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A review on Nrf2 and antioxidant metalloproteins

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ABSTRACT

Nrf2 (nuclear factor erythroid 2–related factor 2) is a crucial transcription factor that regulates cellular defense against oxidative and electrophilic stress. Under basal conditions, Nrf2 binds to Keap1 (Kelch-like ECH-associated protein 1), which promotes its ubiquitination and degradation. The primary cellular response to oxidative or electrophilic stress is mediated through a redox-sensitive protein complex in which actin-associated Keap1 interacts with Nrf2. Upon exposure to stress-inducing agents, critical cysteine residues of Keap1 undergo modification, leading to conformational changes that disrupt the Keap1-Nrf2 interaction. As a result, Nrf2 escapes ubiquitination, stabilizes, and accumulates in the cytoplasm before translocating to the nucleus. Metalloproteins are well recognized as essential reservoirs and protective agents for trace metals such as iron, zinc, and copper, which are critical cofactors for numerous antioxidant enzymes. By controlling the expression of genes encoding metalloproteins and metal-binding proteins, Nrf2 contributes to the precise regulation of intracellular metal balance. This coordination is particularly important because, while essential metals are required for antioxidant defense, their dysregulation can promote oxidative damage through redox cycle reactions. Therefore, Nrf2-mediated regulation of metalloproteins represents a crucial interface between redox homeostasis and metal metabolism, reinforcing its central role in cellular protection.

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1. Introduction

Nrf2 (nuclear factor erythroid 2-related factor 2) plays a well-known role in the activation of antioxidant genes. It acts by binding to other proteins in the cell nucleus, called antioxidant response elements (AREs), which are located in the promoter region of rapidly accelerated fibrosarcoma (RAF) genes. However, these AREs are present not only in the promoter regions of antioxidant genes, but also in genes involved in other cellular protective functions. There are more than 500 genes activated by Nrf2, as well as genes down-regulated by Nrf2, others indirectly regulated through transcription factors regulated by Nrf2, and a small number directly regulated by AREs with inhibitory effects [1]. In short, Nrf2 activates a large number of genes; however, both can increase or decrease gene expression through other transcription factors and even suppress some genes by directly affecting transcription.

During the past 20 years, our understanding of the antioxidant functions of Nrf2 has improved significantly. Nrf2 activates genes containing the antioxidant response element (ARE) in their promoters, leading to an increased synthesis of detoxifying and antioxidant enzymes, such as superoxide dismutase (SOD), which converts superoxide radicals into hydrogen peroxide; catalase (CAT), which decomposes hydrogen peroxide into water and oxygen; and glutathione peroxidase (GPx), which reduces peroxides using glutathione. Nrf2 is a master regulator of the antioxidant response and xenobiotic metabolism through regulation of a wide range of antioxidants [2,3]. Nrf2 protects cells from stressors such as endogenous substances, reactive oxygen species (ROS), radiation, environmental toxins, and xenobiotics derived from the diet. Therefore, activation of the Nrf2 pathway may be a promising strategy for chemoprevention. This perspective is supported by several studies showing that Nrf2 is essential for chemopreventive agents such as sulforaphane and ortho-phenylphenols to inhibit carcinogenesis, and that Nrf2-deficient mice are at increased risk of chemically induced cancer [4-6].

The transcription factor, Nrf2, is primarily responsible for regulating a set of antioxidants and cytoprotective genes in response to oxidative stress. Nrf2 is negatively regulated in the cytoplasm by the Kerch-like ECH-associated protein 1 (Keap1). Keap1 is a substrate adaptor protein that binds to Nrf2 in the cytoplasm and promotes its polyubiquitination by the Cullin3 (Cul3) E3 ubiquitin ligase, thus facilitating its proteasomal degradation. The antioxidant properties of Nrf2 are believed to

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be exerted primarily by stimulating the transcription of antioxidant proteins; however, its effect on intracellular ROS generation is not fully understood. Nrf2 binds to antioxidant response elements (AREs), which are specific sequences present in the promoter regions of target genes, and stimulates the transcription of antioxidant proteins as heterodimers with small Maf proteins. Importantly, intracellular ROS levels are determined not only by the availability of ROS scavengers but also by the systems or enzymes that generate ROS as a major product or byproduct of catalytic reactions. ROS are generated during mitochondrial respiration, and mitochondria are thought to be one of the main sources of ROS generation in cells. Nrf2 affects the availability of substrates for mitochondrial respiration and, therefore, may also affect mitochondrial ROS generation. This process results in the stabilization, accumulation, and nuclear translocation of Nrf2, which heterodimerizes with sMaf proteins and binds to AREs, thus potently inducing the expression of cytoprotective genes encoding enzymes involved in the detoxification of ROS and other oxidants.

One noteworthy observation is that among the antioxidant genes activated by Nrf2, one of the most commonly studied is the haeme oxygenase 1 (HO-1) gene, which converts free haeme, which has pro-oxidant effects, into iron, carbon monoxide (CO), and biliverdin, which is converted into the antioxidant bilirubin via an activity also increased by Nrf2, encoded by two biliverdin reductase genes [7,8]. Iron released by haeme oxygenase is sequestered by ferritin because Nrf2 induces each of the four ferritin genes, preventing iron-induced oxidative stress [7]. This coordinated control of multiple gene-mediating proteins that are functionally linked to produce an important biological response has been repeatedly found in Nrf2-mediated gene regulation. Antioxidant responses are also produced by CO owing to its regulatory role. Heme oxygenase appears to have an important role in producing Nrf2 responses, based on studies using specific enzyme inhibitors or HO-1 gene knockout mice.

The most studied antioxidant gene activated by Nrf2 is quinone oxidoreductase (NQO1), which encodes an enzyme that prevents the redox cycle of semiquinones and thereby reduces oxidative stress [8]. Two superoxide dismutase genes (SOD1 and SOD2) are activated by Nrf2, and each SOD reduces oxidative stress by reducing superoxide levels. The functionally linked catalase and two glutathione peroxidase genes are induced by Nrf2, and these enzymes reduce the H_2O_2 generated from superoxide by SOD. Thus, Nrf2 mediates the coordinated control of multiple antioxidant genes [8].

