25 Bibikova M, Le J, Barnes B, Saedinia-Melnyk S, Zhou L, Shen R *et al.* Genome-wide DNA methylation profiling using infinium assay. *Epigenomics* 2009; **1**: 177–200.
26 Gunderson KL, Steemers FJ, Ren H, Ng P, Zhou L, Tsan C *et al.* Whole-genome genotyping. *Methods Enzymol* 2006; **410**: 359–376.
27 Steemers FJ, Gunderson KL. Whole genome genotyping technologies on the BeadArray platform. *Biotechnol J* 2007; **2**: 41–49.
28 Yang AS, Estecio MR, Doshi K, Kondo Y, Tajara EH, Issa JP. A simple method for estimating global DNA methylation using bisulfite PCR of repetitive DNA elements. *Nucleic Acids Res* 2004; **32**: e38.
29 Okano M, Xie S, Li E. Cloning and characterization of a family of novel mammalian DNA (cytosine-5) methyltransferases. *Nat Genet* 1998; **19**: 219–220.
30 Aimiuwu J, Wang H, Chen P, Xie Z, Wang J, Liu S *et al.* RNA-dependent inhibition of ribonucleotide reductase is a major pathway for 5-azacitidine activity in acute myeloid leukemia. *Blood* 2012; **119**: 5229–5238.
31 Aoyama S, Nakano H, Danbara M, Higashihara M, Harigae H, Takahashi S. The differentiation and apoptotic effects of 2-aza-5'deoxycytidine are dependent on the PU.1 expression level in PU.1-transgenic K562 cells. *Biochem Biophys Res Commun* 2012; **420**: 775–781.
32 Curik N, Burda P, Vargova K, Pospisil V, Belickova M, Vlckova P *et al.* 5-azacitidine in aggressive myelodysplastic syndromes regulates chromatin structure at PU.1 gene and cell differentiation capacity. *Leukemia* 2012; **26**: 1804–1811.
33 Kulis M, Heath S, Bibikova M, Queiros AC, Navarro A, Clot G *et al.* Epigenomic analysis detects widespread gene-body DNA hypomethylation in chronic lymphocytic leukemia. *Nat Genet* 2012; **44**: 1236–1242.
34 Razin A, Riggs AD. DNA methylation and gene function. *Science* 1980; **210**: 604–610.
35 Robertson KD. DNA methylation, methyltransferases, and cancer. *Oncogene* 2001; **20**: 3139–3155.
36 Arand J, Spieler D, Karius T, Branco MR, Meilinger D, Meissner A *et al.* In vitro control of CpG and non-CpG DNA methylation by DNA methyltransferases. *PLoS Genet* 2012; **8**: e1002750.
37 Ley TJ, Ding L, Walter MJ, McLellan MD, Lamprecht T, Larson DE *et al.* DNMT3A mutations in acute myeloid leukemia. *N Engl J Med* 2010; **363**: 2424–2433.
38 Marcucci G, Metzeler KH, Schwind S, Becker H, Maharry K, Mrozek K *et al.* Age-related prognostic impact of different types of DNMT3A mutations in adults with primary cytogenetically normal acute myeloid leukemia. *J Clin Oncol* 2012; **30**: 742–750.
39 Yan XJ, Xu J, Gu ZH, Pan CM, Lu G, Shen Y *et al.* Exome sequencing identifies somatic mutations of DNA methyltransferase gene DNMT3A in acute monocytic leukemia. *Nat Genet* 2011; **43**: 309–315.
40 Yamashita Y, Yuan J, Suetake I, Suzuki H, Ishikawa Y, Choi YL *et al.* Array-based genomic resequencing of human leukemia. *Oncogene* 2010; **29**: 3723–3731.
41 Kim SJ, Zhao H, Hardikar S, Singh AK, Goodell MA, Chen T. A DNMT3A mutation common in AML exhibits dominant-negative effects in murine ES cells. *Blood* 2013; **122**: 4086–4089.
42 Thol F, Damm F, Ludeking A, Winschel C, Wagner K, Morgan M *et al.* Incidence and prognostic influence of DNMT3A mutations in acute myeloid leukemia. *J Clin Oncol* 2011; **29**: 2889–2896.
43 Robertson KD, Uzvolgyi E, Liang G, Talmadge C, Sumegi J, Gonzales FA *et al.* The human DNA methyltransferase (DNMTs) 1, 3a and 3b: coordinate mRNA expression in normal tissue and overexpression in tumors. *Nucleic Acids Res* 1999; **27**: 2291–2298.
