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Research paper

A simple cytofluorimetric score may optimize testing for biallelic CEBPA mutations in patients with acute myeloid leukemia

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ABSTRACT

Acute myeloid leukemia with biallelic mutation of *CEBPA (CEBPA-dm AML)* is a distinct good prognosis entity recognized by WHO 2016 classification. However, testing for *CEBPA* mutation is challenging, due to the intrinsic characteristics of the mutation itself. Indeed, molecular analysis cannot be performed with NGS technique and requires Sanger sequencing. The association of recurrent mutations or translocations with specific immunophenotypic patterns has been already reported in other AML subtypes. The aim of this study was the development of a specific cytofluorimetric score (*CEBPA-dm* score), in order to distinguish patients who are unlikely to harbor the mutation. To this end, the correlation of *CEBPA-dm* score with the presence of the mutation was analyzed in 50 consecutive AML patients with normal karyotype and without *NPM1* mutation (that is mutually exclusive with *CEBPA* mutation). One point each was assigned for expression of HLA DR, CD7, CD13, CD15, CD33, CD34 and one point for lack of expression of CD14.

OS was not influenced by sex, age and *CEBPA-dm* score. Multivariate OS analysis showed that *CEBPA-dm* ($p < 0.02$) and *FLT3-ITD* ($p < 0.01$) were the strongest independent predictors of OS. With a high negative predictive value (100%), *CEBPA-dm* score < 6 was able to identify patients who are unlikely to have the mutation. Therefore, the application of this simple score might optimize the use of expensive and time-consuming diagnostic and prognostic assessment in the baseline work up of AML patients.

1. Introduction

CCAAT/enhancer binding protein alpha gene (*CEBPA*) encodes for a transcription factor that is required for myeloid precursor differentiation [1–4]. *CEBPA* mutations are observed in 10–18% of AML patients [5]. Biallelic mutations occur in half of the cases and play a central role in the development of the disease [6–10]. AML with biallelic mutations of *CEBPA* (*CEBPA-dm*) is now considered a distinct entity by the WHO 2016 classification [11]. The presence of *CEBPA-dm* mutually excludes the presence of other recurrent genetic abnormalities, such as *NPM1* mutation, and is unfrequently associated with *FLT3-ITD* mutation [12,13].

European Leukemia Net (ELN) 2017 includes *CEBPA-dm* AML into the good prognosis subgroup [14,15] so that allogeneic stem cells transplantation in first complete remission is not recommended [16].

The assessment of *CEBPA* gene mutations by Sanger sequencing is challenging due to the great variability of molecular alterations, the lack of hot spots and the presence of single nucleotide mutations [17–21]. Detecting *CEBPA* mutations by Sanger sequencing is therefore expensive and time consuming and should be performed only in experienced centers [18,19]. Moreover, for the same technical reasons, Next Generation Sequencing (NGS) is not optimal for the study of *CEBPA-dm* [17–21]. A novel NGS approach might overcome some of these problems, but the technique is still in an early development phase and needs to be validated and standardized [22].

Immunophenotypic analysis with multi-parametric flow-cytometry

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(IF) is mandatory in the diagnostic work-up of AML [16,23], allowing the identification of the blast cells lineage and leukemia-associated phenotypes [24,25]. In AML, some recurrent chromosomal or molecular abnormalities have been shown to be associated with specific immunophenotypic patterns, as reported for patients with t(8;21) and with mutation of *NPM* [26,27]. Recently, correlations between IF features and presence of *CEBPA-dm* have been described [26–30].

The primary aim of our study was to develop a new IF-score, based only on combined surface antigen expression, in order to assess its correlation with the presence of *CEBPA-dm*. As accessory end points the impact of IF-score on disease outcome was evaluated and compared with other clinical and molecular variables.

