Chi3l1 Regulation of Checkpoint Regulating, Costimulatory, and Coinhibitory Molecules in Primary and Metastatic Lung Cancer

Chitinase 3-like 1 (Chi3l1; also called BRP-39 in the mouse and YKL-40 in man), the prototypic CLP, is expressed in an exaggerated fashion in a variety of diseases characterized by inflammation, injury and remodeling. The levels of Chi3l1 are also increased in the serum and/or tissues from patients with a variety of cancers including lung cancer and their levels are inversely correlated with prognosis of the patients. However, the role(s) of Chi3l1 in the pathogenesis of lung cancer have not been defined. To begin to address this issue, we generated Chi3l1 null mutant (Chi3l1−/−) and lung-specific YKL-40 overexpressing (OE) transgenic (YKL40 Tg) mice and evaluated them in models of metastatic and primary lung cancer.

Preliminary studies using animal models of primary and metastatic lung cancer and Chi3l1 neutralizing antibody demonstrated that Chi3l1 plays an essential role in the regulation of immune checkpoint inhibitors (ICPs) and T cell co-stimulatory molecules. In the first year of grant period, we made a substantial progress in the following areas: 1) Successful generation and preclinical application of effective neutralizing antibodies against Chi3l1 that demonstrated an impressive inhibitory effect on melanoma lung metastasis as well as the primary lung cancer. 2) Effect of Chi3l1 in tumor infiltrating macrophages and CD8 T cells in metastatic and primary lung cancer and its regulation of immune checkpoint inhibitor molecules, such as PD-1 and PD-L1, expression.
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I. Introduction
Recent studies demonstrated that the levels of circulating Chi3l1/YKL-40 are increased in many malignancies including cancers of the prostate, colon, rectum, ovary, kidney, breast, glioblastomas and malignant melanoma (1-13). In these diseases, the levels of YKL-40 frequently correlate directly with disease progression and inversely with disease-free interval and survival (1-13). This is particularly striking in lung cancer where the serum and tissue levels of YKL-40 are impressively increased and correlate with adverse outcomes (14-17). However, the mechanisms of these inductive responses and the roles of Chi3l1/YKL-40 in disease initiation and progression have not been adequately defined. In addition, the utility of serum Chi3l1 assessments as biomarkers for these diseases and the value of interventions that alter Chi3l1 production and or effector responses as cancer therapeutics have not been appropriately addressed. To address these deficiencies we have evaluated the roles of Chi3l1 in primary and metastatic lung cancer. Our preliminary studies demonstrate that (1) Chi3l1/YKL-40 is expressed in an exaggerated manner in human lung cancer where it correlates inversely with survival but does not correlate with the mutations that drive these malignancies; (2) in murine models, Chi3l1 is sequentially induced in normal peritumor and tumor tissues during the early and later stages, respectively, of lung cancer development; (3) Chi3l1 induction via a semaphorin 7a-dependent mechanism plays a critical role in the generation of a metastasis permissive pulmonary microenvironment; (4) in metastatic models, Chi3l1 production and metastatic spread can be inhibited via RIG-like helicase (RLH) innate immunity (18). These findings led us to hypothesize that Chi3l1 plays a major role in the pathogenesis of primary and metastatic lung cancer by augmenting immune checkpoint inhibitors (ICPI; PD-1/PD-L1/L2 and CTLA4) and altering T cell co-stimulation. To address this hypothesis, following specific Aims has been established in this grant proposal:

**Aim #1.** Define the expression and roles of Chi3l1 in the development, growth and metastatic progression of primary lung cancer in vivo.

**Aim #2.** Characterize the mechanism of Chi3l1 induction in primary and metastatic lung cancer and the regulation of these pathways by RLH innate immune activation and the RLH inhibitor NLRX1.

**Aim #3.** Characterize the roles of Chi3l1 in the induction/regulation of ICPI and their ligands and T cell co-stimulators in murine primary and metastatic lung cancer and the mechanisms of these regulatory events.

