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ERK5 Expression in Brain Tumours

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Abstract

Due to the highly invasive nature of glioblastoma multiforme (GBM), one way to combat this disease is to focus on its mechanism of progression and on effectively inhibiting the signalling pathways that control cell proliferation. There is increasing evidence of the role of the MEK5-ERK5 pathway in cancer progression. However, there is a lack of studies investigating the role of ERK5 in glioblastoma and other brain tumours. Exploring the molecular mechanisms that advances glioblastoma cell proliferation may help in identifying potential therapeutic targets. This study examines the expression of ERK5 in GBM and other types and grades of brain tumours. In addition, this study determines whether there exist differences in ERK5 expression between tumour grades, higher vs lower grades, gender, and age. *I hypothesize, that the expression of ERK5 positively correlates with higher grade brain tumour progression.*

ERK5 expression was analyzed by immunohistochemistry in 69 brain tumour tissue samples and a brain tumour tissue microarray (TMA) containing 80 patient samples, The expression of ERK5 was examined using a microscope, and the immunohistochemical reactivity for ERK5 was evaluated and scored according to the percentage of staining. The data was then analyzed by statistical analysis.

This study shows that highly invasive brain tumours, such as GBM, display a high level of expression of ERK5. Furthermore, ERK5 expression appears to increase with advancing tumour stage. My data supports glioblastoma as one of the types of cancer that display abnormal MEK5/ERK5 signalling. Hence, indicating that targeting the MEK5/ERK5 pathway for future targeted therapies to combat glioblastoma may have some potential. However, there is still much that is unknown and has yet to be explored.

Keywords

ERK5, MAPK Signalling pathway, Glioblastoma, Astrocytoma, Brain Tumours. Brain tumour progression

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"All praise is for Allah by whose favor good works are accomplished." There is no God but One God and Prophet Muhammad pbuh is His Messenger. May Allah sw accept.

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Abbreviations

BBB	Blood–Brain Barrier
BDNF	Brain-Derived Neurotrophic Factor
BMK1	Big Mitogen Kinase 1
BPH	Benign Prostatic Hypertrophy
CA	Constitutively Active
CaP	Prostate Cancer
CBTRUS	Central Brain Tumour Registry of the United States
CDKs	Cyclin-Dependent Kinases
CNS	Central Nervous System
CRIC	Clinical Research Impact Committee
DAB	3, 3'-Diaminobenzidine
DCC gene	Deleted-in-Colorectal-Carcinoma gene
DN	Dominant Negative
EGFR	Epidermal Growth Factor Receptor
EMT	Epithelial-Mesenchymal Transition
ERK5	Extracellular-Regulated Protein Kinase 5
F	Female
FGF	Fibroblast Growth Factor
GBM	Glioblastoma Multiforme

GFAP	Glial Fibrillary Acidic Protein
H&E	Hematoxylin and Eosin
H20	Water
H202	Hydrogen Peroxide
HCC	Hepatocellular Carcinoma
IDH Wildtype	Isocitrate Dehydrogenase
IHC	Immunohistochemistry
LHSC	London Health Sciences Centre
LIF	Leukaemia Inhibitory Factor
LOH	Loss of Heterozygosity
M	Male
MAPK	Mitogen Activated Protein Kinase
MAPKK	Mitogen Activated Protein Kinase Kinase
MAPKKK	Mitogen Activated Protein Kinase Kinase Kinase
MGMT	O^6 -Methylguanine-DNA methyltransferase
miRNA	MicroRNA
MRI	Magnetic Resonance Imaging
NaOH	Sodium Hydroxide
NGF	Nerve Growth Factor
NLK	Nemo-Like Kinase
NOS	Not Otherwise Specified

OS	Overall Survival
PBS	Phosphate Buffer Solution
PDGF	Platelet-Derived Growth Factor
PDGF-BB	Fibroblast Mitogen Platelet-Derived Growth Factor -BB
PTEN	Phosphatase and Tensin Homolog
P-value	Probability Value
RB	Retinoblastoma
REB	Research Ethics Board
RT PCR	Reverse Transcription Polymerase Chain Reaction
RTK	Receptor Tyrosine Kinase
STAT3	Signal Transducer and Activator of Transcription 3
TA	Tissue and Archive
TMA	Tissue MicroArray
TMZ	Temozolomide –Alkylating Agent
TNBC	Triple negative Breast Cancer
UTR	Untranslated Region
UWO	University of Western Ontario
VEGF	Vascular Endothelial Growth Factor
WHO	World Health Organiz

Chapter 1

1 Introduction to Gliomas and Astrocytic Tumours

1.1 Epidemiology and Etiology

Glioma is the most common primary neuroepithelial tumour that originates in the brain. Gliomas originate from progenitor of the glial cells that surround and support neurons in the brain, including astrocytes, oligodendrocytes and ependymal cells. Any tumour that arises from these supportive tissues is called a 'glioma'. Astrocytomas arise from the astrocytic cell lineage, oligodendrogliomas from the oligodendrial cell lineage, and ependymomas from the ependymal cell lineage respectively ("Low-Grade Astrocytoma: Background, Pathophysiology, Epidemiology" 2016). Please see (Figure 1) for a diagram of the three different types of glial progenitor cells found in the central nervous system (CNS) and the types of tumours they develop into. It is postulated that gliomas are formed with the progressive accumulation of genetic and/or epigenetic events, which causes cells to deviate from their normal growth mechanisms or are not destroyed by the immune system (Fisher, Schwartzbaum, and Wrensch, n.d.).

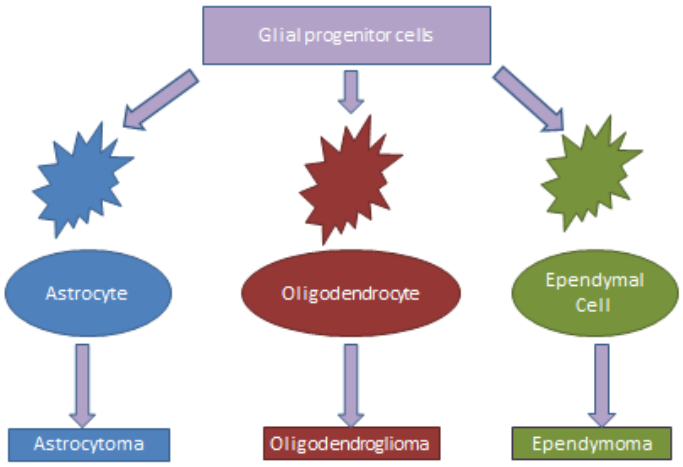


Figure 1 Cells of Origin. Glioma is a type of brain tumour that grows from glial cells. This figure shows the three different types of glial progenitor cells found in the Central Nervous System and the type of tumour they develop into.

Accounting for approximately half of all cases of intracranial neoplasms, primary brain tumours arise from CNS tissue. Metastatic lesions are the cause of the remaining cases of brain tumours. The occurrence of brain tumours in children vs adults differs in the location in which the tumour arises. For instance, in adults, two thirds of primary brain tumours arise from structures above the tentorium (supratentorial), whereas in children, two thirds of brain tumours arise from structures below the tentorium (infratentorial) ("Brain Neoplasms: Practice Essentials, Background, Pathophysiology" 2016). A diagram of the different types and locations of tumours can be found in (Figure 2), and the anatomy of the brain that outlines the supratentorial vs infratentorial areas can be found in (Figure 3).

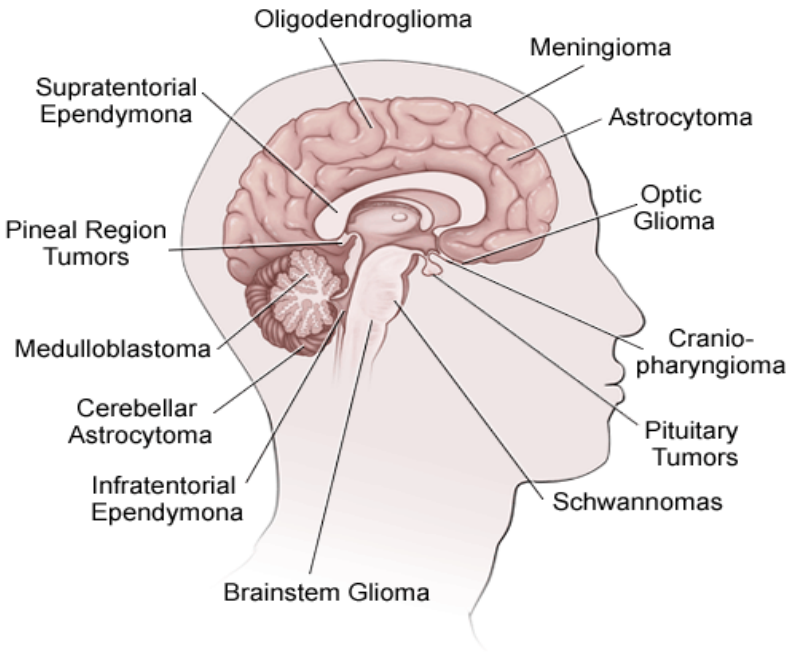


Figure 2 Location of different types of brain tumours. This diagram shows some different types of brain tumours and where they are commonly found in the brain. Glioblastoma, a grade IV tumour, belongs to a group of brain tumours known as astrocytomas.

Source: adapted from www.jvhealth.com

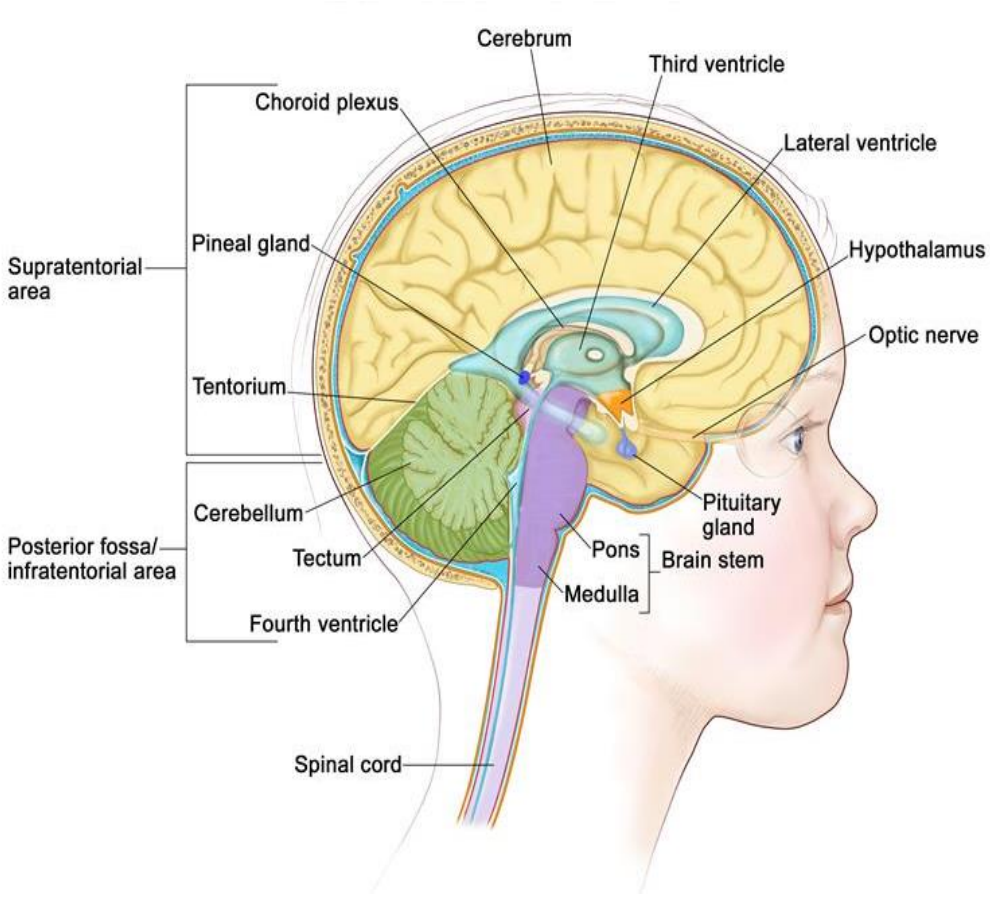


Figure 3 Anatomy of the brain. This figure is an anatomy of the brain in medial view. It shows the areas of the brain that are considered supratentorial and those that are infratentorial. In adults, most brain tumours arise in areas above the tentorium. In children, most brain tumours arise in areas below the tentorium.

Source: Adapted from (https://www.cancer.gov/images/cdr/live/CDR748659-750.jpg).

1.2 Grading of CNS Tumours

1.2.1 Grading across tumour entities

Histological grading is a method used to predict the biological behaviour of a tumour, and is a key factor when a clinician is selecting therapies in which to treat their patients. The WHO (World Health Organization) classification of tumours of the nervous system uses a grading scheme that ranges across a wide variety of tumours (Louis et al. 2007). Tumours are assigned a grade (I, II, III or IV) depending on the amount of abnormality seen in their cells: A lesion is assigned a Grade I if it has low proliferative potential and a possibility of cure following surgical resection alone. A lesion that is designated grade II is typically infiltrative in nature and often recurs. Some type II tumours, such as a low-grade astrocytoma, may progress to higher grades of malignancy, such as anaplastic astrocytoma and glioblastoma. A lesion that is designated grade III is typically assigned to tumours with 'histological evidence of malignancy, including nuclear atypia and brisk mitotic activity'. A lesion that is designated grade IV is assigned to 'cytologically malignant, mitotically active, necrosis-prone neoplasms typically associated with rapid pre- and postoperative disease evolution and a fatal outcome', such as a glioblastoma. Other characteristics of a grade IV tumour may include 'widespread infiltration of surrounding tissue' and a potential for the tumour to affect the craniospinal axis (Louis et al. 2016).

1.2.2 Tumour grade as a prognostic factor

There is a combination of different criteria used to predict a patient's response to therapy and outcome. This includes: clinical findings, for example the patient's age, neurologic status, and the location of the tumour; radiological features such as contrast enhancement; degree of surgical resection; proliferation indices; genetic alterations, and WHO grade. These criteria are all taken into account when prognosis is determined for a particular tumour. In spite of these factors, patients with WHO grade II and III tumors typically survive over 5 years and 2-3 years respectively. Survival outcomes for patients with a grade IV tumour largely depends the treatment regimens that are available but are generally worse than grade III tumours (Louis et al. 2007).

1.2.3 2016 WHO Classification of Tumours of the CNS

The World Health Organization has released an updated version of the "WHO Classification of Tumours of the Central Nervous System". In a meeting held in Haarlem, Netherlands in 2014, a working group of

Neuropathologists established guidelines for how to incorporate molecular findings into brain tumour diagnoses. These guidelines played a role in the major revision of the 2007 CNS WHO classification, which are now reflected in the updated 2016 CNS WHO. The new update uses molecular parameters in addition to histology to characterize numerous tumours. The update introduces newly documented tumours and removes older tumours whose entities, variants and patterns no longer have diagnostic and/or biological relevance. This revised classification includes a recommendation of genes of which routine testing is clinically useful. The 2016 CNS WHO contains major revisions of the diffuse gliomas, medulloblastomas and other embryonal tumours, while also outlining new entities that are defined by both histology and molecular features. For example, these include glioblastoma, IDH-wildtype and glioblastoma, IDH-mutant. Using integrated phenotypic and genotypic parameters for CNS tumour classification will add the objectivity that has been missing from some aspects of the diagnostic process in the past. This in turn will improve the diagnostic process overall, as well as improve patient management, and improve predictions for prognosis and response to treatment (Louis et al. 2016).

1.2.4 Diffuse Gliomas

The category of diffuse gliomas now includes the grade II and grade III astrocytic and oligodendroglioma tumours, which are now divided into IDH-mutant (the majority, if IDH testing is available), IDH-wildtype and NOS categories. The grade IV glioblastomas are now divided into (1) glioblastoma, IDH-wildtype (~90% of the cases, most of which are primary or de novo GBM); (2) glioblastoma, IDH-mutant (about 10 % of cases, most of which are the secondary GBM); and (3) glioblastoma, NOS (those GBM for which full IDH evaluation cannot be done). Epithelioid glioblastoma is a new variant that has been added, and is now under the same umbrella of IDH-wildtype glioblastoma, along with giant cell glioblastoma and gliosarcoma. In addition, glioblastoma with primitive neuronal component was added as a pattern in glioblastoma (Louis et al. 2016).

1.2.5 Nomenclature

The nomenclature of a tumour diagnosis now consists of the histopathological name, followed by the genetic features. For example, *glioblastoma, IDH-mutant*. If a tumour diagnosis has more than one genetic determinant, those are included in the name as well. An example of this is *oligodendroglioma, IDH-mutant and 1p/19q-codeleted*. The term wildtype is used for a tumour lacking a genetic mutation,

for example, *glioblastoma, IDH-wildtype*. A tumour is assigned NOS if there lacks sufficient information to assign a more specific code (Louis et al. 2016).

In the past, all astrocytic tumours had been grouped together. Now, all diffusely infiltrating gliomas (whether astrocytic or oligodendroglial) are grouped together based on their growth pattern, behaviours, and genetic mutations (Louis et al. 2016).

1.3 Glioblastoma Introduction, Pathogenesis and Etiology

GBM belongs to a group of brain tumours known as astrocytomas. Astrocytomas are divided into infiltrating (diffuse) and localized types. Grade I tumours are localized and have a potential for cure after surgical removal. However, the higher-grade tumours (grades II, III, and IV) are far more aggressive and more resistant to treatment. Of the primary brain tumours in adults, GBM is the most frequent and displays an 'aggressive biological behavior' by gradually invading other areas of the brain (Ray-Chaudhury 2010). When a glioma spreads across the corpus callosum into both hemispheres of the brain, the tumour resembles the appearance of a butterfly on a CT or MRI scan, and hence is called a 'butterfly glioma'. Although GBM usually involves the supratentorial compartment, any area of the central nervous system (CNS), as well as multiple areas may be susceptible (Ray-Chaudhury 2010).

1.3.1 GBM clinical subtypes

There are two different forms of GBM, each of which is genetically distinct – primary and secondary GBM (Figure 4). Primary GBM is the most common form occurring in 95% of GBM and typically occurring in older patients. Primary GBM is a newly arising tumour, and develops within 3–6 months. On the other hand, secondary GBM usually progresses from prior low-grade astrocytomas, over a span of 10–15 years, and is typically seen in younger patients (Alifieris and Trafalis 2015). Primary GBM frequently encompasses the loss of heterozygosity (LOH) of 10q (70%) and epidermal growth factor receptor (EGFR) amplification (36%). Secondary GBM exhibits mutation of the TP53 tumour suppressor gene and IDH-1 gene, which can be present in the majority of the precursor low-grade astrocytomas that eventually progress into GBM (Ohgaki and Kleihues 2007). Not surprisingly, due to the accumulation of multiple mutations, glioblastomas contain the most genetic changes of all the astrocytic tumours ("Glioblastoma Multiforme: Practice Essentials, Background, Pathophysiology" 2016).

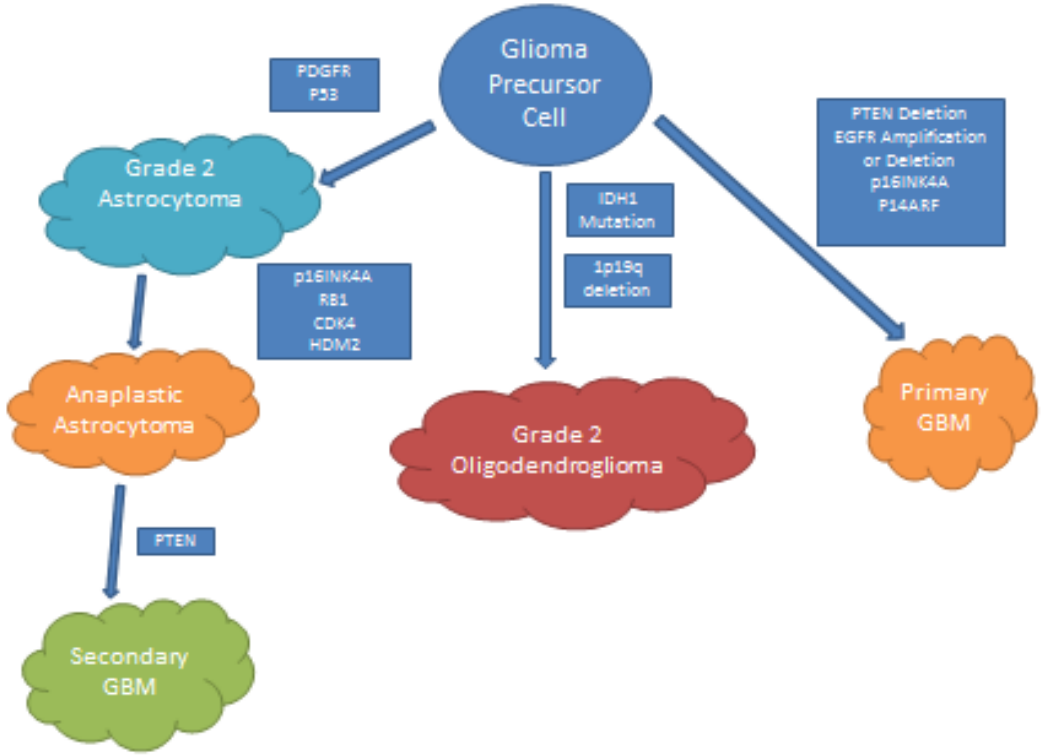


Figure 4 Glioblastoma Pathogenesis. There are two different forms of GBM, each of which is genetically distinct – primary and secondary GBM. Primary GBM is a newly arising tumour, and develops within 3–6 months. Secondary GBM usually progresses from prior low-grade astrocytomas, over a span of 10–15 years. Primary GBM frequently encompasses the LOH of 10q and EGFR amplification. Secondary GBM exhibits mutation of the TP53 tumour suppressor gene and IDH-1 gene, which can be present in the majority of the precursor low-grade astrocytomas that eventually progress into GBM.

1.3.2 Glioblastoma Epidemiology

Approximately 12–16% of all intracranial tumours are glioblastomas (Louis et al. 2007), with an estimated age-adjusted incidence in North America of 3.0 per 100,000 population (Mason et al. 2007).

Detailed and regularly updated population-based survival data for glioma and other primary brain tumours are available through the CBTRUS (Central Brain Tumour Registry of the United States). In the 2008-2012 CBTRUS, the following epidemiology data was reported (Ostrom et al. 2015):

1.3.2.1 Location and histology

According to the CBTRUS, the majority of gliomas occur in the frontal, temporal, parietal, and occipital lobes combined (60.8%). A very small proportion of gliomas occur outside the brain.

1.3.2.2 Survival Rates

In the CBTRUS data, the estimated five- and ten-year survival rates for all malignant brain tumours were 34.4% and 28.8%, respectively. However, depending on tumour histology, the five-year survival rates varied, with 94.2% for pilocytic astrocytoma vs 5.1% for glioblastoma. For most histologies, children and young adults generally have better survival outcomes, as survival generally decreases the older a patient is when diagnosed.

1.3.2.3 Glioblastoma Statistics

According to the CBTRUS data, glioblastoma accounted for the majority of gliomas (55.1%), with astrocytic tumours, including glioblastoma, accounting for 75% of all primary gliomas. In addition, glioblastoma accounted for 15.1% of all primary brain tumours and 46.1% of primary malignant brain tumours. Glioblastoma had the highest incidence rate (3.20 per 100,000 population), followed by diffuse astrocytoma (0.53 per 100,000). The incidence rate of glioblastoma is approximately 2 times greater in whites than in blacks, 1.6 times more common in males than in females, and is primarily diagnosed at older ages (median age of 64 years), with incidence increasing with age (rates highest in those 75-84 years). Glioblastoma is less common in children, with only 2.9% of all brain tumours reported among age 0-19 years.

1.3.3 Glioblastoma Presentation and Diagnosis:

Because glioblastomas can grow rapidly, the most common symptoms are usually caused by increased pressure in the brain. The clinical history depends on the location of the tumour, with headache, nausea, vomiting, and epileptic seizure being common. Personality change, drowsiness, weakness on one side of the body, memory and/or speech difficulties, and visual changes can also occur. The history is usually short due to the rapid growth of the tumour (“Glioblastoma Multiforme Clinical Presentation: History, Physical, Causes” 2016).

The radiological appearance of glioblastomas is often unique. In the post-contrast T1-weighted magnetic resonance imaging (MRI), they appear as an 'enhancing rim around a center (rim-enhancing lesion) with the non-enhancement representing tumour cell necrosis' (Ray-Chaudhury 2010).

1.3.4 Pathology

1.3.4.1 Macroscopy

With the naked eye, glioblastoma is usually seen as quite large and often involves more than one lobe. Glioblastomas are not very well defined due to its diffusely infiltrati**ve** growth pattern. Glioblastoma "Multiforme" was given its name due to its unique gross appearance on cross-section: the viable areas of the tumour appear grayish-white in colour, the non-viable necrotic areas appear yellowish, and many times foci of red-brown discoloration appear due to recent and old hemorrhage (Ray-Chaudhury 2010).

1.3.4.2 Microscopy

Under the microscope, GBM appears as a densely cellular and poorly differentiated tumour that is composed of 'pleomorphic astrocytic elements' (Figure 5). The individual cells appear in one form or appear to be highly variable in size and shape; display a highly stained or 'hyperchromatic' nucleus; and sometimes appear in 'multinucleated' forms (giant cells) (Ray-Chaudhury 2010). Because the tumour cells at the perimeter infiltrate-through the white matter in the normal brain parenchyma, surgical resection is quite impossible, as determining the normal brain from the tumour is quite difficult (Ray-Chaudhury 2010). Several features of glioblastomas include 'increased cellular pleomorphism with brisk mitotic activity', the presence of significant tumour cell necrosis and/or foci of prominent "glomeruloid" microvascular proliferation or angiogenesis (Ray-Chaudhury 2010). Necrosis is present in the center of the tumour, while the periphery contains increased cellularity and microvascular proliferation. Multiple foci of necrosis and microvascular proliferation can also be observed throughout the tumour. The tumour cells also gather at the subpial and subependymal zones, as well as around secondary structures, such as neurons and blood vessels (Ray-Chaudhury 2010).

1.3.4.3 Immunohistochemistry

There are a few immunomarkers of GBM, one of which is GFAP (glial fibrillary acidic protein). Although GFAP is present in both malignant and non-malignant astrocytes and their processes, in many GBM this immunostain is patchy or totally negative (Ray-Chaudhury 2010). GBM's proliferative

capability is recognized by the presence of many mitotic figures within the tumour; however, a more effective way of identifying the proliferative activity is by staining with Ki-67 or MIB-1 antibody as it identifies the growth potential of a tumour. The proliferation index, which varies from tumour to tumour, is calculated by counting the cells that are positive for staining with the antibody as a percentage of all neoplastic cells in the microscopic section (Louis et al. 2007). The Ki-67 stain is useful in differentiating between residual tumour and new growth in patients who have received treatment. However, it is not yet known whether there is a concrete relation between the proliferation index and prognosis (Ray-Chaudhury 2010).

