Ann Hematol
DOI 10.1007/s00277-011-1234-z

ORIGINAL ARTICLE

Fragment length analysis screening for detection of *CEBPA* mutations in intermediate-risk karyotype acute myeloid leukemia

Oscar Fuster · Eva Barragán · Pascual Bolufer · Esperanza Such · Ana Valencia · Mariam Ibáñez · Sandra Dolz · Inmaculada de Juan · Antonio Jiménez · Maria Teresa Gómez · Ismael Buño · Joaquín Martínez · José Cervera · Pau Montesinos · Federico Moscardó · Miguel Ángel Sanz

Received: 9 December 2010 / Accepted: 4 April 2011

Abstract During last years, molecular markers have been increased as prognostic factors routinely screened in acute myeloid leukemia (AML). Recently, an increasing interest has been reported in introducing to clinical practice screening for mutations in the CCAAT/enhancer-binding protein α (CEBPA) gene in AML, as it seems to be a good prognostic factor. However, there is no reliable established method for assessing *CEBPA* mutations during the diagnostic work-up of AMLs. We describe here a straightforward and reliable fragment analysis method based in PCR capillary electrophoresis (PCR-CE) for screening of *CEBPA* mutations; moreover, we present the results obtained in 151 intermediate-risk karyotype AML patients (aged 16–80 years). The method gave a specificity of 100% and sensitivity of 93% with a lower detection limit of 1–5% for *CEBPA* mutations. The series found 19 mutations and four polymorphisms in 12 patients, seven of whom (58%) presented two mutations. The overall frequency of *CEBPA* mutations in AML was 8% (n=12). *CEBPA* mutations showed no coincidence with *FLT3-ITD* or *NPM1* mutations. *CEBPA* mutation predicted better disease-free survival in the group of patients without *FLT3-ITD*, *NPM*, or both genes mutated (HR 3.6, IC 95%; 1.0–13.2, p=0.05) and better overall survival in patients younger than 65 of this group without molecular markers (HR 4.0, IC 95%; 1.0–17.4, p=0.05). In conclusion, the fragment analysis method based in PCR-CE is a rapid, specific, and sensitive method for *CEBPA* mutation screening and our results confirm that *CEBPA* mutations can identify a subgroup of patients with favorable prognosis in AML with intermediate-risk karyotype.

Keywords *CEBPA* · Mutations · Screening · Intermediate risk · Acute myeloid leukemia

O. Fuster · E. Barragán (✉) · P. Bolufer · S. Dolz · I. de Juan
Laboratory of Molecular Biology,
Department of Medical Pathology, Hospital Universitario La Fe,
Escuela de enfermería 7ª planta., Avd. Campanar 21,
Valencia 46009, Spain
e-mail: barragan_eva@gva.es

E. Such · A. Valencia · M. Ibáñez · J. Cervera · P. Montesinos · F. Moscardó · M. Á. Sanz
Hematology Department, Hospital Universitario La Fe,
Valencia, Spain

A. Jiménez
Hematology Department, Hospital Universitario Carlos Haya,
Málaga, Spain

M. T. Gómez
Hematology Department, Hospital Universitario Dr Negrín,
Las Palmas, Spain

I. Buño
Hematology Department, Hospital Gregorio Marañon,
Madrid, Spain

J. Martínez
Hematology Department, Hospital Universitario 12 de Octubre,
Madrid, Spain

Introduction

Somatic-acquired mutations are frequently detected in acute myeloid leukemia (AML). Besides their role in pathogenesis, genetic alterations in AML are known to be major determinants of the patient's response to therapy and its outcome. In fact, assessment of the

Published online: 03 May 2011

presence of internal tandem duplications of the FLT3 receptor gene (*FLT3-ITD*) [1] and mutations in the nucleophosmin gene (*NPM1*) [2] is currently a routine practice in guiding therapeutic decisions in AML patients with normal karyotype [3].

More recently, an increasing interest has been reported in the *CEBPA* gene encoding the CCAAT/enhancer-binding protein α (*CEBPA*), a leucine zipper transcription factor involved in the balance between cell proliferation and terminal differentiation. *CEBPA* consist of a 42-KDa protein which presents in C-terminal a leucine zipper domain (BZIP) mediating homo- or heterodimerization and the DNA-binding domain able to interact with specific DNA sequences; in N-terminal it presents the regulatory and transactivating domains TAD1 and TAD2 [4]. The main role of *CEBPA* protein in hematopoiesis is the development of granulocytes and plays a pivotal role in myeloid differentiation [5, 6]. *CEBPA* knockout mice results in selective block in early granulocyte maturation [7] and accumulation of myeloid blasts in the bone marrow [8] suggesting that *CEBPA* inactivation plays a central role in leukemogenesis.

