Concomitant *WT1* mutations predict poor prognosis in acute myeloid leukemia patients with double mutant *CEBPA*

Acute myeloid leukemia (AML) with double mutant CCAAT/enhancer binding protein α (*CEBPA*^{dm}) is a new entity in the 2016 World Health Organization (WHO) classification with unique biologic features and prognostic implications.[1,2] The incidence of *CEBPA*^{dm} ranges from 7.5% to 11% in AML.[1,3,4] *CEBPA*^{dm} AML patients, when treated with standard chemotherapy, achieve a high complete remission (CR) rate. However, relapse occurs in 40% of patients who attain CR.[1] This has raised the clinically relevant question whether concomitant genetic alterations influence the prognosis of *CEBPA*^{dm} patients. Apart from *GATA2*, the prognostic impact of other concomitant gene mutations is largely unsettled because limited patient numbers preclude informative analyses.[5] Given that AML is a heterogeneous disease, risk-adapted treatment may not only improve the prognosis, but also reduce toxicity from the therapy. Allogeneic hematopoietic stem cell transplantation (allo-HSCT) in first CR is not beneficial for cytogenetically normal AML (CN-AML) patients with *CEBPA*^{dm}.[6] If any concomitant mutations adversely affect the clinical outcome of *CEBPA*^{dm} patients, it will be interesting to know whether allo-HSCT should be performed for these patients. As yet, there is no data to answer this question.

In this study, the aim was to identify additional mutations in *CEBPA*^{dm} AML patients that conferred prognostic significance. Furthermore, we investigated the role of allo-HSCT in *CEBPA*^{dm} patients with concurrent adverse-risk mutations. Mutation analyses in *CEBPA* and 19 other relevant genes, including *FLT3*-ITD, *FLT3*-TKD, *NRAS, KRAS, KIT, PTPN11, RUNX1, GATA2, MLL/PTD, ASXL1, IDH1, IDH2, TET2, DNMT3A, SF3B1, SRSF2, U2AF1, NPM1, WT1*, and *TP53* were performed by Sanger sequencing for patients (n=500) diagnosed from 1994 to 2007.[7,8] For patients (n=256) diagnosed after 2008, Ion Torrent next generation sequencing (NGS) (Thermo Fisher Scientific, MA, USA) was performed. The *WT1* mutations detected by NGS were all confirmed by Sanger sequencing.

We identified 102 (13.5%) *CEBPA*-mutated patients from 756 patients with newly diagnosed *de novo* AML (*Online Supplementary Table S1*); 33 (4.4%) had *CEBPA* single mutation (*CEBPA*^{sm}) and 69 (9.1%), *CEBPA*^{dm}. Sixty-nine *CEBPA*^{dm} patients were found to have 109 distinct mutations (Figure 1A, *Online Supplementary Table S2*). All patients had a combination of one N-terminal and one *C*-terminal mutation. Most (53 of 56, 94.6%) of the N-terminal mutations were frame-shift mutations, while most (42 of 53, 79.2%) of the *C*-terminal mutations were in-frame mutations with internal tandem duplications clustered in the junction between the basic region and the leucine zipper.

CEBPA^{dm} patients were significantly younger and had higher hemoglobin levels at diagnosis than *CEBPA*^{sm} and *CEBPA* wild-type patients. All except one *CEBPA*^{dm} patient had intermediate-risk cytogenetics (P<0.0001) (Figure 1A). The most frequent intermediate-risk cytogenetic change was del(9) (n=4, 5.8%), and CN- AML occurred in 81.2% of *CEBPA*^{dm} patients (n=56).

Fifty (72.5%) of the *CEBPA*^{dm} patients had additional genetic alterations (*Online Supplementary Table S3*). Among them, 29 (58%) had one, 17 (34%) had two, 3 (6%) had three and 1 (2%) had four changes. The most common concurrent molecular event in *CEBPA*^{dm} patients was *GATA2* mutation (33.8%), followed by *FLT3*-ITD (14.5%), *NRAS* (14.5%), *TET2* (13.2%), and *WT1* (11.8%) mutations. *GATA2* was more frequently mutated in *CEBPA*^{dm} patients than in *CEBPA* wild-type patients (33.8% *vs.* 2.8%, P<0.0001). In contrast, *CEBPA*^{dm} patients less frequently harbored *NPM1, ASXL1, IDH2, DNMT3A* and *RUNX1* mutations (Figure 1B).

