GLIAL CELL LINE-DERIVED NEUROTROPHIC FACTOR (GDNF) FAMILY OF LIGANDS IS A MITOGENIC AGENT IN HUMAN GLIOBLASTOMA AND CONFERS CHEMORESISTANCE IN A LIGAND-SPECIFIC FASHION

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SUMMARY

High-grade gliomas are highly malignant tumours. Standard therapy includes surgical resection, radiation therapy and chemotherapy. However, in spite of advances in surgical techniques, medical technology, radiation therapy, chemotherapeutic regimens and other forms of therapy, the overall prognosis remains poor. The median survival of anaplastic astrocytoma and glioblastoma is about 3 years and 1 year respectively. Sadly, the survival outcome has not significantly improved the past 2-3 decades.

Surgery plays an important role in the management of high-grade gliomas. Surgery is critical for histological diagnosis of high-grade gliomas. Aggressive tumour resection can also rapidly reduce the intracranial hypertension associated with bulky disease and provide symptomatic relief and improved quality of life. The most contentious issue surrounds the controversy on whether surgery can improve overall survival and review of the literature shows that there is currently no good data to support this hypothesis.

In vitro experiments however demonstrate that greater tumour loading of glioblastoma cells requires higher levels of the chemotherapeutic agent 1,3-Bis (2-Chloroethyl)-1-Nitrosurea (BCNU) to achieve similar levels of cellular death when compared to a lower tumour loading. Increased tumour burden can therefore confer chemoresistance. Reduction of tumour burden may therefore potentiate adjuvant therapy. It is likely that the chemoresistance properties are potentiated by autocrine and paracrine pathways and facilitated by mitogenic agents.

Local tissue invasion distinguishes high-grade astrocytomas from low-grade tumours and this attribute limits the effectiveness of treatment. High-grade gliomas tend to recur locally until the patient succumbs to microscopic invasion and local compression of vital centres in the brain. The invasive and mitogenic behaviour of gliomas is influenced by proteases, angiogenic factors and growth factors.

Co-expression of growth factors with their corresponding receptors in gliomas may result in complex ligand-receptor interactions. The growth factor receptors expressed on the surface of tumour cells may bind soluble ligand produced by the same (autocrine), or adjacent cells (paracrine). In addition, membrane-anchored growth factor isoforms generated by alternative splicing may bind to the same (juxtacrine) or adjacent tumour cells (paracrine). Intracellular interactions between growth factor receptors and their ligands can also lead to intracrine activation of signaling cascades.

Many different growth factor/receptor systems have been implicated in the proliferative behaviour of gliomas such as vascular endothelial growth factor (VEGF), epidermal growth factor (EGFR), platelet-derived growth factor (PDGF), nerve growth factor (NGF), insulin-like growth factor (IGF), transforming growth factor-beta (TGF-β), brain-derived growth factor (BDGF) and scatter factor/hepatocyte growth factor (SF/HGF).

Glial cell line-derived neurotrophic factor (GDNF) was originally identified in 1993 by Lin et al as a neurotrophic factor. It was isolated from a rat glioma cell line supernatant and was shown to confer increased survival for embryonic midbrain dopamine neurons. Subsequently, it was also found that GDNF also had potent trophic functions in spinal motorneurons and central noradrenergic neurons. The GDNF-family

ligands (GFL) consists of GDNF, neurturin (NRTN), artemin (ARTN) and persephin (PSPN). These GFLs bind to specific GDNF-family receptor-α (GFRα) co-receptors and activate RET. The GFRα receptors are linked to the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor. Four classes of GFRα receptors have been characterised (GFRα1-4), which determine ligand specificity. GDNF binds to GFRα1, NRTN binds to GFRα2, ARTN to GFRα3 and PSPN binds to GFRα4. In addition, NRTN and ARTN may crosstalk weakly with GFRα1 and GDNF with GFRα2 and GFRα3.

Spliced isoforms are also abundant in the GDNF-family receptor-α (GFRα). GFRα1 receptor exists in two highly homologous alternatively spliced isoforms: GFRα1a and GFRα1b. GFRα1b is identical to GFRα1a except for the absence of 5 amino acids (140DVFQQ144), encoded by exon 5. In addition, GFRα2 and GFRα4 receptor spice isoforms have also been identified in mammalian tissue. Three variants of GFRα2 receptors (GFRα2a/2b/2c) have been identified. At least two splice variants of GFRα4 have been identified in rat tissue.

GDNF has been implicated as a mitogenic agent in many cancers such as pancreatic cancer, biliary cancer and phaeochromocytoma. GDNF is ubiquitous in the central nervous system and neural tissue and hence can also play a role in the pathogenesis of high-grade glioma. GDNF and its receptor GDNF-Family Receptor-α1 (GFRα1) have been demonstrated to be strongly expressed in human gliomas. Furthermore, GDNF has also been demonstrated to be a proliferation factor for rat C6 glioma cells by antisense experiments.

GDNF was overexpressed in the glioblastoma cell lines LN-229 and A172. Significantly, the expression of GDNF was also found to be increased in all glioma specimens when compared to adult brain, foetal brain, adult liver and foetal liver. All glioblastoma samples and cell lines demonstrated increased level of expression and the highest expression level was observed in a sample of glioblastoma tissue.

The glioblastoma cell lines had significantly lower levels of expression of GFRα1a compared to human adult and foetal brain samples. 11 out of the 13 human glioma samples had decreased levels of expression of GFRα1a compared to human adult and foetal brain samples. 2 out of the 8 glioblastoma samples had elevated levels of GFRα1a expression.

In the analysis of GFRα1b expression, the 2 glioblastoma cell lines had increased expression of GFRα1b compared to human adult and foetal brain samples. 5 glioma samples had elevated levels of expression of GFRα1b compared to human adult and foetal brain samples. These were all human glioblastoma samples.

On close analysis of the expression levels of GFRα1a and GFRα1b levels, an interesting observation was noted. The glioblastoma cell lines demonstrated much higher levels of GFRα1b expression than GFRα1a expression. For cell line LN-229, the ratio of GFRα1b/GFRα1a was 16.3 and the ratio of GFRα1b/GFRα1a was 14.3 for cell line A172. A similar trend was also noted in 7 out of the 8 human glioblastoma samples. The GFRα1b/GFRα1a ranged from 1.73 to 5.44 in the 7 specimens. Only one human

glioblastoma specimen had a higher GFRα1a/GFRα1b ratio. There exists a differential expression level of GFRα1b and GFRα1a with an elevated GFRα1b/GFRα1a ratio.

The potential role of GDNF in conferring chemoresistance was examined. Glioblastoma cell lines were pre-treated with GDNF and subjected to BCNU chemotherapy and compared to a control group without pretreatment with GDNF. In the analysis for chemotherapy cytotoxicity effects using the MTS assay, GDNF was shown to confer very significant cellular survival in the presence of BCNU chemotherapy. Replicating the experiments in a similar fashion with pretreatment with Neurturin (NRTN) did not demonstrate any survival advantage. This demonstrates that the ability to potentiate chemoresistance is ligand-specific.

GDNF has been found to influence the migration and mitogenic behaviour of low-grade gliomas. Treatment of low-grade Hs683 cells with GDNF significantly increased migration comparable to high-grade C6 cells. The molecular mechanism is mediated by the activation of JNK-1, ERK 1/2 and p38 MAPK. Treatment of Hs683 cells with 60ng/ml of GDNF markedly activated JNK. A kinetic study of GDNF-induced JNK activation showed that JNK was markedly activated within 30 min after GDNF treatment and returned to the basal level at 90 min. ERK 1/2 were activated at 10 min after GDNF treatment and the activated levels remained until 60 min. GDNF markedly increased the active form of p38 MAPK within 10 min, maximally activated at 30 min and decreased at 60 min after the treatment[311].

In the light of the evidence, we examined the modulation of MAPK and Akt signaling pathways in glioblastoma cell lines. LN-229 and A172 human glioblastoma cell

lines were stimulated with BCNU and GNDF and the experiments were studied at 0, 10, 30, 60 and 180 mins respectively.

Western blotting showed that BCNU induces activation of MAP kinases (ERK1/2, JNK and p38) in both LN-229 and A172 human glioblastoma cell lines. BCNU was however found to reduce the background activation of Akt in the A172 human glioblastoma cell line.

GDNF was found to induce the activation of ERK1/2 and Akt in both LN-229 and A172 human glioblastoma cell lines. GDNF was however found to reduce the background activation of JNK and the A172 human glioblastoma cell line in a time-dependent fashion.

The ability of GDNF to promote Akt activity and inhibit JNK activity may contribute to the increased cellular survival to BCNU chemotherapy.

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LIST OF ABBREVIATIONS

AGNIR	Advisory Group on Non-ionising Radiation
AIDS	Acquired Immune Deficiency Syndrome
AML	Acute Myeloid Leukaemia
Artemin	ARTN
ATCC	American Type Culture Collection
BBB	Blood-Brain-Barrier
BCA	Bicinchoninic acid
BCNU	1,3-Bis (2-Chloroethyl)-1-Nitrosurea
BDGF	Brain-Derived growth Factor
bp	Base pairs
BTSG	Brain Tumour Study Group
c-Jun	Proto-oncogene avian sarcoma virus 17
C_t	Threshold Cycles
CCNU	Losmustine
CD	Cluster of Differentiation
Cho	Choline
CNS	Central Nervous System
CO_2	Carbon Dioxide
Cr	Creatine
CSF	Cerebrospinal Fluid
CT	Computed Tomography
Cu^+	Cuprous

Da	Dalton
DAG	Diacylglycerol
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic Acid
ε	Efficiency of target amplification
EBV	Ebstein-Barr Virus
ECM	Extracellular Matrix
EGFR	Epidermal Growth Factor Receptor
EOR	Extent of resection
ErbB2/HER2/Neu	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian)
ERK	extracellular signal-regulated kinase
FBS	Foetal Bovine Serum
^{18}FDG	^{18}F-fluoro-2-deoxyglucose
FGF	Fibroblast Growth Factor
FGFR	Fibroblast Growth Factor Receptor
fMRI	Functional Magnetic Resonance Imaging
G1	Gap 1
GABA	γ-aminobutyric acid
GADPH	Glyceraldehyde-3-phosphate dehydrogenase
GBM	Glioblastoma Multiforme
GDNF	Glial Cell Line-Derived Neurotrophic Factor

GFL	GDNF-Family Ligand
GFR	GDNF-Family Receptor
GPI	Glycosylphosphatidylinositol
GSM	Global System for Mobile Communications
HIV	Human Immunodeficiency Syndrome
HRP	Horseradish Peroxidase
IC	Inhibitory Concentration
ICNIRP	International Commission on Non-ionising Radiation Protection
IEGMP	Independent Expert Group on Mobile Phones
IGF	Insulin-like Growth Factor
IL	Interleukin
IRB	Institutional Review Board
IRF	Interferon Regulatory Factor
IU	International Unit
JC virus	John Cunningham virus
JNK	c-Jun NH(2)-terminal kinase
kDa	kiloDalton
Lac	Lactate
Lip	Lipids
LOE	Level of Evidence
LOH	Loss of Heterozygosity

µg	microgram
µL	microlitre
µM	micromolar
mg	milligram
mL	milliliter
mM	millimolar
MAPK	Mitogen Activated Protein Kinase
MEN	Multiple Endocrine Neoplasia
MGMT	O^6-methylguanine-DNA-methyltransferase
MI	Myoinositol
MMPS	Metalloproteases
miRNA	micro-RNA
mins	minutes
MRI	Magnetic Resonance Imaging
MRS	Magnetic Resonance Spectroscopy
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphnyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt
NAA	N-acetylaspartate
NCAM	Neural Cell Adhesion Molecule
NCCTG	North Central Cancer Treatment Group
Neuturin	NRTN
NF-1	Neurofibromatosis Type-1

NF-2	Neurofibromatosis Type-2
Ng	nanogram
NGF	Nerve Growth Factor
NHG	National Healthcare Group
NNI	National Neuroscience Institute
NRPB	National Radiological Protection Board
NSW	New South Wales
ORF	Open Reading Frame
p53	protein 53 kDa
PAI	Plasminogen Activator Inhibitor
PBS	Phosphate Buffer Solution
PCr	Phosphocreatine
PCR	Polymerase Chain Reaction
PDGF	Platelet-derived Growth Factor
PDGFR	Platelet-derived Growth Factor Receptor
PET	Positron Emission Tomography
pH	potential of hydrogen
Pi	Propidium Iodide
PI3K/AKT-PKB	Phosphoinositide 3-Kinase/AKT-Protein Kinase B
PIP_2	Phosphatidylinositols(4,5)P_2
PIP_3	Phosphatidylinositols(3,4,5)P_3
PKC	Protein Kinase C

PLC-γ/PKC	Phospholipase C-γ/Protein Kinase C
PML	Progressive Multifocal Leukoencephalopathy
pmol	picomole
PSPN	Persephin
PTEN	Phosphatase with tensin homology
PXA	Pleomorphic Xanthoastrocytoma
RB	Retinoblastoma
RCT	Randomised Control Trial
RET	Rearranged during transfection
RF	Radiofrequency
RNA	Ribonucleic Acid
RT	Reverse Transcription
RTK	Receptor Tyrosine Kinase
RTOG	Radiation Therapy Oncology Group
SCC	Squamous Cell Carcinoma
SCID	Severe Combined Immune Deficient
SDS-PAGE	Sodium Dodecyl Sulphate-Polyarylamide Gel Electrophoresis
SF/HGF	Scatter Factor/Hepatocyte Growth Factor
SP	Side population
SPECT	Single Positron Emission Computed Tomography

Taxol	Paclitaxel
TB	Tuberculosis
TBST	Tris buffered saline Tween-20
TGF	Transforming Growth Factor
TIMPS	Tissue Inhibitors of Matrix Metalloproteases
TNF	Tumour Necrosis Factor
tPA	tissue plasminogen activator
TTSH	Tan Tock Seng Hospital
Tyr	Tyrosine
UK	United Kingdom
UPDRS	Unified Parkinson's Disease Rating Scale
US	United States
VEGF	Vascular Endothelial Growth Factor
VHL	Von Hippel-Lindau Disease
VP16	Etoposide
WHO	World Health Organisation

CHAPTER 1

INTRODUCTION

1.1 Brain Tumours

Brain tumour is one of the most devastating forms of human disease. The brain has long been considered sacrosanct and inviolable and the concept of a tumour in the brain is frightening not just to the layman but to medical doctors as well.

Yet brain tumours are one of the most common forms of tumours in humans. They are the second most common form of malignancy in children and the sixth to eight most common form of malignancy in adults. Brain tumours are conventional categorised into primary tumours which originate in the brain and secondary or metastatic brain tumours which originate from a different site such as the lung, breast or colon.

Primary tumours of the brain and spine account for less than 2% of malignancies but are responsible for 7% of the years lost of life lost from cancer prior to 70 years of age. In childhood, these figures are even more dramatic and primary brain tumours account for 20% of malignant tumours diagnosed before 15 years of age[1].

The most common form of primary brain tumours are gliomas which originate from glial cells. There are many forms of gliomas: astrocytomas, ependymomas and oligodendrogliomas are some of the examples.

1.2 Astrocytomas

The term astrocytoma was used as early as the late 19th century by Virchow[2] but was only firmly used in histopathological classification by Baily and Cushing in 1926[3]. Astrocytomas are the most common gliomas and account for more than 60% of all

primary brain tumours[4-5]. They arise from star-shaped glial cells known as astrocytes. In adults, astrocytomas most often arise in the cerebrum. In children, they occur in the brain stem, the cerebellum, and the cerebrum. Astrocytomas are classified by various grading systems. Examples of these are the Kernohan[6], Ringertz[7], St Anne-Mayo[8-9] and the World Health Organisation (WHO)[10] grading systems. The introduction of a grading system marked the beginning of the era of refining different classifications based on histogenesis. The major reason for this shift in philosophy resulted from increasing awareness amongst neuropathologists, neurosurgeons and neuro-oncologists that a meaningful classification schema of central nervous system tumour can give an indication of biologic behaviour and provide a basis for the development of treatment strategies.

The most commonly used grading system currently used is the World Health Organisation (WHO) Classification system. The WHO classification separates the astrocytic tumours into two major categories: the diffusely infiltrating astrocytomas and the relatively more circumscribed, specialised variants of astrocytoma (pilocytic astrocytoma, pleomorphic xanthoastrocytoma and subependymal giant cell astrocytoma)[11].

The diffusely infiltrating group consists of astrocytic tumours which generally infiltrate beyond the macroscopically apparent brain-tumour interface and frequently undergo anaplastic transformation. The more circumscribed group comprises tumours which show limited infiltration into surrounding brain and which infrequently undergo malignant transformation[12].

Four different tumour grades are classified under the WHO grading system: Grade I to Grade IV. Tumours with nuclear atypia alone are designated Grade II; those which in addition to nuclear atypia demonstrate mitotic activity are Grade III; and neoplasms showing atypia, mitosis, endothelial proliferation and/or necrosis are considered Grade IV. WHO Grade I and II tumours are known as low-grade astrocytomas whereas WHO Grade III and IV tumours are known as high-grade or malignant astrocytomas. The most common WHO Grade III astrocytoma is anaplastic astrocytoma and the most common WHO Grade IV astrocytoma is glioblastoma.

High-grade astrocytomas are highly infiltrative and aggressive tumours with marked proliferative potential. Anaplastic astrocytoma may arise from previously low-grade astrocytoma or de novo without an identifiable precursor lesion as a high-grade astrocytoma. The progression of anaplastic astrocytoma to glioblastoma influences the prognosis of the disease. The mean time to progression is 2 years and the mean survival is 3 years[13-15]. Glioblastoma is the most common astrocytoma, accounting for approximately 12-15% of all intracranial neoplasms and 50-60% of all astrocytic tumours[4]. In most European and North American countries, the incidence is in the range of 2-3 new cases per 100,000 population per year[5]. It is however regrettably the most malignant astrocytic tumour. It consists of poorly differentiated neoplastic astrocytes and histological features include cellular polymorphism, nuclear atypia, increased mitotic activity, vascular thrombosis, microvascular proliferation and necrosis. Similar to anaplastic astrocytoma, glioblastoma may arise de novo as glioblastoma or may transform from diffuse astrocytoma (WHO Grade II) or anaplastic astrocytoma. The

diagnostic hallmark is the presence of prominent microvascular proliferation and/or necrosis.

1.3 Malignant Astrocytoma

Malignant astrocytomas are the most frequent primary brain tumours in adults and represent a significant cause of morbidity and mortality. The inherent neurological and mental deterioration with disease progression is a source of great distress not just to the patient but their family members. Family members often feel helpless as they see their loved one progressively and relentlessly deteriorating before their very eyes. The peak incidence of malignant astrocytoma in the 4th and 5th decade of life translates that patients are frequently afflicted at the most productive period of their lives[4]. The high cost of treatment and high fatality rate has obvious serious personal, social and public health consequences.

1.4 Epidemiology of Malignant Astrocytoma

Malignant astrocytomas are the most common primary brain tumours and would generally constitute over 40% of all primary brain tumours. The distribution of malignant astrocytoma in the population is age specific. The probability of histologic malignancy in an astrocytoma is only 0.34 between the years of 30 and 34 and is 0.85 after the age of 60[16]. The incidence per 100,000 population of glioblastoma and astrocytoma rises from 0.2 and 0.5 in the under-14 age group to 4.5 and 1.7 respectively after the age of 45 years[17]. There is a distinct difference in the location of occurrence of these tumours in the different age groups. In the younger age group (<25 years), 67% of astrocytomas are

located in the posterior fossa whereas 90% of the tumours are located in the supratentorial compartment in the older age group (>25 years). The incidence of malignant astrocytoma is more common in males compared to females in a 3:2 ratio[16]. There are trends to show that the incidence of astrocytomas can vary between racial groups or nationalities. This incidence is detected even on incidence rates standardized to the world population for males and females separately. Some of the variation may be due to access to health care and medical technology. Interesting, Japan which has an advanced health care system and modern technology to Western Developed Countries has rates of Central Nervous System (CNS) cancer which are a third or less of those observed in the United States. The incidence in other Asian countries is also low[18]. Some ethic groups such as New Zealand Maoris and New Zealand Pacific Polynesian Islanders have higher incidence rates than Caucasian New Zealanders. In contrast, African Americans have a lower incidence than White Americans in the United States (US). Jews living in the Israel and Jewish populations in the US have elevated rates[19-21]. McCredie et al reviewed the CNS tumour incidence by ethnic group in New South Wales (NSW) and found no stastically significant differences in males but a significantly lower rate in female migrants from Asia[22]. Giles et al similarly also found lower rates of malignant CNS tumours in female migrants from Middle East and Asia[23]. In Singapore Chinese, the age standardized incidence of CNS tumour for adults aged 35-64 years was found to be 2.5 per 100,000 for males and 0.9 per 100,000 for females. This is significantly lower than the age standardized incidence for US Whites which range from 7.4 to 13.7 per 100,000 for males and 4.4 to 12.1 for females[18]. Parkin et al similarly demonstrated that

the incidence of astrocytoma in children aged 0-14 in Singapore was 3.8 per 100,000 compared to 10.3 per 100,000 in New South Wales and 15.7 per 100,000 in Sweden[24].

1.5 Aetiology

In the vast majority of cases, malignant astrocytomas occur sporadically without any identifiable familial tendency or environmental risk factors.

The cell of origin of malignant astrocytomas has traditionally been thought to be the astrocyte based on staining and morphological similarities[4,25]. Malignant transformation, like in all other cancers, has been attributed to genetic aberrations in normal astrocytes leading to dysregulated growth and proliferation. This hypothesis has however been never adequately tested. Furthermore, the concept of dedifferentiation of mature glia cells is questionable and fails to explain the presence of mixed tumours such as oligoastrocytomas.

More than two decades ago, Cairncross proposed a radical hypothesis that glioblastoma may be likened to chronic myelogenous leukaemia, a neoplasm that arises following transformation of a myeloid precursor cell[26]. There is now evidence to support this hypothesis as the presence of pleuripotential neural progenitor cells have been demonstrated in the subventricular zone of mature brain[27], the lining of the lateral ventricles, the dentate gyrus[28], within the hippocampus and subcortical white matter[29]. Lapidot et al had previously demonstrated that cells that from acute myeloid leukaemia (AML) patients exhibiting the haemopoietic stem cell surface phenotype cluster of differentiation (CD) 34^+ and CD 38^- could be injected into severe combined immune-deficient (SCID) mice to generate a leukaemia similar to that of the original patient[30].

Bonnet and Dick subsequently identified a serially transplantable population of human leukaemia cells enriched for tumour-initiating abilities in 1997 and developed an experimental system in which to test the repopulation capacity of normal haemopoietic and leukaemic human cells when injected into mice[31]. Al-Hajj et al demonstrated that isolating cells on the basis of a $CD44^{+}CD24^{-/low}Lineage^{-}$ cell phenotype enriched the tumour-initiating ability of surgically explanted breast cancer cells from a primary site of disease or from metastatic pleural effusions. This constituted the first identification of a cancer stem cell population in solid tumours that could self-renew, proliferate and differentiate to regenerate the phenotypically heterogeneous tumour when injected into mice[32].

In 2003, Singh et al demonstrated that tumour-derived neurosphere cells from human brain tumours expressing the neural stem cell surface marker CD133 had an increased capacity for self-renewal and proliferation in vitro[33]. The presence of pleuripotential neural progenitor stem cells has been identified in glioblastoma tumours[34-37].

Known aetiological agents associated with increase incidence of brain tumours include genetic syndromes, familial clustering and environmental factors.

1.6 Genetic Factors

Several hereditary and congenital diseases have been identified which have a high preponderance of developing not just astrocytoma but also other brain tumours such as meningioma, haemangioblastoma and vestibular schwannoma. Examples of these diseases includes: Neurofibromatosis Type 1 (NF-1), Neurofibromatosis Type 2 (NF-2)[38-

[39], Tuberous sclerosis[40], Von Hippel-Lindau disease (VHL)[41-42], ataxia telangiectasia[43], Gorlin and Turcot syndrome[44]. These diseases are generally inherited in an autosomal dominant fashion but may exhibit varying degrees of penetrance. Other genetic diseases with increased incidence of brain tumours are Li-Fraumeni syndrome[45] and the multiple endocrine neoplasia (MEN) type 1[46].

A familial association of astrocytomas where certain families have increased incidence of astrocytomas has also been proposed[47-49]. This theory is however still controversial, although there is evidence to show that patients newly diagnosed with astrocytomas have a close relative with a verifiable history of a glial tumour. Ikizler reported that 6.7 % of newly diagnosed cases had a significant family history of glial tumour. This data is substantiated with observation that 9.4% of anaplastic astrocytoma patients had at least one first degree relative with an astrocytic tumour[50]. A significant proportion of patients with family history of up to 16% have been reported by Mahaley et al[51]. These figures are significantly higher than the rate of random chance occurrence which is estimated to be 4%.

1.7 Environmental Factors

There are many reports in the literature investigating protean associations between environmental factors and increased risk of brain tumours. However considering the many variable factors, number of studies and low statistical power, it is difficult to definitely prove a direct causation effect and it is expected that many of these factors will be merely chance associations[52].

1.7.1 Radiation

There is evolving evidence that exposure to radiation in utero[53-54], childhood[55] and adulthood[56] may increase the risk of brain tumours. Particularly, the use of radiation therapy to treat children with tinea capitis in Eastern European have demonstrated increased incidence of meningiomas, gliomas and nerve sheath tumours[55]. High dose radiation has been shown to increase the risk of meningioma but not glioma in adults[56-59]. The role of non-ionising radiation in the pathogenesis of brain tumours such as magnetic field radiation is contentious and these forms of radiation are not believed to have any tumour-promoting properties[60]. It has been suggested that residential and occupational exposure to electromagnetic field radiation may relate to the development of CNS tumours in children[61]. This is however highly controversial and Feychting and Ahlbom have failed to find any significant associations between electromagnetic field exposures and CNS tumours in children[62].

1.7.2 Chemicals

Putative carcinogenic chemicals implicated in causing brain tumours include benzene, organic solvents, lubricating oils, acrylonitrile, vinyl chloride, formaldehyde, polycyclic aromatics and phenol[63-64]. Observations in certain occupations with increased exposure such as in the electrical and electronic industry, oil refining, rubber, airplane industry, farming, manufacturing industry, pharmaceutical industry, laboratory professionals, embalmers and chemical industry have shown an associated higher risk of brain tumours[64-68].

1.7.3 Diet

There is no strong association between dietary factors and brain tumours currently. Any association between diet and CNS tumours in humans remain unproven. There have been international correlations between CNS tumours and per capita consumption of total fat, animal protein and fats and oil[69]. These differences can also be easily explained by international differences in technological advancement and ethnic differences in susceptibility. N-nitroso compounds and their precursors might increase brain tumour risk whereas the consumption of orange juice and vitamin supplements (which contain anti-oxidant substances such as ascorbic acid which inhibit endogenous nitrosation activity) has been associated with reduced risk of childhood CNS tumours[70-71]. Most studies however used poor measures of intake and have been too small to detect any significant risks.

The association with alcohol is also sparse and inconsistent. Most studies have shown negative results[72]. In fact, there is literature to support that alcohol consumption can reduce the incidence of brain tumours[59].

1.7.4 Tobacco

The association between smoking and brain tumours is similar to the situation with dietary factors. The findings are difficult to interpret and it is difficult to prove any causation effect. Furthermore, there is conflicting data in the literature. Associations have been shown between passive smoking and childhood CNS tumours[73]. Non-smoking wives of men who smoked more than 20 cigarettes a day were also shown to have a rate

of brain tumour almost fivefold to that of women married to non-smokers[74]. Choi et al and Brownson et al however did not show any risk of CNS tumours associated with smoking[72,75].

1.7.5 Drugs

Long-term uses of drugs such as tranquilizers and anti-epileptic medication have been implicated in the pathogenesis of brain tumours[76]. The regular use of tranquilisers was associated with an increased risk of gliomas[77]. Olsen et al however found that there is an a high rate of CNS tumours in Danish epileptics which subsequently declined on follow-up, indicating that epilepsy rather than anti-epileptic agent was associated with CNS tumours[78]. There is however no definitive epidemiological proof that these associations hold true at the present moment.

1.7.6 Infection

Various forms of infection including bacterial, parasitic and viral infections have been implicated in the aetiology of brain tumours. Viral infections are the most commonly implicated infections. (JC) virus has been reported in high-grade astrocytoma particularly in the setting of multifocal high-grade astrocytoma in Human Immunodeficiency Virus/Acquired Immune Deficiency Syndrome (HIV/AIDS) patients with progressive multifocal leukoencephalopathy (PML) [79]. C-type viruses and human papovavirus have also been detected in a variety of human central nervous system (CNS) tumours[80-81]. Ebstein Barr Virus (EBV) is ubiquitous in cerebral lymphomas occurring in HIV/AIDS patients[82] and the Rous Sarcoma Virus, adenovirus type 12, simian virus 40,

JC papovavirus and both murine and avian sarcoma viruses can induce CNS sarcoma in animal models[83-85].

Associations between Tuberculosis (TB) and glioma have been reported, although there is suggestion that this may be related to an impaired immune response rather than as a direct association[86-87].

Toxoplasmosis gondii infection has a predilection for neural tissue. It has been linked to astrocytoma in one study[88]. However, a study by Ryan et al demonstrated that *Toxoplasmosis gondii* antibodies were associated with meningioma formation but not glioma pathogenesis[89].

1.7.7 Mobile Phone

The recent popularity and widespread use of mobile phones have led to great speculation that brain tumours may be caused by radiofrequency (RF) field from the use of mobile communication devices.

There are currently about 50 million mobile phones in use in the United Kingdom (UK) compared with around 25 million in 2000 and 4.5 million in 1995. These are supported by about 40 000 base stations in the UK network. The majority of these base stations operate under the Global System for Mobile Communications (GSM).

In less than ten years since the first GSM network was commercially launched as the second generation of mobile phones, it has become the world's leading and fastest growing telecommunications system. It is in use by more than one-sixth of the world's population and it has been estimated that at the end of January 2004 there were 1 billion

GSM subscribers across more than 200 countries. The growth of GSM continues unabated with more than 160 million new customers in the last 12 months.

The extensive use of mobile phones suggests that users do not in general judge them to present a significant health hazard. Rather they have welcomed the technology and brought it into use in their everyday lives. Nevertheless, since their introduction, there have been persisting concerns about the possible impact of mobile phone technologies on health.

In 1999, the Independent Expert Group on Mobile Phones (IEGMP) was establish to review the situation. Its report, *Mobile Phones and Health* (the Stewart Report), was published in May 2000. It stated:

" The balance of evidence to date suggests that exposures to RF radiation below NRPB (National Radiological Protection Board) and ICNIRP (International Commission on Non-Ionising Radiation Protection) guidelines do not cause adverse health effects to the general population.

There is now scientific evidence, however, which suggests that there may be biological effects occurring at exposures below these guidelines.

We conclude therefore that it is not possible at present to say that exposure to RF radiation, even at levels below national guidelines, is totally without potential adverse health effects, and that the gaps in knowledge are sufficient to justify a precautionary approach.

We recommend that a precautionary approach to the use of mobile phone technologies be adopted until much more detailed and scientifically robust information on any health effects becomes available."

