Measurement of sensitivity to DNA damaging agents

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Cover illustration: Measuring patient (blood lymphocyte) sensitivity to DNA damaging agents using Cell division assay

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The Lord is my strength and my shield; my heart trusts in Him.

Psalm 28:7

ABSTRACT

There is a large inter-individual variation in intrinsic sensitivity in patients receiving treatment with DNA damaging agents. Cancer therapy exemplifies this problem where patients experience varying degree of normal tissue side effects in response to radiation or chemotherapy. For this reason, it is necessary to develop an assay to predict sensitivity of a patient prior to treatment with DNA damaging agents. This may allow more individualized treatment and improve the therapeutic index. In paper I and II we focused on developing and validating a flow cytometry - based cell division assay (CD) that uses the thymidine analogue EdU (5-ethynyl-2'-deoxyuridine) to measure the proliferative ability after DNA damaging treatment. In paper I, the CD assay measured sensitivity to radiation of human skin fibroblasts with a correlation similar to the standard clonogenic survival assay in a relatively short time frame. Using the easily sampled peripheral blood lymphocytes, the CD assay found variation in intrinsic sensitivity to radiation and detected increased sensitivity in patients with DNA repair defects. In paper II, the CD assay was further validated for measurement of cell sensitivity to DNA damaging drugs. The results indicated that the assay can be used to identify sensitive patients.

Exposure to ionizing radiation generates free radicals that carry out most part of the toxic effects. The cellular antioxidant system regulated by the Nrf2 transcription factor plays a key role in protecting cells against radical induced damage; hence in paper III we have investigated if pretreating cells with Nrf2 activators influence the sensitivity to radiation. Results from paper III demonstrated that repeated treatment using the isothiocyanate sulforaphane protected human skin fibroblasts from toxic effects of ionizing radiation in an Nrf2-dependent manner. In paper IV we found that repeated pretreatment of cells with Nrf2 activators, sulforaphane or synthetic triterpenoid bardoxolone methyl trained the cells to acquire resistance against higher toxic concentrations of both drugs. Together these results indicate that repeated stimulation of Nrf2 system can enhance cytoprotection and that adaptation to stress may be a general feature of the Nrf2 response mechanism.

Keywords: Intrinsic sensitivity, DNA damage, ionizing radiation, cell division, Nrf2, sulforaphane, bardoxolone methyl, cytoprotection

SAMMANFATTNING PÅ SVENSKA

Det finns en stor individuell variation i inneboende känslighet mellan patienter som får behandling med DNA-skadande ämnen. Ett exempel är strålning, där en del av patienterna upplever allvarliga biverkningar trots att behandlingen är samma för alla. Det är därför nödvändigt att identifiera patienter som är extra känsliga innan behandling. Detta kan förbättra behandlingen i resterande patientgrupp samtidigt som allvarliga biverkningar kan undvikas hos känsliga patienter. I artikel I och II har vi fokuserat på att utveckla och validera en flödescytometrisk celldelningsmetod (CD), som använder thymidin-analogen EdU (5-ethynyl-2'-deoxyuridine) för att mäta cellens proliferativa svar på DNA-skadande ämnen. I artikel I mätte CD-metoden strålkänslighet hos humana fibroblaster från hud på relativt kort tid, jämfört med standardmetoden clonogenic assay. Genom att använda lymfocyter, hittade CD-metoden variationer i inneboende känslighet mot strålning och detekterade ökad känslighet hos patienter med defekter i DNA-reparationssystemen. Resultaten från artikel II indikerar att CD-assayn kan mäta patient-känslighet för ämnen som krosslinkar till DNA och kan användas för att identifiera känsliga patienter i en population.

Det cellulära oxidationssystemet som regleras av transkriptionsfaktorn Nrf2 spelar en nyckelroll för att skydda celler mot skadliga fria radikaler. I artikel III och IV fokuserade vi på att studera det Nrf2-medierade cellulära svaret mot toxiska utmaningar. Våra resultat från artikel III demonstrerade att upprepade korta stimuleringar med Nrf2-aktivatorn sulforafan skyddar humana fibroblaster mot joniserande strålning. Denna studie indikerar att Nrf2-systemet kan tränas för att förbättra cellöverlevnaden. I artikel IV demonstrerade vi att upprepade exponeringar med olika Nrf2-aktivatorer, som sulforafan och CDDO-metylester, gjorde att cellerna blev korsresistenta och mer motståndskraftiga mot denna stress. Detta indikerar att anpassning till stress kan vara en generell mekanism bakom Nrf2-systemet.

LIST OF PAPERS

This thesis is based on the following research studies, referred to in the text by their Roman numerals.

I. Sherin T. Mathew*, Pegah Johansson*, Yue Gao, Anders Fasth, Torben Ek, Ola Hammarsten. **A flow cytometry assay that measures cellular sensitivity to DNA-damaging agents, customized for clinical routine laboratories**, Clinical Biochemistry, 2016, 49:566–572.

* equal contribution

II. Pegah Johansson, Sherin T Mathew, Michaela Johansson, Ola Hammarsten. **Validation of Cell division (CD) assay in measuring sensitivity of Fanconi Anemia cells.** Manuscript, 2017

III. Sherin T. Mathew, Petra Bergstrom, Ola Hammarsten. **Repeated Nrf2 stimulation using sulforaphane protects fibroblasts from ionizing radiation**, Toxicology and applied pharmacology, 2014, 276: 188-194.

IV. Sherin T. Mathew, Ola Hammarsten. **Preconditioning cells with sulforaphane or bardoxolone methyl induces adaptation and cross-adaptation in human skin fibroblasts.** Manuscript, 2017

CONTENT

ABBREVIATIONS

AP site	Apurinic/apyramidinic sites
ARE	Antioxidant response element
AT	Ataxia telangiectasia
ATM	Ataxia telangiectasia mutated
ATR	Ataxia Telangiectasia and Rad3-related protein
BARD	Bardoxolone methyl
BRCA1	Breast cancer type 1 susceptibility protein
CD assay	Cell division assay
CdK	Cyclin-dependent kinase
Chk	Checkpoint kinase
DDR	DNA damage response
DMSO	Dimethyl sulfoxide
DNA-PK	DNA dependent protein kinase
DSBs	Double-Strand Breaks
EBV	Epstein Barr Virus
EdU	5-ethynyl-2′-deoxyuridine
FA	Fanconi anemia
H2AX	Histone 2A variant X
HO-1	Heme oxygenase 1
H2DCFDA	2′, 7′-dichlorodihydrofluorescein diacetate

HR	Homologous recombination
ICLs	Interstrand cross links
IR	Ionizing Radiation
Keap1	Kelch–like ECH-associated protein 1
LET	Linear Energy Transfer
LigIV	DNA ligase IV
MEFs	Mouse embryonic fibroblasts
MRN	Mre11-Rad50-Nbs1/Nibrin complex
NHEJ	Non-homologous end joining
NQO1	NAD(P)H:quinone oxidoreductase 1
Nrf2	Nuclear factor erythroid 2- related factor 2 (Nrf2)
NTCP	Normal tissue complication probability
p53	Tumor protein 53
PIKK	Phosphatidylinositol-3-OH kinase-like kinases
qPCR	Quantitative PCR
RBE	Relative biological effectiveness
ROS	Reactive oxygen species
SNP	Single nucleotide polymorphism
SSBs	Single-Strand Breaks
SF	Sulforaphane
TLS	Translesion synthesis

TCP	Tumor control probability
XRCC4	X-ray cross-complementing 4
XLF/Cernunnos	XRCC4-like factor

1 INTRODUCTION

Preserving DNA integrity is critical for normal cellular function and survival. DNA damage inflicted by endogenous and exogenous sources may trigger cellular responses including cell cycle arrest, DNA repair and cell death. But in cancer treatment DNA is targeted and DNA damaging agents are used to induce damage and cell death in tumor cells. This often cause varying levels of normal tissue toxicity in patients, possibly due to individual variation in the intrinsic ability to cope with the DNA damage [1]. However in clinical settings, the intrinsic sensitivity of a patient to DNA damaging agents is not identified and the treatment doses are set to limit adverse toxicity in sensitive patients. As a consequence, majority of the patients who will not develop severe toxicities with higher doses may be undertreated. A predictive assay to detect intrinsic sensitivity of patients in clinical practice is therefore of great importance as it may contribute to increase tumor control in non-sensitive patients while preventing severe toxicity in sensitive patients.

Our cells are continuously exposed to free radicals that are generated during intracellular processes and cells respond to these free radicals by increasing their antioxidant capacity mainly through the Nrf2 transcription factor. As ionizing radiation is known to exhibit most part of cytotoxicity through free radical generation [2], Nrf2 mediated cellular antioxidant system is therefore likely to be involved in maintaining the redox balance after radiation exposure. Nrf2 can be activated by thiol rich chemicals and the cytoprotective effect induced by different Nrf2 activators has been reported [3]. However, relatively little is known about how the Nrf2 signaling system can be tuned to enhance the cellular adaptive ability. It is therefore important to see if pre-activation of Nrf2 influences cellular adaptation to toxic challenges like radiation. Furthermore, the studies on adaptive resistance induced by different Nrf2 activators may open more insights on Nrf2 mediated cytoprotection.

The above topics are discussed in detail in the following sections.

1.1 DNA - damaging agents

Our genomic DNA is constantly encountering lesions occurring as a result of endogenous metabolic reactions, enzymatic conversions and replication errors or from exogenous sources like radiation, tobacco smoke and a wide range of chemicals and chemotherapeutic agents. These DNA lesions may affect fundamental processes and may result in cytotoxicity, gene mutations or genomic instability [4].

1.1.1 Endogenous agents

Although oxygen is important for aerobic life, the chemical nature of oxygen makes it prone to generate free radicals. Free radicals or oxygen derived reactive oxygen species (ROS) that are usually generated within the mitochondria as part of the aerobic metabolism are one of the main source of endogenous DNA damage. ROS are also produced by peroxisomes during phagocytosis and by immune cells like macrophages and neutrophils during inflammation and infections [5, 6]. Spontaneous formation of apurinic /apyraminidnic (AP) sites by cleavage of glycosidic bonds is one of the most frequent lesions. Hydrolytic deamination of cytosine to uracil also leads to damage and mutations [7]

1.1.2 Exogenous agents

DNA is also susceptible to exogenous DNA damaging agents such as UV radiation, ionizing radiation, chemicals and other toxic substances. Ionizing radiation and different chemotherapeutic drugs used in this thesis are discussed below.

Ionizing radiation

As the name indicates ionizing radiation (IR) causes ionization of molecules in its track, leading to chemical alterations. Based on linear energy transfer (LET), IR is subdivided into low LET and high LET radiations. High LET radiation (e.g. alpha and beta particles, heavy ions) generates dense

ionizations and therefore deposits high energy in a small area, causing more complex clustered damage. Low LET radiation (e.g. x-rays, gamma-rays) penetrates deeper into the tissue and therefore causes sparse ionization over long distances. When photons are exposed to matter, they deposit some energy in their path and the unit of this absorbed dose is measured in Gray (Gy) which is equivalent to 1 joule/kg. However, the biological effect of radiation depends on the type of radiation used and the ratio of the radiation dose required to produce the same biological effect between two types of radiation is often represented by the Relative biological effectiveness (RBE) [8].

When ionizing radiation hits a cell, it interacts with cellular targets through direct and indirect actions. The direct effect involves photons interacting with cellular biomolecules including DNA, thereby causing ionization by direct energy deposition. The indirect effect involves ionization of cellular water molecules resulting in the formation of powerful hydroxyl radicals which then damage the DNA and other critical biomolecules including lipids, protein and DNA [9, 10] Most of the damage inferred by IR exposure is through the indirect effect [2]. Through direct and indirect effects, radiation causes different DNA lesions including oxidized bases, apurinic/ apyramidinic sites, sugar modification, single-strand breaks, DNA double-strand breaks, DNA- DNA and DNA- protein cross links [11, 12]. More complex clustered damages occur if multiple lesions in both strands are within a helical turn of the DNA molecule [13].

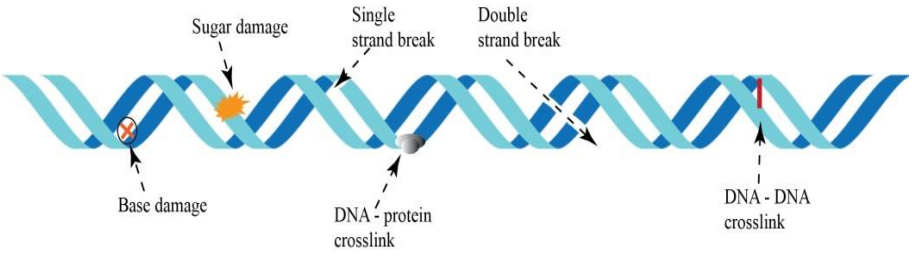

Figure 1. DNA damages caused by ionizing radiation

1.1.3 DNA - damaging drugs

DNA damaging drugs are divided into different groups based on the mechanism of cell killing. Most of these drugs target replication fork progression through direct or indirect interactions and induce cell death.

