BIOLOGICAL MECHANISMS UNDERLYING CANCER RADIO-RESISTANCE

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Abstract:

Radiation therapy is the cornerstone in the treatment of cancer, offering significant benefits in tumour control and patient survival. The efficacy of radiation therapy is limited due to phenomenon of radiation resistance, wherein certain tumour cells exhibit reduced sensitivity to ionizing radiation. Understanding the mechanisms underlying radiation resistance in tumour cells is crucial for enhancing radiotherapy outcome. Our lab has been involved in studying the factors which contribute to radiation resistance, their value in prediction of radiosensitivity and also developing drugs that can overcome this radiation resistance in order to improve the outcome of radiotherapy. We have evaluated the usefulness of different cellular and molecular markers in predicting tumour radiosensitivity and identified that DNA damage responsive genes can be used to predict radiosensitivity of tumour cells. Further, we have also studied the role of different molecular pathways including Nrf2 pathway and NF-κB pathway in radiation resistance of tumour cells. We have also employed synthetic and natural molecules as modulators of these pathways. The study of radiation resistance in tumour cells is a dynamic and evolving field that holds the potential to revolutionize cancer treatment. A deeper understanding of radioresistance mechanisms and development of innovative strategies to circumvent radiation resistance are essential for maximizing the therapeutic benefits of radiation therapy and ultimately improving the prognosis for cancer patients.

1. Introduction:

Radiotherapy is a vital and effective treatment strategy in the comprehensive management of cancer[1]. Around 60% of all cancer patients receive radiation. Radiation therapy can be used as a standalone treatment in many cancers like prostate, head and neck, lung, cervix cancer etc., as a curative therapy. Radiotherapy is also used along with other treatment modalities like surgery and chemotherapy either before or after and thereby reduces the risk of cancer recurrence. Radiation therapy is also used as palliative therapy in many advanced cancers to reduce the pain and bleeding. Radiotherapy has undergone lot of advancements in recent times [2] but these advancements have been mainly related to physics part of the radiotherapy in terms of how accurately the dose can be delivered to the tumour at the same time minimizing the dose to the normal tissues. But beyond a point normal tissue damage cannot be avoided and these improvements cannot improve the radiation targeting. At this juncture, further improvements in radiotherapy can be contributed by studying the biological aspects governing radiotherapy. Understanding the biological underpinnings of cancer and normal tissue responses to radiation can lead to more effective and targeted treatment strategies[3].

Understanding the biological basis of radiation response can help in increasing the therapeutic window (Fig.1) and will be useful in developing more precise radiation therapy approaches[3]. The therapeutic window in radiotherapy of cancer refers to the range of radiation doses that can effectively treat the tumour while minimizing damage to surrounding normal tissues. It represents the balance between delivering a sufficiently high radiation dose to kill or control cancer cells and sparing healthy tissues to reduce treatment-related side effects. The aim of studying and applying biological principles in radiation therapy is to expand the therapeutic window[3].

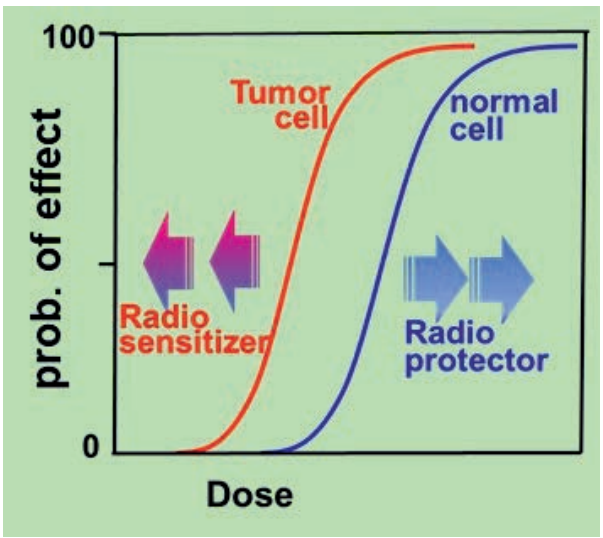


Figure.1: Dose vs effect curve for tumor cells and normal cells depicting the therapeutic window and the role of radiosensitizers and radioprotectors in widening the therapeutic window.

