Loss of Keap1 Function Activates Nrf2 and Provides Advantages for Lung Cancer Cell Growth

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Abstract
Oxidative and electrophilic stresses are sensed by Keap1, which activates Nrf2 to achieve cytoprotection by regulating the expression of drug-metabolizing and antioxidative stress enzymes/proteins. Because oxidative and electrophilic stresses cause many diseases, including cancer, we hypothesized that an abnormality in the Nrf2-Keap1 system may facilitate the growth of cancer cells. We sequenced the KEAP1 gene of 65 Japanese patients with lung cancer and identified five nonsynonymous somatic mutations at a frequency of 8%. We also identified two nonsynonymous somatic KEAP1 gene mutations and two lung cancer cell lines expressing KEAP1 at reduced levels. In lung cancer cells, low Keap1 activity (due to mutations or low-level expression) led to nuclear localization and constitutive activation of Nrf2. The latter resulted in constitutive expression of cytoprotective genes encoding multidrug resistance pumps, phase II detoxifying enzymes, and antioxidative stress enzymes/proteins. Up-regulation of these target genes in lung cancer cells led to cisplatin resistance. Nrf2 activation also stimulated growth of lung cancer–derived cell lines expressing KEAP1 at low levels and in Keap1-null mouse embryonic fibroblasts under homeostatic conditions. Thus, inhibition of NRF2 may provide new therapeutic approaches in lung cancers with activation of Nrf2. [Cancer Res 2008;68(5):1303–9]

Introduction
Oxidative and electrophilic stresses provoke cellular adaptive responses that induce the expression of genes that encode various cytoprotective enzymes. This process is closely related to human diseases, including cancer. Among the cytoprotective gene products, phase II drug-metabolizing enzymes and antioxidative stress enzymes/proteins are encoded by genes that are regulated by a motif called the antioxidant/electrophile-responsive element (1–3). The transcription factor Nrf2 forms a heterodimer with one of the small Maf family proteins, and the complex binds to the antioxidant-responsive element to activate transcription of these cytoprotective genes (4). Keap1 is a newly identified Nrf2-associated protein (5), containing BTB, IVR, and DGR domains (6). These domains contribute to the dual function of Keap1. Keap1 interacts specifically with an adaptor protein, Cullin 3 (Cul3), and forms an E3 ubiquitin ligase to render substrate Nrf2 susceptible to rapid degradation/turnover (7). Keap1 also functions as a sensor for electrophilic/oxidative stresses. In the absence of stress stimuli, Nrf2 is rapidly degraded through the proteasome pathway using ubiquitination by the Keap1-Cul3 E3 ligase (7). On exposure to stress, Keap1 is modified, and Nrf2 is freed from the modified Keap1, translocates into the nucleus, and activates the transcription of various cytoprotective genes (7). The rapid degradation of Nrf2 by Keap1-Cul3 E3 ligase constitutes the molecular basis for induction of cytoprotective enzymes in response to stress.

Nrf2-deficient mice are susceptible to electrophilic/oxidative stresses due to impaired expression of cytoprotective enzymes/proteins (8). This observation strongly supports the essential contribution of Nrf2 to both basal and inducible expression of cytoprotective enzymes and suggests new possibilities for environmental response studies (9). Similarly, Keap1-deficient mice constitutively express cytoprotective and antioxidative enzymes/proteins (10), indicating that the Nrf2-Keap1 system functions physiologically for cytoprotection against environmental stresses. The ubiquitin proteasome system using Cullin-type E3 ligases shares a similar role in critical stress responses. For instance, the transcription factors I-B (11) and hypoxia-inducible factor-1a (12) use Cul1 and Cul2, respectively, to form specific E3 ligases. Inhibition of rapid turnover/repression is a common (and evolutionarily ancient) strategy animals have acquired for rapid responses to environmental changes.

