THE UNIVERSITY OF HULL

Analysis of markers of cell division cycle, apoptosis and autophagy flux in Glioblastoma

being a thesis submitted for the

degree of

DOCTOR OF MEDICINE

in the

UNIVERSITY OF HULL

by

Jawad Yousaf
M.B.,B.S. (King Edward Medical College, Pakistan),
M.R.C.S. (Royal College of Surgeons of England)

September 2012

Abstract

Glioblastomas are the most aggressive and most common type of primary brain neoplasms and are associated with poor prognosis despite advances in surgical and oncological treatments. Currently available treatments include surgical resection, fractionated external beam radiotherapy and chemotherapy. This study aimed to investigate markers of cell division cycle, apoptosis and autophagy flux in an attempt to identify biomarkers with prognostic and/or predictive significance.

The cell cycle markers studied included: Mcm2, expressed throughout the cell cycle; Cyclin A, an S-phase cyclin; Geminin, a protein that prevents re-initiation; and Phosphohistone H3 (PHH3), a marker of mitosis. Apoptotic markers included two anti-apoptotic proteins, Bcl-2 and Bcl-xl; a pro-apoptotic protein, Bak; and a final executioner caspase, caspase 3. Markers of autophagy flux included LC3B, a ubiquitin like protein that form part of the core autophagy machinery; and p62, a mammalian autophagy receptor that binds ubiquitinated proteins.

A total of 66 patients were recruited to the study between 2007 and 2009. Data were collected on patient demographics, pre-operative Karnofsky score, surgical and adjuvant treatment and survival. A tissue micro-array, constructed using glioblastoma tissue was immunohistochemically-stained using antibodies against a panel of markers against the molecules described above. A semiquantitative labelling index (LI) was calculated for cell cycle and apoptotic markers using an average of 18 high power fields (hpf) in three replicate cores. Staining scores were calculated for markers of autophagy flux on the basis of cytoplasmic staining intensity (1-3) and percentage of cells with nuclear staining (1<50%, 2>50%).

Cell cycle marker LI were calculated from a cohort of 66 patients, who were further subdivided into two groups: Group 1 (n=50) underwent surgery and radiotherapy with 24 patients receiving temozolomide; and Group 2 (n=16) received surgical treatment only. In group 1, a LI, higher than the median value for Geminin and Cyclin A correlated with prolonged survival when tumours received adjuvant treatment (Kaplan Meier test, p=0.0046 and p =0.0063 respectively). In group 1, Mcm2 and PHH3 LI did not correlate significantly with survival. There was no relationship between patient survival and LI for any marker in group 2. A reduction in the LI of Mcm2, Geminin and Cyclin A was observed following administration of adjuvant treatment in three patients with recurrent glioblastoma.

Apoptotic marker LI were calculated in 28 patients, due to limited tissue availability; values below the median for Bak expression conferred a survival advantage in these patients by Kaplan Meier analysis (p = 0.0039).

LC3b and p62 staining scores were calculated in 45 patients and correlated significantly with each other. Whilst no significant correlation was observed between LC3b staining score and patient survival, p62 staining above the median conferred a survival disadvantage (Kaplan Meier analysis, p =0.017).

Geminin and Cyclin A, each showed potential as independent prognostic markers in glioblastomas receiving adjuvant treatment. This may reflect the fact that geminin and cyclin A both estimate proliferating cell sub-populations sensitive to radiotherapy/chemotherapy. The addition of these markers could therefore contribute valuable prognostic information if added to the glioblastoma diagnostic panel. The association of high Bak expression with survival advantage suggests a possible, as yet unknown, role of this pro-apoptotic protein in glioblastoma oncogenesis. The association of high p62 expression with decreased survival confirms the important role of autophagy flux in glioblastoma resistance to treatment and suggests a target for future research and targeted therapy.

CONTENTS

FIGURES

TABLES

PUBLICATIONS

Yousaf J, Hill C, Dixit S, Achawal S, O'Brien D, Greenman J and Scott IS. 2012. Markers of cell division cycle in Glioblastoma: significance in prediction of treatment response and patient prognosis. British Journal of Neurosurgery, *in press*.

PRIZES

British Journal of Neurosurgery Prize for best scientific paper, *Markers of cell division cycle: prognostic implications in Glioblastoma*, presented at the Society of British Neurological Surgeons winter meeting at Nottingham, september 2008.

ABSTRACTS PUBLISHED

1) Yousaf J, Hills C, O'Brien D, Greenman J, Scott IS. 2010. Immunohistochemical analysis of autophagy flux in glioblastoma. Neuropathol & Applied Neurobiol. 36: S1:49.
2) Yousaf J, Hills C, Achawal S, Greenman J, O'Brien D, Scott IS. 2009. Immunohistochemical analysis of markers of cell division cycle: Prognostic implications in Glioblastoma. Neuropathol & Applied Neurobiol. 35: S1:34-35.
3) Yousaf J, Hills C, O'Brien D, Greenman J, Scott IS. 2008. Markers of cell division cycle: prognostic implications in Glioblastoma. British Journal of Neurosurgery. 22(4):465.