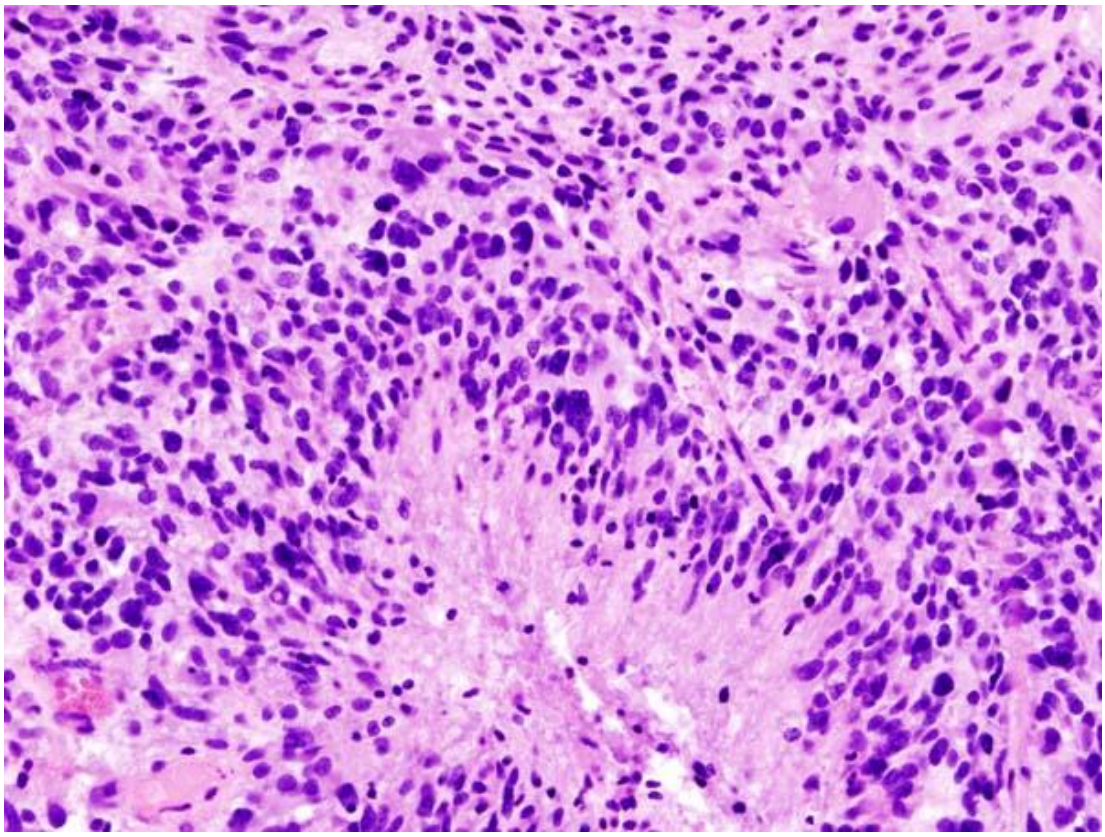


Figure 5 Histopathalogical image of GBM - H&E stain. This is an image of a grade IV Glioblastoma under the microscope. Glioblastoma is polymorphic and can be diagnosed based on two features: endothelial proliferation and necrosis.

1.3.5 Survival and Prognosis:

Through research, clinical trials, and advancing technology, much progress has been made in the fields of surgery, radiation, and chemotherapy for the treatment of glioblastoma. Despite this, the overall survival of glioblastoma patients remains dismal.

In patients with GBM, survival decreases with increasing age at diagnosis. Age has been shown to be an important predictive factor, with younger patients (age less than 50 years) having a better prognosis (Louis et al. 2007). Patients with higher grade tumours, such as GBM, have worse survival than those with lower grade tumours (Chang et al., n.d.).

In patients with GBM, survival depends on a range of different factors. Some of these include: histologic type and grade, age, extent of surgical resection, tumour location, degree of necrosis enhancement on

MRI (Magnetic Resonance Imaging) studies, treatment regimens and patient condition prior to treatment (radiation and chemotherapy), tumour size pre- and post-surgery, Karnofsky Performance Scale score, patient deterioration, and pre-surgical serum albumin level (Chang et al., n.d.). In addition to the above factors, there also exist various genetic disorders which make patients more prone to developing GBM, such as neurofibromatosis, tuberous sclerosis, Von Hippel-Lindau disease, and Li-Fraumeni syndrome (Nieder, Grosu, and Molls 2000).

1.3.6 Genetic Abnormalities

As discussed previously, primary and secondary GBM are shown to differ in their genetic pathways. More recently, determining prognostic factors for GBM are focused on genetic and molecular markers. Newer technologies and tools are allowing researchers to explore the impact of molecular changes in the genes, methylation patterns, chromosome numbers, transcripts, and proteins in these tumours (Chang et al., n.d.). Some of the more common genetic abnormalities are described as follows:

- Loss of heterozygosity (LOH): LOH on chromosome arm 10q is the most frequent gene alteration for both primary and secondary glioblastomas, and unfortunately is associated with poor survival. LOH on 10q is rarely found in other tumour grades, and appears to be exclusive to GBM, playing a major role in its development (von Deimling et al. 1992), (Schmidt et al. 2002).
- p53: This tumour suppressor gene appears in approximately 25-40% of all glioblastoma multiformes. It is among the first mutations to be identified in astrocytic tumours, is more commonly deleted or altered in secondary glioblastoma, and is associated with tumours that develop in younger patients (Ohgaki and Kleihues 2007). One study found that TP53 mutations were a favorable prognostic factor, regardless of whether the glioblastoma was primary or secondary (Schmidt et al. 2002).
- Epidermal growth factor receptor (EGFR) gene: The EGFR is a type of protein that is found on the surface of cells in the body, and is involved in the control of cell proliferation. When this gene is mutated, it results in overexpression and promotes tumour growth. EGFR amplification and mutation in GBM, combined with other genetic alterations, likely plays an important role in the pathogenesis of this disease (Hatanpaa et al. 2010). Amplification of EGFR is found in approximately 43% of primary GBM, but rarely in secondary GBM (Ohgaki and Kleihues 2007).

- MDM2: Overexpression (amplification) of this gene is the second most common gene mutation in GBM. It is observed in 10-15% of patients, and appears to be associated with poor prognosis. Amplification of MDM2 inhibits p53 activity by binding to it and inhibiting its regulation of cell growth (Shapiro et al. 1989).
- Platelet-derived growth factor (PDGF): This is one of the numerous proteins that regulate cell growth and division. Amplification of PDGF is commonly observed in approximately 60% of the pathways leading to secondary glioblastomas (Nazarenko et al. 2012).
- PTEN: PTEN mutations have been found in approximately 20% of glioblastomas and more commonly in primary GBM. Normally, PTEN acts as a cellular phosphatase by turning off signalling pathways and thus suppressing tumour activity. However, when PTEN has a genetic mutation, it loses phosphatase activity, leading to out of control proliferation (Duerr et al. 1998).

There exist many other genetic abnormalities in glioblastomas, some of which include, but are not limited to the following:

- In primary GBM: p16 deletions (30-40%), p16INK4A, and Retinoblastoma (RB) gene protein alterations ("Glioblastoma Multiforme: Practice Essentials, Background, Pathophysiology" 2016).
- In secondary GBM: LOH at chromosome arm 19q (50%), RB protein alterations (25%), and deleted-in-colorectal-carcinoma gene (DCC) loss of expression (50%) ("Glioblastoma Multiforme: Practice Essentials, Background, Pathophysiology" 2016).
- Combined loss of 1p and 10q may lead to a more favorable prognosis for GBM patients (Schmidt et al. 2002).

1.3.7 Treatment

Major challenges in treating glioblastoma are associated with the location of the disease and its complex and heterogeneous biology (Kesari 2011). Treatment of this deadly tumour has progressed over the years, and includes the treatment of the associated toxicities of treatment (Alifieris and Trafalis 2015), (Stupp et al. 2005). Despite this, most patients with GBM will die within 12 months of their diagnosis (Furnari et al. 2007).

However, over the past few decades, there has been much progress in the understanding of glioblastoma's behaviour in the areas of molecular biology, genetics, cytopathology, transcriptional, and genomic aspects

(Ray-Chaudhury 2010). Hence, researchers better understand how the altered genes and pathways in glioma drive GBM, have identified new molecular subtypes in GBM, and have developed new therapeutic strategies for targeting specific genetic lesions and their pathways (Furnari et al. 2007). Moreover, new drugs that are designed to overcome drug resistance are being explored and show promising results in vitro against GBM. However, there has been limited success in vivo, mainly due to their inability to cross the blood–brain barrier (BBB) and to penetrate inside the tumour tissue. This has led to an increased focus on nanocarriers, which have the ability to cross the BBB and transport the drug inside the tumour (Karim et al. 2016).

Despite these advancements, treatment remains challenging due a patient's resistance to traditional therapies, and the inability of many drugs to cross the blood brain barrier (Karim et al. 2016).

1.3.7.1 Chemotherapy:

Chemotherapeutic treatment for newly diagnosed GBM has evolved over the years. One of the current chemotherapy drugs being used is Temozolomide (TMZ), which is an alkylating agent that works by stopping or slowing down brain tumour cell growth, and has an excellent ability to penetrate the central nervous system (Alifieris and Trafalis 2015). Other chemotherapy drugs used to treat GBM include the nitrosureas: carmustine and lomustine, platinum agents, etoposide, irinotecan and the PCV combination (which consists of the chemotherapy drugs Procarbazine, Lomustine and Vincristine) (Alifieris and Trafalis 2015).

1.3.7.2 Radiation Therapy:

Glioblastoma is highly invasive and has the ability to diffusely infiltrate normal brain tissue away from the tumour and recur locally. Because of this, radiation therapy is used to treat the areas of highest risk. There is demonstrated evidence of the benefit of radiation therapy in patients with newly diagnosed GBM. A breakthrough study (Stupp et al. 2005), (Stupp et al. 2009) demonstrated that gross surgical resection followed by concurrent temozolomide and radiation improved overall survival, and established this as being the standard of care for the treatment of newly diagnosed GBM patients. A better understanding of chemotherapy drugs and radiation at the biomolecular and genetic levels will allow treating physicians to identify those patients that will most likely benefit from specific therapeutic regimens (Chao et al. 2009).

Unfortunately, GBMs may acquire resistance to chemotherapy. O^6-methylguanine-DNA methyltransferase (MGMT) is a repair gene that lessens the effects of alkylating drugs. Methylation (silencing) of MGMT is found in 30–60% of GBM cases and is associated with a favorable outcome if treated with alkylating agents (Hegi et al. 2005). One clinical trial demonstrated that knowing the MGMT status affected the overall survival and progression-free survival in patients receiving concurrent temozolomide and radiation, as well as in patients receiving radiation alone. Patients in the radiotherapy group with MGMT methylation had a median survival of 21.7 months versus 15.3 months for those who did not (Hegi et al. 2005).

In elderly patients with GBM above 70 years of age, there exists limited clinical trial data on the management of these patients. However, radiation therapy has been demonstrated to be useful in these patients (Keime-Guibert et al. 2007).

Chapter 2

2 MAPK and ERK5 Signalling

2.1 Introduction to the MAPK Pathway

The mitogen-activated protein kinase (MAPK) signalling pathway includes the signalling molecules Ras, Raf, MEK, and ERK. This pathway is significant in regulating gene expression, cellular growth, and survival. This regulation of cellular functions occurs when the extracellular growth factors in normal cells bind to and activate receptor tyrosine kinases, which in turn cause a downstream signalling cascade, thus leading to the transcription of genes that encode proteins. It is these proteins that regulate these cellular functions (Knight and Irving 2014).

However, when abnormal MAPK signalling occurs, it may lead to increased or uncontrolled cell proliferation, resistance to apoptosis (programmed cell death), and resistance to various therapies. This abnormal MAPK signalling occurs through several mechanisms, such as abnormal expression or activating mutations in receptors and genes (Knight and Irving 2014).

Thus far, four conventional and three unconventional MAPK subfamilies have been identified. The four conventional MAPK subfamilies are: extracellular signal-regulated protein kinases 1/2 (ERK1/2); c-Jun N-terminal kinases 1–3 (JNK1, 2 and 3); p38 MAPKs (p38 a, b, g and d); and ERK5. The three unconventional MAPK subfamilies, ERK3/4, ERK7, and Nemo-like kinase (NLK), are considered unconventional because their N- and C-terminal domain extensions are different than those in the conventional MAPKs (André E. S. Simões, Rodrigues, and Borralho 2016).

The MAPK cascade is activated by several stimuli, such as internal metabolic stress, external mitogens, hormones, or neurotransmitters, cell–matrix and cell–cell interactions (André E. S. Simões, Rodrigues, and Borralho 2016). The MAPK cascade is a three-tiered hierarchical model that is activated when a mitogen-activated kinase kinase kinase (MAPKKK) phosphorylates mitogen-activated kinase kinase (MAPKK), which finally phosphorylates MAPK (André E. S. Simões, Rodrigues, and Borralho 2016).

ERK1/2 is one of the better studied members in the MAPK family and has been demonstrated to play crucial roles in neuronal survival, plasticity and regeneration of many types of brain cells (Miloso et al. 2008). ERK1/2 and ERK5 pathways each respond to specific upstream signals and regulate different sets

of downstream targets (J. E. Cavanaugh et al. 2001). Interestingly enough, during development, it is thought that ERK5 could compensate for ERK1/2 functions when ERK1/2 is not highly expressed or when its activity is low (J. E. Cavanaugh et al. 2001).

ERK5, encoded by the MAPK7 gene, is the most recently identified member of the MAPK family, and was first identified as big mitogen kinase 1(BMK1). It is expressed all over in numerous tissues and is activated by a variety of extracellular stimuli, such as cellular stresses and growth factors, and functions to regulate processes such as cell proliferation and differentiation (Nithianandarajah-Jones et al. 2012). Targeted deletion of ERK5 in mice has revealed that the ERK5 signalling cascade plays a crucial role in cardiovascular development and vascular integrity. More recent data reveals a potential role in cancer and tumour angiogenesis (Nithianandarajah-Jones et al. 2012). ERKs play key roles in cellular proliferation, differentiation, migration, and gene expression (Roux and Blenis 2004).

2.2 MEK5/ERK5 Function

The MEK5/ERK5 pathway is the least studied MAPK signalling pathway. It has been proposed to play a role in the pathology of cancer by contributing to endothelial cell survival, and also functioning as a regulator of tumour cell invasion and migration (Lochhead, Gilley, and Cook 2012).

ERK5 is known to be involved in cell cycle control and cell transformation (Kato et al. 1998), (English et al. 1999), (André E. S. Simões, Rodrigues, and Borralho 2016), (Nithianandarajah-Jones et al. 2012), cardiovascular disease, and neuronal survival (Obara et al. 2008), (Carter et al. 2009), (Cundiff et al. 2009), (Clapé et al. 2009). In the central and peripheral nervous systems, ERK5 is known to be involved in the survival and development of multiple types of brain cells (Jane E. Cavanaugh 2004). Known activators of ERK5 include growth factors such as epidermal growth factor (EGF), NGF, BDNF, NT-3, NT-4, G-protein coupled receptors, and oxidative stress (Fukuhara et al. 2000), (Scapoli et al. 2004), (Jane E. Cavanaugh et al. 2006).

One of the key functions of ERK5 is that it contributes to cell survival mechanisms. Through the analysis of human tumours, it was confirmed that a link exists between abnormal levels of ERK5 and a number of different oncogenes. Furthermore, it is thought that ERK5 mediates the effects of these oncogenes (Wang and Tournier 2006).

We now have a better understanding of the ERK5 signalling pathway's physiological function during normal development and pathogenesis due to the analysis of genetically modified mice. Experiments performed on mice, in which the ERK5 gene have been deleted, has been shown to cause embryonic lethality in these mice (Regan et al. 2002), thus supporting ERK5's crucial role in normal development (Wang and Tournier 2006), (Regan et al. 2002).

2.3 ERK5 Structure and Activation

Both MEK5 and ERK5 are structurally and functionally explicit from other MAPKs (Buschbeck et al. 2002), (J. E. Cavanaugh et al. 2001), (Jane E. Cavanaugh et al. 2006). It has been demonstrated that ERK5 will not interact with either MEK1 or MEK2, indicating that MEK5/ERK5 interaction represents a unique signalling pathway (Zhou, Bao, and Dixon 1995). Structurally, the ERK5 protein contains an N-terminal kinase domain with a similar sequence of 66% with ERK1/2. Unlike other MAPKs, ERK5 contains a larger C-terminus. It is regulated by dual phosphorylation at its tyrosine and threonine sites, which follow a typical 'Thr-Glu-Tyr' pattern. In order for full enzymatic activation to occur, phosphorylation at both Thr and Tyr is necessary. ERK5's C-terminus has two prolin-rich domains (PR1 and PR2), a nuclear localization signal (NLS), autophosphorylation sites, and is thought to allow inhibitors to specifically target without affecting other kinases in the pathway (Kamakura, Moriguchi, and Nishida 1999), (Buschbeck et al. 2002) (Figure 6). MEK5 prompts ERK5 activation by dual phosphorylation of its N-terminal domain (Wang and Tournier 2006). Once active, ERK5 phosphorylates multiple sites in its C-terminal domain, which plays a role in activating transcription (Kato et al. 1998). ERK5 can activate several transcription factors including Sap1, c-FOS, c-MYC, and MEF2 (Pearson et al. 2001).

More recently, two studies have suggested a potentially new way of activating the ERK5 pathway without MEK5. It has been suggested that this activation occurs during mitosis and relies on cyclin-dependent kinases (CDKs) during the G2–M phase transition (Álvarez-Fernández et al. 2013). It has also been suggested that a 'cross-talk mechanism' occurs by members of the MAPK family, where active ERK1/2 phosphorylates ERK5 at the Thr732 residue in the C-terminal domain, instead of through the usual activation of the N-terminal kinase domain, and thus directing ERK5 to the nucleus (Honda et al. 2015).

ERK5 is activated by oxidative and osmotic stresses (Abe et al. 1996), serum (Kato et al. 1997), various growth factors, including vascular endothelial growth factor (VEGF), epidermal growth factor (EGF),

fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), brain-derived neurotrophic factor (BDNF), and nerve growth factor (NGF) (Kato et al. 1998), (Kesavan et al. 2004), (Hayashi and Lee 2004), (Watson et al. 2001), and also by cytokines, such as leukemia inhibitory factor (LIF) (Nicol et al. 2001) and interleukin 6 (IL-6) (Carvajal-Vergara et al. 2005).

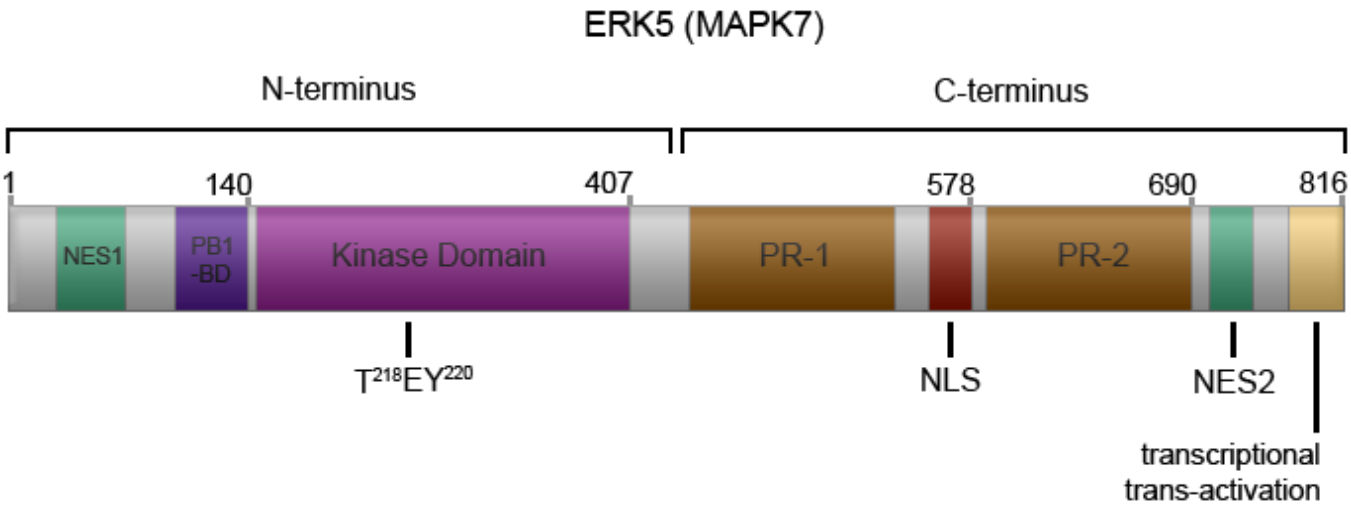


Figure 6 ERK5 (MAPK7) structure. This figure shows the ERK5 structure which has a larger C-terminus than other members of the MAPK family. The C-terminus has two prolin-rich domains (PR1 and PR2) and a nuclear localization signal (NLS). The N-Terminus is 66% similar to ERK 1/2, and contains Thr and Tyr residues in its kinase domain that are phosphorylated by MEK5. The ERK5 antibody used in my experiments were raised against a peptide mapping at the C-terminus of ERK 5.

Source: Adapted from the Atlas of Genetics and Cytogenetics in Oncology and Haematology.

2.4 MAPK/ERK pathway in Cancer

There is an increasing amount of evidence in the literature concerning the role of mitogen activated protein kinases (MAPKs) in the various steps of tumour development and progression. There have been some studies demonstrating that MAPKs regulate cancer cell survival, anti-apoptotic signalling, angiogenesis, and proliferation (Kim and Choi 2010). However, the majority of these studies were not focused on the MEK5-ERK5 pathway and the role it plays in these activities. There are some studies that have demonstrated overexpression or activation of the MEK5-ERK5 pathway in certain types of cancers, such as leukemia, lymphoma, medulloblastoma, and prostate cancer (Sawhney, Liu, and Brattain 2009), (Cronan et al. 2012), (Zuo et al. 2015). However, the MEK5-ERK5 pathway's role in brain tumours and more specifically in glioblastoma has not yet been fully explored.

The RAS-RAF-MEK-ERK signalling pathway is overactivated in several types of cancers (Kim and Choi 2010). RAS and BRAF have been found to carry many cancer-associated mutations that are present in components of the MAPK signalling pathways. Mutations of K-RAS frequently appear in many cancers, including in approximately 50% of colon cancers (Kim and Choi 2010). Mutations in the BRAF gene are detected in approximately 66% of malignant melanomas (Kim and Choi 2010), approximately 10–15% of pilocytic astrocytomas, and in approximately 5–10% of pediatric diffusely infiltrating gliomas (including diffuse astrocytomas, anaplastic astrocytomas, and glioblastomas) (Dougherty et al. 2010). Interestingly, activated mutations of BRAF do not frequently appear in adult gliomas (less than 2%) (Horbinski 2013), (Myung et al. 2012), however when they do, they are associated with the higher-grade more malignant types (Basto et al. 2005). In a study assessing the frequencies of BRAF mutations in various tumours (Schindler et al. 2011), high frequencies of BRAF (V600E) mutations were found in pleomorphic xanthoastrocytomas, gangliogliomas and extra-cerebellar pilocytic astrocytomas, thus implicating that the BRAF(V600E) mutation is a valuable diagnostic marker for these rare tumors. In addition, the study found that there was a low frequency or absence of BRAF mutations in glioblastomas and other gliomas, and no mutations were detected in non-glial tumors, including embryonal tumors, meningiomas, nerve sheath tumors and pituitary adenomas (Schindler et al. 2011).

Mutations in EGFR, which is one growth factor that activates the ERK pathway, are frequently found in lung and colorectal cancers. This abnormal activation appears in approximately 80% of cases of non-small cell lung cancer (Kim and Choi 2010). In gliomas, and especially in glioblastomas, amplification of

EGFR is the most common genetic abnormality seen, and occurs in approximately 50% of cases (Furnari et al. 2007). Dysregulation of EGFR has been shown to enhance tumour growth, migration, angiogenesis, and metastatic spread (Tortora et al. 2007). Further, EGFR overexpression is a poor prognostic factor and correlates with decreased overall survival in GBM patients (Shinojima et al. 2003).

2.4.1 Osteosarcoma and Head and Neck Squamous Carcinoma

The MAPK7 gene was reported to be amplified in high-grade osteosarcoma (van Dartel et al. 2002), however only recently was this gene considered a potential therapeutic target. In osteosarcoma, ERK5 overexpression was correlated with tumour progression, resistance to treatment, and worse overall patient survival (Tesser-Gamba et al. 2012).

ERK5 overexpression has also been found in head and neck squamous carcinoma, and was associated with advanced tumour stage and lymph node metastasis (Sticht et al. 2008).

2.4.2 Breast Cancer

There is clinical evidence that an increase in MEK5/ERK5 signalling may contribute to cancer progression.

In vitro, ERK5 has been linked to the proliferation of breast cancer cells. In one study, researchers used animal and cellular models to investigate the expression, function and prognostic value of ERK5 in human breast cancer. In 84 human breast tumours, the expression of ERK5 was analyzed by immunohistochemistry, active ERK5 (pERK5) was studied by Western blotting, and the correlation of ERK5 with clinicopathological parameters and with disease-free survival in early stage breast cancer patients was investigated. The study found that expression of ERK5 was observed in most patients, and overexpression was observed in 20% of patients. Active ERK5 was observed in a considerable number of human samples, as well as in animal breast cancer samples. Furthermore, overexpression of ERK5 was associated with a decrease in disease-free survival time. The study found that inhibition of ERK5 decreased cancer cell proliferation and also sensitized these cells to the action of anti-HER2 therapies. They concluded that overexpression of ERK5 is an independent predictor of disease-free survival in breast cancer, and may represent a future therapeutic target (Montero et al. 2009).

MEK5 and ERK5 levels were found frequently increased in Triple negative Breast Cancer (TNBC), and correlated with poorer relapse-free survival (Ortiz-Ruiz et al. 2014). In one study, mice were designed to develop TNBC-like tumours, which when analyzed, contained unusually high levels of ERK5 expression compared with normal breast tissue from the same mice (Ortiz-Ruiz et al. 2014).

