Predictive markers of immunotherapy in brain metastatic lung adenocarcinomas

Ph.D Doctoral Theses

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I. INTRODUCTION

It is well-known that the second most common cause of deaths after cardiovascular disease is cancer. Both in Hungary and world wide, the most common malignancy is lung cancer, regarding the incidency and the death rate as well. More than one third of malignant lung tumors are adenocarcinomas (ADC), which are usually peripherally located and the incidence has increased significantly in the past few decades. Brain metastasis occurs in 30-60% of the patients with lung carcinoma, which is the most common intracranial tumor. The incidency of cerebral metastasis is increasing, owing to more effective therapeutic options for primary tumors resulting in longer survival of patients, and more sophisticated imaging techniques. The 5 year survival rate with lung carcinoma was 15.6% in 2010. This number is still under 20%, however, the diagnostical and therapeutical approaches are constantly evolving. The average time between the diagnosis of the primary lung cancer and the brain metastases is 11 months. With the occurance of brain metastasis, the survival of patients become poor: the average survival is 3-4 months even with surgery or radiotherapy.

Platinum based chemotherapy was the main therapeutical approach besides surgical resection. This was followed by a period of targeted therapies, including tyrosine kinase and anaplastic lymphoma kinase inhibitors. However, the use of these agents does not provide satisfactory therapeutic results. Nowadays, immunotherapy is gaining more and more ground, and its initial successes are hopeful.

The cells, which are located in the connective tissue of the tumor, such as immune cells, fibroblasts and endothelial cells are part of the microenvironment of the tumor and may play a role in antitumor immunity. Initially, we thought that immune cells were all present to eliminate the tumor cells. However, it turned out, that the CD8+ T-cells, the CD4+ type 1 helper T-cells, the NK cells and the antigen presenting dendritic cells are part of the anti-tumor immune defense, while the regulatory T-cells, the M2 type macrophages, the myeloid-derived suppressor cells and the CD4+ type 2 helper T-cells promote tumor growth. Activated T cells are the primary mediators of immune effector functions. These cells can express a number of inhibitory receptors, such as cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), programmed cell death protein 1 (PD-1) and lymphocyte-activation gene 3 (LAG3). These are called immune checkpoint molecules because they are involved in regulating T-cell response to self-proteins, chronic infections and tumor antigens. In chronic inflammation and tumor process T-cells are depleted and these inhibitory receptors are expressed. Various immunotherapies, such as adaptive T-cell therapy, CAR-T cell therapy, anti-tumor vaccination or immune checkpoint inhibition are used in most tumor types that act to support the immune

system. PD-L1/PD-1 inhibitors have been introduced in the treatment of lung tumors among immune checkpoint inhibitors. As science progressed, it became evident that the response to PD-L1/PD-1 inhibitors can be inferred from the level of PD-L1 expression on tumor cells prior to treatment. In addition, PD-L1 expression is also a predictive biomarker. Currently, the tumor proportion score (TPS) is used to determine PD-L1 expression of tumor cells, which compares the ratio of PD-L1 positive tumor cells to the number of viable tumor cells. While the applicability of tumor cell PD-L1 expression as a biomarker for immune checkpoint inhibition has been confirmed by numerous clinical studies, the role of the presence of immune cells as well as the expression of PD-L1 and PD-1 on immune cells remains questionable. In addition, there is growing evidence that T-cell receptor (TCR) status can serve as a potential biomarker for both antitumor immunity testing and predicting response to immune checkpoint inhibitors, but the results of previous studies are contradictory. The therapeutic criteria are increasingly outlined, but the appropriate selection criteria for the different stages are still not defined, such as in patients with cerebral metastasis.

II. OBJECTIVES

Our aim was:

- to determine the PD-L1 expression of tumor cells and its correlations with preoperative chemo-, radio- or steroid treatment, as well as with survival data
- to evaluate the presence, distribution and PD-1/PD-L1 expression of tumor-infiltrating immune cells and compare these with the PD-L1 expression of tumor cells, preoperative therapies and survival data in primary lung ADC and brain metastasis samples originate from lung ADC
- to analyse the TCR diversity in paired primary lung ADC and brain metastasis samples

III. MATERIALS AND METHODS

III.1. Samples

227 patients with brain metastatic lung cancer were involved in our retrospective study. Brain metastasis surgeries were carried out in the National Institute of Clinical Neurosciences, Budapest, Hungary in 211 cases. We used the formalin-fixed paraffin-embedded (FFPE) brain metastases samples from the archive of the 1st Department of Pathology and Experimental Cancer Research, Semmelweis University, Budapest, Hungary. In 61 cases surgical resection of primary lung ADC have occured. The diagnosis and management of the primary tumor of

these patients were carried out in the National Korányi Institute of Pulmonology, Budapest, Hungary. In 3 cases the surgical resection of the primary lung ADC were performed in the Bajcsy-Zsilinszky Hospital, Budapest, Hungary. We used the FFPE lung ADC samples from the archive of these institutes. We received 16 primary lung ADC and corresponding brain metastasis FFPE samples from the University of Szeged. Tumors were classified according to the latest WHO Classification. Clinical data from patients were collected from the patient record system of the three institutes.