To our surprise, however, we identified somatic mutations in the KEAP1 gene of Japanese patients with lung cancer and in lung cancer cell lines (13). These KEAP1 mutations affected the repressive activity of Keap1, stimulated nuclear accumulation of Nrf2, and induced constitutive expression of cytoprotective enzymes within the cancer cells. More recently, somatic mutations in KEAP1 were detected in 19% of Caucasian patients with lung cancer (14). Collectively, these observations suggest that the Nrf2-Keap1 pathway acts as a double-edged sword: it protects the body from chemical carcinogenesis but aids the growth and development of cancer cells (13). To examine the characteristics of KEAP1 mutations further and to address whether they provide selective advantages for cancer progression, we analyzed the expression and mutation of the KEAP1 gene in samples from Japanese patients with lung cancer and in established lung cancer cell lines. We
PCR primers were designed to amplify every exon plus high-fidelity DNA polymerase, KOD Plus, and were used for sequencing (15).

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torso was minced and dispersed in 0.25% trypsin/EDTA. MEFs were

at embryonic day 13.5. The head and internal organs were removed, and the

Mouse embryonic fibroblasts (MEF) were prepared from individual embryos

Cell Bank. All cell lines were maintained in DMEM with 10% calf serum, 10%

LC-MS was obtained from the Japanese Collection of Research Bioresources

H1437, H1395, and A549 were obtained from the American Type Culture

National Cancer Center Hospital. Lung cancer cell lines SQ-19, H2126, 

informed consent from patients who received surgical treatment at the

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Materials and Methods

Tissue samples and cell lines. Tumor tissues were obtained with informed consent from patients who received surgical treatment at the National Cancer Center Hospital. Lung cancer cell lines SQ-19, H2126, H1437, H1395, and A549 were obtained from the American Type Culture Collection; II-18 was obtained from Riken Bio Resource Center; and RERF-LC-MS was obtained from the Japanese Collection of Research Bioresources Cell Bank. All cell lines were maintained in DMEM with 10% calf serum, 10% fetal bovine serum (FBS), or 20% FBS containing F12 nutrient mixture. Mouse embryonic fibroblasts (MEF) were prepared from individual embryos at embryonic day 13.5. The head and internal organs were removed, and the torso was minced and dispersed in 0.25% trypsin/EDTA. MEFs were maintained in DMEM containing 10% FBS. Briefly, MEFs at less than five passages were used as early-passage primary MEFs. To immortalize MEFs, the fibroblasts were cultured continuously until the fibroblasts started growing rapidly. Immortalization of the primary MEFs was usually observed after 25 passages.

Sequencing of KEAP1. DNAs extracted from cancer tissues and cell lines were amplified according to a ligation-mediated PCR method using a high-fidelity DNA polymerase, KOD Plus, and were used for sequencing (15). PCR primers were designed to amplify every exon plus ~100 bases flanking each exon. Reference sequence of the KEAP1 gene was retrieved from public database Genbank. Sequencing was performed using the following primer sets: exon 2, 5′-TGAACATGCTCTTCCTTGTTTGAACCGGCAA-3′ (forward) and 5′-TGAGGCCGACACCTCTCTTCAGTTC-3′ (reverse); exon 3, 5′-TTATTGAGATCCCCTTATGGATAGACCTTGA-3′ (forward) and 5′-TAGAATCTCCAGACGAGCTGTCTCTAAATGGA-3′ (reverse); exon 4, 5′-ATGAGCCCATGGGAGGAGACGAGTCACCTTTTGAGGA-3′ (forward) and 5′-TAGAATCTCCAGACGAGCTGTCTCTAAATGGA-3′ (reverse); exon 5, 5′-ATGGACCGCCTCCTCCCTCCTGAGTCA-3′ (forward) and 5′-AATGCCTCCCTCCTGAGTCA-3′ (reverse); and exon 6, 5′-AATGCCTCCCTCCTGAGTCA-3′ (forward) and 5′-AATGCCTCCCTCCTGAGTCA-3′ (reverse).

Plasmid construction and reporter assay. Flag-tagged KEAP1 and its mutations were inserted in pCMV-tag2A (Stratagene). Transfections of expression vectors, the luciferase reporter pRBGP2 containing three copies of Nrf2-binding element (16), and pRL-TK were carried out using Lipofectamine Plus Reagent (Invitrogen). After 36 h of transfection, the cells in 24-well plates were washed by PBS and analyzed for luciferase activities in triplicate in each transfection experiment using Dual-Luciferase Reporter Assay System (Promega). All experiments were repeated at least thrice.

