Nrf2 and Keap1 abnormalities in esophageal squamous cell carcinoma and association with the effect of chemoradiotherapy

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Keywords
Chemoradiotherapy; esophageal squamous cell carcinoma; Keap1/Nrf2; locally advanced.

Abstract
Background: The Keap1-Nrf2 pathway is a key antioxidant and redox signaling cascade. Pathway abnormalities enhance the reactive oxygen species scavenging ability of cancer cells; thus the pathway is involved in carcinogenesis and resistance to chemoradiotherapy (CRT). This retrospective study was conducted to examine the status of the Keap1-Nrf2 pathway in locally advanced esophageal squamous cell carcinoma (ESCC) and to analyze its prognostic value in patients receiving CRT.

Methods: Nrf2 and Keap1 expression were immunohistochemically examined in 152 ESCC and 31 normal esophageal mucosae. All ESCC specimens were obtained from patients with locally advanced ESCC who underwent CRT.

Results: Strong staining of nuclear and cytoplasmic Nrf2 and limited or absent Keap1 expression was uncommon in normal tissues, but frequently observed in ESCC. Interaction between Nrf2 and Keap1 in normal mucosae is negatively correlated, while in tumors there is no negative correlation, indicating that there is little to no interaction between Nrf2 and Keap1 in ESCC. Positive Nrf2 expression in the nucleus was of diagnostic value for predicting ESCC from normal esophageal mucosae, and was significantly associated with poorer clinical response and poor progression-free survival after CRT. The value of Keap1 expression for diagnosis and predicting CRT outcomes was marginal. These different influences of Keap1 and Nrf2 on ESCC indicated that the signaling of this pathway was disturbed and displayed a Keap1-independent pattern.

Conclusion: Ablatory signaling via the Keap1-Nrf2 pathway was common in ESCC and was associated with response and survival after CRT.

Introduction
Esophageal carcinoma is one of the most common malignancies, with almost 500,000 new cases of the disease diagnosed worldwide every year.1 Esophageal squamous cell carcinoma (ESCC) is the major histological type and prevails in developing countries, causing over 200,000 deaths in China annually.2,3 For patients with locally advanced ESCC, definitive chemoradiotherapy (CRT) has been used as an effective treatment and significantly prolongs survival compared with radiotherapy alone.4,5 However, although CRT can initially achieve a considerable response at the cost of severe toxicity, most patients suffer recurrence within three years. Nevertheless, the underlying mechanisms as to how ESCC can resist CRT are not yet known.

Reactive oxygen species (ROS) play a dual role in cancer. Not only are they implicated in the genesis and progression of many cancers including ESCC, they are also involved in the antitumor mechanism of cytotoxic agents.
and radiation.6–18 Therefore, antioxidant and redox signaling has drawn increasing attention in cancer research and the Keap1-Nrf2 pathway is definitely one of the most important signaling cascades.

As a core transcription factor, Nrf2 is bound to Keap1 in cytoplasm and degrades in a proteasome-dependent manner under homeostatic conditions.19 Oxidative stress results in conformational change of the Keap1–Nrf2 complex, allowing Nrf2 to be translocated into the nucleus and activating the transcription of target antioxidant and redox genes.20–23 Aberrant signaling via the Keap1-Nrf2 pathway frequently occurs in cancer cells, leading to the over-activation of Nrf2 and elevation of ROS scavenging ability, facilitating the initiation and development of malignant cells that produce ROS during rapid proliferation.24–30 On the other hand, ROS are also indispensable for the therapeutic effect of platinum complexes, fluorouracil, and X-ray, which means that Nrf2 over-activation could also help cancer cells survive chemotherapy and irradiation and subsequently relapse.9,11–14,26,31

The promoting role of aberrant Keap1-Nrf2 signaling in carcinogenesis and therapy resistance has been well demonstrated in vivo and animal models;7,13,17,25,29,31,32 however, relevant clinical studies remain inadequate. Moreover, Keap1/Nrf2 expression in normal tissues has rarely been reported. In the present study, we examined the expression of Nrf2 and Keap1 in ESCC and normal esophageal mucosa and investigated their prognostic significance for predicting CRT response.

Methods

Patients and tissue samples

Patients with locally advanced ESCC (stage II and III according to the 2002 Union for International Cancer Control Tumor Node Metastasis [TNM] Staging System) diagnosed between January 2010 and February 2014 were enrolled in this study. The Ethics Committees at Shandong Cancer Hospital and Institute approved this study and informed consent was obtained from all participants.

