Response of Non-Small Cell Lung Cancer (NSCLC) Adenocarcinoma Harboring Different Epidermal Growth Factor Receptor (EGFR) Mutations to Ablative Radiation Therapy and Auranofin

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December 2019

A thesis submitted to McGill University in partial fulfilment of the requirements of the degree of Doctor of Philosophy

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Abstract

Lung cancer is the leading cause of cancer death accounting for approximately 26% of all cancer deaths in Canada. Lung cancer can be classified into small cell lung cancer (SCLC) or non-small cell lung cancer (NSCLC). For early stage NSCLC patients who are not eligible for surgery, stereotactic ablative radiation therapy (SABR) serves as an alternative treatment. It is estimated that 15% of adenocarcinoma NSCLC patient carry active mutations in the tyrosine kinase domain (TKD) of epidermal growth factor receptor (EGFR). This frequency can reach up to 62% in non-smokers and Asian NSCLC patients. The most common EGFR mutations are frameshift deletion in exon 19 or a substitution of the leucine amino acid to arginine at codon 858 of exon 21 (L858R). Despite advances in surgery and SBRT, locoregional recurrence and distant metastasis remain as some of the major challenges in treating early stage NSCLC. Following SABR treatment, distant metastasis has been reported to occur as high as 30% in treated patients which can lead to death.

In our study, we investigated the response of isogenic EGFR-mutant NSCLC to SABR and if certain EGFR mutations exhibit better response to SABR compared to others. We also sought to investigate the potential of auranofin (AF), an anti-rheumatoid drug, as a radiosensitizer in radioresistant NSCLC cell lines. To achieve our first objective, our laboratory has used previously developed isogenic-EGFR mutant NSCLC cell lines by stably transfecting A549 cell with constructs carrying different EGFR: wildtype-EGFR (WT), DEL-EGFR (DEL) or L858R-EGFR (L858R). The produced cells were used *in vitro* and *in vivo* to assess their response to SABR. *In vitro* results demonstrated similar pattern in the response to ablative radiation between three cell lines where all cell lines had reduced colony formation, decreased number of viable cells, and increased G2-phase post-treatment (12 or 34Gy). Tumor formation was observed in cells pre-irradiated with a single ablative dose (34Gy) and injected subcutaneously into YFP/SCID mice.

However, cell with DEL-EGFR mutation failed in developing tumor compared to WT-EGFR and L858R-EGFR. To assess response to SABR as a treatment, isogenic EGFR-mutant cells were injected subcutaneously into YFP/SCID mice. Once tumor is formed, animals were selected to be sham-treated (control) or treated with a single fraction of 34Gy. Subcutaneous DEL-EGFR xenograft tumors had a significant decrease in tumor volume nine days post-SABR treatment compared to WT-EGFR and L858R-EGFR xenografts. Furthermore, histological examination on collected xenografts from treated and control groups revealed a significant decrease in apoptotic bodies in DEL-EGFR tumors treated with 34 Gy compared to sham-treated. We were able to report for the first time that the type of EGFR mutations can have an impact on the response to SABR, with DEL-EGFR mutation conveying a better response to SABR compared to WT-EGFR or L858R-EGFR.

To investigate auranofin as a potential radiosensitizer in NSCLC harboring different EGFR status, we used NSCLC established cell lines harboring different EGFR status: WT-EGFR cell lines (**A549 and H460**), EGFR-mutant cell line with deletion mutation (**PC9**), and EGFR-mutant cell line with the acquired resistance mutation T790M (**H1975**). Our results reported a robust effect using auranofin not only in WT-EGFR NSCLC, but also in NSCLC with the acquired resistance mutation to TKIs. EGFR-mutant NSCLC cell lines exhibited much more sensitive profile to auranofin compared to WT-EGFR cell lines.

In our study we reported that response to SABR could be driven by the type of EGFR mutation expressed in the cells with DEL-EGFR mutation exhibiting better response compared to WT-EGFR and L858R-EGFR. We also demonstrated the efficacy of using auranofin to potentiate the effect of radiation in NSCLC cell lines harboring different EGFR status which could be promising at the event of resistance secondary to treatment.

Résumé

Le cancer du poumon est la principale cause de décès dû au cancer et représente environ 26% de tous les décès par le cancer au Canada. Le cancer du poumon peut être classé en cancer du poumon à petites cellules (CPPC) ou cancer du poumon non à petites cellules (CPNPC). La radiothérapie ablative stéréotaxique (SABR) sert de traitement alternatif aux patients de stade précoce de CPNPC qui ne sont pas admissibles à la chirurgie. On estime que 15% des patients atteints de CPNPC à adénocarcinome (ADC) sont porteurs d'une mutation active dans le TKD de l'EGFR. Cette fréquence peut atteindre jusqu'à 62% chez les non-fumeurs et les patients CPNPC asiatiques. Les mutations les plus courantes de l'EGFR sont le décalage du cadre de lecture par délétion dans l'exon 19 ou une substitution de l'acide aminé leucine en arginine au codon 858 de l'exon 21 (L858R). Malgré les progrès de la chirurgie et de la SABR, la récurrence locorégionale et les métastases à distance restent parmi les principaux défis du traitement du CPNPC au stade précoce. À la suite du traitement par SABR, des métastases à distance ont été rapportées à une fréquence aussi élevée que 30% chez les patients traités, pouvant entraîner la mort.

Dans notre étude, nous avons étudié la réponse à la SABR des CPNPC isogéniques mutants pour EGFR afin de voir si certaines mutations d'EGFR présentent une meilleure réponse à la SABR comparées à d'autres. Nous avons également voulu étudier l'effet de l'utilisation d'auranofin (AF), un médicament anti-rhumatoïde, en tant que radiosensibilisateur dans des lignées cellulaires CPNPC radiorésistantes. Pour atteindre le premier objectif, notre laboratoire a utilisé des lignées cellulaires CPNPC isogéniques mutantes pour EGFR, et déjà générées par transfection stable de la lignée A549 avec des constructions de statuts EGFR différents: EGFR de type sauvage (WT), DEL-EGFR (DEL) ou L858R-EGFR (L858R). Les cellules ainsi produites ont été utilisées *in vitro* et *in vivo* pour évaluer leur réponse à la SABR. Les résultats *in vitro* ont démontré une tendance

similaire dans la réponse à la radiation ablative entre les trois lignées cellulaires, où toutes les lignées ont présenté une réduction de la formation de colonies et du nombre de cellules viables, et une augmentation de la phase G2 après le traitement (12 ou 34Gy). La formation de tumeurs a été observée dans les cellules pré-irradiées avec une seule dose ablative (34Gy) avant leur injection sous-cutanée à des souris YFP-SCID. Cependant, la lignée avec la mutation DEL-EGFR a omis de développer des tumeurs par rapport à WT-EGFR et L858R-EGFR. Pour évaluer la réponse à la SABR en tant que traitement, les lignées cellulaires isogéniques mutantes pour EGFR ont été injectées par voie sous-cutanée dans des souris YFP/SCID. Une fois les tumeurs formées, des animaux ont été sélectionnés soit pour simuler le traitement ou pour être traités avec une seule fraction de 34Gy. Les tumeurs sous-cutanées de xénogreffes DEL-EGFR ont présenté une diminution significative du volume tumoral neuf jours après le traitement par SABR par rapport aux xénogreffes WT-EGFR et L858R-EGFR. En outre, l'examen histologique des xénogreffes collectées à partir des groupes traités et témoins a révélé une diminution significative du nombre de corps apoptotiques dans les tumeurs DEL-EGFR traitées avec 34 Gy par rapport à celles traitées avec le traitement simulé. Nous avons pu signaler pour la première fois que les mutations d'EGFR peuvent avoir un impact sur la réponse à la SABR, la mutation DEL-EGFR traduisant une meilleure réponse à la SABR par rapport à WT-EGFR ou L858R-EGFR.

Afin d'étudier l'auranofin en tant que radiosensibilisateur potentiel dans le CPNPC porteur de différents statuts EGFR, nous avons utilisé des lignées cellulaires établies de CPNPC avec différents statuts EGFR: des lignées WT-EGFR (**A549 et H460**), une lignée cellulaire mutante pour EGFR avec une mutation de délétion (**PC9**), et une lignée cellulaire mutante pour EGFR avec la mutation de résistance acquise T790M (**H1975**). Nos résultats ont rapporté un effet robuste en utilisant l'auranofin non seulement dans le CPNPC WT-EGFR, mais également dans le CPNPC

avec la mutation de résistance acquise aux ITK. Les lignées cellulaires CPNPC mutantes pour EGFR ont présenté un profil beaucoup plus sensible à l'auranofin en comparaison aux lignées WT-EGFR.

Dans notre étude, nous avons rapporté que la réponse à la SABR pourrait être fonction du type de mutation de l'EGFR exprimé dans les cellules, avec la mutation DEL-EGFR présentant une meilleure réponse que celle de WT-EGFR et de L858R-EGFR. Nous avons également démontré l'efficacité de l'utilisation d'auranofin pour potentialiser l'effet du rayonnement dans des lignées cellulaires CPNPC portant différents statuts d'EGFR, ce qui pourrait être prometteur en cas de résistance secondaire au traitement.

Acknowledgment

I would to express my sincere gratitude and appreciation to my supervisor Dr. Bassam Abdulkarim whom without his support this thesis would have not seen the light. I cannot thank enough Dr. Abdulkarim for giving me the opportunity to join his translational oncology team and get introduced to the field of oncology. I am very thankful for his help and support and for believing in me and giving me the chance to grow as an independent scientist.

I would like to thank my co-supervisor, Dr. Lisbet Haglund, for her support. I would also like to express my appreciation to the members of my Research Advisory Committee: Dr. Jacques Lapointe, Dr. Francis Rodier, and Dr. Bertrand Jean-Claude. I would also like to thank Dr. Siham Sabri for providing me with her advice and guidance during my PhD. I would like to thank the Faculty of Medicine for their financial support throughout my PhD.

Words are not enough to thank my colleagues and my lab members. I consider myself not only lucky, but also blessed to have you as my friends. Gloria, Nadim, Michele, Zeinab, Rehka, Paul, and Jad …you guys are the best! We laughed together, we cried together, we went through a lot together just like a family, and you guys are indeed my second family. Brian, you were always there for me and you took care of me as one of your lab members, saying “thank you” is not enough!

I would like to thank my parents, Sawsan Al-Mot and Abdullah Al Rabea, for their support, for always being there during my hardships, for believing in me and whom without their support I would not have been able to achieve my dream of pursuing a Ph.D. degree. I love you both beyond any words and I am grateful to have you as my parents. I would also like to thank my brother Hassan Al Rabea, and my sister Tala Al Rabea. Tala, you once wrote my name as one of your stars

during one of your school events. Well, it is my turn now to write both of your names as my shining stars that light up my sky. I am very grateful for your love, support and for always believing in me. This achievement could not have been possible without you. I love you both and I am blessed to have you in my life. You both truly make your older sister very proud!

I am forever in dept to my friends Lina, Lama, Wid, Yusur and Lima. I am very lucky to have you girls in my life. You always had your hearts and homes open for me. You are my support system. Ladies words fail me in expressing my gratitude to your support, I hope that you can count on me as I will always count on you. Thank you for being there for me.

Dedicated to my mother, Sawsan Al-Mot,

because no one other than you deserve it.

Contribution to original knowledge

The work presented in this thesis tried to highlight the impact of driver mutations in non-small cell lung cancer (NSCLC), such as epidermal growth facto receptor (EGFR) mutations, in the response to stereotactic ablative radiation. Data on the outcome of using SABR in NSCLC are very limited and do not provide enough details due to it relatively recent application as a treatment in lung cancer compared to other well investigated treatments (surgery or chemotherapy). In **Chapter 2**, we have used isogenic EGFR-mutant NSCLC to investigate their response to ablative radiation *in vitro* and *in vivo*. This has led to successfully demonstrate differential response to ablative radiation between EGFR subtypes. Our *in vitro* analyses revealed that isogenic EGFR-mutant exhibit similar patter in their response to ablative radiation. *In vivo*, we reported that NSCLC with deletion in the exon 19 of the tyrosine kinase domain (denoted as DEL-EGFR in our study) to exhibit good response to ablative radiation compared to wild-type EGFR NSCLC, or NSCLC with L588R substitution. Our assumption was supported by the inability to develop tumor of pre-irradiated DEL-EGFR when injected subcutaneously. However, pre-irradiated WT-EGFR or L858R-EGFR have successfully formed tumor when injected subcutaneously. Moreover, DEL-EGFR xenografts demonstrated a decrease in tumor volume nine days post-SABR treatment, while xenografts of WT-EGFR and L858R-EGFR had an increase in tumor volume nine days post-treatment. These data confirm that although EGFR-mutant cell lines may exhibit similar responses to radiation *in vitro*, this is not the case *in vivo* which may also reflect SABR outcome in clinical use.

The combination of TKIs and radiation has been used in clinical practice, however; high toxicity has led to limiting its use. EGFR-mutant NSCLC are known to exhibit sensitivity to TKIs and radiation, however; resistance to treatment is predicted to occur following treatment. This raises the question if a treatment can overcome resistance to treatment such as the case of EGFR-mutant

with T790M mutation or NSCLC with wild-type EGFR. In **Chapter 3**, we propose the idea of repurposing the use of auranofin, an anti-rheumatic agent, to overcome resistance in NSCLC cell lines. We reported the efficacy of auranofin in inhibiting tumor formation in H1975 (EGFR-mutant with T790M mutation) and was able to radiosensitize A549 and H460 cell lines (WT-EGFR NSCLC) to radiation. Moreover, combination of auranofin with radiation has led to increased levels of reactive oxygen species (ROS), where ROS is knowns to cause DNA damage which results in cell death. Given that auranofin has been approved to be used clinically, combination of its use with radiation can serve as a therapeutic alternative to overcome resistance to treatment in NSCLC patients.

This thesis has identified DEL-EGFR to exhibit potential good response to SABR when compared to other EGFR status. In addition, this thesis has suggested auranofin as potential therapeutic agent where it has enhanced antitumorigenic effect and increased sensitivity to radiation in NSCLC cells lines.

Contribution to knowledge:

1. Manuscript "**Response of non-small cell lung cancer (NSCLC) harboring different epidermal growth factor receptor mutations to ablative radiotherapy.**" In the process of re-submission.

2. Manuscript "**Auranofin enhances ionizing radiation effect in non-small cell lung cancer (NSCLC) with different epidermal growth factor receptor status**" is in the process of completion for submission.

Contribution of authors

Unless otherwise stated below, I have performed all experiments, designed and coordinated experiments that were not directly conducted by myself, performed of the statistical analysis of generated results, and wrote the manuscripts that constitute this thesis with guidance provided by my supervisor Dr. Bassam Abdulkarim.

Chapter 2 is reproduced from my manuscript "Response of Non-Small Cell Lung Cancer (NSCLC) Harboring Different EGFR Mutations to Ablative Radiotherapy", which is being finalized for submission to the International Journal of Radiation Oncology, Biology, Physics. In this manuscript, Dr. Paul Daniel has helped in the radiation treatment plan and animal treatment with ablative radiation. Mr. Brian Meehan has helped in the subcutaneous injection of the animals and helped in tumor measurement as a blinded scientist to animal groups. Dr. Sophie Camilleri-Broët is a lung pathologist who has helped in the histological scoring of collected tumor while being blinded to the group details. Dr. Janus Rak has helped in providing the YFP/SCID mice to carry *in vivo* experiments. Dr. Chaitanya Nirodi has provided us with construct with different EGFR mutation to generate the isogenic EGFR-mutant NSCLC. Dr. Kolja Eppert has provided us with BFP-Luc viral vector. Dr. Ayman Oweida has helped in designing the treatment plan using ablative radiation. Dr. Siham Sabri and Dr. Bertrand Jean-Claude has helped in reviewing and editing the paper. Dr. Bassam Abulkarim provided support for study design, data analysis, and manuscript review and approval. He has also provided guidance, funding and supervision throughout all carried studies.

Chapter 3 is reproduced from my manuscript "Auranofin causes as radiosensitization effect in non-small cell lung cancer (NSCLC) with wild type-epidermal growth factor receptor (EGFR) or with T790M acquired resistance mutation to tyrosine kinase inhibitor", which is being finalized

for submission to Frontiers in Oncology Journal. In this manuscript Ms. Rehka Whyshnavy Raveendrakumar has helped in the optimization process of the reactive oxygen species (ROS) experiment. Dr. Paul Daniel has helped in the *in-silico* analysis. Dr. Siham Sabri has helped in providing auranofin and necessary materials to carry out ROS experiments. Dr. Bassam Abulkarim provided support for study design, data analysis, and manuscript review and approval. He has also provided guidance, funding and supervision throughout all carried studies.

Other Contributions

1. Oweida A, Sabri S, **Al-Rabea A**, Ebrahimi M, Ruo R, Fraser R, et al. Response to stereotactic ablative radiotherapy in a novel orthotopic model of non-small cell lung cancer. Oncotarget. 2018;9(2):1630-40.

2. Bhosle VK, Rivera JC, Zhou TE, Omri S, Sanchez M, Hamel D, Zhu T, Rouget R, **Rabea A**, et al. Erratum: Nuclear localization of platelet-activating factor receptor controls retinal neovascularization. Cell Discov. 2016;2:16034.

3. Bhosle VK, Rivera JC, Zhou TE, Omri S, Sanchez M, Hamel D, Zhu T, Rouget R, **Rabea A**, et al. Nuclear localization of platelet-activating factor receptor controls retinal neovascularization. Cell Discov. 2016;2:16017.

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List of abbreviations

3D-CRT: Three-dimensional conformal radiation therapy

3D-CT: Three-dimensional computed tomography

4DCT: Four-dimensional computed tomography

ACOSOG: American College of Surgeons Oncology Group

ADC: Adenocarcinoma

AF: Auranofin

AIR: Ablative ionizing radiation

AKT: RAC-alpha serine/threonine-protein kinase

ALK: Anaplastic lymphoma kinase

ALT: Alanine aminotransferase

APCs: Antigen presenting cells

AR: Amphiregulin

Asmase: acid sphingomyelinase

ATM: Ataxia telangiectasia mutated

ATP: Adenosine triphosphate

ATR: ATM- and Rad3-related

BER: Base excision repair

BFP: Blue fluorescent protein

BLI: Bioluminescence imaging

BSA: Bovine serum albumin

BTK: Bruton tyrosine kinase

CAF: cancer associated fibroblasts

CBCT: cone-beam computed tomography

CD25: Cell division cycle 25

CDK: Cyclin-dependent kinase

CHK: checkpoint kinases

CM-H2DCFA: (5-(and 6-)-chloromethyl-2',7'-dichlorodihydrofluoresceine diacetate

CR: Complete response

CRT: Chemo-radiotherapy

CSCs: Cancer stem cells

CT: Contrast-enhanced computed tomography

CTLA4: cytotoxic T lymphocyte-associated antigen 4

DAMP: Damage associated molecular patterns

DC: Dendritic cells

DDR: DNA damage response

DFS: Disease-free survival

DNA-PK: DNA-dependent protein kinase

DSB: Double-strand breaks

EGF: Epidermal growth factor

EGFR: Epidermal growth factor receptor

ERK: Extracellular signal-regulated kinase

ES-NSCLC: Early stage non-small cell lung cancer

FBS: Fetal bovine serum

FDA: Food and drug administration

FGFR: Fibroblast growth factor receptor

FLT3L: FMS-like tyrosine kinase receptor 4 ligand

GR: Glutathione reductase

Grx: Glutaredoxins

GSH: Glutathione

Gy: Gray

H&E: Haemotoxylin and Eosin

H_2O_2: Hydrogen peroxide

HDAC: Histone deacetylase

HER: Human epidermal factor receptor

HIF-1: hypoxia-inducible factor-1

HMGB1: High-motility group box 1

HR: hazard ratio

HR: Homologous recombination

HSP 70: Heat shock protein 70

HSP-90: Heat shock protein-90

ICD: Immunogenic cell death

ICRP: International Commission on Radiological Protection

IGF: Insulin growth factor

IGRT: Image-guided radiation therapy

IL6R: Interleukin 6 receptor

IMRT: Intensity modulated radiation therapy

INF-α: Interferon-alpha

INF-β: interferon beta

INF-γ: Interferon gamma

IO: Immunotherapy

IR: Ionization radiation

KRAS: Kirsten rat sarcoma

LADC: Lung adenocarcinoma

LC: Lung cancer

MAPK: Mitogen-activated protein kinase

MMR: Mismatch repair

mTOR: mechanistic target of rapamycin

MTT: 3-[4,5-dimethylthiazol-2yl]-2,5diphenyltetrazoliumbromide

NADPH: Nicotine adenine dinucleotide phosphate

NER: Nucleotide excision repair

NER: Nucleotide excision repair

NHEJ: nonhomologous end-joining

NRG1: Neuregulin 1

NSCLC: Non-small cell lung cancer

NSCLCs-NOS: Non-small cell lung cancers-not otherwise specified

NTRK1: Neurotrophic tyrosine kinase receptor 1

OER: Oxygen enhancement ratio

OS: Overall survival

PAMP: Pathogen-associated molecular patterns

PARP: poly ADP ribose polymerase

PCR: Polymerase chain reaction

PD: Disease progression

PDL1: programmed cell death ligand 1

p-EGFR: phospho-epidermal growth factor receptor

p-ERK: phosphor- Extracellular signal-regulated kinase

PET/CT: positron emission tomography/computed tomography

PET: Positron emission tomography

PFS: Progression-free survival

PI3K: phosphatidylinositol-3 kinase

PIKKs: phosphoinositide 3-kinase related kinases

PLK1: Polo-like kinase 1

PR: Partial response

PRDX2: Peroxiredoxin2 oligomers

PRR: Pathogen recognition receptor

PTB: phosphotyrosine binding

PTEN: Phosphatase and Tensin Homolog

QL: Quadratic-linear

RNS: Reactive nitrogen species

ROS: Reactive oxygen species

RR: Response rate

RT: Radiation therapy

RTK: Receptor tyrosine kinases

SABR: Stereotactic ablative radiation therapy

SBRT: Stereotactic body radiation therapy

SCC: Squamous cell carcinoma

SCLC: Small cell lung cancer

SDS-PAGE: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

SF: Survival fraction

SF2: Survival fraction at 2 Gy

SH2: Src homology 2

SQC: Squamous cell carcinoma

SSBR: Single strand break repair

SSBs: Single-strand breaks

STAT: Signal transducer and activators of transcription

SUV: Standardized uptake value

T_{reg}: T cells with regulatory function

TAA: Tumor-associated antigens

TCGA: The Cancer Genome Atlas

TGF-α: Transforming growth factor-alpha

TGF-β: Transforming growth factor-beta

Thr: Threonine

TIL: Tumor infiltrating lymphocytes

TKD: Tyrosine kinase domain

TKIs: Tyrosine kinase inhibitors

TMZ: Temozolomide

TNM: Tumor, Node, Metastasis

total-EGFR: Total epidermal growth factor receptor

total-ERK: total-extracellular signal-regulated kinase

Trx: Thioredoxin

TrxR: Thioredoxin reductase

TSR: Tumor stroma ratio

TTF-1: Thyroid transcription factor-1

Tyr: Tyrosine

VEGF: Vascular endothelial growth factor

VEGFR: Vascular endothelial growth factor receptor

WHO: World Health Organization

WT: Wild-type

YFP/SCID: Yellow fluorescent protein/ severe combined immunodeficient

γ-H2AX: Phosphorylated Histone 2AX

Chapter 1: Introduction and Literature Review

1.1 Lung Cancer

Lung cancer remains as the leading cause of death in both men and women worldwide (**Figure 1.1**) [1] with a 5-year survival rate of 15.6% [2, 3] after diagnosis, which is lower compared to the survival rate of breast, colon or prostate cancer [2, 3]. In 1912, inhalation of cigarette smoking has been suggested to be the cause of lung cancer [4]. An investigation on the impact of smoking has been initiated where in the mid-1960s the US Surgeon General has reported tobacco smoking as a direct cause of lung cancer [5]. Second-hand smoking is also considered as an important factor in lung cancer [6]. Approximately, 95 percent of lung cancer can be classified into non-small cell lung cancer (NSCLC) or small cell lung cancer (SCLC) [7]. This classification of lung cancer has a major impact in providing the necessary information for proper staging, treatment, and prognosis.

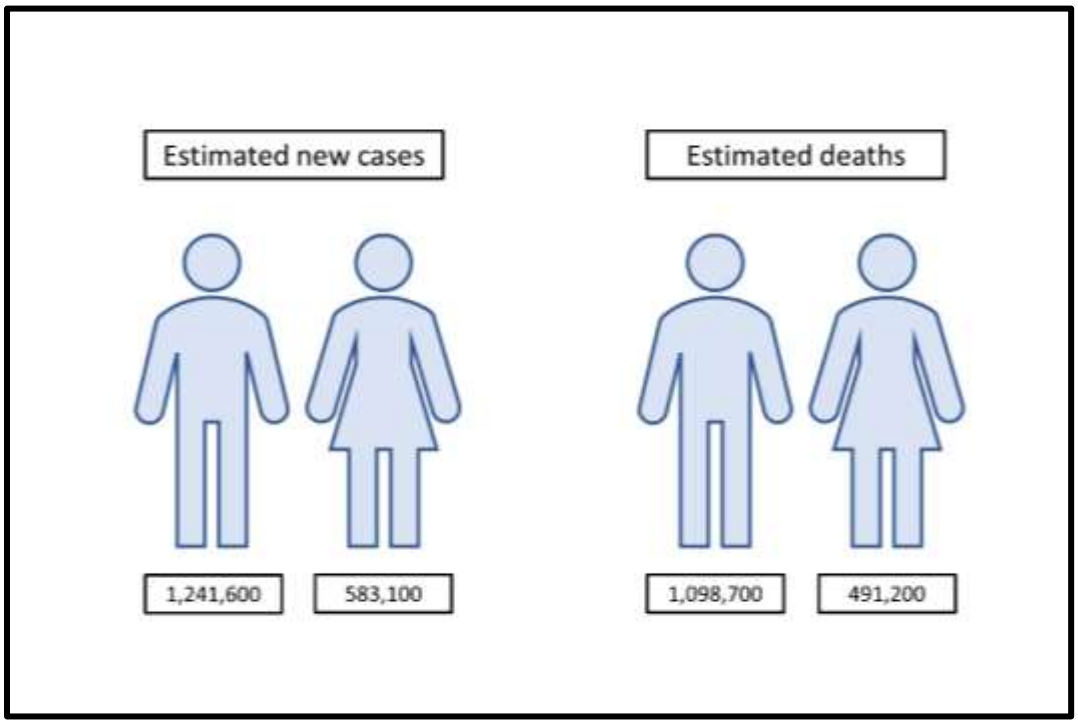

Figure 1.1: Estimated number of new lung cancer cases and deaths caused by lung cancer in males and females.

1.1.1 Diagnosis and staging of Lung Cancer

Several factors are implicated in the choice of treatment for each patient suspected to have lung cancer. Some of these factors are: age, sex, and family history. Initial evaluation and screening for lung cancer include: clinical evaluation of signs and symptoms; radiographic imaging to elucidate the cause of clinical manifestations and to confirm the absence/presence of tumor; and laboratory analyses to screen for abnormalities.

Clinical signs or symptoms presented in lung cancer patients are usually an indication that the disease has reached an advanced stage [8]. The most common clinical signs and symptoms are: cough with 50-75%, hemoptysis with 25-50%, dyspnea with 25% and chest pain with 20% [9-12]. The possibility of lung cancer should always be suspected in current or former smokers with new onset cough or hemoptysis. It is worth mentioning, differentiation between NSCLC and SCLC cannot be easily achieved based on few clinical features and therefore, further assessments need to be considered.

Radiographic imaging provides details that are essential in lung cancer staging and treatment, and usually is one of the initial steps in the diagnosis of lung cancer. Contrast-enhanced computed tomography (CT) in combination with positron emission tomography (PET)/ or alone is usually performed on patients suspected with NSCLC [13-15]. For patients suspected with NSCLC it is recommended that they undergo the following: chest imaging to confirm tumor presence, imaging of the upper abdomen area of liver and adrenal gland to screen for metastasis (or primary site in the case of lung-metastasis), and imaging of potential targeted metastasis sites when symptoms or important findings are present or when CT scan images indicate an advanced stage of the disease [13, 16].

Laboratory assessments are usually done following chest imaging when lung cancer is suspected. These assessments look at complete blood count, electrolytes, calcium, alkaline phosphatase, alanine aminotransferase (ALT), aspartate aminotransferase, creatinine, albumin and lactate dehydrogenase [15, 17]. Furthermore, obtaining a cytopathological or histopathological biopsies, with preference to histopathological specimen, for microscopic examination is crucial in diagnosis and staging of lung cancer.

1.1.2 Staging of Non-Small Cell Lung Cancer

Staging of NSCLC lung cancer relies on the Tumor, Node, Metastasis (TNM) system which is accepted and used internationally for disease characterization. The use of the TNM system allows for accessibility to tumor description and information that can be easily communicated between health care professionals to decide on the best approach to treat the targeted tumor. The way in which the TNM system works is by combining tumor characteristics into stage groups of the disease and correlating it with the survival rate to decide on the recommended treatment. The eighth edition of the updated TNM system has been in effect in the United States and elsewhere starting January 2018 where it replaced the seventh edition of the TNM staging system. The new eighth edition is summarized in **Table 1.1**.

	Overall stage			
8Th edition T/M	**N0**	**N1**	**N2**	**N3**
T1a (≤1 cm)	**IA1** (IA)	**IIB** (IIA)	IIIA	IIIB
T1b (>1 to 2cm)	**IA2** (IA)	**IIB** (IIA)	IIIA	IIIB
T1c (>2 to 3 cm)	**IA3** (IA)	**IIB** (IIA)	IIIA	IIIB
T2a (> 3 to 4 cm)	IB	**IIB** (IIA)	IIIA	IIIB
T2b (>4 to 5 cm)	**IIA** (IB)	**IIB** (IIA)	IIIA	IIIB
T3 (>5 to 7 cm)	**IIB** (IIA)	**IIIA** (IIB)	**IIIB** (IIIA)	**IIIC** (IIIB)
T3 (structures)	IIB	IIIA	**IIIB** (IIIA)	**IIIC** (IIIB)
T4 (>7cm)	**IIIA** (IIB)	IIIA	**IIIB** (IIIA)	**IIIC** (IIIB)
T4 (diaphragm)	**IIIA** (IIB)	IIIA	**IIIB** (IIIA)	**IIIC** (IIIB)
T2a endobronchial: location/ atelectasis 3 to 4 cm	**IB** (IIB)	**IIB** (IIIA)	IIIA	IIIB
T2b endobronchial: location/atelectasis 4 to 5 cm	**IIA** (IIB)	**IIB** (IIIA)	IIIA	IIIB
T4	IIIA	IIIA	IIIB	**IIIC** (IIIB)
M1a	**IVA** (IV)	**IVA** (IV)	**IVA** (IV)	**IVA** (IV)
M1b	**IVA** (IV)	**IVA** (IV)	**IVA** (IV)	**IVA** (IV)
M1c	**IVB** (IV)	**IVB** (IV)	**IVB** (IV)	**IVB** (IV)

Table 1.1: The eighth edition of T, N and M classification stage grouping for lung cancer. Adapted from [18] with modifications.

1.1.3 Histopathology of Non-Small Cell Lung Cancer

In 2015, the World Health Organization (WHO) classification of lung tumor provided modified foundations on which lung tumors should be classified. The classification indicates that lung

tumors can be classified into the following: adenocarcinoma, adenosquamous carcinoma, squamous cell carcinoma, large cell carcinoma, sarcomatoid carcinoma, and neuroendocrine tumors (**Table 1.2**).

Tumor type	Changes from 2004 WHO classification	2015 variants (if applicable)
Adenocarcinoma	▪ Discontinuation of terms bronchioalveolar carcinoma, mixed subtype adenocarcinoma, clear cell and signet ring adenocarcinoma (these subtypes are descriptive features in the 2015 system), and mucinous cystadenocarcinoma (these tumors are reclassified as colloid adenocarcinomas). ▪ Addition of adenocarcinoma in situ and minimally invasive adenocarcinoma. ▪ Classification of invasive adenocarcinoma according to predominant subtype. ▪ Introduction of the term lepidic for a noninvasive component of an invasive adenocarcinoma.	▪ Lepidic ▪ Acinar ▪ Papillary ▪ Micropapillary ▪ Solid ▪ Invasive mucinous (mixed invasive mucinous and non-mucinous) ▪ Colloid ▪ Fetal ▪ Enteric ▪ Minimally invasive ▪ Preinvasive (atypical adenomatous hyperplasia, adenocarcinoma in situ [non-mucinous and mucinous])
Adenosquamous cell carcinoma	▪ No significant changes.	N/A
Squamous cell carcinoma	▪ Papillary, clear cell, small cell, and basaloid carcinoma have been replaced. ▪ Clear cell change is now regarded as a cytologic feature.	▪ Keratinizing ▪ Non-keratinizing ▪ Basaloid
Large cell carcinoma	▪ The following have been reclassified from "large cell carcinomas" to other subgroups: basaloid carcinoma is a subgroup of squamous cell carcinomas, large cell neuroendocrine carcinoma is a neuroendocrine carcinoma, and lymphoepithelioma-like carcinoma belongs to "other and unclassified carcinomas." ▪ Clear cell and rhabdoid are now descriptive features rather than subtypes.	N/A
Sarcomatoid carcinoma	▪ No significant changes, although molecular testing now recommended.	▪ Pleomorphic carcinoma ▪ Spindle cell carcinoma ▪ Giant cell carcinoma ▪ Carcinosarcoma ▪ Pulmonary blastoma
Neuroendocrine carcinoma	▪ Previously, small cell and large cell neuroendocrine tumors were in different categories and are now grouped together.	▪ Small cell carcinoma ▪ Large cell neuroendocrine carcinoma ▪ Carcinoid (typical and atypical)
Diffuse idiopathic pulmonary neuroendocrine cell hyperplasia	▪ No significant changes.	N/A

Table 1.2: 2015 updated WHO classification of invasive malignant epithelial lung tumors compared to 2004 WHO classification. adapted from [19-21].

Differentiation between NSCLC and SCLC is made based on the absence of SCLC pathological features such as: small cell size, nuclear molding, “salt and pepper” chromatin pattern, and nuclear crush artifact [22]. Histologically, NSCLC can be classified into adenocarcinoma, squamous cell carcinoma and large cell carcinoma. Immunohistochemical (IHC) staining using different markers can help be in the classification. For example, adenocarcinoma has a positive staining of thyroid transcription factor-1 (TTF-1), CK7, mucin, napsin-A. Squamous cell carcinoma has a positive staining for p63, and cytokeratin 5/6 (CK 5/6). Large cell carcinoma or adenosquamous carcinoma may have a combination staining positive for both adenocarcinoma and squamous carcinoma stains (**Figure 1.2**).

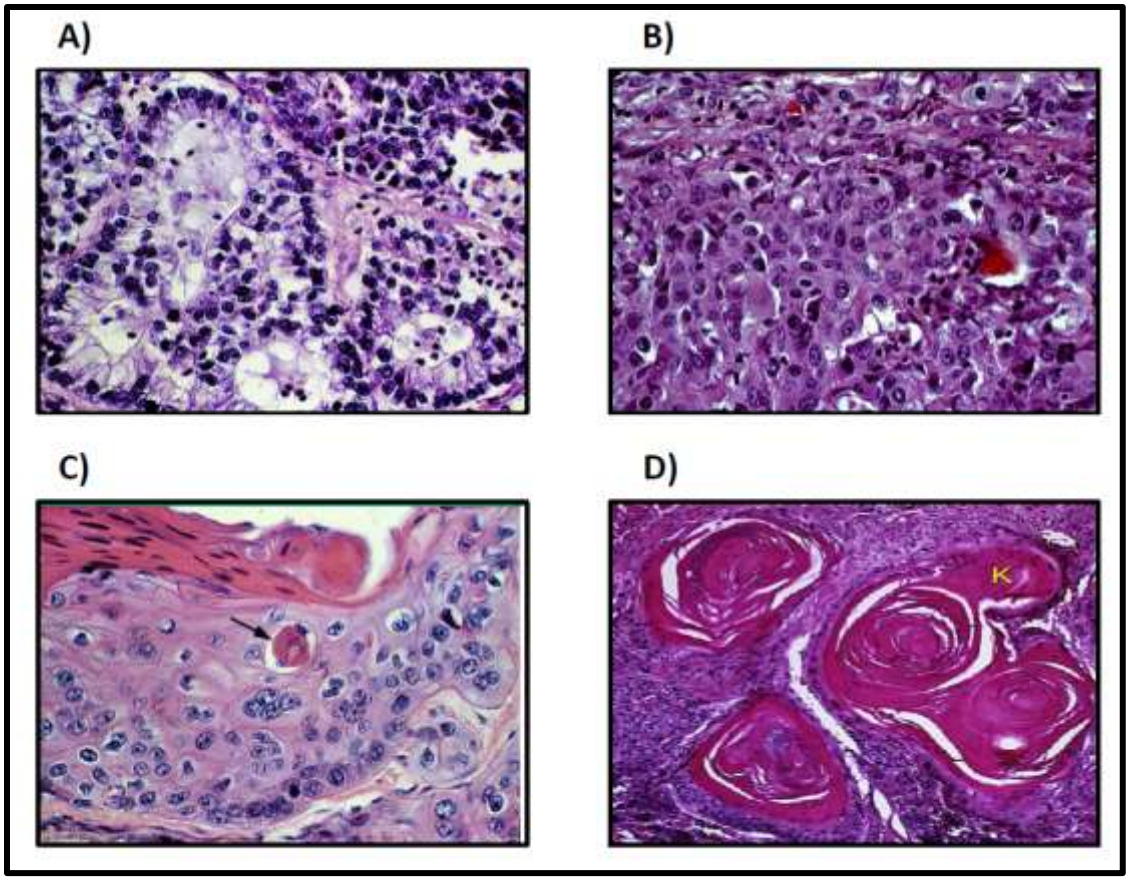

Figure 1.2: Histological representation of NSCLC. A) and B) photomicrograph of lung adenocarcinoma. C) and D) photomicrograph of lung squamous cell carcinoma with keratinization denoted with K in D). Adapted from [22].

1.2 Molecular Drivers in Non-Small Cell Lung Cancer

Oncogene activation plays a crucial role in the development of NSCLC, particularly in lung adenocarcinoma (LADC), where activated genes are called oncogene drivers [23-25]. In LADC, the most well identified oncogene drivers are: *EGFR*, *KRAS*, *BRAF* and *HER2*/*ERBB2* and they are activated by the presence of a point mutation and/or insertion/deletion mutations. In the case

of *ALK* gene, it is activated through fusion with other genes that can be called "partner genes". The percentage of these oncogene drivers to occur in different ethnicities is summarized in **Figure 1.3**.

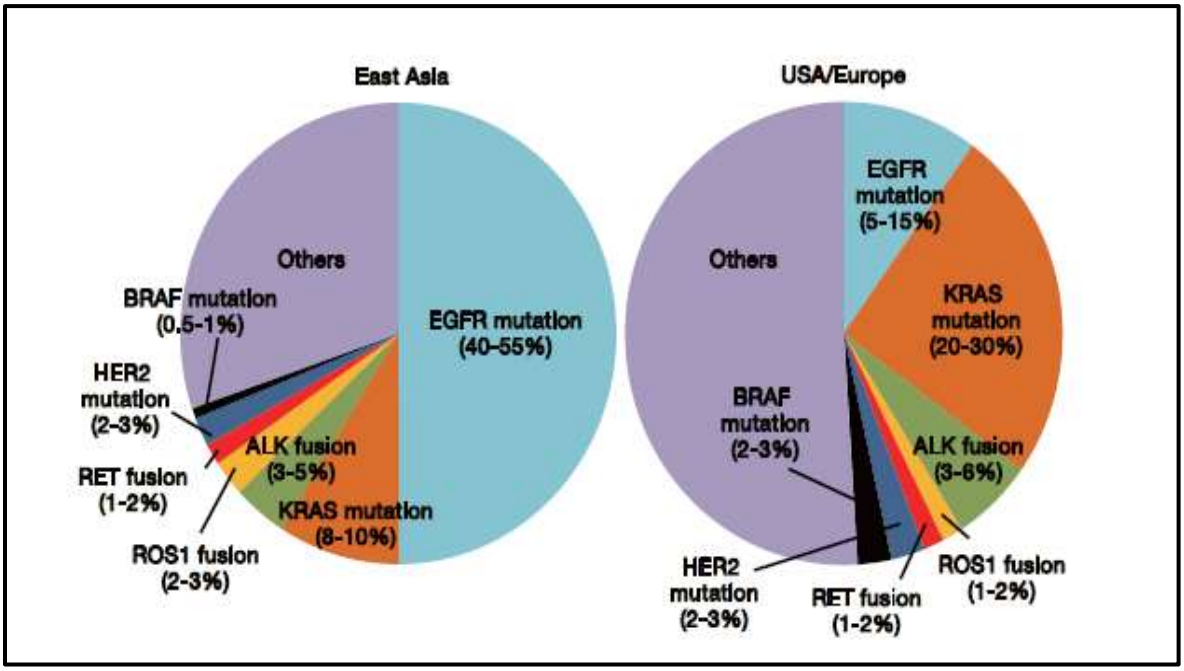

Figure 1.3: Percentage of oncogene drivers in ethnically divers LADC populations. Adapted from [23].

Targeting these abnormally active oncogene drivers has led to the development of drugs that target their site of activation. In the case of LADC harboring EGFR mutations and ALK fusions, tyrosine kinase inhibitors (TKIs) are used to target the activated tyrosine kinase domain. More oncogene drivers have been identified to include RET, ROS1[26-28], NTRK1[29], NRG1 [30, 31], FGFR1/2/3[32-34]. One major molecular driver, epidermal growth factor receptor (EGFR), will be discussed in more details in the upcoming section.

1.2.1 Epidermal Growth Factor Receptor (EGFR)

1.2.1.1. Overview

EGFR (ErbB-1, HER1) is one of the most studied receptor tyrosine kinases in the field of cancer. EGFR is a member of the HER tyrosine kinase family, which also includes: HER2 (ErbB-2), HER3 (ErbB-3) and HER4 (ErbB-4). At the event of binding to its ligands (epidermal growth factor (EGF) and transforming growth factor- alpha (TGF-α), a downstream signaling pathways get activated [35]. Activation of EGFR plays critical role in regulating many important cellular processes such as cell division [36], proliferation [36], migration [37], differentiation [38, 39], transformation and apoptosis [40]. Aberrant EGFR signaling has been reported to have an implication in oncogenic transformation and tumor progression. Abnormal EGFR activation can be due to ligand overexpression, receptor overexpression, or due to constitutive activation of EGFR [41]. Overexpression of EGFR has been reported in many solid tumors including brain, breast, bladder, colon, head and neck, ovarian, prostate and lung cancer [42-48] (**Figure 1.4**).

