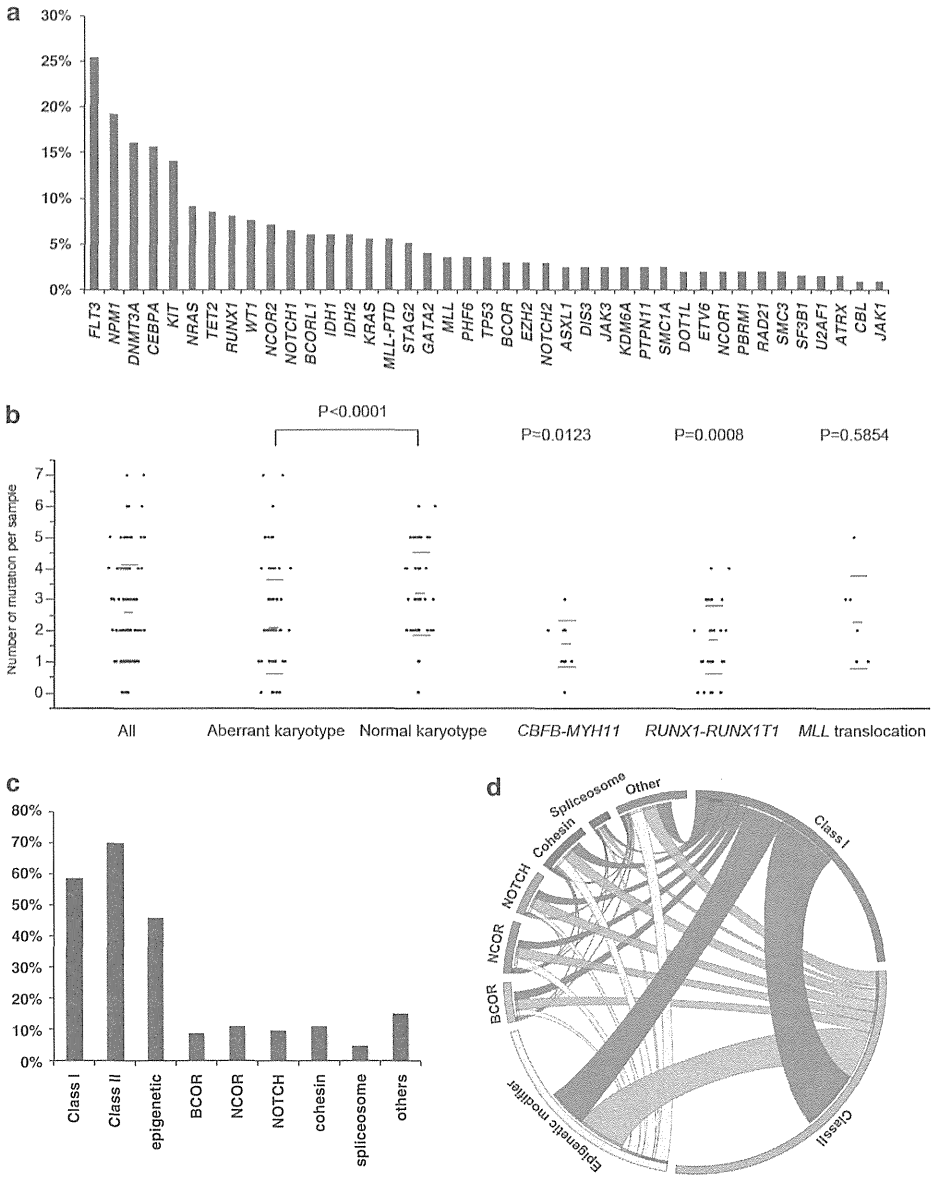


Figure 1. Frequencies and associations of mutated genes. (**a**) Frequencies of analyzed gene mutations. Frequency of each mutated gene is shown. Mutations were identified in 44 genes of 51 genes analyzed in 197 AML patients. Only five genes (*FLT3, NPM1, CEBPA, DNMT3A* and *KIT*) were mutated in more than 10% of the patients. (**b**) Mutated gene numbers according to the cytogenetics. Mean mutation number ± s.d. is indicated by horizontal lines. Mean mutation number per one patient in patients with normal karyotype (3.18 ± 0.16) was significantly higher than in that with aberrant karyotype (2.10 ± 0.15) ($P<0.0001$). Those in AML with *RUNX1-RUNX1T1* (1.68 ± 0.17) and *CBFB-MYH11* (1.57 ± 0.20) were significantly lower than that in all samples ($P=0.0008$ and $P=0.0123$, respectively). That in AML with *MLL*-translocation tended to be lower but not statistically significant (2.28 ± 0.57, $P=0.5854$). (**c**) Frequencies of mutations according to the conceptual classification. Mutations in Class I, Class II and epigenetic modifying genes were frequently identified. (**d**) Association of mutated genes according to the conceptual classification. Circos plot of mutated genes according to the function is shown.[45] Overlap mutations between Class I, Class II and epigenetic modifying genes mutations were frequently observed. These major mutations were widely coexistent with another family genes, such as cohesin complex, BCOR family and spliceosome genes.

achieving CR, and *TP53* mutation was an unfavorable factor; however, multivariate logistic regression analysis including all analyzed mutations showed that only *NPM1* (Hazard ratio (HR): 96.206, 95% Confidence interval (CI): 2.247–411.9, $P=0.0172$) and *TP53* (HR: 22.222, 95% CI: 1.597–333.3, $P=0.0172$) mutations were identified as favorable and unfavorable factors for achieving CR, respectively (Table 2 and Supplementary Table 5).

Importantly, *KIT* mutations were closely associated with *RUNX1-RUNX1T1* or *CBFB-MYH11*, whereas the other mutations that confer the achievement of CR were mutually exclusive (Supplementary Figures 3 and 6). In the patients with *RUNX1-RUNX1T1* or *CBFB-MYH11, NPM1* and *CEBPA*-D mutations, the CR rate (106/112; 94.6%) was significantly higher than for those with the other genotypes (55/85; 64.7%) ($P<0.0001$).

Prognostic impacts of mutations

We next analyzed the prognostic impact of each mutation. By univariate analysis, *FLT3*-ITD (HR: 1.805, 95% CI: 1.130–2.885, $P=0.0135$), *DNMT3A* (HR: 1.696, 95% CI: 1.055–2.725, $P=0.0291$),

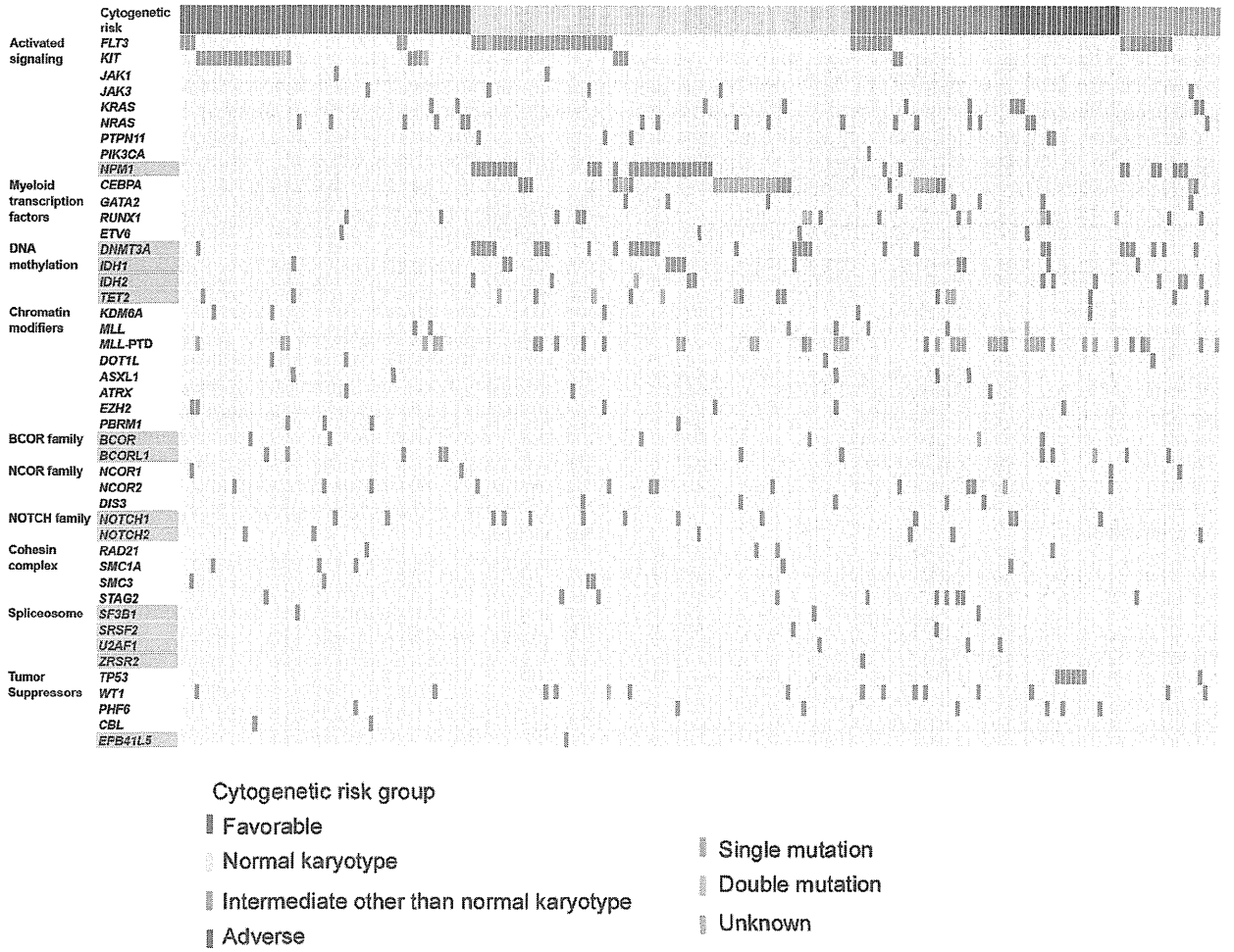


Figure 2. Mutation status according to the cytogetics-risk groups. Identified mutations in analyzed AML patients are shown according to the cytogenetic-risk groups. Pink boxes indicate single mutations and orange boxes indicate double mutations.

