Heterogeneity within AML with CEBPA mutations; only CEBPA double mutations, but not single CEBPA mutations are associated with favourable prognosis

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CCAA/T enhancer binding protein alpha (CEBPA) mutations in AML are associated with favourable prognosis and are divided into N- and C-terminal mutations. The majority of AML patients have both types of mutations. We assessed the prognostic significance of single (n = 7) and double (n = 12) CEBPA mutations among 224 AML patients. Double CEBPA mutations conferred a decisively favourable overall (P = 0.006) and disease-free survival (P = 0.013). However, clinical outcome of patients with single CEBPA mutations was not different from CEBPA wild-type patients. In a multivariable analysis, only double – but not single – CEBPA mutations were identified as independent prognostic factors. These findings indicate heterogeneity within AML patients with CEBPA mutations.

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One of the crucial transcription factors for myeloid cell development is the CCAAT/enhancer binding protein alpha (CEBPA) (Rosenbauer and Tenen, 2007). Targeted disruption of CEBPA results in a selective block of granulocyte maturation (Zhang et al., 1997), whereas conditional expression of CEBPA in precursor cells is sufficient to trigger granulocytic differentiation (Radomska et al., 1998). In AML patients, deregulation of CEBPA function is a common event comprising of genomic mutations (Pabst et al., 2001a; Frohling et al., 2004; Nerlov, 2004), transcriptional and post-transcriptional suppression (Pabst et al., 2001b; Helbling et al., 2004; Helbling et al., 2005), and inactivation by phosphorylation (Radomska et al., 2006).

Two types of CEBPA mutations are predominantly seen: frameshift mutations in the N-terminal truncate the wild-type protein, whereas formation of a dominant-negative 30-kDa peptide initiated from an ATG further downstream is not affected. In contrast, C-terminal CEBPA mutations are in-frame insertions or deletions, thereby affecting DNA binding and homo- or heterodimerisation with other CEBP family members (Nerlov, 2004). The majority of AML patients with CEBPA mutations have both types of mutations, usually on different alleles. However, both types can occur as single CEBPA mutations.

Earlier study has indicated that CEBPA mutations in cytogenetically normal AML patients are associated with favourable prognosis (Frohling et al., 2004; Bienz et al., 2005; Gaidzik and Dohner (2008); Marcucci et al., 2008; Schlenk et al., 2008). Here, we assessed the prognostic significance of the different types of CEBPA mutations among a cohort of 224 consecutive AML patients of all subtypes. By direct sequencing, we identified 12 patients with double and 7 with single heterozygous CEBPA mutations. Patients with double CEBPA mutations represented the combination of C- and N-terminal mutation types. We found that favourable prognosis was exclusively associated with the double CEBPA mutation status, whereas the clinical outcome of patients with single CEBPA mutations did not differ from CEBPA wild-type patients.

MATERIALS AND METHODS

Patients

Malignant cells were collected at diagnosis from the Ficoll-separated mononucleated cells of bone marrow aspirates (132 patients) or peripheral blood (92 patients) from consecutive AML patients seen at the Department of Oncology, University Hospital, Bern, Switzerland between 2001 and 2007. Informed consent from all patients was obtained according to the Declaration of Helsinki, and the studies were approved by decisions of the local ethics committee of Bern, Switzerland. Patients were uniformly treated within the HOVON/SAKK 30/00 protocol.

CEBPA mutational analysis

The entire coding region of the CEBPA gene was amplified using three overlapping PCR primer pairs as described earlier (Bienz et al., 2005). Sequences of the primers are listed in Supplementary Table S1. PCR products were sequenced in both directions. Abnormal sequencing results were repeated twice in both directions, including repetitions of PCR.
Reported gene assays

H1299 cells were transfected with 80 ng of luciferase plasmid encoding an oligomeric CEBPA site, together with 20 ng of pcDNA3-CEBPA or empty pcDNA3 vector along with 1.0 ng of CMV-Renilla plasmid. After 24 h, luciferase activities were determined (Pabst et al, 2001a). Each transfection experiment was repeated at least three times.

Statistical Analysis

Patients alive without progression or relapse by the time of analysis were censored at the time of their last follow-up. Time-to-event curves were constructed according to the Kaplan–Meier analysis were censored at the time of their last follow-up. Time-to-event curves were constructed according to the Kaplan–Meier method and were compared with the log-rank $\chi^2$-test.

RESULTS AND DISCUSSION

By sequencing the entire coding region, we identified 19 patients with CEBPA mutations in our cohort of 224 AML patients at diagnosis. Of these 12 patients had the combination of the N- and the C-terminal type of mutation, further referred here as double CEBPA mutation, and 7 patients had a single CEBPA mutation. Patients with the in-frame insertion polymorphism in the second transactivation domain (Wouters et al, 2007), with base pair variation(s) that did not lead to amino acid changes, or with in-frame sequence variations of unknown significance (one patient), were not considered in the further analysis. All CEBPA mutations are presented in detail in Supplementary Tables S2A and S2B. Two patients with a single point mutation encoding a novel stop codon located downstream of the alternative ATG at position 120 were not of the classic N-terminal mutation type, as formation of the 30-kDa peptide was also affected, but they were classified as having single CEBPA mutations because of the functional relevance of these particular mutations.