CEBPA inactivation may take place by acquired mutations which may occur along the entire coding region. These mutations lead to increase translation of an alternative 30-KDa form with dominant negative activity on the full length 42-KDa protein when occur in N-terminal while C-terminal mutations result in deficient DNA binding and/or homodimerization activities [4, 9]. Mutations in *CEBPA* occur between 5% and 14% of AML [10] and seem to be associated with a good prognosis in AML with intermediate-risk karyotype [11, 12], although recent papers suggest that this favorable outcome could be restricted to those patients harboring double *CEBPA* mutations [13, 14]. *CEBPA* mutations screening has been previously performed by different methods including PCR single strand conformational polymorphism [15], DHPLC [13], fragment analysis [16, 17], and sequencing [11, 18–20], the most commonly used method which is labor extensive, time consuming, and an expensive procedure. AML with mutated *CEBPA* has been included as a provisional entity in the 2008 World Health Organization classification of leukemias [21] and a simple, sensitive, and accurate method for detecting *CEBPA* mutations is required in routine practice. However, the GC-rich coding region of this gene makes it difficult to develop a reliable method to detect *CEBPA* mutations during diagnostic work-up of patients with AML.

We describe here a straightforward and reliable fragment analysis method based in PCR capillary electrophoresis (PCR-CE) for screening *CEBPA* mutations and the results obtained in a cohort of 151AML adult patients with intermediate-risk karyotype.

Materials and methods

Patients and treatment

The study included 151 adult patients (aged 16–80 years) diagnosed with de novo AML with intermediate-risk karyotype in five Spanish institutions (Hospital Universitario La Fe, Hospital Universitario Carlos Haya, Hospital Universitario Dr Negrín, Hospital Universitario Ramón y Cajal and Hospital Universitario 12 de Octubre) between 2002 and 2009. Patients were retrospectively selected according to the availability of DNA and RNA. The leukemia subtypes were established according to the morphological and cytochemical criteria of the French–American–British classification [22], the karyotyping was performed according to standard cytogenetic methods and cytogenetic risk stratification was established according to the refined Medical Research Council (MRC) criteria [23] (Table 1).

One hundred and thirty-two patients were treated with intensive chemotherapy in which induction consisted of a combination of anthracycline plus cytarabine with or without etoposide following the PETHEMA LMA 99 and 2007 trials. Ninety-seven out of the 132 reached complete remission after induction therapy, and 93 of those patients presented follow-up available. The institutional ethics committee for clinical research approved this study, and a written informed consent in accordance with the recommendations of the Declaration of Human Rights, the Conference of Helsinki, and institutional regulations was obtained from all patients.

Samples

The *CEBPA* and *FLT3-ITD* mutation study was performed on DNA and the *NPM1* mutation study on cDNA obtained from bone marrow samples collected at diagnosis. The DNA was isolated using MagNA Pure LC large volume DNA Isolation Kit (Roche, Mannheim, Germany) and mRNA using MagNA Pure LC mRNA HS Kit (Roche) using the automatic MagNA Pure LC System (Roche Molecular Biochemicals, Indianapolis, IN). For cDNA synthesis, 6 μL of mRNA were reverse-transcribed in a 25 μL reaction volume using random hexamer primers with the TaqMan Gold reverse transcription–polymerase chain reaction Kit (PE Applied Biosystems, Foster City, CA, USA) following the manufacturer's protocol.

Molecular analyses

The functional domains of *CEBPA* gene (Gene Bank Accession U34070) were amplified by PCR with forward primers 5′ end-labeled with carboxyfluorescein (FAM). The

Table 1 Clinical characteristics of intermediate-risk karyotype AML patients

Characteristics	Median (range)	No. (%)
Sex		
Male		77 (51)
Female		74 (49)
Age, years	56 (16–80)	
Younger than 65		112 (74)
65 and older		39 (26)
FAB		
M0		14 (9)
M1		28 (19)
M2		38 (25)
M4		18 (12)
M5		21 (14)
M6		7 (5)
M7		2 (1)
Missing data		23 (15)
Karyotype		
Normal		109 (72)
Alterations		42 (28)
+8		9 (21)
t(6; 9)		3 (7)
t(9; 11)		2 (5)
Complex (<4 alterations)		3 (7)
Others[a]		25 (60)
Molecular markers		
FLT3-ITD+/NPM1-		10 (7)
FLT3-ITD-/NPM1+		27 (18)
FLT3-ITD+/NPM1+		17 (11)
FLT3-ITD-/NPM1-		97 (64)
White blood cell count, $\times 10^9$/L	8 (1–342)	
Platelet count, $\times 10^9$/L	67 (3–336)	
Hemoglobin, g/dL	9 (4–17)	
Peripheral blast cell count, %	52 (2–100)	