Histological specimens of tumors were collected endoscopically and fixed in 10% formalin and embedded in paraffin wax. All patients underwent two cycles of cisplatin and 5-fluorouracil regimens (cisplatin 25 mg/m² 3 days, 5-fluorouracil 450–500 mg/m² 5 days) during radiotherapy at Shandong Cancer Hospital and institute (Jinan, China). Radiotherapy doses ranged from 60.4 to 70.0 Gy (median 65 Gy) to planning target volume delivered in 30–38 daily fractions (median 35). Additionally, we collected normal esophageal mucosa from patients who underwent esophagectomy for early stage ESCC. All normal tissues were collected in sites at least 5 cm from carcinoma tissues.

Follow-up and clinical data

Basic information including age, gender, smoking history, alcohol consumption history, tumor location, and TNM staging at the time of diagnosis was collected from medical records. All patients were regularly followed up with physical examinations every three months during the first two years after the last radiotherapy, and every six months thereafter until death or the closing date on 9 February 2017. Clinical response was assessed by standard clinical measurements, esophagography, and computed tomography examinations according to Response Evaluation Criteria in Solid Tumors. Overall survival (OS) was defined as the duration between diagnosis and death from any cause and was censored for survivors at the date of the last follow-up. Progression-free survival (PFS) was defined as the duration between diagnosis and the date of disease progression or death from any cause, and was censored at the date of the last visit for patients without progression.

Immunohistochemistry and evaluation of Nrf2 and Keap1 expression

All 4 µm thick ESCC and normal esophageal mucosal sections were cut from formalin fixed paraffin embedded (FFPE) blocks, deparaffinized in Bond Dewax Solution (Leica Microsystems, Wetzlar, Germany), and rehydrated by graded alcohol. Heat-induced antigen retrieval was achieved under high pressure for 20 minutes at 100°C using Bond Epitope Retrieval Solution 1 (Leica Microsystems). The sections were soaked in 3% hydrogen peroxide solution for 10 minutes to reduce endogenous peroxidase activity and were incubated afterward with primary antibodies against Nrf2 (ab31163, Abcam, Cambridge, UK) and Keap1 (10503-2-AP, Proteintech, Chicago, IL, USA) for two hours at room temperature. Post-primary immunoglobulin G linker reagent was applied for 10 minutes, and the slides were incubated with polymeric horseradish peroxidase immunoglobulin G reagent for 10 minutes to localize the primary antibodies. Diaminobenzidine tetrahydrochloride was used as the substrate to detect antigen–antibody binding. Finally, hematoxylin was applied for five minutes to counterstain nuclei.

Two pathologists independently evaluated the intensity, percentage, and sublocalization of each section. Conflicting results were summarized thereafter and resolved by using a multi-headed microscope. Cytoplasmic Keap1 and nuclear and cytoplasmic Nrf2 were quantified using a four-value intensity score (0, 1+, 2+, or 3+) and the percentage (0–100%) of the extent of reactivity. The quick (Q) score was used to determine expression levels, which was obtained by multiplying the percentage of positive cells (P) by the intensity (I) (Q = P × I; maximum = 300).33 The median values of...
the Q scores were used as cutoff points to classify “negative or low expression” and “positive or high expression.”

Statistical analysis

The differences between Nrf2 and Keap1 expression in normal esophageal mucosa and ESCC samples were assessed using a Mann–Whitney U test. Correlations of Nrf2 and Keap1 expression were evaluated using Spearman’s correlation test and illustrated as scattered plots. The χ² test was performed to evaluate the association of categorical variables. Curves for OS and PFS were obtained using the Kaplan–Meier method, and log-rank tests were performed to analyze differences in survival rates. Hazard ratios and corresponding 95% confidence intervals (CIs) for outcomes were estimated via univariate and multivariate Cox proportion regression models. All two-sided P values > 0.05 were considered statistically significant. Statistical analysis was performed using SPSS version 22.0 (IBM Corp., Armonk, NY, USA).

