Role of KEAP1/NFE2L2 Mutations in the Chemotherapeutic Response of Patients with Non–Small Cell Lung Cancer

Youngtae Jeong[1,2,3,4], Jessica A. Hellyer[1], Henning Stehr[5], Ngoc T. Hoang[1,2,6], Xiaomin Niu[1,7], Millie Das[1,8], Sukhmani K. Padda[1], Kavitha Ramchandran[1], Joel W. Neal[1], Heather Wakelee[1], and Maximilian Diehn[1,2,3]

ABSTRACT

Purpose: Activation of NFE2L2 has been linked to chemoresistance in cell line models. Recently, somatic mutations that activate NFE2L2, including mutations in *NFE2L2, KEAP1*, or *CUL3,* have been found to be associated with poor outcomes in patients with non–small cell lung cancer (NSCLC). However, the impact of these mutations on chemoresistance remains incompletely explored.

Experimental Design: We investigated the effect of *Keap1* deletion on chemoresistance in cell lines from *Trp53*-based mouse models of lung squamous cell carcinoma (LSCC) and lung adenocarcinoma (LUAD). Separately, we identified 51 patients with stage IV NSCLC with *KEAP1, NFE2L2, or CUL3* mutations and a matched cohort of 52 wild-type patients. Time to treatment failure after first-line platinum doublet chemotherapy and overall survival was compared between the two groups.

Results: Deletion of *Keap1* in *Trp53*-null murine LUAD and LSCC resulted in increased clonogenic survival upon treatment with diverse cytotoxic chemotherapies. In patients with NSCLC, median time to treatment failure (TTF) after first-line chemotherapy for the *KEAP1/NFE2L2/CUL3*-mutant cohort was 2.8 months compared with 8.3 months in the control group ($P < 0.0001$). Median overall survival (OS) was 11.2 months in the *KEAP1/NFE2L2/CUL3*-mutant group and 36.8 months in the control group ($P = 0.006$).

Conclusions: *Keap1* deletion confers chemoresistance in murine lung cancer cells. Patients with metastatic NSCLC with mutations in *KEAP1, NFE2L2*, or *CUL3* have shorter TTF and OS after first-line platinum doublet chemotherapy compared with matched controls. Novel approaches for improving outcomes in this subset of patients with NSCLC are therefore needed.

Introduction

Despite significant advances in the treatment landscape for non–small cell lung cancer (NSCLC), the overall survival (OS) of advanced-stage NSCLC remains poor (1, 2). This is due in large part to the development of resistance to chemotherapy by cancer cells. Unfortunately, the molecular causes of intrinsic and acquired chemoresistance remain incompletely understood.

The KEAP1–NFE2L2 pathway regulates redox and metabolic homeostasis and has been implicated in chemoresistance in a variety of cancer types. Nuclear factor (erythroid-derived 2)-like 2 (NFE2L2; also known as NRF2) is a transcription factor that regulates the transcription of antioxidant and drug-detoxifying genes and thus enhances cellular survival. At homeostasis, an adaptor protein called Kelch-like ECH-associated protein 1 (KEAP1) recruits a CUL3-containing E3 ubiquitin ligase complex to NFE2L2 and leads to its proteasome-mediated degradation (3, 4). Under oxidative and toxic stress, NFE2L2 is released from KEAP1, migrates into the nucleus and drives transcription of genes containing antioxidant response element (ARE) in their promoter regions (5). NFE2L2 target genes are involved in antioxidant metabolism and xenobiotic biotransformation reactions and thus protect cells from the effects of cytotoxic chemotherapy.

Recently, large-scale genomic analyses have revealed that genes in the KEAP1–NFE2L2 pathway are mutated in ∼33% of lung squamous cell carcinoma (LSCC; ref. 6) and ∼22% of lung adenocarcinoma (7, 8). Genetically engineered mouse model studies have indicated that *KEAP1* deletion and *NFE2L2* mutations confer a prosurvival phenotype and promote the development and aggressiveness of NSCLCs (9–11), suggesting a potential mechanism for selection of mutations in these genes during tumorigenesis. Previous studies have also suggested that NFE2L2 activation in cancer cells leads to treatment resistance to a variety of anticancer agents. For example, prior work from our group demonstrated that *Keap1* deletion in a *Trp53*-based mouse model of LSCC confers radioresistance by interfering with reactive oxygen species (ROS) generation and that early-stage NSCLC patients with *KEAP1* or *NFE2L2* mutations are at high risk for local recurrence after radiotherapy (11). A role of the KEAP1–NFE2L2 pathway in lung cancer chemoresistance is suggested by preclinical studies showing that *KEAP1* loss or *NFE2L2* overexpression is associated with resistance and *NFE2L2* inhibition with sensitization to chemotherapeutics (12–17). In addition, recent studies have reported a prognostic association between activation of the KEAP1–NFE2L2 pathway and poor OS after chemotherapy. However, these studies

[1]Stanford Cancer Institute, Stanford University School of Medicine, Stanford, California. [2]Institute for Stem Cell Biology and Regenerative Medicine, Stanford University School of Medicine, Stanford, California. [3]Department of Radiation Oncology, Stanford University School of Medicine, Stanford, California. [4]Department of New Biology, DGIST, Daegu, Republic of Korea. [5]Department of Pathology, Stanford University School of Medicine, Stanford, California. [6]Department of Biology, San Francisco State University, San Francisco, California. [7]Department of Shanghai Lung Cancer Center, Shanghai Chest Hospital, Shanghai Jiao Tong University, Shanghai, China. [8]Department of Medicine, VA Palo Alto Health Care System, Palo Alto, California.

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Y. Jeong and J.A. Hellyer contributed equally to this article.

