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Original Article

Identification of novel CEBPA double mutations capable of promoting familial AML via the suppression of myeloid differentiation

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Abstract: CCAAT-enhancer-binding protein α (CEBPA) gene carrying two mutations (CEBPA double mutations) is known to promote familial acute myeloid leukemia (AML). However, the underlying mechanism by which CEBPA double mutations promote AML remains poorly understood. Here we report that a family with three generations suffering from familial AML carries novel double mutations of CEBPA. Seven bases of GCGCGGG were inserted into the N-terminal c.113-114 of CEBPA as germline mutations and three bases of AAG were inserted into the C-terminal c.939-940 as a somatic mutation. To test the functional impact of this double mutation, we constructed plasmid encoding the double mutants of CEBPA and transfected it into the myeloid precursor 32Dcl3 cells. Lentiviral induced overexpression of CEBPA with these double mutations inhibited myeloid differentiation of these 32Dcl3 cells, and led to approximately 4-fold fewer frequency of CD11b expression. Our results confirm that the double mutations of CEBPA at both N- and C-terminals are potentially to induce leukemogenesis of AML.

Keywords: CEBPA mutation, germline mutation, familial AML

Introduction

Acute myeloid leukemia (AML) is a clonal hematopoietic disease that arises from multiple acquired genetic lesions accumulating in hematopoietic progenitors. The concurrent gene mutations are an important indicator of treatment and prognosis of AML, however, the underlying mechanism has not been fully elucidated. Familial AML is a good model for studying the pathogenesis of AML. CCAAT-enhancer-binding protein α (CEBPA) gene plays critical roles in formation of early granulocyte, and the germline mutation of CEBPA is associated with the development of familial AML [1, 2]. Two types of CEBPA mutations have been identified in approximately 10% of AML patients, including the N-terminal mutation and C-terminal mutation. The mutation rate reported in China is higher than that reported in foreign countries [3-5]. The N-terminal mutation induces overexpression of a short protein isoform p30, which interferes with the production of a full-length protein isoform p42 by a dominant-negative effect, promoting AML formation. The C-terminal mutation is found to play a role as a second event in AML. Either mutation or dysregulation of CEBPA in translation or post-translation leads to enhanced cell proliferation while arresting cell differentiation, which is essential for tumor formation [6-8]. Here we report a new family with a previously uncharacterized germline CEBPA N-terminal mutation in a family of three generations that suffered from AML, including grandmother, father and daughter. All these patients showed trend of onset at early age.

Patients and methods

Samples' resource

In this study, a patient with the family history diagnosed as AML was investigated. DNAs were extracted from the patient's bone marrow (BM)

at the initial stage with blast cells at 94% and remission stage. The peripheral blood (PB) from her parents was used as control. This study was approved by the Ethics Committee of the Children's Hospital of Soochow University. The patient and her patients' informed consent was obtained in accordance with the declaration of Helsinki.

Whole-exome sequencing and analysis of variations

Whole-exome sequencing and analysis were conducted by the Cloud Health Genomics limited company. The DNA extraction was performed with the kit (Thermo), the concentration was measured by NanoDrop 2000 (Thermo), and the integrity of DNA was determined by agarose gel electrophoresis. Using covaris ™ S220 (Life Technologies) was used for DNA interruption, end filling and joint. Sequencing platform was Illumina hiseq × ten and Sequencing strategy was 2 × 150 bp. Library Building Kit was truseq nano DNA library prep Kit.

The validation of mutations

Sanger sequencing was employed to verify the mutations [5'-ATGGAGTCGG CCGA CTTC-TACG-3' (foreword) and 5'-TCACGCGCAGTTGC-CCATGGCCT-3' (reverse)]. Polymerase Chain Reaction (PCR) was subjected to one cycle of 95°C for 1 min, 34 cycles of 95°C for 10 s, 55°C for 30 s and 72°C for 1 min, and 72°C for 10 min with the presence of 5% DMSO (Sigma).

