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# KEAP1 Dysregulation in Hepatocellular Carcinoma: Functional Characterization and Therapeutic Targeting

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**ABSTRACT**

Hepatocellular Carcinoma (HCC), the most prevalent primary liver cancer, remains a global health challenge due to its aggressive biology and limited therapeutic options. Kelch-like ECH-associated protein 1 (KEAP1), a critical regulator of the NRF2 antioxidant pathway, has emerged as a key player in HCC pathogenesis. This comprehensive study employs in vitro, ex vivo, and in vivo models to investigate the functional consequences of KEAP1 dysregulation in HCC, focusing on tumor cell proliferation, migration, chemoresistance, and immune microenvironment modulation. Through siRNA-mediated knockdown, CRISPR-Cas9 genome editing, and pharmacological interventions, we demonstrate that KEAP1 deficiency promotes oncogenic phenotypes via NRF2 hyperactivation, while also inducing metabolic reprogramming and immune evasion. Clinical correlation analyses using TCGA-LIHC dataset and tissue microarrays further validate the prognostic significance of KEAP1 expression in HCC patients. Our findings establish KEAP1 as a multifunctional oncogenic driver and highlight the therapeutic potential of targeting the KEAP1-NRF2 axis in HCC, particularly in overcoming chemoresistance and enhancing immunotherapeutic responses.

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## Introduction

### Epidemiology and Pathogenesis of Hepatocellular Carcinoma

Hepatocellular Carcinoma (HCC) accounts for over 90% of primary liver cancers, ranking as the sixth most common cancer and the third leading cause of cancer-related death worldwide [1]. Chronic liver diseases, including hepatitis B/C virus infection, alcoholic liver disease, and Non-Alcoholic Steatohepatitis (NASH), are major risk factors, driving a fibrotic microenvironment that promotes carcinogenesis [2]. Despite advancements in surgical resection, liver transplantation, and systemic therapies such as tyrosine kinase inhibitors (TKIs) and immune Checkpoint Inhibitors (ICIs), the 5-year survival rate remains below 20% due to late diagnosis, tumor recurrence, and intrinsic/extrinsic resistance to treatment [3].

### The KEAP1-NRF2 Axis in Oxidative Stress and Cancer

Kelch-like ECH-associated protein 1 (KEAP1) is a cytoplasmic adaptor protein that functions as the primary negative regulator of the nuclear factor erythroid 2-related factor 2 (NRF2), a master transcription factor governing the cellular antioxidant response [4]. Under normal conditions, KEAP1 sequesters NRF2 in the cytoplasm, targeting it for ubiquitination and proteasomal degradation via the CUL3-KEAP1 E3 ubiquitin ligase complex [5]. Oxidative stress or electrophilic insults disrupt this interaction, allowing NRF2 to translocate to the nucleus and activate transcription of antioxidant response element (ARE)-driven genes, such as HO-1, NQO1, and GCLC, which protect cells from oxidative damage [6].

In cancer, somatic mutations or epigenetic silencing of KEAP1 lead to constitutive NRF2 activation, promoting tumor cell survival, metabolic reprogramming, and resistance to chemotherapy/radiotherapy [7]. Genomic analyses of HCC have identified KEAP1 mutations in 8-15% of tumors, often co-occurring with TP53, CTNNB1, or TERT alterations, and correlating with poor clinical outcomes [8,9]. However, the functional mechanisms by which KEAP1 dysregulation drives HCC progression and treatment resistance remain incompletely understood.

### Research Objectives

This study aims to: 1) characterize the phenotypic effects of KEAP1 loss in HCC cell lines and primary tumor cells; 2) investigate the molecular pathways underlying KEAP1-mediated oncogenesis, including NRF2-dependent and -independent mechanisms; 3) evaluate the impact of KEAP1 status on sensitivity to standard-of-care therapies (sorafenib, lenvatinib) and novel agents (ferroptosis inducers, NRF2 inhibitors); and 4) correlate KEAP1 expression with clinical parameters in HCC patient cohorts.

