MOLECULAR STUDIES ON RADIATION RESPONSE OF HUMAN MALIGNANT CELLS *IN VITRO*

by

JAYAKUMAR SUNDARRAJ

Enrolment No.: Life 01200804012

Bhabha Atomic Research Centre, Mumbai

A Thesis Submitted to the Board of Studies in Life Sciences

In partial fulfilment of requirements

For the Degree of

DOCTOR OF PHILOSOPHY
of
HOMI BHABHA NATIONAL INSTITUTE

OCTOBER, 2014

Homi Bhabha National Institute

Recommendations of the Viva Voce Committee

As members of the Viva Voce Committee, we certify that we have read the dissertation prepared by Jayakumar Sundarraj entitled "Molecular Studies on Radiation Response of Human Malignant Cells *In Vitro*" and recommend that it may be accepted as fulfilling the thesis requirement for the award of Degree of Doctor of Philosophy.

S. Zingde

Chairman – Dr. S. Zingde **Date: 21-10-2014**

B. N. Pandey

Guide / Convener – Dr. B. N. Pandey **Date: 21-10-2014**

Neeta Singh

External Examiner – Dr. Neeta Singh **Date: 21-10-2014**

Member 1 – Dr. M. G. R. Rajan **Date: 21-10-2014**

Member 2 – Dr. J. R. Bandekar **Date: 21-10-2014**

Member 3 – Dr. H. S. Misra **Date: 21-10-2014**

Final approval and acceptance of this thesis is contingent upon the candidate's submission of the final copies of the thesis to HBNI.

I hereby certify that I have read this thesis prepared under my direction and recommend that it may be accepted as fulfilling the thesis requirement.

Date: 21-10-2014

B. N. Pandey

Place: BARC, Mumbai **Guide**

STATEMENT BY AUTHOR

This dissertation has been submitted in partial fulfilment of requirements for an advanced degree at Homi Bhabha National Institute (HBNI) and is deposited in the Library to be made available to borrowers under rules of the HBNI.

Brief quotations from this dissertation are allowable without special permission, provided that accurate acknowledgement of source is made. Requests for permission for extended quotation from or reproduction of this manuscript in whole or in part may be granted by the competent authority of HBNI when in his or her judgment the proposed use of the material is in the interests of scholarship. In all other instances, however, permission must be obtained from the author.

Jayakumar Sundarraj

DECLARATION

I, hereby declare that the investigation presented in the thesis has been carried out by me. The work is original and has not been submitted earlier as a whole or in part for a degree / diploma at this or any other Institution / University.

Jayakumar Sundarraj

List of Publications arising from the thesis

Journal

1. The potential value of the neutral comet assay and the expression of genes associated with DNA damage in assessing the radiosensitivity of tumor cells. Jayakumar S, Bhilwade HN, Pandey BN, Sandur SK, and Chaubey RC. *Mutation Research - Genetic Toxicology and Environmental Mutagenesis* (**2012**) *748*: 52-59.

2. Differential response of DU145 and PC3 prostate cancer cells to ionizing radiation: Role of reactive oxygen species, GSH and Nrf2 in radiosensitivity. Jayakumar S, Kunwar A, Sandur SK, Pandey BN and Chaubey RC. *Biochimica et Biophysica Acta – General Subjects* (**2014**). *1840:* 485–494

Conferences

1. Usefulness of single cell gel electrophoresis in predicting radiosensitivity of tumor cells. Jayakumar S, Thakkar M, Chaubey RC. *Journal of Advanced Biotechnology* (**2010**) *8*:31

2. Predictive Validity of Single Cell Gel Electrophoresis and Expression of Genes Associated with DNA Damage in Assessing Radiosensitivity of Tumor Cells. Jayakumar S, Bhilwade HN, Pandey BN, Santosh Kumar S, and Chaubey RC. International Conference on Advances in Free Radicals, Redox Signaling and Translational Antioxidant Research held by Society for Free Radical Research at Lucknow, Uttar Pradesh (From 30th Jan – 1st Feb 2013).

3. Differential response of DU145 and PC3 prostate cancer cells to ionizing radiation: Role of reactive oxygen species, GSH and Nrf2 in radiosensitivity. Jayakumar S, Santosh Kumar S, and Chaubey RC. Ninth American Association for Cancer Research - Japanese Cancer Association Joint Conference: Breakthroughs in Basic and Translational Cancer Research, held at Maui, Hawaii, USA (From 21st – 25th Feb 2013). Page No:251

Jayakumar Sundarraj

DEDICATION

Dedicated to my Parents …

Acknowledgements

It is my pleasure to thank my guide Dr. B. N. Pandey, Head, Radiation Signalling and Cancer Biology Section, RB & HSD, BARC, for mentoring me throughout the course of this thesis work. I am very fortunate to have such a person with rich scientific acumen, who has provided able guidance, and he was always friendly and approachable. He has been the source of encouragement for me throughout my PhD programme and I thank him for that. I am also indebted to my former guide, Dr. R. C. Chaubey, Former Head, Genetic Toxicology and Chromosome Studies Section, RB&HSD, BARC, who has nurtured me from the day I have joined in BARC, and was instrumental in guiding me for most part of my PhD programme and has always motivated and supported me. I am very fortunate to have such person as mentor in the initial years of my research. I thank him for all the care, advice he has given to me.

I also want to express my heartfelt thanks to Chairmen of my Doctoral Committee, Dr. S. Zingde, Former Dy. Director, ACTREC, for her guidance during the entire course of my PhD work. Her rich experience in cancer biology was a big blessing for me and that helped in improving my work greatly. I also want to place my deepest gratitude to the other members of my Doctoral Committee, Dr. M. Seshadri, Former Head, RB & HSD, Dr. M.G.R. Rajan, Head, RMC, Dr. H.S. Mishra, Head, Molecular Genetics Section, Molecular Biology Division, and Dr. J. R. Bandekar, Head, RB & HSD, for their invaluable guidance and suggestions provided during my doctoral committee meetings, their strong support and co-operation throughout the course of my PhD programme. Any words will not be sufficient to thank them for their advice, support and contribution.

I want to thank Dr. K.B. Sainis, Group Director, Bio-Medical Group, BARC, for his constant support during this course of the PhD. I also want to thank Dr. S. K. Apte, Associate Director, Bio-Medical Group, BARC, for his support during this PhD programme. He has been the source of motivation for me in many ways through his bottomless scientific knowledge, clarity and crystal clear expressions.

I am also very thankful to Dr. S. Santosh Kumar, Head, Free Radical Biology Section, RB & HSD, BARC, for his guidance and support during the course of my PhD programme. He was always there with solution for me whenever I am faced with a hurdle in my journey and without him this thesis would not have seen the light.

I also want to thank my senior colleague, Dr. H. N. Bhilwade, for his constant support and encouragement from the day I have joined in BARC.

I also want to acknowledge my colleagues and friends, Dr. Deepak, Dr. Dharmendra, Vandan, Amit, Ashish, Subhash, Yogendra, Sushanth, Vikas, Thoh, Rahul, Raghav, Gambhir and Swetha for their help and support.

I also want to thank Ms. Jisha Menon and B.A. Naidu for their excellent technical assistance and administrative people for their support.

I want to express my deep gratitude and love to my parents, for their support, motivation and encouragement.

I am also thankful to my brother who has been a role model and the guiding light to my career.

Last but not the least, I want to thank my wife Viji, for her support, affection and encouragement provided to me.

S. Jayakumar

Contents

List of figures

List of tables

ABBREVIATIONS:

ATM	:	Ataxia telangiectasia mutated
ATR	:	ATM Rad3 related
53BP1	:	p53 binding protein1
BRCA1	:	Breast cancer susceptibility gene1
CDKs	:	Cyclin dependent kinases
CHK1/2	:	Check point kinase 1/2
CtIP	:	C-terminal binding protein1 interacting protein
DDR	:	DNA damage response
DEPC	:	Diethyl pyrocarbonate
DMEM	:	Dulbecco's Modified Eagle Medium
DMSO	:	Dimethylsulfoxide
DNA	:	Deoxy Ribonucleic Acid
DNA-PK	:	DNA protein kinase
DNA-PKcs	:	DNA dependent protein kinase catalytic subunit
DSBs	:	Double-strand breaks
EDTA	:	Ethylene diamine tetra acetic acid
EGFR	:	Epidermal growth factor receptor
FCS	:	Fetal calf serum
Gy	:	Gray
GCLC	:	Glutamate-cysteine ligase
GCLM	:	Glutamate-cysteine ligase, modifier subunit
GPx	:	Glutathione peroxidase

GR	:	Glutathione reductase
GSH	:	Glutathione
GSSG	:	Glutathione disulphide
GST	:	Glutathione-S-transferase
HO1	:	Heme oxygenase 1
HRR	:	Homologous recombination repair
HSP70	:	Heat shock proteins
IR	:	Ionizing radiation
KEAP1	:	Kelch-like ECH-associated protein 1
KeV	:	Kilo electron Volt
LIG1	:	DNAligase1
LIG3	:	DNA ligase3
LIG4	:	DNA ligase 4
MAPK	:	Mitogen activated protein kinases
MCL1	:	Myeloid cell leukaemia sequence 1
MeV	:	Mega electron Volt
MRE11	:	Meiotic recombination 11
MRN	:	MRE11/RAD50/NBS1
NBS1	:	Nijmegen breakage syndrome 1
NF-κB	:	Nuclear factor kappa B
Nrf2	:	Nuclear factor (erythroid-derived 2)- like 2
NHEJ	:	NHEJ Non-homologous end-joining
NQO1	:	NAD(P)H dehydrogenase, quinone 1
PARP-1	:	Poly (ADP-ribose) polymerase 1

PCNA	:	Proliferation cell nuclear antigen
PFGE	:	Pulse-field gel electrophoresis
PUMA	:	p53 upregulated modulator of apoptosis
PI3K	:	Phosphatidyl inositol 3-OH kinases
RNA	:	Ribo nucleic acid
ROS	:	Reactive oxygen species
RPA	:	Replication protein A
SF	:	Survival fraction
SOD	:	Superoxide dismutase
SSBs	:	Single-strand breaks
Trx	:	Thioredoxin
TR	:	Thioredoxin reductase

Synopsis

Homi Bhabha National Institute

Ph. D. PROGRAMME

1. **Name of the Student:** Shri S. Jayakumar
2. **Name of the Constituent Institution:** RB&HSD, BARC
3. **Enrolment No.:** Life 01200804012, September 2008
4. **Title of the Thesis:** MOLECULAR STUDIES ON RADIATION RESPONSE OF HUMAN MALIGNANT CELLS IN VITRO
5. **Board of Studies:** Life Sciences

SYNOPSIS

PREAMBLE

Radiotherapy is one of the common treatment modalities for cancer. However, owing to the differences in intrinsic radiosensitivity of the different tumor types, a significant variation in therapeutic response is observed during radiotherapy leading to ineffective killing of tumor cells or adverse side effects associated with damage to normal tissues. Hence, an optimization of radiation dose in clinical practice based on the radiosensitivity of individual patients and tumor types is of paramount importance. This personalization of radiotherapy is possible if we can predict the level of radiosensitivity and then deliver the radiation dose according to the tumor radiosensitivity. But until now there is no clinically applicable assay which can be used in predicting radiosensitivity of tumor cells. Clonogenic assay is the standard assay commonly used to predict the radiosensitivity of the tumor cells. But, clonogenic assay cannot be practiced clinically owing to serious limitations like lack of good clonogenic behaviour in many tumor types/samples and poor plating

efficiency. Requirement of longer time period to obtain the results is another limitation of this assay in clinical scenario. Therefore, there is an urgent need for establishing and evaluating other assays which are fast and reliable in predicting the radiosensitivity of various tumor types.

Multiple factors govern tumor radiosensitivity and a major determinant of radiosensitivity is the interplay of genes which are involved in radiation damage response pathways like DNA repair, redox regulation, cell cycle arrest, and apoptosis. The genes which are involved in these pathways may also prove helpful in predicting tumor radiosensitivity.

With this background, following three objectives were proposed for the thesis:

- Evaluating DNA damage parameters (initial damage, residual damage etc.) as a measure of radiosensitivity in tumor cell lines using comet assay in comparison with clonogenic assay.
- Assessing the usefulness of comet assay in prediction of tumor radiosensitivity after fractionated doses of radiation.
- Analysis of expression pattern of some critical genes involved in apoptosis, DNA repair and cell cycle arrest for their role in assessing tumor radiosensitivity.

The work embodied in the thesis has been divided into following five chapters

Chapter 1: General Introduction

Chapter 2: Assessment of radiosensitivity in human tumor cells using Single cell gel electrophoresis

Chapter 3: Molecular markers in assessment of radiosensitivity of human tumor cells

Chapter 4: Molecular mechanisms governing radiosensitivity of prostate cancer cells

Chapter 5: Summary and future perspectives

Chapters 2-4 describe the experimental results of the thesis. These chapters are further structured in to introduction and the review of literature pertaining to respective chapter followed by material and methods, results and discussion.

CHAPTER 1: General Introduction

This chapter describes the severity of cancer and the importance of radiotherapy. It gives an overview about the interaction of radiation with matter in general and its effect on living cells in terms of damage to the macromolecules and also the about the various pathways activated by the cells in response to radiation induced damage have been outlined. Involvement of DNA damage response pathways in mechanism of cellular radiosensitivity has also been introduced. This chapter also deals about the importance of assessing the tumor radiosensitivity in clinical scenario and in brief about the methods available for this purpose. Further, molecular mechanisms governing the radiation response in tumor cells has been described in light of exploiting them for predicting and modulating their radiosensitivity.

Cancer is one of the leading causes of death worldwide. Cancer causes the death of about 7.6 million people in the world every year. In India about 6.35 lakhs people die every year due to cancer [1]. More than 60% of the cancers are treated using radiotherapy [2]. Radiotherapy is a good alternative for surgical method and helps in long term cure of many cancers like, head and neck, lung, cervix, prostate and bladder [3]. Most of the radiotherapy protocols involve delivering a fixed dose regimen [4]. But because of the difference in intrinsic radiosensitivity of different individuals, radiotherapy develops serious side effects in some individuals and ineffective killing of tumor in some others. Therefore, there is a need for a better understanding about the radiosensitivity of tumor cells which may be helpful in prediction and modulation of tumor radiosensitivity in clinical scenario.

In order to understand the radiosensitivity, it is also very important to understand the interaction of radiation with the biological system. Radiation causes damage to the cells by producing reactive oxygen species (ROS) like hydroxyl radical,

hydrogen peroxide, and superoxide which cause damage to biomolecules. In response to this damage, there is activation of many signal transduction and DNA damage response pathways [5]. These pathways can be either cytoprotective which lead to the repair of the damage, survival and proliferation or cytotoxic, which lead to cell death [6].

Despite the enhanced understanding about the molecular mechanisms governing radiosensitivity, the contribution of other genes and pathways involved in radiosensitivity needs to be investigated to tackle the problem of radiation resistance. The information emerging will help towards potentiating the radiation induced killing of the tumor cells.

CHAPTER 2: Assessment of radiosensitivity in human tumor cells using single cell gel electrophoresis

In this chapter, single cell gel electrophoresis or comet assay was evaluated for its usefulness in assessing the radiosensitivity of tumor cells.

Alkaline and neutral comet assays were evaluated for their usefulness in assessing radiosensitivity by correlating various comet assay parameters with clonogenic survival in different tumor cell lines namely, human fibrosarcoma (HT1080), human colon carcinoma (HT29), human mammary carcinoma (MCF7 and T47D), and human lung adenocarcinoma (A549). Tumor cell lines originated from different tissue types were used to compare their radiosensitivity as well as to evaluate the usefulness of comet assay for determining the radiosensitivity of different cancer types.

Initially the radiosensitivity profile was established in these tumor types using clonogenic survival assay after various doses of gamma-radiation (0.5 Gy to 10 Gy). Values of the survival parameters like SF_2, D_0 and D_{10} were derived from the clonogenic survival curves. These results suggested that amongst the five cell lines used in our experiments, HT1080 cells to be the most radioresistant followed by the MCF7 cells, the T47D cells, the A549 cells and the HT29 cells. In order to know the usefulness of comet assay in assessing radiosensitivity of tumor cells, DNA damage

was estimated in all the five cell lines after exposing to different doses of radiation using both alkaline (measures total DNA damage) and neutral comet assay (measures mainly double strand breaks). Using comet assay, the various parameters like initial DNA damage, repair kinetics, and residual damage were calculated and their correlation with clonogenic survival fraction was analyzed. It was found that initial DNA damage obtained by neutral comet assay correlated well with the radiosensitivity of tumor cells. DNA damage assessed by neutral comet assay showed better correlation than that of the DNA damage obtained by alkaline comet assay. Magnitude of DNA damage assessed immediately after the radiation exposure correlated well with the tumor radiosensitivity than that of other parameters like residual damage. For example, the DNA damage obtained with neutral comet assay after 6 Gy showed better correlation with survival fraction ($r=-0.9$; $p=0.03$) than the corresponding correlation obtained with DNA damage obtained with alkaline comet assay ($r=-0.7$; $p=0.2$). The DNA damage measured immediately after radiation exposure (6 Gy) by neutral comet assay showed better correlation with survival fraction ($r=-0.9$; $p=0.03$) than the corresponding correlation obtained with residual DNA damage ($r=-0.6$; $p=0.3$). Moreover, DNA damage obtained after higher doses (4 Gy or more) showed better correlation with radiosensitivity than the DNA damage obtained at lower doses (<2 Gy).

Fractionated doses are employed during cancer radiotherapy; hence, we also examined whether the radiosensitivity of tumor cells could be assessed by comet assay after fractionated doses of radiation. For this, cells were exposed to fractionated doses of radiation (2 x 2 Gy equivalent to 4 Gy or 3 x 2 Gy equivalent to 6 Gy) followed by clonogenic assay. After fractionation of doses, tumor cells showed higher survival fraction than the respective doses. At fractionated doses, the HT1080 cells had the highest survival fraction, followed by the MCF7 cells, the T47D cells, the A549 cells and the HT29 cells. Moreover, alkaline comet assay and neutral comet assays were also performed in these set of experiments. The radiosensitivities evaluated by the extent of DNA damage observed by the alkaline and the neutral comet assays after the fractionated radiation doses were in the same order as that obtained by the clonogenic assay. But a statistically significant correlation between

DNA damage and survival fraction was observed only with DNA damage obtained by the neutral comet assay and not with the alkaline comet assay. The correlation (r values) obtained between the neutral comet assay and the clonogenic survival are -0.93 (p=0.02) and -0.97 (p=0.004) for 2 x 2 Gy and 3 x 2 Gy, respectively. Similar to the results of acute doses, the total DNA damage, as measured by the alkaline comet assay after fractionated doses, did not show significant correlation with the respective survival fraction. The observed r values were -0.81 (p=0.09) and -0.73 (p=0.16) for 2 x 2 Gy and 3 x 2 Gy, respectively. These results clearly demonstrated the usefulness of DNA damage evaluated by neutral comet assay in assessing the radiosensitivity of tumor cells, after both acute and fractionated doses.

In order to further confirm the usefulness of the standard curve generated using the DNA damage and survival fraction values (from five tumor cell lines) in assessing radiosensitivity, we have chosen two more tumor cells which are of prostate cancer in origin viz., DU145 and PC3. We have performed neutral comet assay immediately after exposing these two tumor cells to either 4 Gy or 6 Gy. The DNA damage values (% DNA in tail) obtained from the neutral comet assay in these cell lines were fitted in the standard curve and survival fraction values were derived. These derived survival fraction values were then compared with actual survival fraction values obtained by performing clonogenic assay. These derived survival fraction values were very close to the actual survival fraction obtained using clonogenic assay. For example, the derived survival fraction for PC3 and DU145 cells at 6 Gy was 0.12 and 0.15 respectively. The actual survival fraction obtained at 6 Gy exposure was 0.10 and 0.13, for PC3 and DU145 cells, respectively.

The major research findings of the chapter are as following:

- A significant correlation exists between the DNA damage assessed by neutral comet assay and clonogenic survival for predicting tumor radiosensitivity. These results are also valid at fractionated doses of irradiation
- The initial DNA damage obtained after radiation exposure is the better indicator of the radiosensitivity than the residual DNA damage

- Correlation between DNA damage obtained by neutral comet assay and radiosensitivity was better than the correlation obtained using alkaline comet assay
- Standard curve obtained (DNA damage vs. survival fraction) from five tumor cell lines could predict the radiosensitivity of prostate cancer cells with good accuracy

CHAPTER 3: Molecular markers in assessment of radiosensitivity of human tumor cells

In previous chapter, we have observed a significant correlation between magnitude of DNA damage and tumor radiosensitivity. Therefore, it is plausible that the genes involved in DNA damage response may also show predictive value in assessing radiosensitivity of tumor cells. DNA damage responses include cell cycle arrest, DNA repair, apoptosis and modulation of cellular redox. Hence in this chapter, the predictive potential of the expression of genes which are involved these pathways in assessing radiosensitivity of tumor cells was investigated. Towards this objective, expression profile of genes involved in cell cycle arrest (*CDKN1A* and *GADD45A),* DNA repair (*KU80, RAD51,* and *HSP70),* apoptosis (*MCL1, BCL2, BAX, FAS, PUMA,* and *MDM2)*, and redox regulation (*NRF2, NQO1, GCLC,* and *HO1)* were investigated after radiation exposure in all six different tumor cells, and their correlation with survival fraction was studied.

Of the 15 genes analyzed, three genes which are involved in DNA repair namely, *KU80, HSP70,* and *RAD51,* showed a significant positive correlation with survival fraction. The r values observed were 0.97 (p=0.02), 0.99 (p=0.01) and 0.97 (p=0.02) for *HSP70, KU80* and *RAD51,* respectively. The other genes did not exhibit a significant correlation with clonogenic survival either at 2 Gy or 6 Gy (The r values ranges from 0.8 to -0.6). Since DNA damage showed correlation with radiosensitivity in our earlier study, we performed a correlation analysis between DNA damage, as assessed by the neutral comet assay, and the relative gene expression of *HSP70, KU80* and *RAD51.* Expression of all three genes after radiation exposure (6 Gy) also showed a significant correlation with the DNA damage, as determined by the neutral

comet assay. The r values obtained from this correlation analysis were -0.98 (p=0.018), -0.92 (p=0.05), and -0.90 (p=0.08) respectively for HSP70, KU80, and RAD51 genes.

Since we have observed the correlation between the DNA repair genes and radiosensitivity, we have further investigated the usefulness of these DNA repair genes in radiosensitization of tumor cells. We have selectively inhibited DNA-PK, a protein involved in non-homologous end joining (NHEJ) followed by radiation exposure and analysis of survival fraction. Inhibition of DNA-PK alone did not cause any significant reduction in survival fraction in control cells. But when combined with radiation it synergistically enhanced the radiation induced killing of the PC3 and DU145 tumor cells at various radiation doses. At 8 Gy survival fraction observed with PC3 and DU145 cells were 0.014 and 0.104 respectively. Whereas the survival fraction obtained with DNA-PK inhibition prior to the radiation exposure (8 Gy) are 0.0017 and 0.0025 respectively for PC3 and DU145 cells.

The major research findings of the chapter are as following:

- Expression of HSP70, KU80 and RAD51 can be useful in assessing the tumor radiosensitivity as they are showing significant correlation with clonogenic survival fraction.
- Significant correlation also was observed between the magnitude of DNA damage obtained by neutral comet assay and extent of expression of DNA repair genes
- Inhibition of DNA-PK can be useful in potentiating the radiation induced killing in tumor cells.

CHAPTER 4: Molecular mechanisms governing radiosensitivity of prostate cancer cells

In chapter 2, prostate cancer cells PC3 and DU145 exhibited differential radioresistance as observed by clonogenic survival. The differential radiosensitivity of these tumor cells was also investigated using other parameters like DNA damage, DNA repair kinetics, and apoptosis. All these results showed that DU145 is more radioresistant than PC3 cells. Hence, in this chapter, we have studied the mechanism

of differential radiosensitivity between these two cancer cells. One of the major consequences of radiation exposure on the cells is change in cellular redox status through generation of reactive oxygen species [7]. Therefore, we have initially measured the cellular ROS levels in these two cells and found that the basal and induced levels of ROS to be significantly lower in radioresistant DU145 cells in comparison to the PC3 cells. Mitochondria was one of the important organelle, which is involved in the radiation induced apoptotic death, the level of ROS in mitochondria also were studied between these two cell types. Similar to the cytosolic ROS, radioresistant DU145 cells showed significantly lower basal and radiation induced level of mitochondrial ROS, in comparison to PC3 cells.

Cells respond to the oxidative stress through intracellular antioxidants like glutathione, thioredoxin reductase, and glutathione peroxidase [8]. These antioxidants help cells in scavenging ROS and salvaging biomolecules from oxidative damage. Therefore, we have examined the level of these anti-oxidant species like GSH, GSH/GSSG ratio, and thioredoxin reductase in these two tumor cells. In the results DU145 cells showed higher level of basal GSH content, GSH/GSSG ratio, and thioredoxin reductase than that of PC3 cells. Since these two tumor cells showed differential ROS level and anti-oxidant response, we hypothesised that the redox regulation in these two cells may be different and that the redox sensitive transcription factor Nrf2 may be playing role in the differential radiosensitivity exhibited by these two cell types.

Under normal conditions, Nrf2 is sequestered in the cytoplasm by binding to KEAP1 protein [9]. In response to oxidative stress, Nrf2 undergoes a rapid translocation into the nucleus, binds to antioxidant response elements in the promoter regions of its target antioxidant genes such as HO1, GCLM, GCLC, thioredoxin reductase 1 and facilitates their transcription [10]. Hence, Nrf2 and its dependent genes may play a crucial role in determining radiosensitivity of tumor cells. Therefore, we have further investigated the role of Nrf2 and its dependent genes in determining radiosensitivity of PC3 and DU145 tumor cells. When we have analysed the nuclear levels of Nrf2 using EMSA, both basal and radiation induced level of Nrf2 was found to be significantly higher in DU145 cells in comparison to PC3

cells. Moreover, we have analysed the expression level of Nrf2 and its dependent genes using the real time q-PCR analysis. These results indicated that the expression of Nrf2 and its dependent genes were significantly higher in DU145 cells in comparison to PC3 cells.

In order to confirm the role of Nrf2 and its dependent genes in radiosensitivity of tumor cells, we examined the survival fraction of PC3 and DU145 cells after radiation exposure, in the presence or absence of inhibitors of Nrf2 and HO1 namely ATRA and SnPP, respectively. Presence of these inhibitors significantly reduced the survival fraction of above tumor cells against the radiation exposure. Treatment with above inhibitors prior to radiation exposure at 4 Gy reduced the survival fraction of PC3 cells from 0.21 to 0.01 and that of DU145 cells from 0.31 to 0.03. Further, to corroborate this evidence of involvement of Nrf2 in radiosensitivity, we have employed knockdown approach by transfecting shRNA against Nrf2 in to DU145 cells. Though Nrf2 knockdown itself has not exhibited any significant reduction in survival, radiation exposure of Nrf2 knockdown cells exhibited synergistic reduction in survival fraction (0.035) in comparison to 4 Gy irradiation group (0.28). Similar results were also observed in the HO1 knockdown studies emphasising the role of Nrf2 and its dependent genes in radiosensitivity of these tumor cells.

The major research findings of the chapter are as following:

- Prostate cancer cells PC3 and DU145 exhibited differential radioresistance.
- Differential redox regulation was observed in DU145 and PC3 cells.
- Nrf2 and its dependent gene HO1 was playing major role in radiation resistance of DU145 cells

CHAPTER 5. General Discussion, Summary and Future Perspectives

In this chapter, a general discussion about the overall findings has been made with respect to the current understanding. This chapter also mentions about the major conclusions and future research directions generated out the thesis.

The major conclusions of the thesis are:

1. DNA double strand breaks as assessed by neutral comet assay can be useful in predicting the tumor radiosensitivity. These results are also valid in fractionated doses of radiation
2. DNA damage obtained by neutral comet assay is a better marker of radiosensitivity than that of the DNA damage obtained by alkaline comet assay
3. DNA damage estimated immediately after the radiation exposure showed better correlation with tumor radiosensitivity than that of other parameters like residual damage
4. Magnitude of expression of DNA repair genes like KU80, RAD51 and HSP70 showed their usefulness in predicting tumor radiosensitivity than the genes involved in other pathways like apoptosis
5. Role of non-homologous end joining pathway in radiosensitization has been shown using DNA-PK inhibitor
6. Role of reactive oxygen species, glutathione, Nrf2 and its dependent genes were shown in the differential radio-response of PC3 and DU145 prostate tumor cells
7. Inhibition of Nrf2 and its dependent genes can be used as strategy in potentiation of radiation induced damage in the tumor cells

Our studies emphasize the usefulness of the neutral comet assay and gene expression of DNA repair genes in the assessment of the radiosensitivity of tumor cells. However, to gain greater confidence in use of these techniques in determining the radiosensitivity of tumor cells, a larger number of cell lines and validation using biopsy samples from cancer patients is needed. Similar to the DNA repair genes, other genes which are involved in the upstream DNA damage signaling (like γ-H2AX, ATM, ATR, CHK1, CHK2 and SMC1) and downstream survival pathways (MAP kinase pathway and NF-κB pathway) can also be evaluated for their usefulness in predicting tumor radiosensitivity. Validation of the DNA-PK as target of radiosensitization using specific novel inhibitors also is necessary to exploit NHEJ as target in

radiosensitization of tumor cells. Apart from NHEJ, we can also inhibit HSP70 and use that as strategy in radiosensitization of tumor cells. Since tumor cells have mutation in DNA repair pathways in most of the cases, drugs targeting the selective DNA repair pathways can selectively sensitize tumor cells to radiation induced damage in comparison to normal cells. Apart from DNA repair pathways, Nrf2 inhibition in radiosensitization should be further validated *in vivo* tumor models.

REFERENCES

[1] I. Ali, Cancer Scenario in India with Future Perspectives, Cancer Therapy, 18 (2011) 56-70.

[2] J.F. Torres-Roca, C.W. Stevens, Predicting response to clinical radiotherapy: past, present, and future directions, Cancer Control, 15 (2008) 151-156.

[3] G. Delaney, S. Jacob, C. Featherstone, M. Barton, The role of radiotherapy in cancer treatment: estimating optimal utilization from a review of evidence-based clinical guidelines, Cancer, 104 (2005) 1129-1137.

[4] J. Shafiq, M. Barton, D. Noble, C. Lemer, L.J. Donaldson, An international review of patient safety measures in radiotherapy practice, Radiotherapy and Oncology, 92 (2009) 15-21.

[5] R.K. Schmidt-Ullrich, P. Dent, S. Grant, R.B. Mikkelsen, K. Valerie, Signal transduction and cellular radiation responses, Radiat Res, 153 (2000) 245-257.

[6] L. Li, M. Story, R.J. Legerski, Cellular responses to ionizing radiation damage, International Journal of Radiation Oncology*Biology*Physics, 49 (2001) 1157-1162.

[7] M.F. Renschler, The emerging role of reactive oxygen species in cancer therapy, European Journal of Cancer, 40 (2004) 1934-1940.

[8] J. Sun, Y. Chen, M. Li, Z. Ge, Role of Antioxidant Enzymes on Ionizing Radiation Resistance, Free Radical Biology and Medicine, 24 (1998) 586-593.

[9] M. Kobayashi, M. Yamamoto, Molecular mechanisms activating the Nrf2-Keap1 pathway of antioxidant gene regulation, Antioxidants & redox signaling, 7 (2005) 385-394.

[10] J.Y. Chan, M. Kwong, Impaired expression of glutathione synthetic enzyme genes in mice with targeted deletion of the Nrf2 basic-leucine zipper protein, Biochim Biophys Acta, 1517 (2000) 19-26.

PUBLICATIONS OF THE THESIS

Journal Publications

1. Jayakumar S, Bhilwade HN, Pandey BN, Sandur SK, and Chaubey RC. (2012). The potential value of the neutral comet assay and the expression of genes associated with DNA damage in assessing the radiosensitivity of tumor cells. *Mutation Research - Genetic Toxicology and Environmental Mutagenesis* **748**: 52-59.

2. Jayakumar S, Kunwar A, Sandur SK, Pandey BN and Chaubey RC. (2014). Differential response of DU145 and PC3 prostate cancer cells to ionizing radiation: Role of reactive oxygen species, GSH and Nrf2 in radiosensitivity. *Biochimica et Biophysica Acta – General Subjects* **1840:** 485–494

Abstracts published in conference proceedings

1. Jayakumar S, Thakkar M, Chaubey RC. 2010. Usefullness of single cell gel electrophoresis in predicting radiosensitivity of tumor cells. Journal of Advanced Biotechnology, **8:**31
2. Jayakumar S, Bhilwade HN, Pandey BN, Santosh Kumar S, and Chaubey RC. (2013). Predictive Validity of Single Cell Gel Electrophoresis and Expression of Genes Associated with DNA Damage in Assessing Radiosensitivity of Tumor Cells. International Conference on International Conference on Advances in Free Radicals, Redox Signaling and Translational Antioxidant Research held by Society for Free Radical Research at Lucknow, Uttar Pradesh (From 30th Jan – 1st Feb 2013)
3. S. Jayakumar S, Santosh Kumar S, and Chaubey RC. (2013). Differential response of DU145 and PC3 prostate cancer cells to ionizing radiation: Role of reactive oxygen species, GSH and Nrf2 in radiosensitivity. Ninth American Association for Cancer Research - Japanese Cancer Association Joint Conference: Breakthroughs in Basic and Translational Cancer Research, held at Maui, Hawaii, USA (From 21-25 Feb 2013). Page No:251

CHAPTER 1

GENERAL INTRODUCTION

1.1. Cancer

Cancer is one of the leading causes of death worldwide. Cancer causes the death of 7.6 million people in the world every year. In India about 6.35 lakh people die every year due to cancer [1, 2]. This represents about 6 % of all death in India. Absolute number of death due to cancer in India is expected to rise because of the increasing population and increase in life expectancy (ICMR report 2012). Among men, the three most common fatal cancers in India are, oral cancer, stomach cancer, and lung cancer with each contributing 22.9 %, 12.6 %, and 11.4 % of cancer deaths respectively. Among women, cervical cancer causes the most death (17.1% of all cancer deaths) followed by stomach (14.1 %) and breast cancer (10.2 %) [1]. Cancer develops as a result of abnormal growth of the normal cells, which have managed to overcome the control mechanisms available in the biological system [3, 4]. Apart from growth and uncontrolled division, cancer cells also have the ability to move through the blood/lymphatic vessels and invade the other tissues of the system [5, 6]. Based on the cells from which it develops, cancer can be called either as carcinoma or as sarcoma. Carcinomas arise from epithelial cells and sarcomas arise from connective tissues and bones. Apart from these two major types, cancer develops from white blood cells are called either as leukemia or lymphoma. Cancers arising from myeloid cells of the blood are called myeloid leukemias. Cancers arise from brain cells are called as glioma, astrocytomas, or medulloblastomas. Out of these types, majority of the cancers are of carcinomas arising from epithelial cells. Probably because, the epithelial cells are the ones, which are frequently exposed to many physical and chemical damages that may favour the development of cancer.

1.2. Radiotherapy and its importance

Cancers are treated by three very important methods. One is the age old surgical method, which is a primary form of treatment and helps in effective cure of a range of non-metastasized tumors [7, 8]. Another useful treatment modality for cancer is chemotherapy. Many patients receive chemotherapy at some point of the cancer treatment and it provides useful symptomatic relief and disease arrest. Number of chemotherapeutic agents is available, which can target different pathways and molecules. There are several recent advances in chemotherapy, which include the molecularly targeted drugs such as the inhibitors of EGFR and Bcr-abl [9, 10] and monoclonal antibodies to cell surface molecules such as EGFR and CD 20. Radiotherapy is another major treatment modality used for treating the cancer. More than 50 % of the cancers are treated using radiotherapy [11, 12]. Radiotherapy is given with or without surgery depending on the clinical and pathological status of the tumor. It helps in long term cure of many cancers like, head and neck, lung, cervix, prostate and bladder. In addition to the curative roles of radiotherapy, many patients gain good palliation by radiation. The proportion of patients who undergo radiotherapy are given in Table 1-1 [12].

Table: 1-1. Percentage of cancer patients treated with radiotherapy [12]

Tumor type	% of all cancers	% of patients from particular cancer receiving radiotherapy	Patients receiving radiotherapy (% of all cancers)
Breast	0.13	83	10.8
Lung	0.10	76	7.6
Melanoma	0.11	23	2.5
Prostate	0.12	60	7.2
Gynecologic	0.05	35	1.8
Colon	0.09	14	1.3
Rectum	0.05	61	3.1
Head and neck	0.04	78	3.1
Gall bladder	0.01	13	0.1
Esophageal	0.01	80	0.8
Stomach	0.02	68	1.4
Pancreas	0.02	57	1.1
Lymphoma	0.04	65	2.6
Leukemia	0.03	4	0.1
Myeloma	0.01	38	0.4
Central nervous	0.02	92	1.8
Renal	0.03	27	0.8
Bladder	0.03	58	1.7
Testis	0.01	49	0.5
Thyroid	0.01	10	0.1
Other	0.06	60	3.4
Total	1.0		52.3

The most widely practiced radiotherapy regimen includes the delivery of 2 Gy /day dose to the tumor for five days in a week [13]. The total treatment time is normally for the period of 4 to 5 weeks. During cancer radiotherapy, the major objective is to deliver maximum possible dose to the tumor cells and minimize the exposure to normal tissues. To achieve this objective, in recent past, significant progress has been made in developing radiotherapy machines, with the help of computerization, automation and precise imaging of tumor volume. Some of these advancements are listed below [14-16].

Stereotactic radiotherapy: In this mode of radiotherapy, radiation is delivered through several beams that are focused precisely on the three-dimensionally localized target [17].

Three-dimensional conformal radiotherapy (3D-CRT): In this technique the dose volume is made to conform closely to the target through the use of 3D-anatomical data acquired from CT or MRI imaging modalities [18].

Intensity modulated radiotherapy (IMRT): It is a refined form of 3D-CRT in which highly conformed dose distribution around the target is provided using non uniform beam intensities [19] to focus radiation to tumor areas.

Image guided radiotherapy (IGRT): This modality integrates the various radiological and functional imaging techniques in order to perform high precision radiotherapy. This modality takes into account target reduction as it occurs during the course of radiotherapy (Tumor volume decrease and weight loss) so as to reduce the internal margins [19].

1.3. Implications of cellular radiosensitivity in radiotherapy

Despite the technological advancements, further improvement of radiotherapy is very difficult in the clinical circumstances due to following factors [20]:

a) Radiation cannot be completely focused and delivered only to the tumor cells
b) Variation in radiosensitivity due to induced radio-resistance in tumor cells involving various molecular pathways during the course of radiotherapy
c) Difference in radiosensitivity of tumor cells from different tissues
d) Difference in radiosensitivity of same tumor type from different individuals
e) Difference in radiosensitivity between the tumors cells and surrounding normal tissues of the same individual

The above said issues are influenced by the fact that most of radiotherapy protocols are not based on radiosensitivity of tumor types / surrounding normal tissues of the particular patient. When the tumor is radioresistant, the radiation delivered may not be sufficient to kill all the tumor cells, which may lead to the tumor recurrence. On the other hand, if the surrounding tissues are more sensitive to radiation, the delivered dose might lead to substantial normal tissue damage, which may lead to lot of side effects including secondary malignancy in long term. Therefore, it is very important to have better understanding about the radiosensitivity of tumor and normal cells in terms of molecular players and biological pathways controlling the radiosensitivity. Such understanding will be helpful in developing molecular markers and assays, which can be useful in predicting/assessing the radiosensitivity prior to radiotherapy (Fig. 1-1). Understanding the radiosensitivity of normal and tumor cells will also pave the way for modulating the radiation responses of tumor cells by selectively targeting certain proteins, which otherwise contribute to radio-resistance.

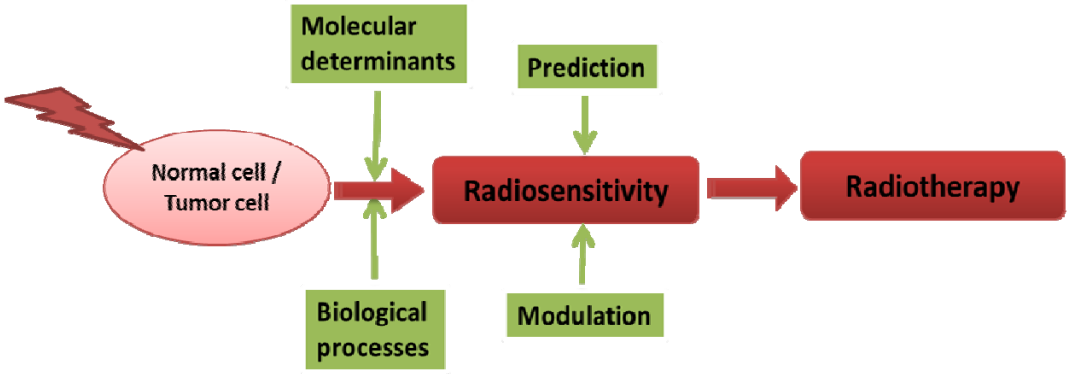


Fig: 1-1. Ways though which radiosensitivity of tumor cells can be addressed, which can ultimately be useful in improving the radiotherapy. Many molecular factors and associated biological processes (in response to radiation) govern the radiosensitivity of a cell. These factors and endpoints can be useful in prediction and modulation of radiosensitivity leading to the improved efficacy of cancer radiotherapy.

1.4. Interaction of radiation with biological systems

To understand the radiosensitivity, it is important to understand the dynamics of radiation interaction with the biological systems. Interaction of radiation with biological system involves a series of events, which vary enormously on time scale. Radiation exposure leads to the excitation and ionization of molecules present in the cells, which results in damage to the various biomolecules, most importantly the DNA [21]. In response to this damage, cells mount a complex network of signaling events, which ultimately determine the cellular radiosensitivity [22-25].

1.4.1. Ionizing radiation and process of energy absorption

Ionizing radiations are either particulate or electromagnetic in nature, which have sufficient energy to remove one or more orbital electrons from the atoms or molecules. When ionizing radiation or the charged particle traverses through the cell, it interacts with the different atoms of the molecules in the cell. During this interaction incoming radiation deposits its energy to the electrons present in the atoms. If the energy is deposited on the outer orbital electrons, it gets excited and if the energy is sufficient, it causes ionization. The emitted electrons will cause further series of ionizing events depending on their energy. These ionization and excitation events occur at the time scale of 10^{-16} seconds and are the first events involved in the radiation effects on the biological system.

When ionizing radiation is traversing through the medium, depending on the energy of the photon and the nature of the medium, energy absorption happens through either one of three following ways: photoelectric absorption, Compton scattering and pair production. When energy is absorbed through photoelectric absorption, the incoming photon deposits all its energy to the electron and eventually ejects it. The ejected electron will have kinetic energy equivalent to the energy of the

photon minus the binding energy of the ejected electron. If the energy of the incoming photon is in the range of 200 KeV to 20 MeV, then the energy absorption occurs through Compton scattering. In this process, the incoming photon deposits a part of its energy and produces fast moving electrons. After this ionization the path of the photon is deflected and it participates in further ionization events. The ejected electrons also further lead to secondary ionizations. Compton scattering is an important process of ionization, as the radiation used for radiotherapy falls in this energy range. Photons with the energy >1.02MeV may also participate in pair production reaction, in which the photon interacts with nucleus and forms electron-positron pair. Positron eventually captured by an electron and annihilation takes place. The annihilation results in production of two photons with the energy of 0.51 MeV, which travel in opposite direction to each other. These photons further participate in Compton scattering or photoelectric absorption in the medium depending on their energy.

1.4.2. Direct and indirect action of radiation

When a particulate or γ-radiation passes through a cell, it will lead to the excitation and ionization of the biomolecules. If the radiation directly deposits its energy to the target biomolecules (DNA, lipid and proteins) and thereby lead to damage, that is called direct effect [26, 27]. Apart from these biomolecules, cells also contain water as their major constituent (~90%). When ionization and excitation takes place in the water molecule, it leads to the formation of free radicals (free radicals are the atom or molecule with an unpaired electron, which makes them highly reactive). These free radicals can diffuse in the medium, react with target molecules, and thereby cause ionization. The ionization in the target molecule through the formation of free radicals is called as indirect action of radiation.

1.4.3. Radiolysis of water

Since water constitutes the major proportion of the cell, most of the energy of the traversing photons is deposited in the water molecule leading to the either excitation and/or ionization of the water molecule. The excited water molecule can further decompose into hydroxyl radical and hydrogen radical. When the water molecule is ionized, it leads to the formation of water radical and aqueous electron. The water radical can readily lose one proton and give rise to hydroxyl radical as given in the following equation:

$$H_2O \longrightarrow H_2O^* \text{ (Excitation)}$$

$$H_2O^* \longrightarrow {}^{\cdot}OH + H^{\cdot}$$

$$H_2O \longrightarrow H_2O^{\cdot +} + e^- \text{ (Ionization)}$$

$$H_2O^{\cdot +} + H_2O \longrightarrow H_3O^+ + {}^{\cdot}OH$$

Since these free radicals form in spurs, they can react with each other and can form measurable products like, H_2O_2 , and H_2

$${}^{\cdot}OH + {}^{\cdot}OH \longrightarrow H_2O_2$$

$${}^{\cdot}OH + {}^{\cdot}H \longrightarrow H_2O$$

$${}^{\cdot}H + {}^{\cdot}H \longrightarrow H_2$$

In the presence of oxygen, hydrogen radical and aqueous electrons react with oxygen and produces superoxide radical as given in the following equation:

$$^{\cdot}H + O^{2} \longrightarrow HO_2^{\cdot} \text{ (hydroperoxyl radical)}$$

$$HO_2^{\cdot} \longrightarrow H^{+} + O_2^{\cdot -} \text{ (superoxide radical)}$$

$$e^{-}_{aq} + O_2 \longrightarrow O_2^{\cdot -}$$

1.4.4. Effect of radiolytic products in the target molecules

The radiolytic products formed from the ionization of water can diffuse and can react with target biomolecules and can initiate biological consequences. These radiolytic products of water mainly react with biomolecules in one of the following ways:

1.4.4.1. Hydrogen abstraction

Hydrogen abstraction is one of the major reaction through which damage to biomolecules is incurred through free radicals. Through this reaction, hydrogen radicals and hydroxyl radicals abstract a hydrogen atom from the reactive hydrogen group attached to a biomolecule and leading to the formation of biomolecule radical. The reaction can be represented using a following equation:

$$R\text{-}H + {}^{\cdot}H \longrightarrow H_2 + R^{\cdot} \text{ (solute radical)}$$

$$R\text{-}H + {}^{\cdot}OH \longrightarrow H_2O + R^{\cdot}$$

Generation of biomolecule radical through hydrogen abstraction is believed to be the major contributor to the biological damage of ionizing radiation.

1.4.4.2. Dissociation reaction

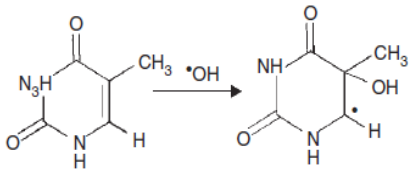
Reaction of aqueous electron and hydrogen electron with amine group attached to biomolecules can lead to the dissociation of amine group leading to the formation of radical, as given in the following equation

$$R\text{-}NH_2 + {}^{\cdot}H \longrightarrow NH_3 + R^{\cdot}$$

$$R\text{-}H + e^-_{aq} \longrightarrow NH_3 + R^{\cdot}$$

1.4.4.3. Addition reaction

In addition reaction, the hydroxyl radical react with the C=C double bonds at diffusion controlled rates. Because of the high electrophilic nature of the C=C, these reactions are largely selective. For example, the ·OH radical can attack the double bond of thymine at C-5 or C-6 and lead to the formation of 5-hydroxythymine intermediate, which can react with oxygen to yield thymine glycol [28].



Thymine 5- hydroxyl thymine radical

1.4.5. Antioxidant enzymes

Cells are always subjected to oxidative insults caused by both endogenous and exogenous generation of free radicals. In order to overcome this oxidative stress, cells have evolved many antioxidant mechanisms. They can be categorized into enzymatic

and non-enzymatic antioxidants [29, 30]. Major antioxidant systems and their functions are described below:

1.4.5.1. Superoxide dismutase

Superoxide dismutase (SOD) is the first antioxidant enzyme to be discovered to have ROS scavenging activity [31] in the year 1969. In the reaction catalysed by SOD, two molecules of superoxide get converted to hydrogen peroxide and molecular oxygen [32, 33].

$$2O_2^{\cdot -} + 2H^{+} \xrightarrow{SOD} H_2O_2 + O_2$$

Three different forms of SOD are present in mammalian system, and among them SOD1 and SOD3 have zinc and redox active copper as their active centre, whereas SOD2 has manganese in their reactive centre. Location wise SOD1 is cytosolic in nature, SOD3 is of extracellular nature and SOD2 is localized in mitochondria. SOD2 is the most crucial anti-oxidant enzyme, which plays a crucial role in disproportionation of superoxide radicals produced in mitochondria. If the SOD2 gene is knocked out it leads to lethality in the very early stages of development, emphasising the important role of SOD2 [34].

1.4.5.2. Catalase

Catalase is the heme-containing enzymes predominantly located in the peroxisomes [35]. Catalases catalyse the dismutation reaction of hydrogen peroxide to molecular oxygen and water as given below.

$$2H_2O_2 \xrightarrow{Catalase} 2H_2O + O_2$$

One of the major roles of catalase is to reduce the risk of $^{\cdot}OH$ formation from hydrogen peroxide through Fenton reaction.

1.4.5.3. Glutathione peroxidase

Glutathione peroxidase (GPx) - a seleno protein, which contains selenium in its active site, acts on hydrogen peroxide and converts it into water. But unlike catalase, GPx needs glutathione (GSH) as a reducing reagent for this reaction. In this process GSH gets oxidised to its disulphide form (GSSG). This GSSG is again reduced to its original form with a help of an enzyme glutathione reductase (GR). For this reaction, NADPH is used as reducing equivalent. The overall reactions of the GSH and GPx system are given below (Fig. 1-2).

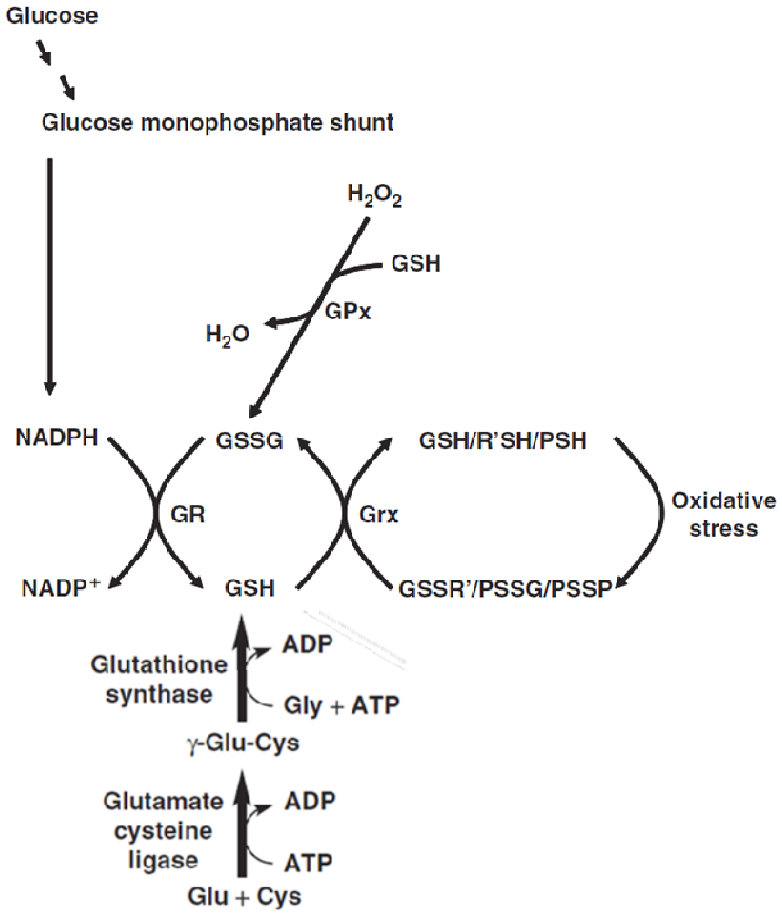


Fig: 1-2. Role of glutathione system in redox homeostasis. Cells are protected from oxidative damage using GSH to detoxify H_2O_2 via glutathione peroxidase (GPx), using GSH-dependent glutaredoxin (Grx) to dethiolate protein disulphides or glutathionylated proteins or nonprotein thiols (R'). The decreased GSH pool is

enriched by glutathione reductase (GR) with NADPH as the electron donor generated from the glucose monophosphate shunt. Glutathione is also replenished by the de novo synthesis with the help of enzymes glutamate cysteine ligase and glutathione synthase. [36]

1.4.5.4. Peroxiredoxins

Peroxiredoxin (Prx) is also an antioxidant enzyme, which catalyses the dismutation of hydrogen peroxide to water. Prx also requires a reducing agent, which is thioredoxin (Trx) in most of the cases. Since, Prx contains two critical cysteine residues, it can act as a good sensor of oxidative stress level in the cell. The oxidised thioredoxin will be converted back to the reduced form by thioredoxin reductase (TR) (Fig. 2-3)

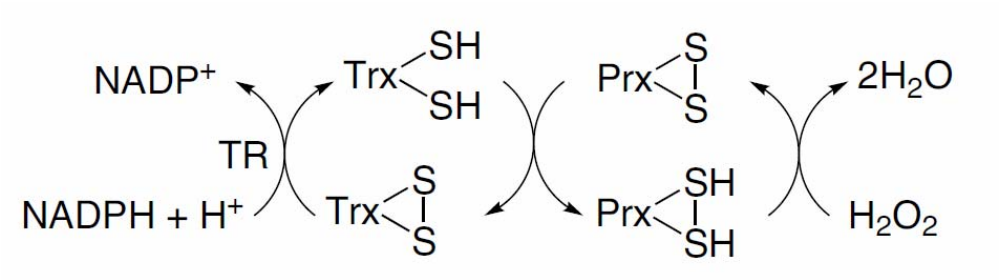


Fig: 1-3. Thioredoxin based anti-oxidant system. Peroxiredoxin dismuates H_2O_2 and thioredoxin helps in recycling of peroxiredoxin. Thioredoxin reductase (TR) reduces the oxidised thioredoxin using NADPH (Adopted from Winyard et al., 2009) [37]

1.4.5.5. Non enzymatic antioxidants

Apart from the above said enzymatic antioxidants, many non enzymatic antioxidants also play crucial role in scavenging free radicals and salvaging the cells from oxidative damage. Ascorbate (vitamin C) is one of the most important water soluble non enzymatic antioxidants present in the cell [38]. This is the most effective radical-scavenger and probably the most abundant free radical scavenger in many cell

types. Ascorbate reacts readily with reactive species such as hydroxyl, alkoxyl, and peroxyl radicals. Another important reactivity of ascorbate is its ability to reduce the tocopheroxyl radical to tocopherol (a lipid soluble non enzymatic antioxidant). Ascorbate and tocopherol together prevent the propagative peroxidation of membrane lipids under oxidative stress conditions.

1.4.6. DNA damage by ionizing radiation

Ionization of DNA leads to the breakage of bonds in the DNA. If the phosphodiester bond connecting the nucleotides gets broken, that leads to the single strand breaks (SSBs). When the phosphodiester bond present in the opposite strands in the close vicinity (within 10 base pair range) gets broken, it results in double strand breaks (DSBs). Apart from the strand breaks, many other damages like base damage, base modification, and sugar modification may also take place after radiation exposure. It is estimated that when mammalian cells are exposed to 1 Gy of γ-radiation, it causes 1000–2000 damaged bases, 1200 damaged sugars, 1000 SSBs, 40 DSBs, and 150 DNA-protein cross-links [28].

1.5. DNA damage response

DNA being the crucial biomolecule, any unrepaired DNA will have severe cellular consequences. In response to DNA damage, cells activates a signal transduction pathway that senses DNA damage and sets in motion a choreographed response to protect the cell and ameliorate the threat to the organism, which is called as DNA damage response (DDR) [23, 39-42]. DDR is a combination of many events, which ultimately determine the cellular fate after the DNA damage. The proteins involved in DDR can be classified into two categories namely, sensors and effectors. Sensor proteins constantly monitor the genome for any DNA damage and in case of any damage they sense it and activate the effector proteins. The effector proteins then

act upon the DNA damage and it ultimately leads to the either one of the following outcomes (Fig. 1-4 and Fig. 1-5) (a) cell cycle arrest (b) DNA repair (c) apoptosis or cell death (d) cytoprotective responses leading to cell survival (e) Mutation or neoplastic transformation

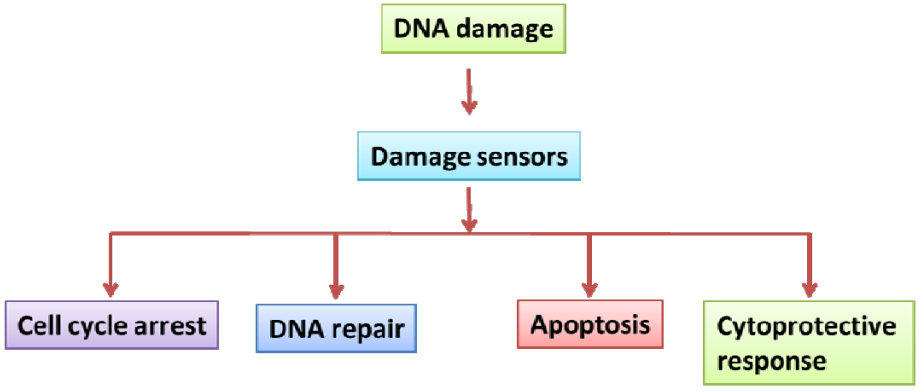


Fig: 1-4. Schematic representation of DNA damage response

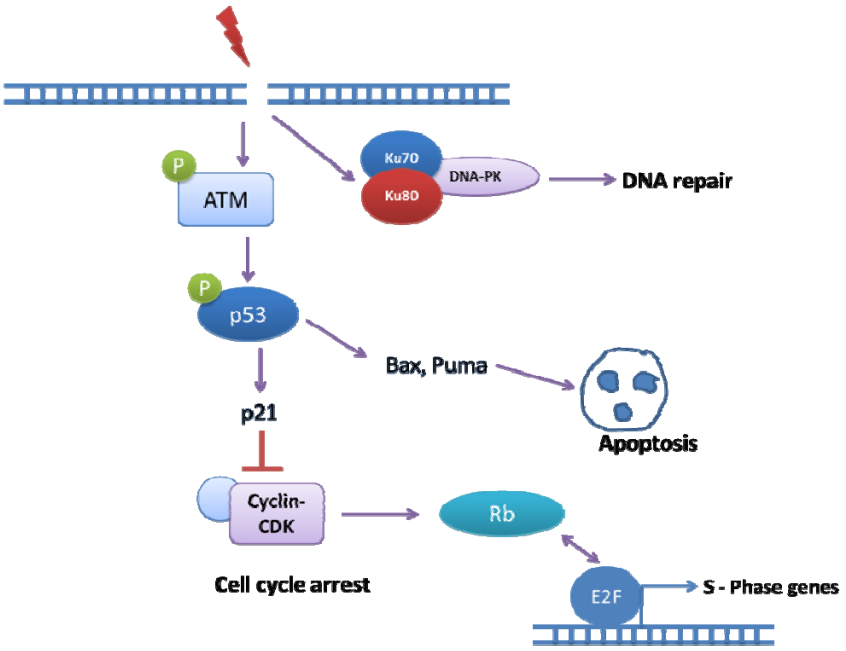


Fig: 1-5. Some of the major players involved in DNA damage response and its outcomes

1.5.1. Sensing of DNA damage

DNA damage is mainly sensed by proteins belonging to the phosphatidylinositol 3- kinases, namely, ATM (Ataxia Telangiectasia mutated), ATR, DNA-PK (DNA-dependent protein kinase). DNA damage is also sensed and bound by members of poly ADP-ribose polymerase (PARP) proteins. ATM and DNA-PK are activated mainly in response to DSBs. Binding of ATM to the damaged site is preceded by binding of MRN (Mre11, RAD50, and NBS1) complex [43-46]. When ATM binds to the damaged site, it leads to the formation of active monomeric forms. Auto-phosphorylation activated ATM leads to the phosphorylation of MRN complex and many other target proteins (53Bp1, H2AX, CHK2, SMC1, p53, BRCA1, Artemis etc.), which are involved in DDR. DNA-PK is a trimeric nuclear serine/threonine kinase composed of a catalytic subunit and two DNA-targeting proteins, KU70 and KU80. The catalytic subunit, by itself, is inactive. It relies on KU70 and KU80 components to direct it to the DNA and trigger its kinase activity. DNA-PK recognizes and initiates repair of DNA double-strand breaks [47]. PARP member of the proteins also play a very important role in sensing DNA damage and activating DDR. Among 16 members of PARP proteins, PARP1, and PARP2 are implicated in DDR. They recognise SSBs, DSBs and catalyse the addition of poly-ADP ribose chain to the proteins, which again recruit proteins involved in the DDR. ATR gets activated in response to SSBs and stalled replication forks.

