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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'deoxyctidine 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 asso-

ciated 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 hematopoietic 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.

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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-*

Myc expression was negatively regulated by *C/EBPα* via 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

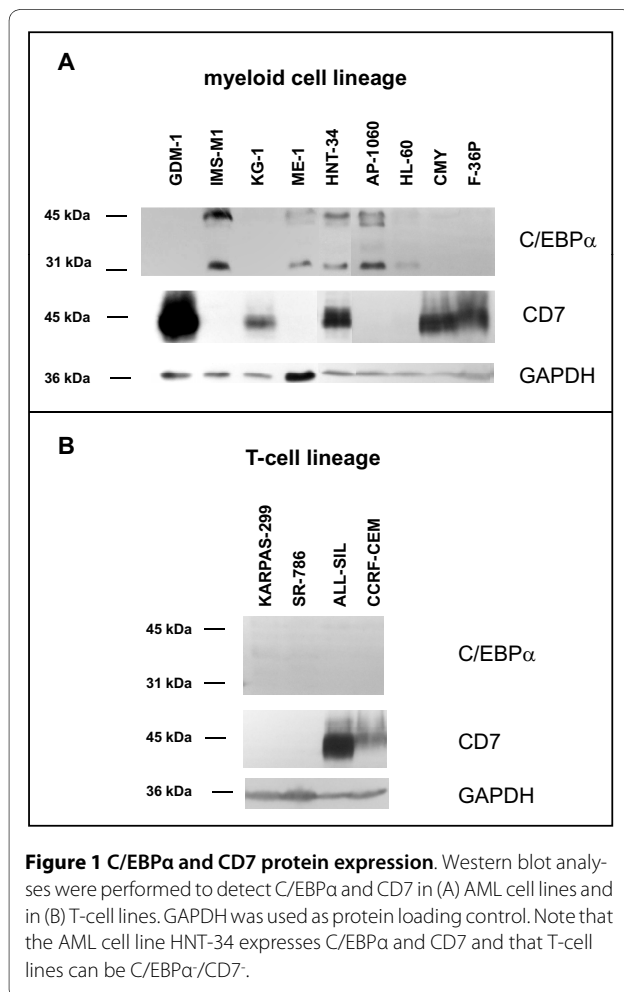


Table 1: C/EBPα and CD7 expression in AML cell lines

| 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).

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-

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: +: $2^{\Delta\Delta\text{ct}} \geq 5.0$; (+) $2^{\Delta\Delta\text{ct}} \geq 0.2, < 5.0$; -: $2^{\Delta\Delta\text{ct}} < 0.2$. The CEBPA-low cell line SET-2 was used as calibrator cell line. CD7 qRT-PCR: +: $2^{\Delta\Delta\text{ct}} > 2.5$; (+) $2^{\Delta\Delta\text{ct}} \geq 1$; -: $2^{\Delta\Delta\text{ct}} < 1$. 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.

