The Grant Review Committee
Give A Breath Research Award
Lung Cancer Canada

Dear members of the committee,

I am an Assistant Professor in the Department of Anatomy, Physiology, and Pharmacology at the University of Saskatchewan (U of S). I received my basic training in pharmacology (undergraduate and master's degrees), cancer biology (PhD) and neurobiology (postdoctoral research). My research investigates the mechanism of nerve-dependent metastasis to develop effective prognostic indicators and therapeutic targets for cancer. Specifically, my lab focuses on the role of parasympathetic neurosignaling in brain metastasis. Thus, lung cancer research is a new priority initiative in my laboratory, especially since lung cancer has a high metastatic tropism to the brain. Patients who develop lung cancer brain metastasis have a low survival rate (< 3 years), and hence, preventive measures should be initiated for high-risk patients at the time of initial diagnosis of the cancer. Unfortunately, effective markers to predict the risk of brain metastasis in lung cancer patients are unavailable. My proposed research for the 'Give A Breath Research Award' addresses this issue and aims to identify a molecular marker to predict the risk of lung cancer brain metastasis. This proposal will also evaluate the potential of therapeutically targeting the muscarinic receptor CHRM1 in suppressing the metastatic abilities of lung cancer cells. Overall, the long-term goal of this research is to improve the quality-of-life of patients affected with advanced stages of lung cancer by identifying ways to prevent the metastatic spread of the disease.

The research proposal and the supporting documents are attached along with this application. I hope the award selection committee will consider this application and support our team's fight against lung cancer.

Sincerely,

Anand Krishnan, MPharm, PhD
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Non-scientific summary

Lung cancer is the leading cause of cancer-related death in men and women in Canada and worldwide. The spreading of lung cancer to the brain [lung cancer brain metastasis (**LCBM**)] contributes to the increased death rate associated with this disease. However, LCBM can be tackled by taking appropriate preventive measures in advance if there are reliable markers available to predict the risk of LCBM at primary disease diagnosis. Unfortunately, there are no markers available to predict the risk of LCBM. We recently found that the receptor CHRM1 is upregulated in lung tumors of patients who did develop LCBM compared to those who did not, suggesting that CHRM1 may be a potential marker for LCBM. Therefore, this research will explore the marker potential of CHRM1 in predicting the risk of LCBM using a large set of human lung cancer samples. Standard immunohistochemical staining, as routinely performed in clinical pathology labs, will be employed to evaluate the marker potential of CHRM1 in LCBM. We will also evaluate the effect of therapeutically targeting CHRM1 in suppressing the metastatic abilities of lung cancer cells using a tumor organoid model. Overall, upon successfully completing this work, we hope to identify a novel marker for predicting the risk of LCBM. Through this work, we also expect to reveal a potential therapeutic target for preventing LCBM. Our long-term goal is to improve the survival and quality-of-life of lung cancer patients affected with advanced stages of the disease.

1.0 BACKGROUND AND PRELIMINARY DATA: Lung cancer **(LC)** is the leading cause of cancer-related death in men and women worldwide[1,2]. The spreading of LC to the brain [**Lung cancer brain metastasis - LCBM**] significantly contributes to LC-related mortality. About 50% of LC patients develop LCBM, and unfortunately, the average survival time of patients affected with LCBM is less than three years[3]. Yet, effective therapies are unavailable, partly due to the inability of drugs to cross the blood-brain barrier and partly due to the limited knowledge of effective therapeutic targets[4,5]. However, predicting the risk of LCBM in advance can better equip the patients with preventive measures. Unfortunately, there is no reliable method available to predict the risk of LCBM. Thus, identifying an effective marker to predict the risk of LCBM is in high demand. Similarly, identifying molecular targets that can be therapeutically exploited to prevent LC dissemination to other organs, including the brain, is also in high demand.

