Ann Hematol (2012) 91:1051–1063
DOI 10.1007/s00277-012-1423-4

ORIGINAL ARTICLE

Monoallelic *CEBPA* mutations in normal karyotype acute myeloid leukemia: independent favorable prognostic factor within *NPM1* mutated patients

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Received: 5 August 2011 / Accepted: 31 January 2012 / Published online: 24 February 2012

Abstract We and others have shown that cytogenetically normal (CN)-AML patients with biallelic *CEBPA* gene mutations (bi*CEBPA*) represent a molecularly distinct group with a favorable prognosis. Patients carrying a monoallelic *CEBPA* mutation (mo*CEBPA*), however, show no different outcome compared to patients with wildtype *CEBPA*, and these mutations are frequently associated with mutated *NPM1* or *FLT3*-ITD. So far, no molecular or clinical hallmark has been identified to prognostically distinguish mo*CEBPA* patients from patients with wildtype *CEBPA*. Therefore, we used the data of 663 CN-AML patients treated within the AMLCG 1999 trial to explore the prognostic value of mo*CEBPA* in the context of concomitant clinical and molecular markers (mutated *NPM1*, *FLT3*-ITD). Multiple Cox regression in 515 patients adjusting for all available potential confounders revealed that the *NPM1* mutation modified the prognostic value of mo*CEBPA* with respect to overall survival (OS, $p=0.017$) and event-free survival (EFS, $p=0.011$). Mo*CEBPA* was beneficial in *NPM1* mutated patients: adjusted OS–hazard ratio (HR) 0.09, 95% confidence interval (CI) 0.01–0.63, $p=0.016$; EFS–HR (95% CI) 0.16 (0.04–0.65), $p=0.010$. In contrast, mo*CEBPA* had no prognostic impact in patients with wildtype *NPM1*: OS–HR (95% CI) 1.08 (0.59–1.97), $p=0.804$; EFS–HR (95% CI)

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1.12 (0.64–1.96), p=0.682. We found no prognostic effect modification for mo*CEBPA* by *FLT3*-ITD. The presence of a mo*CEBPA* mutation was shown to be associated with prolonged survival in *NPM1* mutated CN-AML patients. Confirmation of these results in larger studies will clarify whether an additional mo*CEBPA* mutation influences the risk stratification of patients with an *NPM1* mutated/*FLT3*-ITD positive genotype.

Keywords Monoallelic *CEBPA* mutations · *NPM1* mutation · Normal karyotype · Acute myeloid leukemia

Introduction

CCAAT/enhancer binding protein alpha (CEBPA) is a basic region leucine zipper (bZIP) transcription factor with a crucial role in proliferation and differentiation of myeloid cells [1–3]. *CEBPA* gene mutations occur in approximately 8–14% of patients with acute myeloid leukemia without chromosomal abnormalities (cytogenetically normal AML, CN-AML) [4–6] and can be divided into two main categories: N-terminal frameshift mutations that truncate the p42 wildtype CEBPA (wtCEBPA) protein but preserve a 30-kDa isoform with dominant negative activities on the wtCEBPA protein [7] and C-terminal in-frame mutations in *CEBPA* that disrupt the bZIP region and thereby affect dimerization and DNA binding activities of CEBPA [8, 9].

We and others have recently shown that the favorable prognostic impact originally designated to all patients with *CEBPA* gene mutations is restricted to those patients in whom both alleles in the *CEBPA* gene are disrupted (hereafter called bi*CEBPA* mutated AML) [5, 10–13]. Bi*CEBPA* mutations are typically a combination of an N-terminal mutation on one allele and a C-terminal bZIP mutation on the other allele and result in the lack of wtCEBPA p42 expression [14, 15]. In mice, a combination of an N-terminal and C-terminal disruption of the *CEBPA* gene synergistically resulted in fast and efficient development of leukemia [16–18], and therefore a biallelic disruption of *CEBPA* has been proposed to be sufficient for leukemogenesis. By contrast, mice carrying single N-terminal or C-terminal *CEBPA* mutants developed leukemia with long latencies, and additional mutation events might cooperate with *CEBPA* single mutants in inducing leukemia [16, 17, 19].

Approximately 50% of *CEBPA* mutated patients carry one single heterozygous N-terminal or C-terminal *CEBPA* mutation (hereafter called mo*CEBPA* mutated AML) [5], in whom the expression of wtCEBPA is predicted to be retained at lower levels. Mo*CEBPA* mutated patients have a similar prognostic outcome as wt*CEBPA* patients, and so far no clinical or molecular hallmark of mo*CEBPA* mutant AML has been identified. In contrast to bi*CEBPA* mutated patients, mo*CEBPA* mutated patients have a variety of associated gene mutations [5, 11]. This is most frequently a mutant nucleophosmin (*NPM1*^{mut}) and/or internal tandem duplication mutations of the fms-related tyrosine kinase 3 gene (*FLT3*-ITD). In AML patients, *NPM1*^{mut} has been associated with a favorable outcome [20–22] in the absence of concomitant *FLT3*-ITD [20, 22–24]. Recurrent mutations in the isocitrate dehydrogenase (*IDH*) 1 and 2 and the DNA methyltransferase 3A (*DNMT3A*) gene, both detected by next generation sequencing [25–27], have recently been associated with CN-AML, particularly with the *NPM1*^{mut} subtype [25, 28–32]. So far, the frequency and the prognostic role of these cooperating mutations in the context of an additional mo*CEBPA* mutation are not fully understood. We have previously shown that cooperating *NPM1*^{mut} influences the gene expression profile (GEP) in patients with a mo*CEBPA* mutation [5], and cooperating mutations have been suggested to be responsible for the inability to accurately predict mo*CEBPA* mutated patients by GEP [12, 33].

Therefore, in this study, we explored the role of mo*CEBPA* in the light of concomitant molecular markers within a large series of older and younger CN-AML patients that have been homogeneously treated within the German AMLCG 1999 trial.

