



ESMO Translational Research Fellowship

(Aug, 2015 – July 2017)

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FINAL REPORT

Host Institute: Division of Thoracic Surgery, Medical University of Vienna, Austria Mentor: Prof. Walter Klepetko

Project title: The role of blood and lymph vessels in malignant pleural mesothelioma: From biology to therapy

Home Institute: National Koranyi Institute of Pulmonology, Budapest, Hungary

Introduction

Malignant pleural mesothelioma (MPM) is a devastating thoracic malignancy with constantly rising incidence over the past decades. Currently, the therapeutic management has shifted from single therapy approaches to multimodality treatment strategies including chemotherapy, radical surgery, and radiation. The combination of cisplatin with pemetrexed is the standard first-line systemic therapy.

The recently published phase 3 MAPS study demonstrated that the addition of bevacizumab, a VEGF (vascular endothelial growth factor) neutralizing antibody, to chemotherapy significantly prolongs patients' overall survival (OS). However, the survival benefit achieved with antiangiogenic drugs is modest (can be measured only in a few months). The limited success achieved with these drugs is due in large part to therapy resistance, although the mechanisms of tumor vascularization and resistance against antiangiogenic drugs are poorly understood.

The spread of malignant cells to lymph nodes (LNs) is a common event and LN metastasis is a key prognostic factor in various malignancies, including MPM. However, little is known about the lymphangiogenic process in human MPM and the relationship of its lymphatic network to LN metastasis and prognosis.

Rationale and Aim

This project aimed to clarify the biological and clinical significance of the blood and lymph vasculature in the progression of MPM, a devastating thoracic malignancy with dismal prognosis largely due to resistance to current therapeutic modalities

Experimental design

1. To identify and characterize the role of the (lymph)angiogenic molecules and their receptors that are expressed by the MPM cells and thus may regulate blood and/or lymph vessel formation and/or – even in an autocrine manner - enhance tumor growth.

2. In order to study their potential autocrine effects, overexpression or silencing of (lymph)angiogenic factors will also be studied on the in vitro characteristics of human MPM cells.

3. In vivo growth, tumor vascularization and lymphangiogenesis and lymph node metastasis of orthotopically growing human MPM xenografts formed by genetically modified MPM cells will be assessed as well. By using different microscopy techniques and gene expression profiling, we also plan to better characterize the blood and lymph vasculature of human MPM samples.





Results, Conclusions and Future Perspectives

As proposed in my original research plan, I further analyzed the gene expression profiles (Suppl. Table 1. in the original research plan) of our MPM cell lines and validated the mRNA expressions of the different (lymph)angiogenic molecules (Fig.1) and their receptors (Fig.2).

Next, I measured the protein levels of different (lymph)angiogenic molecules (VEGF-A, VEGF-C, apelin, endothelin-1, FGF2, FGF18) in normal mesothelial cells (NP1, NP2) and in 18 different human MPM cell lines by ELISA. Of note, I found significantly elevated VEGF-A, VEGF-C and decreased endothelin-1 levels in MPM cells (vs. NP1 and NP2 cell lines).

We also established an in vitro vascular/lymphatic model system which allows the quantitative analysis of how diffusive growth factors (like VEGF isoforms) effect vascular sprout and vessel formation. The assay utilizes aggregates made of lymphatic or blood endothelial cells, placed in a 3D fibrin gel environment. Cocultures of endothelial and tumor spheroids reveal how MPM cell lines can modulate endothelial sprouting by diffusive factors. Spheroids of distinct MPM cell lines inhibit endothelial sprout growth to various degrees. By quantitative morphometric analysis we established that M38K spheroids are most repulsive, while p31 spheroids are the most permissive -- with SPC111 spheroids in between these two extremes (Fig.3.)

By using confocal (whole mount samples and frozen sections of the diaphragm) and electron microscopy, we also investigated the vascularisation and growth of orthotopically implanted human P31 and SPC111 MPM cells. Furthermore, we assessed the motility and invasion of P31 and SPC111 cells and spheroids in vitro.