**Aim #4.** Determine if the levels of circulating or tissue Chi3l1 correlate with patient responses to immune checkpoint-based therapeutic interventions (ICPTI) and the relationships between the expression of Chi3l1 and molecules associated with Chi3l1-induced immunosuppression.

II. Keywords

III. Major Accomplishments in First Year of Grant Support
Overall, we made significant progress in many areas of the proposed studies during the first year of grant support. Followings are the description on the major achievements based on the proposed Tasks and Specific Aims:

1. The role and effect of Chi3l1 in tumor lung metastasis and primary lung cancer (Major Task 1, Specific Aim 1). Chi3l1 null mutant (Chi3l1<sup>−/−</sup>) and lung-specific overexpressing YKL-40 Tg mice were used to determine the role and effect of Chi3l1, respectively. After we got ACURO approval, we initiated the mice experiment to determine the expression, role and effect of Chi3l1/YKL-40 in melanoma lung metastasis using wild type and YKL-40 Tg mice. The melanoma cells were prepared as we described previously (19), and B16/F10 melanoma cells (2x10<sup>5</sup> cells/mouse) were delivered to WT and YKL-40 Tg mice via tail vein injection as schematically illustrated in Fig. 1A and evaluated the melanoma colony formation in the lung. With melanoma cell injection, the level of Chi3l1 in the mouse serum were increased (Fig. 1B). In the previous studies, we have reported that melanoma lung metastasis was significantly diminished in the lungs of Chi3l1 null mice compared to wild type mice (19). On the
other hand, overexpression of YKL-40 in the lung of YKL-40 Tg mice significantly enhanced melanoma colonization in the lung compared to WT animals (Fig. 1C). When viewed in combination, these studies demonstrated that Chi3l1/YKL-40 plays an essential role in the melanoma lung metastasis and the induction of Chi3l1 enhances the metastatic lung cancer. The expression of Chi3l1 in the primary lung tumors are also increased mainly in the macrophages and other inflammatory cells surrounding tumor, but not in tumor cells, at early stage (<12 weeks of tumor induct) of tumor development, but advanced stage of tumor development (>20 weeks of tumor induction) tumor cells itself also express increased levels of Chi3l1 (Fig. 2). Similarly to tumor cells, at early stage (<12 weeks of tumor induct) of tumor development, Chi3l1/YKL-40 in primary lung cancer developed in KP mice with YKL-40 Tg mice. However, we did not note with null mutation of Chi3l1 but enhanced with Chi3l1/YKL40 overexpression metastatic lung cancer, primary tumor development is significantly reduced with null mutation of Chi3l1 but enhanced with Chi3l1/YKL40 overexpression (data not shown). We evaluated the primary tumor metastasis to liver, brain, and liver to see potential impact of Chi3l1/YKL-40 in primary lung cancer developed in KP mice with YKL-40 Tg mice. However, we did not note any tumors in other organs outside lung in KP mice even with YKL-40 transgenic overexpression. These results suggest that Chi3l1/YKL-40 may not significantly impact on primary lung tumor metastasis in KP mice. However, we do not exclude the limitation of KP mice in the evaluation of tumor metastasis due to tumors developed in KP or KPL mice may not be appropriate to evaluate its metastatic potential to other organs due to either intrinsic inability of metastasis or early death before reaching distant metastasis. These issues of KP or KPL mice in primary tumor metastasis evaluation has been discussed below in the section of Changes/Problems.

2. Effect of Chi3l1 intervention using neutralizing antibody (Major Task 2, Specific Aim 1). We have successfully developed anti-Chi3l1 monoclonal antibody (called FRG antibody) that effectively block Chi3l1-stimulated MAPK/Erk and Akt activation (data not shown). In present studies, we tested the effectiveness of antibody in vivo first in melanoma lung metastasis model. As shown in Fig 3, the antibody treatment impressively reduced melanoma lung metastasis in a dose and time dependent manners. FRG antibody treatment (i.p, every other day for 13 weeks) also significantly reduced the primary tumor development in KP mice as shown in the Fig. 4. These studies demonstrated that neutralizing Chi3l1 by FRG antibody effectively reduced both in melanoma lung metastasis and primary tumor development or progression. These results strongly suggest that FRG has a substantial therapeutic effect, since antibody treatment after established tumor cells in the lung also significantly reduced the number of melanoma colonization in the lung (Fig. 4 B). Importantly, the degree of FRG inhibition of tumor development is very comparable to anti-PD-1 treatment and it showed synergistic effect when we use both antibodies together (Fig. 5), suggesting that FRG antibody complement or enhance the therapeutic

