Review

Enhancing Tumour Radiosensitivity by Targeting NRF2 Antioxidant System

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Review

Enhancing Tumour Radiosensitivity by Targeting NRF2 Antioxidant System

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Abstract

The challenge of radioresistance in radiotherapy is currently tackled by introducing new radiotherapy facilities with high-quality of ionizing beams, high-precision of radiation delivery to tumour, and optimized treatment plans. This strategy is further enhanced by the development of new therapeutic methods for suppressing radioresistance in tumours of cancer patients by combining radiotherapy with chemo-, immuno-, and targeted therapies tailored to patients' molecular profiles. As a result of numerous preclinical and clinical trials of the combination therapy, the primary molecular mechanisms, driving an increase in radiosensitivity, and the key cellular signalling pathways responsible for radioresistance were identified. One of the established radioresistance mechanisms involves adaptation of cancer cells to an elevated levels of reactive oxygen species (ROS) by activating antioxidant systems (AOS) of cellular protection and survival. Since radiotherapy mainly relies on ROS production that damages DNA, causing cancer cell death, activation of the AOS can mitigate radiotherapy effectiveness. Therefore, suppressing the AOS and its associated adaptation mechanisms may increase tumour radiosensitivity and enhance treatment outcomes. In this review, we discuss the role of one of the key components of the cellular AOS which is under the control of the NRF2 transcription factor (nuclear erythroid factor 2) – a master regulator of cellular redox balance that protects cells from oxidative stress during radiotherapy by governing expression of a battery of antioxidant enzymes. We first outline the molecular mechanism of the redox-sensitive NRF2 AOS and its activation in response to the increased ROS levels following irradiation. We then evaluate experimental and clinical evidence regarding NRF2 activation in various cancer cells and tumours exposed to ionizing irradiation. Furthermore, we discuss results of numerous experimental and clinical investigations demonstrating that suppression of the NRF2 AOS enhances radiosensitivity of various cancers and improves radiotherapy outcomes. Collectively, these findings confirmed the potential of combining radiotherapy with targeted therapy aiming at the suppression of the NRF2 AOS. In this combination therapy NRF2 inhibitors act as radiosensitizers that promote overcoming radioresistance due to extra ROS accumulation and oxidative stress induction in cancer cells by inhibition of the NRF2-dependent antioxidant responses to radiotherapy.

Keywords: NRF2; radiotherapy; radioresistance; radiosensitizer; antioxidant system; targeted therapy

1. Introduction

Over recent decades, the efficiency of radiotherapy has been advanced significantly through the implementation of new radiotherapy units providing high-precise delivery of radiation dose to tumour sites and the design of non-conventional treatment plans in clinical practice. Progress in radiotherapy is further driven by innovative strategies that enhance tumour radiosensitivity in cancer patients. Currently, a promising frontier involves integrating radiotherapy with chemo-, immuno-,

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and targeted therapies guided by molecular diagnostics in patients. This led to the development of effective combined radiotherapy with personalized drug therapies aiming at inhibition of various metabolic and signalling pathways activated in cancer cells by mutations in tumour suppressor genes and oncogenes. Extensive experimental and clinical studies have elucidated the primary molecular mechanisms underlying low cancer radiosensitivity and identified the key signalling and metabolic pathways driving radioresistance (Meehan et al. 2020), (Barker et al. 2015). These investigations have paved the way for the development of novel combination radiotherapy guided by patient-specific genetic and molecular diagnostics. This approach enables clinicians to enhance radiotherapy efficacy by overcoming radioresistance and increasing radiosensitivity index across various cancer types.

One of the signalling pathways activated in various cancer cells is the antioxidant signalling pathway which is under the control of transcription factor, nuclear erythroid factor 2 (NRF2) (Hayes, Dinkova-Kostova, and Tew 2020). Activation of this pathway is associated with the cell response to an increased level of intracellular reactive oxygen species (ROS) generated in cancer cells due to their high metabolic and proliferative activities and mitochondrial dysfunction (Perillo et al. 2020), (Rojo de la Vega, Chapman, and Zhang 2018). Persistent oxidative stress in numerous cancer cells causes high catalytic activity of various cellular redox-sensitive proteins and receptors (e.g. PTEN, EGFR, and others). This, in turn, activates different signalling pathways, that drive cellular proliferation, survival, migration, metastasis, epithelial-mesenchymal transition, and other pathways promoting cancer progression and disease severity (Pizzino et al. 2017).

A high level of ROS in a number of cancer cells are correlated positively with aggressive disease outcomes (Oshi et al. 2022). Furthermore, elevated levels of ROS and oxidative stress cause DNA damage, oxidation of proteins, and lipid peroxidation that lead to chromosomal aberration, lipid peroxidation, and cell death through apoptosis and ferroptosis. The ability of various cancer cell lines to function under oxidative stress condition is ensured by enhanced oxidant protection in cancerous cells by triggering cellular adaptation to oxidative stress (Glorieux et al. 2024). This adaptation to oxidative stress is largely mediated by endogenous antioxidant system (AOS) of cellular defence which is controlled by the redox-sensitive NRF2 transcription factor (Baird and Yamamoto 2020). NRF2 activation at a high level of ROS induces expression of a battery of antioxidant enzymes catalysing ROS degradation such as glutathione peroxidase-2,4 (Gpx), glutathione reductase (GR), thioredoxin (Trx), thioredoxin reductase (TR), peroxiredoxin-1,6 (Prx) and others (Hayes et al., 2020). Additionally, NRF2 transcription factor controls expression of enzymes involved in the biosynthesis of glutathione (GSH), a critical agent of the cellular AOS. Beyond antioxidant defence, NRF2 controls certain signalling pathways that protect cells and tissues from chemical and biological stresses induced by toxins, drugs, and carcinogens (Taguchi, Motohashi, and Yamamoto 2011).

Extensive research has established that activation of the NRF2 AOS in various cancer cells and tumours promotes cancer progression, aggressiveness, metastasis, while confers resistance to chemotherapeutic drugs, that are induce ROS in additional to their primary therapeutic action. (Rojo de la Vega et al. 2018), (Taguchi et al. 2011), (Lau et al. 2008). This activation enables various cancer cells to adapt to chronic oxidative stress. Consequently, cancer cells, functioning in oxidative stress condition, become dependent on the NRF2 AOS as a protective mechanism. This addiction of cancer cells to NRF2 signalling makes the NRF2 AOS a promising therapeutic target for cancer treatment (Kobayashi, Imanaka, and Shigetomi 2022), (Kitamura and Motohashi 2018), (Okazaki, Papagiannakopoulos, and Motohashi 2020). Currently, the NRF2 redox-sensitive system is widely recognized as potential therapeutic target for the development of both activators and inhibitors of NRF2 transcription factor for the treatment of various diseases, including cancer (see reviews (Tufekci et al. 2011), (Mirzaei et al. 2021), (Boutten et al. 2011), (Dinkova-Kostova and Copple 2023), (Li et al. 2023). Numerous comprehensive reviews have summarized results of experimental and clinical studies and confirmed that modulation of the NRF2 AOS by drugs may contribute to the prevention of carcinogenesis and suppression of cancer resistance to chemo- and targeted therapies (Li et al. 2023), (Dinkova-Kostova and Copple 2023), (Dinkova-Kostova and Copple 2023), (Copple 2012a), (Lu et al. 2016), (Zhan, Li, and Zhou 2021), (Zhang et al. 2023).

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In this review, we examine the role of the NRF2 AOS in enhancement of radiotherapy efficiency. We first analyse experimental results demonstrating how NRF2 activation in cancer cells mitigates radiotherapy effect by scavenging radiation-induced ROS and facilitating cancer adaptation to irradiation. Afterwards we discuss findings that characterize activation of the NRF2 AOS as a key adaptive response of cancers to irradiation and a primary molecular mechanisms of radioresistance. Finally, we evaluate a large body of experimental investigations showing that suppressing the NRF2 AOS enhances tumour radiosensitivity and improves radiotherapy outcomes.

2. The Molecular Function of NRF2 Antioxidant System

Endogenous ROS are generated in cells as by-products of various enzymatic reactions in metabolic processes and the respiratory chain in mitochondria (Hayes et al. 2020). As products of radical enzymatic reactions, ROS function as signalling molecules that participate in cellular stress response which is controlled by redox-sensitive proteins containing cysteine residues in their structures. Under homeostatic conditions, intracellular concentration of ROS is maintained at low levels through a balance between ROS generation and its degradation by the cellular AOS which is governed in part by NRF2 transcription factor.

