Letter of Intent

Through my clinical experience as a thoracic surgeon, I have come to deeply appreciate the significant challenges in treating advanced stage lung cancer. There is an increased number of cases that have shown remarkable responses to recent treatment advances, such as immune checkpoint inhibitors and molecular-targeted therapies, specifically for patients with unresectable stage III and IV lung cancer. However, I have also personally encountered a number cases in which disease progression occurs due to the emergence of resistance after first-line therapy. These clinical observations and experiences have become strong personal drivers to develop fundamental research for the discovery of 'next-step' second-line treatments. When second-line therapies achieve sufficient tumor control, complete surgical resection becomes an increasingly feasible option, even for cases that were previously deemed inoperable. Especially, when considering the continued evolution of surgical techniques. This possibility has become a major source of motivation for myself.

Of the various current targets, I have been particularly focused on epidermal growth factor receptor (EGFR) mutation-positive non-small cell lung cancer (NSCLC) and the resistance mechanisms associated with EGFR-tyrosine kinase inhibitor (EGFR-TKI). I am currently engaged in basic research exploring the role of drug-tolerant persister cells (DTP), which are considered to be a key precursor population in the development of acquired resistance. DTP are cancer cells that enter a reversible drug-tolerant state in the early phase of treatment, later giving rise to genetically resistant clones. Targeting these cells is therefore a critical strategy for preventing relapse and potential disease progression.

In parallel, I am developing a novel clinically relevant *in vivo* model by orthotopically transplanting patient-derived lung cancer organoids into the lung of a mouse. This model allows for the investigation of DTP survival mechanisms and drug sensitivity in a microenvironment that more accurately recapitulates human physiology. I believe this system has strong translational potential and will contribute to bridging the gap between laboratory findings and clinical applications.

Drawing on my experience as a clinician, I aim to become a physician-scientist who can lead translational research from the bedside observations, to the benchtop for analyzing, understanding, addressing, and back to the bedside for patient care. With the support of this grant program, I hope to advance the development of innovative therapeutic strategies for EGFR-TKI-resistant NSCLC, and ultimately increase the number of patients who can benefit from curative surgery. Through this work, I aspire to improve outcomes for patients who currently have limited options.

Statement of Objective: Here we aim to elucidate the mechanisms of acquired drug resistance to the third-generation epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI), Osimertinib. Specifically in EGFR-mutant non-small cell lung cancer (NSCLC), we seek to leverage this gained understanding to develop novel therapeutics and strategies to overcome this resistance.

Background and Rationale: Osimertinib, a third-generation EGFR-TKI, was developed to overcome resistance from the EGFR T790M mutation and is now used as a first-line therapy for advanced EGFR-mutant NSCLC, regardless of T790M status (1,2). Although Osimertinib has demonstrated remarkable efficacy in EGFR-mutant NSCLC, acquired resistance occurs in nearly 80% of patients, leading to disease progression and poor outcomes (2). Emerging studies suggest that resistance is not always due to secondary genetic alterations but may originate from a subset of tumor cells [*drug-tolerant persister cells (DTP)]* that survive initial therapy through reversible, non-genetic mechanisms (3). These DTP cells remain in a quiescent and adaptive state, and eventually evolve into fully resistant clones. Understanding and targeting DTP is recognized as a promising approach to delay or prevent therapeutic resistance (4).

To investigate this further, we have focused on *patient-derived cancer organoids*, which provide a three-dimensional culture system that preserves the histological and genetic features of the original tumor. This model has been shown to more accurately predict clinical drug responses than conventional two-dimensional cultures (5). However, organoids typically consist only of epithelial tumor cells and lack components of the *tumor microenvironment (TME)* which are known to influence drug response and tumor progression (6). To overcome this limitation, we aim to develop a novel *in vivo* model by transplanting lung cancer organoids (LCOs) into the lungs of immunodeficient mice. This approach builds upon an orthotopic lung cancer xenograft model using cell lines previously established at our lab (7). This proposed *Patient-Derived Organoid-based Orthotopic Xenograft (PDOOX)* model more accurately recapitulates the native tumor structure and TME within the lung, and addresses the limitation of LCOs.