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**Fig 2.** The kinetic changes Chi3l1 expression in primary lung cancer developed in KP mice. Left panel, IHC staining on the lungs of KP mice with 10 weeks after Cre induction. Right panel, IHC on 22 weeks of tumor induction.

**Fig 3.** The anti-Chi3l1 antibody (monoclonal anti-human Chi3l1 antibody called FRG) effectively blocks melanoma lung metastasis. A. high (200ug/mouse) and low (50ug/mouse) FRG antibody was delivered via I.P injection, to melanoma challenged mice, every other day until sacrifice as indicated. Right panel, melanoma colony counts in the lung of mice with IgG control and FRG antibody. B. FRG treatment before and after melanoma cell challenge to see preventive as well as therapeutic effect anti-Chi3l1 intervention. FRG antibody was delivered via I.P injection, to melanoma challenged mice, every other day until sacrifice as indicated. Right panel, melanoma colony counts in the lung of these mice depending on the time point of FRG treatment.

**Fig 4.** The anti-Chi3l1 antibody (monoclonal anti-human Chi3l1 antibody called FRG) effectively blocks primary lung cancer development/progression. A. Schematic illustration of FRG antibody treatment after adenoviral mediated Cre induction of primary lung cancer in KP mice. 8 weeks of KP mice were subjected to this evaluation. FRG antibody or isotype control (IgG2b) were given every other day for 13 weeks and mice were sacrificed and evaluated the primary tumor development. B. A representative photo of lungs treated with control IgG vs FRG antibody.
effects of anti-PD-1 treatment. These studies highlight potential therapeutic use of anti-Chi3l1 intervention in combination with immune checkpoint inhibition in the treatment of metastatic as well as primary lung cancer. These studies also suggest that developing bi-specific anti-Chi3l1/PD-1 antibody could be a better option to maximize the therapeutic effect of lung cancer. Thus, the successful development and preclinical application of anti-Chi3l1 antibody in metastatic and primary lung cancer is the most significant and impressive major achievement in the first year of grant support.

