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

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ABSTRACT

Purpose: **The transcription factor CCAAT/enhancer binding protein α, encoded by the *CEBPA*, is crucial for the differentiation of immature granulocytes. Mutation of the *CEBPA* may play an important role in leukemogenesis and prognosis. We sought to characterize the *CEBPA* mutation in acute myeloid leukemia (AML) and to clarify if there is a distinct immunophenotype for leukemic cells with the mutation.**

Experiment Design: **One hundred and four patients with *de novo* AML were evaluated for the *CEBPA* mutation and immunophenotype of the leukemic cells.**

Results: **Twenty-two distinct mutations were identified in 16 (15%) of 104 AML patients. Fourteen patients had biallelic mutations, mostly involving both the NH_2-terminal TAD1 region and the COOH-terminal basic leucine zipper domain (bZIP). The mutations in the bZIP region were always tandem duplications and were located at hot-spot regions for topoisomerase II sites. Sequential study of the *CEBPA* mutations showed that the mutations disappeared at complete remission but the same mutations reappeared at relapse. None of the patients developed novel mutations during the follow-up period. Patients with *CEBPA* mutations had significantly higher incidences of CD7 (73%), CD15 (100%), CD34 (93%), and HLA-DR (93%) expression on the leukemic cells.**

Conclusion: **These data revealed that most AML with *CEBPA* mutations were associated with an immunophenotype of HLA-DR$^+$CD7$^+$CD13$^+$CD14$^-$CD15$^+$CD33$^+$CD34$^+$. The close relationship of *CEBPA* mutations with the leukemia status of the patients and the concordance of mutation in presenting and relapse samples implicate the *CEBPA* mutation as a potential marker for monitoring minimal residue disease.**

INTRODUCTION

CCAAT/enhancer binding protein α (C/EBPα) is a 42-kDa transcription factor that possesses a DNA-binding basic leucine zipper domain (bZIP), composed of a basic region and a leucine zipper domain, in the COOH terminus and two transactivation domains TAD 1 and TAD 2 in the NH_2 terminus (1). C/EBPα dimerizes via its leucine zipper domain and then binds DNA via the adjacent basic region. Once bound to DNA, C/EBPα mediates transactivation via its NH_2-terminal TAD (2). C/EBPα expression is selectively maintained during granulocyte differentiation but is markedly down-regulated with monocytic differentiation (3, 4). Microarray analysis showed that C/EBPα could up-regulate those genes important for regulation of hematopoietic stem cell homing and granulocytic differentiation but down-regulate those genes coding for signaling molecules and transcription factors that are implicated in regulation of proliferation of hematopoietic cells (5, 6). It could block progression from the G_1 to S phase and induce terminal maturation of hematopoietic cells (7, 8).

Diminished C/EBPα activity is widely known to contribute to the transformation of myeloid progenitors via reduction of their differentiation potential (9). Recently, *CEBPA* mutations were detected in 7% to 15% of patients with acute myeloid leukemia (AML; refs. 10–14). These mutations largely fall into two major categories: one comprises those mutations that prevent C/EBPα DNA binding via alteration of its COOH-terminal bZIP, and the other comprises those that disrupt translation of the C/EBPα NH_2 terminus, leading to reinitiation of translation at an alternative internal ATG codon located 351 nucleotides downstream of the main AUG initiation codon, and as a result, formation of a 30-kDa C/EBPαp30 isoform. This 30-kDa isoform has the capacity to further reduce wild-type C/EBPα activity by inhibiting its DNA binding and transactivation of the target genes in a dominant-negative effect (10, 15). *CEBPA* mutations were most frequently found in AML M1 or M2 subtype and in those with intermediate-risk cytogenetics. However, the age distribution of the patients with the *CEBPA* mutations and the immunophenotype of their leukemic cells are not known. Sequential studies of these patients at remission and relapse are also limited (15). In the present study, we analyzed entire *CEBPA* sequences in 104 patients with *de novo* AML diagnosed during the period from 1995 to 2000 and 18 selected children diagnosed before the year 1995. We found that the AML patients in this area had a higher frequency of *CEBPA* mutations than those in the West. The children with AML had a probability to have *CEBPA* mutation similar to that of the adults.

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In addition, we showed for the first time that most patients (73%) with *CEBPA* mutations showed expression of all CD7, CD15, CD34, and HLA-DR on the leukemic cells, compared with only 4% in those without the mutation. Most patients with *CEBPA* mutations had biallelic mutations involving both the TAD1 and bZIP regions. The mutations in the bZIP region were always internal tandem duplications and might relate to the presence of the potential topoisomerase II sites in the region.

