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ORIGINAL ARTICLE

Mutational analysis of *DNMT3A* improves the prognostic stratification of patients with acute myeloid leukemia

Satoshi Wakita[1] | Atsushi Marumo[1] | Kaoru Morita[2] | Shinichi Kako[3] | Takashi Toya[4] | Yuho Najima[4] | Noriko Doki[4] | Junya Kanda[5] | Junya Kuroda[6] | Shinichiro Mori[7] | Atsushi Satake[8] | Kensuke Usuki[9] | Toshimitsu Ueki[10] | Nobuhiko Uoshima[11] | Yutaka Kobayashi[11] | Eri Kawata[12] | Kazutaka Nakayama[13] | Yuhei Nagao[14] | Katsuhiro Shono[14] | Motoharu Shibusawa[15] | Jiro Tadokoro[15] | Masao Hagihara[16] | Hitoji Uchiyama[17] | Naoyuki Uchida[18] | Yasushi Kubota[19] | Shinya Kimura[19] | Hisao Nagoshi[20] | Tatsuo Ichinohe[20] | Saiko Kurosawa[21] | Sayuri Motomura[22] | Akiko Hashimoto[23] | Hideharu Muto[24] | Eriko Sato[25] | Masao Ogata[26] | Kenjiro Mitsuhashi[27] | Jun Ando[28] | Haruko Tashiro[29] | Masahiro Sakaguchi[1] | Shunsuke Yui[1] | Kunihito Arai[1] | Tomoaki Kitano[1] | Miho Miyata[1] | Haruka Arai[1] | Masayuki Kanda[1] | Kako Itabashi[1] | Takahiro Fukuda[21] | Yoshinobu Kanda[2,3] | Hiroki Yamaguchi[1]

[1]Department of Hematology, Nippon Medical School, Tokyo, Japan

[2]Division of Hematology, Department of Medicine, Jichi Medical University, Tochigi, Japan

[3]Division of Hematology, Jichi Medical University Saitama Medical Center, Saitama, Japan

[4]Hematology Division, Tokyo Metropolitan Cancer and Infectious Diseases Center, Komagome Hospital, Tokyo, Japan

[5]Department of Hematology and Oncology, Graduate School of Medicine, Kyoto University, Kyoto, Japan

[6]Division of Hematology and Oncology, Kyoto Prefectural University of Medicine, Kyoto, Japan

[7]Hematology Department, St. Luke's International Hospital, Tokyo, Japan

[8]First Department of Internal Medicine, Kansai Medical University, Osaka, Japan

[9]Department of Hematology, NTT Medical Center Tokyo, Tokyo, Japan

[10]Department of Hematology, Nagano Red Cross Hospital, Nagano, Japan

[11]Department of Hematology, Japanese Red Cross, Kyoto Daini Hospital, Kyoto, Japan

[12]Department of Hematology, Panasonic Health Insurance Organization Matsushita Memorial Hospital, Osaka, Japan

[13]Department of Hematology, Yokohama Minami Kyousai Hospital, Yokohama-shi, Japan

[14]Department of Hematology, Chiba Aoba Municipal Hospital, Chiba, Japan

[15]Department of Hematology, IMS group Shinmatsudo Central General Hospital, Chiba, Japan

[16]Department of Hematology, Eiju General Hospital, Tokyo, Japan

[17]Department of Hematology, Japanese Red Cross Kyoto Daiichi Hospital, Kyoto, Japan

[18]Department of Hematology, Federation of National Public Service Personnel Mutual Aid Associations, Toranomon Hospital, Tokyo, Japan

[19]Division of Hematology, Respiratory Medicine and Oncology, Department of Internal Medicine, Faculty of Medicine, Saga University, Saga, Japan

Abbreviations: allo-HSCT, allogeneic hematopoietic stem cell transplantation; *CEBPA*, *CCAAT Enhancer Binding Protein Alpha*; CR, complete remission; CRR, rate of CR in the induction phase; *DNMT3A*, *DNA methyltransferase 3A*; EFS, event-free survival; *FLT3*-ITD, *FMS-like tyrosine kinase 3*; GS-JAML, multicenter collaborative program for gene sequencing of Japanese AML; ITD-AR, *FLT3*-ITD allelic ratio; *NPM1*, *Nucleophosmin1*; OS, overall survival.

Satoshi Wakita and Atsushi Marumo contributed equally to this work.

[20]Department of Hematology and Oncology, Research Institute for Radiation Biology and Medicine, Hiroshima University, Hiroshima, Japan
[21]Department of Hematopoietic Stem Cell Transplantation, National Cancer Center Hospital, Tokyo, Japan
[22]Department of Hematology, Tama-Hokubu Medical Center, Tokyo Metropolitan Health and Medical Treatment Corporation, Tokyo, Japan
[23]Department of Immunology and Hematology, Kobe City Nishi-Kobe Medical Center, Kobe, Japan
[24]Division of Hematology Tokyo Metropolitan Ohtsuka Hospital, Tokyo, Japan
[25]Department of Hematology, Juntendo University Nerima Hospital, Tokyo, Japan
[26]Department of Hematology, Oita University Hospital, Oita, Japan
[27]Department of Hematology, Saitama Red Cross Hospital, Saitama, Japan
[28]Division of Hematology, Department of Internal Medicine, Juntendo University School of Medicine, Tokyo, Japan
[29]Department of Hematology/Oncology, Teikyo University School of Medicine, Tokyo, Japan

Correspondence
Satoshi Wakita, Department of Hematology, Nippon Medical School, 1-1-5 Sendagi, Bunkyo-Ku, Tokyo 113-8603, Japan.
Email: aaa11140@nms.ac.jp

Abstract

Nucleophosmin1 (*NPM1*) mutations are the most frequently detected gene mutations in acute myeloid leukemia (AML) and are considered a favorable prognostic factor. We retrospectively analyzed the prognosis of 605 Japanese patients with de novo AML, including 174 patients with *NPM1*-mutated AML. Although patients with *NPM1*-mutated AML showed a high remission rate, this was not a favorable prognostic factor for overall survival (OS); this is contrary to generally accepted guidelines. Comprehensive gene mutation analysis showed that mutations in codon R882 of *DNA methyltransferase 3A* (*DNMT3A*^{R882} mutations) were a strong predicative factor indicating poor prognosis in all AML ($p < 0.0001$) and *NPM1*-mutated AML cases ($p = 0.0020$). Furthermore, multivariate analysis of all AML cases showed that *DNMT3A*^{R882} mutations and the co-occurrence of internal tandem duplication in *FMS-like tyrosine kinase 3* (*FLT3*-ITD), *NPM1* mutations, and *DNMT3A*^{R882} mutations (triple mutations) were independent factors predicting a poor prognosis related to OS, with *NPM1* mutations being an independent factor for a favorable prognosis (hazard ratios: *DNMT3A*^{R882} mutations, 1.946; triple mutations, 1.992, *NPM1* mutations, 0.548). Considering the effects of *DNMT3A*^{R882} mutations and triple mutations on prognosis and according to the classification of *NPM1*-mutated AML into three risk groups based on *DNMT3A*^{R882}/*FLT3*-ITD genotypes, we achieved the improved stratification of prognosis ($p < 0.0001$). We showed that *DNMT3A*^{R882} mutations are an independent factor for poor prognosis; moreover, when confounding factors that include *DNMT3A*^{R882} mutations were excluded, *NPM1* mutations were a favorable prognostic factor. This revealed that ethnological prognostic discrepancies in *NPM1* mutations might be corrected through prognostic stratification based on the *DNMT3A* status.