Results

Comparison of Nrf2 and Keap1 expression in normal esophageal mucosa and esophageal squamous cell carcinoma (ESCC)

A total of 152 ESCCs and 31 normal esophageal mucosa samples were included in this study. Immunohistochemical staining of normal esophageal mucosa and tumor specimens exhibited different patterns of Nrf2 and Keap1 expression (Fig 1). Cytoplasmic and nuclear staining of normal tissues revealed light to no Nrf2 expression. By contrast, Nrf2 expression in both the cytoplasm and nucleus was frequently observed in ESCC samples (Fig 1a). The medium Q scores of nuclear Nrf2 expression were 0 and 10 for the normal esophageal mucosa and ESCC, respectively. The medium Q scores of cytoplasmic Nrf2 were 0 for both kinds of specimens. While there was little difference between the median scores, Nrf2 expression was significantly more volatile in ESCC, reflecting inter-individual heterogeneity of tumors (Fig 1c). Generally, nuclear and cytoplasmic expression of Nrf2 in ESCC was stronger than in the normal tissue (P < 0.001). The difference in Keap1 expression between the normal esophageal mucosa and ECSS sample was also obvious (Fig 1b,c). Similarly, while the medium Q scores of Keap1 were 270 for both types of specimens, the Keap1 expression level fluctuated much more in ESCC (P = 0.025).

Furthermore, receiver operating characteristic analysis was conducted to evaluate the sensitivity and specificity of Nrf2 and Keap1 expression for predicting ESCC compared to normal tissues (Fig 2). Notably, high nuclear Nrf2 expression displayed considerable diagnostic significance with an area under the curve (AUC) of 0.829 (95% CI 0.771–0.887; P<0.001). High cytoplasmic Nrf2 expression displayed modest diagnostic significance with an AUC of 0.682 (95% CI 0.598–0.765; P = 0.001). The diagnostic value of low Keap1 expression was marginal with an AUC of 0.619 (95% CI 0.514–0.724; P = 0.037).

Correlation of Nrf2 and Keap1 protein expression

Spearman’s correlation testing was performed to examine the relationships of Nrf2 and Keap1 expression in normal esophageal mucosa and ESCC (Fig 3). In normal tissues, Keap1 expression was negatively correlated to both cytoplasmic (rho = −0.344) and nuclear (rho = −0.495) Nrf2 expression. The relationship between cytoplasmic and nuclear Nrf2 expression exhibited no correlation. In ESCC, the relationship between Nrf2 and Keap1 expression disappeared, while nuclear Nrf2 expression was positively correlated to cytoplasmic Nrf2 expression (rho = 0.763).

Relationships between Nrf2 and Keap1 expression and clinicopathologic characteristics of ESCC

The relationships between Nrf2 and Keap1 expression and clinicopathologic characteristics of ESCC are summarized in Table 1. The median Q scores of nuclear and cytoplasmic Nrf2 in ESCC were 10 and 0, respectively, which were subsequently used as cutoff points. Seventy-eight cases (51.32%) were classified as negative nuclear expression, whereas 74 cases (48.68%) were classified as positive. No significant correlations between nuclear Nrf2 and clinicopathologic characteristics were observed. Cases were further grouped into negative (79, 51.97%) and positive (73, 48.03%) Nrf2 expression subgroups. Positive cytoplasmic expression was associated with a heavy smoking history, probably reflecting the enhanced oxygen stress induced by cigarettes.

The median Q score of Keap1 was 270. Using the median score as a cutoff point, 64 (42.11%) and 88 (57.89%) cases were classified as low and high Keap1 expression, respectively. No significant correlations between Keap1 expression and clinicopathologic characteristics were observed.

Relationship between Nrf2 and Keap1 expression and clinical response to CRT

A total of 121 patients (79.61%) achieved a complete response (CR) or partial response (PR), while 31 patients (20.39%) experienced stable disease (SD) or progressive
disease (PD). Positive nuclear Nrf2 expression was associated with a significantly poorer response than negative Nrf2 expression (CR + PR: 71.62% vs. 87.18%; \(P = 0.017\)). By contrast, Keap1 and cytoplasmic Nrf2 expression were not associated with clinical response to CRT (Table 2).

### Association between Nrf2 and Keap1 expression and survival after CRT

Kaplan–Meier survival analysis using log-rank tests indicated that positive nuclear Nrf2 expression was associated with poor PFS (\(P = 0.010\)) (Fig 4). Although the PFS curve of the high Keap1 expression group remained above the low Keap1 expression group, significance was not achieved (\(P = 0.095\)). By contrast, Nrf2 cytoplasmic expression and Keap1 expression were not associated with OS and only Nrf2 staining in the nucleus influenced OS by trend (\(P = 0.075\)). In univariate and multivariate Cox proportional hazard analyses of clinicopathologic characteristics for PFS, nuclear Nrf2 expression was validated as an independent prognostic factor, as well as age and N stage (Table 3).

