TISSUE AND CIRCULATING PROGNOSTIC BIOMARKERS IN MALIGNANT PLEURAL MESOTHELIOMA

PhD thesis

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1 Introduction

Malignant pleural mesothelioma (MPM) is a devastating malignancy, arising from the pleural space. The tumor is known to be a rare disease; however, its incidence is increasing worldwide, probably as a result of widespread exposure to asbestos, known to be the main risk factor for MPM development. In Europe, the average incidence is 2 per 100.000 inhabitants. The frequency is highly dependent on the amount of asbestos removal, asbestos import and industrialization and the peak incidence is to be expected around 2020 due to the long latency period. Inhaled asbestos fibers enter the pleural space through the alveoli or retrograde through the lymphatic vessels, causing cytotoxicity, DNA damage, frustrated phagocytosis and chronic inflammation. Important key mechanisms of the mesothelial cells, such as chromosomal aberrations and epigenetic changes, result in cellular dysfunction at gene, microRNA and protein expression levels. Most of the resulting mutations can be clustered in 4 main intracellular pathways: TP53/DNA repair, cell cycle regulation, mitogen-activated protein kinase (MAPK) and PI3K/AKT. Each of these pathways is known to be important in cell growth, proliferation, and survival, processes that are all altered during tumor development.

Despite many efforts regarding early detection and treatment, outcome remains poor. Even at early stages, minimal tumor burden and lack of distant metastases, median OS ranges from 18 to 23 months, with an expected 5-year survival rate of 15% only. Current treatment guidelines recommend that patients with MPM should be managed by a multidisciplinary team with experience in treating MPM. Treatment options in general include chemotherapy,

radiotherapy and surgery. Selected cases with favorable prognostic parameters (i.e. clinical stage I, medically operable, good performance status, epithelioid subtype) might be candidates for combined multimodality therapy.

Accordingly, new markers are urgently needed to guide selection for new therapeutic compounds and to identify patients for more aggressive treatment approaches. The research conducted in part within this thesis deals with the so far unmet need for molecular prognosticators, which might be easily available, reproducible and detectable and could lead to a better understanding of the underlying mechanisms of resistance to more aggressive multimodal treatment approaches and eventually to improved patient selection to avoid dismal outcome.

2 Objectives

Based on the current literature and on the previous findings of our MPM study group, we intended to investigate the prognostic and predictive value of specific tissue and circulating biomarkers using large cohorts of blood and tissue samples collected in our clinic at the Medical University of Vienna and through our well-established international cooperation with centers specialized in the treatment of this devastating disease.

We intended to study the following biomarkers:

- **Ki-67** is a frequently used IHC marker to determine the fraction of proliferating cells in a given cell population and was increasingly used as a diagnostic tool in different tumor types. Accordingly, we aimed to investigate whether Ki-67 index as determined by

immunolabeling of MPM tissue sections is an independent and, furthermore, reproducible prognostic factor in a large international cohort of MPM samples. We further aimed to study the prognostic power of Ki-67 index compared to other well-established prognostic factors in an independent cohort of MPM patients

- **Complement component 4d (C4d)** is a stable cleavage product of complement protein C4. C4d accumulates following both classical and lectin pathway activation. we aimed to investigate the tissue and circulating levels of C4d in MPM patients and thus compared these data with tumor load, chemotherapy response and clinicopathological parameters. We additionally assessed the impact of chemotherapy on circulating C4d levels and also evaluated the potential prognostic relevance of C4d levels on OS.
- **Activin A (ActA)**, a member of the TGF-beta super-family of growth and differentiation factors, has been shown to play essential roles in mesoderm induction, stem cell biology, reproductive biology, erythroid differentiation, systemic inflammation, cell death induction, wound healing and fibrosis. We aimed to analyze ActA plasma levels in MPM patients and compared them to clinical, radiological and pathological parameters and assessed the prognostic value of ActA in a large cohort of MPM patients.