In this set of mouse experiments, both MPM lines induced the early development of submesothelial microvascular plexuses bulging into the pleural space and covering large areas of the diaphragm including regions distant from tumor colonies. The development of these microvascular networks occurred due to both intussusceptive angiogenesis and endothelial sprouting and was faster when VEGF-A-overexpressing MPM cells were implanted. Importantly, SPC111 cells showed different behavior to P31 cells. P31 colonies invaded and thus incorporated the tumor-induced capillary plexuses from the earliest stages of MPM nodule formation (4-5 days post inoculation) (Fig.4.A). In contrast, SPC111 colonies pushed the capillary plexuses away and thus remained avascular for up to three weeks (Fig.4.B). In support of this, P31 cells and spheroids exhibited significantly higher 2D motility (spreading) on plastic and 3D invasion in collagen and collagen/fibronectin gels. The key event in in vivo SPC111 vascularization was a desmoplastic response beneath the tumor nodules. This desmoplastic matrix was continuously engulfed by the SPC111 nodules resulting in the appearance of intratumoral collagen-containing desmoplastic tissue trunks providing a route for endothelial sprouting from the diaphragm. Based on these in vivo studies, we report two distinct growth and vascularization patterns of orthotopically implanted human MPM tumors in mice. In the invasive growth pattern, MPM cells invade and thus co-opt the peritumoral capillary plexuses of the pleura. In the pushing/desmoplastic growth pattern, MPM cells fail to invade the peritumoral capillary plexuses. Instead, they induce a desmoplastic response within the underlying pleura which allows endothelial sprouting and the development of a nutritive vasculature.



Fig.1. mRNA expression of different (lymph)angiogenic factors

(apelin, adrenomedullin-1, endothelin-1, netrin-4, PDGF-A, PDGF-B, PDGF-C, Ang-1, VEGF-A, VEGF-B, VEGF-C, IGF2) was measured in eight international MPM cell lines (M38K, p31, SPC212, SPC111, CRL5915, CRL5820, I2, I9), in eight MPM cell lines established by our group (VMC12, VMC14, VMC20, VMC23, VMC28, VMC31, VMC33, VMC40) and in the immortalized nonmalignant mesothelial cell line Met5A. Reference gene: β-actin







Fig.2. mRNA expression of different (lymph)angiogenic factor receptors

receptor; EDNRB, endothelin receptor; PDGFR-A,B; TEK, Ang-1 receptor, KDR, VEGF receptor-2; FLT4, VEGF receptor 3; IGF2R, Insulinlike growth factor 2 receptor; FGFR2, fibroblast growth factor receptor-2; FGFR3, fibroblast growth factor receptor-3) was measured in eight international MPM cell lines (M38K, SPC212, SPC111, CRL5915, CRL5820, I2, I9), in eight MPM cell lines established by our group (VMC12, VMC14, VMC20, VMC23, VMC28, VMC31, VMC33, VMC40) and in the non-malignant mesothelial cell line Met5A. Reference gene: β-actin



Fig.3. Spheroids of MPM lines can repel, to various extents, endothelial sprouts. Semaphorins are known regulators of endothelial guidance, and were reported to be produced by MPM cells. Our qPCR analysis revealed that M38K and SPC111 cells produce 10-fold more sema3f and sema3e isoforms, respectively, than p31 cells do. Both sema3f and sema3e are well documented chemorepellents of endothelial cell migration. Thus, several MPM lines can affect the spatial orientation of growing vascular sprouts and vessels. Spheroids of three MPM cell lines chosen on the basis of semaphorin expression profiles were co-cultured with aggregates of lymphatic endothelial cells (LEC) in fibrin gel for 4 days. A: High semaphorin-3b expression of M38K cells repel sprouts and give rise to a highly anisotropic endothelial sprout morphology. B: A lower expression level of SPC111 cells creates a less repellent environment for sprout growth. C: Sprouts approach but do not grow into P31 spheroids, which have the lowest semaphorin-3b expression.







List of Publications and Presentations Resulting from the Translational Research Project "The role of blood and lymph vessels in malignant pleural mesothelioma: From biology to therapy"

Lohinai Z, Klikovits T, Moldvay J, Ostoros G, Raso E, Timar J, Fabian K, Kovalszky I, Kenessey I, Aigner C, Renyi-Vamos F, Klepetko W, Dome B, Hegedus B. KRAS-mutation incidence and prognostic value are metastatic site-specific in lung adenocarcinoma: poor prognosis in patients with KRAS mutation and bone metastasis. *Sci Rep* 2017 Jan 4;7:39721.

