学 位 論 文 内 容 の 要 旨

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学 位 論 文 題 名

Dual inhibition of GPx4 and MEK suppresses pancreatic ductal adenocarcinoma by inducing ferroptosis

（GPx4とMEKの共阻害によるフェロトーシス誘導を介した膵管腺がんの抑制）

[Background and Objectives]

Pancreatic ductal adenocarcinoma (PDAC) is a highly aggressive malignancy with a 5-year survival rate of only 10%. Due to late diagnosis, surgical resection is optional for only 20% of patients, while chemotherapy and radiation demonstrate limited efficacy because of poor response rates and high toxicity. This emphasizes the urgent need for novel treatment regimens.,

Traditional approaches in drug development using cell cultures and mouse models have notable limitations: cultured cells often fail to replicate dynamic microenvironments within the body, while mouse models are costly and time-consuming. In contrast, *Drosophila melanogaster*, the fruit fly, serves as a complementary platform, offering a cost-effective and time-efficient whole-body organism. To investigate PDAC, we developed a '*4-hit*' *Drosophila* model that mirrors genetic alterations of *KRAS*, *TP53*, *CDKN2A*, and *SMAD4*, which are observed in a patient population with the poorest clinical outcome among PDAC patients.

Using the *4-hit* flies, we discovered that reducing the gene dosage of *Riboflavin kinase* (*RFK*) improved the survival of *4-hit* flies, highlighting the significance of riboflavin (RF, also known as vitamin B2) in PDAC. RF and its metabolized forms, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), are critical for metabolic redox and the metabolism of other B vitamins. Hence, I hypothesize that RF-related vitamin metabolism plays a key role in PDAC pathogenesis, although its detailed mechanisms remain unclear. This study aims to identify vitamin metabolism pathways involved in PDAC progression and explore their potential as therapeutic targets.

[Materials and methods]

To investigate vitamin metabolism (VM) pathways in PDAC, I performed genetic screening using *Serrate* (*Ser*) driver-induced *4-hit* flies (*Ser>4-hit*), introducing either heterozygous mutation or siRNA for each VM-related gene. I compared the viability of resulting offspring with that of control flies to identify potential therapeutic targets.

After identifying potential targets, I focused on developing therapeutic strategies for PDAC. I conducted chemical testing by adding small-molecule inhibitors targeting the candidate targets, either alone or in combination with the MEK inhibitor trametinib, into the food for *Ser>4-hit* flies. I compared the viability of *Ser>4-hit* flies treated with these chemicals to that of the vehicle group. I also identified GPx4 as a potential therapeutic target and the combination of ML210, a GPx4 inhibitor, and trametinib as a novel therapeutic strategy. To investigate the role of GPx4 in PDAC, I used RNA interference in human PDAC cells and evaluated the impact of *GPx4* knockdown on cell proliferation.

Given the GPx4's involvement in GSH (glutathione) metabolism and its role in ferroptosis, I assessed its clinical relevance in human PDAC by single-cell RNA sequencing (scRNA-seq). I obtained scRNAseq gene-barcode matrices from Gene Expression Omnibus (GEO) and examined the relative expression of genes involved in GSH metabolism, including *GPx4*, across cell clusters and calculated module scores for GSH metabolism and ferroptosis evaluating the susceptibility of PDAC to GSH interference and ferroptosis induction.

To validate efficacy of ML210 and its combination with trametinib, I employed cultured human PDAC cells. Human PDAC cell lines AsPC-1, Capan-1, and Panc-1 were cultured in spheroid and soft agar assays, and then treated with ML210 and trametinib. Additionally, an orthotopic xenograft mouse model was developed by inoculating AsPC-1 cells labeled with luciferase (AsPC-1-Luc) into the pancreas of immunodeficient mice. Subsequently, the mice were treated with 10 or 40 mg/kg of ML210 intraperitoneally and/or 0.5 mg/kg trametinib

orally for four weeks. Tumor progression was monitored weekly via bioluminescence imaging with luciferin.