Survival analyses were restricted to 530 patients, including 62 *CEBPA*^{dm} patients and 468 others (22 with *CEBPA*^{sm} and 446 *CEBPA* wild-type), who received standard intensive chemotherapy. The CR rate was 90.2% for *CEBPA*^{dm} patients and 72.2% for others (P=0.003). In multivariate analysis, *CEBPA*^{dm} was an independent favorable prognostic factor for OS and DFS (RR 0.420, 95% CI 0.246-0.718, P=0.002 and RR 0.544, 95% CI 0.351-0.842, P=0.006, respectively, *Online Supplementary Table S4*). Of the 56 *CEBPA*^{dm} patients who achieved first CR, 10 received allo-HSCT and 46 had postremission chemotherapy alone. The reasons for frontline allo-HSCT were persistent residual leukemia cells in 4 patients, concurrent *FLT3*-ITD in 3 patients, initial hyperleukocytosis in 2 patients and complex cytogenetics in 1 patient. Intriguingly, the relapse rate was 45.7% in the postremission chemotherapy group and 0% in the allo-HSCT group (P=0.009). DFS was significantly better in

Table 1. Clinical characteristics and treatment outcome of *CEBPA*^{dm} patients with concomitant *WT1* mutations.

UPN	Age/Sex	WBC (k/uL)	Karyotype	*WT1* mutation aa change	Other mutations	Induction response	Relapse	Remission duration (months)	Outcome
24	55M	58.2	CN	P355C	–	CR1	+	9	HSCT at CR2, alive
29	53F	94.6	+21, -x	D377fsX384	–	CR1	+	11	death
32	45F	3.3	Complex	Y402X	–	CR1	–	91	HSCT at CR1, alive
50	59M	387.4	CN	R369G	–	CR1	+	7	death
54	40M	160.0	CN	K399fsX448	–	Refractory	NA	0	death
56	35M	248.0	CN	R458X	*FLT3*-TKD	CR1	+	7	HSCT at PR2, alive
62	28F	17.0	CN	K399fsX400	–	Refractory	NA	0	death
27	69M	227.7	NM	N381fsX450	*TET2*	NA[a]	NA	NA	NA

aa: amino acid; CN: cytogenetically normal; CR: complete remission; NA: not applicable; NM: no mitosis; HSCT: hematopoietic stem cell transplantation; PR: partial response; UPN: unique patient number. [a]UPN27 lost to follow up after diagnosis.

the allo-HSCT group (median, not reached (NR) *vs.* 59.4 months, *P*=0.023) than in the chemotherapy group, while OS was not different (*P*=0.247) (*Online Supplementary Figure S1*).

We further analysed the prognostic significance of concomitant gene mutations with a frequency above 10% in *CEBPA*^{dm} patients. WT1-mutated patients tended to have a lower CR rate (71.4% *vs.* 92.5%, *P*=0.14) and a higher relapse rate (80% *vs.* 34%, *P*=0.047) compared to those with wild-type *WT1* (*Online Supplementary Table S5*). With a median follow up of 69.7 months (range, 1.2-230 months), *WT1*-mutated patients had a significantly shorter OS and DFS than *WT1*-wild patients (median, 14 months *vs.* NR, *P*=0.021; 7.8 months *vs.* NR, *P*=0.008, respectively; Figure 1C). According to the 2017 European LeukemiaNet (ELN) classification, the AML patients were stratified into three risk groups (Figure 2A). Integration of *WT1* mutations could further divide the ELN favorable-risk cohort into three subgroups: *CEBPA*^{dm} *WT1*-mutated patients, *CEBPA*^{dm} *WT1*-wild patients and others. As shown in Figure 2B, *CEBPA*^{dm} patients with *WT1* mutations had worse outcome than other ELN favorable-risk patients. Sequential analyses of *WT1* mutations revealed that the mutations in three *WT1*-mutated patients in the study were lost at CR, but regained at relapse. The mutation burden could either increase or decrease at relapse. Of the 116 *WT1*-wild patients studied, three acquired a novel mutation at relapse (*Online Supplementary Table S6*).

