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Targeted therapy of esophageal squamous cell carcinoma: the NRF2 signaling pathway as a target

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Abstract

Esophageal squamous cell carcinoma (ESCC) is a deadly disease that requires extensive research. Here, we review the current understanding of the functions of the NRF2 signaling pathway in the esophagus. Genomic data suggest that gene mutations and several other mechanisms result in NRF2 hyperactivation in human ESCC. As a consequence, $NRF2^{high}$ ESCC is more resistant to chemoradiotherapy and has poorer survival than $NRF2^{low}$ ESCC. Mechanistically, we believe NRF2, functioning as a transcription factor, causes an esophageal phenotype through regulation of gene transcriptional. We discuss metabolism, mitochondria, proteasomes, and several other signaling pathways as downstream players that may contribute to esophageal phenotype due to NRF2 hyperactivation. Finally, strategies are proposed to target the NRF2 signaling pathway for future therapy of $NRF2^{high}$ ESCC.

Graphical abstract

Genomic data suggest that gene mutations and several other mechanisms result in NRF2 hyperactivation in human esophageal squamous cell carcinoma (ESCC). As a consequence, $NRF2^{high}$ ESCC is more resistant to chemoradiotherapy and has poorer survival than $NRF2^{low}$ ESCC. We discuss metabolism, mitochondria, proteasomes, and other signaling pathways as downstream players that may contribute to phenotypes due to NRF2 hyperactivation

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Competing interests

The authors declare no competing interests.

Keywords

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As a major cellular defense mechanism, the nuclear factor erythroid-derived 2–like 2 (NRF2 or NFE2l2) signaling pathway is known to regulate the expression of enzymes involved in detoxification and the antioxidative stress response. NRF2 forms heterodimers with small Maf proteins and binds to the antioxidant response elements of target genes when cells are exposed to oxidative stress or xenobiotics. Kelch-like ECH-associated protein 1 (KEAP1) inhibits the function of NRF2 by retaining NRF2 in the cytoplasm under normal physiological conditions and allowing nuclear translocation of NRF2 under stress conditions.[1] Although the cancer-preventive function of the NRF2 signaling pathway has been well documented, recent studies have revealed that NRF2 activity is a double-edged sword, as it can be carcinogenic when hyperactive. NRF2 was found to prevent initiation but accelerate the progression of lung carcinogenesis *in vivo*.[2,3] Many studies have repeatedly shown that NRF2 helps cancer cells survive chemoradiation-induced oxidative stress and accelerates drug metabolism[4–7] thus contributing to chemoradioresistance.[8] NRF2 overexpression is associated with poor prognosis in cancer.[9] Genetic targeting of the NRF2 signaling pathway impaired tumorigenesis in the lung, pancreas, and colon.[10–12]

NRF2 in the esophagus

The role of the NRF2 signaling pathway in the esophagus was first revealed in a mouse study by Yamamoto's group in 2003. Genetic activation of NRF2 in *Keap1*$^{-/-}$ mice resulted in esophageal hyperproliferation and hyperkeratosis. These mice died from poor nutrition due to esophageal blockage, and *Nrf2*$^{-/-}$–*Keap1*$^{-/-}$ mice completely rescued the esophageal phenotype.[13] The esophageal phenotype of *Keap1*$^{-/-}$ mice was further attributable to constitutive activation of NRF2 with the assistance of small Maf proteins.[14,15] Tissue-specific deletion of esophageal NRF2 in *Keap1*$^{-/-}$ mice (K5Cre *Nrf2*$^{fl/fl}$ *Keap1*$^{-/-}$) allowed survival until adulthood. However, these mice developed polyuria with low osmolality and bilateral hydronephrosis due to defects in water reabsorption as a result of reduced expression of aquaporin 2 in the kidney.[16] Consistent with these findings using genetic models, chemical activation of NRF2 by tert-butylated hydroxyanisole or its metabolite tert-butylhydroquinone caused hyperkeratosis and squamous cell carcinoma in rodent forestomach.[17–19] It should be noted that hyperkeratosis is a precursor lesion of carcinogen-induced esophageal squamous cell carcinoma (ESCC) in rodents.[20–22] In humans, esophageal hyperkeratosis has also been reported as a result of gastroesophageal reflux, vitamin A deficiency, or tylosis A.[22] On the other hand, *Nrf2*$^{-/-}$ mice were more susceptible to 4-nitroquinoline-1-oxide–induced tongue and esophageal carcinogenesis than wild-type mice, whereas KEAP1 knockdown mice were resistant.[23]