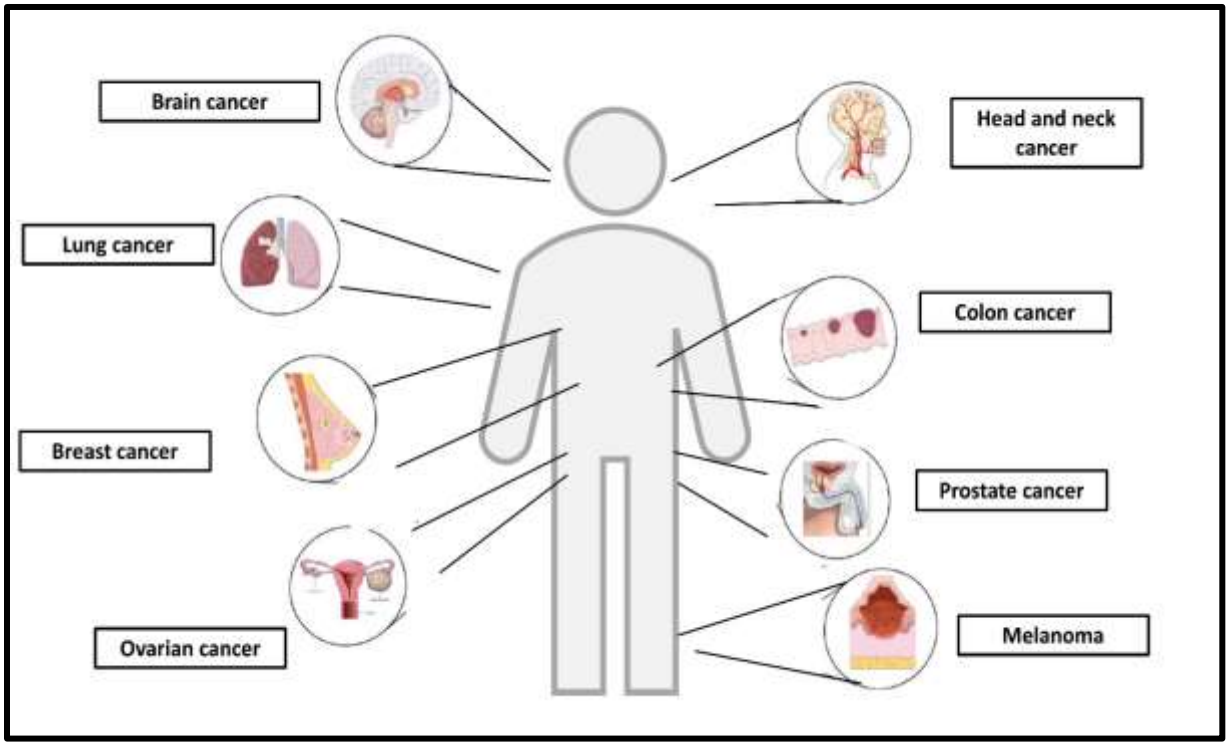

Figure 1.4: Overexpression of EGFR in solid tumors.

1.2.1.2. EGFR protein structure

EGFR is a 170kDa transmembrane protein composed of 1186 residues (**Figure 1.5**). The EGFR protein consists of three main domains: a) the extracellular domain, which is the site of ligand binding; b) the transmembrane domain and c) the intracellular domain, which is the domain of tyrosine kinase activity [49]. The extracellular ligand binding domain is composed of four sub-domains: two domains that are involved in ligand binding L1 (domain I), and L2 (domain III); and two domains that are cysteine rich (CR), known as CR1 (domain II), and CR2 (domain IV) [50].

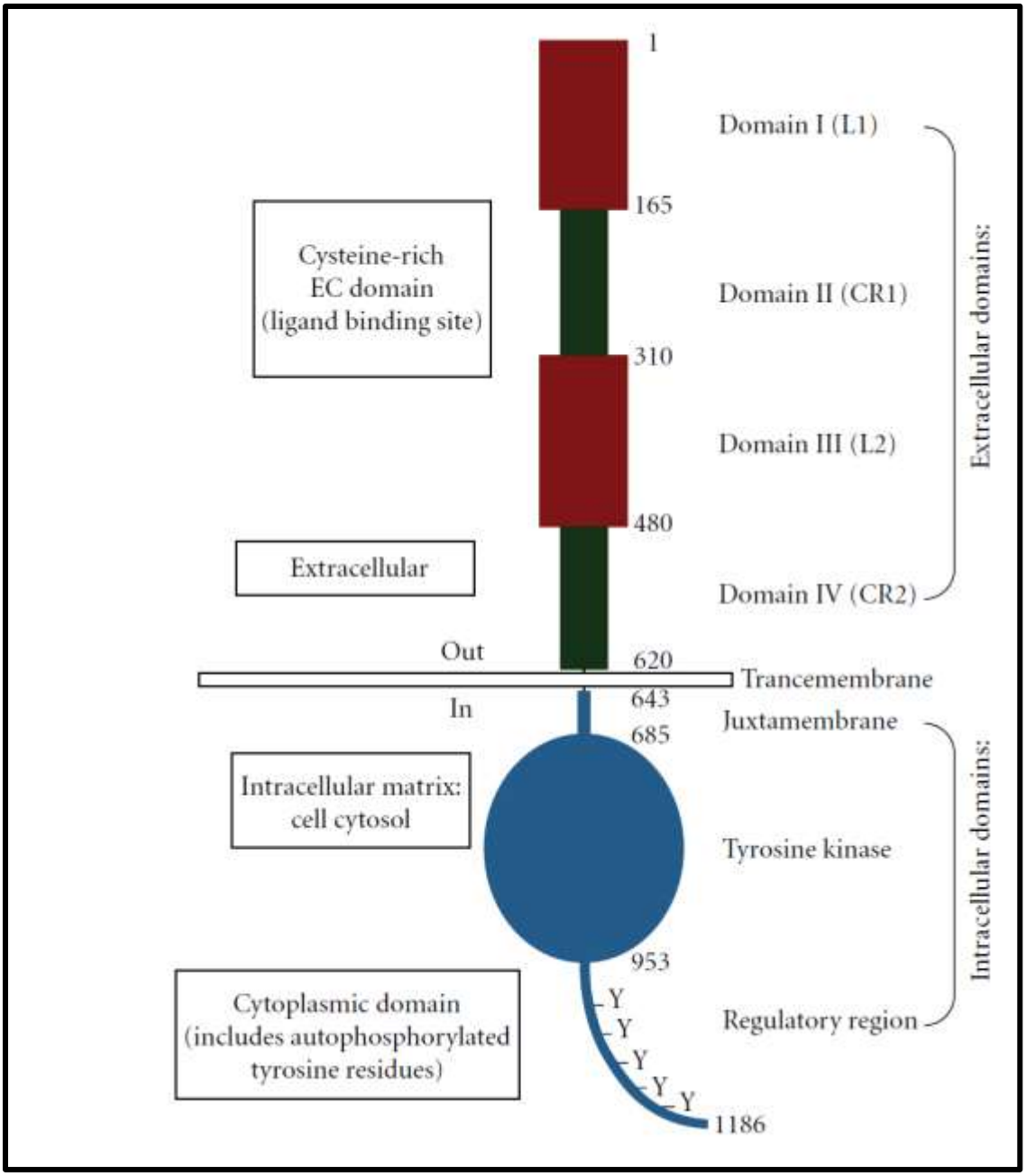

Figure 1.5: Basic structure of EGFR and relevant domain. Adapted from [51].

Epidermal growth factor (EGF) is the first EGFR ligand to be identified, purified and characterized [52, 53]. Subsequently, 12 homologous ligands have been identified for the EGFR family and

include: TGF-α, amphiregulin (AERG), epiregulin (EREG), betacellulin (BTC), heparin-binding EGF-like growth factor (HB-EGF), and the neuregulins (NRGs) [50]. The family of ligands can bind to one or more of the four members of EGFR family (32,33). All these ligands, except for NRGs, can directly bind to EGFR with different affinity. EGFR can form a homodimer, or it can interact with one of the three homologous members (HER2, HER3, and HER4) of the EGFR family leading to heterodimer formation, with HER2 being the favoured partner for this interaction [54].

The transmembrane domain, which is hydrophobic, continues to the juxtamembrane domain which is involved in regulating several processes that include downregulation, ligand dependent internalization of EGFR and protein-protein interactions [55-57]. The intracellular domain is highly conserved among the HER family members of proteins. It holds the tyrosine kinase activity, which plays an important role in the EGFR-mediated signal transduction. Much like other protein kinases, ATP binds between the N-terminal lobe and larger C-terminal lobe in the intracellular domain. The C-terminal lobe contains acceptor tyrosine kinase residues that function as substrates for the phosphotransfer of ATP γ-phosphate groups. Once phosphorylated, the tyrosine residues act as platforms for protein signal transduction that modulate cell signaling responses [58].

1.2.1.3. EGFR signaling pathways

As result of ligand binding to the extracellular domain of EGFR, a homodimer or heterodimer formation of receptor takes place. Receptor dimerization leads to tyrosine kinase activation of the intracellular domain and autophosphorylation of tyrosine residues in the C-terminal lobe. The autophosphorylated tyrosine residues interact with signal transducing proteins which in turn induce activation of signaling pathways in the cell [59].

Induced EGFR tyrosine kinase leads to activation of multiple signaling pathways, such as MAPK, phosphatidylinositol-3-kiansde (PI3K), and JAK/STAT pathways and activation of Src family kinases [60-63]. As depicted in **Figure 1.6**, these activated pathways play an important role in regulating cell growth, cell survival, migration, proliferation, and differentiation in mammalian cells.

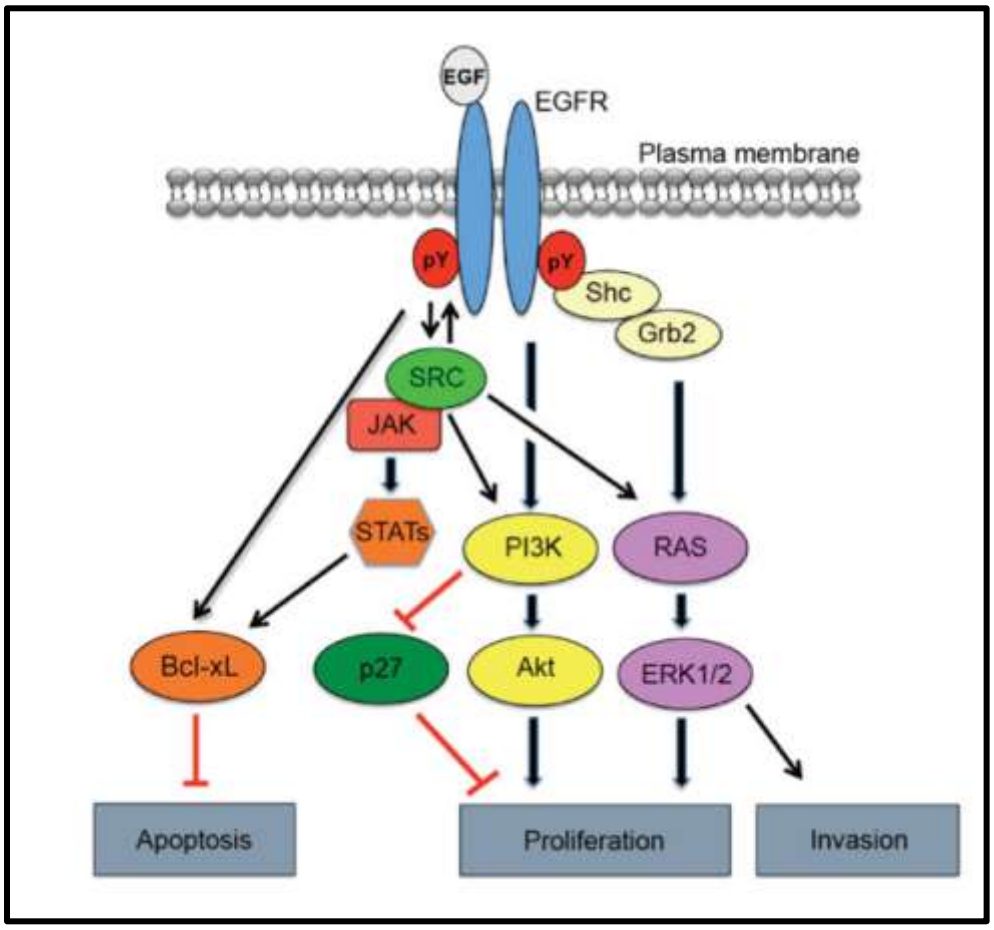

Figure 1.6: EGFR signaling pathways. Activation of the EGFR signaling can induce activation of several signaling pathways that regulate proliferation, growth, survival, and migration. Adapted from [64].

EGFR-mediated MAPK pathway signaling activation is initiated by the adaptor protein GRB2, which interacts with the active tyrosine kinase domain of EGFR. GRB2 can interact with the

receptor either through direct interaction with its phosphotyrosines (Y^{1068} and Y^{1086}) or indirectly through SHC protein, which is phosphorylated by EGFR [65]. Once phosphorylated, GRB2 binds to SOS to form the GRB2/SOS complex, which in turn interacts with membrane-associated RAS protein. As a result of SOS and RAS interaction, an exchange of GDP for GTP takes place leading to activation of RAS [66]. Activated RAS in turn interacts and activates the serine/threonine kinase RAF-1 [67] causing activation of ERK-1/2 through series of intermediate kinases. The activated ERK-1/2 gets translocated into the nucleus where it causes phosphorylation and activation of several nuclear transcription factors [68]. Activated MAPK signaling can also participate in negative feedback loop through phosphorylation of SOS resulting in dissociation of the GRB2/SOS complex, which in turn leads to limited duration of the active RAS [69].

EGFR can interact with c-Src which is a non-receptor tyrosine kinase known to promote proliferation and invasion. In addition to being phosphorylated by active EGFR, c-Src can phosphorylate and activate the tyrosine kinase domain of EGFR (Y^{920}, Y^{891} and Y^{1101}) by itself [70-72]. Activation of EGFR (Y^{920}) through c-Src promotes phosphorylation of p85 sub-unit of PI3K which leads to activation of the PI3K pathway [72]. Phosphorylation of Y^{845} is a key step in the activation of STAT5b [73]. Cells co-overexpressing EGFR/c-Src have higher activity of EGFR substrates, such as phospholipase C-γ (PLCγ) and SHC, compared to cells that overexpress singless kinases [74]. These findings suggest that the crosstalk between EGFR and c-Src can contribute in enhanced EGFR oncogenic signaling in tumor cells.

EGFR signaling can be mediated by the STAT family of proteins. The STAT family of proteins is a family of intracellular signal transducers and transcription factors. These proteins can transmit signals from the cytoplasmic membrane all the way to the nucleus. The STAT family has seven identified proteins: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6 [75].

STATs are usually activated by the JAK tyrosine kinase family, which mediates homo- and heterodimerization of STATs. Active STATs are translocated into the nucleus where they participate in gene transcription [76]. Some the STATs that were reported to be associated with EGFR signaling, are mainly STAT1, STAT3, and STAT5. Activation of STATs through EGFR differs from classical activation mediated by cytokine receptors. STAT activation by EGFR does not require JAK kinases [62].

Moreover, EGFR plays a critical role in lipid metabolism where it can directly activate phospholipase D (PLD), PLCγ, and PI3K. It has been reported that PLD can be activated directly by EGFR [77]. PLD is an enzyme that plays a role in the hydrolysis of phosphatidylcholine, generation of choline and the second messenger phosphatidic acid (PA). PA is an important component of the mammalian target or rapamycin (mTOR) pathway [78].

PLCγ is another enzyme that gets directly activated through EGFR's residues Y^{1173} and Y^{992} [78]. Active PLCγ induces hydrolysis of phosphatidylinositol 4,5-biphosphate (PIP2) resulting in second messengers 1,2-diacylglycerol (DAG) and inositol 1,2,3-triphosphate (IP3). DAG acts as a cofactor in the activation of serine/threonine PKC kinase. This suggests that through PKC, EGFR can participate in the activation of signaling pathways such as MAPK and JNK pathways [79, 80]. Since metabolism of Ca^{2+} is regulated by IP3, therefore, EGFR can contribute in the Ca^{2+}-dependent pathways such NFκB through IP3 [81].

PI3K pathway can be activated as a result of the association of p85 subunit of PI3K with the phosphorylated receptor. HER3 is the preferred partner of p85 subunit of PI3K, however; EGFR can activate PI3K through the formation of EGFR/HER3 heterodimers or through c-Src phosphorylation of EGFR [82]. Activated PI3K induces the formation of phosphatidylinositol-

3,4,5-triphospate (PIP3), which is a well studied second messenger for the activation of the serine/threonine AKT (PKB) kinase. Once AKT is translocated to the plasma membrane, it is phosphorylated (T^{308}) by phosphoinositide-dependent kinase-1(PDK-1). For AKT to be fully activated, it requires phosphorylation at a second site (S^{473}) by the mTOR kinase [83]. Several cellular processes such as cell survival and proliferation are impacted by AKT activation which makes it as one of the major mediators of antiapoptotic effects of EGFR activation [84]. The activation of PI3K pathway is regulated by the phosphatase and tensin homolog (PTEN) which causes dephosphorylation of the second messenger PIP3 yielding to PIP2, which in turn inhibits AKT activity [85].

1.2.1.4. EGFR mutations

Enhanced tyrosine kinase activity can be due to gene amplification, mutation, deletion, or overexpression of EGFR which leads to increased oncogenic properties. Studies have reported the implication of EGFR gene amplification, which causes overexpression of EGFR, in many solid tumors. Increased EGFR expression has been reported clinically to be associated with a decreased survival in patients with bladder, cervical, and head and neck squamous cell carcinoma [86]. In a subset of glioblastoma patients, EGFR amplification has been reported with structural rearrangement which is characterized with an in-frame deletion of the extracellular domain of EGFR. The most common EGFR mutation in glioblastoma is the EGFR mutant variant III (EGFRvIII), which is characterized by lacking the amino acids 6-273 (exon 2-7) with constitutive activation [87].

The first somatic EGFR mutations located within the kinase domain have been reported in NSCLC patients [88-90]. Seven exons (18-24) encode for the tyrosine kinase domain of EGFR (**Figure 1.7**) with EGFR kinase domain mutations being located between the exons 18-21 [88, 89, 91].

Mutations in the tyrosine kinase domain are usually referred to as activating mutations, since they lead to constitutive activation of EGFR kinase. In North America and Europe, EGFR mutations occur in 10% of NSCLC patients, whereas the rate of these mutations is higher in Asia with a rate occurrence of 30-50% in NSCLC patients [91, 92]. Moreover, somatic mutations are more likely to be found in women with adenocarcinoma histology, in individuals with East Asian ethnicity and non-smokers [88, 93]. The most commonly reported EGFR mutations are: in-frame deletion of exon 19 (d746-750) and substitution of leucine at position 858 of exon 21 with an arginine (L858R). These mutations account for almost 90% of all the mutations in EGFR kinase domain [94]. Lynch et al. [89] have reported in their study that exon 19 deletion (d746-750) and L858R mutation exhibit different kinetics of receptor de-phosphorylation compared to wild-type EGFR. A study done by another group concluded that mutations in the tyrosine kinase domain of EGFR are strong stimulator of the PI3K and STAT5 signaling pathways in comparison to wild-type EGFR [95]. Furthermore, these mutations have been reported to possess an increased kcat values and Km for ATP [96, 97]. Decreased ATP affinity in EGFR binding site is most likely to be responsible for increased sensitivity of EGFR-mutant to tyrosine kinase inhibitors (TKIs). In fact, these somatic mutations have correlated with improved clinical response to EGFR-TKIs [88-90, 98, 99].

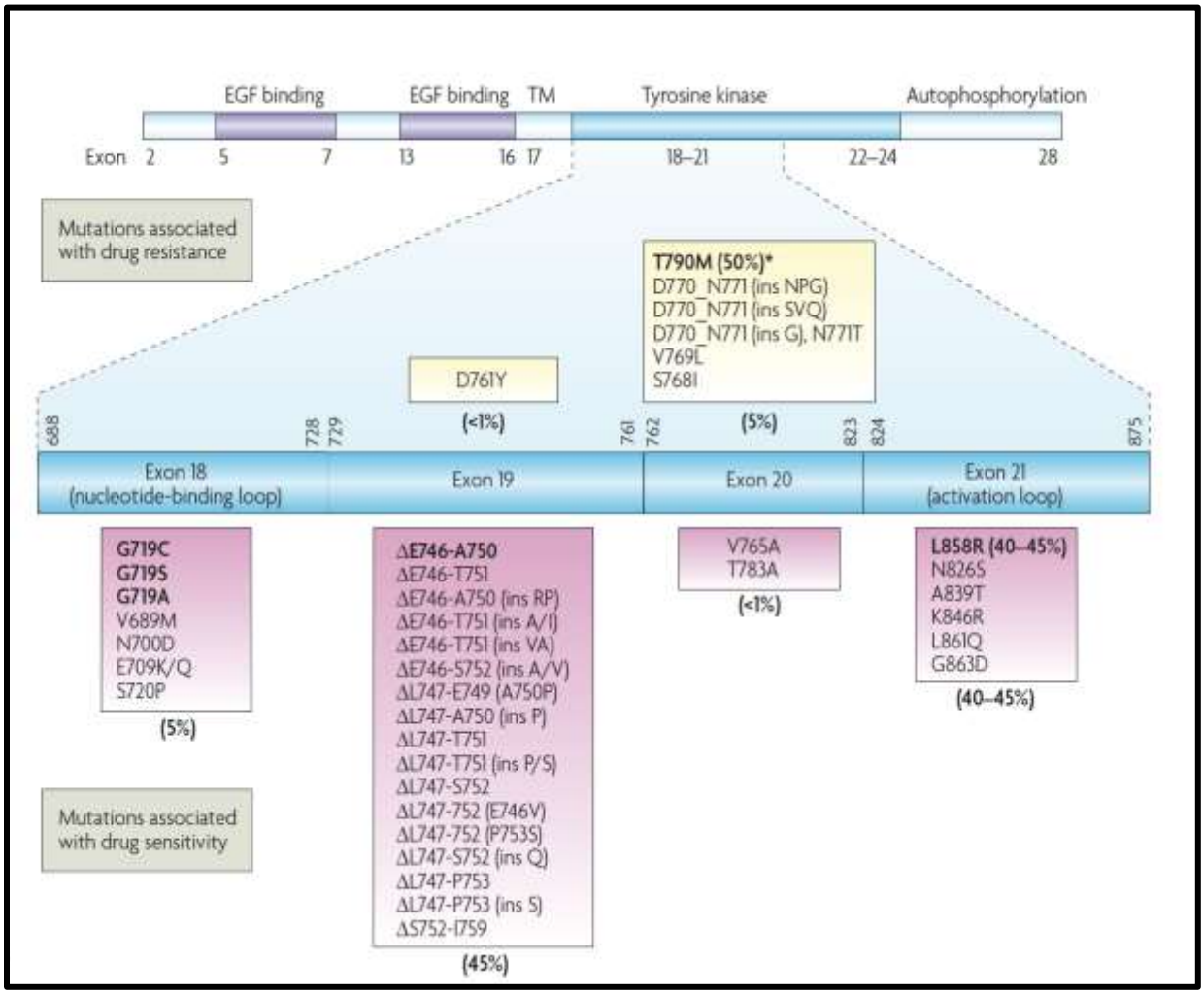

Figure 1.7: Schematic representation of human EGFR protein and mutations in the tyrosine kinase domain (exon 18-21). The most common EGFR mutations are: deletion of exon 19 (d746-750) and L858R substitution (exon 21). Adapted from [100].

1.3 EGFR as therapeutic target in oncology

1.3.1. The mechanism of action of epidermal growth factor receptor-tyrosine kinase inhibitors (EGFR-TKIs)

EGFR-TKIs function by specifically targeting the tyrosine kinase activity of EGFR. Currently, there are three generations of EGFR-TKIs (**Figure 1.8**) that have been approved for clinical use [101-103]. A fourth generation of EGFR-TKIs is emerging and under investigation to combat C797S resistance [104]. First generation of EGFR-TKIs are reversible competitive inhibitors of ATP binding to the active site of EGFR, whereas second- and third-generation are irreversible inhibitors of EGFR [105]. Inhibition of EGFR phosphorylation as result of EGFR-TKIs consequently leads to inhibition of downstream signaling of the receptor. As it was reported by Tracy et al. [106], inhibition of EGFR and its downstream signaling lead to enhanced apoptosis in cells that are dependent on EGFR signaling.

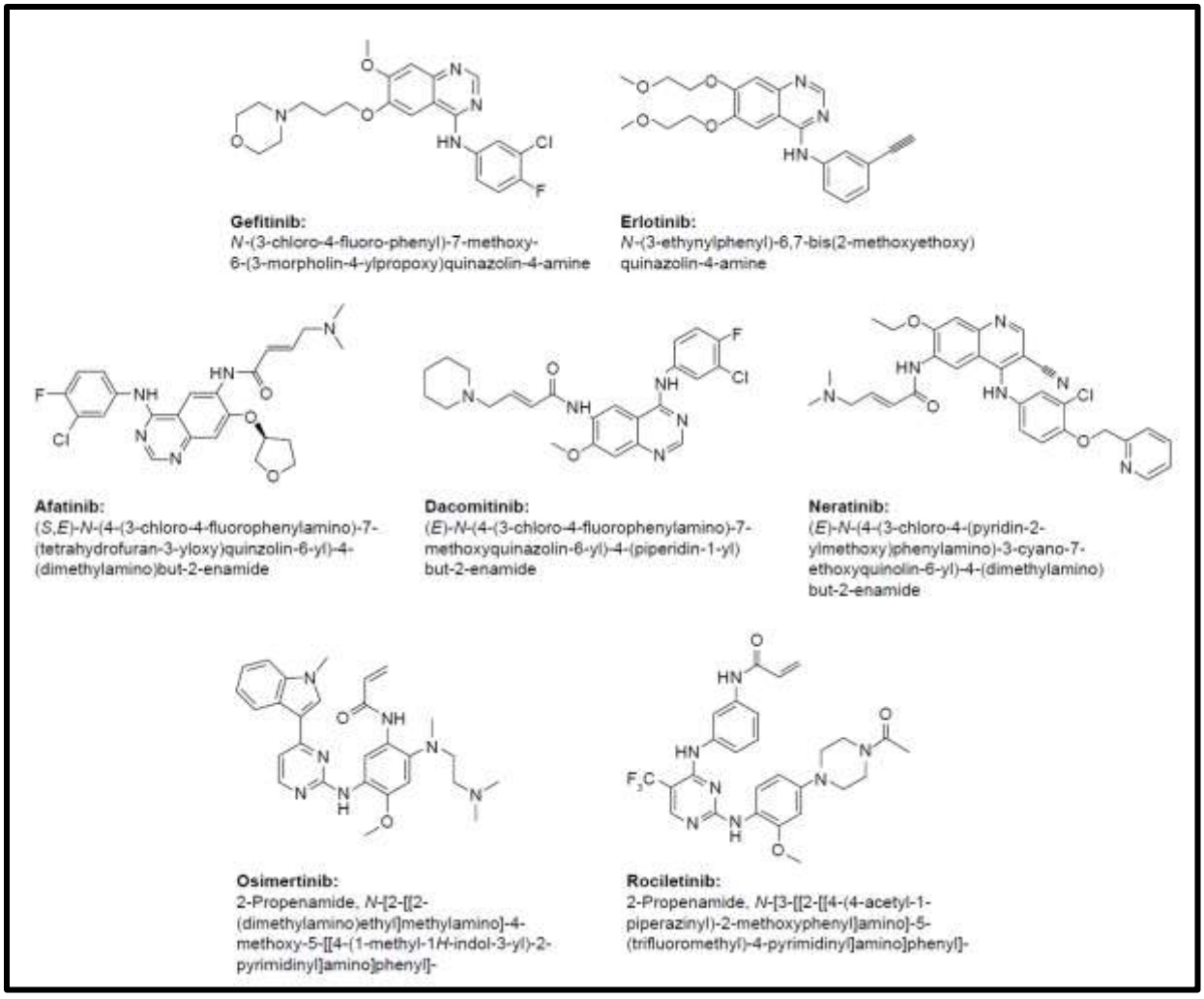

Figure 1.8: Structures and chemical names of EGFR/HER-TKIs. Adapted from [107].

1.3.2. First generation of EGFR-TKIs

Gefitinib and erlotinib both belong to the first generation of EGFR-TKIs and characterized by being two small reversible molecules. In the year of 2003, the FDA has granted a fast-track approval for gefitinib, based on two phase II clinical trials (IDEAL-1 and IDEAL-2), as a treatment for advanced NSCLC patients who did not respond to conventional chemotherapy [108-110]. Subsequent phase III trials (INTACT-1, INTACT-2 and ISEL) have reported that treatment with

gefitinib did not demonstrate any significant overall survival improvement, which has led to restrict the use of gefitinib by FDA [111-113]. The discovery of activating EGFR mutations [88-90] has led researchers to investigate the benefit of using gefitinib in subset of patients who carry these mutations. In the IPASS clinical trial, the use of gefitinib prolonged PFS, which was largely due to the presence of activating EGFR mutations [114]. In the year of 2015, the FDA has approved the use of gefitinib as a treatment for patients with metastatic NSCLC patients whose tumors were positive for EGFR mutations, such as exon 19 deletion or exon 21 (L858R) substitution mutations [101].

In 2004, the FDA has approved the use of erlotinib as a treatment for patients with locally advanced or metastatic NSCLC after failure of at least one prior chemotherapy regimen [115]. In 2010, erlotinib was approved by the FDA as a maintenance treatment of patients with locally advanced or metastatic NSCLC with no disease progression following four cycles of platinum-based first-line chemotherapy [116]. Moreover, in 2013 erlotinib has been approved by the FDA as a first-line treatment for patients with metastatic NSCLC harboring activating EGFR mutations (exon 19 deletion or exon 21 L858R substitution). As of October 2016, the latter indication is the only FDA approved indication of the use of erlotinib [116].

1.3.3. Second generation of EGFR-TKIs

Patients harboring activating EGFR mutations have an initial good response to first-generation EGFR-TKIs, however; acquired resistance to treatment is inevitable [117, 118]. The most commonly reported resistance developed as result of EGFR-TKIs treatment is the acquired T790M mutation in the tyrosine kinase domain of EGFR (exon 20) [117, 118]. The idea of developing a second-generation of EGFR-TKIs was initiated due to detection of acquired resistance to EGFR-TKIs first-generation. Second-generation of EGFR-TKIs function through covalent binding to the

kinase domain of EGFR, which leads to an irreversible inhibition of EGFR phosphorylation [119]. Kwak et al. [120] were the first to report the possibility of overcoming T790M-mediated resistance to first-generation EGFR-TKIs by using irreversible EGFR inhibitors. In their work, they demonstrated using cells with T790M mutation following irreversible inhibition of EGFR to result in an inhibition of downstream signaling of EGFR and enhanced cell killing [120]. This was not observed clinically, where second-generation EGFR-TKIs did not overcome T790M-mediated resistance in NSCLC patients [121, 122]. This was justified by the fact that the therapeutic dose of these irreversible inhibitors, in order to achieve successful inhibition of T790M, was reported to be associated with increased dose-limiting toxicities in patients [105, 123].

Afatinib and dacomitinib, which are irreversible EGFR-TKIs, have reported to be effective in treating non-pretreated NSCLC with activating EGFR mutations (exon 19 deletion and L858R exon 21 substitution) [124, 125]. In January 2018, an approval for afatinib as first-line treatment was extended for patients with metastatic NSCLC whose tumors have other non-resistance EGFR mutations [116]. The approval was granted based on the effectiveness of afatinib in treating patients with metastatic NSCLC with non-resistant EGFR mutations, such as S768I, L861Q, G719X, other than exon 19 deletion or L858R substitution [116].

1.3.4. Third generation of EGFR-TKIs

Due to the inability of second-generation EGFR-TKIs to overcome T790M-mediated resistance, it was essential the development of a novel irreversible third-generation of EGFR-TKIs that can specifically target the T790M and activating EGFR mutations, while paring the wild-type receptor [105]. WZ4002 was the first reported third-generation of EGFR-TKIs [126]. It was reported to be 100-fold more potent towards targeting T790M-EGFR and 100-fold less potent towards wild-type EGFR when compared to aminoquinazoline-based EGFR-TKIs [126]. The promising pre-clinical

outcomes of the use of third-generation EGFR-TKIs has been translated clinically. For instance, a phase III trial investigating the efficacy of Osimertinib has reported a strong efficacy of Osimertinib in comparison to standard chemotherapy in NSCLC patients with T790M mutation [127]. As a result, in March of the year 2017, the FDA has approved the use of Osimertinib as a treatment for NSCLC patients with metastatic T790M-EGFR mutation-positive NSCLC patients whose disease progressed on or after EGFR-TKI treatment [116].

1.3.5. Fourth generation of EGFR-TKIs

T790M-EGFR mutation is the most common mutation that leads to resistance to clinically available EGFR-TKIs. Third-generation EGFR-TKIs are designed to target the T790M mutation [105, 126, 128] have been in constant development and include the following drugs: osimertinib (AZD9291), rociletinib, HM61713, ASP8273, EGF816 and PF-06747775 [104].This class of inhibitors binds covalently to Cys797 and spares wild-type EGFR which leads to decreased toxicity and permits the use of doses that fully supress T790M activity [104]. However, emerging data has reported C797S mutation, located with the tyrosine kinase domain, to be a leading mechanism of resistance to the third-generation of inhibitors (**Figure 1.9**) [129-132]. This has led to the development of EAI001 (EGFR allosteric inhibitor-1) but had a modest potency against individual L858R and T790M mutants [104]. The inhibitor went through medicinal-chemistry-based of optimization leading to the discovery of EAI045, which was found to have high potency and selectivity for L858R/T790M mutation [104]. Its potency was confirmed through a panel of 250 protein kinases [104], which has led to confirm EAI045 to be an allosteric, non-ATP competitive inhibitor of EGFR mutant [133]. Animal studies have confirmed its efficacy in overcoming resistance from acquired T790M and C797S mutations [133]. This makes EAI045 to

be so far the first allosteric EGFR-TKI that can target and overcome T790M and C797S mutations [104].

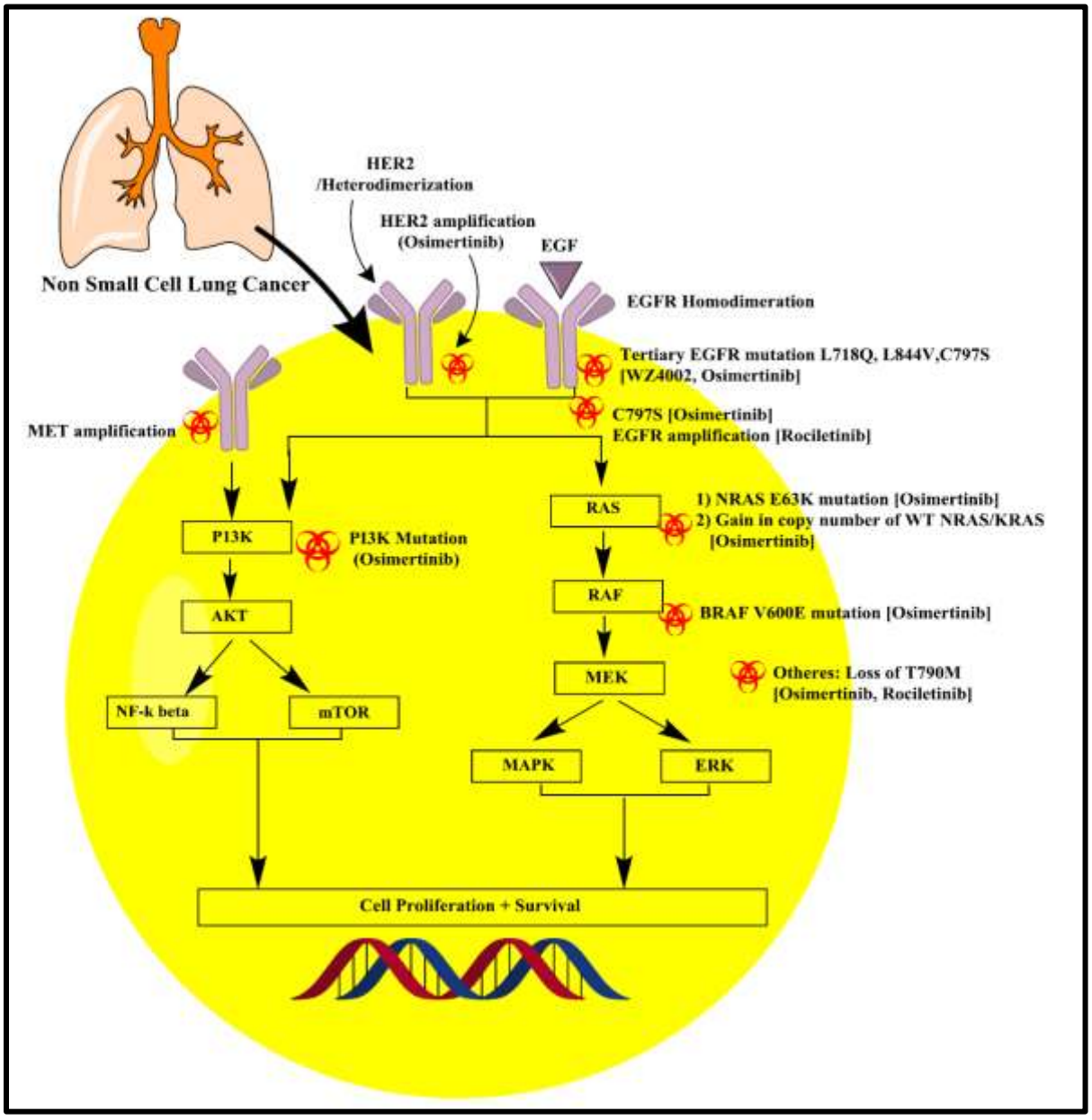

Figure 1.9: Mechanisms of resistance to third generation of EGFR-TKI. adapted from [104].

1.3.6. Resistance to EGFR-TKIs

As mentioned previously, the T790M-EGFR mutation in the exon 20 is the most commonly reported resistance mechanism to aminoquinazoline-based EGFR-TKIs, with more than 50% of NSCLC patients having this resistant mutation [117, 118]. It is known as the gatekeeper mutation because it prevents proper binding of aminoquinazoline-based EGFR-TKIs to the binding site of EGFR's kinase domain [134]. Moreover, the tyrosine kinase domain of T790M-EGFR is characterized by an increased affinity to ATP [135]. Both mechanisms contribute in reduced receptor refractory to inhibition by aminoquinazoline-based EGFR-TKIs.

In addition to T790M resistance mutation, other resistance mechanisms exist such as EGFR amplification [136], MET amplification [137, 138], HER2 amplification [139], NF1 loss [140], PIK3CA [136], B-RAF, K-RAS, N-RAS and MEK1 mutations [141]. Small cell lung cancer (SCLC) transformation and epithelial-to-mesenchymal-transition have also been reported to be associated with resistance to EGFR-TKIs [136].

1.3.7. EGFR mutations co-occurring with other mutations

Although most driver mutations are mutually exclusive, there has been reports of NSCLC patients with double mutations causing dilemma in the decision of choosing the appropriate therapy. This is very critical especially if these aberrations effect two different pathways, with one or both known to exhibit resistance to the drug targeting the aberrant sensitizing alternative pathway. The review by Van Der Steen et al. [142] addresses the complexity of some the reported cases with double mutations in NSCLC patients.

For instance, if a patient presents with double aberrations in EGFR and ALK pathways, different measures should be taken into consideration prior to any treatment recommendations. This is

because activation of EGFR is known to be a mechanism of acquired resistance against crizotinib which is used for the treatment of ALK fusion-positive patients [143]. To address this challenge, it is important to identify which pathway is the main signaling pathway and determine if activation is driven by both pathways or neither. One possibility is by examining the phosphorylation levels of both EGFR and ALK [144]. Obtaining information on phosphorylation status can help in determining which targeted therapy should be recommended as fist-line.

Presence of double mutations in EGFR and KRAS is very challenging as well. The reason behind the complexity of this challenge is due to the fact that KRAS is located downstream of EGFR, which means that mutations in KRAS can bypass EGFR-TKIs leading to resistance to these therapies [145]. Currently, there is no targeted therapies that exist against KRAS mutations. In current clinical practice, when a patient presents with double EGFR and KRAS mutations, the patient will not be treated with EGFR-TKIs [142]. The question associated with this challenge is whether the presence of KRAS mutation will cause an immediate resistance to EGFR-TKIs in all patients or not. Several studies have reported various combination of EGFR and KRAS mutations. In the study done be Lee et al [146], six cases were described with three patients out of the six to be Asian patients whom have been treated with EGFR-TKIs. These patients following treatment with EGFR-TKIs, such as gefitinib or erlotinib, had a PFS ranging from 9 months up to an ongoing PFS of >29 months [146]. Another study by Ulivi et al [147] has reported three Italian cases, two of which received second-line treatment with gefitinib and showed a stable disease for 6 and 14 months. These studies suggest that KRAS mutation does not lead to immediate resistance to EGFR-TKIs. However, the study done by Takeda et al [148] contradicts these findings. In their published work, they reported two male Asians patients both with exon 19 deletion and KRAS G12D mutations, who were both treated with erlotinib to have a PFS of 28 and 32 days with no

response to EGFR-TKIs [148]. Resistance resulted from KRAS mutations might be driven by the percentage of tumor cells carrying KRAS mutation. The use of Next-Generation Sequencing (NGS) has helped in providing a better understanding and estimating cell percentage. Presently, it is not known whether mutations in KRAS and EGFR can occur within the same cell or the tumor contains two cell populations [142]. Since KRAS mutations in EGFR mutant cells can reactivate downstream signaling, thus this causes overcoming inhibition by EGFR-TKIs leading to resistance. Moreover, KRAS mutations in EGFR wild-type cells are not able to bypass inhibition by EGFR-TKI. This emphasizes on further studies being conducted to understand the mechanisms of action behind double mutations.

1.4 EGFR's critical role in DNA repair

1.4.1. Overview

At the event of genotoxic stress, EGFR gets translocated into the nucleus where it plays a critical role in regulating gene transcription, cell signaling and DNA repair mechanism [149]. EGFR is reported to be able to interact with several DNA repair proteins such as DNA-PKcs, RAD51, ATM and BRCA1 [150-156]. MAPK signaling, which is mediated by EGFR, can modulate DNA repair mechanism through increased expression of XRCC1 and ERCC1 in cells [157-159]. In the work published by Hagan et al [160], activation of EGFR-MAPK signaling was shown to be associated with increased PARP activation following exposure to ionizing radiation. At the event of exposure to ionizing radiation, EGFR gets translocated into the nucleus where it participates in the NHEJ repair. EGFR can interact directly with DNA-PKcs which enhances its activity [161, 162]. EGFR can directly phosphorylate DNA-PKcs at T^{2609}, which is an essential part for the NHEJ-mediated DSB repair. Inhibition of T^{2609} phosphorylation has been associated with an increased radiosensitivity profile [163]. DNA-PKcs is a key player in radioprotection mechanism which is

mediated by EGFR activation. In fact, it has been reported that radioprotective effect of EGFR is lost in cells that are DNA-PKcs-deficient [164, 165]. Cells harbouring activating EGFR mutations, such as del746-750 or L858R, have been reported to exhibit a radiosensitive profile following exposure to radiation treatment [165]. This is justified by the defect in radiation-induced nuclear translocation of EGFR preventing interaction between EGFR and DNA-PKcs.

