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CEBPA single mutation can be a possible favorable prognostic indicator in *NPM1* and *FLT3*-ITD wild-type acute myeloid leukemia patients with intermediate cytogenetic risk

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ABSTRACT

The aim of this study was to evaluate the prognostic impact of *CEBPA* single mutation in acute myeloid leukemia (AML) patients with intermediate cytogenetic risk. *CEBPA* single and double mutations were detected in 11 (9.7%) and 17 (15.1%) of 113 *NPM1* wild-type patients, but no *CEBPA* mutations were detected in a group of 44 *NPM1* mutated patients. Among patients with *NPM1*/*FLT3*-ITD wild-type, those with *CEBPA* double mutations ($P = 0.013$ and 0.007 for overall survival and relapse-free survival, respectively) or a single mutation ($P = 0.039$ and 0.020 for overall survival and relapse-free survival, respectively) demonstrated a favorable prognosis compared with *CEBPA* wild-type patients. Subsequent multivariate analysis confirmed the favorable prognostic impact of *CEBPA* single and double mutations. Despite the low statistical power of this study due to the small number of patients, our preliminary data suggest that *CEBPA* single mutation may be associated with favorable clinical outcomes in *NPM1*/*FLT3*-ITD wild-type AML patients with intermediate cytogenetic risk.

1. Introduction

Cytogenetic characteristics are generally accepted as the most important factors that determines the prognosis of patients with acute myeloid leukemia (AML) [1–5]. According to the recently published guidelines of the National Comprehensive Cancer Network (NCCN), approximately half of AML patients are at intermediate cytogenetic risk, as defined by the presence of +8, t(9;11)(p22;q23) and a normal karyotype [6]. In these patients, the prognosis depends on molecular aberrations and demographic findings, such as age and bone barrow (BM) blast proportions at diagnosis. Fms-related tyrosine kinase 3 internal tandem duplications (*FLT3*-ITD) and nucleophosmin (*NPM1*) mutations have been definitively established as molecular aberrations with prognostic value, and several recent studies have shown that other genetic mutations in AML patients also demonstrate prognostic value. These molecular aberrations include mutations in CCAAT/enhancer binding protein α (*CEBPA*) [7–14], isocitrate dehydrogenase (*IDH*) 1/2 [15–18], and DNA methyltransferase 3A (*DNMT3A*) [19,20].

CEBPA encodes a transcription factor that is essential for granulocyte development, and disruption of CEBPA blocks granulocyte maturation [7–14]. *CEBPA* mutations occur in 8–14% of patients with cytogenetically normal AML and are predominantly found in patients with *NPM1* wild-type [7–14]. Patients with *CEBPA* mutations are classified with double or single mutations. *CEBPA* double mutations most frequently involve a combination of N-terminal frameshift mutations and C-terminal in-frame mutations, and have been reported to confer a favorable prognosis in AML patients with normal karyotype [7–14]. *CEBPA* single mutation involves only the N- or C-terminal, and its prognostic value is currently under debate [7–14]. Most studies that have investigated the prognostic impact of *CEBPA* single mutation have reported that it is not an independent factor associated with favorable outcomes [7–14]. In addition, a recent large-scale study concluded that concurrent mutations occur more frequently with *CEBPA* single mutation than double mutations, and the clinical outcomes of *CEBPA* single mutation are not significantly different compared with *CEBPA* wild-type in *NPM1* wild-type AML patients [21]. However, a recent study has reported that *CEBPA* single mutation is an independent favorable prognostic factor in *NPM1* mutated, normal karyotype AML patients [22]. Although a *CEBPA* mutated AML is now classified as a provisional entity by the 2008 World Health Organization classification system [23], the prognostic significance of *CEBPA* single mutation still remains elusive and needs to be evaluated, particularly in association with *NPM1* mutations and other molecular aberrations such as *FLT3*-ITD.

We performed this retrospective study to evaluate the incidence and prognostic impact of *CEBPA* mutations in 157 AML patients with

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intermediate cytogenetic risk who were diagnosed and treated at a single center. In particular, we focused on the prognostic impact of *CEBPA* single mutation and its association with *NPM1* and *FLT3*-ITD mutations.

2. Materials and methods

2.1. Patients

We analyzed a total of 157 AML patients with intermediate cytogenetic risk who were diagnosed between January 2002 and August 2012 at Asan Medical Center (Seoul, Korea). All patients underwent *NPM1* mutation analysis, and our cohort included 113 *NPM1* wild-type and 44 *NPM1* mutated patients. PCR-based amplification and GeneScan analyses were performed on samples as screening test for the detection of *NPM1* mutations, and the results were confirmed by direct sequencing. The sensitivity of the GeneScan analysis was determined by assessing 10-fold serial dilution of the tumor cell lines, and sensitivity was determined to be 2.5%. All procedures used in this study were performed in accordance with the ethical standards of the committee on human experimentation at authors' institution.