Finally, I determined the type of cell death that the treatment induces using the ferroptosis inhibitor Ferrostatin-1 (Fer-1) and the iron chelator Deferoxamine mesylate (DFO). Flow cytometry was used to measure reactive oxygen species (ROS) and iron levels in human PDAC cells. I also evaluated the anti-tumor mechanisms of the seed through immunohistochemical (IHC) and immunofluorescence (IF) staining of tumor samples in mice.

[Results]

I identified 23 VM-related genes whose functional deficiencies rescued the lethality of *Ser>4-hit* flies, indicating their potential as therapeutic targets for PDAC. Notably, 10 of these 23 genes, including GPx4, are part of NAD synthesis pathway, highlighting the critical role of the vitamin B3 (VB3) metabolism pathway in regulating *Ser>4-hit* fly viability. In chemical testing, ML210, a covalent GPx4 inhibitor, significantly improved the survival of *Ser>4-hit* flies when combined with the MEK inhibitor drug trametinib, highlighting GPx4 as a compelling therapeutic target. In human AsPC-1 cells, knockdown of *GPX4* reduced spheroid growth, which was more pronounced in the presence of trametinib.

Additionally, scRNAseq analysis of human PDAC samples revealed high GPx4 expression in cancer epithelial cells. These cells also scored significantly higher in both GSH and ferroptosis modules, indicating that cancer epithelial cells are particularly vulnerable to ferroptosis induction in PDAC.

In soft agar assays, the combination of ML210 and trametinib (hereafter abbreviated as MT), significantly reduced the number and size of AsPC-1 colonies compared to ML210 monotherapy. Similarly, in spheroid assay, MT displayed strong inhibitory effects on PDAC spheroids across various concentration combinations. Moreover, MT significantly suppressed tumor growth in mice with orthotopically implanted AsPC-1 cells compared to monotherapy.

Lastly, it is confirmed that GPx4 and MEK dual inhibition induces ferroptosis. Both Fer-1 and DFO inhibited tumor suppression in trametinib-treated *GPX4*-knockdown AsPC-1 cells. Additionally, Fer-1 and DFO rescued spheroid volume in AsPC-1 cells treated with either ML210 alone or MT. Consistently, MT elevated intracellular ROS and iron levels, effects that were mitigated by Fer-1 or DFO. In mouse xenograft tumors, IF staining of lipid peroxidation marker 4-hydroxynonenal (4-HNE) supported that cancer cells underwent ferroptosis.

[Discussion]

This study identified the pro-tumorigenic role of NAD synthesis pathway in PDAC by genetic screening in *4-hit* flies. The activation of NAD synthesis presents a therapeutic vulnerability, consistent with previous studies linking *NAMPT* overexpression to poor PDAC prognosis.

Moreover, genetic and chemical screening in *Ser>4-hit* flies reinforced the importance of GPx4 as a therapeutic target. GPx4 prevents ferroptosis, an iron-dependent form of cell death driven by lipid peroxidation. Inducing ferroptosis presents a promising strategy for treating PDAC, particularly in drug-resistant cancer cells. However, ferroptosis in PDAC might also harm immune cells, weakening the anti-tumor response. Dual inhibition of GPx4 and MEK could be useful to solve this issue, as MEK inhibition increases iron levels, sensitizing PDAC cells to ferroptosis. Since previous studies have outlined strategies counteracting immunosuppression triggered by ferroptosis, we speculate that the combination of dual GPx4 and MEK inhibition with immunosuppressive countermeasures is a promising strategy to treat PDAC.

[Conclusion]

This research establishes GPx4 as a therapeutic target and demonstrate that dual inhibition of GPx4 and MEK induces ferroptosis, offering a promising treatment strategy for PDAC. The findings demonstrate the versatility and utility of our *Drosophila* screening platform substantiated by mammalian models in developing novel therapeutic approaches for cancer treatment.