STUDIES ON RADIATION INDUCED PRO-SURVIVAL SIGNALING MECHANISMS IN TUMOR MICROENVIRONMENT

By

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A thesis submitted to the

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In partial fulfilment of requirements

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STATEMENT BY AUTHOR

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POONAM YADAV

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.

Poonam Yadav

List of Publications arising from the thesis

Journal

1. “Radio resistance in breast cancer cells is mediated through TGF-β signalling, hybrid epithelial-mesenchymal phenotype and cancer stem cells.” Yadav P and Shankar BS, 2019, Biomed Pharmacother 111:119-130. doi: 10.1016/j.biopha.2018.12.055

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Thank you all!

Abstract

Cancer is a major health concern, and the conventional treatments for cancer include surgery, radiotherapy, chemotherapy, targeted therapies, and immunotherapy [1, 2]. Radiotherapy induces DNA damage either directly or indirectly through the production of reactive oxygen species (ROS) in cancer cells. Radiotherapy plays an important role in the multimodal treatment of breast cancer. A major problem with radiotherapy is the radioresistance acquired by the cells resulting in recurrence of tumor. This is because, in addition to DNA damage, ionizing radiation (IR) activates a number of DNA repair/ survival/ apoptotic pathways which directs the cells to either repair the DNA damage, augment proliferation or to go into apoptosis depending upon the extent of DNA damage. Activation of survival pathways sometimes bypasses minor damage and pushes the cells into proliferation resulting in radioresistant tumors. Radioresistance leads to poor prognosis in cancer patients and it represents the main reason for radiotherapy failure, which can ultimately lead to tumor recurrence and metastasis [3]. Recurrence of tumor is also associated with presence of cancer stem cells (CSCs) [4]. So, it is very important to understand the underlying mechanism of radioresistance to develop an effective therapy against radio resistant cells. The sensitivity of radiation depends on factors like tumor microenvironment hypoxia, presence of cancer stem cells, modulation of repair pathways, soluble growth factors, cytokines and stromal interactions [5, 6]. The hypothesis of the study is that the activation of TGF-β signalling pathways by ionizing radiation leads to radioresistance in breast cancer cells.

The Objectives of this project were

1. To delineate the mechanisms involved in radiation induced survival signalling in cancer cells.

2. To understand the effect of pro and anti-inflammatory cytokines in modulation of radiation mediated signalling in cancer cells.

In this study, breast cancer cell lines MCF-7 and MDA-MB-231 were exposed to radiation (6 Gy) followed by different recovery periods. In cells exposed to 6 Gy followed by a recovery period of 7 days, (D7-6G), an increased proliferation as well as apoptosis was observed along with up regulation of the three TGF-β isoforms TGF-β1, 2 and 3 as well as its receptors TGF-β RI and RII. Increased TGF-β signalling resulted in the increase of its downstream genes Snail-1, ZEB-1 (Zinc finger E-box-binding homeobox-1) and HMGA2 (High Mobility Group AT-Hook 2), which further induced EMT. Expression of *Snail-1*, *ZEB-1* and *HMGA2* was assessed at mRNA level by RT-PCR and confirmed at the protein level by antibody labelling and flowcytometry. Expression of epithelial markers *E-CADHERIN, OCCLUDIN,* and *DESMOPLAKIN* and mesenchymal markers *VIMENTIN, FIBRONECTIN* and *N-CADHERIN* were assessed in these cells. However, there was an increase in both epithelial and mesenchymal markers indicative of a hybrid E/M phenotype. There was an increase in expression of stem cell markers such as *OCTAMER BINDING TRANSCRIPION FACTOR-4 (OCT-4), NANOG, SEX DETERMINING REGION Y-BOX 2 (SOX-2),* and *ALDEHYDE DEHYROGENASE* (*ALDH)*. An increased ALDH level was also confirmed by the assay for its activity. Increased CSCs in D7-6G cells was also confirmed by the enrichment of $CD44^{+}CD24^{-}$ cells. When these D7-6G cells were exposed to another challenge dose of radiation, there was increased proliferation and decreased apoptosis as compared to cells exposed to 6 Gy alone indicating that these cells were radio resistant. TGF-β1-stimulated signalling could be blocked by SB431542, a potent kinase inhibitor of the TGF-β type I receptor ALK5 [7]. Pre- treatment of cells with SB431542 abrogated the radiation induced proliferation of D7-6G cells. Radiation induced TGF-β signalling was also abrogated by pre-treatment of SB431542 along with increased expression of pro- apoptotic genes and

apoptosis. Consequently, there was an inhibition of EMT response and migration in cells pre-treated with SB431542 and 6 Gy IR. Assessment of pro- and anti- inflammatory cytokines TNF-α and IL-10 indicated that there was an increase in TNF-α in D7-6G cells and IL-10 in SB-D7-6G cells. Hence, the cells were pre-treated with either TNF-α or IL-10 to assess the response to radiation. Interestingly, treatment with either of the cytokines completely abrogated the increased proliferation of D7-6G cells.

Growth of radioresistant MCF-7 cells was carried out in the SCID mouse model and proteomic analysis of the resultant tumors was also done. SCID mice carry the scid mutation and are severely deficient in both T cell- and B cell-mediated immunity, as a result of defective V(D)J joining of the immunoglobulin and T-cell receptor gene elements [8]. Athymic mouse has low ovarian estrogen production and therefore tumor growth was induced with β-estradiol supplementation [9]. We established a tumor xenograft model by transplanting normal untreated MCF-7 (MCF UT) and radio resistant MCF-7 (MCF D7-6G) subcutaneously into female SCID mice to study the characteristics of MCF D7-6G grown *in-vivo*. The results demonstrated that MCF D7-6G tumors retains their high proliferation ability *in vivo* i.e. shorter latency and increased tumor burden with time. Serum analysis showed elevated levels of TGF-β isoforms and its receptors in MCF D7-6G tumors as compared to MCF UT tumors. Increased expression of TGF-β downstream genes as well as epithelial and mesenchymal markers was observed in MCF D7-6G tumors at mRNA level. Increased expression of cancer stem cells markers *OCT-4, SOX-2, NANOG* and *ALDH* was also observed. High ALDH levels were also confirmed at the protein level by assay of its activity in cells isolated from the tumor. Label free proteomic analysis of tumor tissue isolated from MCF UT tumor and MCF D7-6G tumor resulted in identification of a total of 649 differentially expressed proteins. Amongst them 17 proteins were significantly up regulated in MCF D7-6G tumors; $p<0.05$. Some of the up-regulated proteins include CCT5, CCT7,

FABP5, RPS21, RPL18, RPL38; Eight proteins were significantly down regulated in MCF D7-6G tumors and include RPS38, RPL9, TXNDL5, RPL8, EIF2S3. Pathway analysis was carried out using online tool Panther classification system which indicated that many of the genes identified were involved in (1) structural molecule activity and binding under molecular functions; (2) metabolic processes, cellular processes and biogenesis under biological process; (3) increased cellular components and nucleic acid binding. Since there was an increase in metabolic processes, Seahorse analyser was used to assess real time changes in oxygen consumption rate (OCR) and extracellular acidification rate (EACR) in MCF UT and MCF D7-6G cells. Results indicated increase in both basal level OCR and EACR indicating increase in glycolysis as well as oxidative phosphorylation. Increased uptake of NBDG by D7-6G cells confirmed increased glycolysis.

This study has shown that even a single dose of radiation exposure followed by a recovery period can result in activation of TGF-β signalling which may further lead to radioresistance. This radioresistant phenotype was characterized by hybrid E/M phenotype and enriched cancer stem cells. Inhibition of TGF-β signalling by SB431542 abrogated radiation induced TGF-β signalling and can be useful in radiosensitization of tumors.

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ABBREVIATIONS

2-D: Two-dimensional gel electrophoresis

2DE-MS: Two-dimensional gel electrophoresis and mass spectrometry

2-NBDG: 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-D-glucose

ABC transporters: ATP binding cassette

ABCB1: ATP-binding cassette subfamily-B member 1

ABCB5: ATP-binding cassette sub-family B-5

ABCG2: ATP-binding cassette subfamily-G member 2

ADCs: Antibody-drug conjugates

AJCC: American Joint Committee on Cancer

ACN: Acetonitrile

AF: Ammonium formate

ALDH: Aldehyde dehydrogenase

ANOVA: Analysis of variance

ARTS: Apoptosis-related protein in TGF-β signaling pathway

ATP: Adenosine triphosphate

BAD: BCL2 associated agonist of cell death

BAX: BCL-2 associated X

BCI: Breast Cancer Index

BCL-2: B-cell lymphoma 2

BCL-XL: B-cell lymphoma-extra large

BCRP: Breast cancer resistance protein

BCS: Breast conserving surgery

BER: Base excision repair

bFGF: Basic fibroblast growth factor

BRCA: Breast cancer gene

BrdU: 5-bromo-2'-deoxyuridine

BMPs: Bone morphogenetic proteins

CAFs: Cancer-associated fibroblasts

CBP: CREB binding protein

CDDP: Cisplatin

CDKs: Cyclin dependent kinase

CGH: Comparative genomic hybridization

CHAPS: 3-(3-cholamidopropyl) dimethylammonio-1-propanesulfonate

CHK1: Checkpoint kinase 1

cIAP1: Cellular inhibitor of apoptosis protein-1

CMF: Cyclophosphamide, methotrexate, and 5-fluorouracil

COX-2: Cyclooxygenase-2

CREB: cAMP response element-binding protein

CSCs: cancer stem cells

CTCs: Circulating tumour cells

CTX: Cyclophosphamide

CV: Co-efficient of variation

D7-4G: breast cancer cells exposed to 4 Gy and kept for recovery period of 7 days

D7-6G: breast cancer cells exposed to 6 Gy and kept for recovery period of 7 days

DCIS: Ductal carcinoma in situ

DMEM: Dulbecco's Modified Eagle Medium

DMSO: Dimethyl sulfoxide

DSBs: Double strand breaks

ECAR: Extracellular acidification rate

EDTA: Ethylenediaminetetraacetic acid

EGFR: Epidermal growth factor receptors

ER: Estrogen Receptor

ELISA: Enzyme-linked immunosorbent assay

EMT: Epithelial–mesenchymal transition

ERCC1: Excision repair cross-complementation group 1

ERK: Extracellular-signal-regulated kinase

EpCAM: Epithelial cell adhesion molecule

ESI: Electrospray ionization

FA: Formic acid

FAK: Focal adhesion kinase

FBS: Fetal bovine serum

FCCP: Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone

FDA: Food and drug administration

FDR: False discovery rate

FEC50 or FEC100: Cyclophosphamide 50 or 100

FITC: Fluorescein isothiocyanate

FT: Fourier transform

FTICR: Fourier-transform ion cyclotron resonance

FWHM: Full width at half maximum

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GBM: Glioblastoma

GGI: Genomic Grade Index

GICs: Glioma-initiating cells

GITR: Glucocorticoid-induced tumor necrosis factor receptor

GO: Gene Ontology

GM-CSF: Macrophage colony stimulating factor

GPCR: G protein-coupled receptors

GRHL2: Grainyhead like transcription factor 2

GRP-78: Glucose-regulated protein

GSK: Glycogen synthase kinase

GST: Glutathione S-transferases

HA: Hyaluronic acid

HDI: Human development index

HER2: Human epidermal growth factor receptor-2

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HIF1α: Hypoxia-inducible factor 1-alpha

HMGA2: High-mobility group AT-hook 2

HPRD: Human protein reference database

HR: Homologous recombination

hTERT: Human telomerase reverse transcriptase

IAP: inhibitor of apoptosis proteins

ICOS: Inducible T-cell costimulator

IDC: Infiltrating ductal carcinoma

IGF-1R: Insulin-like growth factor 1 receptor

ILC: Infiltrating lobular carcinoma

IL-10: Interleukin 10

IPA: Ingenuity Pathway Analysis

iPLA2: Calcium-independent phospholipase A2

iPSCs: Induced pluripotent stem cells

IR: Ionizing radiation

iTRAQ: Isobaric tags for relative and absolute quantitation

JNK: c-Jun N-terminal kinase

KIRC: Kidney renal clear cell carcinoma

KLF4: Kruppel-like factor 4

LAML: Acute myeloid leukemia

LCIS: Lobular carcinoma in situ

LC-MS/MS: Liquid chromatography-tandem mass spectrometry

LDH5: Lactate dehydrogenase 5

LDHA: Lactate dehydrogenase A

LRP5/6: lipoprotein receptor-related protein 5 and 6

LUAD: Lung adenocarcinoma

LUSC: Lung squamous cell carcinoma

MAbs: Monoclonal antibodies

MALDI: Matrix-assisted laser desorption/ionization

MALDI-TOF-MS: Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

MAP: Mitogen-activated protein

MAPK: Mitogen-activated protein kinases

MCF UT: MCF-7 untreated cells

MCF D7-6G: MCF-7 cells exposed to 6 Gy and kept for recovery period of 7 days

MDSCs: Myeloid-derived suppressor cells

M-CSF: Macrophage colony stimulating factor

MEK: Mitogen-activated protein kinase kinase

MEL: Melphalan

MMP-9: Matrix metallopeptidase 9

MET: Mesenchymal–epithelial transition

mFISH: Multicolor fluorescence in situ hybridization

miRNAs: Micro RNAs

MMTS: Methyl methanethiosulfonate

MnSOD: Mn superoxide dismutase

MRM: Multiple reaction monitoring

MRP: Multidrug resistance associated protein

MS2: MS and MS/MS

MSCs: Mesenchymal stem cells

MTT: 3-(4, 5-dimethylthiazol-2-yl)- 2,5-diphenyl tetrazolium bromide

MUC1: Mucin 1

NF-kB: Nuclear factor kappa light chain enhancer of activated B cells

NHEJ: Non-homologous end joining

NK cells: Natural killer cells

NOX2: NADPH oxidase 2

NOX4: NADPH oxidase 4

NSCLC: Non-small cell lung cancer

OCR: Oxygen consumption rate

OCT-4: Octamer-binding transcription factor 4

OS: Overall survival

OV: Ovarian cancer

OVOL2: Ovo like zinc finger 2

PANTHER: Protein annotation through evolutionary relationship

PAM50: Predictor Analysis of Microarray 50

PARP: Poly (ADP-ribose) polymerase

PBI: Partial breast irradiation

PBS: Phosphate buffered saline

PBST: Phosphate buffered saline with tween 20

PD-1: Programmed cell death protein 1

PCR: Polymerase chain reaction

PGK1: Phosphoglycerate kinase 1

PI3K: Phosphatidylinositol 4, 5-bisphosphate 3-kinase

PI: Propidium iodide

PKCδ: Protein kinase C delta

PMRT: Postmastectomy radiotherapy

POD: Peroxidase

PR: Progesterone-receptor

RANKL: Receptor activator of nuclear factor kappa-B ligand

RT: Radiation therapy

ROR: Risk of Recurrence

SAv-HRP: Streptavidin, horseradish peroxidase conjugated

SCID: Severe combined immunodeficiency

SCX: Strong cation exchange

SDS: Sodium dodecyl sulphate

SELDI-TOF MS: Surface-enhanced laser desorption/ionization-TOF MS

SEM: standard error of mean

SERMS: Selective estrogen receptor modulator

SKY: Spectral karyotyping

SMAD: homologues of the Drosophila protein, mothers against decapentaplegic (Mad) and the *Caenorhabditis elegans* protein Sma

SNAI-1: Snail Family Transcriptional Repressor 1

SOX-2: Sex determining region Y- box 2

SP: Side population

SSA: Single-strand annealing

SSBs: Single strand breaks

SWATH-MS: Sequential window acquisition of all theoretical- mass spectra

TAAs: Tumor-associated antigens

TACAs: Tumor-associated carbohydrate antigens

TAMs: Tumor-associated macrophages

TCEP: Tris (2-carboxyethyl) phosphine

TCGA: The cancer genome atlas

TEAB: Triethylammonium bicarbonate buffer

TGF-β: transforming growth factor-beta

TGF-β RI: Transforming growth factor beta receptor I

TGF-β RII: Transforming growth factor beta receptor II

TMB: 3, 3', 5, 5'-Tetramethylbenzidine

TNBC: Triple-negative breast cancer

TNM: Tumor, node, metastasis

TNF-α: Tumor necrosis factor alpha

UNDP: united Nations development programme

uPAR: Urokinase-type plasminogen activator receptor

UT: Untreated

VEGF: Vascular endothelial growth factor

VCAM1: Vascular cell adhesion proteins

WBRT: Whole breast radiotherapy

WT1: Wilms tumor gene

WOWH: Wound-oncogene-wound healing

XIAP: X-linked inhibitor of apoptosis protein

ZEB-1: Zinc finger E-box binding homeobox 1

INDEX

1. INTRODUCTION

1.1 Cancer

Normal cells depend on growth signalling of a tightly-regulated cell cycle to proliferate and maintain tissue homeostasis, which is disrupted in cancer [1]. Transformation can be defined as the process in which different insults continuously act on cells leading to transformative alterations in (epi) genetics, chromosomal numbers and arrangements, and heterotypic interactions which, along the path towards malignancy, undergo cycles of evolutionary clonal selection leading to the acquisition of cancer-competent traits, the hallmarks of cancer [2]. The hallmarks of cancer constitute an organizing principle that provides a conceptual basis that summarizes the complexity of this disease in order to better understand it in its diverse presentations. This conceptualization involves eight biological capabilities—"the hallmarks of cancer"—acquired by cancer cells during the long process of tumor development and malignant progression. Two characteristic traits of cancer cells facilitate the acquisition of these functional capabilities. The eight distinct hallmarks consist of (a) sustaining proliferative signalling, (b) evading growth suppressors, (c) resisting cell death, (d) enabling replicative immortality, (e) inducing angiogenesis, (f) activating invasion and metastasis, (g) deregulating cellular energetics and metabolism, and (h) avoiding immune destruction. The principal facilitators of their acquisition are genome instability with consequent gene mutation and tumor-promoting inflammation. The integration of these hallmark capabilities

involves heterotypic interactions among multiple cell types populating the “tumor microenvironment” (TME), which is composed of cancer cells and a tumor-associated stroma, including three prominent classes of recruited support cells- angiogenic vascular cells, various subtypes of fibroblasts, and infiltrating immune cells. In addition, the neoplastic cells are themselves typically heterogeneous, in that cancer cells can assume a variety of distinctive phenotypic states and undergo genetic diversification during tumor progression [3].

To identify the usefulness of these hallmarks in cancer prognosis, Yu et al., have constructed gene co-expression networks using mRNA expression data of seven cancers [ovarian cancer (OV), breast cancer (BRCA), lung adenocarcinoma (LUAD), acute myeloid leukemia (LAML), lung squamous cell carcinoma (LUSC), pleomorphic glioblastoma (GBM), and kidney renal clear cell carcinoma (KIRC)] from The Cancer Genome Atlas (TCGA). They have integrated this TCGA data to identify conserved gene clusters in all the networks that could reveal the biological mechanisms underlying cancer. This was followed by survival analysis to select the genes that could significantly distinguish between cancer patients in terms of survival regarding multiple types of cancer. This analysis showed that they were mainly related to basic cellular functions, cell cycle, and immune response and in particular, two hallmarks, which were related to “Mitotic cell cycle” and “RNA processing”

could each predict the prognosis of cancer patients regarding four types of cancer and useful for screening for cancer drugs [4].

1.2 Incidences

Cancer burden worldwide is projected to rise from 14 million new cases in 2012 to 24 million in 2035 [5]. It is estimated that nearly one-half of the cases and over one-half of the cancer deaths in the world will occur in Asia in the year 2018, partly because close to 60% of the global population resides there. Europe accounts for 23.4% of the total cancer cases and 20.3% of the cancer deaths, although it represents only 9% of the global population, followed by the Americas' 21% of incidence and 14.4% of mortality worldwide. In contrast to other regions, the shares of cancer deaths in Asia (57.3%) and Africa (7.3%) are higher than the shares of incidence (48.4% and 5.8%, respectively) because of the different distribution of cancer types and higher case fatality rates in these regions [6]. The distribution of number of new cases in 2018 according to human development index is given in Figure 1.1. As can be seen from the Figure 1.2, breast cancer seems to be at the highest percentage across all HDI countries.

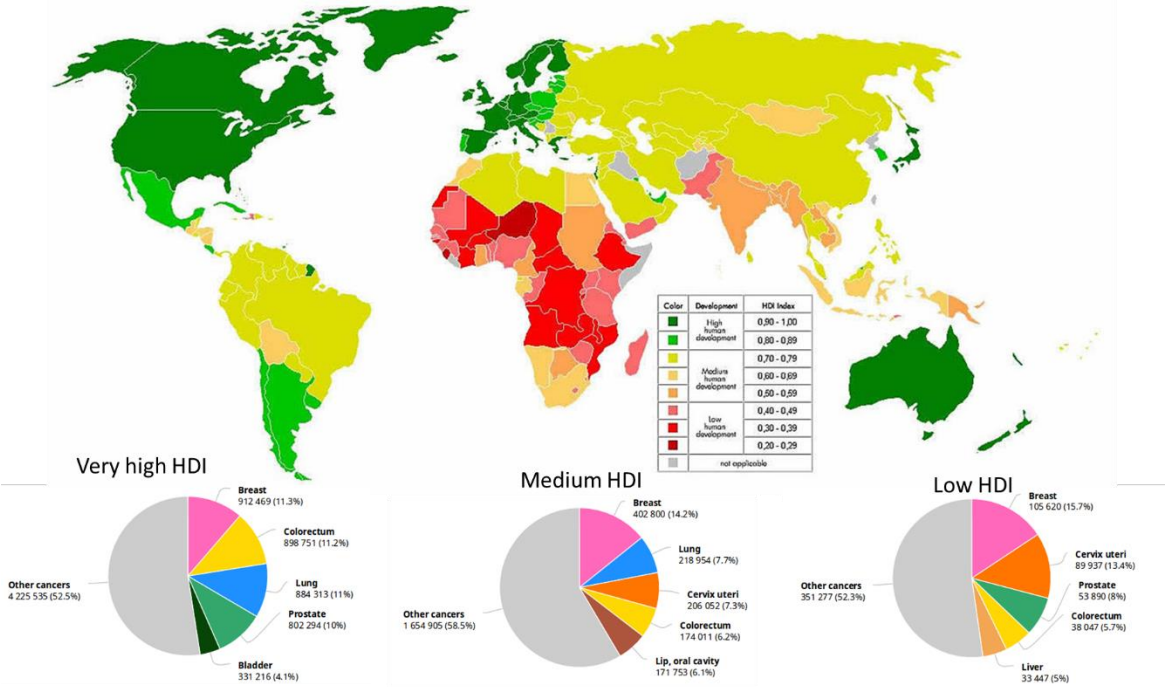


Figure 1.1: Cancer incidences according to levels of the Human Development Index (HDI) across the world in 2018.(GLOBOCAN 2018). HDI is a composite measure of life expectancy, educational attainment, and command over the resources needed for a decent living (UNDP, 2013) and is used in this study to examine the cancer profiles according to four levels of societal development [7, 8].

1.3 Breast cancer

Breast cancer is the most frequently diagnosed neoplastic disease in women around menopause often leading to a significant reduction of these women's ability to function normally in everyday life. Intensive studies conducted over the past several years showed that 20-30% of newly diagnosed breast cancer cases may be associated with the occurrence of various risk factors actively

initiating or modifying the process of neoplastic transformation of breast cells. These include (a) age, (b) family history, (c) reproductive factors, (d) estrogen and (e) life style.

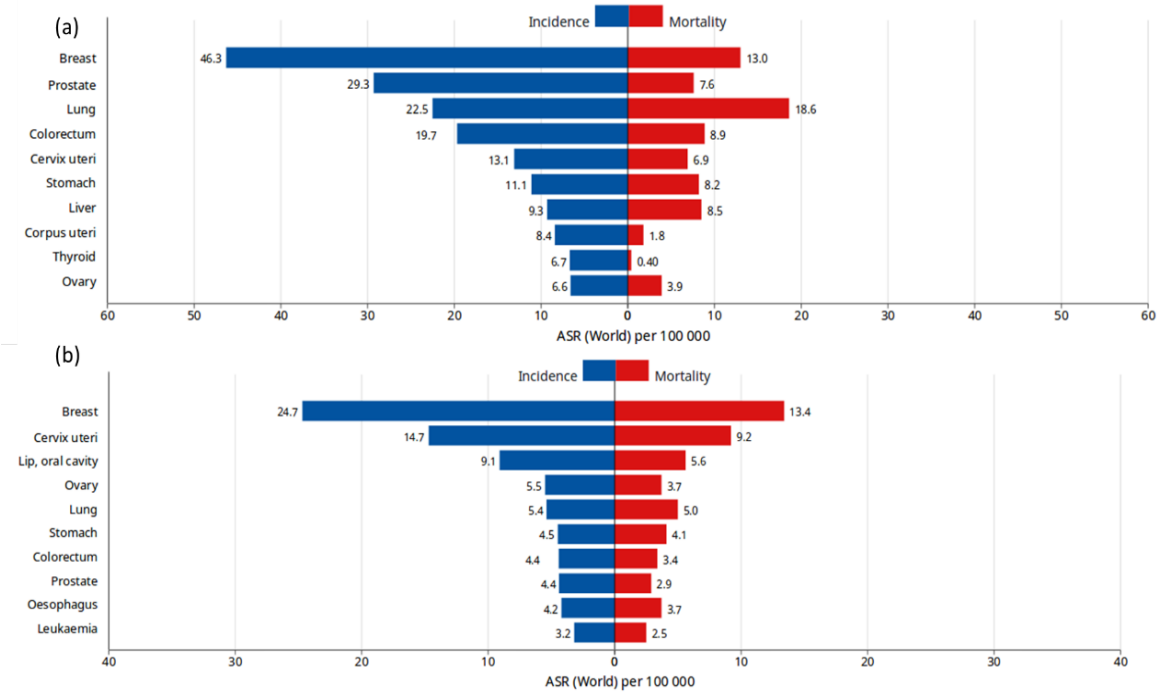


Figure 1.2: Age standardised incidence and mortality of cancer in females in (a) World (b) India [8].

Though the incidence of breast cancer is higher Worldwide (46.3/100,000 cases) as compared to India (24.7/100,000 cases), the mortality is much lower Worldwide (13/100,000) as compared to India (13.4/100,000). Due to this, there is a high mortality to incidence ratio in India (54.3) as compared to worldwide (28.0).

1.3.1 Types of breast cancer

Breast cancer is a compilation of distinct malignancies that manifests in the mammary glands. Carcinomas make up the majority of breast cancers while sarcomas such as phyllodes tumors and angiosarcomas are rarely seen. Breast cancer can be classified into different types according to (I) site, (II) occurrence, (III) histological grading, (IV) TNM staging, (V) expression of biomarkers

1.3.1.1 According to site:

- **Non-Invasive breast cancer:** cells that are confined to the ducts and do not invade surrounding fatty and connective tissues of the breast. Ductal carcinoma in situ (DCIS) is the most common form of non-invasive breast cancer (90%). Lobular carcinoma in situ (LCIS) is less common and considered a marker for increased breast cancer risk.
- **Invasive breast cancer:** cells that break through the duct and lobular wall and invade the surrounding fatty and connective tissues of the breast. Cancer can be invasive without being metastatic (spreading) to the lymph nodes or other organs.

1.3.1.2 According to occurrence:

- **Frequently occurring Breast cancer**

(i) Lobular carcinoma in situ (LCIS, lobular neoplasia): The term, "in situ," refers to cancer that has not spread past the area where it initially

developed. LCIS is a sharp increase in the number of cells within the milk glands (lobules) of the breast.

(ii) Ductal carcinoma in situ (DCIS): DCIS, the most common type of non-invasive breast cancer, is confined to the ducts of the breast. For example, ductal comedo carcinoma.

(iii) Infiltrating lobular carcinoma (ILC): ILC is also known as invasive lobular carcinoma. ILC begins in the milk glands (lobules) of the breast, but often spreads (metastasizes) to other regions of the body. ILC accounts for 10% to 15% of breast cancers.

(iv) Infiltrating ductal carcinoma (IDC): IDC is also known as invasive ductal carcinoma. IDC begins in the milk ducts of the breast and penetrates the wall of the duct, invading the fatty tissue of the breast and possibly other regions of the body. IDC is the most common type of breast cancer, accounting for 80% of breast cancer diagnoses.

❖ **Less commonly occurring Breast cancer**

(i) Medullary carcinoma: Medullary carcinoma is an invasive breast cancer that forms a distinct boundary between tumor tissue and normal tissue. Only 5% of breast cancers are medullary carcinoma.

(ii) Mutinous carcinoma: Also called colloid carcinoma, mutinous carcinoma is a rare breast cancer formed by the mucus-producing cancer cells. Women with mutinous carcinoma generally have a better

prognosis than women with more common types of invasive carcinoma.

(iii) Tubular carcinoma: Tubular carcinomas are a special type of infiltrating (invasive) breast carcinoma. Women with tubular carcinoma generally have a better prognosis than women with more common types of invasive carcinoma. Tubular carcinomas account for around 2% of breast cancer diagnoses.

(iv) Inflammatory breast cancer: Inflammatory breast cancer is the appearance of inflamed breasts (red and warm) with dimples and/or thick ridges caused by cancer cells blocking lymph vessels or channels in the skin over the breast. Though inflammatory breast cancer is rare (accounting for only 1% of breast cancers), it is extremely fast-growing.

(v) Paget's disease of the nipple: A rare form of breast cancer that begins in the milk ducts and spreads to the skin of the nipple and areola, Paget's disease of the nipple only accounts for about 1% of breast cancers.

(vi) Phylloides tumor: Phylloides tumors (also spelled “phyllodes”) can be either benign (non-cancerous) or malignant (cancerous). Phylloides tumors develop in the connective tissues of the breast and may be treated by surgical removal. Phylloides tumors are very rare [9].

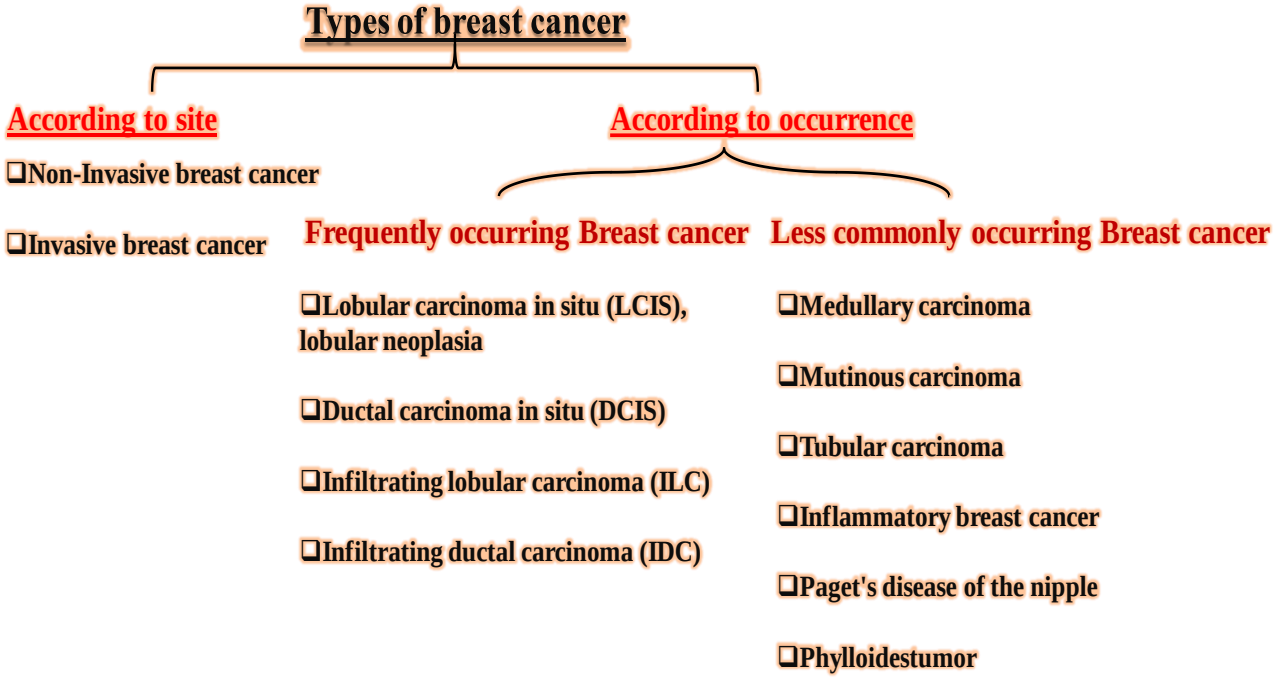


Figure 1.3: Types of breast cancer on basis according to site and occurrence.

(vii) Histological Grading in Breast Carcinoma

(Nottingham Modification of the Bloom-Richardson system)

The histological grade is determined based on the obtained total score from tubule formation, nuclear pleomorphism and mitotic count. Though evaluation by this method is semi-quantitative, it provides very strong prediction for determining patient prognosis

- **Tubule formation:** 1 point if tubule formation constitutes more than 75% of the tumor, 2 points if tubule formation constitutes 10-75% of the tumor and 3 points if tubule formation constitutes less than 10% of the tumor.
- **Nuclear pleomorphism:** 1 point if shape and size difference of nucleus is mild, 2 points if it is moderate and 3 points if it is significant.

Mitotic Count: Mitotic counting process is done at the periphery of the tumor and should be started from the most mitotic active areas. The suggested application is counting within the same field, but it is not necessary to use subsequent fields. Areas rich in tumor that are free of normal breast tissue are preferred as much as possible. Prophase cells should not be counted. Due to differences in image area due to varying brands of microscopes, there are determined and accepted values for the number of mitotic count. Based on these values, mitotic counts are scored as 1, 2 and 3.A total score is obtained by scores on tubule formation, nuclear pleomorphism and mitotic count [10].

(viii) Tumor, node, metastasis (TNM) staging

TNM staging defined by Pierre Denoix, and has gained wide acceptance and is used as a common language among treatment centers widely all over the world, to guide treatment planning, provide a possibility to demonstrate the effectiveness of the treatment during follow-up and predict prognosis [11]. This is based on anatomical staging, which uses the extent of the primary tumor (T), status of the regional llymph nodes (N), and metastasis status (M). The T stage is based on the size and degree of loco-regional invasion by the primary tumor and is categorized from T1 to T4. The N stage is determined by the extent of nodal involvement including axillary, internal mammary and ipsilateral supraclavicular lymph nodes. Distant metastases are evaluated to determine the M stage. Since 1959, the American Joint Committee on Cancer (AJCC) has

published seven editions of the tumor-node-metastasis (TNM) system for cancer staging. In 2017, the 8th edition was announced, in which the revisions were based on the highest level of evidence from newly acquired clinical and pathological data [12, 13]. In a fundamental change, breast cancer is now considered as a group of diseases with different molecular characteristics that indicate different prognoses, patterns of recurrence, disseminations, and sensitivities to available therapies [14]. Therefore, the committee incorporated biomarkers (histologic grade, hormone receptor, HER2 expression, and multigene panels) into the traditional anatomic TNM staging [12, 13].

(ix) Molecular subtypes according to biomarkers

Four biomarkers are tested consistently in invasive breast cancer biopsy and excision specimens because of their potential effect on prognosis and clinical management: estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor-2 (HER2), and nuclear protein involved in cellular proliferation (Ki-67 antigen). Gene expression studies have identified several distinct breast cancer subtypes that differ significantly in prognosis as well as in the therapeutic targets present in the cancer cells. With the advance of gene expression profiling techniques, the list of intrinsic genes that differentiate these subtypes is now made up of several clusters of genes relating to estrogen receptor (ER) expression (the luminal cluster), human epidermal growth factor

2 (HER2) expression, proliferation, and a unique cluster of genes called the basal cluster [15, 16].

Due to the understanding from these studies, breast cancer is usually divided into five defined subtypes based on hormone receptor status and gene expression patterns: (a) luminal A, (b) luminal B, (c) HER2-enriched, (d) triple-negative and (e) normal-like (Table 1.1).

Luminal A tumours are the most common among the luminal types and have the best prognosis [17]. These are characterized by high expression of ER-related genes and low expression of HER2 and proliferation-related genes (eg, Ki-67). Treatment typically involves hormonal therapy [17, 18].

Luminal B tumours are less common and have a slightly worse prognosis than do luminal A tumours. These are characterized by lower expression of ER-related genes, variable expression of HER2 gene clusters, and higher expression of proliferation-related genes [17]. Luminal B cancers grow slightly faster than luminal A cancers, and their prognosis is slightly worse [15, 16].

HER2-enriched breast cancer subtype makes up 10%–15% of breast cancers and is characterized by the absence of ER and PR expression, the high expression of the HER2 and proliferation gene clusters, and the low expression of the luminal and basal clusters. HER2-enriched cancers grow faster than luminal cancers and have a generally worse prognosis. However, they can be

successfully treated with targeted therapies aimed at the HER2 protein, such as Herceptin (or trastuzumab), Perjeta (or pertuzumab), Tykerb (or lapatinib), and Kadcyla (or T-DM1 or ado-trastuzumab emtansine). HER2-enriched subtype is not synonymous with clinically HER2-positive breast cancer. While about 50% of clinical HER2-positive breast cancers are HER2-enriched, the remaining 50% can include any molecular subtype but are mostly HER2-positive luminal subtypes. However, about 30% of HER2-enriched tumors are clinically HER2-negative [15, 16, 19].

Triple-negative/basal-like breast cancer (TNBC) accounts for approximately 20% of all breast cancers, the TNBC subtype is characterized as ER-negative, PR-negative and HER2-negative. TNBC is more common in women with BRCA1 gene mutations as well as among women younger than 40 years of age and African-American women. TNBC usually behaves more aggressively than other types of breast cancer making it a high-grade breast cancer. The most common histology seen in TNBC is infiltrating ductal carcinoma, although a rare histologic subtype, medullary carcinoma, is generally also triple negative. Unlike other breast cancer subtypes with an arsenal of targeted regimens such as ER antagonists and HER2 monoclonal antibodies, TNBC's non-surgical treatment has been limited to conventional chemotherapy, until the recent approval of the PARP inhibitor Olaparib for

BRCA1 and BRCA2 mutation carriers, who are more likely to develop TNBC [15, 16, 19].

Normal-like breast cancer subtype is similar to luminal A disease. It is ER and/or PR-positive, HER2 negative, and has low levels of the protein Ki-67. While normal-like breast cancer has a good prognosis, it is still slightly worse than luminal A cancer [15, 16, 19].

Table 1.1: Molecular subtypes of breast cancer

Subtypes	Molecular Signatures	Characteristics	Treatment options[a]
Luminal A	**ER+, PR±, HER2-, Low Ki67**	**~70%, Most common**	**•Hormonal Therapy •Best prognosis •Targeted Therapy**
Luminal B	**ER+, PR±, HER2±,**	**10%–20%**	**•Hormonal Therapy •High Ki67 •Lower survival than Luminal A •Targeted Therapy**
HER2	**ER-, PR-, HER2+**	**5%–15%**	**•Targeted Therapy**
Triple Negative	**ER-, PR-, HER2-**	**15%–20%**	**•Limited Targeted Therapy •Diagnosed at younger age •Worst prognosis**
Normal-like	**ER+, PR±, HER2-Low Ki67**	**Rare**	**•Hormonal Therapy •Low proliferation gene cluster expression •Targeted Therapy**

[a]These treatment options are in addition to the conventional treatments. Source: [20].

The cellular and molecular heterogeneity of breast cancers mandates the analyses of multiple genetic alterations in concert, which has been made possible by the emergence of next-generation genomics and transcriptomics techniques. Genome wide analyses and transcriptomic profiling have provided critical insights into the nuances of the molecular classification of breast cancers [18, 21], and have helped established several diagnostic and prognostic panels, such as the Oncotype Dx 21-gene Recurrence Score (RS) [22], the Breast Cancer Index (BCI) [23], the Predictor Analysis of Microarray 50 (PAM50) Risk of Recurrence (ROR) score [24-26], the Amsterdam 70-gene prognostic profile (Mammaprint) [27-29], and the Genomic Grade Index (GGI) [30].

1.3.2 Major signalling pathways in breast cancer development and progression

Cancer is driven by genetic and epigenetic alterations that allow cells to escape the mechanisms that normally control their proliferation, survival and migration [31]. Many of these alterations map to signalling pathways that govern cell proliferation and division, cell death, cell differentiation and fate, and cell motility. Thus, activating mutations of proto-oncogenes can cause hyper activation of these signalling pathways, whereas inactivation of tumour suppressors eliminates critical negative regulators of signalling [31].

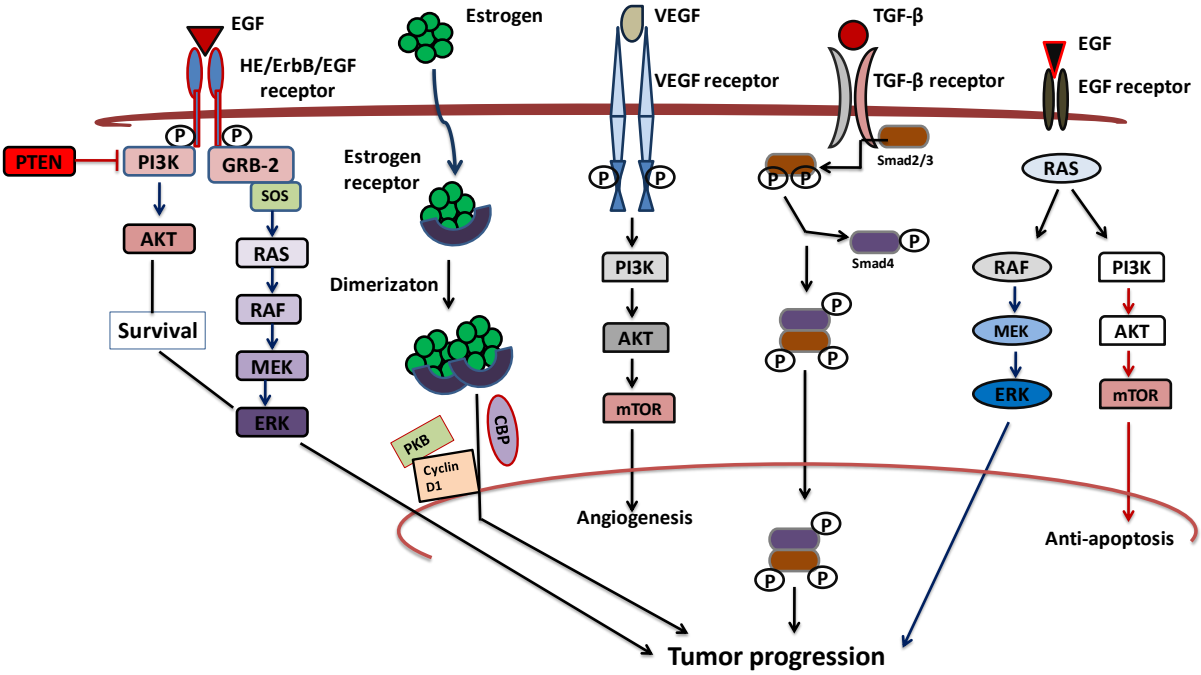


Figure 1.4: Major signalling pathways involved in initiation and progression of tumorigenesis [32].

1.3.2.1 **<u>ER signalling</u>:** Estrogen receptors (ERs) consist of membrane estrogen receptors (mostly G protein-coupled receptors "GPCR") and nuclear estrogen receptors (ERα, ERβ). Both ERα and ERβ are transcriptional factors that either activate or repress the expression of target genes upon ligand binding. ERα (coded by ESR1) and ERβ (coded by ESR2) share common structural features that serve their main functions while upholding receptor-specific signal transduction through exclusive elements [33, 34].

1.3.2.2 **<u>HER2 signaling</u>:** Human epidermal growth factor receptors (EGFRs, or HERs) 1 to 4 constitute a family of tyrosine kinase receptors expressed in normal tissues and in many types of cancer. Human epidermal growth

factor receptor-2 (or HER2/NEU, c-ERBB2) is a member of the EGFRs. Like the others, HER2 is a receptor tyrosine kinase that consists of an extracellular ligand-binding domain, a transmembrane domain, and an intracellular domain [35, 36]. The constitutively active form makes HER2 the preferred component to form dimers with other molecules and grants HER2 the capability of affecting many cellular functions through various pathways. Ligand binding and subsequent dimerization stimulate phosphorylation of tyrosine residues in the intracellular domain of HER2, leading to the activation of multiple downstream signalling pathways such as the mitogen-activated protein kinase (MAPK) and the phosphatidylinositol 4,5-bisphosphate 3-kinase (PI3K) pathways. These signalling pathways are strongly associated with breast tumorigenesis [36, 37].

1.3.2.3 **Wnt/β-catenin signalling:** Wnt proteins are a family of highly glycosylated, secreted proteins with pivotal roles in various developmental processes including embryonic induction, generation of cell polarity, and cell fate specification, as well as in maintaining adult tissue homeostasis. The canonical Wnt/β-catenin signalling is initiated by the binding of these secreted Wnt proteins, which is palmitoylated by Porcupin, to both co-receptors Frizzled and low-density lipoprotein receptor-related protein 5 and 6 (LRP5/6).Wnt receptor interaction leads to recruitment of Axin and Dishelved proteins to the cell membrane and

induces inhibition of glycogen synthase kinase (GSK)-3β protein. GSK-3β is a negative regulator of the Wnt pathway phosphorylating β-catenin thereby targeting it for proteasomal degradation. Inhibition of GSK-3β leads to β-catenin accumulation in the cytoplasm, and its subsequent translocation into the nucleus to act as co-transcriptional activator together with CREB binding protein (CBP) and T-cell factor/lymphoid enhancing factor (TCF/LEF) transcription factors and regulating oncogenes, such as MYC, CCND1 and other target genes [38-40].

1.3.2.4 **Other signalling pathways:** In addition to these three pathways, many other pathways and their crosstalk play important roles in regulating normal mammary development, as well as in breast cancer development if they are dysregulated. These include TGF-β, VEGF, EGF, CDKs (Cyclin dependent kinase), Notch, SHH,PI3K/Akt/mTOR, and others [41].

1.3.3 Treatment modalities

In the management of breast cancer, aim is to preserve quality of life with prolonged life expectancy. The main forms of treatment for cancer in humans are surgery, radiation and chemotherapeutic agents. The drugs can often provide temporary relief of symptoms, lengthening of life and occasionally cures the disease.

1.3.3.1 Surgery

This is the foremost management strategy for individuals whose breast cancer has not extended to further areas of the body and is also a choice for further complex stages of the illness [42]. The kinds of breast cancer surgery vary in the quantity of tissue that is excised with the cancer; this depends on the cancer's characteristics, whether it has extended, and the patient's special feelings. A few of the most familiar kinds of surgery include: (a) lumpectomy or partial mastectomy in which part of the breast that contains malignant tumour along with some healthy tissues and surrounding lymph nodes is removed leaving the major part of the breast intact as possible [43], (b) Mastectomy is the surgical removal of the entire breast and is considered the most effective method of dealing with a diffused case of breast cancer [44].

1.3.3.2 Chemotherapy

Most patients with localized breast cancer may be rendered disease-free with local therapy but distant recurrence is common and is the primary cause of death from the disease.

Adjuvant systemic therapies are effective in reducing the risk of distant and local recurrence, including endocrine therapy, anti-HER2 therapy, and chemotherapy, even in patients at low risk of recurrence. The widespread use of adjuvant systemic therapy has contributed to reduced breast cancer mortality

rates. “Adjuvant!” is a web-based decision aid used by many clinicians to understand the potential benefits of adjuvant therapy (endocrine or cytotoxic), Adjuvant! classifies chemotherapy regimens as first, second and third generation cytotoxic regimens. Anthracyclines (doxorubicin, epirubicin) and/or taxanes (paclitaxel, docetaxel), are the two most active classes of cytotoxic agents used for both early and advanced stage breast cancer [45]. First and second-generation regimens have an important role in clinical practice for tumours with low/intermediate recurrence-risk and include cyclophosphamide, methotrexate, and 5-fluorouracil (CMF), doxorubicin and cyclophosphamide (AC), 5-flourouracil, epirubicin (50 mg/m^2), and cyclophosphamide (FEC50). Second generation chemotherapy regimens include 5-flourouracil, epirubicin (100 mg/m^2), and cyclophosphamide (FEC100), cyclophosphamide, doxorubicin, and 5-fluorouracil (CAF or FAC), sequential doxorubicin/cyclophosphamide followed by paclitaxel (AC-T), sequential epirubicin followed by CMF, or docetaxel plus cyclophosphamide. Third-generation (anthracycline and taxane containing) regimens are commonly used in patients with high recurrence-risk, given their superior efficacy when compared to first or second-generation regimens. Anthracyclines, derivatives of the antibiotic rhodomycin B, were initially isolated in the 1950s from gram-positive *Streptomyces* present in an Indian soil sample. Doxorubicin was isolated from *Streptomyces peucetius* [46], a mutant of the original *Streptomyces* strain found near the Adriatic sea, and was therefore named

Adriamycin. Doxorubicin has been one of the most active single cytotoxic agents in metastatic breast cancer [47]. Paclitaxel was originally isolated from the bark of the Pacific yew tree *taxusbrevifolia*, and its antitumor activity was initially described in 1971 [48]. Paclitaxel binds to microtubules and induces their stabilization by inhibiting their depolymerization, thereby leading to mitotic arrest [49] and chromosome missegregation on abnormal multipolar spindles [50]. Despite its unique mechanism of action, paclitaxel's initial development was slow due to its scarcity and poor solubility. A formulation of paclitaxel solubilized in Cremophor EL was eventually developed but was associated with hypersensitivity reactions to the Cremophor EL vehicle [51], requiring premedication with corticosteroids and histamine blockers. In 1994, Cremophor-EL-paclitaxel was approved by the United States Food and Drug Administration (FDA) for treatment of metastatic breast cancer in patients who had progressed after anthracycline-based combination chemotherapy or who relapsed less than 6 months after adjuvant therapy [51]. Third generation chemotherapy regimens include docetaxel, doxorubicin, and cyclophosphamide (DAC), sequential FEC-taxane therapy, dose dense sequential doxorubicin/cyclophosphamide-paclitaxel (AC-T), sequential AC-weekly paclitaxel or every 3 week docetaxel, sequential versus concurrent taxane administration [52].

