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Relationship Between EGFR Overexpression and Response to Radiation Regimens in Patients with Newly Diagnosed GBM

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Abstract

Glioblastoma multiforme (GBM), the most common infiltrative astrocytic primary brain malignancy remains an incurable disease, despite a multimodal therapy that consists of surgery followed by radiotherapy (RT) with concurrent and adjuvant chemotherapy (TMZ). GBM is a devastating disease with a highly heterogeneous survival between patients. A number of recent large-scale genomic and proteomic studies have shed new light on GBM pathogenesis and molecular diversity. Nonetheless, all patients receive the same treatment of surgery, RT and TMZ. A multitude of targeted therapies have so far failed to demonstrate any survival differences in GBM patients. While certain markers, such as *MGMT* methylation with respect to TMZ treatment, have been found to confer different survival outcomes between patients, there is a need to further stratify patients to investigate optimal treatment regimes. To this end, we constructed a tissue microarray (TMA) for 201 newly diagnosed GBM patients from a single institution, recorded their histopathological and clinical information and assessed expression of major GBM prognostic markers including Ki67, EGFR, p53, PTEN, CD44, and vimentin. *MGMT* promoter methylation has been prospectively performed for 143 (71.1%) patients. We analyzed survival outcomes between patients with EGFR overexpression and non-overexpression. EGFR is known to be involved in the radioresistant phenotype of GBM and activated by RT. Since EGFR overexpression has been found to confer different survival and treatment benefits in GBM and other cancer types, we investigated the different survival outcomes between conventional RT and hypofractionated regimens. We also subdivided tumours into profiles reminiscent of three of the identified molecular based subtypes (Classical, Mesenchymal, Proneural) using immunohistochemistry scoring and analyzed the survival outcomes based on molecular profiles. Further investigation is currently underway to assess the prognostic value of EGFR overexpression in radioresistance with respect to other clinical and histopathological variables.

Our study established a TMA with a clinically annotated database for 201 newly diagnosed GBM patients. This will be of great value for stratification of GBM patients who may derive benefit from a tailored radiation regimen using cost-effective protocols for the implementation of an immunohistochemical-based molecular signature.

Résumé

Le glioblastome multiforme (GBM) est la plus fréquente tumeur astrocytaire primaire maligne du cerveau, et elle demeure une maladie incurable. La thérapie du GBM est multimodale, et elle comprend la chirurgie, la radiothérapie concurrente, et la chimiothérapie au Témozolomide (TMZ), suivie d'un adjuvant TMZ. Malgré cela, le GBM est une maladie dévastatrice avec une survie très hétérogène entre les patients recevant le même traitement. Un certain nombre d'études récentes à grande échelle, génomiques et protéomiques, projette une lumière nouvelle sur la pathogenèse du GBM et sa diversité moléculaire. Cependant, malgré cette connaissance, tous les patients reçoivent le même traitement comprenant la chirurgie, radiothérapie et TMZ. Une multitude de thérapies ciblées ont jusqu'à présent échoué à démontrer les différences de survie chez les patients atteints de GBM. Alors que certains marqueurs, tels que la méthylation du MGMT, ont démontré différents résultats de survie entre les patients par rapport au traitement au TMZ, il est nécessaire de stratifier davantage les patients pour enquêter sur les régimes de traitement optimaux. À cette fin, nous avons construit un microréseau tissulaire (TMA) pour 201 patients atteints de GBM nouvellement diagnostiqués, provenant d'une seule institution, et nous avons complété leurs informations histopathologiques et cliniques, et évalué l'expression des principaux marqueurs de pronostic du GBM dont ki67, EGFR, p53, PTEN, CD44, et vimentine. La méthylation du promoteur de MGMT a été prospectivement analysée pour 201 patients. Nous avons évalué les résultats de survie entre les patients avec surexpression et non-surexpression d'EGFR. L'EGFR est connu pour être impliqué dans le phénotype radiorésistant du GBM, et pour être activé par la radiothérapie. Etant donné que la surexpression d'EGFR confère différents avantages de survie et de traitement dans divers types de cancer, nous avons étudié les différents résultats de survie entre la radiothérapie conventionnelle et les schémas hypofractionnés. Nous avons également subdivisé les tumeurs suivant des profils qui rappellent les sous-types moléculaires (tumeur classique, mésenchymateuse, proneurale) en utilisant l'immunohistochimie, et nous avons finalement analysé les résultats de survie en fonction des profils moléculaires. Une étude plus approfondie est en cours pour évaluer la valeur pronostique de la surexpression d'EGFR en radiorésistance par rapport à d'autres variables cliniques et histopathologiques.

Notre étude a établi un TMA avec une base de données cliniquement annotée pour 201 patients atteints de GBM nouvellement diagnostiqués. Ceci sera d'une grande valeur pour la

stratification des patients atteints de GBM qui peuvent tirer profit d'un schéma de radiothérapie sur mesure, en utilisant des protocoles rentables pour la mise en place d’une signature moléculaire basée sur l’immunohistochimie.

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Firstly, I would like to thank my supervisor, Dr. Siham Sabri, for her unending support and commitment to the lab and her students. Her passion and dedication for research is obvious to everyone around her and she has inspired me numerous times. I am extremely grateful for her mentorship and dedication to my project during my time in her lab.

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I would further like to send my appreciation to all my colleagues for making the lab a place I looked forward to going to every morning. I have had the good fortune of being surrounded by a group of intelligent and critical thinkers that have helped me in all aspects of my research.

Lastly I would like to express my profound gratitude to my family and friends for being a constant source of unwavering support during the completion of my degree. I would especially like to thank my parents for the wealth of opportunities they have offered me. I would not be here without their continued love and support.

Preface

Emma Preston obtained clinical patient information from digital medical records and archived medical records from Montreal University Health Center (MUHC) and the Montreal Neurological Institute (MNI), obtained scores from a tissue microarray with stained markers to form a clinical and immunohistochemical database of GBM patients, analyzed protein expression patterns in the TMA, performed statistical analyses and retrieved tumour tissue for the construction of a new GBM TMA consisting of newly diagnosed patient tissue and their subsequent recurrent tissue at the MNI. Marie-Christine Guiot (MNI) scored immunohistochemically stained markers from our TMA. Fabian Santos aided in the survival statistics. Dr. Bassam Abdulkarim and Dr. Melissa Azoulay established the initial database for 276 newly diagnosed GBM patients. Dr. Siham Sabri was responsible for the conception, design, development of methodology, data analysis, and provided grant-funded financial support to conduct this work.

Contents

Tables and Figures

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Abbreviations

Akt	Protein kinase B
COX-2	Cyclooxygenase 2
CT	Computed Tomography
CTV	Clinical tumour volume
DNA-PKcs	DNA protein kinase catalytic subunit
DSB	Double Strand Breaks
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EGFRvIII	Epidermal Growth Factor Receptor variant III
EMT	Epithelial to Mesenchymal Transition
FISH	Fluorescent in situ Hybridization
Fr	Fractions
GBM	Glioblastoma
GTV	Gross tumour volume
Gy	Gray
ID1	Inhibitor of DNA Binding 1
IDH1	Isocitrate dehydrogenase 1
IHC	Immunohistochemistry
IMRT	Intensity-Modulated Radiotherapy
KPS	Karnofsky Performance Status
MAPK	Mitogen-activated protein kinases
MGMT	O6-methylguanine-DNA methyltransferase
MRI	Magnetic Resonance Imaging
mTORC1	Mammalian target of rapamycin complex 1
mTORC2	Mammalian target of rapamycin complex 2
NF-κB	Nuclear Factor κB
NF1	Neurofibromin 1
OLIG2	Oligodendrocyte transcription factor 2
OSMR	Oncostatin M Receptor

OS	Overall survival
p53	Tumor protein p53
PCNA	Proliferating Cell Nuclear Antigen
PDGFR	Platelet-derived Growth Factor Receptor
PFS	Progression free survival
PI3K	Phosphoinositide 3-kinase
PLC-γ	Phospholipase C Gamma 1
PTEN	Phosphatase and tensin homolg
RTK	Receptor tyrosine kinase
STAT	Signal transducer and activator of transcription
TCGA	The Cancer Genome Atlas
TMA	Tissue microarray
TMZ	Temozolomide
TKI	Tyrosine kinase inhibitor
WHO	World Health Organization
wtEGFR	Wild-type Epidermal Growth Factor Receptor

Chapter 1

1. Introduction

1.1 Glioblastoma Multiforme

Glioblastoma multiforme (GBM), the most frequent and aggressive form of malignant primary brain tumor in adults, represents 45% of all glioma histologies and about 60-75% of all astrocytomas. The traditional classification by the World Health Organization (WHO) as a grade IV for the most aggressive astrocytoma has further integrated recent advances in genetic and molecular subclassification [8]. To date, despite aggressive multimodal treatment including surgery, radiation therapy (RT), and chemotherapy, GBM remains incurable with a dismal median overall survival (OS) of only 15 months and less than 5% of patients surviving more than 5 years after their diagnosis [9].

GBM, typically located in the cerebral hemispheres of the brain, undergoes widespread invasion of surrounding normal brain tissue, aberrant angiogenesis, rapid proliferation and recurrence after resection of the tumour. The WHO classification relies mostly on the histological presence of high-grade astrocytoma with tumour necrosis or microvascular proliferation tumor cells (Figure 1). Since GBM cells are extremely invasive and infiltrative, it is nearly impossible to completely resect the tumour, and the disease almost always recurs (Figure 2) [10].

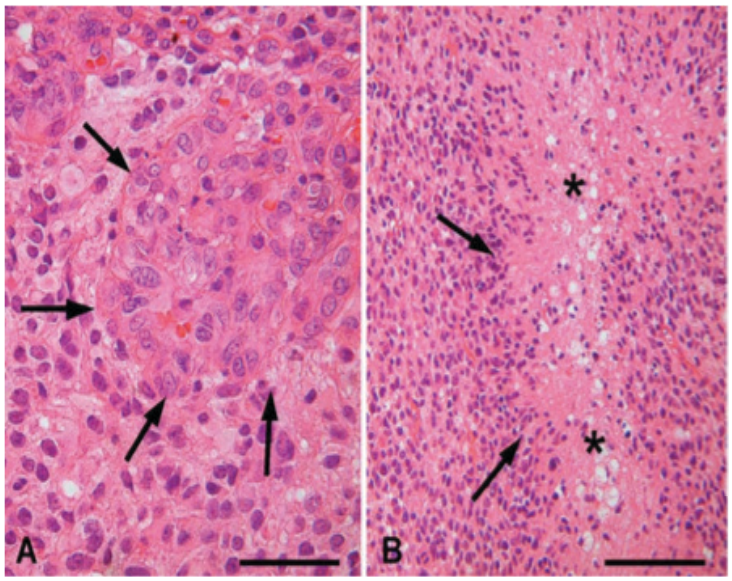

Figure 1. Histological Identification of GBM. A) Vascular proliferation indicated by arrows (70μm scale bar) B) Palisading tumour cells (arrows) surround necrotic area (asterisk) (150μm scale bar). Images from Jansen et al. (2004) [4].

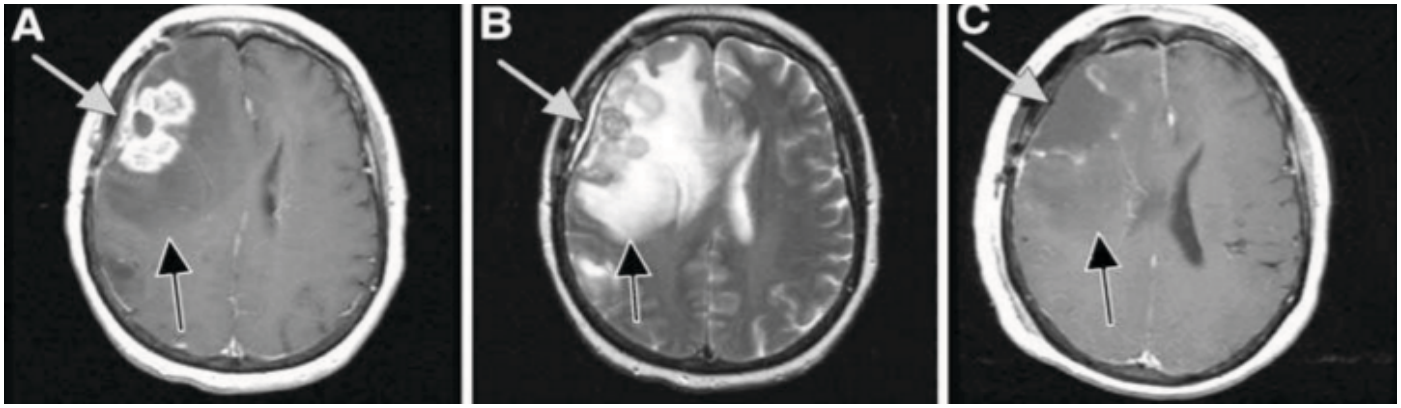

Figure 2. Pre- and Postoperative GBM Visualization through MRI. A) T1-weighted MRI scan of pre-operative GBM tumour with grey arrow pointing to enhancement and black arrow pointing to invading tumour cells. B) T2-weighted MRI scan of the same tumour C) Postoperative T1-weighted scan with removal of tumour (grey arrow) but with remaining infiltrating tumour cells (black arrow). Images from Collignon et al. (2004) [5].

While landmark studies have identified molecular subtypes with different genomic signatures and prognoses in GBM, a clear consensus has yet to be reached on how to precisely sub-classify GBM. In the 2016 Classification of Tumors of the Central Nervous System, however, WHO has officially recognized two variants of GBM based on molecular markers: isocitrate dehydrogenase 1 (IDH1)-wildtype (90% of cases) and IDH1-mutant (10% of cases). IDH-wildtype and mutant patients differ in their clinical characteristics and survival; 15 months for IDH-wildype and 31-months for IDH-mutant when treated with surgery, RT, and chemotherapy. Primary GBM is most common in elderly patients and corresponds most frequently to IDH-wildtype tumours while secondary GBMs often arises in younger patients and corresponds to IDH-mutant tumours. While most GBMs arise de novo and are considered primary tumours, low-grade gliomas can also recur and progress to become secondary GBM [8]. The dismal prognosis of GBM along with its accompanied cognitive deterioration points to an urgent need to identify new therapies and to define subclasses of GBM patients with different prognoses and responses to therapies. It is also crucial to explore the biology behind these differences.

1.2 Tumour Heterogeneity

Cancer, and specifically GBM, consists of a series of genetic and epigenetic aberrations comprising DNA alterations, chromosomal rearrangements and DNA methylation modifications.

One of the most common genetic abnormalities present in GBM is amplification of the epidermal growth factor receptor (EGFR) gene and overexpression of its protein. With the overarching goal of uncovering these alterations, GBM was the first cancer to be genomically profiled by The Cancer Genome Atlas Research Network (TCGA). While decades of molecular studies had uncovered significant altered genes and pathways in GBM, including dysregulation of receptor tyrosine kinase (RTK) genes, phosphoinositide 3-kinase (PI3K) pathway activation, and inactivation of tumour suppressor genes p53 and retinoblastoma, this was the first study to systematically examine the GBM genome. This analysis uncovered recurrent alterations and revealed new insights into the dysregulated pathways present in GBM [11]. Many genetic modifications were found to be involved in the initiation and progression of the disease, causing great heterogeneity between tumours. Based on gene expression, protein expression, and signal transduction pathways, GBM was then classified by multiple groups into different molecular subtypes (Proneural, Neural, Classical and Mesenchymal). These subtypes are mostly based on aberrations and expression of PDGFRA/IDH1, NF1, and EGFR [1, 10]. In a cohort of 206 GBM patients, Verhaak et al. (2010) identified the four molecular subtypes using a classification of 840 signatures genes that displayed a characteristic copy number profile, had different survivals, and different responses to treatment and identified the gene aberrations that were most common to each subtype (Figure 3). Proneural, which includes the focal amplification of PDGFRA and *TP53* mutations and/or loss of heterozygosity, had a lower response to intensive therapy. Additionally, a subset of these tumours were tightly tied to the R132 mutation in *IDH1,* which is a prognostic marker for better prognosis in this disease. The Neural subtype, which is possibly a transitory subtype, had expression of neuronal markers and differentiation, such as NEFL, GABRA1, and SYT1. The Classical subtype is characterized by high expression of EGFR associated with chromosome 7 amplification and focal 9p.21.3 homozygous deletion, comprising CDKN24, and high expression of neural stem cell markers and rarely *TP*53 mutations. The Mesenchymal subtype is defined by focal hemizygous deletions at 17q11.2 that contains NF1, high expression of mesenchymal genes (including YKL-40, MET, CD44, and MERTK and mutant *TP53*, and is correlated to a highly invasive phenotype with short patient survival [1].

