Mutations in the KEAP1-NFE2L2 Pathway Define a Molecular Subset of Rapidly Progressing Lung Adenocarcinoma

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ABSTRACT

Introduction: Molecular characterization studies revealed recurrent kelch like ECH associated protein 1 gene (KEAP1)/nuclear factor, erythroid 2 like 2 gene (NFE2L2) alterations in NSCLC. These genes encode two interacting proteins (a stress response pathway [SRP]) that mediate a cytoprotective response to oxidative stress and xenobiotics. Nevertheless, whether KEAP1/NFE2L2 mutations have an impact on clinical outcomes is unclear.

Methods: We performed amplicon-based next-generation sequencing to characterize the SRP in patients with metastatic NSCLC (Regina Elena National Cancer Institute cohort [n = 88]) treated with first-line chemotherapy. Mutations in the DNA damage response (tumor protein p53 gene [TP53], ATM serine/threonine kinase gene [ATM], and ATR serine/threonine kinase gene [ATR]) were concomitantly analyzed. In lung adenocarcinoma (LAC), we also determined the expression of phosphorylated ataxia telangiectasia mutated kinase and ataxia telangiectasia and Rad3-related protein. Two independent cohorts (the Memorial Sloan Kettering Cancer Center cohort and The Cancer Genome Atlas cohort) with data from approximately 1400 patients with advanced LAC were used to assess the reproducibility of the results.

Results: In the Regina Elena National Cancer Institute cohort, patients whose tumors carried mutations in the KEAP1/NFE2L2 pathway had significantly shorter progression-free survival and overall survival than their wild-type counterparts did (log-rank p = 0.006 and p = 0.018, respectively). This association was driven by LAC in which KEAP1/NFE2L2 mutations were overrepresented in
fast progressors and associated with an increased risk of disease progression and death. LACs carrying KEAP1/ NFE2L2 mutations were characterized by elevated expression of phosphorylated ataxia telangiectasia mutated (pATM) kinase and ataxia telangiectasia and Rad3-related (pATR) protein in association with a pattern of mutual exclusivity with TP53 alterations. The relationship between KEAP1/NFE2L2 mutations and shorter survival was validated in the Memorial Sloan Kettering Cancer Center cohort (n = 1256) (log-rank p < 0.001) and in The Cancer Genome Atlas cohort (n = 162) (log-rank p = 0.039).

**Conclusion:** These findings suggest that a mutant SRP represents a negative prognostic/predictive factor in metastatic LAC and that KEAP1/NFE2L2 mutations may define a molecular subtype of chemotherapy-resistant and rapidly progressing LAC.

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**Keywords:** KEAP1/NFE2L2; Stress response pathway; Lung adenocarcinoma; Fast progressors

**Introduction**

Comprehensive molecular characterization studies carried out to decipher the nature of deregulated pathways in NSCLC conveyed the message that established driver mutations coexist with understudied genomic events. These studies confirmed the remarkable differences existing between lung adenocarcinoma (LAC) and lung squamous cell carcinoma (LSCC). For instance, tumor protein p53 gene (TP53) mutations, which represent the most common alterations in both these histotypes, occur at nearly twice the frequency in LSCC than in LAC (~90% versus ~45%).

However, previously unappreciated similarities also emerged, as exemplified by the genomic alterations in the two main components of the stress response pathway (SRP): kelch like ECH associated protein 1 gene (KEAP1) and nuclear factor, erythroid 2 like 2 gene (NFE2L2 [also known as NRF2]). KEAP1/NFE2L2 alterations were described in 23% of LAC and 31% of LSCC, albeit with a significantly higher prevalence of KEAP1 mutations in LAC and a slightly higher frequency of NFE2L2 alterations in LSCC. Ever since, the SRP has been attracting considerable attention in NSCLC given the mismatch between the frequency of pathway deregulation and the fragmented knowledge about its clinical relevance.

