CD7 in acute myeloid leukemia: correlation with loss of wild-type CEBPA, consequence of epigenetic regulation

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Abstract

Background: CD7 is a negative prognostic marker in myeloid malignancies. In acute myeloid leukemia (AML), an inverse correlation exists between expression of wild-type CEBPA and CD7. Aim of this study was to find out whether C/EBPα is a negative regulator of CD7 and which other regulatory mechanisms might be involved.

Results: As already described for primary AML cells, the majority of AML cell lines tested were either C/EBPα+/CD7- or C/EBPα-/CD7+. However, the existence of isolated CD7+ cell lines expressing wild-type C/EBPα challenges the notion that C/EBPα acts as a unique repressor of CD7. Furthermore, ectopic expression of CEBPA did not reduce CD7 in CD7+ cells and knock-down of C/EBPα failed to induce CD7 in CD7- cells. In contrast, the DNA demethylating agent Aza-2'deoxycytidine triggered CD7 expression in CD7- AML and in T-cell lines suggesting epigenetic regulation of CD7. Bisulfite sequencing data confirmed that CpGs in the CD7 exon1 region are methylated in CD7- cell lines, and unmethylated in CD7+ cell lines.

Conclusion: We confirmed an inverse correlation between the expression of wild-type CEBPA and of CD7 in AML cells. Our results contradict the hypothesis that C/EBPα acts as repressor for CD7, and instead show that epigenetic mechanisms are responsible for CD7 regulation, in AML cells as well as in T-cells, the typical CD7 expressing cell type.

Background

CCAAT/enhancer binding factor alpha (CEBPA), located on chromosome 19q13.1 encodes a transcription factor that is of importance for granulocytic differentiation [1]. C/EBPα is upregulated during myelomonocytic development and positively affects expression of granulocyte differentiation related genes such as the G-CSF receptor (GCSFR), myeloperoxidase and neutrophil elastase (ELA2) [2-4]. CEBPA mutations are found in 5 - 14% of acute myeloid leukemia (AML) cases [5]. C/EBPα mutant proteins block the effect of wild-type C/EBPα on target genes in a dominant-negative manner [6]. This might be the reason why patients with CEBPA mutations and those with a silenced CEBPA promoter are found in the same AML subclass according to gene expression profiling [7]. Also expression of the T-cell marker CD7 has been associated with CEBPA mutations and with CEBPA hypermethylation [7,8].

CD7 is expressed in 30% of AML cases and CD7 positivity is linked with poor prognosis in myeloid malignancies [9,10]. In healthy individuals, CD7 is expressed on thymocytes, T- and natural killer cells, and progenitors of lymphoid and myeloid cells [10]. Conditional knockout experiments in mice suggest that Cebpa is involved in the regulation of Cd7 expression: absence of Cebpa results in upregulation of Cd7 in mouse hematopoetic stem cells, reintroduction of the transcription factor reduces expression of Cd7 [7].

We wanted to find out whether CD7 positivity in AML can be explained as consequence of loss or inactivation of wild-type CEBPA. Therefore, we externally regulated CEBPA expression in AML cell lines and tested whether and how this treatment affected CD7 expression.
Results and discussion

CEBPA expression and CD7 silencing

Quantitative real-time PCR (qRT-PCR) analysis showed that 42% (23/54) of the AML cell lines tested were CD7 positive with expression levels comparable to those of T-cell lines, 28% (15/54) of cell lines were weakly positive and 30% (16/54) were CD7 negative.

On the first view, Western blot analyses confirmed that C/EBPα might be a negative regulator for CD7 expression in AML cell lines: most cell lines showed mutually exclusive expression of these proteins, being either C/EBPα+/CD7- or C/EBPα-/CD7+ (Fig. 1A, Table 1). However, there was one noticeable exception: cell line HNT-34 expressed both proteins, C/EBPα and CD7, challenging the automatic linkage of C/EBPα expression to CD7 repression (Fig. 1A, Table 1).