FAB French–American–British classification

[a] Comprises other cytogenetic alterations included in the intermediate prognostic group of the MRC classification with only one case each

primers used for transactivation domain 1 (TAD1) were F: 5′–TCGGCCGACTTCTACGAGGC–3′ and R: 5′–CCCCGACGCGCTCGTACAGG–3′; for transactivation domain 2 (TAD2) F: 5′–TACCTGGACGGCAGG CTGGA–3′ and R: 5′–TGCAGGTGCATGGTGGTCT–3′; and for the basic and leucine zipper region (BZIP) F: 5′–AGAAGTCGGTGGACAAGAACAGCAA–3′ and R: 5′–AGTTGCCCATGGCCTTGA–3′.

Forty nanograms of DNA was amplified in a 25-μL reaction containing 0.5 μM of each primer, 0.25 mM deoxynucleotide triphosphates (dNTPs), 2 mM $MgCl_2$, 1.5 U Taq Expand High Fidelity (Roche, Mannheim, Germany) and 7% dimethyl sulfoxide (DMSO) for the TAD1 and BZIP fragments, and 0.5 μM of each primer, 0.25 mM dNTPs, 1.5 mM $MgSO_4$, 1× Pfx Amp Buffer, 1× PCR Enhancer Solution, 2 U Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA, USA) and 10% DMSO for the TAD2 fragment.

The PCR program consisted of an initial denaturation at 95°C for 3 min followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 40 s and extension at 72°C for 1 min. A final step of 94°C for 30 s and 60°C for 45 min was performed. Afterwards, 2 μL of PCR products was mixed with 12 μL formamide and 0.5 μL ROX500 internal size standard. The mixtures were heated to 94°C for 5 min and then electrophoresed on an ABI PRISM 3130 Genetic Analyzer. The results were analyzed using the GeneMapper software (Applied Biosystems).

The results were confirmed by sequence analysis using ABI PRISM terminator cycle sequencing kit v1.1 (Applied Biosystems) on the ABI PRISM 3130 Genetic Analyzer.

To assess the lower detection limit of the capillary electrophoresis, we made serial dilutions of mutant samples with normal DNA, and the percentage of mutant was calculated as the area of the mutant peak with respect to the total area (mutant plus wild-type peaks).

The studies of *FLT3-ITD* and *NPM1* mutations were performed according to the methods of Thiede et al. [1] and Schnittger et al. [24], respectively.

Sequencing of PCR products

To confirm the variations detected by capillary electrophoresis, we performed PCR of the fragments using the same primers but without fluorescence. The PCR products were mixed with ExoSap-IT (USB Corporation, Cleveland, OH, USA) to remove the remaining primers and sequenced bidirectionally with forward and reverse primers using ABI PRISM terminator cycle sequencing kit v1.1 (Applied Biosystems) on the ABI PRISM 3130 Genetic Analyzer (Applied Biosystems).

Statistical analysis

The association between the presence of *CEBPA* mutations and patient characteristics was performed with the Mann–Whitney test for continuous variables and with χ^2 or Fisher's exact test for qualitative variables.

A Kaplan–Meier analysis was used to estimate the distributions of disease-free survival (DFS) and overall survival (OS) [25]; the differences between the two survival distributions were analyzed using the log-rank test [26]. Multivariate analysis was performed using the Cox proportional hazards regression model [27].

