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C/EBPβ suppression by interruption of *CUGBP1* resulting from a complex rearrangement of *MLL*

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Abstract

Translocations involving the mixed-lineage leukemia gene (*MLL*) confer a poor prognosis in acute leukemias. In t(1;11)(q21;q23), *MLL* is fused reciprocally with *AF1q*. Here we describe a t(1;11)(q21;q23) with a secondary event involving insertion of the telomeric portion of *MLL* into the p arm of chromosome 11 (11p11). We show that this latter event interrupts the CUG triplet repeat binding protein-1 (*CUGBP1*) gene, a translational enhancer of *C/EBPβ*. We then showed that these cells have reduced expression of CUGBP1 and C/EBPβ when compared to other AML blasts. This is the first report to describe insertional disruption of the *CUGBP1* gene and to suggest a role for the CUGBP1-C/EBPβ pathway in leukemogenesis.

1. Introduction

Translocations of chromosomal band 11q23 are among the most observed rearrangements in leukemia, occurring in 5–6% of primary acute myelogenous leukemias (AML), a majority of secondary leukemias, and nearly all infant acute lymphocytic leukemias (ALL) [1,2]. 11q23 translocations disrupt the mixed-lineage leukemia (*MLL*) gene and usually confer a poor prognosis [1,3]. The *MLL* gene is nonrandomly fused with one of several dozen *MLL* fusion partner genes such as *AF4*, *AF6*, *ELL*, and *ENL* [2,4–6]. Most rearrangements of the *MLL* gene occur within a single 8.5-kb breakpoint cluster region and can be identified by cytogenetic G-banding and fluorescent in situ hybridization (FISH) analyses [6–10]. The t(1;11)(q21;q23) fuses the *MLL* gene to the *AF1q* gene located on chromosomal band 1q21 [6,11]. To our knowledge, fewer than 20 cases of this translocation have been reported this past decade [5,6,11,12].

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CCAAT/enhancer binding proteins (C/EBPs) are transcription factors known to play a critical role in myeloid differentiation. They are often dysregulated in myeloid leukemias and have been identified as targets of BCR/ABL, ETO, and FLT3 mutations. Expression of C/EBP proteins is controlled at several levels, including regulation of translation by certain RNA-binding proteins. One of these proteins, CUG triplet repeat binding protein-1 (CUGBP1), is a translational regulator of *C/EBPβ* that interacts with the 5′ region of *C/EBPβ* mRNA and increases the translation of two C/EBPβ isoforms, liver-enriched activator protein (LAP) and liver-enriched inhibitory protein (LIP) [13].

In this case report, we describe a variant t(1;11)(q21;q23) occurring in a pediatric AML patient (designated AML17) that activates MLL as described previously, but also disrupts *CUGBP1*, leading to decreased translation of *C/EBPβ*.

2. Materials and methods

2.1. Clinical history

The patient described herein (AML17) is a 7-month-old Hispanic female who presented to the local emergency room with a history of 1 week of upper respiratory symptoms, 2 days of fussiness, and fever to 40°C. The presenting white blood cell (WBC) count was 310×10^3 cells/μL with a morphology on peripheral smear suggestive of myeloblasts. Hydration reduced the WBC count to 210.6×10^3 cells/μL. A large-bore pheresis catheter was placed, and leukaphoresis was performed that reduced her WBC to 144.9×10^3 cells/μL. Peripheral blood was sent for routine hematopathologic stains, flow immunophenotyping, and cytogenetics. The smears demonstrated blasts, promonocytes, and reactive-appearing monocytes having fine granules and strong positive neuron-specific enolase (NSE) staining completely inhibited by NaF, consistent with M5 AML. Immunophenotyping revealed positive staining for cell surface markers CD4 (95%), CD11b (90%), CD13 (36%), CD15 (94%), CD33 (96%), CD(16+56) (97%), κ-light chain (86%), and λ-light chain (47%). The cytogenetics findings are described in Table 1. The patient received one dose of rasburicase and was treated according to Pediatric Oncology Group (POG) protocol 9421. The leukemia responded rapidly to therapy, and she remains in remission 1 year off therapy.