Table 1. DNA damaging drugs and the DNA lesions caused by these agents modified from [14]

Drugs	DNA damage
Antimetabolites	Replicative damage, base damage
Alkylating agents	Replicative damage, DNA crosslinks, DNA double strand breaks, DNA adducts
Anthracycline antibiotic	Intercalates into DNA, DNA double strand breaks, DNA crosslinks
Topoisomerase inhibitors	DNA strand breaks, Replicative damage
Radiomimetics	DNA strand breaks, base damage

Different DNA damaging drugs used in this thesis are briefly described below.

Cytarabine

The antimetabolite drug cytarabine (AraC) is a deoxycytidine analogue that interferes with DNA replication and thus induces DNA damage. It is converted to nucleotide analogue AraCTP by deoxycytidine kinases and gets incorporated into DNA during its replication. Once incorporated into the DNA, it inhibits DNA polymerase and prevents further DNA synthesis [15].

Etoposide

Etoposide induce toxicity mainly by inhibiting topoisomerase II enzyme and thus preventing re-ligation of DNA double strand breaks produced by the topoisomerase II enzyme [16]. Topoisomerase enzymes are responsible for the unwinding and rewinding of DNA double helix and prevents tangling of the DNA strands during replication, transcription and recombination [17].

Doxorubicin

Doxorubicin is an anthracycline antibiotic derived from *Streptomyces*. It mainly acts by inhibiting the topoisomerase II enzyme but also intercalates into DNA and form adducts. The quinone moiety present in doxorubicin is oxidized to the unstable semiquinone and its conversion back to quinone releases free radicals that also cause toxicity. It alkylates DNA and forms crosslinks [18, 19].

Calicheamicin

Calicheamicin γ-1 is a radiomimetic antitumor drug that has two radical centers. It binds to the minor groove of the DNA in such a way that its two radical centers become close to the sugar- phosphate backbone of the DNA. The radical centers are activated through trisulfide reduction which in turn abstract hydrogen atoms from the DNA backbone and results in the incision of DNA strands. Most of the DNA strand breaks caused by Calicheamicin γ-1 is double strand breaks [20]

Mitomycin C

Mitomycin C is a bi-functional alkylating agent that induces DNA-protein and DNA-DNA (both intrastrand and interstrand) crosslinks. It is activated by a cycloreduction reaction to a reactive intermediate which then attacks both strands of DNA [21]. Inter-strand cross-links (ICLs) are highly toxic DNA lesions occurring between the two complementary strands of the double helix. A covalent cross linkage formed between nucleotides on opposite strands prevents separation of the DNA strands and thereby blocks DNA replication and transcription [22].

1.2 DNA double strand breaks

Among the different types of DNA lesions, DNA double strand breaks (DSBs) are generally considered to be the most potent [23]. They are the major cause of cell sensitivity after radiation and one gray of radiation dose is believed to cause 20-40 DSBs [24]. Besides radiation, different chemotherapeutic drugs also generate DSBs either directly or from lesions during DNA replication. DSBs are also generated endogenously during DNA replication phase due to damage in the template strand. Endogenous site-specific DSBs are also produced during certain cellular processes such as V(D)J recombination, class-switch recombination and meiosis [25]. DSBs occur when both strands of the DNA double helix are broken. They pose a threat to the genomic integrity and cell survival as any unrepaired DSBs may result in growth arrest, cell death, mutation or chromosomal aberrations [26, 27].

1.3 Cellular response to DNA damage

Cells have evolved specific mechanisms that effectively respond to DNA damage and the signaling network is known as DNA damage response (DDR) pathway. The DDR senses DNA damage, signal the location to transducer kinases and coordinate cellular responses such as cell cycle checkpoint control, DNA repair, cell death or senescence [28]. In response to DNA DSBs, three proteins MRE11, RAD50 and NBS1 (MRN) acts as a complex and play key role in DNA damage detection and signaling. The complex binds to the damaged sites and recruits and activates the ATM signaling kinase [29]. ATM or Ataxia Telangiectasia Mutated, a serine/threonine kinase member of the phosphatidylinositol-3-OH kinase-like kinases (PIKKs) family play a crucial role in signaling DNA double - strand break damage. ATM activates different downstream targets involved in the cell cycle check point control, DNA repair and cell death [30].

As ATM has a crucial role in coordinating cellular response to DNA DSBs, individuals with genetic defect in ATM are highly sensitive to DSBs caused by ionizing radiation and other DSB inducing agents [31]. Loss of ATM function causes the genetic disorder Ataxia telangiectasia (AT). It is a neurodegenerative disorder characterized by neurodegeneration, immune

deficiency and an increased risk of developing cancer. Cells derived from AT patient exhibit hypersensitivity to radiation. [32, 33]

1.3.1 Gamma H2AX

In response to DNA DSBs, the H2A histone variant H2AX becomes phosphorylated at the serine -139 residue [34]. The phosphorylated H2AX or the gamma H2AX (γ-H2AX) formed within seconds after the DSB formation extends to other H2AX molecules within a few mega base pairs of DNA surrounding the DSB [35]. Being a rapid response following DSB formation, γ-H2AX serves as a biomarker of DSB formation [36]. Gamma H2AX signaling is involved in recruiting DNA repair machineries to the damaged site and is usually lost during or after the DNA repair process [37, 38]. H2AX can thus be used to detect the radiosensitivity or the ability to repair damage in cells.

1.3.2 Cell cycle arrest

In response to DNA damage, the cell-cycle checkpoints are activated to prevent cell cycle progression, thereby allowing time for repairing the DNA damage before cell division. However, severe DNA damage can cause prolonged or permanent cell cycle arrest leading to senescence, a state in which cells stop dividing but remain metabolically active[39].

Following sensing the DNA double strand breaks, ATM phosphorylates and activates several downstream proteins such as p53, MDM2,CHK1, CHK2, NBS1 and BRCA1 [40]. The movement of cells through the cell cycle is mainly controlled by cyclin-dependent kinases (CDKs), therefore inhibition of CDK activity through phosphorylation or dephosphorylation is essential to prevent cell cycle progression. ATM induced p53 and CHK 1 /2 plays a major role in inhibiting CDKs.

At the G1/S checkpoint, cells with damaged DNA are prevented entering into the DNA replicating S phase. In response to DNA damage, ATM activated p53 transactivates the cyclin dependent kinase inhibitor, p21 (CIP1/WAF1).

P21 suppress the cyclin E and cyclin A/CDK2 (cyclin dependent kinase 2) complexes necessary for S-phase initiation. In late G1, activated Chk1/Chk2 phosphorylates Cdc25A and targets enhanced degradation of Cdc25A, thereby inhibiting Cdk2 activity and preventing DNA synthesis [41].

The S phase checkpoints activated by damage arising during the DNA replication process slows down the DNA synthesis process by inhibiting the origins of replication. Apart from the ATM activated Chk1/Chk2 -Cdc25A-Cdk2 pathway, the other pathway involved in S-phase checkpoint is activated by ATM-mediated phosphorylation of Nbs1 [42]. Cells with a phenotype defective in degrading Cdc25A or phosphorylating Nbs1 had increased radiosensitivity like ATM-defective cells [43]. The G2/M phase checkpoint prevents cells from entering mitosis if there is a DNA damage and serves to minimize the extent of DNA damage passed on to daughter cells. Analogous to the G1/S checkpoint, Chk1/Chk2-mediated cytoplasmic sequestration of the Cdc25C phosphatase results in the inhibition of cyclin B/Cdk1 and results in G2/M arrest.

1.3.3 DNA repair

The DNA repair process is of crucial importance in maintaining genetic stability and cells have different mechanisms to repair DNA damage. DNA base damages caused by oxidation, alkylation or hydrolysis is excised and repaired by base excision repair. Bulky lesions like DNA adducts are repaired by Nucleotide excision repair where several base pairs containing DNA lesions are removed from the single-stranded DNA, followed by DNA synthesis and ligation. The mismatch or nucleotide errors occurring during DNA replication and recombination are repaired by mismatch repair process [44].

DNA double strand breaks (DSBs) which are the most toxic lesions are mainly repaired through two different mechanisms such as homologous recombination (HR) and non-homologous end joining (NHEJ).

Homologous Recombination

Homologous recombination (HR) requires a homologous DNA sequence from the undamaged sister chromatid as a template to repair the DSB lesion. For this reason, this repair process occurs only during the late S and G2 phase of the cell cycle [45] . It is basically an error-free repair process because it relies on the homologous DNA strand of the undamaged sister chromatid as a template for the repair.

The initial step in HR repair is binding of the Mre11–Rad50–Nbs1 (MRN) complex to the DSB ends and generating 3′ – single - stranded DNA (ssDNA) overhangs capable of invading duplex DNA. These 3′ overhangs gets coated by the ssDNA- binding protein RPA which is subsequently replaced by the RAD51 [46]. RAD51 promotes strand invasion of the homologous sister chromatid forming a Holliday junction structure. After alignment of the homologous sequences, RAD51 is removed followed by DNA synthesis, resolution of the Holliday junction and ligation.

Non-Homologous End joining

Non-homologous end joining (NHEJ) is a conservative end-joining process essential for V (D) J recombination during B cell development. It is also the major pathway for repairing DSB as it allows fast repair throughout the entire cell cycle . NHEJ involves ligation of DNA break ends and therefore does not require sequence homology. The DNA-PK having DNA-binding Ku70/Ku80 heterodimeric subunit (Ku) and DNA-dependent protein kinase catalytic subunit (DNA-PKcs) is a key player in signaling the NHEJ repair process [47]

The repair pathway is initiated by the binding of the Ku complex to the DSB ends followed by recruitment of the DNA-PKcs, a serine/threonine kinase member of the PIKK family [48]. DNA-PKcs tether to the damaged DNA ends and forms a synaptic complex that brings the two DNA ends close to each other. Activated DNA-PKcs becomes autophosphorylated and phosphorylates several other proteins involved in DNA end processing [49]. DNA ends are processed to remove damaged or mismatched bases followed by ligation by the LigIV/XRCC4 complex and XLF/Cernunnos [50]. Several

proteins including Artemis, polynucleotide kinase, DNA polymerases are known to take part in NHEJ repair [51]

Fanconi anemia pathway for repairing DNA interstrand cross links

DNA interstrand cross links are usually detected during the S phase of the cell cycle when the DNA replication fork is blocked due to the covalent cross links between the two DNA strands [52]. Fanconi anemia proteins play a key role in sensing and signaling ICL damage in cells during the S/G2 phase. The stalled replication forks are recognized by FANCM and FAAP24 proteins and the FA core complex containing FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL, FANCM, FANCT, FAAP100, MHF1, MHF2, FAAP20 and FAAP24 proteins are recruited to the damaged site. This core complex proteins catalyzes monoubiquitination of FANCD2 and FANCI [53]. Ubiquitinylated FANCD2 triggers the recruitment of several factors including endonucleases such as ERCC4, MUS81 and FAN1 [54]. The endonucleases coordinates incision on both sides of the cross links resulting in breaking one parental strand and unhooking the ICL from the opposite parental strand. The unhooked cross link on the complementary strand is bypassed by translesion synthesis (TLS) polymerases such as REV1 or DNA polymerase ζ [55]. The incisions can lead to the formation of DNA double strand breaks which are eventually targeted for HR repair [56]. Finally, the FANCD2–I heterodimer is deubiquitylated by the USP1–UAF1enzyme complex and the FA pathway is turned off [57, 58]. Thus the FA pathway is likely to be involved in nucleolytic incision, TLS and HR repair processes.

Mutation in any of the identified FANC genes can result in Fanconi anemia. It is a cancer susceptible genetic disorder characterized by multiple congenital abnormalities, progressive bone marrow failure and increased sensitivity to DNA interstrand crosslinking agents [59]. Due to the repair defect, FA patients are hypersensitive to DNA interstrand cross linking agents.

1.3.4 Cell death

Cells may die if the DNA damage is too high or if the damage cannot be repaired properly.

In response to radiation or DNA damaging drugs, cells may undergo cell death through a programmed mechanism known as apoptosis [60]. Apoptosis occurs through a series of events including chromatin condensation, DNA fragmentation followed by disintegration to apoptotic bodies and subsequent phagocytosis [61]. The tumor suppressor protein, p53 is known to coordinate apoptosis as it activates the pro-apoptotic protein and causes mitochondrial release of cytochrome c followed by activation of caspase 9 leading to apoptotic process.

The prevailing mechanism behind radiation induced cell death is mitotic catastrophe. In this mode of cell death, cells usually undergo several rounds of cell divisions before they die [62]. Cells that undergo cell division without proper repair of DNA damage because of a defective G2/M cell-cycle check point mechanism or cells attempting to enter mitosis before the completion of DNA replication in the S phase can cause a mitotic catastrophe [63]. Mitotic catastrophe is often accompanied by key molecular events of apoptosis that executes cell death. Failure of subsequent cell demise can cause aneuploidy and genomic instability [64, 65].

1.4 Cancer treatment

Cancer treatment mainly involves surgery, chemotherapy, radiotherapy and biologic therapy or their combinations. DNA damaging agents like radiation and chemotherapeutic drugs are used in cancer treatment to induce DNA damage and cell death in tumor cells.