Similarly, the Advisory Group on Non-ionising Radiation (AGNIR) has also concluded that the amount of radiation from radiofrequency field is insufficient to result in carcinogenesis from DNA damage. The most recent report "Mobile Phones and Health 2004" by the National Radiological Protection Board (NRPB) also reported that there was no association between the mobile phone use and brain tumours[90].

The issue of mobile phone usage and brain tumours is however of great current interest in view of the global widespread use and dependence on mobile communication devices. Individual studies have found positive correlations between high grade astrocytoma and phone use ipsilateral to the side of the tumours. Hardell et al identified one cohort study and 13 case-control studies and the data was analysed for mobile phone usage >10 years and ipsilateral exposure if presented. The results showed that the use of mobile phones for >10 years give a consistent pattern of an increased risk for acoustic neuroma and glioma, most pronounced for high-grade glioma. The risk is highest for ipsilateral exposure[91]. A meta-analysis by Lahkola et al however demonstrated no increased risk of brain tumours with mobile phone use greater than 5 years[92]. A large study conducted by Hepworth et al on a large cohort in United Kingdom also did not show any association between mobile phone usage and gliomas[93]. It is cautionary to note that the phenomenon of widespread use of mobile phones is a relatively recent and although there is currently no evidence to support the association between mobile phone usage and brain tumours in the short and medium term, the absolute effects of long-term usage may not be quantifiable at the present moment.

1.8 Clinical Features

The clinical features of high-grade astrocytoma are similar to that of any other space-occupying mass lesion in the brain. The signs and symptoms are a function of the location rather than the actual pathology.

The presentation generally will fall into the following categories:

(A) Signs and symptoms of elevated intracranial pressure - This can result from the tumour mass, cerebral oedema or obstructive hydrocephalus and can manifest as headaches, drowsiness, nausea and vomiting. The headaches are classically worse in the morning and are relieved by vomiting although this relationship is frequently not present. Clinical signs may include evidence of altered consciousness, papilloedema and 6th nerve palsy. In severe cases of herniation syndrome, decerebration and evidence of 3rd nerve palsy and coma will ensue and the condition will rapidly lead to death if no active emergent therapy is instituted.

(B) Focal neurological deficit- This is dependent on the location of the tumour. For instance a tumour located in the speech centre will present with speech disturbance. Examples of focal deficits are cranial nerve deficits, hemiparesis, dysphasia, paraesthesia, visual problems, mental and personality change.

(C) Seizures- Brain tumours constitute 8% of first seizure in adults 15 years of age and older. In a series from the Montreal Neurological Institute, seizures were documented at some stage during the clinical course in 48% of 209 patients with hemispheric astrocytomas[94]. Penfield et al reported a seizure incidence incidence of 37% in glioblastoma, which is nearly half as frequent as low-grade gliomas[95]. The most frequently epileptogenic areas are the frontal, parietal and temporal lobes. Seizures

result from irritation of adjacent cortical structures by the tumour. Morphological and biochemical alterations occur which result for the epileptogenicity of the tumour[96-97]. Epilepsy may be is caused by interference with normal γ-aminobutyric acid (GABA) and glutamate uptake and metabolism in the surrounding cortex. Analysis of human glioma biopsy specimens for the amino acid neurotransmitters and glutamine has shown that gliomas associated with epilepsy have a higher concentration of glutamine[98]. It has also been demonstrated that hyperexcitable cortex surrounding the tumour lesion have significantly reduced populations of GABA and somatostatin containing neurons when compared to adjacent non-tumour, non-epileptogenic cortex from the same patient[99]. Furthermore, comparison of the temporal cortex taken from patients with temporal lobe epilepsy with normal controls showed reduction in thickness of the cortex and reduction in the numbers of nerve cells. This decline was due to cell degeneration and was more severe for non-GABAergic nerve cells. Accordingly, the proportion of the GABA-positive neurons in the otherwise diminished neuronal population increased to 36.4% from the 32% control value. The number of GABAergic terminals, however, decreased even further, explaining the resulting disinhibition during epileptic seizures[100]

Most cancers have the ability to metastasise or spread beyond the normal anatomical confines. This occurs by local infiltration or invasion, lymphatic spread or haematogenous spread. It is noteworthy that malignant gliomas give rise to significant morbidity and mortality by local infiltration and invasion and very rarely metastasise outside the cranium or the central nervous system. The nervous system is also devoid of lymphatic channels. A systematic review of the literature only reviewed 51 cases of

malignant glioma with metastasis to the spine and extraneural structures. The consequences of metastatic spread is however dire with the vast majority of patient not surviving beyond 6 months after the detection of metastatic disease[101].

1.9 Management

When the diagnosis of brain tumour is considered, initial imaging studies should be performed. This initial diagnostic study should be a contrast enhanced computed tomography (CT) or preferably magnetic resonance imaging (MRI) scan. The identification of any mass lesion, particularly in the presence of contrast enhancement is highly suggestive of a high-grade astrocytoma.

There have been recent advances in neuroradiological techniques in functional and metabolic imaging of brain tumours. The functional imaging techniques of positron emission tomography (PET), single positron emission computed tomography (SPECT) and magnetic resonance spectroscopy (MRS) are able to quantify various aspects of brain tumour metabolism. Information regarding tumour blood flow, tumour growth rate, degree of oxygenation, potential of hydrogen (pH) and chemical composition such as lactate (Lac), choline (Cho), N-acetylaspartate (NAA), phosphocreatine (PCr), creatine (Cr) and lipids (Lip) can be obtained.

Increased glucose uptake and glycolysis has long been associated with malignancy[102]. The analog of glucose used in PET is ^{18}F-fluoro-2-deoxyglucose (^{18}FDG). Low uptake of ^{18}FDG has been found to be a good prognostic indicator[103-104]. Herholz et al used ^{18}FDG PET in 36 patients with WHO Grade 2 and 3 tumours and found that 10

out of 11 with a low metabolic index compared to 4 out of 10 patients with a high metabolic index survived during a mean follow-up of 24 months[105-106].

SPECT is a less costly and more investigative tool compared to PET. SPECT scanning uses radioisotopes utilised in nuclear medicine, namely technetium, gallium, thallium and iodine which act as blood flow markers[107]. Thallium is highly sensitive for detecting viable tumour and has even been used to grade astrocytomas[108-112].

Magnetic resonance spectroscopy (MRS) uses the interaction between atomic nuclei and magnetic fields which then detects the resonance spectra of chemical compounds giving a reflection of in situ chemistry. Magnetic nuclear isotopes such as carbon 13, deuterium, fluorine 19, hydrogen 1, phosphorus, sodium 23 or tritium absorb radio frequency energy when placed in a magnetic field. The energy absorption results in resonance of the nuclei of the atoms in the chemical compound studied. Different atoms resonate at different frequencies, and this difference in resonance frequency reveals structural information about the brain metabolites such as choline (Cho), creatine and phosphocreatine (Cr), lactate (Lac), myoinositol (MI), lipids (Lip) and N-Acetylaspartate (NAA). In vivo MRS studies of glial brain tumors have reported increased levels of Cho compared to normal brain[6]. Elevated measurements of Cho/NAA and Cho/Cr ratios have also been found to be an important malignancy marker for histological grading of astrocytoma, and pattern recognition analysis of in vivo MRS has been proposed as a non-invasive method to enhance the diagnosis of human brain tumors grade[113-117]. Apart from Cho, the concentrations of other metabolites such as Lac, Lip and MI can vary even among tumors of similar histological grade, and these chemicals are the subject of active research[118]. Ishimaru has however demonstrated that metastases and glioblastomas

showed definite lipid or lipid/lactate mixture, but anaplastic astrocytomas showed no definite lipid signal[117]. Targeting areas with elevated lipid content may provide the highest diagnostic yield. Ng et al showed that regions with highest lipid content (Lip/Cr ratio) revealed glioblastoma (WHO Grade 4) whereas a region with high Cho/NAA ratio but low Lip/Cr ratio revealed anaplastic astrocytoma (WHO Grade 3)[119]. MRS targeting regions of highest lipid content may therefore be useful in maximizing the diagnostic yield and accuracy when performed stereotactic biopsy for patients with suspected astrocytoma.

At the end of the day, functional and metabolic imaging can only provide a guide on the probabilty of malignancy and has inherent problems with false positivity and negativity as well as issues with sensitivity and specificity. It has an important role in target selection to improve diagnostic yield and reduce sampling error in stereotactic biopsy and also has an important role in the follow-up of patients to help to distinguish between tumour recurrence or treatment effects. However, it has severe limitation in definitive diagnosis and can certainly not provide histological proof of the disease.

Tissue diagnosis is therefore extremely critical as the radiological appearance of malignant astrocytoma may be mimicked by numerous neoplastic and non-neoplastic lesions such as metastatic tumours, lymphoma, bacterial abscess, tuberculosis and cerebral infarction. It is also critical to distinguish between the various forms of primary brain tumours such as oligodendroglioma or ependymoma as this will impact on adjuvant therapy such as radiation therapy and chemotherapy.

The mainstay of therapy is a combination of surgery, radiation therapy and chemotherapy.

The main role of surgery is to obtain tissue diagnosis. Surgery can also significantly and rapidly reduce intracranial hypertension leading to symptomatic relief and recovery of reversible neurological deficits such as hemiparesis. The role of radical surgical resection of high-grade astrocytomas remains controversial although there is some literature to support the role of radical surgery to improve patient survival and disease free progression of the disease.

The type of surgery performed may range from a simple stereotactic biopsy, subtotal debulking of tumour or radical resection of the tumour. The type of surgery performed is dependent on the functional status of the patient, the presence of significant co-morbidities, tumour size and location.

Stereotactic biopsy is especially useful in elderly patients with significant deficits and co-morbidities as well as in situations where the tumour is small and deep-seated which would render more aggressive surgery dangerous.

In most patients with malignant glioma, surgery should be carried out with the aim of removing as much tumour with maximal preservation of structural and functional integrity of surrounding normal anatomy. The aims of radical resection are as follows[120-121]:

(A) Rapid 2-log cell kill

(B) Remove resistant cells

(C) Reduce sampling error associated with a small biopsy

(D) Decrease intracranial hypertension

(E) Improve neurological function

(F) Potentiate adjuvant therapy such as radiation therapy and chemotherapy

(G) Potentially improve survival and disease-free progression

Radiation therapy has for the last 3 decades remained as the single most effective treatment for malignant astrocytoma[122-125]. All patients with high-grade astrocytomas should be given a course of radiation therapy after surgery if they remain in reasonable functional status. The duration and dose of radiation given depends on the functional status of the patient.

The role of chemotherapy is to potentiate and augment radiation therapy. Chemotherapy is greatly limited by penetration into the central nervous system through the blood-brain-barrier, toxicity and chemoresistance to therapeutic agents used. Traditional regimens have been based on the use of nitrosureas. In 1970, two separate groups of investigators reported 40% response rate with 1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU) alone[126-127]. Other agents used include losmustine (CCNU) and procarbazine. Chemotherapy is conventionally used as an adjuvant after surgery and radiation therapy.

The most recent significant advance has been the "Stupp Regimen" 2005. This protocol essentially involves the concomitant administration of temozolamide chemotherapy with radiation therapy followed by temozolamide to glioblastoma patients in one arm of study with a separate arm of study which differed in that no concomitant administration of temozolamide was given. The results of the study showed that the two-year survival rate was 26.5% with radiation and temozolamide and 10.4% with radiation alone[128]. Resulting from these dramatic results and the fact that temozolamide is an orally administered chemotherapy agent with very little adverse effects, this is the current

therapy practised by many units world-wide. The only limiting factor is the extremely high cost of temozolamide which can be prohibitive.

1.10 Limitations in Treatment

Malignant astrocytomas are highly infiltrative and invasive tumours. Conventional imaging can only demonstrate the obvious tumour mass. At the time of diagnosis, there is evidence that tumour cells are present beyond the confines of the main tumour mass seen radiologically and at the time of surgery. A tumour gradient exists with the greatest density of tumour cell number decreasing rapidly at increasing distances from the contrast-enhancing rim of tumour tissue. Therefore the majority of recurrent glioblastoma (more than 90%) occur locally, within 2cm of the original margin of contrast-enhancing tumour[129-132].

However, tumour cells can be found at great distances from the primary site, even on the contralateral hemisphere. Widespread distributions of malignant cells are detected at autopsy and during methodical stereotactic biopsy studies[133-134]. The cells tend to spread along white matter tracts such as long association pathways and corpus callosum[135-136]. This obviously obviates the possibility of more aggressive surgery as that would lead to unacceptable morbidity and mortality.

Renewed growth of astrocytoma following therapy indicates failure to reduce the tumour mass to a size (approximately 10^5 cells) permitting eradication of the remnant cells by the patient's immune system[137-138].

Failure also results from factors which limit the efficacy of each modality. The blood-brain-barrier forms a protective barrier against the ingress of toxins into the central

nervous system. Unfortunately, this protective mechanism conversely also poses a challenge as it impedes the delivery of chemotherapy into the brain tissue. Patients with malignant astrocytomas are also immunocompromised and hence have impaired immunity to eradicate remnant tumour cells. The patient's immune response may be rendered ineffective by the tumour's secretion of factors antagonistic to immune cytokines[139].

Most significantly, malignant astrocytic cells also differ in genetics, morphology, kinetics, metabolism, angiogenesis, oxygenation and antigenic expression which promote their overall survival and render them resistant to therapeutic agents.

These factors all contribute to the current dismal state of affairs. Clinicians are still unable to provide long-term control for malignant astrocytomas and the current prognosis for this group of patients is still uniformly poor with median survival of 1 year for glioblastoma and 3 years for anaplastic astrocytoma[13-15, 140]. Glioblastomas are among the most malignant human and irrespective of aggressive radio- and chemotherapy, only about 2% survived more than 3 years[141-142].

1.11 Molecular Biology

The development of many cancers has directly been attributed to genetic mutations including loss of tumour suppressor genes and mutations in protooncogenes.

The past decade has seen an explosion in the molecular knowledge of astrocytomas. This understanding of the molecular basis for the disease coupled with the failure of current treatment to achieve a satisfactory disease control leaves no doubt that the next major advancement in glioma treatment has to take place in the realm of molecular biology.

Cytogenetic and molecular genetic studies have characterized the various chromosomal and genetic aberrations observed in gliomas.

1.11.1 Multi-step Theory of Tumourigenesis

Many human neoplasms have a tendency to evolve to more malignant states. This concept of malignant progression appears especially applicable to low-grade gliomas. Longitudinal studies have shown that more than 50% of low-grade gliomas will ultimately transform to tumours of higher grade[143]. Müller et al examined 72 patients who had low-grade gliomas at the time of initial surgery. At the time of recurrence, 14% of the tumours were unchanged histologically, whereas 55% were now classified as anaplastic astrocytoma and 30% were now classified as glioblastoma multiforme. The time between the initial diagnosis and the second surgery averaged 31 months[144]. In Soffietti's series, 79% of low-grade patients had grade 3 or 4 tumours at the time of clinical recurrence or death[145].

The progression of low-grade to high-grade astrocytoma involves the progressive loss of genes responsible for the control of cell proliferation, apoptosis and/or invasiveness. The earliest and most common alteration occurs on chromosome 17, which is associated with the tumour suppressor gene TP53[146]. The reported incidence of TP53 gene mutation in astrocytomas is approximately from 30-45%[147-150]. Alterations on chromosome 22 occur in approximately 20-30% of low-grade astrocytomas[148]. Overexpression of the proliferation-promoting platelet-derived growth factors (PDGF-A and PDGF-B) and the PDGFR-α receptor occurs in the vast majority of astrocytomas[151]. The transition of low-grade glioma to anaplastic astrocytoma is believed to be facilitated

by loss of 9p. Approximately 30-50% of gliomas have alterations to the retinoblastoma-1 (RB1) gene. These alterations arise either by mutation in the RB1 gene or by LOH at 13q14[148, 152-154]. Loss of Heterozygosity (LOH) on 19q has been reported in both anaplastic astrocytomas and glioblastomas[155]. Subsequent transformation to glioblastoma is thought to result from deletion of chromosome 10. Tumours of this grade typically demonstrate whole or partial deletion of a copy of chromosome 10 and this occurs in 53-97% of reported cases[156-160]. Amplification of the Epidermal Growth Factor Receptor (EGFR) gene is a late and rare event. This multi-step theory gives rise to the concept of "secondary glioblastoma" which is preceded by the presence of pre-existing low-grade astroctoma.

"Primary glioblastoma" arise de novo without preceding low-grade astrocytoma. It may be argued that progression occurred without detection. However there is molecular genetic evidence that supports that these tumours belong to a separate category. Lang et al characterized de novo glioblastomas as tumours without TP53 mutations but with amplification of EGFR and Loss of Heterozygosity (LOH) of chromosome 10 and secondary glioblastomas as neoplasms with TP53 mutations and LOH of chromosome 17p[161]. Von Deimling also showed that these tumours were more common in the elderly and had more frequent EGFR amplification without LOH on chromosome 17p[162].

The many genetic aberrations result in cell cycle dysregulation, activation of glioma signaling cascades, cellular proliferation, loss of normal apoptosis, invasiveness and angiogenesis.

1.11.2 Glioma Invasiveness

Local tissue invasion distinguishes high-grade astrocytomas from low-grade tumours and this attribute limits the effectiveness of radical resection. High-grade astrocytomas tend to recur locally until the patient succumbs to microscopic invasion and local compression of vital centres in the brain. Although more than 90% of malignant astrocytomas recur within 2cm of the original site of presentation after treatment, some patients may develop recurrence at some distance from the original tumour either in the ipsilateral hemisphere or even on the contralateral hemisphere[129-132]. Single tumour cells that invade the normal brain tissue as far as several centimetres from the macroscopic tumour border escape cytoreductive surgery and even involved-field radiation therapy with a safety margin no more than 2 cm around the presurgical tumour volume and usually results in tumour recurrence within a few months[163].

For gliomas to invade normal brain structures, they must be able to migrate and breakdown the surrounding extracellular matrix (ECM). To migrate, the cell body must be able to modify its shape and stiffness and to interact with the surrounding ECM. Tumour invasion consists of several steps: tumour cell interaction with ECM, hydrolytic degradation of the matrix by the release of proteases and migration of tumour cells into the area of degradation.

There are three major groups of proteases and their inhibitors: (1) matrix metalloproteases (MMPs) and tissue inhibitors of matrix metalloproteases (TIMPs); (2) serine proteases, including urokinase, tissue plasminogen activator (tPA) and plasminogen activator inhibitor (PAI); and (3) cysteine proteases[164-166].

The intracellular mechanisms of glioma migration are dependent on the complex interaction of microtubules, actin microfilaments and intermediate filaments which propel the tumour cell. The ECM also plays an integral role in glioma invasion. Several ECM proteins such as fibronectin, tenscin, laminin, integrins, collagen and vitronectin are utilized by glioma cells for invasion[164-171].

1.11.3 Angiogenesis

Tumours are dependent on angiogenesis, the formation of neo-vessels from existing vessels within the tumour[172]. Tumours secrete diffusible chemicals that stimulate endothelial cells, resulting in neovascularisation of capillaries. A number of angiogenic factors have been identified and examples include vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF), fibroblast growth factor (FGF), transforming growth factor (TGF), interleukin (IL) 8 and tumour necrosis factor-α (TNF-α)[173-174]. Several anti-angiogenic agents have been identified. Examples include interferon α, thalidomide, glioma derived angiogenesis inhibitory factor, angiostatin and endostatin[175]. These inhibitors of angiogenesis may hold promise for the development of novel therapeutic strategies.

1.11.4 Glioma Signaling Pathways and Growth Factors

Tumour stromal interactions play an important role in the mitogenesis of many human malignancies.

In colorectal tumour cells, co-culture of human colonic carcinoma cell lines Caco-2 or HT-29 cells in collagen gels resulted in the formation of a few small solid cell

clusters with no lumina, but when co-cultured with stromal cells, the tumour cells formed glandular structures with central lumina. This fibroblast-induced organization and differentiation of Caco-2 cells (not HT-29 cells) appeared to be mediated by transforming growth factor-beta (TGF-β)[176]. A paracrine mechanism is also seen in squamous cell carcinoma (SCC). Stromal changes with increased expression of proteases and cytokines may promote tumour proliferation. Myofibroblasts are commonly concentrated at the invasive margin of oral SCC. The tumour cells directly induce a myofibroblastic phenotype which is dependent on SCC-derived TGF-β1. In turn, myofibroblasts secrete significantly higher levels of hepatocyte growth factor/scatter factor compared with fibroblast controls which promotes SCC invasion[177]. Malignant melanoma cells are able to express various cytokines and growth factors at different stages of tumour progression, which can confer tumour competence via autocrine and paracrine effects[178].

Likewise, the co-expression of growth factors with their corresponding receptors in gliomas may result in complex ligand-receptor interactions. The growth factor receptors expressed on the surface of tumour cells may bind soluble ligand produced by the same (autocrine), or adjacent cells (paracrine). In addition, membrane-anchored growth factor isoforms generated by alternative splicing may bind to the same (juxtacrine) or adjacent tumour cells (paracrine). Intracellular interactions between growth factor receptors and their ligands can also lead to intracrine activation of signaling cascades[166, 179].

Many different growth factor/receptor systems have been implicated in the proliferative behaviour of gliomas. Vascular endothelial growth factor (VEGF) is considered one of the most potent angiogenic factors in gliomas. It is highly expressed in

malignant gliomas predominantly in areas of necrosis[180-185] and is thought to stimulate angiogenesis in a paracrine manner by binding to tyrosine kinase receptors on endothelial cells[183, 186-188]. The epidermal growth factor receptor (EGFR/c-erb/HER 1) is the mammalian homologue of the v-erbB oncogene from avian erythroblastosis virus[189]. Expression of EGFR has been shown in glioma cell lines and primary tumours with the degree of expression correlating to histological grade. EGFR expression has been linked to higher Ki-67 labelling indices and decreased survival[190-192]. In vitro experiments have demonstrated that expression of EGFR leads to increased proliferation behaviour and reduced apoptosis[193-195]. Co-expression of platelet-derived growth factor (PDGF) and their receptors is a common alteration in glioblastoma which can influence survival and proliferation of tumour cells. All four members of the PDGF family group PDGF-A, B, C and D have been shown to influence malignant behaviour. One of the most consistent cellular signaling defects observed in high-grade gliomas is the presence of an autocrine loop attributable to the co-expression of PDGF-A and PDGF-B and their receptors[196-200]. PDGF-C and PDGF-D expression is also seen in human glioblastoma cell lines and primary human tumour tissues. The use of CT52923, a potent inhibitor of PDGFR blocked PDGF autocrine-mediated phosphorylation of PDGFR, Akt and mitogen-activated protein kinase (MAPK) and was also able to inhibit glioma growth in nude mice models[201].

Nerve growth factor is a member of the family of neurotrophins which are widely expressed in glioma tissue[202]. They bind with different affinity to receptors of the tyr family and can stimulate glioma cell line proliferation in an autocrine fashion[203-204].

Several other putative growth factors have been found to be widely expressed in human glioma cell lines and glioma tissue and can stimulate mitogenic behaviour. These include insulin-like growth factor (IGF)[205], transforming growth factor-beta (TGF-β)[206], brain-derived growth factor (BDGF)[207] and scatter factor/hepatocyte growth factor (SF/HGF)[208-209].

Many of the above ligands are over-expressed due to gene amplification and the receptors can exist in constitutively active mutant forms. The mitogens and their cognate receptors will subsequently regulate several intracellular signaling pathways. The major signaling cascades are: phosphoinositide 3-kinase/AKT-protein kinase B (PI3K/AKT-PKB) pathway, mitogen activated protein kinase (RAS/MAPK) pathway and the phospholipase C-γ/protein kinase C (PLC-γ/PKC) pathway[209-210].

The interplay of these complex signaling pathways resulting from of ligand-receptor interactions induce tumour proliferation, promote angiogenesis, inhibit apoptosis and confer cellular survival and chemoresistance.

1.12 Glial Cell Line-Derived Neurotrophic Factor (GDNF) Family

1.12.1 GDNF Family of Ligands (GFLs)

Glial cell line-derived neurotrophic factor (GDNF) was originally identified in 1993 by Lin et al as a neurotrophic factor. It was isolated from a rat glioma cell line supernatant and was shown to confer increased survival for embryonic midbrain dopamine neurons[211]. Subsequently, it was also found that GDNF also had potent trophic functions in spinal motorneurons and central noradrenergic neurons.

In view of the ability of GDNF to confer neuronal survival and the presence of trophic functions, much research on GDNF has therefore focused on its role as a potential therapeutic agent for neurodegenerative diseases such as Parkinson's Disease. GDNF targeted therapy has reached the realm of clinical trials and application in the field of Parkinson's Disease[212-215].

The GDNF-family ligands (GFL) consist of GDNF, neurturin (NRTN), artemin (ARTN) and persephin (PSPN). The GFLs support midbrain dopamine and motorneurons in the central nervous system. In addition, GDNF, NRTN and ARTN promote the survival and regulate the differentiation of many peripheral neurons such as the sympathetic, parasympathetic, sensory and enteric neurons[216-217].

GFLs belong to the transforming growth factor-beta (TGF-β) superfamily, containing seven cysteine residues with the same relative spacing as other members of the family. GDNF and other structurally characterized members of the TGF-β superfamily have similar conformations[218]. They all belong to the cystine-knot protein family and function as homodimers.

GFLs are produced in the form of a precursor, preproGFL. The sequence is cleaved on secretion and activation of proGFL occurs by proteolytic cleavage. The secreted proneurotrophins may however be biologically active[216, 219-220].

GDNF is secreted as a mature protein of 134 amino acids. In its natural state, it exists as a glycosylated homodimer of approximately 39 kDa.

1.12.2 GDNF-family Signalling

These GFLs bind to specific GDNF-family receptor-α (GFRα) co-receptors and activate RET. The GFRα receptors are linked to the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor. Four classes of GFRα receptors have been characterised (GFRα1-4), which determine ligand specificity. GDNF binds to GFRα1, NRTN binds to GFRα2, ARTN to GFRα3 and PSPN binds to GFRα4. In addition, NRTN and ARTN may crosstalk weakly with GFRα1 and GDNF with GFRα2 and GFRα3.

Rearranged during Transfection (RET) is a single-pass transmembrane protein that contains four cadherin-like repeats in the extracellular domain and a typical intracellular tyrosine kinase domain. The GFL- GFRα binding to the extracellular domain of RET leads to intracellular activation of the tyrosine kinase domain[216,221]. A GDNF dimer binds to a monomeric or dimeric GFRα1, and the GDNF-GFRα1 complex then interacts with two RET molecules, thereby inducing homodimerisation and tyrosine autophosphorylation[222]. However, GDNF mutants that are deficient in GFRα1 binding sites have been shown to be able to activate RET, indicating that at least some RET molecules may be weakly associated with GFRα1 prior to GDNF binding[223].

The lipid micro-environments on the cell surface known as lipid rafts can also take part in the process of signal induction. Lipid rafts contain a given set of proteins, cholesterol and sphingolipids on the outer leaflet of the plasma membrane. They can change their size and composition in response to intra- or extracellular stimuli. This favours specific protein-protein interactions, resulting in the activation of signalling cascades[224-225]. Inactive RET lies outside the lipid rafts and only upon GDNF stimulation does the GFRα receptor incorporate RET into the lipid rafts. Soluble GFRα1 also targets

RET to lipid rafts[226-227]. RET is alternatively spliced to yield at least two spliced isoforms, RET9 and RET51, which differ only in their C-termini. TheRET9 and RET51 signaling cascades differ markedly[228]. Comparison of GFL activation of RET9 and RET51 revealed that phosphorylation of Tyrosine (Tyr) (905) and Tyr(1062) was greater and more sustained in RET9 as compared with RET51. In contrast, Tyr(1015) was more highly phosphorylated over time in RET51 than in RET9. In addition, RET9 and RET51 did not associate with each other in sympathetic neurons after glial cell line-derived neurotrophic factor stimulation, even though they share identical extracellular domains[228]. Using targeted mutagenesis to generate mice that express either RET9 or RET51. Monoisoformic RET9 mice, which lack RET51, are viable and appear normal. In contrast, monoisoformic RET51 animals, which lack RET9, have kidney hypodysplasia and enteric aganglionosis. RET9 transgenes are capable of rescuing the kidney agenesis in RET-deficient mice or causing kidney hypodysplasia in wild-type animals. In contrast, similar RET51 transgenes fail to rescue the kidney agenesis or cause hypodysplasia. Therefore, only the short RET9 isoform can rescue the phenotype of the RET-null mutation in the kidney and enteric system[229].

Recently, the neural cell adhesion molecule (NCAM) was also shown to function as a signaling receptor for members of the GFLs. Significantly, GDNF was shown to stimulate schwann cell migration via NCAM but independently of RET. GDNF was also demonstrated to stimulate axonal growth in primary neurons via NCAM and Fyn Kinase but independently of RET[230].

In addition to being mediators of neuronal survival, neuritic growth and differentiation, the GFLs also have many extra-neuronal functions. GDNF is essential for

renal morphogenesis where it serves as an inductive signal sent from the nephrogenic mesenchyme to the ureteric bud. GDNF is expressed by mesenchyme and is repressed by epithelial conversion of the mesenchymal cells. GDNF releasing beads have been shown to stimulate ureteric branching in cultured kidneys and promote the outgrowth of ectopic ureteric buds from the nephric duct[231-233].

GDNF is also expressed in the testis by Sertoli cells and regulate spermatogenesis in a paracrine fashion. Both GDNF and NRTN stimulate Deoxyribonucleic acid (DNA) synthesis in spermatogonia although transgenic mice experiments demonstrated that GDNF but not NRTN contributes to the regulation of spermatogonial self-renewal and differentiation[234-235].

1.12.3 RET Dysfunction and GDNF in Disease

Mutations in the RET gene can result in many human diseases. There are two groups of RET mutations: gain-in-function mutations and loss-in-function mutations.

Gain-of-function mutations within the receptor tyrosine kinase gene RET cause inherited and non-inherited thyroid cancer. Germline RET mutations are found in virtually all cases of familial medullary thyroid cancer and somatic point mutations occur in nearly half of the sporadic cases. Gene rearrangements of RET, giving rise to chimeric genes have been found in papillary thyroid carcinoma[236-237]. The majority of cases of multiple endocrine neoplasia (MEN) types 2A and 2B are also associated with dysfunction of RET arising from missense mutation, insertions or deletions either in the extracellular or intracellular domain[236-238]. Conversely, loss-of-function mutations are responsible for the development of Hirschsprung's disease, a congenital malformation of

the enteric nervous system. The frequency of RET mutations in Hirschsprung's disease range from 5% to 50% and is more common in the familial form than in the sporadic form[239].

GDNF has immense promise in clinical therapeutics. The area of most intense study is the use of GDNF in Parkinson's Disease. Phase I trials have shown that the delivery of GDNF directly into the putamen can be performed safely and for long periods resulting in a significant increase in dopamine storage in the putamen and improvement in clinical condition. Gill et al reported that after one year, there were no serious clinical side effects. There was a 39% improvement in the off-medication motor sub-score of the Unified Parkinson's Disease Rating Scale (UPDRS) and a 61% improvement in the activities of daily living sub-score. Medication-induced dyskinesias were also reduced by 64% and were not observed off medication during chronic GDNF delivery[240]. Slevin et al reported that the unilateral administration of GDNF results in significant, sustained bilateral benefits in patients with PD. These improvements are lost within 9 months of drug withdrawal[241]. Other areas of potential therapeutic applications are in the realm of sensory regeneration and neuropathic pain, epilepsy, addiction, cerebral ischaemia and male contraception[221, 242-246].