2.2. Patient samples and cell lines

Leukemic myeloblasts were obtained by leukapheresis of newly diagnosed pediatric patients with very high peripheral blood blast counts (Table 1) under an institutional review board (IRB)-approved collection and banking protocol. Mononuclear cells were isolated by density gradient centrifugation over Lymphoprep (Axis-Shield PoC AS, Oslo, Norway). Cells were viably frozen in freezing media [RPMI 1640, 20% fetal bovine serum (FBS), 10% DMSO] and stored in liquid nitrogen. BS LCL is an EBV-transformed lymphoblastoid cell line (LCL) from a normal healthy donor. NIH 3T3 and HeLa cell lines were obtained from American Type Culture Collection (Manassas, VA, USA).

2.3. Conventional cytogenetic analysis

Cytogenetic evaluations were conducted on all AML patient samples as clinically indicated. Routine G-banding karyotype analyses were performed by the clinical laboratory to identify chromosomal translocations and other abnormalities as indicated clinically. Fluorescence in situ hybridization (FISH) was performed on metaphase cells of AML17 using LSI MLL dual color break apart rearrangement probe and TelVysion telomere region-specific probes for chromosomes 1 and 11 (Abbott Molecular/Vysis, Des Plaines, IL, USA).

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2.4. Array comparative genomic hybridization

Genomic DNA was isolated by QIAamp DNA Mini Kit (Qiagen, Inc., Valencia, CA, USA). Array comparative genomic hybridization (aCGH) was performed using the SpectralChip 2600, a bacterial artificial chromosome (BAC) array with an average resolution of 1 MB across the genome. Briefly, genomic DNA was fragmented and labeled by a poplymerase reaction using fluorophore labeled dNTPs and random hexamer primers. DNA was then hybridized to the chip according to the manufacturer's protocol. The chips were scanned using a GenePix 3000B scanner running GenePix Pro 6.0.1 and analyzed using SpectralWare v2.

2.5. Long-distance inverse polymerase chain reaction (LDI-PCR)

The DNA sample was treated and analyzed as described [14]. Briefly, 1 μg of genomic DNA was digested with restriction enzymes and re-ligated to form DNA circles before LDI-PCR using *MLL*-specific primers. Restriction polymorphic PCR amplimers were isolated from the gel and subjected to DNA sequence analyses to obtain the patient-specific fusion sequences [5,14].

2.6. Quantitative reverse-transcription polymerase chain reaction (RT-PCR)

RNA was isolated using the SV Total RNA Isolation Kit (Promega Corp., Madison, WI, USA). Quantitative RT-PCR was performed in triplicate on RNA isolated from each sample (Table 1) using the One-Step RT-PCR Kit (Qiagen) with Taqman probes (Applied Biosystems, Foster City, CA, USA) for *CUGBP1* (Hs00198069_m1), *HOXA9* (Hs00365956_m1), and 18s rRNA (Hs99999901_s1), following the manufacturer's protocol. After 30 minutes of reverse transcription at 48°C and 10 minutes of enzyme activation at 95°C, 40 cycles of amplification were performed (15 seconds of denaturation at 95°C followed by 1 minute of annealing and elongation at 60°C). Fluorescence was measured after each cycle. Quantititative RT-PCR was performed using an Opticon thermocycler (MJ Research, Hercules, CA, USA). The expression levels were normalized to 18s rRNA.

2.7. Western blot analysis

Proteins were isolated from freshly thawed cells and analyzed by Western blotting as described [13,15]. Briefly, cells were homogenized in buffer A containing 20 mmol/L Tris-HCl, pH 7.5, 30 mmol/L NaCl, and 2 mmol/L $MgCl_2$. Nuclei were pelleted by centrifugation at 12,000 rpm for 10 minutes. The supernatant (cytoplasm) was used for the examination of CUGBP1. A nuclear extract was obtained by treatment of nuclei with buffer B (0.42 mol/L NaCl, 20 mmol/L Tris-HCL, pH 7.5, 5 mmol/L DTT, 2 mmol/L $MgCl_2$, and 25% sucrose). Inhibitors of proteases and phosphatases were included in all buffers used for the isolation of proteins. Cytoplasmic and nuclear proteins were separated by denaturing SDS 6–18% gradient gel (BioRad, Hercules, CA, USA) and transferred onto the membrane. Proteins were detected with antibodies against C/EBPβ and CUGBP1 (clones C19 and 3B1, respectively; Santa Cruz Biotechnologies, Inc., Santa Cruz, CA, USA). The membranes were re-probed with antibodies to β-actin and stained with Coomassie to assess for equal protein loading.

