ACER3 supports development of acute myeloid leukemia

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Abstract

No new therapy for acute myeloid leukemia (AML) has been approved for more than 30 years. To effectively treat AML, new molecular targets and therapeutic approaches must be identified. *In silico* analysis of several databases of AML patients demonstrated that the expression of alkaline ceramidase 3 (ACER3) significantly inversely correlates with the overall survival of AML patients. To determine whether ACER3 supports AML development, we employed an shRNA-encoding lentivirus system to inhibit *acer3* expression in human AML cells including NB4, U937, and THP-1 cells. The ACER3 deficiency resulted in decreased cell growth and colony formation, elevated apoptosis, and lower AKT signaling of leukemia cells. Our study indicates that ACER3 contributes to AML pathogenesis, and suggests that alkaline ceramidase inhibition is an option to treat AML.

Introduction

Acute myeloid leukemia (AML) is the most common acute leukemia in adulthood, accounting for approximately 1.2% of all cancer deaths. Its incidence increases in elderly population, and the treatment often yield poor outcomes. Despite continuous treatment, most AML patients relapse within 5 years [1]. There were no new therapies for AML approved in the past 30 years.

We have developed a systematic strategy to identify potential new molecular factors that play important roles in leukemia development [2]. We start with patient databases to identify genes whose expression correlates with the clinical outcomes of AML patients. We then validate the functions of candidate genes in leukemia models in vitro and in vivo and compare these functions to those in normal cells. The signaling pathways identified provide candidate targets for development of therapeutic approaches. Using this approach, we previously identified several ITIM-containing receptors or membrane proteins that support AML development [2-6].

Here we describe our finding that the expression of an intracellular enzyme ACER3 negatively correlated with the overall survival of leukemia patients. Using a loss-of-function approach, we discovered that ACER3 supports the growth, colony formation, and survival of human AML cells, and AKT signaling is decreased upon ACER3 knockdown. ACER3 regulates the metabolism of sphingolipids [7]. Our result suggests that sphingolipid metabolism plays an important role in leukemia pathogenesis, and alkaline ceramidases are potential targets for AML treatment.

Materials and methods

Cell culture

Human leukemia cell lines were grown in RPMI-1640 medium (Hyclone, South Logan, UT, USA) supplemented with 10% FBS (Gibco, Gaithersburg, MD). HEK293T cells were cultured in high glucose Dulbecco's modified Eagle's medium (Hyclone) supplemented with 10% FBS. All cell lines were maintained at 37 °C in 5% CO₂.

shRNAs and primers

Four sets of shRNAs targeting ACER3 were used in our study. The shRNAs were designed based on an online program (<http://rnaidesigner.lifetechnologies.com/rnaiexpress/>). RT-PCR primers were designed online (<http://www.idtdna.com/site>). The oligonucleotide sequences of the shRNAs used are:

Scrambled shRNA: 5'-GATATGTGCGTACCTAGCAT-3'

ACER3 shRNA1: 5' -G TTCCTATCTTCACATCCTTT-3'

ACER3 shRNA2: 5' -GCCAATTCTTTGAAGACCTTT-3'

ACER3 shRNA3: 5' -GCTTAGAGGACTGGGTTATAC-3'

ACER3 shRNA4: 5' -GGACTGGGTTATACATCATTG-3'

Lentiviral shRNA constructs, lentiviral production, and cell transduction

Lentivirus infection was performed essentially as we described [3, 8, 9]. The lentiviral vector PII3.7 was used to express shRNAs designed to target *acer3* mRNA. For the

