

Reactive Oxygen Species Reduction is a Key Underlying Mechanism of Drug Resistance in Cancer Chemotherapy

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Abstract

Reactive Oxygen Species (ROS) management in cancer cells is important for developing successful therapy. Most of the anticancer agents induce ROS generation to kill cancer cells by apoptosis through common molecular pathways. But prolonged treatment with the drug reduces ROS level to confer resistance. Subsequently, drug resistant cells have lower ROS content than drug sensitive cancer cells. Anticancer drugs induce master regulatory genes and these genes act on NFE2L2-KEAP1 antioxidant system to reduce the ROS level in cancer cells. This review focused on the genetic mechanism of drug mediated induction of ROS generation in sensitive cells and the ROS reduction in drug resistant cancer cells.

Keywords: Cancer; ROS; Drug resistance; NFE2L2; Chemotherapy

Introduction

Drug resistance is a devastating problem in cancer chemotherapy because drug resistant cancer cells are harder to kill with the same drug. Despite initial high response rate, a large proportion of patients develop resistance to the drug causing relapse of the disease. This multifaceted problem could be attributed to various factors, mainly on MDR (Multiple Drug Resistance) activation, tissue heterogeneity (including stem cells), microenvironmental context, abnormal mitochondrial respiration, vascularization, DNA repair and apoptosis [1].

The cellular signaling leading to these molecular processes depends on redox signaling in the cell. Reactive oxygen species (ROS) with highly reactive oxygen atom reacts with DNA, amino acids of proteins and unsaturated fatty acids leading to oxidation of these biomolecules. ROS can lead to oxidation [2] of amino acid residues of side chains, formation of protein-protein cross-linkages, and oxidation of the protein backbone resulting in protein fragmentation. These oxidized proteins, in turn, modify normal protein functions, have profound effects in cellular signaling, and create oxidative stresses in the cell, thus compel cells to adopt altered molecular pathways.

Understanding redox regulation and its role in developing drug resistance in cancer cells are immensely important to overcome chemotherapeutic challenges. Modulation of ROS is not only a prerequisite for tumor development but also a measure of drug resistance. Critical ROS level could also be a marker of drug efficacy that would determine the progress of drug response in cancer patients [3].

ROS management in tumorigenesis

Positive ROS signaling is a necessary prerequisite for the development of tumors [4,5]. Cancer cells always have higher ROS content than normal cells [6]. Cancer cells are often found to harbor mitochondrial mutations and consequently abnormal mitochondrial respirations are a general cause for increased ROS generation [7]. Other sources of ROS generation such as from plasma membrane and peroxisomes are not extensively studied in relation to cancers except for peroxiredoxins where *prx^{-/-}* mice develop tumors at many sites and act as tumor suppressor genes [8]. Higher ROS content in cancer cells is necessary to induce tumor development. Therefore one can assume that oxidative stress inducing genes have inductive roles on oncogenes

to induce tumor formation.

An alternative hypothesis suggests that oncogenes increase NFE2L2 (NRF2) expression to activate the antioxidant system for reducing ROS and detoxifying cells to induce tumorigenesis in mice [9]. They showed that oncogenes, K-Ras, B-Raf and c-Myc overexpression increase NFE2L2 expression that subsequently detoxifies cells from increased ROS level and induces tumorigenicity.

Several factors should be considered before explaining this apparent conflict between the ROS generation or reduction during tumorigenesis. The effect of only three oncogenes on an increase of NFE2L2 expression is not sufficient to assume that overall reduction of ROS level certainly induces tumor development [9]. In human lung cancer, several somatic mutations in KEAP1 are identified, although the functions of these individual mutations are not studied [10]. It is believed that these mutations abolish KEAP1 binding with NFE2L2 and free NFE2L2 could activate the antioxidant system to decrease the ROS level in lung cancer but the direct evidences for such an assumption is not verified. Unfortunately, *Keap1^{-/-}* mice expressed NFE2L2 constitutively but did not live more than 21 days, making it impossible to assess its direct role in tumorigenesis [11]. In contrast to oncogenes, tumor suppressor genes, such as p53 downregulation induces ROS generation during tumorigenesis [12] and is also shown to harbor numerous mutations in many cancers [13,14]. Thus, p53 mutation supports the view that positive ROS signaling is necessary for tumorigenesis.

