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DNMT3A R882 Mutation with *FLT3*-ITD Positivity Is an Extremely Poor Prognostic Factor in Patients with Normal-Karyotype Acute Myeloid Leukemia after Allogeneic Hematopoietic Cell Transplantation

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The prognostic relevance of epigenetic modifying genes (*DNMT3A*, *TET2*, and *IDH1/2*) in patients with acute myeloid leukemia (AML) has been investigated extensively. However, the prognostic implications of these mutations after allogeneic hematopoietic cell transplantation (HCT) have not been evaluated comprehensively in patients with normal-karyotype (NK)-AML. A total of 115 patients who received allogeneic HCT for NK-AML were retrospectively evaluated for the *FLT3*-ITD, *NPM1*, *CEBPA*, *DNMT3A*, *TET2*, *IDH1/2*, *WT1*, *NRAS*, *ASXL2*, *FAT1*, *DNAH11*, and *GATA2* mutations in diagnostic samples and analyzed for long-term outcomes after allogeneic HCT. The prevalence rates for the mutations were as follows: *FLT3*-ITD positivity (*FLT3*-ITD^{pos}) (32.2%), *NPM1* mutation (43.5%), *CEBPA* mutation (double) (24.6%), *DNMT3A* mutation (*DNMT3A*^{mut}) (31.3%), *DNMT3A* R882^{mut} (18.3%), *TET2* mutation (8.7%), and *IDH1/2* mutation (16.5%). The 5-year overall survival (OS) and event-free survival (EFS) rates were 57.3% and 58.1%, respectively. A multivariate analysis revealed that *FLT3*-ITD^{pos} (hazard ratio, [HR], 2.23; $P = .006$) and *DNMT3A* R882^{mut} (HR, 2.74; $P = .002$) were unfavorable prognostic factors for OS. In addition, both mutations were significant risk factors for EFS and relapse. People with *DNMT3A* R882^{mut} accompanied by *FLT3*-ITD^{pos} had worse OS and EFS, and higher relapse rates than those with the other mutations, which were confirmed in a propensity score 1:2 matching analysis. These results suggest that *DNMT3A* R882^{mut}, particularly when accompanied by *FLT3*-ITD^{pos}, is a significant prognostic factor for inferior transplantation survival outcome by increasing relapse risk, even after allogeneic HCT.

INTRODUCTION

DNA methylation is a critical process in development, differentiation, genomic stability, X-inactivation, and imprinting, as it regulates the expression of specific genes [1,2]. Novel recurrent mutations involved in DNA methylation have been detected in patients with acute myeloid leukemia (AML) [3,4]. Transfer of the methyl group from S-

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adenosyl-methionine to cytosine is catalyzed by several DNA methyltransferases (DNMTs) during cell replication [2]. *DNMT3A* belongs to a family of DNMTs, including *DNMT1*, *DNMT3A*, and *DNMT3B*. *DNMT3A* is specifically involved in de novo DNA methylation and functions independently during replication [1,5]. *DNMT3A* mutation (*DNMT3A*^{mut}) is observed in 14% to 34% of patients with AML and in 23% to 37% of those with normal-karyotype (NK) AML [1,6-8]. *DNMT3A*^{mut} have also been found in patients in relapsed states, suggesting that these mutations occur early in clonal evolution [1,9-14].

Other epigenetic modifiers that can be mutated in AML include ten-eleven-translocation oncogene family member 2 (*TET2*) and isocitrate dehydrogenase 1/2 (*IDH1/2*). *TET2* demethylates DNA, and *TET2* mutations are leukemogenic in animal models [15]. A *TET2* mutation (*TET2*^{mut}) occurs in 7% to 23% of patients with AML [16-19]. *IDH1/2* are enzymes in the citric acid cycle that catalyze conversion of isocitrate to α-ketoglutarate (α-KG) [3,20]. These mutations confer a neomorphic gain of function to convert α-KG to 2-hydroxyglutarate [4,21]. The frequency of *IDH1* mutation (*IDH1*^{mut}) is 5.5% to 14.0% and that of *IDH2* mutation (*IDH2*^{mut}) is 8.7% to 19.0% in patients with AML [22]. *TET2* catalytic activity depends on α-KG, indicating that *IDH1/2* mutations (*IDH1/2*^{mut}) result in loss of *TET2* function [18], which may explain why *IDH1/2*^{mut} are mutually exclusive with *TET2*^{mut} [3,18].

Cytogenetic and molecular markers have been adopted to enable prognostic stratification of AML and guide the selection of further treatment between consolidative conventional chemotherapy and allogeneic hematopoietic cell transplantation (HCT) [23]. Although mutations in the epigenetic modifying genes (*DNMT3A*, *TET2*, and *IDH1/2*) have been extensively investigated for their prognostic role in patients with AML, their prognostic implications are controversial. A meta-analysis reported that the *DNMT3A*^{mut} or *TET2*^{mut} negatively affects the prognosis of patients with NK-AML and that the prognostic implications of *IDH1/2*^{mut} in patients with NK-AML are unclear [8,22,24]. However, the studies used for this meta-analysis included different patient cohorts with a variety of cytogenetic risk groups. Several studies have shown that fms-related tyrosine kinase 3-internal tandem duplication (*FLT3*-ITD) positivity (*FLT3*-ITD^{pos}) adversely affects treatment outcome in patients with AML after allogeneic HCT because of a higher risk of early relapse [25-27]. However, the prognostic implications of mutations in epigenetic-modifying genes after allogeneic HCT have not been examined in patients with NK-AML.

Herein, we evaluated the prognostic relevance of epigenetic-modifying gene mutations (*DNMT3A*, *TET2*, and *IDH1/2*) after allogeneic HCT in patients with NK-AML in the context of survival and relapse risk.

