KEAP1-Mutant NSCLC: The Catastrophic Failure of a Cell-Protecting Hub

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ABSTRACT

Mutations in the KEAP1-NRF2 pathway are common in NSCLC, albeit with a prevalence of *KEAP1* mutations in lung adenocarcinoma and an equal representation of *KEAP1* and *NFE2L2* (the gene encoding for NRF2) alterations in lung squamous cell carcinoma. The KEAP1-NRF2 axis is a crucial modulator of cellular homeostasis, enabling cells to tolerate oxidative and metabolic stresses, and xenobiotics. The complex cytoprotective response orchestrated by NRF2-mediated gene transcription embraces detoxification mechanisms, ferroptosis protection, and metabolic reprogramming. Given that the KEAP1-NRF2 pathway controls core cellular functions, it is not surprising that a number of clinical studies connected *KEAP1* mutations to increased resistance to chemotherapy, radiotherapy, and targeted agents. More recently, an immune-cold tumor microenvironment was described as a typical feature of *KEAP1*-mutant lung adenocarcinoma. Consistently, a reduced efficacy of immunotherapy was reported in the *KEAP1*-mutant background. Nevertheless, the connection between *KEAP1* and immune resistance seems more complex and dependent on coexisting genomic alterations. Given the clinical implications of deregulated KEAP1-NRF2 pathway in lung cancer, the development of pathway-directed anticancer treatments should be considered a priority in the domain of thoracic oncology.

Keywords: KEAP1; NRF2; Lung cancer; Immunotherapy

Introduction

The KEAP1-NRF2 system represents the major defensive mechanism against oxidative and electrophilic stresses.[1,2] Preclinical and clinical studies in NSCLC revealed that loss-of-function (LOF) mutations in *KEAP1* and gain-of-function mutations in *NFE2L2* (the gene encoding for NRF2) confer resistance to chemotherapy, radiotherapy, and targeted agents.[1] More recently, *KEAP1* mutations were connected to adverse survival outcomes in patients with advanced NSCLC treated with immunotherapy, particularly in the presence of specific co-occurring mutations.[3–5] The increased appreciation of deregulated KEAP1-NRF2 axis in NSCLC is fueling the