Methods

Patients

In this analysis we included diagnostic bone marrow or peripheral blood samples from adult AML patients with a normal karyotype who have been enrolled in the German AML cooperative group (AMLCG) 1999 multicenter treatment trial. Patients with a complete molecular status of *CEBPA*, *NPM1* and *FLT3*-ITD (n=663) were selected out of a total of 802 patients. A subset of these patients (n=467) have also been investigated in a previous publication with a different objective [5]. Available clinical characteristics were age, sex, de novo versus secondary AML, ECOG performance status, French–American–British (FAB) subtypes M1 and M2 versus all other FAB subtypes, white blood cell and platelet counts, the amount of bone marrow blasts, hemoglobin and LDH levels. All patients received intensive induction therapy with thioguanine, cytarabine and daunorubicine (TAD) as standard therapy or high-dose cytarabine and mitoxantrone (HAM) in the experimental arm followed by one course of HAM and consolidation therapy. In patients with an age of ≥60 years, a second induction was only administered in case of an inadequate response to initial intensive induction treatment. Details of the trial protocols have been published elsewhere [34]. The

Table 1 Description of monoallelic *CEBPA* mutations and concurrent prognostic mutations

Pt#	Age (years)/sex	Nucleotide change (protein change)	Description of mutation	Predicted effect	Other gene mutations[a]
1	43/F	c.19dupT (p.Tyr7Leufs*101)	Frameshift duplication before TAD1	Stop before 2nd start site	*NPM1*^{mut}, *FLT3*-TKD, *DNMT3A*^{mut} (c.2644 C > T; p.Arg882Cys)
2	50/F	c.20dupA (p.Tyr7stop)	Nonsense duplication before TAD1	Stop before TAD1	*NPM1*^{mut}, *DNMT3A*^{mut} (c.2645 G > A; p.Arg882His)
3	67/F	c.68dupC (p.His24Alafs*84)	Frameshift duplication before TAD1	Stop before 2nd start site	
4	66/F	c.68dupC (p.His24Alafs*84)	Frameshift duplication before TAD1	Stop before 2nd start site	[b]
5	71/M	c.68dupC (p.His24Alafs*84)	Frameshift duplication before TAD1	Stop before 2nd start site	*NPM1*^{mut}, *FLT3*-ITD
6	69/F	c.68dupC (p.His24Alafs*84)	Frameshift duplication before TAD1	Stop before 2nd start site	*NPM1*^{mut}, *FLT3*-ITD
7	66/M	c.68dupC (p.His24Alafs*84)	Frameshift duplication before TAD1	Stop before 2nd start site	*IDH2*^{mut} (c.419 G > A; p.Arg140Gln)
8	78/M	c.85_91dup (p.Phe31Cysfs*79)	Frameshift duplication before TAD1	Stop before 2nd start site	*FLT3*-ITD
9	18/F	c.178_179insT (p.Thr60Ilefs*48)	Frameshift insertion before TAD1	Stop before 2nd start site	
10	75/F	c.197_200dup (p.Ile68Leufs*41)	Frameshift duplication before TAD1	Stop before 2nd start site	*NPM1*^{mut}, *FLT3*-TKD
11	30/F	c.199dupT (p.Tyr67Leufs*41)	Frameshift duplication before TAD1	Stop before 2nd start site	*NPM1*^{mut}, *FLT3*-ITD
12	64/M	c.245_248dup (p.Gln83Hisfs*26)	Frameshift duplication in TAD1	Stop before 2nd start site	*FLT3*-ITD
13	63/F	c.281_282insGCCGC (p.Val95Profs*67)	Frameshift insertion in TAD1	Stop in TAD2	
14	67/F	c.288_295dup (p.Gly99Alafs*64)	Frameshift duplication before 2nd start site	Stop in TAD2	*NPM1*^{mut}, *FLT3*-ITD
15	48/F	c.332_339del (p.Ala111Glyfs*56)	Frameshift deletion before 2nd start site	Stop in TAD2	*FLT3*-ITD
16	64/F	c.333_343del (p.Pro112Argfs*54)	Frameshift deletion before 2nd start site	Stop in TAD2	[b]
17	68/M	c.335_338del (p.Pro112Argfs*47)	Frameshift deletion before 2nd start site	Stop in TAD2	
18	56/F	c.338dupC (p.Gly116Argfs*54)	Frameshift duplication before 2nd start site	Stop in TAD2	*NPM1*^{mut}, *FLT3*-ITD, *DNMT3A*^{mut} (c.1741 T > C; p.Trp581Arg)
19	53/F	c.350delG (p.Gly117Alafs*43)	Frameshift deletion before 2nd start site	Stop in TAD2	*NPM1*^{mut}, *DNMT3A*^{mut} (c.2645 G > A; p.Arg882His)
20	69/M	c.515_516insCAGC (p.Gln172Hisfs*150)	Frameshift insertion in TAD2	Stop in LZD	*DNMT3A*^{mut} (c.2645 G > A; p.Arg882His), *IDH2*^{mut} (c.419 G > A; p.Arg140Gln)
21	55/M	c.531_532insG (p.Leu178Alafs*143)	Frameshift insertion in TAD2	Stop in LZD	
22	69/F	c.692_713del (p.Pro231Argfs*80)	Frameshift deletion between TAD2 and BR	Stop in LZD	*NPM1*^{mut}, *FLT3*-ITD, *DNMT3A*^{mut} (c.2204A > G; p.Tyr735Cys)
23	66/F	c.922_923ins24bp[c] (p.Val308delins9)	In-frame insertion in the fork of LZD domain	Disruption of LZD	[b]
24	70/M	c.927_928dup (p.Thr310Argfs*9)	Frameshift duplication in LZD	Stop in LZD	*MLL*-PTD[b]
25	47/F	c.934_936dup (p.Gln312dup)	In-frame duplication in LZD	Disruption of LZD	*FLT3*-TKD, *FLT3*-ITD
26	46/F	c.950_951insGTC (p.Leu317_Thr318insSer)	In-frame insertion in LZD	Disruption of LZD	*NPM1*^{mut}, *FLT3*-ITD
27	47/F	c.1027delC (p.Arg343Alafs*79)	Frameshift deletion in LZD	Disruption of LZD	*NPM1*^{mut}, *FLT3*-TKD, *IDH1*^{mut} (c.394 C > T; p.Arg132Cys)
28	78/F	c.1065_1066insGCC (p.Gly355_Asn356insAla)	In-frame insertion after LZD	Elongated protein	