2.4.3 Prostate Cancer

MEK5-ERK5 signalling has also been linked to prostate cancer (CaP); however, the exact mechanism of action is still poorly understood. In prostate cancer, MEK5 is overexpressed and correlates with the presence of bone metastases and less favourable survival (Ramsay et al. 2011). Furthermore, MEK5/ERK5 signalling was shown to encourage prostate tumour cell proliferation by promoting the G1–S cell cycle phase transition (McCracken et al. 2008), and initiation of DNA replication (Dudderidge et al. 2007). Studies have demonstrated the crucial role that ERK5 plays in prostate cancer metastasis, and the promising role that ERK5 inhibitors can play in managing the spread of this cancer (McCracken et al. 2008). One study demonstrated that MEK5 plays a key role in prostate carcinogenesis. Researchers examined MEK5 expression in 127 cases of prostate cancer and 20 cases of benign prostatic hypertrophy (BPH) by immunohistochemistry, and compared the results to clinical parameters. They showed that MEK5 expression is increased in prostate cancer as compared to benign prostatic tissue, thus proving MEK5 does indeed play a role in the spread of this cancer (Mehta et al. 2003).

Recent studies have demonstrated that microRNA (miRNA) mir143 expression plays a role in the regulation of ERK5 expression. In one study, the researchers examined a tissue microarray (TMA) of 530 prostate cancer cores from 168 individual patients and stained for both mir143 and ERK5. They observed a strong inverse relation between ERK5 and mir143, as one increased, the other decreased. The researchers hence confirmed the potential role of mir143 in regulating ERK5 levels in prostate cancer (Ahmad et al. 2013).

2.4.4 Colorectal and Colon Cancer

In regards to colon cancer, one study examined the relevance of MEK5/ERK5 signalling by evaluating MEK5 and ERK5 expression in 323 human colon cancer samples. The researchers evaluated the effects of MEK5/ERK5 signalling on cell cycle progression by flow cytometry. They used an orthotopic xenograft mouse model of colon cancer to assess tumour growth and progression. Their results demonstrated that MEK5 and ERK5 are overexpressed in human adenomas and adenocarcinomas, with

increased ERK5 expression correlating with increased potential for invasiveness and metastasis. In addition, they showed that ERK5 overactivation considerably sped up cell cycle progression and increased cell migration. Their results suggested that the MEK5/ERK5/NF-κB signalling pathway is important for tumour onset, progression and metastasis and could potentially be a therapeutic target in colon cancer treatment (A. E. S. Simões et al. 2015).

2.4.5 Clear Cell Renal Cell Carcinoma

A study looking at fresh samples from human clear cell renal cell carcinoma found that high levels of ERK5 correlated with more aggressive and metastatic stages of the disease (Arias-González et al. 2013).

2.4.6 Hepatocellular Carcinoma

One study examined the role of ERK5 in hepatocellular carcinoma (HCC) in vitro and in vivo. Analysis of ERK5 by Immunohistochemistry (IHC) in human tissues showed more ERK5 activation in patients with HCC or cirrhosis than in normal liver. ERK5 silencing in HCC cells blocked the increase in migration and invasion initiated by EGF or serum. Immunofluorescence experiments demonstrated that ERK5 silencing or inhibition caused a reduction in cell motility. It also demonstrated that ERK5 activation was necessary for the growth of HCC cells. In mice injected with Huh-7 cells silenced for ERK5, the rate of tumour appearance was significantly lower (4/16 mice, 25%) than in animals inoculated with cells unsilenced for ERK5 (9/15 mice, 60%). In addition, at the end of the experiment, tumour volume was smaller in the presence of ERK5 silencing. Hence, the study concluded that the ERK5 pathway plays a critical role in HCC tumour development and growth in vivo, and that further studies looking at blocking the ERK5 pathway should be explored (Rovida et al. 2015).

Another study used high-density oligonucleotide microarrays to investigate DNA copy-number abnormalities in hepatocellular carcinoma (HCC) cell lines. MAPK7, which encodes ERK5, was overexpressed in cell lines in which the gene 17p11 was amplified. An increase in MAPK7 copy number was detected in 53% of HCC tumours. SNU449 is an HCC cell line that has a high level of amplification and overexpression of MAPK7. In this study, downregulation of MAPK7 by siRNA suppressed the growth of this cell line. In addition, mitotic entry of SNU449 cells were found to be regulated by ERK5, which is phosphorylated during the G2/M phases of the cell cycle. Overall, their results suggested that the target of 17p11 amplification is MAPK7, and that ERK5 promotes the growth of HCC cells by regulating mitotic entry (Zen et al. 2009).

2.5 Regulation by miR-143

It has been shown that ERK5 activity is activated by some oncogenes. Recently, protein levels were found to be regulated by tumour-suppressive miRNAs (microRNAs), which suggests that targeted therapies against ERK5 may be of clinical use (Lochhead, Gilley, and Cook 2012). MiRNAs are small non-coding single-stranded RNAs that bind to the 3′-UTR (untranslated region) of target mRNAs. The miRNAs miR-143 and miR-145 are both encoded by the same gene and have been shown to downregulate ERK5 protein levels, thus suggesting a relation between ERK5 and tumour progression (Lochhead, Gilley, and Cook 2012). Hence, the authors of one paper pointed out the possibility that in cancers with down-regulated *miR-143* and *miR-145*, ERK5 may play a role in the development of the cancer (Lochhead, Gilley, and Cook 2012). In addition, one study found that miR-143 and miR-145 were over-expressed in the malignant areas of GBM, and that these miRNAs regulate the progression of glioblastoma (Koo et al. 2012).

2.6 ERK5 in Gliomas

2.6.1 Epithelial to Mesenchymal Transition of Glioma Cells

One important factor that contributes to the invasiveness of high-grade gliomas is the epithelial to mesenchymal transition (EMT) of the glioma cells (Kahlert, Nikkhah, and Maciaczyk 2013). EMT is considered to be the most important malignant process in gliomas (Lehembre et al. 2008). EMT plays an essential role in the development of cancer, including cancer cell migration, invasion, and metastasis. One of the key regulators of EMT is miR-200b-3p (Guarino, Rubino, and Ballabio 2007).

ERK5 is thought to participate in EMT (Drew, Burow, and Beckman 2012). In breast cancer, it was shown that cells treated with ERK5's binding partner, MEK5, can increase factors that regulate EMT. This indicates that the MEK5/ERK5 pathway leads way to mesenchymal-like properties in cells (Zhai et al. 2015). However, the role of ERK5 in EMT in gliomas remains to be uncovered.

2.6.2 miR-200b-3p

Dysregulation of microRNAs (miRNAs) is a common feature in human cancers, including glioma, as miRNAs may function as tumour suppressors or oncogenes. In a recent study, researchers conducted

qRT-PCR analysis on 16 normal brain tissues and 31 glioma tissue samples, and found that the expression of miR-200b-3p was consistently lower in the glioma tissues compared with the normal brain tissues. In addition, the levels of miR-200b-3p expression in the high-grade glioma tissues (grade III and IV) were much lower than those in the low-grade glioma tissues (grade I and II). Their results suggested that miR-200b-3p expression correlated with glioma malignancy, and which further suggested that miR-200b-3p may function as a tumour suppressor gene in the evolution of glioma (Wu et al. 2016). Their results are consistent with other studies that show that miR-200b is downregulated in glioma, and that its downexpression is correlated with poor prognosis in gliomas patients (J. Li et al. 2016), (Peng et al. 2013), and (Men, Liang, and Chen 2014).

Further, this study also demonstrated that miR-200b-3p suppressed migration and invasion of the human glioma cell lines U251 and U87. In addition, ERK5 was identified as a direct target of miR-200b-3p, and both were found to regulate the EMT process, thus affecting cell migration and invasion (Wu et al. 2016). ERK5 was upregulated in glioma specimens and was inversely correlated with miR-200b-3p levels. In vitro, down-regulation of ERK5 decreased cell migration and invasion ability (Wu et al. 2016).

The study confirmed that miR-200b-3p acts as a tumour suppressor gene through various mechanisms, including restraining glioma tumour growth, invasion, and EMT and by direct targeting of ERK5 (Wu et al. 2016). While the researchers suggested that the miR-200b-3p/ERK5 pathway shows much promise as a target for glioma treatment, further research is needed to uncover the mechanism of action and the role of miR-200b-3p in the progression of glioma (Wu et al. 2016).

2.6.3 RAS-RAF-MEK-MAPK pathway

Malignant gliomas do not commonly contain Ras mutations, but due to the amplification or mutations of upstream growth factor receptors, they tend to exhibit increased Ras activity (Knobbe, Reifenberger, and Reifenberger 2004). When Ras is triggered by either EGFR or PDGFR activation or through other pathway alterations, it initiates the MAPK or ERK and phosphatidylinositol 3-kinase (PI3K) pathways through a mitogenic signal (Alifieris and Trafalis 2015). Activation of MAPK is associated with poor outcome in GBM (Pelloski et al. 2006).

2.6.4 PDGF Pathways

Overactivity of the PDGF pathways is found in diseases entailing abundant cell growth, including malignancies (Ostman and Heldin 2007). PDGF activates the MAPK pathways Erk1/2, ERK5, c-Jun N-terminal kinase (JNK), and p38 (Cargnello and Roux 2011). Activated MAP-kinases phosphorylates substrates, such as signalling proteins and transcription factors, which influences cell proliferation, survival and migration (Eger et al. 2014). NR4A1, an orphan nuclear receptor, can be phosphorylated by MAP-kinases including Erk1/2, ERK5 and JNK (To, Zeng, and Wong 2012), is overexpressed in tumour cells, and has demonstrated abilities to both promote and inhibit oncogenesis (Safe et al. 2014).

In a recent study, researchers explored the pathways through which NR4A1 is activated by the fibroblast mitogen platelet-derived growth factor -BB (PDGF-BB) and examined its functional role (Eger et al. 2014). The researchers examined the role of NR4A1 in vitro and found that NR4A1 expression is essential for glioblastoma cell colony formation in soft agar. They found that in the absence of PDGF-BB, NIH3T3 cells did not form colonies in soft agar; but in the presence of PDGF-BB, it induced colony formation in soft agar. Moreover, it was observed that the effect was intensified after overexpression of NR4A1, and that NR4A1 downregulation increased proliferation promoted by PDGF-BB. In addition, in the glioblastoma cell lines U-251MG and U-105MG, an intense overexpression of NR4A1 was observed (Eger et al. 2014). Hence, these results are in keeping with the observation that in various tumours, such as glioblastoma, Erk1/2 activation and NR4A1 expression contribute to migration and invasion (Inaoka et al. 2008), (Z. Li et al. 2013). Furthermore, the researchers found that ERK5, ERK1/2 and NF-κB signalling contributed to an increase in NR4A1 expression via PDGF-BB activation (Eger et al. 2014). Overall, the results of this study concluded that NR4A1 is a potential target in the treatment of various tumours such as glioblastoma (Eger et al. 2014).

2.6.5 Brain-Derived Neurotrophic Factor

Neurotrophins belong to a class of growth factors that signal particular cells to survive, differentiate or grow. They are a family of proteins that are crucial in the development, differentiation, migration, and survival of neuronal and nonneuronal cells (Conover and Yancopoulos 1997). BDNF plays a role in the pathophysiology of certain brain diseases, and its effects are mediated by the MAPK signalling pathway (Numakawa et al. 2010).

In one study, researchers examined whether ERK1/2 and ERK5 played a role in regulating BDNF expression in C6 glial cells and primary astrocytes (Su et al. 2011). Their results showed that the ERK1/2 and ERK5 signalling pathways each regulated the transcription of the BDNF gene in a specific manner, and that ERK5 functioned as a negative regulator of BDNF gene expression in glial cells. Furthermore, their results showed that when regulating the BDNF gene, the ERK1/2 and ERK5 signalling pathways were competing with one another, which is important because both the ERK1/2 and ERK5 signalling pathways can be activated in the same cell (Su et al. 2011). As a whole, their results suggested that ERK5 and ERK1/2 exert opposite effects on BDNF expression and that an imbalance of the ERK5 and ERK1/2 pathways may cause a disruption in neurotrophin expression and/or regulation, thus leading to the development or progression of diseases, including certain types of cancers (Su et al. 2011).

Chapter 3

3 Research Question, Hypothesis, Alternative Hypothesis, and Aims/Objectives

Below are my research question, hypothesis, alternative hypothesis and aims/objectives for my research on ERK5 expression in Brain Tumours.

3.1 Research Question

Does ERK5 play a role in regulating the progression of the highly invasive brain tumour, Glioblastoma?

3.2 Hypothesis:

The expression of ERK5 positively correlates with higher grade brain tumor progression.

3.3 Alternative Hypothesis

A high level of expression of ERK5 is seen in highly invasive brain tumours, such as grade 4 Glioblastomas.

3.4 Aims/Objectives

1. To investigate whether ERK5 is expressed in Glioblastoma Multiforme
2. To investigate whether ERK5 is expressed in other types/grades of brain tumours.
3. To determine whether there exists a difference in ERK5 expression between tumour grades
4. To determine whether there exists a difference in ERK5 expression between higher vs lower grades (1&2 vs 3&4)
5. To determine whether there exists a difference in ERK5 expression between genders
6. To determine whether ERK5 expression is correlated with Age

Chapter 4

4 Materials and Methods

I used immunohistochemistry in my study in order to identify and examine the antigens (ERK5) that are specifically found in the brain tumour tissue samples, using ERK5 antibody. IHC is a technique that can be used to diagnose and follow cancer cells by identifying those antigens that are specifically found in the affected cells ("Immunohistochemistry | Definition of Immunohistochemistry by Medical Dictionary" 2016).

This study was divided into two parts: Study #1 (pilot study) and Study #2. Study #1 was performed on the brain tumour Tissue Microarray (TMA) in order to first determine whether any positive results were obtained before proceeding with the experiments on the samples obtained from the Brain Tumour Tissue Bank (BTTB). After achieving some promising results, Study #2 was performed. The statistical analyses were done on each of Study # 1 and Study #2, and on Study #1 and 2 combined.

The materials and methods used in my study are detailed below.

4.1 Controls and Reagents

Antibody: (ERK 5 (C-20), Santa Cruz Biotechnology, Inc., Cat No. sc-1284). The ERK5 used in these experiments was of goat affinity purified polyclonal antibody. The antibody used in the experiments was raised against a peptide mapping at the C-terminus of ERK 5 of human origin.

Controls: Ameloblastoma (S06-28990) and Cavernous hemangioma (S11-41) tissues

Reagents: Goat Impress (Vector Laboratories Cat No. MP7405) and DAB (DAB Peroxidase Substrate Kit, Vector Laboratories Cat No. SK4100).

The controls, Ameloblastoma and Cavernous hemangioma tissues, were prepared and sectioned according to standard protocol (Appendix A). These tumour samples were used as controls because there are tissue structures in these tissues that are known to be positive for ERK5 staining. Expression of phosphorylated ERK5 was found in ameloblastic tumours, which suggests the involvement of oncogenesis and tumour cell differentiation in these tumours (Kumamoto and Ooya 2007). The ERK5 signalling pathway was found to activate transcription factors that play a role in the development of cavernous hemangiomas

(Cuttano et al. 2016). In addition, these tissues were listed as known positives for ERK5 in the ERK5 antibody specification sheets (Santa Cruz Biotechnology Inc., 2016).

4.2 Samples

Brain Tumour Tissue Microarray:

In order to examine ERK5 expression in Glioblastoma and other types/grades of brain tumours, I first used a tissue microarray (US Biomax Inc., GL803) containing various types and grades of brain tumours. The TMA panel contained a total of 80 cores: 15 astrocytoma, 40 Glioblastoma, 4 ependymoma, 10 oligo astrocytoma, 3 each of medulloblastoma and oligodendroglioma, and 5 adjacent normal tissues, which were used as extra controls. The Brain Tumour Tissue Microarray patient demographics are entailed in (Table 1).

Brain Tumour Tissue Bank samples

100 patient samples of various types and grades of brain tumours were received from the London Health Sciences Centre (LHSC) brain tumour tissue bank. Two slides for each case were provided, one to be used as the positive slide and one for the negative slide. 70 of these 100 slides were used in the experiments as some were to be reserved for potential future studies. A total of 140 slides (70 x 2 – one positive and one negative slide) were stained with ERK5 antibody. The 70 slides were broken down by tumour type as follows: 16 Glioblastoma, 15 Oligodendroglioma, 10 Low Grade Glioma, 16 Anaplastic Astrocytoma, and 13 Ependymoma. The data for 3 tumours were not included in the final analysis due to some missing patient information. Patient sample demographics and details from the patient samples obtained from the BTTB are entailed in (Table 2).

Table 1 Samples from the Brain Tumour Tissue Microarray

Gender	Age	Organ	Pathology	Grade	Staining Score
M	33	Brain	Anaplastic (malignant) ependymoma	3	2
F	48	Brain	Anaplastic (malignant) oligoastrocytoma	3	1
M	33	Brain	Anaplastic (malignant) oligodendroglioma	3	0
F	36	Brain	Astrocytoma	2	0
M	25	Brain	Astrocytoma	1	0
M	31	Brain	Astrocytoma	2	3
M	57	Brain	Astrocytoma	2	0
M	36	Brain	Astrocytoma	2	2
M	41	Brain	Astrocytoma	1	1
F	51	Brain	Astrocytoma	2	2
F	15	Brain	Astrocytoma	1	3
M	46	Brain	Astrocytoma (sparse)	1	3
F	30	Brain	Cancer adjacent normal cerebral tissue	-	0
M	41	Brain	Ependymoma	-	3
M	3	Brain	Ependymoma	-	3
M	65	Brain	Glioblastoma	4	0
M	53	Brain	Glioblastoma	3	1
F	10	Brain	Glioblastoma	4	2
F	16	Brain	Glioblastoma	3	0
F	60	Brain	Glioblastoma	3	0
F	39	Brain	Glioblastoma	3	3
F	50	Brain	Glioblastoma	3	0
F	49	Brain	Glioblastoma	3	2
F	6	Brain	Glioblastoma	4	1
M	59	Brain	Glioblastoma	4	2
M	43	Brain	Glioblastoma	4	0
M	64	Brain	Glioblastoma	3	2
M	25	Brain	Glioblastoma	4	0
M	55	Brain	Glioblastoma	3	3
M	43	Brain	Glioblastoma	3	1
M	40	Brain	Glioblastoma	3	0
F	48	Brain	Glioblastoma	3	3
F	30	Brain	Glioblastoma	3	0
M	68	Brain	Glioblastoma	3	1
F	4	Brain	Glioblastoma	3	3
M	41	Brain	Glioblastoma	3	1

M	59	Brain	Glioblastoma (sparse)	3	3
M	36	Brain	Glioblastoma (sparse) with hemorrhage	3	0
F	22	Brain	Glioblastoma multiforme	4	2
M	58	Brain	Glioblastoma multiforme	4	0
F	52	Brain	Glioblastoma multiforme	4	0
F	14	Brain	Medulloblastoma	-	0
M	11	Brain	Medulloblastoma	-	0
M	3	Brain	Medulloblastoma	-	1
M	29	Brain	Oligodendroglioma	2	1
F	14	Brain	Pilocytic astrocytoma	2	3

Table 2 Patient samples from the Brain Tumour Tissue Bank

Gender	**Age**	**Pathology**	**Grade**	**Staining Score**		
				Cytoplasmic	Perinuclear	Nuclear
M	59	Oligodendroglioma	2	1	0	0
M	29	Oligodendroglioma	2	0	1	0
M	48	Oligodendroglioma	2	1	0	0
F	46	Oligodendroglioma	2	1	0	0
F	50	Oligodendroglioma	2	0	0	0
F	36	Oligodendroglioma	2	1	0	0
M	38	Oligodendroglioma	2	1	0	0
F	56	Oligodendroglioma	2	0	0	0
F	40	Oligodendroglioma	2	1	0	0
M	65	Oligodendroglioma	2	2	0	0
F	28	Oligodendroglioma	2	0	1	0
F	38	Oligodendroglioma	2	0	1	0
M	43	Oligodendroglioma	2	1	1	0
F	31	Oligodendroglioma	2	1	0	0
M	65	Oligodendroglioma	2	2	0	0
F	37	LGG – Astrocytoma	2	0	2	0
M	34	LGG – Astrocytoma	2	0	2	0
F	66	LGG – Astrocytoma	2	0	1	0
M	28	LGG – Astrocytoma	2	0	1	0
F	38	LGG – Astrocytoma	2	0	0	1
M	67	LGG – Astrocytoma	2	0	1	0
M	79	LGG – Astrocytoma	2	0	1	0
M	45	LGG – Astrocytoma	2	0	2	0
F	54	LGG – Astrocytoma	2	0	1	0
F	55	LGG – Astrocytoma	2	1	0	1
F	49	Anaplastic Astrocytoma	3	2	0	0
F	66	Anaplastic Astrocytoma	3	2	0	0
M	57	Anaplastic Astrocytoma	3	2	0	0
M	61	Anaplastic Astrocytoma	3	2	0	0
M	61	Anaplastic Astrocytoma	3	1	0	0
M	66	Anaplastic	3	1	0	0

		Astrocytoma				
M	71	Anaplastic Astrocytoma	3	2	0	0
M	55	Anaplastic Astrocytoma	3	3	0	0
M	85	Anaplastic Astrocytoma	3	2	0	0
F	47	Anaplastic Astrocytoma	3	2	0	0
F	77	Anaplastic Astrocytoma	3	1	0	0
M	69	Anaplastic Astrocytoma	3	0	0	0
M	50	Anaplastic Astrocytoma	3	2	0	0
M	52	Anaplastic Astrocytoma	3	3	0	0
F	28	Anaplastic Astrocytoma	3	3	0	0
F	66	Glioblastoma Multiforme	4	2	0	0
M	88	Glioblastoma Multiforme	4	2	0	0
M	57	Glioblastoma Multiforme	4	3	0	0
F	81	Glioblastoma Multiforme	4	3	0	0
F	82	Glioblastoma Multiforme	4	1	0	0
F	69	Glioblastoma Multiforme	4	3	0	0
M	75	Glioblastoma Multiforme	4	3	0	0
F	76	Glioblastoma Multiforme	4	3	0	0
F	56	Glioblastoma Multiforme	4	2	0	0
M	73	Glioblastoma Multiforme	4	1	0	0
M	50	Glioblastoma Multiforme	4	2	0	0
F	20	Glioblastoma Multiforme	4	3	0	0
M	76	Glioblastoma Multiforme	4	1	0	0

M	72	Glioblastoma Multiforme	4	1	0	0
F	25	Glioblastoma Multiforme	4	1	0	0
F	23	Ependymoma	2	0	0	0
M	35	Ependymoma	2	0	0	0
M	48	Ependymoma	2	0	0	0
F	5	Ependymoma	2	0	0	0
F	17	Ependymoma	2	1	0	0
F	8	Ependymoma	2	0	0	0
F	17	Ependymoma	2	1	0	0
F	50	Ependymoma	2	1	0	0
M	18	Ependymoma	2	0	0	0
M	16	Ependymoma	2	1	0	0
M	16	Ependymoma	2	1	0	0
M	4	Ependymoma	2	0	0	0
F	28	Ependymoma	2	1	0	0

4.3 Methods

Below is a detailed overview of the steps used during each study performed during my experiments. A summary of the methods used can be seen in (Figure 7).

Study #1 (Pilot Study):

The expression of ERK5 was first investigated using a brain tumour tissue microarray. Ameloblastoma (S06-28990) and Cavernous hemangioma (S11-41) tissues were prepared and sectioned according to standard protocol (Appendix A). Immunohistochemistry protocol for ERK 5 (C-20) was performed on positive and negative slides for the brain tumour tissue microarrays, in addition to the controls. The TMA was stained with ERK5 antibody (ERK 5 (C-20), Santa Cruz Biotechnology, Inc., Cat No. sc-1284) according to the IHC protocol for ERK5 (c-20) as described under laboratory procedures below. The expression of ERK5 was analyzed by immunohistochemistry. The reagents used in the experiment were Goat Impress (Vector Laboratories Cat No. MP7405) and DAB (DAB Peroxidase Substrate Kit, Vector Laboratories Cat No. SK4100).

Study #2:

I further investigated the expression of ERK5 using 70 patient samples of brain tumours, which were received from the BTTB at the London Health Sciences Centre, after obtaining the appropriate approvals from Western University's Research Ethics Board (REB), and London Health Sciences Centre's Clinical Research Impact Committee (CRIC) and Tissue and Archive (TA) committee.

The BTTB slides from each case were reviewed to confirm the WHO grade by a neuropathology resident. The neuropathology resident selected the cases to be cut and sent the list of selected cases to the Pathology Lab assistant, who cut the paraffin embedded blocks and prepared the slides. The paraffin processed blocks were sectioned according to the Microtomy protocol (Appendix A). Once the slides were cut, I picked them up and prepared them for the staining process.

The tissues were stained with ERK5 antibody (ERK 5 (C-20), Santa Cruz Biotechnology, Inc. Cat No. sc-1284) according to the IHC protocol for ERK5 (c-20) as described under laboratory procedures below. The expression of ERK5 was analyzed by immunohistochemistry. Ameloblastoma (S06-28990) and Cavernous hemangioma (S11-41) tissues were prepared and sectioned according to standard protocol.

The reagents used in the experiment were Goat Impress (Vector Laboratories Cat No. MP7405) and DAB (Vector Laboratories Cat No. SK4100).

Laboratory Procedures:

Prior to beginning the IHC protocol on the TMA and BTTB samples, the dilution of ERK5 antibody to be used in the experiments was first determined. A dilution range between 1/50-1/500 was recommended on the ERK5 antibody datasheet. After performing titrations to determine what dilution worked best for the ERK5 antibody, a dilution of 1/100 was selected for this experiment as it gave the best staining with minimum background. A pathologist reviewed the slides to confirm that this dilution was the most appropriate for the antibody being used in the experiments.

On Day 1 of the experiment, prior to proceeding with the staining protocol, the slides were de-paraffinized and rehydrated as incomplete removal of paraffin can cause poor staining of the section. Next, the slides were quenched with fresh 3% H2O2 in methanol for 5 minutes, rinsed in running tap water, then rinsed in PBS (Phosphate Buffer Solution) for 5 minutes on the shaker. The slides then underwent heat-mediated antigen retrieval in citrate buffer (2.1 g anhydrous citric acid/L (water) pH to 6.0 with concentrated NaOH) in the decloaking chamber. Most formalin-fixed tissue requires an antigen retrieval step before immunohistochemical staining can proceed. This is due to the formation of methylene bridges during fixation, which cross-link proteins and therefore mask antigenic sites. The antigen retrieval step serves to break the methylene bridges and expose the antigenic sites in order to allow the antibodies to bind.

The slides were then rinsed in running tap water and put in PBS for 5 minutes, blocked in 10% horse serum for 30 minutes at room temperature, then incubated with ERK5 at 1/100 dilution at 4 degrees overnight.