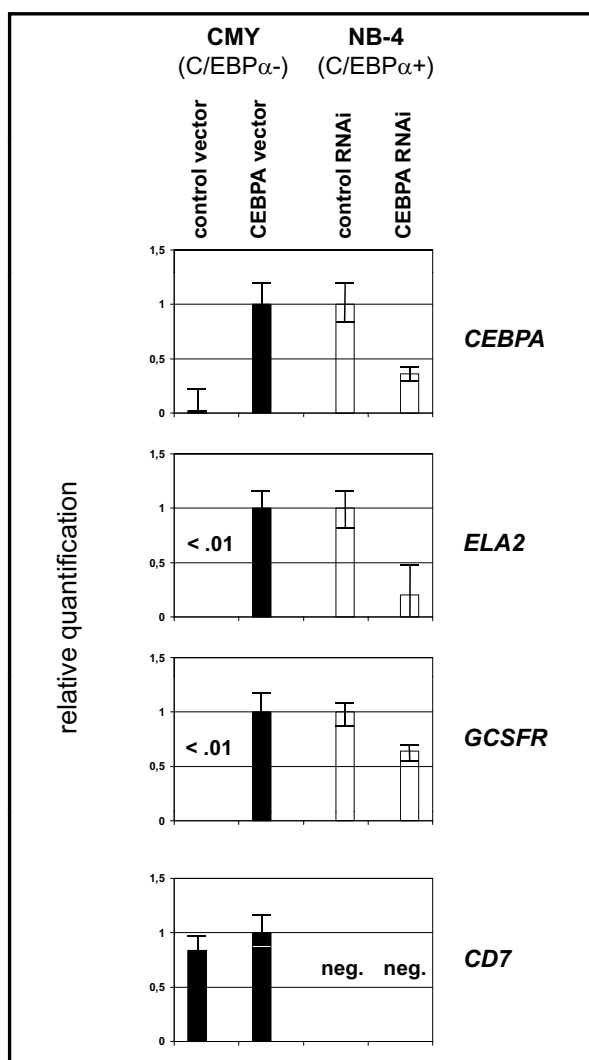


Figure 2 C/EBPα does not affect CD7 mRNA expression. *CEBPA* was ectopically expressed in the *CEBPA*-negative cell line CMY and repressed in the *CEBPA*-positive cell line NB-4. Expression levels were assessed at day 4 (*CEBPA* RNAi) and day 13 (ectopic expression of *CEBPA*) by qRT-PCR. Gene names at the right-hand side of the figure apply for all four columns. Expression of the *C/EBPα* targets *ELA2* and *GCSFR* depends on *CEBPA* expression, *CD7* mRNA levels are unaffected and remain positive in cell line CMY, negative (neg.) in cell line NB-4.

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),

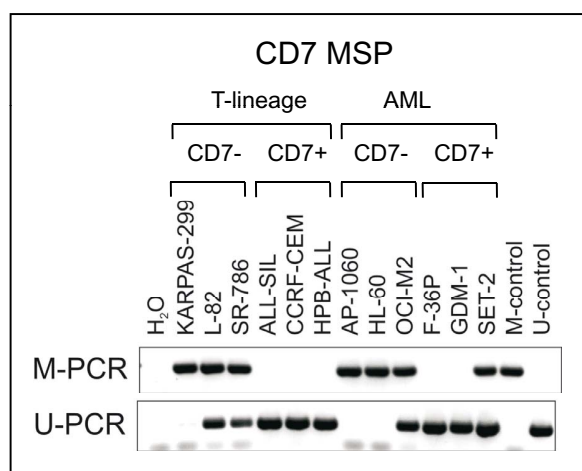


Figure 3 Epigenetic regulation of CD7. *CD7* promoter methylation was analyzed by MSP after bisulfite conversion of the DNA. Agarose gels of *CD7* M- and U-PCR products from T- and AML cell lines are shown as representative results.

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* promoter 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, MONO-MAC-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-

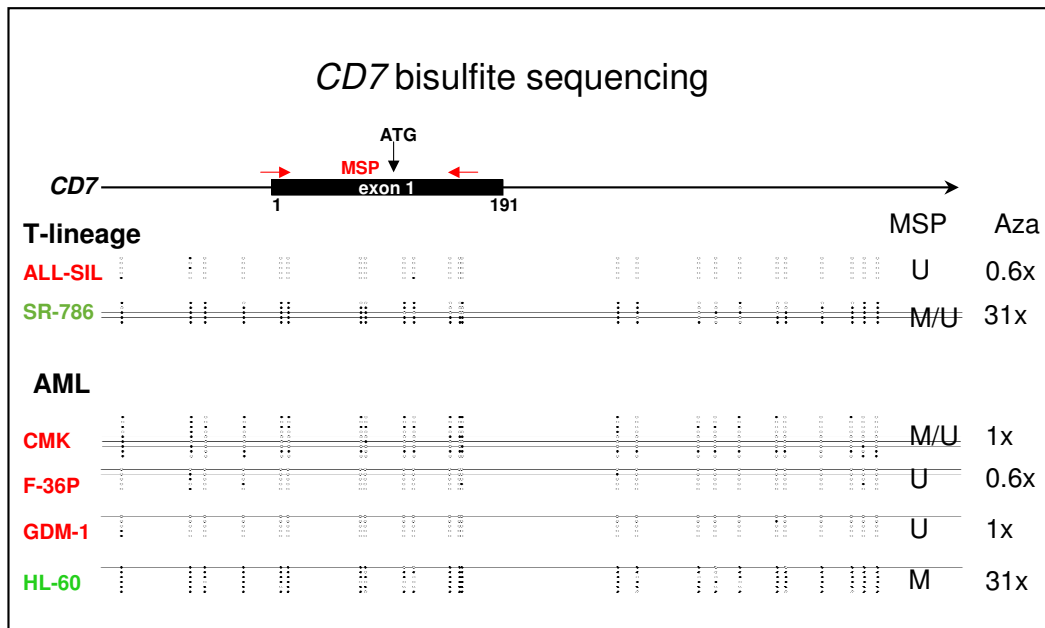


Figure 4 Bisulfite sequencing of CD7 exon 1 region. CpGs are represented as open dots (U = unmethylated) or filled dots (M = methylated). Name in red: CD7⁺ cell line, name in green: CD7⁻ cell line. Numbers on the right hand side show the effect of Aza (3 d, 5 μM) on CD7 mRNA levels, as assessed by qRT-PCR. Note that Aza induces CD7 expression in methylated cell lines (SR-786 and HL-60) only.