Nucleotide numbering for *CEBPA* is according to Genbank Accession No. NM_004364.2, for *DNMT3A* according to NM_175629.1, for *IDH1* according to NM_005896.2 and for *IDH2* according to NM_002168.2

Pt# patient number, *F* female, *M* male, *TAD* transactivation domain, *LZD* leucine zipper domain; *BR* basic region, *NPM1*^{mut} mutations in the *NPM1* gene, *FLT3-ITD* internal tandem duplication of the *FLT3* gene, *FLT3-TKD* tyrosine kinase domain mutations, *MLL-PTD* partial tandem duplications of the *MLL* gene, *DNMT3A*^{mut} mutations in the *DNMT3A* gene, *IDH1/2*^{mut} mutations in the *IDH1/2* genes

[a] Includes mutations in the *NPM1* gene, *FLT3*-ITD, *FLT3*-TKD, *MLL*-PTD and mutations in the *DNMT3A*, *IDH1* and *IDH2* genes

[b] For patient #4, 16, 23 and 24, no *DNMT3A* and *IDH1/2* status could be determined due to insufficient sample material

[c] cgcgacaaggccaagcagcgcaac

Table 2 Patient demographics and survival according to the number of mutated alleles in the *CEBPA* gene

Characteristic	Number	wt*CEBPA* [No. (%)]	mo*CEBPA* [No. (%)]	p^a across wt*CEBPA*/mo*CEBPA*	bi*CEBPA* [No. (%)]	p^b across wt*CEBPA*/mo*CEBPA*/bi*CEBPA*
No. of patients	663	604 (91)	28 (4)		31 (5)	
Age (years)	663					
Median		60	65		61	0.541
Range		17–85	18–78		28–83	
Sex	663					
Female		298 (49)	20 (71)	0.022	16 (52)	0.073
FAB type (*n*)	652	594	28		30	
M0		22 (4)	0 (0)	0.208	1 (32)	0.021
M1		135 (23)	9 (32)		8 (27)	
M2		201 (34)	14 (50)		19 (63)	
M4		143 (24)	4 (14)		1 (3)	
M5		68 (11)	0 (0)		0 (0)	
M6		25 (4)	1 (4)		1 (3)	
FAB M1/M2 (*n*)		336 (57)	23 (82)	0.007	27 (90)	< 0.001
Type of disease	663					
De novo AML		505 (84)	23 (82)		30 (97)	0.258
AML from MDS		80 (13)	5 (18)		1 (37)	
Therapy-related AML		19 (3)	0 (0)		0 (0)	
Hemoglobin (*n*)	653	594	28		31	
Median (g/L)		92	89	0.792	102	0.011
Range (g/L)		42–164	68–132		80–125	
White cell count (*n*)	656	597	28		31	
Median ($\times 10^9$/L)		18	32		21	0.178
Range ($\times 10^9$/L)		0.1–798	1.7–192		0.9–203	
Platelet count (*n*)	656	597	28		31	
Median ($\times 10^9$/L)		59	53	0.897	38	0.037
Range ($\times 10^9$/L)		5–643	5–367		6–176	
Bone marrow blasts (*n*)	627	568	28		31	
Median (%)		80	85		77	0.722
Range (%)		20–100	20–97		20–100	
LDH (*n*)	648	589	28		30	
Median (U/L)		421	426		429	0.887
Range (U/L)		102–14332	152–2666		205–2510	
NPM1	663					
Wildtype		274 (45)	16 (57)	0.229	31 (100)	< 0.001
Mutant		330 (55)	12 (43)		0	
FLT3-ITD	663					
Negative		424 (70)	17 (61)	0.285	28 (90)	0.027
Positive		180 (30)	11 (39)		3 (10)	
NPM1/*FLT3*-ITD	663			0.647		< 0.001
Wildtype/negative		223 (37)	12 (43)		28 (90)	
Wildtype/positive		51 (8)	4 (14)		3 (10)	
Mutant/negative		201 (33)	5 (18)		0	
Mutant/positive		129 (21)	7 (25)		0	
FLT3-TKD (*n*)	595	539	27		29	
Negative		504 (94)	22 (81)	0.081	29 (100)	0.069
Positive		35 (7)	4 (15)		0	
MLL-PTD (*n*)	631	575	28		28	

Table 2 (continued)

Characteristic	Number	wt*CEBPA* [No. (%)]	mo*CEBPA* [No. (%)]	p^a across wt*CEBPA*/ mo*CEBPA*	bi*CEBPA* [No. (%)]	p^b across wt*CEBPA*/ mo*CEBPA*/bi*CEBPA*
Negative		527 (92)	27 (96)		28(100)	0.197
Positive		48 (8)	1 (4)		0	
Complete remission	663					
No. of patients		400	17		24	
Rate (%)		66	61		77	0.350
OS	663					
Median follow-up time (months)		63	72		53	0.682
Median (months)		19.6	22.8	0.408	Not reached	0.007
Events		339	16		11	
EFS	661	602	28		31	
Median (months)		8.1	6.5	0.554	33.8	0.053
Events		464	19		17	
Allogeneic stem cell transplant (*n*)	662	111/603 (18)	6 (21)		6 (19)	0.463
Induction cycles (*n*)	660	601	28		31	
1		221 (37)	10 (36)		10 (32)	0.875
2		380 (63)	18 (64)		21 (68)	
Double induction regimen, ITT	663					
TAD-HAM		311 (52)	13 (46)		14 (45)	0.699
HAM-HAM		293 (49)	15 (54)		17 (55)	
Cumulative dose of cytarabine (mg)	663					
Median		29400	23900		30870	0.318

CN-AML karyotypically normal acute myeloid leukemia, *wtCEBPA* patients with wildtype *CEBPA*, *moCEBPA* patients with monoallelic *CEBPA* mutation, *biCEBPA* patients with biallelic *CEBPA* mutations, *AML* acute myeloid leukemia, *MDS* myelodysplastic syndrome, *LDH* lactate dehydrogenase, *FAB* French–American–British, *NPM1* nucleophosmin, *FLT3-ITD* internal tandem duplication of the *FLT3* gene, *FLT3-TKD* tyrosine kinase domain mutations, *MLL-PTD* partial tandem duplication of the *MLL* gene, *OS* overall survival, *EFS* event-free survival, *ITT* intention to treat, *TAD* 6-thioguanine, cytarabine and daunorubicin, *HAM* high-dose cytarabine and mitoxantrone

[a] Pairwise comparisons between mo*CEBPA* and wt*CEBPA* applying the Mann–Whitney *U* test or χ^2-test/ Fisher's exact test. *p* values are given in case comparison between the three subgroups had a *p* value<0.100

[b] Differences between wt*CEBPA*, mo*CEBPA* and bi*CEBPA* were calculated using the Kruskal–Wallis test or χ^2-test/Fisher's exact test

study protocols were approved by the ethics committees of the participating centers, and all patients provided written informed consent.