Reactive oxygen species (ROS) are by-products of aerobic respiration and are signalling molecules that control various cellular functions. Although ROS are often considered harmful, they act as important signalling molecules at controlled levels. ROS (especially H_2O_2) modify cysteine residues in the regulatory protein Keap1. This modification alters the structure and function of Keap1. Consequently, Nrf2 is released in its repressed state. Nrf2 controls gene expression related to endogenous antioxidant synthesis and ROS-scavenging enzymes in response to various electrophilic compounds that inactivate the negative regulator Keap1. Increasing evidence suggests that mitochondrial ROS (mtROS) production is often mediated by specific protein kinases that activate Nrf2 and induce the expression of antioxidant genes and genes involved in mitochondrial quality/quantity control. Nrf2 is activated or inactivated by mtROS. High levels of mtROS are believed to inactivate the signaling cascade that activates Nrf2, thus inhibiting its activity. However, different locations of ROS generation (matrix or intermembrane space) or differences in the reactive species generated (e.g. secondary generation of hydrogen peroxide, superoxide, or more complex reactive species such as peroxynitrite) may also be involved in Nrf2 inhibition. More research is required to clarify these issues. Since mtCAT expression prevents Nrf2 activation, evidence supports hydrogen peroxide as the culprit of downstream ROS. Nrf2 and other factors (eg., sirtuins, ATF4, and Klf9) reciprocally regulate each other's functions and influence cell fate toward recovery or cell death. The activation of Nrf2 and ATF4 remodels cellular metabolism and upregulates glutathione and NADPH to maintain cytoplasmic and mitochondrial redox homeostasis. Taking antioxidants is not always beneficial for exercise-based health promotion (discussed in this review) or cancer chemoprevention [9], possibly due to impaired mitochondrial excitability (known as "the antioxidant oxidation paradox"). Elucidating the status of Nrf2-centric networks and their associated molecular mechanisms under various pathophysiological conditions is essential to understand healthy homeostasis and predict the outcomes of future Nrf2 activation interventions.

Under oxidative stress, cells maintain redox homeostasis by reprogramming their metabolism and gene expression through the activation of Nrf2 and other stress response pathways. Nrf2 is considered a master regulator of antioxidants, antioxidant biosynthesis, and transcriptional activation of metabolic switch genes [10]. Under non-stress conditions, Nrf2 is ubiquitinated by Keap1 and Cullin-3 E3 ligases and degraded by the 26S proteasome pathway. Keap1 oxidation inhibits Keap1-mediated Nrf2 [11]. When the Keap1 binding site is filled with Nrf2, the newly synthesized Nrf2 translocates to the nucleus and induces the transcriptional activation of target genes, including antioxidant response elements (ARE), within gene regulatory regions [12]. Thus, Nrf2 initiates redox signalling and maintains intracellular redox homeostasis [13]. AREs are important for redox regulation under both stress and nonstress conditions. In a study using mice, Ito *et al.* [2] discovered that the induction of phase II enzymes by ARE is regulated by the protein transcription factor Nrf2.

The efficiency of antioxidant defence and the rate of ROS production are major factors that determine the extent of tissue damage in many diseases [14-22]. The production of reactive oxygen species increases due to exposure to hyperoxia [19,21], which is thought to overwhelm immature antioxidant defence systems and cause oxidative tissue damage, especially in lung tissue [15,23,24]. Antioxidant defence consists of a network of enzymes, proteins, and low-molecular-weight ROS scavengers that protect cellular structures from the harmful effects of ROS. Antioxidant enzymes such as Cu/Zn superoxide dismutase (Cu/Zn SOD), manganese superoxide dismutase (Mn SOD), glutathione peroxidase (GSH-Px), and catalase are generally thought to play important roles in antioxidant defence. This view expands our understanding of the cell's antioxidant defense responses to various forms of oxidative attack. Metalloproteins protect cells from oxidative damage caused by reactive oxygen species (ROS). Metalloproteins play a central role in redox processes. Some metalloproteins bind to and transport, oxygen. Cytochrome c metalloproteins transport electrons in the mitochondrial electron transport chain, and iron-sulfur (Fe-S) proteins are involved in respiration and photosynthesis. Many metalloproteins, such as ceruloplasmin, metallothionein, and ferritin, play important roles in metal homeostasis, acting as reservoirs and chaperones for essential trace metals, such as copper, zinc, and iron. Evidence indicates that these proteins are induced during acute reactions [18,20] and under oxidative stress [16,21]. It has been speculated that these proteins reduce the harmful effects of ROS. The antioxidant properties of these proteins are primarily due to their binding to the redox-active metals, copper and iron, which minimize their ability to catalyse ROS production *via* the Fenton reaction. Over the past decade, several informative review articles on Nrf2 have been published in various journals [7,12,25-32].

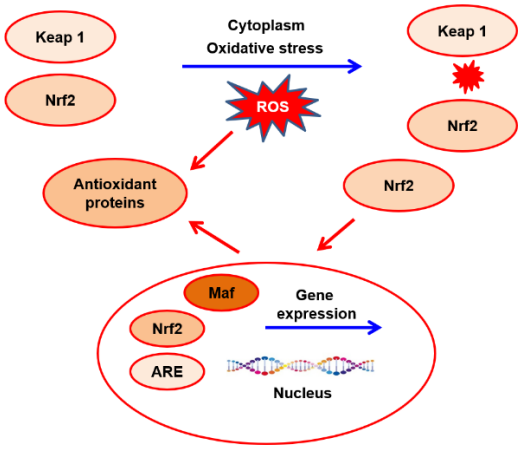

Figure 1. The mechanism of Nrf2 activation in response to oxidative stress. Normally, the Keap1 protein binds to Nrf2 and promotes its degradation, lowering intracellular Nrf2 levels. ROS interact with Keap1 and inhibit its sequestration of Nrf2, thus promoting Nrf2 stabilization and nuclear translocation. In the nucleus, the Nrf2-Maf dimer binds to antioxidant response elements (AREs), thus activating the expression of antioxidant proteins and protecting cells from ROS-induced cellular damage [32].

The authors wrote this review to continue their research on Nrf2 and antioxidant metalloproteins and to contri bute to building an important research network that connects past and present research findings [33-39]. This review is based on both recent and previously published literature.

2. Discussion

2.1. Nrf2 - The transcription factor for oxidative stress response

Transcription factors are proteins that recognize and bind to specific DNA sequences (often the promoter or enhancer region of a gene) and regulate gene transcription, the process by which DNA is copied into RNA. TFs regulate (turn on/off) genes throughout the life of a cell or organism, ensuring that genes are expressed in the right amount at the right time in the right cell. Transcription factors act synergistically to control cell division, proliferation, cell death, cell migration, and tissue formation during embryonic development (body building), sometimes in response to extracellular signals such as hormones. The human genome contains approximately 1,600 transcription factors, half of which are C_2H_2 zinc fingers. Similar to regulons, transcription factors are components of proteomes.