1.4.1 Radiotherapy

Radiotherapy is an important cancer treatment modality with approximately 50 percent of cancer patients are being treated with radiation either as the primary therapy or as part of the combination therapy. Being a localized treatment, it can kill tumor cells in a region but the radiation doses are often limited to avoid toxicities to the surrounding normal tissues. Acute toxicity which may occur within weeks after radiation treatment includes skin and gut tissue damage, inflammation and erythema of the skin and pneumonia. Late tissue damage occurs due to fibrosis, necrosis, atrophy and vascular damage [66-68]. Among patients receiving radiotherapy, severe side effects are reported in five to ten percent while approximately 50 % of patients experience less severe, yet troublesome, effects [69, 70]

The chance of eradicating tumors depends on the treatment doses delivered. The steep dose-response curve indicates that small dose difference can result in clinically relevant tumor control. However, the dose-response relationships for normal tissue toxicity is also steep and any increase in the dose may cause major toxicity [71]. It is represented by standard sigmoid dose–response curves with a narrow therapeutic index between tumor control probability (TCP) and normal tissue complication probability (NTCP).

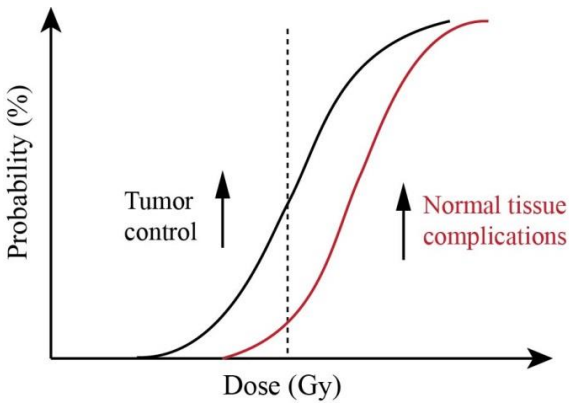

Figure 2. Sigmoid curve showing therapeutic window of radiotherapy. (Figure adapted from Barnett et.al [66])

1.4.2 Chemotherapy

Chemotherapy has been used in the treatment of cancer for more than 50 years. Being a systemic treatment option, the DNA damaging drugs usually affects the dividing ability of normal cells such as bone marrow, digestive tract and hair follicles are also affected resulting in mucositis, alopecia, bone marrow suppression, leukopenia, anemia and thrombocytopenia, increased susceptibility to infections [72]. The individual variation in sensitivity to DNA damaging agents may increase the extent of side effects. For example, patients with mutations in DNA repair genes can be more sensitive to DNA damaging drugs and thus may end up with severe side effects following treatment with conventional chemotherapeutics [73].

1.5 Individual difference in cellular response to DNA damage

There is wide inter-individual variation in response to DNA damaging treatment, also within a group of identically treated patients. The first evidence was published in the 1970s when clinical hyper-sensitivity in ataxia telangiectasia (A-T) patients treated with radiation was reported [74]. Several factors including physical (e.g. total dose, dose per fraction) as well as patient-related factors such as age, smoking, coexisting disease conditions and cellular antioxidant levels influence a patient's risk of developing toxicities [1, 75]. However, studies suggest that genetic predisposition accounts for 70% of the individual differences in radiosensitivity [76]. The exact genetic mechanism behind individual variation in intrinsic sensitivity is not clear however, it is evident that individuals with genetic disorders involving DNA repair defects are more sensitive to radiation.

Table 2. Genetic syndromes associated with DNA repair defects, modified from [77]

Genetic disorder	**Gene**	**DNA repair defect**
Ataxia Telangiectasia (AT)	*ATM*	DSB signaling
Nijmegan breakage syndrome (NBS)	*NBS1*	DSB signaling
A-T like disorder (ATLD)	*MRE11*	DSB signaling
Fanconi Anemia (FA)	*FANC*	Fanconi repair (HR)
Ligase IV syndrome	*LIGIV*	NHEJ repair
Seckel syndrome	*ATR*	DSB signaling

Cells derived from patients with genetic syndromes present with hypersensitivity to radiation due to the mutation in genes involved in DNA damage recognition, signaling and repair [78]. These mutations accounts for only a small subset of the population [79]. However, reports indicate that the radiosensitivity of cells from normal population shows a wide variation in adverse effects and that proportion of patients developing adverse effects after radiotherapy is much high [71, 76, 80-83]. Consequently, more factors influencing radiosensitivity remains to be discovered.

1.5.1 Predicting sensitivity to DNA damage

Due to patient-to-patient variation in intrinsic sensitivity to radiation, several endpoints have been analyzed to predict sensitivity. Since DNA repair is thought to be involved in the mechanism of radiation sensitivity, several methods to detect DNA damage repair are reported including comet assay[84], the gamma H2AX assay[85], pulse field gel electrophoresis[86], DNA end binding complex[87], micronucleus assay [88] and chromosomal aberrations analysis. Gene expression profiling assays such as SNP analysis

and transcriptional profiling have attempted to explore the mechanism behind radiation sensitivity; however, with limited success [89, 90]. The reason for this may be that the individual variation in sensitivity to DNA damaging agents is not based on a general mechanism but may be multifactorial. Due to this, measuring sensitivity from a single endpoint may fail to provide the predictability necessary for clinical settings. In this situation, an assay that measures the net effect of DNA damage in terms of cell survival may be able to predict the intrinsic sensitivity of an individual.

Clonogenic survival assay

Cell survival measured by clonogenic assay has been considered as a gold standard reference endpoint to measure the extent of a patient's normal tissue reaction after radiotherapy. Many studies have found a correlation between clinical radiosensitivity and intrinsic radiation sensitivity measured by clonogenic survival of fibroblasts or lymphocytes [91-93]. However, the assay is quite laborious and time consuming and therefore not clinically applicable in order to test patient's cell sensitivity prior to treatment.

1.6 Free radicals and stress

Free radicals are highly reactive due to the presence of unpaired electrons in their outer shell [10]. Low to moderate levels of ROS are beneficial as cellular signaling molecules but, excessive ROS levels can cause damage to DNA, proteins and lipids [94]. They can oxidize cellular components like DNA, leading to adduct that impair base-pairing, cause base loss, or single-strand breaks (SSBs). However, when in close proximity SSBs on DNA strands may lead to double strand breaks [51]. When the level of free radicals within the body increases and exceeds the body's ability to remove them, a condition known as oxidative stress occurs. Oxidative stress is a deleterious process contributing to various disease conditions including cancer [95], neurodegeneration [96] and ageing [97]. Cellular exposure to free radicals cannot be avoided as they are continuously generated by intracellular processes. Moreover exposure to environmental toxins like UV radiation, ionizing radiation and smoke also give rise to radical production. Cells have

therefore evolved an antioxidant defense network that constantly acts to balance the cellular redox status.

1.7 Nrf2-Keap1 pathway

In humans and several other mammals, the antioxidant defense system is mainly regulated by the basic leucine zipper transcription factor Nuclear factor erythroid 2- related factor 2 (Nrf2) [98]. In response to oxidative or electrophilic stress, activated Nrf2 enter the nucleus and form heterodimer with small Maf protein and initiates transcription of genes containing an antioxidant response element (ARE) in the DNA regulatory region [98]. Nrf2 activates the expression of a series of ARE dependent genes including phase 2 antioxidant enzymes such as heme oxygenase-1, NAD(P)H:quinone oxidoreductase, glutathione peroxidase and other members of the glutathione transferase family. The critical importance of Nrf2 in protecting cells against toxic substances is evidenced in different studies where Nrf2 deletions increased the susceptibility of mice to different toxic chemicals and pathological conditions related to oxidative stress [99-102]

Homeostatic levels of Nrf2 are kept low by its association with the repressor protein Kelch–like ECH-associated protein 1 (Keap1) in the cytoplasm. Keap1 function as a negative regulator of Nrf2 by targeting Nrf2 for ubiquitin dependent proteasomal degradation by Cullin 3-base E3 ubiquitin ligase [103, 104]. Keap1 is a cysteine rich protein consisting of 27 cysteine residues in humans. These cysteine residues act as critical sensors of oxidative or electrophilic stress. Modification of cysteine residues prevents Keap1 mediated Nrf2 ubiquitination, followed by Nrf2 stabilization and nuclear translocation to induce ARE dependent genes. Upon restoring redox homeostasis, Keap1 moves into the nucleus and controls nuclear export of Nrf2 for subsequent proteasomal degradation in the cytoplasm [105]. Thus Keap1 act as a chemical sensor regulating the levels of Nrf2 based on cellular redox status.

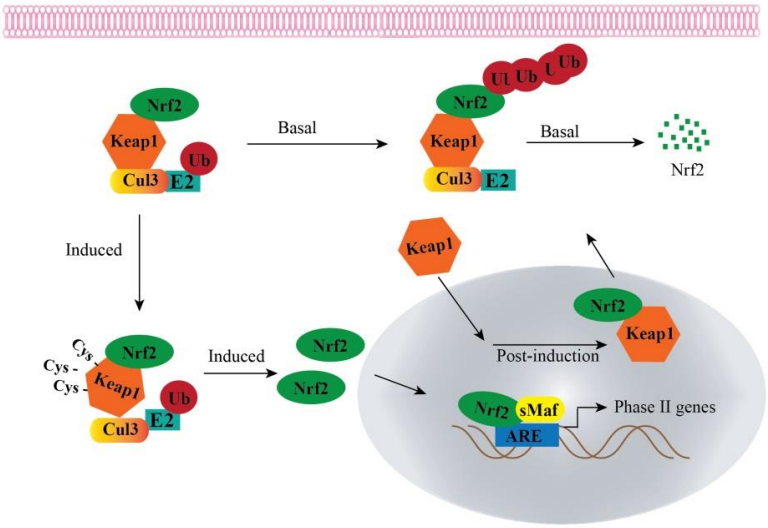

Figure 3. Keap1 mediated Nrf2 regulation.

In basal conditions, Nrf2 is bound to Keap1 in the cytoplasm and undergoes proteasomal degradation. The presence of inducers/stress, cause modification of Keap1 cysteine residues and Nrf2 levels increase. Activated Nrf2 enters the nucleus and dimerizes with Maf to promote transcription of ARE-dependent genes. Finally, Nrf2 is transported out of the nucleus by Keap1 for subsequent proteasomal degradation.

1.8 Activators of Keap1-Nrf2 pathway

In addition to cellular stress different classes of chemicals in table 3 have been shown to induce Nrf2 [3]. These Nrf2 inducing agents are structurally diverse but they share a common property of reacting with sulfhydryl groups of Keap1 cysteine residues and thereby resulting in Nrf2 activation [106, 107]. Nrf2 activators that have been studied in this thesis are discussed later.

Studies have identified critical cysteine residues of Keap1 such as cysteine 273 or cysteine 288 are involved in Keap1 repression of Nrf2 under basal conditions and cysteine 151 for Nrf2 activation in response to electrophilic stress [108-110]. However evidences suggest that different Nrf2 activators

prefer specific cysteine residues to induce Nrf2 activation. Based on the evidences, a "cysteine code" which converts the preferential target cysteine modifications into distinct biological effects has been proposed [111].

Table 3. Different classes of Nrf2 inducers and their example [3]

Classes of Nrf2 activators	**Example**
Michael reaction acceptors	triterpenoids, curcumin
Oxidizable phenols/ quinones	Resveratrol, Tert-butylhydroquinone,
Isothiocyanates /sulfoxythiocarbamates	Sulforaphane, phenethyl isothiocyanate
Dithiolthiones/diallyl sulfides	Oltipraz, diallyl trisulfide
Trivalent arsenicals	Arsenic trioxide, phenylarsine oxide
Dimercaptans	R-lipoic acid, 2,3-dimercaptosuccinic acid
Selenium based compounds	Selenite, organoselenium compounds
Polyenes	Carotenoids, lycopene
Hydroperoxides	Tert-butylhydroperoxide, cumol hydroperoxide
Heavy metals/ metal complexes	Cadmium, auranofin

1.8.1 Sulforaphane

Sulforaphane (SF) is an isothiocyanate abundantly present in broccoli sprouts and other cruciferous vegetables. The glucoraphanin present in these vegetables is broken down by the enzyme myrosinase into the isothiocyanate sulforaphane during cutting or chewing [112]. The isothiocyanate group (-N=C=S) linked to the glucosinolate moiety mainly contributes to the chemopreventive actions of SF. In rats, an oral dose of 50 μmol of SF leads to a peak plasma concentration of approximately 20 μM [113]. In humans, consumption of 200 μmol isothiocyanate prepared from broccoli sprouts lead to peak plasma concentration of ~3.0 μM after 1 hour and declined with a mean half-life of 1.77 ± 0.13 hours [114, 115]. The main urinary excretory products are mercapturic acid and cysteine conjugate forms.

