学 位 論 文 の 要 旨

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学 位 論 文 名　Internal Tandem Duplication in FLT3 Attenuates Proliferation and Regulates Resistance to the FLT3 Inhibitor AC220 by Modulating $p21^{Cdkn1a}$ and Pbx1 in Hematopoietic Cells

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論 文 内 容 の 要 旨

INTRODUCTION

Internal tandem duplication (ITD) mutations in the *Fms-related tyrosine kinase 3 (FLT3)* gene (*FLT3*-ITD) are associated with poor prognosis in patients with acute myeloid leukemia (AML). Due to the development of drug resistance, few FLT3-ITD inhibitors are effective against FLT3-ITD$^+$ AML. In this study, we show that FLT3-ITD activates a novel pathway involving the cyclin-dependent kinase inhibitor $p21^{Cdkn1a}$ (p21) and pre-B cell leukemia transcription factor 1 (Pbx1) that attenuates FLT3-ITD cell proliferation and is involved in the development of drug resistance.

MATERIALS AND METHODS

Animals

Specific pathogen-free female C57BL/6 mice, were purchased from CLEA Japan, Inc. $p21^{-/-}$ mice were provided by Dr. H.E. Broxmeyer of the Indiana University School of Medicine. All experiments with animals in this study were approved by the Ethics Committee for Animal Experimentation of Shimane University and they were handled according to our institutional guidelines.

Cell culture, plasmid transfection, retroviral transduction and shRNA knockdown

Ba/F3 cells expressing wild-type FLT3 or FLT3/ITD (N51) obtained from a patient with AML were provided by Dr. D. G. Gilliland of Harvard Medical School. Retroviral transduction of human wild-type *FLT3* and N51-*FLT3*-ITD in an MSCV-IRES-EGFP vector into mouse bone

marrow cells was performed. After sequential infections, the GFP^+ cells were sorted using a fluorescence-activated cell sorting (FACS) Aria II were cultured in semisolid methylcellulose or 0.3% agar without hematopoietic growth factors. Colony formation was scored after 7 or 14 days. In replicate liquid cultures, cells were stained for c-kit, Sca-1 and standard lineage markers at the time of plating or after incubation in liquid culture. For shRNA-mediated knockdown of *Pbx1*, bone marrow cells from the $p21^{+/+}$ and $p21^{-/-}$ mice were transduced with a control shRNA or *Pbx1* shRNA using a pSINsi-mU6 plasmid. To transduce shRNAs targeted against *p21* and/or *Pbx1* into the FLT3-ITD^+ Ba/F3 cells, non-transfected Ba/F3 cells were transduced with MSCV-*FLT3*-ITD (N51)-EGFP using a retrovirus. The GFP-positive cells were sorted and electroporated with a *p21* shRNA that had been cloned into the pBAsi-mU6 Pur DNA vector. Stable transformants were selected with puromycin. The cells were subsequently transfected with a pSINsi-mU6 plasmid containing a *Pbx1* shRNA, and G418-resistant cells were selected and used for the analyses.

cDNA microarrays and quantitative RT-PCR

Following transduction of N51-*FLT3*-ITD in MSCV-IRES-EGFP into lineage negative bone marrow cells obtained from $p21^{+/+}$ and $p21^{-/-}$ mice, the GFP^+, c-kit^+, Sca-1^+, lineage-negative (KSL) cells were isolated by FACS. Freshly isolated control KSL cells from the same donors were used for the subsequent comparisons. The sorted cells were subjected to a differential mRNA microarray analysis, which was performed by Miltenyi Biotec. In separate experiments, the total RNAs isolated from sorted $p21^{+/+}$ and $p21^{-/-}$ KSL cells, with or without FLT3-ITD, were reverse transcribed and subjected to quantitative RT-PCR (Q-RT-PCR).

Statistical analysis

The data are expressed as the means ± standard errors of the mean (SEM). Significant differences were determined using a two-tailed Student's *t*-test in Microsoft Excel™ .

RESULTS AND DISCUSSION

The ectopic expression of FLT3-ITD significantly enhanced growth factor-independent cell proliferation and up-regulated p21 expression in both mouse bone marrow KSL cells and Ba/F3 cells. The loss of p21 expression enhanced growth factor-independent proliferation and sensitivity to cytarabine (Ara-C) as a consequence of concomitantly enriching the $S+G_2/M$ phase population in FLT3-ITD^+ cells. These data suggest that although FLT3-ITD enhances the growth factor-independent proliferation of the cells, the concomitant increase in p21 expression attenuates their proliferation.

To identify the potential mechanisms by which p21 attenuates the proliferation of FLT3-ITD^+ KSL cells, we compared the gene expression profiles of $p21^{+/+}$ and $p21^{-/-}$ FLT3-ITD^+ KSL cells. P21 deletion in FLT3-ITD^+ KSL cells resulted in the modulation of the expression levels of 111 mRNAs that were either unaffected or differentially regulated in normal bone

marrow KSL cells. Of these 111 genes, 12 were deregulated in human AML stem cells. The transcription factor Pbx1, which regulates the function of hematopoietic stem and progenitor cells and is deregulated in AML stem cells, was up-regulated in FLT3-ITD$^+$ KSL cells upon p21 deletion. However, Pbx1 was not affected by p21 deletion in normal KSL cells. The enhanced cell proliferation following the loss of p21 was partially abrogated when Pbx1 expression was silenced in FLT3-ITD$^+$ primary bone marrow colony-forming cells and Ba/F3 cells. These data suggest that the p21-mediated inhibition of the proliferation of FLT3-ITD$^+$ cells is mediated, at least in part, by Pbx1.