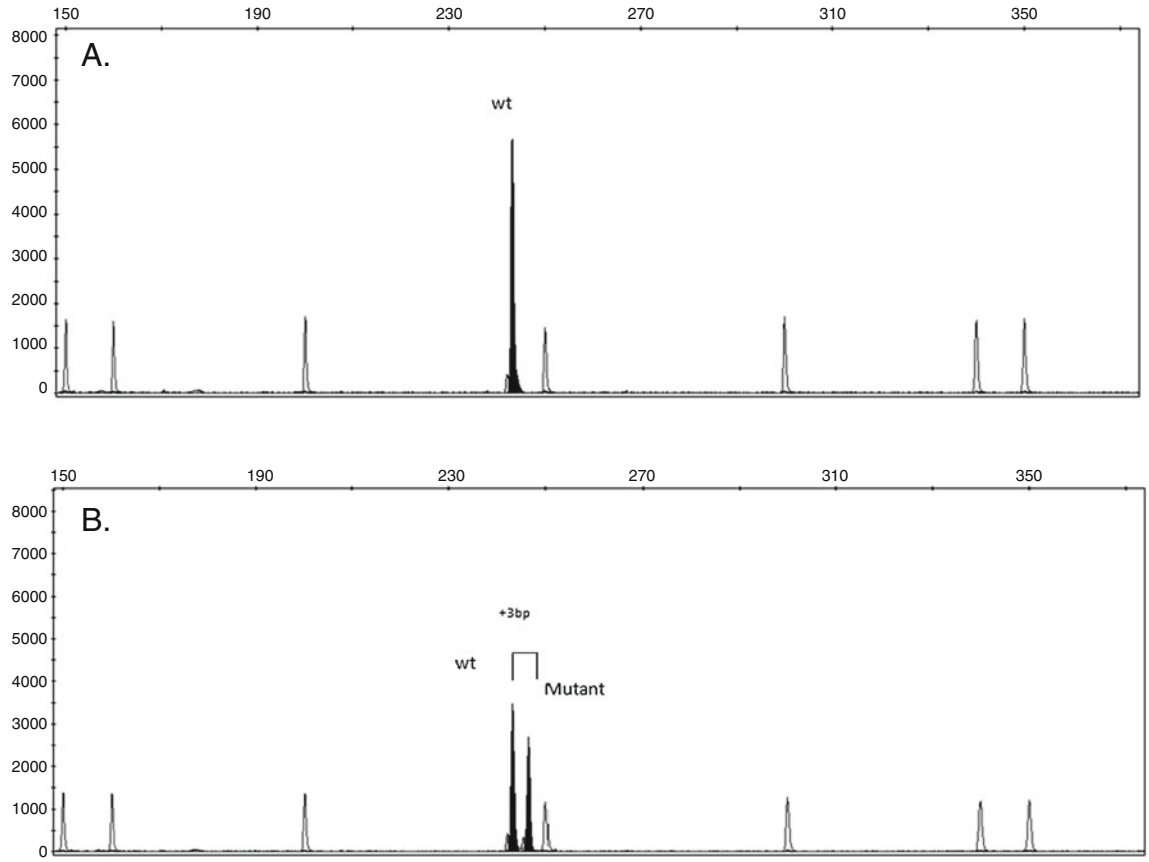

Fig. 1 Fragment analysis method based in capillary electrophoresis for *CEBPA* detection. *Dark peaks* correspond to the *CEBPA* fragment detection and *clear peaks* correspond to the marker size standard. **a** Wild-type patient showing the amplicon size expected. **b** Mutated patient showing 3 bp additional to the wild-type

OS was estimated from the date of diagnosis, while DFS was estimated from the date of complete remission (CR). The endpoints for OS were death (failure) and alive at last follow-up (censored). For DFS, the endpoints were relapse (failure) or death in CR (failure), whichever occurred first, and alive at last follow-up (censored). Patient follow-up was updated on June 30, 2010. All tests were two-sided and a p value$\leq$0.05 was considered significant. Computations were performed using the SPSS v12.0 statistical package (Chicago, IL).

Results

Fragment analysis screening and characterization of *CEBPA* mutations

From 151 patients screened for *CEBPA* mutations by capillary electrophoresis, 138 showed a single peak in the electropherogram, corresponding to the wild-type allele (Fig. 1a). Thirteen patients presented peaks additional to the wild type in one or more *CEBPA* fragments, suggesting the presence of genetic variations (Fig. 1b). Sequencing confirmed seven TAD1 mutations, 11 BZIP mutations, and four TAD2 polymorphisms. These findings indicate that capillary electrophoresis detected 18 mutations in 11 patients. The TAD1 mutations consisted of duplications or insertions/deletions generating a truncated protein, the BZIP mutations included mostly in-frame duplications or in-frame insertions/deletions, and the variation detected in TAD2 fragment was the polymorphism c.1175_1180dup acccgc (Table 2).

All samples were sequenced in parallel with the capillary electrophoresis. Direct sequencing confirmed all *CEBPA* mutations detected by capillary electrophoresis and showed an additional point mutation (c.1583T>G) in the BZIP domain in a patient with a TAD2 polymorphism. In the remaining patients, no additional mutations were detected. Therefore, the series demonstrated a total of 19 mutations in 12 patients. All these mutations were detected with a lower detection limit in capillary electrophoresis of 5% for TAD1, 1% for TAD2, and 2% for BZIP.