We created 3 cohorts: 1) We examined 208 brain metastases, we focused primarily on the relationships between histological parameters and compared these to clinical and survival data as well. 2) We analysed 61 primary lung ADC and corresponding brain metastasis, we focused on the relationships observed between the pairs, considering the effects of each therapy as well. 3) We determined the TCR profile in 19 primary lung ADC and corresponding brain metastasis.

III.2. Tissue microarray

Tissue microarray (TMA) was generated from both the primary and metastatic tumor samples using a computer-controlled automated TMA Master system (3D HISTECH Kft, Budapest, Hungary). Three representative tissue cores with 2 millimeters in diameter were transferred from each sample to the recipient block. Normal liver, tonsilla, placenta and kidney samples were also transferred into each TMA blocks as control tissues for the immunohistochemical reactions. The prepared TMA blocks were cut into 3 μm thick sections.

III.3. Histology and immunohistochemistry

III.3.1. Peritumoral mononuclear infiltration

The presence of peritumoral mononuclear immune cells (mononuclear ring) was determined on H&E stained sections of each primary lung ADC and brain metastasis samples. We created two groups: a) Mononuclear ring was considered present, if a thick or thin layer of mononuclear cell infiltrate was detected at least focally within the brain parenchyma surrounding the metastasis. b) Mononuclear ring was considered absent when only very few scattered or no mononuclear cells were present around the tumor.

III.3.2. Intratumoral mononuclear infiltration

The amount of mononuclear cells, including lymphocytes, histiocytes and plasma cells associated with the primary or metastatic tumor was determined on H&E stained sections by a

semi-quantitative method as the following: (i) <20% or (ii) ≥20% of the tumor stroma contained immune cell.

III.3.3. Immunohistochemistry

Immunohistochemistry has been performed on 3µm thick sections of tissue microarray blocks according to standard laboratory practice on a Leica Bond-Max automated immunostaining system (Leica Biosystems, Wetzlar, Germany). For PD-L1 SP142 clone (dilution 1:100; Spring Bioscience, Ventana; Oro Valley, Arizona, USA) and for PD-1 ab52587 antibody (dilution 1:100; Abcam, Cambridge, UK) was used. The control tissue was placenta and tonsil, respectively.

The amount of positive tumor cells and immune cells were determined by a semi-quantitative method as percentage of positive cells. For tumor cells 1%, 5% and 50%, for immune cells 1%, 5% and 10% cut-off levels were recorded, which are the various thresholds used in different studies. In addition, cases were grouped into four categories based on the PD-L1 expression of tumor cells and immune cells as described in the POPLAR-study: “non-expressors”, “any-expressors”, “medium- and high-expressors” and “high-expressors”.

III.4. T-cell receptor clonality testing

DNS isolation was performed with the Qiagen QIAamp FFPE tissue kit (Qiagen, Hilden, Germany). After that, the TCR clonality testing were carried out with the internationally accepted primers (Integrated DNA Technologies Coralville, Iowa, USA), according to the BioMed2 protocol. Fragment length analysis was performed by focusing on the TRG locus. As a first step, after 10x dilution of the primers, multiplex PCR was performed to enzymatically amplify the DNA sequence (ProFlex PCR System, Life Technologies, Carlsbad, California, USA). The products were detected using an ABI3500 Genetic Analyzer (Life Technologies, Carlsbad, California, USA). Analysis of electropherograms was performed using GeneMapper Sotware 5 (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Sample was considered monoclonal if one of the peak had at least double amplitude than the next peak. Sample was considered polyclonal if several peaks were detectable with a Gaussian distribution. In each case, a positive (monoclonal) and a negative (polyclonal) control were also tested to confirm the results.

III.5. Statistical analysis

III.5.1. Cohort of 208 brain metastatic samples

Categorical variables were compared using Pearson chi-square tests and Pearson's R value was used as a measure of correlation. Survival analysis was performed on overall survival times (OS), survival time from brain metastasis surgery, progression free survival (PFS) and time to brain metastasis occurance (TTBM). For certain analyses, in order to examine clinically more homogenous cohorts, we subdivided the patients into four different groups, including cases with (1) surgery of primary tumor and solitary brain metastasis (n=51), (2) surgery of primary tumor and multiple brain metastases (n=25), (3) no surgery of the primary tumor and solitary brain metastasis (n=90), and (4) no surgery of primary tumor and multiple brain metastases (n=42). Model selection was performed with the 'survival' R package. To build a multivariate model of a selected subset of available variables, initially the SKM(t) Kaplan-Meier estimates of the survival curves were calculated for patient groups of a given value for each variable and log-rank tests were used for comparison. Statistical tests were executed with SPSS version 20.0 (IBM, Armonk, New York, USA).