Under homeostatic conditions, kelch like ECH associated protein 1 (KEAP1) mediates the degradation of nuclear factor, erythroid 2 like 2 (NFE2L2) through the proteasome machinery, acting as an adaptor protein of the culin 3 E3 ubiquitin ligase. Upon oxidative stress, KEAP1 is inactivated, leading to the release of NFE2L2, its accumulation, and nuclear translocation. In the nucleus, NFE2L2 mediates the transcription of genes encoding detoxifying enzymes, antioxidant proteins, and multidrug resistance proteins. Given that this cytoprotective response confers resistance to xenobiotics and oxidative stress, it is not surprising that preclinical studies reported chemotherapy- and radiation-resistant traits upon NFE2L2 activation. Pathway deregulation was also tied to resistance to EGFR-directed therapies.

From a clinical perspective, the immunochemistry (IHC) expression of NFE2L2 and NFE2L2-associated transcriptional signatures seemed to be associated with shorter survival in NSCLC. Nevertheless, the clinical implications of KEAP1/NFE2L2 mutations have remained elusive and to some extent contradictory. Rizvi et al. suggested an association between KEAP1 mutations and efficacy of immune checkpoint inhibitors, consistent with preclinical observations. Conversely, in an attempt to describe the KEAP1/NFE2L2 mutational pattern in NSCLC (e.g., mutational frequencies, co-occurrence with other cancer-associated mutations), Frank et al. noticed the lack of objective tumor response in 30 patients with KEAP1-mutant NSCLC treated primarily with chemotherapy. In this latter case, as the authors correctly pointed out, the small number of patients coupled with the short follow-up hindered any comparisons in terms of survival outcomes between patients whose tumors harbored KEAP1/NFE2L2 mutations and patients with a wild-type disease.

Overall, evidence linking genomic alterations in the KEAP1/NFE2L2 pathway to survival outcomes in metastatic NSCLC has remained scattered. On these grounds, we carried out amplicon-based next-generation sequencing to analyze mutations in SRP genes (KEAP1, NFE2L2, and cullin 3 gene [CUL3]), along with alterations in the DNA damage response (DDR) machinery (TP53, ATM serine/threonine kinase gene [ATM], and ATR serine/threonine kinase gene [ATR]). The logic behind the design of this custom gene panel is rooted in the close connection between oxidative stress and oxidative DNA damage/activation of the DDR. Thus, we specifically investigated two molecularly intertwined processes that are recurrently altered in NSCLC. Molecular analyses were performed in 88 patients with metastatic NSCLC treated with first-line chemotherapy, mostly represented by LAC. To evaluate the relationship between KEAP1/NFE2L2 and the DDR to a deeper extent, in the subgroup of patients with LAC we performed IHC to analyze the expression levels of phosphorylated ataxia telangiectasia mutated (pATM) kinase and ataxia telangiectasia and Rad3-related (pATR) protein. Two independent cohorts that together
encompassed more than 1400 LACs (the Memorial Sloan Kettering Cancer Center [MSKCC] clinical sequencing cohort [with advanced disease (n = 1256)] and The Cancer Genome Atlas [TCGA] cohort [with node-positive disease (n = 162)]) were used to externally validate our findings.2,18-20

Materials and Methods

Patients

In the present study we included 88 patients with histologically confirmed, metastatic NSCLC treated with chemotherapy in the first-line setting. The median follow-up time was 13 months (interquartile range 7–33 months). Patients were considered eligible if complete data on clinical features, treatment outcomes, and mutational profiling were available. Tumor responses were evaluated by the Response Evaluation Criteria in Solid Tumors, version 1.1. Progression-free survival (PFS) was calculated as the time between the first cycle of chemotherapy and radiological evidence of disease progression or death due to any cause. Overall survival (OS) was computed as the time from the first cycle of chemotherapy to death due to any cause. This study was conducted in accordance with the Declaration of Helsinki and approved by the ethics committee of the Regina Elena National Cancer Institute (IRE) of Rome. Written informed consent was obtained from all the participants. This study adheres to the Reporting Recommendations for Tumor Marker Prognostic Studies guidelines.21