1.4.2. Ionizing radiation-mediated EGFR activation

Amplification, overexpression or mutations in EGFR have been reported to be associated with chemo- and radio-resistance in different tumor types [166-169]. Most of these studies have assessed the role of EGFR in the DNA repair mechanism and its role in the response to radiation [167, 169]. Following cell exposure to ionizing radiation, EGFR is phosphorylated and activated which is followed by a subsequent downstream signaling activation [170]. As a result, MAPK and PI3K pathways are activated where their activation has been associated with an increase resistance to chemo- and radiotherapy [171, 172]. Activation of EGFR in the event of ionizing radiation can be stimulated through few mechanisms. One mechanism is through ROS, generated during ionizing radiation, which inactivates a redox-sensitive, cysteine-based protein tyrosine phosphatse which causes instability of the phosphorylated and de-phosphorylated form of the receptor [173]. Another mechanism in which EGFR can be stimulated is through up-regulation of autocrine/paracrine EGFR ligands of the irradiated cells [174, 175]. Post-ionizing radiation exposure, EGFR translocates to the nucleus where it effects gene transcription and DNA repair [149]. Studies have reported an association between EGFR nuclear localization with poor prognosis and resistance to treatment in clinic [176-179].

1.4.3. The combination of EGFR inhibitors and ionizing radiation

Inhibition of EGFR nuclear translocation, which was induced by ionizing radiation, can be achieved by EGFR monoclonal antibody cetuximab. This monoclonal antibody immobilizes EGFR within the cytosol which leads to inhibition of DNA-PKcs activation which prevents DNA repair and cell survival to radiation [161]. We have mentioned previously that EGFR-TKIs function in targeting the tyrosine kinase domain of EGFR leading to inhibition of its activity [149]. This has led to investigate the potential of combining EGFR inhibitors with ionizing radiation to potentiate the antitumor effect. A study has reported that the combination of cetuximab with radiation has significantly increased progression free survival (PFS) compared to radiation alone in head and neck cancer patients [180]. Moreover, several studies have reported an enhanced radiation effect when combined with EGFR-TKIs [153, 181, 182]. One study reported that treatment with gefitinib has modulated the interaction between EGFR and DNA-PKcs in EGFR-expressing cells [183]. Similarly, exposure of head and neck squamous cell carcinoma to radiation had a decreased nuclear expression of DNA-PKcs when combined with gefitinib [184]. Erlotinib has been reported to attenuate ionizing radiation-induced RAD51 leading to an increased sensitivity to radiation [153]. Moreover, erlotinib has been shown to supress HR repair mechanism by targeting nuclear translocation of BRCA1 which results in cytoplasmic retention of BRCA1 and decreased BRCA1 levels in the nucleus [156].

1.5 Treatments of Non-Small Cell Lung Cancer

1.5.1 Surgery as a treatment of NSCLC

For early stage NSCLC (stage I and II), surgical resection is considered as the treatment of choice. Stage I and II patients account for 30% of NSCLC cases [185]. For stage IA patients, surgical resection alone is usually the standard of care. In the case of stage IB patients, treatment with adjuvant chemotherapy remains controversial. One study has investigated the application of

adjuvant chemotherapy treatment of paclitaxel and carboplatin [186]. In their study, they have reported that a significant survival advantage was not observed across the entire cohort [186]. However, a beneficial outcome of adjuvant chemotherapy in stage II NSCLC tumors was reported by the ANITA clinical trial [187].

Stage IIB patients with signs of chest wall invasion (T3N0) are recommended to have en bloc chest wall resection with ribs. It has been associated with a 5-year survival of 40%, however; this survival can decrease to 12% if any mediastinal lymph node was involved [188]. This emphasizes on the need of proper mediastinal staging prior to surgical resection. Improved survival following resection in stage IIB NSCLC has been reported by multiple studies [189, 190].

Patients who are diagnosed with stage IIIA disease are recommended to have surgical resection followed by adjuvant chemotherapy. Diagnosis should be based on the involvement of chest wall, or proximal airways, or due to the presence of satellite nodules within the same lobe as the primary tumor [191]. It has been reported that these patients tend to have a better prognosis when compared to stage IIIA patients with secondary to mediastinal N2 nodal involvement [191]. Some primary exceptions to this treatment include superior sulcus (Pancoast) tumors with hilar lymph node involvement. Typically, patients presented with Pancoast tumors are treated with neoadjuvant chemoradiation followed by surgery [192].

Neoadjuvant chemotherapy or chemoradiation has been reported to be beneficial to patients with clinically resectable stage IIIA (T3N2) disease. In the case where there is no evidence of mediastinal disease or the patient is downstaged to N1 (N2 negative), the patient is considered candidate for surgical resection with increased survival if lobectomy was performed versus pneumonectomy [191, 192]. When pneumonectomy is performed, it is still controversial whether induction will be done using chemotherapy alone or chemoradiation. It is recommended that

preoperative radiation should be omitted due to high risk of perioperative mortality associated with pneumonectomy following chemoradiation. It is preferred to offer preoperative chemotherapy alone followed by pneumonectomy in the event of N2 disease clearance during mediastinal staging for low-risk patients [187, 193]. Patients who are presented with stage IIIB o stage IV NSCLC are typically not suitable candidates for surgery (resection) and should be treated with chemotherapy or chemoradiation.

1.5.2 Radiation therapy as a treatment of lung cancer

Radiation therapy is an essential component of treating all stages of lung cancer. Stereotactic ablative radiation therapy (SABR) has become a standard of care treatment for stage I-II patients who are medically not fit for surgery. Patients with stage IIIA-IIIB disease are typically treated with definitive concurrent chemo-radiotherapy (CRT). Development of intensity modulated radiation therapy (IMRT) has allowed for the delivery of highly potent dose of RT with limited doses to the surrounding normal tissues such as lungs, esophagus and heart. New clinical trials are still investigating SABR potential in treating stage IV patients with the combination of systemic immunotherapies. Gomez et al. have investigated progression-free survival (PFS) and long-term overall survival (OS) in patients with oligometastatic NSCLC that did not progress after front-line systemic therapy [194] where patients were randomly assigned (1:1) to maintenance therapy or observation (MT/O) or to local consolidative therapy (LCT) with radiotherapy or surgery [194]. They reported that LCT has prolonged PFS and OS compared to MT/O in patients with oligometastatic NSCLC that did not progress after front-line systemic therapy [194].

1.5.3 Conventional radiation therapy versus stereotactic ablative radiation therapy in stage I-II disease

Prior to the use of SABR, treatment of small tumors with radiation therapy over 6-7 weeks did not yield very good outcomes, with a local control rate of 30-60% (1-2). During this treatment, patients were treated daily over a period of 6-7 weeks. Delivery of doses greater than 65 Gy were associated with a better local control. Low local control rates can be justified with lack of soft tissue imaging for alignment during treatment, which may cause an under-dosing to the targeted tumor. Inadequate radiation dosing schedules can also have an impact in the low local control rates.

Improvements in radiation delivery and imaging technology have enabled for the application of SABR as an acceptable approach in treating early stage NSCLC. Use of positron emission tomography/computed tomography (PET/CT) and bronchoscopy with the aid of endobronchial ultrasound as part of the pathological nodal staging has led to increased tumor staging accuracy. Proper staging has helped in identifying patients who can benefit of having aggressive treatment. Tumor motion is considered as a major challenge in lung tumors treatment with SABR. Since three-dimensional (3D) CT scans can only capture limited phases of the respiratory cycle, clinicians were required to add larger margins, also known as safety margins, around the gross tumor to ensure that the tumor will be fully targeted. The use of four-dimensional CT (4DCT) scanners was a turning point in the treatment planning process by allowing clinicians to incorporate tumor motion data in designing the radiation field.

Limiting the motion of tumor's patients is another obstacle that clinicians must face to minimize the size of the radiation field. It has been reported that tumor motion is significantly higher when a patient is free-breathing as compared to using abdominal compression device (3). Moreover, ensuring the accuracy of patient's setup during treatment is also considered as another obstacle to

overcome. The use of cone-beam CT (CBCT) machines that are integrated into the linear accelerator device as a single unit has facilitated imaging of the patient's tumor prior to the delivery of each fraction. Image-guided radiation therapy (IGRT) which is the use of imaging during radiation therapy ensures precision and accuracy of the delivered dose of treatment. Published phase I/II trials of SABR (**Table 1**), reported a primary tumor control of 80-100% for T1 tumors. In terms of toxicity, lung SABR is overall associated with very low rates of acute and late toxicity. Side effects that are associated with SBAR include chest wall pain, rib fracture, and decline in pulmonary function tests.

Trial	Years treated, patient number	Tumor stage (n)	Dose/fraction number	Median follow-up (months)	Local control	Overall survival
Timmerman et al. [195]	2000-2003, N = 37	T1: 19 T2: 18	24-60 Gy/3	15.2	87%	1.5yr: 64%
Nagata et al. [196]	1998-2004, N= 45	T1: 32 T2 (<4 cm): 13	48 Gy/4	22-30	98%	3 yr: T1: 83% T2: 72%
Lindberg et al. [197]	2003-2005, N = 57	T1: 72% T2: 28%	45 Gy/3	41.5	4 yr: 79%	5 yr: 30%
Koto et al. [198]	1998-2004, N = 31	T1: 19/31 T2: 12/31	45 Gy/3 for 20 patients, 60 Gy/8 for 11	32	3 yr: T1: 78% T2: 40%	3 yr: 72%
Fakiris et al. [199]	2002-2004, N = 70	T1: 34 T2: 36	T1: 60 Gy/3 T2: 66 Gy/ fxn	50.2	3 yr: 88%	3 yr: 43%

Table 1.3: Published phase I/II trials of SABR. Adapted from [200].

A recently published clinical trial, CHISEL trial, by Ball et al. has compared SABR to standard radiotherapy in stage1 NSCLC [201]. In the study, patients were randomly assigned after stratification for T stage and operability in a 2:1 ratio to SABR or standard therapy [201]. SABR treatment was given as 54 Gy in three fractions of 18 Gy, or 48 Gy in four fractions of 12 Gy if the tumor was smaller than 2 cm from the chest wall [201]. In a standard radiotherapy treatment, the given dose was 66 Gy in 33 daily 2 Gy fractions or 50 Gy in 20 daily 2.5 Gy fractions depending on institutional preference [201]. In their study, they reported that SABR treatment in inoperable peripherally located stage I NSCLC resulted in superior local control of the primary disease without an increase in major toxicity [201]. Due to their findings, they suggested that SABR should be considered as the treatment of choice for these patients [201].

1.5.4 Intensity modulated radiation therapy (IMRT) in stage III NSCLC disease

In stage III NSCLC patients, 4DCT is commonly used in the phase of treatment planning [200]. It allows for motion data to be acquired of both primary lung tumor and mobile lymph node ensuring that the entire trajectory is captured in the target [200]. Increased certainty of tumor location has simplified the use of tighter margins which helped in sparing normal tissues from being damaged [200]. IGRT can also be incorporated to be part of the treatment plan which can help in minimizing margins uncertainty [200]. In the case of a locally advanced disease, intensity modulated radiation therapy (IMRT) can be part of the treatment plan allowing for lower doses to the surrounding normal lung compared to traditional three-dimensional conformal radiation therapy (3D-CRT) [200]. Clinical data have reported a significantly lower rates of grade 3+ pneumonitis when IMRT was used compared to the use of 3D-CRT, even in patients presented with a large tumor size and were treated with IMRT [202]. A study analysis done on 7000 patients using the SEER-Medicare database revealed no difference in overall survival between the use of 3D-CRT and IMRT [203].

In addition to the benefit of sparing regional normal lung tissues, IMRT can also help in sparing other organs such as the heart and esophagus. RTOG 0617 clinical trial has indicated that heart dose and esophageal toxicity can be significant predictors for survival [204]. Therefore, IMRT is the only approach that helps in sparing these critical organs.

1.5.5 Chemotherapy treatment for advanced NSCLC

It is estimated that over 30% of NSCLC cases are diagnosed as stage 4 disease [205]. For advanced-stage NSCLC, chemotherapy would be a long-standing mainstay treatment. In the past 20 years, chemotherapy has shown to have clinical benefits and widespread uptake for advanced NSCLC. Studies have reported that platinum-based doublet regimens not only have prolonged survival in comparison to supportive care but have also improved the quality of life [206]. Ever since, new cytotoxic agents have emerged with the characterization of improving efficacy, better tolerability, or both [207, 208]. The use of antiemetic drugs has also led to improvement in the patient treatment experience [209, 210]. A survival benefit has been associated with the prolongation of the treatment regimens [211]. The introduction of anti-angiogenic agents has shown modest improved outcomes [212, 213].

The combination of chemotherapy with molecularly targeted therapeutics agents has been under investigation in several clinical trials conducted on NSCLC patients with preclinical data supporting its potential use for synergistic efficacy and nonoverlapping toxicities [200]. The list of investigated drugs include: matrix metalloproteinase inhibitors [214], poly ADP ribose polymerase inhibitors (PARP) [215], histone deacetylase (HDAC) inhibitors [216], EGFR inhibitors, anti-angiogenic agents, insulin growth factor (IGF) [217], and heat shock protein (HSP)-90 inhibitors [200, 218]. Up to the present time, drugs targeting VEGF-VEGFR axis and drugs targeting EGFR were the only drugs to show promise in the combined treatment [200].

1.5.6 Epidermal growth factor receptor inhibitors in advanced NSCLC

The FLEX trail [219], an international open-labeled phase III trial, has compared the efficacy of cetuximab (an EGFR inhibitor) plus chemotherapy versus chemotherapy alone in EGFR-positive NSCLC patients. Patients who received cetuximab in addition to chemotherapy had a significant longer survival compared to chemotherapy alone ($P = 0.04$) with a median survival of 11.3 months and 10.1 months, respectively (HR=0.871). The main observed toxicity was an acne-like skin rash, and 10 % of patients on cetuximab experienced severity of grade 3 [219]. In the RTOG 0324 phase II trial [220], the combination of cetuximab with chemoradiation was investigated in unresectable stage III patients. In this single arm trial, the reported median survival was 22.7 months and 2-year overall survival was 49.3%, which is higher than previous reports at the time of the trial [221, 222]. These promising results have led to RTOG 0617 phase III trial [204] which evaluated cetuximab with standard and high-dose chemoradiotherapy. The median survival for patients who received cetuximab was 25 months and 24 months to patients who did not receive cetuximab (HR = 1.07) [204]. Furthermore, the addition of cetuximab to treatment was associated with significantly high rate of toxicity ($P <0.0001$). Grade 3 or higher toxicity rates were reported in 86% of cases treated with cetuximab and 70% without [204]. This has led to the conclusion that addition of cetuximab to concurrent chemoradiation or consolidation treatment did not provide any survival benefit and can result in an increased treatment-related toxicity [223].

Tyrosine kinase inhibitors (TKIs), such as gefitinib and erlotinib, play a critical role in the management of locally advanced NSCLC. Gefitinib is usually used for patients with disease refractory to standard chemotherapy, however; when it is used as a first-line treatment it did not show to improve survival [112, 113, 224]. The INTACT trials have randomized unresectable

locally advanced to metastatic, chemotherapy-naïve patients to receive gefitinib with platinum-doublet chemotherapy of platinum-doublet alone. The median survival, time to progression, or response rate was not improved when gefitinib was added to chemotherapy as a first-line treatment. Moreover, the SWOG S0023 trial [224], has reported that the median survival with gefitinib maintenance following concurrent chemoradiation with cisplatin-etoposide decreased to 23 months compared to 35 months from placebo (P = 0.013). The noted decreased in survival is primarily due to disease progression rather than treatment toxicity. It is worth mentioning that these trials have enrolled patients who are with or without EGFR mutations, which suggests that outcome may differ if patient enrolment was limited to those with EGFR mutations [224].

Erlotinib a TKI that is often used for patients with locally advanced and metastatic disease. In the TRIBUTE study [225], 1059 stage IIIB and IV NSCLC patients were randomized to receive either erlotinib or placebo in combination with six cycles of carboplatin/paclitaxel. They reported that there was no benefit with the addition of erlotinib to overall survival and time to disease progression, there was a survival benefit among patients who never smoked. Erlotinib has caused an increase in the median survival to 22 months compared to 10 months with just carboplatin/paclitaxel alone. In a secondary analysis, patients with EGFR mutations had a better response rate ($P < 0.05$) and a trend toward an improved time to disease progression [226] (P =0.92). However, the overall survival remained the same when erlotinib was added for this subset of patients (P= 0.96).

Radiotherapy combined with immunotherapy could result in enhanced anti-tumor immune response in local and systemic controls. Randomized phase III PACIIFC trial investigated the benefit of adding immune checkpoint inhibitors [227]. Durvalumab, which is a human IgG monoclonal antibody that blocks programmed cell death ligand (PD-L1) binding to programmed

cell death protein (PD-1), was tested following concurrent chemo and radiation therapy (cCRT) with platinum-based doublet versus placebo [227]. The use of Durvalumab has resulted of a better PFS (16.8 months vs. 5.6 months with placebo) [227]. However, 15.4% of the Durvalumab patients group discontinued in the study due to drug adverse events compared to 9.8% of placebo patients group [227]. The combination of radiotherapy and immunotherapy will be discussed in detail in other sections.

1.5.7 Impact of driver mutations on the response to SBRT as a treatment

SBRT is now considered as a treatment option for inoperable or those who refuse surgery, however, recurrence remains as one of the major challenges to be faced following treatment with SBRT. Biological effective dose (BED) and tumor diameters are considered as predictive factors of local recurrence [228]. In the study done by Nakamura et al. [229], they have investigated the pattern of recurrence according to irradiation field after CyberKnife SBRT in early stage NSCLC. In their study, they have reported out-of-field recurrence to be significantly associated with EGFR mutation [229]. Another study by Nakamura et al. [230] has evaluated the failure pattern after definitive chemoradiotherapy in patients with stage III NSCLC harboring EGFR mutations and/or ALK translocation. Their findings reported a significantly lower rate of in-field failure and higher rate of out-of-field failure when compared to wild-type EGFR group [230]. These findings suggest that EGFR mutation is related with out-of-field recurrence after SBRT.

The study conducted by Cassidy et al [231] has assessed the frequency of genetic aberration in early stage NSCLC patients on clinical outcomes after definitive treatment with SBRT. In their study, they have used next-generation sequencing (NGS) and fluorescence in situ hybridization (FISH) to help them in their assessment. They have analyzed 98 samples that were collected from 242 patients. They identified the following mutations: KRAS, BRAF, SMAD family member 4

(SMAD4), EGFR, STK1, TP53, and PTEN [231]. They have also reported gene rearrangements such as: ALK, RET and MET amplification [231]. Their findings report that KRAS-mutated patients were found to have worse local control post-SBRT treatment and patients with MET amplification were found to have worse regional and distant disease control post-SBRT treatment [231].

Association between PIK3CA mutations and outcomes results have been conflicting in literature. While some studies have reported that PIK3CA mutations have demonstrated an improved overall survival in squamous cell lung cancer and breast cancer [232, 233], others have reported a decreased progression-free survival in lung adenocarcinoma [234]. Lockney et al [235] have investigated whether mutations in the EGFR/AKT/PIK3CA signaling pathways are associated with local failure following lung SBRT. They have retrospectively reviewed 166 patients who underwent SBRT and local failure occurred in 16 patients (10%). In univariate analysis, PIK3CA mutations were associated with local failure while tumor histology, tumor size, primary tumor, BED and EGFR mutations were not [235].

Other studies have looked at the benefit of SBRT in more advanced stages. Normally, in stage IV NSCLC patients the first-line of treatment is chemotherapy. However, stage IV patients who are positive for EGFR mutation or ALK rearrangement are given anti-EGFR (gefitinib, erlotinib, afatinib, osimertinib) or anti-ALK (crizotinib, ceritinib, alectinib) tyrosine kinase inhibitors. Borghetti et al. [236] have investigated the association of conventional radiation therapy (RT) or stereotactic body radiotherapy (SBRT) in combination with TKIs for EGFR-mutant or ALK rearrangement-positive stage IV NSCLC. Their study demonstrated that the combination of RT with TKIs was well tolerated and can be a promising treatment option, particularly when stereotactic RT with ablative aim is delivered concomitantly with TKI [236]. Moreover, they

proposed that performing RT concomitantly and without suspension of TKI may lead to an extension of the drug administration which will lead to delaying the switch to a second-line therapy [236]. Their work also suggests that local therapy such as radiotherapy can contribute to optimizing the management of NSCLC with a driver mutation not only in ablative setting, but also in palliative ones [236].

In review by Basler et al [237] has investigated the potential of using SBRT on TKI-resistant sub-clones in oligo-progressive disease (OPD). In their review of retrospective studies, they suggest that the application of aggressive local treatment, such as SBRT or surgery, can help in eradicating TKI-resistant subpopulations and restoring their sensitivity to TKIs. This will enhance PFS and will prolong treatment time with TKI which will result in an increased overall survival [237]. Al-Halabi and his colleagues [238] have analyzed the pattern of failure in TKI-treated metastatic EGFR-mutant NSCLC patients and tried to identify whether a subset of patients would amenable to consolidation SBRT. Their findings, which they advise to be viewed as hypothesis generating, suggest that consolidation SBRT with continued TKI therapy could alter the natural history of disease progression allowing for an extended PFS and overall survival of a subset of patients with stage IV EGFR-mutant NSCLC [238].

1.6 Radiation and tumor radiobiology

1.6.1 Radiation therapy

Radiation therapy uses ionizing radiation to induce cell and tissue destruction. Ionizing radiation (IR) can be classified into two major types: **1. Photon radiation** (x-rays and gamma rays), and **2. Particle radiation** (such as electrons, protons, neutrons, carbon ions, alpha particles, and beta

particles). The energy of each type of IR is different where some can produce higher energy compared to others and higher energy of IR allows for a higher penetration of the tissues. In cancer, radiation is used to cause destruction of malignant diseases. During cancer treatment, radiation deposits energy that leads to cell damage. The amount of energy delivered to tissues is referred to as the absorbed dose. Gray (Gy) is the unit denoted for radiation, where 1 Gy is the equivalent of 1 Joule/kg [239]. In solid tumors, the typical dose range is 60 to 80 Gy and for lymphomas 20 to 40 Gy.

1.6.2 History and evolution of radiation therapy

Since the discovery of x-rays in 1895 by Wilhelm Roentgen and the discovery of radium by Marie and Pierre Curie in 1898, the application of IR has been evolving constantly to keep up with the medical challenges and needs. In 1896, Victor Despeignes has performed the first x-ray treatment ever to a cancer patient [240]. In the same year, Emil Herman Grubbe used x-rays to treat a patient with breast cancer [241]. An increased interest in studying the use of x-rays and radium in medicine started in the beginning of the new century. The most frequently treated cancer type using x-rays was skin cancer due to low radiation penetration in the tissue.

In the 1910s, a new device developed by Coolidge had the ability to emit higher energy x-rays allowing for treatment of deeper cancers [241]. Limited access to information on the properties and mechanism of actions of radiotherapy has led to poor outcomes in terms of effectiveness and benefits which has generated interest among physicians to initiate studies that can provide better understanding of treatments [242]. In the 1920s, physicians started to develop better understanding of how administration of the total dose in fractions was better than a singular treatment session in

terms of cancer control and less side effects [243]. In the 1928, the International Commission on Radiological Protection (ICRP) was created to address questions and concerns regarding radioprotection [244].

The following period, from 1930 to 1950, was characterized by the progress in treating patients effected by deep cancers and was known as the Orthovoltage era. This era was characterized by using the radium-based interstitial irradiation (brachytherapy) and by the development of supervoltage X-ray tubes which enabled the delivery of energy from 50 kV to 200 kV such as electron beam therapy. The use of brachytherapy allowed operators to deliver energy and treat tumors without an external beam source which can limit the side effects on unaffected tissues. In the case of electron beam therapy, it has allowed for the delivery of a higher and variable energy that can be beneficial in treating deeper tumors [241].

1.6.3 The application of quadratic-linear in the assessment of radiotherapy

The use of radiation as a tumor treatment is a double-edged sword where not only damages tumor tissues but also damages healthy ones. This has led researchers to establish a way of maximizing tumor cell kill and avoiding normal tissue toxicities. Colony formation was used as a means of determining cell survival since the publication of the first mammalian radiation survival curve by Puck and Marcus [245]. Investigators fitted survival curve data using multi-target model. In this model, cell survival (**S**) is described in terms of the dose (**D**), a parameter $\mathbf{D_0}$, which is the slope of the exponential portion of the curve, and extrapolation number **n** resulting in the following equation:

$$S=1-(1-e^{-D/D0})^{n}$$

The equation provided a good fit for most experimental data of a wide range of cell killing. However, this equation does not provide a good fit for data obtained at low radiation doses, which predicted zero cell killing at these doses. This has led to replacing this equation with the quadratic-linear (QL) equation which is as follows:

$$S=e^{-(\alpha D+\beta D2)}$$

The use of this model became so successful that it has been used by the radiation oncology community to calculate and determine changes in dose per fraction or in number of fractions to achieve the same radiation effects on normal tissues as a standard fractionation regime. To achieve these calculations, the value α/β is needed where it is usually considered to be ~3Gy for late responding tissues (normal tissues) and ~10 Gy for early responding tissues which includes most tumor tissues [246]. The use of the LQ model has been so successful that it has been used in clinical trials of hyperfractionation to predict superiority of regimes with small doses per fraction (<2 Gy) which aims in decreasing the late effects while maintaining the same early effects (tumor response) [247].

The LQ model has been reasonably a predictive model of *in vitro* and *in vivo* normal tissue dose-response in relation to the dose per fraction ranging from 1.8 to 20 Gy and currently there is no better model to replace it for predicting cell killing. Insufficient clinical evidence at the present time suggest or indicate of a need to modify or replace the current LQ model. There is no model that can describe the dose-time patterns in a complete and correct way. Brenner has investigated the mechanistic uncertainties of the LQ model [248]. Brenner concluded that although the LQ model is attributed to chromosome aberrations other cell killing, such as apoptosis and lethal mutations, can also be accommodated by the LQ model [248].

One major concern of LQ model generality at high doses is repair saturation at high doses. Two arguments were suggesting otherwise. First, the dose response curves for normal tissues fit the LQ model up to a least 20 Gy. Second, the rate and extent of DSB repair is similar in cells after 1 Gy which is usually determined by γ-H2AX loss, and after 80 Gy which is determined by pulsed field gel electrophoresis [249]. Therefore, there is no enough data that supports the idea of repair saturation at high doses of radiotherapy.

Although the LQ model fits the response of both in vitro and in vivo normal tissues, there are some questions that need to be addressed in term of tumor response to radiation. Theses questions need to address whether SBRT provides better results than the standard fractionation. In the following sections, we will address challenges of SBRT application.

1.6.4 The abscopal effect

Advances in radiotherapy technologies have focused on minimizing toxicity while improving therapeutic outcome when treating localized tumor. Interestingly, it was observed that in patients with multiple lesions who were treated with radiotherapy had tumor regression, in rare cases, outside the field of radiation. This phenomenon is known as the "abscopal effect", which is derived from "ab"-away from, and "scopus"-target. In 1953, Mole et al. [250, 251] were the first to describe the "abscopal effect" which describes an immune-mediated response to radiation by tumor cells located distant from the irradiated site [251].

Rare events of abscopal effect have been reported in several cancers including melanoma [252], renal cell carcinoma [253], breast cancer [254], hepatocellular carcinoma [255] and other solid tumors [256]. A recent published review has stated that the abscopal effect has been reported in

46 case reports in the period between 1969 and 2014 [257]. **Figure 1.10** summarizes important development regarding the abscopal effect.

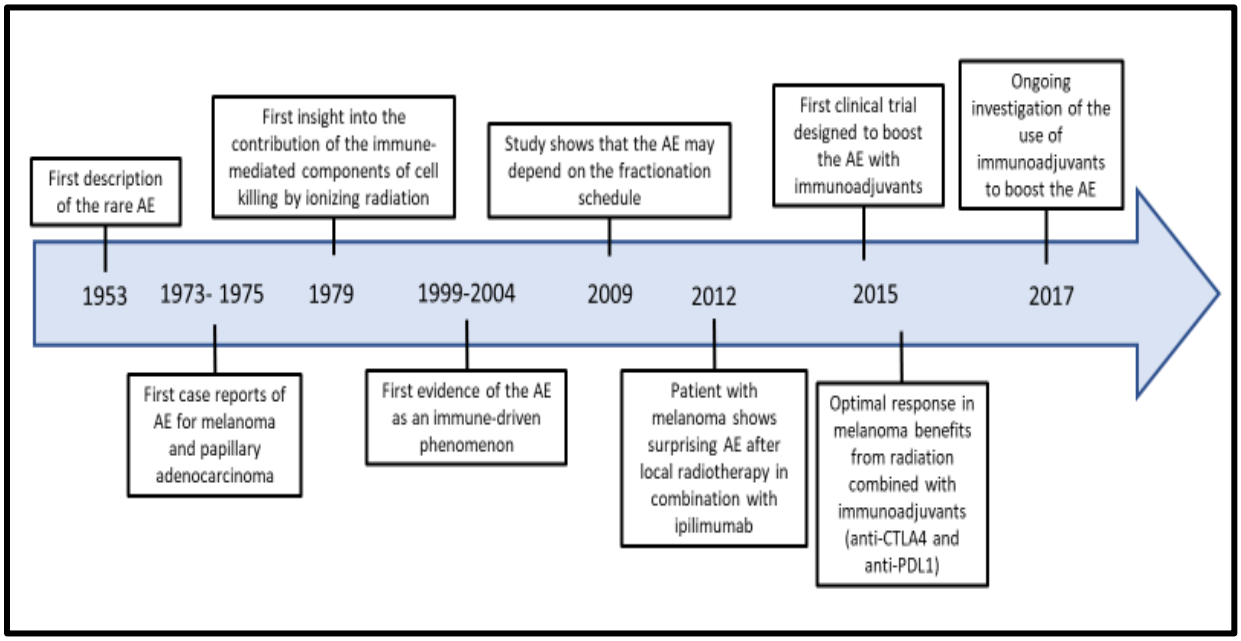

Figure 1.10: Historical timeline of some important developments regarding the abscopal effect. AE: abscopal effect, CTLA4: cytotoxic T lymphocyte-associated antigen 4, PDL1: programmed cell death ligand 1. Adapted from [251].

Initially, radiotherapy has been assumed to be immunosuppressive due to the exquisite radio-sensitivity of leukocytes, however; recent data has shown that radiation therapy can enhance the machineries of antigen processing and presentation pathways [258-260]. In a study done by Reits et al., radiation therapy has caused a dose-dependent increase in the cell-surface MHC-I levels [261]. This could be explained by an increased intracellular peptide pool which resulted from either an increase in protein translation or an increase in protein degradation which can generate a large repertoire of epitope that can be presented following tumor cell death [261].

Increase in antigen release and MHC-I expression is insufficient for the activation of anti-tumor T-cell. For this activation to be achieved, maturation of antigen presenting cells (APCs) is required. Maturation of APCs involves upregulation of MHC-I and -II, increase in the expression of the costimulatory ligands B7-1, B7-2 and cytokines that are involved in T-cell proliferation and phenotypic skewing [262]. Maturation of APCs occurs at the event of APC pathogen recognition receptor (PRR) ligation by non-self-derived adjuvants, pathogen-associated molecular patterns (PAMPs), or endogenous damage associated molecular patterns (DAMPs) [263].

Radiation therapy induces immunogenic cell death (ICD), which unlike apoptosis, causes release of tumor contents such as DAMPs that can be highly pro-inflammatory. Radiation-induced ICD and released DAMPs include high-motility group box 1 (HMGB1), heat shock protein 70 (HSP 70), GP96 and calreticulin membrane exposure [264-266]. Calreticulin is an endoplasmic reticulum resident molecular chaperon which functions in stimulating phagocytosis of cancer cells by dendritic cells (DCs) [267]. HMBG1 on the other hand, is a critical chromatin protein that is responsible in promoting antigen presentation [268]. Radiation-induced calreticulin exposure can enhance the T-cell mediated tumor lysis, and this effect can be abrogated using calreticulin-blocking peptide [269]. Wang et al. have reported that HMBG1 extracellular release and cytoplasmic translocation happen in a dose and time-dependent manner following radiation therapy [270]. A study has reported the critical role of APCs in anti-tumor T-cell priming and abscopal effect. The study has demonstrated in a bilateral syngeneic mouse model of breast cancer wherein immunoadjuvant treatment with FMS-like tyrosine kinase receptor 4 ligand (FLT3L), which promotes dendritic cells development and bone marrow egress [271], has resulted in delayed growth in irradiated flank tumor and the untreated, contralateral tumor [272]. Taken together the

previously mentioned data, there is a strong relationship between an anti-tumor response and radiation therapy mediated tumor cell killing.

1.6.4.1 The biological mechanism of the abscopal effect

The exact biological mechanism of the abscopal effect is not fully understood, however; several studies have proposed possible explanations in which abscopal effects get activated. When a tumor is irradiated, this induces a cellular stress or injury which in turn causes release of neoantigens, also known as tumor-associated antigens (TAAs), in the context of necrotic and apoptotic tumor cells and debris. Increase in the released number of TAAs can trigger a tumor-specific immune response where TAAs get engulfed by APCs and then presented to $CD8^+$-T cells. Subsequently, this causes the $CD8^+$-T cells to recognize and attack both primary and metastatic tumor cells [273] (**Figure 1.11**). As mentioned previously, tumor irradiation causes release of DAMPs and cytokines that promote circulation of immune cells [274]. Collectively, this leads to elimination of tumor cells by primed $CD8^+$-T cells [275, 276].

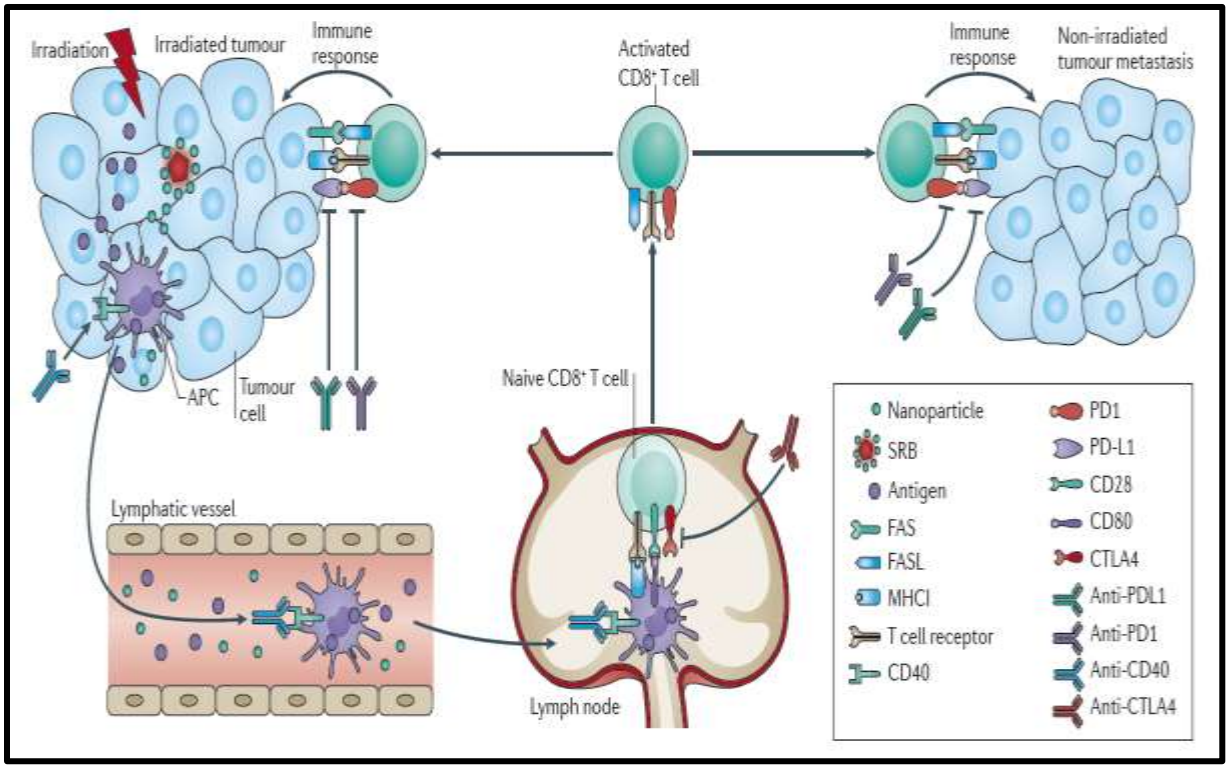

Figure 1.11: The abscopal mechanism. Adapted from [251].

Since the abscopal effect is a rare event, it has been suggested that even primed antitumor $CD8^+$-T cells are incapable in overcoming the suppressive effect of tumor microenvironment [274, 275]. Some of the released cytokines by tumors include: transforming growth factor beta (TGF-β), and surface receptors such as CTLA4, that can lead to inhibition of T cells. T cell functions can also be inhibited by M2 macrophages, myeloid-derived suppressor cells (MDSCs) and immature DCs [274, 275]. Moreover, $CD4^+$ T cells with regulatory function (T_{reg}) can also prevent tumor elimination.

1.6.4.2 The combination of radiotherapy and immunotherapy

As mentioned previously, the cellular mechanisms behind the abscopal effect is not fully understood. Most studies have tried to investigate the abscopal effect mechanism in the combination of radiation therapy (RT) with either anti-CTLA4 or anti-PD-L1 (**Figure 1.12**) to prime the immune system [277-279]. Numerous trials are investigating whether immunotherapy (IO) should be combined with radiation therapy either concurrently or adjuvantly. The key goal of the combination treatment of RT and IO is to enhance or boost abscopal effect.

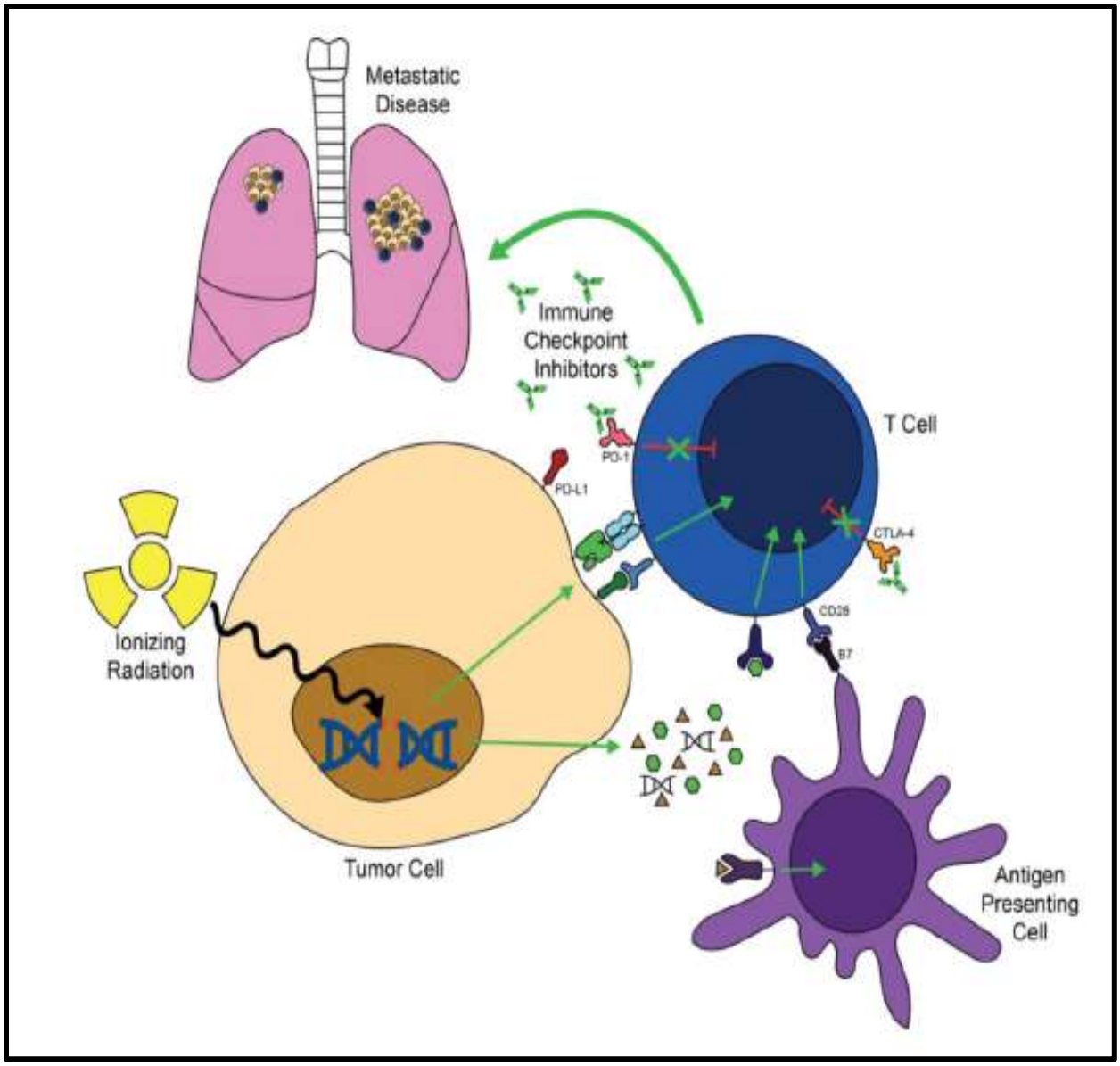

Figure 1.12: Combination of SBRT and immune checkpoint inhibitors. Ionizing radiation (IR) causes release of cytokines and pro-inflammatory molecules from tumor cells that can induce recruitment of antigen presenting cells immune effector cells to the tumor microenvironment. Moreover, IR increases secretion and presentation of tumor antigens that can trigger tumor-specific immune responses. Application of immune checkpoint inhibitors such as anti-PD-1 and anti-CTLA-4 antibodies synergize with radiation and blocking T cell inhibitory signals. Immune cells

can contribute in tumor death at the site of radiation, and radiation can stimulate tumor-specific immune responses that could lead to regression of metastatic disease outside the field of radiation. Adapted from [280].

A study by researchers at the New York University Medical Center was done to investigate the abscopal effect at cellular level using a murine model of breast and colon cancer [278]. In their study, they have implanted tumors in one flank on Day 0, and the contralateral flank on Day 2. The primary tumor received radiation twelve days following implantation, and anti-CTLA4 was as administered two day following radiation. Radiation of the primary tumor was done in a fractionated manner, not in a single fraction, which has led to tumor regression of the secondary tumor when combined with anti-CTLA4 [278]. Furthermore, this effect was observed upon application of IO during radiation, as opposed to administration following completion of radiation. Tissues from both primary and secondary tumors were collected and analyzed and revealed presence of tumor infiltrating lymphocytes (TILs), both CD4+ and CD8+. Moreover, a positive correlation was noted between the expression of interferon gamma (INF-γ) and rejection of the secondary tumor [278]. It was also noted that INF-γ plays a critical role in molecular signaling where CD8+ lymphocytes with tumor specific INF-γ expression were frequently present in rejected secondary specimens [278].

A group of researchers has later demonstrated that the cytoplasm can be a key inhibitor in immune upregulation following radiation [281]. Radiation results in degradation of DNA which accumulates in the cytosol. The cytosolic DNA is then used by cGAS and downstream effector STING to induce stimulation of INF-β by cancer cells and function in activating dendritic cells that are essential for priming of the CD8+ effector cells. Although studies have reported that three to five fractions of radiation can result in the abscopal effect, Vanpouille-Box colleagues have

demonstrated that the presence of cytosolic DNA is essential for the activation of this mechanism [281]. They have reported that activation of DNA exonuclease, Trex1, occurs within radiation doses above 12 to 18 Gy delivered in a single fraction, and this activation did not occur when using three to five fractions of radiation below this dose threshold. Active Trex1 causes degradation of the cytosolic DNA which is required for the downstream activation of CD8+ cells, as mentioned previously [281]. Therefore, radiation can function in both up- and down-regulation of immune system activation through modulation of the intracellular signaling.