Yaroslav and Rebecca [40] published an interesting paper detailing an integrated approach for predicting activators of the transcription factor Nrf2 in response to oxidative stress. Experimental data from several high-throughput screening assays (HTS) for Nrf2 activation were deposited in the PubChem and ICE databases [40]. Typically, the reliability of any model depends heavily on the quality of the input data; in this case, significant effort has been expended on cleaning and processing the collected data. Two approaches were used to construct the predictive model. Furthermore, it has been reported that structural alerts can associate Nrf2 activation with specific molecular fragments. Furthermore, the report identified approximately 151 structural alerts reflecting the structural diversity of Nrf2 activators. Although structural alerts are fast and transparent methods for the identification of potentially active compounds, their scope is limited, accounting for only 61% of all active compounds.

Nrf2 is a transcription factor that regulates the cellular antioxidant defense systems by regulating the transcription of antioxidant proteins and detoxification enzymes. The identification of new Nrf2 activators could lead to improved antioxidant defences that protect cells from cellular damage caused by reactive oxygen species (ROS).

We compiled a dataset of Nrf2 activation from multiple HTS analyses collected from the PubChem and ICE databases. This diverse body of reliable information demonstrates that various environmental and physiological conditions can induce oxidative stress, which can damage cellular components, such as DNA, proteins, and lipids. In addition, oxidative stress has been implicated in many human diseases, including cancer, cardiovascular diseases, neurological diseases, inflammatory diseases, and ageing. There is an urgent need to identify novel compounds that activate Nrf2 and enhance antioxidant defence. To achieve this, we performed high-throughput screening for Nrf2 activators. The best model, using message-passing neural network (MPNN) technology, demonstrated 87% accuracy on a test set of chemicals within a range of 0.3. This integrated approach, which combines structural alerts and MPNN models, was used to screen approved drugs collected from DrugBank and identify potential Nrf2 activators. The authors reported that of the 2,393 compounds tested, 138 were predicted to be Nrf2 activators using two different methods. They stated that the analysis of the compounds revealed some known Nrf2 activators, while others had the potential to be novel activators. They suggested that integrating fragment-based structural alerts and machine learning models into a consensus model improved prediction reliability and provided structural insights. Because these methods are based on different technologies, they can be considered complementary approaches and may yield better prediction results than either method alone.

Nrf2 is a crucial component of the cellular defence against oxidative stress. This study demonstrates that this novel Nrf2 activator is analogous to an antioxidant defence system that protects against reactive oxygen species (ROS)-induced cell damage (Figure 1). The researchers also created a data set of Nrf2 activation from multiple HTS assays collected from the PubChem and ICE databases. This is likely the largest data set to date.

2.2. Nrf2 pathway and related antioxidant compounds

Paunkov *et al.* [32] recently published an article detailing the quantitative analysis of antioxidant compounds involved in the Keap1/Nrf2 pathway. This analysis focuses on measuring how molecules activate, regulate, or are involved in this key oxidative stress response system.

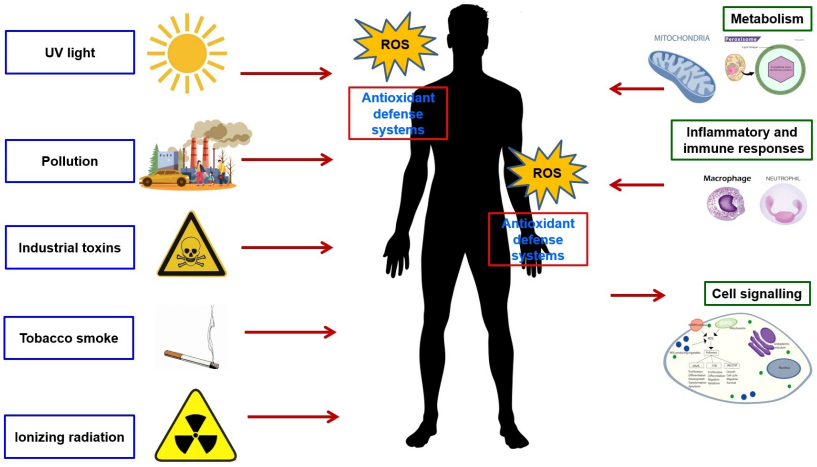

Figure 2. Sources of exogenous and endogenous ROS. ROS can originate from toxic exogenous sources in the environment or they can be produced as by-products of normal cellular metabolism, inflammation, and immune responses. ROS also function as second messengers in cellular signalling pathways [63].

This valuable report, which comprises multiple articles, demonstrates how research focused on Nrf2 has expanded from basic science to numerous clinical applications. The authors provided a technical explanation for their specific clinical focus on natural compounds that modulate Nrf2 activity. The pharmacological effects of natural compounds on health and disease have been extensively studied. The authors cited several published articles [2,31,34,41-48], including representative examples of the applied clinical field into which Nrf2 research is expanding. Furthermore, based on these published reports, the authors documented the chronological evolution of the terminology used in Nrf2 research, highlighting the gradual and steady introduction of Nrf2 into clinical studies. For example, the terms "mouse" and "rat" appeared early in Nrf2-related research, reflecting the use of these models in basic research.

One of the main focus of the Nrf2 research community has been basic research, which has attempted to elucidate the mechanisms of Nrf2 regulation by the cytoplasmic inhibitor Keap1 [49]. Another major research focus has been the identification of the molecular pathways involving Nrf2 as a key transcription factor in cellular homeostasis [50]. Simultaneously, the development of mouse models with loss of Nrf2 function [2] or acquisition [51] has facilitated preclinical research on Nrf2 in various diseases. Examples of this expanding field include protection against acetaminophen toxicity [52], carcinogenesis [53], obesity and diabetes [54], neurodegenerative diseases [34], and kidney diseases [37,55]. Following the discovery that natural compounds, such as sulforaphane found in broccoli sprouts, activate the Nrf2 pathway [56], clinical trials have been started to test these substances as purified drugs or dietary supplements [35,57]. Clinical trials have mainly focused on cancer chemoprevention [58,41], detoxification of environmental pollutants [59], metabolic diseases [60,61], and relapsing forms of multiple sclerosis [62]. Research on synthetic Nrf2 activators in diseases such as diabetic nephropathy, is currently underway [48].

2.3. Nrf2 and oxidative stress: A general overview of mechanisms and implications in human disease

Owing to the versatile properties of Nrf2, it is thought to be involved in many human diseases, which Martin *et al.* briefly discussed in their article (Figure 2) [63]. The researchers noted that oxidative stress is a key factor in most human diseases, ranging from neurodegenerative diseases and neuropsychiatric disorders to cardiovascular diseases, diabetes, and cancer. Antioxidant defence systems have evolved as a means of protection against oxidative stress, and the transcription factor Nrf2 is a key regulator. Previous studies have reported that Nrf2 is primarily responsible for regulating a wide range of antioxidant enzymes involved in detoxification and oxidative stress mitigation, and has been extensively studied in the context of disease. Furthermore, the researchers discussed several published articles related to oxidative stress.