Next-Generation Sequencing

We considered tissue samples collected before the administration of systemic therapies for advanced disease. All specimens were reviewed for histological verification and to ensure a tumor content higher than 50%. Genomic DNA was extracted from 5-μm formalin-fixed paraffin-embedded (FFPE) tissue sections using the All-Prep DNA/RNA FFPE Kit (Qiagen, Valencia, CA). The DNA was quantified with the Qubit fluorometer and the Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific, Waltham, MA). A custom panel using the AmpliSeq technology for Illumina sequencing was developed to analyze ATM, ATR, TP53, KEAP1, NFE2L2, and CUL3 mutations (Illumina, San Diego, CA). The panel consisted of two primer pools that were combined after the initial amplification. The libraries were prepared by following the standard workflow of the manufacturer's instructions and quantified by Qubit and quantitative polymerase chain reaction. The quality of the libraries was assessed with the Agilent 2100 Bioanalyzer and the Agilent DNA 100 kit (Agilent, Santa Clara, CA). The sequencing was performed with the NextSeq 500 instrument (Illumina), sequencing in paired-end mode 151 base pairs from each side.

IHC

The IHC assessment of pATM and pATR was performed on FFPE tissue samples with the following antibodies: anti-phospho-ATM (Ser1981) (clone 7C10D8) mouse monoclonal antibody (Rockland) at a dilution of 1:200 (pH 6) and anti-phospho-ATR (Ser 428) (clone EPR2184) rabbit monoclonal antibody (Abcam) at a dilution of 1:100 (pH 6). Immunoreactions were revealed by a streptavidin-biotin–enhanced immunoperoxidase technique (Super Sensitive MultiLink, Leica, Milan, Italy) in an automated autostainer (Bond III, Leica). pATM and pATR were graded on a four-grade scale (with 0 meaning negative, 1+ meaning weak, 2+ meaning moderate, and 3+ meaning strong) and considered positive in case of a nuclear immunoreactivity grade of 3+. IHC analysis was performed by two investigators (E. G. and S. B.) who were blinded to baseline patient characteristics, mutational data, and treatment outcomes.

Bioinformatic and Statistical Analyses

Primary analysis of sequences was carried out in the Illumina Basespace cloud environment with the DNA Amplicon pipeline. Parameters were set using BWA aligner and Somatic variant caller, with a variant allele frequency threshold of 5% for somatic variants. After variant calling, variant call frequency files were annotated using ANNOVAR software. We called putative somatic variants by exclusively considering protein-damaging events that are rare in the population (Genome Aggregation Database frequency <1%). Furthermore, we interrogated the Catalogue of Somatic Mutations in Cancer database to remove variants indicated as single-nucleotide polymorphisms. Co-occurrence and mutual exclusivity were determined by using the “somatic interactions function” of Maftools.

For statistical analyses, the Pearson chi-square test of independence (two tailed) or the Fisher exact test were used for investigating the relationship between categorical variables. Survival curves were estimated with the Kaplan-Meier product-limit method and compared by using the log-rank test. Multivariate Cox models for PFS and OS were built with variables potentially affecting the outcome of interest. The related estimates were reported as hazard ratio (HR) and 95% confident interval (CI). The nature of the clinical variables included in the multivariate Cox regression models was determined on the basis of the possible association with the
outcome of interest to take into account potential confounding factors. To this end, we applied the “one in 10 rule” (i.e., one variable per 10 events). To further evaluate the stability of the model, we also used stepwise regression (forward selection).

The group of NSCLCs with a mutant SRP was defined by the presence of somatic mutations in either KEAP1 or NFE2L2. Fast progressors (FPs), conventional progressors (CPs), and slow progressors (SPs) were determined on the basis of PFS tertiles. Data related to the MSKCC and TCGA studies were obtained from cBioPortal (available at: http://www.cbioportal.org [downloaded on March 28, 2019]). Regarding the MSKCC data set, we analyzed the whole cohort given that it is represented by advanced LAC (n = 1256). Considering the size of this cohort, we also analyzed the enrichment of KEAP1/NFE2L2 mutations across OS quartiles. Regarding the TCGA cohort, we extracted cases with node-positive disease (n = 162). Indeed, the TCGA cohort largely comprises patients with early-stage tumors (stage I–II), and only a limited number of samples were obtained from patients with stage IV LAC (n = 31). On this basis, we decided to focus our analysis on the subset of patients with node-positive disease, which allowed a fairly reliable comparison with our cohort.