Immunoprecipitation, immunofluorescence, and in vivo ubiquitination assays. Immunoprecipitation, immunofluorescence microscopy, and in vivo ubiquitination assays were performed as described previously (7).

Real-time PCR. Total RNAs were isolated from cell lines by Trizol reagent (Invitrogen). First-strand cDNAs were obtained from total RNA (5 μg) by SuperScript First-Strand Synthesis System (Invitrogen). Real-time PCR was performed by ABI 7000 (Applied Biosystems) using the following primer sets: KEAP1, 5′-TACAGATGGAAGACAAGAGTCTGTTACCTG-3′ (forward) and 5′-TCAACAGGTACAGTCTCCTCCTGCTTCTC-3′ (reverse); NADPH dehydrogenase quinone 1 (NQO1), 5′-AGAAGAGAATTAATGTCTT-3′ (forward) and 5′-CTATATTGCGAGTACGTTGAGGTATTATGTTCT-3′ (reverse); multidrug resistance protein 3 (MRP3), 5′-GACCATCGGCACACGGCCCATCACTATCATGGA-3′ (forward) and 5′-TCACTGA-CTCACATTCTTGAAGAAATTTTTGTC-3′ (reverse); and PRDX1, 5′-AGATTGCTGTCTCIGTCTGATTCTACTCT-3′ (forward) and 5′-TCAC- TCTTCGCTTGAGAAATTTCTTTGTC-3′ (reverse). GAPPD gene was used as an internal control. Reverse transcription-PCR (RT-PCR) was performed using primer sets as same as real-time PCR.

Small interfering RNA and cell growth analysis. For the small interfering RNA (siRNA) experiments, 20 nmol/L of control siRNA (Qiagen) and NRP2-specific siRNA (S03246950; Qiagen) were used. Transfection was performed as described previously (17). RT-PCR was performed after 3 days and cell numbers were counted after 7 days.

Table 1. KEAP1 alterations in lung cancer tissues and cell lines

<table>
<thead>
<tr>
<th>Patients/cell lines</th>
<th>Pathology</th>
<th>Amino acid change</th>
<th>Domain</th>
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<tbody>
<tr>
<td>P1</td>
<td>SCC</td>
<td>A42T (heterozygous)</td>
<td>DGR</td>
</tr>
<tr>
<td>P9</td>
<td>ADC</td>
<td>H200P (heterozygous)</td>
<td>IVR</td>
</tr>
<tr>
<td>P12</td>
<td>ADC</td>
<td>G430C (heterozygous)</td>
<td>DGR</td>
</tr>
<tr>
<td>P23</td>
<td>ADC</td>
<td>R415G (heterozygous)</td>
<td>DGR</td>
</tr>
<tr>
<td>P65</td>
<td>LCC</td>
<td>G476R (homozygous)</td>
<td>DGR</td>
</tr>
<tr>
<td>H2126</td>
<td>ADC</td>
<td>R272C (homozygous)</td>
<td>IVR</td>
</tr>
<tr>
<td>H1648</td>
<td>ADC</td>
<td>G364C (homozygous)</td>
<td>DGR</td>
</tr>
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Abbreviations: SCC, small cell carcinomas; ADC, adenocarcinomas; LCC, large cell carcinomas.

identified the molecular basis of these loss-of-function type somatic mutations in patients with lung cancer and found that they provided advantages to lung cancer cells.

Results and Discussion

To explore somatic mutations in KEAP1 genes of lung cancer patients, we designed a sequencing system for protein-coding
mutations. Thus, the frequency of KEAP1 mutations, all of the mutations found in the current study were missense four missense point mutations and six deletion/insertion mutations, in both Caucasian (4 of 35). Because no deletion/insertion mutations in Japanese lung adenocarcinoma cases (3 of 29) is very similar to that of all lung cancer cases and in 26% (9 of 35) of Caucasian (14), suggesting that the homozygous gene mutations that we identified (Table 1; Fig. 1) might contain LOH at the KEAP1 gene locus.