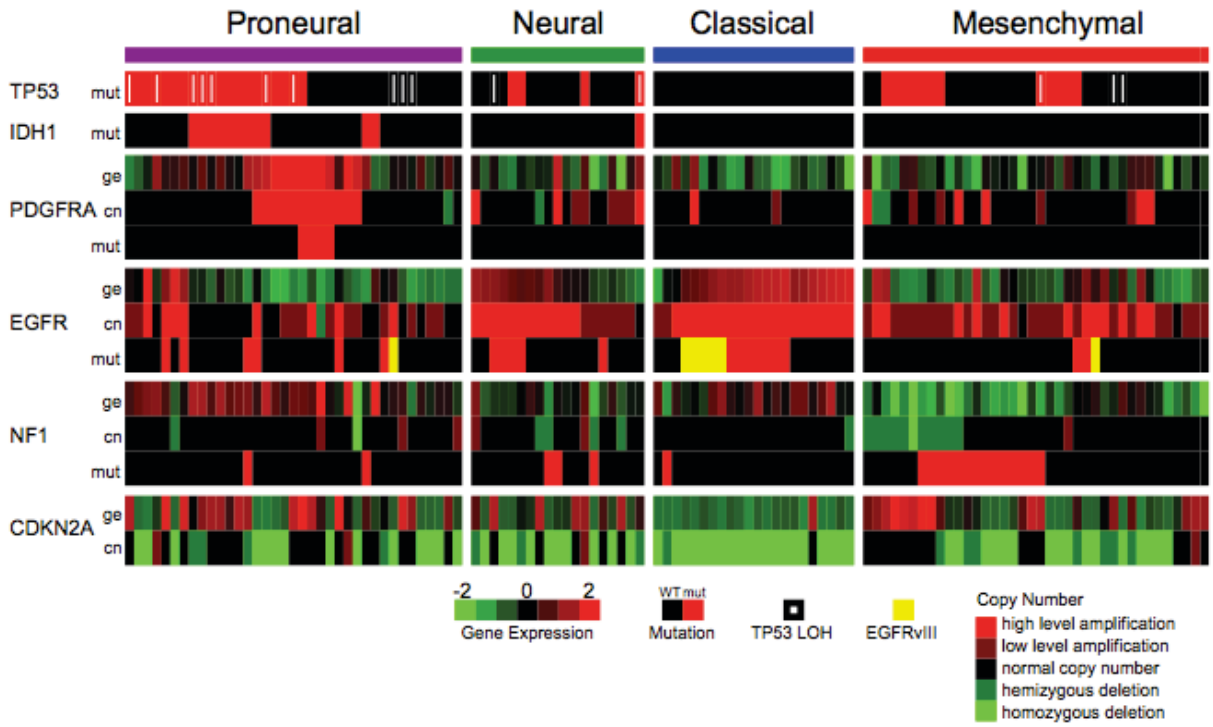

Figure 3. Integrated View of Gene Expression and Genomic Alterations. Data for GBM. Gene expression of commonly mutated genes is standardized in data set of 116 GBM samples that had mutation and copy number data available. Yellow boxes represent EGFRvIII mutation. Figure from Veerhaak et al. (2010) [1].

While it is known that GBM tumors are heterogeneous, treatment is the same for all patients and involves surgery, radiation therapy (RT) and chemotherapy with the alkylating agent temozolomide (TMZ) [2]. Since patients respond differently to treatment, it is of outmost importance to find predictive and prognostic markers in GBM with the ultimate goal to stratify patients into groups to benefit from targeted therapies and prediction of patient outcome. One way to analyze the expression markers in multiple samples at once is by using a tissue microarray (TMA) approach. In 2014, Popova et al. assessed the expression of a number of the proteins that were found to be characteristic of the different GBM subtypes (EGFR, CD44, MERTK, p53, OLIG2) in gliomas of different WHO grades using a TMA approach. The group stratified gliomas into groups reminiscent of the molecular subtypes using three of the four subtypes that had been defined by genetic analyses (Classical, Proneural, Mesenchymal) through immunohistochemistry (IHC) and found similar frequencies as when tmours are separated into the subtypes by molecular data. They were also able to accurately differentiate high from low grade gliomas on the basis of protein expression [12].

1.3 Standard of Care

Due to the infiltrative nature of GBM, complete resection of the tumour is often impossible without causing severe neurological damage. Nonetheless, GBM treatment consists of surgery, followed by RT along with concomitant and adjuvant chemotherapy [2]. RT, which uses ionizing radiation to kill malignant cells, has long been the standard of care in the treatment of GBM. More than 30 years ago, when treatment for high-grade gliomas consisted of surgery and RT, Walker et al. (2010) evaluated patient outcomes differences using RT and the alkylating agents carmustine (1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU)) and semustine either alone or in combination. Carmustine and semustine are nitrosoureas, which are lipophilic DNA alkylating agents that are able to cross the blood-brain barrier. While they found no significant survival differences between the group that received only RT and the group that received RT in combination with either alkylating agents, they found a slight benefit in long-term survival (18 months) when patients received carmustine in combination with RT [13]. Following this study, several groups sought to find an adjuvant chemotherapy that could could significantly increase the dismal median survival. Many chemotherapies have failed to treat GBM due to the inherent complexity of the disease, the inability to cross the blood-brain barrier, and mechanisms of intrinsic and acquired drug resistance [14].

In 2002, a meta-analysis based on 3004 patients enrolled in 12 randomised trials assessed the effect of adjuvant chemotherapy with RT in high-grade glioma. The group found an overall significant increase in survival when RT was combined with chemotherapy. While there was no clear concensus on which type of chemotherapy was most effective, this meta-analysis justified further research into drug treatments for high-grade gliomas. Up until 2005, nitrosoureas were extensively used for newly diagnosed glioblatoma patients along with surgery and RT [15].

TMZ is an alkylating agent that was developed in the 1980s by the UK Cancer Research Campaign. TMZ is spontaneously converted to its active metabolite 5-(3-methyl)1-triazen-1-yl-imidazole-4-carboxamide (MTIC) once administered orally. Before becoming the standard of care for newly diagnosed cases, TMZ was approved for the treatment of recurrent GBM at a daily dose of 150 to 200 mg/m^2 /day for 5 days out of a 28 day cycle [2, 16, 17]. In 2005, a landmark study published by Stupp et al. compared RT alone and RT given in combination with TMZ for newly diagnosed GBM patients (Figure 4). 573 patients from 85 centers were randomly assigned to receive RT alone or RT combined with concomitant followed by adjuvant TMZ. RT

was given at a daily dose of 2 Gy per fraction for a total dose of 60 Gy in both groups. Concomitant chemotherapy was given at a dose of 75mg/ m^2 /day during the course of RT until a maximum of 49 days. After a break of 4-weeks, patients were given up to six cycles of TMZ at a dose of 150 to 200 mg/m^2 /day for 5 days out of a 28 day cycle. Stupp et al. found that concomitant treatment of RT and TMZ resulted in a clinically statistically significant survival benefit, thus making it the standard of care for newly diagnosed GBM patients (Figure 4) [2].

Chemoradiation, however, does not modify the disease course and GBM inevitably recurs and leads to death [18, 19]. As of yet, no consensus has been reached on a standard of care for recurrent GBM despite numerous clinical trials exploring the subject. Patients are often

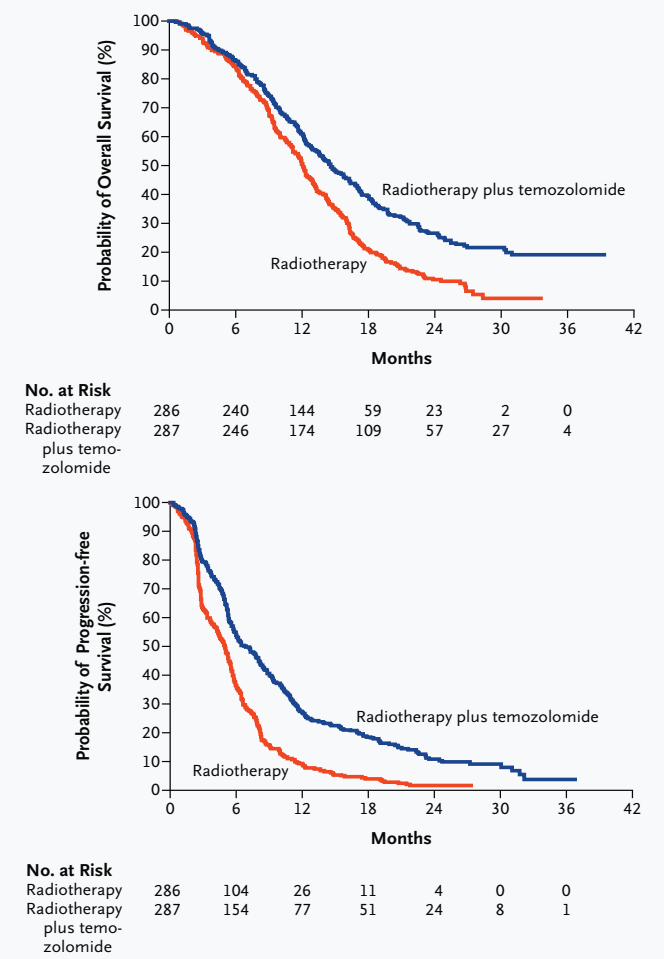

Figure 4. Kaplan-Meier Estimates of OS and PFS in Two Treatment Groups. In the seminal study by Stupp et al. (2005), RT versus RT + TMZ survival outcomes were assessed in a randomized controlled trial in patients newly diagnosed with GBM. RT + TMZ was found to confer a significant survival benefit compared to RT alone. Figure from Stupp et al. (2005) [2].

treated with a repeat surgery and sometimes given re-irradiation, although the survival benefit of both treatments remains controversial. In terms of chemotherapy, patients are often given nitrosoureas (carmustine, lomustine) at recurrence, with TMZ and bevacizumab given less frequently [16]. Recurrent tumours often harbor biologic and genetic changes from the initital primary tumour, in part due to cytotoxic chemotherapy and RT [20].

While surgery followed by RT wih concomitant and adjuvant TMZ is the standard of care for GBM patients, the 5-year survival rate for these patients is less than 9.8%. This low survival rate has been attributed to the complexity and heterogeneity of the disease as well as drug resistance which include DNA damage repair, drug efflux, cancer stem cells and microRNAs. One of the key factors found to determine benefit from TMZ is the promoter methylation of the DNA repair protein 06-methylguanine-DNA methyltransferase (*MGMT)* ([14, 18, 21].

1.3.1 O6-methylguanine-DNA methyltransferase (*MGMT*)

MGMT is ubiquitously expressed in normal tissues and highly conserved phylogenetically. MGMT is critical for the maintenance of cellular integrity and repair of DNA damage and mutagenic lesions [22]. The gene encoding for MGMT (*MGMT)* is often epigenetically silenced in GBM due to promoter methylation in the CpG island of the *MGMT* promoter region. When unmethylated, *MGMT* encodes a protein that removes an alkyl group from the 06 position of guanine, lessening the damage caused by alkylating chemotherapy such as TMZ [21-24]. A study by Hegi et al. (2005) first demonstrated that patients harboring tumours with *MGMT* promoter methylation, considered MGMT negative, had a median survival of 21.7 months versus a median survival of 15.3 months for patients with unmethylated *MGMT* promoter, considered MGMT positive. Similarly, when treated with RT and TMZ, patients with *MGMT* promoter methylation tumors were found to have a 2-year survival rate of 46% compared to 14% for patients with unmethylated MGMT tumors [24]. While MGMT can be considered an important predictor of treatment response, it does not determine whether a patient will receive TMZ treatment or not since there has yet to be chemotherapeutic alternative established for GBM patients with unmethylated *MGMT* promoters. Because of the lack of approved new therapies, all patients with newly diagnosed GBM are treated following the Stupp protocol using RT with concomitant and adjuvant TMZ regardless of *MGMT* methylation status [2, 18].

1.3.2 Improving the Standard of Care

Since few patients survive beyond 5 years with the current standard of care, multiple studies have investigated the effects of changing the dose intensity of TMZ or combination treatment with targeted, cytotoxic, biologic and immunotherapeutic agents to the current chemoradiotherapy approach. Many of these studies aimed to stratify patients based on their molecular profiles to receive targeted therapies in addition to RT and TMZ [20]. Unfortunately, many of these studies have yielded disappointing results. While bevacizumab, an anti-angiogenic humanized monoclonal antibody against vascular endothelial growth factor A, initially showed promise in single-arm studies, it failed to demonstrate a benefit in overall survival when combined with TMZ versus TMZ plus placebo [25, 26]. Although one of the bevacizumab randomized, double blind, placebo-controlled trials demonstrated prolonged progression-free survival, it did not reach the pre-specified improvement target. Similarly, no subgroup of patients defined by *MGMT*-methylation status and prognostic genes derived benefit from the combination treatment. Nonetheless, Bevacizumab has been approved for recurrent GBM since 2009. Other phase II/III clinical trials, such as one that combined cilengitide, an alphavbeta$_3$ and alphavbeta$_5$ integrin inhibitor, have shown no significant improvement in overall survival compared to the standard of care [20, 27].

Another phase III clinical trial examined whether a dose-dense TMZ schedule (75-100mg/m^2 for 21 out of 28 days instead of the standard 150-200mg/m^2 for 5 out of 28 days) improved survival in newly diagnosed GBM patients. The rationale behind this trial was that since dose-dense TMZ has been shown to deplete MGMT levels in blood mononuclear cells, tumour cells with lower MGMT levels would have increased sensitivity to TMZ and result in prolonged survival in GBM patients. Unfortunately, there was no therapeutic advantage for dose-dense TMZ when compared to the standard TMZ dosing schedule regardless of MGMT status. Nonetheless, *MGMT* gene promoter methylation was again validated as a significant prognostic factor for treatment outcome in newly diagnosed GBM patients in this study [28].

1.4 Radiation Therapy

1.4.1 Radiation Overview

RT has long been accepted as a treatment strategy to increase the survival rate of GBM patients after having received maximum debulking surgery. Unfortunately, GBM is known to be

one of the most resistant tumours to RT [29, 30]. Computed tomography (CT) and magnetic resonance imaging (MRI) are used to localize and delineate the tumour for radiation delivery that is specific to the tumour cells and not normal brain tissue. Generally, external beam radiotherapy is delivered to the target defined by CT or MRI scans, the gross tumour volume (GTV), along with a margin of approximately 2.0 cm to form the clinical target volume (CTV). The exact CTV and dose fractionation recommended depends on the organization, with the European Organisation for Research and Treatment of Cancer (EORTC) recommending a single-phase technique of 30 fraction of 2 Gy and the Radiation Therapy Oncology Group (RTOG) favoring a cone-down technique of two different volumes for a total of 60 Gy [31, 32].

RT uses ionizing radiation to kill malignant cells through direct and indirect action and induces a complex set of biological effects and pathways. The direct effect of ionizing radiation on cancerous cells causes DNA damage (double or single stranded breaks) while the indirect effect causes free radicals to form from water molecules. Both actions induce DNA damage and lead to cell death if the cell's DNA repair response pathways are unable to repair the damage. In mammalian cells, DNA damage is mainly repaired by homologous recombination or the more error-prone non-homologous recombination. The two repair pathways require a complex set of factors that may have an effect on differential cell survival depending on the availability of factors and severity of the damage [33]. Both pathways are regulated by members of the phosphatidyl-inositol-3' kinase-related (PI3K) family and are crucial to protection of the genome and cell survival. Cancer cells often harbor extensive and redundant DNA damage response mechanisms that allow the cells to evade death [34].

Despite improvement in control and survival with RT, 85% of GBMs recur within the radiation field, implying that recurrence is due to failure to control the disease at the original site. To analyze recurrence patterns in newly diagnosed GBM patients treated with the standard of care, Minniti et al. (2010) compared patterns of failure between different RT target volume delineations. The group found that the proportion of marginal recurrences was similar using a target delineation based on the post-operative residual disease plus 2 cm margin with or without including peritumoral edema. Minniti et al. also found that recurrences in patients with MGMT-promoter methylation were 64% central and 31% distant, compared to 91% central and 5.4% distant in unmethylated patients. Since larger irradiated brain volumes are associated with higher potential for radiation-induced toxicity, smaller treatment margins in GBM are essential [35].

Recurrence at the surgical site could also be due the inability to precisely visualize the tumour by conventional MRI. Evidence suggests that other imaging techniques, such as magnetic resonance spectroscopy (MRS) and metabolic positron emission tomography (PET), have the ability to better delineate the tumour area [31, 36, 37]. Since GBM is known to be a highly radioresistant tumours, the biological processes behind these mechanisms have been extensively studied to ultimately devise strategies for radiosensitization. Similarly, the use of radiosensitizing agents is an attractive target in GBM since local tumour control is the ultimate therapeutic objective due to the lack of metastasis outside the central nervous system [38].

1.4.2 Radiation Regimens

Another possibility for recurrence at the original tumour site is that the standard dose of 60 Gy in 30 fractions is insufficient for local control [36]. Multiple groups have examined the possibility of increasing the total RT dose and/or dose per fraction to prevent tumor repopulation. Results stemming from trials that examined the role of hyperfractionation (up to a total of 75 Gy in GBM patients) have been disappointing often owing to the increased necrosis in normal brain tissue observed before better local tumour control is seen. In a prospective randomized trial (RTOG 93-05) that compared the effect of adding stereotactic radiosurgery (SRS) to conventional RT with carmustine in GBM patients, no survival benefit was found when the total dose delivered exceeded 60 Gy [39]. Another study that used integrated-boost intensity-modulated radiotherapy (IMRT) local dose escalation found no survival benefit in newly diagnosed GBM patients [40]. Although studies have confirmed the safety of delivering a higher total dose of RT in combination with TMZ, no significant survival benefits were observed when compared to the standard dose of 60 Gy with TMZ [36, 41].

Hypofractionation, which is the delivery of larger but fewer fractions in RT, has been examined as an alternative to the standard fractionation regimen of 60 Gy in 30 fractions. Presently, hypofractionation (60 Gy in 20 fraction or 40 Gy in 15 fractions) is often given to the poor prognosis subgroup (patients over 65 and/or patients with poor performance status) due to their poor tolerance of standard RT, although analyses that retrospectively controlled for the selection bias of elderly patients in hypofractionated regimens found similar survival outcomes between hypofractionated and conventional regimens [42]. In elderly patients, a six-week RT regimen is often associated with higher cases of morbidity and early discontinuation due to deterioration and patient's own choice. Less treatment time is therefore beneficial for these

patients [43]. Hypofractionation has also been given to patients in the hopes of overcoming GBM's own radioresistance [44] . While there is the concern that larger sized fractions will cause increased toxicities, hypofractionation is able to increase cell kill and lower tumour repopulation by increasing the dose per fraction and reducing the treatment time. Investigators have attempted to use hypofractionated RT while also increasing the radiation dose in the immediate vicinity of the tumor and surgical cavity (IMRT) [45]. Groups have also investigated combining hypofractionated RT with concurrent and adjuvant TMZ and reported promising results. Terasaki et al. (2011) found that combining hypofractionated RT (45 Gy in 15 fractions) with TMZ had similar survival rates to standard RT schedules with no severe toxicity observed [46]. Similarly, in a population-based cohort of patients diagnosed with GBM, our lab found that hypofractionation (60 Gy in 20 fractions) was a safe approach with a comparable survival time to the standard RT but with a shorter treatment time [47]. While no survival benefit was found in these studies when a hypofractionated regimen was given, the fact that there was no increased toxicities in these patients justifies the continued study of exploring different radiation fractionation schedules in the hopes of reducing GBM's radioresistant properties.