MATERIALS AND METHODS

Patients. The *CEBPA* mutation was studied in the bone marrow cells from 104 unselected patients with *de novo* AML who had had cells cryopreserved at the National Taiwan University Hospital from 1995 to 2000. Sixty-one were males and 43 were females. There were 96 adults and 8 children (≤15 years). The median age was 46 years, ranging from 1 to 85 years. According to the French-American-British classification (16), 25 were M1, 41 were M2, 17 were M3, 13 were M4, six were M5, and two were M7 subtypes. Excluding the 25 patients who did not receive any chemotherapy or were only treated with low-dose cytosine arabinoside because of old age and/or poor performance status, all other patients with non-M3 subtypes of AML received conventional induction chemotherapy with one of the anthracyclines (doxorubicin or idarubicin) for 3 days and cytosine arabinoside for 7 days. The patients with acute promyelocytic leukemia (M3 subtype) received all-trans retinoic acid with or without concurrent induction chemotherapy. After complete remission was achieved, the patients received consolidation chemotherapy with a conventional dose of cytosine arabinoside and one anthracycline or with high-dose cytosine arabinoside. Sixteen patients received hematopoietic stem cell transplantation.

Immunophenotyping and Cytogenetic Study. A panel of monoclonal antibodies to myeloid-associated antigens including CD13, CD33, CD11b, CD15, CD14, and CD41a, as well as lymphoid-associated antigens including CD2, CD5, CD7, CD19, CD10, and CD20, and lineage nonspecific antigens HLA-DR, CD34, and CD56 was used to characterize the phenotypes of the leukemic cells. Expression of surface antigens on the leukemic cells was shown by an indirect immunoalkaline phosphatase method as described before (17). The cutoff value for positive result of the markers was more than 20%. Chromosome analyses were carried out as described previously (18). Bone marrow cells were harvested directly or after 1 to 3 days of nonstimulated culture. Metaphase chromosomes were banded by the conventional trypsin-Giemsa banding technique and karyotyped according to the International System for Human Cytogenetic Nomenclature (19). Chromosomal abnormalities t(8;21), t(15;17), and inv(16) were considered as good-risk cytogenetics; those of −5/del(5q), −7/del(7q), 3q abnormality and complex karyotype with four or more unrelated abnormalities were grouped as poor-risk cytogenetics, and all other aberrations as intermediate-risk cytogenetics.

Analysis of the Mutation of the *CEBPA*. Mutation of the *CEBPA* was detected by genomic DNA PCR and direct sequencing according to the method described previously (17). The primer sets used were the same as those designed by Pabst et al. (10). Briefly, two overlapping primer pairs were used to amplify the entire coding region of human *CEBPA*: PP1 (550 bp) 5′-TCGCCATGCCGGGAGAACTATAAC-3′ (sense) and 5′-CTGGTAAGGGAAGAGGCCCGCCAG-3′ (antisense), PP2 (680 bp) 5′-CCGCTGGTGATCAAGCAGGA-3′ (sense) and 5′-CACG GTCTGGGCAAGCCTCGAGAT-3′ (antisense). Four alternative primer pairs were used in cases of abnormal or ambiguous results: PP3 (290 bp) 5′-TCG-CCATGCCGGGAGAACTCT A C-3′ and 5′-ACGGCCGCCTT-GGCCTTCTCCTGCT-3′, PP4 (279 bp) 5′-CTTCA ACGA CGAGTTCCTGGCCGA-3′ and 5′-AGCTGCTTGGCTTCATC-CTCCT-3′, PP5 (371 bp) 5′-CCGCTGGTGATCAAGCAGGA-3′ and 5′-CCGGTACTCGTTGCTGTTCT-3′, PP6 (538 bp) 5′-CCGCACCTGCAGTTCCAGAT-3′ and 5′-CACGGTCTGGG-CAAGCCTCGAGAT-3′. PCR reactions were run in a final volume of 50 L containing genomic DNA (100 ng), KCl (50 mmol/L), Tris-HCl (20 mmol/L, pH 8.4), $MgCl_2$ (2.5 mmol/L), 5 volume % DMSO, primers (2 mmol/L of each), nucleotides (0.1 mmol/L of each), and Taq DNA polymerase (1.25 units, Life Technologies, Gaithersburg, MD). The mixture was denatured at 94°C for 1 minute, annealed at 61°C for 40 seconds, and extended at 72°C for 90 seconds for 35 cycles, with a final step for 10 minutes at 72°C. PCR products were electrophoresed on 2% agarose gels, purified (Qiagen, Hilden, Germany) and sequenced using the BigDye Terminator v3.1 Cycle Sequencing kit, which contained AmpliTaq DNA polymerase FS (Applied Biosystems, Foster City, CA), on an automated ABI-3100 Genetic Analyzer (Applied Biosystems). Abnormal sequencing results were confirmed by repeated analyses at least thrice in both directions, including performing different PCR and sequencing with an alternative pair of PCR primers. Because the number of children studied in this series was too low to compare the frequency of *CEBPA* mutation in this age group with that in adults, 18 additional children with AML diagnosed before the study period (1995-2000) were recruited for analysis. The DNA extracted from the peripheral blood mononuclear cells of 19 healthy persons were used as normal controls.