By studying the following approaches related to the radiation sensitivity of the tumours we can achieve expansion in therapeutic window.

a) Studying the radiation sensitivity of the tumours and developing predictive markers: Studying the radiation sensitivity of tumour cells is a crucial aspect of cancer research and treatment. Radiation sensitivity refers to how sensitive tumour cells are to the effects of ionizing radiation, such as X-rays or gamma rays. Since different tumours exhibit different levels of sensitivity to radiation, it is important to develop assays that can assess the level of this sensitivity before starting the treatment. This will help in better treatment planning, personalized medicine, predicting treatment outcomes and developing combination therapies.

b) Studying the molecular determinants of radiation resistance developed by tumour cells: Radiation sensitivity among tumour cells can vary significantly, and different types of tumours may exhibit different levels of sensitivity to ionizing radiation. This difference in radiation resistance exhibited by the tumour cells could be either an intrinsic or acquired phenomena. Intrinsic radiation resistance is the inherent ability of certain tumours to survive and grow despite exposure to ionizing radiation. Acquired radiation resistance is when tumours develop resistance to radiation following radiation therapy. This resistance can develop during the course of treatment and presents a significant challenge in cancer therapy. Studying the molecular determinants of radiation resistance in tumour cells is a critical area of cancer research. Understanding why some tumours are resistant may help in development of strategies to overcome resistance and improve treatment outcomes.

c) Developing drugs that can modulate the factors that contribute to radiation resistance: Overcoming radiation resistance can significantly enhance the effectiveness of radiation therapy and improve therapy outcomes. Developing drugs is a multifaceted process that requires collaboration between researchers, clinicians, pharmaceutical companies, and regulatory agencies. This drug development involves identifying molecular targets that are associated with radiation resistance, validating these targets using *in vitro* and *in vivo* approaches and developing small molecules that can modulate the target.

2. Modalities of cancer therapy:

Advancement in technology over the last few decades has led to considerable evolution in modalities for treatment of cancer and has markedly improved the outcome of cancer therapy. As described below, surgery, chemotherapy, and radiotherapy are extensively used cancer treatment modalities[4]. In addition to these, contemporary modalities include immunotherapy, hormone therapy, and dendritic cell-based immunotherapy

a) **Surgery:** This is the first-line treatment modality of early stage solid tumours with an aim for cure. It includes precise and minimally invasive surgery.

b) **Chemotherapy:** The term “chemotherapy” was coined by the German scientist Paul Ehrlich and this treatment modality was introduced shortly after World War II using nitrogen mustard. Chemotherapy is the most widely used cancer treatment modality used alone or in combination with surgery/radiotherapy.

c) **Radiotherapy:** During radiation treatment, high-energy ionizing radiation deposits energy in the cells it passes through and critical biomolecules of cells. Cancer cells having damaged DNA (deoxyribonucleic acid) are not able to divide which leads to cell cycle arrest and ultimately causes cells to die. However, during tumour radiotherapy,

radiation also causes damage to surrounding normal tissue. Radiotherapy is usually delivered in fraction of 2-4 Gy per sitting as this regimen offers survival advantage to normal cells over tumour cells by virtue of their intact DNA repair system which is triggered by sub-lethal doses of radiation.

Advanced imaging methods and precise targeting of the cancer tissue are being developed with the aim of delivering maximum dose to the tumour.

2.1. Technological advances in cancer radiotherapy:

a) **3D conformal radiotherapy (3DCRT):** It is based on CT imaging for accurate localization of tumour for optimal beam localization and shielding.
b) **Intensity-modulated radiotherapy (IMRT):** In IMRT, computer-controlled intensity-modulation of multiple radiation beams during treatment allows delivery of irregular-shaped radiation doses.
c) **Image-guided radiotherapy (IGRT):** In IGRT, pre-radiotherapy imaging before each treatment helps in avoiding errors such as missing tumour due to organ motion.
d) **Stereotactic body radiation therapy (SBRT):** In SBRT, very high doses are delivered precisely with limited treatment fractions to treat well-defined and small tumor lesions.
e) **Radiation-based surgical knife:**
   - **Stereotactic radiosurgery (SRS):** A small area of the body is exposed to a high dose of very focused radiations beam so that normal tissues are not damaged. It is largely used for treatment of brain tumours when conventional surgical techniques are difficult or unsafe.
   - **Gamma knife:** Multiple centred gamma radiation beams are combined to focus on tumour tissue to ultimately deliver an exceptionally high dose of radiation without a surgical cut.

