EPIDEMIOLOGY AND CLINICAL RELEVANCE OF SUBTYPE-SPECIFIC KRAS AND EGFR MUTATIONS IN LUNG ADENOCARCINOMA

PhD Thesis

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INTRODUCTION

With approximately 1.4 million deaths per year, lung cancer is the leading cause of cancer-related deaths.

The term lung cancer represents a rather heterogeneous group of diseases, including conditions of varying etiology and molecular background. As a result of a new approach to classification based on novel molecular biological methods, the therapeutically relevant main histological categories (small cell lung cancer (SCLC), adenocarcinoma and squamous cell carcinoma) may undergo significant changes in the future.

In addition, identification of the so called ”driver” oncogenic mutations plays a decisive role in the development of different tumor types and open the way to targeted biological therapies. Different amino acid-specific subtype mutations lead to a different downstream signaling and drug sensitivity. Patient responses to chemotherapy within a molecular subgroup may also vary. However, the clinical consequence of amino-acid specific subtypes of these mutations is far less understood.

Molecular epidemiology and clinicopathological characteristics of tumors play an important role in therapy decision and help tumor boards to select patients for molecular analysis. A major obstacle to draw a definitive conclusion is the vast heterogeneity of the studies in terms of ethnicity, histological subtype, tumor stage and treatment modality.

Therefore, in the current studies, we analyzed a well-defined Caucasian, advanced-stage patient cohort within a four-year-long period.

In this thesis, we discuss the epidemiology and clinical relevance of subtype-specific driver oncogenic mutations, especially in an era where there is an urgent, unmet need to include more lung cancer patients in targeted therapy and other effective treatment regimens.

OBJECTIVES

A number of clinicopathological factors influences the incidence and clinical consequence of oncogenic driver mutations. Therefore, in this thesis, we aimed to investigate the epidemiology and clinical relevance of subtype-specific *KRAS* and *EGFR* mutations in lung adenocarcinoma.

1. In advanced-stage lung adenocarcinoma, the clinical significance of amino acid substitution-specific *KRAS* mutational status in terms of tumor progression after chemotherapy and OS has not yet been clearly established. Therefore, in order to better understand the influence of *KRAS* mutations in this setting, we analyzed a large cohort of Caucasian patients with unresected stage III-IV lung adenocarcinoma who were treated with platinum-based chemotherapy.

2. Furthermore, in lung adenocarcinoma, the clinical significance of rare *EGFR* mutations has not yet been fully understood [64, 65]. Therefore, we analyzed a large cohort of Caucasian patients with known *KRAS* and *EGFR* mutational status to compare the epidemiology and clinical consequence of rare and classic *EGFR* mutations.

3. There is limited data available regarding the influence of *KRAS* mutation on the organ specificity of lung adenocarcinoma dissemination. Therefore, the aim of our study was to investigate the metastatic site-specific incidence and prognostic value of *KRAS* mutation in lung adenocarcinoma patients.

METHODS

Study Population

Consecutive patients (n=1247) with cytologically or histologically confirmed, advanced lung adenocarcinoma evaluated at the National Koranyi Institute of Pulmonology and at the Department of Pulmonology, Semmelweis University between 2009-2013 were analyzed in this retrospective study.

Based on the inclusion criteria, we set up three patient cohorts. In the study cohorts, the molecular analysis was performed for potential *EGFR*-TKI therapy indication.
Cohort #1 (n=505 patients) was dedicated to understand the clinical role of amino acid-specific subtype *KRAS* mutations in lung adenocarcinoma. The cohort #2 (n=814 patients) focused on the epidemiology and clinical relevance of rare *EGFR* mutations. The combined cohort (n=903) investigated the site-specific variations in *KRAS* status according to metastatic sites.

Mutation Analysis

Genomic DNA was isolated from formalin-fixed paraffin-embedded (FFPE) tumor tissues. Mutations in *KRAS* and *EGFR* were investigated via polymerase chain reaction restriction fragment length polymorphism and by Sanger (direct) sequencing.

