The N-terminal CEBPA mutant in acute myeloid leukemia impairs CXCR4 expression

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ONLINE SUPPLEMENTARY METHODS SECTION

Antibodies and reagents

Anti-C/EBPα, anti-GAPDH and anti-β-actin antibodies were purchased from Genetex Biotechnology. M2 antibodies and doxycycline were from Sigma-Aldrich.

Cell culture

Human embryonic kidney 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% calf serum. Human myeloid U937 and erythroleukemic K562 cell lines were grown in RPMI 1640 medium (Invitrogen) containing 10% fetal bovine serum (FBS; HyClone). To establish the inducible K562-C/EBPα cells that stably express wild type or mutated C/EBPα, the pTripz-C/EBPα–p42 and p30 plasmids were packaged using HEK293T cells and transduced into K562 cells as described.[1,2] Subclones resistant to puromycin (2 μg/ml) were obtained by limiting dilution.

RT-QPCR analysis of human cell lines

Total RNA was isolated from cells using Trizol reagent (Invitrogen). cDNA was synthesized using ImProm-II reverse transcriptase (Promega). RT-QPCR analysis was performed using 7500 Fast Real-Time PCR System (Applied Biosystems) and Fast SYBER Green Master Mix (Applied Biosystems). *CXCR4* was normalized to *18S rRNA*.

Transfection and luciferase assays

HEK293T cells were transfected using a DNA mixture containing ScreenFect in vitro DNA transfection reagent (InCella). After transfection for 24 h, cells were analyzed for reporter activity assays. Transfected cells were assayed for both firefly and Renilla luciferase activities using the dual luciferase assay system (Promega).

Plasmid construction

The DNA fragment of *CEBPA* was obtained by PCR from K562 genomic DNA and cloned in-frame in the EcoRI and XhoI sites of pCMV-Tag2B plasmid. For generating tetracycline inducible plasmids, wild type *CEBPA* or p30 mutant cDNA were subcloned in the AgeI and XhoI sites of pTRIPZ plasmid. The human *CEBPA* shRNA were cloned into the lentiviral vector pTRIPZ plasmid containing a RFP reporter. The shRNA oligonucleotide sequence targeting *CEBPA* was as described.[3] The pGL2-*CXCR4* plasmid containing human *CXCR4* promoter region fused to the promoterless pGL2 firefly luciferase reporter was a generous gift of Wilhelm Krek.[4] The pGL2-*CXCR4*-0.8 kb was used to PCR amplify to generate a 0.2-kb fragment of the *CXCR4* promoter region, which was subcloned to the pGL2 vector, producing pGL2-*CXCR4*-0.2 kb. Mutant versions of pCMV-tag2B- C/EBPα (pCMV-tag2B-C/EBPα-CTM) and pGL2-*CXCR4*-0.8 kb (pGL2-*CXCR4*-S1, pGL2-*CXCR4*-S2 and pGL2-*CXCR4*-S3) were generated using Quick-Change Site-directed Mutagenesis kit.

Chromatin immunoprecipitation assays (ChIP)

Chromatin immunoprecipitation was performed as described[2]. In brief, cells were treated with formaldehyde at a final concentration of 1% for 10 min at room temperature. Glycine was added at a final concentration of 125 μM to quench cross-linking. Cell lysates were sonicated to generate DNA fragments averaging <1 kb. After pre-clearance, cell extracts were incubated with 1 μg of antibody at 4°C overnight, followed by precipitation with protein A/G Sepharose beads. After washing, elution, deproteination, and heating, immunoprecipitated DNA was extracted and ethanol precipitated. DNA was resuspended in 50 μl of Tris-EDTA buffer. PCR was applied to the immunoprecipitated DNA with the following thermal cycling program: 30 s at 94 ℃, 30 s at 55 ℃ and 1 min at 72 ℃ (30 cycles), followed by an extension time at 72 ℃ for 5 min. PCR products was analyzed on 2.0 % agarose gel and visualized by ethidium bromide staining.

Preparation of nuclear extracts and gel-shift analysis

The preparation of nuclear extracts from HEK293T cells expressing C/EBPα and gel-shift reactions were as described previously[2]. The gel-shift analysis was performed using gel-shift assay kits from Signosis (Sunnyvale, CA). For super-shift assays, antibodies specific for C/EBPα or GAPDH were added to the nuclear extracts for 30 min on ice prior to the DNA binding reaction. After incubation at room

temperature for 30 min with biotin-labeled probes and nuclear extracts (5.0 μg protein), samples were analyzed by electrophoresis at 150 V for 1.5 h through non-denaturing 4% polyacrylamide gels. After separation by 4% non-denaturing polyacrylamide gel electrophoresis, the binding reaction mixtures were transferred onto a nylon membrane, and the membrane was UV-cross-linked. The biotin-labeled DNA was probed with streptavidin-HRP conjugate for chemiluminescence detection.