1.4.3 Mechanisms of Radioresistance

A multitude of *in vitro* studies in the past few decades have led to a deeper understanding of the molecular events involved in radioresistance in glioma cells [7, 34]. One of the major contributing factors to radioresistance in GBM is through increased DNA double strand break (DSB) repair. *TP53*, which encodes the known tumour suppressor p53, is known to be mutated in approximately 30% of primary GBMs and is thought to be involved in the radioresistance of GBM. One of the mechanisms by which it may incur reduced radiosensitivity is through the increased Rad51 levels observed in gliomas. Rad51, one of the proteins vital in mediating homologous recombination repair, is partly controlled by p53-inhibitory signaling, so abnormal p53 function may cause an increase in Rad51 expression. Other proteins, such as the DNA-dependent protein kinase catalytic subunit (DNA-PKcs), ATM, MDC1, and 53PB1 have been implicated in glioma DNA repair after irradiation [33]. Interestingly, using a subpopulation of glioma cells known to be enriched for cancer stem cells (CD133+), Bao et al. (2005) observed that cancer stem cells play a role in glioma radioresistance by activating the DNA damage checkpoint and increasing DNA repair [48]. Likewise, inactivation of the tumor suppressor phosphatase PTEN has been shown to correlate with radioresistance in GBM, likely due to the

upregulation of the PI3K/Akt pathway, since *in vitro* induction of PTEN led to decreased levels of phosphor-Akt and radiosensitization in GBM cells [49].

Using an *in vivo* mouse model of proneural GBM, Holland et al. (2014) found that tumours exhibited a change from the proneural to the mesenchymal gene expression pattern 6 hours after radiation. Since mesenchymally shifted cells have been found to be more radioresistant compared to other GBM subtypes this shift may play an important role in resistance to RT. Similarly, therapeutic radiation is given in multiple fractions over a number of days, so this may have important consequences in the design of fractionation schedules with respect to GBM subtypes [50].

1.5 Epidermal Growth Factor Receptor (EGFR)

1.5.1 EGFR Overview

EGFR overexpression, mutation and/or amplification of the *EGFR* gene represent the most common genetic abnormalities present in GBM. In non-cancerous settings, EGFR is essential for epithelial development and involved in cell differentiation, migration, and growth. In cancer, however, aberrant expression or activity of EGFR is involved in multiple tumorigenic and survival pathways. Since EGFR gene amplification and protein overexpression is relatively rare in low-grade gliomas and secondary GBMs, it is likely involved in the pathogenesis of high grade and not low grade gliomas [7]. The diagnostic identification of *EGFR* amplification by *in situ* hybridization is useful in determining whether a tumour is a grade III or grade IV astrocytoma when the histologic criteria for GBM is not observed as EGFR is rarely expressed in lower-grade astrocytomas. Alternatively, a histologically-defined lower-grade glioma can behave clinically like a GBM when it expresses a mutated form of EGFR, EGFR variant III (EGFRvIII), or exhibits *EGFR* amplification, making the identification of EGFR amplification/expression an important tool in diagnosing GBM [7, 51].

EGFR, also known as ERBB1 or HER1, is a member of the ErbB superfamily consisting of 4 closely related transmembrane receptor tyrosine kinases. EGFR is a cell-surface receptor that is activated by ligands, such as epidermal growth factor (EGF), or in a ligand-independent manner through radiation [34]. Gene amplification and increased protein activation of EGFR often leads to increased angiogenesis, proliferation and compromised apoptosis [52]. Since over 50% of all GBMs exhibit EGFR aberrations and it is known to influence many aspects of tumour

biology, EGFR represents an extremely attractive target for GBM therapy [7, 53].

1.5.2 Signal Transduction

EGFR is a transmembrane glycoprotein with an extracellular region containing ligand binding domains and an intracellular tyrosine kinase domain followed by a C-terminal regulatory region. EGFR has two homologous ligand-binding domains (I and III) and two cystine rich domains (II and IV) in the extracellular component of the receptor (Figure 5A) [54]. EGFR is known to bind to at least 5 mitogenic growth factors (EGF, transforming growth factor-α, heparin binding EGF, amphiregulin, and epiregulin) [55]. EGFR is activated in both a ligand-dependent and ligand-independent manner that consequentially triggers signaling cascades involved in multiple proliferative and survival pathways. Activation of the receptor causes homo- or heterodimerization and induces intracellular tyrosine kinase signaling [54]. EGF ligand binding can cause activation of multiple pathways including Ras, PI3K, STATs, and PLC-γ pathway (Figure 5B) [56].

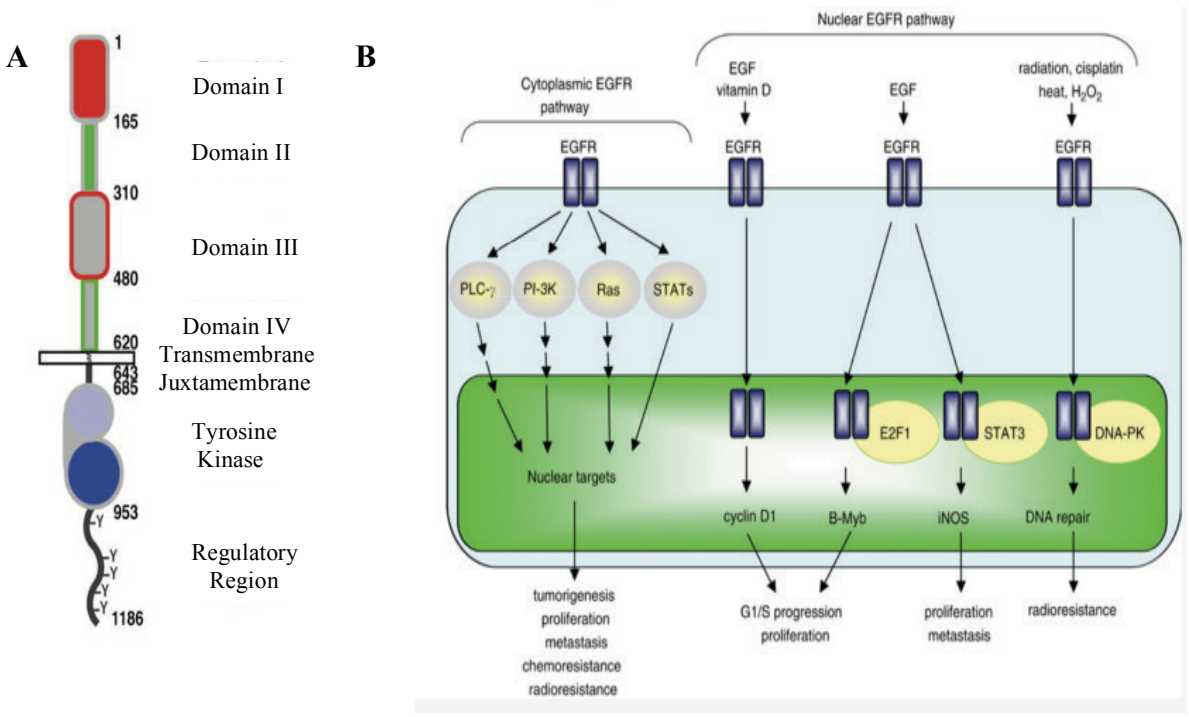

Figure 5. EGFR Signaling. A) Structure of EGFR: numbers denote amino acid number. Figure adapted from Ferguson (2008) [3]. B) Cytoplasmic Oncogenic EGFR and Nuclear EGFR signaling. Figure adapted from Lo et al. (2006) [9].

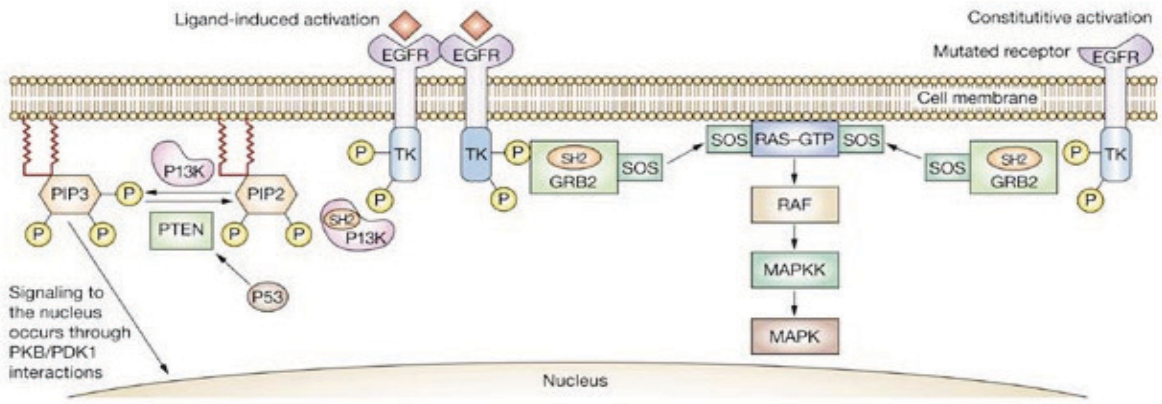

Figure 6. EGFR Signaling Pathways in GBM. Two of the major EGFR signaling cascades in GBM are the PI3K and RAS/MAPK pathway. Ligand-induced activation causes the activation of PI3K pathway which further regulates cell growth, proliferation, and survival. Ras activates a cascade that regulates transcription of genes involved in cell growth. Figure adapted from Collins (2007) [3].

Specifically, EGFR can activate the PI3K and Ras pathways in parallel, which are key signaling pathways of GBM, and cause increased cell invasion, migration, proliferation, and resistance to apoptosis. Once EGFR is activated by ligand binding at its extracellular domain it recruits PI3K which then phosphorylates phosphatidylinositol-4,5-triphosphate (PIP_2) to phosphatidylinositol-3,4,5-triphosphate (PIP_3) resulting in the activation of downstream effectors such as protein kinase B (Akt). Akt promotes the activation of mTORC1, which increases cell growth, and mTORC2, which further activates Akt and other molecules that play a role in cell proliferation and survival (Figure 6). PTEN has the opposite effect on this pathway by dephosphorylating PIP_3 thus downregulating the PI3K signaling cascade [57]. Phosphorylated EGFR regulates Ras by causing the docking of growth-factor-receptor bound-2 (GRB2) to the receptor which then forms a complex with Son of Sevenless (SOS). Activated SOS activates Ras by exchanging guanosine diphosphate (GDP) for guanosine triphosphate (GTP) further triggering a downstream signaling cascade through MAPKs. MAPKs are then able to control gene expression as integral components of transcription factor complexes regulating the transcription of genes responsible for cell growth [3, 6]. Other signaling cascades, such as the STAT3 pathway, are activated through EGFR phosphorylation [58].

1.5.3 Epidermal Growth Factor variant III (EGFRvIII)

The most commonly mutated EGFR variant associated with GBM is EGFRvIII, which

comes from a deletion of exons 2 to 7 of the EGFR gene. The genetic mutation causes a deletion of 267 amino acids from the extracellular domain, rendering it constitutively active without ligand binding and causing increased tumorigenicity in glioma cells [52, 59]. EGFRvIII is tumor-specific and is almost always associated with wild-type (wt) *EGFR* amplification, although tumours with high levels of wt*EGFR* often do not co-express mutant *EGFR.* A possibility for this observation is that *EGFR* amplification occurs first and allows for the propagation and amplification of mutated *EGFRvIII* genes, also validated by the fact that most of the EGFRvIII GBM tumours harboring a detectable EGFRvIII transcript also contain rearranged amplified *EGFR* genes [51, 60]. EGFRvIII has been found to have almost 10-fold lower levels of autophosphorylation than wtEGFR when it is ligand-stimulated. Though the signaling domain is the same for both receptors, EGFRvIII is thought to be more tumorigenic than its wild-type counterpart, possibly due to the altered signaling kinetics in the mutant form. When EGFR binds to its ligand, it undergoes an internalization rate 5-10-fold higher than when it is unbound which leads to a higher rate of degradation/recycling of the receptor [61]. Since EGFRvIII is constitutively active without being ligand-bound, its internalization is slowed which allows it to stay in a state of low-level continuous signaling while also evading negative feedback regulation and degradation. The increased membrane persistence of the receptor is thought to contribute to the tumorigenic potential of the cell and may cause a different set of downstream signaling when compared to wtEGFR, since it activates these signals constitutively when overexpressed [61, 62].

Although both EGFR and EGFRvIII are known to activate the PI3K/Akt and Ras/MAPK pathways, *in vitro* studies suggest that the increased expression of EGFRvIII could result in different cellular responses such as an increase in Bcl-$X_{L,}$ mystoylated alanine-rich protein kinase C, abnormal spindle-like microcephaly-associated (ASPM) protein expression, and matrix metalloproteinase (MMP)-13. Similarly, EGFRvIII preferentially activates the Ras/MAPK pathway over the PI3K/Akt pathway. This differential protein expression profile could partly explain why EGFRvIII cells demonstrate an increased proliferation rate and reduced apoptosis compared to EGFR expressing cells [57]. It has also been proposed that wtEGFR and EGFRvIII may heterodimerize with one another and cause an increase in cell proliferation and survival since they are frequently coexpressed in tumours, although studies have found that no direct physical interaction between EGFR and EGFRvIII occurs in glioma and brain tumour stem cells [7, 63].

One of the mechanisms that increases tumour growth in GBM is through a feed forward mechanism of EGFRvIII, STAT3, and the cytokine receptor Oncostatin M Receptor (OSMR). IHC analyses found that OSMR and EGFRvIII co-localized at the membrane and that no tumours were formed in EGFRvIII-expressing *OSMR* knockdown mice compared to the EGFRvIII-expressing control mice when observed by MRI and by IHC. It is thought that in EGFRvIII-expressing tumours, STAT3 drives tumorigenesis by targeting the transcription of the cytokine receptor OSMR gene, which then acts as a co-receptor of EGFRvIII and together regulate the activation of STAT3 and its transcriptional output, which further upregulates OSMR expression, causing a signaling cascade that leads to activation of tumorigenic genes [63].

1.5.4 Nuclear Localization of EGFR and EGFRvIII

Several groups have reported that EGFR is not only present on the cell surface but also in the nucleus and is involved in the transcriptional activation of multiple oncogenic genes [56, 64]. This finding challenged the idea that membrane-bound RTKs only transduce extracellular signals through multiple activated signaling cascades to get to their transcriptional nuclear targets and instead suggests that extracellular signals can be directly transmitted from the membrane to the nucleus through nuclear localization of these RTKs [65]. Nuclear EGFR was found to be highly expressed in the nuclei of many different types of tumours and is thought to be expressed as the full-length phosphorylated form of EGFR. The exact mechanism by which EGFR translocates to the nucleus remains elusive, although it is thought to be through endocytosis and involves the phosphoinositide kinase PIKfyve and the endosomal marker endosome antigen 1 [66, 67]. Other groups have suggested that chaperone-like factors are able to mask the hydrophobicity of the membrane-domain, making it soluble and able to move through the cytoplasm to get to the nucleus [68].

Nuclear EGFR has been shown to play a role in cancer by transcriptionally activating oncogenic genes, promoting DNA replication and repair, and mediating resistance to various cancer therapies, including RT. In a multitude of cancers, nuclear EGFR has been found to target and promote the transcriptional activation of genes involved in cancer progression, including cyclin D1, inducible nitric oxide synthase (iNOS), B-Myb, Aurora KinaseA, c-Myc, BCRP, and cyclooxygenase 2 (COX-2) (Figure 5) [64]. Although lacking a DNA-binding domain, nuclear EGFR is able to induce the expression of these genes by interacting with other DNA-binding transcription factors, such as STAT3 and E2F1 [69]. *In vitro* studies have demonstrated that

ionizing radiation is able to stimulate EGFR import into the nucleus where it localizes to uncoiled chromatin and increases DNA repair activity, causing resistance to RT [70]. This is likely by associating with proliferating cell nuclear antigen (PCNA) and DNA-PKcs, which play an essential role in survival and cancer progression through DNA replication and repair. Nuclear EGFR has also been shown to promote resistance to various cancer therapeutics, such as cetuximab, gefitinib, cisplatin [64].

While the role and properties of nuclear EGFR has been described in many tumour types, it has only recently been investigated in GBM. In confocal fluorescence microscopy experiments by de la Iglesia et al. (2008), EGFRvIII immunoreactivity, although predominately expressed in the cytoplasm, was also observed in the nucleus of some GBM tumours. Through subcellular fractionation analyses the group also found that EGFRvIII, along with the transcription factor STAT3, was expressed in cytoplasmic and nuclear cell fractions. Interestingly, EGFRvIII was found to complex with STAT3 more efficiently in the nucleus than in the cytoplasm. While STAT3 functions as a tumour-suppressor in the PTEN pathway, the group found that when it formed a complex with EGFRvIII in the nucleus, the two proteins mediated pro-oncogenic glial transformation [68]. Another study found that GBM cell lines express nuclear EGFR/EGFRvIII and that nuclear EGFR/EGFRvIII associates with STAT3 to transcriptionally activate the cyclooxygenase-2 (COX-2 gene), which plays a role in tumor progression, the pro-inflammatory response, and possibly mediating radioresistance in glioma cells [71]. The group identified nuclear EGFR target genes by using U87MG isogenic cell lines with overexpression of wtEGFR (U87MG-EGFR) and nuclear entry-defective EGFR with a deleted nuclear localization signal (U87MG-EGFRdNLS). After EGF stimulation, the group extracted total RNA and identified genes that had a differential expression between the two cell lines using a microarray of over 47,000 human transcripts. Out of the 19 genes that they found to be differentially expressed, the increased expression of COX-2 in U87MG-EGFR cells was validated through quantitative RT-PCR and western blot. Similar results were reported when the group used EGFRvIII instead of wtEGFR [69]. COX-2 may be an attractive target for radiosensitization in GBM tumours with EGFR overexpression or EGFRvIII expression since an *in vitro* study demonstrated that a COX-2 inhibitor increased glioma cell kill when added to radiation exposure compared to radiation alone [71].

