PI: Dae-Kyum Kim, PhD. Assistant Professor. Research Institute of McGill Univesity Cancer Centre

Discovery of novel drug target for cisplatin resistance at late-stage lung cancer with a novel proteome-wide overexpression perturbation method

1. Letter of Intent

Drug resistance is a significant challenge in the treatment of advanced lung cancer (i.e. stage III or IV) in Canada[1]. For instance, cisplatin or carboplatin are popularly used at the early stage of lung cancer, which develops resistance in the later stages (**Aim 1**)[2]. KRAS-G12C inhibitors have shown promising initial response rates but often lose effectiveness as tumors adapt and find alternative pathways to survive[3]. Similarly, immunotherapy, which leverages the body's immune system to fight cancer, faces hurdles due to the tumor's ability to create an immunosuppressive microenvironment[4]. Understanding and addressing drug resistance remains crucial for enhancing the efficacy of treatments for advanced lung cancer and further extending patient survival. For example, researchers are actively exploring combination therapies (**Aim 2**) and new targets, such as Aurora Kinase A (AURKA), to overcome these resistance mechanisms and improve patient outcomes[5].

Cisplatin's rising prominence in late-stage lung cancer compels a deeper investigation into its mechanism of action, especially its impact on intricate biological systems and potential resistance pathways. Recently, extensive 'Omics' approaches allowed us to obtain and explore massive data about individual genotypes and phenotypes for each patient[6]. Sparked by the growing importance of interpreting omics data from patients in advance, pooled CRISPR knockout or siRNA knockdown approaches have been performed extensively[7], even though the complementary gene overexpression screens have been underappreciated[8]. While previously established screening technologies like lentivirus[9] or CRISPRa[10] are useful, they cannot "fairly" capture the "proteome-wide" space due to i) heterogeneous gene overexpression due to random gene insertion, ii) a lack of proteome-wide coverage limited by chromatin accessibility dynamics, and iii) false-positive driver candidates caused by multiple genes being activated per cell, which requires novel screening platform for gene overexpression (**Aim 1**).

In the proposed project (**Fig. 1**), our team aims to address the clinical problem of treating late-stage lung cancers by dissecting resistance-causing mechanisms. **We hypothesized that, by identifying resistance-causing mechanisms of chemotherapy via cisplatin, we can propose combinatorial drug candidates targeting our resistance gene candidates, which in turn could prevent drug resistance for first-line therapy for late-stage lung cancer patients.** Here are our two specific aims:

Aim 1: Understanding novel pathways whose overexpression induces resistance to cisplatin

Aim 2: Discovering candidate inhibitors whose co-treatment with cisplatin can show synergistic drug effect

In **Aim 1**, we will use the Bxb1-landing pad system for inducing gene overexpression "homogeneously" in a "single gene per single cell" fashion to find causal "drivers" for cell proliferation regulation. Specifically, we will apply our novel method to the **A549** cell line (late-stage non-small lung cancer cell line)[11] and investigate its mechanism to gain resistance under cisplatin, one of the first-line therapy drugs. Here, we will not only discover novel drug targets for late-stage lung cancers but also provide a novel system to mimic the heterogeneous genetic backgrounds of patients and tumors with distinct driver candidates. In **Aim 2**, We will test combinatorial therapy with the drug partners that were previously known to target our resistance gene markers found in **Aim 1** to check if we can suppress the resistance in advance. We strongly believe that this research can lay a foundation to interpret patients' proteomic profiles proactively. Furthermore, we can apply our system to various treatments for lung cancer to extend the utility of our proposed research.

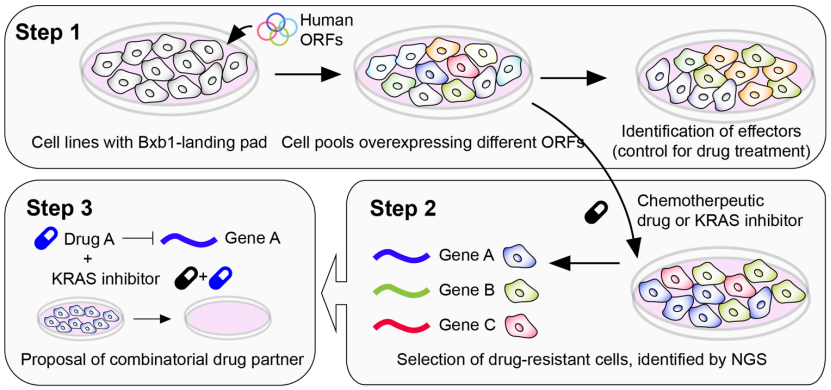


Fig. 1: Schematic overview of screening pipeline. In step 1, we will combine our two novel system of ORFeome and Bxb1-landing pad to create a cell pool with gene overexpression in single gene per cell fashion. In step 2, we will expose our cell pool with proposed drugs to selection for the genes which provides therapeutic resistance. In step 3, the drug target candidates will be further validated by the previously known drugs.