1.5.2. Cell cycle arrest

Cell cycle checkpoint mechanisms are used by cells to verify the DNA integrity before it moves from one stage of cell cycle to the other. Cell cycle checkpoints are also activated in response to DNA damage. During the activation of cell cycle checkpoints, genes for repair pathway and apoptosis are also activated.

When DNA is damaged, activation of cell cycle arrest facilitates the repair process and ensures that cells do not divide during this repair process, which may be detrimental to the cell. During cell cycle checkpoint activation, DNA damage is detected by sensor proteins with the help of mediators. The sensor proteins activate transducers. Transducers then activate the effector proteins which lead to the activation of G1-S arrest, S Phase arrest or G2-M arrest [48-53].

ATM binds to the double strand break sites with the help of MRN complex and because of its protein kinase activity, it phosphorylates in itself at the serine threonine residues. It also acts on many other proteins, like p53, CHK2, NBS1 and BRCA1. This activation of ATM and other proteins result in the initiation of G1-S arrest. Initiation of G1-S arrest takes place when the activated ATM phosphorylates CHK2. Activated CHK2 in turn phosphorylates CDC25A thereby targeting it for ubiquitination and proteasomal degradation (Fig. 1-6). This leads to the accumulation of phosphorylated form of CDK2 (inactive), which is incapable of phosphorylating CDC45 to initiate replication in ORC-ORI complexes. The maintenance of G1 arrest is achieved by phosphorylated p53. Under normal conditions p53 is bound to an inhibitory protein MDM2 and this binding targets p53 to proteasomal degradation. On phosphorylation of p53 by ATM at Ser15, it disrupts its interaction with MDM2 and thereby stabilizing the p53. Activated p53 up regulates the transcription of genes involved in cell cycle arrest, DNA repair, and apoptosis. Some of the prominent genes include p21, GADD45A, PUMA, and BAX [54-56]. Out of which p21 and GADD45A participate in cell cycle arrest. PUMA and BAX help in the cell to undergo apoptosis. Upregulation of p21 leads to it binding with CDK4/CycD complex, thus preventing it from phosphorylating RB2, which will leads to the transcription of S phase genes.

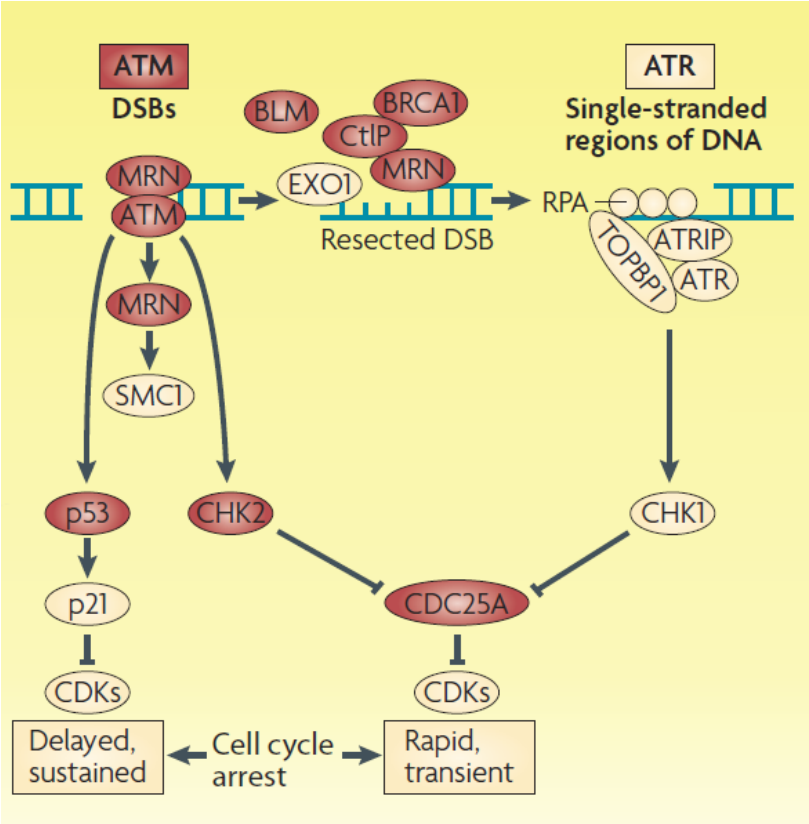


Fig: 1-6. Initiation and maintenance of G1-S checkpoint. Initiation of G1-S arrest happens by binding of ATM to the DSB and activation of CHK2, which in turn lead to the proteasomal degradation of CDC25A and thereby blocking the initiation of replication in ORC-ORI complexes. This G1-S arrest is sustained by p53 mediated activation of p21 (Adopted from Lukas et al., 2010) [57].

1.5.3. DNA repair Pathway

After the DNA damage, cells try to repair the damaged DNA before it further proceeds with the cell cycle. Based on the type of damage, one amongst the following DNA repair pathways are activated for repairing of the damaged DNA:

1. Base excision repair pathway (BER)
2. Nucleotide excision repair (NER)
3. Mismatch repair (MMR)
4. Transcriptional coupled repair (TCR)
5. Non-homologous end joining (NHEJ)
6. Homologous recombination repair (HRR)

1.5.3.1. Base excision repair

BER is responsible for repairing any base damage or single strand breaks in the DNA. In this pathway, initially the damaged base is removed by either one of ten DNA glycosylases [58, 59]. Each of these DNA glycosylases can recognize a specific base damage. Then a single strand break is created at the site of abasic site by AP endonucleases by making a cut in the phosphodiester bond. Then the damaged site is removed and resulting gap is repaired by DNA polymerase.

1.5.3.2. Nucleotide excision repair

Nucleotide excision repair (NER), is the main repair pathway used in repair of any bulky lesion created in a single strand by radiation exposure [60, 61]. Steps involved in NER are, (a) damage recognition by RPA, XPA and XPC-TFIIH, (b) dual incisions bracketing the oligomer with 24-32 long nucleotide using XPF-ERCC1, (c) removal of the oligomer by XPG, (d) repair synthesis with DNA polymerase δ/ε with the other replication accessory proteins PCNA and RFC.

1.5.3.3. Non-homologous end joining

DNA double strand breaks are the life threatening lesions, whose repair is governed by three important pathways, namely, non homologous end joining (NHEJ), alternate NHEJ, and homologous recombination (HR) repair. In mammals NHEJ is the most predominant pathway of DSBs repair.

NHEJ repair starts with binding of KU70/KU80 complex at the damaged site. This heterodimer has a toroidal structure through which it loads on to the ends of the damaged DNA. Binding of KU70 / KU80 encourages the binding of the catalytic subunit of DNA-PK (DNA-PKcs). It stabilizes the ends and initiates the NHEJ repair [62-65]. After binding, DNA-PKcs auto phosphorylate six residues in its ABCDE

cluster and this phosphorylation helps in preventing end resection. Binding of DNA-PKcs at the damaged site promotes the binding of end processing enzyme ARTEMIS and recruitment of XRCC4/LIG4 (Fig.1-7). If the DNA ends are non ligatable, these ends are processed by ARTEMIS and then ligated by XRCC4. As a backup to this classical NHEJ pathway, an alternate NHEJ pathway also is found in mammalian cells. The alt-NHEJ is mediated by the binding of PARP (Poly ADP-Ribose Polymerase) to the damaged ends, which promotes the loading of ligation complex XRCC1/LIG3 to the damaged site, to join the damaged ends.

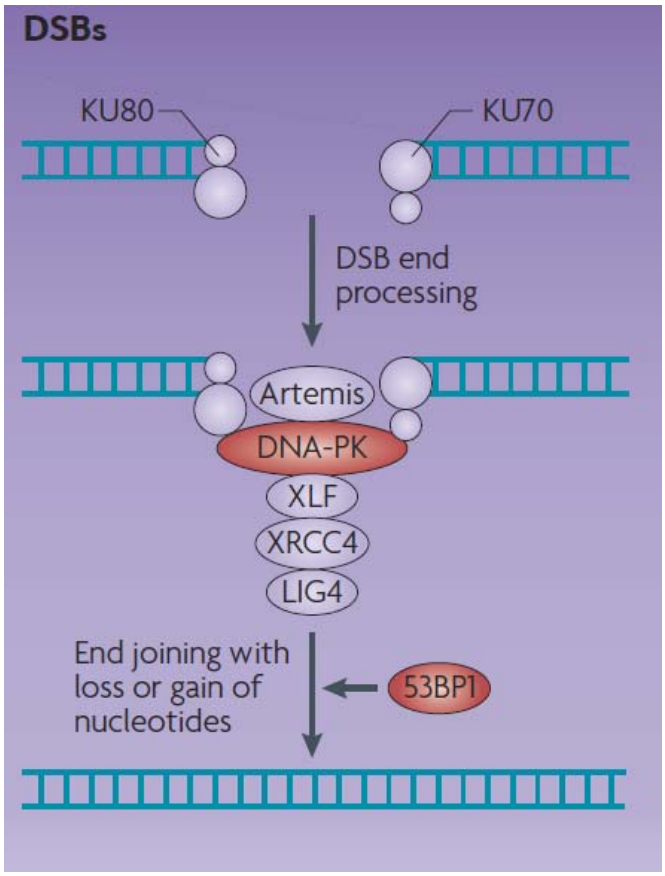


Fig: 1-7. Schematic representation of non-homologous end joining repair pathway (Adopted from Lukas et al., 2010) [57]

1.5.3.4. Homologous recombination repair pathway

Apart from KU70/KU80 and PARP, DNA damage ends can also be recognised and bound by MRN complex. MRN complex binding leads to the binding of ATM to the site, which in turn favour the repair of the damage by HR pathway [66, 67]. MRE11, a component of MRN complex, has both exonuclease and endonuclease activity, which helps in the resection of ends and forms the beginning of the HR pathway. This DNA resection is regulated by ATM through Ctlp, which interacts with BRCA1. DNA resection is induced mainly in S and G2 phase of the cell cycle, which makes HR repair possible. Then RPA complexes bind to the single strand regions of DNA generated during the DNA resection. At this point, RAD51 filaments bind to the RPA bound DNA and it starts the strand invasion mediated by BRCA2. RAD51 activity is also controlled by the phosphorylation by CHK1. Following the RAD51 mediated strand invasion into the sister chromatid, the 3' end is extended using DNA polymerase, which gets ligated with the processed second end of the break (Fig. 1-8).

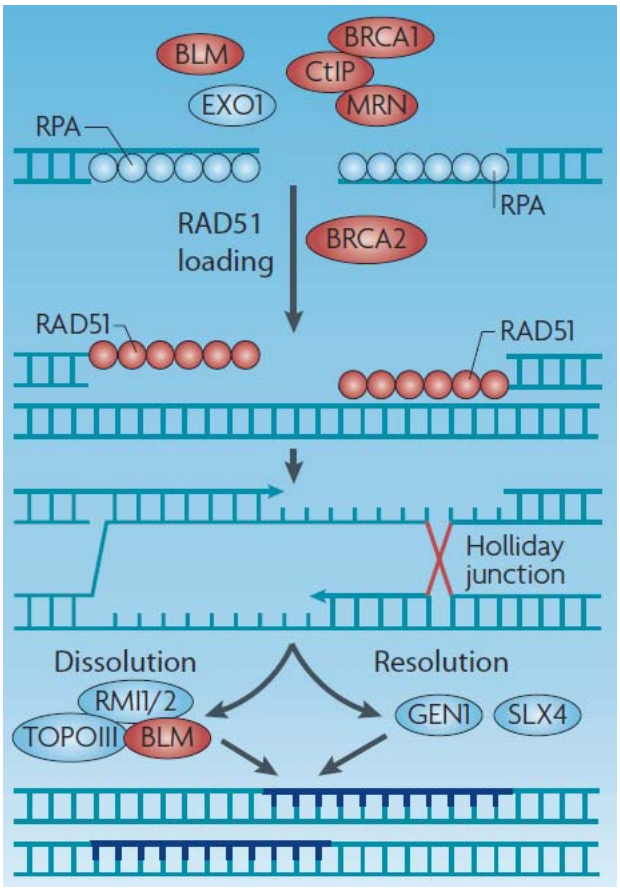


Fig: 1-8. Homologous recombination repair pathway (Adopted from Lukas et al., 2010) [57]

1.6. Apoptosis

One of the key elements in maintenance of genomic stability of an organism is protecting the cells from acquiring and propagating carcinogenic change. When cells are exposed to radiation, and subsequent DNA damage, the cell tries to repair and salvage itself. If the damage is beyond redemption, cells have the ability to take extreme step of programming itself to death, rather than repairing with errors and thereby increasing the chance of accumulating the lesions. This is called programmed cell death or apoptosis. It is a very vital process not only in growth and development but also in maintaining genomic integrity. Apoptosis can be categorised into two broad pathways. One is the extrinsic pathway in which the death signal comes from

the death receptors situated in the plasma membrane, which gets the signal from extracellular milieu. Another pathway is intra cellular pathway, for which the signal emanates within the cell.

Some of the major radiation induced pathways, which is leading to apoptosis is given in the following figure (Fig. 1-9)

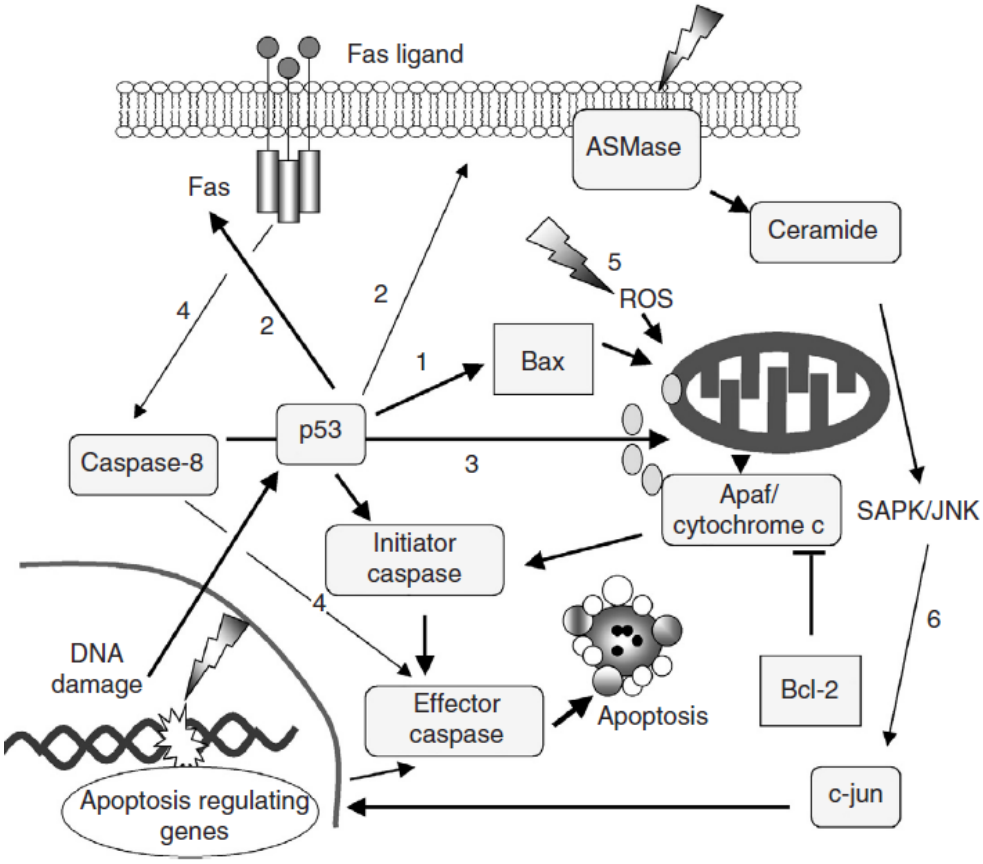


Fig: 1-9. Overview of radiation induced apoptotic pathways. P53 dependent apoptosis was induced either by upregulation of BCL2 related proteins (1), or by upregulation of FAS and FASL (2). Downstream to FAS the apoptosis can be achieved either mitochondrial dependent manner (3), or mitochondrial independent manner (4). ROS mediated release of cytochrome C from mitochondria (5), and p53 independent, ceramide mediated apoptosis caused by membrane damage (6). (Adopted from Lehnerts et al., 2007) [28]

P53 plays a central role in mediating the apoptotic response of irradiated cells. When the p53 was activated after radiation induced DNA damage, it activates the genes like P21 and GADD45A, which are involved in cell cycle arrest predominantly in G1-S phase transition. If the damage is not repaired, p53 also activates genes like BAX and PUMA, which are involved in activation of apoptosis.

Under normal circumstances, the anti-apoptotic protein BCL2 is bound to Apaf thereby retaining in its inactive form. But when cell is perturbed with the radiation induced DNA damage, p53 gets activated via ATM. Activated p53 activates the transcription of Bax- a pro apoptotic protein, which forms a complex by binding to Apaf and cytochrome-c, which is released from the mitochondria during apoptosis. This complex, which is called as apoptosome, acts on procaspase 9 and converts that to an active caspase 9, thereby, triggering a sequential activation of many effector caspases, leading to apoptosis.

When DNA of the cell is damaged, FAS mediated cell death is also possible, which is again regulated through the p53 activation. Expression of FAS gene is up regulated transcriptionally by p53 and that lead to the binding of FADD (Fas Associated Death Domain) to the FAS ligands, which helps in binding and activation of caspase 8. Caspase 8 activates effector caspase 3 and finally it leads to apoptosis.

1.7. Transcriptional activation of NF-κB and Nrf2 pathway after radiation exposure

On irradiation, cells activate many transcriptional factors, which determine the consequences of the cell in terms of its survival and death. Among others two redox sensitive transcription factors, NF- κB and Nrf2 play significant role in survival of cells after radiation exposure.

1.7.1. Nuclear factor kappa B

Nuclear factor kappa B (NF-κB) is a transcription factor, which is expressed primarily under oxidative stress conditions. This transcription factor transcribes many target genes involved in inflammatory response, survival, and antioxidant responses. NF-κB, is a hetero dimer protein, which share an N-terminal DNA binding / dimerization domain, called as Rel-A domain. NF-κB is normally held as inactive form in the cytosol by binding to inhibitory proteins IκBα, IκBβ. Under oxidative stress conditions or in response to stimuli, the inhibitory protein is phosphorylated by IKK (IκB kinase) and leads to the disruption of binding between these two proteins, and thereby NF-κB moves into the nucleus and transcripts its dependent genes.

NF-κB plays an essential role in cell survival, inflammatory response and anti-apoptosis function. Its dysregulation often leads to tumor development, angiogenesis and metastasis. NF-κB induces the expression of several anti-apoptotic genes like BCL2, BCL-xl, c-IAP2 etc. The prosurvival function of NF-κB has been reported in many studies wherein the inhibition of NF-κB leads to the increased apoptosis.

1.7.2. Nrf2 Pathway

Exposure of cells to ionization radiation leads to the accumulation of reactive oxygen species and thereby causes oxidative stress, which in turn may lead to cytotoxicity. The activation of redox sensitive transcription factor NF-E2-related factor 2 (Nrf2) is one of the major systems through which cells respond to oxidative stress. Nrf2 activation leads to activation of genes that are involved in anti-oxidant response. The target genes of Nrf2 has a consensus sequence in their promoter region, which is called as anti-oxidant response elements (ARE) on which Nrf2 binds and activates their transcription. Under normal conditions, Nrf2 is sequestered in the cytoplasm by KEAP1, an adaptor for a Cul3-based E3 ligase that promotes constitutive proteasome mediated Nrf2 degradation. Under oxidative stress, Nrf2 is released from KEAP1 and rapidly translocated into the nucleus, where it upregulates ARE-dependent cytoprotective genes such as glutathione transferases (GST), UDP-glucuronosyltransferases, γ-glutamylcysteine synthetase (γ-GCS), glutathione peroxidase, heme oxygenase-1 (HO-1), catalase, thioredoxin (TRX), Thioredoxin reductase (TR), and NAD(P)H:quinone oxidoreductase-1 (NQO-1). These effector genes help in exerting effective response against oxidative stress (Fig. 1-10).

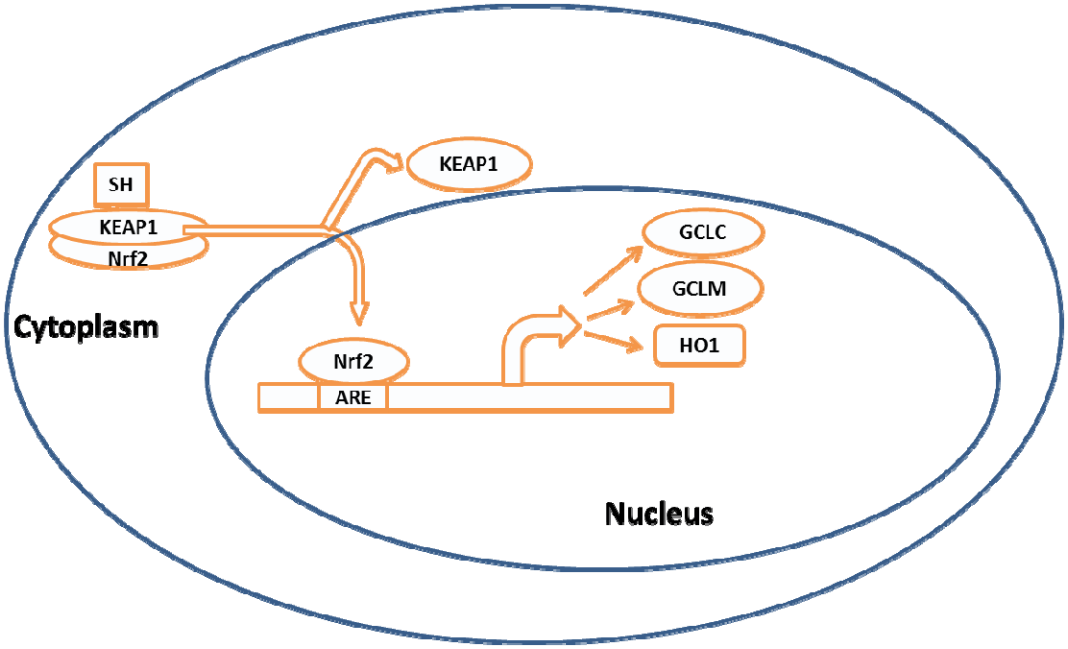


Fig: 1-10. Schematic representation of Nrf2 pathway. Under normal conditions Nrf2 is bound to an inhibitory protein KEAP1. Under oxidative stress Nrf2 translocate into nucleus and activates its target genes.

1.8. Rationale and objectives of the thesis

There is a wealth of information known regarding the response of the cells to DNA damage, which ultimately determines the radiosensitivity of the cells. This information can be useful in determining the radiosensitivity/radio-resistance of the tumor cells. As discussed earlier, the problem of intrinsic difference in radiosensitivity can be addressed from three different angles, with each of them having impact in the improvement of radiotherapy. They are,

1. Prediction of radiosensitivity of the tumor cells
2. Studying about the molecular mechanisms governing radiosensitivity
3. Modulation of the radiosensitivity of tumor cells

From the prediction point of view, if the radiosensitivity levels of the tumor cells can be assessed prior to radiotherapy, that will pave way for personalized radiotherapy. For predicting the radiosensitivity, extent of DNA damage, the extent of expression of genes which are involved in DDR, can be logical candidates. Apart from prediction, new insights into the mechanisms which govern radioresistance of tumor cells can help identifying targets, which can be useful in potentiating the radiation response of tumor cells. A comprehensive study encompassing the above issues of tumor cell radiosensitivity relevant to cancer radiotherapy is not known in literature.

1.9. Objectives of the thesis

With this background, following three objectives were proposed for the thesis:

i. Evaluating DNA damage parameters (initial damage, residual damage etc.) as a measure of radiosensitivity in tumor cell lines using comet assay in comparison with clonogenic assay.

ii. Assessing the usefulness of comet assay in prediction of tumor radiosensitivity after fractionated doses of radiation.

iii. Analysis of expression pattern of some critical genes involved in apoptosis, DNA repair and cell cycle arrest for their role in assessing tumor radiosensitivity.

CHAPTER 2

ASSESSMENT OF RADIOSENSITIVITY IN HUMAN TUMOR CELLS USING SINGLE CELL GEL ELECTROPHORESIS

Assessment of Radiosensitivity in Human Tumor Cells Using Single Cell Gel Electrophoresis

2.1. Introduction

In recent times radiotherapy has undergone considerable technological advances in terms of delivering the doses precisely to the tumor site. In parallel with these technological innovations, better understanding of the radiation response of tumor cells has relevance in improving the efficacy of radiotherapy. Intrinsic radiosensitivity of the tumor cells is one of the major factors, which affect the clinical outcome of the radiotherapy. Malignant tumors are intrinsically and genetically unstable, which lead to large variation in the radiosensitivity of different patients of even within the same tumor type. Owing to this difference in radiosensitivity of tumor cells, in some patients ineffective killing of tumor may result in tumor recurrence. On the other hand, in some of the patients who had undergone radiotherapy, develop severe side effects associated with damage to normal tissues. Hence, optimization of radiation dose in clinical practice based on the radiosensitivity of individual patients and tumor types is of paramount importance, which may help in developing rationale based radiotherapy. Such predictive assays will help in optimizing radiotherapy protocols in cancer patients and also in personalization of radiation dose parameters. Therefore, there is a need to search for a simple, reliable and rapid assay, which can be used for predicting the radiosensitivity of tumor cells.

Clonogenic assay is the standard technique in determining the cellular radiosensitivity [68]. Clonogenic assay measures the ability of a particular cell to divide and produce a macroscopic colony. This simple and effective assay is considered as a standard assay for determining cellular radiosensitivity. Using clonogenic assay, West et al [68] determined the various clonogenic assay parameters

in samples obtained from cervical cancers patients, which were irradiated *ex vivo*. They found SF2 (survival fraction at 2 Gy) to be the most important variable correlated with the clinical outcome. Their study showed that the patients exhibiting higher radiosensitivity (SF2 > 0.42) to have higher 5 year survival rate of 81 % in comparison to 51 % in the patients who have exhibited SF2 <0.42. Similar findings were reported by Björk-Eriksson et al. [69] in 99 head and neck cancer patients. However, clinical usage of clonogenic assay is limited because of practical reasons: (i) the *ex vivo* colony forming ability of many tumors is very low, (ii) low throughput in case a large number of samples have to be analyzed, (iii) the plating efficiency of many tumors in culture conditions is <1 %. Even in the study conducted by Björk-Eriksson et al., [69] more than 30 % of the tumor biopsies did not form colonies (iv) requirement of longer period of time (3-4 weeks) to obtain the results of clonogenic assay [69]. Therefore, there is a need to develop other biological markers or assays that are faster with practical applicability at clinical level across range of tumor types. In this regard, different cellular and molecular assays have been explored to predict the radiosensitivity of normal and tumor cells, however, with varying degrees of success [70-74]. Initially many blood parameters were evaluated for their usefulness in assessing the radiation response of different individuals. Severin et al., [73] analyzed several blood parameters (like cell proliferation, activation of cytokines, antioxidative capacity of blood plasma, level of uric acid, hemoglobin, numbers of total leukocytes of $CD34^+$ hematopoietic blood stem cells and of $CD4^+$ / $CD8^+$ lymphocytes) in leukemic patients undergoing radiotherapy. These parameters were assessed for evaluating the predictive value of these parameters in assessing normal tissue toxicity. Out of these parameters three parameters namely, leukocyte count, damaged lymphocyte score and the antioxidative capacity measured after exposure

were found to have value in predicting the extent of oral mucositis. Because only leukemia patients were analyzed in this study, it is not yet clear whether these results can be applied to other types of cancer patients. Vidyasagar et al. [75] have investigated the extent of glutathione depletion in serum level during the course of radiotherapy and found this parameter to be useful in predicting chemoradioresponse prior to and also at an early stage of treatment of cervical cancers.

Since radiation exposure ultimately leads to the cell death, apoptosis was evaluated for its role in predicting radiation response of tumor cells. Abend et al. [76] examined the percentage of apoptotic cell death as a marker of radiosensitivity in 10 cell lines, but found no meaningful correlation between apoptosis and radiosensitivity.

Since DNA is considered as a primary target of ionizing radiation, DNA damage based end points were widely studied for their value in predicting the radiation response. Cytogenetic approaches like, micronuclei frequency, chromosomal abnormalities, sister chromatid exchange etc., were evaluated for this purpose and found to have correlation with the radiosensitivity [76]. Russell et al., used fluorescence in situ hybridization (FISH) as potential substitute for clonogenic assay in predicting the radiosensitivity of eight normal human fibroblast cell lines with a range of radiosensitivities [77]. In their study, the relationship between surviving fraction and chromosome aberrations varied among cell lines, but a linear regression for all data was observed. But it may be important to mention that many of these cytogenetic techniques need the cells to be in dividing condition, time consuming, and difficult to practice at clinical level.

Therefore, apart from cytogenetic studies, various other assays, which measures DNA damage directly, were evaluated for their use in predicting radiosensitivity. Schwartz et al. [78] through neutral elution technique, which determines extent of

DNA double strand breaks, showed that the radioresistant human tumor cells rejoin DNA double-strand breaks faster than radio sensitive cell lines. Wei et al., [79] used pulsed field gel electrophoresis and analyzed the applicability of DNA damage repair as a marker for predicting radiosensitivity, and reported a positive correlation between the radiosensitivity and the DNA repair capacity. In contrary, El-Awady et al., [80] showed the absence of correlation between the radiosensitivity and DNA repair capacity when pulsed field gel electrophoresis was used to detect DNA damage.

The comet assay, which is widely used to quantify DNA damage after exposure to various genotoxic agents [81], could be a fast and reliable assay system for determining cellular radiosensitivity. Attempts have been made to correlate DNA damage with the clonogenic survival of the tumor cells using either an alkaline or a neutral comet assay. A good correlation between DNA damage (as measured by the comet assay) and clonogenic survival was observed in some studies [82-84]. However, this correlation was not observed in other studies [85, 86], suggesting the need for more studies in this direction for the potential application of the comet assay in determining the radiosensitivity of tumor cells. In these studies, they have taken the tumor cells originated from same tissue type. Moreover, in previous reports there is also lack of consistency in the comet assay parameters tested (initial DNA damage, DNA repair kinetics, residual damage etc.), and those show correlations with the clonogenic survival. Investigators have used only the alkaline comet assay or the neutral comet assay but have not performed side-by-side comparison of both assays. These assays have not been compared with the clonogenic assay after fractionated doses of radiation, which has more relevance with the clinical conditions.

In this chapter, alkaline and neutral comet assays were comprehensively evaluated for their usefulness in assessing radiosensitivity by correlating various comet assay

parameters with clonogenic survival in different tumor cell lines. In the study, tumor cells originated from different tissue types with varied radiosensitivity were taken. This comparison was also performed in radiosensitivity of tumor cells after acute and fractionated doses of radiation.

2.2. Materials and methods

2.2.1. Chemicals

Dulbecco's Modified Eagles Medium (DMEM), antibiotics (streptomycin and penicillin), sodium bicarbonate, crystal violet, Tris-HCl, ethylenediaminetetraacetic acid (EDTA), sodium chloride (NaCl), potassium dihydrogen phosphate, sodium phosphate, potassium chloride, Triton X-100, SYBR Green I, SYBR Green II, dimethylsulfoxide (DMSO) and diethyl pyrocarbonate (DEPC) were purchased from Sigma (Missouri, USA). Fetal bovine serum (FBS) and trypsin-EDTA were obtained from Himedia (Mumbai, India). Sodium hydroxide, boric acid and methanol were purchased from Sisco fine chemicals (Mumbai, India).

2.2.2. Cell lines and cell culture

Human fibrosarcoma (HT1080), human colon carcinoma (HT29), human mammary carcinoma (MCF7 and T47D) and human lung adenocarcinoma (A549) cell lines were procured from the National Centre for Cell Science (Pune, India). Human prostate cancer cell lines (PC3 and DU145) were borrowed from National Institute for Research in Reproductive Health (Mumbai, India). These cells were maintained as exponentially growing monolayers in DMEM supplemented with 10% FBS, streptomycin (0.1 mg/ml) and penicillin (100 U/ml) in a humidified incubator maintained at 37 ^{o}C and 5 % CO_2.

2.2.3. Irradiation (acute and fractionated) conditions

The cells were irradiated (dose rate: 1 Gy/min) using a ^{60}Co teletherapy machine Bhabhatron-II (Panacea Medical Technologies, Bangalore, India). For the comet assay, exponentially growing cells were harvested by trypsinization. The trypsinized cells were suspended in complete medium followed by irradiation at 4 ^{o}C. After irradiation, the samples to be processed immediately after irradiation (0 min) were kept on ice, while those samples for use in repair kinetics studies were incubated at 37 ^{o}C for various time intervals (15, 30, 60 and 120 min). Once all the time points are over, they were all processed together for comet assay. For fractionated γ-irradiation, cells were irradiated either 2 x 2 Gy (4 Gy) or 3 x 2 Gy (6 Gy) with a 2 h time interval between successive doses.

2.2.4. Clonogenic assay

For the clonogenic assay, appropriate cell number required for seeding was standardized based on the plating efficiency and the number of colonies that developed after different doses of radiation. Cells were seeded in 60 mm dishes and allowed to adhere overnight in the culture conditions. These cultures were exposed to various doses of γ-radiation (0.5, 1, 2, 4, 6, 8 and 10 Gy). After 15-20 days, the dishes were washed with PBS, fixed with methanol, stained using 0.5% crystal violet and then rinsed with tap water. The colonies were counted using a stereo microscope. A colony was considered when there were at least 50 or more cells in the particular colony. The survival fraction was calculated using the following formula:

Survival fraction = No. of colonies/ [no. of cells plated X (plating efficiency/100)].

2.2.5. Alkaline comet assay

The alkaline comet assay was used to quantify the total DNA strand breaks [87]. For this assay, the control and the irradiated cells were suspended in 0.8 % low melting agarose and were layered onto frosted slides, which were pre-coated with 1 % normal agarose. After solidification, the slides were kept in lysis buffer (2.5 M NaCl, 10 mM Tris-HCl of pH 10, 100 mM Na2–EDTA, 1 % Triton X-100 and 10 % DMSO) at 4 ^{o}C for 60 min. For equilibration, the slides were transferred to an electrophoretic tank containing alkaline solution (300 mM NaOH, 1 mM EDTA, pH 13.0) for 20 min. The slides were then electrophoresed in the same buffer for 30 min at 0.8 V/cm. After electrophoresis, the slides were neutralized (0.4 M Tris-HCl), stained with 1X SYBR Green II dye and visualized at 40X magnification using a fluorescence microscope (Axioplan, Carl-Zeiss, Germany). For every treatment, two slides were prepared and at least 50 images were taken per slide. The images were analyzed using CASP software (www.casplab.com) to obtain the percentage of DNA within the tail, which is considered to be the best parameter for representing DNA damage in a comet assay [88].

2.2.6. Neutral comet assay

To measure double strand DNA breaks, neutral comet assay was used. The neutral comet assay was performed as previously described [89]. The control and the irradiated cells were suspended in 0.8% low melting agarose and were layered onto frosted slides, which were pre-coated with 1% normal agarose. After solidification, the slides were kept in lysis buffer (2.5 M NaCl, 10 mM Tris-HCl of pH 10, 100 mM Na2–EDTA, 1 % Triton X-100 and 10 % DMSO) at 4 ^{o}C for 60 min. For equilibration, the slides were transferred to an electrophoretic tank containing 0.5X Tris borate EDTA buffer (TBE) for 20 min. The slides were then electrophoresed in

the same buffer for 30 min at 0.8 V/cm. After electrophoresis, the slides were neutralized (0.4 M Tris-HCl), stained with 1X SYBR Green I dye and visualized at 40X magnification using a fluorescence microscope (Axioplan, Carl-Zeiss, Germany). For every treatment, two slides were prepared and at least 50 images were taken per slide. The images were analyzed using CASP software (www.casplab.com) [87] to obtain the percentage of DNA within the tail, which is considered to be the best parameter for representing DNA damage in a comet assay [88].

2.2.7. Statistical analysis

Statistical analysis and the correlation coefficient was calculated using Graphpad Prism 5.0 software (La Jolla, USA). The significance of the correlation coefficient was also calculated by Graphpad Prism using the formula $t= r \sqrt{(n-2)/(1-r^2)}$, where r = correlation coefficient. A Student's t-test was used for comparison of the means of the two groups. Values were considered to be significantly different at $p< 0.05$.

2.3. Results

2.3.1. Establishment of radiosensitivity profiles of different tumor cells using clonogenic assay

To assess the radiosensitivity of the five different tumor cell lines using clonogenic assay, cells were exposed to different doses of radiation and they were allowed to develop macroscopic colonies (Fig. 2-1).

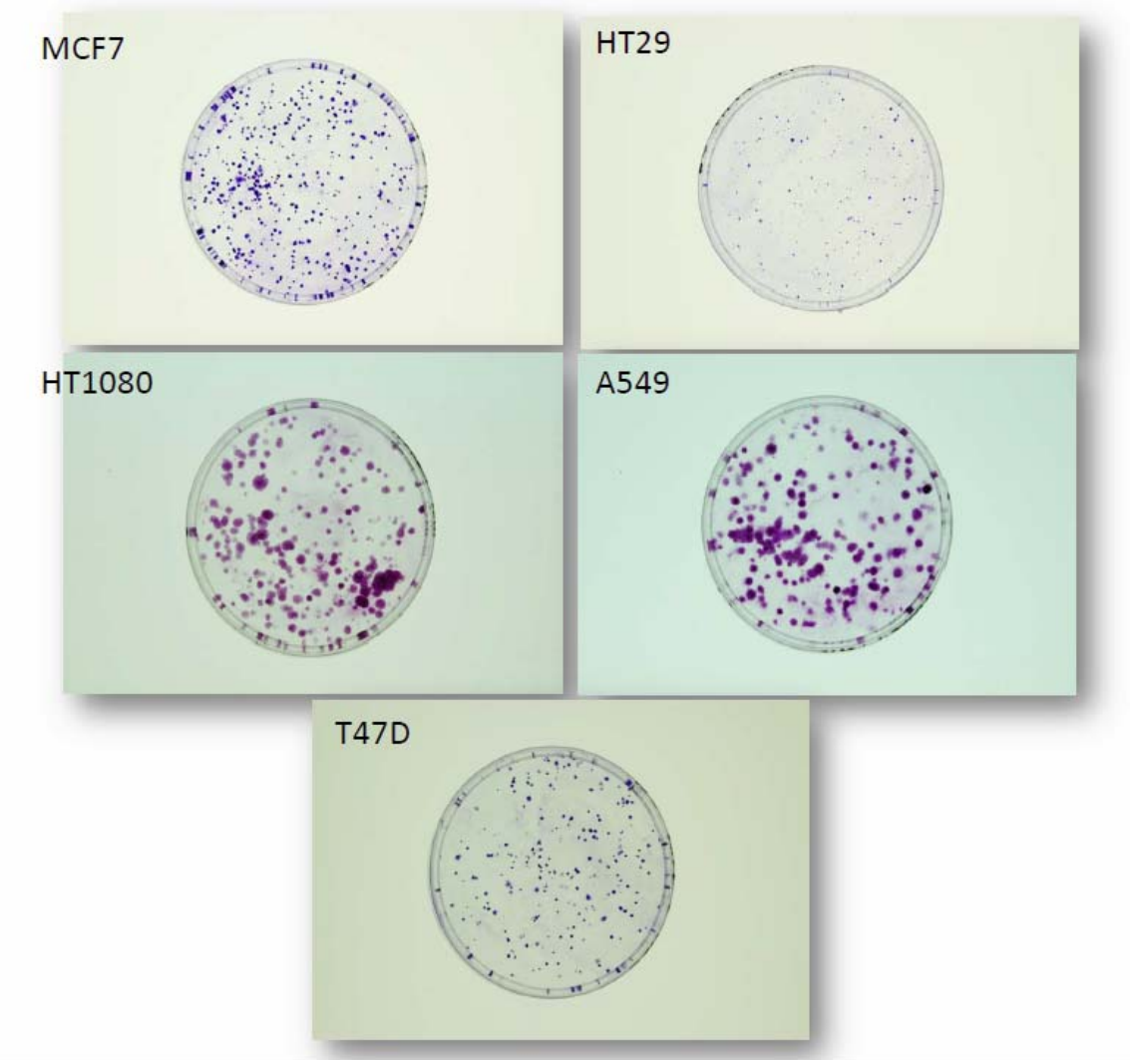


Fig: 2-1. Representative images of the clonogenic dishes of MCF7, HT29, HT1080, A549 and T47D with macroscopic colonies

From the number of colonies developed, the survival fraction was calculated for all the doses and the survival curve were plotted (Fig. 2-2). From the survival curve, SF_2 (survival fraction at 2 Gy) values were calculated. Among the five tumor cells used in the study, the highest SF_2 value (0.71) was found in HT1080 cells followed by MCF7 cells (SF_2: 0.62), T47D cells (SF_2: 0.57), A549 cells (SF_2: 0.54) and HT29 cells (SF_2: 0.52) (Table: 2-1). Similar to the SF_2 values, SF_4 (survival fraction at 4 Gy), SF_6

(survival fraction at 6 Gy), SF_8 (survival fraction at 8 Gy) and SF_{10} (survival fraction at 10 Gy) values were also calculated (Table. 2-1). When the radiation dose was increased, the survival fraction decreased drastically in logarithmic fashion. For example, SF10 values for HT1080, MCF7, T47D, A549 and HT29 cells were 0.0270, 0.0046, 0.0014, 0.0008 and 0.0004 respectively. Apart from SF_2, survival fraction obtained at all the other doses also indicated that among the cell used, HT1080 is the most radio-resistant one followed by MCF7, T47D, A549 and HT29.

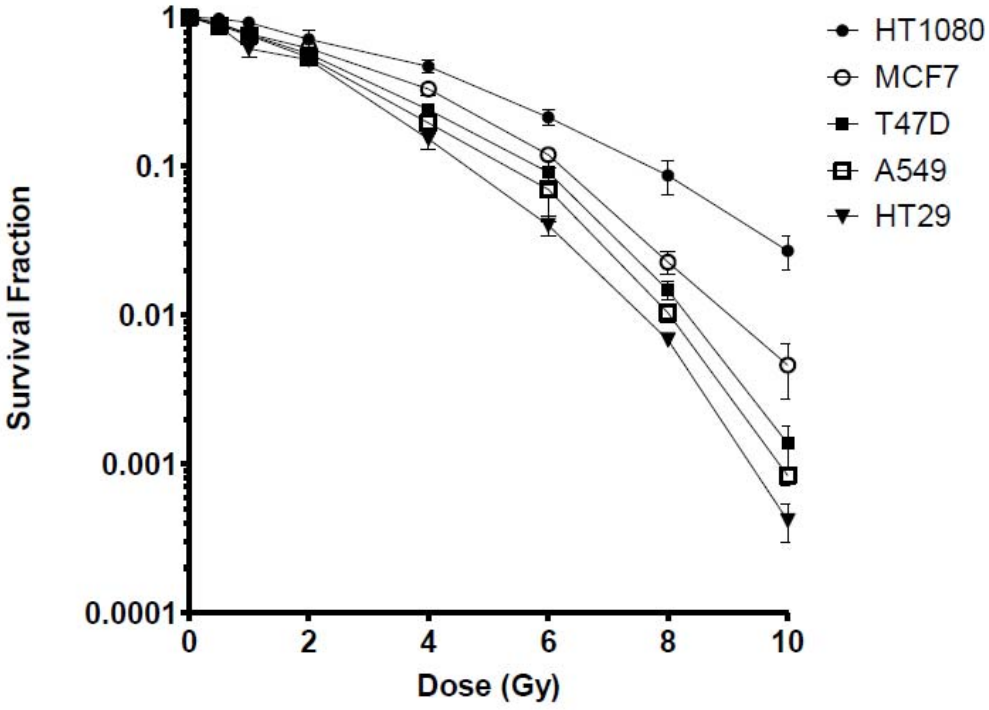


Fig: 2-2. A survival curve for the HT1080, MCF7, T47D, A549 and HT29 cell lines, as determined by clonogenic assay

From the survival curve, D_0 (the dose yielding 37 % survival) and D_{10} (the dose required to kill 90 % of the cells) were calculated (Table 2-2).

Table: 2-1. Survival fraction obtained with different tumor cell lines at 2 Gy (SF2), 4 Gy (SF4), 6 Gy (SF6), 8 Gy (SF8), and 10 Gy (SF10)

Cell line	SF_2	SF_4	SF_6	SF_8	SF_{10}
HT1080	0.708	0.467	0.213	0.086	0.0269
MCF7	0.618	0.329	0.119	0.023	0.0046
T47D	0.565	0.238	0.091	0.015	0.0013
A549	0.536	0.195	0.070	0.010	0.0008
HT29	0.516	0.152	0.040	0.007	0.0004

Table: 2-2. Values of the survival parameters like SF2, D_0 and D_{10} derived from the clonogenic survival curve

Cell line	D_0	D_{10}
HT1080	4.57	7.63
MCF7	3.50	6.20
T47D	2.96	5.75
A549	2.75	5.28
HT29	2.50	4.59

D_{10} values also were highest for HT1080 cells and lowest for HT29 cells. In other cell lines, these survival parameters showed a decreasing trend MCF7 > T47D > A549. All these parameters together suggested that amongst the five cell lines used, HT1080 cells showed the highest radio-resistance, followed by MCF7 cells, T47D cells, A549 cells and HT29 cells.

2.3.2. Measurement of DNA damage and its repair kinetics using alkaline comet assay

In order to evaluate the usefulness of comet assay as an alternative for clonogenic assay in assessing radiosensitivity of tumor cells, after different doses of radiation, DNA damage was estimated in all the five cell lines using comet assay and their correlation with clonogenic assay was analyzed. Initially, alkaline comet assay, which detects the total DNA strand breaks including abasic sites, was performed. All the five cell lines were exposed to 2 Gy, 4 Gy, 6 Gy and 8 Gy of radiation and alkaline comet assay was performed at different time points (0, 15, 30, 60, and 120 min). Gamma-irradiated tumor cells showed a higher percentage of DNA in the tail compared to the controls in all the cells studied. From the comet assay, the percentage of DNA in the tail was obtained. This parameter was found to be the highest in the HT29 cells, as compared to other cell lines, suggesting that the DNA damage was highest in the most radiosensitive HT29 cell line (Fig. 2-3). Among the other cell lines, some overlap was seen in values of % DNA in tail at all the doses. At 4, 6 and 8 Gy, the DNA damage measured by alkaline comet assay graded all the cell lines based on their radiosensitivity except that of A549 which showed less DNA damage than the MCF7 cells and T47D cells (more radioresistant than A549 by clonogenic assay). At the 2 Gy, the DNA damage profiles measured in terms of the percentage of DNA in the tail were not significantly different from each other, although the HT29 cells showed more DNA damage than the other tumor cells.

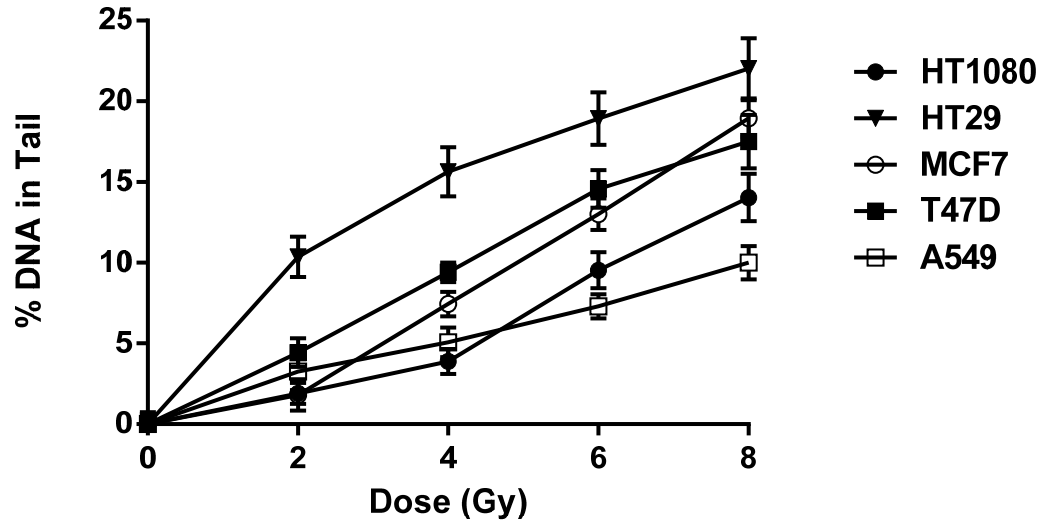


Fig: 2-3. DNA damage assessed by the alkaline comet assay after different doses of radiation

Apart from the initial DNA damage on radiation exposure, the DNA repair capacity of individual cell lines was also measured using alkaline comet assay by incubating the cells at 37 ^{o}C up to 120 min after irradiation (Fig. 2-4 to 2-7). From the repair kinetics, it was observed that though the initial DNA damage for the sensitive cell lines like HT29 is more, they could efficiently repair the DNA damage within 30 min. At 60 min and after, the difference in the DNA damage between the cell lines was significantly narrowed down and at that point in time, it was difficult to grade the cells based on their radiosensitivity using DNA damage by alkaline comet assay.

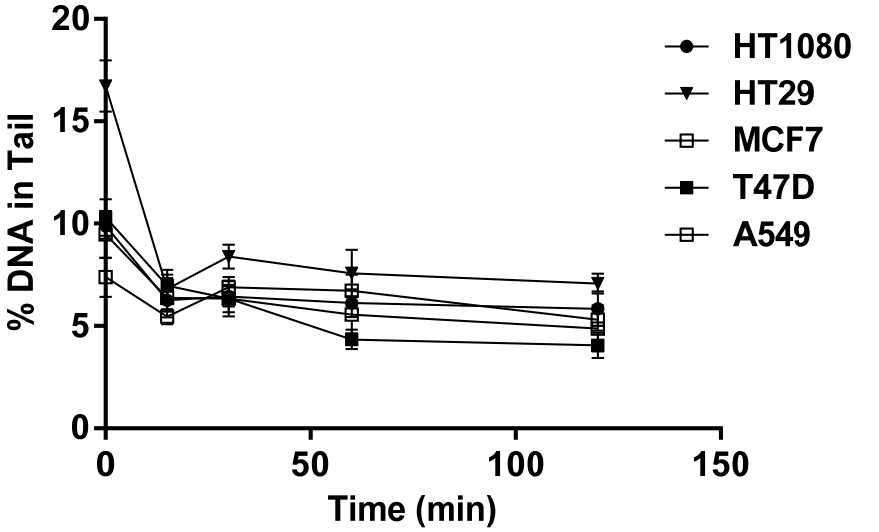


Fig: 2-4. Repair kinetics of DNA damage as assessed by alkaline comet assay after 2 Gy

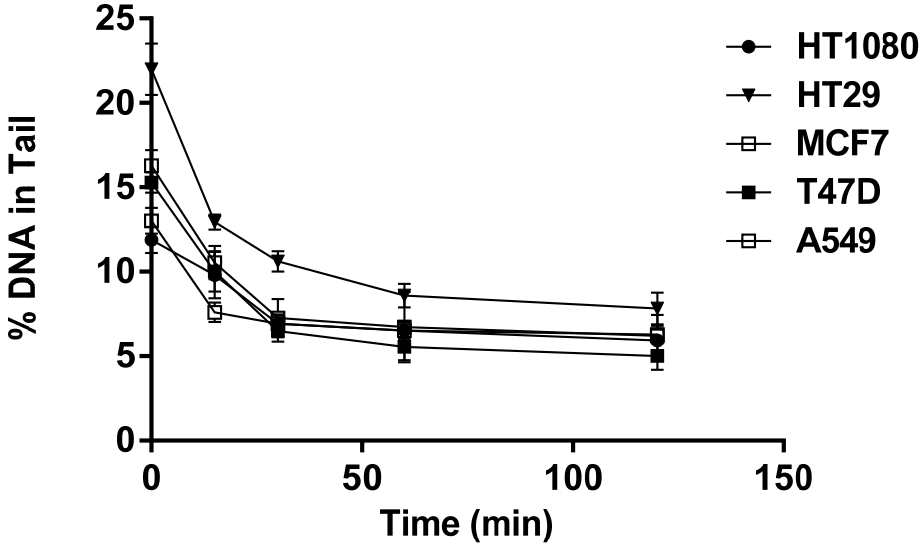


Fig: 2-5. Repair kinetics of DNA damage as assessed by alkaline comet assay after 4 Gy

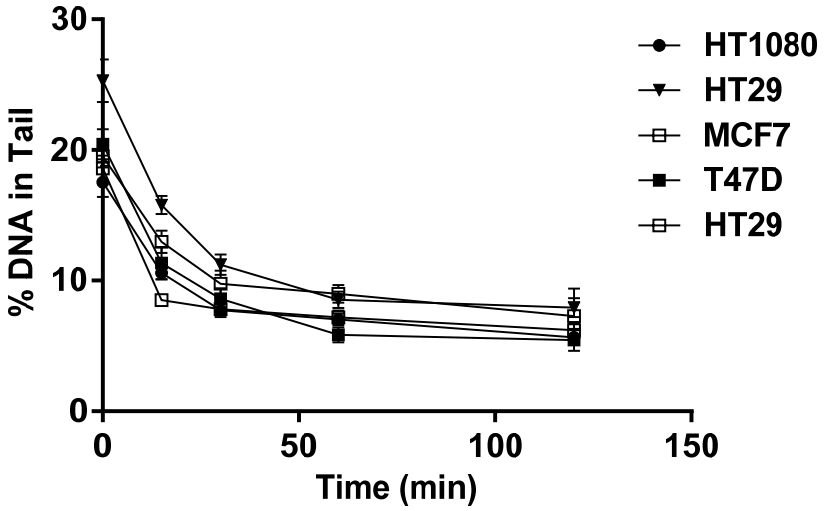


Fig: 2-6. Repair kinetics of DNA damage as assessed by alkaline comet assay after 6 Gy

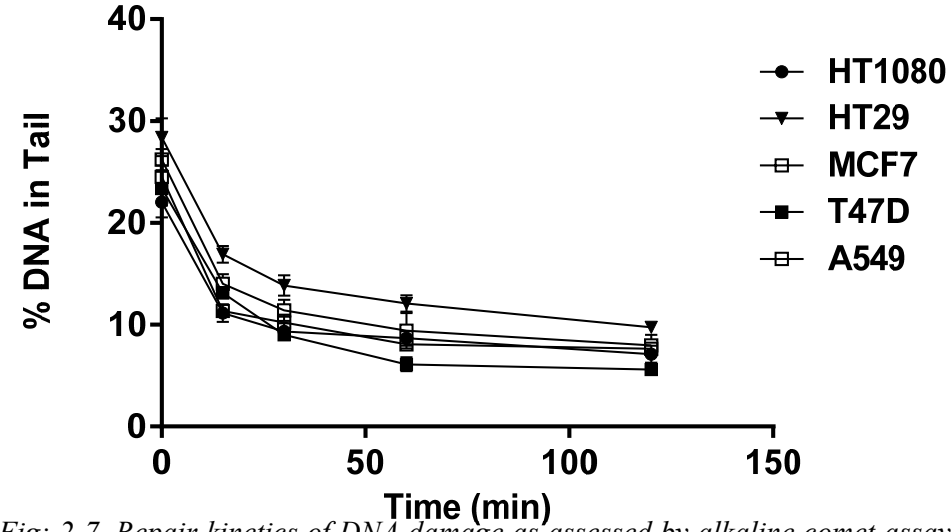


Fig: 2-7. Repair kinetics of DNA damage as assessed by alkaline comet assay after 8 Gy

At 120 min after irradiation maximum DNA damage got repaired and a small residual DNA damage was observed. This unrepaired residual damage was higher in radiosensitive HT29 cells than the other cells. Similarly, radio-resistant HT1080 cells showed the least residual DNA damage at most of the radiation doses. But other than

these two cell lines, the difference in the residual DNA damage of the other cells is very narrow and they were not significantly different to each other.

2.3.3. Correlation between survival fraction and different parameters of DNA damage assessed by alkaline comet assay

After evaluating the radiosensitivity profile and DNA damage profile using clonogenic and comet assay, for five tumor cell lines, the correlation between these two parameters for a given cell line was analysed. This provided a relationship between comet assay and clonogenic assay. All the five cell lines showed a significant correlation between DNA damage observed with alkaline comet assay and clonogenic survival fraction. Significant correlation was seen between these parameters in all the cell lines [HT1080 (Fig. 2-8A), MCF7 cells (Fig. 2-8B), T47D cells (Fig. 2-8C), A549 (Fig. 2-8D), and HT29 (Fig. 2-8E)]. The respective 'r' values and '*p*' values are given in Table 2-3.

Table. 2-3. Correlation coefficient values obtained between the DNA damage and survival fraction for individual cell line at different doses with their 'p' values. These values were obtained from correlation analysis performed between survival fraction and % DNA in tail for different tumor cells (Fig. 2-8 A to E).

Dose	'r ' values	*'p* ' values
HT1080	-0.95	0.014
MCF7	-0.93	0.020
T47D	-0.96	0.007
A549	-0.94	0.014
HT29	-0.98	0.001

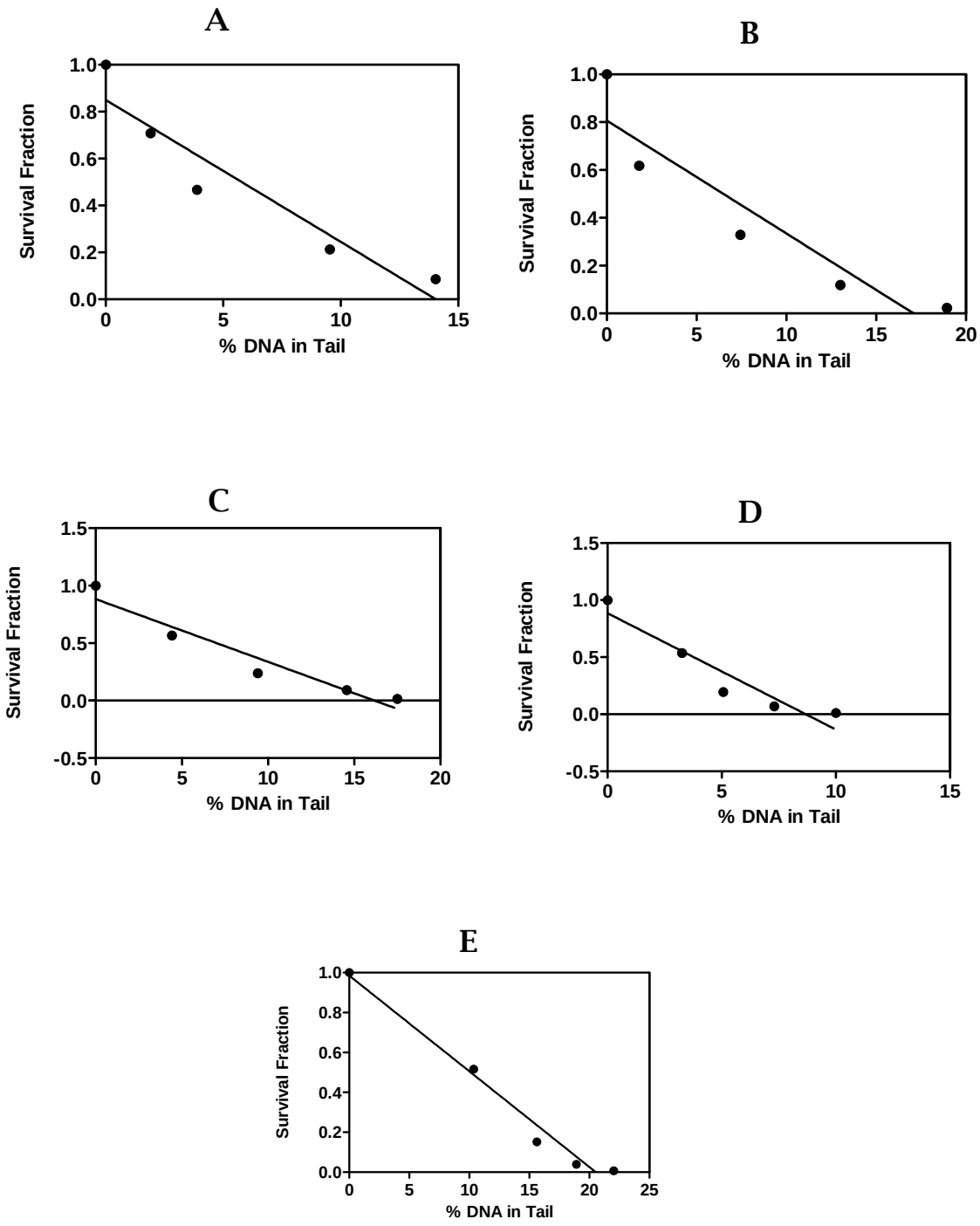


Fig: 2-8. Correlation between DNA damage and survival fraction obtained at different doses with HT1080 cells (A), MCF7 cells (B), T47D cells (C), A549 cells (D), and HT29 cells (E)

Since a significant correlation was obtained between the DNA damage and survival fraction in individual cell lines, the predictive value of DNA damage in assessing radiosensitivity was further studied by examining the correlation between the clonogenic survival and DNA damage in the five cell lines together. The correlation between the total DNA damage obtained after given dose of γ-irradiation and the clonogenic survival at corresponding dose for all the cell lines was calculated. The correlation coefficient obtained between these two parameters at 4 Gy (r=-0.85; Fig. 2-10) was found to be more than the correlation obtained at other doses like 6 Gy (r=-0.67; Fig. 2-9 to 2-12). But these correlations were not statistically significant (Table. 2-4).