In Figure 1, we schematically illustrate the basic components of the NRF2 AOS and its activation following irradiation. According to the established molecular mechanism (McMahon et al. 2006), (Kobayashi et al. 2022) the core of the system is a complex consisting of NRF2 and its repressor, homodimer protein KEAP1 (Kelch-like ECH associating protein 1), which is bound to a Cul3-based E3 ubiquitin ligase. Under physiological redox conditions, the association of *de novo* synthesized NRF2 with the KEAP1-Cul3-E3 complex promotes NRF2 interaction with Cul3-E3 that leads to its subsequent ubiquitination and degradation in proteasomes (Figure 1). This maintains low NRF2 concentration in the cytoplasm and nucleus, limiting the expression of NRF2-regulated genes to constitutive levels.

Under oxidative stress or electrophilic attack, the sulfhydryl groups of KEAP1 cysteine residues undergo to oxidative modification. According to the “hinge and latch” model (McMahon et al. 2006), (Kobayashi et al. 2022) this triggers conformational changes within the NRF2-KEAP1 complex. As a result, the interaction between KEAP1 and the low-affinity DLG ‘latch motif’ is disrupted, while NRF2 remains attached to the second KEAP1 monomer via the high-affinity ‘hinge’ ETGE motif. In this conformation, NRF2 ubiquitination is impaired, and the KEAP1 dimer is blocked from binding newly synthesized NRF2 molecules. This stabilizes NRF2, leading to its cytoplasmic accumulation and subsequent translocation into the nucleus (Figure 1). The resulting nuclear accumulation drives the transcription of NRF2-regulated genes. This molecular mechanism establishes the NRF2-Cul3-KEAP1 complex as a primary sensor of cellular oxidative stress.

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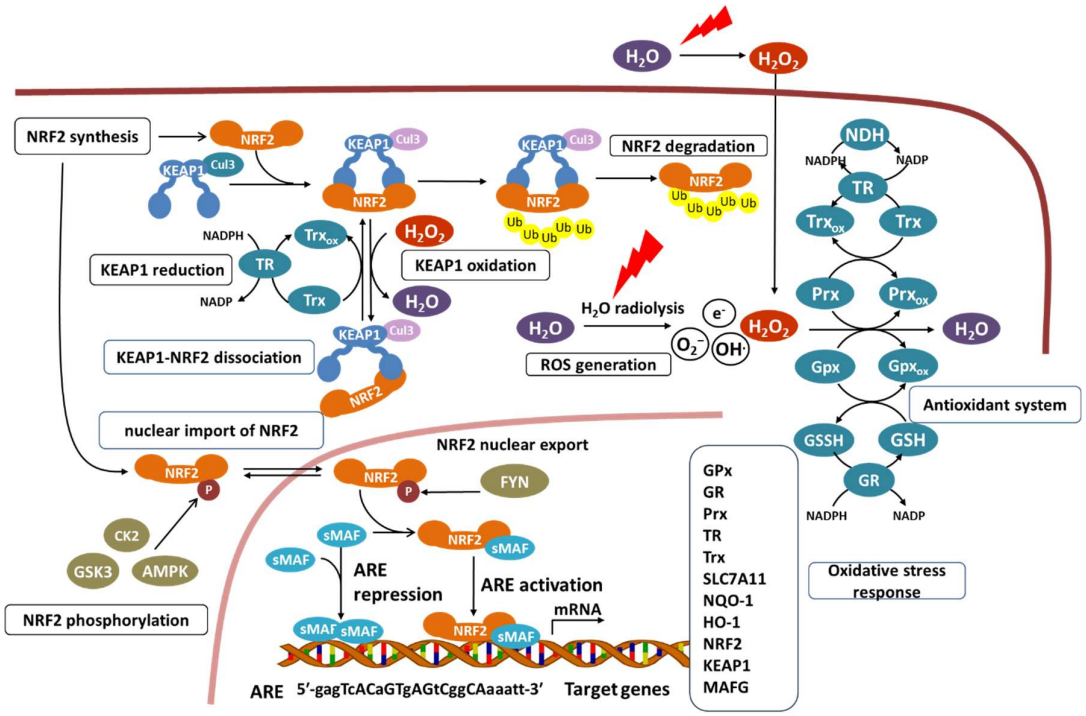

Figure 1. Schematic of molecular function of the NRF2/KEAP1/ARE antioxidant system and its activation in response to irradiation. The main subsystems of the NRF2 AOS are shown. The key NRF2 dependent antioxidant enzymes are listed in the frame: Gpx - glutathione peroxidase 2 and 4, GR-glutathione reductase, Prx - peroxiredoxin 1 and 6, TR - thioredoxin reductase 1, Trx - thioredoxin, NDH - NAD(P)H:quinone oxidoreductase 1, HO-1 - heme oxygenase-1, and SLC7A11-cystine/glutamate transporter.

NRF2 translocates from the cytoplasm to the nucleus, where it binds to small proteins of muscular aponeurotic fibrosarcoma proteins (sMAF-F, sMAF-G, and sMAF-K) to form a transcriptionally active NRF2-sMAF heterodimer. This complex then binds to the cis-acting antioxidant responsive element (ARE) within target gene promoters (Suzuki and Yamamoto 2015) (Figure 1). Otherwise, sMAF-sMAF homodimer forms repressor complexes to the ARE sequence. Thus, the competition between NRF2 and sMAF for dimer formation of either activator (NRF2-sMAF) or repressor (sMAF-sMAF) complexes is other way in activity regulation of the NRF2 AOS.

The NRF2-sMAF transcription complex controls expression of numerous antioxidant enzymes, including glutathione peroxidase (GPx), glutathione reductase (GR), thioredoxin (Trx), and thioredoxin reductase (TR). It also controls enzymes involved in the *de novo* synthesis of glutathione (GSH), the primary reducing substrate of the antioxidant enzymes. The ARE sequences are present in the promoters of the *NFE2L2* (coding NRF2), *sMAF,* and *KEAP1* genes, providing positive and negative feedbacks in the NRF2 AOS. During oxidative stress, NRF2-dependent expression of antioxidant enzymes reduces intracellular ROS levels. This promotes the reduction of KEAP1, triggering the formation of the NRF2-Cul3-KEAP1 complex to resuming NRF2 degradation, thereby completing cellular response to oxidative stress (Figure 1).

NRF2 activity is intricately regulated by its post-translational modification in part by phosphorylation by kinases from various signalling pathways (Figure 1). This regulation enables complex crosstalk between NRF2 signalling and pathways such as PI3K/AKT/mTOR, RAS/ERK, AMPK, DNA repair, and others (Hayes and Dinkova-Kostova 2014). For instance, casein kinase-2 (CK2) phosphorylates NRF2 at multiple serine/threonine residues, yielding an active form of NRF2 required for its nuclear translocation and transcriptional activity (Apopa, He, and Ma 2008). Another activator of NRF2 is AMPK (AMP activated kinase), which phosphorylates NRF2 at Ser550 (Liu et al. 2021). It was proposed that NRF2 phosphorylation at Ser550 prevents NRF2 nuclear export that

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retains its transcriptional activity. In contrast, GSK-3 (Glycogen synthase kinase 3) negatively regulates NRF2 by its phosphorylation at sites Ser344 and Ser347 that leads to the ubiquitination and proteasomal degradation of NRF2 independently from KEAP1 (Liu et al. 2021). Similarly, FYN kinase negatively regulates NRF2 by phosphorylation at Tyr568 in the nucleus that causes NRF2 nuclear export followed by its degradation in the cytoplasm (Kaspar and Jaiswal 2011).

As described above, the NRF2 AOS is a complex signalling system integrated within of the broader signalling network of cell fate regulation. This system maintains cellular redox homeostasis through four functional components: a sensory subsystem, signal conversion module, a network of molecular controllers, and an execution system (Figure 1). These components are interconnected via multiple positive and negative feedback loops that utilize signal amplification to ensure rapid, precise control of intracellular ROS levels. Consequently, effective therapeutic modulation of cellular redox states through pharmacological agents or radiotherapy, requires a detailed understanding of the molecular mechanisms of NRF2/KEAP1 AOS function across diverse cancer types. Alongside experimental studies, theoretical investigation of the complex function and networked control of the NRF2/KEAP1 AOS were carried out in a framework of computational systems biology (Q. Zhang et al. 2010), (Liu, Pi, and Zhang 2022), (Khalil et al. 2015).

3. Activation of the NRF2 Antioxidant System in Cancer Under Oxidative Stress Caused by Ionizing Irradiation

A key mechanism of radiotherapy action is determined by DNA damage in cancer cells, resulting from both the direct and indirect effects of ionizing radiation. Over 70% of this damage occurs due to indirect processes through ROS generation from water radiolysis in cells under ionizing irradiation. When cellular ROS levels rise, the NRF2-regulated antioxidant system is activated. As illustrated Figure 1, elevated H_2O_2 concentration leads to oxidation of KEAP1 in the NRF2-KEAP1-CUL3 complex; this triggers the partial dissociation of NRF2, thereby stopping NRF2 degradation and increasing its cytoplasmic levels. Accumulation of NRF2 transcription factor in the cell nucleus and its binding to ARE sequences in the promoters of NRF2-dependent genes trigger the expression of numerous antioxidant enzymes, catalysing ROS degradation and protecting cells against radiation exposure. NRF2 AOS activation upon irradiation is now considered a key molecular mechanism in the emergence of radioresistance in cancer. Recent clinical trials shown that increased expression of NRF2 and NRF2-dependent genes across various cancer types is associated with radioresistance and is considered a prognostic marker of poor patient survival (Kawasaki et al. 2014).