2. Summary of the proposed research

A. Innovation

A.1. The newly expanded ORFeome allows us to screen the previously unexplored "search space"

As groundwork for this proposal, the following collections of human protein-coding genes (ORFeome) were constructed during my postdoctoral training and are now available at McGill University:

A. Human ORFeome[12] libraries consisting of Gateway entry clones for most protein-coding genes that have been verified by full-length sequencing.

B. Human and yeast barcoded ORFeome (*unpublished*), with a distinct barcode for identifying each gene.

Previous human ORFeome 1.1[13] and 8.1[14] cover only half of all 20K human proteins coding genes, but we recently generated ORFeome 9.1[12] and unpublished ORFeome delta which includes ~95% in total.

A.2. Bxb1-landing pad allows us to "homogeneously" express ORFs in a "single gene per cell" manner to find "resistance drivers"

Recently, the Bxb1-landing pad system[15]—a scalable platform for inducing transgene expression in mammalian cell lines—was developed. Unlike the methods for phage, bacteria, or yeast, pre-existing methods for introducing protein-coding genes into cultured human cells have some challenges, as they can inundate the cells with thousands of plasmids and lentiviruses scramble barcoded cargo and deliver it to genomic sites yielding variable expression levels[14,16]. In the Bxb1-landing pad platform (**Fig. 2A**), a cell line is genomically integrated with a landing pad capable of site-directed recombination, including a constitutive, or inducible promoter-driven expression of mTagBFP. Then, a plasmid with a transgene and a recombination site is transfected into the cell line, which will integrate into the genomic landing pad. Following the integration, the cell will lose mTagBFP and gain mCherry signal, which enables us to select gene-inserted cells with FACS. The recombination site is lost after this integration event, therefore every cell in the pool will express a single gene. In our lab, stable landing pad cell lines were already created for 20 different cell lines including HAP1, HEK293T, HeLa, and **A549** cell lines, which supports the timely feasibility of the proposal. An integration efficiency of 46% was achieved for the HeLa cell line (*unpublished*; **Fig. 2B**), which is enough to overexpress the entire ORFeome collection in a pool at a reasonable cost. Our system allows for "homogenous gene expression" of a single gene at a specific locus with an artificial promoter, which is critical for finding drivers for phenotypic changes on the cell line level such as cell proliferation or drug resistance (**Aims 1**).

A.3. Creative "omics" approaches in human cell lines to suppress drug resistance are necessary.

During our first 2.5 years after we started our lab, our team successfully applied this method in the proteome-wide space in the HeLa cell line with ~90% of expected genes represented with a false discovery rate of 1%. As a proof of concept, we applied our system to find genes regulating cell growth. Genes upregulating proliferation will become more enriched, and be represented more over time, and *vice versa* for down-regulating genes. Among the 112 top hits that help the cell proliferate faster, we found that ~45% of genes already have evidence that when overexpressed (**Fig. 3A**), it will provide an unfavorable effect on cancer patient survival as you can see with the example of our top hit, BRD9, in **lung cancer** (**Aim 1**; **Fig. 3B**) supported by Human Protein Atlas[17]. We further expanded our research to drug resistance against 16 different cytotoxic chemotherapeutic drugs that span all the cell cycle checkpoints. We successfully showed that i) we could reproduce our screening data with five independent batches, ii) drug-resistant datasets show a statistically significant deviation from the three replication controls, and iii) drug resistance markers are enriched with the previously known resistance pathway, such as microtubule regulation with paclitaxel resistance and nucleoside salvage pathway with pyrimidine analog resistance (**Aim 1**). Furthermore, this approach builds on our prior success in identifying resistance mechanisms and therapeutic targets, exemplified by our work with BCL2, a gene conferring resistance to chemotherapeutic TAS102, an FDA-approved drug for metastatic colorectal and pancreatic cancers. In our previous study, bioinformatics analysis revealed BCL2 as a potential resistance marker using an overexpression screening platform (**Aim 2**). Further validation through cytotoxicity assays in Miapaca2 pancreatic cancer cells demonstrated that the combination of TAS102 (TFT analog) and Navitoclax (a clinical drug targeting BCL2), enhanced drug efficacy in cancer cells while sparing normal cells (**Fig. 4**), confirming BCL2 as a valid therapeutic target. These results show that the project is not only feasible but also if the failure rate can be kept low, we will likely be able to expand the range of contexts assayed including late-stage lung cancer we are targeting in this proposal.