The authors clearly stated that reactive oxygen species (ROS) are unstable molecules containing oxygen and/or nitrogen, encompassing both free radicals and nonradical species. The oxygen molecule (O_2) is a weak free radical due to the presence of two unpaired electrons in its valence shell; however, it is less reactive than other oxygen species because of the parallel spin of its electrons. Excessive or prolonged exposure to ROS leads to oxidative stress, a damaging process that impairs cellular lipids, proteins, and nucleic acids, thus hindering their normal function [64]. In this situation, there is an imbalance between the production of ROS and cellular defence mechanisms against oxidative stress, that is, the antioxidant response. Chronic oxidative stress and the resulting oxidative damage have been implicated in numerous human diseases, including cardiovascular and neurodegenerative diseases, diabetes, cancer, and the ageing process [65-69].

The authors discussed antioxidant response enzymes separately. Complex antioxidant defence systems have evolved to protect cells and tissues from oxidative stress. Another author [70] defined antioxidants as 'any substance that, when present in low concentrations relative to the concentration of the oxidizable substrate, significantly delays or inhibits substrate oxidation' [70]. The main antioxidant defences include (1) antioxidants that directly remove reactive oxygen species, such as glutathione, vitamin C, and vitamin E, and (2) antioxidant enzymes, including superoxide dismutase, catalase, and glutathione peroxidase.

Several studies have identified antioxidant response elements (ARE) [71] based on several studies. Also known as the electron response element (EpRE), the ARE is a cis-acting enhancer sequence located within the promoter region of several cell-protective antioxidant genes and phase II enzyme genes. This element consists of the 5′-TGACnnnGC-3′ base sequence and is involved in gene expression stimulated in response to oxidative stress [72]. The authors also reported that AREs are responsible for low-level basal gene expression to mitigate reactive oxygen species (ROS) produced during

cellular respiration. This element is important for regulating redox reactions under both stress and nonstress conditions. Using in vivo studies in mice, Itoh et al. discovered that the activation of phase II enzymes *via* ARE is mediated by the transcription factor Nrf2 [72]. Nrf2-deficient mice show marked decreases in the expression of the GST_1 phase II enzyme and the NQO1 antioxidant subunit [2], and subsequent studies have shown increased sensitivity to carcinogens and impaired acetaminophen detoxification in Nrf2 mice [5,73,74].

The authors cited several references from which they formulated an important hypothesis regarding the antioxidant Nrf2, indicating that Nrf2 is a master transcriptional regulator of the cellular response to oxidative stress [75]. Nrf2 regulates the expression of several antioxidant and phase II enzyme genes and is negatively regulated by Keap1 [49]. Keap1 is a substrate-transforming protein that binds to Nrf2 in the cytoplasm and promotes polyubiquitination via the enzyme Cullin3 (Cul3) E3 ubiquitin ligase, followed by proteasome-catalysed degradation [76-78]. Furthermore, continuous degradation of Nrf2 maintains a moderate baseline expression level under basal conditions. Moreover, under oxidative stress, stress-sensing cysteine residues in Keap1 are altered [79-81], causing a structural change that prevents Keap1 from mediating Cul3-mediated ubiquitin addition to Nrf2 [82]. Previous reports have confirmed that Nrf2 stabilisation, accumulation, and nuclear translocation occur, where it forms a heterodimeric pair with small Maf proteins, binds to AREs, and strongly induces cell-protective genes for enzymes involved in the detoxification of ROS and other oxidants [2].

2.4. Nrf2: A critical participant in the regulation of apoptosis, ferroptosis, and autophagy in gastric cancer

Nrf2 activation promotes the expression of antioxidant enzymes (such as HO-1, NQO1, and GSH-related enzymes), suppressing apoptosis by reducing reactive oxygen species (ROS) levels. Ferroptosis occurs due to lipid peroxidation and iron accumulation. Nrf2 activation is involved not only in defence against ferroptosis but also in tumour progression and treatment resistance. Nrf2 is closely associated with autophagy through p62. The p62 protein plays a crucial role in the activation of the Nrf2 pathway by binding to and degrading Keap1.

Tang *et al.* [83] recently published a detailed discussion on the regulation of apoptosis, ferrous cell death, and autophagy by Nrf2 in gastric cancer. The authors stated that Nrf2 is a specialized transcription factor that maintains oxidative-reductive homeostasis by regulating the expression of genes associated with oxidative stress. They noted that Nrf2 overactivation contributes to tumour progression and is associated with chemotherapy resistance in a significant number of solid tumours. Prophylactic cell death (PCD), including apoptosis, ferrous cell death, and autophagy, plays a crucial role in tumor progression and chemotherapy sensitivity. Increasing evidence suggests that certain antitumor compounds and genes can induce massive production of reactive oxygen species (ROS) by inhibiting Nrf2 expression, thus exacerbating oxidative stress and promoting gastric cancer cell death, and increasing the sensitivity of gastric cancer cells to chemotherapy-induced apoptosis. Researchers clearly demonstrated the role of antitumor drugs in influencing three different types of programmed cell death (apoptosis, anagen, and autophagy) in gastric cancer cells by modulating Nrf2 expression, as well as the molecular mechanisms by which targeting Nrf2 induces apoptosis and enhances the chemotherapy response. They concluded that it is reasonable to believe that Nrf2 represents a potential therapeutic target and that targeting it pharmacologically or genetically could provide a novel strategy for treating gastric cancer.

Furthermore, DeNicola *et al.* [84] argued that Nrf2 is a key transcription factor in the cellular defense signalling pathway against oxidative stress, which protects tumour cells from death by maintaining ROS levels below the toxicity threshold and preserving redox homeostasis in vivo. The authors concluded that pharmacological or genetic knockdown of GRP75 (glucose regulatory protein 75) and Nrf2 impairs the antioxidant capacity and promotes apoptosis in cis-platin-resistant (SGC7901CR) cells, thereby increasing the sensitivity of cis-platin-resistant tumour cells to DDP. In related studies, the AKR1C gene belongs to the ARE gene family and its overexpression has been reported to contribute to resistance to platinum-based drugs. Initial studies have shown that knocking out Nrf2 reduces the expression of AKR1C and increases the sensitivity of gastric cancer cells (GC) to chemotherapeutic agents [85].