Corresponding Authors: Maximilian Diehn, Department of Radiation Oncology, Stanford University, 875 Blake Wilbur Drive, Stanford, CA 94305-5847. Phone: 650-721-2790; Fax: 650-736-2961; E-mail: diehn@stanford.edu and Heather Wakelee, Department of Medicine, Division of Oncology, Stanford Cancer Institute/Stanford University School of Medicine, 875 Blake Wilbur Drive, Stanford, CA 94305. E-mail: hwakelee@stanford.edu

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Translational Relevance

For patients with metastatic, driver mutation–negative, non–small cell lung cancer, chemotherapy remains an integral treatment modality, either in combination with immunotherapy or alone. Although frequently efficacious in the short term, resistance to chemotherapy almost uniformly develops. The mechanisms of intrinsic resistance and therefore strategies to overcome it are poorly understood. The KEAP1–NFE2L2 pathway plays an important role in the management of reactive oxygen species, and mutations in this pathway lead to tumor aggressiveness and enhanced tumor survival. In this study, we investigated the role of the KEAP1–NFE2L2 pathway in intrinsic chemoresistance. To do this, we first exposed lung cancer cells from murine lung cancer models with *Keap1* deletion to cytotoxic chemotherapies and showed that this led to increased clonogenic survival. We then retrospectively evaluated patients with mutations in the KEAP1–NFE2L2 pathway who received upfront chemotherapy and compared their outcomes with patients without mutations. We found that patients with mutations in the KEAP1–NFE2L2 pathway have inferior time on therapy and overall survival, suggesting that these mutations confer chemoresistance.

were limited to patients with NSCLC with KRAS mutations (18) or stratified patients with NSCLC based on protein levels of NRF2 rather than *KEAP1*/*NFE2L2* mutations (19–21).

We therefore set out to explore the impact of *KEAP1*/*NFE2L2* mutations on chemoresistance and prognosis after chemotherapy in NSCLC regardless of *KRAS* mutational status. To this end, we used NSCLC cells from *Trp53*-deletion based lung adenocarcinoma (LUAD) and LSCC mouse models without *Kras* mutations and found that *Keap1* deletion confers resistance to diverse anticancer drugs including platinum reagents. We further demonstrate that *KEAP1*/*NFE2L2* mutations are predictive of worse response to platinum doublet chemotherapy.

Materials and Methods

Animals

Keap1$^{f/f}$ mice (refs. 22, 23; C57BL/6J background) and *Trp53*$^{f/f}$;*R26*^{tdTomato} mice (ref. 24; B6/129 background) were kindly gifted from T. Kensler (University of Pittsburgh, PA) and M. Winslow (Stanford University, CA), respectively. *Trp53*$^{f/f}$;*R26*^{tdTomato} and *Keap1*$^{f/f}$;*Trp53*$^{f/f}$;*R26*^{tdTomato} mice between 4 weeks and 9 months of age were intranasally administered with Ad-Cre viruses for lung adenocarcinoma formation. Mice were housed in a designated pathogen-free area in a facility at the Stanford University School of Medicine accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care. All care and treatment of experimental animals were in accordance with the guidelines of Stanford University School of Medicine institutional animal care and use committee guidelines.

Tumor dissociation and tumorsphere assay

As previously described (11), tumors generated from *Trp53*-null or *Keap1*;*Trp53*-null tracheal or lung cells were minced with a razor blade and suspended in 10 mL of L-15 Leibovitz medium (Thermo Fisher Scientific Inc.) supplemented with 0.5 mL of collagenase/hyaluronidase (Stem Cell Technologies). Tumors were digested for 1.5 to 2 hours at 37°C and 5% CO^2 and manually dissociated by pipetting every 30 minutes. After digestion, 40 mL of blocking buffer was added, and tumor cells were pelleted by centrifugation. Tumor cells were resuspended in 5 mL of trypsin/0.05% EDTA for 5 minutes and centrifuged with the addition of blocking buffer. The cell pellet was incubated with 100 Kunitz units of DNase I (Sigma) and Dispase (Stem Cell Technologies) for 5 minutes at 37°C and added with blocking buffer for centrifugation. After digestion, tumor cells were treated with ACK lysis buffer and filtered through a 40-μm cell strainer. Dissociated tumor cells were resuspended in MTEC/Plus (25) mixed at a 1:1 ratio with growth factor-reduced Matrigel (26). Cell/media/matrigel mixture (100 μL) was plated on top of a 24-well cell culture insert. Media (0.4 mL) were provided to the lower chamber with the treatment of anticancer drugs. Sphere formation and growth were followed for 5 to 7 days. Tumorspheres (>100 μm in diameter) were counted manually.

NSCLC cohort

Patients with NSCLC who had tumor specimens analyzed using the Stanford Solid Tumor Actionable Mutation Panel (STAMP; ref. 27) between January 2014 and August 2018 as part of routine clinical care were included. Two versions of STAMP were used clinically during the era in which these patients were interrogated, one covering 198 genes (302 kb) and the other covering 130 genes (232 kb). We identified a total of 1,021 patients with NSCLC with STAMP results and found 178 patients with *KEAP1, NFE2L2, or CUL3* mutations. From this cohort, patients were included if they had stage IV disease and were treated with a first-line platinum doublet. Patients must have had biopsy confirmed NSCLC and if diagnosed at an early stage, they could not have received chemotherapy in the adjuvant or neoadjuvant setting. Patients who were lost to follow-up, who elected not to receive treatment or who were treated with first-line immunotherapy or molecularly targeted agents were excluded (**Fig. 2**). For the control cohort, 843 patients with available STAMP testing who were wild-type (WT) for KEAP1/NFE2L2/CUL3 mutations were abstracted. Patients with stage IV disease treated with first-line platinum doublet were selected. Patients were matched to the KEAP1/NFE2L2/CUL3 cohort on the basis of gender, age at diagnosis (± 10 years), platinum chemotherapy regimen, smoking history (former, current, never), and race/ethnicity.

Demographic and clinical information, including age at cancer diagnosis, gender, smoking history (former, current, never smoker), ethnicity, performance status, and presence of brain metastases at diagnosis, was abstracted from each patient's medical record. Date of initiation of therapy was defined as the first day of infusional chemotherapy. Date of progression was defined as the date of progression as defined by the treating clinician, or death, whichever came first. Time to treatment failure (TTF) on first-line treatment was calculated by subtracting the date of first-line therapy initiation from the date of treatment discontinuation due to clinical progression, toxicity, patient preference or death, and reported in months. Overall survival (OS) was calculated by subtracting the date of start of chemotherapy from the date of death, also reported in months. Patients who died before radiographic reassessment were deemed to have progressive disease. The study was conducted in accordance with the ethical principles set forward in the Declaration of Helsinki. All patients provided their written consent to participate in a molecular analysis study approved by the Stanford University Institutional Review Board.