44 Girault I, Tozlu S, Lidereau R, Bieche I. Expression analysis of DNA methyltransferase 1, 3A, and 3B in sporadic breast carcinomas. *Clin Cancer Res* 2003; **9**: 4415–4422.
45 Roll JD, Rivenbark AG, Jones WD, Coleman WB. DNMT3b overexpression contributes to a hypermethylator phenotype in human breast cancer cell lines. *Mol Cancer* 2008; **7**: 15.
46 Mizuno S, Chijiwa T, Okamura T, Akashi K, Fukumaki Y, Niho Y *et al.* Expression of DNA methyltransferases DNMT1, 3A, and 3B in normal hematopoiesis and in acute and chronic myelogenous leukemia. *Blood* 2001; **97**: 1172–1179.
47 Hayette S, Thomas X, Jallades L, Chabane K, Charlot C, Tigaud I *et al.* High DNA methyltransferase DNMT3B levels: a poor prognostic marker in acute myeloid leukemia. *PLoS One* 2012; **7**: e51527.
48 Shah MY, Vasanthankumar A, Barnes NY, Figueroa ME, Kamp A, Hendrick C *et al.* DNMT3B7, a truncated DNMT3B isoform expressed in human tumors, disrupts embryonic development and accelerates lymphomagenesis. *Cancer Res* 2010; **70**: 5840–5850.
49 Gopalakrishnan S, Van Emburgh BO, Shan J, Su Z, Fields CR, Vieweg J *et al.* A novel DNMT3B splice variant expressed in tumor and pluripotent cells modulates genomic DNA methylation patterns and displays altered DNA binding. *Mol Cancer Rs* 2009; **7**: 1622–1634.
50 Van Emburgh BO, Robertson KD. Modulation of DNMT3b function in vitro by interactions with Dnmt3L, Dnmt3a and Dnmt3b splice variants. *Nucleic Acids Res* 2001; **39**: 4984–5002.
51 Gopalakrishna-Pilai S, Iverson LE. A DNMT3B alternatively spliced exon and encoded peptide are novel biomarkers of human pluripotent stem cells. *PLoS One* 2011; **6**: e20663.
52 Wu CH, Gordon J, Rastegar M, Ogretmen B, Safa AR. Proteinase-3, a serine protease which mediates doxorubicin-induced apoptosis in the HL-60 leukemia cell line, is downregulated in its doxorubicin-resistant variant. *Oncogene* 2002; **21**: 5160–5174.
53 Aqirre X, Roman-Gomez J, Jimenez-Velasco A, Garate L, Montiel-Duarte C, Navarro G *et al.* ASPP1, a common activator of TP53, is inactivated by aberrant methylation of its promoter in acute lymphoblastic leukemia. *Oncogene* 2006; **25**: 1862–1870.
54 Fazi F, Rosa A, Fatica A, Gelmetti V, De Marchis ML, Nervi C *et al.* A minicircuitry comprised of microRNA-223 and transcription factors NFI-A and C/EBPalpha regulates human granulopoiesis. *Cell* 2005; **123**: 819–831.
55 Taketani T, Taki T, Shibuya N, Kikuchi A, Hanada R, Hayashi Y. Novel NUP98-HOXC11 fusion gene resulted from a chromosomal break within exon 1 of HOXC11 in acute myeloid leukemia with t(11;12)(p15;q13). *Cancer Res* 2002; **62**: 4571–4574.
56 Lin M, Sutherland DR, Horsfall W, Totty N, Yeo E, Nayar R *et al.* Cell surface antigen CD109 is a novel member of the alpha(2) macroglobulin/C3, C4, C5 family of thioester-containing proteins. *Blood* 2002; **99**: 1683–1691.
57 Delhommeau F, Dupont S, Della Valle V, James C, Trannoy S, Masse A *et al.* Mutation in TET2 in myeloid cancers. *N Engl J Med* 2009; **360**: 2289–2301.
58 Mardis ER, Ding L, Dooling DJ, Larson DE, McLellan MD, Chen K *et al.* Recurring mutations found by sequencing an acute myeloid leukemia genome. *N Engl J Med* 2009; **361**: 1058–1066.
59 Carbuccia N, Trouplin V, Gelsi-Boyer V, Murati A, Rocquain J, Adelaide J *et al.* Mutuali exclusion of ASXL1 and NPM1 mutations in a series of acute myeloid leukemias. *Leukemia* 2010; **24**: 469–473.
60 Shih AH, Abdel-Wahab O, Patel JP, Levine RL. The role of mutations in epigenetic regulators in myeloid malignancies. *Nat Rev Cancer* 2012; **12**: 599–612.