Chemotaxis assay

A total of $5x10^5$ cells in a volume of 200 μl were added to the top chamber of Transwell culture inserts (Corning Costar, Lowell, MA) with a pore size of 5 μm. Inserts were placed in wells containing 800 μl of RPMI medium with or without SDF-1 (R&D Systems). Chemotaxis assays were performed at 37℃ for 24 hours. Cells were then counted in triplicates using a hemocytometer.

Flow cytometry

K562 cells were washed with cold PBS, blocked with 5% FBS in PBS on ice for 30 min and then treated with APC-conjugate of mouse anti-human-CXCR4 monoclonal antibody (5 μg/ml, clone 12G5), or with an isotype control, on ice for 1 h. Cells were then washed and analyzed by BD-Canto flow-cytometer (BD Biosciences, San Jose, CA).

ChIP and Quantitative RT-PCR oligonucleotides

human *CXCR4* promoter sense primer 5'-CGCGGGGGAATGGGCGGTTGGAAGC-3', antisense primer 5'-GCCGGACAGGACCTCCCAGAGGCATTTCC -3'; human *albumin* promoter sense primer 5'-CGACGACCCATTCGAACGTCT-3', antisense primer 5'-CTCTCCGGAATCGAACCCTGA-3';

human *β-actin* promoter sense primer 5'-ACGCCAAAACTCTCCCTCCTCCTC-3', antisense primer 5'-CATAAAAGGCAACTTTCGGAACGGC-3'.

human *CXCR4* sense primer 5'-CTGAGAAGCATGACGGACAA-3', antisense primer 5'-TGGAGTGTGACAGCTTGGAG-3';

human *18S rRNA* sense primer 5'-CGACGACCCATTCGAACGTCT-3', antisense primer 5'-CTCTCCGGAATCGAACCCTGA-3'

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Supplementary Figure 1

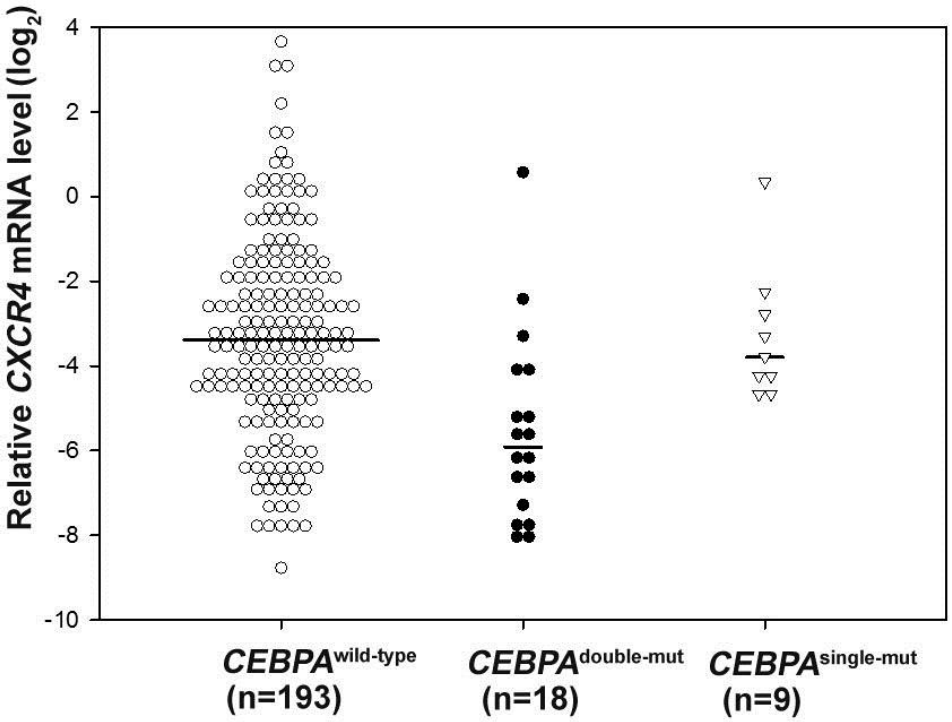

Figure S1. *CXCR4* expression is negatively associated with C/EBPα mutations.

There is negative association of BM *CXCR4* mRNA expression and *CEBPA* mutation (P=0.0019). Intriguingly, there is significant differences in *CXCR4* mRNA expression between patients with *CEBPA* double mutations (*CEBPA*$^{\text{double-mut}}$) and those with single mutation (*CEBPA*$^{\text{single-mut}}$, P=0.0075) or those without mutation (*CEBPA*$^{\text{wild}}$, P=0.0002).