1.3.3.3 Radiotherapy

Radiotherapy in the curative setting for breast cancer has evolved from a one size fits all approach to a personalized, risk-adapted treatment within a multi-disciplinary environment, taking more and more patient factors, the biology of the tumor, and the extent of disease in the lymph nodes into account. Before the year 2000, the procedure was straightforward, with radiotherapy following mastectomy (postmastectomy radiotherapy, PMRT), and whole breast radiotherapy (WBRT) with 50 Gy over 5 weeks after breast-conserving surgery in all patients. PMRT is currently recommended in pre- and postmenopausal patients with locally advanced tumors (T4 or N+ disease, or following R+ resection). PMRT can be avoided following R0 mastectomy in T1–2N0 patients and in T3N0 patients without risk factors [53]. Radiotherapy is an integral part of the breast-conserving therapy approach. No radiotherapy following breast conserving surgery (BCS) may be an option for patients whose life expectancy is shorter than 10 years, with hormone receptor-positive T1N0 tumors without Her2/neu over-expression. Fractionated WBRT is the standard of care for the overwhelming majority of patients following BCS. Hypo fractionated WBRT, i. e., the application of single doses larger than 2 Gy up to reduced total doses, e. g., 15–16 fractions of 2.66 Gy, has been shown in large randomized trials with sufficient follow-up to be at least as effective in terms of local tumour control as conventionally fractionated radiotherapy and results in significantly

less acute and late effects [54]. Hypo fractionated radiotherapy is the preferred radiation schedule in patients who do not need radiotherapy of regional lymph nodes. In younger and high-risk patients, a sequential boost of 10–16 Gy to the tumor bed is recommended, although the improvement in local control is modest and there was no overall long-term survival benefit [55]. Based on long-term results from three randomized studies, (accelerated) partial breast irradiation ((A) PBI) using intraoperative radiotherapy or postoperative multi-catheter brachytherapy or external beam radiotherapy is an option for postmenopausal patients with low-risk tumors (T1N0, R0 resection, hormone receptor positive, non-lobular histology) [56, 57].

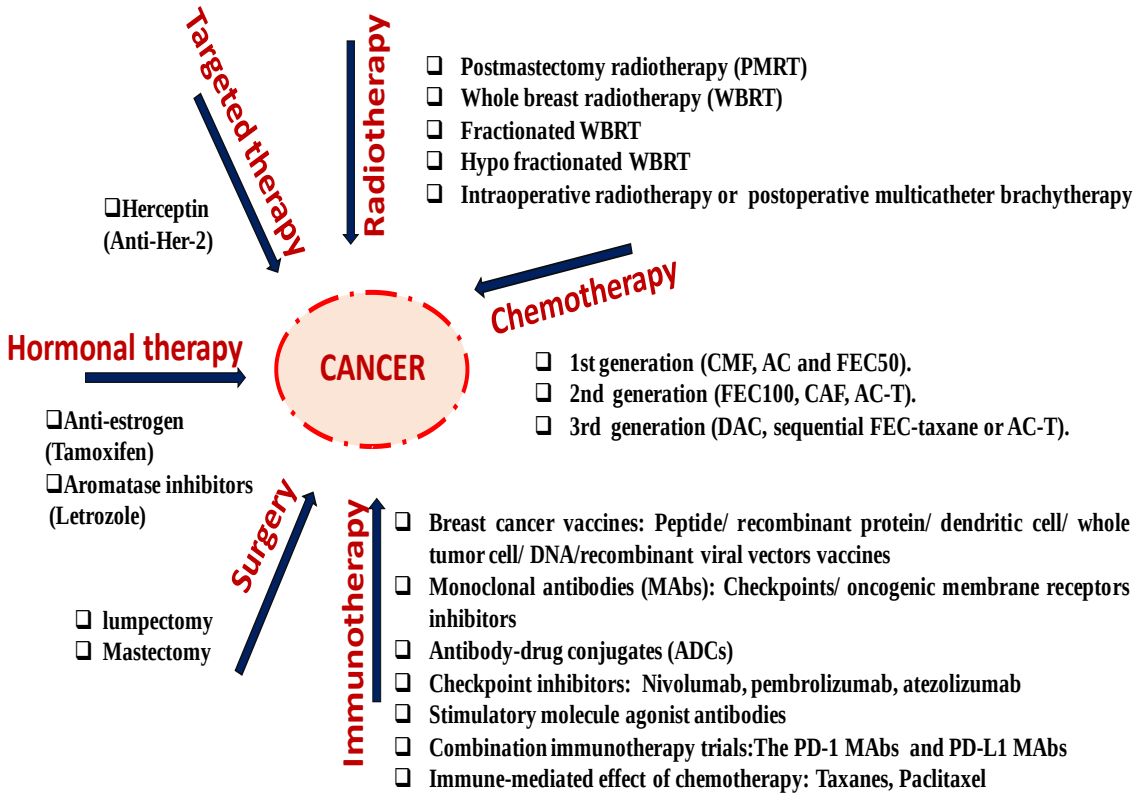


Figure 1.5: Therapeutic armamentarium in cancer. Note: PMRT: Postmastectomy radiotherapy; WBRT: Whole breast radiotherapy; CMF: cyclophosphamide+methotrexate and 5-fluorouracil; FEC100: 5-flourouracil, epirubicin (100 mg/m2); CAF: Cyclophosphamide, doxorubicin, 5-flourouracil; AC-T: a chemotherapy combination of Doxorubicin, cyclophosphamide followed by treatment with paclitaxel; DAC: Docetaxel, doxorubicin, cyclophosphamide; MAbs: Monoclonal antibodies; Antibody-drug conjugates; PD-1 MAbs : Programmed cell death protein 1 monoclonal antibody; PD-L1 Mabs: Programmed cell death ligand protein 1 monoclonal antibody.

1.3.3.4 Hormonal, targeted and Immunotherapy

Anti-estrogen therapy is used in cancers that has hormone receptors such as estrogen receptors and are affected by hormones. The most common category of drugs that are used in breast cancer is anti estrogen, which includes agents like tamoxifen, raloxifene, toremifene etc. Tamoxifen inhibits the hormone oestrogen from entering into cells of the breast cancer. This mechanism inhibits the breast cancer cells from developing. Tamoxifen can be suggested to treat females of any age group. However, tamoxifen is considered as the drug of choice in women who have estrogen receptor positive breast carcinoma. Tamoxifen is a selective estrogen receptor modulator (SERMS) and acts like estrogen on other parts of the body such as uterus. However, it demonstrates anti estrogen properties in breast tissues and competes with estrogen for binding

to the estrogen receptors in the breast [58]. Tamoxifen exhibits its oestrogen antagonist action in numerous tissues such as uterus, liver, bone and breast [59]. Tamoxifen was accepted by the United States Food and Drug Administration (FDA) in 1998 for the impediment of breast cancer for females at elevated danger [60]. There has been a 38% general decline in breast cancer occurrence in females who were at increased danger of breast cancer when administered tamoxifen for a period of 5 years. Tamoxifen inhibits only estrogen receptor positive breast cancers (RR ~ 50%) with no influence on estrogen receptor negative breast cancer [61].

A variety of adverse effects have been reported for females taking tamoxifen, such as venous thrombosis, cataract, endometrial cancer, menstrual disorders and hot flushes. A study indicated that the risk decreasing activity of tamoxifen expands beyond the vigorous management phase of 5 years, and remains for minimum 10 years, whereas the majority of adverse reactions do not carry on beyond the 5 year management duration [62]. Aromatase inhibitors are compounds designed for decreasing estrogen formation by targeting aromatase, the enzyme complex accountable for the last step in the formation of estrogen [63]. The third-generation aromatase inhibitors including letrozole, exemastane and anastrozole are in present utilization [64].

Targeted therapies are drugs prescribed to manage some types of breast cancer. The mainly familiar targeted treatment is the drug Herceptin which is

prescribed to manage HER2 positive breast cancer. It functions by preventing the cancer cells from developing and progressing [65].

Immunotherapy utilizes the immune system of the body to fight against the cancer cells [66]. For a long time, breast cancer was considered non immunogenic. However, the role of the immune system in the emergence of breast cancer has been firmly established [67, 68]. Several strategies have been used to harness the power of the immune system and redirect it to eradicate breast cancer or to induce immune dormancy:

- **Breast cancer vaccines**: Several strategies being studied including peptide vaccines, recombinant protein vaccines, dendritic cell (DC) vaccines, whole tumor cell vaccines, DNA vaccines, and recombinant viral vectors vaccines. They are all designed to stimulate an intrinsic antitumor response targeting tumor-associated antigens (TAAs). Tumor-associated antigens that are specifically recognized by T cells include HER2, mucin 1 (MUC1), carcinoembryonic antigen, sialyl-Tn (STn), human telomerase reverse transcriptase (hTERT), Wilms tumor gene (WT1), and tumor-associated carbohydrate antigens (TACAs).
- **Monoclonal antibodies (MAbs):** Monoclonal antibodies can be divided into those that target the immune system (checkpoint inhibitors) and those that target oncogenic membrane receptors (HER2) or other surface molecules of unknown function (CD20).

❖ **Antibody-drug conjugates (ADCs):** Antibody-drug conjugates are MAbs targeting a cancer-specific antigen and are linked to a payload of a cytotoxic drug by a linker. Antibody-drug conjugates are stable in the systemic circulation and most of the drugs in use have cleavable linkers that, after enzymatic cleavage or the exposure to a reduced pH (potential of hydrogen) or reduction by cytosolic thiols, release the cytotoxic drug inside the antigen-expressing cells. Antibody-drug conjugates using non-cleavable linkers require a thorough catabolism in the lysosomes leading to the release of their cytotoxic drug that should exit the lysosome to cause cell death [69].

❖ **Checkpoint inhibitors**: The agents being tried in breast cancer are based on their application already in melanoma and other malignancies including nivolumab and pembrolizumab (anti-PD-1 antibodies) and atezolizumab (PD-L1 inhibitor). Inhibition of the programmed death-1 and programmed death-ligand 1 (PD-1/PD-L1) pathway is based on the idea of "inhibiting the inhibition" of the immune system [70]. The PD-1 receptor inhibits innate and adaptive immunity when upregulated on immune cells and engaged by its ligand, PD-L1. Cancers take advantage of this mechanism to induce a local immunosuppression by overexpressing PD-L1; CTLA-4 is another immune checkpoint that is being targeted in breast cancer.

❖ **Stimulatory molecule agonist antibodies:** The optimal immune response requires the engagement of costimulatory receptors expressed by CTLs, NK cells, $CD4^{+}$T cells, or APCs. The most relevant receptors are CD27, CD28, CD40, OX40, 4-1BB, GITR, and ICOS. In addition to activating the proliferation and function of the cells carrying these receptors, their activation is associated with suppression of Tregs. The MAbs and fusion proteins produced to target these receptors and many of them are now in phase I or II trials [71].

❖ **Combination immunotherapy:** The PD-1 MAbs (nivolumab and pembrolizumab) and PD-L1 MAbs (atezolizumab, durvalumab, and avelumab) are being tested in many combinations either with chemotherapy or with biological agents targeting HER2-positive or hormone receptor–positive breast cancers. Many combination trials have also been designed in the neoadjuvant and adjuvant settings using different combinations of checkpoint inhibitors with other checkpoint inhibitors, with chemotherapy, cytokines, or with vaccines.

❖ **Immune-mediated effect of chemotherapy**: Traditionally, the effect of chemotherapy has been explained by the induction of cancer cells apoptosis after interrupting their cell cycle apparatus. However, alternative mechanisms involving the immune system have been recently invoked. Taxanes, doxorubicin, and cyclophosphamide,

which are standard chemotherapeutic agents in the treatment of breast cancer, are known to have major effects on the immune system in animals and human experiments [71]. For example, taxanes, as a class, increase serum IFN-γ, IL-2, IL-6, and GM-CSF levels as well as reducing the levels of IL-1 and TNF-α [72]. Paclitaxel given neoadjuvantly increases the levels of tumor infiltrating lymphocytes (TILs) within the tumor itself [73, 74]. This altered cytokine microenvironment can further influence the therapy outcome.

1.4 Resistance, Recurrence and Metastasis

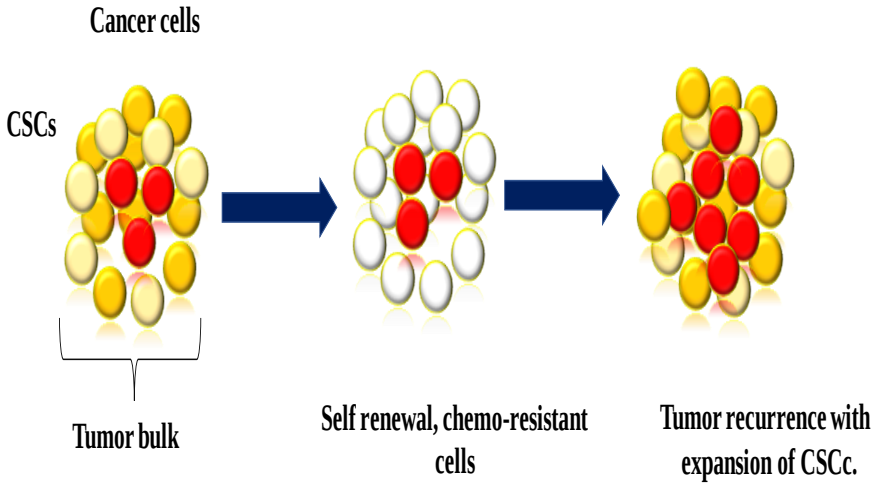
A breast cancer patient has very good chance of a disease-free survival if the cancer is detected and treated early which is very subjective. In many cases, the cancer is thought to have been treated early only to discover its reappearance years after the first intervention. Recurrence of breast cancer is a major clinical manifestation and represents the principal cause of breast cancer-related deaths [75]. A number of researchers have tried to predict some sort of pattern for breast cancer recurrence. This has included studies in various breast cancer subtypes wherein breast cancers are characterized by the presence of receptors such as estrogen receptor (ER), progesterone receptor (PR), and HER2/ErbB2 receptor (HER2) or by the absence of all of them, the triple negative breast cancers (TNBCs). A differential pattern of recurrence between different breast

cancer subtypes has been suggested, [76, 77] and it appears that ER-negative breast cancers are associated with higher risk of recurrence during the initial 5 years after diagnosis, compared to ER-positive breast cancers. Thereafter, the risk of recurrence chronically increases in ER-positive breast cancers for the next 10 years, and at 15 years following diagnosis, the risk appears to be equal for both subtypes. In ductal carcinoma in situ, it has been analysed that the ER-negative/PR-negative but HER2-positive cancers have higher risk of recurrence, compared to ER-positive/PR-positive/HER2-negative cancers [78]. The TNBCs, marked by absence of ER/PR/HER2, are generally associated with high risk of recurrence with particularly high risk of distant recurrences in brain and visceral metastases, compared to receptor positive tumours. Irrespective of the underlying breast cancer subtype, a large number of advanced stage breast cancers are marked by metastases to lymph nodes, and, overall, the presence of axillary lymph node metastases is associated with considerable poor disease-free as well as overall survival [79]. Axillary lymph node metastases remain a very important prognostic variable, and identification of molecular markers for development of lymph node metastases can potentially help intervene early reducing the chances of breast cancer recurrence [80].

There is a differential risk of breast cancer recurrence in patients with different molecular subtypes. Among the patients that underwent breast conserving therapy, patients with ER-positive and PR-positive breast cancers had reduced

instances of recurrence than HER2-overexpressing and TNBC patients. Similar results were observed for mastectomy patients as well where ER-positive and PR-positive patients were again found to be at a lesser risk of recurrence compared to HER2-overexpressing and TNBC patients. Although both HER2-overexpressing and TNBC patients were found to be at a higher risk of recurrence, a direct comparison between the two subtypes revealed that HER2-overexpressing breast cancer patients presented higher risk of recurrence in patients undergoing breast conserving surgery. In the mastectomy patients, the risks of recurrence in HER2-overexpressing and TNBC patients was not found to be statistically different [81].

A



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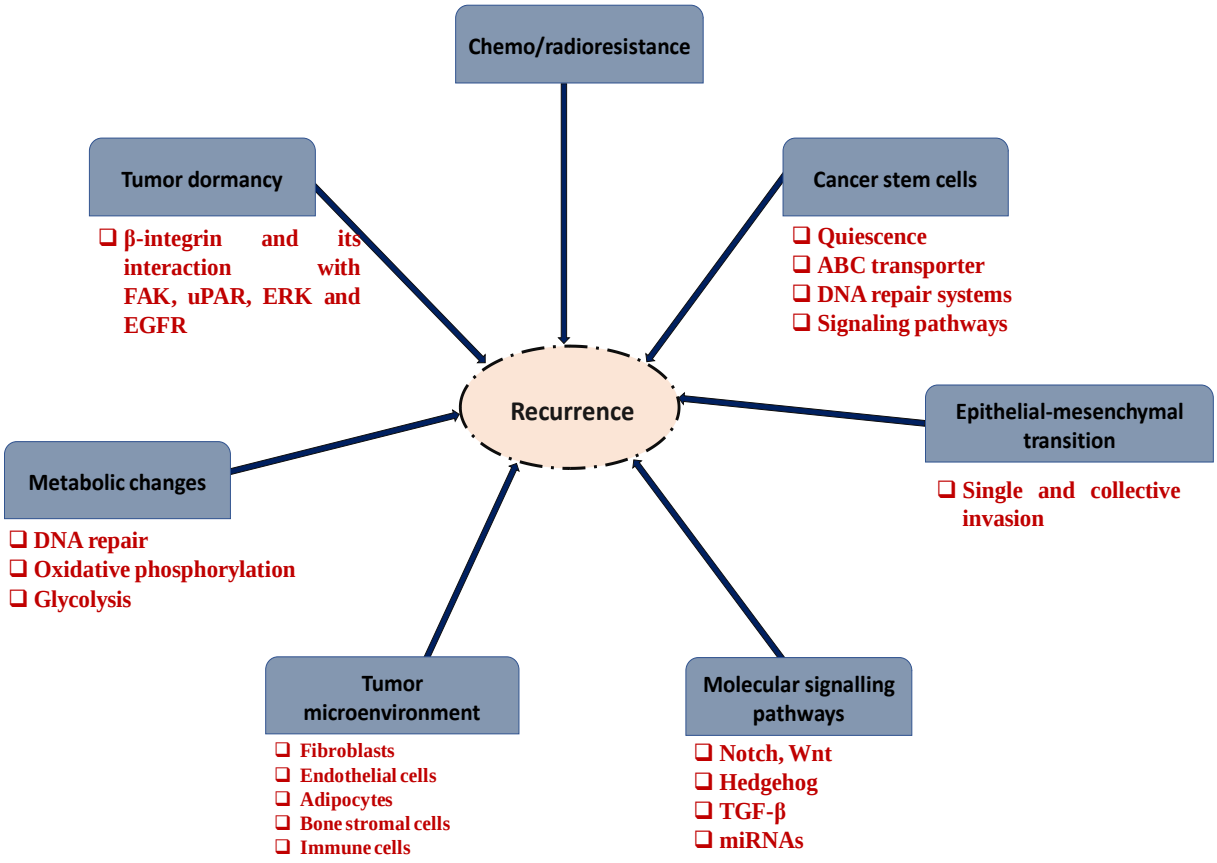


Figure 1.6: Recurrence of cancer cells (A) recurrence through enrichment of CSCs after treatment; (B) different factors responsible for recurrence of tumor.

Two interesting hypotheses have been proposed to better define our understanding of true tumour recurrences. The first one is that the local recurrences might actually initiate long before the diagnosis of primary tumour and may be recorded as multifocal primary tumour at the time of diagnosis, and, that, true local recurrences might actually never metastasize to distant organs [82]. Another theory for cancer recurrence is the wound-oncogene-wound healing (WOWH) hypothesis which is based on the observed interrelationships between precancerous lesions, cancer, oncogenes, wound healing, and cancer recurrence. The essence of this theory is that the “wounds,” exemplified by

physical (such as radiations), chemical (such as carcinogens) and biological (such as inflammation, aging, and reactive oxygen species) damages, trigger the oncogenes to produce cytokines resulting in recruitment of stem cells and tissue remodelling. All this leads to generation of cancer mass, particularly with continued existence of wounds, and ultimately results in death of the organism [83].

1.4.1 Factors that influence breast cancer recurrence

1.4.1.1 Tumor dormancy

Tumor dormancy is the stage where cancer cells, after primary cancer intervention and apparent treatment, enter a state wherein they virtually go undetected waiting for right time and conditions to trigger cancer recurrence [84]. β1-integrin is one molecular factor that has been proposed to play an important role in the switch from dormant state to that of metastatic progression in breast cancer [85]. Interactions of β1-integrin with several factors such as focal adhesion kinase (FAK), urokinase-type plasminogen activator receptor (uPAR), extracellular signal-regulated kinase (ERK), and epidermal growth factor receptor (EGFR), all of which influence tumor microenvironment and have been implicated in breast cancer progression [86, 87].

1.4.1.2 Tumor microenvironment

The tumor microenvironment is now recognized as an important participant of tumor progression and response to treatment. In addition to tumor cells, the tumor microenvironment is composed of several critical normal cell types, including fibroblasts, vascular and lymph endothelial cells, osteoclasts, adipocytes, and immune cells. These cells have important roles in normal homeostasis, which are used by the tumor cells to its advantage. The tumor-associated stromal cells play an important role in contributing to tumorigenesis, tumor progression, and metastasis. Therefore, these host cells can be a potential target in anti-tumor and anti-metastatic therapeutic strategies. This has another advantage that the tumor associated host cells do not mutate and therefore do not develop resistance in response to treatment, which is a major cause of failure in cancer therapeutics targeting neoplastic cells.

Fibroblasts constitute a major portion of stromal cells present in the tumour and their activation is a common feature more than 75% of cancer-associated fibroblasts (CAFs) were found to be in an activated state in breast carcinomas [88]. CAFs influence the initiation and progression of tumours. CAFs, when injected along with breast cancer cells, induced larger tumors as compared to normal fibroblasts. [89]. In addition, injection of non-invasive cells along with CAFs resulted in invasive tumors with metastasis [90].

Endothelial cells are the central regulators of the interaction between the blood and tissues resulting in gas and metabolite transfers and diapedesis in the tumor microenvironment [91]. The endothelium provides support and stability for the blood vessels which are highly organized under normal conditions. On the contrary, the tumour vasculature has no hierarchy and has abnormal sprouting with large gaps [92]. Adipocytes or fat cells were thought to be an inert cell population, but recent evidence suggests that the adipocytes present in the tumor microenvironment can acquire different charcteristics and are called 'cancer-associated adipocytes'. Altered adipocyte metabolites seem to be involved in cancer-associated cachexia, or muscle wasting that is frequently observed in cancer [93].

Bone metastasis is frequently observed in all breast cancers in all molecular subtypes with the exception of basal-like tumours. There is almost a symbiotic relationship between the two cell types, with the tumours secreting growth factors which help in bone remodeling and vice versa with the tumor promoting effect of osteocytes. [94]. Anti-inflammatory cytokines like TGF-β, though have negative growth regulatory functions in normal mammary epithelial cells, promote tumour development in the bone [95]. Breast cancer cells colonize the bone by binding to various adhesion proteins [96]. Pro-inflammatory cytokines alsoactivate osteoclasts through TGF-β and RANKL expression [95].

The cells of the innate immune system, mainly the macrophages, myeloid-derived suppressor cells and dendritic cells contribute to both breast cancer development and progression [97]. Macrophages are recruited by the breast cancer cells through macrophage colony stimulating factor (M- CSF) which polarize into an immunosuppressive or M2 phenotype in the tumor microenvironment. These tumour associated macrophages (TAMs) exhibit immune suppression by secreting anti inflammatory cytokines as well as growth factors [98]. Myeloid-derived suppressor cells (MDSCs) are a mixed population of immature myeloid lineage cells that are found within the breast tumour and peripheral lymph nodes. MDSCs can exhibit immunosuppressive as well as tumor promoting effects and are released from the bone marrow and undergo expansion in response to tumour secreted-growth factors [99]. Dendritic cells are most potent antigen presenting cells and connect the innate and adaptive immune response. Tumor infiltrating dendritic cells captures the tumor antigens from the microenvironment and presents them to T cells. However, in many tumours, dendritic cell function is compromised by cell death of infiltrating dendritic cells, thereby dampening the tumour antigen presentation [100]. Tumour cells avoid detection and destruction by the immune system by ineffective antigen presentation, augmentation of T- regulatory cells, downregulation of co-stimulatory molecules or activation of immune checkpoint inhibitors [101].

1.4.1.3 Cancer stem cells

Many hypotheses have been proposed to explain tumor recurrence such as clonal selection, angiogenic dormancy, and, more recently, cancer stem cells (CSCs) [102, 103]. CSCs are cells within populations of cancer cells or tumours which possess the capacity to self-renew and produce heterogeneous lineages of cancer cells [104]. CSCs, by virtue of being stem cells, have tumor-initiating capabilities. CSCs are now believed to persist in tumors as distinct populations that are fundamentally associated with drug resistance, tumor recurrence, and metastasis. Several molecular pathways have been proposed to play a role in maintenance of CSC phenotype which is further complicated by the observation that none of the molecular markers of CSCs seems to be universally relevant. Most of the research is cancer specific, and the factors/pathways relevant in one cancer may or may not be relevant targets for therapy in other cancers. A number of mechanisms are believed to contribute to the CSCs-induced resistance to drugs and tumor recurrence, and these include quiescence, upregulation of ABC transporters, highly efficient DNA repair systems and upregulation of several signaling pathways [105]. Quiescence is the state of temporary inactivity. The role of quiescence in CSCs activity has its basis in the observation that several chemotherapeutic regimes target rapidly proliferating cancer cells. Thus, CSCs, through their ability to proliferate slowly with intermittent phases of quiescence, can evade the cytotoxic effects of anticancer

drugs. The role of ATP binding cassette (ABC) transporters in drug resistance of cancer cells has long been advocated [106]. CSCs have an enhanced resistance to conventional and targeted therapies. One striking feature of CSCs is they express high levels of specific ABC transporters, whose activity can be measured by using fluorescent dyes, such as Hoechst 33342 and rhodamine 123, which can be exported by ATP-binding cassette subfamily-B member 1 (ABCB1) and ATP-binding cassette subfamily-G member 2 (ABCG2) respectively [107]. This flux can be measured by flowcytometry and during analysis this population of cells can be visualized as a negatively stained population of cells, just to the side of the main population, or the side population (SP). Because CSCs efflux these fluorescent dyes they can be sorted by collecting cells that contain only low levels of Hoechst 33342 fluorescence. When isolated, SP cells are capable of initiating a novel tumour in immune compromised mice in small numbers and give rise to differentiated progeny. However, this isolation method has limitations, because non-CSCs often express ABCB1 and ABCG2 as well. CSCs represent a small population of cells (2–8%) within the bulk tumour; therefore, when isolating CSCs, consideration needs to be given to the right combination of markers [108]. Breast cancer is in the forefront of ongoing studies on the role of CSCs in mediating metastasis as well as resistance to current pharmaceutical regimes, and this is believed to involve a complex interplay of several cell types, cytokines, cell growths, and signalling pathways [109, 110]. Also, cell surface

markers such CD44 (high)/CD24 (low)/ALDH-positive have been associated with the CSCs [111]. The phenomena of drug resistance and tumor recurrence are intricately related because, in order to recur, cancer cells need to overcome the cytotoxic effects of drugs that are used to control the growth of these cancers in clinics. Thus, drug resistance, mediated by CSCs, goes hand in hand with tumor recurrence [112].

1.4.1.4 Epithelial-mesenchymal transition

Epithelial mesenchymal transitions (EMT) is a fundamental biological process by which epithelial cells undergo biochemical shifts to become mesenchymal cells to generate or regenerate tissues that have different polarization from the original epithelia. Progression of most carcinomas towards malignancy is associated with the loss of epithelial differentiation and a switch toward mesenchymal phenotype, which is accompanied by increased cell motility and invasion. The process of EMT, by which epithelial cells undergo remarkable morphological changes, is characterized by a transition from epithelial cobblestone phenotype to elongated fibroblastic. This process involves loss of epithelial cell-cell junction, actin cytoskeleton reorganization, and upregulation of mesenchymal molecular markers such as vimentin, ZEB-1, ZEB-2, fibronectin, and N-cadherin. A disassembly of cell-cell junction, including downregulation and relocation of E-cadherin and zonula occludens-1 as well as downregulation and translocation of β-catenin from cell membrane to nucleus,

is known to be the mechanism for the induction of EMT. Epithelial cells have a regular cell-cell junction and adhesion which inhibits cell movement of individual cells. In contrast, mesenchymal cells have weaker adhesion between cells compared to their epithelial counterparts, which renders mesenchymal cells more motile and confers more invasive characteristics. In addition to classical markers of EMT, such as E-cadherin, vimentin, and ZEB- 1/ZEB-2, the process of EMT is also influenced by several other signalling molecules, particularly those from Notch and Wnt signalling pathways [113, 114].

1.4.1.5 Single cell invasion

Tumor cell invasion is defined as the movement of single cells that break away from the tumor mass and migrate into the blood stream. However, recent studies have shown that there are many modes of single-cell invasion [115]. Tumor cells that have transitioned into mesenchymal type can upregulate proteases, such as matrix metalloproteinases, that degrade ECM proteins [116]. Alternatively, tumor cells can also change their shape and move through gaps in the ECM [117, 118]. A complete mesenchymal state characterized by suppression of E-cadherin and augmentation of vimentin results in the ability of tumor cells to dissociate and invade as individual cells [119].

1.4.1.6 Collective Invasion

Collective invasion is the predominant mode of tumor invasion, as determined by the reconstruction of the primary tumor organization [120], evaluation of tumor explants [121] and intravital imaging [122]. Similar to single cell invasion, there are many modes in collective invasion also. The EMT transitions can confer (a) a trailblazer phenotype (has the ability to initiate collective invasion) [123]. (b) Opportunistic state (motile cells dependent on extrinsic factors for invasion) [124]. (c) A hybrid state (restricted responses, but has the ability to invade under EMT initiating signals). This can be regulated by transcription factors [125]. or micro RNA [126]. This hybrid state can also be sustained by activated transcription factors in collectively invading cells that sustain E-cadherin expression [127].

1.4.1.7 Molecular signalling pathways

Alterations in Notch, Wnt and Hedgehog signalling have been implicated in tumour recurrence [128-130]. Signalling pathways are networks of regulatory proteins and other gene products that act in a coordinated manner to control various biological processes inside the cell. Hedgehog (Hh) signalling is a critical pathway that mainly controls embryonic development, whereas in post-natal life, it is inactive or poorly active, playing a restricted role in stem cell maintenance and tissue homeostasis/repair [131]. Recent studies show that Hh signalling elements talk to several other cofactors belonging to major pathways,

such as Notch, Wnt, and transforming growth factor β (TGF-β), resulting in significant crosstalk between these signalling networks. The integration of several signalling pathways is a key step able to determine a more aggressive behaviour of tumour cells and their resistance to pharmacological approaches [132, 133].

MicroRNAs (miRNAs) are small (19–24 nucleotides) noncoding RNA molecules which down-regulate gene expression by interacting with sequences located in the 3′ untranslated region (UTR) of target mRNAs, resulting in either translational repression or degradation of mRNAs [134]. Regulation of oncogenes/tumour suppressor genes by miRNAs is now recognized as a key step in the progression of human malignancies [135] and it is dependent on sequence complementarities. miRNAs largely function via repression of their target genes; therefore, if the target gene of a miRNA is an oncogene, that particular miRNA will be tumour suppressive. In contrast, an oncogenic miRNA is the one whose target is a tumour suppressor gene [136]. Several investigations have been carried out to uncover the miRNA regulation of breast cancer metastases that might be the reason for tumour relapse. In one such study that focused on bone metastasis of breast cancer, miR-21 and miR-181a were found to be enriched in bone metastatic breast cancers leading to poor prognosis [137]. The last few years have seen an exponential increase in the number of investigations focused on the functionality of miRNAs in breast cancer

progression. Several miRNAs have been reported to be up regulated (miR 9, 21, 31,34c, 122, 125b, 181a) and down regulated (miR 30a, 34a, 92a, 126, 320, 335, 451). However, more robust investigations are needed regarding their target genes and off-target effects to further exploit the potential of these tiny regulatory molecules [138]. Breast cancer recurrence is too complex a problem to be understood entirely through laboratory investigations or the clinical observations alone. Though there is a wealth of accumulated literature, a concerted effort jointly by the basic scientists and clinical investigators is required in order to provide more meaningful, exhaustive collaborative projects that culminate in well-designed clinical trials.

1.4.2 Chemoresistance

The mechanisms of resistance to chemotherapeutic drugs in breast cancer include: (a) cell membrane influenced drug absorption, transport and efflux; (b) membrane glycoproteins that act as efflux pumps; (c) inactivation of anti tumor drugs by altering their metabolism or quantity and affinity of hormone receptors; (d) expression of genes related to DNA repair, proliferation or apoptosis; (e) Cancer stemness; (f) Tumor microenvironment (g) signalling mechanisms.

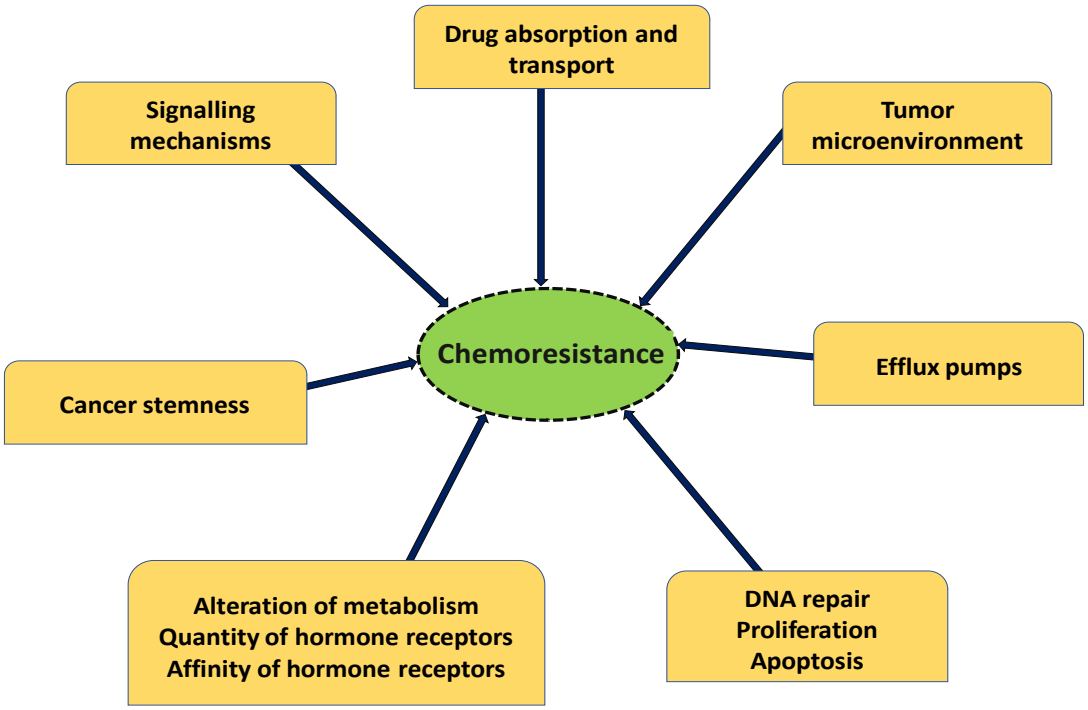


Figure 1.7: Factors responsible for resistance to chemotherapeutic agents.

Membranes from drug-resistant cells have a different lipid composition as compared to the membranes from the parent drug-sensitive cells resulting in altered membrane fluidity, structural order, lipid packing density, membrane potential or the combination of multiple factors. These changes lower the ability of a drug to permeate the membrane [139]. Decreased drug influx is one of the important causes of low intracellular drug accumulation in drug-resistant cells. Most cancer chemotherapeutics are weak bases with pK values between 7.4 and 8.2, lipophilic in neutral form and hence, can traverse the cell membrane which could be altered in a drug resistant cancer cell [140]. Doxorubicin-resistant P388 subline showed a decrease in the membrane's phosphatidylcholine/SM ratio and an increase in membrane order [141]. On the other hand, vinblastine-

resistant leukemia T lymphoblast cells that demonstrated higher levels of cholesterol and phospholipids and a 60% increase in protein/lipid ratio in comparison to membranes of sensitive cells [142].

Drug sequestration is another important drug-resistance mechanisms in which drugs are trapped in intracellular acidic compartments, such as lysosomes, recycling endosomes, and the trans-Golgi network [143], ultimately leading the sequestered drug out of cells via exocytosis. This has been reported due to the difference in pH between cytoplasm and intracellular vesicles [144]. In addition, epigenetic changes in breast cancer drug resistant cells have also been attributed to the altered lipid biosynthesis that directly influenced drug transport and endocytic functions in drug-resistant cells [145].

Membrane transporters play key roles in pharmacology, affecting the entry of drugs into cells and extrusion of drugs from them. In particular, some ATP-binding cassette (ABC) transporters mediate energy-dependent efflux of drugs and thereby play major roles in the development of drug resistance [146]. There are several ABC transporters including MDR-associated protein, such as P-glycoprotein (P- gp), multidrug resistance associated protein (MRP), ABCC subfamily, and breast cancer resistance protein, such as breast cancer resistance protein (BCRP), ABCG subfamily [147]. P-gp is encoded by the gene of ABC subfamily B, member 1 (ABCB1) an predominantly functions through increase of glycoprotein P- 170 and P-150 to promote drug efflux; as well as enhanced

antioxidant status and DNA repair mechanism. ABC transporters increase the drug efflux from tumor cells leading to reduced intracellular drug concentrations which are not cytotoxic enough. Drug resistance mediated by ABC transporters is therefore not drug specific and instead mediate resistance against the entire class of drugs [146, 148].

Another important cause of MDR in cancer cells are the detoxifying enzymes [149]. Glutathione S transferases (GST) improve drug resistance inhibiting pro-survival pathways such as mitogen-activated protein kinase (MAPK) [150] or inactivating several drugs [151] Cancer cells with p53 mutation are resistant to cancer chemotherapy due to the loss of the transcriptional function of wild-type p53 [151]. and possible gain of new function by mutant p53 [152]. Resistance to platinum-based cancer chemotherapy is linked increased DNA repair [153]. and inhibition of apoptosis [154].

Cancer stem cells (CSCs) play an important role in chemoresistance of breast cancer [155]. Furthermore, CSCs can induce drug resistance via increased DNA repair activity and inhibition of apoptosis [156]. The over expression of ALDH1 is also an important cause for drug-resistance of CSCs [156]. In addition some signal transduction pathways such as Notch, Hedgehog [157], and Wnt/β-catenin, may play role in the self-renewal and maintenance as well as drug resistance of CSCs. microRNA mediated regulation of CSC

resulting in drug resistance has also been reported [158]. This has been confirmed in CSCs using a series of markers [159]. The BCSCs exist in two different development statuses: mesenchymal-like (EMT) status and epithelial-like (mesenchymal–epithelial transition [MET]) status [160]. In EMT, BCSCs express CD44+, CD24− and EpCAM+; the BCSCs with MET gene expression profile express aldehyde dehydrogenase (ALDH) [161]. Additionally, ALDH1 expression serves as a predictor of poor prognosis in breast cancers [162].

The tumour microenvironment is composed of extracellular matrix and interstitial tissues and soluble components that include a variety of cytokiens and growth factors. Cellular components of microenvironment include normal stromal cells, lymphocytes, dendritic cells, tumour-associated macrophage (TAM), mesenchymal cells, endothelial cells and cancer-associated fibroblast (CAF). Drug resistance in tumor can be contributed by macrophages [163]. mesenchymal stem cells (MSCs) [164]. endothelial cells [165]. and CAFs [166]. CAFs also contribute to tamoxifen resistance [167]. and other drug resistance in tumor cells [167].

The survival signaling pathways resulting in increased proliferation can result in the resistance to endocrine therapy for breast cancers [168]. Studies show that EGFR, HER-2/neu and PI3K and other growth factor signalling pathways are associated with tamoxifen resistance [169]. Moreover, EGFR nuclear translocation may be one of the mechanisms of resistance to gefitinib in

breast cancer [170]. The role of TGF-β in EMT of breast cancer is well understood [171, 172]. In a study suggestive of adverse effects of anticancer drugs, doxorubicin was induced TGF-β-driven EMT resulting in CSCs and the resistance to chemotherapy [173]. TGF-β also plays a role in the expression of breast cancer resistance protein (BCRP), an ABV drug efflux transporter [174]. This is another indication of a complex relationship between EMT, CSCs, drug resistance, and the breast cancer recurrence.

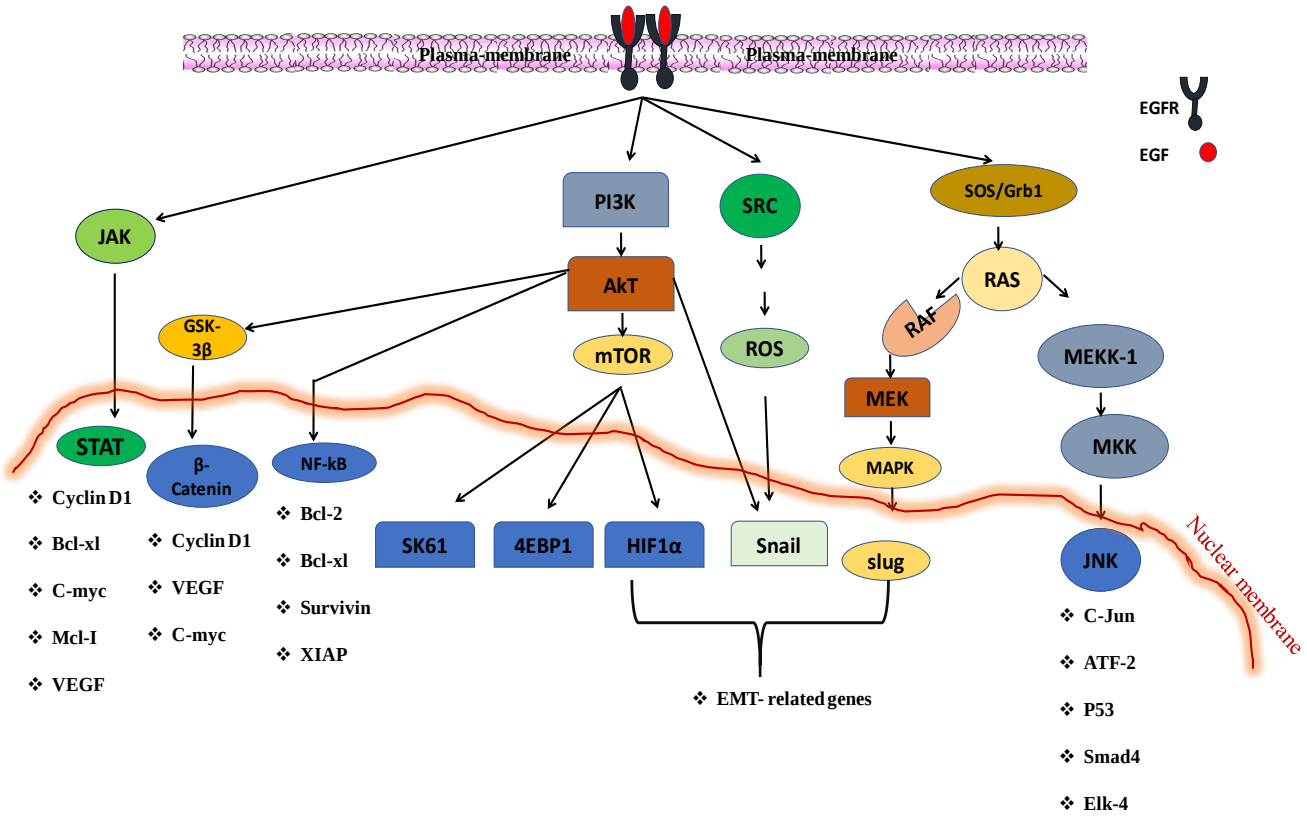


Figure 1.8: Activation of various pro-survival pathways leading to chemoresistance [175, 176].

1.4.3 Radioresistance

Recent improvements in radiation technologies and delivery have substantially increased the efficiency and quality of treatment [177]. Nevertheless, one of the fundamental problems of radiation oncology is tumor resistance to radiation doses which cause an acceptable degree of normal tissue toxicity. Tumor radio resistance leads to loco-regional control failure and disease progression [178, 179]. Meta-analyses have shown that, for women with node-positive disease, post mastectomy radiotherapy reduced the risks of recurrence, breast cancer and overall mortality. By contrast, radiotherapy did not provide any such benefit in women with node-negative disease. The rate of overall recurrence following radiotherapy post mastectomy in node positive women is around 33% during years 0–9 and is significantly lower than the rate of recurrence without administration of radiotherapy (45%). However, these patients experiencing post-radiotherapy loco-regional recurrence were at higher risk of metastases and had poorer overall survival (OS) than patients achieving long-term local control [180].

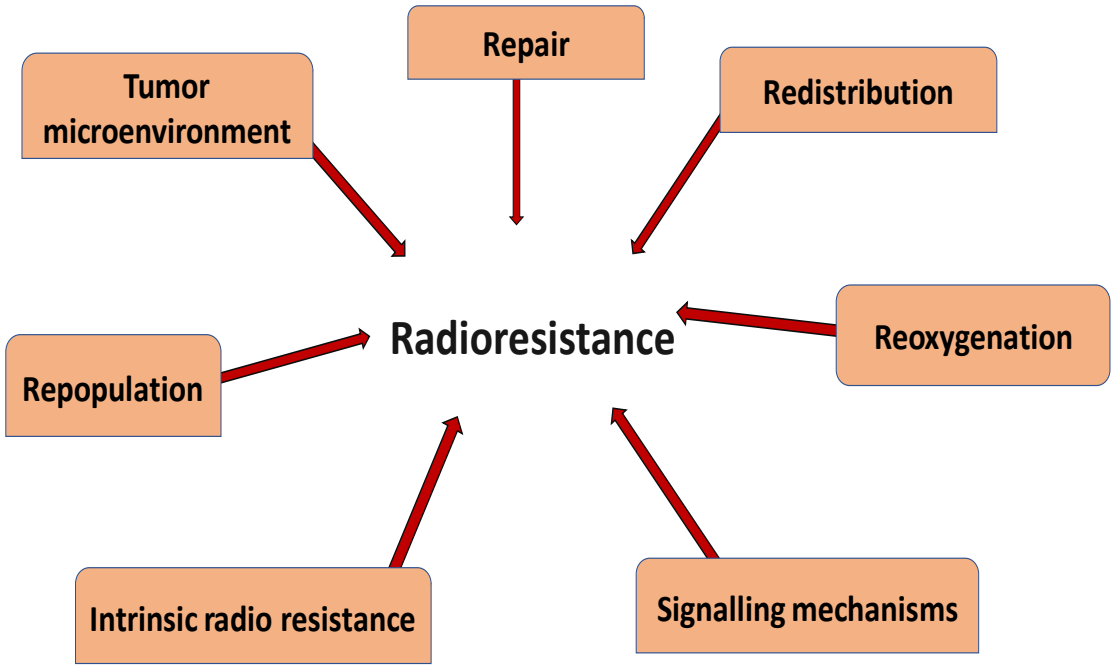


Figure 1.9: Factors responsible for radioresistance in tumors.

The curative potential of radiotherapy depends on its ability to cause reproductive death of tumor cells via accumulation of non-repairable DNA lesions, thereby removing cancer cells from the clonogenic pool [181]. The biology-based patient stratification aims to select potential responders and non-responders to increase the probability of cancer cure by radiation therapy. It is based on the radiobiological concept of the "5 Rs" which are repair, redistribution, repopulation, reoxygenation, and intrinsic radio resistance [182, 183].

The first "R" refers to DNA repair as one of the key determinants of tumour cell survival after radiation therapy. The cellular response to DNA damage is a complex process including activation of the DNA damage response pathways and DNA repair mechanisms [184]. Ionizing radiation (IR) damages

DNA by direct deposition of energy and also indirectly, by ionization of water molecules to produce hydroxyl radicals that attack the DNA. IR induces multiple forms of DNA damage including damage to the bases, and cleavage of the DNA backbone to form DNA single strand breaks (SSBs). These types of DNA damages are detected and repaired by the base excision repair (BER) and SSB repair pathways, respectively [185]. DNA double strand breaks (DSBs) are formed when two SSBs occur on opposite DNA strands approximately 10–20 bp apart. Thus, IR-induced DSBs usually contain overhanging 3' and 5' ends. In addition, the DNA termini frequently contain 3'-phosphate or 3'-phosphoglycolate groups, which must be removed prior to ligation [186]. Moreover, the DNA surrounding the DSB may contain additional forms of DNA damage, producing what are termed complex or clustered lesions [187]. If not repaired, such lesions can result in cell death. If misrepaired, DSBs have the potential to result in chromosomal translocations and genomic instability [188]. DSBs are considered the most cytotoxic type of DNA lesion and to repair DSBs, cells employ two major mechanisms: the more error-prone non-homologous end joining (NHEJ) and the more accurate homologous recombination (HR). In addition, tumour cells also have two extremely error-prone DSB back-up repair mechanisms for both NHEJ and HR, the alt-EJ and single-strand annealing (SSA) [189]. In mammalian cells, HR occurs only in the late synthesis (S) phase and less in the gap 2 (G2) phase of the cell cycle when the DNA template on the sister chromatid is available for recombination,

whereas NHEJ is active throughout the entire cell cycle with highest efficiency during the G2/mitosis (M) stage and is predominant in G0, G1, and early S phases [190, 191]. An activation of these different DNA repair mechanisms at specific phases of the cell cycle results in differences in radio sensitivity throughout the cell cycle, with increased radio resistance in the late S phase and increased radio responsiveness in G2 and M phases. Increased cell radio resistance in the S phase has been attributed to an increased level of DNA replication enabling the HR process [192]. Resistance caused by HR-mediated repair is further enhanced by the presence of all available DNA repair pathways, including those that go beyond the repair of DSBs [193].

Alteration in several cell signalling pathways contributes to the generation of radio resistance. These vary from activation of Wnt/β-catenin [194] long non coding RNAs [195] miRNAs [196] as well as loss of cyclin dependent kinase inhibitor $p27^{KIP1}$ [197]. Radiation induced hypoxia induced factor (HIF1α) activates the transcription of several target genes that regulate various biological processes, including cell proliferation, apoptosis, glucose metabolism, and pH regulation, playing a vital role in the adaptation of cancer cells to hypoxic conditions reviewed in [198].

Several signalling pathways have been reported to contribute to radiation resistance. These include cellular responses to radiation that originate from DNA damage and its consequent repair [199] both non homologous end joining

[200] as well as homologous recombination [201] or various signal transduction pathways like PI3K/AKT, Wnt, EGFR, Notch, operating in the cell [202-206]. Several molecules like survivin, cyclins, NFkB, Her2, manganese superoxide dismutase, peroxiredoxins have been implicated in conferring radio resistance [207-210]

Radiotherapy also modulates antitumor immune responses resulting in the activation of cytokine cascades in the microenvironment. Cytokines, such as TNF-α, IL-1α,IL-1β, IL-6, and TGF-β produced either by the tumor cells or tumor-infiltrating lymphocytes in the microenvironment can greatly influence cellular radio sensitivity and the onset of tissue complications [211-213].

Transforming growth factor- beta (TGF-β) has been reported to be an endogenous, radiation-inducible radio-resistance factor in some cancer cells while not affecting the radio-sensitivity in others [214]. In addition, TGF-β also regulates transcription of various target genes responsible for the pathological changes of late radiation damage in the non-tumour-bearing tissues of previously irradiated patients [215]. TGF-β isoforms, TGF-β1, TGF-β2 and TGF-β3 regulate a wide variety of biological functions including cell proliferation, migration, survival, angiogenesis, immunosurveillance, embryonic stem cell maintenance and differentiation [216]. The multifunctional effects of TGF-β isoforms are elicited through dimerization of the type I (TβRI) and type II (TβRII) serine/threonine kinase receptors. Upon TGF-β binding, the receptor

complex phosphorylates the transcription factors SMAD2 and SMAD3, which then binds to SMAD4 and translocate to the nucleus [217]. In addition to TGF-β, radio-resistance induced by specific protein kinases, transcription factors and microRNAs are also reported [218-221]. Global kinome pathway analysis of radioresistant breast cancer cells has revealed alteration in several kinases involved in cell cycle progression and DNA damage response [222].