Additionally, no germline mutations in NFE2L2 or KEAP1 have been identified in any cancers that would justify the notion that an increase in antioxidants facilitates tumor development. It is believed that cancer stem cells or normal cells when becoming cancerous do need

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Received March 12, 2012; Accepted April 10, 2012; Published April 12, 2012

Citation: Maiti AK (2012) Reactive Oxygen Species Reduction is a Key Underlying Mechanism of Drug Resistance in Cancer Chemotherapy. Chemotherapy 1:104. doi:10.4172/2167-7700.1000104

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increased ROS signaling [5,15], but mature tumor cells cannot sustain excessive ROS generation due to abnormal mitochondrial mutations in cancer cells [7,16]. Any cells, including cancer cells are vulnerable to excess ROS, thus somatic mutations in NFE2L2 or KEAP1 genes observed in cancer cells activate antioxidant systems to reduce the ROS level that actually helps tumor progression, but may not initiate tumor development. Importantly, several NFE2L2 mutations are also observed in lung cancer patients (11/103 patients) and are believed to help tumor progression [10]. It has also been proposed that excess ROS, generated by cancer cells, itself drives cancer cells from their primary site towards the bloodstream and is a molecular basis for metastasis [17]. This assumes that the adherence properties of cancer cells are reduced in the presence of excessive ROS signaling and ROS mediated physiological changes help to prepare them to move from primary sites. Although extensive experimental research is needed to establish this, it appears that tumorigenesis depends on a critical level of ROS in a dose dependant manner but not solely on a decrease or increase of ROS level that activates antioxidant systems or/and metastasis [18,19].

Most anticancer drugs induce ROS generation through common molecular pathways leading to apoptosis

Most of the anticancer agents or drugs initially induce ROS generation to kill cancer cells by apoptosis [15,20,21]. A detailed description of anticancer agent mediated ROS generation has been outlined earlier [22]. However, it is unclear how these anticancer agents influence cellular mechanisms to generate ROS. Direct relationships between drugs and antioxidant modulating genes/proteins are yet to be established. Although it is not known whether ROS generation is the only way to induce cancer cell death, it obviously plays a major role in inducing apoptosis. It is evident that various anticancer agents induce ROS in various cancers activating different genes although the pathways of these genes are studied discretely. But it is not clear whether they lead to the same pathways involving the same set of genes that could induce apoptosis. Evidence suggests that ROS induces a set of genes that are known to induce apoptosis in cancer cells. For example, in breast cancer, rotenone activates ERK1/2, JUN and MAPK8 [23], but sulforaphane inhibits hTERT [24]. Dithiophene induces IL24 in pancreatic cancer cells to activate apoptosis [25]. Similarly, in ovarian cancer cells, chlorambucil and cisplatin activate NFKB and p53 [26] whereas CDDO-ME (C-28 methyl ester derivative methyl-2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oate) downregulates NFE2L2 [27]. PI3K, CFLAR, MAPK8, ERK1/2, PRKDC, p53, NFKB1 and RB1 are the principle genes those are identified to be modulated in various anticancer drug mediated ROS generation [22,28,29]. IPA analysis (Ingenuity Pathway Analysis; www.ingenuity.com) showed that they could activate apoptotic genes, Casapases (CASPs), FADD/MORT and cytochrome C (Figure 1). This ROS-apoptosis model explains that various anticancer agents in most of the cancer cells induce apoptosis through ROS generation in common pathways.

ROS reduction is a general mechanism of drug resistance

Although most of these anticancer drugs induce apoptosis through ROS generation, prolonged treatment with the same drug reduces the ROS level in cancer cells [26]. Thus, drug resistant cells have lower ROS content than the drug sensitive cells and addition of exogenous ROS in conjunction with the drug resensitizes drug resistant cells to sensitive cells. A ROS management cycle in cancer cells could be established, which demonstrates these events (Figure 2). However, it is not known whether a reduced ROS level makes the cancer cells resistant or if resistant cells reduce ROS level in the cell. That is whether

ROS reduction is a primary phenomenon for prolonged treatment with anticancer drug.