Treatments

According to our inclusion criteria, in cohort #1 all patients were treated with platinum-based doublet regimen. Indications for *EGFR* tyrosine kinase inhibitor (TKI) therapy in cohort #2 were: advanced lung adenocarcinoma patients received in 2nd and 3rd lines erlotinib with *KRAS* wild-type tumor, meanwhile 1st line gefitinib was available for Patients with activating *EGFR* mutation.

Statistical Methods

All statistical analyses were performed using the SPSS Statistics 18.0 package.

In vitro experiments

Eight human NSCLC cell lines were used in the experiments. The antiproliferative effect of zoledronic acid (ZA) treatment was evaluated by clonogenic assay. Briefly, 1000 cells were seeded in six well plates and treated 1, 2, 8, and 32 μM ZA for 10 days. Finally, cells were fixed with trichloroacetic acid (10%) and stained with Sulforhodamine B. The protein-bound dye was dissolved in 10 mM Tris and the optical density (OD) was determined.

RESULTS

Molecular epidemiology of driver oncogenic mutations in advanced lung adenocarcinoma

KRAS mutation rate in cohort #1, 2, and combined cohort was 33%, 28%, and 29%, respectively. Furthermore, we found exon 2, codon 12 and 13 *KRAS* mutations in 93% and 7%, respectively. The number of the major KRAS subtypes in cohort #1 was 61 (39%) G12C, 29 (18%) G12V, 27 (17%) G12D, and 8 (5%) G12A. In 31 cases rare KRAS codon 12 and 13 subtype mutations were identified.

In cohort #2, there were 5% classic *EGFR* mutant 6% rare *EGFR* mutant (non-classic mutation where amino acid change occurs), and 3% of the patients carried synonymous (silent) *EGFR* mutations (non-classic mutations without amino-acid change in *EGFR*). Fifty-nine percent of the cases was classified as *KRAS*/*EGFR* double WT.

Of note, in five patients, the G719x or L861Q rare sensitizing mutation was identified. Based on the Catalogue of Somatic Mutations in Cancer (COSMIC) data base, we found synonymous and rare *EGFR* gene mutations already published in lung cancer (N=33mutations) or in malignancies of other organs (N=20 mutations). Additionally, 45 previously unpublished novel mutations were identified.

The T790M resistance mutation was not detected in any patients. Interestingly, in case of 16 patients, 39 mutations were identified within a complex mutation pattern (at least two different *EGFR* mutations within a single sample).

Clinicopathological characteristics of lung adenocarcinoma patients

In all cohort studies, *KRAS* mutational status was significantly associated with smoking. Importantly, the amino acid-specific mutation subtype analysis identified G12V *KRAS* mutation as more frequent in never-smokers than among former and current (or ever) –smokers.
We found that rare *EGFR* mutations were associated with smoking (vs. classic *EGFR* mutations).
Among the 903 consecutive lung adenocarcinoma patients identified, 500 cases were metastatic at the time of diagnosis. We found 362 (72%) single-organ and 138 (28%) multiple-organ metastatic cases. The most frequent metastatic sites included lung (45.6%), bone (26.2%), adrenal gland (17.4%), brain (16.8%), pleura (15.6%), and liver (11%).
There was no difference in the *KRAS* mutation incidence between the single- and multiple-organ or metastatic (28.6%) and non-metastatic cases (28%).

Importantly, patients with brain (29%), bone (28%) or adrenal gland (33%) metastases demonstrated similar *KRAS* mutation frequencies. However, pulmonary metastatic cases demonstrated significantly increased *KRAS* mutation frequency when compared to those with extrapulmonary metastases (35% and 26.5%, respectively) In contrast, pleural dissemination and liver metastasis associated with decreased *KRAS* mutation incidence (vs. all other metastatic sites; 17% ($P<0.001$) and 16% ($P=0.0023$), respectively).

Prognostic factors in advanced lung adenocarcinoma

We found no difference in overall survival (OS) based on smoking habits in cohort #1. In contrast, ever-smoker status was a significant prognostic factor in cohort #2 for reduced OS (vs. never-smoker; $P=0.006$). Disease stage at diagnosis was prognostic in both cohorts.
Of note, we found no effect of *KRAS* mutational status of tumors on OS in neither cohorts. (There was no difference between *KRAS* codon 12, codon 13 mutant or *KRAS* WT patients in OS. Also, we found no difference in OS according to *KRAS* mutation status in patients neither presented with single nor with multiple-organ spreads.
However, classic *EGFR* mutation conferred a significant benefit for OS as compared to *EGFR* and *KRAS* WT or *KRAS* mutation.