Another study by Wang and colleagues [277] has demonstrated that RT can abrogate resistance to anti-PD-1 immunotherapy by increasing the expression of MHC-I. In their study, they have created a murine model that is resistant to anti-PD-1 by passaging 344SQ parental (Kras-mutated, p53 deficient) murine lung cancer cell line in a host with multiple rounds of anti-PD-1 antibodies [277]. While MHC-I was expressed on the parental 344SQ_P cell line, it was absent in the 344SQ_R cell line. To examine if RT can re-sensitize the resistant 344SQ_R cells, mice inoculated with 344SQ_R cells received a cumulative dose of 36 Gy over three fractions. Six days post-treatment, mice were sacrificed, and tumor cells were analyzed using flow cytometry. It was noted a significant increase of both MHC-I and -II following radiation. Researchers have also looked at the combination of radiation and IO, where they have treated 344SQ_R mice with RT followed by anti-PD-1 therapy. They have reported that tumor regression in both primary (irradiated) and secondary (non-irradiated) tumors, suggesting that RT can abrogate resistance in an anti-PD-1 resistant tumor model [277].

Current clinical trials are trying to investigate the optimal timing of treatments. A phase II clinical trial, NCT02239900 trial, of patients with lung and liver cancers is being conducted where patients will receive ipilimumab with concurrent or sequential RT [282]. In the sequential group, SBRT

will begin at the second cycle of ipilimumab, while the concurrent group will receive SBRT immediately after the first cycle of ipilimumab [282].

1.6.5 Implication of tumor burden and stroma in the response to radiation and immunotherapy

1.6.5.1 Tumor stroma can have negative effects on tumor response

Although the combination of RT and immunotherapy have led to promising outcomes, the mechanisms by which they synergize are not fully explained. Researchers have identified other critical factors that can have an impact on tumor response to RT and immunotherapy. These factors are: overall tumor bulk and tumor stroma [283, 284].

Stromal tissue is considered one of the major components of the tumor microenvironment, including NSCLC [285]. Tumor stroma involves mostly cancer associated fibroblasts (CAFs) and is phenotypically different and can be metabolically active [286]. Within the tumor microenvironment, abnormal stroma can have many implications in tumor response to treatment. Stroma involvement includes: supporting malignant proliferation of cancer cells, preventing penetration of systemic therapies, inducing resistance to radiation through hypoxia and upregulation of hypoxia-inducible factor-1-alpha (HIF1-α), and stimulating tumor cells to metastasize [286-288].

Zhang and colleagues have examined resected tissues of patients with NSCLC and highlighted the prognostic value of tumor stroma ratio (TSR) [285]. TSR can be defined as the ratio of tumor volume to stroma volume where hematoxylin and eosin staining was done on resected tissues and was used for the assessment. Patients were divided into stroma-rich (tissue with >50% stroma volume) group, and stroma-poor (tissue with <50% stroma by volume) group. They have reported

that the stroma-rich patient group had significantly the poorest outcomes. Their multivariate analysis of 5-year overall survival reported statistically significant hazard ratio (HR) and disease-free survival (DFS) of 1.748 and 1.570, respectively. Moreover, correlations of low TSR and poor outcomes have been reported in histology of other cancers such as breast, colorectal, esophageal, hepatocellular, but not in cervical cancer [284].

1.6.5.2 Implication of tumor burden decrease on improving outcomes

Traditionally, radiation was used as a palliative treatment. However, recent developments in image guidance and stereotactic delivery have provided clinicians the ability to precisely define tumor location and geometry. This has allowed radiation oncologists to deliver ablative doses to metastases with minimal damage to surrounding healthy tissues. The application of SBRT in the setting of oligometastatic disease has increased to achieve local control and extend survival [289-291]. Unfortunately, patients show distal failure with an average time of 12 months. This emphasizes on the rationale for combining stereotactic radiation with systemic agents.

Other studies have suggested that treatment of oligometastatic disease with stereotactic radiation can have different mechanisms in causing an effective immunomodulation. Huang and his colleagues have investigated potential factors that can influence re-invigoration of exhausted T-cells in patients with metastatic melanoma receiving pembrolizumab [283]. They have reported that the overall tumor burden contributes in the clinical outcomes of these patients. In their study, they stated that the ratio of the degree of T-cell reinvigoration to tumor burden was directly proportional to progression-free survival (PFS) (**Figure 1.13**).

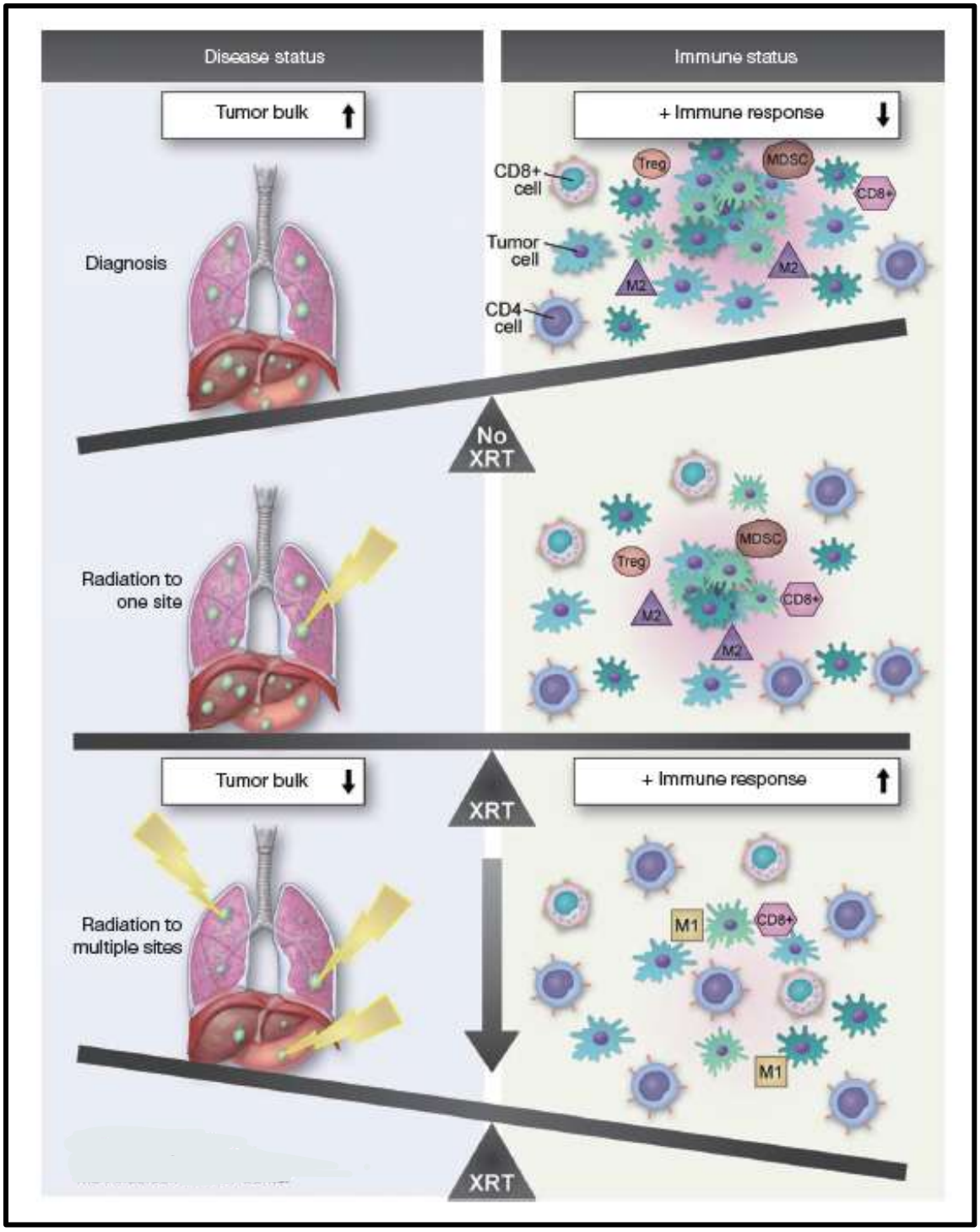

Figure 1.13: Relationship between tumor bulk and radiation. Radiation of multiple sites versus single site, and impact on the systemic immune response to treatment. adapted from [282].

The importance of tumor burden in the response to immunotherapy has been investigated by several studies that are showing combination of RT with immunotherapy even in sequential order can have a good outcome. Shaverdian and colleagues [292] have mentioned that previous treatment with radiotherapy in advanced NSCLC patients had resulted in a longer progression free and overall survival when treated with pembrolizumab in their phase I KEYNOTE-001 clinical trial.

1.6.6 SBRT and the 5Rs of radiobiology

Radiation can cause cell death by triggering double-strand breaks (DSB) in DNA, which prevents cells from reproducing. The response of tumors to radiation has been characterized by factors that are implicated in the ability of radiation to induce DNA damage, and the ability of certain tumor cell populations to recover from such a damage. Biological studies on the response to radiation have indicated five factors (**Figure 1.14**) that can influence the effect of radiation therapy on tumor response. These five factors are: **1.** Repair of cellular damage, **2.** Repopulation of cells following exposure to irradiation, **3.** Redistribution of cells within the cell cycle, **4.** Reoxygenation of the surviving cells, and **5.** Radiosensitivity of cells. We will review these factors in more details in the following section.

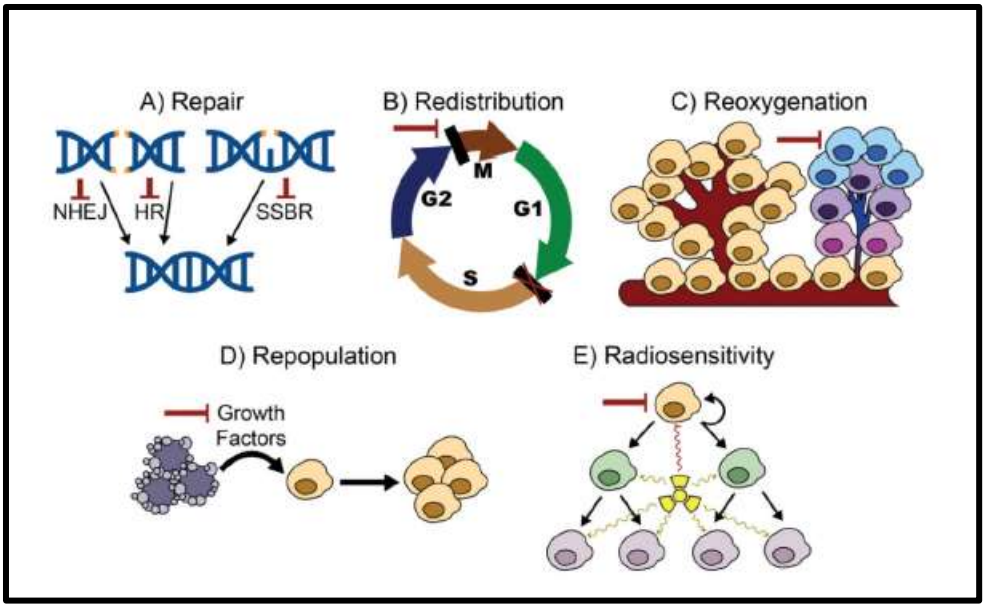

Figure 1.14: The 5R's of radiation biology. A) Non-homologous end joining (NHEJ), homologous recombination (HR), and single strand break repair (SSBR) are critical for repairing double-strand and single-strand DNA breaks that result from SBRT. Proteins that are involved in these responses can be targeted leading to radiosensitization effect. B) Blocking cell cycle checkpoints such as the G2/M transition may induce mitotic catastrophe following SBRT. Tumor cells that are deficient in checkpoints due to mutations or altered gene expression have an increased effect of mitotic catastrophe. C) Hypoxic cells are resistant to radiation, therefore, targeting these cells could lead to a better local control with SBRT. D) Dying cells may secrete growth factors that induce proliferation of surviving tumor cells. Blocking the formation of these factors or their interaction with surviving cells may prevent repopulation of tumor cells following SBRT. E) The radiosensitivity of tumor cells and their ability to regenerate following radiation therapy vary within a tumor. Targeting cancer stem cells, which may be radioresistant, could increase tumor eradication with SBRT. Adapted from [280].

1.6.6.1 Repair of cellular damage

When cells are exposed to stressful events, such as IR, cells detect and repair their damaged DNA through diverse machinery. DNA is usually the main target of IR. Exposure to IR in tumor cells can generate reactive oxygen species (ROS), such as hydroxyl radicals, which can directly damage the DNA. We will discuss ROS in more details in other sections. In brief, hydroxyl radicals result through interaction of IR with water with the cell. The hydroxyl radical then get incorporated into the DNA at the sugar bases leading to DNA strand breaks. Studies have suggested that high doses of radiation can cause cell death by damaging cell membrane or other cellular compartments [293]. In ablative radiation such as stereotactic ablative radiotherapy (SBRT), which uses high dose per fraction (>8Gy), can kill cells by DNA damage that is similar to conventional fractionation [294].The resulted DNA damage can be in the form of double-strand breaks (DSB), single-strand breaks (SSB), cross-linking, chromosomal rearrangements, and base mismatch [295, 296]. DSB due to IR is the primary means by which radiation kills cells. DNA damage can trigger activation of the repair machinery or cell death. Outcomes that can occur as part of the DNA damage response (DDR) are summarized in (**Figure 1.15**). Larger radiation dose that are delivered over a short time period such as the one used in SABR causes an increased amount of DNA damage and may lead to more complex alterations that are more difficult to repair. Response to DNA damage depends on the cell type, type of damage, and cell cycle phase.

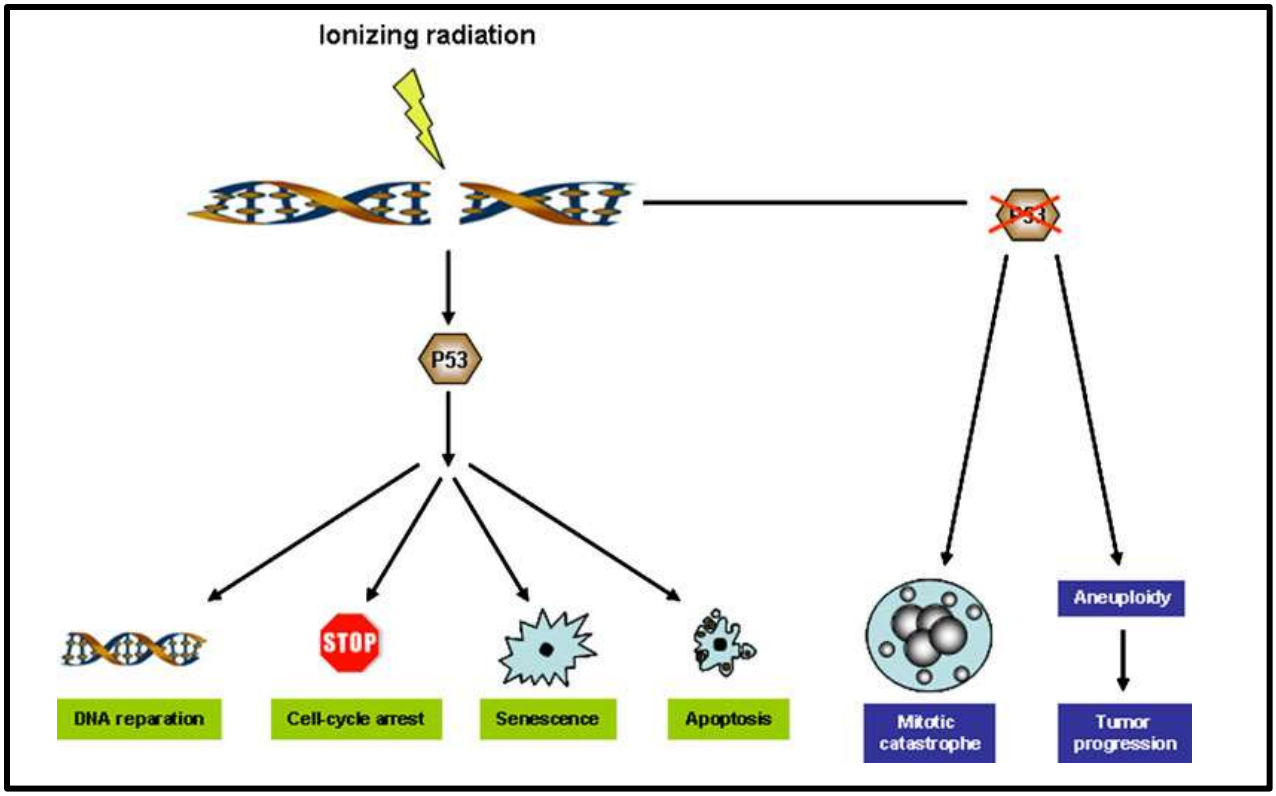

Figure 1.15: Summary of possible outcomes after DNA damage due to IR. Cellular outcomes are represented depending on p53 status. Adapted from [297].

It is worth mentioning that p53, a tumor suppressor, plays a major role in regulating DDR. DNA damage such as DSBs and SSBs are sensed by ataxia telangiectasia mutated (ATM), and ATM- and Rad3-related (ATR) causing stabilization of p53 which in turn triggers proteins that are involved in regulating proliferation, cell cycle checkpoints, apoptosis and DNA repair (**Figure 1.11**). During exposure to IR, cells with a functional p53 can go through apoptosis or induce activation of DNA repair. If the cell is unable to repair damaged DNA, it can remain in a permanent cell cycle arrest, known as senescence, or can go through apoptosis. In the event where p53 is mutated, which is present in 50% of cancers, exposure to IR causes DNA damage which leads to

cell survival through aneuploidy or cell death through mitotic catastrophe [297]. Tumors with mutated p53 have been reported to exhibit resistance to IR which suggests that tumor with mutated p53 have a worse prognosis compared to tumors with wild type p53 [298].

One key regulator of the cell cycle progression is the cell division cycle 25 (CDC25) phosphatase which is responsible in activating cyclin-dependent kinase (CDK) complexes. CDC25 functions by dephosphorylating two residues in the ATP binding loop of CDKs [299]. This indicates that CDC25 can function as an early sensor of DNA damage and cell cycle progression. The ATM/ATR kinases regulate CDC25 activity through phosphorylation and activation of checkpoint kinases CHK1 and CHK2 which in turn result in inhibition or degradation of CDC25 [300].

In cell cycle arrest, the cell cycle checkpoints get activated to participate in DNA repair machineries which can range from homologous recombination (HR), non-homologous end joining (NHEJ), base excision repair (BER), nucleotide excision repair (NER) to mismatch repair (MMR) (**Figure 1.16**). The choice of DNA repair pathways depends on several factors such as the nature and severity of the damage, the mode of damage and the cell cycle phase in which the damage took place [301, 302].

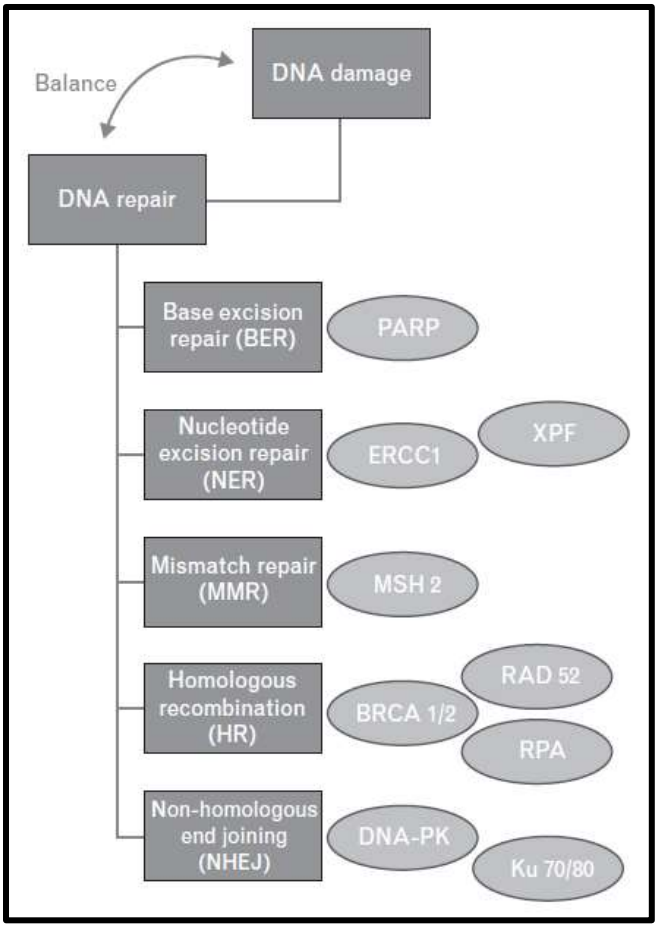

Figure 1.16: Representation of DNA repair pathways (in blocks) and proteins involved in each pathway (in circles). Adapted from [303].

In higher eukaryotes, the main DSBs repair pathways are: nonhomologous end-joining (**NHEJ**) and homologous recombination (**HR**) [304]. During all phases of cell cycle, NHEJ is the main repair mechanism of DSBs, especially during G0 and G1 phases [304]. During the late S/G2 phases, HR is main repair mechanism and plays a supportive role in the sister chromatid repair [304]. Both repair pathways have a close relationship with phosphoinositide 3-kinase related kinases (PIKKs) such as ATM, ATR and DNA-dependent protein kinase (DNA-PK) [304].

Activation of HR takes place with the activation of ATM and subsequent phosphorylation of H2AX at the DSB site. As a result of H2AX phosphorylation, BRCA1 and NBS1 repair proteins get recruited which then get phosphorylated by ATM [305]. In NHEJ (**Figure 1.17**), the process begins with binding of Ku heterodimers to DSBs and recruitment of DNA-PK catalytic subunits [306]. This leads DNA-PK catalytic subunits to bind to Ku and form complexes that recruit nucleases, polymerases and ligases to participate in the repair process. The extent of the repair mechanism following exposure to IR varies and depends on several factors such as dose per fraction, total dose and dose rate [307-309].

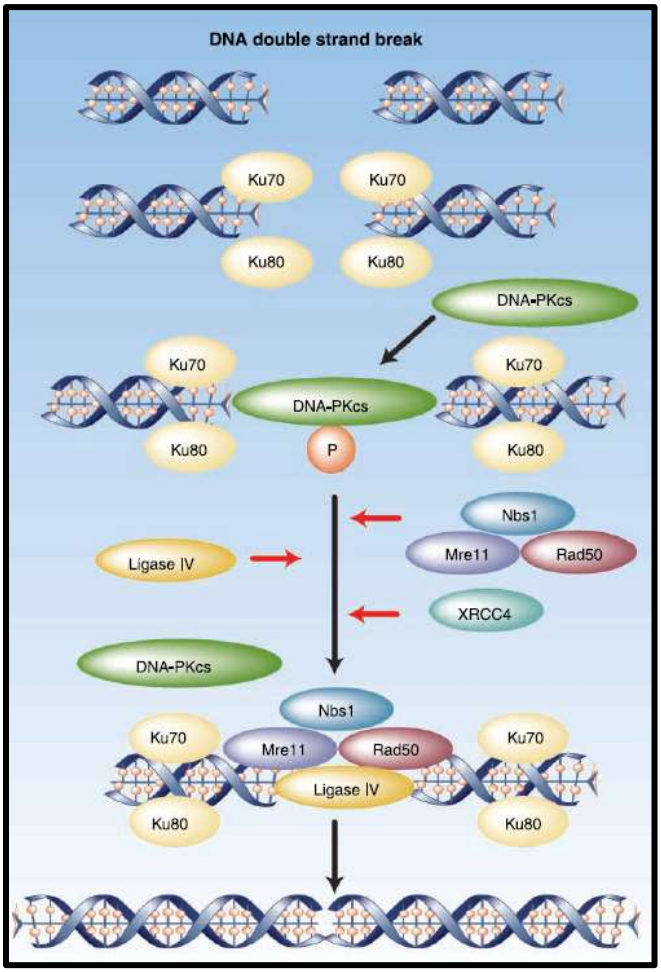

Figure 1.17: Radiation induces activation of the nonhomologous end-joining (NHEJ) DNA repair pathway. DSBs resulted from ionizing radiation bind to regulatory subunits (Ku70 and Ku80) and promote recruitment of catalytic subunit DNA-PKcs that prevents premature processing of DNA ends. Adapted from [310].

1.6.6.2 Repopulation of cells following exposure to irradiation

One of the major challenges regarding the use of radiation therapy is to minimize damage to normal tissues. Radiation using fractions of 1-3 Gy can allow healthy tissues to recover from sublethal doses of radiation. Unfortunately, this is also observed in tumor cells where they can survive radiation and repopulate. Repopulation of tumor cells is a major obstacle of using fractionated radiotherapy and is a serious challenge for tumor control [311, 312]. Moreover, studies have reported accelerated repopulation of tumor cells following exposure to fractionated IR [313]. It has been suggested that accelerated repopulation happens because of a combination of an increased proliferation of clonogenic cells and a reduced cell loss due to acquired radioresistance [314]. Comparison of cell survival curves of cells exposed to increasing doses of radiation compared to cells exposed to fractionated doses of radiation is shown in (**Figure 1.18**). Increased radiosensitivity was observed in cell survival curve of a single-dose of radiation compared to cell survival curve of cells exposed to multiple fractions of IR. Increased radioresistance is associated with the delivery of fractioned doses of IR and is considered a major contributor in cell repopulation and recovery from sublethal damage.

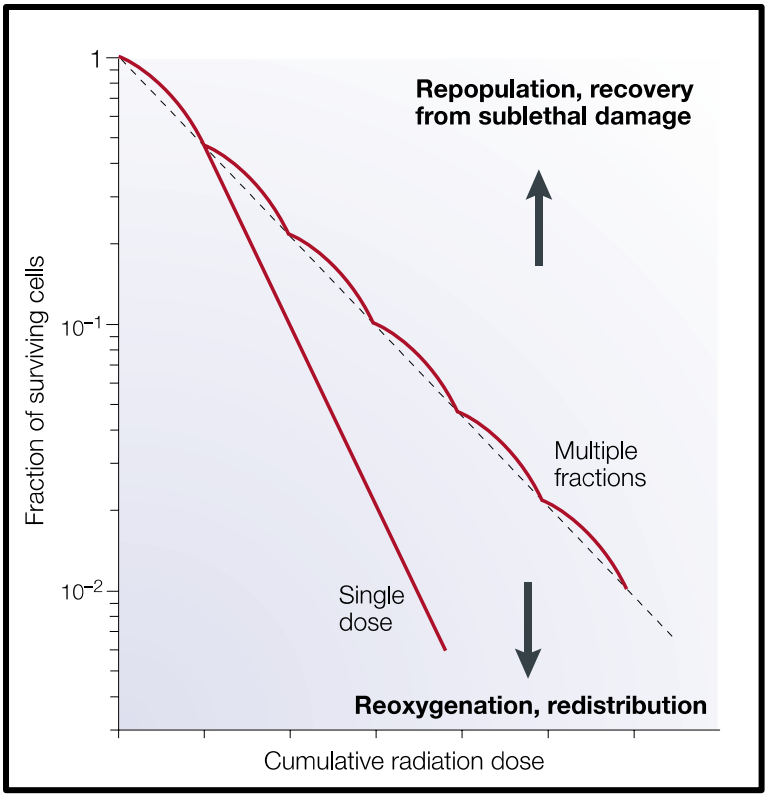

Figure 1.18: Cell survival curves after exposure to a single-dose radiation versus exposure to multiple fractions of radiation. Adapted from [312].

1.6.6.3 Redistribution of cells within the cell cycle

The cell cycle activity is regulated by a family of cyclin-dependent kinases (CDKs). The CDKs family functions in ensuring cells do not progress to the next cell cycle phase unless they have completed the previous phase. Cyclins are synthesized during cell cycle progression and then bind to CDKs to form a cyclin-CDK complex. The formed complex then causes cell cycle progression and subsequent degradation of the cyclins. When DNA damage is detected by ATM and stabilization of p53, the CDK inhibitor, p21 binds to the CDK-cyclin complexes and reduces their affinity for pRb, which in turn results in halting of cell cycle progression. Cells at different phases

of the cell cycle have different profile of radiosensitivity. For instance, cells in the mitotic and G2 phases are considered to be in a most sensitive state to radiation, whereas cells in the G1 and S phases are considered to be relatively more resistant to radiation [315, 316]. Reports on the variability of cells radiodensity in different cell cycle phases have been consistent across many cell lines and include lung cancer cell as well [316, 317] (**Figure 1.19**). Cells that are exposed to radiation doses of 1 Gy or more become arrested at G2 phase. Cell arrest at different phases of cycle happens due to activation of the cell cycle checkpoints which prevents progression to the next phase of the cell cycle before damaged DNA gets repaired. If a cell becomes arrested at G2 phase because of activation of the G2/M checkpoint and enters mitosis without damaged DNA being repaired, the cell may not be able to complete the process of mitosis and can go through a form of cell death known as mitotic catastrophe.

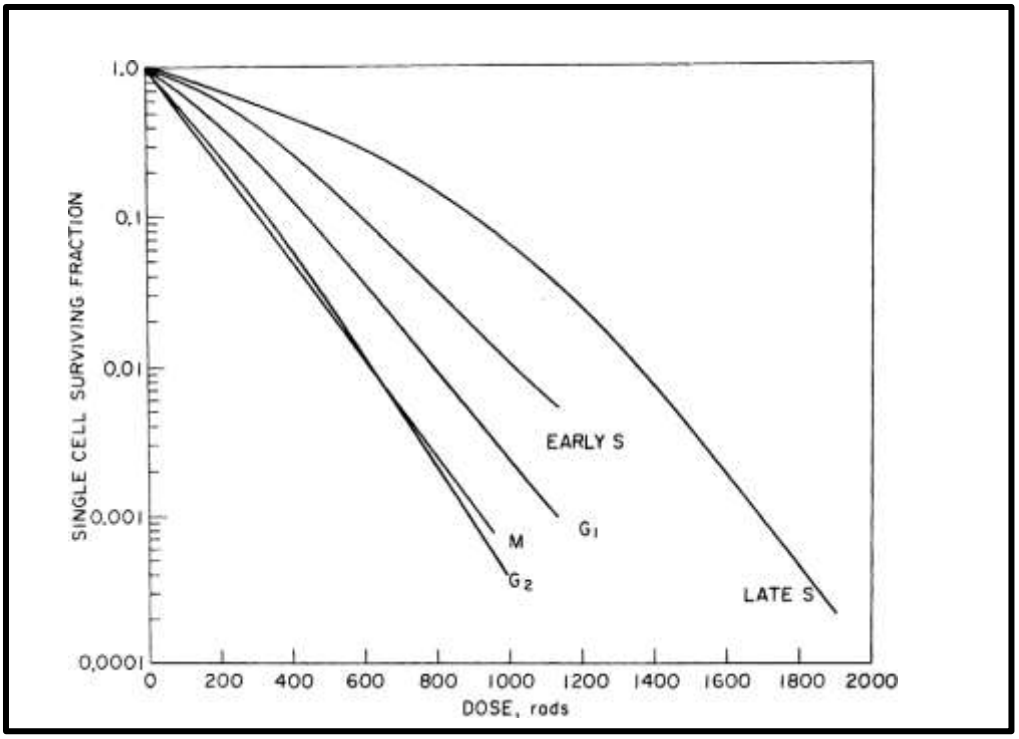

Figure 1.19: Representation of cell survival at different phases of cell cycle in Chinese hamster lung cells. Adapted from [318].

1.6.6.4 Reoxygenation of the surviving cells

Abnormality in tumor microcirculatory can lead to tumor hypoxia and poor oxygen diffusion. Tumor hypoxia is associated with tumor proliferation, malignant progression and an increased resistance to therapy. Tumor cells can adjust to hypoxic environment by expressing angiogenic factors, glycolytic enzymes, and stress proteins that work cooperatively to allow for cellular adaptation [319]. Adaption of tumor cells to hypoxic environment is regulated at the transcriptional level through hypoxia-inducible factor-1 (HIF-1), which can upregulate the expression of more than 400 genes that can include pro-angiogenic factors, such as vascular endothelial growth factor

(VEGF). The cellular mechanism of how HIF-1 can regulate hypoxia is demonstrated in (**Figure 1.20**).

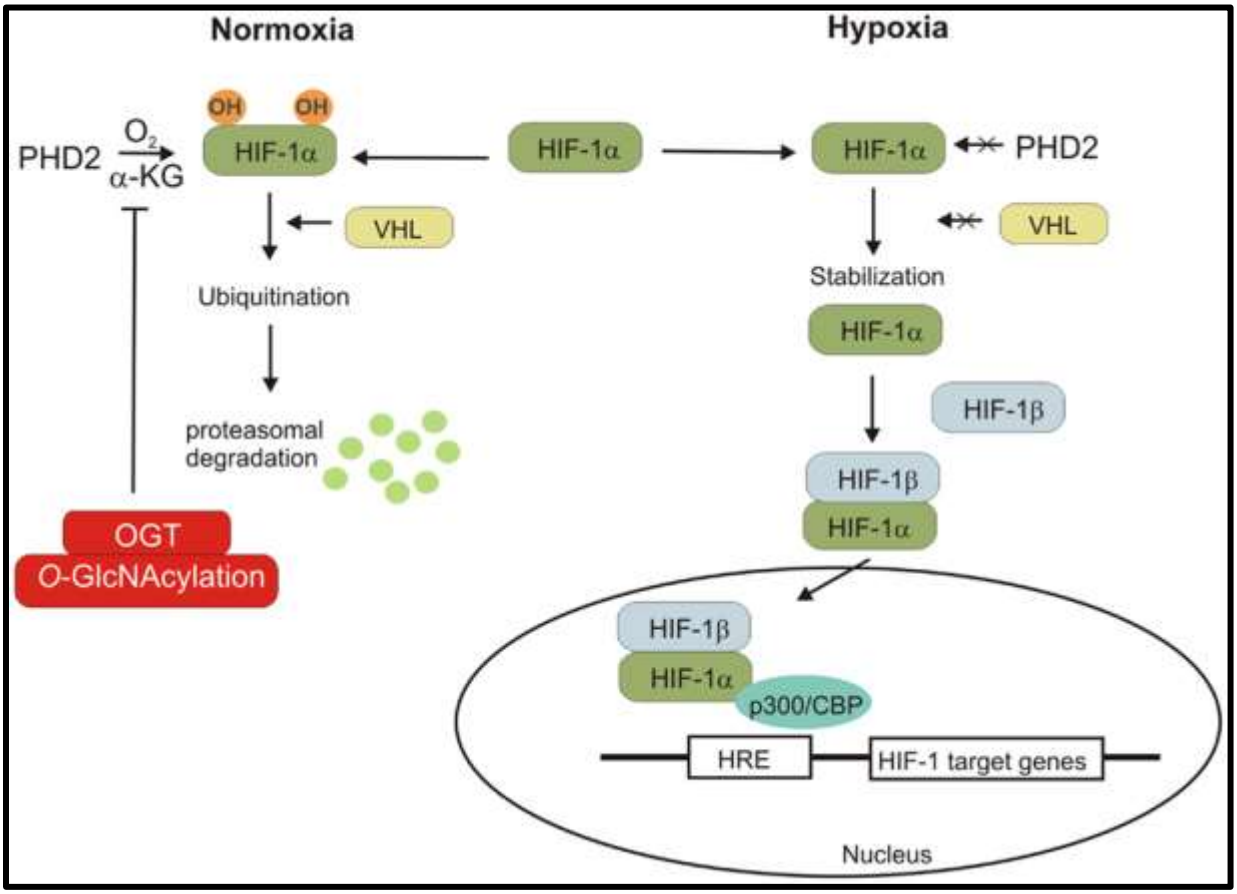

Figure 1.20: Regulation of HIF-1 during normoxic and hypoxic conditions. Adapted from [320].

During normal oxygen conditions (normoxia), HIF-1 is transcribed and degraded by the Von Hippel Lindau pathway [321]. However, under hypoxic conditions HIF-1 is stabilized and translocated into the nucleus where it binds with its beta units to promote activation of downstream genes that include VEGF and glucose transporters [322].

At the event where a tumor is exposed to fractionated radiation, a proportion of the hypoxic population cells get reoxygenated between fractions due to fluctuations in tumor perfusion within

blood vessels which allow for an increase in interstitial fluid pressure (**Figure 1.21**) [323, 324]. Oxygen can enhance the radiosensitivity of tissues by increasing production of free reactive oxygen species (ROS) in addition to stabilizing existing ROS [325-327]. One way to measure the effect of oxygen in enhancing radiosensitivity is through the oxygen enhancement ratio (OER), which is defined as the ratio of the dose to hypoxic cells over the dose to aerobic cells that can lead to decrease in cell survival to the same level. In NSCLC tumors, the OER is typically around 2.8 [328]. When doses above 8-10 Gy per fraction are delivered, or when a large dose of radiation is delivered in a single fraction to the targeted tumor, severe vasculature damage takes place and consequently oxygenation of the vasculature in not possible due to death of endothelial cells [329].

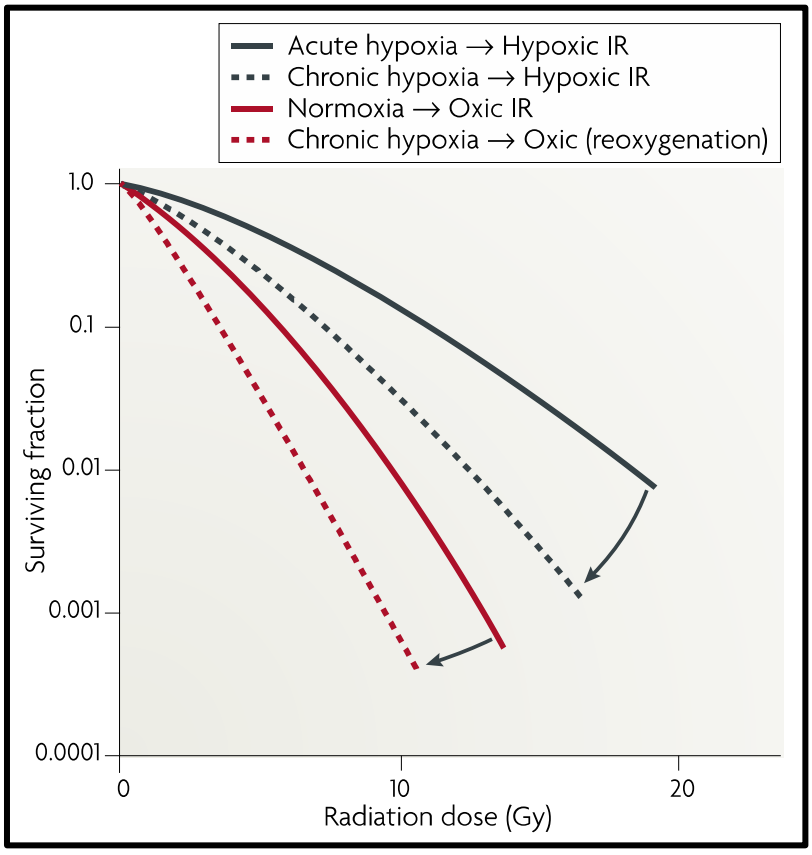

Figure 1.21: Cell survival curves of cells exposed to radiation under acute and chronic hypoxia versus normoxic conditions and reoxygenated tissues. Adapted from [330].

1.6.6.5 Radiosensitivity of cells

In the 1975, Rod Withers has published a paper with the title "The 4 R's of Radiotherapy" [331], which included: repair, repopulation, redistribution, repopulation and reoxygenation (discussed above in details). In the 1980s, the understanding of human tumor radiobiology has shifted to recognize that cells from different tumors exhibit variable radiosensitivity [332]. Fertil and Malaise were the first to investigate this idea and prove it through survival curve [333], which was later on confirmed by others [334]. Steel et al. reported that cell lines established from tumors with good response to radiation had a significantly steeper slope at 2Gy compared to less curable tumors [335]. Steel and colleagues went on to demonstrate that even at low dose rate the difference in radiosensitivity was greatly noticeable between human tumor cell lines (**Figure 1.22**). This has lead to the initiation of "Radiosensitivity" to be the fifth R of radiobiology, which indicates the response to radiation in tumors [332].

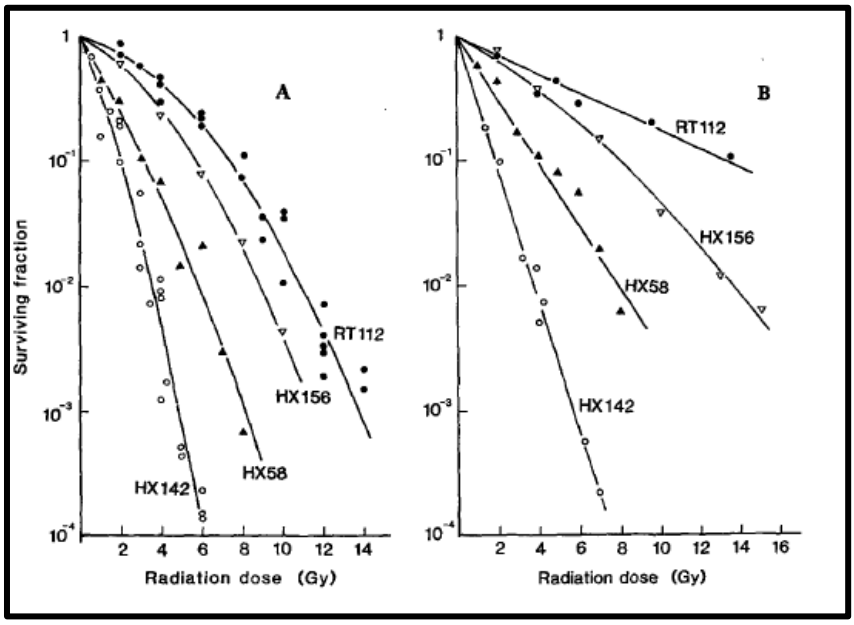

Figure 1.22: Steel and colleagues work which demonstrated differences in radiosensitivity between different human tumor cell lines. The cell survival curve was done on four different

human tumor cell lines: HX142 (neuroblastoma); HX58 (pancreatic cancer); HX156 (cervical cancer); and RT112 (bladder cancer). A) Cell survival curve at high dose rate (150cGy/min); B) Cell survival curve at low dose rate (2cGy/min). adapted from [332].

As mentioned earlier, assessment of radiosensitivity is done through obtaining the survival fraction at 2 Gy (SF2) measured from cell survival curves of clonogenic assays [334, 336]. Clonogenic assay helps in providing an idea about the cell's reproductive potential [336]. Reported SF2 measurements of *in vitro* studies have shown well correlation *in vivo* in terms of response to radiation in mouse models [337]. **Figure 1.23** exhibits differences in SF2 values of 29 human lung cancer cell lines with different histological subtypes.