![Figure 1](image-url)  
**Figure 1** Nrf2 and Keap1 immunohistochemical stains in normal esophageal mucosa and esophageal squamous cell carcinoma (ESCC). (a) Representative cases of Nrf2 staining. Neither cytoplasmic nor nuclear expression of Nrf2 was common in normal esophageal mucosa (intensity = 0). ESCC samples displayed increased Nrf2 staining in both the cytoplasm and nucleus. (b) Representative cases of Keap1 staining. Keap1 expression was high in the normal esophageal mucosa (intensity = 3). ESCC showed various staining patterns of Keap1 and limited expression was common. (Original magnification = 400; Scale bar 50 μm). Black arrows indicate positive nuclear staining and white arrows indicate positive cytoplasmic staining. (c) Comparison of immunohistochemical Q scores of Nrf2 and Keap1 between ESCC and normal esophageal mucosa. The medium lines of boxes show the median value, the top and bottom lines of boxes represent the 75th and 25th percentiles, respectively, and the ends of whiskers represent the 10th and 90th percentiles.
Discussion

In this study, Nrf2 and Keap1 protein expression differed between normal esophageal mucosa and ECSS samples. In normal esophageal mucosa, stable expression of Keap1 and little to no Nrf2 expression in the nucleus reflected the homeostatic condition of normal cells. By contrast, positive expression of Nrf2 and limited immunohistochemical staining of Keap1 were much more common in the ESCC samples, which implied that Keap1/Nrf2 signaling might be disturbed during the development or progression of ESCC. Moreover, the diagnostic significance of nuclear Nrf2 positive expression was well displayed in receiver operating characteristic analysis, indicating that excessive nuclear translocation of Nrf2 exclusively occurred in tumors. These findings are inconsistent with the results of previous studies of in vitro and mouse models, which found that Keap1 dysfunction and Nrf2 over-activation
could facilitate normal cells to gain histological and molecular features of cancers.\textsuperscript{17,32}

The different correlation patterns of Nrf2 and Keap1 protein expression in the normal esophageal mucosa and ECSS samples also present aberrant signaling of the Keap1/Nrf2 pathway. Keap1, the inhibitor of the pathway, also acts as an adaptor in the Cul3-based E3 ligase complex which ubiquitinates Nrf2 binding with Keap1.\textsuperscript{19} Thus, in functional signaling, Nrf2 always degrades in a Keap1-dependent manner and Nrf2 expression should be negatively correlated with Keap1. Our Spearman test in normal esophageal mucosa confirmed this point. We also found that the relationship between Keap1 and nuclear Nrf2 is stronger than that of Keap1 and cytoplasmic Nrf2, probably because of the cytoplasmic anchoring effect of Keap1 on Nrf2. By contrast, in the ECSS samples, the negative correlation between the two proteins disappeared. A reasonable explanation is the disrupted interaction between Keap1 and Nrf2, which could result from somatic mutation. While ECSS rarely harbored Keap1 mutations,

| Characteristic       | N  | Negative | Positive | P  | Negative | Positive | P  |
|----------------------|----|----------|----------|----|----------|----------|----|
| All cases            | 152| 78       | 74       |    | 79       | 73       |    |
| Age                  |    |          |          |    |          |          |    |
| ≤ 65                 | 81 | 42       | 39       | 0.890 | 40       | 41       | 0.495 |
| >65                  | 71 | 36       | 35       |    | 39       | 32       |    |
| Gender               |    |          |          |    |          |          |    |
| Male                 | 108| 55       | 53       | 0.880 | 56       | 52       | 0.962 |
| Female               | 44 | 23       | 21       |    | 23       | 21       |    |
| Smoking index        |    |          |          |    |          |          |    |
| <400                 | 94 | 53       | 41       | 0.112 | 55       | 39       | 0.040* |
| ≥400                 | 58 | 25       | 33       |    | 24       | 34       |    |
| Alcohol intake       |    |          |          |    |          |          |    |
| Yes                  | 59 | 30       | 29       | 0.927 | 26       | 33       | 0.363 |
| No                   | 93 | 48       | 45       |    | 43       | 40       |    |
| Location             |    |          |          |    |          |          |    |
| Upper                | 66 | 37       | 29       | 0.305 | 37       | 29       | 0.377 |
| Lower                | 86 | 41       | 45       |    | 42       | 44       |    |
| T                    |    |          |          |    |          |          |    |
| T2                   | 18 | 9        | 9        | 0.921 | 9        | 9        | 0.642 |
| T3                   | 111| 58       | 53       | 0.867 | 60       | 51       | 0.465 |
| T4                   | 23 | 11       | 12       |    | 10       | 13       |    |
| N                    |    |          |          |    |          |          |    |
| N0                   | 32 | 16       | 16       | 0.867 | 20       | 12       | 0.180 |
| N1                   | 120| 62       | 58       |    | 59       | 61       |    |
| Stage                |    |          |          |    |          |          |    |
| II A                 | 30 | 15       | 15       | 0.900 | 19       | 11       | 0.256 |
| II B                 | 11 | 5        | 6        |    | 4        | 7        |    |
| III                  | 111| 58      | 53       |    | 56       | 55       |    |