III.5.2. Cohort of 61 primary lung ADC and corresponding brain metastasis

Spearman correlation between different variables was calculated using python version 3.5.3 with the help of the scipy.stats statistical package. As true associations between quantitative variables are best detected without applying artificial cut-off levels, we aimed at establishing the significance of correlations using the original semi-quantitative scale of the histology parameters described above. However, as all the values of this scale are commonly used as cut-off levels to separate patients into two distinct groups both in previous studies and clinical practice, preliminary investigations were carried out to determine significant correlations when patients are stratified based on a given threshold level. Thus for all investigated parameters, the cut-off level with the most significant result was chosen and Bonferroni-corrections were applied. Changes in the different investigated parameters between primary lung ADC and the corresponding brain metastasis were defined as either positive (+1), negative (-1) or neutral (0), based on whether the value of the given parameter increased, decreased or did not change in the brain metastasis, compared to the primary tumor. The mean of the direction of changes in differently treated groups were compared using unequal variances t-tests from the scipy.stats python package. Whenever multiple cut-off levels were available, the one with the lowest p-value for the given parameter was chosen for further investigation. This filtered set of p-values

was corrected for multiple testing with the Holm–Šidák method, given the non-independent nature of the tests.

III.5.3. Cohort of 19 primary lung ADC and corresponding brain metastasis

Categorical variables were compared using Pearson chi-square tests and Pearson's R value was used as a measure of correlation.

The $p<0.05$ was considered as statistically significant.

III.6. Ethical aspects

Permissions to use the archived tissue have been obtained from the Local Ethical Committee of the Semmelweis University (#155/2012, #510/2013, #86/2015) and the study was conducted in accordance with the Declaration of Helsinki.

IV. RESULTS

IV.1. Cohort of 208 brain metastatic samples

IV.1.1. Survival data

The OS was 29.7 (±24.2), the survival from the brain metastasis surgery was 14.9 (±13.5) months. The PFS was 15.1 (±16.4) months, the average time to cerebral metastasis was 22.0 (±21.5) months. In 78 cases the brain metastasis was first diagnosed.

IV.1.2. Peri- and intratumoral mononuclear cells

56% of the cases presented with peritumoral mononuclear ring and 44% without. 69% of the cases had <20% and 31% of the cases had ≥20% intratumoral stromal immune cells. There was a positive correlation between the presence of peritumoral mononuclear ring and the amount of intratumoral mononuclear cells.

IV.1.3. PD-L1 and PD-1 expression

Among the brain metastasis samples 41.2% showed ≥1%, 31.5% showed ≥5% and 21.8% showed ≥50% PD-L1 expression on the tumor cells. Regarding the immune cells 63.8% showed ≥1%, 48.1% showed ≥5% and 25.1% showed ≥10% PD-L1 positivity and 75.2% showed ≥1%, 26.1% showed ≥5% and 11.5%showed ≥10% PD-1 positivity. According to the POPLAR-study 26.4% of the cases were "non-expressors" and 73.6% of the cases were expressors. 38.0% of the cases were "any-expressors", 20.3% were "medium- and high-expressors" and 41.7% were "high-expressors" according to the degree of the expression. There was a positive correlation between the amount of PD-L1 positive tumor- and immune cells and the amount of PD-1

positive immune cells. Moreover, “higher PD-L1 expressors” according to the POPLAR-study were associated with more PD-1 positive immune cells, at any cut-off levels.

IV.1.4. The effect of different treatment on the amount, distribution and PD-1/PD-L1 expression of immune cells

The proportion of cases with mononuclear ring or ≥20% intratumoral immune cells was the same in the group with or without preoperative chemo- or steroid therapy. Steroid therapy before the surgery of brain metastasis significantly correlated with the lower PD-L1 expression of immune cells with 10% cut-off level. There was no correlation between the steroid therapy and the PD-L1 expression of tumor cells and PD-1 expression of immune cells. Chemotherapy before the surgery of brain metastasis did not influence the PD-L1 expression of tumor cells and the PD-1 and PD-L1 expression of immune cells.

IV.1.5. Correlation of the amount of mononuclear cells and PD-L1/PD-1 expression

Sparse stromal immune cells in a brain metastasis samples correlated with low levels of PD-L1 positivity of tumor cells at any cut-off levels. Regarding PD-L1 positivity of immune cells, ≥20% stromal immune cell correlated with higher levels of PD-L1 positive immune cells for every cut-off level. In addition, the presence of mononuclear ring correlated with more PD-L1 positive immune cells at every cut-off level as well. More than 1% PD-1 positivity of immune cells showed significant correlation with ≥20% of stromal immune cell and the presence of mononuclear ring. Regarding the groups used in the POPLAR-study, “higher PD-L1 expressors” were associated with the presence of mononuclear ring and ≥20% stromal immune cells of brain metastases, as compared to “lower PD-L1 expressors” (or “non-expressors”).

IV.1.6. Correlation of the different therapies with survival

Early clinical stages (I-II) at the diagnosis of lung ADC, surgical resection of the primary tumor and chemotherapy showed a significant association with better OS with univariate log-rank test. Multivariate Cox regression analysis identified surgery as positive and the lack of chemotherapy as negative independent prognostic factors. However, these parameters did not significantly correlate with survival after brain surgery by log-rank test.

IV.1.7. Correlation of the distribution and the amount of mononuclear cells with survival

The lack of peritumoral mononuclear ring was associated with worse survival after brain metastasis surgery, and a borderline significant tendency towards worse survival remained with multivariate Cox regression analysis. Cases with ≥20% of stromal immune cells only showed a tendency for better OS.