Figure 4. Chemical structure of sulforaphane

Sulforaphane activates Keap1-Nrf2 pathway through its direct interaction with Cys-151, a cysteine residue of Keap1 [116]. It has been shown to induce expression of phase II enzymes and antioxidant proteins including heme oxygenase 1 (HO-1), NAD(P)H:quinone oxidoreductase-1 (NQO1) and glutathione transferases (GSTs) [117]. Thus SF induces a protective effect against various toxic chemicals [118]. As reported, SF inhibits cancer cell proliferation by inducing cell cycle arrest and apoptosis [119-121]. In a recent clinical trial on patients with recurrent prostate cancer, daily administration 200 μmol/day sulforaphane rich extracts for up to 20 weeks did not lead to any signs of adverse effects but decreased the PSA levels to 50% in 1 out of 20 patients and PSA doubling time was reduced with sulforaphane treatment [122]. Further clinical studies such as the POUDER trial are underway to evaluate the feasibility of SFN as an adjuvant for chemotherapy in patients with advanced pancreatic cancer [123].

1.8.2 Bardoxolone methyl

Bardoxolone methyl or BARD (also known as CDDO methyl ester) is a synthetic derivative of triterpenoid oleanolic acid and a well-known potent Nrf2 inducer activating Nrf2 at nanomolar concentrations. It belongs to the class of Michael reaction acceptors and is known to activate downstream targets of Nrf2 including HO-1 and NQO1 and thereby induce chemopreventive properties [124].

BARD has been shown to protect cells from ionizing radiation as demonstrated by Kim,et.al [125] and others[126]. It has been shown to enhance the estimated glomerular filtration rate (eGFR) in patients with chronic kidney disease [127]. However, a Phase III clinical trial with treatment of advanced chronic kidney disease (CKD) in patients with type 2 diabetes mellitus was terminated due to fatal side effects [128].

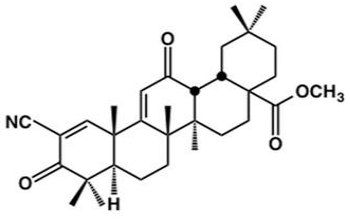

Figure 5. Chemical structure of bardoxolone methyl

1.9 Hormesis

Hormesis is described as a biphasic dose-response phenomenon where an agent inducing stimulatory effect at moderate doses can produce an inhibitory effect at high doses [129, 130]. The hormetic effect has been observed with different stress inducers including oxidants, phytochemicals, exercise and calorie restriction.

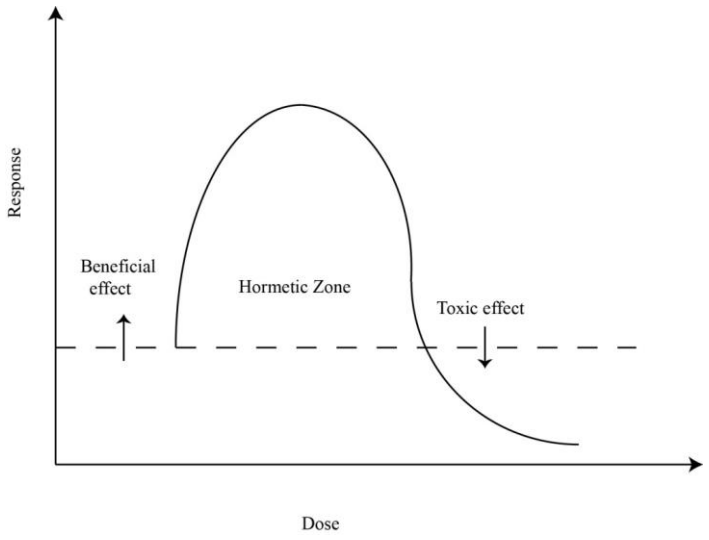

Figure 6. Illustration of hormetic response.

1.9.1 Hormetic effect of Nrf2 inducers

It has become increasingly clear that Nrf2 activation is often tightly regulated and Nrf2 inducing agents follow a hormetic dose-response curve [131]. The electrophilic characteristic of Nrf2 inducing agents enable them to act as pro-oxidants and thus the protective effects can only be observed at particular nontoxic doses. Sulforaphane is a well-known hormetic agent that has chemopreventive and cytoprotective effect at lower concentrations but cytotoxic and antiproliferative effect at higher concentrations [132, 133]. The chemoprevention is mainly due to Nrf2 based Phase II gene induction [134] while the cytotoxic effect is reported to be mediated by cell cycle arrest and apoptosis. Other Nrf2 inducers including a BARD analog may also be considered as hormetic moieties as they induce protective effects at low nano - molar concentrations but higher concentrations are reported to be toxic to cells [135].

2 AIM

The main aim of the research work presented in this thesis was to develop and validate a flow cytometry based method to measure intrinsic sensitivity to DNA damaging agents. How the pre-activation of Nrf2 influenced inherent radiosensitivity and cellular adaptation was also studied.

2.1 Specific aims:

In paper I, we aimed to develop a flow cytometry based cell division assay that can be used in clinical settings to measure patient sensitivity to radiation and chemotherapeutic agents.

In paper II, the aim was to validate the cell division assay in predicting sensitivity to DNA inter-strand crosslinking agent and thereby identifying sensitive patients in a population.

The study presented in paper III investigated whether repeated treatment using the Nrf2 activator sulforaphane protects skin fibroblasts from the toxic effects of ionizing radiation.

In paper IV, the adaptive response after pretreatment with the Nrf2 activators, sulforaphane and bardoxolone methyl was studied.

3 MATERIALS AND METHODS

This section provides an overview of the methods used in this study. A detailed description of all the experimental methods can be found in the attached papers.

Patient samples

Blood samples from healthy individuals described in paper I and II were collected from the Hematology lab at the Clinical Chemistry department at Sahlgrenska University hospital. Blood samples from Ataxia telangiectasia (AT) and Fanconi anemia (FA) patients were obtained from other hospitals in Sweden. This study was approved by the ethical committee and informed consent was obtained for the FA and AT patients. Peripheral blood mononuclear cells (PBMNCs) were isolated from blood samples through density gradient centrifugation using Lymphoprep (Axis shield).

Cell types

Human primary skin fibroblasts bought from ATCC (CRL- 2091) were used in the study described in paper I, III and IV. The Nrf2 wild type (Nrf2 +/+) and Nrf2 knock out (Nrf2 -/-) mouse embryonic fibroblasts (MEFs) used in paper III was a kind gift by Professor John D. Hayes at the University of Dundee, UK.

In paper IV, lymphoblasts derived from Fanconi anemia patients (GM13071-A -complementation group B, GM16749 -A– complementation group A and GM16756 -A–complementation group D2 from the Coriell Institute for Medical research) and normal lymphoblasts (MTB-B-1 and SAC-B-1) immortalized with the B95-8 strain of the EBV virus was used to study the sensitivity to cytotoxic drugs. All the cells were grown in appropriate growth medium in a humidified incubator with 5 % CO_2 at 37 ° C.

Radiation

Cells were irradiated using the Gammacell 3000 Elan instrument (Best Theratronics) at the dose rate of 128 mGy/s. In paper I and III, cells were treated with clinically relevant doses of γ-radiation.

Cytotoxic drugs

DNA damage was chemically induced using different chemotherapeutic agents as in paper I and II. Stock solutions of 100 mM etoposide, 3.4 mM doxorubicin, 10 mM cytarabine, 20 μM calicheamicin, 30 mM mitomycin C, 10 mM DNA-PK inhibitor Nu7441 and 10 mM ATM inhibitor Ku55933 (Merck Millipore) were prepared in dimethyl sulfoxide (DMSO) and stored at −80 °C (4 °C for Mitomycin C). The drugs were diluted in DMSO and working concentrations were added to the media. In all the treatments, the final concentration of the DMSO solvent was 0.1 %.

Nrf2 activating agents

Nrf2 activating agents such as sulforaphane (100 mM), BARD (10 mM), curcumin and tBHQ used in paper III and IV were prepared in DMSO and stored at −80 °C. Working concentrations were prepared in DMSO and 0.1 % DMSO was used as a vehicle control.

METHODS

Clonogenic assay

The clonogenic assay or colony assay which is considered as the ''gold standard'' method for measuring intrinsic sensitivity was used in paper I to measure the radiosensitivity of human skin fibroblasts. Cells were allowed to divide and form colonies under normal growth conditions. After 10-12 days of incubation, cell colonies were stained and manually counted. Colonies with 50 or more cells were considered as a colony [136]. Thus the results represent long term dividing ability of cells in response to radiation. However, the long time required in completing an experiment and the

manual labor in counting the colonies makes this assay less practical in clinical settings.

Cell division (CD) assay

In this thesis, the proportion of cells that have undergone cell division after cytotoxic treatments were EdU labeled and detected with the help of flow cytometer.

EdU labeling

The thymidine analogue, 5-ethynyl 2- deoxyuridine (EdU) was used to label proliferating cells. EdU incorporated into newly synthesized DNA during S-phase of the cell cycle can be easily identified through the Click-iT staining [137, 138]. The terminal alkyne group in EdU specifically reacts with fluorescently labeled azide dye and form a triazole bond between alkyne and azide groups in the presence of copper sulphate. This allows the detection of EdU labeled DNA in the divided cells [139]. In all the papers, 10 μM EdU was used for cell labeling.

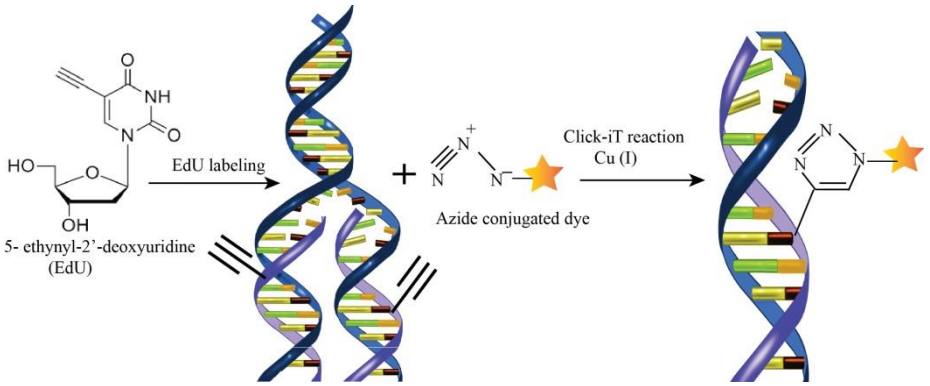

Figure 7. EdU detection using specific click-iT staining. EdU incorporates into DNA during the replication phase. The ethynyl group of the incorporated EdU in the DNA reacts with the azide group of the dye in a copper catalyzed Click-iT reaction and forms a triazole bond.

EdU staining

Cells are allowed to grow in appropriate growth medium for 48 or 72 h after which 10 μM EdU was added. After 16 h incubation in the presence of EdU, cells are harvested and fixed using formaldehyde and permeabilized using saponin. EdU labeled cells were stained using the Click-iT reaction mix where the azide conjugated fluorescent dye forms covalent bond with the alkyne group in the EdU in the presence of CuSO4 and a reducing agent. Finally, cells were stained using a cell cycle dye to analyze the DNA content in the cells. CountBright absolute counting beads (Life technologies) were added to the samples prior to a flow cytometry analysis as an internal control for the sample volume analyzed by the flow cytometer.

Measurement of DNA double strand breaks

Phosphorylation of the histone protein H2AX or gamma H2AX is considered to be a biomarker for DNA double-strand breaks (DSBs) formation [36, 140]. Therefore DSBs can be detected by staining cells with Gamma H2AX antibody using immunofluorescence, flow cytometry or western blot. According to evidences, the number of γ-H2AX foci is equivalent to the number of DSBs formed and therefore disappearance of these foci are likely to represent repair of DSBs [141]. In paper I, gamma H2AX in patient blood lymphocytes was measured using γ-H2AX flow cytometry assay [142]. The ability of lymphocytes treated with ATM or DNA PK inhibitors to recover from radiation induced damage was validated by measuring γ-H2AX fluorescence at different time points after radiation. In paper III, gamma H2AX foci formed after radiation in 10 μM sulforaphane treated cells was detected and quantified by immunofluorescence [143].

Measurements of reactive oxygen species

Fluorescent probes have been used to quantify intracellular reactive oxygen species (ROS). The cell permeable fluorescent probe 2′, 7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) has been used to detect radiation induced ROS [144]. It is cleaved by cellular esterases to DCFH which is converted to fluorescent 2',7'-dichlorofluorescein (DCF) upon oxidation by several ROS including hydroxyl radicals (OH) and peroxynitrite

[145]. In paper III, we used the chloromethyl derivative of H_2DCFDA to quantify the basal and radiation induced ROS levels in sulforaphane treated cells. The ROS levels were detected using a flow cytometer.

Gene expression analysis

Quantitative analysis of the messenger RNA (mRNA) expression patterns was done using quantitative PCR or qPCR. In paper III, the Nrf2 target gene expressions after SFN treatment was studied using qPCR.