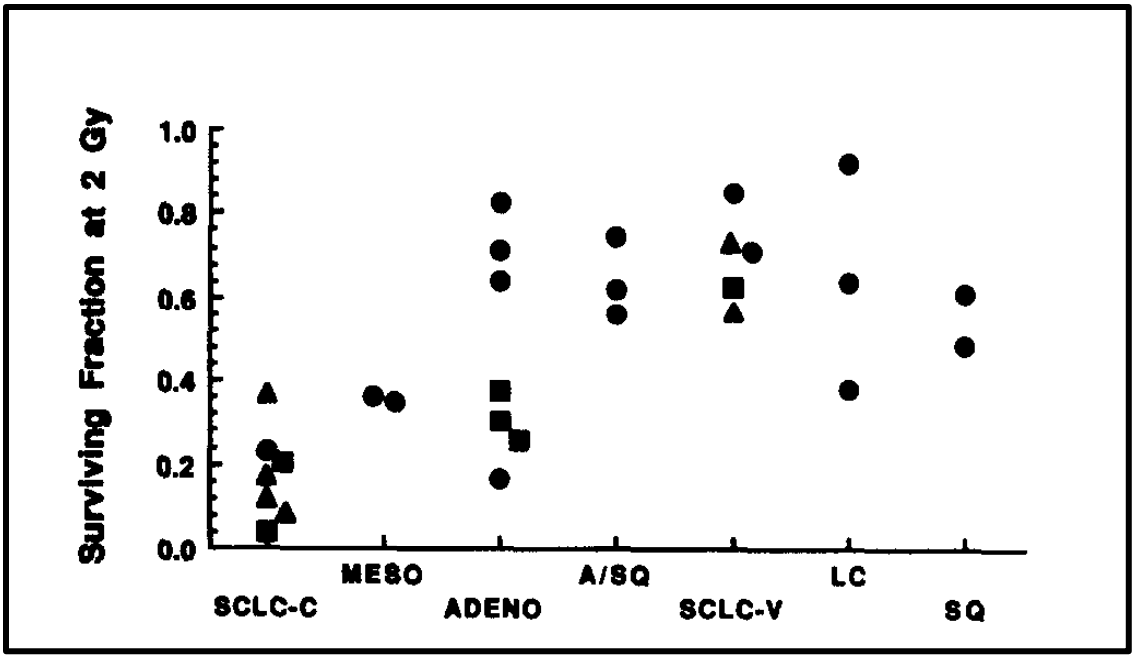

Figure 1.23: Radiosensitivity of 29 human lung cancer cell lines based on the surviving fraction at 2Gy. SF2 values were derived from clonogenic cell survival assays. The histological subtypes are: SCLC-C, classic small cell lung cancer; MESO, mesothelioma; A/SQ, adenosquamous; SCLC-V, variant small cell. Adapted from [338].

1.6.7 Vascular damage in Endothelial Cells and Enhanced Immunity

1.6.7.1 Endothelial cell damage may increase the cytotoxic effect of irradiated tumor cells

Studies have proposed that tumor endothelial cells contribute in tumor sensitivity to radiation of 10 Gy or more. The work of Zvi Fuks and Richard Kolesnick reported that the same tumors were more sensitive to radiation in mice sensitive to radiation-induced endothelial cells apoptosis than in mice resistant to endothelial cell apoptosis [329, 339]. However, they have suggested as an explanation is that may be due to bone marrow's implication in the response to radiation. They have demonstrated that mice with wild type asmase$^{+/+}$ (acid sphingomyelinase) can be converted from sensitive to resistant by bone marrow transplant derived from mice with endothelial apoptosis resistant (asmase$^{-/-}$) [339]. The idea that endothelial cells were derived from the transplanted bone marrow has been challenged by other studies where they doubted the incorporation of bone marrow into tumor endothelium [340, 341], or where others suggested that the incorporation of bone marrow cells into tumor endothelium is very low [342]. This indicates that the asmase$^{-/-}$ character of the bone marrow to have an implication in tumor resistance, and not in endothelial cells of the tumor (**Figure 1.24**).

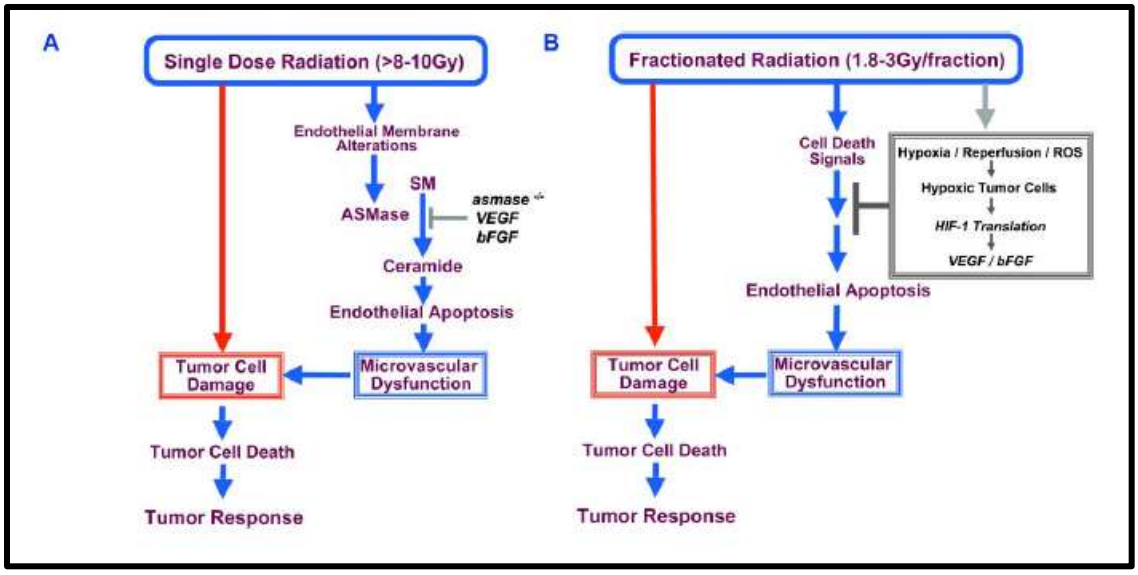

Figure 1.24: Models of microvascular endothelial engagement in tumor response to single-dose or fractionated radiotherapy. Radiation-induced endothelial cell damage following exposure to A) a single-dose (> 8-10 Gy) of radiotherapy, and B) and exposure to fractionated radiotherapy. Adapted from [339].

There has been no enough data on endothelial cell apoptosis theory, in fact most publications have reported modest changes to tumor vasculature with gradual loss of tumor endothelial cells following irradiation [343, 344]. Therefore, the rapid post-irradiation endothelial damage concept cannot be confirmed to be implicated in increased tumor cell kill following SBRT.

1.6.7.2 Vascular damage due to high doses can result in secondary cell killing

Song and colleagues [345] have proposed that radiation using doses higher than 10 Gy induces vascular damage that leads to indirect tumor cell death. However, there is very limited data that supports this hypothesis [346]. Data from Barendsen and Broese work on cell survival in rat

rhabdomyosarcoma as a function of time after single doses of both 10 and 20 Gy showed no evidence of increased cell kill as a function of time after irradiation [347]. Therefore, further investigation should be done to provide more details on the mechanisms that play role in tumor sensitivity after high dose per fraction radiotherapy.

1.6.7.3 Tumor irradiation can enhance antitumor immunity

Some studies have tried to answer some challenging questions regarding the "abscopal effect", one of these questions is the antigenicity of tumors. Preclinical data from Demaria laboratory reported that the antigenicity of tumors has been greater using fractionated irradiation than for single fraction doses [278]. However, radiation schedules used in this study were not comparable to standard fraction [278]. The tested schedules were: 20 Gy x 1, 8 Gy x 3, and 6 Gy x 5 fractions in consecutive days, with the fractionated 8 Gy being the most effective, followed with 6 Gy having an intermediate effect and the 20 Gy being the least effective [278]. Therefore, all tested schedules can be considered very similar to SBRT. Another preclinical study done by Weichselbaum laboratory [250] has reported an enhanced antitumor immunity of local tumor irradiation with 20 Gy x 1 than with 5 Gy x 4 over two weeks. It is worth mentioning that in the preclinical Demaria study, radiation was combined with anti-CTLA-4 antibody with no indication of enhanced antitumor immunity by radiation alone [278]. In the case of Weichselbaum study, antitumor immunity was achieved by irradiation alone [250]. The previously mentioned data suggests the need for further investigation on the recommended dose per fraction and timing of the radiation regimen to provide an optimal effect.

1.6.8 The relationship between ionizing radiation (IR) and reactive oxygen species (ROS) in inducing DNA damage

Ionizing radiation (IR) can cause double strand breaks (DSBs) through direct high-energy damage to the sugar backbone of the DNA, and through release of free radicals generated in cells-mostly ·OH from water [348]. Chemotherapeutics, such as doxorubicin and cisplatin, can also increase ROS levels which contribute to their genotoxicity [349, 350]. The way in which ROS participate in DNA damage is by oxidizing the nucleoside bases (e.g. formation of 8-oxo-gunaine) [351], which can lead to G-T or G-A transversion if not repaired. The newly oxidized bases are usually recognized and repaired through base excision repair (BER) pathway, but if they occur simultaneously on opposing strands, BER can result in generation of DSBs [352]. Accumulation of ROS can also induce mitochondrial DNA lesions, strand breaks and degradation of the mitochondrial DNA [353].

1.6.8.1 Overview of ROS

ROS is a family of short-lived molecules like O_2^-, H_2O_2, and ·OH and was initially described in skeletal muscle as free radicals [354]. Initially, ROS was thought to be a hazardous by-product released through mitochondrial respiration, however; discoveries in the last few decades revealed functional cellular role to ROS such as aiding immunity (e.g. oxidative bursts in phagocytes to eliminate pathogen) [355], and triggering cell signaling (e.g. H_2O_2 participate in regulating NFκB. MAPK pathways) [356]. ROS production happens endogenously by (i) mitochondria (O_2 functions as a terminal electron acceptor for electron transport chain) [357], (ii) NADPH oxidases, which is a cell a membrane bound enzyme [358], (iii) peroxisomes (which contain enzymes that can produce H_2O_2 e.g. ploy amine oxidase) [359], (iv) endoplasmic reticulum (which can produce H_2O_2

as a by-product during the process or protein folding); or when exposed to exogenous stress such as IR, chemotherapeutic drugs and environmental insults that can have an implication on organelles functions and enzymes [360].

ROS plays a critical role in mediating chemotherapy and radiotherapy responses by regulating downstream cell survival or death signaling cascades [361-363]. This has led researchers to investigate the use of ROS in cancer prevention or enhancing the response to therapy [363, 364]. Despite the extensive effort in studying ROS, very little progress is seen regarding ROS knowledge from labs to clinics. *In vitro* data on antioxidants showed promising results, however; trials on cancer prevention have demonstrated negative results [365, 366] which highlights the need for further investigation and understanding of ROS implication in cells.

1.6.8.2 Oncogenic replication stress due to ROS-induced DNA damage

Oncogene induced replication stress is considered an important cause of endogenous DNA damage and generator of DSB in cancer [367]. Proto-oncogenes help in cell growth and proliferation, but mutations or overexpression can lead to continuous cell growth and carcinogenesis. Aberrant replication fork progression and DNA synthesis are usually associated with oncogenic cell cycle [368]. Replication stress causes a genomic instability which allows for tumor development through additional accumulation of pro-carcinogenic changes [367, 369]. DNA damage response (DDR) limits the expansion of abnormally replicating cells which leads to selective pressure for DDR defects in carcinogenesis [370].

Replication stress can happen due to aberrant origin firing, decoupling of DNA polymerase-helicase activity, or physical obstacles to the replication fork [368]. Active oncogene causes an upregulation of ROS which in turn influences the occurrence of replication stress [369, 371]. ROS

cause oxidization of dNTPs which affects polymerase activity and reduces replication fork velocity in vitro [372, 373]. Another way in which ROS can affect the replication fork progression is by dissociating peroxiredoxin2 oligomers (PRDX2). PRDX2 functions as a replisome-associated ROS sensor where it can bind to the fork accelerator TIMELESS when exposed to low levels of ROS. High ROS levels can lead to dissociation of PRDX2 and TIMELESS complex, which slows the replication fork progress [374].

1.6.8.3 ROS effect on cell cycle progression

Cell cycle arrest is a key modulator in DDR where it prevents cells with damaged DNA to proceed with cell division. It has been reported in Hela cells that asperlin induced-ROS causes an ATM-Chk2 mediated G2/M arrest [375]. Similarly, in colorectal cancer cells ROS induced Chk1 activation led to a p53 independent G2/M arrest [376]. In addition to their ability in activating cell cycle checkpoints, ROS can also promote cell cycle arrest by direct action on the Cdc25 family of protein phosphatases (Cdc25A, B and C). The Cdc25 phosphatases family promotes progression of the cell cycle by removing inhibitory phosphates on cyclin dependent kinases (CDK) [299], and their levels/activity can be regulated by ROS. For example, ROS can downregulate Cdc25C protein levels which leads to G2/M arrest [377]. Caulibugulone A, a family of isoquinoline quinones) induces ROS production and causes reduction of the total Cdc25A levels [378]. Similarly, oxidation of Cdc25A and reduction in its activity is observed by 17β-Oestradiol-induced ROS [379].

Mitotic entry and recovery from the G2/M arrest are regulated by mitotic kinases, such as Polo-like kinase 1 (PLK1) and AURORA-A. In cancer, these kinases are usually overexpressed. PLK1 phosphorylates glucose-6-phosphate dehydrogenase, which leads to an increase in PPP and

production of NADPH, thus causing an increase of the antioxidant capacity of the cell. Oxidative stress with H_2O_2 increases the expression of PLK1 in a p53 dependent manner [380, 381], but maintains the G2/M arrest phase. On the other hand, accumulation of ROS inhibits Aurora kinase A [382], although PLK1 and Aurora-A are epistatic in the pathway. Due to the previously mentioned information, PLK1 and Aurora-A kinase inhibitors are being investigated in clinical trials in the hope of providing a better understanding between ROS and these proteins.

1.6.8.4 Cellular antioxidant enzymes

In addition to DNA damage, ROS can induce damages in the lipids [383, 384] and proteins [385, 386]. In the case of lipid peroxidation, polyunsaturated fatty acids serve as an excellent target for free radicals due to their multiple double bonds. This oxidation is critical for the generation of atherosclerosis plaques [387, 388]. In proteins, ROS can react with several amino acid residues *in vitro*, which can lead to anything from modification and less enzyme activity to denatured, non-functioning proteins [386, 389]. Some of the most susceptible amino acids are the ones that contain sulfur- or selenium-residues. Antioxidant systems such as Thioredoxin (Trx), glutaredoxins (Grx), or glutathione (GSH) systems function in protecting protein from ROS modification [390]. In the following sections, we will discuss the Trx system and its role in cancer in further details.

1.7 Thioredoxin System in Cancer Biology

For healthy cells it is essential to maintain a redox homeostasis, a balance between the generation of reactive oxygen species (ROS) and their elimination by cellular antioxidant networks. Mitochondrial metabolism is the major source of ROS [391]. ROS is an important molecule in regulating several redox signaling pathways that promote many biological processes such as cell

survival, growth and proliferation [392-395]. Cancer cells usually have an elevated ROS due to their uncontrolled proliferation and high metabolic rate. Cancer cells tend to maintain redox balance through upregulation of antioxidant system to counteract the increased ROS levels [391]. Although elevated ROS is essential for maintaining tumor phenotypes, it also reduces cancer cells that are vulnerable to oxidative stress [396]. The idea of altering the redox environment of cancer cells is being explored for the development of cancer therapy and potential strategy for cancer treatment [396-402].

1.7.1 The Thioredoxin System

Thioredoxin (Trx) system consists of: thioredoxin reductase (TrxR), thioredoxin (Trx) and NADPH [391]. This system is ubiquitous and present in all levels of life and can be found in plants, archaea and humans. It has a critical role in regulating redox signaling pathways through antioxidant defense, selenium metabolism and regulation of gene transcription (**Figure 1.25**) [403-406]. Trx proteins are small (~12kDa) with a conserved Trp-Cys-Gly-Pro-Cys-Lys sequence which have been identified and described since 1960s [407, 408].

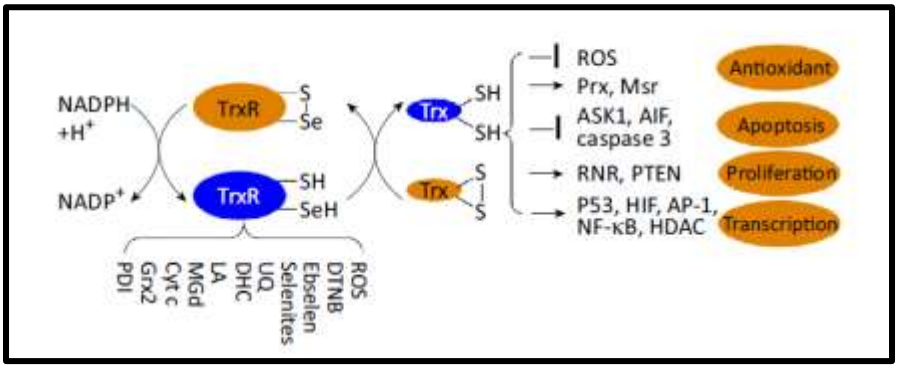

Figure 1.25: Thioredoxin system and its function in regulating biological pathways. Adapted from [391].

TrxR enzymes are part of the falavoprotein family of pyridine nucleotide-disulfide oxidoreductases which has a FAD prosthetic group, an NADPH binding site, and a redox active site containing a dithiol/disulfide motif [391]. This family also includes glutathione reductase (GR), lipoamide dehydrogenase, trypanothione reductase, and mercury reductase [409]. Mammalian TrxRs are large (~55kDa), compared to those of lower organisms, and share similar structures and catalytic activity of GR [410, 411]. The first clone of human TrxR1 was done from human placenta [412]. There are three isoforms of TrxR in humans: TrxR1 which is present in the cytosol and nucleus, TrxR2 is present in mitochondria, and TrxR3 (also known as thioredoxin glutathione reductase) is present in testis tissue [391].

1.7.2 Thioredoxin in Cancer

TrxR is a key player in tumor-associated redox process which is critical in cancer pathology [413-415]. A study, where a panel of 60 human cancer cell lines were screened for TrxR1, has reported TrxR1 to be over expressed in multiple types of tumor [405, 416-421]. As mentioned previously, the Trx system is a key player in multiple metabolic pathways including: selenium metabolism, DNA synthesis, and oxidation resistance in cancer cells [422, 423]. Various cancer cell lines and tissues exhibit an elevated expression of TrxR1 [419, 424]. In human breast cancer and hepatocellular cancer, over-expression of TrxR1 has been identified as a marker of poor prognosis in patients [413, 425]. Furthermore, men with high expression of TrxR1 are at high risk of prostate cancer [426].

1.7.3 Thioredoxin in Non-Small Cell Lung Cancer

Previous studies investigated the outcome of TrxR1 depletion and reported a reverse in tumorigenicity of lung carcinoma cells [427]. Other studies have investigated the anti-tumor effect of TrxR1 using TrxR1-specific inhibitors [428-432]. One study has reported an induced apoptosis following the use of 1,2- [bis (1,2-Benzisoselenazolone-3 (2H)-ketone)] ethane (BBSKE), a specific inhibitor of TrxR1, causing suppression of TrxR/Tr-Tr-NF-κB pathway [433-435] (16-18). TrxR1 involvement in cancer proliferation has led to the hypothesis of suggesting TrxR1 as a potential diagnostic marker [436].

One study has looked at TrxR1 levels in plasma as a diagnostic marker in cancer patients and suggested TrxR1 level of 7.1 U/ml as the cut-off-value of diagnosis [436]. In the study, TrxR1 levels were measured and evaluated in in healthy individuals and cancer patients, where the measured TrxR1 values were 0.65 U/ml and 9.85 U/ml ($p<0.05$), respectively indicating elevated levels of TrxR1 in cancer patients. They have also reported an elevated level of TrxR1 in lung cancer patients compared to patients with benign diseases. Moreover, analysis of TrxR1 level in pathology specimens from NSCLC patients revealed high TrxR1 activity in the carcinoma tissues than in the para-carcinoma tissues. Their work has provided evidence that TrxR1 levels are elevated in cancer patients and NSCLC patients and could be used as a diagnostic marker in NSCLC [436].

1.7.4 Auranofin as an Inhibitor of Thioredoxin Reductase (TrxR)

Auranofin is a potent inhibitor of TrxR where it functions in inhibiting the enzyme activity [437]. Inhibition of TrxR causes overproduction of ROS which in turn activates the apoptotic pathway

[429, 438-442]. Induced strong cytotoxicity by auranofin was reported in chronic leukemia and gastric cancer cells in which an endoplasmic reticulum stress is observed and associated with ROS overproduction [438, 439]. It has also been reported that auranofin was effective in inducing apoptosis in drug-resistant myeloma and chronic leukemia through ROS-dependent and ROS-independent mechanisms [429, 441]. The use of auranofin in combined modality for cancer treatment is being tested in leukemia, lung cancer and epithelial ovarian cancer [443].

In the work of Yan et al., it has been reported that loss of expression or reduced activity of glutathione reductase (GSR) gene in NSCLC cell lines leads to an increased sensitivity to auranofin [444]. The work of Liu et al. has demonstrated that auranofin can enhance chemosensitivity of small cell lung cancer (SCLC) *in vitro* and *in vivo* when combined with cisplatin, a standard chemotherapy in the treatment of SCLC [445]. Moreover, they reported that auranofin was capable in sensitizing cells to cisplatin by inducing overproduction of ROS which led to mitochondrial dysfunction and DNA damage in SCLC [445]. In current clinical trials, auranofin is being tested in combination with sirolimus, an mTOR inhibitor, to treat patients with advanced or recurrent NSCLC and SCLC [445]. Given the previously mentioned findings, the use of auranofin as a drug to target TrxR in NSCLC with different EGFR status, particularly NSCLC that are know to exhibit resistance to treatments (TKIs or radiation) such as T790M-EGFR or wild-type EGFR, may be a promising treatment in overcoming resistance and enhancing anti-tumorigenic effect.

1.8 Research rationale and objectives

1.8.1 Research rationale

Stereotactic ablative radiation therapy (SABR) became an alternative treatment for inoperable early stage non-small cell lung cancer patients (ES-NSCLC). Despite advances in SABR and surgery, locoregional recurrence and distant metastasis remain as the most common challenges to be faced during treatment of ES-NSCLC. In fact, it has been estimated that the occurrence rate of distant metastasis in patients treated with SABR can reach up to 30% which leads to death [446, 447].

Our lab has previously assessed the response of three genetically distinct adenocarcinoma cell lines A549, HCC827 and H1975 to ablative radiation and fractionated radiation [448]. A549 cell line is characterized by having wild-type EGFR, whereas HCC827 is characterized by having deletion in exon 19 of EGFR, and H1975 is characterized by having double EGFR mutations (L858R and T790M). Our findings indicated that ablative radiation was able to significantly reduce cell proliferation and clonogenic survival in A549 compared to fractionated radiation [448]. Moreover, we reported that ablative radiation had significantly enhanced invasiveness of A549 cell line but not in HCC827 or H1975 cell lines [448].

Our group has also developed an orthotopic NSCLC animal model to allow in investigating the response of clinically-relevant doses of stereotactic ablative radiotherapy (SABR) [449]. In this model, intra-thoracic injection of A549 cell line was done using image-guided technique. A single dose of 34 Gy was delivered to the developed tumor [449]. We reported that the majority of animals who received SABR had a complete response (67%), while 33% had local failure [449]. Furthermore, we observed that 50% of animals with complete response failed distantly [449]. Gene

analysis done on treated and untreated tumors revealed that SABR had significantly modulated genes expression [449]. For instance, high levels of interleukin-8 (IL-8) which has a critical role in promoting tumor invasion was observed following SABR [449].

Due to its relatively young age as a treatment, in comparison to other treatments, there is an urgency and need to have an in-depth understanding of possible outcomes associated with SABR application. Newly emerging studies, such as the one by Nakamura et al [229], have reported a significant association between EGFR mutation and out-field recurrence post-SBRT treatment. Another study by Nakamura et al [230], has reported a significantly lower in-field failure and higher out-of-field failure in EGFR-mutant group compared to wild-type EGFR group post-SBRT. In their study they have evaluated the patter of failure following definitive chemoradiotherapy in patients with stage III NSCLC harboring EGFR mutations and/or ALK translocation. Poor outcomes have also been reported to be associated in NSCLC patients with KRAS mutation and MET amplification post SBRT/SABR treatment [231]. Another study has indicated that the efficacy of SABR can vary depending on tumor histology where squamous cell carcinoma (SCC) had a significantly higher rate of local failure (2-fold) compared to ADC following treatment [450].

Implication of EGFR mutations in the response to treatment such as chemotherapy and TKIs has been investigated. Rosell et al have looked at the outcomes of using erlotinib versus standard chemotherapy in stage IV NSCLC harbouring EGFR mutations [451]. They have reported an increased PFS (9.7 months) in erlotinib treated group compared to standard chemotherapy (5.2 months) [451]. What was interesting in their reported work, is the difference in PFS between different EGFR subtypes following treatment with erlotinib. NSCLC patients with in-frame shift deletion in the exon 19 had a higher PFS (11.0 months) versus L858R mutation group (8.4 months) [451]. This has led us to question the implication of different EGFR mutations in driving a

differential response to ablative radiation. To our knowledge, there are no studies that have reported the response of isogenic EGFR-mutant NSCLC to ablative radiation *in vivo*. Moreover, up to our knowledge, no study has reported the implication of EGFR subtypes in the differential response to ablative radiation. This has led us to investigate the response to SABR *in vitro* and *in vivo* using isogenic EGFR-mutant cell lines as a model to help in providing insights on the differential response.

As previously mentioned, ionizing radiation can cause double strand breaks (DSBs) by damaging the sugar backbone of the DNA through generation of reactive oxygen species (ROS) [348]. Chemotherapy such as doxorubicin and cisplatin can also lead to an increased ROS levels, which contribute to their genotoxicity [349, 350]. Elevated ROS levels have been reported to activate apoptotic pathways [429, 438-442]. ROS production is regulated by antioxidant systems such as the Thioredoxin system which consists of: Thioredoxin (Trx), thioredoxin reductase (TrxR), and NADPH [391]. Auranofin, an FDA approved drug used in treating rheumatoid arthritis, is a potent inhibitor of TrxR (thioredoxin reductase) causing an inhibition of the enzyme activity [437]. Inhibition of TrxR results in increased levels of ROS which in turn will result in activation of apoptotic pathway [429, 438-442]. This suggests that auranofin can be used as a potential therapeutic drug targeting tumor cells, where it induces an increase in ROS levels, causing activation of apoptotic pathway and leading to cell death. The use of auranofin in combined modality of cancer treatment is currently being tested in different cancers [443]. Relapses and resistance to treatment are very common to occur as a result of first-line treatment. This stresses on the need to develop and explore therapeutic approaches as potential alternatives that can be accessible during these challenges and complications. Consequently, we thought of assessing the efficacy of auranofin in lung adenocarcinoma cell lines harbouring different EGFR mutations that

are known to be resistant to treatments (TKIs or radiation) such as EGFR-mutant lung adenocarcinoma with T790M mutation or wild-type EGFR lung adenocarcinoma. In our assessment, we tested the potential of auranofin as an as antitumorigenic drug combined with radiation or alone to induce maximum tumor inhibition.

1.8.2 Research objectives

The aims of this thesis are divided into the following main objectives:

1. Investigate the response of isogenic EGFR-mutant lung adenocarcinoma cell lines to ablative radiation therapy *in vitro* and *in vivo* (**Chapter 2**).

2. Identify if certain EGFR mutations exhibit better response to ablative radiation therapy compared to others (**Chapter 2**).

3. Examine the efficacy of auranofin as an antitumorigenic drug in lung adenocarcinoma cell lines with different EGFR status (**Chapter 3**).

4.Assess the potential of auranofin as a radiosensitizer in radioresistant lung adenocarcinoma (**Chapter 3**).

We hypothesize that lung adenocarcinoma with different EGFR status will exhibit a discrete response to SABR, which will help in identifying EGFR subtype that exhibit good response to SABR. In addition, we hypothesize that auranofin treatment combined with radiation will enhance radiosensitivity in NSCLC.

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Chapter 2: Response of lung adenocarcinoma harbouring different epidermal growth factor receptor mutations to ablative radiotherapy

Response of lung adenocarcinoma harbouring different epidermal growth factor receptor mutations to ablative radiotherapy

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Authors declare no conflict of interest.

Areej Al Rabea was supported by McGill University Graduate Student Award. This work was funded by Montreal General Foundation.

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Word count: 3,489

Number of figures: 5 figures

2.1 Abstract:

2.1.1 Purpose

Stereotactic ablative radiation therapy (SABR) provides an alternative treatment strategy for early stage lung adenocarcinoma patients who are inoperable. Response of lung adenocarcinoma patients carrying epidermal growth factor receptor (EGFR) mutations is not well investigated. This study aimed to investigate response of isogenic EGFR-mutant lung adenocarcinoma to SABR.

2.1.2 Materials and Methods

A549 lung adenocarcinoma cell line was stably transfected with either wild type-EGFR (WT), DEL-EGFR (DEL) or L858R-EGFR (L858R) constructs to generate isogenic cell lines. *In vitro* assessment included colony formation, cell viability and proliferation assays. Tumor formation was assessed by subcutaneous injection of pre-irradiated cells in YFP/SCID mice. ABRT response was evaluated in mice injected subcutaneously with isogenic WT-EGFR or EGFR-mutant cells either sham-treated or treated with a single fraction of 34 Gy. The effect of ABRT in altering tumor morphology and protein expression was evaluated in tumors collected from sham-treated and irradiated groups.

2.1.3 Results

EGFR-mutant lung adenocarcinoma cell lines displayed similar in vitro response to ablative radiation (ABR) including: reduced colony formation, cell viability and cell cycle arrest in G2, post-treatment. Pre-irradiated WT-EGFR and L858R-EGFR lung adenocarcinoma cell lines maintained their ability to initiate tumor growth *in vivo*, whilst pre-irradiated DEL-EGFR cells were unable to form tumor upon injection. Subcutaneous DEL-EGFR xenograft tumors had a significant decrease in tumor volume post-ABRT treatment compared to WT-EGFR and L858R-

EGFR xenografts. Histological assessment demonstrated less tumor necrosis and a significant decrease (p = 0.05) of apoptotic cells in DEL-EGFR treated tumors compared to L858-EGFR treated tumor.

2.1.4 Conclusions

We report for the first time that EGFR mutations impact response to ABRT, with DEL-EGFR mutation imparting better response to ABRT compared to WT-EGFR or L858R-EGFR mutations. Fractionation should be further tested to assess the optimal ABRT regimen in the context of lung adenocarcinoma with EGFR mutations.

2.2 Introduction:

Lung cancer (LC) remains the leading cause of cancer mortality worldwide in both men and women [1]. LC can be classified into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), which represents 85% of LC and can be divided histologically into squamous cell carcinoma (SQC), adenocarcinoma (ADC), and non-small cell lung cancers-not otherwise specified (NSCLCs-NOS) [2]. Surgical resection is the standard treatment for stage I and II NSCLC patients [3]. In patients who are not candidates for surgery or prefer alternative treatments, ABRT is offered as the treatment of choice. ABRT is the delivery of a single or limited number of high-dose fractions of RT and can be used for LC patients with lesions up to 5 cm in size [4].

Several randomized controlled trials have attempted to compare the outcome of ABRT to surgical resection in operable early stage NSCLC (ES-NSCLC) patients [5]. However, these trials had to be terminated prematurely due to poor recruitment. Pooled analysis of two prematurely closed trials comparing ABRT to lobectomy reported outcome in 58 patients, in which 31 patients were treated

with ABRT and 27 patients with surgery. This analysis suggested clinical equipoise with estimated overall survival at 3-years to be 95% in the ABRT group, compared to 79% in the surgery group. The risk of recurrence after ABRT is not well explored, but findings suggest that outcomes are dependent on tumor size, location, histology, pre-treatment positron emission tomography (PET)/ computed tomography (CT) standardized uptake value (SUV), and age [6,7]. One study looked at the efficacy of ABRT with regards to histological subtypes of LC and suggested that squamous cell carcinoma (SCC) had a significantly higher rate of local failure (2 fold) after ABRT compared to ADC [8].

Analysis of ABRT outcomes in EGFR-mutant LC is not yet explored. It is estimated that approximately 15% of lung adenocarcinoma patients carry mutations in the tyrosine kinase domain (TKD) of EGFR with a high frequency (up to 62 percent) in non-smokers and Asians [9-11]. The most common EGFR mutations include: frameshift deletion in exon 19 or a substitution of the leucine amino acid to arginine at codon 858 of exon 21 [12]. Stage IV lung adenocarcinoma with EGFR mutations are associated with good response to tyrosine kinase inhibitors (TKIs), such as gefitinib and erlotinib as a first line treatment compared to standard chemotherapy [13]. Few studies have addressed the role of EGFR mutations in the response to treatment. In 2012, Rosell et. al. conducted a randomised clinical trial comparing erlotinib versus standard chemotherapy in stage IV lung adenocarcinoma patients with EGFR mutation-positive [14]. EGFR mutation-positive patients treated with erlotinib had a median progression-free survival (PFS) of 9.7 months compared to a 5.2 month in the chemotherapy group. Moreover, they have reported that the group with deletion in exon 19 (DEL-EGFR) had a better PFS when treated with erlotinib (11.0 months) compared to the group with L858R-EGFR mutation (8.4 months).

Our group has developed a pre-clinical orthotopic animal model to assess tumor response to ABRT [15]. In this model, human lung adenocarcinoma A549 cell line was injected intrathoracically into nude rats. After treatment with a single fraction of 34 Gy, a complete response to ABRT was observed in 4 out of 6 treated animals; however, 50% of treated animals developed distant metastasis [15]. The A549 being EGFR-expressing cells, these results prompted us to investigate the relationship between the observed effects and the mutation status of EGFR. While the relationship between EGFR mutation status and response to EGFR inhibitors is now well documented, its direct correlation with ABRT outcomes remains to be elucidated. To date, no study addressed the impact of different EGFR-mutations on lung adenocarcinoma response to ABRT *in vitro* and *in vivo*. Here we demonstrate that EGFR-DEL lung adenocarcinoma tumors are highly responsive compared to lung adenocarcinoma tumors with WT-EGFR or L858R-EGFR, suggesting that assessment of EGFR status is crucial for choosing the delivered ABRT treatment.

2.3 Materials and methods:

2.3.1 Cell Culture and transduction of isogenic EGFR-mutant cell lines

Isogenic EGFR-mutant lung adenocarcinoma cell lines were generated using A549 (ATCC, VA) transfected with lentivirus carrying gene construct of wildtype-EGFR (WT), EGFR frameshift deletion E746-A750 (DEL), or point-substitution mutation at amino acid position 858 (L858R) [16]. Constructs were kindly provided by Dr. Chaitanya Nirodi. Cells were cultured at 37 °C and 5% CO_2 in RPMI supplemented with 5% FBS and blasticidin for selection.

2.3.2 Colony formation assay and cell viability

Colony formation assay was performed similarly to previously published work [17]. Briefly, exponentially growing cells were irradiated with increasing dose of irradiation (0-8 Gy). To

investigate EGFR-mutant cell lines ability to overcome ablative radiation, isogenic cells were seeded at density of 3000 cells and irradiated with single dose of 0 Gy (Control), 12, or 34Gy. Faxitron X-ray machine (Faxitron X-ray Corporation, IL) was used for irradiation at a voltage of X-ray tube set to 160 kVp, current of 6.3 mA and a dose rate of 0.66Gy/min. Cells were then cultured 37 °C and 5% CO_2 for 8-10 days, fixed with formalin, and stained with methylene blue.

2.3.3 Cell viability assessment

For cell viability measurement, cells were seeded and irradiated with single dose of 12 or, 34Gy, or 0 Gy (Control), 24 hrs post-irradiation cells were washed with 1X PBS, trypsinized and analyzed for viability, total cell count, and measurement of cell diameter using Vi-Cell Cell Counter.

2.3.4 Cell Cycle and cellular proliferation analysis

Cell cycle and cellular proliferation analysis was done as described in Supplementary Methods.

2.3.5 Generation of luciferase-expressing EGFR-mutant lung adenocarcinoma cell lines

For *in vivo* experiments, EGFR-mutant cells were transfected with a Lentiviral vector, kindly provided by Dr. Kolja Eppert, that expresses blue fluorescent protein (BFP)-luciferase. The transfected cells were sorted and used for subcutaneous injection (**Supplementary Figure 1 a-c**). BLI using IVIS Lumina (PerkinElmer, MA) was performed to confirm viability of injected cells [18]. Mice were anaesthetized by 2% isoflurane inhalation followed by an intraperitoneal (IP) injection (10ul/gram of animal weight) of D-Luciferin (15mg/ml in PBS, Cedarlane, Ontario, CA). Bioluminescent images were acquired at 5 minutes intervals.

2.3.6 Tumor formation of pre-irradiated EGFR-mutant lung adenocarcinoma

Isogenic EGFR-mutant cells were irradiated *in vitro* with a single fraction of 34Gy and injected subcutaneously at concentration of $2.0x10^6$ cells mixed with matrigel in 1:1 ratio for a total of 200 ul per mouse for a total of 18 mice (n=6 per cell line). These groups of mice are denoted throughout the study as Pre-IR-WT, Pre-IR-DEL or Pre-IR-L858R. Yellow fluorescent protein-severe combined immunodeficiency (YFP/SCID) mice with the age of 6 to 8 weeks were used for subcutaneous injection.

2.3.7 Cone beam computed tomography (CBCT) and ablative radiotherapy

In contrast to the previous experiments, EGFR mutated cell lines were injected subcutaneously at concentration of $2.0x10^6$ cells mixed with matrigel in 1:1 ratio for a total of 200 ul per mouse for a total of 36 mice (n=12 per cell line). Following injection, caliper measurements were carried out to determine tumor growth, which is reported as tumor volume calculated using the formula $width^2$ x length/2. Tumor size was measured by a research assistant blinded to the animal study. Mice were randomly assigned to receive a single fraction of 34 Gy (treated group), or not (control group). Tumor volume was measured at the day before (Day 0) and nine days post-treatment. Change in the tumor volume was measured relative to day 0.

CBCT scans were performed using X-RAD 225Cx (Precision X-Ray Inc., CT) to confirm tumor localization and size. Mice were anaesthetized by 2% isoflurane inhalation and positioned on the CBCT bed. Images were captured at an isotropic voxel size of 45 µm (40 kV, 500 µA and 400 ms integration time) (**Supplementary Figure 2)**. CBCT images were acquired using the X-RAD 225Cx small animal irradiator and processed using the Small Animal Radio Therapy (SmART) plan treatment system (Precision X-ray Inc.) for contouring [18,19]. A treatment of a single fraction of 34 Gy, was imported, calculated and delivered to animal (**Supplementary Figure 3**). All animal

procedures were carried out with accordance and approval of McGill University Animal Care Committee.

2.3.8 Histological tissue processing and protein extraction

To investigate whether ABRT has altered the tumor morphology of isogenic EGFR-mutant cells, a histological assessment of % of necrosis, architectural pattern and number of apoptotic cells was performed. Once animals reached the end point of the experiments, tumors were collected and fixed using 10% formalin for at least 48 hrs. Tissues were routinely processed and paraffin embedded. Five µm slides were stained by Haemotoxylin and Eosin (H&E) and assessed by (SCB), blinded to the EGFR mutation information. The following information was gathered: the tumor necrosis (estimated semi-quantitatively 10% increment), number of apoptotic cells (counted from 10 consecutive high-power fields, distant from necrotic areas) and architectural pattern (percentage of solid tumor estimated semi-quantitatively with 10% increment). Protein extraction and immunoblotting was performed as described in the Supplementary Methods.

2.3.9 Statistical analysis

All *in vitro* experiments were performed independently at least three times. One-way ANOVA or two-tailed student's t-test was used to compare between treated and non-treated groups. Statistical significance was set at $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

2.4 Results:

2.4.1 Response to ABRT in isogenic EGFR-mutant *lung adenocarcinoma*

To assess the differential response to radiation in EGFR mutant lung adenocarcinoma, A549 transfected with lentivirus carrying gene construct of WT, DEL or L858R were used in clonogenic

assay and revealed no significant difference between the cell lines (**Figure 2.1a**). Cell viability using Vi-Cell (**Figure 2.1b**) indicated a decrease in the number of viable cells at 12 and 34 Gy by: 54% and 63% in WT; 45% and 50% in DEL; and 46% and 56% in L858R, respectively. We further validated ability of isogenic EGFR-mutant cells to overcome exposure to ABR by forming colonies following treatment with a single fraction of 12 or 34G (**Figure 2.1c**). An increase in the G2-phase of cell cycle was also noted in all the three cell lines following ABR with 12 or 34 Gy (**Figure 2.1d)**. Measurements of the proliferation rate post-ABR, showed a significant decrease in DEL-EGFR at 48 and 72 hrs post radiation (p-values of <0.05 and <0.01, respectively), and of L858R-EGFR at 72hrs (p-value <0.05) (**Figure 2.1e**).

2.4.2 lung adenocarcinoma harbouring EGFR-DEL mutation exhibit a better response to ABRT compared to EGFR-WT and EGFR-L858R mutation in vivo

To investigate the ability of EGFR-mutant cells to overcome radiation-induced stress and develop tumor following ABR, cells were irradiated at 34Gy and injected subcutaneously in the flank of YFP/SCID mice (Pre-IR group)**.** Tumor size was weekly measured and BLI was used to confirm viability of injected cells (**Figure 2.2**). Tumor measurements showed animals injected with Pre-IR-L858R had the highest tumor volume with an average tumor volume of 7.89 cm^3 and tumor range of 0.8-34.11 cm^3 compared to Pre-IR-WT and Pre-IR-DEL with an average tumor volume of 0.66 cm^3 and 0.02 cm^3, and tumor range of 0-1.64 cm^3 and 0-0.07 cm^3, respectively. In fact, animals injected with Pre-IR-DEL showed no tumor development despite BLI confirmation of their viability 12 months following injection.

We reported a complete response to ABRT in an orthotopic animal model injected with A549 cell line [15]. To assess the response to ABRT in isogenic mutant cells, animals were injected subcutaneously in the left flank of YFP/SCID mice and randomly chosen to receive a single

fraction of 34Gy. Median survival of mice with tumors with EGFR WT-treated and EGFR L858R-treated were similar, 51 and 43 days, respectively (Supplementary Figure 4). However, nine days post-treatment, a significant decrease in tumor volume was noted in DEL-treated group compared to WT-treated and L858R-treated groups. In fact, WT and L858R treated groups exhibited an increased tumor volume post-ABRT treatment (**Figure 2.3**).

2.4.3 Histological assessment of isogenic EGFR-mutant lung adenocarcinoma following ABRT treatment

Tumors collected from both control and treated animal groups were assessed for percentage of necrosis, percentage of solid tumor and number of apoptotic cells. The average necrosis percentage was 33% in WT-control, 27% in WT-treated, 35% in DEL-control, 0% in DEL-treated, 50% in L858R-control, and 22% in L858R-treated (**Figure 2.4a-c**). Solid tumor percentage was 32% in WT-control, 28% in WT-treated, 30% in DEL-control, 20% in DEL-treated, 30% in L858R-control, and 25% in L858R-treated (**Figure 2.4d-f**). The average measured apoptotic cells was 78 in WT-control, 92 in WT-treated, 51 in DEL-control, 21 in DEL-treated, 86 in L858R-control, and 63 in L858R-treated (**Figure 2.4g-i**). A significant decrease ($p = 0.05$) in the number of apoptotic cells was noted in DEL-treated compared to DEL-control.

2.4.4. Protein expression in tumor derived from isogenic EGFR-mutant post ablative radiation

We have also evaluated change in protein expression as a response to ABRT in tumor tissues from treated and control group by performing immunoblotting. We assessed the expression of phospho-EGFR (p-EGFR), total-EGFR, p-AKT, total-AKT, p-ERK, total-ERK (**Figure 5a**), proteins implicated in cell survival, proliferation and apoptotic processes. We observed a decrease in p-

ERK levels in DEL-treated group by 0.7-fold change (**Figure 5b**) compared to WT-treated and L858R-treated groups where both had an increase of p-ERK levels of 4.9- and 3.2-fold change, respectively (**Figure 5**). These data correlate with reduced cell proliferation and tumor growth observed in DEL-EGFR-mutant lung adenocarcinoma post-ABRT compared to WT- and L858R-EGFR-mutant tumors.