PATIENTS AND METHODS

Patients and Treatment

The enrolled patients were diagnosed from October 1998 to September 2012 at 7 institutions that had diagnostic archived samples available for genetic study. The patient diagnostic DNA samples were routinely archived regardless of cytogenetic results. Patients with NK-AML were retrospectively selected before selecting only those patients who received initial induction chemotherapy using a standard protocol (3-day course of anthracycline with a 7-day course of cytosine arabinoside) and achieved first complete remission (CR1) before allogeneic HCT. Patients who only received consolidative chemotherapy were excluded. The selection process is illustrated in Figure S1.

A total of 115 patients were included in the analysis. Molecular genetic information was not used to determine the allogeneic HCT treatment modality. Patients who achieved CR received consolidation chemotherapy until an available donor was identified. Before 2006, HLA typing at low resolution was used for HLA-A, -B, -C, and -DRB1, whereas high-resolution HLA typing was used after 2006. Sibling donors with HLA-A, -B, -C and -DRB1 8/8—matched related donors were defined as *matched related donors*. For unrelated transplantation, HLA-A, -B, -C, and DRB1 8/8—matched unrelated donors were defined as matched unrelated donors, and transplantation from HLA-A, -B, -C, and -DRB1 7/8 or 6/8—matched unrelated donors was defined as *mismatched unrelated donors*. The conditioning regimens were classified as *myeloablative* if total body irradiation $>$ 8 Gy (n = 22), oral busulfan $\geq$ 9 mg/kg, or intravenous busulfan $\geq$ 7.2 mg/kg (n = 67) was included in the conditioning regimen, whereas other conditioning regimens were classified as *reduced-intensity conditioning regimens*. Informed consent for genetic analyses of the samples taken at the time of diagnosis was obtained from all subjects. This study was approved by the institutional review board of Chonnam National University, Hwasun Hospital, Korea.

Gene Mutation Analyses

Cryopreserved bone marrow or peripheral blood samples taken at diagnosis were archived, and genomic DNA was extracted using QIAamp DNA blood mini-kits (Qiagen, Valencia, CA), following the manufacturer's protocol. The mutation analysis was performed using Sanger sequencing and a PCR methodology. *TET2*, *FLT3*-ITD, and nucleophosmin 1 (*NPM1*) mutation testing was performed as described previously [28,29]. *TET2* missense mutations were included in the analysis only when they were located within 1 of 2 evolutionarily conserved domains (amino acids 1104 to 1478 or 1845 to 2002); identical *TET2* mutations in both alleles were defined as homozygous [30,31]. The *DNMT3A*, Wilms tumor gene 1 (*WT1*), neuroblastoma RAS viral oncogene homolog (*NRAS*), additional sex combs like transcriptional regulator 2 (*ASXL2*), and *IDH1/2* mutation analyses were performed as reported previously [30-36]. CCAAT/enhancer binding protein α (*CEBPA*), FAT Atypical Cadherin 1 (*FAT1*), dynein axonemal heavy chain 11 (*DNAH11*), and guanine-adenine-thymine-adenine 2 (*GATA2*) were amplified by genomic PCR; overlapping PCR products covering the entire coding sequence were generated and sequenced using the PCR primers shown in Table S1. Amplification featured initial denaturation at 95°C for 5 minutes, followed by 40 cycles of 94°C for 30 seconds, 62°C for 30 seconds, and 72°C for 1 minute, and a final extension at 72°C for 10 minutes. The amplification products were sequenced on an ABI 3100 platform using a cycle sequencing kit (BigDye Terminator, Applied Biosystems, Foster City, CA).

Response and Survival Endpoints

The definition of complete remission (CR) followed recommended criteria [37]. *Relapse incidence* (RI) was defined as the time from the date of allogeneic HCT to the date of relapse in all patients, considering competing risks of death without relapse. *Nonrelapse mortality* (NRM) was defined as death occurring in the absence of relapse. *Event-free survival* (EFS) was defined as the time from the date of allogeneic HCT to the date of death from any cause or relapse, whichever occurred first. *Overall survival* (OS) was defined as the time from the date of allogeneic HCT to the date of last follow-up or death from any cause. Acute graft-versus-host disease (aGVHD) and chronic graft-versus-host disease (cGVHD) grading were recorded as described previously [38,39].

Statistical Analysis

Descriptive statistics are presented as frequencies with percentages for categorical variables and as medians with ranges for continuous variables. Clinical characteristics and treatment outcomes were compared with each mutation. The chi-square test was used to compare differences in the categorical data distributions and the Student *t*-test was used to evaluate differences between continuous variables.

EFS and OS were calculated using the Kaplan-Meier method, and differences among groups were compared using the log-rank test for the univariate analysis and the Cox's proportional hazard model for the multivariate analysis. cGVHD was treated as a non—time-dependent covariate. The incidences of relapse, NRM, aGVHD, and cGVHD were calculated using the cumulative incidence method, considering competing risks. Gray's test was used for univariate comparisons and the Fine-Gray test was used for multivariate comparisons [40]. A backward step-wise selection procedure was used for the final model construction in the multivariate analysis, in which covariates with *P* values $<$ .10 in the univariate analysis were included. *P* values $<$ .05 were considered to indicate significance. Hazard ratios (HRs) and 95% confidence intervals (CIs) were estimated using a predetermined reference risk unity value.

Significantly lower outcomes were noted in the group with the *FLT3*-ITD^{pos}/*DNMT3A* R882^{mut} when compared with those in the other groups. However, as expected, there was an imbalance in the pretransplantation characteristics between the 2 groups, which necessitated excluding the effects of other imbalanced confounding variables, except mutation status. Pretransplantation clinical factors were included in the propensity score