With continued advancements in targeted therapies, attention has been increasingly focused on treatments that target Nrf2. Several researchers have argued that inhibiting Nrf2-related antioxidant processes through antitumor drugs or gene regulation could be a realistic and promising therapeutic strategy, and systematic research is underway to regulate Nrf2 activity to induce programmed cell death in gastric cancer. Most studies have revealed that inhibition of Nrf2 expression induces apoptosis and ferroptosis, enhances tumor sensitivity to chemotherapy drugs, and exerts antitumor effects. However, autophagy plays a dual role in tumors, both promoting tumor growth and having antitumor effects, which has been clearly verified by these authors [84]. For example, C-2 promotes autophagy by activating the JNK/ERK pathway, thereby upregulating the p62/Keap1/Nrf2 pathway and antagonizing the anti-proliferative effects of C-2. In contrast, the report mentions that BDH2 inhibits Nrf2 expression, which in turn inhibits autophagy induced by the PI3K/AKT/mTOR pathway, suppressing the proliferation of GC cells. The report states that inhibition of Nrf2 activity by antitumor drugs or gene regulation may play an important role in the treatment of GC and that combination with other chemotherapy drugs is a particularly promising therapeutic strategy.

The authors found that cell regulatory genes, such as DJ-1 and vascular endothelial growth factor (VEGF), promote oxidative stress, inhibit cell apoptosis and ferroptosis, and promote cell proliferation by regulating Nrf2 expression. Therefore, targeting the Nrf2 signaling pathway to regulate programmed cell death (PCD) has proven to be an effective treatment strategy for GC to combat uncontrolled cell proliferation and chemotherapy resistance. In their study, the authors systematically and thoroughly examined the effects of several antitumor drugs on PCD and chemotherapy resistance in GC cells by modulating the Nrf2 signalling pathway, providing valuable insights for future research targeting Nrf2 in GC treatment.

Tang *et al.* [83] concluded that a deeper understanding of the effect of Nrf2 on tumor PCD processes is needed, as it will provide a strong rationale for overcoming drug resistance during GC treatment. Nrf2 is poised to become a promising pharmacological target in Gastric cancer (GC) breakthrough research.

A few years ago, Li and Kong [86] published a report on the molecular mechanism of Nrf2-mediated antioxidant responses. Initially, the authors made significant comments on their reports. They reported that Nrf2 signaling is suppressed by Keap1 under basal conditions and is induced by oxidative stress. Keap1 has recently been identified as a Cullin 3-dependent substrate adaptor protein (Figure 3).

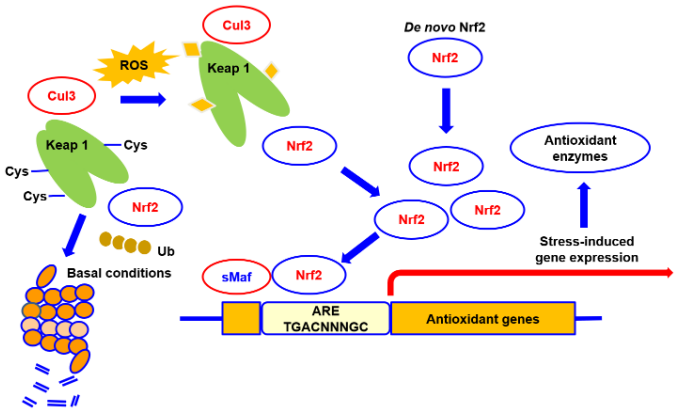

Figure 3. Keap1-Nrf2 pathway. In the normal state, Keap1 is bound to Nrf2, which is ubiquitinated by the Cul3 E3 ubiquitin ligase and degraded by the proteasome. Under oxidative stress, the sensor cysteine of Keap1 is modified by ROS, leading to the stabilization, accumulation, and translocation of Nrf2 to the nucleus, where it forms a heterodimer with sMaf and binds to ARE, activating the transcription of antioxidant genes [63].

The authors also clearly stated that oxidative perturbations can inhibit Keap1-mediated Nrf2 ubiquitination but cannot disrupt the binding between Nrf2 and Keap1. Nrf2 is an intrinsically redox-sensitive transcription factor. Based on these findings, a new model of Nrf2-mediated redox signalling has been proposed. The authors suggested that the free suspended Nrf2 protein functions as a redox sensitive probe and that Keap1 acts as a gatekeeper, regulating the availability of the Nrf2 probe, thus controlling the overall sensitivity to redox signaling.

Usually, oxidative damage usually inhibits the ubiquitination of Nrf2 via Keap1, which is a central mechanism in cellular antioxidant defence. Under normal basal conditions, Nrf2 (nuclear factor erythrocyte 2-related factor 2) is bound to Keap1 (Kelch-like ECH-related protein 1) in the cytoplasm, and Keap1 functions as an adapter for the Cullin-3 (Cul3) E3 ubiquitin ligase complex. As a result, both continuous ubiquitination and rapid proteasomal degradation of Nrf2 are observed.

The authors provide a brief summary, noting the increasing evidence that Keap1-mediated ubiquitination of Nrf2 is highly redox-sensitive. In contrast, Keap1/Nrf2 degradation is relatively less redox-sensitive. Due to the low redox sensitivity of Keap1/Nrf2 degradation, the "relay" step is unlikely to occur in conventional models. Furthermore, the translocation of Nrf2 to the nucleus is highly redox-sensitive. The authors proposed a novel model in which two Nrf2 protein pools exist within the cell: free Nrf2 (fNrf2) and Keap1-bound Nrf2 (kNrf2), both of which undergo ubiquitination and proteasomal degradation. Under homeostatic conditions, the continuous synthesis of new Nrf2 proteins and ubiquitin-dependent degradation of Nrf2 occur. Nrf2 synthesis and degradation are in equilibrium. Consequently, only the pool of fNrf2 required for basal or sustained activity is retained. When cells are exposed to oxidative stress, redox-sensitive Nrf2 ubiquitination is inhibited. As a result, Keap1's Nrf2 binding capacity is saturated, and Keap1 is degraded by autoubiquitination [87,88]. However, Nrf2 translation was promoted [89].

The observation of Nrf2 translocation to the nucleus suggests that the relative amount of fNrf2 may determine the magnitude of the antioxidant response. A higher amount of fNrf2 in the cell leads to a higher number of Nrf2 cells entering the nucleus, along with the same amount of reactive oxygen species/nitrogen species, resulting in a stronger antioxidant response. In this model, redox signals are encoded by fNrf2 and translocated to the nucleus. The cell's overall sensitivity to redox reactions is determined by the size of the fNrf2 pool, which is regulated by Keap1. Thus, Keap1 acts as a gatekeeper of redox sensitivity. The main difference between the old and new models is that the redox signals are not transmitted from Keap1 to Nrf2. Both Nrf2 and Keap1 are highly sensitive to redox signalling. This new model also explains the acute effects of phase II stimulation. Low concentrations of phase II inducers inhibit ubiquitination to Nrf2 via Keap1, increasing the αNrf2 band. Thus, Phase II inducers produce a "priming" effect that alters the overall redox sensitivity of cells, thereby promoting a more effective antioxidant response when cells are exposed to oxidative stress.