On Day 2 of the experiment, the slides were rinsed in PBS for 5 minutes on the shaker, then incubated with secondary goat impress reagent for 30 minutes at room temperature. The slides were then rinsed in PBS for 5 minutes on the shaker, and then incubated with DAB solution for 10 minutes.

DAB (3, 3 -diaminobenzidine) HRP substrate produces a dark brown reaction product, which was indeed observed in the experiments.

After incubation, the slides were then rinsed with H20, counterstained with hematoxylin, then rehydrated using the rehydrating protocol. The final step was to mount and coverslip the slides in cytoseal under the fume hood.

Scoring and Analyses:

ERK5 staining was examined using a microscope. ERK5 expression, as a percentage of cells staining for total tumour cells, was scored by a neuropathology resident and myself. The cells were scored according to the percentage and intensity of cytoplasmic, nuclear and perinuclear staining, if any. A quantitative analysis was based on the percentage of stained cells and intensity of the staining, and was defined as follows: 0, no appreciable staining in cells (0 to <10%); 1, weak intensity in cells (between 10% and 30%); 2, intermediate intensity of staining (30% to 60%); and 3, strong intensity of staining (>60%). The scoring criteria used are entailed in (Table 3).

I confirmed the histological diagnoses of each tumour sample received from the BTTB by reviewing the surgical pathology report for each of the patient tissue specimens. Unfortunately I did not have access to these reports for the samples in the TMA.

Images of the slides were taken using the Aperio Digital Pathology Slide Scanner, available at the Pathology Department at the London Health Sciences Centre, University Hospital Campus. An application called 'ImageScope' was the software used to view the slide images taken by the Slide Scanner. Images of the slides are seen below (Figures 10 to 29).

Table 3 ERK5 Staining Scoring Key

Scoring Key	
Score	Criteria
0	no appreciable staining in cells (0 to <10%)
1	weak intensity in cells (between 10% and 30%)
2	intermediate intensity of staining (30% to 60%)
3	strong intensity of staining (>60%)

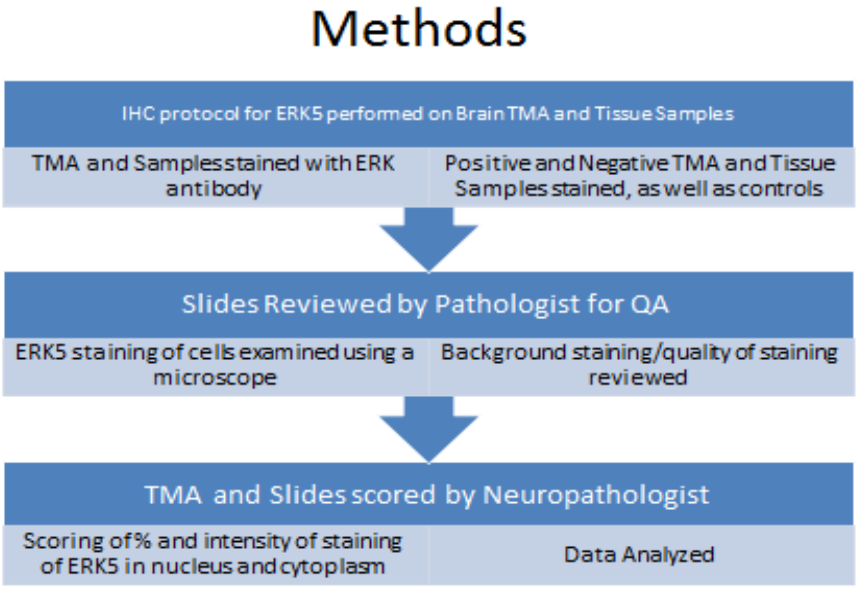


Figure 7 Methods summary. This is a simple summary of the methods used during my experiments. I used immunohistochemistry in my study on a brain tumour TMA and pathology samples from the BTTB in order to examine the expression of ERK5 in those samples. The slides were reviewed by a neuropathologist for QA, then examined under a microscope and scored for % and intensity of ERK5 staining in the nucleus and cytoplasm. The data was then analyzed by statistical analysis.

4.4 Statistical Analysis

Statistical analysis was performed by Dr. Michael Miller, a Research Consultant Statistician with Western University, and the Department of Paediatrics, Children's Hospital, London Ontario. Statistical analysis was performed using the SPSS v.24 Software (IBM Corporation, Armonk, NY, USA).

Analyses were performed on the data from:

- Study 1 only
- Study 2 only
- Study 1 & 2 combined

The frequency and percent of ERK5 staining in various types and grades of brain tumours were captured and are displayed in (Tables 7, 12 and 17). The Kruskal-Wallis test was used to determine whether differences in ERK5 staining existed between tumour grade. The Mann-Whitney U test was used to

determine whether differences in ERK5 staining existed between the higher vs lower grades, as well as between ERK5 staining and gender. Spearman's Rho was used to determine whether there was a correlation between ERK5 staining and age. A probability (P) value <.05 was considered significant.

Chapter 5

5 Results

The result for both Study #1 and Study #2 are detailed below.

5.1 Results for Study #1 (Pilot Study)

In my first study, I reported the expression of ERK5 in various brain tumours using TMA and IHC. I found that ERK5 was present in a substantial number of the samples in the TMA. ERK5 staining was present in 57.3% of all brain tumour samples examined, with some level of ERK5 staining present in 57.5% of Glioblastoma samples (Table 4). 'Intermediate' and 'strong' staining were present in 27.5% of Glioblastomas and 50% of Astrocytomas respectively (Figure 8). ERK5 staining was very minimal in normal brain tissue controls, with significant staining only seen in 1 of 5 healthy brain tissue samples (Table 5).

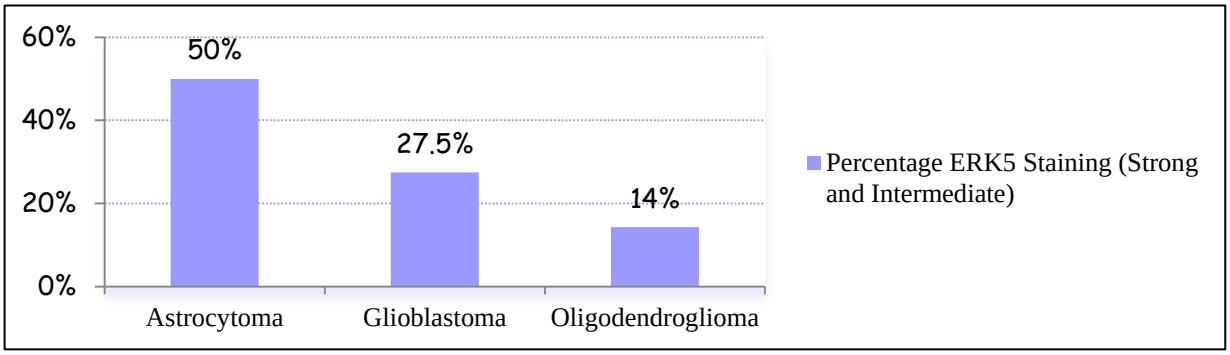


Figure 8 Percentage ERK5 staining in brain tumour samples (Study #1). The percentage of ERK5 staining (both intermediate and Strong staining combined) were present in 27.5% of Glioblastomas, 50% of Astrocytomas, and 14% of Oligodendrogliomas.

Table 4 Glioblastoma Staining Results

All Glioblastomas		
Score	Criteria	
0	no appreciable staining in cells (0 to <10%)	16
1	weak intensity in cells (between 10% and 30%)	12
2	intermediate intensity of staining (30% to 60%)	6
3	strong intensity of staining (>60%)	5
	Total	40

Table 5 ERK5 Staining % from Study #1 (Pilot)

Diagnosis	No. of TMA cores	% ERK5 staining
Normal	5	1/5 (20%)
Astrocytoma	14	7/14 (50%)
Glioblastoma	40	11/40 (27.5%)
Oligodendroglioma	7	1/7 (14%)

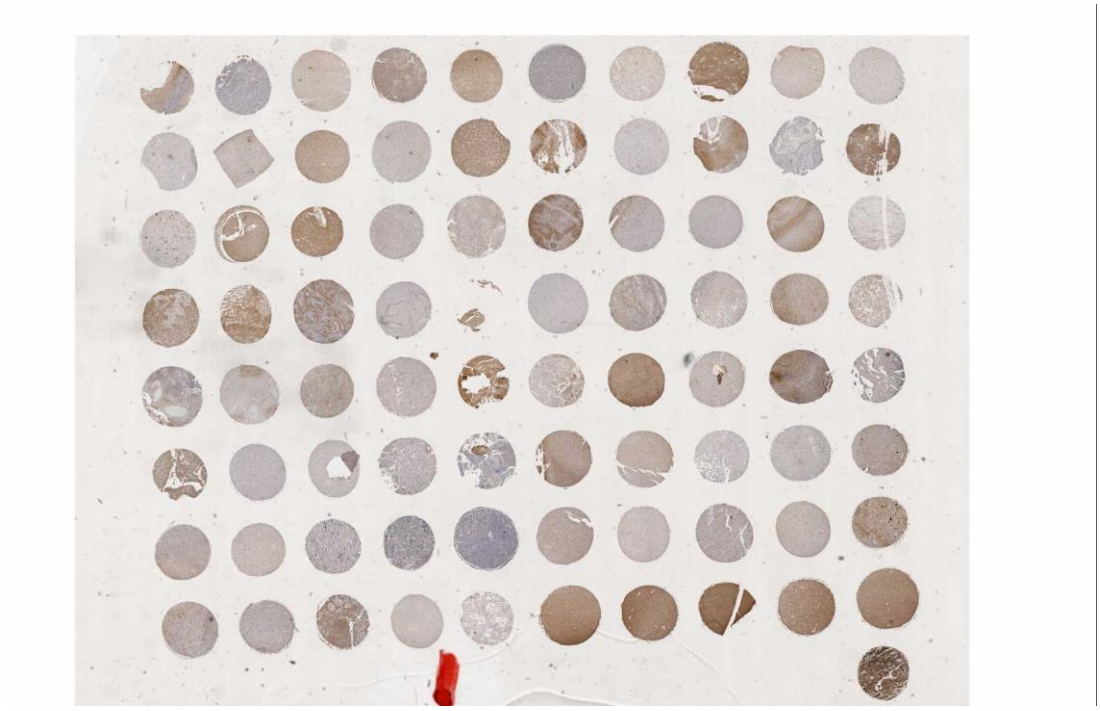


Figure 9 Brain Tumour Tissue Microarray stained with ERK5 antibody. This image shows the brain tissue microarray slide after it has been stained with ERK5 antibody. There are 80 cores of various types of brain tumours in this TMA, all of which were analyzed and scored according to the percentage of staining and the intensity of staining of ERK5.

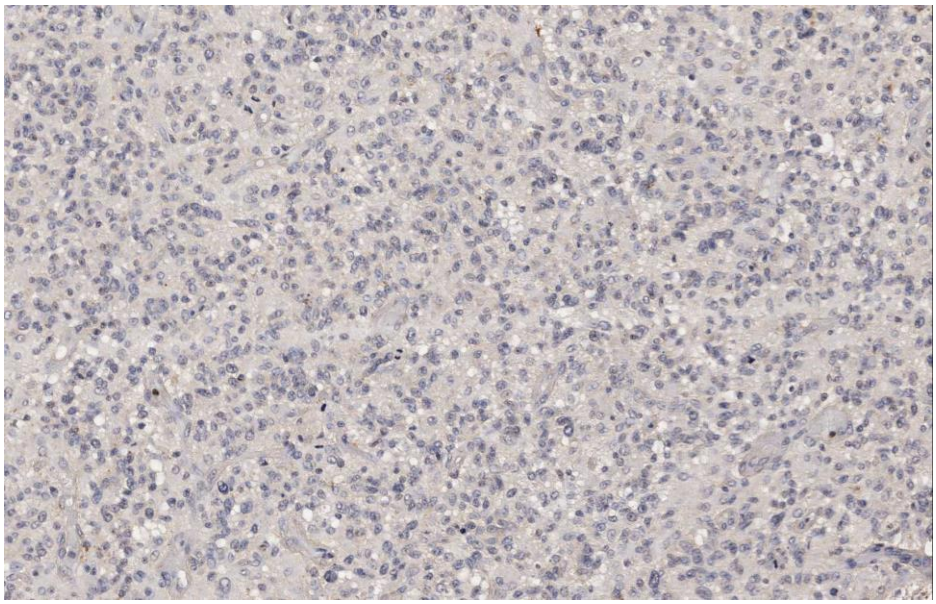


Figure 10 GBM with ERK5 staining score = 0. This figure shows a GBM sample that has a staining score of 0. After staining this tissue with ERK5 antibody, there was no appreciable staining seen in the tumour cells, with between 0 to < 10% of tumour cells stained.

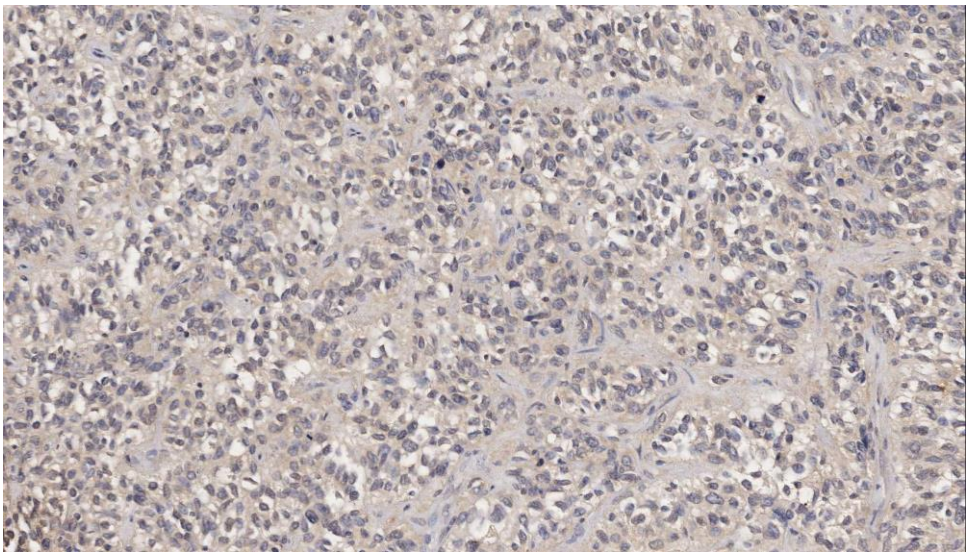


Figure 11 GBM with ERK5 staining score = 1. This figure shows a GBM sample that has a staining score of 1. After staining this tissue with ERK5 antibody, there was a weak intensity of staining seen in the tumour cells, with between 10 to 30 % of tumour cells stained.

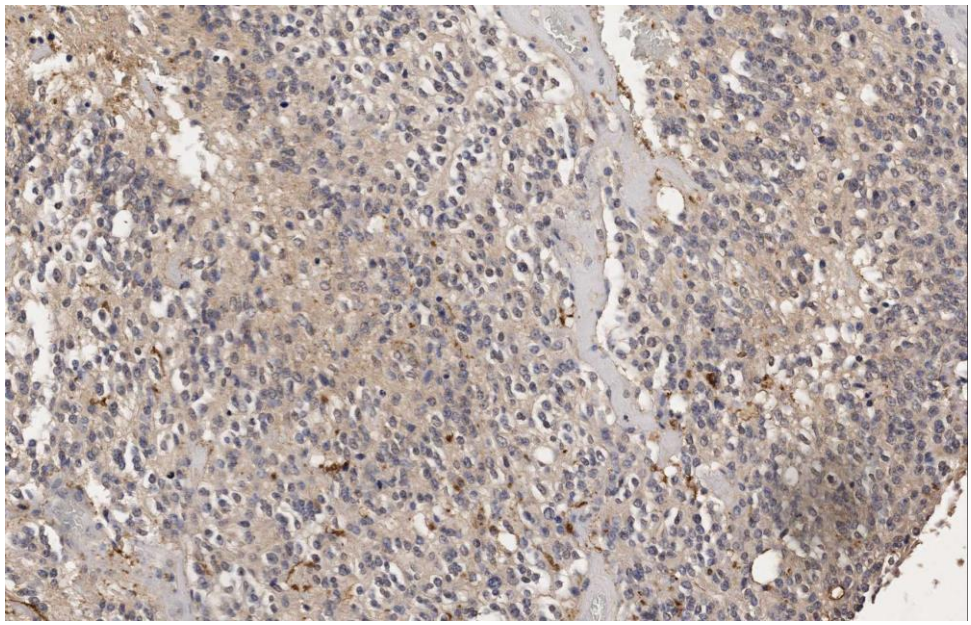


Figure 12 GBM with ERK5 staining score = 2. This figure shows a GBM sample that has a staining score of 2. After staining this tissue with ERK5 antibody, there was an intermediate intensity of staining seen in the tumour cells, with between 30 to 60% of tumour cells stained.

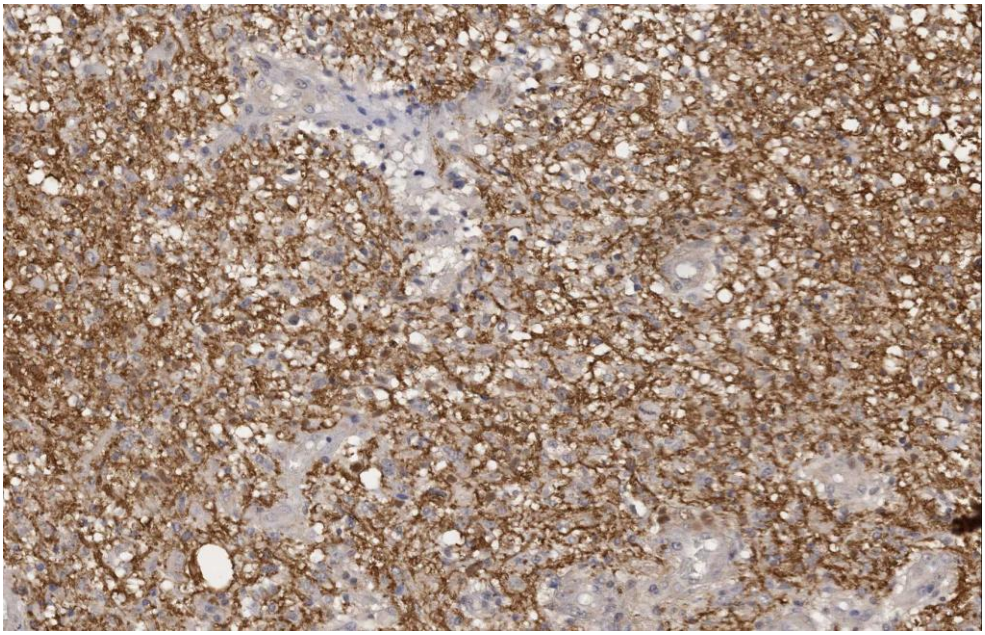


Figure 13 GBM with ERK5 staining score = 3. This figure shows a GBM sample that has a staining score of 3. After staining this tissue with ERK5 antibody, there was a strong intensity of staining seen in the tumour cells, with > 60 % of tumour cells stained.

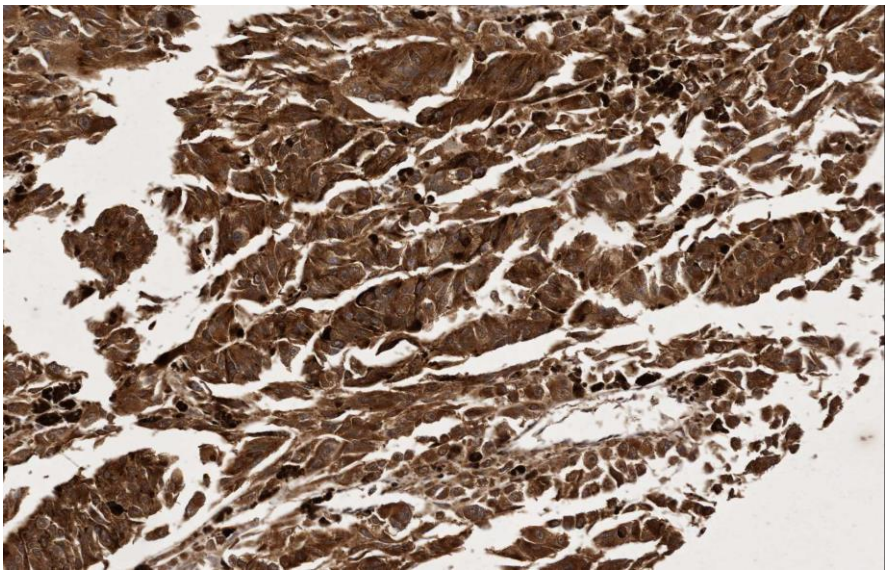


Figure 14 Control (skin) stained with ERK5. This is a tissue sample from a 58 year old male patient with malignant melanoma. This skin sample was included in the brain tumour TMA and was used as an additional control. It was stained with ERK5 antibody and as can be seen in the image, showed significant staining of ERK5.

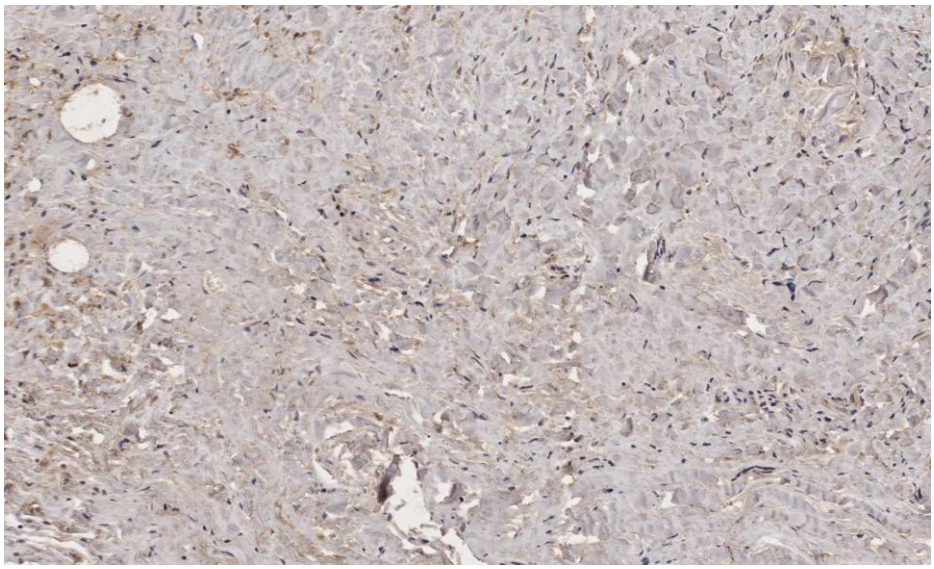


Figure 15 Negative slide stained with ERK5. This is an example of one of the negative controls used in my IHC experiments. This is a negative reagent control slide that was stained in the same manner as the positive control. The primary antibody, ERK5, was omitted on all the negative slides so as to ensure specificity and sensitivity of the ERK5 antibody and rule out additional/non-specific staining.

5.2 Results for Study # 2

My results from study #2 were consistent with my previous results in study #1 in that they showed that ERK5 was present in the cytoplasms of 65.2% of all brain tumour samples examined, with some level of ERK5 staining present in the cytoplasms of 100% of all of the Glioblastoma samples examined (Table 6). 'Weak', 'Intermediate' and 'strong' staining of ERK5 were present in 33.3%, 26.7% and 40% of the cytoplasms of Glioblastoma samples respectively (Table 6). 'Weak', 'Intermediate' and 'Strong' staining of ERK5 was present in 20%, 53.3% and 20% of the cytoplasms of the grade III Anaplastic Astrocytoma samples respectively (Table 6).

ERK5 was only present in the nucleus of 2.9% of all brain tumours examined. Specifically, ERK5 staining was only seen in the nucleus of the grade II LGG Astrocytoma. No other staining was seen in the nucleus of any of the other samples.

In the Grade II LGG Astrocytomas, 'Weak' and 'intermediate' perinuclear staining was seen in 50% and 30% of the samples respectively. 'Weak' staining was seen in the cytoplasm of 10% of the samples, and in the nucleus of 20% of the grade II LGG samples (Table 6).

'Weak' and 'Intermediate' staining of ERK5 was present in 50% and 12.5% of the cytoplasms of the Oligodendroglioma samples respectively. 'Weak' perinuclear staining was seen in 37.5% of the Oligodendroglioma samples. "Weak" ERK5 staining of the cytoplasm was only seen in 42.9% of the Ependymoma samples examined. No nuclear or perinuclear ERK5 staining was seen in the Ependymoma samples (Table 6).

ERK5 staining was not present in the normal parts of the brain, but only in areas where tumour was present.