1.5.5 EGFR-targeted Therapy

Since EGFR expression is associated with multiple tumorigenic pathways including radioresistance, it is regarded as an attractive target for therapeutic interventions. EGFR-targeted therapies are either tyrosine kinase inhibitors (TKIs) that bind to EGFR's ligand binding site, monoclonal antibodies, immunotherapeutic agents, and recently, RNA therapies. While multiple EGFR-targeted therapies have found success in clinical trials for other cancers, none have yet proven their efficacy in GBM. EGFR-targeted therapies in GBM clinical trials have encountered several problems such as crossing the blood-brain-barrier, resistance to therapy, hypersensitivity, and increased patient toxicities [57]. Most of these trials have used monoclonal antibodies or TKIs to target EGFR, although rindopepimut, which is a vaccine that elicits an immune response against cells expressing EGFRvIII, has recently garnered interest and has entered clinical trials. Since EGFRvIII is almost exclusively present in tumour and not normal cells, this strategy presents a highly targeted method of killing only tumour cells using the patient's own immune system [57, 72].

Functional diversity and redundancy is a key problem in EGFR-targeted therapy since EGFR can cross-communicate with other pathways, leading to alternative oncogenic cascades and eventually leading to tumour progression despite EGFR inhibition (Figure 7) [6]. *In vitro* studies have demonstrated that EGFRvIII expression can result in activation of the RTK/RAS/PI3K pathway even when EGFR is inhibited, since EGFRvIII signals through mTOR2 whereas EGFR signals through mTOR1 which leads to increased cell survival and proliferation [73]. Similarly, targeting EGFR may result in selection pressure for other mutations that cause resistance to therapy, such as loss of the tumour suppressor PTEN, leading to the upregulation of the PI3K signaling cascade [57]. It has also been observed in GBM stem cells that the PI3K pathway can still be activated when EGFR is inhibited due to the compensatory activation of other tyrosine kinases, such as ERBB2 and ERBB3, which have similar downstream signaling targets, causing functional resistance to EGFR-inhibitors [74]. The disappointing history of clinical trials targeting EGFR in GBM points to the need for patient stratification based on the presence of EGFR/EGFRvIII overexpression or amplification as demonstrated in a study showing that EGFR overexpression was strongly correlated to improved survival in patients treated with the metronomic schedule of TMZ, and the use of drug combinations based on individual tumour characteristics [75, 76]. Interestingly, in a study that

examined cetuximab, an anti-EGFR therapy that targets both EGFR and EGFRvIII, there was still a difference in response between EGFR and EGFRvIII-expressing tumour cells when treated with a multimodal approach of TMZ, cetuximab, and RT. This was likely due to the differences in sensitivity between the two EGFR variants to TMZ, which emphasizes the need to stratify patients based on EGFR and EGFRvIII protein expression in order to receive the most beneficial treatment [77].

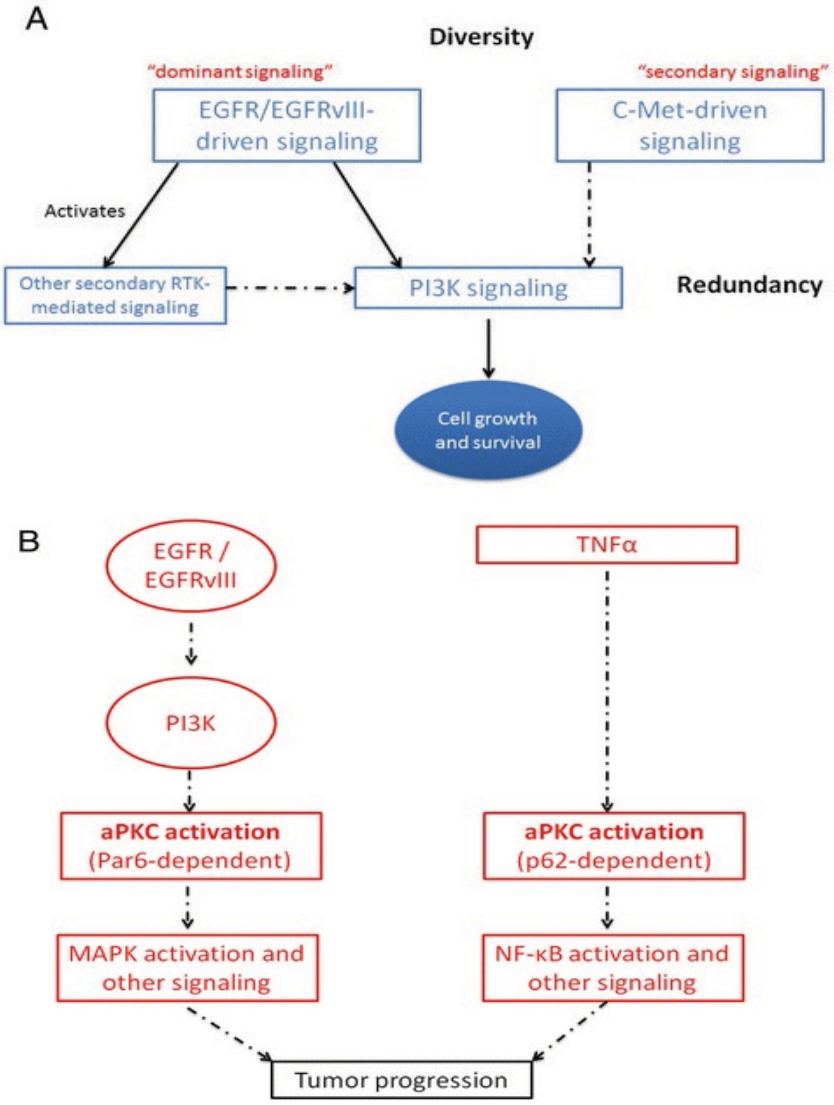

Figure 7. Cross-communication in the EGFR Signaling Pathway. A) Redundancy and diversity in the EGFR-induced signaling pathway contribute to tumor progression and clinical failure of EGFR-targeted agents in GBM. B) Diverse pathways, such as the TNFα signaling cascade, are able to initiate oncogenic pathways leading to tumour progression when EGFR is inhibited. Figures by Azuaje, Tiemann, and Niclou (2015) [6].

1.5.6 Prognostic Value

The prognostic value of EGFR and EGFRvIII has been widely debated and seems to rely on a multitude of other factors. In a study examining the relationship between EGFR and survival, immunohistochemically-determined EGFR positivity was found to correlate to reduced survival and increased local failure after irradiation in patients with astrocytic tumours [78]. While multiple studies have found that EGFR overexpression/amplification has no effect on prognosis in GBM, one group reported that EGFRvIII overexpression with *EGFR* amplification was a negative prognostic factor for overall survival [79, 80]. Although Heimberger et al. (2005) found that neither EGFR nor EGFRvIII overexpression was a prognostic factor for GBM patients who had undergone surgery, they identified EGFRvIII as a significant negative predictor of survival for patients who survived longer than 1 year. Their patient population, however, may have a selection bias since they only included patients with gross total resection. I*n vitro* studies have demonstrated increased invasiveness for EGFRvIII-positive GBM so tumours that were deemed unresectable due to widespread invasion that were excluded from the study may have been more likely to express EGFR or EGFRvIII [80, 81]. Other studies have found that the prognostic significance of EGFR overexpression or gene amplification depends on the age group, with overexpression/amplification often associated with a worse prognosis in younger patients and a better prognosis among older patients [82]. It is also important to note that EGFR amplification in GBM patients becomes more common with increasing age. Interestingly, Simmons et al. (2001) uncovered a complex relationship between age, EGFR expression and p53 status. As demonstrated in other studies, the group found that EGFR positivity was associated with better survival in older patients and worse survival in younger patients. In the younger patient subgroup, however, Simmons et al. found that EGFR only predicted a worse prognosis in tumours that were also negative for p53, which is another commonly altered gene in GBM with an unclear prognostic value. This study highlighted the genetic complexity of markers in GBM and that subgroups based on patient characteristics and different markers is needed to achieve maximal benefit from EGFR-targeted therapies [83].

1.5.7 EGFR and Radioresistance

In a study that compared EGFR immunoreactivity to radiographically assessed radiation response in GBM, EGFR overexpression was found to be a predictor of poor radiation response [84]. A number of studies in different cancers have found that cells with high EGFR expression

are more insensitive to radiation-induced apoptosis and more resistant to cytotoxic agents. Akimoto et al. (1999) explored the relationship between EGFR expression and tumor radioresponse in nine murine carcinomas and found that levels of EGFR inversely correlated to radiation-induced apoptosis, and tumors with high EGFR expression had a lower *in vivo* radiocurability. Autophosphorylation of EGFR occurred in high but not low EGFR-expressing tumours. Although the group did not explore this relationship in glioma cells, this study implied that EGFR expression could be a predictor of radiation response and in selecting an appropriate therapeutic treatment in patients based on EGFR expression [85]. Similarly, another group found that GBM U87MG cells exhibited enhanced radiosensitivity when they were treated with an EGFR inhibitor and exposed to various doses of irradiation [86]. Further *in vitro* studies found that gefitinib, an EGFR-tyrosine kinase inhibitor, sensitized GBM cells to irradiation. Possible mechanisms of EGFR-induced radioresistance includes reduced apoptosis -which is a common response to DNA damaging ionizing radiation- and increased cellular proliferation [87]. Ligand-independent radiation induced EGFR activation has been found to rapidly repair DNA double strand breaks (DSB) after radiation, possibly through activation of DNA protein kinase catalytic subunit (DNA-PKcs) by EGFR nuclear localization or through the PI3K/Akt pathway [34].

Ionizing radiation can induce intrinsic or acquired resistance to treatment by activating signal transduction pathways such as NF-κB and pathways regulated by receptor tyrosine kinases (RTKs) like EGFR. EGFR and EGFRvIII expression has been shown to correlate to increased radioresistance in GBM cells. EGFR regulates multiple downstream signaling pathways including the PI3K-AKT-mTOR, a pathway known to be implicated in RT resistance [38, 88]. EGFRvIII has also been shown to stimulate the repair of DSB breaks by promoting DNA-PKs activation, possibly through the PI3K-AKt pathway (Figure 8). A possible mechanism for this observation is that once activated through EGFRvIII signaling after irradiation, Akt impedes cell apoptosis by inhibiting proapoptotic factors (BCL2 antagonist of cell death and procaspase-9) and may translocate to the nucleus and associate with DNA-PK, an important factor in the non-homolougous end joining repair pathway, at sites of DNA damage [89]. Another possibility is that EGFR and EGFRvIII are able to translocate to the nucleus after irradiation and interact with DNA-PKs to stimulate DNA repair, although the experimental evidence for this in GBM has yet to be uncovered (Figure 8) [7].

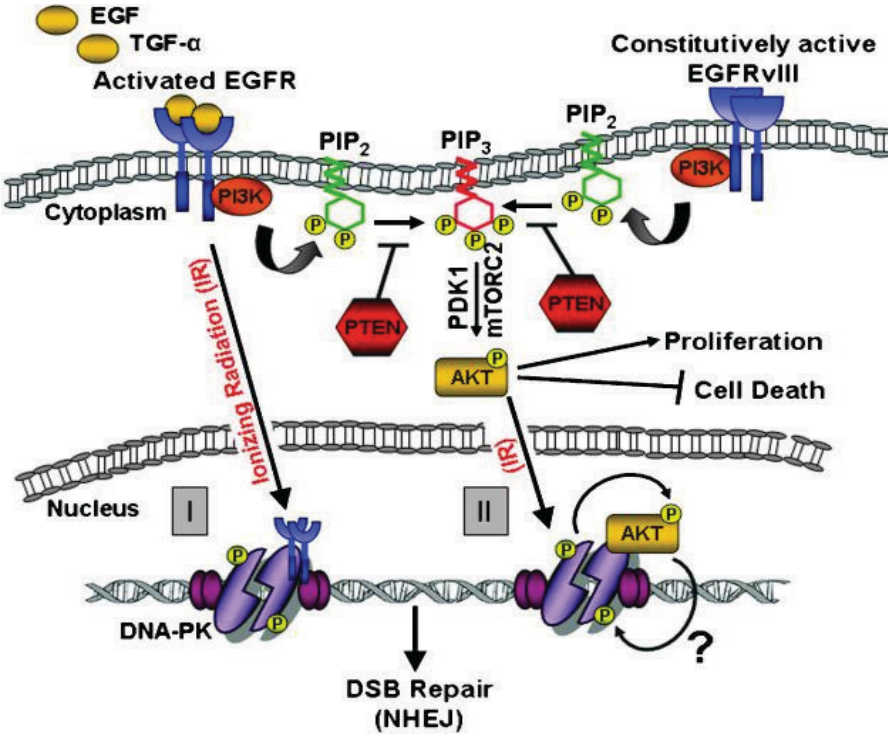

Figure 8. Proposed Mechanisms of EGFR in Radioresistance. Activated EGFR is proposed to cause increased DNA repair after irradiation through the PI3K-Akt pathway (II) or through the nuclear localization of EGFR and subsequent interaction with DNA-PKcs (I). Evidence for nuclear localization of EGFR/EGFRvIII and interaction with DNA-PKcs in GBM has yet to be elucidated. Figure from Hatanpaa et al. (2010) [7].

Nuclear EGFR translocation has been found to be induced by RT and play a role in resistance to therapy. Nuclear EGFR is able to associate with and possibly phosphorylate PCNA, a DNA clamp that is used to recruit proteins during DNA replication, which leads to its stabilization and promotion of DNA replication after radiation-induced damage. Similarly, after irradiation, EGFR has been shown to localize to the nucleus and interact with DNA-PK, a protein kinase involved in DNA repair of double-stranded breaks [64, 70]. In GBM, nuclear EGFR/EGFRvIII has so far only been found to associate with STAT3 and transcriptionally activate the COX-2 gene, which plays a role in tumor progression, the pro-inflammatory response cascade, and, interestingly, possibly mediates radioresistance in glioma cells. *In vitro* experiments have found that COX-2 inhibitors increased the glioma cell kill when added to radiation exposure compared to radiation alone in a radioresistant cell line with high COX-2 expression [71]. *In vivo* models have also demonstrated that celecoxib, a selective COX-2 inhibitor, increased radiosensitivity and reduced clonogenic survival in mice with implanted GBM cells [90]. Similarly, prostaglandin E_2 (PGE_2), which is derived from COX-2, has been

shown to activate the ERK1/2 MAPK pathway and induce Id1, which resulted in increased self-renewal and resistance to radiation-induced DNA damage in GBM [91]. A possible mechanism for the observation that COX-2 inhibition increases radiosensitivity may be through the COX-2 induced expression of Id1. Id1, a functional marker of glioma-initiating cells (GICs) and inhibitor of DNA binding/differentiation, is suggested to be involved in DNA damage pathways and an effector of the p53 DNA damage response pathway [92]. It is also important to note that cytoplasmic EGFR has been shown to regulate COX-2 expression through p38-MAPK signaling and activation of the Sp1 transcription factor [93, 94]. Taken together, one of the ways that nuclear and cytoplasmic EGFR may induce radioresistance in GBM could be through the regulation of COX-2 which causes the activation of the ERK/MAPK pathway and induces Id1.

Rationale and Hypothesis

Despite the aggressive multimodal treatment given to patients, the prognosis of GBM remains extremely dismal. Different patients exhibit variable benefits to treatment, pointing to the need to stratify patients based on gene/protein expression to elucidate which subgroup of patients will benefit most from different therapies. While several studies have explored the effects of hypofractionated RT in newly diagnosed GBM patients, there has yet to be a clear answer as to whether a different radiation regimen could be more effective than conventional RT or whether a specific subgroup of patients harboring common genetic alterations may benefit more from different regimens. Since EGFR is activated by radiation in GBM and known to play a role in radioresistance, its overexpression may potentially hold a prognostic value to stratify patients for a given radiation regimen that could be associated with a better treatment outcome.

We hypothesized that patients with high EGFR expression would have a better survival outcome when treated with hypofractionated radiation compared to the conventional radiation regimen since the higher dose per fraction may increase cell kill and disrupt EGFR's proliferative abilities. We aimed to explore the effect of EGFR overexpression and its relationship to other commonly overexpressed proteins with regards to clinical characteristics in a cohort of 201 newly diagnosed GBM patients from a single institution. We also analyzed the prognostic value of EGFR in GBM patients, since different studies have found conflicting results, and explore its relationship to other known markers in GBM. Additionally, we used IHC expression patterns to subdivide tumours into groups reminiscent of molecular based GBM subtypes and investigated whether the subtypes had an effect on survival outcomes. We used a

TMA of 201 patient samples with an annotated clinical database to accomplish our aims.

The following chapter summarizes our ongoing study in a manuscript style.

Chapter 2. EGFR Overexpression and Response to Different Radiation Regimens in Newly Diagnosed GBM Patients

2.1 Introduction

Glioblastoma multiforme (GBM), the most frequent and aggressive form of malignant primary brain tumor has an extremely dismal prognosis of less than 2 years despite treatment of surgery and chemoradiation [2]. GBM consists of a series of genetic and epigenetic aberrations composed of DNA alterations, chromosomal rearrangements and DNA methylation modifications. EGFR overexpression, one of the most commonly observed protein alteration in GBM, plays a role in multiple pro-tumorigenic pathways, such as increased cell proliferation, reduced apoptosis, increased angiogenesis and increased radioresistance. EGFR is overexpressed in approximately 54% of all GBM tumours with *EGFR* gene amplification present in 34% of all tumours. 31% of all tumours overexpress both EGFR and its commonly mutated form, epidermal growth factor receptor variant III (EGFRvIII) [7]. EGFR expression has been correlated to a poor radiation response in GBM and multiple *in vitro* studies have found that EGFR and EGFRvIII play a role in radioresistance in GBM cells, likely due to the upregulation of the PI3K-AKT pathway and increase in the repair of DNA double strand breaks after irradiation [7, 84, 86, 89].

