EVALUATION OF PREDICTIVE AND PROGNOSTIC BIOMARKERS IN THORACIC MALIGNANCIES

PhD Thesis

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1. Introduction

In recent decades, with the accessibility and application of different anti-tumor treatments, the survival of cancer patients has improved. Nevertheless, the overall survival rate in general is still relatively poor. Therefore, in order to improve the therapeutic outcomes through a better patient selection, tumor researchers continuously strive to identify novel prognostic- and predictive biomarkers (BMs).

Prognostic BMs offer information about a patient's overall cancer outcome regardless of therapy. Therefore, the absence or presence of such prognostic markers may help to select patients for a particular treatment but does not predict the therapeutic response.

Lung cancer is a heterogeneous malignancy with several histological subtypes. Importantly, these subtypes have widely different pathological and clinical features. Histologically, non-small cell lung cancer (NSCLC) is the predominant lung cancer subtype, and more than 40% of all NSCLCs are lung adenocarcinomas (LADC). However, not all LADCs are the same, and inter-tumoral heterogeneity exists both in terms of pathological and molecular characteristic. epidermal growth factor receptor (EGFR) mutations are the second most common oncogenic driver alterations in LADC, accounting for approximately 15% of all LADCs in Caucasian patients and about 40% to 50% in Asian patients. About 90% of activating EGFR mutations are short in-frame deletions in exon 19 or point mutations in exon 21 (often called "classical" EGFR mutations). Exon 18 mutations are rare and relatively homogenous

(compared to other rare mutations such as EGFR exon 20 insertions) as they represent about 4% of all EGFR mutations. Importantly, in LADC, these EGFR-sensitizing mutations confer sensitivity to first-, and next-generation EGFR tyrosine kinase inhibitors (EGFR-TKIs) such as gefitinib, erlotinib, dacomitinib, afatinib and osimertinib in patients with advanced-stage disease. The objective response rate to EGFR-TKIs in patients carrying EGFR-sensitizing mutations is only 70% to 80%, and while some patients show a clear survival benefit to TKIs, others fail to respond properly. Different EGFR mutation subtypes and molecular characteristics can determine various predictive and prognostic features. In addition, differences in the proportion of tumor cells (TCs) harboring EGFR mutations might also contribute to therapy response since only a fraction of cancer cells carry heterozygous activating mutations, whereas other tumor cells have wild-type EGFR.

Malignant pleural mesothelioma (MPM) is a rare and aggressive malignancy arising from the pleural mesothelium. Recent advances in multidisciplinary therapeutic approaches, including surgery, chemotherapy (CHT), and radiation therapy (RT) have improved the overall survival (OS) in highly selected patients. Moreover, recent phase I/II trials have shown some benefit of immunotherapy in MPM. Still, single-agent checkpoint inhibitors were so far not demonstrated to be superior to standard CHT in more extensive phase III trials. Nevertheless, a recent phase III study investigating the efficacy of first-line nivolumab plus ipilimumab (vs. platinum doublet CHT) showed promising results

regarding OS. Of note, however, the progression-free survival (PFS) was similar between the treatment arms even in case of combination immunotherapy. Altogether, selecting MPM patients for appropriate therapeutic approaches remains a crucial problem, resulting in an unmet need to identify prognostic BMs which can predict the OS. In recent years, immunotherapy strategies against cancer have emerged as a powerful tool for the treatment of different tumoral entities. Programmed cell death 1 (PD-1) plays a crucial role in inhibiting the immune reactions and stimulating self-tolerance by activating antigen-specific T cell apoptosis, inhibiting regulatory T cell apoptosis and modulating T cell activity. Meanwhile, programmed death ligand 1 (PD-L1) is a transmembrane protein that is considered to be a co-inhibitory factor of the immune response. Besides their potential to predict the efficacy of immunotherapy, PD-L1 and PD-1 expressions have shown conflicting results regarding their prognostic significance. The prognostic significance of PD-L1 is rather controversial in lung cancer. With regards to MPM, currently, only limited data is available on the prevalence and prognostic role of PD-L1 and PD-1 expression. The exact role of these tissue BMs in predicting MPM outcome remains thus controversial.