KEYWORDS
acute myeloid leukemia, *DNMT3A*, *NPM1*, prognostic factor, triple mutation

1 | INTRODUCTION

Exon12 frameshift mutations in *NPM1* are found in ~35% of de novo AML patients. This *NPM1* mutation is frequently detected in an overlapping manner with internal tandem duplications of *FLT3*-ITD and mutations in *DNMT3A*.[1,2] Multiple large cohort trials conducted in Europe and the USA showed that *NPM1* mutations are a strong factor indicating a favorable prognosis.[2-6] In addition, according to the European LeukemiaNet 2017 (ELN 2017) classification, which is a commonly used prognostic model, this mutation is considered an absolute factor of a favorable prognosis that complements the prognostic value of the *FLT3*-ITD allelic ratio (AR) and lowers the one-step risk category.[7,8] Meanwhile, the clinical significance of *NPM1* mutations in Asia and the Middle East remains unclear. Although

several studies have targeted cohorts in Asia and the Middle East, most of them have not been able to show the effect of *NPM1* mutations based on univariate analyses.[9–14]

DNMT3A mutations are genetic abnormalities detected in 10%–30% of de novo AML cases, with ~60% of them occurring at the R882 site. Their frequent detection in AML was first reported in 2010[15] and, since then, they have been reported as a factor indicative of a poor prognosis in many cohort analyses.[16–18] However, their ability to serve as an independent prognostic factor has been debated because they are detected in an overlapping manner with various genetic abnormalities, and in fact, *DNMT3A* mutations were excluded among major prognostic factors even in the ELN 2017 classification.[7] Interestingly, mutations at the R882 site of *DNMT3A* (*DNMT3A*^{R882}; which are the most common among *DNMT3A* mutations)[19] overlap with other genetic abnormalities. Alternatively, overlapping *DNMT3A* mutations with *NPM1* mutations or *FLT3*-ITD have been suggested to be stronger factors indicating a poor prognosis,[2,20] and suggesting the need for a more detailed subgroup analysis.

We previously conducted a retrospective prognostic analysis of 744 patients with AML enrolled between 2001 and 2019 based on the data from the GS-JAML, which was a clinical sequencing program that was conducted as a multicenter joint study in Japan.[21] However, patients with *NPM1*-mutated AML did not show a favorable prognosis. In this study, we sought to clarify the factors that influence this "unexpected prognosis of *NPM1*-mutated AML" by targeting only patients aged 70 years or younger who were diagnosed after 2010 and who received standard induction therapy based on GS-JAML data. We also analyzed the effects of *NPM1* and concurrent mutations, particularly *DNMT3A* mutations, on the prognosis.

2 | METHODS

2.1 | Study population

All patients in this analysis were enrolled and selected by the GS-JAML and conducted by the Nippon Medical School. Briefly, Japanese residents aged ≥16 years with de novo AML since 2001 were enrolled in this observational study after obtaining their consent. The following test results of patients with AML, since 2009, were provided by the investigators to the physicians within 1 month: *FLT3*-ITD (PCR assay from 2009, fragment analysis from 2018), *NPM1* exon12 (from 2009), *CEBPA* (from 2009), and *DNMT3A*^{R882} (from 2017). This analysis included patients with de novo AML (excluding the FAB-M3 subtype) enrolled in the GS-JAML study from 2010 to 2019.

2.2 | Mutation screening and target-capture sequencing of the AML gene panel

Patient samples were collected at the time of diagnosis, and genomic DNA was extracted from patients with ≥20% blasts in bone marrow or peripheral blood. Screening for cytogenetic abnormalities was performed through G-band analysis, and fluorescence in situ hybridization analysis was used to additionally search for t(8;21)(q22;q22.1), t(15;17)(q22;q21), and inv(16)(p13;q22)/t(16;16)(p13;q22). The cytogenetic prognosis was then designated according to the ELN 2017 classification. Screening for *FLT3-ITD*, mutations in exon 12 of *NPM1*, *DNMT3A*^{R882} mutations, and the entire exon of *CEBPA*, was performed as previously described.[21,22] For this study, high-AR and low-AR cases were defined as ITD-AR >0.5 and <0.5, respectively. Using cryopreserved samples, target-capture sequencing of the AML gene panel (Table S1) was performed retrospectively, as previously described.[21] The sequences of all exons of *DNMT3A* were confirmed through this target-capture sequencing.

2.3 | Statistical analysis

Definitions of response criteria were in accordance with the system recommended by the ELN 2017.[7] The primary outcome was OS. Secondary outcomes included OS censored at the time of allo-HSCT and EFS. The induction phase was defined as the period of 60 days after the start of initial remission induction therapy. Induction failure was defined as failure to achieve CR in the induction phase. OS was calculated from the time of diagnosis until death from any cause. Events related to EFS included induction failure, first relapse, and death resulting from any cause. For patients without an event, all survival end-points were censored at the date of the last follow-up.

Chi-squared and Fisher's exact tests were used to test the association between categorical variables and the presence or absence of mutations. The non-parametric Mann–Whitney *U*-test was used to assess the difference in the distributions of a non-proportional continuous variable. All statistical tests were two-sided. The Kaplan–Meier method and log-rank test were used to analyze OS and EFS. Regarding the prognostic factors, multivariate analysis was conducted using the Cox proportional hazards model. A backward and forward stepwise procedure selection model using the Akaike information criterion was used to identify independent prognostic factors. Statistical analyses were performed using GraphPad Prism version 9.00 for Windows (GraphPad Software) and EZR (version 1.36; Saitama Medical Center, Jichi Medical University).[23]

3 | RESULTS

3.1 | Patient characteristics

Among 919 patients who registered with the GS-JAML from 2010 to 2019 and who were 70 years of age or younger, we targeted those who underwent the 7+3 induction regimen, which is a standard induction therapy consisting of 7 days of standard-dose cytarabine (100–200 mg/m^2 continuous infusion) and 3 days of an anthracycline antibiotic infusion (idarubicin 12 mg/m^2 or daunorubicin 60–90 mg/m^2), and evaluated the results of their comprehensive gene analysis. Patients who used FLT3 inhibitors for induction therapy, low-dose cytarabine treatment (for unfit patients), or azacitidine

were excluded because these comprised a small number of cases. In total, 605 patients were included in this analysis. Table S2 shows the clinical backgrounds of patients. The median observation period for all 605 patients was 830.5 days. Among all patients, we found that 127 (20.9%) exhibited *FLT3*-ITD and, of these, 44 were low-AR, whereas 80 were high-AR cases. We detected ITD alleles in three patients by PCR, but fragment analysis via capillary electrophoresis was difficult, and the ITD-AR was unknown. We also detected *NPM1* mutations in 174 patients (28.8%), whereas *DNMT3A* mutations were detected in 125 patients (20.7%). Of the 125 *DNMT3A* mutation-positive patients, 67 had a *DNMT3A*^{R882} mutation, whereas 58 had a *DNMT3A*$^{non\text{-}R882}$ mutation; there was no overlap between *DNMT3A*^{R882} mutations and *DNMT3A*$^{non\text{-}R882}$ mutations. We identified 83 patients who had both *NPM1* and *DNMT3A* mutations, of whom 49 had both *NPM1* and *DNMT3A*^{R882} mutations, whereas 34 had both *NPM1* and *DNMT3A*$^{non\text{-}R882}$ mutations. Figure S1 shows the gene abnormality distribution, including the overlap in mutations between AML-related genes included in the ELN 2017 classification and *DNMT3A* mutations. We mainly detected *FLT3*-ITD and mutations in *NPM1* and *DNMT3A* in the intermediate-risk karyotype group, especially in patients with a normal karyotype, in agreement with previous reports. Additionally, we observed that these gene mutations had little overlap with mutations in *ASXL1*, *RUNX1*, and *TP53*.