PRESENTATIONS

1) Yousaf J, Hills C, O'Brien D, Greenman J, Scott IS. Immunohistochemical analysis of autophagy flux in glioblastoma. Poster presentation at British Neuropathology Society meeting January 2010.
2) Yousaf J, Hills C, Achawal S, Greenman J, O'Brien D, Scott IS. Immunohistochemical analysis of markers of cell division cycle: Prognostic implications in Glioblastoma. Poster presentation at British Neuropathology Society meeting January 2009.
3) Yousaf J, Hills C, O'Brien D, Greenman J, Scott IS. Markers of cell division cycle: prognostic implications in Glioblastoma. Oral presentation at Society of British Neurological Surgeons winter meeting September 2008.

Chapter 1

Introduction

Glioblastoma: Background and challenges - Cell division cycle - Cell cycle: phases - Cyclins, CDKs and CDKIs - G1 phase and restriction point - S phase - G2 and M phases – Markers of cell division cycle and role in cancer - Cell Cycle phase distribution in Glioblastoma - Apoptosis - Pathways of apoptosis - Intrinsic pathway - Extrinsic pathway - Apoptosis and Glioblastoma - Autophagy - Autophagy Flux - Autophagy in cellular homeostasis - Autophagy in cell death - Autophagy pathway - AKT/mTOR pathway - p62 and autophagy – Autophagy in glioma - Measurement of autophagy flux – Project aims

1:1) Glioblastoma: Background and challenges

Primary brain tumours account for approximately 2% of all primary tumours. Glioblastomas are the most aggressive and most common type of primary brain neoplasms (figure 1.1) representing 12-15% of all primary intracranial neoplasms with an annual incidence of 3-4/100000 population per year (Louis *et al.*, 2007). Although they can present at any age the peak incidence is between 45 and 75 years of age with a slight male preponderance.

The presentation of patients with glioblastoma can be highly variable and depends on the location and size of the tumour. Symptoms can occur secondary to raised intracranial pressure (headaches, nausea, vomiting), focal mass effect (hemiparesis, aphasia/dysphasia, hemiparesis, sensory symptoms, visual disturbance), mood and personality changes or seizures (Preusser *et al.*, 2011).

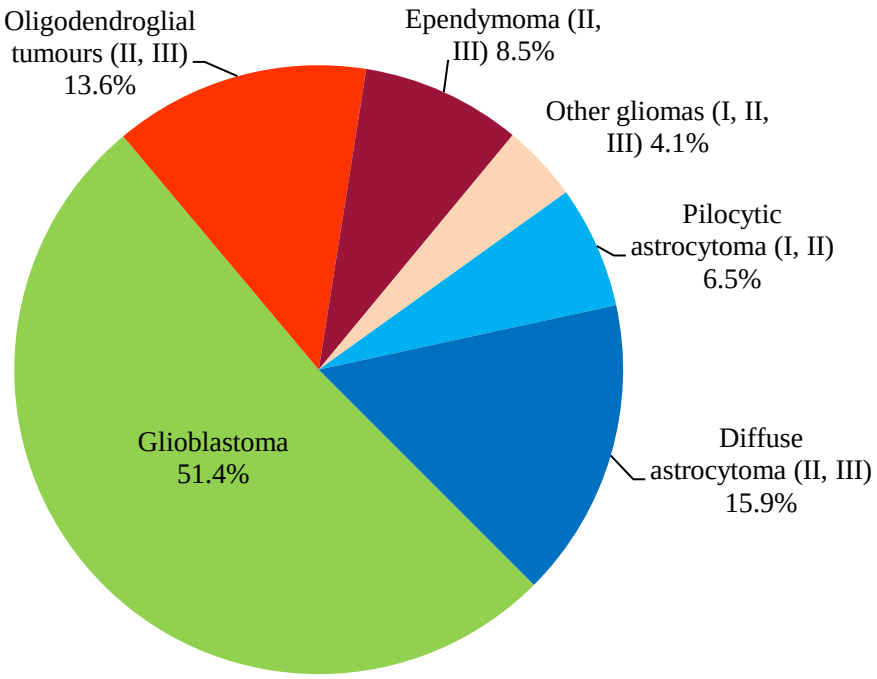


Figure 1.1: Relative frequencies of gliomas. Adapted from Preusser *et al.*, 2011)

Primarily on the basis of their clinical presentation, glioblastomas are classified as primary or secondary. This distinction between primary and secondary glioblastomas was first described in 1940 by a german neuropathologist, Hans-Joachim Scherer (Peiffer and Kleihues, 1999). Primary glioblastomas represent the vast majority (more than 90%) of glioblastomas, usually in older patients, and these have a relatively short clinical presentation (Ohgaki and Kleihues, 2007); occuring without clinical or histopathological evidence of pre-existing less malignant precursor lesion. Secondary glioblastomas develop through progressive transformation from diffuse astrocytoma (WHO Grade II) or anaplastic astrocytoma (WHO Grade III) (Ohgaki *et al*., 2004; Ohgaki and Kleihues, 2007). Secondary glioblastomas are less frequent (less than 10% of all glioblastomas) and tend to occur in younger patients (Ohgaki *et al*., 2004; Ohgaki and Kleihues, 2005). Approximately 70% of diffuse astrocytomas will progress to

glioblastoma over an interval which varies from 1-10 years with a mean period of 4-5 years (Ohgaki and Kleihues, 2007). Primary and secondary glioblastomas are clinically and morphologically indistinguishable and when adjusted for patient age, carry a similar prognosis (Ohgaki *et al*., 2004; Ohgaki and Kleihues, 2005). Ohgaki and Kleihues have described the genetic alterations that occur during the evolution of primary and secondary glioblastoma (Ohgaki and Kleihues, 2007); these are summarized in figure 1.2 More recently Nobusawa *et al*. (2009) have described IDH1 mutations in primary and secondary glioblastoma. In their study, IDH1 mutation was found in 3.7% of primary glioblastomas whereas 73% of secondary glioblastomas exhibited IDH1 mutation. Nobusawa et al. further reported that IDH1 mutation as a genetic marker of secondary glioblastoma corresponded to respective clinical diagnosis in 95% of the cases (Nobusawa *et al*., 2009).