Supplementary Information accompanies this paper on the Leukemia website (http://www.nature.com/leu)

Leukemia (2014) **28**, 1586–1595

www.nature.com/leu

ORIGINAL ARTICLE

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

R Kihara[1,27], Y Nagata[2,3,27], H Kiyoi[1], T Kato[1], E Yamamoto[1], K Suzuki[1], F Chen[1], N Asou[4], S Ohtake[5], S Miyawaki[6], Y Miyazaki[7], T Sakura[8], Y Ozawa[9], N Usui[10], H Kanamori[11], T Kiguchi[12], K Imai[13], N Uike[14], F Kimura[15], K Kitamura[16], C Nakaseko[17], M Onizuka[18], A Takeshita[19], F Ishida[20], H Suzushima[21], Y Kato[22], H Miwa[23], Y Shiraishi[24], K Chiba[24], H Tanaka[25], S Miyano[24,25], S Ogawa[2,3] and T Naoe[1,26]

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.

Leukemia (2014) **28** 1586–1595; doi:10.1038/leu.2014.55

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

[1]Department of Hematology and Oncology, Nagoya University Graduate School of Medicine, Nagoya, Japan; [2]Cancer Genomics Project, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan; [3]Department of Pathology and Tumor Biology, Graduate School of Medicine, Kyoto University, Kyoto, Japan; [4]Department of Hematology, Kumamoto University School of Medicine, Kumamoto, Japan; [5]Department of Clinical Laboratory Science, Kanazawa University Graduate School of Medical Sciences, Kanazawa, Japan; [6]Division of Hematology, Tokyo Metropolitan Ohtsuka Hospital, Tokyo, Japan; [7]Department of Hematology, Atomic Bomb Disease Institute, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan; [8]Leukemia Research Center, Saiseikai Maebashi Hospital, Maebashi, Japan; [9]Department of Hematology, Japanese Red Cross Nagoya First Hospital, Nagoya, Japan; [10]Division of Clinical Oncology and Hematology, Department of Internal Medicine, Jikei University School of Medicine, Tokyo, Japan; [11]Department of Hematology, Kanagawa Cancer Center, Kanagawa, Japan; [12]Department of Hematology, Tokyo Medical University, Tokyo, Japan; [13]Department of Hematology, Sapporo Hokuyu Hospital, Sapporo, Japan; [14]Department of Hematology, National Hospital Organization Kyushu Cancer Center, Fukuoka, Japan; [15]Division of Hematology, Department of Internal Medicine, National Defense Medical College, Saitama, Japan; [16]Division of Hematology, Ichinomiya Municipal Hospital, Ichinomiya, Japan; [17]Department of Hematology, Chiba University Hospital, Chiba, Japan; [18]Department of Hematology and Oncology, Tokai University School of Medicine, Isehara, Japan; [19]Department of Internal Medicine, Hamamatsu University School of Medicine, Hamamatsu, Japan; [20]Division of Hematology, Department of Internal Medicine, Shinshu University School of Medicine, Matsumoto, Japan; [21]Department of Hematology, Kumamoto Shinto General Hospital, Kumamoto, Japan; [22]Department of Neurology, Hematology, Metabolism, Endocrinology, and Diabetology (DNHMED), Yamagata University School of Medicine, Yamagata, Japan; [23]Department of Internal Medicine, Division of Hematology, Aichi Medical University School of Medicine, Nagakute, Japan; [24]Laboratory of DNA Information Analysis, Human Genome Center, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan; [25]Laboratory of Sequence Analysis, Human Genome Center, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan and [26]National Hospital Organization Nagoya Medical Center, Nagoya, Japan. Correspondence: Dr H Kiyoi, Department of Hematology and Oncology, Nagoya University Graduate School of Medicine, Tsurumai-cho 65, Showa-ku, Nagoya, Aichi 466-8550, Japan or Professor S Ogawa, Department of Pathology and Tumor Biology, Graduate School of Medicine, Kyoto University, Kyoto, Japan.
E-mail: kiyoi@med.nagoya-u.ac.jp or sogawa-tky@umin.ac.jp
[27]These authors contributed equally to this work.
Received 3 December 2013; revised 21 January 2014; accepted 24 January 2014; accepted article preview online 3 February 2014; advance online publication, 14 February 2014

