Development of customized strategies for improving the efficacy of cancer radiotherapy

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Abstract
We have developed DNA damage and DNA damage based gene expression as predictive markers of radiosensitivity in cancer cells which would find application in customized radiotherapy. We have identified Nrf2 as a target for modulating radiosensitivity in cancer cells and normal cells. We have also developed Nrf2 centric drugs for mitigation of radiation injury.

Introduction
Cancer is one of the major causes of death worldwide. Radiation therapy is an important treatment modality employed for cancer. But due to the inter-individual differences in radiosensitivity of cancer cells, the efficacy of the radiotherapy is affected. Further, normal tissue toxicity is also a major concern to the clinicians during radiotherapy. The efficacy can be improved by prediction of radiosensitivity of cancer cells and through modulation of radiation responses of normal and cancer cells. Clonogenic assay is considered as gold standard for predicting radiosensitivity. However, this assay cannot be used under clinical settings because the tumor samples derived from patients seldom grow in a petri dish to form colonies. Hence there is a need for development of rapid and reliable assay for predicting the radiosensitivity, which will help in designing personalized radiotherapeutic regimen. Apart from prediction, modulation of radiosensitivity is also important for improving the outcome of radiotherapy. For modulation of radiation response, understanding the molecular players governing radiosensitivity of cell is pivotal. Drugs which target the proteins that are contributing to radioresistance of cancer cells can be effective sensitizers and the drugs which activate survival proteins specifically in normal cells can be potential radioprotectors. When cells are exposed to gamma radiation, free radicals are formed as a result of water radiolysis and these free radicals in turn cause damage to cellular macromolecules like protein and DNA. Damage to DNA (double strand breaks) is the most critical lesion, which if unrepaired can lead to cell death. Cells activate many signaling mechanisms and repair pathways which help in salvaging the DNA damage. These factors can be used as targets for predicting and modulating the radiosensitivity. One ubiquitous transcription factor which gets activated during the radiation exposure is Nrf2. Under normal conditions, this transcription factor is sequestered in the cytoplasm and during oxidative stress, Nrf2 is translocated to the nucleus and thereby transactivates the target genes which are involved in anti-oxidant response leading to the survival of the cell. Targeting Nrf2 can be a good strategy for radiosensitization of tumor cells and radioprotection of normal cells. Drugs which target this molecule can be good radio-modulators.

Materials and Methods
Tumor cells were obtained from National Centre for Cell Science, Pune. Chemicals and reagents were procured from Sigma-Aldrich.

Clonogenic assay
Clonogenic assay determines the ability of a cell to divide multiple times and form a macroscopic colony. Tumor cells were cultured in the petri dish for 12 h and then they were exposed to radiation. They were further cultured for two weeks for the colony development. The colonies were fixed using
methanol, stained with crystal violet and counted using a colony counter.

Assessment of DNA damage by comet assay
When a cell containing DNA damage is subjected to electrophoresis the movement of DNA will be higher as compared to that from control cell. Comet assay measures the DNA damage using this principle. For this assay, the cells are mixed with agarose gel and layered on to the slide. After solidification, the cells were lysed using lysis buffer, and electrophoresis was carried out in Tris-Borate-EDTA buffer. The cells were stained with a SYBR-Green dye (a DNA binding fluorescence dye), visualized using fluorescence microscope and the images were used for calculating the extent of DNA damage by analyzing the tail formation.

Assessment of DNA damage by gamma-H2AX foci
H2AX is a histone variant found as a part of the nucleosome, on which DNA is coiled around. When the DNA is damaged, this histone gets phosphorylated and it can be detected as foci using antibody staining. By analyzing the number of foci present at different time points after radiation, the extent of DNA repair was studied. For performing this assay, cells were grown on a coverslip, given respective treatments, fixed using 4% paraformaldehyde, permeabilized by treating with Triton-X-100, stained with γ-H2AX antibody and secondary antibody labelled with fluorescent dye. The cells were counterstained with DAPI and visualized under fluorescence microscope and average number of foci present in each nucleus was counted as a marker for DNA damage.

Results and Discussion
Correlation between clonogenic assay and comet assay for predicting the radiosensitivity of tumor cells
To know the usefulness of comet assay in predicting the radiosensitivity of tumor cells, the radiosensitivity profiles of seven different cancer cells were established using clonogenic assay indicating their respective survival fraction. The radiation induced DNA damage was quantified using comet assay (% DNA in tail). The correlation between survival fraction and % DNA in tail was calculated and we found that these two parameters showed significant correlation (Fig.1). This correlation was also valid under fractionated dose regimens. Out of several time points analysed, the comet assay performed immediately after irradiation showed best correlation with radiosensitivity. Moreover, neutral comet assay, which preferably assesses DNA double strand breaks showed better correlation with radiosensitivity than the alkaline comet assay which quantifies total DNA damage (single and double strand breaks). In conclusion, our results established that the neutral comet assay performed immediately after radiation exposure can be a good predictive marker for radiosensitivity.