Table 2. Gene mutations affecting the CR achievement

Mutations	*CR rate (%)*		P-*value*
	Positive	*Negative*	
Fisher's exact test			
NPM1	97	78	0.0041
CEBPA-D-Mt.	100	80	0.0273
KIT	96	79	0.0326
RUNX1-RUNX1T1 or *CBFB-MYH11*	91	78	0.0409
TP53	14	84	0.0002

Mutations	*HR (95% CI)*	P-*value*
Multivariate analysis		
Wild-*NPM1*	96.206 (2.247–411.9)	<0.0001
TP53 mutation	22.222 (1.597–333.3)	0.0172

Abbreviations: CI, confidence interval; CR, complete remission; HR, hazard ratio. By the Fisher's exact test, *RUNX1-RUNX1T1* or *CBFB-MYH11, KIT, NPM1* and *CEBPA*-D mutations were identified as the favorable factor for achieving CR, and *TP53* mutation was for the unfavorable factor. The multivariate logistic regression analysis including all analyzed mutations showed that only wild-*NPM1* and *TP53* mutation were identified as unfavorable factors for achieving CR.

TP53 (HR: 15.167, 95% CI: 6.555–35.094, $P<0.001$), *MLL*-PTD (HR: 3.782, 95% CI: 1.948–7.346, $P<0.001$) and *RUNX1* (HR: 2.301, 95% CI: 1.278–4.146, $P=0.0055$) mutations and the karyotypes other than *RUNX1-RUNX1T1* or *CBFB-MYH11* (HR: 2.786, 95% CI: 1.608–4.831, $P=0.0003$) were identified as unfavorable prognostic factors for OS (Table 3 and Supplementary Figure 7). Multivariate Cox regression analysis with stepwise selection showed that *TP53* (HR: 14.803, 95% CI: 6.259–35.009, $P<0.001$), *MLL*-PTD (HR: 2.853, 95% CI: 1.401–5.810, $P=0.0039$) and *RUNX1* (HR: 1.965, 95% CI: 1.054–3.663, $P=0.0336$) mutations and the karyotypes other than *RUNX1-RUNX1T1* or *CBFB-MYH11* (HR: 2.353, 95% CI: 1.342–4.132, $P=0.0028$) were independent poor prognostic factors for OS (Table 3).

In this cohort, mutations of NOTCH family, the cohesin complex, BCOR family and spliceosome genes were frequently identified. NOTCH family and BCOR family genes were not associated with the CR rate, OS and DFS. Although mutations of cohesin complex genes were not associated with the CR rate and DFS, the patients harboring those mutations revealed better OS than those without mutations ($P=0.0274$) (Figure 3). The CR rate and DFS of patients with spliceosome gene mutations tended to be lower than for those without mutations, although both differences were not statistically significant: the CR was achieved in five of the nine (55.6%) and 156 of the 188 (83.0%) patients ($P=0.0601$), and 3-year DFS were 0% and 38.9% ($P=0.1117$) in those with and

without mutations, respectively (Supplementary Table 3 and Supplementary Figure 8).

When the patients were stratified into the risk groups recommended by ELN, that is, FR, IR-I, IR-II and AR groups included 92, 35, 42 and 28 patients, respectively. The ELN system well stratified the long-term prognosis of adult AML patients, whereas the OSs of IR-I and AR groups were the same in the present cohort (Supplementary Figure 9). Therefore, we analyzed whether another mutations could further stratify the prognosis in each ELN-risk group. *MLL*-PTD, *GATA2* and *TP53* mutations were identified as further poor prognostic factors in IR-I, IR-II and AR groups, respectively. Furthermore, we identified that the *DNMT3A* mutation was a poor prognostic factor in the FR group except for the AML with t(8;21) (q22;q22); *RUNX1-RUNX1T1* or inv(16) (p13q22); *CBFB-MYH11* (CBF-AML) (Figure 4).

Table 3. Unfavorable prognostic factors for overall survival (OS)

Mutations	*HR (95% CI)*	*P-value*
Univariate analysis		
TP53	15.167 (6.555–35.094)	<0.0001
MLL-PTD	3.782 (1948–7.346)	<0.0001
Non CBF	2.786 (1.608–4.831)	0.0003
RUNX1	2.301 (1.278–4.146)	0.0055
FLT3-ITD	1.805 (2.247–4119)	0.0135
DNMT3A	1.696 (1.055–2.725)	0.0291
Multivariate analysis		
TP53	14.803 (6.259–35.009)	<0.0001
MLL-PTD	2.853 (1.4017–5.810)	0.0039
Non CBF	2.353 (1.342–4.132)	0.0028
RUNX1	1.965 (1.054–3.663)	0.0336

Abbreviations: CI, confidence interval; HR, hazard ratio. By the univariate analysis, *FLT3*-ITD, *DNMT3A*, *TP53*, *MLL*-PTD and *RUNX1* mutations and the karyotypes other than *RUNX1-RUNX1T1* or *CBFB-MYH11* were identified as adverse prognostic factors for OS. Multivariate Cox regression analysis with stepwise selection showed that *TP53*, *MLL*-PTD and *RUNX1* mutations and the karyotypes other than *RUNX1-RUNX1T1* or *CBFB-MYH11* were independent poor prognostic factors for OS.

In addition, we also evaluated two recently reported risk stratification systems on the basis of genetic status in our cohort.[5,39] Patel *et al.*[5] reported a risk stratification system on the basis of cytogenetics and genetic status According to their system, our patients were clearly stratified into three risk groups, although the patients in the intermediate cytogenetic risk with a favorable mutational risk profile and those in the favorable cytogenetic-risk profile showed the same probability of OS (Supplementary Figure 10a). Grossmann *et al.*[39] reported a prognostic model solely on the basis of molecular mutations. Although our cohort did not include AML with *PML-RARA*, our patients were clearly stratified into four risk groups. However, the patients in the very favorable group and those in the favorable group showed the same probability of OS (Supplementary Figure 10b).

On the other hand, *KIT* mutations were frequently identified in CBF-AML, while they were not a poor prognostic factor for either OS or DFS (Supplementary Figure 11a). According to the types of *KIT* mutations, CBF-AML patients harboring mutations in exon 17 of the *KIT* gene showed worse prognosis than those harboring the other types of *KIT* mutation, although this was not statistically significant (Supplementary Figure 11b). Notably, in the CBF-AML patients harboring *KIT* mutations, OS and DFS of those treated with three courses of HiDAC consolidation therapy tended to be better than those treated with four courses of conventional standard-dose multiagent therapy (Supplementary Figure 11c).

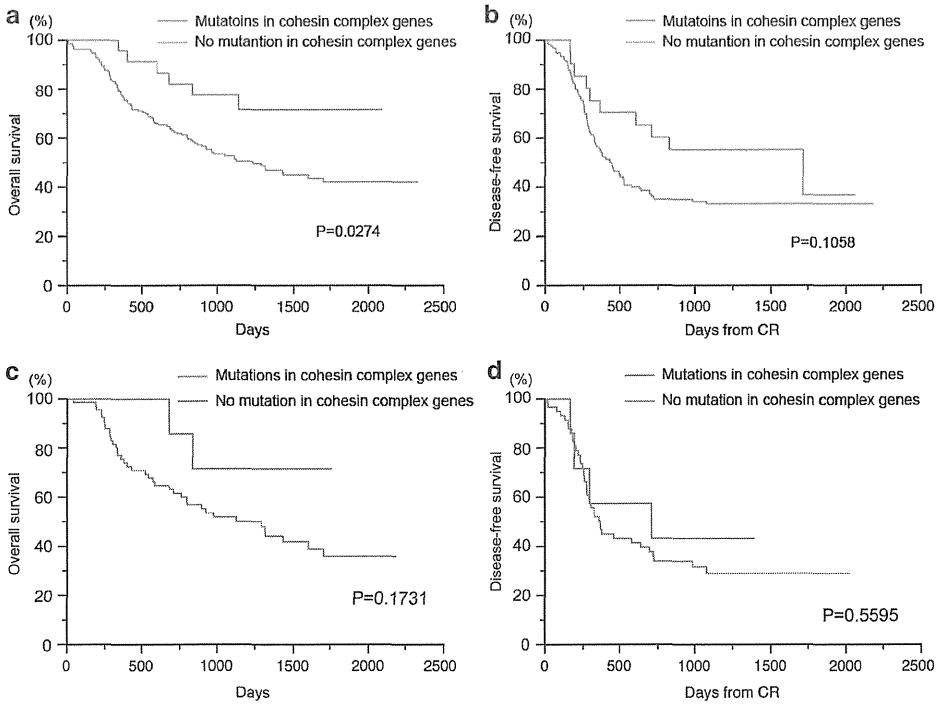


Figure 3. Prognostic impact of mutations in cohesin complex genes. Kaplan–Meier curves for OS and DFS according to the mutations in cohesin complex genes are shown. (**a**) OS in the total patients, (**b**) DFS in the total patient, (**c**) OS in the patients with normal karyotype, (**d**) DFS in the patients with normal karyotype. Although mutations of cohesin complex genes were not associated with the CR rate and DFS, the patients harboring those mutations revealed better OS than those without mutations ($P = 0.0274$). In the patients with normal karyotype, OS of the mutated patients tended to be better that that of unmutated patients, though statistical significance was not observed ($P = 0.1731$).

