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、令和元年 11 月 19 日、学位申請者 Bureta Costansia Anselim 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。

Question 1) You selected three GBM cell lines, are they the only cell lines that you had checked? Or you selected three cell lines from other cell lines, because they were a good model for the experiment?

Answer) We used only this three cell lines, because we wanted to use the cell lines that have been reported to express Hedgehog genes.

Question 2. a) You used 300μM and 1000μM Temozolomide (TMZ), these concentrations are too high, too toxic. Most of the literatures use 100μM. Have you ever tried less concentrations?

Answer) We started the experiment with lower concentrations, unfortunately they could not inhibit the proliferation of the cell lines.

Question 2. b) But the cells survived even if you used 1000μM?

Answer) Yes, Our drug was not effective at low concentration.

Question 3) Usually the function of TMZ is not cytotoxicity, its cell arrest during cell cycle. After 72hrs, did you check the cell proliferation? In presentation slides No. 12, This figure shows that the cells after 72hrs begin to proliferate again, because cell cycle is arrested during the treatment. In your situation how was the cell proliferation after 72hrs.

Answer) We did not proceed with the experiment after 72hrs.

Question 4) In figure 2 & 3, You selected 640μM of TMZ, how did you decide this concentration?

Answer) This concentration was used in combination studies (ATO: TMZ). So as to use the calcusyn model, five fixed constant ratios are need. Because our ratio was 1:320, our maximum drug concentration used was 2:640.

Question 5) In figure 2. (B) What is X-axis?

Answer) It is TMZ.

Question 6. a) In figure 4, when you use combined treatment, there is DNA damage, and apoptosis pathway is activated. That is the mechanism of the drug combination?

Answer) Yes.

Question 6. b) In your idea, DNA damage is evident in the combination? Why is it evident in combined treatment and not single treatment?

Answer) Because of more dsDNA breaks, it was not evident on single treatment because in western blot experiments, you have to titrate the protein concentrations. Hence after titration, the strongest bands will be more evident.

Educative suggestion; The definition has changed several years ago. We don't say Primary and Secondary GBM. Primary GBM is now designated as "Glioblastoma, IDH -wildtype", and Secondary GBM is designated as "Glioblastoma, IDH -mutant".

Question 7) Figure 3. Even in high dose of TMZ combination, about half amount of the cells are still alive. Even in the combination therapy, how do you think about the cells that are still alive? They already get resistance? Or have an activity of Hh pathway? Are they already into the apoptotic pathway?

Answer) Vismodegib was a bit resistant in inhibiting the proliferation of the cells even when combined with TMZ, and the remaining cells, might be resistant to the drugs. But they are already going into the apoptotic pathway.

Question 8) How can human GBM cell lines proliferate in nude mice?

Answer) Since T cell immune system is disrupted in the nude mice, human cancer cells can live in its body even in the different species from human.

Question 9) Figure 5. Cleaved Caspase 3 band and Caspase 3 bands should be detected in the same gel, why did you use different gels?

Answer) When the molecular weight is a bit close together, it's difficult to assess the proteins using the same gel.

Question 10) Is Hh pathway still active in normal cells? How many times is Hh activated in GBM cells rather than normal cells?

Answer) Hh pathway is usually activated in normal cells especially embryonic cells, but when the pathway is impaired that is when tumor cells arise. In this experiment we did not assess the expression levels of Hh genes. But if we could use normal brain cells, and GBM cells, then assess (by RT-PCR or western blot) the expression of GLI1/GL2 and PTCH1, we could see the expression

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levels of these genes.
Question11. a) Figure 4, you showed caspase activation by cleavage. But you just showed activation of caspase. How did you know that the cells really undergo apoptosis? You just showed enzymatic activation but not direct activation in your experiment. If you do additional experiments to show apoptosis, what kind of experiments will you do?
Answer) We did not do other experiments, but with the inhibition in proliferation we believed apoptosis will have occurred. Additional experiment that we could have done include; Flow cytometry assay.
Question11. b) What kind of results will you obtain from flow cytometer?
Answer) Asses the cell cycle, and see Sub G/D1 increase in Flow cytometry.
Question11. c) Any other experiments?
Answer) Tunnel stain, electron microscopic observation to see the condensation of the chromatin.
Question 12) Is there any biomarker for the TMZ response?
Answer) Methylation of MGMT promotor is a useful predictor of glioma response to alkylating agents such as TMZ and the presence of Alkykguanine alykyltransferase (AGT) gene in brain tumors predicts poor response to TMZ.
Question 13) Terminology; in your paper you said synergistic effect, so this is really synergistic effect or just additive?
Answer) We used the term "Synergistic effect" because when assessing synergism by calcusyn software. If the Combination Index (CI) is <1 it indicates Synergism if CI=1 indicates additivity and CI>1 is antagonism. Our CI values were <1 indicating synergism.
Question 14) In vivo experiment in Fig 5. Do you think proliferation is inhibited, or cell death is induced in the combination treatment?
Answer) There was inhibition of proliferation, followed by cell death.
Question 15) Is it possible to do similar in vivo experiments in the brain?
Answer) Yes, it is possible, but we lacked Imaging modalities to help visualize the tumors in the brain, that's why we inoculated them in the flanks.
Question 16) Resistance to TMZ is partly due to expression/function of MGMT. Did you check whether MGMT is expressed in your cell lines?
Answer) No, we did not check the expression of MGMT in these cell lines. But they have been reported to express MGMT.
Question 17) In this study you showed that the combination of TMZ and ATO/VIS is effective, is it possible that ATO or VIS inhibit the expression of MGMT?
Answer) We did not investigate if ATO and VIS could inhibit MGMT, we couldn't find reports showing that, ATO and VIS can inhibit MGMT.
Question 18) You chose a ratio of 1:320 ATO: TMZ, how did you choose this ratio?
Answer) After assessing the single effect of each drug. We calculated the 50% effective dose concentration ED50. We used the ED50 ratios of the two drugs and we also performed several pilot studies.
Question 19) In Figure 2, the effect of ATO saturate at very low concentration, I thought you can use higher ratio, 1:1000
Answer) 1:1000 will be too toxic for the cells.
Question 20) In Figure 2, why can't you see the synergistic effect against U251MG in lower effect fraction, in other cells the combination therapy is very effective from low effect to high effect.
Answer) U251MG in combination treatment there was synergistic effect. But in single drug treatment, 1μM was not effective in inhibiting the cell proliferation unlike in other cells.
Question 21) In the content you said these cells have a high activity of Hh pathway, and in other papers you find that they express MGMT. Are there any evidences that these inhibitors can inhibit MGMT expression?
Answer) There is no evidence that ATO and VIS can inhibit MGMT expression.
Question 22) Don't you have other idea than MGMT, can relate to the synergistic effect of these drugs?
Answer) There is a high likelihood that the pleiotropic effect of ATO and off-target effects of SMO have a high possibility of affecting the growth inhibition of GBM and cause the synergistic effect of the drugs.
Question 23) Other cells those don't express MGMT, but high Hh pathway genes, do you think they don't have any synergistic effect on these cells?
Answer) There are no previous studies that have reported these drug combinations in cells which don't express MGMT. In our study, we assessed only cells which express MGMT.
Question 24) Do you know P53 status of these cells?
Answer) p53 of U87MG is wild-type, and U251MG and U138MG have mutated p53.
Question 25) Did you try the combination of ATO and VIS?
Answer) Yes, we performed the experiment on the combination of ATO and VIS. Yes, there was an inhibition of cell proliferation. But we did not report them in our study, because our main focus was the combination of Hh inhibitor and alkylating agent.

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。