Statistical analysis

Statistical analysis was performed using Excel Version 14.7.3, RStudio version 1.1 and Prism 8. The Kaplan–Meier method was used to estimate PFS and OS. For analysis of PFS, patients who were

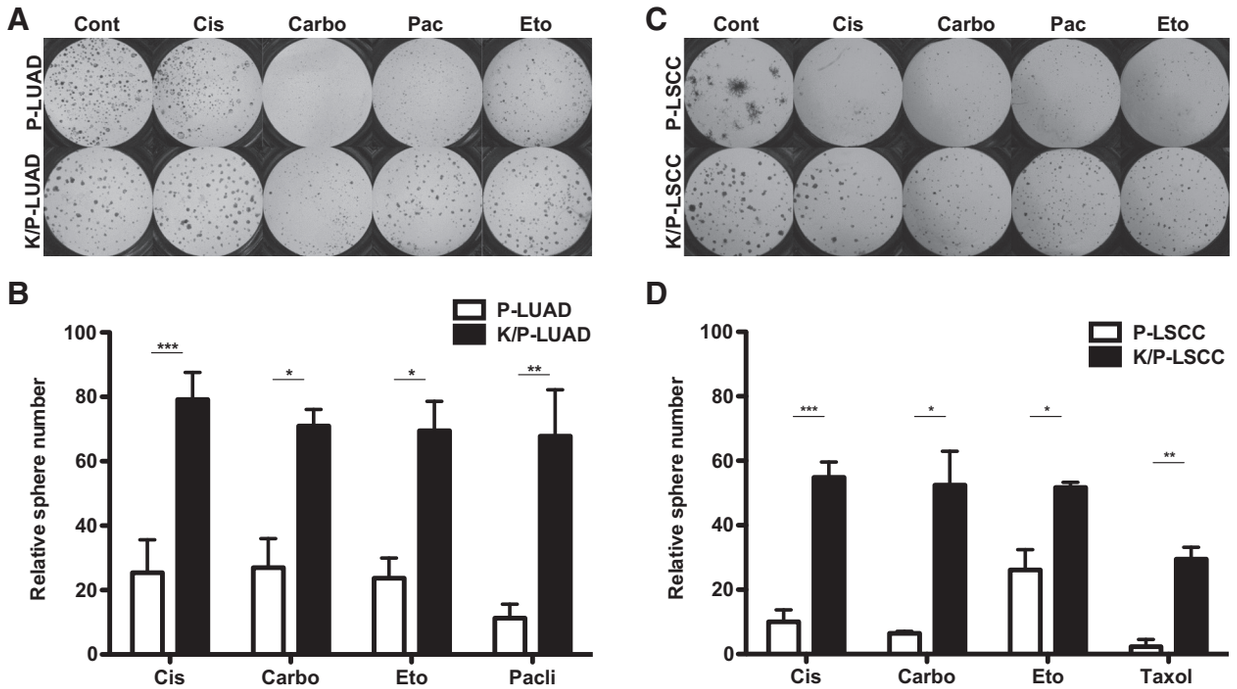

Figure 1.

KEAP1/NFE2L2/CUL3 mutations confer NSCLC chemoresistance. **A** and **B,** Relative number of P-LUAD and K/P-LUAD tumorspheres treated with vehicle or cisplatin (Cis, 20 μmol/L), carboplatin (Carbo, 10 μmol/L), paclitaxel (Pac, 0.5 μmol/L), and etoposide (Eto, 0.3 μmol/L). $N = 3$ biological replicates. **C** and **D,** Relative number of P-LSCC and K/P-LSCC tumorspheres treated with vehicle or cisplatin (Cis, 20 μmol/L), carboplatin (Carbo, 10 μmol/L), paclitaxel (Pac, 0.5 μmol/L), and etoposide (Eto, 0.3 μmol/L). $N = 3$ biological replicates. Data in **B** and **D** are presented as mean ± SEM (***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$).

alive with no evidence of disease progression at the time of the data abstraction (November 30, 2018) or who were lost to follow-up were censored. For OS, patients who were alive or lost to follow-up at the time of data abstraction (November 30, 2018) were censored. Comparison of survival curves was done using the log-rank (Mantel–Cox) test. Significance was defined as $P < 0.05$ and hazard ratio (HR) with 95% CI were reported. Univariate and multivariate analyses were performed with RStudio. A forward selection method was used in which variables with a $P \leq 0.1$ on univariate analysis were selected for input into multivariate analysis.

Results

Keap1 deletion confers NSCLC chemoresistance

In a previous study, we demonstrated that combined deletion of *Trp53* and *Keap1* in airway basal stem cells or peripheral lung cells leads to LSCC and LUAD, respectively (11). Additionally, we found that deletion of *Keap1* confers radioresistance in mouse NSCLC, and that *KEAP1/NFE2L2* mutations are predictive of local failure and recurrence of NSCLC after radiotherapy in human patients. In this study, we set out to investigate whether *Keap1* deletion in NSCLC also confers chemoresistance, because, like irradiation, many anticancer drugs kill cancer cells via generation of ROS and subsequent DNA damage. Additionally, transcriptional targets of NFE2L2 include genes involved in electrophile detoxification and drug efflux (28). We therefore hypothesized that *Keap1* deletion in NSCLC will lead to chemoresistance. To test this hypothesis, we treated cancer cells from *Trp53* deletion–based *Keap1*-WT ($Keap1^{WT}$;$Trp53^{-/-}$, "P-") and *Keap1*-deleted ($Keap1^{-/-}$;$Trp53^{-/-}$, "K/P-") LUADs with several anticancer drugs, including cisplatin, carboplatin, paclitaxel, and etoposide. In *in vitro* tumorsphere assays, K/P-LUAD cells were significantly more resistant than P-LUAD cells to all drugs tested, suggesting that *Keap1* deletion confers resistance to anticancer drugs (**Fig. 1A** and **B**). Similarly, K/P-LSCC tumorspheres also displayed resistance to all drugs tested as compared with the P-LSCC cells (**Fig. 1C** and **D**). These data demonstrate that *Keap1* deletion, which mimics *KEAP1/NFE2L2* mutations found in human tumors, leads to *in vitro* chemoresistance in NSCLC models.