To determine whether double mutations in different regions of the *CEBPA* of these patients were in different alleles, the entire coding region was amplified with the primers PP1 sense and PP2 antisense shown above. The PCR products were then cloned into the TA-cloning vector pGEM-T Easy (Promega, Madison, WI) and 7 to 13 clones were sequenced in each patient with the primers used to amplify the corresponding regions of the *CEBPA*.

Analysis of the Internal Tandem Duplication of the FLT. Genomic DNA was amplified by PCR using the primer pairs of 5′-CAATTTAGGTATGAAAGCC-3′ (forward) and 5′-GTACCTTTCAGCATTTTGAC-3′ (reverse), which covered the justamembrane domain through the TK1 domain (20). The PCR products were then electrophoresed on 4% agarose gel and visualized with ethidium bromide under an UV lamp. The abnormal PCR products were purified and cloned into the TA-cloning vector pGEM-T Easy and sequenced.

Statistics. Continuous variables were compared by Wilcoxon rank-sum test, and discrete ones, by χ^2 or Fisher′s exact test. Curves of survival and complete remission (CR) duration were plotted by the Kaplan-Meier method; differences between curves were analyzed by the log-rank test. All statistical analyses were done using the SPSS 8.0 for Windows (SPSS, Chicago, IL). The significance of results was defined as a level of $P < 0.005$ at both tails.

RESULTS

Characterization of *CEBPA* Mutations. Twenty-eight mutations were identified in the 104 AML patients, which were mainly located in the TAD1 region or its upstream and in the bZIP domain (Table 1). Of these, four were one-nucleotide substitutions without alteration of amino acid and resulted in silent mutations (P1-P4; Table 1). They occurred in one, one, one, and two patients, respectively. The other two mutations in the TAD2 region, TAD2-B and TAD2-C (Table 1), were in-frame mutations, which did not generate a shortened 30-kDa isoform. TAD2-B had 3-bp insertion, resulting in eight instead of seven GCC repeats and eight instead of seven proline repeats. It was detected in one AML patient (patient 86; Table 2) and did not disappear at CR. TAD2-C was an in-frame mutation with 6-bp insertion resulting in four GCACCC repeats instead of three GCACCC repeats in the genomic DNA and four histidine-proline repeats instead of three in the protein (Table 1). This mutation was detected in 7 (39%) of 19 healthy volunteers and 20 (20%) of the AML patients and remained positive at CR in the patients studied (patients 25, 39, 76, and 95; Table 2). These six mutations were considered as insignificant changes for leukemogenesis and the patients with these six mutations were not included in the group of *CEBPA*-mutated patients in the following analyses.

Excluding the mutations stated above, 16 AML patients (15%) were found to have 22 distinct mutations (Table 3). Fourteen patients were shown to have biallelic mutations by cloning and subsequent nucleotide sequence analyses. In 11 of them, the mutations were heterozygous, with one allele containing a mutation in the TAD1 region and the other allele, a mutation in the bZIP regions, although one or two cloned alleles from two patients showed both mutations on the same alleles. In one patient (patient 52), the two alleles had different bZIP mutations; in another (patient 78), the two alleles carried a TAD1 mutation and a TAD2 mutation, respectively; and in the remaining one (patient 23) the mutation was homozygous in the bZIP region (Table 3). The nine NH_2-terminal mutations comprised of TAD1-A to TAD1-I (Table 1), were all frame-shift mutations and were predicted to generate a shortened dominant-negative 30-kDa (C/EBPαp30) isoform instead of the 42-kDa wild form by using alternative internal initiation codon (10, 15). Most of the COOH-terminal mutations (9 of 12), comprising bZIP-B to bZIP-H, bZIP-J, and bZIP-K, were in-frame mutations with internal tandem duplications clustered in the junction between the basic region and the leucine zipper (Table 1). The remaining TAD2-A mutation was a frame-shift mutation located in TAD2, downstream of the alternative initiation site for C/EBPαp30