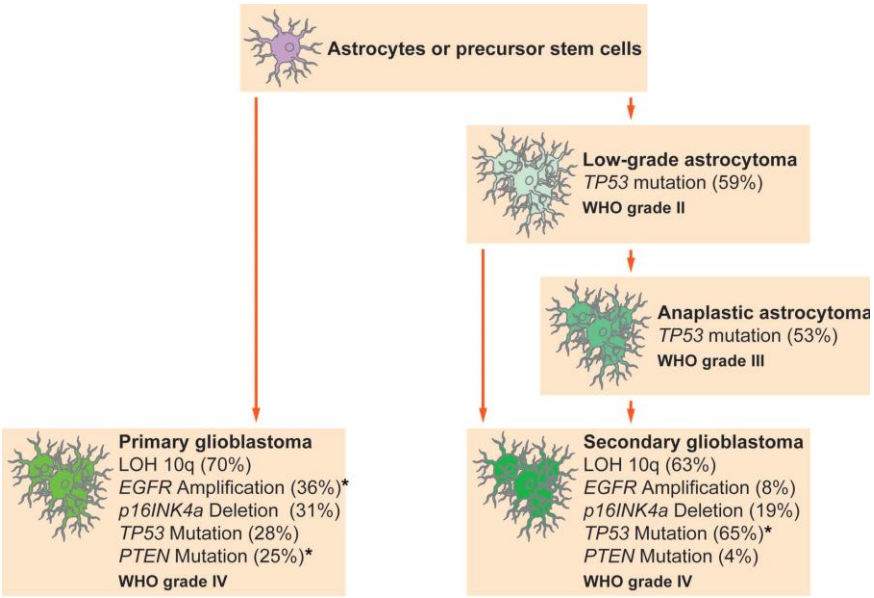
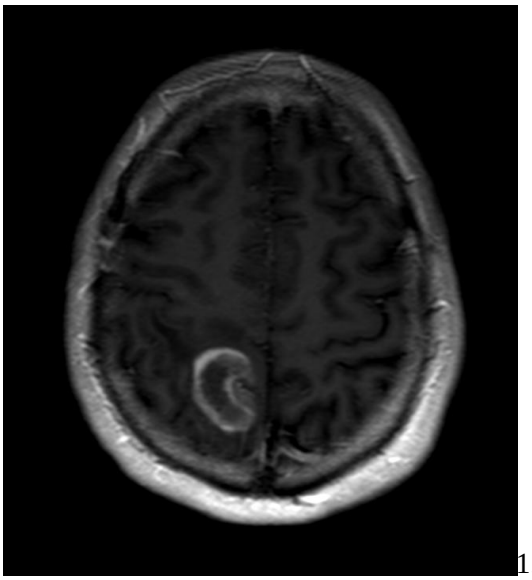
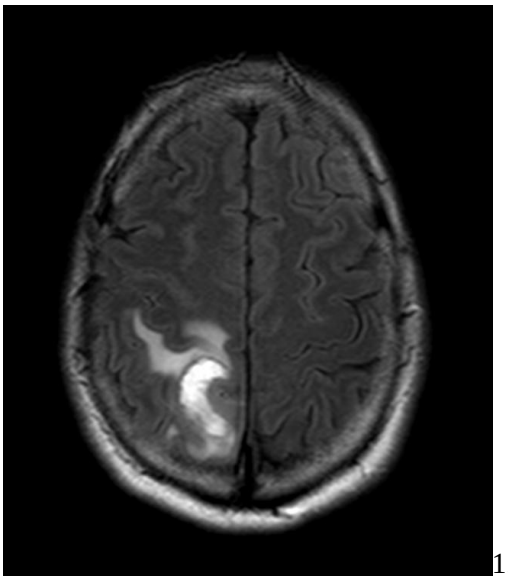


Figure 1.2: Genetic pathways to primary and secondary glioblastomas at the population level (Ohgaki and Kleihues, 2007).

Histologically, glioblastoma is characterized by poorly differentiated astrocytic tumour cells exhibiting nuclear atypia, cellular pleomorphism and brisk mitotic activity. The tumours are

also associated with vascular thrombosis, microvascular proliferation and necrosis (Louis *et al*., 2007). While most glioblastomas are unilateral occupying much of the lobe, those located in corpus callosum and brain stem can be bilaterally symmetrical. They are also characterized by rapid spread through neighbouring structures particularly the corpus callosum, fornix, internal capsule, anterior commissure and optic radiations (Louis *et al*., 2007). Another clinically important feature is the location of invading cells outside the contrast-enhancing rim of the tumour as seen on cranial imaging (Figure 1.3a and 1.3b). This allows these cells to escape surgical resection and evade the higher doses of radiation during radiotherapy (Louis *et al*., 2007). This feature has implications in term of future local recurrence and hence poor outcome.