Evidence suggests that drug resistant cells have a higher expression of catalase at the plasma membrane that could reduce some of the ROS level [30]. Recent observations also indicate that overexpression of NFE2L2, that mechanistically should reduce ROS, actually confers resistance to lung and ovarian epithelial cancer for platinum based drugs [31,32]. Depletion of glutathione-S-transferase (GSH), an antioxidant producing enzyme, through phenyl isothiocyanate (PEITC) induces apoptosis in MCF7 breast cancer cells [33]. In fibrosarcoma cells, p21 mediated apoptosis could be blocked by overexpression of catalase at the mitochondria of these cells [34]. APEX1, a DNA transcription factor and DNA repair gene, confers drug resistance through ROS and MDR activation [35]. Recently, Li et al. [36] showed that APE1 regulates mitochondrial membrane potential and ROS production after photodynamic therapy of lung cancer cells and induces apoptosis. Thus, evidence is accumulating that the reduced ROS level could be the primary reason for acquired drug resistance in various cancers.

The molecular mechanism of ROS reduction

NFE2L2-KEAP1 is the most potent antioxidant regulatory system that reduces ROS level in cancer cells. NFE2L2, a transcription factor, remains bound in cytoplasm with another protein called KEAP1 (Figure 3). Oxidative stress releases NFE2L2 from the KEAP1 complex [37]. Free NFE2L2 is phosphorylated and travels to the nucleus to bind at the Antioxidant Responsive Element (ARE) which is located at the promoter of a series of antioxidant genes and activates transcription. The minimum sequence requirement for ARE is 5'-gagTcACaGTgAGtCggCAaaatt-3' or TMAAnRTGAYnnnGCRwww or TGA(C/T)nnnGCA [38,39] and the presence of two or more copies of the ARE in close proximity to each other often serves as a bona fide ARE [40]. Increase of NFE2L2 in the nucleus facilitates transcription of antioxidant genes, such as catalase (CAT) or glutathione -S-transferase (GST) that reduces ROS level in the cell [41].