Multiple studies have investigated the prognostic value of EGFR and EGFRvIII in patient survival and reported contrasting results. Although some studies have found that EGFR is of no prognostic value in GBM, others have found that EGFR is prognostic when the patient population is grouped by other characteristics such a p53 status, age, and survival time [80, 82, 83]. In a study by Shinojima et al. (2003), the group retrospectively analyzed the relationship between *EGFR* gene status and EGFR expression and treatment outcomes in newly diagnosed GBM patients. The group found that tumours with EGFRvIII overexpression and *EGFR* amplification was a strong indicator for poor survival [79]. Other studies have found that *EGFR* gene amplification is often associated with EGFR protein overexpression, though some tumours with EGFR overexpression do not have gene amplification [95, 96].

EGFR expression has been correlated to patient survival with respect to different treatment regimens in GBM and other cancers. Cominelli et al. (2015) identified long-term

survivors (>35 months) in a group of GBM patients that had been treated with standard postsurgical concomitant RT and adjuvant chemotherapy. They explored molecular and histopathological features associated with survival and found that EGFR overexpression was strongly correlated to improved survival (PFS and OS) but only in patients treated with the metronomic schedule (75mg/m^2 daily until progression) and not the standard schedule of TMZ. This study validates the need to explore subgroups of patients that might benefit more from different therapy regimens [76]. Interestingly, in a randomized controlled trial that explored the relationship between EGFR expression and fractionated RT in head and neck squamous cell carcinoma (HNSCC), Bentzen et al. (2005) compared the locoregional tumour control in patients receiving continuous accelerated RT (4.5 Gy per day for 12 days) versus those receiving the conventional fractionation schedule of 2 Gy per day for 45 days. The group then assessed EGFR status of these patients using immunohistochemistry and found that patients with high EGFR expression had higher locoregional tumour-control when given accelerated RT compared to the conventional fractionation schedule. By contrast, patients with low EGFR expression showed no difference in benefit between the two treatment arms. A possible explanation for this observation is that since EGFR is involved in the rapid repopulation of tumor cells, having a longer treatment time allows the tumor cells to repopulate, so by shortening the treatment time and increasing the radiation dose more tumour cells are killed [97].

Considering the radioresistant properties of EGFR and its ability to be activated by radiation in GBM, we aimed to investigate in this study whether EGFR overexpression holds a prognostic value for GBM in our cohort and if different radiation regimens confer a difference in survival outcomes for patients with EGFR overexpression. We also aimed to investigate whether there was a relationship between EGFR overexpression and other commonly aberrantly expressed proteins by analyzing co-expression of EGFR with other markers with respect to response to different radiation regimens.

2.2 Materials and Methods

2.2.1 Patient Population

GEN-Research Ethics Board approval was obtained and we compiled a list of adult patients who underwent surgery for GBM between January 2001 and December 2012 at the Montreal General Hospital and Montreal Neurological Institute. Clinical details, including

patient information, treatment details and tumor pathology reports were obtained using hospital charts and an electronic Clinical Information System (OACIS). In some cases, clinical information was missing due to patients changing hospitals (lost to follow-up). Patients were selected for the TMA on the basis of having histologically-confirmed GBM from tissue sample at the time of surgery. 201 patients were selected for the study. Clinical information (imaging, focality and location of the tumour, age, Karnofsky Performance Status (KPS) score, comorbidities, toxicities, radiation, chemotherapy, recurrence, and date of death) was obtained as of the date of diagnosis. Pathological information, include expression of GFAP, amplification of *EGFR, PTEN* loss, expression of p53, and methylation status of *MGMT* (through methylation-specific polymerase chain reaction (PCR)) was available for a portion of the patients. *EGFR* amplification was determined by fluorescent in situ hybridization (FISH).

2.2.2 Patient Treatment

Maximum safe resection, based on patient characteristics and extent and location of the tumour was given to all patients. In most cases, presence of residual tumour was assessed by magnetic resonance imaging (MRI) or computed tomography (CT) 24 to 72 hours post-operatively. Surgery was considered to be a gross total resection (GTR) if no residual tumour was seen by MRI or CT, and subtotal resection (STR) if residual tumour was noted. Most patients were given post-operative RT and chemotherapy. RT planning was CT-based with 3 mm slices. The surgical cavity along with any post-operative residual disease was contoured with a margin of 2 cm. The majority of patients either received the conventional fractionation schedule of 60 Gy in 30 fractions, the hypofractionated schedules of 60 Gy in 20 fractions or 40 Gy in 15 fractions, with a few patients receiving alternate schedules. RT was delivered 5 days a week.

Most patients received concomitant and adjuvant TMZ although some patients received alternate first-line therapy (BCNU, PCV, Irinotecan, Everolimus, Avastin) either because TMZ had yet to become the standard of care or the patient was enrolled in a clinical trial. Patients received concomitant TMZ at the standard dose of 75mg/m2 daily during radiation treatment and adjuvant TMZ at a dose of 150-200mg/m^2 for 5 days every 28 days for up to 12 months (or until recurrence). A few patients were enrolled in a clinical trial and received a dose-intensive schedule of adjuvant TMZ 21 out of 28 days [98].

After completing their radiation treatment, patients were assessed by MRI and clinical evaluation every three months. Recurrence was defined as when residual enhancement or new

areas of tumour were observed radiologically. Recurrence was considered central if the disease was in the initial surgical cavity and distal if it recurred more than 2 cm away from the cavity. After recurrence, patients were often given second line chemotherapy treatment (procarbazine, lomustine, avastin, TMZ) and/or surgery. Re-irradiation was given in a few cases. Progression free survival (PFS) was measured from the date of diagnosis to the date of when recurrence occurred radiologically. Overall survival (OS) was defined as the time from the date of diagnosis to date of death, or to the last clinical follow-up if the patient was alive at the time of analysis.

Pseudoprogression was defined as post-radiation radiological progression followed by regression or stability of the tumour if the patient was not receiving treatment when the progression was observed. Cases where the patient underwent surgery for recurrence but was found to have no tumour cells on pathological observation were considered to have pseudoprogression as well.

2.2.3 Tissue Microarray (TMA)

Tumor tissue paraffin blocks were collected from 201 newly GBM patients (Figure 9). 3 cores were taken from every patient's tumour tissue sample. 13 patients had tissue samples taken from more than one surgery. Tissue microarray (TMA) sections (3 cores) were immunohistochemically stained for on BenchMark XT (Ventana Medical Systems) using the technical protocol XT ultraView DAB v3. Antigen retrieval was performed using an extended CC2 protocol or standard CC1 protocol (Ventana Medical Systems). Antigen detection was carried out using Ultra-View diaminobenzidine chromogen (Ventana Medical Systems). Primary antibody was omitted in the negative control. Immunohistochemistry (IHC) staining was performed for mutated IDH1 (R132H mutation; clone H09; Ventane), CD44 (clone SP37; Ventana), Ki67 (SP6 ab16667; Abcam), EGFR (E3138; clone F4; Dianova), PTEN (clone 138G6; Cell Signaling Technology), p53 (clone Bp53-11; Ventana), and Vimentin (clone EPR3776; Abcam). IHC staining was assessed and scored by a neuropathologist (MCG) who was unaware of the patient's clinical features. IDH1 staining was scored by positive versus negative staining for the mutation R132H, Ki67 by the percentage of cells stained, PTEN by whether there was expression in the tissue sample (negative versus positive), CD44 by the staining intensity and number of cells stained, p53 by positive or negative expression, and vimentin by negative, moderate or positive staining. Ki67 and CD44 staining were considered to have high expression when the staining was above 10% and 50%, respectively [12]. EGFR

staining was scored based on the intensity of staining and the number of stained cells in all three cores: 0 (no staining), 1 (light staining), 2 (moderate), 3 (strong). Scores of 2 and 3 were defined as overexpression while scores of 0 and 1 as no overexpression [79, 99]. Using an algorithm based on separating tumours into the molecularly-defined GBM subtypes, expression of EGFR, p53, CD44, PTEN, and IDH1 were used to subdivide our cohort into groups reminiscent of 3 of the molecularly defined GBM subtypes. Briefly, this was performed by grouping tumours with high EGFR and negative p53 expression (Classical-like subtype), and then grouping the tumours that did not fall into the high EGFR/low p53 category into a high p53 and/or IDH1 and/or PTEN expression group (Proneural-like), and then the remaining tumours with high CD44 into the Mesenchymal-like subtype [1, 12].

2.2.4 Endpoints and Statistical Analysis

The endpoints of this study were overall survival (OS) and progression free survival (PFS) between patients with overexpressing and non-overexpressing EGFR tumours. Statistical analysis was performed using SAS version 9.3 (SAS Institute, Cary, NC, USA) and Prism 7 GraphPad. Correlations between EGFR and other markers were performed by chi square test. Descriptive statistics were performed for the patient population. Multivariate cox proportional hazard regression models were used to assess OS and PFS in the patient population. For OS, the index date was date of death and date of first recurrence/progression for PFS. The effects of EGFR overexpression were quantified by hazard ratio (HRs) with 95% confidence intervals (95% CIs). Analyses were performed for other possible prognostic factors such as age (dichotomous: ≥65 versus <65), KPS (dichotomous: ≥70 versus 70), extent of initial surgery, receiving chemotherapy before the index date, repeat surgery before the index date, and methylation status of MGMT. Statistical significance for the Kaplan-Meier curves were determined using the log-rank test, and all analyses were two-sided with significance considered at $p < 0.05$.

2.3 Results

2.3.1 Patient Population Characteristics

A total of 201 patients with histologically confirmed GBM were included in this population-based study. A summary of their characteristics is presented in Table 1. In this population, 117 (58.2%) patients were male and 84 (41.8%) were female, with 126 of the

patients under 65 years old (62.7%) and 75 patients over 65 years old (37.3%). 183 tumours were considered primary (91%), while 16 were considered secondary (8.0%), and 2 were unknown (1%). 191 (95%) patients had unifocal tumours while 10 were multifocal (5.0%). In terms of KPS, which is a measure of functional impairment, 19 (9.5%) patients had a KPS score of <70, 127 patients were above 70 (63.2), and 55 patients (27.4) had unknown KPS scores. At surgery, 61 patients (30.3%) received a total resection, 133 patients (66.2%) received a subtotal resection, and 7 patients (3.5%) had an unknown extent of surgery due to missing clinical information. 166 (82.6%) patients received RT, 26 (12.9%) patients did not, and 9 (4.5%) patients could not be determined due to missing clinical information. Out of the 166 patients that received RT, 75 patients received the conventional fractionation schedule of 60 Gy/30 Fr (46.4%), 41 patients received the fractionated schedule of 60 Gy/20 Fr (24.7%), 22 patients received 40 Gy in 15 fractions (13.3%), 14 patients (8.4%) received a different fractionation schedule than the ones previously mentioned, and 12 patients (7.2%) received RT although we could not determine their dosage or fractionation schedule due to missing clinical information. 136 patients (67.7%) received chemotherapy while 53 patients (26.4%) did not, and 17 patients (6.0%) could not be determined. 141 patients (70.1%) had a recurrence, 43 patients (21.4%) did not and 17 patients (8.5%) could not be determined due to missing clinical information. 67 patients had a repeat surgery (33.3%) while 109 patients (54.2%) did not and 25 patients (12.4%) were unknown. *MGMT* methylation information was available in 143 cases, and 50.0% of these patients were found to be *MGMT* methylated (Table 2).

Survival of patients receiving RT versus RT and chemo was examined with RT+ chemo conferring a statistically better survival outcome than RT alone (Figure 10). For PFS, mean and median for was RT: 5.4 months and 4.2 months; RT and Chemo was 12.8 months and 8.1 months (n=126; Log-rank *p-value* = 0.0002). For OS mean and median for RT was 12.5 months and 9.1 months; RT and Chemo was 25.2 months and 16.7 months (n=164; Log-rank *p-value = 0.0002).* When comparing survival outcomes for patients between each radiation regimen, we found a significant difference in OS but not PFS (Figure 11). Overall survival was significantly increased in the 60 Gy/30 Fr and 60 Gy/20 Fr groups compared to the 40 Gy/15 Fr and other regimens group (Log-rank *p value* <0.0001).

In the adjusted PFS/OS multivariate analysis, we found that for OS, higher age compared to lower age significantly correlated to lower survival (HR 2.11; CI, 1.26-3.53; P=0.0044), with

having a higher KPS also correlating to having a better OS (HR 0.51; CI, 0.26-0.87; P=0.0453). Having chemotherapy in the correlated to better OS (HR 0.50; CI, 0.25-0.99; P=0.0496) as was having a repeat surgery (HR 0.57; CI, 0.37-0.87; P=0.00101). For PFS, having chemotherapy was correlated to a better PFS compared to not having chemotherapy (HR 0.43; CI, 0.22-0.86; P=0.00177), as was having a higher KPS (HR 0.31; CI, 0.16-0.63; P=0.0012) (Table 3).

2.3.2 TMA Protein Expression

In the TMA of 201 newly diagnosed patients, 111 patients (56.1%) were found to have no EGFR overexpression while 87 patients (43.9%) had EGFR overexpression, and 3 patient tissue samples (1.5%) were unable to be scored for EGFR due to folded or missing cores (Table 4). EGFR overexpression was defined as scores of 2+ (moderate staining) or 3+ (strong staining), while no EGFR overexpression was defined as scores of 0 (no staining) or 1 (light or focal) (Table 5). Information about *EGFR* amplification from patient medical records, determined by FISH staining, was available for 82 patients with 44 patients (53.7%) patients determined to have *EGFR* amplification, and 38 (46.3%) with no amplification (Table 5). Out of the 82 patients with information on *EGFR* amplification and EGFR overexpression, 73% of *EGFR*-amplified tumours were found to also have EGFR overexpression, and 84% of the EGFR-overexpressed tumours also had *EGFR* amplification ($p < 0.0001$). Expression of mutated IDH1, PTEN, p53, vimentin, Ki67, and CD44 is summarized in Table 6. While a few cases were not able to be scored due to folded or missing cores, IDH1-mutant was found to be positive in 6.0% of the samples, PTEN expression was observed in 8.0% of the tumours, p53 expression was observed in 13.9% of the patients, vimentin staining was high in 85.0% of the samples, Ki67 was high in 58.2%, and CD44 in 86.6% of the cases. We compared EGFR expression to each marker and found a significant negative correlation with IDH1-mutant expression and p53 expression to EGFR overexpression ($p<0.005$) (Table 7). By separating the tumours into groups reminiscent of molecular defined GBM subtypes, we found that 79 (39.3%) tumour samples fell into the Classical-like subtype (high EGFR and low p53), 40 (19.9%) in the Proneural-like subtype (high p53 and/or IDH1-mut and/or PTEN expression), 72 (35.8%) in the Mesenchymal-like subtype (high CD44), and 10 (5.0%) could not be categorized (Figure 15). Survival outcomes with regards to groups reminiscent of the molecular subtypes were analyzed but no correlation was observed (data not shown).

2.3.3 EGFR Overexpression

In terms of radiation regimen (Table 8), we found no trend in EGFR expression with relation to which radiation regiment they were given ($p > 0.05$). By splitting the population of 198 patients with known EGFR expression status into EGFR overexpressing and non-EGFR overexpressing subgroups, we examined the clinical characteristics of patients and whether there were any observable trends (Table 9). No clinical factors (age, sex, KPS, extent of surgery, chemotherapy, recurrence, repeat surgery, *MGMT* methylation) were found to be significantly correlated to EGFR overexpression ($p > 0.05$). In most cases, the patients in each subgroup exhibited a similar proportion of each variable independent of EGFR expression (above or under 65 years old, male versus female, KPS score of under 70 or above 70, etc.).

2.3.4 EGFR Overexpression and Survival

When looking at the survival outcomes (OS and PFS), we found no significant correlation between the EGFR overexpressing and non-overexpressing subgroups. In the EGFR-overexpressing subgroup, the mean OS was 18 months and the median was 13.2 months, while the mean PFS was 9.8 months and the median was 6.5 months. In the non-EGFR overexpressing subgroup, the mean OS was 22 months, the median is 14 months, and the mean PFS was 12 months while the median was 7 months (Figure 12). While there is a slight trend for lower median and mean survival in the EGFR overexpressing compared to the non-EGFR overexpressing subgroup, this proved to be non-significant (log-rank *p-value* of 0.1526 for OS and 0.1540 for PFS) (Figure 12). When assessing patient survival within the different radiation regimens, we found that patients with no EGFR overexpression had a survival advantage for PFS within the 60Gy/30 Fr subgroup compared to the EGFR overexpressing patients within the same subgroup (n= 32; Log-rank *p-value* = 0.0357) (Figure 13). Within the other radiation regimens and for OS, however, we found no association between EGFR expression and survival time ($p > 0.05$) (Figure 13). We also compared survival outcomes to EGFR overexpression in just the 60 Gy/30 Fr (ConvRT) and 60 Gy/20 Fr (HFRT) regimens but it was not significant (Figure 14). Similarly, we compared all markers, age, and KPS score data to EGFR expression but found no significance in survival outcomes (data not shown).