Regarding other concomitant gene mutations, *GATA2* mutation was correlated with a trend of longer DFS (median, NR *vs.* 16.1 months, *P*=0.078). *FLT3*-ITD, *NRAS* and *TET2* mutations seemed not to have implications on the clinical outcome (*Online Supplementary Table S7* and *Online Supplementary Figure S2*).

To our knowledge, this is the first study to demonstrate the prognostic impact of concurrent *WT1* mutations on *CEBPA*^{dm} patients. *WT1* mutation occurs in 6-10% of AML patients and is associated with poor prognosis in CN-AML and non-selective AML patients.[9,10] Intriguingly, *WT1* mutations are frequently identified in *CEBPA*^{dm} patients.[11] We distinctly found that *WT1* mutations were associated with poor clinical outcome in *CEBPA*^{dm}

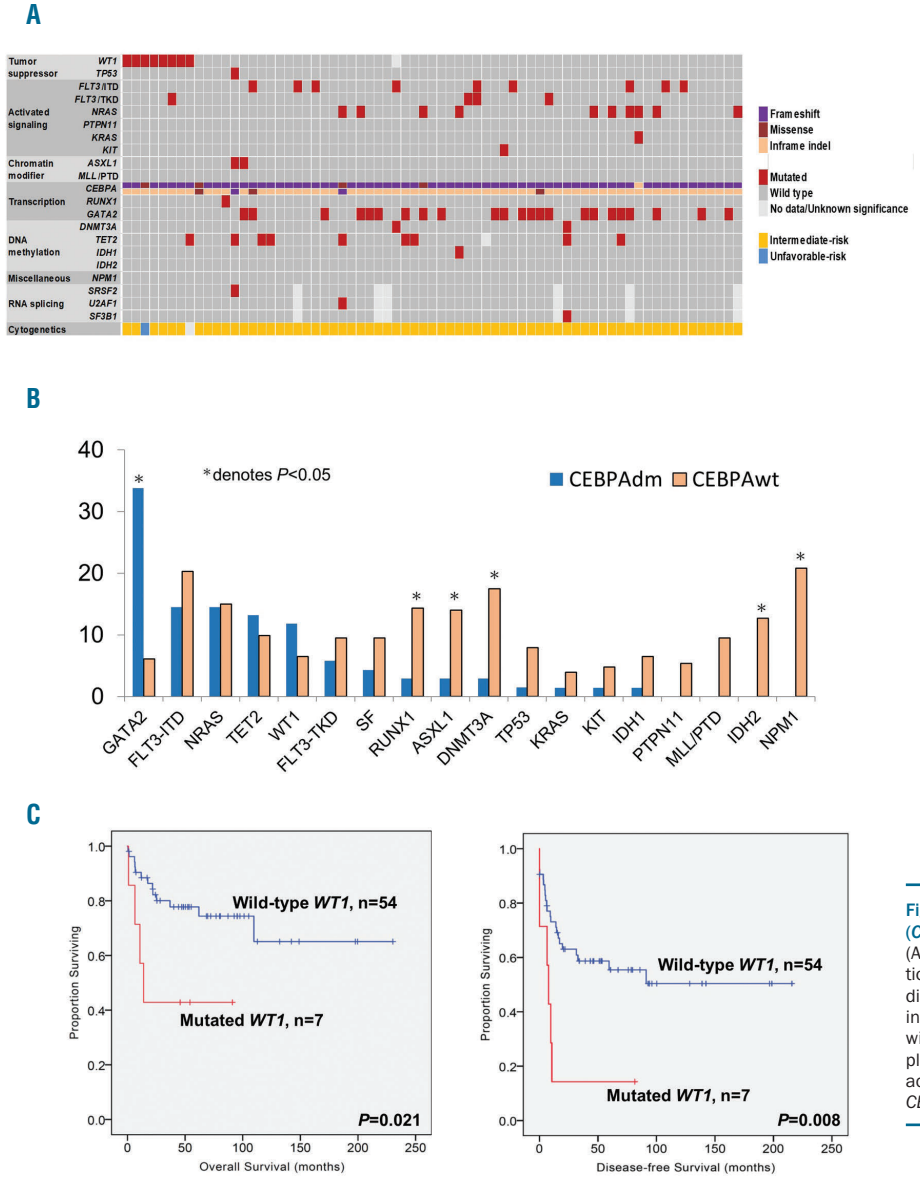

Figure 1. *CEBPA* double mutations (*CEBPA*^{dm}) in *de novo* AML patients. (A) The diagram of concurrent mutations in patients with *CEBPA*^{dm}. (B) The distribution of concomitant mutations in AML patients with either *CEBPA*^{dm} or wild-type *CEBPA*. (C) Kaplan-Meier plots for OS (left) and DFS (right) according to *WT1* mutation status in *CEBPA*^{dm} patients.