3. Role and effect of Chi3l1 in tumor infiltrating inflammatory cells with expression of immune checkpoint molecules (ICPs) (Major Task 2, Specific Aim 1; Part of Major Task 4 in Specific Aim 3). The Chi3l1 regulation of inflammatory cells and immune cells expressing immune checkpoint molecules such as PD-1, PD-L1 or CTLA4 are one of the major focus of this project. To begin to understand the role and effect of Chi3l1 in tumor infiltrating inflammatory and immune cells, we specifically evaluated the CD11b+ or CD68+ cells (targeting tumor associated macrophages) and CD8+ T cells (activated cytotoxic T cells) since these cells are reported as the most significantly implicated cells in the regulation of lung cancer development and progression in relation to the expression of ICPs (20, 21). Our studies demonstrate that Chi3l1/YKL-40 itself regulates the expression of ICPs, since PD-L1, PD-L2 and CTLA4 expression levels are significantly increased in the lungs of YKL-40 Tg mice compared to WT controls (Fig. 6). In these mice, subset of Cd11b+/PD-L1 expressing cells are decreased in the cells isolated from Chi3l1-/- mice while significantly increased in the cells from YKL-40 Tg mice compared to WT controls, further supporting a significant role and effect of Chi3l1/YKL-40 in the regulation of inflammatory cells expressing ICPs (Fig. 7A). It is also interesting to note that number of CD68+ PD-L1 expressing cells are also significantly reduced in the mice lung treated with FRG antibody compared to IgG control treated ones (Fig 7B). The immunohistochemical staining on the lungs with melanoma metastasis demonstrated that the lung of Chi3l1-/- mice showed significantly less number of CD68+/PD-L1 positive inflammatory cell infiltration compared to WT animals, further supporting that Chi3l1 contribute to the recruitment of tumor-associated macrophages expressing PD-L1 (Fig. 8A). Similarly, primary lung cancers developed in KP mice also showed increased levels of PD-L1 stained cells with YKL-40 overexpression (YKL-40Tg) compared to WT or null mutation of Chi3l1 (Chi3l1-/-) (Fig. 8B).
Overall, FACs evaluation on the B16-F10 melanoma challenged WT and Chi3l1−/− mice showed less Cd3(+)/PD-1, CTLA-4 positive T cells and Cd68+PD-L1+ macrophages while increased levels of stimulatory ICPs such as ICOS, CD80 and CD86 (+) compared to WT mice, supporting an important role of Chi3l1 in tumor infiltrating inflammatory cells expressing ICPs in these cells (Table 1). Similar to Chi3l1 null mutant mice, FRG anti-Chi3l1 antibody treatment also significantly reduced CD68+/PD-L1+ positive macrophages compared to mice treated with only control IgG (Fig. 9), suggesting a specific role of Chi3l1 in the regulation of tumor-associated macrophages expressing PD-L1. On the other hand, FRG treatment enhances CD8(+) T cell infiltration into primary tumor developed in KP mice compared to IgG control treated ones, suggesting a role of Chi3l1 in CD8 T cell infiltration into lung cancer (Fig. 10).

4. Acquisition of patient’s samples (Major Task 5, Specific Aim 4). After we got HPRO approval, we initiated sample collection from the patients from Yale and start to evaluate serum levels Chi3l1 in the patients with and without anti-PD-1 or anti-PD-L1 immunotherapy. Currently, a total of 27 serum samples form the patients at Yale New Haven Hospital Yale University and 20 samples from Rhode Island Lifespan Hospital Brown University are being collected and will be subjected to evaluation on the levels of Chi3l1 and its correlation with patient’s response to immune therapy (before and after anti-PD1 or anti-PD-L1 immunotherapy) including tumor progression, and overall survival. Thus, we will be able to have enough number of subjects to evaluate the relationship between the levels of Chi3l1 and clinical parameters with ICP intervention of lung cancer patients as proposed.

5. Automated Quantitative Analysis (AQUA) of Chi3l1 on patient’s tissue samples (Major Task 5, Specific Aim 4). An anti-goat polyclonal anti-human Chi3l1 antibody (R&D Systems) was selected and tested whether it is suitable for AQUA staining. The antibody showed reasonable staining pattern for AQUA evaluation with 1:10 to 1:100 range of dilution, since Chi3l1 staining was well located either within epithelial cells/tumors (counterstained with Pan-cytokeratin stains) or other stromal cells (Fig. 11). An archived lung cancer cohort of YTMA 250 will be subjected to the AQUA staining with Chi3l1 and Pan-cytokeratin antibodies (see Appendices for cohort Characteristics).

IV. Impact
In the first year of grant period, we demonstrated that neutralization of Chi3l1 using newly generated anti-Chi3l1 monoclonal antibody called “FRG” antibody specifically and impressively reduced the melanoma lung metastasis and primary lung cancer development in both preventive and therapeutic manners. This is a significant accomplishment for future clinical application of anti-Chi3l1 antibody for lung cancer intervention. In addition, we identified that Chi3l1 regulates tumor infiltrating inflammatory cells expressing immune checkpoint molecules (ICPs), such as PD-1 and PD-L1, that are frequently implicated in lung and other type of cancers. Interestingly, our studies identified that Chi3l1 antibody treatment showed comparable levels of tumor inhibition by PD-1 antibody treatment, and that further synergistically enhances the effect of anti-PD-1 treatment when we treat together. These studies highlighted that intervention of Chi3l1 and immune check point molecules at the same time could be better option to inhibit tumor development and progression in the lung. We also identified Chi3l1 significantly affect immune cell recruitment and infiltration to the lung, specifically macrophages as well as CD8 T cells, in metastatic and primary lung cancer, supporting Chi3l1 is one of major modulators of immune and inflammatory cell responses implicated in the pathogenesis of lung cancer.