Furthermore, it remained open as to how the transcription factor C/EBPα could inhibit expression of CD7. The search for transcription factor binding sites using bioinformatic databases (TFSEARCH and TESS) did not reveal a potential C/EBPα binding site in the CD7 promoter region (-713 to +624). A report describing that C/EBPα might be a negative regulator for transcription factor C/EBPα could inhibit expression of CD7- or C/EBPα-/CD7+ (Fig. 1A, Table 1).

However, there was an E2F binding site [11] indicates the possibility of a C/EBPα-mediated transcriptional gene regulation by protein/protein interaction. CD7 exon 1 also contains an E2F binding site according to TFSEARCH results. To test whether C/EBPα acts as direct or indirect transcriptional repressor for CD7 - by protein/DNA or by protein/protein interaction - we checked our cell lines for any C/EBPα/CD7 mRNA correlation. Analysis of C/EBPα protein and CD7 mRNA expression showed an even weaker correlation than the protein/protein analysis: 3/25 cell lines (HNT-34, IMS-M1, ME-1) were C/EBPα-positive and still showed high expression levels of CD7 mRNA (Table 2).

We sequenced the CEBPA gene to find out whether CD7 expression in these three cell lines might result from inactivating CEBPA mutations. Two of the three C/EBPα+/CD7+ cell lines (HNT-34, IMS-M1) carried and expressed an in-frame CEBPA mutation resulting in four (instead of three) histidine-proline repeats in the transactivation domain 2 of the protein. However, this mutation is considered insignificant for leukemogenesis as it was detected in 39% of healthy volunteers and in 20% of AML patients who remained positive after complete remission [8]. Accordingly, 7/25 (28%) cell lines in our study carried this length polymorphism. Sequencing revealed that none of the three C/EBPα positive and CD7 mRNA positive cell lines showed an inactivating CEBPA aberration. Furthermore, cell line ME-1 did not carry any mutation at all, showing that the CD7 gene could be transcribed despite expression of wild-type C/EBPα.

No direct influence of C/EBPα on expression of CD7

We had started this project to find out whether CD7 positivity in AML might be due to loss or inactivation of wild-type CEBPA. In line with the idea of a repressor function for C/EBPα was the observation that most cell lines showed an inverse correlation between C/EBPα and CD7 expression (Table 2). However, 3/25 cell lines (HNT-34, IMS-M1, ME-1) were C/EBPα+ and still expressed CD7 mRNA. C/EBPα/CD7 double positivity does not necessarily contradict a repressor function of C/EBPα. Cell lines HNT-34, IMS-M1 and ME-1 might carry additional genetic or epigenetic alterations not allowing a "normal" repressor function of C/EBPα in these cell lines.

To experimentally test whether C/EBPα has a direct inhibitory effect on CD7 expression, we first ectopically expressed CEBPA in the C/EBPα+/CD7+ cell line CMY and then knocked down C/EBPα in the C/EBPα+/CD7+ cell line NB-4 (Table 2). In both cell lines, expression of the transcriptional C/EBPα targets GCSFR and ELA2 was positively correlated with CEBPA expression levels (Fig. 2). In contrast, CD7 mRNA levels were neither positively nor negatively affected by C/EBPα (Fig. 2). These results
contradict the hypothesis that C/EBPα acts as CD7 suppressor.

**Epigenetic regulation of CD7**

Study of T-cell lines confirmed that CD7 repression can occur in the absence of C/EBPα: T-cell lines are C/EBPα-negative, but not all T-cell lines express CD7 (Fig. 1B). The CD7 promoter region does not match the criteria of a standard CpG island with a GC content > 50% and an observed CpG/expected CpG ratio > 0.6 [12]. However, according to the criteria of Weber et al. [13] the CD7 exon 1 region contains a subthreshold CpG island (inter-

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**Table 1: C/EBPα and CD7 expression in AML cell lines**

| C/EBPα | CD7 neg | CD7 low | CD7 high |
|--------|---------|---------|----------|
| neg    | 0       | 1       | 9        |
| low    | 1       | 1       | 1        |
| high   | 10      | 1       | 1        |

Inverse correlation between C/EBPα and CD7 protein expression in 25 AML cell lines as assessed by Western blot analysis. Cell lines classified as “low” showed faint signals, “high” denotes all positive signals in Fig. 1. GAPDH was used as protein loading control. Note that one cell line (HNT-34) expressed C/EBPα (high) and CD7 (high).