lentiviral supernatant production, HEK-293T cells maintained in DMEM supplemented with 10% FBS and antibiotics were grown on 10-cm culture plates to ~60% confluence. For transfection, Polyjet (SigmaGen, Rockville, MD, USA) was used according to the manufacture's protocol. For *acer3* inhibition, 5.5 µg of the envelop plasmid *pMD2G*, 2 µg of the packaging plasmid *PsPAX2*, 7.5 µg of the shRNA lentiviral construct were dissolved in 0.5 mL of DMEM medium (serum and antibiotic free), Polyjet was added, and the solution was incubated for 15 min before addition to HEK293T cells. After 5 hours, the supernatant was discarded and replaced with 10 mL of DMEM with 10% FBS without antibiotics. After 48 hours, the supernatant was collected into a 15-mL tube, and 10 mL of medium were added to the plate. This was repeated 72 hours later. The lentivirus-containing supernatant was filtered through a 20-µm pore filter, and quickly frozen in liquid nitrogen for storage at -80 °C. Cells were cultured in 6-well plates (5×10^5 cells/well), and 16 hours later were transduced with virus by addition of 1 mL of lentiviral supernatant diluted in 1 mL of DMEM complete medium and 4 µL of protein sulfate. Cells were centrifuged at 2,000 rpm (Heraeus Biofuge Stratos centrifuge, ThermoScientific, Waltham, MA, USA) for 120 min at 37 °C. Cells were then cultured at 37 °C in 5% CO₂ for 5 hours. Culture medium was discarded, and cells were cultured in complete 1640 medium with 10% FBS and antibiotics for 19 hours. This procedure was repeated for a second infection.

Cell growth assays

GFP⁺ shRNA-encoding lentivirus-infected cells were sorted by flow cytometry two days post-infection and 10,000 cells were plated in 1.55-cm wells. Cell numbers were

determined at indicated days in triplicate wells. The experiment was repeated three times with similar results.

Colony formation assay

The colony formation assay for human cell lines was performed essentially as described [10]. The cells were plated at 1000 cells/ml RPMI medium with FBS in 0.35% agar over 0.5% agar base layer. Colonies were grown for 2 weeks before counting.

Apoptosis assay

The apoptosis assay was performed using the Annexin V-PE apoptosis detection kit (eBioscience, San Diego, CA, USA) as we described previously [2-4]. Fluorescence signals from at least 10,000 cells were collected by FACS (Beckman, CA, USA) to determine the percentage of apoptotic cells.

Quantitative real-time RT-PCR

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was applied to total RNA extracted from cells or patient samples. Reverse transcription was performed using M-MLV reverse transcriptase cDNA Synthesis Kit (Takara Bio, Otsu, Shiga, Japan). Real-time RT-PCR was carried out on ABI 7900HT Fast Real-Time PCR System (Foster City, CA, USA) with SYBR-Green PCR Master Mix (Toyobo, Kita-ku, Osaka, Japan). A comparative CT method ($2^{-\Delta\Delta CT}$) was used to quantify gene expression.

Gene expression and survival analysis

We analyzed publicly available gene expression datasets from human AML studies as described [2]. Data were obtained from the TCGA AML database (<https://tcga-data.nci.nih.gov/tcga/>, n = 187), the GSE6891 database (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE6891>, n = 520), and the GSE10358 database (n = 91). Expression was normalized to total mRNA. Patients were separated into two groups based on whether they had higher or lower than the average expression levels of the ACER3 to perform Kaplan-Meier survival analysis (GraphPad Prism, version 5.0, GraphPad, San Diego, CA, USA). *Acer3* mRNA levels in different types of human lymphoid leukemia and myeloid leukemia samples were determined from GEO dataset GSE13159 (n = 2096).

Statistical analyses

Data are expressed as means $\pm$ SEM. Data were analyzed by Student's *t*-test and were considered statistically significant if $p < 0.05$. The survival rates of the two groups were analyzed using a log-rank test and were considered statistically significant if $p < 0.05$. *p* values are represented as precise *p* values or generally as * $p < 0.05$.

Results

ACER3 expression negatively correlates with the overall survival of AML patients

To follow our systematic approach to identify genes that potentially support leukemia development [2-4], we performed an *in silico* analysis of the relationship between gene expression and the overall survival of AML patients using data from three independent databases: the TCGA AML database (<https://tcga-data.nci.nih.gov/tcga/>; n = 187), the GSE6891 database (n = 520), and the GSE10358 database (n = 91). We found that the expression of ACER3 negatively correlates with the overall survival of AML patients in all these datasets (Fig. 1A-C).

