主論文の要旨

Co-expression of wild-type FLT3 attenuates the inhibitory effect of FLT3 inhibitor on FLT3 mutated leukemia cells

（正常 FLT3 の共発現は FLT3 変異陽性白血病細胞に対する FLT3 阻害剤の阻害効果を減弱する）

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Introduction

FLT3-ITD mutation has been reported in about 30% of acute myeloid leukemia (AML) patients and is associated with poor prognosis. Many agents have been investigated as FLT3 inhibitors and evaluated for efficacy and safety in clinical trials, but the clinical efficacy was lower than expected and no FLT3 inhibitors have been approved to date. Several resistance mechanisms, such as potency, plasma protein binding and emergence of secondary mutation in the kinase domain have been revealed. Recently, it was reported that increased plasma FLT3 ligand (FL) concentration after chemotherapy impeded the efficacies of FLT3 inhibitors. However, the mechanism of reduced inhibitory effects of FLT3 inhibitors caused by FL has not been fully elucidated. Since most AML cells harboring *FLT3* mutation co-express wild type (Wt)-FLT3, we hypothesized that FL stimulation to the Wt-FLT3 and activation of its downstream molecules mainly conferred resistance to FLT3 inhibitors in FLT3 mutated AML cells.

Method

The impact of FL stimulation on the efficiency of FLT3 inhibitors was investigated in established 32D cells, sole Wt-FLT3 expressing cells, ITD-FLT3-Myc and ITD-FLT3-FLAG co-expressing cells, Wt-FLT3-Myc and ITD-FLT3-FLAG co-expressing cells, Wt-FLT3-FLAG and cyITD-FLT3-Myc co-expressing cells, and Wt- and ITD-FLT3 co-expressing 32D cells as well as primary AML samples *in vitro and in vivo*. Informed consent was obtained from all patients according to the Declaration of Helsinki for banking and molecular analysis. Approval was also obtained from the ethical committees of Nagoya University.

Results

We evaluated growth inhibitory effects of FLT3 inhibitors in the presence or absence of exogenous FL using established 32D cells (Figure 1A). Although FL stimulation weakly impaired the inhibitory effect of quizartinib against sole ITD-FLT3 cells, the GI_{50} value was significantly increased 4.5-fold with FL stimulation in Wt- and ITD-FLT3 co-expressing cells. A similar pattern was observed with other FLT3 inhibitors, sorafenib and KW-2449. Moreover, addition of soluble FL significantly decreased the quizartinib-induced apoptosis in Wt- and ITD-FLT3 co-expressing 32D cells, but not in sole ITD-FLT3 cells (Figure 1B). The membrane-bound form FL is expressed on stromal cells in the bone marrow microenvironment and it is proposed that a bone marrow niche protects leukemia cells from chemotherapy and FLT3 inhibitor treatment. We therefore established membrane-bound form FL-expressing COS-7. In co-culture with FL-COS-7 cells, the inhibitory effect of quizartinib on Wt- and ITD-FLT3 co-expressing cells was significantly decreased, but not on sole ITD-FLT3 cells in both MTT assay (Figure 1C)

and apoptosis assay (Figure 1D). Then, we examined whether FL reduced the inhibitory effects of FLT3 inhibitors *in vivo*. We inoculated subcutaneously sole ITD-FLT3 cells and Wt- and ITD-FLT3 co-expressing cells into NOD/SCID mice, and treated them with quizartinib or vehicle control. Oral administration of quizartinib showed a potent anti-tumor effect on sole ITD-FLT3 cell-inoculated mice, while the inhibitory effect of quizartinib was significantly lower in Wt- and ITD co-expressing cell-inoculated mice (Figure 2A-2B).

We next investigated the effects of FL on FLT3 and its downstream molecules. Although ITD-FLT3 was re-phosphorylated by the addition of FL, downstream molecules were not affected by FL, FL activated Wt-FLT3, in Wt- and ITD-FLT3 co-expressing cells in the presence of quizartinib. In paralleled with the activation of Wt-FLT3, AKT and MAPK, but not STAT5, were re-phosphorylated by FL (Figure 3). In primary AML cells expressing both Wt- and ITD-FLT3, the addition of FL induced upward shifts in the dose-response curve, indicating increased cell viability and resistance to quizartinib (Figure 4, upper panels). However, this FL-induced upward shift in the dose-response curve was not observed in the samples K405-2 and I994-2 with a homozygous ITD allele obtained after disease progression in a xenograft mouse model (Figure 4, lower panels).