1.12.4 GDNF and Cancer

There are carcinogenic consequences of dysregulated RET activation. GDNF has been implicated as a mitogenic agent in many cancers.

RET mutations are common in MEN syndromes 2A and 2B[236-238]. Patients with MEN 2 develop hyperplasia of adrenal chromaffin cells which are often followed by

phaechromocytomas[247]. GDNF and NRTN were found to be mitogens for normal adult rat chromaffin cells in vitro. NRTN was the more potent mitogen and caused increased phosphorylation of extracellular signal-regulated kinases 1 and 2 in cultured chromaffin cells[248].

In pancreatic cancer, RET overexpression was seen in pancreatic tumour tissue. GDNF and ARTN were strongly expressed in all intrapancreatic nerves and intrapancreatic neural invasion was significantly related to the expression of GDNF. In invasion assays, the migration of pancreatic cancer cells are markedly induced by co-culture with human glioma cells capable of secreting GDNF. In all intrapancreatic nerves of pancreatic cancer tissue, GDNF and artermin were expressed strongly. The expression of RET was stronger than that seen in normal ductal cells and was significantly related to the survival rate after resection[249-250]. Perineural invasion is an important prognostic factor for patients with bile duct carcinoma and is not surprising that GDNF can play a role in the pathogenesis of bile duct cancer. Using immunohistochemistry, moderate to strong staining of GDNF in tumour cells was observed more frequently in sections with perineural invasion. Cell migration was also seen to be enhanced by conditioned media from GDNF-treated cells[251].

GDNF can promote proliferation of neuroblastoma cells and can have a role in promoting resistance to differentiation or cytotoxic therapy of neuroblastoma[252].

1.12.5 GDNF and Malignant Astrocytoma

Malignant astrocytomas are the most common form of primary brain cancer. Many growth factors such as PDGF, VEGF, IGF and BDGF have been demonstrated to

promote proliferation, inhibit apoptosis and induce angiogenesis in malignant astrocytomas. GDNF is ubiquitous in the central nervous system and neural tissue and hence can also play a role in the pathogenesis of high-grade glioma.

GDNF and its receptor GFRα1 have been demonstrated to be strongly expressed in human gliomas. In an analysis of 20 human astrocytomas (14 glioblastomas, 1 gliosarcoma and 5 astrocytomas), GDNF protein concentrations were found to be present in high amounts compared to postmortem human frontal lobe and rat cortex. Immunohistochemistry of GFRα1 demonstrated strongly positive staining in 5 out of 15 high-grade glioma samples, weakly positive staining in 4 specimens and no staining in 6 specimens[253].

Additional evidence for the role of GDNF as a proliferation factor for glioma comes from experiments with rats. GDNF is found to be highly expressed in rat glioma cell lines B49 and C6. Knockdown experiments with antisense oligonucleotides performed with rat C6 glioma cells demonstrated that knockdown of C6 cells with GDNF and GFRα1 significantly reduced the number of C6 glioma cells and also inhibited the incorporation of bromodeoxyuridine as a sign of DNA synthesis. Persephin antibodies did not however result in any difference when compared to control experiments. This therefore suggests that GDNF but not persephin is a potent proliferation factor for rat glioma cells[254].

1.12.6 GFRα Splice Isoforms/Variants

Multicellular eukaryotic cells contain multiple introns. The presence of multiple introns permits the expression of multiple, related proteins from a single gene by alternative

splicing producing different forms of a protein, known as spliced isoforms or spliced variants. The formation of a mature mRNA involves the removal of introns and splicing together with exons. Splicing occurs at short, conserved sequences in pre-mRNAs via two transesterification reactions. Johnson et al used microarrays to monitor splicing at every exon-exon junction in more than 10,000 multi-exon human genes in 52 tissues and cell lines and concluded that at least 74% of human multi-exon genes are alternatively spliced[255]. Alternative splicing is therefore responsible for much of the protein diversity in humans.

Cancer cells often acquire aberrant profiles of alternative spliced isoforms that can promote cell proliferation and invasion and inhibit apoptotic cell death. The CD99 gene encodes two distinct transmembrane proteins by alternative splicing of its transcript. Two spliced variants, the major wild-type form (type I) and the minor spliced variant (type II) are present. Expression of CD99 type II but not type I is associated with markedly elevated invasiveness of breast cancer cell lines[256]. Ras activating mutations are important in colorectal cancer. K-ras encodes two splice variants, K-ras 4A and 4B. Plowman et al has investigated the expression of K-ras in colorectal cancer and found that K-ras 4B was expressed ubiquitously and was the predominant spliced variant whereas K-ras 4A was expressed differentially with detection in colorectal tumours and cell lines and normal colon, pancreas and lung. They postulate that altered splicing of either the K-ras proto-oncogene, in favour of K-ras 4B, may modulate tumour development[257]. Alternative splicing variants of interferon regulatory factor (IRF-1) have also been found in cervical cancer. Five variants lacking some combination of exons 7, 8 and 9 have been identified and their expression levels were higher in malignant samples.

Alternative splicing in exons 7, 8, and 9 may therefore be an important mechanism for negatively regulating IRF-1 in cervical cancer[258]. Fibroblast growth factor receptor 3 (FGFR3) is a major family member expressed in both normal human urothelium and cultured normal urothelial cells and is expressed as the IIIb isoform. A spliced variant, FGFR3 Delta8-10, lacking exons encoding the COOH-terminal half of immunoglobulin-like domain III and the transmembrane domain have been identified by Tomlinson et al. In culture, cells overexpressing FGFR IIIb showed FGFR1-induced proliferation which was inhibited by addition of FGFR3 Delta8-10[259]. Interleukin-17 receptor-like protein (IL-17RL) spliced isoforms have also been identified in prostate cancer and may play a role in the mitogenesis of prostatic cancer. Extensive alternative splicing is present with more than 90 different IL-17RL isoforms detected. The three most abundant isoforms account for approximately half the total transcripts[260]. Alternative splicing of interleukin-7 (IL-7) is also found abundantly in malignant haemopoietic cells and the diverse IL-7 protein isoforms may play an important role in the pathogenesis of leukemia[261].

Spliced isoforms are also abundant in the GDNF-family receptor-α (GFRα). GFRα1 receptor exists in two highly homologous alternatively spliced isoforms: GFRα1a and GFRα1b[262-264]. GFRα1b is identical to GFRα1a except for the absence of 5 amino acids (140DVFQQ144), encoded by exon 5. In addition, GFRα2 and GFRα4 receptor spice isoforms have also been identified in mammalian tissue. Three variants of GFRα2 receptors (GFRα2a/2b/2c) have been identified[265-266]. At least two splice variants of GFRα4 have been identified in rat tissue[267-269].

Alternatively spliced variants of RET[229, 270-271] and NCAM[272-274] also exist and have been demonstrated to subserve distinct biochemical and physiological functions.

The presence of multiple spliced isoforms increases the permutational possibility and complexity of ligand-receptor interactions and can potentially result in diverse biological and physiological processes. The spliced variants have been shown to have differing levels of expression in different tissue. Tissue expression of alternatively spliced GFRα1, NCAM and RET isoforms were studied by Yoong et al in various murine adult tissue and whole embryo. Both GFRα1a and GFRα1b were expressed at similar levels in all tissues examined, except in the brain where, GFRα1a is the predominant spliced isoform. The expression of both GFRα1 isoforms were expressed at similar levels but were significantly higher than in other tissues. RET was most highly expressed in the brain and testis and RET9 was the predominant spliced variant. NCAM120, 140 and 180 were expressed at equivalent levels in the brain. DNA microarray was used to analyse NRTN stimulation of GFRα1 isoforms in transfected Neuro2A cells. Both GFRα1 isoforms demonstrated rapid phosphorylation and nuclear localization of ERK1/2 when stimulated by GDNF or NRTN. An early time point chosen to examine the gene profile of NRTN stimulation showed that stimulation with NRTN resulted in specific gene changes not shared by the spiced isoforms, indicative of distinct functional roles[275].

1.13 Objective of Research Project

GDNF and its receptor GFRα1 have been demonstrated to be strongly expressed in human gliomas by Wisenhofer et al[253]. GDNF has also been demonstrated to be a proliferation factor for rat C6 glioma cells by antisense experiments[254].

Emerging data showing varying levels of expression of spliced variants in various tissues raises the question that differing spliced isoform expression levels may be present

in pathological states. Such differing levels of isoform expression can also impact on the pathogenesis of disease.

There is no literature quantifying the expression level of the GFRα receptor splice isoforms in malignant gliomas. There have also been no experiments investigating the effects of GDNF on human glioma model. Studies investigating possible mechanisms of GDNF action on chemoresistance and cellular survival are also lacking.

The objectives of this research are:

1. To identify the expression of GDNF and its receptors in glioblastoma cell lines and human glioma specimens. Specifically, we endeavoured to ascertain if there were differing levels of expression of the various spice isoform variants.
2. To investigate the role of GDNF in promoting tumour growth and proliferation.
3. To elucidate putative signaling pathways of GDNF in tumourigenesis.

CHAPTER 2

MATERIALS AND METHODS

2.1 Cell Culture

(a) Cell lines

The cell lines with obtained from American Type Culture Collection (ATCC®). The cell lines were selected based on their histological subtypes. All the cell lines selected were *Homo sapiens* (human) glioblastoma (WHO Grade IV) cell lines.

The following cell lines were selected:

1. Cell Line Designation: LN-229 (ATCC® Catalog No. CRL-2611)
2. Cell Line Designation: A172 (ATCC® Catalog No. CRL-1620)

(b) Cell Stock

The cell lines were seeded and cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 100 IU/ml penicillin and 100 µg/ml streptomycin in 75mL flasks. Cultures were maintained at 37°C in an atmosphere of 5% CO_2. After 24 hours the solutions were replaced with fresh culture. The cell lines were expanded and trypsinised at 80 to 90% confluency and stocked up in cryotubes and stored at -80 degrees Celsius.

(c) Maintenance of Cell Lines

All cell lines were cultured in DMEM supplemented with 10% fetal bovine serum, 100 IU/ml penicillin and 100 µg/ml streptomycin in 75 mL flasks. Cultures were maintained

at 37°C in an atmosphere of 5% CO_2. The cells were trypsinised at 80 to 90% confluency and reseeded into 75 mL flasks.

2.2 Human Glioma Specimens

(a) Ethics approval

The project was submitted to the Hospital Ethics Committee and was approved by the Tan Tock Seng Hospital (TTSH) Ethics Committee on 3 Dec 2003 (Ref: CR/ETHICS/03/950).

The oversight of the research project was transferred to the National Healthcare Group (NHG) Domain Specific Review Board on 23 Apr 2004 (DSRB-A-00-37) and subsequently to the National Neuroscience Institute (NNI) Institutional Review Board (IRB) on 30 May 2005.

The approved period for the study is from 30 May 2005 to 29 May 2007. The study reference number assigned is NNI-IRB/00/029.

(b) Patient Consent

Informed consent was obtained from a member of the clinical staff who was not actively involved in the research conducted. Tumour samples were only analyzed on superfluous tissue after the completion of all diagnostic tests and procedures. This was performed to ensure that research activity did not compromise the clinical management of patients. Details of the consent procedure are detailed in Appendix 1.

(c) Specimens

Patients with pre-operative diagnosis of glioma on MRI scan were approached to obtain informed consent for the research. Small tumours which underwent biopsy were excluded from the study as all the tissues were sent for diagnostic tests. In large tumours which were subjected to extensive tumour resection, excess fresh tumour was kept aside and stored at -80 ^{o}C until the diagnostic tests were completed before the tissue is used for research. This protocol was followed to ensure optimal care to the patient and the patient's well-being is given top priority at all times in accordance with the Hospital Ethics Committee.

A total of 13 specimens were collected. The histological subtypes consisted of the following:

(a) 8 cases of glioblastoma (WHO Grade IV)

(b) 2 cases of Pleomorphic Xanthoastrocytoma (PXA) (WHO Grade II) (1 case was a recurrent tumour with previous history of PXA)

(c) 1 case of anaplastic ependymoma (WHO Grade III)

(d) 1 case of oligodendroglioma (WHO Grade II)

(e) 1 case of pilocytic astrocytoma (WHO Grade I)

2.3 Quantitative Real Time Polymerase Chain Reaction (PCR)

(a) Reverse Transcription (RT) Reaction

Total ribonucleic acid (RNA) was prepared with preparation with guanidinium isothiocyanate, followed by delipidation. RNA is isolated by differentially partitioning DNA into the organic phase, followed by precipitation with ethanol. Total RNA was

obtained from the cell lines and human glioma samples. Further purification to remove genomic DNA was performed using Nucleospin RNA II (Macherey-Magel, Germany) accordingly to the manufacturer's specifications. The integrity of total RNA was validated by denaturing agarose gel electrophoresis. Five micrograms of total RNA from each tissue specimen was reverse-transcribed using 400 U of ImpromII and 0.5 μg of random hexamer (Promega, Madison, WI, USA) for 60 min at 42 ^{o}C according to the manufacturer's specifications The reaction was terminated by heating at 70 ^{o}C for 5 min and used directly for quantitative real-time PCR.

(b) Sequence Independent Real-Time PCR using SYBR Green I Plasmids Construction

Unless otherwise stated, all templates were generated by reverse transcription (RT)-PCR using human brain cDNA subcloned into pGEM-T (Promega), and all clones were verified by DNA sequencing. The complete open reading frame (ORF) of human GFRα1a (GenBank accession number: NM_005264), GFRα1b (GenBank accession number: NM_145793), Ret 9 (GenBank accession number: NM_020630), Ret 51 (GenBank accession number: NM_020975), NCAM (GenBank accession number: NM_000615) and GFRα2 (GenBank accession number: NM_001495) were generated by PCR and subcloned into pIRESneo (clontech). The complete ORF of GADPH was generated using the GenBank sequence (accession number NM_008084). DNAMAN software package was used for the design of the primers.

The following primers were used:

GFR1a: (150bp)

hGFR1a (Forward Primer): CATATCAGATGTTTTTCAGCAAGTGGA

hGFR1(Reverse Primer): CAGACATCGTTGGACACGCT

GFR1b: (145bp)

hGFR1b (Forward Primer): TGGTCCCATTCATATCAGTGGA

hGFR1(Reverse Primer): CAGACATCGTTGGACACGCT

GFR2

Common:

hGFR2GPI (Forward Primer): CAGTGACAGTACCAGCTTGGG

hGFR2GPI (Reverse Primer): AAGGCCTGTTTCAGCATCAG

Ret 9: (83bp)

hRet9 (Forward Primer): GGATTGAAAACAAACTCTATGGTAGA

hRet9 (Reverse Primer): AGGAAGGATAGTGCARAGGGGAC

Ret 51: (142bp)

hRet51 (Forward Primer): AAACAAACTCTATGGCATGTCAGAC

hRet51 (Reverse Primer): CGCTGAGGGTGAAAGCATC

hNCAM (Forward Primer): CAGCAGCGGATCTCAGTGGT

hNCAM (Reverse Primer): CATCACACACAATCACGGCA

GDNF: (175bp)

hGDNF (Forward Primer): TCACTGACTTGGGTCTGGG

hGDNF (Reverse Primer): TCAAAGGCGATGGGTCTGC

GAPDH: (92bp)

hGAPDH (Forward Primer): AAACCTGCCAAATATGATGAC

hGAPDH (Reverse Primer): ACCTGGTGCTCAGTGTAG

(c) Sequence Independent Real-Time PCR

Real-time PCR was performed on the iCycler iQ (Biorad, Hercules, USA) using Sybr Green I. The threshold cycles (Ct) were calculated using the Optical interface v3.0B. Real-time PCR was performed after an initial denaturation for 3 min at 95 degree Celsius followed by 40 to 60 cycles of 60 sec denaturation at 95 ^{o}C, 30 sec annealing at 60 ^{o}C and 60 sec extension at 72 ^{o}C. Fluorescent detection was carried out at the annealing phase. The reaction was carried out in a total volume of 50 µl in 1x XtenseMixtuSG^{TM} (BioWORKS), containing 2.5 mM Mg Cl_2, 10pmol of primers and 1.25 U platinum DNA polymerase (Invitrogen). Melt curve analyses and agarose gel electrophoresis were carried out at the end of PCR to verify the identity of the products. All real-time PCR quantification was carried out simultaneously with linearised plasmid standards and a negative water control. The concentrations of GFRα1, GFRα2, GDNF, RET and NCAM in all the tissues were interpolated from standard curves and then normalized to the expressions of Glyceraldehyde-3-phosphate dehydrogenase (GADPH) in the same tissues. Differences in the expression levels were analysed using paired Student's *t* test with a level of significance taken as $p<0.05$. The specificity of the assay ($\Delta Ct/\varepsilon$) is determined by the difference in the Ct values of the defined target and test templates normalised to the efficiency of target amplification (ε).

2.4 1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU)

BCNU was obtained from Sigma-Aldrich. It is a DNA alkylating agent causing DNA interstrand crosslinks[276]. It is stored at a temperature of −20°C. BCNU is highly hygroscopic and hence fresh preparations are produced at the commencement of each set of experiments[277].

BCNU was selected as it is a nitrosurea which is the drug used as in traditional regimens for malignant gliomas. In 1970, two separate groups of investigators reported 40% response rate with 1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU) alone[126-127].

Recently, the use of temozolamide has been popularised as part of the "Stupp Regimen". This protocol essentially involves the concomitant administration of temozolamide chemotherapy with radiation therapy followed by temozolamide to glioblastoma patients in one arm of study with a separate arm of study which differed in that no concomitant administration of temozolamide was given. The results of the study showed that the two-year survival rate was 26.5% with radiation and temozolamide and 10.4% with radiation alone[128]. Temozolamide is prohibitively costly and hence BCNU was selected as the cytotoxic agent of choice in view of its long history of efficacy and significantly lower cost.

2.5 Ligands

The GDNF-Family ligands used for stimulation were GDNF and NRTN. GDNF binds to GFRα1 and NRTN binds to GFRα2. NRTN may crosstalk weakly with GFRα1 and GDNF with GFRα2 and GFRα3.

The concentration of GDNF and NRTN used for stimulation experiments was 50ng/ml.

2.6 Cytotoxicity assay

(a) MTS assay

Promega CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay was used to determine cytotoxicity effects of BCNU. This is a colorimetric method for determining the number of viable cells in culture in 96-well plate format. The Promega CellTiter 96® AQ_{ueous} One Solution Reagent contains a tetrazolium compound [3-(4,5-dimethylthiazol -2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt; $MTS^{(a)}$] and an electron coupling reagent. The quantity of formazan as measured by the amount of 490 nm absorbance is directly related to the number of living cells in culture. 100 µL of Promega CellTiter 96® AQ_{ueous} One Solution was added to each well and incubated at 37 ^{o}C for one hour and then absorbance was read at 490 nm with a spectrophotometric plate reader. Tumour cells were treated with 2% Triton X-100 to obtain baseline absorbance value which was subtracted from the various readings obtained.

(b) Normalisation of MTS Assay

Different concentrations of cells were seeded at 5,000 cells per well, 10,000 cells per well, 15,000 cells per well and 20,000 cells per well in DMEM supplemented with 2% fetal bovine serum. Cell loading beyond 20,000 cells per well resulted in rapid cell proliferation and saturation of the assay.

After plating, the cells were incubated at 37 ^{o}C for 24 hours before treatment with varying concentrations of BCNU from 25-175 µg/mL in 25 µg/mL increments. The control tumour cells not treated with BCNU were treated with 1% alcohol. The cells were

incubated at 37 ^{o}C in an atmosphere of for 24 hours before being subject to the MTS assay.

(c) Propidium Iodide (Pi) staining

After completion of treatment of the cells with BCNU, the culture media is aspirated and 800 μL of 75% cold ethanol is added. The cells were then incubated for 10 min at 4 ^{o}C Celsius. Re-aspiration of the media is performed and staining done using 1 μg/mL of Propidium Iodide (Pi)/0.1% Triton X-100/10 μg/ml RNAaseA in phosphate buffer solution (PBS). The solution is then incubated for 5 min at 37 ^{o}C and observed under the microscope.

2.7 Western Blot

(a) Protein quantification by bicinchoninic acid (BCA) assay

LN-229 and A172 cell lines were stimulated with BCNU and GNDF and the experiments were studied at 0, 10, 30, 60 and 180 mins respectively.

BCA^{TM} Protein Assay Reagent Kit (Pierce, USA) was used to determine protein concentration. The protein assay is based on bicinchoninic acid (BCA) for the colorimetric detection and quantification of total protein. This method combines the reduction of Cu^{2+} to Cu^{+} by protein in alkaline medium with the highly sensitive and selective colorimetric detection of the cuprous (Cu^{+}) using bicinchoninic acid. The macromolecular structure of protein, and number of peptide bonds and the presence of four amino acids (cysteine, cystine, tryptophan and tyrosine) have been reported to be responsible for color formation in protein sample when assayed with BCA[278]. The purple

reaction product, formed by the interaction of two molecules of BCA with one cuprous ion (Cu^{+}), is water-soluble and exhibits a strong absorbance at 562 nm. This allows the spectrophotometric quantification of protein in aqueous solutions.

Working reagent was prepared by combining 50 parts of reagent A (sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.2 M sodium hydroxide) with one part of reagent B (4 % cupric sulfate solution). Serial dilutions (20-2000 μg/ml) of protein standard and samples were assayed in parallel. Twenty five microlitres of each standard or unknown sample was pipetted into the appropriate microtiter plate wells and then 200 μl of working reagent was added to each well and mixed for 30 sec. The plate was incubated at 37°C for 30 min and absorbance was read at 562 nm with a microtiter plate reader (Model 680, Bio-Rad, USA). Standard curve was prepared by plotting the absorbance at 562 nm vs. protein concentration and protein concentration of each unknown samples were interpolated.

(b) Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Discontinuous one-dimensional gel electrophoresis was carried out using Bio-Rad Mini-Protean apparatus (Bio-Rad Laboratories, USA) with an acrylamide resolving gel and 5% (w/v) stacking gel. The percentage of acrylamide – bisacrylamide (Bio-Rad Laboratories, USA) in resolving gel was chosen depending on the molecular weight of the protein of interest. For the present study, a 10-12 % (w/v) of resolving gel was used.

Samples were suspended in SDS sample buffer, heated at 95°C for 5 min, briefly centrifuged, loaded and underwent electrophoresis at 100 volts until the dye front

migrated to the resolving gel, then the voltage was increased to 180 volts until the dye reached the bottom of the resolving gel.

(c) Western blotting and detection

Following SDS-PAGE, the gel was removed and equilibrated in transfer buffer for 10 min at room temperature with constant shaking. A piece of nitrocellulose membrane (Hybond-C Extra, Amersham Pharmacia Biotech UK) was placed on top of the gel and covered by a filter paper which was then placed between a semidry transfer apparatus (Bio-Rad Laboratories, USA). The transfer was carried out at 100 volts for 60 min. The membrane was then incubated in TBST solution with 5 % fat-free skimmed milk (w/v, Diploma Instant, Melbourne Australia) for 1 hr at room temperature to reduce non-specific binding of antibody in subsequent steps. Immunoblots were probed to the following: Anti-phospho-erk1/2, anti-erk1/2, anti-phospho-jnk, anti-actin, anti-phospho-p38 and anti-phospho-akt. The membrane was then washed 3 times in TBST and incubated with the primary antibody (1 : 1000 dilution in TBST, 1% (w/v) skimmed milk) for 1 hr at room temperature or overnight at 4ºC on a rocking platform. After the incubation, the membrane was washed for 3 x 5 min in 1 % (w/v) skimmed milk in Tis buffered saline Tween-20 (TBST). The secondary antibody conjugated with horseradish peroxidase (HRP) (DAKO, Denmark) at 1:1000 dilution in 1 % skimmed milk (w/v) /TBST was then added and incubated for 1 hr at room temperature. The membrane was then washed for 3 x 5 min in TBST and detected by Super Signal® West Dura Extended Duration chemiluminescent substrate (Pierce, USA) according to manufacturer's instruction. The chemiluminescent image was captured on Biomax film (MR 1, Kodak).

2.8 Study on the Impact of Cell Tumour Burden on Chemoresistance

Three human glioblastoma cell lines (ATCC® Catalog No. CRL-2611, Designation LN-229; ATCC® Catalog No. CRL-1620, Designation A-172) were used for the experiments. The cell lines were cultured in 75ml flasks with Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 100 IU/ml penicillin and 100µg/ml streptomycin. Cultures were maintained at 37^{o}C in an atmosphere of 5% CO_2.

When the cells were 90% confluent, they were trysinised, counted in a haematocytometer and seeded into 96 well plates. The cells were plated in the following concentrations with 150 µl of 2% FBS media: 5,000 cells per well; 10,000 cells per well and 20,000 cells per well. Cell loading beyond 20,000 cells per well resulted in overcrowding which would cause cells to enter into Gap 1 (G1) arrested state.

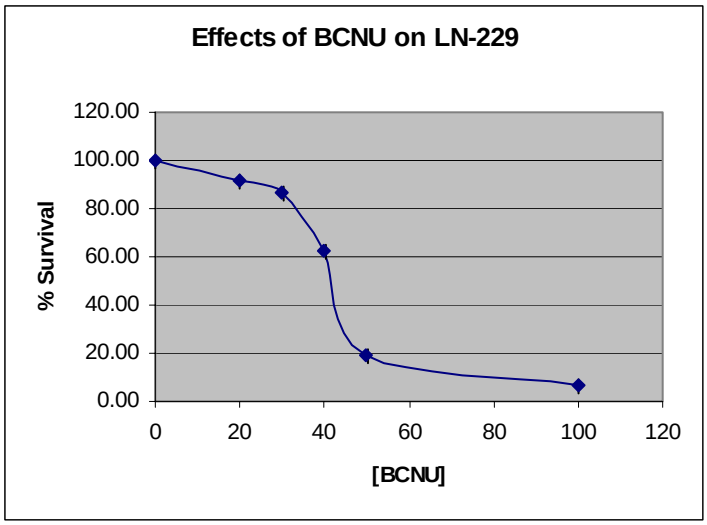
After plating, the cells were incubated at 37 ^{o}C in an atmosphere of 5% CO_2 for 24 hours before being treated with varying concentrations of 1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU).

After 24 hours of treatment with BCNU, the cells were subject to Promega CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay was used to determine cytotoxicity effects of BCNU as described above. Pi staining was also performed as described above.

2.9 Study on the effects of GDNF and NRTN on BCNU chemotherapy and its role in chemoresistance

LN-229 and A172 were grown to 90% confluency and trypsinised. For MTS assay, 10,000 cells were seeded into 96 well plates and grown in DMEM enriched with 2% foetal calf serum. The experimental group were seeded with GDNF or NRTN enriched media at 50ng/ml concentration. The control group was not treated with GDNF or NRTN. After 24 hours, the LN-229 cells were treated with 1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU) at concentrations at 40 µg/ml and 50µg/ml and the A172 cells were treated at 75 µg/ml. The cells were then examined morphologically and underwent MTS assay after 24 hours of treatment with BCNU. The BCNU was obtained from Sigma Aldrich.

The BCNU concentration used was based on experiments demonstrating the cytotoxic effects of varying concentrations of BCNU on LN-229 and A172. 50µg/ml and 75 µg/ml were the concentrations that exhibited significant (>70%) cell death (Figure 1). Higher concentrations of BCNU resulted in complete cell death.



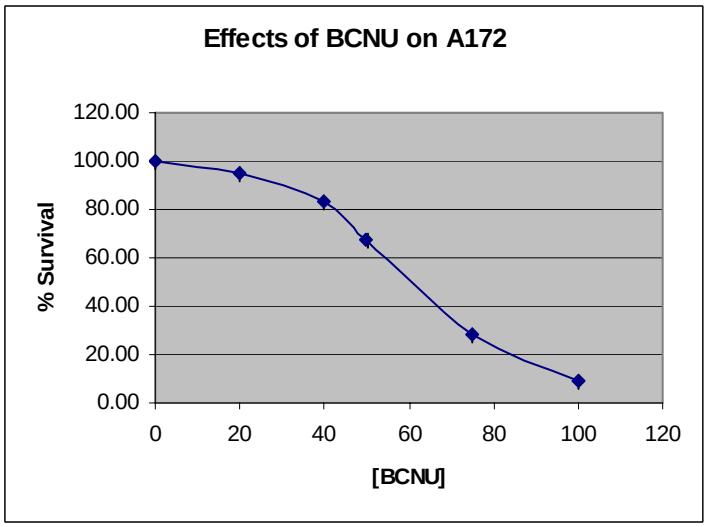


Figure 1: Effects of BCNU on cytotoxicity of LN-229 and A172 Cell Lines

BCNU concentration of 50µg/ml resulted in >70% cell death on Promega CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay.

For Pi index, 5 x 10^3 were seeded per well into 12 well plates. Similarly, the experimental group were pre-treated with GDNF or NRTN for 24 hours, treated with BCNU at the same concentrations and subsequently underwent Pi staining.

All the experiments were performed and repeated in triplicates.

CHAPTER 3

RESULTS

3.1 Higher glioblastoma cell loading required higher concentration of BCNU to achieve similar cell cytotoxicity

The human glioblastoma cell lines LN-229 and A-172 were plated in 2% FBS media in 96-well plates at concentrations of 5,000 cells per well, 10,000 cells per well, 15,000 cells per well and 20,000 cells per well and incubated at 37 ºC in an atmosphere of 5% CO_2 for 24 hours. They were then subjected to varying concentrations of BCNU from 25-175 μg/mL in 25 μg/mL increments. After 24 hours of treatment, the cells were examined morphologically under the microscope and cell proliferation assay with Promega CellTiter 96® AQ_{ueous} One Solution Proliferation Assay was performed.

3.2 Morphology of Cells

Figure 2 shows the normal appearance of the human glioblastoma cells (LN-229). On morphological examination under x100 and x400 magnification, it is observed that higher concentration of BCNU were required to produce a pyknotic appearance of the glioblastoma cells indicative of cellular death (Figure 3). BCNU concentration at 50 μg/mL resulted in the vast majority of cells showing a pyknotic appearance for glioblastoma cells plated at 5,000 cells per well (Figure 3). For glioblastoma cells seeded at 10,000, 15,000 and 20,000 cells per well, the corresponding concentration of BCNU reproducing similar morphological appearance were 75 μg/mL, 100 μg/mL and 125 μg/mL respectively. A similar trend was observed with cell line A-172.

Propidium Iodide (Pi) staining showed DNA fragmentation which demonstrates the presence of apoptotic cell death (Figure 4).