\*P < 0.05. ESCC, esophageal squamous cell carcinoma.

| Protein     | Expression | CR + PR | SD + PD | \(\chi^2\) | P       |
|-------------|------------|---------|---------|------------|---------|
| Nrf2 in nucleus | Negative (%) | 68(87.18%) | 10(12.82%) | 5.661 | 0.017* |
|             | Positive   | 53(71.62%) | 21(28.34%) |      |        |
| Nrf2 in cytoplasm | Negative (%) | 64(81.01%) | 15(18.99%) | 0.201 | 0.654 |
|             | Positive | 57(78.08%) | 16(21.92%) |      |        |
| Keap1       | Low (%)    | 48(75.00%) | 16(25.00%) | 1.444 | 0.229 |
|             | High (%)  | 73(82.95%) | 15(17.05%) |      |        |
| Total       |            | 121(79.61%) | 31(20.39%) |      |        |

\*P < 0.05. CR, complete response; PD, progressive diseases; PR, partial response; SD, stable disease.
Nrf2 mutations were frequent, at a rate of 11.4–22%.²⁹,³⁴ All mutations impaired the binding affinity of motifs, which are the binding sites with Keap1.²⁹,³⁴ Hence, Keap1 lost function in Nrf2 mutated cells and Nrf2 was constitutively translocated into the nucleus. Unfortunately because every single endoscopically collected specimen was limited, DNA extraction and sequencing was unavailable. However, the positive nuclear Nrf2 staining rate of ECSS was 48.68%, much higher than Nrf2 mutation rates reported in previous studies, indicating the existence of other involved mechanisms. Indeed, recent studies have proven this point. Several disruptor proteins were identified, including p62 and PALB2, which can compete with Nrf2 to bind Keap1, thus resulting in Nrf2 over-activation.³⁵–³⁹ K-ras, B-raf, and Myc

Table 3 Cox regression analyses for progression-free survival

| Characteristic          | Univariate analysis |                 | Multivariate analysis |                 |
|-------------------------|---------------------|-----------------|-----------------------|-----------------|
|                         | HR (95% CI)         | P               | HR (95% CI)           | P               |
| Age                     | ≤ 65 vs. > 65 years | 0.692 (0.484–0.990) | 0.044*                | 0.618 (0.420–0.909) | 0.015*          |
| Gender                  | Female vs. male     | 0.992 (0.665–1.481) | 0.992                 | 0.868 (0.559–1.348) | 0.529           |
| Location                | Lower vs. upper     | 0.939 (0.655–1.345) | 0.730                 | 0.972 (0.673–1.405) | 0.881           |
| T                       | T4 vs. T2/T3        | 1.090 (0.667–1.781) | 0.731                 | 0.923 (0.561–1.519) | 0.753           |
| N                       | N1 vs. N0           | 1.905 (1.176–3.086) | 0.009*                | 2.077 (1.278–3.374) | 0.003*          |
| Nrf2 in nucleus         | Negative vs. positive | 0.629 (0.439–0.900) | 0.011*                | 0.606 (0.419–0.877) | 0.008*          |
| Keap1                   | Low vs. high        | 1.356 (0.946–1.942) | 0.097                 | 1.314 (0.903–1.910) | 0.154           |

*P < 0.05. CI, confidence interval; HR, hazard ratio.
oncogene activation and PTEN anti-oncogene disruption could upregulate the transcription of Nrf2.\textsuperscript{40,42} Furthermore, Keap1 promoter methylation and microRNA-targeting Keap1 have also been found in several cancers.\textsuperscript{43–47} In summary, Nrf2 hyperactivity could be induced in diverse ways during the carcinogenic progress. Therefore, the positive correlation between cytoplasmic and nuclear Nrf2 immunostaining in ECSS could reflect Keap1-independent upregulation of Nrf2.

