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FLT3-ITD drives Ara-C resistance in leukemic cells via the induction of RUNX3

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ABSTRACT

Internal tandem duplication (ITD) mutations of the *FLT3* gene (*FLT3*-ITD) are well known to correlate with a poor prognosis in acute myeloid leukemia (AML). We previously reported that *FLT3*-ITD confers resistance to cytosine arabinoside (Ara-C), a key cytotoxic agent in AML treatments. In order to elucidate the detailed molecular mechanisms underlying the Ara-C resistance induced by *FLT3*-ITD, we performed a microarray gene expression analysis of the human leukemic cell line K562 transduced with *FLT3*-ITD (K562/FLT3-ITD) and identified *RUNX3* as a downstream target of *FLT3*-ITD. The transcriptional induction of the *RUNX3* expression by FLT3-ITD was noted on a Luciferase assay. The knockdown of the *RUNX3* expression in the K562/FLT3-ITD cells increased the sensitivity to Ara-C, and the exogenous expression of *RUNX3 per se* resulted in the enhancement of Ara-C resistance in the K562 cells. A relationship between the *FLT3*-ITD-induced *RUNX3* expression and Ara-C resistance was also observed in AML cells with an endogenous *FLT3*-ITD expression. Collectively, these findings demonstrate that RUNX3 is a prerequisite for Ara-C resistance via FLT3-ITD signaling.

1. Introduction

Acute myeloid leukemia (AML) is a group of heterogeneous hematological malignancies that includes 24 subtypes according to the 2008 World Health Organization (WHO) classification. The current standard induction therapy for AML, except acute promyelocytic leukemia, is composed of Ara-C and anthracyclines such as daunorubicin and idarubicin [1]. These regimens are effective in eliminating the bulk of leukemic blasts and inducing AML remission in most cases; however, the disease often relapses in patients with certain cytogenetics. Therefore, chromosomal aberrations are thought to affect the AML prognosis [2,3].

Cytogenetically normal AML (CN-AML) represents approximately 45% of cases and is considered to have an intermediate risk. However, it is further divided by gene mutations into subsets with different outcomes [4–6]. For example, internal tandem duplication of the juxtamembrane domain of fms-related tyrosine kinase 3 (*FLT3*-ITD) is found in 25–35% of CN-AML patients and carries a poor prognosis, with a high risk of relapse after standard chemotherapy and a median survival of less than five months [7–10].

Class III receptor tyrosine kinase FLT3, expressed in the hematopoietic stem cell fraction, plays an important role in cellular survival and proliferation. Functional analyses have revealed that ITD mutations constitutively activate multiple downstream targets of FLT3, including PI3K/AKT, MAPK/ERK and STAT3/5 [11]. A number of tyrosine kinase inhibitors (TKIs) against FLT3 have been developed and introduced in clinical trials [12]. However, the treatment effectiveness has not yet reached satisfactory levels.

We previously reported Ara-C resistance in *FLT3*-ITD leukemic cells via reduced intracellular Ara-C uptake [13]. In order to clarify the detailed mechanisms, we performed a microarray gene expression analysis in *FLT3*-ITD-transduced K562 cells and found that *RUNX3* is upregulated by *FLT3*-ITD transduction. Moreover, *RUNX3* was found to play an important role as a downstream molecule in Ara-C resistance in leukemic cells with FLT3-ITD.

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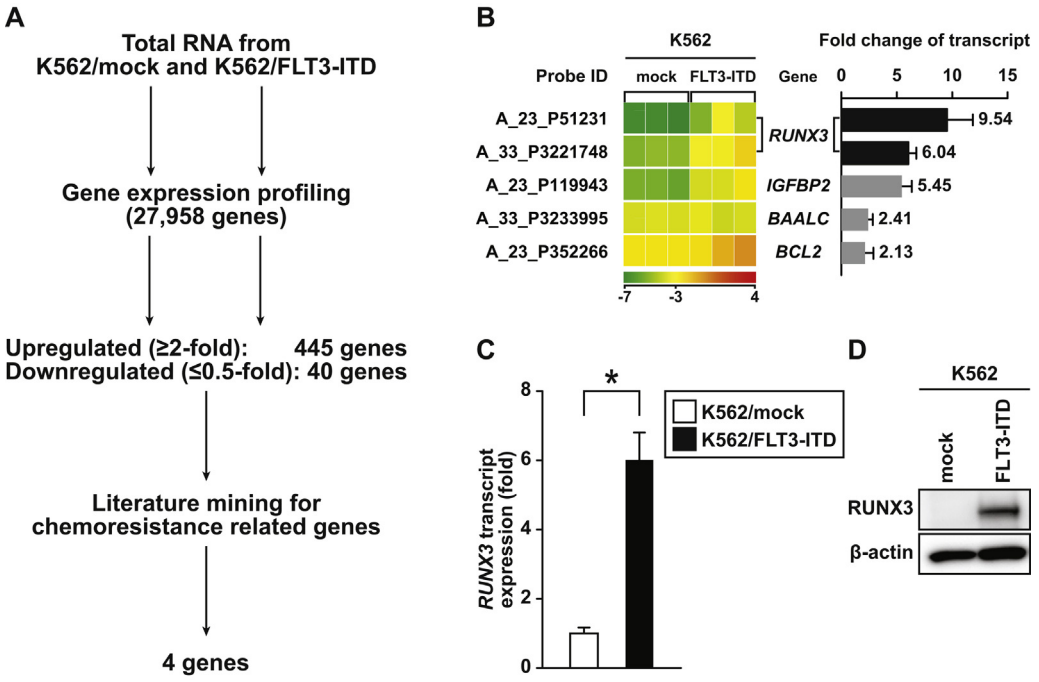