Molecular analyses

CEBPA mutational screening was performed by multiplex PCR-based fragment length analysis and confirmation sequencing as previously described [35]. Patients with two different mutations were categorized as bi*CEBPA*. In 20 of the bi*CEBPA* patients, cloning analysis was performed and revealed that the distribution of the mutations was biallelic [5]. Patients with single heterozygous mutations as determined by sequencing analysis were categorized as mo*CEBPA*. Within our patient cohort, we did not identify patients with homozygous mutations or patients with more than two mutations in *CEBPA*.

Patients were further characterized at the molecular level with regards to *NPM1* mutations [22, 36], *FLT3*-ITD [37], point mutations in the *FLT3* gene at codons D835 and I836 (*FLT3*-TKD) [38], and partial tandem duplications within the MLL gene (*MLL*-PTD) [39] as described. These analyses were performed on complementary DNA as described [35]. Furthermore, genomic DNA was obtained with the QIamp DNA Mini Kit (Qiagen, Hilden, Germany) of all mo*CEBPA* patients in order to screen for *IDH1*, *IDH2* and *DNMT3A* mutations. Mutation analysis of codon 132 of the *IDH1* gene and codon 140 and 172 of the *IDH2* gene was performed by bidirectional sequencing using primers spanning the mutation hotspots as previously described [40]. Due to limited sample availability, we confined *DNMT3A* mutation screening to exons 15–23 in which most mutations have been detected in AML so far [25, 41]. Exon-spanning primers were designed, and amplicons were scanned by LightCycler High Resolution

Table 3 Comparison of patient demographics and survival in N-terminally versus C-terminally located mo*CEBPA* mutations

Characteristic	Number	N-terminal mutation [No. (%)]	C-terminal mutation [No. (%)]	p^a
No. of patients	28	19 (68)	9 (32)	
Age (years)	28			
Median		64	66	0.554
Range		18–78	46–78	
Sex	28			
Female		13 (68)	7 (77)	1.000
Type of disease	28			
De novo AML		17 (89)	6 (67)	0.290
*S*econdary AML		2 (11)	3 (33)	
Hemoglobin (*n*)	28			
Median (g/L)		99	84	0.061
Range (g/L)		72–131	68–112	
White cell count (*n*)	28			
Median ($\times 10^9$/L)		44	31	0.363
Range ($\times 10^9$/L)		1.8–128.9	1.7–192	
Platelet count (*n*)	28			
Median ($\times 10^9$/L)		53	51	0.731
Range ($\times 10^9$/L)		10–223	5–367	
Bone marrow blasts (*n*)	27	18		
Median (%)		82.5	85	0.896
Range (%)		40–95	20–97	
LDH (*n*)	28			
Median (U/L)		423	428	0.417
Range (U/L)		152–1331	186–2666	
NPM1	28			
Wildtype		10 (53)	6 (67)	0.687
Mutant		9 (47)	3 (33)	
FLT3-ITD	28			
Negative		11 (58)	6 (67)	1.000
Positive		8 (42)	3 (33)	
FLT3-TKD (*n*)	28	17	9	
Negative		15 (88)	7 (78)	0.591
Positive		2 (12)	2 (22)	
MLL-PTD (*n*)	27	18	9	
Negative		18 (100)	8 (89)	0.333
Positive		0	1 (11)	
DNMT3A (*n*)	24	18	6	
Wildtype		14	4	0.618
Mutant		4	2	
IDH1 R132 (*n*)	24	18	6	
Wildtype		18	5	0.250
Mutant		0	1	
IDH2 R140 (*n*)	24	18	6	
Wildtype		17	5	0.446
Mutant		1	1	
Complete remission				
No. of patients		12	5	0.507
Rate (%)		36.2	55.6	
OS	28			
Median (months)		24.3	22.8	0.646
Events		10	6	
EFS	28			
Median (months)		6.5	21.4	0.796
Events		13	6	

Abbreviations: *moCEBPA* patients with monoallelic *CEBPA* mutation, *AML* acute myeloid leukemia, *LDH* lactate dehydrogenase, *FAB* French–American–British, *NPM1* nucleophosmin, *FLT3-ITD* internal tandem duplication of the *FLT3* gene, *FLT3-TKD* tyrosine kinase domain mutations, *MLL-PTD* partial tandem duplication of the *MLL* gene, *OS* overall survival, *EFS* event-free survival

[a] Pairwise comparisons were calculated using the Mann–Whitney *U* test or χ^2 -test/Fisher's exact test

Melting master (Roche Diagnostics, Penzberg, Germany) following the manufacturer's instructions. Differences in the fluorescence of the high resolution melting profile in comparison to wildtype genes in HL-60 cells were confirmed by bidirectional DNA sequencing.

Statistical analyses

Outcome variables were overall survival (OS) and event-free survival (EFS). OS was calculated from randomization to death from any cause or to the latest follow-up date. EFS was defined as the period from the start of therapy until lack of a complete remission (CR), relapse after CR or death without relapse. Independent prognostic factors were examined by multivariable Cox regression including all available clinical characteristics and molecular markers. All survival analyses were performed with and without censoring time to event at the date of allogeneic transplantation, if performed. To explore modifying effects, the interaction terms between $NPM1^{mut}$ and *FLT3*-ITD,