Cells were lysed and mRNA was extracted using oligo (d)T-covered magnetic beads which attract the poly adenine tails of mRNA. To ensure greater stability, the single stranded mRNA was converted to double-stranded complementary DNA or cDNA using a reverse transcriptase enzyme. The expression of the Nrf2 target genes Heme oxygenase (human HMOX1and mouse Hmox1) and NAD(P)H dehydrogenase quinone 1 (human NQO1and mouse Nqo1) was studied using the TaqMan® Gene Expression Assay. In TaqMan® assays the target gene specific probes are coupled to a fluorophore and a quencher and the exponential amplification of DNA is identified by the accumulation of the fluorescent signal from the probe. If the probe remains intact, the quencher prevents fluorescence. During the amplification process, the probes hybridized to the target sequences are cleaved by the DNA polymerase enzyme resulting in the fluorescence signal allowing quantification of amplified DNA. A reference gene that is thought to be evenly expressed is measured in all the samples. Using the relative quantification ($\Delta\Delta C_T$) method [146], the fluorescence from the target gene was related to the reference gene in each sample and finally normalized to the control sample.

Nuclear localization of Nrf2

In response to oxidative or electrophilic stress, Nrf2 is activated and moves to the nucleus to induce response gene transcription [147]; nuclear accumulation of Nrf2 therefore reflects Nrf2 activation. In paper III, the nuclear

translocation of Nrf2 in sulforaphane-treated cells was assessed using immunofluorescence technique.

Statistical analysis

Data in this thesis is presented as mean standard deviation of the replicates. The ratio of standard deviation to mean was calculated and represented as coefficient of variation (CV) in paper I and II. Further statistical significance between different groups was analyzed using unpaired student's *t*-test, one-way ANOVA or two-way ANOVA as indicated in the papers. In paper I, the relationship between two different groups was analyzed using Pearson correlation coefficient. All the graphs were plotted and statistical analysis was done using the Graphpad prism software.

4 RESULTS AND DISCUSSION

PAPER I

Radiation sensitivity of human skin fibroblasts measured using cell division assay correlates to the clonogenic assay

Although it is quite evident that the intrinsic sensitivity to DNA damaging agents varies among individuals, this is not considered when prescribing treatment with DNA damaging agents. In radiation therapy, the treatment is based on population averages and this may result in severe side effects in the extremely sensitive patients while non-sensitive patients may not receive an adequate dose for tumor control. Finding methods to identify intrinsic sensitivity of patients prior to treatment with DNA damaging agents is therefore of critical importance. Identifying sensitive patients prior to treatment can prevent adverse effects and at the same time provide a chance to use higher doses in non-sensitive patients to achieve better tumor control.

The cell division (CD) assay was developed to detect intrinsic sensitivity to DNA damaging agents in patients. The method utilizes the thymidine analogue EdU (5-ethynyl-2'- deoxyuridine) to label the cells that divide in response to treatment with DNA damaging agents and the divided cells are specifically stained using an EdU-reactive dye and detected with the aid of a flow cytometer. Prior to EdU labeling, cells were allowed adequate time to exhibit cytotoxicity or growth arrest caused by DNA damaging agents. Since EdU induce cell cycle arrest and apoptosis [148], the cells usually divide only once in the presence of EdU. Our results indicated that 16 h incubation of the cells in EdU-containing medium allows detection of almost all the dividing cells in the culture.

Skin fibroblasts have been widely used in measuring sensitivity to radiation and studies have shown a positive correlation between fibroblasts sensitivity measured by clonogenic assay and radiotherapy toxicities [91, 149]. The radiosensitivity of human skin fibroblasts measured using the CD assay

showed a similar correlation to the clonogenic assay. The clonogenic survival assay is considered as the gold standard method to measure cell sensitivity [150] but when compared with the practical aspects of the clonogenic assay, the CD assay is simpler and less time-consuming (4 days) than the colony assay (10–14 days).

The cell division assay detected lymphocyte sensitivity to radiation and chemotherapeutic drugs

Peripheral blood lymphocytes can be easily obtained from patients with less pain and it has been widely used in biological dosimetry studies. We optimized the CD assay on peripheral blood lymphocytes and measured CD3/CD28 specific T-cell proliferation in response to ionizing radiation and chemotherapeutic drugs treatment. Chemicals like NU7441and KU55933 are potent and selective inhibitors of DNA-PKcs [151] and ATM [152] and treating cells with these agents can sensitize the cells to DNA double strand break inducing agents. Using the CD assay we measured the increased radiosensitivity of lymphocytes treated with DNA-PK and ATM inhibitors. Furthermore, the CD assay was able to detect the increased radiosensitivity in two patients with the Ataxia telangiectasia (AT), a genetic disorder due to defective ATM signaling [153].

The method also measured lymphocyte sensitivity to different chemotherapeutic drugs including drugs that induce DNA damage during the DNA replication process. Many DNA damaging agents induce toxicity during replication however; it is not possible to measure sensitivity to such agents using short-term assays that measure DNA damage and repair in the absence of cell division. An assay which measured apoptosis of T-lymphocyte has shown positive correlation to radiation-induced late toxicity; however this assay cannot predict sensitivity to agents that induce toxicity during replication [154]. Our results indicated that the CD assay is able to detect patient sensitivity to drugs that specifically induce damage in dividing cells. Using the CD assay, we were able to detect hypersensitivity to the DNA interstrand cross linking agent mitomycin C in a patient with suspected Fanconi anemia.

We also measured the radiosensitivity of blood lymphocytes from healthy controls. The results showed up to twofold inter- individual variation with marked sensitivity in one patient. This also indicates that the number of radiosensitive patients may be much higher than the patients with rare genetic syndromes. Inter-individual variation in intrinsic sensitivity to radiation has been reported in several previous research studies [71, 81]. Moreover, heterozygous carriers of genetic disorders like AT may also have an increased risk to developing cancer and may have increased sensitivity to radiation [155, 156].

As most of the DNA repair disorders are characterized by sensitivity to DNA damaging agents and cancer predisposition, these sensitive individuals run a potential risk of accidental injury during cancer treatment. The common phenotypic characteristics can help physicians identify the sensitive population before cancer treatment is initiated. However, the heterozygotes for these disorders with no symptoms can be left unidentified before treatment and may end up in severe side effects due to increased sensitivity. Thus there is a need for rapid screening of patient sensitivity prior to DNA damaging therapy in clinical settings. Our results indicate that the CD assay can measure sensitivity to these agents quickly and conveniently.

PAPER II

The cell division assay is able to detect increased sensitivity to DNA inter- strand cross linking agent

Individuals with DNA repair defects have increased sensitivity to DNA damaging agents and Fanconi anemia (FA) is one such genetic syndrome caused by a defect in any of the Fanconi repair genes specialized at repairing DNA interstrand cross links [157]. These patients are likely to have congenital abnormalities, developmental delays, bone marrow failure and increased susceptibility to hematological malignancies and squamous cell carcinoma [53]. DNA interstrand cross links are deleterious lesions inhibiting DNA replication and transcription and cells from FA patients are therefore extremely sensitive to DNA interstrand cross linking agents (ICLs) like

mitomycin C. Therefore sensitivity to mitomycin C has been used as a diagnostic tool to identify these patients [158].

The CD assay is able to measure the proliferative response to different DNA damaging agents including those that induce damage during cell division. In the previous study, it has been shown that the CD assay can detect the hypersensitivity to mitomycin C in Fanconi anemia patient. To further validate the CD assay, we measured the mitomycin C sensitivity of Epstein Barr Virus (EBV) transformed lymphoblastoid cells (LCLs) from FA patients. The CD assay detected increased sensitivity in the all FA cell types with defects in FANCD2, FANCA and FANCB complementation compared to cells from healthy controls. FA patients are prone to bone marrow failure and are likely to undergo treatment with different chemotherapeutic drugs during bone marrow transplantation process. Antimetabolite drugs like cytarabine and its analogues are used in the bone marrow transplant conditioning process and we; therefore measured the sensitivity of the FA- LCLs to cytarabine. When compared to the control LCLs, we observed increased sensitivity in cell types derived from FA patients with complementation group FANCA and FANCD2 but not in FANCB suggesting that the sensitivity of FA patients varies based on the gene mutation. We also observed no increased sensitivity in FA- LCLs (FANCD2 and FANCB) treated with the radiomimetic drug calicheamicin. Although FA patients are known to have increased sensitivity to radiation, there are reports demonstrating no increased radiosensitivity in FA patient cells [159, 160]. Further analysis of lymphocyte sensitivity to mitomcyin C showed a wide variation in intrinsic sensitivity among healthy controls emphasizing the importance of measuring sensitivity to DNA damaging drugs prior to treatment.

Due to hypersensitivity to ICL inducing drugs, FA patients must avoid treatment with these drugs. However, the diagnosis based on clinical symptoms is not always possible due to the wide variation in patient phenotype. [161]. Therefore patients lacking congenital abnormalities or a positive family history of Fanconi anemia may be left undiagnosed until they develop severe toxicity upon treatment with a bone marrow transplant conditioning regimen or cancer therapy [73, 162, 163]. Heterozygous FA

carriers with an estimated frequency of 1 in 181[164] have also been reported to suffer severe complications after chemo/radiotherapy [165, 166]. For this reason, it is quite important to identify the sensitivity of patients prior to the use of DNA damaging agents. Currently the diagnosis of Fanconi anemia is based on chromosomal breakage analysis in lymphocytes exposed to ICL generating drugs. However, this test is only available in specific laboratories. Our results indicate that the CD assay can measure patient sensitivity to mitomycin C and can therefore be used to identify FA patients.

PAPER III

Repeated treatment using Nrf2 activating sulforaphane protects cells from ionizing radiation

Being a DNA damaging agent, ionizing radiation (IR) interrupts cell homeostasis and causes cytotoxicity mainly through the generation of reactive oxygen species (ROS). Since Nrf2 is involved in cytoprotection against radicals, we investigated if pre-activation of Nrf2 influence intrinsic cell radiosensitivity. A previous study from our laboratory showed that Nrf2 response levels are increased in cells exposed to repeated brief sulforaphane treatment [167]. We therefore wanted to investigate if repeated pretreatment with sulforaphane could protect cells from radiation induced toxicity. To test this hypothesis, human skin fibroblasts were treated with different concentrations (0 – 30 μM) of sulforaphane (SF) for a single four hour (single treatment) or four hour repeatedly for 3 days (repeated treatment) prior to radiation. The plasma half-life of sulforaphane in humans who ate broccoli is approximately two hour [114]. In our experimental settings, we used the four hour SF treatment to mimic this brief exposure time point and tried to investigate how repeated daily exposure to SF influence the Nrf2 response and cellular adaptation to radiation induced cell damage.

The cell proliferative response after 2 Gy and 4 Gy radiation doses showed increased protection in repeatedly treated cells but not in single four hour treated cells. However, the protective effect was observed at intermediate

sulforaphane concentrations with a maximum effect at 10 μM and declined at higher concentrations indicating the hormetic response. ROS levels measured using a fluorescent probe showed decreased levels of ROS in sulforaphane treated cells at basal conditions and after exposure to 2Gy and 4 Gy radiation doses suggesting that the repeated sulforaphane stimulations increased the intracellular antioxidant capacity. Moreover, in cells repeatedly treated with 10 μM SFN, γ-H2AX foci formed after 1Gy radiation tend to decrease with time and at 4h the number of foci were significantly fewer compared to the vehicle treated control. Reports suggest that the disappearance of γ-H2AX foci is linked to the repair of DNA double strand breaks after low dose of radiation [168]. Therefore the faster clearance of γ-H2AX foci observed in sulforaphane treated cells may be due to repeated sulforaphane treatment having a positive influence on radiation induced DSB repair.

Protective effect with repeated sulforaphane treatment is Nrf2 dependent

To evaluate the role of Nrf2 in mediating the SF induced protective effect, the gene expressions of two Nrf2 response genes heme oxygenase 1 (HO-1), NAD(P)H:quinone oxidoreductase-1 (NQO1) that are known to be induced by sulforaphane was measured after single and repeated treatment [169]. HO-1 expression was induced in cells treated with SF (both single and repeated treatment). The maximum induction was at 10 μM and decreased at higher sulforaphane concentrations, thus indicating a hormetic response. In line with the previous study [167], dose-dependent induction of NQO1 was observed only in repeatedly treated cells but not in single treated cells.

The role of Nrf2 was further investigated using Nrf2 +/+ (WT) and Nrf2 -/- (KO) mouse embryonic fibroblasts (MEFs). Cells were treated with 0 -10μM concentrations of SF (single and repeated treatment) prior to radiation and cell division was measured. In Nrf2 WT MEFs, a single 4 h SF treatment failed to show cytoprotection against radiation while enhanced protection was observed with the repeated treatment that tend to decrease at higher concentrations. However, sulforaphane treatment resulted in increased toxicity in MEF cells lacking the functional Nrf2 gene and this was more

pronounced with repeated sulforaphane treatment and/or irradiation. This is in line with another study which has reported increased intrinsic radiosensitivity in Nrf2 KO MEFs [170]. From our results it was clear that sulforaphane mediated cytoprotective effect against radiation requires a functional Nrf2 response

Together our results showed that repeated sulforaphane treatment can protect cells from radiation induced toxicities at moderate concentrations. However, the radioprotective effect started to decline at higher SF concentrations and SF itself was toxic to cells. Thus SF treatment induced hormetic effect on cells; that is at moderate doses it showed cytoprotective effect while at higher doses it induced cytotoxic effect.

