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Review Article

Nrf2-Keap1 Activation, A Promising Strategy in the Prevention of Cancer

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ABSTRACT

Oxidative stress has been implicated in many human diseases such as aging, neurodegenerative disorders and many in cancer. Nrf2 (a master transcription factor that regulates antioxidant response element (ARE) mediated expression of antioxidant enzymes and cyto protective proteins) plays an important role in the response to intrinsic oxidative stress. Excess reactive oxygen species (ROS) causes oxidative damage to cellular DNA, and other bio molecules such as lipids and proteins; genetic changes and/or epigenetic alterations can lead to the deregulation of oncogenes and tumour suppressor genes, ultimately contributing to carcinogenesis. To alleviate this oxidative stress, there are several anti oxidative stress response, most of which are regulated by Nrf2. Keap1 (a regulator of Nrf2 activity) is a cyto plasmic, cysteine-rich, actin-bound protein that chelate NRF2 in the cytoplasm for degradation. Upon contact with ROS, NRF2 is stabilized and translocate into the nucleus and activate the transcription of many cyto protective genes encoding detoxification and antioxidant protein and exerts a protective function against xenobiotics and oxidative stresses. Increasing attention has been paid to the role of Nrf2 in cancer cells because the constitutive stabilization of Nrf2 has been observed in many human cancers with poor prognosis. Better understanding of the molecular mechanism underlying Nrf2 function in response to ROS and electro philes will provide bases for the development of a new strategy aimed at preventing oxidative stress as well as attenuating oxidative-induced cyto toxicity associated with carcinogenesis. Recent advances are highlighted in the understanding of Nrf2-Keap1 gene, transcriptional regulation, complex binding and function and Nrf2-keap1 transcription regulation pathway as a promising prevention of cancer.

Key words: Antioxidant response element (ARE), Cancer, Nrf2, Keap1, Oxidative stress.

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INTRODUCTION

Bodies are inevitably and constantly insulted by different environmental stresses caused by toxic chemicals, free radicals, carcinogens, and xeno biotic metabolites, which can induce the pathogenesis of many diseases, including cancer, diabetes, atherosclerosis, Alzheimer's disease, and arthritis.[1-4]

The expression of an array of antioxidant and phase 2 drug metabolizing enzymes protects cells from various oxidative stresses.[5-8] The induction of these cyto protective genes are regulated at the transcriptional level by a specific cis-acting element, the antioxidant/electro phile response element (ARE/EpRE) found in the promoter regions of these genes.[9,10] Nrf2 (nuclear factor E2-related factor 2) has been found to be the central transcription factor that interacts with the ARE/Ep RE to trans activate cyto protective gene expression constitutively or to induce the expression in response to oxidative stress signals.[11]

Nrf2 belongs to the Cap'n'Collar (CNC) family of transcription factors that contain a conserved basic region-leucine zipper structure.[12] By comparing the human and chicken Nrf2 amino acid sequences, six highly homologous regions have been defined in Nrf2 (Neh1 to Neh6 domains).[13] Of these Neh domains; Neh1, Neh3, and Neh6 are located in the N-terminal half of Nrf2. Neh1 contains a CNC-type basic-leucine zipper DNA binding motif, and Neh6 contains a serine-rich conserved region. In the N-terminal half, there are two acidic trans activation domains, Neh4 and Neh5, which have been shown to interact with the KIX and CH3 domains of CBP for trans activation.[14] Neh2 located at the N-terminus of Nrf2 acts as the regulatory domain for cellular stress response. Neh2 interacts with a cyto plasmic protein Keap1 (Kelch-like ECH-associated protein 1).[13] Keap1 possesses four functional domains: BTB (Broad complex, Tram track, and Bric-a-Brac) (Bardwell and Treisman, 1994), IVR (intervening region), DGR (double glycine repeat or Kelch repeat),[15] and CTR (C-terminal region).