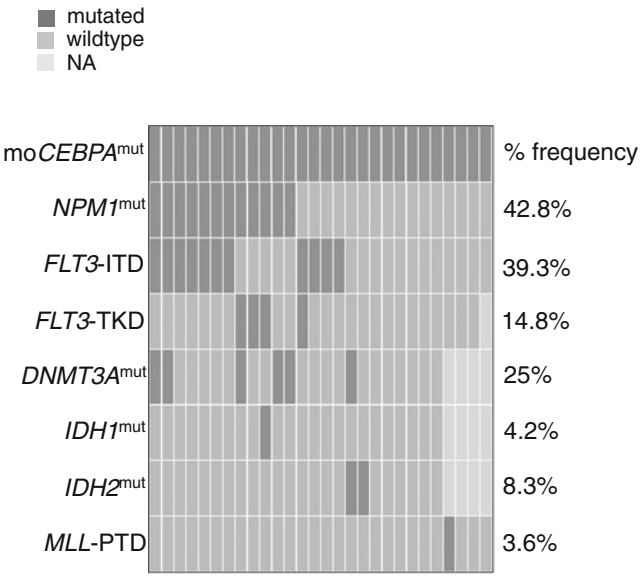

Fig. 1 Frequency and distribution of concurrent mutations in mo*CEBPA* mutated patients. Each *box* in a column represents one patient and in rows the genotypes *NPM1*^{mut}, *FLT3*-ITD, *FLT3*-TKD, *DNMT3A*^{mut}, *IDH1*^{mut}, *IDH2*^{mut} or *MLL*-PTD (*dark grey*), wildtype (*grey*) and missing values (*light grey*). Abbreviations: *moCEBPA* monoallelic *CEBPA* mutation, *NPM1*^{mut} mutations in the nucleophosmin gene, *FLT3*-ITD internal tandem duplications in the *FLT3* gene, *FLT3-TKD* tyrosine kinase mutations in the *FLT3* gene, *MLL-PTD* partial tandem duplications in the *MLL* gene, *DNMT3A*^{mut} mutations in the DNA methyltransferase 3A gene, *IDH1/2*^{mut} mutations in the *IDH1/2* genes

NPM1^{mut} and *moCEBPA*, *FLT3*-ITD and *moCEBPA*, and *FLT3*-ITD and *biCEBPA* were included in the models. The interaction term between *NPM1*^{mut} and bi*CEBPA* could not be included, since no bi*CEBPA* patient carried an *NPM1* mutation. For mo*CEPBA* patients [5] and *NPM1*^{mut} patients [22, 42], a higher frequency of female sex has previously been reported. Therefore, we tested whether female sex prognostically modified the outcome of patients with a mo*CEBPA*, *NPM1*^{mut}, *FLT3*-ITD or bi*CEBPA*. Non-significant interaction terms were eliminated backwards. In case of significant interactions, stratified hazard ratios were reported.

Survival curves were calculated according to the Kaplan–Meier method and compared with the log rank test. For comparisons of different categoric parameters in two, respectively, three subgroups were performed using the χ^2-test. For continuous variables, we used the Mann–Whitney *U* test for the comparison between two different groups and the Kruskal–Wallis test for the comparison between the three *CEBPA* subgroups. All tests were two-tailed, and p-values<0.05 were considered statistically significant.

Statistical computations were performed using SPSS software, version 16.0 (SPSS, Chicago, IL) and the R 2.7.2 software package (R Foundation for Statistical Computing, Vienna, Austria).

Results

CEBPA mutation status

We had a complete molecular status of 663 CN-AML patients with a median age of 60 years (range 17–85 years). Survival parameters did not significantly differ between selected and unselected patients ("Appendix"). *CEBPA* gene mutations were detected in 59 patient samples (8.7%). Thirty-one patients (4.7%) had bi*CEBPA* mutations among which most patients had a combination of an N-terminal frameshift mutation and a C-terminal mutation in the bZIP domain of CEBPA (28/31). Twenty-eight patients (4.1%) carried mo*CEBPA* mutations which were predominantly N-terminal frameshift mutations (19/28) (Pts #1–19, Table 1). Nine of 28 mo*CEBPA* patients had in-frame and frameshift mutations affecting the C-terminus of CEBPA (Pts #20–28, Table 1).

The clinical and molecular characteristics of the study cohort according to the number of mutated alleles in the *CEBPA* gene are shown in Table 2. The percentage of patients that received allogeneic bone marrow transplantation or treatment regimens was not significantly different in the three subgroups (Table 2).

Characterization of mo*CEBPA* mutated patients

Different leukemogenic capacities and molecular mechanisms have been suggested between *CEBPA* mutations located either in the N-terminus or in the C-terminus of the *CEBPA* gene [16, 17]. However, no significant differences in outcome were observed when comparing the 19 patients with N-terminally located mo*CEBPA* mutations with the nine patients containing C-terminal *CEBPA* mutations (Table 3). Also, several clinical parameters and the frequency of concomitant molecular mutations were not significantly different between patients with N-terminally or C-terminally located mo*CEBPA* mutations (Table 3).

Therefore, for subsequent analysis, the location of the mo*CEBPA* mutations was not further taken into consideration.

The only significant clinical difference of mo*CEBPA* mutated patients in comparison to patients with wt*CEBPA* was a higher percentage of female patients and, as in all *CEBPA* mutated patients, a higher percentage of FAB M1 or M2 (Table 2). Clinical outcome parameters and the frequency of additional *NPM1*^{mut}, *FLT3*-ITD or MLL-PTD were statistically not different when compared to patients with wt*CEBPA* (Table 2).

In addition, we determined the mutation status for *IDH1*, *IDH2* and *DNMT3A* genes in 24 out of 28 mo*CEBPA* patients of which we had sufficient material left. One mo*CEBPA* patient had an *IDH1* R132C mutation (1/24, 4.2%), two patients had *IDH2* R140Q mutations (8.3%)

and six patients had missense mutations in the *DNMT3A* gene (25%). A more detailed description of these mutations is given in Table 1.

The genotype and the association of the different molecular markers investigated in mo*CEBPA* patients are described in Fig. 1. In short, mo*CEBPA* mutated patients most frequently had an additional *NPM1*^{mut} (12/28: 42.8%). All of these mo*CEBPA*/*NPM1*^{mut} patients were further associated with at least one of the following aberrations: *FLT3*-ITD in seven cases, *FLT3*-TKD in three cases, *DNMT3A* in five cases and an *IDH1* mutation in one case. Of the 16 mo*CEBPA*/*NPM1*^{unmut} patients (16/28: 57.1%), four patients had a concurrent *FLT3*-ITD, one an *FLT3*-TKD, one an *MLL*-PTD, two an *IDH2* R140 mutation and one a *DNMT3A* mutation.