Radiation-induced activation of the NRF2 AOS in cancer cells has been experimentally confirmed by measuring the expression of NRF2 target genes. Most studies have utilized markers such as *NQO1* (encoding NAD(P)H dehydrogenase), *HO-1* (haemoxygenase-1), *GST2* (glutathione S-transferase A2) genes to evaluate this activation (Singh et al. 2010). Additionally, increased transcriptional activity of NRF2 was detected using ARE-luciferase reporter assays (Tang et al. 2022). In this review, we examine the role of the NRF2 AOS in cancer radioresistance by comparing experimental data on the responses of parental cancer cells and gene-modified ones, where NRF2 expression were either constitutively activated or silenced.

Investigation on the role of the NRF2 AOS in radioresistance mechanism in non-small cell lung cancer (NSCLC) cell lines A549 and H460 showed constitutive activation of NRF2 transcription factor (Singh et al. 2010). This was confirmed by the measuring the expression of several NRF2-regulated genes (*NQO1*, *GCL*, *GSR*, *TXN*, and *TXNRD1*). Knockdown of NRF2 using small interfering RNA (siRNA) reduced the expression of these genes by approximately 50% following 10 Gy irradiation. Consequently, transfected cells exhibited a significant increase in ROS levels 24 hours post-irradiation, compared to controls. Furthermore, measurement of the dose dependence of clonogenic cell survival showed an increase in radiosensitivity of these cells to radiation. In Figure 2A and 2B, we provided the experimental dose dependencies of survival fraction *SF*(*D*) for both cell lines and plotted approximating survival curves according to the equation of the linear-quadratic (LQ) model: $SF(D) = e^{-(\alpha D+\beta D^2)}$, where *D* is the exposure dose, α and β are parameters of the LQ model (McMahon 2018). Calculation of the α/β ratio, characterizing cell radiosensitivity, showed increased

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radiosensitivity of the NRF2 siRNA transfected cells, A549-siRNA (α/β = 4.25 Gy) compared to parental A549 cells with normal functioning NRF2 AOS (α/β = 0.15 Gy) (Table 1). Similarly, inhibition of NRF2 expression by NRF2 siRNA transfection in H460 cells (H460-siRNA) increased their radiosensitivity (α/β = 1.6 Gy) relative to parental H460 cells (α/β = 0.97 Gy) (Figure 2B and Table 1).

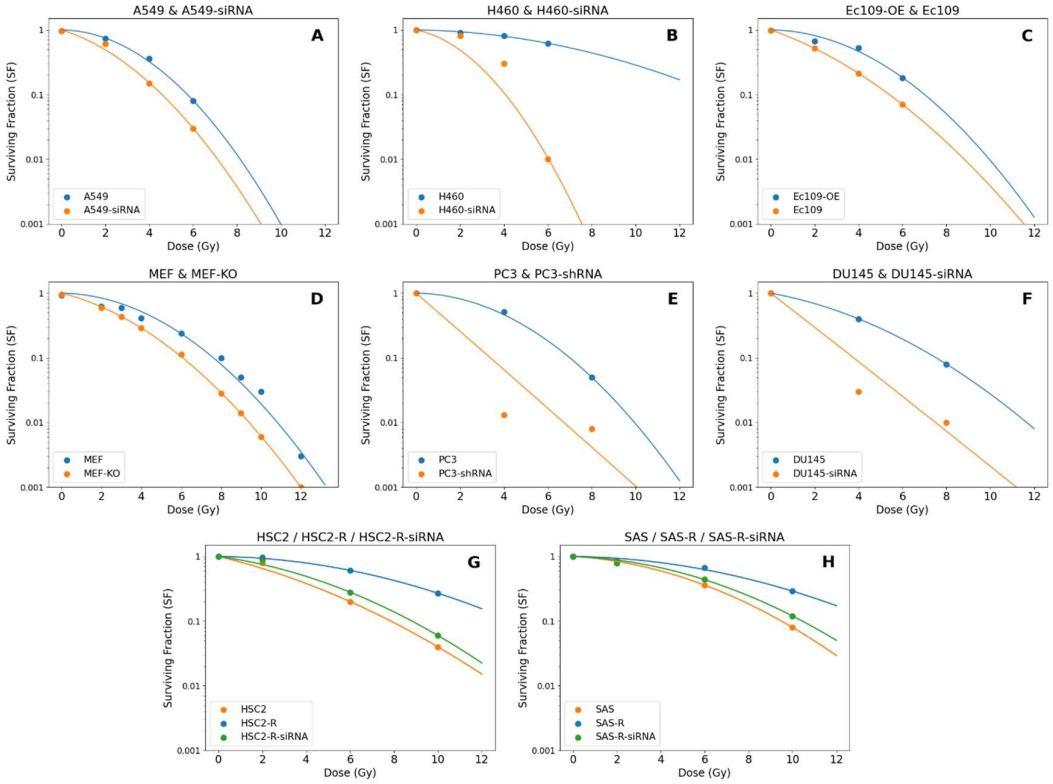

Figure 2. Changes in dose dependencies of clonogenic survival of cancer cells upon inhibition/activation of the NRF2 antioxidant system. (A) Non-small cell lung cancer cells A549 and A549-siRNA; (B) H460 and H460-siRNA (Singh et al. 2010); (C) Ec109 and Ec109-OE esophageal squamous carcinoma cells (Xia et al. 2020); (D) mouse fibroblast cell lines MEF and MEF-KO (McDonald et al. 2010); (E) prostate cancer cell lines PC3 and PC3-shRNA (Jayakumar et al. 2014); (F) DU145 and DU145-shRNA cells (Jayakumar et al. 2014); (G) oral squamous cell carcinoma cells HSC, HSC-R, and HSC-R-siRNA; (H) SAS, SAS-R, and SAS-R-siRNA cells (Matsuoka et al. 2022). Points - experimental data; lines - approximating curves according to the LQ model.

Investigation of human embryonal (RD) and alveolar (RH30) rhabdomyosarcoma (RMS) cell lines revealed increased ROS levels (mainly superoxide anion in mitochondria) alongside elevated expression of NRF2 mRNA and NRF2-dependent genes (*SOD-2*, *CAT* (catalase), and *Gpx4*) immediately following cell exposure by proton beam at doses of 1 Gy - 5 Gy (Marampon et al. 2019). The activation of the NRF2 AOS in RMS cells in response to radiation-induced ROS elevation rapidly restored ROS levels to below the basal state. This suggests that the NRF2 AOS is highly sensitive to ROS accumulation and efficiently counteracts oxidative stress. The authors suggested that a highly sensitive NRF2-dependent antioxidant response to radiation may be a key protective mechanism responsible for acquired radioresistance of RMS tumours. Notably, the lower expression of NRF2-dependent genes in RH30 cells compared to that in RD cells was found to result in their higher radiosensitivity. Furthermore, blocking NRF2 expression upon transfection of cells with NRF2 siRNA significantly increased radiation-induced ROS levels and enhanced radiosensitivity compared to cells with the functional NRF2 AOS. These findings demonstrated that the radioresistance mechanism in

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RMS cells is driven by their ability to mitigate oxidative stress through the activation of the NRF2 AOS.

Table 1. Coefficients α, β, and ratio α/β of the LQ model obtained by approximation of dose dependencies of clonogenic survival of cells with activated or suppressed NRF2 AOS (Figure 2).