An association between radiation and EMT has been reported by many investigators [223-226]. Similarly, radiation induced enrichment of cancer stem cells in xenografts exposed to radiation [227] as well as induction of stem cell-like properties in non-stem cancer cells have also been reported [155, 228, 229]. Tumor heterogeneity, in particular, the existence of cancer stem cells (CSC) plays an important role in radio-resistance. Altered DNA repair to involvement of Notch, TGF-β and Wnt signaling pathways have been demonstrated to have a role in CSC maintenance [202, 230-235]. However, the relationship between radiation induced EMT processes and cancer stem cells has conclusively not been established. Though many studies indicate an association between EMT and the gain of CSC properties, the signalling pathways linking them are still not explicit and could be triggered by TGFβ, Wnt/β-catenin, Hedgehog, Notch, and others [236].

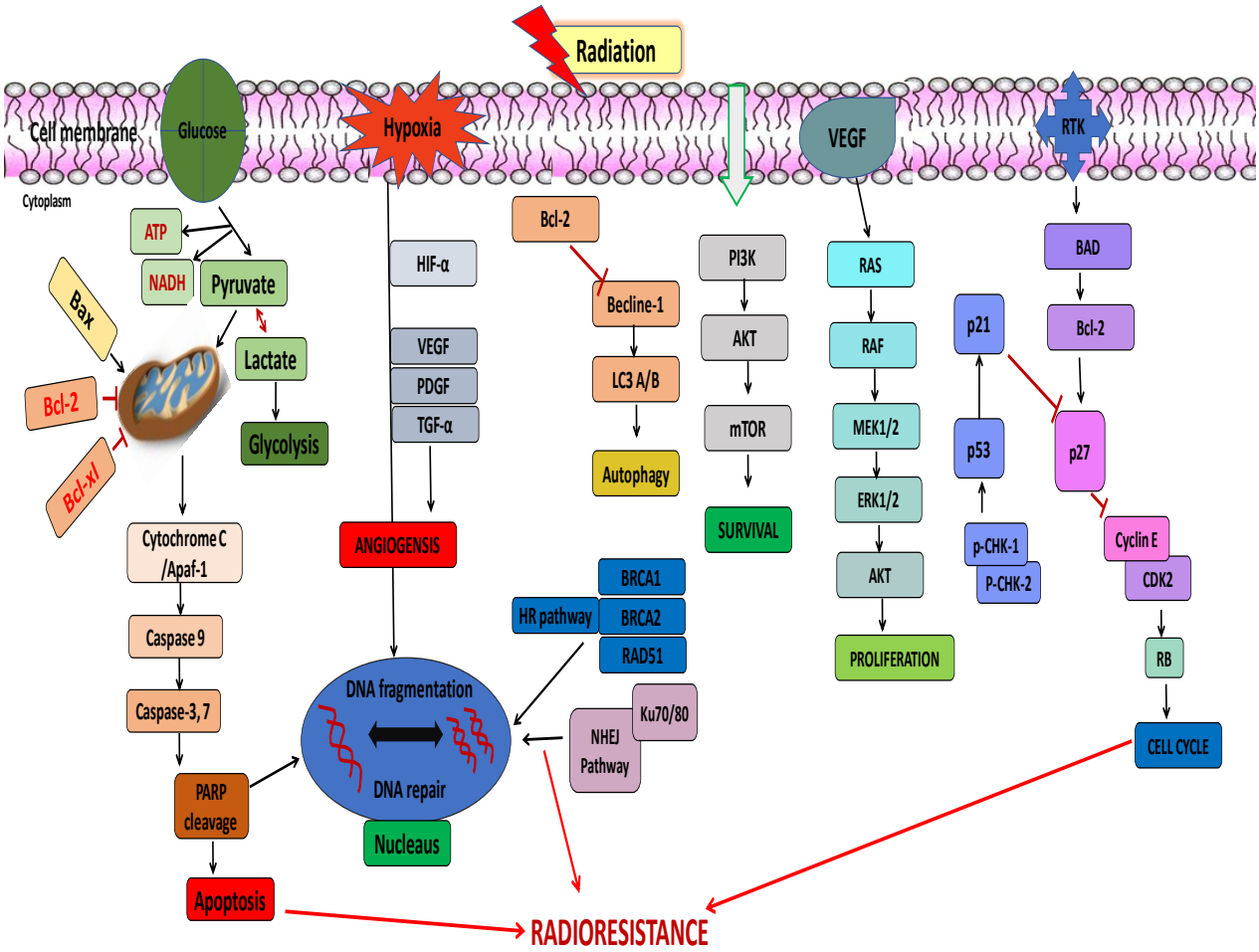


Figure 1.10: Radiation induced pro-survival pathways responsible for development of radioresistance

Cancer radioresistance is associated with the activation of several pro-survival pathways (PI3K/Akt/mTOR, ERK, glycolysis, VEGF, autophagy, NHEJ and HR DNA repairs), the induction of cell cycle redistribution and inactivation of apoptosis pathway after exposure to radiation. ERK: Extracellular signal-regulated kinases, HR: Homologous recombination, NHEJ: Non-homologous end joining, VEGF: Vascular endothelial growth factor

1.4.4 Metabolic changes

DNA repair, mitochondrial oxidative phosphorylation and glycolysis: Breast cancer has been associated with deficiency in DSB break repair in many studies which indicate that the functions of key breast cancer susceptibility genes, BRCA1 and BRCA2, ATM and TP53 their products play important roles in DSB repair and chromosome stability [237-239]. Also, increased frequencies of chromatid breaks and gaps after exposure to radiation in G2-phase have been observed in cultured cells from predisposed individuals and breast cancer patients [240, 241]. On the other hand, there are also studies that demonstrate that sporadic breast cancers are not associated with a deficiency in DSB repair, but rather with upregulation of the HR pathway [242]. Radioresistance is considered to partly occur through their extensive ability of repairing DNA damage that has been provoked through radiation. This enhancement of DNA repair capacity can be either direct, through elevated DNA repair mechanisms, or indirect, through delayed cell-cycle progression. Several approaches are being used to target DNA double-strand break repair molecules for radio sensitization, including small interfering RNA, aptamers, antisense and small-molecule inhibitors [243-247]. Alterations in metabolism have been reported to be mediated by STAT [248] or AKT [249]. Many studies indicate that interfering with glycolytic or mitochondrial metabolism may improve radiosensitivity [250, 251].

1.5 Proteomic approaches to identify biomarkers of therapy resistance

Many studies have been conducted to elucidate the mechanisms of therapy resistance and to identify predictive biomarkers. The use of global analytical techniques to study complex phenomena, such as resistance to anti cancer therapy, enable the simultaneous analysis of whole genomes and/or proteomes and potentially allow all genes and/or proteins that are associated with a specific disease phenotype to be identified. The major advantage being that the regulation of previously unknown genes and/or proteins can be implicated in a particular disease state.

Genomic approaches include cytogenetics techniques, such as multicolor fluorescence in situ hybridization (mFISH), spectral karyotyping (SKY), and comparative genomic hybridization (CGH) which exploit advanced fluorescent technology to study the whole chromosomal complement of cells [252]. Transcriptomic approaches use microarray-based technology, which explores the relative levels of RNA expression from thousands of known genes simultaneously or RNA sequencing for gene expression profiling. Gene-expression profiling has been used to analyze both chemotherapy and radiotherapy resistances in various tumor types [253, 254].

The proteins within a cell are responsible for key biologic processes and also make up the bulk of pharmaceutical targets. Unfortunately, the expression

levels of mRNA and the corresponding protein are often not comparable and posttranslational modifications and alternative splicing events cannot be inferred from genomic technologies. Therefore, in order to gain a more comprehensive understanding of intricate biologic systems, the information extracted from genome studies must be complemented with information on the proteins themselves. Recent technological advances have enhanced the analysis of the human proteome.

A number of proteome analysis methods have been utilized in cancer research. They include (a) Two-Dimensional Gel Electrophoresis and Mass Spectrometry (2DE-MS) (b) Matrix-Assisted Laser Desorption/Ionization (MALDI) (c) (Electrospray Ionization (ESI) based MS (d) Mass Spectrometry Instrumentation and Protein Identification (e) Quantitative Proteomics Using Labeling Techniques (f) Surface-Enhanced Laser Desorption/Ionization-TOF MS (SELDI-TOF MS) (g) Liquid Chromatography with tandem mass spectrometry.

There is a variety of mass spectrometers based on different ionization, mass analysing, and detection methods. While ionization methods determine the classes of substances available for measurement, it is a combination of the mass analyser with the detector that ultimately determines the quality and reliability of analysis. Depending on the physics of mass analysis, analysers could belong to quadrupole, magnetic sector, ion trap, time-of-flight (TOF), or Fourier

transform (FT) generic types. They could be further combined together to allow analysis of both analytes and their fragments (MS/MS), most popular combinations being triple quadrupole and quadrupole/time-of-flight hybrids. Alternatively, the same analyser can perform MS and MS/MS (MS2) analysis, sometimes to a high MSn stage, such as a radiofrequency ion trap (Paul trap) or a static electromagnetic trap (Penning trap) [255].

The Orbitrap mass analyser, the newest addition to the family of high-resolution mass spectrometry analysers consists essentially of three electrodes. Outer electrodes have the shape of cups facing each other and electrically isolated by a hair-thin gap secured by a central ring made of a dielectric. A spindle-like central electrode holds the trap together and aligns it via dielectric end-spacers. When voltage is applied between the outer and the central electrodes, the resulting electric field is strictly linear along the axis and thus oscillations along this direction will be purely harmonic. At the same time, the radial component of the field strongly attracts ions to the central electrode. Ions are injected into the volume between the central and outer electrodes essentially along a tangent through a specially machined slot with a compensation electrode (a “deflector”) in one of the outer electrodes. With voltage applied between the central and outer electrodes, a radial electric field bends the ion trajectory toward the central electrode while tangential velocity creates an opposing centrifugal force. With a correct choice of parameters, the ions remain

on a nearly circular spiral inside the trap, much like a planet in the solar system. At the same time, the axial electric field caused by the special conical shape of electrodes pushes ions toward the widest part of the trap initiating harmonic axial oscillations. Outer electrodes are then used as receiver plates for image current detection of these axial oscillations. The digitized image current in the time domain is Fourier-transformed into the frequency domain in the same way as in FTICR and then converted into a mass spectrum [255].

Many studies have reported the identification of novel biomarkers of radioresistance in breast cancer cells following proteomic analysis. These include 26s proteasome, focal adhesion kinase, peroxiredoxins, cathepsins, gelsolin, arginino-succinate synthase 1 and C-type mannose receptor 2, Maspin, 14-3-3σ, glucose-regulated protein (GRP-78), and Mn superoxide dismutase (MnSOD) [210, 256-259]. Proteomic studies on drug and radiation resistant xenograft tumors have also been reported [260-262].

Research Hypothesis

As discussed above, activation of various pro-survival signalling pathways play a crucial role in emergence of chemo and radio resistance. Most of the studies on radio resistance reported in the literature involve the use of radioresistant cell lines generated after exposure of cell lines to incremental and/or multiple doses of radiation amounting to several Gy. Our hypothesis is that "radiation induces pro-survival signalling mechanisms in cancer cells, and hence helps in acquiring radioresistant properties. These radiation induced changes are reverse or modulates if pro-survival signalling is blocked or if there is any change in tumor microenvironment". We have studied the emergence of radio resistance in breast cancer cell lines after an acute exposure of radiation following a long recovery period and the importance of TGF-β signalling pathway in this radioresistance.

- **Objective of the present study**:

✓ To delineate the mechanisms involved in radiation induced survival signalling in cancer cells.

✓ To understand the effect of pro and anti-inflammatory cytokines in modulation of radiation mediated signalling in cancer cells.

2. MATERIALS AND METHODS

The studies reported in this thesis include (1) *in-vitro* experiments conducted in cell lines (2) *in vivo* experiments conducted in SCID mice. Methodologies used in cell lines for *in-vitro* experiments were (a) cell culture and maintenance, (b) treatment of cells, (c) assays for proliferation, (d) MTT assay, (e) BrdU incorporation, (f) clonogenic assay, (g) assays for apoptosis, (h) annexin labelling, (i) sub G1 DNA content, (j) migration assay, (k) ELISA, (l) RT-PCR, (m) Flowcytometry, (n) Estimation of Glucose Uptake Using 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose (2-NBDG), (o) ALDH assay, (p) Detection of metabolic changes: Sea horse analyser. Methodologies used in tumors generated in SCID mice for *in-vivo* experiments were (a) animal maintenance: generation of radioresistant cells in SCID mice model, (b) preparation of single cell suspension from tumor mass, (c) protein estimation by Bradford assay, (d) proteomic analysis of tumor samples, (e) denaturation and reduction of protein samples, (f) liquid chromatography, (g) high-resolution mass spectrometry, (h) analysis of MS-data, (i) PANTHER Analysis, (j) statistical analysis, some techniques like RT-PCR, ALDH assay and ELISA were used in cell lines as well as in tumors.

2.1 *In-vitro* experiments

2.1.1 Cell culture and maintenance

The breast cancer cell lines used in this study, MCF-7 and MDA-MB-231 were obtained from National Centre for Cell Sciences, Pune, India.

MCF-7 is a human breast cancer cell line (ATCC Number HTB-22) derived from the pleural effusion of a 69-year-old Caucasian metastatic breast cancer (adenocarcinoma) by Dr. Soule of the Michigan Cancer Foundation, Detroit, MI in 1970 [263]. MCF-7 cell line has estrogen, progesterone and glucocorticoid receptors and also called as first hormone responding breast cancer cell line.

MDA-MB-231 cell line (ATCC Number HTB-26) is an epithelial, human breast cancer cell line that was established from a pleural effusion of a 51-year-old Caucasian female with a metastatic mammary adenocarcinoma. MDA-MB-231 cell line is highly aggressive, invasive and poorly differentiated triple-negative breast cancer (TNBC) cell line. It lacks estrogen receptor (ER) and progesterone receptor (PR) and HER2 receptors (human epidermal growth factor receptor 2) [264, 265].

These cell lines were maintained in Dulbecco's Modified Eagle's medium (DMEM) (high glucose: 4 mM L-glutamine, 4.5 g/L glucose and 3.7 g/L sodium bicarbonate in 25 mM HEPES buffer with sodium pyruvate) supplemented with 10% fetal bovine serum (FBS), 100 units/ml of penicillin

and 100 μg/ml of streptomycin (complete medium). Cells were passaged 2 times a week on attaining confluence by treatment with trypsin-EDTA (0.25% porcine trypsin, 0.02% EDTA) solution. The resulting single cell suspension was diluted and centrifuged at 101 g/minute to remove trypsin, re-suspended in complete medium and used for further experiments or incubated at 37^0C in a humidified incubator with 5% CO_2 atmosphere. Preservation of cell lines: Cells were cryopreserved in 1 ml of freezing solution i.e.10% dimethyl sulfoxide (DMSO), 40% FBS (fetal bovine serum) and 50% DMEM in cryo vials in -80^0C overnight and subsequently stored in liquid nitrogen at -196^0C. When needed, cells were revived by thawing the frozen stocks by washing twice with DMEM to remove all traces of DMSO and cells were re-suspended in complete DMEM in 100 mm dishes. Cells were maintained in humidified incubators with 5% CO_2 at 37^0C.

2.1.2 Treatment of cells

MCF-7 and MDA-MB-231 cells were plated in 60 mm dishes at the density of 10^5cells /5 ml complete medium for 6 h. Complete medium was replaced with serum free medium for overnight prior to exposure to ionizing radiation (IR). Cells were irradiated using Bhabhatron, a ^{60}Co source (Panacea Biotech Ltd, New Delhi, India) with a dose rate of 1 Gy/min. Medium was changed to complete medium after irradiation. Figure 2.1 describes the treatment protocol followed. Breast cancer cells were exposed to different doses of ionizing

radiation (2–10 Gy). The cells were placed in a humidified incubator at 37^0C in a 5% CO_2 atmosphere and allowed to recover for days 1, 4 and 7. Following the recovery period, they were trypsinized and re-plated immediately for MTT assay. All other experiments like BrdU incorporation, clonogenic assay, apoptosis, RT-PCR, flowcytometry analysis of different genes etc., were carried out in cells exposed to 6 Gy and recovered for 7 days (D7-6G).

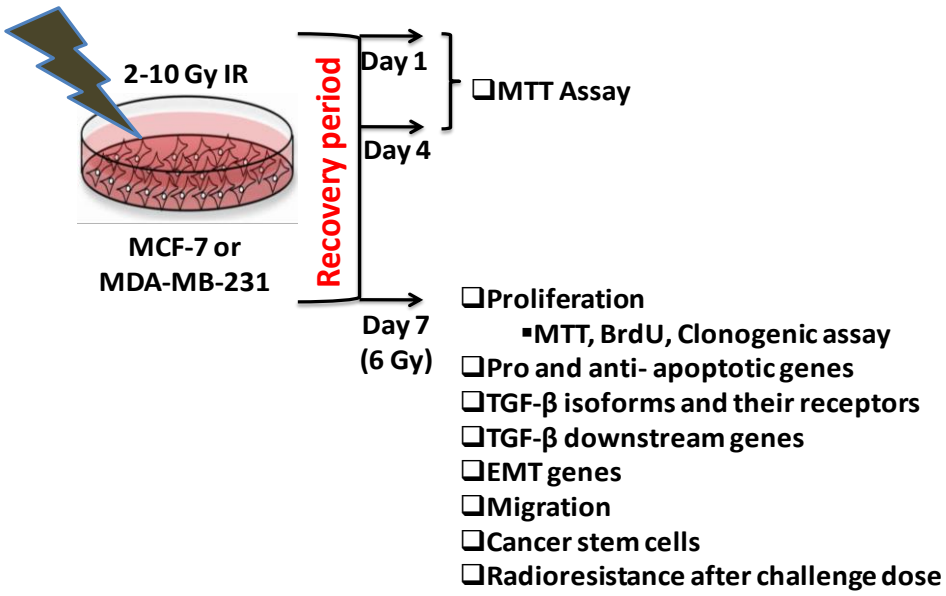


Figure 2.1: Scheme of the experimental protocol

2.1.3 Assay for proliferation

2.1.3.1 MTT assay

MTT [3-(4, 5-dimethythiazol2-yl)-2, 5-diphenyltetrazolium bromide] is yellow in colour, enters the cells and passes into the mitochondria where it is reduced by mitochondrial succinate dehydrogenase to an insoluble, coloured (dark purple) formazan product. The cells are then solubilised with 10% sodium

dodecyl sulphate (SDS) and the released, solubilised formazan reagent is measured spectrophotometrically [266]. Since reduction of MTT can only occur in metabolically active cells, the level of activity is a measure of the viability of the cells. This assay was carried out in all different treatment groups: (i) cells exposed to 6Gy IR, (ii) cells pre-treated with TGF-β receptor inhibitor SB431542: 10 µM for 2 h followed by exposure to IR or (iii) cells pre-treated with cytokines, either TNF-α or IL-10 for 24 h followed by exposure to IR. On the day of the experiment, cells were trypsinized, re-suspended in compete medium, counted and again seeded at different densities of 10^3, $5X10^4$ and 10^4 in 96 well plate for a period of 48 h. After 48 h, MTT (10 µM) was added and incubated for 4 h in a humidified incubator at 37^0C and 5% CO_2. After 4 h, formazan crystals were solubilised with 10% SDS (100 µl/well) overnight. Next day the absorbance was measured at 550 nm in a microplate reader.

2.1.3.2 BrdU incorporation

BrdU (5-bromo-2'-deoxyuridine) is an analogue of the DNA precursor thymidine. When BrdU is added to the cell culture, proliferating cells will incorporate it into their DNA and the amount of BrdU in the DNA of cells was detected by cellular ELISA with monoclonal antibodies against BrdU [267]. MCF-7 and MDA-MB-231 cells were seeded at a density of 10^5 cells/5 ml in 60 mm dishes in complete medium for 6 h, following which the cells were kept in plain medium, and serum starved overnight. Cells were exposed to ionizing

radiation 6 Gy IR and allowed for a recovery period of 7 days in humidified incubator at 37^0C and 5% CO_2. The supernatant was discarded and adherent cells were subsequently trypsinized, re-suspended in compete medium, counted and again seeded at different densities of 10^3, $5X10^4$ and 10^4 in 96 well plate for a period of 48 h. Later 10 µM BrdU was added. After 2 h, excess BrdU solution was removed thoroughly by flicking the plate. After removal of the labelling medium, FixDenat solution was added to the cells and incubated for half an hour after which it was removed by thoroughly flicking the plate. Anti-BrdU-POD solution (100 µl/well) was added and incubated at 25^0C for 90 minutes. Plate was rinsed thrice with 200 µl washing solution. Substrate solution (100 µl) was added and incubated until sufficient colour was formed. The colour development was stopped using 25 µl of stop solution, 1 M H_2SO_4. Absorbance was measured in a micro plate reader at 450 nm (BioTek, Winooski, VT, USA).

2.1.3.3 Clonogenic assay

Clonogenic assay or colony formation assay is an *in-vitro* cell survival assay which is based on the ability of a single cell to grow into a colony. It determines the cell reproductive death after treatment with ionizing radiation or other cytotoxic agents. The colony is defined as a group of 50-100 cells [268]. After treatment, adherent cells were trypsinized, re-suspended in complete medium, counted and seeded at a density of 100 cells/well in 6 well plates for a week. Once colonies are visible, then supernatants were discarded and plates were

washed twice with PBS and fixed with acetone: methanol (70:30) overnight at -20^{0}C. Colonies were stained with crystal violet (0.5% w/v). Stereo microscope was used to capture the colonies and were manually counted. After counting clones, plating efficiency (PE) and survival fraction (SF) can be calculated using the following equation:

PE= No. of colonies formed/ No. of cells seeded x 100

SF= No. of colonies formed after treatment / No. of cells seeded x PE.

2.1.4 Assays for Apoptosis

2.1.4.1 Annexin labelling

Apoptosis is a physiological process which plays an essential role in the development and homeostasis of all multi-cellular organisms. It is characterized by DNA fragmentation and, consequently, loss of nuclear DNA content. Cells undergo distinct morphological changes depending on the pathway. Annexin V and propidium iodide (PI) labelling of cells is used to identify cell death, and distinguish between its different pathways: apoptosis, or programmed cell death, and necrosis. Apoptosis was assessed in the following treatment groups: (i) cells exposed to 6 Gy IR. After treatment and 7 days recovery period, adherent cells were trypsinized, washed with PBS and re-suspended in Annexin V binding buffer along with the floating cells. Cells were centrifuged at 101 g for 5 minutes and supernatant was discarded. Cells were re-suspended in 100 µL

1X Annexin V binding buffer. Annexin V– FITC was added according to the manufacturer's recommendations, incubated at RT for 15 minutes. Propidium iodide (PI) solution diluted in binding buffer was added to the final concentration of 2 μg/mL in each sample and incubated in dark for 15 minutes. Cells were washed with PBS and resuspended in 1X Annexin V binding buffer and fixed with 1% formaldehyde (fixative) solution for 10 minutes on ice. Cells were washed with PBS and acquired in a PartecCyFlow Space™ flowcytometer and data were analysed using FCS Express™ software.

2.1.4.2 Sub G1 DNA content

Apoptosis is characterized by DNA fragmentation and, consequently, loss of nuclear DNA content. Use of a fluorochrome PI (propidium iodide: an intercalating agent), which is capable of binding and labelling DNA, followed by flow cytometric evaluation [269] gives an estimate of the DNA content of the cells. Since apoptosis is involved with DNA fragmentation, the DNA content of apoptotic cells is lower and therefore cells with less than G1 DNA content are enumerated as apoptotic cells [270]. Apoptosis was assessed in the following treatment groups: (i) cells exposed to 6 Gy IR, (ii) cells treated with TGF-β receptor inhibitor SB431542: 10 μM for 2 h followed by exposure to IR. After treatment, adherent cells were trypsinized, re-suspended in compete medium, counted and again seeded at a density of 10^5 cells/well in 6 well plates for 48 h. After 48 h, cells were trypsinized, washed with PBS and re-suspended

in propidium iodide (PI) staining solution (50 µg/ml PI, 0.1% Triton-X 100 and 0.1% sodium citrate in dH_2O) at 37^0C for 30 min. Samples were acquired in a PartecCyFlow Space™ flowcytometer and data were analysed using FCS Express™ software. Cells with less than G1 DNA content were enumerated as apoptotic cells.

2.1.5 Migration assay

The movement of individual cells, cell sheets and clusters of cells from one location to another is called as cell migration [271]. Migration assay was carried out in the following treatment groups: (i) cells exposed to 6Gy IR, (ii) cells treated with TGF-β receptor inhibitor SB431542: 10 µM for 2 h followed by exposure to IR. After treatment, the adherent cells were trypsinized, re-suspended in compete medium, counted and again seeded at a density of 10^3 cells/insert in transwell inserts (with 8 µm membrane pore size) and incubated for 72 h. The cells on the upper side of the membrane were removed with a cotton swab. All cells that migrated to the bottom of the membrane were fixed in methanol: acetone (7:3) for 20 min at -20^0C and stained with crystal violet, photographed in a Nikon Eclipse Ti™ inverted microscope equipped with a Nikon digital camera and counted using NIS elements™ software.

2.1.6 ELISA

Secretion of cytokines by breast cancer cells was measured by sandwich ELISA technique. In this method, the cytokine is captured between two antibodies specific for two different epitopes. To detect antigen (cytokine), the wells of microtiter plates are coated with specific (capture) antibody followed by incubation with test solutions containing antigen [272]. Free or unbound antigen is washed out by wash buffer (PBST) and a different antigen specific antibody conjugated to enzyme (i.e., developing reagent) is added, followed by incubation. Unbound conjugate is washed out by PBST and substrate is added. When a measurable amount of colour is developed, stop solution is added to stop the reaction. The absorbance was measured at 450 nm in a micro plate reader. The amount of substrate hydrolysed is proportional to the amount of antigen in the test solution. ELISA was carried out in the different treatment groups: (i) cells exposed to 6 Gy IR, (ii) cells treated with TGF-β receptor inhibitor SB431542: 10 μM for 2 h followed by exposure to IR After treatments, the supernatants were collected and to remove dead cells or debris, they were centrifuged at 101 g for 5 minutes, collected in different tubes and used for cytokine measurements. Supernatants were stored at -80^{0}C until use. ELISA was carried out for different cytokines like TGF-β1, β2, β3 as well as TNF-α and IL-10. The range of standards used for these cytokines are given below.

Table no: 2.1. Range of standards used in ELISA

Cytokine	Standard range
TGF-β1	**62.5 pg/ml -1000 pg/ml**
TGF- β2	**10 pg/ml -10000 pg/ml**
TGF- β3	**10 pg/ml -10000 pg/ml**
TNF-α	**62.5 pg/ml -10000 pg/ml**
IL-10	**15.625 pg/ml -500 pg/ml**

Activation of sample: For detection of TGF-β isoforms in supernatant, the samples were first activated by acidification using 1 N HCl at 1:25 (100 µl supernatant + 4 µl of 1 N HCl) for 10 minutes at 4^0C. Acidification reaction was neutralized by addition of 1 N NaOH at 1:25 (4 µl) to activated sample.

Microwell plate was coated with capture antibody prepared in coating buffer: 0.1 M sodium carbonate buffer, pH 9.5. Capture antibody (2 mg/ml) was used at a dilution of 1: 250 in coating buffer and 100 µl was added in 96 well plates and incubated overnight at 4^0 C. Plates were washed with wash buffer (PBS with 0.05% Tween-20). After washing, assay diluent (PBS with 10% FBS) was added and incubated for an hour. Plates were washed again with wash buffer. Samples (supernatants for TNF-α and IL-10 and activated supernatants for TGF-β isoforms), and the respective standards in the concentration ranges as

given in the Table 2.1 were added in the plates and incubated for 2 h, followed by washes with wash buffer. Then biotinylated detection antibody diluted 1:250 and enzyme reagent: streptavidin-horseradish peroxidase conjugate (SAv-HRP) diluted 1:250, was added to the plate and incubated for 2 h. Plate was washed and substrate tetramethylbenzidine (TMB) was added till colour developed. Reaction was stopped by adding stop solution (2 N $H2SO_4$). Absorbance was measured in microplate reader at 450 nm.

2.1.7 RT-PCR

Real-time PCR is a quantitative method for determining copy number of PCR templates, such as DNA or cDNA. Intercalator-based method was used which requires a double-stranded DNA dye (SYBR Green) in the PCR which binds to newly synthesized double-stranded DNA and generates fluorescence. Total RNA was isolated from 10^6 cells by using RNA isolation kit (HiPurA™ Total RNA Miniprep Purification Spin Kit, HiMedia, India). Concentration of total RNA isolated was determined by measuring its absorbance at wavelength of A260nm/A280nm with Picodrop™ spectrophotometer. Optical density ratio of pure RNA preparations is 2.0.

Reverse transcription: It involves the synthesis of DNA from RNA by using an RNA-dependent DNA polymerase followed by PCR (polymerase chain reaction). Total RNA was reverse transcribed to cDNA in a reaction mixture which contained: dNTPs, reverse transcriptase and random hexanucleotide

primers. This was further used for PCR amplification of target genes. RT-PCR was carried out in the different treatment groups: (i) cells exposed to ionizing radiation (IR 6 Gy), (ii) cells treated with TGF-β receptor inhibitor SB431542: 10 μM for 4 h followed by exposure to IR or (iii) UT and D7-6G tumors isolated from SCID mice. After treatment, the expression of the target genes in the different sample groups was assessed using Real-time PCR. Total RNA (250 ng) was reverse transcribed to cDNA using first strand cDNA synthesis kit. Equal amount of cDNA (0.4 ng) was used for PCR amplification of the genes using specific primers. Concentration of gene specific primers used was 2.5 pmoles. qRT-PCR was carried out in a LightCycler® 480 System (Roche Applied Science, Penzberg, Upper Bavaria, Germany). All reactions were performed with SYBR green in triplicates. Relative mRNA levels were calculated by the $2^{-\Delta\Delta Ct}$ method ($\Delta Ct = Ct_{Target} - Ct_{Ref}$, $\Delta\Delta Ct = \Delta Ct_{treatment} - \Delta Ct_{untreated}$), using GAPDH or 18sRNA as the reference gene.

Table 2.2: List of primers used in the study

Genes	Forward primer5'-3'	Reverse primer3'-5'
18s RNA	CTACCACATCCAAGGAAGGCA	TTTTTCGTCACTACCTCCCCG
ALDH	TGAATGGCACGAATCCAAGAG	CACGTCGGGCTTATCTCCT
Bad	GTTCCAGATCCCAGAGTTTG	CCTCCATGATGGCTGCTG
Bax	TTTCTCACGGCAACTTCAAC	GGAGGAAGTCCAATGTCCAG
Bcl-2	GAGGATTGTGGCGTTCTTT	CCCAGCCTCCGTTATCCT
Bcl-Xl	ACATCCCAGCTCCACATCAC	CGATCCGACTCACCAATACC

Desmoplakin	**GCTTGCCAACTTCAGAGGTTCT**	**TTGGAGAATAGCCTGGAGCAGT**
E-Cadherin	**TTCCTCCCAATACATCTCCC**	**TTGATTTTGTAGTCACCCACC**
Fibronectin	**CCCCATTCCAGGACACTTCTG**	**GCCCACGGTAACAACCTCTT**
GAPDH	**ATGACATCAAGAAGGTGGTG**	**CATACCAGGAAATGAGCTTG**
HMGA2	**AAGTTGTTCAGAAGAAGCCTGCTCA**	**TGGAAAGACCATGGCAATACAGAAT**
N-Cadherin	**GAGGAGTCAGTGAAGGAGTCA**	**GGCAAGTTGATTGGAGGGATG**
NANOG	**AGGCAAACAACCCACTTCTG**	**TCTGCTGGAGGCTGAGGTAT**
Occludin	**ATGTCATCCAGGCCTC**	**ATAGACAATTGTGGCA**
OCT-4	**CGCAAGCCCTCATTTCAC**	**CATCACCTCCACCACCTG**
SNAIL-1	**CACTATGCCGCGCTCTTTC**	**GCTGGAAGGTAAACTCTGGATTAGA**
SOX-2	**TGTCATTTGCTGTGGGTGAT**	**GGGGTGCAAAAGAGGAGAGT**
TGF-β1	**GGCCCTGCCCCTACATTT**	**CCGGGTTATGCTGGTTGTACA**
TGF-β2	**TCAAGAGGGATCTAGGGTGGAA**	**GGCARGCTCCAGCACAGAA**
TGF-β3	**CAGCTCTAAGCGGAATGAGCAG**	**TATAGCGCTGTTTGGCAATGTG**
TGF-β RI	**AAGTCATCACCTGGCCTTGGT**	**TGCGGTTGTGGCAGATATAGA**
TGF-BRII	**AATATCCTCTGAAGAACGACCTAA**	**TCCCACCTGCCCACTGTTA**
Vimentin	**CTCTTCCAAACTTTTCCTCCC**	**AGTTTCGTTGATAACCTGTCC**
ZEB-1	**AGTGATCCAGCCAAATGGAA**	**TTTTTGGGCGGTGTAGAATC**

2.1.8 Flowcytometry

Flowcytometry is a laser based instrument which is used to count and sort cells, detect biomarkers and engineered proteins, by suspending cells in a stream of fluid and illuminated by laser as the light source. The light scattered in forward and right angles are measured as FSC and SSC. If a fluorescent label, or fluorochrome, is specifically and stoichiometrically bound to a cellular component, the fluorescence intensity will ideally represent the amount of that particular cell component. Flowcytometry for assessment of different markers was carried out in the following treatment groups (i) cells exposed to ionizing radiation (IR 6 Gy), (ii) cells treated with TGF-β receptor inhibitor SB431542: 10 μM for 4 h followed by exposure to IR.

For surface labelling, cells were washed with PBS and conjugated E-Cadherin antibodies were added to the cells, incubated for 1 h, and washed with PBS. Isotype control was used to account for non- specific binding. For intracellular labelling, cells were fixed with 1 ml of 70% chilled ethanol in -20^0C O/N. Cells were washed with cold PBS and kept in blocking solution (5% FBS in PBS) for 30 min to prevent non-specific binding. The cells were then washed with PBS and primary antibodies (1 μg/10^6 cells) were added to cells and incubated for 1 h. After two washes with PBS, the cells were incubated with fluorochrome conjugated secondary antibody for 30 min. Cells labelled only with secondary antibody served as a negative control. Twenty thousand cells were acquired in a

PartecCyFlow® Space flowcytometer using FloMax 2.1™ software and data were analysed using FCS Express™ software. Direct primary antibodies against Snail-1, ZEB-1, HMGA2 and Vimentin, Bax and Bcl-2 were used.

2.1.9 Estimation of Glucose Uptake Using 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose (2-NBDG)

Cancer cells show increased glycolysis and glucose uptake as a result of reprogrammed glucose metabolism [273]. Breast cancer cells are known to overexpress Glut5, a sugar transporter responsible for the transfer of sugar across the cell membrane but not over express in the normal breast cells [274]. Evaluation of glucose uptake ability in cells was carried out by flow cytometric detection of fluorescence produced by the cells following incubation with a fluorescent D-glucose analogue 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-D-glucose (2-NBDG). It's a sensitive and non-radioactive assay for direct and rapid measurement of glucose uptake in single, living cells. MCF D7-6G cells were trypsinized, washed with PBS and resuspended in 2-NBDG (5 µM) labelling solution prepared in PBS with 2% serum, and incubated for an hour at 37^0C in dark. After an hour, cells were washed with PBS and acquired PartecCyFlow Space™ flowcytometer and data were analysed using FCS Express™ software.

2.1.10 ALDH assay

The NAD-dependent Aldehyde Dehydrogenase (ALDH) plays a vital role in cellular detoxification. It oxidizes various aldehydes and generates the corresponding carboxylic acid. In this assay, acetaldehyde is oxidized by ALDH generating NADH which then reduces a colourless probe to a coloured product with strong absorbance at 450 nm [275]. ALDH assay was carried out in the following treatment groups: (i) untreated MCF-7 cells and cells exposed to ionizing radiation (IR 6 Gy), (ii) cells isolated from UT and D7-6G tumors in SCID mice. Cells were harvested using trypsin, counted and 1 x 10^6 cells were rapidly homogenized with ~ 200µl ice cold ALDH assay buffer for 10 minutes on ice, and then spun down at 101 g for 5 min to remove nuclei and insoluble material. Fifty µl of the collected supernatant was added into a 96 well plate, incubated at room temperature for 5 min and absorbance of samples and sample backgrounds was measured at 450 nm (A1 & A1B) and then again measured after 20 minutes (A2 & A2B). The absorbance of NADH standards (4 nM to 20 nM) was measured at the beginning and end point.

Calculation: Absorbance values of the standards corrected for the blank was used for the construction of the standard curve.

ALDH activity in the supernatant was calculated using the formula:

Sample ΔOD 450 nm [(A2 - A2B) - (A1 - A1B)].

These values were applied on the standard curve to get B nmol of NADH generated during the reaction time (ΔT= T2 - T1).

ALDH activity = (B/ (ΔT X V)) x Dilution Factor = nmol/min/ml = mU/ml.

2.1.11 Detection of metabolic changes: Sea horse analyser

Cancer cell uses different strategies to meet their energy requirementsand anabolic needs [276]. Mitochondria are structurally and functionally different from their non-cancerous counterparts andhave emerged as a potential target for anticancer therapy [277]. Mitochondria plays a key role in energy metabolism and cell cycle regulation of cells. Metabolic status can be detected by measuring oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) in living cells, utilizing the Seahorse XF24 Extracellular Flux Analyzer. The Seahorse XF24 Extracellular Flux Analyzer continuously measures (Milli-pH unit changes) oxygen concentration and proton flux in the cell supernatant in real time. These measurements are converted into OCR and ECAR values and helps in direct quantification of mitochondrial respiration and glycolysis [278]. OCR and ECAR were measured in Untreated MCF-7 cells and MCF D7-6G cells. Cells were plated in XF24 cell culture plates and maintained for 24 h. Sensor cartridge was hydrated overnight with calibrant in an incubator at 37^0 C. Pyruvate (100 mM), glutamine (200 mM) and glucose (2.5 mM), were added to XF basal medium. Growth medium was changed to assay medium pH 7.4 and cells were then allowed to equilibrate in an incubator. Then carbonyl cyanide-4-

(trifluoromethoxy) phenylhydrazone (FCCP), an ionophore (14.4 nmol) and oligomycin ATP synthase inhibitor (12.6 nmol) were mixed in XF assay medium, loaded into cartridges and assay was run. Analysis was done using XF analyser. It allows precise measurements of milli-pH unit changes. OCR and ECAR were measured under basal conditions and after injection of compounds through drug injection ports.

2.2 *In-vivo* experiments

2.2.1 SCID Mice

SCID mice carry the scid mutation and are severely deficient in both T cell- and B cell-mediated immunity, as a result of defective V(D)J joining of the immunoglobulin and T-cell receptor gene elements [279]. These mice have low ovarian estrogen production and therefore tumor growth was induced with β-estradiol supplementation [280].

2.2.2 Animal maintenance

Eight to ten-week-old SCID female mice were used. They were reared in the animal house facility of Advanced Centre for Treatment, Research and Education in Cancer (ACTREC), Navi Mumbai. SCID mice were transported in sterile plastic cages and were housed in plastic cages at sterile environment and constant temperature (23^0C) with a 12 / 12 hour light / dark cycle. Female mice were used in the experiments. Maintenance and dissection of mice were strictly

followed according to the ethics guidelines issued by the Institutional Animal Ethics Committee of Bhabha Atomic Research Centre, Government of India (BAEC/06/17).

2.2.3 Generation of radioresistant cells in SCID mice model

SinceD7-6G cells showed high proliferation, the tumor forming ability of these cells *in-vivo* was examined. MCF-7 require estrogen for their growth and so for the better growth of MCF-7 cells *in vivo,* mice were injected with β-estradiaol subcutaneously prior to injection of tumor cells [281]. Untreated and D7-6G MCF-7 cells ($10X10^6$) were injected subcutaneously in SCID female mice. β-estradiaol (2 mg/ml) injection was given subcutaneously after every 72 h. Tumor size was monitored by vernier caliper from day 8 onwards and the animals were sacrificed on day 18 and tumors excised out. Tumor weight was measured and tumor volume was calculated by the formula $V = 1/2(\text{Length} \times \text{Width}^2)$, where V is tumor volume, W is tumor width, L is tumor length [282].

2.2.4 Preparation of single cell suspension from tumor mass

Tumor excised from untreated group of mice and D7-6G group were chopped into small pieces and homogenized using a tissue dissociator kit and gentleMACS™ tissue dissociator. Proprietary Enzymes D, R and A were used to dissociate tumor mass into single cells. Necrotic tissue and fat cells were removed from tumor mass andtumor was cut into small pices of 2-4 mm size.

Enzyme mix was prepared in gentleMACS^{TM} C Tube (prepared in RPMI 1640 2.35 ml + enzyme D: 100µl + enzyme R: 50 µl and enzyme A: 12.5 µl) tumor pieces were transferred into gentleMACS^{TM} C Tube. Incubated for 45 minutes at 40^0C on rocker. The tube was then taken out and loaded onto the gentleMACS^{TM} tissue dissociator and programme "Soft and intermediated tumor: 37c_m_TDK_1" was run. Pre-wet SmartStrainer was pre-wetted with RPMI 1640 medium. The samples were transferred to gentleMACS^{TM} M tube and the programme "Soft and intermediated tumor: 37c_m_TDK_2" was run. After the run was completed, the cell suspension from gentleMACS^{TM}M tube was filterd through Pre-wet SmartStrainer and collected into 15 ml Falcon tube, spun at 137 g for 7 minutes, supernatant was removed and cell pellet was resuspended in complete medium. This procedure resulted in a single cell suspension. These cells were washed thrice with serum containing medium and counted. These tumor cells were further maintained in incubator at 37^0C in 5% CO_2 at the density of $5X10^6$/10 ml of complete medium in 100 mm dishes. These tumor cells were further used for ALDH assay.

2.2.5 Protein estimation by Bradford assay

The assay measures the concentration of total protein in a sample. Under acidic conditions, coomassie dye binds to protein molecules, resultingin a color change from brown to blue. It measures the presence of the basic amino acid residues, arginine, lysine and histidine, which contributes to formation of the protein-dye

complex [283]. For estimation of proteins, 10 µl of unknown protein sample was diluted in 800 µl of water. Standard BSA: (Bovine serum albumin 1 mg/ml) solution was taken in the range of 0 µg to 50 µg. Equal volumes of protein and bradford solution were mixed and incubated in dark for 10 min. Absorbance was measured at 595 nm.

2.2.6 Proteomic analysis of tumor samples

Tissues storedin RNA later were homogenized and used for MS work. Proteins were extracted from tumor tissues in buffer (6 M urea, 2 M thiourea, 2% CHAPS, 0.5% SDS in HPLC grade water), by sonication at 20% amp., 1 sec on/ 2 sec off cycle for 10 min followed by centrifugation at 2195g for 30 min at 4^0C. Supernatant was collected and 6 volume of ice cold acetone was added slowly and kept overnight at -20^0C. Precipitate formed was collected after centrifugation. Acetone was allowed to evaporate from the samples. The pellets were dissolved in 1 M triethylammonium bicarbonate (TEAB) + 6 M urea buffer. Protein estimation was carried out by Bradford method and 100 µg of total protein was used for further process.

2.2.7 Denaturation and reduction of protein samples

Samples were denatured and reduced according to manufacturer's protocol. One hundered µg of proteins from MCF UT and MCF D7 6G tumors were denatured with 2% Sodium dodecyl sulphate (SDS) and reduced with (tris(2-

carboxyethyl) phosphine) (TCEP) at 60^0C for 1 h and cysteine residues were blocked with methyl methanethiosulfonate (MMTS) for 30 minutes in dark at room temperature (RT), followed by trypsin digestion overnight at 37°C. The peptides were dried in a vacuum centrifuge and fractionated by cation exchange (SCX) cartridge system (Sciex, USA). The dried sample was reconstituted with 8 mM ammonium formate (AF)/25% acetonitrile (ACN) (pH 3.0) and eluted from the column using 50 to 500 mM AF/25% ACN (pH 3.0). Three fractions were obtained. The dried fractions were purified through ZipTip pipette tips C18 (Merck-Millipore, USA), eluted with 0.1% formic acid (FA) in 80% ACN and dried with vacuum centrifuge. The samples were resuspended in 0.1% formic acid in H_2O and analyzed by LC MS/MS. LC-MS/MS analysis was performed on O-HRLCMS-72 and Thermo EASY-nLC from SAIF facility at IIT BOMBAY.

2.2.8 Liquid chromatography

With the development of electrospray ionisation (ESI), liquid chromatography-mass spectrometry (LC-MS) gives a simple and robust interface. Wide range of biological molecules and the use of tandem MS and stable isotope internal standards allows highly sensitive and accurate assays to be developed. Single analytical run can give measures of several compounds and high degree of multiplexing with the fast scanning speeds [284].

Samples were introduced into the EASY-nLC 1200 instrument (Thermo scientific, USA), using an Analytical Column: PepMap RSLC C18 2µm, 100A x 50 cm , Pre-column: Acclaim PepMap 100, 100 µm x 2 cm nanoviper. Mobile phase consisted of Solvent A: 0.1% formic acid in milli Q water Solvent B: 0.1% formic acid in a mixture of 80% acetonitrile in milli Q water, wash solvent 3:0.1% formic acid in milli Q water. Temperature was maintained at 30^0C and the flow rate was 4.0 µl/min. The samples were injected (12 µL) into the HPLC system in acetonitrile.

2.2.9 High-resolution mass spectrometry

The MS and MS/MS studies were performed on Thermofisher Q-exactive mass spectrometer (Thermo Electron, Bremen, Germany) using electrospray ionization source and orbitrap mass analyzer. For ionization, heated electrospray ionization source was used and heater temperature was 450^0C and capillary of the ESI interface was 250^0C. As sheath gas and auxiliary gas, nitrogen was used. The electro spray set at 4.5 kv and tube lens was set at 90 V. The mass spectrometer was operated in full scan MS. The selected range was from 100 to 1000 m/z and the resolution was 70,000 full width half maximum (FWHM) with an isolation window applied [278].

2.2.10 Analysis of MS-data

Proteome Discoverer software suite (Thermo Fisher Scientific, Bremen, Germany) was used for MS/MS searches and protein quantitation. SEQUEST algorithm was used for database searches with NCBI RefSeq human protein database. The search parameters included trypsin as the protease with maximum of 2 missed cleavages allowed; oxidation of methionine was set as a dynamic modification while static modifications included carbamidomethyl (alkylation) at cysteine. Precursor mass tolerance was set to 10 ppm and fragment mass tolerance was set to 0.6 Da. The false discovery rate (FDR) was calculated by carrying out decoy database searches and peptides scoring better than 1% FDR score cut-off were considered for further analysis. Bioinformatics analysis of differentially expressed proteins from UT and D7-6G tumors was done to classify proteins based on subcellular localization and biological function. Classification wascarried outbased on annotations in the Human Protein Reference Database (HPRD; www.hprd.org) [285], which is in compliance with Gene Ontology (GO) standards. Pathway and network analysis of differentially expressed proteins was done using Ingenuity Pathway Analysis (IPA).

2.2.11 PANTHER Analysis

The PANTHER (protein annotation through evolutionary relationship) classification system (http://www.pantherdb.org/) is a comprehensive system that combines gene function, ontology, pathways and statistical analysis tools to

analyze large-scale, genome-wide data from sequencing, proteomics or gene expression experiments [286]. The PANTHER system is composed of three functional module. The core module: Large protein library from 82 organisms; second module is PANTHER pathway module: it contains 176 expert curated pathways; the third module is: website tool suit, contains bioinformatics tools and software for analysis of large scale data [287]. Proteins having abundance ratio fold change more than 5 and less than 1 were used to input in PANTHER tools for analysis. Entrez Gene IDs were entered. PANTHER Go-slim Molecular function tool, PANTHER Go-slim Biological Process, PANTHER Go-slim Cellular Processes, and PANTHER Protein Class tools and PANTHER Pathway tools were used to compare changes between MCF UT tumors and MCF D7-6G tumors.

2.2.12 Statistical analysis

Graph Pad Prism was used to perform statistical analyses. For column analyses, statistical significance between the groups was assessed using one- way ANOVA with Dunnett's post test comparing all columns to control untreated "UT" column, and a p value less than 0.05 was considered significant. For grouped analyses, statistical significance was assessed using two- way ANOVA with Bonferroni post-tests to compare replicate means by row and compare each column to "UT" column, All results are expressed as mean ± standard error of mean (SEM) and a p value less than 0.05 was considered significant.

3. RESULTS

Radiotherapy (RT) is a major treatment modality in the treatment of cancer. The major problem with radiotherapy is the resistance acquired by cancer cells, resulting in recurrence of tumour, poor prognosis thus leading to treatment failure. This is a complex process involving multiple mechanisms, genes and factors. The mechanisms underlying the development of radioresistance have been studied by many researchers, and the major factors involved in this process seem to be (a) increased cell cycle arrest and rate of DNA damage response [288, 289], (b) changes in the tumour microenvironment [290], (c) autophagy and tumour metabolism changes [249, 291], (d) clonal selection, angiogenic dormancy, and cancer stem cells (CSCs) [102]. Work embodied in this thesis describes the alterations in TGF-β signalling pathway in breast cancer cell lines that acquire radioresistance following recovery after an acute exposure of radiation.

The results chapter is subdivided into three parts. Part I describes the findings of the role of TGF-β signalling, hybrid epithelial-mesenchymal phenotype and cancer stem cells in acquired radioresistance of breast cancer cells. The breast cancer cell lines MCF-7 and MDA-MB-231 were used as a model system to study the effects of radiation. MCF-7 and MDA-MB-231 cells exposed to radiation followed by different recovery periods generated radioresistant phenotype. Part II of the results chapter describes the effect of TGF-β receptor I (TGF-β RI) inhibitor SB431542 and pro- and anti-inflammatory cytokines TNF-

α/IL-10 on radioresistance of breast cancer cells. Pre-treatment with SB431542 and/or pro- and anti-inflammatory cytokine completely abrogated the radiation induced characteristics in breast cancer cells. Part III of the results chapter describes the growth of radioresistant MCF-7 cells in the SCID mouse model, TGF-β signalling, hybrid epithelial-mesenchymal phenotype and cancer stem cells in cells obtained from these tumours, proteomic analysis of the tumours and changes in metabolic processes in radioresistant MCF-7 cells.

