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Impact of *KEAP1/NFE2L2/CUL3* mutations on duration of response to EGFR tyrosine kinase inhibitors in *EGFR* mutated non-small cell lung cancer

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ABSTRACT

Objectives: For patients with *Epidermal Growth Factor Receptor* (*EGFR*)-mutated non-small cell lung cancer (NSCLC), frontline EGFR-tyrosine kinase inhibitor (TKI) therapy compared to chemotherapy improves outcomes. However, resistance to these agents uniformly develops. Recently, mutations in the *KEAP1-NFE2L2* pathway have been implicated as a potential mechanism of acquired EGFR TKI resistance.

Materials and methods: We examined all patients with metastatic NSCLC with mutations in both *EGFR* and *KEAP1/NFE2L2/CUL3* identified on next generation sequencing from 2015 - 2018. These patients were compared to a NSCLC control cohort with mutations in *EGFR* and wild type in *KEAP1/NFE2L2/CUL3* matched on the basis of sex, smoking status, age and race. Time to treatment failure on EGFR TKI therapy and overall survival were examined.

Results: Among 228 *EGFR* mutant NSCLCs, 17 (7%) also carried mutations in *KEAP1, NFE2L2, or CUL3*. The most common co-mutation in both the *KEAP1/NFE2L2/CUL3* mutant and wild-type cohort was *TP53*. Patients with *KEAP1/NFE2L2/CUL3* mutations had a shorter median time to treatment failure on EGFR TKI (4.7 months) compared with the wild-type matched cohort (13.0 months), $p = 0.0014$. There was no difference in overall survival.

Conclusion: For NSCLC patients with mutations in *EGFR*, co-mutations in *KEAP1/NFE2L2/CUL3* are associated with significantly decreased time to treatment failure. Our results suggest that these mutations represent a mechanism of intrinsic resistance to TKI treatment.

1. Introduction

Epidermal Growth Factor Receptor (*EGFR*) mutations occur in approximately 30% of cases of non-small cell lung cancer (NSCLC) and are important drivers in the development of lung adenocarcinoma [1]. The last few years have seen the development of several FDA approved EGFR targeted therapies, which have significantly improved progression free survival while achieving more favorable side effect profiles compared to chemotherapy for patients with sensitizing mutations in *EGFR* [2–4]. However, despite the development of new therapies, acquired resistance to EGFR tyrosine kinase inhibitors (TKIs) is inevitable and progression usually occurs within 10–20 months of starting treatment [5].

Although development of EGFR TKI resistance is virtually universal, the mechanisms of resistance are incompletely understood. First generation TKIs (erlotinib, gefitinib) were the standard of care for many years and continue to be widely used despite development of second and third generation drugs. For patients on first generation TKIs, observed mechanisms of resistance include development of T790 M resistance mutations in the EGFR domain in up to two-thirds, up-regulation of proteins in "bypass" pathways such as HER3, MET, IGF-1R, or mutations in *HER2* or *PIK3CA* [1].

Recently, the KEAP1-NFE2L2 pathway has been implicated in the development of EGFR TKI resistance. The KEAP1-NFE2L2 pathway

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plays a key role in cellular stress response by driving expression of anti-free radical defense genes and detoxification enzymes. When a cell is stressed, key residues in the KEAP1 protein are oxidized, rendering it unable to bind to the transcription factor NFE2L2 (also known as NRF2). NFE2L2 then migrates to the nucleus and is able to transcribe its targets, leading to expression of genes containing antioxidant response elements (ARE) in their promoters [6]. Mutations in *KEAP1* or *NFE2L2* occur in approximately 20–25% of lung adenocarcinomas [7,8] and lead to constitutive activation of NFE2L2. This results in pro-survival signals [9], resistance to chemotherapy in *KRAS* mutant tumors [10], and radioresistance [11].