2.5. Chemical protection against oxidative stress of Nrf2

Nrf2 is a key transcription factor that protects cells from oxidative and electrophilic stresses. Nrf2-mediated chemoprotection is achieved by activating the antioxidant, detoxification, and cytoprotective pathways. Normally, Nrf2 binds to Keap1 (Kelch-like ECH-associated protein 1) in the cytoplasm. Nrf2 is activated by a Keap1-dependent, evolutionarily conserved, de-inhibitory signalling mechanism. Under basal conditions, Nrf2 is inhibited by Keap1-regulated ubiquitination and proteasomal degradation, but is activated by oxidants and electrophiles through the modification of critical cysteine thiol groups on Keap1 and Nrf2. Activated Nrf2 mediates the expression of a series of enzymes and signaling proteins that control drug metabolism, antioxidant defense, and oxidative signaling, thus affecting oxidative physiology and pathology. Nrf2 regulates oxidation levels and oxidative signaling, and is involved in the control of multiple program functions, including autophagy, inflammasome signalling, UPR, apoptosis, mitochondrial biogenesis, and stem cell regulation. Nrf2 exerts various protective effects against toxic and chronic diseases through natural and pharmacological mechanisms, opening new avenues for drug development. An interesting study on the role of Nrf2 in oxidative stress has been published [90]. Nrf2 essentially controls intracellular oxidant levels and signalling by regulating the expression of three groups of ARE (antioxidant response element (ARE)-dependent genes (ARE-): drug-metabolising enzymes/transporters, antioxidant enzymes/proteins, and oxidative signalling proteins. The authors also pointed out that since its discovery more than a decade ago as a transcription factor that mediates the induction of ARE-dependent drug metabolizing enzymes (DMEs), Nrf2 has rapidly emerged as a key regulator of oxidative stress, suggesting its involvement in various chronic and toxic diseases.

Niture *et al.* [91] published a detailed study on this topic. The Nrf2 complex acts as a sensor of oxidative stress induced by chemicals and radiation in cells.

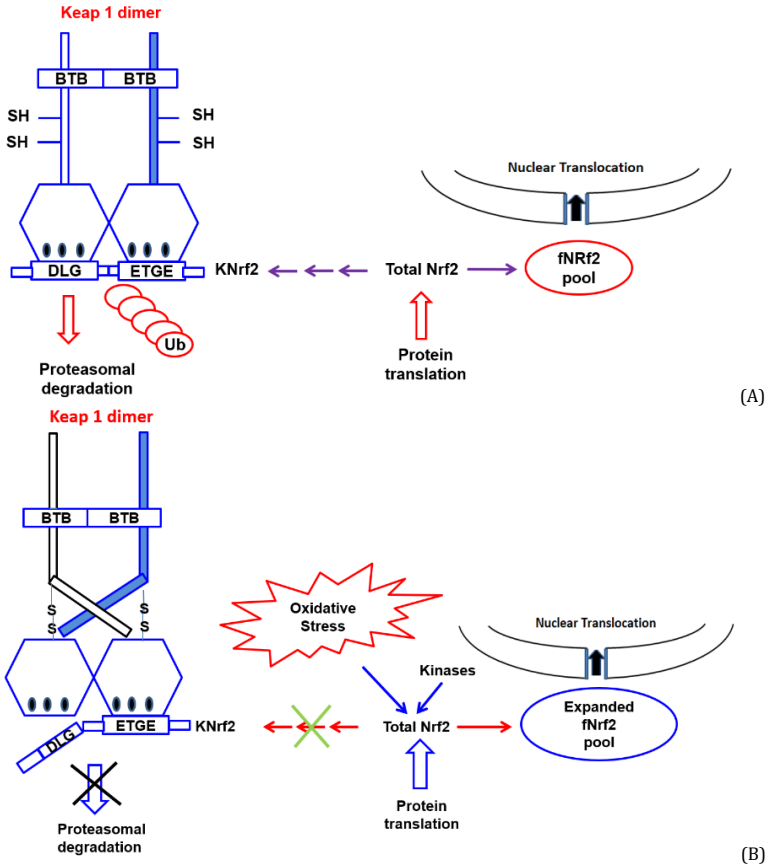

Figure 4. Hypothetical model of redox signaling mediated by Nrf2. The Keap1 dimer functions as a substrate adapter protein involved in ubiquitination (Ub). The Nrf2 protein has two pools: free suspension Nrf2 (fNrf2) and Keap1-bound Nrf2 (kNrf2). Under homeostatic conditions (A), kNrf2 binds to the Keap1 dimer *via* a high-affinity ETGE (hinge) motif and a low-affinity DLG (latch) motif [86]. Self-ubiquitination of Keap1 reduces its binding ability to Nrf2, and this ability is further diminished. However, Nrf2 translation is promoted. As a result, the size of the fNrf2 pool expands (B).

Antioxidants are potent Nrf2 activators because they generate trace amounts of oxidative stress after metabolism, which stimulates Nrf2 activation. Antioxidants are frequently used to study the signaling mechanisms of signal transduction from antioxidants to coordinated induction of protective gene expression in cells, which is essential for cell protection and survival. It should be noted that antioxidants not only function as "scavengers" that remove reactive oxygen species, but also as signalling molecules that activate coordinated gene expression programs for cell protection. This process occurs primarily through redox-sensitive signalling pathways and transcription factors. The signalling mechanism of antioxidants to Nrf2 is a complex phenomenon involving the baseline, pre-induction, induction, and post-induction stages.