3.2 | Rate of complete remission in the induction phase

Table S3 shows the CRR in each subgroup classified based on *FLT3*-ITD, *NPM1*, and *DNMT3A* mutations. The overall CRR was 74.5%. In particular, we detected that the CRRs for the chromosomal risk groups were 92.4% for the favorable-risk karyotype, 75.4% for the intermediate-risk karyotype, and 50.0% for the adverse-risk karyotype, resulting in clear stratification based on chromosomal prognostic classification. We also found that the CRRs based on the *FLT3*-ITD status were 77.1% for *FLT3*-ITD-negative, 72.1% for low-AR, and 60.0% for high-AR cases. Meanwhile, the CRRs based on the normal karyotype only were 80.8% for *FLT3*-ITD-negative, 72.7% for low-AR, and 59.7% for high-AR cases, with CRR gradually decreasing. Furthermore, we noticed that for the *FLT3*-ITD negative, ITD low-AR, and ITD high-AR groups, the *NPM1* mutation-positive cases tended to have a higher CRR than that of the negative cases (ITD negative: *NPM1* mutation positive vs. negative = 87.1% vs. 74.6%; low-AR: *NPM1* mutation positive vs. negative = 76.9% vs. 64.7%; high-AR: *NPM1* mutation positive vs. negative = 68.8% vs. 46.9%).

3.3 | Prognostic value of *FLT3*-ITD, *NPM1*, and *DNMT3A* mutations

Kaplan–Meier plots of OS, OS censored at allo-HSCT, and EFS for the entire cohort are shown in Figure S2. Kaplan–Meier plots comparing the following are shown in Figure S3 with OS, Figure S4 with OS censored at allo-HSCT, and Figure S5 with EFS, respectively: (a) all patients within the subgroups were classified based on the *FLT3*-ITD AR (*FLT3*-ITD-negative, low-AR, or high-AR), (b) all patients with *NPM1* mutation-positive or -negative disease, (c) all patients with *DNMT3A* wild-type, *DNMT3A*^{R882} mutation positive, or *DNMT3A*$^{non\text{-}R882}$ mutation positive, (d) normal karyotype AML patients with subgroups classified based on the *FLT3*-ITD AR (*FLT3*-ITD-negative, low-AR, or high-AR), (e) normal karyotype AML patients with *NPM1* mutation-positive or -negative disease, (f) normal karyotype AML patients with *DNMT3A* wild-type, *DNMT3A*^{R882} mutation-positive, or *DNMT3A*$^{non\text{-}R882}$ mutation-positive disease. As shown in Figures S3–S5a,d, we identified the *FLT3*-ITD high-AR status as a strong factor indicative of a poor prognosis related to the OS, OS censored at allo-HSCT, and EFS in the analysis of all patients and those with a normal karyotype. Meanwhile, as shown in Figures S3–S5b,e, we could not establish the *NPM1* mutation as a factor indicating a favorable prognosis for OS, OS censored at allo-HSCT, or EFS in analyses of all patients or those with a normal karyotype. Likewise, *NPM1* mutations were not a prognostic factor in subgroup analysis based on *FLT3*-ITD (data not shown).

As shown in Figures S3–S5c,f, we identified *DNMT3A* mutations as strong factors indicative of a poor prognosis. In particular, we found that, compared with the wild-type *DNMT3A*, the *DNMT3A*^{R882} mutation was strongly correlated with a worse OS, OS censored at allo-HSCT, and EFS for all patients and for those with a normal karyotype. Meanwhile, the *DNMT3A*$^{non\text{-}R882}$ mutation-positive group showed an intermediate prognosis between that of the *DNMT3A* wild-type group and that of the *DNMT3A*^{R882} mutation-positive group. Further, when comparing the *DNMT3A*$^{non\text{-}R882}$ mutation-positive group and *DNMT3A* wild-type group, we found that *DNMT3A*$^{non\text{-}R882}$ mutations were significantly correlated with a poor OS, OS censored at allo-HSCT, and EFS in the analysis of patients with a normal karyotype.

3.4 | Different prognostic values of *DNMT3A* mutations in *NPM1*-mutated and *NPM1* wild-type AML

We assumed that the profiles of concurrent genetic abnormalities were involved in the "unexpected prognosis of *NPM1*-mutated AML." Therefore, we performed univariate analyses of OS, OS censored at allo-HSCT, and EFS according to the mutation status of the markedly recurring mutated genes in *NPM1*-mutated AML. *DNMT3A* mutations were frequently detected in *NPM1*-mutated AML (47.7%; 83/174) and were strongly associated with a poor prognosis (Table S4). Figure 1A–C shows Kaplan–Meier plots comparing the *DNMT3A* genotype subgroups (*DNMT3A* wild-type group, *DNMT3A*^{R882} mutation-positive group, and *DNMT3A*$^{non\text{-}R882}$ mutation-positive group) for all karyotypes/*NPM1*-mutated AML (Figure 1A: OS; Figure 1B: OS censored at allo-HSCT; Figure 1C: EFS), whereas Figure 1D–F shows the Kaplan–Meier plots comparing the *DNMT3A* genotype subgroups for normal karyotypes/*NPM1*-mutated AML (Figure 1D: OS; Figure 1D: OS censored at

allo-HSCT; Figure 1F: EFS). We observed that for patients with all karyotypes/*NPM1*-mutated AML and normal karyotypes/*NPM1*-mutated AML, *DNMT3A*^{R882} mutations were strong predictive factors of a poor prognosis and were associated with a shortened OS, OS censored at allo-HSCT, and EFS. Furthermore, we found that *DNMT3A*$^{non\text{-}R882}$ mutation-positive patients tended to have a shorter OS censored at allo-HSCT and EFS but a similar OS compared with those of the *DNMT3A* wild-type patients. These results suggested that the presence of *DNMT3A*^{R882} mutations was primarily related to the "unexpected poor prognosis of *NPM1*-mutated AML" tendency observed in the present study cohort.

We further found that the combined rate of *DNMT3A* mutations in *NPM1* wild-type AML was 9.74% (42/431). Figure 2A–C shows Kaplan–Meier plots comparing *DNMT3A* genotype subgroups for all karyotypes/*NPM1* wild-type AML (Figure 2A: OS; Figure 2B: OS censored at allo-HSCT; Figure 2C: EFS), whereas Figure 2D–F shows Kaplan–Meier plots comparing *DNMT3A* genotype subgroups for normal karyotypes/*NPM1* wild-type AML (Figure 2D: OS; Figure 2E: OS censored at allo-HSCT; Figure 2F: EFS). We determined that *DNMT3A*^{R882} mutations were a significant factor indicating a poor prognosis with respect to the OS, OS censored at allo-HSCT, and EFS for *NPM1* wild-type AML and with respect to the OS and EFS for normal karyotype/*NPM1* wild-type AML. Meanwhile, we found that *DNMT3A*$^{non\text{-}R882}$ mutations were a significant factor indicating a poor prognosis with respect to the OS and EFS for normal karyotype/*NPM1* wild-type AML. As the sample size of *DNMT3A* mutation-positive cases among those with *NPM1* wild-type AML was small and the genetic background was diverse, the ability of *DNMT3A* mutations to serve as independent prognostic factors needs to be further investigated.