Table. 2-4. Correlation coefficient values obtained between the DNA damage (obtained using alkaline comet assay) and survival fraction with different cell lines at different doses. These correlation coefficients obtained at all the doses were analyzed for their statistical significance using the formula $t = r\sqrt{(n-2)/(1-r^2)}$, *where r = correlation coefficient. The 'p' values derived from the calculation are also given in the table.*

Dose	'r ' values	*'p* ' values
2 Gy	-0.51	0.37
4 Gy	-0.85	0.06
6 Gy	-0.67	0.21
8 Gy	-0.74	0.15

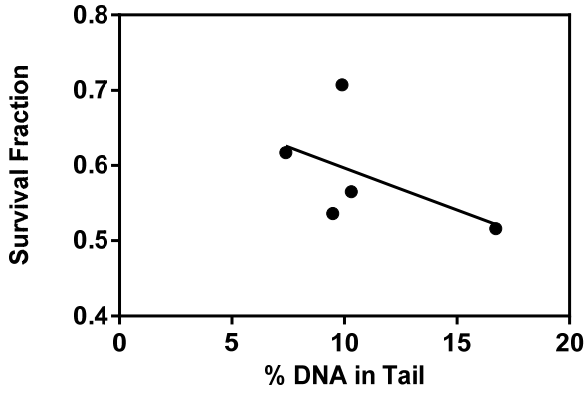


Fig: 2-9. Correlation between the survival fraction and DNA damage assessed by comet assay obtained at 2 Gy

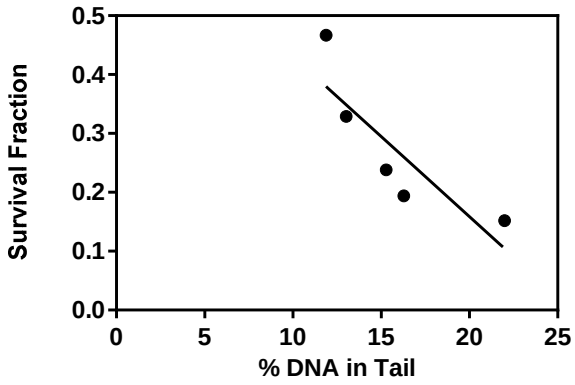


Fig: 2-10. Correlation between the survival fraction and DNA damage assessed by comet assay obtained at 4 Gy

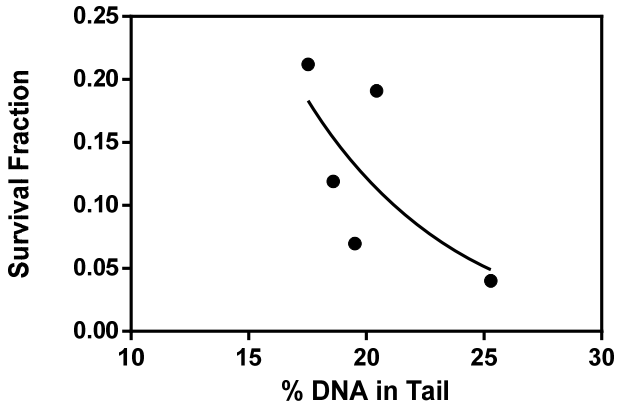


Fig: 2-11. Correlation between the survival fraction and DNA damage (obtained by alkaline comet assay) at 6 Gy.

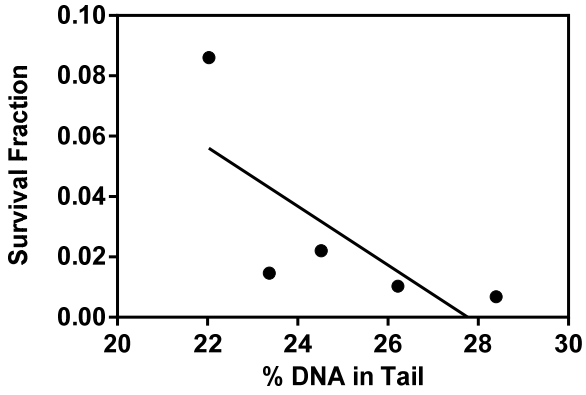


Fig: 2-12. Correlation between the survival fraction and DNA damage assessed by comet assay obtained at 8 Gy

Similar to the initial DNA damage values, the correlation between the residual DNA damage obtained after the repair and the survival fraction was also analyzed at all the dose points. They also did not give significant correlation (Table. 2-5).

Table. 2-5. Values of the correlation coefficient obtained between the residual DNA damage obtained after repair using alkaline comet assay and survival fraction with different cell lines at different doses.

Dose	'r ' values	*'p* ' values
2 Gy	0.62	0.27
4 Gy	0.24	0.7
6 Gy	-0.17	0.78
8 Gy	0.18	0.7

2.3.4. Measurement of DNA damage using neutral comet assay and its repair kinetics after different doses

Since alkaline comet assay measures the total DNA damage including base damage, this might be leading to the reduced sensitivity of the assay as observed previously in this chapter. Neutral comet assay measures mainly the DNA double strand breaks in the cells. Number of studies has reported that the DSBs can be potent in exerting cellular responses than the other lesions. It was hypothesized that measuring the DNA double strand breaks and correlating them with survival fraction might give better correlation between them. Therefore, the extent of DSBs were estimated by neutral comet assay in all the five different tumor cell types after exposing them to different dose radiation doses. All the cell lines showed a dose-dependent increase in DNA damage, as reflected by an increase in the percentage of DNA in the tail obtained from neutral comet assay (Fig. 2-13).

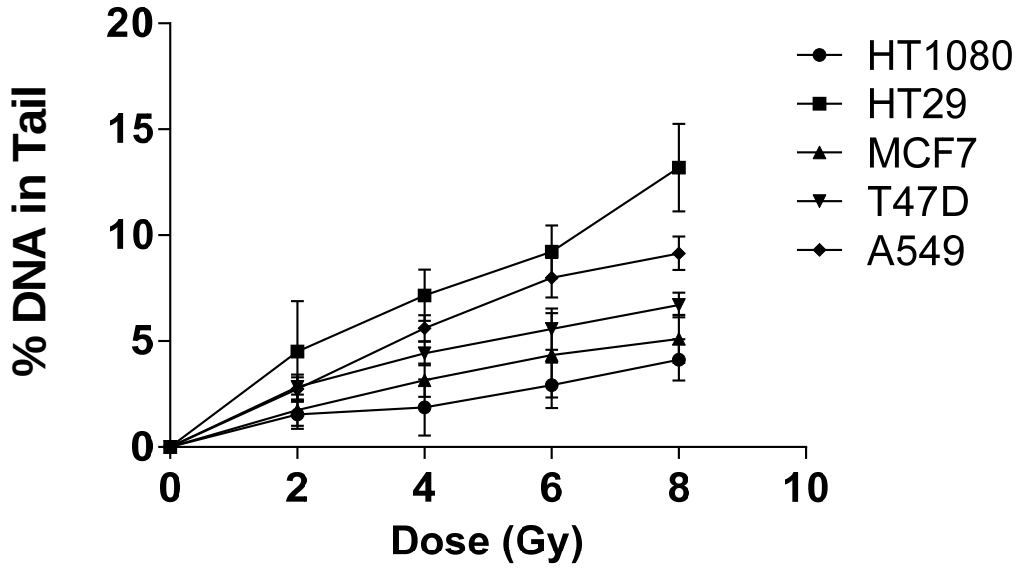


Fig: 2-13. DNA damage assessed by the neutral comet assay after different doses of radiation in different tumor cells

Similar to alkaline comet assay, in the neutral comet assay gave maximum DNA damage in HT29 cells at all the doses. The observed % DNA in tail at 2 Gy for HT29 cells was 4.51 and the corresponding value at 10 Gy for HT29 cells was 13.1. In the other cells DNA damage parameters (assessed by neutral comet assay) showed a descending trend A549 > T47D > MCF7 > HT1080. This gradation of cell lines based on DNA damage by neutral comet assay was similar with that of the order determined by clonogenic assay (Table. 2-1).

Apart from the initial DNA damage, the DNA repair kinetics was also analyzed by neutral comet assay after irradiating the cells to 2, 4, 6 and 8 Gy and allowing them to repair for 2 h. But in contrast to the initial DNA damage (measured in terms of % DNA in tail), repair kinetics analysis, could not segregate the cells according to their radiosensitivity at all the dose points (Fig. 2-14 to 2-17)

.

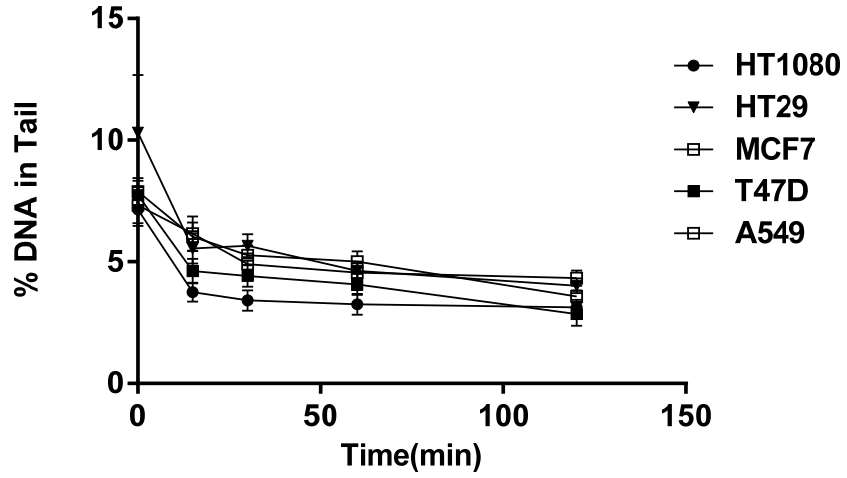


Fig: 2-14. Repair kinetics of DNA damage as assessed by neutral comet assay after 2 Gy

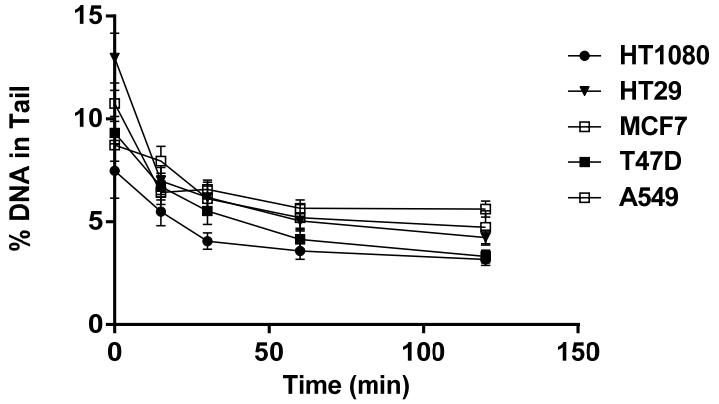


Fig: 2-15. Repair kinetics of DNA damage as assessed by neutral comet assay after 4 Gy

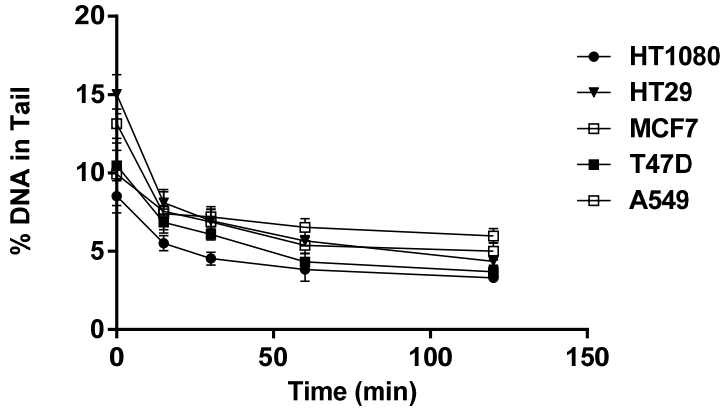


Fig: 2-16. Repair kinetics of DNA damage as assessed by neutral comet assay after 6 Gy

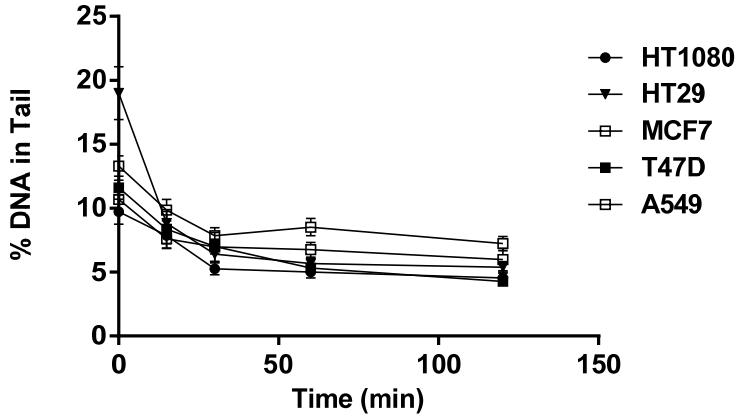


Fig: 2-17. Repair kinetics of DNA damage as assessed by neutral comet assay after 8 Gy

The HT29 cells, which showed the highest DNA damage immediately after irradiation, showed faster repair and after 60 min of irradiation, the DNA damage observed in HT29 was not significantly different from that of the other cell lines. In residual DNA damage observed after 2 h, the radioresistant HT1080 showed the least

DNA damage. But other cell lines did not show gradation in their residual damage based on their radiosensitivity.

2.3.5. Correlation between survival fraction and DNA damage obtained by neutral comet assay

To establish the value of the neutral comet assay in determining the radiosensitivity of tumor cells, the correlation between the initial DNA damage and the clonogenic survival fraction was analyzed. The correlation between DNA damage and survival fraction obtained at different doses, was calculated individually for each cell line (Fig. 2-18A to E) and is tabulated in Table. 2-6. Correlation coefficient obtained in all the cell lines was significant and these correlations were better than that of the corresponding correlation obtained in alkaline comet assay.

Table. 2-6. Correlation values obtained between the DNA damage obtained by neutral comet assay and survival fraction for individual cell line at different doses with their 'p' values. These values were derived from survival fraction vs. % DNA in tail curves for different cell lines (Fig. 2-18A to E)

Dose	'r ' values	*'p* ' values
HT1080	-0.975	0.004
MCF7	-0.997	0.001
T47D	-0.990	0.001
A549	-0.974	0.005
HT29	-0.940	0.017

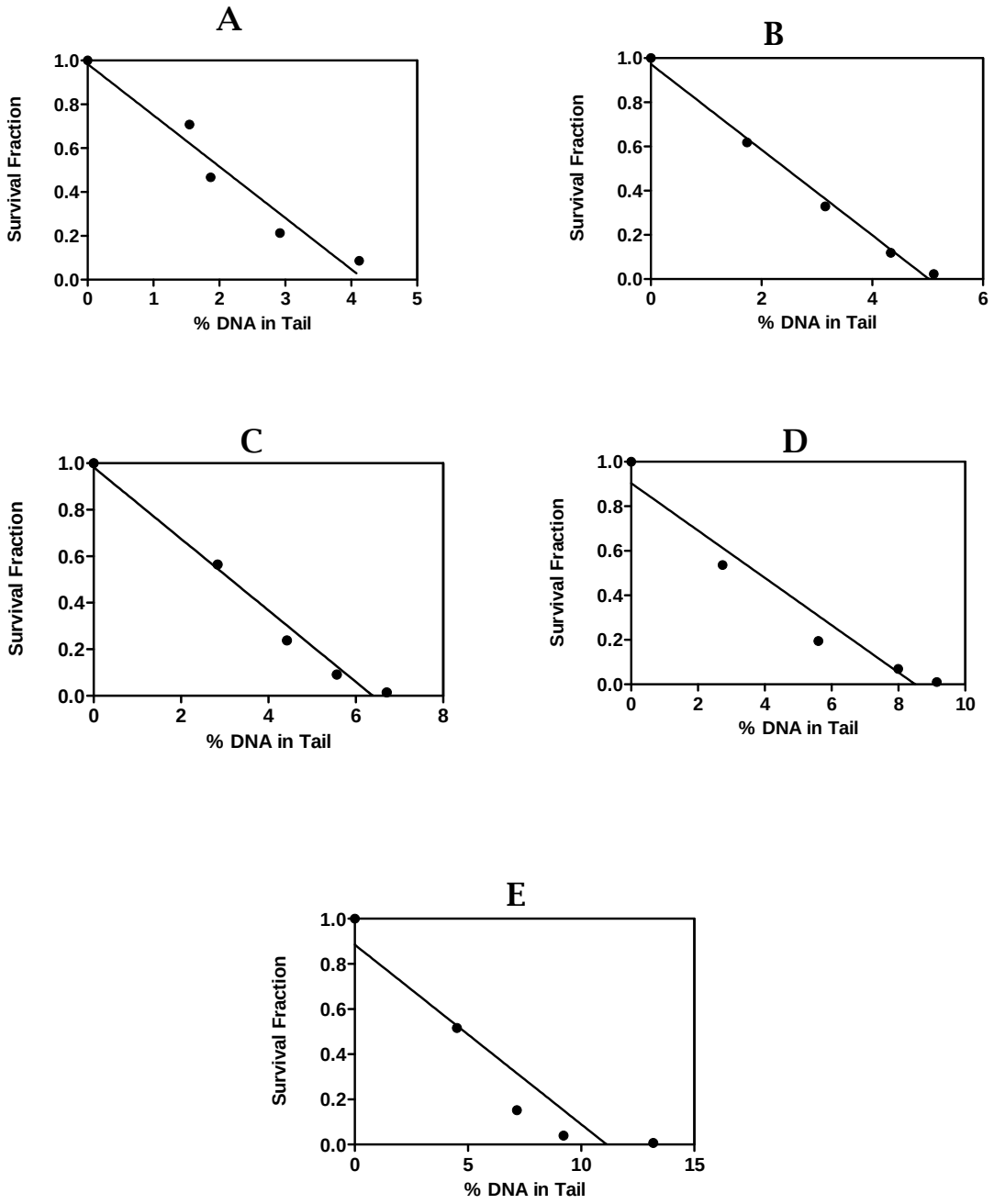


Fig: 2-18. Correlation between DNA damage (obtained using neutral comet assay) and survival fraction obtained at different doses with HT1080 cells (A), MCF7 cells (B), T47D cells (C), A549 cells (D), and HT29 cells (E)

Having observed a significant linear correlation between DNA damage and survival fraction for individual cell lines, the predictive potential of comet assay was evaluated in assessing the radiosensitivity, by examining the correlation between DNA damage and clonogenic survival obtained in all the cell lines together at corresponding radiation doses. At all the doses, the DNA damage and survival fraction showed a significant correlation, with the exception of 2 Gy ($r = -0.7$; $p = 0.14$) (Fig. 2-19). The initial DNA damage observed at 4 Gy (Fig. 2-20) showed significant correlation ($r = -0.9$; $p = 0.03$) with survival fraction. Similarly at 6 Gy (Fig. 2-21) also, the initial DNA damage obtained using neutral comet assay exhibited significant correlation ($r = -0.91$; $p = 0.03$) with clonogenic survival.

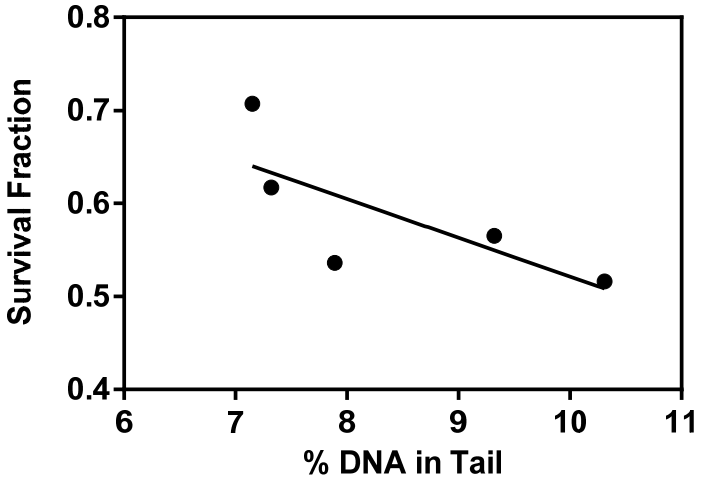


Fig: 2-19. Correlation between the survival fraction and DNA damage assessed by neutral comet assay obtained at 2 Gy

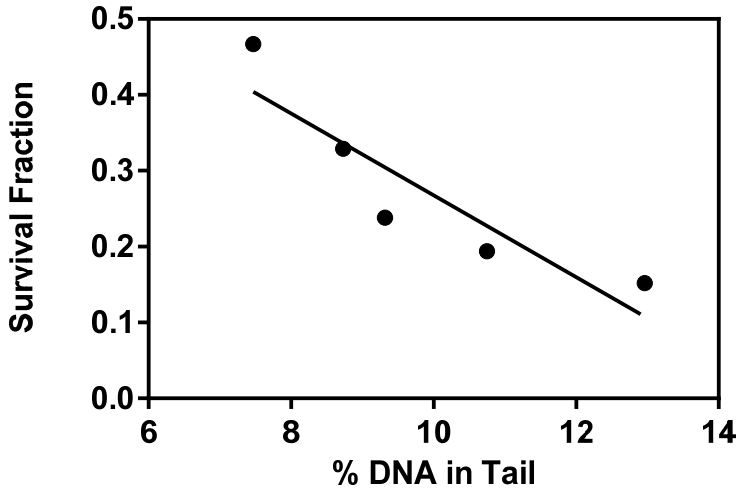


Fig: 2-20. Correlation between the survival fraction and DNA damage assessed by neutral comet assay obtained at 4 Gy

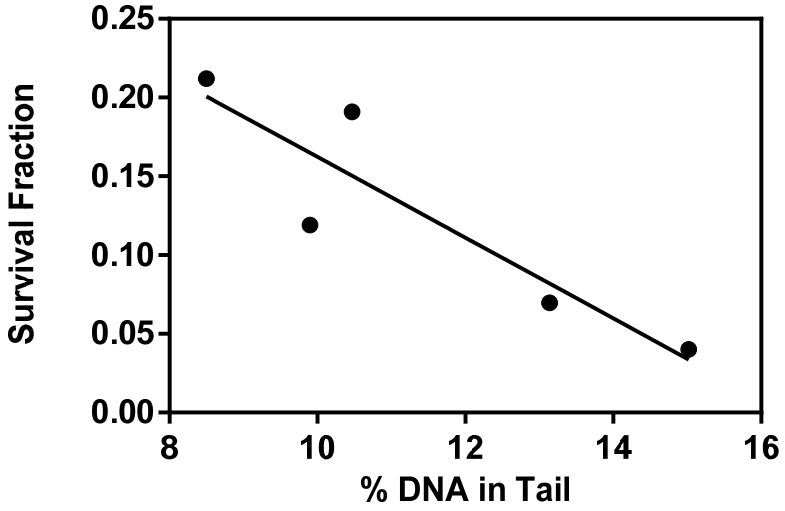


Fig: 2-21. Correlation between the survival fraction and DNA damage assessed by neutral comet assay obtained at 6 Gy

After studying the correlation between the initial DNA damage and clonogenic survival, the correlation between the residual DNA damage and survival fraction was also studied. Residual DNA damage did not show significant correlation with clonogenic survival fraction. At 8 Gy the correlation value observed between residual DNA damage and survival fraction was -0.44 (p =0.454; Fig. 2-22C), at 6 Gy the correlation observed was -0.545 (p = 0.341; Fig. 2-22B), and the corresponding correlation observed at 4 Gy was -0.498 (p =0.392; Fig. 2-22A).

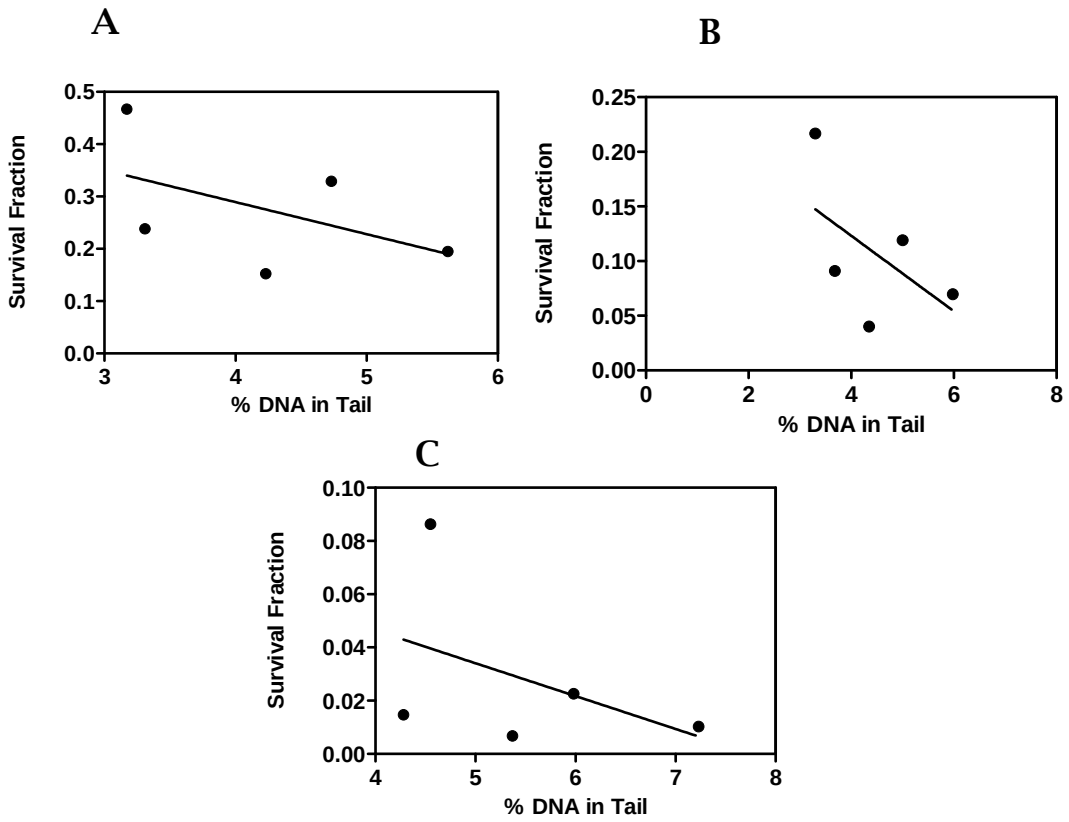


Fig: 2-22. Correlation between the survival fraction and residual DNA damage assessed by neutral comet assay at 4 Gy (A), 6 Gy (B), and 8 Gy (C)

2.3.6. Correlation between survival fraction and DNA damage assessed by comet assay after fractionated irradiation conditions

Fractionated doses are employed during cancer radiotherapy; hence, the predictive potential of comet assay in assessing the radiosensitivity was examined after fractionated doses of radiation. The magnitude of DNA damage was determined by both the alkaline and the neutral comet assay after irradiating the cells with fractionated doses of radiation (2 x 2 Gy or 3 x 2 Gy ; interval of 2 h between the fractions). Clonogenic assay was also performed after fractionated radiation dose exposure. Cell lines exhibited less radiosensitivity after fractionated irradiation than that of observed at corresponding acute doses (Fig. 2-23).

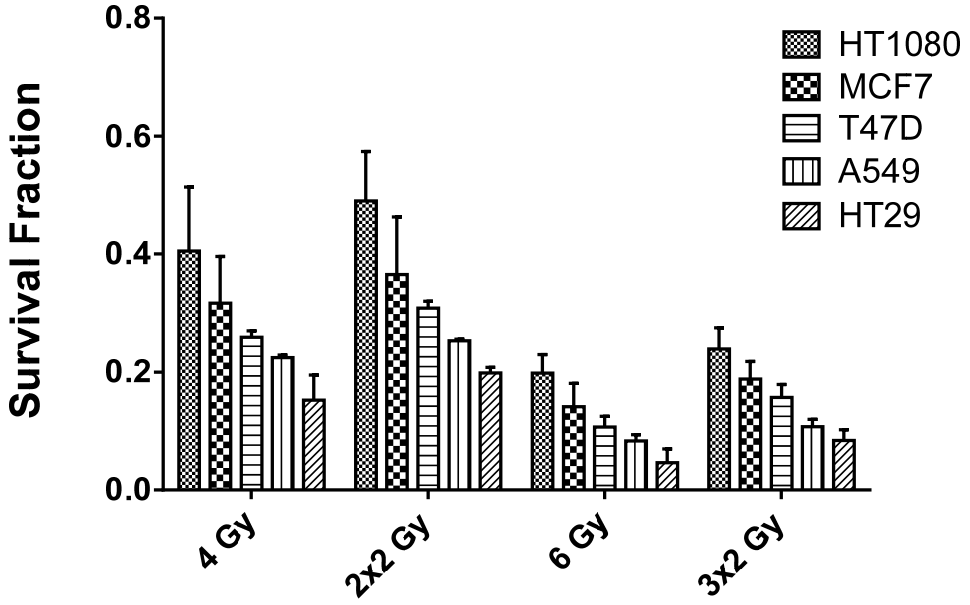


Fig: 2-23. Survival fraction obtained by irradiating the cells to either an acute dose of 4 Gy / 6 Gy or a fractionated dose 2 x 2 Gy / 3 x 2 Gy of gamma radiation

But gradation of cell lines according to the radiosensitivity was similar in both fractionated doses and the acute doses (Fig. 2-23).

At both fractionated doses, the HT1080 cells had the highest survival fraction, followed by the MCF7 cells, the T47D cells, the A549 cells and the HT29 cells. Interestingly, the radiosensitivities evaluated by the extent of DNA damage observed by the alkaline and the neutral comet assays after the fractionated radiation doses were in the same order as that obtained by the clonogenic assay (Fig. 2-24).

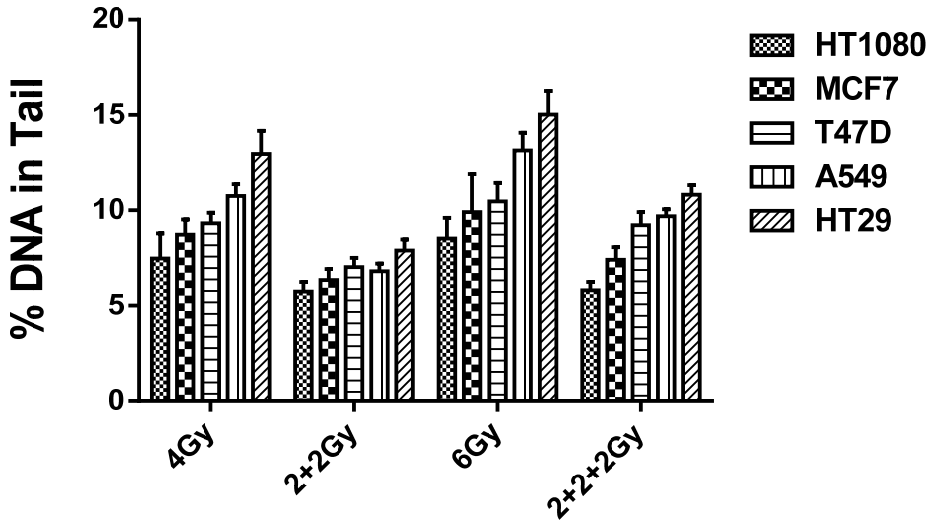


Fig: 2-24. DNA damage obtained by neutral comet assay after exposing the cells to either an acute dose of 4 Gy / 6 Gy or fractionated dose 2 x 2 Gy / 3 x 2 Gy of gamma radiation

A statistically significant correlation was observed between the DNA damage (as assessed by the neutral comet assay) and the clonogenic survival of five cell lines after fractionated doses of radiation (Fig. 2-25 and 2-26) with r values of -0.97 (p = 0.019) and -0.93 (p = 0.015) for 2 x 2 Gy and 3 x 2 Gy, respectively.

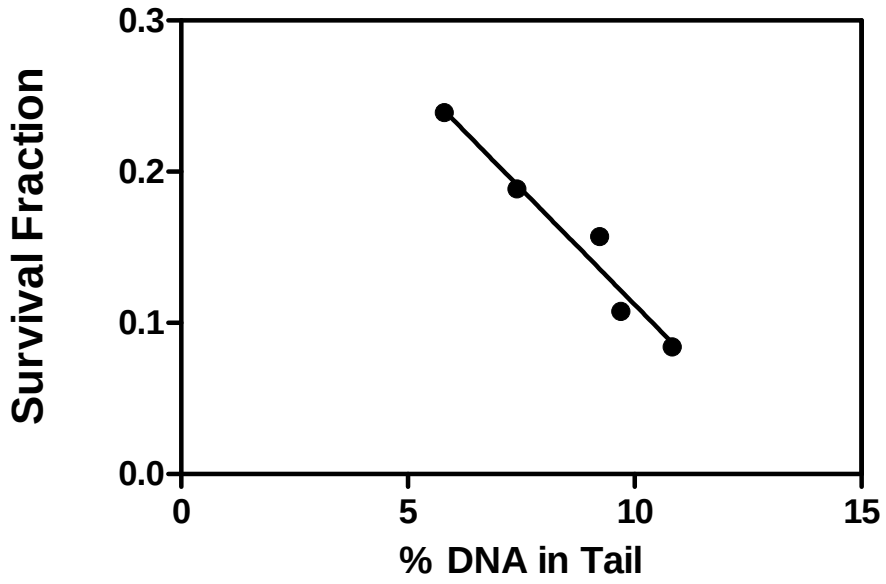


Fig: 2-25. DNA damage obtained by a neutral comet assay after exposure to 2 x 2 Gy fractionated dose and its correlation with clonogenic survival fraction obtained at the corresponding fractionated radiation dose exposure in five cell lines

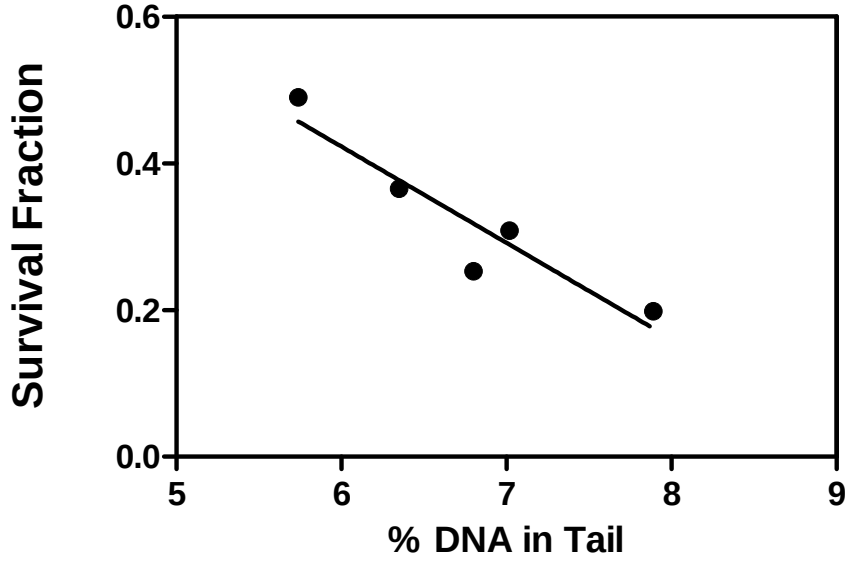


Fig: 2-26. DNA damage obtained by a neutral comet assay after exposure to 3 x 2 Gy fractionated dose and its correlation with clonogenic survival fraction obtained at the corresponding fractionated radiation dose exposure in five cell lines

Similar to the results of acute doses, the total DNA damage, measured by the alkaline comet assay after fractionated doses, did not show significant correlation with the respective survival fraction (Fig. 2-27 and 2-28). The observed r values were -0.81 (p = 0.09) and -0.73 (p = 0.16) for 2 x 2 Gy and 3 x 2 Gy, respectively.

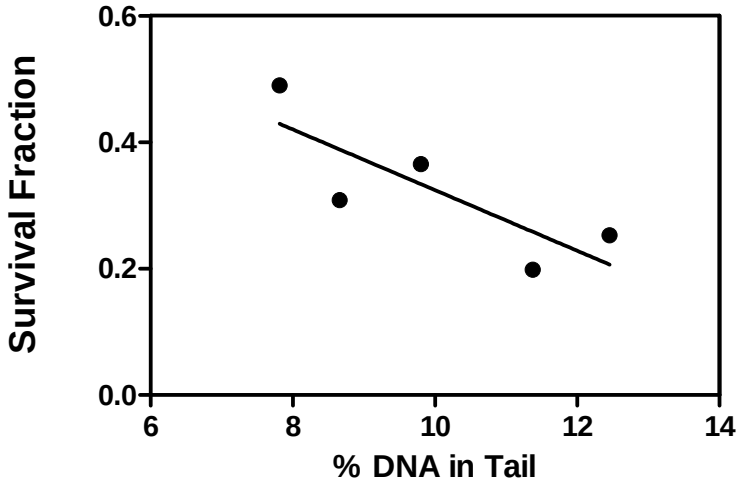


Fig: 2-27. DNA damage obtained by alkaline comet assay after exposure to 2 x 2 Gy fractionated dose and its correlation with clonogenic survival fraction obtained at the corresponding fractionated radiation dose exposure in five cell lines

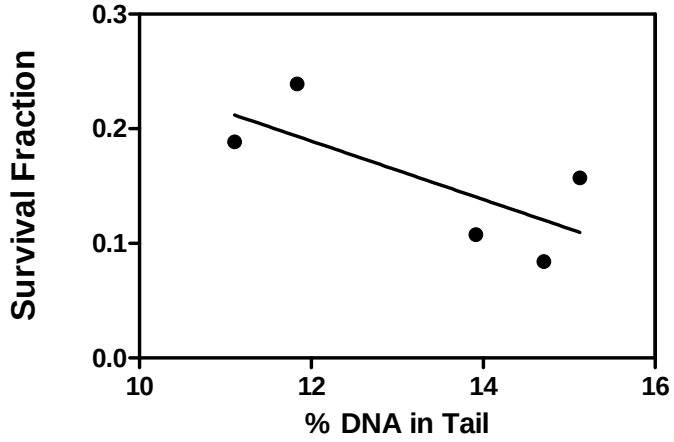


Fig: 2-28. DNA damage obtained by alkaline comet assay after exposure to 3 x 2 Gy fractionated dose and its correlation with clonogenic survival fraction obtained at the corresponding fractionated radiation dose exposure in five cell lines

2.3.7. Validation of the utility of DNA damage as an indicator of comet assay using PC3 and DU145 cells from the standard curve established by other cell lines

In order to validate the usefulness of the standard curve generated using the DNA damage values obtained using the five different tumor cell lines, two more tumor cells of prostate cancer origin were chosen (DU145 and PC3). For the validation, neutral comet assay was performed immediately after irradiating these two tumor cells to either 4 Gy or 6 Gy. From the neutral comet assay, % DNA in tail values was determined. These values were fitted in the standard curve, which has been generated using the five different tumor cells previously (Fig. 2-20 and 2-21). Using the DNA damage values survival fraction values were derived from the standard curve. This predicted survival fraction values were then compared with actual survival fraction values obtained by performing clonogenic assay.

After 4 Gy of radiation exposure, values of %DNA in tail obtained using neutral comet assay were 9.94 and 9.33 for PC3 and DU145 cells, respectively.

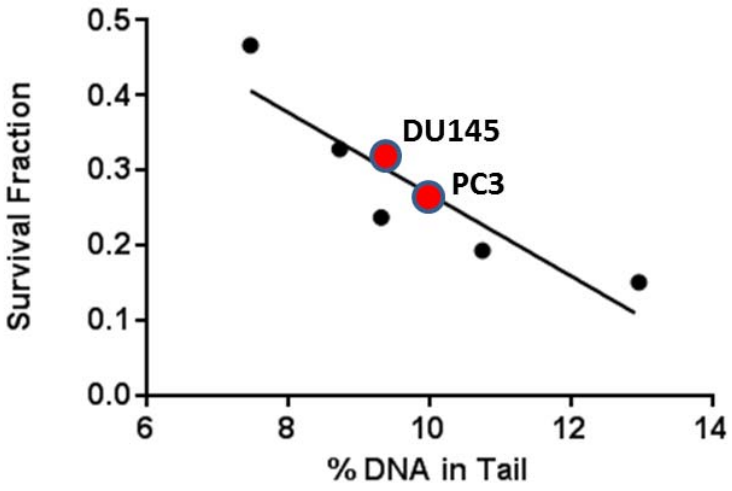


Fig: 2-29. The DNA damage values obtained from PC3 and DU145 cells were plotted on the standard curve obtained from five different cell lines after 4 Gy radiation exposure

Using these values (% DNA in tail from neutral comet assay), the survival fractions for these two cell lines were derived (Fig. 2-29). The derived survival fraction for these two cells at 4 Gy was 0.278 and 0.308, respectively for PC3 and DU145 cells. These derived survival fraction values were very close to the actual survival fraction values obtained using clonogenic assay. The actual survival fraction values obtained at 4 Gy exposure was 0.265 and 0.320 for PC3 and DU145 cells, respectively.

Similar to 4 Gy, after 6 Gy also PC3 cells showed higher DNA damage (% DNA in tail: 11.66) than the DU145 cells (% DNA in tail: 10.39). Using these values, the predicted survival fraction was derived from the standard curve, generated using the five different tumor cell lines (by plotting survival fraction and DNA damage obtained at 6 Gy) (Fig. 2-30). The derived survival fraction for these two cells at 6 Gy was 0.12 and 0.149 for PC3 and DU145 cells, respectively. These derived survival fraction values were also close to the actual survival fraction values obtained using clonogenic assay at 6 Gy i.e. 0.086 and 0.125 for PC3 and DU145 cells, respectively.

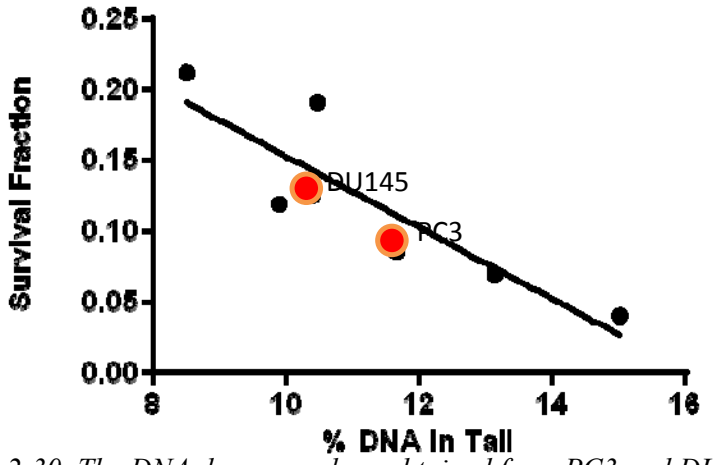


Fig: 2-30. The DNA damage values obtained from PC3 and DU145 cells were plotted on the standard curve obtained from five different cell lines after 6 Gy

2.4. Discussion

Assessment of tumor radiosensitivity prior to radiotherapy will be of great help in improving the efficacy of radiotherapy. However, cellular radiosensitivity is an interplay of several factors such as tissue type, the physiological state of the cell, hypoxia [90], the anti-oxidant capacity of the cell [91], mutations present in different genes and the expression pattern of certain critical radio-responsive genes. Under these different circumstances, finding a suitable assay or parameter that will reflect the radiosensitivity is a challenging task. DNA damage parameters may serve as a good indicator of radiosensitivity. In the present study, radiosensitivities of different tumor cell lines were established using clonogenic assay and this was correlated with DNA damage assessed by comet assay.

Five tumor cell lines were selected namely, HT1080, HT29, MCF7, T47D, and A549. Out of which MCF7 and T47D are breast carcinoma and other cell lines HT1080 (fibrosarcoma), A549 (lung adenocarcinoma) and HT29 (colon carcinoma) are of different tissue origin. The different cell types were used to ensure that the assay was able to distinguish the radiosensitivity across different tumor types. In the clonogenic assay, the HT1080 cells showed the highest radioresistance, followed by the MCF7 cells, the T47D cells, the A549 cells and the HT29 cells. Out of these five cell lines, the T47D cells and the HT29 cells have a mutation in the *TP53* gene. *TP53* status is known to determine the cellular radiosensitivity. In present study, the T47D cells showed higher radiosensitivity than the MCF7 cells (*TP53* wild type), which is consistent with previous clonogenic assay reports [92]. It is likely that apart from *TP53,* mutations present in other genes may determine the radiosensitivity in these cell lines.

The alkaline comet assay is a widely used technique to detect total DNA strand breaks. Therefore, the results of an alkaline comet assay were compared with those of a clonogenic assay in determining the radiosensitivity of the tumor cell lines. Although the alkaline comet assay could differentiate between the most radioresistant and least radioresistant cell lines, it failed to differentiate the narrow differences in the radiosensitivities of the other cell types. As the alkaline comet assay measures the total stand breaks, including alkali labile sites, it may mask the critical DNA damage differences exhibited in different tumor types, thereby compromising the predictive value of the alkaline comet assay. Interestingly, the neutral comet assay was able to grade all the tumor cell lines according to their radiosensitivities, as determined by the clonogenic assay and indicated a significant correlation with the clonogenic survival. It was also observed that the neutral comet assay provided a stronger correlation with clonogenicity at higher doses (4 Gy and 6 Gy) than at 2 Gy, which may be attributed to a lower occurrence of double strand breaks at 2 Gy. For all the cell lines, the DNA repair kinetics, as measured using either neutral or alkaline comet assay, failed to strongly correlate with the radiosensitivity, which is in agreement with some of the earlier reports [80]. But in a recent study [84], the difference in DNA repair was resolved by neutral comet assay. But in this study [84], they have selectively chosen radiosensitive human lymphoblastoid cell lines from individuals with DNA repair disorder, whereas in the current study, different tumor cell lines have been used. Though the residual DNA damage, as measured by the neutral comet assay, is able to segregate the most radioresistant and radiosensitive cell lines, it is not able to grade all the cell types according to their radiosensitivity. Though DNA repair capacity is one major determinant of the radiosensitivity of the cell, a comet assay may not be sensitive enough to resolve the smaller differences in the residual DNA damage

between the cell lines. Therefore, the DNA damage measured immediately after the radiation exposure could be a better reflection of the radiosensitivity than the residual DNA damage. After acute and fractionated doses, the neutral comet assay measurement of DNA damage were negatively correlating with the clonogenicity of the tumor cells, suggesting that this technique may be useful at clinically relevant fractionated doses. A time gap of 2 h between the two fractions was chosen based on earlier reports [93], showing that maximum repair occurred within 2 h. These results clearly indicate that the neutral comet assay could predict the radiosensitivity of different tumor cells, which is in line with the results of the clonogenic survival assay that is considered to be the 'gold standard' method in assessing radiosensitivity. However, the clonogenic assay will be difficult to implement under clinical settings because of the low plating efficiency, the lack of proper colony formation and the time-consuming nature of the protocol. Using the neutral comet assay, some of these drawbacks can be overcome. Comet assay has lot of advantages like simplicity, reliability, sensitivity, and it is amenable for automation. Therefore, with further validation with clinical samples, the neutral comet assay can be a promising assay parameter, which can be used as surrogate marker of radiosensitivity in tumor cells.

CHAPTER 3

MOLECULAR MARKERS IN ASSESSMENT OF RADIOSENSITIVITY OF HUMAN TUMOR CELLS

Molecular Markers in Assessment of Radiosensitivity of Human Tumor Cells

3.1. Introduction

One of the major factors governing the therapeutic efficacy during cancer radiotherapy is intrinsic radiosensitivity of the tumor cells. This intrinsic radiosensitivity is determined by the mutations present in the genome of the tumor cells and also the expression level of many genes/proteins, which are involved in different crucial radiation response pathways. These genes can also be used as predictive markers for radiosensitivity of tumor cells [94]. Therefore, in this study, genes which are activated in DNA damage response (DDR) were chosen to evaluate their usefulness in predicting tumor radiosensitivity. DNA damage response involves the sensing of the DNA damage, activation of signal transducers, expression of effector molecules, and thereby determining the cell fate decision. In all these steps number of proteins/genes are involved in determining different cellular consequences (Fig. 3-1).

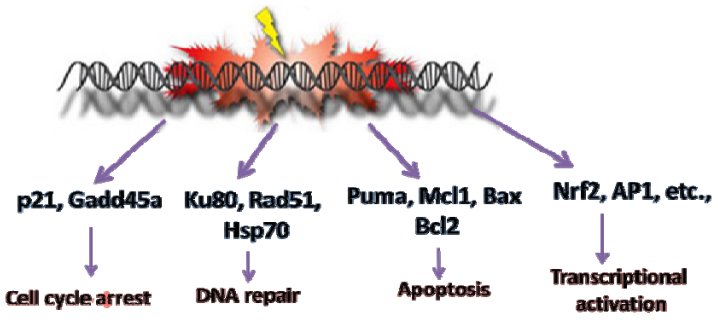


Fig. 3-1. Various genes expressed in response to DNA damage and their involvement in various cellular consequences

When cells are exposed to radiation, it causes damage to the bio-molecules including DNA. DNA damage elicits a complex cellular response by modulation of diverse genes/proteins involved in repair of DNA damage [95, 96]. If the extent of DNA damage is high, repair of that DNA has the risk of acquiring mutations, which can lead to diseases including cancer. Therefore, in these circumstances, cells may programme itself towards apoptotic death. Apart from activating DNA repair and apoptotic genes, cells also activate many redox sensitive transcription factors upon DNA damage. These transcription factors, upregulate many of its target genes that are involved in the cytoprotection and cell survival.

The genes, which are involved in these pathways, can be used in predicting as well as modulating the radiosensitivity of the tumor cells. There are studies which have used this strategy to assess the radiosensitivity of normal cells [97-101] and thereby use these markers for biodosimetry. However, not much comprehensive study has been done to identify genes, which are the markers of radioresistance/radiosensitivity in tumor cells. Since gene expression analysis can be easily automated, and can be performed in high throughput, applicability of this strategy under clinical scenario would be easier. From this perspective, it is important to identify the genes, whose expression show predictive value in tumor radiosensitivity. Results of the previous Chapter (Chapter 2), revealed the significant correlation between DNA damage and radiosensitivity. Therefore, it is plausible that the genes involved in DDR can also be a potential marker of tumor radiosensitivity.

Therefore, the expression pattern of 15 genes, which are involved in DNA repair, cell cycle arrest, apoptosis and redox regulation was evaluated in six different cancer cell lines (HT1080, DU145, MCF7, PC3, A549 and HT29), after exposure to 2 Gy or 6 Gy of γ-radiation. Then the magnitude of expression was correlated with the

clonogenic survival fraction to find out the ability of genes in predicting the radiosensitivity of tumor cells.

3.2. Materials and Methods

3.2.1. Chemicals

Dulbecco's Modified Eagles Medium (DMEM), antibiotics (streptomycin and penicillin), sodium bicarbonate, crystal violet, Tris-HCl, ethylenediaminetetraacetic acid (EDTA), sodium chloride (NaCl), dimethylsulfoxide (DMSO) and diethyl pyrocarbonate (DEPC) were purchased from Sigma (Missouri, USA). Fetal bovine serum (FBS) and trypsin-EDTA were obtained from Himedia (Mumbai, India).

3.2.2. Cell lines and irradiation

Human fibrosarcoma (HT1080), human colon carcinoma (HT29), human mammary carcinoma (MCF7), human lung adenocarcinoma (A549) cell lines were procured from the National Centre for Cell Science (Pune, India). Prostate carcinoma (PC3 and DU145) cell lines were obtained from National Institute for Research in Reproductive Health, Mumbai. These cells were maintained as exponentially growing monolayers in DMEM supplemented with 10% FBS, streptomycin (0.1 mg/ml) and penicillin (100 U/ml) in a humidified incubator maintained at 37 ^{o}C and 5% CO_2. The cells were irradiated (dose rate: 1 Gy/min) using a ^{60}Co teletherapy machine Bhabhatron II (Panacea Medical Technologies, Bangalore, India). For RNA isolation, the exponentially growing cells (2 x 10^6 cells) were seeded overnight in a culture dish, then irradiated (either 2 or 6 Gy) and incubated for 4 h before RNA isolation.

3.2.3. The clonogenic assay

For correlating the gene expression with radiosensitivity, the clonogenic survival fraction of cells at corresponding doses was taken from the clonogenic survival curve from Chapter 2, Section 3.1.

3.2.4. Quantitative real time RT-PCR

To quantify the mRNA expression of the relevant genes, quantitative real time reverse transcriptase polymerase chain reaction (quantitative PCR) was used [87]. The total RNA was isolated using a Triazol reagent (Sigma, St. Louis, MO, USA) and was suspended in 50 µl of deionized DEPC-treated water. Two µg of total RNA was used for the synthesis of cDNA by reverse transcription (cDNA synthesis kit, Sigma). The PCR reactions were composed of 10X SYBR green PCR mix with 5 µl of twice-diluted cDNA templates, 1 µl each of the forward and the reverse primers (0.5 µM ; Table 1), and 3 µl of PCR-grade water in a 20 µl reaction mixture. The above reaction mixtures were amplified with a denaturation step at 95 ^{o}C for 5 min and 40 cycles of amplification including 95 ^{o}C for 15 s, 57 ^{o}C for 15 s and 72 ^{o}C for 20 s, followed by a melting curve analysis on a Rotor Gene 3000 (Corbett Life Science, Australia). The specificity of the respective amplicons was confirmed from the melting curve analysis. The amplification of each gene was performed in triplicate on two biological replicates. The threshold cycle values obtained from the runs were used for calculating the fold change in gene expression using REST-384 version 2 software [102]. The expression of the genes were normalized to a housekeeping gene, β-actin and the relative change in the expression was plotted with respect to the control group.

Table: 3-1. *Primer sequences of different genes whose expression has been evaluated by quantitative real time RT-PCR*

genes	Forward primer	Reverse primer
β-*ACTIN*	CTGGAACGGTGAAGGTGACA	AAGGGACTTCCTGTAACAATGCA
HSP70	ATGCAAGTGGACCAGGAGGAA	TGATTCTCGATTGGCAGGTCCACA
KU80	CAGCGACAGGTGTTTGCTGAGA	AGCCTGTTGAGAACCTGGTTGGAT
RAD51	TCATCGCCCATGCATCAACACC	AGTCTTTGGCATCTCCCACTCCAT
CDKN1A	CCTCATCCCGTGTTCTCCTTT	GTACCACCCAGCGGACAAGT
GADD45A	TCAGCGCACGATCACTGTC	CCAGCAGGCACAACACCAC
BCL2	TGTGGCCTTCTTTGAGTTCGGTG	GTACAGTTCCACAAAGGCATCCC
SNAIL	TACAGCAGGGCAGGACTCTAAT	AGGACAGAGTCCCAGATGAGCTTT
MDM2	ACCTCACAGATTCCAGCTTCG	TTTCATAGTATAAGTGTCTTTTT
PUMA	CCTGGAGGGTCCTGTACAATCT	GCACCTAATTGGGCTCCATCT
FAS	AGCTTGGTCTAGAGTGAAAA	GAGGCAGAATCATGAGATAT
NRF2	GCATGCCCTCACCTGCTACTTTA	CTGAGTGTTCTGGTGATGCCACA
HO1	AGGGAATTCTCTTGGCTGGCTT	ATGCCATAGGCTCCTTCCTCCTTT
GCLC	GGAAGTGGATGTGGACACCAGA	AACTCCCTCATCCATCTGGCAACT
MCL1	AAAGAGGCTGGGATGGGTTT	CAAAAGCCAGCAGCACATTC
NQO1	GGGATCCACGGGGACATGAATG	ATTTGAATTCGGGCGTCTGCTG

3.2.5. Statistical analysis

Statistical analysis was performed and the correlation coefficient was calculated using Graphpad Prism 5.0 software (La Jolla, USA). The significance of the correlation coefficient was also calculated by Graphpad Prism using the formula $t= r \sqrt{(n-2)/(1-r^2)}$, where r = correlation coefficient. A Student's t-test was used for comparison of the means of the two groups. Values were considered to be significantly different at $p< 0.05$.

3.3. Results

3.3.1. Expression pattern of DNA repair genes, RAD51, KU80 and HSP70 genes in the various tumor cell lines after radiation exposure

Different repair pathways are activated, which are involved in the repair of radiation induced DNA damage. Majority of the double strand breaks are repaired by either non homologous end joining (NHEJ) or homologous recombination repair (HRR). Double strand breaks are considered as the most crucial lesions, which lead to cell death if left unrepaired. Moreover, in the previous chapter, a significant correlation between DSBs and tumor radiosensitivity was observed. Therefore, the genes which are involved in DSB repair like KU80, HSP70 and RAD51 were selected, and their expression was analyzed after 2 Gy and 6 Gy of radiation exposure.

KU80 gene showed upregulation in its expression after radiation exposure in a dose dependent manner. Among the six cell lines, the expression of KU80 gene was highest in radioresistant HT1080 cells and the expression was lowest in most radiosensitive HT29 cells at 6 Gy. The fold change in expression of KU80 genes in most radioresistant HT1080 cells was 2.1 and 3.8 at 2 Gy and 6 Gy, respectively (Fig. 3-2A and 3-2B). Followed by HT1080 cells, DU145 and MCF7 cells showed comparable level of KU80 expression. Increased KU80 expression to the range of 2.0 and 2.2 folds was observed in DU145 cells at 2 Gy and 6 Gy, respectively. The relative expression of KU80 gene in MCF cells was 1.4 and 2.4 folds after 2 Gy and 6 Gy respectively. Similarly, expression ratio observed in A549 cells was 1.0 and 1.5 at 2 Gy and 6 Gy respectively. Relative expression of 0.6 at 2 Gy and 1.5 at 6 Gy was observed in relatively radiosensitive PC3 cells. In HT29, which is a least radioresistant among the tumor cell lines used, KU80 expression showed 1.3 and 1.5 folds upregulation after 2 Gy and 6 Gy respectively in comparison to control.

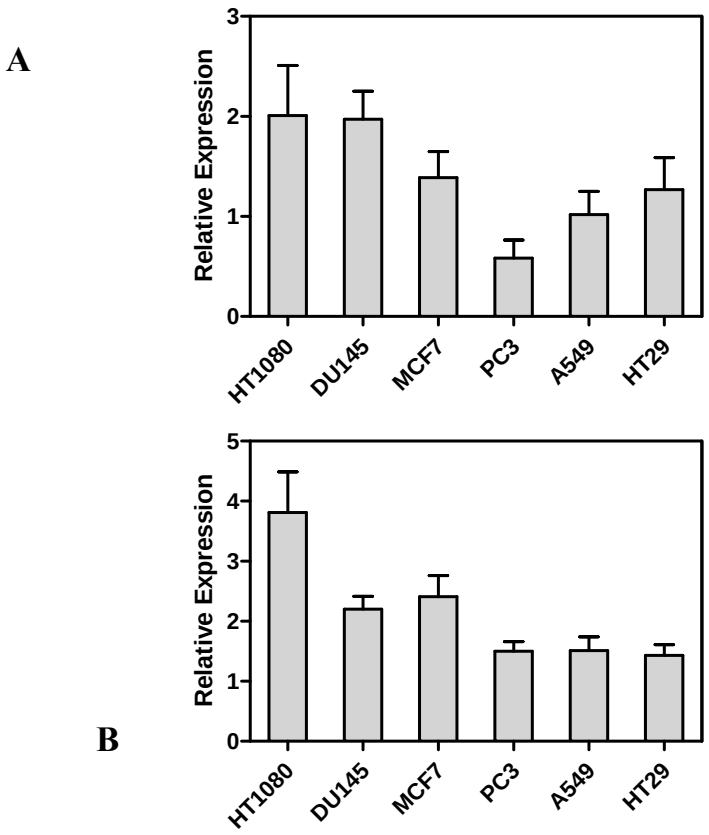


Fig: 3-2. Relative expression of KU80 gene in different tumor cells after exposing to either 2 Gy (A) or 6 Gy (B)

Expression of another gene RAD51, which is involved in the homologous recombinant repair pathway, was also examined in these six tumor cells after exposure to either 2 Gy or 6 Gy. After 6 Gy, maximum expression was seen in HT1080 (4.9 folds) followed by DU145 (3.4 folds), MCF7 (2.7 folds), PC3 (2.0 folds), HT29 (1.5 folds) and A549 (1.6 folds) (Fig. 3-3B). At 2 Gy, the magnitude of expression seen in all the cell lines was lower than the 6 Gy. At 2 Gy, HT1080, DU145 and MCF7 cells showed upregulation to the range of 2 folds in comparison to 1 fold increase in remaining other cell lines namely, PC3, A549 and HT29 cells (Fig. 3-3A).