Lohinai Z, Dome P, Szilagyi Z, Ostoros G, Moldvay J, Hegedus B, Dome B, Weiss GJ. From Bench to Bedside: Attempt to Evaluate Repositioning of Drugs in the Treatment of Metastatic Small Cell Lung Cancer (SCLC). *PLoS One* 2016 Jan 6;11(1):e0144797.

Hoda MA, Rozsas A, Lang E, Klikovits T, **Lohinai Z**, Torok S, Berta J, Bendek M, Berger W, Hegedus B, Klepetko W, Renyi-Vamos F, Grusch M, Dome B, Laszlo V. High circulating activin A level is associated with tumor progression and predicts poor prognosis in lung adenocarcinoma. *Oncotarget.* 2016; 7:13388-13399.

Klikovits T, **Lohinai Z**, Fabian K, Gyulai M, Fodor A, Varga J, Baranya E, Pipek O, Csabai I, Szallasi Z, Timar J, Hegedus B, Dome B, Moldvay J. Primary location of lung adenocarcinoma correlates with metastatic site and sequence – central tumors give rise to early metastases and are associated with bone involvement and decreased survival (manuscript under review in the journal "Lung Cancer")

Zoltan Lohinai¹, Laura Bonanno², Aleksei Aksarin³, Alberto Pavan², Balazs Santa¹, Virag Hollosi¹, Balazs Hegedus⁴, Judit Moldvay¹, PierFranco Conte⁵, Michael Ter-Ovanesov⁶, Evgeny Bilan³, Balazs Dome⁷, Glen J. Weiss⁸. Neutrophil-Lymphocyte Ratio is Prognostic in Early-Stage Resected Small-Cell Lung Cancer (manuscript submitted to JTO)

Lohinai Z, Dome B, Weiss GJ. Next-generation Sequencing May Discriminate Extreme Long-term versus Short-term Survival in Patients with Metastatic Small-Cell Lung Cancer (SCLC). (manuscript submitted to JECCR)





Ghimessy AK, Hegedus B, Timar J, Ostoros G, Renyi-Vamos F, **Lohinai Z**, Dome B. The effect of KRAS mutation on anti-VEGF treatment in advanced lung adenocarcinoma. Oral presentation at the ERS Lung Science Conference, 23-26 March, 2017 Estoril, Portugal

Lohinai Z, Oo HZ, Kumar G, Allen JW, Tran NL, Dome B, Moldvay J, Weiss GJ, Daugaard M. High Oncofetal Chondroitin Sulfate Expression is an Independent Prognostic Factor of Poor Survival in Early-Stage NSCLC. Oral presentation at the IASLC World Conference on Lung Cancer, December 2016, Vienna

Hollosi V, Moldvay J, Bendek M, Ostoros G, Hegedus B, Dome B, Weiss GJ, **Lohinai Z**. Neutrophil-Lymphocyte and Platelet-Lymphocyte Ratios Predict Prognosis in Early-Stage Resected Small-Cell Lung Cancer Patients. Poster presentation at the IASLC World Conference on Lung Cancer, December 2016, Vienna

Lohinai Z, PhD Thesis: Epidemiology and Clinical Relevance of Subtype-specific KRAS and EGFR Mutations in Lung Adenocarcinoma. 2016, Semmelweis University, Clinical Medicine PhD School. Supervisors: Dr. Balázs Hegedűs, PhD. Dr. Balázs Döme, MD, PhD.

Kovacs I, Bugyik E, Dezso K, Tarnoki-Zach J, Mehes E, Czirok A, Lang E, Grusch M, Berger W, Hegedus B, **Lohinai Z**, Gieszer B, Hoda MA, Klikovits T, Klepetko W, Laszlo V, Paku S, Dome B. How pleural mesothelioma nodules remodel their surroundings to vascularize and grow: findings from orthotopic mouse models (abstract accepted at iMig 2018)

"How pleural mesothelioma nodules remodel their surroundings to vascularize and grow: findings from orthotopic mouse models" (manuscript under preparation)

(as requested by ESMO, I have stated in each paper/presentation that I am a recipient of an ESMO translational fellowship)

Selection of Courses and Workshops Attended During the Fellowship IASLC World Conference on Lung Cancer, December 2015, Denver, USA ERS European Respiratory Society Annual Meeting, 2016, London, UK IASLC World Conference on Lung Cancer, December 2016, Vienna, Austria

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References

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Research Mentor full name	Signature and Date
Prof. Walter Klepetko, MD	19.04.2018