1.3a


1.3b

Figures 1.3a and 1.3b: Axial T1 Gadolinium enhanced (figure 2a) and axial FLAIR (figure 2b) MRI images demonstrating a right parietal glioblastoma (confirmed on histology). The tumour demonstrates characteristic peripheral contrast enhancment with Gadolinium (Figure 2a). Note the extension of FLAIR signal beyond the contrast enhancing tumour margins in figure 2b suggesting the presence of invading tumour cells.

Histologically up to 2.4% of all glioblastomas are truly multifocal (Batzdorf and Malamud, 1963; Russel and Rubinstein, 1989). Distinct histological subtypes of Glioblastoma are recognized and described (Louis *et al*., 2007). These include Giant cell glioblastoma, Gliosarcoma and Glioblastoma with oligodendroglial components.

Giant cell glioblastoma accounts for up to 5% of glioblastoma (Homma *et al*., 2006) and is histologically characterized by predominance of multinucleated giant cells with an abundant stromal reticulin network. Giant cell glioblastomas carry a relatively poor prognosis (Huang *et al*., 1996) although some studies (Shinojima *et al*., 2004) suggest a better outcome in comparison to usual glioblastoma, possibly due to less infiltrative nature.

Gliosarcoma is a histological variant of glioblastoma characterized by glial and mesenchymal differentiation conferring a biphasic tissue pattern. It comprises of 2% of all glioblastomas and has been suggested to carry a favourable prognosis as compared to the usual glioblastomas (Maiuri *et al*., 1990).

Glioblastoma with oligodendroglial component has been recognized as a distinct entity in WHO classification 2007. As the name suggests, these tumours exhibit foci resembling oligodendrogliomas and have been suggested to carry a better prognosis as compared to standard glioblastoma (Kraus *et al*., 2001), possibly due to relative chemosensitivity of the oligodendroglial component.

Glioblastoma is associated with a poor prognosis as compared to other common malignancies including prostate, breast, lung and colon cancer and has a median survival of approximately 12 months, despite recent advances in surgical and oncological interventions (Westermark, 2012). Currently available treatment options include surgical resection (where feasible), fractionated focal external beam radiotherapy and chemotherapy.

Gross total resection (GTR), where feasible, remains the first and most important step in treatment of glioblastoma (Mineo *et al*., 2007; Filipini *et al*., 2008; Gorlia *et al*., 2008) and has been shown to enhance patient survival (Stummer *et al.*, 2008; McGirt *et al*., 2008; Sanai and Berger, 2008; McGirt *et al*., 2009; Dea *et al*., 2012). Surgery provides a cytoreductive treatment, helps to alleviate tumour mass effect, reduces intracranial pressure and contributes to increase the efficacy of oncological treatments. Blurring of margin between tumour tissue and healthy brain in the infiltration zone and the presence of tumour cells beyond the contrast enhancing tumour margin make the achievement of GTR very challenging. At the same time, the advantages associated with attempts at GTR have to be balanced with the risk of causing neurological deficits especially when the tumour is located close to or in an eloquent location. Recent advances in preoperative and intraoperative advanced MRI imaging (Gonzalez-Darder, 2010), use of high resolution intraoperative ultrasonography, 5-Aminolevulinic acid aided tumour resection (Stummer *et al*., 2006; Feigl *et al.*, 2010) and intraoperative neurophysiological monitoring (Feigl *et al.*, 2010) have all helped to achieve GTR particularly when the tumour is located in eloquent areas.

Before the widespread clinical use of temozolomide chemotherapy for glioblastoma over the past 7 years, radiotherapy following surgery was the only available treatment. The addition of radiotherapy following surgery improves survival as compared with radiotherapy alone having been demonstrated to improve patient survival from 3-4 months to 7-12 months (Stupp *et al*., 2005). Currently, radiotherapy with concomitant and adjuvant temozolomide is the mainstay of oncological treatment for glioblastoma and has been demonstrated to enhance median patient survival by more than two months when compared with radiotherapy alone (Stupp *et al*., 2005; Stupp *et al*., 2009). Temozolomide is an oral alkylating agent with good penetration across the blood brain barrier and causes DNA damage by generating methylation at N-7 or O-6 position of guanine residues. Some tumour cells are able to repair this

methylation by expressing an enzyme O-6-methylguanine-DNA methyltransferase (MGMT). Epigenetic silencing of MGMT by promoter methylation compromises tumour cells' ability to repair DNA and increases their susceptibility to temozolomide. MGMT promoter methylation can occur in approximately 45% of glioblastomas and has been shown to confer a survival advantage when treated with radiotherapy and temozolomide (Hegi *et al*., 2005).

In addition to Temozolomide, surgically implanted BCNU (Carmustine) polymer wafers (Gliadel) have been approved by NICE and have been shown to improve patient survival in Glioblastoma (Westphal *et al*., 2006; Dixit *et al*., 2011; Barr and Grundy, 2012). Recently a few retrospective and prospective trials (Lechapt-Zalcman *et al.*, 2012; Noel G *et al.*, 2012; Bock HC *et al.*, 2010; McGirt MJ *et al*., 2009) have reported the safety and efficacy of the use of BCNU at the time of primary surgery followed by radiotherapy with concomitant and adjuvant temozolomide. Most of these trials have reported no significant increase in serious toxicity and an incremental gain of 2-3 months in median survival in comparison with published results using carmustine wafers or concomitant and adjuvant temozolomide alone (Dixit *et al.*, 2011). However, these studies have small numbers of patients and in the absence of any phase III trials, the sequential use of carmustine wafers followed by radiotherapy with concomitant and adjuvant temozolomide is currently not the standard of care in the treatment of glioblastoma.