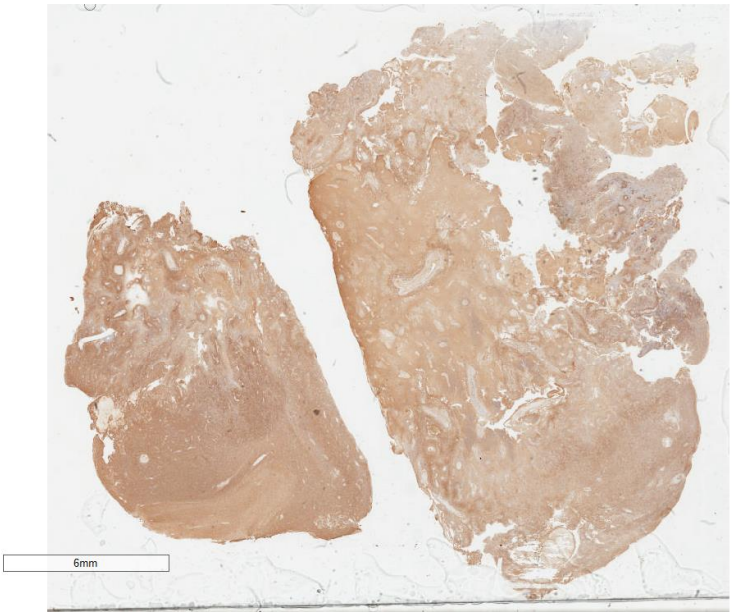
Table 5 ERK5 Staining Results From Study # 2

Staining of ERK5 in Cytoplasm						
Grade	Type	None	Weak	Intermediate	Strong	Total %
IV	Glioblastoma	0	33.3	26.7	40	100
III	Anaplastic Astrocytoma	6.7	20	53.3	20	100
II	Astrocytoma, LGG	90	10	0	0	100
II	Oligodendroglioma	37.5	50	12.5	0	100
I	Ependymoma	57.1	42.9	0	0	100

Staining of ERK5 in Nucleus						
Grade	Type	None	Weak	Intermediate	Strong	Total %
IV	Glioblastoma	100	0	0	0	100
III	Anaplastic Astrocytoma	100	0	0	0	100
II	Astrocytoma, LGG	80	20	0	0	100
II	Oligodendroglioma	100	0	0	0	100
I	Ependymoma	100	0	0	0	100

Perinuclear Staining of ERK5						
Grade	Type	None	Weak	Intermediate	Strong	Total %
IV	Glioblastoma	100	0	0	0	100
III	Anaplastic Astrocytoma	100	0	0	0	100
II	Astrocytoma, LGG	20	50	30	0	100
II	Oligodendroglioma	62.5	37.5	0	0	100
I	Ependymoma	100	0	0	0	100

A)



B)

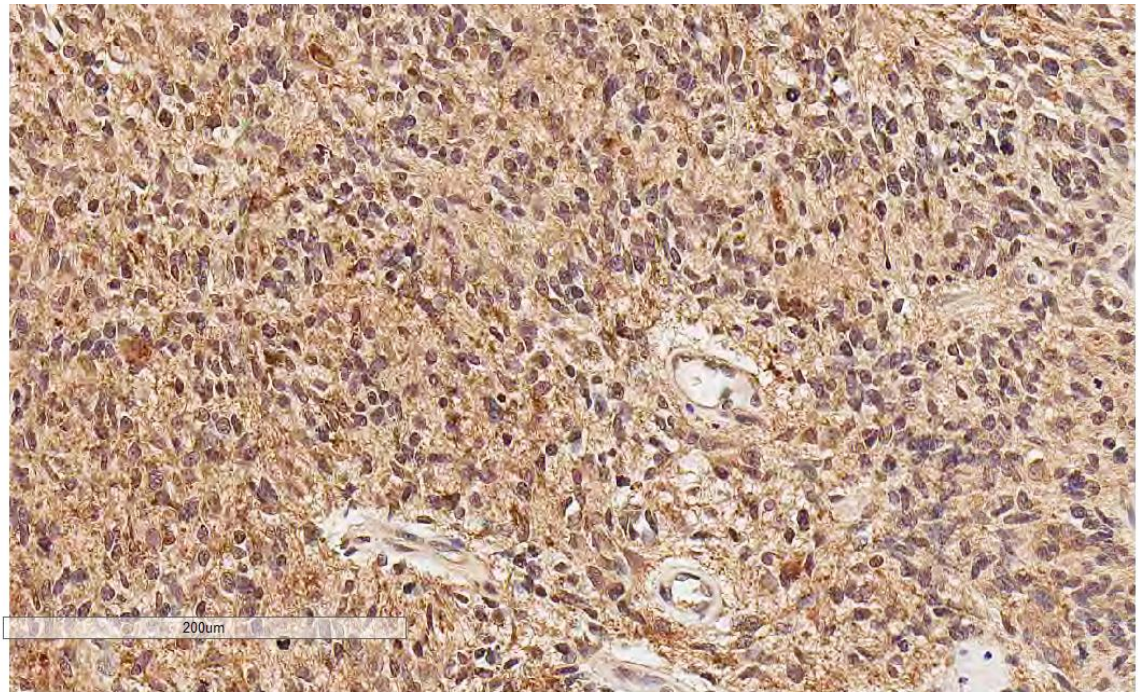
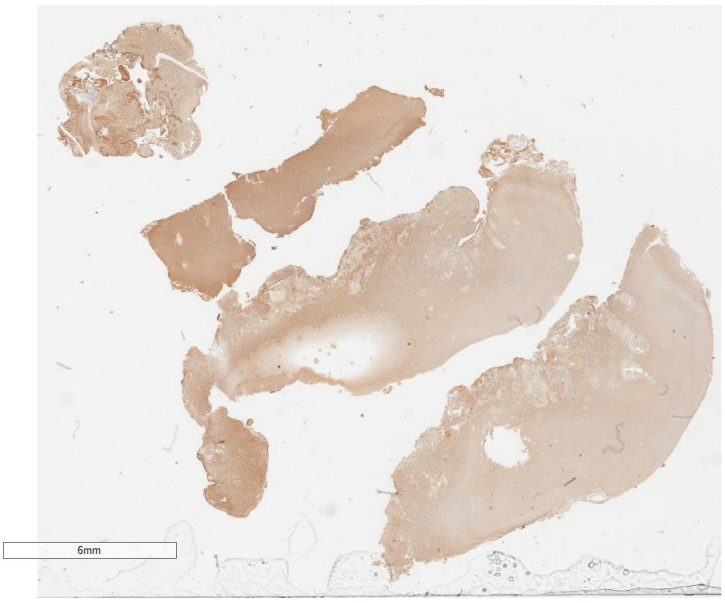


Figure 16 GBM (grade IV), ERK5 staining score = 3. A) This figure shows a GBM sample from a 75 year old male that was stained with ERK5 antibody, with a scale of 6mm. B) As seen in this image with a scale of 200um, this GBM sample was assigned an ERK5 staining score of 3. There was a strong intensity of staining seen in the tumour cells, with > 60% of tumour cells stained.

A)



B)

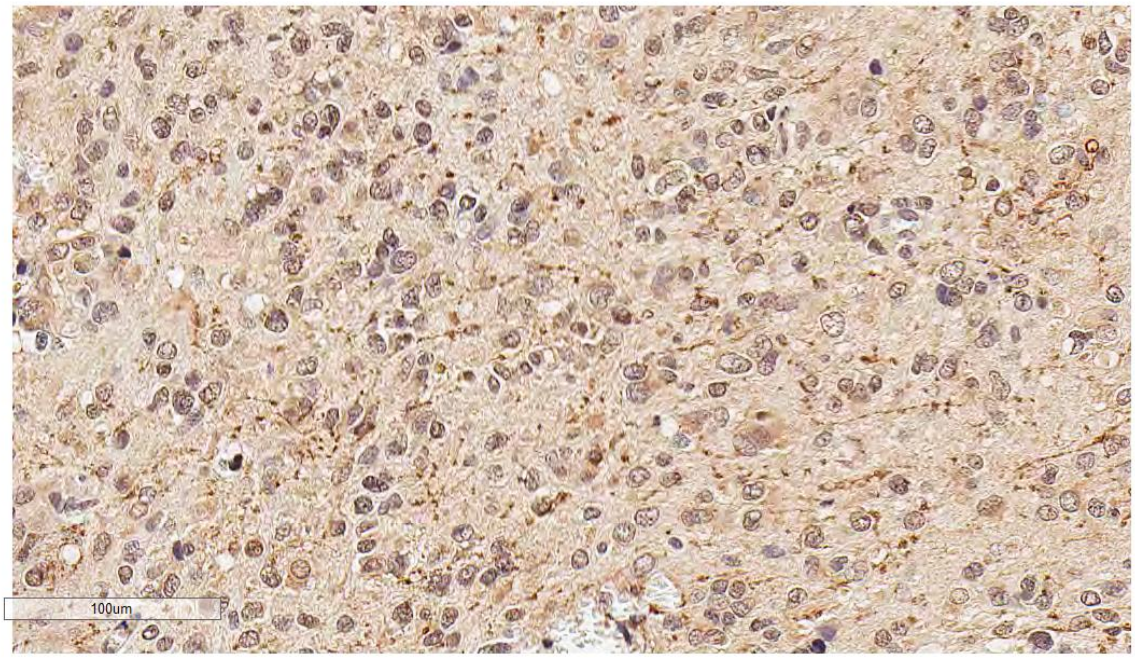
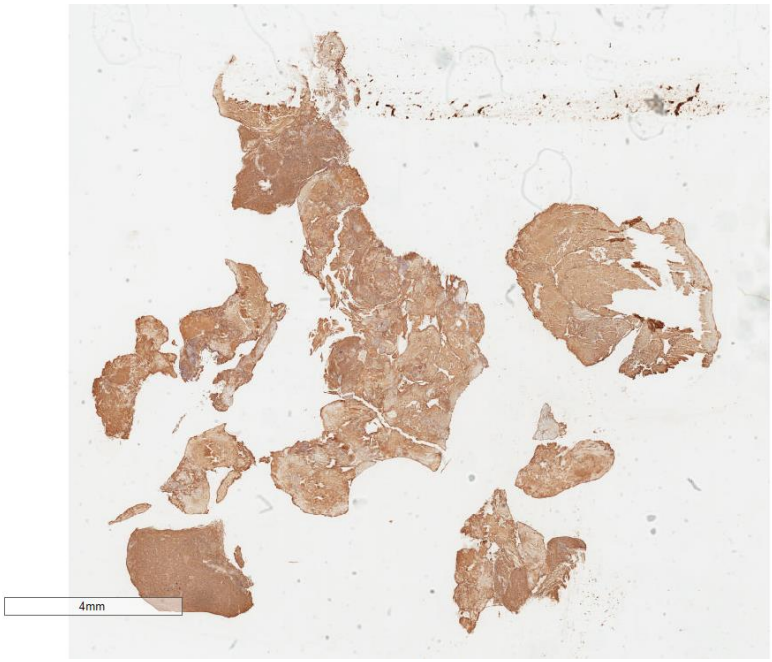


Figure 17 GBM (grade IV), ERK5 staining score =3. A) This figure shows a GBM sample from a 57 year old male that was stained with ERK5 antibody, with a scale of 6mm. **B)** As seen in this image with a scale of 100um, this GBM sample was assigned an ERK5 staining score of 3. There was a strong intensity of staining seen in the tumour cells, with > 60% of tumour cells stained.

A)



B)

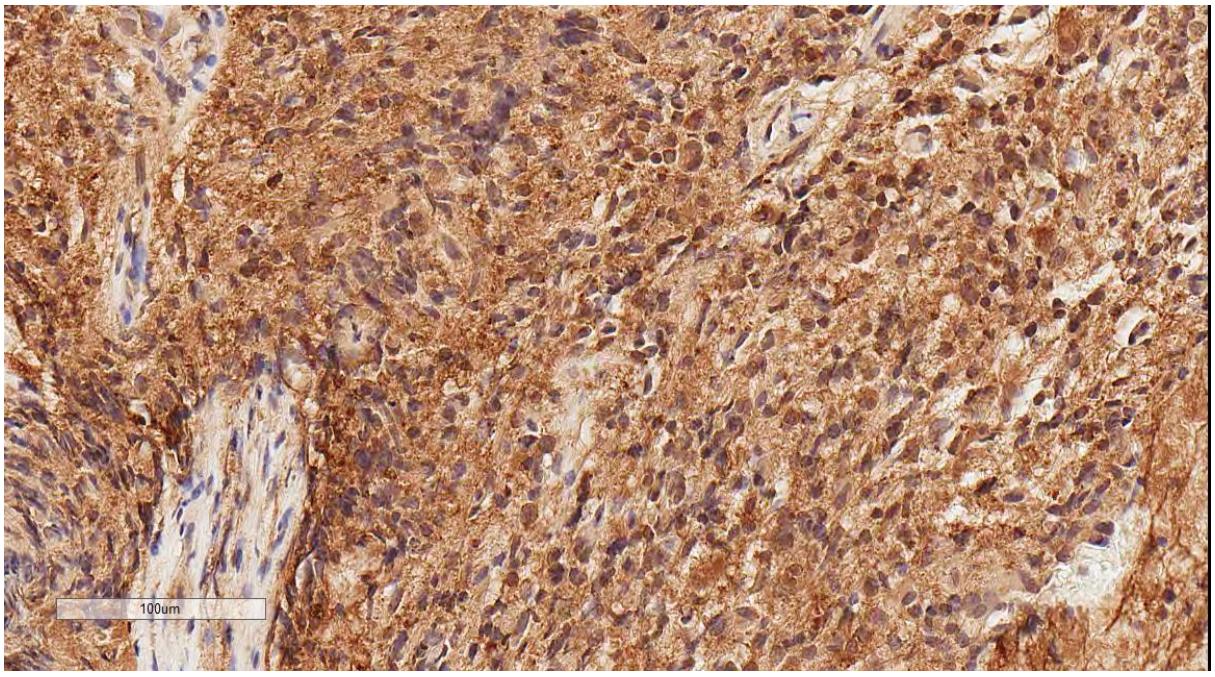
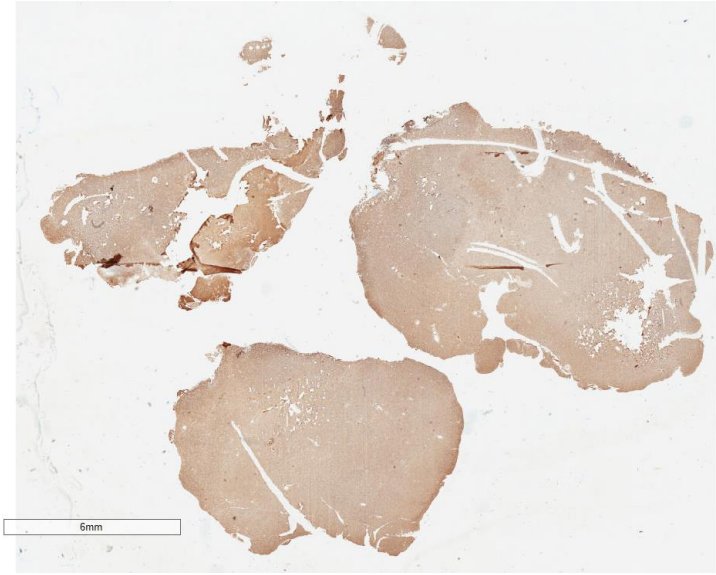


Figure 18 GBM (grade IV), ERK5 staining score = 3. A) This figure shows a GBM sample from a 69 year old female that was stained with ERK5 antibody, with a scale of 4mm. **B)** As seen in this image with a scale of 100um, this GBM sample was assigned an ERK5 staining score of 3. There was a strong intensity of staining seen in the tumour cells, with > 60% of tumour cells stained.

A)



B)

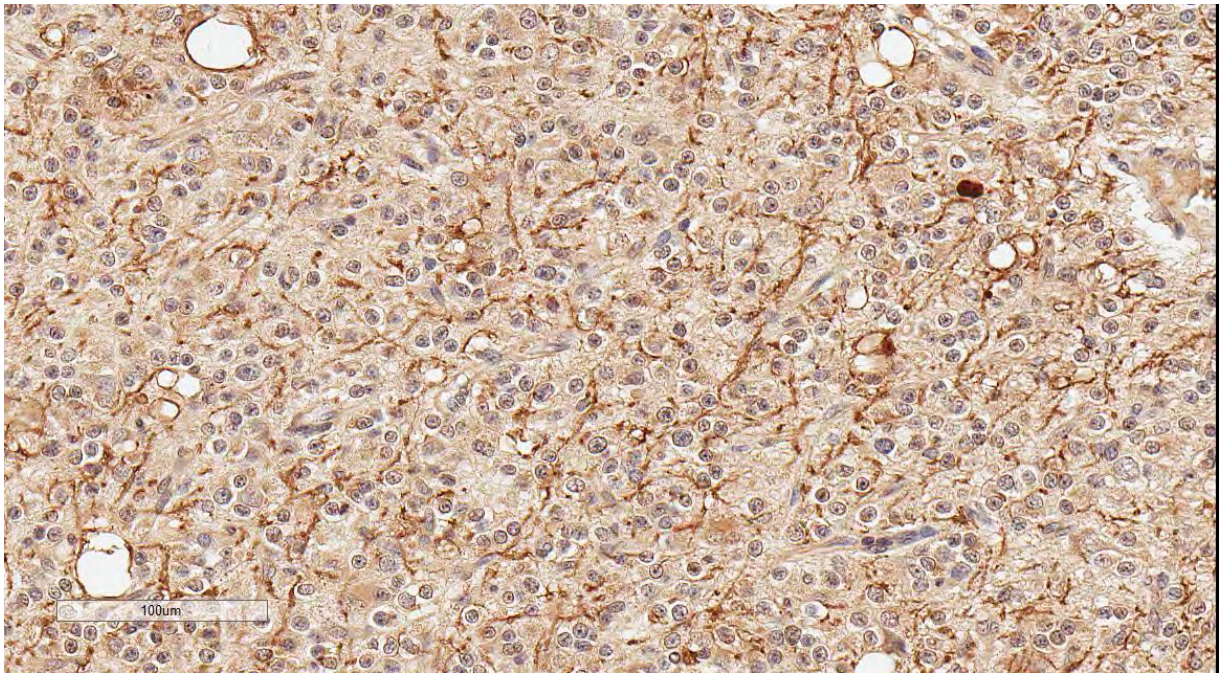
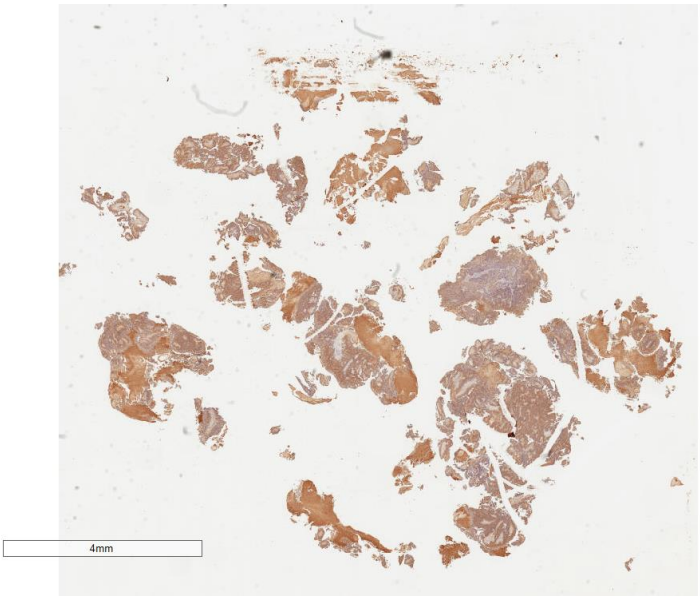


Figure 19 Oligodendroglioma (grade II), ERK5 staining score = 2. A) This figure shows an oligodendroglioma sample from a 46 year old male that was stained with ERK5 antibody, with a scale of 6mm. **B)** As seen in this image with a scale of 100um, this oligodendroglioma sample was assigned an ERK5 staining score of 2. There was an intermediate intensity of staining seen in the tumour cells, with between 30 to 60% of tumour cells stained.

A)



B)

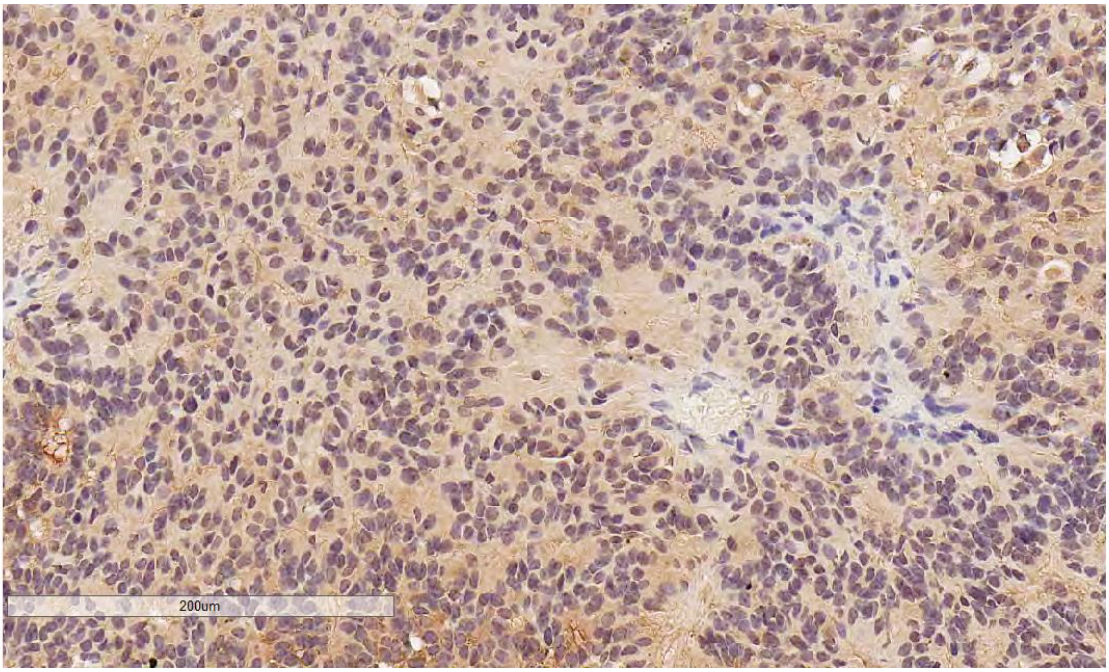
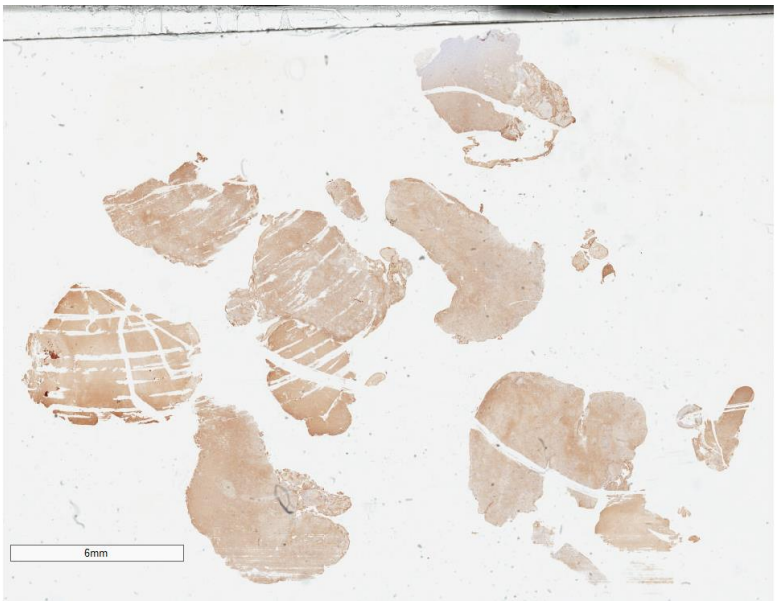


Figure 20 Ependymoma (grade I), ERK5 staining score = 1. A) This figure shows an ependymoma sample from a 17 year old female that was stained with ERK5 antibody, with a scale of 4mm. **B)** As seen in this image with a scale of 200um, this ependymoma sample was assigned an ERK5 staining score of 1. There was a weak intensity of staining seen in the tumour cells, with between 10 and 30% of tumour cells stained.

A)



B)

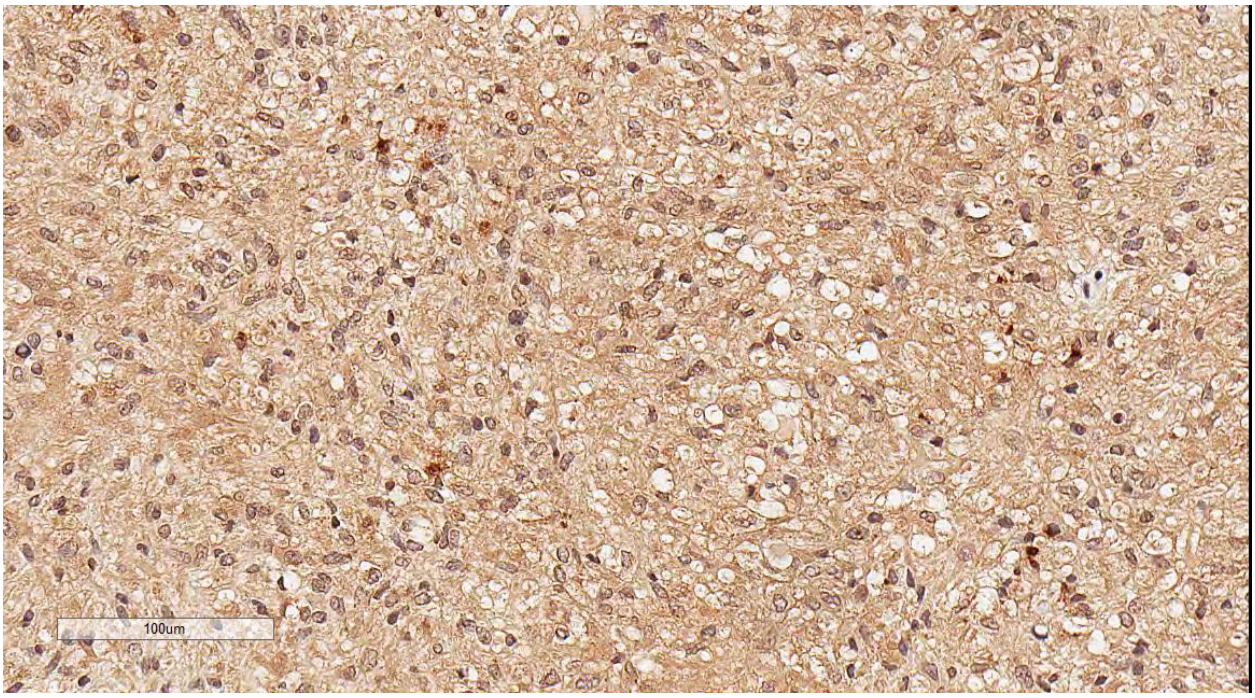
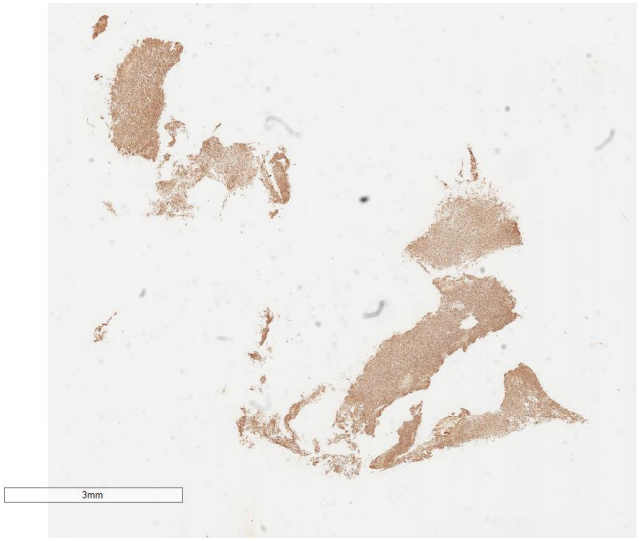


Figure 21 GBM (grade IV), ERK5 staining score =3. A) This figure shows a GBM sample from a 76 year old female that was stained with ERK5 antibody, with a scale of 6mm. **B)** As seen in this image with a scale of 100um, this GBM sample was assigned an ERK5 staining score of 3. There was a strong intensity of staining seen in the tumour cells, with > 60% of tumour cells stained.