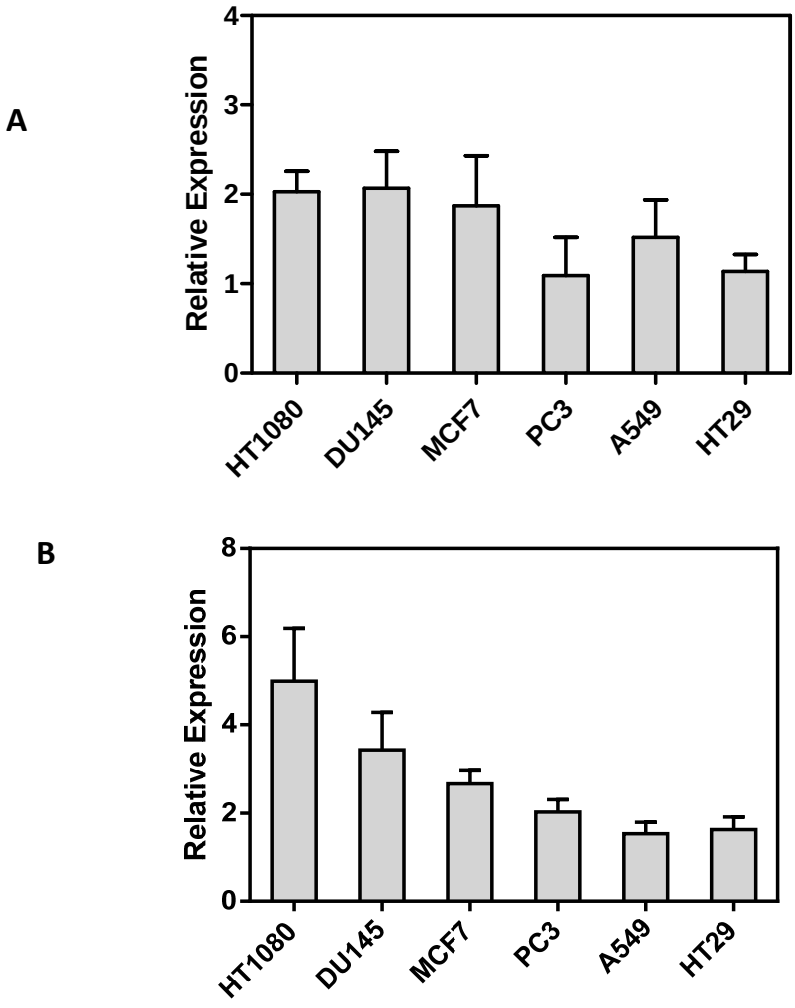


Fig: 3-3. Relative gene expression of RAD51 gene in the different tumor cells after irradiated to either 2 Gy (A) or 6 Gy (B)

Heat shock proteins (HSP) are the proteins which are upregulated under different stress conditions and they were reported to be upregulated during radiation exposure also. Heat shock proteins are the chaperons, which play a role in proper folding of the proteins and more so under stress conditions including oxidative stress. Two major types of HSP are known in human cells and they are HSP90 and HSP70. HSP90 protein has been widely studied and they have been investigated as a target for radiosensitization in cancer therapy [103, 104]. Recent studies have indicated the role

of HSP70 proteins in DNA repair [105]. But the predictive value of HSP70 was not studied for determining the tumor radiosensitivity. At 2 Gy, radioresistant HT1080 cells showed maximum upregulation (two folds increase) in comparison to control, followed by DU145 and MCF7 cells, which showed 1.5 fold increase in their expression. Remaining three cell lines namely, PC3, A549, and HT29 showed an increase of one fold upregulation in comparison to control cells (Fig. 3-4A). At 6 Gy, similar trend in the expression in HSP70 gene was observed in comparison to expression at 2 Gy. But the magnitude of expression at 6 Gy was marginally higher than the 2 Gy (Fig. 3-4B). At 6 Gy, maximum increase in the expression (2.75 folds) was seen in HT1080 cells, followed by MCF7 cells (2.3 folds), DU145 (1.7 folds), A549 (1.6 folds), PC3 (1.4 folds) and HT29 (1.4 folds).

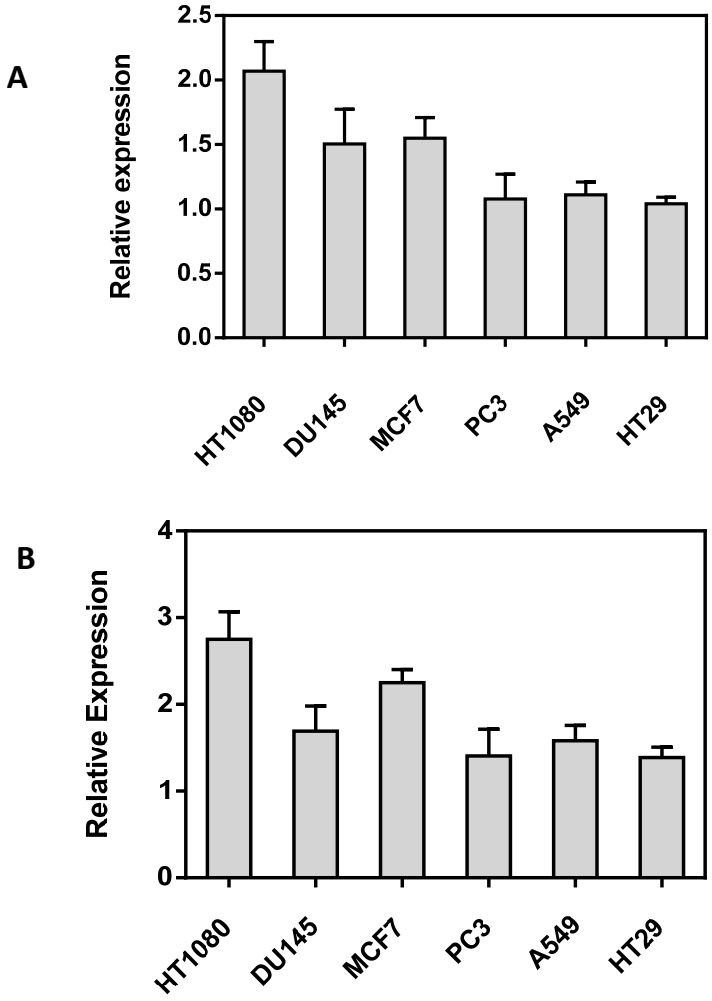


Fig: 3-4. Relative expression of HSP70 gene in different tumor cells after exposing to either 2Gy (A) or 6 Gy (B)

3.3.2. Expression pattern of *P21* and *GADD45A* genes in the various tumor cell lines after radiation exposure

Cell cycle arrest is a vital response against genotoxic stress like radiation to facilitate the repair of the damaged DNA. Tumor suppressor protein p53 has been described to act as a critical effector in cell cycle arrest [50, 52, 54]. Upon stress, p53 becomes transcriptionally active and upregulates the transcription of downstream effector genes, which contain p53 recognition sites in their regulatory regions. *P21* and *GADD45A* are two such important targets of p53 and each of them independently exhibits cell cycle arrest activity. On activation, *P21* inhibits the cyclin dependent kinases and leads to the G1 cell cycle arrest [106]. GADD45A protein causes G2 arrest through its interaction with Cdc2 protein [107]. GADD45A also interact with p21 and act upon different cellular pathways to exert their growth-suppressive function.

We have evaluated the expression of these genes in control and radiation exposed cells. After 2 Gy, there was no significant difference in the expression of *P21* gene between the different cell lines (Fig. 3-5A). All the cell lines showed almost the same level of expression (1.5 to 2 folds upregulation). At 6 Gy, significant upregulation was observed in A549 cells (5.7 folds) compared to other cell lines (Fig. 3-5B).

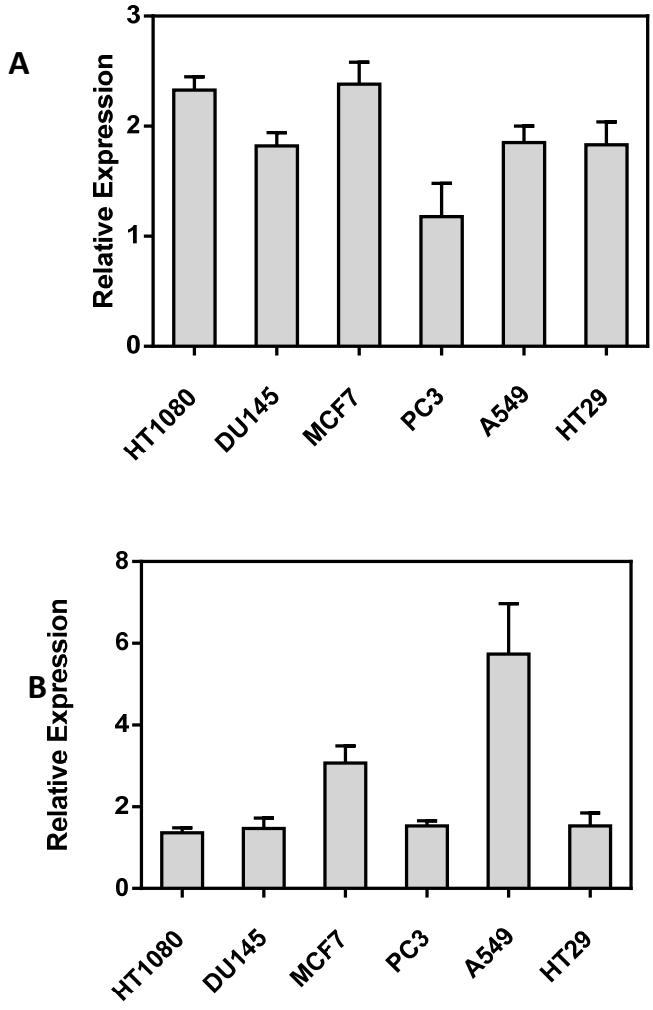


Fig: 3-5. Relative expression change observed in the expression of p21gene in different tumor cells after exposing to either 2Gy (A) or 6 Gy (B)

Similar to *P21* expression, A549 cells also showed significant upregulation in expression of *GADD45A* after 6 Gy (Fig. 3-6B). Five folds increase in expression was observed in A549 cells followed by 3 folds increase in DU145 cells. Apart from A549 and DU145, other cell lines showed same level of expression with the relative expression of around 1.5 folds. At 2 Gy, DU145, MCF7 and A549 cells showed positive expression with 1.5 to 2 folds upregulation (Fig. 3-6A). But, HT1080 and

HT29 cells showed down regulation in expression of *GADD45A* with 2 folds and 1.3 folds, respectively.

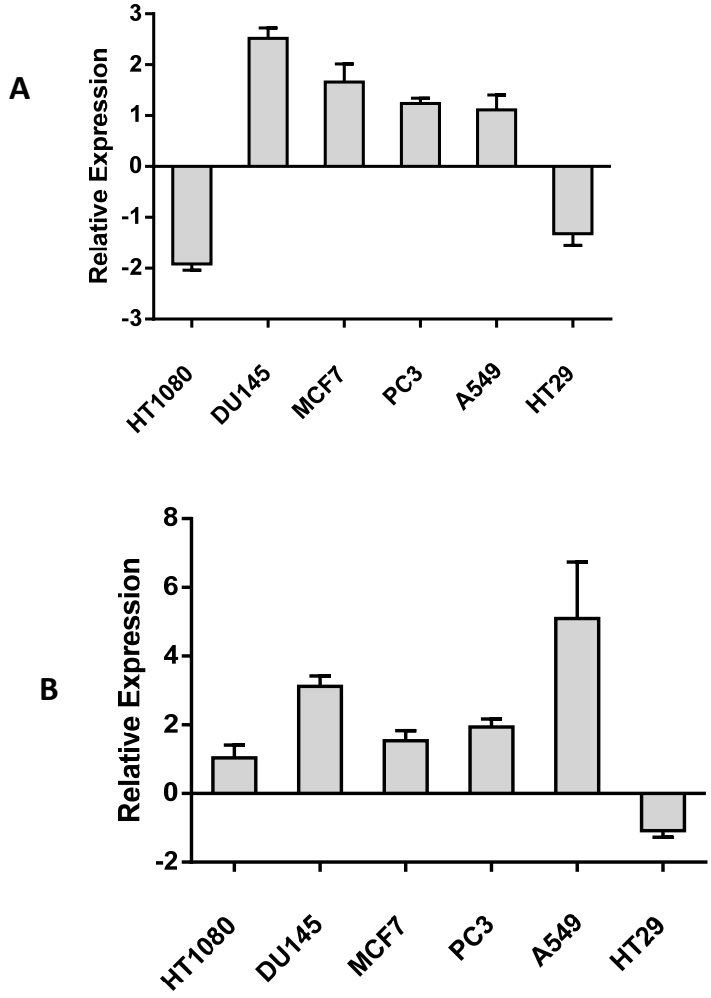


Fig: 3-6. Relative expression change observed in GADD45A gene in different tumor cells after exposing to either 2 Gy (A) or 6 Gy (B)

3.3.3. Expression pattern of apoptotic pathway genes in irradiated tumor cell lines

When the extent of DNA damage caused by ionizing radiation is beyond the threshold level, cells programme themselves to die in order to avoid the risk of accumulating mutations. The cell death decision is the result of interaction between of many proteins and their balance. *MCL1, PUMA, FAS, BCL2, MDM2* and *SNAIL* are some of the major players which determine the cell to undergo apoptosis and the execution of apoptosis [108, 109]. Therefore, the expression patterns of these genes were examined in the tumor cells exposed to various doses of radiation.

After 2 Gy, A549 cells showed 4.1 folds upregulation in PUMA gene expression followed by DU145, PC3 and MCF7 cells showed two folds upregulation (Fig. 3-7A). But HT1080 and HT29 cells showed -2.2 and -4.2 folds down regulation, respectively in expression of PUMA gene (Fig. 3-7A). Similar trend of expression was also seen after 6 Gy in all the four cell lines. But at 6 Gy, A549 cells showed 7.8 folds upregulation in PUMA expression, which is significantly higher than the expression observed at 2 Gy (Fig. 3-7B).

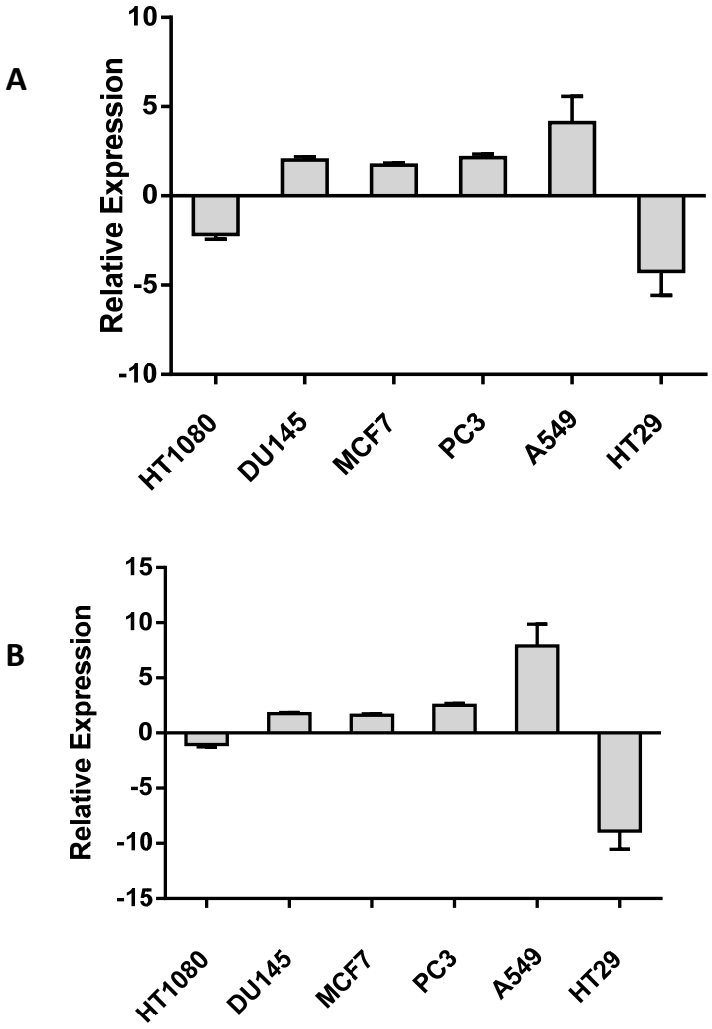


Fig: 3-7. Relative expression change observed in the expression of PUMA gene in different tumor cells after exposing to either 2Gy (A) or 6Gy (B)

Expression of *MCL1* - an anti-apoptotic gene, was studied among these six tumor cell lines irradiation. After 6 Gy, all the tumor cells, except PC3 and A549 showed the expression in *MCL1* in accordance with their radiosensitivity. Highest upregulation was seen in A549 cells (3.8 folds). Among the other cell lines, radioresistant HT1080 cells showed 2.5 folds upregulation in *MCL1* expression followed by DU145 cells (1.4 fold), MCF7 cells (1.3 fold), and the least radioresistant HT29 cells (1.2 fold) at 6 Gy (Fig. 3-8B). But at 2 Gy, no significant upregulation was seen in HT1080 cells. MCF7 and HT29 cells showed upregulation in the range of two fold (Fig 3-8A).

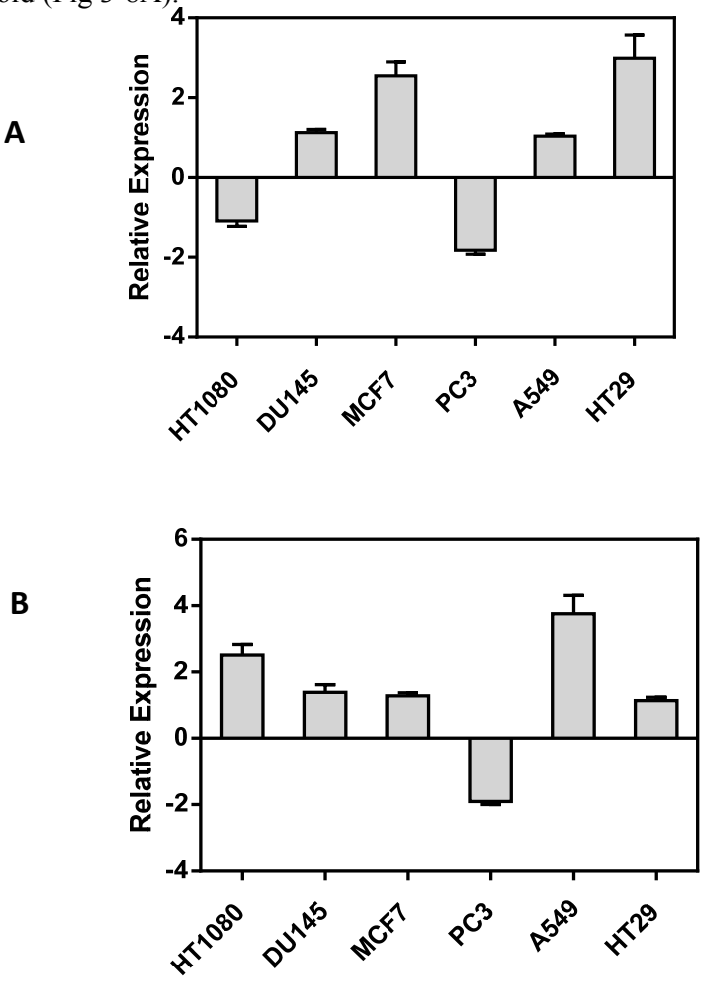


Fig: 3-8. Relative expression of MCL1 gene in different tumor cells after exposing to either 2 Gy (A) or 6 Gy (B)

Other genes which are involved in apoptosis like *MDM2, BCL2, FAS* and *SNAIL* have also been investigated for their usefulness in assessing radiosensitivity of the tumor cells. But these genes did not show gene expression in accordance with the radiosensitivity of the tumor cells. At 6 Gy all the cell lines showed almost equal upregulation of *BCL2* gene (2 folds) except A549 cells, which showed 4.3 folds induction (Fig. 3-9B and 3-9A). But at 2 Gy radiation exposure, DU145, PC3 and HT29 cells showed two folds down regulation of *BCL2* in their expression while other cell lines showed a two folds upregulation in expression.

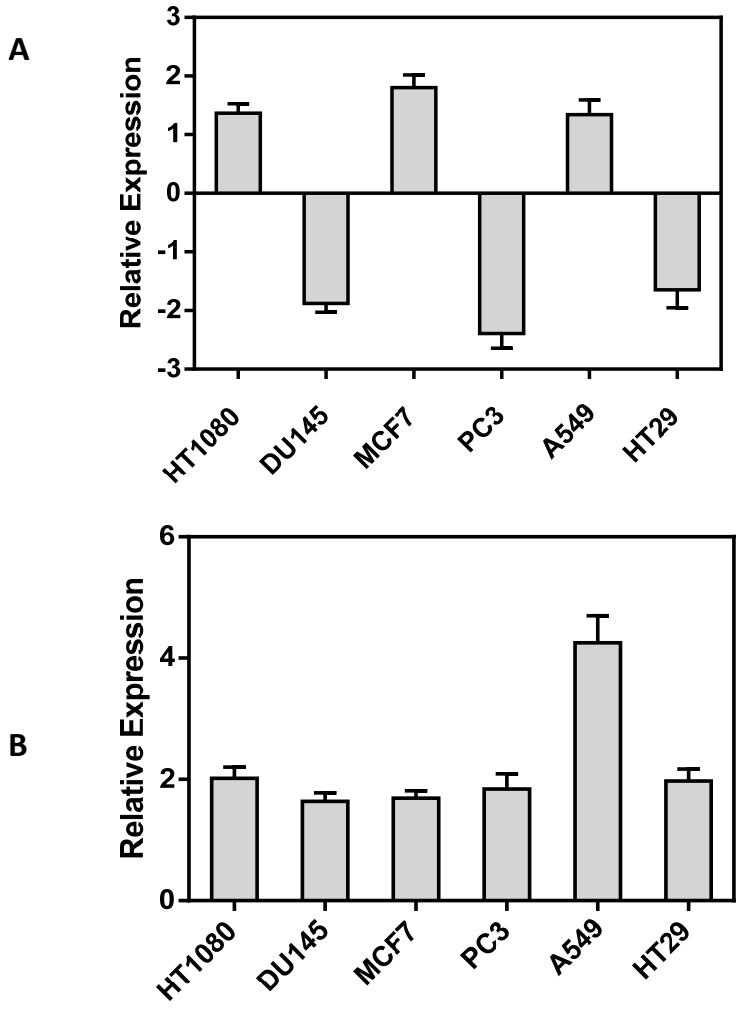


Fig: 3-9. Relative expression observed in BCL2 gene in different tumor cells after exposing to either 2 Gy (A) or 6 Gy (B)

Similarly all the cell lines except A549, showed around 1.5 fold upregulation (HT1080, DU145 and MCF7) or 1.5 fold down regulation (PC3 and HT29 cells) in *FAS* gene expression at 6 Gy. At the same radiation exposure A549 cells showed more than 5 folds upregulation in the expression of *FAS* (Fig. 3-10B). After 2 Gy, HT1080, DU145, MCF7 and A549 cells have showed positive regulation in the expression of FAS gene (1 to 2 folds) (Fig. 3-10A). Whereas, PC3 and HT29 cells showed marginal down regulation in their *FAS* expression (-1 to -2 fold) (Fig. 3-10A).

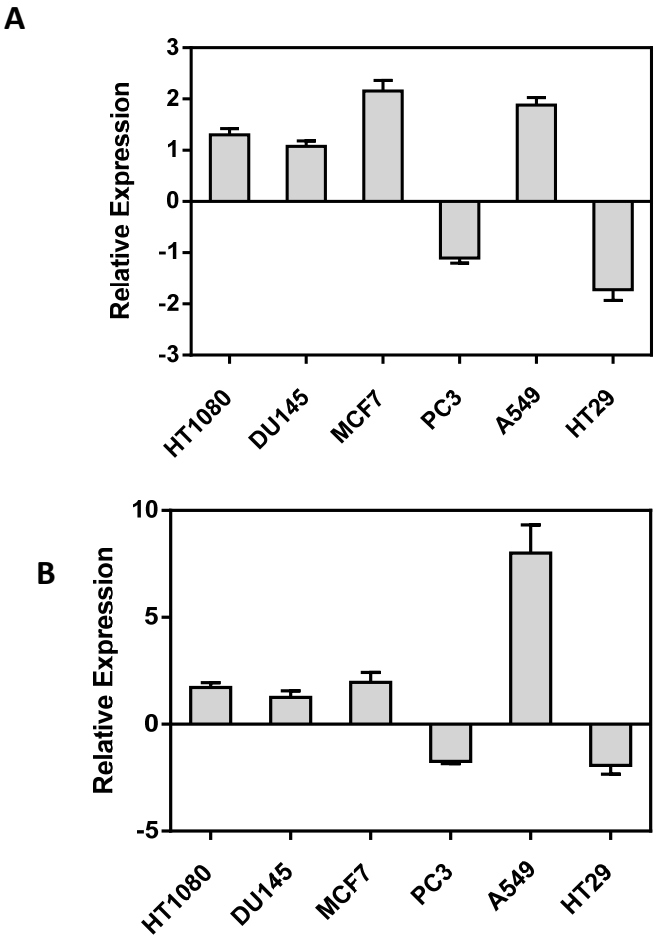


Fig: 3-10. Relative expression of FAS gene in different tumor cells after exposing to either 2 Gy (A) or 6 Gy (B)

Expression of another transcription factor *SNAIL*, which is involved in apoptosis and epithelial mesenchymal transition was also evaluated for its expression in these tumor cells irradiated with 2 or 6 Gy. Out of the six tumor cells *SNAIL* expression was slightly upregulated in the range of 2 folds in HT1080, DU145, MCF7, PC3, and A549 cells. But in HT29 cells, the change was significantly higher than the other cells (4.4 folds) at a given radiation dose of 6 Gy (Fig. 3-11B). Similar trend was also seen after 2 Gy radiation exposure (Fig. 3-11A).

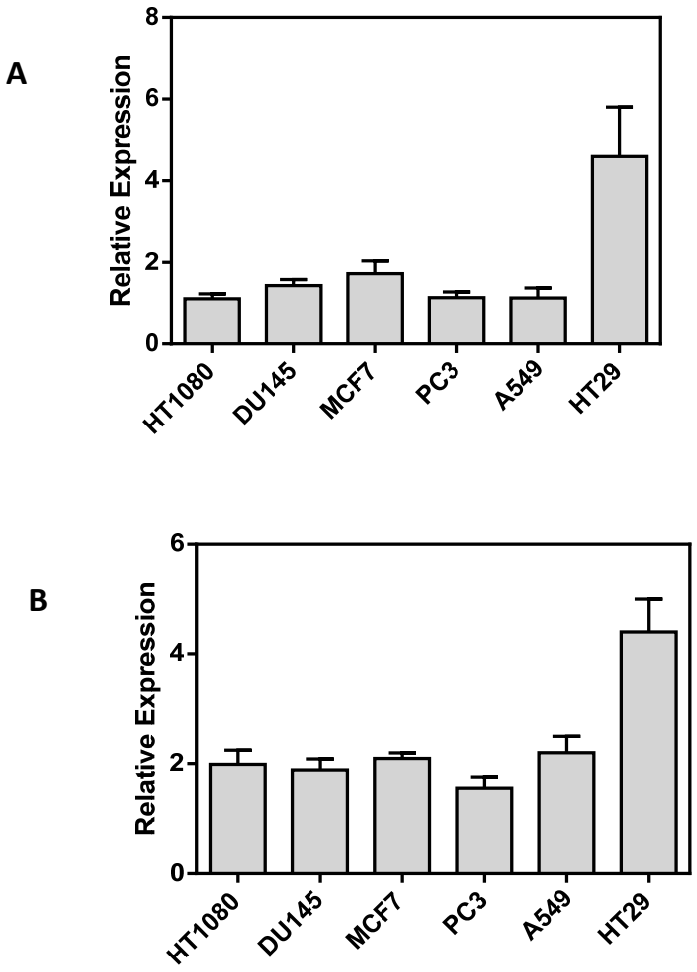


Fig: 3-11. Relative expression of SNAIL gene observed in different tumor cells after exposing to either 2 Gy (A) or 6 Gy (B)

3.3.4. Expression pattern of antioxidant pathway genes in the various tumor cell lines after radiation exposure

In order to maintain the redox balance, cells employ antioxidant pathways to overcome the oxidative stress and to maintain the redox homeostasis. During the radiation exposure, redox balance of the cells tilts towards oxidative condition and in order to counter that, cells upregulate many antioxidant enzymes and proteins. These enzymes have an antioxidant responsive element in their promoter (ARE) region and that will be bound and recognized by a redox transcription factor *Nrf2* and will be transcriptionally activated. These upregulated proteins and enzymes will be employed in bringing back the cellular oxidative homeostasis. Therefore, *Nrf2* and its dependent genes like *HO1, GCLC* and *NQO1* were chosen and their differential expression after radiation exposure was studied to assess the radiosensitivity of the tumor cells.

At 2 Gy, there was no significant difference in the mRNA level of *Nrf2* was observed (Fig. 3-12A). But at 6 Gy, all the cells showed significant upregulation in expression of *Nrf2* (Fig. 3-12B). HT1080 cells, which are radioresistant in comparison to other cells, showed 2.7 folds increase in expression of *Nrf2* followed by DU145, A549, MCF7, and HT29 cells. (Fig. 3-12B). Among all the six cell lines, least expression was observed in PC3 cells (1.1 fold)

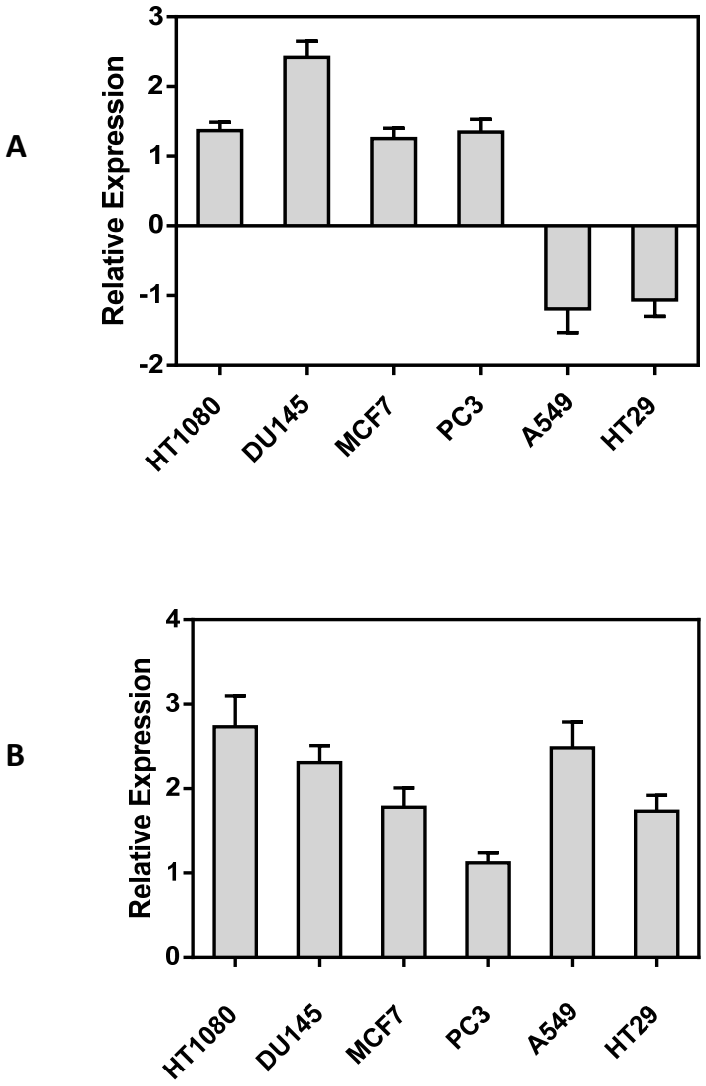


Fig: 3-12. Relative expression of Nrf2 gene in different tumor cells after exposing to either 2 Gy (A) or 6 Gy (B)

Apart from *Nrf2*, the expression of its dependent genes, *HO1, NQO1* and *GCLC* was determined in the six different tumor cells. Expression analysis done after exposing the cells to 2 Gy revealed that the maximum of 2.5 folds upregulation in the expression of HO1 was observed in DU145 cells followed by MCF7 cells (1.5 fold) and PC3 cells (1.2 fold). HT1080 and HT29 cells showed down regulation in their HO1 expression at 2 Gy (Fig. 3-13A). But after 6 Gy, A549 and DU145 cells showed

3 folds increase in their expression over control. HT1080 and PC3 cells showed 1.6 and 2.5 fold upregulation, respectively. But at 6 Gy, HT29 cells showed down regulation in *HO1* expression (3-13B).

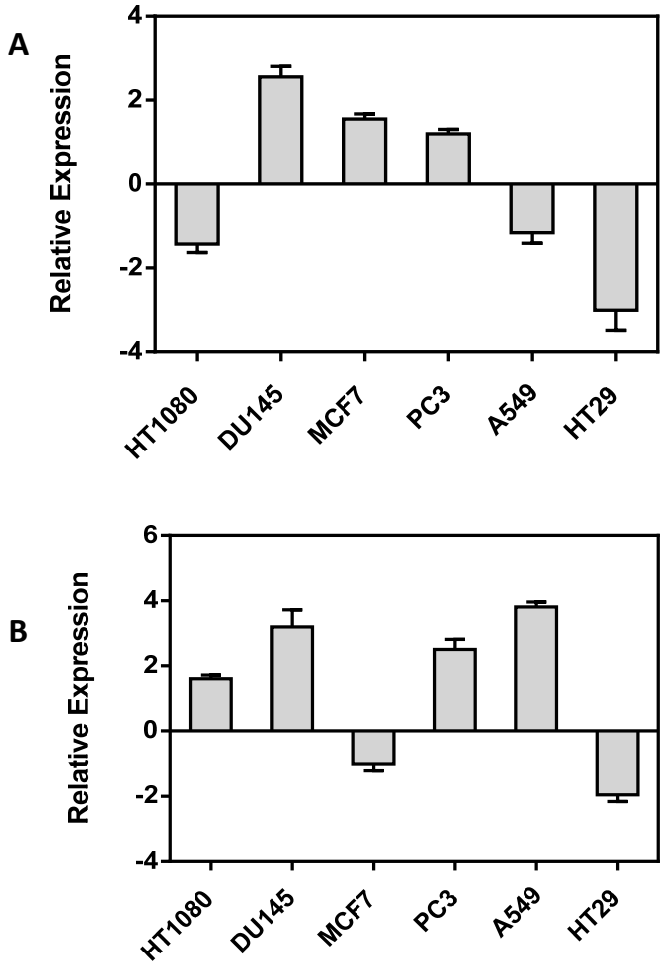


Fig: 3-13. Relative expression of HO1 gene in different tumor cells after exposing to either 2 Gy (A) or 6 Gy (B)

Out of six cell lines, DU145 cells showed the highest upregulation in *GCLC* expression both at 2 Gy and 6 Gy treatments. All the other cell lines showed comparable levels of *GCLC* expression (two folds upregulation) both at 2 Gy and 6 Gy (Fig. 3-14A and 3-14B).

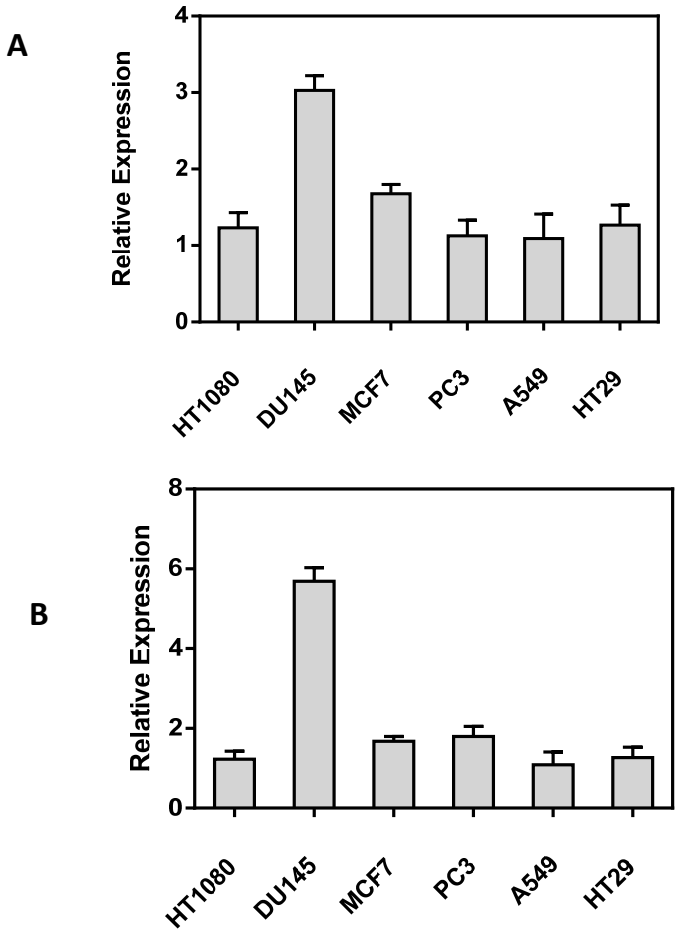


Fig: 3-14. Fold expression change observed in the expression of GCLC gene in different tumor cells after exposing to exposing to either 2 Gy (A) or 6 Gy (B)

3.3.5. Correlation between survival fraction and expression of DNA repair genes RAD51, KU80, and HSP70

After investigating the expression profile of 15 genes (*MCL1, BCL2, KU80, RAD51, NRF2, NQO1, GCLC, HSP70, FAS, PUMA, CDKN1A, HO1, SNAIL, GADD45A* and *MDM2)* in five tumor cell lines (HT1080, DU145, MCF7, PC3, A549 and HT29 cells) (Fig. 3-2 to 3-14), the correlation between the expression and survival fraction (obtained from Chapter 2; Table. 2-1) was examined and tabulated in Table 3-2. Of the 15 genes analyzed, only three genes (*HSP70, KU80* and *RAD51*) showed an expression pattern that was related to the radiosensitivity of the cell line. The other genes did not exhibit a significant correlation with clonogenic survival at either 2 Gy or 6 Gy (Table 3-2). The expression of *HSP70, KU80* and *RAD51* observed at 6 Gy radiation exposure showed a significant positive correlation with the survival fraction. The 'r' values observed were 0.91 (p = 0.013), 0.97 (p =0.0014) and 0.97 (p = 0.0013) for *HSP70, KU80* and *RAD51,* respectively (Fig. 3-15A, B and C*)*.

Table 3-2. Correlation coefficient obtained for various genes by correlating expression with the survival fraction

Gene	r - value	*p*-values
HSP70	0.905	0.0130
RAD51	0.970	0.0013
KU80	0.969	0.0014
Cdkn1a	0.499	0.310
GADD45A	0.100	0.900
MDM2	-0.605	0.202
BCL2	-0.299	0.560
MCL1	0.203	0.699
PUMA	0.118	0.820
FAS	0.492	0.320
SNAIL	-0.538	0.270
Nrf2	0.569	0.238
HO1	0.195	0.710
NQO1	-0.461	0.356
GCLC	-0.218	0.677

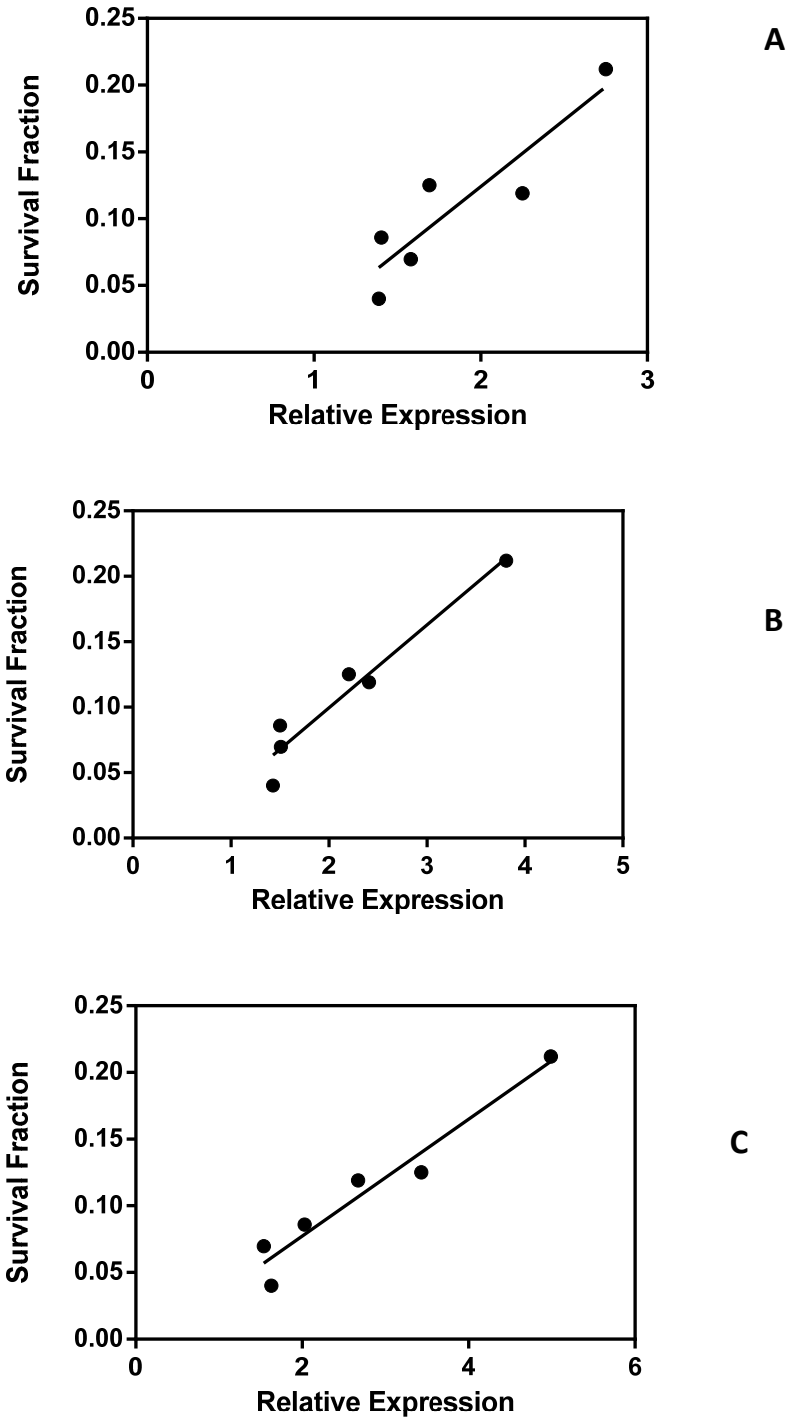


Fig: 3-15. Correlation between the survival fraction and the mRNA expression of HSP70 (A) KU80 (B) and RAD51 (C) at 6 Gy in six tumor cell lines.

Apart from correlating the gene expression with the clonogenic survival fraction, further correlation analysis was performed between DNA damage (as assessed by the neutral comet assay) and the relative gene expression of *HSP70, KU80* and *RAD51*. Expression of all three genes after radiation exposure (6 Gy) showed a significant correlation with the DNA damage, measured by the neutral comet assay (Fig. 3-16A, B and C). The correlation coefficients for *HSP70, KU80* and *RAD51* in this analysis were -0.83 (p = 0.040), -0.85 (p = 0.033) and -0.86 (p = 0.028) respectively.

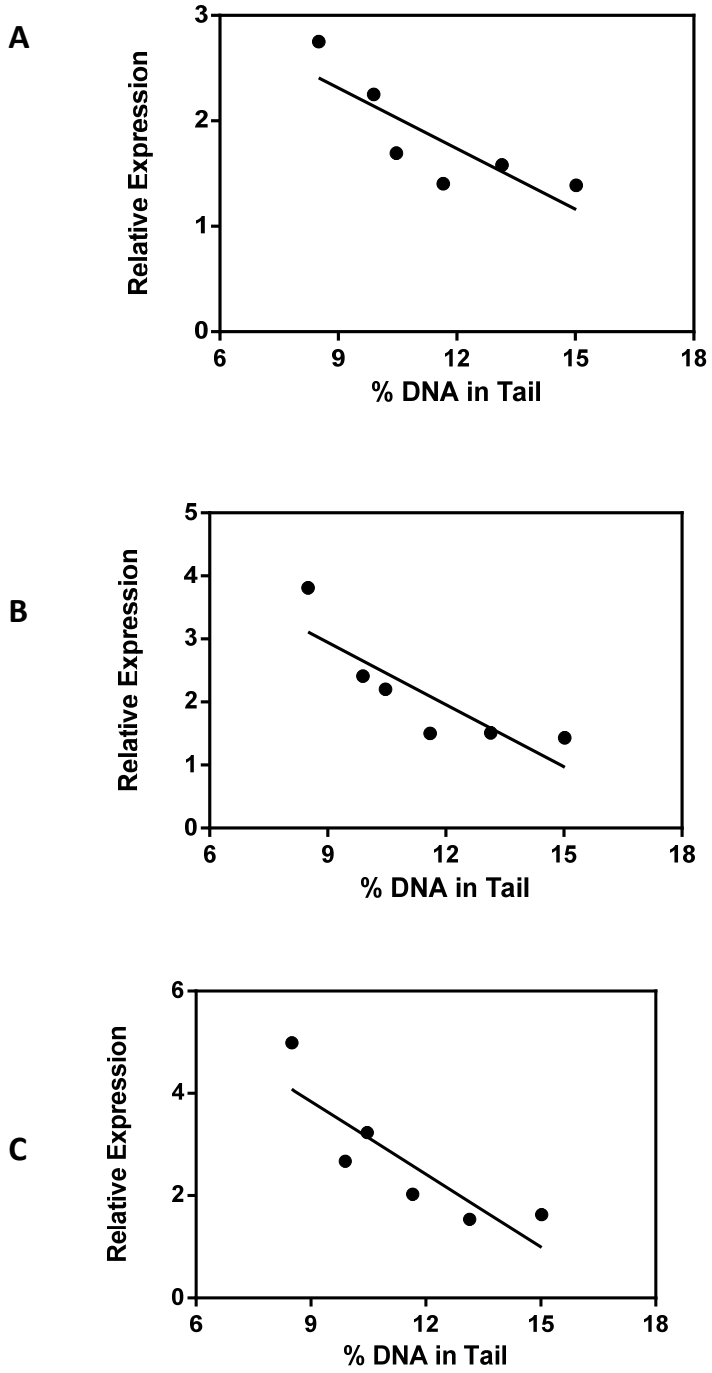


Fig: 3.16. Correlation between gene expression of HSP70 (A), KU80 (B) and RAD51 (C) with DNA damage obtained by neutral comet assay at 6 Gy in different tumor cells.

3.3.6. Radiosensitization of tumor cells using DNA-PK inhibitor NU7026

This study has shown that the expression of genes which are involved in double strand DNA repair pathways especially DNA-PK (KU70, KU80 and DNA-PKcs) can be good predictive markers for tumor radiosensitivity. Therefore, the effect of inhibiting DNA-PK (DNA-PK consists of KU70, KU80 and DNA-PKcs); a protein involved in the non-homologous end joining was investigated using a pharmacological inhibitor (NU7026), in potentiating the radiation induced damage in tumor cells. For this experiment, a relatively radioresistant DU145 cells were treated with 10 μM NU7026 for 2 h prior to the radiation treatment (2, 4 and 8 Gy) and the survival fraction was calculated. From the survival fraction it was found that the pretreatment of NU7026 alone did not have any significant effect on survival fraction of the DU145 cells. But in the cells which are treated together with NU7026 and radiation showed a synergistic reduction in survival fraction. Survival fraction observed in the cells, which are treated with NU7026 followed by 8 Gy is 0.002 in comparison to survival fraction of 0.1 with 8 Gy alone (Fig. 3.17 and 3.18). Similar results were found with other doses of radiation also. The DNA damage after inhibiting DNA-PK in cells which are exposed to radiation was also analysed. DNA-PK inhibition using NU7026 treatment resulted in significantly slower repair kinetics (Fig. 3. 19).

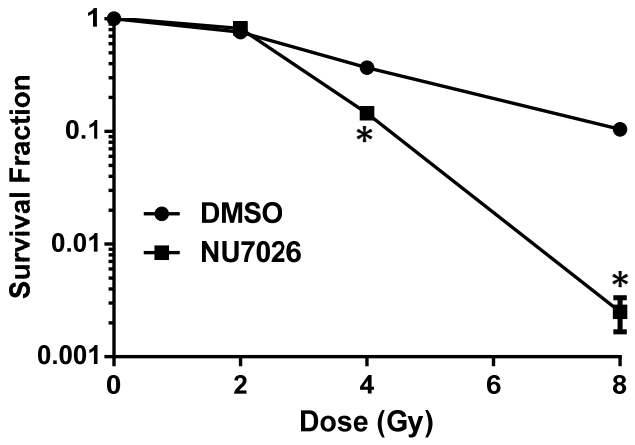


*Fig: 3.17. Survival fraction observed in DU145 cells which are treated with DNA-PK inhibitor. *p<0.05 in comparison to DMSO control group*

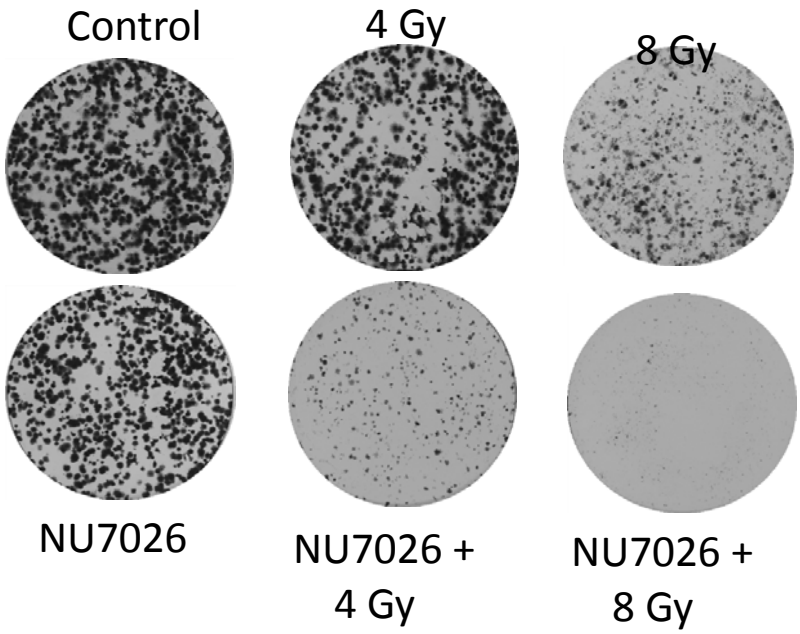


Fig: 3.18. Representative images of clonogenic survival assay of DU145 cells which are treated with DNA-PK inhibitor (NU7026)

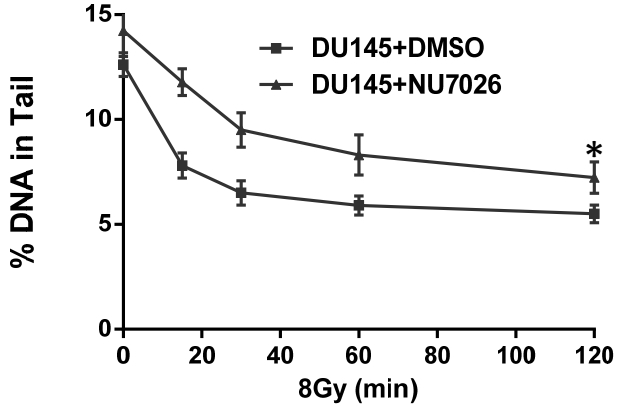


*Fig: 3.19. DNA repair kinetics analysis of DU145 cells which are treated with DNA-PK inhibitor (NU7026) followed by exposure to 8 Gy of radiation. *p<0.05 in comparison to DMSO control group.*

3.4. Discussion

Cellular radiosensitivity is mainly governed by expression of genes/proteins involved in radiation response pathways like DNA repair, cell cycle arrest, oxidative mechanism and apoptosis. Differential expression of these genes exhibited in different tumor cells form the basis of differential intrinsic radiosensitivity observed in different tumor cells. Therefore, it is plausible that the genes associated with these pathways can be useful as predictive markers to assess the tumor radiosensitivity and also in modulating radiosensitivity. Hence, expression of genes involved in various radiation response mechanisms was studied for their predictive validity in assessing radiosensitivity. Out of the 15 genes studied, expression of *KU80, HSP70,* and *RAD51* were showed significant correlation with the survival fraction of the irradiated tumor cells. KU80 is a protein that, make up the KU heterodimer with KU70, which binds at

ends of DNA double-strand breaks and is required for the non-homologous end joining pathway of DNA repair. DSBs resulting from ionizing radiation are among the most severe types of DNA damage, with unrepaired DSBs leading to oncogenic transformation and genetic instability. Results showed the potential of *KU80* expression in assessment of tumor radiosensitivity. In a similar study Ye J et al., have reported the differential expression of *KU80* in different tumor cells and its upregulation at mRNA level on irradiation [110]. In another study by Moeller et al. [111] showed relationship between expression of *KU80* with treatment response / mortality following radiotherapy in head and neck cancer patients. But the usefulness of KU80 expression in predicting the tumor radiosensitivity was not found in the literature and the present study has demonstrated the same. Studies have reported a significantly high level of KU80 expression in tumor cells than the corresponding normal cells [110]. The higher expression of KU80 seen in tumor cells in comparison to corresponding normal cells may be one of the reasons for the acquired radio-resistance in many of the tumor cells. These observations, combined with my results (that there is a gradation in KU80 expression within the tumor cells according to their radiosensitivity of tumor cells) lead us to hypothesise that the KU80 can be a good target for sensitizing the tumor cells against radiation induced damage. Therefore, an inhibitor of DNA-PK (KU80 is a subunit of DNA-PK) was used to examine the value of DNA-PK as the target to potentiate the radiation induced damage. It was found that the DNA-PK inhibition as such had no significant impact on the tumor cell survival, but with radiation, it had a synergistic effect in reducing the survival of the tumor cells. In the presence of DNA-PK inhibitor, the ability of the cells to repair their DSB was greatly hindered and that might have led to mitotic catastrophe and thereby a reduction in survival. This strategy of inhibiting one of the DNA repair pathway can

be a very good strategy in selectively radiosensitizing tumor cells in comparison to the normal cells. Because, tumor cells have mutation in one or more DNA repair pathways and they heavily dependent on some of the DNA repair pathways [112-114]. Similar to *KU80, RAD51* is the protein involved in double strand DNA repair, but through homologous recombination. The mutations in *RAD51* gene of normal cells were reported to have increased risk of cancer development [115]. At the same time, cancers with mutation in *RAD51* genes were also reported to exhibit more radiosensitivity [116]. Therefore, there was strong reason to speculate that the expression levels of *RAD51* may be one of the determining factors in radiosensitivity of tumor cells [117, 118]. Hence, the value of *RAD51* expression in predicting the radiosensitivity was studied in tumor cells and a significant correlation was seen between the expression of *RAD51* and radiosensitivity of different tumor cells. Out of 15 genes evaluated for their use in predicting radiosensitivity of tumor cells, in addition to KU80 and RAD51, expression of *HSP70* also showed significant correlation with the radiosensitivity of tumor cells. Though *HSP70* known to be expressed and regulated under elevated temperature, radiation also known to induce the expression of this gene. *HSP70* and its family members are also known to interact with the proteins involved in DNA double strand breaks [119] and implicated in radiosensitivity [120, 121]. Heat shock proteins have been widely studied as target in anticancer research. Many of the inhibitors of HSP90 are under various stages of clinical stages for their use in cancer treatment [122-124]. Apart from the fact that there is overexpression of HSP70 seen in the tumor cells, the current study also showed the upregulation of HSP70 on radiation treatment. This may be one of the plausible mechanisms of acquired radiation resistance. Inhibitors of HSP70 can also be one of the targets which can be used for radiosensitization and to overcome the

radiation resistance during radiotherapy. Since the acquired radiation resistance is contributed by many factors (like upregulation in the expression of KU80, HSP70 etc. on ionizing radiation) combinatorial inhibition of more than one gene can be a better alternative for overcoming the radiation resistance.

The results using neutral comet assay showed a good correlation of double strand breaks with radiosensitivity of tumor cells, which seems to be associated with over expression of genes like *KU80, RAD51* and *HSP70,* which are involved in repair of double strand breaks. Therefore, it may be plausible that the cells which are capable of activating DNA repair genes show less DNA damage. Moreover, expression of *GADD45A*, *P21*, and *PUMA* genes, which are known to be involved in cell cycle arrest, SSB repair, and apoptosis, failed to exhibit significant correlation with tumor radiosensitivity. The fact that many tumor types have mutations in p53, and these genes (*GADD45A, P21, and BAX) are* controlled by p53 transcription factor, may be the reason for the absence of correlation between these genes and radiosensitivity. However, the relationship between expressions of other genes and radiosensitivity needs further systematic investigation.

CHAPTER 4

MOLECULAR MECHANISMS GOVERNING RADIOSENSITIVITY OF PROSTATE CANCER CELLS

Molecular Mechanisms Governing Radio-sensitivity of Prostate Cancer Cells

4.1. Introduction

Intrinsic radiosensitivity of the tumor cells is the major determining factor of the outcome of radiotherapy, which is determined by the interaction between networks of signalling pathways. Better understanding of these signalling cascades and their role in radiosensitivity will enable us to exploit them in radiosensitization of tumor cells. The molecular biological approaches in addressing this radiobiological quest have helped in unravelling mechanisms and signalling cascades involved in radiosensitivity of the tumor cells.

Exposure of biological system to γ-radiation leads to the damage of biomolecules directly or indirectly through ROS generated by radiolysis of water. Cells possess many defence mechanisms, which help the cells to overcome from the oxidative damage. Intracellular antioxidants/antioxidant enzymes like glutathione, thioredoxin reductase, glutathione peroxidase, catalase and superoxide dismutase etc., form the first line of defence against ROS induced oxidative stress in the cells [125, 126]. Apart from these antioxidant enzymes, many cytoprotective genes like NF-κB, AP1, MAP kinases, and EGFR etc., are also activated by the cells during the oxidative damage. These antioxidants and detoxifying proteins facilitate cells in scavenging ROS, salvaging biomolecules and in recovery of cells from oxidative damage. Redox and antioxidant systems possessed by cancer cells are superior to the normal cells [127] and are known to be involved in mechanism of radioresistance or chemoresistance of tumor cells. Number of studies have revealed that the blocking of the ROS defence mechanisms [128] and detoxifying enzymes [30] could sensitize tumor cells against ionizing radiation and chemotherapeutic agents.

Intracellular levels of most of the above enzymatic and non-enzymatic anti-oxidants are regulated by a redox sensitive transcription factor Nrf2 (Nuclear factor-E2-related factor 2) [129, 130]. Under the normal conditions, Nrf2 is sequestered in the cytoplasm by binding to KEAP1 (Kelch-like ECH-associated protein1) protein. KEAP1 protein acts as a functional adopter for cullin3-dependent E3-ubiquitin ligase complex and thereby promotes the degradation of Nrf2 protein. In response to oxidative stress, Nrf2 undergoes a rapid translocation into the nucleus, binds to antioxidant response elements, which are present in the promoter regions of its target antioxidant genes such as heme oxygenase 1 (HO1), NADH quinone oxidoreductase 1 (NQO1), the glutamate cysteine ligase catalytic subunit (GCLC), thioredoxin reductase 1 (TXRD1), etc. [131]. Nrf2 and its dependent genes may play a crucial role in determining radiosensitivity of tumor cells. Indeed few recent studies carried out in non-small-cell cell lung cancer [132] and esophageal squamous cancer cells [133] showed the involvement of Nrf2 in radioresistance of tumor cells.

In the previous chapters (Chapter 2 and 3) the differential radiosensitivity of seven different tumor cell lines was evaluated. Even the two cell lines form same tissue of origin (PC3 and DU145) showed significantly different DNA damage immediately after the radiation exposure. Therefore, it would be interesting to investigate the involvement of mechanisms at cellular and molecular level in the observed differential radiosensitivity of these two prostate cancer cells

Prostate cancer is one of the most commonly diagnosed non cutaneous malignancies in men. Surgical removal, hormone ablation therapy and radiotherapy are the major treatment modalities for the prostate cancer. Radiotherapy can be used as curative treatment of clinically localized prostate cancer. However, the radiation resistance has become a practical impediment to the radiotherapy of prostate cancers.