When FLT3-ITD was antagonized with AC220, a selective inhibitor of FLT3-ITD, *p21* expression was decreased and a rapid decline in the number of viable FLT3-ITD$^+$ Ba/F3 cells; however, the cells eventually became refractory to AC220. Overexpressing p21 in FLT3-ITD$^+$ Ba/F3 cells delayed the emergence of cells refractory to AC220, whereas p21 silencing accelerated their development, suggesting that blocking p21 by FLT3-ITD inhibition facilitates the emergence of FLT3-ITD$^+$ cells that are refractory to AC220. These data indicate that targeting FLT3-ITD can eradicate growth-inhibitory signals by inhibiting p21 expression, thereby potentially contributing to FLT3-ITD$^+$ AML progression.

Our data suggest that the deregulation of p21 expression in FLT3-ITD$^+$ cells contributes to resistance to chemotherapy in two distinct ways. FLT3-ITD$^+$ cells undergo quiescence by up-regulating p21 expression, which enhances their resistance to conventional chemotherapeutic drugs, such as Ara-C. In contrast, the disruption of the expression and/or function of p21 resulting from FLT3-ITD inhibition contributes to the emergence of FLT3-ITD$^+$ cells that are refractory to FLT3-ITD inhibitors. This result suggests that antagonizing p21 function can sensitize FLT3-ITD$^+$ cells to chemotherapy, whereas activating p21 may aid in inhibiting the development of FLT3-ITD$^+$ cells that are refractory to FLT3-ITD inhibitors.

CONCLUSION

We demonstrate that FLT3-ITD attenuates FLT3-ITD$^+$ cell proliferation by modulating the p21/Pbx1 axis. Although the FLT3-ITD-mediated increase in p21 expression increases resistance to Ara-C by reducing cell cycle progression, disruption of p21 expression using an FLT3-ITD inhibitor accelerates the development of refractory FLT3-ITD$^+$ cells. These results suggest that the deregulated expression of p21 and/or Pbx1 by FLT3-ITD likely contributes to the refractory phenotype of FLT3-ITD$^+$ AML cells. P21 and/or PBX1 may represent additional therapeutic targets for patients with FLT3-ITD$^+$ AML, particularly those who are refractory to FLT3-ITD inhibitors.

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論文審査の結果の要旨

FMS-like tyrosine kinase 3 internal tandem duplications（*FLT3*-ITD）変異は急性骨髄性白血病（AML）の20%に出現し、この遺伝子変異をもつAMLは予後不良であることが知られている。

現在、開発中である*FLT3*-ITDをターゲットとした薬剤（AC220）は、治療開始時は効果があるが、*FLT3*-ITD抑制薬に対する耐性細胞の出現の為、次第に治療に抵抗性となる。また、*FLT3*-ITDはサイクリン依存性キナーゼ抑制分子p21 ^{CDKN1a}(p21)の発現を上昇させることも報告されている。今回、申請者は*FLT3*-ITD陽性細胞の増殖と*FLT3*-ITD抑制剤耐性におけるp21機能を解析した。

①*FLT3*-ITD は、マウス造血細胞の増殖と p21 発現を亢進した。しかし、p21 を欠損させると、その増殖は更に亢進した。したがって、*FLT3*-ITD によって上昇する p21 は、*FLT3*-ITD 陽性細胞の増殖を抑制する。

②p21 を欠損させた *FLT3*-ITD 陽性細胞では、転写因子 Pbx1 の発現が上昇した。一方、p21 欠損によって生じる *FLT3*-ITD 陽性細胞の増殖亢進は、Pbx1 の発現阻害により有意に抑制された。したがって、p21 が惹起する *FLT3*-ITD 陽性細胞の増殖抑制は、Pbx1 の発現減少を介する。

③*FLT3*-ITD 抑制薬 AC220 によって、*FLT3*-ITD 陽性細胞の増殖と p21 発現は有意に減少したが、数日後、細胞は AC220 存在下でも再増殖した。一方、p21 を過剰発現させると、AC220 耐性細胞の出現は遅延した。したがって、AC220 による p21 減少が、AC220 耐性 *FLT3*-ITD 細胞の増殖を促す。

これらのデータは、p21 と Pbx1 は *FLT3*-ITD 陽性細胞の増殖を抑制するが、AC220 は p21 を抑制することで AC220 耐性細胞出現を促すことを示す。したがって、p21 は *FLT3*-ITD 陽性 AML に対する新たな治療標的となりうることを示した。本研究は新規抗白血病薬の開発にもつながる重要な知見をもたらすものであり、博士（医学）の学位授与に値すると判断した。