Using this model, we will investigate the mechanisms by which DTP emerges under EGFR-TKI treatment and identify potential therapeutic targets to eliminate these cells. The PDOOX model will serve as a powerful tool to bridge the gap between *in vitro* findings and clinical application, ultimately contributing to the development of novel treatment strategies to overcome Osimertinib resistance and improve outcomes for patients with EGFR-mutant NSCLC.

Research Overview and Implementation Plan:

1. Establishment of EGFR-mutant PDOOX model harboring DTP: Dr. Tsao lab, where we conduct collaborative research, is one of the few global leaders in LCO research. Together we have developed a proprietary protocol for generating LCOs and have also contributed significantly to the field of DTP research (4,8). His group has successfully established DTP models using EGFR-mutant LCOs and patient-derived xenografts (PDXs). Building upon this knowledge, this study aims to establish PDOOX models incorporating DTP. We will first introduce a synthetic luciferase gene (Akaluc) into EGFR-mutant LCOs using lentiviral transduction. The AkaLumine-HCl bioluminescence imaging (AkaBLI) system allows for non-invasive, real-time tumor monitoring *in vivo* (9). The Akaluc-labeled LCOs will then be orthotopically implanted into the lungs of immunocompromised mice via a transbronchial approach (7). Osimertinib will be administered orally using a protocol similar to that used in PDXs, to induce a DTP state in the PDOOX models. Tumor dynamics, a critical indicator for DTP modeling, will be monitored using a Bioluminescence imaging system (Xenogen) as well as a preclinical small-animal CT (Mediso nanoScan).

2. Characterization of DTP-harboring PDOOX model: Tumor tissues from PDOOX mice with acquired DTP will be harvested and compared to pre-treatment PDOOX tumors using Whole Exome Sequencing, Whole Genome Bisulfite Sequencing, bulk RNA Sequencing, and single-cell RNA Sequencing to identify key resistance-associated factors. These candidate factors will then be suppressed in LCOs using shRNA or CRISPR/Cas9 gene editing. New PDOOX models will be re-established with these modified organoids, and their sensitivity to Osimertinib will be reassessed.

Clinical Implications: This study will comprehensively elucidate the molecular mechanisms underlying DTP emergence in EGFR-mutant NSCLC following Osimertinib treatment, using a physiologically relevant orthotopic model. Our approach is expected to yield novel therapeutic targets that may help overcome resistance to EGFR-TKI. Furthermore, by validating these targets using genetic approaches at a preclinical level, we aim to develop actionable strategies to prevent or counteract acquired resistance. This is particularly important for patients with EGFR-mutant NSCLC who initially respond to Osimertinib but subsequently relapse and face limited treatment options. Our findings will contribute to the foundation for precision medicine approaches that may improve therapeutic durability and overall survival in this difficult-to-treat population.

Appendix of Summary of the proposed research: References

1. Yu HA, Arcila ME, Rekhtman N, Sima CS, Zakowski MF, Pao W, et al. Analysis of tumor specimens at the time of acquired resistance to EGFR-TKI therapy in 155 patients with EGFR-mutant lung cancers. Clin Cancer Res 2013;19:2240-7

2. Soria JC, Ohe Y, Vansteenkiste J, Reungwetwattana T, Chewaskulyong B, Lee KH, et al. Osimertinib in Untreated EGFR-Mutated Advanced Non-Small-Cell Lung Cancer. N Engl J Med 2018;378:113-25

3. Leonetti A, Sharma S, Minari R, Perego P, Giovannetti E, Tiseo M. Resistance mechanisms to osimertinib in EGFR-mutated non-small cell lung cancer. Br J Cancer 2019;121:725-37

4. Mikubo M, Inoue Y, Liu G, Tsao MS. Mechanism of Drug Tolerant Persister Cancer Cells: The Landscape and Clinical Implication for Therapy. J Thorac Oncol 2021;16:1798-809

5. Vlachogiannis G, Hedayat S, Vatsiou A, Jamin Y, Fernández-Mateos J, Khan K, et al. Patient-derived organoids model treatment response of metastatic gastrointestinal cancers. Science 2018;359:920-6

6. Bejarano L, Jordão MJC, Joyce JA. Therapeutic Targeting of the Tumor Microenvironment. Cancer Discov 2021;11:933-59

7. Nakajima T, Anayama T, Matsuda Y, Hwang DM, McVeigh PZ, Wilson BC, et al. Orthotopic lung cancer murine model by nonoperative transbronchial approach. Ann Thorac Surg 2014;97:1771-5