Table 2 *CEBPA* mutations detected in AML with intermediate-risk karyotype

UPN	TAD1		TAD2		BZIP	
	Nucleotide change	Amino acid change	Nucleotide change	Amino acid change	Nucleotide change	Amino acid change
1					c.1499_1516dup(18 pb)	A303_V308dup
2					c.1525_1527dup(3 pb)	Q312dup
3			c.1175_1180dup(6 pb)	P194_H195dup	c.1583 T>G	L331R
4			c.1175_1180dup(6 pb)	P194_H195dup	c.1525_1527dup(3 pb)	Q312dup
5					c.1532dup T	L315fsX320
6	c.652_653dup(2 pb)	S21fsX160			c.1525_1527del(3 pb)	Q312fsX358
7	c.779_782dup(3 pb)	I68fsX108			c.1520_1521ins(3 pb)	T310_Q311insL
8	c.838del(1 pb)	Q83fsX159			c.1518_1553dup(36 pb)	E309_D320dup
9	c.790_791dup(2 pb)	I68fsX160			c.1497_1538dup(42 pb)	A303_L315dup
					c.1538_1539ins(3 pb)	L315_E316insDR
10	c.858 del(1 pb)	K90fsX159	c.1175_1180dup(6 pb)	P194_H195dup	c.1500_1501ins(3 pb)	A303_K304insF
11	c.698del(1 pb)	G36fsX159			c.1507_1530dup (24 pb)	R306_K313dup
12	c.868del(1 pb)	A93fsX158			c.1528_1530dup(3 pb)	K313dup
13			c.1175_1180dup(6 pb)	P194_H195dup		

AML acute myeloid leukemia, *UPN* unique patient number, *TAD*1 transactivation domain 1, *TAD*2 transactivation domain 2, *BZIP* leucine zipper domain

Incidence of *CEBPA* mutations and relationship with molecular markers and clinical characteristics

The 12 patients with *CEBPA* mutations in some of the fragments represented an overall incidence of 8% of the AML patients with intermediate-risk karyotype. Seven of 12 patients (58%) had two mutations, combining TAD1 and BZIP mutations, and the remaining five patients (42%) had a single mutation in the BZIP domain.

Twenty-eight patients (19%) harbored *FLT3-ITD*, 44 (29%) *NPM1* mutations and 55 (36%) patients had at least one mutation in *NPM1* or *FLT3-ITD*. The presence of *CEBPA* mutations showed no coincidence with the presence of *NPM1* or *FLT3-ITD*. We did not find an association between *CEBPA* mutations and age, sex, white blood cell count or blast cell count.

CEBPA mutations and disease outcome

CR was achieved in 74% of patients with AML intermediate-risk karyotype: 100% in those with mutated *CEBPA* and 72% in those with no mutation (p=0.11).

With a median follow-up of 18 months (range, 1–103 months), the OS and DFS at 2 years were 39% and 37%, respectively. The OS of patients with mutated and nonmutated *CEBPA* was 67% and 41%, respectively (p=0.07), while the DFS was 65% and 39% (p=0.15). When the analysis of OS and DFS was restricted to patients without *FLT3-ITD* and *NPM1* mutations, patients with *CEBPA* had a significantly higher OS than those without the mutation (p=0.04) (Fig. 2) and showed a trend to higher DFS (p=0.06).

Cox's multivariate analysis showed that age (>65 years) influenced OS (HR, 2.2, IC 95%; 1.1–4.4, p=0.02). In patients younger than 65 years, the presence of *CEBPA* mutations was the only independent favorable prognostic factor for OS (HR 4.0, IC 95%; 1.0–17.4, p=0.05). For DFS, *CEBPA* mutation was the only prognostic factor identified (HR 3.6, IC 95%; 1.0–13.2, p=0.05).