B. Approach

B.1. Aim 1: Understanding novel pathways whose overexpression induces resistance to cisplatin

B.1.1. Construction of cell pool with proteome-wide gene overexpression in a single gene per cell fashion

By combining the ORFeome collections[12] (**Section B.1.**) and Bxb1-landing pad system[15] (**Section B.2.**), we can establish a novel platform to evaluate the effects of gene overexpression perturbation, and model tumor heterogeneity. To move our ORFeome entry clones to the destination vector containing recombination sites for the Bxb1-landing pad, we will use an *en masse* cloning strategy (**Fig. 5**) previously arranged for HeLa cell[18]. We will divide the proteome-wide space (18,000 ORFs) into 9 subspaces (2,000 ORFs) and then perform Gateway LR cloning of Entry clones as a pool into the destination plasmid and aim for ~3 million transformed bacterial colonies, representing each gene ~150x. Using maxi-prepped DNA from bacteria transformed with the cloned plasmid pool, a pooled transfection of mammalian cells will be performed, with the same coverage of ~150x. Given that the gene insertion efficiency of this Bxb1-landing pad system is ~46% (*unpublished*; **Fig. 2B**), ~3M cells (10 x 15cm confluent plates) are required to be transfected for each gene to be represented 150 times. Sorted cells will be outgrown to 200x on 100 x 15 cm plates (to ~20B cells), and then frozen in aliquots so that we can use the same pool of mammalian cells to screen multiple conditions, minimizing batch variability. We aim to include more than 90% of the target ORFs in our **A549** cell pool, as demonstrated by our success in the HeLa cell line (**Section A.3**).

B.1.2. Screening of cell pool with first-line therapy: cisplatin

We chose to screen for **cisplatin** as our preliminary condition as it is among the most commonly prescribed first-line chemotherapy options for non-small-cell lung cancer (NSCLC)[19]. We chose the concentration of IC_{50} to find not only gene candidates for drug resistance but also for drug sensitization to increase the impact of our screening[20]. To determine an appropriate treatment dose (IC_{50}) for **cisplatin** in the **A549** cell line, we will follow the protocol we used for HeLa cell lines (**section A.3.**). We will plate 3,000 cells in a 96-well plate and test viability using a resazurin assay[20] after 72 h of treatment, and interpolate the IC_{50} from 10 varying concentrations. We will further verify the IC_{50} concentration in a 15-cm dish format (the same format that we will perform the actual screening) to determine the accurate concentration that leads to 50% inhibition of cell growth. Once the appropriate dose for selection had been established, we thawed cells expressing the ORFeome library that previously had been generated and frozen and grew them in culture for three days. To ensure reproducibility, we screen three replicates each for cisplatin and DMSO control. We plated 3M cells into each 15-cm dish and selected for 5 days, maintaining 3M cells throughout each passage. Cells are grown without treatment for 3 additional days and harvested to ensure we can purify sufficient genomic DNA for library preparation.

B.2. Aim 2: Discovering candidate inhibitors whose co-treatment with cisplatin can show synergistic drug effect

B.2.1. Detection of statistically significant drug resistance marker by next-generation sequencing (NGS)