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'-TTT TTG AGT TTT GAG CGT TTG C-3', CD7 M rev 5'-AAA CAA ACC GCG AAC CAA CG-3', CD7 U fwd 5'-GTT TTT

Table 3: CD7 promoter methylation and CD7 mRNA expression in AML cell lines

| CD7 MSP | CD7 mRNA negative | CD7 mRNA low | CD7 mRNA high |
|---------|-------------------|--------------|---------------|
| U | 0 | 0 | 2 |
| U/M | 1 | 2 | 11 |
| M | 8 | 1 | 0 |

CD7 methylation was assessed by MSP. U: products with primers recognizing unmethylated CD7 only; U/M: U and M (methylated) products; M: M products only. CD7 mRNA expression was assessed by qRT-PCR and evaluated as described in Table 2. Note that 2/2 unmethylated (U only) cell lines are CD7-positive and that 8/9 methylated (M only) cell lines are CD7-negative. Methylation-specific (M) and unmethylation-specific (U) PCR were very sensitive, allowing the detection of one methylated cell in 1000 unmethylated cells and vice versa. Therefore, a meaningful correlation between CD7 methylation and expression was not apparent in U/M cells.

TTT GAG TTT TGA GTG TTT GT-3', *CD7* U rev 5'-CCA AAC AAA CCA CAA ACC AAC 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://biq-analyzer.bioinf.mpi-sb.mpg.de> 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 *CEBPA* (Hs 00269972s1), *ELA2* (Hs 00357734 m1) and *GCSFR* (Hs 01114427 m1) expression levels with *TBP* as endogenous control. Expression of *CD7* was assessed using the SYBR GREEN PCR Master Mix (Applied Biosystems) with *GAPDH* as internal control. *CD7* forward: 5'-GTG CTG GCG AGG ACA CAG-3'; *CD7* reverse: 5'-TCG TAC ACC ACA CAT GCC G-3'. *GAPDH* forward: 5'-TGG GTG TGA ACC ATG AGA AG-3'; *GAPDH* reverse: 5'-TCC ACG ATA CCA AAG TTG TCA-3'. Relative expression levels were calculated using the $\Delta\Delta C_t$ -method.

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 5×10^5 cells/ml, Aza was added at a final concentration of 5 μ M. Control cells were treated with 0.05% DMSO. After 2 d, half of the medium was replenished with medium

with/without Aza (5 μ M). 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/EBP α 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" protocol (DuPont, Bad Homburg, Germany).

Bioinformatic database search for C/EBP α 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/EBP α 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'-*Bgl*II and a 3'-*Sal*I 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: FP*CEBPA*: 5'-GATCCCCGGCCAAGAAGTCGGTGGACTTCAAGA GAGTCCACCGACTTCTTGGCCTTTTTTGAAG-3'; RPC *EBPA*: 5'-CGACTTCCAAAAAAGGCCAAGAAGTCG GTGGACTCTCTTGAAGTCCACCGACTTCTTGGCCGGG-3'.

The non-complementary 9-nt loop sequences are underlined, and each sense oligonucleotide harbors a stretch of T as a *Pol*III transcription termination signal. The oligonucleotides were annealed and inserted 3' of the H1-RNA promoter into the *Bgl*II/*Sal*I-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 *Sma*I and *Hinc*II and blunt-end ligated into the *Sna*BI 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 $5-10 \times 10^8$ 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.

Acknowledgements

We thank Beatrice Mueller and Thomas Pabst (University Hospital Bern, Switzerland) for providing *CEBPA* plasmid, and Rod MacLeod for critically reading the manuscript.

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Received: 8 March 2010 Accepted: 14 April 2010

Published: 14 April 2010

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doi: 10.1186/1756-8722-3-15

Cite this article as: Röhrs et al., CD7 in acute myeloid leukemia: correlation with loss of wild-type CEBPA, consequence of epigenetic regulation *Journal of Hematology & Oncology* 2010, **3**:15

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