Heterogeneous Patterns of CEBPα Mutation Status in the Progression of Myelodysplastic Syndrome and Chronic Myelomonocytic Leukemia to Acute Myelogenous Leukemia

Lee-Yung Shih,1,2 Chein-Fuang Huang,1 Tung-Liang Lin,1 Jin-Hou Wu,1 Po-Nan Wang,1 Po Dunn,1,2 Ming-Chung Kuo,1 and Tzung-Chih Tang1

1Division of Hematology-Oncology, Department of Internal Medicine, Chang Gung Memorial Hospital, Taipei, Taiwan and 2School of Medicine, Chang Gung University, Taoyuan, Taiwan

ABSTRACT

Purpose: We aimed to assess the role of CEBPα mutations in the progression of myelodysplastic syndrome (MDS) to acute myelogenous leukemia (AML) and their cooperating mutations.

Experimental Design: Mutational analysis of CEBPα with direct sequencing for each PCR product was done on matched bone marrow samples obtained from 50 adult patients with MDS at diagnosis and at AML transformation. Cloning analysis was used to determine the allelic distribution.

Results: CEBPα mutations were identified in four patients at diagnosis of MDS, including one with refractory anemia with excess blasts and three with chronic myelomonocytic leukemia. At AML transformation, three patients retained the identical mutant clones as their initial diagnosis, and one lost the CEBPα mutation when she gained FLT3-ITD mutation. Together, seven mutations were identified in four patients with MDS (6, 7).

Conclusions: Our results show that CEBPα mutations play a role in a subset of patients with MDS, especially in chronic myelomonocytic leukemia. The mutation status was heterogeneous, exhibiting identical clone, clonal change, or clonal evolution during the progression to AML.

INTRODUCTION

Myelodysplastic syndrome (MDS) encompasses a heterogeneous group of clonal hematopoietic stem cell disorders characterized by ineffective hematopoiesis, refractory cytopenia, and a tendency to progress to acute myelogenous leukemia (AML; ref. 1). Over the last decade, genetic aberrations in patients with MDS have been a subject of investigation; cytogenetic and molecular data suggest that MDS involves a stepwise genetic progression (2). Evidence has been accumulated that genetic alterations are closely associated with the progression of MDS to AML (3–6). MDS serves as a useful model for studying the abnormal genetic events that occur in leukemogenesis. Efforts are being made to determine the prognostic significance of various genetic aberrations in adult patients with MDS. We have recently shown that one third of patients with MDS acquired internal tandem duplication of fms-like tyrosine kinase (FLT3-ITD) or N-ras mutations during AML evolution, and FLT3-ITD mutation predicted a poor outcome in patients with MDS (6, 7).

Transcription factor CCAAT/enhancer binding protein α (C/EBPα), encoded by CEBPα gene that is located at chromosome 19q13.1 (8), was originally isolated from rat liver that bound to viral enhancer sequences CCAAT (9, 10). It has been shown that C/EBPα is specially expressed in human myelomonocytic cell lines (11). Further study showed that C/EBPα expression is maintained during granulocytic differentiation. C/EBPα is essential for the granulocytic differentiation of common myeloid progenitors and its disruption results in a state in which granulocyte differentiation is blocked (12, 13). Recently, CEBPα mutations have been described in 7% to 10% of patients with newly diagnosed AML (14–19) and were associated with a favorable prognosis (16, 18). CEBPα mutations affect either the NH2-terminal part of the protein that specifically abolish translation of full-length (42 kDa) C/EBPα, leading to overexpression of a shorter (30 kDa) form or the basic region leucine zipper (bZIP) domain that affects dimerization and DNA binding (14, 15). Two previous studies on CEBPα mutations in MDS analyzed the samples exclusively obtained from patients at MDS phase (15, 19), and another group of investigators focused on AML samples in which a very limited number of samples derived from MDS were included (14). Data of systematic analysis comparing the mutation status of CEBPα gene in the matched paired samples at both MDS and AML phases have not been available. Furthermore, the clinical significance and prognostic relevance of CEBPα mutations in MDS have not yet been assessed. In the present study, we analyzed matched paired marrow samples obtained from 50...
patients at initial diagnosis of MDS and at AML transformation. In addition, the mutation status was analyzed for correlation with clinicohematologic features and outcome. To our knowledge, the current study is by far the largest to systematically assess the role of CEBPα mutations in the progression of MDS to AML.