Table 1 Characterization of the mutations of the *CEBPA* coding region

	Mutation	Nucleotide feature	Protein feature
Upstream to TAD1			
TAD1-A	nt641-647(−7 bp)	−GCCACCT	H18fsX157
TAD1-B	nt655 (−CC)	6C → 4C	P23fsX159
P1	nt654 (C → A)		normal, S21S
TAD1-C	nt715 (−C)	2C → 1C	P42fsX159
TAD1-D	nt773 (−C)	2C → 1C	I62fsX159
TAD1-E	nt775/776 (+A)	1A → 2A	I62fsX107
TAD1-F*	nt800/801 (+C)	3C → 4C	A71fsX107
TAD1-G	nt838 (−C)	2C → 1C	Q83fsX159
TAD1			
TAD1-H	nt855/861 (−5 bp)	−GGAGAAG, +CA	Q88H, S89fsX100
	nt872-877 (−6 bp)	−CCGTGG	
	nt896-904 (−9 bp)	6GCG → 3GCG	
TAD1-I	nt875/876 (+10 bp)	+2T, nt868-875 duplication	G96fsX110
TAD2			
P2	nt978 (C → T)		normal, P129P
TAD2-A	nt1045 (−G)	4G → 3G	R152fsX159
P3	nt1146 (G → T)		normal, P185P
TAD2-B	nt1157/1158 (+3 bp)	7GCC → 8GCC	P189-190ins, 7P → 8P
TAD2-C	nt1178/1179 (+6 bp)	3GCACCC → 4GCACCC	HP196-197ins, 3HP→4HP
Upstream to bZIP			
P4*	nt1281 (G → T)		normal, T210T
bZIP			
bZIP-A*	nt1507/1508 (+3 bp)	2AGC → 3AGC	Q305-306ins, 1Q → 2Q
bZIP-B	nt1511/1512 (+9 bp)	nt1503-1511 duplication	QRN307-308ins
bZIP-C	nt1518/1519 (+3 bp)	nt1516-1518 duplication	E309-310ins
bZIP-D	nt1520/1521 (+21 bp)	nt1500-1520 duplication	KQRNVET310-311ins
bZIP-E*	nt1530/1531 (+3 bp)	nt1528-1530 duplication	K313-314ins
bZIP-F*	nt1530/1531 (+24 bp)	nt1507-1530 duplication	RNVETQQK313-314ins
bZIP-G*	nt1533/1534 (+3 bp)	nt1531-1533 duplication	V314-315ins
bZIP-H	nt1538/1539 (+36 bp)	+T, nt1504-1538 duplication	DQRNVETQQKVL315-316ins
bZIP-I	nt1552/1554 (+3 bp)	−A, +TCAT	321 N → SY
bZIP-J	nt1552/1553 (+33 bp)	nt1520-1552 duplication	TQQKVLELTSD320-321ins
bZIP-K	nt1568/1569 (+57 bp)	nt1512-1568 duplication	VETQQKVLELTSDNDRLRK327-328ins
bZIP-L	nt1595 (T → A)		335 L → Q

NOTE. Sequence numbering is according to Genbank accession no. U34070.
*Mutations which have been reported before (10–14, 21).

Table 2 Sequential follow up of *CEBPA* mutations and karyotypic changes

Patient	Date	Status	Karyotype	NH_2-terminal*	COOH-terminal*
Patients with distinct mutations					
2	1995/1/14	new	N	TAD1-E	bZIP -K
	1995/4/22	CR	N	N	N
	1996/11/19	Relapse	add(17)(p13)	TAD1-E	bZIP -K
6	1996/5/10	New	N	TAD1-B	P4†
	1996/6/17	CR	ND	N	P4†
14	1998/4/27	New	N	TAD1-F	bZIP -J
	1998/7/27	CR	ND	N	N
18	1998/8/20	New	N	TAD1-G	bZIP -C
	1998/10/27	CR 1	N	N	N
	1999/12/2	Relapse	inv(12)(q13q24)	TAD1-G	bZIP -C
	2003/8/19	CR 2	N	N	N
52	1998/6/22	New	N	N	bZIP -F, -L
	1998/9/18	CR1	N	N	N
	1999/11/19	Relapse	N	N	bZIP -F, -L
	2000/4/25	CR2	N	N	N
61	1998/6/15	New	+21	TAD1-G	bZIP -E
	1998/7/16	CR	N	N	N
62	2000/1/20	New	N	TAD1-C	N
	2000/11/14	CR	N	N	N
69	1996/1/13	New	N	TAD1-A	bZIP -H
	1996/7/10	CR	N	N	N
78	1995/6/19	New	+8, +4	TAD1-D, TAD2-A	N
	1994/4/14	CR	N	N	N
91	1999/8/21	New	del(9q)	TAD1-I	bZIP -A
	1999/9/17	CR	N	N	N
93	1999/8/19	New	N	TAD1-G	bZIP -D
	1999/12/30	CR	N	N	N
	2000/12/15	Relapse 1	ND	TAD1-G	bZIP -D
	2001/6/12	Relapse 2	del(9q)	TAD1-G	bZIP -D
Patients with insignificant mutations					
25	1995/6/30	New	+21	N	TAD2-C
	1995/9/13	CR	N	N	TAD2-C
	1997/3/21	Relapse	N	N	TAD2-C
39	1996/6/4	New	t(9;11)	N	TAD2-C
	1996/10/30	CR	N	N	TAD2-C
55	1998/2/18	new	del(16q)	P2†	N
	1998/4/15	CR	N	P2†	N
76	1995/6/30	New	inv(16)	N	TAD2-C
	1998/2/13	CR	N	N	TAD2-C
86	2000/4/12	New	N	N	TAD2-B
	2000/7/13	CR	N	N	TAD2-B
	2000/10/5	Relapse	N	N	TAD2-B
95	1999/12/15	New	+8, t(16;21)	N	TAD2-C
	2000/2/9	CR	N	N	TAD2-C
	2000/9/29	Relapse	−7, der(18)t(7;18)	N	TAD2-C