To determine the proliferation effect of overexpressed genes, we will use next-generation sequencing (NGS) to determine the change in the frequency of genes in a pool. Genes providing drug resistance will become more enriched after selection and drug-sensitizing genes will become depleted, relative to DMSO control. We will collect genomic DNA from 3M cells for both DMSO and cisplatin-treated samples with three replicates, then amplify the inserted genes by PCR using primers targeting the conserved site flanking the integration site. Subsequently, we will use Tn5 transposase and indexing PCR for demultiplexing to allow the detection of exogenous genes with NGS. We will aim for 3 million reads to maintain ~150x coverage and analyze our results using a pipeline adapted from an RNA-seq analysis pipeline[21] to compute quantitative measures for each gene (RPKM; Reads Per Kilobase of transcript, per Million mapped reads). Using three replicates, we will apply an empirical testing pipeline, which was used previously[12] to select for statistically significant gene markers. Alternatively, we will use other established tools for differential gene analysis such as edgeR[22] and drugZ[23]. To test the robustness of our approach, 20 randomly selected gene markers for cisplatin-resistance will be tested individually with two parallel gene overexpression methods: i) Bxb1-landing pad-based gene insertion and ii) lentiviral gene overexpression. Moreover, for the gene markers which have previously known targeting drugs, we will treat those in the target gene-overexpressing cell lines to check whether we can suppress the drug resistance in advance.

B.2.2. Proposing inhibitor candidates based on the previously developed inhibitors.

For the candidate gene markers we found in section **B.2.1.**, we will use the Therapeutic Target Database (TTD)[24] to propose possible inhibitors to overcome cisplatin resistance.

C. Supporting figures (Figure 2 to 5)

Fig. 2: Schematics of integration via Bxb-landing pad works (A) and efficiency of integration into HeLa cell (B). AAVSI, adeno-associated virus integration site 1 which guarantees stable expression as a safe harbor locus; ORF, open reading frame; hEF1a, constitutively active human EF-1α promoter.

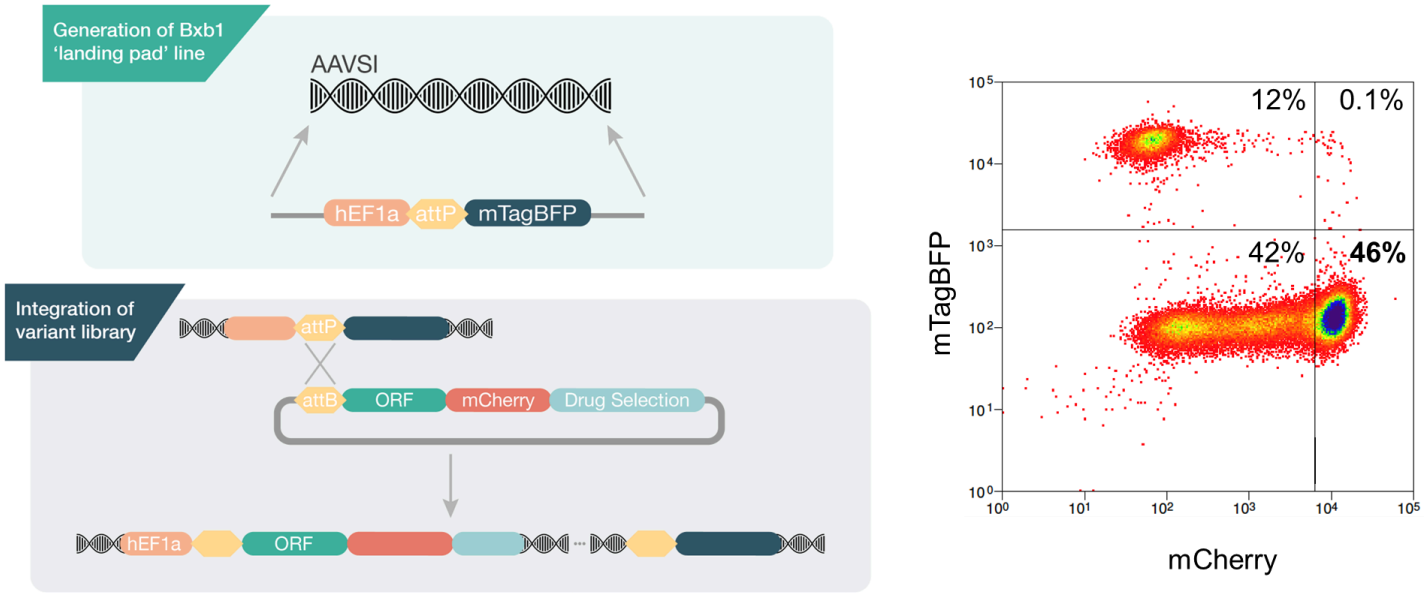


Fig. 3: Systematic analysis of the candidate which promote cell proliferation. Random set was chosen 1000 times by random permutation