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

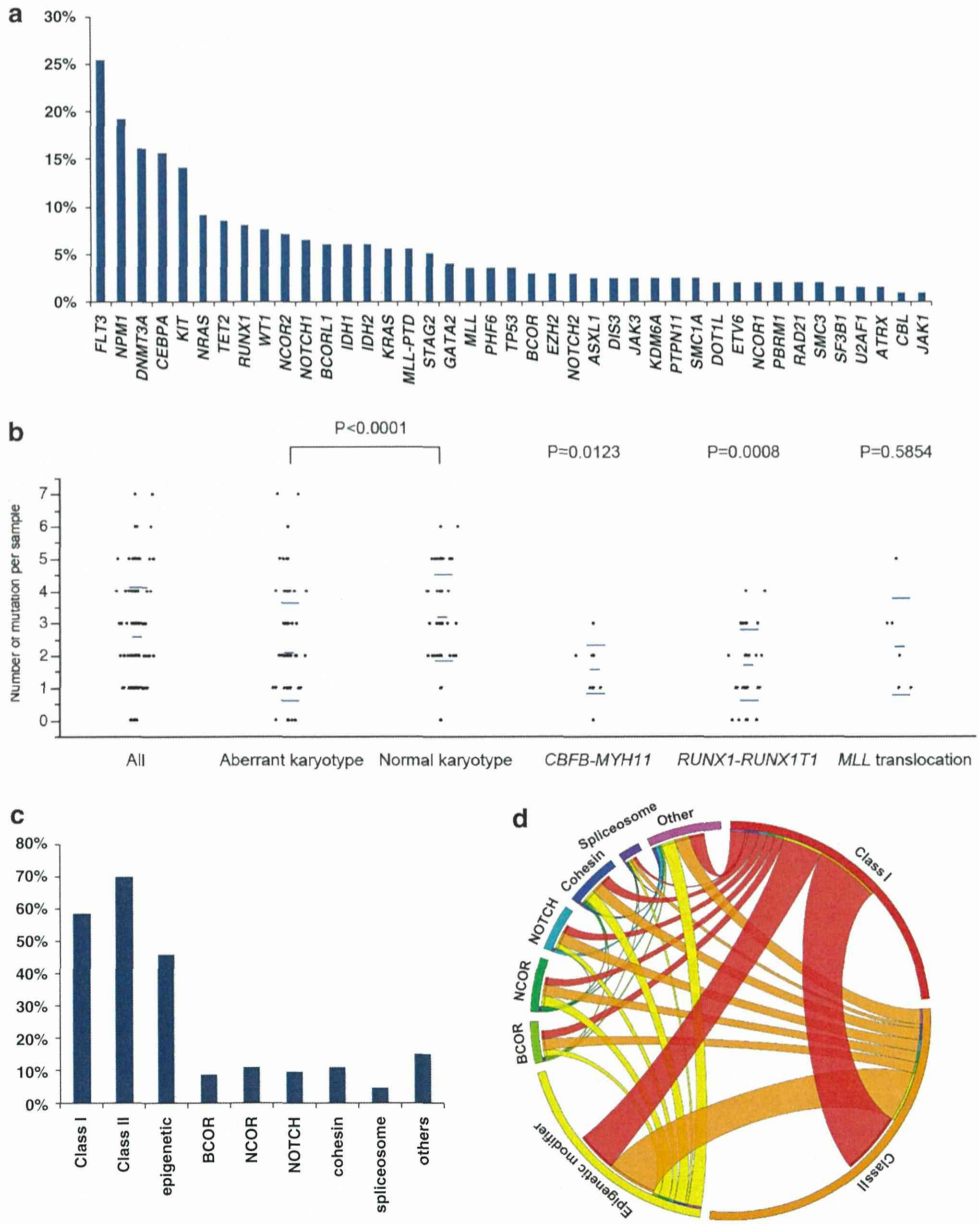


Figure 1. Frequencies and associations of mutated genes. (**a**) Frequencies of analyzed gene mutations. Frequency of each mutated gene is shown. Mutations were identified in 44 genes of 51 genes analyzed in 197 AML patients. Only five genes (*FLT3, NPM1, CEBPA, DNMT3A* and *KIT*) were mutated in more 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, $P=0.0291$),