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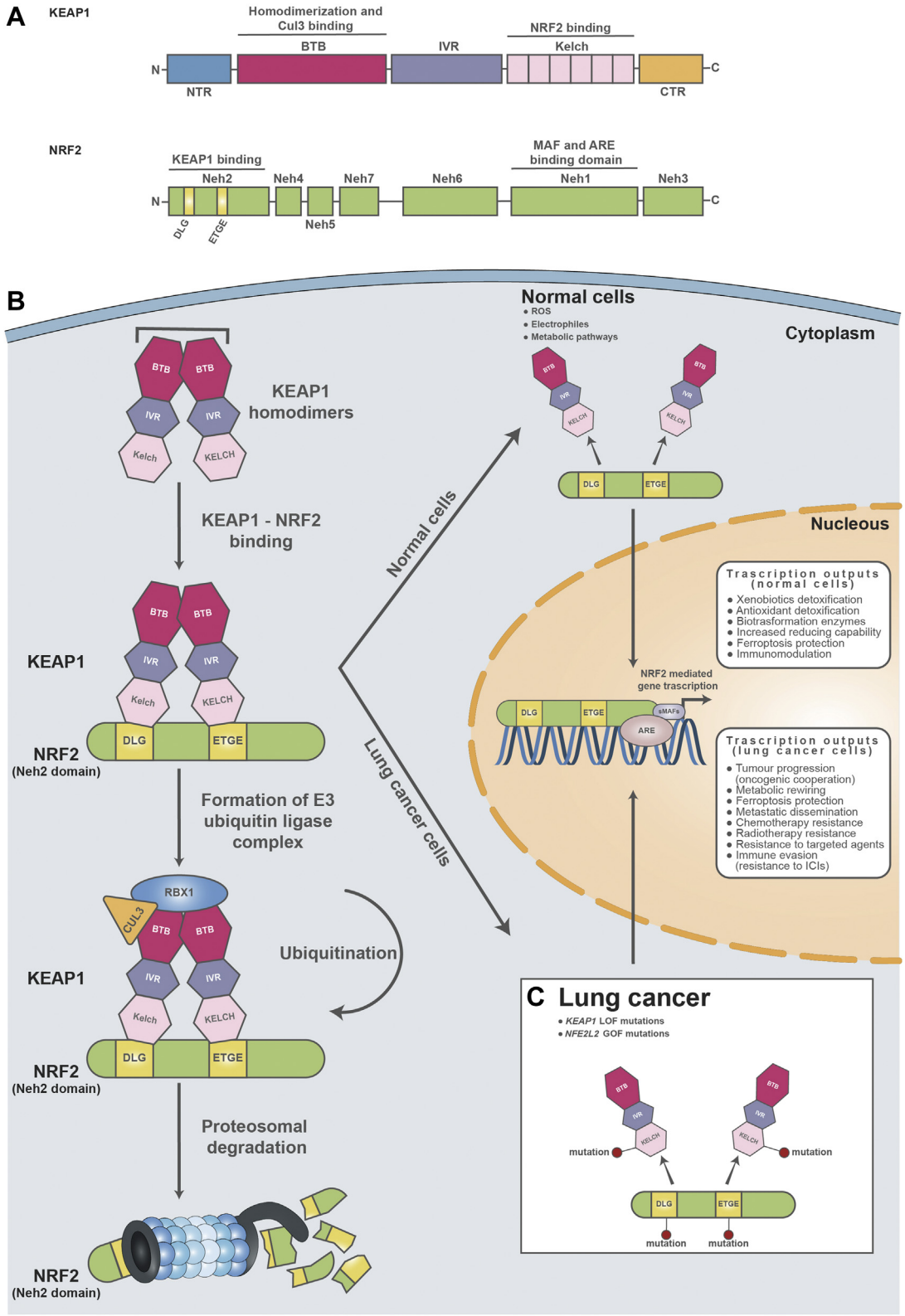

Figure 1. Schematic overview of the KEAP1-NRF2 pathway. (*A*) NRF2 and KEAP1 protein domains. (*B*) Activation and regulation of NRF2 in normal cells. In unstressed cells, KEAP1 binds NRF2 mediating the proteasome-dependent degradation of NRF2. ROS/nitrogen species and metabolic intermediates lead to conformational changes in KEAP1, resulting in impaired NRF2 targeting. In the nucleus, NRF2 modulates the transcription of target genes (genes containing antioxidant-responsive elements in their promoter), orchestrating a cytoprotective and genoprotective program. (*C*) Deregulated KEAP1-NRF2 pathway in NSCLC. In NSCLC, *KEAP1* LOF mutations and *NFE2L2* GOF mutations mediate an array of tumor-promoting

search of pharmacologic strategies for targeting aberrant pathway activation, and early phase clinical trials with compounds targeting metabolic vulnerabilities are ongoing. Here, we discuss the molecular functions of the KEAP1-NRF2 pathway, its role in lung tumorigenesis, evidence linking *KEAP1* and *NFE2L2* mutations to reduced efficacy of established anticancer treatments in patients with NSCLC, and the strategy proposed for targeting deregulated KEAP1-NRF2 activity.