3. Results

3.1. A variant t(1;11)(q21;q23) identified by cytogenetics and FISH

The rearrangement of chromosomal bands 1q21 and 11q23 was identified in the routine G-banding analysis (Fig. 1a). Metaphase FISH using region-specific probes for chromosome 1 shows normal 1p and 1q telomeric hybridization signals on one copy of chromosome 1 but

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only a 1p signal on the homologous chromosome. The remaining 1q signal is located on the derivative chromosome 11 (Fig. 1b). Similarly, metaphase FISH using region-specific probes for chromosome 11 shows normal 11p and 11q telomeric hybridization signals on one copy of chromosome 11, but an 11p only on the homologous pair and the remaining 11q on the derivative chromosome 1 (Fig. 1c), suggesting a balanced translocation. However, FISH analysis for the *MLL* gene using a break-apart rearrangement probe shows the telomeric hybridization signal of the *MLL* probe (orange) is located in the p-arm of chromosome 11 (Fig. 1d) rather than on chromosome 1, where it is expected. Array comparative genomic hybridization (aCGH) reveals no genomic losses or gains within the detection limits of this assay (Fig. 2).

3.2. Genomic breakpoints determined by LDI-PCR

To determine the genomic breakpoints of the t(1;11) in AML17, the fusion region was amplified by LDI-PCR for the 5′ *MLL* fusion (Fig. 3a, lane 2) and the 3′ *MLL* fusion (Fig. 3a, lane 3). The upper band in lane 2 is consistent with the wild-type *MLL*. The sequence of the lower band in lane 2 reveals a fusion of 5′ *MLL* at intron 10/11 with *AF1q* at intron 1/2, linked by two filler nucleotides (Fig. 3b). Sequencing of the band in lane 3 reveals a fusion of 3′ *MLL* at intron 10/11 with *CUGBP1* at intron 9/10, linked by two filler nucleotides (Fig. 3c). This sequence indicates that *CUGBP1* and *MLL* are fused in opposing transcriptional directions.

3.3. Expression of CUGBP1 is reduced and HOXA9 is elevated

Since the *CUGBP1-MLL* fusion did not result in a transcribable fusion but, rather, disrupted the *CUGBP1* gene, we evaluated whether transcription of *CUGBP1* was affected. In addition, to confirm that *MLL-AF1q* was an activating fusion product, we assessed transcriptional levels of *HOXA9* as a known target of MLL. The mRNA levels of *CUGBP1* and *HOXA9* in AML17 were compared to four other AML samples (Table 1) and one LCL by quantitative RT-PCR using Taqman probes. AML17 has decreased *CUGBP1* (Fig. 4a) and increased *HOXA9* expression (Fig. 4b) compared to the other cells tested.

3.4. Expression of C/EBPβ isoforms are reduced

CUGBP1 binds to the 5′ region of *C/EBPβ* mRNA and increases the translation of the C/EBPβ-LIP and C/EBPβ-LAP isoforms [16]. The decrease in *CUGBP1* expression suggests that its leukemogenic role in this patient might be through a loss of post-transcriptional regulation of *C/EBPβ*. Therefore we assessed the protein expression of these genes in AML17 and compared them to AML13, a sample with high mRNA levels of *CUGBP1*. The reduced protein level of CUGBP1 as determined by Western blot probe (Figure 5a) is consistent with the reduced mRNA levels shown by quantitative RT-PCR (Figure 4a). Moreover, Western blot for C/EBPβ showed that the protein levels of both the LIP and LAP isoforms of C/EBPβ are markedly decreased in AML17 compared to the levels of these isoforms in AML13 (Figure 5b). This inhibition of *C/EBPβ* translation is consistent with the hypothesis that the interrupted *CUGBP1* gene leads to a low level of CUGBP1 and thus to reduced translation of *C/EBPβ*.