KEAP1/NFE2L2/CUL3 mutation status is a predictor of outcomes after first-line platinum doublet chemotherapy

Based on these preclinical data, we hypothesized that patients with *KEAP1, NFE2L2*, and *CUL3*-mutant metastatic NSCLC will have worse prognosis after first-line platinum doublet chemotherapy than their WT counterparts. To test this hypothesis, we identified a cohort of 178 patients with NSCLC with *KEAP1/NFE2L2/CUL3* mutations on the Stanford STAMP. Of these, 51 patients met our inclusion criteria (**Fig. 2**) and were matched to 52 *KEAP1/NFE2L2/CUL3* WT patients as described above (**Table 1**). Of the 51 patients with *KEAP1/NFE2L2/CUL3* mutations, 35 had somatic mutations in *KEAP1*, 12 patients in *NFE2L2*, and 4 patients in *CUL3*. The average age was 69 in the *KEAP1/NFE2L2/CUL3*-mutant group and 65 in the control group. Across both groups, the majority of patients were male, white, former smokers, diagnosed with *de novo* metastatic disease and had adenocarcinoma histology. The most common first-line chemotherapy regimen was carboplatin or cisplatin plus pemetrexed. There were a similar number of

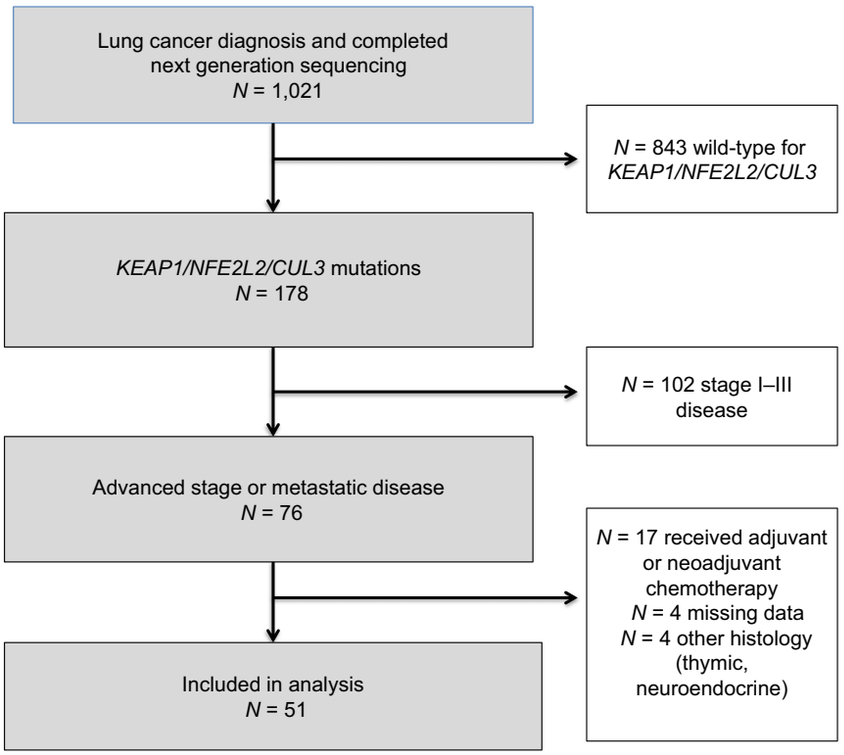

Figure 2.
Consort diagram detailing the identification of *KEAP1/NFE2L2/CUL3*-mutant patients included in this study. WT patients were identified from the 843 patients without *KEAP1/NFE2L2/CUL3* mutations and were matched on gender, age at diagnosis (± 10 years), platinum chemotherapy regimen, smoking history (former, current, never), and race/ethnicity.

patients with brain metastases at diagnosis in both cohorts, and Eastern Cooperative Oncology Group performance status was well matched. The median follow-up was 39 months.

We next explored the distribution of mutations in other genes within our cohort (**Fig. 3**). The most frequently mutated genes in the *KEAP1/NFE2L2/CUL3* group were classic NSCLC driver genes, including *TP53* ($N = 34$; 67%), *KRAS* ($N = 13$; 25%), *STK11* ($N = 7$; 14%), and *epidermal growth factor receptor (EGFR; N* = 5; 10%). Similarly, the most common mutations in the control group were found in *TP53* ($N = 29$; 55%), *KRAS* ($N = 14$; 26%), and *EGFR* ($N = 8$; 15%). These frequencies are in line with those observed in large tumor genotyping studies (6–8), suggesting our cohort is representative of the molecular diversity within NSCLC. We observed similar rates of *EGFR* and *KRAS* mutations across both cohorts. MET (8% vs. 0%, unadjusted $P = 0.01$) and NRAS (6% vs. 0%, unadjusted $P = 0.03$) mutations were slightly more common in the control group, and APC mutations were more common in the *KEAP1/NFE2L2/CUL3* cohort (8% vs. 0%, unadjusted $P = 0.01$), although these differences were not significant upon multihypothesis testing correction. Other targetable mutations such as *ALK, ROS1*, or *BRAF* were uncommon across both cohorts (range, 2%–4%; data not shown).

Next, we compared treatment outcomes in patients with or without *KEAP1, NFE2L2*, or *CUL3* mutations. The average TTF for first-line chemotherapy was 5.0 months in the *KEAP1/NFE2L2/CUL3*-mutant cohort versus 11.4 months in the WT group ($P < 0.001$). The median TTF on first-line chemotherapy for the *KEAP1/NFE2L2/CUL3*-mutant cohort was 2.8 months compared with 8.3 months in the control group ($P < 0.0001$; **Fig. 4A**). The median OS was 11.2 months in the *KEAP1/NFE2L2/CUL3*-mutant group and 36.8 months in the control group ($P = 0.006$; **Fig. 4B**). Given prior reports of inferior outcomes in *KRAS* and *KEAP1* comutated patients, we further examined the effect of *KEAP1/NFE2L2/CUL3* mutations in *KRAS* WT patients. In *KRAS* WT patients, *KEAP1/NFE2L2/CUL3* mutations were associated with shorter time on treatment (2.6 vs. 8.5 months, $P = 0.0001$) and shorter OS (11.2 vs. 38.1 months, $P = 0.0039$) compared with *KEAP1/NFE2L2/CUL3* WT patients, suggesting that the association between *KEAP1/NFE2L2/CUL3* mutations and worse prognosis is not limited to patients with *KRAS* mutations (**Fig. 5A** and **B**).

