Clinical and Pathological Characteristics of KEAP1- and NFE2L2-Mutated Non–Small Cell Lung Carcinoma (NSCLC)

Rieke Frank1,2, Matthias Schefller1,2, Sabine Merkelbach-Bruse2,3, Michaela A. Ihle2,3, Anna Kron1,3, Michael Rauer4,5, Frank Ueckeroth1,2,3, Katharina König2,3, Sebastian Michels1,2, Rieke Fischer1,2, Anna Eisert1,2, Jana Fassunke2,3, Carina Heydt2,3, Monika Serke2,6, Yon-Dschun Ko2,7, Ulrich Gerigk2,8, Thomas Geist2,9, Britta Kaminsky2,10, Lukas C. Heukamp2,3, Mathieu Clement-Ziza4,5, Reinhard Büttner2,3, and Jürgen Wolf1,2

Abstract

Purpose: KEAP1 and NFE2L2 mutations are associated with impaired prognosis in a variety of cancers and with squamous cell carcinoma formation in non–small cell lung cancer (NSCLC). However, little is known about frequency, histology dependence, molecular and clinical presentation as well as response to systemic treatment in NSCLC.

Experimental Design: Tumor tissue of 1,391 patients with NSCLC was analyzed using next-generation sequencing (NGS). Clinical and pathologic characteristics, survival, and treatment outcome of patients with KEAP1 or NFE2L2 mutations were assessed.

Results: KEAP1 mutations occurred with a frequency of 11.3% (n = 157) and NFE2L2 mutations with a frequency of 3.5% (n = 49) in NSCLC patients. In the vast majority of patients, both mutations did not occur simultaneously. KEAP1 mutations were found mainly in adenocarcinoma (AD; 72%), while NFE2L2 mutations were more common in squamous cell carcinoma (LSCC; 59%). KEAP1 mutations were spread over the whole protein, whereas NFE2L2 mutations were clustered in specific hotspot regions. In over 80% of the patients both mutations co-occurred with other cancer-related mutations, among them also targetable aberrations like activating EGFR mutations or MET amplification. Both patient groups showed different patterns of metastases, stage distribution and performance state. No patient with KEAP1 mutation had a response on systemic treatment in first-, second-, or third-line setting. Of NFE2L2-mutated patients, none responded to second- or third-line therapy.

Conclusions: KEAP1- and NFE2L2-mutated NSCLC patients represent a highly heterogeneous patient cohort. Both are associated with different histologies and usually are found together with other cancer-related, partly targetable, genetic aberrations. In addition, both markers seem to be predictive for chemotherapy resistance. Clin Cancer Res; 24(13); 3087–96. ©2018 AACR.

Introduction

Systemic therapies targeting oncogenic aberrations in non–small cell lung cancer (NSCLC) have dramatically improved the outcome of genetically defined subgroups. Exemplarily, NSCLC patients with activating EGFR mutations, ALK or ROS1 rearrangements benefit from tyrosine kinase inhibitor (TKI) treatment in terms of response and survival (1–6). It thus is a critical need to further identify and characterize genomic aberrations in NSCLC, which could either act as therapeutic targets themselves or modify response to targeted treatment.

The KEAP1–NRF2 (protein encoded by the NFE2L2 gene) pathway plays a critical role in oxidative stress response by triggering antioxidant and anti-inflammatory effects (7). In healthy tissue KEAP1 counteracts NRF2 by leading to its degradation (7–11). Being exposed to oxidative stress, KEAP1 is inactivated and no longer able to bind and control NRF2, which is subsequently stabilized and translocates into the nucleus (7, 9, 12). There, KEAP1 promotes transcription of genes encoding detoxifying enzymes and antioxidative stress proteins (13–15).