Cells	α (Gy^{-1})	β (Gy^{-2})	α/β (Gy)	R^2 (%)
A549	0.010 ± 0.068	0.068 ± 0.011	0.15	99.6
A549-siRNA	0.242 ± 0.037	0.057 ± 0.006	4.25	99.3
H460	0.011±0.006	0.0114±0.001	0.97	99.5
H460-siRNA	0.160± 0.557	0.0100±0.094	1.60	90.2
HSC2	0.187 ± 0.008	0.013 ± 0.001	13.8	99.4
HSC2-R	0.010 ± 0.001	0.012 ± 0.0001	0.84	99.9
HSC2-R-siRNA	0.108 ± 0.005	0.017 ± 0.001	6.23	99.4
Ec109-OE	0.010 ± 0.084	0.045 ± 0.014	0.22	96.4
Ec109	0.273± 0.010	0.028 ± 0.002	9.7	100
MEF	0.010 ± 0.069	0.038 ± 0.006	0.26	98.2
MEF-KO	0.178 ± 0.006	0.033 ± 0.0004	5.35	100
SAS	0.047±0.001	0.021±0.0001	2.27	100
SAS-siRNA	0.138±0.005	0.014±0.006	9.68	99.7
SAS-R	0.01±0.023	0.010±0.002	0.88	98.2
SAS-R-siRNA	0.03±0.01	0.02±0.001	1.47	99.9
PC3	0.211±0.001	0.020±0.001	10.34	100
DU145	0.142±0.001	0.022±0.001	6.58	100

The impact of increased NRF2 levels on cancer cell radiosensitivity was investigated in esophageal squamous cell carcinoma (ESCC) cells Ec109 and KYSE-30, transfected with a lentivirus vector carrying the *NFE2L2* gene (Xia et al. 2020). Analysis of dose-dependent clonogenic survival revealed that NRF2-overexpressing cells (Ec109-OE and KYSE-30-OE) exhibited lower radiosensitivity (α/β=0.22 Gy for Ec109-OE cells) compared to the parental cells (α/β=9.7 Gy for Ec109 cells) (Figure 2C). The authors suggested that this acquired radioresistance stems from the high expression of Ca^{2+}/calmodulin-dependent protein kinase (CaMKII) observed in these cells. This NRF2-dependent expression of CaMKII was proposed to be attributed to the presence of ARE sequence within the promoter of *CAMK2* gene. CaMKII expression triggers autophagy activation that facilitates the degradation of ROS when they reach elevated levels. Survival analysis of ESCC patients showed that high levels of both NRF2 and CaMKII correlate with a significant decrease in overall survival, suggesting their potential as prognostic markers for severe outcome of the disease. Consequently, radioresistance of esophageal squamous cell carcinoma may be associated with NRF2-dependent CaMKII expression and subsequent activation of autophagy. This mechanism effectively neutralized ROS, protecting cancer cells from the cellular oxidative stress typically induced by radiation.

Studies of NRF2-dependent radioresistance have identified another mechanism of cancer cell death aside from apoptosis, namely ferroptosis which is a programmed cell death driven by iron-dependent lipid peroxidation (LPO) of the cellular membranes (Feng et al. 2021). Immunohistochemical analysis of ESCC tissues from 127 patients revealed elevated levels of NRF2 and its downstream target, the cysteine/glutamate transporter protein (SLC7A11), which is critical for the GSH synthesis. High expression of both NRF2 and SLC7A11 correlated with lower overall survival and disease free-progression. To confirm the role of ferroptosis in cell death, irradiation

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experiments were conducted using KYSE30 and NRF2-upregulated KYSE150 cell lines. The modified cells exhibited reduced levels of ROS, LPO, and ferroptosis compared to parental cells. These findings suggest that radioresistance in ESCC is driven by the NRF2/SLC7A11 axis, which effectively inhibits ferroptosis.

The effect of radiation on NRF2 AOS activation in non-cancerous cells was investigated in mouse fibroblast cells (MEF). These cells exhibited a dose-dependent increase in ROS levels and ARE transcriptional activity on the fifth day after irradiation (McDonald et al. 2010). Measurement of basal ROS levels revealed its high levels in NRF2 knockout cells (MEF-KO) compared to that in WT MEF cells. The action of irradiation with doses of 8 Gy and 10 Gy caused a delayed two-fold increase in ROS in WT MEF cells, whereas MEF-KO cells experienced a more than ten-fold increase in ROS immediately after exposure. These findings indicate that blocking the NRF2 AOS in MEF-KO cells results in an uncontrolled radiation-induced increase in ROS, whereas the NRF2 AOS in WT MEF cells maintains ROS homeostasis during and after irradiation. Furthermore, this study identified a five-day delay in expression of the markers of NRF2 AOS activation, specifically the *HO-1* and *GSTA2* genes. Analysis of dose dependent clonogenic cell survival showed that blocking the NRF2 AOS in MEF-KO cells significantly increased radiosensitivity compared to that in WT MEF cells with the functional NRF2 AOS. Experimental data on dose dependence of cell survival for both cells and fitting curves using the LQ model are shown in Figure 2D. Calculation of the α/β ratio confirmed increased radiosensitivity of MEF-KO cells (α/β=5.35 Gy) relative to WT MEF cells (α/β=0.26 Gy). Consistent results were obtained in other non-cancerous cells, specifically NIH-3T3 fibroblast and DC2.4 dendritic cell lines.

The effect of *KEAP1* and *NFE2L2* gene knockouts on the radiosensitivity of MEF cells was investigated (Singh et al. 2010). Analysis of dose dependent clonogenic cell survival revealed an increase in radiosensitivity of cells with double knockout of $KEAP^{-/-}$ compared to that in wild type. Cells with knockouts of either $KEAP^{-/-}$ or $NFE2L2^{-/-}$ and knockout of both $NFE2L2^{-/-}$ and $KEAP^{-/-}$ exhibited NRF2 accumulation in the nucleus and NRF2 AOS activation, evidenced by the high expression of genes under the control of NRF2 transcription factor. Notably, $KEAP^{-/-}$ cells demonstrated decreased radiosensitivity and higher growth rate compared to that of both parental cells and $NRF2^{-/-}$ cells. This suggested that robust NRF2 AOS activation and subsequent reduction of ROS drive these effects.

High expression of NRF2 mRNA, NRF2 protein, and its active phosphorylated form pNRF2 was detected in radioresistant oral squamous cell carcinoma (OSCC) cells HSC2-R and SAS-R. These resistant cell lines were obtained by gradual exposure to incremental X-ray doses from 0.5 Gy/day to 2 Gy/day (Matsuoka et al. 2022). Dose dependencies of clonogenic cell survival of HSC2 and HSC2-R cells are shown in Figure 2G, and the corresponding parameters of the LQ model are given in Table 1. Notably, radiosensitivity of HSC2-R cells is significantly lower (α/β=0.84 Gy) in comparison with that of parental cells HSC2 (α/β=13.8 Gy). Furthermore, siRNA-mediated knockdown of NRF2 expression in HSC2-R-siRNA cells was shown to sensitise resistant HSC2-R cells (α/β=6.23 Gy) (Figure 2G).

Investigation of OSCC cells SAS showed that knockdown of NFE2L2 gene significantly increased radiosensitivity of SAS-siRNA cells (α/β=9.68 Gy) relative to control SAS cells (α/β= 2.27 Gy, Figure 2H and Table 1) (Matsuoka et al. 2022). In contrast, knockdown of *NFE2L2* gene in SAS-R cells (SAS-R-siRNA cells) did not significantly change radiosensitivity (α/β=0.88 Gy for SAS-R cells vs. α/β=1.47 Gy for SAS-R-siRNA cells, Figure 2H and Table 1). Unlike HSC2-R cells, SAS-R cells exhibited a decrease in NRF2 expression after irradiation in comparison with parental cells. At the same time, an increase in phosphorylated form pNRF2 and its nuclear localization indicated functional activation of the NRF2 transcription factor in these cells.

In this study, immunohistochemical analysis of biopsy specimens of patients with OSCC revealed elevated levels of pNRF2 following preoperative chemoradiotherapy. Population analysis of overall survival data indicated that patients with high pNRF2 expression had lower overall survival compared to that in patients with low pNRF2 levels. Furthermore, progression-free survival

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data suggested that high pNRF2 levels serve as a prognostic marker of poor five-year survival in OSCC patients (Matsuoka et al. 2022).

Immunohistochemical analysis also revealed increased NRF2 expression after irradiation in glioma patient tissues (Tang et al. 2022), which correlated with high levels of the hypoxia protein HIF-1α. Consistent with these clinical findings, *in vitro* experiments confirmed upregulation of NRF2 during radiation action of doses from 1 Gy to 8 Gy on human glioma cell lines U251 and U87 grown under hypoxic conditions. Furthermore, the dose dependence of *NFE2L2* expression, measured by the ARE-luciferase reporter activity, was evaluated four-day post-irradiation in cells cultured under both hypoxic and normoxic conditions. Knockout of the *NFE2L2* gene increased radiosensitivity in cells compared to that in parental cells. This effect correlated with a significant rise in ROS levels, a depletion of NRF2-dependent enzymes (NQO1 and NO1), and reduced GSH biosynthesis in the transfected cells. Notably, the molecular mechanism undelaying observed delay in NRF2 activation following irradiation in certain cancer cell lines remains unexplained.