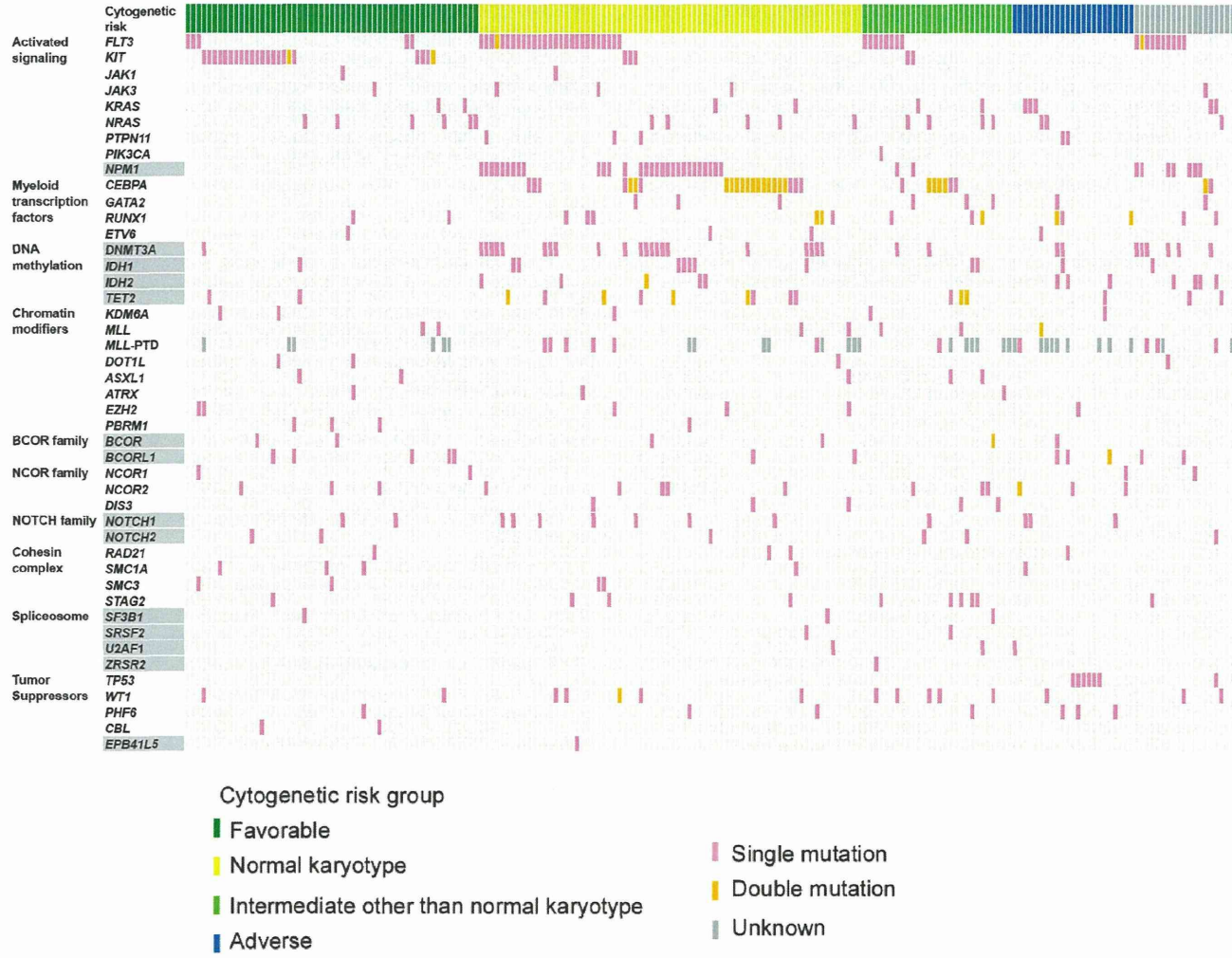


Figure 2. Mutation status according to the cytogetics-risk groups. Identified mutations in analyzed AML patients are shown according to the cytogenetic-risk groups. Pink boxes indicate single mutations and orange boxes indicate double mutations.

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).

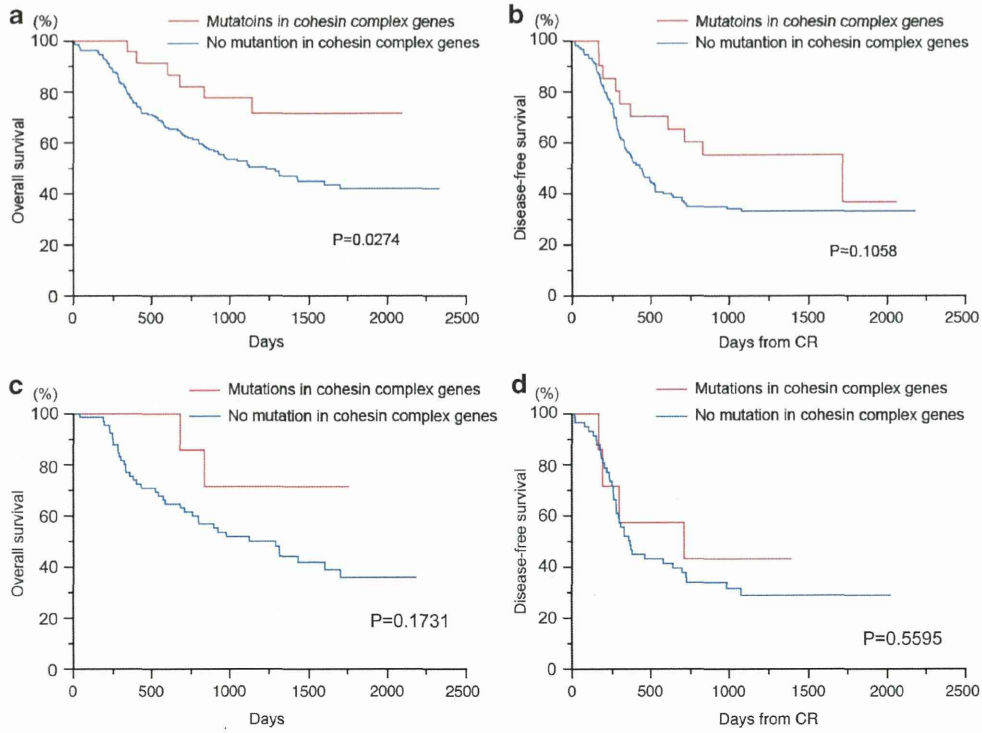


Figure 3. Prognostic impact of mutations in cohesin complex genes. Kaplan–Meier curves for OS and DFS according to the mutations in cohesin complex genes are shown. (**a**) OS in the total patients, (**b**) DFS in the total patient, (**c**) OS in the patients with normal karyotype, (**d**) DFS in the 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 ($P = 0.1731$).

