Novel Slit/Robo and CXCL12/CXCR4-mediated signaling mechanisms that modulate small cell lung cancer progression and metastasis. S. Sassi, D. Ahirwar, T. Wilkie, L. Elgaddafi, Ramesh K. Ganju

Abstract: Small cell lung cancer (SCLC) represents 20% of lung cancers and is characterized by early dissemination, development of chemoresistance and a poor prognosis. Small cell lung cancer (SCLC) is a highly aggressive malignancy with a limited spectrum of therapeutic options. Therefore, identifying early biomarkers and targets may lead to the development of innovative therapies that will improve the survival of SCLC patients. Slit2, a secreted glycoprotein, has been shown to be suppressed in a number of cancers. Slit2 has recently emerged as an important tumor suppressor gene and acts through Roundabout Homolog1 (Robo1) receptor. Slit2/Robo1 signaling has been reported to inhibit the migration of a variety of cancer cells including non-small cell lung cancer (NSCLC). The chemokine receptor CXCR4 and its cognate chemotactic ligand CXCL12 play an important role in cell migration, cancer growth, angiogenesis, and metastasis. However, the molecular mechanism by which the Slit/Robo complex inhibits the migration of small cell lung cancer is not well defined. Aims: Determine Slit2and Robo1 expression in a wide range of pulmonary neuroendocrine carcinomas (NEC), including SCLC and in human SCLC patient samples 2. Analyze the role of Slit2 in tumor growth and metastasis in vivo using a Small cell lung cancer mouse model.3. Investigate the role of Slit2/Robo1 signaling pathway modulates the CXCL12/CXCR4-induced chemotaxis and metastasis in Small cell lung cancer.

Materials and Methods: Here, we evaluate the anti-cancer and anti-metastatic potential of Slit2 protein using mouse models. **Cell lines**, we used highly metastatic SBC5 and SBC3 small cell lung cancer cell lines. Slit2 and Robo1expression were analyzed by immunofluorescence, western blot and Fluorescence-activated cell sorting of live cells (FACS) by using anti-Slit2 (Abcam) and anti-Robo1 (Chemocentryx) antibodies. Further in vitro **cell migration, invasion, and colony assays**, Modified Boyden chambers (Coster) were coated with 0.1% gelatin (for migration assays) or extracellular matrix components (for invasion assays). **Experimental Design:** To evaluate the potential role of Ad-Slit2 in tumor growth and metastasis in small cell lung cancer in vivo. **Animal studies**, all animal work was done in accordance with protocols approved by the Institutional Animal Care and Use Committee at the Ohio State University. Female athymic nude mice, SBC5 cell lines were transduced with lentivirus-encoding luciferase were injected subcutaneous injection and through Tail –vein. **Quantitative real-time PCR**, The total RNA from either cell lines or tumor tissue was extracted using a QIAGEN RNeasy kit. **Immunostaining**, all staining was performed in formalin-fixed, paraffin embedded 8-mm-thick tumor. Slides were incubated with primary antibody for Ki67, Cytokeratin, and CD31.

Results: Slit2 regulates metastatic potential of small cell lung cancer cells in vitro. To understand the role of Slit in SCLC pathogenesis, we first assessed the expression of Robo1 and Slit2 in SBC5, a highly metastatic SCLC cell line and SBC3, a low metastasizing SCLC cell line. By western blot analysis and immunofluorescence, we found that Slit2 expression was very low in SBC5 compared to SBC3 and normal epithelial cell line, BEAS. In addition, we showed that 90% of SBC5 showed expression for Robo1, while 39% cells showed expression in SBC3 by FACS Expression of typical SCLC markers Slit2, Robo1 of the SBC3 and SBC5 cell lines were tested in RT-qPCR relatively to RNA from normal lung cell line. Since Slit2 is a secretory protein, we demonstrated the presence of Slit2 in the conditioned media of SBC3 cells. Recombinant soluble Slit2 was shown to inhibit cell migration, invasion and wound healing in SBC5 and SBC3 cells. These effects were mediated through Robo1. Furthermore, we found CXCR4 expression was high in SBC5 compared to SBC3. Also, we found that soluble Slit2 treatment significantly reduced the number of tightly packed colonies in a colony formation assay using SBC5 cells, suggesting that Slit2 acts as a tumor suppressor in SCLC. In addition, we observed that Slit2 inhibited CXCL12-induced cell migration, chemoinvasion, and chemotaxis To further analyze the molecular mechanism of Slit2 -mediated signaling, we observed characterized Slit-2- mediated inhibition of the CXCL12/CXCR4 chemotactic pathway and found that Slit-2 can block the CXCL12- induced activation of the Src and PI3K kinases. In addition, Slit-2 inhibited the CXCL12-induced activation of MAPKs, it inhibited the Akt phosphorylation and Rac activation induced by this chemokine. Slit2 reduces small cell lung tumor growth and inhibits metastasis in vivo. To evaluate the potential role of Ad-Slit2 in the development of tumor and metastasis in mice model. We first established a mouse model of small cell lung cancer. Then, we injected luciferase-labeled SBC5 SCLC subcutaneous injection and Tail-vein and after confirmed of tumor establishment with bioluminescence imaging, mice were treated control group (Ad-Null) and another group (Ad- Slit2) treatment twice weekly. Both groups underwent weekly bioluminescence imaging to monitor tumor growth. All mice were subsequently sacrificed and their tumors collected and analyzed. Compared with the Ad-Null group, Ad-Slit2 mice had significantly lower tumor burden in 1/6 than controls 6/6. Using syngeneic mouse model, we reported that the ectopic expression of Slit2 using Adeno-Slit2 transduction successfully restrains tumor growth and also significantly inhibited liver, bone, and brain metastasis compared to the Adeno-null control group. Subsequently, Immunohistochemistry of Ki67 and CD31 depicted that Slit2 decreases angiogenesis and proliferation. It is thus curious that Slits found to function as a chemoattractant for vascular endothelial cells. **Conclusion**, these studies reveal that Slit2 and Robo1 may play an important role in small cell lung tumor progression and metastasis and could be used for the development of novel and targeted therapies for highly aggressive small cell lung cancer