Discussion

This study presents a reliable fragment analysis method based in the PCR-CE for screening for *CEBPA* mutations

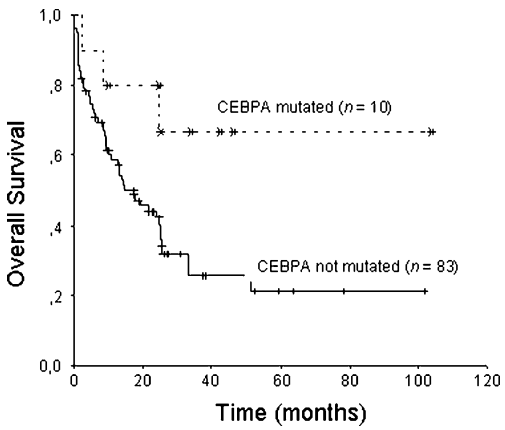

Fig. 2 Kaplan–Meier curve for overall survival according to *CEBPA* status in AML patients with intermediate-risk karyotype and without *FLT3-ITD* and *NPM1* mutations

and confirms the good prognosis of AML intermediate-risk karyotype patients with *CEBPA* mutations. Our data show that the fragment analysis method reported here allows the distinction of wild-type DNA from other DNA harboring *CEBPA* mutations in the N-terminal TAD1 and TAD2 fragments and the C-terminal BZIP.

The method is specific and sensitive enough for *CEBPA* mutation screening because no false positive or negative results were found when nucleotide length variations were considered. Taking into account point mutations, the method had a specificity and sensitivity of 100% and 93% (12/13), respectively. The only false negative detected was a single nucleotide substitution c.1583T>G in a patient who also had a TAD2 polymorphism. The low incidence of nucleotide substitutions (0.7%), in line with that previously reported in other series [16, 20], indicates that the sensitivity of the method is adequate for *CEBPA* mutation screening in the clinical laboratory.

The lower detection limit of the method described here (5% for TAD1, 1% for TAD2, and 2% for BZIP mutations) is even lower than that of nucleotide sequencing (20%), which is the most commonly used method for *CEBPA* mutation detection [9, 20]. Other relevant advantages of the fragment analysis are rapidity and low cost. In contrast, nucleotide sequencing is a time-consuming and expensive method, requiring purification, two bidirectional sequencing reactions, and the interpretation of the electropherogram.

Our study confirms that *CEBPA* mutations are clustered around two major hotspots: the N-terminus including TAD1 and the region before TAD2, and the C-terminus comprising BZIP. In TAD2, however, the polymorphism c.1175_1180dup acccgc was the only variation detected, which indicates that mutations in this fragment are uncommon [16, 20, 28, 29]. We have also confirmed that frameshift mutations are preferentially clustered in the TAD1 N-terminal while in-frame mutations are located more frequently in the BZIP C-terminal fragments [13, 20]. Additional advantages of our method, compared with other fragment analysis methods based in capillary electrophoresis, are the coverage of the area between TAD1 and TAD2 [17] where many mutations have been reported [16, 29] and the requirement for fewer primer pairs, making it cheaper and less time consuming [16].

The incidence of *CEBPA* mutations found in this study in patients with intermediate-risk karyotype AML (8%) was similar to that previously reported [13, 14, 20, 28], increasing to 12% in the subgroup of patients without *FLT3-ITD* and *NPM1* mutations. The lack of association of mutated *CEBPA* in patients harboring *FLT3-ITD* or *NPM1* mutations makes the routine screening of *CEBPA* in these particular patients unnecessary, as suggested by others [4, 13, 30].

The favorable outcome in the present study of patients harboring *CEBPA* mutations, particularly those younger than 65 years, is in line with previous reports [11, 18, 20, 30]. We were unable to demonstrate the distinct impacts of single and double mutations of *CEBPA* in the outcomes, as has been recently reported [13, 14], probably because of the relatively low incidence of mutations and the limited sample size.

In conclusion, fragment analysis is a rapid, specific, and sensitive method for *CEBPA* mutation screening. The interest in implementing this technique in routine practice lies in the favorable prognosis of patients with AML-harboring *CEBPA* mutations, especially in those with normal or intermediate karyotype and without *FLT3-ITD* and *NPM1* mutations.

Acknowledgments This study was partially supported by the grants "Fondo de Investigación Sanitaria, Instituto de Salud Carlos III" 06/0657, "Red Temática de Investigación Cooperativa en Cancer" RD06/0020/0031, "Ministerio de Ciencia e Innovación" BES2008-0080539, "Contrato de técnicos de apoyo a la investigación en el Sistema Nacional de Salud" CA08/00141, and "X Contratos de investigación para postresidentes-Instituto de Investigación Sanitaria, Fundación de Investigación Hospital La Fe-". We also are grateful to Biobanco La Fe for supplying biological samples (RD09/0076/00021).