IV.1.8. Correlation of PD-L1 and PD-1 expression with survival

No correlation was observed by log-rank test between PD-L1 expression of tumor cells or immune cells, and PD-1 expression of immune cells at any cut-off levels and OS or survival after brain metastasis surgery, and the results remained non-significant with multivariate Cox regression analysis. For patients who underwent removal of the primary tumor and also had multiple brain metastasis, <10% PD-1 expression of immune cells was associated with a tendency towards better OS with Kaplan-Meier analysis. In addition, lower PD-L1 expression of tumor cells at any cut-off levels was associated with better survival after brain surgery, but Kaplan-Meier analyses revealed significantly better post brain metastasis surgery survival only by using 1% cut-off level. The analysis of different PD-L1 expressor groups used in the POPLAR-study showed better survival after brain metastasis surgery in the "non-expressors" compared to the "any-expressors". Kaplan-Meier analyses also showed better OS in the "lower-expressors" compared to the "high-expressors".

IV.2. Cohort of 61 primary lung adenocarcinoma and corresponding brain metastasis

IV.2.1. Survival data

The OS was 41.2 (±26.0), the survival from the brain metastasis surgery was 16.4 (±12.9) months. The PFS was 21.1 (±18.1) months, the average time to cerebral metastasis was 26.3 (±23.5) months. In 5 cases the brain metastasis was first diagnosed.

IV.2.2. Peri- and intratumoral mononuclear cells

In the primary lung ADC cases 78% presented with peritumoral mononuclear ring and 22% without; 84% of the cases had <20% and 16% of the cases had ≥20% intratumoral stromal immune cells. Among the brain metastases 63% of the cases presented with peritumoral mononuclear ring and 37% without; 69% of the cases had <20% and 31% of the cases had ≥20% intratumoral stromal immune cells.

IV.2.3. PD-L1 and PD-1 expression

36.1% of the primary lung ADC showed ≥1%, 24.6% showed ≥5% and 6.6% showed ≥50% PD-L1 expression on the tumor cells. Regarding the immune cells 44.3% showed ≥1%, 9.8% showed ≥5% and 3.3% showed ≥10% PD-L1 positivity and 83.6% showed ≥1%, 45.9% showed ≥5% and 21.3%showed ≥10% PD-1 positivity. Among the brain metastasis 34.4% of the cases showed ≥1%, 27.9% showed ≥5% and 18% showed ≥50% PD-L1 expression on the tumor cells. Regarding the immune cells 35.6% showed ≥1%, 6.8% showed ≥5% and 6.8% showed ≥10%

PD-L1 positivity and 63.9% showed ≥1%, 24.6% showed ≥5% and 11.5%showed ≥10% PD-1 positivity.

IV.2.4. Correlation of the intratumoral stromal and peritumoral immune cells between the primary lung ADC and the corresponding brain metastasis samples

There was no correlation regarding the amount of intratumoral stromal or peritumoral immune cells between the paired primary lung ADC and brain metastatic samples. In addition, no significant correlation was detected between the peritumoral and stromal immune cells within the tumor samples.

IV.2.5. Correlation of PD-1/PD-L1 expression between the primary lung ADC and brain metastatic pairs

There was a significant positive correlation regarding the PD-L1 expression of tumor cells between the paired primary lung ADC and brain metastatic samples with all cut-off levels. The most prominent correlation was observed when using no cut-off levels; however, correlations with all cut-off levels remained significant when corrected for multiple comparisons. A similar, albeit much weaker trend of positive correlation could be observed for the PD-L1 expression of immune cells in lung ADC samples and brain metastases for the 10% cut-off level, but this tendency did not remain significant when correcting for multiple comparisons. There was no significant correlation regarding the PD-1 expression of immune cells between the paired samples, however a lower level of PD-1 expression was visible in the brain metastases.

IV.2.6. Correlation of the amount of mononuclear cells and PD-L1/PD-1 expression in primary lung ADC

Sparse stromal immune cells in a lung ADC sample correlated with low levels of PD-L1 positivity of immune cells. Regarding PD-1 positivity a tendency appeared between sparse stromal immune cell and low PD-1 expression. There was no correlation between the amount of stromal immune cells and PD-L1 positivity of tumor cells. The presence of mononuclear ring correlated with more PD-1 positive immune cells, however it did not correlate with the PD-L1 expression of tumor cells or immune cells.

IV.2.7. Correlation of the distribution and the amount of mononuclear cells and the PD-1/PD-L1 expression with survival

There was no correlation between the distribution and the amount of mononuclear cells and survival, nor between the PD-L1 expression of tumor cells or immune cells or PD-1 expression of immune cells and survival.

IV.2.8. Effects of various treatments on the direction of changes in the amount of intratumoral stromal and peritumoral immune cells from primary lung ADC to the corresponding brain metastasis

The direction of changes (increase/decrease/no change) in the amount of intratumoral stromal and peritumoral immune cells from primary lung ADC to the corresponding brain metastasis were similar in groups of patients who have or have not received radio-, chemo- or steroid therapy before the surgical resection of lung ADC or brain metastasis.