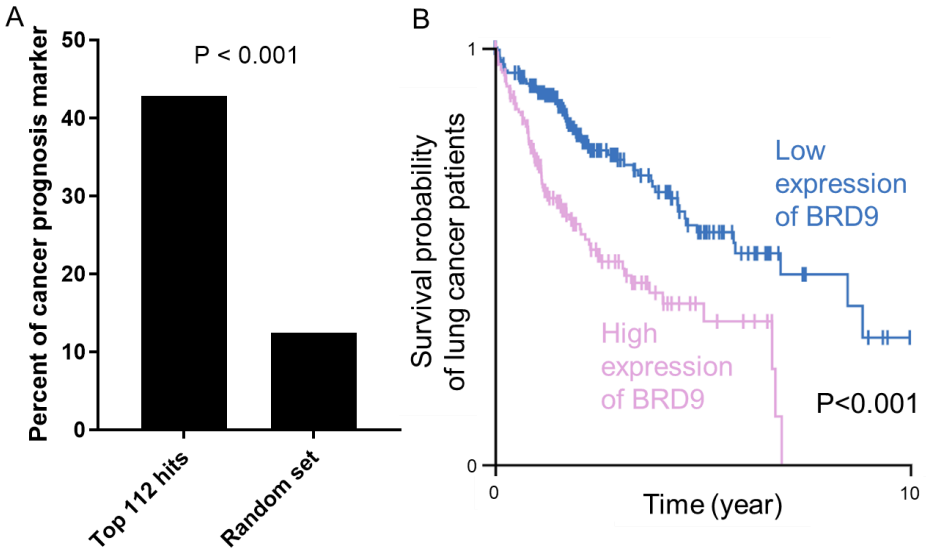


Fig. 4: Cytotoxicity testing of combinatorial drugs in the colon cancer model (Miapaca2) and epithelial cells (HPNE).

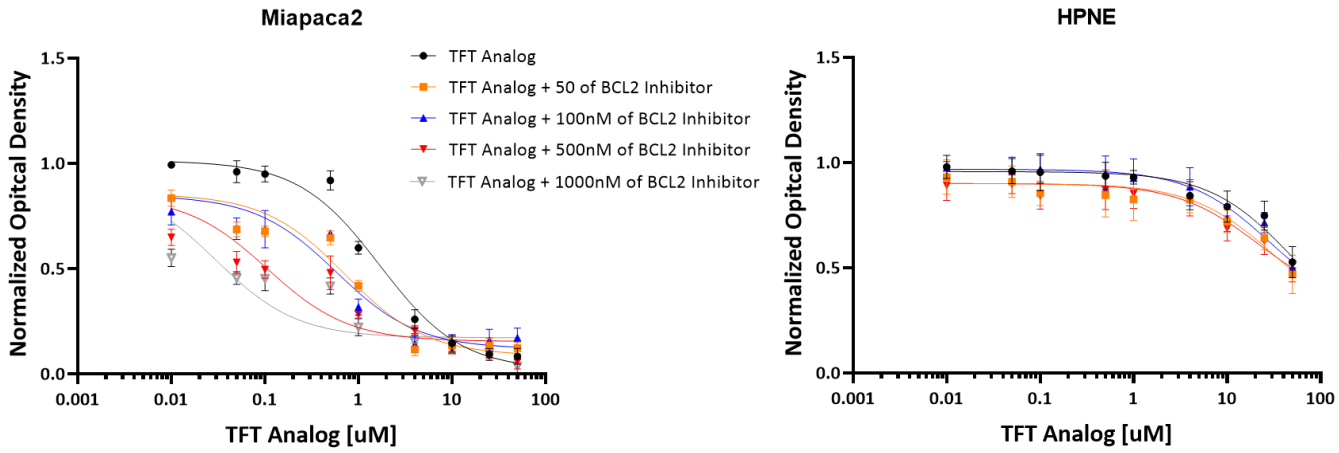
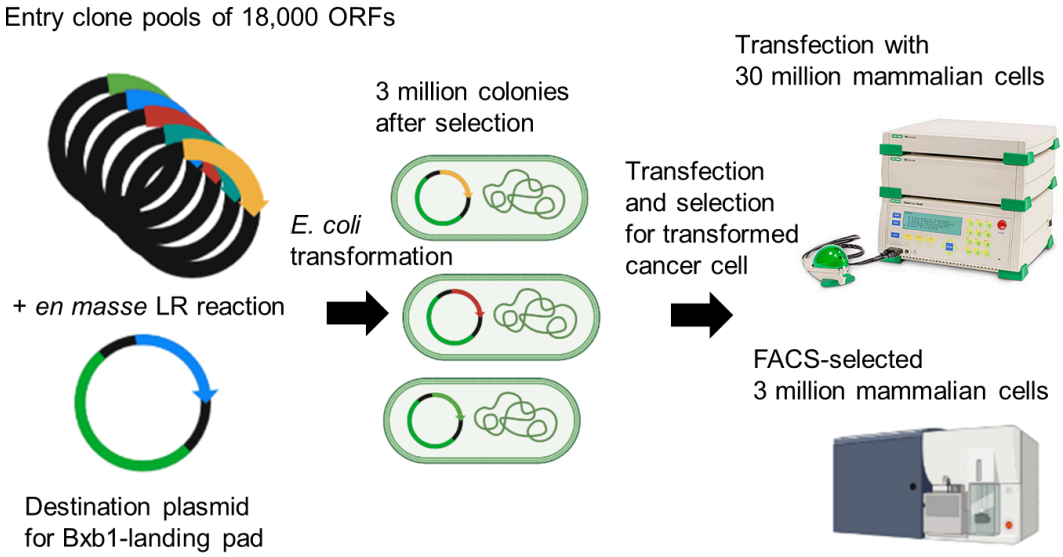