Pre-clinical data suggest that the KEAP1-NFE2L2 pathway may play an important role in EGFR-TKI resistance [12,13]. Early studies have shown that NFE2L2 pathway is activated not only by KEAP1, but also through the EGFR signaling pathway and mutations in the KEAP1 pathway significantly reduce sensitivity to EGFR TKIs *in vitro* and enhance tumor aggressiveness [12,14]. Therefore, we hypothesized that patients with *EGFR* and *KEAP1/NFE2L2* mutated NSCLC will have shortened time to treatment failure on EGFR TKI compared with their *KEAP1/NFE2L2* wild-type (WT) counterparts.

2. Materials and methods

Stage IV NSCLC patients who had tumor specimens analyzed using the Stanford Solid Tumor Actionable Mutation Panel (STAMP) [15] as part of routine clinical care between 2015 and 2018, and who provided their consent to participate in a molecular analysis study approved by the Stanford University Institutional Review Board, were included. STAMP was performed on tumor specimens, the timing of which was determined by the treating clinician.

Patients were selected if they had biopsy-proven stage IV NSCLC, sensitizing *EGFR* driver mutations, and were treated with an EGFR TKI. Patients with recurrent disease after treatment for early stage disease could not have received EGFR TKI in the adjuvant setting during their initial treatment. Patients who were lost to follow up or who elected not to receive treatment were excluded. Demographic information including age at cancer diagnosis, gender, smoking history (former, current, never smoker) and race/ethnicity were abstracted from each patient's medical record. Date of initiation of therapy was defined as the first day of TKI administration. Date of progression was defined as the start of subsequent treatment or death, whichever came first. Time to treatment failure (TTF) was calculated from the date of therapy initiation to the date of progression as defined by the treating clinician. Overall survival (OS) was calculated by subtracting the date of diagnosis from the date of death, also reported in months. Patients who died before radiographic reassessment were considered to have OS events.

For the control cohort, all patients with *EGFR* mutations with available next generation sequencing from 2015 to 2018 who were wild type for *KEAP1/NFE2L2/CUL3* mutations were abstracted. Patients were matched to the *KEAP1/NFE2L2/CUL3* cohort in a ratio of 1:3 on the basis of gender, age at diagnosis, EGFR TKI, smoking history (never, former, current) and race/ethnicity.

2.1. Sample size calculations

One thousand twenty-five patients with NSCLC had available next generation sequencing by STAMP analysis. Approximately 40% of this cohort has *de novo* metastatic or recurrent disease. Assuming an *EGFR* mutation rate of 30% [16] and a *KEAP1/NFE2L2* mutation rate of 15% based off of prior literature [17], we estimated a sample size of 240 patients. The planned sample size of 240 patients would provide at least 80% power to detect a difference in TTF with a hazard ratio of ≥ 2 and a type I error of 5%.

2.2. Statistical analysis

Statistical analysis was performed using Excel Version 14.7.3 and Prism 7. The Kaplan-Meier method was used to estimate TTF and OS. Comparison of survival curves was done using the Log-rank test. Significance was defined as $P < 0.05$. Cox regression was performed and hazard ratio (HR) with 95% CI were reported.

3. Results

We identified a cohort of 228 metastatic *EGFR* mutant NSCLC patients who had undergone tumor genotyping using the Stanford Solid Tumor Actionable Mutation Panel. Of these patients, 17 (7%) had mutations in *KEAP1/NFE2L2/CUL3*. Nine patients received first line EGFR TKI with erlotinib (n = 8) or osimertinib (n = 1). Of the eight patients who did not receive front line TKI therapies, three went on to receive second line TKI and were included in the analysis. Out of twelve patients, 4 had mutations in *KEAP1*, 7 in *NFE2L2,* and 1 in *CUL3* (Cohort mutations in Supplemental Table 1). Cohort characteristics included average age of 68 years old, and a predominance of females (67%), Asians (67%) and never-smokers (83%). There was no significant difference between the *KEAP1/NFE2L2/CUL3* mutated cohort and control cohort in regards to age, gender, smoking status, race, histology or first line therapy (Table 1).