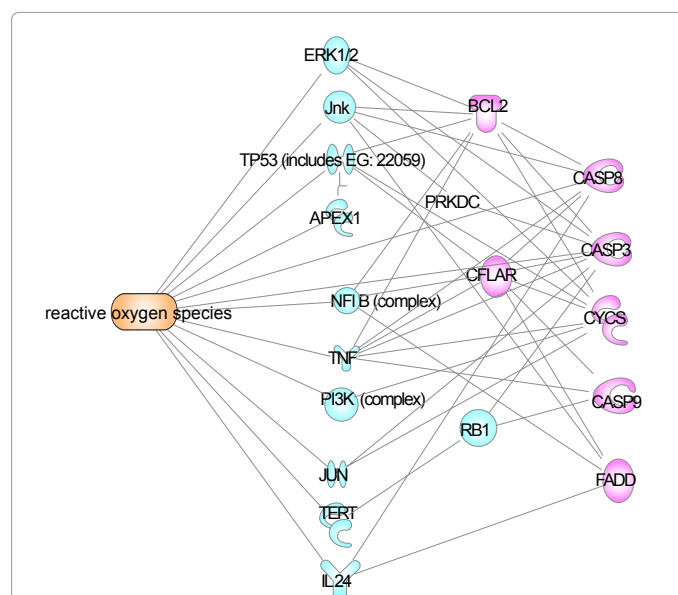
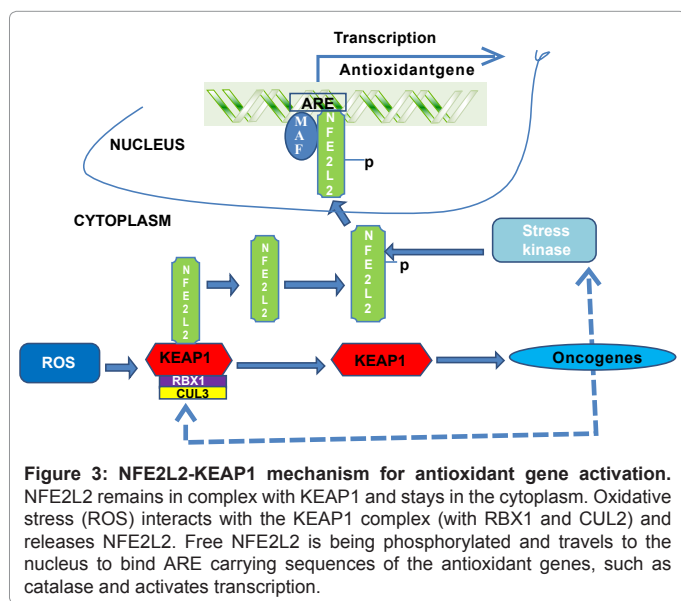
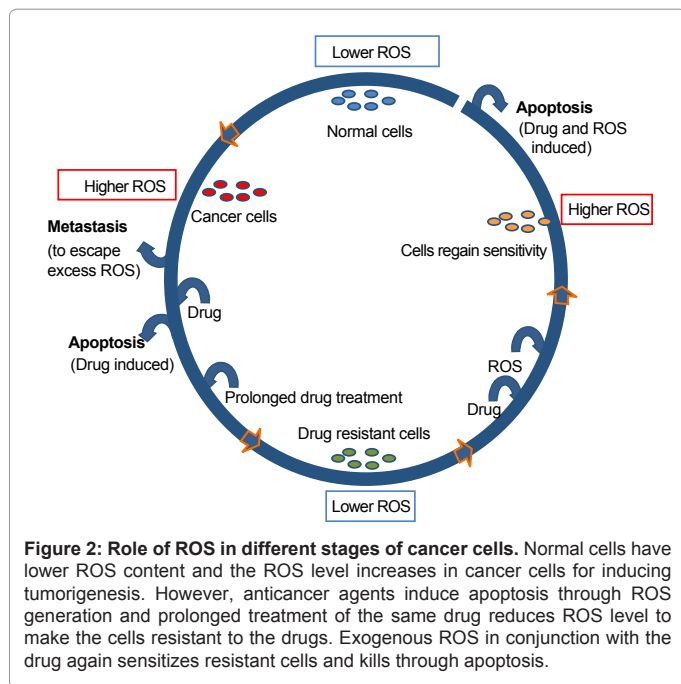


Figure 1: ROS inducing genes induce apoptosis in common pathways. In various cancer cells with various anticancer agents, ROS induces a set of master regulatory genes which in turn act on apoptotic genes, thus inducing apoptosis through common pathways. Sky blue-master regulatory genes, pink-apoptotic genes, orange-small molecules.



Master regulatory genes could act on upstream of NFE2L2-KEAP1 system to activate antioxidant system

Similar set of genes, such as ARHGEF6, MAPK8, p53, CYR61, PRKDC, CDK6 and others [23,26,28,29] those induce apoptosis through ROS generation are also involved to modulate the antioxidant system through NFE2L2-KEAP1 and reduce ROS. Some of them are common (p53, PRKDC, MAPK8 etc) and are believed to regulate NFE2L2 and KEAP1 expression or phosphorylation in positive or negative way thus play dual role in apoptosis or ROS reduction. IPA analysis with these master regulatory genes suggests that they could modulate NFE2L2-KEAP1 expression (Figure 4). Therefore these genes are not the target of NFE2L2. ARHGEF6, a prominent gene that is identified in drug resistance in ovarian cancer [26], could interact with NFE2L2 through MAPK8, implying MAPK8 could cause phosphorylation of NFE2L2