Fig. 5: Preparation of cell pools with gene overexpression by en masse approach. ORF, open reading frame; LR, LR clonase to move Gateway Entry clones to destination plasmid.



3. Impact statement

A. Proteome-wide overexpression perturbation will enable 'proactive' precision medicine for patients with rare overexpressing biomarkers.

In this proposal (**Fig. 6**), we propose a novel platform to mimic cancer heterogeneity with simplified driver candidate overexpression at a proteome-wide scale (**Aim 1&2**). In traditional 'reactive' testing, we must begin with a patient-derived sample. Several independent models must be created to study the underlying molecular drivers, and the development of effective therapy for the driver may not be efficient enough to benefit the initial patient. Furthermore, cancer heterogeneity severely limits this approach in proposing alternative strategies to treat each unique case. In the current era of personalized medicine, where we have a vast amount of genomic, transcriptomic, and proteomic data, we can find novel treatment strategies through functional genomics rather than relying on patient-derived information. Our proposal will contribute to the new 'proactive' testing by providing a proteome-scale gene overexpression effect map, as well as novel drug targets that can circumvent resistance (**Aim 1&2**). Thus, when a (rare) overexpressed biomarker is discovered in a patient, we can immediately interpret the biomarker to predict the expected outcomes for particular drugs. As a further study, we will perform a small-molecule screen to find novel drugs that target our resistance gene candidates which broadens treatment options and allows clinicians to avoid providing the patient with an ineffective therapy.

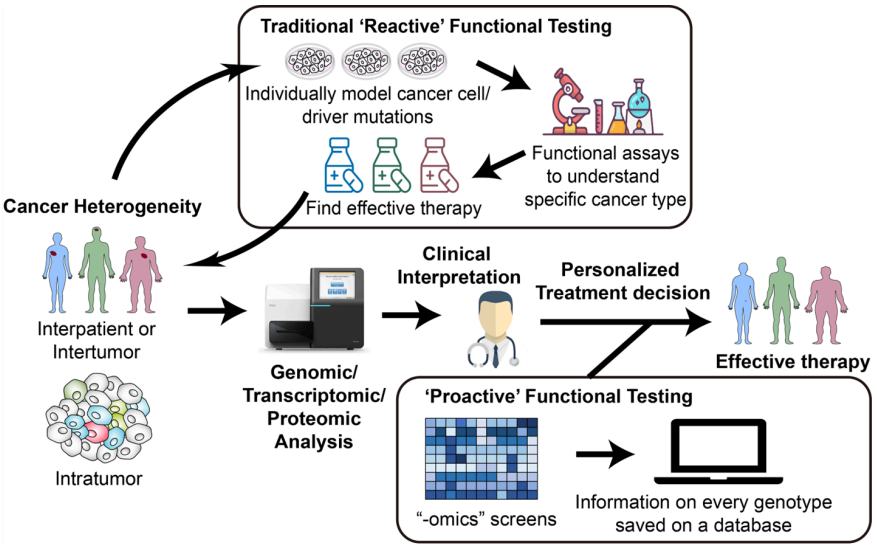


Fig. 6: Summary of expected outcome to interpret patient data in a proactive manner.