4. Discussion

The germline *MLL* gene encodes a protein of 3,972 amino acids (431 kD), which is a common target for chromosomal translocations in acute leukemias [11,17]. The human *MLL* gene is homologous to *Drosophila trithorax*, which is a homeotic transcriptional regulator and contains PHD finger motifs along with DNA-binding motifs such as AT hooks and a DNA methyltransferase homology region [11,18–20]. Translocations involving the *MLL* gene form various in-frame fusion transcripts with many different partner genes throughout

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the genome [5,17]. The N-terminal portion of the *MLL* gene that contains the AT hook DNA-binding motifs and the methyltransferase homology is necessary for the oncogenic effect when fused with the C-terminal portion of the partner gene [11,18,20,21]. In AML17, the *MLL-AF1q* fusion contains this oncogenic region of MLL, as expected. The second rearrangement in AML17 fuses the telomeric end of the *MLL* gene with the 5′ segment of the CUG triplet repeat binding protein-1 gene (*CUGBP1*) in opposing transcriptional directions.

CUGBP1 is a downstream target of the epidermal growth factor receptor (EGFR) signaling pathway and a translational regulator that interacts with the CUG repeats in the 5′ region of *C/EBPβ* mRNA [13,22]. The binding of CUGBP1 to the mRNA of *C/EBPβ* increases translation of the C/EBPβ-LIP and C/EBPβ-LAP isoforms [13,16,22]. C/EBPβ, also known as nuclear factor of interleukin-6 (NF-IL6), regulates the transcriptional activities of many processes, including the production of several cytokine genes and myeloid differentiation. Although C/EBPβ is not required for steady-state granulopoiesis [23,24], it is required for normal and emergency myeloid differentiation [25,26]. In AML17, disruption of *CUGBP1* by the C-terminal portion of the *MLL* gene and subsequent loss of *C/EBPβ* translation suggests a role for *CUGBP1* mutations in leukemogenesis.

These data suggest that the complex rearrangement in AML17 caused both oncogenic conversion of *MLL* by fusion to *AF1q* and decreased C/EBPβ expression by the loss of translational enhancement mediated by CUGBP1. This rearrangement could have resulted from (1) two sequential events (independent translocation and deletion/insertion events) or (2) a single cross-over event involving four recombination sites. The sequences obtained by LDI-PCR showed complete conservation of the genomic *MLL* sequence, suggesting the latter mechanism. This is the first report showing a possible role for the CUGBP1-C/EBPβ pathway in leukemogenesis.

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W.T.C. performed the RT-PCR experiments and was the primary author of the manuscript. M.F. performed the array CGH, and M.A. perfomed the G-banding analysis and metaphase FISH. R.N. coordinated and interpreted the clinical cytogenetic data. C.M. and E.K. performed the LDI-PCR and interpreted the data with the assistance of R.M. N.T. performed and interpreted the Western blot. D.A.L. coordinated the research direction of the project, collected and banked the patient samples under an IRB-approved research protocol, and assisted in writing the manuscript.

