学位論文の要旨

氏名　平出 智裕

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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著者名　Tomohiro Hirade, Mariko Abe, Chie Onishi, Takeshi Taketani, Seiji Yamaguchi, Seiji Fukuda

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.

論文審査及び最終試験又は学力の確認の結果の要旨

㊙ ・ 乙	氏 名	平出 智裕
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学位論文審査委員	主 査	松本 健一 印
	副 査	松崎 有未 印
	副 査	京 哲 印

論文審査の結果の要旨

受容体型チロシンキナーゼFLT3はホモ二量体を形成することで造血幹細胞の未分化性維持や生存に必須のシグナルを伝達する。FLT3にはいくつかの変異が存在するが、FLT3/ITDは膜透過部位直下にリピート配列が挿入されることによりFLT3が持続的に活性化する変異である。急性骨髄性白血病（AML）においては小児で10%、成人で30%がFLT3/ITD変異を持ち、これらの患者は予後不良であることが知られている。これまでに多くのFLT3/ITD阻害薬が開発されてきたが、初期には有効であるものの、長期的には耐性となり治癒に至らないケースが多い。

本研究では網羅的遺伝子解析を行い、FLT3/ITD陽性AML患者白血病細胞とFLT3/ITDを導入した細胞株においては、FLT3/ITD陰性細胞に比べ、RUNX1の発現が有意に上昇していることを明らかにした。RUNX1は主に未熟な造血細胞の分化を制御する転写因子であるが、その機能喪失がAMLの発症に関与するために癌抑制遺伝子の役割を持つと考えられてきた。しかしながら、FLT3/ITD存在下でその発現が上昇していることは、逆にRUNX1が癌遺伝子として機能する可能性を示唆する。そこで、IL-3依存性に増殖する造血細胞(32D)に対し白血病患者に由来するFLT3/ITDを遺伝子導入することで、FLT3/ITDシグナル下流におけるRUNX1の機能を明らかにし、RUNX1が新たな治療標的になりうるかを検証した。

① FLT3/ITDを遺伝子導入後、32DはIL-3非依存的な増殖性を獲得した。
② この細胞にRunx1 shRNAを導入しノックダウンすると、細胞増殖が抑制されると共に顆粒球系への分化を促進し、自己複製能の指標である2次コロニー形成能が抑制された。
③ Runx1ノックダウンに加えてFLT3/ITD阻害薬AC220を併用すると、より効果的に細胞増殖が抑制された。
④ 低AC220存在下で人工的に誘導したAC220抵抗性FLT3/ITD導入細胞の増殖はRunx1ノックダウンにより完全に抑制された。

以上のことからFLT3/ITDは、RUNX1の発現上昇を介して細胞増殖、分化抑制、自己複製能を増強すること、AC220抵抗性の獲得にはRUNX1の発現が必須であることが明らかとなった。これらのデータは、FLT3/ITD下流ではRUNX1が癌遺伝子として機能しており、FLT3/ITD阻害剤抵抗性FLT3/ITD陽性AMLにおいて、RUNX1は新たな治療標的となりうることを示唆する。

最終試験又は学力の確認の結果の要旨

申請者は、AML患者にみられるFLT3/ITD変異による細胞増殖や薬剤耐性の亢進、分化能の抑制にはRUNX1が関与することを分子生物学的手法により明確に示し、臨床的にも重要な知見を示した。また関連知識も豊富で質疑応答も的確なため学位授与に値するものと判断した。（主査：松本健一）

申請者は変異型受容体FLT3/ITDがRUNX1の発現上昇を誘導し、細胞の自立増殖・分化抑制およびチロシンキナーゼ抑制剤に対する抵抗性獲得の主要因であることを明らかにした。審査時の質疑応答も適切で背景の知識も充分であることから学位授与に値すると判断した。（副査：松崎有未）

申請者はAML患者に認められるFLT3/ITD変異によってもたらされるシグナル下流のRUNX1の発現誘導が細胞増殖と分化抑制に作用し、FLT3/ITD阻害剤抵抗性の要因となり得ることを示した。これによりRUNX1が治療分子標的となり得ることが示された点に臨床的な意義が認められる。研究内容、プレゼンテーション、質疑応答のいずれも十分なレベルに達しており学位授与に値する。

（副査：京哲）