2.5 Discussion:

ABRT treatment has become a standard of care for ES-NSCLC patient who are medically inoperable or those who decline surgery [20]. Although radiation offers a high rate of local control (nearly 90% at 5-year), the high rate of distant metastasis (up to 30%) remains the most common cause of death. Very few models exist that address the fate of cells *in vivo* after ABRT exposure. Here we addressed the response of LC cells harboring EGFR-mutation to ablative radiation treatment *in vivo*. We have selected common EGFR mutations that are known as biomarkers for the response to TKI.

Active EGFR mutations have been the primary targets for therapeutic intervention against lung adenocarcinoma, the two most common being: exon 19 deletion (60%), and L858R point mutation (35%) where leucine is replaced by arginine at position 858 of exon 21. Despite the importance of these biomarkers for EGFR-targeted therapies and the critical role of radiation therapy in the clinical management of lung cancer, no previous studies have compared the fate of cells harbouring EGFR mutations *in vivo* following exposure to radiation. Our response analyses were based on: a) subcutaneous injection of pre-irradiated cells, and b) measurements of tumor volume *in vivo* followed by ABRT treatment.

Our *in vivo* results indicate that responses to radiation treatment vary with the mutational status of the cells. In contrast, *in vitro* cellular responses (e.g. colony formation, cell viability, cellular proliferation, and cell cycle analysis) to ABR analysis were not mutation dependent. We believe that this marked difference may be based on microenvironmental events. *In vitro,* cells are cultured in a monolayer, whereas *in vivo* they are influenced by their surrounding environment and its different stimuli (e.g. growth factors, metabolites, angiogenesis) that may trigger different signaling pathways.

Interestingly, DEL-EGFR mutant was incapable of forming tumor *in vivo* when cells were pre-irradiated prior to injection. Furthermore, a decreased tumor volume nine days post-ABRT treatment was noted in DEL-EGFR group, whereas WT-EGFR and L858R-EGFR had an increased tumor volume nine days post treatment. We also report a sustained ERK activation, a key factor in cell proliferation and tumor growth [21], in tumors collected from WT-EGFR and L858R-EGFR treated groups versus DEL-EGFR treated group, which had decreased ERK activation levels. Differences in ERK activation could explain differential tumor proliferation and apoptosis profile.

The pathology behind local failure following stereotactic body radiotherapy (SBRT/ABRT) remains unknown. Information is not reported because patients treated with SBRT are usually not candidate for surgery. Palma et al. [22] have assessed the pathologic complete response (pCR) in ES-NSCLC patients who had neo-adjuvant SBRT followed with surgery [22]. They report a pCR of 60%, which was lower than their estimated rate 90% pCR after SBRT [22]. Other investigators have reported the histology of surgically resected tumors initially treated with SBRT [23,24]. A significant increase in necrosis [23], or fibrosis associated with necrosis and less dense tumor [24] have been reported in tumors with partial response to SBRT. These results support our preclinical findings suggesting necrosis as an indication of poor response post-SBRT. In our results, DEL-treated

group had no necrosis when compared to WT-treated and L858R-treated groups and this correlate with excellent response to ABRT. Differential histological response to ABRT can be due to EGFR status. These results are in agreement with our *in silico* analysis of publicly available TCGA data (See Supplementary Figures) of early stage-NSCLC patients [25-27] that showed low overall survival of patients with L858R-EGFR mutation compared to DEL-EGFR. Likewise, L858R-EGFR showed poorer survival rates than those expressing WT-EGFR.

Our work gives *prima facie* evidence of a differential response to ABRT in EGFR-driven LC with different EGFR mutation status and suggest the presence of necrosis as poor response to ABRT. This study suggests that practitioners should be cautious in patients with different EGFR status prior to considering clinical management of ES-NSCLC patients with ABRT. Further studies are warranted to confirm our finding in cohort of lung cancer patients with different EGFR status and treated with ABRT.

2.6 Figure legends:

Figure 2.1: Response to ablative radiation in isogenic EGFR-mutant cell lines. a) Clonogenic assay of isogenic EGFR-mutant cells. b) Total number of viable cells in isogenic EGFR mutant cells following ABRT. c) Colony formation assay with colonies staining positive for methylene blue. d) Cell cycle analysis. e) Proliferation rate analysis. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

Figure 2.2: Tumor development of irradiated EGFR mutant cell lines. Bioluminescence imaging (BLI) of animals injected with EGFR mutant cells that were pre-irradiated at 34 Gy.

Figure 2.3: Response to ABRT treatment in animals injected with cells harbouring different EGFR mutations. a) animals were injected subcutaneously with either WT, DEL or L858R cell lines and divided into two groups: Control and treated group with a single dose of 34 Gy. b) BLI measurements at day 9 after treatment in Ctrl and treated groups. c) Tumor volume following nine days of treatment. **: $p < 0.01$

Figure 2.4: **Histological assessment of collected tumors from control and treated groups of isogenic EGFR mutant cell lines**. a-b) Representation of necrotic area, a) representation of 60% of necrosis present in the collected tumor at 20x, b) representation of absence of necrosis at 20x. c) Percentage of necrotic area in control and treated groups of all three cell lines. d-e) Percentage of solid tumor control and treated groups, d) representation of 20% of solid tumor at 40x, e) representation of 60% of solid tumor at 40x. f) Percentage of solid tumor in control and treated groups of all three cell lines. g-h) Apoptotic cells present in collected tumors, black arrows pointing to apoptotic cells. *: $p < 0.05$

Figure 2.5: Proteins expression assessment of collected tumor tissues. a) Tissues were collected from WT-EGFR (control n=3, treated n=6), DEL-EGFR (control n=2, treated n=6), and L858R-EGFR (control n=3, treated n=6). b) Densitometry analysis of p-ERK/total-ERK ratio normalized to beta-actin. M#: mouse number.

2.7 References

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Figure 2.1:

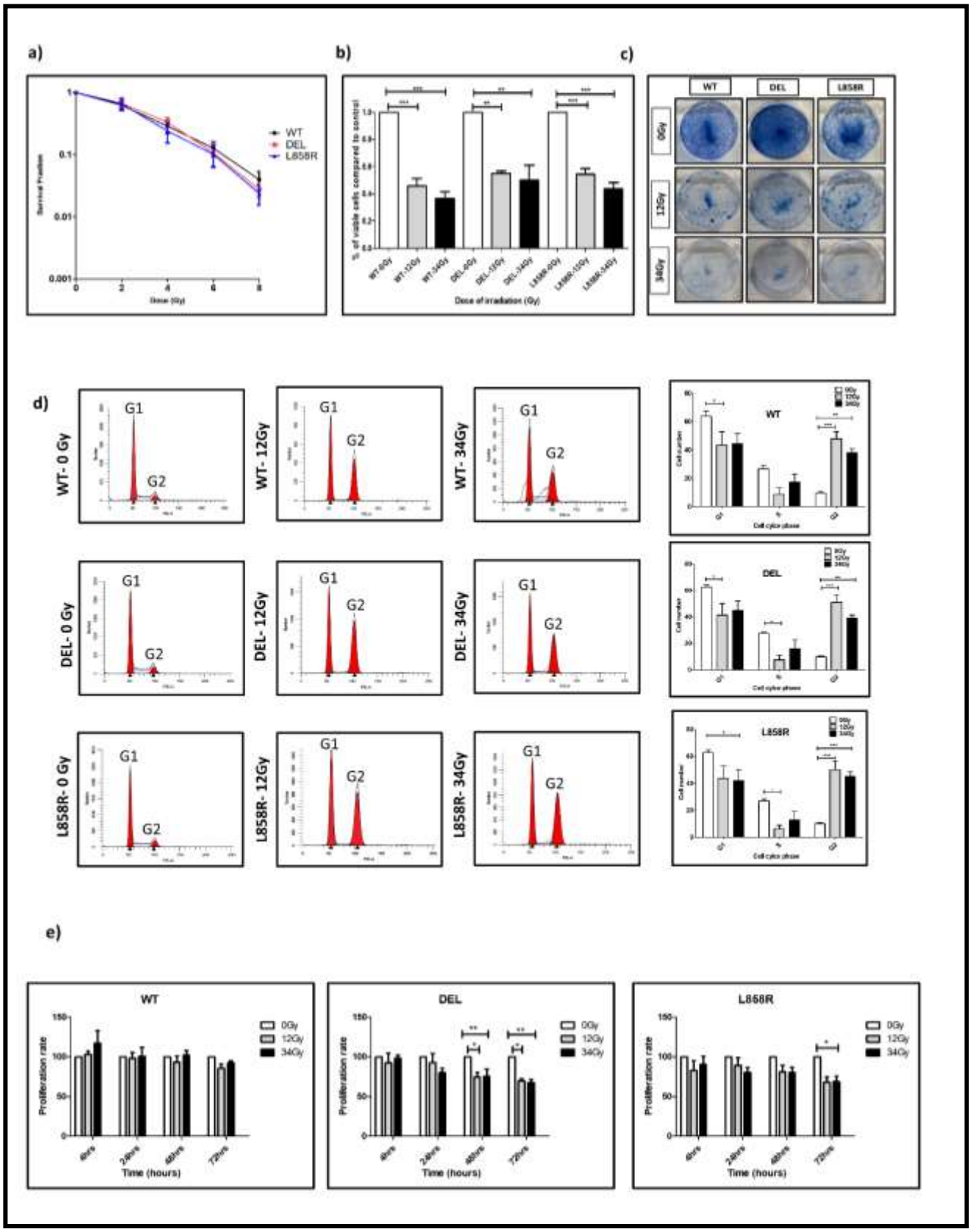
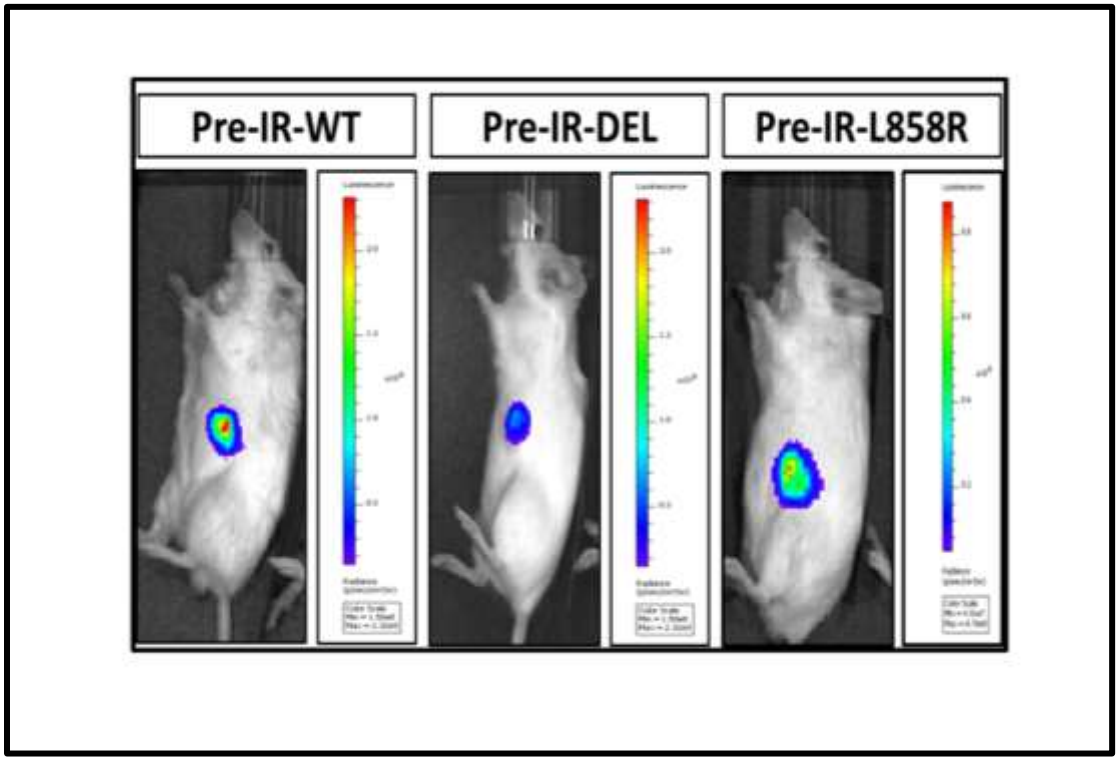

a)
b)
c)
WT
DEL
L858R
Survival Fraction
Dose (Gy)
% of viable cells compared to control
Dose of irradiation (Gy)
0Gy
12Gy
34Gy
d)
WT- 0 Gy
WT- 12Gy
WT- 34Gy
DEL- 0 Gy
DEL- 12Gy
DEL- 34Gy
L858R- 0 Gy
L858R- 12Gy
L858R- 34Gy
G1
G2
Cell cycle phase
e)
Proliferation rate
Time (hours)
4hrs
24hrs
48hrs
72hrs

Figure 2.2:



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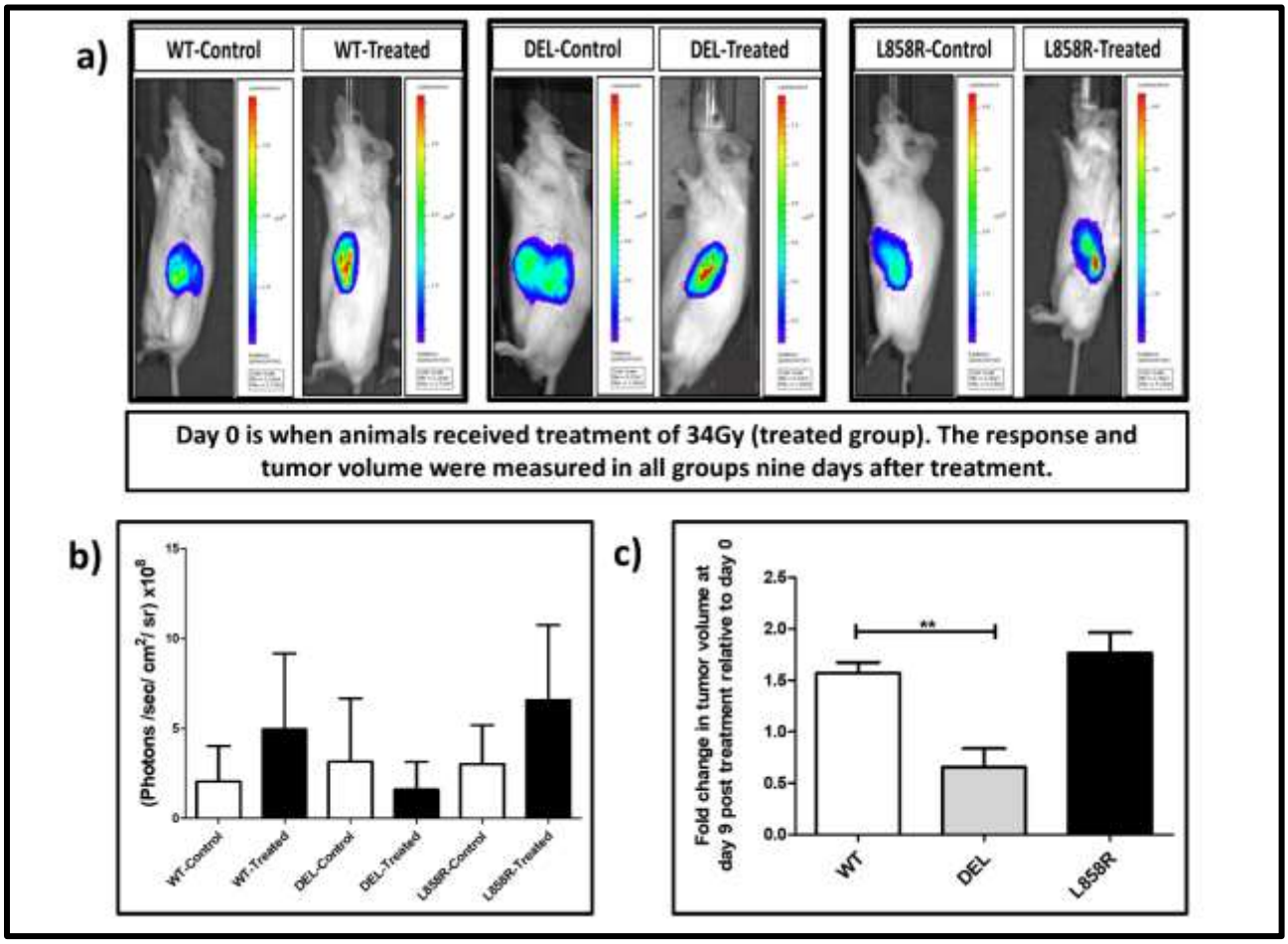
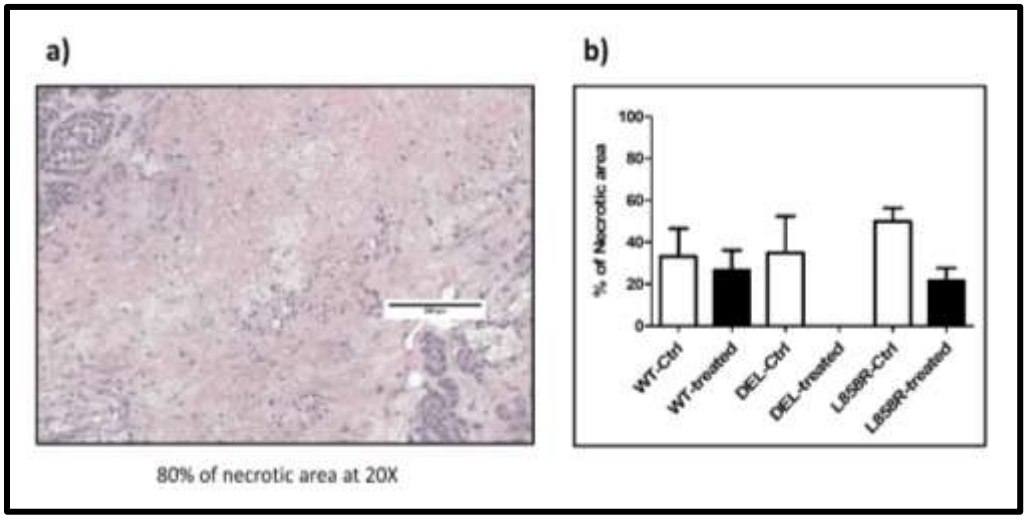
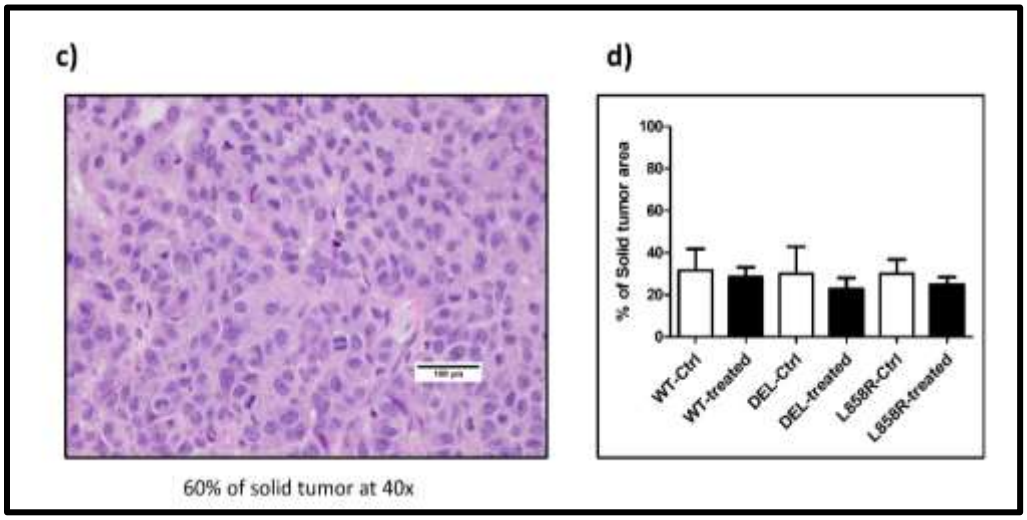

a)
WT-Control
WT-Treated
DEL-Control
DEL-Treated
L858R-Control
L858R-Treated
Day 0 is when animals received treatment of 34Gy (treated group). The response and tumor volume were measured in all groups nine days after treatment.
b)
(Photons /sec/ cm²/ sr) x10⁸
WT-Control
WT-Treated
DEL-Control
DEL-Treated
L858R-Control
L858R-Treated
c)
Fold change in tumor volume at day 9 post treatment relative to day 0
**
WT
DEL
L858R

Figure 2.4:


a)
b)
% of Necrotic area
100
80
60
40
20
0
WT-Ctrl
WT-treated
DEL-Ctrl
DEL-treated
L858R-Ctrl
L858R-treated
80% of necrotic area at 20X


c)
d)
% of Solid tumor area
100
80
60
40
20
0
WT-Ctrl
WT-treated
DEL-Ctrl
DEL-treated
L858R-Ctrl
L858R-treated
60% of solid tumor at 40x

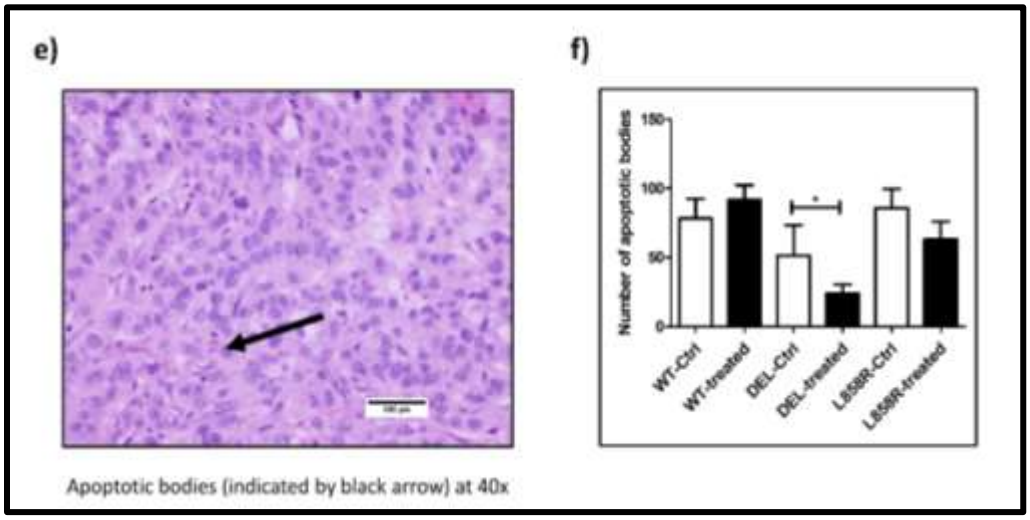
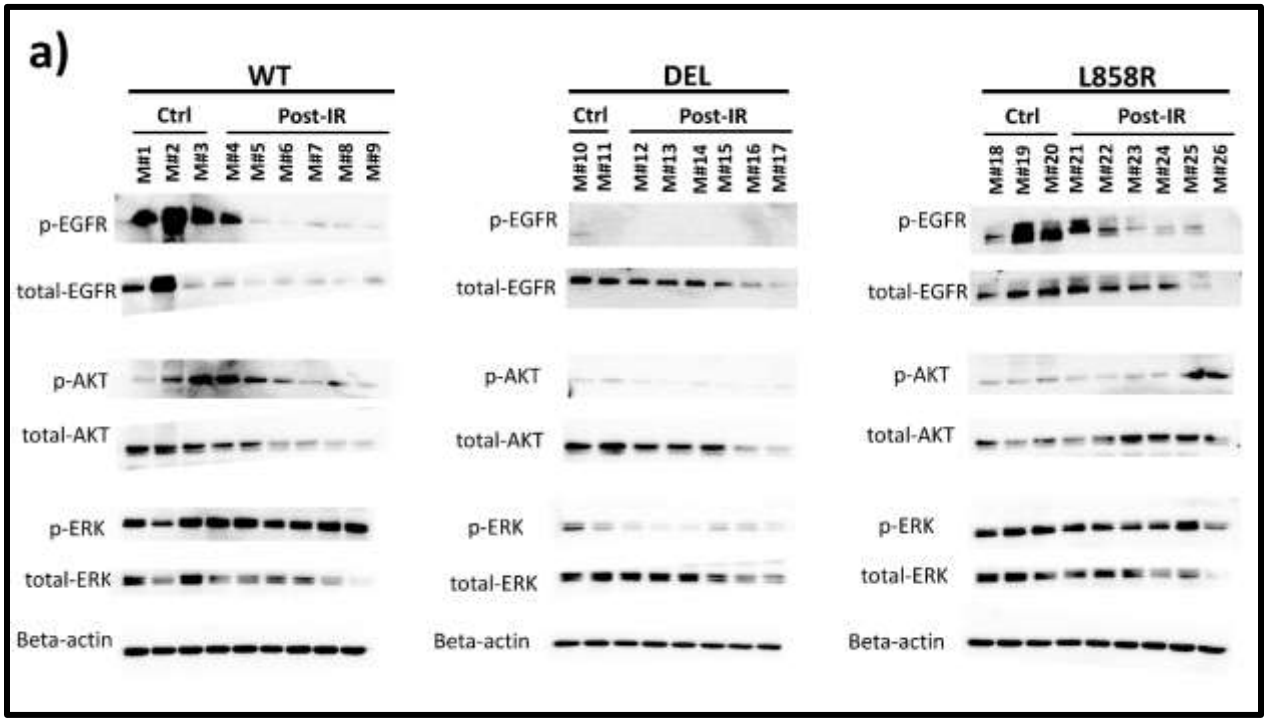

e)
f)
Number of apoptotic bodies
150
100
50
0
*
WT-Ctrl
WT-treated
DEL-Ctrl
DEL-treated
L858R-Ctrl
L858R-treated
Apoptotic bodies (indicated by black arrow) at 40x

Figure 2.5:


a)
WT
DEL
L858R
Ctrl
Post-IR
M#1
M#2
M#3
M#4
M#5
M#6
M#7
M#8
M#9
M#10
M#11
M#12
M#13
M#14
M#15
M#16
M#17
M#18
M#19
M#20
M#21
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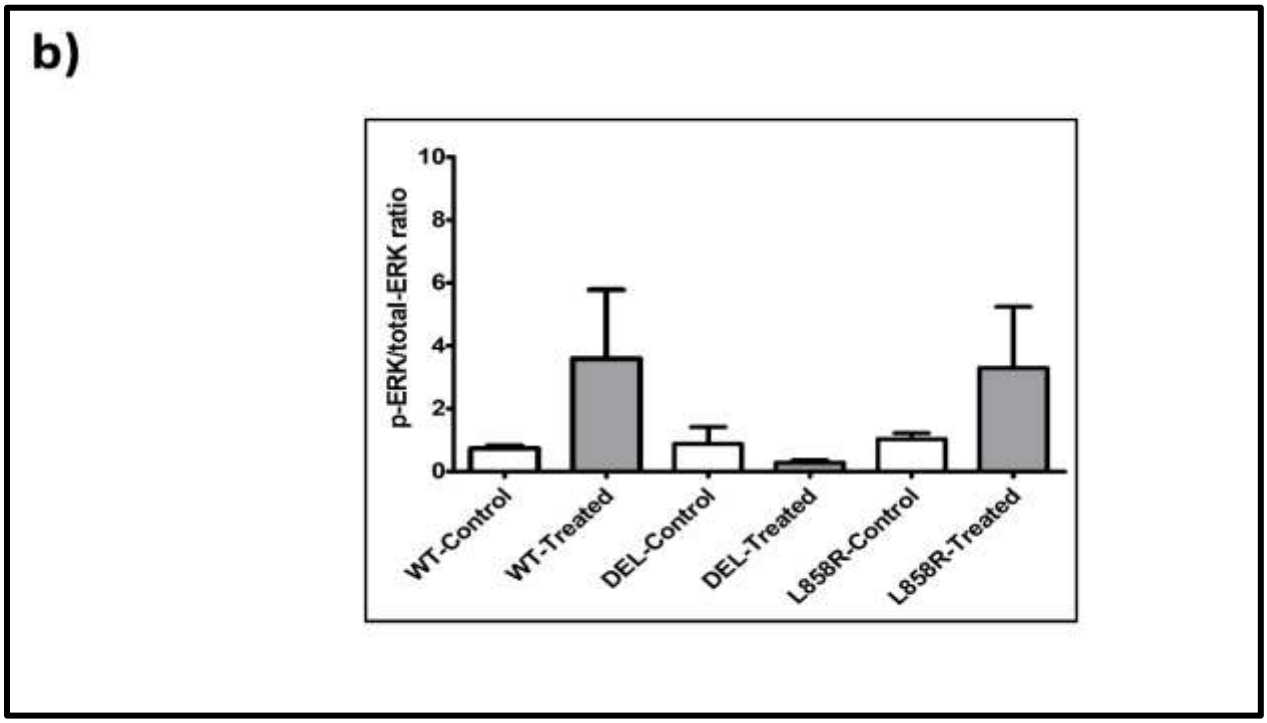
p-AKT
total-AKT
p-ERK
total-ERK
Beta-actin


b)
p-ERK/total-ERK ratio
10
8
6
4
2
0
WT-Control
WT-Treated
DEL-Control
DEL-Treated
L858R-Control
L858R-Treated

2.8 Supplementary Materials and Methods

2.8.1 Cell Cycle, and proliferation analysis

Cells were irradiated with single dose of 12 or 34 Gy, trypsinized 24hrs post-radiation, fixed using 70% ethanol and kept at -20 °C until staining. For staining, cells were washed with 1xPBS and stained with a final concentration of 50ug/ml propidium iodide and 100ug/ml RNAse A and kept at 4°C for at least 1hr before flow cytometry analysis. Cell cycle analysis was done at the Immunophenotyping Platform using BD FACSCanto II.

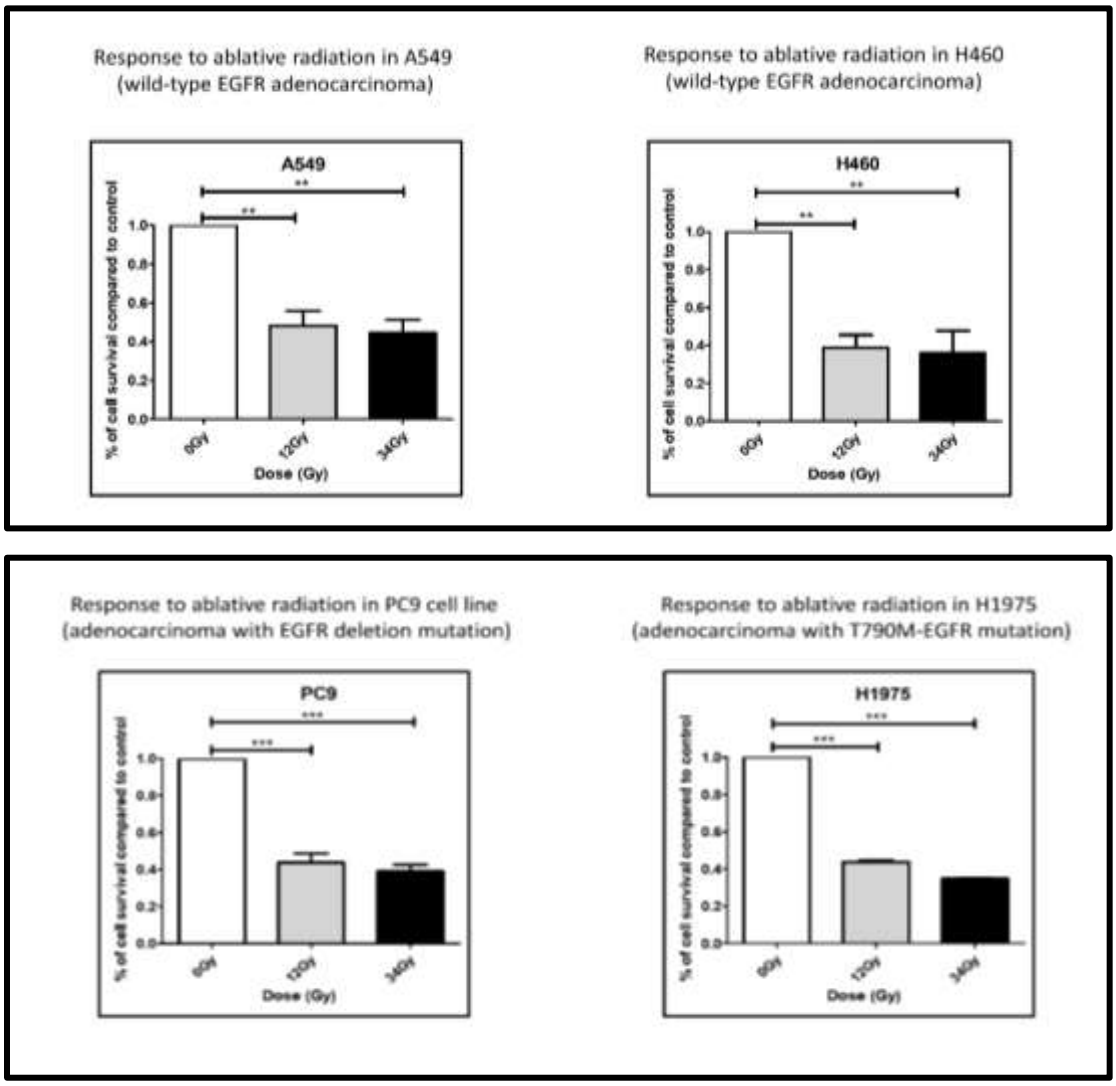
To assess cell proliferation, Vybrant 3-[4,5-dimethylthiazol-2yl]-2,5diphenyltetrazoliumbromide (MTT) assay was performed. Cells were irradiated at 0, 12 or 34 Gy and MTT was added at 4, 24, 48, and 72 hrs post-irradiation. MTT was added to cells and incubated for 4hrs, DMSO was added and absorbance was measured following incubation at 560 nm using a standard microplate reader (Thermo Scientific, Multiskan Spectrum).

2.8.2 Protein extraction and immunoblotting

Tissues were lysed in RIPA buffer (Cedarlane, Ontarion, Canada) supplemented with phosphatase (Sigma Aldrich, Ontario, Canada) and protease inhibitors (Sigma Aldrich, Ontario, Canada) using the Speed Mill Plus Homogenizer. Homogenates were centrifuged for 15 min at 1000 rpm. The supernatant containing the protein lysates was collected and proteins were quantified using BCA protein quantification method. Equal amounts of protein were separated by SDS-PAGE under reducing conditions and blotted onto polyvinylidene difluoride membrane. Membranes are blocked with 5% non-fat milk or 5% bovine serum albumin (BSA) and probed with primary directed against DEL-EGFR, L858R-EGFR, total-EGFR, phospho-EGFR, total-Akt, phospho-Akt, total-Erk 1/2, phosphor- Ersk ½ (Cell Signaling Technology, MA), beta-actin (Sigma,

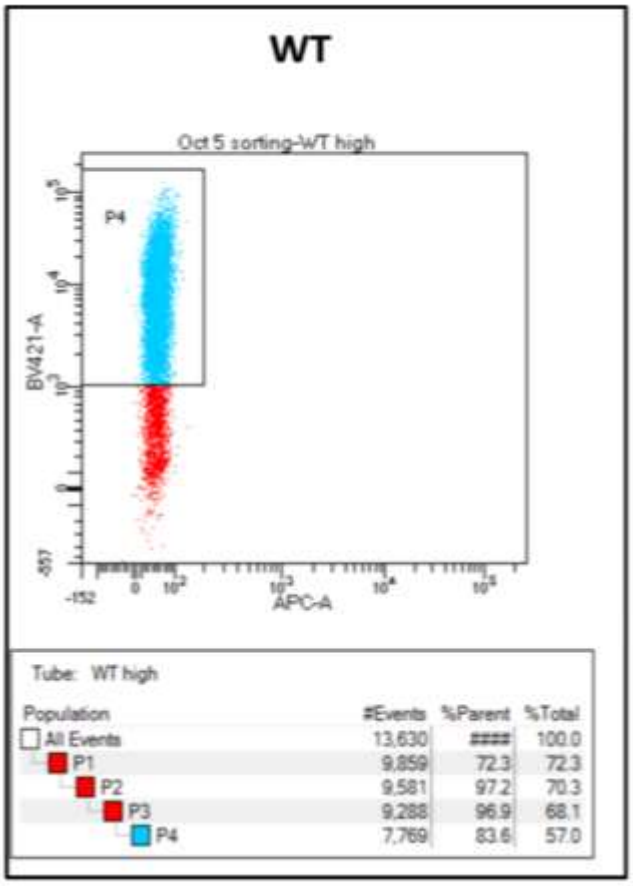
Ontario, CA) served as a loading control. All primary antibodies were used at a dilution of 1:1000 in 5% (BSA) except for beta-actin which was used at a dilution of 1:250 in 5% non-fat milk.

2.9 Supplementary Figures:

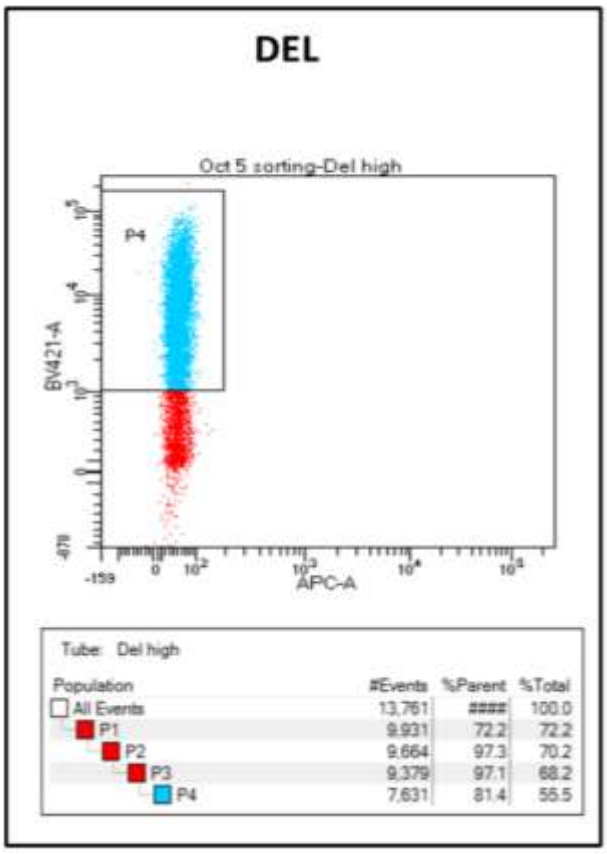


Supplementary figure 2.1: Response to ablative radiation in adenocarcinoma established cell lines with different EGFR status.

