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 (RR) in radiotherapy (RT) is currently being tackled, on one hand, by introducing into clinical practice new RT equipment with high quality of ionizing beams, high precision of radiation delivery to tumour, and optimization of treatment plans. On the other hand, new therapeutic methods for suppressing RR in tumours of cancer patients are being developed through a combination of RT with chemo-, immuno- and targeted therapies based on molecular diagnostic data. As a result of numerous preclinical and clinical trials of the combination therapy, the main molecular mechanisms, driving an increase in radiosensitivity (RS), were found out, and the key cellular signalling pathways responsible for RR were established. One of the established mechanisms is in adaptation of cancer cells to an elevated level of reactive oxygen species (ROS) by activation of antioxidant systems (AOS) of cellular protection and survival. Considering that RT relies mainly on the production of ROS that damage DNA and cause cancer cell death. Consequently, the activation of the AOS can mitigate the RT effectiveness, and it is assumed that suppression of the AOS and cellular adaptation mechanisms to a high ROS level can increase tumour RS and enhance the effectiveness of radiotherapy. In this review we discussed a role of one of the key components of the cellular AOS being under the control by a transcription factor NRF2 (nuclear erythroid factor 2) which governs the expression of a battery of antioxidant enzymes and protects cells from oxidative stress induced by RT. First, we briefly discussed the molecular function of the redox-sensitive the NRF2 AOS, which is activated in cells following the increased ROS level due to irradiation. Second, we reviewed the experimental and clinical data on activation of the NRF2 AOS in some cancer cells and tumour under ionizing irradiation. Third, we discussed results of numerous experimental and clinical investigations which clearly showed that suppression of the NRF2 AOS leads to an increase in radiosensitivity of various cancers and an enhancement in the effectiveness of RT in cancer patients. These data confirm the potential of combining RT with targeted therapy aiming at the suppression of the NRF2 cytoprotective AOS. Based on multiple experimental and clinical studies, we advocated a role of NRF2 inhibitors as radiosensitizers that promote overcoming radioresistance due to extra ROS accumulation and oxidative stress induction by inhibition of the NRF2-dependent antioxidant responses to radiotherapy.

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

1. Introduction

The efficiency of radiotherapy has been significantly improved in recent decades by introducing new radiotherapy units providing precise delivery of radiation dose to tumour sites and designing non-conventional treatment plans in clinical practice. The successful development of innovative therapeutical strategies for enhancement of tumours radiosensitivity (RS) in cancer patients also

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determines progress in radiotherapy. Combining radiotherapy with chemo-, immuno-, and targeted therapies based on molecular diagnostics data now is a promising direction in the advancement of radiotherapy. It led to the development of effective combined radiotherapy with personalized drug therapies aimed at inhibiting certain metabolic and signalling pathways activated in cancer cells due to mutations in tumour suppressors and oncogenes. A large body of experimental and clinical investigations have elucidated some of the main molecular mechanisms of low radiosensitivity of malignant tumours and identified some of the main signalling and metabolic pathways and mutations that cause radioresistance in tumours (Meehan et al. 2020), (Barker et al. 2015). These investigations let to the development of new combined radiotherapy, based on genetic and molecular diagnostics data of patients. This makes it possible to enhance the radiotherapy effectiveness by suppressing radioresistance (RR) and increase radiosensitivity index of various types of cancer.

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

A high level of ROS in a number of cancer cells are positively correlated with aggressive disease outcome (Oshi et al. 2022). Further, elevated levels of ROS and OS cause DNA damage, oxidation of proteins and lipid peroxidation leading to chromosomal aberration, lipid peroxidation, and cell death like apoptosis and ferroptosis. The ability of many cancer cell lines to function under OS condition is ensured by enhanced oxidant protection in cancerous cells by triggering a mechanism of cellular adaptation of cancer cells to OS (Glorieux et al. 2024). Cancer cells adapt to OS due to the endogenous antioxidant system (AOS) of cellular defence, which is controlled by the redox-sensitive NRF2 TF (Baird and Yamamoto 2020). Its activation at a high level of ROS induces expression of a battery of antioxidant enzymes catalysing ROS degradation. NRF2 TR being a key regulator of the cellular AOS controls expression of such antioxidant enzymes 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). NRF2 TR also controls expression of enzymes participating in biosynthesis of glutathione (GSH) being of a significant agent of the AOS. Besides antioxidant enzymes, NRF2 NF controls certain signalling pathways that protects cells and tissues from chemical and biological stresses induced by toxins, drugs and carcinogens (Taguchi, Motohashi, and Yamamoto 2011).

Activation of the NRF2 AOS that promotes cancer progression, aggressiveness, metastasis, and confers resistance to chemotherapeutic drugs by inducing high ROS level has been detected in various cancer cells and tumours (Rojo de la Vega et al. 2018), (Taguchi et al. 2011), (Lau et al. 2008). In most cases, activation of the NRF2 AOS leads to cancer adaptation to the oxidative stress experienced by cancer cells. Thus, cancer cells, functioning under condition of oxidative stress, depend on the NRF2 AOS as a protective cellular system. Addiction of some cancer cells to the NRF2 signalling pathway 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 generally considered as a potential therapeutic target for the development of both activators and inhibitors of the NRF2 system 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). Many systematic and comprehensive reviews have summarized results of experimental and clinical studies which showed that modulation of the NRF2 AOS by drugs may contribute to prevention of

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cancerogenesis and suppression of cancer resistance to chemo- and targeted therapy (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).

In this review we discuss a role for the NRF2 AOS of cancer cells in radiotherapy efficiency modulation. We analyse experimental results which showed that activation of the NRF2 AOS in cancer cells mitigates effectiveness of radiotherapy due to scavenging radiation-induced ROS and cancer adaptation to irradiation. We discuss the results of experimental studies in which authors consider the activation of the NRF2 AOS as an adaptive response of cancers to irradiation and as one of the molecular mechanisms of radioresistance that protect cancer cells from damage produced by radiation. Finally, we review a large body of experimental works that revealed that suppression of the NRF2 AOS increases tumour radiosensitivity and enhance radiotherapy effectiveness.

2. Molecular Mechanism of the Function of NRF2 Antioxidant System

ROS are formed in cells and organs as by-products of enzymatic reactions in metabolic processes and the respiratory chain in mitochondria (Hayes et al. 2020). Being products of radical enzymatic reactions, ROS at low concentrations play a role of signalling molecules and mainly participate in stress signalling which are controlled by redox-sensitive proteins containing cysteine residues in their structure. Under normal conditions, intracellular concentration of ROS is kept at a low level due to the balance between its generation and detoxification by the cellular AOS under the control of NRF2 transcription factor.

According to the molecular mechanism established to date (McMahon *и др.* 2006; Kobayashi et al., 2004; Horie et al. 2021), the main element of the NRF2 AOS of the cell is a complex of the NRF2 with a dimer repressor protein KEAP1 (Kelch-like ECH associating protein 1) binding with a Cul3-based E3 ubiquitin ligase. Under normal cell redox conditions, binding of *de novo* synthesized NRF2 with the KEAP1-Cul3-E3 complex occurs that promotes NRF2 interaction with Cul3-E3 leading to subsequent ubiquitination and degradation of NRF2 in proteasomes (Fig. 1). This leads to a low concentration of NRF2 in the cell cytoplasm and nucleus that ensures constitutive expression of NRF2-regulated genes and proteins of the AOS.

Under oxidative stress or electrophile attack, oxidative modification of the sulfhydryl groups of KEAP1 cysteine residues occurs according to “the hinge and latch” model (McMahon *и др.* 2006; Kobayashi et al., 2004; Horie et al. 2021), that leads to conformational changes in the structure of the NRF2-KEAP1 dimer complex. As a result, the binding of NRF2 to the KEAP1 dimer is altered: the binding of one molecule of KEAP1 to the low-affinity motif (DLG, latch motif) of NRF2 is broken, and NRF2 remains bound only to the second molecule of the KEAP1 dimer via the high-affinity motif (ETGE hinge motif). In this conformation, NRF2 ubiquitination is disrupted and the KEAP1 dimer is blocked from binding to other NRF2 molecules. This leads to blocking of KEAP1-dependent proteosomal degradation of NRF2 and, as a consequence, causes an increase in NRF2 levels in the cell cytoplasm due to *de novo* synthesized NRF2 proteins (Fig. 1). Subsequent enhanced transport of NRF2 from cytoplasm into the cell nucleus and its accumulation in the nucleus leads to activation of transcription of NRF2-regulated genes. Thus, the NRF2-Cul3-KEAP1 complex acts as a sensor of oxidative stress in cells.