3 Methods

Tumor tissue, blood samples and patient data were collected in the following institutions:

- Division of Thoracic Surgery, Medical University of Vienna, Austria (AUT)
- National Koranyi Institute of Pulmonology, Budapest, Hungary (HUN)
- Department for Respiratory Diseases Jordanovac, School of Medicine, University of Zagreb, Croatia (CRO)
- Department for Pulmonology, University Clinic Golnik, Slovenia (SLO)
- Concord Repatriation General Hospital and Strathfield Private Hospital in Sydney, Australia (AUS)

3.1 Evaluation of Ki-67 index as a prognostic parameter in MPM

In order to evaluate Ki-67 IHC in MPM, we investigated 285 patients. The test cohort consisted of 187 patients from three institutions. 91 patients were included in CRO. The HUN cohort consisted of 42 cases. 54 cases were included in AUT. In addition, 98 patients from SLO were analyzed as an independent validation cohort. All patients were referred to one of the four institutions between 1994 and 2012. All tumor samples were fixed in formalin and embedded in paraffin (FFPE). One 4-µm section from a representative, tumor-rich FFPE block was stained by hematoxylin/eosin (HE) to confirm and locate areas of definitive tumor content and consecutive sections were used

for Ki67 IHC. Evaluation of IHC staining was independently performed by one pathologist in the test cohort (AUT/CRO/HUN) and by another independent pathologist in the validation cohort (SLO). Results of IHC scoring were correlated with clinicopathological patient data and survival.

3.2 Evaluation of circulating C4d as a prognostic parameter in MPM

Clinical data and plasma samples of 55 consecutive, histologically verified MPM patients were collected at the time of diagnosis (n=30) or before curative intent surgery after induction chemotherapy (n=25) at the center in AUT between May 2011 and December 2014. In 12 patients, plasma samples at the time of diagnosis as well as before surgery were available, representing pre- and post-chemotherapy samples. Of 32 of these 55 patients, FFPE tissue specimens were collected for IHC analysis. Furthermore, plasma samples from an age-matched cohort of 21 healthy volunteers (HV) as well as from 14 patients diagnosed with non-malignant pleural diseases (NMPD) were included. 32 FFPE MPM tissue specimens were collected and analyzed by C4d IHC. Additionally, we performed C1q IHC in 14 FFPE tissue specimens deriving from patients with high (n = 7) and low (n = 7) circulating C4d levels. Circulating C4d and C3a plasma levels were measured using ELISA. In 20 of 55 patients, tumor volumetry measurement was achievable. Results of IHC staining, ELISA and tumor volumetry were correlated with clinicopathological characteristics and patient outcome.

3.3 Evaluation of circulating ActA as a prognostic parameter in MPM

Plasma samples from 129 MPM patients from AUT, AUS and CRO were analyzed for circulating ActA by ELISA. 16 patients who were diagnosed with pleuritis or pleural fibrosis and demonstrated no sign of MPM were also included. Additionally, plasma samples were collected from an age- and gender-matched cohort of 45 healthy individuals. Circulating Act was correlated with clinicopathological variables.

4 Results

4.1 Ki-67

The test cohort consisted of 40 female (21.4%) and 147 male (78.6%) histologically verified MPM patients. Median OS in the test cohort was 12.0 months (CI 9.3–14.7). In univariate survival analyses, histology and treatment were found to have prognostic value. Gender, age and disease stage had no significant impact on OS in univariate survival analyses of the test cohort. The median percentage of Ki67-positive tumor cells was 15.0% (range: 0–60%) for the entire test cohort. There was no significant difference in the distribution of Ki67 index with regard to age, sex, histology or stage. Furthermore, there was no association with histology. Accordingly, the distribution of the histological subtypes within the high and low Ki67 index groups did not differ significantly (Table 7). No association of Ki67 index and disease stage was found in the test cohort. However, patients that were treated in a multimodality setting had lower Ki67 index when

compared with those in other treatment groups. Accordingly, the Ki67 index was significantly lower in patients treated by induction therapy.