MATERIALS AND METHODS

Patients and Samples. Beginning in November 1991, patients with de novo MDS were diagnosed, treated, and had been followed until death or for periods up to 112.5 months in the single center (Division of Hematology-Oncology, Chang Gung Memorial Hospital, Taipei, Taiwan). As of March 2004, 70 patients had matched paired samples both at initial diagnosis and at AML transformation available for comparative analysis. The study was approved by the Institutional Review Board of Chang Gung Memorial Hospital. The bone marrow samples were obtained with informed consent. Bone marrow samples were enriched by Ficoll-Hypaque (1.077 g/mL, Amersham Pharmacia, Buckinghamshire, United Kingdom) density-gradient centrifugation and cryopreserved in 10% DMSO and 20% fetal bovine serum at −70°C or in liquid nitrogen until use. Morphologic subtypes of MDS were initially classified according to the French-American-British (FAB) criteria (20); this classification was subsequently modified with the introduction of the new WHO system (21). As a result, 20 patients with >20% blasts in bone marrow at initial diagnosis were excluded from the current study; the remaining 50 patients formed the basis of the present study. Seven patients had refractory anemia, one had refractory anemia with ringed sideroblasts, 27 had chronic myelomonocytic leukemia (CMML). In the current study, transformation to AML was defined by the presence of >30% blasts in bone marrow.

Cytogenetic findings were available in 43 patients and were divided into three categories: good (normal, −Y, del [5q], del [20q]), poor (complex or chromosome 7 anomalies), and intermediate (all other abnormalities), according to the criteria developed by the International MDS Risk Analysis Workshop (22). Data allowing the calculation of risk scores according to the International Prognostic Scoring System (IPSS) were available for 43 patients (22). All patients with MDS were managed with supportive care after initial diagnosis except for four young patients who had CMML; the mutation was absent in all eight patients with CMML.

After AML transformation, 21 patients received standard induction chemotherapy with daunomycin and cytarabine (Ara-C) with two overlapping pairs of primers: PP1F 5′-TCGCC-ATGCCGGAGAACTCCTAAC-3′ and PP1R 5′-CTGGTA-AGGGAAGGCCGCAG-3′, and PP2F 5′-CCGGCTGTGATCAACAGGA-3′ and PP2R 5′-CACGGTCTGGGAACCCCGAGAT-3′, which cover the entire coding region of human CEBPα (14), on a DNA thermal cycler (Applied Biosystems 9600) using a program consisting of 35 cycles at 94°C for 60 seconds, 62°C for 40 seconds, and 72°C for 90 seconds, with an initial preheating at 95°C for 10 minutes and a final step for 10 minutes at 72°C.

RESULTS

Detection of N-ras and FLT3 Activation Mutations. The DNA PCR assay for the detection of point mutations at codons 12, 13, and 61 of N-ras gene, FLT3/ITD and point mutations at tyrosine kinase domain of FLT3 (FLT3/TKD), Genescan-based analysis of the FLT3/ITD mutant level, and sequencing of the duplicated fragments of FLT3/ITD were done as previously described (6, 23).

Statistical Analysis. Fisher’s exact test, χ² analysis, unpaired t test, and Wilcoxon’s rank-sum test were used as appropriate to make comparisons between groups. Kaplan-Meier analysis was used to evaluate survival. Differences in survival were assessed using the log-rank test. Statistical analyses were done using SPSS software version 8.0 for Windows (SPSS Inc., Chicago, IL). In all analyses, P values were two-tailed and values <0.05 were considered statistically significant.