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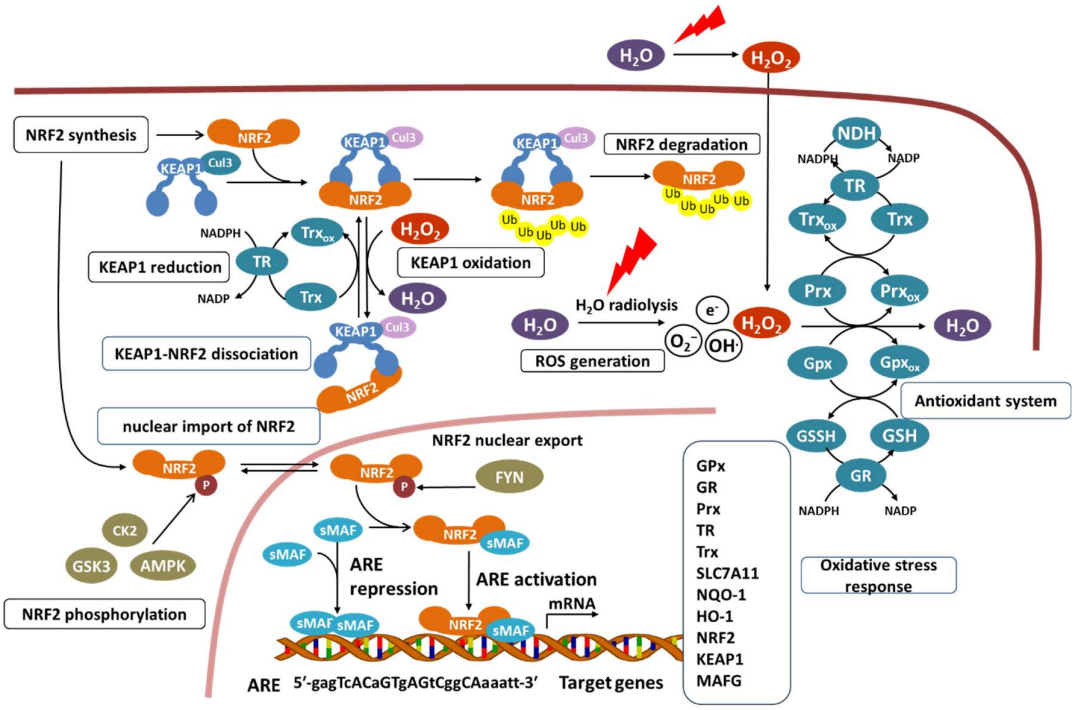

Figure 1. Schematic of the activation of the NRF2/KEAP1/ARE cellular antioxidant system in response to irradiation. The main subsystems of the NRF2 AOS are shown. The key NRF2 dependent antioxidant enzymes are listed in the frame: Gpx - glutathione peroxidase 2 and 4, GR-glutathione reductase, Prx - 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 is transported from cytoplasm to the nucleus and binds to small proteins of muscular aponeurotic fibrosarcoma (sMAF-F, sMAF-G, sMAF-K) to form a transcriptionally active NRF2-sMAF heterodimer, which, in turn, binds to the cis-acting antioxidant responsive element (ARE) in the promoters of the target genes (Suzuki and Yamamoto 2015) (see Fig. 1). Otherwise, formation of the sMAF-sMAF homodimer leads to the production of a repressor complex upon binding to the ARE sequence. Thus, the competition of NRF2 for binding to sMAF and the formation of activator (NRF2-sMAF) and repressor (sMAF-sMAF) complexes is other way in regulation of NRF2 AOS activation.

The NRF2-sMAF transcription complex controls expression and production of many antioxidant enzymes such as glutathione peroxidase (GPx), glutathione reductase (GR), thioredoxin (Trx), thioredoxin reductase (TR) as well as enzymes participating in the *de novo* synthesis of glutathione (GSH), which a reductant substrate of many antioxidant enzymes. The ARE sequences was found in the promoters of *NFE2L2* (coding NRF2), *sMAF,* and *KEAP1* genes that provides positive and negative feedbacks in the NRF2 AOS. During oxidative stress, NRF2-dependent expression of antioxidant enzymes leads to a fall in intracellular ROS level and reduction of KEAP1 that causes formation of the NRF2-Cul3-KEAP1 complex, resuming of NRF2 degradation, and then completion of the cellular response to oxidative stress (Fig. 1).

NRF2 activity is intricately regulated by its post-translational modification, in part, by phosphorylation by kinases from other signalling pathways (see scheme in Fig. 1). This regulation realizes a complex crosswalks of the HRF2 pathway with such pathways as PI3K/AKT/mTOR, RAS/ERK, AMPK, DNA repair pathway and others (Hayes and Dinkova-Kostova 2014). NRF2 is phosphorylated by casein kinase-2 (CK2) at multiple serine/threonine residues that produces an active form of NRF2 necessary for its nuclear translocation and transcriptional activity (Apopa, He, and Ma 2008). Other 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 may prevent NRF2 nuclear export that retains its transcriptional activity. GSK-3 (Glycogen synthase kinase 3) negatively

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regulates NRF2 by its phosphorylation at sites Ser344 and Ser347 that mediates the ubiquitination and proteasomal degradation of NRF2 independently from KEAP1 (Liu et al. 2021). FYN kinase also negatively regulates NRF2 by phosphorylation at Tyr568 in the nucleus causing NRF2 nuclear export followed by its degradation in the cytoplasm (Kaspar and Jaiswal 2011).

As is seen, the NRF2 AOS is a complex signalling system being a part of a bigger signalling network of cell fate regulation. It controls cell redox balance and includes a sensory subsystem, signal conversion subsystem, system of molecular controllers, and an execution system (Pic.1) . All subsystems are interconnected by numerous positive and negative feedbacks with signal amplification regime to provide fast and strict control of ROS homeostasis in cells. Thus, for effective therapeutic intervention on the redox state of cells by drug and radiation therapies, it is necessary a detailed understanding of the molecular mechanisms of functioning of the NRF2/KEAP1 AOS in various cancer cells. Along with experimental studies, theoretical investigation of the complex function and networked control of the NRF2/KEAP1 AOS were carried out by methods 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 Irradiation

A key mechanism of radiotherapy action is damaging of DNA of cancer cells by direct and indirect effects of ionizing radiation. More than 70% of DNA damages occur due to indirect processes through ROS generation during radiolysis of water in cells. When the ROS level increases in cells, the AOS controlled by NRF2 TF is activated. As shown in Fig. 1, an increase in the concentration of H_2O_2 leads to the oxidation of KEAP1 in the NRF2-KEAP1-CUL3 complex and partial dissociation of NRF2, that stops its degradation and increases its level in the cytoplasm. 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. The emergence of radioresistance induced by NRF2 AOS activation upon irradiation has been found in many cancer cell lines. In clinical trials, it has been shown that increased NRF2 expression in different cancer types contributes to radioresistance in patients and may be a prognostic marker of poor patient survival (Kawasaki et al. 2014).

The activation of the NRF2 AOS in cancer cells under radiation was experimentally established by the measurement of genes expression being under the control of NRF2 transcription factor. In the majority of the works, expression of *NQO1* (encoding NAD(P)H dehydrogenase), *HO-1* (haemoxygenase-1), *GST2* (glutathione S-transferase A2) and other genes, which are considered to be markers of NRF2 AOS activation, were measured (Singh et al. 2010). Also, an increase in transcriptional activity of NRF2 is detected by a change in ARE-luciferase reporter signalling (Tang et al. 2022). In a study of a role of NRF2 AOS activation in cancer radioresistance, we compared the responses of original cancer cells and gene-modified cells, in which either activation or blocking of NRF2 expression were modelled.