3.5 | Identification of prognostic factors through multivariate analysis

To investigate the effect of *DNMT3A* mutations on the prognosis of AML, we targeted all patients with AML, as well as those patients with *NPM1*-mutated and *NPM1* wild-type disease, and conducted

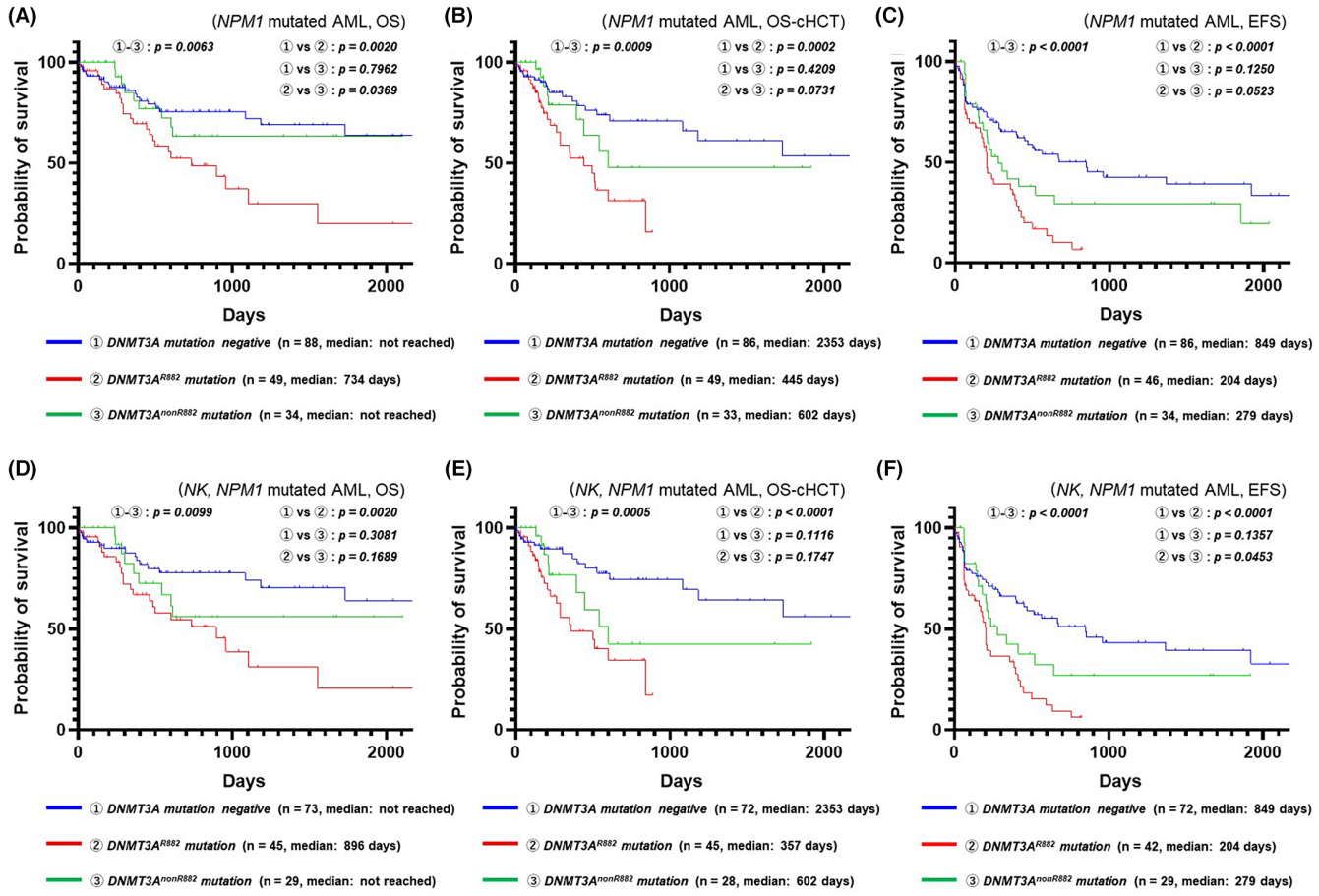

FIGURE 1 Effect of *DNMT3A* mutations on the prognosis of *NPM1*-mutated acute myeloid leukemia (AML). (A) Kaplan–Meier plots (overall survival; OS) comparing *DNMT3A* genotype subgroups for all karyotypes/*NPM1*-mutated AML. (B) Kaplan–Meier plots (OS censored at allo-HSCT) comparing *DNMT3A* genotype subgroups for all karyotypes/*NPM1*-mutated AML. (C) Kaplan–Meier plots (event-free survival; EFS) comparing *DNMT3A* genotype subgroups for all karyotypes/*NPM1*-mutated AML. (D) Kaplan–Meier plots (OS) comparing *DNMT3A* genotype subgroups for normal karyotype (NK)/*NPM1*-mutated AML. (E) Kaplan–Meier plots (OS censored at allo-HSCT) comparing *DNMT3A* genotype subgroups for NK/*NPM1*-mutated AML. (F) Kaplan–Meier plots (EFS) comparing *DNMT3A* genotype subgroups for NK/*NPM1*-mutated AML

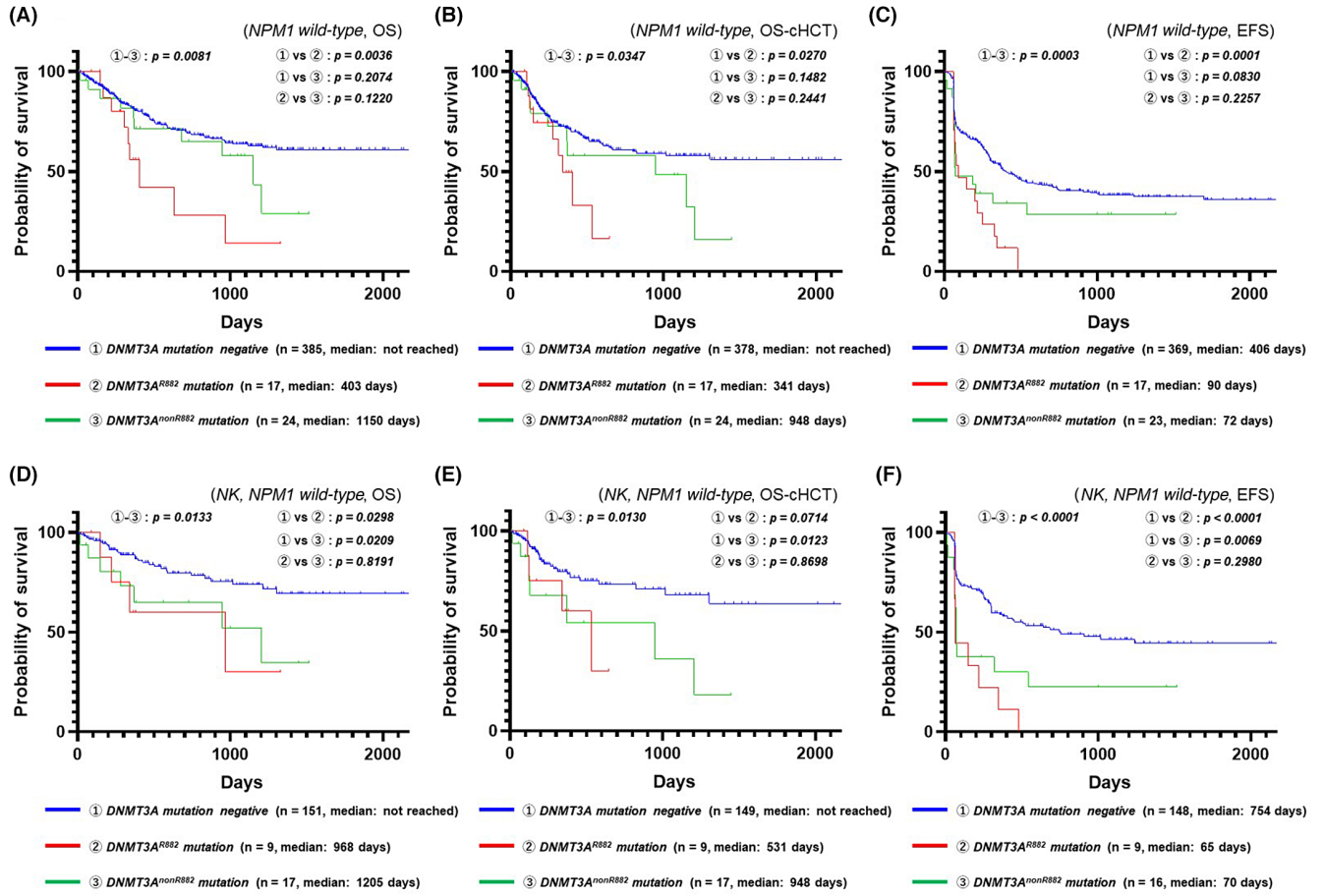

FIGURE 2 Effect of *DNMT3A* mutations on the prognosis of *NPM1* wild-type acute myeloid leukemia (AML). (A) Kaplan–Meier plots (overall survival; OS) comparing *DNMT3A* genotype subgroups for all karyotypes/*NPM1* wild-type AML. (B) Kaplan–Meier plots (OS censored at allo-HSCT) comparing *DNMT3A* genotype subgroups for all karyotypes/*NPM1* wild-type AML. (C) Kaplan–Meier plots (event-free survival; EFS) comparing *DNMT3A* genotype subgroups for all karyotypes/*NPM1* wild-type AML. (D) Kaplan–Meier plots (OS) comparing *DNMT3A* genotype subgroups for normal karyotype (NK)/*NPM1* wild-type AML. (E) Kaplan–Meier plots (OS censored at allo-HSCT) comparing *DNMT3A* genotype subgroups for NK/*NPM1* wild-type AML. (F) Kaplan–Meier plots (EFS) comparing *DNMT3A* genotype subgroups for NK/*NPM1* wild-type AML