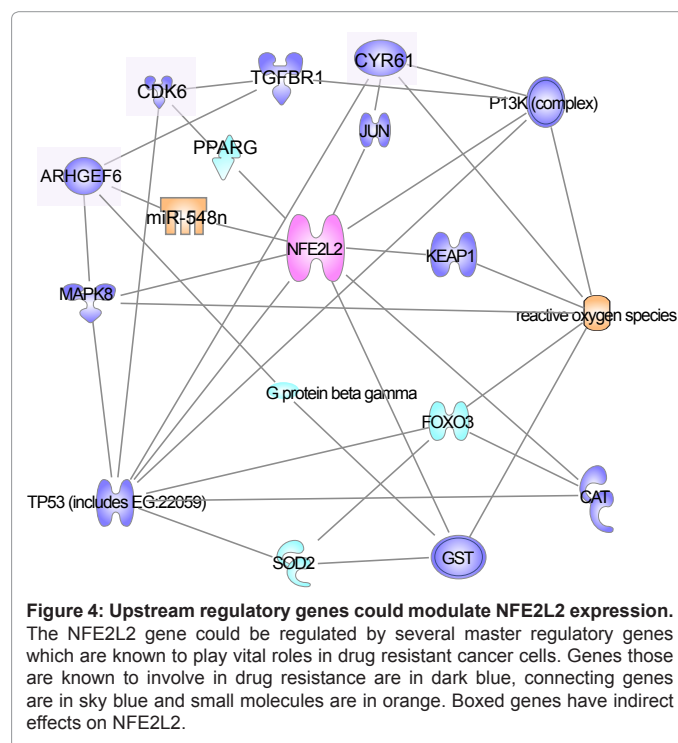
allowing it to travel to the nucleus [42]. Increased CAT expression could also reduce ROS level to make cancer cells drug resistant, which is observed by Bechtel and Bayer [30]. Similarly, CDKs are also predicted to be involved in phosphorylating NFE2L2 [31], although the specific role of CDK6 in phosphorylating NFE2L2 has not been demonstrated. However, CYR61, a ROS inducing angiogenic gene could also modulate NFE2L2 expression through the oncogene, JUN. Although direct relationships between p53 and NFE2L2 have not been demonstrated, p53 acts on MAPK8 and CDKs (Cyclin Dependent Kinase), which are essentially needed to phosphorylate NFE2L2, thus having an indirect role in NFE2L2 functions [43]. It has been also suggested that p53 and NFE2L2 act on different pathways in neuroblastoma cells and their activities depend on the type of oxidative stress a cell faces, such as diamide or H₂O₂ [29,44]. Diamide activates the NFE2L2 antioxidant system that protects the cell from oxidative stress, but p53 induces apoptosis when exposed to H₂O₂.

Master regulatory genes could also act on Non-ARE containing antioxidant genes to reduce ROS

However, all antioxidant genes do not possess ARE sequences at their promoters, and are not regulated by the NFE2L2-KEAP1 system. The extensive networking suggests that anticancer agents inducing master regulatory genes could also interact with other antioxidant modulatory genes (Figure 5). Here we observe that MAPK8 directly regulates peroxiredoxins (PRDX5) and CAT, ARHGEF6 interacts with SOD2 through PAK2, APEX1 interacts with PRDX6 and the NFKB complex interacts with many antioxidant producing/regulatory genes such as SOD1, PRDX2, PRDX4, GPX4, SOD2, CAT etc. Thus, the ROS regulation system in cancer cells is not limited by only NFE2L2-KEAP1 antioxidant system and master genes could also regulate non-ARE containing genes to reduce total cellular ROS in the cell.