We next examined the most frequent co-mutations in the two cohorts. In both the *KEAP1/NFE2L2/CUL3* mutated and wildtype cohorts, *TP53* was the most common mutation (75% and 50%, respectively). *PIK3CA* mutations occurred in 13% of the control cohort but did not appear in the *KEAP1/NFE2L2/CUL3* mutated cohort. Additional mutations in *MET, RB1, BAP1* and *PTEN* occurred at a frequency of 8.3% each in the *KEAP1/NFE2L2/CUL3* cohort, while PTEN and RB1 occurred in the control cohort at a frequency of 5.3 and 2.6%, respectively (Fig. 1). The most commonly observed *EGFR* mutations across both cohorts were exon 19 deletion and L858R mutation in exon 21, which is in line with other published reports [18,19].

There was a statistically significant difference in time to treatment failure in patients with *EGFR* and *KEAP1/NFE2L2/CUL3* mutations treated with EGFR TKI compared to patients with *EGFR* mutations and wild-type for *KEAP1/NFE2L2/CUL3* (4.7 vs. 13 months), HR 2.8, 95% CI (1.1–7.2), $p = 0.0014$. There was no difference in overall survival between the two cohorts, HR 1.3 (95% CI 0.56–3.1), $p = 0.48$ (Fig. 2). As *TP53* was a commonly mutated gene overall we analyzed TTF and

Table 1
Patient Characteristics.

	KEAP1/NFE2L2/CUL3 mutant (n = 12)	*KEAP1/NFE2L2/CUL3* Wild-Type Control (n = 39)	*p-value*
Age, mean (years)	68	64	*0.33*
Gender			
Male	4	14	*0.87*
Female	8	25	
Smoking status			
Former	2	9	*0.64*
Current	0	0	
Never	10	30	
Race			
White	4	14	*0.87*
Asian	8	25	
Histology			
Adenocarcinoma	12	39	*1.0*
First line therapy			
Erlotinib	8	30	*0.69*
Afatinib	0	1	
Gefitinib	0	1	
Osimertinib	1	1	
Chemotherapy	3	6	