In addition, as patients with EGFR mutations on average have superior outcomes compared with *EGFR* WT patients, we additionally performed a subset analysis in which *EGFR*-mutant patients were removed from both cohorts (five from the *KEAP1/NFE2L2/CUL3*-mutant cohort and eight from the *KEAP1/NFE2L2/CUL3* WT cohort). TTF remained shorter in *KEAP1/NFE2L2/CUL3*-mutant patients compared with *KEAP1/NFE2L2/CUL3* WT patients (2.7 vs. 8.2 months, $P = 0.0003$). Similarly, *KEAP1/NFE2L2/CUL3*-mutant patients continued to display shorter OS than *KEAP1/NFE2L2/CUL3* WT patients (10.1 months vs. 34.1 months; $P = 0.02$; Supplementary Fig. S1).

Finally, we also analyzed OS and PFS associations of other genes that were frequently mutated in our cohort. Univariate Cox regression analysis was performed on *STK11, TP53*, and *KRAS* mutations (**Table 2**). Genes with $P \leq 0.1$ in univariate analysis (UVA) were forward selected for multivariate analysis (MVA). *STK11* mutations were negatively associated with TTF on UVA (HR, 2.36; 95% CI, 1.09–5.08; $P = 0.03$) but not significant in MVA with *KEAP1/NFE2L2/CUL3* (HR, 2.07; 95% CI, 0.48–0.89; $P = 0.09$). *KEAP1/NFE2L2/CUL3* mutations were significant predictors of TTF (HR, 2.19; 95% CI, 1.42–3.38, $P = 0.00036$) and OS (HR, 2.17; 95% CI, 1.34–3.52; $P = 0.0016$). Thus, KEAP1–NFE2L2 pathway mutations are strongly associated with poor outcomes to first-line chemotherapy in advanced NSCLC.

Table 1. Patient and tumor characteristics.

Characteristics	*KEAP1/NFE2L2/CUL3* mutant ($n = 51$)	Control ($n = 52$)	*P*
Age, mean (y)	69	65	0.06
Gender			
Male	31	28	0.82
Female	20	24	
Race			
White	31	34	0.96
Asian	14	14	
Black or African American	5	2	
Other	1	2	
Smoking history			
Current	3	2	0.97
Previous	44	41	
Never	4	9	
Stage at diagnosis			
Recurrent stage I	4	0	0.97
Recurrent stage II	2	1	
Recurrent stage III	0	0	
De novo stage IV	45	51	
Brain metastases at diagnosis			
Yes	20	17	0.76
No	31	35	
ECOG at diagnosis			
0	15	7	0.94
1	18	26	
2	9	8	
3	1	4	
Not documented	8	7	
Histology			
Adenocarcinoma	46	46	0.98
Squamous	4	4	
Mixed	1	1	
Significant comutations			
TP53	34	29	0.89
KRAS	13	14	
STK11	7	3	
EGFR	5	8	
Chemotherapy			
Platinum[a]/pemetrexed	40	40	0.97
Platinum[a]/etoposide	4	2	
Platinum[a]/taxol	3	4	
Platinum[a]/gemcitabine	4	6	

[a]Carboplatin or cisplatin. ECOG; Eastern Cooperative Oncology Group.

Table 2. Univariable (UVA) and multivariable (MVA) competing risk regression adjusted for the competing risk of death.

Mutation		Time to treatment failure		Overall survival	
		UVA	MVA	UVA	MVA
KEAP1/ NFE2L2/ CUL3	HR	**2.19**	**2.11**	**2.17**	
	95% CI	**1.42–3.38**	**1.37–3.26**	**1.34–3.52**	
	P	**$3.6e^{-04}$**	**$7.2e^{-04}$**	**$1.6e^{-03}$**	
STK11	HR	**2.36**	2.07	1.57	
	95% CI	**1.09–5.08**	0.48–0.89	0.65–3.8	
	P	**0.03**	0.09	0.32	
TP53	HR	1.17		1.08	
	95% CI	0.80–1.72		0.67–1.73	
	P	0.41		0.75	
KRAS	HR	1.02		1.09	
	95% CI	0.67–1.56		0.63–1.89	
	P	0.91		0.75	

Boldface indicates $P < 0.05$.Abbreviations: HR, hazard ratio; CI, confidence interval.

Discussion

In this study, we demonstrated that deletion of KEAP1 confers chemoresistance in preclinical models of LUAD and LSCC and that patients with metastatic NSCLC with *KEAP1/NFE2L2/CUL3* mutations have significantly shorter TTF and OS when treated with first-line platinum doublet chemotherapy. Our prior work demonstrated that *KEAP1* deletion also confers NSCLC resistance to ionizing radiation and that *KEAP1* and *NFE2L2* mutations are associated with worse prognosis after radiotherapy. Together, our findings indicate that *KEAP1/NFE2L2/CUL3* mutations induce resistance to conventional cancer therapies and could serve as a biomarker to predict therapeutic responses for patients with both localized and metastatic NSCLC.

Our findings build upon prior work showing that activation of the KEAP1–NFE2L2 pathway is associated with worse outcomes after chemotherapy. Prior studies have mostly focused on immunohistochemical NFE2L2 and/or KEAP1, and some have shown the association of expression with outcomes in patients with early- or advanced-stage NSCLC (18–20). Given the rapid adoption of next-generation sequencing-based tumor genotyping, our demonstration that mutations in *KEAP1/NFE2L2/CUL3* are associated with worse outcomes after first-line chemotherapy has immediate clinical relevance.

In line with previous studies reporting the frequency of *KEAP1/NFE2L2/CUL3* mutations in 15% to 30% of NSCLCs (6–8, 29), we identified mutations in these genes in 178 of 1,021 patients (17%). Notably, there was no significant difference in the frequency of driver mutations such as *KRAS, EGFR, ALK, ROS1, or BRAF* in between the two cohorts. Our results are also consistent with a recent study reporting that *KEAP1/NFE2L2* mutations are associated with worse outcomes after platinum doublet chemotherapy in patients with NSCLC with *KRAS* mutations (18). This study found that at a median follow-up of 1.5 years, advanced NSCLC patients with mutations in *KRAS* and *KEAP1/NFE2L2* had shorter OS and duration of platinum-based therapy than patients with only mutations in *KRAS*. Our findings expand upon these observations by demonstrating that patients with *KEAP1/NFE2L2/CUL3* mutations have shorter time on treatment and OS regardless of *KRAS* mutation status and extending the median follow-up duration to over 3 years. Due to the small number of patients with NSCLC with *KRAS* mutations in our cohort, we were not powered to detect differences in outcomes within the *KRAS*-mutant cohort.