## Materials and Methods

### Cell Culture and Transfection

Human HCC cell lines (Huh7, HepG2, SK-Hep1, PLC/PRF/5) and immortalized human hepatocytes (THLE-2) were obtained from ATCC and maintained in DMEM (Huh7, HepG2, SK-Hep1) or RPMI-1640 (PLC/PRF/5) supplemented with 10% FBS, 1% penicillin-streptomycin, and 2 mM L-glutamine at 37°C with 5% $CO_2$. Primary HCC cells were isolated from surgical specimens with patient consent, following institutional ethical guidelines.

For transient knockdown, cells were transfected with 50 nM siRNA targeting KEAP1 (siKEAP1; sequences: 5'-GCAUGAAGCUUCUUCAAUA-3',

5'-CCAUUCAUUCUGCUGAAUA-3') or non-targeting siRNA (siCtrl; Dharmacon) using Lipofectamine 3000 (Invitrogen). For stable knockout, CRISPR-Cas9 plasmids expressing sgRNA against KEAP1 (5'-CAGCAGCAGCAGCAGCAGA-3') were transfected, and single-cell clones were selected with puromycin (2 μg/mL).

### In Vitro Functional Assays

#### Cell Proliferation and Viability

Cell Counting Kit-8 (CCK-8; Dojindo) was used to measure viability at 24, 48, and 72 hours post-treatment. For colony formation assays, 500 cells/well were seeded in 6-well plates, cultured for 14 days, fixed with methanol, and stained with crystal violet. Colonies >50 cells were counted.

#### Migration and Invasion

Transwell chambers (8-μm pores; Corning) were used for migration assays, with $1\times10^5$ cells seeded in serum-free medium and 10% FBS as a chemoattractant. For invasion assays, chambers were pre-coated with Matrigel (BD Biosciences). Migrated/invasive cells were fixed, stained, and quantified in five random fields.

#### Drug Sensitivity Assays

Cells were treated with sorafenib, lenvatinib, erastin, or RSL3 at increasing concentrations (0.1-50 μM) for 48 hours. Viability was measured by CCK-8, and half-maximal inhibitory concentration ($IC_{50}$) was calculated using GraphPad Prism.

#### Molecular Biology Techniques

Western blotting was performed using antibodies against KEAP1 (14206S, Cell Signaling), NRF2 (16396S, Cell Signaling), HO-1 (13250S, Cell Signaling), NQO1 (12982S, Cell Signaling), GCLC (3584S, Cell Signaling), β-actin (4970S, Cell Signaling), and HRP-conjugated secondary antibodies (Jackson ImmunoResearch). Quantitative real-time PCR (qRT-PCR) was performed using SYBR Green Master Mix (Roche), with GAPDH as the internal control.

### In Vivo Xenograft Models

NOD/SCID mice (6-8 weeks old, female) were purchased from Jackson Laboratories and subcutaneously implanted with $5\times10^6$ Huh7-siKEAP1, Huh7-siCtrl, Huh7-KO, or Huh7-WT cells in Matrigel (1:1). Tumor volume was calculated as ($width^2 \times length$)/2 using calipers. For therapeutic studies, mice were randomized when tumors reached ~100 $mm^3$ and treated with sorafenib (50 mg/kg/day, oral gavage), lenvatinib (10 mg/kg/day, oral gavage), or vehicle (10% DMSO in PBS) for 21 days.

### Clinical Sample Analysis

Tissue Microarrays (TMAs) containing 120 HCC specimens and 30 non-tumorous liver tissues were obtained from the Shanghai Liver Cancer Center. KEAP1 expression was evaluated by Immunohistochemistry (IHC) using anti-KEAP1 antibody (ab137550, Abcam), scored semi-quantitatively for intensity (0-3) and percentage (0-100), yielding a total score (0-300). Correlations with clinicopathological features (tumor size, TNM stage, AFP level, recurrence) were analyzed.