All of the mutations identified in this study were located within the IVR and DGR domains of Keap1 (Table 1; Fig. 1A). The IVR domain is responsible for the ubiquitination of Nrf2 (7), and the DGR domain interacts with Nrf2 (5). To examine the molecular defects caused by these mutations, we asked whether mutant Keap1 has the ability to interact with Nrf2 and sequester it in the cytoplasm. To this end, we transfected enhanced green fluorescent protein (EGFP)-fused Nrf2 (Nrf2-GFP) with a Flag-tagged Keap1 mutant into NIH3T3 cells and examined the cellular localization of Nrf2-GFP. We also used an immunohistochemical procedure with an anti-Flag antibody.

To elucidate whether these Keap1 mutants interacted physically with Nrf2, we performed an immunoprecipitation analysis. When homozygous gene mutations in KEAP1 in one cancer tissue and in two lung cancer–derived cell lines (Table 1). Microsatellite-based genotyping of lung cancer tissues showed loss of heterozygosity (LOH) at 19p13.2 (containing the KEAP1 gene locus) in 41% of cases (14), suggesting that the homozygous gene mutations that we identified (Table 1; Fig. 1B) might contain LOH at the KEAP1 gene locus.
Flag-tagged Keap1 or Keap1 mutants were transfected into NIH3T3 cells concomitant with hemagglutinin (HA)-tagged Nrf2, WT Keap1 and Keap1 H200P and R272C mutants (Fig. 2B, lanes 1–3) were immunoprecipitated with Nrf2 by an anti-Flag antibody. In contrast, Nrf2 was not coimmunoprecipitated efficiently with Keap1 R415G and G430C mutants (Fig. 2B, lanes 4 and 5). These results are consistent with the subcellular localization analysis and show that R415G and G430C mutants in the DGR domain impair the association of Keap1 with Nrf2.

To examine how those Keap1 mutations affected the repressive activity of Keap1 against Nrf2, we performed a reporter cotransfection-transactivation analysis. Keap1 or individual Keap1 mutants were cotransfected into NIH3T3 cells with an Nrf2 expression plasmid and a luciferase reporter pRBGP2 (16). Luciferase activity was repressed significantly when WT Keap1 or the H200P mutant was transfected simultaneously (Fig. 2C, lanes 3 and 4). We did not detect any effects of the H200P mutant. In contrast, cotransfection of R272C, R415G, or G430C mutants (Fig. 2C, lanes 5–7) had no effect on luciferase reporter activity, suggesting that the latter three mutations affected the capacity of Keap1 to repress Nrf2. Significantly, the loss of repressive activity in R415G and G430C mutants correlated well with the loss of interaction with Nrf2.

Because we identified R415G and G430C mutants as heterozygous gene mutations in lung cancer, we asked whether the Keap1 mutant affected WT Keap1 activity. Toward that end, pRBGP2 reporter activity was examined in NIH3T3 cells after cotransfection with WT Keap1 and graded doses of the R415G or G430C mutants. The repressive activity of WT Keap1 decreased with increments of the R415G or G430C mutants (Fig. 2D, lanes 3–8), suggesting that these mutants repressed WT Keap1 in a dominant-negative manner. Thus, heterozygous mutation of KEAP1 can also activate Nrf2 through its dominant-negative function. In this regard, it is interesting to note the recently proposed two-site substrate recognition model for Keap1-Nrf2 interaction (18). In this model, one Keap1 homodimer associates with two sites of Nrf2, and this interaction is essential for ubiquitination of Nrf2. We surmise that the R415G or G430C mutant dimerizes with WT Keap1 to form a nonfunctional Keap1 heterodimer.

In contrast to the R415G and G430C mutants, the R272C mutant retained binding activity to Nrf2 but lost Nrf2 repressive activity (Fig. 2A–C). We hypothesized that this mutant in IVR might impair Nrf2 degradation, as the IVR domain of KEAP1 is responsible for the degradation of Nrf2 (7). To test this hypothesis, we expressed Nrf2 and WT Keap1 or the R272C mutant in NIH3T3 cells, and the stability of Nrf2 protein was determined. Whereas the Nrf2 level was very low in the cells cotransfected with WT Keap1, treatment of cells with the proteasome inhibitor MG132 stabilized the Nrf2 protein (Fig. 3A, lanes 1 and 2). The Nrf2 level in cells cotransfected with the R272C mutant was very high and was not induced effectively by MG132 (Fig. 3A, lanes 3 and 4). These results indicate that the R272C mutant impaired Nrf2 degradation.