A)



B)

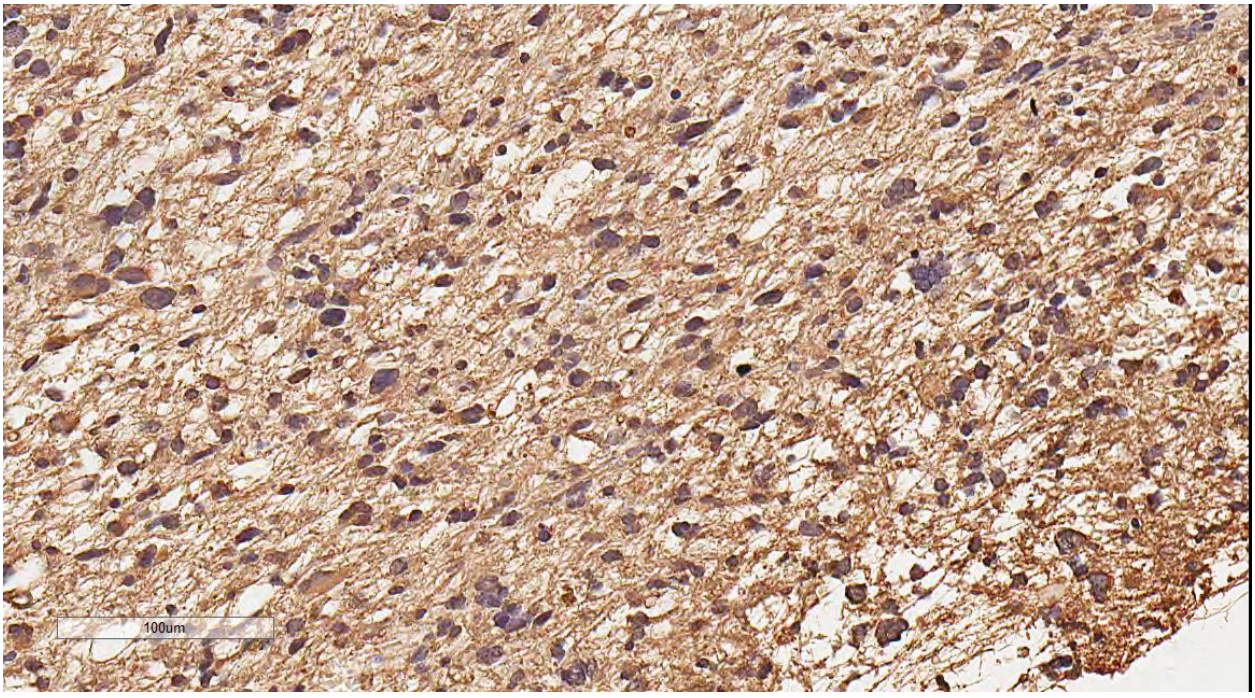
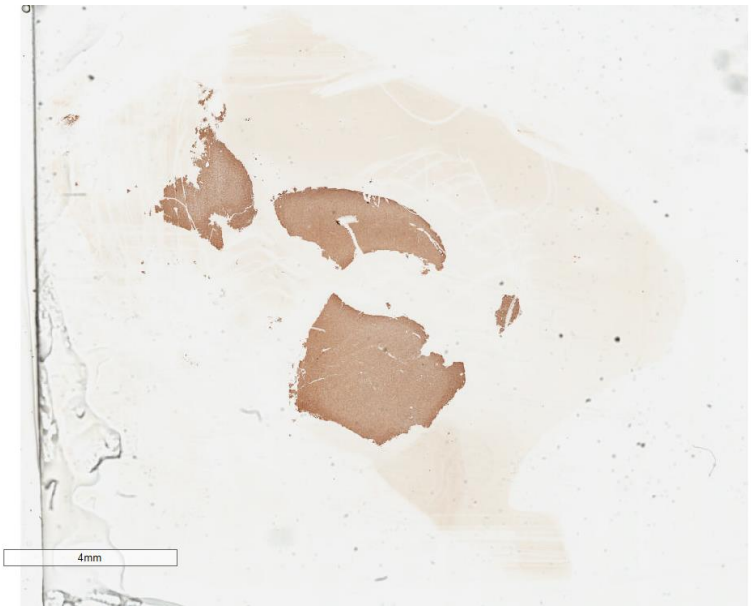


Figure 22 Anaplastic Astrocytoma (grade III), ERK5 staining score = 2. A) This figure shows an anaplastic astrocytoma sample from a 47 year old female that was stained with ERK5 antibody, with a scale of 3mm. **B)** As seen in this image with a scale of 100um, this anaplastic astrocytoma sample was assigned an ERK5 staining score of 2. There was an intermediate intensity of staining seen in the tumour cells, with between 30 to 60% of tumour cells stained.

A)



B)

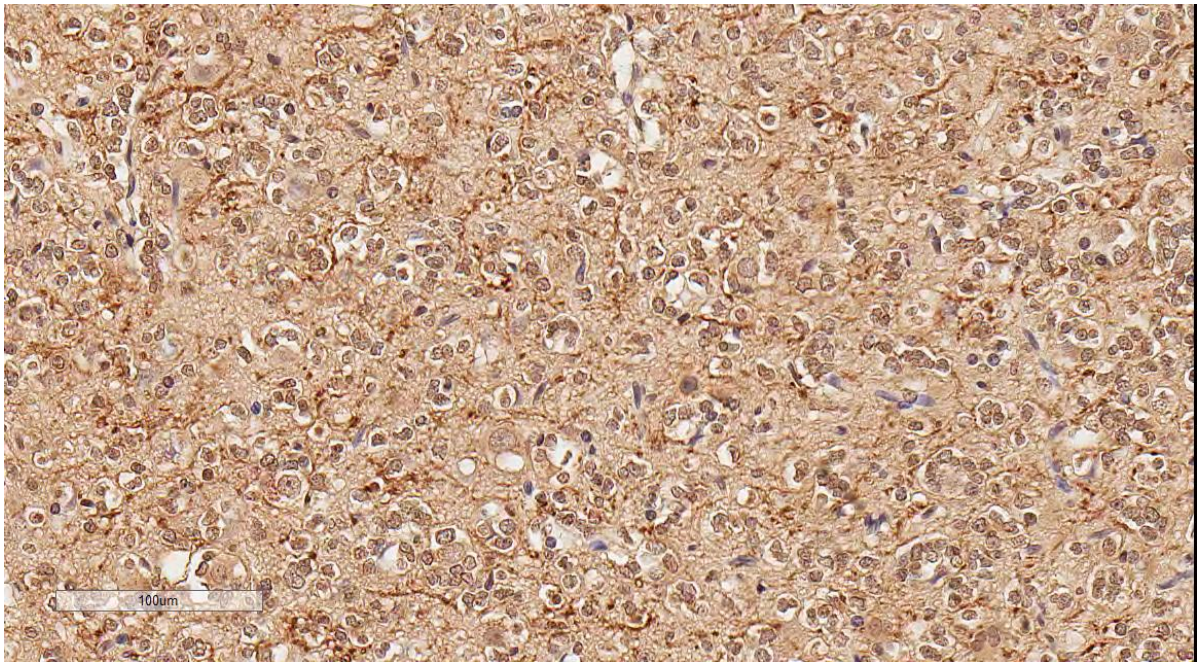
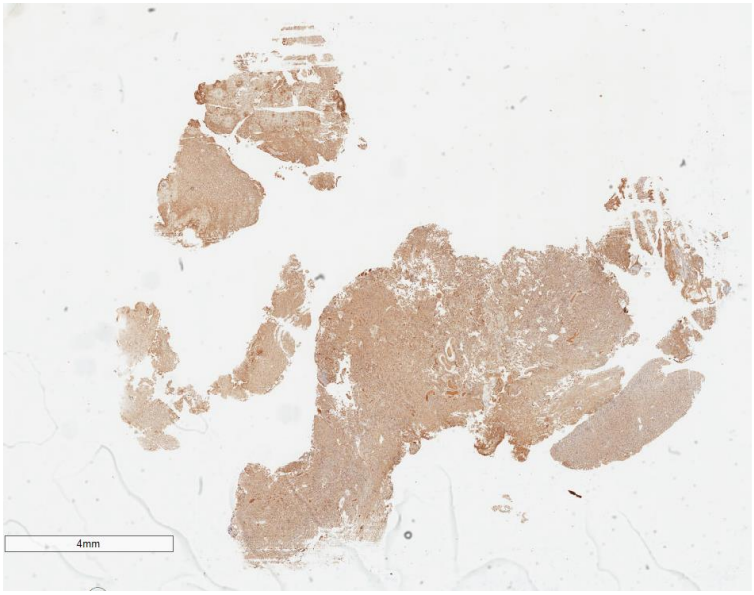


Figure 23 Oligodendroglioma (grade II), ERK5 staining score = 2. This figure shows an oligodendroglioma sample from a 65 year old male that was stained with ERK5 antibody, with a scale of 4mm. **B)** As seen in this image with a scale of 100um, this oligodendroglioma sample was assigned an ERK5 staining score of 2. There was an intermediate intensity of staining seen in the tumour cells, with between 30 to 60% of tumour cells stained.

A)



B)

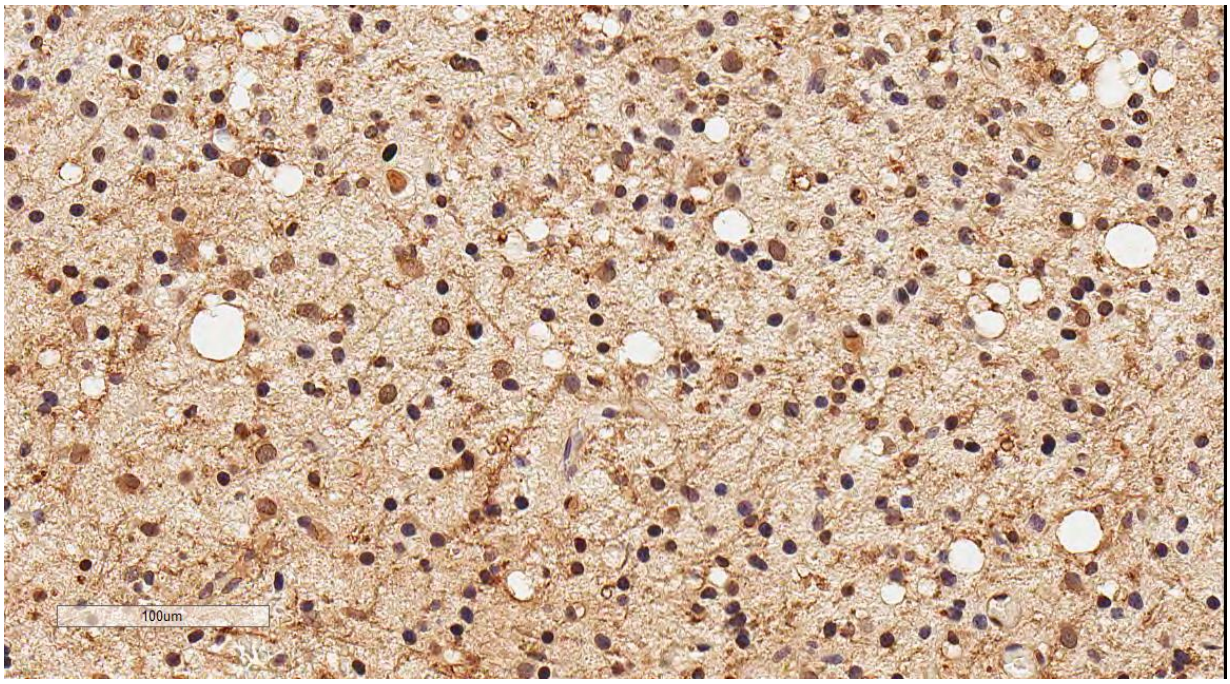


Figure 24 LGG (grade II astrocytoma), ERK5 staining score = 2. A) This figure shows a LGG sample from a 34 year old male that was stained with ERK5 antibody, with a scale of 4mm. **B)** As seen in this image with a scale of 100um, this LGG sample was assigned an ERK5 staining score of 2. There was an intermediate intensity of staining seen in the tumour cells, with between 30 to 60% of tumour cells stained.

A)

B)

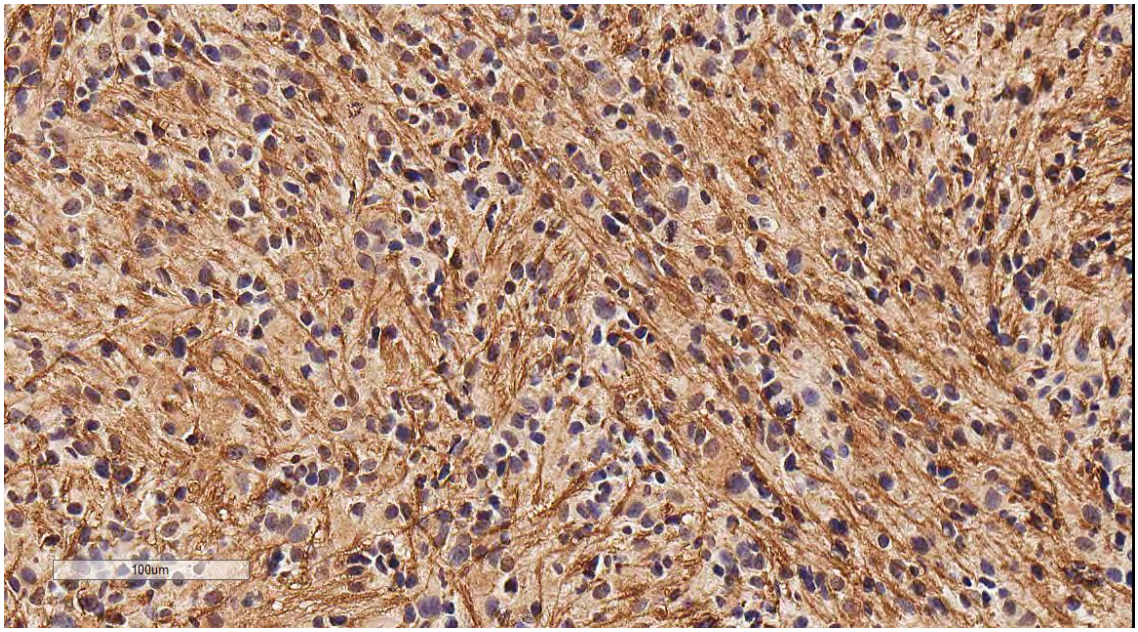
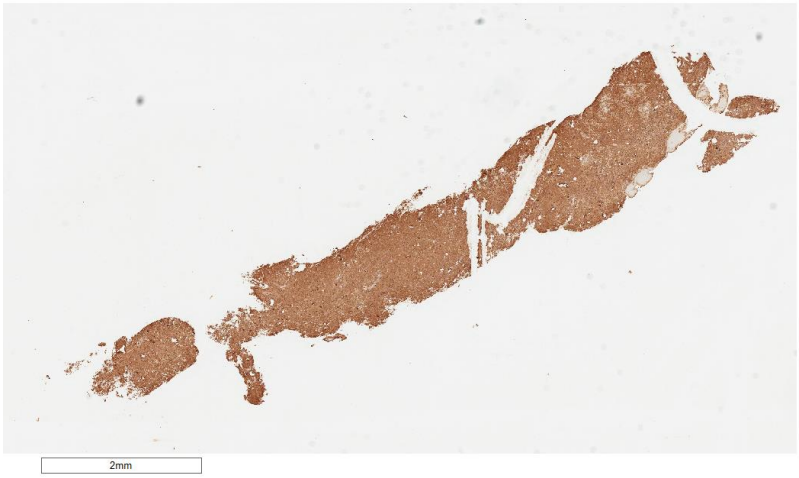


Figure 25 Anaplastic Astrocytoma (grade III), ERK5 staining score = 3. A) This figure shows an anaplastic astrocytoma sample from a 28 year old female that was stained with ERK5 antibody, with a scale of 2mm. **B)** As seen in this image with a scale of 100um, this anaplastic astrocytoma sample was assigned an ERK5 staining score of 3. There was a strong intensity of staining seen in the tumour cells, with > 60% of tumour cells stained.

A)



B)

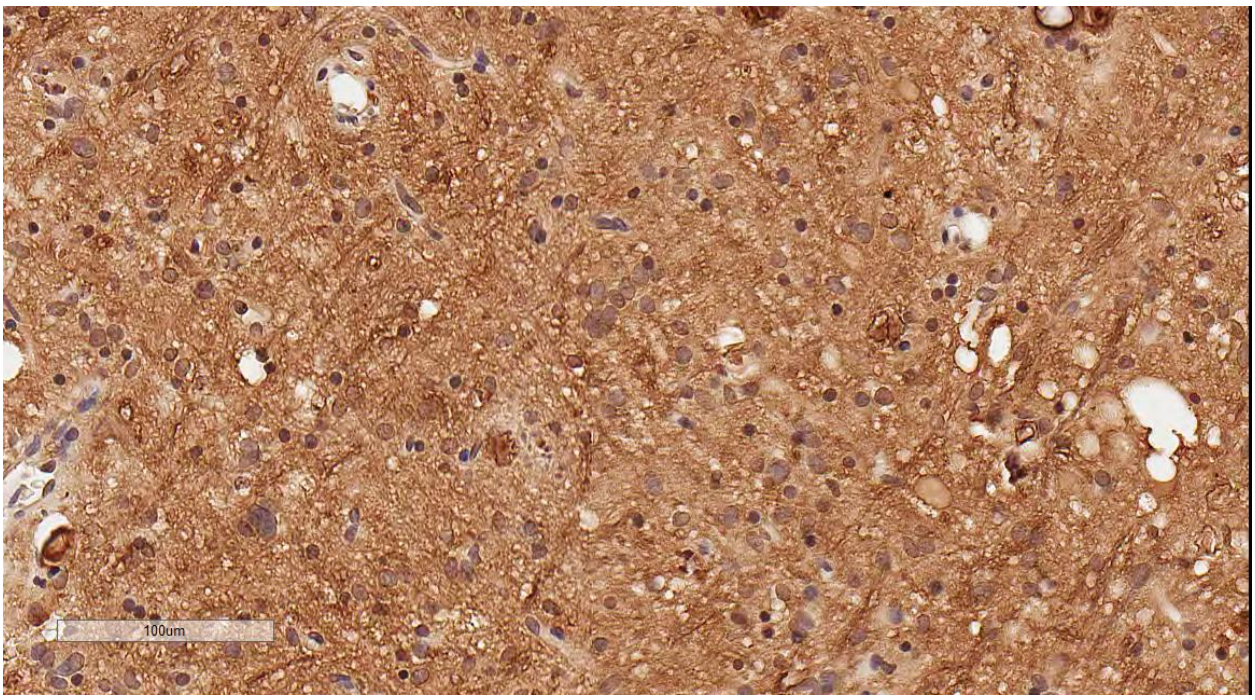
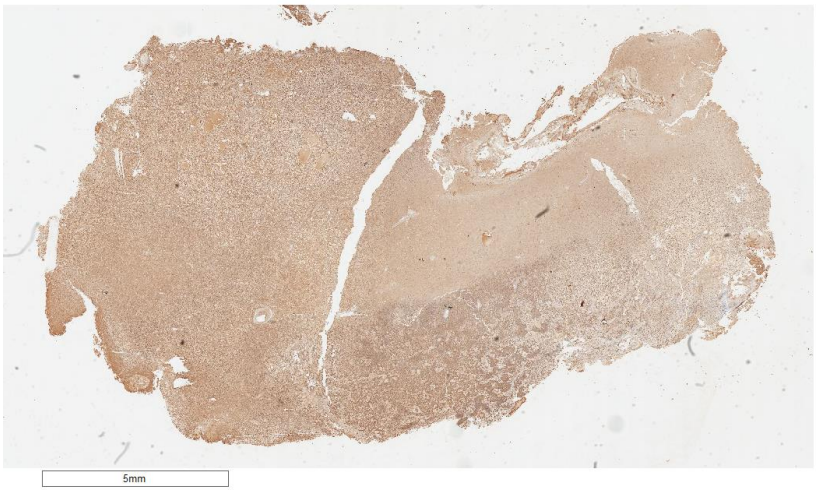


Figure 26 LGG (grade II), ERK5 staining score = 1. A) This figure shows a LGG from a 66 year old female that was stained with ERK5 antibody, with a scale of 2mm. **B)** As seen in this image with a scale of 100um, this LGG sample was assigned an ERK5 staining score of 1. There was a weak intensity of staining seen in the tumour cells, with between 10 and 30% of tumour cells stained.

A)



B)

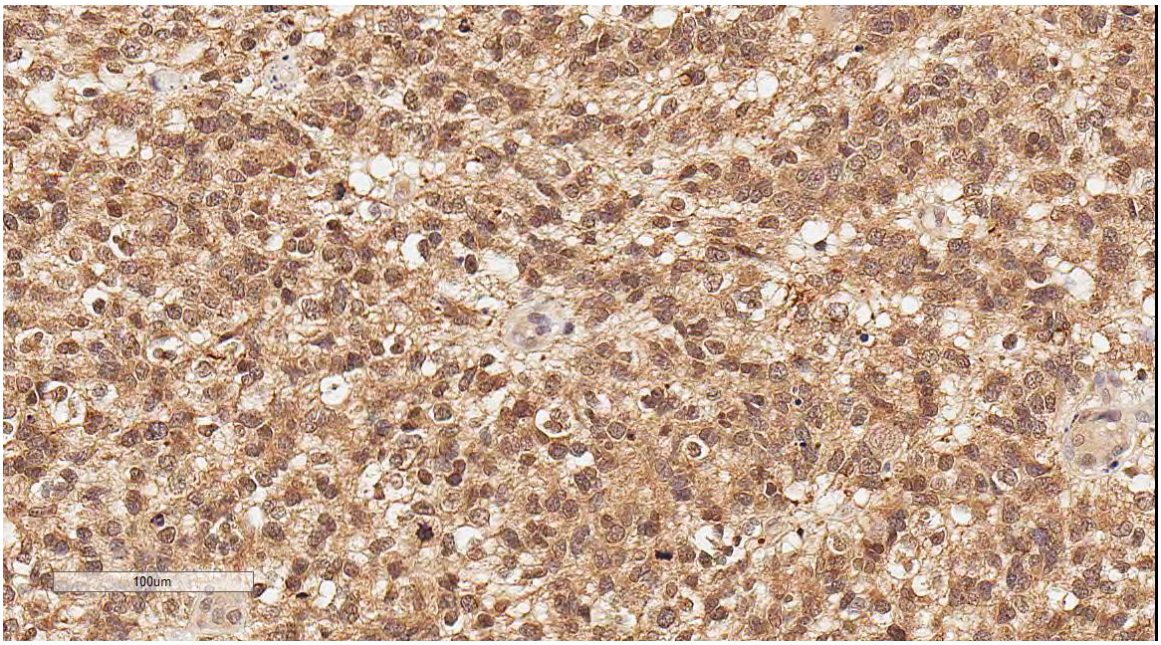
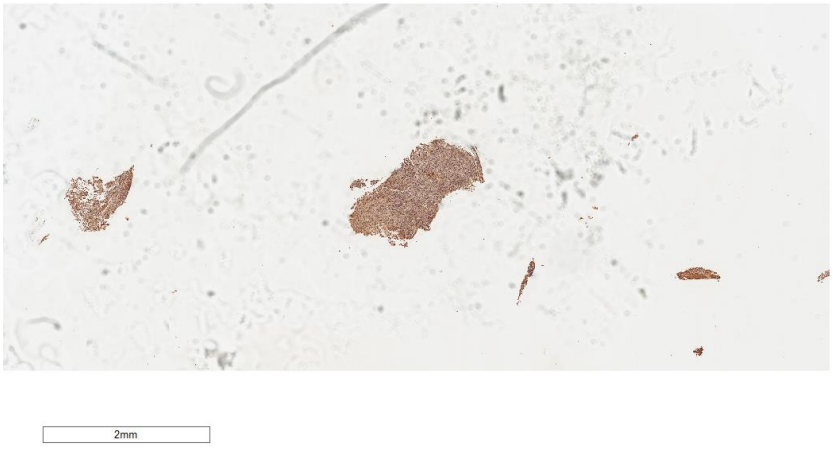


Figure 27 Anaplastic Astrocytoma (grade III), ERK5 staining score = 3. A) This figure shows an anaplastic astrocytoma sample from a 55 year old male that was stained with ERK5 antibody, with a scale of 5mm. **B)** As seen in this image with a scale of 100um, this anaplastic astrocytoma sample was assigned an ERK5 staining score of 3. There was a strong intensity of staining seen in the tumour cells, with > 60% of tumour cells stained.

A)



B)

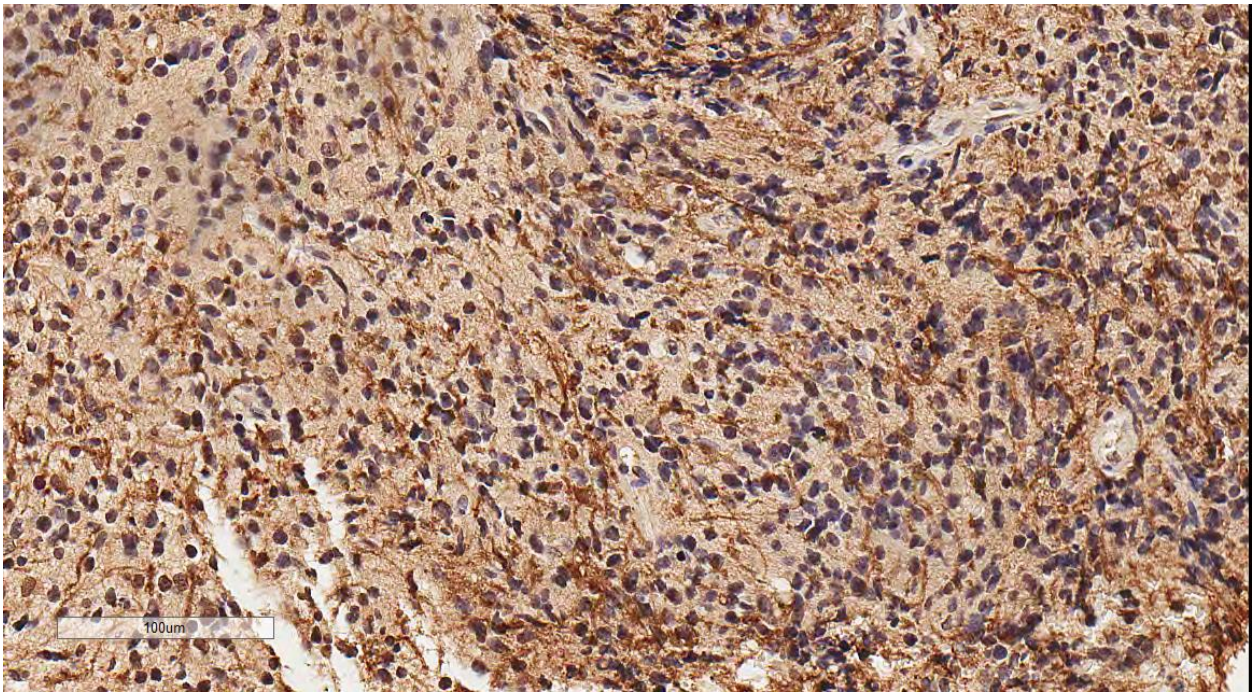
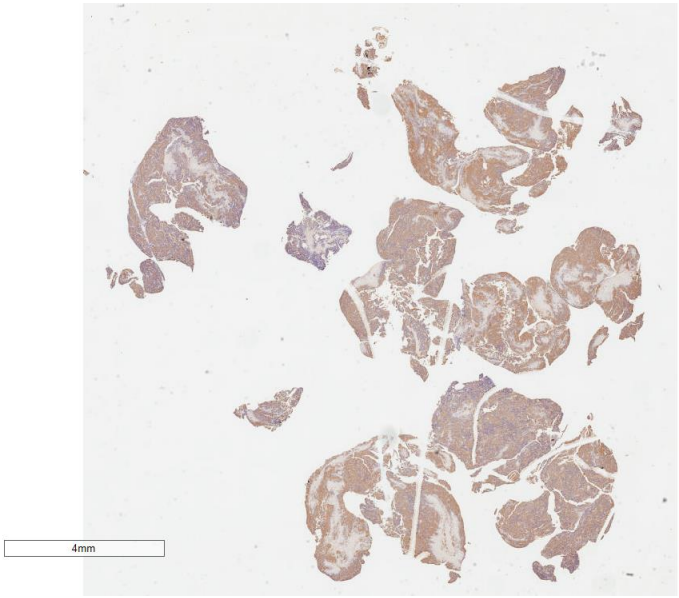


Figure 28 Anaplastic Astrocytoma (grade III), ERK5 staining score = 3. A) This figure shows an anaplastic astrocytoma sample from a 52 year old male that was stained with ERK5 antibody, with a scale of 2mm. **B)** As seen in this image with a scale of 100um, this anaplastic astrocytoma sample was assigned an ERK5 staining score of 3. There was a strong intensity of staining seen in the tumour cells, with > 60% of tumour cells stained.