To ensure consistency with our molecular characterization, we did not consider copy number variations. The level of significance was defined as a p value less than 0.05. Statistical analyses were carried out using SPSS software (version 21.0, IBM, Armonk, NY).

Results

Characteristics of the Patients and Mutational Pattern

We first analyzed tissue samples from 88 patients with metastatic NSCLC (the IRE cohort) who received chemotherapy in the first-line setting (Supplementary Table 1). The mutational frequencies of the investigated genes were consistent with those reported in comprehensive characterization studies (Fig. 1A).1,2 Regarding mutations in the SRP, we confirmed the higher representation of KEAP1 mutations in LAC and a comparable mutational frequency of KEAP1 and NFE2L2 in LSCC (Fig. 1B and C). A detailed list of the KEAP1/NFE2L2 mutations is provided in Supplementary Table 2, and their nature represented on the linear proteins is illustrated in Supplementary Figure 1. Among the 11 patients with LAC who were carrying alterations in EGFR

Figure 1. Oncoprints showing the distribution of mutations in the whole Regina Elena National Cancer Institute cohort (A), in lung adenocarcinoma (LAC) (B), and in lung squamous cell carcinoma (LSCC) (C). Beside each oncoprint a mutation summary (including variant classification, single-nucleotide variant [SNV] class, and number of variants per sample) is provided. TP53, tumor protein p53 gene; KEAP1, kelch like ECH associated protein 1 gene; ATM, ATM serine/threonine kinase gene; NFE2L2, nuclear factor, erythroid 2 like 2 gene; ATR, ATR serine/threonine kinase gene; CUL3, cullin 3 gene.
or ALK receptor tyrosine kinase gene (*ALK*), one single KEAP1 mutation was detected (see Fig. 1B). The suggestion of mutual exclusivity between KEAP1/NFE2L2 and EGFR mutations is also reported in the TCGA and MSKCC data sets (available at: http://www.cbioportal.org), indicating that LAC with a mutant SRP may represent a defined subset of tumors rarely treatable with EGFR-directed therapies. We did not observe any significant association between KEAP1/NFE2L2 mutations and baseline clinical features, including programmed death ligand 1 expression (Supplementary Table 3).

**KEAP1/NFE2L2 Mutations Are Associated with Inferior Survival Outcomes in Advanced NSCLC**

We next investigated whether KEAP1/NFE2L2 mutations had an impact on PFS and OS in the IRE cohort. Patients whose tumors harbored mutations in the SRP had significantly shorter PFS (log-rank *p* = 0.006) and OS (log-rank *p* = 0.018) than did patients with a wild-type disease (Fig. 2A and B). Univariate Cox models showed that patients with SRP-mutant tumors were at increased risk of disease progression and death (Supplementary Table 4). Moreover, multivariate Cox regression models indicated that KEAP1-NFE2L2 mutations are independent predictors of adverse survival outcomes (for PFS, HR = 2.09, 95% CI: 1.20–3.65, *p* = 0.009; for OS, HR = 1.80, 95% CI: 1.03–3.13, *p* = 0.037) (Fig. 2C).

**KEAP1/NFE2L2 Mutations Are Overrepresented in FPs and Confer Inferior Survival Outcomes in LAC**

To better define the clinical significance of KEAP1/NFE2L2 mutations in metastatic NSCLC, we verified whether SRP pathway alterations displayed a differential distribution throughout the three prespecified groups of patients. **Figure 2.** Kaplan-Meier survival curves of progression-free survival (PFS) (A) and overall survival (OS) (B) comparing kelch like ECH associated protein 1 gene (*KEAP1*)/nuclear factor, erythroid 2 like 2 gene (*NFE2L2*)–positive cases with their wild-type (Wt) counterparts (the whole Regina Elena National Cancer Institute cohort). (C) Forest plot illustrating the multivariate Cox regression analyses for PFS and OS. Mut, mutant; CI, confidence interval; PS, performance status; LSCC, lung squamous cell carcinoma; LAC, lung adenocarcinoma; PR, partial response; PD, progressive disease; TKI, tyrosine kinase inhibitor; ICI, immune checkpoint inhibitor.
patients in terms of disease evolution, namely, FPs, CPs, and SPs. These groups were defined on the basis of PFS tertiles, as detailed in the Bioinformatic and Statistical Analyses section. In the entire IRE cohort, a nonsignificant association was recorded between KEAP1/NFE2L2 mutations and FPs (chi-square \( p = 0.061 \)) (Fig. 3A).