IV.2.9. Effect of various treatments on the direction of changes in the PD-1/PD-L1 expression of tumor and immune cells from primary lung ADC to the corresponding brain metastasis

There was no significant difference in the direction of changes of PD-L1 expression of tumor or immune cells and PD-1 expression of immune cells from primary lung ADC to the corresponding brain metastasis in virtually all of the various treatment groups of patients when corrected for multiple testing. Of note, cases having received radiotherapy before surgical resection of lung ADC showed a significant increase in PD-1 expression of immune cells in their brain metastasis compared to the primary lung ADC. However, this correlation could only be observed at 1% cut-off value, and the group with radiotherapy comprised only 2 cases vs. 59 cases without.

IV.3. Cohort of 19 primary lung ADC and corresponding brain metastasis samples

IV.3.1. Tumor infiltrating lymphocytes

On H&E stained sections of the 38 samples we determined with a semi-quantitative method the amount of lymphocytes within and around the tumor tissue to see if the sample is eligible for further analysis. All of the tumor samples contained >1% lymphocyte, thus we found the samples suitable for TCR clonality testing.

IV.3.2. T-cell receptor clonality

In the 38 samples 2 primary lung ADC and 1 brain metastasis did not contain sufficient amount of DNA, thus the analysis has failed. One among the 17 remaining primary lung ADC samples and 3 out of the 16 brain metastasis samples were detected as monoclonal. We observed change in the TCR clonality in 2 brain metastases compared to the corresponding primary lung adenocarcinoma.

IV.3.3. Correlations of TCR clonality and the different therapies

Chemotherapy administration before primary lung ADC did not correlate with TCR status in primary lung ADC samples. Chemo- or steroid therapy before the brain metastasis surgery

alone appears not to disturb the metastatic tumor samples' TCR diversity, however chemo- and steroid therapy together may induce change towards monoclonality in brain metastases compared to the corresponding primary lung adenocarcinoma.

V. DISCUSSION

In our work, we determined the relationship between the distribution and amount of immune cells, PD-L1 expression of tumor cells and PD-L1 and PD-1 expression of immune cells in 61 primary lung ADC and 208 lung ADC brain metastases. Furthermore, we analysed the TCR clonality in 19 primary lung ADC and corresponding brain metastases. We examined only lung ADC and those metastases, as previous results show that the effect of PD-L1 expression on survival is also controversial in studies solely based on lung ADC patients. Systemic chemotherapy or radiotherapy is often used in the treatment of primary lung cancer, and steroid therapy is used in brain metastasis. Analysing the effect of these therapies is crucial, as it is not known exactly how the current biomarker, the PD-L1 expression of tumor cells in the brain metastases, and thus the therapeutic indication of immune-checkpoint inhibitors will be affected. Further parameters were investigated to identify potential new biomarkers. We raised the question whether we can deduce the characteristics and the therapeutic sensitivity of brain metastasis from the characteristics of the primary tumor, and how these relationships are influenced by the oncotherapy.

V.1. PD-L1 expression of tumor cells

According to our data, at least 34-41% of patients with brain metastatic lung ADC may receive nivolumab or pembrolizumab monotherapy in second line, as for these agents more than 1% PD-L1 expression of tumor cells is required. From 2017 pembrolizumab therapy can be used as first line in combination with pemetrexed or carboplatin chemotherapy regardless of PD-L1 expression level. However, only 22% of the patients with brain metastatic lung ADC can receive atezolizumab monotherapy in second line regarding the PD-L1 expression of tumor cells. In other studies, different expression levels have been reported by examining primary lung ADC samples. Pawelczyk *et al*. characterized 364 lung ADC samples and they found <1% PD-L1 expression in 29.1% of the cases and >50% in 4.4%. Kim *et al.* observed <1% PD-L1 expression in 71.9% of the cases and >50% in 13% of the cases in 146 lung ADC patients. Different levels of PD-L1 expression between cohorts may also result from the use of different antibodies.

In lung ADC brain metastases PD-L1 expression of tumor cells was not affected either by the chemotherapy used during the course of the disease or the steroid therapy directly used before surgery of the brain metastasis. It has been reported in melanoma brain metastases that steroid therapy has no effect on PD-L1 expression of tumor cells. However, the results are contradictory when examining primary non-small cell lung cancer (NSCLC) samples. Shin *et al.* showed a significant increase in PD-L1 expression, but our research group described a decrease in PD-L1 expression following platinum-based chemotherapy similarly to others. While chemotherapy for primary tumor may affect PD-L1 expression of tumor cells in primary localization, in the case of brain metastases, presumably due to the protective role of the blood-brain barrier, in addition to the lack of therapeutic effect, no effect on PD-L1 expression occurs. Positive correlation was observed between primary lung ADC and corresponding brain metastasis in PD-L1 expressions of tumor cells. Our results are consistent with the results of other primary NSCLC and cerebral metastatic studies. A similar relationship was described using the 5% threshold by Mansfield *et al.* and Takamori *et al.*. Kim *et al.* reported correlation between paired samples of PD-L1 expression using the 1% and 50% cut-off levels. Since brain metastases are believed to arise from only a few cells, we have to consider two hypotheses to explain this remarkable and robust concordance. The first assumes that brain metastases are developing from a "clump" of primary cells that by random chance selection reflect the proportion of PD-L1 positive cells. The second hypothesis assumes that the proportion of PD-L1 positive cells in the brain metastases are recreated from the one or few metastasizing primary cells independently from their initial PD-L1 expression status. This hypothesis is supported by experimental evidence that cell populations exist in equilibria in various transcriptomic states and when those subpopulations are isolated the same equilibria are reached again from each subpopulation. The correlation between PD-L1 expressions of tumor cells in tumor pairs was not influenced by preoperatively applied chemo-, radio- and steroid therapies. Although Takamori *et al.* have investigated a small number of cases, they did not detect chemotherapy-induced effects on PD-L1 expression of brain metastases of NSCLC either.