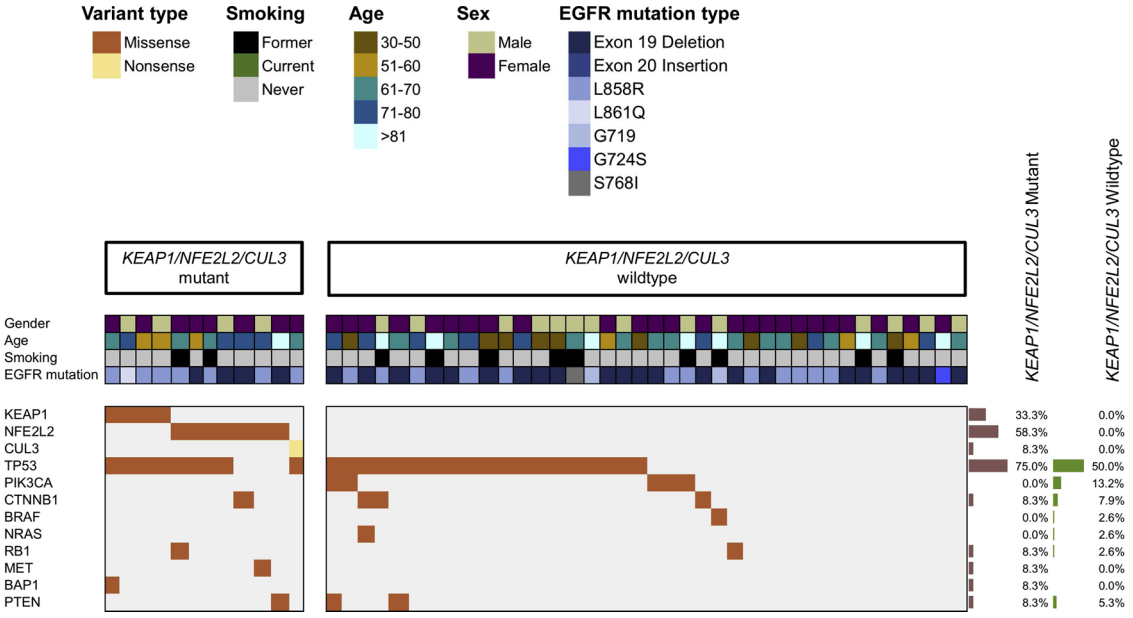

Fig. 1. Oncoprint of EGFR-mutated patients with mutations in *KEAP1/NFE2L2/CUL3* and matched *KEAP1/NFE2L2/CUL3* wildtype cohort.

OS for *TP53* mutant compared with *TP53* wildtype and found no difference in TTF or OS between the two cohorts (Supplemental Fig. 1).

4. Discussion

We found that 7% of *EGFR* mutant lung adenocarcinoma patients in our cohort also had mutations in *KEAP1/NFE2L2/CUL3,* which is similar to the rates found in The Cancer Genome Atlas cohort [7]. Furthermore, patients with *EGFR* mutant tumors and co-mutations in *KEAP1/NFE2L2/CUL3* had a shorter time on TKI therapy than controls. The average TTF on TKI therapy in the *KEAP1/NFE2L2/CUL3* mutated cohort was 4.7 months, which is significantly shorter than the 12.5 months observed in the control cohort and the median progression-free survival of 9–19 months in the seminal EGFR TKI clinical trials [3–5]. This difference in TTF on EGFR TKI therapy did not translate to differences in overall survival, but our study was underpowered to detect such differences.

Recently *KEAP1* pathway mutations have been implicated in leading to clinical resistance to several types of cancer therapy. In a study by Arbour et al., lung adenocarcinoma patients with *KRAS* mutations and co-occurring *KEAP1* mutations were found to have shorter overall survival after initial treatment with chemotherapy [10]. Separately, we previously showed that patients with localized NSCLC and *KEAP1/NEF2L2* mutations have a significantly elevated local failure rate [11]. However, to our knowledge, the impact of *KEAP1/NFE2L2/CUL3* mutations on response to TKI therapy has not been extensively examined. That said, a potential role for *KEAP1* mutations as a resistance mechanism to EGFR TKIs was supported by a recent study which found acquisition of a new *KEAP1* deletion in one patient out of 38 patients at the time of EGFR TKI resistance [13]. Taken together with the intrinsic resistance to EGFR TKIs suggested by the shorter TTF of *KEAP1/NFE2L2/CUL3* mutant patients in our study, these findings support an important role of these mutations in EGFR TKI resistance.

The mechanism by which *KEAP1/NFE2L2/CUL3* mutations lead to TKI resistance in EGFR-mutant NSCLC is currently unclear. Resistance to cytotoxic therapy in patients with *KEAP1/NFE2L2/CUL3* mutations is thought to occur via up-regulation of genes involved in free radical defenses, thus conferring resistance to the reactive oxygen species generated by these agents. It is possible that this mechanism is also at least partially involved in the development of EGFR TKI resistance. However, for patients with *EGFR* mutations treated with TKIs, the mechanism of resistance may also rely on the close association between