Despite the significant advances in the treatment modalities, prostate cancer is one of the leading causes of cancer death in men. The treatment of androgen independent prostate cancers has become a challenging aspect as they invariably show chemoresistance and radioresistance [134]. The molecular mechanisms and the factors, which determine the radioresistance of these tumor cells is not clear.

Therefore, in this Chapter, the role of oxidative stress and associated pathways redox axis was investigated for its role in determining radiosensitivity of prostate cancer. In this study two androgen independent cell lines viz., PC3 and DU145 were used which are known to exhibit differences in chemosensitivity [127].

4.2. Materials and Methods

4.2.1. Chemicals

Dulbecco's Modified Eagles Medium (DMEM), antibiotics (streptomycin and penicillin), sodium bicarbonate, crystal violet, Tris-HCl, ethylenediaminetetraacetic acid (EDTA), sodium chloride (NaCl), dimethylsulfoxide (DMSO), dihydrodichloroflourescin diacetate (H_2-DCFDA), dihydrorhodamine 123 (DHR 123), propidium iodide (PI), sodium citrate, triton X-100, ribonuclease A, all-trans retinoic acid (ATRA), tin protoporphyrin (SnPP), glutathione (reduced and oxidized) and diethyl pyrocarbonate (DEPC) were purchased from Sigma-Aldrich (MO, USA). Fetal bovine serum (FBS) and trypsin-EDTA were from Himedia (Mumbai, India). Lipofectamine was purchased from Invitrogen (Bangalore, India) and short hairpin RNA (shRNA) plasmids were purchased from Origene (MD, USA).

4.2.2. Cell lines and irradiation

PC3 and DU145 cells were obtained from National Institute for Research in Reproductive Health, Mumbai. Cells were maintained as exponentially growing monolayer in DMEM supplemented with 10% FBS, penicillin and streptomycin in humidified incubator maintained at 37^{o} C with 5 % CO_2.

The cells were irradiated (dose rate: 1 Gy/min) using a ^{60}Co teletherapy machine Bhabhatron II (Panacea Medical Technologies, Bangalore, India). For comet assay, exponentially growing cells were harvested by trypsinization. Cells thus obtained were suspended in complete medium followed by irradiation at 4 ^{o}C. After irradiation, samples to be processed immediately after irradiation (0 min) were kept in ice, whereas, for repair kinetics studies, the samples were incubated at 37 ^{o}C for various time intervals (15, 30, 60, and 120 min). For RNA isolation, exponentially growing cells (2 x 10^6 cells) were seeded overnight in culture dish (BD Falcon, USA), irradiated (either 4 or 8 Gy) and cultured for 24 h before RNA isolation.

4.2.3. Clonogenic assay

Clonogenic assay was performed as mentioned previously [135] and in chapter 2.2.4. Briefly, exponentially growing cells were seeded on 60 mm dishes and allowed to adhere overnight in culture conditions. These cultures were exposed to required doses of γ-radiation (1, 2, 4, 6, 8 and 10 Gy). For studies involving the inhibition of Nrf2 and HO1 activity, cells were treated with the respective inhibitors (ATRA: 10 μM or SnPP: 15 μM) for 1 h before irradiation. After irradiation dishes were kept in the incubator for 15 days for the colony development. After the colony development, they were processed and surviving fraction was calculated as mentioned in Chapter 2 and section 2.4.

4.2.4. Apoptosis assay by PI staining

For apoptosis assay, exponentially growing cells were harvested and plated overnight followed by irradiation (either 4 Gy or 8 Gy). Forty eight hours after radiation exposure, cells were harvested, fixed with 70% ethanol and stained using PI staining solution (0.5 µg/ml propidium iodide, 10 µg/ml ribonuclease A, 0.1% sodium citrate and 0.1% Triton X-100) at 4^{o} C for 24 h. A total of 20,000 cells were acquired using Partec flow cytometer and were analysed using FlowJo software for pre G1 peak.

4.2.5. Homogenous caspase assay

Homogenous caspase assay was performed using homogenous caspase assay kit (Roche, Germany). With this assay the combined activity of caspase 3 and caspase 7 were measured. This assay is based on the fluorescence emanated due to the cleavage of the pro fluorescent substrate attached to a peptide, by caspase 3 or caspase 7. Exponentially growing cells were plated and allowed to adhere for overnight followed by radiation exposure. At 48 h after irradiation, cells were harvested and 40000 cells were incubated along with the incubation buffer containing substrate peptide attached to Rhodamine 110. One hour after the incubation, Rhodamine 110 fluorescence was measured using a flourimeter (λex 485 nm, λem 521 nm) and relative caspase activity was calculated in comparison to control cells.

4.2.6. Comet assay

To determine the magnitude of DNA damage, neutral comet assay was performed as mentioned previously [136] and in chapter 2.2.6.

4.2.7. Electrophoretic mobility shift assay

In order to assay the DNA binding ability of Nrf2 in PC3 and DU145 cells, electrophoretic mobility assay (EMSA) was carried out [137]. For EMSA, exponentially growing cells were harvested and plated for overnight. Next day dishes were irradiated and then kept for incubation. Twenty four hours after irradiation, cells were harvested and incubated with 100 µl of cytoplasmic extraction buffer (10 mM HEPES with pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, with protease inhibitor cocktail) for 45 min in ice. After incubation, 4 µl of 10% NP-40 was added to the tube followed by vortexing for 30 seconds. After vortexing, nuclear pellet was separated from the mix by centrifugation at 13, 000 rpm for 2 min at 4^{o} C. Nuclear extract was prepared from the pellet by repeated vortexing after dissolving the pellet in nuclear extraction buffer (20 mM HEPES with pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, with protease inhibitor cocktail). Protein was quantified in the nuclear extracts, and 10 µg of protein was incubated with the ^{32}P labelled Nrf2 binding consensus sequence (5'-TGG GGA ACC TGT GCT GAG TCA CTG GAG-3', Santa Cruz Biotechnology, CA, USA) for 1 h. The mixture was loaded on to 7.6 % polyacrylamide gel and electrophoresed at 70 mA. After the electrophoresis, gel was vacuum dried and the signal was developed using phosphor imager.

4.2.8. Quantitative real time RT-PCR

To quantify the mRNA expression of genes, quantitative real time reverse transcriptase polymerase chain reaction (RT q-PCR) was used [87]. Twenty four hours after irradiation, total RNA was isolated using TRI reagent (Sigma, MO, USA) as per the manufacturer's instructions. Two µg of total RNA was used for the synthesis of cDNA by reverse transcription (cDNA synthesis kit, Sigma, MO, USA). PCR reactions were setup by mixing 10X SYBR green PCR mix with 5 µl of 2 times

diluted cDNA templates, 1 µl each of forward and reverse primers (0.5 µM ; Table. 4-1), and 3 µl of PCR-grade water in 20 µl reaction mixture. The above reaction mixtures were amplified in the following steps: step 1—denaturation at 95 ^{o}C for 5 min; step 2—denaturation at 95 ^{o}C for 15 s; step 3—annealing at 57 ^{o}C for 15 s; step 4—extension at 72 ^{o}C for 20 s; step 5—melting curve analysis. Steps 2–4 were repeated for 40 cycles using the Rotor Gene 3000 (Corbett Life Science, Australia). The threshold cycle values obtained from above runs were used for calculating the fold change in gene expression by REST-384 version 2 software. The expressions of genes were normalized against that of a housekeeping gene, β-actin, and the relative change in the expression was plotted with respect to control group.

Table: 4-1. *Primer sequences of different genes whose expression has been checked by quantitative real time RT-PCR*

Genes	Forward primer	Reverse primer
β-ACTIN	CTGGAACGGTGAAGGTGACA	AAGGGACTTCCTGTAACAATGCA
NRF2	AGCATGCCCTCACCTGCTACTTTA	ACTGAGTGTTCTGGTGATGCCACA
HO1	AGAGGGAATTCTCTTGGCTGGCTT	ATGCCATAGGCTCCTTCCTCCTTT
GCLC	ATGGAAGTGGATGTGGACACCAGA	AACTCCCTCATCCATCTGGCAACT
TXRD1	TCCTATGTCGCTTTGGAGTGC	GGACCTAACCATAACAGTGACGC
KEAP1	CTGCAGGATCATACCAAGCAGG	GAACATGGCCTTGAAGACAGG

4.2.9. ROS measurement

For measuring intra-cellular cytosolic ROS levels, 1x10^{5} cells were plated in 24 well plates for overnight. Then cells were treated with oxidation sensitive DCF-DA (a final concentration of 10µM) for 30 min at 37 ^{o}C prior to irradiation. After irradiation, the fluorescence of DCF in cells was measured (λ_{ex} - 485 nm, λ_{em} - 535 nm) at different time intervals using a flourimeter [138]. Similarly for measuring

mitochondrial ROS, dihydrorhodamine at a final concentration of 10 μM was added to the cells in suspension, incubated for 30 min prior to irradiation and the rhodamine fluorescence was measured (λ_{ex} 511 nm, λ_{em} 536 nm) at various time intervals after irradiation using flourimeter (BioTek Synergy H1) [139].

4.2.10. Measurement of GSH and GSSG levels

Glutathione (GSH) and glutathione disulphide (GSSG) level were measured in control and treated cells as described previously [140]. Measurement of GSH by this method involves the oxidation of GSH by the sulfhydryl reagent 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) to form the yellow derivative 5'-thio-2-nitrobenzoic acid (TNB), measurable at 412 nm. Irradiated cells were lysed by repeated freezing and thawing followed by centrifugation at 3000 x g for 4 min at 4 ^{o}C. From the supernatant 50 μl was aliquated in a 96 well plate, mixed with freshly prepared DTNB, absorbance was measured at 412 nm, and the GSH was expressed as nmoles/mg of protein. For measuring GSSG, cell extract was treated with 2-vinyl-pyridine, which covalently reacts with GSH (but not with GSSG). Then the GSSG present in the cell was recycled to GSH by glutathione reductase in the presence of NADPH and then measured by DTNB reduction method.

4.2.11. Measurement of thioredoxin reductase activity

Thioredoxin reductase 1 (TXRD1) activity was measured using a kit (Thioredoxin reductase assay kit, Cayman chemical company, USA) by following manufacturer's protocol. Briefly, DTNB reduction was measured in the absence and presence of aurothiomalate, a specific TXRD1 inhibitor that allows for the correction of TXRD1 independent DTNB reduction. By calculating the difference between the above two conditions, TXRD1 activity was estimated.

4.2.12. Knock down of Nrf2 expression using short hairpin RNA (shRNA)

Nrf2 expression was knocked down by transfecting cells with shRNA. For this purpose, exponentially growing cells were transfected using lipofectamine-2000 (Invitrogen, Bangalore, India) with either Nrf2 or random sequence shRNA. Cells were harvested after 24 h of transfection and were plated for clonogenic survival assay. At 48 h after transfection, cells were exposed to various doses of irradiation and their clonogenic survival was seen after 15 days.

4.2.13. Statistical analysis

Statistical analysis was performed using Graphpad Prism 5.0 software (La Jolla, CA, USA). Student's t-test was used for comparing the means of two groups. Values were considered significantly different if $p < 0.05$.

4.3. Results

4.3.1. DU145 cells exhibits higher magnitude of radioresistance than PC3 cells

Results from previous chapter (Chapter 2, Section 3.8) showed differential radiosensitivity between two prostate cancer cells, PC3 and DU145. Out of these two cell lines, DU145 was found to be more radioresistant than the PC3 cells measured in terms of clonogenic survival at 6 Gy (Fig. 2-27), which was found to be correlated with magnitude of DNA damage measured by comet assay (Fig. 2-26 and 2-27). In order to further confirm their differential radiation response, clonogenic assay was performed by exposing the cells to various doses of gamma radiation (1 - 10 Gy). In this clonogenic survival assay, DU145 cells showed more radioresistance than that of PC3 cells. Though the radiosensitivity of PC3 was comparable to that of DU145 at 0.5 and 2 Gy, PC3 cells showed a relative decrease in their survival fraction in comparison to DU145 cells at higher doses of radiation (8 and 10 Gy) (Fig. 4-1A and B). The same was shown in a representative image of culture dish (Fig. 4-1C). For example, survival fractions estimated for PC3 cells at 2 and 10 Gy were 0.83 and 0.004, respectively. Whereas in DU145 cells, survival fraction was 0.89 and 0.02, after 2 Gy and 10 Gy, respectively. The dose required to bring down the survival fraction from 0.1 to 0.01 for PC3 and DU145 cells was calculated to be 3 and 4.5 Gy, respectively. Although both these tumor cells are from same tissue of origin, a significant difference in their radiosensitivity was evident as shown by clonogenic survival assay.

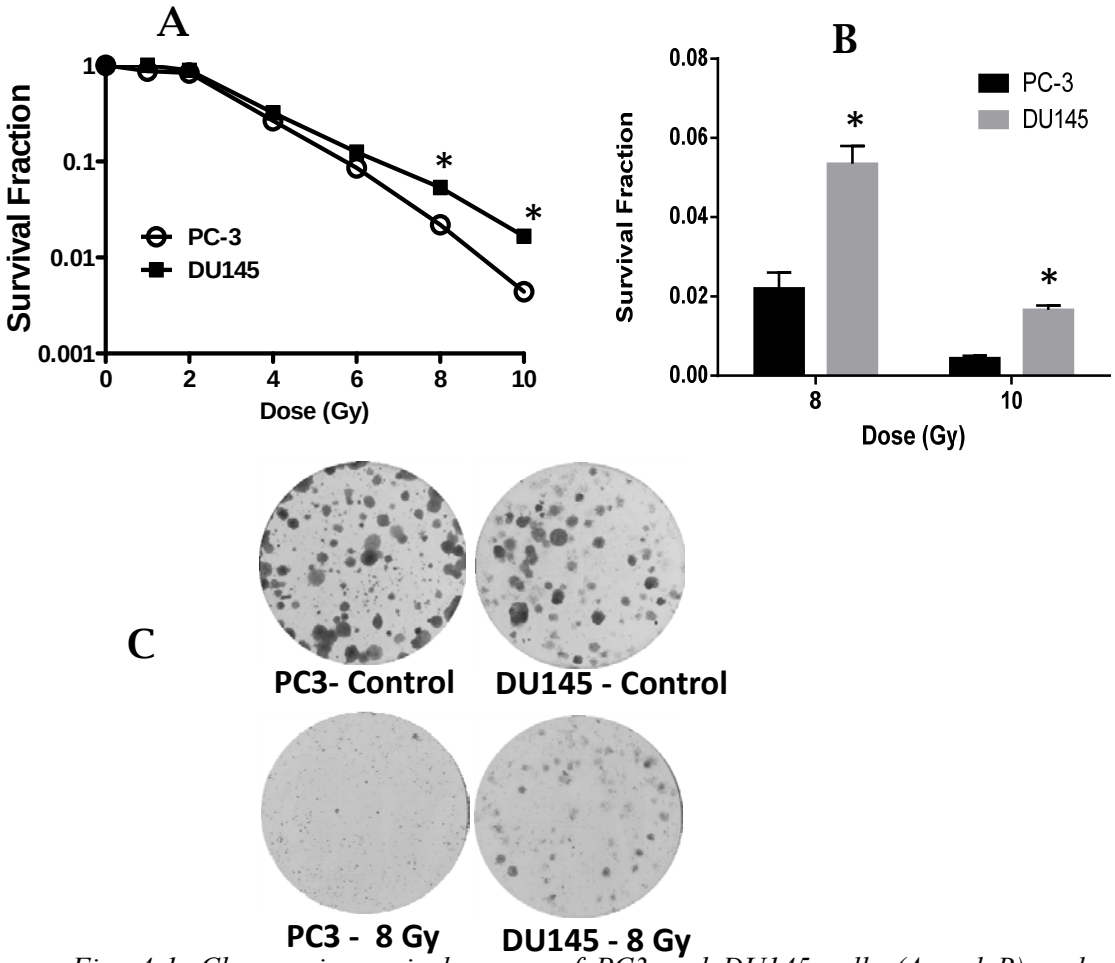


*Fig: 4-1. Clonogenic survival curves of PC3 and DU145 cells (A and B) and a representative clonogenic dish of PC3 and DU145 cells after exposure to 8 Gy (C). For clonogenic assay, exponentially growing cells have been irradiated, grown for two weeks, stained and the number of colonies were counted and the survival fraction has been plotted. Three independent experiments were performed in triplicates each time and mean ± SEM has been plotted. * $p < 0.05$ in comparison to respective PC3 group.*

Differential radiation response of these two cells was also evidenced by estimating cell death using propidium iodide staining followed by flow cytometry. For example, after 8 Gy of radiation exposure, 24% of PC3 cells underwent apoptosis (% cells in pre G1 peak) as compared to 12% of DU145 cells (Fig. 4-2A and 4-2B).

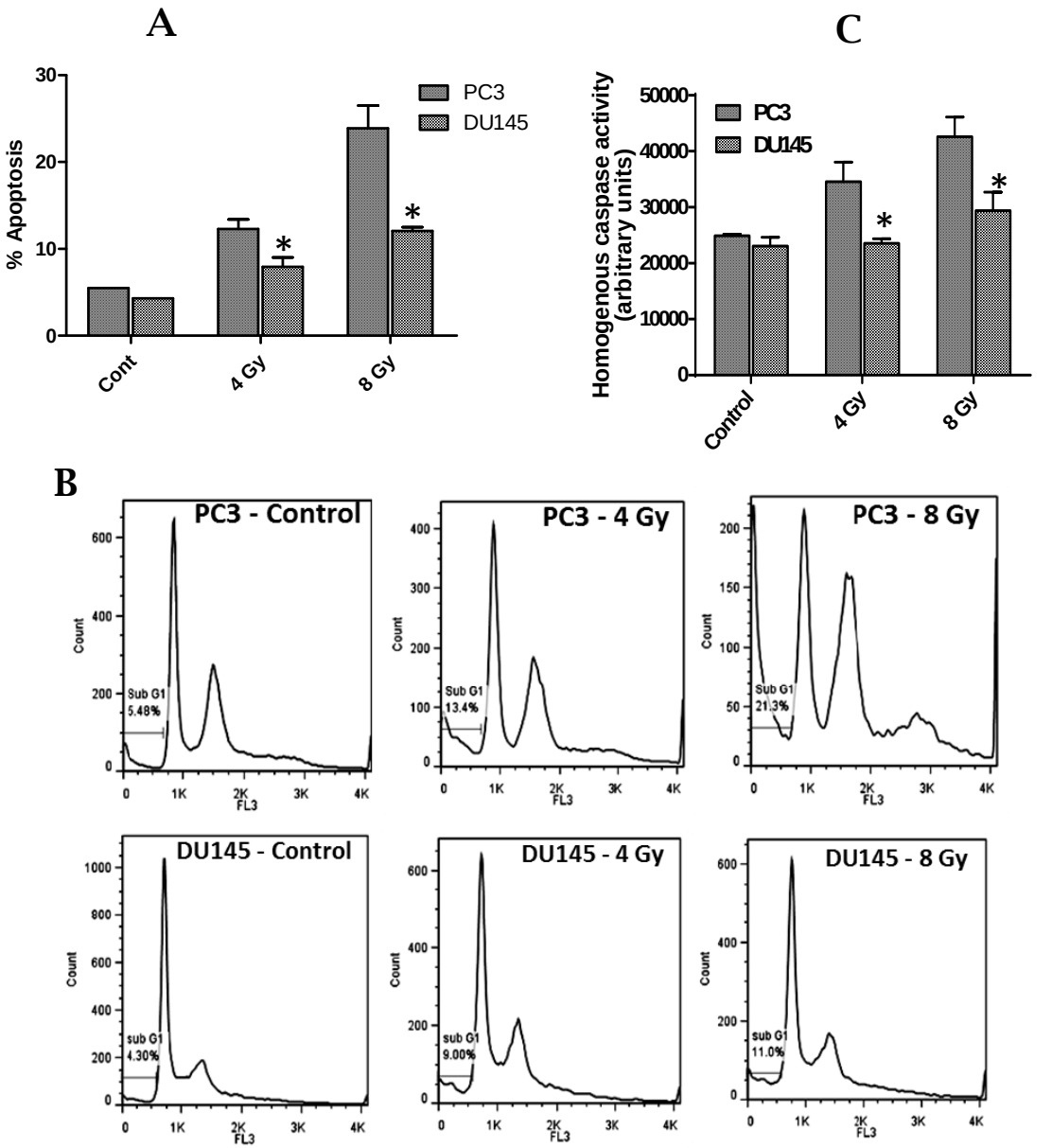


*Fig: 4-2. Radiosensitivity of PC3 and DU145 cells, as determined by apoptosis measured by PI staining (A and B), and homogenous caspase assay (C). For PI assay, forty eight hours after radiation exposure, cells were harvested, fixed and stained using PI staining solution. A total of 20,000 cells were acquired using Partec flow cytometer and were analysed using FlowJo software. Percentages of cells in pre G1 peak were represented as cells undergoing apoptosis. Mean ± SEM obtained from two independent experiments done in triplicates, has been plotted. For homogenous caspase assay, cells were harvested at 48 h after the radiation exposure and processed accordingly using homogenous caspase assay kit. Arbitrary units of mean fluorescence obtained in two experiments done in triplicates have been plotted with SEM. * $p < 0.05$ in comparison to respective PC3 group.*

To confirm the results obtained by propidium iodide assay, caspase activity was also evaluated in these two cell lines after 4 Gy and 8 Gy radiation exposures. Homogenous caspase activity (activity of caspase 3 and 7) was observed to be significantly higher in PC3 cells in comparison to DU145 cells after 4 Gy and 8 Gy (Fig. 4-2C).

In order to further evaluate the differential radiosensitivity of above tumor cells, the DNA damage was estimated by neutral comet assay after exposing the cells to various doses of gamma radiation (2-8 Gy). The results revealed increased magnitude of DNA damage in PC3 cells in comparison to DU145 cells at all the doses with more significant differences at the higher doses (Fig. 4-3A). The % DNA in tail observed in PC3 cells after 8 Gy was 8.95±0.72 as against 6.92±0.43 in DU145 cells.

The DNA damage repair kinetics was also studied by assessing the extent of DNA damage neutral comet assay at different time intervals (up to 2 h) after radiation exposure by comet assay. DU145 cells showed faster recovery of DNA damage in comparison to PC3 cells (Fig. 4-3B and 4-3C). However, both these cell types showed no significant difference in the residual DNA damage at the end of 2 h. Thus, from clonogenic survival, apoptosis and DNA damage assay, it was clearly evident that among the two cell lines, DU145 is more radioresistant than that of PC3 cells.

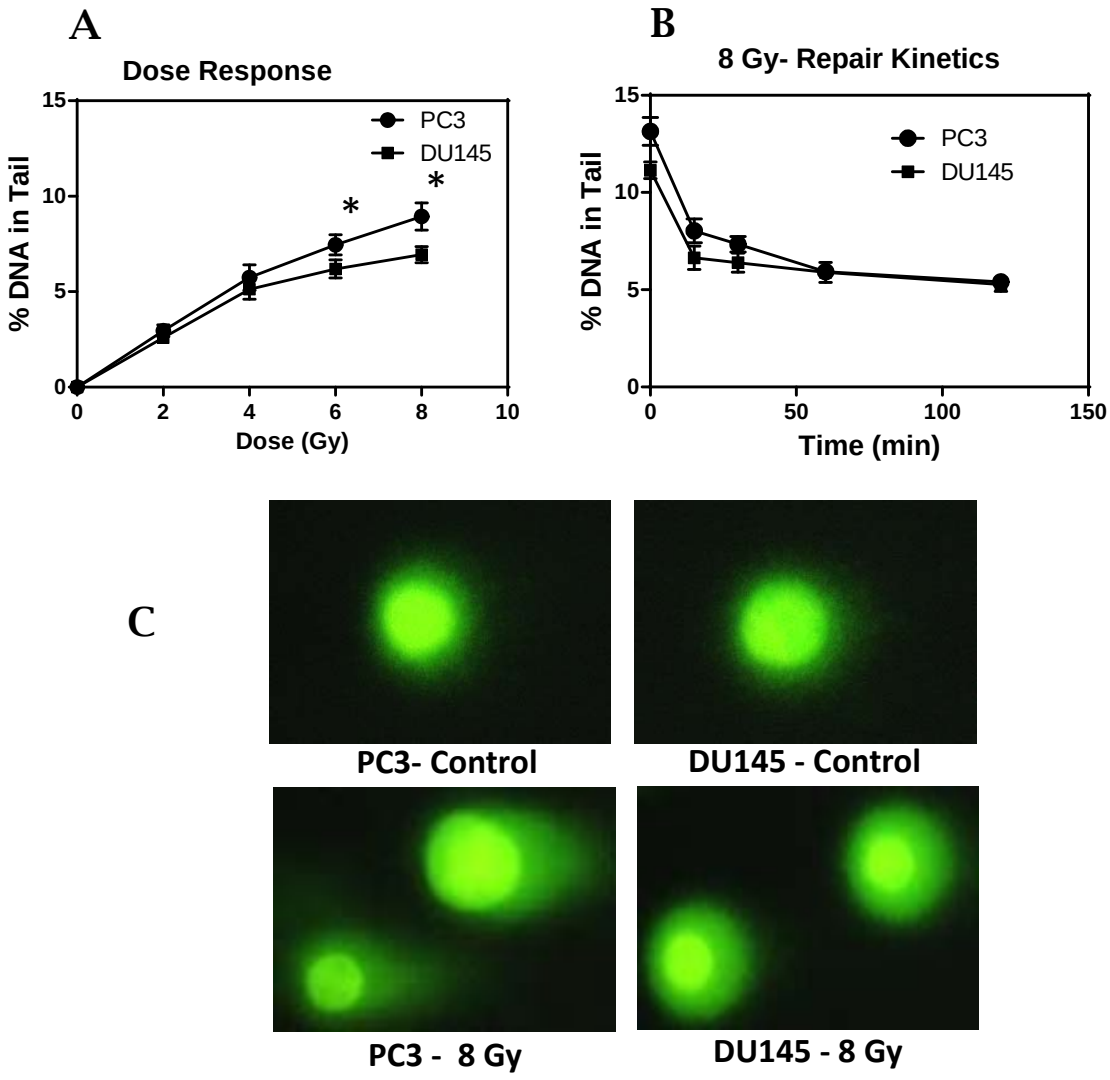


*Fig: 4- 3. DNA damage of PC3 and DU145 cells assessed by neutral comet assay (A), its repair kinetics (B) and the representative images of the comets obtained in neutral comet assay. Neutral comet assay was performed in the control as well as irradiated cells at different time points after irradiation as described in the materials and methods section. From the neutral comet assay, at least 50 cells per slide were microscopically grabbed and analyzed for DNA damage. Two slides were prepared from every treatment, and the mean ± SEM of three independent experiments was plotted. (C) Representative images of PC3 and DU145 cells obtained using comet assay after exposure to 4 Gy of radiation dose. * $p < 0.05$ in comparison to respective PC3 group.*

4.3.2. Determination of cytosolic and mitochondrial reactive oxygen species in PC3 and DU145 cells

Since PC3 and DU145 exhibited differences in radiosensitivity, the redox status of these cells was investigated by estimating ROS and GSH level under control and irradiated (4 and 8 Gy) conditions. Interestingly, radiosensitive PC3 cells showed higher basal as well as radiation induced level of cytosolic ROS (measured by DCF fluorescence) in comparison to that of DU145 cells (Fig. 4-4A to 4-4C). The basal DCF fluorescence observed in radiosensitive PC3 cells was 7183±189 (A.U / 10^5 cells) in comparison to 5073±124 (A.U / 10^5 cells) in DU145 cells.

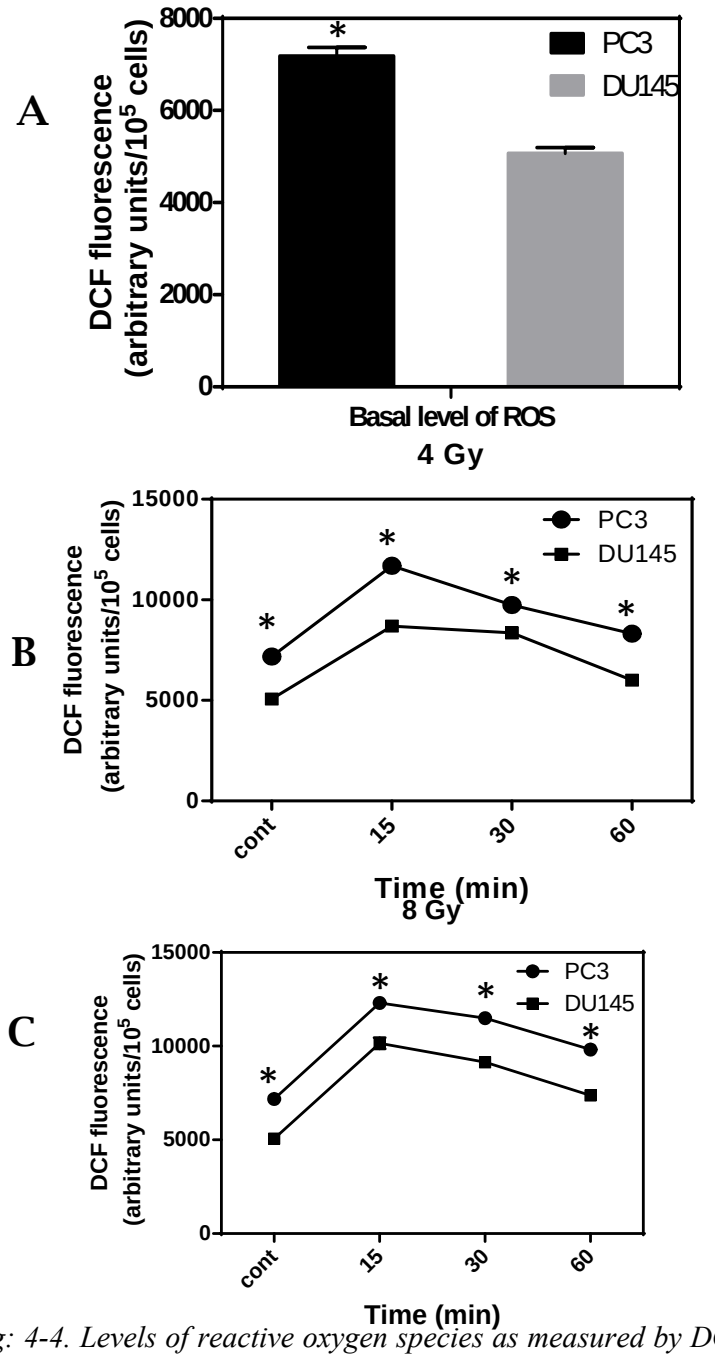


*Fig: 4-4. Levels of reactive oxygen species as measured by DCF fluorescence in PC3 and DU145 cells in control (A), after 4 Gy (B) and 8 Gy (C). For measuring ROS, either DCHFDA or DHR was added to cells 30 min prior to irradiation, fluorescence of DCF or rhodamine recorded at different time intervals and the mean ± SEM was plotted. Three independent experiments in triplicates at each time point were performed. * $p < 0.05$ in comparison to respective DU145 group.*

After irradiation, maximum intracellular ROS was observed at 15 min in both the tumor cell lines, which declined with progress of time. Unlike DCF, the rhodamine fluorescence, which measures mitochondrial ROS showed time dependant increase following irradiation. As observed with DCF, the rhodamine fluorescence was lower in DU145 cells compared to PC3 cells at all the time points studied suggesting increased level of mitochondrial ROS in PC3 cells compared to DU145 cells (Fig. 4-5).

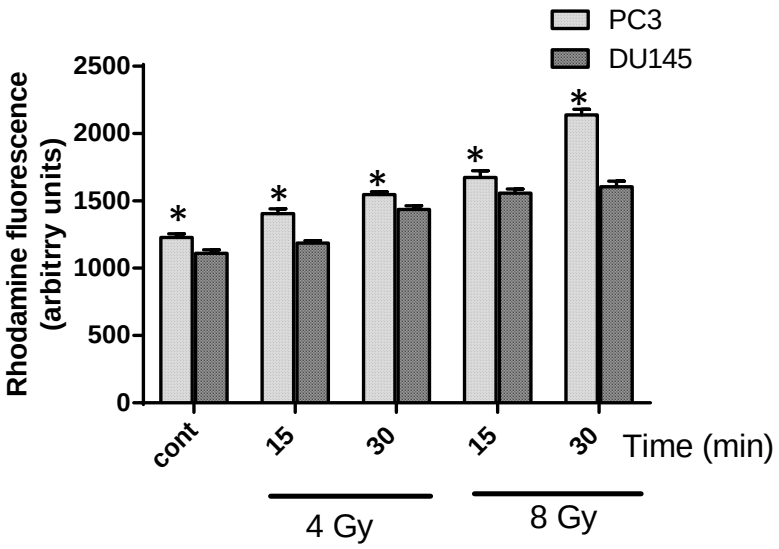


*Fig: 4-5. Levels of mitochondrial ROS as estimated by dihydrorhodamine (DHR) in PC3 and DU145 cells after radiation exposure of 4 Gy and 8 Gy. DHR was added to cells 30 min prior to irradiation, fluorescence of rhodamine recorded at different time intervals and the mean ± SEM was plotted. Three independent experiments in triplicates at each time point were performed. * $p < 0.05$ in comparison to respective DU145 group.*

4.3.3. Determination of GSH (glutathione) and GSSG ratio in PC3 and DU145 cells

Cellular levels of glutathione determine the redox balance and maintain redox homeostasis in the cell. At higher oxidative stress, GSH gets oxidised to GSSG. The ratio between GSH to GSSG will be affected when the cells are under oxidative stress. During the process of homeostasis, the GSSG will be again converted to GSH through an enzyme dependent pathway. Therefore, the radiosensitivity of a particular cell is highly dependent on the level of GSH and the ability of the cell to again re-synthesis the GSH and bring their level back to *de novo* levels. Hence, a higher ratio of GSH/GSSG indicates stronger capacity of cells to overcome oxidative stress.

Out of the two cell lines used in the study, DU145 cells showed more radioresistance than the PC3 cells. DU145 cells also showed higher intracellular ROS than the PC3 cells. In this regard, the GSH and GSSG levels in PC3 and DU14 5 cells were also estimated. In agreement to ROS level, DU145 cells showed significantly higher basal GSH content than that of PC3 cells (Fig. 4-5A). On irradiation both the cells showed depletion in their GSH level but with progress of time, DU145 cells showed much faster recovery (at 8 Gy) of GSH content than that of PC cells (Fig. 4-6A). The ratio of GSH and GSSG was also significantly higher in DU145 cells than that of the PC3 cells and it was maintained even at 24 h after irradiation at different doses (Fig. 4-6B). Taken together these results suggest higher reducing type of redox environment in DU145 cells compared to PC3 cells and this could be the reason for the enhanced tolerance of DU145 against radiation exposure.

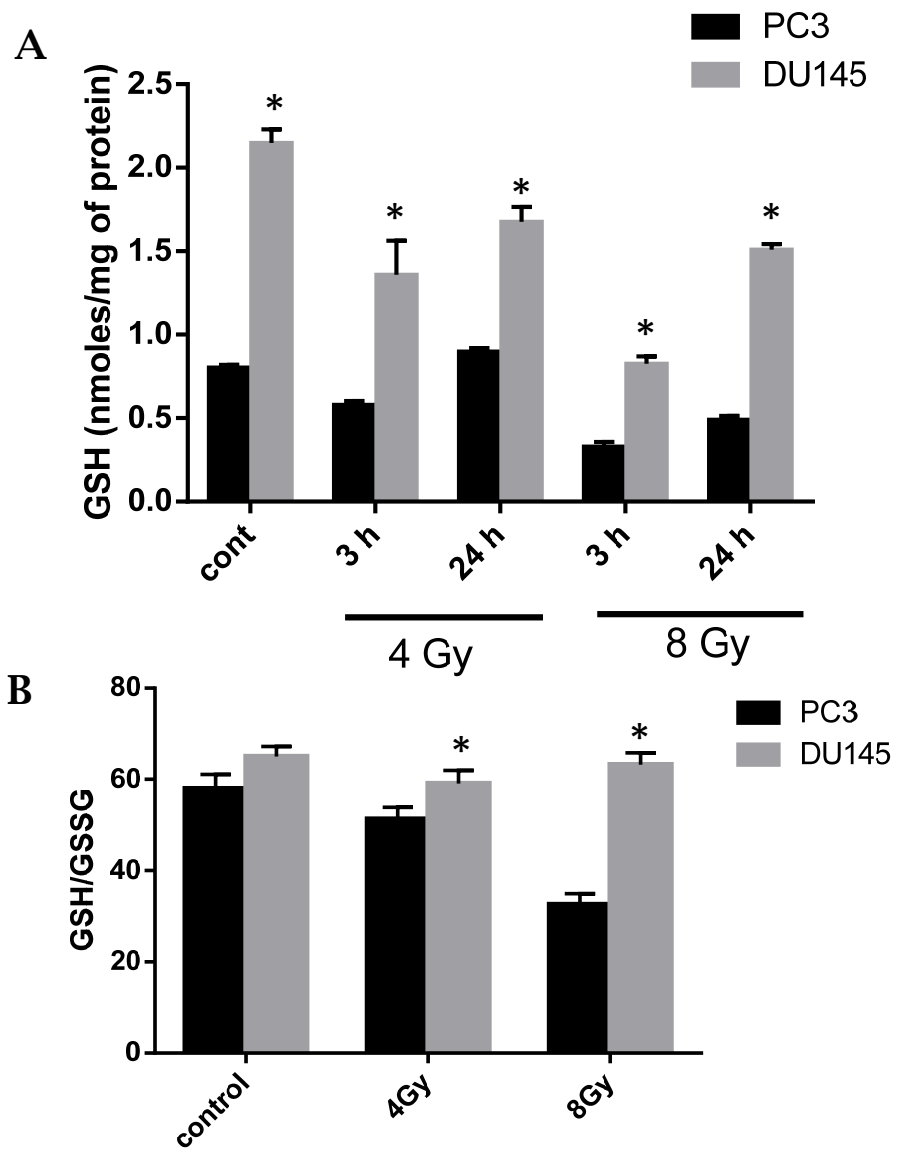


*Fig: 4-6. GSH level and GGH/GSSG ratio in irradiated PC3 and DU145 cells in comparison to control. GSH level were measured at 3 and 24 h (A) after radiation exposure. GSH and GSSG level measured after 24 h (B) after radiation exposure. Three independent experiments were performed in triplicates at each time point and mean ± SEM has been plotted. * $p < 0.05$ in comparison to respective PC3 group.*

4.3.3. DU145 cells exhibits elevated levels of Nrf2 and its dependent transcripts than PC3 cells after radiation exposure

Since difference in the level of antioxidants and ROS was observed between the two cell lines after irradiation, the involvement of redox sensitive transcription factor Nrf2

in the observed difference of radiosensitivity of above two cell lines was anticipated. In order to confirm that, the activation of Nrf2 was measured using EMSA in these cells after irradiation. DU145 cells showed higher basal level of Nrf2 in comparison to PC3 cells (Fig. 4-7A and 4-7B). In irradiated DU145 and PC3 cells, the nuclear level of Nrf2 has significantly increased in comparison to unirrradiated control cells. The increase observed in DU145 cells was significantly higher than the PC3 cells. Similarly after 8 Gy, the nuclear level of Nrf2 was higher than the levels observed in control and 4 Gy. Interestingly, in PC3 cells which exhibited higher radiosensitivity at 8 Gy showed significantly lower level of Nrf2 at 8 Gy by EMSA assay. DU145 cells showed higher basal and radiation induced Nrf2 in comparison to PC3 cells (Fig. 4-7A and 4-7B).

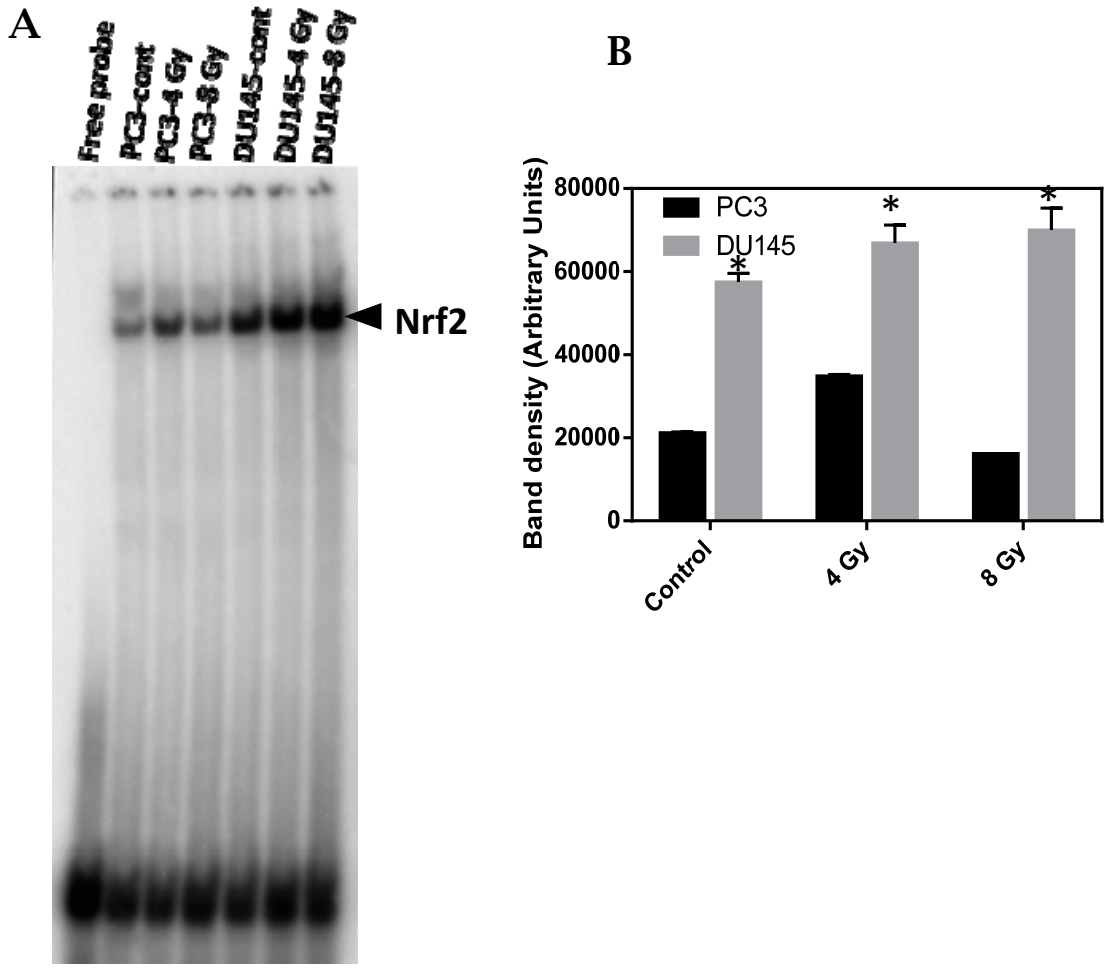


*Fig: 4-7. Nuclear level of Nrf2 in PC3 and DU145 cells as assessed by EMSA (A) and their densitometric analysis (B). For EMSA, protein lysate was made from nuclear pellets, and equal amount of protein was incubated with the ^{32}P labelled Nrf2 binding consensus sequence and followed by electrophoresis. After the electrophoresis, gel was vacuum dried and the signal was developed using phosphor imager. Representative image of the three experiments has been shown. The band intensity (marked with an arrow) was measured using densitometric analysis using gel documentation system. * $p < 0.05$ in comparison to respective PC3 group.*

The expression of Nrf2 at mRNA level was also measured in these cells by RT q-PCR after 24 h after radiation exposure. DU145 cells showed two folds higher induction of Nrf2 level in comparison to PC3 cells after 4 and 8 Gy (Fig. 4-8).

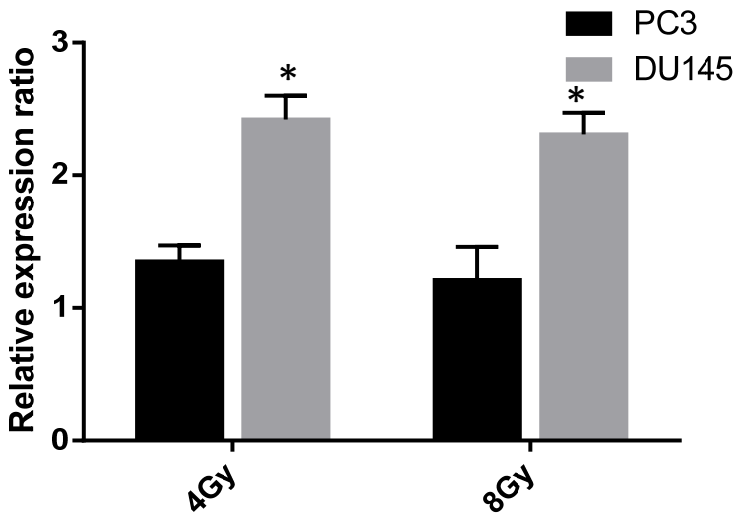


*Fig: 4-8. mRNA levels of Nrf2 in PC3 and DU145 cells as assessed by Real time q-PCR. Gene expression was analyzed (24 h after radiation exposure) by real-time q-PCR, after exposing the cells to 4 Gy/8 Gy of gamma irradiation. The bars represent the mean ± SEM obtained from two independent experiments done in triplicates. * $p < 0.05$ in comparison to respective PC3 group.*

As there was a difference seen in Nrf2 level between these two cell lines, the expression level of Nrf2 dependent genes like HO1 (Heme Oxygenase), GCLC (Glutamine Cysteine Ligase Catalytic subunit), GCLM (Glutamine Cysteine Ligase Modifier Subunit), TXRD1 (Thioredoxin Reductase 1), TXRD2 (Thioredoxin Reductase 2), TXN1 (Thioredoxin 1) and TXN2 (Thioredoxin 2), was evaluated after irradiating to either 4 Gy or 8 Gy. Following radiation exposure, PC3 and DU145 showed an upregulation in all these Nrf2 dependent genes. For same dose of radiation, the magnitude of upregulation seen in the DU145 cells was higher than in PC3 cells for GCLC, GCLM and HO1 genes. After 8 Gy of radiation, DU145 cells showed 5.7,

4.1 and 3.2 folds upregulation in the expression of GCLC, GCLM and HO1 genes, respectively over unirradiated controls (Fig. 4-9A to 4-9C). However in PC3 cells, the expression of GCLC, GCLM and HO1 genes showed 1.8, 2.86 and 2.5 folds increase respectively after 8 Gy irradiation, which was significantly lower than that of the increase observed in DU145 cells (Fig. 4-9A to 4-9C).

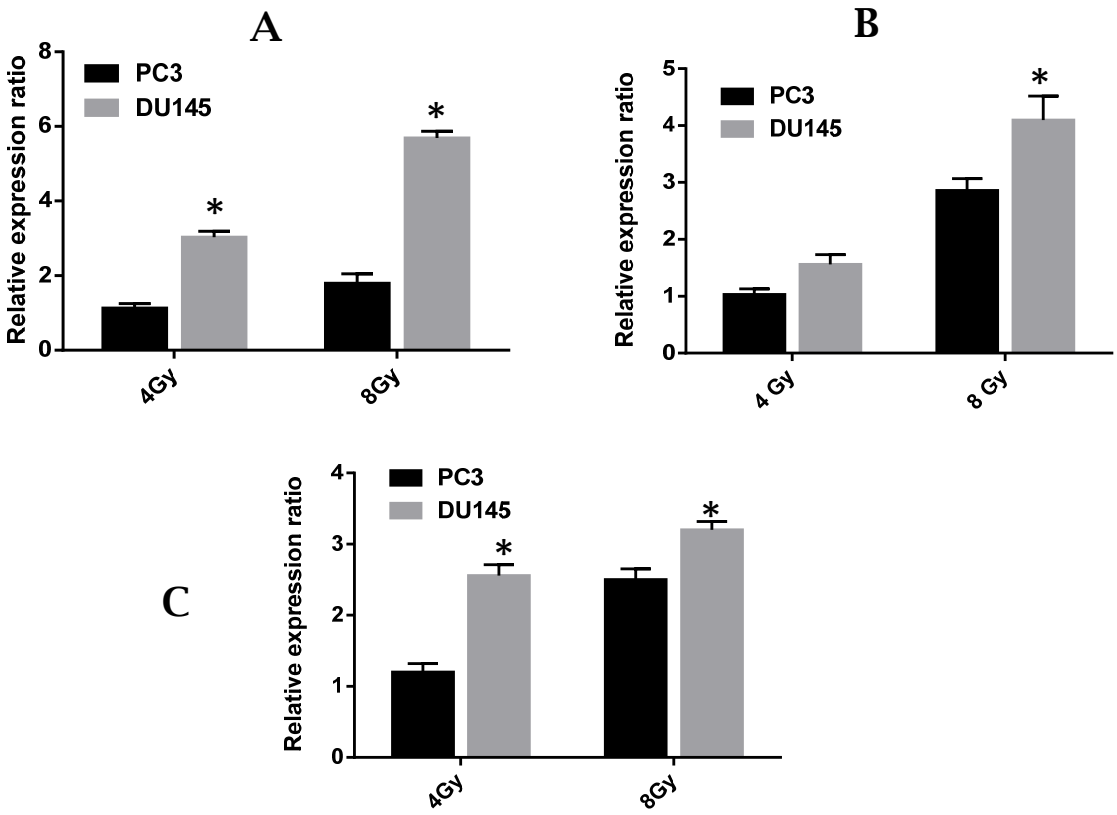


*Fig: 4-9. mRNA levels of Nrf2 dependent genes GCLC (A), GCLM (B), and HO1 (C) in PC3 and DU145 cells as assessed by Real time q-PCR. Real-time q-PCR was performed after exposing the cells to 4 Gy/8 Gy of gamma irradiation. The bars represent the mean ± SEM obtained from two independent experiments done in triplicates. * $p < 0.05$ in comparison to respective PC3 group.*

Similar to the enzymes involved in glutathione synthesis, proteins involved in thioredoxin cycle are also under the transcriptional control of Nrf2. Therefore, the expression levels of TXN1, TXN2, TXRD1 and TXRD2 were investigated. These genes showed significant upregulation in DU145 cells after radiation in comparison to PC3 cells (Fig. 4-10A to 4-10D).

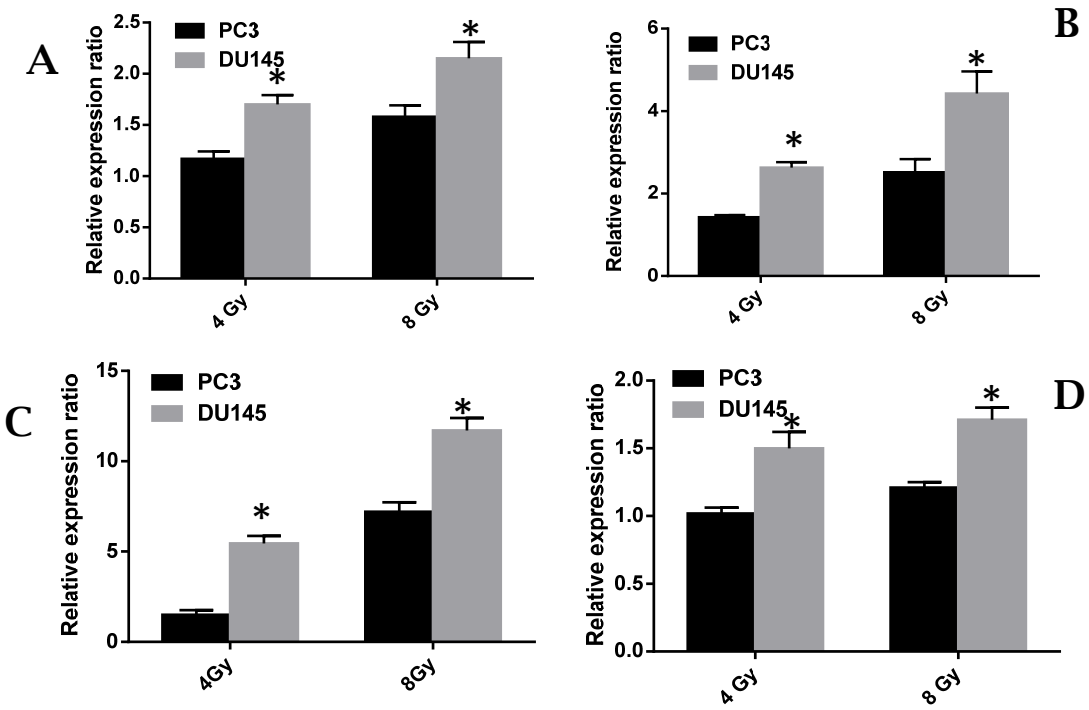


*Fig: 4-10. mRNA levels of Nrf2 dependent genes TXN1 (A), TXN2 (B), TXRD1 (C) and TXRD2 (D) in PC3 and DU145 cells as assessed by Real time q-PCR. Real-time q-PCR was performed after exposing the cells to 4 Gy/8 Gy of gamma radiation. The bars represent the mean ± SEM obtained from two independent experiments done in triplicates. * $p < 0.05$ in comparison to respective PC3 group.*

Moreover, biochemical activity of TXRD1 was also measured in the cell lysate at 24 h after irradiation, which showed a significant increase in DU145 cells than that of PC3 cells after 4 Gy of irradiation (Fig. 4-11A).

Since KEAP1 protein plays a key role in regulating the Nrf2 pathway, mRNA level of KEAP1 in PC3 and DU145 cells was evaluated. Basal level of KEAP1 gene in DU145 cells was found to be two fold lower than that of the PC3 cells (Fig. 4-11B). Further, the changes in mRNA level of KEAP1 were also examined after exposure to radiation and found that both PC3 and DU145 cells showed a down regulation of KEAP1 gene. Extent of this down regulation was marginally higher in PC3 cells than that observed in DU145 cells. But the differences were not statistically significant (Fig. 4-11C).

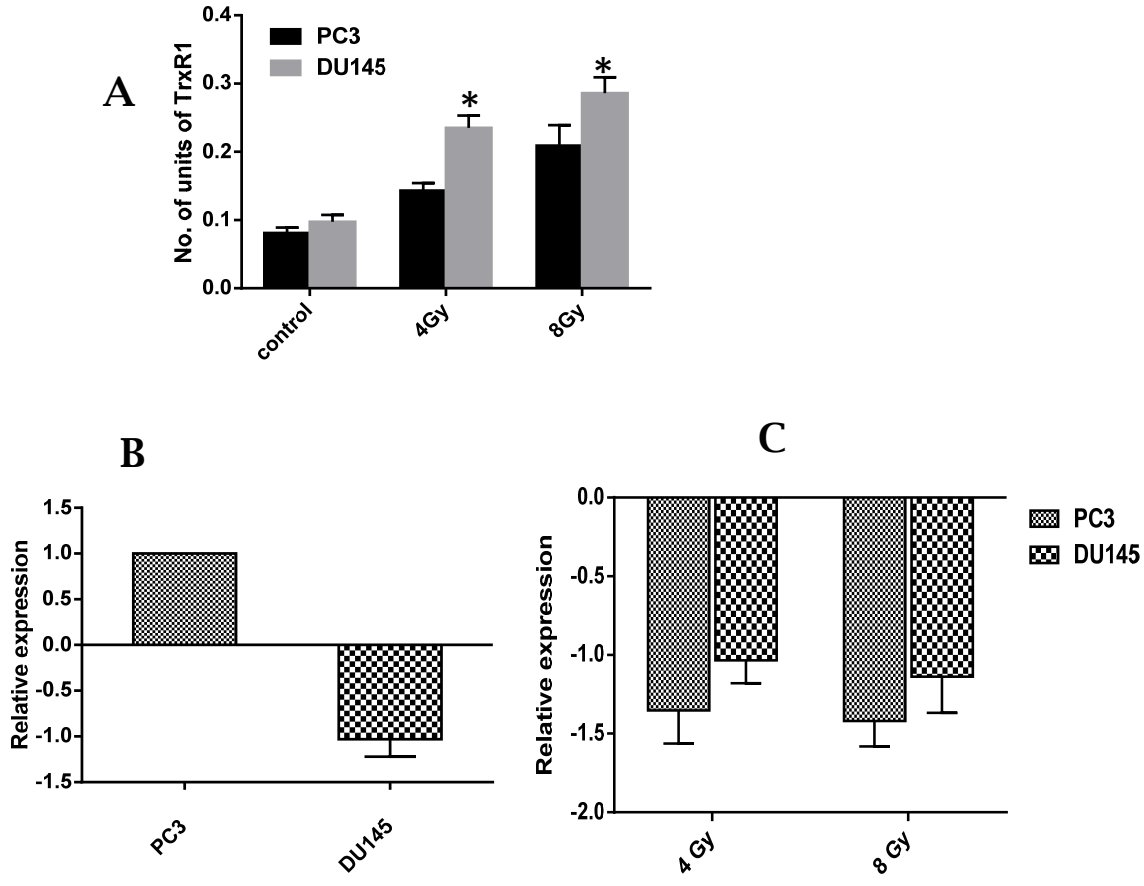


*Fig: 4-11. (A) Biochemical activity of TXRD1 in PC3 and DU145 cells. The bars represent the mean ± SEM obtained from two independent experiments done in triplicates. (B) mRNA levels of KEAP1 in unirradiated PC3 and DU145 cells (C) Keap1 expression after radiation exposure in PC3 and DU145 cells. Gene expression was analyzed by real-time q-PCR, at 24 h after exposing the cells to 4 Gy/8 Gy of gamma irradiation. The error bars represent the mean ± SEM obtained from two independent experiments done in triplicates. * $p < 0.05$ in comparison to respective PC3 cells.*

4.3.4. Nrf2 and HO1 level determine radiosensitivity in DU145 and PC3 cells

In order to confirm the role of Nrf2 and its dependent genes in radiosensitivity of tumor cells, the survival fraction of irradiated PC3 and DU145 cells was examined, in the presence or absence of inhibitors of Nrf2 and HO1 namely all-*trans* retinoic acid (ATRA) [141] and Tin protoporphyrin (SnPP) [142], respectively. Treatment of

cells with these inhibitors prior to irradiation significantly reduced the survival fraction of irradiated tumor cells. Treatment with above inhibitors prior to 4 Gy irradiation reduced the survival fraction of PC3 cells from 0.21 to 0.01 and that of DU145 cells from 0.31 to 0.03 (Fig.4-12A and 4-12B).

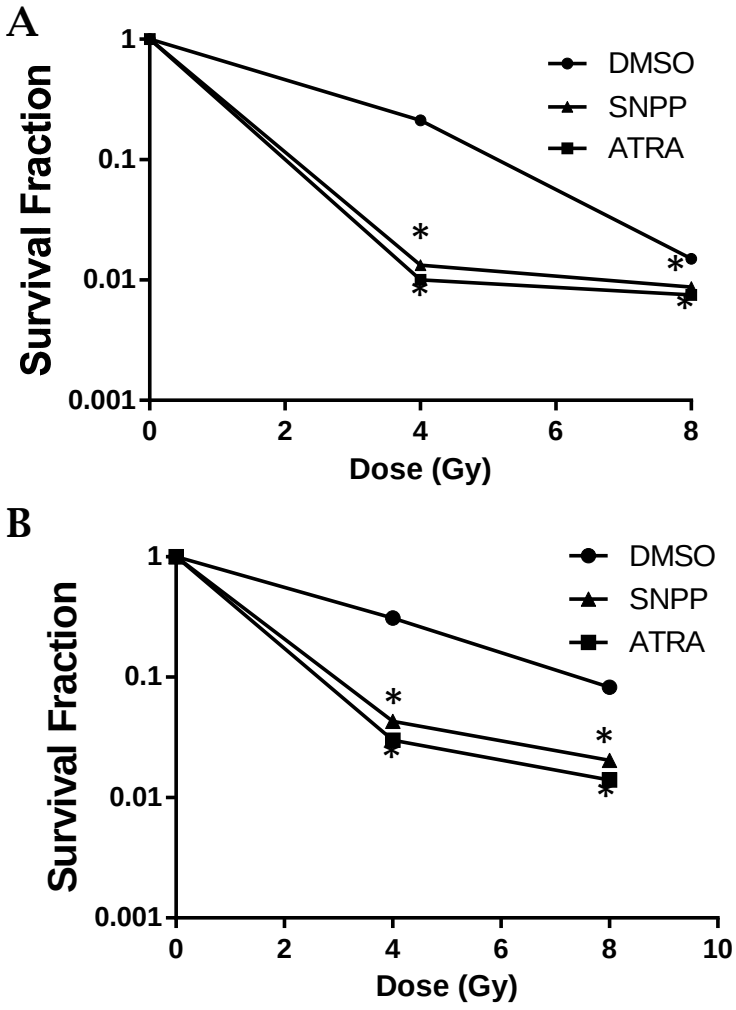


*Fig: 4-12. Survival fraction of PC3 (A) and DU145 (B) cells after exposure to radiation in the presence of 10 μM ATRA (Nrf2 inhibitor) or 5 μM SNPP (HO1 inhibitor). Exponentially growing cells were treated with inhibitors followed by irradiation and clonogenic assay. Three independent experiments were performed in triplicates each time and mean ± SEM has been plotted. * $p < 0.05$ in comparison to DMSO control group.*

Similarly, in DU145 cells, SNPP and ATRA treatment decreased the survival fraction from 0.08 to 0.01 after 8 Gy, whereas the survival fraction of PC3 cells reduced from 0.015 to 0.007. Further, to corroborate the evidence of involvement of Nrf2 in radiosensitivity, knockdown approach was employed by transfecting shRNA against Nrf2 in DU145 cells. Initially shRNA was used at the concentration of 1µg/ml of medium and these cells were transfected with the shRNA. But at this concentration, the Nrf2 knockdown in the cells led to significantly lower survival fraction (0.3) in comparison to the control (Fig. 4-13A). Therefore, the concentration of shRNA targeting Nrf2 was reduced to 0.5µg/ml of medium. At this concentration the Nrf2 knockdown alone did not cause significant reduction in survival fraction (Fig. 4-13B). A nonspecific shRNA having scrambled sequence was used as a control. The transfected cells were irradiated (4 Gy) and their survival fraction was analysed. The cells transfected with shRNA targeting Nrf2, showed a drastic decrease in survival fraction (0.03), in comparison to the cells transfected with scrambled shRNA (0.28) after 4 Gy irradiation (Fig. 4-13B and 4-13C).