To determine whether the R272C mutant binds Cul3 in the cells, we then performed an immunoprecipitation analysis. When Flag-tagged Keap1 or the R272C mutant was transfected with HA-tagged Cul3 into 293T cells, Cul3 was coimmunoprecipitated with both the WT Keap1 and the R272C mutant (Fig. 3B, lanes 1 and 2), indicating that R272C binds to Cul3 as WT Keap1 does. We also
performed an in vivo ubiquitination analysis to determine how the 
R272C-Cul3 complex contributed to ubiquitination of Nrf2. For this 
purpose, either Flag-tagged WT Keap1 or the R272C mutant was 
transfected into 293T cells concomitant with HA-tagged Nrf2 and 
His-tagged ubiquitin, and HA-Nrf2 was immunoprecipitated by an 
anti-HA antibody. Nrf2 ubiquitination was detected by immunoblot 
analysis with an anti-histidine antibody. Nrf2 ubiquitination was 
detected by immunoblot analysis with His-tagged ubiquitin, and HA-Nrf2 was 
immunoprecipitated by the R272C mutant (Fig. 3). We previously found that CysT and 
CysC288 of Keap1 are essential for the ubiquitination of Nrf2 (7). Therefore, we speculated that the association between the R272C mutant and Cul3 may be inadequate for the ubiquitination of Nrf2.

The molecular basis by which the R272C mutant lost the capacity to degrade Nrf2 is unclear. However, further studies in this area should help elucidate the mechanism by which Keap1-Cul3 E3 ligase degrades Nrf2. In this regard, it is interesting to note that endogenous Nrf2 is located in the nucleus of H2126 cells harboring the R272C mutation, even in the absence of stress stimuli (Fig. 3D), suggesting that the stabilized Nrf2 protein could enter the nucleus in homeostatic conditions.

Next, we used real-time PCR to analyze the expression level of the KEAP1 gene in primary lung cancer tissues and normal tissues from adenocarcinoma patients. We found lower levels of expression of KEAP1 in cancer tissue (Fig. 4A, lane 1). We next analyzed the expression level of the KEAP1 gene in six lung cancer cell lines by real-time PCR and found a reduction of KEAP1 gene expression in two lung adenocarcinoma cell lines, H1437 and II-18 (Fig. 4B, lanes 2 and 5). These observations imply that reduced expression of the KEAP1 gene may occur frequently in lung cancer, as is the case for the somatic mutations. To examine the subcellular localization of Nrf2 in cells expressing lower levels of KEAP1, we identified endogenous Nrf2 using confocal laser scanning microscopy. We found that endogenous Nrf2 was localized in the nuclei of H1437, II-18, and A549 (G333C; ref. 14) cells with the KEAP1 mutation (Fig. 4C). However, it was not located in the nuclei of SQ-19 cells with normal KEAP1 gene (Fig. 4C). Showing very good agreement, high-level expression of the two target genes, NQO1 (19) and MRP3 (20), was detected by real-time PCR analysis in H1437, II-18, and A549 cells but not in SQ-19 cells (Supplementary Fig. S1B). These results show that loss of Keap1 activity in these cells consistently enhanced the nuclear accumulation of Nrf2 and contributed to the increased expression of cytoprotective enzymes/proteins.

Because Nrf2 induces detoxifying enzymes and multidrug-resistant pumps, cell lines expressing lower levels of KEAP1

