学位論文の要旨

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学位論文名　Internal Tandem Duplication of FLT3 Deregulates Proliferation and Differentiation and Confers Resistance to the FLT3 Inhibitor AC220 by Up-regulating RUNX1 Expression in Hematopoietic Cells

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論文内容の要旨

INTRODUCTION

FMS-like tyrosine kinase 3 (FLT3) plays crucial roles in the survival, proliferation and differentiation of hematopoietic stem cells. The most common form of FLT3 mutation is Internal Tandem Duplication in the juxtamembrane domain of the FLT3 gene (FLT3/ITD) that induces constitutive activation of the FLT3-kinase by destroying the auto-inhibitory function of the kinase domain. FLT3/ITDs are detected in 10-15% of children and 30% of adult patients with acute myeloid leukemia (AML) and are associated with extremely poor prognoses. Although a number of antagonists against FLT3/ITD have been developed, few inhibitors are effective for the treatment of FLT3/ITD$^+$ AML because of the emergence of drug-resistant cells. AC220, a second-generation class III tyrosine kinase inhibitor (TKI) used in phase II clinical trials, is a very potent and specific inhibitor for FLT3/ITD compared to other TKIs, however, FLT3/ITD$^+$ cells can become refractory to AC220. These findings underscore the need to develop additional therapeutic strategies to overcome the resistance of FLT3/ITD$^+$ AML to TKIs.

We found that RUNX1, a transcription factor that regulates normal hematopoiesis, is up-regulated in patients with FLT3/ITD$^+$ AML. While RUNX1 can function as a tumor suppressor, recent data demonstrate that RUNX1 is required for AML cell survival.

In this study, we investigated the functional role of RUNX1 in aberrant cell proliferation, differentiation and drug resistance to FLT3 inhibitor in FLT3/ITD$^+$ cells.

MATERIALS AND METHODS

cDNA microarray

Ba/F3 cells expressing wild-type FLT3 or FLT3/ITD (N51 and N78) obtained from two different patients with AML were provided by Dr. D. G. Gilliland of Harvard Medical School. Ba/F3 cells containing wild-type FLT3, N51-FLT3/ITD and N78-FLT3/ITD were subjected to cDNA microarray performed by Miltenyi Biotec.

The 40 modulated genes shared by FLT3/ITD$^+$ Ba/F3 cells, human FLT3/ITD$^+$ AML cells and human AML stem cells were classified based on their biological process as defined by Gene Ontology terms using the DAVID program.

Retroviral transduction of FLT3/ITD and shRNA knockdown

The FLT3/ITD (N51)$^+$ 32D cells and FLT3/ITD$^-$ (wild-type FLT3$^+$) 32D cells were generated by retroviral transduction. For shRNA knockdown of RUNX1, FLT3/ITD$^+$ 32D cells were electroporated with shRNA specific to RUNX1 cloned into the pSingle-tTS-shRNA vector using a Nucleofector Kit V. To activate the RNA interference of RUNX1, doxycycline was added to the culture medium. The reduction of RUNX1 mRNA and protein was validated by quantitative RT-PCR and intracellular flow cytometry. To generate the AC220-resistant cells, FLT3/ITD$^+$ 32D cells were exposed to stepwise increasing concentrations of AC220. To determine the cell proliferation, the viable cells were counted with the FACSCalibur cytometer based on the light scatter.

Statistical analysis

The data are expressed as the mean ± standard error of the mean (SEM), and statistical significance was evaluated using the two-tailed Student t-test in Microsoft Excel.

RESULTS AND DISCUSSION

We first identified the shared molecules that are deregulated by FLT3/ITD in patients with AML and in murine cell lines by microarray analysis. A comparison of these molecules with those deregulated by human AML stem cells identified 40 molecules. In these 40 molecules, RUNX1, a core-binding transcription factor and plays an important role in hematopoietic homeostasis, particularly differentiation and proliferation, was significantly up-regulated.

Silencing of RUNX1 expression by RUNX1 shRNA significantly decreased proliferation as well as secondary colony formation and partially abrogated the impaired myeloid differentiation of FLT3/ITD$^+$ 32D cells. Furthermore, silencing of RUNX1 expression enhances the cytotoxic effects of AC220, a second-generation class III tyrosine

kinase inhibitor (TKI), in FLT3/ITD$^+$ 32D cells. While AC220 significantly decreased the number of FLT3/ITD$^+$ 32D cells, the cells re-proliferate in the presence of AC220 after a few weeks, indicating that FLT3/ITD$^+$ cells became resistance to AC220. Interestingly, the expression of RUNX1 mRNA or protein was significantly higher in AC220 resistant FLT3/ITD$^+$ cells compared to AC220 sensitive parental cells. We therefore determined whether silencing RUNX1 could abolish the proliferation of AC220-resistant FLT3/ITD$^+$ cells.

The FLT3/ITD$^+$ 32D cells containing two different doxycycline-inducible shRNA for RUNX1 or control shRNA were incubated with stepwise increasing concentration of AC220 in the absence of doxycycline for 4 weeks. These AC220-resistant cells were then incubated with AC220 in the presence of doxycycline to induce the shRNA against RUNX1. The number of FLT3/ITD$^+$ 32D cells containing control shRNA gradually increased as expected; however, the number of those with two different shRNAs against RUNX1 was significantly reduced, indicating that silencing RUNX1 abrogates the proliferation of AC220-resistant FLT3/ITD$^+$ cells. The data indicate that up-regulation of RUNX1 is responsible for resistance to AC220 in FLT3/ITD$^+$ cells. A previous report demonstrated that the resistance to AC220 by FLT3/ITD$^+$ AML cells was induced by the additional mutations in the kinase domain of FLT3 gene. However, the sequence of the kinase domain of ectopic FLT3 in the AC220-resistant FLT3/ITD$^+$ 32D cells was identical to the parental FLT3/ITD$^+$ 32D cells. Moreover, the RUNX1 gene sequence including all exons as well as exon/intron boundaries in AC220-resistant FLT3/ITD$^+$ 32D cells was identical to the parental FLT3/ITD$^+$ 32D cells. Moreover, AC220 withdrawal significantly decreased RUNX1 expression in the AC220 refractory FLT3/ITD$^+$ 32D cells, which was comparable to those in parental FLT3/ITD$^+$ cells. The reversible up-regulation of RUNX1 in AC220-resistant cells suggests that an epigenetic mechanism is likely involved in the up-regulation of RUNX1 by the AC220-resistant cells rather than mutational mechanism.

CONCLUSION

Our data indicate that FLT3/ITD deregulates cell proliferation and differentiation and confers resistance to AC220 by up-regulating RUNX1 expression.

These findings suggest the oncogenic role of RUNX1 in FLT3/ITD$^+$ cells and that antagonizing RUNX1 represents a potential therapeutic strategy in patients with refractory FLT3/ITD$^+$ AML to FLT3/ITD inhibitors.