NOTE. The data of the serial studies in another 10 patients without *CEBPA* mutation were not shown. There was also an absence of mutation at relapse in these patients after a median interval of 15 months (range, 4-58 months). Three of them had karyotypic evolution at relapse.

Abbreviations: CR, complete remission; N, normal karyotype or normal *CEBPA*; ND, not determined.

*The details of the mutations were shown in Table 1.

†Silent mutation.

(Table 1); therefore, the TAD2-A was not expected to have the dominant-negative effect.

Sequential Studies of *CEBPA* Mutations in Acute Myeloid Leukemia Patients. *CEBPA* mutations were serially studied in 27 patients (Table 2), including 11 patients with distinct *CEBPA* mutations at diagnosis and another 16 patients with no mutation (10 patients) or with insignificant mutations (6 patients). None developed a new mutation during the follow-up period. Among the 11 *CEBPA*-mutated patients, all mutations disappeared at CR. The same mutations as those at diagnosis were detected at first relapse in all four patients who relapsed (patients 2, 18, 52, and 93) and also at second relapse in patient 93, although cytogenetic evolution was found in three of them (patients 2, 18, and 93). Serial studies of allelic frequency and distribution of the mutations at diagnosis and relapse by cloning and sequencing were done in three patients with biallelic mutations (Table 4). One cloned allele with double mutations emerged at relapse in patient 18. On the contrary, double mutations detected at diagnosis were not found at relapse in patient 93. In the six patients with insignificant mutations, including one with TAD2-B, four with TAD2-C, and one with P2 silent mutation, the mutations remained the same at CR in all of them and also at relapse in the three patients studied. One patient (patient 95) showed chromosomal abnormalities at relapse that were different from those at diagnosis. In the 10 patients without mutation at diagnosis, there was also an

Table 3 The patterns of *CEBPA* mutations and immunophenotypes in the 16 AML patients

Patient					% Leukemic cells with the antigen expression*								
No	FAB	Cytogenetics	NH_2-terminal†	COOH-terminal†	HLA-DR	CD34	CD13	CD33	CD11b	CD14	CD15	CD7	CD56
2	M2	N	TAD1-E	bZIP -K	94	81	54	0	0	0	38	0	ND
10	M2	N	TAD1-G	bZIP -G	99	36	41	0	0	0	68	15	ND
14	M2	N	TAD1-F	bZIP -J	61	68	59	67	ND	0	42	8	ND
15	M4	N	TAD1-H	bZIP -B	80	1	45	89	42	32	100	53	0
16	M1	N	TAD1-B, P1	bZIP -E	100	41	96	65	12	10	95	98	0
18	M2	N	TAD1-G	bZIP -C	100	36	75	93	25	12	95	50	0
34	M2	nm	TAD1-G	bZIP -I	100	51	64	100	18	26	24	99	ND
61	M1	sp, +21	TAD1-G	bZIP -E	98	63	52	62	8	4	36	54	48
69	M2	N	TAD1-A	bZIP -H	100	74	95	51	0	0	55	90	ND
91	M1	sp, del(9q)	TAD1-I	bZIP -A	100	100	60	82	24	8	98	85	3
93	M1	N	TAD1-G	bZIP -E	100	100	80	89	0	0	75	94	0
78	M2	sp, +8,+4	TAD1-D, TAD2-A	N	ND								
23	M2	sp, +21	N	bZIP -D‡	58	24	0	75	0	0	93	82	ND
62	M2	N	TAD1-C	N	2	42	5	54	ND	2	99	0	0
6	M1	N	TAD1-B	P4	91	30	54	50	3	0	35	60	ND
52	M1	N	N	bZIP -F, -L	92	47	48	85	8	2	92	68	65

Abbreviations: N, normal karyotype or normal sequence; ND, not determined; nm, no metaphase; sp, simple (<4) chromosomal abnormalities excluding t(8;21), t(15;17), inv(16), aberrations at 11q23, −5/del(5q), −7/del(7q), and 3q abnormalities; +8, +4, +21, gain of the chromosome 8, 4, or 21.

*CD2, CD5, CD19, and CD10 were all negative in these patients.

†The details of the mutations are shown in Table 1.

‡Homozygous for bZIP-D.

absence of mutation at relapse after a median interval of 15 months (range, 4-58 months). Three of them had karyotypic evolution at relapse.