a Cox regression analysis of OS using the following factors as input: age (>60 or ≤60 years old); allo-HSCT at first CR (yes or no); favorable-risk karyotype (yes or no); adverse-risk karyotype (yes or no); *FLT3*-ITD high-AR (positive or negative); *FLT3*-ITD (regardless of ITD-AR, positive or negative); *NPM1* mutation (positive or negative); *CEBPA* double mutation (positive or negative); *ASXL1* mutations (positive or negative); *RUNX1* mutations (positive or negative); *TP53* mutations (positive or negative); *DNMT3A*^{R882} mutations (positive or negative); *DNMT3A* mutations (regardless of position, positive or negative); triple mutation in *NPM1*, *FLT3*-ITD, and *DNMT3A* (yes or no); and triple mutation in *NPM1*, *FLT3*-ITD, and *DNMT3A*^{R882} (yes or no). A backward and forward stepwise procedure selection model using the Akaike information criterion was used to extract independent events. Table 1 shows a summary of the analysis. Using Cox regression, we extracted the *DNMT3A*^{R882} mutation and triple mutation in *NPM1*, *FLT*-ITD, and *DNMT3A*^{R882} as independent factors of poor prognosis. Simultaneously, removing these confounding factors resulted in the *NPM1* mutation being an independent factor for a favorable prognosis. Using Cox regression analysis for only *NPM1*-mutated AML, we identified the *DNMT3A*^{R882} mutation, *FLT3*-ITD mutation, and age >60 years as factors indicative of a poor prognosis, whereas allo-HSCT at first CR was identified as a factor associated with a favorable prognosis. Meanwhile, using Cox regression analysis for only *NPM1* wild-type AML, we identified *TP53* mutations, the adverse-risk karyotype, *FLT3*-ITD high-AR, *DNMT3A* mutations, and age >60 years as factors related to a poor prognosis. Interestingly, all *DNMT3A* mutations were identified as independent factors correlated with OS in *NPM1* wild-type AML, regardless of the mutation site.

3.6 | Effects of *FLT3*-ITD and *DNMT3A*^{R882} mutations on the prognosis of *NPM1*-mutated AML

From our multivariate analysis, we speculated that treating *DNMT3A*^{R882} mutation-positive patients as a separate prognostic

group would correct the discrepancy between the *NPM1*-mutated AML cohort and the conventional prognostic model. To show the effect of *DNMT3A*^{R882} mutation/*FLT3*-ITD AR genotypes on the prognosis of *NPM1*-mutated AML, we demonstrated the prognosis of each *FLT3*-ITD subgroup in terms of *NPM1* mutation-positive/*DNMT3A*^{R882} wild-type and *NPM1* mutation-positive/*DNMT3A*^{R882} mutation-positive patients using Kaplan–Meier plots (*NPM1* mutation-positive/*DNMT3A*^{R882} wild-type: OS, Figure 3A; OS censored at allo-HSCT, Figure 3B; EFS, Figure 3C; *NPM1* mutation-positive/*DNMT3A*^{R882} mutation-positive: OS, Figure 3D, OS censored at allo-HSCT, Figure 3E; EFS, Figure 3F). As shown in Figure 3A–C, *NPM1* mutation-positive/*DNMT3A*^{R882} wild-type patients tended to have a favorable prognosis in all *FLT3*-ITD subgroups. Focusing on the prognostic value of the *FLT3*-ITD AR for *NPM1* mutation-positive/*DNMT3A*^{R882} wild-type patients, a comparison of OS and EFS showed more early deaths and early events in *FLT3*-ITD high-AR patients than in *FLT3*-ITD-negative or low-AR patients (OS: *FLT3*-ITD high-AR vs. *FLT3*-ITD-negative or low-AR, $p = 0.1045$; EFS: *FLT3*-ITD high-AR vs. *FLT3*-ITD-negative or low-AR, $p = 0.0558$). Furthermore, a comparison of OS censored at allo-HSCT among *NPM1* mutation-positive/*DNMT3A*^{R882} wild-type patients showed that *FLT3*-ITD-negative patients had increased survival compared with *FLT3*-ITD-positive patients (*FLT3*-ITD-negative vs. *FLT3*-ITD-positive, $p = 0.0086$). These facts suggested that *FLT3*-ITD-positive cases should routinely be assigned to allo-HSCT even for patients with *FLT3*-ITD low-AR disease. Meanwhile, as shown in Figure 3D–F, *NPM1* mutation-positive/*DNMT3A*^{R882} mutation-positive patients showed a poor prognosis for all subgroups classified based on *FLT3*-ITD. In particular, we noticed that *FLT3*-ITD-positive patients had an extremely poor prognosis with respect to all prognostic indices, namely OS, OS censored at allo-HSCT, and EFS, regardless of the ITD-AR. This result suggests the importance of triple mutations for a short-term prognosis.

TABLE 1 Cox proportional hazards regression analysis on OS

Overall survival	Hazard ratio	Lower 95% CI	Upper 95% CI	*p*-value
All cases	***n* = 597, number of events = 177 (8 observations deleted due to missingness) Concordance index = 0.728**			
TP53 mutation	2.802	1.668	4.705	<0.001
Adverse karyotype	2.249	1.435	3.525	<0.001
Triple mutation in *NPM1*, *FLT3*-ITD, and *DNMT3A*^{R882}	1.992	0.925	4.288	0.078
Age > 60 years old	1.95	1.427	2.665	<0.001
DNMT3A^{R882} mutation	1.946	1.133	3.341	0.016
FLT3-ITD	1.835	1.209	2.786	0.004
Favorable karyotype	0.548	0.316	0.95	0.032
NPM1 mutation	0.548	0.356	0.842	0.006
Allo-HSCT at 1st CR	0.498	0.333	0.744	0.001
CEBPA double mutation	0.321	0.162	0.633	0.001
***NPM1*-mutated AML**	***n* = 171, number of events = 53 (3 observations deleted due to missingness) Concordance index = 0.687**			
DNMT3A^{R882} mutation	2.823	1.583	5.035	<0.001
FLT3-ITD	2.678	1.495	4.797	0.001
Age > 60 years old	2.227	1.239	4.004	0.007
Allo-HSCT at 1st CR	0.543	0.256	1.151	0.111
***NPM1* wild-type AML**	***n* = 426, number of events = 124 (5 observations deleted due to missingness) Concordance index: 0.742**			
TP53 mutation	2.899	1.674	5.022	<0.001
Adverse karyotype	2.401	1.504	3.834	<0.001
FLT3-ITD high-AR	1.862	1.056	3.281	0.032
DNMT3A mutation	1.803	1.076	3.021	0.025
Age > 60 years old	1.757	1.213	2.544	0.003
Favorable karyotype	0.56	0.319	0.982	0.043
Allo-HSCT at 1st CR	0.493	0.307	0.792	0.003
CEBPA double mutation	0.332	0.167	0.662	0.002