KEAP1-NRF2 Function

In unstressed cells, the redox-sensitive KEAP1 protein binds NRF2 at DLG and ETGE degron motifs (conserved amino acid motifs) in the Neh2 domain, triggering NRF2 proteasomal degradation by means of the CUL3-RBX1 E3 ubiquitin ligase complex (Fig. 1*A* and *B*).[1,2]

The accumulation of reactive oxygen and nitrogen species modifies sensor cysteines in KEAP1, leading to conformational changes in KEAP1 homodimers and impaired NRF2 ubiquitination.[1,2] Comparable effects are elicited by metabolic intermediates produced during glycolysis, tricarboxylic acid cycle, or lipid metabolism (Fig. 1*B*).[6–9] Beyond chemical and metabolic cues, oncogenic stimuli intersect the KEAP1-NRF2 axis (RAS/MAPK, p62).[10,11]

When NRF2 is released from KEAP1 inhibition, it translocates to the nucleus, dimerizes with small MAF proteins, and induces the expression of target genes containing antioxidant response elements in their promoter.[1,2] The transcriptional program orchestrated by NRF2 aims at re-establishing the redox homeostasis and at protecting cells from xenobiotics (Fig. 1*B*).[1,2] Indeed, target genes encode for mediators of antioxidant detoxification, biotransformation enzymes, enzymes increasing the cellular reducing capability, and multidrug efflux pumps (Fig. 1*B*). A specific gene module is deputed to the prevention of ferroptosis, a nonapoptotic and iron-dependent cell death modality triggered by the accumulation of lipid reactive oxygen species.[12,13]

Given that redox cycling mechanisms require NADPH and other substrates, the biological output of NRF2 activation also envisions the redirection of glucose, glycolytic intermediates, and glutamine toward anabolic pathways to fulfill this increased demand.[14–16] As a result, NRF2 activation, elicited by redox, metabolic, or xenobiotic stressors, culminates in a profound reorganization of core cellular processes, coupling genoprotective and cytoprotective pathways to metabolic rewiring. Intersecting a number of processes lying at the centerpiece of cell fate decision, the KEAP1-NRF2 pathway was also connected with the DNA damage response machinery, the system balancing DNA damage repair, tolerance and apoptosis.[17,18]

A further branch of KEAP1-NRF2–regulated processes refers to immunomodulation.[2] The immune-associated function of the pathway gained attention in tumors owing to the success of immune checkpoint inhibitors (ICIs) and the adverse survival outcomes of patients with NSCLC whose tumors harbored *KEAP1* mutations.[18,19] Evidence indicates that NRF2 interferes with the transcription of cytokines, chemokines, and the type I interferon-inducing cGAS/STING signaling.[20–22] Moreover, metabolites abnormally consumed or secreted after metabolic reprogramming modify the tumor microenvironment composition, in processes that may generate a hostile milieu for antitumor T-cell functions. Consistently, an immune-desert tumor microenvironment is emerging as a hallmark of *KEAP1*-mutant lung adenocarcinoma (LUAD).[3] Likewise, low $CD8^+$ tumor-infiltrating lymphocyte density, assessed by immunohistochemistry, was noticed in *KEAP1*-mutant lung squamous cell carcinoma (LUSC).[23]

Role of the KEAP1-NRF2 Pathway in Tumorigenesis

Carcinogen-induced models and genetically engineered mouse models (GEMMs) have been instrumental in understanding KEAP1-NRF2 pathway function in neoplastic diseases. In particular, a "Janus-faced" role during carcinogenesis was proposed, which is, protumorigenic and antitumorigenic in a stage- and context-dependent manner. In normal cells, NRF2 activation ensures protection against cancer initiation by preventing cellular damage induced by chemicals and radiation (the canonical, protective role).[24–27] For instance, in a chemical carcinogenesis model, an increased tumor formation was observed in $Nrf2^{-/-}$ mice as compared with wild-type animals.[26] Likewise, constitutive NRF2 activation protected mice from radiation-induced skin carcinogenesis.[27] Conversely, in cancer cells, NRF2 activation promotes disease progression, metastatic dissemination, and resistance to cytotoxic agents (the "dark side" of NRF2; Fig. 1*C*).[28–32] In this setting, it was described that, after urethane exposure, $Nrf2^{-/-}$ mice developed a higher number of microscopic nodules than the $Nrf2^{+/+}$ counterparts.[29] Nevertheless, on long-term exposure, lung tumors were more frequently observed in $Nrf2^{+/+}$ mice and associated with *Kras* mutations.[29]