B. Wide application scope of this method to tackle late-stage lung cancer treatment

Here in this application, due to the budget and the one-year timeline, we focused on the resistance against cisplatin as a first proof-of-concept. However, our method can be used to tackle various drugs in further studies like carboplatin, KRAS G12C inhibitors, immunotherapy, etc. Especially, since the applicant (Dr. Dae-Kyum Kim) is an early career researcher who just started his lab 3 years ago, support for Lung Cancer Canada can be super beneficial to further expand this research. With this support, we are planning to also apply this method to study different aspects of late-stage lung cancer, such as autophagy, metastasis, transcription factor-promoter interaction, etc. which we already have a good amount of preliminary data with the HeLa cell line model.

4. A public, non-scientific summary

Although drug resistance mechanisms are regulated largely by ectopic overexpression of genes, our knowledge of the phenotypic outcomes of overexpressed genes, especially under various anticancer drugs, remains largely incomplete. To efficiently evaluate gene overexpression effects in late-stage lung cancer including drug-specific effects, we propose to apply a new strategy to cancer cell line models, by integrating a comprehensive human gene collection with an innovative gene overexpression system. We expect our screening approach will lead to the discovery of novel therapies and prognostic biomarkers for late-stage lung cancer patients with rare gene overexpression to overcome resistance using gene overexpression screening.

Appendix for items 1 to 4: List of References

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5. Budget (total of 25K CAD)

A. Personel: 14K CAD

Kyeong Beom Jo, M.Sc. (PhD candidate, 50% of his time will be devoted to this project). Mr. Jo will coordinate the daily management of the study, mainly focused on the experimental procedures while being trained in computational analysis under the supervision of Dr. Kim.

B. Supplies: 5K CAD

Based on experience with related large-scale genomics efforts, consumable costs are estimated at about $5,000. This is a bit higher than standard molecular genetics lab work, as it includes items like plasticware for large-scale robotic arraying (e.g. $1,000 to thaw and copy one full ORFeome collection) and extensive PCR to prepare next-generation sequencing libraries ($100 use of Q5 enzyme to prepare each of the samples). In addition, we will order drugs, lab reagents, disposable tissue culture ware, media, and related supplies.

C. Other expenses: 6K CAD

Next-generation sequencing will be performed through Genome Quebec. It is anticipated that $4,000 will be required to support sequencing. We estimated that we need at least 20 million reads to detect 20 thousand protein-coding genes we will cover with 1,000x coverage, which cost us 400 CAD. Considering that we will do several replicates (five replicates for each control and drug), we need **4K CAD in total**.

Cell sorting will be performed through the Flow & Image Cytometry Shared Resource at the Research Institute of McGill University Health Centre. It is anticipated that about $2,000 will be required to support cell sorting. Mainly, we will use the core facility to bulk sort cells with successful gene overexpression from a pool of cells after transfection. We will use the Sony MA900 machine, which will cost us about $50 per hour. We aim to sort and collect a total of 20 million cells, which is 20,000 ORFs represented 1,000 times each. Assuming that integration efficiency is about 10%, we need to sort 200 million cells, which will cost us about 40 hours of sorting with a regular speed of 3,000 events per second, accounting for three replicates for each drug and cell line, and troubleshooting. Therefore, we estimated that we would need **2K CAD in total**.

6. The names of the investigators

Dae-Kyum Kim, PhD.

Assistant Professor, Division of Thoracic and Upper Gastrointestinal Surgery, Department of Surgery, Faculty of Medicine and Health Sciences, McGill University

Junior Scientist, Cancer Research Program, Research Institute of McGill University Health Centre

Aune Foundation Endowed Chair in Translational Oncology, Montreal General Hospital Foundation, McGill University Health Centre

Address: 1001 Decarie Blvd, Room # E02.6244 (RI-MUHC Glen site, Cancer Research Program), Montreal, Quebec H4A 3J1, Canada

E-mail: dae.k.kim@mcgill.ca

Phone: +1 (514) 412-4400 ext. 76319

Web site: https://kimlab.us

Centre universitaire de santé McGill
Institut de recherche

McGill University Health Centre
Research Institute

Rhian Touyz
MBBCh MSc(Med) PhD FRCP FRSE FMedSci FCAHS

Directrice exécutive et scientifique en chef
Institut de recherche du Centre universitaire de santé McGill
Chaire Dr Phil Gold en médecine
Université McGill