Mutations in the KEAP1/NRF2-pathway are known to be involved in malignant transformation in various cancer types (16–24). Somatic loss-of-function mutations of KEAP1 lead to
Translational Relevance

In the present study, we show that KEAP1 and NFE2L2 mutations represent a heterogeneous NSCLC subgroup. Despite preclinical models showing a close interaction of these mutations in transformation, they occur nearly mutually exclusive in NSCLC and are associated with different histologies. Their frequent co-occurrence with other cancer-related mutations in line with the clinical heterogeneity argues against a role as "driver" mutations and stimulates further experiments investigating a modifier role, particular in tumors carrying already established drivers. In addition, our results suggest using NGS-based molecular multiplex diagnostics in clinical research to cover not only already established driver mutations but also potentially modifying co-occurring mutations. Finally, this work provides further evidence for a role of both KEAP1 and NFE2L2 mutations in chemoresistance.

an increase of NRF2 in the nucleus (16, 19, 25). Somatic gain-of-function mutations of NFE2L2 are found near or within pivotal binding motifs (17) and interrupt binding of NRF2 to KEAP1 dimers (26). This leads to an increase of (i) intracellular NRF2, (ii) the synthesis of antioxidant and detoxification enzymes, and (iii) the production of drug efflux pumps in cancer cells (16, 19, 21, 26).

KEAP1 or NFE2L2 mutations promote cell proliferation in tumors and may also participate in causing resistance to chemotherapy (19, 21, 27). Downregulation of NFE2L2 or overexpression of KEAP1 both triggered chemotherapy sensitivity (21, 26–29). In a squamous-cell carcinoma lung cancer (LSCC) mouse model, KEAP1 mutations were associated with carcinoma formation and tumor aggressiveness, and both KEAP1 and NFE2L2 mutations promoted resistance against radiotherapy (RT; ref. 30).

It has recently been demonstrated that NSCLC patients with KEAP1 mutation in addition to an activating KRAS mutation have a worse prognosis compared with KRAS-mutated patients without KEAP1 mutation (31). The co-occurrence of KEAP1 mutations generally describes a biologically unique subtype of KRAS-mutated NSCLCs (32). In adenocarcinoma of the lung (AD), patients with a high mutational load and benefit from anti-PD1 treatment also showed a high prevalence of KEAP1 mutations (33). Furthermore, NFE2L2 mutations were found to be associated with high PD-L1 expression in LSCC (34). It thus suggests a link between these mutations and a possible benefit from immune-checkpoint inhibition.

So far, however, little is known about the clinical presentation of NSCLC patients harboring mutations in either KEAP1, NFE2L2, or both as well as on their impact on systemic NSCLC treatment. In the present study, we describe and analyze the clinical and genetic characteristics of NSCLC patients with KEAP1 or NFE2L2 mutations and compare both groups with each other. We further evaluated the survival of these patients and their responses on systemic treatments.

Materials and Methods

Patients

Patient data was analyzed consecutively within the Network Genomic Medicine (NGM) Lung Cancer. NGM is a German health care provider network where next-generation sequencing (NGS)-based molecular diagnostics of lung cancer is performed centrally for about 280 hospitals and private-practices-based oncologists (www.ngm-cancer.com). Incoming formalin-fixed paraffin-embedded (FFPE) lung cancer samples were analyzed from 2011 to 2013 and from May 2015 to August 2015 at the Institute of Pathology, University Hospital of Cologne (Cologne, Germany). Screening procedures and data assessment were performed in accordance with local standards. Data assessment was approved by the responsible ethics committee (ref. number 10-242), and all patients consented for data analysis.

The cohorts consisted of both an establishment cohort and a validation cohort for the implementation of NGS in routine lung cancer diagnostics (35). Patients of both cohorts were not pre-selected regarding smoking history, age, stage, or sex.

Samples and immunohistochemistry

Histopathologic diagnostics was performed centrally per local standard operating procedures. The histopathologic differentiation between AD and SQCC was based on immunohistochemical staining [CK5/6, CK7, p40, and thyroid transcription factor 1 (TTF1)] as previously published (36).

Next-generation sequencing (NGS) and FISH diagnostics

NGS was performed using a MiSeq benchtop sequencer as described previously (Illumina; ref. 33; Supplementary Table S1). We used an in-house algorithm to call for genomic variants of the targeted sequences (37). The variants were then stored in a FileMaker (Filemaker GmbH, Germany) database for further analyses. After reporting, we used COSMIC (http://cancer.sanger.ac.uk/cosmic), OncoKB (http://oncokb.org), and CancerHotspots (http://cancerhotspots.org) databases for further annotation. In silico evaluation on the impact of the detected mutations was made with PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2).

