平成 28年度　技術交流助成　成果報告（日本留学）

2018　年　05　月　10　日
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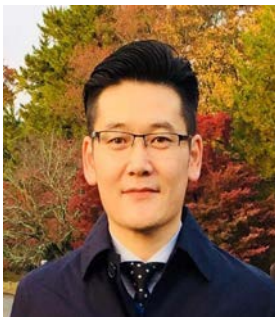


留　　学　　先　東海大学大学院

受入先担当者　教授　宮地　勇人

留　学　期　間　2016年04月01日～2018年03月31日

1.　留学中に実施した研究テーマ

“Fibronectin mediates Ara-C resistance in FLT3-ITD positive leukemia”

Activating mutations in Fms-like tyrosine kinase 3 (FLT3) are present in one-third of AML patients, and are associated with a poor prognosis of the disease. FLT3 is a membrane-bound receptor tyrosine kinase, which activates mitogenic downstream signaling pathways such as Ras/MAPK, JAK/phosphorylated Stat 5 (p-Stat5), and phosphatidylinositol 3-kinase-Akt. FLT3-ITD inhibitors, such as sorafenib, AC220, and crenolanib, showed efficacy for therapy in preclinical models of AML. We have reported a specific resistance against Ara-C in the leukemic cells, harboring FLT3-ITD. Additionally, it is known that the extracellular matrix (ECM) in the bone marrow provides a safe haven not only for the normal hematopoietic progenitors, but for leukemic stem cells as well. To address this, we have studied the roles of cell adhesion on FLT3-ITD-induced chemotherapy resistance using the components of ECM.

2.　留学期間中の研究成果

2.1 Fibronectin 1 expression was upregulated in the K562/FLT3-ITD cells

We performed to transfection FLT3-ITD mutation into K562 cells for transiently transfected by electroporation. Transfection of the FLT3-ITD in K562 cells induced significant upregulation of the fibronectin 1 (FN) gene, as compared to the control cells transfected with the empty vector. The *FN1* expression was attenuated after exogenous fibronectin or collagen type IV exposure.

2.2 Exogenous fibronectin mediated the Ara-C resistance in the K562 cells

We then studied whether exogenous fibronectin or collagen type IV influenced Ara-C resistance in *FLT3*-ITD transfected K562 cells.　Upon

exposure to cytotoxic agents, the cells were grown onto fibronectin or collagen type IV pre-coated plates. Both K562/mock and K562/FLT3-ITD cells incubated on the fibronectin-coated plate showed a higher viability as compared to those in the absence of fibronectin, even without anticancer drugs. The leukemic cells exhibited enhanced resistance to Ara-C in the presence of fibronectin, but not in the collagen type IV. An enhanced resistance against Ara-C was reversed by specific inhibition of the fibronectin adhesion using the monoclonal antibody. Extracellular matrix proteins did not affect the IC_{50} values for the other cytotoxic agents such as methotrexate, vincristine and idarubicin.

2.3 Fibronectin-mediated Ara-C resistance of the FLT3-ITD$^+$ leukemic cells with FLT3-ITD inhibition by crenolanib

To investigate the interaction between the *FN1* expression and FLT3-ITD mutation, we used crenolanib, which is a highly selective tyrosine kinase inhibitor of both wild-type and mutated FLT3 receptors. FLT3 inhibitor treatment reduced the *FN1* transcript expression in K562/FLT3-ITD cells compared to untreated cells. The FLT3 inhibitor treatment did not affect the IC50 for Ara-C in the K562/FLT3-ITD cells with exogenous fibronectin, and substantially restored the Ara-C sensitivity with vehicle and collagen type IV.

2.4 Leukemic cells with hydrophilic surface treatment (non-specific binding)

We examined growth of leukemic cells when maintained in an unattached state via hydrophilic surface treatment. We found that hydrophilic surface treatment significantly prevented the cell attachment, and did not affect to the IC50 of Ara-C in the K562/mock, K562/FLT3-ITD, MOLM-14, and MV4:11 cells.

2.5 Comparison of gene expression in leukemic cells with ECM proteins

We finally analyzed VLA-4 ($\alpha 4\beta 1$-integrin), VLA-5 ($\alpha 5\beta 1$-integrin), and Deoxycytidine Kinase (DCK) expression levels on K562/mock and K562/FLT3-ITD cells using RT-qPCR. These cells showed significant expression of alpha 4 subunit of VLA-4 receptor (*ITGA4*), but not any other integrins in the presence of fibronectin compared to COL4 or absence of ECM. DCK was overexpressed in the presence of collagen type IV, but not in the presence of fibronectin or vehicle.

3. 今後の研究計画

Our current data suggest that the adhesion of FLT3-ITD leukemic cells with the element of the extracellular matrix - fibronectin has enhanced the

resistance to Ara-C. Based on our results we need to clarify underlying molecular mechanism of FN-mediated Ara-C resistance.