The BTB domain, like some of its structural homologs,[16] has been shown to serve as a dimerization domain, and dimerization of Keap1 appears to be important for effective function of Keap1.[17] Being a substrate adaptor of the Cul3-based E3 ligase machinery[18,19] and an oxidative stress sensor,[20] Keap1 utilizes the DGR and CTR domains to interact with the Neh2 domain of Nrf2. This intermolecular interaction allows Keap1 to regulate the rate of Nrf2 protein turnover through ubiquitin signalling and proteasomal proteolysis. Oxidative/ electrophilic stress signals are transduced by modification of the sulfhydryl groups of reactive cysteines within the IVR domain,[20] which attenuates both polyubiquitination and proteasomal degradation of Nrf2[18] and results in an enhanced nuclear accumulation of Nrf2 for trans activating ARE dependent cellular protective enzymes,[21] such as heme oxygenase 1 and NAD(P)H-quinone oxido educates 1.

The nuclear factor erythroid 2-related factor 2 or (Nrf2) Kelch-like ECH-associated protein 1 system represents an important mechanism by which mammalian cells can sense and adapt to chemical and oxidative stresses.[22-24] Normally, Keap1 targets Nrf2 for ubiquitylation, leading to its proteasomal degradation.[25] In response to chemical or oxidative stress, the interaction between Nrf2 and Keap1 is perturbed, resulting in the stabilization and nuclear accumulation of Nrf2.[24,26] Nrf2 localised in the nucleus interacts with antioxidant response elements in the promoter regions of a plethora of genes coding for phase 2 detoxifying enzymes (e.g. glutathione-S-transferases and NAD(P)H quinone oxido reductase), antioxidant proteins (e.g. glutathione synthetic enzymes) and transporters (e.g. ABCC2, ABCC3, ABCG2 and xc - subunit.[27-31] Elevated

Nrf2 levels have been observed in head and neck,[32] gall bladder[33] and lung cancer,[34] and evidence indicates that a dysregulated Nrf2/Keap1 system may protect against the deleterious effects of oxidative stress, whilst also conferring properties of enhanced cellular proliferation and a drug-resistant phenotype, in certain cancers,[34-36] effectively acting as a double edged sword.[35]

Studies have demonstrated that Nrf2 promotes the survival of not only normal cells but also cancer cells. Accumulation of Nrf2 in cancer cells creates an environment conducive for cell growth and protects against oxidative stress, chemotherapeutic agents, and radiotherapy. This phenomenon has been termed the "dark side of Nrf2".[37,38] This discovery has opened up a broad spectrum of research geared toward a better understanding of the role of Nrf2 signalling in cancer and has set a new paradigm for the development of pharmacological reagents targeting Nrf2 for cancer prevention and treatment.

The Nrf2–Keap1 (Kelch-like erythroid cell-derived protein with CNC homology [ECH]-associated protein 1)–ARE (antioxidant response).

Nrf2 contains seven functional domains, known as Neh1– Neh7 (Figure 1). Of these, the Neh2 domain, located in the N terminus of Nrf2, is the major regulatory domain. Neh2 contains seven lysine residues that are responsible for ubiquitin conjugation[19] as well as two binding sites (termed ETGE and DLG motifs) that help regulate Nrf2 stability.[26] The ETGE and DLG motifs interact with Keap1, which is a substrate adaptor protein for the Cullin 3 (Cul3)- dependent E3 ubiquitin ligase complex that represses Nrf2 by promoting its ubiquitination and subsequent protease mal degradation.[18,19,29,30] The Neh1 and Neh6 domains have also been reported to regulate the stability of Nrf2. Neh1 contains a CNC-type bZIP DNA-binding motif that allows Nrf2 to bind DNA and dimerize with other transcription factors.[41] Additionally, the Neh1 domain has been shown to interact with UbcM2, a ubiquitin-conjugating enzyme, to regulate the stability of Nrf2.42 The Neh6 domain contains two binding sites (DSGIS and DSAPGS motifs) for the b-transducin repeat- containing protein (b-TrCP). b-TrCP acts as a substrate adaptor for the Skp1–Cul1–Rbx1/Roc1 ubiquitin ligase complex. Phosphorylation of the DSGIS motif by GSK-3 increases the ability of b-TrCP to ubiquitinate Nrf2 and promote its rapid turnover.[43-45] The Neh3, Neh4, and Neh5 domains interact with co activators to enable the transactivation of Nrf2 target genes. The Neh3 domain binds to the chromo-ATPase/ helicase DNA-binding protein family member CHD6, which functions as an Nrf2 transcriptional coactivator.[46] The Neh4 and Neh5 domains have been shown to interact with the CH3 domains of CBP (CREB binding protein) to facilitate the transactivation of Nrf2 target genes.[14,47] Recently, however, a seventh Neh domain (Neh7, amino acids 209–316) was identified and shown to interact with the retinoic X receptor a, an Nrf2 repressor, and repress Nrf2 target gene transcription.[48]