Lentivirus vectors

Plu-CMV-MCS-iGFP lentivirus vector was used to build up two mutant CEBPA constructs: one carrying a N-terminal mutation (c.113-114ins-GCGCGGG) of CEBPA and the other harboring a C-terminal mutation (c.939-940insAAG), the two mutations: N^m and C^m were subcloned into upstream of Plu-CMV-MCS-iGFP lentiviral vector and sanger sequencing as part 2.3 described was used to confirm the successful construction of mutants. The mutant forms of the CEBPA construct were further transfected into myeloid precursor 32Dcl3 cells. Anti-CEBPA (CST) antibody was used to examine the expression level of CEBPA protein in cells using Western-blot.

Cell proliferation and differentiation assay

32Dcl3 cells transfected with different mutants or vector control in the logarithmic growth phase were planted in 96-well plates at a density of 5 × 10^4 cells/ml, and the growth level of transfected 32Dcl3 cells were estimated by quantitating luminescence (FR-200, Bio-red) after 24, 48, 72, 96 hours. 32Dcl3 cell transfected with different mutants were cultured in RIPM-1640 medium supplemented with 10% fetal bovine serum and 2.5 ng/ml interleukin 3 (IL-3).

32Dcl3 cells transfected with different mutants in the logarithmic growth phase were planted in 6-well plates at a density of 1 × 10^6 cells/ml. Six days later, cells were collected and $CD11b^+$ positive cells were tested using flow cytometer (FACSAria cell sorter, BD Bioscience) using phycoerythrin(PE)-conjugated anti-CD11b antibody (BioLegend). Cells were cultured with 2.5 ng/ml IL-3 or 50 ng/ml granulocyte colony-stimulating factor (G-CSF) for differentiation assay as previously described [9]. Lentivirus infection was performed following the published reference [10]. Student *t* test was performed to examine the statistical significance. Results were from three independent experiments. *P* values <0.05 were considered significant.

Results

Patient characteristics

A 3-years-old female patient (IV.1 in **Figure 1A**) was admitted to the hospital due to fever for 2 days and a continuous cough for over 10 days. Physical examination showed hepatosplenomegaly, no other obviously abnormal symptoms. Complete blood count (CBC) showed hemoglobin of 103 g/L, leukocytes of 59.04 × 10^9/L and platelets of 115 × 10^9/L. Myelomonoblastic cells accounted for 45% in her PB and 80.5% cells in the BM. Immunophenotyping analysis showed the blasts were positive for CD33, CD34, CD7, CD117, and CD13. Chromosome analysis showed 46,XX. FISH examination confirmed her normal karyotype.

Family history revealed that her father (III.1 in **Figure 1A**) developed AML-M2 at the age of 13 years old and her grandmother (II.2 in **Figure**