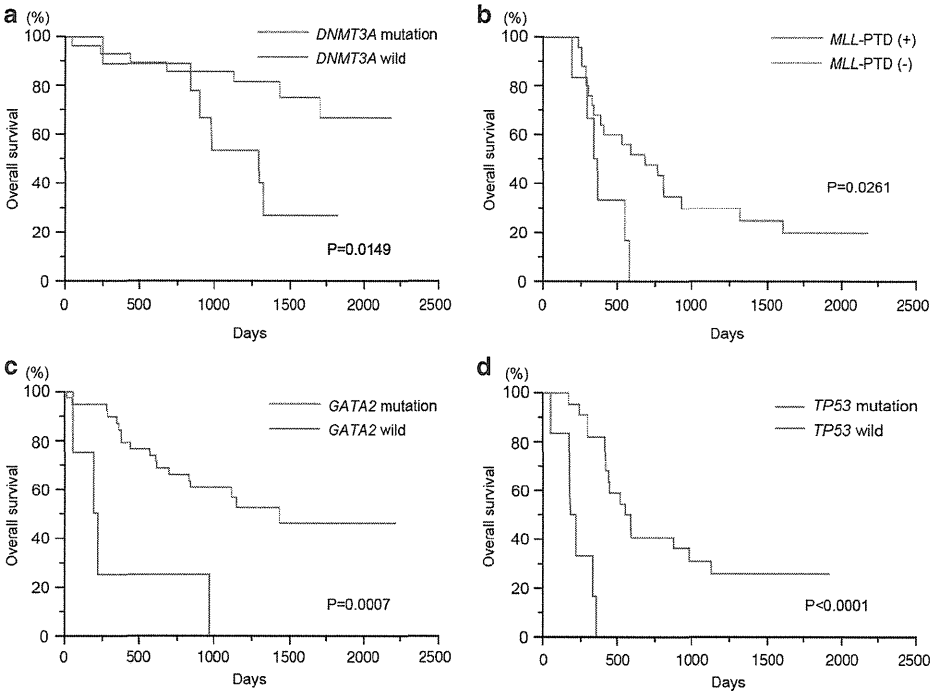


Figure 4. Mutations that could further stratify the ELN-risk groups into two risk groups. (**a**) *DNMT3A* mutation was a poor prognostic factor in the FR group except for the AML with t(8;21) (q22;q22); *RUNX1-RUNX1T1* or inv(16) (p13q22); *CBFB-MYH11* (CBF-AML). (**b**) *MLL*-PTD was a poor prognostic factor for the OS in the ELN IR-I group. (**c**) *GATA2* mutation was a poor prognostic factor for the OS in the ELN IR-II group. (**d**) *TP53* mutation was a poor prognostic factor for the OS in the ELN AR group.

In CBF-AML, the accumulated mutation number was lower than in the other types of AML, although many kinds of mutation were identified (Supplementary Figure 12). However, we could not identify a gene that affects the prognosis of CBF-AML.

Taking these results together, we tried to modify the genetic criteria for the ELN stratification system. When the CN-AML patients with *DNMT3A* mutations of the FR group and the patients with *MLL*-PTD of the IR-I group were included in the IR-I and the AR group, respectively, we could more clearly stratified the patients into four risk groups for OS than the original ELN system (Figures 5a and b). Furthermore, as the prognosis of the patients with *TP53* mutations were very unfavorable as previously reported,[39] we could more clearly stratify the patients into five risk groups for OS by classifying the *TP53*-mutated patients as the very adverse-risk group (Figures 5c and d).

DISCUSSION

In this study, we comprehensively analyzed mutations of 51 genes by the targeting sequence, and identified a total of 505 mutations in 44 genes in 197 adult *de novo* AML patients except for APL. The whole-genome and -exome analysis demonstrated recurrent mutations in a total of 260 genes in 200 AML patients, suggesting that another mutations might be accumulated in the presently analyzed AML cells.[40] However, frequencies of most mutated genes were reportedly less than 10%. In consistence, only five genes (*FLT3, NPM1, CEBPA, DNMT3A* and *KIT*) were mutated in more than 10% patients in our study, and each mutation frequency was almost the same as previous reports.[5,17,19,39] The frequency of *KIT* mutation was relatively higher in our study than previous reports,[5,40] while this is caused by the higher frequency of CBF-AML (28.0%) in the Japanese patients, in which *KIT* mutations are frequently identified. Our study, therefore, essentially includes major genetic regions, which may affect the pathogenesis and prognosis of AML. However, mutation analyses were not thoroughly performed in all subtypes of AML, such as acute erythroid leukemia and acute megakaryoblastic leukemia because of their lower frequencies. Further analyses are required to fully clarify the genetic alterations in AML.

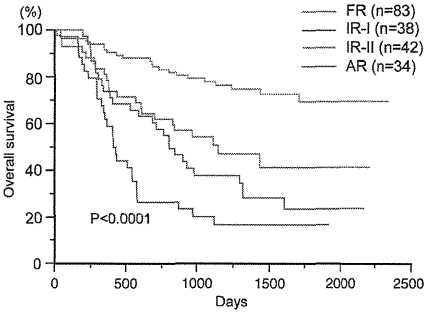
The whole-genome and -exome analysis demonstrated that an average of mutated genes in coding regions per sample was 5.24.[40] Of note was that there were significant differences in mutated gene numbers among the types of cytogenetics and mutations: the mean mutation numbers were higher in AML with *RUNX1-RUX1T1*, and are lower in that with *PML-RARA* and *MLL* translocations than that of all samples. As analyzed gene numbers were limited, mean mutated gene number per sample (2.56 ± 0.11) was low in our study; however, there were different features from the previous report. In our study, higher mutation number was observed in CN-AML (3.18 ± 0.16), and lower was in CBF-AML. Furthermore, the mutation number in AML with *MLL*-translocation tended to be lower (2.28 ± 0.57, $P = 0.5854$). These results collectively suggested that recurrent cytogenetic abnormalities, such as *RUNX1-RUNX1T1, CBFB-MYH11* and *MLL*-translocation, have a strong potential for the initiation of AML, and that most of accumulated mutations in AML with these cytogenetics might be passenger mutations.

It has been reported that common mutations in AML, such as *DNMT3A, NPM1, CEBPA, IDH1/2* and *RUNX1*, were mutually exclusive of the transcription-factor fusions, indicating the high potential for leukemia initiation.[10,11,13,40,41] Consistently, *DNMT3A, NPM1* and *CEBPA* mutations were not identified in CBF-AML, but frequent in CN-AML. In addition, we identified that *MLL*-PTD mutation was also exclusive of CBF-AML. In CBF-AML, *KIT* mutations were preferentially identified, whereas several types of mutations were also accumulated, suggesting that many mutations could act as a driver mutation for the clonal expansion of the initiating clone with *RUNX1-RUNX1T1* and

a

Genetic group	Subsets
Favorable	• CBF-AMLs • CN-AMLs with mutated *NPM1* without *FLT3*-ITD and mutated *DNMT3A* (normal karyotype) • CN-AMLs with mutated *CEBPA* without mutated *DNMT3A* (normal karyotype)
Intermediate-I	• CN-AMLs with mutated *DNMT3A* • CN-AMLs except for those included in the FR and AR
Intermediate-II	• AMLs included in the IR-II of the ELN stratification
Adverse	• CN-AMLs with *MLL*-PTD • AMLs included in the AR of the ELN stratification

b



c

Genetic group	Subsets
Favorable	• CBF-AMLs • CN-AMLs with mutated *NPM1* without *FLT3*-ITD and mutated *DNMT3A* (normal karyotype) • CN-AMLs with mutated *CEBPA* without mutated *DNMT3A* (normal karyotype)
Intermediate-I	• CN-AMLs with mutated *DNMT3A* • CN-AMLs except for those included in the FR and AR
Intermediate-II	• AMLs included in the IR-II of the ELN stratification except for those with mutated *TP53*
Adverse	• CN-AMLs with *MLL*-PTD • AMLs included in the AR of the ELN stratification except for those with mutated *TP53*
Very Adverse	• AMLs with mutated *TP53*

d

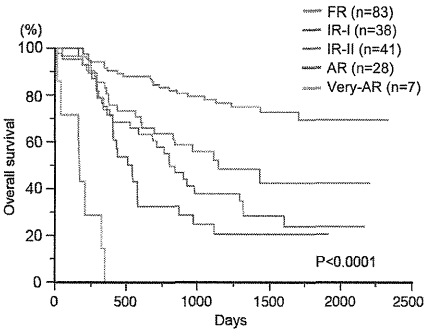


Figure 5. Risk stratification by modifying the ELN stratification system. When the CN-AML patients with *DNMT3A* mutations of the FR group and the patients with *MLL*-PTD of the IR-I group were included in the IR-I and the AR group, respectively (**a**), we could more clearly stratify the patients into four risk groups for OS than the original ELN system (**b**). When the patients with *TP53* mutations were classified as the very adverse-risk group (**c**), we could more clearly stratify the patients into five risk groups for OS (**d**).