3. Assessing the radiosensitivity of tumour cells:

Different tumours exhibit different sensitivity to radiation and hence their therapeutic response is different. If we can assess the radiosensitivity of the tumours before radiation therapy, optimal dose as per the radiosensitivity level of the tumour can be worked out in order to achieve good therapeutic ratio. Hence it is important to have a rapid and reliable assay that can be employed to predict tumour radiosensitivity. Clonogenic assay- an assay that measures cell's ability to consistently divide and form a colony, has been shown to assess the radiosensitivity of the tumours close to its original levels[5]. But clonogenic assay cannot be used under clinical settings for the following reasons:

a) Many times the tumours from the patient do not grow in the dish and form colony
b) Plating efficiency - ratio between number of colonies formed to the cell number that was plated. It is extremely low for many tumours.
c) It takes many weeks to obtain the result and hence time consuming.

So there is a need to develop a surrogate assay/marker that matches with the results of the clonogenic assay. In this regard different research groups are evaluating the role of different end points in evaluating the radiosensitivity[6]. Since DNA damage is the major cause of cell death caused by radiation, we have evaluated DNA damage caused by radiation as a marker of radiosensitivity. To address this question, we have selected different cancer cell lines and first established their radiosensitivity profile using standard clonogenic assay[7]. In clonogenic assay, as expected different cancer cells showed varying levels of radiosensitivity after exposure to different doses of radiation (Fig.2). Among the seven different cancer cells used, HT1080 (human fibrosarcoma cells) showed most radio resistance and HT29 (human colon cancer) showed least radiation resistance. Then we have estimated the extent of DNA damage caused by different doses of radiation in these cells using comet assay and correlated the extent of DNA damage with survival fraction obtained through clonogenic assay.

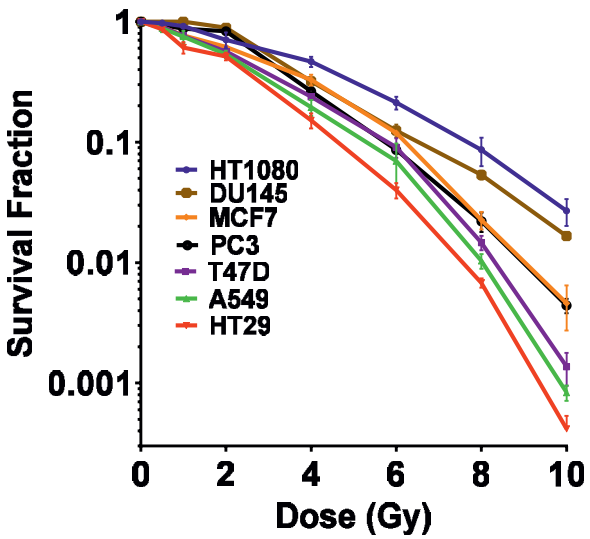


Figure.2: Radiosensitivity profile of different cancer cells as established by clonogenic survival assay.

We have used neutral and alkaline comet assay and found that DNA damage as obtained through neutral comet assay showed significant correlation with radiosensitivity as predicted by clonogenic assay, implying the predictive value of DNA double strand breaks and comet assay in predicting the radiosensitivity of cancer cells. Since radiation is given in fractionated form in clinical settings, we gave fractionated doses of radiation to cells and under fractionated radiation condition also, neutral comet assay showed very good correlation with the radiosensitivity estimated by clonogenic assay ($r = -0.97$), showing its usefulness in predicting radiosensitivity (Fig.3).

Cells respond to radiation induced DNA damage by activating certain genes involved in DNA repair and cyto-protection. Since DNA damage is showing good correlation with radiosensitivity, we studied the expression levels of these different DNA damage responsive

genes and studied their correlation with clonogenic radiosensitivity. Among many genes studied, we found that the gene expression signatures of Ku80, Rad51 and HSP70 showed significant correlation with radiosensitivity as well[7].