We identified CEBPA mutations exclusively in patients below the age of 61 years at diagnosis. Thus, only AML patients in this age range were studied in the control group of CEBPA wild-type patients, allowing comparison of patients treated within the same protocol. We detected no differences between patients with single and double CEBPA mutations with regard to FAB subtypes, leukocytes at diagnosis, percentage of peripheral blasts at diagnosis, bone marrow infiltration at diagnosis, and LDH levels (Supplementary Table S3).

**Figure 1**

(A) Overall survival of AML patients without CEBPA mutations (wt; $n = 205$), with a single ($n = 7$), and with the combination of C- and N-terminal CEBPA mutations (double; $n = 12$). Patients who are alive were censored at the last follow-up. X-axis indicates months, Y-axis is probability of survival. (B) Disease-free survival of AML patients without (wt; $n = 205$), with a single ($n = 7$), or with double ($n = 12$) CEBPA mutations. (C) Transient transfection experiments in H1299 cells using equal amounts of pcDNA3 expression plasmids encoding human CEBPA wild-type (wt), the N-terminal frame-shift mutation 245delC (as present in patient #3s in Supplementary Table S2), the C-terminal in-frame mutation 1079–1080insTCT (as present in patient #5s), and the combination of both plasmids. V: pcDNA3 expression plasmid alone. The luciferase reporter construct encodes an oligomeric CEBPA site. (D) Western blot analyses for CEBPA protein using whole-cell lysates of patients #27 (AML-M1 with a normal karyotype and no abnormalities in CEBPA, FLT3, and NPM1), #3s (AML-M2 with the N-terminal frame-shift mutation 245delC), #11d (AML-M1 with both the N-terminal 213insAG mutation and the C-terminal 1088-1089insCCG mutations), and #5s (AML-M1 with the C-terminal in-frame mutation 1079–1080insTCT). (E) Schematic presentation of CEBPA wild-type protein (upper panel) and the 30-kDa peptide initiated at the ATG at amino acid 120 (lower panel). Black bars indicate the two transactivation domains, and grey bars represent the region for DNA binding and homo-/heterodimerisation.
Remarkably, additional molecular abnormalities were exclusively detected in the single CEBPA mutation group, including one patient with FLT3-ITD and one with NPM1 mutation. All patients with CEBPA mutations had a normal karyotype with the exception of one patient with monosomy 7 in the single CEBPA mutation group and one with del6q24 in the double CEBPA mutation group. Results of karyotype and molecular analyses are given in Supplementary Table S4.

Comparing patients with single vs double CEBPA mutations, we found that the clinical outcome differed markedly (Supplementary Table S5). A complete remission after induction chemotherapy was achieved in all the 19 AML patients with CEBPA mutations. However, patients with single CEBPA mutations had a significantly worse median overall survival (OS) of 15 months (Figure 1A; \( P = 0.006 \)) and disease-free survival (DFS) of 12 months (Figure 1B; \( P = 0.013 \)) compared with patients with double CEBPA mutations in whom both median DFS and OS were not reached after a median follow-up of 34 months. Moreover, the course of the disease of patients with single CEBPA mutations was not different both for OS and DFS from CEBPA wild-type patients. Finally, in a multivariable analysis discriminating white blood cell count, age, NPM1 mutations, and FLT3-ITD, only double CEBPA mutations, but not single CEBPA mutations, were identified as independent prognostic factors (Table 1). The CEBPA mutation status per se turned out to be of independent prognostic significance.

To illustrate the consequences of single vs double CEBPA mutations in terms of CEBPA function, we transfected H1299 cells with expression plasmids encoding CEBPA wild-type, an N-terminal frame-shift mutation, a C-terminal in-frame mutation, or the combination of both plasmids, and we determined the potential of these CEBPA peptides to activate a target promoter sequence as present in the G-CSF receptor promoter. As illustrated in Figure 1C, both the N- and the C-terminal CEBPA mutant peptides inhibited wild-type CEBPA protein in a dominant-negative manner, by reducing its activation potential by roughly 70%. However, it is important that the combination of both mutant constructs – in the absence of wild-type protein – failed to activate at all. This suggests that CEBPA activity is completely abolished in malignant cells of patients with double CEBPA mutations, whereas it is retained to some degree in patients with single CEBPA mutations. In addition, Figure 1D presents the various CEBPA peptides made in malignant cells from AML patients with different types of CEBPA mutations. It indicates that N-terminal frame-shift CEBPA mutations, in fact, decisively decrease the amount of wild-type CEBPA protein, whereas the 30-kDa peptide is detectable at a higher amount.

In conclusion, our findings indicate that there is relevant prognostic heterogeneity within AML patients with CEBPA mutations. Double CEBPA mutations are associated with distinctly favourable prognosis, whereas clinical outcome of AML patients with single CEBPA mutations is not different from CEBPA wild-type patients. However, the number of patients with a single CEBPA mutation is limited in our collection of patients, and larger series are needed to definitively assess its prognostic significance.

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