CBFB-MYH11. Further study is required to clarify which combination is necessary for the clonal expansion, and whether different combinations cause clinical and phenotypical varieties.

After the completion of genetic alterations in AML, the most important issue is to clarify the prognostic impact of each mutation and/or co-occurring mutations.[7,42] The recently recommended ELN classification system is the first system that includes both cytogenetics and mutation status. Several groups reported that the ELN system clearly stratified the long-term prognosis of AML patients. However, the prognosis of FR groups except for the CBF-AML is still controversial. Paschka *et al.*[43] reported that the *IDH1/2* mutation was a poor prognostic factor in CN-AML with mutated *NPM1* without *FLT3*-ITD. In contrast, Patel *et al.*[5] reported that the *IDH1/2* mutation was a favorable prognostic factor in AML with mutated *NPM1* without *FLT3*-ITD. Furthermore, it has been reported that the *TET2* mutation was an adverse prognostic factor in AML with mutated *NPM1* or *CEBPA* without *FLT3*-ITD.[22] In the present cohort, we could not observe the statistically significant effects of *IDH1/2* and *TET2* mutations on the prognosis of CN-AML with mutated *NPM1* or *CEBPA* without *FLT3*-ITD, while we identified that *DNMT3A* mutation is an adverse prognostic factor in CN-AML with mutated *NPM1* or *CEBPA* without *FLT3*-ITD. In addition, we could not find the better prognostic impact of the *CEBPA* double mutations on the FR group recommended by the ELN in contrast to previous reports.[39,44]

Although different mutations might further stratify the prognosis of AML with mutated *NPM1* or *CEBPA* without *FLT3*-ITD, it was noteworthy that all mutations belonged to the class modifying methylation status.[13,38] These results collectively suggested that the epigenetic deregulation might contribute the pathogenesis of AML with mutated *NPM1* or *CEBPA* without *FLT3*-ITD. Prospective and large-scale study is necessary to clarify what genetic alterations influence the prognosis of AML with these genotypes.

In this study, we demonstrated that the prognosis of adult AML patients could be more clearly stratified by including the *DNMT3A* and *MLL*-PTD mutation status than the original ELN system, and that *TP53* mutations have a very adverse effect on the prognosis of AML patients. However, as most recurrently identified mutations were observed less than 5% of AML, it is highly expected to refine the genetic-based risk stratification system by much larger-scale studies. In addition, it is also important to evaluate the prognostic effects according to the functions of mutated genes rather than each sole mutation.

In the JALSG AML201 study, patients were randomized to the standard dose of IDR + Ara-C or HiDNR + Ara-C induction therapy, and the CR patients were again randomized to three courses of HiDAC or four courses of conventional standard-dose multiagent consolidation therapy. Therefore, we analyzed whether therapeutic regimens affect the CR rate and long-term survivals according to the mutation status and risk groups on the basis of the genetic status, while we could not observe any significant differences between therapeutic regimens and genetic status. Furthermore, we could not demonstrate that allo-SCT could improve the prognosis of the patients falling in the intermediate- and adverse-risk groups because of the small number of patients who underwent allo-SCT in the first CR in this cohort. It is, therefore, required to evaluate whether therapeutic regimens and allo-SCT affect the prognosis according to the genetic status.

In conclusion, we comprehensively analyzed 51 genes mutations in 197 *de novo* adult AML patients who were registered to a single prospective clinical study, and demonstrated that cooperative and exclusive mutation patterns and their prognostic impacts. Furthermore, we demonstrated that the prognosis of adult AML patients could be more clearly stratified by including the *DNMT3A*, *MLL*-PTD and *TP53* mutation status than the original ELN system. However, prognostic impacts of some mutation status are different from the previous reports. We must refine the risk

stratification system by considering all known-risk factors in a large-scale and well-established cohort, although molecular genetic status has a strong impact on the prognosis of AML patients. We are now conducting a prospective large-scale study to confirm the present results.

CONFLICT OF INTEREST

H Kiyoi: Research funding from Bristol-Myers Squibb, Novartis Pharma, Chugai Pharmaceutical Co., Ltd. and Kyowa Hakko Kirin Co., Ltd. YM: Honoraria from Bristol-Myers Squibb, Novartis Pharma, Chugai Pharmaceutical Co., Ltd., Kyowa Hakko Kirin Co., Ltd. and Celgene Japan; Research funding from Bristol-Myers Squibb, Novartis Pharma, Chugai Pharmaceutical Co., Ltd. and Kyowa Hakko Kirin Co., Ltd. NU: Consultant for Kyowa Hakko Kirin Co., Ltd.; Honoraria from Bristol-Myers Squibb, Novartis Pharma and Chugai Pharmaceutical Co., Ltd.; Research funding from Bristol-Myers Squibb, Novartis Pharma, Chugai Pharmaceutical Co., Ltd. and Kyowa Hakko Kirin Co., Ltd. TN: Research funding from Bristol-Myers Squibb, Novartis Pharma, Chugai Pharmaceutical Co., Ltd., Kyowa Hakko Kirin Co., Ltd., Dainippon Sumitomo Pharma and Zenyaku Kogyo. The other authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

H Kiyoi, S Ogawa and TN designed the study, interpreted the data and wrote the manuscript; RK, YN, T Kato, EY, KS and FC performed molecular analysis and interpreted the data; YN, YS, KC, HT, SM and S Ogawa performed bioinformatics; NA, S Ohtake, SM, YM, TS, YO, N Usui, H Kanamori, T Kiguchi, KI, N Uike, FK, KK, CN, MO, AT, FI, HS, YK and HM collected samples and clinical data, contributed to the interpretation of the data and critically reviewed the draft; and all authors approved the final version submitted for the publication.

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up to 80 years of age supporting the use of modern anti-myeloma therapy independent of age.[16,17]

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Recurrent genetic defects on chromosome 7q in myeloid neoplasms

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Monosomy 7 (-7) and deletion of the long arm (del(7q)) of chromosome 7 (chr7) belong to the most commonly acquired karyotypic abnormalities in myeloid neoplasms and are associated with poor prognosis. Although somatic mutations or decreased expression of *EZH2* have a role,[1–3] the molecular mechanisms behind chr7 alterations in most cases remain undefined. It is likely that other pivotal target genes contribute to -7/del(7q) pathogenesis. Somatic mutations may either be unique to del(7q) hemizygous inactivation, or be shared between 7q diploid and haploid cases. In addition to the hypothetical mutations in hemizygous configuration, del(7q) alone may result in haploinsufficient gene expression. Corresponding heterozygous hypomorphic mutations or epigenetic inactivation of genes located on chr7q may phenocopy the haploinsufficient expression resulting from del(7q). To identify somatic mutations and haploinsufficiently expressed genes located on chr7q, we applied whole-exome sequencing (WES) and expression analysis both in deletion cases and in those with an apparently normal diploid chr7. Here we report the recurrent genetic defects in *CUX1*, *LUC7L2* and *EZH2*, and other results of the comprehensive molecular screen of chr7q.

Using metaphase cytogenetics and SNP-array-based karyotyping, loss of heterozygosity (LOH) lesions affecting 7q were identified in 171 of 1131 cases (15%), including low-risk myelodysplastic syndromes (MDS) (14%), high-risk MDS (34%), secondary acute myeloid leukemia (sAML) (18%), MDS/myeloproliferative neoplasms (MPN) (13%), MPN (6%) and primary acute myeloid leukemia (pAML) (8%) (Supplementary Table S1). Minimal commonly deleted regions (CDRs) were defined as 7q22 (100847518–101872055; CDR1), 7q34 (138190944–139672739; CDR2) and 7q35-q36 (144707068–148942012; CDR3), as previously described.[4] Analysis of WES results for all exons on chr7 for 428 cases with different myeloid neoplasms (Figure 1a) identified 490 nonsilent alterations in 306 genes located on chr7 (5% of all alterations found; Supplementary Table S2). After stringent filtering and bioanalytic processing to avoid false positives (Supplementary Figure S1),[5] we narrowed the focus of our investigations to 'tier 1' mutations; 155 mutated genes were found in 25% (17/68) of -7/del(7q) cases, in 100% (6/6) of uniparental disomy for chr7q (UPD7q) and in 30% (107/354) of diploid for chr7 (Figure 1b). All mutations were validated by

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Sanger sequencing and targeted deep sequencing of DNA from both tumor and germline ($CD3^+$ T cells or buccal mucosa) cells. UPD7q was more frequently affected by somatic mutations than -7 or del(7q). Notably, 17% (39/228) of the somatic mutations were located in one of the CDRs with marker genes *CUX1* in 7q22, *LUC7L2* in 7q34, and *CUL1* and *EZH2* in 7q35-36.

