Chapter 7

Summary & Conclusion
The main findings of the study are as follows –

- We have successfully established, three radioresistant sublines from their parental oral cancer cell lines of different oral subsites i.e. two tongue and one buccal mucosa cell line.

- These radioresistant sublines show increased $D_0$ values compared to their parental cells and expressed some of the reported radioresistance related markers that validate its acquired radioresistance character and make them a prospective in-vitro podium for studying acquired radioresistance related molecular changes.

- To the best of our knowledge, this is the first such study from India regarding the establishment of radioresistant sublines by clinically admissible low dose fractionated ionizing radiation.

- The differential proteomic profiling of these radioresistant cells compared to their respective parental cells by two dimensional gel electrophoresis followed by mass spectrometry analysis has identified 102 differentially expressed proteins.

- Expression of some of the mass spectrometry based identified proteins was validated by western blotting.

- The standard PD quest analysis on the three technical replicates for each of the parental and radioresistant set of cells provided groups of differentially expressed proteins in all the sets that belong to different cellular functions; affirming the probable role of distinct pathways related to radioresistance.

- Further, a total of 08 proteins were found to be common among all the three parental and radioresistant set of cells. On validating the same by western blotting, it was found that STIP 1 and PKM 2 are common up & down regulated in all the three set of cells; thus are worthy of further analysis.
- Also, among these eight common proteins; STIP 1, PKM 2 and PGP expression were validated by western blotting in two of the radioresistant set of cells that belong to different oral subsites. The expression of six proteins (out of eight) was found to be validated in one of the established radioresistant set of cells; thus implying their possible association with cellular radioresistance.

- Apart from this, there are array of proteins that does not came as common among all the three set of parental and radioresistant cells (may be due to dissimilar response of these different set of cells towards radiation), but they can also be the prospective molecules for further investigations.

- Two of the established radioresistant cells exhibit elongated morphology, less cell to cell contact, increased filopodia on cell surface and markers associated with epithelial to mesenchymal transition; suggesting the acquisition of EMT phenotype in the radioresistant cells.

- One of the established tongue radioresistant cells show increased migratory and invasive behaviour compared to its parental cell. Further, from its differential 2D profile; an actin binding- ERM family member protein Moesin was found to be up regulated, which is further validated by western blotting.

- Increased co-expression of Moesin with its cell surface receptor CD44 was also found at the leading migratory edges of these radioresistant cells that hint towards its role in the acquired migratory and invasive behaviour of these cells.

- siRNA mediated knock down of Moesin protein in these radioresistant cells resulted in its decreased migration and invasion, thus associating the role of Moesin protein with the acquired migratory and invasive property.
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- Further, Moesin knockdown also results in the reduction of the acquired radioresistance character of these cells compared to the control siRNA treated cells. To the best of our knowledge, this is the first such study showing the role of Moesin with the cellular radioresistance and therefore makes it one of the target proteins for further studies.

- All the radioresistant cells exhibited increase in soft agar colonies (both in area and number of colonies) and suggest the acquisition of anchorage independent growth property in these cells.

- These radioresistant cells also exhibit increased ability to form 3D spheroids compared to their respective parental cells. Spheroid forming potential is one of the properties that are reported to be associated with stem cell features.

- All the established radioresistant cells exhibit increased expression of stem cell associated markers. Therefore, these observations i.e. anchorage independent growth, spheroid formation & stem cell markers together suggest the enrichment of stem cell like properties in them, post multiple doses of fractionated ionizing radiation.

- Finally, these radioresistant cells also show increased tumorogenic potential in immuno-compromised mice compared to their respective parental cells. This is the gold standard method to test the stemness and thus suggests the enrichment of cancer stem cell like features in these established radioresistant cells.

- Distinct Raman spectral profile for two of the established radioresistant sublines of buccal mucosa origin suggest the feasibility of Raman spectroscopy as a potential non-invasive tool for predicting radiation response through spectral markers in oral cancer patients.
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- To the best of our knowledge, the Raman spectroscopic study is the first report that represents the utility of Raman spectroscopy in acquired radioresistant oral cancer sublines, established from parental oral cancer cell lines by clinically administered fractionated ionizing radiation. As, other reported studies were related to radiation induced biochemical changes in cancer cell lines at single doses of radiation only.

- The cDNA microarray profile of two of the established radioresistant sublines (tongue & buccal oral subsites) gave 1190 and 1378 as significantly up regulated while 750 and 1152 as down regulated genes with > 2 fold change respectively.

- The microarray result validated by real time PCR; done independently on the cDNA of parental and radioresistant cells for some of the randomly selected differentially expressed genes.

- To interpret the biological functions of the differentially expressed genes; the functional annotation of the same was carried out by three different curated databases i.e. Panther, Reactome & KEGG for both the parental and radioresistant set of cells.

- Panther analysis revealed pathways related to Angiogenesis, Apoptosis signalling, Plasminogen activating cascade & p38 MAPK pathways as significantly up regulated while Wnt signalling, cadherin signalling as significantly down regulated pathway in radioresistant cells.

- Reactome analysis identify; Hemostasis, Integrin cell surface interactions, Telomere Maintenance as significantly up regulated, while Signaling in Immune system pathways as significantly down regulated in the radioresistant cells compared to the parental cells.

- The KEGG enrichment analysis revealed; Pathways in cancer, Focal adhesion, mTOR signalling, Cell cycle, Chemokine signaling, Insulin signaling and ECM-receptor interaction,
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Cytokine-cytokine receptor interaction and Toll-like receptor signaling pathways as significantly upregulated. Whereas, Cell adhesion molecules, Hedgehog signalling, Nucleotide excision repair, Ubiquitin mediated proteolysis and TGF-beta signaling pathways as the significantly down regulated pathways.

- This integrated analysis from three different databases on two different set of established radioresistant set of cells may help in understanding the underlying molecular differences between parental and radioresistant oral cancer cells.

In conclusion, our findings indicate that clinically admissible fractionated ionizing radiation induced different in-vitro characteristics will help in understanding radioresistance in oral cancer. The identified molecules both at proteomic & transcriptomic levels and spectral profiles may have important therapeutic implications in the treatment of oral cancer.