When we compared differential gene expression between the normal esophagus and Barrett's esophagus,[24] NRF2 was found to be one of the transcriptional factors enriched in normal esophageal squamous epithelium. We then studied how the NRF2 signaling pathway regulates morphogenesis of the esophageal epithelium in mice by comparing gene

expression profiles in wild-type, *Nrf2*$^{-/-}$, *Keap1*$^{-/-}$ and *Nrf2*$^{-/-}$ *Keap1*$^{-/-}$ esophagi using gene microarrays. We found that the NRF2 signaling pathway had a baseline activity at the early stage and was further activated later during the development of mouse esophageal squamous epithelium. *Keap*1$^{-/-}$ esophagus had an increased expression of keratinization genes, PI3K/Akt pathway genes, and PPARβ/δ.[22] Since the keratinized layer is the major protective layer against physical stress and chemical injuries,[25] and terminally differentiated keratinocytes express proteins that can provide protection by quenching reactive oxygen species,[26] we hypothesized that NRF2 may be involved in esophageal epithelial barrier function and may therefore play a protective role during gastroesophageal reflux. Indeed, NRF2 deficiency reduced transepithelial electrical resistance and increased intercellular space in the esophageal epithelium through downregulation of claudin 4 (CLDN4). Chromatin immunoprecipitation (ChIP) analysis clearly showed binding of NRF2 to the predicted sites in the promoter region of mouse *Cldn4*. Meanwhile, NRF2 target genes and gene sets associated with oxidoreductase activity, mitochondrial biogenesis, and energy production were downregulated in *Nrf2*$^{-/-}$ esophagus. Consistent with these observations, ATP biogenesis and CoxIV (a mitochondrial marker) were also downregulated.[27] These data suggested that energy-dependent tight junction integrity was subject to NRF2 regulation. Activating NRF2 may potentially strengthen esophageal epithelial barrier function as a therapeutic approach for gastroesophageal reflux disease.[28–30]

Que's group further demonstrated that basal progenitor cell–specific expression of constitutively active bone morphogenetic protein (BMP) promoted squamous differentiation in mouse esophagus. The action of BMP was mediated through increased intracellular oxidative stress and an NRF2-mediated antioxidative response. This mechanism is further involved in the development of eosinophilic esophagitis, in which reduced squamous differentiation is associated with high levels of follistatin (a BMP inhibitor) and disrupted BMP/NRF2 pathways.[31]

Gene mutations and NRF2 hyperactivation in human ESCC

With the recent technological advances in next-generation sequencing, human ESCC samples from North and South America, China, Japan, Vietnam, and Malawi have been sequenced.[32–46] ESCC shares similar genomic profiles with head and neck SCC and lung SCC, but not esophageal adenocarcinoma, suggesting common etiological factors.[47–49] In fact, *NRF2* and *KEAP1* have been classified among 291 high-confidence cancer-driver genes acting on 3205 tumors from 12 different cancer types.[50]

Among many gene mutations, *NRF2* mutations were commonly seen, with a frequency over 5%, even up to ~ 20% in certain reports. Mutations in other genes of the NRF2 signaling pathway (*KEAP1* and *CUL3*) were relatively less common. *NRF2* mutations were mostly located in the DLG and ETGE motifs (KEAP1-binding domain) and the DNA-binding domain, while *KEAP1* mutations tended to be scattered across the whole gene (Fig. 1). *NRF2* mutations and *KEAP1* mutations were mutually exclusive in human lung cancer cell lines.[51] In human ESCC tissue samples, such mutual exclusivity was also suggested.[33,52]