2.3.5 Figures

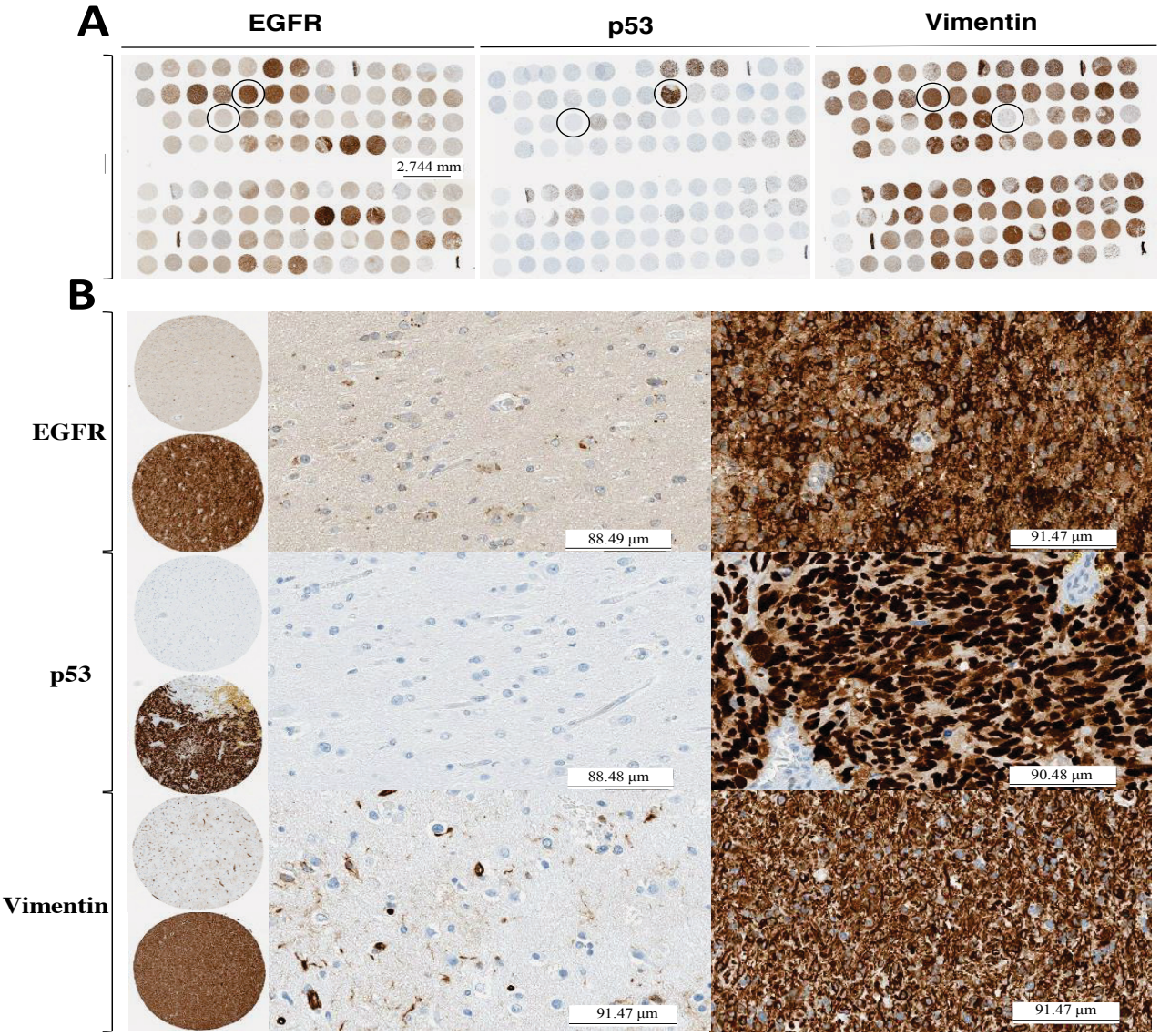

Figure 9. Tissue Microarray (TMA). Tissue samples were collected from 201 GBM patients at surgery A) Triplicate cores from each patient's tumour tissue were constructed into a TMA B) Magnification of H&E staining in each core for EGFR, p53, and Vimentin. Representative images of staining to score high expression (right side) versus non-expression (left side) of EGFR, p53, and vimentin.

	Patients	
	n=201	**%**
Age		
<65	126	62.7
≥65	75	37.3
Sex		
Males	117	58.2
Females	84	41.8
Tumour		
Primary	183	91.0
Secondary	16	8.0
n/a	2	1.0
Focality		
Unifocal	191	95.0
Multifocal	10	5.0
KPS		
<70	19	9.5
≥70	127	63.2
n/a	55	27.4
Extent of Surgery		
GTR	61	30.3
STR	133	66.2
n/a	7	3.5
RT		
Yes	166	82.6
No	26	12.9
n/a	9	4.5
Radiation Regimen		
60 Gy/30 Fr	77	46.4
60 Gy/20 Fr	41	24.7
40 Gy/15 Fr	22	13.3
Other	14	8.4
n/a	12	7.2
Chemotherapy		
Yes	136	67.7
No	53	26.4
n/a	12	6.0
Recurrence		
Yes	141	70.1
No	43	21.4
n/a	17	8.5
Repeat Surgery		
Yes	67	33.3
No	109	54.2
n/a	25	12.4

Table 1. Patient Characteristics. Summary of clinical characteristics of patients included in the TMA (n=201). Abbreviations: *GTR* Gross total resection, *STR* Subtotal resection *Gy* Gray *Fr* Fraction

	Patients	
MGMT	**n=201**	%
Unmethylated	72	35.8
Methylated	71	35.3
n/a	58	28.9

Table 2. *MGMT* Methylation Status. Information from patient pathology reports used for diagnostic purposes.

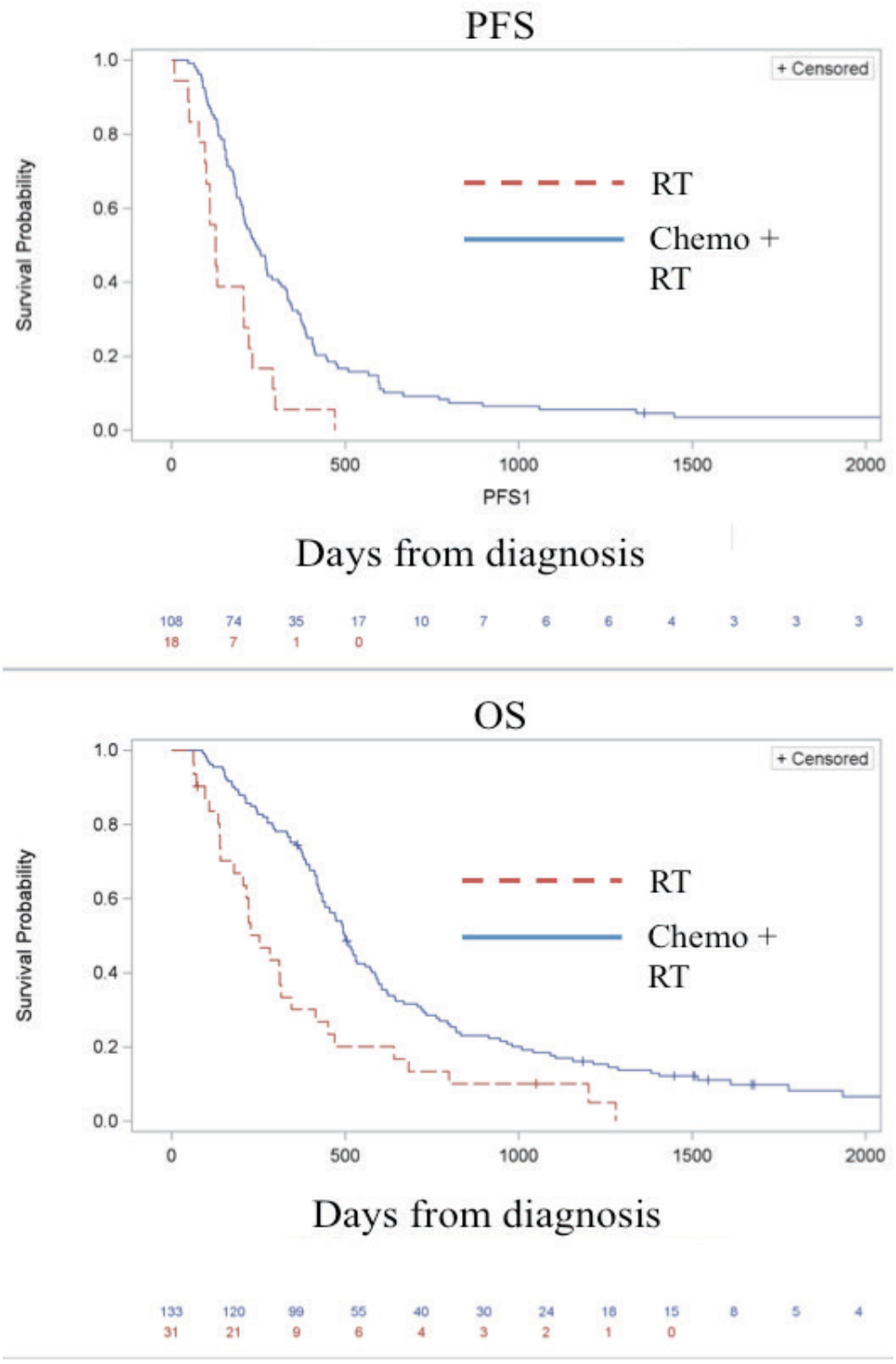

Figure 10. Kaplan Meier Curves Comparing Type of Treatment to Survival. Significant relationship between RT versus RT + Chemo in survival. For PFS, median survival for RT: 4.2 months. Median survival for RT + Chemo: 8.1 months (n=126; Log-rank *p-value* = 0.0002). For OS, median survival for RT: 9.1 months. Median survival for RT + Chemo: 16.7 months (n=164; Log-rank *p-value* = *0.0002).* Abbreviations: *RT* Radiation therapy *Chemo* Chemotherapy

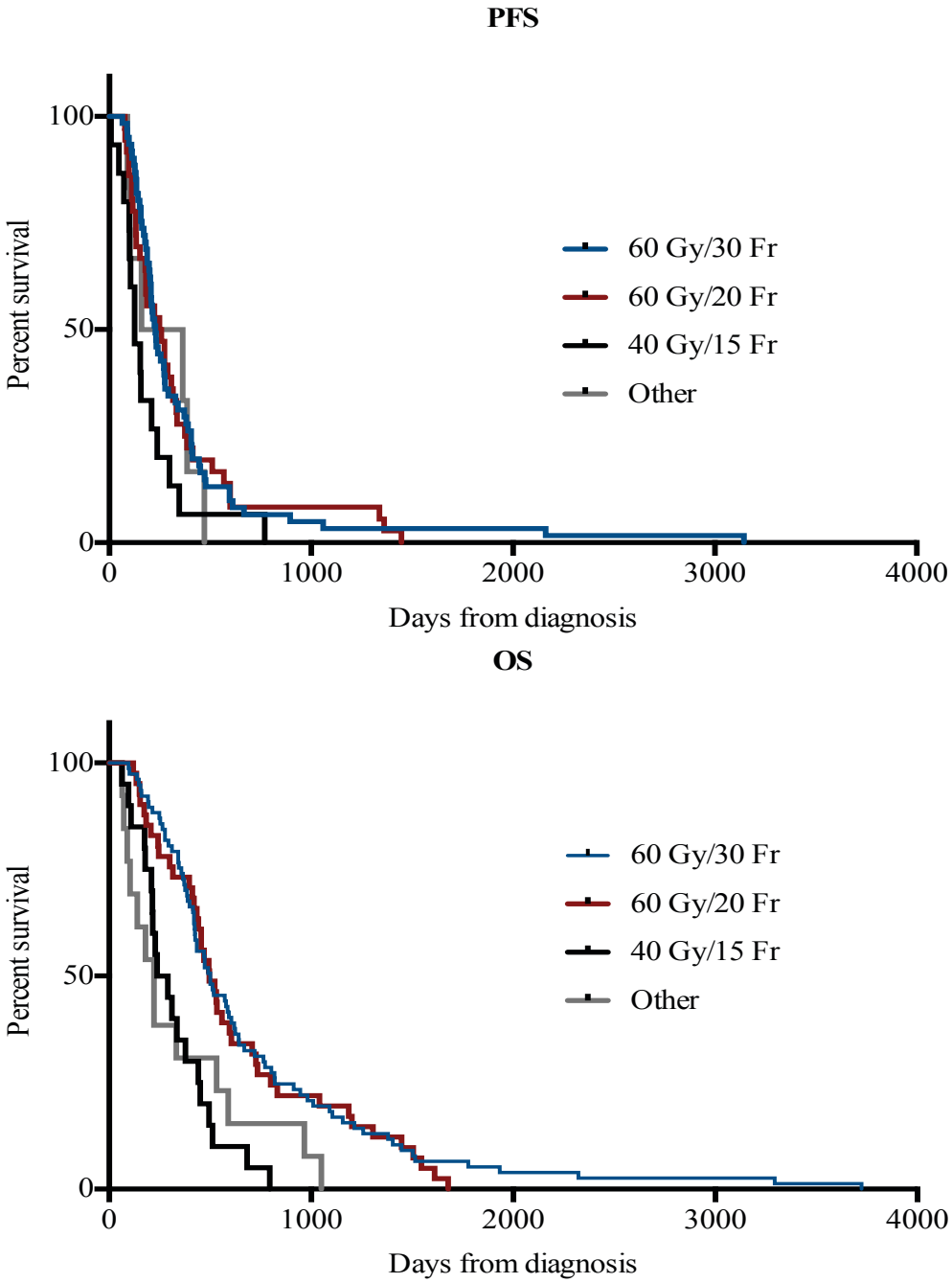

Figure 11. Survival in Different RT Regimens. For PFS, median survival for 60Gy/30 Fr: 228 days; 60Gy/20 Fr: 253 days; 40 Gy/15 Fr: 126 days; Other regimens: 262 days (n=118; Log-rank *p*-value = 0.0944). OS was found to correlate to different radiation regimens. For OS, median survival for 60 Gy/30 Fr: 501 days, 60 Gy/20 Fr: 496 days, 40 Gy/15 Fr: 264 days, Other regimens: 222 days. (n=151; Log-rank *p-value* <0.0001). Abbreviations: *Gy* Gray *Fr* Fractions

	Progression Free Survival			Overall Survival		
	Hazard Ratio	*95% CI*	*p-value*	Hazard Ratio	95% CI	*p-value*
Radiation Regimen						
60 Gy/30 Fr	1			1		
60 Gy/20 Fr	0.86	(0.53-1.39)	0.5515	0.76	(0.47-1.23)	0.2720
40 Gy/15 Fr	1.37	(0.65-2.88)	0.4048	0.77	(0.38-1.54)	0.4646
Other Regimens	0.48	(0.15-1.53)	0.2209	0.85	(0.34-2.11)	0.7266
KPS						
<70	1			1		
≥70	0.31	(0.16-0.63)	0.0012	0.51	(0.26-0.98)	0.0453
Age						
<65	1			1		
≥65	1.12	(0.63-1.98)	0.6881	2.11	(1.26-3.53)	0.0044
Extent of surgery						
Total	1			1		
Subtotal	0.62	(0.39-0.97)	0.3830	0.74	(0.48-1.14)	0.1832
Chemotherapy						
No	1			1		
Yes	0.43	(0.22-0.86)	0.0177	0.50	(0.25-0.99)	0.0496
Repeat surgery						
No				1		
Yes				0.57	(0.37-0.87)	0.0101

Table 3. Adjusted PFS/OS Multivariate Analysis. For OS, higher age significantly correlated to lower survival (HR 2.11; CI, 1.26-3.53; *P*=0.0044), with having a higher KPS also correlating to having a better OS (HR 0.51; CI, 0.26-0.87; *P=0.0453)*. Having chemotherapy was correlated to better OS (HR 0.50; CI, 0.25-0.99; *P*=0.0496) as was having a repeat surgery (HR 0.57; CI, 0.37-0.87; *P*=0.00101). For PFS, having chemotherapy was correlated to a better PFS than not having chemotherapy (HR 0.43; CI, 0.22-0.86; *P*=0.00177), as was having a higher KPS (HR 0.31; CI, 0.16-0.63; *P*=0.0012).

	Patients	
	n=201	%
EGFR Overexpression	87	43.3
No EGFR Overexpression	111	55.2
n/a	3	1.5

Table 4. IHC Expression of EGFR in GBM Patients. EGFR staining was scored based on the intensity of staining and the number of stained cells in all three cores. 3 tissue samples were unable to be scored due to folded or missing cores (marked as n/a) (n=201).

***EGFR* Amplification**	**EGFR Protein Expression (IHC Score)**				
	0	**1+**	**2+**	**3+**	**Total**
No	31	1	4	2	38
Yes	12	1	7	24	44
Total	43	2	11	26	82

Table 5. Correlation between *EGFR* Amplification and EGFR Overexpression. *EGFR* amplification was determined by fluorescent in situ hybridization (FISH) and EGFR expression was determined immunohistochemically. IHC scores of 2+ and 3+ are considered to be EGFR-overexpressing. Correlation of EGFR overexpression to *EGFR* amplification is significant (*P<0.0001* by chi-square test) (n=82).

IHC Protein Expression (n=201)												
	IDH1(mt)		**PTEN**		**p53**		**Vimentin**		**Ki67**		**CD44**	
	Patients	**%**	**Patients**	**%**	**Patients**	**%**	**Patients**	**%**	**Patients**	**%**	**Patients**	**%**
-	187	93.0	178	88.6	159	79.1	22	10.9	74	36.8	19	9.5
+	12	6.0	16	8.0	28	13.9	171	85.0	117	58.2	174	86.6
n/a	2	1.0	7	3.5	14	7.0	8	4.0	10	5.0	8	4.0

Table 6. IHC Expression of IDH1, PTEN, p53, Vimentin, Ki67, and CD44. Scoring of markers commonly expressed in GBM. Tissue samples that were unable to be scored due to folded or missing cores are marked as n/a. IDH1 (mt) and PTEN were scored as neg/pos and the rest of the markers as high/low expression.

	CD44			p53			Vimentin		
	Low	**High**	***p-value***	**Low**	**High**	***p-value***	**Low**	**High**	***p-value***
EGFR -	13	93		80	21	0.0157	14	93	
EGFR+	5	81	0.1273	79	7		7	78	0.2850
Total	18	174		159	28		21	171	

	IDH1-mutant			Ki67			PTEN		
	Neg	**Pos**	***p-value***	**Low**	**High**	***p-value***	**Neg**	**Pos**	***p-value***
EGFR-	99	12		47	58		97	12	
EGFR+	87	0	0.0016	27	59	0.0592	81	4	0.1133
Total	186	12		74	117		178	16	

Table 7. Correlation of EGFR to Other Markers. EGFR + denotes EFGR overexpression; EGFR– denotes no EGFR Overexpression. IDH1-mutant and p53 expression were negatively correlated to EGFR overexpression (*p<0.05).* Chi square tests were used for correlation statistics (n=201).

