論文の内容の要旨

論文題目　Analysis of the process of Bcr-Abl-triggered hematopoietic transformation using a ligand-controllable variant

和訳　リガンド制御型変異体を用いたBcr-Ablによる造血細胞形質転換過程の解析

指導教員　東條　有伸　教授

東京大学大学院医学系研究科

平成　19　年4月入学

医学博士課程　内科専攻

氏名　ハーンプラソップワット、ラタナカニット

(HARNPRASOPWAT, Ratanakanit)

Introduction: In Philadelphia chromosome (Ph), which is a hallmark of both chronic myeloid leukemia (CML) and a subgroup of acute lymphoblastic leukemia (ALL), the ABL gene is juxtaposed to the BCR gene, resulting in a constitutively active tyrosine kinase BCR-ABL fusion gene. Bcr-Abl is responsible for the clonal expansion of Ph+ leukemic cells by inhibition of apoptosis and/or stimulation of cell cycling through the phosphorylation cascades of a number of signal transducers. Accordingly, it is unlikely to delineate the individual process of Bcr-Abl-induced transformation of hematopoietic

cells. Therefore, the purpose of this study is to revisit the mechanism of Bcr-Abl-induced leukemogenesis and especially to delineate the initial step in this leukemogenic process using ligand-controllable Bcr-Abl.

Materials and Methods: Ligand-controllable Bcr-Abl was constructed by deleting first 63 amino acids from p190WT, which are indispensable for oligomerization and activation of Bcr-Abl and then, ER-LBD was fused to the C-terminus of p190Δcc to form p190ΔccER, a $p190^{Bcr\text{-}Abl}$ mutant including ER-LBD at the C-terminus but not CC region at the N-terminus. GM-CSF-dependent human TF-1 cells were transduced with p190ΔccER as well as wild-type p190 (WT), p190Δcc and vector control, respectively, and a series of transformants were subjected to biological assays as well as biochemical analysis.

Results: To investigate the biological consequences of p190ΔccER as well as p190Δcc, human GM-CSF-dependent TF-1 cells were transduced with retroviral vectors encoding these Bcr-Abl mutants. Viable cell number of TF-1/p190ΔccER cells, but not TF-1/p190Δcc cells was maintained during a few days after switch from GM-CSF to 4-HT, and thereafter proliferated at the comparable rate to GM-CSF-supported cells. The growth of 4-HT-treated TF-1/p190ΔccER cells was more sensitive to imatinib than

that of TF-1/p190WT cells, suggesting the absolute Bcr-Abl kinase dependency of their growth.

To elucidate the primary event that is directly and/or rapidly influenced by p190, the profile of phosphotyrosine (pY)-containing proteins in response to 4-HT treatment was determined by immunoblot analysis. Whole amount of p190ΔccER increased in a time-dependent manner and reached the plateau level after 4 days of 4-HT treatment where the pattern of pY-containing proteins quite resembled TF-1/p190WT cells. This can be explained by the observation that ligand-free EGFP-Abl:ER was highly unstable, and upon 4-HT binding, its stability increased over time. Such a stabilizing mechanism might be adapted to substrate proteins including CrkL which are directly bound to and phosphorylated by Bcr-Abl. Phosphorylated CrkL could not be found within several hours after 4-HT stimulation. On the contrary, 4-HT-induced tyrosine phosphorylation of Stat5 could be observed within 10 min, suggesting its apparent role in the initial anti-apoptotic phase supported by p190ΔccER.

Both NF-κB and Stat5 are the representative signal transducers activated in Ph+ leukemia and involved in transcriptional activation of anti-apoptotic molecules. I next investigated the changes in NF-κB activity during 4-HT treatment of TF-1/ p190ΔccER cells. NF-κB activity only began to be upregulated at Day 2 of 4-HT treatment. To search for induced genes in the present context, I performed gene

expression profiling on TF-1/p190ΔccER cells before and after 4-HT treatment and confirmed the result by QR-PCR and found 7 genes (Bcl-xL, HIF-1α, HSPA1A, WT1, GATA2, PRAME and BAG3) were differentially expressed. Of these 7 genes 5, except for BAG3 and GATA2, can be upregulated by Stat5 as shown by inducing TF-1 cells carrying DOX-inducible lentiviral expression system for constitutively active Stat5 mutant (mStat5A1*6) with doxycycline.

Discussion: This is the first description of ligand-controllable Bcr-Abl. TF-1/p190ΔccER cells showed autonomous growth in the presence of 4-HT, while there was a delay of ~3 days until they began to proliferate in response to 4-HT. Such a time-lag can be explained by the observation that unliganded p190ΔccER was highly unstable and became stabilized upon 4-HT binding in a time-dependent manner. By analogy with other chimeric proteins fused to ER-LBD, unstability of p190ΔccER may be attributed to rapid degradation by the ubiquitin-proteasome pathway. However, most of other ER-fusion proteins turned to be unstable upon ligand binding, and p190ΔccER is a rare exception. Since Bcr-Abl physically interacts with multiple substrate proteins via inherent interfaces including phosphorylated tyrosine residues, SH2 and SH3 domains, unstability of p190ΔccER is likely to inhibit such a physical interaction and to compromise downstream signaling pathways. This assumption is partly supported by

delayed phosphorylation of CrkL, a major substrate bound to Bcr-Abl. On the contrary, 4-HT-induced rapid phosphorylation of Stat5 suggests another mode of close interaction between Bcr-Abl and Stat5.

From a viewpoint of anti-apoptotic activity, I focused on NF-κB in addition to Stat5. NF-κB activity per viable cell did not change until Day 2 after 4-HT treatment indicating that NF-κB is activated substantially behind Stat5 after 4-HT treatment of TF-1/p190ΔccER cells. Thus, Stat5 may rationally play a dominant role in the initial anti-apoptotic step triggered by Bcr-Abl. Intriguingly, there is a functional and/or physical interaction between individual Bcr-Abl-inducible genes or proteins in this study. HSPA1A encodes heat shock 70kDa protein 1A (Hsp70), a member of molecular chaperones, which are involved in the regulation of resistance to apoptosis in different tumors. BAG3 interacts with Hsp70/Hsc70 through its BAG domain. BAG3 is constitutively expressed in some leukemia cells and has been shown to sustain their survival and downmodulate their apoptotic responses to drugs through Hsp70-dependent or -independent mechanism. Furthermore, Cesaro et al. have recently reported that WT1 is a direct transcriptional activator of the BAG3 gene and RNAi-mediated knockdown of WT1 enhances apoptosis in K562 cells. In accordance with my data, Zhao et al. reported that higher expression of Bcr-Abl correlated with a nonhypoxic induction of HIF-1α, which was required for the survival and proliferation

of imatinib-resistant cells. PRAME was described as a corepressor of retinoic acid signaling in solid tumor cells, and the recent paper suggests that PRAME inhibits myeloid differentiation in normal and leukemic hematopoiesis. My present data provided more definitive evidence for Bcr-Abl-induced expression of WT1 and PRAME probably through the Stat5 signaling pathway. Apart from Bcl-xL, other target genes for Stat5 have been newly identified in this study. We are now searching for Stat5-binding sites in the transcription regulatory regions in these target genes.

In conclusion, the Bcr-Abl/Stat5 pathway is likely to integrate multiple effector molecules to prevent apoptosis, and they are potential therapeutic targets in Ph-positive leukemias.