Investigation on the role of the NRF2 AOS in radioresistance 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 studied by the measurement of expression of several genes (*NQO1*, *GCL*, *GSR*, *TXN*, and *TXNRD1*) being under the control of NRF2. Inhibition of NRF2 expression by NRF2 small interfering RNA (siRNA) transfection of cells caused a decrease in the expression of these genes by about 50% when exposed to irradiation with a dose of 10 Gy. It resulted in a significant increase in ROS level in the transfected cells after 24 h post-irradiation, compared to that in control cells. Measurement of the dose dependence of clonogenic cell survival showed an increase in radiosensitivity of cells under inhibition of NRF2 expression siRNA transfection. In Fig. 2A and 2B, we provided the experimental dose dependencies of survival fraction $SF(D)$ for both cells and plotted approximating survival curves according to the equation of the linear-quadratic model (LQ): $SF(D) = e^{-(\alpha D+\beta\ ^2)}$, where D is the exposure dose, α and β – parameters of the LQ model (McMahon 2018). Calculation of the α/β ratio, which characterizes cell radiosensitivity, showed

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increased radiosensitivity of the NRF2 siRNA transfected cells A549-siRNA ($\alpha/\beta = 4.25$ Gy) compared to cells A549 with normal functioning NRF2 AOS ($\alpha/\beta = 0.15$ Gy) (see Table 1). Inhibition of NRF2 expression by NRF2 siRNA transfection in H460 cells (H460-siRNA) led to an increase in their radiosensitivity as compared with that of parental H460 cells (see Table 1).

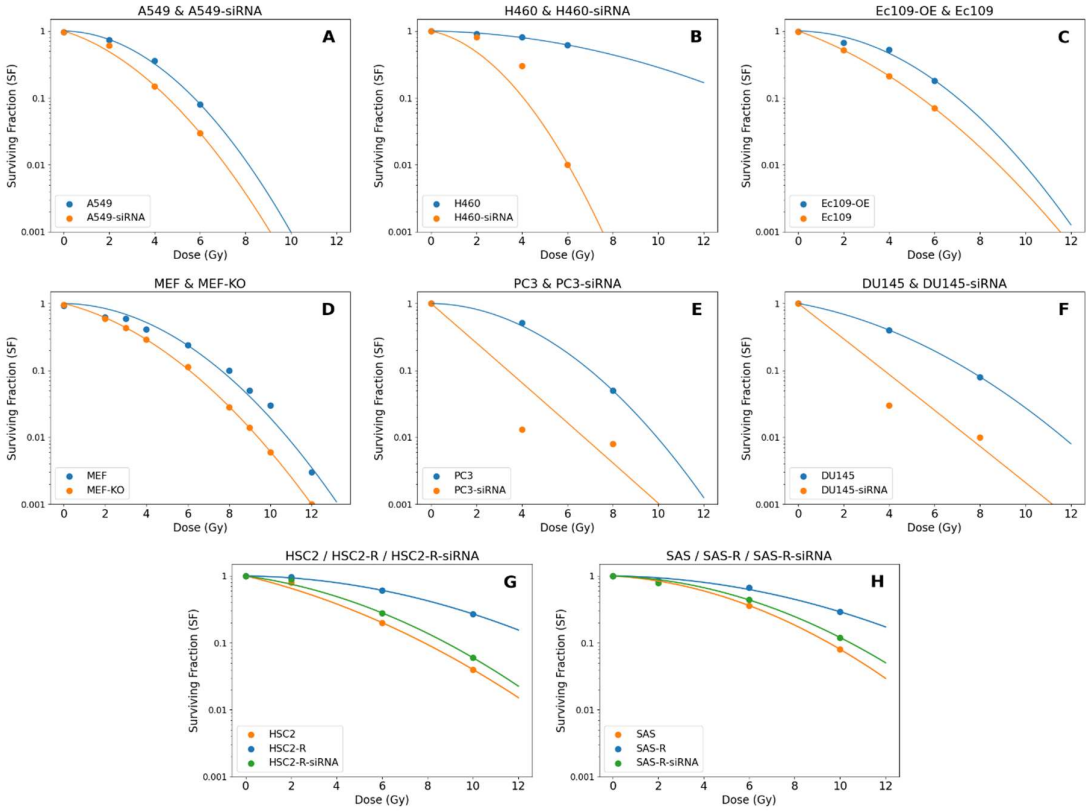

Figure 2. Changes in dose dependencies of clonogenic survival of cancer cells upon inhibition/activation of the NRF2 dependent antioxidant system. (A) Non-small cell lung cancer cells A549 and A549-siRNA; (B) H460 and H460-siRNA (Singh et al. 2010); (C) Ec109 and Ec109-OE esophageal squamous carcinoma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

Investigation of human embryonal (RD) and alveolar (RH30) rhabdomyosarcoma (RMS) cell lines showed an increase in ROS level (mainly superoxide anion in mitochondria), expression of NRF2 mRNA, and NRF2 dependent genes (*SOD-2*, *CAT* (catalase), *Gpx4*) that was detected immediately after irradiation of cells with proton beam at doses of 1 Gy - 5 Gy (Marampon et al. 2019). Activation of the NRF2 AOS in RMS cells followed by fast ROS elevation under irradiation was found to restore ROS level just lower than basal level in these cells. It means that the NRF2 AOS is highly sensitive to ROS accumulation and is able to bring back high ROS concentration which increased due to radiation action. The authors suggested that highly sensitive NRF2 dependent antioxidant response to radiation may be one of the protective mechanism responsible for acquire radioresistance of RMS tumours. It was found that lower expression of NRF2 dependent genes in RH30 cells compared with that in RD cells caused its higher radiosensitivity. Blocking of NRF2 expression upon transfection of cells with NRF2 siRNA resulted in a significant increase in the radiation-induced ROS level and enhanced radiosensitivity compared to cells with normal functioning NRF2 AOS. This finding demonstrated that radioresistance mechanism of RMS cells is related to their ability to overcome radiation-induced ROS by activation of the NRF2 AOS.

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Table 1. Coefficients α, β, and ratio α/β of the LQ model, obtained by approximation of dose dependencies of clonogenic cancer cells survival with active and/or suppressed NRF2 AOS (Fig.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 effect of increased NRF2 level on cell radiosensitivity was investigated in Ec109 and KYSE-30 esophageal squamous cell carcinoma (ESCC) cells in which a lentivirus vector carrying *NFE2L2* gene was transfected (Xia et al. 2020). Measured dose dependence of clonogenic cell survival showed that cells with overexpressed NRF2 (Ec109-OE and KYSE-30-OE) exhibited lower radiosensitivity (α/β=0.22 Gy for Ec109-OE cells) compared to parental cells (α/β=9.7 Gy for Ec109 cells) (Fig. 2C). Authors suggested that the mechanism of acquired radioresistance in cells with an increased level of NRF2 relates to high expression of Ca^{2+}/calmodulin dependent protein kinase (CaMKII), that was observed in these cells. NRF2-dependent expression of CaMKII is due to the occurrence of ARE sequence in the promoters of *CAMK2* genes. Expression of CaMKII leads to the autophagy activation that promotes degradation of ROS at their elevated levels. Survival analysis of ESCC patients showed that high level of NRF2 and CaMKII resulted in a significant decrease of patients' survival, and it might be a prognostic factor of severe outcome of the disease. Thus, radioresistance of esophageal squamous cell carcinoma cells may be associated with NRF2-dependent expression of CaMKII and activation of autophagy leading to degradation of ROS and protection of cancer cells from oxidative stress during radiation therapy.

A study of NRF2-dependent radioresistance has identified another mechanism of cancer cell death different from apoptosis, namely ferroptosis which is a programmed cell death due to lipid peroxidation (POL) of the cellular membranes in the presence of Fe^{2+} atoms (Feng et al. 2021). Immunohistochemical analysis of ESCC tissues from 127 patients revealed an increased level of NRF2 and NRF2-dependent cysteine/glutamate transporter protein (SLC7A11) which plays an important role in the GSH synthesis. Patients with a high level of NRF2 and SLC7A11 had lower overall survival and disease progression-free survival. To confirm the role of ferroptosis in cell death, experiments were performed on the ESCC cell lines KYSE30 and modified cells KYSE150, in which NRF2 was upregulated by gene modification. The results of cell irradiation experiments showed that the levels of ROS, POL, and ferroptosis were lower in the modified cells than in the parental cells. The radioresistance in ESCC cells was shown to be due to NRF2/SLC7A11-dependent inhibition of ferroptosis.

