4. GENERAL DISCUSSION

Despite considerable advances in the areas of genomics, proteomics and metabolomics, targeting tumor specific genotype/phenotype for developing effective therapy of cancer continues to be a challenge to the biomedical scientists. While some success has been achieved in targeting specific genotypic/phenotypic alterations in a limited number of tumors, more efforts are required in developing effective therapies for many tumors. Otto Warburg postulated that change in metabolism as the fundamental phenotypic change as a cause of cancer (Warburg, 1956), a claim now known as the Warburg hypothesis and forms a part of the metabolic reprogramming that is widely discussed today (Lu and Thompson, 2012, Soaga 2013, Wu and Zhao 2013). Mutations in oncogenes and tumor suppressor genes are known to be responsible for malignant transformation and the Warburg effect has been considered to be more a result of these mutations and an associated characteristic of cancer cells, than a cause (Bertram, 2000 Grandér, 1998). However, emerging evidences suggest that metabolic reprogramming may need drive the process of carcinogenesis, rather than being a consequence of it (Bertram, 2000). These changes in metabolism of cancer cells viz. enhanced glycolytic pathway has been used as a biochemical basis for the diagnosis of malignant lesions using 18FDG based Positron Emission Tomography (PET) scan as well as the design of therapeutic strategies to preferentially kill cancer cells by inhibitors of glycolysis, where cytotoxicity as well as radio- and chemo-sensitization of tumor cells has been reported in vitro (Averill-Bates et al., 1994; Jelluma et al., 2006; Lee et al., 1997; Coleman et al., 2008). However, the utility of this approach particularly with reference to variations in other biologic behavior of tumors needs to be critically examined.

2-DG inhibits the growth of neoplastic cells and enhances treatment-induced death in vitro (Dwarakanath & Jain, 1989, Zhang et al., 2006; Aft et al., 2002), by exerting cytotoxic effect in cancer cells (Dwarakanath 2009), while sparing the normal cells (Jain et al., 1979; Kalia et al., 1982). Many mechanisms have been postulated to contribute to the antitumor effect of 2-DG, including inhibition of glucose transport (Nelson et al., 1996) and hexokinase II activity, depletion of cellular ATP, blockage of cell cycle progression, induction of apoptosis (Maher et al., 2004), induction of
endoplasmic reticulum stress (Heminger et al., 2006; Pahl et al., 1996), and/or induction of oxidative stress (Lin et al., 2003; Coleman et al., 2008; Simons et al., 2007). 2-DG sensitizes cancer cells to radiation through mechanisms such as inhibition of DNA repair processes and recovery from potential lethal damage (Dwarkanath et al., 2001; Jha et al., 1993), and induction of oxidative stress (Lin et al., 2003; Coleman et al., 2008).

There is ample evidence supporting the hypothesis that glycolytic inhibitor like 2-DG leads to an inhibition of DNA repair pathways after exposure to radiation due to impairment of ATP production and energy metabolism (Jain et al., 1982). However, effects on various other cell signaling pathways appear to be equally, if not more important than the inhibition of DNA repair that is responsible for radio- and chemosensitization (Dwarakanath, 2009). Radiosensitization has also been suggested to be due to disruption of thiol metabolism resulting in oxidative stress related cell death in the form of apoptosis (Lin et al., 2003) that could be reduced by the addition of N-acetyl cysteine (Coleman et al., 2008) and enhanced by inhibiting glutamate cysteine ligase activity (Andringa et al., 2006). Alterations in the expression of many genes involved in damage response pathways including DNA repair and apoptosis, transcriptional regulators, cell signaling, besides energy metabolism has been reported that could significantly influence the radiosensitization of tumor cells (Heminger et al., 2006). A robust UPR is also induced by 2-DG that contributes to the radiosensitization (Heminger et al., 2006). The 2-DG induced enhancement of radiation damage has been found to be directly proportional to the glucose usage, presence of hypoxia and doses of 2-DG and radiation (Dwarkanath et al., 2001, Khaitan et al., 2006). Clinical trials (phase I/II) using a combination of hypofractionated radiotherapy and oral administration of 2-DG on human gliomas have been shown to be well tolerated with minimal acute and late radiation effects (Mohanti et al., 1996, Singh et al, 2005). Improvement in survival and quality of life have also been reported (Singh et al, 2005; Dwarkanath et al, 2009), which may possibly linked to protection of normal tissues (Prasanna et al, 2012).