The role of cellular redox status and the NRF2-dependent AOS in the radiosensitivity of prostate cancer cell lines PC3 and DU145 was investigated detailed using genomic, proteomics and phenotypic approaches (Jayakumar et al. 2014). Analysis of clonogenic survival assays revealed different radiosensitivity between the two lines (α/β=10.34 Gy for PC3 vs. α/β=6.58 Gy for DU145, Figures 2E, 2F and Table 1). (Note, that the three experimental data points on the dose dependence of PC3 and DU145 cells in Figures 2E and 2F are insufficient to derive reliable α/β values according to the LQ model). Assessment of radiation-induced DNA damage in these cells showed that DU145 cells exhibited both a higher magnitude of DNA damage and faster repair kinetics compared to PC3 cells. Furthermore, while PC3 cells displayed higher basal and radiation-induced ROS levels, DU145 cells maintained elevated basal GSH levels and a higher GSH/GSSG ratio, contributing to reduced oxidative stress and lower radiosensitivity. Moreover, DU145 cells exhibited significantly higher activation of the NRF2 AOS and increased expression of NRF2 downstream antioxidant genes (*HO1*, *GCLC*, *TXRD1*, and others) compared to PC3 cells. Based on these results, authors assumed that NRF2 activity determines different radiosensitive phenotypes of these cells. To test this, they evaluated clonogenic survival of PC3-shRNA and DU145-shRNA sublines, in which NRF2 expression was knocked down by transfection with short hairpin RNA (shRNA). Silencing NRF2 transcription factor significantly reduced survival in both transfected lines (Figure 2E and Figure 2F). Notably, the dose dependent survival curves for PC3-shRNA and DU145-shRNA cells do not follow the LQ model. Approximation of the experimental dose dependencies by the LQ model gave zero values of α that means a straight-line approximation (Figures 3E and 3F). This suggests that inhibition of the NRF2 AOS in these cells may induce low-dose hypersensitivity at doses below 4 Gy. However, more precise measurements of the dose dependence are required to fully characterize this low- dose behaviour.

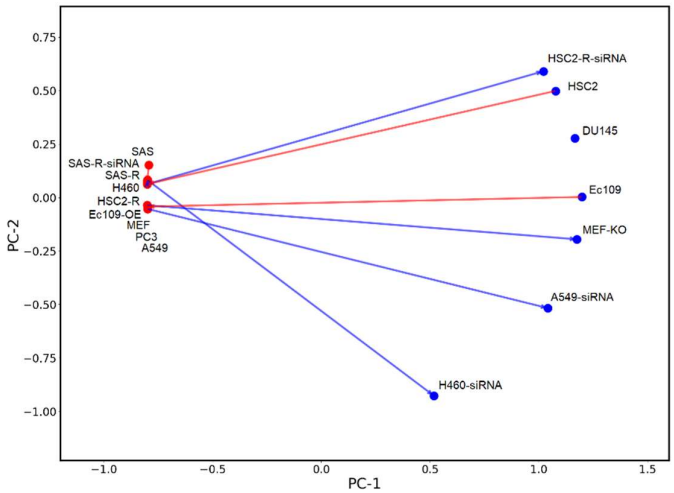

Figure 3. Clustering of cancer cells according their radiosensitivity, represented on the principal components PC-1 and PC-2 axes. A cluster of radioresistant cancer cell lines with inhibition of the

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NRF2 AOS (red points) and a cluster of their radiosensitive parental or transfected cells (blue points) are showed. Arrows indicate the transitions of cells from the cluster of radioresistant to that of radiosensitive cells as a result of inhibition of the NRF2 AOS.

Principal component analysis (PCA) and k-means clustering (Wannouss, Golyshev, and Goltsov 2023) were applied to a set of the obtained parameters α/β and α (Table 1). This allowed clustering radiosensitive and radioresistant cells in the principal component space of PC-1 and PC-2. Figure 3 illustrates the classification of radiosensitive and radioresistant cells with normal versus suppressed NRF2 AOS functioning with blue and red points representing radiosensitive and radioresistant cells, respectively. Arrows indicate the transitions of the cells from the radioresistant to radiosensitive clusters following inhibition of the NRF2 AOS. Furthermore, hierarchical clustering based on the same parameters of the LQ model (α/β and α) yielded results consistent with the PCA-based clustering (Figure 4).

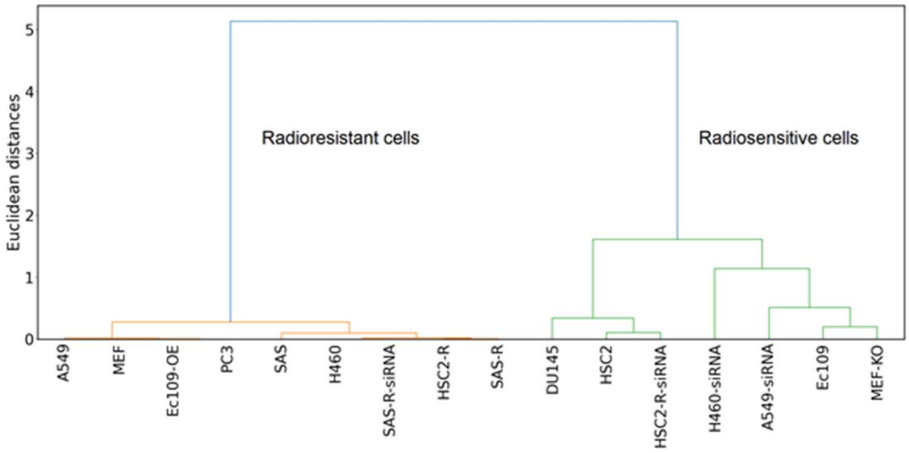

Figure 4. Dendrogram of agglomerative hierarchical clustering of radioresistance (left arm) and radiosensitive (right arm) cancer cells with normally functional or modulated NRF2 AOS.

One notable unresolved issue in NRF2 AOS function is the temporal discrepancy between NRF2 activation and the onset of ARE-dependent gene expression following irradiation. In various cancer cells discussed above, NRF2 activation occurred immediately after irradiation. For example, NRF2 activation in rhabdomyosarcoma cell lines was detected directly after irradiation (Marampon et al. 2019). In contrast, in MEF cells, an increase in ROS levels and ARE transcriptional activity were registered on the fifth day after irradiation (McDonald et al. 2010). Similarly, radiation-induced NRF2 activation in NSCLC cell lines H1299 and A549 was observed within 6 hours after irradiation and persisted for 2 to 4 days in H1299 as well as until day 2 in A549 cells (Lee et al. 2012). This significant lag was suggested to relate to delayed ROS generation after irradiation, a process potentially driven by cycles of cell death and pro-inflammation cytokine production (McDonald et al. 2010). It has been also proposed that radiation-mediated NRF2 activation is cyclic process initiated by the primary exposure (Lee et al. 2012). Note that oscillation regime in NRF2 activation was observed in human vascular endothelial HMEC-1 cells stimulated by sulforaphane, suggesting a potential mechanism for persistent NRF2 activity (Xue et al. 2015). The data reviewed here suggest that delayed radiation-induced NRF2 activation is inherent to radioresistant cells such as A549, H1299, and MEF cells. In contrast, radiosensitive NRF2 knockout MEF-KO cells exhibit a more than 10-fold increase in ROS immediately after irradiation (McDonald et al. 2010). Further investigation is required to fully elucidate the mechanisms underlying these distinct temporal patterns in NRF2 AOS activation.

To summarize experimental findings discussed in this section, we schematically presented in Figure 5 several scenarios of radiation-induced responses of cancer cells with various different levels of basal ROS and NRF2 AOS activity (Trachootham et al. 2008), (Ribeiro 2023). Curve 1 in Figure 5 shows a case when radiation-induced increase of ROS over the basal level and above NRF2 activation

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threshold, inducing oxidative stress in cancer cells. The subsequent activation of the NRF2 AOS and the upregulation of the antioxidant enzymes' expression suppress oxidative stress and restore ROS homeostasis. The robust antioxidant capacity enables cells to overcome radiation-induced oxidative stress, mitigates therapeutic radiation effect, resulting in low radiosensitive phenotype. The scenario discussed above can be applied to prostate cancer cell line DU145 (Jayakumar et al. 2014) and rhabdomyosarcoma RMS cells (Marampon et al. 2019), both of which exhibit strong antioxidant responses and low radiosensitivity. In Figure 5, curve 2 corresponds to the case of NRF2 AOS inhibition, bringing about an uncontrolled radiation-induced ROS rise, leading to high radiosensitivity. Across all cancer cell lines, discussed above, NRF2 AOS inhibition by knockout of the *NFE2L2* gene resulted in a significant increase in ROS levels and high radiosensitivity. Conversely, curve 3 represents a case of cancer cells with *de novo* activation of the NRF2 AOS due to *KEAP1* mutations or *NFE2L2* overexpression, where radiation-induced oxidative stress is suppressed by active NRF2 AOS. Examples of this case include OSCC and HSC2 cells, which exhibit high expression of NRF2, NRF2 mRNA, and pNRF2 alongside a radioresistant phenotype (Matsuoka et al. 2022). Similarly, ESCC cells, overexpressing NRF2 (Ec109-OE and KYSE-30-OE), demonstrated reduced radiosensitivity (Xia et al. 2020). The reduced KEAP1 levels may account for the high basal NRF2 levels in radioresistant DU145 cells (Jayakumar et al. 2014). Curve 4 in Figure 5 illustrates a scenario where cancer cells possess a high basal ROS level, leading to increased radiation-induced ROS and high radiosensitivity. We propose that this occurs when to cellular antioxidant capacity is overwhelmed and the NRF2 AOS response to oxidative stress is limited. This case may be exemplified by the prostate cancer cell line PC3, which exhibits high radiosensitivity (α/β=10.34 Gy, Table 1) alongside high basal and radiation-induced ROS levels (Jayakumar et al. 2014). We also added case 5 in Figure 5, representing cancer cells that adapt to high ROS levels and resist to radiotherapy. An example of this case may be a radioresistance phenotype of prostate cancer cell PC-3 cells, which are characterized by high aggressiveness, increased cellular proliferation, and invasive potential (Sideri et al. 2022). It has been proposed that radioresistant PC-3 cells adapt to oxidative stress through an enhanced capability to repair DNA double-strand breaks caused by ROS.