3.1

Radio resistance in breast cancer cells is mediated through TGF-β signalling, hybrid epithelial-mesenchymal phenotype and cancer stem cells

The studies were conducted in cell lines chosen to represent different molecular subtypes of breast cancer and included MCF-7 (ER^+, PgR^+, $HER2^-$), which is hormone-dependent, and the MDA-MB-231 cell line, which is triple negative (ER^-, PgR^-, $HER2^-$) and consequently hormone-independent. Development of radioresistant cell lines: the standard model employed for studying radio-resistance *in-vitro* is to repeatedly expose cell lines to radiation amounting to a total dose of 30-60 Gy [292]. This thesis describes the development of radioresistant cell lines after exposing breast cancer cells to acute doses of radiation and allowing them to recover for different time periods.

3.1.1 Recovery response of breast cancer cells MCF-7 and MDA- MB-231 to radiation

3.1.1.1 Effect of radiation recovery response on viability of breast cancer cells (MTT assay)

To assess the response of breast cancer cells after radiation treatment, MCF-7 and MDA-MB-231 cells were exposed to 2 Gy, 4 Gy, 6 Gy and 10 Gy of ionizing radiation and allowed a recovery period of 1, 4 and 7 days. In MCF-7, treatment with IR resulted in decreased viability on day 1 following IR exposure with all the doses. On day 4 post IR, significant increase in survival was observed with exposure of 2 Gy which further decreased with increase in dose. On day 7 post IR, slight increase with 2 Gy followed by significant increase in survival (around 1.5 fold) with 4 and 6 Gy was seen, and a decrease with 10 Gy

(Figure3.2a). In MDA-MB-231, on day 1 post IR, increase in survival was observed with 4 Gy which decreased with increase in IR dose. On day 4, survival decreased with all the doses of IR in comparison to untreated and on day 7 post IR, survival was lower with a dose of 2 Gy and 4 Gy which significantly increased with 6 Gy (1.2 fold) and further decreased with 10 Gy (Figure3.2b). Cells recovered for 7 days post radiation exposure of 6 Gy are termed as D7-6G and this nomenclature will be followed throughout this thesis. MCF-7 cells exposed to 6 Gy and recovered for 7 days were named as MCF D7-6G whereas MDA-MB-231 cells exposed to 6 Gy and recovered for 7 days were named as MDA D7-6G. Further studies were carried out on D7-6G cells.

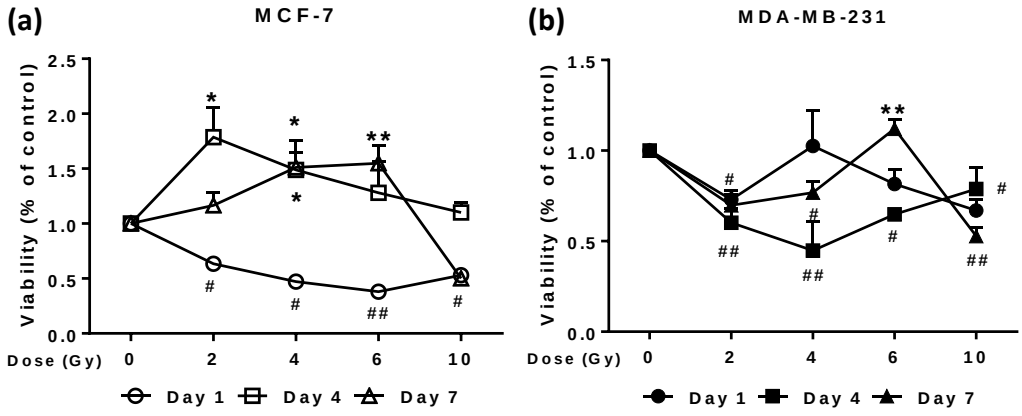


Figure 3.1: Recovery response of breast cancer cells. Viability of (a) MCF-7 and (b) MDA-MB-231 cells on different days following exposure to ionizing radiation (IR). Adherent cells were exposed to IR (2 to 10 Gy) and incubated for different days (Days 1, 4, and 7). On each of these days following recovery after exposure to IR, live adherent cells were trypsinized, counted and re-plated for

MTT assay. (a) Represents viability after days 1, 4 and 7 of MCF-7 cells and (b) depict viability after days 1, 4 and 7 of MDA-MB-231 cells. The experiments were repeated three times and a representative figure is shown. # is decrease in proliferation in comparison to untreated and * is increase in proliferation in comparison to untreated: *p≤0.05; **p≤0.01; #p≤0.05; ##p≤0.01.

3.1.1.2 Effect of radiation recovery response on proliferation ability of breast cancer cells (BrdU incorporation)

Since MTT assay indicated increased viability of D7-6G cells, the growth of breast cancer cells MCF-7 and MDA-MB-231 cells exposed to 6 Gy of IR and allowed to recover for 7 days was assessed by means of BrdU incorporation. MCF D7-6G cells showed around 3.8 fold increase in proliferation in comparison to untreated cells (Figure3.2a). Whereas MDA D7-6G cells showed 1.2 fold increase in proliferation in comparison to untreated cells (Figure3.3b).

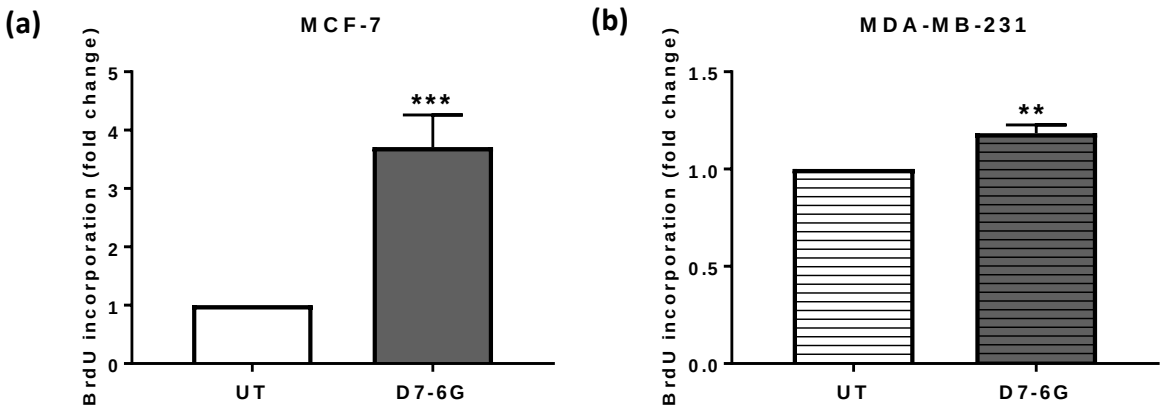


Figure 3.2: Incorporation of BrDU in D7-6G breast cancer cells (a) MCF-7 and (b) MDA-MB-231. MCF D7-6G and MDA D7-6G cells were re-plated

immediately for 48 h and proliferation of UT and D7-6G cells was assessed by BrdU incorporation. The data are represented as fold change and are mean ± S.E.M of values of three independent experiments. * is increase in proliferation in comparison to untreated. **p≤0.01; ***p≤0.001

3.1.2 Effect of radiation recovery response on colony forming capacity

To assess the colony forming ability, clonogenic assays were performed in MCF-7 and MDA-MB-231 cells exposed to 6 Gy of IR and allowed to recover for 7 days. There was increased colony forming ability of both MCF D7-6G (1.7 fold) & MDA D7-6G (1.5 fold) as compared to untreated cells (Figure3.3).

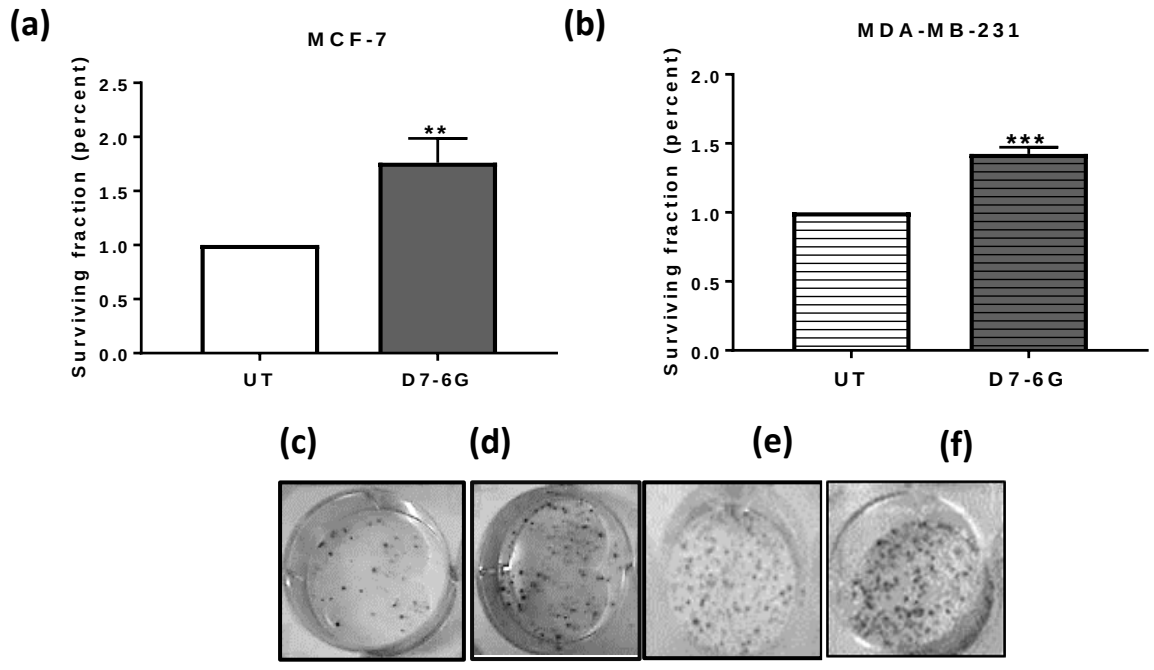


Figure 3.3: Colony forming ability of D7-6G breast cancer cells (a) MCF-7, (b) MDA-MB-231 cells. (c), (d), (e) and (f) are images of colonies formed by

untreated MCF-7, MCF D7-6G, untreated MDA-MB-231 and MDA D7-6G respectively. D7-6G cells were trypsinized, counted and plated in 6 well plates to assess their colony forming ability. The data are represented as fold change and are mean ± S.E.M of values of three independent experiments. * is increase in proliferation in comparison to untreated. **p≤0.01; ***p≤0.001

3.1.3 Effect of radiation recovery response on cell death

3.1.3.1 Effect of radiation recovery response on expression of pro-apoptotic and anti-apoptotic genes

Since there was increased proliferation in D7-6G cells, we assessed the expression of some pro- and anti-apoptotic genes. An increase was seen in both pro- and anti apoptotic genes in D7-6G cells of both cell lines (Figure3.4a-b). The expression of pro-apoptotic genes was around 5 fold (*BAX*), and 2 fold higher (*BAD*) as compared to untreated and those of anti-apoptotic genes was around 2 fold (*BCL-2*) and 2.5 fold (*BCL-Xl*) higher in MCF-7; whereas for MDA-MB-231 it was 15 fold higher (*BAX*), and 16 fold higher (*BAD*) as compared to untreated and those of anti-apoptotic genes was around 19 fold (*BCL-2*) and 30 fold (*BCL-XL*) higher. This mRNA expression pattern of *BAX* and *BCL-2* was then confirmed by image cytometric analysis. Bax and Bcl-2 proteins followed the same pattern as mRNA and demonstrated an increase in D7-6G cells (Figure3.4c: MCF-7; Figure3.5d: MDA-MB-231).

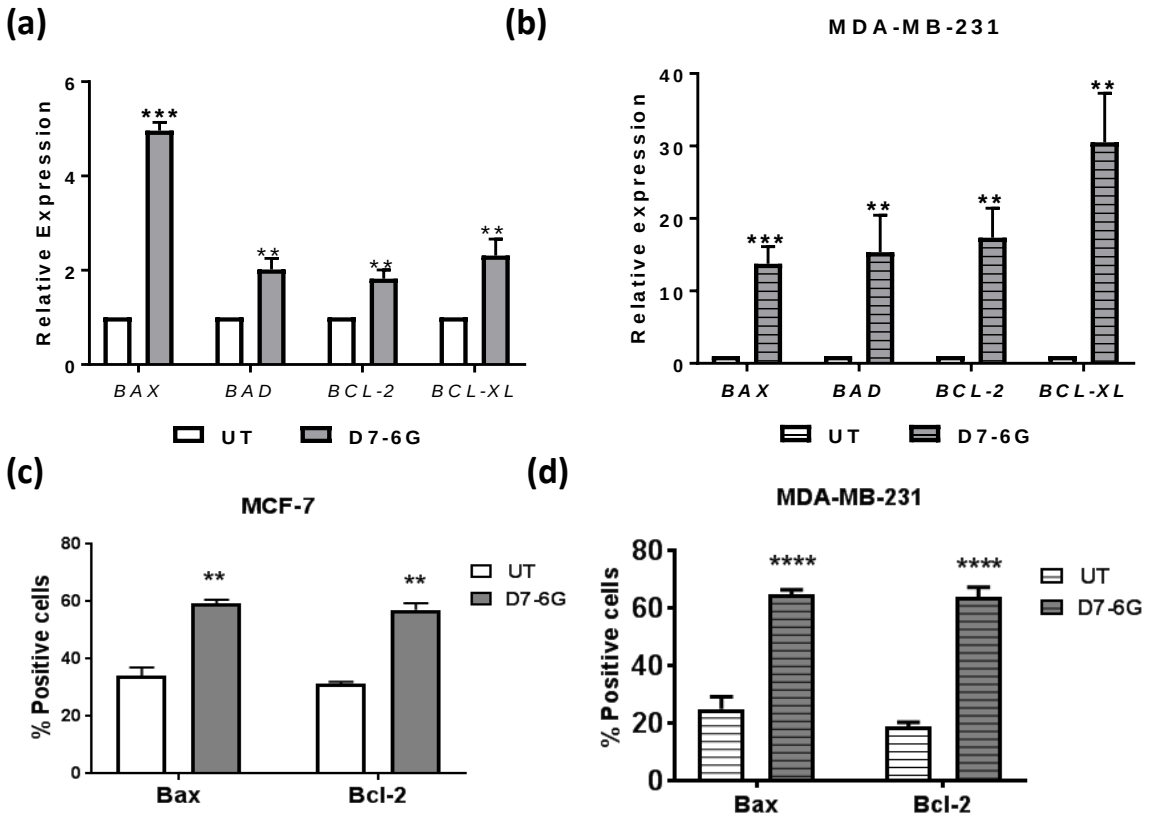


Figure 3.4: Effect of radiation recovery response on expression of pro-apoptotic and anti-apoptotic genes. Breast cancer cells were exposed to 6 Gy radiation and allowed to recover for 7 days. Expression of pro- and anti- apoptotic genes in radiation recovered (a) MCF-7 and (b) MDA-MB-231 cells was assessed by RT PCR using specific primers. Image cytometric analysis of Bax and Bcl-2 proteins in UT and D7-6 G cells of (c) MCF-7 (d) MDA-MB-231 cells.; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$ increase in comparison to UT. The values represented are mean ± S.E.M of values from one representative experiment. Three such experiments were carried out.

3.1.3.2 Effect of radiation recovery response on apoptosis and necrosis

Since an increase in cell proliferation and clonogenic capacity was observed, the role of radiation in cell death: both apoptosis and necrosis were studied in MCF-7 and MDA-MB-231 cells after radiation exposure. There was an increase in cell death of D7-6G cells, both apoptosis (only annexin positive) and necrosis (annexin/propidium iodide dual positive), as compared to untreated cells in both MCF D7-6G (Figure3.5a: UT MCF; Figure3.5b: MCF D7-6G and Figure3.5c: UT MDA; Figure3.5d: MDA D7-6G cells).

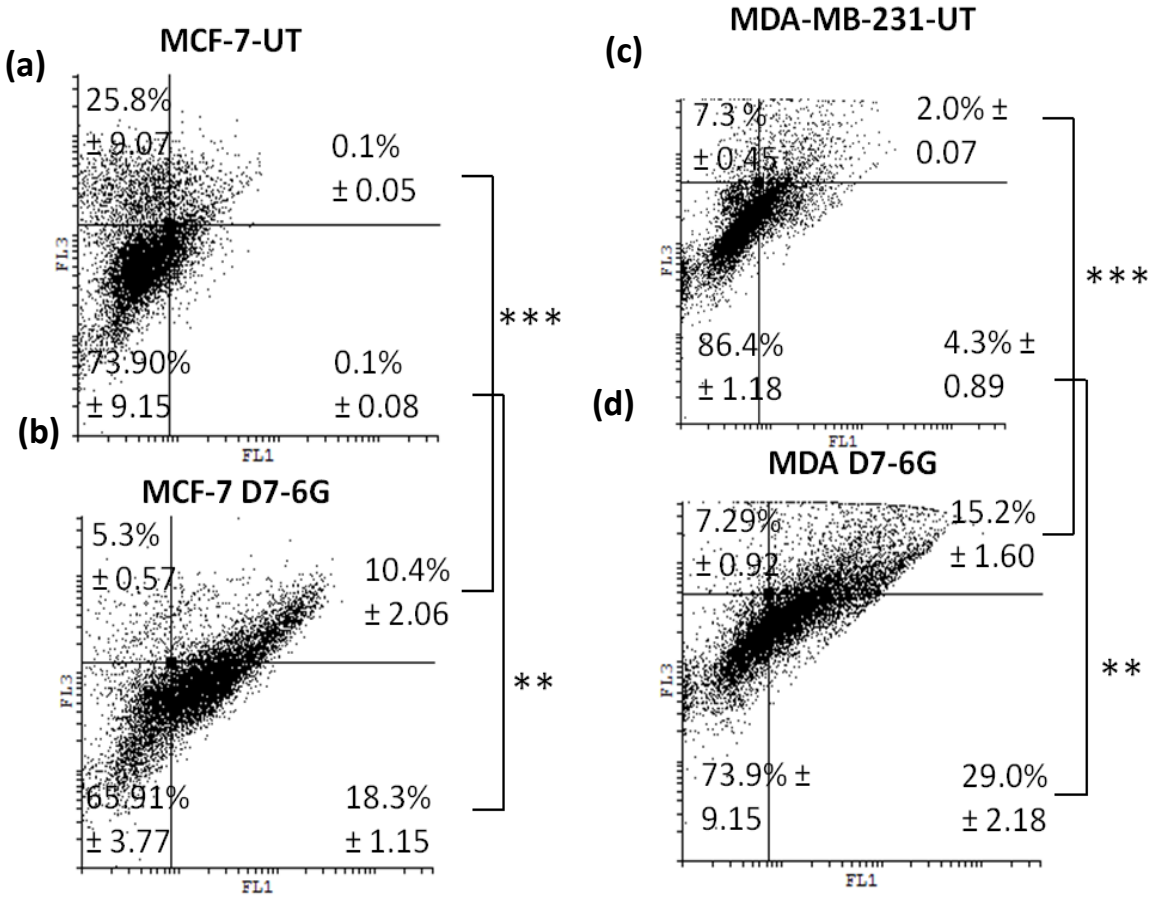


Figure 3.5: Effect of radiation recovery response on apoptosis and necrosis. The percentage of cells undergoing apoptosis/necrosis was assessed by Annexin-V/ propidium iodide staining and flowcytometry in (a) UT MCF-7; (b) MCF:D7-6G cells; (c) UT MDA-MB-231 and (d) MDA:D7-6 G cells. **$p \leq 0.01$; ***$p \leq 0.001$; increase in comparison to UT. The values represented are mean ± S.E.M of values from one representative experiment. Three such experiments were carried out.

3.1.4 Effect of radiation recovery response on expression of TGF-β isoforms and their receptors

Since both pro and anti apoptotic genes were up-regulated and both proliferation and apoptosis were high, these results suggested involvement of TGF-β signalling which is known for its duality of function [293]. We assessed the levels of TGF-β isoforms and its receptors in D7-6G cells. MCF D7-6G cells showed around 1.8, 4.2 and 2.1 fold increase in *TGF-β* isoforms *1*, *2* & *3* respectively (Figure3.6a) and *TGF-β receptors I* and *II* showed around 3.3 and 2.9 fold increase respectively as compared to untreated cells (Figure3.6b). Whereas, MDA-D7-6G cells showed around 2.0, 12.0 and 21.0 fold increase in *TGF-β1, 2* & *3* respectively (Figure3.6c) and *TGF-β receptors I* and *II* showed around 18 and 17 fold increase in comparison to untreated (Figure3.6d). Cytokines levels of TGF-β1, TGF-β2 and TGF-β3 in the supernatant of UT and D7-6G cells were assessed. In both cell lines, there was a significant increase in

all three isoforms of TGF- β in the supernatant of D7-6G cells of (Figure3.6c: MCF-7; Figure3.6d: MDA-MB-231). However, the fold increase of TGF- β2 and TGF- β3 was much higher as compared to TGF- β1.

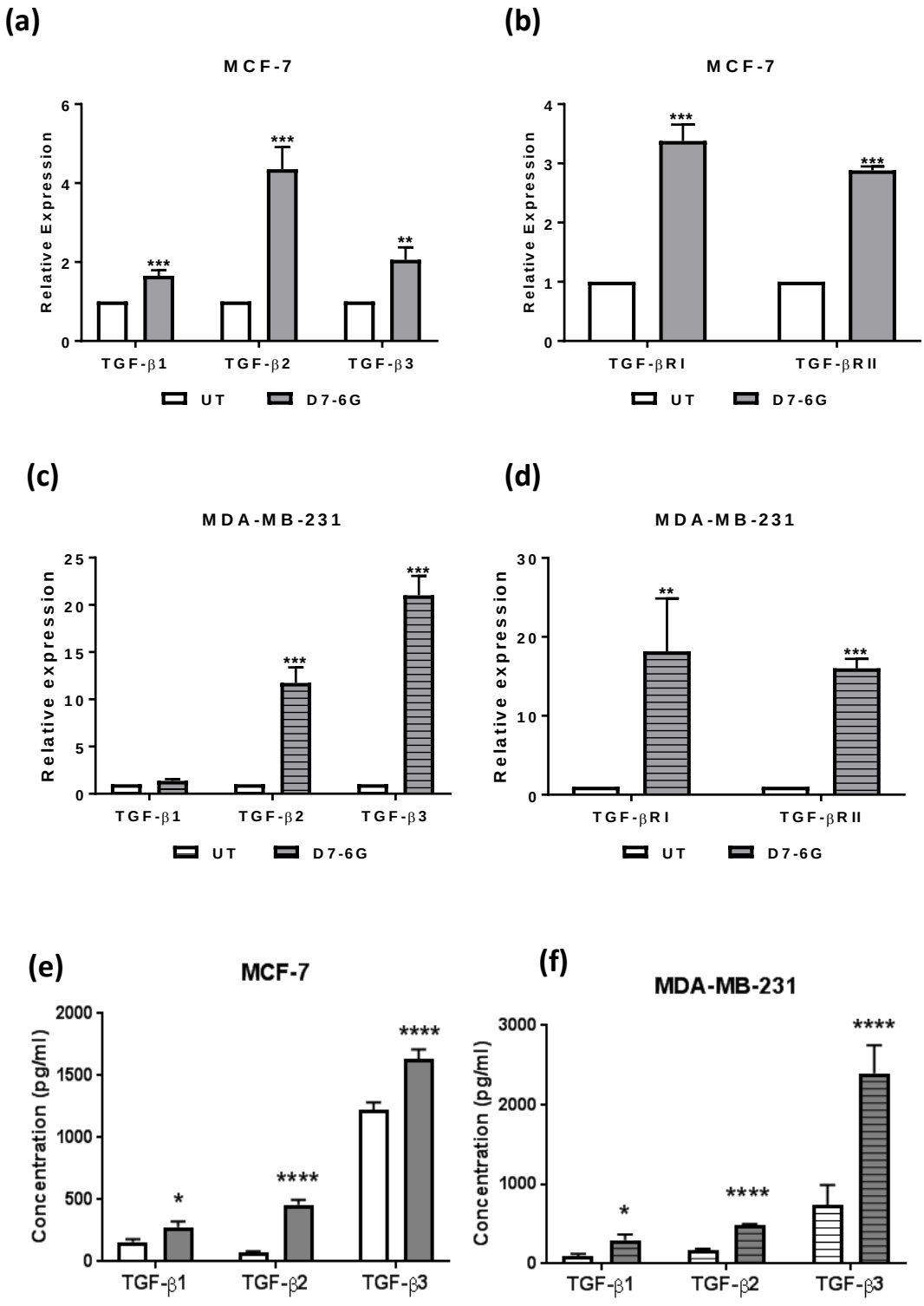


Figure 3.6: Expression of TGF-β isoforms and their receptors in D7-6G breast cancer cells. Expression of TGF-β isoforms and its receptors was assessed by RT-PCR using specific primers. (a) and (c) are expression of different isoforms of TGF-β of MCF-7 and MDA-MB-231 respectively whereas, (b) and (d) are expression of TGF-β receptors in MCF-7 and MDA-MB-231 respectively. Cytokines TGF-β1, 2 and 3 were estimated in culture supernatant of MCF and MDA D7-6G cells. Secreted cytokines TGF-β1, 2 & 3 in (e) MCF D7-6G cells and (f) MDA D7-6G cells. The values represented are mean ± S.E.M from three independent experiments.* is increase in comparison to untreated. *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001.

3.1.5 Effect of radiation recovery response on TGF-β downstream genes

Since increase in TGF-β isoforms and its receptors were increased in response to radiation, we assessed the downstream genes of TGF-β. Expression of TGF-β downstream genes were altered in both MCF D7-6G and MDA D7-6G cells. MCF D7-6G cells showed around 2.3 fold increase in expression of *SNAIL-1* and *HMGA2* in comparison to untreated (Figure3.7a). Whereas MDA D7-6G cells showed around 17, 7 and 13 fold increase in expression of *SNAIL-1*, *ZEB-1* and *HMGA2* respectively (Figure3.7b). The changes in mRNA expression were also confirmed by analysis of their protein levels by flowcytometry of antibody labelled cells. Flowcytometry analysis showed increase in Snail-1 and

ZEB-1in MCF D7-6G as compared to untreated (Figure3.7c-d). MDA D7-6G cells showed significant increase in ZEB-1 and HMGA2 as compared to untreated (Figure3.7g-h).

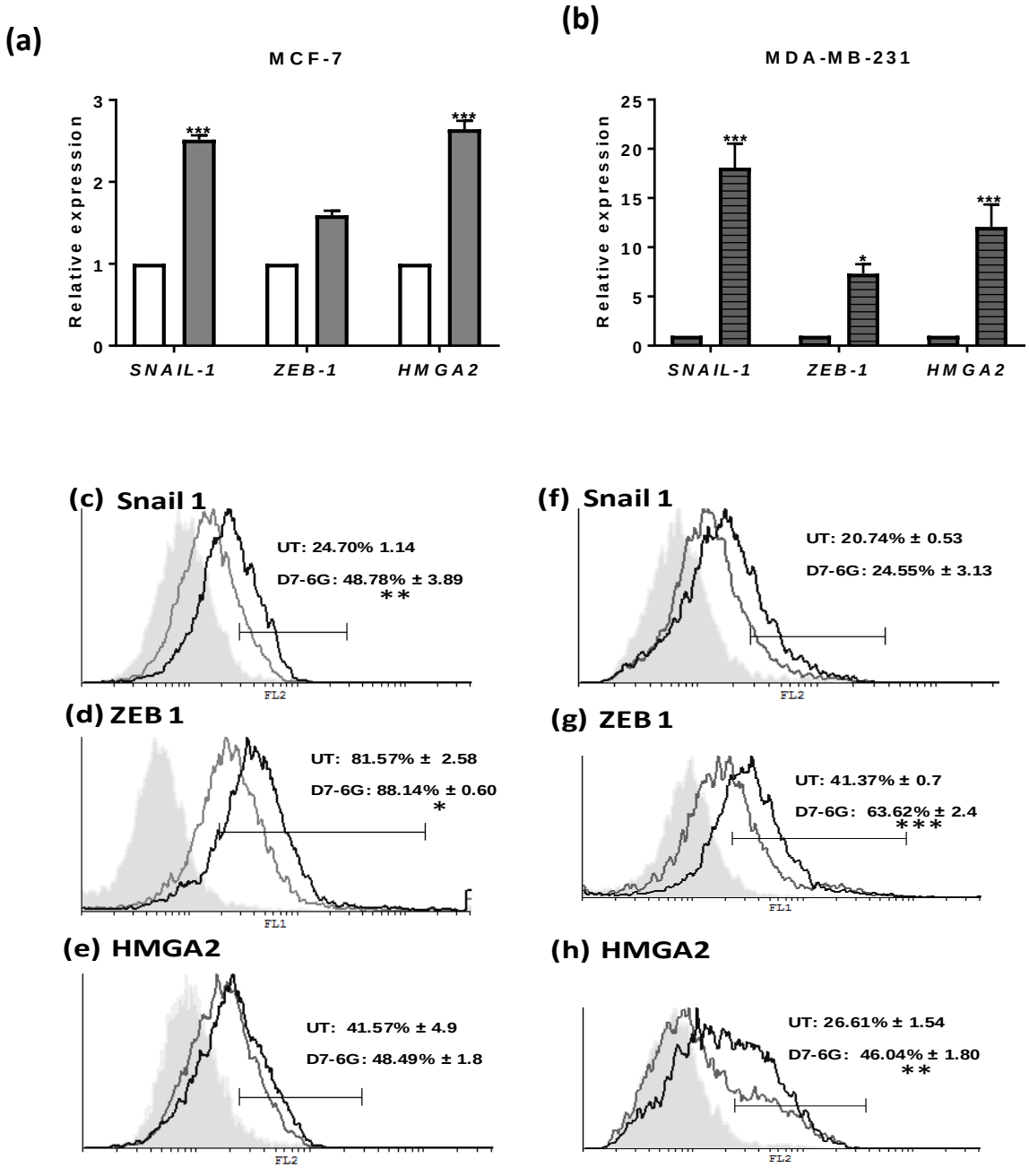


Figure 3.7: Expression of TGF-β downstream genes in D7-6G breast cancer cells. Cells were exposed to 6 Gy IR and allowed to recover for 7 days. On day 7, adherent live cells were used to assess the relative expression of TGF-β

downstream genes *SNAIL-1*, *ZEB-1* and *HMGA2* in (a) MCF-7; (b) MDA-MB-231 by RT-PCR using specific primers. Following labelling of treated cells with antibodies, flowcytometric analysis of TGF-β downstream genes (b) Snail-1 (d) ZEB-1 and (e) HMGA2 in MCF-7 cells and (f) Snail-1 (g) ZEB-1 and (h) HMGA2 in MDA-MB-231 was carried out. The values represented are mean ±S.E.M of values from one representative experiment. Two experiments were carried out. * is increase in comparison to untreated. *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$.

3.1.6 Effect of radiation recovery response on epithelial-mesenchymal transition

Since we observed increase in the TGF-β downstream genes *SNAIL-1*, *ZEB-1* and *HMGA2* which are known inducers of EMT responses, we assessed the epithelial and mesenchymal markers in both the D7-6G cell lines. MCF D7-6G cells showed more than 2 fold significant increase in epithelial markers *E-CADHERIN*, *OCCLUDIN* and *DESMOPLAKIN* (Figure3.8a) and around 1 – 2 fold in *VIMENTIN* and *FIBRONECTIN* and around 3.4 fold increase in *N-CADHERIN* as compared to untreated cells (Figure3.8b). MDA D7-6G cells showed 12, 7, 19 fold increase in expression of epithelial markers *E-CADHERIN*, *OCCLUDIN* and *DESMOPLAKIN* (Figure3.8c), but increase in *VIMENTIN* and *N-CADHERIN* and decrease in *FIBRONECTIN* as compared to

untreated (Figure3.8d). Protein levels of E-cadherin and vimentin were higher in D7-6G cells in both MCF-7 (Figure3.8e) and MDA-MB-231 (Figure3.8f).

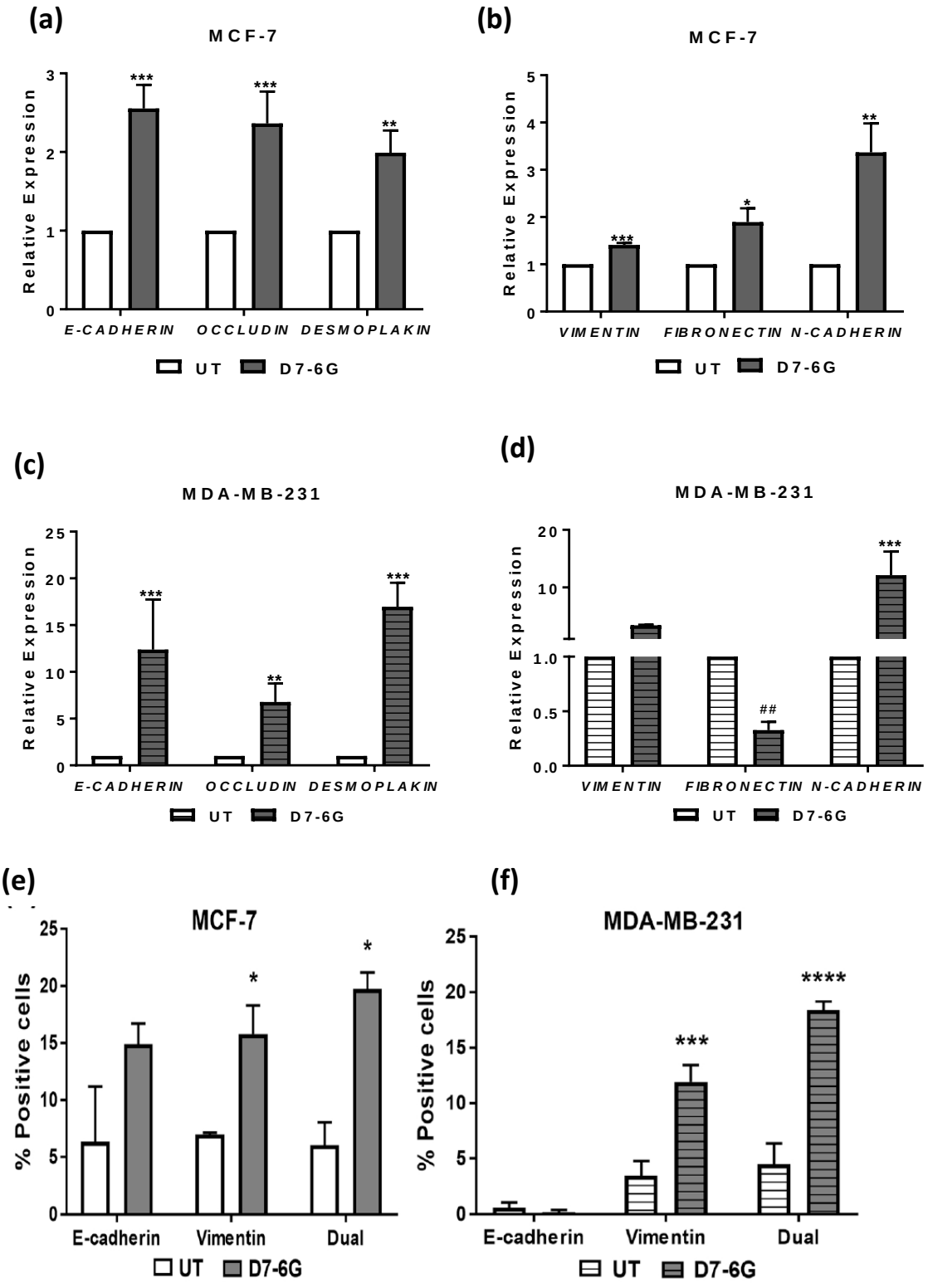


Figure 3.8: Expression of epithelial and mesenchymal markers in D7-6G breast cancer cells. MCF-7 & MDA-MB-231 cells were exposed to 6 Gy IR and allowed to recover for 7 days. On day 7, adherent live cells were used to assess the relative expression of EMT genes. MCF-7 cells: (a) epithelial markers, (b) mesenchymal markers. MDA-MB-231 cells, (c) epithelial markers, (d) mesenchymal markers. Image cytometric analysis of single and dual positive population of E-cadherin and vimentin in UT and D7-6G cells of (e) MCF-7 and (f) MDA-MB-231. The values represented are mean ±S.E.M of values from one representative experiment. Three experiments were carried out. * is increase in comparison to untreated. *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; $^{\#\#}p \leq 0.01$ decrease in comparison to untreated.

3.1.7 Effect of radiation recovery response on migration

Since we observed increase in TGF-β downstream genes *SNAIL-1, ZEB-1* and *HMGA2* and an increase in expression of epithelial and mesenchymal markers in both the cell lines in D7-6G cells, we assessed the migration ability of D7-6G cells through transwell inserts.

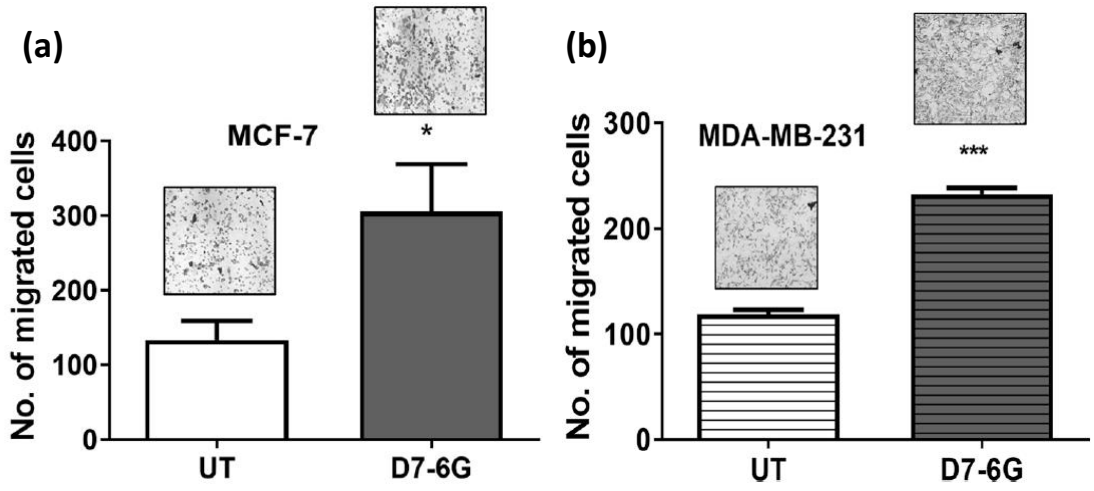


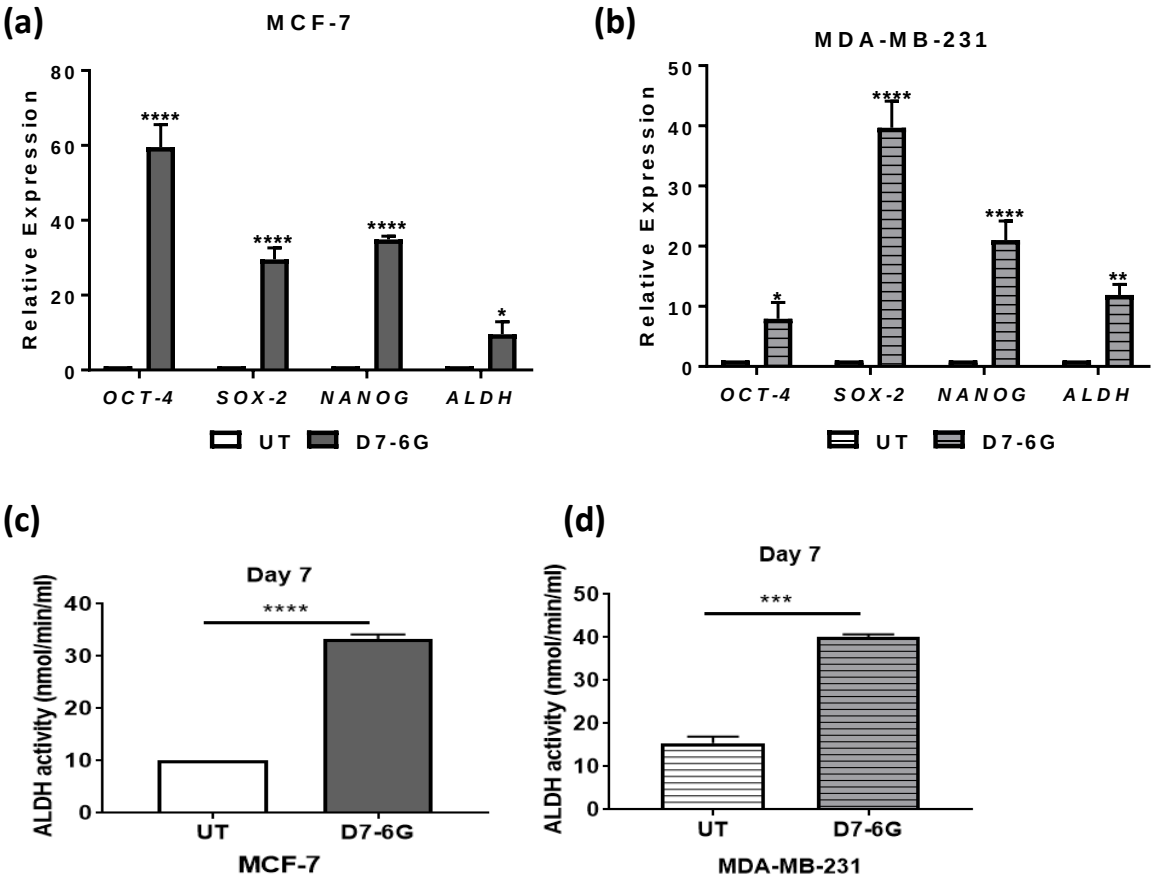
Figure 3.9: Migration ability of D7-6G breast cancer cells. MCF-7 & MDA-MB-231 cells were exposed to 6 Gy IR and allowed to recover for 7 days. On day 7, migration of UT and D7-6G was assessed in (a) MCF-7 (b) MDA-MB-231 cells through an 8 μm transwell insert for 72 h. The migrated cells present in the bottom of the membrane were stained and counted. Representative images of the migrated cells are shown above the respective histograms. The values represented are mean ± S.E.M of values obtained from three independent experiments. *$p \leq 0.05$; ***$p \leq 0.001$ is increase in comparison to UT.

3.1.8 Effect of radiation recovery response on cancer stem cells

There was increase in proliferation and migration of D7-6G cells. Although the exact mechanisms of cancer radioresistance have not been fully understood, evidences from multiple studies support the idea that cancer stem cells (CSCs) and different signaling pathways play important roles in regulating radiation response [294]. Therefore we assessed the presence of CSCs in D7-6G cells.

Both the cell lines showed significant increase in expression of stem cell markers octamer-binding transcription factor 4 (*OCT-4*), sex determining region Y-box 2 (*SOX-2*), *NANOG* and *ALDH*.

Since an increased expression of *ALDH* was observed, we also evaluated the activity of ALDH enzyme which was increased in D7-6 G cells. Increased CSCs in MCF D7-6G (Figure3.10e) and MDA D7-6G (Figure3.10f) cells was also confirmed by the enrichment of $CD44^{+}CD24^{-}$ cells.



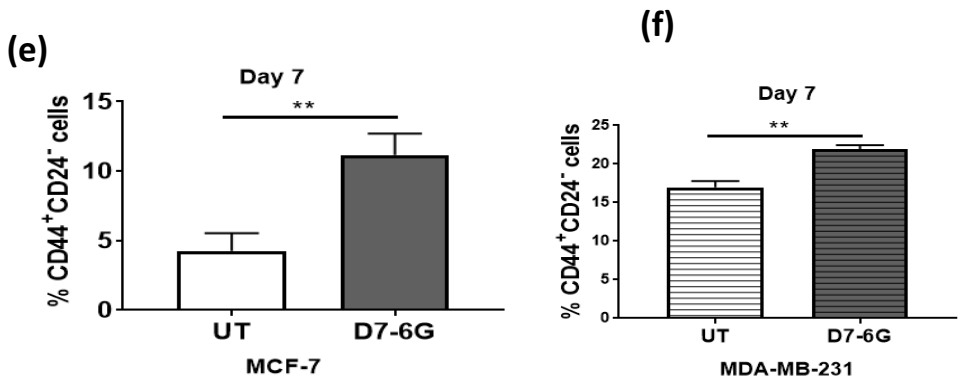


Figure 3.10: Cancer stem cell markers in D7-6G breast cancer cells. Breast cancer cells were exposed to 6 Gy IR and allowed to recover for 7 days. On day 7, relative expression of *OCT-4*, *SOX-2*, *NANOG* and *ALDH* was assessed by RT-PCR in (a) MCF-7 & (b) MDA-MB-231 cells. ALDH activity was assessed in D7-6G cells in (c) MCF D7-6G and (d) MDA D7-6G cells and percentage of CSCs was assessed as $CD44^{+}CD24^{-}$ cells by flowcytometry. The values represented are mean ± S.E.M from three independent experiments. *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$ increase in comparison to UT.

3.1.9 Effect of challenge dose of radiation on radiation recovered cells

Since D7-6G cells had shown increased proliferation, higher migration, presence of CSCs, and elevated TGF-β signalling, we assessed the viability of these D7-6G cells when exposed to a challenge dose (D7-6G + 6Gy). Cells exposed to only the challenge dose of 6 Gy served as the control. There was decreased apoptosis (Figure3.11a) and increased viability (Figure3.11c) in MCF D7-6 G + 6 Gy cells as compared to cells exposed to 6 Gy alone. Similar pattern

was observed with MDA D7-6G + 6 Gy cells i. e. decreased apoptosis (Figure3.11b) and increased viability (Figure3.11d).

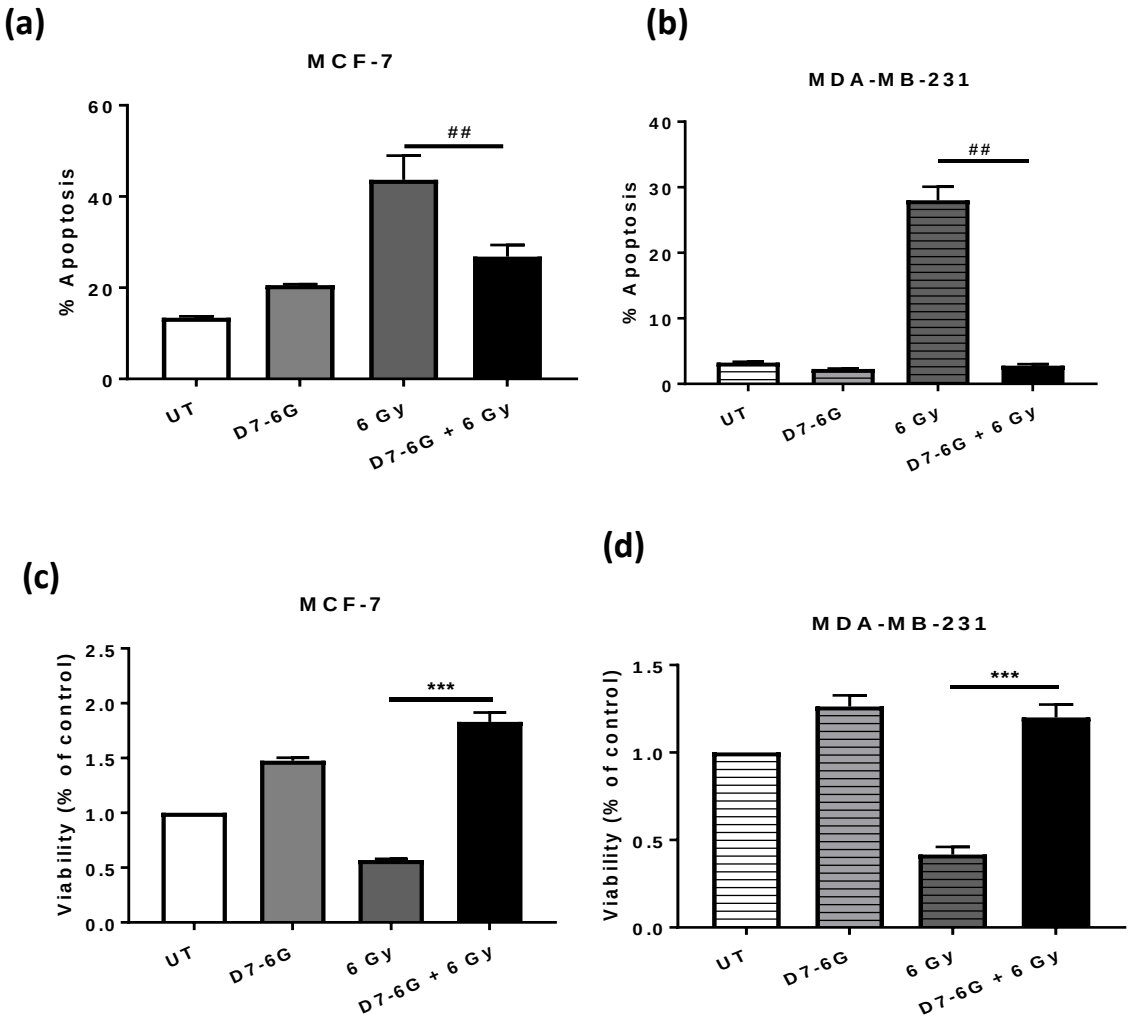


Figure 3.11: Effect of challenge dose of radiation toD7-6G breast cancer cells. MCF-7 & MDA-MB-231 cells were exposed to 6 Gy and allowed to recover for 7 days. On Day 7, adherent D7-6 G cells were trypsinized, counted and again exposed to a challenge dose of 6 Gy. Cells exposed only to the challenge dose served as control. After 48 h, cell death and viability were assessed: apoptosis in (a) MCF-7 and (b) MDA-MB-231cells, viability in (c) MCF-7 and (d) MDA-

MB231. The values represented are mean ± S.E.M from three independent experiments. ***$p \leq 0.001$: increase; $^{\#\#}p \leq 0.01$ decrease in comparison to UT.

3.2

Effect of TGF-β inhibitor SB431542 and pro- and anti-inflammatory cytokines TNF-α/IL-10 on radioresistance of breast cancer cells

1.2.1 Effect of TGF-β 1 receptor inhibitor SB431542 pre-treatment on viability of D7-6G cells

As we observed that there was elevated TGF-β signalling in radioresistant cells, we wanted to study the effects of blocking this signalling with TGF-β receptor I inhibitor (SB431542). As seen from the figure (Figure12a: MCF-7; Figure12b: MDA-MB-231), there was increased proliferation in D7-6G cells. However, when they were pre-treated with different concentrations of SB431542, this increased proliferation was not observed any longer and this effect was abrogated even at the lowest concentration. However, at 5 and 10 µM concentration the viability of D7-6G cells decreased further as compared to the control cells treated with drug alone indicating radiosensitization. Hence a concentration of 10 µM was used for all further experiments.