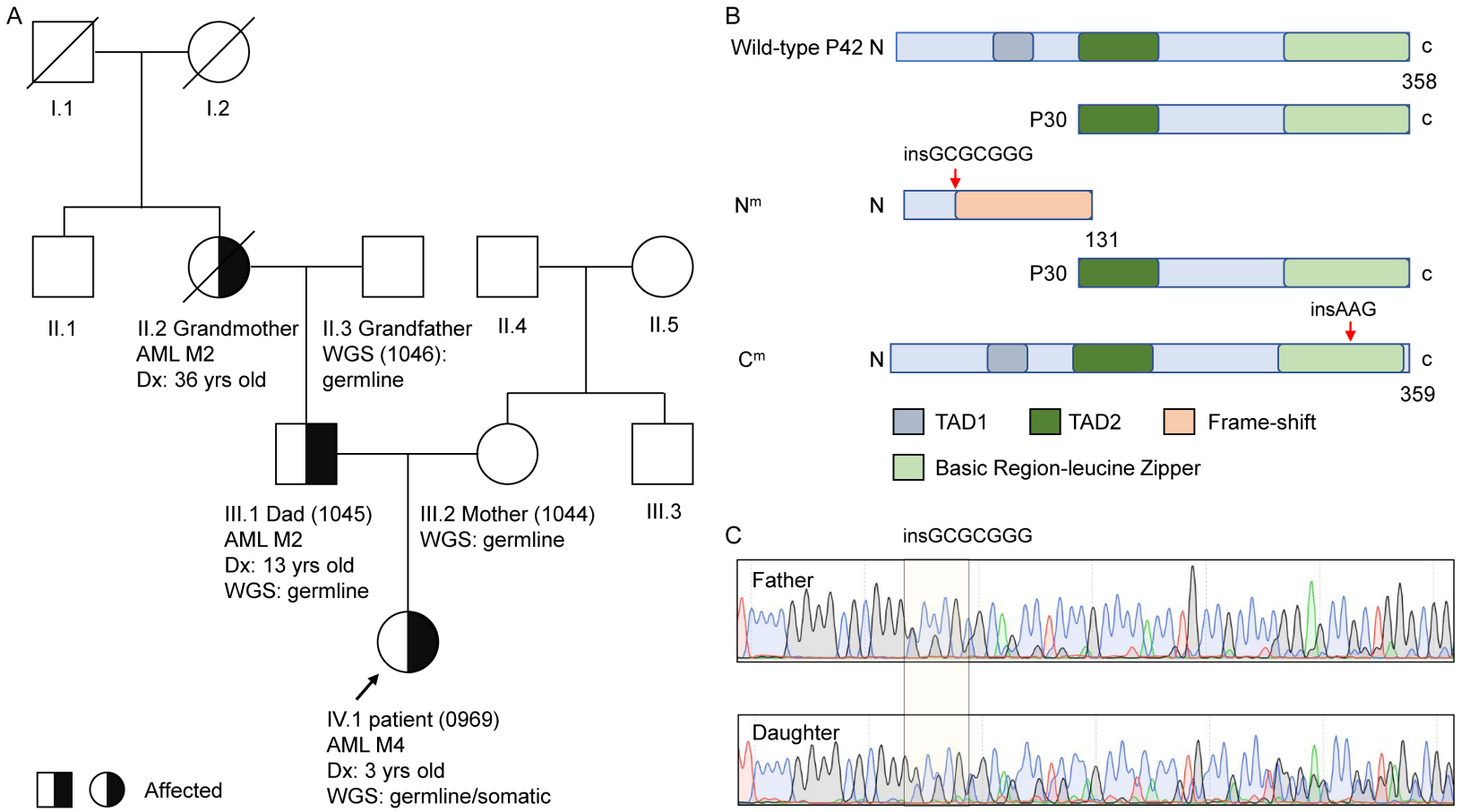


Figure 1. Identification of an N^m CEBPA mutation in patients with familial AML: A: Different types of AML occurred in three generations of one family, accounting grandmother (II.2), father (III.1), and daughter (IV.1). B: Both of N^m and C^m caused the frameshift mutation, but only N^m induced early termination of gene coding which resulted in the absence of P42 protein. C: The Sanger sequencing verification of germline mutation in father's, mother's and daughter's samples: N^m was detected in sample from father (III.1) and daughter (IV.1), confirming that the N^m was germline mutation.

Table 1. Chemotherapy protocols

Treatment Stage	Drug	Dose	Usage	Time (d)
Induction 1	Daunomycin	50 mg/m2.d	Ivgtt	1-3
	Cytarabine	100 mg/m2.q12h	Ivgtt	1-7
	Etoposide	100 mg/m2.d	Ivgtt	1-5
Induction 2	Homoharringtonine	3 mg/m2.d	Ivgtt	1-7
	Cytarabine	100 mg/m2.q12h	Ivgtt	1-7
	Etoposide	100 mg/m2.d	Ivgtt	1-5
Consolidation 1	Mitoxantrone	10 mg/m2.d	Ivgtt	1-3
	Cytarabine	3000 mg/m2.q12h	Ivgtt	1-3
Consolidation2	Etoposide	150 mg.m2.d	Ivgtt	1-3
	Cytarabine	3000 mg/m2.q12h	Ivgtt	1-3