The effect of radiation on NRF2 AOS activation in non-cancerous cells was investigated in the experiments on mouse fibroblast cells (MEF) which showed a dose-dependent increase in ROS and ARE transcriptional activity on the fifth day after irradiation (McDonald et al. 2010). Measurement of

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the basal ROS level in cells showed an increased ROS level in NRF2 knockout cells (MEF-KO) cells compared to that in WT MEF cells. The action of irradiation with doses of 8 Gy and 10 Gy caused delayed 2-fold ROS rise, while MEF-KO cells showed a more than 10-fold ROS increase immediately after irradiation. Thus, blocking the NRF2 AOS in MEF-KO cells resulted in an uncontrolled radiation-induced ROS increase, whereas in WT MEF cells, the NRF2 AOS maintains a low ROS level during irradiation. In this work, five-day delayed expression of the markers of NRF2 AOS activation (HO-1 and GSTA2) was found. Measurement of dose dependence of clonogenic survival of cells showed that blocking of the NRF2 AOS in MEF-KO cells caused an increased radiosensitivity compared to that in WT MEF cells with normally functioning NRF2 AOS. Experimental dose dependence of cell survival of both cells and approximating curves according to the LQ model are shown in Fig. 2D. Calculation of the α/β ratio showed an increased radiosensitivity of MEF-KO cells (α/β=5.35 Gy) compared to WT MEF cells (α/β=0.26 Gy). Similar results were obtained for non-cancerous fibroblast and dendritic cell lines NIH-3T3 and DC2.4, respectively.

The effect of *KEAP1* and *NFE2L2* gene knockouts on the radiosensitivity of MEF cells was investigated (Singh et al. 2010). Dose dependence measurement showed a radiosensitivity increase in 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 nucleus and the NRF2 AOS activation that was confirmed by observation of an increase in the expression of genes under the control of NRF2 transcription factor. The results of dose dependence measurement showed a decrease in radiosensitivity of $KEAP^{-/-}$ knockout cells. Measurement of cell population growth revealed higher growth rate of $KEAP^{-/-}$ cells compared to that of the parental cells and $NRF2^{-/-}$ knockout cells. This was proposed to be due to a high degree of NRF2 AOS activation and a decrease of ROS in these cells.

High expression of NRF2 mRNA, NRF2, and its active phosphorylated form, pNRF2 was detected in radioresistant oral squamous cell carcinoma (OSCC) cells HSC2-R and SAS-R which were obtained by gradual exposure of HSC2 cells to increasing X-ray radiation doses of 0.5 Gy/day - 2 Gy/day (Matsuoka et al. 2022). Dose dependencies of clonogenic cell survival of HSC2 and HSC2-R cells are shown in Fig. 2E, and parameters of the LQ model are given in Table 1. Radiosensitivity of HSC2-R cells dropped dramatically (α/β=0.84 Gy) in comparison with that of parental cells HSC2 with α/β=13.8 Gy. Suppression of NRF2 expression in HSC2-R-siRNA cells was shown to sensitise resistant HSC2-R cells by increasing sensitivity to α/β=6.23 Gy.

Investigation of OSCC cells SAS showed a significant increase of radiosensitivity in the result of knockdown of *NFE2L2* gene (α/β= 2.27 Gy for SAS cells against α/β=9.68 Gy for SAS-siRNA cells, see Table 1) (Matsuoka et al. 2022). Knockdown of *NFE2L2* gene in SAS-R-siRNA cells did not cause a significant increase in their radiosensitivity (α/β= 0.88 Gy for SAS-R cells against α/β=1.47 Gy for SAS-R-siRNA cells, see Fig. 2F and Table 1). In contrast to 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 an active form, pNRF2 and in its nuclear localization were observed that indicated activation of NRF2 transcription factor.

In this research, immunohistochemical analysis of the biopsy specimens of patients with OSCC showed an increased level of pNRF2 after preoperative chemoradiotherapy. A population analysis of overall survival data revealed a lower survival of a cohort with high pNRF2 expression compared to that in patients with low pNRF2 level. Progression free survival data showed that high pNRF2 level may be a prognostic marker of poor 5-year survival in patients with OSCC (Matsuoka et al. 2022).

An increased NRF2 expression after irradiation was also detected in glioma tissues of patients using immunohistochemical analysis (Tang et al. 2022). This was accompanied by a high level of hypoxia protein HIF-1α. *In vitro* experiments confirmed the increased level of NRF2 during the action of radiation with doses of 1 Gy - 8 Gy on human glioma cell lines U251 and U87 grown under hypoxic conditions. Dose dependence of *NFE2L2* expression measured by ARE-luciferase reporter signal was measured on the fourth day after irradiation in cells grown under both hypoxic and normoxic

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conditions. Knockout of the *NFE2L2* gene resulted in increasing radiosensitivity in the cells compared to that in parental cells that correlated with a significant increase in ROS level, a drop in a level of NRF2-dependent enzymes (NQO1 and NO1), and GSH biosynthesis in transfected cells. Note, at present, there is no explanation of the molecular mechanism of the detected delay of NRF2 activation under irradiation in a number of cancer cell lines.

The role of cellular redox status and the NRF2 dependent AOS in the radiosensitivity of prostate cancer cell lines PC3 and DU145 was detailed investigated using genomic, proteomics and phenotypic approaches (Jayakumar et al. 2014). Analysis of clonogenic survival assays showed that both cells possess different radiosensitivity (α/β=10.34 Gy for PC3 and α/β=6.58 Gy, see Figs. 2G, 3H and Table 1). (Note, that three experimental points on the dose dependence of PC3 and DU145 cells in Figs. 2G and 3H are not enough to provide reliable values of α/β according to the LQ model). Investigation of DNA damage in cells under irradiation showed an increased magnitude of DNA damage and faster DNA damage repair kinetics in DU145 cells in comparison to that in PC3 cells. PC3 cells showed higher basal and radiation-induced ROS levels, while DU145 cells had an elevated basal GSH level and a higher GSH/GSSG ratio that contributed to their reduced oxidative stress and weak radiosensitivity. Increased activation of the NRF2 AOS and its downstream antioxidant genes (HO1, GCLC, TXRD1, and others) in DU145 cells compared to that in PC3 cells was observed. Based on these results, authors assumed that the different status of NRF2 transcription factor activity determines different radiosensitive phenotypes of these cells. To confirm this assumption, authors investigated clonogenic survival of DU145 and PC3 cells, in which NRF2 expression was knocked down by transfection of cells with short hairpin RNA (DU145-shRNA and PC3-shRNA). Blocking of NRF2 transcription factor caused significant reduction of transfected cell survival (see Fig. 2G and Fig. 2H). Note, that dose dependencies of DU145-shRNA and PC3-shRNA cells with knocked down for NRF2 are not described by LQ model. Approximation of the experimental dose dependencies by the LQ model gave zero values of β that led to a straight-line approximation (Figs. 3G and 3H). It can be assumed that inhibition of the NRF2 AOS in these cells leads to hypersensitivity to radiation at doses less 2 Gy. The detailed measurement of the dose dependence is needed to investigate in detail this behaviour at low doses.

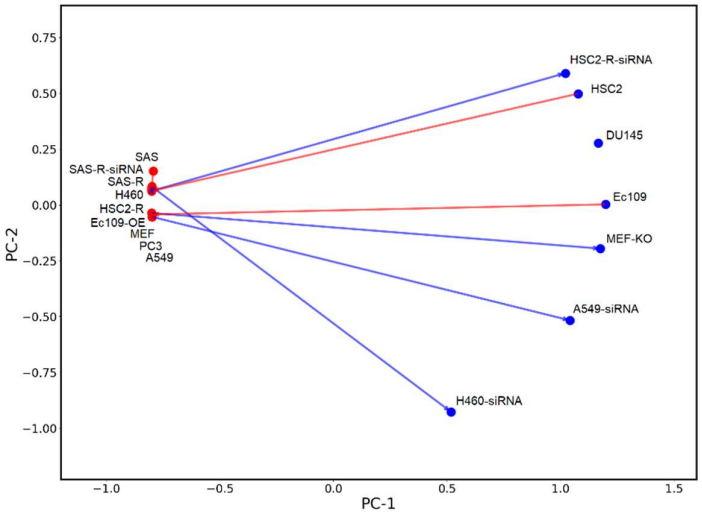

Figure 3. Clustering of cancer cells according their radiosensitivity represented on the principal components PC-1 and PC-2 axes. Claster of radioresistant cancer cell lines with inhibition of the NRF2 AOS (red dots) and cluster of their radiosensitive parental or transfected cells (red dots) are showed. 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.