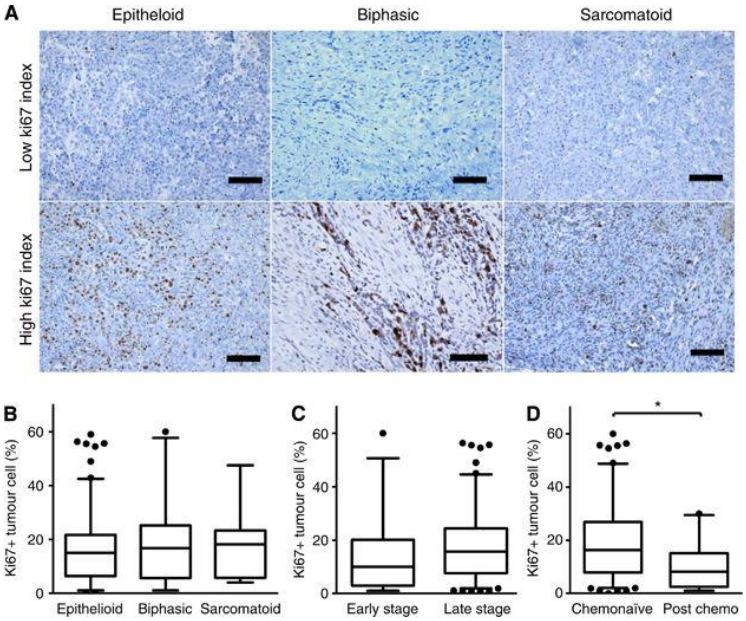

Figure 1: Ki67 index in MPM tissue samples.

Outcome of MPM patients with high Ki67 index was significantly worse compared to the low Ki67 index group (median OS 7.5 vs. 19.1 months, HR 2.3, CI 1.7–3.2, P<0.001) in univariate survival analyses. In multivariate survival analyses, Ki67 index remained to be a significant prognostic parameter (HR: 2.1, CI: 1.4–3.1, P<0.001) independently from histology (P=0.013) and treatment modality (P=0.001), whereas age, gender and stage had no significant independent prognostic impact on OS. In subgroup analyses, Ki67 showed prognostic power only in the epithelioid subgroup whereas it had no prognostic impact in the non-epithelioid subtype. In all other subgroup analyses (for age, gender, stage and treatment), Ki67 proved

to be a prognostic marker in all except the surgery-alone subgroup. Moreover, Ki-67 proved to be a prognostic parameter in the validation cohort as well.

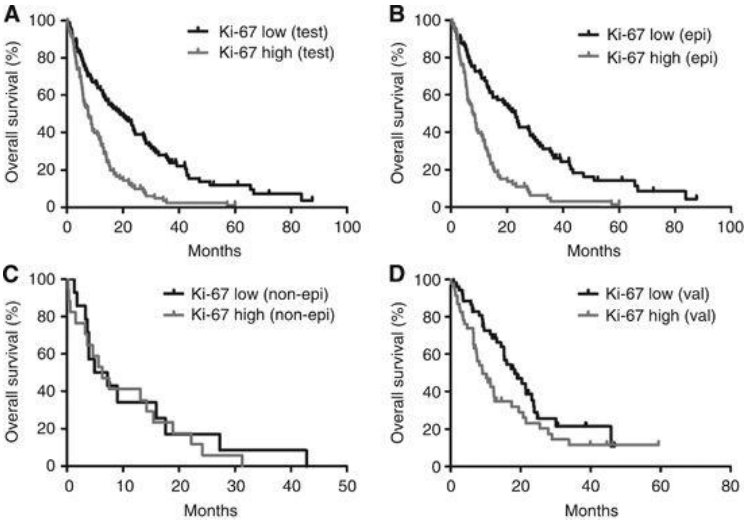

Figure 2: Prognostic power of Ki67 index in MPM

Furthermore, we performed a ROC curve analysis to test the sensitivity and specificity for detecting early death within one year after diagnosis. For this analysis, all epithelioid MPM patients with clinical follow-up for at least 12 months (n=221) were included. Ki67 showed an area under the curve of 0.68 for predicting 1-year survival. The sensitivity and specificity at 15% Ki-67 positivity were 0.69 and 0.57, respectively.