Table 2. Demographic characteristics of patients

Characteristics	Description
Age, year	3
Gender	Female
Phenotype for AML	M4
Karyotype	46, XX
Mutations	Double
Conditioning regimen	BuCy + cytarabine
Donor-recipient sex pairs	F-F
Donor-recipient HLA match	6/10
Graft type	Bone marrow +PBSC
Mononuclear cells	5.37×10^8/kg
CD34+	7.59×10^6/kg
GVHD prophylaxis	ATG+CsA+MTX+
GVHD treatment	Methylprednisolone
Neutrophil engraftment	+14 d
Platelet engraftment	+16 d
IV degree of aGVHD	+20 d
cGVHD	Yes

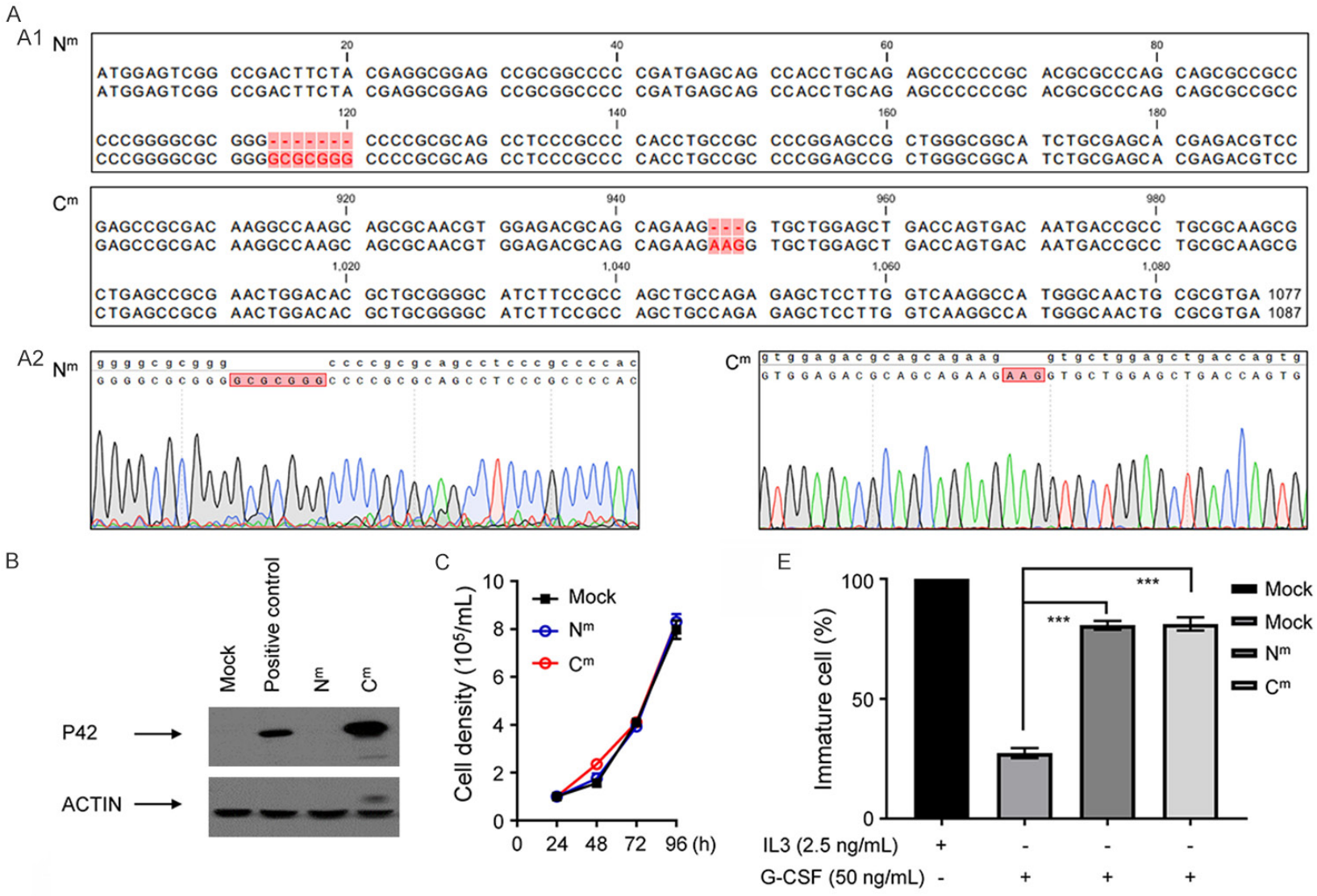
Note: BuCy: busulphan cyclophosphamide, CsA: cyclosporin A, MTX: methotrexate, PBSC: peripheral blood stem cell, aGVHD: acute graft versus host disease, cGVHD: chronic graft versus host disease.