However, whereas in LSCC KEAP1/NFE2L2 alterations were significantly more common in the group of CPs (chi-square \( p = 0.033 \)) (Fig. 3B), in LAC they were significantly overrepresented in FPs (chi-square \( p = 0.012 \)) (Fig. 3C). Importantly, with the exception of EGFR status, we did not record any significant imbalance when comparing the distribution of clinical variables in FPs, CPs, and SPs, thus indicating that hyperprogressions are intimately tied to SRP mutations (Supplementary Table 5). Consistently, when LAC was considered specifically, KEAP1/NFE2L2 mutations were associated with an increased risk of disease progression and death in multivariate Cox regression models (for PFS, HR = 2.34, 95% CI: 1.16–4.71, \( p = 0.017 \); for OS, HR = 1.96, 95% CI: 1.01–3.80, \( p = 0.048 \)) (Fig. 4A). Comparable results were obtained in multivariate Cox regression models for PFS and OS generated by stepwise regression with forward selection (see Fig. 4A). For completeness, univariate Cox regression models are presented in Supplementary Table 6. Conversely, mutations in the SRP did not apparently affect PFS and OS in LSCC (log-rank \( p = 0.118 \) and log-rank \( p = 0.701 \), respectively) (Fig. 4B and C).

It is worth mentioning that we did not include CUL3 in our genomic signature. Indeed, a three-gene predictor did not improve the performance of the original model (Supplementary Fig. 2), given that this more complex signature reclassified only two cases.

Moreover, cullin 3 cannot properly be considered a pathway-specific component, given that it belongs to the ubiquitin-proteasome system and is involved in an array of biological processes.

**IHC Evaluation of pATR/pATM and the Analysis of Somatic Interactions Revealed the Relationship between KEAP1/NFE2L2 and the DDR**

Given that we hypothesized a connection between KEAP1/NFE2L2 mutations and key pathways involved in cell cycle checkpoints and DNA repair, we also tested the expression of activated (phosphorylated) ATR and ATM by IHC in LAC (Fig. 5A). First, we noticed a significant association between the KEAP1/NFE2L2 genomic signature and high expression of both pATR and pATM (Fisher \( p = 0.024 \) and chi-square \( p = 0.039 \), respectively. Fig. 5B). Second, we reported a significant enrichment of pATM expression in the subgroup of FPs (chi-square \( p = 0.032 \) [Fig. 5B]). Moreover, the analysis of somatic

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**Figure 3.** Oncoprints summarizing the distribution of mutations in the investigated genes across the three different patterns of disease evolution (fast progressors [FPs] [green], conventional progressors [CPs] [sky blue], and slow progressors [SPs] [orange]) in the whole study cohort (A), in lung squamous cell carcinoma (LSCC) (B), and in lung adenocarcinoma (LAC) (C). TP53, tumor protein p53 gene; KEAP1, kelch like ECH associated protein 1 gene; ATM, ATM serine/threonine kinase gene; NFE2L2, nuclear factor, erythroid 2 like 2 gene; ATR, ATR serine/threonine kinase gene; CUL3, cullin 3 gene.
interactions revealed that KEAP1 and TP53 mutations tend to be mutually exclusive, as further confirmed in the TCGA and MSKCC cohorts (Fig. 5C). Collectively, data related to the different expression of pATR and pATM in KEAP1/NFE2L2-mutant and wild-type LAC, along with the mutual exclusivity between KEAP1/NFE2L2 and TP53 mutations, suggest that tumors harboring genomic alterations in the SRP are characterized by a conserved, and supposedly overactive, DDR machinery. In turn, this specific molecular background translates into adverse therapeutic and survival outcomes.