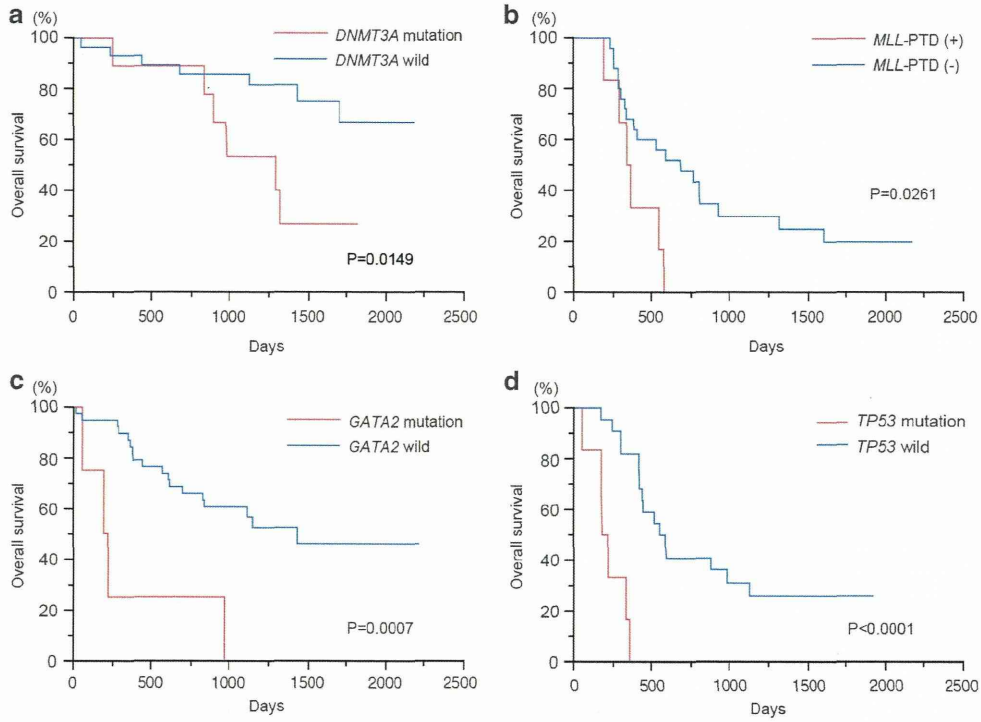


Figure 4. Mutations that could further stratify the ELN-risk groups into two risk groups. (**a**) *DNMT3A* mutation was a poor prognostic factor in the FR group except for the AML with t(8;21) (q22;q22); *RUNX1-RUNX1T1* or inv(16) (p13q22); *CBFB-MYH11* (CBF-AML). (**b**) *MLL*-PTD was a poor prognostic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

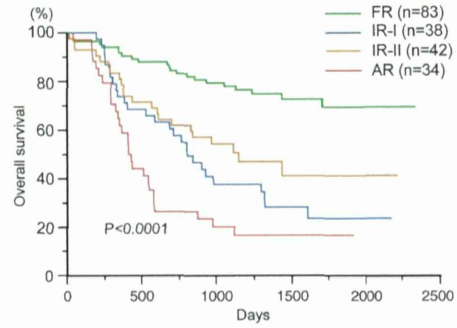
The whole-genome and -exome analysis demonstrated that an average of mutated genes in coding regions per sample was 5.24.[40] Of note was that there were significant differences in mutated gene numbers among the types of cytogenetics and mutations: the mean mutation numbers were higher in AML with *RUNX1-RUX1T1*, 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

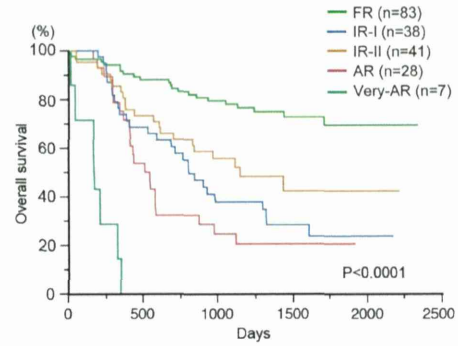


Figure 5. Risk stratification by modifying the ELN stratification system. When the CN-AML patients with *DNMT3A* mutations of the FR group and the patients with *MLL*-PTD of the IR-I group were included in the IR-I and the AR group, respectively (**a**), we could more clearly stratify the patients into 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.