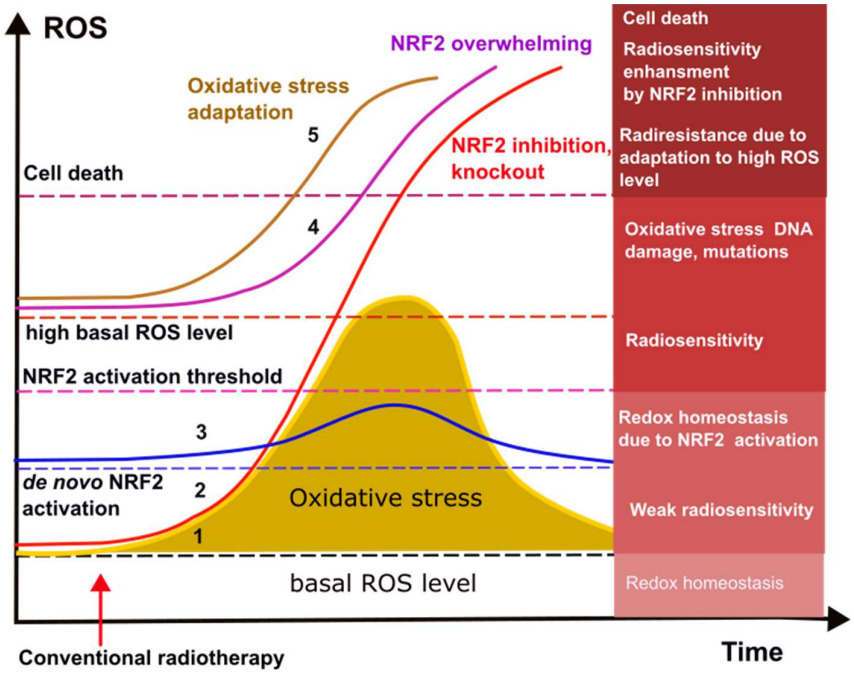

Figure 5. Schematic representation of the radiation-induced ROS dynamics in cancer cells with various basal ROS and NRF2 activity levels. Curve 1 (yellow) corresponds to suppression of radiation-induced oxidative stress in cancer cells with low basal ROS and high antioxidant capacity resulting in low radiosensitivity. Curve 2 (red) displays uncontrolled ROS rise following NRF2 AOS inhibition, leading to high radiosensitivity. Curve 3 (blue) corresponds to mitigation of radiation-induced

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oxidative stress via *de novo* activation of the NRF2 AOS due to *KEAP1* mutations or *NFE2L2* overexpression, resulting in low radiosensitivity. Curve 4 (magenta) represents a scenario when high basal ROS levels predispose to increased radiation-induced ROS and high radiosensitivity. Curve 5 (brown) shows a case of cancer cell adaptation to chronic high ROS levels, leading to an acquired radioresistant phenotype.

4. NRF2 Activation as a Factor of Radioresistance

The preceding review of experimental and clinical studies demonstrates that ionizing radiation activates the NRF2 AOS in various cancer cells thereby attenuating their radiosensitivity. At the same time, it has been found that blocking the NRF2 transcription factor reversibly enhance radiosensitivity. Given these findings, in this section we analyse studies on radiosensitivity of various cancer cells and tumour tissues, where genetic mutations or epigenetic modifications activate the NRF2 AOS, leading to tumour adaptation to radiation-induced oxidative stress.

As we discussed above, the increased basal expression of NRF2 transcription factor in DU145 prostate carcinoma cells has been investigated at the genomic, proteomics and phenotypic levels (Jayakumar et al. 2014). Analysis of clonogenic survival data on cells following irradiation with doses of 4 Gy and 8 Gy showed that DU145 cells exhibit low radiosensitivity, which correlates with less DNA damage, compared to radiosensitive PC3 cells, and its lower NRF2 levels. Examination of cellular redox status revealed low basal ROS levels in DU145 cells and its reduced levels upon irradiation. These findings associated with higher expression of the *NFE2L2* gene and NRF2-dependent genes (*GCLC*, *HO1*, and *TXRD1*) in DU145 cells compared to PC3 cells. Furthermore, DU145 cells exhibited a higher basal GSH level and GSH/GSSG ratio than PC3 cells, correlating with elevated levels of GCLC (glutamate-cysteine ligase catalytic subunit) participating in GSH synthesis. Measurement of basal KEAP1 levels, which regulates NRF2 degradation (Figure 1), showed its lower levels in DU145 cells than that in PC3 cells; furthermore, irradiation further reduced KEAP1 levels. This study demonstrated that radioresistance of DU145 cells can be suppressed through either *NFE2L2* gene knockout or the use of NRF2 or HO1 inhibitors. The authors concluded that activation of the NRF2 AOS, driven by high NRF2 expression and low KEAP1 levels, enhances the cellular recovery status under irradiation, thereby conferring radioresistance to prostate carcinoma cells.

A comprehensive investigation into the role of mutations within the NRF2/KEAP1 pathway in the development of radioresistance was carried out in squamous cell lung cancer (SCLC) (Jeong et al. 2017). Mutations in this pathway were identified in 30% of the patients under investigation, correlating with high tumour aggressiveness and an increased risk of recurrence and distant metastases following radiotherapy. The role of these mutations was investigated in P-LSCC cells with *Trp53* gene deletion and K/P-LSCC cells with double deletion of *Keap1* and *Trp53* genes. Histologically, these models closely resemble poorly differentiated squamous cell carcinoma. Experiments were conducted *in vitro* on a population of these cells and in mice model using tumour xenografts. Results showed higher activation of NRF2 transcription factor in K/P-LSCC cells compared to P-LSCC cells. This activity was accompanied by the increased expression of NRF2-dependent enzymes and corresponding decreased ROS levels. Furthermore, K/P-LSCC cells with *Keap1* and *Trp53* gene deletion exhibited high proliferation and metastasis rates. Clonogenic analysis of cell survival across a radiation dose range of 1 Gy-10 Gy demonstrated enhanced radioresistance of K/P-LSCC cells compared to P-LSCC cells. Concurrently, significantly less DNA damage was detected in the *Keap1*-deleted cells. These findings were further validated by *in vivo* experiments, where K/P-LSCC tumour in mice following irradiation at a dose of 6 Gy showed significant attenuation of the irradiation-induced DNA damage effect on tumour growth compared to P-LSCC tumours.

In the same study, a method to suppress radioresistance in SCLC cells with *KEAP1* gene deletion was proposed (Jeong et al. 2017). It was found that K/P-LSCC cells overexpress *SLC7A11* gene which is under the control of NRF2 transcription factor and encodes protein SLC7A11 (Solute Carrier Family 7 Member 11), a key subunit of the cystine/glutamate antiporter system playing the essential role in the GSH synthesis. Pharmacological inhibition of the cysteine import system by sulfasalazine resulted

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in sensitization of K/P-LSCC cells to ionizing radiation and restoration of radiosensitivity inherent to their parental cells.

Translating these *in vitro* and *in vivo* findings into a clinical study, the clinical trial was conducted involving 42 patients with Stages I-III prostate carcinoma receiving radiation therapy (Jeong et al. 2017). The trial included patients with mutations in the Kelch domain of *KEAP1* gene, which disrupt NRF2 binding. In this group, higher localized relapse rate of 70% was observed compared to 18% in patients without mutations in the NRF2/KEAP1 AOS. The authors demonstrated that *KEAP1* mutations could be identified non-invasively using circulating tumour DNA (ctDNA) blood test. The results of radiation therapy in this group of patients confirmed that this genetic profile is a strong predictor of poor radiation response and high recurrence rates.