Apart from temozolomide and BCNU, other anti-neoplastic agents have been used in the treatment of glioblastoma but with limited success at best. PCV chemotherapy is a combination chemotherapy of Procarbazine, Lomustine (CCNU) and Vincristine and has been used extensively for the treatment of glioblastoma for over 30 years (Levin and Wilson, 1976). A Medical Research Council randomized control trial involving 674 patients with grade 3 and grade 4 astrocytomas (67% with histologically confirmed glioblastoma) showed

no significant survival benefit with PCV chemotherapy after radiotherapy versus radiotherapy alone (MRC brain tumour working party, 2000). Kappelle *et al*. (2001) have reported the results of PCV chemotherapy in 63 patients with recurrent glioblastoma. In this study, PCV chemotherapy resulted in complete response in 3%, partial response in 8%, stable disease in 25% of the patients and progression free survival at 6 months in 29%. Schmidt F *et al.* (2006) however reported only 3 partial responses in 86 patients with recurrent glioblastoma and progression-free survival at 6 months in 38.4%. More recently Brada M *et al.* (2010) have reported a prospective randomized trial comparing the results of temozolomide (in two different dose regimens) versus PCV chemotherapy in 447 chemotherapy-naive patients with recurrent high grade glioma. Brada M et al. did not show any clear benefit of temozolomide over PCV chemotherapy. Currently PCV chemotherapy is mostly used as a second line chemotherapy agent in recurrent glioblastoma.

In summary, patient age, Karnofsky performance score, tumour location, gross total resection, radiotherapy, temozolomide, MGMT status are factors known to influence outcome in glioblastoma (Lamborn *et al.*, 2004; Mineo *et al*., 2007). The overall prognosis however continues to remain poor with most patients dead within 15 months of diagnosis (Westermark, 2012).

The focus of this thesis is to study markers of cellular proliferation, survival and cell death in glioblastomas and to identify specific biological markers which could be used on paraffin-embedded surgical biopsy specimens in an effort to predict the likely response of individual tumours to adjuvant treatment and thus aid in clinical decision making and better targeting of currently available treatments.

1:2) Cell division cycle

The cell division cycle is a series of exquisitely controlled processes leading to cell reproduction (Murray and Hunt, 1993; Nasmyth, 1996; Stillman, 1996; Csikasz-Nagy A, *et al*. 2006; Budirahardja and Gonczy, 2009; Uhlmann *et al.*, 2011). The timing and coordination of these events is controlled by a complex regulatory network to ensure that cell division is accomplished without the introduction of deleterious mutations, chromosomal abnormalities and unequal distribution of chromatin between daughter cells (Bell and Dutta, 2002; Coverley and Laskey, 1994; Sun and Kong, 2010; Uhlmann *et al*. 2011). Defective cell cycle arrest at the respective checkpoints is associated with genomic instability and oncogenesis (Loeb, 1991; Bartek and Lucas, 2001; Nyberg *et al*., 2002; Aguilera and Gomez-Gonzales, 2008; Langerak aand Russell, 2011).

1:3) Cell cycle: phases

Cells in active state of proliferation are said to be in cell cycle. The cell cycle has four distinct phases namely G1, S, G2 and M. G1 and G2 are gap phases which prepare the cell for the next phase and precede S and M phases respectively. Chromosomal duplication occurs in the S (for synthesis) phase and chromosomal segregation occurs in the M (for Mitosis) phase (Murray and Hunt, 1993; Morgan, 2007).

Following the cell division cycle, the cell either enters a quiescent or specialized resting phase called G0 or re-enters the cell cycle. Most cells in terminally differentiated tissues are in G0 phase and re-enter the cell cycle upon appropriate stimulation including cellular injury and growth factors.

The M phase has four distinct phases namely prophase, metaphase, anaphase and telophase. DNA chromosomes condense and the mitotic spindle is assembled in prophase. The

chromosomes align upon the spindle in metaphase. The chromosomes then segregate by releasing cohesion and reel in the spindle (anaphase) until they decondense and form nuclei (telophase). Together G1, S and G2 phases are also called interphase (Morgan, 2007).

Progression through the cell cycle follows an organized pattern to ensure that DNA replication occurs once and only once through the cell cycle and precedes chromosomal segregation which in turn precedes cytokinesis. Throughout the cell cycle, inbuilt mechanisms in the form of checkpoints ensure that the genetic information is transferred completely and correctly to the daughter cells (Murray and Hunt, 1993; Morgan 2007) (figure 1.3). Detection of irreparable genetic damage at these checkpoints results in activation of apoptosis or programmed cell death. Cells lacking effective checkpoints display genomic instability, faulty DNA replication or aberrant segregation (Loeb, 1991).