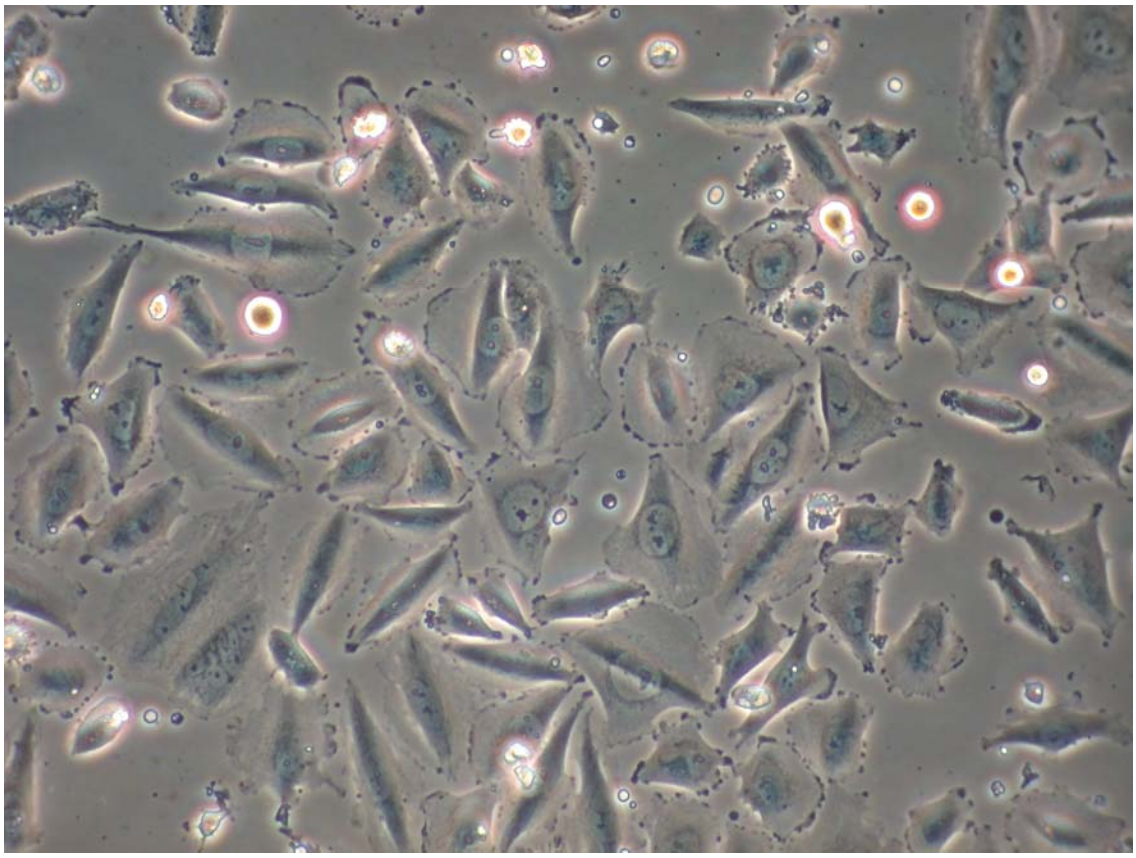


Figure 2: Normal Appearance of LN-229 Cell Line (400X magnification)

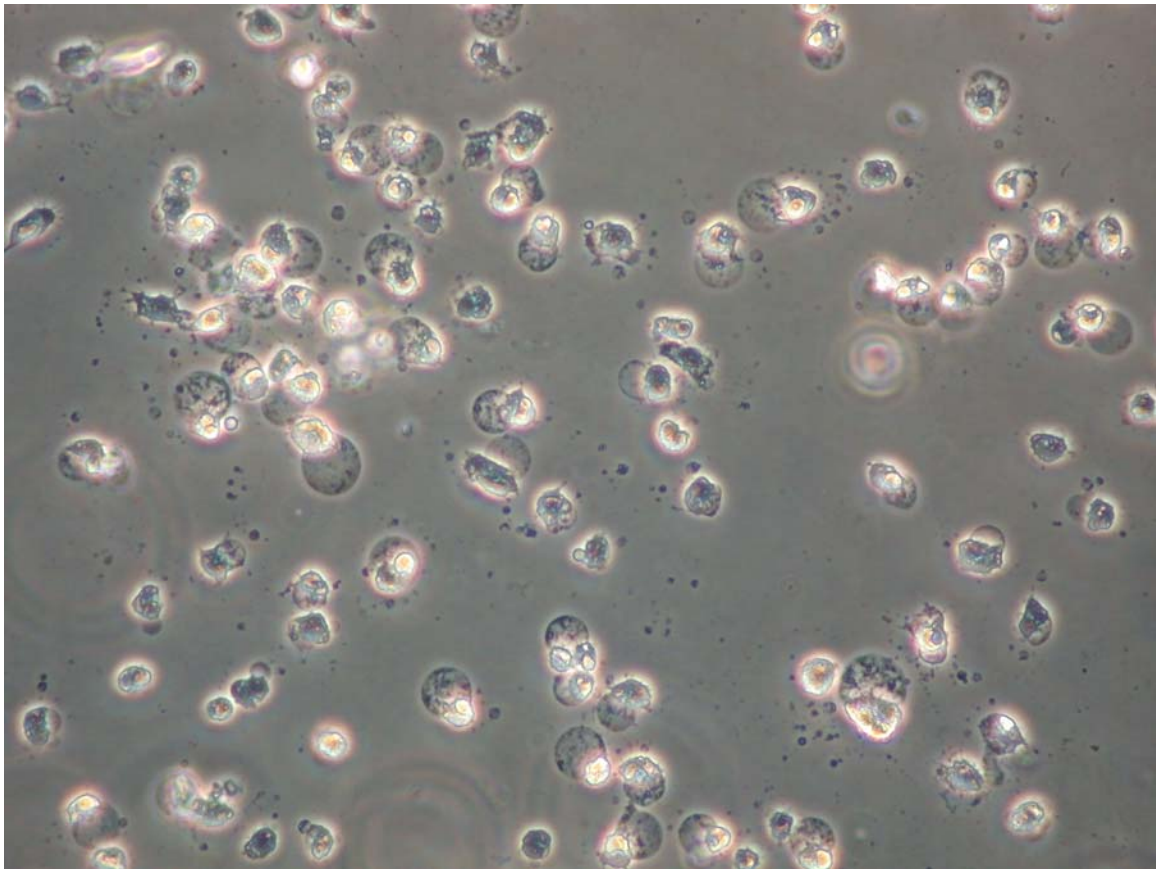


Figure 3: Pyknotic Appearance of LN-229 Cell Line after treatment with BCNU (400X magnification)

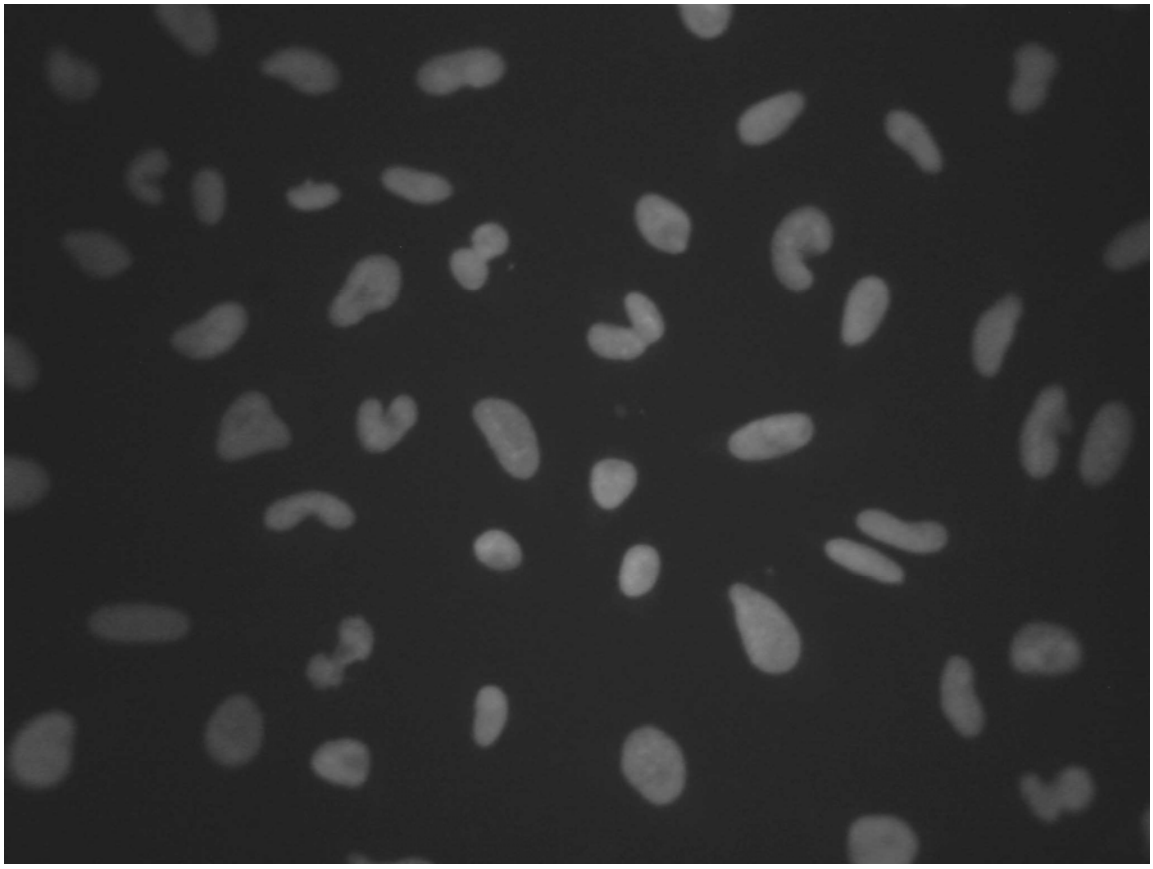


Figure 4: Propidium Iodide (Pi) staining showing DNA fragmentation (400X magnification)

3.3 Cell Proliferation Assay

Promega CellTiter 96® AQ_{ueous} One Solution Proliferation Assay was used to determine cytotoxicity effects of BCNU. The drug concentration required to achieve inhibitory concentration (IC) greater than 90% growth inhibition (IC_{90}) was taken as the reference for comparison of efficacy of chemotherapy dose.

For LN-229 cells seeded at 5,000 per well density, the drug concentration required to achieve greater than 90% growth inhibition was 75 μg/mL. Cells seeded at densities of 10,000, 15,000 and 20,000 per well required BCNU concentrations of 100 μg/mL, 150 μg/mL and 175 μg/mL respectively (Figure 5).

For A172 cell line, the drug concentration required to achieve IC_{90} for cells seeded at 5,000, 10,000, 15,000 and 20,000 cells per well were 100 μg/mL, 125 μg/mL, 150 μg/mL and 175 μg/mL respectively (Figure 6).

The survival curves for the various cell lines demonstrate a consistent trend of larger chemotherapy doses required to achieve similar levels of growth inhibition for higher tumour loading in all the cell lines.

This demonstrates that increased glioblastoma cell loading in the well requires significantly higher concentration of chemotherapeutic agent to achieve similar cytotoxicity effects.

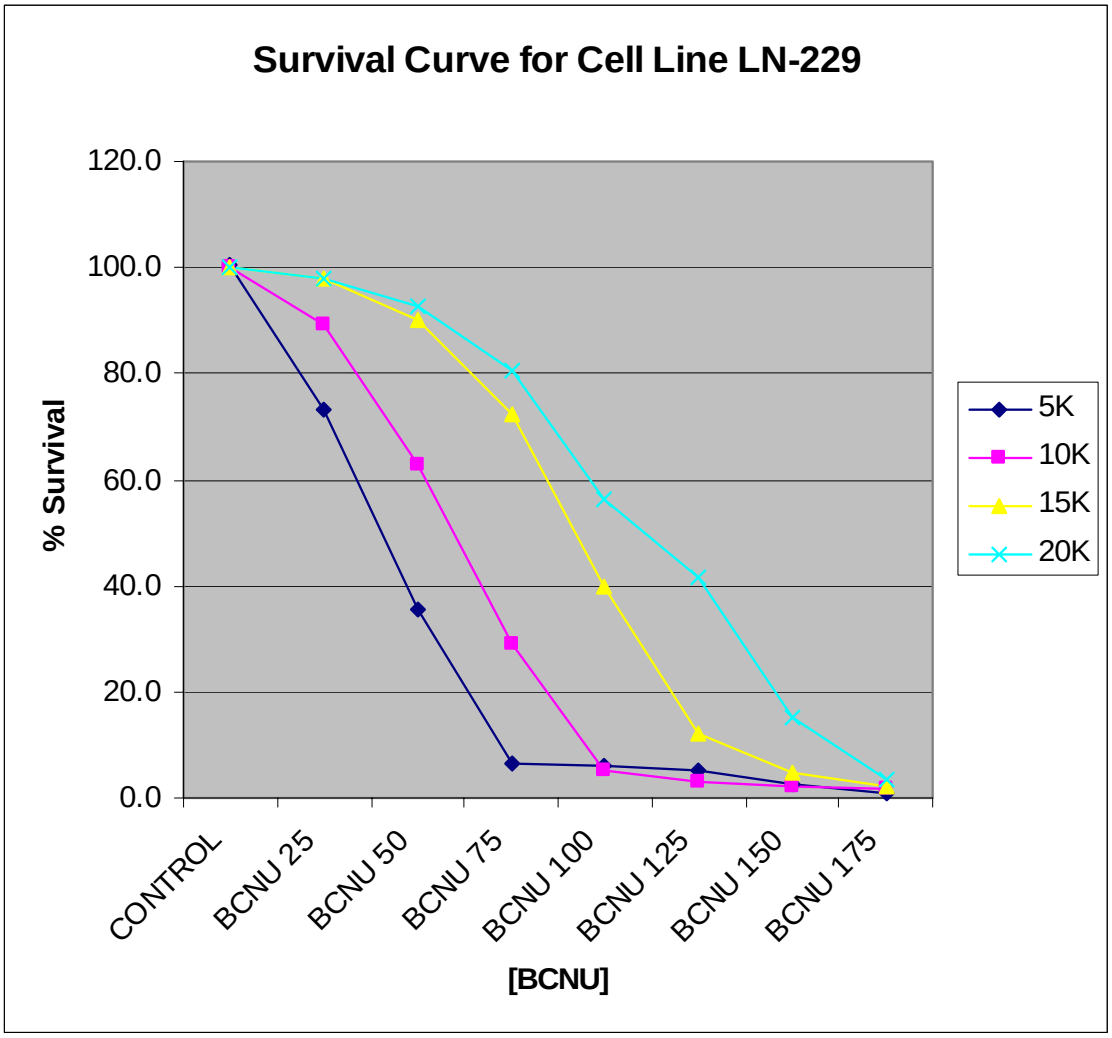


Figure 5: Survival Curve for Cell Line LN-229

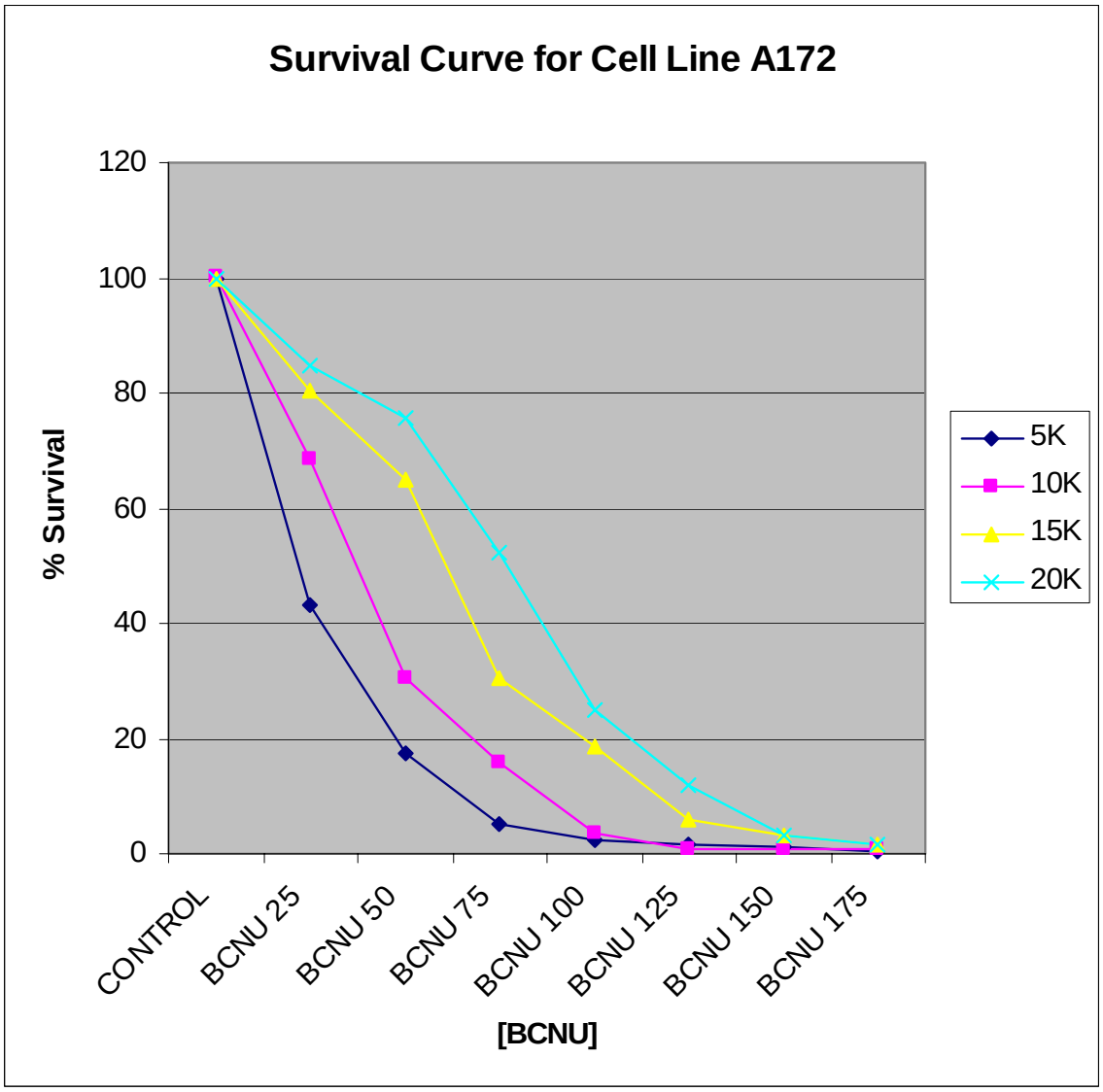


Figure 6: Survival Curve for Cell Line A172

3.4 GDNF Expression Level

GDNF was overexpressed in the two cell lines LN-229 and A172. Significantly, the expression of GDNF was also found to be increased in all glioma specimens when compared to adult brain, foetal brain, adult liver and foetal liver.

All glioblastoma samples and cell lines demonstrated increased level of expression. The highest expression level was observed in a sample of glioblastoma tissue. Other gliomas studied (oligodendroglioma, PXA, recurrent PXA, anaplastic ependymoma and pilocytic astrocytoma) also demonstrated increased levels of expression of GDNF. The pilocytic astrocytoma and PXA had the lowest level of GDNF expression of all the glioma samples. The results are summarised in Figure 7.

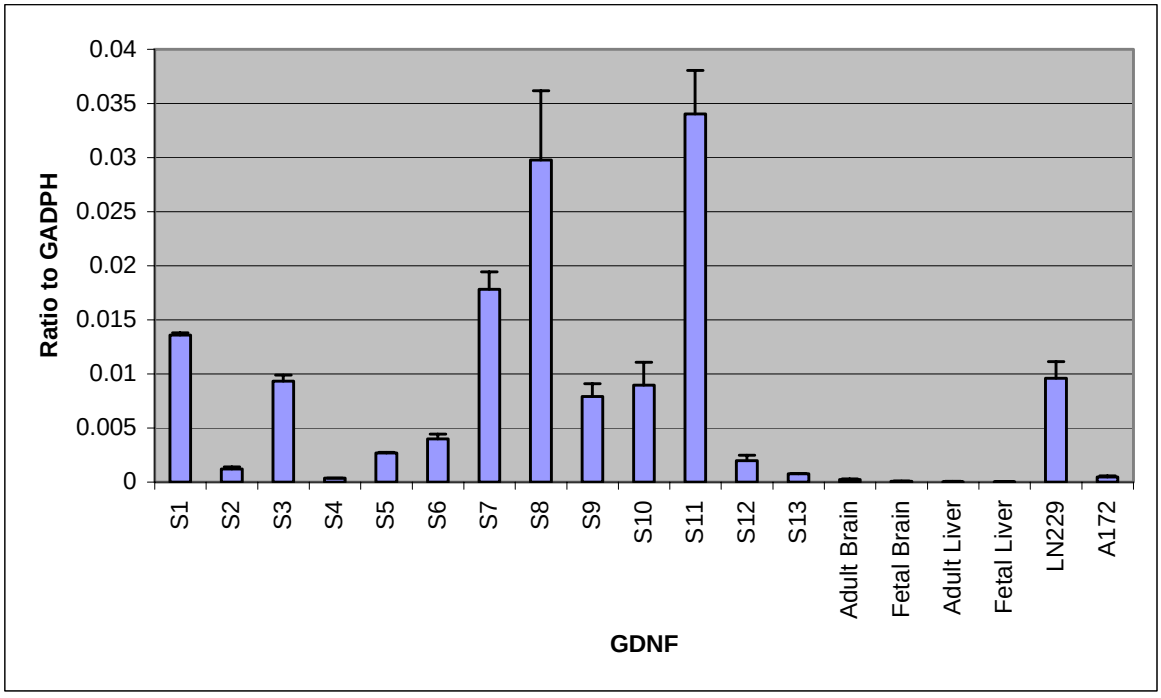


Figure 7: Expression Levels of GDNF

Key to samples:

S1: Oligodendroglioma (WHO Grade II)
S2: Glioblastoma (WHO Grade IV)
S3: Glioblastoma (WHO Grade IV)
S4: Pleomorphic Xanthoastrocytoma (WHO Grade II)
S5: Glioblastoma (WHO Grade IV)
S6: Glioblastoma (WHO Grade IV)
S7: Glioblastoma (WHO Grade IV)
S8: Recurrent Pleomorphic Xanthoastrocytoma (WHO Grade II)
S9: Glioblastoma (WHO Grade IV)
S10: Anaplastic Ependymoma (WHO Grade III)
S11: Glioblastoma (WHO Grade IV)
S12: Glioblastoma (WHO Grade IV)
S13: Pilocytic Astrocytoma (WHO Grade I)

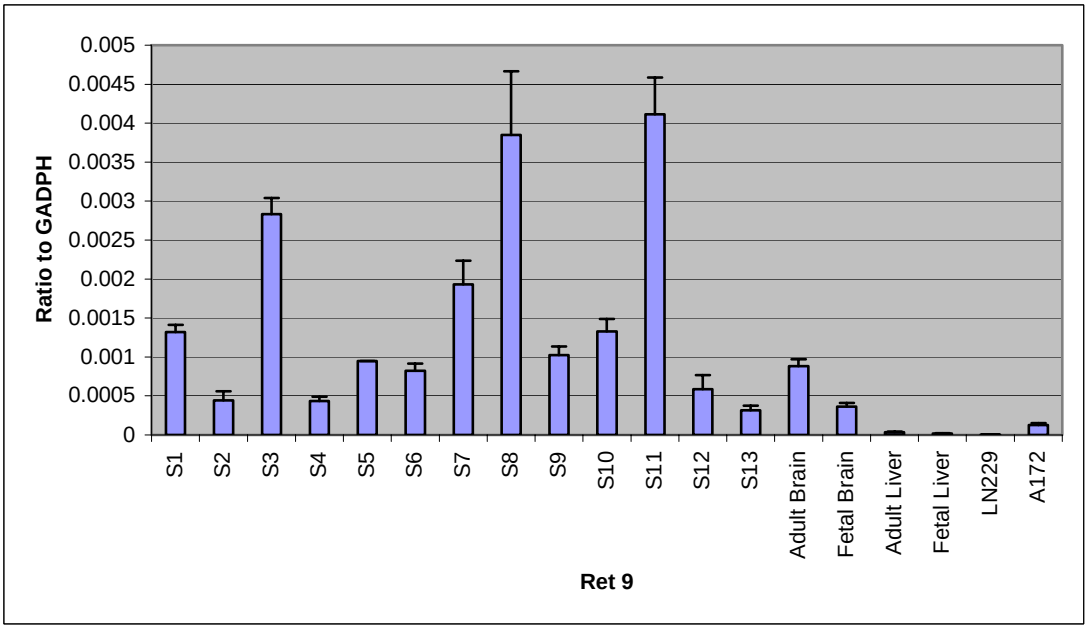
3.5 Expression of RET Isoforms (RET 9 and RET 51)

Ret 9 expression was reduced in the 2 glioblastoma cell lines investigated.

Ret 9 was however more highly expressed in 8 out of 13 of the glioma samples when compared to the expression level in human adult brain. The level of expression was lower than human adult brain in three glioblastoma samples, one PXA and the case of pilocytic astrocytoma. Incidentally, these 5 samples with the lowest Ret expression also had the lowest corresponding GDNF expression.

The level of Ret 51 expression was noted to be fairly homogeneous amongst the all the glioma samples as well as human adult and foetal brain. The expression level is however significantly reduced for the two cell lines studied.

Comparing the ratio of Ret9/Ret51 showed that Ret 9 is the predominant splice isoform in glioma samples and in brain and liver tissue. The results are summarised in Figure 8.



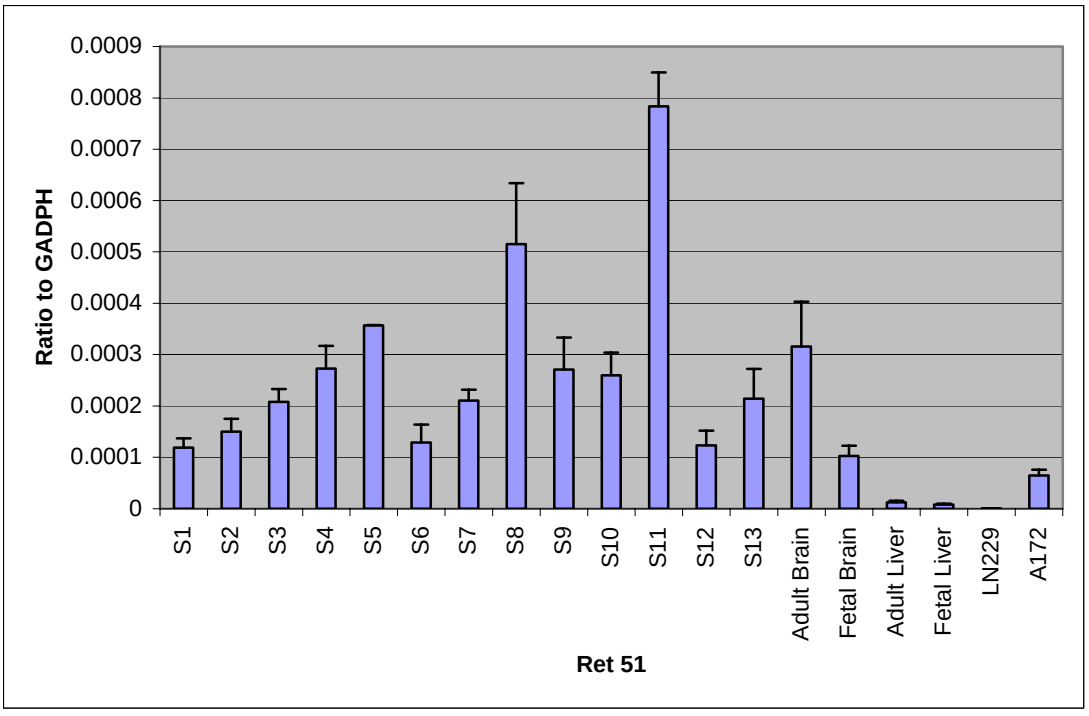


Figure 8: Expression Levels of RET 9 and RET 51

Key to samples:

S1: Oligodendroglioma (WHO Grade II)
S2: Glioblastoma (WHO Grade IV)
S3: Glioblastoma (WHO Grade IV)
S4: Pleomorphic Xanthoastrocytoma (WHO Grade II)
S5: Glioblastoma (WHO Grade IV)
S6: Glioblastoma (WHO Grade IV)
S7: Glioblastoma (WHO Grade IV)
S8: Recurrent Pleomorphic Xanthoastrocytoma (WHO Grade II)
S9: Glioblastoma (WHO Grade IV)
S10: Anaplastic Ependymoma (WHO Grade III)
S11: Glioblastoma (WHO Grade IV)
S12: Glioblastoma (WHO Grade IV)
S13: Pilocytic Astrocytoma (WHO Grade I)

3.6 Expression of NCAM

The glioblastoma cell lines had significantly lower expression of NCAM when compared to human adult and foetal brain samples. All glioma specimens had lower NCAM expression compared to human foetal brain and 11 of the 13 glioma samples had reduced expression compared to adult brain. 2 out of the 8 glioblastoma samples had slightly higher expression than adult brain (Figure 9).

As the expression level of NCAM in all the glioblastoma cell lines and human glioma samples were lower than human foetal brain samples, expression levels of the various NCAM splice isoform variants were not quantified.

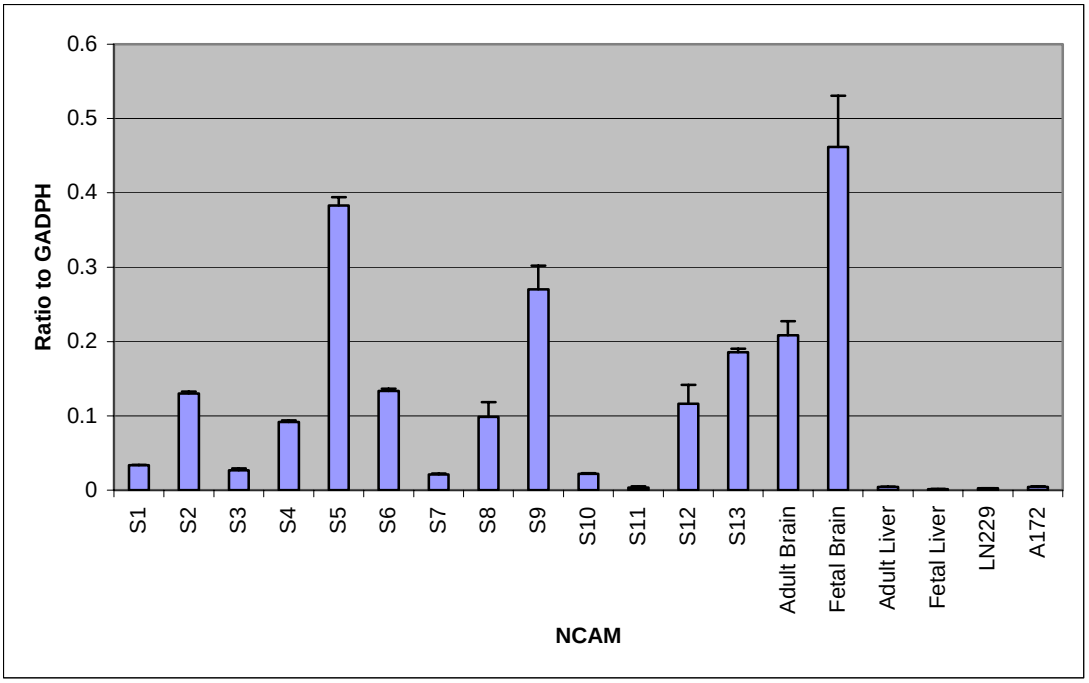


Figure 9: Expression Levels of NCAM

Key to samples:

S1: Oligodendroglioma (WHO Grade II)
S2: Glioblastoma (WHO Grade IV)
S3: Glioblastoma (WHO Grade IV)
S4: Pleomorphic Xanthoastrocytoma (WHO Grade II)
S5: Glioblastoma (WHO Grade IV)
S6: Glioblastoma (WHO Grade IV)
S7: Glioblastoma (WHO Grade IV)
S8: Recurrent Pleomorphic Xanthoastrocytoma (WHO Grade II)
S9: Glioblastoma (WHO Grade IV)
S10: Anaplastic Ependymoma (WHO Grade III)
S11: Glioblastoma (WHO Grade IV)
S12: Glioblastoma (WHO Grade IV)
S13: Pilocytic Astrocytoma (WHO Grade I)

3.7 Expression of GFRα1a

The glioblastoma cell lines had significantly lower levels of expression of GFRα1a compared to human adult and foetal brain samples. 11 out of the 13 human glioma samples had decreased levels of expression of GFRα1a compared to human adult and

foetal brain samples. 2 out of the 8 glioblastoma samples had elevated levels of GFRα1a expression (Figure 10).