The TTF and OS we observed in the *KEAP1/NFE2L2/CUL3* WT cohort significantly exceeded the expected outcomes after first-line platinum-based chemotherapy in advanced NSCLC (30). We believe this likely reflects referral bias in the types of patients seen at tertiary institutions, with patients generally being younger and healthier. In addition, our center also treats a higher percentage of Asian patients (~30% of our cohort) and prior studies have shown that Asians have improved progression-free and OS after chemotherapy compared with Caucasian patients (31).

Limitations of our study include its retrospective nature and the single-institution origin of the patients. Furthermore, there were not enough patients in our cohort who received immunotherapy to explore

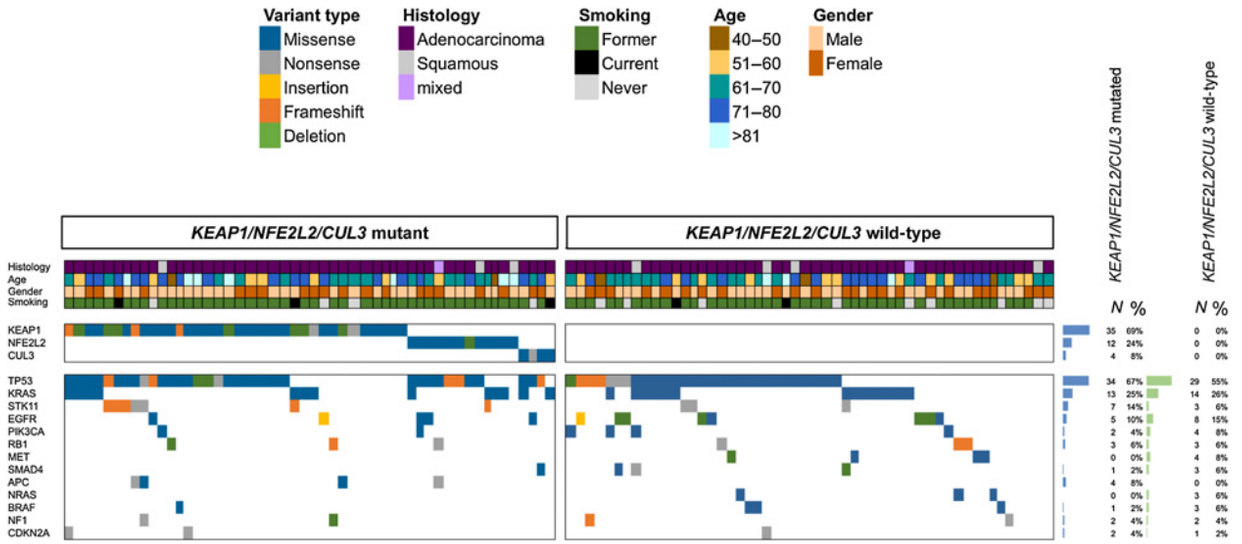

Figure 3.
Co-occurring genetic events in *KEAP1/NFE2L2/CUL3*-mutant and WT cancers.

the potential effect of *KEAP1, NFE2L2*, and *CUL3* mutations on treatment with such agents, which is a significant limitation given that chemoimmunotherapy combinations are now standard of care for first-line treatment of advanced NSCLC. However, prior work on *KRAS*- and *KEAP1/NFE2L2*-mutated adenocarcinoma showed shorter progression-free survival after first-line immunotherapy (18). Therefore, it is likely that the poor prognosis to chemotherapy conferred by KEAP1/NFE2L2 mutations would extend to chemoimmunotherapy combinations. However, further studies in this space are needed. Additionally, due to their low frequency of occurrence, we had insufficient power to test if *NFE2L2* or *CUL3* mutations are individually associated with outcome.

In conclusion, we show that *Keap1* deletion results in chemoresistance in isogenic mouse models of LUAD and LSCC and that patients with mutations in the KEAP1–NEF2L2 pathway have significantly worse outcomes after first-line platinum-based chemotherapy. Our findings add to the growing body of evidence that these mutations identify a particularly resistant subtype of NSCLC and have potential clinical implications. Future work should endeavor to understand if patients with *KEAP1/NFE2L2/CUL3* mutations could benefit from more aggressive upfront therapy. Additionally, our work supports the importance of developing novel strategies to overcome treatment resistance conferred by *KEAP1/NFE2L2/CUL3* mutations (32, 33).

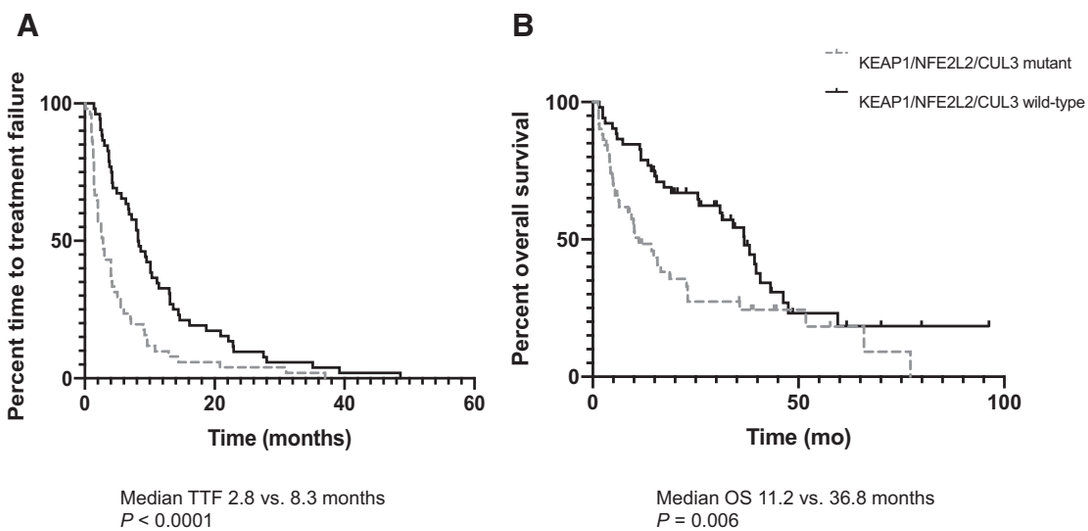

Figure 4.
Association of prognosis and *KEAP1/NFE2L2/CUL3* mutations. Kaplan–Meier survival analyses for (**A**) TTF and (**B**) OS for patients with metastatic NSCLC after first-line platinum doublet chemotherapy.