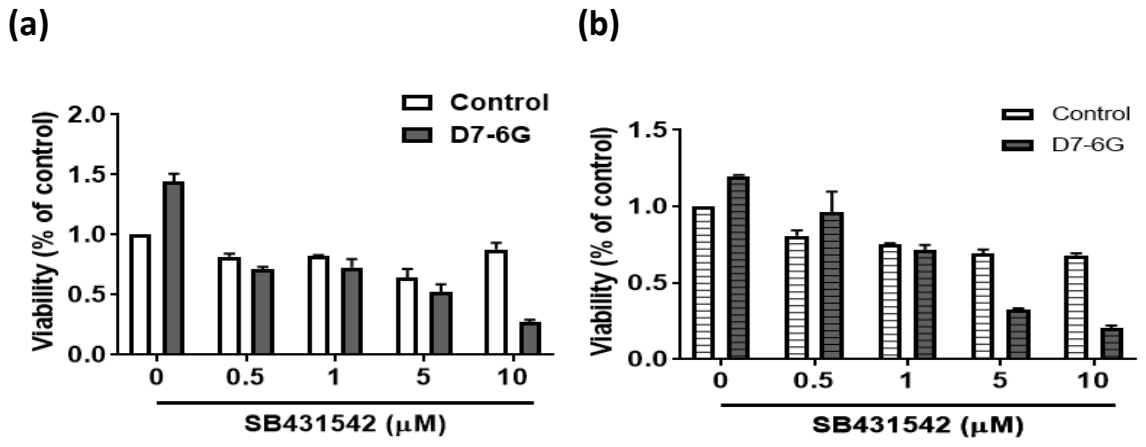
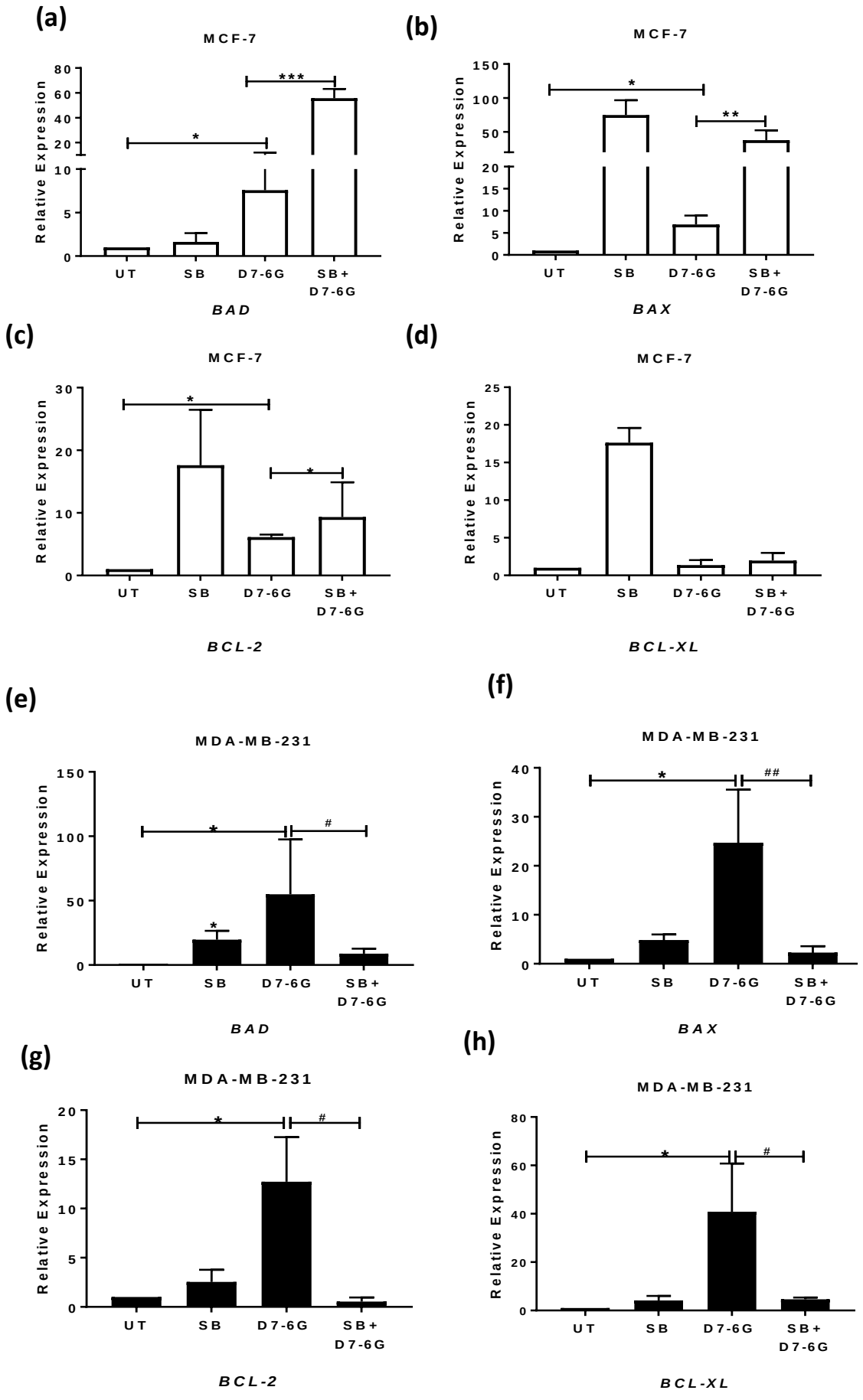


Figure 3.12: Effect of TGF-β 1 receptor inhibitor SB431542 pre-treatment on radiation recovery response of breast cancer cells. (a) MCF-7 cells and (b)

MDA-MB-231. Both cells were pre-treated with different concentrations of SB431542 followed by exposure to 6 Gy and recovery period of 7 days. On day 7, adherent D7-6G from all groups were trypsinized and MTT assay was performed to assess the viability.

1.2.2 Effect of SB431542 pre-treatment on apoptosis of D7-6G cells.

In MCF-7 cells, pre-treatment with SB431542 followed by exposure to 6 Gy (SB+D7-6G) increased mRNA levels of pro-apoptotic genes *BAD* and *BAX* around 50 fold (Figure3.13a and Figure3.13b respectively), *BCL-2* around 9 fold (Figure3.13c), with no change in *BCL-XL* (Figure3.13d). The fold increase in pro-apoptotic genes was several folds higher than the changes observed in anti apoptotic genes. In MDA-MB-231, there was more than 50% decrease in both pro-apoptotic genes *BAD* (Figure3.13e) *and BAX* (Figure3.13f) and anti apoptotic genes *BCL-2* (Figure3.13g) *and BCL-XL* (Figure3.13h) as compared to D7-6G cells. In addition, there was an increase in the expression of these genes following treatment with only SB431542 also. When apoptosis was assessed in these cells 48 h after re-plating, there was an increase in apoptosis of D7-6G cells pre-treated with SB431542 (Figure3.13i-j).


(a)
MCF-7
Relative Expression
80
60
40
20
10
5
0
*
***
UT
SB
D7-6G
SB+ D7-6G
BAD
(b)
MCF-7
Relative Expression
150
100
50
20
15
10
5
0
*
**
UT
SB
D7-6G
SB+ D7-6G
BAX
(c)
MCF-7
Relative Expression
30
20
10
0
*
*
UT
SB
D7-6G
SB+ D7-6G
BCL-2
(d)
MCF-7
Relative Expression
25
20
15
10
5
0
UT
SB
D7-6G
SB+ D7-6G
BCL-XL
(e)
MDA-MB-231
Relative Expression
150
100
50
0
*
*
#
UT
SB
D7-6G
SB+ D7-6G
BAD
(f)
MDA-MB-231
Relative Expression
40
30
20
10
0
*
##
UT
SB
D7-6G
SB+ D7-6G
BAX
(g)
MDA-MB-231
Relative Expression
20
15
10
5
0
*
#
UT
SB
D7-6G
SB+ D7-6G
BCL-2
(h)
MDA-MB-231
Relative Expression
80
60
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20
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#
UT
SB
D7-6G
SB+ D7-6G
BCL-XL

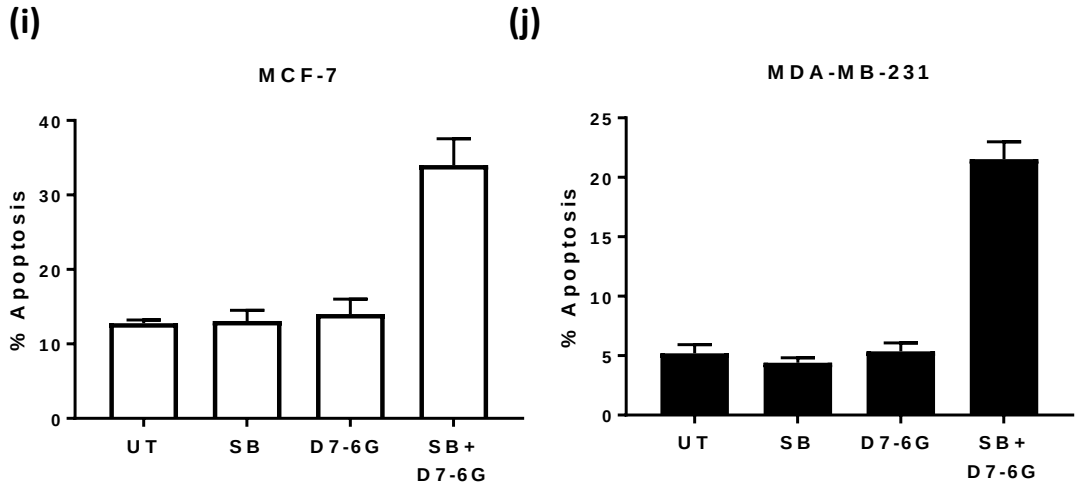


Figure 3.13: Effect of SB431542 pre-treatment on expression of pro- and anti-apoptotic genes and apoptosis of D7-6G cells: MCF-7 & MDA-MB-231 cells were pre-treated with SB431542 for 2 h followed by exposure to 6 Gy and recovery period of 7 days. On day 7, the expression of pro and anti- apoptotic genes was assessed by RT PCR using specific primers. (a-d) expression of *BAD, BAX, BCL-2* and *BCL-XL* in MCF-7 cells (e-h) expression of *BAD, BAX, BCL-2* and *BCL-XL* in MDA-MB-231 cells (i) and (j) are apoptosis in MCF-7 and MDA-MB-231 respectively. *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; $^{\#}p \leq 0.05$; $^{\#\#}p \leq 0.01$ decrease in comparison to D7-6G. The values represented are mean ± S.E.M of values from one representative experiment. Three such experiments were carried out.

1.2.3 Effect of SB431542 pre-treatment on TGF-β signalling in D7-6G cells

There was an increase in expression of *SNAIL-1* (2.3 fold), *ZEB-1* (1.3 fold), and no change in *HMGA2* in MCF D7-6G cells. Pre-treatment of MCF-7 to

SB431542 down regulated radiation induced TGF-β signalling as expression of all the three TGF-β downstream genes *SNAIL-1* (Figure3.14A), *ZEB-1* (Figure3.14b) and *HMGA2* (Figure3.14c) were lower as compared to D7-6G cells. Though the levels of *SNAIL-1* reduced back to control levels, *ZEB-1* and *HMGA2* levels remained marginally higher. Interestingly, treatment with only SB431542 marginally increased expression of these genes indicating a TGF-β independent regulation of these genes.

In MDA-MB-231 cells, there was an increase in expression of *ZEB-1* (2 fold) and *HMGA2* (1.5 fold). Pre-treatment with SB431542 in MDA-MB-231 cells decreased expression of *ZEB-1* (Figure3.14e) and *HMGA2* (Figure3.14f) to control levels. There was no significant change in the expression of these genes due to treatment with only SB431542.

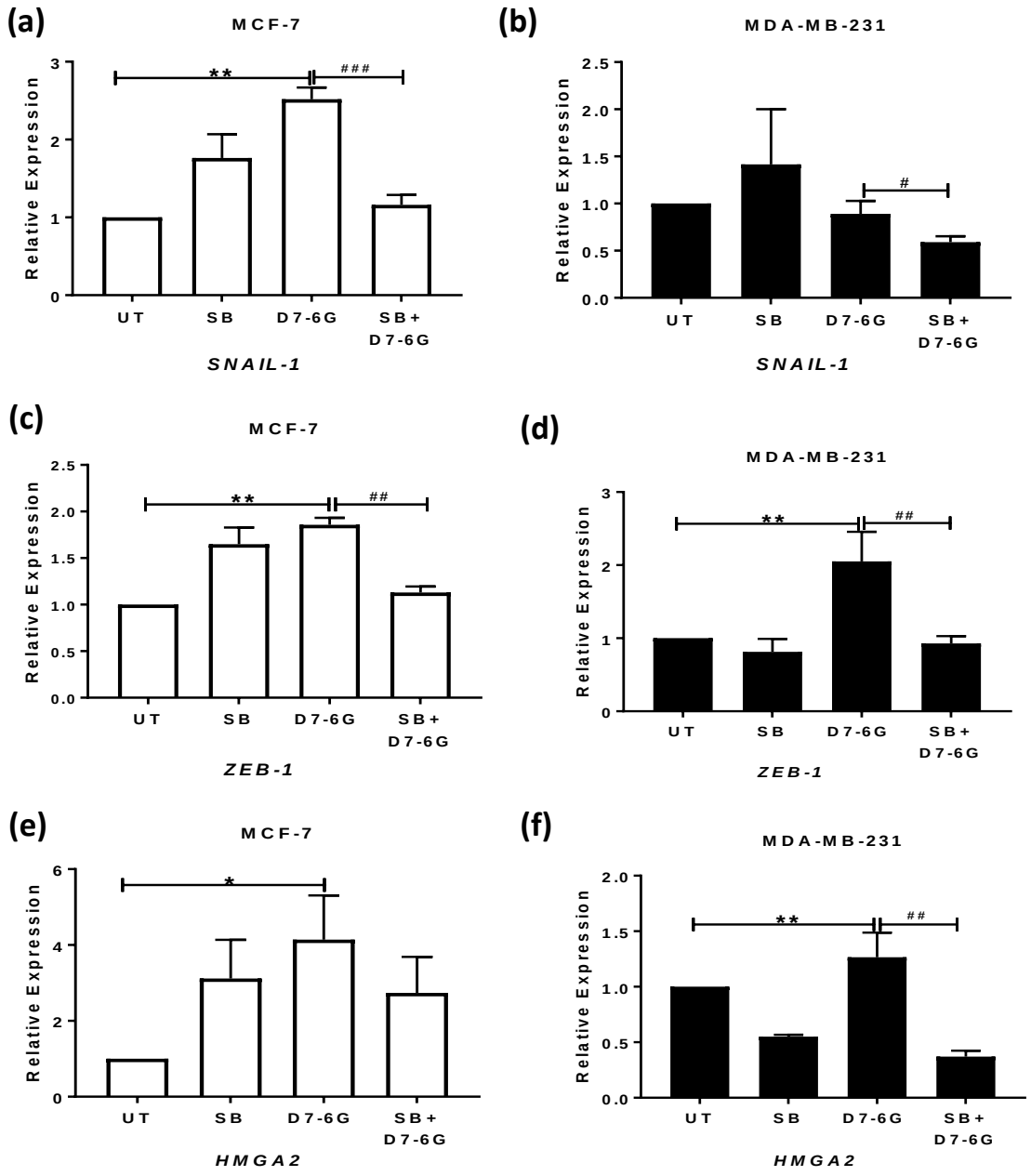
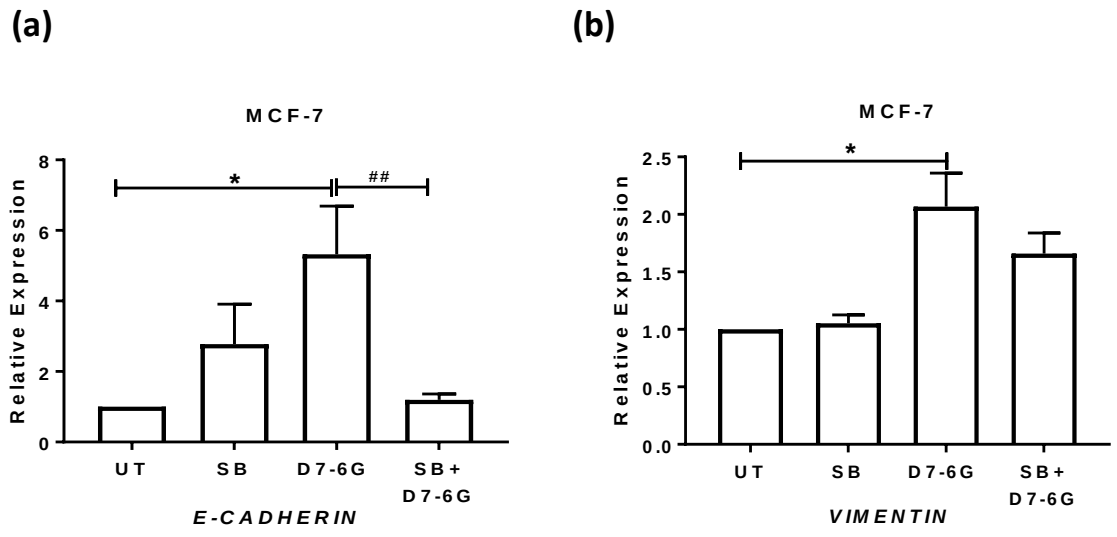


Figure 3.14: Effect of SB431542 on TGF-β signalling in D7-6G cells. MCF-7 & MDA-MB-231 cells were pre-treated with SB431542 for 2 h followed by exposure to 6 Gy and recovery period of 7 days. On day 7, expression of TGF-β downstream genes (a) *SNAIL-1*, *(b) ZEB-1* and (c) *HMGA2* in MCF 7 and MDA-MB-231: (d) *SNAIL-1*, *(e) ZEB-1* and (f) *HMGA2* genes was assessed by RT PCR using specific primers. *$p \leq 0.05$; **$p \leq 0.01$; * is increase and # is

decrease $^{\#}p \leq 0.05$; $^{\#\#}p \leq 0.01$; $^{\#\#\#}p \leq 0.001$. The values represented are mean ± S.E.M of values from three representative experiments.

1.2.4 Effect of SB431542 pre-treatment on hybrid E/M phenotype in MCF D76G cells

Since there was an increase in radiation induced migration and hybrid epithelial -mesenchymal phenotype in D7-6G cells, we assessed the effect of SB431542 in the same. As observed earlier there was a hybrid E/M phenotype with increase observed in both *E-CADHERIN* (1.8 fold) and *VIMENTIN* (2 fold). Pre- treatment with SB431542 followed by IR results in the significant decrease in both the radiation induced EMT markers, E-*CADHERIN* (Figure3.15a) and *VIMENTIN* (Figure3.15b). Migration assay with membrane inserts also confirmed a decrease in hybrid E/M phenotype with a decrease in the number of migrated cells in SB+D7-6G cells as compared to D7-6G cells (Figure3.15c).



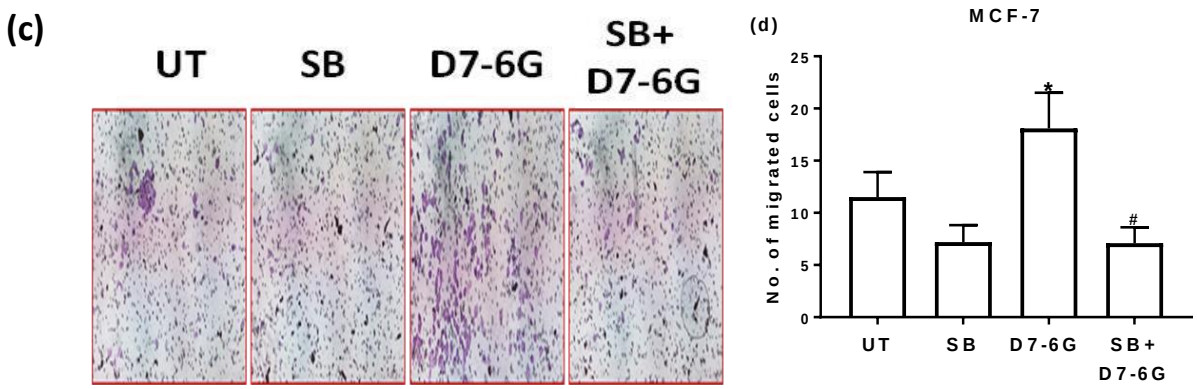
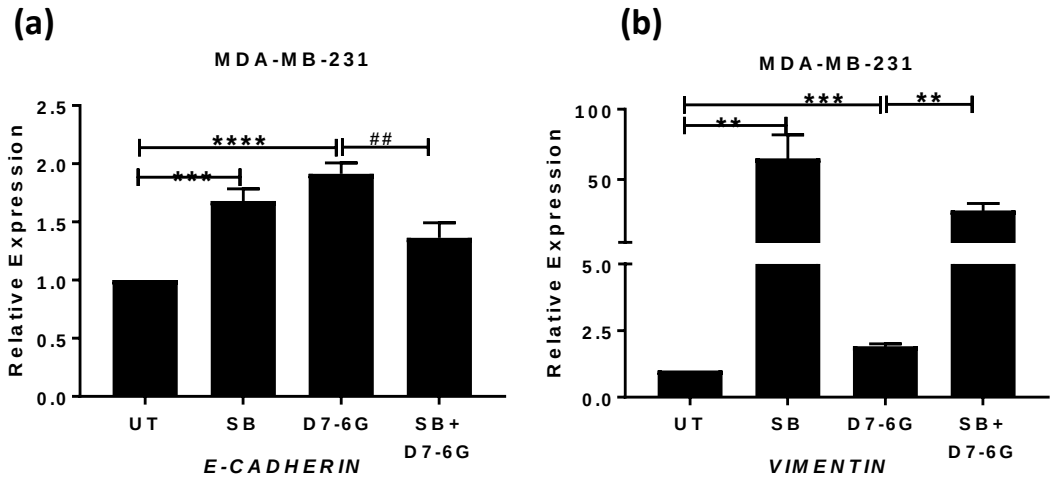


Figure 3.15: Effect of SB431542 pre-treatment on hybrid E/M phenotype in MCF D76G cells. MCF-7 cells were pre-treated with SB431542 for 2 h followed by exposure to 6 Gy and recovery period of 7 days. On day 7, the expression of (a) epithelial gene *E-CADHERIN* and (b) mesenchymal gene *VIMENTIN* was assessed by RT PCR using specific primers. On day 7, the cells were re-plated on membrane inserts (8µm) for 72 h. After 72 h inserts were taken out fixed with methanol: acetone (7:3) and stained with crystal violet, and counted, (c) Representative images of MCF-7 cells with different treatments, (d) Histogram representation of the number of migrated cells in different treatments groups. $^{*}p \leq 0.05$; $^{\#}p \leq 0.05$; $^{\#\#}p \leq 0.01$; * is increase and # is decrease. The values represented are mean ± S.E.M of values from three representative experiments.

1.2.5 Effect of SB431542 pre-treatment on hybrid E/M phenotype in MDA D76G cells

Since there was increase in radiation induced migration and hybrid E/M phenotype in D7-6G cells, we assessed the effect of SB431542 in these cells. There was an increase in E-*CADHERIN* and increase in *VIMENTIN* in D7-6G cells. Treatment with SB+ D7-6G resulted in marginal increase in *E-CADHERIN* (1.3 fold) but further increase in *VIMENTIN* (30 fold). Though there was a marginal increase in E-*CADHERIN* levels upon SB431542 treatment in MCF-7 cells, this effect was many folds in MDA-MB-231 cells. Relative expression of epithelial marker *E-CADHERIN* was 1.8 fold high in MDA-MB-231 cells pre-treated with SB431542 in comparison to untreated cells (Figure3.16a) whereas mesenchymal marker *VIMENTIN* showed 55 fold increase (Figure3.16b) in the same. However, as a contradiction, migration assay with membrane inserts showed that the number of migrated cells in SB+D7-6G cells were lower in comparison to D7-6G cells (Figure3.16d).



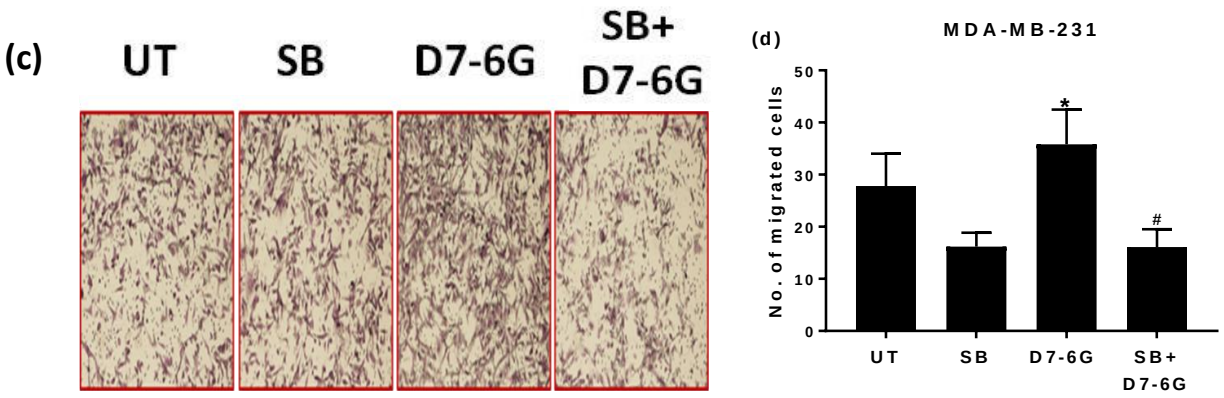


Figure 3.16: Effect of SB431542 pre-treatment on hybrid E/M phenotype in MDA D76G cells. MDA-MB-231 cells were pre-treated with SB431542 for 2 h followed by exposure to 6 Gy and recovery period of 7 days. On day 7, the expression of (a) epithelial marker *E-CADHERIN* and (b) mesenchymal marker *VIMENTIN* was assessed by RT PCR using specific primers. On day 7, cells were replated on membrane inserts (8µm) for 72 h. After 72 h, inserts were taken out, fixed with methanol: acetone (7:3) and stained with crystal violet and counted, (c) representative images of MDA-MB-231 cells with different treatments, (d) histogram representation of migrated cells in different treatments group. *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$; $^{\#}p \leq 0.05$; $^{\#\#}p \leq 0.01$; * is increase and # is decrease. The values represented are mean ± S.E.M of values from three representative experiments.

1.2.6 Secretion of pro-inflammatory cytokine TNF-α and anti-inflammatory cytokine IL-10 in D7-6G cells

There is a dysregulation of the physiological cytokine milieu in tumour microenvironment since it is contributed by tumour cells as well as a broad

range of immune cells, prompting pro- or anti-inflammatory responses based on their combination [295]. In the tumour microenvironment, cytokines interact with a plethora of biomolecules, such as cancer stem cells, microRNA, epithelial-mesenchymal transition markers, transcription factors and are involved in processes such as epigenetic regulation, autophagy, immunoediting, and inflammation related to tumour progression, ultimately tipping the balance between immunosuppression and immunostimulation, thus influencing disease progression [296]. In addition, therapeutic modalities like ionizing radiation also induce an inflammatory response in tumour cells as well as cells of the innate immune system, especially macrophages leading to chronic inflammation through tissue damage and fibrosis [297, 298]. Hence we assessed the supernatants of D7-6G cells to evaluate if there are any alterations in pro-and anti- inflammatory cytokine secretion profile. TNF-α was chosen as a signature pro-inflammatory cytokine and IL-10 as an anti-inflammatory cytokine. D7-6G cells showed increased secretion of TNF-α in both the cell lines (Figure3.17a MCF-7; Figure3.17b MDA-MB-231) which was abrogated when pre- treated with SB431542. With respect to anti-inflammatory cytokine IL-10, there was a marginal decrease in MCF D7-6G cells whereas there was no change in MDA-MB-231 cells (Figure3.17c MCF-7; Figure3.17d MDA-MB-231). Interestingly, pre- treatment with SB431542 resulted in a drastic increase in IL-10 secretion in SB+D7-6G of both the cell lines.

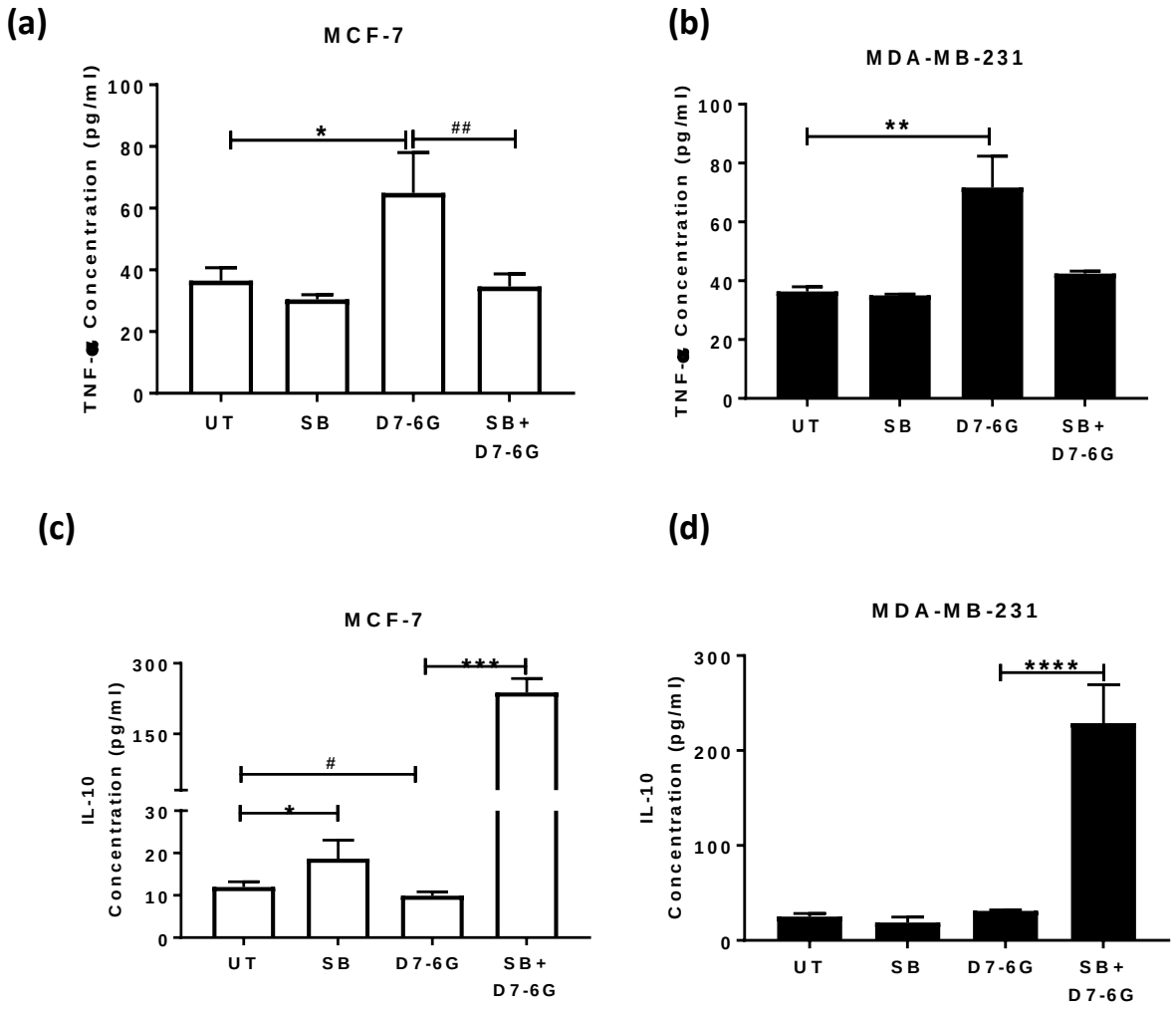


Figure 3.17: Effect of SB431542 and radiation recovery response on secretion of pro-inflammatory cytokine TNF-α and anti-inflammatory cytokine IL-10. MCF-7 and MDA-MB-231 cells were pre-treated with SB431542 for 2 h followed by exposure to 6 Gy and recovery period of 7 days. On day 7, supernatants were collected and used for detection of cytokines TNF-α and IL-10 by ELISA. TNF-α in culture supernatant of (a) MCF-7 and (b) MDA-MB-231; IL-10 in culture supernatant of (c) MCF-7 and (d) MDA-MB-231 (*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$; $^{\#}p \leq 0.05$; $^{\#\#}p \leq 0.01$. * is

increase and # is decrease. The values represented are mean ± S.E.M of values from three representative experiments.

1.2.7 Pre- treatment with TNF-α or IL-10 modulates the radiation induced proliferation of D7-6G cells

Since there was an increase in TNF-α in D7-6G cells and IL-10 in SB+D7-6G groups, the cells were pre-treated with either TNF-α or IL-10 to assess the response to radiation. It was observed that treatment with either of the cytokines completely abrogated the increased proliferation of D7-6G cells. TNF-α pre-treated cells (Figure3.18a MCF-7; Figure3.18b MDA-MB-231), IL-10 pre-treated cells (Figure3.18c MCF-7; Figure3.18d MDA-MB-231).

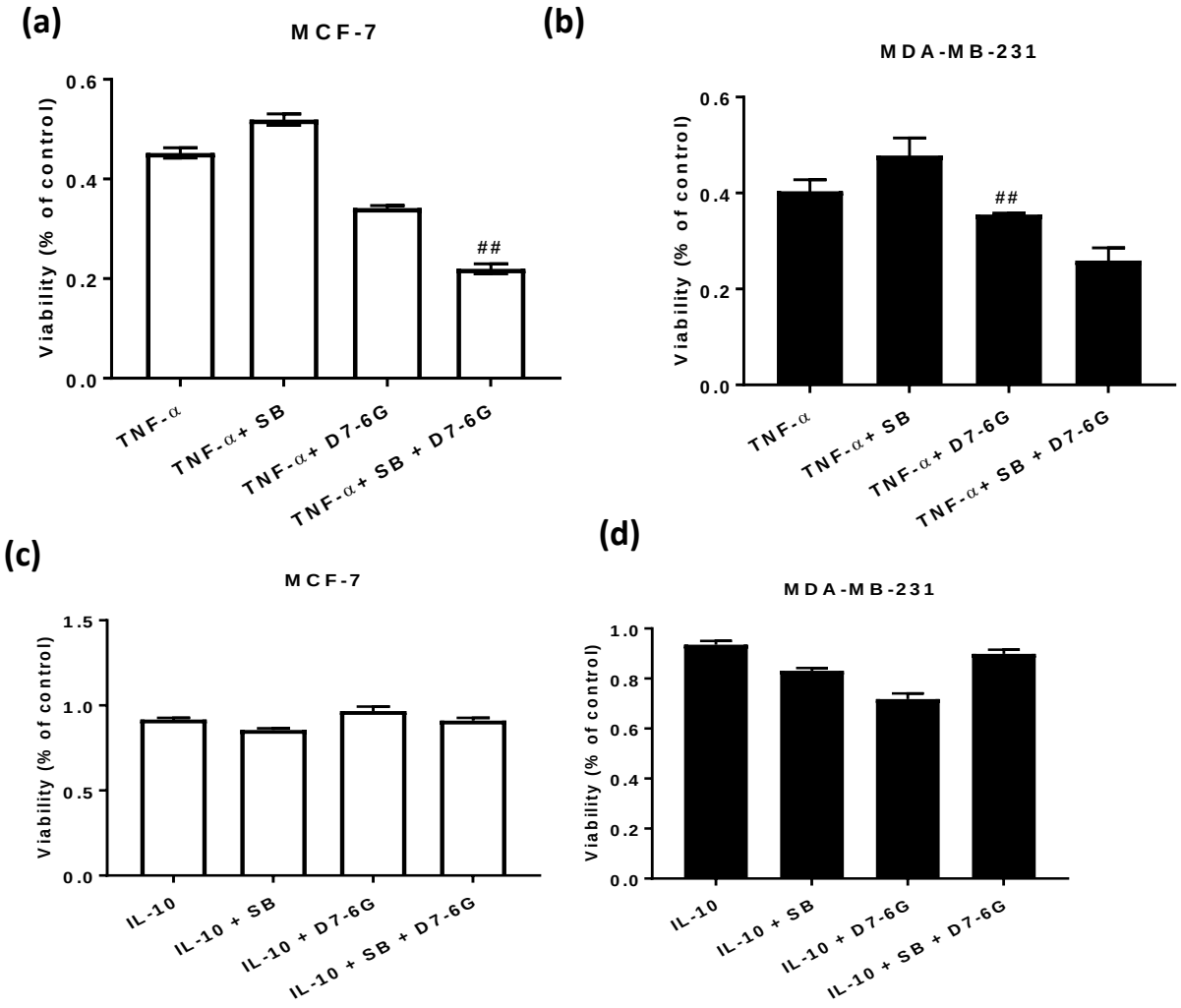


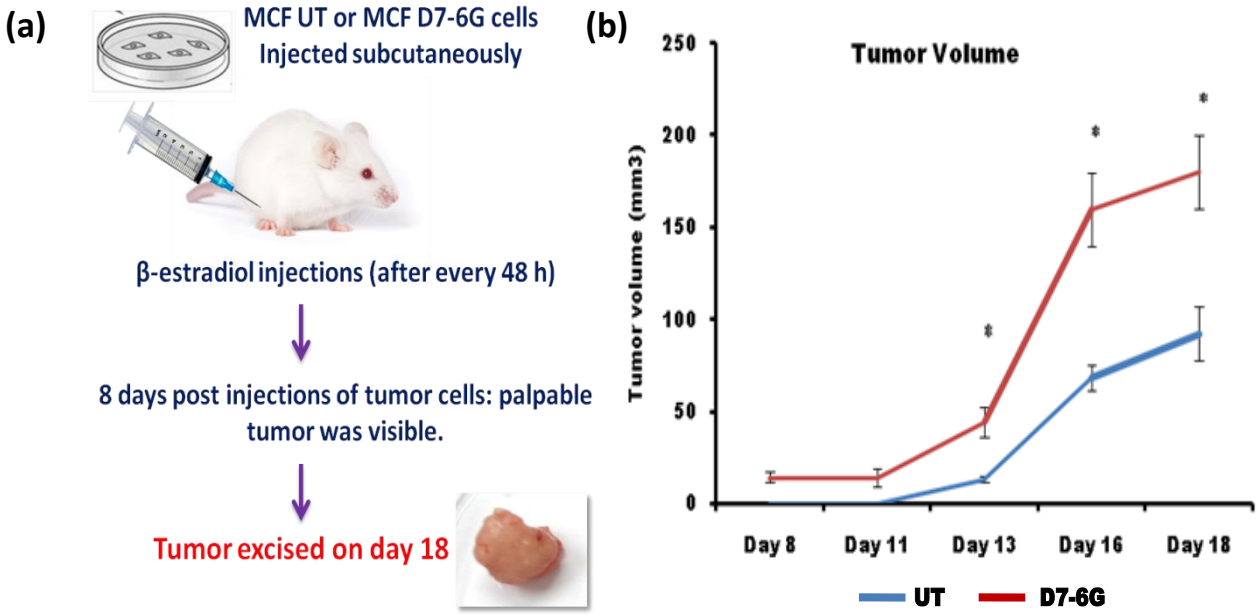
Figure 3.18: Effect of pro-inflammatory cytokine TNF-α and inhibitory cytokine IL-10 on the radiation induced proliferation of D7-6G cells. MCF-7 and MDA-MB-231 cells were pre-treated with SB431542 for 2 h followed by TNF-α and IL-10 treatments for 24 h, further followed by exposure to 6 Gy IR and recovery period of 7 days. On day 7, adherent cells were trypsinized, counted and replated for MTT assay for 48 h. $^{\#\#}p \leq 0.01$; decrease in comparison to D7-6G. The values represented are mean ± S.E.M of values from one representative experiment. Two such experiments were carried out.

1.3

Growth and proteomic analysis of radioresistant MCF-7 cells in the SCID mouse model

1.3.1 Generation of MCF D7-6G tumour model in SCID mice

Since MCF-7 D7-6G cells showed radioresistant phenotype, we established a tumour xenograft model by transplanting untreated MCF-7 (MCF UT) and radioresistant MCF-7 (MCF D7-6G) cells subcutaneously into female SCID mice to study the characteristics of MCF D7-6G grown *in-vivo*. Since MCF-7 requires estrogen for their growth, β-estradiol injections were given subcutaneously (Figure3.19a). The results demonstrated that MCF D7-6G tumours retains their high proliferation ability *in vivo*. These cells demonstrated shorter latency and increased tumour burden with time (Figure 3.19b).



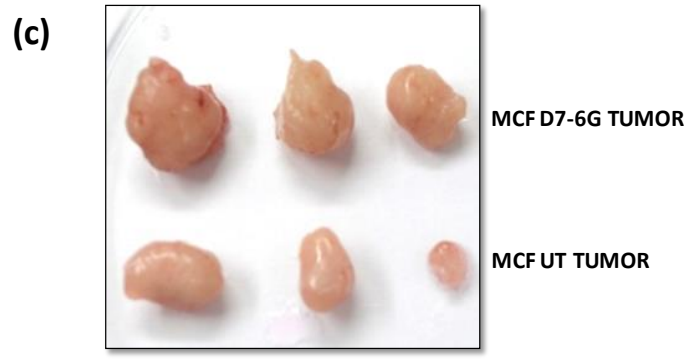


Figure 3.19: Growth of MCF-7 UT and MCF D7-6G cells in SCID mice. (a) Schematic for establishment of tumor in SCID mice. (b) Tumor growth kinetics of UT and D7-6G tumors. SCID mice were injected with β-estradiol for one week followed by subcutaneous injection of MCF UT and MCF D7-6G cells. (c) Representative images of MCF UT and MCF D7-6G tumors in SCID mice. Tumor size was measured from day 8 onwards in subcutaneously xenografted tumors *in-vivo* by external calliper. Tumor volume was calculated by use of the modified ellipsoid formula 1/2(Length × $Width^2$) [299]. The values represented are mean ± S.E.M of values from one representative experiment. Two such experiments were carried out. *$p \leq 0.05$; increase in comparison to UT (N=10 mice per group).

1.3.2 Mixed apoptotic phenotype in MCF D7-6Gtumors

Since there was mixed apoptotic phenotype *in-vitro,* we assessed the pro and anti-apoptotic genes in MCF UT and MCF D7-6G tumors. Mixed expression with an increase seen in both pro-and anti- apoptotic genes was observed similar to *in-vitro* conditions. Pro apoptotic gene *BAD* showed 15 fold increase in

comparison to untreated, but no change in *BAX* expression (Figure3.20a). Ani-apoptotic genes *BCL-2* and *BCL-XL* in MCF D7-6G tumors showed 11 fold and 19 fold increase respectively in comparison to MCF UT tumors (Figure3.20b).

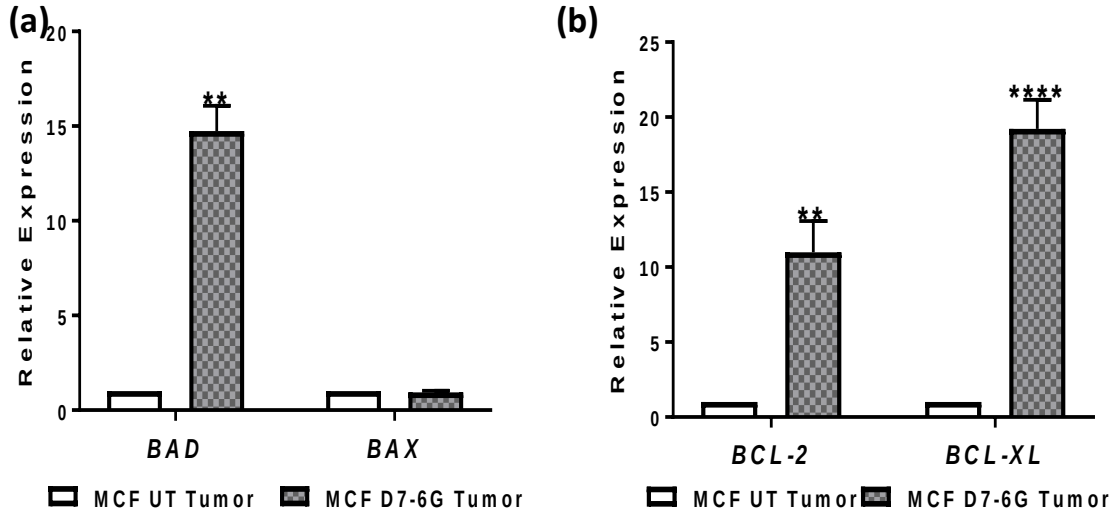


Figure 3.20: Mixed apoptotic phenotype in MCF D7-6G tumors (a) pro-apoptotic and (b) anti-apoptotic genes in MCF D7-6G tumors. On day 18 following injection of breast cancer cells, the tumors from MCF UT SCID mice and MCF D7-6G SCID mice were removed and chopped into small pieces. From chopped tumor tissue, total RNA was extracted according to manufacturer's protocol and the expression of pro and anti apoptotic genes was assessed. **$p \leq 0.01$; ****$p \leq 0.0001$ increase in comparison to UT. The values represented are mean ± S.E.M of values from three pooled samples.

1.3.3 Increased TGF-β signalling in MCF D7-6G tumors

Since there was increased TGF-β signalling *in-vitro*, we assessed the mRNA levels of TGF-β isoforms and its receptors in MCF UT and MCF D7-6G tumors. Increased expressions of TGF-β isoforms 1, 2 and 3 and their receptors TGF-β RI and RII was observed in MCF D7-6G tumors (Figure 3.21) and increase in secreted TGF-β isoforms I and II were observed in sera of MCF D7-6G tumor bearing mice (Figure21c).

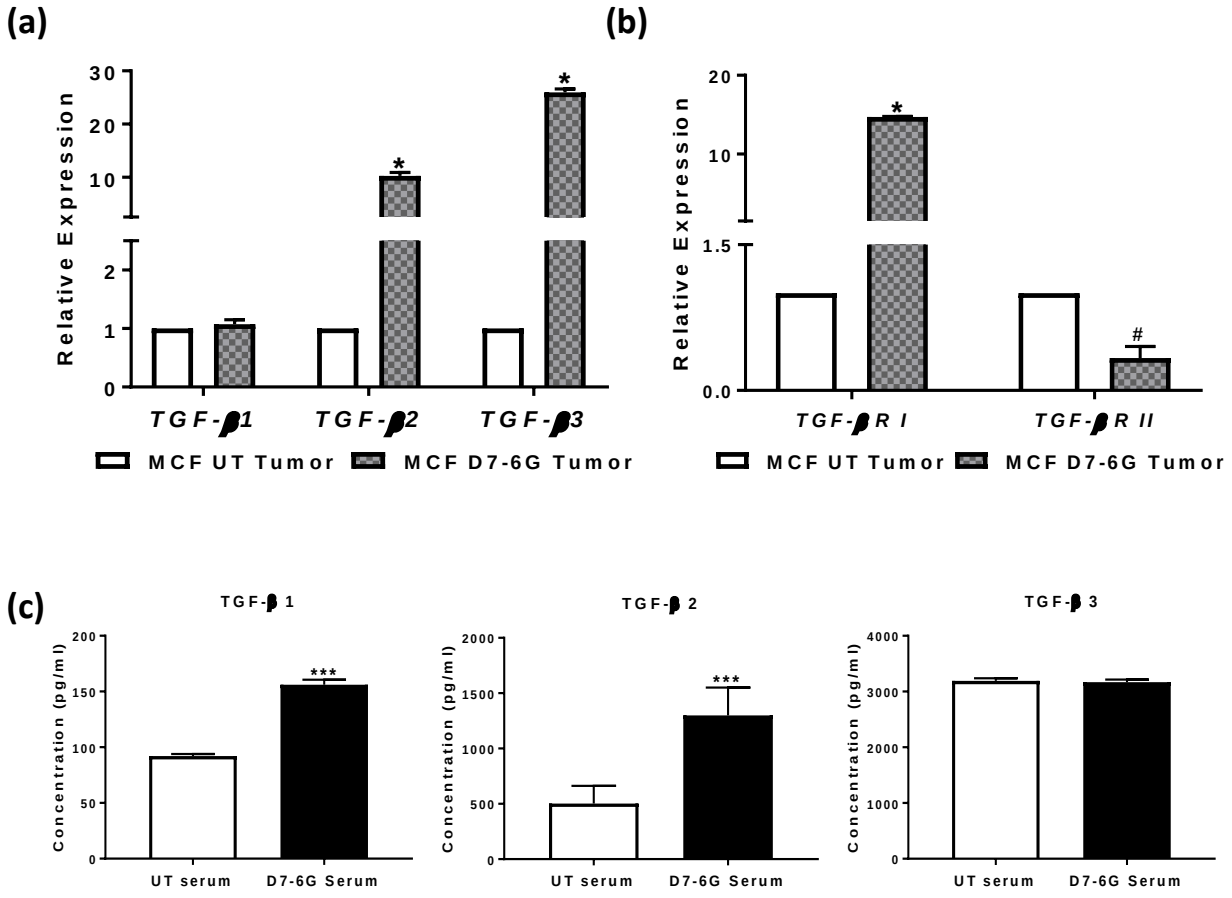


Figure 3.21: Increased TGF-β isoforms and their receptors in MCF D7-6G tumors. On day 18 after injection of breast cancer cells, the tumors from MCF UT SCID mice and MCF D7-6G SCID mice were removed and chopped into

small pieces. From chopped tumor tissue, total RNA was extracted according to manufacturer's protocol and the expression of TGF-β isoforms 1, 2 and 3 and TGF-β RI, RII were assessed. (a) TGF-β isoforms 1, 2 and 3, (b) TGF-β R I and TGF-β R II, (c) Cytokines TGF-β 1, 2 and 3 in serum of D7-6G SCID mice. *p ≤0.05; ***p ≤ 0.001 increase and $^{\#}$p ≤ .05; decrease in comparison to UT. The values represented are mean ± S.E.M of values from three pooled samples.

1.3.4 Increase in TGF-β downstream genes in MCF D7-6G tumors.

Since increase in TGF-β isoforms and its receptors were increased in MCF UT and MCF D7-6G tumors, we assessed the downstream genes of TGF-β: *SNAIL-1, HMGA2* and *ZEB-1*. Expression of TGF-β downstream genes increased significantly in MCF D7-6G tumors. *SNAIL-1* showed 4.5 fold, *HMGA2* showed 3 fold and *ZEB-1* showed around 2.7 fold increases in comparison to MCF UT tumors (Figure3.22).

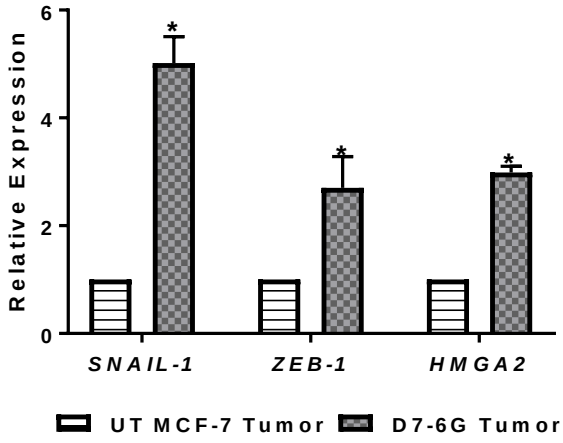


Figure 3.22: Increased expression of TGF-β downstream genes in MCF D7-6G tumors. On day 18 following injection of breast cancer cells, the tumors from MCF UT SCID mice and MCF D7-6G SCID mice were removed and chopped into small pieces. From chopped tumor tissue, total RNA was extracted according to manufacturer's protocol and the expression of *SNAIL-1; HMGA2* and *ZEB-1* was assessed. *$p \leq 0.05$; increase in comparison to UT. The values represented are mean ± S.E.M of values from three pooled samples.