The clinical significance of the NRF2/KEAP1 system in oncotherapy outcome is demonstrated by the high frequency of mutations in *NFE2L2* and *KEAP1* genes found across various malignancies. Furthermore, it has been shown that NRF2/KEAP1 mutations constitutively activate the NRF2 AOS and confer *de novo* resistance to various chemotherapy drugs including cisplatin, etoposide, paclitaxel, bortezomib, gemcitabine, 5-fluorouracil, etc., which therapeutic mechanism in particular link to ROS generation with subsequent DNA damage. Given these results presented in the review, it is hypothesized that various mutations in the NRF2/KEAP1 AOS found in numerous cancer cells significantly contribute to tumour adaptation to ionizing radiation and radioresistance emergence. To suppress these unwanted processes weakening radiotherapy efficiency, pharmacological inhibition of the NRF2/KEAP1 AOS by drug intervention can be employed. The following section summarizes the main mutations in the NRF2/KEAP1 system across various cancers.

The primary molecular mechanisms driving expression of NRF2 transcription factor in cancer cells include: somatic mutations in *KEAP1* or *NFE2L2* genes; epigenetic modifications of the *KEAP1* gene, that suppress its expression; post-transcriptional regulation by microRNA expression that regulates *NFE2L2* and *KEAP1* transcripts; and increased expression and accumulation of proteins that compete with NRF2 for KEAP1 binding (Fabrizio et al. 2018), (Sparaneo et al. 2025).

Somatic mutations in *KEAP1* were initially identified in lung tumour tissue samples (19%) and lung cell lines (50%), which they represent the second most frequent and significant genetic alterations in lung cancer (Singh et al. 2006). *KEAP1* mutations have also been found in other human malignancies, including ovarian (19%) (Konstantinopoulos et al. 2011), prostate (8%) (P. Zhang et al. 2010), gastric (11%), liver (9%), colorectal (8%), and breast cancer (2%) (Yoo et al. 2012). Distributed across several KEAP1 domains, these mutations impair its function, triggering nuclear accumulation of NRF2 and the constitutive activation of the NRF2/KEAP1 antioxidant system.

The most frequent genetic alterations affecting the function of the KEAP1-NRF2 complex in solid tumours are point mutations accompanied by loss of heterozygosity (LOH). They typically occur within exon encoding either the Kelch domains in KEAP1 responsible for the binding of KEAP1 with NRF2 or the IVR and BTB domains, which contain cysteine residues critical for redox regulation of NRF2. Consequently, these mutations impair KEAP1-mediated ubiquitination of NRF2. In non-small cell lung cancer (NSCLC), these alterations lead to the overexpression of NRF2-dependent antioxidant enzymes and increased chemoresistance(Singh et al. 2006).

Somatic mutations of *NFE2L2* occur mainly in the ETGE (57%) or DLG (43%) motifs that cause dissociation of the KEAP1-NRF2 complex. Mutations in the ETGE motif disrupt the high-affinity interaction between KEAP1 and NRF2, while those in the DLG motif abolish the low-affinity interaction. These mutations were identified in tissue samples of patients with papillary renal cell carcinoma (PRCC) exhibiting constitutive activation of the KEAP1/NRF2 AOS (Ooi et al. 2013). Additionally, deletion mutations in the *CUL3* ubiquitin ligase gene results in a complete loss of enzyme function; this inhibits NRF2 degradation, leading to its accumulation and subsequent activation of the KEAP1/NRF2 AOS.

An elevated prevalence of *NFE2L2* mutations in endometrial carcinoma compared to healthy cells was detected by immunohistochemical analysis of various malignant and benign tissue samples (Jiang et al. 2010). Subsequent studies using endometrial carcinoma cell lines demonstrated resistance to oxidative stress-inducing chemotherapeutic drugs, such as cisplatin and paclitaxel. Furthermore,

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in xenograft mouse models, the inhibition of NRF2 significantly reduced tumour volume when combined with chemotherapy.

Notably, NRF2 overexpression in KEAP1 mutant human NSCLC cell lines was found to be detrimental to cell proliferation, viability, and anchorage-independent colony formation. Collectively, these findings establish a context dependence activity for NRF2 during the lung tumorigenesis (DeBlasi et al. 2023).

An additional mechanism of NRF2 activation involves its upregulation through the crosstalk between the NRF2 AOS and other oncogenic signalling pathways (Khalil et al. 2016), (Tao et al. 2014). For example, in 20–30% of lung carcinomas, NFE2L2 transcription is driven by KRAS mutations that activate the MAPK proliferative pathway, leading to chemoresistance (Tao et al. 2014). In NSCLC xenograft models, KRAS-induced increase in NFE2L2 transcription was shown to confer resistance to cisplatin. Notably, treatment with the NRF2 inhibitor brusatol effectively reversed this drug resistance.

Beyond somatic mutations in *NFE2L2*, *KEAP1*, and *CUL3* that disrupt the NRF2-KEAP1 interaction, other NRF2 activator function through a similar mechanism. For instance, the cyclin-dependent kinase inhibitor p21 competitively binds to the DLG motif of NRF2, completely impeding its two-site binding to KEAP1. This interference leads to nuclear NRF2 accumulation and, consequently, enhances cell survival under oxidative stress (Chen et al. 2009).

Beyond mutations, the KEAP1/NRF2 pathway is frequently activated through epigenetic modifications, most notably the aberrant methylation of the *KEAP1* gene promoter (Fabrizio et al. 2018). It was found that epigenetic modification is the primary cause of *KEAP1* suppression in solid tumours. *KEAP1* hypermethylation confers a growth advantage to cancer cells and correlates with poor clinical outcomes (Copple 2012b). *KEAP1* methylation was found in 51% of early-stages lung cancer patients, significantly increasing risk of recurrence following surgery (Fabrizio et al. 2018). Similarly, a group of patients with estrogen- and HER2-negative status was found to have the highest risk of recurrence after chemotherapy with a hazard ratio of HR=14.73 (Barbano et al. 2013).

Epigenetic modulation is a primary driver of *KEAP1* deregulation in renal cancer patients (48.6%) and serves as a predictive marker for patient survival (Fabrizio et al. 2018). Similarly, increased methylation of *KEAP1* gene promoter in glioma cells was observed in 60% of the patient tissue samples under investigation (Muscarella et al. 2011). Analysis of the survival rate of glioma patients without disease progression after radiotherapy showed a higher survival rate in a group of patients with methylated *KEAP1* compared to those with unmethylated *KEAP1*. This increased radiosensitivity, occurring despite low KEAP1 expression and subsequent NRF2 activation, contradicts the general observation that NRF2 signaling typically decreases radiosensitivity. It can be assumed that the mechanism of radiosensitivity increase in this case relates to the peculiarities of NRF2/KEAP1 AOS activation in glioma cells which are predominantly grow under hypoxic conditions.

5. The NRF2 Antioxidant System as a Therapeutic Target to Enhance Radiosensitivity of Cancer Cells

Activation of the NRF2/KEAP1 AOS in specific cancer cells at increasing ROS level promotes cell adaptation to oxidative stress induced by ionizing radiation. This response directly promotes radioresistance. As discussed in the previous section, this activation result from *de novo* mutations within the NRF2/KEAP1 signalling system during carcinogenesis or a cellular protective response to radiation-induced oxidative stress. Activation of the NRF2/KEAP1 AOS in various malignancies suggests critical dependence of some cancers on the function of the cellular antioxidant system. NRF2-dependent tumours harbouring NRF2, KEAP1, or CUL3 mutations exhibit high therapeutic resistance and serve as markers of poor prognosis in patients with non-small cell lung cancer, esophageal cancer, and head and neck cancer (Kitamura and Motohashi 2018). This dependency further amplified during radiation therapy, when radiation-induced ROS generation in tumour environment triggers an intensive antioxidant response, allowing cancer cells to neutralize ROS and minimize DNA damages caused by radiation. As established in the previous section, extensive

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experimental data confirmed the dose-dependent activation of the NRF2/KEAP1 AOS and upregulation of NRF2 following irradiation. These findings allow considering the NRF2/KEAP1 AOS as a promising potential target of therapeutic intervention in combination with radiation therapy. Currently, NRF2 transcription factor was included in a list of other transcription factors along with STAT, NF-κB, Notch, and others which are recognised as therapeutic targets in cancer treatment (Darnell 2002), (Bushweller 2019). Unlike many other transcription factors, NRF2 is unique because it is considered as a target for action of both inhibitors and activators, depending on specific diseases and cellular redox status. At present, NRF2 is recognized alongside other transcription factors as a promising target for novel therapeutic strategies in combination with radiotherapy to overcome radioresistance (Galeaz, Totis, and Bisio 2021).

In clinical oncology, the efficacy of targeted therapy is determined by drugs inhibiting certain signalling pathways that are abnormally activated by mutations in oncogenes and tumour suppressor genes. Targeted therapies have achieved great success when combined with other oncological treatments. Given the frequent aberrant activation of the NRF2 transcription factor in tumours, NRF2 may be considered as a critical pharmacological target. Specifically, inhibition of the NRF2/KEAP1 AOS in combination with radiotherapy represents a potent strategy for enhancing tumour radiosensitivity. Radiosensitivity enhancement by inhibition of the NRF2/KEAP1 AOS in tumour cells is determined by elevating basal ROS levels and extra generation of ROS under the action of ionizing radiation during radiotherapy (Figure 5). Furthermore, blocking NRF2 transcription factor represses *NFE2L2* gene and NRF2-dependent downstream genes, which control various metabolic and signalling pathways regulating proliferation, growth, amino acid synthesis (serine and glycine), systems maintaining self-renewal, and pluripotency of cancer stem cells (Rojo de la Vega et al. 2018). Suppression of these pathways increases radiosensitivity by inhibition of cancer cell growth and enhancement of cancer stem cell differentiation.