Treatment strategies were administered according to NCCN guidelines [6]. All patients received standard induction chemotherapy that consisted of cytarabine and daunorubicin. This regimen included the continuous intravenous infusion of 200 mg/m^2/day cytarabine (100 mg/m^2/day for patients >60 years) on days 1–7 and 45 mg/m^2/day daunorubicin on days 1–3. Complete remission (CR) was defined as the presence of <5% blast cells and >20% cellularity in standardized BM aspirates after the first or second course of induction chemotherapy. Patients who failed to achieve CR but demonstrated partial remission received a second identical cycle of induction chemotherapy. Relapse was defined as the presence of >5% leukemic blasts in BM aspirates for patients who had previously achieved CR. When relapse occurred, patients received the same induction chemotherapy regimen that was used following the initial diagnosis. During the first CR state, patients assessed as better risk received post-remission therapy that consisted of high-dose cytarabine (patients < 60 years) or standard-dose cytarabine (patients > 60 years) followed by maintenance therapy. Patients assessed as intermediate or poor risk were preferably invited to receive allogeneic stem cell transplantation (SCT) depending on the patient's age and availability of a suitable donor. Although autologous SCT is mentioned as a treatment modality in the NCCN guidelines, autologous SCT was not performed in our cohort. Because *CEBPA* mutation data were not available at the time of patient treatment, the *CEBPA* mutation status did not affect the treatment options.

We analyzed the distributions of sex and age, French-American-British (FAB) classifications, laboratory findings at diagnosis (e.g., complete blood cell count, proportion of blasts in BM aspirates, karyotype) in all patients. We also determined the dates when allogeneic SCT was performed, CR was achieved, and relapse/death occurred. We also investigated the mutation results for *FLT3*-ITD and *FLT3* D835 by reviewing the electronic medical records. *FLT3*-ITD and *FLT3* D835 mutation analysis was performed using two different methods: (1) multiplex PCR using the Seeplex *FLT3* genotyping kit (Seegene, Seoul, Korea) for specimens obtained prior to 2011; or (2) GeneScan analysis followed by direct sequencing for specimens obtained after 2011. The sensitivity of the multiplex PCR kit was assessed using 10-fold serial dilutions of the quality control material (1000 copies/reaction), which was provided by the manufacturer, and estimated at 1% (10 copies/reaction). The sensitivity of the GeneScan analysis for the detection of *FLT3* mutations was the same as the *NPM1* mutation analysis (2.5%).

2.2. CEBPA mutation analysis

Direct sequencing was performed to analyze the *CEBPA* mutations, as previously described [13] (except for different primer sequences and PCR conditions that were used). Four sets of in-house-designed primers were used and their sequences were as follow: AF, 5′-CGC CAT GCC GGG AGA ACT CT-3′; AR, 5′-CTT CTC CTG CTG CCG GCT GT-3′; BF, 5′-GCC GCC TTC AAC GAC GAG TT-3′; BR, 5′-CTT GGC TTC ATC CTC CTC GC-3′; CF, 5′-CCG CTG GTG ATC AAG CAG GA-3′; CR, 5′-CCG GTA CTC GTT GCT GTT CT-3′; DF, 5′-CAA GGC CAA GAA GTC GGT GGA CA-3′; DR, 5′-CAC GGT CTG GGC AAG CCT CGA GAT-3′. The following PCR conditions were used: incubation at 94 °C for 5 min (initial denaturation), 34 cycles at 94 °C for 60 s (denaturation), 56 °C for 60 s (annealing), and 72 °C for 60 s (extension), followed by the final extension at 72 °C for 7 min. The Sequencher program (GeneCodes Corp., Ann Arbor, MI, USA) was used to align the derived and reference sequences (NM_004364.3). The identified mutations were described according to the guidelines of the Human Gene Variation Society (HGVS; http://www.hgvs.org).