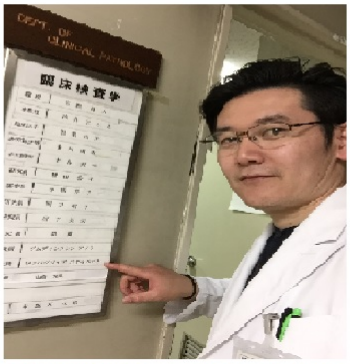
1. To determine the interaction between the components of the ECM of bone marrow and the resistance of the anticancer drugs of FLT3-ITD positive leukemic cells, various culture conditions that promote mutual reaction between the cells and the extracellular matrix environment (in the presence of ECM and stromal cells). To conduct an anticancer drug susceptibility test, MTT assay is used to evaluate for cell viability evaluation. As an ECM, FN, COL4 are used as an integrin ligand which interacts with adhesion receptors VLA-4 and VLA-5 on the surface of leukemic cells. Changes in resistance to anticancer drugs are investigated using monoclonal antibodies or competitive peptides that suppress the interaction between cells and ECM environment.
2. To see the pharmacological molecular mechanism of anticancer drug resistance in FLT3-ITD positive leukemic cells and its influence, and to analyze gene mutations and functions, membrane transport of anticancer agents, intracellular signaling pathways,under the above-mentioned culture conditions.
3. To investigate the variations of the above integrins and integrin ligands as gene expression following interaction of cells with the extracellular matrix. Expression and phosphorylation of FAK/PI3K/ERK signaling and proliferation signals are examined as intracellular signals.
4. In order to clarify the regulatory mechanism of the candidate gene, select a molecule capable of binding to the promoter region and investigate a change in expression in the treatment of a specific inhibitor.
5. To develop detection assay of leukemia treatment reactivity using the gene which have been found contributing to anti-cancer drug resistance.

4. その他と謝辞（日本での生活・交流の様子など）

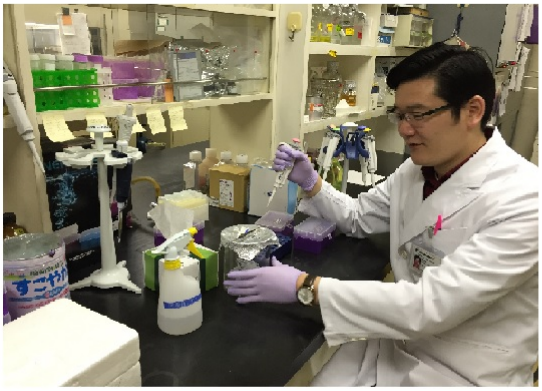
I would like to express my heartily thanks of gratitude to the Nakatani Foundation as well as Professor Hayato Miyachi who give me the golden opportunity to do this wonderful research project on the bone microenvironment mediated drug resistance in leukemia, which also helped me in doing a lot of research and I came to know about so many new things I am really thankful to them. The success and outcome of this research project required a lot of guidance and assistance from many people and I am extremely privileged to have got this all along the completion of this study. All that I have done is only due to such supervision and assistance and I would not forget to thank them.

TOKAI UNIVERSITY

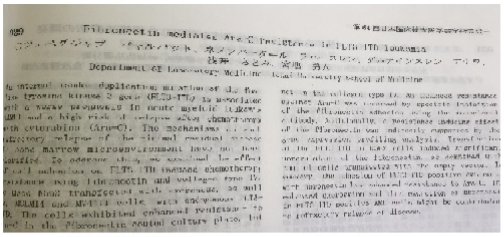
Research Life in the Tokai University School of Medicine



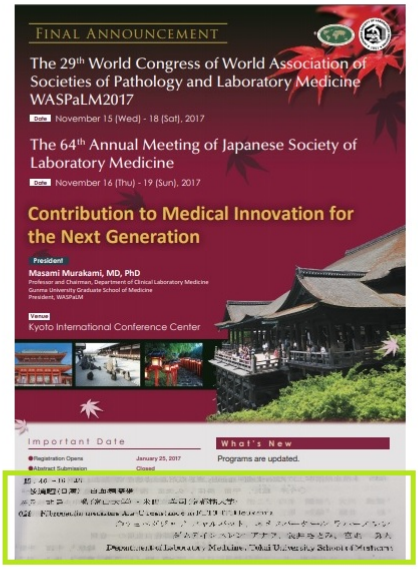
Visiting Researcher at the Department of Laboratory Medicine



My daily research life in the experimental laboratory



We published an abstract on The Official Journal of Japanese Society of Laboratory Medicine



We presented oral presentation at the Annual meeting of Japanese Society of Laboratory Medicine