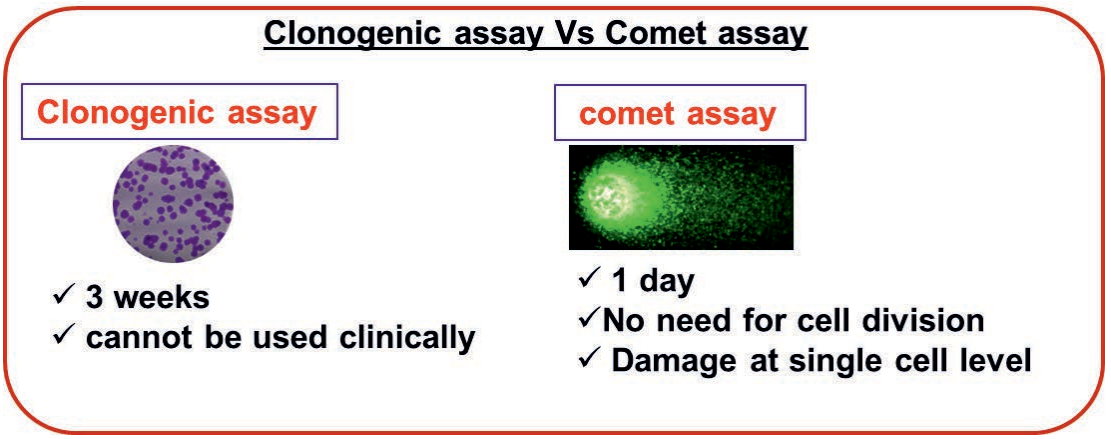


Figure.3: Advantage of comet assay over clonogenic assay in predicting radiosensitivity of cancer cells.

4. Mechanisms involved in radiation resistance:

Radiation resistance in tumour cells is a significant challenge in radiation therapy, and it can limit the effectiveness of this treatment modality[8]. Studying the molecular determinants of radiation resistance is crucial for tailoring radiation therapy to individual patients, improving treatment outcomes, reducing toxicity, and advancing our understanding of cancer biology. It is an essential component of personalized cancer care and has the potential to significantly impact cancer treatment strategies[1].

4.1. Role of Nrf2 in radiation resistance: To determine the molecular determinants of radiation resistance, we used PC3 and DU145 cells. Despite having the same tissue of origin, these two androgen independent prostate cancer cells showed difference in radiosensitivity[9]. DU145 cells showed more survival in clonogenic assay and less DNA damage in comet assay as compared to PC3 cells after radiation exposure (Fig.4).

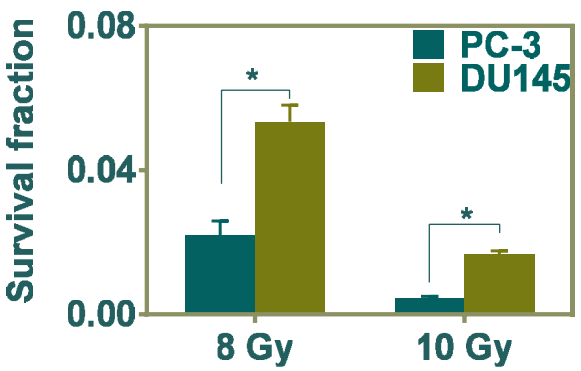
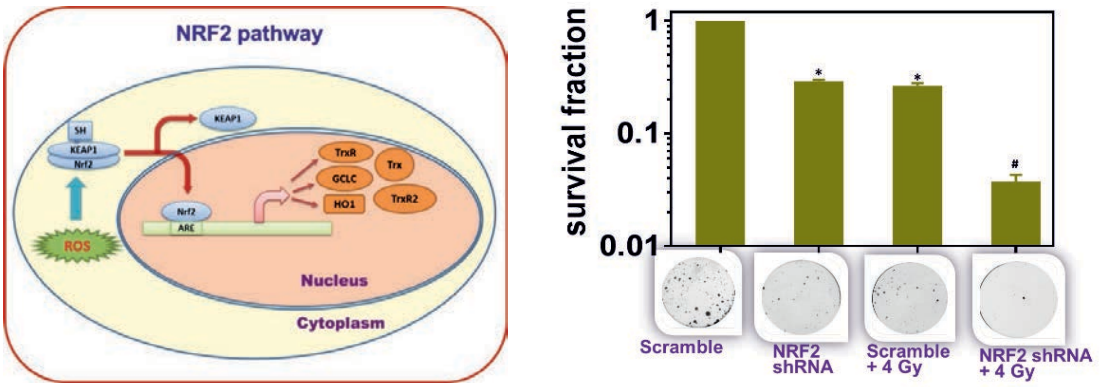