Figure 4. Low-level expression of KEAP1 in lung cancer cells provides advantages to lung cancer cell progression. A, expression of KEAP1 gene in normal lung tissues and lung cancer tissues. The KEAP1 expression of normal lung tissues (N) and lung cancer tissues (T) from five Japanese patients with adenocarcinomas (P1 to P5) were analyzed by real-time PCR. Level of KEAP1 gene expression in each normal tissue was set at 1.0. GAPD gene was used as an internal control. B, expression of KEAP1 gene of six lung cancer cell lines. The expression level of the cell lines RERF-LC-MS (lane 1), II-18 (lane 2), SQ-19 (lane 3), A549 (lane 4), H1437 (lane 5), or H1395 (lane 6) were analyzed by real-time PCR. Level of KEAP1 gene expression in RERF-LC-MS cell line was set at 1.0. GAPD gene was used as an internal control. C, nuclear localizations of Nrf2 in lung cancer cell lines with KEAP1 lower expression and mutation. Nrf2 in SQ-19 (normal KEAP1), II-18 (low expression of KEAP1), H1437 (low expression of KEAP1), or A549 (C333A mutant of KEAP1) cells was stained by anti-Nrf2 antibody. Nrf2 signals (green) were observed within the nucleus with a punctate pattern and in regions with little blue 4',6-diamidino-2-phenylindole signal in II-18, H1437, and A549 cells but not in SQ-19 cells. D, Nrf2-mediated gene expression provides drug resistance in lung cancer cell lines. SQ-19, II-18, H1437, or A549 cells were exposed to incremental amounts of cisplatin (0, 3, 6 and 12 μmol/L), and after 10 d, viable cells were determined. Data are presented as percentage of viable cells relative to the DMSO-treated control cells.
should show resistance to chemotherapy reagents. To test this possibility, we analyzed the sensitivity of these cell lines to cisplatin. Cell viability was measured 10 days after exposure to various concentrations of cisplatin. We found that cell lines expressing lower levels of **KEAP1** (H1437 and II-18) and **KEAP1** mutant cells (A549) showed greater resistance to cisplatin than SQ-19 cells with normal **KEAP1** (Fig. 4D), indicating that constitutive activation of Nrf2 enhances resistance to chemotherapy reagents.

Another intriguing possibility is that the constitutive activation of Nrf2 in cancer cells may confer selective growth advantages to cancer cells. Indeed, an Nrf2 target gene, **PRDX1** (21), enhances cell growth and radioresistance in the lung cancer cell line A549 (22), which harbors a mutation in the **KEAP1** gene (14). We therefore used real-time PCR to determine the **PRDX1** expression level in cell lines expressing lower levels of **KEAP1**. We found that it was higher in H1437, II-18, and A549 cells than in SQ-19 cells (Supplementary Fig. S1B). These results support the notion that some of the Nrf2 target genes in lung cancer cells may enhance cancer cell growth. Therefore, to determine whether knockdown of **NRF2** expression affects the growth of cancer cells with **KEAP1** mutation or reduced expression, we analyzed cell growth for 7 days after transfection of SQ-19 cells (**KEAP1** normal), II-18 cells (low expression of **KEAP1**), and A549 cells (**KEAP1** mutation) with **NRF2**-specific siRNA. We found that knockdown of **NRF2** expression (Fig. 5A, lanes 1–6) reduced expression of its target genes (Fig. 5A, lanes 9–12 and 15–18) and induced growth inhibition (Fig. 5B, lanes 3–6) of II-18 and A549 cells but not of SQ-19 cells [Fig. 5A, lanes 7, 8, 13 and B 1 and 2]. These results suggest that activation of Nrf2 through reduced Keap1 activity, either through mutation or reduced expression, provides growth advantages to lung cancer cells under homeostatic conditions. To further examine the advantages of cancer cells harboring the **KEAP1** mutation, we studied the growth of **Keap1**+/− MEFs. Both **Keap1**+/− and **Keap1**−/− MEFs grew at similar rate during the first few passages (Supplementary Fig. S2). However, following spontaneous immortalization of MEF cells and after several passages, **Keap1**−/− cells grew faster than **KEAP1**+/− and **Keap1**+/− cells (Fig. 5C). This result supports our contention that reduced Keap1 activity facilitates cell growth. The molecular basis of activated Nrf2-stimulated cell growth is unclear. Thus, further exploration of Nrf2 activation and its effect on cancer cell growth is necessary.

In summary, we identified somatic mutations of **KEAP1** and low-level expressions of **KEAP1** in lung cancer tissues and cell lines. These results indicate a high incidence/frequent occurrence of loss of Keap1 function in patients with lung cancer. The loss of Keap1 function enhanced the nuclear accumulation of Nrf2 and elevated the expression of antioxidative and antixenobiotic stress enzymes and drug efflux pumps, suggesting that cancer cells with weakened Keap1 function acquire multiple advantages for proliferation.
Therefore, disruption of Nrf2 by specific inhibitors may provide new therapeutic approaches against lung cancers with high Nrf2 activity.

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