2.6. Mechanism of chemical activation of Nrf2

The transcription factor Nrf2 plays a central role in the antioxidant response. Similar to many other transcription factors, Nrf2 signalling is regulated by compartmental segregation. Under basal conditions, Nrf2 is primarily localised in the cytoplasm. Upon oxidative stress caused by the accumulation of ROS [86,92,93] or reactive nitrogen species (RNS) [94,95], Nrf2 rapidly translocates to the nucleus, where it induces an antioxidant response [96]. At least four components are essential for the antioxidant response: Nrf2, Keap1, and a group of small musculoaponeurotic fibrosarcoma (Maf) proteins known as antioxidant response elements (ARE) or electrophile response elements (EpRE). Nrf2 is a basic leucine zipper (bZIP) transcription factor with a cap-and-collar (CNC) structure [75]. Nrf2 contains a highly conserved domain known as the Nrf2-ECH homology domain (Neh). The Neh1 domain is a CNC-bZIP domain that allows Nrf2 to form a heterodimer with the ZIP domains of small Maf proteins. The Neh2 domain contains a degron involved in ubiquitin-dependent degradation [97]. These domains mediate cooperative transcriptional activation of Nrf2 [98]. Recently, the Neh3 domain, located at the carboxyl terminus of Nrf2, was shown to play a permissive role in Nrf2 transcriptional activation [99]. Due to the lack of structural information on Nrf2, it is unclear how Neh3 cooperates with the Neh4-Neh5 domain to exert its transcriptional activation activity. The Neh6 domain is located in an intermediate region that connects the Neh5 and Neh1 domains. The Neh6 domain of Nrf2 is rich in serine residues and contains two β-transducin repeat-containing protein (β-TrCP) degrons, DSGIS and DSAPGS, that are involved in Nrf2 degradation. These degrons play an important role in regulating Nrf2 stability under oxidative stress and contribute to its degradation in complex cellular environments. Understanding these mechanisms will provide information on the complex regulation of Nrf2 and its role in antioxidant gene expression [100].

Two common features have been consistently observed in various antioxidant responses induced by different compounds: an increase in steady-state Nrf2 levels and Nrf2 nuclear translocation. Furthermore, the mechanisms underlying Nrf2 inhibition and activation vary widely. Significant progress has been made in our understanding of Keap1/Nrf2 interactions and the regulation of antioxidant responses. However, several intriguing issues remain unresolved.

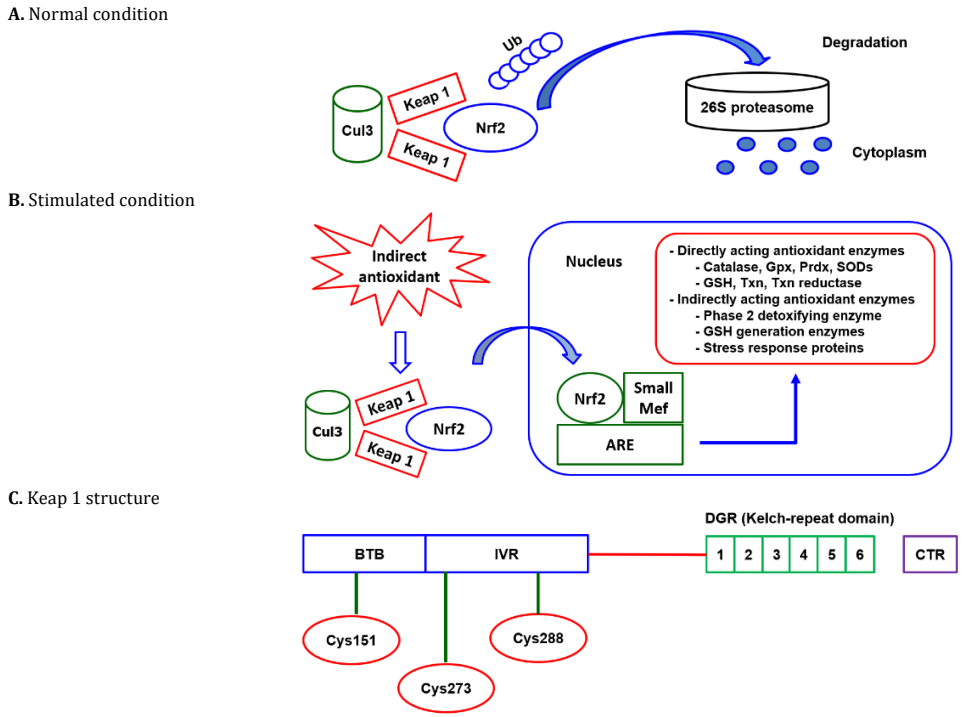

Figure 5. Regulation by Nrf2 and Keap1. (A) Under normal conditions, Nrf2 is cleaved *via* a Keap1-Cul3-dependent pathway. (B) In the presence of indirect antioxidants, the binding of Keap1 and Nrf2 is inhibited and Nrf2 translocates to the nucleus and activates ARE-driven genes. (C) Protein structure of Keap1 and reactive cysteine residues involved in Nrf2 activity [103].

Rapid advances in technologies such as protein crystallography, LC-MS/MS analysis, genetic manipulation (transgenic mice, gene knockdown using siRNA or shRNA), and DNA/RNA microarrays may lead to the elucidation of several issues in the near future. As the proposed "hinge and latch" model demonstrates, the combination of structural and functional studies is highly beneficial (Figure 4). The "hinge and latch" model is a widely accepted mechanism that explains how the transcription factor Nrf2 is regulated by the inhibitor Keap1 under normal and oxidative stress conditions. When both the hinge (ETGE) and latch (DLG) are connected: Nrf2 is ubiquitinated and degraded. Disruption of the latch protects Nrf2, and Nrf2 is protected from degradation.

In the future, by solving the crystal structure of the entire Keap1 molecule and combining mutations and artificial thiol modifications, we may be able to visualise whether zinc coordination can stabilize the Keap1/Nrf2 bond, whether the formation of an intermolecular disulfide bond between C273 and C288 changes the distance of the arginine triad between the two different Kelch domains, and the mechanisms that switch Nrf2 ubiquitination to Keap1 self-ubiquitination. The crystal structure of Nrf2 may enable the visualisation of whether disulfide bond formation or other types of thiol modifications alter its structure and function of Nrf2. Overall, progress in these areas will not only enable a deeper understanding of the mechanisms of Nrf2 signalling but may also aid in the design and optimisation of more potent drugs for the chemo-prevention of cancer, aging, inflammation, and neurodegenerative diseases [101].

2.7. Nrf2 system and development of indirect antioxidants

Indirect antioxidant strategies can be developed by targeting the Keap1 cysteine, optimising their selectivity, increasing their bioavailability, and regulating Nrf2 activation. The design of electrophilic molecules, selective modification of reactive thiol groups, avoidance of excessive toxicity, fine-tuning of reactivity to Keap1, improved bioavailability, enhanced solubility and stability, the use of prodrugs or delivery systems, and avoidance of chronic hyperactivation (associated with cancer progression) are key factors in the development of indirect antioxidants.

The Nrf2 system is a central regulatory network that controls cellular defence against oxidative and electrophilic stress, forming a biological basis for the development of indirect antioxidants. The development of indirect antioxidants has focused on activating Nrf2, thereby enhancing the body's antioxidant, detoxification, and repair systems. This strategy offers a more physiologically relevant and sustainable approach to oxidative stress management than direct antioxidant supplementation.