1A) suffered from AML-M2 at the age of 36 years old. Her father (III.1 in **Figure 1A**) survived after autologous hematopoietic stem cell transplantation (auto-HSCT) and her grandmother (II.2 in **Figure 1A**) died from the disease without treatment. In order to understand the mechanism of this familial leukemia, we performed whole-genome sequencing and found that her blast cells possessed double mutations on the CEBPA gene (IV.1 in **Figure 1A**, a N-terminal mutation (c.113-114insGCGCGGG) and a C-terminal mutation (c.939-940insAAG). In her remission blood, only one mutation located on N-terminal mutation (c.113-114insGCGCGGG) was identified (**Figure 1B**). We further sequenced her father's (III.1 in **Figure 1A** blood and found that her father has only one mutation located on the N-terminal of CEBPA (c.113-114insGCGCGGG) (**Figure 1C**). Her mother hadn't any mutations of CEBPA. We didn't get her grandmother's (II.2 in **Figure 1A**) tissue because of her early death. To the best of our knowledge, these two mutations in CEBPA have not been previously reported in AML patients.

The patient (IV.1 in **Figure 1A**) received daunorubicine, cytarabine, and etoposide as her first induction therapy and got CR, followed by homoharringtonine, cytarabine, and etoposide for the second induction therapy (**Table 1**). After two cycles of consolidation therapy (**Table 1**), she was given haploid hematopoietic stem cell transplantation because of lack matched unrelated donor. The donor was her mother. The conditional regimen and prevention of graft versus host disease (GVHD) were illustrated in **Table 2**. She recovered from IV degree of GVHD that involved skin and gastrointestinal tract. Now she is stable for more than 6 years.

Novel CEBPA double mutations are capable of promoting familial AML via the suppression of myeloid differentiation

The sequencing data indicated that the mutation of (N^m) c.113-114insGCGCGGG was inherited because the father and the daughter had the same mutation. The N-terminal mutation (N^m) c.113-114insGCGCGGG was presented in the onset and CR samples of the patient and her father's sample. Notably, the daughter had another mutation at the C-terminal: (C^m) c.939-940insAAG. However, this later mutation disappeared in her CR sample. Her father also didn't show any other mutations in CEBPA under his healthy condition. It suggests that C-terminal: (C^m) c.939-940insAAG probably plays a synergistic effect on the development of AML primarily induced by N-terminal mutation (N^m) c.113-114insGCGCGGG. The case further told us that the N^m was a germline mutation and C^m was a somatic mutation.