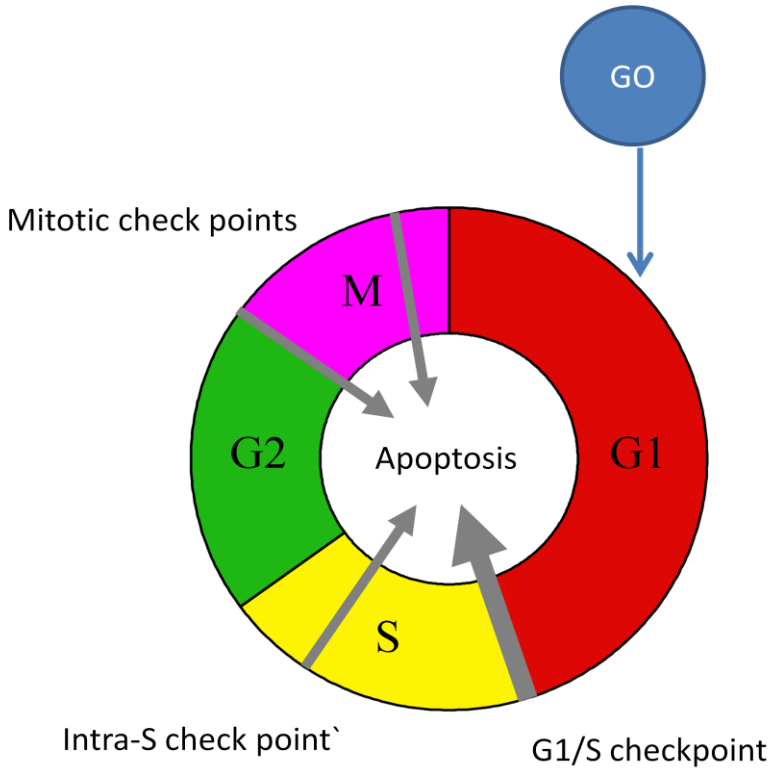


Figure 1.4: Sites of important cell cycle checkpoints in mammalian cell cycle.

1:4) Cyclins, CDKs and CDKIs

Progression through the cell cycle is controlled by the synthesis, degradation and state of phosphorylation of proteins called cyclins (Evans *et al*., 1983). The cyclins form complexes with Cyclin dependent kinases (CDKs) and these cyclin-CDK complexes then control progression through the cell cycle by activating a variety of proteins by phosphorylation (Budirahardja and Gonczy, 2009). The cyclin-CDK complexes are the core controlling mechanism that drives the eukaryotic cell cycle (Budirahardja and Gonczy, 2009). The cyclins display periodic oscillatory changes in concentration during different phases of the cell cycle and this helps to generate CDK activity that forms the foundation of the cell cycle control system cells and also ensures a unidirectional cell cycle progression (Budirahardja and Gonczy, 2009; Morgan, 1997; Nurse, 1990). CDK regulation is achieved by association with cyclins and inhibitor proteins, phosphorylation-dephosphorylation and cyclin synthesis or degradation. The cyclins are divided into four classes based on their expression, timing in the cell cycle and their function: G1, G1/S, S and M phase cyclins. The cyclin concentration in turn is regulated by cyclin gene expression and destruction of cyclins by proteolysis. The CDK activity is modulated by a variety of cyclin dependent kinase Inhibitors (CDKI). These include p21, p27 and p57 with a broad inhibition activity and p15, p16 and p19 (INK family) with specific inhibition of CDK4 and CDK6.

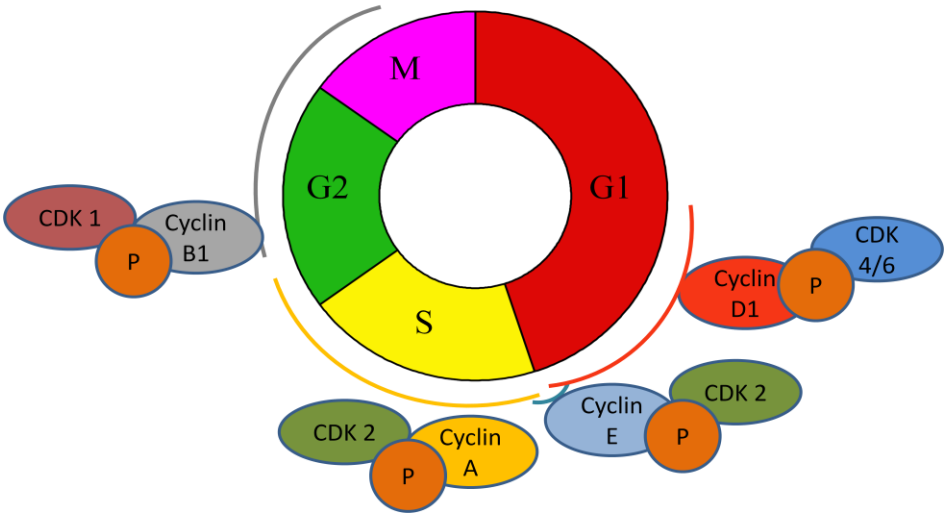


Figure1.5: Cyclin-CDK complexes and their distribution at different phases of the cell cycle. Cyclin D1 is expressed in the latter third of G1 and together with cyclins E and A is involved in progression through the G1/S transition. Cyclin A is expressed in S and to a variable extent into G2. In S-phase, cyclin A is complexed to CDK2, however, in G2 and early M, it is complexed to CDK1. Cyclin B1 is expressed in both G2 and prophase of M. In G2, cyclinB1 is identified as a cytoplasmic molecule, in prophase of M the molecule is found in the nucleus until, with breakdown of the nuclear membrane, staining becomes diffuse.