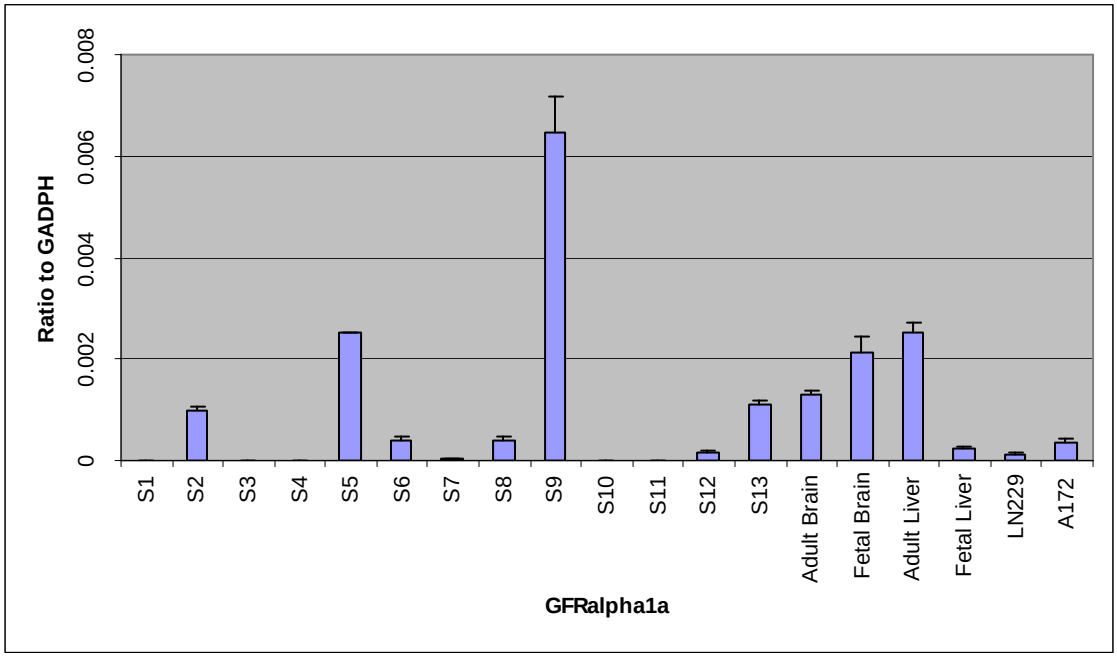


Figure 10: Expression Levels of GFRα1a

Key to samples:

S1: Oligodendroglioma (WHO Grade II)
S2: Glioblastoma (WHO Grade IV)
S3: Glioblastoma (WHO Grade IV)
S4: Pleomorphic Xanthoastrocytoma (WHO Grade II)
S5: Glioblastoma (WHO Grade IV)
S6: Glioblastoma (WHO Grade IV)
S7: Glioblastoma (WHO Grade IV)
S8: Recurrent Pleomorphic Xanthoastrocytoma (WHO Grade II)
S9: Glioblastoma (WHO Grade IV)
S10: Anaplastic Ependymoma (WHO Grade III)
S11: Glioblastoma (WHO Grade IV)
S12: Glioblastoma (WHO Grade IV)
S13: Pilocytic Astrocytoma (WHO Grade I)

3.8 Expression of GFRα1b

The 2 glioblastoma cell lines had increased expression of GFRα1b compared to human adult and foetal brain samples. 5 glioma samples had elevated levels of expression of GFRα1b compared to human adult and foetal brain samples. These were all human glioblastoma samples (Figure 11).

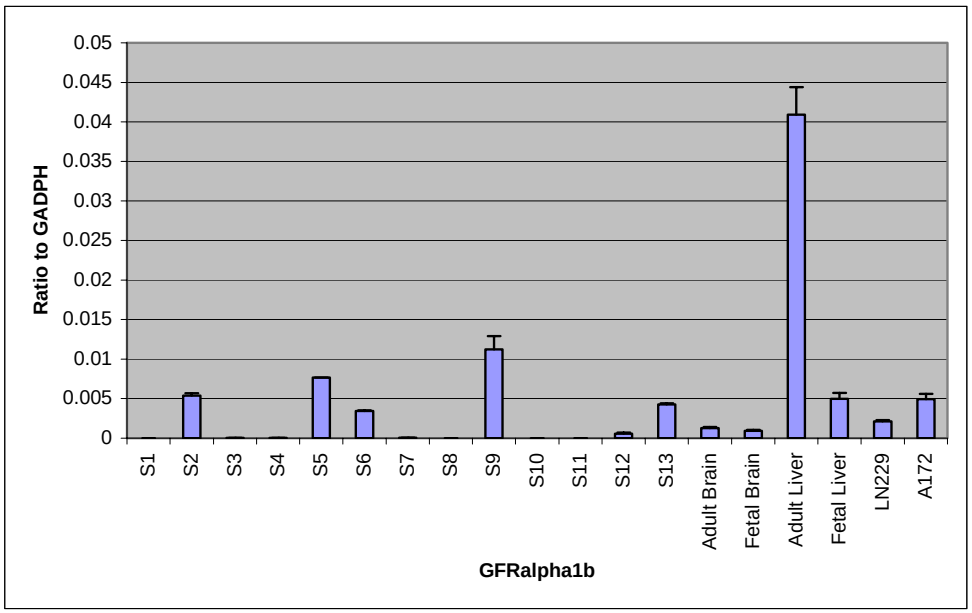


Figure 11: Expression Levels of GFRα1b

Key to samples:

S1: Oligodendroglioma (WHO Grade II)
S2: Glioblastoma (WHO Grade IV)
S3: Glioblastoma (WHO Grade IV)
S4: Pleomorphic Xanthoastrocytoma (WHO Grade II)
S5: Glioblastoma (WHO Grade IV)
S6: Glioblastoma (WHO Grade IV)
S7: Glioblastoma (WHO Grade IV)
S8: Recurrent Pleomorphic Xanthoastrocytoma (WHO Grade II)
S9: Glioblastoma (WHO Grade IV)
S10: Anaplastic Ependymoma (WHO Grade III)
S11: Glioblastoma (WHO Grade IV)
S12: Glioblastoma (WHO Grade IV)
S13: Pilocytic Astrocytoma (WHO Grade I)

3.9 Differential Expression Levels of GFRα1b and GFRα1a

On close analysis of the expression levels of GFRα1a and GFRα1b levels, an interesting observation was noted.

The glioblastoma cell lines demonstrated much higher levels of GFRα1b expression than GFRα1a expression. For cell line LN-229, the ratio of GFRα1b/GFRα1a was 16.3 and the ratio of GFRα1b/GFRα1a was 14.3 for cell line A172.

A similar trend was also noted in 7 out of the 8 human glioblastoma samples. The GFRα1b/GFRα1a ranged from 1.73 to 5.44 in the 7 specimens. Only one human glioblastoma specimen had a higher GFRα1a/GFRα1b ratio.

The GFRα1b/GFRα1a ratio for the pilocytic astrocytoma and PXA are 3.88 and 4.20 respectively. The oligodendroglioma sample had higher level of expression of GFRα1a with a GFRα1a/GFRα1b ratio of 5.99. GFRα1b expression was undetectable for the recurrent PXA specimen.

The results are summarised in Figure 12.

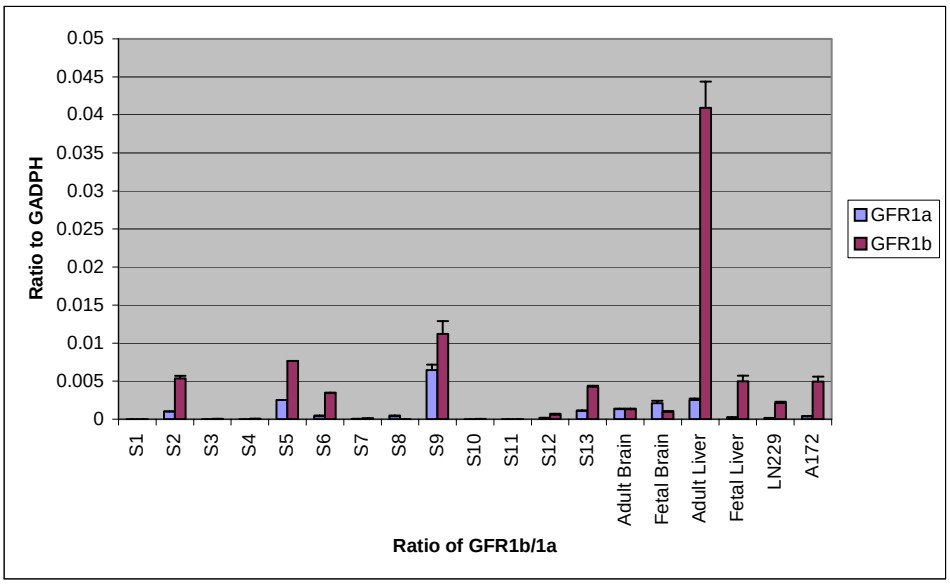


Figure 12: Differential expression levels of GFRα1a and GFRα1b

Key to samples:

S1: Oligodendroglioma (WHO Grade II)
S2: Glioblastoma (WHO Grade IV)
S3: Glioblastoma (WHO Grade IV)
S4: Pleomorphic Xanthoastrocytoma (WHO Grade II)
S5: Glioblastoma (WHO Grade IV)
S6: Glioblastoma (WHO Grade IV)
S7: Glioblastoma (WHO Grade IV)
S8: Recurrent Pleomorphic Xanthoastrocytoma (WHO Grade II)
S9: Glioblastoma (WHO Grade IV)
S10: Anaplastic Ependymoma (WHO Grade III)
S11: Glioblastoma (WHO Grade IV)
S12: Glioblastoma (WHO Grade IV)
S13: Pilocytic Astrocytoma (WHO Grade I)

3.10 Expression of GFRα2

All human glioma samples and glioblastoma cell lines demonstrated significantly decreased levels of expression of GFRα2 compared to human adult brain and human foetal brain samples (Figure 13). As the level of expression of GFRα2 was shown to be much lower than in the normal controls, expression levels of GFRα2 splice isoform variants were not measured.

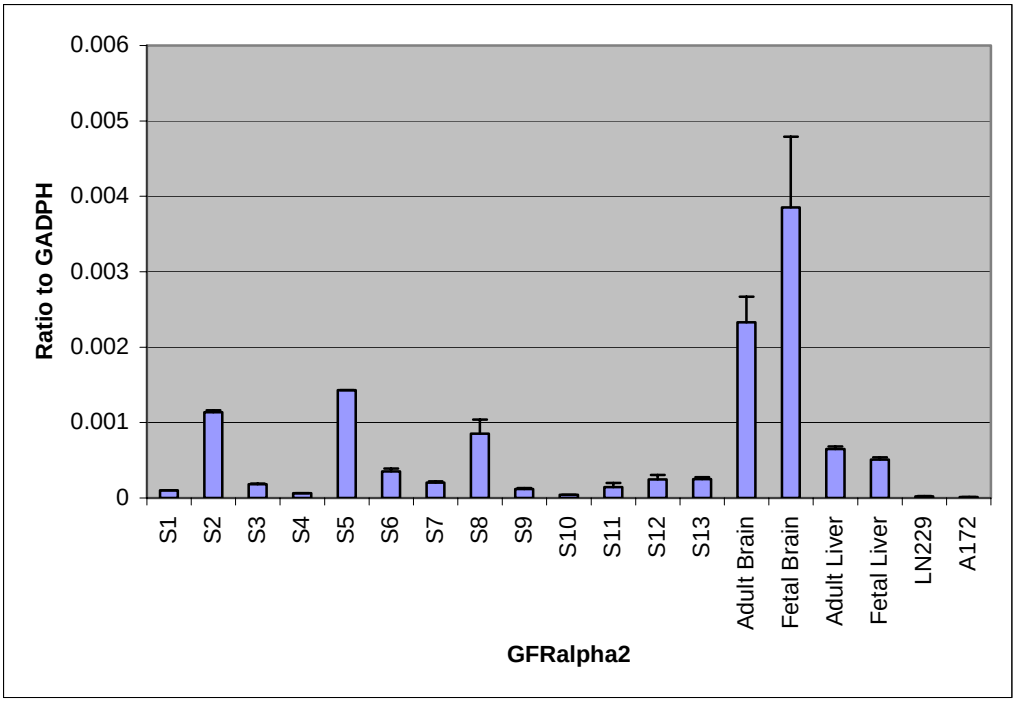


Figure 13: Expression Levels of GFRα2

Key to samples:

S1: Oligodendroglioma (WHO Grade II)
S2: Glioblastoma (WHO Grade IV)
S3: Glioblastoma (WHO Grade IV)
S4: Pleomorphic Xanthoastrocytoma (WHO Grade II)
S5: Glioblastoma (WHO Grade IV)
S6: Glioblastoma (WHO Grade IV)
S7: Glioblastoma (WHO Grade IV)
S8: Recurrent Pleomorphic Xanthoastrocytoma (WHO Grade II)
S9: Glioblastoma (WHO Grade IV)
S10: Anaplastic Ependymoma (WHO Grade III)
S11: Glioblastoma (WHO Grade IV)
S12: Glioblastoma (WHO Grade IV)
S13: Pilocytic Astrocytoma (WHO Grade I)

3.11 Study of Effects of GDNF on BCNU chemotherapy

BCNU is a nitrosurea and is the chemotherapy drug used as in traditional regimens for malignant gliomas. Pre-treatment with GDNF was performed to elucidate the possible effects of GDNF in promoting tumour proliferation by antagonising the cytotoxic effects of chemotherapy. GDNF may therefore play a role in conferring chemoresistance.

LN-229 and A172 were grown to 90% confluency and trypsinised. The experimental groups were seeded with GDNF enriched media at 50ng/ml concentration. The control group was not treated with GDNF. 24 hours, the LN-229 cells were treated with 1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU) at concentrations at 40 µg/ml and 50µg/ml and the A172 cells were treated at 75 µg/ml.

After 24 hours of BCNU treatment, the morphology of the glioblastoma cells was observed. A larger proportion of the cells pre-treated with GDNF had normal morphology whereas a larger proportion of the cells in the control arm appeared rounded and pyknotic in appearance (Figure 14).

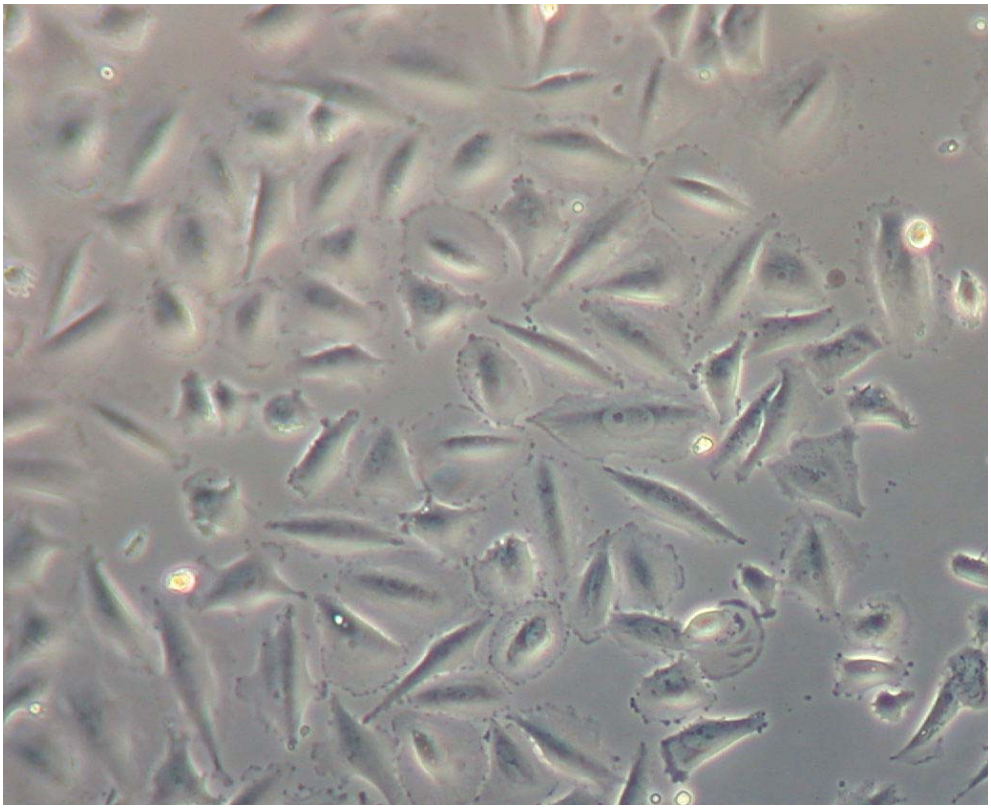


Figure 14a: Morphology of LN-229 *with* pre-treatment with GDNF prior to treatment with BCNU at 50µg/ml (400X magnification)

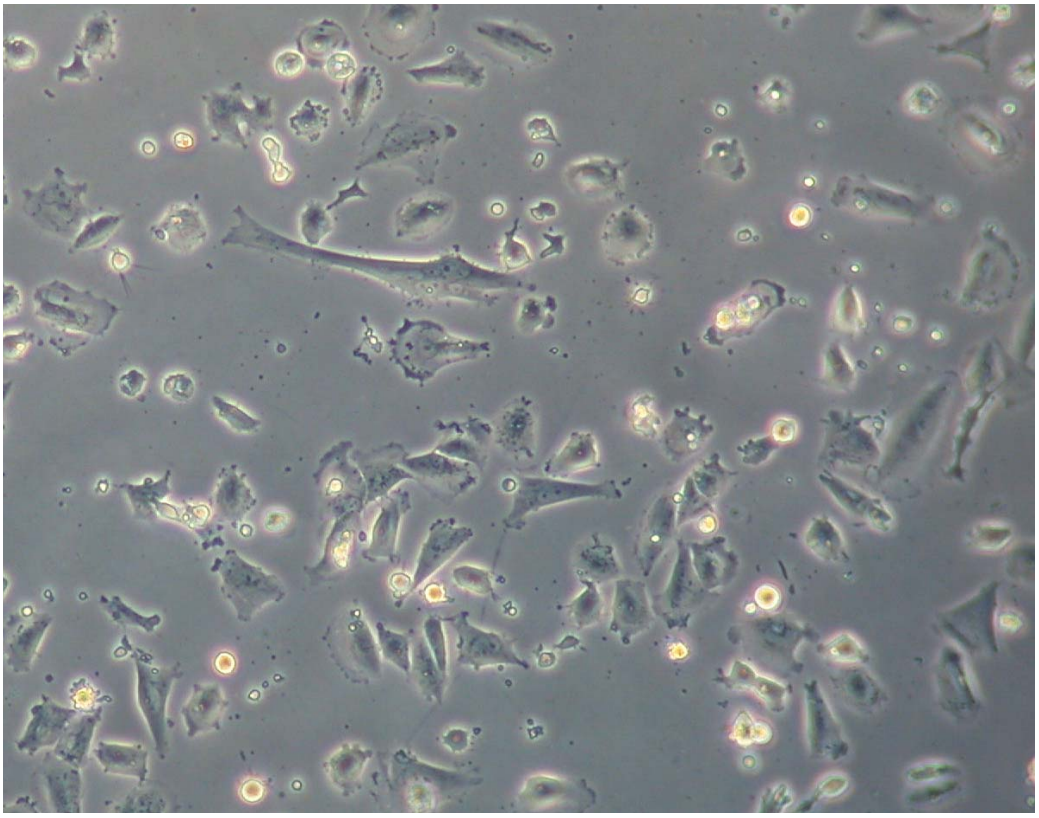


Figure 14b: Morphology of LN-229 *without* pre-treatment with GDNF prior to treatment with BCNU at 50µg/ml (400X magnification)

In the analysis for chemotherapy cytotoxicity effects using the MTS assay, GDNF was shown to demonstrate very significant cellular survival in the presence of BCNU chemotherapy.

When the LN-229 cells were treated with BCNU at 40µg/ml concentration, the cellular survival on MTS assay were 31.7%, 41.0% and 52.1% for the group not pre-treated with GDNF. For the group pre-treated with GDNF, the cellular survival on MTS assay were 71.4%, 77.9% and 79.1% respectively. This difference was statistically significant ($P<0.05$). The mean cellular survival on MTS assay in the group not pre-treated with GDNF was 41.6% and 75.8% in the group pre-treated with GDNF. This difference was statistically significant ($p<0.05$).

A similar trend was also noticed when the LN-229 cells were treated with BCNU at 50µg/ml concentration. The cellular survival on MTS assay were 14.0%, 30.6% and 35.4% for the group not pre-treated with GDNF. For the group pre-treated with GDNF, the cellular survival on MTS assay were 50.8%, 56.2% and 57.2% respectively. This difference was statistically significant ($P<0.05$). The mean cellular survival on MTS assay in the group not pre-treated with GDNF was 26.7% and 54.7% in the group pre-treated with GDNF. This difference was statistically significant ($p<0.05$). The results are summarised in Figures 15.

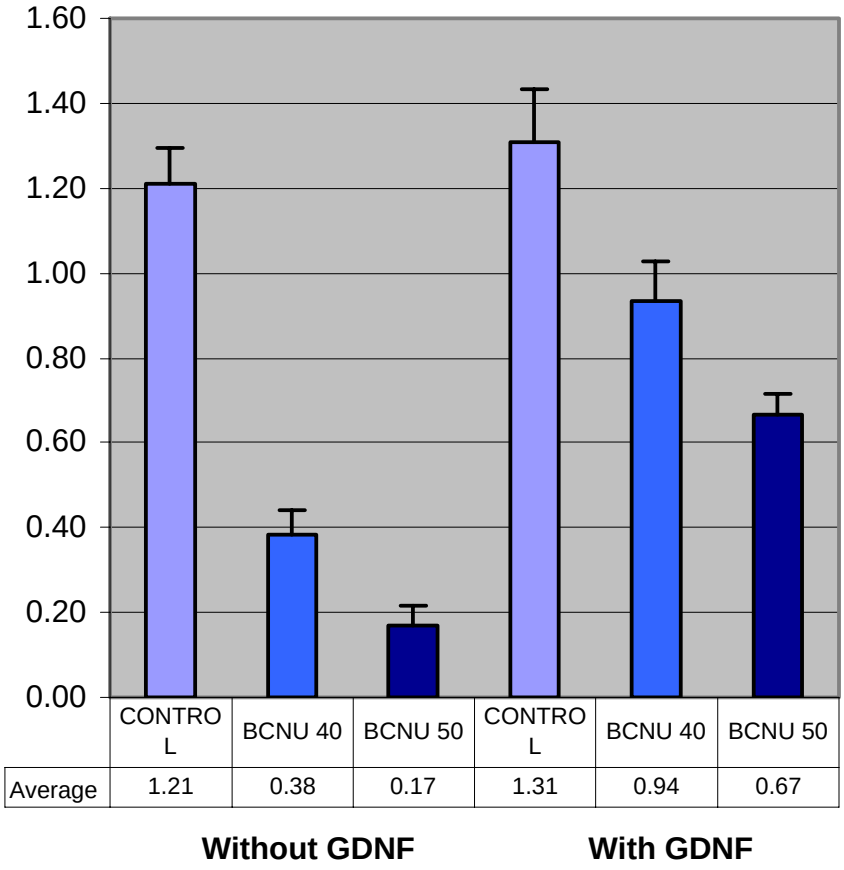


Figure 15a: Study of the effects of BCNU chemotherapy with and without pre-treatment with GDNF on cell line LN-229 (Experiment 1)

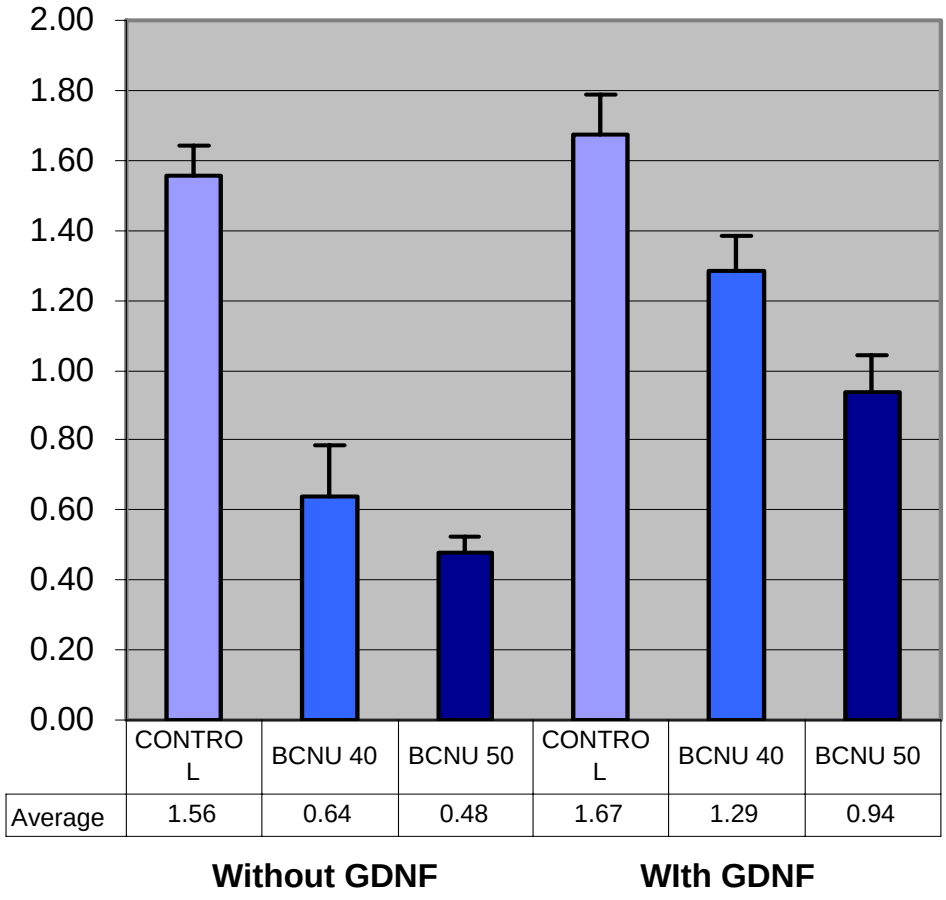


Figure 15b: Study of the effects of BCNU chemotherapy with and without pre-treatment with GDNF on cell line LN-229 (Experiment 2)

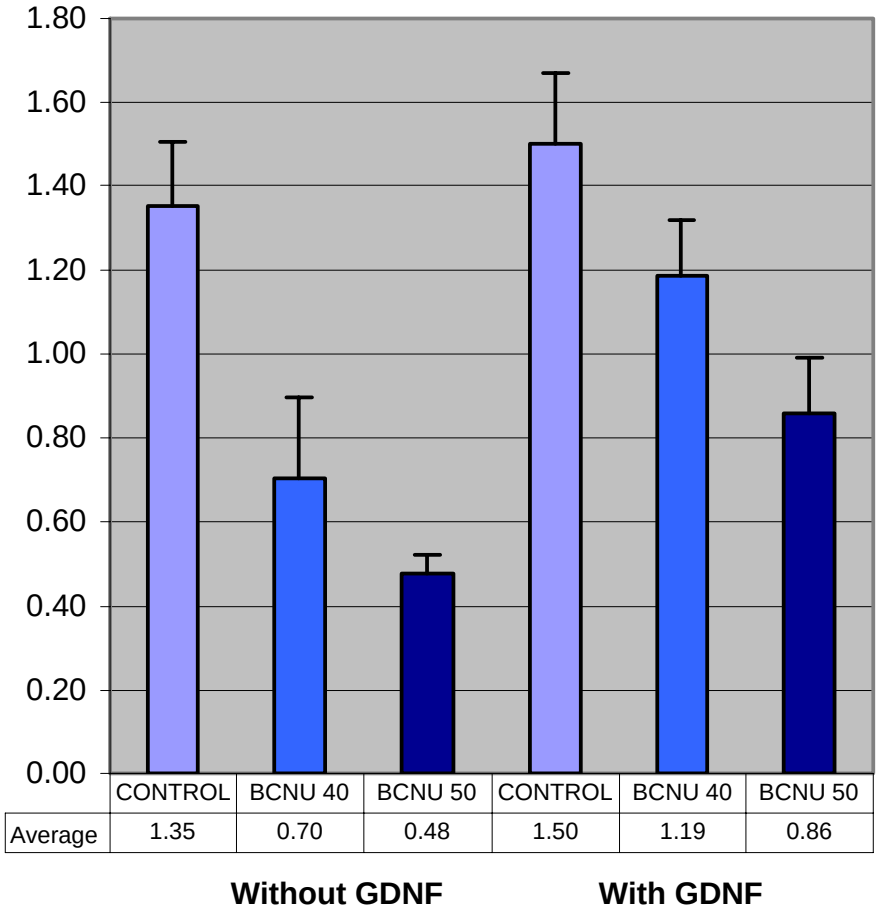


Figure 15c: Study of the effects of BCNU chemotherapy with and without pre-treatment with GDNF on cell line LN-229 (Experiment 3)

The experimental protocol was replicated for glioblastoma cell line A172. In this cell line, GDNF also demonstrated very significant cellular survival in the presence of BCNU chemotherapy.

When the A172 cells were treated with BCNU at 75µg/ml concentration, the cellular survival on MTS assay were 30.9%, 26.0% and 31.4% for the group not pre-treated with GDNF. For the group pre-treated with GDNF, the cellular survival on MTS assay were 61.0%, 58.3% and 68.0% respectively. This difference was statistically significant ($P<0.05$). The mean cellular survival on MTS assay in the group not pre-treated with GDNF was 29.4% and 62.4% in the group pre-treated with GDNF. This difference was statistically significant ($p<0.05$). The results are summarised in Figure 16.