2. Material and methods

2.1. Patients

One-hundred consecutive younger (< 60 yrs), de-novo AML patients, treated between January 2006 and January 2016, with available cytofluorimetric, cytogenetic and molecular assessment at diagnosis were included in the present study. All patients received fludarabine-high dose cytarabine-idarubicine intensive induction followed by a risk-adapted consolidation strategy [25,31]. Molecular assessment included RT-PCR for *NPM1* mutation, *FLT3-ITD* and *CEBPA*. All patients with cytogenetic abnormalities and/or with *NPM1* mutation were excluded from the analysis, as those abnormalities are not found in patients with *CEBPA-dm* [17,32,33].

Fifty patients were therefore included in the study. Median age at diagnosis was 51.5 years (range 19–60 years); 27 patients (54%) were male; median WBC count at diagnosis was 14400/μl (range 1–380000/μl), high allelic burden *FLT3-ITD* mutation was found in 6 patients (12%), *CEBPA-dm* in 9 patients (18%). ELN 2017 risk assessment [15] was favorable or intermediate in 44 patients (88%) and high in 6 (12%).

2.2. Statistical methods

Continuous variables were compared using Student's *t*-test or, where necessary, Wilcoxon's Rank test. Dichotomous variables were compared using the Chi-square test or, where necessary, Fisher's exact test.

Survival curves were built using the Kaplan Meier method, and univariate survival analysis was performed using the Log-rank test. A landmark analysis was performed at day 90 for DFS evaluation, both in the whole cohort of patients and in patients undergoing allo-BMT in CR1, including all patients alive and achieving CR after one or two induction cycles. A Cox Proportional Hazard Model was built for multivariate survival analysis, including only the variables that respected proportional risk assumption. All two-tailed p-values < 0.05 were considered statistically significant [34].

All analysis has been performed on IBM SPSS® v22 running on Debian (Linux) operating system.

2.3. Molecular analysis

NPM1 mutation (*NPM1-A*, *B* and *D* mutation) was measured using Muta Quant Kit Ipsogen from Qiagen [35].

FLT3-ITD allelic burden was determined as ratio of Time PCR were performed on DNA Engine Opticon 2 - BIORAD. *FLT3-ITD* mutations were searched using polymerase chain reaction (PCR the area under the curve "*FLT3-ITD*" divided by AUC "FLT3-wild type" (low allelic ratio < 0.5; high allelic ratio > 0.5) [36,37].

CEBPA-dm were detected by genomic DNA PCR and direct sequencing. The primer sets are those designed by Pabst et al. [6] There are three overlapping primer pairs were used to amplify the entire coding region of human CEBPA: CEBPA AF-TCGCCATGCCGGGAGAACTCT AAC, CEBPA ARAGCTGCTTGGCTTCATCCTCCT (548bp); CEBPA BF-CCGCTGGTGATCAAGCAGGA, CEBPA BR-CCGGTACTCGTTGCTGTTCT (390bp); CEBPA CFCAAGGCCAAGAAGTCGGTGGACA, CEBPA CR-CACGGTCTGGGCAAGCCTCGAGAT (356bp). PCR reactions were made in a final volume of 50 μL containing genomic DNA (300 ng), KCl (50 mmol/L), Tris-HCl (20 mmol/L, pH 8.4), MgCl2 (2.5 mmol/L), 5 vol % DMSO, primers (2 mmol/L of each), nucleotides (0.1 mmol/L of each), and Taq DNA polymerase (1U). PCR conditions were 94 °C for 45 s, 62 °C for 45 s and 72 °C for 45 s for 45 cycles, with a final step for 10 min at 72 °C. PCR products were sequenced using BigDye.

Terminator Cycle Sequencing Kit o v1.1 kit (Applied Biosystems) on ABI 3730 Genetic Analyzer (Applied Biosystems).