Since our data showed that MAPK was the dominant pathway in the decreased inhibitory effect of FLT3 inhibitors, we investigated whether inhibition of MAPK by the MEK inhibitor U0126 canceled the FL-induced resistance to FLT3 inhibitors. Addition of U0126 to quizartinib nullified the increased GI_{50} value by FL stimulation in Wt- and ITD-FLT3 co-expressing cells (Figure 5, left panel). We confirmed the same results in Wt- and cyITD-FLT3 co-expressing cells (Figure 5, right panel). It is known that maturation of FLT3 with glycosylation is necessary for cell surface expression. We examined whether blocking of FLT3 receptor maturation and translocation to the cell surface impede FL-dependent activation of Wt-FLT3. Wt- and ITD-FLT3 co-expressing cells were treated with the glycosylation inhibitors, tunicamycin or brefeldin (BFA). Flow cytometry analysis showed that both inhibitors reduced surface expression of FLT3 in Wt- and ITD-FLT3 co-expressing cells. Furthermore, FL-induced resistance to quizartinib was cancelled out by combination with tunicamycin or BFA in Wt- and ITD-FLT3 co-expressing cells and Wt- and cyITD-FLT3 co-expressing cells (Figure 6A-B).

Discussion

We demonstrated that the FL-dependent Wt-FLT3 signal is an essential mechanism in the reduced inhibitory effects of FLT3 inhibitors in clinical use. In addition, we showed that the continuous FL stimulation induced by the contact with

FL-expressing stromal cells, also provides AML cells with resistance to FLT3 inhibitors in bone marrow and gives rise to disease relapse. Therefore, the interaction between co-expressed Wt-FLT3 on AML cells and FL expressing stromal cells in bone marrow microenvironment should be considered for total eradication of *FLT3*-ITD positive AML cells.

In our observation, MAPK was the dominant pathway in the decreased inhibitory effects of FLT3 inhibitors induced by FL. Persistent MAPK activation leads to BAD phosphorylation, resulting in the inhibition of cell apoptosis. It was also reported that growth factors widely induced resistance to primary kinase inhibitors by persistent activation of AKT and MAPK pathways in other cancers, which highlighted the importance of ligand-dependent Wt-RTKs activation in kinase inhibitor resistance.

Since FL has been considered one of the important mechanisms leading to lower clinical efficacies of FLT3 inhibitors, several strategies can be applied to restore the potency of FLT3 inhibitors. Our results suggested that the combination therapy with inhibitors to specific downstream molecules activated by FL can be a better therapeutic strategy. We also propose a strategy to overcome FL-mediated resistance by blocking glycosylation. Our results showed that blocking of FLT3 receptor maturation and translocation to the cell surface by glycosylation inhibitors impede FL-dependent activation of Wt-FLT3 and resistance to FLT3 inhibitors in Wt- and ITD-FLT3 co-expressing cells. These results give us further evidence that Wt-FLT3 activation by FL on the cell surface is vital for FLT3 inhibitor resistance. Since AML cells with *FLT3*-ITD mutation co-express not only Wt-FLT3, but also other Wt-receptor tyrosine kinases (RTKs) such as KIT and AXL, blocking glycosylation may be a promising strategy to overcome other RTK ligand dependent resistance in AML.

Conclusion

We elucidated that FL impeded the inhibitory effects of FLT3 inhibitors mainly through the activation of Wt-FLT3, but not mutated FLT3, in the Wt- and ITD-FLT3 co-expressing cells. Furthermore, FL-induced activation of Wt-FLT3-MAPK axis was the dominant pathway for the resistance, and the glycosylation of Wt-FLT3 was also vital for FL-dependent kinase activation and following resistance to FLT3 inhibitors.