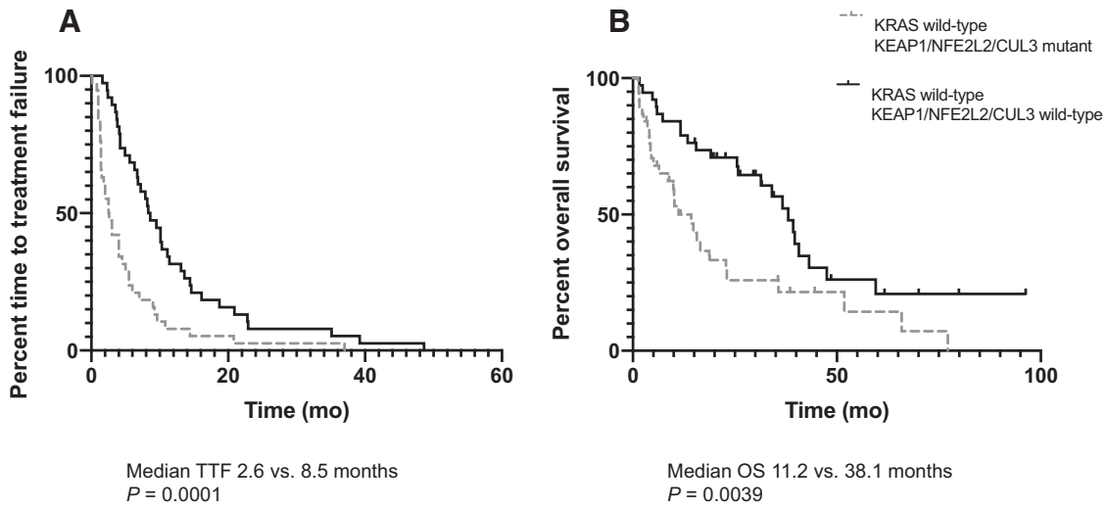

Figure 5.
A, TTF and (**B**) OS for *KRAS* wild-type/*KEAP1/NFE2L2/CUL3*-mutant and *KRAS* wild-type/*KEAP1/NFE2L2/CUL3* WT patients.

Disclosure of Potential Conflicts of Interest

M. Das is an employee/paid consultant for Bristol-Myers Squibb and AstraZeneca, and reports receiving commercial research grants from Verily, United Therapeutics, AbbVie, Varian and Celgene. S.K. Padda is an employee/paid consultant for AstraZeneca, AbbVie, G1 Therapeutic, Janssen Pharmaceuticals, and Pfizer, and reports receiving commercial research grants from Epicentrix, Bayer, Boehringer Ingelheim and 47 Inc. J.W. Neal is an employee/paid consultant for AstraZeneca, Genentech/Roche, Exelixis, Jounce Therapeutics, Takeda Pharmaceuticals and Eli Lilly and Company, and reports receiving commercial research grants from Genentech/Roche, Merck, Novartis, Boehringer Ingelheim, Exelixis, Nektar Therapeutics, Takeda Pharmaceuticals, Adaptimmune and GlaxoSmithKline. M. Diehn is an employee/paid consultant for Roche, AstraZeneca, BioNTech, reports receiving commercial research grants from Varian Medical Systems, and holds ownership interest (including patents) in CiberMed. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: Y. Jeong, J.A. Hellyer, K. Ramchandran, H. Wakelee, M. Diehn
Development of methodology: Y. Jeong, J.A. Hellyer, H. Stehr, M. Diehn
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Jeong, J.A. Hellyer, N.T. Hoang, X. Niu, K. Ramchandran, J.W. Neal, H. Wakelee, M. Diehn
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Jeong, J.A. Hellyer, H. Stehr, N.T. Hoang, X. Niu, S.K. Padda, H. Wakelee, M. Diehn
Writing, review, and/or revision of the manuscript: Y. Jeong, J.A. Hellyer, H. Stehr, N.T. Hoang, X. Niu, M. Das, S.K. Padda, K. Ramchandran, J.W. Neal, H. Wakelee, M. Diehn
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J.A. Hellyer, X. Niu, M. Diehn
Study supervision: X. Niu, M. Diehn

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References

1. Society AC. 2018 cancer facts and figures. Atlanta, GA:ACS; 2018.
2. Gandhi L, Rodríguez-Abreu D, Gadgeel S, Esteban E, Felip E, De Angelis F, et al. Pembrolizumab plus chemotherapy in metastatic non-small-cell lung cancer. N Engl J Med 2018;378:2078–92.
3. Ma Q. Role of nrf2 in oxidative stress and toxicity. Annu Rev Pharmacol Toxicol 2013;53:401–26.
4. Cullinan SB, Gordan JD, Jin J, Harper JW, Diehl JA. The Keap1-BTB protein is an adaptor that bridges Nrf2 to a Cul3-based E3 ligase: oxidative stress sensing by a Cul3-Keap1 ligase. Mol Cell Biol 2004;24:8477–86.
5. Itoh K, Wakabayashi N, Katoh Y, Ishii T, Igarashi K, Engel JD, et al. Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. Genes Dev 1999;13:76–86.
6. Cancer Genome Atlas Research N. Comprehensive genomic characterization of squamous cell lung cancers. Nature 2012;489:519–25.
7. Cancer Genome Atlas Research N. Comprehensive molecular profiling of lung adenocarcinoma. Nature 2014;511:543–50.
8. Imielinski M, Berger AH, Hammerman PS, Hernandez B, Pugh TJ, Hodis E, et al. Mapping the hallmarks of lung adenocarcinoma with massively parallel sequencing. Cell 2012;150:1107–20.
9. Satoh H, Moriguchi T, Takai J, Ebina M, Yamamoto M. Nrf2 prevents initiation but accelerates progression through the Kras signaling pathway during lung carcinogenesis. Cancer Res 2013;73:4158–68.
10. Satoh H, Moriguchi T, Saigusa D, Baird L, Yu L, Rokutan H, et al. NRF2 intensifies host defense systems to prevent lung carcinogenesis, but after tumor initiation accelerates malignant cell growth. Cancer Res 2016;76:3088–96.
11. Jeong Y, Hoang NT, Lovejoy A, Stehr H, Newman AM, Gentles AJ, et al. Role of KEAP1/NRF2 and TP53 mutations in lung squamous cell carcinoma development and radiation resistance. Cancer Discov 2017;7:86–101.