THE KEAP1

Nrf2 is primarily regulated by Keap1, a substrate adaptor for a Cul3-containing E3 ubiquitin ligase. Keap1 possesses three functional domains, including a broad complex/tram track/bric-a-brac (BTB) domain, an intervening region (IVR), and a Kelch domain, also known as the double glycine repeat (DGR) domain (Figure 1). The BTB domain binds Cul3 and is required for Keap1 dimerization.[50,51] The Kelch/ DGR domain is critical for maintaining the interaction between Nrf2 and Keap1 by interacting with the Neh2 domain of Nrf2.[13,43] The IVR links the BTB and Kelch/DGR domains and contains several cysteine residues that have been proposed to regulate Keap1 activity.[18,52] Thus, each of the three domains is thought to play a unique role in mediating Nrf2 ubiquitination and repression. Under basal conditions, Nrf2 is primarily localized in a complex with Keap1 via direct protein–protein interactions between the Keap1 Kelch domain and the ETGE and DLG motifs on the Neh2 domain of Nrf2 (Figure 2). Keap1 has been shown to bind to the ETGE motif with a higher affinity than to the DLG motif.[25,26] Based on these observations, a two-site substrate recognition hinge-and-latch model describing the interaction between Nrf2 and Keap1 was developed.[25,26] The model suggests that Keap1 recruits Nrf2 via the ETGE motif (hinge), and once this interaction has been established, the DLG motif (latch) docks onto an adjacent unoccupied Kelch repeat domain on Keap1. Two Keap1 molecules position the seven ubiquitin-accepting lysine residues that are located between the DLG and ETGE motifs of Nrf2 in a favorable position and promote Nrf2 polyubiquitination and its subsequent proteasomal degradation by the 26S proteasome.[19,25,53] Therefore, the Keap1–Cul3–E3 ubiquitin ligase complex tightly regulates Nrf2 protein to maintain it at a low level. Conversely, recent evidence has demonstrated that USP15 (ubiquitin-specific peptidase 15), a deubiquitinating enzyme, also plays an important role in mediating the ubiquitination and degradation of Nrf2. USP15 deubiquitinates Keap1, stabilizes the Keap1– Cul3–E3 ligase complex, and enhances its E3 ligase activity, which ultimately leads to the degradation of Nrf2.[54]

Nrf2–Keap1 signaling pathway

In response to a diverse array of stimuli, it has been proposed that critical cysteine residues, especially Cys151, within Keap1 can be covalently modified, allowing Nrf2 to evade Keap1-mediated ubiquitination (Figure 3). The human Keap1 protein contains 27 cysteine residues that can be oxidized to sulfenic acid, form disulfides, or be covalently adducted by electrophiles.[55-57] The modification of thiols on Keap1 is thought to alter its conformation and results in the release of Nrf2 from the low-affinity binding site (DLG motif); however, Nrf2 remains attached to Keap1 by the ETGE motif. These changes are thought to prevent Nrf2 ubiquitination.[25-26] Consequently, Keap1 molecules become saturated with Nrf2 that is no longer targeted for degradation, and newly synthesized, free Nrf2 trans locates to the nucleus. In the nucleus, Nrf2 dimerizes with members of the masculoaponeurotic fibro sarcoma (Maf) protein family that have been shown to facilitate the binding of Nrf2 to AREs located within the regulatory regions of a wide variety of genes involved in cytoprotection and metabolism.[27,58-60] The ARE is a cis-acting DNA enhancer sequence with the consensus sequence 59-RTGABnnnGCR-39, where conserved nucleotides are in capitals, and the "n" represents any nucleotide.61 The Nrf2–Maf heterodimer recruits transcriptional co activators that promote the transcription of genes involved in[1] regulating the synthesis and metabolism of glutathione, such as glutamate–cysteine ligase catalytic subunit (GCS);[2] antioxidant proteins specializing in neutralizing reactive species such as glutathione peroxidase (GPX);[3] drug vmetabolizing enzymes like UDP-glucuronosyl-transferase 1A1;[4] xenobiotic transporters, including multidrug resistance protein 1 (MRP1); and[5] numerous other stress response proteins. By inducing the expression of this battery of genes, Nrf2 is able to augment a wide range of cell defense processes, thereby enhancing the overall capacity of cells to detoxify potentially harmful entities. As such, the Nrf2–Keap1 pathway is generally considered a major cellular defense pathway.