Chemoradiotherapy with 5-fluorouracil and cisplatin is the standard treatment for locally advanced ECSS.\textsuperscript{4,5} ROS formation is indispensable in the mechanism underlying its therapeutic effect. About two-thirds of X-ray damage is caused by ROS generation via ionization of water molecules,\textsuperscript{18} and both 5-fluorouracil and cisplatin induce apoptosis in a ROS-dependent fashion.\textsuperscript{5,9,12} Therefore, in cancer cells with high Nrf2 activity, the cytotoxic efficacy of CRT should be heavily impaired as a result of antioxidant enzyme upregulation. A series of previous studies proved this viewpoint in cells and animal models. Tian et al. found that modification of Nrf2 and Keap1 expression changed cancer cell line sensitivity to platinum-based drugs.\textsuperscript{31} Lee et al. demonstrated that the functional inhibition of Nrf2 led to radiosensitivity enhancement in cells and mice xenografts.\textsuperscript{13} Other laboratory research has reached similar conclusions.\textsuperscript{8,17,29} Moreover, although rare, Kawasaki et al. conducted a relevant clinical trial and found that Nrf2 expression was related to CRT outcomes in patients with ECSS.\textsuperscript{30}

In regard to prognostic analysis, our findings were similar to those of Kawasaki et al. Using a much larger sample, we show that positive nuclear Nrf2 staining is associated with poor prognosis after CRT. The results demonstrate that CRT could induce a significantly higher objective response rate in patients with negative Nrf2 expression in the nucleus. In survival analysis, nuclear Nrf2 status only influenced a trend of OS after CRT and positive nuclear Nrf2 was significantly associated with poor PFS. Moreover, Nrf2 in the nucleus was identified as an independent prognostic factor of PFS. All of our results are consistent with those of previous studies and suggest that excessive nuclear translocation of Nrf2 indicates an impaired therapeutic effect of CRT.

We also analyzed the role of cytoplasmic Nrf2 and Keap1 expression in CRT for ECSS. In contrast to nuclear Nrf2, Nrf2 in the cytoplasm had no effect on response or survival rates after CRT. Perhaps high cytoplasmic expression of Nrf2 reflects downregulated degeneration and/or enhanced transcription of the protein, but not activation. Similarly, the prognostic value of Keap1 was marginal and its low expression indicated slightly poorer PFS, while high Keap1 expression indicated survival in lung squamous cell carcinoma.\textsuperscript{26} The lack of Keap1 influence on CRT in ECSS is probably a result of Nrf2, which can be activated in diverse ways, many of which are independent of Keap1 regulation. The loss of a negative correlation between nuclear Nrf2 and Keap1 in ESCC specimens indicates this is the case.

We examined the differences in Nrf2 and Keap1 expression between normal esophageal mucosa and ECSS samples. The promoting role of aberrant Keap1-Nrf2 signaling in carcinogenesis was proven in a clinical setting. Positive Nrf2 expression in the nucleus was associated with poor prognosis of ESCC after CRT. The results of this study imply that hyperactivity of Nrf2 contributes to cancer genesis and resistance of CRT in ESCC. Therefore the Keap1/Nrf2 pathway should be a key target of novel therapy in future and deserves more attention.

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Disclosure

No authors report any conflict of interest.

Reference

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin 2011; 61: 69–90. Published erratum appears in CA Cancer J Clin 2011:61:134.
2. Enzinger PC, Mayer RJ. Esophageal cancer. N Engl J Med 2003; 349: 2241–52.
3. Chen W, Zheng R, Baade PD et al. Cancer statistics in China, 2015. CA Cancer J Clin 2016; 66: 115–32.
4. Ishida K, Ando N, Yamamoto S, Ide H, Shinoda M. Phase II study of cisplatin and 5-fluorouracil with concurrent radiotherapy in advanced squamous cell carcinoma of the esophagus: A Japan Esophageal Oncology Group (JEOG)/Japan Clinical Oncology Group trial (JCOG9516). Jpn J Clin Oncol 2004; 34: 615–9. Published erratum appears in Jpn J Clin Oncol 2005:35:108.
5. Kato K, Muro K, Minashi K et al. Phase II study of chemoradiotherapy with 5-fluorouracil and cisplatin for stage II–III esophageal squamous cell carcinoma: JCOG trial (JCOG 9906). Int J Radiat Oncol Biol Phys 2011; 81: 684–90.
6. Schumacker PT. Reactive oxygen species in cancer: A dance with the devil. Cancer Cell 2015; 27: 156–7.
7. Shibata T, Saito S, Kokubu A, Suzuki T, Yamamoto M, Hirohashi S. Global downstream pathway analysis reveals a dependence of oncogenic NF-E2-related factor 2 mutation on the mTOR growth signaling pathway. Cancer Res 2010; 70: 9095–105.
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8 Itoh T, Terazawa R, Kojima K et al. Cisplatin induces production of reactive oxygen species via NADPH oxidase activation in human prostate cancer cells. *Free Radic Res* 2011; 45: 1033–9.