Prognostic value of a mo*CEBPA* mutation in 515 CN-AML patients

In a multiple Cox regression analysis in 515 complete cases adjusting for all candidate prognostic factors, we looked for different prognostic values of mo*CEBPA* according to the most frequently occurring concurrent markers *NPM1*^{mut} and *FLT3*-ITD.

Besides the known prognostic effect modification of *NPM1*^{mut} by *FLT3*-ITD, we found that the prognostic value of mo*CEBPA* was modified by *NPM1*^{mut} with regard to OS: HR (95% CI) 0.08 (0.01–0.64), p=0.017 and EFS: HR (95% CI) 0.14 (0.03–0.64), p=0.011 (Table 4). Stratified calculation of HR revealed that in *NPM1*^{unmut} patients,

Table 4 Multiple Cox regression analysis for overall and event-free survival in 515 CN-AML patients

Variable	Comparison	Stratum	OS–HR (95% CI)	p	EFS–HR (95% CI)	p
mo*CEBPA*	mo vs. wt*CEBPA*	*NPM1*^{unmut}	1.08 (0.59–1.97)	0.804	1.12 (0.64–1.96)	0.682
	mo vs. wt*CEBPA*	*NPM1*^{mut}	0.09 (0.01–0.63)	0.016	0.16 (0.04–0.65)	0.010
NPM1^{mut}/mo*CEBPA*	mut/mo vs. others	All	0.08 (0.01–0.64)	0.017	0.14 (0.03–0.64)	0.011
NPM1^{mut}/*FLT3*-ITD	mut/pos vs. others	All	1.85 (1.10–3.10)	0.020	1.94 (1.21–3.11)	0.006
NPM1^{mut}/female sex	mut/female vs. others	All	2.40 (1.49–3.86)	< 0.001	2.46 (1.60–3.79)	< 0.001
NPM1^{mut}	mut vs. wt	*wtCEBPA/ FLT3*-ITD^{neg}/male	0.20 (0.13–0.31)	< 0.001	0.19 (0.13–0.28)	< 0.001
	mut vs. wt	*moCEBPA/ FLT3*-ITD^{neg}/male	0.02 (0.00–0.13)	< 0.001	0.03 (0.01–0.13)	< 0.001
	mut vs. wt	*wtCEBPA/ FLT3*-ITD^{neg}/female	0.49 (0.32–0.75)	0.001	0.47 (0.32–0.69)	< 0.001
	mut vs. wt	*moCEBPA/ FLT3*-ITD^{neg}/female	0.04 (0.01–0.31)	0.002	0.07 (0.02–0.30)	< 0.001
	mut vs. wt	*wtCEBPA/FLT3*-ITD^{pos}/male	0.40 (0.22–0.63)	< 0.001	0.37 (0.23–0.60)	< 0.001
	mut vs. wt	*moCEBPA/FLT3*-ITD^{pos}/male	0.03 (0.00–0.25)	0.001	0.05 (0.01–0.25)	< 0.001
	mut vs. wt	*wtCEBPA/FLT3*-ITD^{pos}/female	0.90 (0.55–1.46)	0.663	0.92 (0.58–1.44)	0.709
	mut vs. wt	*moCEBPA/FLT3*-ITD^{pos}/female	0.07 (0.01–0.61)	0.016	0.13 (0.03–0.63)	0.011
FLT3-ITD	pos vs. neg	*NPM1*^{unmut}	0.91 (0.59–1.39)	0.647	0.78 (0.53–1.15)	0.211
	pos vs. neg	*NPM1*^{mut}	1.67 (1.18–2.38)	0.004	1.51 (0.10–2.09)	0.012
Sex	Female vs. male	*NPM1*^{unmut}	0.62 (0.45–0.85)	0.003	0.57 (0.43–0.77)	< 0.001
	Female vs. male	*NPM1*^{mut}	1.47 (1.04–2.09)	0.030	1.41 (1.03–1.94)	0.034
bi*CEBPA*	bi vs. wt*CEBPA*	All	0.24 (0.12–0.49)	< 0.001	0.33 (0.18–0.58)	< 0.001
FLT3-TKD	pos vs. neg	All	1.43 (0.91–2.25)	0.122	1.33 (0.86–2.05)	0.202
MLL-PTD	pos vs. neg	All	0.94 (0.63–1.40)	0.763	1.03 (0.71–1.49)	0.874
Age (years)	+10	All	1.41 (1.28–1.56)	< 0.001	1.21 (1.11–1.32)	< 0.001
ECOG PS	2–4 vs. 0/1	All	1.20 (0.94–1.54)	0.154	1.15 (0.91–1.44)	0.248
De novo AML	vs. non-de novo	All	0.91 (0.66–1.25)	0.555	0.99 (0.75–1.34)	0.987
FAB M1/M2	vs. others	All	1.05 (0.82–1.35)	0.682	1.12 (0.89–1.40)	0.341
WBC ($\times 10^9$/L)	10-fold	All	1.41 (1.11–1.79)	0.005	1.35 (1.08–1.68)	0.008
Platelet count ($\times 10^9$/L)	10-fold	All	0.72 (0.53–0.99)	0.043	0.78 (0.59–1.04)	0.088
Hemoglobin (g/L)	+1	All	0.99 (0.99–1.01)	0.860	1.00 (1.00–1.01)	0.438
BM blasts (%)	+1	All	1.01 (0.99–1.01)	0.126	1.01 (0.99–1.01)	0.078
LDH (U/L)	10-fold	All	1.83 (1.14–2.94)	0.012	1.64 (1.07–2.53)	0.025

p values were calculated using the Wald test

OS overall survival, *EFS* event-free survival, *HR* hazard ratio, *CI* confidence interval, *biCEBPA* biallelic *CEBPA* mutations, *moCEBPA* monoallelic *CEBPA* mutations, *NPM1*^{mut} mutations in the nucleophosmin gene, *NPM1*^{unmut} no mutations in the nucleophosmin gene, *FLT3-ITD* internal tandem duplications in the fms-like tyrosine kinase-3 gene, *MLL-PTD* partial tandem duplications in the *MLL* gene, *ECOG PS* ECOG performance status, *FAB* French–American–British, *WBC* white blood cell count, *LDH* lactate dehydrogenase

mo*CEBPA* was of no prognostic impact: OS–HR (95% CI) 1.08 (0.59–1.97), p=0.804; EFS–HR (95% CI) 1.12 (0.64–1.96), p=0.682, whereas it was beneficial in *NPM1*^{mut} patients: OS–HR (95% CI) 0.09 (0.01–0.63), p=0.016; EFS–HR (95% CI) 0.16 (0.04–0.65), p=0.010. These same results were retained in a multivariable model with censoring transplanted patients at the date of allogeneic transplantation: OS–HR (95% CI) 0.09 (0.01–0.63), p=0.016; EFS–HR (95% CI) 0.15 (0.04–0.62), p=0.009 (data not shown).