Current interest in marker determination is enhanced by discovering pathological genes that have proven to be of clinical significance, such as EGFR mutations, PD-1 and PD-L1 proteins. The aim of our study was to assess the clinicopathological relevance of the aforementioned BMs in thoracic malignancies.

2. Objectives

Targeting EGFR is a promising strategy for treating LADC patients since numerous studies over the past decade have shown that TKI inhibitors gefitinib and erlotinib are effective in advanced-stage NSCLCs harboring EGFR sensitizing mutations. Previous studies on Asian patients suggest that higher relative EGFR mutational abundance might predict the efficacy of EGFR-TKI treatment. However, the biological and clinical relevance of adjusted tumoral EGFR variant allele frequency (EGFR-aVAF) in disease prognosis and clinical response to EGFR-TKIs is still mostly unclear. Therefore, to improve patient selection and better understand the influence of EGFR-aVAF in this setting regarding therapeutic approaches, we aimed to assess the relationship between EGFR-aVAF and response to EGFR-TKIs in a homogenous patient cohort of Caucasian LADC patients.

Currently, only limited data is available on the prevalence and prognostic role of PD-L1 and PD-1 expression in MPM. Previous studies suggest that high PD-L1 expression might be associated with impaired survival outcomes in MPM, yet the prognostic value and clinicopathological significance of both PD-L1 and PD-1 are still controversial. To further explore the expression and prognostic impact of PD-L1 and PD-1 of tumor cells (TCs) and tumor-infiltrating lymphocytes (TILs), our multi-institutional study aimed to investigate the expression patterns of these molecules and their relationship with clinicopathologic parameters and long-term outcome in human MPM.

3. Results

In our first study, 89 LADC patients with known EGFR gene mutations were enrolled in the study. All patients had an advanced-stage disease and Caucasian background. The median age of all cases was 67 (range, 34–92) years and patients were predominantly female (71.9%). A total of 46 (51.7%) patients had exon 19 deletion, while 41 (46.1%) and 2 (2.2%) patients had exon 21- and exon 18-point mutations, respectively. As for therapeutic approaches, 58 (65.2%) patients received gefitinib, while 31 (34.8%) patients were treated with erlotinib. In order to study the clinical relevance of the mutational percentage of tumoral tissue, we performed comparative statistical analyses of EGFR-aVAF and clinicopathological variables. Out of all 89 cases, 72 cases showed EGFR-aVAF between 5% and 94% and 17 patients exhibited EGFR-aVAF ≥95%. In case of six patients, the EGFR-aVAF of tumoral tissue was <20%. Interestingly, the adjusted VAF was significantly higher in patients harboring EGFR exon 19 mutations than those with exon 21 mutant tumors ($p<0.001$). There were no statistically significant differences in the mean EGFR-aVAF according to age ($p=0.93$), gender ($p=0.809$), or smoking history ($p=0.467$).

The median PFS and OS of the total cohort was 38 and 72 weeks, respectively. At the closing date of the clinical follow-up, all patients with EGFR exon 18 mutations, 42 patients with exon 19 mutations and 39 patients with exon 21 mutations had experienced disease progression after EGFR-TKI therapy. Due to the small number of patients in the

EGFR exon 18-mutated subgroup, statistical analyses were performed solely by comparing the median PFS and OS of exon subgroups 19 and 21. Accordingly, LADC patients with tumors harboring EGFR exon 19 mutations had significantly improved median PFS than those with exon 21 mutations (median PFSs were 44 vs. 25 weeks, respectively; p=0.003). In line with the PFS data, EGFR exon 19 mutations were significantly associated with longer OS as well (vs. exon 21 mutation, median OSs were 76 vs. 57 weeks, respectively; p=0.02). Regarding the administered therapeutic agents, no significant differences have been observed either in PFS (p=0.654) or in OS (p=0.665) in patients treated with gefitinib vs. erlotinib. Of note, the treatment line of EGFR-TKI did not influence the survival outcomes either. As for smoking history, there was no significant difference in PFS between never-smoker versus ever-smoker patients (p=0.099). Interestingly, however, Kaplan-Meyer curves demonstrated significantly longer median OS in never-smoker patients (vs. ever-smokers, median OSs were 106 vs. 52 weeks, respectively, p=0.007).