RESULTS

Frequencies and Patterns of CEBPα Mutations in Patients with Myelodysplastic Syndrome at Initial Diagnosis and at Acute Myelogenous Leukemia Transformation. Of the 50 MDS patients at initial diagnosis, CEBPα mutations were detected in 1 of 27 patients with RAEB and in 3 of 15 patients with CMML; the mutation was absent in all eight patients with refractory anemia or refractory anemia with ringed sideroblasts.
AML transformation occurred at a median of 9.1 months (range, 1-112.5 months) following the diagnosis of MDS. At time of AML transformation, three patients retained the identical mutant clones as their initial diagnosis, whereas one lost CEBPα mutation, and another three acquired the mutations. Taken together, seven patients had CEBPα mutations during their disease courses. As shown in Table 1, four patients (patients 1, 4, 6, and 7) had NH2-terminal mutations resulting in frameshift and truncation of the proteins, three of them also had additional mutations: patient 1 had an insertion in transactivation domain (TAD) 2 inducing a frameshift and stop in TAD 2 at codon 169, patient 4 had a 4 bp duplication in TAD 1 and stop at codon 108, and patient 7 had a 17 bp duplication between TAD 1 and TAD 2 resulting in a termination in TAD 2 at codon 165. Two patients had missense mutations, one (patient 2) with a substitution in bZIP, 971T > A (L324Q), and the other (patient 3) with a substitution between TAD 1 and TAD 2, 365G > A (G122E). The remaining patient (patient 5) had a deletion of the first nucleotide of codon 286 in bZIP domain that induced a frameshift and an eventual termination at codon 317. All the mutations were heterozygous. Cloning analysis was done to determine the allelic distribution in the three samples that carried two different mutations. Patient 1, of the 20 clones of MDS sample analyzed, 14 harbored only single mutations, either 179 deletion or 384_385 insertion of G, whereas one clone carried combined mutations on the same allele. In all the alleles carrying single mutation of 179 deletion at MDS phase, acquired 384_385 insertion of G during the progression to AML. In patient 4, 8 of 10 clones harbored only single mutations and one clone each harbored combined mutations and wild-type sequence. The combined mutations on the same allele resulted in an NH2-terminal stop (H246fsX161). In patient 7, all of the 10 cloned alleles harbored single distinct mutations.

**Cooperating Mutations in Myelodysplastic Syndrome/Acute Myelogenous Leukemia Patients.** Patients with CEBPα mutations were also analyzed for activation mutations of FLT3 and N-ras genes (Table 2). None of CEBPα mutation(+) patients had FLT3-TKD mutations either at MDS or AML phase. One patient (patient 7) had a mutation at codon 12 of N-ras gene (Gly12Val) at both phases. Another patient (patient 4) had CEBPα mutation at initial diagnosis of RAEB for which she initially received low-dose Ara-C without response. One cycle of combination chemotherapy with daunomycin and Ara-C was given later. She was then lost to follow-up until AML transformation when she acquired FLT3-ITD but lost CEBPα mutation.

**Clinicohematologic Characteristics of Myelodysplastic Syndrome/Acute Myelogenous Leukemia Patients with CEBPα Mutations.** The clinicohematologic features, IPSS scores, and outcome of the seven patients with MDS/AML who carried CEBPα mutations are summarized in Table 1. Chromosome analysis was successfully done for 43 patients; 17 were found to have normal karyotypes, 20 had intermediate cytogenetics, and 6 had unfavorable cytogenetics. Of the four patients with CEBPα mutations at diagnosis of MDS, two had intermediate cytogenetics and one patient each had normal karyotype and unfavorable cytogenetics. IPSS scores were low in one patient and high in three patients. Table 3 summarizes the initial clinicohematologic characteristics and time to AML transformation according to CEBPα mutation status for patients with MDS. There was no difference in age, sex, hemoglobin level, WBC counts, percentage of blasts in bone marrow, morphologic subtype, cytogenetic subgroup, IPSS score (≤1.5 versus ≥2.0), or time to AML transformation between patients with CEBPα mutations and patients without the mutations. Patients carrying the CEBPα mutations had a higher percentage.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Clinicohematologic characteristics and outcome of patients with CEBPα mutations at MDS and/or AML stages</th>
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<tbody>
<tr>
<td>Patient</td>
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<tr>
<td>1</td>
<td>71/M</td>
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*Genbank accession no. NM_004364.2.
†Two mutations on the same allele.
of circulating blasts \( (P = 0.039) \) and a lower platelet count \( (P = 0.002) \). No significant difference in overall survival \( (P = 0.148; \text{Fig. } 1A) \) was observed between the two groups. A comparison of clinicohematologic features at AML stage between patients with and without \( CEBP\alpha \) mutations was also made (Table 3). Patients with \( CEBP\alpha \) mutations had a higher frequency of FAB AML-M2 phenotype. No difference was observed with respect to age, sex, hemoglobin level, platelet count or percentage of blasts in bone marrow or peripheral blood between \( CEBP\alpha \) mutation(+) and \( CEBP\alpha \) mutation(-) groups. There was no difference in survival after AML transformation according to the mutation status of \( CEBP\alpha \) \( (P = 0.917; \text{Fig. } 1B) \).