In a subset of patients, FISH were carried out to determine rearrangements and/or amplifications (Supplementary Table S6). MET and FGFR1 amplifications were tested and classified as reported (38, 39). FGFR1 was analyzed routinely in all LSCC patients, whereas MET amplification testing by FISH was routinely introduced for AD in June 2014.

Clinical characteristics

We assessed age, sex, tumor stage (UICC), TNM status, smoking status (qualitatively following CDC reporting; ref. 40, and in pack-years), exposure to other known pulmonary carcinogens, histology, metastases and treatment modalities and their outcome, if available. Outcome was provided by the network partners and followed Response Evaluation Criteria in Solid Tumors V1.0 (RECIST), proposing that stable disease (SD) and responses [partial response (PR), complete response (CR)] need to be confirmed by a CT scan not earlier than 4 weeks after the initial response assessment. If restaging was not performed or available, we asked for investigators’ assessments. Staging and restaging procedures were performed in accordance with local standards from each partner, usually including CT scans of the involved areas and MRI scans for brain metastases in all patients.

Statistics and software

Qualitative variables (e.g., sex or smoking status) were summarized by count and percentage, quantitative variables by mean...
and standard deviation or median and range. Distribution of time-to-event was described by the Kaplan–Meier estimator. Follow-up was assessed using “reversed” Kaplan–Meier statistics. Association between categorical variables was assessed using χ² tests or Fisher exact tests, if appropriate. Statistical analyses were performed using SPSS v23.0 (IBM Corp.). Plots were generated using GraphPad Prism 6 (GraphPad Software Inc.). For the 'lollipop charts,' we used cBioPortal for Cancer Genomics’ MutationMapper (http://www.cbioportal.org/mutation_mapper.jsp).

Results

KEAP1 mutations

Tumor tissue of 1,391 NSCLC patients was analyzed using NGS (Fig. 1; Supplementary Table S1). KEAP1 mutations were detected in 157 patients (11.3%). Up to 134 different mutations were identified (Supplementary Table S2). They are spread over the whole KEAP1 length (Fig. 3A). The most common mutations were Q620 deletions (n = 5), R320L (n = 4), R320W (n = 4), and V369A (n = 3). No hotspot mutations could be identified (Fig. 3A).

NFE2L2 mutations

Forty-nine patients (3.5%) had 30 different NFE2L2 mutations (Supplementary Table S3). The most common mutations were W24R (n = 5), R34Q (n = 5), W24C (n = 4), E79K (n = 4), and R34G (n = 3). Clusters of mutations were observed in region E79 (n = 5), R34 (n = 11), and W24 (n = 9; Fig. 3A). R34 and E79 have been described as hotspot regions in human cancer, whereas W24 has not (41). These hotspot regions are near or within the ETGE and DLG motifs, which are the KEAP1 binding domains of NFE2L2 (ref. 17; Fig. 3A).

Clinical characteristics of patients with KEAP1 and NFE2L2 mutations

Detailed clinical characteristics of the cohort are provided in Table 1. A complete listing of the control group is provided in Supplementary Table S8. In both patients with KEAP1 mutation (KEAP1 group) and in patients with NFE2L2 mutation (NFE2L2 group), more males were affected. The same was true for the control group (Fig. 2A). There was no gender bias in this dataset (P = 0.51). The median age at diagnosis was 63.7 years (range, 40–84) for the KEAP1 group and 66.6 years (range, 30–81) for the NFE2L2 group.

Smoking status was assessable in 112 patients with KEAP1 mutation and 35 patients with NFE2L2 mutation. Both groups were characterized by strong smoking exposure, with only 6 never smokers in the KEAP1 group and three in the NFE2L2 group. In contrast, the control group consisted of 19.4% never-smokers (Fig. 2B). Median quantity of pack-years was 35 for KEAP1 (range, 0–100) and 40 for NFE2L2 (range, 0–120). Three patients in the NFE2L2 group had a history of asbestos exposure and one had been exposed to quartz dust, while there was not a comparable exposure documented within the KEAP1 group.