A)



B)

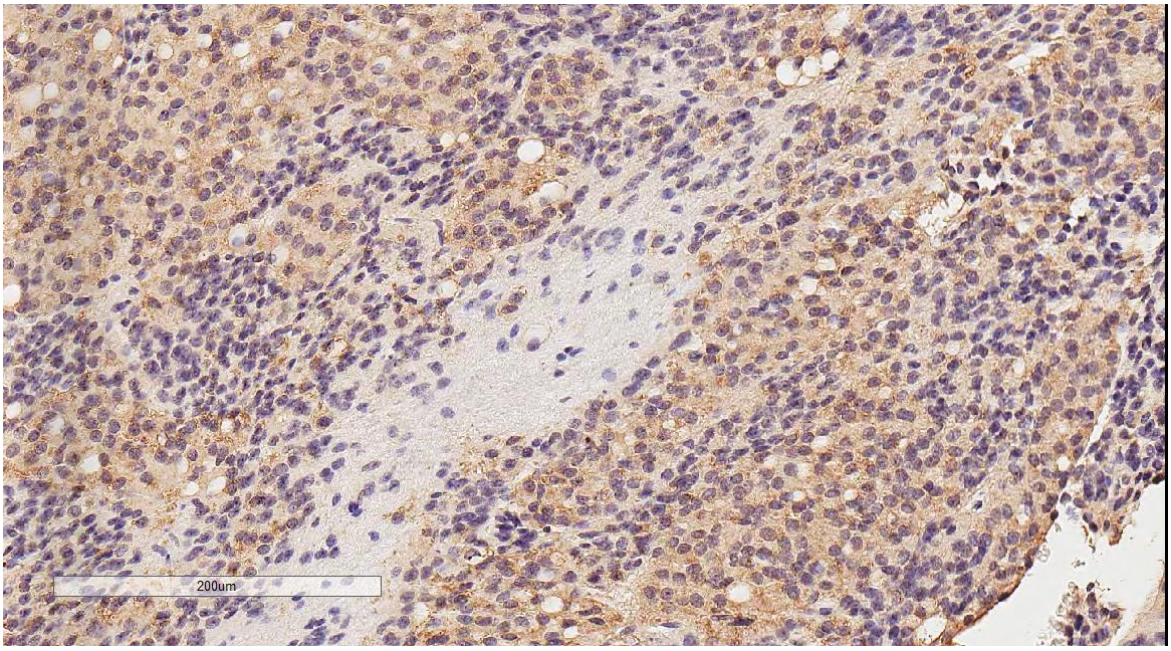


Figure 29 Ependymoma (grade I), ERK5 staining score = 1. A) This figure shows an ependymoma sample from a 16 year old male that was stained with ERK5 antibody, with a scale of 4mm. **B)** As seen in this image with a scale of 200um, this ependymoma sample was assigned an ERK5 staining score of 1. There was a weak intensity of staining seen in the tumour cells, with between 10 and 30% of tumour cells stained.

Chapter 6

6 Statistical Analyses

In this study, I sought to address the Aims/Objectives stated previously, the results of which were answered by using statistical analyses as detailed in this section.

6.1 Statistical Tests Utilized

Statistical analysis was performed by a statistician using the SPSS v.24 Software (IBM Corporation, Armonk, NY, USA). The frequency and percent of ERK5 staining in various types and grades of brain tumours were captured and are displayed in (Table 7). The Kruskal-Wallis test was used to determine whether differences in ERK5 staining existed between tumour grades. The Mann-Whitney U test was used to determine whether differences in ERK5 staining existed between the higher vs lower grades, as well as between ERK5 staining and gender. Spearman's Rho was used to determine whether there was a correlation between ERK5 staining and age. A probability (P) value <.05 was considered significant.

Analyses were performed on the data from:

- Study 1 only
- Study 2 only
- Study 1 & 2 combined

There were no significant results for Study 1. There were some significant results for Study 1 and 2 combined, but the best results were with Study 2 only.

6.2 STUDY 1

Aim/Objective # 1: To investigate whether ERK5 is expressed in Glioblastoma Multiforme

Aim/Objective # 2: To investigate whether ERK5 is expressed in other types/grades of brain tumours.

In order to address the above two objectives, the frequency and percentage of ERK5 staining expressed in GBM and the other types and grades of brain tumours are summarized in the below table:

Table 7 Study #1: Expression of ERK5 in different types/grades of Brain Tumours

Pathology			Frequency	Percent	Valid Percent	Cumulative Percent
Anaplastic Astrocytoma	Valid	0	1	33.3	33.3	33.3
		1	1	33.3	33.3	66.7
		2	1	33.3	33.3	100.0
		Total	3	100.0	100.0	
Ependymoma	Valid	3	2	100.0	100.0	100.0
Glioblastoma	Valid	0	11	42.3	42.3	42.3
		1	5	19.2	19.2	61.5
		2	5	19.2	19.2	80.8
		3	5	19.2	19.2	100.0
		Total	26	100.0	100.0	
LGG – Astrocytoma	Valid	0	3	33.3	33.3	33.3
		1	1	11.1	11.1	44.4
		2	2	22.2	22.2	66.7
		3	3	33.3	33.3	100.0
		Total	9	100.0	100.0	
Medulloblastoma	Valid	0	2	66.7	66.7	66.7
		1	1	33.3	33.3	100.0
		Total	3	100.0	100.0	
Oligodendroglioma	Valid	1	1	100.0	100.0	100.0
Pilocytic Astrocytoma	Valid	3	1	100.0	100.0	100.0

In study # 1, I reported the expression of ERK5 in various brain tumours using TMA and IHC. I found that ERK5 staining was present in a substantial number of the samples in the TMA. ERK5 staining was present in 57.3% of all brain tumour samples examined, with some level of ERK5 staining present in 57.5% of glioblastoma samples. 'Intermediate' and 'strong' staining were present in 38.4% of glioblastomas.

'Weak' and 'Intermediate' staining of ERK5 was present in 33.3% and 33.3% of the cytoplasms of the grade III anaplastic astrocytoma samples respectively. However, there were only a small amount of these samples as seen in the above table.

There was some ERK5 staining observed in some of the samples of oligodendroglioma, pilocytic astrocytoma, medulloblastoma, ependymoma, and LGG – astrocytoma. However, the sample size for these types of brain tumours was very small. Of note, there was some level of ERK5 staining observed in 66.6% in the 9 available samples of the LGG – astrocytoma. There was 'Intermediate' and 'Strong' staining seen in 55.5% of the LGG samples.

ERK5 staining was very minimal in normal brain tissue controls, as significant staining was only seen in 1 of 5 healthy brain tissue samples.

Based on the statistical analyses performed in study #1, there were no differences in ERK5 staining between tumour grades, higher vs. lower tumour grades, or gender. ERK5 staining was not correlated with age. The statistical tests performed were not significant.

Aim/Objective # 3: To determine whether there exists a difference in ERK5 expression between tumour grades

Table 8 Study #1: Difference in ERK5 between tumour grades (Kruskal-Wallis test) - not significant

Descriptive Statistics

Grade		N	Minimum	Maximum	Mean	Std. Deviation
.	ERK5	5	0	3	1.40	1.517
	Valid N (listwise)	5				
1	ERK5	4	0	3	1.75	1.500
	Valid N (listwise)	4				
2	ERK5	7	0	3	1.57	1.272
	Valid N (listwise)	7				
3	ERK5	20	0	3	1.30	1.218
	Valid N (listwise)	20				
4	ERK5	9	0	2	.78	.972
	Valid N (listwise)	9				

Test Statistics[a,b]

	ERK5
Chi-Square	2.628
Df	3
Asymp. Sig.	.453

a. Kruskal Wallis Test

b. Grouping Variable: grade

<u>Aim/Objective # 4:</u> To determine whether there exists a difference in ERK5 expression between higher vs lower grades (1&2 vs 3&4)

Table 9 Study #1: Difference in ERK5 between higher vs lower grades (Mann-Whitney U test) - not significant

Descriptive Statistics

high_grade		N	Minimum	Maximum	Mean	Std. Deviation
.	ERK5	5	0	3	1.40	1.517
	Valid N (listwise)	5				
1&2	ERK5	11	0	3	1.64	1.286
	Valid N (listwise)	11				
3&4	ERK5	29	0	3	1.14	1.156
	Valid N (listwise)	29				

Test Statistics[a]

	ERK5
Mann-Whitney U	123.000
Wilcoxon W	558.000
Z	-1.152
Asymp. Sig. (2-tailed)	.249
Exact Sig. [2*(1-tailed Sig.)]	.280[b]

a. Grouping Variable: high_grade

b. Not corrected for ties.

Aim/Objective # 5: To determine whether there exists a difference in ERK5 expression between genders

Table 10 Study #1: Difference in ERK5 between genders (Mann-Whitney U test) - not significant

Descriptive Statistics

Sex		N	Minimum	Maximum	Mean	Std. Deviation
Male	ERK5	27	0	3	1.22	1.188
	Valid N (listwise)	27				
Female	ERK5	18	0	3	1.39	1.290
	Valid N (listwise)	18				

Test Statistics[a]

	ERK5
Mann-Whitney U	228.000
Wilcoxon W	606.000
Z	-.363
Asymp. Sig. (2-tailed)	.717

a. Grouping Variable: sex

Aim/Objective # 6: To determine whether ERK5 expression is correlated with Age

Table 11 Study #1: ERK5 correlated with age (Spearman's rho) - not significant

Correlations

			ERK5	age_yrs
Spearman's rho	ERK5	Correlation Coefficient	1.000	-.078

		Sig. (2-tailed)	.	.612
		N	45	45
	age_yrs	Correlation Coefficient	-.078	1.000
		Sig. (2-tailed)	.612	.
		N	45	45

6.3 STUDY 2

<u>Aim/Objective # 1:</u> To investigate whether ERK5 is expressed in Glioblastoma Multiforme

<u>Aim/Objective # 2:</u> To investigate whether ERK5 is expressed in other types/grades of brain tumours.

In order to address the above two objectives, the frequency and percentage of ERK5 staining expressed in GBM and the other types and grades of brain tumours are summarized in the below table:

Table 12 Study #2: Expression of ERK5 in different types/grades of brain tumours

Pathology			Frequency	Percent	Valid Percent	Cumulative Percent
Anaplastic Astrocytoma	Valid	0	1	6.3	6.7	6.7
		1	3	18.8	20.0	26.7
		2	8	50.0	53.3	80.0
		3	3	18.8	20.0	100.0
		Total	15	93.8	100.0	
	Missing	System	1	6.3		
	Total		16	100.0		
Ependymoma	Valid	0	7	53.8	53.8	53.8

		1	6	46.2	46.2	100.0
		Total	13	100.0	100.0	
Glioblastoma	Valid	1	5	31.3	33.3	33.3
		2	4	25.0	26.7	60.0
		3	6	37.5	40.0	100.0
		Total	15	93.8	100.0	
	Missing	System	1	6.3		
	Total		16	100.0		
LGG – Astrocytoma	Valid	0	9	90.0	90.0	90.0
		1	1	10.0	10.0	100.0
		Total	10	100.0	100.0	
Oligodendroglioma	Valid	0	5	33.3	33.3	33.3
		1	8	53.3	53.3	86.7
		2	2	13.3	13.3	100.0
		Total	15	100.0	100.0	

The results show that ERK5 staining was present in the cytoplasms of 55% of all brain tumour samples examined, with some level of ERK5 staining present in the cytoplasms of 100% of all of the glioblastoma samples examined. ‘Weak’, ‘Intermediate’ and ‘strong’ staining of ERK5 were present in 33.3%, 26.7% and 40% of the cytoplasms of glioblastoma samples respectively. ‘Weak’, ‘Intermediate’ and ‘Strong’ staining of ERK5 was present in 20%, 53.3% and 20% of the cytoplasms of the grade III anaplastic astrocytoma samples respectively.

ERK5 was only present in the nucleus of 2.9% of all brain tumours examined. Specifically, ERK5 staining was only seen in the nucleus of the grade II LGG astrocytoma. No other staining was seen in the nucleus of any of the other samples. Perinuclear staining was seen in the Grade II oligodendrogliomas.

'Weak' staining of ERK5 was present in 10% of the cytoplasms of the grade II LGG astrocytoma samples.

'Weak' and 'Intermediate' staining of ERK5 was present in 53.3% and 13.3% of the cytoplasms of the oligodendroglioma samples respectively.

'Weak' staining of ERK5 was present in 46.2% of the cytoplasms of the ependymoma samples.

Aim/Objective # 3: To determine whether there exists a difference in ERK5 expression between tumour grades

Table 13 Study #2: Difference in ERK5 between tumour grade (Kruskal-Wallis test) - significant difference

Descriptive Statistics

Grade		N	Minimum	Maximum	Mean	Std. Deviation
2	ERK5	38	0	2	.50	.604
	Valid N (listwise)	38				
3	ERK5	15	0	3	1.87	.834
	Valid N (listwise)	15				
4	ERK5	15	1	3	2.07	.884
	Valid N (listwise)	15				

Ranks

	Grade	N	Mean Rank
ERK5	2	38	22.54

	3	15	48.37
	4	15	50.93
	Total	68	

In this study, I examined whether there was a significant difference in ERK5 amongst grades. Using the Kruskal-Wallis H test, the results show that there exists a significant difference in ERK5 amongst grades. When examining the level of ERK5 staining between grades 2 & 3, there exists a significant difference. Similarly, when examining the level of ERK5 staining between grades 2 & 4, there too exists a significant difference. There was no significant difference in level of ERK5 staining when examining grades 3 & 4.

The following table shows a significant difference in ERK5 amongst grades. Looking at the means above, the differences exist between grades 2 & 3 and between grades 2 & 4 (but no significant difference between grades 3 & 4). Report as chi-square=34.519, df=2, p<0.001.

Test Statistics[a,b]

	ERK5
Chi-Square	34.519
Df	2
Asymp. Sig.	.000

a. Kruskal Wallis Test

b. Grouping Variable: grade

<u>Aim/Objective # 4:</u> To determine whether there exists a difference in ERK5 expression between higher vs lower grades (1&2 vs 3&4)

The Mann-Whitney U test is used to compare differences between two independent groups when the dependent variable is either ordinal or continuous, but not normally distributed. In this study, I examined whether there was a difference in ERK5 amongst low and high grades. According to the statistical analyses performed, there was a significant difference in ERK5 amongst low and high grades, with grades 3 & 4 having a higher mean ERK5 value compared to grades 1 & 2.

Table 14 Study #2: Difference in ERK5 between higher vs lower grades (Mann-Whitney U test) - significant difference

Descriptive Statistics

high_grade		N	Minimum	Maximum	Mean	Std. Deviation
1&2	ERK5	38	0	2	.50	.604
	Valid N (listwise)	38				
3&4	ERK5	30	0	3	1.97	.850
	Valid N (listwise)	30				

The following table shows a significant difference in ERK5 amongst low and high grades, with grades 3&4 having a higher mean ERK5 value compared to grades 1&2. Report as Mann-Whitney U=115.50, p<0.001.

Test Statistics[a]

	ERK5
Mann-Whitney U	115.500
Wilcoxon W	856.500
Z	-5.864
Asymp. Sig. (2-tailed)	.000

a. Grouping Variable: high_grade

<u>Aim/Objective # 5:</u> To determine whether there exists a difference in ERK5 expression between genders

In order to determine whether there was a difference in ERK5 expression between males or females in this set of data, the Mann-Whitney U test was performed. There was no significant difference found in this analysis and hence, there was no difference in ERK5 expression between males or females.

Table 15 Study #2: Difference in ERK5 expression between genders (Mann-Whitney U test) - not significant

Descriptive Statistics

Sex		N	Minimum	Maximum	Mean	Std. Deviation
.	ERK5	1	2	2	2.00	.
	Valid N (listwise)	1				
Male	ERK5	34	0	3	1.15	1.019
	Valid N (listwise)	34				
Female	ERK5	33	0	3	1.12	1.053
	Valid N (listwise)	33				

Test Statistics[a]

	ERK5
Mann-Whitney U	548.500
Wilcoxon W	1109.500
Z	-.164
Asymp. Sig. (2-tailed)	.870

a. Grouping Variable: sex

Aim/Objective # 6: To determine whether ERK5 expression is correlated with Age

In order to determine whether ERK5 expression is correlated with age in this study, the Spearman's rho analysis was performed. The Spearman's rho analysis is used to discover the strength of a link between two sets of data. In this study, it was found that there was a significant relation between ERK5 expression and age. As the age of the patient increased, the level of ERK5 staining observed also increased.

The following table shows that as age increases, ERK5 score also increases. Report as Spearman's r=0.38, p=0.001.

Table 16 Study #2: ERK5 correlated with age (Spearman's rho) - significant relation

Correlations

			ERK5	age_yrs
Spearman's rho	ERK5	Correlation Coefficient	1.000	.382**
		Sig. (2-tailed)	.	.001
		N	68	67
	age_yrs	Correlation Coefficient	.382**	1.000
		Sig. (2-tailed)	.001	.
		N	67	69

**. Correlation is significant at the 0.01 level (2-tailed).

6.4 STUDY 1 & 2 COMBINED

Aim/Objective # 1: To investigate whether ERK5 is expressed in Glioblastoma Multiforme

Aim/Objective # 2: To investigate whether ERK5 is expressed in other types/grades of brain tumours.

In order to address the above two objectives, the frequency and percentage of ERK5 staining expressed in GBM and the other types and grades of brain tumours are summarized in the below table:

Aim/Objective # 1: To investigate whether ERK5 is expressed in Glioblastoma Multiforme

Aim/Objective # 2: To investigate whether ERK5 is expressed in other types/grades of brain tumours.

In order to address the above two objectives, the frequency and percentage of ERK5 staining expressed in GBM and the other types and grades of brain tumours are summarized in the below table:

Table 17 Study # 1&2: ERK5 Expression in different types/grades of brain tumours

Pathology			Frequency	Percent	Valid Percent	Cumulative Percent
Anaplastic Astrocytoma	Valid	0	2	10.5	11.1	11.1
		1	4	21.1	22.2	33.3
		2	9	47.4	50.0	83.3
		3	3	15.8	16.7	100.0
		Total	18	94.7	100.0	
	Missing	System	1	5.3		
	Total		19	100.0		
Ependymoma	Valid	0	7	46.7	46.7	46.7
		1	6	40.0	40.0	86.7
		3	2	13.3	13.3	100.0
		Total	15	100.0	100.0	
Glioblastoma	Valid	0	11	26.2	26.8	26.8
		1	10	23.8	24.4	51.2
		2	9	21.4	22.0	73.2
		3	11	26.2	26.8	100.0
		Total	41	97.6	100.0	
	Missing	System	1	2.4		
	Total		42	100.0		
LGG – Astrocytoma	Valid	0	12	63.2	63.2	63.2

		1	2	10.5	10.5	73.7
		2	2	10.5	10.5	84.2
		3	3	15.8	15.8	100.0
		Total	19	100.0	100.0	
Medulloblastoma	Valid	0	2	66.7	66.7	66.7
		1	1	33.3	33.3	100.0
		Total	3	100.0	100.0	
Oligodendroglioma	Valid	0	5	31.3	31.3	31.3
		1	9	56.3	56.3	87.5
		2	2	12.5	12.5	100.0
		Total	16	100.0	100.0	
Pilocytic Astrocytoma	Valid	3	1	100.0	100.0	100.0

When combining the data from Study #1 and Study #2, there are some results that are found to be statistically significant, although not as strong as with the results of Study #2 only. These results are seen in the below tables, and are summarized as follows:

The results show that ERK5 staining was present in the cytoplasms of 65.5% of all brain tumour samples examined, with some level of ERK5 staining present in the cytoplasms of 85.4% of all of the glioblastoma samples examined. When combining all glioblastoma samples from both studies, ‘Weak’, ‘Intermediate’ and ‘strong’ staining of ERK5 were present in 24.4%, 22% and 26.8% of the cytoplasms of glioblastoma samples respectively. ‘Weak’, ‘Intermediate’ and ‘Strong’ staining of ERK5 was present in 22.2%, 50% and 16.7% of the cytoplasms of the grade III anaplastic astrocytoma samples respectively.

‘Weak’, ‘Intermediate’ and ‘Strong’ staining of ERK5 was present in 10.5%, 10.5% and 15.8% of the cytoplasms of the grade II LGG astrocytoma samples respectively.

'Weak' and 'Intermediate' staining of ERK5 was present in 56.3% and 12.5% of the cytoplasms of the oligodendroglioma samples respectively.

'Weak' and 'Strong' staining of ERK5 was present in 40% and 13.3% of the cytoplasms of the ependymoma samples respectively.

There was some ERK5 staining observed in some of the samples of pilocytic astrocytoma and medulloblastoma. However, the sample size of these tumours was very low.

Aim/Objective # 3: To determine whether there exists a difference in ERK5 expression between tumour grades

Table 18 Study # 1&2: Difference in ERK5 between tumour grades (Kruskal-Wallis test) - significant difference

Descriptive Statistics

Grade		N	Minimum	Maximum	Mean	Std. Deviation
.	ERK5	5	0	3	1.40	1.517
	Valid N (listwise)	5				
1	ERK5	4	0	3	1.75	1.500
	Valid N (listwise)	4				
2	ERK5	45	0	3	.67	.826
	Valid N (listwise)	45				
3	ERK5	35	0	3	1.54	1.094
	Valid N (listwise)	35				
4	ERK5	24	0	3	1.58	1.100
	Valid N (listwise)	24				

I combined the results for studies # 1 and #2 and examined whether there was a significant difference in ERK5 amongst grades. Using the Kruskal-Wallis H test, the results show that there exists a significant difference in ERK5 amongst grades. When a standard ANOVA was run to look at individual differences, the differences only exist between grades 2 & 3. There was no significant difference between grades 2 & 4 or between grades 3 & 4.

The following table shows a significant difference in ERK5 amongst grades. Report as chi-square=17.86, df=3, p<0.001.

Test Statistics[a,b]

	ERK5
Chi-Square	17.859
Df	3
Asymp. Sig.	.000

a. Kruskal Wallis Test

b. Grouping Variable: grade

<u>Aim/Objective # 4:</u> To determine whether there exists a difference in ERK5 expression between higher vs lower grades (1&2 vs 3&4)

Table 6 Study # 1&2: Difference in ERK5 between higher vs lower grades (Mann-Whitney U test) - significant difference

Descriptive Statistics

high_grade		N	Minimum	Maximum	Mean	Std. Deviation
.	ERK5	5	0	3	1.40	1.517
	Valid N (listwise)	5				
1&2	ERK5	49	0	3	.76	.925

	Valid N (listwise)	49				
3&4	ERK5	59	0	3	1.56	1.087
	Valid N (listwise)	59				

I combined the data from both Study # 1 and #2 and examined whether there was a difference in ERK5 amongst low and high grades. According to the statistical analyses performed, there was a significant difference in ERK5 amongst low and high grades, with grades 3 & 4 having a higher mean ERK5 value compared to grades 1 & 2. This is similar to the results seen in the data from Study #2 only.

The following table shows a significant difference in ERK5 amongst low and high grades, with grades 3&4 having a higher mean ERK5 value compared to grades 1&2. Report as Mann-Whitney U=848.00, p<0.001.

Test Statistics[a]

	ERK5
Mann-Whitney U	848.000
Wilcoxon W	2073.000
Z	-3.838
Asymp. Sig. (2-tailed)	.000

a. Grouping Variable: high_grade

Aim/Objective # 5: To determine whether there exists a difference in ERK5 expression between genders

Table 20 Study # 1&2: Difference in ERK5 between genders (Mann-Whitney U test) - not significant

Descriptive Statistics

Sex		N	Minimum	Maximum	Mean	Std. Deviation
.	ERK5	1	2	2	2.00	.
	Valid N (listwise)	1				
Male	ERK5	61	0	3	1.18	1.088
	Valid N (listwise)	61				
Female	ERK5	51	0	3	1.22	1.137
	Valid N (listwise)	51				

Test Statistics[a]

	ERK5
Mann-Whitney U	1537.000
Wilcoxon W	3428.000
Z	-.113
Asymp. Sig. (2-tailed)	.910

a. Grouping Variable: sex

Aim/Objective # 6: To determine whether ERK5 expression is correlated with Age

Table 21 Study # 1&2: ERK5 correlated with age (Spearman's rho) - not significant

Correlations

		ERK5	age_yrs

Spearman's rho	ERK5	Correlation Coefficient	1.000	.179
		Sig. (2-tailed)	.	.059
		N	113	112
	age_yrs	Correlation Coefficient	.179	1.000
		Sig. (2-tailed)	.059	.
		N	112	114

Based on the statistical analyses performed on the data from both study #1 and #2, there was no correlation seen between ERK5 staining and age, and there were no differences in ERK5 between males or females. The statistical tests performed were not significant.