References

1. Thiede C, Steudel C, Mohr B, Schaich M, Schäkel U, Platzbecker U, Wermke M, Bornhäuser M, Ritter M, Neubauer A, Ehninger G, Illmer T (2002) Analysis of FLT3-activating mutations in 979 patients with acute myelogenous leukemia: association with FAB subtypes and identification of subgroups with poor prognosis. Blood 99:4326–4335
2. Falini B, Mecucci C, Tiacci E, Alcalay M, Rosati R, Pasqualucci L, La Starza R, Diverio D, Colombo E, Santucci A, Bigerna B, Pacini R, Pucciarini A, Liso A, Vignetti M, Fazi P, Meani N, Pettirossi V, Saglio G, Mandelli F, Lo-Coco F, Pelicci PG, Martelli MF (2005) GIMEMA acute leukemia working party. Nucleophosmin in acute myelogenous leukemia with a normal karyotype. N Engl J Med 352:254–266
3. Löwenberg B (2008) Diagnosis and prognosis in acute myeloid leukemia—the art of distinction. N Engl J Med 358:1960–1962
4. Renneville A, Roumier C, Biggio V, Nibourel O, Boissel N, Fenaux P, Preudhomme C (2008) Cooperating gene mutations in acute myeloid leukemia: a review of the literature. Leukemia 22:915–931
5. Radomska HS, Huettner CS, Zhang P, Cheng T, Scadden DT, Tenen DG (1998) CCAAT/enhancer binding protein alpha is a regulatory switch sufficient for induction of granulocytic development from bipotential myeloid progenitors. Mol Cell Biol 18:4301–4314
6. Tenen DG, Hromas R, Licht JD, Zhang DE (1997) Transcription factors, normal myeloid development, and leukemia. Blood 90:489–519
7. Zhang DE, Zhang P, Wang ND, Hetherington CJ, Darlington GJ, Tenen DG (1997) Absence of granulocyte colony-stimulating factor signaling and neutrophil development in CCAAT enhancer binding protein alpha-deficient mice. Proc Natl Acad Sci USA 94:569–574