Supplementary Figure 2

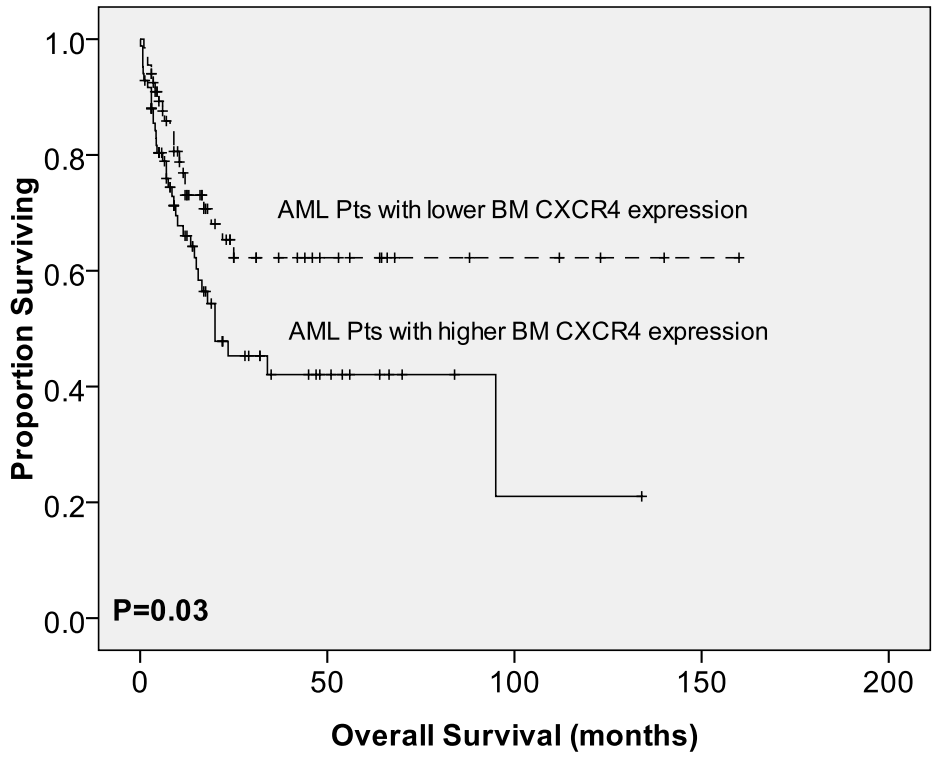

Figure S2. Kaplan–Meier survival curves for overall survival stratified by BM *CXCR4* mRNA expression. A total of 151 patients with AML who received conventional intensive chemotherapy showed that patients with higher BM *CXCR4* expression had shorter overall survival than those with lower expression (P=0.03).

Supplementary Figure 3

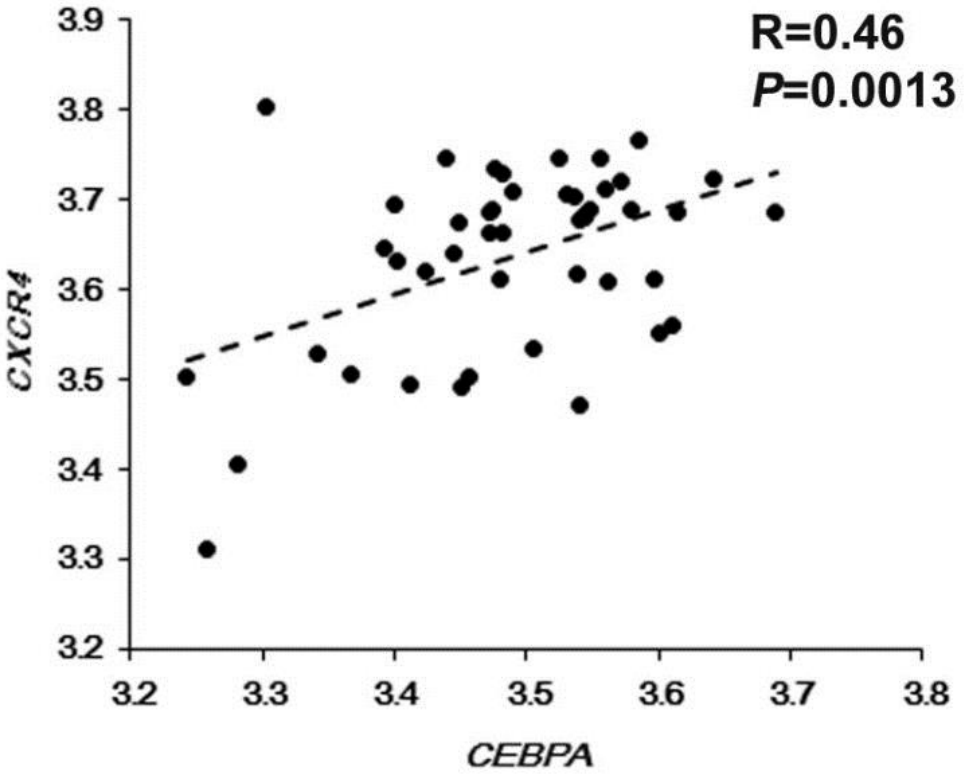

Figure S3. Correlation of *CEBPA* and *CXCR4* mRNA levels in AML patients. Scatter plot of log_2-transformed intensities between *CEBPA* and *CXCR4* expression in the patients with wild-type *CEBPA* from the German AMLCG cohort reported by Dufour et. al.[5] The Pearson correlation coefficient (R), level of statistical significance (P), and regression line (dash line) are shown.