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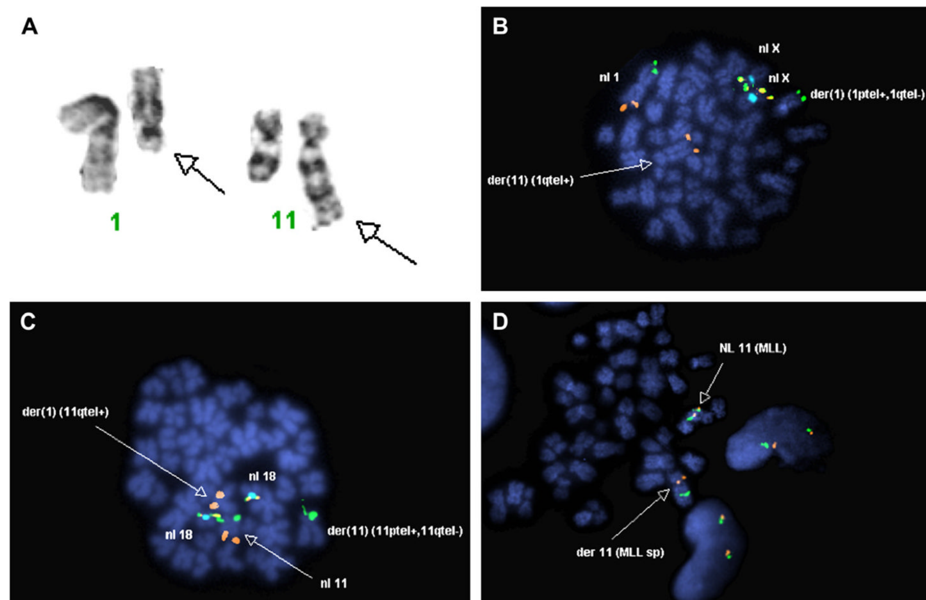

Fig. 1.

Cytogenetics of AML17. (A) G-banding analysis shows derivative chromosomes 1 and 11 (arrows). Metaphase FISH using telomere region–specific probes for chromosomes 1 (B) and 11 (C) shows a pattern consistent with a balanced reciprocal translocation. (D) FISH using a break-apart rearrangement probe for the *MLL* gene shows both portions of *MLL* on the derivative chromosome 11.

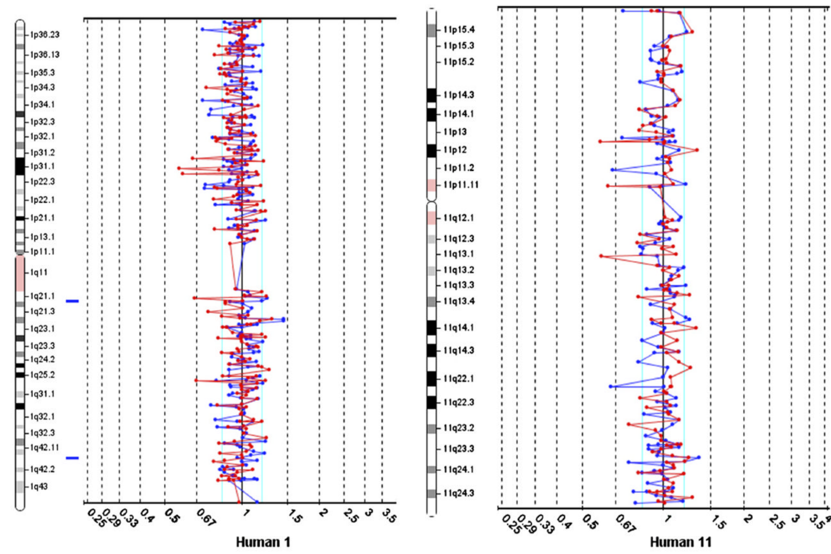

Fig. 2.

Array CGH of AML17. DNA was purified from banked samples of AML17 and hybridized to the BAC-based human genome array as described, showing no apparent losses or gains of genetic material. Chromosomes 1 (left) and 11 (right) are shown.