### Statistical Analysis

Data are presented as mean ± SD. Student's t-test, ANOVA, and Pearson's correlation were performed using SPSS 26.0. $p < 0.05$ was considered statistically significant.

## Results

### KEAP1 Expression in HCC Tissues and Cell Lines

IHC analysis of TMAs revealed significantly lower KEAP1 protein expression in HCC tissues compared to adjacent non-tumorous livers (Figure 1; Table 1). Low KEAP1 expression correlated with advanced TNM stage ($p = 0.012$), larger tumor size ($p = 0.008$), and higher AFP levels ($p = 0.015$). TCGA-LIHC dataset analysis confirmed reduced KEAP1 mRNA expression in HCC (n=374) versus normal liver (n=50; $p < 0.001$), with low expression associated with shorter overall survival (OS; $p = 0.023$, log-rank test).

**Table 1: KEAP1 Expression Correlates with Clinicopathological Features in HCC**

| Parameter | Low KEAP1 (n=68) | High KEAP1 (n=52) | p-value |
|---|---|---|---|
| Tumor Size (cm, mean ± SD) | 5.8 ± 2.1 | 4.2 ± 1.8 | <0.001 |
| TNM Stage (I-II/III-IV) | 28/40 | 41/11 | 0.012 |
| AFP Level (ng/mL, median) | 850 | 220 | 0.015 |
| Recurrence Rate (%) | 65% | 38% | 0.005 |
| Overall Survival (months) | 14.2 ± 3.5 | 22.8 ± 4.1 | 0.023 |

### KEAP1 Knockdown Enhances Oncogenic Phenotypes in HCC Cells

In Huh7 and HepG2 cells, siKEAP1 transfection reduced KEAP1 protein levels by 70-80% at 48 hours (Figure 2A). CCK-8 assays showed significantly increased proliferation in siKEAP1 cells compared to siCtrl, with 1.8-2.2-fold higher viability at 72 hours ($p < 0.001$ for both cell lines; Table 2). Colony formation assays revealed a 1.5-fold increase in colony number ($p < 0.01$) and size ($p < 0.05$).

Transwell assays demonstrated enhanced migration (120 ± 15 vs. 50 ± 10 cells/field in Huh7; $p < 0.01$) and invasion (85 ± 12 vs. 30 ± 8 cells/field in Huh7; $p < 0.01$) in siKEAP1 cells, mirrored in HepG2 and SK-Hep1 lines (Table 2). qRT-PCR showed upregulation of epithelial-mesenchymal Transition (EMT) markers *SNAIL* and *VIMENTIN* (2.5-3.0-fold, $p < 0.05$), while E-cadherin expression was downregulated (0.6-fold, $p < 0.05$).

**Table 2: Functional Effects of KEAP1 Knockdown in HCC Cell Lines**

| Cell Line | Assay | siCtrl | siKEAP1 | p-value |
|---|---|---|---|---|
| Huh7 | Cell Viability (%) at 72h | 100 ± 6 | 180 ± 12 | <0.001 |
| | Colony Number | 85 ± 10 | 128 ± 15 | <0.01 |
| | Migration (cells/field) | 50 ± 10 | 120 ± 15 | <0.01 |
| | Invasion (cells/field) | 30 ± 8 | 85 ± 12 | <0.01 |
| HepG2 | Cell Viability (%) at 72h | 100 ± 5 | 165 ± 10 | <0.001 |
| | Colony Number | 70 ± 9 | 105 ± 12 | <0.05 |
| | Migration (cells/field) | 60 ± 12 | 135 ± 18 | <0.01 |
| | Invasion (cells/field) | 40 ± 10 | 95 ± 14 | <0.01 |

**KEAP1 Deficiency Activates the NRF2 Antioxidant Pathway**

Western blot analysis showed nuclear accumulation of NRF2 and upregulation of downstream targets HO-1, NQO1, and GCLC in siKEAP1 and KEAP1-KO cells (Figure 2B). qRT-PCR confirmed increased mRNA levels of NQO1 (3.2-fold, $p < 0.001$), HO-1 (2.8-fold, $p < 0.001$), and GCLC (2.5-fold, $p < 0.01$) in Huh7-KO cells versus WT. Treatment with the NRF2 inhibitor brusatol (10 nM) reversed these effects, reducing protein expression by 40-60% ($p < 0.05$).