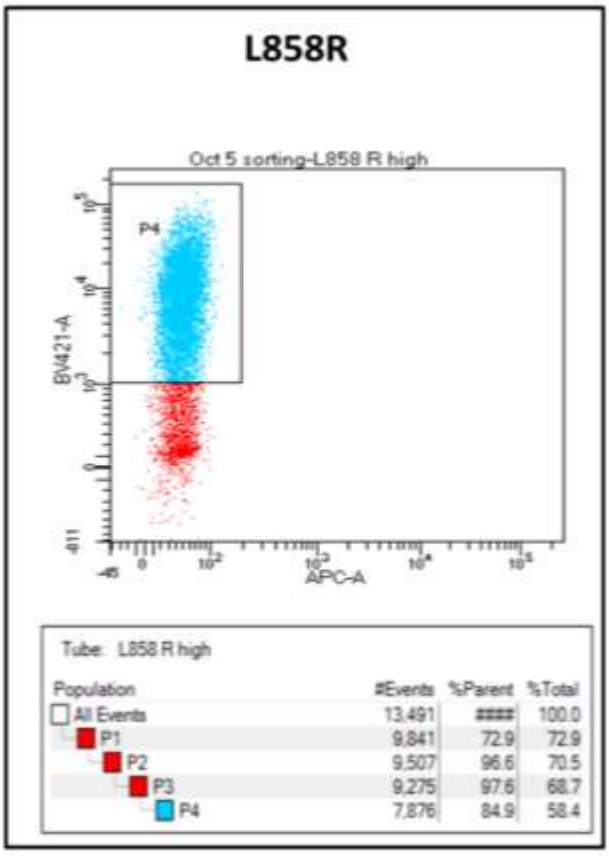
a)


WT
Oct 5 sorting-WT high
P4
BV421-A
APC-A
-557
-152
Tube: WT high
Population #Events %Parent %Total
All Events 13,630 #### 100.0
P1 9,859 72.3 72.3
P2 9,581 97.2 70.3
P3 9,288 96.9 68.1
P4 7,769 83.6 57.0

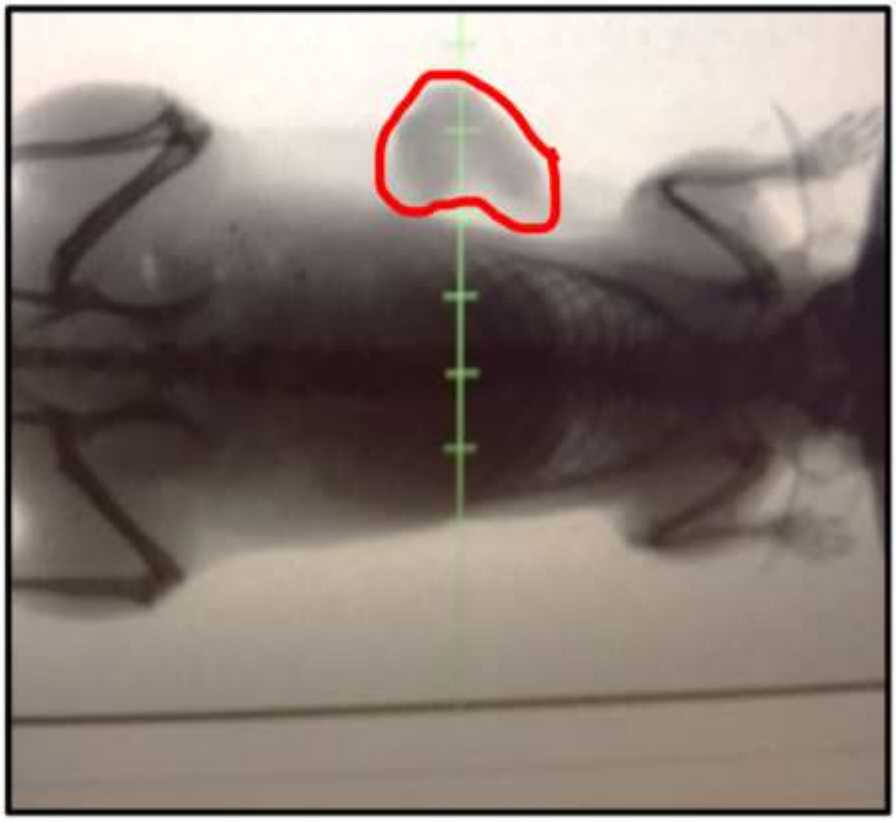
b)


DEL
Oct 5 sorting-Del high
P4
BV421-A
APC-A
-159
-878
Tube: Del high
Population #Events %Parent %Total
All Events 13,761 #### 100.0
P1 9,931 72.2 72.2
P2 9,664 97.3 70.2
P3 9,379 97.1 68.2
P4 7,631 81.4 55.5

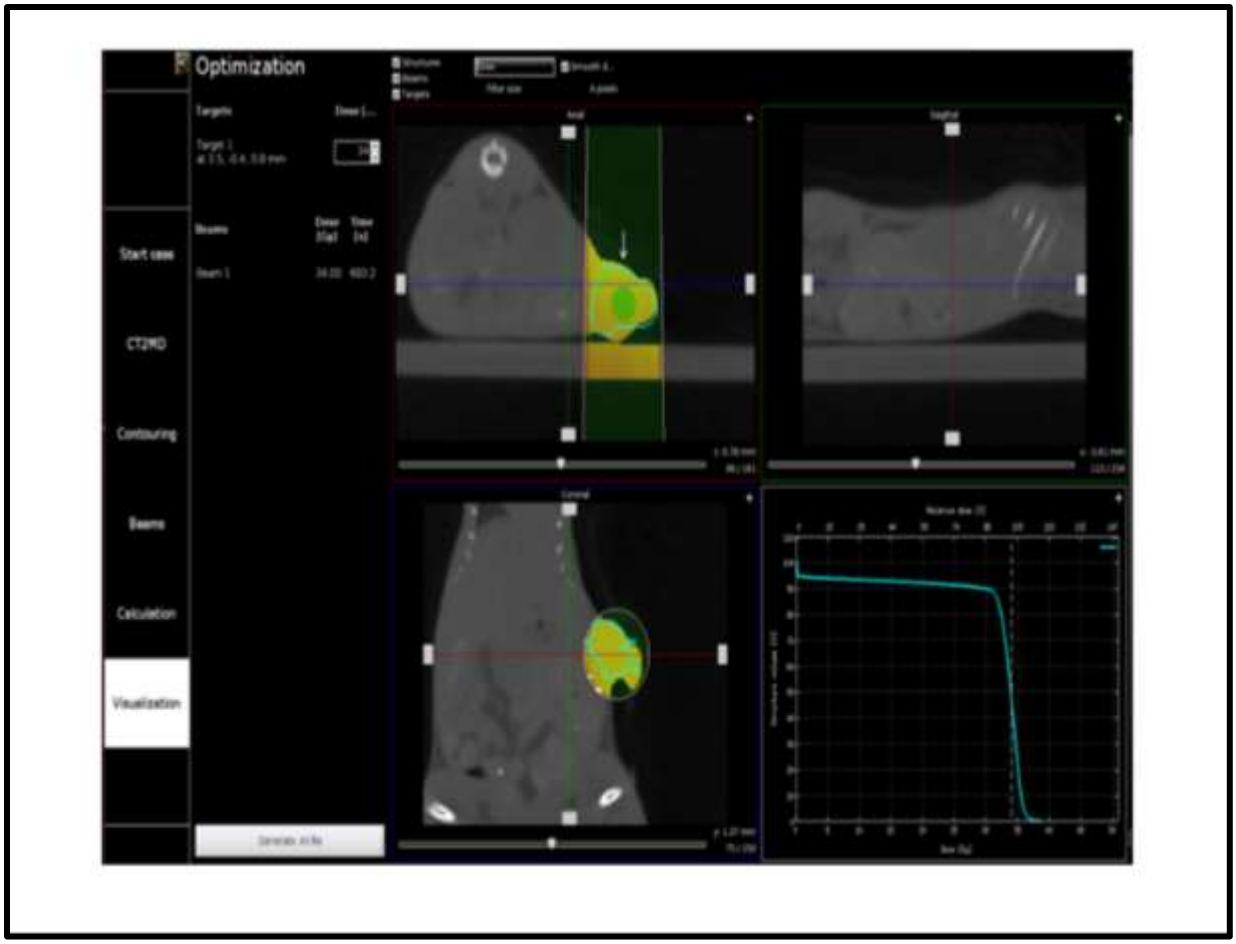
c)



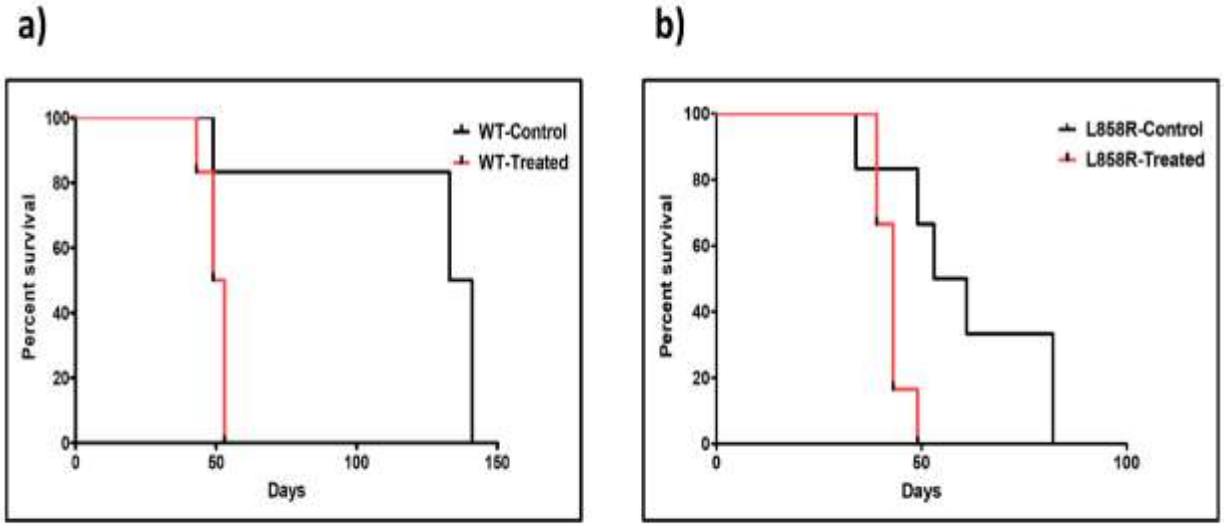
Supplementary figure 2.2: Cell sorting of BFP-Luciferase positive population of A549 transfected with either a) WT-, b) DEL-, or c) L858R-EGFR.



Supplementary figure 2.3: CT-scan image of tumor formation following subcutaneous injection of EGFR-mutant lung adenocarcinoma into YFP-SCID mice.



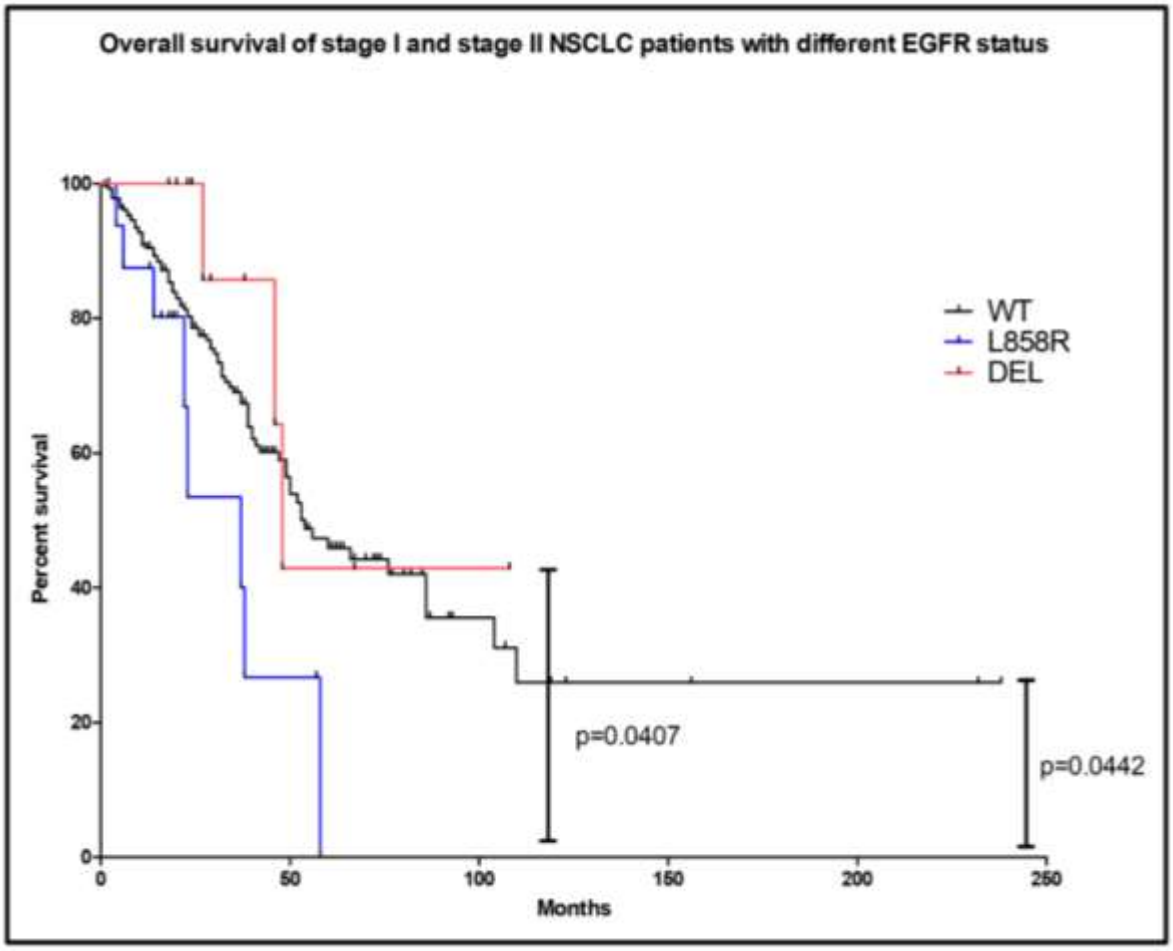
Supplementary figure 2.4: Treatment plan of animals treated with a single fraction of 34 Gy.



Supplementary figure 2.5: Overall survival data of control and ABRT-treated YFP/SCID mice injected with isogenic EGFR-mutant lung adenocarcinoma.

Supplementary table 2.1: The Cancer Genome Atlas data of early stage non-small cell lung cancer included in the overall survival analysis.

EGFR STATUS	WT	L858R	DEL	TOTAL
TOTAL REPORTED CASES	294	18	12	348
GENDER				
FEMALE	153	14	9	190
MALE	141	4	3	158
RACE				
WHITE	228	16	10	268
BLACK OR AFRICAN AMERICAN	31	1		38
ASIAN	4	1	1	7
AMERICAN INDIAN OR ALASKA NATIVE	1			1
NOT REPORTED	30		1	34
TUMOR STAGE				
I (INCLUDING IA AND IB)	189	12	6	225
II (INCLUDING IIA AND IIB)	105	6	6	122
NR				1
VITAL STATUS				
ALIVE	201	10	9	237
DEAD	93	8	3	111



Supplementary figure 2.6: Survival analysis of TCGA data of adenocarcinoma lung cancer patients. Patients with L858R-EGFR mutation have a lower overall survival when compared to patients carrying WT- and DEL-EGFR.

Connecting text

The work described in the previous chapter demonstrated implication of epidermal growth factor receptor (EGFR) in the response to stereotactic ablative radiation therapy (SABR). In the previous chapter, we described that the response to SABR may be influenced not only by the presence of a driver mutation such as EGFR, but also by the type of mutation expressed. We presented that deletion in the exon 19 exhibited better response to SABR compared to wildtype (WT)-EGFR and EGFR with L858R mutation. studies have indicated that EGFR-mutant NSCLC exhibit a much more sensitive profile to TKIs, or radiation compared to NSCLC with WT-EGFR. Moreover, almost all patients eventually develop progressive disease, requiring further treatment which is to switch to a second-line of treatment. This has led to ask the question if we can overcome resistance in lung adenocarcinoma due to WT-EGFR or EGFR mutations secondary to a treatment (T790M or C797S mutations). Thirodoxin reductase (TrxR) which is part of the thirodoxin (Trx) system is involved in regulating ROS levels. Inhibition of TrxR enzyme activity result in elevated ROS levels which can lead to apoptotic pathway activation. This has led us to explore repurposing auranofin, which is an inhibitor of TrxR, as a potential drug that can enhance antitumorigenic activity and sensitivity to radiation in lung adenocarcinoma known to exhibit resistance to treatment in NSCLC with different EGFR status. In the upcoming chapter, we explore auranofin as potential radiosensitizer in treatment-resistant lung adenocarcinoma cell lines such as wild-type EGFR lung adenocarcinoma or EGFR-T790M mutant lung adenocarcinoma.

Chapter 3: Auranofin enhances ionizing radiation effect in lung adenocarcinoma with different epidermal growth factor receptor (EGFR) status

Auranofin enhances ionizing radiation effect in lung adenocarcinoma with different epidermal growth factor receptor (EGFR) status

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3.1 Abstract

3.1.1 Background: For early stage non-small cell lung cancer (NSCLC) patients, surgery serves as the treatment of choice. However, for non-surgical candidates or for patients with advanced stages there are different therapeutic approaches that include the use of one or combined modality of chemotherapy, radiotherapy, and tyrosine kinase inhibitors (TKIs). Reactive oxygen species (ROS) result during ionizing radiation (IR) known to cause DNA damage are usually eliminated by antioxidant networks such as Thioredoxin (Trx) system. The Trx system consists of thioredoxin reductase (TrxR), thioredoxin (Trx) and NADPH. Overproduction of ROS by inhibiting TrxR activity has been associated with an increased cell death. This study aimed to investigate the potential use of auranofin, an anti-rheumatoid drug and a potent inhibitor of TrxR activity, as a radiosensitizer in lung adenocarcinoma with different epidermal growth factor receptor (EGFR) status in combination with ionizing radiation (IR).

3.1.2 Methods: lung adenocarcinoma harboring different EGFR status were used. The following lung adenocarcinoma cell lines were used: A549 and H460 both are WT-EGFR; PC9 which has an EGFR deletion mutation, and H1975 which has double EGFR mutation (L858R and acquired resistance to TKIs mutation T790M). Cells were treated with gefitinib or auranofin followed by ionising radiation (IR). The effect of combined treatment was assessed by clonogenic survival assay and ability to produce reactive oxygen species (ROS). Differential expression of TrxR1 in lung adenocarcinoma was evaluated by western blotting.

3.1.3 Results: Differential expression levels of TrxR1 and radiosensitivity were observed in lung adenocarcinoma cell lines with H1975 cell line exhibiting low TrxR1 levels and higher sensitivity

to radiation compared to A549, H460 and PC9. Treatment of EGFR-mutant lung adenocarcinoma, PC9 and H1975, with auranofin induced an increased inhibition of colony formation compared to gefitinib treatment. Decreased colony formation in lung adenocarcinoma was observed when treated with auranofin alone, as it was observed in H1975 cell line, or when combined with radiation as it was observed in PC9, A549 and H460. We have also reported an increase in ROS levels in PC9, H1975 and A549 when auranofin was combined with radiation compared to auranofin alone. Cell lysate collected from lung adenocarcinoma following different treatment exhibit differential levels of expressed TrxR1.

3.1.4 Conclusion: We report that the use of auranofin induces an antitumorigenic effect in lung adenocarcinoma harbouring EGFR-mutant and radiosensitizes lung adenocarcinoma with wild-type EGFR. We report differential levels of produced ROS and change in TrxR1 expression levels following treatment.

3.2 Introduction:

Lung cancer (LC) remains as the leading cause of cancer deaths worldwide [1] with 85% of the cases are classified into non-small cell lung cancer (NSCLC) [2]. Treatment of NSCLC depends on several factors that include the presence of a driver mutation. It is estimated that 10% to 17% of NSCLC cases will carry an active mutation in the tyrosine kinase domain (TKD) of epidermal growth factor receptor (EGFR) [3-6] and this frequency can increase up to 65% in Asian population [7-9]. EGFR-mutant lung cancer cells exhibit an initial good response to tyrosine kinase inhibitor (TKI), such as gefitinib [10,11] and erlotinib [12] which has led to using these agents as first line treatment for EGFR-positive metastatic lung cancer patients. Despite initial response to TKIs, the development of an acquired resistance in EGFR-positive NSCLC patients is inevitable and reported to occur in a median of 10 to 16 months post-treatment [13].

Radiation is one of the main treatments of NSCLC. In fact, the use of ablative radiotherapy has become an alternative treatment for early stage (ES)-NSCLC patients who are not surgical candidates [14]. Ionizing radiation (IR) main biological effect is the generation of reactive oxygen species (ROS) [15]. ROS is a key modulator of several biological processes that are involved in both cell survival and cell death. Elevated levels of ROS can result in significant DNA damage and multiple cellular responses such as cell cycle arrest, senescence and apoptosis [16]. However, it has been reported that basal levels of generated ROS as a response to low-dose ionizing radiation (LDIR) can cause beneficial cellular responses [17]. Studies have indicated that cancer stem cells have lower levels of ROS compared to their more mature progeny and are associated with increased expression of free radical scavenging systems, reduced DNA damage and spared cells after irradiation compared to non-tumorigenic cells [18].

ROS levels are regulated by antioxidant systems such as Thioredoxin (Trx), glutaredoxins (Grx), or glutathione (GSH) systems [19]. The thioredoxin (Trx) system, which consists of Trx protein, thioredoxin reductase enzyme (TrxR) and NADPH is a major regulator of ROS levels and redox cellular function [20]. TrxR enzyme has been reported to be involved in tumor-associated redox process which is critical in cancer pathology [21-23]. A study of a panel of 60 human cancer cell lines has screened for TrxR1 levels and reported TrxR1 to be over expressed in multiple types of tumor [24-30]. NSCLC is one of the cancers to be reported with the highest levels of TrxR which was supported by data obtained *in vitro* and *in vivo* [25,27,31]. Upregulation of TrxR in NSCLC is reported to be part of several biological processes such as redox balance, transcription factor activities and tumor growth [24,32]. A retrospective study conducted by Chen et al. reported TrxR as an independent poor prognostic factor in EGFR wild type and ALK negative NSCLC patients [33]. Other studies [34] have reported a significant decrease in TrxR levels in NSCLC who underwent surgery, which suggests TrxR activity in NSCLC as a promising biomarker to monitor treatment outcome and response [34].

Auranofin, which is a potent inhibitor of TrxR enzyme activity [35], has been under investigation in several preclinical studies on lung cancer. A study by Yan et al reported that reduced or lost expression of glutathione reductase (GSR) gene in NSCLC cell lines increases cell lines sensitivity to auranofin [36]. Another group, Liu et al. [37], has reported that the use of auranofin enhances chemosensitivity of small cell lung cancer (SCLC) cells in vitro and in vivo when combined with cisplatin in treating SCLC. The reported that auranofin sensitized SCLC to cisplatin by stimulating overproduction of ROS which led to mitochondrial dysfunction and DNA damage in SCLC [37]. Currently, auranofin is being tested in combination with sirolimus, an mTOR inhibitor, to treat patients with advanced solid tumors or recurrent NSCLC [37]. Based on the previously mentioned

findings, TrxR serves as a potential biomarker and target in treating NSCLC [38-40]. In the current study, our aim is to investigate the efficacy of auranofin *in vitro* as antitumorigenic and radiosensitizer drug in lung adenocarcinoma that are resistant to treatment and determine if combination of auranofin with radiation will induce a ROS-dependent response.

3.3. Materials and Methods:

3.3.1 Cell culture. lung adenocarcinoma cell lines were used: A549 and H460 cell lines, both carry wild-type (WT) EGFR; PC9 cell line with E746-A750 in-frame shift deletion; and H1975 cell line harbouring the T790M somatic mutation resistant to TKIs. PC9 and H1975 cell lines were kindly provided by Dr. Sidhong Huang. lung adenocarcinoma with wild-type EGFR (A549 and H460) were obtained from ATCC. All cell lines were maintained in RPMI supplemented with 10% FBS and 1% Penicillin-streptomycin, except for A549 which was maintain in DMEM-F12 supplemented with 10% FBS and 1% Penicillin-streptomycin. Cells were cultured at 37°C in humidified atmosphere containing 5% CO_2 and 95% air.

3.3.2 Clonogenic assay. The colony-forming assay was performed as reported previously by our group [41]. Lung adenocarcinoma cells were seeded and irradiated with increasing does of irradiation (0-8 Gy) and cultured until colonies of at least 50 cells have appeared (8-10 days). The surviving fraction was determined by dividing the number of formed colonies by the number of plated cells.

For colony forming assay done with combined treatment using gefitinib or auranofin with radiation, cells were seeded at different density and allowed to adhere overnight. The following day cells were treated with different doses of gefitinib or auranofin. PC9 and H1975 cells were treated with gefitinib or auranofin at final concentration of 0.25, 0.5, 1 or 2 µmolar/ml. A549 and H460 cells were treated with auranofin at concentration of 1 or 2 µmolar/ml. 24hrs post-drug-

treatment cells were irradiated at different doses using Faxitron X-ray machine. The voltage of X-ray tube was set to 160 kVp, current of 6.3 mA and a dose rate of 0.63Gy/min. Cells were then cultured at 37 ℃ and 5% CO_2 until colonies of at least 50 cells have appeared (8-10 days), cells were then fixed with formalin, and stained with methylene blue. Non-treated cells served as control.

3.3.3. Reactive oxygen species (ROS). For ROS measurements, cells were seeded and left to adhere over night. The following day, cells were treated with auranofin and non-treated cells served as a control. PC9 and H1975 were treated with auranofin at a final concentration of 0.25 µM, A549 and H460 were treated with auranofin at a final concentration of 0.5 µM. 24hrs post-treatment with auranofin, cells were trypsinized, washed with 1X PBS twice and stained with CM-H2DCFA (5-(and 6-)-chloromethyl-2',7'-dichlorodihydrofluoresceine diacetate (Invitrogen Molecular Probes, Eugene, OR) at a final concentration of 10mM in phenol red free media for 30 minutes at 37°C. Cells were then irradiated at 2 Gy and fluorescence intensity was then measured using fluorescent microplate reader at an excitation of 490 nm and emission of 525 nm (Tecan, Infinite M2000). Relative produced ROS levels (measured by fluorescence intensity) was calculated using the following formula: (fluoresce intensity treated - fluoresce intensity blank)/ (fluoresce intensity control - fluoresce intensity blank). Non-treated cells served as control.

3.3.4 Immunoblotting and analysis. Immunoblotting of collected lysate was done as mentioned previously by our group [41]. Briefly, lung adenocarcinoma were cultured and allowed to adhere overnight. To assess for TrxR1 levels, lysate was collected the following day using RIPA buffer (Cedarlane, Ontario, Canada) supplemented with phosphatase (Sigma Aldrich, Ontario, Canada) and protease inhibitors (Sigma Aldrich, Ontario, Canada). Cell lysates were centrifuged for 15 min at 1000 rpm. The supernatant containing the protein lysates was collected and proteins were

quantified using BCA protein quantification method. Equal amounts of protein were separated by a 12% SDS-PAGE under reducing conditions and blotted onto polyvinylidene difluoride membrane. Membranes were then blocked in 5% fat-free milk and probed with primary antibody against TrxR1 (Santa Cruz Biotechnology, TX) and normalized to beta-actin (Cell Signaling, MA). Protein densitometric measurements were done using ImageJ software.

For collection of cell lysate following treatment, cells were cultured and allowed to adhere overnight. The following day, cells were then treated or not with auranofin or gefitinib for 24hrs, at concentration of 0.25 µM for PC9 and H1975 and 0.5 µM for A549 and H460. For combined treatment of auranofin with radiation, cells were treated with auranofin for 24hrs, at concentration of 0.25 µM for PC9 and H1975 and 0.5 µM for A549 and H460 and irradiated at 2Gy. Cell lysate was collected one day post-treatment using RIPA buffer.

3.3.5 Statistical analysis. All experiments were performed in triplicates and repeated independently at least three times. All generated data were analyzed using GraphPad Prism software (version 5.01). Comparison of survival fraction at 2 Gy in lung adenocarcinoma compared to A549 cell line was done using two-tailed Student's t-test. Data are reported as average ± standard error of the mean (SEM). Statistical significance was set at $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

3.4 Results:

3.4.1 Expression of thioredoxin reductase 1 (TrxR1) in lung adenocarcinoma correlated negatively with radiosensitivity

Assessment of TrxR1 protein levels was done in A549, H460, H1975 and PC9, where we noted that H1975 cell line exhibited lower levels of TrxR1 compared to other cell lines. TrxR1 levels in H1975 was 0.02-fold while A549, H460 and PC9 had TrxR1 levels of 0.87-fold, 1.61-fold, and 0.78-fold, respectively (**Figure 3.1 a-b**). Moreover, we noted that lung adenocarcinoma cell lines exhibited different sensitivity to radiation (**Figure 3.1 c**). Survival fraction at 2 Gy (SF2) of lung adenocarcinoma following exposure to radiation indicated a decrease in the total number of formed colonies by 33% in A549, 40% in H460, 55% in PC9, and 60% in H1975 (**Figure 3.1 d**) when compared to non-treated cells (0 Gy). A significant decrease in colony formation was observed in EGFR-mutant PC9 (P = 0.041) and H1975 (P = 0.022) cell lines when compared to WT-EGFR A549 cell line (**Figure 3.1 d**). Interestingly, H1975 cell line, which has the lowest levels of TrxR1, exhibited higher sensitivity to radiation compared to A549, H460 and PC9.

3.4.2 Colony formation assessment in EGFR-mutant lung adenocarcinoma cells suggests high potency of auranofin in inhibiting colony formation compared to TKI

Response of EGFR-mutant lung adenocarcinoma to auranofin was assessed in PC9 and H1975 cell lines and its efficacy was compared to gefitinib. PC9 cell line has an in-frame shift deletion, while H1975 cell line has T790M mutation which is known to exhibit resistance to TKIs. PC9 and H1975 were treated with 0.25µM, 0.5µM, 1µM or 2µM of gefitinib or auranofin and response to treatment was assessed using colony formation assay. PC9 cells exhibited a complete inhibition of colony formation when treated with gefitinib even at a low dose of 0.25µM (**Figure 3.2 a**). PC9 treatment with auranofin induced a complete inhibition of colony formation at a dose of 0.5µM (**Figure 3.2 b**). In the case of H1975, treatment with gefitinib did not inhibit colony formation despite higher doses of gefitinib (**Figure 3.2 c**). H1975 treated with auranofin had a complete inhibition of colony formation following treatment with doses as low as 0.25µM (**Figure 3.2 d**). Inhibition of colony

formation following treatment with gefitinib or auranofin in PC9 and H1975 are summarized in (**Figure 3.2 e**).

3.4.3 Auranofin exhibits high antitumorigenic effect in wild-type EGFR lung adenocarcinoma

Since our previous assessment of auranofin suggested high potency in inhibiting colony formation in EGFR-mutant lung adenocarcinoma, we wanted to assess its potency in WT-EGFR lung adenocarcinoma such as A549 and H460. A549 and H460 cell lines were treated with auranofin at a dose of 1µM and 2µM and formation of colonies was used to assess response to treatment. Auranofin caused a decrease in the number of formed colonies where A549 had a 32% (at 1µM) and 14% (at 2µM) of formed colonies compared to non-treated cells (**Figure 3.3 a**). H460 cells also exhibited a decrease in the number of formed colonies following treatment with auranofin where it had 29% (at 1µM) and 17% (at 2µM) of formed colonies compared to non-treated cells (**Figure 3.3 b**).

3.4.4 Auranofin exhibits higher radiosensitization profile in EGFR-mutant lung adenocarcinoma compared to gefitinib

To test the efficacy of gefitinib and auranofin in radiosensitizing EGFR-mutant lung adenocarcinoma cells, PC9 and H1975 cell lines were pre-treated with either gefitinib or auranofin followed with radiation and assessed their response to combined treatment through colony formation assay. Treatment of PC9 with gefitinib has caused a complete inhibition of PC9 cells to form colonies (**Figure 3.4 a**). The complete inhibition (or 100% inhibition) was observed as different doses of gefitinib ranging from 0.25µM to 2.0µM, suggesting high sensitivity to gefitinib. Radiation alone (0 µM of gefitinib) caused a 54.5% of inhibition in PC9 cells. In the case of H1975,

higher doses of gefitinib (2.0μM) combined with radiation resulted in a significant inhibition (P = 0.006) of colony formation with 70% colony formation inhibition at SF2 compared to radiation alone (0 μM of gefitinib) which had 59.5% of colony formation inhibition at SF2 (**Figure 3.4 b**).

Pre-treatment of PC9 and H1975 cells with auranofin has enhanced the efficacy of radiation. We observed a significant inhibition (P = 0.0001) of colony formation when PC9 cells were pre-treated with 0.25μM of auranofin followed with radiation which resulted in an inhibition of colony formation of 99.98% with an increase in inhibition of colony formation at higher doses of auranofin (0.5, 1.0, and 2.0 μM of auranofin) (**Figure 3.5 a**). Radiation alone resulted in an inhibition of colony formation of 54.55% at SF2 (**Figure 3.5 a**). H1975 cells pre-treated with auranofin had a complete inhibition of colony formation (100% inhibition) at different doses of auranofin ranging from 0.25μM to 2.0μM suggesting high sensitivity to the combined treatment. H1975 treated with radiation alone had an inhibition of 59.5% at SF2 (**Figure 3.5 b**).

3.4.5 Auranofin radiosensitizes wild-type EGFR lung adenocarcinoma to radiation

We then evaluated the use of auranofin combined with RT in WT-EGFR lung adenocarcinoma cells. A549 and H460 cells were pre-treated with 1μM or 2μM of auranofin and was followed with RT. Pre-treatment of A549 with 2μM of auranofin resulted in significant (P = 0.003) inhibition of colony formation with an inhibition of 86.5% compared to radiation alone which had an average inhibition of 32.8% at SF2 (**Figure 3.6 a**). Furthermore, pre-treatment of H460 cells with auranofin resulted in significant colony inhibition at 1μM and 2μM with an inhibition of 71.4% (P = 0.035) and 83.2% (P= 0.003), respectively versus radiation alone which had an inhibition of colony formation of 40.5% at SF2 (**Figure 3.6 b**).

3.4.6 Production of reactive oxygen species (ROS) as a response to treatment in lung adenocarcinoma

To determine whether auranofin alone or combined with radiation can influence the levels of produced ROS in lung adenocarcinoma with different EGFR status, were measured ROS levels using a specific stain (CM-H2DCF) in lung adenocarcinoma cell lines (**Figure 3.7**). In PC9 cells, a significant increase of ROS levels was observed when cells were exposed to ionizing radiation alone 1.43-fold (P = 0.017) or in combination with auranofin1.32-fold (P =0.03) compared to control, while auranofin resulted in 0.92-fold of produced ROS (**Figure 3.7 a**). H1975 cells exhibited a significant increase of produced ROS levels when exposed to radiation alone 1.72-fold (P = 0.001) while combination of auranofin with radiation resulted in 1.28-fold compared to control, treatment with auranofin alone resulted in 0.88-fold of released ROS (**Figure 3.7 b**). A significant increase in ROS levels was also observed in A549 cells, which are wild type EGFR, with an increase of 1.5-fold (P = 0.0032) when exposed to radiation alone and 1.63-fold (P = 0.0013) increase when auranofin was combined with radiation compared to the control, while auranofin alone resulted in 1.11-fold of released ROS (**Figure 3.7 c**). In the case of H460 cells, although there was no significant increase in ROS levels, we have noticed a trend increase of 1.1-fold following radiation alone, and 1.06-fold following combined treatment of auranofin and radiation compared to the control, while auranofin alone resulted in 0.93-fold of ROS levels (**Figure 3.7 d**). These results suggest that the levels of produced ROS are not only impacted by EGFR status, but also by the type of treatment in which cells were exposed to.

3.4.7 Expression of TrxR1 in lung adenocarcinoma cells with different EGFR status

TrxR1 plays an important role in regulating redox processes in tumors, particularly in regulating ROS production, we wanted to investigate if TrxR1 expression in lung adenocarcinoma will be

altered due to treatment exposure such as auranofin, ionizing radiation or combination of both. We performed an immunoblotting assay on A549, H460 and PC9 cells following treatment with gefitinib, auranofin, ionizing radiation at 2 Gy or combined treatment of auranofin followed with radiation at 2 Gy (**Figure 3.8**). Combined treatment of auranofin followed with radiation at 2 Gy resulted did not alter the expression of TrxR1 levels where we observed TrxR1 levels of 0.95-fold in A549, 0.68-fold in H460, and 1.1-fold in H1975 compared to control. This suggests that combined treatment of auranofin with radiation is effective in inhibiting the enzyme activity but not its expression levels.

3.5 Discussion:

Auranofin, which is a clinically approved drug for the treatment of arthritis [42], is being considered as an antitumor agent in treating several cancers. Previous studies have reported that auranofin can inhibit thioredoxin reductase (TrxR) activity which is part of the Trx antioxidant system, leading to enhanced production of ROS levels [43,44]. Since upregulation of TrxR and its involvement in several biological processes has been reported in NSCLC [24,32], targeting or altering its activity may help in inhibiting tumor cells survival and leads to tumor cell death. In our study, we investigated the potential use of auranofin as antitumor drug and as a radiosensitizer that can enhance antitumor activity by altering ROS levels in lung adenocarcinoma with different EGFR status.

Our results support the hypothesis that auranofin exhibits an antitumor activity by its ability in inhibiting colony formation as a single drug or when combined with ionizing radiation depending on the status of EGFR in lung adenocarcinoma cells. In our study, we reported that treatment of lung adenocarcinoma cells with auranofin and radiation induced an increase of ROS levels in PC9, H1975 and A549 cells compared to auranofin alone. In the case of H1975, ionizing radiation has led to the highest levels of released ROS. The antitumor effect caused by auranofin can inhibit the

activity of TrxR1 which impacts the ROS levels and enhance response to treatment. Furthermore, we have observed that several factors impact ROS levels such as EGFR status, type of treatment, dose and duration of treatment.

In lung cancer, EGFR has been a key target for treatment and prevention. However, chronic treatment with tyrosine kinase inhibitors (TKIs) has led to drug resistance which triggered interest in finding alternatives to overcome resistance to TKIs. Chronic exposure to low ROS levels can stimulate several biological processes such as mitosis, cell survival, cell growth, cell proliferation and angiogenesis in cancers [45]. On the other hand, high ROS levels have toxic effects on cancer cells and can lead to cell cycle arrest, cell death and enhance sensitivity to TKI [22,46]. In the study by Leung et al., it has been reported that the use of sanguinarine, a powerful ROS inducer, in EGFR-TKI-resistant H1975 cells caused an accumulation of ROS through activation of NOX3 leading to inactivation of MsrA and overoxidation of EGFR ^{T790M} mutation [22]. Furthermore, overproduction of ROS through induction of superoxide and hydrogen peroxide has been reported to induce cell death in NSCLC cells [47]. Overall, the previously mentioned information suggests that inducing overproduction of ROS can help in overcoming resistance in lung adenocarcinoma cells.

Unlike normal cells, cancer cells have higher levels of ROS and antioxidant activity that are meant to keep a balance status. At the event where cancer cells are unable to overcome additional oxidative stress, they become more vulnerable to ROS [48,49] where high levels of ROS can induce DNA damage, cell cycle arrest and cell death [15]. Studies have investigated the idea of stimulating overproduction of ROS as a way of targeting tumor cells. One study has reported that breast cancer stem cells were sensitive to glycolysis inhibitor 2-DG, a ROS inducer, by inhibiting the antioxidant pathways thioredoxin (TXN) and glutathione (GSH) [50]. A study by Yan et al. have investigated

the sensitivity of human lung cancer cell lines to auranofin following knock out or knock down of glutathione (GSH)/GSH reductase (GSR) [36]. In their study, analysis of a panel of 129 NSCLC cell lines reported a correlation between auranofin sensitivity and expression levels of GSR, glutamate-cysteine ligase catalytic subunit (GCLC), and NAD(P)H quinone dehydrogenase 1 (NQO1) genes[36]. Their findings demonstrated that lung cancers with compromised enzyme expression, such as the ones needed for glutathione homeostasis (e.g. GSR), may be targeted by thioredoxin/thioredoxin reductase inhibitors such as auranofin. Other studies have reported that auranofin can inhibit both thioredoxin and glutathione antioxidant systems, such as the case when auranofin was combined with Vitamin C (a ROS inducer) where it resulted in a synergistic antitumor activity in triple-negative breast cancer [51].

In conclusion, we report that the anti-arthritic drug auranofin can be used as an antitumor drug and a radiosensitizer in lung adenocarcinoma wild type and EGFR-mutant cell lines. The idea of repurposing this drug in combination with standard treatment could lead to better response and may be an alternative option at the time of progression and development of resistance.

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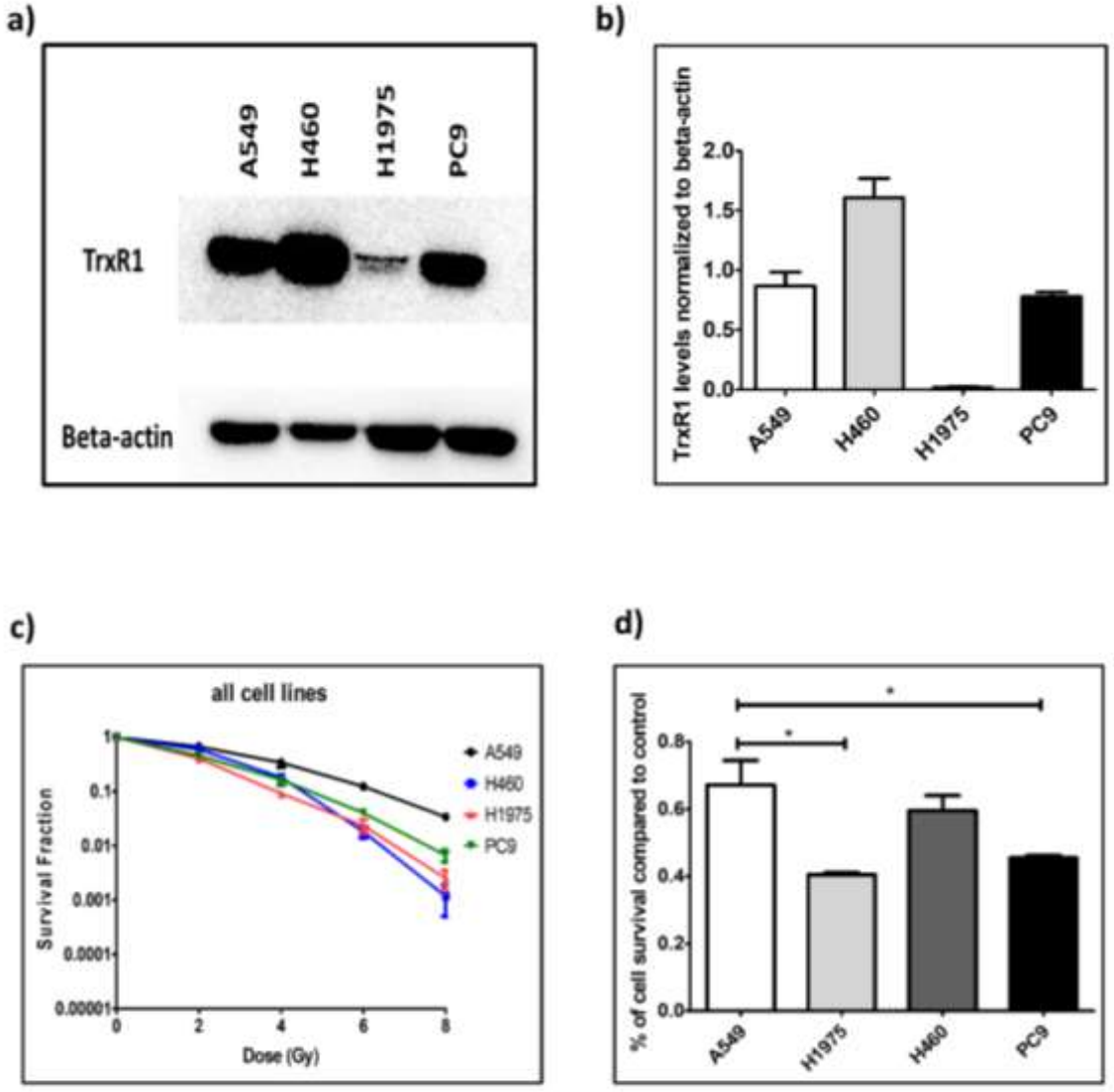
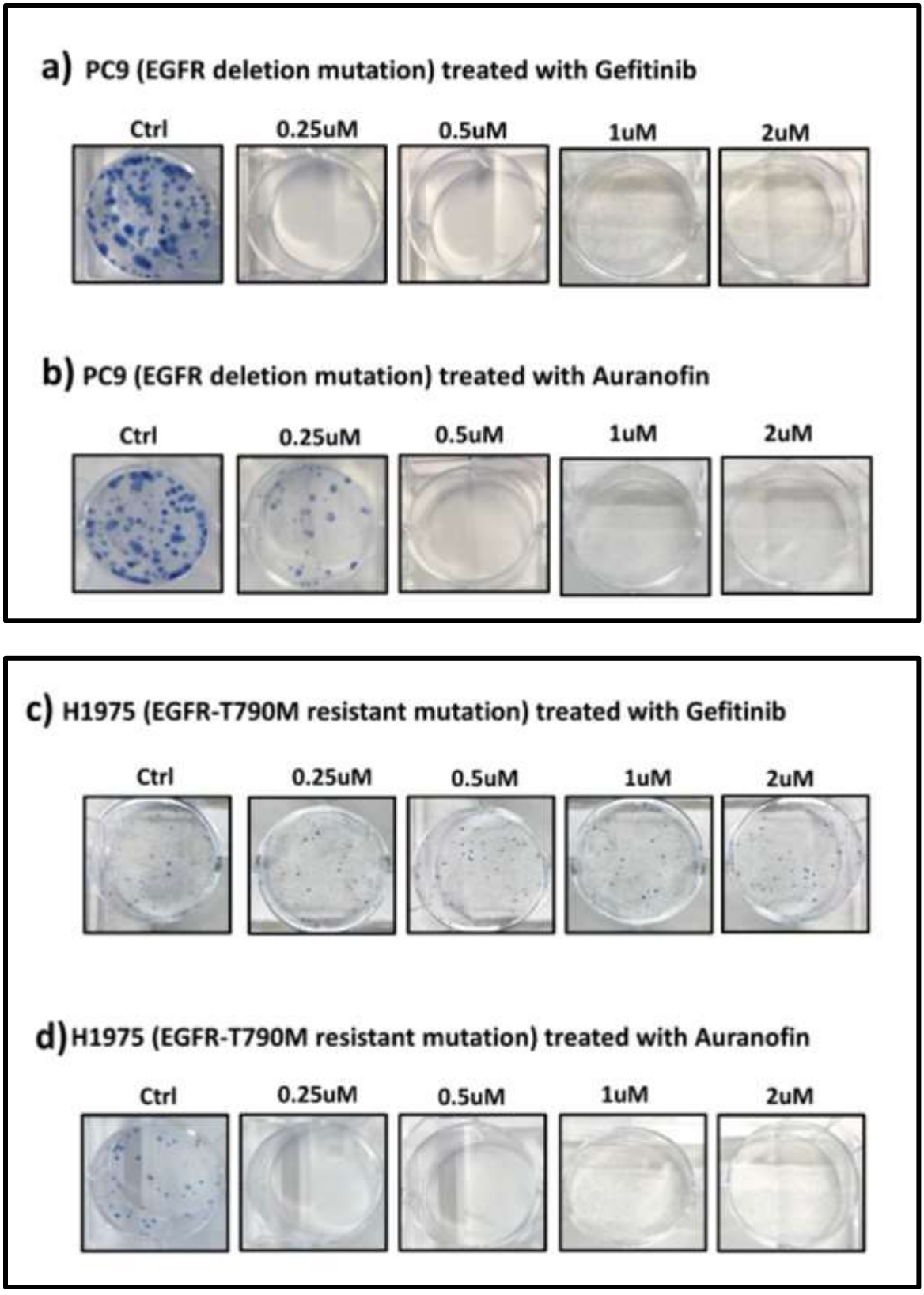


Figure 3.1: Differential TrxR1 expression and response to ionizing radiation in lung adenocarcinoma with different EGFR status. a) Differential TrxR1 expression in lung adenocarcinoma harbouring different EGFR. b) TrxR1 levels in lung adenocarcinoma cell lines normalized to beta-actin. c) Clonogenic survival of lung adenocarcinoma with different EGFR status. d) Cell survival at SF2 of lung adenocarcinoma compared to control (nontreated cells) with significant decrease in cell survival in H1975 (60%) and PC9 (55%). N = 3, *: *P* <0.05.


a) PC9 (EGFR deletion mutation) treated with Gefitinib
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c) H1975 (EGFR-T790M resistant mutation) treated with Gefitinib
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d) H1975 (EGFR-T790M resistant mutation) treated with Auranofin
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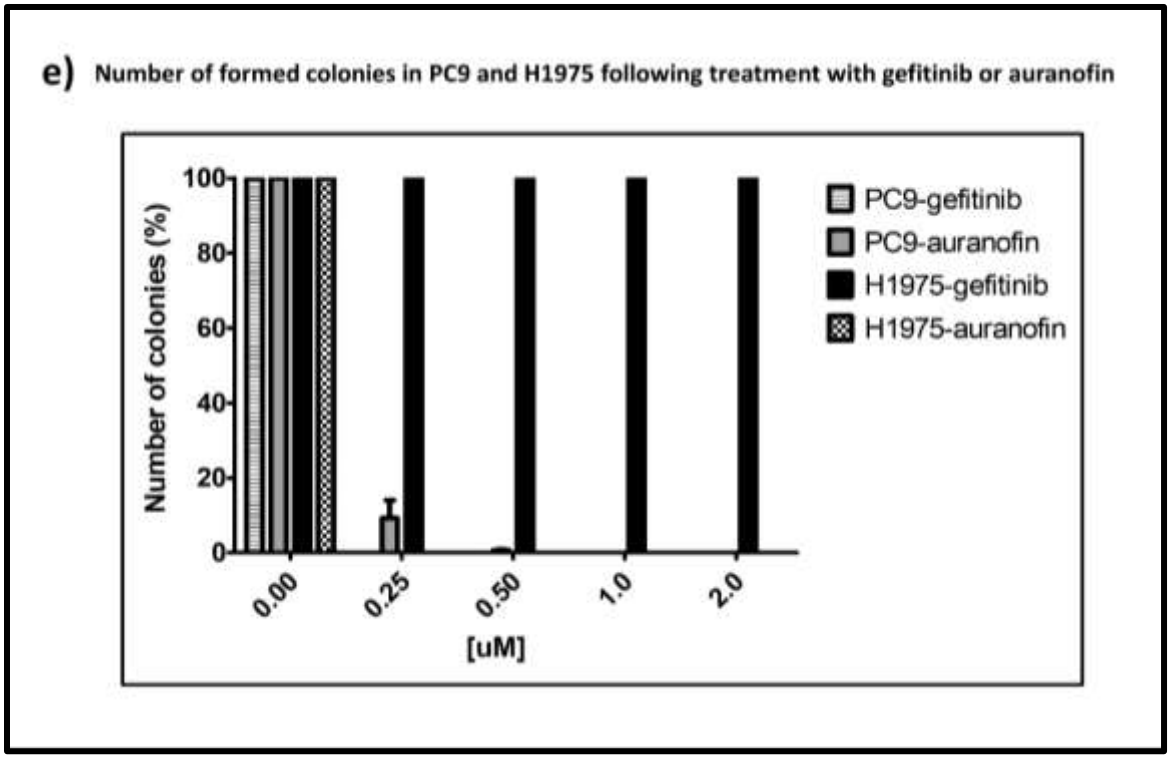

Figure 3.2: Response to gefitinib and auranofin in EGFR-mutant lung adenocarcinoma. a) Complete colony formation inhibition (100%) in PC9 cells following exposure to gefitinib at different doses. b) Colony formation in PC9 cells following exposure to auranofin at different doses. c) Colony formation in H1975 cells following exposure to gefitinib at different does. d) Complete colony formation inhibition (100%) in H1975 cells following exposure to auranofin at different doses. e) Percentage of formed colonies in PC9 and H1975 cells following exposure to gefitinib or auranofin. N = 3.