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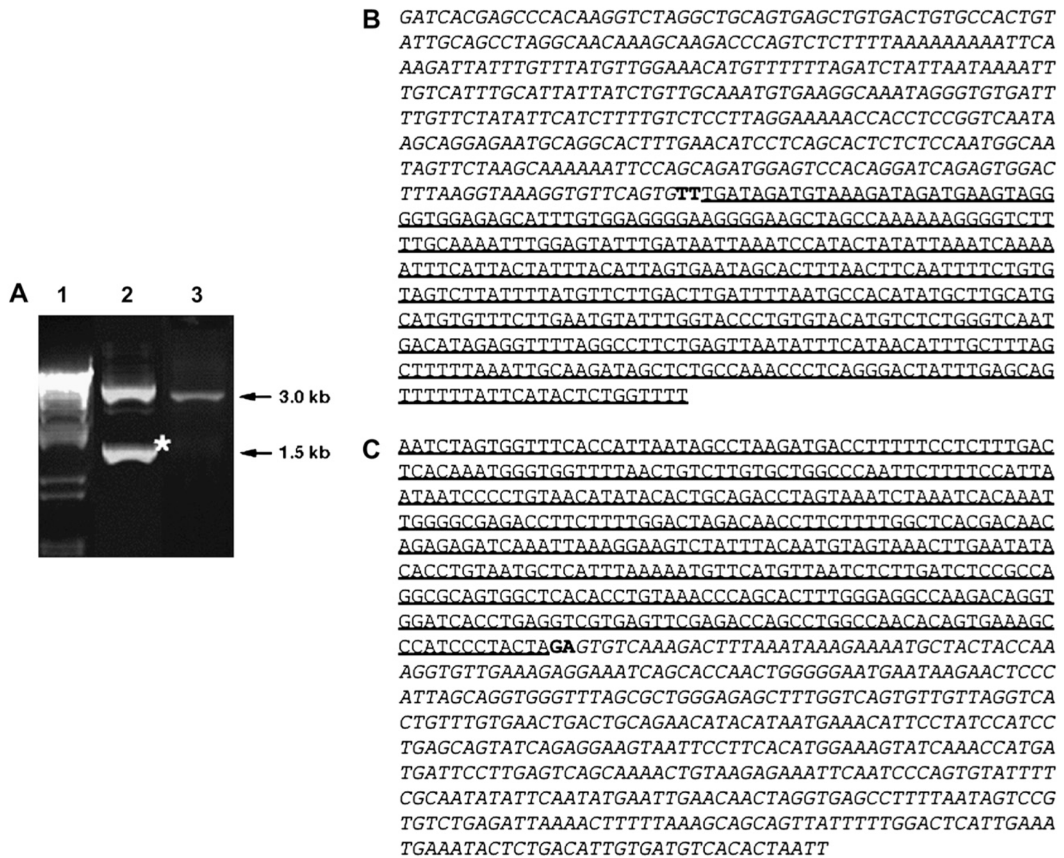

Fig. 3.

Genomic breakpoints within *MLL*. (A) LDI-PCR analysis of AML17 genomic DNA. Lane 1, marker; lane 2, LDI-PCR analysis of *MLL*-translocation partner (TP) fusion showing the wild-type band and the additional *MLL*-TP band (asterisk); lane 3, LDI-PCR analysis of TP-*MLL* fusion (in this case, only the TP-*MLL* amplimer appeared). (B) The sequence of the *MLL*-TP amplimer reveals a fusion between intron 10/11 of *MLL* (italic) and intron 1/2 of *AF1q* (underline) linked by two filler nucleotides (bold). (C) The sequence TP-*MLL* reveals a fusion between intron 9/10 of *CUGBP1* (underline) and intron 10/11 of *MLL* (italic) linked by two filler nucleotides (bold).

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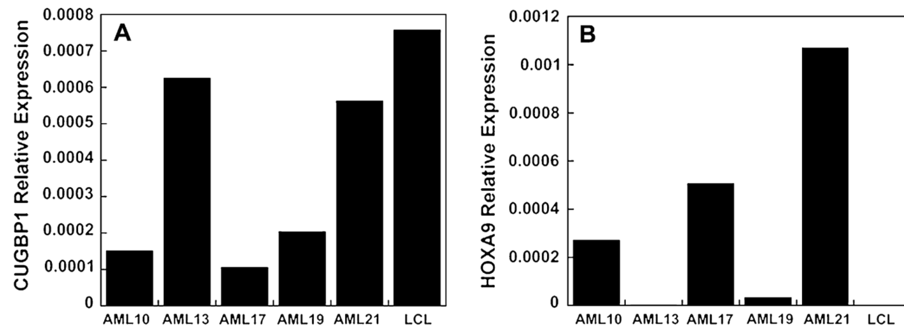

Fig. 4.