2.3. Mutation analysis of IDH1/IDH2/DNMT3A R882

Direct sequencing was used to detect *IDH1*, *IDH2*, or *DNMT3A* R882 mutations. We focused on previously described mutation hotspots (codon 132 in exon 4 of *IDH1*, codon 140/172 in exon 4 of *IDH2*, codon 882 at exon 23 of *DNMT3A*) and designed the following primer sets for these regions: F, 5′-GAG AAG AGG GTT GAG GAG TTC A-3′ and R, 5′-TTC ATA CCT TGC TTA ATG GGT GT-3′ for *IDH1*; F, 5′-GCT GCA GTG GGA CCA CTA TT-3′ and R, 5′-GTG CCC AGG TCA GTG GAT-3′ for *IDH2*; F, 5′-CTG CCC TCT CTG CCT TTT CT-3′; R, 5′-TCT CCA TCC TCA TGT TCT TGG-3′ for *DNMT3A*. The PCR conditions were identical to those used to analyze the *CEBPA* mutations, except that different annealing temperatures were applied (60 °C for *IDH1*/*2* and 62 °C for *DNMT3A*).

2.4. Data analysis

A total of 157 patients were initially categorized as *NPM1* wild-type (n = 113) or *NPM1* mutated (n = 44). Patient demographics and molecular abnormalities were compared between two subgroups. Comparisons were subsequently performed to evaluate the association between the *CEBPA* mutation status and clinical findings and prognosis in the 113 *NPM1* wild-type patients. For this analysis, the patients were categorized into three subgroups: *CEBPA* wild-type (n = 85), *CEBPA* single mutation (n = 11), or *CEBPA* double mutations (n = 17). As preliminary prognostic endpoints, the CR, relapse, and death rates were compared between these three patient groups.

To further evaluate the prognostic impact of *CEBPA* mutations, comparisons of overall survival (OS) and relapse-free survival (RFS) were performed. Since the incidences of *CEBPA* mutations in patients with *NPM1* wild-type/*FLT3*-ITD mutation were only 8.7% (2 of 23 patients) and those in patients with *NPM1* mutated were 0% (0 of 44 patients), 90 patients with *NPM1*/*FLT3*-ITD wild-type were included in the survival analysis and categorized into the three subgroups with respect to *CEBPA* mutation status as follows: *CEBPA* wild-type (n = 64), *CEBPA* single mutation (n = 10), or *CEBPA* double mutations (n = 16). As the primary prognostic endpoints, the 1- and 2-year OS and RFS rates were calculated and compared between three subgroups. OS was defined as the time from diagnosis to death or the last follow-up. RFS was defined as the time from CR to relapse (for patients who experienced relapse), time from CR to death (for non-relapsed patients who did not survive), or time from CR to last follow-up (for non-relapsed patients who survived). Patients who received allogeneic SCT were censored from the survival analysis at the time of transplantation.

Finally, multivariate analysis of the OS and RFS was performed on a total of 157 patients to confirm the prognostic impacts of the two different types of *CEBPA* mutation. In this analysis, the *FLT3*-ITD mutation status, *NPM1* mutation status, age, and BM blast proportion were included as covariables.

2.5. Statistical analysis

Pearson Chi-square and Fisher exact tests (for small numbers less than 5 in each subgroups) were used to compare categorical variables between patient groups. Comparisons of the continuous variables were performed using the Student's t-test (between two subgroups) or analysis of variation (ANOVA, among three subgroups). Estimates of survival (OS and RFS) were generated using the Kaplan–Meier method and compared using the log-rank test. Multivariate analyses of OS and RFS were performed using a Cox's proportional hazards model. For all analyses, tests were two-tailed and P-values $\leq$0.05 was considered statistically significant. P-values between 0.05 and 0.1 were considered marginally significant. Statistical computations were performed using SPSS software (version 13.0.1 for Windows; SPSS Inc, Chicago, IL, USA).

3. Results

3.1. Comparison of the demographic and molecular abnormalities in the total study cohort of 157 AML patients with respect to NPM1 mutation status

The demographic findings were not significantly different between *NPM1* wild-type and *NPM1* mutated patients, with the exception of a female predominance (P = 0.002) and higher BM blast proportions at diagnosis (P = 0.006) in *NPM1* mutated patients. A normal karyotype was determined in 126 patients (80.3%), and trisomy 8 and t(9;11)(p22;q23) was determined in the remaining 19 and 12 patients, respectively. The incidence of a normal karyotype in *NPM1* wild-type patients was significantly lower than in *NPM1* mutated patients (75.2% vs. 93.2%; P = 0.013). However, allogeneic SCT performance and CR, relapse, and death rates did not differ significantly between our patient groups.

As expected, the molecular aberration profiles were found to be significantly different between two patient groups. *CEBPA* mutations were detected in 28 of 113 *NPM1* wild-type patients (24.8%). A single mutation was found in 11 patients (9.7%) and double mutations were found in 17 patients (15.1%). However, no *CEBPA* mutations were detected in 44 *NPM1* mutated patients. Single polymorphic variations (c.584_589dupACCCGC;