Although we did not find the PD-L1 expression of tumor cells as a prognostic marker in primary lung ADC, Pawelczyk *et al.* examining 364 lung ADC samples showed better survival in cases with <50% PD-L1 expression. PD-L1 expression of tumor cells in brain metastases was not prognostic in the whole cohort, however, when investigating the clinical subgroups separately, <1% PD-L1 expression correlated with better survival after brain metastasis surgery among patients who underwent removal of the primary tumor and also had multiple brain metastasis.

In these cases, it can be assumed that PD-L1/PD-1 inhibitors may themselves have a significant effect on the reactivation of the immune response.

V.2. Tumor-associated immune cells

V.2.1. Correlations of the distribution and amount of tumor-associated immune cells

We observed less than 20% intratumoral mononuclear cells in 84% of the lung ADC cases and in 69% of the brain metastases. Lack of mononuclear ring was detected in 22% of the primary tumors and 44% of brain metastases. In cases of tumors with low immune cell infiltrates, it is assumed that those patients do not have an efficient immune response to eliminate the tumor. These patients are unlikely to have a beneficial effect when using immune-checkpoint inhibitor monotherapy. In combination with agents that increase the migration of immune cells into the tumor microenvironment, such as the CTLA-4 inhibitor ipilimumab, a more favorable response may be achievable.

Comparing the primary lung ADC and corresponding brain metastasis, there was no correlation between the location and amount of immune cells in the tumor microenvironment. Mansfield *et al.* examined 73 primary lung carcinoma - brain metastatic pairs and found significantly more tumor infiltrating lymphocytes in the primary tumor compared to brain metastasis. In the study of 12 primary lung carcinoma - brain metastatic pairs, Kim *et al.*, did not found difference in the location and amount of immune cells between the paired samples.

When investigating the effects of preoperatively applied therapies, such as chemotherapy, radio- and steroid therapy, it has been found that none of the therapeutic agents affects the location and amount of immune cells in the brain metastases compared to the primary tumor. Berghoff *et al.* in cerebral metastatic cohorts of mixed origin have also been showed that the distribution of immune cells in the tumor microenvironment was independent of radio- and steroid therapy. Based on all of these, none of the general oncotherapies has effect on the migration of immune cells into the tumor microenvironment.

There was a positive correlation between the amount of stromal immune cells and the presence of the mononuclear ring in brain metastases. Literature data are contradictory when examining non-homogeneous patient groups. In certain studies more inflammatory cells have been reported around cerebral metastases than intratumorally, but in contrast, higher rate of immune cell infiltration in tumor stroma has also been reported. We observed a similar trend in the primary lung ADC cases as in brain metastases.

There was no correlation between the amount and distribution of immune cells and the PD-L1 expression of tumor cells in primary lung ADC samples. However, the amount of stromal

immune cells and the PD-L1 expression of the tumor cells showed a positive correlation in the brain metastases. In other studies, brain metastases originating from melanoma and NSCLC also showed a significant positive correlation between the amount of tumor infiltrating lymphocytes and the PD-L1 expression of tumor cells. This result is consistent with the observation that PD-L1 expression of tumor cells reflects an immunologically active microenvironment.

The amount and distribution of immune cells in the primary lung ADC samples were not prognostic. However, earlier studies have shown that tumor infiltrating lymphocytes or lymphocytes in the tumor stroma are independent prognostic markers in NSCLC. A high proportion of immune cell infiltration is associated with a more favorable prognosis. This correlation was not detected in our cohort due to the low number of primary lung ADC samples. According to our results, the observed mononuclear cell infiltration in lung ADC brain metastases, mainly in peritumoral localization, was associated with better survival, both from primary tumor diagnosis and brain metastasis surgery. In these cases, immunoreactivation by immune-checkpoint inhibitors may have additional beneficial clinical effects.

V.2.2. Correlations of the PD-L1 and PD-1 expression of tumor-associated immune cells

PD-L1 expression of immune cells showed a similar correlation as PD-L1 expression of tumor cells between the paird samples. However, PD-1 expression of immune cells was different in brain metastasis compared to primary lung ADC. Similar to the works of Kim *et al.* and Berghoff *et al.* the reduction of PD-1 positivity of immune cells was seen in brain metastases. In brain metastases, the amount of PD-L1 positive tumor and immune cells correlated positively with the amount of PD-1 positive immune cells. Duchnowska *et al.* did not observed any correlation between PD-1 and PD-L1 expression in in brain metastases of breast carcinoma. Our results suggest that there is a dynamic communication between tumor cells and immune cells, and that depleted, PD-1-expressing immune cells induce PD-L1 expression in their surroundings.