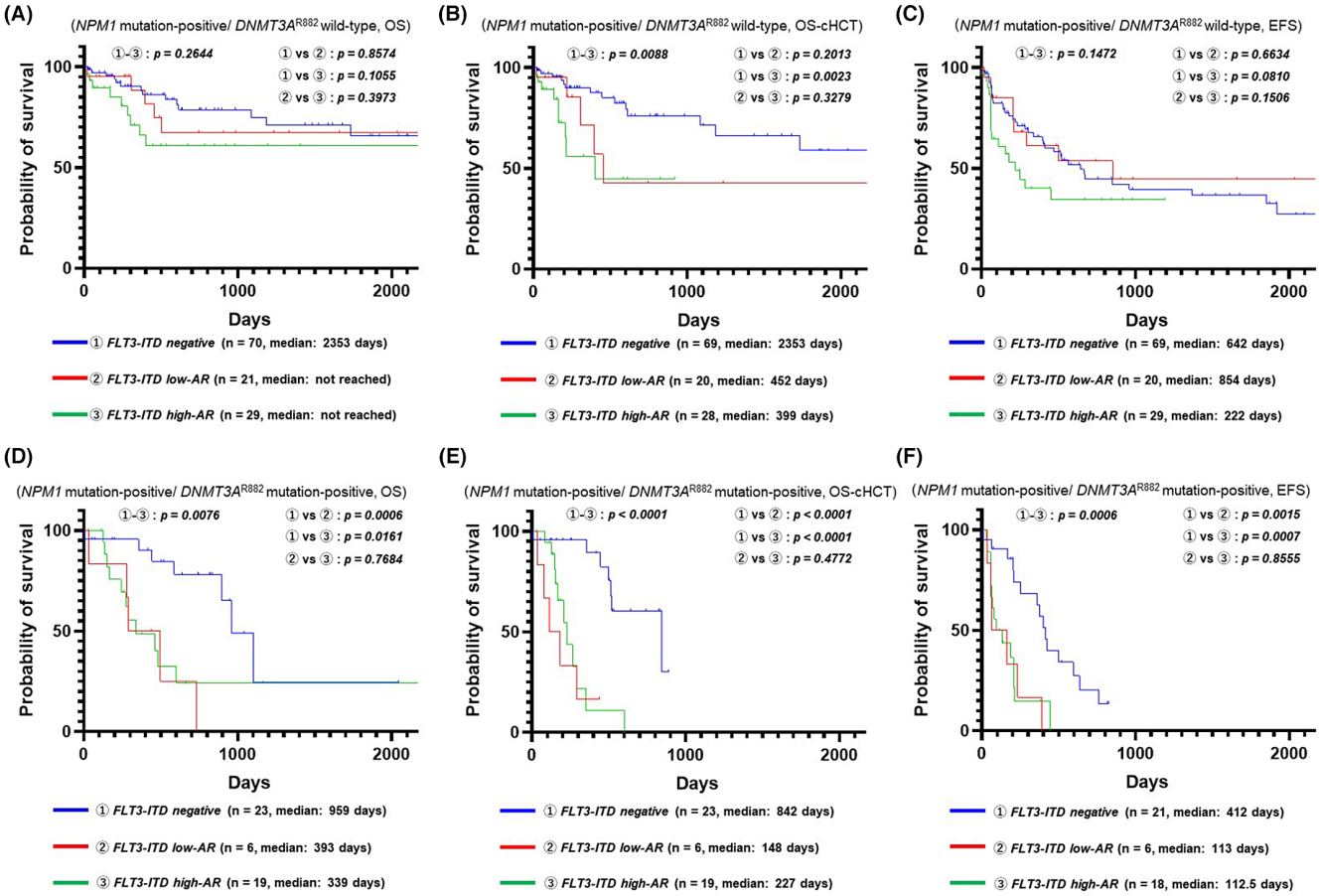

FIGURE 3 Effect of *FLT3*-ITD allelic ratio on the prognosis of *NPM1* mutation-positive/*DNMT3A*^{R882} wild-type acute myeloid leukemia (AML) and *NPM1* mutation-positive/*DNMT3A*^{R882} mutation-positive AML. (A) Kaplan–Meier plots (overall survival; OS) comparing *FLT3*-ITD subgroups for *NPM1* mutation-positive/*DNMT3A*^{R882} wild-type patients. (B) Kaplan–Meier plots (OS censored at allo-HSCT) comparing FLT3-ITD subgroups for NPM1 mutation-positive/DNMT3AR882 wild-type patients. (C) Kaplan–Meier plots (event-free survival; EFS) comparing FLT3-ITD subgroups for NPM1 mutation-positive/DNMT3AR882 wild-type patients. (D) Kaplan–Meier plots (OS) comparing FLT3-ITD subgroups for NPM1 mutation-positive/DNMT3AR882 mutation-positive patients. (E) Kaplan–Meier plots (OS censored at allo-HSCT) comparing FLT3-ITD subgroups for NPM1 mutation-positive/DNMT3AR882 mutation-positive patients. (F) Kaplan–Meier plots (EFS) comparing FLT3-ITD subgroups for NPM1 mutation-positive/DNMT3AR882 mutation-positive patients

TABLE 2 Risk stratification by *DNMT3A*^{R882} mutation/*NPM1* mutation/*FLT3*-ITD allelic ratio genotypes

NPM1 mutation[a]	*DNMT3A*^{R882} mutation	*FLT3*-ITD	Patient number (HSCT at 1st CR)	2-year OS	2-year OS censored at HSCT	2-year EFS	ELN 2017	GS-JAML (2022)
Positive	Negative	Negative	73 (4)	78.7%	76.0%	44.8%	Favorable	Favorable
		Low AR	21 (10)	67.4%	42.8%	53.6%	Favorable	Favorable[b]
		High AR	29 (13)	60.9%	44.8%	34.3%	Intermediate	Intermediate
	Positive	Negative	23 (4)	78.2%	60.4%	20.5%	Favorable	Intermediate
		Low AR	6 (2)	25.0%	16.7%	0.0%	Favorable	Adverse
		High AR	19 (4)	24.3%	0.0%	0.0%	Intermediate	Adverse

[a]*TP53* mutation was detected in one patient with normal karyotype/*NPM1* mutation positive/*FLT3*-ITD negative/*DNMT3A*^{R882} mutation positive and in one patient with normal karyotype/*NPM1* mutation positive/*FLT3*-ITD negative/*DNMT3A* mutation negative. Furthermore, a complex karyotype was detected in one patient with *NPM1* mutation positive/*FLT3*-ITD high-AR/*DNMT3A* mutation negative. These patients are included in the *NPM1* mutation-positive AML analysis data.

[b]The *NPM1* mutation positive/*DNMT3A*^{R882} negative/*FLT3*-ITD low-AR group exhibited a short survival in the analysis of OS censored at HSCT, and allogeneic HSCT should be actively indicated when FLT3 inhibitors are not used.

3.7 | Prognostic model for *NPM1*-mutated AML

We attempted to modify the conventional prognostic stratification system using the *FLT3*-ITD status for *NPM1*-mutated AML. Table 2 shows a newly modified risk classification, as well as the 2-year OS, OS censored at allo-HSCT, and EFS of each subgroup, sorted based on the *DNMT3A*^{R882} mutation/*NPM1* mutation/*FLT3*-ITD AR genotype. As with the ELN 2017 classification, we classified *DNMT3A*^{R882} wild-type patients into a favorable prognosis group if they were *FLT3*-ITD negative or had a low AR and into an intermediate prognosis group if they had a high AR. Meanwhile, we classified *DNMT3A*^{R882} mutation-positive patients into an intermediate prognosis group if they were *FLT3*-ITD negative and into a poor prognosis group if they were *FLT3*-ITD positive, regardless of their low- or high-AR status. Notably, there were only six patients in the poor prognosis group (i.e., patients with triple-mutated AML) of the 25 patients who underwent transplantation during the first CR. The 19 patients who did not undergo transplantation comprised a higher number of patients who were resistant to the initial treatment or had an early relapse; thus, the low rate of transplantation seemed to reflect unsuccessful bridging therapy to the transplantation.