Fig. 1. *FLT3*-ITD upregulated the RUNX3 expression in the K562 cells.
(A) Selection strategy of the genes differently expressed in the K562/FLT3-ITD cells using a microarray gene expression analysis. (B) Heat-map representation and the quantitative expression levels of four identified genes associated with chemotherapy resistance in the K562/FLT3-ITD cells. The mean gene expression values were calculated using the data obtained from three independent experiments. (C), (D). The expression of RUNX3 in the K562/mock and K562/FLT3-ITD cells evaluated by RT-qPCR (C) and a Western blot analysis (D). * indicates $p < 0.01$.

2. Materials and methods

2.1. Cell lines and reagents

Human leukemia cell lines K562, MOLM-14 (kindly provided by Hayashibara Biochemical Laboratories, Okayama, Japan) [14] and MV4:11 (ATCC, Manassas, VA, USA) were cultured in RPMI-1640 medium (Wako, Osaka, Japan) containing 10% Fetal Bovine Serum (FBS; Wako) and 1% Penicillin/Streptomycin (PC/SM; Sigma–Aldrich, St. Louis, MO, USA) at 37 °C and 5% CO_2. PLAT-gp packaging cells (kindly provided by Dr. Toshio Kitamura) and HEK293T cells were cultured in DMEM medium (Wako) with 10% FBS, 1% PC/SM at 37 °C and 5% CO_2. Primary leukemic blasts were obtained from AML patients with *FLT3*-ITD (supplementary Table S1), after their written informed consent was provided, according to the Declaration of Helsinki and with approval from the Tokai University Committee on Clinical Investigation (Permit number: #13I-24). The blasts were cultured in StemPro-34 SFM medium (Life Technologies, Carlsbad, CA, USA), supplemented with StemPro-34 Nutrient Supplement (Life Technologies), 2 mM L-Glutamine (Life Technologies), 50 ng/ml recombinant human thrombopoietin (rhTPO; Kirin Brewery, Gunma, Japan), 50 ng/ml recombinant human stem cell factor (rhSCF; Wako), and 50 ng/ml recombinant human FLT3 ligand (rhFL; Wako).

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Stock solutions of 2 mM cytosine arabinofuranoside (cytarabine, Ara-C; Wako) and 20 mM vincristine (VCR; Wako) were prepared with physiological saline solution. Methotrexate (MTX; Wako) and idarubicin (IDR; Sigma–Aldrich) were diluted with RPMI-1640 and ethanol to prepare 20 mM and 0.5 nM stock solutions, respectively. Stock solutions of FLT3 inhibitors PKC412 (5 mM) (Alexis, Lausen, Switzerland) and Crenolanib (100 μM) (ChemieTek, Indianapolis, IN, USA) were prepared in DMSO.

2.2. Generation of FLT3-ITD, RUNX3-overexpressed and RUNX3-knockdown cells

The pMY-puro-FLT3-ITD plasmid was kindly provided by Dr. Tetsuya Nosaka (Mie University, Tsu, Japan) [15]. Human *RUNX3* cDNA (purchased from RIKEN Bioresource Center, Tsukuba, Japan) [16] was cloned into the pMY-puro vector (kindly provided by Dr. Toshio Kitamura), yielding pMY-puro-RUNX3. These retroviral vectors were used to generate recombinant retroviruses, as previously described [17]. K562 cells infected with these viruses were selected using 2 μg/ml of puromycin.

The pLKO.1 lentiviral vectors encoding two different target sequences of pre-designed shRNA for *RUNX3* and the negative control were obtained from Sigma–Aldrich. The puromycin resistance gene was replaced with the enhanced green fluorescent protein (*EGFP*) gene. After packaging, generated recombinant lentiviruses were infected into both K562/FLT3-ITD and MOLM-14 cells; EGFP-expressing cells were subsequently sorted via FACSAria (BD Biosciences, San Jose, CA, USA). The target sequences of shRNA were: negative control, 5′-GCGCGATAGCGCTAATAATTT-3′; RUNX3_KD-1, 5′-GGCTAGCAGCATGCGGTATTT-3′; RUNX3_KD-2, 5′-CGCCTTCAAGGTGGTGGCATT-3′.

2.3. RNA extraction, reverse transcription and quantitative real-time PCR

Total RNA was extracted using Isogen (Nippon Gene, Tokyo, Japan), and cDNA was generated from 2 μg of RNA using the VILO SuperScript cDNA synthesis kit (Life Technologies). PCR was performed using Phusion FLEX HS (New England Biolabs, Ipswich, MA, USA) polymerase, and quantitative real-time PCR (RT-qPCR) was performed with SYBR Premix Ex Taq II (Tli RNase H Plus) (Takara Bio, Otsu, Japan) on ABI StepOnePlus thermal cycler (Applied Biosystems, Foster City, CA, USA).