ACER3 is an alkaline ceramidase and *acer3* gene is located at 11q13.5 of the human genome and 7E2 of the mouse genome respectively. ACER3 is localized to the endoplasmic reticulum and Golgi apparatus and catalyzes the hydrolysis of phytoceramide into phytosphingosine and free fatty acid [11]. The role of ACER3 in sphingolipid metabolism and signaling is under investigation [12, 13]. However, its potential function in leukemia is unknown.

We analyzed gene expression of ACER3 in a number of human AML and lymphoblastic cell lines. *acer3* mRNA is highly expressed in AML cell lines NB4, U937, and THP-1. It is also expressed in lymphoblastic leukemia (ALL) cell lines Kasumi-2, NALM-6, RCH-ACV, KOPN-8, and 697, though at lower levels (Fig. 1D). Concordantly, the *acer3* mRNA levels were relatively high in various cells from patients with myeloid

malignancies including AML (Fig. 1E). Together, these gene expression analyses suggest that ACER3 is highly expressed by AML cells and may support AML development.

ACER3 is essential for the growth of human acute leukemia cells

We employed the loss-of-function approach to study the potential function of ACER3 in human leukemia cells. To this end, we sought to silence the expression of ACER3 by introducing lentivirus-encoded shRNAs designed to target *acer3*. Of the four shRNAs tested, we selected shRNA3 and shRNA4 that showed greater knockdown efficiencies (Fig. 2A) for functional study.

The ACER3 deficiency resulted from shRNA 3 or 4 significantly decreased the *in vitro* growth of three AML cell lines – NB4, U937, and THP-1 – which have relatively high ACER3 expression levels (Fig. 2B-D). For instance, at day 2, 4, and 6 post-infection of shRNAs, the NB4 cells infected by shRNA3 or shRNA4 had 39.6%, 19.2%, and 27.5% or 61.5%, 39.7%, and 34.6% respectively of that of control cells treated with a scrambled shRNA (Fig. 2B).

To determine the underlying mechanism by which ACER3 supports the growth of these leukemia cells, we compared the cell cycle status and apoptosis of NB4 cells treated with these shRNAs or scrambled control shRNA. No significant difference was observed in cell cycle distribution in these two treatment conditions (data not shown). However, ACER3-deficient NB4 cells infected with shRNA3 or shRNA4 had significantly increased levels of apoptosis compared to cells treated with the control shRNA (Fig. 2E;

15.2% early apoptotic cells and 5.1% late apoptotic cells in scramble control-treated cells vs. 39.3% and 18.8% in ACER3-deficient cells at 72 hr after shRNA infection). Similar effects of inhibition of *acer3* expression on apoptosis were observed in THP-1 and U937 cells (Fig. 2F-G). These results indicate that ACER3 supports leukemia cell growth by suppressing apoptosis.

We further tested the effect of ACER3 knockdown on colony forming ability of THP-1 cells. THP-1 cells infected with shRNA3 or shRNA4 had significantly decreased colony formation (Fig. 3A-B). This result, consistent with those of the cell growth and apoptosis analyses, indicates that ACER3 supports AML development.

The inhibition of *acer3* expression decreased p-AKT but not total AKT in NB4 cells (Fig. 4). Concordantly, the expression of *Bax* was increased in cells deficient in ACER3 compared to control cells (Fig. 4). Our results clearly indicate that ACER3 is essential for survival of human AML cell lines, and suggest that ACER3 acts through AKT/BAX signaling pathway.

Discussion

In this study, we demonstrated that the expression of ACER3 negatively correlates with the overall survival of AML patients. ACER3 is highly expressed in human AML cell lines. Importantly, using a loss-of-function approach, we showed that ACER3 supports leukemia development by decreasing apoptosis of AML cells. This is the first demonstration of ACER3 function in leukemia development and in the hematopoietic system.