Executive Director and Chief Scientific Officer
Research Institute of the McGill University Health Centre
Dr. Phil Gold Chair in Medicine
McGill University

E S1.5066.6 - 1001 boul. Décarie
Montréal QC H4A 3J1
CANADA
Tél 514 934-1934, 71608
rhian.touyz@mcgill.ca
ircusm.ca rimuhc.ca

January 29, 2025

Re: Statement of Support for Dr. Dae-Kyum Kim's Application to Lung Cancer Canada

Dear Review Committee of the Lung Cancer Canada's Give A Breath Research Award,

I am pleased to provide this Letter of Support for Dr. Dae-Kyum Kim's application to the Lung Cancer Canada's Give A Breath Research Award.

As requested, I confirm that Dr. Kim's first Faculty appointment at McGill University and as a Junior Scientist at the Research Institute of the McGill University Health Centre (The Institute) in Canada began on September 1, 2024. Here, in his application, he proposes to discover combinatorial therapeutic options for advanced lung cancer in Canada. As a new researcher here, Dr. Kim received a generous start-up package from the Montreal General Hospital Foundation Thoracic Surgery Fund of $1,350,000 over five years: $1,000,000 for lab operation (200,000 per year for five years of 2024-2029) and $350,000 for equipment.

In addition, Dr. Dae Kyum Kim's laboratory is located at the Research Institute of the McGill University Health Centre (The Institute), one of the top research institutes in Canada. The RI-MUHC is a modern facility fully integrated into the MUHC hospital aiming to facilitate investigator-initiated and discovery-driven research and speed up biomedical, patient-oriented, and applied clinical research. The Institute's environment is structured into 8 thematic programs and Dr. Kim is affiliated with the Cancer Research Program (CRP), which provides a highly collaborative research environment and envisions transforming existing medical practices through precision oncology holistic approaches.

a. Dr. Kim has assigned office (27 sq. ft) and laboratory space (200 sq. ft) within the CRP area at the Centre for Translation Biology (CTB), an open-space wet lab facility, located at the Glen site. Dr. Kim's assigned space could accommodate up to 6 people and is strategically located next to CRP teams. His laboratory space includes 2 workbenches which each can accommodate four individual desks or experimental benches (18 ft. each), a shared chemical hood, and an equipment zone for fridges, freezers and liquid nitrogen tanks.

b. Dr. Kim's team has unrestricted access to a shared tissue culture room (190 sq. ft) fully equipped with 4 certified biological safety cabinets, CO2 incubators, inverted microscopes, and centrifuges. Dr. Kim's specific equipment includes an automated robot for handling his comprehensive clone collection for yeast and human protein-coding genes, acquired with his generous start-up fund. Dr. Kim's team has also access to common shared areas housing all equipment required to conduct the proposed project including -80°C freezers, centrifuges, ultracentrifuges, plate readers, shakers, cold rooms, gel imaging systems, qPCR, cell counters, etc.

c. As a member of the Institute, Dr. Kim has unrestricted access to innovative technologies and high-quality services through the Technologies Platforms located at the

Glen site including the Histopathology, Immunophenotyping, Proteomics, Molecular Imaging, and Bioinformatics platforms. Each provides privileged access to recent technologies and instrumentation, top-level scientific expertise, and training support to conduct experimentation for a competitive price.

d. The Institute administrative centre offers full support to Dr. Kim's team such as Human Resources, Research Grant and Data Management, Finance, Business Development and Contract Services, Environmental Health and Safety, Communication as well as Infrastructures of IT and Technical Support. Students and staff have access to shared student rooms, and computers are linked to the McGill University network. Through the CRP support team, Dr. Kim has access to personalized day-to-day administrative, technical, and scientific support. The CRP assists scientists with grant applications, facilitates new partnerships, acts as a liaison between scientists and other instances, and organizes scientific events such as seminars, networking events, and annual research day.

We believe that this support from our Institute can enable his proposal to be feasible and further continue after your foundation's 1-year support.

Sincerely,

Rhian M Touyz MB ChB, M. Sc. (Méd), Ph.D., FRCP, FRSE, FMedSci, FCAHS
Executive Director and Chief Scientific Officer
Research Institute of the McGill University Health Centre (RI-MUHC)