8. Shi R, Radulovich N, Ng C, Liu N, Notsuda H, Cabanero M, et al. Organoid Cultures as Preclinical Models of Non-Small Cell Lung Cancer. Clin Cancer Res 2020;26:1162-74

9. Iwano S, Sugiyama M, Hama H, Watakabe A, Hasegawa N, Kuchimaru T, et al. Single-cell bioluminescence imaging of deep tissue in freely moving animals. Science 2018;359:935-9

Impact Statement

Molecular targeted therapy using epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI) has brought a paradigm shift in the treatment of advanced non-small cell lung cancer (NSCLC). However, acquired resistance remains a major clinical challenge, significantly limiting the long-term efficacy of these therapies. This study proposes the development and application of a patient-derived organoid-based orthotopic xenograft (PDOOX) model, which recapitulates the native tumor microenvironment (TME). Notably, this model is designed to enable non-invasive, real-time *in vivo* tracking of drug-tolerant persister cells (DTP), a key precursor of resistance, by labeling lung cancer organoids with a synthetic luciferase (Akaluc) and utilizing high-sensitivity and -specificity bioluminescence imaging.

Leveraging this cutting-edge platform, we will perform comprehensive molecular profiling of DTP populations at the pre-resistance stage, identifying critical molecular mechanisms that sustain their survival and proliferation. These findings will make it feasible to develop therapeutic interventions that specifically target the earliest stages in the process of resistance acquisition, a field that has remained largely unexplored due to technical limitations. Furthermore, through CRISPR/Cas9-based functional validation, we aim to rapidly identify and assess actionable molecular targets with therapeutic potential.

The outcomes of this research will, in the short-term, lay the critical groundwork for novel therapeutic strategies aimed at overcoming EGFR-TKI resistance. In the medium-term, it will accelerate the clinical implementation of more effective and durable treatments, thereby contributing directly to the long-term extension of patient survival and improvement of quality of life. In addition, enhanced tumor control may also broaden surgical eligibility for patients previously deemed unresectable, thereby creating new opportunities for curative-intent treatment. Importantly, the PDOOX model and associated analytical framework developed in this project are broadly applicable to studying resistance mechanisms in other molecular-targeted therapies. As such, the impact of this research has potential to reach beyond lung cancer, promoting the advancement of precision oncology across multiple cancer types.

We are confident that this work will drive a new translational research paradigm, exerting a sustained and powerful influence on the reduction of lung cancer mortality and alleviation of patient burden through innovation in resistance-targeted therapy.

Public Non-Scientific Summary

Lung cancer remains one of the leading causes of cancer related deaths worldwide. While recent advances in treatment, such as targeted therapies, have significantly improved outcomes for some patients, many still face relapse due to drug resistance. In particular, patients with a type of lung cancer that carries a mutation in the epidermal growth factor receptor (EGFR) gene often initially respond well to the first-line treatment with a drug called Osimertinib. Unfortunately, over time, cancer can become resistant to this drug, leading to disease progression.

One key cause of this resistance is the presence of a small group of cancer cells called drug-tolerant persister cells (DTP). These cells evade the drug and survive in a dormant state, allowing them to slowly adapt and eventually grow back in a drug-resistant form. Understanding these DTP cells, why they survive, and how they evolve could be the key to preventing drug resistance.

To study this problem more effectively, we are developing a new experimental model that closely mimics how lung cancer behaves in the human body. We use tiny three-dimensional structures (7-8 mm, when aggregated) called organoids grown from patient tumor samples. These organoids are then implanted into the lungs of mice in a way that recapitulates the natural tumor environment, including blood vessels and surrounding tissue. This method allows us to observe how cancer cells respond to treatment in real time using a special imaging system based on light.

Our approach is unique because it not only recreates the complexity of human lung tumors but also allows us to track the early stages of drug resistance as it happens. We can analyze the genetic and molecular features of DTP and test what happens when we block certain genes using advanced tools like CRISPR/Cas9. This helps us identify which molecules are essential for the survival of these cells.

The ultimate goal of this research is to find new ways to eliminate DTP before they cause complete resistance. By doing so, we aim not only to develop new therapeutics but also to enhance the long-term effectiveness of existing treatments such as Osimertinib. This could allow more patients to remain in remission longer and possibly make surgery an option for those previously considered inoperable.

