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SPG6 supports development of acute myeloid leukemia by regulating BMPR2-Smad-Bcl-2/Bcl-xl signaling

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ABSTRACT

Acute myeloid leukemia (AML) is the most common acute leukemia affecting adults. To effectively treat AML, new molecular targets and therapeutic approaches must be identified. In silico analysis of several available databases of AML patients showed that the expression of Spastic Paraplegia 6 Protein (SPG6) significantly inversely correlates with the overall survival of AML patients. To determine whether SPG6 supports AML development, we employed an shRNA-encoding lentivirus system to inhibit SPG6 expression in human AML cells including NB4 and MV4-11 cells. Knockdown expression of SPG6 resulted in decreased cell growth and elevated apoptosis of these leukemia cells. Notably, the SPG6 deficiency resulted in higher BMPR2 expression indicating that BMPR2 signaling contributes to AML pathogenesis. Furthermore, SPG6 deficiency promoted phosphorylation of Smad1/5/9 and decreased transcription of Bcl-2 and Bcl-xl. Our study suggests that SPG6 contributes to AML pathogenesis, and suggests that inhibition of SPG6 may be novel strategy for treating human AML.

1. Introduction

Acute myeloid leukemia (AML) is a heterogeneous disease characterized by the proliferation of immature transformed myeloid blasts in the bone marrow and peripheral blood. Treatments for AML yield poor outcomes, especially for the elderly patients. For those older than 60 years, who made up of the majority of AML cases, the 5-year OS was only 10–20% [1]. Seriously, few of patients who relapsed after complete remission could survive for more than 5 years [2]. Despite advances in understanding the pathogenesis of AML, the standard therapy remained nearly unchanged over the past three decades. Therefore, new molecular targets and therapeutic approaches are urgently required for effectively treatment of human AML.

To identify potential therapeutic targets in AML, a number of groups have identified cell surface proteins preferentially expressed on leukemia cells including CD47 [3], CD44 [4], CD96 [5], TIM3 [6] and CD123 [7] et al. These specific receptors mediate signaling that differs in leukemia cells from that in normal hematopoietic cells, which may lead to the development of novel antileukemia-specific agents.

We have developed a systematic strategy to identify potential new molecular factors that play important roles in leukemia development. Using this approach, we previously identified several ITIM-containing receptors or membrane proteins that support AML development [8–12]. Here we describe our finding that the expression of an intracellular enzyme SPG6 negatively correlated with the overall survival of AML patients. Using a loss-of-function approach, we discovered that SPG6 supports the growth and survival of human AML cells, and type II BMP receptor (BMPR2) signaling is increased upon SPG6 knockdown.

2. Materials and methods

2.1. Cell culture

Human leukemia cell lines were grown in RPMI-1640 medium (Hyclone, South Logan, UT, USA) supplemented with 10% FBS (Gibco, Gaithersburg, MD). HEK-293 T 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 a humidified atmosphere of 5% CO2.

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2.2. Gene expression and survival analysis

We analyzed publicly available gene expression datasets from human AML studies as described [8]. Data were obtained from the TCGA AML database (https://tcga-data.nci.nih.gov/tcga/, n = 187). Expression was normalized to total mRNA. Patients were separated into two groups based on whether they had higher or lower than the medium expression levels of the SPG6 to perform Kaplan-Meier survival analysis (GraphPad Prism, version 5.0, GraphPad, San Diego, CA, USA). SPG6 mRNA levels in different types of human myeloid leukemia (GSE13159) and normal hematopoiesis (GSE42519) samples were determined from GEO dataset.