	EGFR Overexpression		No EGFR Overexpression		*P-value*
	Number	**%**	**Number**	**%**	
60 Gy/30 Fr	32	19.6	43	26.4	
60 Gy/20 Fr	18	11.0	22	13.5	
40 Gy/15 Fr	12	7.4	10	6.1	0.696
Other	5	3.1	9	5.5	
n/a	6	3.7	6	3.7	

Table 8. Radiation Regimen Received per EGFR Expression. 163 patients in the population received RT with known EGFR expression status. Abbreviations: *Gy* Gray *Fr* fractions

	EGFR Overexpression		No EGFR Overexpression		*P value*
	Number	**%**	**Number**	**%**	
Age					
<65	55	27.8	68	34.3	0.778
≥65	32	16.2	43	21.7	
Sex					
Male	46	23.2	68	34.3	0.236
Female	41	20.7	43	21.7	
KPS					
<70	10	5.1	9	4.5	0.634
≥70	58	29.3	66	33.3	
n/a	19	9.6	36	18.2	
Extent of Surgery					
Subtotal	60	30.3	72	36.4	0.675
Total	25	12.6	34	17.2	
n/a	2	1.0	5	2.5	
Chemotherapy					
Yes	57	28.8	77	38.9	0.358
No	26	13.1	26	13.1	
n/a	4	2.0	8	4.0	
Recurrence					
Yes	62	31.3	77	38.9	0.751
No	18	9.1	25	12.6	
n/a	7	3.5	9	4.5	
Repeat Surgery					
Yes	27	13.6	38	19.2	0.676
No	50	25.3	59	29.8	
n/a	10	5.1	14	7.1	
MGMT					
Unmethylated	35	17.7	36	18.2	0.267
Methylated	28	14.1	42	21.2	
n/a	24	12.1	33	16.7	

Table 9. Patient Characteristics in Relation to EGFR Expression. Patients were separated by characteristics and EGFR overexpression. None of the characteristics are correlated to EGFR overexpression (*p value* > 0.05).

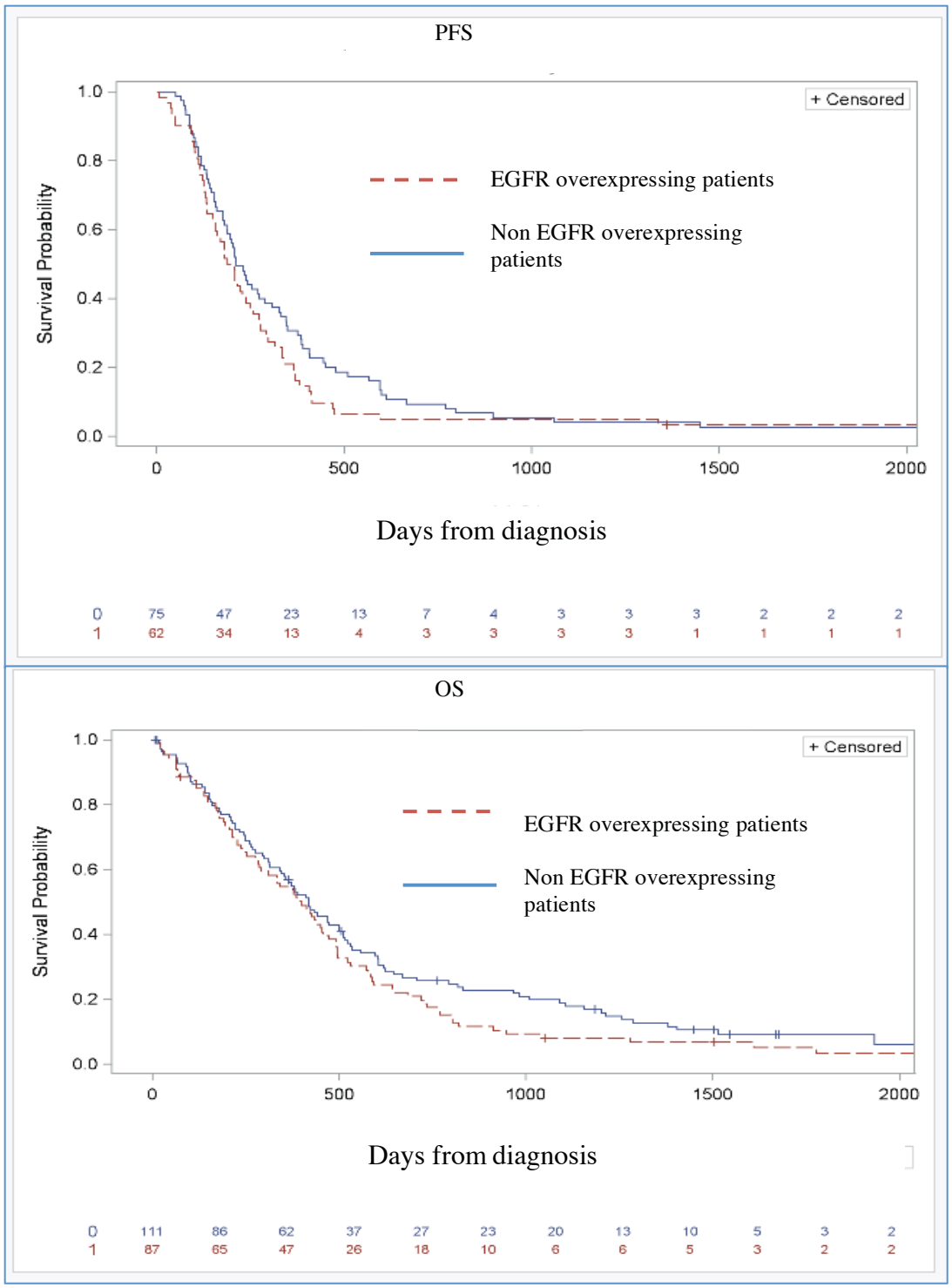

Figure 12. Survival Outcome with EGFR Overexpression. No significant survival difference was found between EGFR overexpressing and non-overexpressing patients. For PFS, median survival for EGFR overexpressing patients: 6.5 months. Median survival for non EGFR overexpressing patients: 7.0 months (n=137; Log-rank *p-value* = 0.1540). For OS, median survival for EGFR overexpressing patients: 13.2 months. Median survival for non EGFR overexpressing patients: 14.0 months (n=198; Log-rank *p-value* = 0.1526).

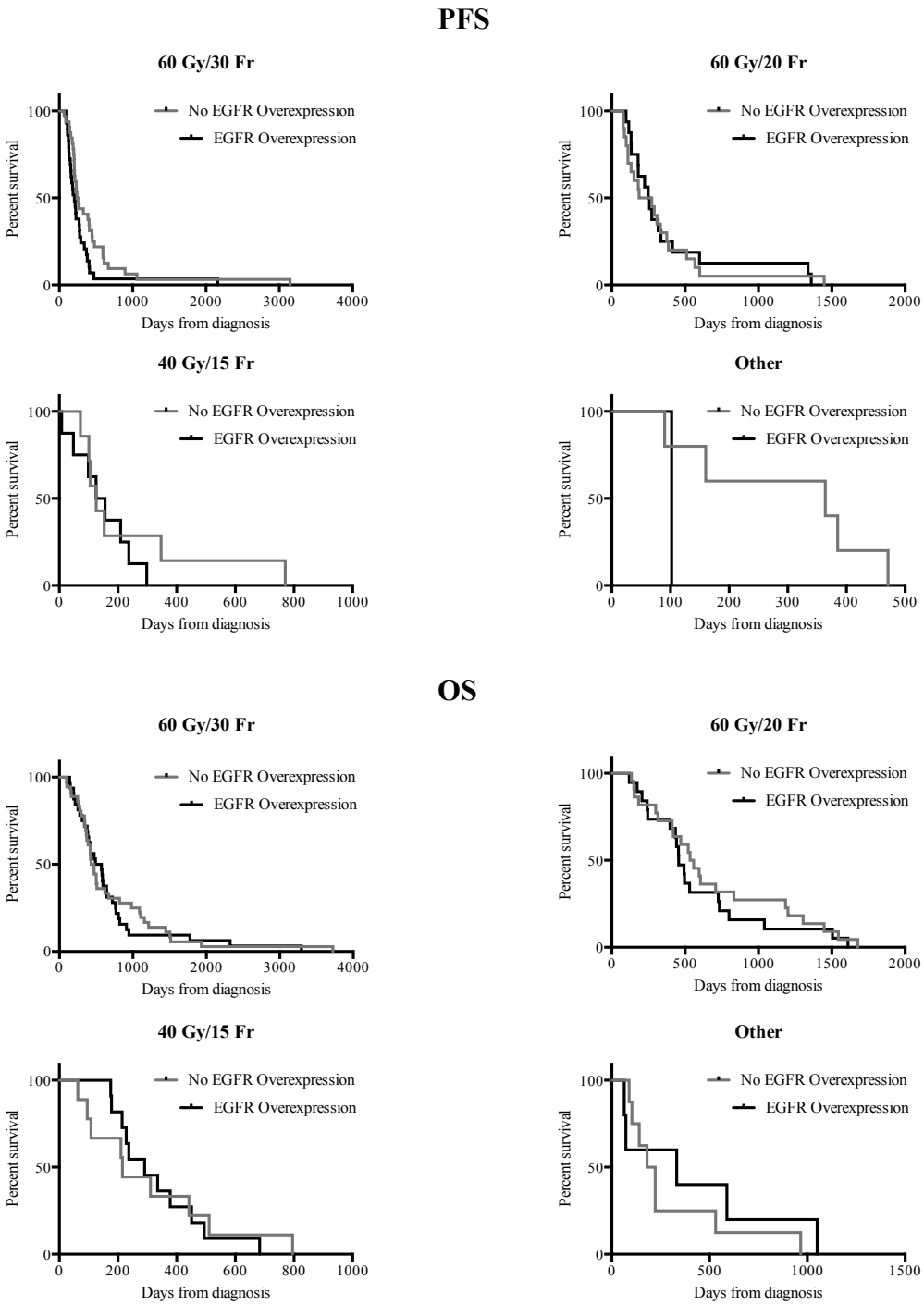

Figure 13. Survival and EGFR Overexpression within Different RT Regimens. Kaplan Meier survival outcomes for all patients that received radiation treatment with known EGFR expression is shown (n=151). Patients with no EGFR overexpression were found to have a survival advantage for PFS within the 60Gy/30 Fr subgroup (median survival at 248 days versus 206 days for the EGFR overexpressing patients within the subgroup) (n= 32; Log-rank *p-value* = 0.0357). No significant correlation between EGFR expression was found within any other radiation regimen.

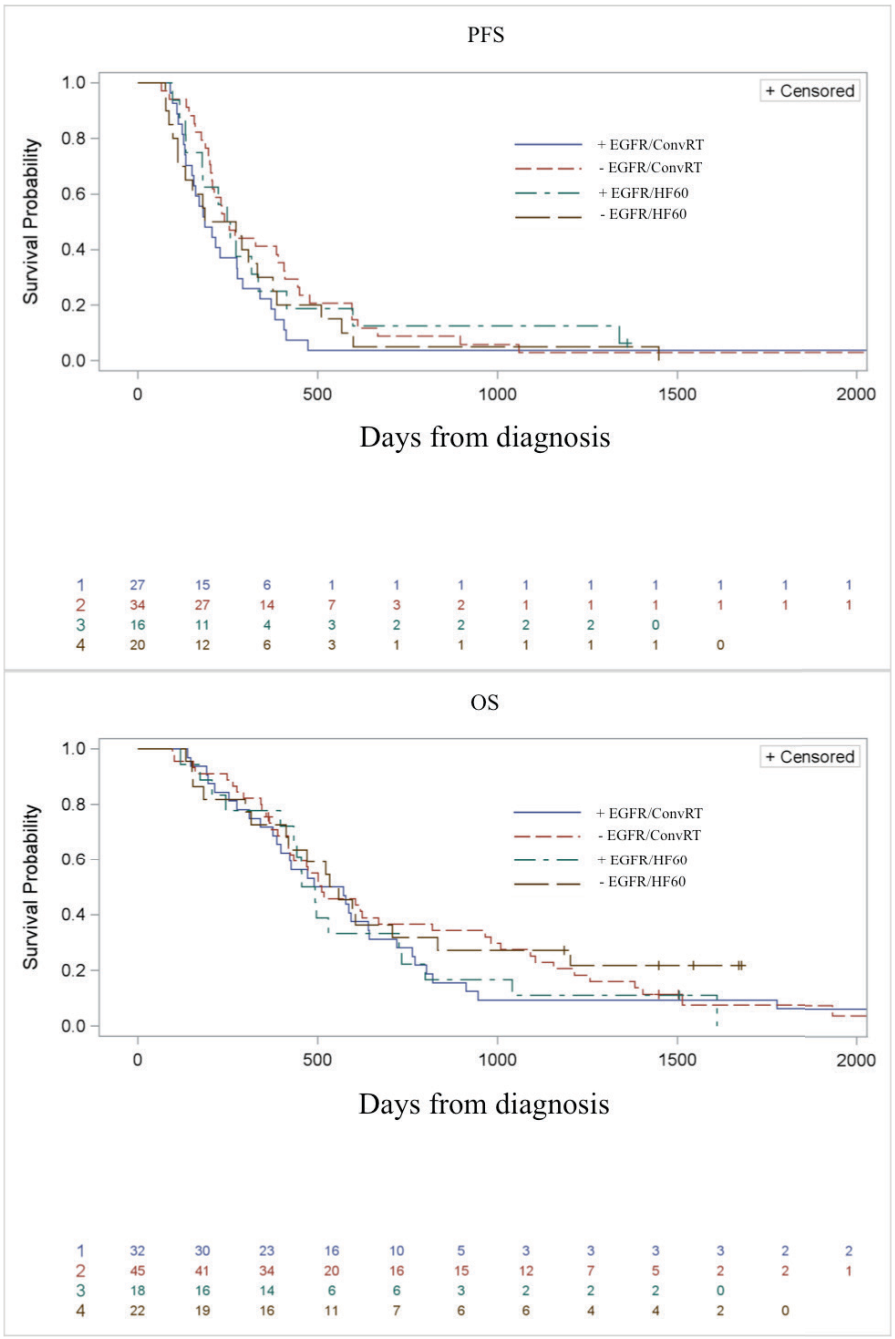

Figure 14. Survival and EGFR Overexpression in Two RT Regimens. No significant relationship was found. +EGFR represents overexpression and –EGFR represents no overexpression. For PFS, median survival for + EGFR/ConvRT: 6.2 months; for - EGFR/ConvRT: 8.2 months; for + EGFR/HF60: 8.4 months; for - EGFR/HF60: 7.6 months (n=137; Log-rank *p-value* = 0.2804). For OS, median survival for +EGFR/ConvRT: 17.7 months; for - EGFR/ConvRT: 17 months; for + EGFR/HF60: 15.8 months; for - EGFR/HF60: 7.6 months (n=198; Log-rank *p-value* = 0.5870). Abbreviations: *ConvRT* Conventional RT (60 Gy/ 30 Fr) *HF60* Hypofractionated RT (60 Gy in 20 Fr).

2.4 Discussion

EGFR and Resistance to Therapy

RT has been a constant in GBM treatment for over thirty years despite the fact that GBM is a highly radioresistant tumour. Strategies to lessen this effect are critical for the treatment of this disease. As of yet, not treatment modalities confer a bigger survival benefit than the standard of care consisting of surgery, RT and chemotherapy with the alkylating agent TMZ despite major improvements in imaging techniques, RT, and chemotherapy techniques [2]. Years of research have focused on understanding the biology behind this disease in the hopes of finding new therapies and on strategies to reduce the treatment resistance in GBM. Despite the fact that the main signaling pathways (p53, tumor suppressor retinoblastoma pRB, PI3K/Akt/mTOR, Ras/MAPK, and STAT3 cascades) and the genetic aberrations involved in GBM tumorigenesis have been well described, newly developed GBM therapies have been mostly disappointing [6, 52, 57]. Since EGFR overexpression is involved in common tumorigenic and radioresistance-conferring pathways including the PI3K, Ras, and STAT3 pathways, multiple therapies (gefitinib, erlotinib, lapatinib) have been developed to target and inhibit EGFR in the hopes of down-regulating these pathways. Unfortunately these trials have had disappointing results likely due to the redundancy and diversity in the EGFR signaling cascade, as the inhibition of EGFR does not block its major signaling cascades [6, 81]. To overcome GBM's resistance to treatment, however, phase I/II trials have examined the effect of adding an EGFR-inhibitor to the standard therapy of RT and TMZ, some with promising results [100]. The addition of EGFR-inhibitors to the standard approach represents an attractive strategy that is currently being investigated by multiple groups.

Another way to tackle the issue of treatment resistance in GBM is by stratifying patients based on treatment outcome. GBM is an extremely heterogeneous disease, which likely causes the variability in treatment outcomes. Stratification of patients based on gene or protein expression may be useful in determining which patients will incur the greatest survival benefit from GBM therapies and which is the optimal treatment regimen for a given patient. In our study we examined the expression of EGFR, one of the most commonly overexpressed proteins in GBM, and its relation to survival in different radiation regimens. Since EGFR has been found to play a role in radioresistance through the PI3K pathway and/or DNA-PKcs activation and has been shown to become activated when exposed to irradiation, we wanted to explore its

relationship to different radiation regimens and observe whether overexpression had an effect on survival. As previous studies have found that EGFR overexpression within different treatment regimens confers different survival outcomes, we hoped to elucidate a fractionation schedule that provided a better survival outcome to a subgroup of patients based on EGFR expression [76, 97]. In our cohort of 201 GBM patients, we found a significant survival advantage for PFS in the conventional radiation regimen subgroup for patients that did not have EGFR overexpression compared to those that did. The number of patients in this subgroup, however, was small (n=30) and we did not find any difference in survival for OS within any radiation regimen with respect to EGFR overexpression. Other factors, such as age, KPS, or treatment could partly explain the PFS survival difference found within the conventional radiation regimen subgroup for EGFR overexpression. Additionally, we did not find any survival differences by looking at the expression patterns of other markers with relation to EGFR overexpression in different radiation regimens (data not shown). Since the 40 Gy/15 Fr subgroup had a lower survival than the conventional (60 Gy/30 Fr) and hypofractionated regimen of 60 Gy/20 Fr, we compared the relationship between EGFR overexpression and survival in the two regimens. Again, however, we found no significant relationship. One possibility for this lack of association between EGFR expression and radiation regimens in survival could be due to the heterogeneity of our population and differences in treatment that were not accounted for. Since we included patients treated for GBM from 2001 to 2012, a portion of our patients were not given the primary GBM treatment consisting of RT and concurrent and adjuvant TMZ since the standard of care was only implemented in 2005. These patients were often given other chemotherapeutic agents such as BCNU or procarbazine [2]. Similarly, some of our patients treated after 2005 were enrolled in clinical trials and given other treatment agents such as Irinotecan, Everolimus, or Avastin. This could affect the survival outcomes of the patients within the same RT regimen groups. Another reason for the overall lack of association between EGFR and survival with respect to different radiation regimens could be due to the fact that nuclear EGFR was not assessed in our study, as nuclear EGFR has been found to be induced by RT and play a role in resistance to therapy [69, 101].