**External Validation of the KEAP1/NFE2L2 Genomic Signature in the MSKCC and TCGA Cohorts**

Finally, we sought to externally validate our findings in two independent cohorts. In the MSKCC cohort (n = 1256), KEAP1/NFE2L2 mutations were associated with inferior OS (log-rank \( p < 0.001 \)) (Fig. 6A). This relationship was not modified when patients with EGFR mutations or ALK rearrangements were excluded (log-rank \( p < 0.001 \)) (Fig. 6B). Given that we observed an overrepresentation of KEAP1/NFE2L2 mutations in FPs in the IRE cohort, we verified the distribution of KEAP1/NFE2L2 mutations across OS quartiles in the MSKCC cohort. Again, we noticed a preferential and statistically significant distribution of KEAP1/NFE2L2 mutations in the lowest quartile (Q1), which contained more than 40% of all the detected mutations (chi-square \( p < 0.001 \) [Fig. 6C]). The robustness of the KEAP1/NFE2L2 genomic signature was confirmed in a multivariate Cox regression model that included sex, smoking history, EGFR status, ALK status, KRAS, phosphatase and tensin homolog gene (PTEN), and phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha gene (PIK3CA) mutations (HR = 1.64, 95% CI: 1.26–
2.13, \( p < 0.001 \) (Fig. 6D). Moreover, subgroup analysis revealed that KRAS status did not modify the association between KEAP1/NFE2L2 mutations and shorter survival (Supplementary Fig. 3). The connection between KEAP1/NFE2L2 mutations and shorter survival was also observed in the TCGA node-positive LAC cohort \((n = 162)\) (log-rank \( p = 0.039 \)) (Fig. 6E).

In the TCGA study, we also verified the impact of biallelic inactivation of KEAP1 by using a classification according to which homozygous deletion (GISTIC-2) or combined heterozygous deletion (GISTIC-1) and mutation were considered biallelic inactivation and heterozygous deletion (GISTIC-1) or mutation was considered monoallelic loss. However, this classification apparently does not hold any prognostic significance (Supplementary Fig. 4). Nevertheless, we cannot draw any definitive conclusion from this analysis, given that we did not specifically evaluate other mechanisms of inactivation (e.g., epigenetic silencing).

Regarding LSCC, in the TCGA node-positive cohort survival curves did not significantly differ when SRP-mutant cases were compared with wild-type cases, whereas the analysis of the MSKCC cohort yielded the opposite results (Supplementary Fig. 5A and B). Given that the two cohorts are comparable in size, we cannot draw any definitive conclusion regarding the association between KEAP1/NFE2L2 mutations and survival outcomes in LSCC.

**Discussion**

In the present study, we investigated the clinical implications of KEAP1/NFE2L2 mutations in metastatic NSCLC, with particular emphasis placed on LAC. The logic behind our study was that although deregulation of the SRP is gaining considerable attention in NSCLC owing to its frequent deregulation, whether this alteration translates into clinically relevant information is unclear. We sought to address this issue according to a straightforward strategy, evaluating mutations in SRP genes and in central components of the DDR machinery in first cohort (the IRE cohort) and then verifying our conclusions in two independent cohorts, which overall included data from approximately 1400 patients with advanced LAC. We are aware that the retrospective design invites caution. Nevertheless, the reproducibility of our findings in two independent and clinically comparable cohorts significantly strengthens our conclusions. Moreover, our data are consistent with two very
recently published reports, the first of which was intended to delineate the mutational pattern of KEAP1/NFE2L2 and the second of which specifically focused on the subset KRAS-mutant tumors, which represents approximately 25% of all NSCLC.23