Advanced chemotherapy by modulation of ROS

Modulation of ROS in combination with the drug is a useful strategy



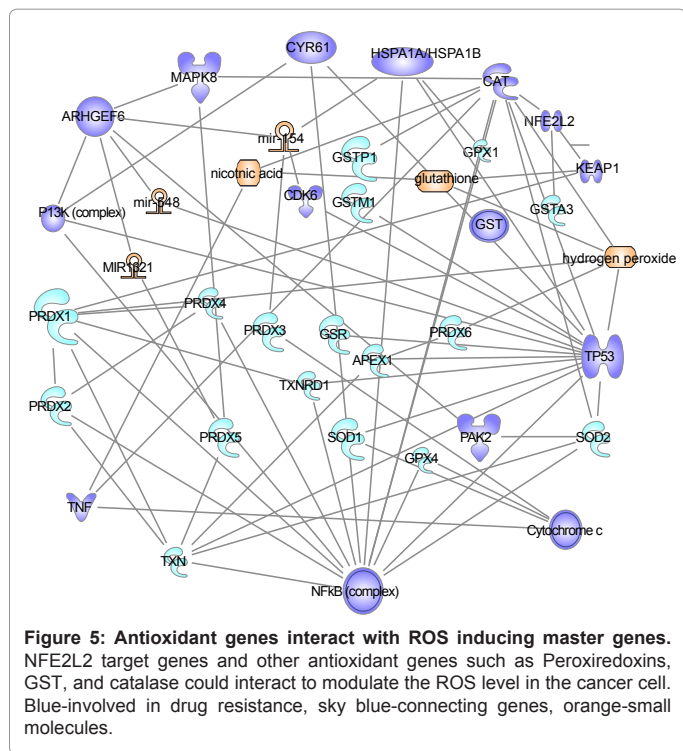


Figure 5: Antioxidant genes interact with ROS inducing master genes. NFE2L2 target genes and other antioxidant genes such as Peroxiredoxins, GST, and catalase could interact to modulate the ROS level in the cancer cell. Blue-involved in drug resistance, sky blue-connecting genes, orange-small molecules.

for ‘combinational chemotherapy’. ROS level could be modulated in several ways:

- i) by targeting mitochondrial ROS generation and maintenance, such as, optimal concentration of catalase inhibitor, 3-aminotriazole (3-AT) could increase ROS level in the cell [30]. Copper chelating complex, casiopeinas induce mitochondrial damage and increase ROS generation in lung cancer cells, eventually lead to apoptosis [45].
- ii) by modulating ROS regulatory genes, such as, NFE2L2 or KEAP1 have significant impact in modulating ROS level, thus could be helpful for overcoming drug resistance. The function of these target proteins could be impaired by designing or screening small molecules. SiRNA or triplex oligo mediated gene silencing could also be useful for knocking down antioxidant genes that would elevate ROS level in the cell. Efficient delivery of oligo through lipoplexes and polyplexes has recently been developed to be useful for efficient gene silencing [46]. Systematic knockdown of KEAP1 by siRNA confers resistance to carboplatin treated epithelial ovarian cancer cells [32]. However, it is not known whether KEAP1 downregulation overcomes complete resistance in these cells. It would be worthwhile to modulate other antioxidant regulatory genes upstream of NFE2L2 such as ARHGEF6, p53, MAPK8, CDK6 or CYR61 to overcome complete drug resistance in cancer cells. It would also be useful for combined manipulation of several genes that would be necessary for overcoming complete resistance in drug treated cancer cells. As most cancers share common pathways for antioxidant regulation to induce apoptosis, these strategies could be useful for developing advanced chemotherapy for many cancers.
- iii) by direct delivery of ROS into tumor cells through designing nanoparticles conjugated with ROS generating enzymes that

increases ROS level in cancer cells and induces apoptosis [47].

In summary, I discussed here that tumorigenesis depends on the level of ROS in the cell and that positive ROS signaling is necessary during initial tumor development. Advanced tumor cells try to lower ROS level through the NFE2L2-KEAP1 antioxidant system. Anticancer agents induce apoptosis in most of the cancer cells through excess ROS generation by activating a few master genes which act upstream of antioxidant system maintaining genes, such as NFE2L2-KEAP1. Prolonged treatment with the same drug reduces ROS level in resistant cells and confer resistance to the drug. A set of master regulatory genes controls the ROS level in drug resistance cancer cells and reduces the ROS level to confer drug resistance. Manipulating these master regulatory genes could help to overcome drug resistance in most of the cancer cells.

Acknowledgement

I thank Ms. Beth Mikkola for critically reading this manuscript. I thank reviewers for their helpful comments to improve the manuscript.

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