In our cohort, we found that the fractionated radiation regimen of 40 Gy/15 Fr conferred a significantly lower survival outcome than the 60 Gy/30 Fr and 60 Gy/20 Fr regimens. This has been found in other studies and is likely due to the fact the the 40 Gy/15 Fr is often given to

patients with limited tumour resection and a lower KPS score, factors that have been shown to confer a lower patient survival, to lower their overall treatment time [47]. In a study that retrospectively controlled for the selection bias of elderly GBM patients in the hypofractionated radiation group compared to the standard fractionation schedule, Arvold et al. (2015) found that both regimens conferred similar survival when also given concurrent TMZ. The lower survival outcomes that we observed in the fractionated regimen is likely due to the selection bias of elderly and worse performance status patients [42]. Since hypofractionated radiation regimens likely confer no worse survival outcomes or increase toxicities compared to the conventional regimen, the possible benefit of hypofractionated radiation regimens within subgroups of patients remains to be investigated. Similarly, it has been observed that RT can induce damage to non-tumor cells; a phenomenon deemed the "bystander effect". Through multiple pathways including microenvironmental signaling and cytokine cellular toxicities, irradiation is able cause DNA damage to neighboring non-targeted cells and induce genomic and molecular instabilities in these cells [38]. Since the hypofractionation regimen of 60 Gy/20 Fr delivers the same dose as the conventional regimen but in a shorter treatment time and confers the same survival as the conventional regimen, the role of the bystander effect in different regimens warrants further studies.

Population Characteristics

By comparing the survival outcomes between patients that received only RT versus those that received RT with concurrent chemotherapy, we found a significant survival benefit with the multimodal treatment. Our cohort confirmed the established finding that RT and chemotherapy is more effective than RT alone as published by multiple groups [2, 102]. *MGMT* methylation, which is considered a prognostic factor for treatment outcome with TMZ in GBM, was methylated in 50% of the 173 cases with available *MGMT* methylation information from patient records. This is similar to what has been reported of approximately half of all GBMs exhibiting *MGMT* methylation [21].

Protein Expression

In our cohort of GBM patients, we found that 43.3% of all tumours exhibited EGFR overexpression. This percentage is similar to other reports implying that our cohort had a representative EGFR expression profile as what is seen in all GBMs [51, 53]. We also found that

73% of *EGFR*-amplified tumours were found to also have EGFR overexpression, and 84% of the EGFR-overexpressed tumours also had *EGFR* amplification (*p<0.0001)*. This finding mirrors other studies that have found a strong correlation between EGFR overexpression and *EGFR* amplification [95]. Other markers, such as IDH1 positivity which represents IDH1 mutation was found at a lower proportion (6%) than what is found in most studies (10.0%) [103]. PTEN, a tumour suppressor that is often deleted due to loss of heterozygosity (LOH) in GBM and has been considered a prognostic marker for survival, was found to be expressed in only 8.0% of our cohort. Other studies have found that PTEN is deleted in 50%-70% of GBM cases [104].

When EGFR expression was compared to expression of each marker, we found that both IDH1-mutant and p53 expression were negatively correlated to EGFR overexpression. The *IDH1* gene, which encodes an enzyme involved in the citric acid cycle, is often mutated in secondary tumours and is known to be a prognostic factor for favourable survival. Since *IDH1* mutations are commonly seen in low-grade gliomas and secondary GBMs, they are thought to be involved in the early events of glioma development and precede *TP53* mutations in grade II astrocytomas. Current evidence suggests that the most commonly mutated form of *IDH1,* which consists of a missense mutation of arginine to histidine, drives several oncogenic pathways through hypoxia-inducible factors (HIFs), post-translational modifications of collagen, and the maintenance of a hypermethalator phenotype. In primary GBMs, however, mutated IDH1 is rarely observed when other common genetic aberrations, such as EGFR, are present, leading to the idea that they are only involved in tumour initiation in secondary, and not primary GBM [103]. In our cohort, although the proportion of IDH1-mutant tumours was small, none of them had EGFR overexpression (*p<0.005).* We, along with other groups that assessed the IHC expression of EGFR and IDH1-mutant positivity, have validated the finding that mutated IDH1 and EGFR co-expression in GBM is rare [105].

Similarly to *IDH1* mutations, *TP53* mutations are involved in the early events of glioma development and are more commonly observed in secondary as opposed to primary GBMs. *TP53* encodes the tumor suppressor p53, which regulates the cell cycle and activates apoptosis or proliferative arrest when the cell has DNA damage or an unstable genome [106]. The negative correlation between p53 and EGFR overexpression that we found in our cohort has been validated in our studies that have found that the overexpression of the two proteins are mutually exclusive events in GBM development [96, 107].

Further analyses of our protein markers are needed to fully illustrate the prognostic value of co-expression of proteins within our cohort.

IHC-Assessed Subtypes

After The Cancer Genome Atlas (TCGA) provided a comprehensive view of the genomic alterations driving the tumorigenesis of GBM, multiple groups used this dataset to subdivide tumours into groups with common genomic abnormalities and different phenotypes [11]. Verhaak et al. (2010) used this dataset along with previously published datasets to subdivide the samples into four molecular defined subtypes: Classical, Proneural, Neural, and Mesenchymal [1]. Recently, Popova et al. (2014) used a TMA to immunohistochemically assess the expression of a portion of the proteins that have been identified in the subtyping of GBM. Although they were not able to compare each sample's IHC profile to its molecular signature, they found that the frequency of each protein subtype was consistent with the frequency of each subtype obtained using molecular techniques. The group also found that EGFR was the most significant protein for identifying a subtype of glioma using IHC [12]. Previous studies have also demonstrated that assessment of EGFR IHC is an accurate reflection of its molecular signature [79, 108]. EGFR IHC is therefore a useful tool to assess EGFR expression in tumours in TMAs. Additionally, an advantage of using a TMA to analyze a high number of tissue samples at one time is that it removes any experimental variability in the immunostaining protocol between samples since each sample is handled in the same way [109]. It is also economically feasible to assess thousands of patient samples at one time. In our study, we used the expression of EGFR, IDH1-mut, PTEN, CD44, and p53 and followed an algorithm based off the molecular subtypes established by Veerhaak et al. (2010) and similar to the IHC algorithm by Popova et al. (2014) to divide our patient samples into categories reminiscent of the molecularly defined subgroups, although we did not define the expression of proteins MERTK, PDGFRA, and OLIG2 as Popova et al. did we did add PTEN expression data (Figure 15).

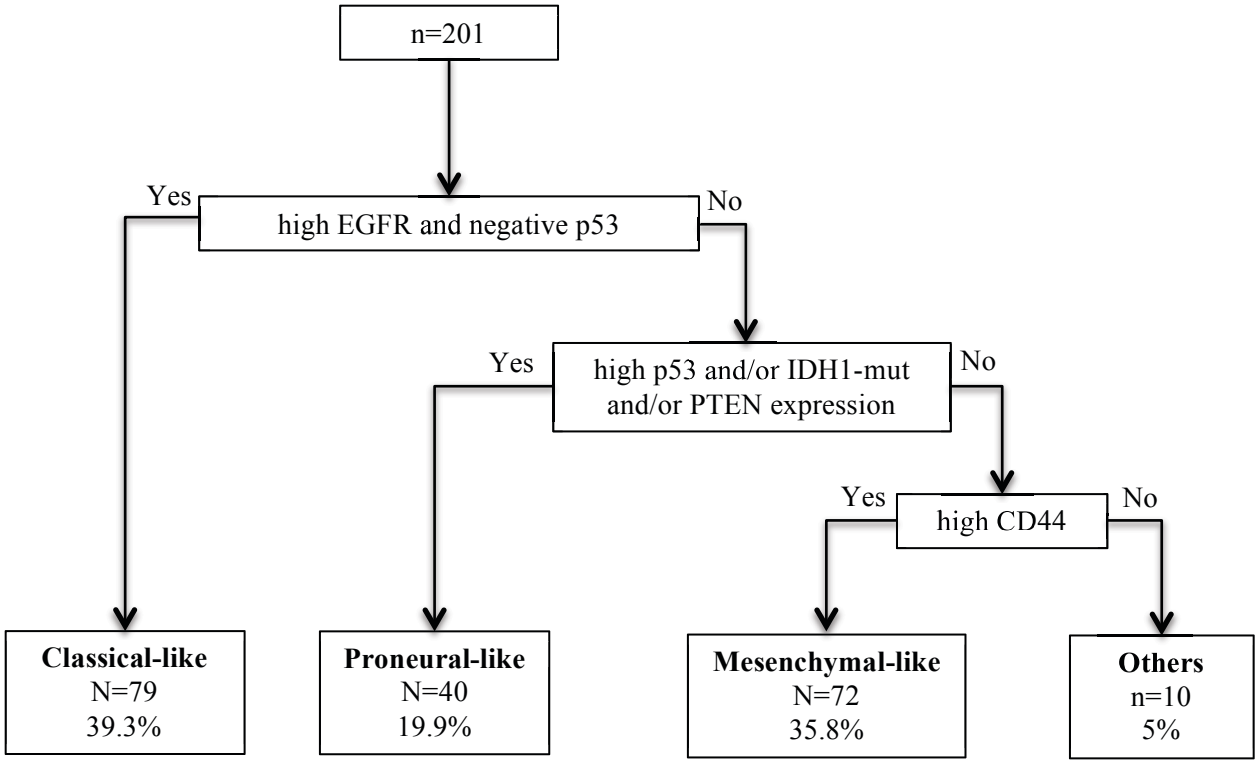

Figure 15. GBM IHC Grouping Based on Molecular Subtypes. Algorithm used in our study to separate GBM tumours into groups reminiscent of GBM molecular subtypes. Similar to the algorithm used by Popova et al. (2014).

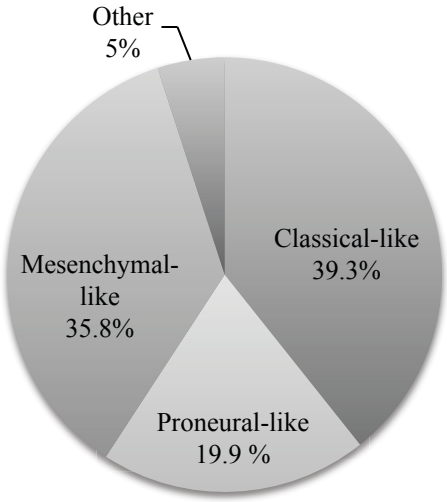

Figure 16. Histologically Defined Molecular Subtypes in GBM. Using expression data of EGFR, p53, PTEN, IDH1, and CD44, we subdivided our cohort of 201 tumours into three categories reminiscent of the previously molecularly defined subtypes: Classical (high EGFR expression and low p53 expression), Proneural (high p53 and/or IDH1 expression and/or PTEN expression) and Mesenchymal (high CD44 expression).

We found that 39.3% of our population had an IHC profile reminiscent of the Classical subtype, which was almost exactly the same as the proportion found by Popova et al (39.0%) (Figure 16). For the other subtypes, our proportions were similar but not exact. We had 35.8% of our population with IHC profiles reminiscent of the Mesenchymal subtype while Popova et al. had 29.0%. For the Proneural subtype, we only had 19.9% of our samples of reminiscent of the subtype whereas they reported 29% [12]. A possible reason for this discrepancy could be that we did not have expression data for OLIG2 and PDGFRA, which the group used to subdivide tumours into the Proneural-like subgroup. In another study that characterized 100 GBM tumour samples into subtypes reminiscent of Classical and Proneural molecular subtypes, Le Mercier et al. (2012) was able to differentiate between treatment outcomes by IHC-based profiling of EGFR, p53, and PDGRA and found that 37.6% of GBM tumours were classified in the Classical-like subtype and 60.2% in the Proneural-like subtype. The group found that adding TMZ to RT significantly improved survival for patients in Classical-like subtype but not the Proneural-like subtype [110]. Additionally, Holland et al. (2013) found that radiation triggered a shift from the proneural to the mesenchymal subtype, with mesenchymally shifted cells reported to be more radioresistant than other subtypes [50]. This offers a possible explanation to the radioresistance seen in GBM and has important implications for targeted therapies with respect to tumour subtypes. Although our study may be missing markers, specifically PDGFRA, to accurately subdivide tumours into molecular defined subtypes, IHC is a much more economical and feasible approach to subtype than the molecular approach and warrants further studies. Specifically, the assessment of treatment outcomes could be investigated on a large scale with IHC TMAs to determine whether certain subtypes have better outcomes in different treatment regimens.

EGFR Prognostic Value

Although we noticed a trend of decreased median and mean survival in EGFR-overexpressing tumours, there was no significantly different survival outcome between the two subgroups. While certain studies have found a prognostic value in EGFR or EGFRvII overexpression and *EGFR* amplification, many studies have found no difference in survival outcomes [78, 80, 111]. However, certain groups have found that when factors such as p53 expression, age, and survival time to further stratify patients, EGFR becomes a prognostic factor

[82]. Further stratification may be useful to uncover the complicated relationship EGFR may have with other markers.

We found that lower age, higher KPS, receiving chemotherapy, and having a repeat surgery all correlated to a better survival outcome, which has been reported in the literature [2, 9, 112]. While our study validates the finding that EGFR is not useful as a prognostic factor, further stratification of our patient population may lead to significantly different survival outcomes between EGFR-overexpressing and non-overexpressing patients and is worth exploring.

We acknowledge the limitations of this study, which include the heterogeneity of the cohort and possible heterogeneity of the TMA cores. Heterogeneity within GBM tumours is a hallmark of this disease [113]. This may hinder assessment of protein expression in TMAs since only a small part of the tumour is being scored. While we constructed TMAs with three cores per patient, it is possible that this was not representative of the tumour and could result in variable stratification. Similarly, different groups use different IHC cutoffs to study EGFR overexpression. While we defined EGFR overexpression through a commonly used scoring system based on the intensity of the staining (scored from 0-3), other groups have uses a 30% cell staining cutoff or consider immunopositivity of the EGFR antibody as EGFR overexpression [76, 79]. This could cause differences in tumours deemed EGFR overexpressing.

Chapter 3

3.1 Conclusions and Perspectives

While there has been a long debate as to whether EGFR is of prognostic value, our study found that in terms of overall and progression free survival, it is of no prognostic value. This finding was slightly surprising since EGFR is one the most commonly overexpressed proteins in GBM and is known to be an important factor in GBM tumorigenesis, though reports have validated this finding [111]. Although we noticed a slight trend in increased median and mean survival for patients with no EGFR-overexpression, this was not found to be significant. Since EGFR has been implicated in radioresistance, we investigated the relationship between different radiation regimens and survival outcomes with respect to EGFR overexpression to identify patients that would benefit more from specific regimens. Thus far, our analysis found a significant relationship between EGFR overexpression and lower survival in the conventional radiation regimen, although the sample size was small. Further analyses are needed to elucidate

the possibility that co-expression of EGFR with other GBM markers that are important for response to RT may be required for the identification of subgroups with differential response to different radiation regimens and treatment.

Despite aggressive therapy involving surgery and chemoradiation, the survival of GBM remains extremely dismal. Although there has been an extensive amount of research on the subject, no significant changes in therapy or survival outcome have occurred since the Stupp protocol was established eleven years ago [2].

In the last few years we have seen a shift in the understanding of cancer, and specifically of GBM, to a more molecular approach. GBM is now seen as a group of disease with multiple altered pathways and genes. This also led to a shift in attempts to develop targeted therapies to block pathways involved in the survival and proliferation of GBM cells, although thus far, no therapies have yet proven to be successful in clinical trials in GBM. In our study, we aimed to further analyze the role of RT as a previously well-established component of standard GBM treatment and stratify patients to identify those who may benefit most from a particular RT regimen.

TMA analysis is an efficient and feasible cost-effective way to assess the expression of different markers using IHC, based on available routine diagnostic settings. Although we did not find significant results from EGFR expression with respect to RT for survival outcomes, it is imperative to analyze additional expression patterns and survival outcomes in order to better stratify patients for optimal disease treatment.

Similarly, since it is known that most GBM tumours eventually recur and lead to death, we are currently designing a new TMA for paired-matched newly diagnosed and recurrent cases with a clinical annotated database for 67 patients from a single institution. We will examine the expression of different molecular markers for recurrent cases to investigate expression changes with respect to treatment and recurrence. Specifically, since RT has been shown to increase EGFR/EGFRvIII expression and increase EGFRvIII nuclear localization, we will analyze the expression of EGFR and EGFRvIII before and after radiation [114]. In a preliminary investigation of EGFR expression at recurrence, we found that 1 out of 13 recurrent patients (7.7%) shifted from non-EGFR overexpressing to EGFR overexpressing after chemoradiation at recurrence. We hope to investigate this finding in a larger cohort of patients and examine protein expression to further elucidate the role of treatment, and specifically RT, in tumour protein

profiles.

In summary, our study established a TMA with a clinically annotated database for 201 newly diagnosed GBM patients. This will be of a great value for stratification of GBM patients who may derive benefit from a tailored radiation regimen using cost-effective protocols for the implementation of an IHC-based molecular signature. Further investigation is currently underway to assess the prognostic value of EGFR overexpression in radioresistance with respect to other clinical and histopathological variables. Assessing the role of EGFR and other biomarkers will hopefully guide therapy developments and aid in selecting patients who will benefit most from different treatments, leading to a better survival outcome for those diagnosed with this devastating disease.

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