Both in the primary lung ADC and brain metastasis samples the >20% stromal immune cell infiltrate and the presence of the mononuclear ring were associated with higher PD-L1 and PD-1 expression of immune cells. Since PD-L1 and PD-1 expression of immune cells also showed a positive correlation with PD-L1 expression of tumor cells in brain metastases, co-observed protein expressions indicate a potentially immunosuppressive environment.

Studies of cerebral metastases from various primary tumors have reported that steroid therapy has no effect on the amount of PD-L1 expression of brain metastasis. In contrast, in our work,

the PD-L1 expression of immune cells in brain metastases was significantly reduced by steroid therapy used immediately prior to surgery of brain metastasis. The direction of change observed between the primary lung ADC and corresponding brain metastases showed a similar tendency in case of steroid therapy. Based on these, steroid therapy can reduce PD-L1 expression without altering the amount of immune cells. Preoperatively applied systemic chemotherapy and radiotherapy did not affect PD-L1 and PD-1 expression in brain metastases. Since steroid therapy may have an effect on the PD-L1 expression of immune cells in brain metastases but it does not affect PD-L1 expression of tumor cells and PD-1 expression of immune cells, in case of introducing the combined positive score (CPS) it will be necessary to test the brain metastasis before the immune-checkpoint inhibitor therapy.

We analysed our brain metastases data according to the POPLAR-study. As it takes both PD-L1 expression of tumor cells and immune cells into account, it can provide a biologically more relevant information. 26.4% of the cases were in the “non-expressing” group, meaning that neither tumor cells nor immune cells expressed PD-L1, so these patients would most likely not benefit from PD-L1/PD-1 inhibitor treatment. 41.7% was “high expressors” of the samples expressing PD-L1, representing 30% of all patients. These patients are likely to have significant benefit from PD-L1/PD-1 inhibition. PD-L1 expression in the “high-expressor” group showed significant correlation with the presence of mononuclear ring, as well as with >20% stromal immune cells. In the same patient group, higher PD-1 expression of immune cells was also observed. There was no correlation with survival data in subgroups according to the POPLAR-study. Among the clinically homogenized patient groups, the survival of patients who underwent removal of the primary tumor and also had multiple brain metastasis was better after brain metastasis surgery in the “non-expressors” compared to the “any-expressors” and after primary lung ADC diagnosis in the “lower-expressors” compared to the “high-expressors”. Although initially it seemed that the combined score according to the POPLAR-study could be useful in the therapeutic proposal, its use has not become widespread. However, in some studies, this evaluation system can be found. For example, Gadget *et al.* examining 360 patients with brain metastasis from NSCLC described 42.8% “non-expressing” and 17.5% 'high expressing' ratio.

V.3. T-cell receptor clonality

Based on the clinical studies, it is becoming increasingly evident that the TCR status can serve as a potential biomarker for the research of anti-tumor adaptive immunity and for predicting

response to immune-checkpoint inhibitors in each solid tumor. Most often, the sequencing of the CDR3 region is used to determine the TCR status.

Reuben *et al.* in 11 lung ADC samples not receiving neodajuvant therapy have shown that high intratumoral TCR heterogeneity is associated with increased recurrence, progression, and shorter disease-free survival. In our work 17 primary lung ADC were suitable for TCR analysis and 16 proved to be polyclonal, which is not surprising, as these diseases have already progressed.

Anti-CTLA-4 treatment for melanoma and prostate carcinoma increased the TCR diversity in the samples, and CTLA-4 inhibitor therapy in patients with originally high TCR diversity was associated with better OS. Melanoma samples were characterized by a high TCR diversity of tumor infiltrating lymphocytes in patients responding to PD-1 inhibitor therapy. Based on these it is likely that CTLA-4 inhibition prior to PD-1 inhibition may increase its therapeutic effect by inducing polyclonal TCR status in the tumor. In our samples, more than 90% of the primary lung ADC and more than 80% of the brain metastases would be expected to have a therapeutic effect from PD-1 inhibitor therapy, which can be further enhanced by pre-treatment with CTLA-4 inhibitors. However, other studies suggest that reduced TCR diversity may indicate tumor-antigen-specific T-cell accumulation in the microenvironment of the tumor. Thus, by examining the diversity of TCR, potential tumor-reactive T-cell clones can be identified. Echchakir *et al.* recorded 3 oligoclonal samples from 9 NSCLC samples, which means a dominant T-cell clone associated with the relative expansion of certain T-cell subpopulations. Only 1 of our lung ADC samples and 3 of the brain metastases showed monoclonality. To clarify whether the presence of the dominant T-cell clone in these samples can indeed indicate tumor-specific immunity further studies are required.

Comparing the TCR status of the paired primary lung ADC – brain metastasis samples, changes were observed in two cases, which correlated with chemotherapy and steroid therapy applied prior to brain metastasis surgery. In these two cases, the presumably more favorable polyclonal status became monoclonal in brain metastasis compared to primary lung ADC. Thus, if the TCR status is accepted as a therapeutic biomarker, it is assumed that it will be necessary to examine each tumor sample prior to treatment.