ACKNOWLEDGEMENTS

This study was performed as a research program of the Project for Development of Innovative Research on Cancer Therapeutics (P-Direct), Ministry of Education, Culture, Sports, Science and Technology of Japan, and was also supported by Grants-in-Aid from the Scientific Research Program from the Japanese Ministry of Education, Culture, Sports, Science, and Technology, the Ministry of Health, Labor and Welfare for Cancer Research (Clinical Cancer Research H23-004 and H25-006), the National Cancer Center Research and Development Fund (23-A-23) and from KAKENHI (22134006 and 23249052).

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.

REFERENCES

1 Estey E, Dohner H. Acute myeloid leukaemia. *Lancet* 2006; **368**: 1894–1907.
2 Dohner H, Estey EH, Amadori S, Appelbaum FR, Buchner T, Burnett AK *et al.* Diagnosis and management of acute myeloid leukemia in adults: recommendations from an international expert panel, on behalf of the European LeukemiaNet. *Blood* 2010; **115**: 453–474.
3 Koreth J, Schlenk R, Kopecky KJ, Honda S, Sierra J, Djulbegovic BJ *et al.* Allogeneic stem cell transplantation for acute myeloid leukemia in first complete remission: systematic review and meta-analysis of prospective clinical trials. *JAMA* 2009; **301**: 2349–2361.
4 Grimwade D, Hills RK, Moorman AV, Walker H, Chatters S, Goldstone AH *et al.* Refinement of cytogenetic classification in acute myeloid leukemia: determination of prognostic significance of rare recurring chromosomal abnormalities among 5876 younger adult patients treated in the United Kingdom Medical Research Council trials. *Blood* 2010; **116**: 354–365.
5 Patel JP, Gonen M, Figueroa ME, Fernandez H, Sun Z, Racevskis J *et al.* Prognostic relevance of integrated genetic profiling in acute myeloid leukemia. *N Engl J Med* 2012; **366**: 1079–1089.
6 Shen Y, Zhu YM, Fan X, Shi JY, Wang QR, Yan XJ *et al.* Gene mutation patterns and their prognostic impact in a cohort of 1185 patients with acute myeloid leukemia. *Blood* 2011; **118**: 5593–5603.
7 Ofran Y, Rowe JM. Genetic profiling in acute myeloid leukaemia—where are we and what is its role in patient management. *Br J Haematol* 2013; **160**: 303–320.
8 Delhommeau F, Dupont S, Della Valle V, James C, Trannoy S, Masse A *et al.* Mutation in TET2 in myeloid cancers. *N Engl J Med* 2009; **360**: 2289–2301.
9 Langemeijer SM, Kuiper RP, Berends M, Knops R, Aslanyan MG, Massop M *et al.* Acquired mutations in TET2 are common in myelodysplastic syndromes. *Nat Genet* 2009; **41**: 838–842.
10 Mardis ER, Ding L, Dooling DJ, Larson DE, McLellan MD, Chen K *et al.* Recurring mutations found by sequencing an acute myeloid leukemia genome. *N Engl J Med* 2009; **361**: 1058–1066.
11 Ley TJ, Ding L, Walter MJ, McLellan MD, Lamprecht T, Larson DE *et al.* DNMT3A mutations in acute myeloid leukemia. *N Engl J Med* 2010; **363**: 2424–2433.
12 Nikoloski G, Langemeijer SM, Kuiper RP, Knops R, Massop M, Tonnissen ER *et al.* Somatic mutations of the histone methyltransferase gene EZH2 in myelodysplastic syndromes. *Nat Genet* 2010; **42**: 665–667.
13 Shih AH, Abdel-Wahab O, Patel JP, Levine RL. The role of mutations in epigenetic regulators in myeloid malignancies. *Nat Rev Cancer* 2012; **12**: 599–612.
14 Chou WC, Huang HH, Hou HA, Chen CY, Tang JL, Yao M *et al.* Distinct clinical and biological features of *de novo* acute myeloid leukemia with additional sex comb-like 1 (ASXL1) mutations. *Blood* 2010; **116**: 4086–4094.
15 Grossmann V, Tiacci E, Holmes AB, Kohlmann A, Martelli MP, Kern W *et al.* Whole-exome sequencing identifies somatic mutations of BCOR in acute myeloid leukemia with normal karyotype. *Blood* 2011; **118**: 6153–6163.
16 Li M, Collins R, Jiao Y, Ouillette P, Bixby D, Erba H *et al.* Somatic mutations in the transcriptional corepressor gene BCORL1 in adult acute myelogenous leukemia. *Blood* 2011; **118**: 5914–5917.