Under basal conditions, Keap1 binds to the ETGE and DLG motifs on Nrf2 and brings Nrf2 into Keap1–Cul3–E3 ubiquitin ligase complex, leading to ubiquitination and subsequent degradation of Nrf2. Oxidative stress or electrophiles can cause a conformational change in the Keap1–Cul3–E3 ubiquitin ligase by acting on specific cysteine residues in Keap1. These changes disrupt Nrf2–Keap1 binding at the DLG domain. Nrf2 is stabilized, and free Nrf2 translocates to the nucleus, where it dimerizes with members of the small Maf family and binds to AREs (59-RTGABNNNGCR-39) within regulatory regions of a wide variety of cell defense genes, including NQO1, GCLM, HO-1, and MRP1. (E) ETGE; (D) DLG.[49]

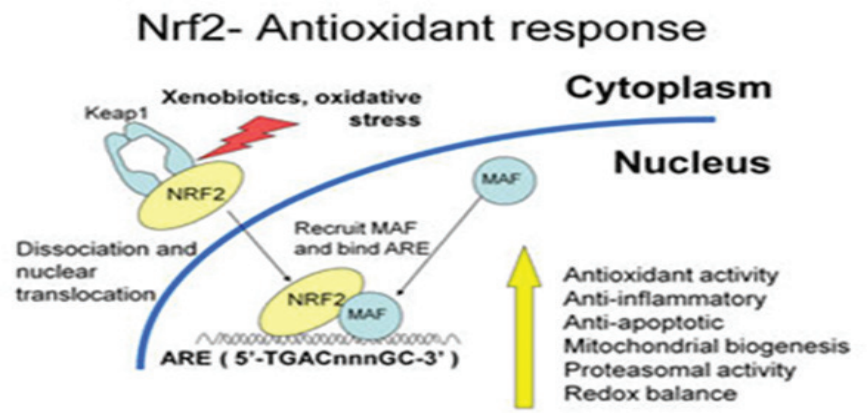

Figure 1: Nrf2 antioxidant response (Zhang *et al.*,)[19] NRF2.

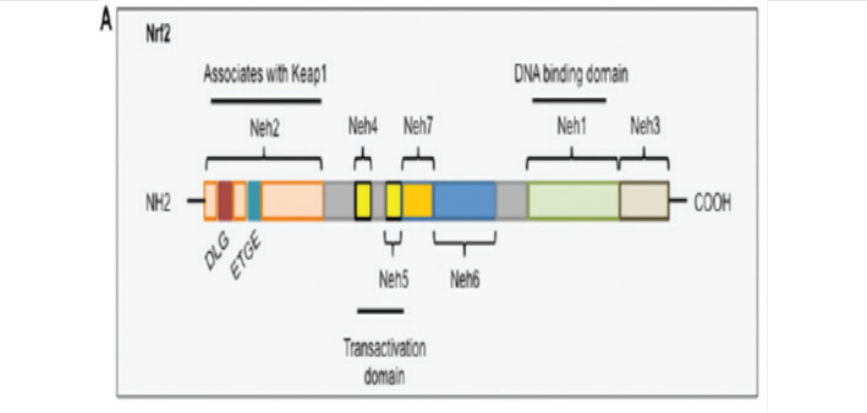

Figure 2: Conserved domains of Nrf2. (A) Nrf2 contains seven domains, known as Neh1–Neh7. The Neh2 domain contains two binding motifs, DLG and ETGE, which interact with Keap1. The Neh4, Neh5, and Neh3 domains are important for the transactivation activity of Nrf2. The Neh6 domain is a serine-rich region that regulates Nrf2 stability. The Neh1 domain is a basic region leucine zipper motif that is important for its stability, DNA binding, and dimerization with Maf. (Jaramilloi *et al.*,).[49]