PAPER IV

Preconditioning cells with sulforaphane or BARD induces adaptation to toxic challenge

The cellular network to adapt to oxidative and electrophilic stress is mainly regulated by the Nrf2 transcription factor [171]. In paper III, we have observed a hormetic dose response with SF treatment where moderate concentrations of SF showed protection against damage caused by ionizing radiation but higher SF concentrations appeared to be toxic to the cells [172]. Therefore we tried to explore whether pretreatment of cells with nontoxic concentration of sulforaphane can permit adaptation to toxic concentrations of the same or other Nrf2 activating chemical. In order to validate the extent of Nrf2 stimulation required to induce cellular adaptation, human skin fibroblasts were treated with Nrf2 activating agents such as sulforaphane (SF) and synthetic triterpenoid bardoxolone methyl (BARD) for 4 h daily for 5 days (5-day protocol), 4 h daily for 3 days (3-day protocol), 24 h for 2 days (2-day protocol) and 4h for 1 day (1-day protocol).

Cells treated with 10 μM SF or 30 nM BARD were exposed to higher toxic concentrations of the same substance along with the non-pretreated control.

The dividing ability of cells measured by the CD assay showed several folds increase in percentage cell division in pretreated cells compared to the cells which had no pretreatment. This indicated that the adaptive resistance to toxicity can be acquired if preceded by pretreatment with the same stressor. The adaptive ability was more enhanced in cells repeatedly treated for short time period (4h daily) for 3 days and 5 days. Moreover, pretreatment with higher concentrations (30 μM SF) resulted in relatively decreased adaptation indicating hormesis where protective effects tend to lose at higher concentration. Similar adaptation has been reported in rodents where regular exercise activated Nrf2 signaling however, no Nrf2 induction was observed when the exercise was continued until exhaustion [173, 174]. The cells were able to withstand the toxic challenge even one week after pretreatment with SF or BARD. However the cells that were freeze-thawed and grown in the absence of SF lost the resistance to higher toxic concentration. This suggests that the adaptive effect may be transient but not long term based on selection.

Preconditioning cells with sulforaphane or BARD induces cross resistance

In many cases, the tolerance developed through adaptation cause resistance against toxic doses of the same stressor and cross-adaptation to other stress factors [175]. Therefore we have checked whether preconditioning with one chemical activator could induce cross resistance to the other substance. The results from the cell division assay showed adaptive cross resistance to higher toxic concentrations with both SF and BARD pretreatment. Similar cross-adaptation was reported in another study where pretreatment with different Nrf2 inducers protected mouse embryonic fibroblasts against challenging dose of hydrogen peroxide [27].

Although SF and BARD are Nrf2 activating agents, they belong to different classes of structurally different Nrf2 activators. SF is an isothiocyanate and BARD is a synthetic triterpenoid. The cross-resistance observed in preconditioned cells suggest that the chemical structure of the electrophilic compound is not an important factor in modulating transient adaptation to

stress. Therefore the protective effect of Nrf2 against stress is a more general response that is not based on chemical nature of the stress.

5 CONCLUSION AND FUTURE PERSPECTIVES

The cell division assay described in paper I and II in this thesis can detect patient's intrinsic sensitivity to radiation and other DNA damaging agents. Using this assay the sensitivity of a patient can be easily identified with a small volume of blood sample and results can be obtained in 4 days. However, more clinical validation studies in patients undergoing treatment with radiation and other DNA damaging agents are required to confirm the predictive ability of the method before implementing it in the routine laboratories to test patient sensitivity prior to DNA damaging therapies.

The work presented in paper III of this thesis demonstrated that repeated treatment with isothiocyanate sulforaphane protects human skin fibroblasts and mouse embryonic fibroblasts from cellular damage caused by ionizing radiation in an Nrf2 dependent manner. Based on the results, it is plausible that sulforaphane could be used to protect normal tissue damage caused by radiation during radiation therapy. However, further studies are needed to investigate whether sulforaphane could selectively protect the normal cells but not tumor.

In paper IV we found that repeated pretreatment with structurally different Nrf2 activators, sulforaphane and bardoxolone methyl trained the skin fibroblasts to acquire resistance against higher toxic concentrations of both the drugs. Our results suggest that adaptation to stress is a general feature of Nrf2 response and that usually follows a hormetic pattern.

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REFERENCES

1. Bentzen, S.M. and J. Overgaard, *Patient-to-Patient Variability in the Expression of Radiation-Induced Normal Tissue Injury.* Semin Radiat Oncol, 1994. **4**(2): p. 68-80.
2. Riley, P.A., *Free radicals in biology: oxidative stress and the effects of ionizing radiation.* Int J Radiat Biol, 1994. **65**(1): p. 27-33.
3. Magesh, S., Y. Chen, and L. Hu, *Small molecule modulators of Keap1-Nrf2-ARE pathway as potential preventive and therapeutic agents.* Med Res Rev, 2012. **32**(4): p. 687-726.
4. Ciccia, A. and S.J. Elledge, *The DNA damage response: making it safe to play with knives.* Mol Cell, 2010. **40**(2): p. 179-204.
5. Davies, K.J., *Oxidative stress: the paradox of aerobic life.* Biochem Soc Symp, 1995. **61**: p. 1-31.
6. Jackson, A.L. and L.A. Loeb, *The contribution of endogenous sources of DNA damage to the multiple mutations in cancer.* Mutat Res, 2001. **477**(1-2): p. 7-21.
7. Tubbs, A. and A. Nussenzweig, *Endogenous DNA Damage as a Source of Genomic Instability in Cancer.* Cell, 2017. **168**(4): p. 644-656.
8. *Introduction.* Annals of the ICRP, 1989. **20**(4): p. 1-6.
9. Halliwell, B.a.G., J.M., *Free Radicals in Biology and Medicine* 4ed. 2007.
10. Cheeseman, K.H. and T.F. Slater, *An introduction to free radical biochemistry.* Br Med Bull, 1993. **49**(3): p. 481-93.
11. von Sonntag, C., *The chemistry of free-radical-mediated DNA damage.* Basic Life Sci, 1991. **58**: p. 287-317; discussion 317-21.
12. Dizdaroglu, M. and P. Jaruga, *Mechanisms of free radical-induced damage to DNA.* Free Radic Res, 2012. **46**(4): p. 382-419.
13. Goodhead, D.T., *Initial events in the cellular effects of ionizing radiations: clustered damage in DNA.* Int J Radiat Biol, 1994. **65**(1): p. 7-17.
14. Helleday, T., et al., *DNA repair pathways as targets for cancer therapy.* Nat Rev Cancer, 2008. **8**(3): p. 193-204.
15. Parker, W.B., *Enzymology of purine and pyrimidine antimetabolites used in the treatment of cancer.* Chem Rev, 2009. **109**(7): p. 2880-93.
16. Montecucco, A., F. Zanetta, and G. Biamonti, *Molecular mechanisms of etoposide.* EXCLI J, 2015. **14**: p. 95-108.
17. Binaschi, M., F. Zunino, and G. Capranico, *Mechanism of action of DNA topoisomerase inhibitors.* Stem Cells, 1995. **13**(4): p. 369-79.

18. Cutts, S.M., et al., *The power and potential of doxorubicin-DNA adducts.* IUBMB Life, 2005. **57**(2): p. 73-81.
19. Tewey, K.M., et al., *Adriamycin-induced DNA damage mediated by mammalian DNA topoisomerase II.* Science, 1984. **226**(4673): p. 466-8.
20. Elmroth, K., et al., *Cleavage of cellular DNA by calicheamicin gamma1.* DNA Repair (Amst), 2003. **2**(4): p. 363-74.
21. Tomasz, M., *Mitomycin C: small, fast and deadly (but very selective).* Chem Biol, 1995. **2**(9): p. 575-9.
22. Deans, A.J. and S.C. West, *DNA interstrand crosslink repair and cancer.* Nat Rev Cancer, 2011. **11**(7): p. 467-80.
23. Ward, J.F., *The yield of DNA double-strand breaks produced intracellularly by ionizing radiation: a review.* Int J Radiat Biol, 1990. **57**(6): p. 1141-50.
24. Lomax, M.E., L.K. Folkes, and P. O'Neill, *Biological consequences of radiation-induced DNA damage: relevance to radiotherapy.* Clin Oncol (R Coll Radiol), 2013. **25**(10): p. 578-85.
25. Hartlerode, A.J. and R. Scully, *Mechanisms of double-strand break repair in somatic mammalian cells.* Biochem J, 2009. **423**(2): p. 157-68.
26. Iliakis, G., *The role of DNA double strand breaks in ionizing radiation-induced killing of eukaryotic cells.* Bioessays, 1991. **13**(12): p. 641-8.
27. Olive, P.L., *The role of DNA single- and double-strand breaks in cell killing by ionizing radiation.* Radiat Res, 1998. **150**(5 Suppl): p. S42-51.
28. Harper, J.W. and S.J. Elledge, *The DNA damage response: ten years after.* Mol Cell, 2007. **28**(5): p. 739-45.
29. Lee, J.H. and T.T. Paull, *ATM activation by DNA double-strand breaks through the Mre11-Rad50-Nbs1 complex.* Science, 2005. **308**(5721): p. 551-4.
30. Shiloh, Y., *ATM and related protein kinases: safeguarding genome integrity.* Nat Rev Cancer, 2003. **3**(3): p. 155-68.
31. Kuhne, M., et al., *A double-strand break repair defect in ATM-deficient cells contributes to radiosensitivity.* Cancer Res, 2004. **64**(2): p. 500-8.
32. Lavin, M.F., *Ataxia-telangiectasia: from a rare disorder to a paradigm for cell signalling and cancer.* Nat Rev Mol Cell Biol, 2008. **9**(10): p. 759-69.
33. Khanna, K.K., *Cancer risk and the ATM gene: a continuing debate.* J Natl Cancer Inst, 2000. **92**(10): p. 795-802.

34. Rogakou, E.P., et al., *DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139.* J Biol Chem, 1998. **273**(10): p. 5858-68.
35. Rogakou, E.P., et al., *Megabase chromatin domains involved in DNA double-strand breaks in vivo.* J Cell Biol, 1999. **146**(5): p. 905-16.
36. Rothkamm, K. and S. Horn, *gamma-H2AX as protein biomarker for radiation exposure.* Ann Ist Super Sanita, 2009. **45**(3): p. 265-71.
37. Paull, T.T., et al., *A critical role for histone H2AX in recruitment of repair factors to nuclear foci after DNA damage.* Curr Biol, 2000. **10**(15): p. 886-95.
38. Chowdhury, D., et al., *gamma-H2AX dephosphorylation by protein phosphatase 2A facilitates DNA double-strand break repair.* Mol Cell, 2005. **20**(5): p. 801-9.
39. Campisi, J., *Cellular senescence as a tumor-suppressor mechanism.* Trends Cell Biol, 2001. **11**(11): p. S27-31.
40. Schmitt, E., et al., *DNA-damage response network at the crossroads of cell-cycle checkpoints, cellular senescence and apoptosis.* J Zhejiang Univ Sci B, 2007. **8**(6): p. 377-97.
41. Sexl, V., et al., *A rate limiting function of cdc25A for S phase entry inversely correlates with tyrosine dephosphorylation of Cdk2.* Oncogene, 1999. **18**(3): p. 573-82.
42. Yazdi, P.T., et al., *SMC1 is a downstream effector in the ATM/NBS1 branch of the human S-phase checkpoint.* Genes Dev, 2002. **16**(5): p. 571-82.
43. Falck, J., et al., *The DNA damage-dependent intra-S phase checkpoint is regulated by parallel pathways.* Nat Genet, 2002. **30**(3): p. 290-4.
44. Iyama, T. and D.M. Wilson, 3rd, *DNA repair mechanisms in dividing and non-dividing cells.* DNA Repair (Amst), 2013. **12**(8): p. 620-36.
45. Arnaudeau, C., C. Lundin, and T. Helleday, *DNA double-strand breaks associated with replication forks are predominantly repaired by homologous recombination involving an exchange mechanism in mammalian cells.* J Mol Biol, 2001. **307**(5): p. 1235-45.
46. Chen, H., M. Lisby, and L.S. Symington, *RPA coordinates DNA end resection and prevents formation of DNA hairpins.* Mol Cell, 2013. **50**(4): p. 589-600.
47. Jackson, S.P. and P.A. Jeggo, *DNA double-strand break repair and V(D)J recombination: involvement of DNA-PK.* Trends Biochem Sci, 1995. **20**(10): p. 412-5.
48. Dynan, W.S. and S. Yoo, *Interaction of Ku protein and DNA-dependent protein kinase catalytic subunit with nucleic acids.* Nucleic Acids Res, 1998. **26**(7): p. 1551-9.