A
A1
Nm
ATGGAGTCGG CCGACTTCTA CGAGGCGGAG CCGCGGCCCC CGATGAGCAG CCACCTGCAG AGCCCCCCGC ACGCGCCCAG CAGCGCCGCC
ATGGAGTCGG CCGACTTCTA CGAGGCGGAG CCGCGGCCCC CGATGAGCAG CCACCTGCAG AGCCCCCCGC ACGCGCCCAG CAGCGCCGCC
CCCGGGGCGC GGG-------
CCCGGGGCGC GGGGCGCGGG
CCCCGCGCAG CCTCCCGCCC CACCTGCCGC CCCGGAGCCG CTGGGCGGCA TCTGCGAGCA CGAGACGTCC
CCCCGCGCAG CCTCCCGCCC CACCTGCCGC CCCGGAGCCG CTGGGCGGCA TCTGCGAGCA CGAGACGTCC
Cm
GAGCCGCGAC AAGGCCAAGC AGCGCAACGT GGAGACGCAG CAGAAG---G TGCTGGAGCT GACCAGTGAC AATGACCGCC TGCGCAAGCG
GAGCCGCGAC AAGGCCAAGC AGCGCAACGT GGAGACGCAG CAGAAGAAGG TGCTGGAGCT GACCAGTGAC AATGACCGCC TGCGCAAGCG
CTGAGCCGCG AACTGGACAC GCTGCGGGGC ATCTTCCGCC AGCTGCCAGA GAGCTCCTTG GTCAAGGCCA TGGGCAACTG CGCGTGA 1077
CTGAGCCGCG AACTGGACAC GCTGCGGGGC ATCTTCCGCC AGCTGCCAGA GAGCTCCTTG GTCAAGGCCA TGGGCAACTG CGCGTGA 1087
A2
Nm
Cm
B
Mock
Positive control
Nm
Cm
P42
ACTIN
C
Cell density (10⁵/mL)
Mock
Nm
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0 24 48 72 96 (h)
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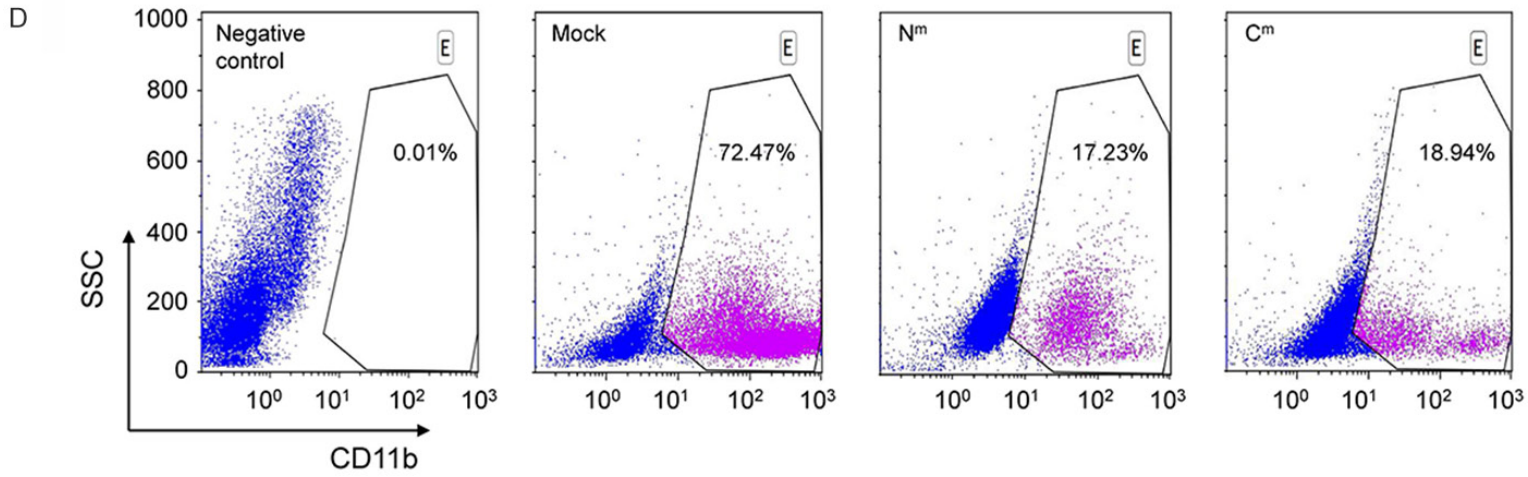