2.4. Flow cytometry

Erythrocyte-lysed whole BM samples obtained at diagnosis were analyzed with a broad panel of monoclonal antibodies to define lineage according to WHO classification and to identify the most relevant aberrations described in blasts (leukemia-associated immunophenotype (LAP), as described elsewhere [23,24]. A broad combination of monoclonal antibodies in eight color staining (FITC, fluorescein isotiocyanate/PE phycoerythrin/ PerCP-Cy™5.5, peridinin-chlorophyll proteins-cychrome 5.5/APC, allophycocyanin/BD™ APC-H7, allophycocyanin/BD Horizon V450™/BD Horizon V500™) were used at diagnosis. An expression on more than 25% of leukemic cells was considered as positive. All antibodies were purchased from BD Biosciences (San Jose, CA, USA) except for Polyclonal Rabbit Anti-Human lysozyme from DAKO (Milan, Italy). At least two antibody combinations found relevant at diagnosis, were used to track residual leukemic cells during follow up. Data were acquired using BD FACSCAnto II Flow Cytometer (BD Bioscience, San Jose, CA). Instrument performance over time was assessed by BDTM Cytometer Setup and Tracking Beads.

We define a positive expression of an antigen if expressed > 20%. The antigens were considered expressed if present in at least one of the leukemic populations.

Basing on previous reports on IF features of *CEBPA-dm* AML, a comprehensive flow-cytometry-based *CEBPA-dm* score was created, assigning one point each for expression of HLA DR, CD7, CD13, CD15, CD33, CD34 and one point for lack of expression of CD14 [8,17].

3. Results

CEBPA-dm score was 7 in 2 patients, (4%), 6 in 16 (32%), 5 in 22 (44%), 4 in 6 (24%), 3 in 2 (4%), and 2 in 2 cases (4%). The flow cytometry data from a representative case of a patient with a *CEBPA-dm* score of 7/7 is depicted in Fig. 1.

A score of 6 or greater was significantly correlated with the presence of *CEBPA-dm* ($p < 0.05$), whereas no *CEBPA-dm* was recorded among patients with a score less than 6.

The positive predictive value (PPV) for a score greater than 5 was 9/18 (50%), whereas the negative predictive value (NPV) for a score lower than 6 was 100% (Table 1).

Four out of the 6 patients with a *CEBPA* score of 6 with a positive CD7 had *CEBPA-dm*. The PPV for a score > 5 including the CD7 expression was 75%. In this series, 60-day mortality was 6%. Main causes of early death were uncontrolled infections or bleedings. In surviving patients, CR rate was 40/47 (85%).

Among 9 patients with biallelic mutation, 6, 2 and 1 patients had N + C, C + C, and N + N mutations, respectively. The two patients with a CEBPA score of 7/7 had a N + C and a C + C mutation, whereas the patients with a score of 6/7 had a N + C, C + C and N + N in 5, 1 and 1 cases, respectively.

None of the analyzed variables significantly correlated with complete response (CR) probability, although *CEBPA-dm* patients and patients with *CEBPA-dm* score ≥ 6 had a trend toward higher CR rate (Table 2).