49. Chan, D.W., et al., *Autophosphorylation of the DNA-dependent protein kinase catalytic subunit is required for rejoining of DNA double-strand breaks.* Genes Dev, 2002. **16**(18): p. 2333-8.
50. Drouet, J., et al., *DNA-dependent protein kinase and XRCC4-DNA ligase IV mobilization in the cell in response to DNA double strand breaks.* J Biol Chem, 2005. **280**(8): p. 7060-9.
51. Lieber, M.R., *The mechanism of double-strand DNA break repair by the nonhomologous DNA end-joining pathway.* Annu Rev Biochem, 2010. **79**: p. 181-211.
52. Zhang, J., et al., *DNA interstrand cross-link repair requires replication-fork convergence.* Nat Struct Mol Biol, 2015. **22**(3): p. 242-7.
53. Ceccaldi, R., P. Sarangi, and A.D. D'Andrea, *The Fanconi anaemia pathway: new players and new functions.* Nat Rev Mol Cell Biol, 2016. **17**(6): p. 337-49.
54. Smogorzewska, A., et al., *A genetic screen identifies FAN1, a Fanconi anemia-associated nuclease necessary for DNA interstrand crosslink repair.* Mol Cell, 2010. **39**(1): p. 36-47.
55. Budzowska, M., et al., *Regulation of the Rev1-pol zeta complex during bypass of a DNA interstrand cross-link.* EMBO J, 2015. **34**(14): p. 1971-85.
56. Kim, H. and A.D. D'Andrea, *Regulation of DNA cross-link repair by the Fanconi anemia/BRCA pathway.* Genes Dev, 2012. **26**(13): p. 1393-408.
57. Nijman, S.M., et al., *The deubiquitinating enzyme USP1 regulates the Fanconi anemia pathway.* Mol Cell, 2005. **17**(3): p. 331-9.
58. Cohn, M.A., et al., *A UAF1-containing multisubunit protein complex regulates the Fanconi anemia pathway.* Mol Cell, 2007. **28**(5): p. 786-97.
59. Alter, B.P., *Diagnosis, genetics, and management of inherited bone marrow failure syndromes.* Hematology Am Soc Hematol Educ Program, 2007: p. 29-39.
60. Nicholson, D.W. and N.A. Thornberry, *Apoptosis. Life and death decisions.* Science, 2003. **299**(5604): p. 214-5.
61. Wyllie, A.H., J.F. Kerr, and A.R. Currie, *Cell death: the significance of apoptosis.* Int Rev Cytol, 1980. **68**: p. 251-306.
62. Chang, W.P. and J.B. Little, *Delayed reproductive death in X-irradiated Chinese hamster ovary cells.* Int J Radiat Biol, 1991. **60**(3): p. 483-96.
63. Castedo, M., et al., *Cell death by mitotic catastrophe: a molecular definition.* Oncogene, 2004. **23**(16): p. 2825-37.

64. Galluzzi, L., et al., *Molecular definitions of cell death subroutines: recommendations of the Nomenclature Committee on Cell Death 2012.* Cell Death Differ, 2012. **19**(1): p. 107-20.
65. Vitale, I., et al., *Mitotic catastrophe: a mechanism for avoiding genomic instability.* Nat Rev Mol Cell Biol, 2011. **12**(6): p. 385-92.
66. Barnett, G.C., et al., *Normal tissue reactions to radiotherapy: towards tailoring treatment dose by genotype.* Nat Rev Cancer, 2009. **9**(2): p. 134-42.
67. Brush, J., et al., *Molecular mechanisms of late normal tissue injury.* Semin Radiat Oncol, 2007. **17**(2): p. 121-30.
68. Stone, H.B., et al., *Effects of radiation on normal tissue: consequences and mechanisms.* Lancet Oncol, 2003. **4**(9): p. 529-36.
69. Vale, C., et al., *Late complications from chemoradiotherapy for cervical cancer: reflections from cervical cancer survivors 10 years after the national cancer institute alert.* Clin Oncol (R Coll Radiol), 2010. **22**(7): p. 588-9.
70. Syndikus, I., et al., *Late gastrointestinal toxicity after dose-escalated conformal radiotherapy for early prostate cancer: results from the UK Medical Research Council RT01 trial (ISRCTN47772397).* Int J Radiat Oncol Biol Phys, 2010. **77**(3): p. 773-83.
71. Turesson, I., *Individual variation and dose dependency in the progression rate of skin telangiectasia.* Int J Radiat Oncol Biol Phys, 1990. **19**(6): p. 1569-74.
72. Goodman, M., *Managing the side effects of chemotherapy.* Semin Oncol Nurs, 1989. **5**(2 Suppl 1): p. 29-52.
73. Gyger, M., et al., *Unsuspected Fanconi's anemia and bone marrow transplantation in cases of acute myelomonocytic leukemia.* N Engl J Med, 1989. **321**(2): p. 120-1.
74. Gotoff, S.P., E. Amirmokri, and E.J. Liebner, *Ataxia telangiectasia. Neoplasia, untoward response to x-irradiation, and tuberous sclerosis.* Am J Dis Child, 1967. **114**(6): p. 617-25.
75. Barnett, G.C., et al., *The Cambridge Breast Intensity-modulated Radiotherapy Trial: patient- and treatment-related factors that influence late toxicity.* Clin Oncol (R Coll Radiol), 2011. **23**(10): p. 662-73.
76. Turesson, I., et al., *Prognostic factors for acute and late skin reactions in radiotherapy patients.* Int J Radiat Oncol Biol Phys, 1996. **36**(5): p. 1065-75.
77. McKinnon, P.J., *DNA repair deficiency and neurological disease.* Nat Rev Neurosci, 2009. **10**(2): p. 100-12.
78. Chistiakov, D.A., N.V. Voronova, and P.A. Chistiakov, *Genetic variations in DNA repair genes, radiosensitivity to cancer and*

susceptibility to acute tissue reactions in radiotherapy-treated cancer patients. Acta Oncol, 2008. **47**(5): p. 809-24.
79. Gatti, R.A., *The inherited basis of human radiosensitivity.* Acta Oncol, 2001. **40**(6): p. 702-11.
80. Little, J.B., et al., *Survival of human diploid skin fibroblasts from normal individuals after X-irradiation.* Int J Radiat Biol, 1988. **54**(6): p. 899-910.
81. Safwat, A., et al., *Deterministic rather than stochastic factors explain most of the variation in the expression of skin telangiectasia after radiotherapy.* Int J Radiat Oncol Biol Phys, 2002. **52**(1): p. 198-204.
82. Hopwood, P., et al., *Comparison of patient-reported breast, arm, and shoulder symptoms and body image after radiotherapy for early breast cancer: 5-year follow-up in the randomised Standardisation of Breast Radiotherapy (START) trials.* Lancet Oncol, 2010. **11**(3): p. 231-40.
83. Miller, R.C., et al., *Mometasone furoate effect on acute skin toxicity in breast cancer patients receiving radiotherapy: a phase III double-blind, randomized trial from the North Central Cancer Treatment Group N06C4.* Int J Radiat Oncol Biol Phys, 2011. **79**(5): p. 1460-6.
84. Brammer, I., M. Zoller, and E. Dikomey, *Relationship between cellular radiosensitivity and DNA damage measured by comet assay in human normal, NBS and AT fibroblasts.* Int J Radiat Biol, 2001. **77**(9): p. 929-38.
85. Ismail, I.H., T.I. Wadhra, and O. Hammarsten, *An optimized method for detecting gamma-H2AX in blood cells reveals a significant interindividual variation in the gamma-H2AX response among humans.* Nucleic Acids Res, 2007. **35**(5): p. e36.
86. Zhou, P.K., et al., *The radiosensitivity of human fibroblast cell lines correlates with residual levels of DNA double-strand breaks.* Radiother Oncol, 1998. **47**(3): p. 271-6.
87. Ismail, S.M., et al., *Predicting radiosensitivity using DNA end-binding complex analysis.* Clin Cancer Res, 2004. **10**(4): p. 1226-34.
88. Encheva, E., et al., *Investigating micronucleus assay applicability for prediction of normal tissue intrinsic radiosensitivity in gynecological cancer patients.* Rep Pract Oncol Radiother, 2011. **17**(1): p. 24-31.
89. Svensson, J.P., et al., *Analysis of gene expression using gene sets discriminates cancer patients with and without late radiation toxicity.* PLoS Med, 2006. **3**(10): p. e422.
90. Wilding, C.S., et al., *Influence of polymorphisms at loci encoding DNA repair proteins on cancer susceptibility and G2 chromosomal radiosensitivity.* Environ Mol Mutagen, 2007. **48**(1): p. 48-57.
91. Johansen, J., et al., *Evidence for a positive correlation between in vitro radiosensitivity of normal human skin fibroblasts and the*

occurrence of subcutaneous fibrosis after radiotherapy. Int J Radiat Biol, 1994. **66**(4): p. 407-12.
92. Mann, B.D., et al., *Clinical correlations with drug sensitivities in the clonogenic assay: a retrospective study.* Arch Surg, 1982. **117**(1): p. 33-6.
93. West, C.M., et al., *Lymphocyte radiosensitivity is a significant prognostic factor for morbidity in carcinoma of the cervix.* Int J Radiat Oncol Biol Phys, 2001. **51**(1): p. 10-5.
94. Finkel, T., *Signal transduction by reactive oxygen species.* J Cell Biol, 2011. **194**(1): p. 7-15.
95. Trachootham, D., J. Alexandre, and P. Huang, *Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach?* Nat Rev Drug Discov, 2009. **8**(7): p. 579-91.
96. Andersen, J.K., *Oxidative stress in neurodegeneration: cause or consequence?* Nat Med, 2004. **10 Suppl**: p. S18-25.
97. Haigis, M.C. and B.A. Yankner, *The aging stress response.* Mol Cell, 2010. **40**(2): p. 333-44.
98. Itoh, K., et al., *An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements.* Biochem Biophys Res Commun, 1997. **236**(2): p. 313-22.
99. Walters, D.M., H.Y. Cho, and S.R. Kleeberger, *Oxidative stress and antioxidants in the pathogenesis of pulmonary fibrosis: a potential role for Nrf2.* Antioxid Redox Signal, 2008. **10**(2): p. 321-32.
100. Iizuka, T., et al., *Nrf2-deficient mice are highly susceptible to cigarette smoke-induced emphysema.* Genes Cells, 2005. **10**(12): p. 1113-25.
101. Ramos-Gomez, M., et al., *Sensitivity to carcinogenesis is increased and chemoprotective efficacy of enzyme inducers is lost in nrf2 transcription factor-deficient mice.* Proc Natl Acad Sci U S A, 2001. **98**(6): p. 3410-5.
102. Rangasamy, T., et al., *Genetic ablation of Nrf2 enhances susceptibility to cigarette smoke-induced emphysema in mice.* J Clin Invest, 2004. **114**(9): p. 1248-59.
103. Zhang, D.D., et al., *Keap1 is a redox-regulated substrate adaptor protein for a Cul3-dependent ubiquitin ligase complex.* Mol Cell Biol, 2004. **24**(24): p. 10941-53.
104. Kobayashi, A., et al., *Oxidative stress sensor Keap1 functions as an adaptor for Cul3-based E3 ligase to regulate proteasomal degradation of Nrf2.* Mol Cell Biol, 2004. **24**(16): p. 7130-9.
105. Sun, Z., et al., *Keap1 controls postinduction repression of the Nrf2-mediated antioxidant response by escorting nuclear export of Nrf2.* Mol Cell Biol, 2007. **27**(18): p. 6334-49.