Indirect antioxidants are compounds that, instead of directly scavenging free radicals, stimulate the body's own antioxidant defense system by activating Nrf2, which is important for the following reasons: (1) reactive oxygen species (ROS) play a role in physiological signalling; (2) direct antioxidants can inhibit redox signalling; and (3) indirect antioxidants provide sustained protective effects, tissue-specific gene stimulation, and reduce the risk of oxidative damage. Most indirect antioxidants are mild electrophilic or redox-active compounds that modify cysteine residues (Cys151, Cys273 and Cys288) in the Keap1 protein (Figure 5). Their mechanisms of action are primarily based on covalent modification of the KEAP1 protein, inhibition of ubiquitin in addition to Nrf2, stabilization and accumulation of Nrf2 in the nucleus, and stimulation of gene expression by antioxidant response elements (ARE).

A new concept, indirect antioxidants, has emerged from recent discoveries in cellular antioxidant systems in cell biology [102]. Indirect antioxidants enhance the antioxidant capacity of cells by increasing gene expression. Jung *et al.* [103] discussed the role of the transcription factor Nrf2 in antioxidant gene regulation and its importance in various pathological conditions and disease models [103]. In this article, the authors

mentioned that small molecule Nrf2 activators might be promising indirect antioxidants for the prevention and treatment of a wide range of human diseases. Proteins such as superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), and the small thiol molecules glutathione (GSH) and thioredoxin (Txn) are directly involved in reactive oxygen species (ROS) scavenging. Catalase, superoxide dismutase (SOD), and glutathione peroxidase (GPx) directly neutralize reactive oxygen species (ROS). Mammalian catalase, with a molecular weight of 240 kDa, is a tetramer consisting of four identical subunits that contain a porphyrin haeme group [102]. It is expressed in all tissues, but is particularly concentrated in the liver, erythrocytes, and kidneys [104]. Catalase catalyzes the decomposition of two hydrogen peroxide molecules into two water molecules and one oxygen molecule, thereby preventing the production of highly reactive hydroxyl radicals from hydrogen peroxide. Human GPx is a selenoprotein that exists in five isotypes (GPx 1, 2, 3, 4, and 6) and can reduce hydrogen peroxide and soluble fatty acid hydroperoxides using GSH2 as a co-substrate [105]. The antioxidant role of GPx has been demonstrated by gene knockout studies in animal models, and GPx1 deficiency in mice results in abnormalities in endothelial and cardiomyocyte function due to severe oxidative stress [106].

The usefulness of direct antioxidants has been questioned by multiple randomised clinical trials showing that vitamins C, E, and β-carotene do not reduce human cancer incidence. Furthermore, direct antioxidants have short half-lives, requiring frequent administration and relatively high doses to maintain their physiological effects. Indirect antioxidants are small molecules that upregulate the expression of genes encoding antioxidant proteins via Nrf2. Ultimately, this affects physiological, biochemical, and/or cellular processes that inactivate free radicals or inhibit chemical reactions initiated by free radicals. In contrast to the short half-life of direct antioxidants, indirect antioxidants act through gene regulation, resulting in long-lasting physiological effects. Additionally, indirect antioxidants are less likely to cause pro-oxidative effects, which are problematic with high-dose vitamin E therapy. In particular, accumulating evidence suggests that small-molecule Nrf2 activators exert multifaceted effects. These include cancer prevention, reduction in inflammatory damage, protection from protein toxicity, promotion of liver regeneration after injury, and maintenance of balanced lipid metabolism.

For the development of indirect antioxidants, Keap1 is now accepted as a direct molecular target for antioxidant gene inducers. However, because of the limited understanding of the structural biology of Keap-Nrf2 proteins, the detailed mechanism by which specific chemicals react with specific sulfhydryl residues of Keap1 remains to be elucidated. In general, inducers of antioxidant genes have diverse structures and chemical properties; however, one common feature is their high reactivity toward sulfhydryl groups through oxidation or alkylation reactions. Talalay et al. defined nine chemical classes of sulfhydryl-reactive gene inducers, including isothiocyanates, dithiolethiones, a variety of Michael reaction acceptors, arsenic and heavy metals, hydroperoxides, vicinal dimercaptans, oxidized diphenols, phenylene diamines, and quinones [107]. A recent study by Kobayashi et al. developed a zebrafish model of Keap1 mutation and classified several sulfhydryl reactive chemicals into distinct categories depending on the reactive cysteine residues required for their action [108]. In their classification, sulforaphane, D3T, and GSH depleting agent diethylmaleate are classified into the same class based on the requirement of Cys151 for Nrf2 activation, while Prostaglandin A2 and 15-deoxy-Δ12,14-prostaglandin J2, in a different class, require Cys273 for their action. On the contrary, an independent study reported that hydrogen peroxide modified multiple cysteine residues of Keap1, including Cys77, Cys297, Cys319, Cys369, and Cys434, indicating more nonspecific modifications [109]. These results suggest that specific cysteine residues of Keap1 respond differently to different signals. Therefore, an accurate understanding of the cysteine reactivity of Keap1 will promote the development of more specific antioxidants for the activation of Nrf2. In conclusion, we propose that the development of specific small-molecule Nrf2 activators may be a successful strategy to control or prevent a wide range of human diseases associated with oxidative injuries [102].

3. Conclusions

Nrf2 is a key transcription factor that regulates the expression of several antioxidant and detoxification genes. Nrf2 plays both a gene regulatory and biochemical protective role through the regulation of metalloproteins by directly upregulating genes encoding antioxidant metalloproteins such as SOD1, GPx, catalase, metallothionein, and heme oxygenase-1 (HO-1). This review primarily discusses Nrf2 as a transcription factor, the Nrf2 system, and the development of indirect antioxidants during the oxidative stress response. Furthermore, it highlights the crucial role of Nrf2 in regulating apoptosis, ferroptosis, and autophagy in gastric cancer and other diseases. In other words, Nrf2 (nuclear factor erythroid-2 related factor 2) and antioxidant metalloproteins function together as a central cellular defence system against oxidative stress. These functions can be explained at two levels: regulation (Nrf2) and execution (metalloproteins).

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CRediT authorship contribution statement

Conceptualization: Akitsu Takashiro; Data Curation: Akitsu Takashiro, Abul Monsur Showkot Hossain, Obilova Malika Qudratovna, Ayumu Odaka, Daisuke Nakane; Writing - Original Draft: Akitsu Takashiro, Abul Monsur Showkot Hossain, Qobilova Malika Qudratovna, Ayumu Odaka, Daisuke Nakane; Writing - Review and Editing: Akitsu Takashiro, Abul Monsur Showkot Hossain, Qobilova Malika Qudratovna, Ayumu Odaka, Daisuke Nakane; Supervision: Akitsu Takashiro; Project Administration: Akitsu Takashiro.

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