1.3.5 Hybrid E-M phenotype in MCF D7-6G tumors

Since MCF D7-6G tumors had shown increase in mRNA levels of *SNAIL-1*, *HMGA2* and *ZEB-1*, which induces EMT, we assess the expression of EMT genes in MCF UT and MCF D7-6G tumors. Epithelial markers *E-CADHERIN*, *OCCLUDIN* and *DESMOPLAKIN* showed 20, 12 and 6 fold increase respectively in comparison to MCF UT tumors (Figure3.23a) along with increase in mesenchymal markers *FIBRONECTIN*, *N-CADHERIN* and *VIMENTIN:* 22, 1.6 and 2 fold respectively in comparison to MCF UT tumors (Figure3.23b).

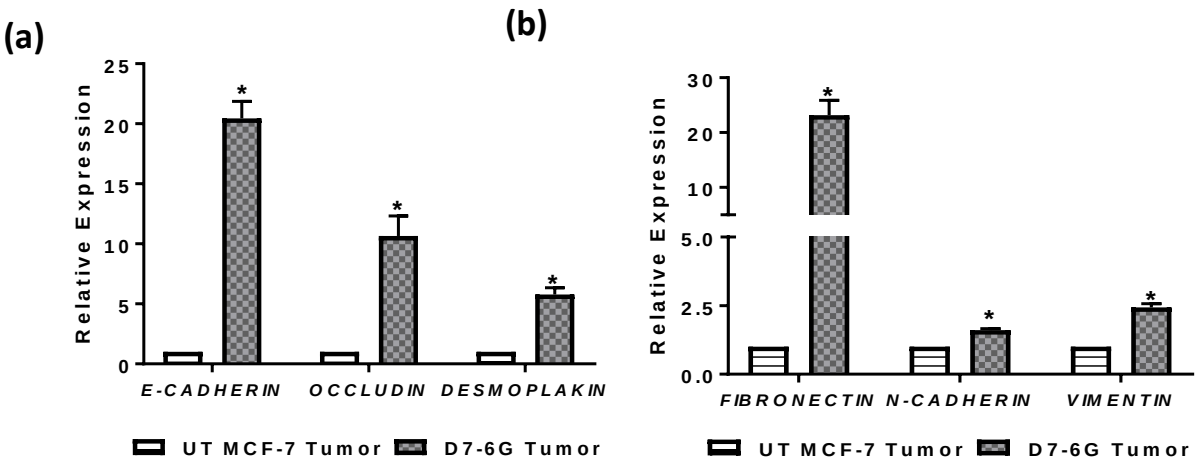


Figure 3.23: Hybrid E-M phenotype in MCF D7-6G tumors. SCID mice were injected with β-estradiol for one week followed by s.c. injection of MCF UT and MCF D7-6G cells. Tumor was removed and total RNA was isolated and epithelial and mesenchymal markers were assessed by RT PCR using specific primers. The values represented are mean ± S.E.M of values from one representative experiment. *p < 0.05; increase in comparison to MCF UT tumors. Two such experiments were carried out.

1.3.6 Cancer stem cells in MCF D7-6G tumors.

Since MCF D7-6G tumor had shown radioresistant properties, we assessed the CSCs markers and ALDH activity in these tumors. MCF D7-6G tumor had higher mRNA expression of *OCT-4, SOX-2, NANOG* and *ALDH* (Figure3.24a). Since mRNA levels of *ALDH* were very high along with other CSCs markers, we assessed the ALDH activity in MCF UT SCID tumors and MCF D7-6G SCID tumors. Results indicate around 2.5 times increase in ALDH activity of MCF D7-6G SCID tumors than that of untreated control group (Figure3.24b).

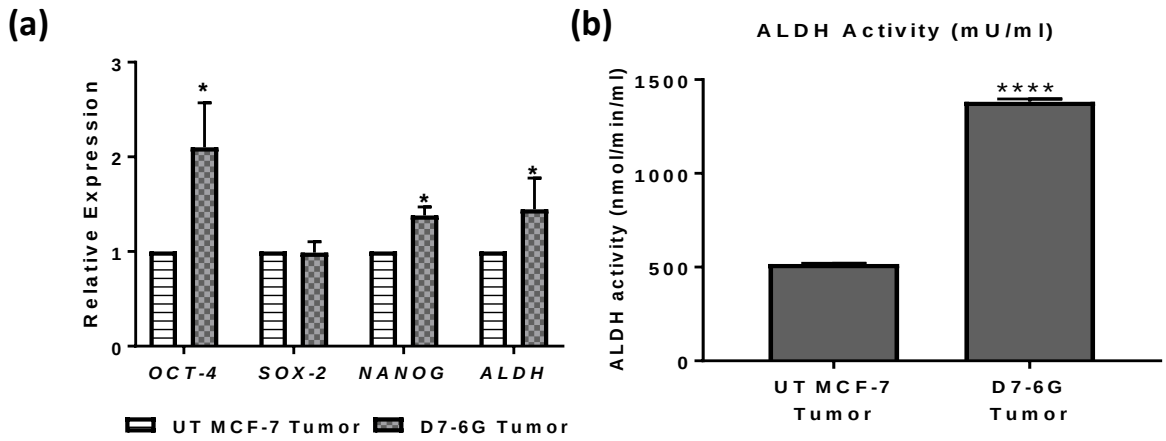


Figure 3.24: Cancer stem cell markers in D7-6G tumors. SCID mice were injected with β-estradiol for one week followed by s.c. injection of MCF UT and MCF D7-6G cells. Cells were isolated from tumor samples. Total RNA was extracted and relative expressions of (a) *OCT-4, SOX-2, NANOG* and *ALDH* was assessed by RT-PCR, (b) ALDH activity was assessed in MCF D7-6G tumors according to manufacturer's protocol. The values represented are mean ± S.E.M of values from one representative experiment. Two such experiments were carried out. *$p \leq 0.05$; increase in comparison to UT.

1.3.7 Label free proteomic analysis of MCF D7-6G tumors

Since there were changes in TGF-β signalling in D7-6G tumors, we carried out proteomic analysis in these tumors to identify alteration in other signalling pathways. Label free proteomic analysis of tumor tissue isolated from MCF UT tumor and MCF D7-6G tumor resulted in identification of a total of 649 differentially expressed proteins. The LC/MS profiles of protein samples from both the groups are given in Figure3.25a-b. Analysis of the data through

Proteome Discoverer software revealed the abundance of various proteins in the two groups, their abundance ratio as well as their co-efficient of variation (CV). The null hypothesis for the statistical tests was that there is no difference between the two-sample group means of a protein, and the alternative hypothesis was that the sample group means of a protein differ from each other. The means of two groups of proteins were calculated using t-test [300].

With an assumption of equal group variances, the t-statistic was calculated as

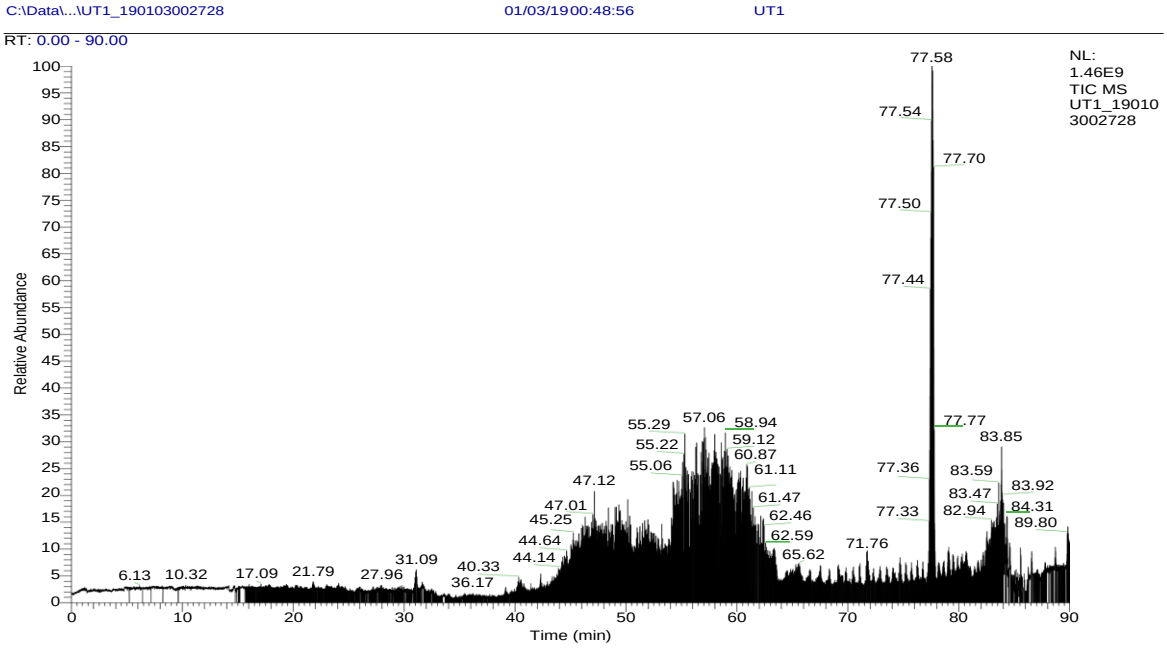
$$t(i) = \frac{\overline{x}_1(i) - \overline{x}_2(i)}{s(i)}$$

Where $\overline{x}j(i)$ is the average abundance level of protein i in sample group j and s (i) is the pooled standard error for the expression of the protein i estimated as

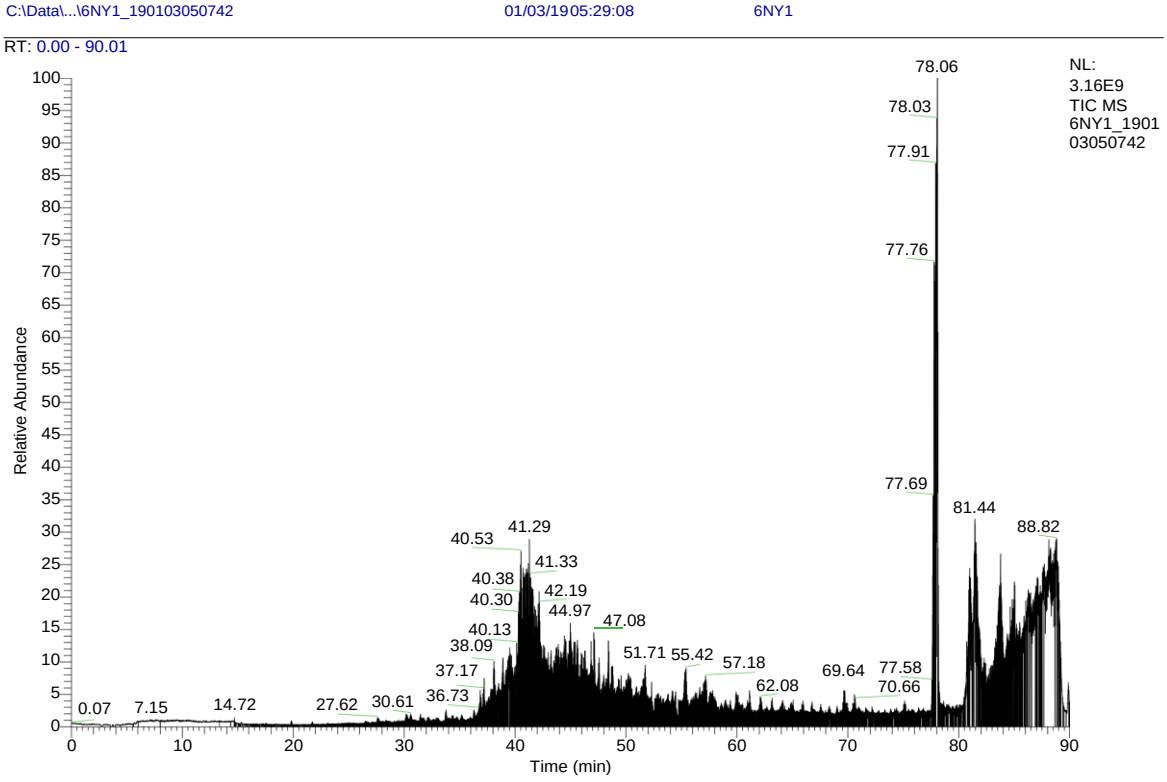
$$s(i) = \sqrt{\left(\frac{1}{n_1} + \frac{1}{n_2}\right)\frac{(n_1 - 1)s_1^2(i) + (n_2 - 1)s_2^2(i)}{n_1 + n_2 - 2}}$$

Following t-test, it was found that 17 proteins were significantly up regulated in MCF D7-6G tumors; $p \leq 0.05$ (Table 3.1). The log fold change in their abundance is given. Eight proteins were significantly downregulated (Table 3.2).

(a)


C:\Data\...\UT1_190103002728
01/03/1900:48:56
UT1
RT: 0.00 - 90.00
NL:
1.46E9
TIC MS
UT1_19010
3002728
Relative Abundance
Time (min)
77.58
77.54
77.70
77.50
77.44
77.77
83.85
83.59
77.36
83.92
83.47
84.31
82.94
77.33
89.80
71.76
55.29
57.06
58.94
55.22
59.12
60.87
55.06
61.11
47.12
61.47
47.01
62.46
45.25
62.59
44.64
65.62
44.14
31.09
40.33
36.17
6.13
10.32
17.09
21.79
27.96

(b)


C:\Data\...\6NY1_190103050742
01/03/1905:29:08
6NY1
RT: 0.00 - 90.01
NL:
3.16E9
TIC MS
6NY1_1901
03050742
Relative Abundance
Time (min)
78.06
78.03
77.91
77.76
77.69
81.44
88.82
41.29
40.53
41.33
40.38
42.19
40.30
44.97
47.08
40.13
38.09
51.71
55.42
57.18
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27.62
30.61

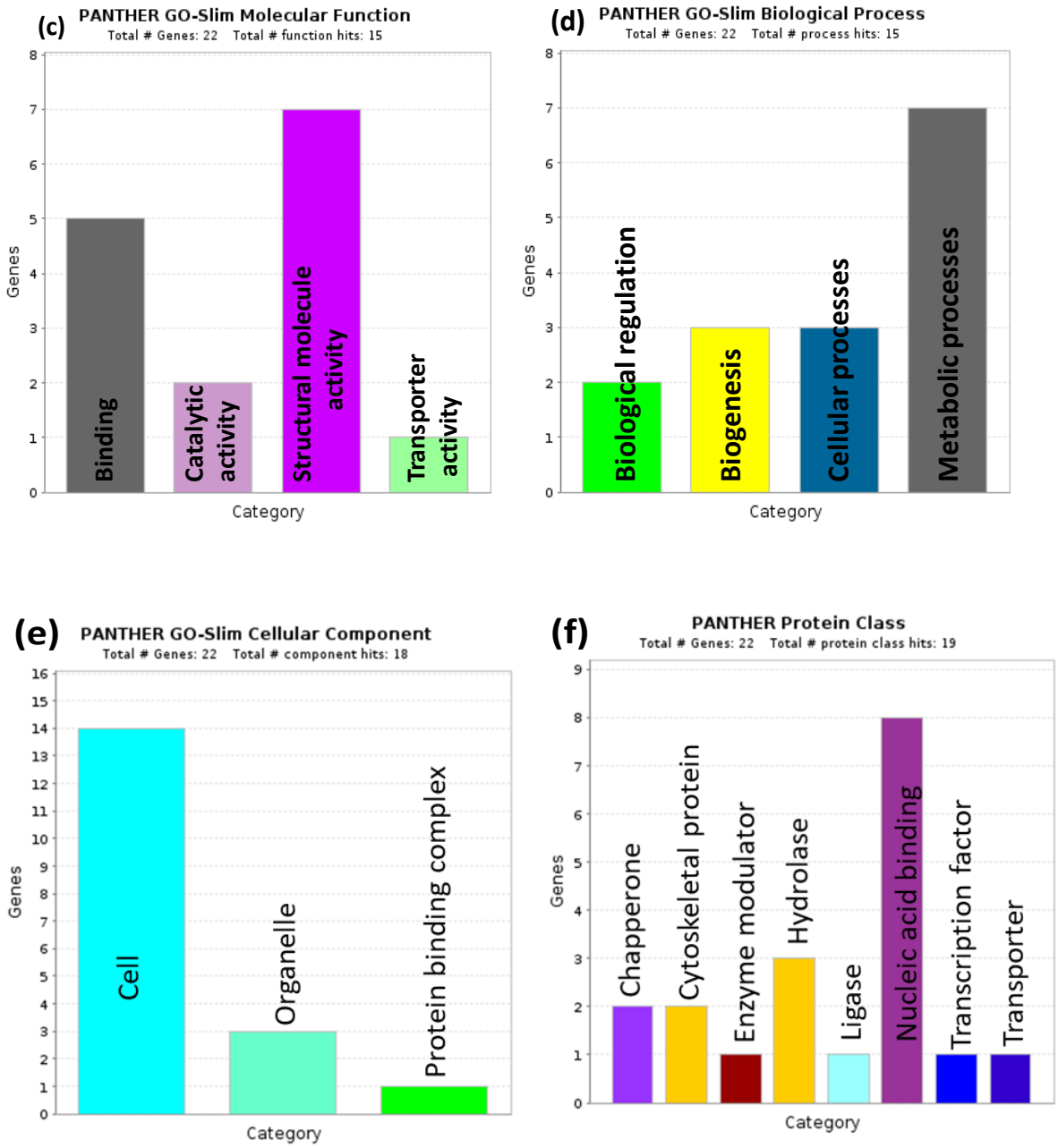


Figure 3.25: LC-MS/MS profile of (a) MCF UT and (b) MCF D7-6G SCID tumors spectra, (c) PANTHER Go-slim Molecular Function tool, (d) PANTHER Go-slim Biological Process, (e) PANTHER Go-slim Cellular Processes, and (f) PANTHER protein class tools showing diffrential expression of genes in two groups. From tumor samples, total protein was isolated in 6 M urea buffer by sonication followed by centrifugation at 1613g for 30 min at 4^0C.

Sample was cleaned up by ice cold acetone precipitation overnight at -20^{0}C. Precipitate formed was collected after centrifugation. The pellets were dissolved in 1M triethylammonium bicarbonate (TEAB) + 6 M urea buffer followed by denaturation and reduction and digestion using 0.1% trypsin. Purification of samples was done through ZipTip pipette tips, followed by speed vac of samples to near dryness followed by resuspension in formic acid and analysed by LC-MS/MS.

Table 3.1: List of up-regulated genes in MCF D-6G tumors

Gene	Gene name	Function	Fold change T/UT
CCT5	T-complex protein 1 subunit epsilon	Molecular chaperone; Unfolded polypeptides enter the central cavity of the complex and are folded in an ATP-dependent manner. The complex folds various proteins, including actin and tubulin.	1.2
PSMC3	26S proteasome regulatory subunit 6A	ATPase 3 subunit of 26s proteasome, a member of the triple-A family of ATPases that have chaperone-like activity.	2.0
CCT7	T-complex protein 1 subunit eta (Fragment)	A molecular chaperone that is a member of the chaperonin containing TCP1 complex (CCT)	2.4
ACTN1	Actinin, alpha 1, isoform CRA_a	Alpha actinins belong to the spectrin gene superfamily. In nonmuscle cells, the cytoskeletal isoform is found along microfilament bundles and adherens-type junctions, where it is involved in binding actin to the membrane.	2.5

FABP5	Fatty acid-binding protein 5	Fatty acid binding proteins are a family of small, highly conserved, cytoplasmic proteins that bind long-chain fatty acids and other hydrophobic ligands. FABPs may play roles in fatty acid uptake, transport, and metabolism.	2.9
TUBB	Tubulin beta chain	beta tubulin protein; forms a dimer with alpha tubulin and acts as a structural component of microtubules.	3.5
CKAP4	Cytoskeleton-associated protein 4, isoform CRA_c	cytoskeleton associated protein 4	3.9
RPS21	40S ribosomal protein S21	ribosomal protein S21; component of the 40S subunit; belongs to the S21E family of ribosomal proteins. It is located in the cytoplasm.	4.1
MYH9	Myosin, heavy polypeptide 9, non-muscle, isoform CRA_a	conventional non-muscle myosin; myosin IIA heavy chain that contains an IQ domain and a myosin head-like domain which is involved in several important functions, including cytokinesis, cell motility and maintenance of cell shape.	5.0
RPL18	60S ribosomal protein L18 (Fragment)	Ribosomal protein L18, a member of the L18E family of ribosomal proteins that is a component of the 60S subunit.	5.0
ATP2A2	ATPase Ca++ transporting cardiac muscle slow twitch 2 isoform 1 (Fragment)	ATPase sarcoplasmic/endoplasmic reticulum Ca2+ transporting 2; intracellular pumps located in the sarcoplasmic or endoplasmic reticulum	5.1
TPM1	Epididymis secretory protein Li 265	tropomyosin family of highly conserved, widely distributed actin-binding proteins involved in the cytoskeleton of non-muscle cells.	5.2
MTHFD1	cDNA FLJ56016, highly similar to C-1-tetrahydrofolate synthase,	protein that possesses three distinct enzymatic activities, 5,10-methylenetetrahydrofolate dehydrogenase, 5,10-methenyltetrahydrofolate cyclohydrolase and 10-formyltetrahydrofolate synthetase. Each of these activities catalyzes one of three sequential reactions in the interconversion of 1-carbon derivatives of tetrahydrofolate, which are substrates for methionine,	5.2

	cytoplasmic	thymidylate, and de novo purine syntheses.	
RPL38	60S ribosomal protein L38	ribosomal protein L38; a ribosomal protein that is a component of the 60S subunit; located in cytoplasm	6.6

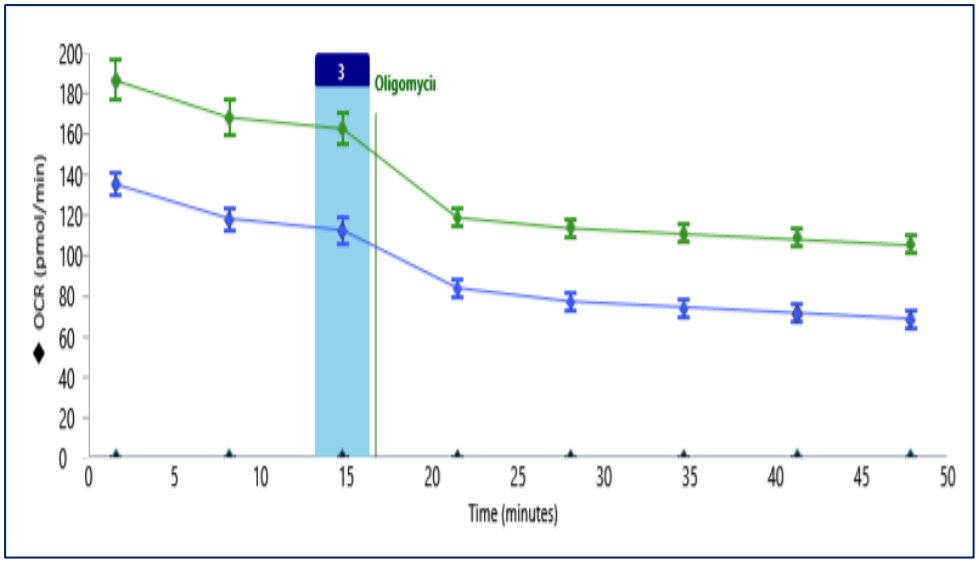
Table 3.2: List of down regulated genes in MCF D7-6G tumors.

Gene	**Gene name**	**Function**	**Fold change T/UT**
RPS8	40S ribosomal protein S8	ribosomal protein S8; a component of the 40S subunit; located in cytoplasm	-6.6
RPL9	60S ribosomal protein L9	ribosomal protein L9; a component of the 60S subunit; located in cytoplasm	-6.4
TXNDC5	Thioredoxin domain-containing protein 5	thioredoxin domain containing 5; member of the disulfide isomerase (PDI) family of endoplasmic reticulum (ER) proteins that catalyze protein folding and thiol-disulfide interchange reactions	-4.3
RPL8	60S ribosomal protein L8	ribosomal protein L8; a component of the 60S subunit; located in cytoplasm	-3.2
SBDS	SBDS ribosome maturation factor	SBDS ribosome maturation factor; a highly conserved protein that plays an essential role in ribosome biogenesis.	-2.8
EIF2S3	Eukaryotic translation initiation factor 2 subunit 3	eukaryotic translation initiation factor 2 subunit gamma; largest subunit of a heterotrimeric GTP-binding protein involved in the recruitment of methionyl-tRNA (i) to the 40 S ribosomal subunit	-1.6
PGAM1	Phosphoglycerate mutase	mutase that catalyzes the reversible reaction of 3-phosphoglycerate (3-PGA) to 2-phosphoglycerate (2-PGA) in the glycolytic pathway	-0.9
ANXA5	Annexin A5	annexin family of calcium-dependent phospholipid binding proteins some of which have been implicated in membrane-related events along exocytotic and endocytotic pathways	-0.8

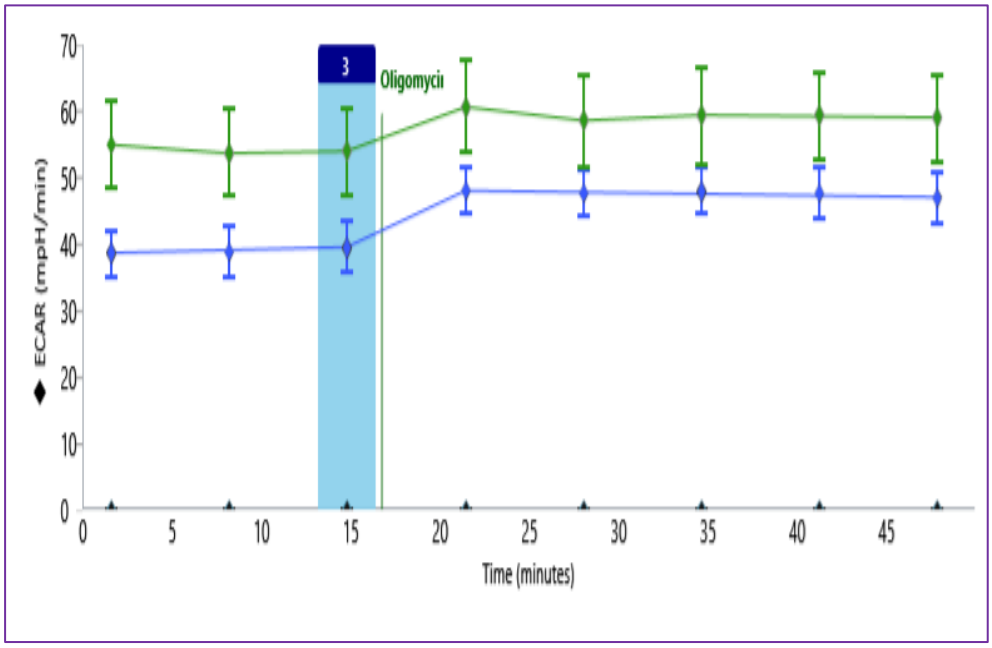
1.3.8 Metabolic changes in MCF D7-6G cells

Analysis of proteomics data by PANTHER Go-slim Biological Process indicated that maximum number of genes altered belonged to metabolic processes. Hence, we assessed the basal metabolic status of MCF D7-6G cells by SeaHorse metabolic analyzer. MCF D7-6G cells showed significant increase in the basal oxygen consumption ratio (OCR) and extracellular acidification rate (ECAR) as compared to MCF UT. Stressor mix (oligomycin and carbonyl cyanide-p-trifluoromethoxyphenylhydrazone "FCCP") injection significantly reduced the OCR but the reduction was observed in both the groups and the difference between MCF UT and MCF D7-6G cells remained significant. Similarly, there was an increase in ECAR with the injection of the stressor mix but that was observed in both the groups. These results indicated that there was an increase in basal oxidative phosphorylation (OCR) and basal glycolysis (ECAR) in MCF D7-6G cells which were highly energetic as compared to MCF UT cells.

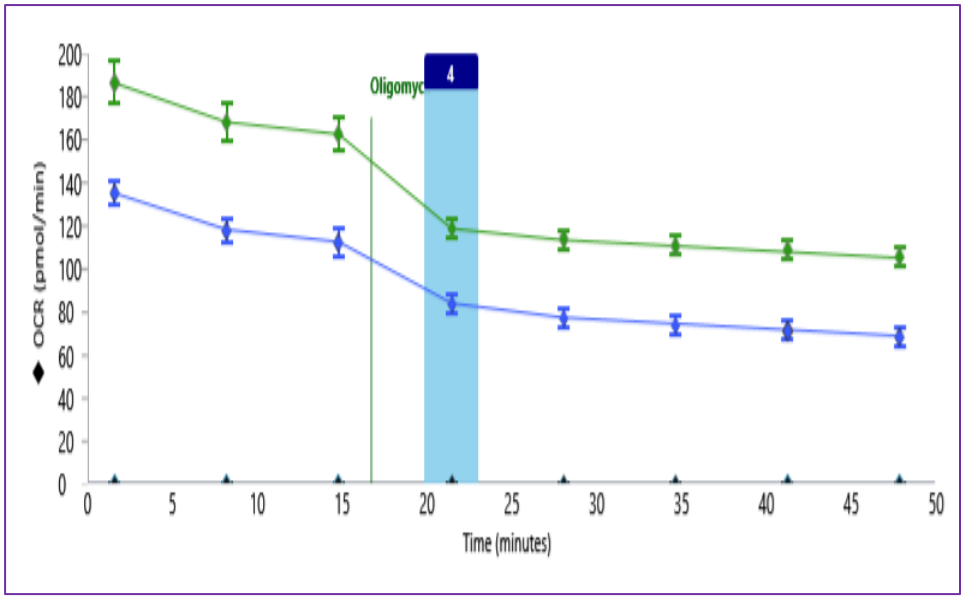
(a)


3
Oligomycin
OCR (pmol/min)
200
180
160
140
120
100
80
60
40
20
0
0
5
10
15
20
25
30
35
40
45
50
Time (minutes)

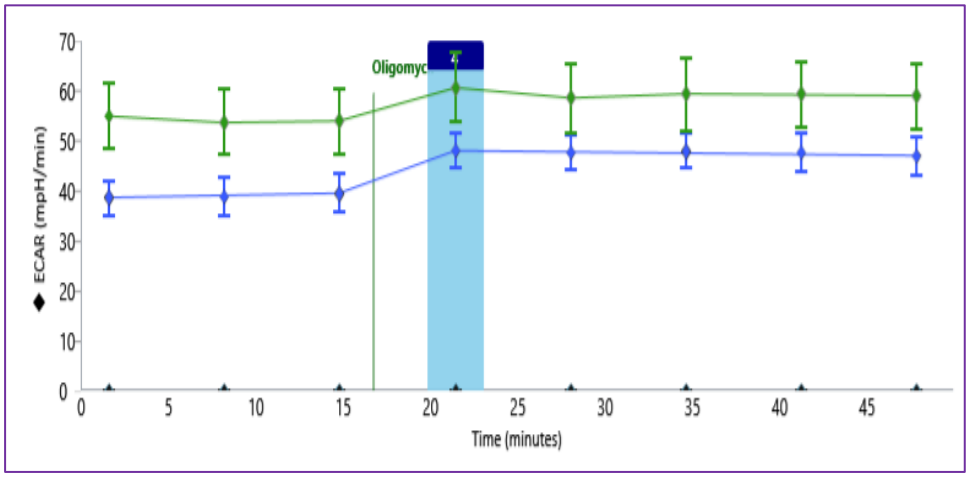
(b)


3
Oligomycin
ECAR (mpH/min)
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
35
40
45
Time (minutes)

(c)



(d)



(e)

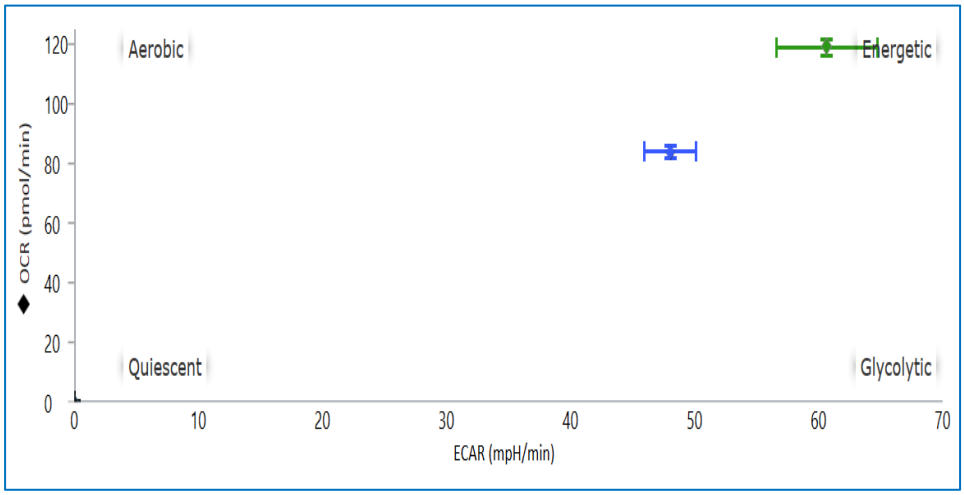


Figure 3.26: Metabolic changes in D7-6G breast cancer cells. Cell energy phenotype: OCR in MCF UT (blue) and MCF D7-6G cells (green) (a) before

addition of stressor mix, (b) after injection of stressor mix; (c) ECAR before addition of stressor mix, (d) ECAR after injection of stressor mix. (e) Basal level energy map of MCF UT and MCF D7-6G cells.

1.3.9 Increased glycolytic rate in MCF D7-6G cells

Since there was an increase in ECAR, which indicated increased glycolysis, we assessed the uptake of a fluorescent analogue of glucose, NBDG by D7-6G cells. MCF D7-6G cells showed increased uptake of NBDG in comparison to MCF UT (Figure 3.27).

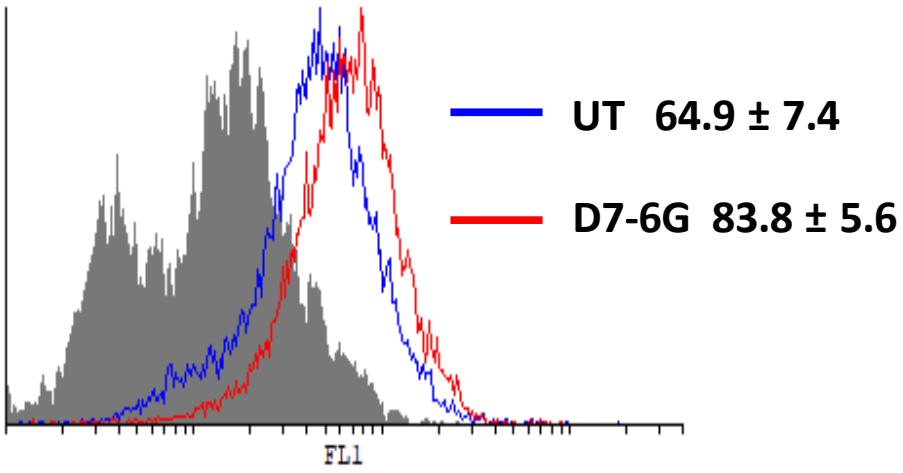


Figure 3.27: Increased glycolytic rate in MCF D7-6G cancer cells. NBDG uptake in (blue) MCF UT, (red) MCF D7-6G and grey filled histogram represent unlabelled cells.

4. DISCUSSION AND CONCLUSIONS

4.1 Discussion

Development of resistance is one of the major barriers of successful radiotherapy in breast cancer and has been previously reported in cell lines following exposure to fractionated irradiation amounting to a total dose of 30-60 Gy[208, 218, 222, 301, 302]. Berton et al, 2017 have also reported such development of radio-resistance following loss of p27 gene [197]. Candidate molecules derived from studies of such cell lines include miR668, as well as inhibitors of COX-2, BCL-2 and CHK1 [220, 221, 301, 303]. Many investigators worldwide are also trying to predict the response of radioresistant tumors using interferon, hypoxia or cell cycle and DNA damage related and other gene signatures [304-306].

In this study, we wanted to identify the minimum dose of radiation required for the development of such a radioresistant phenotype. Therefore, we irradiated cells with different doses of radiation and allowed them to recover for various time periods. We demonstrate that a single exposure of 6 Gy followed by a recovery period of 7 days results in a radioresistant phenotype with increase in proliferation ability as well as apoptosis. The phenotype of increased proliferation observed *in-vitro* could be carried forward *in-vivo* also in SCID mice model further validates these observations. Though expression of anti-

apoptotic genes were highly elevated, and *BAX* levels did not change, there was elevated *BAD* mRNA, a pro-apoptotic gene in D7-6G tumors. Though this appears contradictory, such increase in both apoptosis and proliferation has been reported to increase with increasing tumor size and lesion grade clinically in breast and cervical cancer [307, 308]. These results also suggest that there could exist a close relationship between the radiation doses and recovery periods which could be an important contributing factor for emergence of radio-resistance. Early studies of genetically programmed cell death demonstrated that the selective activation of caspases induces apoptosis and the precise elimination of excess cells, thereby sculpting structures and refining tissues. However, over the past decade, there has been a fundamental shift in our understanding of the roles of caspases during cell death—a shift precipitated by the revelation that apoptotic cells actively engage with their surrounding environment throughout the death process, and caspases can trigger a myriad of signals, some of which drive concurrent cell proliferation regenerating damaged structures and building up lost tissues [309]. Recently, evidence has suggested that pro-apoptotic proteins mostly caspases can induce proliferation of neighbouring surviving cells to replace dying cells. This process, called as "apoptosis-induced proliferation," or "compensatory proliferation" may be critical for stem cell activity and tissue regeneration [310]. Depending on the caspases involved, at least two distinct types of apoptosis-induced proliferation can be distinguished. One of these types have been studied using a model in

which cells have initiated cell death, but are prevented from executing it because of effector caspase inhibition, thereby generating “undead” cells that emit persistent mitogen signalling and overgrowth. Early evidence that apoptotic cells induce mitogens has come from studies in Drosophila. The proliferation pathway in P35-expressing undead cells induces the expression of the Wnt family member wingless and the TGF-β/BMP member dpp [311-313] which encode secretory proteins with strong mitogenic properties. At least in the wing imaginal discs, wingless and dpp were attributable to the overgrowth phenotype as elimination of these genes blocked abnormal growth [314, 315]. In other models of regeneration, such as planarians and newt, Wnt, TGF-β, and Hh signalling have been implicated in regeneration responses [316-318]. Such conditions are likely to contribute to certain forms of cancer andis now established in different types of cancers such as melanoma, glioma and pancreatic ductal adenocarcinoma [319-321]. In addition to undead tumor cells, “genuine” apoptotic tumor cells can also promote apoptosis-induced proliferation. Recently, it was shown that tumor cells that were induced to die by radiotherapy stimulate tumor regrowth [322]. In this case, dying tumor cells were not in an undead condition, but were able to secrete signaling molecules for tumor regrowth. Caspase-3 was required for tumor regrowth as Caspase-3 deficiency rendered the tumors more sensitive to radiotherapy. This proliferation- and tumor-promoting activity of Caspase-3 was found to be mediated through cleavage and activation of cytosolic calcium-independent

phospholipase A2 (iPLA2) that ultimately produces prostaglandin E2 [322, 323]. Caspase-7, while first linked to activation of iPLA2, seems to be more important for activation of protein kinase C delta (PKCδ), which in turn mediates phosphorylation of Akt, p38 and JNK1/2 leading to mitogen production and tumor repopulation following radiation therapy [321]. Simultaneous increases in proliferation and apoptosis have been shown to be associated with selective activation of MAPKs in vascular cells in vein graft [324]. Thus, apoptosis induced proliferation or compensatory proliferation during radio- and chemotherapy may be ineffective or even counter-productive because apoptotic tumor cells can induce proliferation of surviving tumor cells. This in turn can lead to emergence of radio and chemoresistance, the scenario which is presented in this thesis. After irradiation, cells were cultured in the same dish for the recovery period. It is possible that the increased population of dead cells could be acting as a mitogenic signal and inducing proliferation of the remaining cancer cells.

These D7-6G cells are also characterised by an enrichment of TGF-β (isoforms 1/2/3) signalling, hybrid E/M phenotype and increased cancer stem cells. Inhibition of TGF-β signalling prior to radiation exposure prevents the development of radio resistance. Similar TGF-β signalling was found to be enriched in breast cancer cells following recovery after chemotherapy as well as in biopsies after chemotherapy [325-327]. TGF-β1 has been shown to be

increased in serum of tumor bearing animals following exposure to radiation [212, 328] and inhibition of TGFβ1 increased the radio-sensitivity of breast cancer and glioblastoma *in-vitro* and *in-vivo* [328-331]. However, this increase in serum TGF-β1 in tumor bearing animals exposed to radiation could have been contributed by the normal tissues also in response to radiation [332, 333]. In comparison, in the D7-6G tumors developed in SCID mice, *TGF-β2* and *3* were very highly elevated with not much change in *TGF-β1*. Similarly, there was increase in *TGF-βR I* and not in *TGF-βR II* indicating that these isoforms have a major role in the radioresistance. However, when the levels of these secreted TGF-β isoforms were tested in sera, there was elevation in TGF-β1 and TGF-β2 and no change in TGF-β3. Hence it is reasonable to speculate that TGF-β2 could be a major player in radioresistance.TGF-β2 overexpression has been identified as candidate determinants in models of acquired resistance to combination treatment of mitogen-activated protein kinase kinase (MEK) and phosphoinositide 3-kinases (PI3K) inhibitors indicating that it could play an important role in drug resistance too and not only in radiation resistance [334].

Complete killing of tumor cells is required to prevent recurrence, and this will be determined by the radio-resistance of different subpopulations and the number of radio resistant cells. Our study indicates the changes occurring in radiation exposed cells during the subsequent recovery period and the time course experiment indicates at least a minimum period of 7 days is required for

this development to occur. This seems to be mediated by TGF-β signalling as there was an increase in the expression of the ligands as well as receptors and downstream transcription factors. TGF-β levels are positively associated with tumor resistance to radiotherapy or chemotherapy; this positive association may attribute to treatment-initiated EMT of tumor cells. Zhao et al. [335] observed that increased TGF-β levels during radiation therapy are strongly correlated with poor prognosis among patients with non-small cell lung cancer. In addition, poor prognosis of glioblastoma (GBM) routinely treated with ionizing radiation has been attributed to the relative radioresistance of glioma-initiating cells (GICs). GICs are sensitive to treatment, but response is mediated by undefined factors in a microenvironment. Resistance of GIC to radiation, which is mediated by the tumor microenvironment, can be abolished by inhibiting TGF-β/Smad signaling pathway [336]. Tas et al. [337] showed that patients with chemotherapy-unresponsive epithelial ovarian cancer present higher serum TGF-β levels than responsive patients (P=0.02). These studies support the current hypothesis that a subtle relationship exists among TGF-β, EMT phenotype, and therapy resistance.

Increased TGF-β signalling results in EMT leading to increased metastasis [338]. During EMT, epithelial cells lose cell-cell adhesion and gain migratory and invasive traits either partially or completely, leading to a hybrid epithelial/mesenchymal (hybrid E/M) or a mesenchymal phenotype

respectively. Mesenchymal cells move individually, but hybrid E/M cells migrate collectively as observed during gastrulation, wound healing, and the formation of tumor clusters detected as Circulating Tumor Cells (CTCs) [125]. Our results also indicate such a hybrid phenotype with upregulation of both epithelial and mesenchymal markers resulting in increased migration. This was observed both under *in-vitro* conditions as well as in the tumors developed in SCID mice. Cells in a hybrid E-M phenotype retain at least some levels of E-cadherin—the loss of which is considered a hallmark of EMT—and co-express epithelial and mesenchymal markers and display an amalgamation of adhesion and migration to migrate collectively [339]. Snail1 and Zeb-1 are E-cadherin-transcriptional repressors induced during EMT [340]. An exact correlation between the expression of transcripts and protein levels were not found and D7:6G cells from both the cell lines and there was an upregulation of at least two of the three transcription factors. A mathematical model that considers the dynamics of miR-200, Zeb mRNA, Zeb protein, GRHL2 protein and SNAIL protein has shown that the levels of these proteins in the cell determine the shift between E to E/M to M phenotype [125]. This hybrid E/M state has been suggested to reflect stemness and has been associated with poor prognosis, independent of cellular origin [341]. In a study involving ovarian cancer cultures from biopsies/as cites of grade III and IV carcinomas, more than 60% of the clonal cultures were found to be of hybrid E/M type. Interestingly, only cultures containing E/M cells that also co-stained with stem cell markers were

able to form tumors in SCID-beige mice within 4 months [342]. Similar hybrid E/M phenotype has been associated with resistance to the epidermal growth factor receptor inhibitor, erlotinib, in HCC827 derived cell lines with an enrichment of TGF-β pathway [343]. Recently it has been demonstrated that tumorigenicity depends on individual cells residing in this E/M hybrid state and cannot be phenocopied by mixing two cell populations that reside stably at the two ends of the spectrum, i.e., in the E and in the M state. Hence, residence in a specific intermediate state along the E–M spectrum rather than phenotypic plasticity appears critical to the expression of tumor-initiating capacity [344]. The E-M spectrum has been characterized in ovarian and lung adenocarcinoma [345, 346].

Studies have revealed underlying feedback loops that can regulate phenotypic plasticity in ovarian cancer. Forty two ovarian carcinoma cell lines were characterized as epithelial (E-Cad+/Pan-CK+/Vim−), mesenchymal (E-Cad−/Pan-CK−/Vim+), hybrid E/M -intermediate E (E-Cad+/Pan-CK+/Vim+) or hybrid E/M intermediate M (E-Cad−/Pan-CK+/Vim−). The intermediate M ovarian carcinoma cell line exhibited significantly higher spheroidogenic efficiency, migratory and invasive potential relative to the ovarian carcinoma cell lines with other phenotypes. [347]. In lung adenocarcinoma, Schliekelman et al. analyzed the cell morphologies and the ratios of surface localized E-cadherin to vimentin of 38 non-small cell lung cancer (NSCLC) cell lines out of

which nine were binned as epithelial, nine as mesenchymal, and 20 as hybrid E/M [346]. The hybrid E/M cell lines identified at a population level can therefore contain purely individually hybrid E/M cells that stably co-express E-cadherin and vimentin [125] or alternatively express either only E-cadherin or only vimentin, resulting in cell lines largely a mixture of epithelial and mesenchymal cells [348]. It is difficult to predict which of these two types the D7-6G cells would belong to. The mRNA data from *in-vitro* D7-6G cells as well as those of D7-6G tumors clearly indicate increase in both epithelial and mesenchymal markers. Image cytometry data indicates increase in single positive as well as dual positive cells. So, it can be speculated that there could be a mixture of epithelial, mesenchymal as well as hybrid E/M cells that co-express the markers.

Recent evidence indicates that cancer stem cells (CSCs), which have an unlimited potential of cell division and an ability to repopulate the whole tumor[349] also have intrinsic radio resistance [350]. CSCs share some of the critical properties with embryonic stem cells such as unlimited self-renewal, multi-lineage differentiation potential and maintenance of the stemness state. Elevated expression levels of genes associated with stemness and pluripotency, such as OCT4, Nanog, SOX2, and kruppel-like factor 4 (KLF4) have been reported in cisplatin resistant ovarian cancer and chemoradiation-resistant pancreatic cancer [351, 352]. Caspases 3 and 8, two proteases associated with

apoptotic cell death, have been shown to play critical roles in induction of induced pluripotent stem cells (iPSCs) from human fibroblasts. Activation of caspases 3 and 8 occurs soon after transduction of iPSC-inducing transcription factors. Oct-4, a key iPSC transcription factor, is responsible for the activation. Inhibition of caspase 3 or 8 in human fibroblast cells partially or completely prevents the induction of iPSCs, respectively [353]. Several inhibitors of apoptosis proteins (IAP) have also been implicated to play an important role in the regulation of apoptosis in cancer stem cells. IAP proteins comprise a family of endogenous caspase inhibitors that block apoptosis signaling pathways at key nodes [354]. For example, (a) the CD133 positive fraction in glioblastoma has been shown to harbor higher levels of X-linked inhibitor of apoptosis protein (XIAP) and cellular inhibitor of apoptosis protein-1 (cIAP1) compared to the CD133-negative population [355], (b) survivin was amongst the set of three stem cell-associated genes that were identified in soft-tissue sarcomas to correlate with poor outcome [356], (c) Apoptosis-related protein in TGF-β signaling pathway (ARTS/septin 4 isoform 2) is an endogenous antagonist of IAP proteins that has been implied in the control of stem cells. While this protein was originally named according to its role in promoting TGF-β-induced apoptosis, it has subsequently been shown to be broadly implicated in regulating apoptosis signaling via direct binding and antagonizing XIAP [357]. Interestingly, ARTS-deficient mice were found to harbor increased numbers of stem and progenitor cells associated with an elevated susceptibility for tumor

formation, which has at least in part been linked to defective apoptosis in the absence of ARTS [358]. Due to these complexities, the mechanisms leading to radio resistance have not been completely understood. In fact, it has been demonstrated that engineering immortalized mammary epithelial cells to stably express Snail or Twist or stimulating them with TGF-β produced a post-EMT population of cells that displayed the markers (e.g., $CD44^{high}/CD24^{low}$) and features (e.g., mammosphere and tumor-initiating behaviours) of stem-like cells. [359, 360]. CD44 is the major receptor for hyaluronic acid (HA) and the binding has been shown to initiate the metastatic spread of tumor cells [361]. HA-CD44 interaction initiates a downstream cascade of events that modulate cell adhesion, motility, proliferation, and survival [362]. The standard form of CD44, CD44s, lacks the 10 variably spliced exons and codes for a typical type1 transmembrane protein [363]. HA binding initiates extracellular clustering of CD44s, resulting in the activation of kinases [364]. CD44s can serve as, a co-receptor physically linked to other classical signaling receptors [365], a docking protein for other proteins [366] and the trans-membrane domain of CD44s can be cleaved and translocated to the nucleus, where it functions as a transcription factor [367].Recent studies provide strong evidence, using both *in-vitro* and *in-vivo* models, that CD44s-mediated adhesion and signaling are required for cell growth and the dissemination of breast-derived tumor [368, 369]. Bourguignon et al (2008) have established a molecular link between CD44-signaling and TGF-β2 [370]. The study provided evidence that activation of TGF-β2 is an

essential CD44-downstream event required for tumor cell survival and metastasis. Furthermore, a polymorphism in the promoter of TGF-β2 that enhances expression of the protein was associated with lymph node metastasis in breast cancer patients, pointing to a role of TGF-β2 in the process of invasion [371]. TGF- β2, has been confirmed as a CD44s-downstream transcriptional target gene, involved in CD44-promoted breast cancer cell motility [372]. It can be speculated from our results that radiation induced TGF-β2 signalling and further enrichment of CD44 could drive a feedback loop of more TGF-β2 synthesis.CD44 induced transcription of TGF-β2 has been shown to be through activation of the transcription factor cAMP response element-binding protein (CREB) [373].