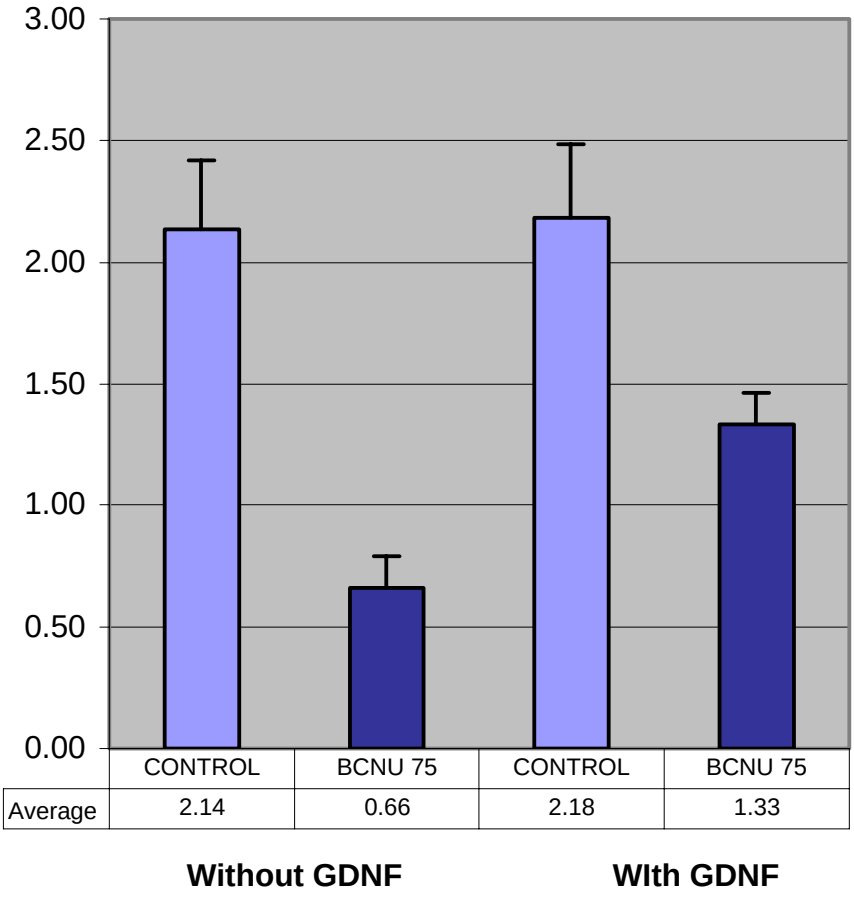


Figure 16a: Study of the effects of BCNU chemotherapy with and without pre-treatment with GDNF on cell line A172 (Experiment 1)

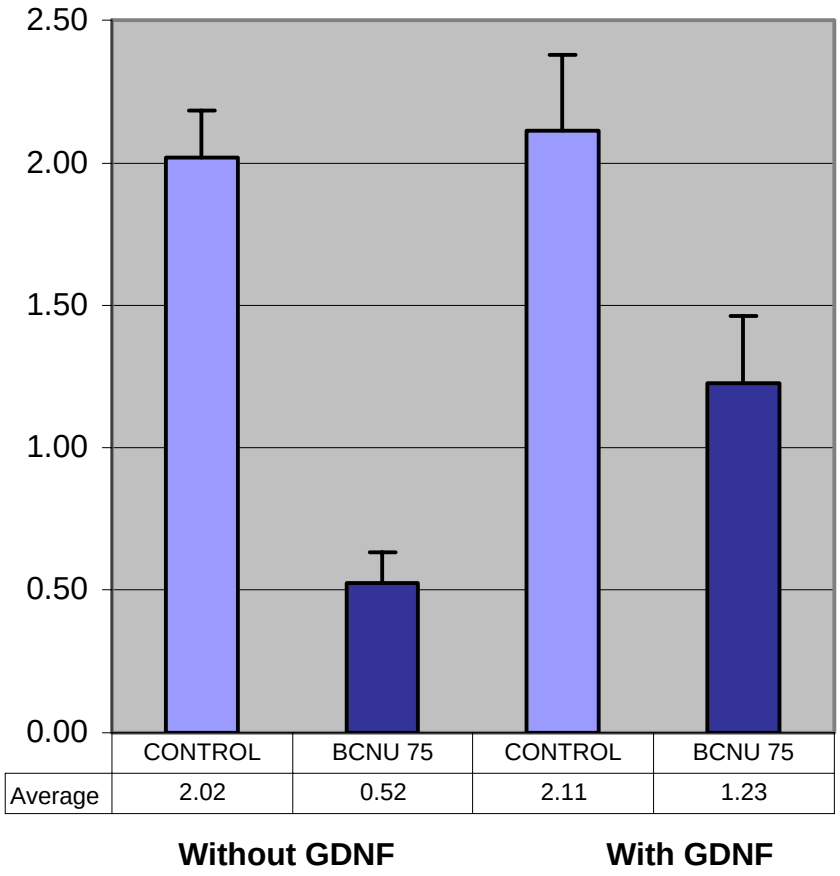


Figure 16b: Study of the effects of BCNU chemotherapy with and without pre-treatment with GDNF on cell line A172 (Experiment 2)

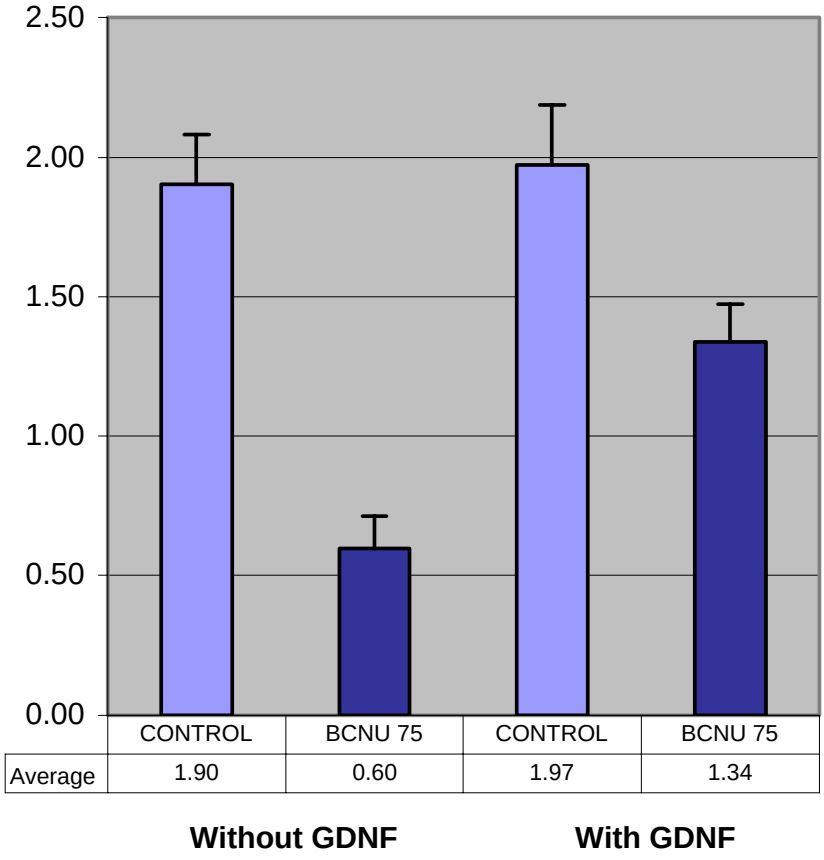


Figure 16c: Study of the effects of BCNU chemotherapy with and without pre-treatment with GDNF on cell line A172 (Experiment 3)

The results therefore demonstrate that GDNF can confer significantly cellular survival in the presence of BCNU chemotherapy. This ability to confer chemoresistance is also demonstrated in two different human glioblastoma cell lines: LN-229 and A172.

3.12 Study on the Effects of NRTN on BCNU chemotherapy

GDNF binds to GFRα1 and NRTN binds to GFRα2. In addition, NRTN may crosstalk weakly with GFRα1 and GDNF with GFRα2. This ability to crosstalk with other GFRα receptors is the basis to investigate the role of NRTN on BCNU chemotherapy and aims to elucidate if NRTN has a similar effect on chemoresistance and cellular survival.

LN-229 and A172 were grown to 90% confluency and trypsinised. The experimental group was seeded with NRTN enriched media at 50ng/ml concentration. The control group was not treated with NRTN. 24 hours, the LN-229 cells were treated with 1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU) at concentrations at 40 μg/ml and 50μg/ml and the A172 cells were treated at 75 μg/ml.

After 24 hours of BCNU treatment, the morphology of the glioblastoma cells was observed. There was no discernable difference in the morphology of the glioblastoma cells in the cells pre-treated with NRTN and in the cells not pre-treated with NRTN (Figure 17).

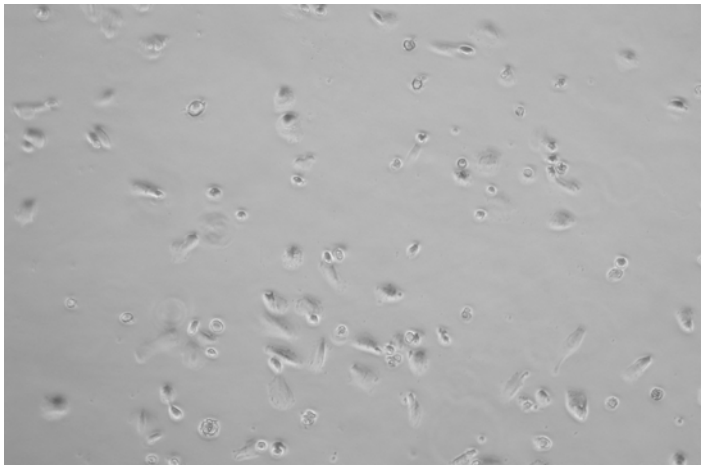


Figure 17a: Morphology of LN-229 *with* pre-treatment with NRTN prior to treatment with BCNU at 50µg/ml (100X magnification)

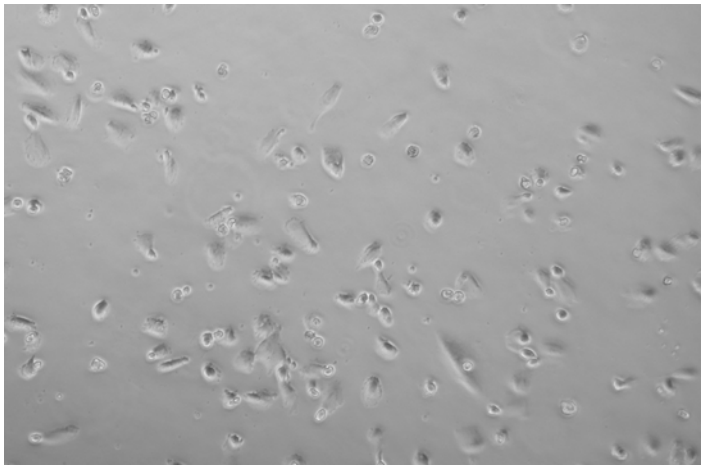


Figure 17b: Morphology of LN-229 *without* pre-treatment with NRTN prior to treatment with BCNU at 50µg/ml (100X magnification)

In the analysis for chemotherapy cytotoxicity effects using the MTS assay, NRTN did not demonstrate any significant cellular survival advantage in the presence of BCNU chemotherapy.

When the LN-229 cells were treated with BCNU at 40μg/ml concentration, the cellular survival on MTS assay were 69.2%, 71.9% and 57.4% for the group not pre-treated with NRTN. For the group pre-treated with NRTN, the cellular survival on MTS assay were 70.9%, 66.4% and 57.4% respectively. There was no statistically significant difference ($p>0.05$). The mean cellular survival on MTS assay in the group not pre-treated with NRTN was 66.2% and 64.9% in the group pre-treated with NRTN. This difference was not statistically significant ($p>0.05$).

A similar trend was also noticed when the LN-229 cells were treated with BCNU at 50μg/ml concentration. The cellular survival on MTS assay were 43.6%, 37.7% and 29.5% for the group not pre-treated with NRTN. For the group pre-treated with NRTN, the cellular survival on MTS assay were 44.3%, 38.7% and 30.9% respectively. This difference was not statistically significant ($p>0.05$). The mean cellular survival on MTS assay in the group not pre-treated with NRTN was 36.9% and 38.0% in the group pre-treated with NRTN. This difference was not statistically significant ($p>0.05$). The results are summarised in Figure 18.

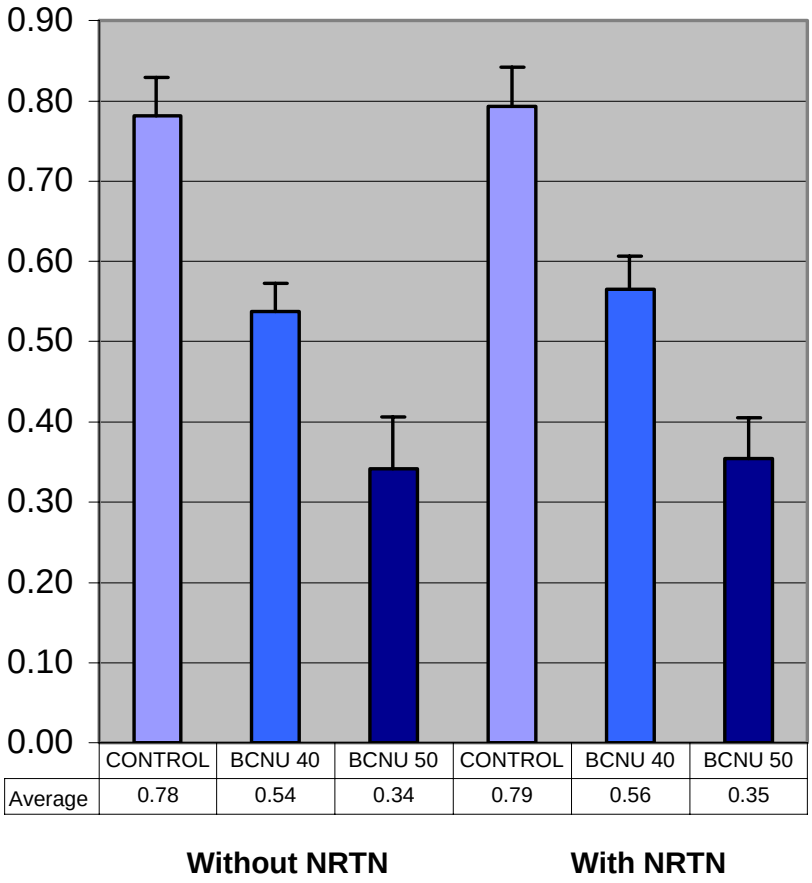


Figure 18a: Study of the effects of BCNU chemotherapy with and without pre-treatment with NRTN on cell line LN-229 (Experiment 1)

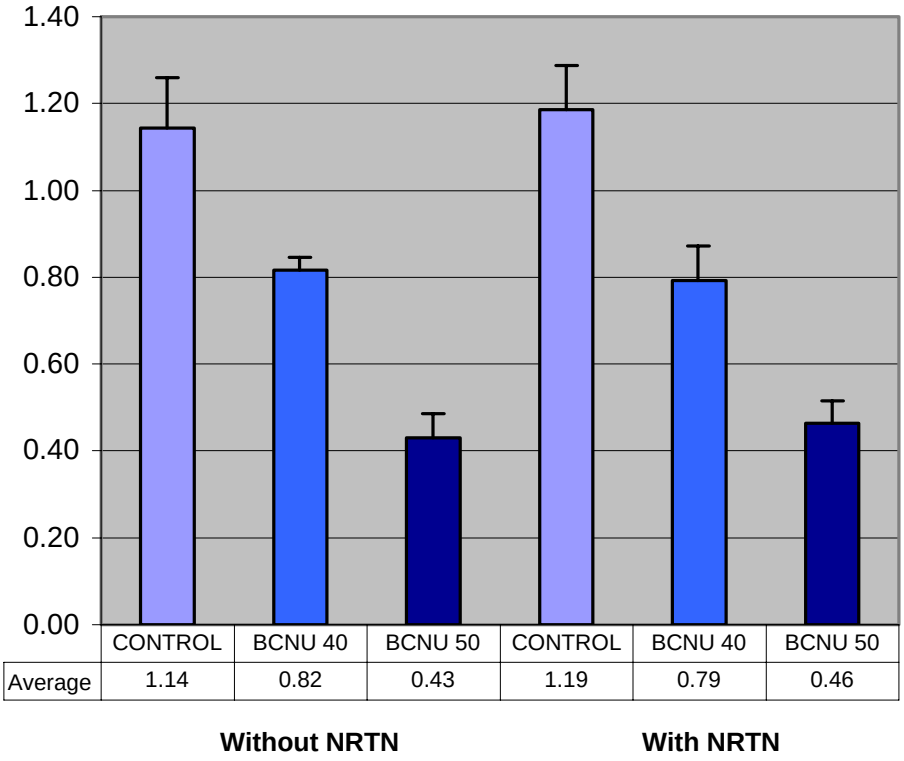


Figure 18b: Study of the effects of BCNU chemotherapy with and without pre-treatment with NRTN on cell line LN-229 (Experiment 2)

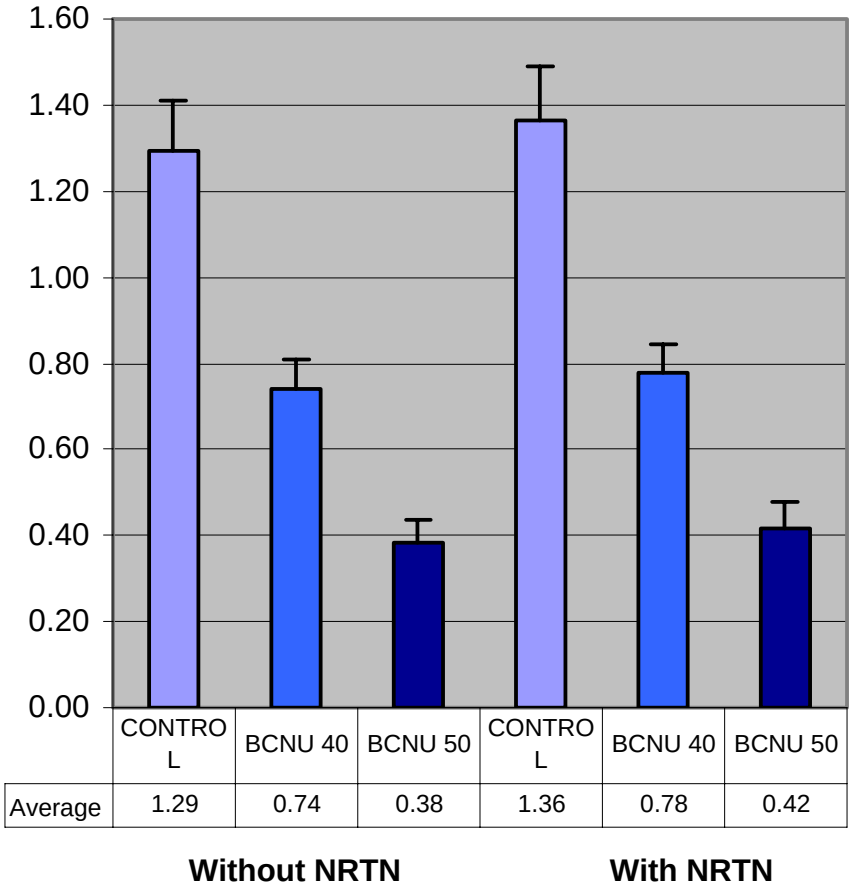


Figure 18c: Study of the effects of BCNU chemotherapy with and without pre-treatment with NRTN on cell line LN-229 (Experiment 3)

The experimental protocol was replicated for glioblastoma cell line A172. In this cell line, NRTN also did not demonstrate very significant cellular survival in the presence of BCNU chemotherapy.

When the A172 cells were treated with BCNU at 75µg/ml concentration, the cellular survival on MTS assay were 32.3%, 32.5% and 33.3% for the group not pre-treated with NRTN. For the group pre-treated with NRTN, the cellular survival on MTS assay were 35.3%, 31.0% and 35.5% respectively. There was no statistical difference between the values in the two groups ($p>0.05$). The mean cellular survival on MTS assay in the group not pre-treated with GDNF was 32.7% and 33.9% in the group pre-treated with NRTN. There was no statistically significant difference between the two groups ($p>0.05$). The results are summarised in Figure 19.

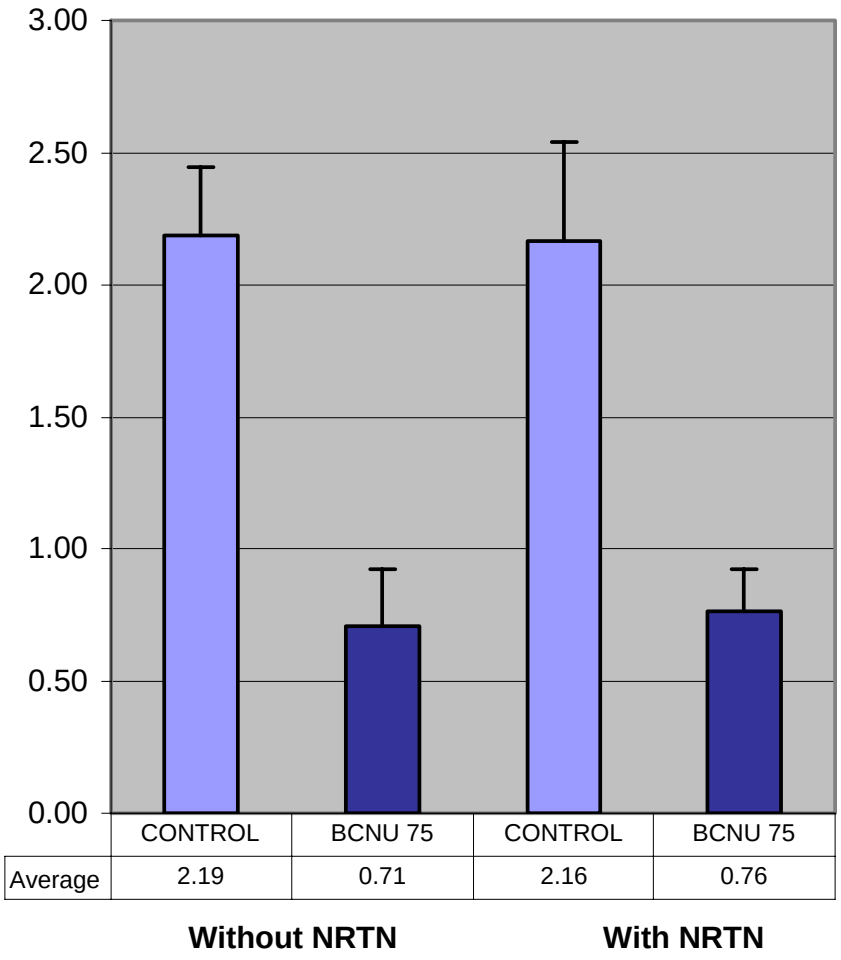


Figure 19a: Study of the effects of BCNU chemotherapy with and without pre-treatment with NRTN on cell line A172 (Experiment 1)

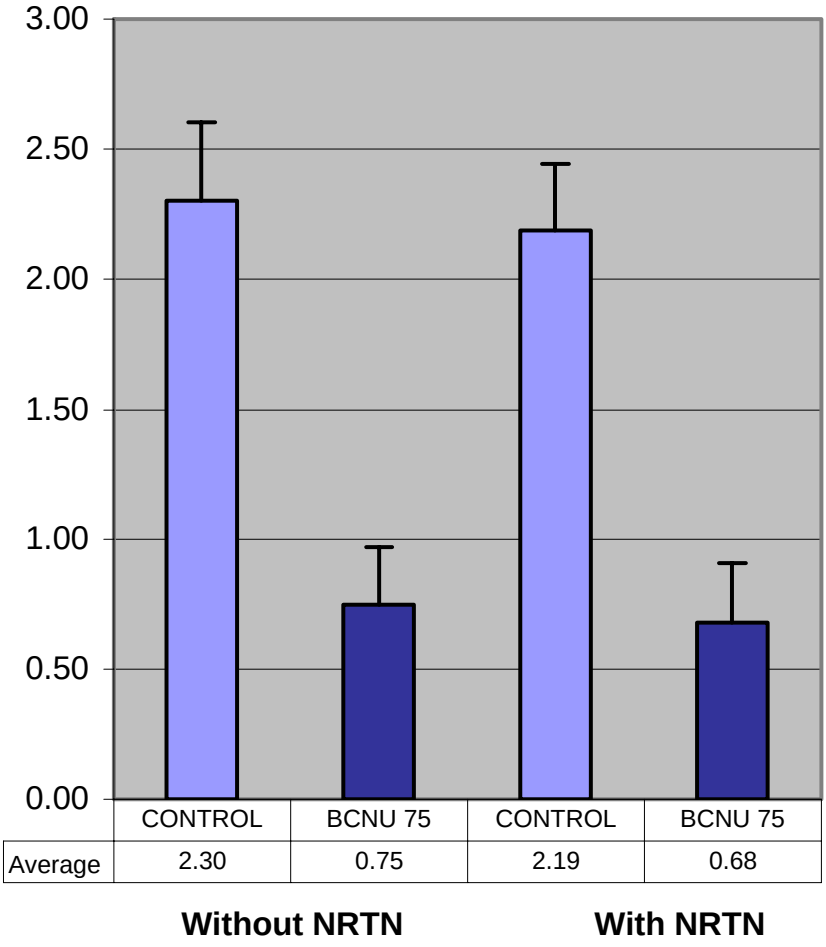


Figure 19b: Study of the effects of BCNU chemotherapy with and without pre-treatment with GDNF on cell line A172 (Experiment 2)

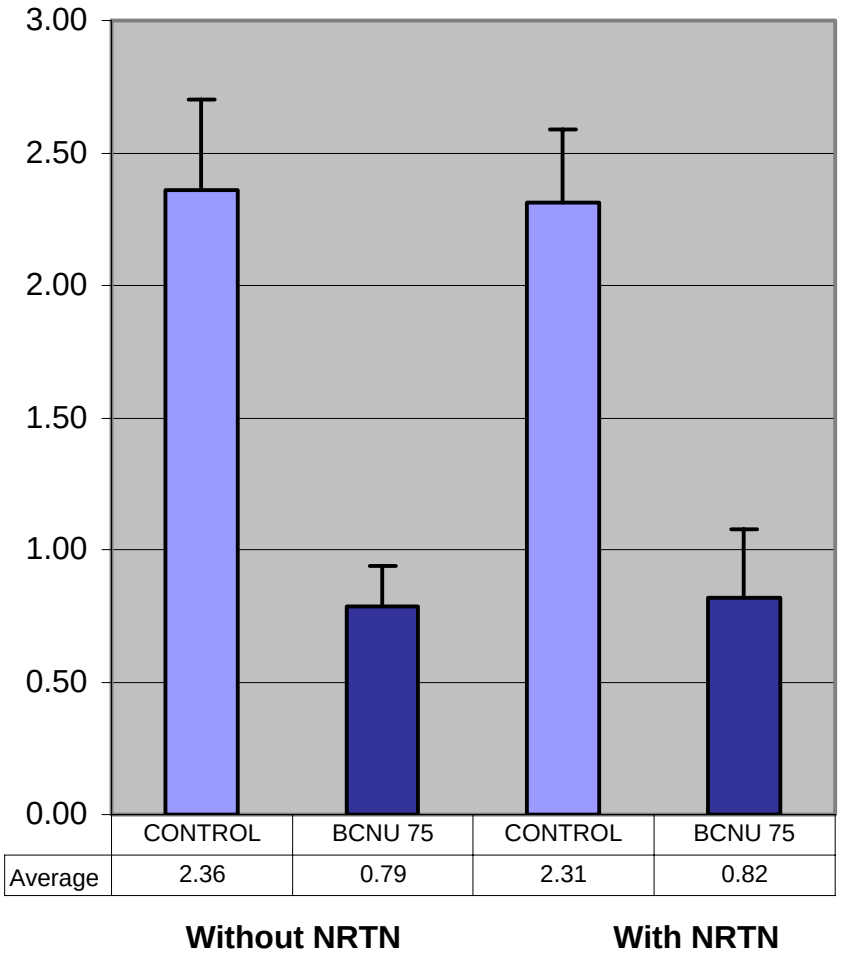


Figure 19c: Study of the effects of BCNU chemotherapy with and without pre-treatment with GDNF on cell line A172 (Experiment 3)

The results therefore demonstrate that NRTN, unlike GDNF does not confer any cellular survival advantage to BCNU chemotherapy. This absence of significant chemoresistance property is demonstrated in two different human glioblastoma cell lines: LN-229 and A172.

3.13 Signalling Mapping on stimulation with BCNU and GDNF for LN-229 and A172

Many different growth factor/receptor systems have been implicated in the proliferative behaviour of gliomas. Examples of such growth factors are: Vascular Endothelial Growth Factor (VEGF), Nerve Growth Factor (NGF) and Platelet-Derived Growth Factor (PDGF). The results above have also convincing demonstrated that GDNF but not NRTN has a significant role in conferring chemoresistance to BCNU chemotherapy.

These mitogens and their cognate receptors will subsequently regulate several intracellular signaling pathways. The major signaling cascades in activated in malignant gliomas are: phosphoinositide 3-kinase/AKT-protein kinase B (PI3K/AKT-PKB) pathway, mitogen activated protein kinase (RAS/MAPK) pathway and the phospholipase C-γ/protein kinase C (PLC-γ/PKC) pathway.

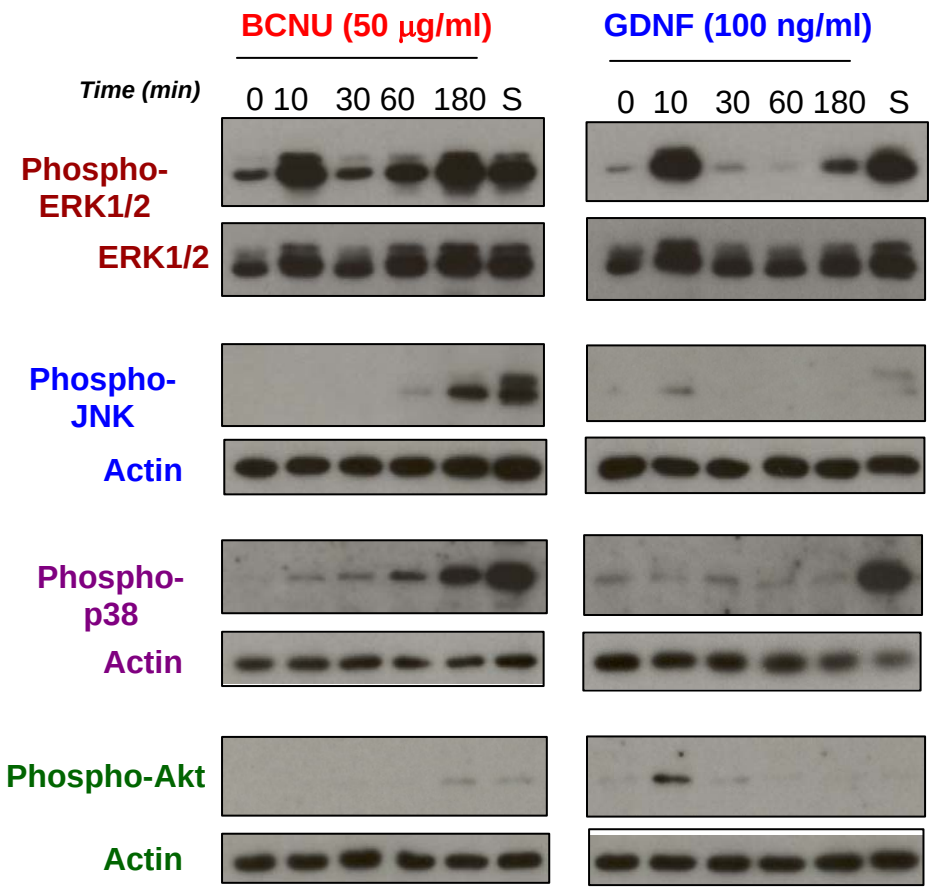
The modulation of MAPK and Akt signaling pathways in glioblastoma cell lines was investigated. LN-229 and A172 human glioblastoma cell lines were stimulated with BCNU and GNDF and the experiments were studied at 0, 10, 30, 60 and 180 mins respectively. Experiments were terminated with 2% SDS.