Among many rare somatic events, eight genes (*EZH2*, *CUX1*, *LUC7L2*, *CFTR*, *PLXNA4*, *DYNC1I1*, *NRCAM* and *CUL1*) corresponding to regions affecting del(7q) were affected by multiple mutations, totaling 44 events (Supplementary Figure S2). For example, mutations of a core component of E3 ubiquitin ligase complex *CUL1*[6,7] were detected only in cases with -7/del(7q) ($N=2$, hemizygous mutations). Conversely, somatic mutations of *PLXNA4*, encoding a component of the neuropilin–plexin complex,[8] were observed only in cases with diploid 7q. In cases with diploid 7q, we observed 23 different heterozygous alterations, of which 63% (five of the eight genes) were also affected in -7/del(7q) or UPD7q (hemizygous/homozygous) (Figure 1b). Previously described *EZH2* (7q36.1) mutations, located in CDR3, were seen in either heterozygous, homozygous or hemizygous configurations. However, *EZH2* mutations were most common in UPD7q cases (67%, 4/6), and in only 5% (4/88) of -7/del(7q) cases. The other CDRs also contained genes recurrently affected by mutations including *CUX1* (7q22, $N=5$) and *LUC7L2* (7q34, $N=8$). Somatic mutations were also identified on 7p (*HDAC9*, *IKZF1* and *EGFR*), which may account for the differences between -7 and del(7q).

In addition to somatic defects in the genes on CDRs of 7q, copy number loss of the wild-type genes might result in haploinsufficiency and the resultant effects may be similar to the heterozygous hypomorphic or loss of function mutations. To investigate haploinsufficient gene expression due to -7/del(7q), we compared relative mRNA levels between cases with and without -7/del(7q). Underexpressed genes (< -2 s.d. of 17 healthy controls) were more common in -7/del(7q), in particular within the three CDRs (Supplementary Figure S3). Furthermore, cases with deletion of *CUX1*, *LUC7L2* and *EZH2* (all located in CDRs) showed a significantly lower mRNA expression compared with those with diploid 7q ($P<0.001$, <0.001 and <0.05, respectively, Figure 2a). When mutated in case diploid for 7q, these genes were commonly affected by frameshift and nonsense mutations (Supplementary Table S3), implying loss of function. Thus, it is likely that either a hypomorphic mutation, and/or haploinsufficiency may lead to the downstream pathological consequences. Conversely, there was no significant haploinsufficiency identified in the genes located outside CDRs (*NRCAM* (7q31.1), *PLXNA4* (7q32.3) and *CFTR* (7q31.2) (data not shown).

To elucidate the clinical impact of recurrent deficiency in specific genes on chr7, we correlated clinical characteristics to either deletion or corresponding somatic inactivating mutations. Mutations and deletions in *CUX1*, *LUC7L2* and *EZH2* were observed in 11%, 11% and 12% of cases, respectively (Figure 2b) and were less prevalent in cases with MPN compared with MDS ($P=0.02$,

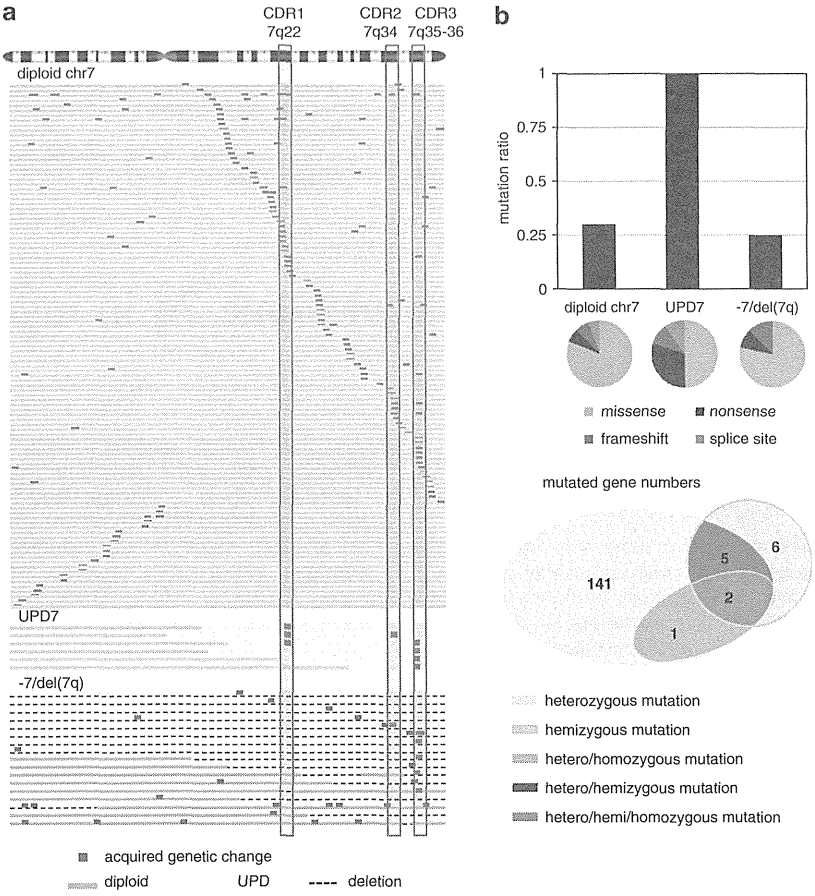

Figure 1. Mutations and LOH on chr7. (**a**) Mutations of chr7 detected by whole-exome sequencing in the whole cohort ($N=428$) are shown in red. LOH on chr7 is demonstrated as follows: a normal diploid, blue and orange; a uniparental disomy (UPD7), double blue lines; and a deletion for chr7, dashed line. Three distinct CDRs, indicated by vertical rectangles, are identified on 7q by mapping of SNP-A karyotyping. (**b**) Frequency of the cases with mutations in each chr7 status (diploid, UPD and deletion), and mutational types (missense, nonsense, frameshift and splice site) are demonstrated by bar graph and pie chart, respectively (upper panel). Venn diagram shows the number of mutated genes categorized by their configuration of zygosity (heterozygous, homozygous and heterozygous) (lower panel).

<0.01 and =0.02, respectively) and sAML ($P=0.01$, <0.01 and =0.01, respectively). Deletions involving these three gene loci were more prevalent in high-risk MDS than in low-risk MDS ($P<0.001$) and in sAML than in pAML ($P<0.001$). Consistent with this finding, mutations in any one of three genes were significantly associated with shorter survival ($P<0.0001$; HR=2.55; 95% CI, 1.98 to 7.64; Figure 2c): *CUX1* mutations were linked to compromised survival, as were mutations in *LUC7L2* or *EZH2* (HR=2.32 and HR=3.16, respectively). Furthermore, the presence of low *CUX1*, *LUC7L2* or *EZH2* expression correlated with a significantly shorter survival ($P=0.001$; HR=2.05, 95% CI, 1.45 to 4.45; Figure 2d) in pAML patients. We performed additional clinical investigations, but no relationship between the low expression of these three genes and other clinical parameters (for example, age, cytogenetics and blood counts) was found (data not shown).

We also investigated the potential relationship of somatic mutation events observed on other chromosomes to concomitant -7/del(7q) and UPD7 (Supplementary Figure S4a). There were clear differences between both LOH7 groups, in which -7/del(7q) was more associated with accessory chromosomal events (that is, del(5q), del(17p) or trisomy 8) than cases with UPD7q. Although well-known, frequent mutations in *U2AF1*, *TET2* and *TP53* were commonly found in both LOH7 groups, some specific genes, including the CSMD family, were uniquely observed in -7/del(7q). LOH7 was also associated with somatic mutations in *SETBP1* ($P=0.02$, Supplementary Figure S4b). When we investigated concomitant mutations and genetic events in mutation cases with *CUX1*, *LUC7L2* and *EZH2*, these three most common mutations were not mutually exclusive (Supplementary Figure S4c). It is possible that concomitant mutations exert synergetic effects analogous to the defect created by a long