Quantitative RT-PCR analysis. Quantitative RT-PCR for *CUGBP1* (A) and *HOXA9* (B) was performed on RNA from representative primary AML blasts and a control LCL line. Expression levels are normalized to 18S rRNA.

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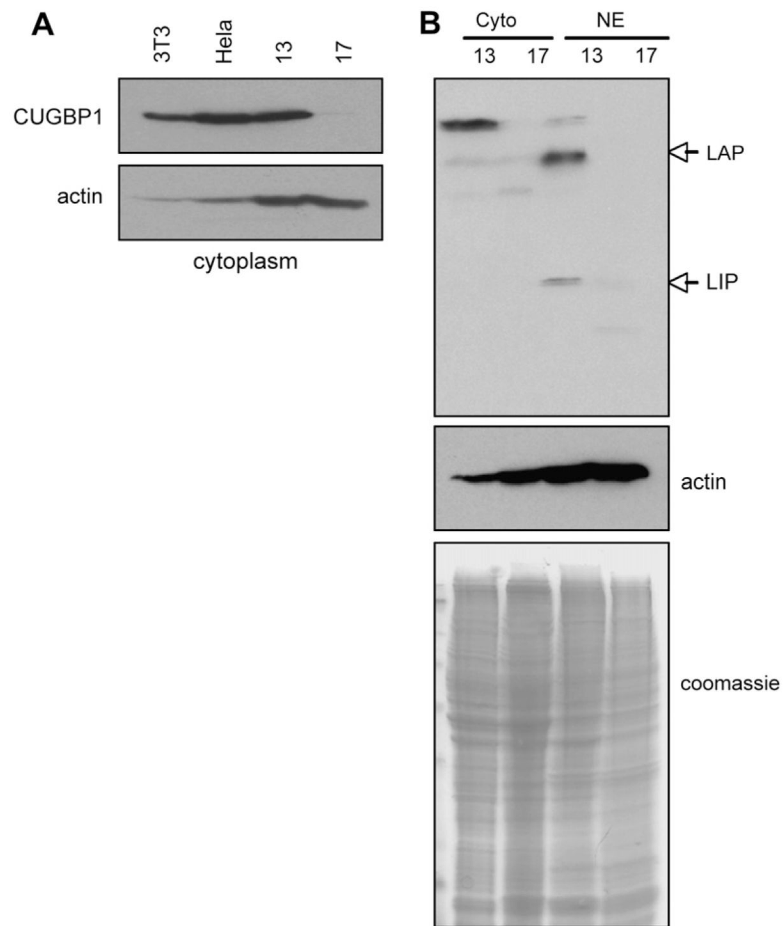

Fig. 5.

Expression of CUGBP1 and C/EBPβ proteins. (A) Western blot analysis of CUGBP1 in protein lysates from AML13 and AML17 with actin-loading controls. Protein lysates from 3T3-L1 and HeLa cells were used as controls. (B) Western blot analysis of C/EBPβ in cytoplasmic (Cyto) and nuclear extract (NE) proteins from AML13 and AML17. Positions of LAP and LIP isoforms are shown by arrows. Coomassie staining and actin show equal protein loading of all lanes.

Table 1

Patient characteristics of primary AML samples used in quantitative RT-PCR study

Sample	Age at diagnosis	FAB	Reported clinical karyotype
AML10	6 years	M5	45,X,add(X)(q?25),t(2;7)(p21;q36),t(2;11)(q23;p13),del(3)(q21),del(5)(q15q31),del(14)(q?24),-17,add(22)(q11.2)[18]46,X
AML13	11 years	M2	46,XX,t(4;20)(q31;q11)[19]/46,XX[1]
AML17	7 months	M5	46,XX,t(1;11)(q21;q23),inv(11)(q13q23)[20]
AML19	8 years	M1, MDS-related	47,XX,t(1;19)(p22;q13),del(2)(q33),del(3)(q21),+i(5)(p10),add(6)(p2?3)[14]
AML21	12 years	M1/M2	46,XY[20]

Leukocytes were obtained by leukopheresis from patients with AML at initial diagnosis. Hematopathologic subtypes and cytogenetic abnormalities were determined by the clinical laboratory.

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