![Graph showing correlation between survival fraction and DNA in tail](image)

Fig.1: Correlation between clonogenic assay (survival fraction) and neutral comet assay (% DNA in tail) was obtained in seven different cancer cell lines.

Role of Nrf-2 in radioresistance of cancer cells
Apart from predicting radiosensitivity, understanding the underlying molecular mechanisms is also important for identifying druggable targets in the cells. For this purpose, a microarray analysis was performed in two cancer cell lines which exhibited differential radiosensitivity measured in terms of survival fraction and apoptosis. From the microarray data, the genes which are differentially expressed in radioresistant cells were identified. Thioredoxin family of genes was upregulated in radioresistant cells as compared to radiosensitive cells. Since thioredoxin family genes are regulated by Nrf2, we analysed the expression of Nrf2 in these cells. The radioresistant cells showed higher expression of basal as well as radiation induced Nrf2. Accordingly, the levels of radiation induced reactive
oxygen species were higher in radiosensitive cells as compared to radioresistant cells. To further confirm the role of Nrf2 in radioresistance, we have used chemical inhibitor (all-trans retinoic acid) or knocked down approach by shRNA and then examined their effect on the radiosensitivity of cancer cells. Pharmacological inhibition or knockdown of Nrf2 synergistically enhanced the radiosensitivity of cancer cells implying that Nrf2 overexpression in tumor cells can lead to radioresistance.

**Involvement of Nrf2 in DNA repair**

To further understand the possible mechanism through which Nrf2 is regulating radioresistance, the effect of Nrf2 on DNA repair was studied. When Nrf2 was inhibited or knocked down, DNA repair was slowed down, as found by γ-H2AX foci (Fig.2). The influence of Nrf2 on DNA repair was not dependent on its downstream genes involved in antioxidant activity. This was confirmed by studying the DNA repair in the presence of a known anti-oxidant N-acetyl-cysteine. Further, involvement of the non-homologous end joining pathway was ruled out by combining DNA-PK inhibitor along with Nrf2 inhibitor. When the upstream regions of DNA repair genes were probed, RAD51 family of genes (involved in homologous DNA repair) showed

![Fig:2. Effect of Nrf2-knockdown (Nrf2-KD) on DNA damage repair as compared to control (DMSO) as analysed by γ-H2AX foci formation.](image)

![Fig:3. Role of Nrf-2 in the DNA repair and in radioresistance of cancer cells.](image)
the presence of antioxidant response elements (ARE) in their promoter region. The expression of RAD51 family of genes was decreased in Nrf2 knockdown cells, suggesting that the Nrf2 is involved in DNA repair through the homologous recombination repair. Hence our results clearly indicated that apart from involvement in antioxidant response, Nrf2 is also modulates DNA repair and thereby contributes to radiation resistance in cancer cells (Fig.3).

**Development of new drugs for modulation of cellular radio-sensitivity**

Many phytochemicals and their derived compounds were screened to modulate the radiosensitivity of cancer cells and normal cells. We found that dimethoxy curcumin (DIMC), a synthetic analogue of curcumin, modulated radiosensitivity of cancer cells. The bio-availability and stability of DIMC is more as compared to curcumin. Our studies revealed that DIMC at 2.5 µM concentration increased the radiosensitivity of the cancer cells and cancer stem cells. This effect was due to inhibition of thioredoxin reductase- an Nrf2 dependent gene. We have also identified another drug which is a derivative of green plant pigment chlorophyll (BARC Radio Modifier-BRM). Preclinical studies using BRM demonstrated its radioprotective efficacy against lethal doses of radiation in mice. Safety and toxicological studies in multiple species of rodents have been completed under GLP conditions. BRM is well tolerated up to 5g/Kg body weight. This technology has been incubated with Innovative Drug Research Solutions Ltd., Bangalore. The formulation has been optimized and 2 lakh tablets have been manufactured under GMP conditions for human clinical trials.

**Conclusion**

Our efforts have identified a simple, fast and efficient protocol for predicting the radio-sensitivity of patient derived cancer cells. This protocol will be useful for customized radiotherapy. Further, we have identified Nrf-2 and thioredoxin reductase as druggable targets for modulation of radiosensitivity. Based on these studies, we have developed new radio-modifying drugs for use in clinic.

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