Figure.4: Differential radiosensitivity exhibited by two prostate cancer cells in clonogenic assay

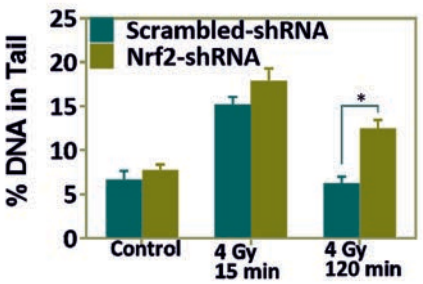
We performed a microarray analysis to find out the global change in the gene expression between these two cells and analysed the differentially expressed genes. In DU145 cells thioredoxin pathway genes, genes involved in redox regulation and Nrf2 dependent genes were differentially expressed. Nrf2 is bound to an inhibitor protein called Keap-1 under normal condition and during oxidative stress conditions, they get translocated to the nucleus and transactivate genes involved in anti-oxidant response and cyto-protection (Fig.5A)[10]. When we estimated the levels of Nrf2, DU145 had more Nrf2 than PC3.



(A) (B)

Figure.5: Scheme depicting the Nrf2 pathway (A) and knocking down of Nrf2 increases the radiosensitivity (B)

Further, pharmacological inhibition and knockdown of Nrf2 in DU145 cells led to significant increase in radiosensitivity, validating the causal role of Nrf2 in radiation resistance (Fig.5B). Moreover, in our studies, DU145 cells showed increased DNA repair capacity than PC3. Hence, we studied the relationship between Nrf2 and increased DNA repair [11] and (Fig.6A) found a novel role of Nrf2 in DNA repair.



(A)

Name of the gene	Matching sequence of the ARE sequence	Position of matching sequence
RAD51	GTGACTCAGCAG	15822-15835
DMC1	GTGACTCAGCA	6614-6624
RBBP8	GGTGACTCAGCA	1878-1867
RAD51C/XRCC3	GGTGACTCA	2406-2398
RAD52	GTGACTCAG	1819-1827

(B)

Figure.6: Repair of DNA damage estimated after knocking down Nrf2 (A). The list of genes involved in DNA repair with ARE sequences in upstream of the gene (B).

Further, we analysed the upstream sequences of all the known DNA repair genes. Several genes of homologous repair possessed Antioxidant Responsive Elements (ARE) in upstream regions (Fig.6B). ARE elements being the consensus regions situated in the upstream regions of the genes where Nrf2 binds and transactivate their expression. DNA repair genes were down regulated in Nrf2 knockdown cells confirming the role of Nrf2 in DNA repair through its influence on HR pathway[11]. This finding can be exploited in Nrf2 mutated cancer cells by treating them using PARP1 inhibitors to induce synthetic lethality.

4.2. Role of NF-κB in radiation resistance: NF-κB is a pro-survival transcription factor, which regulates the expression of more than 200 genes [12]. Increased basal levels of active NF-κB is associated with resistance of tumour cells to killing by chemo and radiotherapy[13]. We observed that NF-κB plays a central role in regulating responses of normal cells to radiation exposure. We observed that repair of radiation induced DNA damage was faster in cells exposed to radiation in vitro as compared to cells obtained from irradiated mice. Mechanistically it was found that NF-κB activation was increased and caspase activation was less in cells exposed to radiation *in vitro* as compared to *in vivo* [14].