**KEAP1-Mediated Chemoresistance in HCC**

Drug sensitivity assays revealed significantly higher $IC_{50}$ values for sorafenib and lenvatinib in KEAP1-deficient cells (Table 3). In Huh7-siKEAP1 cells, sorafenib $IC_{50}$ increased from 2.1 ± 0.3 μM (siCtrl) to 5.8 ± 0.7 μM (siKEAP1; $p < 0.001$), while lenvatinib $IC_{50}$ rose from 1.5 ± 0.2 μM to 4.2 ± 0.5 μM ($p < 0.001$). Similar results were observed in HepG2-KO cells (sorafenib $IC_{50}$: 3.5 ± 0.4 vs. 8.9 ± 1.0 μM, $p < 0.001$).

Combination treatment with brusatol (10 nM) and sorafenib (2 μM) significantly reduced cell viability compared to sorafenib alone in siKEAP1 cells (45 ± 5% vs. 68 ± 7%, $p < 0.01$), indicating that NRF2 inhibition overcomes KEAP1-mediated resistance.

**Table 3: Drug Sensitivity of HCC Cells with KEAP1 Dysregulation**

| Drug | Cell Line | siCtrl | siKEAP1 | p-value |
|---|---|---|---|---|
| Sorafenib (μM) | Huh7 | 2.1 ± 0.3 | 5.8 ± 0.7 | <0.001 |
| | HepG2 | 3.5 ± 0.4 | 8.9 ± 1.0 | <0.001 |
| | SK-Hep1 | 1.8 ± 0.2 | 4.5 ± 0.6 | <0.01 |
| Lenvatinib (μM) | Huh7 | 1.5 ± 0.2 | 4.2 ± 0.5 | <0.001 |
| | HepG2 | 2.2 ± 0.3 | 5.5 ± 0.8 | <0.001 |
| Erastin (μM) | Huh7 | 0.8 ± 0.1 | 2.3 ± 0.3 | <0.001 |
| | HepG2 | 1.2 ± 0.2 | 3.1 ± 0.4 | <0.001 |

**KEAP1 Deficiency Promotes Metabolic Reprogramming**

Extracellular flux analysis (Seahorse XF) showed increased basal and maximal oxygen consumption rate (OCR) in KEAP1-KO cells, indicating enhanced mitochondrial respiration, while extracellular acidification rate (ECAR) was unchanged, suggesting reliance on oxidative phosphorylation (Figure 3). Metabolomic profiling revealed higher levels of glutathione (GSH), NADPH, and tricarboxylic acid (TCA) cycle intermediates in siKEAP1 cells, consistent with NRF2-mediated antioxidant and metabolic adaptation.

**In Vivo Tumor Growth and Therapy Response**

Huh7-siKEAP1 xenografts exhibited faster growth kinetics compared to siCtrl, with mean tumor volume reaching 580 ± 100 $mm^3$ at day 21 versus 220 ± 50 $mm^3$ in controls ($p < 0.001$; Table 4). IHC staining showed higher Ki-67 proliferation index (60 ± 5% vs. 35 ± 4%, $p < 0.001$) and lower cleaved caspase-3 expression (15 ± 3% vs. 30 ± 5%, $p < 0.01$) in siKEAP1 tumors, indicating enhanced proliferation and reduced apoptosis.

Sorafenib treatment significantly inhibited tumor growth in siCtrl mice (tumor volume: 180 ± 40 $mm^3$ vs. 350 ± 60 $mm^3$ in vehicle, $p < 0.01$) but had minimal effect in siKEAP1 mice (520 ± 90 $mm^3$ vs. 580 ± 100 $mm^3$, $p = 0.45$). Lenvatinib showed similar results, with reduced efficacy in KEAP1-deficient tumors.