Chapter 7

7 Discussion

Below, I discuss each of the aims/objectives from my study and attempt to support each idea with support from a thorough review of the literature.

7.1 Tumour Grade and higher vs lower grades

The following discussion addresses the below two aims/objectives:

To determine whether there exists a difference in ERK5 expression between tumour grades

To determine whether there exists a difference in ERK5 expression between higher vs lower grades (1&2 vs 3&4)

Abnormal expression of MEK5/ERK5 has already been reported in several human cancers. The relevance of MEK5/ERK5 signalling in cancer is becoming very pertinent as it has been demonstrated that ERK5 plays a role in angiogenesis, migration and tumour metastasis (Wang and Tournier 2006). Of interest, abnormal ERK5 signalling is found to be associated with more aggressive phenotypes and a poorer disease prognosis in several tumour types, partly due to a tumour cell's ability to migrate (Wang and Tournier 2006).

There exist many studies in the literature that correlate an increased expression of ERK5 with a poorer prognosis in many types of cancers. However, there exists a lack of data in the literature that correlate an increased expression of ERK5 with brain tumours, and particularly higher grade brain tumours, such as glioblastoma. Due to this lack of available data, along with previous findings that demonstrate a role of ERK5 in some more aggressive types of cancers, I chose to study ERK5 expression in glioblastoma and other grade and types of tumours as these are more aggressive tumours with a known poorer prognosis.

There are several studies in the literature that report findings that ERK5 expression is correlated with disease outcome. One study found that ERK5 overexpression correlated with tumour progression, resistance to treatment, and worse overall patient survival (Tesser-Gamba et al. 2012). Another study found that ERK5 overexpression was associated with advanced tumour stage and lymph node metastasis (Sticht et al. 2008). In a study looking at CCRCC (Clear Cell Renal Cell Carcinoma), the researchers

observed that a lack of ERK5 expression resulted in decreased cell motility *in vitro*, which they thought correlated with low metastatic ability (Arias-González et al. 2013). Also, in breast cancer, expression of ERK5 was found to correlate with a worse prognosis (Montero et al. 2009). In one study looking at ERK5 in early stage Breast Cancer, overexpression of ERK5 was found to be an independent predictor of disease-free survival (Montero et al. 2009). In addition, through examining breast tumour pathology specimens, it was found that ERK5 was overexpressed in 20% of patients with breast cancer (Esparis-Ogando et al. 2002).

In prostate cancer, it was shown that higher levels of ERK5 expression were associated with bone metastasis and poorer prognosis (Mehta et al. 2003). In oral squamous cell carcinoma, higher levels of p-ERK5 expression were correlated with more advanced tumour stage and the presence of lymph node involvement (Sticht et al. 2008).

In patients with brain tumours, a higher grade tumour results in a poorer prognosis. However, there exists minimal data to support the association between ERK5 overexpression and metastasis and poorer prognosis in these higher grade brain tumours. One paper stated that MEKK2 is involved in JNK and ERK5 activation, and JNK activation can promote apoptosis in GBM cells (Su et al. 2011), (L. Li et al. 2008). Another paper showed that activated components of the Akt and MAPK pathway are associated with decreased overall survival in glioblastoma (Pelloski et al. 2006). One paper highlighted the findings that patients with a lower grade glioma had much better survival than those glioma patients with a higher grade. Also, patients with the highest grade brain tumours, such as GBM, had the poorest survival (Fisher, Schwartzbaum, and Wrensch, n.d.).

As referenced above, there exist many studies in the literature that correlate an increased expression of ERK5 with a poorer prognosis in many types of cancers. Tumours develop their resistance and aggressiveness due to the morphological and molecular changes that tumour cells experience (Wang and Tournier 2006), (Rovida et al. 2015). This aggressiveness leads to a poorer outcome and prognosis in many types of cancers, but may also be the case in higher grade brain tumours such as GBM. However, there exists a lack of data in the literature that demonstrate the relationship between increased ERK5 expression and higher grade brain tumours, such as glioblastoma. Due to this lack of available data, along with previous findings that demonstrate a role of ERK5 in some more aggressive types of cancers, I

chose to study ERK5 expression in glioblastoma and other grade and types of tumours as these are more aggressive tumours with a known poorer prognosis.

In the present study, I showed that ERK5 levels are increased in the higher grade brain tumours, glioblastoma and anaplastic astrocytoma. My results demonstrated that there exists a difference in ERK5 expression amongst tumour grade, and that higher levels of ERK5 are associated with more advanced cancer stages. This suggests the importance of MEK5/ERK5 signalling in the more aggressive tumours. These results are congruent with data reported in the literature that indicate more aggressive tumours display higher levels of ERK5. Thus, it is possible that the higher grade brain tumour samples (3 & 4) showed an increase in ERK5 expression due to its link to tumour cell proliferation.

According to the statistical analyses performed, the results show that there exists a significant difference in ERK5 amongst grades. In addition, there was a significant difference in ERK5 levels amongst low and high grades, with grades 3 & 4 having a higher mean ERK5 value compared to grades 1 & 2.

ERK5 was also found to be expressed in some of the lower grade brain tumours, thus suggesting that ERK5 may be a key factor in the transition from lower grade brain tumours to higher grade brain tumours. As previously discussed, lower grade brain tumours, such as grade II astrocytomas, may progress from lower grades to higher grades over time, potentially resulting in a secondary GBM. As the tumours progresses through various stages of development, the percentage of ERK5 staining appears to increase, and there appeared to be evident staining of the process. ERK5 did not appear to differentiate between primary and secondary GBM, however this needs to be investigated further as there was limited data available to reach a definite conclusion.

Thus, perhaps the results of the ERK5 staining seen in these experiments show that ERK5 plays a role in the progression of lower grade brain tumours to secondary glioblastomas later in life. In addition, the increased ERK5 staining seen in the higher grade brain tumours could be a result of the advanced pathological stage of this disease. This is in keeping with previously reported data on other types of cancers, and may also indicate that this is true of certain types of brain tumors as well. Specifically, this appears to be the case with GBM.

7.2 ERK5 expression in Glioblastoma and other types/grades of brain tumours

The following discussion addresses the below aim/objective:

To determine if ERK5 is expressed in other types/grades of brain tumours

There was very little information in the literature regarding the expression of ERK5 in brain tumours. There was also very little information available on the MAPK/ERK5 signalling pathway and its role in GBM and other types of brain tumours. I found several papers that discussed the role of the MEK5/ERK5 signalling pathway in cancers of other types. Hence, I wanted to first examine not only ERK5 expression in GBM, but also to find out if various other types and grades of brain tumours also exhibited ERK5 expression. As shown in my experiments there was certainly staining of the process in brain tumours, and in particular in the higher grade tumours.

In both the studies I performed combined, there were a total of 46 GBM samples examined for ERK5 expression. When combining the data from Study #1 and Study #2, there are some results that are found to be statistically significant, although not as strong as with the results of Study #2 only. The results showed that ERK5 staining was present in the cytoplasms of 65.5% of all brain tumour samples examined, with some level of ERK5 staining present in the cytoplasms of 85.4% of all of the glioblastoma samples examined. When combining all glioblastoma samples from both studies, 'Weak', 'Intermediate' and 'strong' staining of ERK5 were present in 24.4%, 22% and 26.8% of the cytoplasms of glioblastoma samples respectively.

In Study #1, the results show that ERK5 staining was present in the cytoplasms of 55% of all brain tumour samples examined, with some level of ERK5 staining present in the cytoplasms of 100% of all of the glioblastoma samples examined. 'Weak', 'Intermediate' and 'strong' staining of ERK5 were present in 33.3%, 26.7% and 40% of the cytoplasms of glioblastoma samples respectively. 'Weak', 'Intermediate' and 'Strong' staining of ERK5 was present in 20%, 53.3% and 20% of the cytoplasms of the grade III anaplastic astrocytoma samples respectively.

ERK5 was only present in the nucleus of 2.9% of all brain tumours examined. Specifically, ERK5 staining was only seen in the nucleus of the grade II LGG astrocytoma. No other staining was seen in the

nucleus of any of the other samples. Of note, perinuclear staining was seen in the Grade II oligodendrogliomas.

'Weak' staining of ERK5 was present in 10% of the cytoplasms of the grade II LGG astrocytoma samples.

'Weak' and 'Intermediate' staining of ERK5 was present in 53.3% and 13.3% of the cytoplasms of the oligodendroglioma samples respectively.

'Weak' staining of ERK5 was present in 46.2% of the cytoplasms of the ependymoma samples.

In study #1, ERK5 staining was very minimal in normal brain tissue controls, and significant staining was only seen in 1 of 5 healthy brain tissue samples.

My data supports glioblastoma as yet another type of cancer that displays abnormal MEK5/ERK5 signalling, as can be seen by the increased expression of ERK5 in this tumour. This indicates that targeting the MEK5/ERK5 pathway may be of potential benefit for future targeted therapies to combat this disease. However, there is still much that has yet to be explored.

7.3 There is no difference in ERK5 expression between males or females.

The following discussion addresses the below aim/objective:

To determine whether there exists a difference in ERK5 expression between genders

According to the CBTBUS for 2008-2012, GBM is 1.6 times more common in males than in females (Ostrom et al. 2015).

Of the 16 GBM patient samples obtained from our pathology department, 8 samples were from male patients and 8 samples were from female patients. Of the 26 GBM patient samples in the tissue microarray, 14 samples were from male patients and 12 samples were from female patients. Overall, in the total of 42 GBM samples, 22 samples were from male patients and 20 samples were from female patients.

Nonetheless, after reviewing the literature, I did not find any reports or data supporting an increased expression of ERK5 in either gender. This is consistent with the results of my study in which I found there to be no difference in ERK5 expression between males or females.

7.4 ERK5 expression is correlated with Age

The following discussion addresses the below aim/objective:

To determine whether ERK5 expression is correlated with age

I reviewed some limited clinical characteristics of the patients whose samples I obtained from our Pathology department and investigated whether age was correlated with the level of ERK5 staining. Of the 16 GBM patient samples obtained from our pathology department, 14 patients were over the age of 50 years (minimum of 50 and maximum of 88, with a mean age of 70.7 years). The data from one patient sample was not included in the statistical analyses as the data was incomplete. The remaining two patients were 20 and 25 years of age. Of the 26 GBM patient samples in the tissue microarray, 11 patient samples were 50 years of age or older (minimum age of 50, maximum age of 68, and mean age of 58.5 years of age), and 15 patients were under 50 years of age (minimum age of 4, maximum age of 49, and mean age of 30.1 years of age).

I did find a significant relation between ERK5 expression and age. As the age of the patient increased, the level of ERK5 staining observed also increased. This is in line with GBM prognosis being worse as the age of the patient increases. Survival estimates of adult glioblastoma patients are quite low, with 5.1%, of patients surviving five years post diagnosis. Those patients who are diagnosed with GBM under the age of 20 have better survival estimates. Thus, survival of these patients decreases with increasing age at diagnosis (Ostrom et al. 2015).

In breast tumour patients of older age, there was found to be an increase in ERK5 activity. One study investigated the roles of ERK1/2 and ERK5 in breast tumour proliferation in aged patients using a new 'benzimidazole inhibitor compound'. The researchers used transgenic MMTV *neu* mouse model to examine ERK1/2 and ERK5 activity with age. Their results showed that in middle and older aged mice, there were higher levels of ERK5 expression compared to that seen in the younger mice. In addition, they used the BT474 human breast cancer cell line and the MMTV *neu* cell line (MMC) to examine ERK5 activity and proliferation with age, and found that at earlier ages, ERK5 was constitutively active under basal conditions and ERK5 levels increased with increasing age (Monlish and Cavanaugh 2009).

Hence, I would argue that the results of my experiments are consistent with the above studies in other types of cancers, where higher grade brain tumours were seen to have an increased ERK5 activity with increasing age.

Chapter 8

8 Summary and Conclusion

8.1 Summary

In the present study, I evaluated the expression levels of ERK5 in 115 human brain tumour tissues and 5 non-neoplastic brain tissue specimens. 42 of the 115 samples were glioblastoma samples. The present study revealed that ERK5 was expressed at low levels in non-cancerous and low-grade glioma tissues, whereas high-grade gliomas expressed ERK5 at increased levels. This result was consistent with previous studies on other types of tumours, such as breast cancer and triple negative breast cancer (TNBC), where ERK5 expression has been correlated with patient outcome (Ortiz-Ruiz et al. 2014).

However, the association between ERK5 expression and the clinical prognosis of glioblastoma patients has not been fully defined or thoroughly studied. ERK5 is one of the least studied members of the MAPK group and in addition, there is even less data available on ERK5 and its role in GBM. In the present study, GBM patients with a high expression of ERK5 appear to be associated with a poor outcome. I have shown that ERK5 expression was present in all 15 of the GBM patient samples obtained from the tumour bank. In terms of disease outcome, 11 out of 15 died within 2 years of being diagnosed with GBM (73.3%), 2 out of 15 were lost to follow up (13.3%), and 3 out of 15 had no information available (20%). Hence, it would appear that those with a poorer prognosis had some level of ERK5 staining in their tumour, thus suggesting that ERK5 expression may be correlated with disease outcome. However, I was unable to obtain the survival data for all GBM patients, as this information was not easily accessible for all patients. GBM patients in general have a worse prognosis and shorter overall survival than patients with less aggressive and lower grade tumours.

In summary, the present study has demonstrated that ERK5 is overexpressed in higher grade gliomas, and particularly in the Grade IV glioblastoma and Grade III anaplastic astrocytomas. This result is based on a both samples from a Tissue Microarray and patient samples obtained from the Brain Tumour Tissue Bank. The present study identified that in various types of brain tumours, ERK5 expression increases with advancing grades. This increase in ERK5 expression was particularly the case with glioblastoma. Therefore, ERK5 may be a key player in the intrusive progression of glioblastomas. In a clinical setting, targeting ERK5 may be useful in these higher grade brain tumours such as glioblastoma. Thus, in order

to uncover the role that ERK5 plays in targeting brain tumours and GBM specifically, more research is needed that examines the role of ERK5-specific inhibitors and other drugs that interfere with ERK5 signalling. In addition, endeavours to better characterize the molecular mechanisms involved in GBM progression may assist in our understanding of the behaviour of the tumour, as well as pin-point potential therapeutic targets. Moreover, further exploration is needed in order to understand and better characterize the relationship between ERK5 expression and the prognosis and overall survival of glioblastoma patients.

8.2 Conclusion

The hypothesis of this study was to determine if there is a presence of a novel mechanism of ERK5 regulation of glioblastoma progression. This study showed that highly invasive brain tumours, such as glioblastoma, display a high level of expression of the ERK5 signalling pathway, and hence, ERK5 can be a potential target for glioblastoma therapy. Furthermore, my results suggest that the ERK5 signalling pathway could potentially be used as a biomarker, or as a prognostic factor for GBM progression. The abundant evidence of the expression of ERK5 in various types of tumours, together with the lack of studies and data investigating the role of ERK5 expression in glioblastoma and other grades of brain tumours shows that there is much yet to be explored with regards to the role of ERK5 in glioblastoma. In a clinical setting, targeting ERK5 may be useful in these higher grade brain tumours such as glioblastoma. Thus, in order to uncover the role that ERK5 plays in targeting brain tumours and GBM specifically, more research is needed that examines the role of ERK5-specific inhibitors and other drugs that interfere with ERK5 signalling. In addition, endeavours to better characterize the molecular mechanisms involved in GBM progression may assist in our understanding of the behaviour of the tumour, as well as pin-point potential therapeutic targets. Moreover, further exploration is needed in order to understand and better characterize the relationship between ERK5 expression and the prognosis and overall survival of glioblastoma patients.

My data supports glioblastoma as yet another type of cancer that displays abnormal MEK5/ERK5 signalling, as can be seen by the increased expression of ERK5 in this tumour. This indicates that targeting the MEK5/ERK5 pathway may be of potential benefit for future targeted therapies to combat this disease. However, there is still much that has yet to be explored.

My specific Aims/Objectives were as follows:

1. **To investigate whether ERK5 is expressed in Glioblastoma Multiforme.** I have successfully stained ERK5 antibody on 42 glioblastoma samples using IHC. I showed that there was some level of ERK5 staining in the cytoplasms of 85.4% of all of the glioblastoma samples examined, with 'Intermediate' and 'Strong' staining in 48.8% of the cytoplasms of all glioblastoma samples. In the glioblastoma pathology tissues from the tumour bank, 100% of the tumour samples of GBM had some level of ERK5 staining (>10% ERK5 staining in the cells).

2. **To investigate whether ERK5 is expressed in other types/grades of brain tumours.** I have successfully stained ERK5 antibody on 115 human brain tumour samples using IHC. I showed that there was ERK5 staining in the cytoplasms of 65.5% of all brain tumour samples examined.

3. **To determine whether there exists a difference in ERK5 expression between tumour grades.** I showed that there is a significant difference in ERK5 expression amongst grades.

4. **To determine whether there exists a difference in ERK5 expression between higher vs lower grades (1&2 vs 3&4).** I showed that there was a significant difference in ERK5 amongst low and high grades, with grades 3 & 4 having a higher mean ERK5 value compared to grades 1 & 2.

5. **To determine whether there exists a difference in ERK5 expression between genders.** Through statistical analyses, I showed that there was no difference in ERK5 expression between males or females in my study.

6. **To determine whether ERK5 expression is correlated with Age.** Through statistical analyses, I showed that in the tissue microarray samples, ERK5 expression was not correlated with age. In the Tumour bank tissue samples, I showed that there was a significant relation between ERK5 expression and age. As the age of the patient increased, the level of ERK5 staining observed also increased.

8.3 Limitations

Although I have reached my aims and objectives in this research, there are several limitations that I have faced as I undertook this research.

Sample Size - The sample size was not as large as I had initially planned for. I had hoped to be able to stain and analyze a greater number of grade 4 brain tumour samples, however due to time restrictions, this proved to be very difficult.

Follow up and survival data – there was very limited follow up and survival data on some of the patients whose brain tumour samples I examined. In order to better correlate survival and overall outcome with ERK5 staining in these patients' samples, it would have been beneficial to have data available for all patients. In addition, some patient samples had cytogenetics pathology results available, while other patient samples did not. If cytogenetics were available for all samples, it would have been interesting to analyze some of the results and further investigate the relevance of such results and the role they may play in the progression of GBM.

Patient data from TMA - In addition, there was very limited patient data available from the tissue microarray of 80 cores. The data was limited to age, gender and diagnosis, but no further clinical data was provided. It would have been beneficial to analyze and further explore this data.

Slide scoring – for each of the studies performed, a different neuropathology resident helped me score the slides. It would have been much better to have the same neuropathology resident help me score the slides as it would have contributed to more consistent data. Unfortunately though, this was not possible as the first neuropathology resident was no longer available.

Chapter 9

9 Future Directions

There exist significant gaps in the literature and of our understanding of the role of ERK5 in glioblastoma progression. However, researchers are beginning to uncover the clinical and biological behavior of glioblastomas, as well as the genetic pathways involved in the progression of these tumours. They are also starting to uncover the role of various genetic alterations in these pathways, as well as their mechanisms. Further research and data, as well as a better overall understanding of the nature of glioblastomas and the role the ERK5 signalling pathway plays in the progression of this tumour will hopefully lead to more effective targeted GBM therapies.

Further exploration of some key genetic alterations in glioblastoma should be targeted as areas for future research, as it will allow us to better understand which genetic alterations should be targeted and which should not. Further exploring the area of targeted therapies and selecting patients whom will likely benefit from a particular targeted therapy seems to be a promising area for future research. For example, looking at glioblastoma patients with methylated MGMT promotors and how they benefit from specific chemotherapeutic agents. In addition, further exploration is needed in order to fully understand the mechanism of action and the role that MicroRNAs play in the development of glioblastoma. For example, more research is needed on miR-200b-3p and the role it plays as a tumour suppressor gene in glioblastomas. Another area that requires further exploration and understanding is the role of the ERK5 signalling pathway as a regulator of the BDNF gene in gliomas.

My results suggest that the ERK5 signalling pathway could potentially be targeted for the role that it plays in GBM progression. While the mechanism of action is not yet fully understood, we do find some indications that ERK5 is involved in the progression of GBM. The ERK5 signalling pathway may be an ideal target for therapeutic intervention by developing drugs that specifically block ERK5 activity, particularly in higher grade brain tumours such as glioblastoma. In order to explore the value of ERK5 targeting in glioblastoma and other higher grade brain tumours, further studies are needed.

Based on the results from my experiment, I believe the next steps would be to further investigate the role of ERK5 in glioblastoma and other high grade brain tumours, specifically grades III and IV. It would be interesting to compare ERK5 staining to EGFR staining in these very same tumour samples as the EGFR gene is found to be amplified in approximately 43% of primary glioblastomas.

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Appendix A: Microtomy Procedure

Purpose: This procedure gives instructions on using a microtome and producing 4µm (or less) section from paraffin processed blocks. Slides and blocks are verified a minimum of 3 times to ensure errors are reduced.

Equipment: Microtome, Glass slides, Staining rack, Slide tray, Ice tray, Water bath (approx. 47 degrees Celsius).

Specimen: Paraffin processed block

Procedure:

1. Prior to cutting, verify that the surgical numbers on the slides correspond to the blocks received in the tote.
2. Initial slides using a chemical resistant marker in the bottom right hand corner of the frosted section of the slide.
3. Place paraffin processed block securely in the microtome chuck.
4. Rough trim into block until full face or appropriate level reached.
5. Place trimmed block on ice. Note: cooled block sections are much easier to work with.
6. If calcium deposits are present, perform surface decal procedure
7. Prior to placing trimmed block in chuck, perform a visual check to verify the surgical number on the block with the surgical number on the slide.
8. Segregate the slides needed for the block to be cut from the remaining slides.
9. Set microtome increment at 4µm unless otherwise requested.
10. Cut sections according to schedule of cutting and staining.
11. Float sections on the water bath. Paraffin will become warm and tissue will lay flat, removing wrinkles.
12. Select a good quality section.
13. Mount on correctly labeled glass slide.
14. Allow slide to drain.

15. Prior to placing slide in staining rack, visually check to verify the surgical number on the slide against the block.
16. Place rack in appropriate drying oven.

Reference: Lynch's Medical Laboratory Technology, p906.

Appendix B - Curriculum Vitae

Name: Reem Ansari

Post-secondary Education and Degrees:

University of Western Ontario
London, Ontario, Canada
2010-2017 MSc. Pathology

The University of Western Ontario
London, Ontario, Canada
2006-2007 Clinical Trials Management

University of Western Ontario
London, Ontario, Canada
2000-2004 BSc.
Honours Foods and Nutrition

Certifications and other programs:

SoCRA (Society of Clinical Research Associates)
Exam and Certification
2007

SoCRA (Society of Clinical Research Associates)
Recertification
2010, 2013, 2016

London Health Sciences Centre
London, Ontario, Canada
2016 Emerging Leader Program

Honours and Awards:

Deans Honour Roll – The University of Western Ontario
2003-2004

Facilitator/Speaker for Workshop on "Grading Toxicities"
2012 POGO (Paediatric Oncology Group of Ontario) Satellite Education Day
May 28, 2012, Toronto, Ontario.

Related Work Experience

Clinical Research Coordinator – Lead
Children's Hospital, London Health Sciences Centre
2007 - Present

- Coordinating Clinical Trials for Children's Hospital, LHSC with the C17 Research Network and the Canadian Pediatric Brain Tumour Consortium.
- Coordinating Clinical Trials with the Children's Oncology Group

Study Clinical Research Associate (CRA)
ACNS0621 Research Study Protocol
Cilengitide (EMD 121974) (IND# 59073) in Recurrent or Progressive and Refractory Childhood High-Grade Glioma
Children's Oncology Group
July 2010 – Present

Study Clinical Research Associate (CRA)
AALL08B1 Research Study Protocol
Classification of Newly Diagnosed Acute Lymphoblastic Leukemia (ALL)
Children's Oncology Group
July 2014 – Present

Diabetes Team Research Assistant
Centre for Studies in Family Medicine, Department of Family Medicine
University of Western Ontario
May, 2005 – November 2007

- Diabetes clinical trials and research projects, with primary duties involving the CANOE (CAnadian Normoglycemia Outcomes Evaluation) study and coordinating the epi-CANOE study.
- Collaborated with Researchers in writing several research grant proposals on Dietary Calcium intake and the prevention of childhood obesity.

Clinical Trials Management Practicum Student
ACCORD (Action to Control Cardiovascular Risk in Diabetes) Clinical Trial
St. Joseph's Health Care, London
Jan 2005 - Nov 2007

Workshop Facilitator/Speaker:

- Chair of the monthly Paediatric Hematology/Oncology Research Rounds for our Multi-Disciplinary team
- Facilitator/Speaker for Workshop on "Grading Toxicities" at the 2012 POGO Satellite Education Day, May 28, 2012 in Toronto, Ontario.
- Conducted training for Research Rounds staff on **"Good Documentation Practices"**- July 15, 2014.
- Conducted training for new Oncology Nursing Staff, along with my two Colleagues, Leslie Paddock and Julie Nichols: **"Haematoloy/Oncology CRAs at Children's Hospital"** – October 10, 2014.

COG CRA Research Committee Member:
2013-Present

- Promoting research endeavors within and collaboratively between disciplines in the Children's Oncology Group
- Bring forth ideas that will improve education, research and practice.
- Work together to design, implement and complete evidence based projects.
- Disseminate research findings through journal clubs, poster presentations, symposiums and publications.

SoCRA London Chapter Education Committee Member
2014-Present

- Facilitates Educational activities for the local London SoCRA chapter.

CRA Poster Session Judge at the Children's Oncology Group Meetings:

- CRA Poster Session, "Conquering Common Issues at Institutional Sites". September 13-16, 2011 – Atlanta.
- CRA Poster Session: "Trials and Tribulations of Using Electronic Medical Records (EMR) in Clinical Research". September 11-15, 2012 – Atlanta.
- CRA Poster Session: ""Tool Sharing: Implementing an Evidence-Based Approach to Clinical Research Practice". October 7-11, 2013 – Dallas, Tx.
- CRA Poster Session: "Promoting Education and Excellence at Sites: Professional Development and Career Paths for Clinical Research Professionals". September 16-19, 2014 - Dallas, TX.

Research Related Regulatory/HC/Lawson Training:

- HC Division 5 Training – March 18, 2014; July 2016
- NIH Human Participant Protections Education course certificate – February 9, 2005
- TCPS2 Core Training – Health Canada – January 8, 2015
- GCP Training – May 7, 2010
- GCP Training – April 19, 2009
- CITI GCP Training – March 11, 2014; July 2016
- Lawson Core and additional SOPs – February 18, 2014