The primary research in my lab investigates the mechanism of nerve-dependent metastasis to identify effective prognostic indicators and therapeutic targets for cancer. Previous pre-clinical studies showed that the cholinergic receptor muscarinic (**CHRM**) promotes LC progression and metastasis[6-10]. CHRMs are specific receptors for the parasympathetic neurotransmitter Acetylcholine. Among the different CHRMs, the **CHRM1 and CHRM3** were shown to contribute to several cancers [11]. Given the potential roles of CHRMs in LC metastasis, we, for the first time, examined the expression of CHRM1 and CHRM3 in primary non-small cell lung carcinoma (NSCLC) samples from patients who did develop and did not develop LCBM *[24 samples (12 males and 12 females)/group]*. **Our preliminary data showed that the expression of CHRM1 was significantly increased in primary tumors of patients who did develop LCBM (Met group) compared to those who did not (NM group) (Figure 1-** *this data is presented at the Saskatchewan Cancer Research Conference*)**.** Although a trend for CHRM3 upregulation was seen in the Met group, it was not statistically significant. We then grouped the Met samples into the low-stage (< stage III) and high-stage (≥ stage III) tumors and reanalyzed the data. The CHRM1 expression was significantly high in ≥ stage III tumors compared to the low stages, substantiating its potential to serve as a true marker for LC progression. In contrast, CHRM3 expression showed no significant change between the low and high-stage tumors. Further, we reanalyzed the CHRM1expression data in Met and NM samples from male and female patients and found a significant increase in its expression in the Met samples of both male and female patients, further signifying its potential to serve as a true predictive marker for LCBM (**Figure 1**).

2.0 HYPOTHESIS AND OBJECTIVES: Based on our preliminary data showing the consistent upregulation of CHRM1 in patients who developed LCBM, I hypothesize that CHRM1 will serve as a predictive marker for LCBM. Therefore, **I propose to examine the expression of CHRM1 in 200 human NSCLC samples representing the NM and Met groups (100 samples/group) using immunohistochemistry (IHC) to systematically evaluate its performance as a predictive marker for LCBM.** Although our preliminary data showed increased expression of CHRM1 in Met samples, the functional implications of its upregulation, like whether it is a promoter or suppressor of LCBM, is unknown. Therefore, **we will also evaluate the effect of pharmacological interventions of CHRM1 on the metastatic abilities of LC cells using a lung tumor organoid model. Overall, this is novel research and a new initiative from my lab.**

Objectives

- *Objective 1: Evaluate the expression of CHRM1 in 200 NSCLC samples using IHC staining and assess its marker potential in predicting the risk of LCBM.*
- *Objective 2: Evaluate the effect of pharmacological interventions of CHRM1 in suppressing the metastatic potential of LC cells using a tumor organoid model.*

Rationale for the IHC analysis: Interpretation of IHC staining by a qualified anatomical pathologist is a standard clinical practice for molecular staging of cancers. While our western blot data shows that CHRM1 expression is high in Met samples, it is critical to ensure that such results can be captured in IHC staining

for its application in the clinics to predict the risk of LCBM. We considered a total sample size of 100 per group *(50 males and 50 females for biological sex-specific analysis*) to arrive at stronger conclusions with appropriate statistical strength.

Rationale for the tumor organoid model: Metastasis involves complex cellular events wherein cancer cells initially undergo epithelial-mesenchymal transition (**EMT**) to become extra motile, enter the vasculature (intravasation), survive in the vasculature, and extravasate (exit the vessels) and, finally, form colonies in the secondary site[12]. While no available experimental models capture all these events, a tumor organoid model can efficiently capture EMT and intravasation of cancer cells, the two critical initial events for metastasis, within a natural tumor microenvironment[13,14]. It is thus considered as the best available model.

3.0 EXPERIMENTAL PLAN: We already purchased **60** human NSCLC samples from the Alberta Cancer Research Biobank, with 30 samples *(15 males/females)* each representing NM and Met groups. We will purchase an additional 70 NSCLC samples *(35 males/females)* for each group ***(140 additional samples)*** and profile the expression of CHRM1 in a total of **200 samples** using IHC staining. A postdoctoral fellow, Dr. Hanrong, joining my lab in March 2025, will perform the IHC staining. My collaborator, Dr. Hui Wang, an anatomical pathologist in the Saskatchewan Health Authority and USask, will blindly score the IHC slides *(collaboration letter attached)*. The IHC scoring *(mild, moderate, and intense staining and % positive cells)* will be tabulated against NM and Met cases. Further, ROC curve analysis will be performed to assess the strength of the model, and Youden's Index will be calculated to define a threshold value for IHC-based CHRM1 expression in predicting the risk of LCBM[15]. Independent *(males and females separate)* and combined analysis will be done to identify the influence of biological sex on the association between CHRM1 and LCBM.