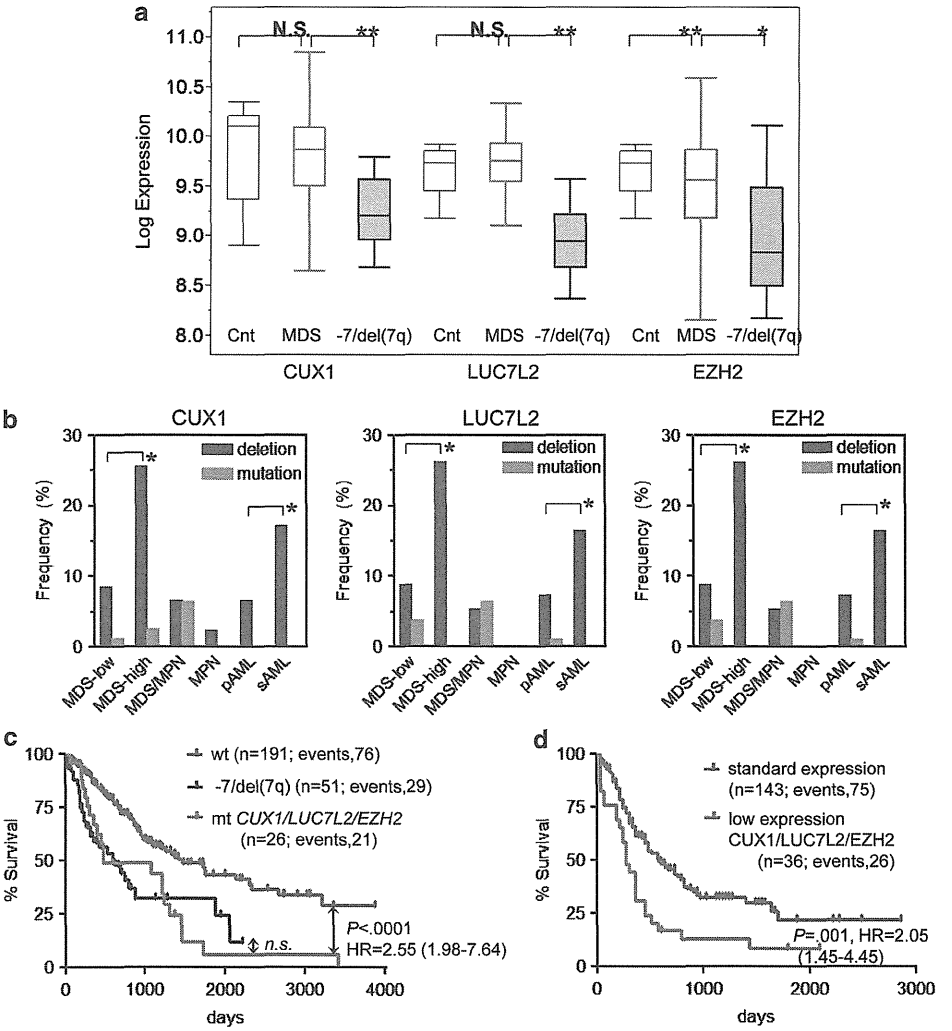

Figure 2. Gene expression of *CUX1*, *LUC7L2* and *EZH2*. (**a**) By expression array analysis, -7/del(7q) patients' samples ($n=9$) show significantly decreased expressions of all *CUX1*, *LUC7L2* and *EZH2* genes relative to those from MDS patients who possess normal diploid chr7 ($n=174$). Statistics analysis was performed using Student's *t*-test with a two-sided test. *$P<0.05$, **$P<0.001$, N.S.; not significant. (**b**) Frequency of deletion and somatic mutation in *CUX1*, *LUC7L2* and *EZH2*. In the cohort of patients with myeloid malignancies, SNP array karyotyping and metaphase cytogenetics ($n=1559$) were applied for the detection of 7q deletion affecting CUX1, LUC7L2 and EZH2 loci. Mutations of each gene were searched in 428 cases by whole-exome and validation sequencing. Frequency of the deletions and mutations was calculated in each disease phenotypes. MDS-low includes refractory cytopenia with unilineage dysplasia, refractory cytopenia with multilineage dysplasia, MDS with isolated del5q, MDS unclassifiable and refractory anemia with ring sideroblasts. MDS-high includes refractory anemia with excess blasts. *$P<0.001$ (χ^2-test). (**c**) Overall survival was compared among patients with mutations (mt) of *CUX1*, *LUC7L2* or *EZH2*, wild type (wt) and del(7q). (**d**) Overall survival was compared between AML patients with low expression of *EZH2*, *CUX1* and *EZH2* and those with normal expression of the corresponding genes. *P*-values presented correspond to the Cox regression between the groups indicated. n.s.; not significant.

deletion on chr7q (involving multiple CDRs). *TET2* mutations were most prevalent in *CUX1* or *EZH2* mutant cases ($P<0.001$, Supplementary Figure S4d), suggesting that *TET2* lesions constitute the driver events. In addition to *LUC7L2*, we found mutations in other spliceosomal genes (*U2AF1* or *U2AF2*) in *CUX1* or *EZH2* mutation cases, which were also mutated in cases of LOH7q without these mutations. Of note is that we could not find any concomitant *TP53* mutations in cases with mutated *CUX1*, *LUC7L2* and *EZH2*. In sum, our findings indicate that diverse additional molecular events might have a significant role in hypomorphic gene defects on chr7.

Our results demonstrate the various associations of the LOH7q (including UPD) with or without concomitant mutations involving genes located in 7q region. Regardless of their causes (deletion or normal diploid chr7) or heterozygous hypomorphic mutation, haploinsufficient expression of *CUX1*, *LUC7L2* and *EZH2* is associated with a poor survival suggesting that hypomorphic mutations and haploinsufficiency both lead to deficient function of these genes in -7/del(7q) myeloid neoplasms. Hypomorphic *EZH2* mutations (located in SET domain) found in myeloid neoplasms have been implicated in premature termination or direct abrogation of histone methyltransferase activity. It remains unclear why *EZH2* mutations occur more commonly in the context of UPD7q rather than -7/del(7q),[2] but likely, as reported here and recently,[9] loss of EZH2 function may occur through multiple pathways, including homozygous mutations with UPD7q, haploinsufficiency and through phenocopy by splicesome mutations. The function and likely the consequences of *LUC7L2* mutations are distinct from *EZH2*; LUC7L2 is one of the splicesome subunits interacting with U1 snRNP to recognize non-consensus splice donor sites.[10,11] We identified eight mutant cases, interestingly, mostly *LUC7L2* mutations resulted in premature stop codons. The biological effect of wild-type and mutant *LUC7L2* on myeloid neoplasms has not been studied. CUX1 as a transcription factor regulates a large number of genes and microRNAs involved in DNA replication, DNA damage response and cell cycle progression.[12,13] In agreement with clinical observations, including not only MDS/MPN overlap but also MPN cases,[14] *Cux1* transgenic mice developed an MPN-like myeloid leukemia with massive expansion of neutrophils.[15]

In conclusion, no single gene defect explains the pathogenesis of LOH, but some specific molecular events, including recurrent somatic mutations in *CUX1*, *LUC7L2* and *EZH2*, are recurrent in -7/del(7q). While del(7q) results in low expression in these genes, down modulation and hypomorphic mutations are also seen in some cases diploid for 7q and all are uniformly associated with poor survival.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Detection of the G17V RHOA Mutation in Angioimmunoblastic T-Cell Lymphoma and Related Lymphomas Using Quantitative Allele-Specific PCR

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Abstract

Angioimmunoblastic T-cell lymphoma (AITL) and peripheral T-cell lymphoma, not otherwise specified (PTCL-NOS) are subtypes of T-cell lymphoma. Due to low tumor cell content and substantial reactive cell infiltration, these lymphomas are sometimes mistaken for other types of lymphomas or even non-neoplastic diseases. In addition, a significant proportion of PTCL-NOS cases reportedly exhibit features of AITL (AITL-like PTCL-NOS). Thus disagreement is common in distinguishing between AITL and PTCL-NOS. Using whole-exome and subsequent targeted sequencing, we recently identified G17V *RHOA* mutations in 60–70% of AITL and AITL-like PTCL-NOS cases but not in other hematologic cancers, including other T-cell malignancies. Here, we establish a sensitive detection method for the G17V *RHOA* mutation using a quantitative allele-specific polymerase chain reaction (qAS-PCR) assay. Mutated allele frequencies deduced from this approach were highly correlated with those determined by deep sequencing. This method could serve as a novel diagnostic tool for 60–70% of AITL and AITL-like PTCL-NOS.