Tumor suppressor p53 plays a role in energy metabolism by regulating metabolic processes (Kern et al., 1987). p53 stimulates oxidative phosphorylation after sensing
decrease of ATP through up regulation of the synthesis of the cytochrome C oxidase 2 (SCO2) gene that encodes a copper chaperone protein required for the assembly of mitochondrial cytochrome c oxidase (complex IV) (Matoba et al., 1990; Liu et al., 2002) as well as transcriptional activation of subunit I of cytochrome c oxidase (Bensaad et al., 2007). Furthermore, p53 activates TP53-induced glycolysis and the apoptosis regulator (TIGAR), which functions to direct glucose to the pentose phosphate pathway (PPP). The increase in PPP results in the stimulation of nucleotide synthesis and production of NADPH, which is an important component of the antioxidant defense system (Bensaad et al., 2006). In addition to the role of p53 in the regulation of mitochondrial respiration, p53 inhibits glycolysis by repressing the transcription of GLUT1, GLUT4 genes that encode glucose transporters (Okamura et al., 1999) and the phosphoglycerate mutase (PGM) gene that encodes a glycolytic enzyme responsible for the rearrangement of phosphoglycerate (Kondoh et al., 2005). Mutation(s) leading to alterations in the functioning of p53 is expected to disturb the p53 mediated regulation of a balance between oxidative phosphorylation and glycolysis, as well as the redox balance. Such mutations are likely to influence the viability and proliferation of cells as well as the response to the inhibition of glycolysis (caused by inhibitors like 2-DG). Therefore, a good understanding about the response of tumor cells that harbor such mutations (or any other changes in the p53 status) to a combined treatment of 2-DG and anticancer therapeutics (like radiation and chemotherapeutic drugs is likely to help in designing therapeutic protocols for enhancing efficacy as well as individualizing the treatment. It is highly desirable that such studies deploy models that are realistic with reference to the clinical scenario rather than simplistic systems. Towards this end, the present studies employed cells that carry both wild and mutated p53 with mutations in certain regions. Indeed some of these genotypes (and phenotypes) are reported to be prevalent in oral cancers analyzed from patients (Madan et al., 2011). Recent studies have shown that a combination of ionizing radiation and adenoviral p53 gene therapy can enhance the radiosensitivity of both p53-mutant and wild-type cancer cells (Harris et al., 1996; Nielsen et al., 1998; Colletier et al., 2000).
Results of the present studies show that both the mutant cell lines (KB 68 and KB 110) exhibit enhanced oxidative stress in the form of elevated ROS levels (Fig 3.1.6) and increase in NADP+/NADPH ratio (Table 3.1.8) suggesting alterations in the redox balance possibly due to disturbed balance in the p53 mediated regulation of mitochondrial function (including ROS generation) and glycolysis linked anti-oxidant defense. This enhanced oxidative stress resulted in a reduction in the clonogenic efficiency of the mutant cells, where moderate reduction (20 -30 %) in the clonogenic capacity (plating efficiency) was noted. However, there could be other contributions besides enhancement in the oxidative stress, which needs to be investigated further. Irrespective of the reasons for reduced clonogenic proficiency, these observations suggest that tumors with such geno/phenotype may occasionally show auto-regression. This however, needs to be systematically investigated in animal tumors.

Cancer cells have a fundamental defect in their electron transport chains, leading to increased ROS production, relative to normal cells, for this cancerous cells have to increase their glucose metabolism to enhance the metabolic decomposition of ROS formed from defective mitochondrial electron transport chains (Spitz et al., 2000). Glucose deprivation seems to be more severe in cancer cells than normal due to higher oxidative stress produced by defective mitochondrial respiration. Exposure of radiation causes free radical formation, which also contribute in formation of ROS species. We observed that endogenous ROS was higher in p53 mutant cell lines and treatment with the combination (i.e. 2-DG and radiation) further enhanced ROS production particularly in both the mutant cells (KB68 and KB110) (Fig 3.2.8b). These results support the proposition that mutation in p53, glucose deprivation and irradiation together play a major role in the accumulation of higher ROS in these (mutant) cells subjected to the combined treatment (2-DG + radiation) (Fig 3.2.8b) resulting in a higher cell killing in both the mutant cell lines as compared to p53 wt KB cells. Thus, it appears that combining 2-DG with ionizing radiation may be beneficial in enhancing the tumors that carry geno/phenotypes with reference to p53- as studied here (p53 mutations at proline rich regions). Whether this is applicable in tumors with all types of mutations in the p53 status or is restricted to tumors that are similar to KB68 and KB 110 needs to be systematically investigated. Undoubtedly
this understanding will be helpful in individualizing therapy using 2-DG (or other glycolytic inhibitors) as adjuvant in radio- and/or chemotherapy.