With a median follow-up of 62 months (IC 95%: 39.89–84.10

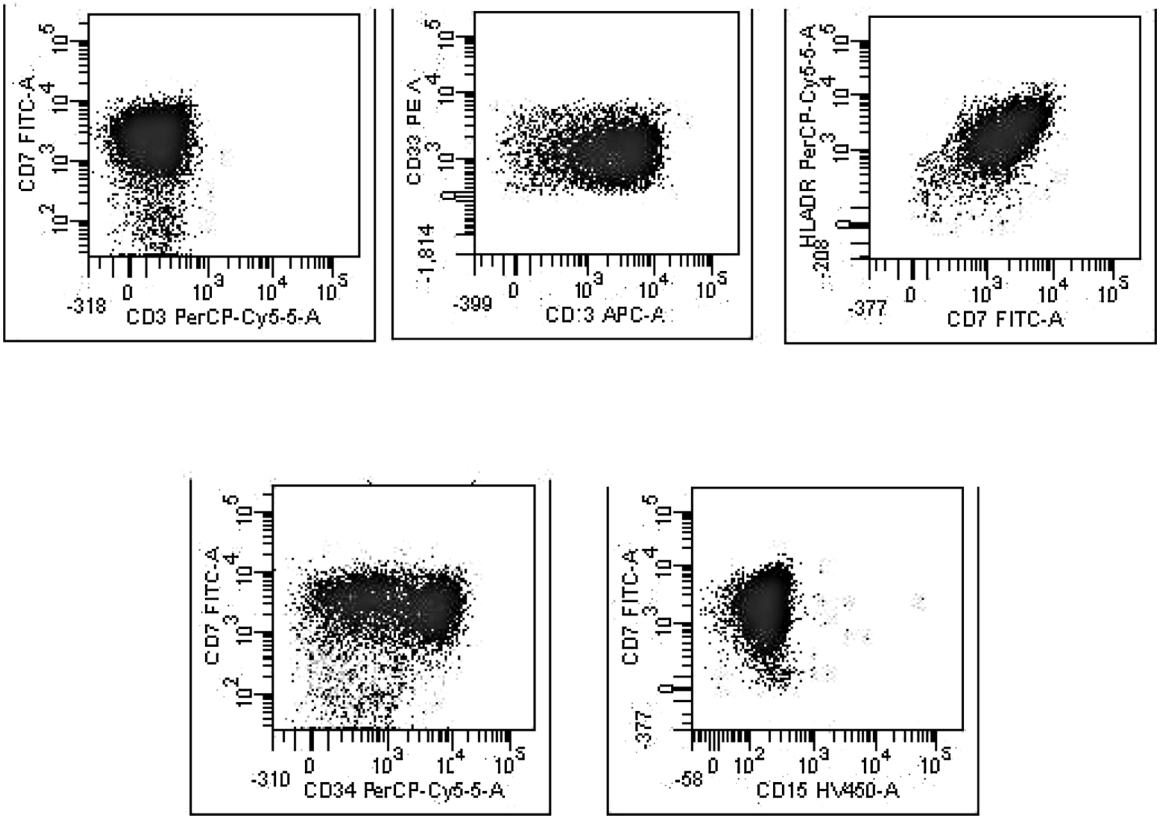

Fig. 1. Flow cytometry data from a patient with CEBPA-*dm* score of 7/7.

Table 1
Correlation between *CEBPA-dm* score and biallelic *CEBPA* mutation.

CEBPA-dm Score	Number pts.	*CEBPA* MUTATED
7	2 (4%)	2/2 (100%)
6	16 (32%)	7/16 (43.8%)
5	22 (44%)	0
4	6 (12%)	0
3	2 (4%)	0
2	2 (4%)	0

Table 2
CR probability in evaluable patients.

	CR-RATE	p
ALL PATIENTS	**40/47**	
CEBPA-dm	10/10 (100%)	0.318
***CEBPA* wild type**	30/37 (80.1%)	
***FLT3-ITD* -negative**	36/42 (85.7%)	0.571
***FLT3-ITD* -positive**	4/5 (80.0%)	
***CEBPA-dm* score ≥ 6**	16/17 (94.1%)	0.396
***CEBPA-dm* score < 6**	24/30 (80.0%)	
Sex -male	22/26 (84.6%)	1.00
Sex -female	18/21 (85.7%)	
Age < 45	12/15 (80.0%)	0.664
Age > 45	28/32 (87.5%)	
WBC < 30000/µl	30/34 (88.2%)	0.377
WBC > 30000/µl	10/13 (76.9%)	

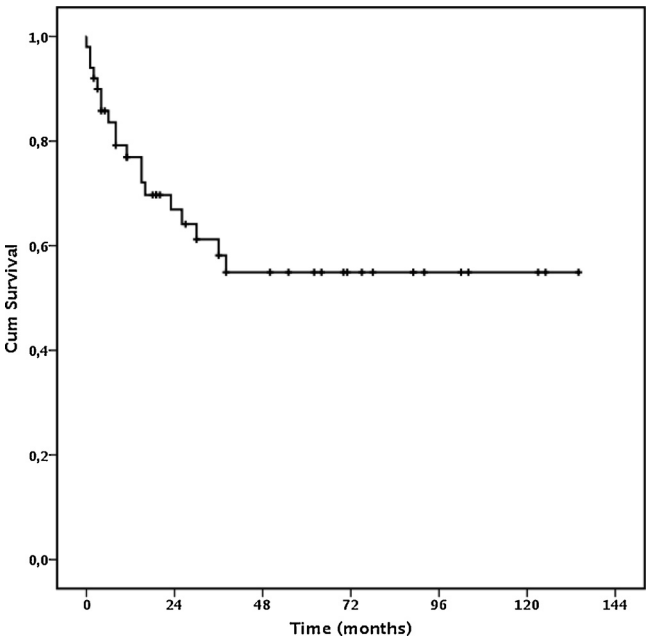

Fig. 2. Overall Survival in all patients.