On the basis of the genomics data, ESCC can be clustered into three subtypes, with subtype 1 (56%, 50/90) characterized by genomic alterations in the NRF2 signaling pathway. This subtype had a higher frequency of SOX2 and/or p63 amplification and potential involvement of the Hippo pathway, similar to head and neck SCC and lung SCC.[44] Asian patients tended to be clustered in subtype 1, whereas Eastern European and South American patients clustered in subtype 2, and North American patients in subtype 3.[44] ESCC in African American patients also tended to involve the NRF2 signaling pathway.[53] Similar to ESCC, a molecular subtype with NRF2 activation has also been reported in head and neck SCC based on microarray data.[54,55]

NRF2 mutations have not been reported in esophageal squamous hyperplasia and non-tumorous dysplasia. However, they were present in low-grade dysplasia and high-grade dysplasia associated with ESCC.[36–38] Phylogenetic analysis showed that *NRF2* mutation as a driver mutation tended to be located on the branches of the tumor phylogenetic tree, while mutations of tumor suppressor genes (e.g., p53) tended to be located on the trunk, suggesting that *NRF2* mutation may be a relatively late event during the development of ESCC.[37,46]

Many point mutations in *NRF2* have been shown to activate NRF2 as a result of altered interaction between NRF2 and KEAP1[56–58] and increased nuclear localization of NRF2.[59] Certain *KEAP1* mutations, when heterozygous, had a dominant-negative effect on the wild-type KEAP1 and thus gave rise to NRF2 activation.[60] Genomic mutations of the NRF2 signaling pathway correlated with the transcriptional activity of the NRF2 signaling pathway in ESCC.[45] We also found that human ESCC can be clustered into $NRF2^{high}$ and $NRF2^{low}$ cases according to gene microarray data of several esophagus-specific NRF2 target genes.

Other than mutations, at least five additional mechanisms are known to activate NRF2 in cancer: hypomethylation of *KEAP1*, accumulation of disruptor proteins, increased production of NRF2, electrophoretic attack of KEAP1 by oncometabolites, and downregulation of *NRF2*-targeting microRNAs (miRNAs).[61,62] This explains why there is a much higher percentage of ESCC with NRF2 hyperactivation than with point mutations.

Consequences of NRF2 hyperactivation in ESCC

Significant correlations were found between positive NRF2 expression and unfavorable response to chemoradiotherapy in ESCC patients. NRF2 overexpression was significantly correlated with lymph node metastasis, postoperative recurrence, and overall survival.[36,63,64] Multivariate analysis showed that NRF2 expression status was an independent prognostic factor.[64] Even the molecular signatures due to *NRF2* mutations were significantly predictive and prognostic for clinical response. Mutant NRF2 conferred increased cell proliferation, attachment-independent survival, and resistance to 5-fluorouracil and γ-irradiation.[36] Blockage of NRF2 suppresses the migration and invasion of ESCC cells in a hypoxic microenvironment.[63] These data support the notion that NRF2 hyperactivation plays an important role in ESCC and can be targeted to improve the therapeutic efficacy of conventional therapy.

How does NRF2 hyperactivation contribute to ESCC?

NRF2, working as a transcription factor, regulates gene transcription.[65] Previous ChIP-Seq experiments have shown that NRF2 potentially up- or downregulates transcription of hundreds of genes.[66–69] We hypothesized that NRF2 hyperactivation caused esophageal hyperproliferation and hyperkeratosis through gene transcriptional regulation in esophageal squamous epithelial cells. Due to the nature of cell and tissue specificity in transcription factor binding,[70–72] the ChIP-Seq experiment will need to be repeated with esophageal samples. Several pathways are known to be regulated by NRF2.