The 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.

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The results of the classification of radiosensitive and radioresistant cells with normal and suppressed functioning of the NRF2 AOS are shown in Fig. 3, where blue and red points indicate radiosensitive and radioresistant cells, respectively. Arrows point out the transitions of the cells from the radioresistant to radiosensitive clusters upon inhibition of the NRF2 AOS. Additionally, we carried out hierarchical clustering of the selected cancer cells on their parameters of the LQ model (α/β and α) (Fig. 4) and obtained the same clusters of radioresistant and radiosensitive cells as by using PCA clustering (Fig. 3).

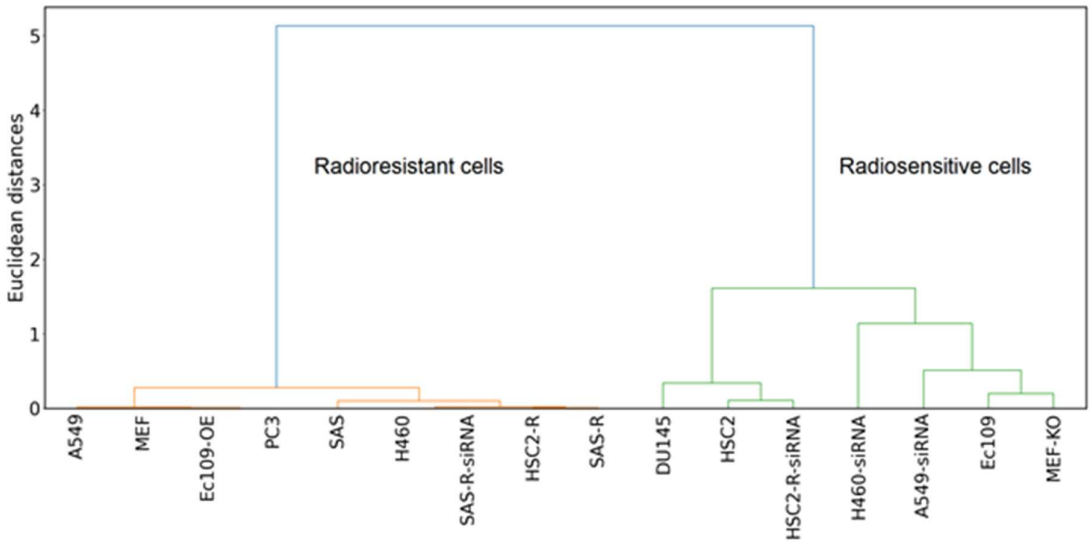

Figure 4. Dendrogram of agglomerative hierarchical clustering of radioresistance (left arm) and radiosensitive (right arm) cancer cells with normal or modulated NRF2 AOS.

It is worth to note an unresolved problem of NRF2 activation that was mentioned above. This relates to the different time of activation of NRF2 and the start of ARE-dependent gene expression after irradiation. In various cancer cells, discussed above, NRF2 activation was observed immediately after irradiation. For example, NRF2 activation in rhabdomyosarcoma cell lines was detected directly after irradiation (Marampon et al. 2019). In contrast, in the experiments on MEF cells, an increase in ROS level and ARE transcriptional activity were registered on the fifth day after irradiation (McDonald et al. 2010). Radiation-induced NRF2 activation in NSCLC cell lines H1299 and A549 cells was observed within 6 h after irradiation and persisted from day 2 to day 4 in H1299 and until day 2 in A549 (Lee et al. 2012). This significant delay was suggested to relate to the delayed ROS generation after irradiation, the mechanism of which is unknown and likely related to cyclical events of cell death and pro-inflammation cytokine production (McDonald et al. 2010). It was also proposed that radiation-mediated NRF2 activation is cyclic process which starts with irradiation (Lee et al. 2012). Note, oscillation regime in NRF2 activation was observed in human vascular endothelial HMEC-1 cells stimulated by sulforaphane that can explain persistent NRF2 activity (Xue et al. 2015). Based on literature data reviewed here, we can mention that the delayed radiation-induced NRF2 activation was found in radioresistant cells such as A549, H1299, and MEF cells, while radiosensitive MEF-KO cells with NRF2 knockout showed a more than 10-fold ROS increase immediately after irradiation (McDonald et al. 2010)

To summarize experimental findings discussed above in this section, we schematically presented several scenarios of the radiation-induced responses of cancer cells having different levels of basal ROS and activity of the NRF2 AOS (Fig. 5). Curve 1 in Fig. 5 shows an increase of ROS level over the basal ROS one and above the NRF2 activation threshold that leads to radiation-induced oxidative stress in cancer cells. Activation of the NRF2 AOS and upregulation of the antioxidant enzymes' expression result in suppression of oxidative stress and restoration of the basal ROS level. The strong antioxidant capacity enables the cells to overcome radiation-induced oxidative stress,

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mitigates therapeutic radiation effect and leads to weak radiosensitivity of cancer cells. In the above discussion, this scenario can be applied to prostate cancer cell line

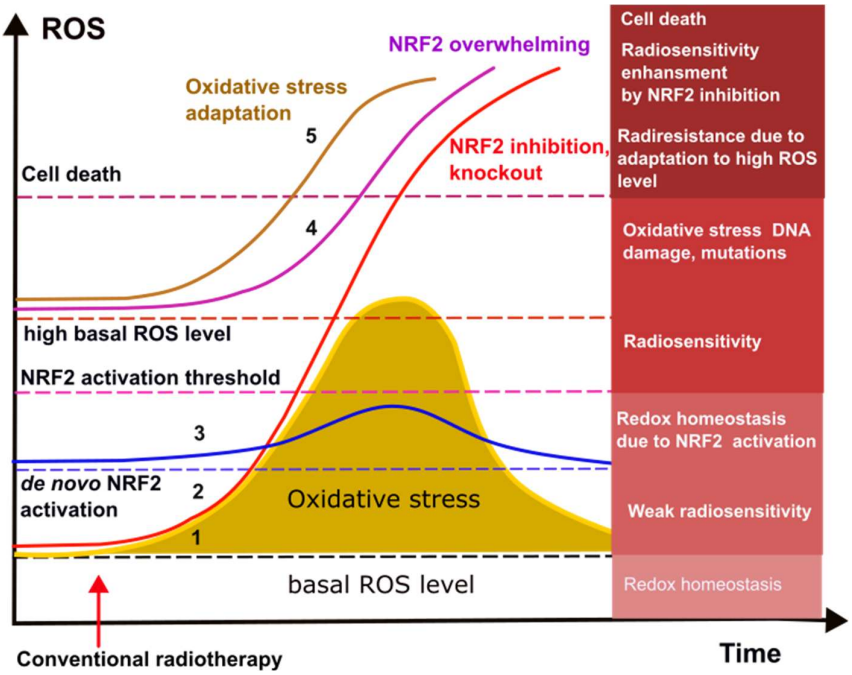

Figure 5. Schematic representation of the radiation-induced ROS dynamics in cancer cells with different basal ROS and NRF2 activity levels. Curve 1 (yellow) corresponds to the suppression of the radiation-induced oxidative stress in cancer cells with a low basal ROS level and strong antioxidant capacity that results in low radiosensitivity. Curve 2 (red) displays an uncontrolled ROS rise in cancer cells under inhibition of the NRF2 AOS that leads to high radiosensitivity. Curve 3 (blue) corresponds to mitigation of radiation-induced oxidative stress in cancer cells with *de novo* NRF2 AOS activation due to KEAP1 mutations or *NFE2L2* overexpression that causes low radiosensitivity. Curve 4 (mafgenta) represents a scenario when cancer cells have a high basal ROS level that leads to an increased radiation-induced ROS level and high radiosensitivity. Curve 5 (brown) shows a case of cancer cell adaptation to a high level of ROS that leads to radioresistance.