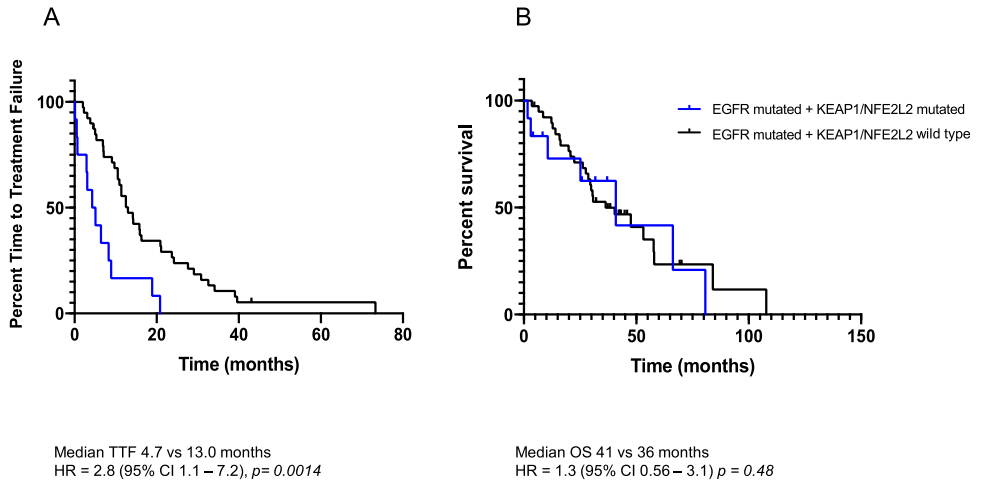

Fig. 2. A) Time to Treatment Failure and B) Overall survival for patients with *EGFR* and *KEAP1/NFE2L2/CUL3* mutations compared with *EGFR* mutations alone.

KEAP1 and EGFR signaling. For example, a recent study by Yamadori et al. found that EGFR signaling activates NFE2L2 and that reduction of KEAP1 mRNA decreased the efficacy of EGFR TKIs in an *EGFR*-mutant cell line [12]. In another study, a gefitinib resistant cell line was found to have acquired a *KEAP1* mutation which mediated resistance [14]. In a third study, Krall et al. performed a CRISPR-Cas9 gene deletion screen and found that loss of KEAP1 promoted survival in an *EGFR*-mutant cell line treated with erlotinib [20].

Limitations of our study include a relatively small sample size, which is only powered to detect a hazard ratio of greater than or equal to two for overall survival. Furthermore, the clinical genotyping assay used did not include sequencing of germline DNA and therefore a subset of the mutations we identified may have been single nucleotide polymorphisms. In addition, the definition of TTF we used relied on determination of progression by treating clinicians and was not assessed using validated criteria such as RECIST [21]. Finally, the retrospective nature of our study introduces the potential of unmeasured confounders.

5. Conclusion

In summary, we show that mutations in the *KEAP1/NFE2L2* pathway occur in approximately 7% of lung adenocarcinomas with activating *EGFR* mutations at diagnosis. Patients with *KEAP1/NFE2L2/CUL3* mutations display relative intrinsic resistance to EGFR TKIs with reduced time to treatment failure. Therefore, more aggressive upfront therapy such as combination treatments with EGFR TKI and chemotherapy and/or angiogenesis inhibitor may be warranted.

Conflict of interest

Dr. Das has received research funding from Celgene, United Therapeutics, AbbVie, Verily and serves on advisory board for Bristol-Myers Squibb and AstraZeneca.

Dr. Padda has received research funding from EpicentRx, Forty Seven Inc, Bayer and consults for Astra Zeneca, AbbVie, G1 Therapeutics and Janssen Phamaceuticals.

Dr. Neal has received research funding from Genentech/Roche, Merck, Novartis, Boehringer Ingelheim, Exelixis, ARIAD/Takeda, Nektar and has served in a consulting role for ARIAD/Takeda, AstraZeneca, Genentech/Roche, Lilly, Exelixis. Loxo Oncology and Jounce Therapeutics.

Dr. Diehn has received research funding from Varian Medical Systems, has served as a consultant for Roche, AstraZeneca, and BioNTech, and has equity in CiberMed.

Dr. Wakelee has received research funding from Astra Zeneca, Novartis, ACEA Biosciences, Bayer, BMS, Celgene, Clovis Oncology, Exelixis, Genentech/Roche, Gilead, Lilly, Merck, Pfizer, Pharmacyclics, Xcovery and serves on an advisory board for AstraZeneca.

The remaining authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.lungcan.2019.05.002.

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