Metabolism

In addition to their involvement with the metabolic phenotype of NRF2 hyperactivation in the esophagus, mitochondria also regulate oxidative stress, cell signaling, and cell death during carcinogenesis.[73,74] Our previous study showed that the number of mitochondria was decreased in *NRF2*$^{-/-}$ esophagus compared with wild-type esophagus. Mitochondrion-related gene sets were downregulated in *NRF2*$^{-/-}$ esophagus. Reduction of mitochondria was confirmed by downregulation of a mitochondrial marker protein (Cox IV). These data were consistent with other studies showing that NRF2 regulates mitochondrial biogenesis and cellular bioenergetics.[75–77] It is also known that KEAP1 and NRF2 are tethered to mitochondria through PGAM5 and p62.[78,79] NRF2 regulates productions of reactive oxygen species and thus protects against mitochondrial decay.[80,81] Therefore, mitochondria may be potentially critical for the phenotypes attributable to NRF2 hyperactivation.[82,83]

Proteasome

Proteasomal subunits are known to be regulated by the NRF2 signaling pathway.[84] Proteasome inhibitors may be effective for NRF2^{high} ESCC.[85] It has been reported that NRF2 contributes to colon carcinogenesis through its regulation of proteasomes.[86] Inhibition of NRF2 by the alkaloid trigonelline rendered pancreatic cancer cells more susceptible to apoptosis through decreased proteasomal gene expression and proteasome activity.[87]

Notch signaling

Recent literature suggests a cross talk between the NRF2 and Notch signaling pathways.[88,89] We also showed that expression of both NICD1 and HES1 in oral squamous epithelial cells were regulated by NRF2,[90] consistent with previous findings in hematopoietic stem cells,[91] airway basal stem cells,[92] and mouse embryonic fibroblasts.[93] Although the Notch signaling pathway is believed to be anti-carcinogenic through its regulation of squamous epithelial cell differentiation,[94,95] several recent studies indicated that upregulation of the Notch signaling pathway may contribute to the malignant phenotype in these cells as well.[96–98] It remains to be determined whether NRF2-activated Notch signaling pathway plays a dual role in ESCC.

PI3K/Akt signaling

The epidermal growth factor (EGF) signaling pathway has long been associated with human ESCC. With eight ligands and four receptors, this pathway has several downstream signaling paths, one of which is the PI3K/Akt pathway.[99–101] Recent next-generation sequencing studies have confirmed *PIK3CA* mutations as drivers in ESCC.[32–34] Phospho-Akt levels

were increased in the *KEAP1*$^{-/-}$ esophagus.[22] In the literature, transgenic overexpression of EGF ligand or receptor (AREG, ERBB2) and a constitutively active Akt or transgenic knockout of PTEN (an inhibitor of the PI3K/Akt pathway) caused esophageal hyperkeratosis in mice.[102–105] It appears that NRF2 and PI3K/Akt regulate each other in a reciprocal manner. Loss of PTEN increased NRF2 activity.[106] Since PI3K/Akt mutations and activation are commonly seen in human ESCC,[33,52] it would be interesting to further understand how these two signaling pathways interact with each other. More importantly, targeting both signaling pathways may have synergistic effects on ESCC.

The NRF2 signaling pathway as a therapeutic target in ESCC

We believe that the esophagus is a unique organ site for studies on NRF2 hyperactivation owing to the strong esophageal phenotype in the *KEAP1*$^{-/-}$ esophagus.[13] Targeting the NRF2 signaling pathway in the esophagus will not only help us develop a better therapy for NRF2^{high} ESCC but also potentially contribute to therapy of NRF2^{high} cancers of other organ sites (e.g., head and neck, lung). However, tissue of origin and environment are critical factors implicated in carcinogenesis driven by genetic alterations. It will be essential to focus on the esophagus to develop therapeutic strategies for NRF2^{high} ESCC. Additionally, *in vivo* studies will likely be more reliable than *in vitro* cell culture studies, as seen in recent cancer metabolism studies.[107,108]