Ceramidases catalyze the hydrolysis of ceramides to sphingosine and fatty acids. Five human ceramidases are classified as acid, neutral, and alkaline types [11]. ACER3 acts as an alkaline ceramidase, and is localized to the Golgi complex and ER. Compared to other two alkaline ceramidases, ACER3 is highly expressed in a number of different tissues [11]. Consistent with its role in sphingolipid metabolism and neuronal protection [12], ACER3 is associated with partial fetal alcohol syndrome and farber lipogranulomatosis, and childhood leukodystrophy [14].

The roles of ceramidases in cancer are unclear. It was reported that ceramides enhance the production of pro-inflammatory cytokines in innate immune cells and ACER3 has an anti-inflammatory function [7]. On the other hand, it was demonstrated that the acid ceramidase is transcriptionally suppressed by interferon regulatory factor 8, which is a factor to support myeloid cell differentiation. ACER knockdown or treatment by an inhibitor enhanced FASL-induced apoptosis of chronic myeloid leukemia (CML) cells, whereas forced expression of ACER had the opposite effect [15]. The effect of ACER3

on AML cells as we demonstrated here echoes that of the acid ceramidase on CML cells. Because the lack of major developmental defect of ACER3 knockout mice [12], the anti-ACER3 strategy may effectively inhibit leukemia growth yet with minimal toxicity to normal cells. It may be desirable to test the effect of ACER inhibitor and ceremide for AML treatment. It will also be interesting to study the potential different roles of ACER3 in different types of cancer.

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Competing interest disclosure

The authors declare no competing financial interests.

Author contributions

C.C. and Y.Y. contributed to experimental design, performed experiments, interpreted data, and writing of the manuscript. L.Z. contributed to *in silico* analysis. C.L., J.C., J.X., M.L., Y.W. and C.C.Z. contributed to experimental design and writing of the manuscript.

Figure 1. ACER3 expression negatively correlates with overall survival of AML patients. (A-C) *acer3* mRNA expression data from (A) the TCGA AML database (n = 187), (B) the GSE6891 database (n = 520), and (C) the GSE10358 database (n = 91) were normalized to total mRNA expression. Patients were separated into two groups based on whether expression of *acer3* was higher or lower than the average expression levels, and % survival vs. time was plotted. (D) *acer3* mRNA expression (relative fold changes to that in Kasumi-2 cells) as determined by real-time RT-PCR plotted for human monocytic and monoblastic AML and B-ALL cell lines. Technical replicate n = 3. (E) *Acer3* mRNA levels in different types of human lymphoid leukemia and myeloid leukemia samples (GEO dataset GSE13159, n= 2096). The *acer3* level in CLL was set as 1.0.

Figure 2. Inhibition of *acer3* expression significantly decreased AML cell growth. (A) Endogenous *acer3* expression was inhibited in 293T cells transfected with shRNAs targeting *acer3* mRNA but not with a scrambled control shRNA as determined by real-time RT-PCR at 48 hours after lentiviral infection (n = 3). (B-D) Inhibition of *acer3* expression by two different shRNAs significantly decreased growth of NB4 (B), U937 (C), and THP-1 (D) cells at 6 days post-infection when compared to growth of cells that express a scrambled control shRNA (n = 4). * p < 0.05. (E-G) ACER3 deficiency induced apoptosis of NB4 (E), THP-1 (F), and U937 (G) cells at 72 hr post treatment with shRNA3, shRNA4, or a scrambled control shRNA. Shown are representative flow cytometry plots and data summary. Technical replicate n = 3.

Figure 3. ACER3 deficiency decreased colony formation of AML cells. (A) ACER3 deficiency decreased colony formation of THP-1 cells as shown by the plate images at 2 weeks after plating. (B) Summary of colony formation of THP-1 cells infected by shRNA3, shRNA4, or a scrambled control shRNA at 2 weeks after plating. * $p < 0.05$.

Figure 4. Lack of ACER3 significantly decreased AKT activation in AML cells. Expression of indicated molecules was quantified in NB4 cells at 96 hr post transfection with shRNA3, shRNA4, or a scrambled control shRNA by Western blotting.

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Figure 1

Figure 2

Figure 3

A

Scramble

shRNA3

shRNA4

B

Figure 4