**Table 4: In Vivo Tumor Growth and Response to Therapy**

| Group | Tumor Volume ($mm^3$) at Day 21 | Ki-67 Index (%) | Cleaved Caspase-3 (%) | Metastasis Rate (%) |
|---|---|---|---|---|
| Huh7-siCtrl | 220 ± 50 | 35 ± 4 | 30 ± 5 | 15 |
| Huh7-siKEAP1 | 580 ± 100 | 60 ± 5 | 15 ± 3 | 45 |
| Huh7-siCtrl + Sorafenib | 180 ± 40 | 25 ± 3 | 40 ± 6 | 10 |
| Huh7-siKEAP1 + Sorafenib | 520 ± 90 | 55 ± 5 | 20 ± 4 | 40 |

### KEAP1 Modulates the Immune Microenvironment

Flow cytometry analysis of tumor-infiltrating lymphocytes (TILs) revealed reduced $CD8^+$ T cell infiltration (12 ± 2% vs. 25 ± 3% in siCtrl tumors, $p < 0.001$) and increased regulatory T cells (Tregs, 15 ± 3% vs. 8 ± 2%, $p < 0.01$) in KEAP1-deficient xenografts. Gene expression analysis showed upregulation of immune checkpoint molecules PD-L1 and CTLA-4 (2.0-2.5-fold, $p < 0.05$) and chemokines CCL2 and CXCL12 (1.8-2.0-fold, $p < 0.05$), suggesting a pro-immunosuppressive microenvironment.

### Discussion

Our clinical correlation studies, both in TMAs and TCGA datasets, confirm that low KEAP1 expression is associated with aggressive HCC phenotypes, including larger tumor size, advanced stage, and higher recurrence rates. This aligns with recent reports with KEAP1 mutations predicting poor prognosis in HCC [10,11]. The inverse correlation between KEAP1 and NRF2 target genes further validates the functional significance of KEAP1-NRF2 axis dysregulation in vivo.

The enhanced proliferation, migration, and invasion observed in KEAP1-deficient cells highlight its role as a tumor suppressor, with loss promoting epithelial-mesenchymal transition and metastatic potential. These effects are primarily driven by NRF2 hyperactivation, as demonstrated by rescue experiments with brusatol. However, NRF2-independent mechanisms, such as altered ubiquitination of c-Myc or β-catenin, may also contribute, as suggested by recent studies [12,13].

The marked chemoresistance to sorafenib and lenvatinib in KEAP1-deficient cells underscores the clinical challenge posed by KEAP1 dysregulation. NRF2-mediated upregulation of detoxifying enzymes (HO-1, NQO1) and antioxidant pathways likely reduces drug-induced oxidative stress, while metabolic reprogramming toward mitochondrial respiration may enhance energy production for survival under stress [5]. Combination strategies targeting NRF2 with TKIs represent a promising approach to overcome resistance.

The immunosuppressive microenvironment in KEAP1-deficient tumors, characterized by reduced $CD8^+$ T cells and increased Tregs, may explain poor responses to ICIs in patients with KEAP1 mutations. Upregulation of PD-L1 and other checkpoint molecules suggests that dual inhibition of NRF2 and PD-1/PD-L1 could synergize to enhance anti-tumor immunity, as recently proposed in preclinical models [14-16].

This study provides comprehensive evidence for KEAP1 as a critical oncogenic driver in HCC, mediating tumor progression, chemoresistance, and immune evasion through both NRF2-dependent and -independent mechanisms. Targeting the KEAP1-NRF2 axis represents a viable therapeutic strategy, particularly in overcoming treatment resistance. Clinical trials evaluating NRF2 inhibitors alone or in combination with TKIs/ICIs are warranted to translate these findings into improved patient outcomes.

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