functions, spanning from tumor progression (by means of oncogenic cooperation) and metabolic reprogramming to resistance to chemotherapy, radiotherapy, and targeted agents. An immune-cold microenvironment characterizes *KEAP1-NFE2L2*-mutant NSCLC, which may account for an increased ability to tolerate immune checkpoint inhibitors. CTR, C-terminal region; GOF, gain-of-function; LOF, loss-of-function; NTR, N-terminal region; ROS, reactive oxygen species; sMAF, small MAF.

Moreover, a tumor-promoting and oncogene-directed (e.g., *KRAS*, *MYC*) increased NRF2 activity was described in lung and pancreas tumorigenesis models,[10] whereas *Nrf2* loss hindered tumor initiation.[33,34] Regarding *KEAP1*, its tumor-suppressive functions were clarified exploiting a CRISPR-Cas9–based approach in a GEMM of *Kras*-driven LUAD.[35] *Keap1* LOF resulted in higher tumor burden and faster tumor growth kinetics when compared with control animals. Likewise, combined inactivation of *Keap1* and *Pten* promoted LUAD formation, suggesting the existence of an oncogenic cooperation between NRF2 and the PI3K/AKT pathway.[36] Last, combined loss of *Keap1* and *Trp53* resulted in the onset of tumors having the histologic and molecular features of LUSC.[37] Recollecting the aforementioned evidence and considering that KEAP1-NRF2 alterations in NSCLC are significantly more common in smokers than in non-smokers, it is plausible that although chronic exposure to tobacco smoking induces a cytoprotective NRF2 activation, a switch toward tumor-enhancing functions occurs through oncogenic cooperation mechanisms. Thus, although available evidence indicates that *KEAP1* and *NFE2L2* alterations do not represent cancer-initiating events, their onset after a first mutational hit confers a fitness advantage by supporting tumor growth, dissemination, and therapeutic resistance.

KEAP1 and *NFE2L2* Mutations in NSCLC

KEAP1 and *NFE2L2* mutations occur in approximately 20% of LUAD and 25% to 30% of LUSC (available at https://genie.cbioportal.org). While in LUAD the majority of alterations are observed in *KEAP1*, a fairly equal representation of *KEAP1* and *NFE2L2* mutations is recorded in LUSC. In both settings, *CUL3* alterations are uncommon (~2%–3%). *KEAP1* mutations have been detected, along with *TP53*, *KRAS*, and *STK11* (also known as LKB1), in the normal airway epithelium in patients with early stage NSCLC, thus providing hints on the driver nature of these alterations.[38] Taking into account the key molecular function of *KEAP1* and *NFE2L2*, it is not surprising the association between their mutations, smoking history, and mutual exclusivity with some actionable alterations (particularly *EGFR*).[18,39] Regardless of pathological subtype, *KEAP1* and *NFE2L2* alterations are mutually exclusive. Whether this mutual exclusivity is rooted in the detrimental effects of a double mutational hit on the same pathway or, rather, it reflects the existence of different disease entities remains an issue yet to be addressed.