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**Table 2: C/EBPα and CD7 expression in individual AML cell lines**

| Cell lines   | CEBPA mRNA | C/EBPα protein | CD7 mRNA | CD7 protein |
|--------------|------------|----------------|----------|-------------|
| AP-1060      | +          | +              | -        | -           |
| CMK          | (+)        | -              | +        | +           |
| CMY          | -          | -              | +        | +           |
| F-36P        | -          | -              | +        | +           |
| GDM-1        | (+)        | -              | +        | +           |
| HL-60        | +          | +              | -        | -           |
| HNT-34       | +          | +              | +        | +           |
| HT-93A       | +          | +              | -        | -           |
| IMS-M1       | +          | +              | +        | -           |
| KG-1         | (+)        | -              | +        | +           |
| M-07e        | -          | -              | +        | +           |
| ME-1         | +          | +              | +        | -           |
| MEGAL        | (+)        | -              | +        | +           |
| MOLM-16      | -          | -              | +        | +           |
| MONO-MAC-6   | +          | +              | (+)      | (+)         |
| MUTZ-8       | +          | +              | (+)      | (+)         |
| NB-4         | +          | +              | -        | -           |
| OCI-AML2     | +          | +              | -        | -           |
| OCI-AML5     | +          | +              | -        | -           |
| OCI-M1       | (+)        | (+)            | -        | (+)         |
| OCI-M2       | (+)        | -              | -        | (+)         |
| SET-2        | (+)        | (+)            | +        | +           |
| SIG-M5       | +          | +              | -        | -           |
| SKNO-1       | (+)        | (+)            | (+)      | -           |
| TF-1         | -          | -              | +        | +           |

C/EBPα and CD7 Western blot analysis: + positive, (+) weakly positive, - negative. GAPDH was used as protein loading control. CEBPA qRT-PCR: \( +: 2^{-\Delta\Delta C_{T}} \geq 5.0; (+): 2^{-\Delta\Delta C_{T}} > 2.5; -, 2^{-\Delta\Delta C_{T}} < 5.0; < 2^{-\Delta\Delta C_{T}} < 0.2 \). The CEBPA-low cell line SET-2 was used as calibrator cell line. CD7 qRT-PCR: \( +: 2^{-\Delta\Delta C_{T}} > 2.5; (+): 2^{-\Delta\Delta C_{T}} \geq 1; -, 2^{-\Delta\Delta C_{T}} < 1 \). The CD7-low cell line MUTZ-8 was used as calibrator cell line. Note that cell lines HNT-34, IMS-M1 and ME-1 (bold) express C/EBPα and CD7 mRNA.
mediate CpG promoter) with moderate CpG richness (observed CpG/expected CpG ratio > 0.2) and high GC content (>60%) suggesting that the gene might be epigenetically regulated. Methylation-specific PCR (MSP) and sequencing of bisulfite-converted DNA revealed that this site was methylated in CD7-negative T-cell lines, while CD7-positive T-cell lines were not methylated around the transcriptional start site (Fig. 3, 4). These data support a recent study linking CD7 expression to chromatin modifications in CML [14]. Also in AML, unmethylated cell lines (unmethylated signal U only) expressed CD7 (2/2), while methylated cell lines (methylated signal M only) were CD7-negative (8/9) or weakly positive (1/9) (Table 3). Furthermore, a DNA demethylating agent induced CD7 expression in CD7-methylated cell lines, independent of histological origin: the T-anaplastic large cell lymphoma-derived cell line SR-786 and the AML cell line HL-60 each showed a ca. 30-fold increase of CD7 expression after treatment with 5-Aza-2’-deoxycytidine (Aza), while unmethylated cell lines (ALL-SIL, F-36P, GDM-1) were unaffected (Fig. 4). These results suggest that epigenetic mechanisms play a role in the regulation of CD7, both in T-cell lines and in AML cell lines: (i) we found a negative correlation between CD7 methylation and gene expression, and (ii) observed that a demethylating agent induced CD7 expression in silenced cell lines.