2.3. shRNAs and primers

Two sets of shRNAs targeting SPG6 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:

Scramble shRNA: 5′-GATATGTGCGTACCTAGCAT-3′
SPG6 shRNA1: 5′- GCCGATGATCTTCTCTATAA -3′
SPG6 shRNA2: 5′- GAATACATGTGGCTAACAA -3′

2.4. Lentiviral shRNA constructs, lentiviral production, and cell transduction

Lentivirus infection was performed essentially as we described [13,14]. The lentiviral vector PLL3.7 was used to express shRNAs designed to target SPG6 mRNA. For the lentiviral supernatant production, HEK-293 T cells maintained in DMEM supplemented with 10% FBS and antibiotics were grown on 10-cm culture plates to~60% confluence. For transfection, Polyjet (SignaGen, Rockville, MD, USA) was used according to the manufacture's protocol. For SPG6 inhibition, 5.5 mg of the envelop plasmid pMD2G, 2 mg of the packaging plasmid PsPAX2, 7.5 mg of the shRNA lentiviral construct were dissolved in 0.5 mL of DMEM medium (serum and antibiotic free), Polyjet was added, and the solutionwas incubated for 15 min before addition to HEK293T cells. After 5 h, the supernatant was discarded and replaced with 10 mL of DMEM with 10% FBS without antibiotics. After 48 h, the supernatant was collected into a 15-mL tube, and 10 mL of medium were added to the plate. This was repeated 72 h later. The lentivirus-containing supernatant was filtered through a 20-mM 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 h later were transduced with virus by addition of 1 mL of lentiviral supernatant diluted in 1 mL of DMEM complete medium and 4 mL of protein sulfate. Cells were centrifuged at 1800 rpm (Heraeus Biofuge Stratos centrifuge, Thermo-Scientific, Waltham, MA, USA) for 120 min at 37 °C. Cells were then cultured at 37 °C in 5% CO_2 for 5 h. Culture medium was discarded, and cells were cultured in complete 1640 medium with 10% FBS and antibiotics for 19 h. This procedure was repeated for a second infection.

2.5. 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.

2.6. Apoptosis assay

The apoptosis assay was performed using the Annexin V-PE apoptosis detection kit (eBioscience, San Diego, CA, USA) as we described previously [10,15,16]. Fluorescence signals from at least 10,000 cells were collected by FACS (Beckman, CA, USA) to determine the percentage of apoptotic cells.

2.7. Quantitative real-time RT-PCR

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was applied to total RNA extracted from leukemia cells. Reverse transcription was performed using M-MLV reverse transcriptase cDNA Synthesis Kit (Takara Bio, Otsu, Shiga, Japan). Real-time 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^{-ddct}) was used to quantify gene expression.

2.8. Western blot

The following antibodies were used for western blot assay: anti-*p*-Smad1/5/9 (#13820 T).

(Cell Signaling Technology, Beverly, MA); anti-SPG6 (#ab128640) (Shatin, N.T., Hong Kong); BMPR2 (#14376-I-AP), Smad1 (66559-I-Ig), Smad 5 (12167-I-AP), c-Caspase 3 (66470−1-Ig) and GAPDH (60004−1-Ig) (Proteintech Group, Chicago, IL). Cells were lysed with ice-cold RIPA lysis buffer containing protease inhibitors (PMSF, Aprotinin and phosSTOP). Lysate was incubated on ice for 30 min and then centrifuged for 20 min at 12,000 g to remove debris. Proteins were boiled in 1 × loading buffer for 10 min, 30 μg proteins were resolved by SDS-PAGE and proteins were transferred electrophoretically to PVDF membrane (250 mA, 90 min). Membranes were incubated with primary antibodies overnight at 4 °C and appropriate HRP-secondary antibodies for 1 h at room temperature. Detection was performed with chemiluminescent agents. Images were gathered by Alpha Innotech's FluorChem imaging system. Densitometric analysis of blots was performed with Image J.

2.9. Statistical analyses

Data are expressed as means ± 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 $^*p < 0.05$.

3. Results

3.1. SPG6 expression negatively correlates with the overall survival of AML patients

To explore the potential role of SPG6 in AML, we first performed an in-silico analysis of the expression level of SPG6 in human AML patients. Result showed that SPG6 expression was significantly up-regulated in different types of human myeloid leukemia patients compared to that in healthy bone marrow samples (Fig. 1A). We further examined gene expression of SPG6 in a number of human AML and lymphoblastic cell lines. Concordantly, SPG6 mRNA is highly expressed in AML cell lines MV4-11, NB4, and THP-1. It is also expressed in lymphoblastic leukemia (ALL) cell lines RCH-ACV, KOPN-8 and 697, though at lower levels (Fig. 1B). Importantly, SPG6 expression was more elevated in AML patients with

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