Next, we evaluated the survival outcomes of TKI-treated EGFR-mutant LADC patients regarding adjusted tumoral variant allele frequencies. Notably, a statistically significant positive linear correlation was found between EGFR-aVAF and PFS (r=0.319; p=0.002, Spearman's correlation). In contrast, no significant correlation was found between EGFR-aVAF and OS, although the correlation coefficient was clinically notable (r=0.208; p=0.061, Spearman's correlation). In order to rule out

the potential confounding effects of Spearman's correlation and to evaluate the survival outcomes with Kaplan-Meier methods, patients were categorized by the median EGFR-aVAF (70%) of tumoral tissue. Therefore, we grouped patients into low (<70%) and high (≥70%) EGFR-aVAF categories and found that patients with high adjusted tumoral EGFR-VAF had significantly longer PFS than those in the low EGFR-aVAF group (median PFSs were 52 vs. 26 weeks, respectively; P<0.001). Additionally, patients with high EGFR-aVAF also had significantly improved OS (vs. those with low EGFR-aVAF; median OSs were 94 vs. 57 weeks, respectively; p=0.011).

In order to assess if the predictive value of tumoral EGFR-aVAF was independent of other clinicopathological factors, we performed a multivariate Cox regression analysis. The model was adjusted for clinicopathological variables such as EGFR-aVAF, age, gender, EGFR exon mutation, therapeutic agents and treatment line. Importantly, we found that EGFR-aVAF of tumoral tissue remained a significant prognostic factor for PFS [continuous variable, hazard ratio (HR): –0.009, 95% confidence interval (CI): 0.982–0.999; p=0.042]. Besides, Cox regression analysis revealed that the specific exon mutations (nominal variable, HR: 0.284, 95% CI: 1.017–1.735; p=0.037) also influence the PFS independently.

203 MPM patients were enrolled in our second study. The full cohort comprised 151 (75%) epithelioid and 39 (19%) non-epithelioid (i.e.

biphasic or sarcomatoid) MPMs. Thirteen (6%) cases were classified as MPM not otherwise specified (NOS). The median age of all cases was 64 years (range 27-86) and patients were predominantly male (71.4%). At diagnosis, 63 (31%) and 99 (49%) cases had IMIG/TNM stage I-II and stage III-IV disease, respectively. Twenty-nine (14%) patients MMT, including surgery, while 113 (56%) patients underwent other therapeutic approaches such as CHT, RT, CHT/RT or BSC. In case of 61 patients, treatment-related data was not available. PD-L1 expression was measured in both of the TC and TIL populations. Meanwhile, PD-1 expression was analyzed solely in TILs because we did not observe any positivity on TCs. Out of all 203 cases, 152 (75%) cases did not show any TC PD-L1 expression. Of the 51 (25%) cases who were categorized as TC/PD-L1 positive (≥1%), the tumor samples of 33 (16%) and 18 (8%) patients were categorized by TC/PD-L1 scores "low" and "high", respectively. Positive staining (PD-L1 TIL expression ≥1%) was found in 13 (8%) patients, and only 1 case exhibited a PD-L1 TIL expression >10%. PD-1 expression of TILs could be measured in 164 patients. TIL PD-1 positivity (i.e. ≥1%) was found in 83 (50%) patients. A higher than 10% TIL PD-1 expression was observed in 39 (24%) patients.

Next, we studied the correlation between clinicopathological parameters and PD-L1 and PD-1 expression of TCs and TILs. No significant correlation was found between PD-L1 or PD-1 TC or TIL expressions and clinical variables such as age, gender, histological subtype or tumor stage when patients were dichotomized into PD-L1 and PD-1 negative

(no staining) vs. positive (≥ 1% staining) categories. Of note, using cut-off values of 10% or 50% for PD-L1 or PD-1 expressions did not yield significant associations either. It is also important to mention that we did not find significant associations between TC or TIL PD-L1/PD-1 expressions and histological subtypes or therapeutic modality.