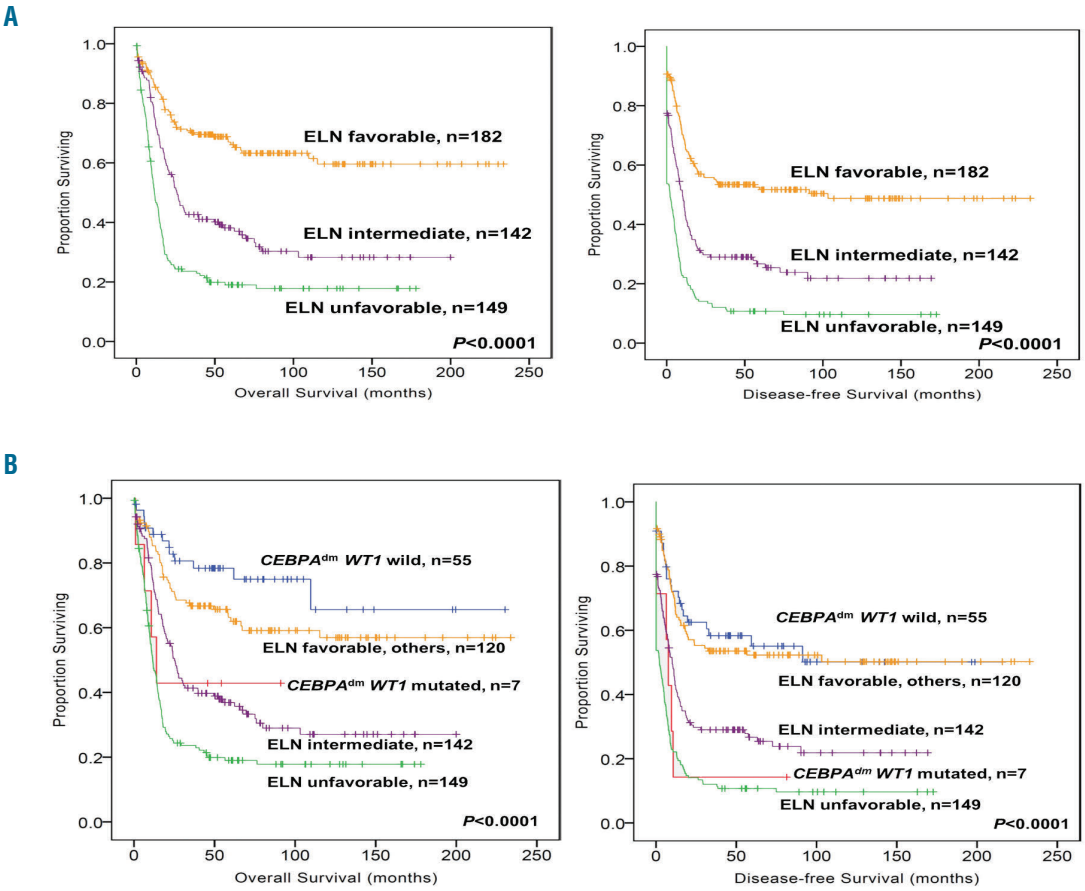

Figure 2. Risk stratification of the ELN favorable group according to the status of *CEBPA* and *WT1* mutations. (A) Kaplan-Meier plots for OS and DFS stratified by the 2017 ELN risk categories. (B) ELN favorable group could be further separated into three subgroups according to the status of *CEBPA* and *WT1* mutations. *CEBPA*^{dm} patients with concurrent *WT1* mutations had OS and DFS poorer than other ELN favorable-risk patients, but similar to those with the ELN intermediate (CR 76.8%, relapse rate 56.0%, median OS 26.0 months, median DFS 10.2 months) or unfavorable-risk category (CR 53.7%, relapse rate 61.3%, median OS 11.6 months, median DFS 2.1 months). ELN:European LeukemiaNet.

patients. Furthermore, we showed that integration of *WT1* mutations could refine the ELN risk stratification in favorable-risk subgroups. The prognostic impact of concomitant mutations in *CEBPA*^{dm} patients have not been widely assessed with the exception of *GATA2* and *TET2* mutations.[3,5,12] Grossmann *et al.* showed that the presence of *TET2* mutations correlated with worse survival. In contrast, we did not find the prognostic impact of *TET2* mutations in *CEBPA*^{dm} patients.

A high frequency of *TET2* co-mutation (around 34%) in *CEBPA*^{dm} patients was reported previously,[3,5] while it was only 13.2% in our study. The reason that our results were very different from those reported in other geographical areas might partly be explained by the difference in patient characteristics. The *CEBPA*^{dm} patients in the studies of Grossmann *et al.*[5] and Fasan *et al.*[3] were significantly older than ours (median age, 57.5 and 56.3 *vs.* 40 years). It is well documented that *TET2* mutations occur more frequently in elderly AML patients than younger ones[13] and this was reflected in the different prevalence of *TET2* mutations between our cohort and the other two. Furthermore, for *TET2* missense mutations, the missense mutations with unknown biologic significance were censored, which would possibly lead to lower frequency of *TET2* mutations in this study.