Hanrong will perform the tumor organoid experiments. She recently filed a patent application for her newly developed vascularized tumor organoid model, testifying to her ability to perform the proposed experiment *(https://d.wanfangdata.com.cn/patent/CN202410964815.5)*. LC cell lines derived from male *(NCI-H1770)* and female *(NCI-H2009)* patients, representing stage IV cancer, will be used for the organoid model. We will examine the effect of CHRM1 agonist *(Xanomeline)* and antagonist *(Pirenzepine)* in EMT transition and intravasation of LC cells in the organoid model. EMT characteristics will be evaluated by examining the expression of EMT markers *(E-Cadherin, slug, snail, twist)* in cancer cells using immunostaining and western blotting. Intravasation of cells will be visualized by live cell imaging of red fluorescent protein-labelled capillaries and green fluorescent protein-labelled cancer cells in the organoid cultures using the spinning disk confocal microscope available in our department.

4.0 POTENTIAL PITFALLS AND MITIGATION STRATEGIES: Tumor is a heterogeneous tissue. Hence, the fractions of the tumors we collected from the tumor bank may not represent the exact molecular pathology ***(please note that this is not true for all samples).*** However, this is a general caveat for human tissue studies. We considered 100 samples/group *(50 males and 50 females),* which falls within the sample size range recommended for biomarker studies, to arrive at statistically powerful conclusions.

5.0 ALIGNMENT OF THE PROPOSAL WITH THE SCOPE OF THE COMPETITION: The proposed research addresses an unmet need to identify risk predictors and therapeutic targets for advanced and metastatic lung cancers forming LCBM. The competition seeks applications focusing on advanced or metastatic lung cancer, and thus, this proposal is a best fit for this competition. Additionally, I propose a clinically translational approach (IHC technique) for CHRM1-based molecular characterization of NSCLC and risk prediction of LCBM. As such, this proposal has direct practical relevance to Canadians with advanced lung cancer, which is another priority of this competition.

6.0 FINANCIAL REQUIREMENT: Although I have active funding from the BCSC (for breast cancer brain metastasis studies) and NSERC (for nerve regeneration studies), they are not dedicated to lung cancer research. The preliminary data for this proposal was generated from my start-up fund, and I will be able to cover part of the expenses for this work from my CoMBRIDGE fund. However, without the kind support from 'Give a Breath Research Award', this new research in my lab will not progress. I hope the committee will see the merit of the proposal and support this new initiative in my young lab.