Currently, the different subsystems of the NRF2/KEAP1 AOS (Figure 1) are recognized as druggable targets under active investigation (Zhou et al. 2013), (Glorieux et al. 2024), (Copple 2012b), (Paramasivan et al. 2019). Substantial progress has been made in the development of activators of the NRF2 AOS as cytoprotective drugs for the therapy of various oxidative stress-related diseases, including cancer. Many drugs activating NRF2 transcription factor have already been approved by the FDA for clinical use (Lu et al. 2016). In contrast, relatively few effective inhibitors with high specificity towards the NRF2/KEAP1 AOS have been developed. The development of NRF2 inhibitors are currently at the stages of preclinical and clinical trials (Telkoparan-Akillilar et al. 2021). However, given the mounting evidence that NRF2 activation and mutations in the NRF2/KEAP1 AOS drive carcinogenesis and therapeutic resistance, the development of multi-level strategies to block this signalling pathway is rapidly accelerating.

NRF2 inhibitors comprise a broad range of natural and synthetic compounds. Natural NRF2 inhibitors include compounds such as flavonoids (apigenin, luteolin), alkaloids (halofuginone, trigonelline, and berberine), quassinoids (brusatol), and others (triptolidine, gensenosides) extracted from plants (Pouremamali et al. 2022), (Zhang et al. 2023). While the FDA has not yet approved NRF2-targeted drugs for cancer treatment, clinical trials indicated that these inhibitors can suppress tumour growth and sensitize malignancies to chemotherapy. By chronically elevating ROS levels in tumours, these agents showed promise in treating patients with colorectal carcinoma, lung, gastric, and squamous cell tongue cancers (Pouremamali et al. 2022).

Application of NRF2 inhibitors in combination with radiation to treat cancer demonstrated their significant potential as enhancers of radiosensitivity of cancer cells. For instance, the proposed compound 4-(2-Cyclohexylethoxy) aniline (IM3829) was found to inhibit radiation-induced NRF2 activity and expression of the NRF2 dependent downstream genes (Lee et al. 2012). Notably, combination of IM3829 with radiation exhibited a synergistic effect, i.e. while IM3829 and radiation separately did not cause noticeable effect on apoptosis, ROS levels, and tumour growth, their combination markedly increased apoptosis, ROS production, and decreased tumour volume in H1299 or A549 lung cancer xenografts compared with control. Authors suggested that NRF2 inhibitor IM3829 acts as a radiosensitizer by blocking NRG2-dependent antioxidant response, thereby

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promoting ROS accumulation and reversing radioresistance. Notably, the synergistic effect of NRF2-targeting drugs is a key benefit of this combination strategy in radiotherapy. Another advantage is their selectivity, i.e. these drugs primarily affect cancer cells with active NRF2. Consequently, they act locally in the irradiated area of tumour, minimizing side effects on healthy tissue.

Beyond their primary therapeutic effects, many anticancer drugs used in the clinic were found to inhibit the NRF2 AOS, rendering cells vulnerable to oxidative stress (El-Naggar et al. 2019). These known drugs were thought to exert an indirect therapeutic effect through NRF2 inhibition, providing additional mechanisms of action on cancer cells. For example, various chemotherapeutic drugs, including temozolomide and homoharringtonin (elongation inhibitor), suppress NRF2 expression at the transcription level, effectively sensitizing lung and bladder cancer cells to chemotherapeutic drugs (El-Naggar et al. 2019).

Targeted drug therapy can suppress activity of NRF2 transcription factor at various levels of the NRF2 AOS signalling pathway. For instance, anticancer drug entinostat, which is a histone deacetylation inhibitor, inhibited NRF2 synthesis at the translation level. It achieves this by acetylating and reducing the activity of YB-1 protein, which is essential for NRF2 mRNA translation (El-Naggar et al. 2019) and exhibits high activity in sarcoma cells. This additional therapeutic effect of this drug induced oxidative stress in sarcoma cells *in vitro* and significantly suppressed metastasis *in vivo*. Similarly, the dual PI3K/mTOR inhibitor omipalisib inhibited NRF2 synthesis by suppressing NRF2 mRNA translation, showing therapeutic effects in gastric cancer, sarcoma, and osteosarcoma. Additionally, the dual-action PI3K-DNA PK inhibitor PIK-75 induced NRF2 degradation, aiding to overcome the resistance of pancreatic cancer to gemcitobin (Telkoparan-Akillilar et al. 2021). The Bcl-2 inhibitor, venetoclax suppressed increased expression of NRF2 caused by *NFE2L2* gene demethylation; this triggered ROS generation in mitochondria that induced apoptosis in myeloid leukaemia cells. Furthermore, the targeted therapies trastuzumab and pertuzumab (HER2 inhibitors) suppressed NRF2 transcription factor activity at the epigenetic level (Khalil et al. 2016). Their combined action promoted methylation of the *NFE2L2* promoter, leading to epigenetic inhibition of *NFE2L2* expression. This resulted in subsequent generation of ROS and the suppression of glutathione synthesis.

Clinical application of NRF2 targeted agents in combination with radiotherapy requires identification of specific cancer types most likely to benefit. The potential candidates for this combined therapy are mainly malignant tumours that exhibit a dependency on the NRF2 AOS, typically characterized by elevating NRF2 transcription factor activity and acquired radioresistance. This cohort primarily may include cancers harbouring mutations in *NFE2L2*, *KEAP1*, or *CUL3* genes, as well as those with epigenetic modifications in *NFE2L2* that drive NRF2 activation.

6. Conclusions

Extensive experimental and clinical studies have demonstrated that the NRF2/KEAP1 antioxidant system of cancer cells is one of the promising targets for personalized therapy combined with radiotherapy. The synergistic effect of this combined approach is driven by the addiction of certain cancer cells to the NRF2/KEAP1 AOS – a dependency often resulting from a high frequency of mutations in this system that leadi radioresistance. A strong antioxidant response of cancer cells upon activation of NRF2/KEAP1 AOS enhances ROS scavenging, allowing cells to adapt to oxidative stress and survive under ionizing radiation. The adaptive cell response diminishes the radiosensitivity of cancer cells. Inhibiting the NRF2 AOS suppresses the antioxidant defences of cancer cells, triggering an uncontrolled accumulation of ROS beyond the homeostatic threshold. This shift induces the oxidative stress and subsequent apoptosis of cancer cells. Consequently, the development of therapeutic strategies to block the activation of the NRF2/KEAP1 AOS during radiotherapy could enhance efficacy of personalized radiotherapy for a broad range of cancer patients.

To determine the effectiveness of radiotherapy in combination with this targeted therapy, further studies are required to confirm the association between mutations and epigenetic modifications in the NRF2/KEAP1 axis and radioresistance. Experimental and clinical studies in this

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direction will clarify the NRF2-dependent mechanism of radioresistance, enabling selection of patient cohorts who will benefit most from personalized combination radiotherapy. In this case, fractionated regimens with escalated radiation doses may be appropriate. A promising strategy will be to identify cancer types that exhibit synergistic effect between inhibition of the NRF2/KEAP1 AOS and radiotherapy, thereby defining the population most sensitive to this combined therapy.

Another promising avenue for developing this combined radiotherapy involves identification of critical targets for effective inhibition of the NRF2 AOS. As shown in this review, numerous signalling proteins, functioning in various subsystems of the NRF2 AOS, could be therapeutic targets. Blocking of the NRF2 transcription factor can be achieved at the proteomic, genetic or epigenetic levels. A specific target for therapeutic action should be guided by molecular and genetic data regarding dysregulation of the NRF2 axis, which drives its high activity in various cancers. This approach will facilitate the development of personalized radiosensitization strategies tailored patients with specific mutations in the NRF2 AOS.

The efficacy of radiotherapy combined with suppression of the NRF2/KEAP1 AOS significantly depends on the activation status of the cellular antioxidant system and its response to ionizing radiation across different tumours types. Integration of proteomic and genomic data on mutations and activation of the NRF2/KEAP1 AOS into further research and clinical trials will facilitate selection of optimal personalized protocol of combining radiotherapy and help identify patient cohorts with NRF2-dependent radioresistance.

Intensive experimental and clinical studies currently underway in these areas highlight the clear potential for the development of effective combining radiotherapy that inhibit the NRF2/KEAP1 antioxidant system in cancer cells to enhance efficacy of personalized radiotherapy.

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