V. Changes/ Problems
Although we achieved most of the major goals proposed in the first year of grant, we encountered unexpected delay in the generation of KP or KPL mice. As noted above, we did not note any significant primary lung cancer metastasis in organs other than lung even with YKI-40 overexpression, suggesting potential limitation in the use of KP or KPL mice for the evaluation of metastasis of primary lung tumors. These issues will be specifically addressed in the 2nd year of grant period as discussed below.

1. **Breeding problem in the generation of double mutant mice of KP and KPL mice.** Without any specific reason, we are currently experiencing difficulties in the generation of pups from KP and KPL mice. Accordingly, we are not able to generate enough number of mice after crosses between Sema7a\(^{-/-}\) and NLRX\(^{-/-}\) with KP or KPL mice and we could not complete the evaluation on the effect of poly(I:C) treatment on the tumors developed in KP or KPL mice as proposed as Major Task 3/Specific Aim2. Since we are starting to establish new KP and KPL breeding colonies by reintroduction of these mice from original source, we will be able to use these mice in 6-8 months of 2nd year of grant period to complete proposed studies. Due to this unexpected breeding issue of KP and KPL mice, in vivo siRNA silencing of Sem7a and NLRX will be considered for characterization on the role of Sema7a and NLRX1 in KP or KPL mice instead of multiple mutant breeding. In this way we are able to complete proposed studies without further delay with minimal genetic burden resulted from multiple mutant crosses.

2. **Absence of primary tumor metastasis to other organs in KP or KPL mice.** Since we did not note any distant metastasis other than lung in KP or KPL mutant mice even with crosses with YKL-40 Tg mice that significantly enhances primary lung cancer development and progression in these mice, we speculated that current KP or KPL mice may not be appropriate for the evaluation of distant tumor metastasis to other organs. Although we do not know exact underlying cause of this limitation, we speculate several possibilities of this lack of distant metastasis including: 1) With advancement of tumors, the mice are too sick with significant tumor burden, lead to early death (we noted that most of the mice with tumors in KP or KPL mice died around 14 weeks of tumor induction) before distant metastasis of tumor metastasis in other organs. 2) the tumor cells developed in the lung with Kras/P53 mutation have intrinsically low ability to metastasis to outside the lung. To overcome this limitation in the evaluation of distant metastasis of primary lung cancer developed in KP or KPL mice, we will use ectopic colonization of lung tumor cells between WT, Chi3l1\(^{+/+}\) and YKL-40 Tg mice (Cre-Rosa26(YKL-40) Tg mice, overexpress Chi3l1 in cell specific way, recently developed in our laboratory) after i.v or subcutaneous injection of primary tumor cells established from KP or KPL mice. In this way, we will be able to determine the role and effect of Chi3l1/YKL-40 in primary lung cancer cell metastasis to other organs including skin, liver, or brain as well as lung.

VI. **Products**


VII. **Participants and other collaborating organizations:**

**Participants:**
- Jack A. Elias, MD (PI, Brown University)
- Chun Geun Lee, MD, PhD (Co-Investigator, Brown University)
- Bing Ma, MD (Co-Investigator, Brown University)
- Roy Herbst, MD, PhD (Co-investigator, Yale University)
- Ja Seok Koo, PhD (Co-investigator, Yale University)
- Howard Safran, MD (Co-investigator, Brown University)
- Bedia Akosman (Graduate Student, Brown University)

VIII. **Special Reporting Requirement:** N/A
IX. References Cited


X. Appendices

Cohort Characteristics of YTMA 250:
Block YTMA 250-2

Total spots on the array: 314
Patient samples: 298, 8 patient samples represented in 2 fold redundancy, 8 controls

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