Our data consistently indicate that the TGF-β downstream genes or EMT genes were upregulated to a greater extent in MDA-MB-231 as compared to MCF-7 cells indicating differences in ionizing radiation induced signalling. Direct inhibition of TGF-βRI using small molecule inhibitor or neutralizing antibodies have been reported to interfere with radiation responses. TGF-β receptor (TGF-βR) I kinase inhibitor LY2109761reduced clonogenicity and increased radiosensitivity in glioblastoma (GBM) cell lines and cancer stem–like cells, augmenting the tumor growth delay produced by fractionated radiotherapy in a supra-additive manner *in-vivo*. In an orthotopic intracranial model, LY2109761 significantly reduced tumor growth, prolonged survival, and

extended the prolongation of survival induced by radiation treatment [329]. TGF-β inhibitors have also been preclinically evaluated; some of these inhibitors are in early stage clinical studies either using monoclonal antibodies against TGF-β1 (CAT192, GC1008, ID11) or antisense oligodeoxynucleotide specific for TGF-β1 mRNA (AP11014) or TGF-β2 mRNA (AP12009) [374-377]. These trials suggest that TGF-β inhibition exhibit promising efficacy and safety. However, large clinical trials are required to clarify the feasibility and safety of treatments. In addition, small molecule inhibitors indirectly affecting TGF-β /Smad signalling pathway also can be used for enhancing radio sensitivity. For e.g. screening of a miRNA expression library in glioblastoma resulted in identification of 4 miRNAs: miR125a, miR150, miR1, and miR425 that induced radio resistance. Investigation of the factors/pathways that regulate the expression of these miRNAs, revealed a correlation of these miRNAs with the TGF-β pathway in glioblastomas and manipulating TGF-β signalling influenced their expression [378]. Silencing of CDP138, a CDK5 binding partner, inhibited TGF-β/Smad signalling resulting in impaired radio resistance and metastasis via GDF15 in lung cancer [379]. SB431542 used in our studies is a selective inhibitor of endogenous activin and TGF-beta signaling but has no effect on BMP signalling. SB-431542 also has no effect on components of the ERK, JNK, or p38 MAP kinase pathways or on components of the signaling pathways activated in response to serum [380].

SB431542 has also been shown to attenuate the tumor-promoting effects of TGF-β, including TGF-β-induced EMT, cell motility, migration and invasion, and vascular endothelial growth factor secretion in human cancer cell lines[381].Interestingly, SB431542 induced anchorage independent growth of cells that are growth-inhibited by TGF-β, whereas it reduced colony formation by cells that are growth-promoted by TGF-β. However, SB431542 had no effect on a cell line that failed to respond to TGF-β. The authors thus suggest that SB431542 can thus be used as therapeutic agent for blocking tumor invasion, angiogenesis, and metastasis, when tumors are refractory to TGF-β-induced tumor-suppressor functions but responsive to tumor-promoting effects of TGF-β [381]. In our studies, we also had observed increased expression of some markers by the drug SB431542 alone. This has to be confirmed with different concentrations and could indicate TGF-β independent signalling. In addition, SB431542 enhanced the antitumor effect of radiofrequency ablation on bladder cancer cells [382] and p53 dependent radiosensitization of non small cell lung cancer cells [383]. However, SB431542 has been shown to alleviate IR-induced BM suppression, partially through the inhibition of IR-induced NADPH oxidase 2 (NOX2) and NADPH oxidase 4 (NOX4)expressions [384].

In addition to TGF-β, there was increased secretion of TNF-α also in D7-6G cells. HeLa cells exposed to TNF-α following chronic treatment with TGF-β exhibited EMT, self-renewal and high mobility and this was mediated by NF-

κB/Twist signaling axis [385]. Breast cancer cells have been shown to induce stromal fibroblasts to express matrix metelloproease-9 (MMP-9) via secretion of TNF-α and TGF-β [386]. The balance between pro-inflammatory and anti-inflammatory cytokines is critical in determining a positive or a negative outcome, adverse reaction and resistance to radiation treatment [387]. Many different factors can influence the cytokine profiles produced after radiation exposure. For example, radiation dose, tissue type and the inborn characteristics of tumor cells can influence the local response into a pro- or anti-tumor effect [298, 388]. In addition, it is important to realize that *in-vivo* and *in-vitro* cytokine expression profiles change greatly [389]. Moreover, the pathogenesis of *in-vivo* radiation damages has a genetic basis also, such as polymorphisms in cytokine genes which contribute to the considerable diversity between individuals both in terms of efficacy and adverse reactions [390].

There was an increase in IL-10 in SB+D7-6G cells observed. This is the first report that inhibition of TGF-β signalling can lead to an increase in IL-10 secretion. The biological effects of IL-10 on tumor growth have ranged from modulating tumor growth (via indirect effects on the immune system) to inhibiting tumor angiogenesis and metastasis.IL-10 secreted by tumor cells have been shown to inhibit macrophage-derived angiogenic factors [391] or sensitize tumor cells to NK cells thereby indirectly blocking tumor growth and metastasis [392]. Contradictory reports of IL-10 down-regulating the antitumor activities

of monocytes and macrophages thereby blocking production of antitumor effector molecules and enabling metastasis have also been reported [393, 394]. Tumor-secreted TGF-β have been shown to induce IL-10 production by the macrophages, suppressing the antitumor activities of the macrophages [395]. Transfection of primary human prostate tumor cells with TGF-β1 gene was shown to stimulate anchorage-independent growth and promoted tumor growth, angiogenesis, and metastasis after orthotopic implantation in severe combined immunodeficiency mice. In contrast, IL-10 transfected cells or cells co-transfected with these two genes exhibited reduced growth rates and significantly reduced angiogenesis and metastasis after 8, 12, and 16 weeks. Increased mouse survival was correlated with IL-10 activity and inversely correlated with TGF-β1 expression and the authors suggest that IL-10 might be of therapeutic value in treating patients with cancer who have a high probability of metastasis [396]. On the other hand, it also has been reported that the relatively large amount of IL-10 secreted by tumor-associated macrophages (TAMs) to be responsible for breast cancer drug resistance through the IL-10/STAT3/Bcl-2 signaling pathway [397]. The role of IL-10 in breast cancer therefore seems to be controversial. Clinically also, IL-10 has been reported to be a poor prognostic factor as well as associated with disease free survival [398, 399].

Pre-treatment with either TNF-α or IL-10 abrogated the increased proliferation of D7-6G cells indicating abrogation of radioresistance. TNF-α has been reported to be the most significantly up-regulated molecule in radioresistant lung cancer cells [400]. Antagonistic effects of TNF-α on TGF-β signaling have been reported through the down-regulation of TGF-β receptor type II or by counteracting TGF-β stimulation of type I collagen gene expression [401, 402]. On the other hand, TNF-α has been shown to induce TGF-β1 through the ERK pathway primarily via a post-transcriptional mechanism that involves stabilization of the TGF-β1 transcript [403]. TGF-β and IL-10 are inhibitory cytokines that have key role in immune homeostasis, and mice deficient for either of these regulatory cytokines develop severe inflammatory diseases [404, 405]. Both cytokines have synergistic effects and are major contributors to immune tolerance by Tregs but their therapeutic application remains limited due to their pleiotropic and context-dependent effects [406-408]. These results thus indicate that the tumor microenvironment plays an important role in therapy outcome and a pro-inflammatory microenvironment can antagonize radiation induced TGF-β signalling and therefore development of radioresistance.

The radioresistant phenotype generated *in-vitro* was also carried forward *in-vivo*. Shorter latency and larger tumors validate the changes that have occurred in D7-6G cells. For example hybrid E/M phenotype and enrichment of cancer stem cells. Ogawa et al have evaluated the role of tumor cell and tumor stroma

sensitivity as determinants of radiation-induced tumor growth delay. They used DNA double-strand break repair defective DNA-PKcs−/− tumor cell line and its radioresistant DNA-PKcs+/+–transfected counterpart to initiate tumors in nude and hypersensitive severe combined immunodeficient (SCID) mice. They have concluded that the tumor cell radiosensitivity was the major determinant of tumor response in nude mice. However, tumor response measured in terms of growth delay was greater in SCID than in nude mice, probably due to the substantial vascular damage observed in SCID mice following irradiation [409]. Similar increased growth of radioresistant cells in immune compromised mice have been shown for glioblastoma, head and neck and gastric cancers [162, 410, 411].

Proteomic analysis of these radio resistant tumors grown in SCID mice were carried out to evaluate the alterations in other pathways apart from TGF-β signalling. The results demonstrate that there were alterations found in several pathways, the most prominent being those in metabolism and cytoskeletal regulation. We also confirmed changes in metabolism by means of OCR and EACR which indicate there is an increase in both glycolysis as well as oxidative phosphorylation. Increased NBDG uptake further confirms increased glycolysis in these radioresistant cells. During the past few years, accumulating number of MS proteomics studies have been applied to identify potential biomarkers associated with cancer radioresistance. These proteomics techniques consist of

matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS), isobaric tags for relative and absolute quantitation (iTRAQ), liquid chromatography-tandem mass spectrometry (LC-MS/MS), multiple reaction monitoring (MRM) as well as Sequential Window Acquisition of all Theoretical Mass Spectra (SWATH-MS) [412]. LC-MS/MS (LC-based separation techniques directly coupled to automated MS/MS) strategies offer high-throughput analyses resulting in the acquisition of hundreds of thousands of MS/MS fragmentation spectra in a single experiment [413]. Label-free quantification through spectral counting is based on the principle that highly abundant peptides will generate a higher number of MS/MS spectra [414]. Proteomic analysis of radioresistant breast cancer cells showed a decrease in the expression of the 26S proteasome in all radioresistant derivatives when compared with the respective parent cells [256]. Similar to our studies, proteomic analysis of prostate cancer radioresistant cells identified PI3K/Akt, vascular endothelial growth factor (VEGF) and glucose metabolism as the main pathways associated with radio resistance [415]. This was found to be the case in the proteomic analysis of radio resistant cells in xenograft model also and the authors suggest that lactate dehydrogenase A (LDHA) is an ideal therapeutic target to develop combination therapy for overcoming radioresistance [261]. Studies carried out in biopsy samples from 83 patients with prostate cancer undergoing radical hypofractionated and accelerated radiotherapy showed that lactate dehydrogenase 5 (LDH5) overexpression was significantly linked to

highly proliferating prostate carcinomas clinically and with biochemical failure and local relapse following radiotherapy [416]. Similarly, hypoxia-inducible factor 1-alpha (HIF1α) and LDH5 have been shown to be markers of poor outcome in patients with bladder cancer [417] head and neck cancer [418] treated with radiotherapy.2D-LC-MS/MS approach in radioresistant and radiosensitive astrocytoma patients identified two markers-cofilin-1 and phosphoglycerate kinase 1 (PGK1) to be significantly up-regulated in the radioresistant astrocytomas [419].

The findings from this thesis highlight that the signalling events that lead up to radio resistance can occur even after acute exposures in the recovery period. Parallel situations can be present in the clinical set up with discontinuation of radiation schedules by the patient or even in the recovery periods present within the schedule. These studies thus bring to the forefront that radiation resistance need not necessarily happen after the cumulative exposure but can happen even after acute radiation exposures if sufficient recovery period is present. Another important feature was that the radioresistant phenotype observed was co-existent with hybrid E/M phenotype and cancer stem cells. Research on the plasticity of the cancer cells to convert between epithelial and mesenchymal phenotype or to co-express both markers creating a phenotype that can culminate in collective migration of cells is upcoming and much more need to be done to get a complete understating of this phenomenon.

Similarly, the role of pro- or anti-inflammatory cytokines on development of radioresistance needs to be studied further and can give us a clue regarding the inter individual responses to radiation therapy.

4.2 Conclusions

- Breast cancer cells MCF-7 and MDA-MB-231 cells showed increased ability for proliferation following acute exposure of 6 Gy with a recovery period of 7 days. These cells were termed as D7-6G cells and had the following characteristics:
- Along with proliferation, there was an increase in both pro-and anti apoptotic genes both at mRNA as well as protein levels and increase in both necrotic and apoptotic population.
- Increase in all three isoforms of TGF-β was observed at transcript level and by ELISA.
- Increase in TGF-β RI and RII at transcript level.
- Increase in TGF-β downstream genes Snail-1, ZEB-1 and HMGA2 both at mRNA and protein levels.
- Increased ability of migration.
- Demonstrated hybrid epithelial - mesenchymal phenotype and increase in the expression of stem cell markers at transcript level.
- Increase activity of ALDH enzyme.

- Exhibited radio resistance when exposed to another challenge dose of radiation 6 Gy.
- Decreased apoptosis and increased proliferation in D7-6G cells exposed to challenge dose.
- Pre-treatment of SB431542 abrogates increased proliferation of D7-6G cells.
- Pre-treatment with SB431542, increased pro-apoptotic genes and apoptosis of D7-6G cells in both the cell lines MCF-7 and MDA-MB-231.
- Pre-treatment of SB431542 decreases radiation induced increased expression of epithelial marker in both MCF-7 and MDA-MB-231.
- Pre-treatment of SB431542 blocked radiation induced TGF-β signalling in both MCF-7 and MDA-MB-231.
- Increased secretion of TNF- α in D7-6G cells and increased IL-10 in D7-6G cells pre-treated with SB431542.
- Pre-treatment with TNF-α and IL-10 abrogates increased proliferation of D7-6G cells.
- Shorter latency and increased tumor burden of MCF D7-6G cells in SCID mice as compared to tumors generated from MCF UT cells.
- Increased expression of pro-apoptotic genes in D7-6G tumors.

- MCF D7-6G tumors shows significant increase in all three isoforms of TGF- β and their receptors at mRNA level and secreted TGF-β2 and TGF-β3 in the serum.
- Increased TGF-β downstream genes Snail-1, ZEB-1 and HMGA2 in MCF D7-6G tumors.
- Expression of both epithelial and mesenchymal genes resulting in a hybrid epithelial –mesenchymal phenotype in MCF D7-6G tumors.
- Increase in the expression of stem cell markers as well as activity of ALDH was observed in MCF D7-6G tumors.
- Label free proteomic analysis of tumor tissue isolated from MCF UT tumor and MCF D7-6G tumor resulted in 649 differentially expressed proteins.
- Seventeen proteins were significantly up regulated and eight proteins were significantly down regulated in MCF D7-6G tumors.
- Pathway analysis carried using online tool PANTHER classification system indicated increased metabolism of MCF D7-6G tumors.
- Seahorse analysis shows increase in both basal level OCR and EACR indicating increase in glycolysis as well as oxidative phosphorylation in MCF D7-6G cells.
- Increased uptake of NBDG by MCF D7-6G cells confirmed increased glycolysis.

4.3 Summary

Radiotherapy dates back to the late 19th century and plays an important role in the treatment of breast cancer. It kills and destroys cancer cells either by necrosis or activation of programmed cell death or apoptosis. But all cells are not killed by radiotherapy the surviving cells activated DNA damage response. Along with repair pathways there is an activation of several pro-survival pathways that can result in proliferation of cells ultimately resulting in radio resistance. This phenomenon of radio resistance has been studied using cell culture techniques by several investigators in 70s and 80s [420, 421]. This was termed as potentially lethal damage repair (PLDR) and was one of the most important factors that related cell culture studies of human tumors and their radio sensitivities to their clinical responses. Potentially lethal damage was thought of as a cellular injury damage that can lead to cell death under some circumstances but if conditions are modified to allow for repair can result in cell survival and proliferation. However, at that time, the underlying molecular mechanisms for this was not known. Studies undertaken in this thesis has revisited this phenomenon with a deeper understanding of the underlying molecular mechanisms. Results outlined in this thesis demonstrates that even an acute exposure of radiation to breast cancer cells MCF 7 and MDA-MB-231 can result in increased proliferation with activation of TGF-β signalling, hybrid E/M phenotype and enrichment of cancer stem cells. When these cells were exposed

to a challenge dose of radiation, it resulted in radioresistance indicating the changes that have happened during the recovery period are the primary reasons for development of radio resistance. Clinically, this radioresistance leads recurrence of tumor and causes treatment failure. This resistance is achieved by activation of pro-survival pathways, one of them being TGF-β signalling pathway. D7-6G cells showed elevated TGF-β signalling in terms of expression of TGF-β isoforms 1, 2 an 3 at RNA as well as at protein level and increased expression of its receptors TGF-β R1 and R2.TGF-β downstream genes Snail-1, ZEB-1 and HMGA2 were also up regulated which cause EMT that help in increased migration. Radiation induces enrichment of CSCs in D7-6G cells, which further make them radioresistant. Cancer cells can acquire a spectrum of stable hybrid epithelial/mesenchymal (E/M) states during EMT. Cells in these hybrid E/M phenotypes often combine epithelial and mesenchymal features and tend to migrate collectively commonly as small clusters. Such collectively migrating cancer cells play a pivotal role in seeding metastases and their presence in cancer patients indicates an adverse prognostic factor. Increasing experimental evidence suggests a strong association of EMT with stemness which was also observed in D7-6G cells. The importance of TGF-β signalling in increased proliferation of D7-6G cells were further confirmed with the abrogation of this phenotype by pre-treatment with SB431542. Another interesting observation was that there was an enhanced secretion of TNF-α along with TGF-β in D7-6G cells. However in cells pre-treated with SB431542,

this entire pattern changed with an elevation of IL-10 secretion. So when cells were pre-treated with either TNF-α and IL-10, interestingly, there was again an abrogation of radiation induced proliferation of D7-6G cells. These results thus highlight the importance of tumor microenvironment in development of radioresistance. Shorter latency period with higher tumor burden D7-6G tumors confirm the *in-vitro* observations on these cells that they do have an enrichment of cancer stem cells. Elevated TGF-β signalling, hybrid E/M phenotype and cancer stem cell markers in these tumors confirmed the same. Proteomic analysis shows increase in metabolism in D7-6G tumors. High OCR and ECAR observed in MCF D7-6G cells reveals that these cells have an augmented energy requirement and therefore an up-regulation in both glycolysis and oxidative phosphorylation. Increased 2-NBDG uptake in D7-6G cells further confirm the increased glycolysis in MCF D7-6G cells.

4.4 Highlights of the study

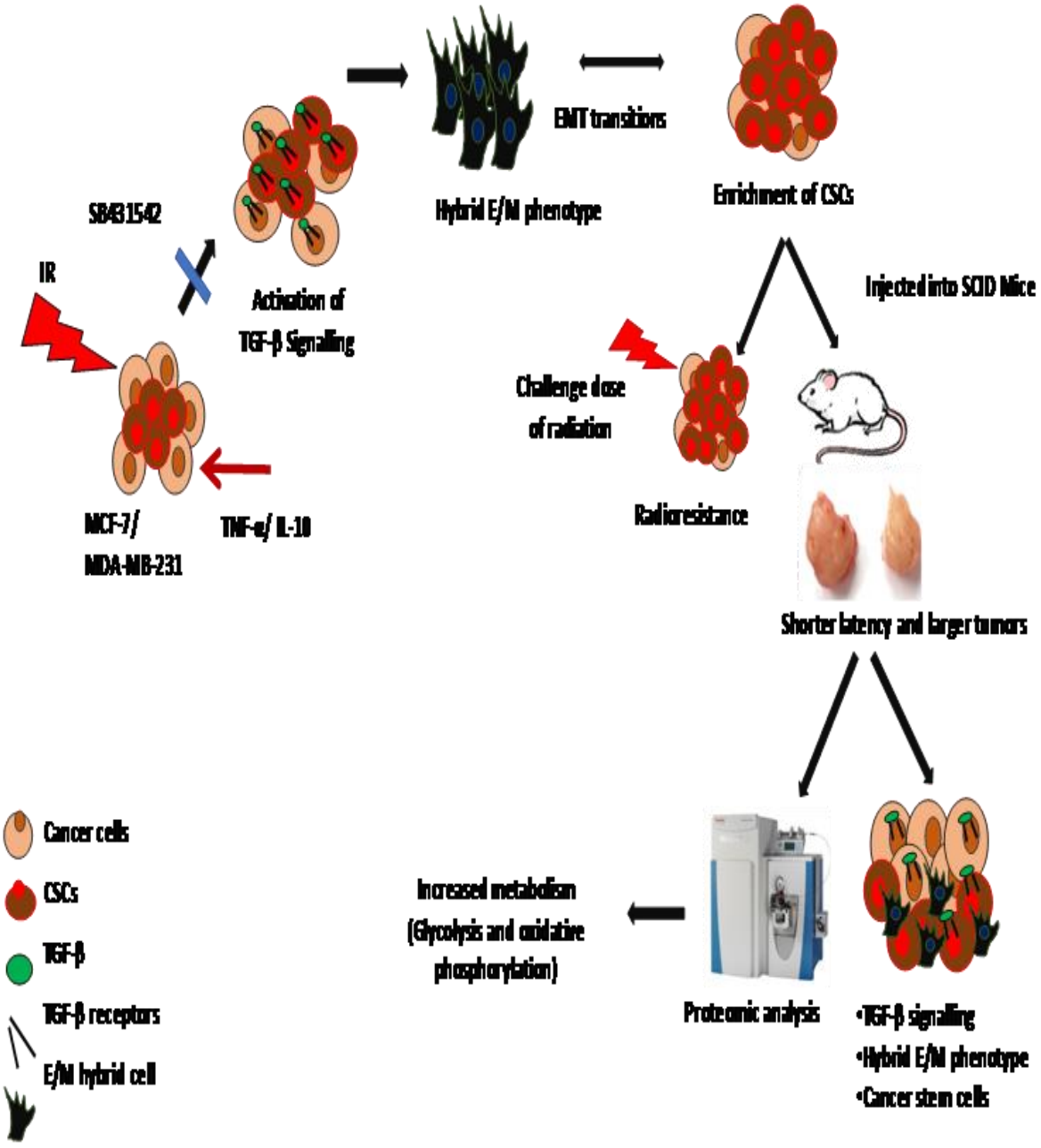


Figure 4.1: Pictorial summary of the role of TGF-β signalling in emergence of radioresistance phenotype. Activation of TGF-β signalling leads to hybrid E/M phenotype, enrichment of CSC and radioresistance phenotype which could be blocked by pre-treatment with SB431542/TNF-α/IL-10. These cells formed

larger tumors in SCID mice which also showed a similar phenotype. Proteomic analysis indicated an upregulation of metabolic pathways in these cells which were confirmed by increased OCR and EACR.

4.5 Future Directions

- To study the activation of other pro-survival pathways mediated by AKT, ERK and ATM/ATR etc. in D7-6G cells and their cross-talks.
- Assessment of other activated pro-survival signalling pathways by blocking them with their specific inhibitors on D7-6G cells.
- To study the effects of cytokines TNF-α and IL-10 on D7-6G cells and their downstream signalling.
- To study the transitions of E-M hybrid phenotype of D7-6G cells and its effects of radioresistance.
- Radiation dose response curves by clonogenic assay for MCF-D7-6G and MDA-D7-6G cells influenced by the TNF-α, IL-10as well as TGF-β inhibitor.
- To study the effect of TNF-α and IL-10 on other pro-survival pathways like MAPK, AKT and Smad pathways in D7-6G cells.
- Characterization of MCF UT and MCF D7-6G tumor cells.
- To study the radiation induced cytotoxicity and its effects on radiosensitization.

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2. Annexure

Name	Source	Catalogue number
2-(5-Bromo-2-pyridylazo)-5-(diethylamino) phenol (BP blue stain)	Sigma(St. Louis, MO, USA)	180017
2-Mercaptoethanol		M6250
Acetonitrile (ACN)		271004
Bovine Serum Albumin		A2153
Bradford reagent		B6916
Crystal Violet		C3886
Dimethylsulfoxide (DMSO)		D2650
Dithiothreitol (DTT)		D0632
Ethylenediaminetetraacetic acid (EDTA)		E6758
Propidium iodide (PI)		P4170
HRP chemiluminescent substrate		WBKLS0500
Hydrogen Peroxide Solution 30%w/v		18755
Non-fat milk	HiMedia (Mumbai, India)	M530

Recombinant Human IL-10	MiltenyiBiotec, BergischGladbach, Germany	4674484
Recombinant Human TNF-α		130-094-015
Protector RNase A inhibitor	Roche Applied Science, (Germany)	03335407001
SB431542	Santa Cruz Biotechnology (Santa Cruz, CA,USA)	204255
SDS	Sigma (St. Louis, MO, USA)	L3771
Sodium bicarbonate		S5761
SYBR green		S9430
TEMED		T9281
Thiourea		T8656
Thiazolyl Blue Tetrazolium Bromide (MTT)		M5655
Triton X 100		X100
Tween 20		P2287
Urea		U5378

Antibodies

Name	Source	Catalogue number
Alexa Fluor 488 Goat Anti- rabbit Antibody	BD Biosciences, San Jose, CA	BD555742
Anti-mouse FITC IgG2bk antibody		BD555742
Alexa Fluor 488 conjugated Anti-BrdU Antibody, clone BU-1, antibody	Sigma(St. Louis,MO, USA)	FCMAB101A4
E-Cadherin Antibody (G-10)	Santa Cruz Biotechnology (Santa Cruz, CA,USA)	SC 8426
Anti-vimentin antibody (clone RV202)		SC32322
CD24	BD Biosciences (Franklin	555428

	Lakes, NJ,USA)	
CD44		555478
Rabbit Anti-Human HMGA2 (C-term)	Pacific Science, Bangplad Bangkok	RBT-102-16480
SNAI1 Polyclonal Antibody	Elabsciences, Houston, Texas	E-AB-32931
Human IL-10 ELISA Set	BD Biosciences (Franklin Lakes, NJ,USA)	555142
Human TNF-α ELISA Set		557953
Human TGF-β 1 ELISA Set	Thermo Fisher Scientific (Waltham, MA, USA)	15531227
Human TGF-β 2 Quantikine ELISA kit	R&D Systems, (Minneapolis, Minnesota, USA)	DB250
Human TGF-β 3 ELISA Set		DY243
ZEB 1 Antibody	Novus	2A8A6

(2A8A6)	Biologicals,Centennial, Colorado	

Tissue culture reagents

Name	Source	Catalogue number
DMEM	HiMedia (Mumbai, India)	AL151A
Heat inactivated fetal bovine serum		RM9955-100ML
Penicillin-Streptomycin		P4333
RPMI-1640		AL060A
Trypsin-EDTA solution		T3924

Molecular biology reagents:

Name	Source	Catalogue number
cDNA synthesis kit	Roche Applied Science (Germany)	05081963001
LightCycler® 480 SYBR		04707516001

Green I Master		
Deoxynucleotides Set	5 Prime GmbH (Hilden, Deutschland)	2201230266
Perfect Pure RNA isolation Kit		2302340
Primers	Sigma (St. Louis, MO, USA)	
Taq polymerase	Invitrogen (Grand Island, NY, USA)	10342-053

Miscellaneous

Name	Source	Catalogue number
Cell culture inserts (8 μm)	BD Biosciences (Franklin Lakes, NJ, USA)	353097
5-Bromo-2′-deoxy-uridine Labeling and Detection Kit I	Roche Applied Science (Germany)	11296736001
Annexin V-FITC Apoptosis Detection Kit	Sigma (St. Louis, MO, USA)	APOAF-50TST

Buffers and solutions

- Phosphate Buffered Saline (PBS): 137mM NaCl (8 g), 10 mM Phosphate (Sodium hydrogen phosphate: 1.44 g and potassium dihydrogen phosphate: 0.24 g), 2.7 mM KCl (0.2 g) pH of 7.4.
- PBST: 0.05 % Tween 20 in PBS
- Flowcytometry: Propidium Iodide (PI) Solution: 50 µg/ml PI, 0.1% Triton-X 100 and 0.1% sodium citrate in dH2O
- Fixation buffer: 2 % paraformaldehyde in 1x PBS
- Permeabilization buffer : 0.1 % Triton X-100 in 1Xpbs
- Lysis buffer: 6 M urea, 2 M thiourea, 2% CHAPS, 0.5% SDS in HPLC grade water
- Dissolution Buffer: 1M triethylammonium bicarbonate (TEAB) + 6 M urea

ELISA

- Coating carbonate buffer: 0.15 M sodium carbonate, 0.35 M sodium bicarbonate, pH 9.6
- Phosphate Buffered Saline (PBS): 137 mM NaCl (8 g), 10 mM Phosphate (Sodium hydrogen phosphate: 1.44 g and potassium dihydrogen phosphate: 0.24 g), 2.7 mM KCl (0.2 g) pH of 7.4
- Blocking buffer: PBS, 1% BSA
- Wash solution: PBS, 0.05% Tween-20
- Dilution buffer: PBS, 0.05% Tween-20, 0.1% BSA
- Stop Solution: 2 N H_2SO_4

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Radio resistance in breast cancer cells is mediated through TGF-β signalling, hybrid epithelial-mesenchymal phenotype and cancer stem cells

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ABSTRACT

Aims: A major obstacle for effective cancer treatment by radiation therapy is the development of radio-resistance and identification of underlying mechanisms and activated pathways will lead to better combination therapies.

Main methods: Irradiated MCF-7 and MDA-MB-231 breast cancer cell lines were characterised following different recovery periods. Proliferation was assessed by MTT, BrdU and clonogenic assays and apoptosis by Annexin V/propidium iodide staining and flow cytometry. Gene expression was monitored by real time PCR/ELISA/antibody labelling and migration using transwell inserts.

Key findings: Breast cancer cell lines exposed to 6 Gy followed by recovery period for 7 days (D7-6 G) had increased ability for proliferation as well as apoptosis. D7-6 G from both cell lines had increased expression of transforming growth factor isoforms (TGF)-β1, β2 and β3, their receptors TGF-βR1 and TGF-βR2 which are known for such dual effects. The expression of downstream transcription factors Snail, Zeb-1 and HMGA2 also showed a differential pattern in D7-6 G cells with upregulation of at least two of these transcription factors. D7-6 G cells from both cell lines displayed hybrid epithelial-mesenchymal (E/M) phenotype with increased expression of E/M markers and migration. D7-6 G cells had increased expression of cancer stem cells markers Oct4, Sox2, and Nanog; aldehyde dehydrogenase expression and activity; proportion of $CD44^{+}CD24^{-}$ cells. This was accompanied by radio resistance when exposed to a challenge dose of radiation. Treatment with TGF-βRI inhibitor abrogated the increase in proliferation of D7-6 G cells.

Significance: Blocking of TGF-β signalling may therefore be an effective strategy for overcoming radio resistance induced by radiation exposure.

1. Introduction

Radiotherapy (RT) is a major therapeutic modality in the management of early breast cancer. Though over 50% of patients receive RT at some time during the treatment of their disease, not all patients derive therapeutic benefit due to radio resistance as evidenced by distant metastatic spread and local recurrence [1]. The sensitivity of cancer to radiation depends on many factors like negative hormone receptor status, the number of cancer stem cells present before initiation of radiation therapy, ability of these stem cells to increase in number during the course of radiation therapy due to repopulation, effects of the tumour microenvironment such as hypoxia, stromal interaction and variations in the intrinsic sensitivity of cells to radiation, modulation of DNA repair or other cell survival pathways [2,3].

Transforming growth factor -beta (TGF-β) has been reported to be an endogenous, radiation-inducible radio-resistance factor in some cancer cells while not affecting the radio-sensitivity in others [4]. In addition, TGF-β also regulates transcription of various target genes responsible for the pathological changes of late radiation damage in the non-tumour-bearing tissues of previously irradiated patients [5]. TGF-β

Abbreviations: TGF, transforming growth factor; RT, radiotherapy; EMT, epithelial mesenchymal transitions; CSCs, cancer stem cells; E/M, hybrid epithelial-mesenchymal; DMEM, Dulbecco's Modified Eagle Medium; FBS, fetal bovine serum; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide; PI, propidium iodide; D7-4G, cells that have recovered for 7 days following exposure to 4 Gy; D7-6G, cells that have recovered for 7 days following exposure to 6 Gy; PBS, phosphate buffered saline; HMGA2, high mobility group protein; UT, untreated; 6G, cells exposed to 6 Gy alone; IR, ionizing radiation; OCT4, octamer-binding transcription factor 4; SOX2, sex determining region Y-box 2; KLF4, kruppel-like factor 4

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isoforms, TGF-β1, TGF-β2 and TGF-β3 regulate a wide variety of biological functions including cell proliferation, migration, survival, angiogenesis, immunosurveillance, embryonic stem cell maintenance and differentiation [6]. The multifunctional effects of TGF-β isoforms are elicited through dimerization of the type I (TβRI) and type II (TβRII) serine/threonine kinase receptors. Upon TGF-β binding, the receptor complex phosphorylates the transcription factors SMAD2 and SMAD3, which then binds to SMAD4 and translocate to the nucleus [7]. In addition to TGF-β, radio-resistance induced by specific protein kinases, transcription factors and microRNAs are also reported [8–11]. Global kinome pathway analysis of radioresistant breast cancer cells has revealed alteration in several kinases involved in cell cycle progression and DNA damage response [12].

Epithelial mesenchymal transitions (EMT) is a fundamental biological process by which epithelial cells undergo biochemical shifts to become mesenchymal cells to generate or regenerate tissues that have different polarization from the original epithelia. An association between radiation and EMT has been reported by many investigators [13–16]. Similarly, radiation induced enrichment of cancer stem cells in xenografts exposed to radiation [17] as well as induction of stem cell-like properties in non-stem cancer cells have also been reported [18–20]. However, the relationship between radiation induced EMT processes and cancer stem cells has conclusively not been established. Though many studies indicate an association between EMT and the gain of CSC properties, the signalling pathways linking them are still not explicit and could be triggered by TGFβ, Wnt/β-catenin, Hedgehog, Notch, and others [21].

The standard model employed for studying radio-resistance in vitro is to repeatedly expose cell lines to radiation amounting to a total dose of 30–60 Gy. We report that exposure to a single dose of 6 Gy followed by a subsequent recovery period of 7 days resulted in increased TGF-β signalling in breast cancer cells leading to hybrid epithelial-mesenchymal (E–M) phenotype as well as increase in cancer stem cells. This further resulted in radio-resistance that could be prevented by TGF-βRI inhibitor SB431542.

2. Materials and Methods

2.1. Chemicals and reagents

Dulbecco's Modified Eagle Medium (DMEM), heat-inactivated fetal bovine serum (FBS) and RNA isolation kit were procured from HiMedia (Mumbai, MH, India). First strand cDNA synthesis kit, SYBR green master mix kit and Cell Proliferation ELISA, (Bromo deoxy uridine (BrdU))-colorimetric were from Roche Applied Science (Penzberg, BY, Germany). MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide), Annexin V-FITC, propidium iodide (PI), RNase A and crystal violet were purchased from Sigma (St. Louis, MO, USA). Human/Mouse TGFβ-1 (2nd Gen) ELISA Kit was purchased from ebiosciences, Inc. (San Diego, CA, USA). Human TGF-beta 2 Quantikine ELISA Kit and Human TGF-beta 3 DuoSet ELISA kit were purchased from R&D systems (Minneapolis, MN, USA). Anti-CD24-PE and anti-CD44-FITC conjugated antibodies and transwell inserts (8 μm) were purchased from BD Biosciences (Franklin Lakes, NJ, USA). Aldehyde Dehydrogenase Activity Colorimetric Assay Kit was purchased from BioVision Incorporated (Milpitas, CA, USA). Antibodies against Snail was from Elabscience (Houston, TX, USA) and HMGA2 was from RayBiotech (Norcross, GA, USA). Anti-Zeb-1 was purchased from Novus Biologicals (Centennial, CO, USA). Anti-Bax and Bcl-2 were from Cell Signaling Technology (Danvers, MA, USA).

2.2. Cell lines and treatment

Human breast cancer cell lines MCF-7 and MDA-MB-231 were obtained from National Center for Cell Sciences, Pune, India and maintained in DMEM containing 10% FBS (complete medium) at 37 °C in a 5% CO_2 atmosphere. Cells were serum starved overnight prior to exposure to ionizing radiation (IR) using Bhabhatron, a Co^{60} source (Panacea Biotech Ltd, New Delhi, India) with a dose rate of 1 Gy/min. The cells were allowed to recover for different time points in complete medium at 37 °C in a 5% CO_2 atmosphere. The nomenclature used for cell lines after these treatments is given in the following table:

Dose	Recovery time	Nomenclature
4 Gy	7 days	D7-4G
6 Gy	7 days	D7-6 G

2.3. Assays for proliferation

2.3.1. MTT assay

Cell viability was measured by MTT dye conversion to formazan. On different days of recovery following radiation exposure, cells were trypsinised and plated in 96 well plates (10^3) for 48 h. Four replicates were taken for each group and the absorbance of solubilized formazan crystals was read in a microplate reader at 550 nm. The viability is expressed as fold change over control.

2.3.2. BrDU assay

Untreated cells and those that have recovered for 7 days following exposure to 6 Gy (D7-6 G) were seeded at a density of 5×10^3 cells / well in 96 well plate for a period of 48 h. After pulsing the cells with 10 μM BrdU for 2 h, they were treated with FixDenat solution followed by anti-BrdU-POD solutions from the kit. The plate was incubated at 25 °C for 90 min followed by addition of tetramethyl benzidine substrate (TMB). After sufficient colour development, 25 μl of stop solution (1 M H_2SO_4) was added. Absorbance was read on microplate reader at 690 nm Biotek synergy H1 microplate reader (Winooski, VT, USA).

2.3.3. Clonogenic assay

Untreated cells and those that have recovered for 7 days following exposure to 6 Gy (D7-6 G) were seeded at a density of 200 cells / well in a 6 well plate in complete medium for a period of 7 days. Once colonies were formed, cells were washed with phosphate buffered saline (PBS) and fixed with methanol: acetone (7:3) at −20 °C. Fixed colonies were stained with crystal violet. The stained colonies were counted using stereo microscope and the results are expressed as surviving fraction. Each experiment was carried out in triplicates/group and was repeated three times.

2.4. Assay for apoptosis

Untreated and D7-6 G cells were harvested using trypsin and suspended in Annexin-V-FITC labelling buffer at 37 °C for 30 min. Samples were acquired in a Partec CyFlow Space™ flow cytometer (Partec, Munich, Germany) using the FloMax 2.1™ software and data were analysed using Cyflogic™ software.

2.5. Flow cytometry/image cytometry

For labelling of surface markers, untreated and D7-6 G cells were harvested and washed twice with PBS. Cells were suspended in minimum volume and anti CD24 and anti CD44 antibodies were added and incubated for 40–60 min. Cells were washed twice with PBS and resuspended in PBS. For labelling of intracellular proteins like Snail, Zeb-1, HMGA2, Bax and Bcl-2: untreated and D7-6 G cells were harvested and washed twice with PBS. Later, cells were fixed with 100 μl of 4% paraformaldehyde and then permeabilized with 100 μl of permeabilization buffer (0.05% Tween 20 in PBS), cells were incubated for 60 min with anti-Snail, anti Zeb-1 and anti HMGA2 antibodies. Cells were washed twice with PBS and incubated with respective secondary

antibodies for 60 min; Cells labelled with anti- CD24, CD44, Snail, Zeb-1 and HMGA2 were acquired in a flow cytometer and were analysed using Cyflogic™ software. Cells labelled with anti-Bax/Bcl-2, E-cadherin and vimentin were acquired in Amnis™ image cytometer and analysed using Ideas™ software.

2.6. Assay for aldehyde dehydrogenase

Activity of aldehyde dehydrogenase in untreated cells and D7-6 G of both the cell lines MCF-7 and MDA-MB-231 were measured according to manufacturer's instructions.

2.7. Migration assay

Untreated and D7-6 G cells (10^3 cells/insert) were plated in transwell inserts (with 8 μm pore membrane) and incubated for 72 h. The bottom of the plate contained complete medium. The cells on the upper side of the membrane were removed with a cotton swab. All cells that migrated to the bottom of the membrane were fixed in methanol: acetone (7:3) for 20 min at −20 °C and stained with crystal violet, photographed in a light microscope and counted using NIS elements™ software.

2.8. ELISA

The cytokines TGF-β1, TGF-β2 and TGF-β3 were estimated using ELISA kits according to manufacturer's instructions. Samples were treated with 1 N HCl followed by neutralisation with NaOH (1.2 N/ 0.5 M HEPES) to activate the TGF- β isoforms present in the supernatant.

2.9. RT-PCR

The expression of all the genes in untreated cells and those treated with different doses of radiation and allowed to recover for different days was assessed using Real-time PCR. Total RNA was extracted using RNA isolation kit and one microgram of total RNA was reverse transcribed to cDNA using first strand cDNA synthesis kit. Equal amount of cDNA (2.5 ng) was used for PCR amplification of the genes using specific primers (Table 1). qRT-PCR was carried out on a LightCycler® 480 System (Roche Applied Science, Penzberg, Upper Bavaria, Germany). All reactions were performed with SYBR green in triplicates. Relative mRNA levels were calculated by the $2^{-\Delta\Delta Ct}$ method ($\Delta Ct = Ct_{Target} - Ct_{Ref}$, $\Delta\Delta Ct = \Delta Ct_{treatment} - \Delta Ct_{untreated}$), using GAPDH or 18sRNA as the reference gene.

2.10. Statistical analysis

All results are expressed as mean ± standard error of mean (SEM). Graph Pad Prism was used to perform statistical analyses. For column analyses, statistical significance between the groups was assessed using one- way ANOVA with Dunnett's post-test comparing all columns to control untreated "UT" column, and a p value less than 0.05 was considered significant. For grouped analyses, statistical significance was assessed using two- way ANOVA with Bonferroni post-tests to compare replicate means by row and compare each column to "UT" column, and a p value less than 0.05 was considered significant.

3. Results

3.1. Increased proliferation ability of D7-6 G cells

The scheme of the irradiation protocol as well as recovery period and the different assays carried out are summarised in Fig. 1a. MCF-7 and MDA-MB-231 cells irradiated with different doses of radiation were incubated for recovery period of either 4 or 7 days before re-plating for another 48 h to assess their proliferation by MTT assay. After recovery periods of 4 and 7 days, there was a change in the ability of the irradiated cells to proliferate (Figs. 1b and 1c) in MCF-7 and MDA-MB-231 cells. In MCF-7 cells, on day 4 after recovery, there was increased proliferation in 2 and 4 Gy, whereas on day 7 there was an increase after 4 or 6 Gy (Fig. 1b). In contrast, this effect was observed only after 6 Gy and day 7 recovery in MDA-MB-231 cells (Fig. 1c). However, with very high doses like 10 Gy, this was not observed in both cell lines (Fig. 1 b,c). This was further confirmed with clonogenic assays (Fig. 1d) as well as BrDU incorporation (Fig. 1e) for both cell lines exposed to 6 Gy and allowed to recover for 7 days (D7-6 G).

Table 1
List of primers used in the study:

Primer name	Forward sequence 5'-3'	Reverse sequence 3'-5'
GAPDH	ATGACATCAAGAAGGTGGTG	CATACCAGGAAATGAGCTTG
Bax	TTTCTCACGGCAACTTCAAC	GGAGGAAGTCCAATGTCCAG
Bad	GTTCCAGATCCCAGAGTTTG	CCTCCATGATGGCTGCTG
Bcl-2	GAGGATTGTGGCGTTCTTT	CCCAGCCTCCGTTATCCT
Bcl-Xl	ACATCCCAGCTCCACATCAC	CGATCCGACTCACCAATACC
TGF-β1	GGCCCTGCCCCTACATTT	CCGGGTTATGCTGGTTGTACA
TGF-β2	TCAAGAGGGATCTAGGGTGGAA	GGCARGCTCCAGCACAGAA
TGF-β3	CAGCTCTAAGCGGAATGAGCAG	TATAGCGCTGTTTGGCAATGTG
TGF-βR1	AAGTCATCACCTGGCCTTGGT	TGCGGTTGTGGCAGATATAGA
TGF-BR2	AATATCCTCTGAAGAACGACCTAA	TCCCACCTGCCCACTGTTA
Snail I	CACTATGCCGCGCTCTTTC	GCTGGAAGGTAAACTCTGGATTAGA
ZEB-1	AGTGATCCAGCCAAATGGAA	TTTTTGGGCGGTGTAGAATC
HMGA2	AAGTTGTTCAGAAGAAGCCTGCTCA	TGGAAAGACCATGGCAATACAGAAT
E-Cadherin	TTCCTCCCAATACATCTCCC	TTGATTTTGTAGTCACCCACC
Occludin	ATGTCATCCAGGCCTC	ATAGACAATTGTGGCA
Desmoplakin	GCTTGCCAACTTCAGAGGTTCT	TTGGAGAATAGCCTGGAGCAGT
Vimentin	CTCTTCCAAACTTTTCCTCCC	AGTTTCGTTGATAACCTGTCC
Fibronectin	CCCCATTCCAGGACACTTCTG	GCCCACGGTAACAACCTCTT
N-Cadherin	GAGGAGTCAGTGAAGGAGTCA	GGCAAGTTGATTGGAGGGATG
OCT4	CGCAAGCCCTCATTTCAC	CATCACCTCCACCACCTG
SOX2	TGTCATTTGCTGTGGGTGAT	GGGGTGCAAAAGAGGAGAGT
NANOG	AGGCAAACAACCCACTTCTG	TCTGCTGGAGGCTGAGGTAT
ALDH	TGAATGGCACGAATCCAAGAG	CACGTCGGGCTTATCTCCT
18SrRNA	CTACCACATCCAAGGAAGGCA	TTTTTCGTCACTACCTCCCCG

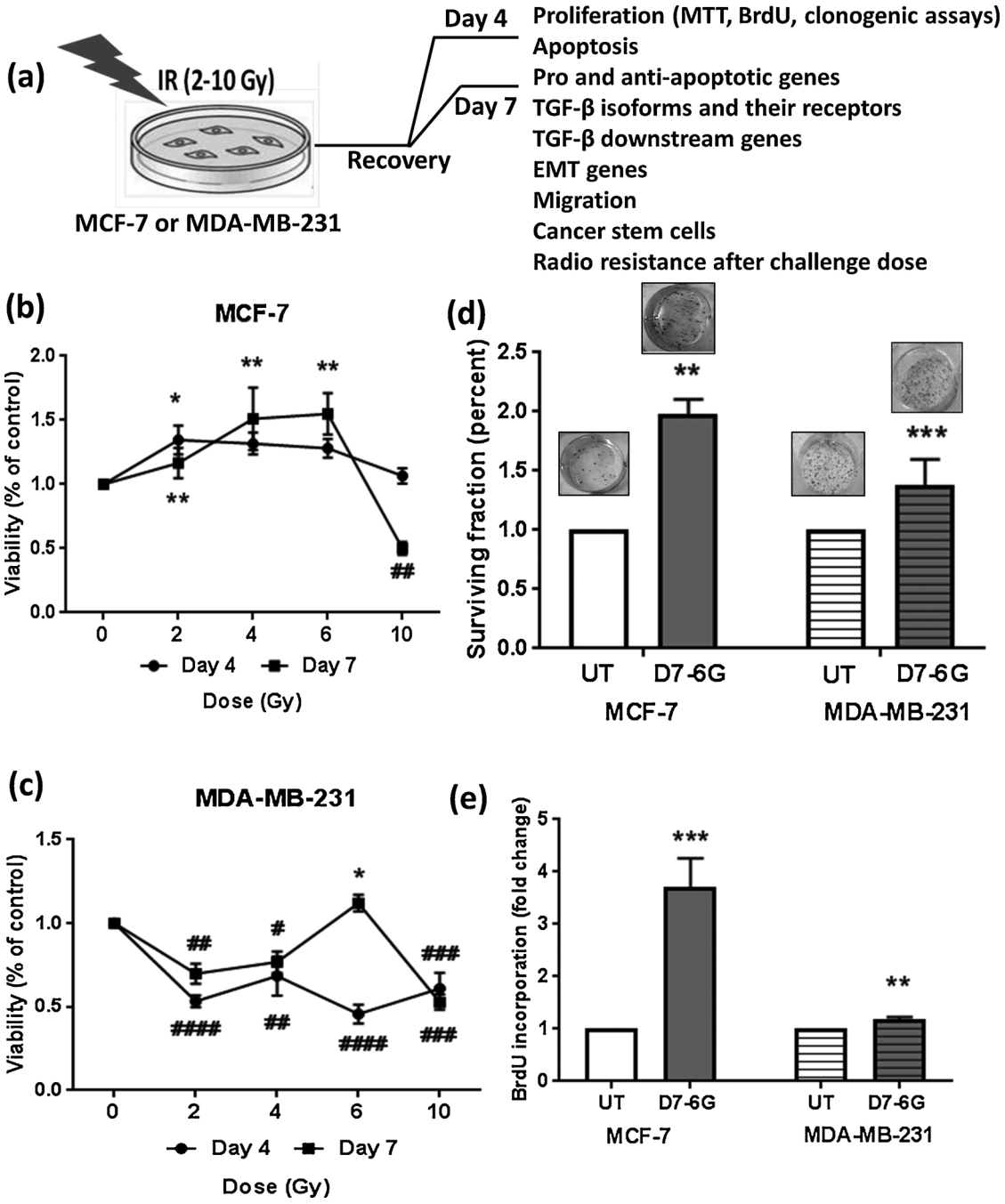

Fig. 1. Increased proliferation ability of D7-6 G cells: (a) The scheme of the experimental protocol. Breast cancer cells were exposed to different doses of ionizing radiation (0–10 Gy) and re-plated immediately for MTT assay on (b) day 4 and day 7: MCF-7 (c) day 4 and day 7: MDA-MB-231. (d) proliferation of UT and D7-6 G cells by clonogenic assay. (e) proliferation of UT and D7-6 G cells by BrdU incorporation. The data are represented as fold change and are mean ± S.E.M of values of three independent experiments. # $p < 0.05$; ## $p < 0.01$; ### $p < 0.001$ decrease in comparison to UT; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$increase in comparison to UT.

3.2. Mixed apoptotic phenotype of D7-6 G cells

As there was increased proliferation in D7-6 G cells, we assessed the expression of some pro- and anti-apoptotic genes. Interestingly, there was a mixed expression, with an increase seen in both pro- and anti-apoptotic genes in D7-6 G cells of both cell lines (Fig. 2a and e). The increase of anti-apoptotic genes was several fold higher than pro-apoptotic genes in MDA-MB-231. This pattern of expression of Bax and Bcl-2 was also confirmed by image cytometric analysis of UT and D7-6 G cells labelled with respective antibodies (Fig. 2b and f). Expression of proteins Bax and Bcl-2 followed the same pattern as mRNA. We also assessed the cell death (apoptosis and necrosis) in these cells by Annexin-V/propidium iodide staining. There was an increase in cell death of D7-6 G cells, both apoptosis (only annexin positive) and necrosis (annexin/propidium iodide dual positive), as compared to untreated cells in both the cell lines (Fig. 2c, d, g and h).