9 Schweyer S, Soruri A, Heinzte A, Radzun HJ, Fayazy A. The role of reactive oxygen species in cisplatin-induced apoptosis in human malignant testicular germ cell lines. *Int J Oncol* 2004; 25: 1671–6.

10 Zhang B, Wang Y, Su Y. Peroxiredoxins, a novel target in cancer radiotherapy. *Cancer Lett* 2009; 286: 154–60.

11 Matsunaga T, Tsuji Y, Kaai K et al. Toxicity against gastric cancer cells by combined treatment with 5-fluorouracil and mitomycin c: Implication in oxidative stress. *Cancer Chemother Pharmacol* 2010; 66: 517–26.

12 Lamberti M, Porto S, Marra M et al. 5-Fluorouracil induces apoptosis in rat cardiocytes through intracellular oxidative stress. *J Exp Clin Cancer Res* 2012; 31: 60.

13 Lee S, Lim MJ, Kim MH et al. An effective strategy for increasing the radiosensitivity of human lung cancer cells by blocking Nrf2-dependent antioxidant responses. *Free Radic Biol Med* 2012; 53: 807–16.

14 Gupta S, Singh KK, Vyas VJ, Chaturvedi VN, Reddy MV, Harinath BC. Assessment of oxidative stress and effect of antioxidant supplementation during radiotherapy in carcinoma of upper digestive tract. *Indian J Clin Biochem* 2000; 15: 52–5.

15 Ozben T. Oxidative stress and apoptosis: Impact on cancer therapy. *J Pharm Sci* 2007; 96: 2181–96.

16 Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. *Int J Biochem Cell Biol* 2007; 39: 44–84.

17 Jeong Y, Hoang NT, Lovejoy A et al. Role of KEAP1/NRF2 and TP53 mutations in lung squamous cell carcinoma development and radiation resistance. *Cancer Discov* 2017; 7: 86–101.

18 Borek C. Antioxidants and radiation therapy. *J Nutr* 2004; 134: 3207S–98.

19 Kobayashi A, Kang MI, Okawa H et al. Oxidative stress sensor Keap1 functions as an adaptor for Cul3-based E3 ligase to regulate proteasomal degradation of Nrf2. *Mol Cell Biol* 2004; 24: 7130–9.

20 Zhang DD. Mechanistic studies of the Nrf2-Keap1 signaling pathway. *Drug Metab Rev* 2006; 38: 769–89.

21 Ishii T, Itoh K, Takahashi S et al. Transcription factor Nrf2 coordinately regulates a group of oxidative stress-inducible genes in macrophages. *J Biol Chem* 2000; 275: 16023–9.

22 Katoh Y, Itoh K, Yoshida E, Miyagishi M, Fukamizu A, Yamamoto M. Two domains of Nrf2 cooperatively bind CREB, a CREB binding protein, and synergistically activate transcription. *Genes Cells* 2001; 6: 857–68.

23 Banning A, Deubel S, Kluth D, Zhou Z, Brigelius-Flohe R. The G1-GPx gene is a target for Nrf2. *Mol Cell Biol* 2005; 25: 4914–23.

24 Stacy DR, Ely K, Massion PP et al. Increased expression of nuclear factor E2 p45-related factor 2 (NRF2) in head and neck squamous cell carcinomas. *Head Neck* 2006; 28: 813–8.

25 Ohta T, Iijima K, Miyamoto M et al. Loss of Keap1 function activates Nrf2 and provides advantages for lung cancer cell growth. *Cancer Res* 2008; 68: 1303–9.

26 Solis LM, Behrens C, Dong W et al. Nrf2 and Keap1 abnormalities in non-small cell lung carcinoma and association with clinicopathologic features. *Clin Cancer Res* 2010; 16: 3743–53.