Western blotting showed that BCNU induces activation of MAP kinases (extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun NH(2)-terminal kinase (JNK)

and p38) in both LN-229 and A172 human glioblastoma cell lines. BCNU was however found to reduce the background activation of Akt in the A172 human glioblastoma cell line.

GDNF was found to induce the activation of ERK1/2 and Akt in both LN-229 and A172 human glioblastoma cell lines. GDNF was however found to reduce the background activation of JNK and the A172 human glioblastoma cell line in a time-dependent fashion (Figure 20).

LN229 Cell line



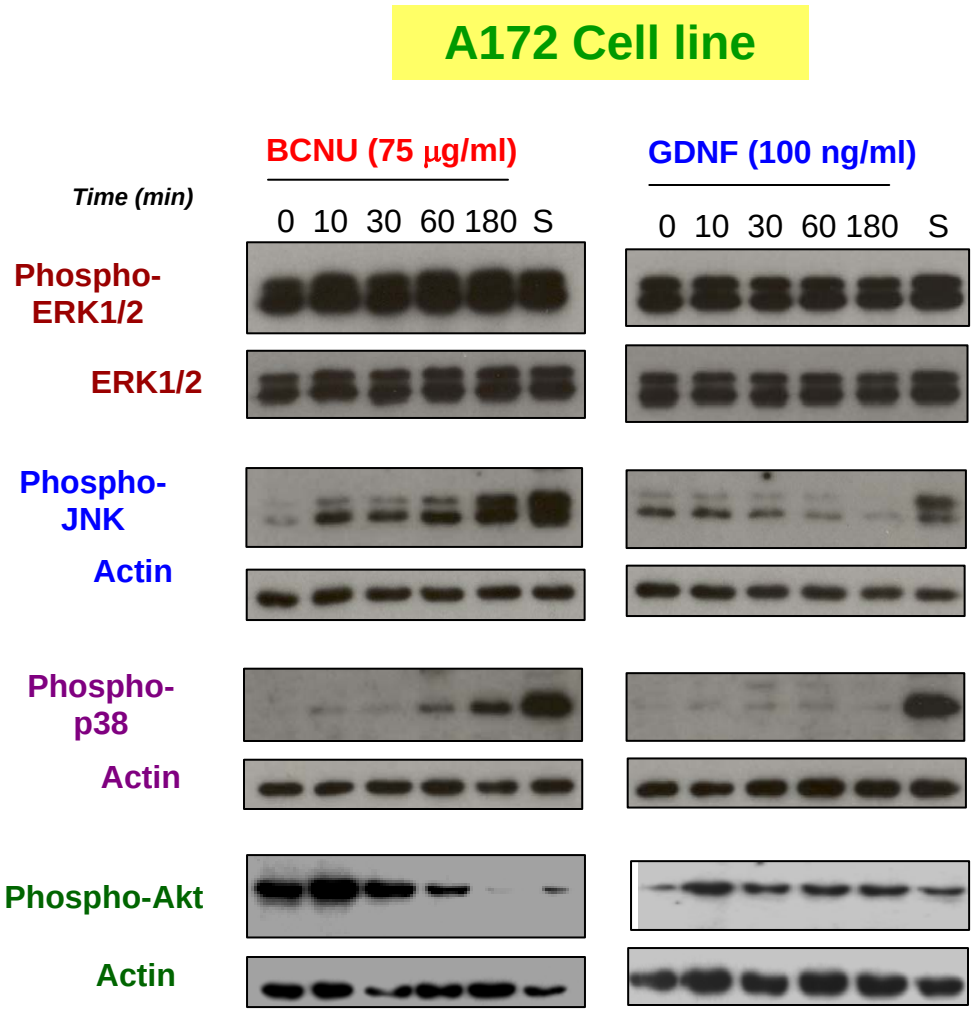


Figure 20: Western Blotting showing activation of phospho-ERK1/2, ERK 1/2, phospho-JNK, phsopho-p38, phospho-Akt on cell lines LN-229 and A172 on stimulation with BCNU and GNDF

CHAPTER 4

DISCUSSION

4.1 Role of Radical Surgery

Surgery plays an important role in the management of high-grade gliomas. Surgery is critical for histological diagnosis of high-grade gliomas. Aggressive tumour resection can also rapidly reduce the intracranial hypertension associated with bulky disease and provide symptomatic relief and improved quality of life.

Surgical management can range from a simple biopsy with the express purpose of histological diagnosis, debulking of tumour to relief the pressure effects effects exerted by the tumour mass to more radical surgery. There is little argument that extensive surgical resection can provide higher diagnostic yield by obviating the problems of sampling error from a simple biopsy. There is also little controversy that more radical tumour resection can reduce intracranial hypertension and lead to symptomatic relief and recovery of reversible neurological deficits. However, the role of radical surgery in improving overall survival remains highly contentious and controversial.

The invasive and widely infiltrative nature of high grade glioma makes curative resection impossible[279]. This has been supported by the fact that even hemispherectomy was associated with survival rate of less than two years. Hemispherectomy as a means to achieve total glioma removal was pioneered by Walter Dandy in 1928[280]. Patient survival following hemispherectomy reported in the early 1930's as less than 2 years[280-284]. Eight decades on, even when near total excision is achieved and corroborated with post resection magnetic resonance imaging (MRI), median survival rate remains at a dismal 13 months[285].

Tumour recurrence commonly occurs near to resection margin[129-132, 286]. This is attributed to the sharp drop in tumour cell density with increasing distance from resection cavity. These ideas underlie the belief that a wider resection margin, coupled with adjuvant therapy would delay recurrence and prolong survival.

Four extensive reviews of the available literature have been carried out to date in an attempt to resolve the controversy of whether surgical resection improves survival time in malignant gliomas (see Table 1). Two publications evaluated existing evidence prior to 1990[287-288]. Another reviewed publications from 1991 to 1999[289]. The most recent update was from the Cochrane database of systematic reviews[290]. This systematic review identified only one study with adequate scientific rigour. It was a randomised trial, which enrolled 23 patients and compared biopsy to surgical excision. The results showed no difference in outcome between the two treatment options; however, these findings may not be conclusive because of the small sample size of the study. All four reviews unanimously bemoaned the lack of well conducted studies and arrived at a similar conclusion: that there is of absence of good scientific evidence to support claims of survival benefit from surgical resection of malignant gliomas.

Lacroix et al retrospectively analysed 416 consecutive cases of glioblastoma multiforme (GBM) treated at the M.D. Anderson Cancer Centre[285]. This however included 44% of previously treated cases at other institutions prior to referral (cytoreductive surgery or biopsy only, with or without adjuvant chemotherapy or radiation therapy). No comparison group was available (matched untreated group). The patients' tumour volumes were quantified prospectively based on preoperative and postoperative MR images. Tumour volume was defined based on contrast enhancement

on T1-weighted imaging and increased signal intensity on T2-weighted imaging. Volumetric measurement was then performed using a software program. This method of assessing extent of resection is quantitative and reliable compared to other methods which were based on intraoperative surgeon judgement and/or radiologist assessment of preoperative and postoperative MR imaging without quantification of tumour volume, both of which are prone to subjectivity and variability. The authors reported longer survival in patients with at least 98% tumour resection (median survival 13 vs. 8.8 months). In the selected group without prior surgical intervention (56%) the reported survival was 13 months for patients with ≥ 98% resection and 10.1 months for less than 98% glioma resection. The survival benefit for ≥ 98% resection was much better in the previously not treated group compared to the previously surgically treated (at a separate institution) group. There was also a trend for survival benefit once tumour resection exceeds 89%. Although none of this data constitutes Class I evidence, it does show a trend that aggressive tumour resection can be performed with low morbidity with better survival compared to more conservative surgery. Furthermore, it is not likely that a randomised controlled trial can be conducted in the future because of ethical considerations.

In summary there is no good evidence that aggressive surgical resection significantly improves survival outcome. On the contrary recent evidence from a series a trials of glioma treatment suggests that any survival benefit, if present, is likely to be marginal. Radical resection may therefore be attempted if the incidence of surgical morbidity is reasonably low.

Table 2 summarises additional studies which support improved survival following glioma resection. Most are retrospective in nature with no matched control, let alone randomised control; thus there is no strong evidence that aggressive surgical resection per se significantly improves survival outcome.

4.2 Why surgical resection then?

Failure to demonstrate prolonged survival should not detract physicians from considering surgical resection due to the numerous other benefits or tumour removal.

(A) Symptomatic relief and neurological improvement

Symptomatic relief from mass effect and obstructed cerebrospinal fluid (CSF) circulation are obvious benefit of glioma resection. Distortion of brain structure and compression of neural pathways contribute to both general symptoms and focal deficits, which may have some degree of improvement following surgical resection.

Global symptoms such as headache, nausea, vomiting and general malaise often show dramatic improvement after surgery[291]. Relief of local compression may contribute to partial reversal of neurological deficit[292].

An often used indicator of potential neurological improvement following surgical glioma resection is a trial course of dexamethasone (16 mg per day). Patients with improved functional status after steroid use are usually the ones who will also show improvement in their quality of life after aggressive surgical resection, provided there is low postoperative morbidity[293].

Brown et al evaluated the quality of life following 3 separate high grade glioma treatment regimes for high grade gliomas[294]. In this trial, patients with gross total glioma resection and adjuvant therapy had improved overall quality of life at 2 and 4 month follow-up. There was no survival benefit.

Ammirat et al[295] and Sawaya et al[296] found that gross total resections are associated with better patient neurological performance scores than those observed after more limited resections. Furthermore, partial resection, with significant residual tumour, may lead to an increased risk of post operative bleeding and oedema exacerbation.

(B) Oncologic reduction to augment adjuvant therapy

Oncologic reduction is another benefit of aggressive surgical resection. A 99% excision would reduce the amount of neoplastic cells by a factor of two, from 10^9 to 10^7 cells. A lower tumour load increases the efficacy of adjuvant therapy. Stewart et al[297] performed a systematic review and meta-analysis of the effect of systemic chemotherapy on high grade glioma. They showed improved survival with a combined modality of surgical resection, radiotherapy and chemotherapy, as compared to surgery and radiotherapy. Surgical treatment included biopsy only, incomplete resection or complete resection. In the biopsy group survival at one year improved from 36% to 42%. Subgroup analysis did not show evidence of a differential effect of chemotherapy on extent of resection; however there was a trend towards improved survival in the patients who underwent complete and incomplete resection, compared to those in the biopsy group, although the improvement was not statistically significant.

(C) More accurate diagnosis

Accuracy of histological diagnosis is dependent on tissue sample size. This is especially true in the setting of false negatives associated with stereotactic biopsy as a result of limited tissue sample, which is estimated to be around 10%[120]. Jackson et al[298] reported a discrepancy rate of 38% between biopsy and subsequently resected specimens in 81 patients. This discrepancy was found to affect treatment in 26% of cases. The prognosis was altered in 38% of cases.

(D) Aid in research

The collection of large human tumour samples allows comprehensive molecular analysis and fingerprinting of each tumour and this may lead us to individually-tailored molecular therapies. Only through further understanding of the biology of gliomas can we hope to find a cure in the future.

There is therefore inconclusive evidence to support aggressive resection in prolonging survival in patients with high grade glioma. Most studies were insufficiently powered (or power of study not reported) to refute this issue once and for all.

With radical excision the reported outcome, at best, is a mean survival benefit of about 13 months[285]. In the elderly (>65 years old), this is further reduced to 3 months[299]. However such data suffer selection bias, as patients with expected poor outcome would not have undergone surgery. Even when survival benefit may be possible this is only seen with radical excision in excess of 98%[285].

As such we advocate the following guideline for glioblastoma resection[300]:

1. Tumour resection should be considered for histological confirmation, cytoreduction and to alleviate mass effect.
2. Aggressiveness of tumour resection is limited by the risk of incurring further new neurological deficit, in particular deficits which delays post operative radiotherapy and chemotherapy.
3. Adjuvant intraoperative procedures to facilitate safe tumour resection should be encouraged.

Surgical Adjuncts that Exist to Limit Surgical Morbidity

Because of the limited lifespan of high-grade glioma patients, it is crucial that surgical debulking does not compound any existing neurological deficit. Otherwise, any potential gain from the surgical resection would be offset by the morbidity. Many techniques have been developed to identify eloquent cortex, especially language, motor and sensory cortex. These adjuncts aid in defining the resection limit, and further debulking beyond this limit will likely increase the risk of surgical morbidity.

Functional MRI (fMRI) helps to identify language and motor centres. Mueller et al [301] compared the location of the fMRI activation with positive responses to intraoperative cortical stimulation and showed that in patients with more than 2 cm between the margin of the tumour and the activation, no decline in motor function occurred from surgical resection. fMRI of tactile, motor and language tasks is feasible in patients with tumours that are near the eloquent cortex, and shows promise as a means of determining postoperative motor deficit risk following surgical resection of frontal or parietal lobe tumours.

Intraoperative MRI potentially permits greater safety during aggressive resection of tumours by providing real-time images of residual tumour and the surrounding brain. It also leads to greater surgical accuracy by reducing neuronavigation errors due to intraoperative brain shift. In a study of 137 patients with WHO Grade III-IV gliomas, Nimsky et al[299] found that 66% of patients with Grade III tumours and 28% of patients with Grade IV tumours underwent extended resection with the guidance of intraoperative MRI, thereby increasing the percentage of complete resections by 15% in Grade III gliomas and by 12% in Grade IV gliomas. Unfortunately, this increase is only marginal because in many cases, the tumour extends into the eloquent brain areas and could not be excised safely.

The integrated application of functional navigation on top of intraoperative MRI resulted in a lower postoperative morbidity rate, e.g., a transient new neurological deficit of 10.2% and a permanent neurological deficit of 2.9%. Oh et al[302] went on to suggest that this may become the standard of care in due time owing to the fact that patients with less residual tumour may respond more favourably to adjuvant chemotherapy with temozolomide.

Awake craniotomy with cortical mapping can localise eloquent motor cortex more reliably than anatomical landmarks. Employing identification techniques developed by doing awake craniotomy in 65 patients at the Mayo Clinic, Meyer et al[303] found that resecting tumour until the onset of neurological deficits resulted in slightly more than half (52%) of the patients having a greater than 90% reduction in T2 signal postoperatively. At the same time, these techniques allow for good functional recovery. 94% of the 48 patients who developed intraoperative deficits achieved a modified Rankin grade of 2 or

less at 3-month follow up. Combining awake craniotomy with intraoperative cortical stimulation could reduce early neurological deterioration.

We performed a 3-year retrospective review of patients who underwent awake craniotomy under local anaesthesia at the National Neuroscience Institute, Singapore. All patients had tumours in close proximity to eloquent cortex, including speech areas in the dominant hemisphere as well as primary sensory and motor cortex in either hemisphere. Brain mapping was performed by direct cortical stimulation using the Ojemann stimulator to identify a safe corridor for surgical approach to the tumour. Intraoperative physiological monitoring was carried out with physiological monitoring of speech, motor and sensory functions during the process of surgical resection. All resections were evaluated and verified by postoperative imaging and reviewed by an independent assessor. Postoperative complications and neurological deficits, as well as extent of tumour resection, were evaluated.

A total of 20 patients underwent stereotactic resection over a period of 3 years from July 2003 to August 2006. There were 7 male patients and 13 female patients, with a mean age of 39.8 years. The average length of stay was 5.5 days. There were no major anaesthetic complications and no perioperative deaths. Postoperative neurological deficits were seen in 6 patients (30%) and this was permanent in only 1 patient (5%). The degree of cytoreduction achieved was greater than 90% in 58% of patients and a further 21% had greater than 80% cytoreduction[304].

Awake craniotomy is therefore a safe technique that allows maximal resection of lesions in close relationship to eloquent cortex and has a low risk of neurological deficit.

Based on the current literature, there is still a lack of evidence on whether surgical resection improves patient survival. However, there are benefits, albeit short-term ones, to be had from surgical resection, and these should be borne in mind. Technological advances in the form of intraoperative and functional MRI along with awake craniotomy techniques may be employed to reduce surgical morbidity and improve the extent of surgical resection. Ideally, a controlled randomized trial would best answer the perennial question of whether surgical resection improves patient outcome and survival, but these will not be possible because of inherent ethical concerns. Well-controlled retrospective studies with a multivariate analysis of all potential confounding factors can answer further questions but unfortunately will not provide Class I evidence.

Table 1: Systematic reviews of the extent of resection influencing outcome

Authors	Studies included in literature reviewed	Clinical question	Study descriptions	Conclusions
Nazzaro and Neuwelt, Quigley and Maroon	Prior to 1990 (all reported trials)	Extent of high grade glioma resection vs survival outcome	No RCT Lack of prospective observational data. Confounding factors not accounted for.	Flawed study designs. Little evidence to support hypothesis that aggressive surgical management significantly prolonged survival.
Hess	1991 to 1999 (all reported trials)		Retrospective data except for one prospective study. Only 4 adjusted for confounding factors.	No reliable clinical study. Little scientific evidence to support assertion that aggressive surgical resection prolongs survival
Grant	Up to 2006 (Randomised and clinically controlled trials only)	Effect of surgical resection vs biopsy on survival, time to progression or quality of life	1 RCT Inequalities among groups Underpowered Radiological misdiagnosis (30 randomised, only 23 with high grade gliomas)	Single small, underpowered study. Unable to conclude if one form of treatment (surgical excision or biopsy only) is superior to another.

RCT: randomised control trial

Table 2: Studies (1999 – 2006) excluded by the Cochrane review

Paper	Study type	Results reported
Lacroix 2001	Retrospective. N=416 44% had prior treatment elsewhere. Multivariate analyses	Improved survival associated with 98% or more total resection. (median survival 13 vs 8.8 months). Adjusted rate ratio 1.6 (95% CI 1.3-2, p<0.0001)
Buckner 2003	a. large cooperative trials: BTSG, NCCTG, RTSG Retrospective data, multivariate analysis including recursive partitioning analysis (RPA)	Survival advantage for patients underwent resection
Laws 2003	Retrospective outcome data of 788 patients over 4 years (1997-2001), multivariate analysis of resection vs biopsy	P<0.0001 Increased survival time even after eliminating 'poor' risk patients who may have been over-represented in biopsy group
Proescholdt 2003	120 articles up to 2003	No studies with high LOE (52.5% of studies had Level IIIb evidence which formed the majority). 72.5% of studies observed a positive effect of total resection but they contain

		methodological limitations which may significantly influence results.
Bucci 2004	Retrospective Pediatric population. MRI validated total resection (defined as >90% resection) Small group(n=39) Median follow-up 47.6 months	Median survival of patients with total resection vs residual disease, 122.2 vs 21.3 months (p<0.005)
Brown 2005	Phase II trial. No matched control cohort Gross total resection group had better initial quality of life assessment.	On multivariable analyses performed patients with gross total resection were less likely to be depressed and had improved quality of life at 2 month follow-up.
Schneider 2005	Prospective (n=31) Resection extent measured by post op MRI. Unadjusted for known prognostication factors	Median survival for complete vs incomplete resection, 537 vs 237 days (p=0.0037)
Stark 2005	Retrospective (n=267) Univariate analysis of survival time (Not adjusted) Resection extent measured by post op CT with contrast	Gross total resection associated with prolonged survival P=0.014

RCT – randomised control trial
LOE – level of evidence
EOR – extent of resection

BTSG – Brain Tumour Study Group

NCCTG – North Central Cancer Treatment Group

RTOG – Radiation Therapy Oncology Group

4.3 Higher glioblastoma tumour burden reduces efficacy of BCNU chemotherapy: in vitro evidence to support radical surgery for malignant gliomas

The in vitro experiments were designed to ascertain whether tumour burden can independently affect the efficacy of the standard chemotherapy agent BCNU.

On analysis of the experimental data, it was shown that significantly higher doses of BCNU are required to bring about tumour death in a setting of high tumour burden. Conversely, a smaller dose of BCNU is necessary to bring about tumour death in the presence of lower tumour burden.

For LN-229 cells seeded at 5,000 per well density, the drug concentration required to achieve greater than 90% growth inhibition (IC_{90}) was 75μg/mL. Cells seeded at densities of 10,000, 15,000 and 20,000 per well required BCNU concentrations of 100 μg/mL, 150 μg/mL and 175 μg/mL respectively.

For A172 cell line, the drug concentration required to achieve IC_{90} for cells seeded at 5,000, 10,000, 15,000 and 20,000 cells per well were 100 μg/mL, 125 μg/mL, 150 μg/mL and 175 μg/mL respectively.

For T98G cell line, the drug concentration required to achieve IC_{90} for cells seeded at 5,000, 10,000, 15,000 and 20,000 cells per well were 75 μg/mL, 100 μg/mL, 125 μg/mL and 150 μg/mL respectively.

The survival curves for the various cell lines demonstrate a consistent trend of larger chemotherapy doses required to achieve similar levels of growth inhibition for higher tumour loading in all the cell lines.

This vitro study has therefore shown increased chemotherapy resistance with higher glioblastoma load. A four fold increase in GBM tumour load requires a two fold

increase in BCNU concentration to achieve the same effect. Radical resection of high-grade gliomas may therefore facilitate adjuvant therapy by reducing oncologic load. This effect has also been in clinical studies. Stewart et al[297] performed a systematic review and meta-analysis of the effect of systemic chemotherapy on high-grade gliomas. They showed improved survival with a combined modality of surgical resection, radiotherapy and chemotherapy, as compared to surgery and radiotherapy. Surgical treatment included biopsy only, incomplete resection or complete resection. Subgroup analysis did not show evidence of a differential effect of chemotherapy on extent of resection; however, there was a trend towards improved survival in the patients who underwent complete and incomplete resection, compared to those in the biopsy group, although the improvement was not statistically significant.

Our in vitro experimental data therefore shows that increased tumour burden can reduce the efficacy of chemotherapy in human glioblastoma call lines. Radical surgery to reduce tumour burden can therefore potentiate adjuvant therapy. This lends further credence to support aggressive surgery for high-grade gliomas.

4.4 Growth Factors

The control of cell proliferation is highly regulated by growth factors. Growth factors are high affinity ligands for membrane-spanning cell surface receptors belonging to the family of receptors tyrosine kinase (RTK). Activation of the receptor results in complex multistep signal transduction pathway which includes ligand binding and receptor dimerisation, intermolecular phosphorylation of the intracellular domain on tyrosine residues, recruitment and activation of cytoplasmic signaling molecules that transmit

signals to the nucleus. In malignant transformed cells, the receptor tyrosine kinase is dysregulated and constitutively activated. Activation can occur by ligand overexpression, mutations within the RTK protein or overexpression of the receptor due to genetic aberrations such as translocation or gene amplification. Overexpression can result in RTK activation by increasing the concentration of the protein at the plasma membrane leading to ligand independent dimerisation[305].

Activation of growth factors or their receptors is evident in many human cancers. For instance, the ErbB family of receptors includes GFR, v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian) (ErbB2/HER2/Neu). ErbB3 and ErbB4 can be bound and activated by ligands. Their activation can contribute to the proliferative behaviour of a variety of cancers, including breast, ovarian, colorectal, small cell lung cancer and glioblastoma[306].

Many mitogens and their cognate receptors are present in overactive forms in gliomas. Examples of these mitogens are: PDGF, EGF and EGFR. Many of the ligands are over-expressed due to gene amplification and the receptors exist in constitutively active forms. The cognate receptors contain tyrosine kinase activity regulating several intracellular signaling cascades.

4.5 Cellular Signalling

The fundamental feature of all cancer cells is their ability to grow, survive and proliferate beyond a normal homeostatic environment. Recent advances in molecular research have shown complex interactions between cell surface receptors and intracellular signaling proteins. Multiple cellular pathways have been elucidated which promote cell

proliferation, growth and anti-apoptosis, ultimately contributing to the survival and mitogenesis of tumour cells. Growth factor receptors are membrane-spanning proteins characterised by innate tyrosine kinase activity. The receptor tyrosine kinases (RTKs) catalyse the transfer of the γ phosphate of ATP to hydroxyl groups of tyrosines on target proteins. Specific growth factors or ligands are over-expressed due to gene amplification and the receptors can exist in constitutively active mutant forms. Many growth factors have been found to be widely expressed in human glioma cell lines and glioma tissue and can stimulate mitogenic behaviour. These include vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), epidermal growth factor receptor (EGFR/c-erb/HER 1), insulin-like growth factor (IGF), transforming growth factor-beta (TGF-β), brain-derived growth factor (BDGF) and scatter factor/hepatocyte growth factor (SF/HGF)[173-174, 189-209].

Binding of growth factors induces conformational changes in the extracellular domain of the receptor and facilitates dimerisation or clustering of receptor tyrosine kinases. Dimeric ligands such as PDGF induce a symmetric ligand/receptor interaction whereas monomeric ligands such as EGF induce receptor dimerisation.

Ligand binding, receptor dimerisation and consequent conformational change in the growth factor receptor brings about union of two catalytic domains, resulting in autophosphorylation of tyrosine residues within the catalytic domain and non-catalytic regulatory regions of the cytoplasmic domain. Phosphorylation of key residue within the kinase activation loop induces the opening of the catalytic site and allows the ingress of ATP and substrates, while phosphorylated residues in non-catalytic regions create

docking sites for downstream signaling molecules that are essential for signal propagation[306].

In gliomas, the cognate receptors activate several intracellular signaling cascades: P13K/AKT-PTB (phosphoinositide 3-kinase/AKT-protein kinase B) pathway, RAS/MAPK (mitogen activated protein kinase) pathway and the PLC-γ/PKC (phospholipase C-γ/protein kinase C) pathway[209-210].

The MAPK regulate highly conserved signaling pathways in all eukaryotic cells. All MAPK pathways include a core three-tiered signaling unit, in which MAPKs are activated by the sequential activation of linked serine/threonine kinases.

Phosphoinositides are phospholipids of cell membranes that are dynamically regulated in response to growth factor signaling[307]. They contribute to signal propagation by serving as precursors of the second messengers IP3 and Diacylglycerol (DAG) or by binding to signaling proteins that contain specific phosphoinositide binding modules. Overall, the most important downstream signaling pathways involved are the P13K/Akt and RAS/MAPK pathways.

4.6 Paracrine and Autocrine Loops in Cancer

Tumour-stromal interactions can influence tumour differentiation and invasion in many tumours. In colorectal tumour cells, co-culture of Caco-2 or HT-29 cells in collagen gels resulted in the formation of a few small solid cell clusters with no lumina, but when co-cultured with stromal cells, the tumour cells formed glandular structures with central lumina. This fibroblast-induced differentiation of Caco-2 cells (nit HT-29 cells) appeared to be mediated via transforming growth factor-beta (TGF-β)[176]. A paracrine mechanism is

also seen in squamous cell carcinoma (SCC). Stromal changes with increased expression of proteases and cytokines may promote tumour proliferation. Myofibroblasts are commonly concentrated at the invasive margin of oral SCC. The tumour cells directly induce a myofibroblastic phenotype which is dependent on SCC-derived TGF-β1. In turn, myofibroblasts secrete significantly higher levels of hepatocyte growth factor/scatter factor compared with fibroblast controls which promotes SCC invasion[177]. Malignant melanoma cells are able to express various cytokines and growth factors at different stages of tumour progression, which can confer tumour competence via autocrine and paracrine effects[178].

4.7 Paracrine and Autocrine Loops in Gliomas

Likewise, the co-expression of growth factors with their corresponding receptors in gliomas may result in complex endogenous ligand-receptor interactions. The growth factor receptors expressed on the surface of tumour cells may bind soluble ligand produced by the same (autocrine) or adjacent cells (paracrine). In addition, membrane-anchored growth factor isoforms generated by alternative splicing may bind to the same (juxtacrine) or adjacent tumour cells (paracrine). Intracellular interactions between growth factors and their ligands can also lead to intracrine activation of signaling cascades[166, 179].

Many different growth factor/receptor systems have been implicated in the proliferative behaviour of gliomas. Vascular endothelial growth factor (VEGF) is considered one of the most potent angiogenic factors in gliomas. It is highly expressed in

malignant gliomas predominantly in areas of necrosis[180-185] and is thought to stimulate angiogenesis in a paracrine manner by binding to tyrosine kinase receptors on endothelial cells[183, 186-188]. The epidermal growth receptor (EGFR/c-erb1/HER1) is the mammalian homologue of the v-erbB oncogene from avian erythroblastosis virus[189]. Expression of EGFR has been shown in glioma cell lines and primary tumours with the degree of expression correlating with histological grade. EGFR expression has been linked to higher Ki-67 labelling indices and decreased survival[190-192]. In vitro experiments have demonstrated that expression of EGFR leads to increased proliferation and reduced apoptosis[193-195]. Co-expression of platelet-derived growth factor (PDGF) and their receptors is a common alteration in glioblastomas which can influence survival and proliferation of tumour cells. All four members of the PDGF family group PDGF-A, B, C and D have been shown to influence malignant behaviour. One of the most consistent cellular signaling defects observed in high-grade gliomas is the presence of an autocrine loop attributable to the coexpression of PDGF-A and PDGF-B and their receptors[196-200]. PDGF-C and PDGF-D expression is also seen in human glioblastoma cell lines and primary human tumour tissues. The use of CT52923, a potent inhibitor of PDGFR blocked PDGF autocrine-mediated phosphorylation of PDGFR, Akt, and mitogen-activated protein kinase (MAPK) and was also able to inhibit glioma growth in nude mice models[201].

Nerve growth factor is a member of the family of neurotrophins which are widely expressed in glioma tissue[202]. They bind with different affinity to receptors of the trk family and can stimulate glioma cell line proliferation in an autocrine fashion[203-204]. Several other putative growth factors have been found to be widely expressed in human

glioma cell lines and glioma tissue and can stimulate mitogenic behaviour. These include insulin-like growth factor (IGF)[205], transforming growth factor-beta (TGF-β)[206], brain-derived neurotrophic factor (BDNF)[207] and scatter factor/hepatocyte growth factor (SF/HGF)[208-209].

A highly complex system of ligand-receptor interactions are therefore present to stimulate tumour proliferation, promote invasiveness, inhibit apoptosis and confer cellular survival and chemoresistance.

4.8 Glial Cell Line-Derived Neurotrophic Factor (GDNF) Family

Glial cell line-derived neurotrophic factor (GDNF) was originally identified in 1993 by Lin et al as a neurotrophic factor[211]. It was isolated from a rat glioma cell line supernatant and was shown to confer increased survival for embryonic midbrain dopamine neurons. Subsequently, it was also found that GDNF also had potent trophic functions in spinal motorneurons and central noradrenergic neurons.

Due to its ability to promote neuronal survival, in the realm of neuroscience research, much of the research on GDNF has therefore been focused on its role as a potential therapeutic agent for neurodegenerative diseases such as Parkinson's Disease[212-215].

The GDNF-family ligands (GFL) consist of GDNF, neurturin (NRTN), artemin (ARTN) and persephin (PSPN). The GFLs support midbrain dopamine and motorneurons in the central nervous system. In addition, GDNF, NRTN and ARTN promote the survival and regulate the differentiation of many peripheral neurons such as the sympathetic, parasympathetic, sensory and enteric neurons[216-217].

GFLs belong to the transforming growth factor-beta (TGF-β) superfamily, containing seven cysteine residues with the same relative spacing as other members of the family[218]. They all belong to the cystine-knot protein family and function as homodimers.