In our opinion, the results presented herein have important elements of novelty that hold the potential to open up novel therapeutic scenarios, adding a further level of molecular segmentation of LAC. First, KEAP1/NFE2L2 mutations are associated with adverse survival outcomes in patients with metastatic LAC and are significantly enriched in FPs. Thus, KEAP1/NFE2L2 mutations plausibly define a molecular subset of LAC characterized by intrinsic chemotherapy resistance and a hyperprogressive behavior. Second, the pattern of mutual exclusivity between KEAP1/NFE2L2 mutations and EGFR mutations/ALK rearrangements denotes that KEAP1/NFE2L2-mutant LAC may represent a distinct...
molecular subtype of tumors untreatable with EGFR- and ALK receptor tyrosine kinase–directed therapies. This observation, coupled with the rapidly progressive nature of LAC carrying KEAP1/NFE2L2 mutations, calls for intense research efforts aimed toward developing novel pharmacological strategies capable of achieving a selective targeting of the SRP pathway. Third, LAC carrying KEAP1/NFE2L2 mutations plausibly harbor highly efficient DNA-protecting mechanisms. DNA damage can be of an endogenous and exogenous nature, being correlated with the elevated production of reactive oxygen species stemming from increased metabolic demands (oxidative DNA damage), the replicative stress originating from activating mutations in oncogenes that control cell proliferation and exposure to some chemotherapeutics.24 Our idea is that the combination of SRP mutations with an extremely proficient genome-protecting machinery generates a “superfit” status characterized by an extreme resistance to current therapeutics and ultrarapid evolution.

The biological output of aberrant NFE2L2-mediated gene transcription provides a robust preclinical ground to our observations. As already mentioned, NFE2L2 drives a transcriptional program that enhances cytoprotection.3,4,25,26 Moreover, our data related to the association between SRP mutations and the DDR suggest that KEAP1/NFE2L2-mutant LAC efficiently handles DNA damage.

At the same time, NFE2L2 promotes a metabolic reprogramming that sustains cellular proliferation under overactive phosphoinositide 3-kinase–Akt signaling.27 Thus, deregulation of the KEAP1/NFE2L2 pathway enables cancer cells to withstand chemotherapy while undergoing rapid proliferation. These combined features easily explain the adverse survival outcomes conferred by KEAP1/NFE2L2 mutations and the enrichment of these alterations in rapidly progressing LAC.

Next, our choice to concomitantly characterize the SRP and the DDR pathway deserves to be mentioned. We believed that mutations in central orchestrators of the DDR would have been instrumental to better frame dysregulated SRPs in the clinical setting. When evaluating survival outcomes, we did not observe any potential interaction between these two oncogenic routes given that more complex genomic model that integrated DDR features into the KEAP1/NFE2L2 signature did not improve its predictive performance. Nevertheless, in the subgroup of patients with metastatic LAC we recorded a significant association between KEAP1/NFE2L2 mutations and nuclear expression of pATR and pATM. This co-occurrence, coupled with the mutual exclusivity between KEAP1/NFE2L2 and TP53 mutations, raised the hypothesis that two molecular avenues (SRP and DDR) actively cooperate in generating a “hybrid” phenotype characterized by intrinsic chemotherapy resistance and rapid proliferation.

Our previous data suggested that a full appreciation of DDR activity requires the combined assessment of pathway mutations along with protein-level analysis of activated DNA damage markers and kinases.28 On this basis, we have initiated a deeper characterization of the DDR to understand whether DDR markers may further improve the predictive ability of the KEAP1/NFE2L2 signature.

To this end, we also planned the analysis of mutations in genes promoting cellular proliferations, such as KRAS, BRAF, PIK3CA, and EGFR, to take into account an important source of replicative stress and endogenous DNA damage.

In conclusion, the data presented herein suggest that KEAP1/NFE2L2 mutations may delineate a novel molecular subgroup of LAC, conferring extremely adverse survival outcomes when treated with chemotherapy. The potential “Janus-faced” role of the mutant SRP pathway, namely, a detrimental role when tumors are treated with chemotherapy but also a potentially predictive significance of increased efficacy of immune checkpoint inhibitors, deserves increased consideration given the rapidly evolving therapeutic landscape of metastatic NSCLC. On this basis, post hoc analyses of randomized phase III trials comparing chemotherapy with immunotherapy or chemoimmunotherapy with chemotherapy are strongly advised to better quantify the impact of KEAP1/NFE2L2 mutations in LAC and, more importantly, to address whether the presence of these alterations can add a further level of therapeutically exploitable molecular stratification. Finally, we cannot rule out the possibility that a mutant KEAP1/NFE2L2 pathway represents a negative prognostic factor in LSCC also. To this end, more focused investigations are warranted at both the preclinical and clinical levels.

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Supplementary Data
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