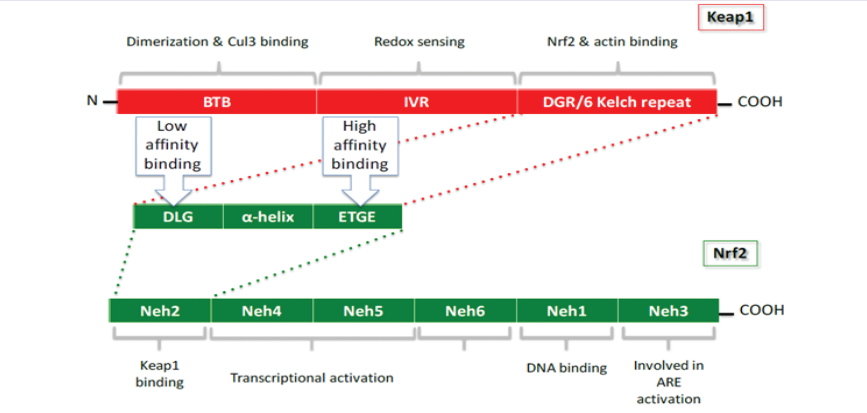

Figure 3: Schematic representation of the interaction between Nrf2 and Keap1 (Itoh).[13]

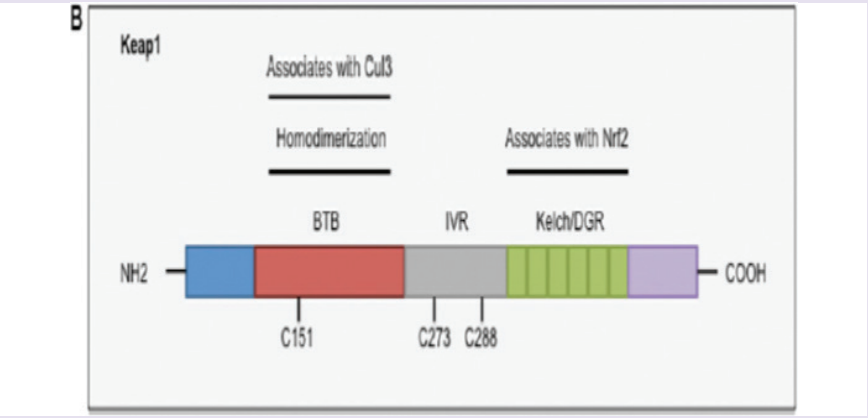

Figure 4: Conserved domains Keap1. (B) Keap1 contains three major domains. The BTB domain mediates Keap1 homodimerization and associates with Cul3. The IVR domain contains critical cysteine residues and connects the BTB domain with the C terminus Kelch/DGR domain. The Kelch/DGR domain mediates binding with the Neh2 domain of Nrf2.[49]

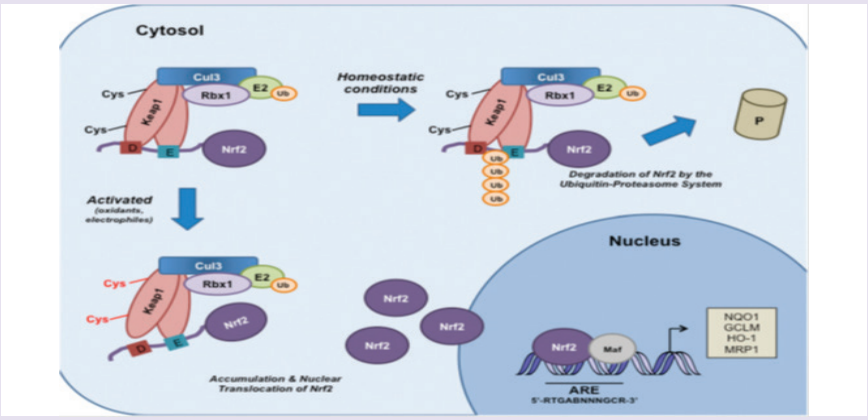

Figure 5: (Schematic model of the Nrf2–Keap1 signaling pathway.[62]