Figure 4A–C shows the Kaplan–Meier plots when patients with *NPM1*-mutated AML were stratified according to the ELN 2017 classification (OS: Figure 4A; OS censored at allo-HSCT: Figure 4B, EFS: Figure 4C), whereas Figure 4D–F shows the Kaplan–Meier plots when patients with *NPM1*-mutated AML were stratified according to the new system that was reorganized based on information of the GS-JAML cohort (OS: Figure 4D; OS censored at allo-HSCT: Figure 4E; EFS: Figure 4F). We found that the new stratification system could predict the recurrence of *NPM1*-mutated AML more clearly than the ELN 2017 classification and succeeded in identifying the patients with a poor prognosis.

4 | DISCUSSION

In this study, we investigated the effect of *NPM1* mutations on the prognosis of 605 patients with AML, aged ≤70 years, who underwent standard induction therapy. Although patients with *NPM1*-mutated AML showed a high CRR, this was not a factor predicting a favorable prognosis in terms of OS, in contrast with that shown in previous studies and key guidelines. Furthermore, we demonstrated that *DNMT3A*^{R882} mutations were an independent factor for poor prognosis; when confounding factors that include *DNMT3A*^{R882} mutations were excluded, *NPM1* mutations were a strong factor predicting a favorable prognosis. As *DNMT3A* mutations frequently occur in patients with *NPM1* mutations and *FLT3*-ITD, which are strong predictive factors of prognosis, it is often affected by Simpson's Paradox in univariate analysis, which presumably explains how its effects on prognosis and its importance are underestimated.[24] In addition, the stratification of prognosis by ITD-AR introduced in the ELN 2017 classification has resulted in the subclassification of *NPM1*-mutated AML, obscuring the value of *DNMT3A* mutations in *NPM1*-mutated AML. The results of this study demonstrate that *DNMT3A* mutations are a factor indicative of a poor prognosis even with stratification based on ITD-AR, proving its clinical significance.

Moreover, the results of this study call attention to the importance of *DNMT3A* mutations in our Japanese cohort. Interestingly, JALSG conducted a comprehensive gene mutation analysis of 197 patients with de novo AML who were registered in the JALSG AML201 in 2014. *NPM1* mutations were not identified as a prognostic factor in this analysis, either in univariate or multivariate analyses in which abundant gene data were inputted.[20] Moreover, they demonstrated that the prognosis of *NPM1*-mutated AML patients could be more clearly stratified by including the *DNMT3A* mutation status compared with that with the original ELN system. These findings were in agreement with those of our study. The fact that JALSG, which is the largest prospective cohort study group in Japan, and our study, which is the largest retrospective cohort study conducted in Japan, were in agreement is particularly important. The current study and the JALSG study indicated that ethnological prognostic discrepancies in *NPM1* mutations might be corrected by stratifying prognosis based on the *DNMT3A* status.

We also highlighted the importance of *DNMT3A*^{R882} mutations as a factor predicting a poor prognosis in *NPM1*-mutated AML. However, we did not manage to establish the clinical significance of *DNMT3A*$^{non\text{-}R882}$ mutations. An analysis of the OS for *NPM1*-mutated AML showed that the *DNMT3A*$^{non\text{-}R882}$ mutation-positive group had an intermediate prognosis between that of the *DNMT3A* wild-type and that of *DNMT3A*^{R882} mutation-positive group, and analysis of OS for *NPM1* wild-type patients with normal karyotypes showed a tendency for a poor prognosis, similar to that observed in the *DNMT3A*^{R882} mutation-positive group. Previously, the AML Study Group (AMLSG) analyzed 1770 patients with de novo AML and reported that, among *DNMT3A* mutations, *DNMT3A*^{R882} mutations were often detected in AML with a normal karyotype and *NPM1*-mutated AML and were factors indicative of a poor prognosis related to OS. In contrast, *DNMT3A*$^{non\text{-}R882}$ mutations had a low detection frequency, overlapped with chromosomal abnormalities, and had little association with the prognosis of *NPM1*-mutated AML patients.[19] This reported difference in clinical significance depending on the site of the *DNMT3A* mutation is in good agreement with our findings.

We would like to propose a rearrangement of the genetic risk stratification that includes *DNMT3A* mutations. Recently, Herold et al. analyzed the long-term follow-up data of the target cohort of ELN 2017 by adding an extension cohort and reported that *DNMT3A* mutations were associated with a worse OS and relapse-free survival in each stratified risk group according to *NPM1*/*FLT3*-ITD genotypes.[25] The importance of *DNMT3A* mutations has been reassessed as a factor predicting a poor prognosis. Based on our results, we created a newly modified risk classification. This new prognostic model clarifies the importance of *DNMT3A*^{R882} mutations and emphasizes the importance of triple-mutated AML with duplicate *FLT3*-ITD, *NPM1* mutations, and *DNMT3A* mutations. In recent years, several large cohort studies have reported a poor