DU145 (Jayakumar et al. 2014) and rhabdomyosarcoma RMS cells (Marampon et al. 2019) which show a strong antioxidant response and weak radiosensitivity. Curve 2 in Fig. 5 corresponds to the case of the NRF2 AOS inhibition leading to the radiation-induced uncontrolled ROS rise causing high radiosensitivity. In all cancer cell lines, discussed above, the NRF2 AOS inhibition by knockout of the *NFE2L2* gene resulted in a significant increase in ROS level and high radiosensitivity. Curve 3 represents the case of cancer cells with *de novo* NRF2 AOS activation due to *KEAP1* mutations or *NFE2L2* overexpression, when radiation-induced oxidative stress is suppressed by active NRF2 AOS. An example of this case may be OSCC cells HSC2 which exhibit high expression of NRF2, NRF2 mRNA, pNRF2, and a radioresistant phenotype (Matsuoka et al. 2022). ESCC cells with overexpressed NRF2 (Ec109-OE and KYSE-30-OE) also showed lower radiosensitivity (Xia et al. 2020). The reduced level of KEAP1 may be the reasons for a high basal level of NRF2 in DU145 cells possessing radioresistivity (Jayakumar et al. 2014). Curve 4 in Fig. 5 corresponds a scenario when cancer cells have a high basal ROS level that leads to the increasing radiation-induced ROS level and high radiosensitivity. We proposed that this scenario is realized due to overwhelming cellular antioxidant capacity and a limited response to oxidative stress by the NRF2 AOS. This case may be represented by prostate cancer cell line PC3 with high radiosensitivity (α/β=10.34 Gy, see Table 1) which showed high basal and radiation-induced ROS levels (Jayakumar et al. 2014). We also added case 5 in Fig. 5 which corresponds to cancer cells which adapt to a high level of ROS and resist to radiotherapy. An example of this case may be a radioresistance phenotype of prostate cancer cell PC-

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3 cells which are characterized by high aggressiveness, increased cellular proliferation, and invasive potential (Sideri et al. 2022). It was proposed that radioresistant PC-3 cells adapt to oxidative stress due to increased capability to repair DNA double-strand breaks caused by ROS.

4. NRF2 Activation as a Factor of Radioresistance

The analysis of experimental and clinical studies carried out in the previous section showed that ionizing radiation induces activation of NRF2 AOS in numerous cancer cell lines leading to attenuation of their radiosensitivity. At the same time, it was found that blocking of the NRF2 transcription factor can reversibly enhance radiosensitivity. Given these findings, in this section we analyse results of the studies on radiosensitivity of various cancer cells and tumour tissues, in which genetic mutations or epigenetic modifications in NRF2 AOS were found to lead to NRF2 AOS activation and tumour adaptation to radiation-induced oxidative stress.

An increased basal expression of NRF2 transcription factor was detected in DU145 prostate carcinoma cells (Jayakumar et al. 2014). Analysis of clonogenic cell survival data at cell irradiation with doses of 4 Gy and 8 Gy showed low radiosensitivity of DU145 cells that correlated with less damage of DNA compared to radiosensitive PC3 cells and its lower level of NRF2. Examination of cellular redox status displayed a low basal level of ROS in DU145 cells and its reduced level upon irradiation. These findings associated with the observation of increased expression levels of *NFE2L2* gene and NRF2-dependent genes (*GCLC*, *HO1*, and *TXRD1*), which were significantly higher in radioresistant DU145 cells than in radiosensitive PC3 cells. Also, DU145 cells exhibited higher basal GSH level and GSH/GSSG ratio than PC3 cells that correlated with high level of GCLC (glutamate-cysteine ligase catalytic subunit) participating in GSH synthesis. Measurement of a basal level of KEAP1, which regulates NRF2 degradation (Fig. 1), showed its lower level in DU145 cells than that in PC3 cells, and irradiation of the cells further reduced KEAP1 level. The study showed that it possible to suppress the radioresistance of DU145 cells by either knockout of the *NFE2L2* gene or using NRF2 or HO1 inhibitors. Authors concluded that activation of the NRF2 AOS in the prostate carcinoma cells caused by the high expression of NRF2 and low level of KEAP1 led to an enhanced recovery status of cells under irradiation and conferring their radioresistance.

A thorough investigation of the role of mutations within the NRF2/KEAP1 system in the development of radioresistance has been carried out in squamous cell lung cancer (SCLC) (Jeong et al. 2017). Mutations in this system were found in 30% of the patients under investigation who were found to have a high degree of tumour aggressiveness with a high risk of recurrence and distant metastases after radiation therapy (Jeong et al. 2017). The role of these mutations was investigated in P-LSCC cells with *Trp53* gene deletion and double deletion of *Keap1* and *Trp53* genes (K/P-LSCC) in mouse cancer which were found to closely resemble poorly differentiated squamous carcinoma cells by their histology. Experiments were performed *in vitro* on a population of these cells and in *in vivo* experiments in mice with tumours grown by transplantation of these cells. Increased activation of NRF2 transcription factor was observed in K/P-LSCC cells compared to that in P-LSCC cells. This was accompanied by an increased level of NRF2-dependent enzymes and decreased ROS level. Also, cells with *Keap1* and *Trp53* gene deletion (K/P-LSCC) were characterized by high proliferation and metastasis rate. Clonogenic analysis of cell survival under the radiation with doses of 1 Gy-10 Gy showed improved radioresistance of K/P-LSCC cells when compared to P-LSCC cells. At the same time, significantly less damage of DNA was detected in cells with *Keap1* deletion. These results were confirmed by *in vivo* experiments which showed a significant attenuation of the irradiation damage effect at a dose of 6 Gy on K/P-LSCC tumour growth in mice than with P-LSCC tumours.

In the same study (Jeong et al. 2017), a method to suppress radioresistance in SCLC cells with *KEAP1* gene deletion was proposed. K/P-LSCC cells showed increased expression of the cystine/glutamate transporter gene *SLC7A11* which, as previously discussed, is under the control of NRF2 and encodes a protein that plays an important role in the GSH synthesis pathway. Inhibition of the cysteine import system by sulfasalazine resulted in sensitization of K/P-LSCC cells to ionizing radiation and restoration of radiosensitivity inherent to the parental cells.

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Based on the results obtained in *in vitro* and *in vivo* experiments, clinical trials were performed with 42 patients at stages I-III of prostate carcinoma who underwent radiation therapy (Jeong et al. 2017). Patients with mutations in the Kelch domain of *KEAP1* gene, which is responsible for NRF2 binding, were selected. In this group, localized relapses were reported in 70% of patients compared to 18% of patients in the group without mutations in the NRF2/KEAP1 AOS. In order to non-invasively identify patients with *KEAP1* mutations, a circulating tumour DNA (ctDNA) blood test was performed, and patients with *KEAP1* mutations were successfully identified. The results of radiation therapy in this group of patients showed a significantly high recurrence rate.

The clinical significance of the NRF2/KEAP1 system in oncotherapy is demonstrated by the high frequency of mutations in *NFE2L2* and *KEAP1* genes found in cells and tissues of various types of cancer. It has been shown that NRF2/KEAP1 mutations leading to NRF2 AOS activation in cancer cells caused *de novo* resistance of cells to some chemotherapy drugs (cisplatin, etoposide, paclitaxel, bortezomib, gemcitabine, 5-fluorouracil, etc.) which therapeutic mechanism, in particular, link to ROS generation with subsequent DNA damage. It has been also demonstrated that radioresistance can be successfully suppressed by inhibition of the NRF2/KEAP1 AOS. Based on the results presented in this review, it can be hypothesized that various mutations in the NRF2/KEAP1 AOS found in cancer cells and tissues also significantly contribute to tumour adaptation to ionizing radiation and radioresistance that can be suppressed by blocking of the NRF2/KEAP1 AOS with drug intervention. Below we summarize the main types of mutations in the NRF2/KEAP1 system found in various cancers.

The main molecular mechanisms for the increased expression of NRF2 transcription factor in cancer cells are as follows: somatic mutations of *KEAP1* or *NFE2L2*; epigenetic modifications of the *KEAP1* gene leading to suppression of its expression; microRNA expression that regulates *NFE2L2* and *KEAP1* genes at the post-translational level; and increased expression and accumulation of proteins which compete with NRF2 for binding with KEAP1 (Fabrizio et al. 2018), (Sparaneo et al. 2025).