27 Adam J, Hatipoglu E, O’Flaherty L et al. Renal cyst formation in Fh1-deficient mice is independent of the Hif/Phd pathway: Roles for fumarate in KEAP1 succination and Nrf2 signaling. *Cancer Cell* 2011; 20: 524–37.

28 Ooi A, Wong JC, Petillo D et al. An antioxidant response phenotype shared between hereditary and sporadic type 2 papillary renal cell carcinoma. *Cancer Cell* 2011; 20: 511–23.

29 Shibata T, Kokubu A, Saito S et al. Keap1/Nrf2 regulator for predicting the effect of chemoradiation therapy on esophageal squamous cell carcinoma. *Ann Surg Oncol* 2014; 21: 2347–52.

30 Kawasaki Y, Okamura H, Uchikado Y et al. Nrf2 is useful for predicting the effect of chemoradiation therapy on esophageal squamous cell carcinoma. *Ann Surg Oncol* 2014; 21: 2347–52.

31 Tian Y, Wu K, Liu Q et al. Modification of platinum sensitivity by KEAP1/NRF2 signals in non-small cell lung cancer. *J Hematol Oncol* 2016; 9: 83.

32 Umemura A, He F, Taniguchi K et al. p62, upregulated during preneoplasia, induces hepatocellular carcinogenesis by maintaining survival of stressed HCC-initiating cells. *Cancer Cell* 2016; 29: 935–48.

33 Charafe-Jauffret E, Tarpin C, Bardou VJ et al. Immunophenotypic analysis of inflammatory breast cancers: Identification of an ‘inflammatory signature’. *J Pathol* 2004; 202: 265–73.

34 Kim YR, Oh JE, Kim MS et al. Oncogenic NRF2 mutations in squamous cell carcinomas of oesophagus and skin. *J Pathol* 2010; 220: 446–51.

35 Jain A, Lamark T, Sjøttem E et al. p62/SQSTM1 is a target protein through competitive binding to KEAP1 protein. *J Biol Chem* 2010; 285: 22576–91.

36 Ichimura Y, Waguri S, Sou YS et al. Phosphorylation of p62 activates the Keap1-Nrf2 pathway during selective autophagy. *Mol Cell* 2013; 51: 618–31.

37 Camp ND, James RG, Dawson DW et al. Wilms tumor gene on X chromosome (WTX) inhibits degradation of NRF2 protein through competitive binding to KEAP1 protein. *J Biol Chem* 2012; 287: 6539–50.

38 Hast BE, Goldfarb D, Mulvany KM et al. Proteomic analysis of ubiquitin ligase KEAP1 reveals associated proteins that inhibit NRF2 ubiquitination. *Cancer Res* 2013; 73: 2199–210.
39 Ma J, Cai H, Wu T et al. PALB2 interacts with KEAP1 to promote NRF2 nuclear accumulation and function. *Mol Cell Biol* 2012; 32: 1506–17.
40 DeNicola GM, Karreth FA, Humpton TJ et al. Oncogene-induced Nrf2 transcription promotes ROS detoxification and tumorigenesis. *Nature* 2011; 475: 106–9.
41 Mitsuishi Y, Taguchi K, Kawatani Y et al. Nrf2 redirects glucose and glutamine into anabolic pathways in metabolic reprogramming. *Cancer Cell* 2012; 22: 66–79.
42 Yamadori T, Ishii Y, Homma S et al. Molecular mechanisms for the regulation of Nrf2-mediated cell proliferation in non-small-cell lung cancers. *Oncogene* 2012; 31: 4768–77.
43 Wang R, An J, Ji F, Jiao H, Sun H, Zhou D. Hypermethylation of the Keap1 gene in human lung cancer cell lines and lung cancer tissues. *Biochem Biophys Res Commun* 2008; 373: 151–4.
44 Muscarella LA, Barbano R, D’Angelo V et al. Regulation of KEAP1 expression by promoter methylation in malignant gliomas and association with patient’s outcome. *Epigenetics* 2011; 6: 317–25.
45 Hanada N, Takahata T, Zhou Q et al. Methylation of the KEAP1 gene promoter region in human colorectal cancer. *BMC Cancer* 2012; 12: 66.
46 Barbano R, Muscarella LA, Pasculli B et al. Aberrant Keap1 methylation in breast cancer and association with clinicopathological features. *Epigenetics* 2013; 8: 105–12.
47 Eades G, Yang M, Yao Y, Zhang Y, Zhou Q. miR-200a regulates Nrf2 activation by targeting Keap1 mRNA in breast cancer cells. *J Biol Chem* 2011; 286: 40725–33.