Correlation of *CEBPA* Mutations with Biological and Clinical Features. Comparison of the clinical and laboratory features between the 16 patients with and 88 without *CEBPA* mutation is summarized in Table 5. All but one patient with *CEBPA* mutations had M1 or M2 subtypes of AML. Overall, 15 (23%) of 66 patients with M1 or M2 subtypes of AML showed *CEBPA* mutations, compared with 1 (3%) of 38 patients with other subtypes of AML who did so ($P = 0.001$). One hundred and two patients had cytogenetic data. Fifteen (25%) of 61 patients with intermediate-risk cytogenetics and 11 (35%) of 31 patients with normal karyotype showed *CEBPA* mutations; however, none of those with good- and poor-risk cytogenetics showed these. Immunophenotyping of the leukemic cells was done in 97 patients. The patients with *CEBPA* mutations had significantly higher incidences of CD7 (73%), CD15 (100%), CD34 (93%), and HLA-DR (93%) expression than other patients (Tables 3 and 5). Eleven (73%) of 15 patients with *CEBPA* mutations showed expression of all these four antigens, compared with only 3 (4%) of 82 patients without *CEBPA* mutation ($P < 0.001$). The expression of other antigens in the *CEBPA*-mutated patients was similar to that of the patients without *CEBPA* mutations.

The patients with *CEBPA* mutations had significantly higher hemoglobin levels and a trend of lower platelet counts than did those without the mutation (Table 5). The adults with *CEBPA* mutations had trends of longer median CR duration and survival time than those without the mutation (19 versus 9 months, $P = 0.212$ and not reached versus 32 months, $P = 0.225$, respectively). If only the patients with intermediate-risk cytogenetics were analyzed, the median CR duration and survival time were 17 months and not reached respectively in the patients with *CEBPA* mutations, compared with 7 and 26 months, respectively, in those without the mutation ($P = 0.071$ and 0.265, respectively).

Internal Tandem Duplication of the *FLT3*. Internal tandem duplication of the *FLT3* was detected in 18 (17%) of 104 AML patients. Only two patients with *CEBPA* mutations showed *FLT3*-internal tandem duplication.

DISCUSSION

In this study, *CEBPA* mutations were detected in 15% (16 of 104) of the total AML patients, 25% (15 of 61) of the

Table 4 Serial studies of allelic frequency and distribution of mutations in three patients with biallelic mutations

Patient	Status	No. clones evaluated	No. clones with			
			Mutation 1*	Mutation 2*	Double mutations	Wild type
18	Diagnosis	7	2	3	0	2
	Relapse	11	5	2	1	3
93	Diagnosis	10	6	2	2	0
	Relapse 1	11	2	4	0	5
	Relapse 2	10	3	5	0	2
52	Diagnosis	13	6	6	0	1
	Relapse	11	2	2	0	7

NOTE. No new mutation was detected at relapse.

*Mutation 1 and Mutation 2 stand for leftward and rightward mutation, respectively.

Table 5 Correlation of the *CEBPA* mutations with clinical features, FAB subtypes, cytogenetics, and immunophenotypes

Variable	Total (n = 104)	Distinct *CEBPA* mutated (n = 16)	Others (n = 88)	P
Age (y)				
>15	96	16	80	0.254
≤15	8	0	8	
Sex (male/female)	61/43	11/5	50/38	0.587
Hb (g/dL)*		9.8±1.9	7.6±2.7	0.036
WBC (×1,000/μL)*		16.5±123.7	19.4±84.7	0.293
Platelet (×1,000/μL)*		21.0±19.9	33.5±46.9	0.065
BM blast (%)*		66.2±24.2	51.0±31.7	0.126
LDH (IU)*		997±1287	1009±1211	0.992
FAB subtype				
M1 + M2	66	15 (23%)	51	0.001
Others	38	1	37	
Cytogenetics†				
Good	31	0	31	0.004
Intermediate‡	61	15 (25%)	46	
Poor	10	0	10	
Antigen expression§				
CD7	22%	73%	12%	<0.001
CD15	71%	100%	65%	0.005
CD34	64%	93%	58%	0.008
HLA-DR	71%	93%	67%	0.034

Abbreviations: FAB, French-American-British; BM, bone marrow; Hb, hemoglobin.

*Mean ± SD.

†Cytogenetic data are available from 102 patients. Good, including t(8;21), t(15;17), and inv(16); Poor, −5/del(5q), −7/del(7q), 3q anomaly, and complex (≥4) abnormalities. Intermediate, normal karyotype and other chromosomal abnormalities.

‡Thirty-one patients showed normal karyotype; 11 (35%) of them had *CEBPA* mutations.