The median follow-up time for all 203 patients was 12.8 months. The Median OS of the total cohort was 13.2 months (95% CI 10.6-15.8). First, we performed a univariate survival analysis in order to identify clinical prognostic factors for OS. We found that patients with epithelioid histological subtype exhibited significantly improved OS compared to those with non-epithelioid MPM (median OSs were 13.2 vs. 12.7 months, respectively; HR 0.64, p=0.012). Patients with stage I/II MPM (vs. stage III/IV, respectively, HR 0.66, p=0.01) and patients receiving multimodality treatment (vs. other therapies, HR 0.32, p<0.001) were also associated with significantly improved OS. There were no significant associations between OS and gender or age (dichotomized at a cut-off of 65 years, data not shown).

Next, we examined the prognostic value of PD-L1 and PD-1 expression of TCs and TILs. Our initial statistical analyses indicated that patients whose TCs did not express PD-L1 (median OS 14 months) had comparable OS to those with PD-L1 TC expressions between 1% and 10% (median OS 16 months, p=0.194). We grouped patients accordingly into low (≤10%) and high (>10%) PD-L1 TC categories and found that low PD-L1 expression was significantly associated with improved OS

(HR 0.39, p<0.001). PD-L1 was rarely expressed by TILS, and there was no difference in the OS of patients whose tumor samples were categorized by a PD-L1 TIL score <1% (n=152) vs. ≥1% (median OSs were 15.1 vs. 11.8 months, HR 0.82, p=0.508). Similarly, we could not show prognostic information from the PD-1 expression of TILs when patients were grouped into PD-1 TIL <1% vs. ≥1% and ≤10% vs. >10% categories.

In order to assess if the prognostic value of PD-L1 TC expression was independent from significant clinical prognostic factors, we performed a multivariate Cox regression analysis with available data from 126 (62%) patients. The model was adjusted for clinical factors such as age, gender, histological subtype, tumor stage at diagnosis and treatment. We found that PD-L1 TC expression at a 10% cut-off remained a significant prognostic factor for OS (low vs. high expression; HR 0.405, p=0.005). Histological subtype (epithelioid vs. non-epithelioid; HR 0.504, p=0.009), tumor stage (I-II vs. III-IV; HR 0.545, p=0.007) and treatment (MMT vs. other therapies, HR 0.351, p<0.001) also independently influenced OS. As 126 (62%) patients only had completely available data for the multivariate model, we performed an exploratory multivariate Cox regression analysis, using a dataset after multiple imputations by MICE approach, including all 203 cases, in order to avoid the omission of data. In this exploratory analysis, PD-L1 TC expression remained as a significant prognostic factor for OS (HR 0.443, p=0.004), independent from age, gender, histologic subtype, stage and treatment.

4. Conclusions

We found that high tumoral (≥70%) EGFR-aVAF can be used as a positive predictive BM for PFS in EGFR-TKI-treated LADC patients, and high (>10%) TC PD-L1 expression is an independent negative prognostic BM for OS in MPM. Moreover, our first study also proposes that EGFR-aVAF is considerably higher among patients with exon 19 deletions, thus confirming these patients' longer PFS and OS. These results might explain why the duration of response in some patients with EGFR-sensitizing mutations is not as long as expected when no resistance related abnormality is detected. Altogether, by shedding light on the predictive and prognostic relevance of EGFR-aVAF, our results might help to improve patient selection and treatment in advanced-stage LADC patients harboring EGFR-sensitizing mutations.

In our second study, besides confirming the prognostic role of TC PD-L1 expression, we also found that both TCs and TILs uniformly express PD-L1 in MPM. Furthermore, this was the most extensive study that comprehensively evaluated the prognostic value of PD-1 by TILs in a multicenter cohort of MPM patients. Consequently, our results concerning PD-1 and PD-L1 expression in MPM might as well contribute to the development of new therapeutic and follow-up strategies in this devastating disease.

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