4.2 C4d

We found no tumor cell-specific C4d expression in these samples. However, several germinal centers of ectopic lymphoid structures within the tumor strongly stained positive for C4d. To investigate the

relevance of circulating C4d as a potential diagnostic biomarker, we compared C4d plasma levels of MPM patients (n=55), HVs (n=21) and with NMPD (n=14) and found no significant differences between these three cohorts. Patients presenting with epithelioid subtype tended to have lower circulating C4d levels. Moreover, patients presenting with advanced disease showed a tendency to have higher circulating C4d plasma levels when compared to patients with early-stage disease. High circulating C4d levels were significantly associated with a higher tumor load. Moreover, higher circulating C4d levels were associated with higher plasma levels of fibrinogen and CRP. We detected no correlation between C4d and white blood count (WBC). Importantly, patients with stable disease or progressive disease (SD/PD) after CHT had significantly higher C4d levels compared to those with a partial or major response.

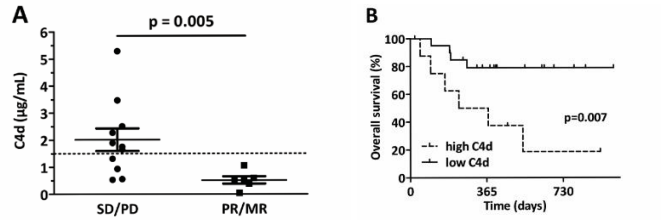

Figure 3: Circulating plasma level of C4d correlates with chemotherapy response and prognosis in MPM patients.

Patients with high C4d had significantly worse prognosis compared to patients with low plasma levels. Importantly, a multivariate cox regression analysis revealed that plasma C4d levels at diagnosis influenced OS independently from histological subtype, IMIG stage and type of treatment.

4.3 ActA

Circulating ActA levels were significantly elevated in MPM patients compared to HVs or patients with NMPD. Patients with NMPD also showed a trend for elevation of ActA levels. Patients with ActA levels below the median were significantly younger, of epithelioid subtype, and had lower stage and more often MMT including surgery. There was a significant difference in ActA levels between the 3 main histological subtypes of MPM. Moreover, ActA levels were significantly decreased in patients below the median age of 66 years. Patients with high plasma ActA levels had significantly worse outcome compared to patients with low levels. With regards to age, plasma ActA proved to be a prognostic marker only in patients under the age of 66 years. Importantly, plasma ActA had a prognostic impact only in the subgroup of patients with epithelioid MPM. In patients with non-epithelioid histology, there was a non-significant trend for different OS regarding ActA levels only.

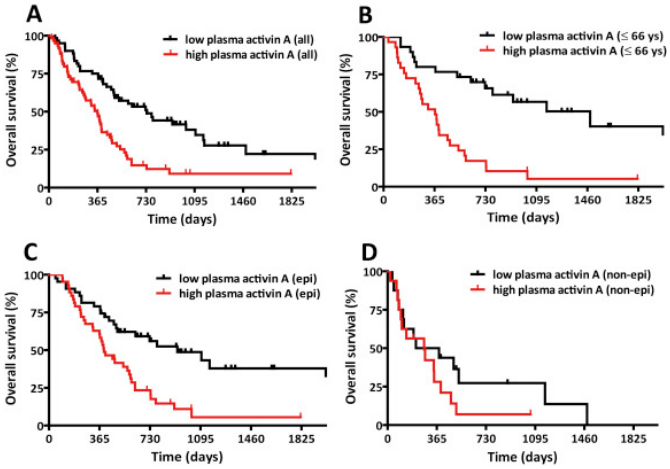

Figure 4: Prognostic power of circulating plasma ActA in MPM.