months), 3-year Overall Survival (OS) was 54.9% (median not reached, Fig. 2).

Patients with *CEBPA-dm* had better outcome if compared to un-mutated patients (3-year OS was 74.1% and 51.5% in patients with or without *CEBPA-dm*, respectively, $p < 0.02$, Fig. 3).

Patients with *FLT3-ITD* had a shorter survival (3-year OS was 16.7% and 64.2% in patients with or without *FLT3-ITD* mutation, respectively $p < 0.003$, Fig. 4).

WBC count at diagnosis only exerted a borderline influence on OS (3-year OS was 37.8 and 66.7%, in patients with WBC at diagnosis higher or lower than 30000/µl, respectively, $p = 0.057$). OS was not influenced by sex, age and *CEBPA-dm* score.

Multivariate OS analysis showed that *CEBPA-dm* ($p < 0.02$) and *FLT3-ITD* ($p < 0.01$) were the strongest independent predictors of OS.

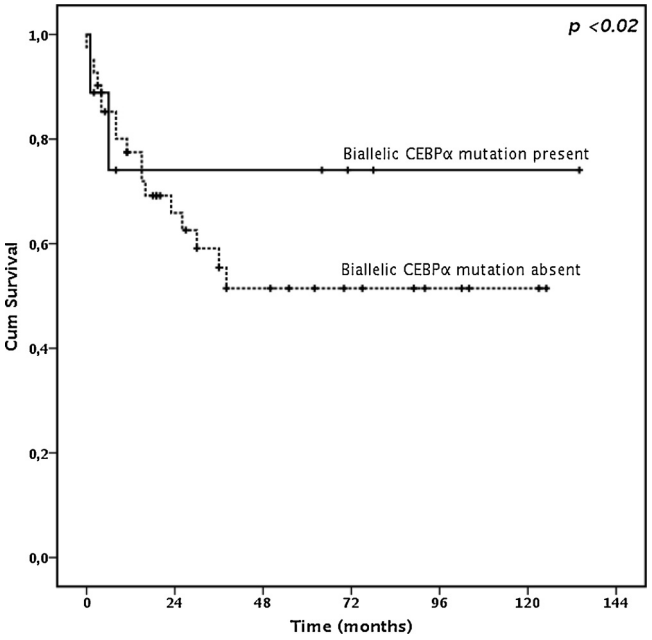

Fig. 3. Overall Survival according to biallelic *CEBPA* mutation status.

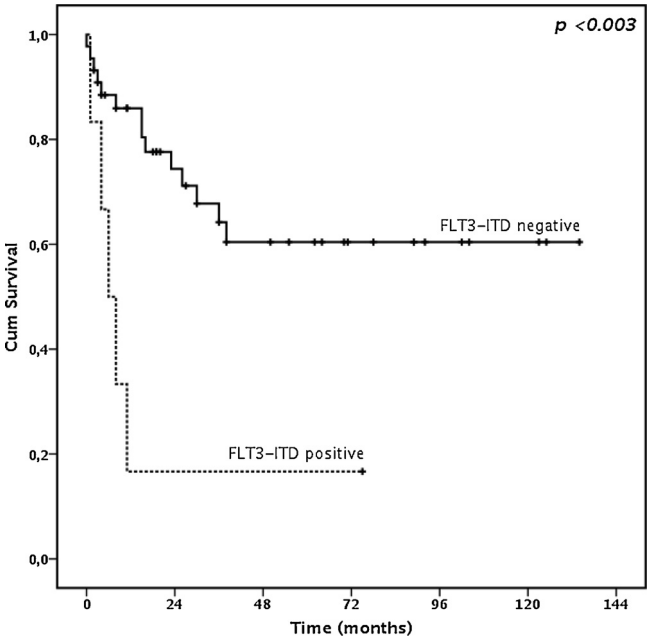

Fig. 4. Overall survival according to *FLT3-ITD* mutational status.