Interestingly, four cell lines (IMS-M1, ME-1, MONOMIC-6, SKNO-1) were CD7 mRNA positive but did not express CD7 protein (Table 2). Future studies might show which posttranscriptional mechanisms – including possibly translational inhibition by microRNAs - are responsible for this phenomenon.

**Conclusions**

An inverse correlation between CD7 methylation and CD7 expression was observed in T-cell lines as well as in AML cell lines suggesting that in both lineages epigenetic mechanisms underlie CD7 regulation. Two observations imply that other factors are also important for CD7 expression: (i) the stimulating effect of Aza on CD7 expression levels varied substantially across different CD7 methylated cell lines, and (ii) even cell lines that were clearly responsive to Aza with respect to CD7 mRNA induction did not show upregulation of CD7 pro-
tein as assessed by Western blot and FACS analysis (data not shown). Although transfection studies did not indicate that C/EBPα acts as CD7 repressor, the inverse correlation between CEBPA and CD7 expression reported for primary AML cases was confirmed for most AML cell lines. Thus, future studies should address whether C/EBPα is a second factor responsible for repression of CD7 besides promoter methylation.

Methods

Human cell lines

The continuous cell lines were either taken from the stock of the cell bank (DSMZ - German Collection of Microorganisms and Cell Cultures) or were generously provided by the original investigators. Detailed references and cultivation protocols have been described previously [15].

Methylation-specific polymerase chain reaction (MSP)

Bisulfite conversion of DNA was performed as described by the supplier (EpitTect Bisulfite Kit, Qiagen, Hilden, Germany). For detecting CD7 promoter methylation, we performed nested PCR with first round primers (CD7 BSP fwd 5’-TTT TGT GGA GAT GTA GGG GTA-3’, CD7 BSP rev 5’-CAC CAT CAA TCT AAC CAA AAA AAC-3’) amplifying converted DNA independently of the methylation status (bisulfite-specific PCR, BSP), while second round primers (CD7 M fwd 5’-TCA CAT CAA TCT AAC AAA AAC-3’) amplifying converted DNA independently of the methylation status (bisulfite-specific PCR, BSP), while second round primers (CD7 M fwd 5’-TCA CAT CAA TCT AAC AAA AAC-3’) amplifying converted DNA independently of the methylation status (bisulfite-specific PCR, BSP), while second round primers (CD7 M fwd 5’-TCA CAT CAA TCT AAC AAA AAC-3’) amplifying converted DNA independently of the methylation status (bisulfite-specific PCR, BSP), while second round primers (CD7 M fwd 5’-TCA CAT CAA TCT AAC AAA AAC-3’) amplifying converted DNA independently of the methylation status (bisulfite-specific PCR, BSP), while second round primers (CD7 M fwd 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TTC GAG TTT TGA GTG TTC-G3, CD7 U rev 5'-CCA AAC AAA CCA AAA ACC A-3') for M- and U-PCR specifically recognized the methylated or unmethylated versions of the promoter. PCR products of the initial BSP were diluted 1:100 for subsequent M- and U-PCR. Annealing temperatures were 53°C for BSP with 35 cycles and 63°C for M- and U-PCR with 30 cycles each. Epitect PCR Control DNA (Qiagen) was used as control for methylated and unmethylated templates.

**Bisulfite sequencing**

To confirm the methylation status of the CD7 promoter, DNA of cell lines was bisulfite converted according to the manufacturer's instructions (Qiagen). Subsequently, amplification of the CD7 exon 1 region (760 bp) was performed using primers CD7 BSP fwd and CD7 BSP rev, specifically binding bisulfite converted DNA (for primer sequence and PCR conditions see MSP section). Resulting CD7 fragments were purified, cloned into pGEM-TEasy vector (Promega, Madison, WI, USA) and sequenced. Sequences were evaluated using BiQ Analyzer http://www.jhoonline.org/content/3/1/15 and had to conform to at least 90% bisulfite conversion rate [16]. In addition, identical clones were excluded from the analysis.