The mutational pattern of *KEAP1* is consistent with its tumor-suppressive function. Indeed, pathogenic mutations are scattered throughout the whole gene length, and approximately one-third of them are stop-gain variants.[1] To some extent, *KEAP1* displays similarities with *TP53*. For instance, some *KEAP1* variants exhibited dominant-negative effects, which is, the encoded protein negatively interferes with the wild-type one.[40] Furthermore, *KEAP1* loss of heterozygosity was reported.[41] Adding a further level of complexity, *KEAP1* epigenetic silencing has been described.[42] Conversely, the oncogenic nature of *NFE2L2* is mirrored by hotspot mutations clustering at the Neh2 domain.[1] Given that *NFE2L2* mutations mostly occur at KEAP1 binding sites (DLG and ETGE motifs), they hinder KEAP1-mediated NFR2 degradation, thus leading to the constitutive activation of NRF2-driven gene transcription.[43–45]

KEAP1 and *NFE2L2* mutations have a distinct comutation repertoire. *KEAP1* alterations often co-occur with *STK11* and *KRAS* in LUAD, whereas *NFE2L2* and *TP53* mutations coexist in LUSC. The tendency toward *KEAP1* and *STK11* coalteration deserves particular mention. *STK11* encodes a serine/threonine kinase (LKB1) acting upstream AMPK family members, which are involved in cellular energy regulation.[46] This suggests that *KEAP1* and *STK11* co-mutant LUAD configures a metabolically addicted phenotype. Moreover, a sharpened capability to tolerate ferroptosis was described in *KEAP1* and *STK11* double-mutant LUAD.[13]

KEAP1/NFE2L2 and Immunotherapy

The interest surrounding *KEAP1* was fueled by pioneering molecular characterization studies shedding light on the recurrent nature of *KEAP1* and *NFE2L2* mutations in NSCLC, coupled with the deleterious effects of *KEAP1* in NSCLC treated with chemotherapy and radiotherapy.[18,32,47,48] Moreover, *KEAP1* inactivation was associated with reduced sensitivity to EGFR-directed therapies (osimertinib) and agents targeting the RTK-RAS-MAPK pathway and ALK.[49,50] The advent of ICIs, a broader understanding of the pathway, and the increased use of sequencing technologies in clinical practice prompted a wave of novel studies striving to elucidate the relationship between *KEAP1* and immunotherapy. The same holds true for its comutational background, relying on the concept of epistatic interactions.[51] Coexisting mutations in *KEAP1*, *STK11*, *SMARCA4*, or *PBRM1* have been noticed in a subset of ICI-treated LUAD patients with shorter survival outcomes when compared with single-mutant and wild-type cases.[3] The subset of tumors with coexisting mutations had high tumor mutational burden, indicating the quality of alterations has greater predictive capability than the overall number of nonsynonymous mutations. Recently, two independent studies linked *KEAP1* to *TP53* in LUAD (immunotherapy-treated population on the first study,

and early and advanced settings on the second one).[4,52] The message conveyed was that *KEAP1* and *TP53* co-mutant LUAD shares clinical features of "pure" *TP53*-mutant tumors (which is, tumors carrying *TP53* mutations in the absence of co-occurring *KEAP1* mutations). Indeed, *KEAP1* and *TP53* double-mutant LUAD had intermediate prognosis, comparable with that of *TP53*-mutant tumors. Conversely, *KEAP1* single-mutant LUAD had the shortest survival. In this context, the mutual exclusivity between *TP53* and *KRAS* mutations suggests an enrichment for *KRAS* alterations in the *KEAP1* single-mutant subgroup and raises the idea that including *KRAS* in this genomic predictor may further refine its prognostic and predictive capabilities.[4,53] Next, the comparison between *KEAP1* single-mutant and *KEAP1* and *TP53* double-mutant LUADs revealed distinct immunogenomic features and evolutionary trajectories, despite sharing an active super enhancer element sustaining the expression of ferroptosis-preventing genes (AKR).[4] Furthermore, it has been reported that *KEAP1* and *STK11* mutations negatively affected clinical outcomes in immunotherapy-treated patients with *KRAS*-mutant LUAD, but not in the *KRAS* wild-type setting.[5] This enforces the idea that an efficient subtyping can be achieved through the interrogation of multiple genomic markers, instead of a single feature.