Different models of cell cycle control have been proposed (Chen *et al*, 2004; Csikasz-Nagy *et al*., 2006). More recently, Gerard and Goldbeter have proposed an integrated computational model for the network of CDKs that control the dynamics of the cell cycle (Gerard and Golbeter, 2009 and 2011). The CDK network consists of four coupled members including cyclin D/CDK4-6, cyclin E/CDK2, cylcin A/CDK2 and cyclin B/CDK1 and sequential activation of these complexes respectively control progression through G1, transition to DNA replication in S, transition to G2 and finally G2/M transition allowing entry into M phase (figure 1.4). The model also includes retinoblastoma gene (pRb) and transcription factor E2F which inhibit and promote progression through cell cycle respectively. Another feature of the model is the existence of restriction point beyond which the cell cycle progression continues independent of growth factors.

1:5) G1 phase and restriction point

During G1 phase, nucleotide and proteins of the replicative machinery are synthesized and the expression of genes associated with deoxynucleotide synthesis are upregulated in preparation for DNA synthesis. G1 cyclin (Cyclin D in vertebrates) helps coordinate cell growth with entry into the new cell cycle and together with the G1/S cyclin (Cyclin E) triggers progression into a new cell cycle at the restriction point (start point in Yeast). This leads to processes culminating in DNA replication. The level of cyclin D rises early in G1 and is limited to late G1 whereas the expression of cyclin E is limited to late G1 and early S phase. Cyclin D forms complexes with Cdk4 or Cdk6 to yield active protein kinase (Hunter and Pines, 1994) which phosphorylates Rb which is a key event in G1/S transition (Buschges *et al*. 1999; Hunter and Pines, 1994; Lei *et al*., 1997; Sawa *et al*., 1998). There is also increasing evidence to suggest that cyclin D activity is required for activation of cyclin E whose complex with CDK2 is required for transition through G1/S checkpoint (Coverley *et al*., 2002). During early G1 the hypophosphorylated form of pRb binds and inactivates E2F transcription factor. Following phosphorylation of pRb, E2F is released which then stimulates transcription of genes required for DNA replication. In particular E2F leads to further expression of cyclin E in a positive feedback loop fashion.

G1 DNA damage results in a strong and often irreversible block of cell cycle progression at restriction point. The G1 DNA damage response is of two types. The "Rapid" response occurring in minutes is mediated by inactivation of Cyclin E-CDK2 causing cell cycle arrest in G1 by preventing progression past the restriction point. p53 activation constitutes the "Delayed" or maintenance response. p53 is a gene regulatory protein that plays a central role in cellular response to DNA damage and other stressors, with activation leading to apoptosis (Levine., 1997; Harris and Levine, 2005). It binds directly to the promoters of its target

genes and alters the rate at which their transcription in initiated. In most cases the expression of target genes is stimulated and the overall result of p53 is increased production of proteins that stimulate cell cycle arrest and promote apoptosis. At the same time, p53 increases transcription of genes encoding apoptosis inhibitors. In the absence of DNA damage, Mdm2, an E3 ubiquitin-protein ligase ubiquitinates p53 thereby promoting its destruction by the proteasome. Upon DNA damage, phosphorylation of Mdm2 and p53 disrupts their association, resulting in stabilization and activation of p53. Phosphorylation of p53 also increases its interaction with histone acytylase, p300. This results in increased acetylation of histones and p53, both of which act to increase p53 dependent gene expression.

1:6) S phase

During the late M and early G1 phase, a complex of initiator proteins, the Pre-replicative complex (Pre-RC), assembles at discrete sites on the chromosome called origins of replication (figure 1:5). Pre-RC is composed of Origin Recognition complex (ORC), Cdt1 (Chromosomal licencing and DNA replication factor 1), Cdc6 (cell division cycle 6) and the Mini-chromosomal protein complex (MCM2-7). Pre-RC prepares the origins for "licensing" (Bell and Dutta, 2002; Coverley and Laskey; 1994, Lei and Tye, 2001). During the S phase, the Pre-RC is transforms into an active Pre-initiation complex by the activity of Cyclin E/Cdk2 and Cyclin A/CDK2 complexes. Once activated, MCM2-7 acts as a DNA helicase and unwinds the DNA enabling access to the DNA synthesis machinery (figure 1.5). Once the MCM2-7 complex initiates DNA unwinding and DNA replication commences, re-replication is prevented by Cyclin A/CDK2 complex which phosphorylates MCM2-7 rendering it inactive. Another key factor in preventing re-replication is Geminin, this molecule binds to Cdt1, inhibits its association with Cdc6 and prevents reloading of MCM2-7 complex at the origins (Madine and Laskey, 2001; Wohlschegel *et al*., 2000; Wohlschlegel *et*

al., 2002). In mammals, Cyclin A expression is limited to S phase whereas Geminin in expressed in S, G2 and M until the anaphase/telophase transition when it is degraded by proteolysis mediated by the Anaphase Promoting Complex (APC). This inactivation then releases Cdt1 which is available for the following round of the cell cycle. These mechanisms ensure that Pre-RC reassembly is prevented until the next G1 phase and thus DNA replication occurs once and only once during the cell cycle.