4.3. Role of cellular redox balance in radiation resistance:
Redox homeostasis is a critical aspect of cancer cells wherein ROS have been shown to exert paradoxical effects on cancers progression and metastasis. Elevated ROS levels are known to stimulate tumorigenesis and help in cancer progression, whereas excessive ROS levels result in cell death[15]. Although, high ROS levels are a characteristic of cancer cells, they modulate their redox regulatory systems to combat oxidative stress and this tight regulation of redox balance is essential for their survival. In this direction, studies were performed to comprehend the role of redox regulatory systems in regulating tumour radio-resistance. Tumour cells were found to respond to radiation exposure in a biphasic manner i.e. early (0–6 h) and late (16–48 h) responses. The constitutive glutathione (GSH) and thioredoxin (Trx) systems contribute towards restoring the redox balance during the early phase and also activate redox-sensitive transcription factor Nrf2. We observed an increase in the levels of GSH and Trx during the late phase which mitigates radiation induced damage and disruption of these pathways hamper the ability of cells to counter radiation induced damage. Interestingly, in cancer cells, disruption of these pathways increased the sensitivity of cells to radiation induced cell death. These findings highlight the importance of cellular thiols and antioxidant pathways in regulating the responses of cells to radiation-induced redox imbalance[16].

In another study, we elucidated the mechanism of acquired radiation resistance in lung cancer cells. The radiation resistant (RR) lung cancer cells exhibited enrichment of cell with cytosolic ROS, increase in redox regulatory transcription factor Nrf2 signalling and cell cycle delay as compared to parental cells. This study identified a novel subpopulation of radio-resistant cells, which exhibit radio-resistance by maintaining high levels of ROS along with increased Nrf2 signaling. These results also indicate the putative application of Nrf2 pathway as a druggable target to overcome radio-resistance of lung cancer cells[17].

5. Strategies to overcome radiation resistance:

5.1. Synthetic molecules: After identifying the targets that determine radiation resistance, it is important to identify molecules and drugs that can be used to modulate the targets[18-19]. In our studies we have identified the role of Nrf2 pathway and its dependent genes in radiation resistance. TRXR catalyses the reduction of thioredoxin and thereby play crucial role in maintaining redox balance in the cells[20]. TRXR is present in two isoforms and one is present in cytosol (TRXR1) and another one is in mitochondria (TRXR2). TRXR is overexpressed in many cancers and is responsible for radiation resistance. Studies show that curcumin can be a good inhibitor of TRXR. But curcumin has less stability and low bioavailability in the cells. Hence we used dimethoxycurcumin (DIMC)-a stable analogue of curcumin with better bioavailability, to evaluate its ability to inhibit TRXR. In a cell free and cellular system, treatment with DIMC inhibited TRXR (Fig.7). Further, DIMC treated cells caused synergistic increase in radiation sensitivity of different cancer cells like A549, MCF7 and PC3 cells[21]. The DIMC treatment led to increased apoptosis and increased caspase activation. Further, we overexpressed thioredoxin in the cells followed by DIMC treatment. In the cells overexpressed with thioredoxin showed significant reversal in DIMC mediated radiosensitization. We also did a molecular docking study in which the binding of DIMC was studied with TRXR. The free energy change indicated the strong binding of DIMC with the active site of TRXR meaning that DIMC could be a potent inhibitor of TRXR.

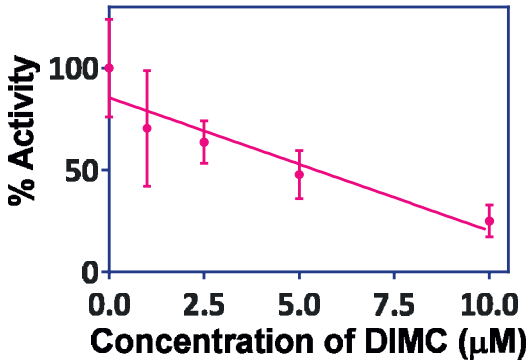


Figure.7: Dose dependent inhibitory effect of DIMC on TRXR in a cell free system

5.2. Natural molecules: Troxerutin, found naturally in several vegetables, is known for various beneficial biological activities [22]. In view of its ability to protect normal cells against radiation damage, we explored its effects on radiation induced killing of tumour cells. We observed that Troxerutin could bind strongly to DNA in vitro via interaction with DNA minor groove which was supported by computational studies which also indicated docking of troxerutin with mammalian DNA. Interestingly, treatment of cancer cells with Troxerutin prior to radiation exposure, could enhance radiation induced cancer cell death. This ability of Troxerutin to increase radiation induced cancer cell death was found to be due to its ability to synergistically augment radiation induced generation of ROS in radio-resistant cells along with its ability to induce strand breaks via its interaction with DNA minor groove[23].