Several strategies have been proposed to target the NRF2 signaling pathway for cancer therapy: transcriptional downregulation of NRF2; increased degradation of *NRF2* mRNA or decreased translation; enhancement of NRF2 degradation through upregulation/activation of KEAP1–CUL3, β-TrCP-SCF, or HRD1; blocking the dimerization of NRF2 with small Maf proteins; and blocking the NRF2–sMaf DNA-binding domain.[109] In addition, NRF2 downstream pathways may also be targeted if they can be shown to be functionally critical for NRF2^{high} ESCC.[110] For example, a recent study used chemical proteomics to map druggable proteins that are selectively expressed in NRF2^{high} lung cancer. NR0B1 was identified as a downstream druggable target, and small molecules were found to disrupt NR0B1 protein complexes and thus inhibit NRF2-dependent lung cancer.[111] Several small molecule NRF2 inhibitors—halofuginone,[112] brusatol,[113] AEM1,[114] and ML385[115]—have been identified by high-throughput screening. We are also in the process of screening NRF2 inhibitors from chemical libraries for NRF2^{high} ESCC. Yet, targeting a transcription factor can be a challenge. Most known NRF2 inhibitors may actually target mechanisms other than NRF2 itself. In addition to small molecule inhibitors, miRNA can be an alternative approach. A reporter-coupled miRNA library screen identified four miRNAs (miR-507, -634, -450a, and -129-5p) that negatively regulate the NRF2 signaling pathway. Administration of miR-507 alone or in combination with cisplatin inhibited tumor growth *in vivo*.[62]

It should be noted that the location of *NRF2* mutations on the branches of tumor phylogenetic trees suggests that targeting NRF2 may not be as effective as targeting the trunks (e.g., p53). Targeting branches may even lead to growth acceleration of non-mutated subpopulations.[46] In fact, Clemons proposed targeting the glutathione biosynthesis pathway

(or NRF2 signaling pathway) in p53-mutanted cancers, considering that more than 80% of ESCCs harbor mutations in the p53 gene.[116]

Conclusions

NRF2 hyperactivation is one of the commonly seen molecular alterations in human ESCC. Multiple studies have clearly shown a poor prognosis in cases with hyperactive NRF2. Therefore, it is critical to understand the molecular mechanisms of ESCC associated with hyperactive NRF2 and develop targeted therapy directed at NRF2 signaling. We believe NRF2 as a transcription factor causes an esophageal phenotype through gene transcriptional regulation. Several strategies have been proposed to target the NRF2 signaling pathway for future therapy of $NRF2^{high}$ ESCC.

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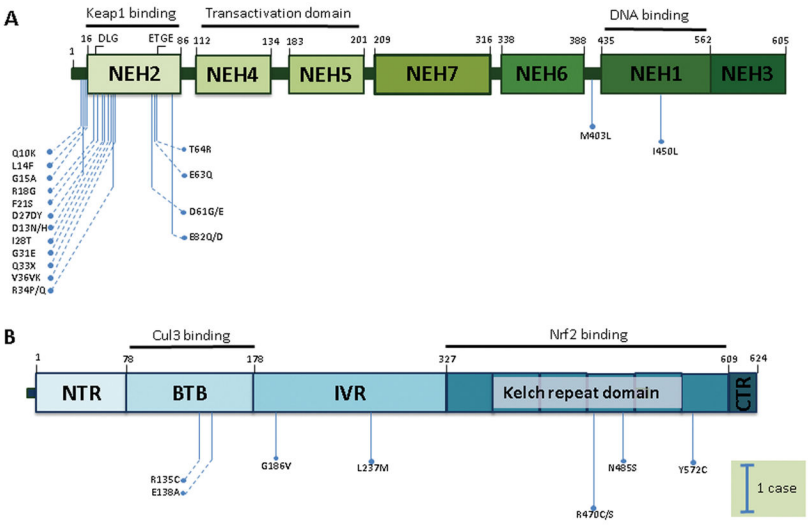

Figure 1.

Point mutations in *NRF2* and *KEAP1* in human ESCC based on original data from four recent next-generation sequencing studies from China.[32–34,40]