3.3. Increased TGF-β signalling in D76 G cells

Since TGF-β has a dual role in proliferation and apoptosis, we examined the gene expression of different isoforms of TGF-β namely, TGF-β1, 2 and 3 and their receptors, TGF-βR1 and R2. In MCF-7: D7-6 G

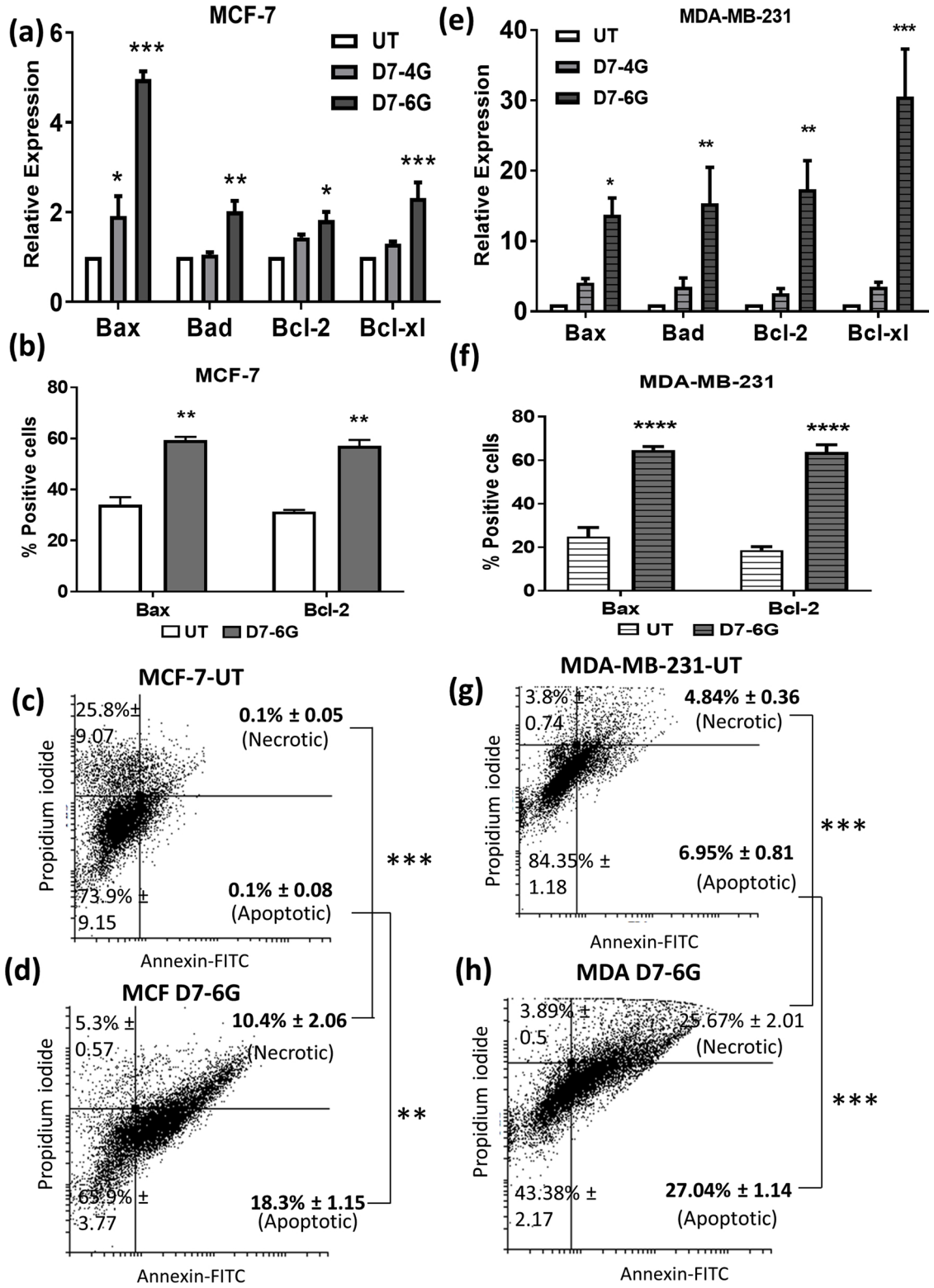

Fig. 2. Mixed apoptotic phenotype of D7-6 G cells: Breast cancer cells were exposed to 6 Gy radiation and allowed to recover for 7 days. Expression of pro- and anti- apoptotic genes in radiation recovered (a) MCF-7 and (e) MDA-MB-231 cells were assessed by RT-PCR using specific primers. Image cytometric analysis of Bax and Bcl-2 proteins in UT and D7-6 G cells of (b) MCF-7 (f) MDA-MB-231 cells. The percentage of cells undergoing apoptosis/necrosis was assessed by Annexin-V/ propidium iodide staining and flow cytometry in (c) UT MCF-7; (d) MCF:D7-6 G cells; (g) UT MDA-MB-231 and (h) MDA:D7-6 G cells. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$ increase in comparison to UT. The values represented are mean ± S.E.M of values from one representative experiment. Three such experiments were carried out.

cells, there was 2-fold increase of TGF-β1 expression. In addition, there was an increase of 4-fold in expression of TGF-β2, 2-fold in TGF-β3 and around 3-fold in TGF-βR1 and R2 in D7-6 G cells (Fig. 3a). In MDA: D7-6 G cells, there was no change in expression of TGF-β1 and more than 10-fold increase in TGF-β2, TGF-β3 and its receptors TGF-βR1 and TGF-βR2 (Fig. 3b). To confirm this, the levels of TGF-β1, TGF-β2 and TGF-β3

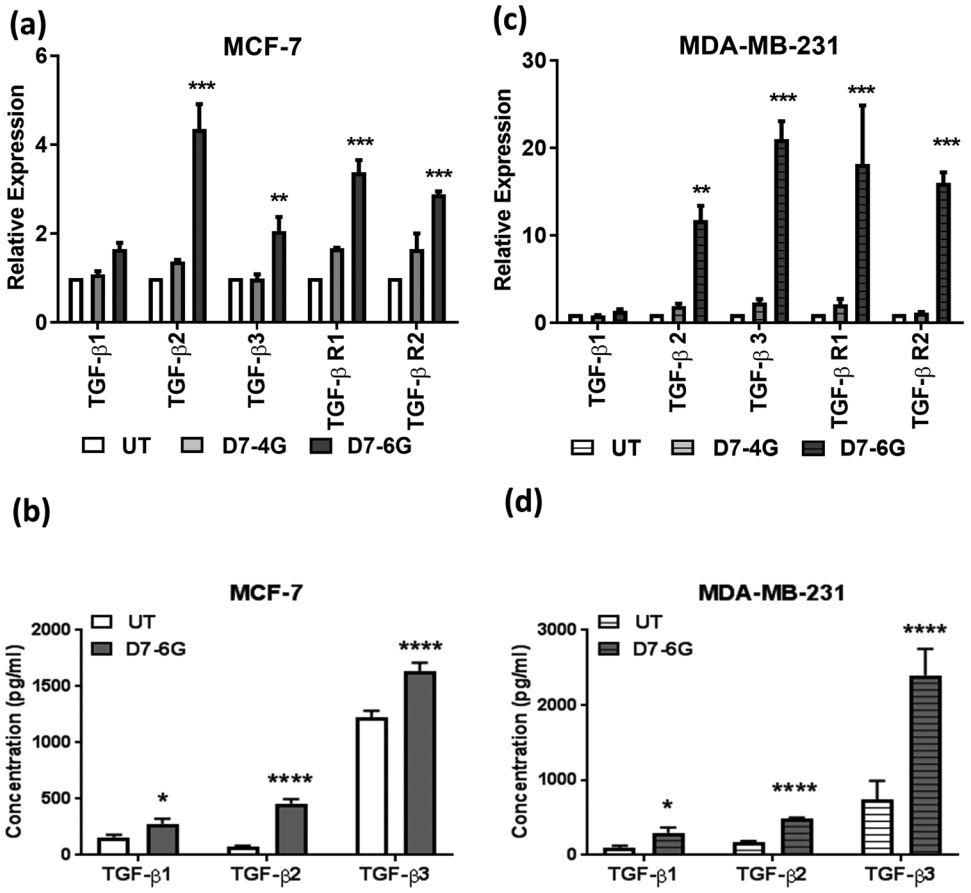

Fig. 3. Increased TGF-β signalling in D7-6 G cells: Expression of different isoforms of TGF-β and its receptors in radiation recovered cells of (a) MCF-7 cells (c) MDA-MB-231 cells. Estimation of TGF-β1, TGF-β2 and TGF-β3 in culture supernatant of UT and D7-6 G cells of (b) MCF-7 (d) MDA-MB-231 cells. The values represented are mean ± S.E.M from three independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001 increase in comparison to UT.

in the supernatant of UT and D7-6 G cells were assessed (Fig. 3b and d). There was a significant increase in all three isoforms of TGF- β in the supernatant of D7-6 G cells of both cell lines (Fig. 3c and d). However, the fold increase of TGF- β2 and TGF- β3 was much higher as compared to TGF- β1. Since there was no significant change in TGF-β1 transcript level in MDA: D7-6 G cells, this increase can possibly be attributable to changes in secretion of the active form or some post-translational modification. When we next examined the expression of TGF-β downstream genes, again, such a differential pattern was observed with a significant increase in Snail and HMGA2 transcripts in MCF-7: D7-6 G cells (Fig. 4a) and Snail, Zeb-1 and HMGA2 in MDA: D7-6 G cells (Fig. 4b). Expression of the TGF-β downstream genes was altered only in D7-6 G of both cell lines and no appreciable change was seen in D7-4 G cells. The expression of these genes was also confirmed by labelling the cells with antibodies specific to these proteins followed by flow cytometric analyses. However, these protein data were not parallel to the mRNA changes and showed increased expression of Snail in MCF-7:D7-6 G cells (Fig. 4b), Zeb-1 in D7-6 G of both cell lines (Fig. 4c and g) and HMGA2 in MDA:D7-6 G (Fig. 4d and h). These results thus demonstrate that regulation of these transcription factors is complex and probably have the involvement of miRNAs as well as post translational modifications [22,23].

3.4. Hybrid epithelial-mesenchymal (E-M) phenotype and increased invasion

A panel of epithelial and mesenchymal markers were assessed in these breast cancer cell lines. In MCF-7 cells recovering from 4 and 6 Gy exposure (D7-4 G and D7-6 G), there was an increase in epithelial markers E-cadherin and occludin. Along with this, there was an increase in desmoplakin and a 3-fold increase in mesenchymal marker N-cadherin in D7-6 G cells. There was no statistically significant difference in the expression of mesenchymal markers vimentin and fibronectin in both D7-4 G and D7-6 G cells (Fig. 5a). In MDA-MB-231 cells recovering from 6 Gy exposure (D7-6 G), there was an increase in epithelial markers E-cadherin and occludin. This was accompanied by marginal changes in vimentin and > 10-fold increase in mesenchymal marker N-cadherin (Fig. 5d). These results suggest development of a mixed or hybrid E/M phenotype consisting of increased expression of both epithelial markers, E-cadherin, occludin and desmoplakin as well as mesenchymal marker N- cadherin. The expression of proteins E-cadherin and vimentin was confirmed by labelling the UT and D7-6 G cells with specific antibodies followed by image cytometric analysis (Fig. 5b and d). Dot plot analyses was carried out to assess single and double positive cells. Fig. 5b and e demonstrate that in MCF: D7-6 G as well as MDA:D7-6 G cells there was a significant increase in vimentin positive and dual positive cells. Since there was increased TGF-β signalling and development of hybrid epithelial –mesenchymal phenotype, the ability of D7-6 G cells to migrate was assessed in transwell inserts and found to increase as compared to the untreated cells (Fig. 5c and f).

3.5. Increased cancer stem cells leading to radio resistance of D7-6 G cells

MCF-7 and MDA-MB-231 cells along with their D7-4 G and D7-6 G counterparts were assayed for the presence of cancer stem cell markers Oct4, Sox2, Nanog as well as ALDH. There was an increase in the expression of all these stem cell markers in MCF-7:D7-6 G cells (Fig. 6a) and MDA:D7-6 G cells (Fig. 6d) as compared to the control unirradiated cells. In comparison, there was no significant change in the D7-4 G cells (Fig. 6a and d). We also analysed the activity of ALDH in these cells which also showed a significant change (Fig. 6b). Fig. 6e shows the expression of another stem cell marker $CD44^{+}CD24^{-}$ which also showed a statistical significant increase in D7-6 G of both cell lines (Fig. 6e). When MCF-7:D7-6 G cells were further exposed to a challenge

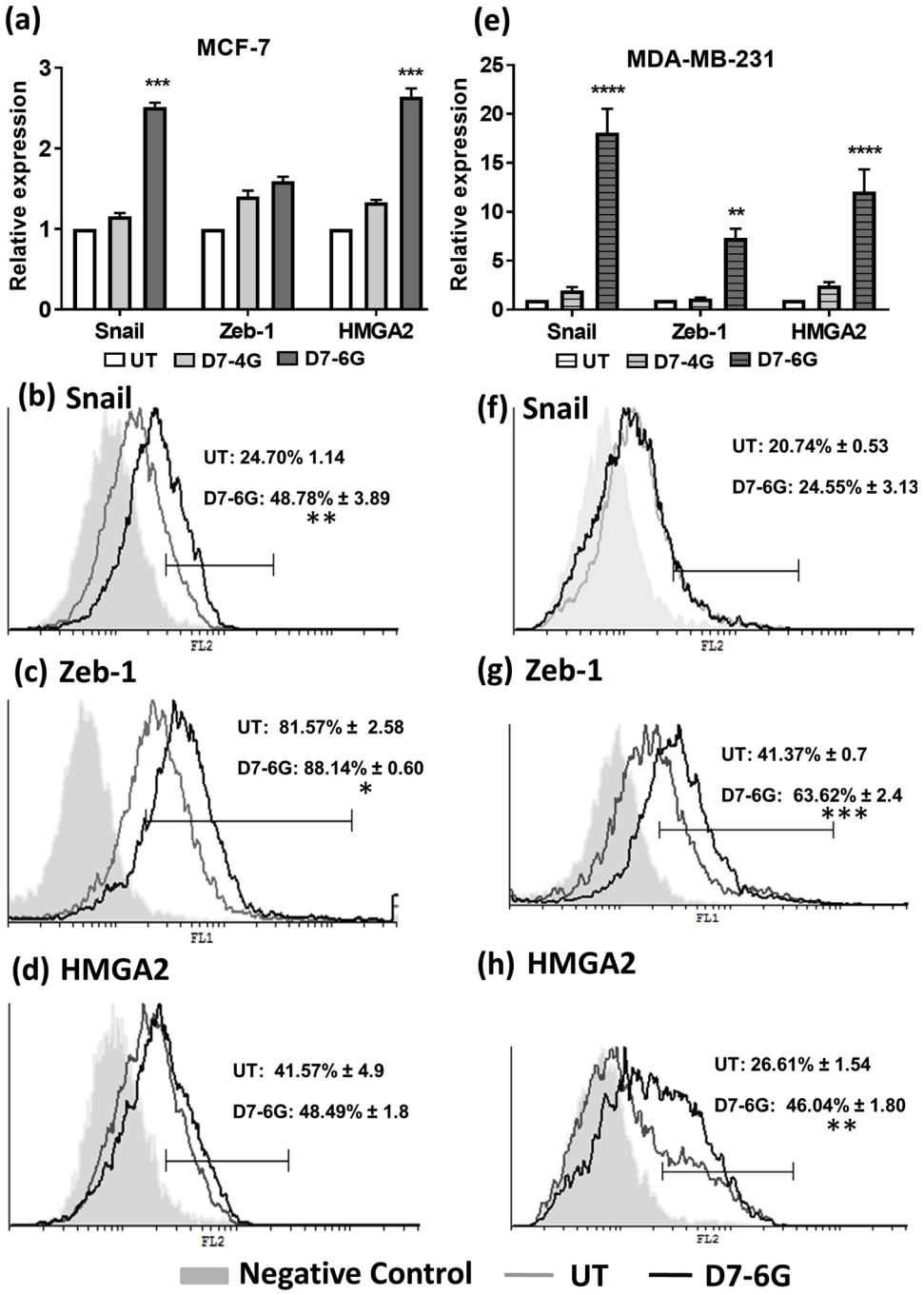

Fig. 4. Expression of TGF-β downstream gens: Expression of TGF-β downstream genes by RT-PCR using specific primers in radiation recovered cells of (a) MCF-7 cells (e) MDA-MB-231 cells. Flow cytometric analysis of TGF-β downstream genes (b) Snail, (c) Zeb-1 and (d) HMGA2 in MCF-7 cells and (f) Snail, (g) Zeb-1 and (h) HMGA2 in MDA-MB-231. The values represented are mean ± S.E.M of values from one representative experiment. Two such experiments were carried out. *p < 0.05; **p < 0.01; ***p < 0.001 increase in comparison to UT.

dose of 6 Gy radiation (D7-6 G + 6 G) and their ability to undergo apoptosis and proliferation was assessed and compared to cells exposed to 6 Gy alone (6 G). There was an increase in apoptosis of D7-6 G cells as compared to UT cells as observed earlier. There was a 3-fold increase in apoptosis in 6 G alone. As compared to 6 G, there was decreased apoptosis of D7-6 G + 6 G cells (Fig. 6c). A similar pattern was observed in terms of proliferation (Fig. 6f). An increase in D7-6 G cells as compared to UT cells as observed earlier. A decrease in proliferation of 6 G cells and in comparison, a significant increase in proliferation of D7-6 G + 6 G cells (Fig. 6f). Similar pattern of radio resistance was observed in MDA: D7-6 G cells in terms of apoptosis and proliferation (Fig. 6c and f).

3.6. TGF- βRI inhibitor SB431542 prevents increased proliferation of D7-6 G breast cancer cells

Breast cancer cells MCF-7 and MDA-MB-231 were treated with different concentration of TGF- βRI inhibitor SB431542 for 1 h followed by exposure to 6 Gy. These cells were then allowed to recover for 7 days and proliferation assessed by MTT assay. SB431542 was able to abrogate the increased proliferation ability of D7-6 G cells that probably contributed to radio resistance in both these cell lines (Fig. 7a and b). The signalling events postulated to lead to the development of radio resistance is summarised in Fig. 7c. Radiation induced TGF-β signalling results in hybrid E/M phenotype as well as increase in cancer stem cells.

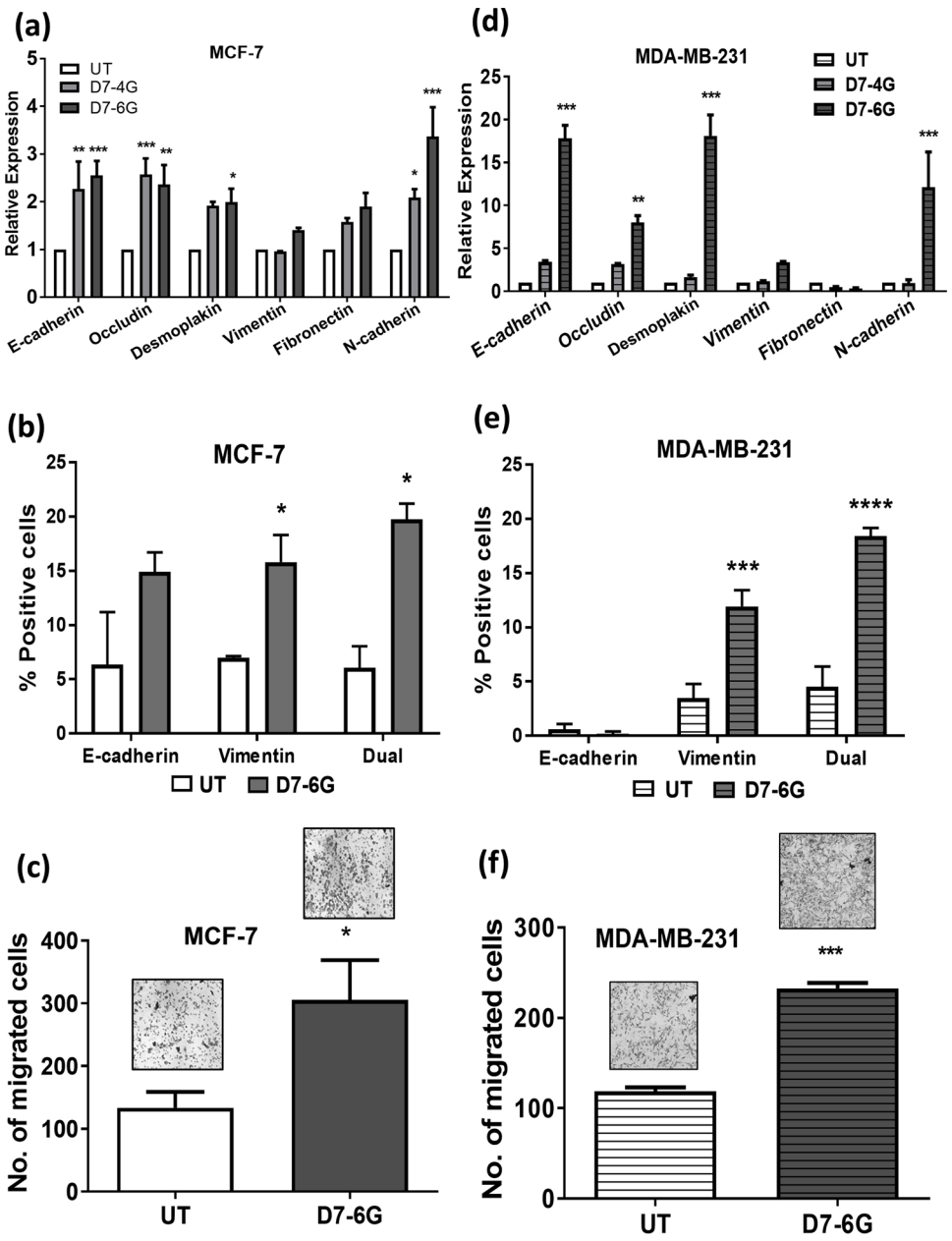

Fig. 5. Hybrid epithelial-mesenchymal (E/M) phenotype and increased migration: Expression of epithelial and mesenchymal markers by RT-PCR using specific primers in radiation recovered cells of (a) MCF-7 cells (d) MDA-MB-231 cells. Image cytometric analysis of E-cadherin and vimentin in UT and D7-6 G cells of (b) MCF-7 and (e) MDA-MB-231. Migration of UT and D7-6 G in (c) MCF-7 (f) MDA-MB-231 cells through an 8 μm transwell insert for 72 h. The migrated cells present in the bottom of the membrane were counted. The values represented are mean ± S.E.M of values obtained from three independent experiments. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$ increase in comparison to UT.

Both these phenotypes ultimately result in the development of radio resistance. However, whether hybrid E/M phenotype is directly responsible for the induction of cancer stem cells is not known.

4. Discussion

Development of resistance is one of the major barriers of successful radiotherapy in breast cancer and has been previously reported in cell lines following exposure to fractionated irradiation amounting to a total dose of 30–60 Gy [8,12,24–26]. Berton et al, 2017 have also reported such development of radio-resistance following loss of p27 gene [27]. Candidate molecules derived from studies of such cell lines include miR668, as well as inhibitors of COX-2, BCL-2 and CHK1 [10,11,25,28]. Many investigators worldwide are also trying to predict the response of radioresistant tumours using interferon, hypoxia or cell cycle and DNA damage related and other gene signatures [29–31].

In this study, we wanted to identify the minimum dose of radiation required for the development of such a radioresistant phenotype. Therefore, we irradiated cells with different doses of radiation allowed them to recover for various time periods. We demonstrate that a single exposure of 6 Gy followed by a recovery period of 7 days results in a radioresistant phenotype with increase in proliferation ability as well as apoptosis. Though this appears contradictory, such increase in both apoptosis and proliferation has been reported to increase with increasing tumour size and lesion grade clinically in breast and cervical cancer [32,33]. These results also suggest that there could exist a close relationship between the radiation doses and recovery periods which could be an important contributing factor for emergence of radio-resistance.

These D7-6 G cells are also characterised by an enrichment of TGF-β (isoforms 1/2/3) signalling, hybrid E/M phenotype and increased cancer stem cells. Inhibition of TGF-β signalling prior to radiation exposure prevents the development of radio resistance. Similar TGF-β signalling was found to be enriched in breast cancer cells following recovery after chemotherapy as well as in biopsies after chemotherapy [34–36]. TGF-β1 has been shown to be increased in serum of tumour bearing animals following exposure to radiation [37,38] and inhibition of TGFβ1 increased the radio-sensitivity of breast cancer and

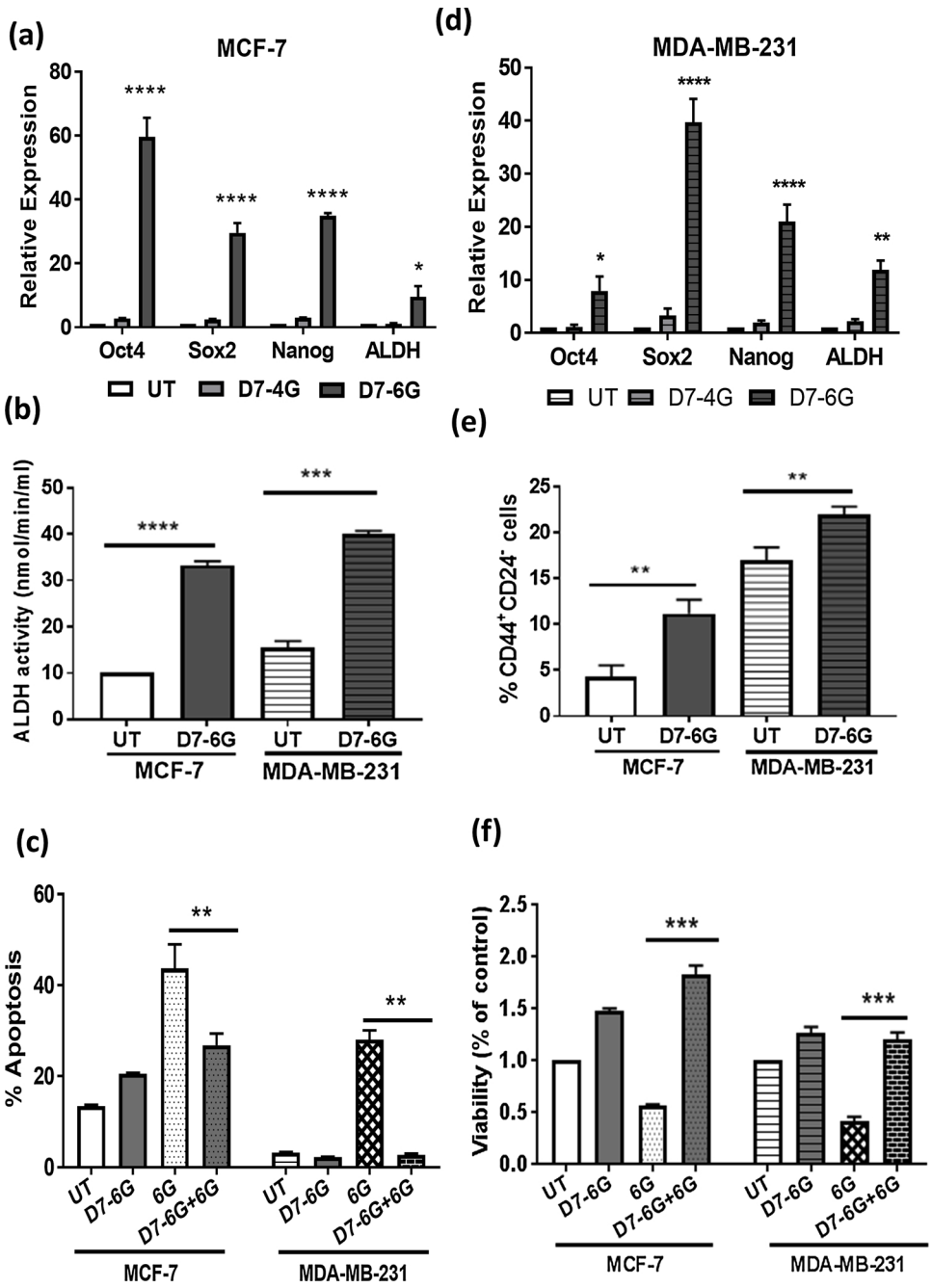

Fig. 6. Increased cancer stem cells and radio resistance of D7-6 G cells: Stem cells markers Oct4, Sox2, Nanog and ALDH were assessed in radiation recovered cells of (a) MCF-7 and (d) MDA-MB-231 cells (b) Aldehyde dehydrogenase activity in UT and D7-6 G cells of both cell lines (e) Flow cytometric analysis of $CD44^{+}CD24^{-}$ population in UT and D7-6 G cells of both cell lines. Radio-resistance was assessed in UT and D7-6 G cells in terms of (c) apoptosis (f) proliferation in both cell lines following a challenge dose (6 Gy) of radiation and further re-plated for 48 h. The values represented are mean ± S.E.M from three independent experiments. $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$ increase in comparison to UT.

glioblastoma in vitro and in vivo [37,39–41]. However, this increase in serum TGF-β1 in tumour bearing animals exposed to radiation could have been contributed by the normal tissues also in response to radiation [42,43].

Complete killing of tumour cells is required to prevent recurrence, and this will be determined by the radio-resistance of different subpopulations and the number of radio resistant cells. Our study indicates the changes occurring in radiation exposed cells during the subsequent recovery period and the time course experiment indicates at least a minimum period of 7 days is required for this development to occur. This seems to be mediated by TGF-β signalling as there was an increase in the expression of the ligands as well as receptors and downstream transcription factors. Increased TGF-β signalling results in epithelial mesenchymal transition (EMT) leading to increased metastasis [44]. During EMT, epithelial cells lose cell-cell adhesion and gain migratory and invasive traits either partially or completely, leading to a hybrid epithelial/mesenchymal (hybrid E/M) or a mesenchymal phenotype respectively. Mesenchymal cells move individually, but hybrid E/M cells migrate collectively as observed during gastrulation, wound healing, and the formation of tumour clusters detected as Circulating Tumour Cells (CTCs) [45]. Our results also indicate such a hybrid phenotype with upregulation of both epithelial and mesenchymal markers resulting in increased migration. Cells in a hybrid E–M phenotype retain at least some levels of E-cadherin—the loss of which is considered a hallmark of EMT—and co-express epithelial and mesenchymal markers and display an amalgamation of adhesion and migration to migrate collectively [46]. Snail1 and Zeb-1 are E-cadherin-transcriptional repressors induced during EMT [47]. An exact correlation between the expression of transcripts and protein levels were not found and D7:6 G cells from both the cell lines and there was an upregulation of at least two of the three transcription factors. A mathematical model that considers the dynamics of miR-200, Zeb mRNA, Zeb

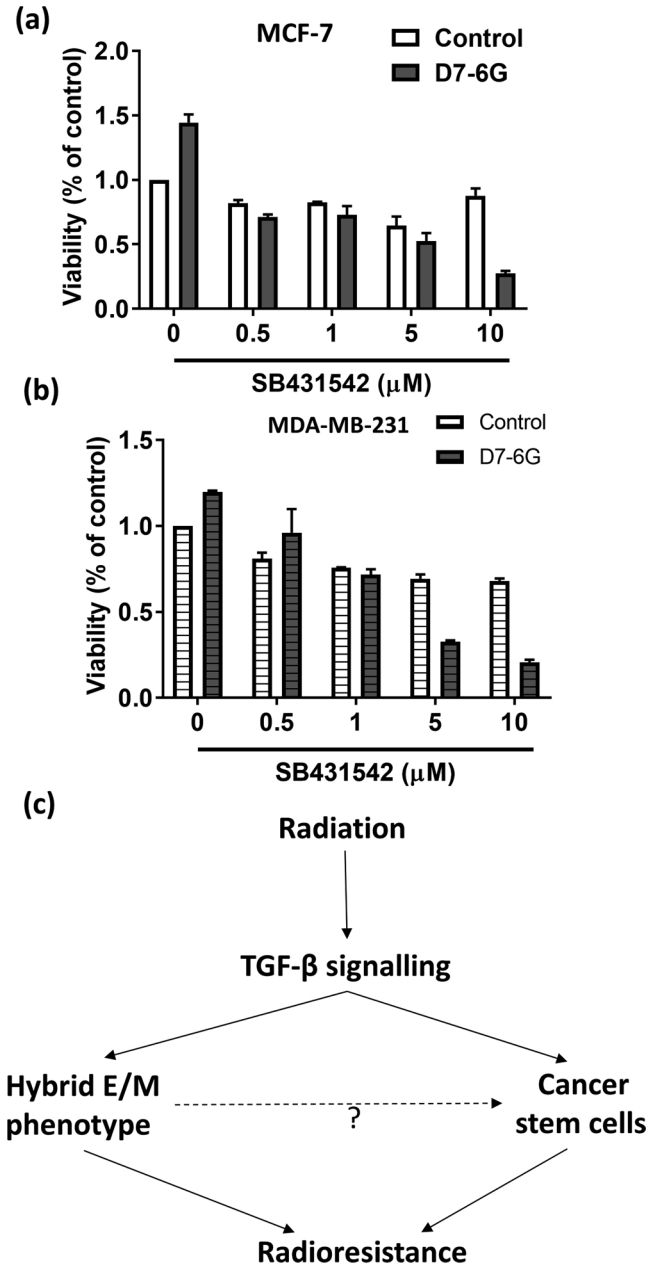

Fig. 7. TGF-βRI inhibitor SB431542 abrogate increased proliferation of D7-6 G in MCF-7 and MDA-MB-231 cells. Breast cancer cells (a) MCF-7 and (b) MDA-MB-231 cells were pre-treated with different concentrations of TGF-βRI inhibitor SB431542 followed by exposure to 6 Gy and recovery of 7 days. Viability was assessed by MTT assay. The values represented are mean ± S.E.M of values from one representative experiment. Three such experiments were carried out. (c) Schematic representation of the proposed signalling following exposure to ionizing radiation resulting in radio-resistance.

protein, GRHL2 protein and SNAIL protein has shown that the levels of these proteins in the cell determine the shift between E to E/M to M phenotype [45]. This hybrid E/M state has been suggested to reflect stemness and has been associated with poor prognosis, independent of cellular origin [48]. In a study involving ovarian cancer cultures from biopsies/ascites of grade III and IV carcinomas, more than 60% of the clonal cultures were found to be of hybrid E/M type. Interestingly, only cultures containing E/M cells that also co-stained with stem cell markers were able to form tumors in SCID-beige mice within 4 months [49]. Similar hybrid E/M phenotype has been associated with resistance to the epidermal growth factor receptor inhibitor, erlotinib, in HCC827 derived cell lines with an enrichment of TGF-β pathway [50].

Recent evidence indicates that cancer stem cells (CSCs), which have an unlimited potential of cell division and an ability to repopulate the whole tumour [51] also have intrinsic radio resistance [52]. CSCs share some of the critical properties with embryonic stem cells such as unlimited self-renewal, multi-lineage differentiation potential, and maintenance of the stemness state. Elevated expression levels of genes associated with stemness and pluripotency, such as octamer-binding transcription factor 4 (OCT4), Nanog, sex determining region Y-box 2 (SOX2), and kruppel-like factor 4 (KLF4) have been reported in cisplatin resistant ovarian cancer and chemoradiation-resistant pancreatic cancer [53,54]. Due to these complexities, the mechanisms leading to radio resistance have not been completely understood. In fact, it has been demonstrated that engineering immortalized mammary epithelial cells to stably express Snail or Twist or stimulating them with TGF-β produced a post-EMT population of cells that displayed the markers (e.g., $CD44^{high}/CD24^{low}$) and features (e.g., mammosphere and tumour-initiating behaviours) of stem-like cells. [55,56]. Our data consistently indicate that the TGF-β downstream genes or EMT genes were upregulated to a greater extent in MDA-MB-231 as compared to MCF-7 cells indicating differences in ionizing radiation induced signalling. Direct inhibition of TGF-βRI using small molecule inhibitor or neutralizing antibodies have been reported [35]. In addition, small molecule inhibitors indirectly affecting TGF-β /Smad signalling pathway also can be used for enhancing radio sensitivity. For e.g. screening of a miRNA expression library in glioblastoma resulted in identification of 4 miRNAs: miR125a, miR150, miR1, and miR425 that induced radio resistance. Investigation of the factors/pathways that regulate the expression of these miRNAs, revealed a correlation of these miRNAs with the TGF-β pathway in glioblastomas and manipulating TGF-β signalling influenced their expression [57]. Silencing of CDP138, a CDK5 binding partner, inhibited TGF-β/Smad signalling resulting in impaired radio resistance and metastasis via GDF15 in lung cancer [58].

5. Conclusion

TGF-β signalling is very crucial for development of radio resistance and targeting TGF-β signalling could be useful in eliminating metastasis as well as radiation resistance and improve patient survival in breast cancer.

Conflict of interest statement

The authors declare no conflict of interest.

Author contributions

Ms. Poonam Yadav was involved in the design of the study and acquisition of data, analysis and interpretation of data. Dr. Bhavani Shankar was involved in the conception and design of the study; analysis and interpretation of data, drafting the article or revising it critically for important intellectual content and final approval of the version to be submitted.

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ABBREVIATIONS

2-D: Two-dimensional gel electrophoresis

2DE-MS: Two-dimensional gel electrophoresis and mass spectrometry

2-NBDG: 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-D-glucose

ABC transporters: ATP binding cassette

ABCB1: ATP-binding cassette subfamily-B member 1

ABCB5: ATP-binding cassette sub-family B-5

ABCG2: ATP-binding cassette subfamily-G member 2

ADCs: Antibody-drug conjugates

AJCC: American Joint Committee on Cancer

ACN: Acetonitrile

AF: Ammonium formate

ALDH: Aldehyde dehydrogenase

ANOVA: Analysis of variance

ARTS: Apoptosis-related protein in TGF-β signaling pathway

ATP: Adenosine triphosphate

BAD: BCL2 associated agonist of cell death

BAX: BCL-2 associated X

BCI: Breast Cancer Index

BCL-2: B-cell lymphoma 2

BCL-XL: B-cell lymphoma-extra large

BCRP: Breast cancer resistance protein

BCS: Breast conserving surgery

BER: Base excision repair

bFGF: Basic fibroblast growth factor

BRCA: Breast cancer gene

BrdU: 5-bromo-2'-deoxyuridine

BMPs: Bone morphogenetic proteins

CAFs: Cancer-associated fibroblasts

CBP: CREB binding protein

CDDP: Cisplatin

CDKs: Cyclin dependent kinase

CGH: Comparative genomic hybridization

CHAPS: 3-(3-cholamidopropyl) dimethylammonio-1-propanesulfonate

CHK1: Checkpoint kinase 1

cIAP1: Cellular inhibitor of apoptosis protein-1

CMF: Cyclophosphamide, methotrexate, and 5-fluorouracil

COX-2: Cyclooxygenase-2

CREB: cAMP response element-binding protein

CSCs: cancer stem cells

CTCs: Circulating tumour cells

CTX: Cyclophosphamide

CV: Co-efficient of variation

D7-4G: breast cancer cells exposed to 4 Gy and kept for recovery period of 7 days

D7-6G: breast cancer cells exposed to 6 Gy and kept for recovery period of 7 days

DCIS: Ductal carcinoma in situ

DMEM: Dulbecco's Modified Eagle Medium

DMSO: Dimethyl sulfoxide

DSBs: Double strand breaks

ECAR: Extracellular acidification rate

EDTA: Ethylenediaminetetraacetic acid

EGFR: Epidermal growth factor receptors

ER: Estrogen Receptor

ELISA: Enzyme-linked immunosorbent assay

EMT: Epithelial–mesenchymal transition

ERCC1: Excision repair cross-complementation group 1

ERK: Extracellular-signal-regulated kinase

EpCAM: Epithelial cell adhesion molecule

ESI: Electrospray ionization

FA: Formic acid

FAK: Focal adhesion kinase

FBS: Fetal bovine serum

FCCP: Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone

FDA: Food and drug administration

FDR: False discovery rate

FEC50 or FEC100: Cyclophosphamide 50 or 100

FITC: Fluorescein isothiocyanate

FT: Fourier transform

FTICR: Fourier-transform ion cyclotron resonance

FWHM: Full width at half maximum

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GBM: Glioblastoma

GGI: Genomic Grade Index

GICs: Glioma-initiating cells

GITR: Glucocorticoid-induced tumor necrosis factor receptor

GO: Gene Ontology

GM-CSF: Macrophage colony stimulating factor

GPCR: G protein-coupled receptors

GRHL2: Grainyhead like transcription factor 2

GRP-78: Glucose-regulated protein

GSK: Glycogen synthase kinase

GST: Glutathione S-transferases

HA: Hyaluronic acid

HDI: Human development index

HER2: Human epidermal growth factor receptor-2

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HIF1α: Hypoxia-inducible factor 1-alpha

HMGA2: High-mobility group AT-hook 2

HPRD: Human protein reference database

HR: Homologous recombination

hTERT: Human telomerase reverse transcriptase

IAP: inhibitor of apoptosis proteins

ICOS: Inducible T-cell costimulator

IDC: Infiltrating ductal carcinoma

IGF-1R: Insulin-like growth factor 1 receptor

ILC: Infiltrating lobular carcinoma

IL-10: Interleukin 10

IPA: Ingenuity Pathway Analysis

iPLA2: Calcium-independent phospholipase A2

iPSCs: Induced pluripotent stem cells

IR: Ionizing radiation

iTRAQ: Isobaric tags for relative and absolute quantitation

JNK: c-Jun N-terminal kinase

KIRC: Kidney renal clear cell carcinoma

KLF4: Kruppel-like factor 4

LAML: Acute myeloid leukemia

LCIS: Lobular carcinoma in situ

LC-MS/MS: Liquid chromatography-tandem mass spectrometry

LDH5: Lactate dehydrogenase 5

LDHA: Lactate dehydrogenase A

LRP5/6: lipoprotein receptor-related protein 5 and 6

LUAD: Lung adenocarcinoma

LUSC: Lung squamous cell carcinoma

MAbs: Monoclonal antibodies

MALDI: Matrix-assisted laser desorption/ionization

MALDI-TOF-MS: Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

MAP: Mitogen-activated protein

MAPK: Mitogen-activated protein kinases

MCF UT: MCF-7 untreated cells

MCF D7-6G: MCF-7 cells exposed to 6 Gy and kept for recovery period of 7 days

MDSCs: Myeloid-derived suppressor cells

M-CSF: Macrophage colony stimulating factor

MEK: Mitogen-activated protein kinase kinase

MEL: Melphalan

MMP-9: Matrix metallopeptidase 9

MET: Mesenchymal–epithelial transition

mFISH: Multicolor fluorescence in situ hybridization

miRNAs: Micro RNAs

MMTS: Methyl methanethiosulfonate

MnSOD: Mn superoxide dismutase

MRM: Multiple reaction monitoring

MRP: Multidrug resistance associated protein

MS2: MS and MS/MS

MSCs: Mesenchymal stem cells

MTT: 3-(4, 5-dimethylthiazol-2-yl)- 2,5-diphenyl tetrazolium bromide

MUC1: Mucin 1

NF-kB: Nuclear factor kappa light chain enhancer of activated B cells

NHEJ: Non-homologous end joining

NK cells: Natural killer cells

NOX2: NADPH oxidase 2

NOX4: NADPH oxidase 4

NSCLC: Non-small cell lung cancer

OCR: Oxygen consumption rate

OCT-4: Octamer-binding transcription factor 4

OS: Overall survival

OV: Ovarian cancer

OVOL2: Ovo like zinc finger 2

PANTHER: Protein annotation through evolutionary relationship

PAM50: Predictor Analysis of Microarray 50

PARP: Poly (ADP-ribose) polymerase

PBI: Partial breast irradiation

PBS: Phosphate buffered saline

PBST: Phosphate buffered saline with tween 20

PD-1: Programmed cell death protein 1

PCR: Polymerase chain reaction

PGK1: Phosphoglycerate kinase 1

PI3K: Phosphatidylinositol 4, 5-bisphosphate 3-kinase

PI: Propidium iodide

PKCδ: Protein kinase C delta

PMRT: Postmastectomy radiotherapy

POD: Peroxidase

PR: Progesterone-receptor

RANKL: Receptor activator of nuclear factor kappa-B ligand

RT: Radiation therapy

ROR: Risk of Recurrence

SAv-HRP: Streptavidin, horseradish peroxidase conjugated

SCID: Severe combined immunodeficiency

SCX: Strong cation exchange

SDS: Sodium dodecyl sulphate

SELDI-TOF MS: Surface-enhanced laser desorption/ionization-TOF MS

SEM: standard error of mean

SERMS: Selective estrogen receptor modulator

SKY: Spectral karyotyping

SMAD: homologues of the Drosophila protein, mothers against decapentaplegic (Mad) and the *Caenorhabditis elegans* protein Sma

SNAI-1: Snail Family Transcriptional Repressor 1

SOX-2: Sex determining region Y- box 2

SP: Side population

SSA: Single-strand annealing

SSBs: Single strand breaks

SWATH-MS: Sequential window acquisition of all theoretical- mass spectra

TAAs: Tumor-associated antigens

TACAs: Tumor-associated carbohydrate antigens

TAMs: Tumor-associated macrophages

TCEP: Tris (2-carboxyethyl) phosphine

TCGA: The cancer genome atlas

TEAB: Triethylammonium bicarbonate buffer

TGF-β: transforming growth factor-beta

TGF-β RI: Transforming growth factor beta receptor I

TGF-β RII: Transforming growth factor beta receptor II

TMB: 3, 3', 5, 5'-Tetramethylbenzidine

TNBC: Triple-negative breast cancer

TNM: Tumor, node, metastasis

TNF-α: Tumor necrosis factor alpha

UNDP: united Nations development programme

uPAR: Urokinase-type plasminogen activator receptor

UT: Untreated

VEGF: Vascular endothelial growth factor

VCAM1: Vascular cell adhesion proteins

WBRT: Whole breast radiotherapy

WT1: Wilms tumor gene

WOWH: Wound-oncogene-wound healing

XIAP: X-linked inhibitor of apoptosis protein

ZEB-1: Zinc finger E-box binding homeobox 1

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6. Annexure

Name	Source	Catalogue number
2-(5-Bromo-2-pyridylazo)-5-(diethylamino) phenol (BP blue stain)	Sigma(St. Louis, MO, USA)	180017
2-Mercaptoethanol		M6250
Acetonitrile (ACN)		271004
Bovine Serum Albumin		A2153
Bradford reagent		B6916
Crystal Violet		C3886
Dimethylsulfoxide (DMSO)		D2650
Dithiothreitol (DTT)		D0632
Ethylenediaminetetraacetic acid (EDTA)		E6758
Propidium iodide (PI)		P4170
HRP chemiluminescent substrate		WBKLS0500
Hydrogen Peroxide Solution 30%w/v		18755
Non-fat milk	HiMedia (Mumbai, India)	M530

Recombinant Human IL-10	MiltenyiBiotec, BergischGladbach, Germany	4674484
Recombinant Human TNF-α		130-094-015
Protector RNase A inhibitor	Roche Applied Science, (Germany)	03335407001
SB431542	Santa Cruz Biotechnology (Santa Cruz, CA,USA)	204255
SDS	Sigma (St. Louis, MO, USA)	L3771
Sodium bicarbonate		S5761
SYBR green		S9430
TEMED		T9281
Thiourea		T8656
Thiazolyl Blue Tetrazolium Bromide (MTT)		M5655
Triton X 100		X100
Tween 20		P2287
Urea		U5378

Antibodies

Name	Source	Catalogue number
Alexa Fluor 488 Goat Anti- rabbit Antibody	BD Biosciences, San Jose, CA	BD555742
Anti-mouse FITC IgG2bk antibody		BD555742
Alexa Fluor 488 conjugated Anti-BrdU Antibody, clone BU-1, antibody	Sigma(St. Louis,MO, USA)	FCMAB101A4
E-Cadherin Antibody (G-10)	Santa Cruz Biotechnology (Santa Cruz, CA,USA)	SC 8426
Anti-vimentin antibody (clone RV202)		SC32322
CD24	BD Biosciences (Franklin	555428

	Lakes, NJ,USA)	
CD44		555478
Rabbit Anti-Human HMGA2 (C-term)	Pacific Science, Bangplad Bangkok	RBT-102-16480
SNAI1 Polyclonal Antibody	Elabsciences, Houston, Texas	E-AB-32931
Human IL-10 ELISA Set	BD Biosciences (Franklin Lakes, NJ,USA)	555142
Human TNF-α ELISA Set		557953
Human TGF-β 1 ELISA Set	Thermo Fisher Scientific (Waltham, MA, USA)	15531227
Human TGF-β 2 Quantikine ELISA kit	R&D Systems, (Minneapolis, Minnesota, USA)	DB250
Human TGF-β 3 ELISA Set		DY243
ZEB 1 Antibody	Novus	2A8A6

(2A8A6)	Biologicals,Centennial, Colorado	

Tissue culture reagents

Name	Source	Catalogue number
DMEM	HiMedia (Mumbai, India)	AL151A
Heat inactivated fetal bovine serum		RM9955-100ML
Penicillin-Streptomycin		P4333
RPMI-1640		AL060A
Trypsin-EDTA solution		T3924

Molecular biology reagents:

Name	Source	Catalogue number
cDNA synthesis kit	Roche Applied Science (Germany)	05081963001
LightCycler® 480 SYBR		04707516001

Green I Master		
Deoxynucleotides Set	5 Prime GmbH (Hilden, Deutschland)	2201230266
Perfect Pure RNA isolation Kit		2302340
Primers	Sigma (St. Louis, MO, USA)	
Taq polymerase	Invitrogen (Grand Island, NY, USA)	10342-053

Miscellaneous

Name	Source	Catalogue number
Cell culture inserts (8 µm)	BD Biosciences (Franklin Lakes, NJ, USA)	353097
5-Bromo-2′-deoxy-uridine Labeling and Detection Kit I	Roche Applied Science (Germany)	11296736001
Annexin V-FITC Apoptosis Detection Kit	Sigma (St. Louis, MO, USA)	APOAF-50TST

Buffers and solutions

- Phosphate Buffered Saline (PBS): 137mM NaCl (8 g), 10 mM Phosphate (Sodium hydrogen phosphate: 1.44 g and potassium dihydrogen phosphate: 0.24 g), 2.7 mM KCl (0.2 g) pH of 7.4.
- PBST: 0.05 % Tween 20 in PBS
- Flowcytometry: Propidium Iodide (PI) Solution: 50 µg/ml PI, 0.1% Triton-X 100 and 0.1% sodium citrate in dH2O
- Fixation buffer: 2 % paraformaldehyde in 1x PBS
- Permeabilization buffer : 0.1 % Triton X-100 in 1Xpbs
- Lysis buffer: 6 M urea, 2 M thiourea, 2% CHAPS, 0.5% SDS in HPLC grade water
- Dissolution Buffer: 1M triethylammonium bicarbonate (TEAB) + 6 M urea

ELISA

- Coating carbonate buffer: 0.15 M sodium carbonate, 0.35 M sodium bicarbonate, pH 9.6
- Phosphate Buffered Saline (PBS): 137 mM NaCl (8 g), 10 mM Phosphate (Sodium hydrogen phosphate: 1.44 g and potassium dihydrogen phosphate: 0.24 g), 2.7 mM KCl (0.2 g) pH of 7.4
- Blocking buffer: PBS, 1% BSA
- Wash solution: PBS, 0.05% Tween-20
- Dilution buffer: PBS, 0.05% Tween-20, 0.1% BSA
- Stop Solution: 2 N H_2SO_4