17 Welch JS, Ley TJ, Link DC, Miller CA, Larson DE, Koboldt DC *et al.* The origin and evolution of mutations in acute myeloid leukemia. *Cell* 2012; **150**: 264–278.
18 Yoshida K, Sanada M, Shiraishi Y, Nowak D, Nagata Y, Yamamoto R *et al.* Frequent pathway mutations of splicing machinery in myelodysplasia. *Nature* 2011; **478**: 64–69.
19 Naoe T, Kiyoi H. Gene mutations of acute myeloid leukemia in the genome era. *Int J Hematol* 2013; **97**: 165–174.
20 Rollig C, Bornhauser M, Thiede C, Taube F, Kramer M, Mohr B *et al.* Long-term prognosis of acute myeloid leukemia according to the new genetic risk classification of the European LeukemiaNet recommendations: evaluation of the proposed reporting system. *J Clin Oncol* 2011; **29**: 2758–2765.
21 Mrozek K, Marcucci G, Nicolet D, Maharry KS, Becker H, Whitman SP *et al.* Prognostic significance of the European LeukemiaNet standardized system for reporting cytogenetic and molecular alterations in adults with acute myeloid leukemia. *J Clin Oncol* 2012; **30**: 4515–4523.
22 Metzeler KH, Maharry K, Radmacher MD, Mrozek K, Margeson D, Becker H *et al.* TET2 mutations improve the new European LeukemiaNet risk classification of acute myeloid leukemia: a Cancer and Leukemia Group B study. *J Clin Oncol* 2011; **29**: 1373–1381.
23 Miyawaki S. Clinical studies of acute myeloid leukemia in the Japan Adult Leukemia Study Group. *Int J Hematol* 2012; **96**: 171–177.
24 Ohtake S, Miyawaki S, Fujita H, Kiyoi H, Shinagawa K, Usui N *et al.* Randomized study of induction therapy comparing standard-dose idarubicin with high-dose daunorubicin in adult patients with previously untreated acute myeloid leukemia: the JALSG AML201 Study. *Blood* 2011; **117**: 2358–2365.
25 Miyawaki S, Ohtake S, Fujisawa S, Kiyoi H, Shinagawa K, Usui N *et al.* A randomized comparison of 4 courses of standard-dose multiagent chemotherapy versus 3 courses of high-dose cytarabine alone in postremission therapy for acute myeloid leukemia in adults: the JALSG AML201 Study. *Blood* 2011; **117**: 2366–2372.
26 Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DA, Gralnick HR *et al.* Proposed revised criteria for the classification of acute myeloid leukemia. A report of the French-American-British Cooperative Group. *Ann Intern Med* 1985; **103**: 620–625.
27 Kiyoi H, Naoe T, Nakano Y, Yokota S, Minami S, Miyawaki S *et al.* Prognostic implication of FLT3 and N-RAS gene mutations in acute myeloid leukemia. *Blood* 1999; **93**: 3074–3080.
28 Ozeki K, Kiyoi H, Hirose Y, Iwai M, Ninomiya M, Kodera Y *et al.* Biologic and clinical significance of the FLT3 transcript level in acute myeloid leukemia. *Blood* 2004; **103**: 1901–1908.
29 Yamamoto Y, Kiyoi H, Nakano Y, Suzuki R, Kodera Y, Miyawaki S *et al.* Activating mutation of D835 within the activation loop of FLT3 in human hematologic malignancies. *Blood* 2001; **97**: 2434–2439.
30 Osumi K, Fukui T, Kiyoi H, Kasai M, Kodera Y, Kudo K *et al.* Rapid screening of leukemia fusion transcripts in acute leukemia by real-time PCR. *Leuk Lymphoma* 2002; **43**: 2291–2299.
31 Makishima H, Yoshida K, Nguyen N, Przychodzen B, Sanada M, Okuno Y *et al.* Somatic SETBP1 mutations in myeloid malignancies. *Nat Genet* 2013; **45**: 942–946.
32 Kon A, Shih LY, Minamino M, Sanada M, Shiraishi Y, Nagata Y *et al.* Recurrent mutations in multiple components of the cohesin complex in myeloid neoplasms. *Nat Genet* 2013; **45**: 1232–1237.
33 Sakaguchi H, Okuno Y, Muramatsu H, Yoshida K, Shiraishi Y, Takahashi M *et al.* Exome sequencing identifies secondary mutations of SETBP1 and JAK3 in juvenile myelomonocytic leukemia. *Nat Genet* 2013; **45**: 937–941.
34 Ishikawa Y, Kiyoi H, Tsujimura A, Miyawaki S, Miyazaki Y, Kuriyama K *et al.* Comprehensive analysis of cooperative gene mutations between class I and class II in *de novo* acute myeloid leukemia. *Eur J Haematol* 2009; **83**: 90–98.