**DISCUSSION**

\( CEBP\alpha \) mutations have been described in a subset of patients with AML (14–18) and were associated with favorable outcome (16, 18). The clinical and prognostic significance of \( CEBP\alpha \) mutations in MDS is not clear. The incidence of \( CEBP\alpha \) mutations in our patients with MDS who later developed AML was 8% at initial diagnosis; of note, three of the four MDS patients carrying \( CEBP\alpha \) mutations had CMML. According to WHO classification system, CMML is categorized to be an MDS/myeloproliferative disease rather than MDS per se (21). Two groups have studied the \( CEBP\alpha \) mutations in adult patients with MDS (15, 19); both used the PCR–single-strand conformational polymorphism approach. Kaeferstein et al. (19) found that none in the 52 patients with MDS, which included five patients with CMML, had \( CEBP\alpha \) mutation. Gombart et al. (15) analyzed 92 patients with MDS and detected only one patient with RAEB in transformation carrying \( CEBP\alpha \) mutation; however, the distribution of FAB subtype for the 92 MDS patients was not available in that study. As direct sequencing is the most unambiguous method for detecting \( CEBP\alpha \) mutations, we used direct sequencing for each PCR product in the present study. In addition, samples with abnormal PCR products were repeatedly assayed with alternative primer pairs. Moreover, three of seven samples were further subjected to cloning analysis. The low incidence of \( CEBP\alpha \) mutations in the previous studies was probably attributed to the fact that the sensitivity of PCR–single-strand conformational polymorphism approach may not allow the detection of all mutations. In the present series, 3 of 15 patients with CMML at diagnosis had \( CEBP\alpha \) mutations; the 20% incidence in CMML was contrast to none in the five CMML patients in the previous report (19). More work specifically looking at \( CEBP\alpha \) mutations in CMML or MDS/myeloproliferative diseases is warranted.

Regarding to the frequency of \( CEBP\alpha \) mutations at AML transformation, one group of investigators had examined nine patients at time of AML progression. However, in that series, only one patient had matched samples at both MDS and AML phases; that patient did not have \( CEBP\alpha \) mutation at diagnosis of RAEB but acquired the mutation at AML progression (19). Another group reported that none in the six patients with AML derived from MDS had \( CEBP\alpha \) mutation (14). In the present study, we specially did a comparative analysis of the matched paired marrow samples on \( CEBP\alpha \) mutations at diagnosis of MDS and at AML transformation. We found that the frequency of \( CEBP\alpha \) mutations at AML transformation was 12%. Three of four MDS patients carrying \( CEBP\alpha \) mutations at diagnosis retained the identical mutant clones at AML transformation, suggesting that \( CEBP\alpha \) mutations were important for the leukemogenesis of MDS throughout the disease course in this subset of patients. On the other hand, we observed that one patient (patient 4) lost the mutation when she gained \( FLT3-ITD \) at the time of AML transformation. The patient had received

![Fig. 1](image-url) Kaplan-Meier estimates of survival according to \( CEBP\alpha \) mutation status in patients with MDS (A) and after AML transformation (B).