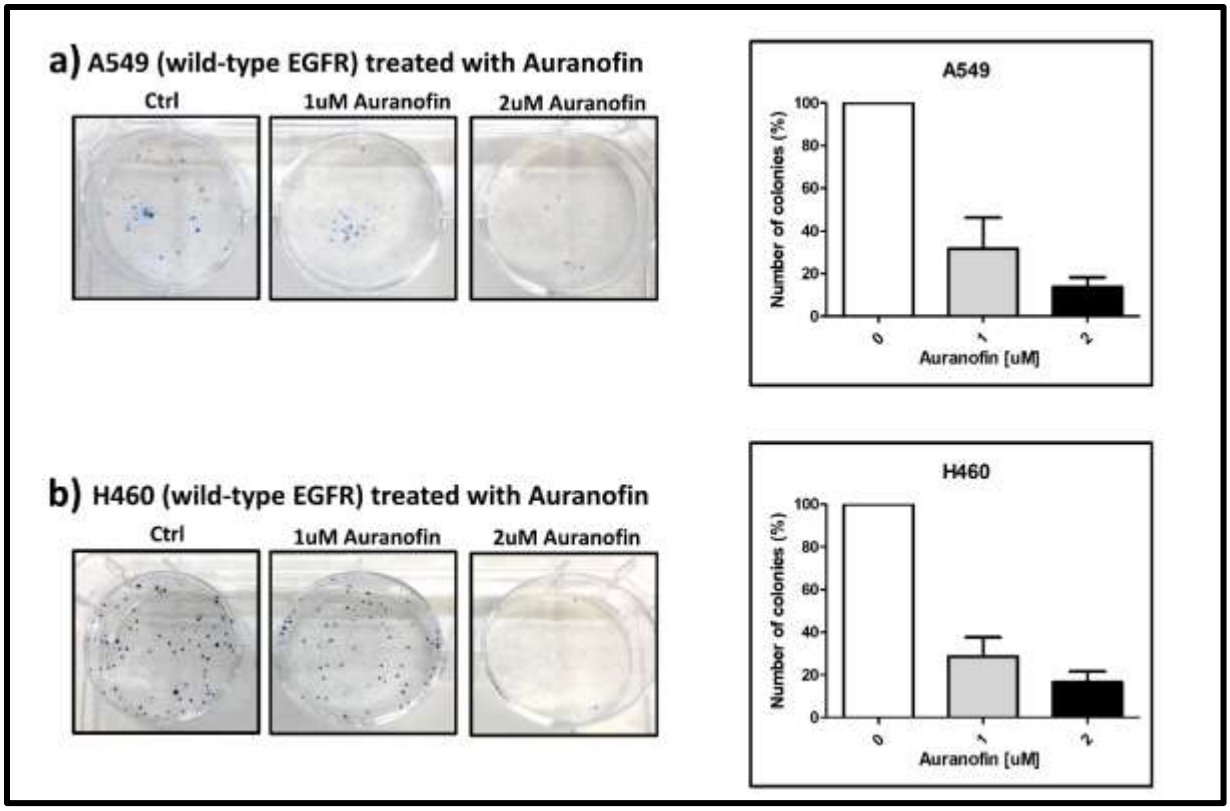

Figure 3.3: Exposure to auranofin in wild type-EGFR lung adenocarcinoma induces decrease in colony formation. a) Colony formation in A549 cells with 34% and 14% colonies formed following exposure to auranofin at 1µM and 2 µM, respectively. b) Colony formation in H460 cells with 29% and 17% colonies formed following exposure to auranofin at 1µM and 2 µM, respectively. c) Number of formed colonies in A549 and H460 following exposure to auranofin at 1µM and 2 µM compared to control. N = 3.

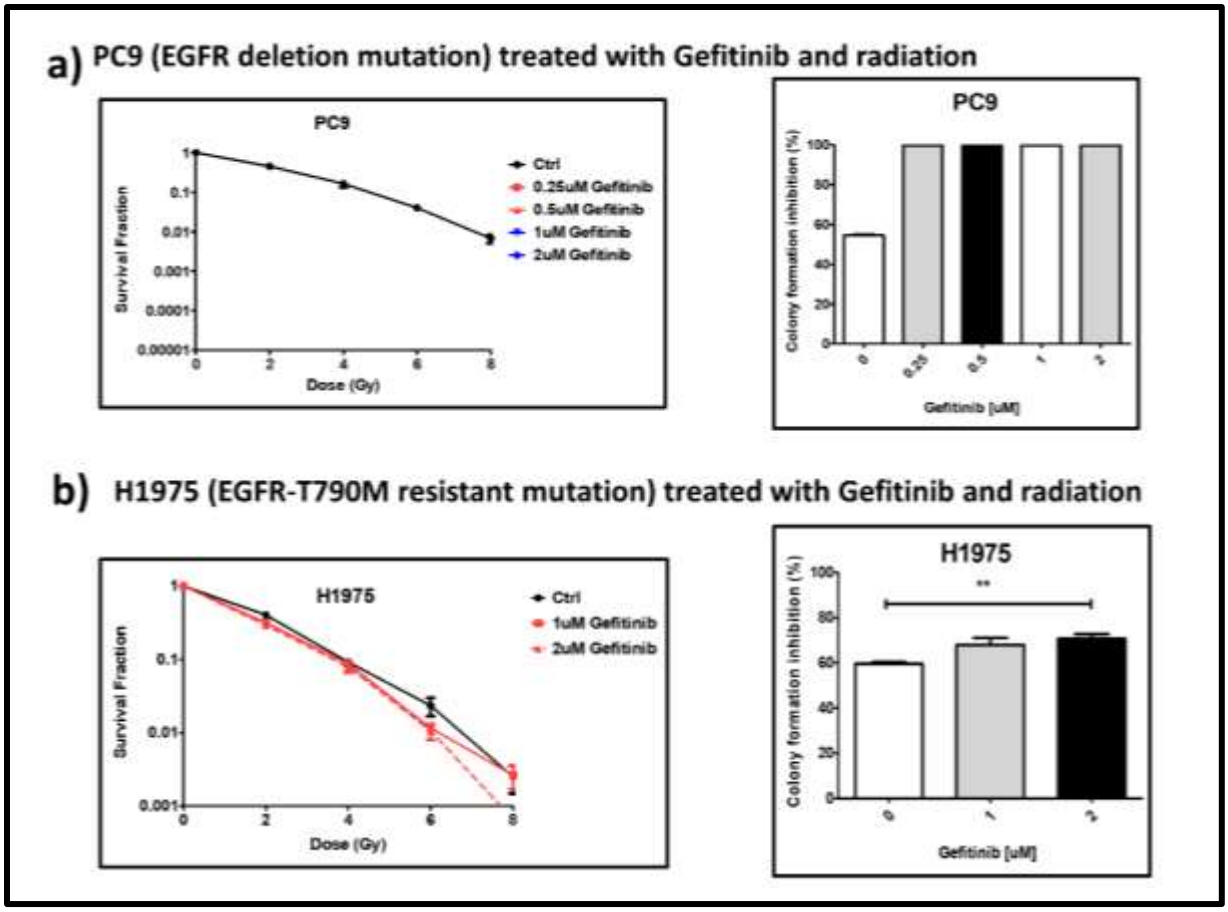

Figure 3.4: Combined treatment of gefitinib followed with radiation induced a differential inhibition of colony formation in EGFR-mutant lung adenocarcinoma. Treatment of PC9 cell lines with gefitinib followed with radiation increased inhibition of colony formation up to 100% inhibition when combined with radiation versus radiation alone (a-b). Treatment of H1975 cell lines with gefitinib followed with radiation caused a significant inhibition of colony formation of 71% at 2µM of gefitinib compared to radiation alone (c-d). N = 3 *: *P* <0.05.

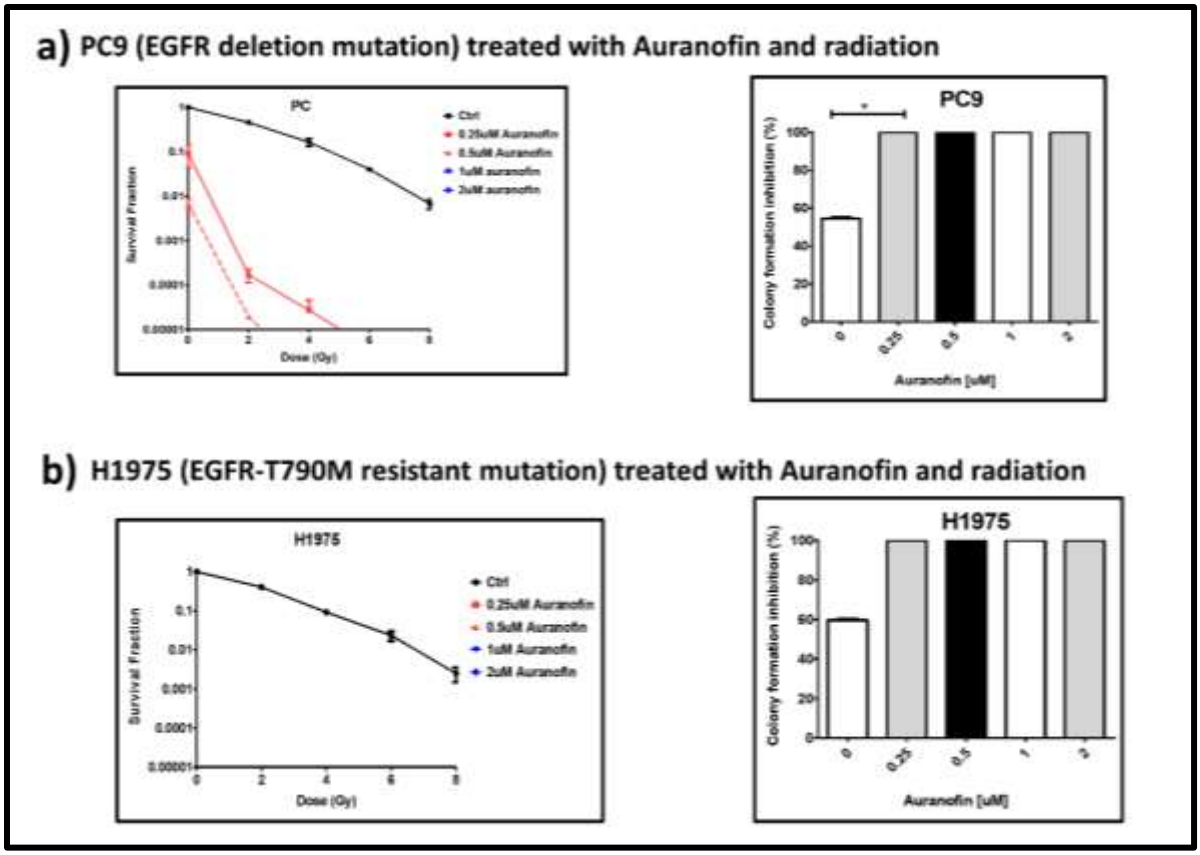

Figure 3.5 EGFR-mutant lung adenocarcinoma cell lines exhibit a good response to pre-treatment with auranofin followed with radiation. Pre-treatment of PC9 cell line with auranofin induced a radiosensitization effect and increased inhibition of 99.8% of colony formation compared to radiation alone (a-b). Pre-treatment of H1975 cell line with auranofin caused an increase in colony formation inhibition with pre-treatment of auranofin followed with radiation versus radiation alone (c-d). N = 3 *: *P* <0.05.

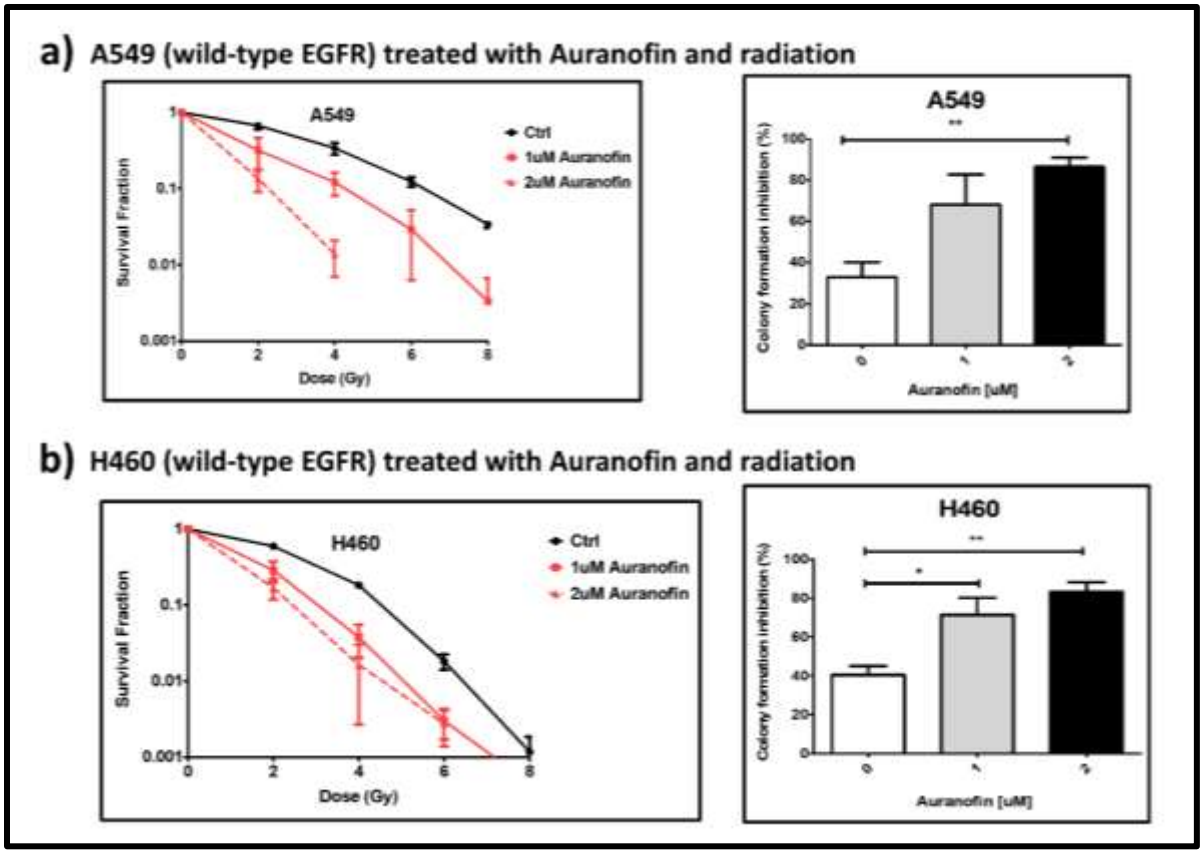

Figure 3.6: Treatment with auranofin induced a radiosensitization effect in WT-EGFR lung adenocarcinoma. Pre-treatment of A549 with auranofin followed with radiation induced a significant inhibition of colony formation at 2µM of auranofin with 86% inhibition of colony formation compared to 33% of inhibition using radiation alone (a-b). Pre-treatment of H460 with auranofin followed with radiation induced a significant inhibition of colony formation of 71% and 83% at 1µM or 2µM, respectively compared to radiation alone which had an inhibition of 40% (c-d). N = 3. *: *P* <0.05, **:*P* <0.01.

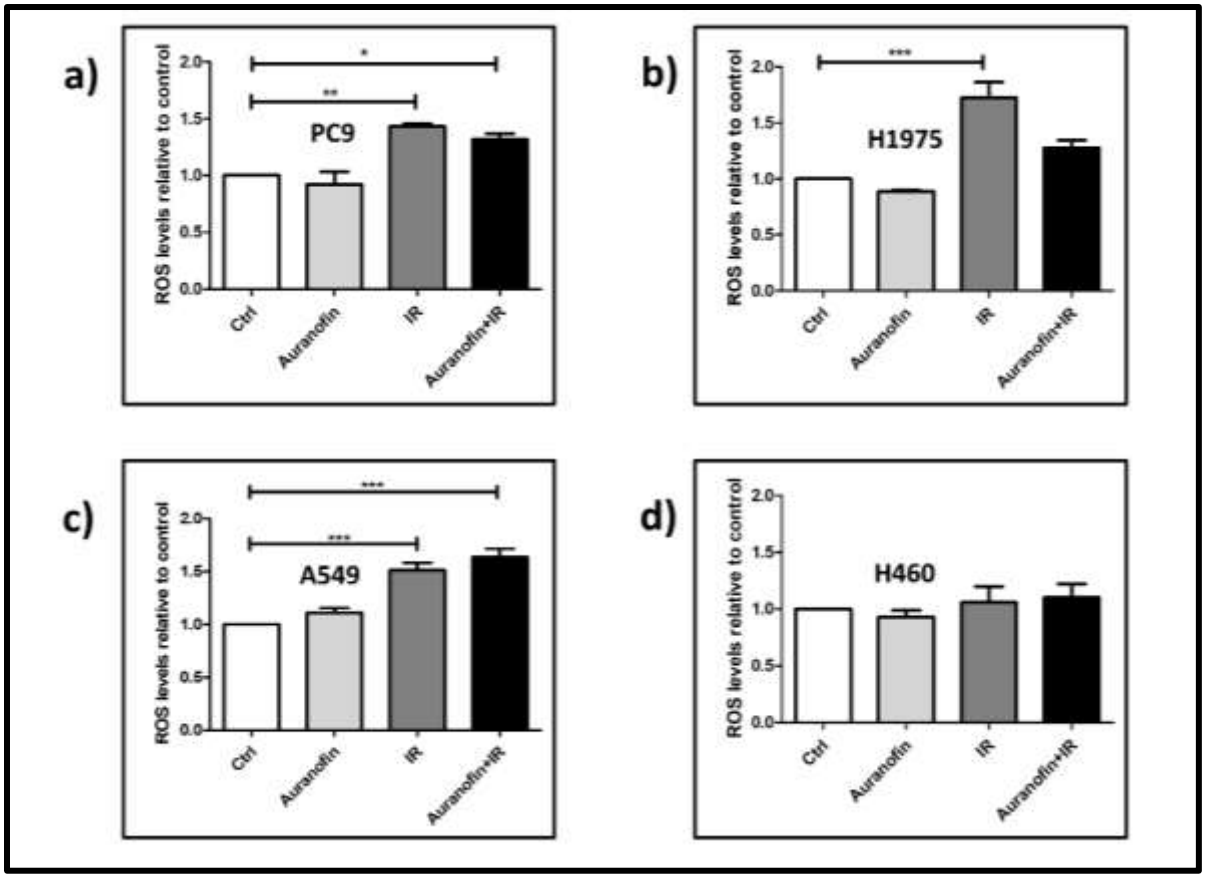

Figure 3.7: Lung adenocarcinoma harboring different EGFR mutation have variable levels of released reactive oxygen species (ROS). Measurements of ROS levels were done on NSCLC cell lines that were treated with either auranofin, 2 Gy of ionizing radiation (IR), or pre-treated with auranofin followed with radiation (auranofin +IR). Radiation treatment induced significant increase of ROS levels in PC9 (a), H1975 (b), and A549 (c), whereas pre-treatment with auranofin followed with radiation resulted in elevated ROS levels in PC9 (a) and A549 (c) cell lines. N = 3. *: *P* <.05, **: *P* <.01, *: *P* <.001.**

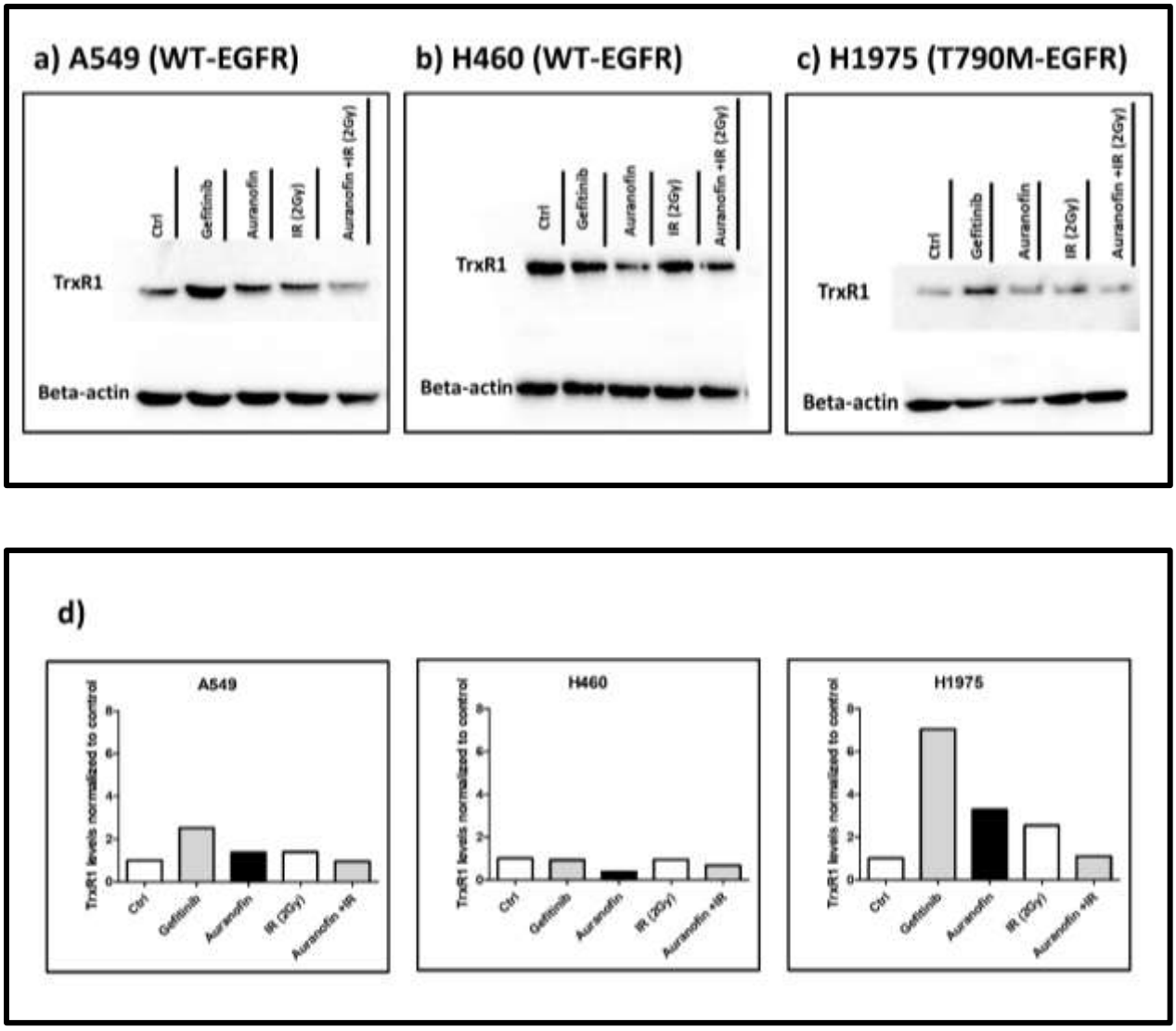

Figure 3.8: TrxR1 expression in lung adenocarcinoma harboring different EGFR following treatment with gefitinib, auranofin, ionizing radiation (IR) at 2Gy, or auranofin followed with ionizing radiation (IR) at 2 Gy: a) A549, b) H460, and c) H1975. d) TrxR1 level in lung adenocarcinoma following treatment normalized to control (non-treated cells).

Chapter 4: General discussion and future direction

4.1 Discussion

Our results emphasize on the impact of EGFR mutation type in lung adenocarcinoma on the response to ablative radiation. We report that E746-E750 deletion in the exon 19 (denoted as DEL in our discussion) of the tyrosine kinase domain of EGFR to exhibit a better response to ablative radiation compared to wild-type EGFR and compared to EGFR-mutant with L858R substitution mutation. Even though all three cell lines (WT-EGFR, DEL-EGFR and L858R-EGFR) had a similar pattern of response to ablative radiation *in vitro*, we observed a differential response to ablative radiation *in vivo*. This suggests that the mechanisms activated, or inhibited, post-ablative radiation are dependent on the type of mutation within the tyrosine kinase domain of EGFR. We also propose auranofin as potential drug to enhance that antitumor effect in lung adenocarcinoma. Particularly, cells that exhibit resistance to TKIs, such as lung adenocarcinoma with T790M-EGFR mutation, or cells that are radioresistant such as lung adenocarcinoma with wild-type EGFR. Our findings shed the light on the importance of EGFR mutations and their impact on the response to treatment and propose auranofin as potential therapeutic in lung adenocarcinoma cells that are resistant to treatment.

This Ph.D. thesis is an extension to our previous reported findings suggesting that lung adenocarcinoma cell lines with different EGFR status exhibit a variable response to ablative and fractionated doses of ionizing radiation (IR) [1]. In our previous work, A549 (wild type-EGFR), HCC827 (EGFR-mutant with in-frame shift deletion), and H1975 (EGFR-mutant with L858R and T790M double mutations) were used for assessing the response to radiation [1]. In A549 cell line, our findings demonstrated that the use of ablative ionizing radiation (AIR) compared to fractionated ionizing radiation (FIR) has caused a significant reduction in cellular proliferation and clonogenic survival [1]. HCC827 and H1975 cell lines did not exhibit similar response [1].

Moreover, a significantly enhanced invasive profile was observed in A549 cells following AIR, which was not observed in HCC827 or H1975 cells [1]. These results suggest that differential proliferative and invasive responses to AIR can be dependent on genetic subtype.

In addition, we have previously assessed the response to stereotactic ablative radiotherapy (SABR) in an orthotopic lung adenocarcinoma animal model which was developed to help in evaluating the response to clinically-relevant doses of SABR [2]. In our published work, we used image-guiding approach to help in performing an intra-thoracic injection of A549 cell line in the right lung of nude rats [2]. Once a tumor was developed, a dose of 34 Gy was delivered in a single fraction with the aid of clinical photon energies and animals were sacrificed 10-60 days post-treatment [2]. In four out of the six treated animals, a complete response (CR) to treatment was observed in 30 days of treatment [2]. one animal had a partial response (PR) to treatment and one animal had disease progression (PD). Half of the animals with initial complete response had indications of metastasis using CT and was confirmed by pathological assessment [2]. Examination of the cancer-related genes indicated a significant difference between tumors that were subjected to SABR and untreated tumors [2]. Treatment with SABR has caused a significant alteration in the expression of genes involved in adhesion, migration, and angiogenesis. These findings raise the importance of investigating and developing targeted therapies to prevent pro-invasive outcome of SABR in NSCLC.

4.2 Contribution #1 (Chapter 2):

Our findings on the implication of EGFR mutations in the response to ablative radiation could provide insight on the possible outcomes of such treatment in EGFR-mutant lung adenocarcinoma

which require further clinical validation in the future. We suggest that ablative radiation can be beneficial in lung adenocarcinoma that are positive for DEL-EGFR mutation and may have a countereffect in the case of L858R-EGFR positive or WT-EGFR. Our recommendations are based on the following observations. First, DEL-EGFR cells exposed to a single fraction of 34Gy and injected subcutaneously into mice failed in developing tumors, although BLI confirmed their viability 12 months post-injection. This proposes that ablative radiation could have altered the mechanism of proliferation or other mechanisms that prevented these cells from overcoming radiation and may have caused these cells to become in "dormant" state. Since the cells were detectable using BLI indicating their viability status, the idea that these cells have went through apoptosis has been ruled out. In the case of WT-EGFR and L858R-EGFR that were also exposed to a single fraction of 34Gy and injected subcutaneously into mice, a tumor formation was observed in animals injected with either irradiated cell line. This indicates that both cell lines had the required machinery to overcome exposure to ablative radiation and induce proliferation. Additionally, since the structure of L858R-EGFR is very close to WT-EGFR (compared to DEL-EGFR) where only one amino acid is replaced by another one, this suggests that WT-EGFR and L858R-EGFR may share similar pattern of cellular activation.

Second, a decrease in tumor volume was observed in animals that were injected subcutaneously with DEL-EGFR cells and treated with single fraction of 34Gy once a tumor was observed. Decrease in tumor volume was observed nine days post-treatment. However, animals injected with WT-EGFR or L858R-EGFR cells followed with a treatment of a single fraction of 34Gy had an increased tumor volume nine days following treatment compared to the day when radiation was performed (day 0). These results indicate that cellular signaling in both WT-EGFR and L858R-EGFR cells can overcome radiation and activate their proliferation signaling supporting our

previous finding. Very few animal studies have tried to explore the biology behind stereotactic body radiation therapy in pre-clinical lung cancer animal models. Pre-clinical studies done on studying the response to ABRT in lung cancer models have compared the response to treatment in general, regardless of the type of EGFR mutation. The study done by Zhang et al had compared the effect of delivering a single fraction of 12 Gy to three fractions of 12 Gy (a total of 36 Gy) in orthotopic rat lung tumor model [3]. They have demonstrated a complete tumor ablation by delivering 36 Gy in three fractions of 12 Gy [3]. Our group has also studied the response to ABRT in an orthotopic animal model where we reported a 67% complete response in animals treated with a single fraction of 34 Gy [2]. Clinically, one study done Nakamura et al [4] has looked at the pattern of recurrence after CyberKnife SBRT in early stage NSCLC. They have reported out-of-field recurrence to be significantly associated with EGFR mutation [4]. However, they did not specify in their work if recurrence was associated with a specific type of EGFR mutation which suggests for future investigation if recurrence is mutation-specific.

Our third observation was based on histological assessment of tissues collected from both control (untreated) and treated groups of animals injected with either cells. This assessment was done by a lung cancer pathologist, Dr. Sophie Camilleri-Broet. In our assessment we looked at differences between the three cell line in terms of percentage of necrotic area, percentage of solid tumor, and number of apoptotic bodies as response to ablative radiation. Interestingly, tissues collected from DEL-EGFR treated group did not show necrosis compared to its control group and compared to treated groups of other cell lines (WT-EGFR and L858R-EGFR). In a study of 593 NSCLC patients who underwent surgical resection, it was reported that tumor necrosis can be an indicator of poor prognosis [5]. Two smaller studies done on stage I of NSCLC and non-neuroendocrine large cell carcinoma have also shown correlation between tumor necrosis and poor prognosis [6,

7]. Our assessments where we demonstrated a decreased necrotic area in DEL-EGFR treated group could be an indication of a good response. Although there was no significance in the percentage of solid tumor in between treated and control groups, there was a significant decrease in the number of apoptotic bodies in DEL-EGFR treated group compared to its control. There have been conflicting reports whether a correlation between apoptosis and proliferation with poor prognosis exist [8, 9]. Others have reported no correlation between apoptosis and poor prognosis [10, 11]. Our preliminary results demonstrate difference between the three cell lines histologically which should be further investigated in the future for correlation between poor prognosis and specific mutation of EGFR.

Protein assessment of collected tumor from both control and treated groups were done to check for alteration in protein expression post-ABRT. Proteins that were assessed include: phosphor-EGFR (p-EGFR), total-EGFR, p-AKT, total-AKT, p-ERK, total-ERK. We observed that there was a decrease in the phosphorylated levels of ERK in DEL-treated group by 0.7-fold change compared to WT- and L858R-treated groups where both had increased p-ERK levels of 4.9-fold and 3.2-fold change, respectively. Protein assessment suggests that ABRT/SABR was capable in altering phosphorylation mechanisms in DEL-EGFR but was not effective in altering phosphorylation of WT-EGFR and L858R-EGFR. ERK and its isoforms are known to be implicated in cell proliferation [12] where phosphorylation of both threonine (Thr202) and tyrosine (Tyr204) residues are required for full kinase activation [12]. Active phosphorylation of ERK has been reported in up to one-third of NSCLCs with an inconsistent association with prognosis [13-15]. Moreover, increased levels of phosphorylated ERK has been reported in emphysema compared to healthy lung tissues [16], suggesting that a sustained ERK activation may be a key factor in the progression of emphysema [17, 18].

It has been reported by many retrospective and prospective studies that were done on NSCLC patients treated with various lines of EGFR-TKIs that a longer progression-free survival (PFS), and occasionally a more favorable overall survival (OS), in patients with deletion mutation in the exon 19 than those with L858R or other EGFR mutations [19-21]. On the other hand, other clinical studies have reported that there was no difference in the effectiveness of EGFR-TKIs according to the type of EGFR mutations [22-25]. We investigated if overall survival in lung cancer patients was dependent on the type of EGFR mutation. We used publicly available TCGA data of early stage (stage I and stage II) adenocarcinoma lung cancer patients [26-28]. A total of 348 reported cases were included in the analysis. The included cases were then grouped into either WT group (no reported EGFR mutation), DEL group (E746-A750 deletion mutation), and L858R group (point-substitution mutation). A summary of patients' information and tumor characteristics are summarized in **Chapter 2, Supplementary Table 1**. Our analysis revealed that there is a significant decrease in the overall survival in the group with L858R mutation compared to WT or DEL groups which proposes that L858R mutation might be associated with poor prognosis compared to DEL mutation (**Chapter 2, Supplementary figure 6**).

Several types of exon 19 deletion in the tyrosine kinase domain of EGFR have been reported in NSCLC. Most of these deletions have been found to encompass the amino acids codons L747 to E749 (LRE fragment) [29]. Other deletions that do not included the LRE fragment have been reported as well. The most frequent deletions in the exon 19 of EGFR is del746-A750 with a frequency of 66.1% [30]. Structural analyses demonstrated allosteric mechanism that is critical for EGFR kinase domain activation [31]. Deletions in the exon 19 of EGFR, that are located between strand β3 and helix αC, could be implicated in altering the inactive conformation of EGFR kinase domain and aid in enhancing the effectiveness of EGFR TKIs [31-34]. A study that was done to

assess the response rate (RR) and PFS in NSCLC with different exon 19 deletions suggested that patients with non-LRE exon 19 deletions could have lower RR and relative shorter median PFS compared to LRE deletions [35]. Taken together all of the previously mentioned data, LRE deletion in the exon 19 may also contribute in enhanced sensitivity to ablative radiation compared to L858R mutation.

4.3 Contribution #2 (Chapter 3)

Activating EGFR mutations have been associated with increased sensitivity to TKIs, such as gefitinib or erlotinib, however; resistance secondary to treatment is inevitable. Moreover, our results that were mentioned previously indicate that even at the event where different EGFR-mutant lung adenocarcinoma cell lines are exposed to the same dose of ablative radiation, their response to treatment is different. This has led us to investigate the potential of using auranofin as a drug that will enhance antitumor effect combined with radiation (or alone) in lung adenocarcinoma cell lines that are known to exhibit resistance to TKIs such as H1975 which has T790M-EGFR and L858R-EGFR double mutations, or radioresistant cells such as A549 and H460 which have WT-EGFR and compare their response to PC9 cell line which is an EGFR-mutant lung adenocarcinoma known to be sensitive to both treatment. The antioxidant Trx system, which regulates ROS production, consists of the Trx protein, thioredoxin reductase (TrxR) enzyme and NADPH. Auranofin functions as a potent inhibitor of TrxR leading to inhibition of the enzyme activity [36], in which this inhibition causes overproduction of ROS and subsequently activation of the apoptotic pathway [37-42].

Assessment of TrxR1 (one of TrxR isoforms) levels in lung adenocarcinoma with different EGFR status showed a negative association with sensitivity to radiation. For instance, A549, H460 and PC9 cell lines had high levels of TrxR1 compared to H1975 cell line which exhibited a more

sensitive profile to radiation compared to the other cell lines. Studies have reported that deficiency in TrxR1 has caused a reversed tumor phenotype and tumorigenicity of lung carcinoma cells [43]. It is worth mentioning that high levels of TrxR1 was suggested as a diagnostic marker in NSCLC. Our results report the efficacy of auranofin in inhibiting colony formation in EGFR-mutant lung adenocarcinoma (PC9 and H1975) at lower doses (0.25-0.5 μM), where as WT-EGFR lung adenocarcinoma (A549 and H460) needed higher doses (1.0-2.0 μM) to achieve partial inhibition of colony formation. Interestingly, when auranofin was used as a radiosensitizer it enhanced the effect of radiation causing a complete inhibition of colony formation in both A549 and H460 cell lines.

Chemotherapy remains as a key treatment for NSCLC patients, where it is estimated that 40% of NSCLC patients will receive chemotherapy as a treatment modality [44]. Some of the anticancer that are used include paclitaxel, 5-fluorouracil and gemcitabine which are used in combination with radiation to help in improving the effectiveness of radiotherapy [45-51]. Currently, platinum-based chemoradiation treatment method presents a 5-year survival rate of 5-15% in NSCLC [52]. Some of the major hindrances in the treatment of NSCLC are resistance to therapy and toxicity issues [53, 54]. In our study, we compared the effectiveness of auranofin to gefitinib as antitumor drug alone or in combination with radiation in lung adenocarcinoma harboring different EGFR status and demonstrate the efficacy auranofin an antitumorigenic drug in lung adenocarcinoma regardless of their EGFR status.

4.4 Study limitations

The current study has several strengths and limitations. Its major strength is its novelty; to the best of our knowledge, the current study is the first to investigate the impact of EGFR-mutant type on the response to ablative radiation and propose auranofin as a potential radiosensitizer in radioresistant lung adenocarcinoma cell lines. The major limitation of the current study is the introduction of EGFR-mutant construct into A549 cell line. A549 has a K-RAS mutation, which is downstream of the EGFR signaling and could impact the cell signaling. However, this the only isogenic EGFR-mutant lung adenocarcinoma model and has been used previously in the work of Das et al [55], where they have used these cells to investigate their response to ionizing radiation in vitro. Another limitation to our study is investigating the efficacy of auranofin in vivo but could be something to be investigated in the future by others. Despite these confounders, we believe that our current study is novel in the literature because to our knowledge it is the first to investigate the response of isogenic EGFR-mutant lung adenocarcinoma cell lines in vivo and the first to propose the use of auranofin as a radiosensitizer for lung adenocarcinoma cells. An additional limitation to our study, is unavailable *in vivo* data on the efficacy of auranofin as a radiosensitizer or as as a drug that can overcome resistant resulted from initial treatment

4.5 Future directions

To strengthen our study there are few concepts that need to be further investigated. For example, it would help to investigate the mechanism(s) downstream of EGFR mutations leading to the observed differential response to ablative radiation. This can be assessed through transcriptomic and proteomic analysis which will give an idea of how cell signaling participate in the differential

response. Clinically, it would be interesting to design a study where patients are screened for not only for the presence/absence of driver mutation, but also for the type of mutation they carry. This will help in validating if the response to ablative radiation is driven by the type of mutation. This will stress on the need of more personalized treatment that benefit the patient as an individual and not as part of a population.

Another idea that can be addressed in the future is to investigate the efficacy of auranofin combined with radiation *in vivo*. Our in vitro results demonstrate a promising potential of auranofin, however; this needs to be further analyzed in vivo under stimulus of the microenvironment. It would be also interesting to test different regimen of auranofin, before radiation or combined with radiation, that can lead to a maximized tumor inhibition. Moreover, the efficacy of auranofin should be tested in the event of resistance secondary to treatment. Further studies should be intended to examine auranofin efficacy in other driver mutations that remain challenging to be overcome such as KRAS, ALK or at the event where resistance is developed secondary to treatment such T790M and C797S mutations. Answers that address these concerns will provide insights on auranofin's potential as therapeutic drug as first-line treatment and at the event of resistance and relapses.

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