106. Dinkova-Kostova, A.T., et al., *Direct evidence that sulfhydryl groups of Keap1 are the sensors regulating induction of phase 2 enzymes that protect against carcinogens and oxidants.* Proc Natl Acad Sci U S A, 2002. **99**(18): p. 11908-13.
107. Dinkova-Kostova, A.T. and P. Talalay, *Direct and indirect antioxidant properties of inducers of cytoprotective proteins.* Mol Nutr Food Res, 2008. **52 Suppl 1**: p. S128-38.
108. Zhang, D.D. and M. Hannink, *Distinct cysteine residues in Keap1 are required for Keap1-dependent ubiquitination of Nrf2 and for stabilization of Nrf2 by chemopreventive agents and oxidative stress.* Mol Cell Biol, 2003. **23**(22): p. 8137-51.
109. Wakabayashi, N., et al., *Protection against electrophile and oxidant stress by induction of the phase 2 response: fate of cysteines of the Keap1 sensor modified by inducers.* Proc Natl Acad Sci U S A, 2004. **101**(7): p. 2040-5.
110. Yamamoto, T., et al., *Physiological significance of reactive cysteine residues of Keap1 in determining Nrf2 activity.* Mol Cell Biol, 2008. **28**(8): p. 2758-70.
111. Kobayashi, M., et al., *The antioxidant defense system Keap1-Nrf2 comprises a multiple sensing mechanism for responding to a wide range of chemical compounds.* Mol Cell Biol, 2009. **29**(2): p. 493-502.
112. Fahey, J.W., Y. Zhang, and P. Talalay, *Broccoli sprouts: an exceptionally rich source of inducers of enzymes that protect against chemical carcinogens.* Proc Natl Acad Sci U S A, 1997. **94**(19): p. 10367-72.
113. Hu, R., et al., *In vivo pharmacokinetics and regulation of gene expression profiles by isothiocyanate sulforaphane in the rat.* J Pharmacol Exp Ther, 2004. **310**(1): p. 263-71.
114. Ye, L., et al., *Quantitative determination of dithiocarbamates in human plasma, serum, erythrocytes and urine: pharmacokinetics of broccoli sprout isothiocyanates in humans.* Clin Chim Acta, 2002. **316**(1-2): p. 43-53.
115. Cornblatt, B.S., et al., *Preclinical and clinical evaluation of sulforaphane for chemoprevention in the breast.* Carcinogenesis, 2007. **28**(7): p. 1485-90.
116. Hu, C., et al., *Modification of keap1 cysteine residues by sulforaphane.* Chem Res Toxicol, 2011. **24**(4): p. 515-21.
117. Fahey, J.W. and P. Talalay, *Antioxidant functions of sulforaphane: a potent inducer of Phase II detoxication enzymes.* Food Chem Toxicol, 1999. **37**(9-10): p. 973-9.
118. Higgins, L.G., et al., *Transcription factor Nrf2 mediates an adaptive response to sulforaphane that protects fibroblasts in vitro against the*

cytotoxic effects of electrophiles, peroxides and redox-cycling agents. Toxicol Appl Pharmacol, 2009. **237**(3): p. 267-80.
119. Choi, S., et al., *D,L-Sulforaphane-induced cell death in human prostate cancer cells is regulated by inhibitor of apoptosis family proteins and Apaf-1.* Carcinogenesis, 2007. **28**(1): p. 151-62.
120. Chiao, J.W., et al., *Sulforaphane and its metabolite mediate growth arrest and apoptosis in human prostate cancer cells.* Int J Oncol, 2002. **20**(3): p. 631-6.
121. Qazi, A., et al., *Anticancer activity of a broccoli derivative, sulforaphane, in barrett adenocarcinoma: potential use in chemoprevention and as adjuvant in chemotherapy.* Transl Oncol, 2010. **3**(6): p. 389-99.
122. Alumkal, J.J., et al., *A phase II study of sulforaphane-rich broccoli sprout extracts in men with recurrent prostate cancer.* Invest New Drugs, 2015. **33**(2): p. 480-9.
123. Lozanovski, V.J., et al., *Pilot study evaluating broccoli sprouts in advanced pancreatic cancer (POUDER trial) - study protocol for a randomized controlled trial.* Trials, 2014. **15**: p. 204.
124. Wang, Y.Y., et al., *Bardoxolone methyl (CDDO-Me) as a therapeutic agent: an update on its pharmacokinetic and pharmacodynamic properties.* Drug Des Devel Ther, 2014. **8**: p. 2075-88.
125. Kim, S.B., et al., *Targeting of Nrf2 induces DNA damage signaling and protects colonic epithelial cells from ionizing radiation.* Proc Natl Acad Sci U S A, 2012. **109**(43): p. E2949-55.
126. El-Ashmawy, M., et al., *CDDO-Me protects normal lung and breast epithelial cells but not cancer cells from radiation.* PLoS One, 2014. **9**(12): p. e115600.
127. Pergola, P.E., et al., *Bardoxolone methyl and kidney function in CKD with type 2 diabetes.* N Engl J Med, 2011. **365**(4): p. 327-36.
128. de Zeeuw, D., et al., *Bardoxolone methyl in type 2 diabetes and stage 4 chronic kidney disease.* N Engl J Med, 2013. **369**(26): p. 2492-503.
129. Mattson, M.P., *Hormesis defined.* Ageing Res Rev, 2008. **7**(1): p. 1-7.
130. Calabrese, E.J., *Converging concepts: adaptive response, preconditioning, and the Yerkes-Dodson Law are manifestations of hormesis.* Ageing Res Rev, 2008. **7**(1): p. 8-20.
131. Davies, K.J., *Adaptive homeostasis.* Mol Aspects Med, 2016. **49**: p. 1-7.
132. Zanichelli, F., et al., *Low concentrations of isothiocyanates protect mesenchymal stem cells from oxidative injuries, while high concentrations exacerbate DNA damage.* Apoptosis, 2012. **17**(9): p. 964-74.

133. Zanichelli, F., et al., *Dose-dependent effects of R-sulforaphane isothiocyanate on the biology of human mesenchymal stem cells, at dietary amounts, it promotes cell proliferation and reduces senescence and apoptosis, while at anti-cancer drug doses, it has a cytotoxic effect.* Age (Dordr), 2012. **34**(2): p. 281-93.
134. Talalay, P., et al., *Chemoprotection against cancer by phase 2 enzyme induction.* Toxicol Lett, 1995. **82-83**: p. 173-9.
135. Vaziri, N.D., et al., *Dose-dependent deleterious and salutary actions of the Nrf2 inducer dh404 in chronic kidney disease.* Free Radic Biol Med, 2015. **86**: p. 374-81.
136. Franken, N.A., et al., *Clonogenic assay of cells in vitro.* Nat Protoc, 2006. **1**(5): p. 2315-9.
137. Salic, A. and T.J. Mitchison, *A chemical method for fast and sensitive detection of DNA synthesis in vivo.* Proc Natl Acad Sci U S A, 2008. **105**(7): p. 2415-20.
138. Buck, S.B., et al., *Detection of S-phase cell cycle progression using 5-ethynyl-2'-deoxyuridine incorporation with click chemistry, an alternative to using 5-bromo-2'-deoxyuridine antibodies.* Biotechniques, 2008. **44**(7): p. 927-9.
139. Rostovtsev, V.V., et al., *A stepwise huisgen cycloaddition process: copper(I)-catalyzed regioselective "ligation" of azides and terminal alkynes.* Angew Chem Int Ed Engl, 2002. **41**(14): p. 2596-9.
140. Kuo, L.J. and L.X. Yang, *Gamma-H2AX - a novel biomarker for DNA double-strand breaks.* In Vivo, 2008. **22**(3): p. 305-9.
141. Rothkamm, K. and M. Lobrich, *Evidence for a lack of DNA double-strand break repair in human cells exposed to very low x-ray doses.* Proc Natl Acad Sci U S A, 2003. **100**(9): p. 5057-62.
142. Muslimovic, A., et al., *An optimized method for measurement of gamma-H2AX in blood mononuclear and cultured cells.* Nat Protoc, 2008. **3**(7): p. 1187-93.
143. Johansson, P., et al., *In-solution staining and arraying method for the immunofluorescence detection of gammaH2AX foci optimized for clinical applications.* Biotechniques, 2011. **51**(3): p. 185-9.
144. Korystov, Y.N., et al., *Detection of reactive oxygen species induced by radiation in cells using the dichlorofluorescein assay.* Radiat Res, 2007. **168**(2): p. 226-32.
145. Hafer, K., K.S. Iwamoto, and R.H. Schiestl, *Refinement of the dichlorofluorescein assay for flow cytometric measurement of reactive oxygen species in irradiated and bystander cell populations.* Radiat Res, 2008. **169**(4): p. 460-8.
146. Livak, K.J. and T.D. Schmittgen, *Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method.* Methods, 2001. **25**(4): p. 402-8.

147. Itoh, K., et al., *Keap1 regulates both cytoplasmic-nuclear shuttling and degradation of Nrf2 in response to electrophiles.* Genes Cells, 2003. **8**(4): p. 379-91.
148. Zhao, H., et al., *DNA damage signaling, impairment of cell cycle progression, and apoptosis triggered by 5-ethynyl-2'-deoxyuridine incorporated into DNA.* Cytometry A, 2013. **83**(11): p. 979-88.
149. Geara, F.B., et al., *Prospective comparison of in vitro normal cell radiosensitivity and normal tissue reactions in radiotherapy patients.* Int J Radiat Oncol Biol Phys, 1993. **27**(5): p. 1173-9.
150. Oppitz, U., et al., *The in vitro colony assay: a predictor of clinical outcome.* Int J Radiat Biol, 2001. **77**(1): p. 105-10.
151. Zhao, Y., et al., *Preclinical evaluation of a potent novel DNA-dependent protein kinase inhibitor NU7441.* Cancer Res, 2006. **66**(10): p. 5354-62.
152. Hickson, I., et al., *Identification and characterization of a novel and specific inhibitor of the ataxia-telangiectasia mutated kinase ATM.* Cancer Res, 2004. **64**(24): p. 9152-9.
153. Taylor, A.M., et al., *Ataxia telangiectasia: a human mutation with abnormal radiation sensitivity.* Nature, 1975. **258**(5534): p. 427-9.
154. Ozsahin, M., et al., *CD4 and CD8 T-lymphocyte apoptosis can predict radiation-induced late toxicity: a prospective study in 399 patients.* Clin Cancer Res, 2005. **11**(20): p. 7426-33.
155. Thorstenson, Y.R., et al., *Contributions of ATM mutations to familial breast and ovarian cancer.* Cancer Res, 2003. **63**(12): p. 3325-33.
156. Angele, S., et al., *ATM haplotypes and cellular response to DNA damage: association with breast cancer risk and clinical radiosensitivity.* Cancer Res, 2003. **63**(24): p. 8717-25.
157. Nagel, Z.D., I.A. Chaim, and L.D. Samson, *Inter-individual variation in DNA repair capacity: a need for multi-pathway functional assays to promote translational DNA repair research.* DNA Repair (Amst), 2014. **19**: p. 199-213.
158. Oostra, A.B., et al., *Diagnosis of fanconi anemia: chromosomal breakage analysis.* Anemia, 2012. **2012**: p. 238731.
159. Duckworth-Rysiecki, G. and A.M. Taylor, *Effects of ionizing radiation on cells from Fanconi's anemia patients.* Cancer Res, 1985. **45**(1): p. 416-20.
160. Alter, B.P., *Radiosensitivity in Fanconi's anemia patients.* Radiother Oncol, 2002. **62**(3): p. 345-7.
161. Alter, B.P., *Fanconi's anaemia and its variability.* Br J Haematol, 1993. **85**(1): p. 9-14.
162. Giampietro, P.F., et al., *Diagnosis of Fanconi anemia in patients without congenital malformations: an international Fanconi Anemia Registry Study.* Am J Med Genet, 1997. **68**(1): p. 58-61.

163. Glanz, A. and F.C. Fraser, *Spectrum of anomalies in Fanconi anaemia.* J Med Genet, 1982. **19**(6): p. 412-6.
164. Rosenberg, P.S., H. Tamary, and B.P. Alter, *How high are carrier frequencies of rare recessive syndromes? Contemporary estimates for Fanconi Anemia in the United States and Israel.* Am J Med Genet A, 2011. **155A**(8): p. 1877-83.
165. Sirak, I., et al., *Hypersensitivity to chemoradiation in FANCA carrier with cervical carcinoma-A case report and review of the literature.* Rep Pract Oncol Radiother, 2015. **20**(4): p. 309-15.
166. Berwick, M., et al., *Genetic heterogeneity among Fanconi anemia heterozygotes and risk of cancer.* Cancer Res, 2007. **67**(19): p. 9591-6.
167. Bergstrom, P., et al., *Repeated transient sulforaphane stimulation in astrocytes leads to prolonged Nrf2-mediated gene expression and protection from superoxide-induced damage.* Neuropharmacology, 2011. **60**(2-3): p. 343-53.
168. Bouquet, F., C. Muller, and B. Salles, *The loss of gammaH2AX signal is a marker of DNA double strand breaks repair only at low levels of DNA damage.* Cell Cycle, 2006. **5**(10): p. 1116-22.
169. Dinkova-Kostova, A.T., et al., *Induction of the phase 2 response in mouse and human skin by sulforaphane-containing broccoli sprout extracts.* Cancer Epidemiol Biomarkers Prev, 2007. **16**(4): p. 847-51.
170. McDonald, J.T., et al., *Ionizing radiation activates the Nrf2 antioxidant response.* Cancer Res, 2010. **70**(21): p. 8886-95.
171. Espinosa-Diez, C., et al., *Antioxidant responses and cellular adjustments to oxidative stress.* Redox Biol, 2015. **6**: p. 183-97.
172. Mathew, S.T., P. Bergstrom, and O. Hammarsten, *Repeated Nrf2 stimulation using sulforaphane protects fibroblasts from ionizing radiation.* Toxicol Appl Pharmacol, 2014. **276**(3): p. 188-94.
173. Aguiar, A.S., Jr., et al., *Moderate-Intensity Physical Exercise Protects Against Experimental 6-Hydroxydopamine-Induced Hemiparkinsonism Through Nrf2-Antioxidant Response Element Pathway.* Neurochem Res, 2016. **41**(1-2): p. 64-72.
174. Malaguti, M., et al., *Sulforaphane treatment protects skeletal muscle against damage induced by exhaustive exercise in rats.* J Appl Physiol (1985), 2009. **107**(4): p. 1028-36.
175. Milisav, I., B. Poljsak, and D. Suput, *Adaptive response, evidence of cross-resistance and its potential clinical use.* Int J Mol Sci, 2012. **13**(9): p. 10771-806.