Somatic mutations in *KEAP1* were initially discovered in lung tumour tissue samples (19%) and lung cell lines (50%) which are the second most common and important genetic alterations in lung cancer (Singh et al. 2006). Also, *KEAP1* mutations have been found in other human cancers such as ovarian cancer (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). These mutations were found in several *KEAP1* domains, resulting in its inactivation and NRF2 accumulation in the nucleus of cancer cells and leading to activation of the NRF2/KEAP1 AOS.

The most common type of genetic alterations affecting the function of the KEAP1-NRF2 complex in solid tumours are the point mutations with loss of heterozygosity. They usually occur in exon regions that encode Kelch domains in KEAP1, which are responsible for the binding of KEAP1 with NRF2, and in the IVR and BTB domains, which contain cysteine bases and are responsible for the redox regulation of NRF2. Also, these mutations cause a general failure of KEAP1-mediated NRF2 ubiquitination. High expression of NRF2-dependent AOS enzymes and resistance to chemotherapy drugs have been observed in NSCLC cells with these mutations (Singh et al. 2006).

Somatic mutations of *NFE2L2* occur mainly in either the ETGE (57%) or DLG (43%) motifs that lead to dissociation of the KEAP1-NRF2 complex. When mutations in the ETGE motif take place, the high-affinity interaction between KEAP1 and NRF2 is destroyed, whereas mutations in the DLG motif result in the destruction of the low-affinity interaction. These mutations were identified in tissue samples of patients with papillary renal cell carcinoma with activation of the KEAP1/NRF2 AOS (Ooi et al. 2013). Deletion mutations in the *CUL3* ubiquitin ligase gene were also found that lead to complete loss of the enzyme function causing inhibition of NRF2 degradation, its accumulation, and activation of the KEAP1/NRF2 AOS.

An increased prevalence of *NFE2L2* mutations in endometrial carcinoma as compared to healthy cells was detected by immunohistochemical analysis of numerous tissue samples from malignant and benign tumours (Jiang et al. 2010). Further study based on endometrial carcinoma cells showed their

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resistance to chemotherapeutic drugs (cisplatin and paclitaxel) which induce oxidative stress in cells. Inhibition of NRF2 resulted in a reduction of tumour size in mice with xenotransplantation of endometrial carcinoma cancer cells under the effect of chemotherapeutic drugs.

Note, NRF2 overexpression in KEAP1 mutant human NSCLC cell lines was detrimental to cell proliferation, viability, and anchorage-independent colony formation. Collectively, these results established the context dependence and activity threshold for NRF2 during the lung tumorigenic process (DeBlasi et al. 2023).

An additional mechanism of NRF2 transcription factor activation is an increase of NRF2 expression due to the coupling of the NRF2 AOS with other cellular signalling pathways that are activated due to oncomutations (Khalil et al. 2016), (Tao et al. 2014). For example, in lung carcinoma tissues, activation of *NFE2L2* gene in 20-30% of cases is associated with activation of MAPK proliferation signalling pathway by mutation in *KRAS* oncogene leading to chemoresistance (Tao et al. 2014). In xenograft tumour models of NSCLC cancers with *KRAS* gene mutation, it was shown that NRF2 activation was due to KRAS-induced increase in *NFE2L2* transcription. This resulted in increased resistance to cisplatin. The action of the NRF2 inhibitor, brusatol led to suppression of drug resistance.

In addition to somatic mutations in *NFE2L2*, *KEAP1,* and *CUL3* genes leading to disruption of the interaction of NRF2 with KEAP1, other activator of NRF2 was found that acts by similar mechanism. p21 protein, a cyclin-dependent kinase inhibitor binds to the DLG motif of NRF2 and impedes two-site binding of NRF2 to KEAP1, causing NRF2 accumulation in the cell nucleus and, as a consequence, enhancing cell survival under oxidative stress (Chen et al. 2009).

Another mechanism of KEAP1/NRF2 AOS activation relates to epigenetic modifications, such as aberrant methylation of the *KEAP1* gene promoter (Fabrizio et al. 2018). It was found that epigenetic modifications are the most common causes of the suppression of *KEAP1* gene expression in solid tumours. *KEAP1* hypermethylation confers a growth advantage to cancer cells and correlates with poor clinical prognosis in cancer patients with this abnormality (Copple 2012b). Epigenetic modifications in *KEAP1* gene were found in 51% of patients with early stages of lung cancer and an increased risk of disease progression after surgical tumour removal (Fabrizio et al. 2018). A group of patients with estrogen- and HER2-negative status was found to have the highest risk of recurrence after chemotherapy with a relative risk value of HR=14.73 (Barbano et al. 2013). Epigenetic modulation of *KEAP1* has also been shown to be one of the leading mechanisms of KEAP1 deregulation (48.6%) in renal cancer patients and can be considered as a predictive marker of patient survival (Fabrizio et al. 2018). Further, increased methylation of the *KEAP1* gene promoter in glioma cells was detected 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 the group of patients with methylated *KEAP1* than for the group with unmethylated *KEAP1* gene. Note, that the observation of the increased radiosensitivity of glioma in patients with NRF2 activation at low *KEAP1* expression is not consistent with the above discussed data that demonstrated a decrease in radiosensitivity when increased NRF2 expression in cancerous cells and tissue samples. It can be assumed that the mechanism of radiosensitivity increase is related to the peculiarities of NRF2/KEAP1 AOS activation in glioma cells which are predominantly 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 and, as a consequence, leads to radioresistance of cancer cells. As demonstrated in the previous section, the activation of the NRF2/KEAP1 AOS can occur as a result of both *de novo* mutations in the NRF2/KEAP1 signalling system during carcinogenesis and as a protective cellular response to radiation-induced oxidative stress. Increased activity of the NRF2/KEAP1 AOS, found in many types of cancer cells, suggests

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strong dependence of some cancers on functioning of the cell antioxidant system. NRF2-dependent cancers with NRF2, KEAP1, and CUL3 mutations exhibit high therapeutic resistance and these mutations are 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 greatly increases with radiation therapy causing generation of ROS in the tumour environment. The intensive antioxidant response of cancer cells is directed to neutralize ROS that allows cancer cells to minimize the damage caused by radiation. As shown in the previous section, a large amount of experimental on the dose-dependent activation of the NRF2/KEAP1 AOS and increased expression level of NRF2 under radiation has been accumulated to date. This allows considering the NRF2/KEAP1 AOS as a promising potential target of therapeutic intervention in combination with radiation therapy. Currently, NRF2 transcription factor is 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). Uniqueness of NRF2 transcription factor as against other transcription factors is that NRF2 is considered as a target for action both inhibitors and activators depending on diseases and cell redox status. NRF2 along with other transcription factors is treated as a promising candidate for novel therapeutic strategies in combining with radiotherapy to aim at suppression of radioresistance (Galeaz, Totis, and Bisio 2021).

Success of targeted therapy in clinical oncology is determined by the inhibitory effect of drugs on certain cellular signalling pathways that are abnormally activated in cancers due to mutations in oncogenes and onco-suppressor genes. Targeted therapies have achieved great success in combinations with other types of oncotherapies. Given the large number of examples of tumours showing aberrant activation of NRF2 transcription factor, NRF2 should be considered as an important pharmacological target for drug therapy. Clinical application of targeted therapy aiming at the inhibition of the NRF2/KEAP1 AOS in combination with radiotherapy appears to be a promising strategy to enhance radiosensitivity of malignancies. Radiosensitivity enhancement by inhibition of the NRF2/KEAP1 AOS in tumour cells is determined by chronic exceeding of a basal ROS level in a number of cancer cells and by extra generation of ROS under the action of ionizing radiation during radiotherapy (see Fig. 5). Additionally, blocking of NRF2 transcription factor results in repression of *NFE2L2* gene and NRF2-dependent genes that control various metabolic and signalling pathways of cancer cell survival, 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 cellular pathways further leads to increased radiosensitivity through inhibition of cancer cell growth and enhanced cancer stem cell differentiation.