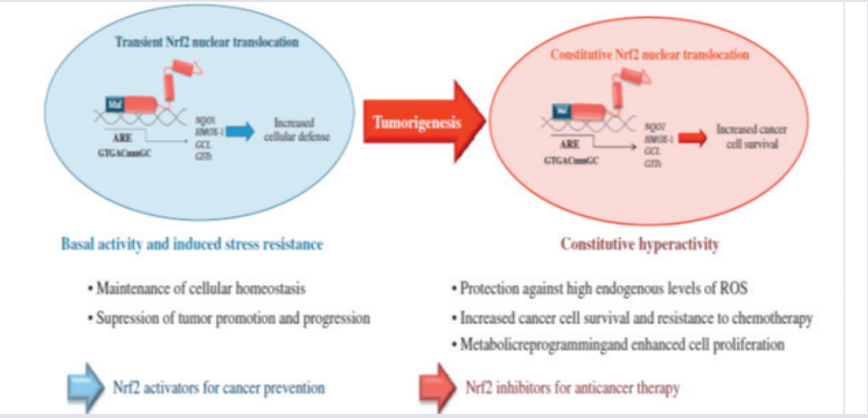

Figure 6: Dual role of Nrf2.Several mechanisms have been reported for the increased activity of Nrf2 in cancers, including[1] somatic mutations in KEAP1, CUL3, or NRF2;[2] epigenetic silencing of Keap1;[3] aberrant accumulation of proteins that disrupt the interaction between Nrf2 and Keap1;[4] transcriptional up-regulation of NRF2 through oncogene-dependent signaling; and[5] modification of Keap1 by metabolic intermediates.[72]

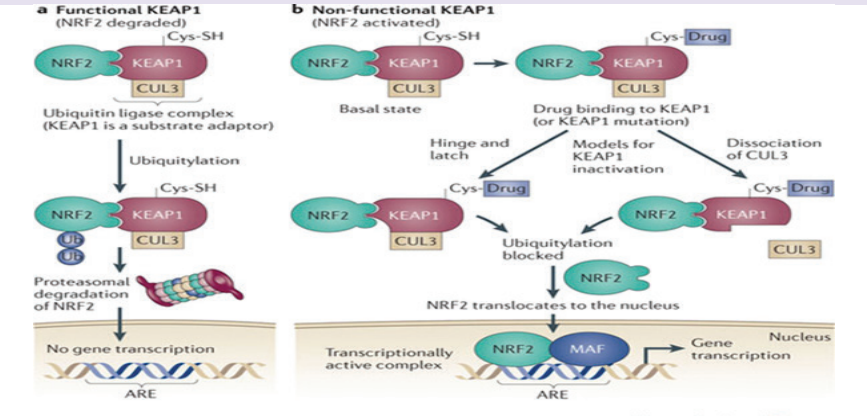

Figure 7: General pathway for the mechanism of Nrf2-Keap1.[31]

THE DUAL ROLE OF NRF2 IN CANCER

Tumor suppressor functions of Nrf2: 'the good side of Nrf2'

Several studies using Nrf2 knockout mice (Nrf2) shows that Nrf2 protects against chemical carcinogen-induced tumour formation in the stomach, bladder, and skin. For example, Nrf2-null mice are more likely to develop gastric neoplasia after exposure to benzo(a)pyerene compared with wild-type mice.[63] Higher tumor burdens were reported in the intestines of Nrf2-deficient mice challenged with azoxymethane followed by dextran sodium sulfate compared with wildtype mice.[64] In addition, Nrf2-deficient mice had a higher incidence of bladder tumors following exposure to N-nitrosobutyl(4- hydroxybutyl)amine[65] as well as an increased incidence of skin tumors after exposure to 7,12-dimethylbenz(a)anthracene or 12-O-tetradecanoylphorbol- 13-acetate, two potent carcinogens.[66] The mechanism by which Nrf2 protects against chemical induced carcinogenesis may be due in part to its ability to reduce the amount of reactive oxygen species (ROS) and DNA damage in cells.[67] Further evidence supporting the protective role of Nrf2 comes from studies with mice harboring a single-nucleotide polymorphism (SNP) in the promoter region of the mouse Nrf2 gene. Mice with this SNP have reduced expression of Nrf2 and are more susceptible to hyperoxiainduced lung damage.[68] The human NRF2 gene also harbors a SNP in its promoter region (rs6721961).[69] Individuals with this SNP have significantly lower NRF2 messenger RNA (mRNA) levels and an increased risk of developing non-small-cell lung cancer (NSCLC).[70]

Oncogenic functions of Nrf2: 'the dark side of Nrf2'