In contrast, there was no significant difference in the OS of rare *EGFR* mutation positive patients compared to patients with WT *KRAS*/*EGFR* or with mutant *KRAS*.
Next, we investigated the impact of *KRAS* mutation on OS in different organ-specific metastases in lung adenocarcinoma patients. We found a clinically relevant and significant increase in OS in patients presented with *KRAS* WT bone metastasis (vs. *KRAS* mutants, median OS 9.7 vs. 3.7 months; P=0.003). Importantly, we found no statistically significant information in any other organ-specific comparison.

Therapeutic consequences of subtype-specific oncogenic mutations in advanced lung adenocarcinoma.

We evaluated the RR and PFS of platinum-based chemotherapy treated locally advanced or metastatic lung adenocarcinoma patients. There was no difference in RR or PFS among tumors carrying *KRAS* codon 12, codon 13 mutations or *KRAS* WT. We found that G12V *KRAS* mutant patients are significantly more frequent among never-smokers than other codon 12 *KRAS* mutant (G12x) cases. This subgroup of patients had a non-significantly increased RR to platinum-based chemotherapy (P=0.077). Furthermore, there was a non-significant modest increase in PFS. (233 vs. 175 days).

Next, we evaluated TKI-treated advanced lung adenocarcinoma patients with classic and rare *EGFR*. There was a significantly increased RR among patients with classic *EGFR* mutations compared to those with rare *EGFR* mutations (RR 71% vs 37%, respectively; P=0.039).

Oncogenic driver dependent in vitro zoledronic acid sensitivity of lung adenocarcinoma cells

In order to investigate the factors contributing the poor prognosis of *KRAS* mutant bone metastatic patients we performed experiments to test the sensitivity of *KRAS* mutant and *KRAS* WT lung adenocarcinoma cells to ZA, a frequently administered therapeutic regimen in bone metastatic patients.

Therefore, we performed clonogenic assay in lung adenocarcinoma cells following bisphosphonate treatment with ZA. All cell lines demonstrated sensitivity. Interestingly, resistance was not found in any of the cell lines including *KRAS* mutant cells.

CONCLUSIONS

Considering the results of this thesis the following main conclusions can be drawn in order to answer the questions formulated as the aims of the thesis.

1. The G12V subtype of *KRAS* mutations is associated with different clinicopathological characteristics and patients carrying G12V mutations may show increased response to platinum-based doublet regimens.

2. In our study, the majority of rare *EGFR* mutations was associated with smoking, shorter overall survival, and decreased EGFR-TKI response when compared with classic *EGFR* mutations. Studies characterizing the TKI sensitizing effect of individual rare mutations are indispensable to prevent the exclusion of patients with sensitizing rare *EGFR* mutations who may benefit from EGFR-TKI therapy.

3. Our study is the first that showed metastatic site-specific variation of the prognostic value of *KRAS* status in lung adenocarcinoma. We suggest the *KRAS* mutation may have important implications for diagnostic strategies and treatment decisions.

4. Based on our results, we suggest that *KRAS* mutation has a strong prognostic value in bone metastatic patients associated with decreased OS. Nevertheless, further studies are needed to evaluate whether *KRAS* mutation can be used to risk stratify patients with bone metastasis or even might predict response to various treatment options for bone metastatic patients.

5. The effect of zoledronic acid treatment on the clonogenic potential of lung adenocarcinoma cell was not dependent on *KRAS* mutant status and thus prenylation inhibition may not depend on the driver oncogenic mutations present in the tumor. Importantly, prenylation inhibition may be able to inhibit both *KRAS* mutant and *KRAS* wild-type lung cancer cells. The worse outcome of bone metastatic *KRAS* mutant patients in our combined cohort might not be due to the decreased sensitivity of tumor cells to zoledronic acid.

PUBLICATIONS

Publications related to the thesis

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