Therapeutic Targeting of *KEAP1*- or *NFE2L2*-Mutant NSCLC

The deleterious impact of *KEAP1* mutations on survival outcomes of patients with NSCLC is fueling an intense search of therapeutic strategies for targeting NRF2-addicted tumors. These efforts are mostly capitalizing on the concept of metabolic vulnerabilities, which is, the increased dependency on a given metabolic avenue stemming from NRF2-driven metabolic rewiring. For instance, NRF2-addicted tumors deplete intracellular glutamate pools, thus becoming dependent on extracellular glutamine. Thus, the inhibition of glutaminase, the enzyme that catalyzes the conversion of glutamine to glutamate, was proposed as a therapeutic strategy against NRF2-addicted NSCLC.[15] On this basis, the glutaminase inhibitor telaglenastat (CB-839) is being evaluated in phase 2 trials, either in combination with chemoimmunotherapy or alone, in patients with advanced NSCLC whose tumors harbor *KEAP1* or *NFE2L2* mutations (KEAPSAKE and BeGIN trials; NCT04265534 and NCT03872427). Likewise, the dual mTORC1/2 inhibitor sapanisertib is being evaluated in the advanced setting, given that preclinical evidence suggested that *NFE2L2* mutations induce mTOR pathway dependency (NCT02417701 and NCT04250545).[54] Finally, the use of GEMMs and CRISPR/Cas9 screens is shedding light on novel vulnerabilities. For instance, the endoplasmic reticulum-associated protein Slc33a1 was identified as a *KEAP1* mutant-specific dependency.[55]

Concluding Remarks

After nearly two decades of success with tyrosine kinase inhibitors (and more recently ICIs), the advent of *KEAP1* (and *STK11*) in the NSCLC mutational landscape is raising the "battlefield" to an entirely new and more complex level, dominated by pharmacologically orphan events shaping the natural history of the disease. We believe that future research should encompass three main domains. First, refining our current knowledge on oncogenic cooperation and lethal interactions. Preclinical models (e.g., GEMMs) and loss-of-function genetic screens hold the potential to significantly advance our understanding in KEAP1-NRF2 biology and uncover novel genotype-specific vulnerabilities. Second, mutational co-occurring models may represent a first generation of user-friendly genomic tools for routine clinical practice (e.g., *KEAP1*, *STK11* and *KRAS*, *KEAP1*, and *TP53*). To some extent, this approach oversimplifies the complexity of genetic interactions. Consistently, we are investigating the clonal dynamics characterizing *KEAP1*-mutant LUAD with the aim of improving molecular subtyping. Finally, a first generation of ongoing clinical trials attempts to target *KEAP1*- and *NFE2L2*-mutant NSCLC. Although this mirrors an increased awareness on the biological and clinical relevance of the pathway, at the same time the proposed strategies mostly rely on an indirect targeting (metabolic dependencies). A direct inhibition of NRF2 should actively be pursued, as this strategy may more efficiently turn off the multiple oncogenic routes fueled by aberrant NRF2 activity. Overall, the new threat of driver tumor-suppressor genes with pleiotropic effects calls for global collaborations and academia-industry partnerships.

CRediT Authorship Contribution Statement

Stefano Scalera, Marco Mazzotta, Clelia Cortile, Eriseld Krasniqi, Marcello Maugeri-Saccà: Conceptualization, Writing - original draft.

Ruggero De Maria, Federico Cappuzzo, Gennaro Ciliberto: Investigation, Writing - review & editing.

Stefano Scalera, Marco Mazzotta, Clelia Cortile, Eriseld Krasniqi: Visualization.

Marcello Maugeri-Saccà: Supervision, Wrote the final version of the manuscript.

Stefano Scalera, Marco Mazzotta, Clelia Cortile, Eriseld Krasniqi, Ruggero De Maria, Federico Cappuzzo, Gennaro Ciliberto, Marcello Maugeri-Saccà: Drafting the manuscript, Read and approved the final

version of the manuscript, Agree to be accountable for all aspects of the work.

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