There was no prognostic effect modification of mo*CEBPA* by *FLT3*-ITD (OS, p=0.294; EFS, p=0.688) or for bi*CEBPA* by *FLT3*-ITD (OS, p=0.924; EFS, p=0.460).

We further tested whether the higher frequency of female sex in mo*CEBPA* patients is of prognostic importance. However, female sex did not modify the prognosis of mo*CEBPA* (OS, p=0.399), bi*CEBPA* (OS, p=0.510) or *FLT3*-ITD (OS, p=0.375). We, however, identified a modifying prognostic effect for *NPM1*^{mut} by female sex: OS–HR (95% CI) 2.40 (1.49–3.86), p<0.001, such that female sex was identified as an unfavorable prognostic marker within *NPM1*^{mut} patients and as a favorable prognostic marker within *NPM1*^{unmut} patients (Table 4).

The favorable prognostic effect of *NPM1*^{mut} was therefore modified by *FLT3*-ITD, mo*CEBPA* and female sex resulting in eight strata with beneficial effect to a different extent. In all of these subgroups, a mo*CEBPA* mutation positively enhanced the effect of *NPM1*^{mut} independently of *FLT3*-ITD or sex with hazard ratios by a factor of 0.12. Other independent prognostic factors for OS and EFS were the presence of a bi*CEBPA* mutation, younger age, white blood cell count and lactate dehydrogenase levels (Table 4). There were no qualitative differences in the results with censoring time to event at the date of allogeneic transplantation (data not shown).

For graphical demonstration of the different prognostic effects of *NPM1*^{mut} according to the presence of a mo*CEBPA*, we compared *NPM1*^{mut}/mo*CEBPA* patients (n=12) to *NPM1*^{mut}/wt*CEBPA* patients (n=330) irrespective of the presence of additional markers in univariate Kaplan–Meier analysis. We found a strong trend towards an additional favorable effect of a mo*CEBPA* mutation on OS and EFS of *NPM1*^{mut} patients: median OS for mo*CEBPA*/*NPM1*^{mut} versus wt*CEBPA*/*NPM1*^{mut} was not reached versus 34.3 months, respectively, p=0.071 (Fig. 2a); median EFS was not reached versus 13.6 months, respectively, p=0.054 (Fig. 2b). The favorable trend of mo*CEBPA* mutations on the

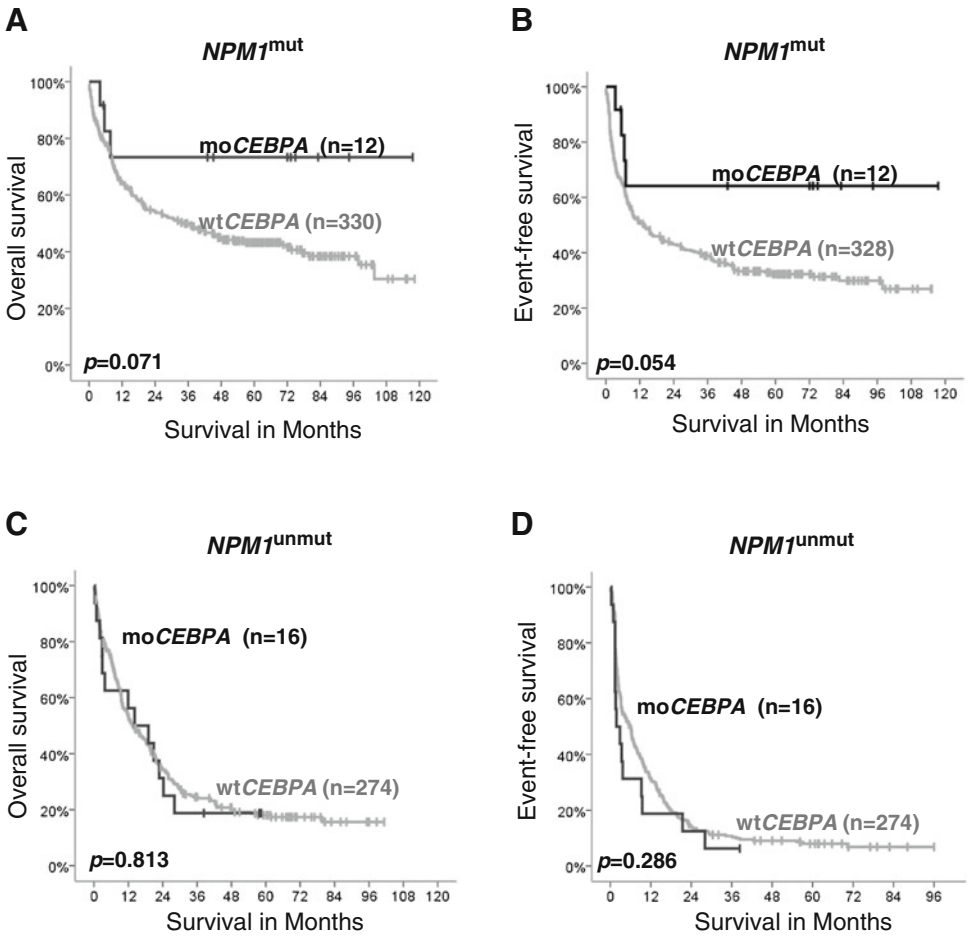

Fig. 2 Kaplan–Meier analysis for overall and event-free survival investigating the prognostic impact of a mo*CEBPA* mutation versus wt*CEBPA* in all *NPM1*^{mut} patients (**a** + **b**) and in all *NPM1*^{unmut} patients (**c** + **d**), irrespective of other prognostic markers. Abbreviations: *moCEBPA* monoallelic *CEBPA* mutation, *wtCEBPA* wildtype *CEBPA*, *NPM1*^{mut} mutations in the *NPM1* gene, *NPM1*^{unmut} no mutations in the *NPM1* gene. *p*: log rank test for pairwise comparison

outcome of *NPM1*^{mut} patients was retained when censoring transplanted patients (median OS: not reached versus 40.7 months, p=0.106; median EFS: not reached versus 13.5, p=0.061). No prognostic effect of a mo*CEBPA* was observed in *NPM1*^{unmut} patients (Fig. 2c–d).