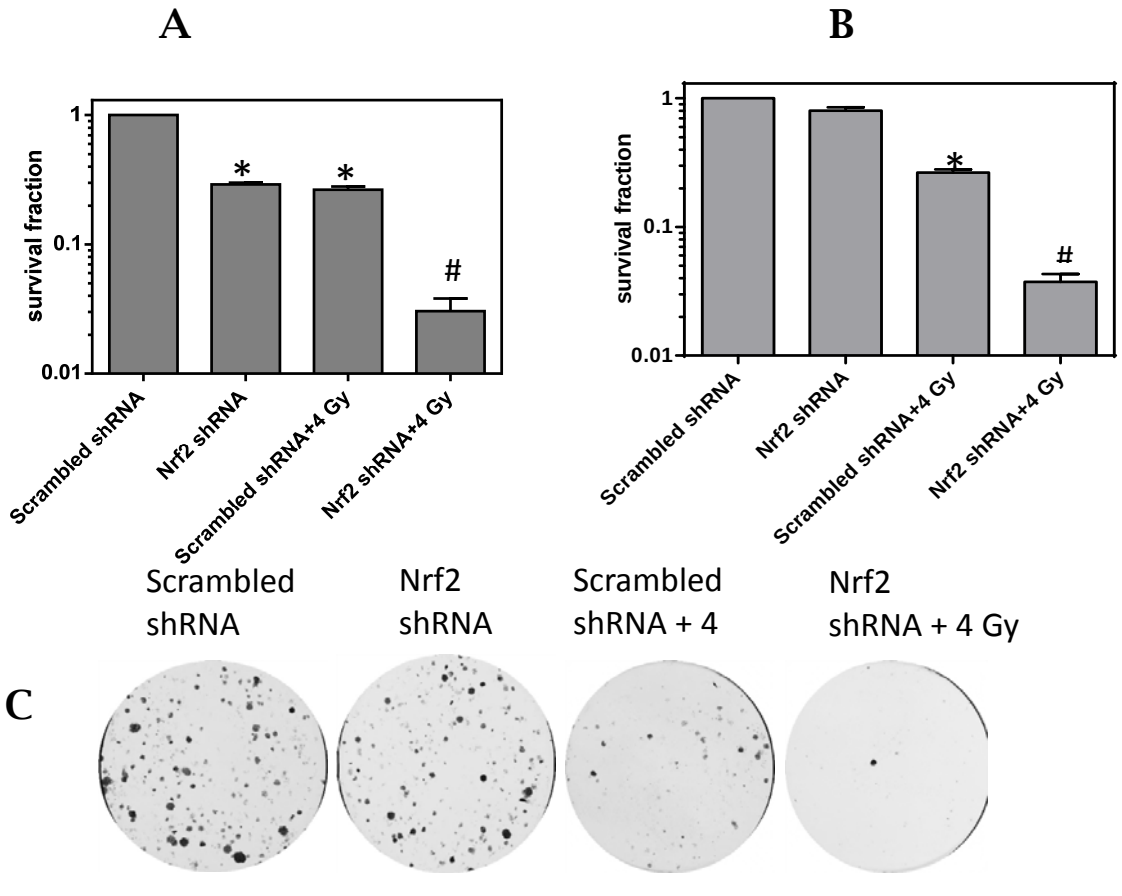


*Fig: 4-13. Survival fraction of DU145 cells after knocking down the expression of Nrf2 expression followed by radiation exposure either with Nrf2 shRNA concentration of 1µg/ml (A) or 0.5µg/ml (B) and the representative image of clonogenic dishes (C). Survival fraction was calculated in DU145 cells after knocking down the expression of Nrf2 which are exposed to radiation, 48 h after transfection with shRNA targeting Nrf2. Survival fraction was assessed by clonogenic assay by counting the colonies developed at 15 days after treatment and radiation exposure. A nonspecific shRNA containing scrambled sequences was used as control. Two independent experiments were performed with triplicates each time. Mean ± SEM has been plotted. Representative clonogenic dishes belonging to various treatment groups have been shown. * $p < 0.05$ in comparison to scrambled shRNA group. # $p < 0.05$ in comparison to scrambled shRNA + 4 Gy group.*

Clonogenic survival assay was also performed in DU145 cells after knocking down the expression of HO1 (an Nrf2 dependent gene). Though HO1 knockdown itself has not exhibited any significant reduction in survival, radiation exposure of HO1 knockdown cells exhibited significant reduction in survival fraction (Fig. 4-14A and 4-14B). Knocking down of HO1 from cells prior to 4 Gy irradiation reduced the survival fraction to 0.26 in comparison to the survival fraction of 0.07, which was observed in cells transfected with scrambled shRNA followed by exposure to 4 Gy. These results suggested the involvement of redox regulated transcription factor Nrf2 and associated genes in determining radiosensitivity of prostate tumor cells.

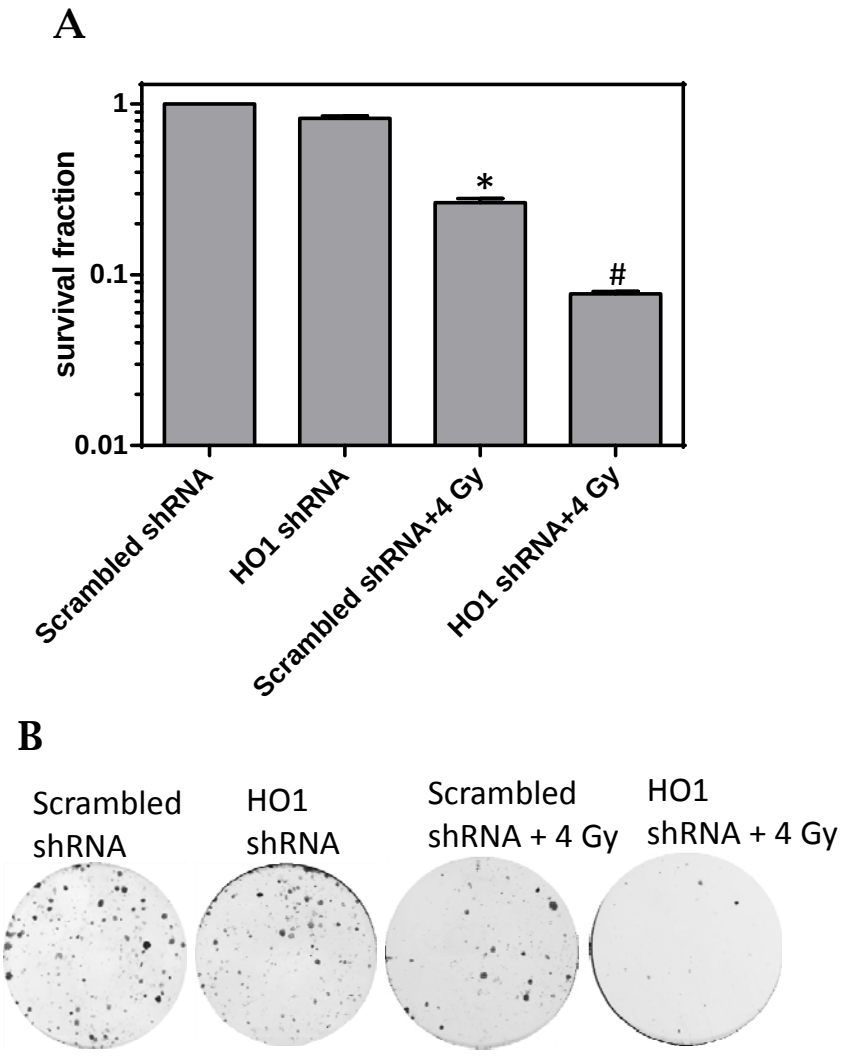


*Fig: 4-14. Survival fraction of DU145 cells after knocking down the expression of HO1 expression followed by radiation exposure. Survival fraction was calculated in DU145 cells after knocking down the expression of HO1 which are exposed to radiation, 48 h after transfection with shRNA targeting HO1. Survival fraction was assessed by clonogenic assay by counting the colonies developed at 15 days after treatment and radiation exposure. A nonspecific shRNA containing scrambled sequences was used as control. Two independent experiments were performed with triplicates each time. Mean ± SEM has been plotted. Representative clonogenic dishes belonging to various treatment groups have been shown (B). * $p < 0.05$ in comparison to scrambled shRNA group. # $p < 0.05$ in comparison to scrambled shRNA + 4 Gy group.*

4.4. Discussion

Molecular mechanisms governing the radiosensitivity/radioresistance of tumor cells are not clearly understood and there is a need for further research in this area. In order to study the molecular players which determine radiosensitivity, two prostate tumor cells namely, PC3 and DU145 were chosen, which are androgen independent cells, known to have differential chemotherapeutic response [143, 144]. However, differential radiosensitivity and underlying mechanism have not been investigated in these two prostate cancer cell lines. In this study, the radiosensitivity of these two prostate cancer cells was investigated and an attempt was made to rationalize molecular differences between them contributing to their differential radiosensitivity. These two cell lines showed difference in their radiosensitivity as measured by clonogenic survival fraction after irradiation (1-10 Gy) and PC3 cells were found to be more radiosensitive than that of DU145 cells. This difference was more prominent at higher doses (>8 Gy) than at the lower doses (<4 Gy) of radiation exposure. In apoptosis analysis also PC3 cells showed more sensitivity towards radiation than the DU145 cells. Radiosensitive PC3 cells also showed more DNA damage than that of DU145 cells. Though these two tumor cells exhibited significant difference in DNA damage after the radiation exposure, following DNA repair kinetics, there was no significant difference in residual DNA damage between these two cells. These results are in corroboration with earlier observation that the initial DNA damage observed by neutral comet assay to be a good marker of radiosensitivity than that of the residual DNA damage [136].

Since ROS is known to play an important role in the cytotoxic action of ionizing radiation, basal and inducible level of ROS in these two tumor cells was evaluated. PC3 cells, which are radiosensitive among the two cell lines, showed

higher basal as well as radiation induced ROS on radiation exposure. Similar to cytosolic ROS, mitochondrial ROS level also was found to be more in radiosensitive PC3 cells after the radiation exposure. Cells employ many enzymatic and non-enzymatic antioxidants to counter the effect of ROS and to bring back cell homeostasis [145]. One of the most versatile protectors of such antioxidants is GSH. GSH protects cells from radiation damage by several mechanisms including radical scavenging, restoration of damaged molecules by hydrogen donation, reduction of peroxides and maintenance of protein thiols in the reduced state [146]. Therefore, the level of GSH, and GSH to GSSG ratio was evaluated in both PC3 and DU145 tumor cells under control and irradiated conditions. A relatively radiosensitive PC3 cells showed low basal level of GSH and also showed faster depletion of GSH after radiation exposure. The radioresistant DU145 cells showed faster recovery (especially at the higher doses) from oxidative stress and this was also supported by their high GSH to GSSG ratio in comparison to that of PC3 cells. Increased accumulation of ROS combined with the faster depletion of GSH may be responsible for higher DNA damage observed in PC3 cells after radiation exposure, which is supported by study showing that thiol depletion can lead to higher radiation induced apoptosis [147].

Since differences in cellular redox environment was observed between these two cell lines after irradiation, we hypothesised that the transcription factor, which controls the enzymes involved in GSH synthesis may be playing role in determining radiosensitivity. Nrf2 is the transcription factor involved in the transcription of the enzymes for GSH biosynthesis [148, 149]. Apart from the GSH biosynthesis enzymes (GCLC and GCLM), glutathione peroxidase enzymes (GPX1, GPX2 and GPX3) and thioredoxin family proteins (Thioredoxin, TXRD1, Peroxiredoxin) have also been shown to be the transcriptional targets of Nrf2 [132, 150, 151]. The basal level of

Nrf2 was found to be higher in radioresistant DU145 cells in comparison to the PC3 cells. One of the reasons for this higher Nrf2 level in DU145 cells may be that DU145 cells are reported to have hypermethylation in the KEAP1 promoter region leading to the lower synthesis of KEAP1 protein [131]. Lower level of KEAP1 lead to the diminished sequestering of Nrf2 in the cytosol and higher level of Nrf2 in the nucleus. Apart from high basal level of Nrf2, DU145 cells also showed higher inducible level of radiation induced Nrf2 activation and its dependent genes in comparison to PC3 cells. GCLC and GCLM are the enzymes, which are involved in biosynthesis of GSH and have shown significant upregulation in radioresistant DU145 cells in comparison to PC3 cells. This could have played a role in restoring the thiol balance in these cells quickly contributing to their radioresistance as compared to PC3 cells. Moreover, when the Nrf2 was inhibited by inhibitors as well as shRNA a drastic reduction was observed in survival fraction of radioresistant DU145 cells after the radiation exposure.

In conclusion, in this study, the role of Nrf2 and its dependent genes was demonstrated in radioresistance of prostate tumor cells through management of intracellular ROS and GSH level (Fig. 4-15). As the radioresistance is the serious impediment in radiotherapy, Nrf2 inhibition can be used as a target to enhance the ionizing radiation induced tumor killing.

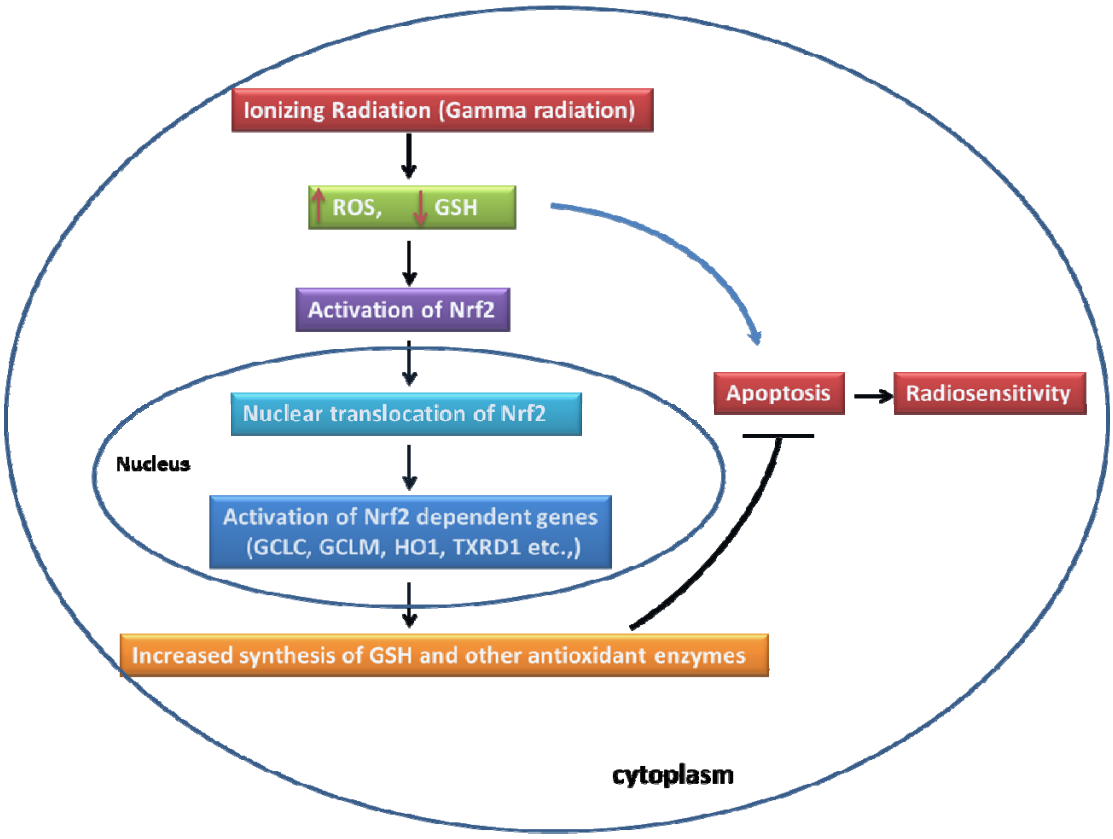


Fig.4-15. Schematic diagram representing the role of ROS, GSH and Nrf2 pathway contributing to the radioresistance of tumor cells. In radiosensitive PC3 cells, radiation exposure led to higher accumulation of ROS in comparison to DU145 cells. Since DU145 cells have high expression of Nrf2 it leads to more accumulation of GSH. The induced levels of Nrf2 and its dependent enzymes are also more in DU145 cells which may be leading to the higher radioresistance of DU145 cells.

CHAPTER 5

GENERAL DISCUSSION, SUMMARY AND FUTURE PERSPECTIVES

General Discussion, Summary and Future Perspectives

5.1. General Discussion

Some of the major issues which are affecting the efficacy of radiotherapy can be overcome by addressing the issue of radiosensitivity of tumor cells. The issue of radiosensitivity in cancer radiotherapy can be addressed from three angles, namely, prediction, understanding the molecular mechanisms, and its modulation. Finding out an assay which can be used in predicting the radiosensitivity is the need of the hour, as that can have huge impact in improving the efficacy of the radiotherapy, by way of delivering personalized doses to the patients undergoing radiotherapy. Since DNA is the major cellular target of radiation, DNA damage based assays can be a good surrogate marker of radiosensitivity. Therefore, usefulness of comet assay was evaluated for predicting radiosensitivity of tumor cells. Comet assay is a simple, reliable, quantitative, easy to perform, and can be automated for high throughput DNA damage analysis of large number of samples. It can be performed in any type of cells and it doesn't need the cells to be in dividing condition. Therefore, comet assay was chosen for assessing the DNA damage. Through comet assay various parameters of DNA damage can be obtained. Moreover, there is a need to standardise the optimum parameter, which can reflect the radiosensitivity of tumor cells. Clonogenic survival is known to be an ideal assay, which can reflect the radiosensitivity of tumor cells. But this assay cannot be used for prediction at clinical level. Therefore, another parameter, which can correlate with the clonogenic survival, could be a good alternative to clonogenic assay in assessing the radiosensitivity of tumor cells. Therefore, the approach followed in the thesis was, to establish the radiosensitivity

profile of the different tumor cells using clonogenic assay, then assess the DNA damage parameters by comet assay and analyse the correlation between these two parameters. For the study, cells from different tissue origin was selected, so as to see if the comet assay can be used with different tumor cells of different tissue of origin.

The radiosensitivity of different tumors cells were analysed by clonogenic assay, which showed that the HT1080 cells to be the most radioresistant cells followed by MCF7, A549, T47D, and HT29 cells. Then amongst these cells, the magnitude of DNA damage was analyzed after radiation treatment by alkaline and neutral comet assay. Out of various parameters of DNA damage, the initial DNA damage (immediately after radiation exposure) obtained by comet assay graded the cells according to their radiosensitivity. Therefore, correlation between the initial DNA damage obtained by comet assay was correlated with clonogenic survival fraction of irradiated tumor cells. Out of neutral and alkaline comet assays, the initial DNA damage obtained by neutral comet assay showed significant correlation with clonogenic survival fraction than that of the correlation obtained with survival fraction and DNA damage obtained by alkaline comet assay. Neutral comet assay mainly detects the DNA double strand breaks, whereas, alkaline comet assay detects the total DNA damage including the single strand breaks, alkali labile sites etc. DNA damage assessed by neutral comet assay has shown higher and significant correlation coefficient values than the alkaline comet assay, corroborating the fact that the DSBs are the significant lesions, which can lead to the cell death. Using comet assay, different DNA damage parameters like, initial DNA damage, DNA repair kinetics, and residual DNA damage can be determined. The correlation between each of these parameters with radiosensitivity of tumor cells was analysed in this study to find out the best parameter, which will reflect the radiosensitivity of tumor cells. Out of these

parameters, initial DNA damage showed a significant correlation than that of the correlation obtained with other parameters like residual DNA damage. Though the difference in DNA damage repair is the major determinant of radiosensitivity, the comet assay could not resolve these differences with significance. This finding in this regard is in corroboration with the other studies [80], wherein DNA repair kinetics analysed by constant- and graded-field gel electrophoresis failed to provide correlation with radiosensitivity of the tumor cells.

Since the radiation dose during radiotherapy is delivered in fractions, the usefulness of comet assay was also examined in predicting the radiosensitivity of tumor cells after exposing them to fractionated dose of radiation. The correlation exhibited by comet assay in predicting radiosensitivity under fractionated dose regimen is similar to that of the correlation obtained with an acute radiation dose. The neutral comet assay exhibited significant correlation than the alkaline comet assay under fractionated radiation exposure.

Since DNA damage was showing significant correlation with radiosensitivity, it is also plausible that the response elicited by the cell in response to the DNA damage at molecular level, might also show correlation with the radiosensitivity. The analysis of gene expression as marker of tumor radiosensitivity can have advantages in terms of automation and complementing with the other assays for radiosensitivity prediction. Therefore, the set of genes which are involved in critical DNA damage response pathways (DNA repair, cell cycle arrest, apoptosis and antioxidant response) was selected and their correlation with the radiosensitivity of tumor cells was examined. Radiation induced expression of 15 genes which were involved in these pathways were analysed and their correlation with radiosensitivity of tumor cells were analysed. Out of these 15 genes, three genes, namely, *HSP70, KU80* and *RAD51* showed

significant correlation with radiation sensitivity. These genes are involved in the repair of DNA damage especially DNA double strand breaks. Since the extent of DNA damage (DSBs) was showing correlation with radiosensitivity, the genes which were involved in DSB repair also showed their usefulness in predicting the radiosensitivity of tumor cells. Apart from these three genes other genes namely *P21* and *NRF2* were showing gene expression pattern in accordance to radiosensitivity of the cell but the correlation between their expression and radiosensitivity was not statistically significantly. Since the genes involved in DNA repair showed correlation with radiosensitivity, targeting the DNA repair pathway can be a good strategy for radiosensitization. For validating this hypothesis, DNA-PK was pharmacologically inhibited, and its effect was evaluated in radiosensitivity of the tumor cells. DNA-PK is a key protein which is involved in the repair of DNA DSBs. DNA-PK is a complex containing KU70, KU80 and DNA-PKcs. When DNA-PK was inhibited using a specific inhibitor NU7026, it showed a synergistic reduction in the radioresistance of tumor cells. This indicates the fact that the genes involved in the DNA repair pathways can not only be used in predicting the radiosensitivity of the tumor cells but also to sensitize the tumor cells against radiation induced DNA damage.

Apart from DNA repair, genes involved in the maintenance of cellular redox homeostasis also are important determinants of radiosensitivity of the tumor cells. In order to validate this hypothesis, two prostate cancer cell lines namely PC3 and DU145 cells were chosen, which showed significant difference in their radiosensitivity. Using clonogenic survival, DNA damage and apoptosis assays, it was found that among these two cells lines, PC3 is more radiosensitive than the DU145 cells. In these two cells the redox status was examined after the radiation exposure. The basal and radiation induced of ROS were found to be higher in radiosensitive

PC3 cells than DU145 cells. Similarly, level of glutathione and the ratio of GSH to GSSG were found to be higher in radioresistant DU145 cells than the PC3 cells. The expressions of anti-oxidant genes including the genes which are involved in glutathione synthesis are governed by a redox-sensitive transcription factor Nrf2. Therefore, the level of Nrf2 was evaluated in these two cell lines and it was found that, the basal and radiation inducible level of Nrf2 in radioresistant DU145 cell were significantly higher than the PC3 cells. Consequently, the Nrf2 dependent genes like, *HO1, GCLC, GCLM, TXN1, TXN2, TXRD1* and *TXRD2* were also significantly higher in radioresistant DU145 cells. Under normal conditions Nrf2 is tightly bound to KEAP1 protein and that leads to the targeting of Nrf2 to the proteasomal degradation. It is plausible that the deregulations in KEAP1 may be playing role in the higher Nrf2 level observed in DU145 cells. Therefore, the Keap1 expression was evaluated in these two cell types and it was found that the relative level of KEAP1 in DU145 was lower in comparison to PC3 cells. Previous studies [131] have reported that in DU145 cells the KEAP1 promoter regions are hypermethylated and this may be the reason for the lower level of Keap1 and higher level s of Nrf2 observed in DU145 cells. The role of Nrf2 and its dependent gene HO1 in radiosensitivity of these cells, was further confirmed by pharmacological intervention and knockdown studies. From these studies, it was found that the Nrf2 and HO1 can be good targets for radiosensitization of tumor cells.

Apart from the intrinsic radiosensitivity, the hypoxia also plays important role in governing tumor radiosensitivity. It has been known that the hypoxic cells are radioresistant because of the lower oxygen concentration present in that region thereby production of decreased ROS level after low LET radiation exposure. But interestingly, it was also found that hypoxia plays a major role in tumor progression

and angiogenesis. This hypoxia induced angiogenesis is mediated by the induction of a transcription factor HIF1-α in the hypoxic regions. HIF1-α transactivates many genes, which encode angiogenic growth factors [152]. HIF1-α has been implicated in radioresistance of tumor cells. [153]. Recently, evidences have shown that Nrf2 inhibition can lead to the down regulation of HIF1-α, suggesting a crosstalk between the Nrf2 and HIF1-α [154]. Therefore, it can be assumed that Nrf2 mediated HIF1-α signalling can lead to radioresistance in hypoxic tumors, and Nrf2 inhibition can be a good target for radiosensitizing hypoxic cells also.

The radiation induced ROS production, DNA damage, DNA damage response by the cell, and Nrf2 pathway has been depicted in the form of a scheme (Fig. 5.1).

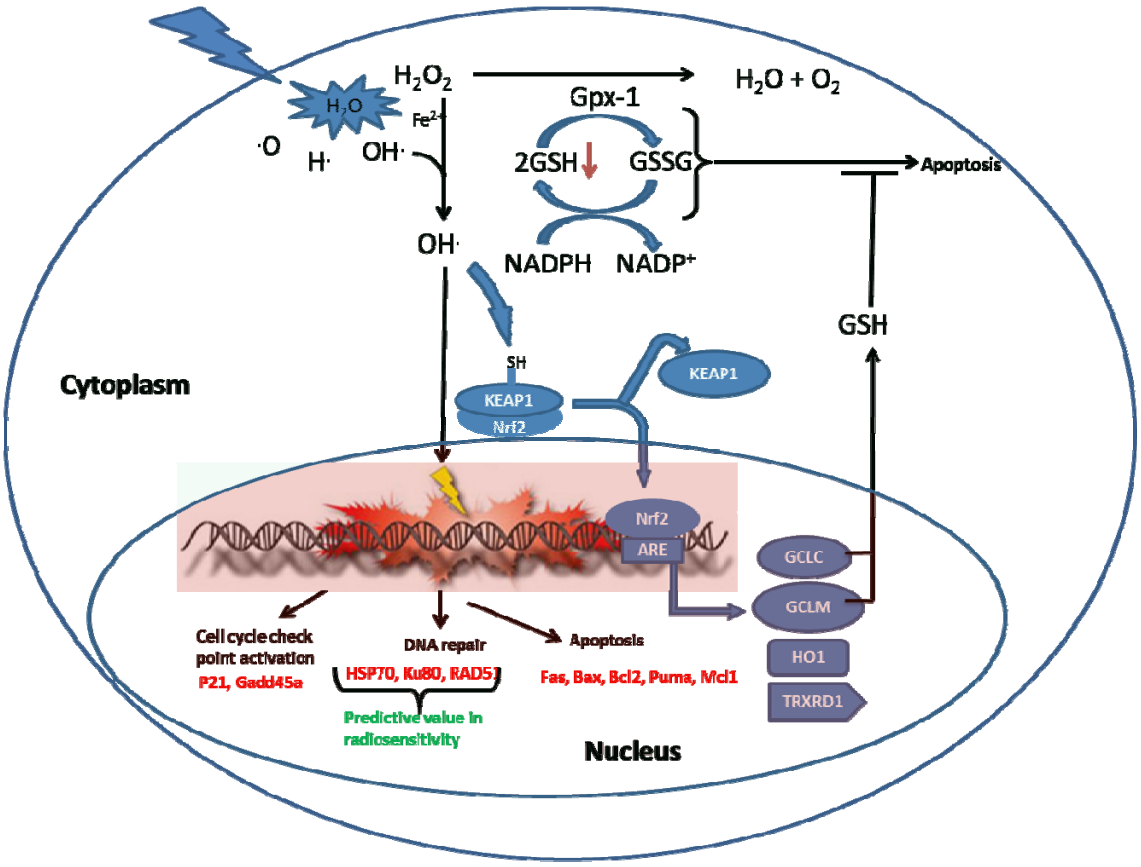


Fig: 5.1. Schematic representation of radiation induced ROS production and their effect on the cell. The ROS produced by radiation exposure, leads to the DNA damage and in response to DNA damage, cells activate DNA damage response pathways like, cell cycle arrest, DNA repair, apoptosis, Nrf2 pathway etc. The critical genes (like KU80, RAD51 and HSP70) which are involved in these pathways can be a good candidate for the purpose of predicting the radiosensitivity and also as targets of radiosensitization.

5.2. Summary

- DNA damage assessed by neutral comet assay showed significant correlation with the radiosensitivity of tumor cells
- The correlation exhibited by neutral comet assay with radiosensitivity is better than the correlation exhibited by alkaline comet assay.
- DNA damage assessed immediately after the radiation exposure showed better correlation with the tumor radiosensitivity than that of other parameters like DNA repair kinetics and residual damage
- DNA damage obtained after higher doses 4, 8 Gy showed better correlation with radiosensitivity than the DNA damage obtained at the lower dose (2 Gy)
- DNA damage measured by neutral comet assay after exposure of tumor cells to fractionated doses of radiation also showed significant correlation with their survival fraction
- The standard curve depicting the correlation between the tumor radiosensitivity and DNA damage was again validated using two other cell lines namely PC3 and DU145. The predicted radiosensitivity level of these cell lines using the standard curve was very close to the actual radiosensitivity of these cell lines.
- Usefulness of expression of genes associated with DNA damage response in assessing radiosensitivity was investigated and out of 15 genes evaluated, *KU80, RAD51* and *HSP70* showed significant correlation with radiosensitivity, which was further validated using inhibitor of DNA-PK, which showed a very good radiosensitizing potential.
- Prostate cancer cells PC3 and DU145 showed differential response to radiation corroborated with difference in their ROS status and GSH/ GSSG ratio.

- Basal as well as inducible level of Nrf2 and its dependent genes in DU145 cells (radioresistant) were significantly higher than the radiosensitive PC3 cells
- Role of Nrf2 and associated genes in determining the radiosensitivity was further studied by using inhibitors and shRNA of Nrf2 and HO1 significantly reduced the survival fraction of these tumor cells against the radiation exposure.

5.3. Future Perspectives

Predicting the radiosensitivity of tumor cells prior to radiotherapy will immensely help in optimising the dose of radiotherapy, so as to reduce the normal tissue toxicity and to improve the efficacy of radiotherapy. In this study, the potential of comet assay (a simple and reliable assay which can assess the DNA damage) in assessing the radiosensitivity of tumor cells was investigated. Neutral comet assay showed a significant correlation with the radiosensitivity of tumor cells. This correlation study was performed in five different tumor cells which have originated from different tissue of origin. But these study needs to be extended with many more number of tumor cells with wide variation in radiosensitivity. After validating with more number of cell lines *in vitro*, the usefulness of neutral comet assay in predicting the radiosensitivity needs to be evaluated under clinical scenario using biopsy samples from cancer patients.

Apart from the tumor cells the evaluation of normal tissue toxicity also is equally important and that can also play a major role in improving the efficacy of radiotherapy. The neutral comet assay can also be evaluated for its use in radiosensitivity of normal cells. Since the normal cells in the form of peripheral blood lymphocytes can be easily obtained, correlation between the normal tissue radiosensitivity and treatment prognosis / normal tissue side effects can be studied.

Analysis of radiosensitivity in both normal and tumor cells would give better picture regarding the differences in the radiosensitivity between the normal and tumor cells if any, and that can help in devising personalised radiotherapeutic regimen leading to the higher therapeutic efficacy.

Expression of the genes involved in DNA damage repair was found to have better correlation with radiosensitivity than the genes associated with other biological processes. But in this current study, the genes selected were not exhaustive and only limited genes were selected, which were representing major DNA damage response pathways. But in order to use this strategy in predicting the radiosensitivity with more confidence, more number of genes, which are involved in DNA damage response pathways need to be examined for their value in predicting the radiosensitivity of tumor cells. With the use of high throughput micro array techniques, the gene expression signature of many pathways can be examined and that will pave the way for accurate prediction of radiosensitivity and also it can be automated easily. Apart from having predictive value, these genes can be targeted for their use in radiosensitization of tumor cells. One such target which was investigated for radiosensitization of tumor cells is, Nrf2 and its dependent genes. In this study the role of Nrf2 and HO1 was examined in prostrate tumor cells and the inhibition or knockdown of these genes potentiated the radiation induced cell killing. Since Nrf2 is overexpressed in many of the tumors, it can be a very good target of radiosensitization for evaluating it in clinical conditions. Apart from the inhibition of either DNA-PK or Nrf2 the combinatorial inhibition of both these proteins needs to be evaluated as a better strategy for radiosensitization of tumor cells. Apart from intrinsic radiosensitivity, hypoxia is another important factor which determines the

radiosensitivity of the tumor. It will be interesting to study the effect of inhibition of Nrf2 and its dependent genes in hypoxic cells.

REFERENCES

REFERENCES

[1] R. Dikshit, P.C. Gupta, C. Ramasundarahettige, V. Gajalakshmi, L. Aleksandrowicz, R. Badwe, R. Kumar, S. Roy, W. Suraweera, F. Bray, M. Mallath, P.K. Singh, D.N. Sinha, A.S. Shet, H. Gelband, P. Jha, Cancer mortality in India: a nationally representative survey, Lancet, 379 (2012) 1807-1816.

[2] W.A. Imran Ali, Wani, Kishwar Saleem, Cancer Scenario in India with Future Perspectives, Cancer Therapy, 8 (2011) 56-70.

[3] R. Ramaswami, V. Harding, T. Newsom-Davis, Novel cancer therapies: treatments driven by tumour biology, Postgrad Med J, 89 (2013) 652-658.

[4] M. Jang, S.S. Kim, J. Lee, Cancer cell metabolism: implications for therapeutic targets, Exp Mol Med, 45 (2013) e45.

[5] D. Spano, C. Heck, P. De Antonellis, G. Christofori, M. Zollo, Molecular networks that regulate cancer metastasis, Seminars in Cancer Biology, 22 (2012) 234-249.

[6] C.P. Neal, D.P. Berry, Basic principles of the molecular biology of cancer II: angiogenesis, invasion and metastasis, Surgery (Oxford), 24 (2006) 120-125.

[7] C.J. Reade, L.R. Eiriksson, A. Covens, Surgery for early stage cervical cancer: How radical should it be?, Gynecologic Oncology, 131 (2013) 222-230.

[8] P.J. Lovrics, S.D. Cornacchi, R. Vora, C.H. Goldsmith, K. Kahnamoui, Systematic review of radioguided surgery for non-palpable breast cancer, European Journal of Surgical Oncology (EJSO), 37 (2011) 388-397.

[9] P.R. Dutta, A. Maity, Cellular responses to EGFR inhibitors and their relevance to cancer therapy, Cancer Letters, 254 (2007) 165-177.

[10] J. Cadranel, A.-M. Ruppert, M. Beau-Faller, M. Wislez, Therapeutic strategy for advanced EGFR mutant non-small-cell lung carcinoma, Critical Reviews in Oncology/Hematology.

[11] K.M. Prise, G. Schettino, M. Folkard, K.D. Held, New insights on cell death from radiation exposure, The lancet oncology, 6 (2005) 520-528.

[12] G. Delaney, S. Jacob, C. Featherstone, M. Barton, The role of radiotherapy in cancer treatment: estimating optimal utilization from a review of evidence-based clinical guidelines, Cancer, 104 (2005) 1129-1137.

[13] J. Shafiq, M. Barton, D. Noble, C. Lemer, L.J. Donaldson, An international review of patient safety measures in radiotherapy practice, Radiother Oncol, 92 (2009) 15-21.

[14] M. Elshaikh, M. Ljungman, R. Ten Haken, A.S. Lichter, Advances in radiation oncology, Annual review of medicine, 57 (2006) 19-31.

[15] F. Khan, The physics of radiation therapy. , Baltimore, MD: Williams and Wilkins; , (1994).

[16] S.R. Mehta, V. Suhag, M. Semwal, N. Sharma, Radiotherapy: Basic concepts and recent advances, Medical Journal Armed Forces India, 66 (2010) 158-162.

[17] M. Dahele, J.D. Zindler, E. Sanchez, W.F. Verbakel, J.P.A. Kuijer, B.J. Slotman, S. Senan, Imaging for Stereotactic Spine Radiotherapy: Clinical Considerations, International Journal of Radiation Oncology*Biology*Physics, 81 (2011) 321-330.

[18] S. Korreman, C. Rasch, H. McNair, D. Verellen, U. Oelfke, P. Maingon, B. Mijnheer, V. Khoo, The European Society of Therapeutic Radiology and Oncology–European Institute of Radiotherapy (ESTRO–EIR) report on 3D CT-based in-room image guidance systems: A practical and technical review and guide, Radiotherapy and Oncology, 94 (2010) 129-144.

[19] R.B. Den, A.P. Dicker, IMRT, IGRT, SBRT: Advances in Treatment Planning and Delivery of Radiotherapy, International Journal of Radiation Oncology*Biology*Physics, 83 (2012) e3.

[20] M.L.K. Chua, K. Rothkamm, Biomarkers of Radiation Exposure: Can They Predict Normal Tissue Radiosensitivity?, Clin Oncol-Uk, 25 (2013) 610-616.

[21] C.F. Dunne-Daly, Principles of radiotherapy and radiobiology, Seminars in Oncology Nursing, 15 (1999) 250-259.

[22] J. Vignard, G. Mirey, B. Salles, Ionizing-radiation induced DNA double-strand breaks: A direct and indirect lighting up, Radiotherapy and Oncology, (2013).

[23] D.R. Raleigh, D.A. Haas-Kogan, Molecular targets and mechanisms of radiosensitization using DNA damage response pathways, Future Oncology, 9 (2013) 219-233.

[24] L.M. Martin, B. Marples, T.H. Lynch, D. Hollywood, L. Marignol, Exposure to low dose ionising radiation: Molecular and clinical consequences, Cancer Letters, 338 (2013) 209-218.

[25] M.L. Chua, K. Rothkamm, Biomarkers of radiation exposure: can they predict normal tissue radiosensitivity?, Clinical Oncology (R Coll Radiol), 25 (2013) 610-616.

[26] S. Tapio, Ionizing radiation effects on cells, organelles and tissues on proteome level, Adv Exp Med Biol, 990 (2013) 37-48.

[27] J.B. Little, Cellular effects of ionizing radiation, The New England journal of medicine, 278 (1968) 369-376 concl.

[28] S. Lehnert, Biomolecular Action of Ionizing Radiation, (2007).

[29] J.F. Weiss, M.R. Landauer, Protection against ionizing radiation by antioxidant nutrients and phytochemicals, Toxicology, 189 (2003) 1-20.

[30] J. Sun, Y. Chen, M. Li, Z. Ge, Role of Antioxidant Enzymes on Ionizing Radiation Resistance, Free Radical Biology and Medicine, 24 (1998) 586-593.

[31] J.M. McCord, I. Fridovich, Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein), Journal of Biological Chemistry, 244 (1969) 6049-6055.

[32] R.A. Greenwald, Superoxide dismutase and catalase as therapeutic agents for human diseases a critical review, Free Radical Biology and Medicine, 8 (1990) 201-209.

[33] L.W. Oberley, Mechanism of the tumor suppressive effect of MnSOD overexpression, Biomedicine & Pharmacotherapy, 59 (2005) 143-148.

[34] A.P. Kudin, E. Taheizadeh-Fard, W.S. Kunz, Mitochondrial dysfunction in MnSOD knockout mice, Biochimica et Biophysica Acta (BBA) - Bioenergetics, 1817, Supplement (2012) S100.

[35] W. Zhong, T. Yan, R. Lim, L.W. Oberley, Expression of superoxide dismutases, catalase, and glutathione peroxidase in glioma cells, Free Radical Biology and Medicine, 27 (1999) 1334-1345.

[36] R. Banerjee, Redox Biochemistry, (2008).

[37] C.J.a.P.G. Winyard, Redox Signaling and Regulation in Biology and Medicine, (2009).

[38] J. Du, J.J. Cullen, G.R. Buettner, Ascorbic acid: Chemistry, biology and the treatment of cancer, Biochimica et Biophysica Acta (BBA) - Reviews on Cancer, 1826 (2012) 443-457.

[39] B.D. Price, A.D. D'Andrea, Chromatin remodeling at DNA double-strand breaks, Cell, 152 (2013) 1344-1354.

[40] J.N. Kavanagh, K.M. Redmond, G. Schettino, K.M. Prise, DNA double strand break repair: a radiation perspective, Antioxidants & redox signaling, 18 (2013) 2458-2472.

[41] J.M. Furgason, M. Bahassi el, Targeting DNA repair mechanisms in cancer, Pharmacology & therapeutics, 137 (2013) 298-308.

[42] E. Fokas, R. Prevo, E.M. Hammond, T.B. Brunner, W.G. McKenna, R.J. Muschel, Targeting ATR in DNA damage response and cancer therapeutics, Cancer Treat Rev, (2013).

[43] F.A. Derheimer, M.B. Kastan, Multiple roles of ATM in monitoring and maintaining DNA integrity, FEBS letters, 584 (2010) 3675-3681.

[44] D. Durocher, S.P. Jackson, DNA-PK, ATM and ATR as sensors of DNA damage: variations on a theme?, Current Opinion in Cell Biology, 13 (2001) 225-231.

[45] E.U. Kurz, S.P. Lees-Miller, DNA damage-induced activation of ATM and ATM-dependent signaling pathways, DNA Repair, 3 (2004) 889-900.

[46] Y. Shiloh, ATM and ATR: networking cellular responses to DNA damage, Current Opinion in Genetics & Development, 11 (2001) 71-77.

[47] S.J. Collis, T.L. DeWeese, P.A. Jeggo, A.R. Parker, The life and death of DNA-PK, Oncogene, 24 (2005) 949-961.

[48] V.R. Gogineni, A.K. Nalla, R. Gupta, D.H. Dinh, J.D. Klopfenstein, J.S. Rao, Chk2-mediated G2/M cell cycle arrest maintains radiation resistance in malignant meningioma cells, Cancer Letters, 313 (2011) 64-75.

[49] P.A. Jeggo, M. Löbrich, Contribution of DNA repair and cell cycle checkpoint arrest to the maintenance of genomic stability, DNA Repair, 5 (2006) 1192-1198.

[50] A. Maity, W.G. McKenna, R.J. Muschel, The molecular basis for cell cycle delays following ionizing radiation: a review, Radiotherapy and Oncology, 31 (1994) 1-13.

[51] K.I. Park, H.S. Park, A. Nagappan, G.E. Hong, D.H. Lee, S.R. Kang, J.A. Kim, J. Zhang, E.H. Kim, W.S. Lee, S.C. Shin, Y.S. Hah, G.S. Kim, Induction of the cell

cycle arrest and apoptosis by flavonoids isolated from Korean Citrus aurantium L. in non-small-cell lung cancer cells, Food Chemistry, 135 (2012) 2728-2735.

[52] T.M. Pawlik, K. Keyomarsi, Role of cell cycle in mediating sensitivity to radiotherapy, International Journal of Radiation Oncology*Biology*Physics, 59 (2004) 928-942.

[53] M.S. Rudoltz, G. Kao, K.R. Blank, R.J. Muschel, W. Gillies McKenna, Molecular biology of the cell cycle: Potential for therapeutic applications in radiation oncology, Seminars in Radiation Oncology, 6 (1996) 284-294.

[54] R.G. Bristow, S. Benchimol, R.P. Hill, The p53 gene as a modifier of intrinsic radiosensitivity: implications for radiotherapy, Radiotherapy and Oncology, 40 (1996) 197-223.

[55] V. Dulić, W.K. Kaufmann, S.J. Wilson, T.D. Tisty, E. Lees, J.W. Harper, S.J. Elledge, S.I. Reed, p53-dependent inhibition of cyclin-dependent kinase activities in human fibroblasts during radiation-induced G1 arrest, Cell, 76 (1994) 1013-1023.

[56] F. Zölzer, S. Hillebrandt, C. Streffer, Radiation induced G1-block and p53 status in six human cell lines, Radiotherapy and Oncology, 37 (1995) 20-28.

[57] J.B.a.J. Lukas, The DNA damage response in tumorigenesis and cancer treatment Nature Reviews Cancer, (2010).

[58] M. Dizdaroglu, Base-excision repair of oxidative DNA damage by DNA glycosylases, Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 591 (2005) 45-59.

[59] N. Yang, H. Galick, S.S. Wallace, Attempted base excision repair of ionizing radiation damage in human lymphoblastoid cells produces lethal and mutagenic double strand breaks, DNA Repair, 3 (2004) 1323-1334.

[60] S. Adimoolam, J.M. Ford, p53 and regulation of DNA damage recognition during nucleotide excision repair, DNA Repair, 2 (2003) 947-954.

[61] E.C. Friedberg, Nucleotide Excision Repair: Biology, in: J.L. Editors-in-Chief: William, M.D. Lane (Eds.) Encyclopedia of Biological Chemistry, Academic Press, Waltham, 2013, pp. 337-340.

[62] S. Burma, B.P.C. Chen, D.J. Chen, Role of non-homologous end joining (NHEJ) in maintaining genomic integrity, DNA Repair, 5 (2006) 1042-1048.

[63] S.P. Lees-Miller, K. Meek, Repair of DNA double strand breaks by non-homologous end joining, Biochimie, 85 (2003) 1161-1173.

[64] E. Mladenov, G. Iliakis, Induction and repair of DNA double strand breaks: The increasing spectrum of non-homologous end joining pathways, Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 711 (2011) 61-72.

[65] F.V. Rassool, DNA double strand breaks (DSB) and non-homologous end joining (NHEJ) pathways in human leukemia, Cancer Letters, 193 (2003) 1-9.

[66] A. Dudáš, M. Chovanec, DNA double-strand break repair by homologous recombination, Mutation Research/Reviews in Mutation Research, 566 (2004) 131-167.

[67] P.A. Jeggo, V. Geuting, M. Löbrich, The role of homologous recombination in radiation-induced double-strand break repair, Radiotherapy and Oncology, 101 (2011) 7-12.

[68] C.M. West, S.E. Davidson, S.A. Roberts, R.D. Hunter, The independence of intrinsic radiosensitivity as a prognostic factor for patient response to radiotherapy of carcinoma of the cervix, Br J Cancer, 76 (1997) 1184-1190.

[69] T. Bjork-Eriksson, C. West, E. Karlsson, C. Mercke, Tumor radiosensitivity (SF2) is a prognostic factor for local control in head and neck cancers, Int J Radiat Oncol Biol Phys, 46 (2000) 13-19.

[70] J.F. Torres-Roca, S. Eschrich, H. Zhao, G. Bloom, J. Sung, S. McCarthy, A.B. Cantor, A. Scuto, C. Li, S. Zhang, R. Jove, T. Yeatman, Prediction of radiation sensitivity using a gene expression classifier, Cancer Res, 65 (2005) 7169-7176.

[71] P.L. Olive, J.P. Banath, Phosphorylation of histone H2AX as a measure of radiosensitivity, Int J Radiat Oncol Biol Phys, 58 (2004) 331-335.

[72] N.E. Crompton, M. Ozsahin, A versatile and rapid assay of radiosensitivity of peripheral blood leukocytes based on DNA and surface-marker assessment of cytotoxicity, Radiat Res, 147 (1997) 55-60.

[73] E. Severin, B. Greve, E. Pascher, N. Wedemeyer, U. Hacker-Klom, G. Silling, J. Kienast, N. Willich, W. Gohde, Evidence for predictive validity of blood assays to evaluate individual radiosensitivity, Int J Radiat Oncol Biol Phys, 64 (2006) 242-250.

[74] S. Folkvord, K. Flatmark, S. Dueland, R. de Wijn, K.K. Groholt, K.H. Hole, J.M. Nesland, R. Ruijtenbeek, P.J. Boender, M. Johansen, K.E. Giercksky, A.H. Ree, Prediction of response to preoperative chemoradiotherapy in rectal cancer by multiplex kinase activity profiling, Int J Radiat Oncol Biol Phys, 78 (2010) 555-562.

[75] M.S. Vidyasagar, M. Kodali, P.P. Saxena, D. Upadhya, C.M. Krishna, B.M. Vadhiraja, D.J. Fernandes, S.R.B. Sadashiva, Predictive and Prognostic Significance of Glutathione Levels and DNA Damage in Cervix Cancer Patients Undergoing Radiotherapy, Int J Radiat Oncol, 78 (2010) 343-349.

[76] M. Abend, K. Kehe, M. Riedel, D. Van Beuningen, Correlation of micronucleus and apoptosis assays with reproductive cell death can be improved by considering other modes of death, Int J Radiat Biol, 76 (2000) 249-259.

[77] N.S. Russell, C.F. Arlett, H. Bartelink, A.C. Begg, Use of Fluorescence in-Situ Hybridization to Determine the Relationship between Chromosome-Aberrations and Cell-Survival in 8 Human Fibroblast Strains, International Journal of Radiation Biology, 68 (1995) 185-196.

[78] J.L. Schwartz, R. Mustafi, M.A. Beckett, R.R. Weichselbaum, Prediction of the radiation sensitivity of human squamous cell carcinoma cells using DNA filter elution, Radiat Res, 123 (1990) 1-6.

[79] K. Wei, E. Wandl, K.H. Karcher, X-ray induced DNA double-strand breakage and rejoining in a radiosensitive human renal carcinoma cell line estimated by CHEF electrophoresis, Strahlenther Onkol, 169 (1993) 740-744.

[80] R.A. El-Awady, E. Dikomey, J. Dahm-Daphi, Radiosensitivity of human tumour cells is correlated with the induction but not with the repair of DNA double-strand breaks, Br J Cancer, 89 (2003) 593-601.

[81] D.J. McKenna, S.R. McKeown, V.J. McKelvey-Martin, Potential use of the comet assay in the clinical management of cancer, Mutagenesis, 23 (2008) 183-190.

[82] S.R. McKeown, T. Robson, M.E. Price, E.T. Ho, D.G. Hirst, V.J. McKelvey-Martin, Potential use of the alkaline comet assay as a predictor of bladder tumour response to radiation, Br J Cancer, 89 (2003) 2264-2270.

[83] M.A. Moneef, B.T. Sherwood, K.J. Bowman, R.C. Kockelbergh, R.P. Symonds, W.P. Steward, J.K. Mellon, G.D. Jones, Measurements using the alkaline comet assay predict bladder cancer cell radiosensitivity, Br J Cancer, 89 (2003) 2271-2276.

[84] S.A. Nahas, R. Davies, F. Fike, K. Nakamura, L. Du, R. Kayali, N.T. Martin, P. Concannon, R.A. Gatti, Comprehensive profiling of radiosensitive human cell lines with DNA damage response assays identifies the neutral comet assay as a potential surrogate for clonogenic survival, Radiat Res, 177 (2012) 176-186.

[85] P.L. Olive, J.P. Banath, H.S. MacPhail, Lack of a correlation between radiosensitivity and DNA double-strand break induction or rejoining in six human tumor cell lines, Cancer Res, 54 (1994) 3939-3946.

[86] W.U. Muller, T. Bauch, C. Streffer, F. Niedereichholz, W. Bocker, Comet assay studies of radiation-induced DNA damage and repair in various tumour cell lines, Int J Radiat Biol, 65 (1994) 315-319.

[87] A. Kunwar, S. Jayakumar, H.N. Bhilwade, P.P. Bag, H. Bhatt, R.C. Chaubey, K.I. Priyadarsini, Protective effects of selenocystine against gamma-radiation-induced genotoxicity in Swiss albino mice, Radiat Environ Biophys, 50 (2011) 271-280.

[88] B. Burlinson, R.R. Tice, G. Speit, E. Agurell, S.Y. Brendler-Schwaab, A.R. Collins, P. Escobar, M. Honma, T.S. Kumaravel, M. Nakajima, Y.F. Sasaki, V. Thybaud, Y. Uno, M. Vasquez, A. Hartmann, Fourth International Workgroup on Genotoxicity testing: Results of the in vivo Comet assay workgroup, Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 627 (2007) 31-35.

[89] Y. Yu, W. Zhu, H. Diao, C. Zhou, F.F. Chen, J. Yang, A comparative study of using comet assay and γH2AX foci formation in the detection of N-methyl-N′-nitro-N-nitrosoguanidine-induced DNA damage, Toxicology in Vitro, 20 (2006) 959-965.

[90] D.J. Carlson, P.J. Keall, B.W. Loo, Jr., Z.J. Chen, J.M. Brown, Hypofractionation results in reduced tumor cell kill compared to conventional fractionation for tumors with regions of hypoxia, Int J Radiat Oncol Biol Phys, 79 (2011) 1188-1195.

[91] B. Shankar, S.S. Kumar, K.B. Sainis, Generation of reactive oxygen species and radiation response in lymphocytes and tumor cells, Radiat Res, 160 (2003) 478-487.

[92] Y.-H. Zhong, Z.-K. Liao, F.-X. Zhou, C.-H. Xie, C.-Y. Xiao, D.-F. Pan, Z.-G. Luo, S.-Q. Liu, Y.-F. Zhou, Telomere length inversely correlates with radiosensitivity in

human carcinoma cells with the same tissue background, Biochemical and Biophysical Research Communications, 367 (2008) 84-89.

[93] M.M. Elkind, H. Sutton-Gilbert, W.B. Moses, T. Alescio, R.W. Swain, Radiation Response of Mammalian Cells Grown in Culture. V. Temperature Dependence of the Repair of X-Ray Damage in Surviving Cells (Aerobic and Hypoxic), Radiat Res, 25 (1965) 359-376.

[94] D.G. Hirst, T. Robson, Molecular biology: the key to personalised treatment in radiation oncology?, Br J Radiol, 83 (2010) 723-728.

[95] S. Nowsheen, E.S. Yang, The intersection between DNA damage response and cell death pathways, Exp Oncol, 34 (2012) 243-254.

[96] R.K. Schmidt-Ullrich, P. Dent, S. Grant, R.B. Mikkelsen, K. Valerie, Signal transduction and cellular radiation responses, Radiat Res, 153 (2000) 245-257.

[97] A.N. Filiano, H.M. Fathallah-Shaykh, J. Fiveash, J. Gage, A. Cantor, S. Kharbanda, M.R. Johnson, Gene expression analysis in radiotherapy patients and C57BL/6 mice as a measure of exposure to ionizing radiation, Radiat Res, 176 (2011) 49-61.

[98] L.A. Henriquez Hernandez, P.C. Lara, B. Pinar, E. Bordon, C. Rodriguez Gallego, C. Bilbao, L. Fernandez Perez, A. Flores Morales, Constitutive gene expression profile segregates toxicity in locally advanced breast cancer patients treated with high-dose hyperfractionated radical radiotherapy, Radiat Oncol, 4 (2009) 17.

[99] S. Kabacik, A. Mackay, N. Tamber, G. Manning, P. Finnon, F. Paillier, A. Ashworth, S. Bouffler, C. Badie, Gene expression following ionising radiation: identification of biomarkers for dose estimation and prediction of individual response, Int J Radiat Biol, 87 (2011) 115-129.

[100] K. Kato, K. Takahashi, S. Monzen, H. Yamamoto, A. Maruyama, K. Itoh, I. Kashiwakura, Relationship between radiosensitivity and Nrf2 target gene expression in human hematopoietic stem cells, Radiat Res, 174 (2010) 177-184.

[101] T.A. Kato, P.F. Wilson, H. Nagasaw, Y. Peng, M.M. Weil, J.B. Little, J.S. Bedford, Variations in radiosensitivity among individuals: a potential impact on risk assessment?, Health Phys, 97 (2009) 470-480.

[102] M.W. Pfaffl, G.W. Horgan, L. Dempfle, Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR, Nucleic Acids Res, 30 (2002) e36.

[103] X. Lu, L. Xiao, L. Wang, D.M. Ruden, Hsp90 inhibitors and drug resistance in cancer: The potential benefits of combination therapies of Hsp90 inhibitors and other anti-cancer drugs, Biochemical Pharmacology, 83 (2012) 995-1004.

[104] J.-C. Ko, H.-J. Chen, Y.-C. Huang, S.-C. Tseng, S.-H. Weng, T.-Y. Wo, Y.-J. Huang, H.-C. Chiu, M.-S. Tsai, R.Y.Y. Chiou, Y.-W. Lin, HSP90 inhibition induces cytotoxicity via down-regulation of Rad51 expression and DNA repair capacity in non-small cell lung cancer cells, Regulatory Toxicology and Pharmacology, 64 (2012) 415-424.

[105] P. Kotoglou, A. Kalaitzakis, P. Vezyraki, T. Tzavaras, L.K. Michalis, F. Dantzer, J.U. Jung, C. Angelidis, Hsp70 translocates to the nuclei and nucleoli, binds to XRCC1 and PARP-1, and protects HeLa cells from single-strand DNA breaks, Cell Stress Chaperones, 14 (2009) 391-406.

[106] O. Cazzalini, A.I. Scovassi, M. Savio, L.A. Stivala, E. Prosperi, Multiple roles of the cell cycle inhibitor p21CDKN1A in the DNA damage response, Mutation Research/Reviews in Mutation Research, 704 (2010) 12-20.

[107] Q. Zhan, Gadd45a, a p53- and BRCA1-regulated stress protein, in cellular response to DNA damage, Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 569 (2005) 133-143.

[108] B.A. Callus, D.M. Moujallad, J. Silke, R. Gerl, A.M. Jabbour, P.G. Ekert, D.L. Vaux, Triggering of Apoptosis by Puma Is Determined by the Threshold Set by Prosurvival Bcl-2 Family Proteins, Journal of Molecular Biology, 384 (2008) 313-323.

[109] S. Maddika, S.R. Ande, S. Panigrahi, T. Paranjothy, K. Weglarczyk, A. Zuse, M. Eshraghi, K.D. Manda, E. Wiechec, M. Los, Cell survival, cell death and cell cycle pathways are interconnected: Implications for cancer therapy, Drug Resistance Updates, 10 (2007) 13-29.

[110] J. Ye, Z. Ren, Q. Gu, L. Wang, J. Wang, Ku80 is differentially expressed in human lung carcinomas and upregulated in response to irradiation in mice, DNA and cell biology, 30 (2011) 987-994.

[111] B.J. Moeller, J.S. Yordy, M.D. Williams, U. Giri, U. Raju, D.P. Molkentine, L.A. Byers, J.V. Heymach, M.D. Story, J.J. Lee, E.M. Sturgis, R.S. Weber, A.S. Garden, K.K. Ang, D.L. Schwartz, DNA repair biomarker profiling of head and neck cancer: Ku80 expression predicts locoregional failure and death following radiotherapy, Clin Cancer Res, 17 (2011) 2035-2043.

[112] D. Zhang, H.B. Wang, K.L. Brinkman, S.X. Han, B. Xu, Strategies for targeting the DNA damage response for cancer therapeutics, Chinese journal of cancer, 31 (2012) 359-363.

[113] E. Mladenov, S. Magin, A. Soni, G. Iliakis, DNA double-strand break repair as determinant of cellular radiosensitivity to killing and target in radiation therapy, Frontiers in oncology, 3 (2013) 113.

[114] A.J. Chalmers, M. Lakshman, N. Chan, R.G. Bristow, Poly(ADP-ribose) polymerase inhibition as a model for synthetic lethality in developing radiation oncology targets, Semin Radiat Oncol, 20 (2010) 274-281.

[115] R.E. Flores-Obando, S.M. Gollin, C.C. Ragin, Polymorphisms in DNA damage response genes and head and neck cancer risk, Biomarkers : biochemical indicators of exposure, response, and susceptibility to chemicals, 15 (2010) 379-399.