Ultimately, the insights gained from this research could extend beyond lung cancer to other cancers that face similar resistance problems. Our model and methods may serve as a foundation for the development of better and more personalized cancer treatments, improving quality of life, and survival for many patients.

Budget justification

A. Personal

Research Fellow (0.6 FTE) – Salary: $31,800 / Benefits: $8,000

Total: $39,800

The applicant is the sole research fellow responsible for executing the entire project, dedicating 60% of their research time (0.6 FTE). With an MD background and extensive expertise in lung cancer biology, organoid and xenograft models, the fellow will lead the study design, perform all experimental procedures, analyze data, and present findings.

B. Equipment and Facility Access

- Animal facility usage: $2,090

- Imaging cores (bioluminescence and CT): $3,236

Total: $5,326

These facilities are critical for orthotopic transplantation and non-invasive monitoring of tumor growth and therapeutic response *in vivo*.

C. Experimental Animals

Mice (immunodeficient): $3,000

Required for establishing the PDOOX model, including purchase, housing, and care.

D. Materials and supplies

Medium	**$3680**
Dishes	**$1212**
Matrigel	**$3200**
TrypLE Express	**$990**
PBS	**$456**
Tips	**$410**
Centrifuge tubes	**$416**
Inhibitors	**$2176**
Antibodies	**$2107**
DNA Assay Kit	**$1080**
PCR reagent	**$1088**
Western Blotting	**$1504**
Whole Exome Sequencing	**$1730**

Whole Genome Bisulfite Sequencing	**$1875**
bulk RNA Sequencing	**$4000**
Single cell RNA Sequencing	**$6000**

Total: $31,924

These consumables support the full experimental pipeline: organoid culture, genetic editing, molecular profiling, and mechanistic studies.

Other Sources of Funding

Preliminary studies were supported in part by the William Coco Chair in Surgical Innovation for Lung Cancer and the Grants-in-Aid for Scientific Research JSPS KAKENHI. These supports were specifically allocated to the acquisition of non-consumable equipment that is already installed in our laboratory.

The current proposal requests funding solely for the execution of new experimental work, including animal facility usage, imaging core access and materials required for *in vivo* and *in vitro* studies. No part of this proposed project is supported by any other source.

Total Budget:

A. Personnel: $39,800
B. Equipment and Facility Access: $5,326
C. Experimental Animals: $3,000
D. Materials and Supplies: $31,924

Grand Total: $80,050

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September 19, 2025

To the Give A Breath Research Award Review Committee, Lung Cancer Canada

I am pleased to provide this letter of institutional support for Dr. Yusuke Fujibayashi in connection with his application to the Give A Breath Research Award from Lung Cancer Canada. Dr. Fujibayashi, a thoracic surgeon and current postdoctoral fellow, focuses his research on understanding and overcoming acquired resistance to EGFR-tyrosine kinase inhibitor (EGFR-TKI) in non-small cell lung cancer (NSCLC). The proposed project, which focuses on targeting drug-tolerant persister cells (DTP) using a patient-derived organoid-based orthotopic xenograft (PDOOX) model, is both innovative and feasible within the infrastructure and collaborative environment of our institute.

I confirm that the University Health Network (UHN) provides a world-class research setting that supports this project in full. Specifically, Dr. Fujibayashi has access to all translational research facilities at the UHN, including:

- Established capabilities in patient-derived organoid culture and CRISPR/Cas9 gene editing
- Access to UHN's advanced animal research and preclinical imaging cores
- A collaborative research community with expertise in molecular oncology, lung cancer biology, and resistance mechanisms
- Mentorship from leaders in the field of thoracic oncology, including surgeons and researchers at the Toronto General Hospital and Princess Margaret Cancer Centre

Dr. Fujibayashi's project aligns strongly with UHN's commitment to fostering translational research that directly impacts patient care. The insights gained through this work are expected to accelerate the development of next-generation therapeutic strategies for EGFR-mutated NSCLC and have the potential to improve outcomes for patients who currently have limited treatment options.

Our division and institution are fully committed to supporting Dr. Fujibayashi's proposed research through both research resources and academic mentorship. We are confident that Dr. Fujibayashi will carry out this work with the highest scientific and ethical standards, and that the outcomes will contribute meaningfully to the goals of Lung Cancer Canada.

Please feel free to contact me if you have any questions or concerns.

Sincerely,

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