Appendix I

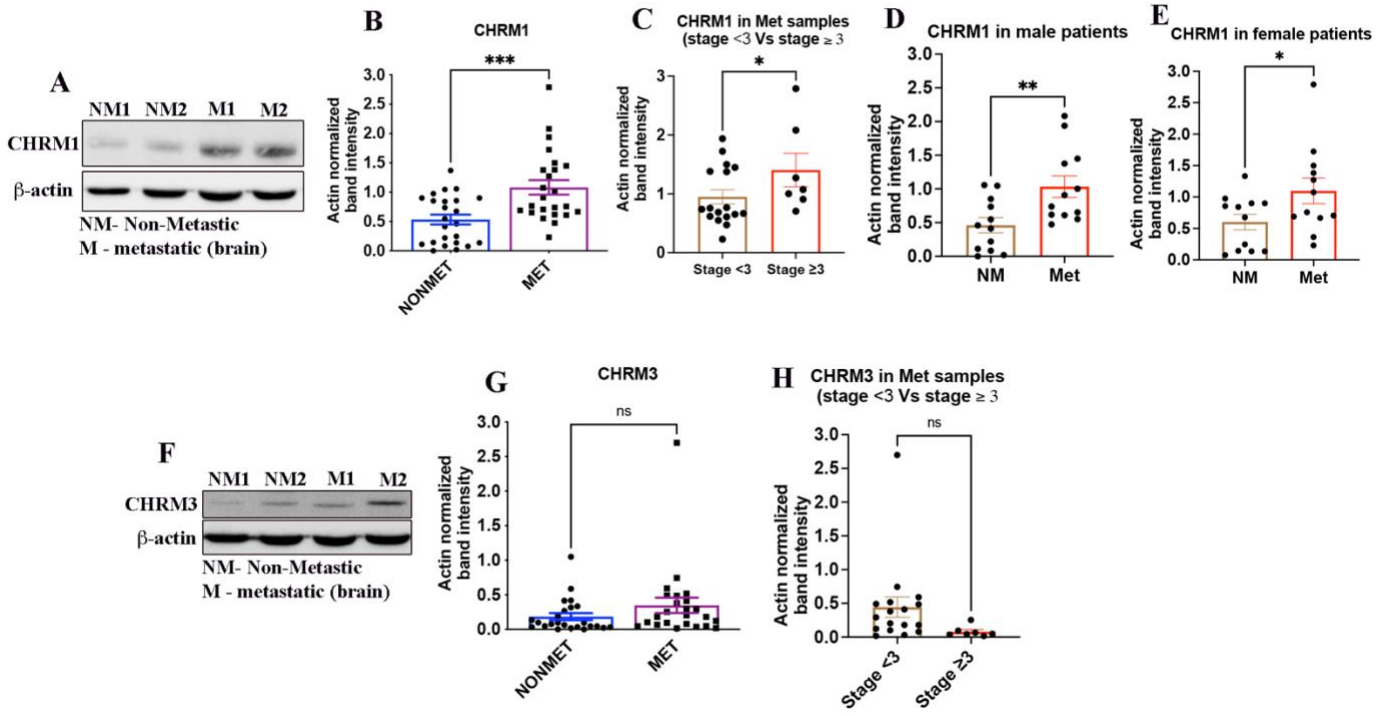

Figure 1: (A) *Western blot shows the expression of CHRM1 in primary non-metastatic (NM) and metastatic (M) NSCLC samples. (**B**) Quantification of CHRM1 band intensities in non-metastatic (NONMET) and metastatic (MET) samples shows significant upregulation of CHRM1 in the metastatic group [n=24 (12 males and 12 females)/group]. (**C**) Quantification of CHRM1 band intensities in <stage 3 Vs ≥ stage 3 subgroups of the metastatic cases shows significant upregulation of CHRM1 in ≥ stage 3 samples (n=17<stage 3, n= 7 ≥ stage 3). (**D**) Quantification of CHRM1 band intensities in non-metastatic (NONMET) and metastatic (MET) samples from male patients shows significant upregulation of CHRM1 in the metastatic group ((n=12/group). (**E**) Quantification of CHRM1 band intensities in non-metastatic (NONMET) and metastatic (MET) samples from female patients shows significant upregulation of CHRM1 in the metastatic group ((n=12/group). **(F)** Western blot shows the expression of CHRM3 in primary non-metastatic (NM) and metastatic (M) NSCLC samples. (**G**) Quantification of CHRM3 band intensities in non-metastatic (NONMET) and metastatic (MET) samples shows no significant change in CHRM3 expression [n=24 (12 males and 12 females /group]. (**H**) Quantification of CHRM3 band intensities in <stage 3 Vs ≥ stage 3 subgroups of the metastatic cases shows no significant change in CHRM3 expression (n=17<stage 3, n= 7 ≥ stage 3). Data presented as mean± SE; Standard 't' test; *p<0.05; **p<0.01; ***p<0.001.*

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IMPACT STATEMENT

Patients affected with **lung cancer brain metastasis (LCBM**) have an average survival time of less than three years due to the lack of effective treatments. Therefore, **prevention is the key to tackling LCBM**. Patients at high risk of LCBM should be identified earlier so that preventive measures can be taken in advance. For example, prophylactic cranial irradiation is a preventive measure for patients who are at high risk of brain metastasis. Unfortunately, there are no molecular markers available to reliably predict the risk of LCBM. In this study, we are hopeful to identify a reliable marker to predict the risk of LCBM. This would eventually ensure early identification of patients at high risk of LCBM for timely preventive measures. Such measures will improve the survival and quality-of-life of patients and reduce the clinical and socioeconomic burden associated with managing lung cancers. Therefore, by identifying a novel predictive marker for LCBM, our work can positively impact lung cancer management, reduce mortality, and improve patients' quality of life.