GFLs are produced in the form of a precursor, preproGFL. The sequence is cleaved on secretion and activation of proGFL occurs by proteolytic cleavage. The secreted proneurotrophins may however be biologically active[216, 219-220].

GDNF is secreted as a mature protein of 134 amino acids. In its natural state, it exists as a glycosylated homodimer of approximately 39 kDa.

4.9 GDNF and Malignant Gliomas

Besides the nervous system, GDNF is produced in many other organ systems in the human body. There is an increasing trend that GDNF is implicated as a mitogenic agent for cancers in the various organ systems where it is found to be present.

GDNF and NRTN were found to be mitogens for normal adult rat chromaffin cells in vitro. The mitogenic behaviour is potentiated by binding and activation of protein kinase C (PKC). Although both ligands contributed to increased mitogenesis, NRTN was the more potent mitogen and caused increased phosphorylation of extracellular signal-regulated kinases 1 and 2 in cultured chromaffin cells[248].

In pancreatic cancer, RET overexpression was seen in pancreatic tumour tissue. GDNF and ARTN were strongly expressed in all intrapancreatic nerves and intrapancreatic neural invasion was significantly related to the expression of GDNF. In vitro invasion assays, the migration of pancreatic cancer cells are markedly induced by

co-culture with human glioma cells capable of secreting GDNF. Immunohistochemical assessment of GDNF, NRTN, PSPN, ARTN, GDNF family receptor alpha-1 and alpha-2 and RET in 51 cases of surgically resected pancreatic cancer demonstrated that GDNF and ARTN were expressed strongly in all intrapancreatic nerves. In pancreatic cancer tissues, the expression of RET was stronger than that seen in normal ductal cells and was significantly related to the survival rate after resection and lymphatic invasion[249-250]. Intrapancreatic neural invasion was also significantly related to the expression of GDNF The expression of RET in pancreatic cancer tissues may therefore be a useful prognostic marker and GDNF may play an important role in neural invasion. RET expression in pancreatic cancer cells may also be a potential target for anti-invasion therapy. Treatment of pancreatic carcinoma cell lines with GDNF resulted in activation of the monomeric GTPases N-Ras, Rac1, and RhoA, in activation of the mitogen-activated protein kinases extracellular signal-regulated kinase (ERK) and c-Jun NH(2)-terminal kinase (JNK) and in activation of the phosphatidylinositol 3-kinase/Akt pathway. Both inhibition of the Ras-Raf-MEK (mitogen-activated protein/ERK kinase)-ERK cascade by either stable expression of dominant-negative H-Ras(N17) or addition of the MEK1 inhibitor PD98059 as well as inhibition of the phosphatidylinositol 3-kinase pathway by LY294002 prevented GDNF-induced migration and invasion of pancreatic carcinoma cells. These results demonstrate that pancreatic tumor cell migration and possibly perineural invasion in response to GDNF is critically controlled by activation of the Ras-Raf-MEK-ERK and the phosphatidylinositol 3-kinase pathway. (Activation of phosphatidylinositol 3-kinase and extracellular signal-regulated kinase is required for

glial cell line-derived neurotrophic factor-induced migration and invasion of pancreatic carcinoma cells[308].

Perineural invasion is an important prognostic factor for patients with bile duct carcinoma and is not surprising that GDNF can play a role in the pathogenesis of bile duct cancer. Using immunohistochemistry, moderate to strong staining of GDNF in tumour cells was observed more frequently in sections with perineural invasion. Cell migration was also seen to be enhanced by conditioned media from GDNF-treated cells[251].

GDNF can promote proliferation of neuroblastoma cells and can have a role in promoting resistance to differentiation or cytotoxic therapy of neuroblastoma. (Glial cell line-derived neurotrophic factor (GDNF) family ligands reduce the sensitivity of neuroblastoma cells to pharmacologically induced cell death, growth arrest and differentiation[309].

Immunohistochemistry studies in vestibular schwannoma have shown that co-expression of transforming growth factor-beta 1 (TGF-β1) and GDNF in vestibular schwannoma may demonstrate trophic synergism in this tumour.

GDNF is produced by astrocytes and is ubiquitous in the central nervous system and neural tissue and hence can potentially play an important role in the pathogenesis of high-grade glioma.

GDNF and its receptor GFRα1 have been demonstrated to be strongly expressed in human gliomas by Wiesenhofer et al. In an analysis of 20 human astrocytomas (14 glioblastomas, 1 gliosarcoma and 5 astrocytomas), GDNF protein concentrations were found to be present in high amounts compared to postmortem human frontal lobe and rat

cortex. Immunohistochemistry of GFRα1 demonstrated strongly positive staining in 5 out of 15 high-grade glioma samples, weakly positive staining in 4 specimens and no staining in 6 specimens[253].

In murine experiments, GDNF is found to be highly expressed in rat glioma cell lines B49 and C6. Furthermore, knockdown experiments with antisense oligonucleotides performed with rat C6 glioma cells demonstrated that knockdown of C6 cells with GDNF and GFRα1 significantly reduced the number of C6 glioma cells and also inhibited the incorporation of bromodeoxyuridine as a sign of DNA synthesis[254].

In our study, the use of Real-Time PCR to quantify the level of expression of GDNF demonstrated that GDNF was more highly expressed in glioma samples and glioblastoma cell lines than in adult brain, foetal brain, adult liver and foetal liver samples. All glioblastoma samples and cell lines demonstrated increased level of expression. The highest expression level was observed in a sample of glioblastoma tissue. Other gliomas studied (oligodendroglioma, PXA, recurrent PXA, anaplastic ependymoma and pilocytic astrocytoma) also demonstrated increased levels of expression of GDNF. The pilocytic astrocytoma and PXA had the lowest level of GDNF expression of all the glioma samples studied. Pilocytic astrocytoma is a benign tumour classified as WHO Grade I tumour. PXA is a low-grade glial tumour (WHO Grade II). Although, it is tempting to postulate that GDNF expression may be correlated to the WHO Grading of the tumour, this theory is not tenable at this stage as the oligodendroglioma (WHO Grade II) sample demonstrated very high level of GDNF expression. Obviously, a much larger sample size to compare the difference in expression of GDNF between the low-grade and high-grade gliomas may be able to ascertain a

statistically significant difference. In practical terms, we are limited by the small numbers of low grade gliomas in our population and would therefore not have sufficient clinical numbers to test this hypothesis.

Significantly, in vitro experiments comparing the level of GDNF expression in high-grade C6 glioma cells and low-grade Hs683 cells revealed that the high-grade cells secrete more GDNF than the low-grade cells. GDNF signaling is also more highly activated in C6 cells than in Hs683 cells. Treatment of the Hs683 cells with GDNF significantly increased migration comparable to C6 cells[311].

It is noteworthy that the case of recurrent PXA showed the second highest level of expression of GDNF. PXA is a rare primary astrocytic tumour of the nervous system usually involving the superficial temporal cortex of children and young adults. Although the tumour may exhibit histological features of pleomorphism or cellular atypia, the overall prognosis is good compared with other glial tumours, and only 30% of PXA recur and 15-20% undergo anaplastic transformation[312-317]. It would be interesting to study the varying levels of GNDF expression to ascertain whether increased level of GDNF expression is correlated with recurrence of PXA, more aggressive behaviour or malignant transformation.

4.10 Splice Isoforms/Variants

Alternative splicing is responsible for much of the protein diversity in humans. Approximately 60% of human genes express multiple mRNAs and approximately 80% of these alternative splicing events lead to variation in the encoded protein[255].

RET pre-mRNA alternative splicing leads to the production of two isoforms: RET 9 and RET 51, which possess different biochemical and biological properties. Alternatively spliced variants NCAM also exist and have been demonstrated to subserve distinct biochemical and physiological functions.

Splice isoforms are also abundant in the GDNF-family receptor-α (GFRα). Several variants have been observed for GFRα1, GFRα2 and GFRα4 pre-mRNAs. GFRα1 receptor exists in two highly homologous alternatively spliced isoforms: GFRα1a and GFRα1b[262-264].

GFRα1b is identical to GFRα1a except for the absence of 5 amino acids (140DVFQQ144), encoded by exon 5. In addition, GFRα2 and GFRα4 receptor splice isoforms have also been identified in mammalian tissue. Three variants of GFRα2 receptors (GFRα2a/2b/2c) have been identified[265-266]. At least two splice variants of GFRα4 have been identified in rat tissue[267-269]. The presence of multiple spliced isoforms increases the permutational possibility and complexity of ligand-receptor interactions and can potentially result in diverse biological and physiological processes.

Quantification of expression levels of GFRα1a and GFRα1b in murine brain, heart, ileum, kidney, liver and testis showed that both spliced isoforms were expressed at fairly equivalent levels except in the brain, where GFRα1a is the predominant isoform. Expression levels of RET9 were significantly higher than that of RET51 in murine brain, heart, ileum and testis. All three NCAM spliced variants (NCAM120, 140 and 180) were highly expressed in the murine brain but at low levels in most peripheral tissues[275].

Neuro-2a cell lines expressing either GFRα1 isoforms demonstrated that GDNF and NRTN bind to GFRα1b isoform more efficiently than to the GFRα1a isoform.

Investigation of the capacity of each GFRα1 isoform to activate the RET receptor showed that at low ligand concentrations GFRα1b mediated RET phosphorylation to a larger extent than GFRα1a. At ligand saturation, RET activation was maximal and no difference between the GFRα1 isoforms was observed[318].

Using transfected Neuro2A which expressed both NCAM and RET endogenously as a model, Yoong et al showed that when stimulated with either GDNF or NRTN, both GFRα1a and GFRα1b induced the phosphorylation of ERK1/2 potently. Microarray analyses of GFRα1 isoforms transfected cells stimulated with NRTN however showed distinct and non-overlapping gene profiles, providing evidence that the spliced GFRα1 isoforms may have different functions[275].

Measurement of the expression of miRNA precursors in human BE(2)-C cells that express GFRα2 but not GFRα1 with quantitative real-time PCR demonstrated that GDNF and NTN differentially regulate the expression of distinct micro-RNA (miRNA) precursors through the activation of mitogen-activated protein kinase. The expression of distinct miRNA precursors is can therefore also differentially regulated by specific ligands through the activation of GFRα2.

4.11 Splice Variants in Gliomas

Real-Time PCR studying the expression levels of RET showed a consistent trend that RET9 is the predominant spliced isoform in all human glioma samples and glioblastoma cell lines. RET9 was also the predominant spliced isoform found in brain and liver samples.

The glioblastoma cell lines had significantly lower levels of expression of GFRα1a compared to human adult and foetal brain samples. 11 out of the 13 human glioma samples had decreased levels of expression of GFRα1a compared to human adult and foetal brain samples. 2 out of the 8 glioblastoma samples had elevated levels of GFRα1a expression.

The 2 glioblastoma cell lines had increased expression of GFRα1b compared to human adult and foetal brain samples. 5 glioma samples had elevated levels of expression of GFRα1b compared to human adult and foetal brain samples. These were all human glioblastoma samples.

On close analysis of the expression levels of GFRα1a and GFRα1b levels, an interesting observation was noted. The glioblastoma cell lines demonstrated much higher levels of GFRα1b expression than GFRα1a expression. For cell line LN-229, the ratio of GFRα1b/GFRα1a was 16.3 and the ratio of GFRα1b/GFRα1a was 14.3 for cell line A172. A similar trend was also noted in 7 out of the 8 human glioblastoma samples. The GFRα1b/GFRα1a ranged from 1.73 to 5.44 in the 7 specimens. Only one human glioblastoma specimen had a higher GFRα1a/GFRα1b ratio

The GFRα1b/GFRα1a ratio for the pilocytic astrocytoma and PXA are 3.88 and 4.20 respectively. The oligodendroglioma sample had higher level of expression of GFRα1a with a GFRα1a/GFRα1b ratio of 5.99. GFRα1b expression was undetectable for the recurrent PXA specimen.

GFRα1b is therefore the predominant spliced isoform in human glioblastoma samples and human glioblastoma cell lines. This is especially significant in the setting that GFRα1a is the usual predominant spliced isoform in the brain.

GFRα2 is alternatively spliced into at least three isoforms (GFRα2a, GFRα2b and GFRα2c). Yoong et al hypothesized that the spliced isoforms may have differing functional properties. When transfected Neuro2A cells were stimulated with GDNF and NRTN, both the GFRα2a and GFRα2c transfected cell lines, but not the GFRα2b transfected cell line, promoted neurite outgrowth. In addition, the GFRα2 isoforms were shown to regulate different early-response genes when stimulated with GDNF or NRTN. In coexpression studies, GFRα2b transfected cell line was found to inhibit ligand-induced neurite outgrowth by GFRα2a and GFRα2c. Stimulation of GFRα2b also inhibited the neurite outgrowth induced by GFRα1a. Furthermore, activation of GFRα2b inhibited neurite outgrowth induced by retinoic acid and activated RhoA[319].

This shift in spliced isoform expression in glioblastoma suggests that the GFRα1 spliced isoforms may subserve different functions and this altered state of expression may contribute to the mitogenic behaviour of the tumour.

4.12 Potentiation of Chemoresistance

The mainstay of therapy for malignant gliomas is a combination of surgery, radiation therapy and chemotherapy. The effectiveness of standard chemotherapy regimens such as procarbazine, lomustine (CCNU), vincristine and 1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU) are limited by their toxicities and inability to penetrate the blood-brain-barrier (BBB). The BBB consists of endothelium with tight junctions, vascular cells and astrocytic foot processes and it is estimated that 98% of all known small molecules are unable to breach the BBB. For a chemotherapy agent to cross the BBB in significant

concentration, the molecular size must be less than 400-500 Da and the drug must be highly lipid-soluble.

It is now also known that human cancer populations are not homogeneous. Cancer stem cells have an intrinsic capacity for unlimited self-renewal and possess the ability to initiate and drive tumour progression in an animal model[320]. Thus, they would seem the most probable candidates responsible for tumour chemoresistance and recurrence.

Hirschmann-Jax et al have shown that the "side population" (SP) of neuroblastoma cells not only had the characteristics of tumour stem cells (multipotentiality and self-renewal), but were also more resistant to the effects of drugs such as mitoxantrone, and may contribute to the overall drug resistance phenotype of relapsed or resistant cancers[321-322]. Neuroblastoma cells cultured in the presence of mitoxantrone showed a progressive increase in the frequency of the SP fraction, indicating that their ability to expel mitoxantrone offered a survival advantage to these putative stem cells. Sorted SP cells, unlike non-SP cells, were also able to proliferate and establish new colonies in the presence of mitoxantrone, whereas non-SP cells could not, demonstrating stem-cell-like properties. Taken together, the data confirm the link between SP and drug resistance, disease persistence and relapse. A study by Liu and colleagues demonstrated an increased resistance of $CD133^+$ brain tumour stem cells in response to treatment with chemotherapeutic agents such as temozolomide, carboplatin, paclitaxel (Taxol) and etoposide (VP16) as compared to autologous $CD133^-$ cells[323]. Gene expression studies revealed a higher expression of multidrug resistance gene BCRP1, and DNA-mismatch repair genes such as O^6-methylguanine–DNA methyltransferase (MGMT), as well as

genes that inhibited apoptosis in the CD133-expressing cancer stem cells. Furthermore, the work showed that CD133 gene expression was significantly higher in recurrent GBM tissue specimens as compared to their respective newly diagnosed tumours. Clinically, it is observed that tumours respond to chemotherapies only to recur with renewed resilience and aggression. Although chemotherapy kills most of the cells in a tumour, these results suggest that cancer stem cells may be left behind, which then recur due to their enhanced chemoresistance.

Growth factors can also potentiate chemoresistance. Brain-Derived Neurotropic Factor (BDNF) and TrkB are expressed in many poor-prognosis neuroblastoma tumours. Activation of the BDNF-TrkB signal transduction pathway was investigated in two neuroblastoma cell lines, 15N and SY5Y. 15N cells lack the high-affinity receptor p145TrkB and express BDNF; 15N cells were used along with 15N-TrkB cells, a sub-line transfected with a TrkB expression vector. In cytotoxicity assays, 15N-TrkB cells were consistently 1.4-2 fold more resistant to vinblastine than 15N cells. BDNF can therefore confer chemoresistance to vinblastine treatment[324].

In oesophageal cancer, insulin-like growth factor-I (IGF-I) prevented the apoptosis of CE81T/VGH oesophageal carcinoma cell lines induced by chemotherapeutic drugs, such as cisplatin, 5-fluorouracil and camptothecin[325].

Treatment of neuroblastoma cell lines BE(2)-C and SY5Ywith GDNF or NRTN caused cells to grow at a more rapid rate compared to untreated cells. GDNF and NRTN also overcame the growth inhibitory effects of all-*trans*-retinoic acid (aRA) on BE(2)-C cells respectively, which expressed both RET and GFRα1. Furthermore, cytotoxicity assays showed that BE(2)-C cells treated with cytotoxic agent and GDNF demonstrated

enhanced cell growth at low concentrations of cisplatin, etoposide and vincristine. This ability of GDNF and NRTN to promote chemoresistance raised the possibility of a similar phenomenon in glioblastoma[326].

When the LN-229 cells were treated with BCNU at 40μg/ml concentration, the cellular survival on MTS assay were 31.7%, 41.0% and 52.1% for the group not pre-treated with GDNF. For the group pre-treated with GDNF, the cellular survival on MTS assay were 71.4%, 77.9% and 79.1% respectively. This difference was statistically significant ($P<0.05$). The mean cellular survival on MTS assay in the group not pre-treated with GDNF was 41.6% and 75.8% in the group pre-treated with GDNF. This difference was statistically significant ($p<0.05$).

A similar trend was also noticed when the LN-229 cells were treated with BCNU at 50μg/ml concentration. The cellular survival on MTS assay were 14.0%, 30.6% and 35.4% for the group not pre-treated with GDNF. For the group pre-treated with GDNF, the cellular survival on MTS assay were 50.8%, 56.2% and 57.2% respectively. This difference was statistically significant ($P<0.05$). The mean cellular survival on MTS assay in the group not pre-treated with GDNF was 26.7% and 54.7% in the group pre-treated with GDNF. This difference was statistically significant ($p<0.05$).

The same experimental protocol was replicated for glioblastoma cell line A172. In this cell line, GDNF also demonstrated very significant cellular survival in the presence of BCNU chemotherapy.

When the A172 cells were treated with BCNU at 75μg/ml concentration, the cellular survival on MTS assay were 30.9%, 26.0% and 31.4% for the group not pre-treated with GDNF. For the group pre-treated with GDNF, the cellular survival on MTS

assay were 61.0%, 58.3% and 68.0% respectively. This difference was statistically significant ($P<0.05$). The mean cellular survival on MTS assay in the group not pre-treated with GDNF was 29.4% and 62.4% in the group pre-treated with GDNF. This difference was statistically significant ($p<0.05$). This ability to confer increased survival can be directly attributed to the chemoresistance property of GDNF as GDNF did not exhibit significant direct proliferative advantage in the control experiments.

Significantly, when the exact experiments were repeated with NRTN pre-treatment instead of GDNF pre-treatment, no significant survival advantage was detected. The results therefore demonstrate GDNF but not NRTN can confer cellular survival advantage to BCNU chemotherapy. This ability to confer chemoresistance is demonstrated in two different human glioblastoma cell lines: LN-229 and A172.

The GDNF-family ligands (GFL) can therefore confer chemoresistance to BCNU chemotherapy in glioblastoma in a ligand-specific fashion.

Lee et al investigated the differing effects of GDNF and NRTN in RET/GFRα1-expressing cells in a specific cell line NG108-15, which endogenously expressed RET and GFRα1 but not GFRα2-4. Immunoblot data showed that GDNF caused a transient activation whereas NRTN caused a sustained activation of both p44/p42 MAP kinases and PLCγ. Under serum starvation, NG108-15 cells differentiate to form euritis. NRTN but not GDNF stimulated neurite overgrowth, which could be blocked by the selective PLC inhibitor U73122. On the other hand, GDNF but not NRTN promoted cell survival and this could not be blocked by the p44/p42 MAK kinase inhibitor PD98059. Activation of GFRα1 with different ligand can therefore result in differing biological responses[327].

4.13 Signalling Mapping

Combined modality treatment such as concomitant radiation and chemotherapy is frequently used in the treatment of cancers to improve overall efficacy of treatment. The combination of BCNU chemotherapy and radiation therapy in glioblastoma has however failed to produce any additive or synergistic effects observed in other tumour types. In vitro experiments have shown actual antagonistic effect between radiation and chemotherapy in three primary human glioblastoma cell lines (GBME3-5). Irradiation of the three cell lines from 2 to 10Gy followed by BCNU chemotherapy ranging from 10 to 50μM in the presence of exogenous epidermal growth factor (EGF). Compared with cells treated with BCNU alone at virtually each BCNU concentration, the apoptotic index was significantly lower in the cells pre-treated by radiation compared with BCNU alone. Reversal of the sequential order by administering chemotherapy prior to radiation therapy showed similar findings demonstrating that this antagonistic effect between radiation and BCNU was present irrespective of the sequential order of administration. Elucidating the downstream pathways mediating this phenomenon showed that MAPK and PI3-K/Akt activities were up-regulated after either radiation or chemotherapy. Further, BCNU-mediated stimulation of PI3-K/Akt via EGFR appears to most significantly increase resistance to radiation-induced apoptosis[328].

GDNF has been found to influence the migration and mitogenic behaviour of low-grade gliomas. Treatment of low-grade Hs683 cells with GDNF significantly increased migration comparable to high-grade C6 cells. The molecular mechanism is mediated by the activation of JNK-1, ERK 1/2 and p38 MAPK. Treatment of Hs683 cells with 60ng/ml of GDNF markedly activated JNK. A kinetic study of GDNF-induced JNK

activation showed that JNK was markedly activated within 30 min after GDNF treatment and returned to the basal level at 90 min. ERK 1/2 were activated at 10 min after GDNF treatment and the activated levels remained until 60 min. GDNF markedly increased the active form of p38 MAPK within 10 min, maximally activated at 30 min and decreased at 60 min after the treatment[311].

In the light of the evidence, we examined the modulation of MAPK and Akt signaling pathways in glioblastoma cell lines. LN-229 and A172 human glioblastoma cell lines were stimulated with BCNU and GNDF and the experiments were studied at 0, 10, 30, 60 and 180 mins respectively.

Western blotting showed that BCNU induces activation of MAP kinases (ERK1/2, JNK and p38) in both LN-229 and A172 human glioblastoma cell lines. BCNU was however found to reduce the background activation of Akt in the A172 human glioblastoma cell line.

GDNF was found to induce the activation of ERK1/2 and Akt in both LN-229 and A172 human glioblastoma cell lines. LN-229 cell line has a wild-type phosphatase with tensin homology (PTEN) gene. PTEN is a phosphatidylinositols(3,4,5)P_3 (PIP_3) phosphatase and negatively regulates the PI3-K/Akt pathway by converting PIP_3 back to phosphatidylinositols(4,5)P_2 (PIP_2)[332]. GDNF is therefore able to activate Akt even in the presence of PTEN gene.

GDNF was however found to reduce the background activation of JNK and the A172 human glioblastoma cell line in a time-dependent fashion.

The ability of GDNF to promote Akt activity and inhibit JNK activity may contribute to the increased cellular survival to BCNU chemotherapy. The interplay of the

signaling pathways activated by BCNU and GDNF which result in cellular survival or death is summarised in Figure 21.

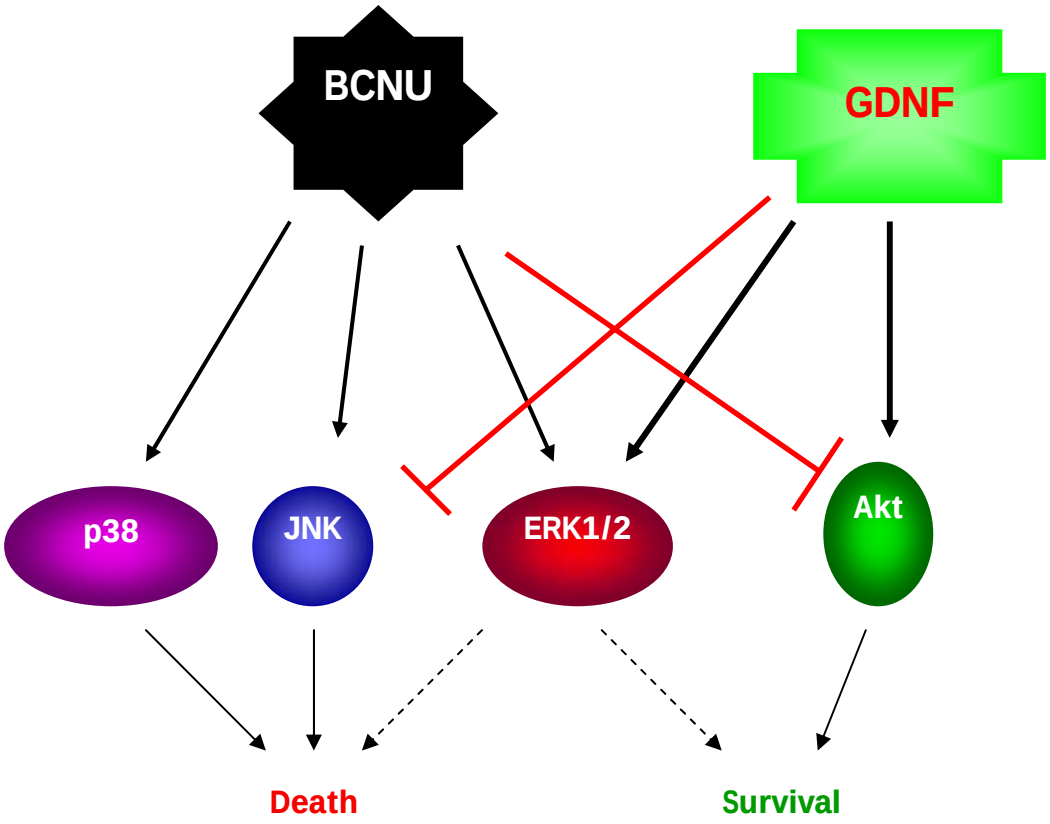


Figure 21: Diagram summarising the interplay between BCNU and GDNF stimulation pathways influencing survival and death pathways in GBM cell lines

CHAPTER 5

FUTURE STUDIES

GDNF was demonstrated in the above studies that it can promote mitogenic behaviour of glioblastoma. This is promoted by its ability to confer chemoresistance property to glioblastoma cells in the presence of conventional chemotherapy agent (BCNU).

This finding has significant clinical implications as GDNF is ubiquitous in the central nervous system and glial tumours are therefore constantly exposed to and stimulated by GDNF. Significantly, the studies showed that only GDNF, but not NRTN, was able to confer chemoresistance.

GDNF binds to specific GDNF-family receptor-α1 (GFRα1) co-receptor and activate RET. The GFRα1 receptor is linked to the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor.

Cancer cells often acquire aberrant profiles of alternative spliced isoforms that can promote cell proliferation and invasion and inhibit apoptotic cell death. Spliced isoforms are also abundant in the GDNF-family receptor-α (GFRα). GFRα1 receptor exists in two highly homologous alternatively spliced isoforms: GFRα1a and GFRα1b[262-264]. GFRα1b is identical to GFRα1a except for the absence of 5 amino acids (140DVFQQ144), encoded by exon 5. GFRα1b was found to be the predominant spliced isoform in the human glioblastoma samples and human glioblastoma cell lines in the study.

Similar to GFRα1, GFRα2 is alternatively spliced into at least three isoforms (GFRα2a, GFRα2b and GFRα2c). When transfected Neuro2A cells were stimulated with GDNF and NRTN, both the GFRα2a and GFRα2c transfected cell lines, but not the

GFRα2b transfected cell line, promoted neurite outgrowth. GFRα2 isoforms were also demonstrated to regulate different early-response genes when stimulated with GDNF or NRTN. Furthermore, in coexpression studies, GFRα2b transfected cell line was found to inhibit ligand-induced neurite outgrowth by GFRα2a and GFRα2c[319]. All these data taken together suggests that GFRα spliced isoforms regulate distinct biological functions. As GFRα1b is the predominant spliced isoform in glioblastoma cell lines and human samples and GDNF has been shown to confer chemoresistance in glioblastoma cells, it would be fascinating to examine the role of the spliced isoforms in conferring mitogenic behaviour.

Post-transcriptional gene silencing (PTGS), which was initially a bizarre phenomenon thought to be limited to petunias and a few other plant species has now become one of the most important molecular techniques in biology. Most importantly, the emerging use of PTGS, particularly RNA interference (RNAi) as a tool to knockout or knockdown expression of specific genes can facilitate the study of the specific gene[329-331]. Future studies with RNAi to knockdown GFRα1a and GFRα1b specifically can potentially provide us with information on the precise roles of each spliced isoform with respect to mitogenesis. Investigation of the downstream signaling pathways with knockdown experiments will also provide important details on the differing signaling pathways involved.

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APPENDICES

Appendix 1

CONSENT FOR USE OF BRAIN TUMOUR TISSUE FOR RESEARCH

Background of project

Astrocytomas are the most common primary brain tumours. They are classified into two broad groups: low-grade astrocytomas and high-grade astrocytomas.

The biological behaviour can range markedly. Generally, low-grade astrocytomas are tumours that exhibit slow, progressive growth whereas high-grade astrocytomas tend to grow more rapidly.

Many factors can influence this growth rate. One such factor is the influence of growth factors. There are numerous growth factors which have been shown to influence the behaviour of these tumours. Glial Cell-Line Derived Neurotrophic Factor (GDNF) is an important growth factor which has not been studied extensively.

GDNF is produced in normal brain tissues. We believe that it is produced in excessive quantities in patients with astrocytomas and that the excessive levels of GDNF stimulate tumour growth.

We hope that understanding the role of GDNF on tumour growth will allow us to understand tumour biology better and ultimately be able to develop new treatment strategies for the future.

How can you help?

At the time of surgery, tumour tissue is removed. Some tissue is collected for histology and at times microbiological tests if indicated. These tests allow us to make an accurate diagnosis. Excess tissue is discarded. We hope to be able to use this tissue for our research. Only some of this tissue will be used for this research project and extra tissue will be stored in freezers. Should we require the tissue for future research projects, we will obtain consent from you again.

The research data will be coded and your identity will not be revealed and your confidentiality maintained at all times.

You can be assured that your care is the top priority. In situations where only small amounts of tissue are obtained, they will be sent for the relevant tests and not for research. We will not compromise on your care.

What if I choose not to participate in the research project?

Participation in this project is totally voluntary. Non-participation will not influence your care in any way and you will receive the same level of care as a patient who chooses to participate in the project.

Who can I contact for more information?

You can contact the following persons if you have any queries:

Dr Ng Wai Hoe (Principle Investigator)

Dr Yeo Tseng Tsai (Collaborator)

Ms Emily Ang (Neuro-oncology Nurse Clinician)

Contact Number: 63577191

I have read and understood the information regarding consent for the use of brain tumour tissue for research. I understand that:

1. Participation in this research project is totally voluntary
2. Only excess brain tumour tissue that will normally be discarded will be used for research
3. My clinical care is the first priority and non-participation will not influence my care in any way

I give consent for the use of any excess brain tumour tissue for research.

_____ _____

(Signature) (Name/NRIC)

I have explained the background and purpose of the research project to the patient and answered all queries.

_____ _____

(Signature) (Name/Designation)