An increase in the radiation-induced micronuclei expression (manifestation of chromosomal damage in post-mitotic daughter cells) caused by 2-DG in wt, 68 and 110 cells observed in the present studies suggests that 2-DG disturbs the repair of radiation-induced DNA damage resulting in enhanced chromosomal damage, thereby enhancing the mitotic death. This indeed is expected as 2-DG induced DNA repair has been widely reported (Jain et al, 1982, Kalia et al, 1982, Dwarakanath & Jain, 1989, Dwarakanath et al, 2001, Dwarakanath 2009). These results suggest that mitotic death (which is linked to cytogenetic damage) has a significant contribution in enhanced radiation-induced cell death by 2-DG in all the three cell lines. However, this does not seem to be strongly influenced by the p53 status, suggesting thereby that the repair of DNA damage influencing the residual damage (responsible for mitotic death linked to cytogenetic damage) is perhaps less susceptible to alterations in the p53 status. Whether this is true for tumor cells with all types of p53 mutations or valid only for the type of mutations studied here needs further investigations.

Treatment induced oxidative stress has the potential to cause cell death by inducing apoptosis, which has been exploited in many anticancer therapies. The susceptibility of tumors to this approach depends both on the phenotype (including the metabolism) and microenvironment of the tumor cells as well as the nature of the anticancer therapeutics. Low LET ionizing radiation induced cytotoxicity depends to a large extent on the causation of oxidative damage both to the DNA as well as other vita biomolecules like lipid and protein. Oxidative stress results in the dysfunction of mitochondria, which leads to the collapse of Δψm and release of pro-apoptotic factors such as cytochrome c (Liu et al., 2003). In the present studies we observed an elevation in Δψm and higher level of cytosolic cytochrome C levels in mutant cells treated with 2-DG and radiation treated in comparison to the wild type cells (Figs 3.2.10 & 3.2.13a, b). This was accompanied by a higher Bax/BCl2 ratio that facilitates the release of cytochrome C in to the cytosol. A higher degree of apoptosis observed in the mutant cell lines cell lines appears to be driven by the higher level of oxidative stress induced in these cells due a synergism between the endogenously
higher level (seen in these cells), radiation-induced oxidative stress and 2-DG induced oxidative stress, thereby eliciting a strong oxidative stress induced cell death. Therefore, tumor cells with mutated p53 (like 68 and 110 studied here), that show enhanced glycolysis as well as oxidative stress, may be more susceptible to treatment with a combination of 2-DG and radiation/chemotherapeutic drugs. However, it is pertinent to note that these mutant cells (68 and 110) studied here showed both a moderate increase in glycolysis (Table 3.1.1) and endogenous oxidative stress (Fig 3.1.6), while enhanced glycolysis is generally associated with reduced oxidative stress (Kondoh et al., 2007). Therefore, whether the radiosensitizing effects observed are specific to these mutants studied here or can be generalized with reference to other mutations in p53 needs further investigations.

Overall, these results strongly support the hypothesis that treatment of human head and neck cancer cells having particular p53 mutations with a combination of 2-DG and radiation enhances clonogenic cell killing by a mechanism that involves oxidative stress mediated by hydroperoxides suggesting that inhibitors of glucose and hydroperoxide metabolism can be used in combination with radiation to enhance therapeutic responses in head and neck cancer having particular p53 mutations. Taken together, results from the present studies suggest that the fate of head and neck cancer cells is strongly influenced by the positions of p53 mutations as well as exposure and concentration of the glycolytic inhibitor 2-DG and doses of radiation. Results also support the proposition that head and neck carcinoma cells which bear mutations in p53 in the proline rich regions at positions 68 and 110 are more susceptible to cytotoxicity and radiosensitivity induced by glycolytic inhibitor than the parental cells possibly due to enhanced metabolic oxidative stress mediated by 2-DG and radiation linked to mutations in p53. Therefore, the proposition that use of glycolytic inhibitors like 2-DG as potential therapeutic agents individually as well as an adjuvant with radiation merits consideration as it induces oxidative stress mediated cell death selectively in head and neck carcinoma cells with p53 mutations (particularly in the proline rich region like 68 and 110 positions studied here).

Taken together, findings of the present studies suggest that it may be possible to stratify patients based on the p53 status of the tumors, who could benefit from the
combined therapy (2-DG + radiation/chemotherapy) by evaluating the p53 status in tumor cells with minimally invasive procedures (fine needle biopsy) or non-invasive methods (optical imaging) that may become available in future. From that perspective, our findings suggest that p53 status could be used as a predictive marker of the sensitivity of tumors to the combined treatment of 2-DG (or other glycolytic inhibitors) and radiation/chemotherapeutic drugs.