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Introduction

Based on the classification proposed by the World Health Organization (WHO), Angioimmunoblastic T-cell lymphoma (AITL) is a distinct subtype of T-cell lymphoma that accounts for 20% of peripheral T-cell lymphoma cases [1]. AITL is characterized by generalized lymphadenopathy, hyperglobulinemia, and autoimmune-like manifestations [1,2]. Pathologic examination of AITL tumors reveals polymorphous infiltration of reactive cells, including endothelial venules and follicular dendritic cells [3,4]. Based on gene expression profiling and immunohistochemical staining, the normal counterparts of AITL tumor cells are proposed to be follicular helper T cells (TFHs) [5]. Peripheral T-cell lymphoma, not otherwise specified (PTCL-NOS) is a more heterogenous type of lymphoma, one that shows variation even in CD4 and CD8 expression. Some PTCL-NOS cases share features of AITL, such as immunohistochemical

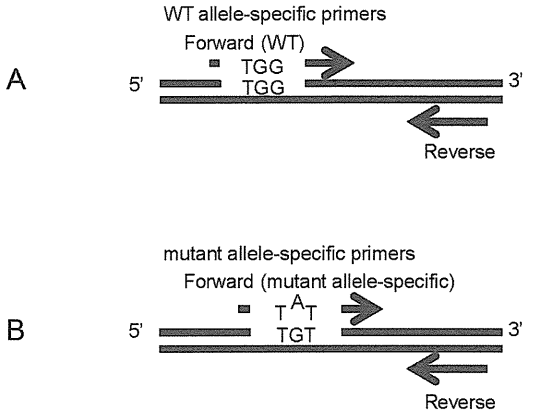

Figure 1. Design of primers used in the study. A WT allele-specific primer forward primer (Upper), a mutant allele-specific forward primer (Lower), and a common primer were designed. The 3′ end of the forward mutant primer was specific to the mutant site (G to T) and an internal mismatch at the second nucleotide from 3′ end (G to A) was introduced to improve specificity.
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staining patterns resembling those seen in AITL (AITL-like PTCL-NOS) [6].

Expertise is required to diagnose AITL and PTCL-NOS because generally low tumor cell content obscures the neoplastic nature of some cases, and large reactive B-cells are often confused with tumor cells [7]. Clonal rearrangement of the T-cell receptor gene is undetectable in 10–25% of AITL cases due to low tumor cell frequency [1]. In addition, clonal growth of Epstein-Bar virus-infected B-cells is not uncommon in these kinds of cancers, causing detection of clonal immunoglobulin gene rearrangement in 20% of these case. [1].

Mutations in *TET2, IDH2*, and *DNMT3A* are frequently seen in AITL and AITL-like PTCL-NOS [8,9], although these mutations are also common to various myeloid malignancies [10,11]. We and others reported a large cohort of AITL and PTCL-NOS patients revealing that the G17V *RHOA* mutation was highly specific to AITL and AITL-like PTCL-NOS and very frequent (seen in 60–70% of cases) in these T-cell lymphomas [12,13]. This observation suggests that detection of the G17V *RHOA* mutation could serve as a new diagnostic tool to discriminate these lymphomas from other diseases. One difficulty, however, is that *RHOA* mutation allele frequencies in these lymphomas are generally as low as <0.2 or often <0.1, reflecting low tumor cell content. Therefore, diagnosis of these conditions requires development of sensitive and cost-efficient methods that are as accurate as deep sequencing, which is expensive and not commonly used in most clinical testing facilities.

To meet this need, we developed a quantitative allele-specific polymerase chain reaction (qAS-PCR) method that sensitively detects the G17V *RHOA* mutation in a highly accurate manner. This assay should provide a realistic way to conduct laboratory testing to diagnose AITL and AITL-like PTCL-NOS.

Materials and Methods

Primer design

We designed two forward primers that discriminate wild-type (WT) from G17V *RHOA* for use with one common reverse primer. The mutant forward primer was designed using a previously described algorithm [14]. The 3′ end is specific to the mutant site and an internal mismatch at the second nucleotide from the 3′ end was introduced to improve specificity (Figure 1 and Table 1). We performed local alignment analysis using the BLAST program (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) to confirm primer specificity.

Preparation of plasmids containing WT and mutant cDNA and standard curve generation

WT or G17V mutant *RHOA* cDNA was subcloned into pBluescript (pBS/wtRHOA or pBS/mutRHOA, respectively; Agilent Technologies, Santa Clara, CA). qPCR reactions were performed in a final volume of 20 μl using 10 nM primers and the SYBR-Green mix (Roche Applied Science, Mannheim, Germany), and amplicons were subjected to either the ABI7500 or 7900 Fast Sequence Detection Systems (Life Technologies, Carlsbad, CA). Use of either the WT or mutant forward primer plus the common primer generated a 73-bp PCR product. The following PCR conditions were used: 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C and 60 sec at 60°C.

Standard curves of amplicon levels were created by qPCR using serially-diluted pBS/wtRHOA or pBS/mutRHOA with WT or mutant primers, respectively.

Preparation of template plasmid DNA mixtures

pBS/mutRHOA was mixed with pBS/wtRHOA in 100, 10, 1.0, 0.1, 0.01 and 0% ratios. Overall DNA concentration was adjusted to 1.0 ng/well of a plate. All mixtures were then serially-diluted 1:10 for 4 cycles. qPCR was performed with these templates plus primers using conditions described above.

Patients and samples

Tumor samples were collected from 53 patients with AITL, 55 with PTCL-NOS, 19 with B-cell malignancies, 129 with myeloid malignancies, and 5 with another T-cell lymphoma (for a total of 261), according to WHO classification. Twenty-seven non-tumor samples, including bone marrow mononuclear cells and buccal cells from lymphoma patients, were also analyzed as controls. The Ethics Committee University of Tsukuba Hospital approved the protocol and consent procedure, according to which written informed consent was provided by the participants. Genomic DNA was extracted from 13 formalin-fixed/paraffin-

Table 1. Sequence of allele-specific primers used for this study.

Primer	Sequence
Forward (WT*[1])	ATTGTTGGTGATGGAGCCTGTGG
Forward (MUT*[2])	ATTGTTGGTGATGGAGCCTGTAT
Reverse (common)	ACACCTCTGGGAACTGGTCCT

*[1] WT, wild-type; *[2] MUT, mutant.
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Table 2. Analysis of genomic DNA samples.

Disease	Frozen amp*[1]	Frozen not-amp*[2]	PLP not-amp	FFPE not-amp	Total
AITL	14	10	19	10	53
PTCL-NOS	16	8	28	3	55
B-cell lymphoma	1	18			19
Myeloid malignancies	129				129
Other T-cell lymphomas		5			5
Control samples	27				27
Total	187	41	47	13	288

*[1]amp, amplified; *[2]not-amp, not-amplified.
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embedded (FFPE), 47 periodate/lysine/paraformaldehyde (PLP)-fixed, and 228 fresh frozen specimens, using an FFPE tissue kit (QIAGEN, Hilden, Germany) for FFPE and PLP samples and a Puregene DNA blood kit (QIAGEN) for fresh frozen specimens, according to manufacturer's instructions.

One hundred and one DNA samples were original, while 187 were whole genome-amplified by either GenomiPhi (GE, Fairfield, CT) or a RepliG mini kit (Qiagen) (Table 2). For DNA extracted from FFPE samples, we also prepared PCR amplicon with AmpliTaq Gold 360 (Life technologies) in a final volume of 20 μl with 20 ng genomic DNA, 5 nM primers (Table 3), 5 μl of AmpliTaq gold master mix, and 0.3 μl of 360 GC Enhancer. For this amplicon preparation, the following PCR conditions were used: one cycle of 15 min at 95°C, 4 min at 60°C, and 1 min at 72°C, next 35 cycles of 1 min at 95°C, 1 min at 60°C, and 1 min at 72°C, and finally 10 min at 72°C and kept at 4°C. Amplicons were purified using PCR purification kit (QIAGEN).

Each DNA sample was quantified using the Qubit dsDNA HS Assay kit and a Qubit fluorometer (Life Technologies, Carlsbad, CA). Extracted DNA samples were stored at −20°C until use.

For 108 of the total 288 genomic DNA samples, data sets for mutant allele frequencies obtained by deep sequencing using the MiSeq System (Illumina, San Diego, CA), which were used in our previous report [12], were reanalyzed.

qPCR of patient samples

qPCR reactions using duplicate patient samples were performed in a final volume of 20 μl with 50 ng of original or whole genome-amplified genomic DNA or 1.0×10^{-2} ng PCR-amplified DNA as a template, 10 nM primers, and the SYBR-Green mix (Roche, Basel, Switzerland) in conditions similar to those used for plasmid templates described above.

Levels of amplicons generated using either the WT or mutant primer, calculated with reference to respective standard curves, were designated [wt] and [mut], respectively.

Table 3. Primer sequences for making PCR amplicons of FFPE samples.

Primer	Sequence
Forward	GCCCCATGGTTACCAAAGCA
Reverse	GCTTTCCATCCACCTCGATA

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Statistical analysis

Statistical analysis was conducted using SPSS software (Japan International Business.

Machines Corporation, Tokyo). A P-value <0.05 was considered statistically significant.