In a multivariate cox regression model, ActA was the only significant cofactor, independently influencing OS apart from gender, age, MPM subtype and stage of disease. Tumor volumetry analysis was performed in 19 patients and correlated with circulating ActA levels. Tumor volume showed a significant positive correlation with the corresponding plasma ActA levels. In an exploratory analysis, we compared matched chemo-naïve and post-chemotherapy samples (n = 14). Increased levels were found in ten patients whereas plasma ActA level decreased after treatment in four patients. Of note, the patient with the dramatic decrease in plasma ActA level after CHT experienced a major response.

5 Conclusions

1. Ki-67 index is a reproducible and easily available prognostic tissue derived biomarker in MPM. We could demonstrate that high Ki-67 expression is associated with significantly worse prognosis in a large international tissue collection and this result was additionally reproduced in an independent validation cohort. Importantly, Ki-67 was exclusively prognostic for OS in epithelioid MPM. Moreover, we showed that chemotherapy significantly decreased tumor proliferation (as measured by Ki-67 expression) in MPM and thus Ki-67 might be used as marker to monitor response to chemotherapy.

2. Complement activation might play a role in the formation and progression of MPM. Here we report for the first time that C4d, a marker for complement cascade activation, is significantly

elevated in late stage MPM and patients with high tumor volume. Furthermore, high circulating C4d levels were significantly associated with lack of clinical response to chemotherapy and with decreased OS. Considering these results, we suggest that compliment activation plays an important role in MPM, partly influencing prognosis. We also conclude that assessing circulating C4d levels might help to select patients for surgery following induction chemotherapy.

3. The biological function and protumorigenic effect of ActA in MPM has been well described. In our study we demonstrate that circulating ActA is significantly elevated in MPM, especially in cases with non-epithelioid subtype. As in the case of C4d, high circulating ActA was associated with tumor volume and worse prognosis. Similar to Ki-67 index of MPM tissue, circulating ActA was exclusively prognostic in epithelioid cases. In summary, circulating ActA may support the histological classification of MPM and at the same time help to identify epithelioid MPM patients with poor prognosis.

6 Students publications related to the thesis

Klikovits T*, Stockhammer P*, Laszlo V, Dong Y, Hoda M, Ghanim B, Opitz I, Frauenfellner T, Nguyen-Kim T, Weder W, Berger W, Grusch M, Aigner C, Klepetko W, Dome B, Renyi-Vamos F, Oehler R, Hegedus B
Circulating level of the complement component 4d (C4d) correlates with tumor volume, chemotherapeutic response and survival in patients with malignant pleural mesothelioma
Scientific Reports 2017; 7:16456, *shared first authorship KT & SP

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Hoda M, Dong Y, Rozsas A, Klikovits T, Laszlo V, Ghanim V, Stockhammer P, Jakopovic M, Samarzija M, Brcic L, Bendek M, Szirtes I, Reid G, Kirschner M, Kao S, Opitz-Schmitz I, Weder W, Frauenfelder T, Nguyen-Kim T, Aigner C, Klepetko W, van Zandwijk N, Berger W, Dome B, Grusch M, Hegedus B
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Eur J Cancer 2016; 63:64-73

Ghanim B*, Klikovits T*, Hoda M, Lang G, Szirtes I, Setinek U, Rozsas A, Renyi-Vamos F, Laszlo V, Grusch M, Filipits M, Scheed A, Jakopovic M, Samarzija M, Brcic L, Stancic–Rokotov D, Kern I, Rozman A, Dekan G, Klepetko W, Berger W, Glasz T, Dome B, Hegedus B
Ki67 index is an independent prognostic factor in epithelioid but not in non-epithelioid malignant pleural mesothelioma: a multicenter study
Br J Cancer 2015; 112:783-92, *shared first authorship GB & KT