**Table 2** Cooperating mutations in MDS/AML patients with \( CEBP\alpha \) mutations

<table>
<thead>
<tr>
<th>Patient</th>
<th>( CEBP\alpha )</th>
<th>( FLT3\alpha -ITD )</th>
<th>( FLT3\alpha -TKD )</th>
<th>( N-ras )</th>
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*Gly12Val/Gly12Val.
combination chemotherapy before AML transformation. It was conceivable that the mutant clone carrying CEBPα mutations in this patient was eradicated by chemotherapy and leukemia was outgrown by an evolved clone harboring FLT3-ITD mutation. CEBPα mutations were not detected at diagnosis of MDS but were present at AML transformation in another three patients, indicating the emergence of a novel CEBPα mutant clone in the progression of AML. These observations suggest that CEBPα mutations can be an early or late event in the disease course of MDS.

Four of seven patients had NH2-terminal mutations that resulted in the production of 30 kDa protein that lacks the transactivation ability and has dominant-negative effect on transcription (14, 15). One patient had a mutation in the bZIP domain, which was predicted to inhibit dimerization and DNA binding, thus reducing the transactivation of CEBPα target genes (14, 15). The functional effect of the two missense mutations of 365G > A and 971T > A remained to be determined. By cloning analysis, we showed that the two CEBPα mutations in the same individual patients could occur on the same allele or on separate alleles. Two patients had combined mutations that occurred on the same alleles, resulting in different mutant proteins; one of them had an RSH-terminal mutation at AML progression, suggesting that acquisition of CEBPα mutation could exert the collaborative effect on leukemia progression in MDS patients carrying N-ras mutation.

The use of matched patient samples in the current study allowed us to define the role of CEBPα mutations in the progression of MDS to AML. Our results showed that the frequency of CEBPα mutations was 8% at initial diagnosis of MDS and 12% at AML transformation. These results indicate that CEBPα mutations play a role in the pathogenesis in a subset of patients with MDS/AML. We also observed that one patient (patient 7) harbored both CEBPα and N-ras mutations at AML progression, suggesting that acquisition of CEBPα mutation could exert the collaborative effect on leukemia progression in MDS patients carrying N-ras mutation.

All the mutant alleles were present at AML transformation in another three patients, including one patient (patient 7) who harbored two mutations in the same individual patients could occur on the same allele or on separate alleles. Two patients had combined mutations that occurred on the same alleles, resulting in different mutant proteins; one of them had an NH2-terminal mutation at AML progression, suggesting that acquisition of CEBPα mutations could exert the collaborative effect on leukemia progression in MDS patients carrying N-ras mutation.

The outcome of our patients with MDS who later developed AML, CEBPα mutations did not influence outcome either at the MDS or AML stage.

Cooperating genetic alterations were investigated in MDS/AML patients with CEBPα mutations. None of our patients with CEBPα mutations had coexistence of FLT3-ITD or FLT3-TKD mutations. It was interesting to note that patient 4 had a clonal change during the progression of MDS to AML (i.e., CEBPα mutant clone was lost when FLT3-ITD clone was acquired). The observations suggested that alterations of these genes played a role in a noncooperative manner in a subset of patients with MDS/AML. We also observed that one patient (patient 7) harbored both CEBPα and N-ras mutations at AML progression, suggesting that acquisition of CEBPα mutation could exert the collaborative effect on leukemia progression in MDS patients carrying N-ras mutation.

The use of matched patient samples in the current study allowed us to define the role of CEBPα mutations in the progression of MDS to AML. Our results showed that the frequency of CEBPα mutations was 8% at initial diagnosis of MDS and 12% at AML transformation. These results indicate that CEBPα mutations play a role in the pathogenesis in a subset of patients with MDS throughout the disease course. We also showed that the patterns of CEBPα mutations were heterogeneous during the progression of MDS to AML, including retaining of identical clones, loss of mutant clone, or emergence as novel clones.

REFERENCES


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