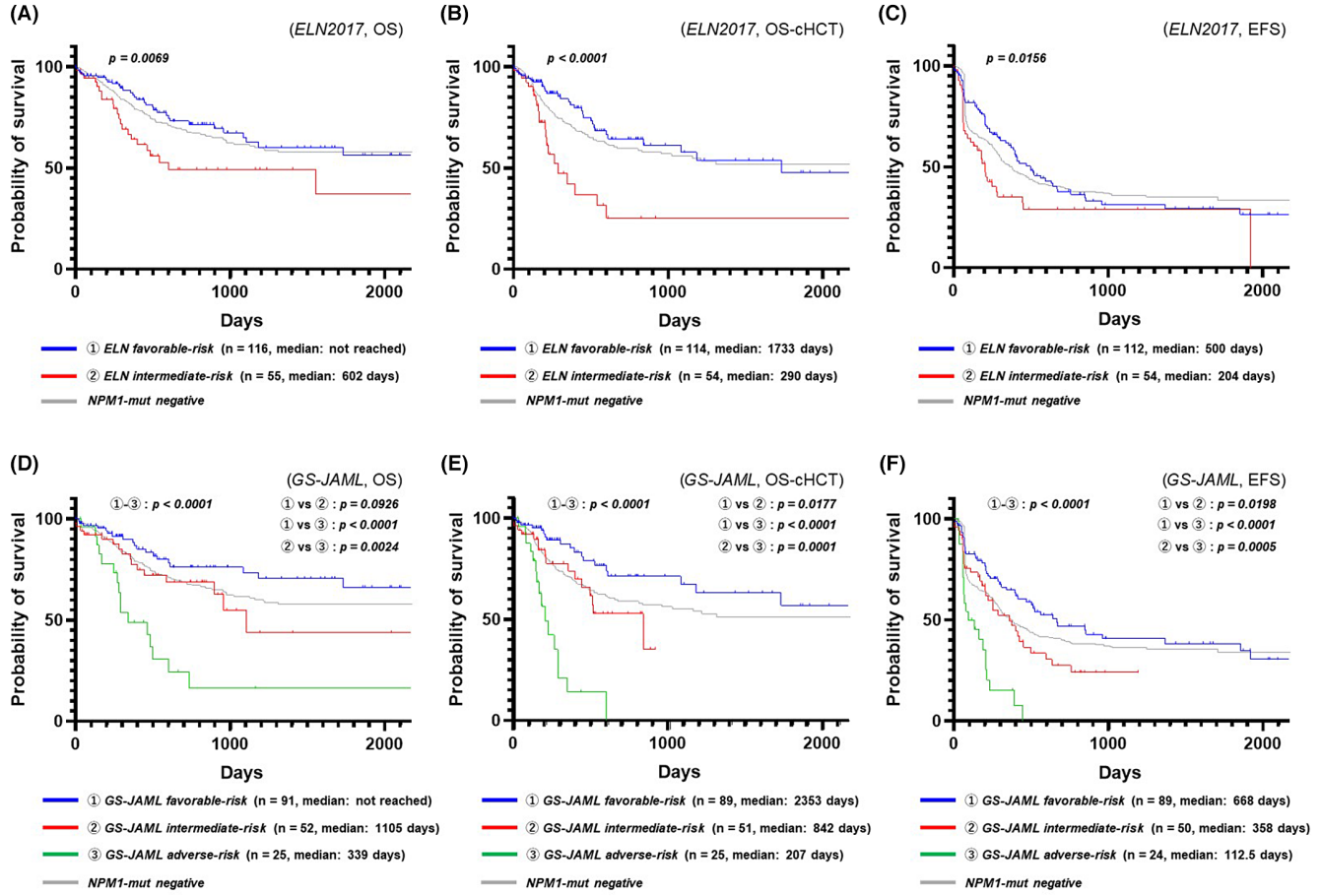

FIGURE 4 Prognostic model based on *DNMT3A*^{R882} mutation/*NPM1* mutation/*FLT3*-ITD allelic ratio genotypes. (A) Prognostic stratification using *NPM1*/*FLT3*-ITD genotypes (overall survival; OS) according to the European LeukemiaNet 2017 (ELN 2017) classification of *NPM1*-mutated acute myeloid leukemia (AML). (B) Prognostic stratification using *NPM1*/*FLT3*-ITD genotypes (OS censored at allo-HSCT) according to the ELN 2017 classification of *NPM1*-mutated AML. (C) Prognostic stratification using *NPM1*/*FLT3*-ITD genotypes (event-free survival; EFS) according to the ELN 2017 classification of *NPM1*-mutated AML. (D) Prognostic stratification using DNMT3AR882/NPM1/FLT3-ITD genotypes (OS) according to the multicenter collaborative program for gene sequencing of Japanese AML (GS-JAML) classification of NPM1-mutated AML. (E) Prognostic stratification using DNMT3AR882/NPM1/FLT3-ITD genotypes (OS censored at allo-HSCT) according to the GS-JAML classification of NPM1-mutated AML (F) Prognostic stratification using DNMT3AR882/NPM1/FLT3-ITD genotypes (EFS) according to the GS-JAML classification of NPM1-mutated AML

prognosis in triple-mutated AML.[2,26–28] Our study further supports these studies. The recently released ELN 2022 risk classification did not adopt the *DNMT3A* mutation as a poor prognostic factor, because it might have been thought that the efficacy of an FLT3 inhibitor combined with chemotherapy must have overcome the poor prognosis associated with the triple-mutant type.[29] The Ratify trial revealed that midostaurin, as an adjunct to daunorubicin-cytarabine induction and high-dose cytarabine consolidation therapy, improved the long-term survival rate.[30] Based on this result, it has become standard to incorporate the FLT3 inhibitor midostaurin into first-line therapy for patients with *FLT3*-mutated AML. Certainly, combination chemotherapy using FLT3 inhibitors and subsequent bridging to allo-HSCT might be the key to treatment success for triple-mutated AML that is treatment refractory in the early treatment phase, but there is no evidence that FLT3 inhibitors can improve the prognosis of triple-mutated AML to date. In fact, considering that the prognosis of triple-mutated AML is extremely poor, as reflected by the data of this study, the use of FLT3 inhibitors would most likely also result in inadequate therapeutic outcomes, hence implying the importance of thoroughly validating the recommendation proposed in the ELN 2022 guidelines. Early prediction using subsequent molecular responses to *NPM1* mutations can help to develop improved strategies for the treatment of *NPM1*-mutated AML harboring *DNMT3A* mutations. Recently, Onate et al. analyzed 164 patients diagnosed with *NPM1*-mutated AML enrolled in the Spanish Cooperative Group for the Diagnosis and Treatment of Acute Myeloid Leukemia and Myelodysplastic Syndromes (CETLAM) protocols. The authors reported that *DNMT3A* mutations did not modify the prognostic value of *FLT3*-ITD AR in *NPM1*-mutated AML, although patients with the *DNMT3A* mutation had a worse clearance of measurable residual disease (MRD) for *NPM1* mutation.[31] The adverse effect of the *DNMT3* mutation may be counteracted by a closer follow-up of *NPM1*-mutation MRD and a pre-emptive therapeutic intervention.

In conclusion, we showed that *DNMT3A*^{R882} mutations are an independent factor that can predict a poor prognosis; when confounding factors that include *DNMT3A*^{R882} mutations were excluded, *NPM1* mutations were a strong factor associated with a favorable prognosis. These findings indicate that ethnological prognostic discrepancies in *NPM1* mutations might be corrected by stratifying prognosis based on the *DNMT3A* status. In addition, the triple-mutant disease was associated with an inferior response to early-phase AML therapy, which could provide new insights for individualized AML chemotherapy.

AUTHOR CONTRIBUTIONS

Satoshi Wakita and Atsushi Marumo were the principal investigators and take primary responsibility for the paper. Hiroki Yamaguchi designed the study and supervised the experiments; Satoshi Wakita and Atsushi Marumo designed and performed the experiments, interpreted the data, and wrote the manuscript; Kaoru Morita, Shinichi Kako, Takashi Toya, Yuho Najima, Noriko Doki, Junya Kanda, Junya Kuroda, Shinichiro Mori, Kensuke Usuki, Toshimitsu Ueki, Nobuhiko Uoshima, Yutaka Kobayashi, Eri Kawata, Kazutaka Nakayama, Yuhei Nagao, Katsuhiro Shono, Motoharu Sibusawa, Jiro Tadokoro, Masao Hagihara, Hitoji Uchiyama, Naoyuki Uchida, Yasushi Kubota, Shinya Kimura, Hisao Nagoshi, Tatsuo Ichinohe, Saiko Kurosawa, Sayuri Motomura, Akiko Hashimoto, Hideharu Muto, Eriko Sato, Masao Ogata, Kenjiro Mitsuhashi, Jun Ando, Haruko Tashiro, Takahiro Fukuda, and Yoshinobu Kanda contributed to patient recruitment and data extraction; Kunihito Arai, Tomoaki Kitano, Miho Miyata, Haruka Arai, Masayuki Kanda, and Kako Itabashi performed the laboratory work for the study; Masahiro Sakaguchi and Shunsuke Yui interpreted molecular data and performed statistical analysis. All authors reviewed and approved the manuscript.

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CONFLICT OF INTEREST

No authors have a financial interest with respect to the conduction or reporting of the study. S. Kimura is an editorial board member of *Cancer Science*.

ETHICS STATEMENT

Approval of the research protocol by an Institutional Reviewer Board: This study was reviewed and approved by the Human Subjects Institutional Review Board (project approval number 29-07-783) of the Nippon Medical School (Tokyo, Japan).

Informed Consent: Informed consent was obtained in accordance with the Declaration of Helsinki from all participants.

Registry and the Registration No. of the study/trial: N/A.

Animal Studies: N/A.

ORCID

Satoshi Wakita https://orcid.org/0000-0002-5810-5433
Yuho Najima https://orcid.org/0000-0001-8910-0021
Junya Kanda https://orcid.org/0000-0002-6704-3633
Junya Kuroda https://orcid.org/0000-0001-6130-1550
Kensuke Usuki https://orcid.org/0000-0002-1216-4470
Masao Hagihara https://orcid.org/0000-0002-7143-4813
Shinya Kimura https://orcid.org/0000-0003-1717-6208
Sayuri Motomura https://orcid.org/0000-0002-6960-0800

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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