[13] The ethnic difference might be another influencing factor. Recently, *CSF3R* mutation was found closely associated with *CEBPA* mutation in both adult and pediatric AML patients.[14] Unfortunately, *CSF3R* mutation was not included in our panel.

According to the current ELN guidelines, allo-HSCT is not routinely recommended in *CEBPA*^{dm} patients in first CR. Indeed, though postremission chemotherapy alone in first CR correlated with a significantly higher relapse rate and shorter DFS as compared with allo-HSCT, the high relapse rate in the chemotherapy subgroup did not translate into a significant inferior OS because relapsed patients still showed a high second CR rate.[6,15] The relapse rate of *CEBPA*^{dm} patients after first CR in this study was 37.5% in total *CEBPA*^{dm} patients and 45.7% in the postremission chemotherapy subgroup, which was comparable with that reported previously (36.2%-41%).[1,6] Surprisingly, all *WT1*-mutated *CEBPA*^{dm} patients, if not transplanted in first CR, encountered disease relapse (Table 1). The second CR rate was only 25% after re-induction, which was much lower than that (around 80%) in the total *CEBPA*^{dm} cohort.[6] Taken together, it is suggested that *CEBPA*^{dm} patients with *WT1* co-mutation

receive HSCT in first CR given the high relapse rate and gravid prognosis if relapse occurs. Further prospective randomized studies are warranted to validate the point.

This study clearly demonstrates the heterogeneous clinical outcome of *CEBPA*^{dm} patients and provides useful clinical information on refining the 2017 ELN risk categorization. Concomitant *WT1* mutations suffice to be a marker for dismal prognosis in *CEBPA*^{dm} patients and help in our understanding of the process of leukemogenesis in this group. More importantly, allo-HSCT in first CR may be indicated for long-term disease control of this poor-risk entity.

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