As discussed in previous section, NF-κB activation plays a role in radio-resistance by upregulation of genes involved in suppression of cell death. Hence, it is prudent that

suppression of NF-κB signaling could help overcome radiation resistance in cancer cells. In this direction, we employed Curcumin (diferuloylmethane), which is a major constituent of the yellow spice turmeric. We found that Curcumin inhibited clonogenic potential of cancer cells exposed to radiation (Fig. 8). Curcumin was able to suppress radiation induced activation of NF-κB signaling pathway. Curcumin also inhibited the activity of multiple activators of NF-κB signaling pathway such as IKK activity and Akt phosphorylation. This resulted in decreased expression of NF-κB dependent genes responsible for radio-resistance such as anti-apoptotic proteins. These studies revealed that curcumin can be used to overcome inducible radiation resistance pathways in cancer cells[24].

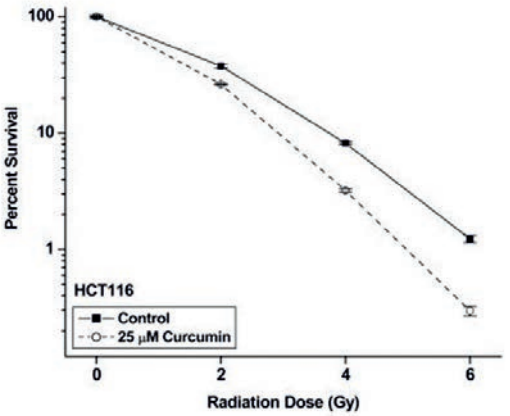


Figure.8: Curcumin enhances radiation-induced cytotoxicity in colon cancer cells (HCT 116)

6. Conclusions and future perspectives:

The radiation resistance of tumour cells remains a formidable challenge in the field of radiation oncology. Prediction of radiation sensitivity prior to radiotherapy can be helpful in personalization of radiotherapy and hence it is important. We studied the correlation between DNA damage caused by radiation and radioresistance. To study DNA double strand breaks, comet assay was used which is quick, reliable and sensitive technique that can be a very good alternative to clonogenic assay. The extent of DNA damage assessed by comet assay and the gene expression signatures of certain DNA repair genes showed significant correlation with radiation sensitivity of tumour cells. Further validation of the usefulness of comet assay and gene expression signatures in patient samples in prediction of radiosensitivity will help in further deployment of these assays and markers in clinical settings. Apart from prediction, understanding the mechanisms and pathways involved in radiation resistance is also very important in combating radiation resistance in tumour cells. Nrf2 regulates cellular antioxidant response and we found a role of Nrf2 and thioredoxin reductases in radiation resistance. In many cancers, Nrf2 is hyperactivated and excessive Nrf2 activation has been associated with drug resistance. Targeting Nrf2 can be a prudent strategy to killing cancer cells. The Nrf2 inhibitor development is still in its infancy and we do not have any Nrf2 inhibitor that is approved for clinical use. One of the major reasons for this bottleneck is that it is difficult to develop inhibitor against Nrf2 as it does not have active site or deep groove structure. Moreover, all the available inhibitors have the issue of non-specificity and normal tissue toxicity. Hence, we have targeted one of its dependent protein TRXR and developed

inhibitors. TRXR is also known to be overexpressed in many cancers and is linked with radiation resistance in patients and dimethyl curcumin could effectively inhibit TRXR and thereby increase the radiation sensitivity of the tumour cells. Further experiments in in vivo models and its safety study will be important for taking this molecule further up in the drug development ladder.

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