§Percentage of patients with the antigen expression. There were no statistical differences in expression of other antigens, including myeloid associated antigens CD13, CD33, CD11b, CD14, and CD41 and lymphoid-associated antigens CD10, CD19, CD20, CD2, CD5, and CD56 between the two groups of patients.

AML patients with intermediate-risk cytogenetics, and 35% (11 of 31) of those with normal karyotype. These frequencies were higher than those reported in western countries: around 7% in total AML and 15% in those with intermediate-risk cytogenetics or those with normal karyotype (10, 11, 14, 21). Uneven geographic distribution of nonrandom chromosome abnormalities in malignant disorders has been reported before (17, 22, 23). It is suggested that the heterogeneity in the incidence of nonrandom cytogenetic abnormalities in various areas may reflect differences in racial or environmental factors (24). The distribution of the genetic changes in AML that can not be detected in cytogenetic studies, such as *CEBPA* mutations, may also show geographic differences. The studies of the *CEBPA* mutation in the literature were all done on adults, and the gene mutation in pediatric AML has not been characterized yet. Because the number of AML children in this study was very limited, the incidence of the *CEBPA* mutation in these children (0 of 8) could not reflect the true condition. To clarify the occurrence of the *CEBPA* mutation in pediatric AML, 18 additional children with AML diagnosed before the year 1995 were recruited for analysis. Three (12%) of a total of 26 children were found to have *CEBPA* mutations (data not shown), an incidence similar to that in the adults (16 of 96, 17%), indicating there is no discrepancy in *CEBPA* aberration in children and in adults.

AML patients with some specific chromosomal abnormalities showed distinct immunophenotypes (17, 25, 26). However, the immunophenotype of the *CEBPA*-mutated AML has not been reported before. In the present study, the leukemic cells from 11 of 15 *CEBPA*-mutated patients expressed all the HLA-DR, CD7, CD15, and CD34 antigens; in addition, the majority of *CEBPA*-mutated leukemic cells expressed CD13 (87%) and CD33 (87%), but not CD14 (13%), delineated an immunophenotype of HLA-DR$^+$ CD7$^+$ CD13$^+$ CD14$^-$ CD15$^+$ CD33$^+$ CD34$^+$. The *CEBPA* mutations occur most frequently in AML M1 or M2 subtypes (10–12, 14). CD15 is usually detected in the more differentiated subtype M2 of AML, and can also be present in other AML, including M0 and M1 subtypes (27, 28). Interestingly, all patients with t(8;21), a chromosome translocation associated with down regulation of C/EBPα expression and function (29), showed CD15 expression on the leukemic cells, as shown in our previous study (17) and in this study (data not shown). CD7 is a T cell–associated antigen but can also be detected on leukemic cells in around 13% to 37% of AML cases (30–33). In this study, a very high frequency (73%) of CD7 expression was found in the *CEBPA*-mutated AML. Recently, Valk et al., using Affymetrix U133A GeneChips analysis, also showed clustering of up-regulated CD7 with *CEBPA* mutations in AML cells (34). Whether expression of one of the dominant-negative *CEBPA* mutations induces CD7 expression in a myeloid cell line is a subject that requires further study.

Several reports have linked *CEBPA* mutations with a favorable outcome in AML (11, 13, 14). In this study, whereas among the patients with intermediate-risk cytogenetics, the patients with *CEBPA*-mutations tended to have a longer remission duration and survival time than those without the mutation, the difference did not reach statistical significance. Snaddon et al. also found that *CEBPA* mutation was not of prognostic importance in their AML patients (12). Furthermore, although the patients in this study did not receive the same consolidation treatment, the percentage of the patients treated

with conventional doses of cytosine arabinoside, high doses of cytosine arabinoside, or hematopoietic stem cell transplantation was similar between the patients with and without *CEBPA* mutations (data not shown). Therefore, the treatment heterogeneity might not influence the results of prognostic analysis. Moreover, other genomic abnormalities, such as internal tandem duplication of FLT3 and MLL and other genetic alteration, may accompany *CEBPA* mutations and have a prognostic impact in the *CEBPA*-mutated patients (35). Further prospective studies of the genetic alterations in more patients are needed to clarify this point. Functional studies of the C/EBPα protein affected by various mutations, alone, or in combination, may also be helpful to evaluate their influence on clinical outcome.

There were 22 distinct mutations identified in this study. From further evaluation of these *CEBPA* mutant sequences, two major types of mutants were revealed. One was repeat-number changes of simple sequence repeats, such as TAD1-B to TAD1-H, TAD2-A to TAD2-C, and bZIP-A mutations; the other was internal tandem duplications, such as TAD1-H, TAD1-I, bZIP-B to bZIP-H, bZIP-J, and bZIP-K mutations. The former was supposed to correlate with mismatch repair defect or insufficiency (36); the latter was supposed to correlate with topoisomerase II–induced double strand breaks and subsequent errors in DNA repair through overactive nonhomologous end joining systems (37). After the use of FUZZNUC nucleic acid pattern searches in the EMBOSS program, several potential topoisomerase II sites (38) were found within bZIP regions in the *CEBPA* (Fig. 1), supporting this hypothesis. The significance of topoisomerase II sites for other AML-related mutations, *PML-RARA*, *FLT3*-internal tandem duplication, and *MLL* duplication, has been revealed (39–41). Further topoisomerase II binding activity to these topoisomerase II sites in the *CEBPA* should be clarified to ensure the significance of topoisomerase II in leukemogenesis of patients with AML.