Figure 2. The function of our identified CEBPA double mutations in myeloid leukemogenesis: A1, A2: Construction of N^m and C^m expression plasmids and sanger sequencing was employed to verify the mutations. B: Expression of CEBPA in 32Dcl3 cells transfected with empty Plu-CMV-MCS-iGFP lentivirus vector (Mock) or mutations (N^m and C^m). C: 32Dcl3 cells transfected with different plasmids were cultured in RIPM-1640 medium with 2.5 ng/ml IL-3 and data derived from the mean and standard deviation of 3 independent experiments. There is no difference in growth rate of 32Dcl3 cells expressing N^m or C^m. D: The effects of N^m and C^m on the differentiation of myeloid precursor cells to neutrophils. 32Dcl3 cells transfected with N^m or C^m plasmids. After two days, these cells were cultured in RIPM-1640 medium with 50 ng/ml G-CSF for 6 days. CD11b positive cells were measured by flow and the percentage of mature cells was calculated. E: Histogram of cell differentiation experiment results. CD11b negative cells were immature, statistical signification of 3 independent experiments were measured by Student *t* test. ***means P<0.001.

To investigate the biological function of the CEBPA double mutations, we constructed two mutant CEBPA constructs and transfected into myeloid precursor 32Dcl3 cells using Plu-CMV-MCS-iGFP lentivirus vector, which was confirmed by sequencing: one carrying a N-terminal mutation (c.113-114insGCGCGGG) of CEBPA and the other harboring a C-terminal mutation (c.939-940insAAG) (**Figure 2A**). Western blot revealed that the size of the transfected mutants was 42 KD which further confirmed the vector constructions (**Figure 2B**). Transfected 32Dcl3 cells were cultured in the presence of IL-3 followed by the treatment of G-CSF at 50 ng/ml for 6 days. Cell proliferawtion assays using CCK8 method showed that the mutants didn't influence the growth of transfected cells (**Figure 2C**). Notably, 32Dcl3 cells expressing CEBPA-N^m or CEBPA-C^m exhibited lower level of CD11b expression compared to control cells without expressing CEBPA mutants. CD11b was expressed in 17.23% N^m mutant cells and 18.94% C^m mutant cells. This was about 4-fold fewer than wild-type cells (72.47%). These results indicated that while wild-type cells were relatively mature (**Figure 2D**), the mutant 32Dcl3 cells transfected with either CEBPA-N^m or CEBPA-C^m showed significantly increased proportion of immature cells (**Figure 2E**). Thus, both CEBPA-N^m and CEBPA-C^m variations can block the differentiation of 32Dcl3 cells into polymorphonucleated neutrophils (**Figure 2D**, **2E**).

Discussion

Familial AML with CEBPA mutation has been reported in a few number of families [1, 2, 4, 10-13]. In our case, three generations from this family developed a different type of AML. We confirmed these mutations using Sanger sequencing in patient's DNA sample during CR and her father's DNA sample after auto-HSCT. There was no mutations found in her mother's DNA sample from the PB. We observed that her father had the same gene mutation as his daughter. Notably, the Whole-exome sequencing showed the daughter had additional mutation. This indicated that the N-terminal mutation in the patient and her father was germline mutation. In contrast, the C-terminal mutation in the patient was a somatic mutation. It is reported that the N-terminal mutation alone was not enough for the onset of disease, and the supplementary mutation was necessary for leukemogenesis, which is consistent with our results [2, 14-16]. In fact, her father survived well after auto-HSCT and has not relapsed or a tendency to develop new malignancy. In contrast, she developed AML with double CEBPA mutations, and her onset age was even earlier than her father.

Our functional experiment results of the 32Dcl3 cells indicated that those mutations affected myeloid cells differentiation. Thus, both the CEBPA gene N-terminal mutation (c.113-114insGCGCGGG) and C-terminal mutation (c.939-940insAAG) may act as new CEBPA double mutations with the potential to induce malignant diseases. Up to now, our patient had received haplo-HSCT from her mother and survived for 7 years. Since three generations with AML in this family showed an early trend of onset age, it is possible that other factors in the patient may contribute to an early disease onset, such as other undiscovered somatic mutations, life style and environmental factors.

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Disclosure of conflict of interest

None.

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