Detailed analysis of OS is provided in Table 3.

Table 3
Overall Survival Analysis.

	Median OS (months)	3-Year OS (% alive)	p univariate (multivariate)
ALL PATIENTS	NR	54.9	–
CEBPA-dm	NR	74.1%	0.015
***CEBPA* - wild type**	NR	51.5%	(0.015)
***FLT3-ITD* -negative**	NR	64.2%	0.002
***FLT3-ITD* -positive**	6	16.7%	(0.005)
***CEBPA* score ≥ 6**	NR	58.8%	0.700
***CEBPA* score < 6**	NR	55.9%	(-)
Sex male	NR	61.2%	0.397
Sex female	38	54.9%	(-)
Age < 45 yo	NR	60.8%	0.469
Age > 45 yo	NR	57.1%	(-)
WBC < 30000/µl	NR	66.7%	0.057
WBC > 30000/µl	15	37.8%	(0.165)

4. Discussion

In some AML subgroups, the correlation between cytogenetic and molecular alterations and IF has been reported, [29,31]. Few studies have specifically correlated immunophenotypic features with *CEBPA* mutations [17,26,28,30].

In this paper, we combined the single phenotypic aberrations, already reported in patients with *CEBPA-dm,* and developed a simple 7-antigens IF score correlating with the presence of *CEBPA-dm* in a cohort of cytogenetically normal, de novo AML patients. A score of 7 had a very high sensitivity in identifying *CEPBA-dm* but most patients with *CEBPA-dm* had a score of 6. Our data seem to indicate that CD7 expression is more important than other IF markers, as patients with a score of 6 including CD7 expression had an increased probability of harboring *CEBPA-dm*. This observation is consistent with previous reports showing that biallelic *CEBPA* mutation by itself could lead to aberrant expression of CD7 in myeloid cells [17,28,38].

Most importantly, no patients with a *CEBPA-dm* score lower than 6 had the mutation (NPV 100%). Early studies mainly proposed the use of *CEBPA-dm* associated immunophenotypic features for subsequent minimal residual disease evaluation [7,8]. More recently Mannelli et al. disclosed a strong positive correlation between a combination of six antigens and a particular side scatter value with the presence of *CEBPA-dm* and suggested the use of IF analysis to promptly identify patients harboring the mutation [28]. Our seven-points score, albeit the small number of patients included in our series, was able to identify patients harboring CEBPA-dm but proved most useful in disclosing AML patients with no probability of having *CEBPA-dm*. Moreover, a combination based only on antigen expression may potentially improve the reproducibility. A screening strategy based on *CEBPA-dm* score, thanks to its very high NPV, has a very low risk of missing AML patients with *CEBPA-dm* and may be helpful in centers where the molecular screening for *CEBPA-dm* cannot be promptly performed in all newly diagnosed patients. Furthermore, as IF is routinely performed at diagnosis, the application of the *CEBPA-dm* score will not increase cost or time expenditure.

Differently from what was described in other AML subtypes [23,29,39], the *CEBPA-dm* score alone did not show prognostic significance in our study cohort. Conversely, our results underline that the presence of molecular aberrations such as *FLT3-ITD* mutation or *CEBPA-dm* are more relevant than the combined expression of surface markers.

Overall, our study suggests that patients with a *CEBPA-dm* score ≥ 6 should immediately undergo Sanger sequencing for CEBPA-dm, whereas in the other patients this complex evaluation might be delayed (or even omitted?) due to very low probability of finding the mutation. This could optimize prognostic stratification work up by giving the right priority to the sample evaluation.

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