According to the results of our research the traditional oncotherapy methods used in the treatment of cancer disease, such as chemotherapy and radiotherapy, as well as steroid therapy, which is almost routinely used in cerebral metastases, do not affect the important biomarker, PD-L1 expression level in brain metastases. However, PD-L1 expression of immune cells was

significantly reduced by steroid therapy. Although the TCR status could be tested only in 16 paired samples, chemotherapy and steroid therapy applied together prior to brain metastasis surgery may affect the TCR clonality of brain metastases, but further studies are needed to assess this more accurately. In summary, it can be assumed that PD-L1 expression of tumor cells is a specific feature of the tumor that is not affected by routine oncotherapy. If the PD-L1 expression of tumor cells remains the only therapeutic criterion for PD-L1/PD-1 inhibitor therapy, it is likely that the determination of PD-L1 expression in the primary tumor sample may be sufficient to establish therapeutic indication in metastatic patients. It would be of great help in the presence of inoperable brain metastases. However, if CPS or TCR status determination is introduced as therapeutic criterion, it will be necessary to test each tumor sample to start treatment.

VI. CONCLUSIONS

- The PD-L1 expression of tumor cells is not affected by the applied chemotherapy during the disease, nor by the steroid therapy before brain metastasis surgery in brain metastasis of lung ADC.
- The positive correlation between the PD-L1 expression of tumor cells in the primary lung ADC and the corresponding brain metastasis samples is not affected by the preoperative chemo-, radio- or steroid therapy.
- For patients who underwent removal of the primary tumor and also had multiple brain metastasis, <1% PD-L1 expression of tumor cells was associated with better survival after brain metastasis surgery.
- Preoperative chemo-, radio- or steroid therapy has no effect on the distribution and the amount of mononuclear cells in the brain metastases.
- The presence of peritumoral mononuclear ring is associated with better OS and survival after brain metastasis surgery.
- Preoperative steroid therapy may reduce the PD-L1 expression of immune cells without altering the amount of immune cells in barin metastases.
- In subgroups according to the POPLAR-study PD-L1 expression was a prognostic marker in lung ADC brain metastases.
- Dual chemo- and steroid therapy may change the TCR status in brain metastases compared to primary lung ADC.

VII. PUBLICATION RECORD

VII. I. Articles related to the subject of the dissertation

1. **Vanda Téglási**, Lilla Reiniger, Katalin Fábián, Orsolya Pipek, Irén Csala, Attila G Bagó, Péter Várallyai, Laura Vízkeleti, Lívia Rojkó, József Tímár, Balázs Döme, Zoltán Szállási, Charles Swanton, Judit Moldvay. Evaluating the significance of density, localization, and PD-1/PD-L1 immunopositivity of mononuclear cells in the clinical course of lung adenocarcinoma patients with brain metastasis. Neuro Oncology 2017 Aug 1;19(8):1058-1067.
   doi: 10.1093/neuonc/now309. IF: 9,384
2. **Vanda Téglási**, Orsolya Pipek, Rita Lózsa, Kinga Berta, Dávid Szüts, Tünde Harkó, Pál Vadász, Lívia Rojkó, Balázs Döme, Attila G Bagó, József Tímár, Judit Moldvay, Zoltán Szállási, Lilla Reiniger. PD-L1 expression of lung cancer cells, unlike infiltrating immune cells, is stable and unaffected by therapy during brain metastasis. Clinical Lung Cancer.
   doi: 10.1016/j.cllc.2019.05.008 IF: 4,204

VII. II. Articles in different subject

3. Dóra Marosvári, **Vanda Téglási**, Irén Csala, Márta Marschalkó, Csaba Bödör, Balázs Timár, Judit Csomor, Judit Hársing, Lilla Reiniger. Altered microRNA expression in folliculotropic and transformed mycosis fungoides. Pathology and Oncology Research 2015 Jul;21(3):821-5.
   doi: 10.1007/s12253-015-9897-8. IF: 1,960
4. Lívia Rojkó, Lilla Reiniger, **Vanda Téglási**, Katalin Fábián, Orsolya Pipek, Attila Vágvölgyi, László Agócs, János Fillinger, Zita Kajdácsi, József Tímár, Balázs Döme, Zoltán Szállási, Judit Moldvay. Chemotherapy treatment is associated with altered PD-L1 expression in lung cancer patients. Journal of Cancer Research and Clinical Oncology 2018 Jul;144(7):1219-1226.
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5. **Vanda Téglási**, Dániel T Csűry, Katalin Dezső, Edina Bugyik, Vanessza Szabó, Zoltán Szállási, Sándor Paku, Lilla Reiniger. Origin and distribution of connective tissue and pericytes impacting vascularization in brain metastases with different growth patterns. Journal of Neuropathology and Experimental Neurology 2019 Feb 28. pii: nlz007.
   doi: 10.1093/jnen/nlz007. IF: 3,490

6. Lilla Reiniger, **Vanda Téglási**, Orsolya Pipek, Lívia Rojkó, Tibor Glasz, Attila Vágvölgyi, Ilona Kovalszky, Marcell Gyulai, Zoltán Lohinai, Erzsébet Rásó, József Tímár, Balázs Döme, Zoltán Szállási, Judit Moldvay. Tumor necrosis correlates with PD-L1 and PD-1 expression in lung adenocarcinoma. Acta Oncologica 2019 Apr 19:1-8.
   doi: 10.1080/0284186X.2019.1598575. IF: 3,473