This research will also evaluate the potential of therapeutically targeting the muscarinic receptor CHRM1 in suppressing the metastatic capabilities of lung cancer cells in a tumor organoid model. If the proposed experiments are successful in the organoid model, the immediate future step will be to evaluate the therapeutic target potential of CHRM1 in LCBM in animal models. My clinical collaborator at the Saskatchewan Cancer Agency (Dr. Shahid Ahmed) will help us initiate clinical studies in the province if the follow-up animal studies are also successful. The long-term goal of this second objective is to develop an effective preventive therapy to tackle LCBM and, thus, improve the survival and quality-of-life of lung cancer patients. Please note that CHRM1-based therapy for LCBM is a novel approach and is thus considered well beyond the first-line therapy for lung cancer. Therefore, our research aligns well with the 'scope' of this research award, which states that *'work that will explore research initiatives beyond first-line therapy'* will be supported.

Please also note that we will publish the results in high-impact journals and present the data to broader audiences, including researchers, clinicians, the public, and other stakeholders. We will acknowledge the support of 'Give A Breath Research Award' and 'Lung Cancer Canada' in our publications and other correspondence to bring additional visibility to this prestigious funding program.

BUDGET REQUEST

1.0 RESEARCH PERSONNEL SALARY

Postdoctoral fellow *(Dr. Hanrong Li: 0.25 salary for one year)*
Hanrong is an MD PhD, joining my lab in March 2025. She has done her PhD in cancer research and has excellent expertise in IHC technique, cell culture, and tumor organoid model. Hanrong will perform the IHC staining and tumor organoid experiment as proposed in the research proposal.

- *I request 0.25 salary for Hanrong for one year:* ***$ 15, 000*** *(Hanrong's rest of the salary will be covered from my CoMBRIDGE fund).*

2.0 REAGENT COST

The cost of reagents for IHC staining and tumor organoid model: CHRM1 primary antibody, xanomeline, pirenzepine, IHC development kit, xylene, ethyl alcohol, antigen retrieval buffer, DPX mountant, slides, coverslips, and cell culture reagents.
This cost (~$5000) will be covered from my CoMBRIDGE fund.

3.0 COST OF TUMOR TISSUES

140 tumor samples will be purchased from the Alberta Cancer Research Biobank ($75/tumor sample and associated de-identified clinical information- *please see the previous invoice attached)*.

Cost required for purchasing the tumor samples: $10,500

- *Cost requested for purchasing the tumor samples:* ***$10,000*** *(additional $500 will be covered from my CoMBRIDGE fund)*

TOTAL BUDGET REQUESTED: $25,000

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January 22nd, 2025

Lung Cancer Canada

133 Richmond St. W., Suite 208
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Dear Members of the Give a Breath Research Award Committee,

I am writing to provide my full support for Dr. Anand Krishnan's application for the **Give a Breath Research Award**. Dr. Krishnan is a dedicated member of the research faculty at the University of Saskatchewan, where they lead impactful research in basic and translational science.

The proposed study, *"Prognostic and therapeutic exploitation of muscarinic signaling in lung cancer brain metastasis"* is both innovative and feasible within our institution. The University of Saskatchewan is equipped with state-of-the-art laboratory facilities, advanced infrastructure for basic and translational research, and access to specialized equipment required to support this project. Additionally, Dr. Krishnan benefits from a collaborative research environment and institutional resources that will ensure the successful execution of this study.

We are confident in Dr. Krishnan's ability to carry out this important work and are committed to providing the necessary support to facilitate the proposed research.

Thank you for considering Dr. Krishnan's application for this prestigious award.

Sincerely,

Marek Radomski

Dr. Marek Radomski
Vice Dean Research
College of Medicine

Saskatchewan Health Authority

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Saskatoon City Hospital
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To
The Grant Review Committee
Lung Cancer Canada

Re: Collaboration with Dr. Krishnan's research group for lung cancer research

I am pleased to collaborate with Dr. Krishnan's research on 'prognostic and therapeutic exploitation of muscarinic signaling in lung cancer brain metastasis' and support this research proposal for the Give a Breath Research Award. I am particularly interested in providing my expertise to score the IHC slides and evaluate the performance of CHRM1 as a marker for lung cancer brain metastasis. I have 3.5 years of experience as an anatomical pathologist and I regularly evaluate lung cancer specimens as part of my professional commitment.

The proposed research has high clinical relevance, and I am very excited to be part of this research.

I wish Dr. Krishnan the very best with this application.

Sincerely

Dr. Hui Wang MD, PhD, FRCPC
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