12. Ohta T, Iijima K, Miyamoto M, Nakahara I, Tanaka H, Ohtsuji M, et al. Loss of Keap1 function activates Nrf2 and provides advantages for lung cancer cell growth. Cancer Res 2008;68:1303–9.
13. Singh A, Boldin-Adamsky S, Thimmulappa RK, Rath SK, Ashush H, Coulter J, et al. RNAi-mediated silencing of nuclear factor erythroid-2-related factor 2 gene expression in non-small cell lung cancer inhibits tumor growth and increases efficacy of chemotherapy. Cancer Res 2008;68:7975–84.
14. Zhang P, Singh A, Yegnasubramanian S, Esopi D, Kombairaju P, Bodas M, et al. Loss of Kelch-like ECH-associated protein 1 function in prostate cancer cells causes chemoresistance and radioresistance and promotes tumor growth. Mol Cancer Ther 2010;9:336–46.
15. Ren D, Villeneuve NF, Jiang T, Wu T, Lau A, Toppin HA, et al. Brusatol enhances the efficacy of chemotherapy by inhibiting the Nrf2-mediated defense mechanism. Proc Natl Acad Sci U S A 2011;108:1433–8.
16. Tao S, Wang S, Moghaddam SJ, Ooi A, Chapman E, Wong PK, et al. Oncogenic KRAS confers chemoresistance by upregulating NRF2. Cancer Res 2014;74:7430–41.
17. Wang XJ, Sun Z, Villeneuve NF, Zhang S, Zhao F, Li Y, et al. Nrf2 enhances resistance of cancer cells to chemotherapeutic drugs, the dark side of Nrf2. Carcinogenesis 2008;29:1235–43.
18. Arbour KC, Jordan E, Kim HR, Dienstag J, Yu HA, Sanchez-Vega F, et al. Effects of co-occurring genomic alterations on outcomes in patients with KRAS-mutant non-small cell lung cancer. Clin Cancer Res 2018;24:334–40.
19. Inoue D, Suzuki T, Mitsuishi Y, Miki Y, Suzuki S, Sugawara S, et al. Accumulation of p62/SQSTM1 is associated with poor prognosis in patients with lung adenocarcinoma. Cancer Sci 2012;103:760–6.
20. Yang H, Wang W, Zhang Y, Zhao J, Lin E, Gao J, et al. The role of NF-E2-related factor 2 in predicting chemoresistance and prognosis in advanced non-small-cell lung cancer. Clin Lung Cancer 2011;12:166–71.
21. Solis LM, Behrens C, Dong W, Suraokar M, Ozburn NC, Moran CA, et al. Nrf2 and Keap1 abnormalities in non-small cell lung carcinoma and association with clinicopathologic features. Clin Cancer Res 2010;16:3743–53.
22. Wakabayashi N, Shin S, Slocum SL, Agoston ES, Wakabayashi J, Kwak MK, et al. Regulation of notch1 signaling by nrf2: implications for tissue regeneration. Sci Signal 2010;3:ra52.
23. Okawa H, Motohashi H, Kobayashi A, Aburatani H, Kensler TW, Yamamoto M. Hepatocyte-specific deletion of the keap1 gene activates Nrf2 and confers potent resistance against acute drug toxicity. Biochem Biophys Res Commun 2006;339:79–88.
24. Madisen L, Zwingman TA, Sunkin SM, Oh SW, Zariwala HA, Gu H, et al. A robust and high-throughput Cre reporting and characterization system for the whole mouse brain. Nat Neurosci 2010;13:133–40.
25. You Y, Richer EJ, Huang T, Brody SL. Growth and differentiation of mouse tracheal epithelial cells: selection of a proliferative population. Am J Physiol Lung Cell Mol Physiol 2002;283:L1315–21.
26. Dontu G, Abdallah WM, Foley JM, Jackson KW, Clarke MF, Kawamura MJ, et al. In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells. Genes Dev 2003;17:1253–70.
27. Chuang JC, Stehr H, Liang Y, Das M, Huang J, Diehn M, et al. ERBB2-mutated metastatic non-small cell lung cancer: response and resistance to targeted therapies. J Thorac Oncol 2017;12:833–42.
28. Kensler TW, Wakabayashi N, Biswal S. Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. Annu Rev Pharmacol Toxicol 2007;47:89–116.
29. Frank R, Scheffler M, Merkelbach-Bruse S, Ihle MA, Kron A, Rauer M, et al. Clinical and pathological characteristics of *KEAP1*- and *NFE2L2*-mutated non–small cell lung carcinoma (NSCLC). Clin Cancer Res 2018;24:3087–96.
30. Wakelee H, Belani CP. Optimizing first-line treatment options for patients with advanced NSCLC. Oncologist 2005;10Suppl 3:1–10.
31. Soo RA, Loh M, Mok TS, Ou SH, Cho BC, Yeo WL, et al. Ethnic differences in survival outcome in patients with advanced stage non-small cell lung cancer: results of a meta-analysis of randomized controlled trials. J Thorac Oncol 2011;6:1030–8.
32. Barrera-Rodriguez R. Importance of the Keap1-Nrf2 pathway in NSCLC: is it a possible biomarker? Biomed Rep 2018;9:375–82.
33. Cuadrado A, Rojo AI, Wells G, Hayes JD, Cousin SP, Rumsey WL, et al. Therapeutic targeting of the NRF2 and KEAP1 partnership in chronic diseases. Nat Rev Drug Discovery 2019;18(4):295–317.