Patients with KEAP1 mutation had predominantly AD (72.2%), comparable to the control group with 73.8% AD. In contrast, LSCC was the most frequent histology subtype with 59.2% in the NFE2L2 group, followed by AD (32.7%; Fig. 2C, P < 0.01). In both groups, most patients had metastasized disease (stage IV) at first diagnosis, significantly more frequent in the KEAP1 group (71.0% vs. 51.2%, P = 0.03).

Eastern Cooperative Oncology Group’s Performance status (ECOG) was documented in 105 patients in the KEAP1 group and 26 in the NFE2L2 group. While ECOG 1 and ECOG 2 had a similar distribution pattern in both groups, there were differences for ECOG 0 (21.9% vs. 26.9%), ECOG 3 (7.6% vs. 3.8%), and ECOG 4 (1.9% vs. 0%). The difference in ECOG 3 plus 4 in both groups was not significant (P = 0.46). In the control group, nearly half of the patients presented with ECOG 0 (Fig. 2D).

In the KEAP1 group, a high frequency of distant metastases affecting bones, brain, liver, skin, and spleen could be seen in stage IV patients, whereas patients in the NFE2L2 mutation more frequently suffered from local spread (i.e., lung, pleura, mediastinal lymph nodes; Fig. 2E and F).

There were 4 patients with both a KEAP1 mutation and a NFE2L2 mutation. All four patients had a history of smoking and three patients had LSCC, while only 1 patient had AD. There were no further similarities regarding clinical characteristics and co-occurring aberrations between those patients. We performed a Fisher exact test, which revealed a z-pooled P > 2.2–16 for mutually exclusivity.

Co-occurring aberrations

We found additional genomic aberrations in 87.3% of the KEAP1 group and 83.7% of the NFE2L2 group (Fig. 3B; Supplementary Tables S4 and S5). TP53 mutations were the most common co-occurring mutations in both groups (44.9%, KEAP1; 40.8%, NFE2L2). EGFR mutations also showed a similar distribution pattern occurring in 6.3% (KEAP1) and 6.1% (NFE2L2). In both patient groups MET amplifications occurred at high frequency (18.3%, KEAP1; 26.3%, NFE2L2; Supplementary Table S6). Fitting to the correspondent histological distributions, we detected differences regarding the co-occurrence of KRAS

Figure 1.
Analytical flow sheet of the study. Euler diagram showing the proportions of mutated patients compared with the total cohort.

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Table 1. Comparison of clinical characteristics of patients with KEAP1 mutations versus patients with NFE2L2 mutations

<table>
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(P = 0.03), PTEN (n.s.), PIK3CA (n.s.) mutations and FGFR1 amplifications (Supplementary Table S6; Fig. 3B). Out of the reported mutations, we identified only one TP53 polymorphism (P72R) in both groups, six proposed MET polymorphisms (T1010I), and one EGFR P848L. The differences in mutational profile is strongly supported by data on gene expression differences and mutational data (42, 43).

Survival

Median follow-up for stage IV patients was only 1.8 months (95% CI, 0.0–3.7 months) for the KEAP1 group (n = 80) and 5.3 months (95% CI, 2.1–8.5 months) for the NFE2L2 group (n = 21). Thus, we abstained from further survival analyses. The median OS of these patients with KEAP1 mutation was 19.1 months (95% CI, 1.8–36.3 months) and 14.0 months...
(95% CI, 5.6–22.3 months) for patients with NFE2L2 mutation (Fig. 3C).