Currently, the different subsystems of the NRF2/KEAP1 AOS are considered as druggable targets and is being actively investigated (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 diseases associated with the progress of oxidative stress, including cancer. Many drugs activating NRF2 transcription factor have already been approved by the FDA for clinical use (Lu et al. 2016). In contrast to NRF2 activators, relatively few effective inhibitors with high specificity towards the NRF2/KEAP1 AOS have been developed. Currently, the development of NRF2 inhibitors is at the stage of preclinical and clinical trials (Telkoparan-Akillilar et al. 2021). However, given the large data conforming an important role of NRF2 activation and mutations in the NRF2/KEAP1 AOS in carcinogenesis and cancer therapeutic resistance, the development of effective strategies to block the NRF2/KEAP1 AOS at different levels of the complex regulation of this signalling pathway is underway.

A large group of NRF2 transcription factor inhibitors are either natural or synthetic compounds. Natural NRF2 inhibitors include compounds such as flavonoids (apegeni, luteoli), alkaloids (halofuginone, trigonelline, and barberine), quassinoids (brusatol), and others (triptolidine, gensenoside) extracted from plants (Pouremamali et al. 2022), (Zhang et al. 2023). Although no NRF2-targeted drugs have been approved for cancer treatment by the FDA, the results of clinical trials on targeting NRF2 inhibition have shown promising results for these inhibitors to suppress tumour

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growth and enhance sensitivity to anticancer drugs by chronically increasing ROS in tumours in 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 showed their potentiality as enhancers of radiosensitivity of cancer cells. For example, proposed compound 4-(2-Cyclohexylethoxy) aniline (IM3829) was found to inhibit the increase in NRF2 activity and expression of the NRF2 dependent genes induced by radiation (Lee et al. 2012). Importantly, combination of NRF2 inhibitor IM3829 with radiation exerted a synergetic effect, i.e. IM3829 and radiation separately did not cause noticeable effect on neither apoptosis, nor ROS level, nor tumour growth. Yet the combination of IM3829 with radiation synergistically increased apoptosis, ROS level, and decreased tumour volume in H1299 or A549 lung cancer xenografts compared with control. Authors suggested that NRF2 inhibitor IM3829 plays a role of radiosensitizer that overcomes radioresistance by promoting ROS accumulation by blocking NRG2-dependent antioxidant responses. Note, the synergetic effect of the drugs targeting NRF2 is one of the benefits of this combination strategy in radiotherapy. Another advantage of these drugs is that they affect only cancer cells having high level of active NRF2 induced by radiation. Thus, these drugs act locally in the irradiation area without side effects on healthy tissue.

Many anticancer drugs used in the clinic, in addition to their direct therapeutic effects, were found to exert inhibitory effects on the NRF2 AOS, making cells vulnerable to oxidative stress (El-Naggar et al. 2019). The indirect therapeutic effects of known drugs were suggested to possess the additional mechanisms of action on cancer cells through NRF2 inhibition. For example, various chemotherapeutic drugs were shown to inhibit the NRF2 AOS. Drugs such as temozolomide-1 and homoharringtonin (elongation inhibitor) suppress NRF2 expression at the transcription level and effectively increase sensitivity of lung and bladder cancer cells to chemotherapeutic drugs (El-Naggar et al. 2019).

Personalized drug therapy has the ability to suppress activity of NRF2 transcription factor at different levels of the NRF2 AOS signalling pathway. Anticancer drug entinostat, which is a histone deacetylation inhibitor, inhibited NRF2 synthesis at the translation level by acetylating and reducing the activity of YB-1 protein that is involved in NRF2 mRNA translation (El-Naggar et al. 2019) and exhibits high activity in sarcoma cells. This additional therapeutic effect of the drug induced oxidative stress in sarcoma cells *in vitro* and significantly suppressed sarcoma metastasis *in vivo*. Also, the antitumor drug omipalisib (a dual PI3K/mTOR inhibitor) inhibited NRF2 synthesis by suppressing NRF2 mRNA translation and exerted therapeutic effects in gastric cancer, sarcoma, and osteosarcoma. The dual-action PI3K-DNA PK inhibitor, PIK-75, induced NRF2 degradation and helped to overcome the resistance of pancreatic cancer to gemcitobin (Telkoparan-Akillilar et al. 2021). The Bcl-2 inhibitor, venetoslax suppressed increased expression of NRF2 caused by *NFE2L2* gene demethylation and induced ROS generation in mitochondria that led to apoptosis in myeloid leukaemia cells. Suppression of NRF2 transcription factor activity at the epigenetic level is induced by targeted therapy drugs, trastuzumab and pertuzumab (HER2 receptor inhibitors). Their combined action caused methylation of the *NFE2L2* promoter that led to epigenetic inhibition of *NFE2L2* expression with subsequent generation of ROS and suppression of glutathione synthesis (Khalil et al. 2016).

Clinical application of NRF2 targeted therapy for radiation therapy requires identification of cancer types, for which this combination will be most effective. The potential candidates for this combined therapy are mainly malignant tumours exhibiting dependency on the NRF2 AOS that is manifested by increased NRF2 transcription factor activity and acquisition of radioresistance. Primarily, this should include cancer with mutations in *NFE2L2*, *KEAP1*, and *CUL3* genes as well as with epigenetic modifications in *NFE2L2* leading to NRF2 activation.

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6. Conclusions

A large number of experimental and clinical studies has shown that the NRF2/KEAP1 antioxidant system of cancer cells is one of the potential targets for personalized therapy combined with radiation therapy. Synergetic effect of the combination of two therapies is proposed to be determined by addiction of cancer cell to the NRF2/KEAP1 AOS that causes the high frequency of mutations in the NRF2/KEAP1 AOS in cancer leading to radioresistance. An A strong antioxidant response of cancer cells upon activation of NRF2/KEAP1 AOS enhances ROS degradation and allows cells to adapt to oxidative stress when exposed to ionizing radiation. The adaptive cell response leads to a weakening of the radiosensitivity of cancer cells. When the NRF2 AOS is inhibited, the antioxidant protection of cancer cells is suppressed that causes an uncontrolled excess of ROS above the threshold level, leading to the development of oxidative stress and apoptosis of cancer cells. The development of therapeutic strategies to block the activation of the NRF2/KEAP1 AOS during radiotherapy can enhance effectiveness of personalized radiation therapy for a large number of cancer patients.

To determine the effectiveness of radiotherapy in combination with this targeted therapy, further studies are needed to confirm association of the mutations and epigenetic modifications in the NRF2/KEAP1 AOC with radioresistance. Experimental and clinical studies in this direction will allow understanding the NRF2-dependent mechanism of radioresistance and subsequently selecting the appropriate groups of patients who will benefit by personalized combination radiotherapy. In this case, fractionation regimens with an increased radiation dose may be defined for the selected groups of patients during radiotherapy. Most promising strategy will be searching for types of cancer and cancer cells displaying synergetic effect between inhibitions of the NRF2/KEAP1 AOS and radiotherapy, that will allows defining the most vulnerable cancers to this combined therapy.

Another promising direction in the development of this type of combined radiotherapy is the identification of critical targets for effective inhibition of the NRF2 AOS. As shown in this review, many signalling proteins functioning in the various subsystems of the NRF2 AOS can be therapeutic targets. At the same time, blocking of the NRF2 AOS can be implemented both at the proteomic level ether at the genetic or epigenetic levels. A specific target for therapeutic action should be selected based on molecular and genetic data on disturbances in the NRF2 AOS, leading to its high activity in various types of cancer. This will also allow development of various methods for personalized radiosensitization of tumours in patients with certain mutations in the NRF2 AOS.

The efficacy of a combined action of radiotherapy with suppression of the NRF2/KEAP1 antioxidant system significantly depends on the activation status of the antioxidant system and its response to ionizing radiation in various types of cancer tumours. Inclusion of proteomic and genomic data on mutations and activation of the NRF2/KEAP1 AOS in further research and clinical trials can be used to select the best option for personalized combination radiotherapy and to determine groups of patients with NRF2-dependent mechanism of radioresistance.

Intensive experimental and clinical studies are currently underway in these areas that indicates undoubted potential for the development of effective combination radiotherapy with blocking of the NRF2/KEAP1 antioxidant system of cancer cells in order to enhance efficacy of personalized radiotherapy.

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