**Gene expression analyses**

Quantitative PCR was performed on a 7500 Applied Biosystems (Darmstadt, Germany) real-time PCR system using the manufacturer's protocol. RNA was prepared using the RNeasy Mini kit (Qiagen). For mRNA quantification, reverse transcription was performed using the SuperScript II reverse transcriptase kit (Invitrogen, Karlsruhe, Germany). TaqMan probes (Applied Biosystems) were used to quantify human GAPDH as internal control.

**Treatment with DNA demethylating agent Aza**

5-Aza-2'-deoxycytidine (Aza) (Sigma) dissolved in DMSO was used to verify the effect of methylation on expression of CD7. Cells were seeded at a cell density of 5 x 10^5 cells/ml, Aza was added at a final concentration of 5 μM. Control cells were treated with 0.05% DMSO. After 2 d, half of the medium was replenished with medium with/without Aza (5 μM). After 3 d, respectively 4 d, cells were harvested to prepare RNA or protein.

**Western blot analysis**

Samples were prepared as described previously [17]. Anti CD7 antiserum was purchased from Santa Cruz (Heidelberg, Germany), anti C/EBPα antiserum was obtained from Cell Signaling/New England Biolabs (Frankfurt, Germany). Specific bands on nitrocellulose membranes were visualized with the biotin/streptavidin-horseradish peroxidase system (Amersham, Freiburg, Germany) in combination with the "Renaissance Western Blot Chemoluminescence Reagent" (DuPont, Bad Homburg, Germany).

**Bioinformatic database search for C/EBPα binding sites**

The genomic sequence of the CD7 promoter region from -713 to +624 was analyzed with the database search tools TFSEARCH ver.1.3 http://www.cbrc.jp/research/db/TFSEARCH.html and TESS http://www.cbil.upenn.edu/cgi-bin/tess/tess for the existence of potential C/EBPα binding sites (Factor ID in TESS: T00105).

**Plasmid construction**

For generating the anti-CEBPA shRNA, DNA oligonucleotides corresponding to position 818–836 of the sequence of the human CEBPA gene (GenBank accession no. NM_004364.3) were subjected to BLAST homology search, and thereafter chemically synthesized including overhang sequences from a 5'-BglII and a 3'-SalI restriction site for cloning purposes (BioSpring, Frankfurt, Germany). The numbering of the first nucleotide of the shRNA refers to the ATG start codon. The oligonucleotide sequences were as follows: FPCEBPA: 5'-GATCCGGCGCCAAGAAATGCGGACTTCAAGAAGTCCACCGACTTCTTGGCCTTTTTTGGAGAAG-3'; RPEBPA: 5'-CGAATTCGGCGCCAAGAAATGCGGACTTCAAGAAGTCCACCGACTTCTTGGCCTTTTTTGGAGAAG-3'. The non-complementary 9-nt loop sequences are underlined, and each sense oligonucleotide harbors a stretch of T as a PolIII transcription termination signal. The oligonucleotides were annealed and inserted 3' of the H1-RNA promoter into the BglII/SalI-digested pBluescript-derived pH1-plasmid to generate pH1-CEBPA as described [18]. The control plasmid pH1-GL4 has been described earlier [18]. Finally, the H1-CEBPA expression cassette was excised by digestion with SmaI and HincII and blunt-end ligated into the SmaBI site of the pdc-SR lentiviral vector to generate pdch1-CEBPA-SR plasmid. The lentiviral plasmid encodes RFP,express as reporter gene.
Preparation of recombinant lentiviral supernatants and lentiviral transduction

Preparation of recombinant lentiviral supernatants and transduction were performed as described previously [18]. The titers were averaged and typically ranged between 5-10 × 10^8 IU/ml. Concentrated viral supernatants were used for transduction of NB-4 cells in 48-well plates as described [18].

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SR designed parts of the study and performed MSP analysis, sequencing of bisulfite-converted DNA and co-wrote the manuscript, MS performed knock-down and expression experiments, JR performed Western blot analysis, MZ performed quantitative real-time PCR, HGD provided cell lines and critically read the manuscript, HQ designed the study and wrote the manuscript. All authors read and approved the final manuscript.

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