[116] N. Pratesi, M. Mangoni, I. Mancini, F. Paiar, L. Simi, L. Livi, S. Cassani, M. Buglione, S. Grisanti, C. Almici, C. Polli, C. Saieva, S.M. Magrini, G. Biti, M. Pazzagli, C. Orlando, Association between single nucleotide polymorphisms in the XRCC1 and RAD51 genes and clinical radiosensitivity in head and neck cancer, Radiother Oncol, 99 (2011) 356-361.

[117] S.J. Collis, A. Tighe, S.D. Scott, S.A. Roberts, J.H. Hendry, G.P. Margison, Ribozyme minigene-mediated RAD51 down-regulation increases radiosensitivity of human prostate cancer cells, Nucleic Acids Res, 29 (2001) 1534-1538.

[118] T. Ohnishi, T. Taki, S. Hiraga, N. Arita, T. Morita, In vitro and in vivo potentiation of radiosensitivity of malignant gliomas by antisense inhibition of the RAD51 gene, Biochem Biophys Res Commun, 245 (1998) 319-324.

[119] C.E. Tambini, K.G. Spink, C.J. Ross, M.A. Hill, J. Thacker, The importance of XRCC2 in RAD51-related DNA damage repair, DNA Repair, 9 (2010) 517-525.

[120] X.L. Du, T. Jiang, Z.Q. Wen, R. Gao, M. Cui, F. Wang, Silencing of heat shock protein 70 expression enhances radiotherapy efficacy and inhibits cell invasion in endometrial cancer cell line, Croat Med J, 50 (2009) 143-150.

[121] P. Niu, L. Liu, Z. Gong, H. Tan, F. Wang, J. Yuan, Y. Feng, Q. Wei, R.M. Tanguay, T. Wu, Overexpressed heat shock protein 70 protects cells against DNA

damage caused by ultraviolet C in a dose-dependent manner, Cell Stress Chaperones, 11 (2006) 162-169.

[122] Q. Zhang, S. Zhai, L. Li, X. Li, H. Zhou, A. Liu, G. Su, Q. Mu, Y. Du, B. Yan, Anti-tumor selectivity of a novel Tubulin and HSP90 dual-targeting inhibitor in non-small cell lung cancer models, Biochemical Pharmacology, 86 (2013) 351-360.

[123] F. Zagouri, T.N. Sergentanis, D. Chrysikos, C.A. Papadimitriou, M.-A. Dimopoulos, T. Psaltopoulou, Hsp90 inhibitors in breast cancer: A systematic review, The Breast, 22 (2013) 569-578.

[124] M.M. Centenera, A.K. Fitzpatrick, W.D. Tilley, L.M. Butler, Hsp90: Still a viable target in prostate cancer, Biochimica et Biophysica Acta (BBA) - Reviews on Cancer, 1835 (2013) 211-218.

[125] D. Gius, Redox-sensitive signaling factors and antioxidants: how tumor cells respond to ionizing radiation, The Journal of nutrition, 134 (2004) 3213S-3214S.

[126] H.C. Lee, D.W. Kim, K.Y. Jung, I.C. Park, M.J. Park, M.S. Kim, S.H. Woo, C.H. Rhee, H. Yoo, S.H. Lee, S.I. Hong, Increased expression of antioxidant enzymes in radioresistant variant from U251 human glioblastoma cell line, International journal of molecular medicine, 13 (2004) 883-887.

[127] P. Pettazzoni, E. Ciamporcero, C. Medana, S. Pizzimenti, F. Dal Bello, V.G. Minero, C. Toaldo, R. Minelli, K. Uchida, M.U. Dianzani, R. Pili, G. Barrera, Nuclear factor erythroid 2-related factor-2 activity controls 4-hydroxynonenal metabolism and activity in prostate cancer cells, Free Radic Biol Med, 51 (2011) 1610-1618.

[128] G. Stevens, M. Joiner, B. Joiner, H. Johns, J. Denekamp, Radiosensitization of Mouse Skin by Oxygen and Depletion of Glutathione, Int J Radiat Oncol, 33 (1995) 399-408.

[129] T. Nguyen, P. Nioi, C.B. Pickett, The Nrf2-antioxidant response element signaling pathway and its activation by oxidative stress, J Biol Chem, 284 (2009) 13291-13295.

[130] N. Wakabayashi, S.L. Slocum, J.J. Skoko, S. Shin, T.W. Kensler, When NRF2 talks, who's listening?, Antioxidants & redox signaling, 13 (2010) 1649-1663.

[131] P. Zhang, A. Singh, S. Yegnasubramanian, D. Esopi, P. Kombairaju, M. Bodas, H. Wu, S.G. Bova, S. Biswal, Loss of Kelch-like ECH-associated protein 1 function in prostate cancer cells causes chemoresistance and radioresistance and promotes tumor growth, Mol Cancer Ther, 9 (2010) 336-346.

[132] A. Singh, M. Bodas, N. Wakabayashi, F. Bunz, S. Biswal, Gain of Nrf2 function in non-small-cell lung cancer cells confers radioresistance, Antioxidants & redox signaling, 13 (2010) 1627-1637.

[133] T. Shibata, A. Kokubu, S. Saito, M. Narisawa-Saito, H. Sasaki, K. Aoyagi, Y. Yoshimatsu, Y. Tachimori, R. Kushima, T. Kiyono, M. Yamamoto, NRF2 mutation confers malignant potential and resistance to chemoradiation therapy in advanced esophageal squamous cancer, Neoplasia, 13 (2011) 864-873.

[134] K.J. Niermann, L. Moretti, N.J. Giacalone, Y. Sun, S.M. Schleicher, P. Kopsombut, L.R. Mitchell, K.W. Kim, B. Lu, Enhanced radiosensitivity of androgen-resistant prostate cancer: AZD1152-mediated Aurora kinase B inhibition, Radiat Res, 175 (2011) 444-451.

[135] N.A.P. Franken, H.M. Rodermond, J. Stap, J. Haveman, C. van Bree, Clonogenic assay of cells in vitro, Nature Protocols, 1 (2006) 2315-2319.

[136] S. Jayakumar, H.N. Bhilwade, B.N. Pandey, S.K. Sandur, R.C. Chaubey, The potential value of the neutral comet assay and the expression of genes associated with DNA damage in assessing the radiosensitivity of tumor cells, Mutation Research/Genetic Toxicology and Environmental Mutagenesis.

[137] S.K. Sandur, H. Ichikawa, G. Sethi, K.S. Ahn, B.B. Aggarwal, Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) suppresses NF-kappaB activation and NF-kappaB-regulated gene products through modulation of p65 and IkappaBalpha kinase activation, leading to potentiation of apoptosis induced by cytokine and chemotherapeutic agents, J Biol Chem, 281 (2006) 17023-17033.

[138] B.N. Pandey, K.P. Mishra, Modification of thymocytes membrane radiooxidative damage and apoptosis by eugenol, J Environ Pathol Toxicol Oncol, 23 (2004) 117-122.

[139] B. Kumar, A. Kumar, S. Ghosh, B.N. Pandey, K.P. Mishra, B. Hazra, Diospyrin derivative, an anticancer quinonoid, regulates apoptosis at endoplasmic reticulum as well as mitochondria by modulating cytosolic calcium in human breast carcinoma cells, Biochem Biophys Res Commun, 417 (2012) 903-909.

[140] I. Rahman, A. Kode, S.K. Biswas, Assay for quantitative determination of glutathione and glutathione disulfide levels using enzymatic recycling method, Nat Protoc, 1 (2006) 3159-3165.

[141] X.J. Wang, J.D. Hayes, C.J. Henderson, C.R. Wolf, Identification of retinoic acid as an inhibitor of transcription factor Nrf2 through activation of retinoic acid receptor alpha, Proc Natl Acad Sci U S A, 104 (2007) 19589-19594.

[142] C.S. Simionatto, K.E. Anderson, G.S. Drummond, A. Kappas, Studies on the mechanism of Sn-protoporphyrin suppression of hyperbilirubinemia. Inhibition of heme oxidation and bilirubin production, J Clin Invest, 75 (1985) 513-521.

[143] S. Singh, D. Chitkara, R. Mehrazin, S.W. Behrman, R.W. Wake, R.I. Mahato, Chemoresistance in Prostate Cancer Cells Is Regulated by miRNAs and Hedgehog Pathway, PLoS One, 7 (2012) e40021.

[144] J.T. Lee, Jr., L.S. Steelman, J.A. McCubrey, Phosphatidylinositol 3'-kinase activation leads to multidrug resistance protein-1 expression and subsequent chemoresistance in advanced prostate cancer cells, Cancer Res, 64 (2004) 8397-8404.

[145] J. Sun, Y. Chen, M. Li, Z. Ge, Role of antioxidant enzymes on ionizing radiation resistance, Free Radic Biol Med, 24 (1998) 586-593.

[146] E.A. Bump, J.M. Brown, Role of glutathione in the radiation response of mammalian cells in vitro and in vivo, Pharmacology & therapeutics, 47 (1990) 117-136.

[147] N. Mirkovic, D.W. Voehringer, M.D. Story, D.J. McConkey, T.J. McDonnell, R.E. Meyn, Resistance to radiation-induced apoptosis in Bcl-2-expressing cells is reversed by depleting cellular thiols, Oncogene, 15 (1997) 1461-1470.

[148] R. Brigelius-Flohe, L. Flohe, Basic principles and emerging concepts in the redox control of transcription factors, Antioxidants & redox signaling, 15 (2011) 2335-2381.

[149] M. Tsukimoto, N. Tamaishi, T. Homma, S. Kojima, Low-dose gamma-ray irradiation induces translocation of Nrf2 into nuclear in mouse macrophage RAW264.7 cells, Journal of radiation research, 51 (2010) 349-353.

[150] T.W. Kensler, N. Wakabayashi, S. Biswal, Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway, Annual review of pharmacology and toxicology, 47 (2007) 89-116.

[151] M. Tanito, M.P. Agbaga, R.E. Anderson, Upregulation of thioredoxin system via Nrf2-antioxidant responsive element pathway in adaptive-retinal neuroprotection in vivo and in vitro, Free Radic Biol Med, 42 (2007) 1838-1850.

[152] G.L. Semenza, Targeting HIF-1 for cancer therapy, Nature reviews. Cancer, 3 (2003) 721-732.

[153] B.J. Moeller, Y. Cao, C.Y. Li, M.W. Dewhirst, Radiation activates HIF-1 to regulate vascular radiosensitivity in tumors: role of reoxygenation, free radicals, and stress granules, Cancer cell, 5 (2004) 429-441.

[154] T.H. Kim, E.G. Hur, S.J. Kang, J.A. Kim, D. Thapa, Y.M. Lee, S.K. Ku, Y. Jung, M.K. Kwak, NRF2 blockade suppresses colon tumor angiogenesis by inhibiting hypoxia-induced activation of HIF-1alpha, Cancer Res, 71 (2011) 2260-2275.

Mutation Research 748 (2012) 52–59

Contents lists available at SciVerse ScienceDirect

Mutation Research/Genetic Toxicology and Environmental Mutagenesis

journal homepage: www.elsevier.com/locate/gentox
Community address: www.elsevier.com/locate/mutres

The potential value of the neutral comet assay and the expression of genes associated with DNA damage in assessing the radiosensitivity of tumor cells

Sundarraj Jayakumar, Hari N. Bhilwade, Badri N. Pandey, Santosh K. Sandur, Ramesh C. Chaubey*

Radiation Biology and Health Sciences Division, Bhabha Atomic Research Centre, Mumbai 400 085, India

ARTICLE INFO

Article history:
Received 16 February 2012
Received in revised form 27 June 2012
Accepted 30 June 2012
Available online 9 July 2012

Keywords:
Ionizing radiation
Comet assay
Radiosensitivity
Predictive assay
DNA damage

ABSTRACT

The assessment of tumor radiosensitivity would be particularly useful in optimizing the radiation dose during radiotherapy. Therefore, the degree of correlation between radiation-induced DNA damage, as measured by the alkaline and the neutral comet assays, and the clonogenic survival of different human tumor cells was studied. Further, tumor radiosensitivity was compared with the expression of genes associated with the cellular response to radiation damage. Five different human tumor cell lines were chosen and the radiosensitivity of these cells was established by clonogenic assay. Alkaline and neutral comet assays were performed in γ-irradiated cells (2–8 Gy; either acute or fractionated). Quantitative PCR was performed to evaluate the expression of DNA damage response genes in control and irradiated cells. The relative radiosensitivity of the cell lines assessed by the extent of DNA damage (neutral comet assay) immediately after irradiation (4 Gy or 6 Gy) was in agreement with radiosensitivity pattern obtained by the clonogenic assay. The survival fraction of irradiated cells showed a better correlation with the magnitude of DNA damage measured by the neutral comet assay ($r = -0.9$; $P < 0.05$; 6 Gy) than evaluated by alkaline comet assay ($r = -0.73$; $P < 0.05$; 6 Gy). Further, a significant correlation between the clonogenic survival and DNA damage was observed in cells exposed to fractionated doses of radiation. Of 15 genes investigated in the gene expression study, *HSP70*, *KU80* and *RAD51* all showed significant positive correlations ($r = 0.9$; $P < 0.05$) with tumor radiosensitivity. Our study clearly demonstrated that the neutral comet assay was better than alkaline comet assay for assessment of radiosensitivities of tumor cells after acute or fractionated doses of irradiation.

© 2012 Elsevier B.V. All rights reserved.

1. Introduction

Radiotherapy is one of the common treatment modalities for cancer. However, owing to the difference in intrinsic radiosensitivity of the different tumor types and individual genetic makeup, a significant variation in therapeutic response is observed during radiotherapy [1], leading to ineffective killing of tumor cells and occasional adverse effects in normal tissues. Hence, an optimization of radiation dose in clinical practice based on the radiosensitivity of individual patients and tumor types is of paramount importance.

The clonogenic assay is the gold standard technique in determining the cellular radiosensitivity [2]. However, clinical usage of the technique is limited because of the time required to develop colonies and low plating efficiency of many tumor biopsies [3]. Moreover, it is difficult and laborious to establish cultures from many of the tumor biopsies. Therefore, there is a need to develop biological markers or assays that are faster and can be more readily applied to different tumor types. Towards this objective, different cellular and molecular assays have been explored with varying degrees of success [4–8]. DNA is considered as a primary target of ionizing radiation, an estimation of the DNA damage would seem a likely surrogate marker for radiosensitivity. The comet assay, which is widely used to quantify DNA damage after exposure to various genotoxic agents [9], can be a fast and reliable assay system for determining cellular radiosensitivity. Attempts have been made in the past to correlate DNA damage with the clonogenic survival of the tumor cells using either an alkaline or a neutral comet assay. A good correlation between DNA damage, as measured by the comet assay and clonogenic survival has been observed in some studies [10–12]. However, this correlation was not observed in other studies [13,14], suggesting for more number of studies in this direction for the potential application of the comet assay in determining the radiosensitivity of tumor cells. Moreover, in previous reports there is also lack of consistency in the comet assay parameters tested (initial DNA damage, DNA repair kinetics, residual damage, etc.), and those show correlations with the clonogenic survival. Investigators have used only the alkaline comet assay or the neutral comet assay but have not performed side-by-side comparison of both assays. These assays have not been compared with the clonogenic assay after fractionated doses of

* Corresponding author. Tel.: +91 22 25590417; fax: +91 22 25505151.
E-mail address: rcchaubey@yahoo.com (R.C. Chaubey).

1383-5718/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.mrgentox.2012.06.008

radiation, which more closely matches with the clinical conditions. Hence, in the present study, alkaline and neutral comet assays were evaluated for their usefulness in assessing radiosensitivity by correlating various comet assay parameters with clonogenic survival in different tumor cell lines. This comparison was performed in tumor cells after acute and fractionated doses of radiation. Additionally, the radiation-induced changes in the expression of genes involved in radiation response pathways was measured using quantitative PCR and correlated with the survival fraction to understand the predictive validity of these genes in assessing the intrinsic radiosensitivity of tumor cells.

2. Materials and methods

2.1. Chemicals

Dulbecco's Modified Eagles Medium (DMEM), antibiotics (streptomycin and penicillin), sodium bicarbonate, crystal violet, Tris–HCl, ethylenediaminetetraacetic acid (EDTA), sodium chloride (NaCl), dimethyl sulfoxide (DMSO) and diethyl pyrocarbonate (DEPC) were purchased from Sigma (Missouri, USA). Fetal bovine serum (FBS) and trypsin–EDTA were obtained from Himedia (Mumbai, India). Sodium hydroxide, boric acid and methanol were purchased from Sisco fine chemicals (Mumbai, India).

2.2. Cell lines and irradiation

Human fibrosarcoma (HT1080), human colon carcinoma (HT29), human mammary carcinoma (MCF7 and T47D) and human lung adenocarcinoma (A549) cell lines were procured from the National Centre for Cell Science (Pune, India). These cells were maintained as exponentially growing monolayers in DMEM supplemented with 10% FBS, streptomycin (0.1 mg/ml) and penicillin (100 U/ml) in a humidified incubator maintained at 37 °C and 5% CO_2.

The cells were irradiated (dose rate: 1 Gy/min) using a ^{60}Co teletherapy machine (Panacea Medical Technologies, Bangalore, India). For the comet assay, exponentially growing cells were harvested by trypsinization. The trypsinized cells were suspended in complete medium followed by irradiation at 4 °C. After irradiation, the samples to be processed immediately after irradiation (0 min) were kept on ice, while those samples for use in repair kinetics studies were incubated at 37 °C for various time intervals (15, 30, 60 and 120 min). The fractionated γ-irradiation conditions involved the exposure of cells to either 2 × 2 Gy (4 Gy) or 3 × 2 Gy (6 Gy), with a 2 h time interval between successive doses. For RNA isolation, the exponentially growing cells (2×10^6 cells) were seeded overnight in a culture dish and then irradiated (either 2 or 6 Gy) and cultured for 4 h before RNA isolation.

2.3. The clonogenic assay

For the clonogenic assay, appropriate cell number required for seeding was standardized based on the plating efficiency and the number of colonies that developed after different doses of radiation. Cells were seeded on 60 mm dishes and allowed to adhere overnight in culture conditions. These cultures were exposed to various doses of γ-radiation (0.5, 1, 2, 4, 6, 8 and 10 Gy). After 15–20 days, the dishes were washed with PBS, fixed with methanol, stained using 0.5% crystal violet and then rinsed with tap water. The colonies were counted using a stereo microscope. A colony was considered when there were at least 50 or more cells. The survival fraction was calculated using the following formula:

$$\text{Survival fraction} = \frac{\text{no. of colonies}}{\text{no. of cells plated} \times (\text{plating efficiency}/100)}.$$

2.4. The comet assay

To measure DNA damage, both alkaline comet assay and neutral comet assays were performed. The alkaline comet assay was used to quantify the total DNA strand breaks, as previously mentioned [15]. Briefly, the control and the irradiated cells were suspended in 0.8% low melting agarose and were layered onto frosted slides, which were pre-coated with 1% normal agarose. After solidification, the slides were kept in lysis buffer at 4 °C for 60 min. For equilibration, the slides were transferred to an electrophoretic tank containing alkaline solution (300 mM NaOH, 1 mM EDTA, pH 13.0) for 20 min. The slides were then electrophoresed in the same buffer for 30 min at 0.8 V/cm. After electrophoresis, the slides were neutralized (0.4 M Tris–HCl), stained with 1× SYBR Green II dye and visualized at 40× magnification using a fluorescence microscope (Axioplan, Carl-Zeiss, Germany). For every treatment, two slides were prepared and at least 50 images were taken per slide. The images were analyzed using CASP software (www.casplab.com) [15] to obtain the percentage of DNA within the tail, which is considered to be the best parameter for representing DNA damage in a comet assay [16]. A neutral comet assay was performed as previously described [17]. The assay procedure is essentially the same as the alkaline comet assay, with the exception that the equilibration and electrophoresis steps were performed in a 0.5× Tris–borate EDTA buffer.

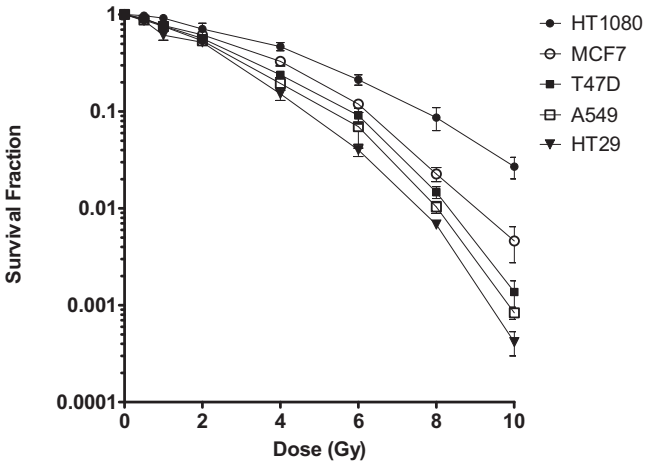


Fig. 1. A survival curve for the HT1080, MCF7, T47D, A549 and HT29 cell lines, as determined by clonogenic assay. Exponentially growing cells were plated and irradiated, following which a clonogenic assay was performed, from which the mean ± SEM (standard error of means) was plotted. Three independent experiments were performed with 5 replicates in each experiment.

2.5. Quantitative real time RT-PCR

To quantify the mRNA expression of the relevant genes, quantitative real time reverse transcriptase polymerase chain reaction (quantitative PCR) was used [18]. The total RNA was isolated using a TRI reagent (Sigma, St. Louis, MO, USA) according to the manufacturer's instructions. The RNA was suspended in 50 μl of deionized DEPC-treated water and 2 μg of total RNA was used for the synthesis of cDNA by reverse transcription (cDNA synthesis kit, Sigma). The PCRs were composed of 10× SYBR green PCR mix with 5 μl of twice-diluted cDNA templates, 1 μl each of the forward and the reverse primers (0.5 μM; Supplementary Table S1), and 3 μl of PCR-grade water in a 20 μl reaction mixture. The above reaction mixtures were amplified with a denaturation step at 95 °C for 5 min and 40 cycles of amplification including 95 °C for 15 s, 57 °C for 15 s and 72 °C for 20 s, followed by a melting curve analysis on a Rotor Gene 3000 (Corbett Life Science, Australia). The specificity of the respective amplicons was confirmed from the melting curve analysis. The amplification of each gene was performed in triplicate on two biological replicates. The threshold cycle values obtained from the runs were used for calculating the fold change in gene expression using REST-384 version 2 software [19]. The expressions of the genes were normalized to a housekeeping gene, β-actin and the relative change in the expression was plotted with respect to the control group.

2.6. Statistical analysis

Wherever required, statistical analysis was performed and the correlation coefficient was calculated using Graphpad Prism 5.0 software (La Jolla, USA). The significance of the correlation coefficient was also calculated by Graphpad Prism using the formula $t = r\sqrt{(n-2)}/(1-r^2)$, where r = correlation coefficient. A Student's t-test was used for comparison of the means of the two groups. Values were considered to be significantly different at $P < 0.05$.

3. Results

3.1. The clonogenic survival of tumor cell lines

To assess the radiosensitivity of the different tumor cell lines, a clonogenic assay was performed and the survival fraction was plotted (Fig. 1). From the survival curve, SF_2 (survival fraction at 2 Gy), D_0 (the dose yielding 37% survival) and D_{10} (the dose required to kill 90% of the cells) were calculated (Supplementary Table S2). The SF_2 value for HT1080 cells was 0.71 and for HT29 cells was 0.52. Similar to the SF_2 values, the D_0 and the D_{10} values were highest for HT1080 cells and lowest for HT29 cells. In other cell lines, these clonogenic assay parameters showed a decreasing trend MCF7 > T47D > A549. These results suggest that amongst the five cell lines used in our experiments, HT1080 cells showed the highest radioresistance,

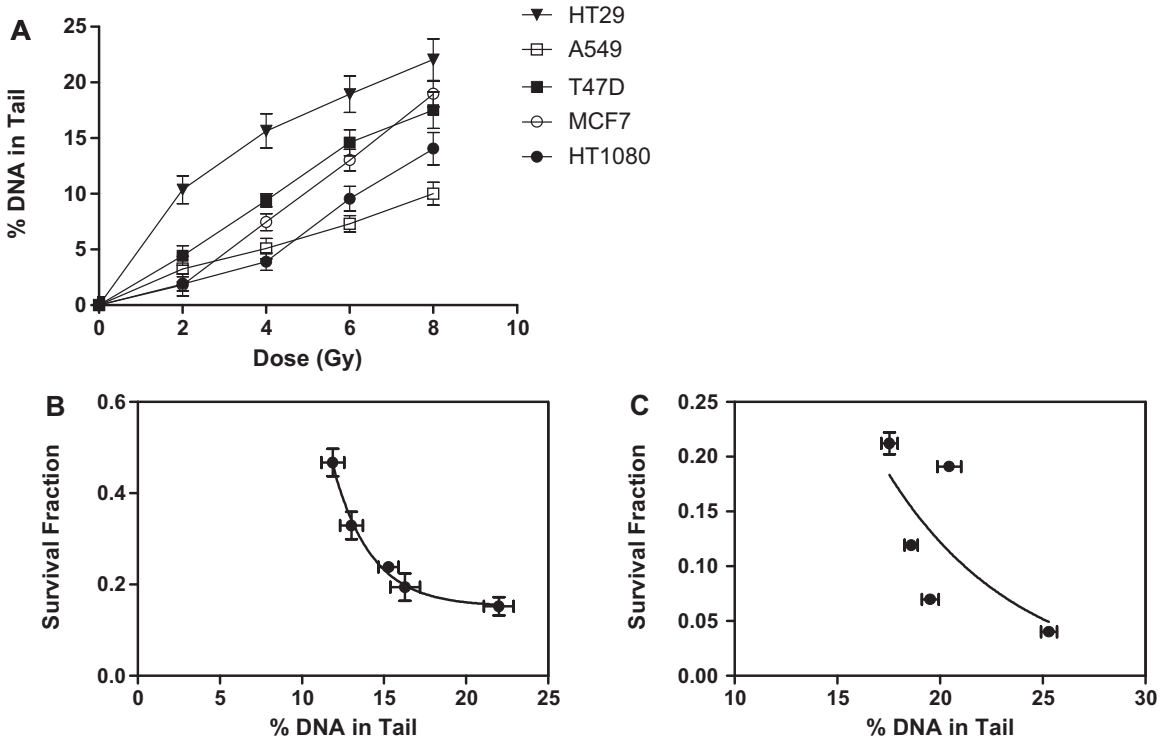


Fig. 2. DNA damage assessed by the alkaline comet assay after different doses of radiation (A), along with its correlation with clonogenic survival at 4 Gy (B) and 6 Gy (C). Exponentially growing cells were harvested and irradiated (2, 4, 6 and 8 Gy) at 4 °C. Immediately after irradiation, an alkaline comet assay was performed as described in Section 2. From the alkaline comet assay, at least 50 cells per slide were microscopically grabbed and analyzed for DNA damage. Two slides were prepared from every treatment, and the mean ± SEM of three independent experiments was plotted. Correlation analysis between DNA damage and survival fraction was performed by taking the values from survival curves (Fig. 1) at each dose.

followed by the MCF7 cells, the T47D cells, the A549 cells and the HT29 cells.

3.2. The correlation between the clonogenic survival and total DNA strand breaks in the irradiated tumor cells

To determine the correlation between the total DNA damage and the clonogenic assay results, an alkaline comet assay was performed on the irradiated tumor cell lines. γ-Irradiated tumor cells showed a higher percentage of DNA in the tail when compared to the controls. The percentage of DNA in the tail was found to be the highest in the HT29 cells, as compared to other cell lines, suggesting that the DNA damage was highest in the most radiosensitive HT29 cell line (Fig. 2A). In the other cell lines, the DNA damage profiles measured in terms of the percentage of DNA in the tail were not significantly different from each other, although the HT1080 cells and the MCF7 cells showed less DNA damage than the T47D cells and the A549 cells at some of the doses. The correlation between the total DNA damage after γ-irradiation and the clonogenic survival of each of the tumor cell lines at corresponding doses was calculated. A negative correlation was observed between these two parameters and 4 Gy ($r = -0.85$; Fig. 2B) was found to be more strongly correlating with survival than 6 Gy ($r = -0.73$; Fig. 2C). However, these correlation coefficients were not statistically significant. Apart from the initial DNA damage, we also analyzed the repair kinetics at 2, 4, 6 and 8 Gy for up to 2 h (Supplementary Fig. S1A–D) in the different cancer cell lines; among them, the HT1080 cells showed faster repair kinetics and less residual DNA damage after 2 h than the other tumor cells. However, neither the repair kinetics nor the residual DNA damage, as measured by the alkaline comet assay, could grade the tumor cells according to their radiosensitivity.

3.3. The correlation between the DNA damage observed by a neutral comet assay and the survival fraction in irradiated tumor cells

As there was no significant correlation between the total DNA strand breaks and clonogenic survival, further comparisons using the neutral comet assay and clonogenic survival after different radiation doses were undertaken. All the cell types showed a dose-dependent increase in DNA damage, as reflected by an increase in the percentage of DNA in the tail obtained from neutral comet assay (Fig. 3A). The HT1080 cells showed the least DNA damage, followed by the MCF7 cells, the T47D cells, the A549 cells and the HT29 cells when measured immediately after irradiation. However, it was interesting to observe that each tumor cell line used in our study showed a characteristic dose response pattern. From the DNA damage repair analysis, which was performed by measuring the DNA damage after different time intervals (15, 30, 45, 60 and 120 min) at every dose (Supplementary Fig. 2A–D), the HT1080 cells showed a faster recovery and less residual damage. The other tumor cell lines did not show any significant differences in these parameters.

To establish the value of the neutral comet assay in determining the radiosensitivity of tumor cells, the correlation between the initial DNA damage and the clonogenic survival fraction was analyzed. At all the doses, we observed that the DNA damage and survival fraction displayed a significant negative correlation, with the exception of 2 Gy ($r = -0.7$). The initial DNA damage observed after 4 Gy (Fig. 3B) or 6 Gy (Fig. 3C) was correlated ($r = -0.9$) with the survival fraction at the respective doses and was statistically significant ($P < 0.05$). There was no significant correlation observed between the residual DNA damage and radiosensitivity.

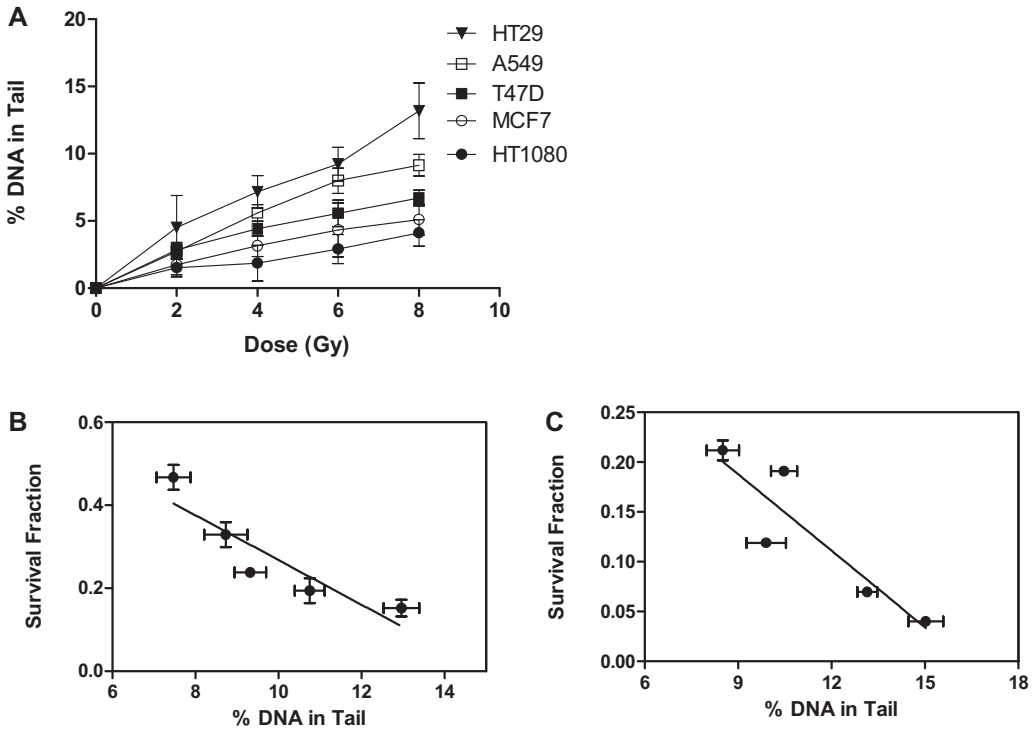


Fig. 3. DNA damage assessed by the neutral comet assay after different doses of γ-radiation (A) and the correlation with clonogenic survival at 4 Gy (B) and 6 Gy (C). Exponentially growing cells were harvested and irradiated (2, 4, 6 and 8 Gy) at 4 °C. Immediately after irradiation, a neutral comet assay was performed as described in Section 2. From the neutral comet assay, at least 50 cells were microscopically grabbed and analyzed for DNA damage. Two slides were prepared from every treatment and the mean ± SEM of three independent experiments was plotted. Correlation analysis between DNA damage and survival fraction was performed by taking the values from survival curves (Fig. 1) at each dose.

3.4. The correlation between the clonogenic assay and comet assay after fractionated doses of radiation exposure

Fractionated doses are employed during cancer radiotherapy; hence, we also examined whether the radiosensitivity of tumor cells could be assessed by comet assay after fractionated doses of radiation. The magnitude of DNA damage was determined by both the alkaline and the neutral comet assay after irradiating the cells with fractionated doses of radiation (2 × 2 Gy or 3 × 2 Gy; interval of 2 h between the fractions). A clonogenic assay was performed after fractionated radiation dose exposure and graded the cell lines according to their sensitivity and this order matched with the corresponding acute doses (Fig. 4A). At both fractionated doses, the HT1080 cells had the highest survival fraction, followed by the MCF7 cells, the T47D cells, the A549 cells and the HT29 cells. Interestingly, the radiosensitivities evaluated by the extent of DNA damage observed by the alkaline and the neutral comet assays after the fractionated radiation doses were in the same order as that obtained by the clonogenic assay. A statistically significant correlation was observed between the DNA damage, as assessed by the neutral comet assay and the clonogenic survival after fractionated doses of radiation (Fig. 4D and E) with r values of −0.97 and −0.93 for 2 × 2 Gy and 3 × 2 Gy, respectively. In agreement with the results of acute doses, the total DNA damage, as measured by the alkaline comet assay after fractionated doses, did not show significant correlation with the respective survival fraction (Fig. 4B and C). The observed r values were −0.81 and −0.73 for 2 × 2 Gy and 3 × 2 Gy, respectively. Our results clearly demonstrate that the neutral comet assay can assess the radiosensitivity of tumor cells, after both acute and fractionated doses.

3.5. The correlation between radiosensitivity and gene expression

The cellular radiosensitivity depends on alterations in the expression of genes involved in DNA damage repair and the subsequent regulation of pro- and anti-survival signaling pathways [20]. We have investigated the expression profile of 15 genes involved in the crucial pathways of DNA damage response like repair, apoptosis, and redox regulation. The expression patterns of *MCL1*, *BCL2*, *KU80*, *RAD51*, *NRF2*, *NQO1*, *GCLC*, *HSP70*, *FAS*, *PUMA*, *CDKN1A*, *HO1*, *SNAIL*, *GADD45A* and *MDM2* in HT1080 cells, MCF7 cells, A549 cells and HT29 cells were analyzed by quantitative PCR 4 h after irradiation with either 2 Gy or 6 Gy. The fold change in expression was calculated for each gene and correlated with the clonogenic survival of the cell line. After irradiation (2 and 6 Gy), expression levels of genes examined in our study varied differently in the different cell lines (Supplementary Figs. S3 and S4). Of the 15 genes analyzed, only three genes (*HSP70*, *KU80* and *RAD51*) showed an expression pattern that was related to the radiosensitivity of the cell line. There was a higher expression of *HSP70*, *KU80* and *RAD51* in the radio-resistant cell line HT1080 (a 2.07, 2.81 and 4.9 fold increase respectively at 6 Gy) than in the radiosensitive cell line HT29 (1.04, 1.43 and 1.64 respectively at 6 Gy) (Fig. 5A–C). A similar trend was also observed at 2 Gy; however, the magnitude of the fold increase was lower. The fold increase in expression of these genes in the other two cell lines was also proportional to their radiosensitivity, as assessed by the clonogenic assay. The expression of these three genes showed a significant positive correlation with survival fraction. The r values observed were 0.97, 0.99 and 0.97 for *HSP70*, *KU80* and *RAD51*, respectively ($P < 0.05$; Fig. 5D–F). The other genes did not exhibit a significant correlation with clonogenic survival at either 2 Gy or 6 Gy (Table 1).

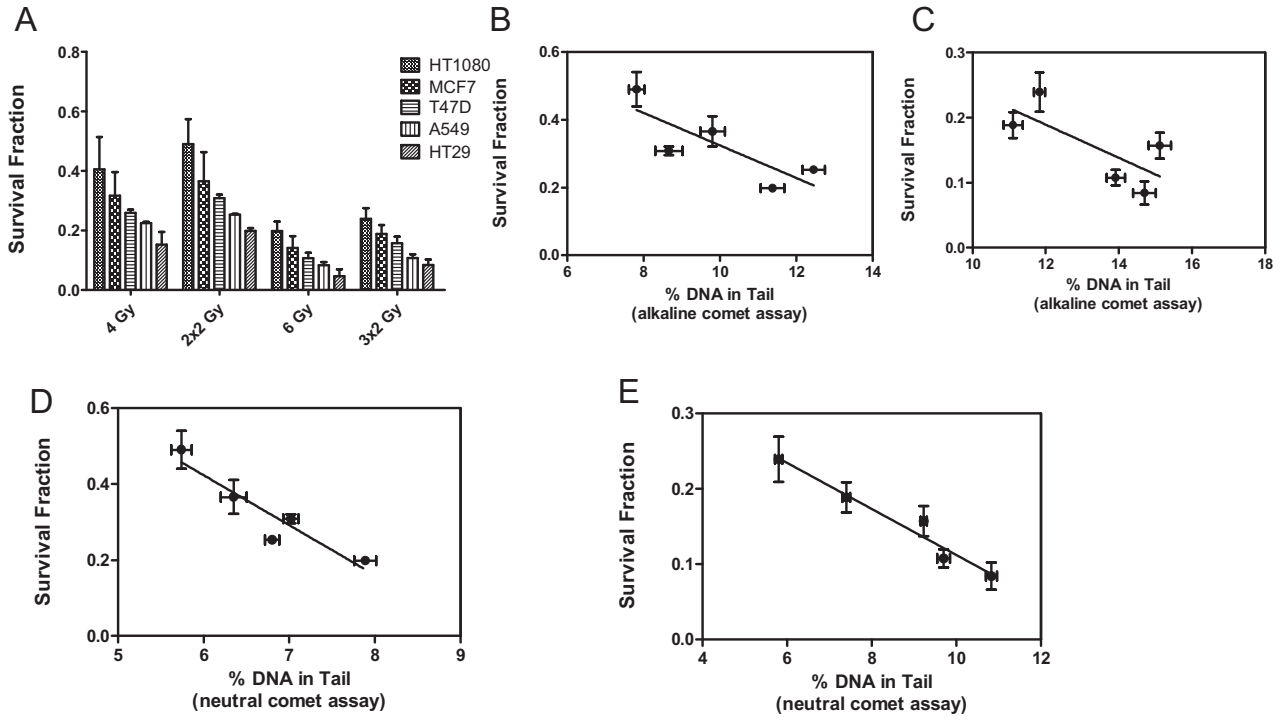


Fig. 4. Survival fraction obtained by exposing the cells to either an acute dose of 4 Gy/6 Gy or a fractionated dose 2 × 2 Gy/3 × 2 Gy of γ-radiation (A) and the correlation with DNA damage, as obtained by the alkaline comet assay after exposure to either 2 × 2 Gy (B) or 3 × 2 Gy (C) or with the DNA damage obtained by a neutral comet assay after exposure to either 2 × 2 Gy (D) or 3 × 2 Gy (E). The survival fraction was calculated by a clonogenic assay. The values represent the mean ± SEM derived from three independent experiments. For the comet assay, exponentially growing cells were harvested and exposed to a fractionated dose (2 Gy), with a 2 h interval between each fraction, before processing for the alkaline or the neutral comet assay.

Apart from correlating the gene expression with the clonogenic survival fraction, we performed a correlation analysis between DNA damage, as assessed by the neutral comet assay, and the relative gene expression of *HSP70*, *KU80* and *RAD51*. Expression of all three genes after radiation exposure (6 Gy) showed a significant correlation with the DNA damage, as indicated by the neutral comet assay (Supplementary Fig. S5A–C). The correlation coefficients for *HSP70*, *KU80* and

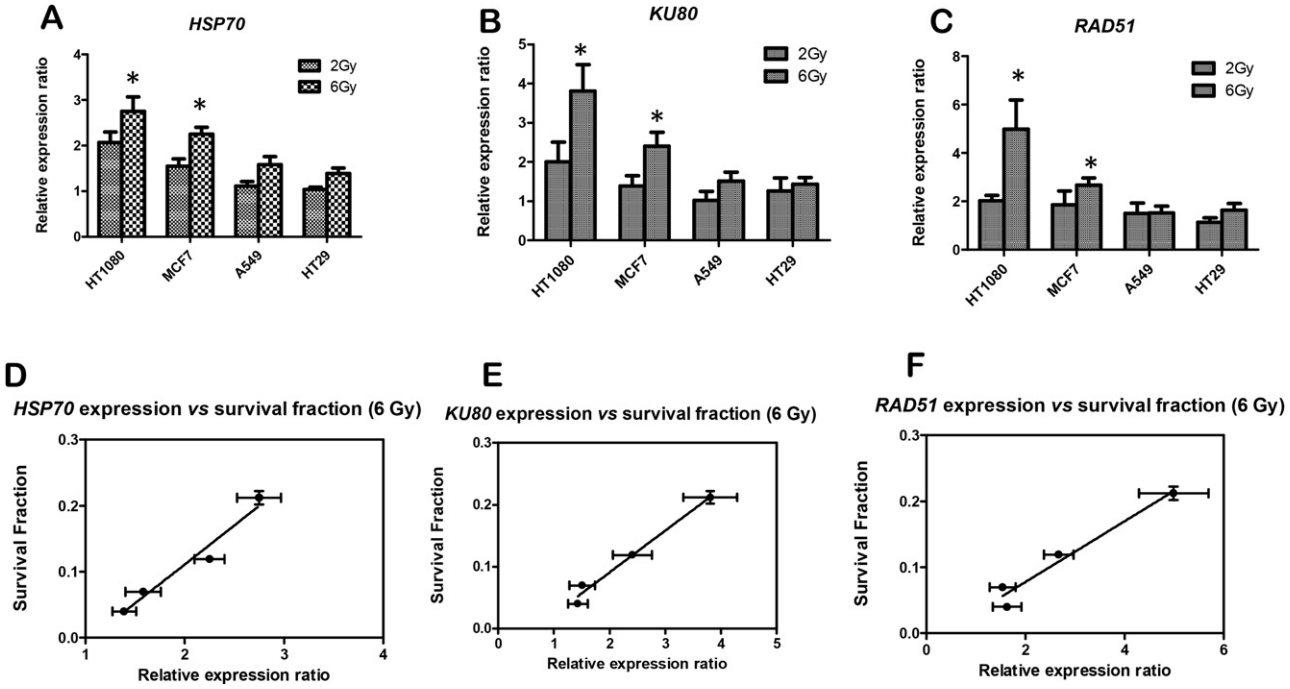


Fig. 5. The relative mRNA expression ratio of *HSP70* (A), *KU80* (B) and *RAD51* (C) and the correlation with cell survival fraction (D–F). Gene expression was analyzed by quantitative PCR, after exposing to 2 Gy/6 Gy of γ-irradiation. The bars represent the mean ± SEM obtained from six replicates. * Groups are significantly different in comparison to the HT29 group ($P < 0.05$). For correlation studies, the clonogenic survival values were taken from Fig. 1 and the gene expression values were taken from Fig. 5A–C.

Table 1
Correlation coefficient obtained for various genes by correlating expression with the survival fraction.

Gene	Correlation parameters			
	r-Value	95% confidence interval	*p*-Values from two sided test	*p*-Value significance
HSP70	0.97	0.31–0.99	0.02	Yes
RAD51	0.97	0.29–0.99	0.02	Yes
KU80	0.99	0.63–0.99	0.09	Yes
Cdkn1a	0.81	−0.67 to 0.99	0.18	No
GADD45A	−0.02	−0.96 to 0.95	0.98	No
MDM2	−0.6	−0.99 to 0.85	0.39	No
BCL2	−0.33	−0.98 to 0.92	0.66	No
MCL1	0.12	−0.95 to 0.96	0.87	No
PUMA	0.13	−0.94 to 0.97	0.86	No
FAS	0.48	−0.89 to 0.98	0.51	No
SNAIL	−0.67	−0.99 to 0.81	0.32	No
Nrf2	0.63	−0.83 to 0.99	0.36	No
HO1	0.23	−0.93 to 0.97	0.76	No
NQO1	−0.73	−0.99 to 0.77	0.26	No
GCLC	−0.1	−0.96 to 0.95	0.9	No

RAD51 in this analysis were −0.98, −0.91 and −0.87 respectively.

4. Discussion

Assessment of tumor radiosensitivity prior to radiotherapy will be of great help in improving the efficacy of radiotherapy. However, cellular radiosensitivity is an interplay of several factors such as tissue type, the physiological state of the cell, hypoxia [21], the anti-oxidant capacity of the cell [22], mutations present in different genes and the expression pattern of certain critical radioresponsive genes. Under these different circumstances, finding a suitable assay or parameter that will reflect the radiosensitivity is a challenging task. The DNA damage parameters may serve as a good indicator of radiosensitivity. In the present study, radiosensitivities of different tumor cell lines were established using a time consuming technique clonogenic assay and this was correlated with DNA damage assessed by comet assay and gene expression to know the predictive validity of these parameters in assessing radiosensitivity.

We selected five tumor cell lines, out of which MCF7 and T47D are breast carcinoma and other cell lines HT1080 (fibrosarcoma), A549 (lung adenocarcinoma) and HT29 (colon carcinoma) are of different tissue origins. The different cell types were used to ensure that the assay was able to distinguish the radiosensitivity across different tumor types. In the clonogenic assay, the HT1080 cells showed the highest radioresistance, followed by the MCF7 cells, the T47D cells, the A549 cells and the HT29 cells (Fig. 1). Out of these five cell lines, the T47D cells and the HT29 cells have a mutation in the *TP53* gene. *TP53* status is known to determine the cellular radiosensitivity. In present study, the T47D cells showed a higher radiosensitivity than the MCF7 cells (*TP53* wild type), which is consistent with previous clonogenic assay reports [23]. It is likely that apart from *TP53*, mutations present in other genes may determine the radiosensitivity.

The alkaline comet assay is a very widely used technique to detect total DNA strand breaks. Therefore, the results of an alkaline comet assay were compared with those of a clonogenic assay in determining the radiosensitivity of the tumor cell lines. Although the alkaline comet assay could differentiate between the most radioresistant and least radioresistant cell lines, it failed to differentiate the narrow differences in the radiosensitivities of the other cell types (Fig. 2A–C). As the alkaline comet assay measures the total stand breaks, including alkali labile sites, it may mask the critical DNA damage differences exhibited in different tumor types, thereby compromising the predictive value of the alkaline comet assay. Interestingly, the neutral comet assay was able to grade all the tumor cell lines according to their radiosensitivities, as determined by the clonogenic assay and indicated a significant correlation with the clonogenic survival (Fig. 3A–C). It was also observed that the neutral comet assay provided a stronger correlation with clonogenicity at higher doses (4 Gy and 6 Gy) than at 2 Gy, which may be attributed to a lower occurrence of strand breaks at 2 Gy. For all the cell lines, the DNA repair kinetics, as measured using either neutral or alkaline comet assay, failed to strongly correlate with the radiosensitivity, which is in agreement with some of the earlier reports [24] but not with other studies [12]. Though the residual DNA damage, as measured by the neutral comet assay, is able to segregate the most radioresistant and radiosensitive cell lines, it is not able to grade all the cell types according to their radiosensitivity. Though DNA repair capacity is one major determinant of the radiosensitivity of the cell, a comet assay may not be sensitive enough to resolve the smaller differences in the residual DNA damage between the cell lines. Therefore, the DNA damage measured right after the radiation exposure could be a better reflection of the radiosensitivity than the residual DNA damage. After acute and fractionated doses, the neutral comet assay measurements of DNA damage were negatively correlating with the clonogenicity of the tumor cells (Fig. 4D and E), suggesting that this technique may be useful at clinically relevant fractionated doses. A time gap of 2 h between the two fractions was chosen based on earlier reports [25], showing that maximum repair occurred within 2 h. We also performed an experiment to examine the difference in the survival between 2 h fractionation and 24 h fractionation and found that there was no difference between these fractionations (data not shown). Therefore, we used a 2 h interval between each fractionated dose.

Our results clearly indicate that the neutral comet assay could predict the radiosensitivity of different tumor cells, which is in line with the results of the clonogenic survival assay that is considered to be the gold standard method in assessing radiosensitivity. However, the clonogenic assay will be difficult to implement under clinical settings because of the low plating efficiency, the lack of proper colony formation and the time-consuming nature of the protocol. Using the neutral comet assay, some of these drawbacks can be overcome.

Cellular radiosensitivity is governed by the expression of genes/proteins involved in radiation response pathways, such as DNA damage, repair, oxidative mechanisms and apoptosis. Hence, in addition to the comet assay, we studied the fold change in expression of genes involved in various radiation response mechanisms for their predictive validity in radiosensitivity. Of the 15 genes studied, the expression of *HSP70*, *KU80* and *RAD51* was

positively correlated with the survival fraction of the irradiated tumor cells (Fig. 5D–F). KU80 makes a heterodimer with KU70, which binds at ends of DNA double-strand breaks and is required for the non-homologous end joining pathway of DNA repair. Our results showed the potential for *KU80* expression in the assessment of tumor radiosensitivity. This was further supported by a recent study by Moeller et al. [26], who showed a relationship between the expression of *KU80* with treatment response and mortality following radiotherapy in head and neck cancer patients. Similar to *KU80*, *RAD51* is involved in DNA double-strand break repair, but through homologous recombination. Our results show a significant correlation between the expression of *RAD51* and radiosensitivity, which is in agreement with previous studies showing a role for *RAD51* in radiosensitivity [27,28]. Even though *HSP70* is known to be involved in protein folding as chaperons, radiation is also known to induce the expression of this gene. *HSP70* and its family members are also known to interact with the proteins involved in DNA double strand breaks [29] and are implicated in radiosensitivity [30,31]. Moreover, no significant correlation was observed between radiosensitivity and the expression of *GADD45A*, a gene that is known to be involved in cell cycle arrest and repair of single-strand breaks. Expression of genes involved in other pathways, such as apoptosis and redox mechanism, which are late events after radiation exposure, did not show a significant correlation with the radiosensitivity. Further, a good correlation between the fold change of genes associated with DNA damage and cellular radiosensitivity may be due to the fact that DNA is a primary target of radiation damage and is involved in the regulation of subsequent cellular processes. However, the relationship between the expression of other genes and radiosensitivity needs further systematic investigation.

In conclusion, our studies emphasize the usefulness of the neutral comet assay in the assessment of the radiosensitivity of tumor cells as compared to the alkaline comet assay. In line with this observation, the expression of genes involved in the repair of DNA double strand breaks were also shown to be useful in determining the radiosensitivity of tumor cells. However, to gain greater confidence in use of these techniques in determining the radiosensitivity of tumor cells, a larger number of cell lines and validation in a clinical scenario using biopsy samples may be needed.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgements

The authors wish to thank Mr. Sanjay Shinde for his help in irradiation and Mr. B.A. Naidu for technical help. The authors also would like to thank the Department of Atomic Energy, India, for funding this research.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.mrgentox.2012.06.008.

References

[1] L.J. Peters, The ESTRO Regaud lecture, Inherent radiosensitivity of tumor and normal tissue cells as a predictor of human tumor response, Radiother. Oncol. 17 (1990) 177–190.
[2] C.M. West, S.E. Davidson, S.A. Roberts, R.D. Hunter, The independence of intrinsic radiosensitivity as a prognostic factor for patient response to radiotherapy of carcinoma of the cervix, Br. J. Cancer 76 (1997) 1184–1190.
[3] T. Bjork-Eriksson, C. West, E. Karlsson, C. Mercke, Tumor radiosensitivity (SF2) is a prognostic factor for local control in head and neck cancers, Int. J. Radiat. Oncol. Biol. Phys. 46 (2000) 13–19.
[4] J.F. Torres-Roca, S. Eschrich, H. Zhao, G. Bloom, J. Sung, S. McCarthy, A.B. Cantor, A. Scuto, C. Li, S. Zhang, R. Jove, T. Yeatman, Prediction of radiation sensitivity using a gene expression classifier, Cancer Res. 65 (2005) 7169–7176.
[5] P.L. Olive, J.P. Banath, Phosphorylation of histone H2AX as a measure of radiosensitivity, Int. J. Radiat. Oncol. Biol. Phys. 58 (2004) 331–335.
[6] N.E. Crompton, M. Ozsahin, A versatile and rapid assay of radiosensitivity of peripheral blood leukocytes based on DNA and surface-marker assessment of cytotoxicity, Radiat. Res. 147 (1997) 55–60.
[7] E. Severin, B. Greve, E. Pascher, N. Wedemeyer, U. Hacker-Klom, G. Silling, J. Kienast, N. Willich, W. Gohde, Evidence for predictive validity of blood assays to evaluate individual radiosensitivity, Int. J. Radiat. Oncol. Biol. Phys. 64 (2006) 242–250.
[8] S. Folkvord, K. Flatmark, S. Dueland, R. de Wijn, K.K. Groholt, K.H. Hole, J.M. Nesland, R. Ruijtenbeek, P.J. Boender, M. Johansen, K.E. Giercksky, A.H. Ree, Prediction of response to preoperative chemoradiotherapy in rectal cancer by multiplex kinase activity profiling, Int. J. Radiat. Oncol. Biol. Phys. 78 (2010) 555–562.
[9] D.J. McKenna, S.R. McKeown, V.J. McKelvey-Martin, Potential use of the comet assay in the clinical management of cancer, Mutagenesis 23 (2008) 183–190.
[10] S.R. McKeown, T. Robson, M.E. Price, E.T. Ho, D.G. Hirst, V.J. McKelvey-Martin, Potential use of the alkaline comet assay as a predictor of bladder tumour response to radiation, Br. J. Cancer 89 (2003) 2264–2270.
[11] M.A. Moneef, B.T. Sherwood, K.J. Bowman, R.C. Kockelbergh, R.P. Symonds, W.P. Steward, J.K. Mellon, G.D. Jones, Measurements using the alkaline comet assay predict bladder cancer cell radiosensitivity, Br. J. Cancer 89 (2003) 2271–2276.
[12] S.A. Nahas, R. Davies, F. Fike, K. Nakamura, L. Du, R. Kayali, N.T. Martin, P. Concannon, R.A. Gatti, Comprehensive profiling of radiosensitive human cell lines with DNA damage response assays identifies the neutral comet assay as a potential surrogate for clonogenic survival, Radiat. Res. 177 (2012) 176–186.
[13] P.L. Olive, J.P. Banath, H.S. MacPhail, Lack of a correlation between radiosensitivity and DNA double-strand break induction or rejoining in six human tumor cell lines, Cancer Res. 54 (1994) 3939–3946.
[14] W.U. Muller, T. Bauch, C. Streffer, F. Niedereichholz, W. Bocker, Comet assay studies of radiation-induced DNA damage and repair in various tumour cell lines, Int. J. Radiat. Biol. 65 (1994) 315–319.
[15] S. Jayakumar, H.N. Bhilwade, P.S. Dange, H.D. Sarma, R.C. Chaubey, B.N. Pandey, Magnitude of radiation-induced DNA damage in peripheral blood leukocytes and its correlation with aggressiveness of thymic lymphoma in Swiss mice, Int. J. Radiat. Biol. 87 (2011) 1113–1119.
[16] B. Burlinson, R.R. Tice, G. Speit, E. Agurell, S.Y. Brendler-Schwaab, A.R. Collins, P. Escobar, M. Honma, T.S. Kumaravel, M. Nakajima, Y.F. Sasaki, V. Thybaud, Y. Uno, M. Vasquez, A. Hartmann, Fourth International Workgroup on Genotoxicity testing: Results of the in vivo Comet assay workgroup, Mutat. Res. Genet. Toxicol. Environ. Mutagen. 627 (2007) 31–35.
[17] Y. Yu, W. Zhu, H. Diao, C. Zhou, F.F. Chen, J. Yang, A comparative study of using comet assay and γH2AX foci formation in the detection of N-methyl-N′-nitro-N-nitrosoguanidine-induced DNA damage, Toxicol. In Vitro 20 (2006) 959–965.
[18] A. Kunwar, S. Jayakumar, H.N. Bhilwade, P.P. Bag, H. Bhatt, R.C. Chaubey, K.I. Priyadarsini, Protective effects of selenocystine against gamma-radiation-induced genotoxicity in Swiss albino mice, Radiat. Environ. Biophys. 50 (2011) 271–280.
[19] M.W. Pfaffl, G.W. Horgan, L. Dempfle, Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR, Nucleic Acids Res. 30 (2002) e36.
[20] Y.S. Lee, J.H. Oh, S. Yoon, M.S. Kwon, C.W. Song, K.H. Kim, M.J. Cho, M.L. Mollah, Y.J. Je, Y.D. Kim, C.D. Kim, J.H. Lee, Differential gene expression profiles of radioresistant non-small-cell lung cancer cell lines established by fractionated irradiation: tumor protein p53-inducible protein 3 confers sensitivity to ionizing radiation, Int. J. Radiat. Oncol. Biol. Phys. 77 (2010) 858–866.
[21] D.J. Carlson, P.J. Keall, B.W. Loo, Z.J. Chen Jr., J.M. Brown, Hypofractionation results in reduced tumor cell kill compared to conventional fractionation for tumors with regions of hypoxia, Int. J. Radiat. Oncol. Biol. Phys. 79 (2011) 1188–1195.
[22] B. Shankar, S.S. Kumar, K.B. Sainis, Generation of reactive oxygen species and radiation response in lymphocytes and tumor cells, Radiat. Res. 160 (2003) 478–487.
[23] Y.-H. Zhong, Z.-K. Liao, F.-X. Zhou, C.-H. Xie, C.-Y. Xiao, D.-F. Pan, Z.-G. Luo, S.-Q. Liu, Y.-F. Zhou, Telomere length inversely correlates with radiosensitivity in human carcinoma cells with the same tissue background, Biochem. Biophys. Res. Commun. 367 (2008) 84–89.
[24] R.A. El-Awady, E. Dikomey, J. Dahm-Daphi, Radiosensitivity of human tumour cells is correlated with the induction but not with the repair of DNA double-strand breaks, Br. J. Cancer 89 (2003) 593–601.
[25] M.M. Elkind, H. Sutton-Gilbert, W.B. Moses, T. Alescio, R.W. Swain, Radiation response of mammalian cells grown in culture. V. Temperature dependence of the repair of X-ray damage in surviving cells (aerobic and hypoxic), Radiat. Res. 25 (1965) 359–376.
[26] B.J. Moeller, J.S. Yordy, M.D. Williams, U. Giri, U. Raju, D.P. Molkentine, L.A. Byers, J.V. Heymach, M.D. Story, J.J. Lee, E.M. Sturgis, R.S. Weber, A.S. Garden, K.K. Ang, D.L. Schwartz, DNA repair biomarker profiling of head and neck cancer: Ku80 expression predicts locoregional failure and death following radiotherapy, Clin. Cancer Res. 17 (2011) 2035–2043.
[27] S.J. Collis, A. Tighe, S.D. Scott, S.A. Roberts, J.H. Hendry, G.P. Margison, Ribozyme minigene-mediated RAD51 down-regulation increases

radiosensitivity of human prostate cancer cells, Nucleic Acids Res. 29 (2001) 1534–1538.
[28] T. Ohnishi, T. Taki, S. Hiraga, N. Arita, T. Morita, In vitro and in vivo potentiation of radiosensitivity of malignant gliomas by antisense inhibition of the RAD51 gene, Biochem. Biophys. Res. Commun. 245 (1998) 319–324.
[29] C.E. Tambini, K.G. Spink, C.J. Ross, M.A. Hill, J. Thacker, The importance of XRCC2 in RAD51-related DNA damage repair, DNA Repair 9 (2010) 517–525.
[30] X.L. Du, T. Jiang, Z.Q. Wen, R. Gao, M. Cui, F. Wang, Silencing of heat shock protein 70 expression enhances radiotherapy efficacy and inhibits cell invasion in endometrial cancer cell line, Croat. Med. J. 50 (2009) 143–150.
[31] P. Niu, L. Liu, Z. Gong, H. Tan, F. Wang, J. Yuan, Y. Feng, Q. Wei, R.M. Tanguay, T. Wu, Overexpressed heat shock protein 70 protects cells against DNA damage caused by ultraviolet C in a dose-dependent manner, Cell Stress Chaperones 11 (2006) 162–169.