Response assessment
For 30 patients with KEAP1 mutation, details about systemic therapy were assessed. Twenty-seven patients had additional aberrations (Supplementary Table S7). These patients received between one and three lines of chemotherapy or TKIs as systemic therapy. Most patients (28) did not respond to first-line chemotherapy [progressive disease (PD)]. Both two patients with SD had an activating EGFR mutation and one of them received erlotinib and bevacizumab, the other had SD under carboplatin/paclitaxel, representing the only non-PD KEAP1 patient treated with conventional chemotherapy. Due to the short duration of first-line treatment, 17 patients (56.7%) received systemic first-line therapy. Outcome analysis was available for 8 patients, which all developed PD regardless of therapy regimen. In third-line, 3 patients received therapy. Two of them had a PD but the outcome of the other patient is not known (Supplementary Table S7). There was no responder in none of the therapy lines.
Eight patients with NFE2L2 mutation receiving systemic therapy were evaluable for outcome analysis. Only one patient did not present an additional aberration. Four patients developed PD during first-line therapy, leading to a significantly better outcome regarding non-PD first-line as compared with the KEAP1 group ($P = 0.01$, Fig. 4B). Two patients had a SD as best outcome.
and two patients had a partial response (PR). Of these 4 patients with benefit from therapy, 2 patients received erlotinib and bevacizumab and 1 of these 2 patients had an activating EGFR mutation and responded. The third patient harbored an additional STK11 mutation and received carboplatin and etoposide resulting in a PR, representing the only responder to a platinum-based regimen in the cohort. The fourth patient did not harbor a detectable comutation and received cisplatin, pemetrexed, and bevacizumab, developing a SD. Two patients received both second- and third-line treatments without response (Fig. 4A).

Out of the 38 patients with an outcome analysis 16 patients harbored an additional TP53 mutation. Twelve out of 32 patients (37.5%) with a PD during first-line therapy had a TP53 mutation. In contrast, also 4 out of 6 patients (66.6%) with a non-PD during first-line therapy had TP53 mutations. We therefore conclude that

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**Figure 4.**

Treatment outcome. **A,** Responses to systemic therapy according to the therapy line. **B,** PD vs. non-PD in first-line systemic therapy. **C,** Regimen used in first-line therapy, second-line therapy, and third-line therapy. **D,** Progressive disease (PD) versus non-progressive disease (non-PD) of chemotherapy-based and targeted therapy-based therapies regardless of therapy line.
chemoresistance in our cohort is not biased by the co-occurrence of TP53 mutations.

Comparing treatment with regard to the therapy used regardless of therapy line, 62 regimens could be identified in 38 patients, whereof 50 were chemotherapy treatments and 10 erlotinib-based treatments (± bevacizumab). Two lines could not be identified retrospectively. In each group, we found one PR and two SD, respectively (Fig. 4D).

**Discussion**

To the best of our knowledge, this is the largest cohort of NSCLC patients with KEAP1 and NFE2L2 mutations analyzed so far regarding clinical and pathologic characteristics. Both mutations are discussed to play a pivotal role in cancer formation and maintenance. We detected KEAP1 mutations with a frequency of 11.3% and NFE2L2 mutations with a frequency of 3.5% among a European all-comer NSCLC cohort.

Surprisingly, even though both types of mutations affect the same pathway and show a close functional interaction, they only exceptionally co-occur within the same tumor. Thus, only 4 patients in our cohort harbored a KEAP1 and a NFE2L2 mutation simultaneously. Moreover, both mutations are associated with different NSCLC histologies, as well as their known expression patterns (43). While most KEAP1 mutations occurred in patients with AD, patients with NFE2L2 mutations mostly presented with LSCC. On a molecular level, KEAP1 mutations were not found in specific hotspot regions, but heterogeneously spread over the whole protein, whereas NFE2L2 mutations were found in specific hotspot regions, in line with previous reports (17, 41). Regarding clinical presentation, KEAP1 patients had a significantly higher frequency of stage IV at primary diagnosis than NFE2L2 patients. Further, although not significantly, the KEAP1 group presented in worse performance state and tended to present with a higher frequency of hematogenous spread.

In a small subset of our cohort in which reproducible response on systemic therapies was assessable, response on different lines of hematogenous spread.

**Authors’ Contributions**

Conception and design: R. Frank, M. Schefler, M. Serke, R. Büttner, J. Wolf


Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): R. Frank, M. Schefler, S. Merkelbach-Bruse, M. Ihle, K. König, R. Fischer, J. Fassunke, C. Heydt, M. Serke, Y.-D. Ko, T. Geist, B. Kaminski, L. C. Heukamp, R. Büttner, J. Wolf


Writing, review, and/or revision of the manuscript: R. Frank, M. Schefler, S. Merkelbach-Bruse, M. Ihle, A. Kron, M. Rauer, S. Michels, R. Fischer, A. Eisert,
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Rieke Frank, Matthias Scheffler, Sabine Merkelbach-Bruse, et al.


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