Discussion

This is, to our knowledge, the first study investigating the prognostic value of a single monoallelic *CEBPA* mutation within homogeneously treated CN-AML patients in the context of other molecular markers. We and others have previously shown that a mo*CEBPA* mutation as a single marker had no independent prognostic impact in the unstratified cohort of CN-AML [5, 10, 11, 13].

Here we show for the first time a modification of prognostic effects for mo*CEBPA* by *NPM1*^{mut} such that mo*CEBPA* had a positive influence in *NPM1*^{mut}, but no effect in *NPM1*^{unmut} patients (Table 4, Fig. 2). A previous study in younger AML patients including patients with complex karyotypes did not find a statistically significant benefit of a single *CEBPA* mutation in *NPM1*^{mut} AML, but the authors have not investigated a prognostic effect modification of mo*CEBPA* by *NPM1*^{mut} in a multivariable analysis [10].

A cooperative favorable effect of concurrent mo*CEBPA*/*NPM1*^{mut} is somewhat surprising, as both *NPM1*^{mut} and *CEBPA* mutations have typical features of primary genetic lesions impairing hematopoietic differentiation [43]. Interestingly, ten out of 12 patients with concurrent mo*CEBPA* and *NPM1*^{mut} had an additional mutation in the *FLT3* gene, an additional *FLT3*-ITD in seven cases and an additional *FLT3*-TKD in three cases (Table 1). For both mutated *CEBPA* and *NPM1*^{mut}, an additional *FLT3*-ITD has shown to be a potential secondary event [44] important for disease progression [45, 46]. A potential pathogenetic relevance of *FLT3*-ITD^{pos} in *CEBPA* mutated AML has recently been demonstrated in a bone marrow transplantation model in which a C-terminal *CEBPA* mutant collaborated with an *FLT3*-ITD in the process of leukemogenesis [17]. A co-occurrence of *NPM1* or *CEBPA* mutations with an *FLT3*-TKD has shown to be prognostically favorable [38].

In our patient cohort including patients over 60 years, we did not find a prognostic effect modification of mo*CEBPA* or bi*CEBPA* by *FLT3*-ITD. A negative prognostic influence of an *FLT3*-ITD in *CEBPA* mutant AML has previously been described in younger AML patients [12]. These discrepancies might be due to the small incidences of concurrent *FLT3*-ITD and *CEBPA* mutations. Alternatively, in *NPM1*^{mut} patients, there are indications that the prognostic impact of an additional *FLT3*-ITD might be influenced by age. Several studies including patients over the age of 60 years have shown a favorable effect of *NPM1*^{mut} irrespective of the presence or absence of an *FLT3*-ITD [47]. We show here that a mo*CEBPA* mutation had a favorable effect in *NPM1*^{mut} patients irrespective of the presence or absence of *FLT3*-ITD (Table 4, Fig. 2a, b). In accordance to these data, a recently reported meta-analysis in CN-AML patients observed a favorable trend for all *NPM1*/*FLT3*-ITD genotypes in *CEBPA* single mutant patients in comparison to patients with wt*CEBPA* [12]. Further large prospective trials are required to clarify whether the favorable shift of *NPM1*^{mut}/*FLT3*-ITD^{pos} patients in the presence of a mo*CEBPA* mutation may have therapeutic implications regarding the need for transplantation recommended for *FLT3*-ITD^{pos} patients [23].

The frequency of combined *IDH1/IDH2* mutations that we detected within the subgroup of mo*CEBPA* mutations (12.5%) was similar as previously reported in *CEBPA* mutated CN-AML patients (13–17%) and less than reported in CN-AML patients (23–33%) [30]. The incidence of *DNMT3A* mutations in mo*CEBPA* patients (25%) was slightly less than that reported in CN-AML (27–34%) [25, 32, 41] but well in accordance to a recent report and comment to this report by Thol et al. who detected 21% (five of 24) of *DNMT3A* mutations in CN-AML patients with a single *CEBPA* mutation [32]. Due to the relatively low percentages of these mutations in mo*CEBPA* mutated patients, their prognostic role in this subgroup is not clear yet. Clarification will require larger patient cohorts.

A higher female prevalence was the only clinical difference in all mo*CEBPA* mutated patients as compared to wt*CEBPA* patients (Table 2) also when limited to *NPM1*^{mut} patients (data not shown). In a recent study in younger AML patients, the favorable prognostic impact of patients with *CEBPA* double mutations was predominately seen in younger and female patients [10]. However, we did not find a prognostic association of mo*CEBPA* or bi*CEBPA* with female sex.

In summary, we have shown that within *NPM1*^{mut} CN-AML patients, mo*CEBPA* was associated with an independent favorable outcome, similarly as previously shown for bi*CEBPA* mutated patients. Our results might prompt further larger analyses to clarify the clinical and therapeutic relevance of these findings.

Acknowledgements The authors would like to thank Evelyn Zellmeier and Gudrun Mellert for excellent technical assistance.

Conflict of interest The authors indicate no potential conflict of interest.

Appendix

Selection

Patients with a complete molecular status of *CEBPA*, *NPM1* and *FLT3*-ITD were selected out of a total of 802 patients. The

selected patients (n=663) had a significantly higher percentage of bone marrow blasts and leucocytes compared to non-selected patients (n=139). Other clinical parameters and the frequency of molecular markers were similar in selected versus unselected patients. OS, EFS and RFS did not significantly differ between both tested and untested groups (p=0.342, p=0.219, p=0.600, respectively) (data not shown).

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