Biochimica et Biophysica Acta 1840 (2014) 485–494

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

Differential response of DU145 and PC3 prostate cancer cells to ionizing radiation: Role of reactive oxygen species, GSH and Nrf2 in radiosensitivity

Sundarraj Jayakumar [a], Amit Kunwar [b], Santosh K. Sandur [a,*], Badri N. Pandey [a], Ramesh C. Chaubey [a,1]

[a] Radiation Biology and Health Sciences Division, Bhabha Atomic Research Centre, Mumbai 400 085, India
[b] Radiation and Photochemistry Division, Bhabha Atomic Research Centre, Mumbai 400 085, India

ARTICLE INFO

Article history:
Received 10 May 2013
Received in revised form 14 September 2013
Accepted 1 October 2013
Available online 9 October 2013

Keywords:
Ionizing radiation
Radiosensitivity
Prostate cancer
Nrf2
PC3
DU145

ABSTRACT

Background: Radioresistance is the major impediment in radiotherapy of many cancers including prostate cancer, necessitating the need to understand the factors contributing to radioresistance in tumor cells. In the present study, the role of cellular redox and redox sensitive transcription factor, Nrf2 in the radiosensitivity of prostate cancer cell lines PC3 and DU145, has been investigated.
Materials and methods: Differential radiosensitivity of PC3 and DU145 cells was assessed using clonogenic assay, flow cytometry, and comet assay. Their redox status was measured using DCFDA and DHR probes. Expression of Nrf2 and its dependent genes was measured by EMSA and real time PCR. Knockdown studies were done using shRNA transfection.
Results: PC3 and DU145 cells differed significantly in their radiosensitivity as observed by clonogenic survival, apoptosis and neutral comet assays. Both basal and inducible levels of ROS were higher in PC3 cells than that of DU145 cells. DU145 cells showed higher level of basal GSH content and GSH/GSSG ratio than that of PC3 cells. Further, significant increase in both basal and induced levels of Nrf2 and its dependent genes was observed in DU145 cells. Knock-down experiments and pharmacological intervention studies revealed the involvement of Nrf2 in differential radio-resistance of these cells.
Conclusion: Cellular redox status and Nrf2 levels play a causal role in radio-resistance of prostate cancer cells.
General significance: The pivotal role Nrf2 has been shown in the radioresistance of tumor cells and this study will further help in exploiting this factor in radiosensitization of other tumor cell types.
© 2013 Elsevier B.V. All rights reserved.

1. Introduction

Radiotherapy is one of the major treatment modalities for cancers. More than 60% of the cancers are treated by radiation therapy either alone or in combination with chemotherapy or surgery. But the therapeutic efficacy of radiotherapy is hindered by the difference in the intrinsic radiosensitivity of different tumor cells. Therefore, it is very important to understand about the radiosensitivity, its molecular determinants, and outcome of such studies would be useful in prediction and modulation of radiosensitivity [1].

Radiation kills the tumor cells by producing reactive oxygen species (ROS) like hydroxyl radical, hydrogen peroxide, and superoxide which cause damage to biomolecules including DNA (indirect effect) or by directly causing DNA damage (direct effect). The proportion of direct and indirect damage depends on the quality and type of radiation. Gamma radiation kills cancer cells mainly by indirect damage. Intracellular antioxidants like glutathione, thioredoxin reductase, glutathione peroxidase, catalase, superoxide dismutase, etc. form the first line of defense against ROS induced oxidative stress in the cells [2,3]. These antioxidants help cells in scavenging ROS and salvaging biomolecules from oxidative damage.

Prior studies have established that the intracellular levels of most of the above enzymatic and non-enzymatic anti-oxidants are regulated by a redox sensitive transcription factor nuclear factor-E2-related factor 2 (Nrf2) [4,5]. Under normal conditions, Nrf2 protein is bound to an inhibitor protein Kelch-like-ECH-associated protein (Keap1). This binding of Keap1 protein to Nrf2 leads to ubiquitination and degradation of Nrf2 by proteasomal pathway [6]. Under oxidative stress conditions, critical cysteine residues present on the Keap1 protein get oxidised and thereby disrupting the binding of Keap1 to Nrf2. Then Nrf2 undergoes a rapid translocation into the nucleus, binds to antioxidant response elements (ARE), which are present in the promoter regions of its target antioxidant genes such as heme oxygenase 1 (HO1), NADH quinone oxidoreductase 1 (NQO1), the glutamate cysteine ligase catalytic subunit (GCLC), and thioredoxin reductase 1 (TXRD1), and facilitates their transcription [6]. Therefore, Nrf2 and its dependent genes may play a crucial

* Corresponding author at: Radiation Biology & Health Sciences Division, Bhabha Atomic Research Centre, Mumbai 400085, India. Tel.: +91 22 25595356; fax: +91 22 25505151.
E-mail address: sskumar@barc.gov.in (S.K. Sandur).
[1] Present address: C-11-6, Kendriya Vihar, Kharghar, Navi Mumbai, India.

0304-4165/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bbagen.2013.10.006

role in determining radiosensitivity of tumor cells. Indeed few recent studies carried out in non-small-cell lung cancer [7] and esophageal squamous cancer cells [8] reporting the involvement of Nrf2 in radioresistance of tumor cells support the above hypothesis. However, such studies need to be performed under different scenarios and in different cell types in order to exploit Nrf2 as one of the targets for improving the effectiveness of radiotherapy.

Prostate cancer is one of the most commonly diagnosed non-cutaneous malignancies in men. Surgical removal, hormone ablation therapy and radiotherapy are the major treatment modalities for the prostate cancer. Radiotherapy can be used as curative treatment of clinically localized prostate cancer [9]. However, the radiation resistance has become a practical impediment to the radiotherapy of prostate cancers. Despite the significant advances in treatment modalities, prostate cancer is one of the leading causes of death due to cancer in men. Especially the treatment of androgen independent prostate cancers has become a challenging aspect. The tumors which are androgen independent also invariably show chemoresistance and radioresistance [10,11]. Therefore understanding the molecular mechanisms and the factors which determine the radioresistance of this tumor type is very important and would be helpful in improving the therapeutic efficacy of the radiotherapy of prostate cancer. Many studies have dwelled into this aspect of radioresistance of prostate cancer cells and have demonstrated the role aurora kinase B [11], P21 activated kinase-6 [12] and 12-lipoxygenase [13] in radioresistance of prostate cancer cells. Furthermore, Zhang et al. [6] observed point mutations in Keap1 protein of prostate cancer cells and found transcriptional and post-transcriptional regulation of Keap1 protein which affects the treatment response in prostate tumor cells. But the role of Nrf2 and its dependent genes in radiation resistance of androgen independent prostate cancer is not reported. This may help in exploiting Nrf2 as a target in improving therapeutic efficacy. Among the various prostate tumor cells, PC3 and DU145 cells which lack androgen receptors are useful as many prostate cancer patients show androgen independent tumor growth [11].

In the present study, we investigated the role of Nrf2 and its dependent genes in determining radiosensitivity of prostate cancer cells using two well-characterised androgen independent cell lines viz., PC3 and DU145 which are known to exhibit differences in their chemotherapeutic response.

2. Materials and methods

2.1. Chemicals

Dulbecco's modified Eagles medium (DMEM), antibiotics (streptomycin and penicillin), sodium bicarbonate, crystal violet, Tris–HCl, ethylenediaminetetraacetic acid (EDTA), sodium chloride (NaCl), dimethyl sulfoxide (DMSO), dihydrodichloroflourescin diacetate (H_2-DCFDA), dihydorhodamine 123 (DHR 123), propidium iodide (PI), sodium citrate, triton X-100, ribonuclease A, all-trans retinoic acid (ATRA), tin protoporphyrin (SnPP), glutathione (reduced and oxidized) and diethyl pyrocarbonate (DEPC) were purchased from Sigma-Aldrich (MO, USA). Fetal bovine serum (FBS) and trypsin-EDTA were from Himedia (Mumbai, India). Lipofectamine was purchased from Invitrogen (Bangalore, India) and short hairpin RNA (shRNA) plasmids were purchased from OriGene (MD, USA).

2.2. Cell lines and irradiation

PC3 and DU145 cells were obtained from the National Institute for Research in Reproductive Health, Mumbai. Cells were maintained as exponentially growing monolayer in DMEM supplemented with 10% FBS, penicillin and streptomycin in humidified incubator maintained at 37 °C with 5% CO_2 in air.

Cells were irradiated (dose rate: 2.5 Gy/min) using ^{60}Co gamma cell 220 irradiator (AECL, Canada). For comet assay, exponentially growing cells were harvested by trypsinization. Cells obtained were thus suspended in complete medium followed by irradiation at 4 °C. After irradiation, samples to be processed immediately (0 min) were kept in ice, whereas, for repair kinetic studies, samples were incubated at 37 °C for various time intervals (15, 30, 60, and 120 min). For RNA isolation, exponentially growing cells (2×10^6 cells) were seeded overnight in culture dish (BD Falcon, USA), irradiated (either 4 or 8 Gy) and cultured for 24 h before RNA isolation. For electrophoretic mobility shift assay (EMSA), exponentially growing cells were seeded overnight, irradiated and incubated for 24 h before performing EMSA.

2.3. Clonogenic assay

Clonogenic assay was performed as mentioned previously [14]. Exponentially growing cells were seeded on 60 mm dishes and allowed to adhere overnight in culture conditions. These cultures were exposed to various doses of γ-radiation (1, 2, 4, 6, 8 and 10 Gy). For studies involving the inhibition of Nrf2 and HO1 activities, cells were treated with the respective inhibitors (10 μM ATRA or 15 μM SnPP respectively) for 1 h before irradiation. After irradiation dishes were kept in the incubator for 15 days for the colony development. After the colony development dishes were washed with PBS, fixed with methanol and then stained using 0.5% crystal violet followed by rinsing the dishes with tap water. Colonies were counted using a stereo microscope. A colony was considered when there were at least 50 or more cells. Survival fraction was calculated using following formula:

$$\text{Survival fraction} = \text{No.of colonies}/[\text{no.of cells plated X}(\text{plating efficiency}/100)].$$

2.4. Apoptosis assay by PI staining

For apoptosis assay, cells were plated for overnight followed by radiation exposure. Forty eight hours after radiation exposure, cells were harvested, fixed with 70% ethanol and stained using propidium iodide (PI) staining solution (0.5 μg/ml PI, 10 μg/ml ribonuclease A, 0.1% sodium citrate and 0.1% Triton X-100). A total of 20,000 cells were acquired using the Partec flow cytometer and were analyzed using FlowJo software.

2.5. Homogenous caspase assay

Homogenous caspase assay was performed using homogenous caspase assay kit (Roche, Germany). With this assay the combined activities of caspase 3 and caspase 7 were measured. This assay is based on the fluorescence emanated due to the cleavage of the pro-fluorescent substrate attached to a peptide, by caspase 3 or caspase7. Exponentially growing cells were plated and allowed to adhere for overnight followed by radiation exposure. At 48 h after radiation exposure, cells were harvested and 40,000 cells were incubated along with the incubation buffer containing substrate peptide attached to Rhodamine 110. One hour after the incubation, Rhodamine 110 fluorescence was measured using a flourimeter (λ_{ex} — 485 nm, λ_{em} — 521 nm) and relative caspase activity was calculated in comparison to control cells.

2.6. Comet assay

To determine the magnitude of DNA damage, neutral comet assay was performed as mentioned previously [15]. Briefly, control and irradiated cells were suspended in 0.8% low melting agarose and were layered on to frosted slides pre-coated with 1% normal agarose. After solidification, slides were kept in the lysis buffer at 4 °C for 60 min. For equilibration, the slides were transferred to electrophoretic tank containing 0.5× Tris–Borate–EDTA buffer. Slides were electrophoresed in the same buffer for 30 min at 0.8 V/cm. After electrophoresis, slides were neutralised (0.4 M

Table 1
Primer sequences of different genes whose expression has been checked by quantitative real time RT-PCR.

Genes	Forward primer	Reverse primer
β-Actin	CTGGAACGGTGAAGGTGACA	AAGGGACTTCCTGTAACAATGCA
Nrf2	AGCATGCCCTCACCTGCTACTTTA	ACTGAGTGTTCTGGTGATGCCACA
HO1	AGAGGGAATTCTCTTGGCTGGCTT	ATGCCATAGGCTCCTTCCTCCTTT
GCLC	ATGGAAGTGGATGTGGACACCAGA	AACTCCCTCATCCATCTGGCAACT
TXRD1	TCCTATGTCGCTTTGGAGTGC	GGACCTAACCATAACAGTGACGC
Keap1	CTGCAGGATCATACCAAGCAGG	GAACATGGCCTTGAAGACAGG

Tris–HCl), stained (1× SYBR Green I dye) and visualised at 40× magnifications using fluorescence microscope (Axioplan, Carl-Zeiss, Germany). For every treatment, two slides were prepared and at least 50 images were grabbed per slide. The images were analyzed using CASP software (www.casplab.com) to obtain % DNA in tail, which is considered as the reliable parameter for representing the DNA damage in comet assay.

2.7. Electrophoretic mobility shift assay

In order to assay the DNA binding ability of Nrf2 in PC3 and DU145 cells, electrophoretic mobility assay (EMSA) was carried out as mentioned previously [16]. Briefly, at 24 h after irradiation, nuclear extract was prepared from the nuclear pellet by repeated vortexing. Protein was quantified from the nuclear extracts, and equal amount of protein was incubated with the ^{32}P labeled Nrf2 binding consensus sequence (5′-TGG GGA ACC TGT GCT GAG TCA CTG GAG-3′, Santa Cruz Biotechnology, CA, USA) for 1 h and then it was loaded onto 7.6% polyacrylamide gel and electrophoresed at 70 mA. After the electrophoresis, gel was vacuum dried and the signal was developed using phosphor imager.

2.8. Quantitative real time RT-PCR

To quantify the mRNA expression of genes, quantitative real time reverse transcriptase polymerase chain reaction (RT q-PCR) was used [17]. Total RNA was isolated using TRI reagent (Sigma, MO, USA) as

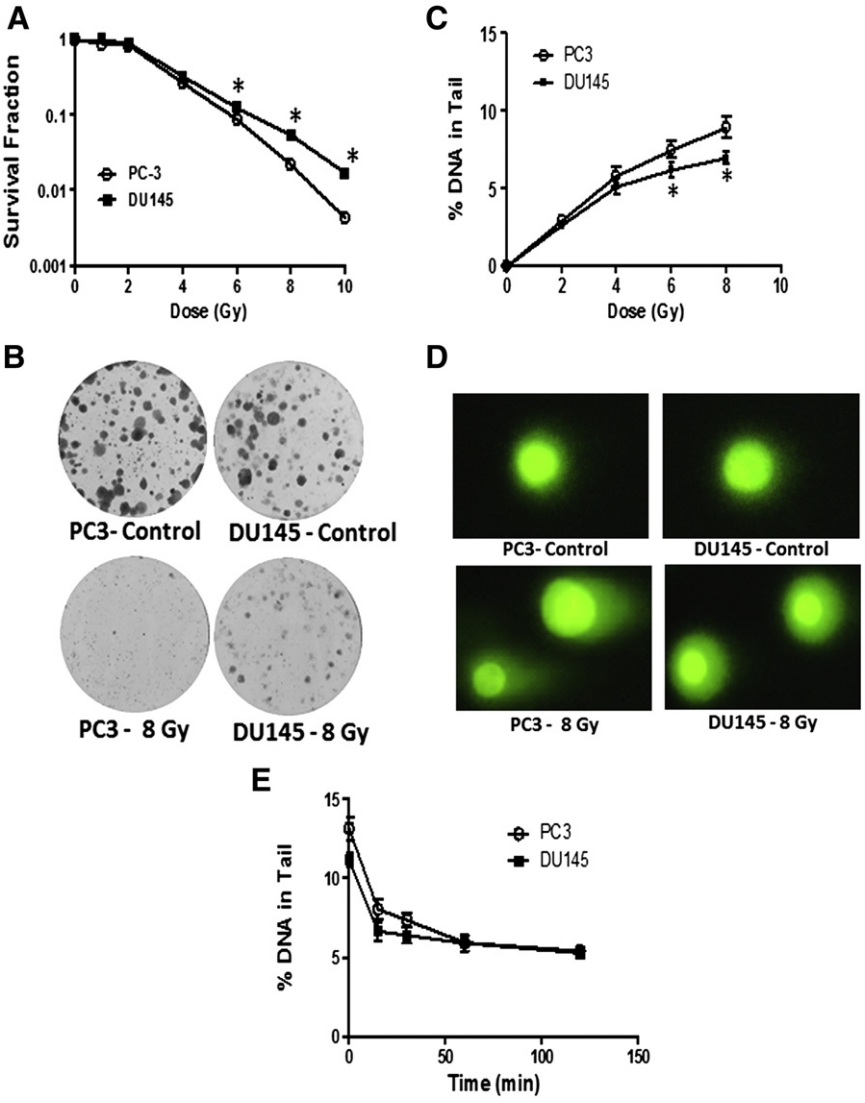


Fig. 1. Radiosensitivity profile of PC3 and DU145 cells, as determined by clonogenic assay (A and B), DNA damage (C and D) and DNA repair kinetics (E) obtained by neutral comet assay. For clonogenic assay, exponentially growing cells have been irradiated, grown for two weeks, stained and the number of colonies was counted and the survival fraction has been plotted. Three independent experiments were performed in triplicates each time and mean ± SEM has been plotted. Representative image of clonogenic assay of PC3 and DU145 cells after 8 Gy radiation exposure has been shown (B). Neutral comet assay was performed in the control as well as irradiated cells at different time points after irradiation as described in the Materials and methods section. From the neutral comet assay, at least 50 cells per slide were microscopically grabbed and analyzed for DNA damage. Two slides were prepared for every treatment, and the mean ± SEM of three independent experiments was plotted. Representative images of PC3 and DU145 cells obtained after exposure to 8 Gy of radiation has been shown (D). *$P < 0.05$ in comparison to respective PC3 group.

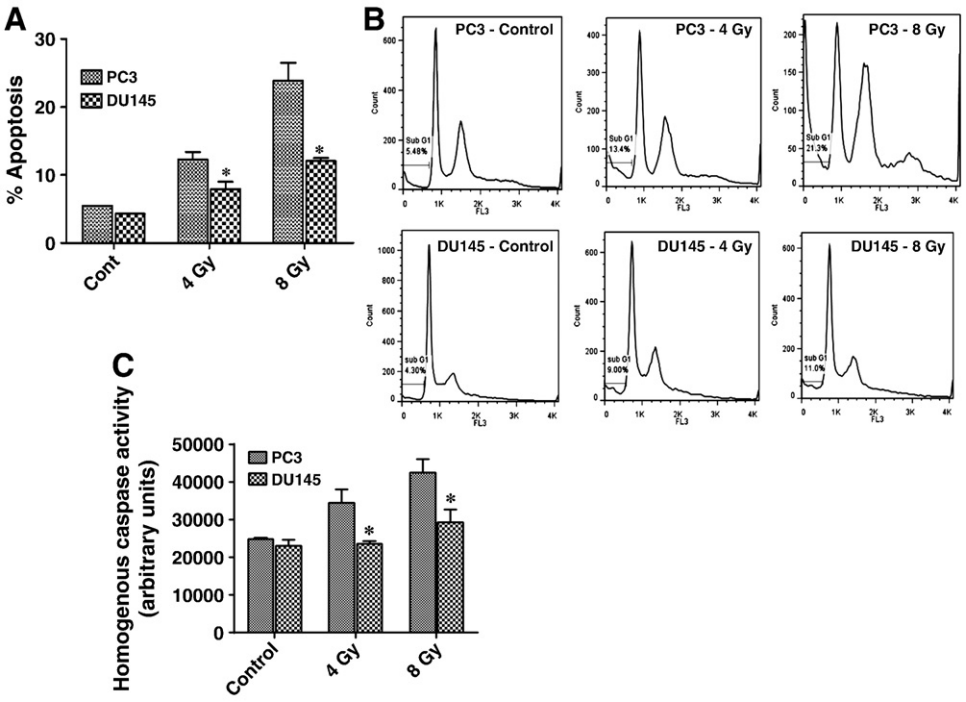


Fig. 2. Radiation response of PC3 and DU145 cells, as determined by apoptosis enumerated by PI staining (A and B), and homogenous caspase assay (C). For PI assay, cells were plated for overnight followed by radiation exposure. Forty eight hours after radiation exposure, cells were harvested, fixed and stained using PI staining solution. A total of 20,000 cells were acquired using the Partec flow cytometer and were analyzed using FlowJo software. Percentages of cells in pre-G1 peak were represented as cells undergoing apoptosis. Mean ± SEM obtained from two independent experiments done in triplicates, has been plotted. For homogenous caspase assay, cells were harvested at 48 h after the radiation exposure and processed accordingly using homogenous caspase assay kit. Arbitrary units of mean fluorescence obtained in two experiments done in triplicates have been plotted with SEM. *$P < 0.05$ in comparison to respective PC3 group.

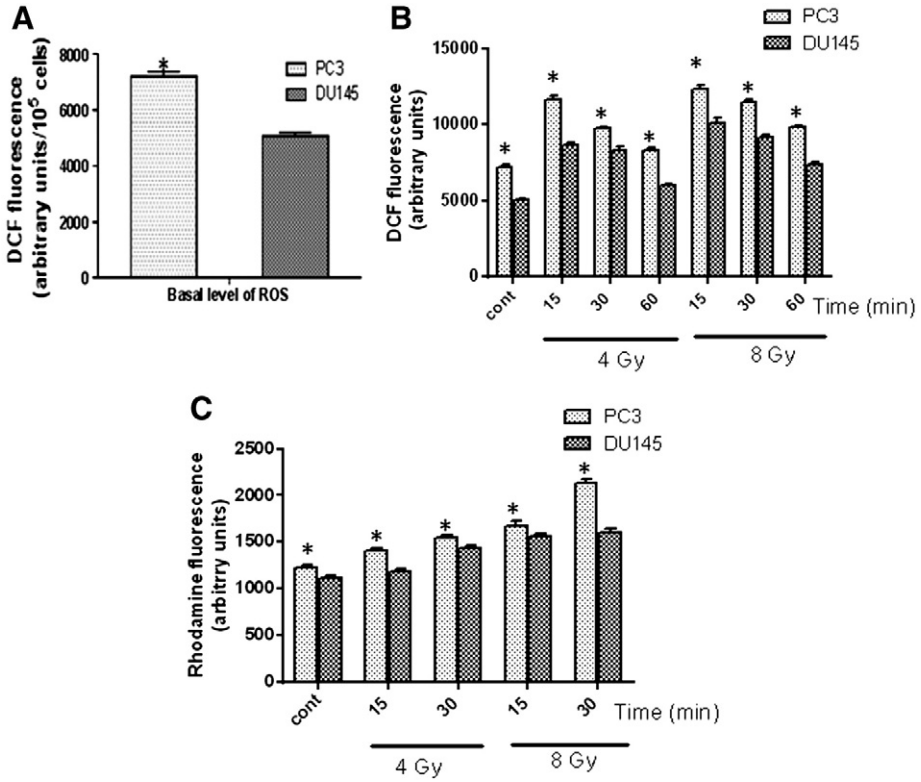


Fig. 3. Levels of reactive oxygen species as measured by DCF fluorescence in PC3 and DU145 cells in control (A), after 4 Gy and 8 Gy (B). Mitochondrial ROS as estimated by rhodamine in PC3 and DU145 cells after radiation exposure of 4 Gy and 8 Gy (C). For measuring ROS, either DCFDA or DHR was added to cells 30 min prior to irradiation, fluorescence of DCF or rhodamine recorded at different time intervals and the mean ± SEM was plotted. Three independent experiments in triplicates at each time point were performed. *$P < 0.05$ in comparison to respective DU145 group.

per manufacturer's instructions. Two micrograms of total RNA was used for the synthesis of cDNA by reverse transcription (cDNA synthesis kit, Sigma, MO, USA). PCR reactions were set up by mixing 10× SYBR green PCR mix with 5 μl of 2 times diluted cDNA templates, 1 μl each of forward and reverse primers (0.5 μM; Table 1), and 3 μl of PCR-grade water in 20 μl reaction mixture. The above reaction mixtures were amplified in the following steps: step 1—denaturation at 95 °C for 5 min; step 2—denaturation at 95 °C for 15 s; step 3—annealing at 57 °C for 15 s; step 4—extension at 72 °C for 20 s; and step 5—melting curve analysis. Steps 2–4 were repeated for 40 cycles using the Rotor Gene 3000 (Corbett Life Science, Australia). The threshold cycle values obtained from the above runs were used for calculating the fold change in gene expression by REST-384 version 2 software. The expressions of genes were normalized against that of a housekeeping gene, β-actin, and the relative change in the expression was plotted with respect to control group.

2.9. ROS measurement

For measuring intra-cellular cytosolic ROS levels, 1×10^5 cells were plated in 24 well plates for overnight. Then cells were treated with oxidation sensitive DCF-DA (a final concentration of 10 μM) in culture medium for 30 min at 37 °C prior to irradiation. After irradiation, the fluorescence of DCF in cells was measured (λ_{ex} — 485 nm, λ_{em} — 535 nm) at different time intervals [18]. Similarly for measuring mitochondrial ROS, dihydrorhodamine 123 at a final concentration of 10 μM was added to the cells, incubated for 30 min prior to irradiation and the rhodamine fluorescence was measured (λ_{ex} — 511 nm, λ_{em} — 536 nm) at various time intervals after irradiation [19].

2.10. Measurement of GSH and GSSG levels

Glutathione (GSH) and glutathione disulphide (GSSG) levels were measured as described previously [20]. Measurement of GSH by this method involves the oxidation of GSH by the sulfhydryl reagent 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) to form the yellow derivative 5′-thio-2-nitrobenzoic acid (TNB), measurable at 412 nm. For measuring GSSG, the GSSG formed was recycled to GSH by glutathione reductase in the presence of NADPH and then measured by DTNB reduction method.

2.11. Measurement of thioredoxin reductase activity

Thioredoxin reductase 1 (TXRD1) activity was measured using a kit (Thioredoxin reductase assay kit, Cayman Chemical Company, USA) by following manufacturer's protocol. Briefly, DTNB reduction was measured in the absence and presence of aurothiomalate, a specific TXRD1 inhibitor that allows for the correction of TXRD1 independent DTNB reduction. By calculating the difference between the above two conditions, TXRD1 activity was estimated.

2.12. Knockdown of Nrf2 and HO-1 expression using short hairpin RNA (ShRNA)

Nrf2 and HO-1 expression was knocked down by transfecting cells with shRNA. For this purpose, exponentially growing cells were transfected with either NRF2 or random sequence shRNA, using lipofectamine-2000 as mentioned in the manufacturer's protocol (Invitrogen, Bangalore, India). Cells were harvested after 24 h of transfection and were plated for clonogenic survival assay. At 48 h after transfection, cells were exposed to various doses of irradiation and their clonogenic survival was seen after 15 days.

2.13. Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0 software (La Jolla, CA, USA). Student's *t*-test was used for comparing the means of two groups. One-way ANOVA was performed to test the significance when more than two groups were involved. Values were considered significantly different if $P < 0.05$.

3. Results

3.1. DU145 cells exhibits higher magnitude of radioresistance than PC3 cells

Clonogenic assay was used to evaluate the radiosensitivity of PC3 and DU145 prostate tumor cells. Cells were exposed to various doses of gamma radiation ranging from 1 to 10 Gy and their survival fraction was calculated after 15 days. Among the two cell lines DU145 cells showed more radioresistance than PC3 cells. Though the radiosensitivity of PC3 was comparable to that of DU145 at 0.5 and 2 Gy, PC3 cells showed a relative decrease in their survival fraction in comparison to DU145 cells as the dose of radiation increased (Fig. 1A and B). For example survival fractions estimated for PC3 cells at 2 and 10 Gy were 0.83 and 0.004 respectively, whereas that for DU145 0.89 and 0.02 respectively. The dose required to bring down the survival fraction from 0.1 to 0.01 for PC3 and DU145 cells was calculated to be 3 and 4.5 Gy respectively. Although both these tumor cells are from the same tissue of origin, a significant difference in their radiosensitivity was apparent in clonogenic survival assay. Differential radiation response of these two cells was also evaluated by DNA damage by neutral comet assay after exposing the cells to various doses of gamma radiation (2–8 Gy). The results revealed increased magnitude of DNA damage in PC3 cells in comparison to that of DU145 cells at all the doses with more significant differences at the higher doses (Fig. 1C and D). We also studied the DNA damage repair kinetics by assessing the extent of DNA damage at different time intervals (up to 2 h) after radiation exposure, by neutral comet assay. DU145 cells showed faster recovery of DNA damage in comparison to PC3 cells (Fig. 1E). In order to further evaluate the differential radiosensitivity of the above tumor cells, we have estimated the

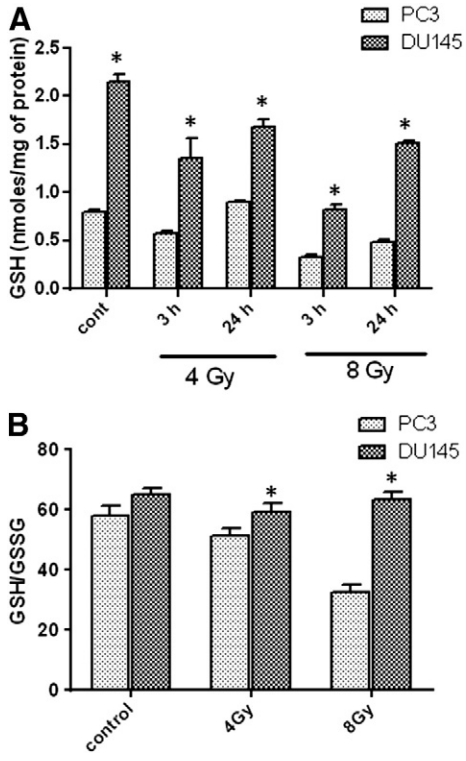


Fig. 4. Level of GSH and GGH/GSSG ratio in PC3 and DU145 cells. GSH levels were measured at 3 h and 24 h (A) after radiation exposure. GSH to GSSG ratio measured after 24 h (B) after radiation exposure. Three independent experiments were performed in triplicates at each time point and mean ± SEM has been plotted. *$P < 0.05$ in comparison to respective PC3 group.

cell death using propidium iodide staining followed by flow cytometry. For example, after 8 Gy of radiation exposure, 24% of PC3 cells underwent apoptosis (% cells in pre-G1 peak) as compared to 12% of DU145 cells (Fig. 2A and B). To confirm the results obtained by PI assay, caspase activity was also evaluated in these two cell lines after 4 Gy and 8 Gy radiation exposures. Homogenous caspase activity (activity of caspases 3 and 7) was observed to be significantly higher in PC3 cells in comparison to that of DU145 cells after 4 Gy and 8 Gy radiation exposures (Fig. 2C). Thus from clonogenic survival assay, apoptosis assay and DNA damage assay, it was clearly evident that among the two cell lines, DU145 cells showed more radioresistance than PC3 cells.

3.2. Intracellular redox environment of DU145 cells is more reducing than that of PC3 cells

Since PC3 and DU145 exhibited differences in radiosensitivity, we further investigated the redox state of these cells by estimating ROS and GSH levels under both the control and irradiated (4 and 8 Gy) conditions. Interestingly PC3 cells which were observed to be radiosensitive showed higher basal as well as induced level of cytosolic ROS (measured by DCF fluorescence) in comparison to that of DU145 cells (Fig. 3A and B). In both the tumor cell lines, maximum intracellular ROS was observed at 15 min following irradiation, which declined with progress of time. Unlike DCF, rhodamine fluorescence, which measures mitochondrial ROS showed time dependent increase following irradiation. As observed with DCF, the rhodamine fluorescence was lower in DU145 cells compared to that of PC3 cells at all the time points studied suggesting increased level of mitochondrial ROS in PC3 cells compared to that of DU145 cells (Fig. 3C).

In agreement with ROS levels, DU145 cells showed significantly higher basal GSH content than that of PC3 cells (Fig. 4A). On irradiation both the cells showed depletion in their GSH but with progress of time, DU145 cells showed much faster recovery of GSH content than that of PC cells (Fig. 4A). The ratio of GSH and GSSG was also significantly higher in DU145 cells than that of the PC3 cells and it was maintained even at 24 h after irradiation at different doses (Fig. 4B). Taken together these results suggest a reducing type of redox environment in DU145 cells compared to that of PC3 cells and this could be the reason for the enhanced tolerability of DU145 against radiation exposure.

3.3. DU145 cells exhibits elevated levels of Nrf2 and its dependent transcripts than PC3 cells after radiation exposure

Anticipating the involvement of redox sensitive transcription factor Nrf2 in the observed differences in redox environment of the above two cell lines, we measured the activation of Nrf2 in these cells under irradiated condition using EMSA. DU145 cells showed higher basal levels of Nrf2 in comparison to PC3 cells (Fig. 5A). Moreover, PC3 cells, which exhibited high radiosensitivity, showed significantly lower level of Nrf2 than DU145 cells after radiation exposure (4 and 8 Gy). We also measured the mRNA expression level of Nrf2 in these cells by RT q-PCR at

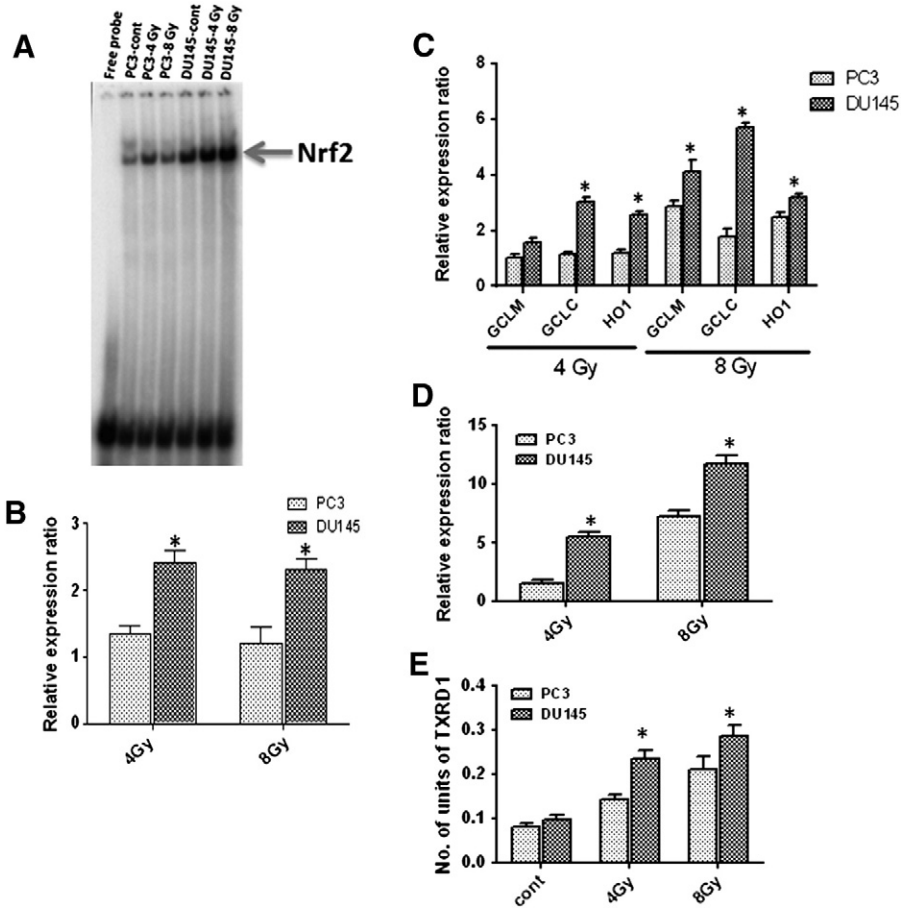


Fig. 5. Nuclear levels of Nrf2 as assessed by EMSA in nuclear extract (A), mRNA levels of Nrf2 (B) and its dependent genes GCLM, GCLC, HO1 (C) and TXRD1 (D) in PC3 and DU145 cells after 4 Gy and 8 Gy of radiation exposure. Biochemical activity of TXRD1 enzyme in PC3 and DU145 cells after exposure to 4 Gy and 8 Gy of radiation (E) was also estimated. For EMSA protein lysate was made from nuclear pellets, and equal amount of protein was incubated with the ^{32}P labeled Nrf2 binding consensus sequence and followed by electrophoresis. After the electrophoresis, gel was vacuum dried and the signal was developed using phosphor imager. Representative image of the three experiments has been shown. Gene expression was analyzed by real-time q-PCR, at 24 h after exposing the cells to 4 Gy/8 Gy of gamma irradiation. The bars represent the mean ± SEM obtained from two independent experiments done in triplicates. *$P < 0.05$ in comparison to respective PC3 group.

24 h after radiation exposure. DU145 cells showed two fold higher induction of Nrf2 levels in comparison to PC3 cells after 4 and 8 Gy of radiation exposure (Fig. 5B).

As there was a difference seen in Nrf2 levels between these two cell lines, we also evaluated the expression levels of Nrf2 dependent genes like HO1, GCLC and TXRD1 after exposing to either 4 Gy or 8 Gy. Following 4 Gy of radiation exposure, DU145 cells showed 5.69, 2.6 and 5.46 fold upregulation in the expression of GCLC, HO1 and TXRD1 genes over control respectively (Fig. 5C and D). However in PC3 cells, these genes did not show significant upregulation after radiation exposure. Biochemical activity of TXRD1 was also measured in the cell lysate at 24 h after radiation exposure, which indicated a significant increase in DU145 cells than in PC3 cells after 4 Gy radiation exposure (Fig. 5E).

Since Keap1 protein plays a key role in regulating the Nrf2 pathway, we have evaluated the mRNA levels of Keap1 in PC3 and DU145 cells. Basal levels of Keap1 gene in DU145 cells were found to be two fold lower than that of the PC3 cells (Fig. 6A). Further, the changes in mRNA levels of Keap1 were also examined after exposure to radiation and found that both PC3 and DU145 cells showed a negative regulation of Keap1 gene. The extent of this negative regulation was marginally higher in PC3 cells than that observed in DU145 cells. But the differences were not statistically significant (Fig. 6B).

3.4. Nrf2 and HO1 levels determine radiosensitivity in DU145 and PC3 cells

In order to confirm the role of Nrf2 and its dependent genes in radiosensitivity of tumor cells, we examined the survival fraction of PC3 and DU145 cells after radiation exposure, in the presence or absence of inhibitors of Nrf2 and HO1 namely ATRA and SnPP, respectively. The presence of these inhibitors significantly reduced the survival fraction of the above tumor cells against the radiation exposure. Treatment with the above inhibitors prior to radiation exposure at 4 Gy reduced the survival fraction of PC3 cells from 0.21 to 0.01 and that of DU145 cells from 0.31 to 0.03 (Fig. 7A and B). Similarly, in DU145 cells, SNPP and ATRA treatments decreased the survival fraction from 0.08 to 0.014 after 8 Gy radiation exposure, whereas the survival fraction of PC3 cells reduced from 0.015 to 0.007. Further, to corroborate this evidence of involvement of Nrf2 in radiosensitivity, we have employed knockdown approach by transfecting shRNA against Nrf2 into DU145 cells. A nonspecific shRNA having scrambled sequence was used as a control. The transfected cells were exposed to 4 Gy of radiation and their survival fraction was analyzed. The cells, which were transfected with shRNA targeting Nrf2 prior to radiation exposure, showed a drastic decrease in survival fraction (0.035), in comparison to the cells transfected with scrambled shRNA (0.28) after 4 Gy irradiation (Fig. 7C and D). It was also observed that the cells transfected with Nrf2 specific shRNA showed significant reduction in survival even without the radiation treatment. Clonogenic survival assay was also performed in DU145 cells after knocking down the expression of HO1 (an Nrf2 dependent gene). Though HO1 knockdown itself has not exhibited any significant reduction in survival, radiation exposure of HO1 knockdown cells exhibited significant reduction in survival fraction (Fig. 7E and F). These evidences suggested the involvement of redox regulated transcription factor in determining radiosensitivity of prostate tumor cells.

4. Discussion

Molecular mechanisms governing the radiosensitivity/radioresistance of tumor cells are not clearly understood and there is a need for further research in this area. In order to study the molecular players which determine radiosensitivity, we have chosen two prostate tumor cells namely, PC3 and DU145, which are androgen independent cells, known to have differential chemotherapeutic response [21,22]. However, differential radiosensitivity and underlying mechanism have not been investigated in these two prostate cancer cell lines. In this study, we have investigated the radiosensitivity of these two prostate cancer cells and made an attempt to rationalize molecular differences between them contributing to their differential radiosensitivity. These two cell lines showed difference in their radiosensitivity as measured by clonogenic survival fraction after irradiation (1–10 Gy) and PC3 cells were found to be more radiosensitive than DU145 cells. This difference was more prominent at higher doses (>8 Gy) than at the lower doses (<4 Gy) of radiation exposure (Fig. 1A). In apoptosis analysis PC3 cells also showed more sensitivity towards radiation than DU145 cells. Radiosensitive PC3 cells also showed more DNA damage than DU145 cells. Though these two tumor cells exhibited significant difference in DNA damage after the radiation exposure, following DNA repair kinetics, there was no significant difference in residual DNA damage between these two cells. These results are in corroboration with earlier observation that the initial DNA damage observed by neutral comet assay is a good marker of radiosensitivity than the residual DNA damage [15].

Since ROS is known to play an important role in the cytotoxic action of ionizing radiation, we have evaluated basal and inducible levels of ROS in these two tumor cells. PC3 cells, which are radiosensitive among the two cell lines, showed higher basal as well as inducible levels of ROS on radiation exposure (Fig. 3A–C). High levels of ROS accumulation can lead to increased DNA damage and a variety of other cellular responses including cell cycle arrest, senescence and apoptosis [23]. Similar to cytosolic ROS, mitochondrial ROS levels was also found to be more in radiosensitive PC3 cells after the radiation exposure. Cells employ many enzymatic and non-enzymatic antioxidants to counter the effect of ROS and to bring back cell homeostasis [24]. One of the most versatile protectors of such antioxidants is GSH. GSH protects cells from radiation damage by several mechanisms including radical scavenging, restoration of damaged molecules by hydrogen donation, reduction of peroxides and maintenance of protein thiols in the reduced state [25]. Therefore, we have evaluated the level of GSH, and GSH to GSSG ratio in both PC3 and DU145 tumor cells under control and irradiated conditions. A relatively radiosensitive PC3 cells showed low basal

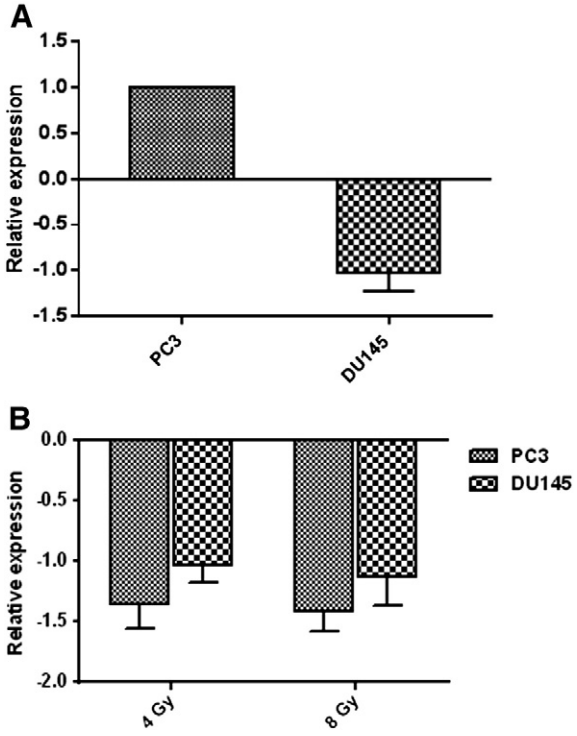


Fig. 6. mRNA levels of Keap1 in control (A) and after radiation exposure (B) in PC3 and DU145 cells. Gene expression was analyzed by real-time q-PCR, at 24 h after exposing the cells to 4 Gy/8 Gy of gamma irradiation. The error bars represent the mean ± SEM obtained from two independent experiments done in triplicates.

level of GSH and also showed faster depletion of GSH after radiation exposure (Fig. 4A and B). The radioresistant DU145 cells showed faster recovery from oxidative stress and this was also supported by their high GSH to GSSG ratio in comparison to that of PC3 cells. Increased accumulation of ROS combined with the faster depletion of GSH may be responsible for higher DNA damage observed in PC3 cells after radiation exposure. It has also been shown that thiol depletion can lead to higher radiation induced apoptosis [26].

Since we have observed differences in cellular redox environment between these two cell lines after irradiation, we hypothesised that the transcription factor which controls the enzymes involved in GSH synthesis may be playing key role in determining radiosensitivity. Nrf2 is the transcription factor which involved in the transcription of the enzymes involved in GSH biosynthesis [27,28]. Apart from the enzyme which is involved in GSH biosynthesis (GCLC and GCLM), glutathione peroxidase enzymes (GPX1, GPX2 and GPX3) and thioredoxin families (thioredoxin, TXRD1, peroxiredoxin) have also been shown to be the transcriptional targets of Nrf2 [7,29,30]. In the present study, we have observed that Nrf2 activation and its dependent genes were upregulated in DU145 cells in comparison to those in PC3 cells (Fig. 5A–E). Keap1 protein plays a major role in regulating the nuclear Nrf2 levels. Therefore we have evaluated the Keap1 mRNA levels in these two cell types and found that relative levels of Keap1 were lower in DU145 cells than that in PC3 cells. These reduced levels of Keap1 may be one of the reasons for higher basal levels of Nrf2 in DU145 cells. Reduced levels of Keap1 in DU145 cells may be attributed to the reported hyper-methylation in the Keap1 promoter of the DU145 cells [6]. GCLC, an enzyme which is involved in biosynthesis of GSH, has shown significant upregulation in radioresistant DU145 cells in comparison to that of PC3 cells. This could have played a role in restoring the thiol balance in these cells quickly contributing to their radioresistance as compared to PC3 cells. Moreover, when we inhibited the Nrf2 by inhibitors as well as shRNA we observed a drastic reduction in survival fraction of radioresistant DU145 cells after the radiation exposure. It was also observed that the cells transfected with Nrf2 specific shRNA showed significant reduction in survival even without the

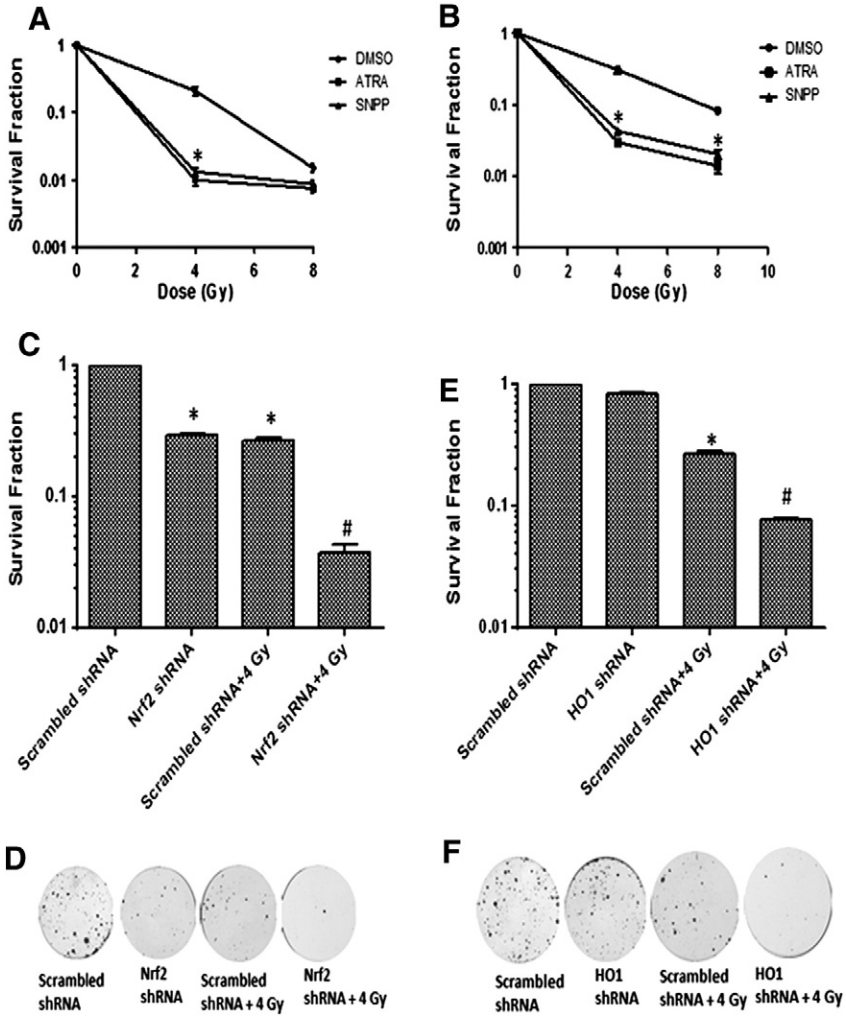


Fig. 7. Survival fraction of PC3 (A) and DU145 (B) cells after exposure to radiation in the presence of ATRA (Nrf2 inhibitor) or SnPP (HO1 inhibitor). Exponentially growing cells were plated, treated with inhibitors, irradiated and followed by clonogenic assay. Three independent experiments were performed in triplicates each time and mean ± SEM has been plotted. $^{*}P < 0.05$ in comparison to DMSO control group. Survival fraction was also calculated in DU145 cells after knocking down the expression of Nrf2 expression (C) or HO1 expression (D) which is exposed to radiation, 48 h after transfection with shRNA targeting Nrf2 or HO1 expression. Survival fraction was assessed by clonogenic assay by counting the colonies developed at 15 days after treatment and radiation exposure. A nonspecific shRNA containing scrambled sequences was used as control. Two independent experiments were performed with triplicates each time. Mean ± SEM has been plotted. Representative clonogenic dishes belonging to various treatment groups have also been shown (E and F). $^{*}P < 0.05$ in comparison to scrambled shRNA group. $\#P < 0.05$ in comparison to scrambled shRNA + 4 Gy group.

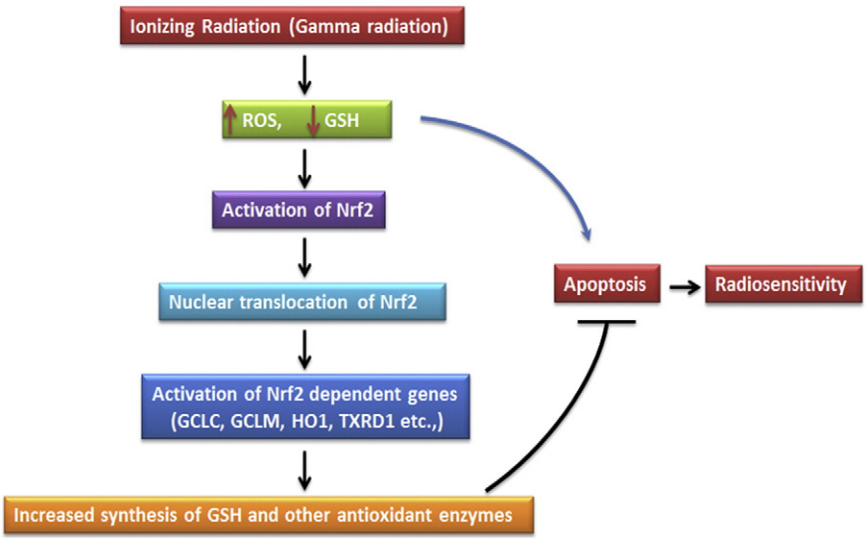


Fig. 8. Schematic diagram representing the role of ROS, GSH and Nrf2 pathway contributing to the radioresistance of tumor cells.

radiation exposure. This may be due to the fact that some basal levels of Nrf2 are required for maintaining the cellular oxidative homeostasis. When Nrf2 is knocked down, even under normal circumstances, it will lead to the accumulation of higher levels of ROS and that may affect the cell survival [31].

In conclusion, in this study, we have demonstrated the role of Nrf2 and its dependent genes in radioresistance of prostate tumor cells through management of intracellular ROS and GSH levels (Fig. 8). As the radioresistance is the serious impediment in radiotherapy, Nrf2 inhibition can be used as a target to enhance the ionizing radiation induced tumor killing.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgements

Authors would like to thank Mr. B.A. Naidu for technical assistance and Ms. Jisha Menon for help in flow cytometry studies. Authors would also like to thank Dr. Geetanjali Sachdeva from the National Institute for Research in Reproductive Health, for providing us both PC3 and DU145 cell lines and the Department of Atomic Energy, India, for funding this study.

References

[1] L. Peters, M. McKay, Predictive assays: will they ever have a role in the clinic? Int. J. Radiat. Oncol. Biol. Phys. 49 (2001) 501–504.
[2] D. Gius, Redox-sensitive signaling factors and antioxidants: how tumor cells respond to ionizing radiation, J. Nutr. 134 (2004) 3213S–3214S.
[3] H.C. Lee, D.W. Kim, K.Y. Jung, I.C. Park, M.J. Park, M.S. Kim, S.H. Woo, C.H. Rhee, H. Yoo, S.H. Lee, S.I. Hong, Increased expression of antioxidant enzymes in radioresistant variant from U251 human glioblastoma cell line, Int. J. Mol. Med. 13 (2004) 883–887.
[4] T. Nguyen, P. Nioi, C.B. Pickett, The Nrf2–antioxidant response element signaling pathway and its activation by oxidative stress, J. Biol. Chem. 284 (2009) 13291–13295.
[5] N. Wakabayashi, S.L. Slocum, J.J. Skoko, S. Shin, T.W. Kensler, When NRF2 talks, who's listening? Antioxid. Redox Signal. 13 (2010) 1649–1663.
[6] P. Zhang, A. Singh, S. Yegnasubramanian, D. Esopi, P. Kombairaju, M. Bodas, H. Wu, S.G. Bova, S. Biswal, Loss of Kelch-like ECH-associated protein 1 function in prostate cancer cells causes chemoresistance and radioresistance and promotes tumor growth, Mol. Cancer Ther. 9 (2010) 336–346.
[7] A. Singh, M. Bodas, N. Wakabayashi, F. Bunz, S. Biswal, Gain of Nrf2 function in non-small-cell lung cancer cells confers radioresistance, Antioxid. Redox Signal. 13 (2010) 1627–1637.
[8] T. Shibata, A. Kokubu, S. Saito, M. Narisawa-Saito, H. Sasaki, K. Aoyagi, Y. Yoshimatsu, Y. Tachimori, R. Kushima, T. Kiyono, M. Yamamoto, NRF2 mutation confers malignant potential and resistance to chemoradiation therapy in advanced esophageal squamous cancer, Neoplasia 13 (2011) 864–873.
[9] M.J. Szostak, N. Kyprianou, Radiation-induced apoptosis: predictive and therapeutic significance in radiotherapy of prostate cancer (Review), Oncol. Rep. 7 (2000) 699–706.
[10] P. Pettazzoni, E. Ciamporcero, C. Medana, S. Pizzimenti, F. Dal Bello, V.G. Minero, C. Toaldo, R. Minelli, K. Uchida, M.U. Dianzani, R. Pili, G. Barrera, Nuclear factor erythroid 2-related factor-2 activity controls 4-hydroxynonenal metabolism and activity in prostate cancer cells, Free Radic. Biol. Med. 51 (2011) 1610–1618.
[11] K.J. Niermann, L. Moretti, N.J. Giacalone, Y. Sun, S.M. Schleicher, P. Kopsombut, L.R. Mitchell, K.W. Kim, B. Lu, Enhanced radiosensitivity of androgen-resistant prostate cancer: AZD1152-mediated Aurora kinase B inhibition, Radiat. Res. 175 (2011) 444–451.
[12] M. Zhang, M. Siedow, G. Saia, A. Chakravarti, Inhibition of p21-activated kinase 6 (PAK6) increases radiosensitivity of prostate cancer cells, Prostate 70 (2010) 807–816.
[13] J. Lovey, D. Nie, J. Tovari, I. Kenessey, J. Timar, M. Kandouz, K.V. Honn, Radiosensitivity of human prostate cancer cells can be modulated by inhibition of 12-lipoxygenase, Cancer Lett. 335 (2013) 495–501.
[14] N.A.P. Franken, H.M. Rodermond, J. Stap, J. Haveman, C. van Bree, Clonogenic assay of cells in vitro, Nat. Protoc. 1 (2006) 2315–2319.
[15] S. Jayakumar, H.N. Bhilwade, B.N. Pandey, S.K. Sandur, R.C. Chaubey, The potential value of the neutral comet assay and the expression of genes associated with DNA damage in assessing the radiosensitivity of tumor cells, Mutat. Res. 748 (2012) 52–59.
[16] S.K. Sandur, H. Ichikawa, G. Sethi, K.S. Ahn, B.B. Aggarwal, Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) suppresses NF-kappaB activation and NF-kappaB-regulated gene products through modulation of p65 and IkappaBalpha kinase activation, leading to potentiation of apoptosis induced by cytokine and chemotherapeutic agents, J. Biol. Chem. 281 (2006) 17023–17033.
[17] A. Kunwar, S. Jayakumar, H.N. Bhilwade, P.P. Bag, H. Bhatt, R.C. Chaubey, K.I. Priyadarsini, Protective effects of selenocystine against gamma-radiation-induced genotoxicity in Swiss albino mice, Radiat. Environ. Biophys. 50 (2011) 271–280.
[18] B.N. Pandey, K.P. Mishra, Modification of thymocytes membrane radiooxidative damage and apoptosis by eugenol, J. Environ. Pathol. Toxicol. Oncol. 23 (2004) 117–122.
[19] B. Kumar, A. Kumar, S. Ghosh, B.N. Pandey, K.P. Mishra, B. Hazra, Diospyrin derivative, an anticancer quinonoid, regulates apoptosis at endoplasmic reticulum as well as mitochondria by modulating cytosolic calcium in human breast carcinoma cells, Biochem. Biophys. Res. Commun. 417 (2012) 903–909.
[20] I. Rahman, A. Kode, S.K. Biswas, Assay for quantitative determination of glutathione and glutathione disulfide levels using enzymatic recycling method, Nat. Protoc. 1 (2006) 3159–3165.
[21] S. Singh, D. Chitkara, R. Mehrazin, S.W. Behrman, R.W. Wake, R.I. Mahato, Chemoresistance in prostate cancer cells is regulated by miRNAs and Hedgehog pathway, PLoS One 7 (2012) e40021.
[22] J.T. Lee Jr., L.S. Steelman, J.A. McCubrey, Phosphatidylinositol 3′-kinase activation leads to multidrug resistance protein-1 expression and subsequent chemoresistance in advanced prostate cancer cells, Cancer Res. 64 (2004) 8397–8404.
[23] B.E. Lehnert, R. Iyer, Exposure to low-level chemicals and ionizing radiation: reactive oxygen species and cellular pathways, Hum. Exp. Toxicol. 21 (2002) 65–69.
[24] J. Sun, Y. Chen, M. Li, Z. Ge, Role of antioxidant enzymes on ionizing radiation resistance, Free Radic. Biol. Med. 24 (1998) 586–593.

[25] E.A. Bump, J.M. Brown, Role of glutathione in the radiation response of mammalian cells in vitro and in vivo, Pharmacol. Ther. 47 (1990) 117–136.
[26] N. Mirkovic, D.W. Voehringer, M.D. Story, D.J. McConkey, T.J. McDonnell, R.E. Meyn, Resistance to radiation-induced apoptosis in Bcl-2-expressing cells is reversed by depleting cellular thiols, Oncogene 15 (1997) 1461–1470.
[27] R. Brigelius-Flohe, L. Flohe, Basic principles and emerging concepts in the redox control of transcription factors, Antioxid. Redox Signal. 15 (2011) 2335–2381.
[28] M. Tsukimoto, N. Tamaishi, T. Homma, S. Kojima, Low-dose gamma-ray irradiation induces translocation of Nrf2 into nuclear in mouse macrophage RAW264.7 cells, J. Radiat. Res. 51 (2010) 349–353.
[29] T.W. Kensler, N. Wakabayashi, S. Biswal, Cell survival responses to environmental stresses via the Keap1–Nrf2–ARE pathway, Annu. Rev. Pharmacol. Toxicol. 47 (2007) 89–116.
[30] M. Tanito, M.P. Agbaga, R.E. Anderson, Upregulation of thioredoxin system via Nrf2–antioxidant responsive element pathway in adaptive-retinal neuroprotection in vivo and in vitro, Free Radic. Biol. Med. 42 (2007) 1838–1850.
[31] J.T. McDonald, K. Kim, A.J. Norris, E. Vlashi, T.M. Phillips, C. Lagadec, L. Della Donna, J. Ratikan, H. Szelag, L. Hlatky, W.H. McBride, Ionizing radiation activates the Nrf2 antioxidant response, Cancer Res. 70 (2010) 8886–8895.