最終試験の結果の要旨

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主査および副査の5名は、令和4年3月14日、学位申請者 ALMITRA RINDIARTI 君に面接し、学位申請論文の内容について説明を求めると共に、関連事項について試問を行った。具体的には、以下のような質疑応答がなされ、いずれについても満足すべき回答を得ることができた。

質問 1) Is there any patient showing characteristics of nelarabine resistance as shown in your study in the clinical setting? (回答) Our previous study (Sripornsawan et al, 2016) examined blast cells from T-ALL patients and showed a correlation between the ratio of (ENT1xDCK/CDAxRRM1) with LCso of nelarabine. Lonetti et al (2016) using primary cells from relapsed T-ALL patients showed upregulated PI3K/AKT signaling in nelarabine resistance.

質問 2) What is the molecular mechanism related to gene changes in nelarabine metabolism? Is there a master gene that regulates them? (回答) Literature showed the role of genetic changes and epigenetic regulation in gene expression. Our study showed changes in many genes; suggesting mechanisms might relate to upstream regulation such as transcriptional factors related to nelarabine activation genes e.g. Sp1, KLF5, STAT1. Future direction should be aimed to elucidate the master gene regulator.

質問 3) Your discussion stated that the MAPK/ERK pathway didn't induce cell death. Clone 2 showed decrease in cell viability not accompanied by PARP cleavage. What is the mechanism? (回答) Clone 2 has higher activity of p-ERK thus it might be more susceptible to MEK/ERK inhibition. PARP cleavage was not seen, but we did not check other death markers. The role of PARP in apoptosis is dispensable and cell death might still occur independently of PARP; such as by AIF or NAD^+ depletion.

質問 4) Did you check the genomic database of the CCRF-CEM cell line? (回答) We did not check the genomic data of CCRF-CEM before experiments. ATCC database showed mutations in CDKN2A, FBXW7, KRAS, MYB, NOTCH1 (HD domain), PTEN, and TP53. We checked the whole-genomic sequence (data not published) to compare the genome of Clone 1 and Clone 2 with CCRF-CEM as a reference for mutation in known cancer driver genes. Mutational status as seen in the cell line database was confirmed in all cells. We found additional changes in CREBBP, WHSC1, PRPS2, MSH6, IKZF1, and NRAS in resistant clones; but have not done direct sequencing to confirm it. These results could guide further study regarding genetic changes in resistant subclones.

質問 5) Why did you not check the mRNA and protein expression of the BCl-2 family and p-Akt/Akt together? (回答) We checked the mRNA expression of Bcl2 family genes based on the literature that they are transcriptionally regulated, especially pro-apoptotic genes that are transcriptionally activated upon response to death stimuli. However, there is post-transcriptional regulation of Bcl2 family genes, and checking protein is important to give comprehensive information. We checked the protein expression of phosphorylated and total protein of Akt to see the activation of Akt, as Akt is a kinase that depended on phosphorylation for activity.

質問 6) Could you explain the morphological changes in apoptosis? (回答) Apoptosis is programmed cell death characterized by shrinkage of cells, chromatin condensation and fragmentation, cytoplasmic bleb, and apoptotic bodies. We can also observe phagocytosis of apoptotic bodies by macrophages.

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質問 7) Were Clone 1 and Clone 2 generated or just selected from the sensitive population? (回答) The original CCRF-CEM cells consist of a heterogeneous population. We used the selection method with an increasing dose of nelarabine to select resistant cells from a mixed population, then cloning with limiting dilution to isolate cells that may be more resistant than others. T-ALL relapse might be mediated by oncogenic hits that are already present in minor subclones at diagnosis or mutations of specific drug-resistance genes. Considering a relatively short time to develop Clone 1 and Clone 2; the resistant subclones might be a small population of cells selected from sensitive CCRF-CEM.

質問 8) Can you explain the mechanism regarding T-cell lineage selective cytotoxicity of nelarabine? (回答) A higher accumulation of ara-GTP is seen in T-cell than B-cell. Ara-G is not significantly degraded by purine nucleoside phosphorylase (PNP) in T-cell. Elevated levels of ara-GTP resulted in inhibition of DNA synthesis and cell death to a greater extent in T-cell.

質問 9) Do you have any information regarding the chromosomal abnormality in CCRF-CEM? (回答) We did not check chromosomal abnormality in cells used in the study. Literature showed variability of CCRF-CEM karyotype: *Kadioglu et al (2016*

質問 10) Is nelarabine resistance maintained after incubation of cells without nelarabine? (回答) Resistance of our subclones up to one month in nelarabine-free condition assessed with MTT assay remained stable. Resistance is also maintained after a freeze-thaw cycle of cells. We have not checked the resistance stability for longer than a month.

質問 11) Nelarabine in converted to ara-G in plasma by adenosine deaminase (ADA). How about in vitro? (回答) Fetal Bovine Serum (FBS) added in cell culture medium contains ADA thus it is able to convert nelarabine to ara-G in vitro. Our result also showed a comparable degree of cell viability between nelarabine treatment and ara-G treatment in vitro.

質問 12) Can overexpression of ENT1, DCK, and DGUOK in resistant clones reverse the nelarabine resistance? (回答) Overexpressing genes related to nelarabine metabolism could be used as resistance reversal strategies. Several studies have shown gene overexpression with several compounds could reverse resistance such as vorinostat (DCK), bryostatin (DCK/5'-NU activity ratio), and zidovudine (ENT1). Our preliminary data using zidovudine showed upregulation of ENT1, DCK, and DGUOK. Combination therapy with zidovudine and nelarabine showed increased cytotoxicity in Clone 1 but was not consistent in Clone 2.

質問 13) Have you checked Akt upstream (PTEN and PI3K gene) in resistant cells? (回答) We haven't checked the upstream of Akt. Literature showed PTEN (15-20%) and PI3KCA (~2%) mutation in T-ALL. Additional experiment (genome sequencing) in our cells showed all cells had a mutation in PTEN, with an additional frameshift mutation in PIK3R1 seen in the resistant clone. Confirmation regarding Akt upstream will give better information on nelarabine resistance and resistance reversal strategies.

質問 14) Why is there no difference in the effect of PKI-587 as single versus combination therapy with nelarabine? (回答) PKI-587 is a dual PI3K/mTOR pathway inhibitor; the multi-kinase blockade led to increased toxicity and may be potent in masking the effects of combination therapy. The effect of PI3K/Akt inhibitors also depends on underlying molecular mechanism; PKI-587 also showed an important effect in loss-of-function in PTEN, TSC1/2, and STK11 that we have to confirm in further study.

質問 15) Are there any direct evidence from your study that showed changes in genes related to nelarabine intracellular activation? And what is the mechanism of Akt activation in resistant subclones? (回答) Our additional experiment with whole-genome sequence (data not published) showed no mutations in genes related to nelarabine metabolism so we have not found any direct evidence so far. We also did not check their cellular activity. Constitutive activation of Akt is related to cell survival effect on cells. We could check Akt upstream (RTK, PTEN, PI3K, PDK1) or Akt target to assess downstream pathway (GSK3β, mTOR, FOXO, etc.). Further studies are needed to elucidate a comprehensive pathway for Akt activation related to nelarabine resistance.

以上の結果から、５名の審査委員は申請者が大学院博士課程修了者としての学力・識見を有しているものと認め、博士（医学）の学位を与えるに足る資格を有するものと認定した。