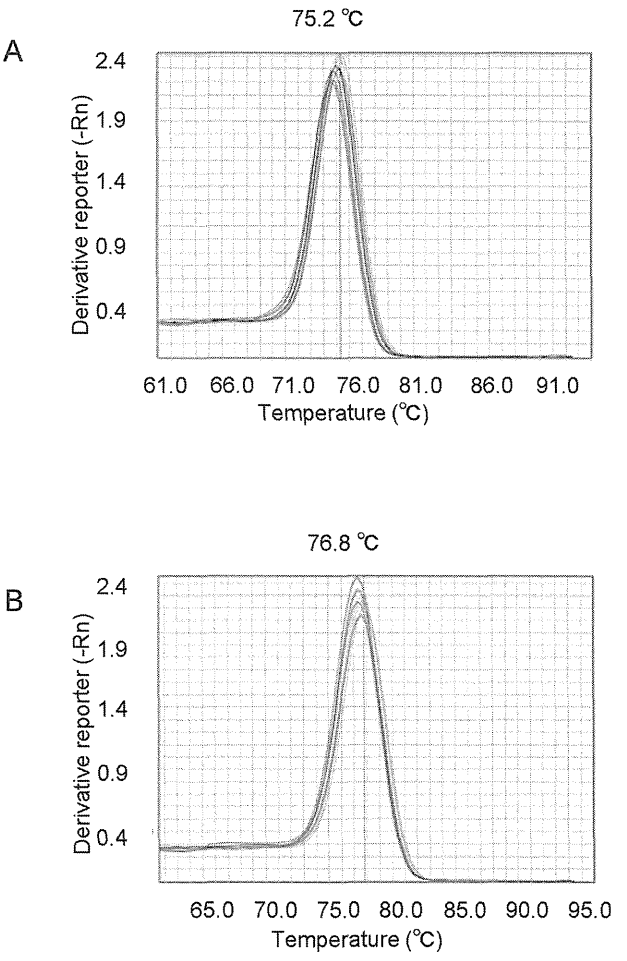

Figure 2. Melting curve analysis. A. Melting curve constructed using WT allele-specific primers. B. Melting curve constructed using mutant allele-specific primer set.
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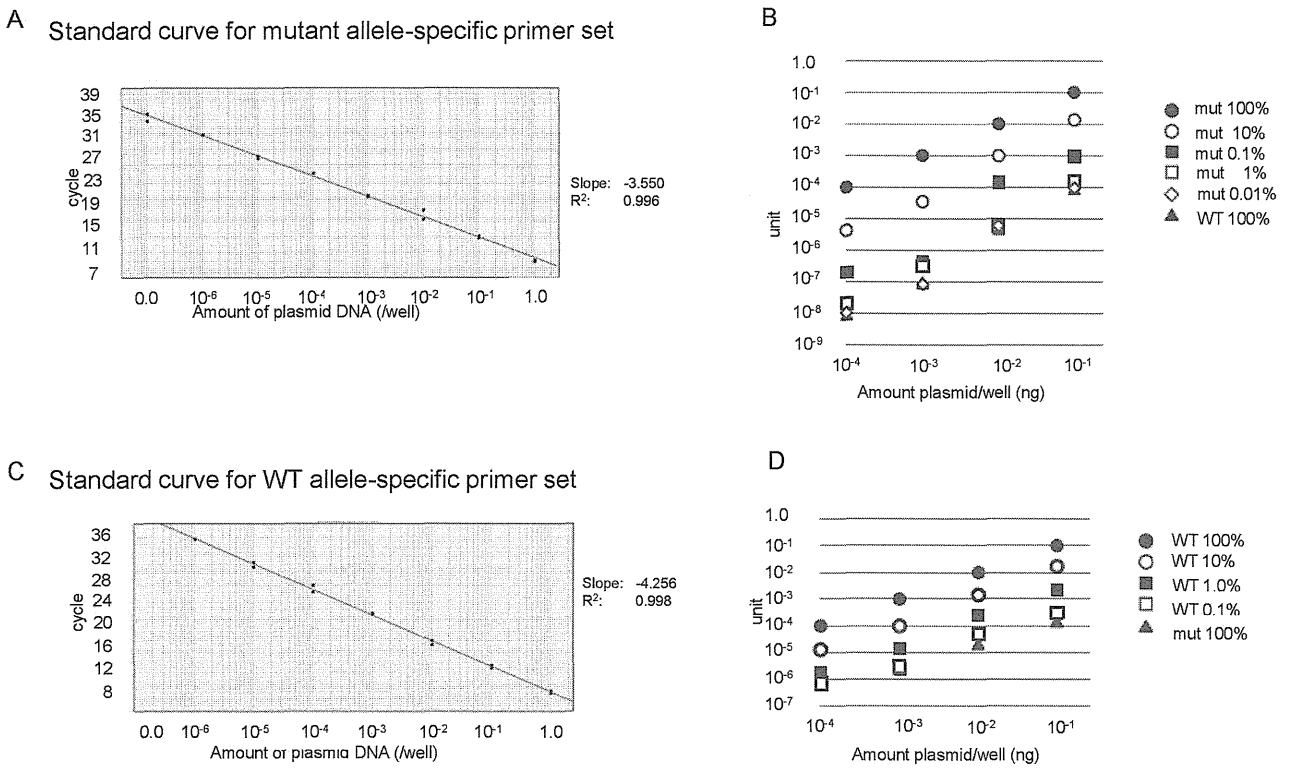

Figure 3. Standard curve showing linearity of quantitative allele-specific PCR. A standard curve was generated by serial dilution of WT or G17V cDNA that had been subcloned into pBluescript. A. Serial dilution of pBS/mutRHOA. Black dots correspond to 1.0×10^{-9}~1.0 unit of mutant plasmid (duplicate samples). The titration slope is −3.550 and R^2 is 0.996. B. pBS/mutRHOA was mixed with pBS/wtRHOA at 100%, 10%, 1.0%, 0.1%, 0.01% and 0%. Mix concentrations were adjusted to 1.0 ng/well and diluted 1:10 4 times for quantitative PCR analysis with allele-specific mutant primers. Horizonal axis indicates the amount of DNA per well. Vertical axis indicates unit for each sample. Black dot, MUT 100%; open dot, MUT 10%; square, MUT 1%; open square, MUT 0.1%; diamond, MUT 0.01%; triangle, MUT 0% (WT 100%) C. Serial dilution of pBS/wtRHOA. Black dots correspond to 1.0×10^{-6}~1.0 unit of WT cDNA (duplicate samples). The titration slope is −4.256, and R^2 is 0.998. D. pBS/wtRHOA was mixed with pBS/mutRHOA at 100%, 10%, 1.0%, 0.1%, 0.1% and 0%. Mix concentrations were adjusted to 1.0 ng/well and diluted 1:10 4 times for quantitative PCR analysis with WT allele-specific primers. Black dot, WT 100%; open dot, WT 10%; square, WT 1%; open square, WT 0.1%; triangle, WT 0% (MUT 100%).
doi:10.1371/journal.pone.0109714.g003

Results

Primer specificity

Melting curve analysis revealed that amplicons generated using either WT or mutant primers melted at 76.8°C or 75.3°C, respectively. Non-specific amplicons were not observed in either pBS/wtRHOA/WT primer or pBS/mutRHOA/mutant primer combinations (Figures 2A and 2B).

Linearity of amplicon generation

We then varied either the ratio of pBS/mutRHOA to pBS/wtRHOA or the concentration of total input DNA, and measured the amounts of PCR product generated using the mutant primer. Because we observed a nearly linear relationship between the amounts of generated amplicon and input DNA in the range of 10^4 (1–0.0001 ng DNA/well) at each ratio of pBS/mutRHOA to pBS/wtRHOA (Figure 3A), we defined the amount of amplicon derived from 100% pBS/mutRHOA template at 0.1 ng/well as 0.1 unit, and tested whether linearity was maintained with varying ratios of pBS/mutRHOA to pBS/wtRHOA. The template samples of 0.1 ng/well containing 10, 1, 0.1, and 0.01% pBS/mutRHOA were measured as 1.0×10^{-2} unit (C.I. (confidence interval), $0.8–1.3\times10^{-2}$; S.F. (scaling factor), 0.95–1.06), 1.2×10^{-3} unit (C.I., $0.8–1.6\times10^{-3}$; S.F., 0.96–1.07), 2.2×10^{-4} unit (C.I., $1.5–3.0\times10^{-4}$; S.F., 1.05–1.14), and 1.0×10^{-5} unit (C.I., $0.4–1.6\times10^{-5}$; S.F., 0.92–1.04), indicative of linearity in the range of 10^4 (100–0.01%). Taken together, linearity was maintained in the range of 10^9 (Figures 3A and 3B).

Similarly, when we assessed the WT primer using various ratios of pBS/wtRHOA to pBS/mutRHOA and concentrations of input DNA, linearity between the amounts of amplicon and template were maintained between 100–0.1% (a range of 10^3) and 1–0.001 ng DNA/well (a range of 10^3). This analysis indicated a total dynamic range of 10^6 (Figures 3C and 3D).

qAS-PCR of T-cell lymphoma samples

qAS-PCR with 50 ng of genomic DNA was performed using 106 AITL and PTCL-NOS samples including 11 FFPE samples. The [wt] and [mut] values were distributed between 7.9×10^{-5} and 1.8×10^{-1} units, and 2.0×10^{-7} and 7.6×10^{-2} units, respectively. Nevertheless, it was not possible to use absolute values of [mut] for levels of G17V *RHOA* alleles, due to variation in DNA quality. Therefore, we undertook relative measures to assess G17V *RHOA* allele frequency. To do so, we calculated a [mut]/([wt]+[mut]) value and compared it with mutant variant allele frequencies determined by MiSeq. [mut]/([wt]+[mut]) values were distributed between 3.2×10^{-4} and 3.0×10^{-1}. Among samples judged to harbor a G17V *RHOA* mutation by deep sequencing using the MiSeq System (cut-off level, 0.02), which was defined in previous paper [12], [mut]/([wt]+[mut]) values of DNA