Most patients (14 of 16) with *CEBPA* mutations in this study showed genetic alterations in both alleles, most frequently (11 patients) with one allele containing a mutation in bZIP DNA-binding domain and the other, a TAD1 frame-shift mutation. A similar finding has been reported before (13). Sequential analyses in this study showed that the *CEBPA* mutations detected at diagnosis disappeared at CR, but the same mutations reappeared at relapse. No novel mutation was detected during the follow-up period. Cloning and sequencing studies showed that the patients with biallelic mutations at presentation also disclosed the same pattern of mutations at relapse, although a few cloned alleles might have double mutations either at diagnosis or at relapse. Another serial analysis of the *CEBPA* has been reported (42), one in which no new mutation was detected at relapse, either. These findings suggest that the *CEBPA* mutation is probably a primary change in the development of AML, but does not play a role in the progression of the disease. Because of the close relationship between the *CEBPA* mutation and the disease status of the leukemia and the concordance of mutations in presentation and relapse samples, examination of *CEBPA* mutation may be a useful marker for monitoring minimal residual disease. Furthermore, since all but one *CEBPA*-mutated patient had bZIP mutants, the detection of minimal residual disease can be simplified by examining the bZIP region using PCR coupled with a DNA fragment analyzer.

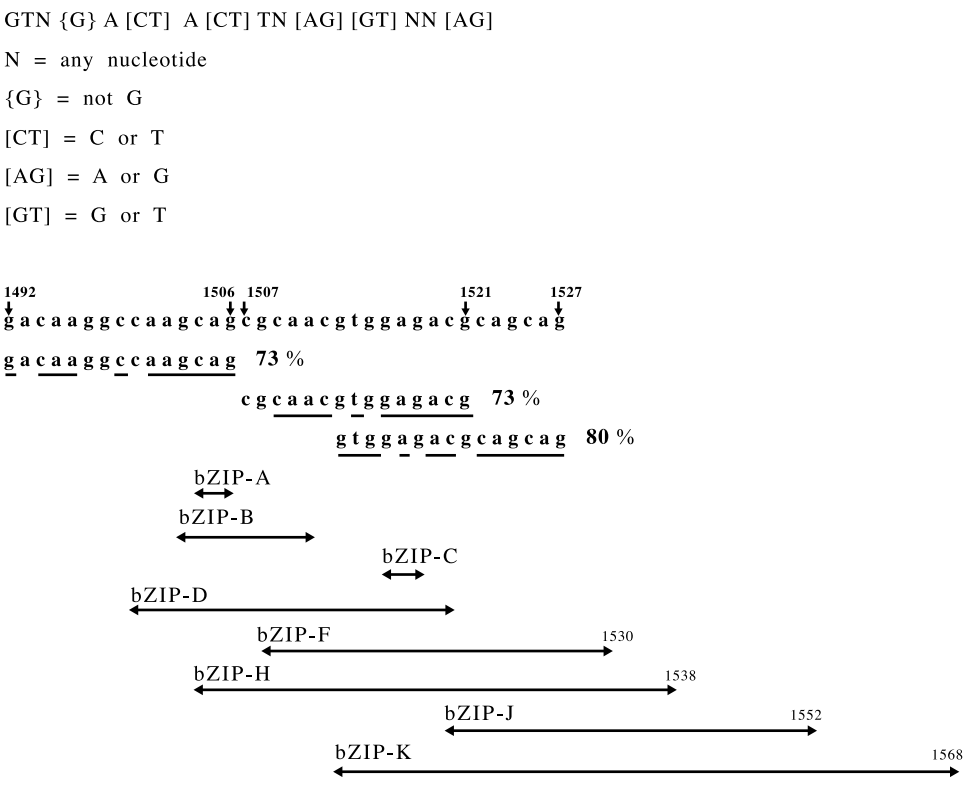

Fig. 1 Potential topoisomerase II sites in bZIP region of *CEBPA* gene. Top, consensus topoisomerase II recognition sequence (38, 39). Middle, partial bZIP sequence from nt1492 to nt1527, numbering according to Genbank accession no. U34070. Percentages of homologous rate at right side of potential topoisomerase II sites, showing hot spot at this area. Homologous nucleotides (*underlined*). Bottom, duplicated fragments of various bZIP mutants (↔).

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