Although a wide body of evidence indicates that activation of Nrf2 protects against a variety of toxicants and diseases, the prolonged activation of Nrf2 has been shown to favor the progression of several types of cancers. Nrf2 has been shown to be constitutively elevated in lung, breast, head and neck, ovarian, and endometrial carcinomas. The prognosis of patients with tumors expressing high levels of Nrf2 in the clinic is poor (Shibata *et al.*, 2008; Solis *et al.*, 2010; Sasaki *et al.*, 2012) partly due to Nrf2's ability to enhance cancer cell proliferation and promote chemo-resistance and radio-resistance. In addition, Nrf2 expression is induced during the course of drug resistance. Collectively, these studies suggest that Nrf2 contributes to both intrinsic and acquired chemo-resistance.[34-71]

Mechanisms of Nrf2-Keap1-ARE transcription regulation

Comprehensive studies have been devoted to elucidate molecular mechanisms responsible for activation of Nrf2. Under normal physiological conditions, Keap1 physically entrapsinactive Nrf2 in the cytoplasm, thereby repressing its translocation to the nucleus.[13] During electrophilic stress, Nrf2 translocates into the nucleus, thus initiating Nrf2-ARE transcriptional activation.[72] While the molecular mechanisms involved in the Nrf2 transcriptional activation of antioxidant enzymes are still debated, studies have in consensus demonstrated that Keap1 is the major repressor of Nrf2 through various regulatory mechanisms. From *in vitro* experimental studies, Keap1 co-expression in cells has been demonstrated to prevent Nrf2 trans activation activity.[13] The suppressive effect of Keap1 can be abolished significantly by antioxidant treatments, indicating that Keap1-Nrf2 complex may be destabilized by alterations in cellular redox state.[20]

How can Nrf2 fight against cancer?

- Protect cells against cellular stress/free radical damage.
- Safeguard cell from effects of inflammatory stress.
- Enhance production/activity of the bodies potent antioxidant enzymes.
- Restricts/modulate underlining mechanisms involved in carcinogenesis.
- Positively influence genes involved in formation of cancer cells.
- Shows ability to slow progression of and 'kill' cancer cell (Mitsuishi *et al.*,2012).

CONCLUSION

Reactive oxygen species (ROS) can contribute to cancer by damaging macromolecules like DNA and proteins, and the beneficial impact of antioxidant molecules in reducing cancer risk is well appreciated. However, constitutive activation of the antioxidant master regulator NRF2 via somatic mutations has also been implicated in carcinogenesis. Therefore, the role of NRF2-initiated antioxidant activity in etiology, progression, and treatment of disease continues to increase in complexity.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBEREVIATION USED

ARE: Antioxidant response element; **BTB:** Broad complex/tram track/bric-a-brac; **CNC:** Cap'n'Collar; **Cul3:** Cullin 3; **DRG:** Double glycine repeat; **GCS:** glutamate–cysteine ligase catalytic subunit; **EpRE:** Electrophile response element; **GPx:** Glutathione peroxidase; **Keap1:** Kelch-like ECH-associated protein 1; **Maf:** Masculoaponeurotic fibro sarcoma; **MPR1:** Multidrug resistance protein 1; **mRNA:** Messenger RNA; **Nrf2:** nuclear factor E2-related factor 2; **ROS:** Reactive oxygen species.

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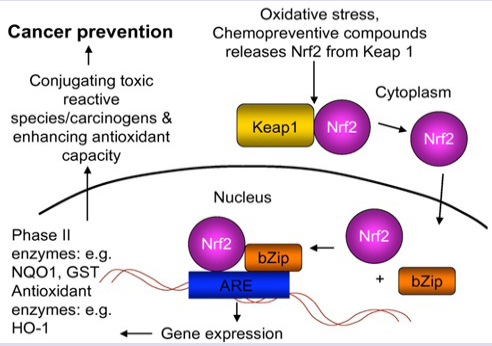
PICTORIAL ABSTRACT



SUMMARY

- Nrf2 fight against cancer by restricting or modulating the underlining mechanisms involved in carcinogenesis.
- Nrf2 enhance production and activity of the bodies potent antioxidant enzymes against cancer.
- Keap1 is a major repressor of Nrf2 through various regulatory mechanism
- Nrf2-Keap1 activation stimulation initiates cancer chemoprevention and promote overall health.

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