8. Zhang P, Iwasaki-Arai J, Iwasaki H, Fenyus ML, Dayaram T, Owens BM, Shigematsu H, Levantini E, Huettner CS, Lekstrom-Himes JA, Akashi K, Tenen DG (2004) Enhancement of hematopoietic stem cell repopulating capacity and self-renewal in the absence of the transcription factor C/EBP alpha. Immunity 21:853–863
9. Pabst T, Mueller BU, Zhang P, Radomska HS, Narravula S, Schnittger S, Behre G, Hiddemann W, Tenen DG (2001) Dominant-negative mutations of CEBPA, encoding CCAAT/enhancer binding protein-alpha (C/EBPalpha), in acute myeloid leukemia. Nat Genet 27:263–270
10. Pabst T, Mueller BU (2007) Transcriptional dysregulation during myeloid transformation in AML. Oncogene 26:6829–6837
11. van Waalwijk B, van Doorn-Khosrovani S, Erpelinck C, Meijer J, van Oosterhoud S, van Putten WL, Valk PJ, Berna Beverloo H, Tenen DG, Löwenberg B, Delwel R (2003) Biallelic mutations in the CEBPA gene and low CEBPA expression levels as prognostic markers in intermediate-risk AML. Hematol J 4:31–40
12. Marcucci G, Maharry K, Radmacher MD, Mrózek K, Vukosavljevic T, Paschka P, Whitman SP, Langer C, Baldus CD, Liu CG, Ruppert AS, Powell BL, Carroll AJ, Caligiuri MA, Kolitz JE, Larson RA, Bloomfield CD (2008) Prognostic significance of, and gene and microRNA expression signatures associated with, CEBPA mutations in cytogenetically normal acute myeloid leukemia with high-risk molecular features: a Cancer and Leukemia Group B Study. J Clin Oncol 26:5078–5087
13. Green CL, Koo KK, Hills RK, Burnett AK, Lynch DC, Gale RE (2010) Prognostic significance of CEBPA mutations in a large cohort of younger adult patients with acute myeloid leukemia: impact of double CEBPA mutations and the interaction with FLT3 and NPM1 mutations. J Clin Oncol 28:2739–2747
14. Wouters BJ, Löwenberg B, Erpelinck-Verschueren CA, van Putten WL, Valk PJ, Delwel R (2009) Double CEBPA mutations, but not single CEBPA mutations, define a subgroup of acute myeloid leukemia with a distinctive gene expression profile that is uniquely associated with a favorable outcome. Blood 113:3088–3091
15. Snaddon J, Smith ML, Neat M, Cambal-Parrales M, Dixon-McIver A, Arch R, Amess JA, Rohatiner AZ, Lister TA, Fitzgibbon J (2003) Mutations of CEBPA in acute myeloid leukemia FAB types M1 and M2. Genes Chromosom Cancer 37:72–78
16. Benthaus T, Schneider F, Mellert G, Zellmeier E, Schneider S, Kakadia PM, Hiddemann W, Bohlander SK, Feuring-Buske M, Braess J, Spiekermann K, Dufour A (2008) Rapid and sensitive screening for CEBPA mutations in acute myeloid leukaemia. Br J Haematol 143:230–239
17. Lin LI, Lin TC, Chou WC, Tang JL, Lin DT, Tien HF (2006) A novel fluorescence-based multiplex PCR assay for rapid simultaneous detection of CEBPA mutations and NPM mutations in patients with acute myeloid leukemias. Leukemia 20:1899–1903
18. Fröhling S, Schlenk RF, Stolze I, Bihlmayr J, Benner A, Kreitmeier S, Tobis K, Döhner H, Döhner K (2004) CEBPA mutations in younger adults with acute myeloid leukemia and normal cytogenetics: prognostic relevance and analysis of cooperating mutations. J Clin Oncol 22:624–633
19. Lin LI, Chen CY, Lin DT, Tsay W, Tang JL, Yeh YC, Shen HL, Su FH, Yao M, Huang SY, Tien HF (2005) Characterization of CEBPA mutations in acute myeloid leukemia: most patients with CEBPA mutations have biallelic mutations and show a distinct immunophenotype of the leukemic cells. Clin Cancer Res 11:1372–1379
20. Preudhomme C, Sagot C, Boissel N, Cayuela JM, Tigaud I, de Botton S, Thomas X, Raffoux E, Lamandin C, Castaigne S, Fenaux P, Dombret H (2002) Favorable prognostic significance of CEBPA mutations in patients with de novo acute myeloid leukemia: a study from the Acute Leukemia French Association (ALFA). Blood 100:2717–2723
21. Vardiman JW, Thiele J, Arber DA, Brunning RD, Borowitz MJ, Porwit A, Harris NL, Le Beau MM, Hellström-Lindberg E, Tefferi A, Bloomfield CD (2009) The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes. Blood 114:937–951
22. Benett JM, Catovsky D, Daniel MT, Flandrin G, Galton DAG, Gralnick HR, Sultan C (1976) Proposed revised criteria for the classification of acute myeloid leukemia. A report of the French–American–British Cooperative group. Br J Haematol 33:451–458
23. Grimwade D, Hills RK, Moorman AV, Walker H, Chatters S, Goldstone AH, Wheatley K, Harrison CJ, Burnett AK, National Cancer Research Institute Adult Leukaemia Working Group (2010) Refinement of cytogenetic classification in acute myeloid leukemia: determination of prognostic significance of rare recurring chromosomal abnormalities among 5876 younger adult patients treated in the United Kingdom Medical Research Council trials. Blood 116:354–365
24. Schnittger S, Schoch C, Kern W, Mecucci C, Tschulik C, Martelli MF, Haferlach T, Hiddemann W, Falini B (2005) Nucleophosmin gene mutations are predictors of favorable prognosis in acute myelogenous leukemia with a normal karyotype. Blood 106:3733–3739
25. Kaplan EL, Meier P (1958) Nonparametric estimations from incomplete observations. J Am Stat Assoc 53:457–481
26. Mantel N (1966) Evaluation of survival data and two new rank order statistics arising in its consideration. Cancer Chemother Rep 50:163–170
27. Cox DR (1972) Regression models and life tables (with discussion). JR Stat Soc B 34:187–220
28. Leroy H, Roumier C, Huyghe P, Biggio V, Fenaux P, Preudhomme C (2005) CEBPA point mutations in hematological malignancies. Leukemia 19:329–334
29. Leecharendkeat A, Tocharoentanaphol C, Auewarakul CU (2008) CCAAT/enhancer binding protein-alpha polymorphisms occur more frequently than mutations in acute myeloid leukemia and exist across all cytogenetic risk groups and leukemia subtypes. Int J Cancer 123:2321–2326
30. Schlenk RF, Döhner K, Krauter J, Fröhling S, Corbacioglu A, Bullinger L, Habdank M, Späth D, Morgan M, Benner A, Schlegelberger B, Heil G, Ganser A, Döhner H (2008) Mutations and treatment outcome in cytogenetically normal acute myeloid leukemia. N Engl J Med 358:1909–1918