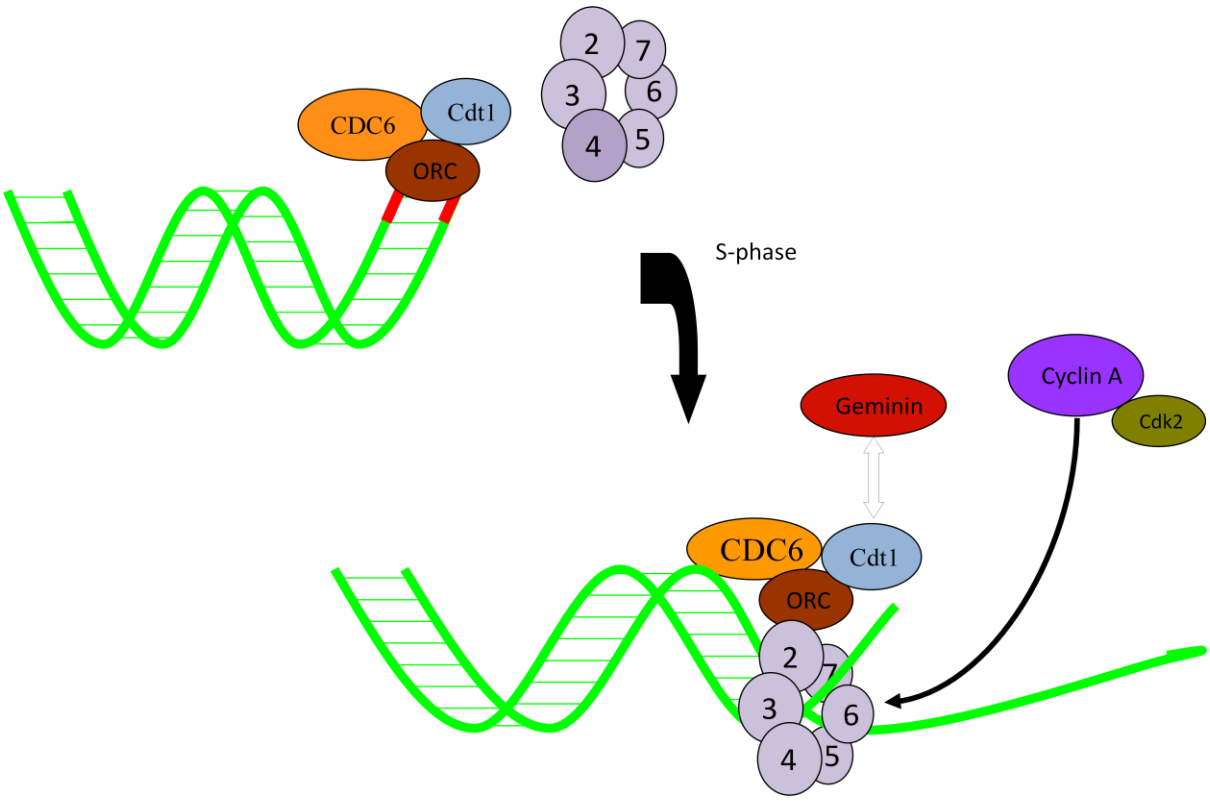


Figure 1.6: Mcm proteins and licencing of DNA for replication. In G1 phase, the pre-replication complex is assembled which allows Mcm proteins 2-7 to form a hexamer at the origins of replication. Mcm complex acts as a DNA helicase and unwinds the DNA. Cyclin A/Cdk2 then inactivates Mcm complex. Geminin inhibits Cdt1and helps to prevent re-initiation.

1:7) G2 and M phases

During G2, regulatory factors for mitosis are synthesised and modifications occur in the chromatin structure to support mitotic chromosomal condensation. At the onset of mitosis, chromosome condensation is mediated by cyclin B/CDK1 Complexes. Phosphorylation of histone H1 by CDK1/cyclin B modifies chromatin structure through alterations in nucleosome interactions (Nigg, 1998). CDK1/cyclin B promotes completion of chromosomal condensation (Murray and Hunt, 1993) by phosphorylating and consequently activating casein kinases and phosphorylating condensins. In addition it is also thought to stimulate centrosome separation and nuclear envelope breakdown and spindle assembly during prophase. The effects of Cyclin B-CDK1 are irreversible once set in motion. This is due to the "all or none" irreversible nature of cyclin B-CDK1 activation (Morgan, 2007).

At the end of S phase, the cells contain a duplicate set of chromosomes in a tightly associated pair called sister chromatids. During the M phase the sister chromatids are separated and one of each pair is distributed to each daughter cell (Nasmyth *et al*., 2000). During prophase, the chromosomes undergo condensation and the sister chromatid cohesion is loosened by removal of DNA catenation and partial loss of cohesion protein that hold the sister chromatids together.

Also in prophase, the centrosome separates, each nucleating its own radial microtubule array eventually resulting in the formation of the bipolar microtubule array of mitotic spindle. Prometaphase begins when the nuclear envelope breaks down and lasts until the sister chromatids are completely attached to the spindle and have migrated to the central spindle. Specialised regions of the chromatin called kinetochores form sites by which the sister chromatids become attached to the spindle microtubules (Alberts *et al.,* 2002; Murray &

Hunt, 1993). When all the chromosomes are lined up on the mitotic spindle, the cell is said to be in metaphase and the structure is referred to as a metaphase plate.

Cyclin B is the primary M phase cyclin. Cyclin B/CDK 1 activation begins in G2-phase when Wee1 (a nuclear kinase) phosphorylates CDK1. When phosphorylated at three sites (amino acids 14, 15 and around 160) CDK1 is inactive. However, at the G2/M transition, Cdc25 phosphatase dephosphorylates the cyclin B/CDK1 complex (at amino acids 14 and 15 but not at 160), thus activating it by removing the two phosphate groups from the active site of the CDK1 kinase. Cdc25 is activated by at least two sets of protein kinases. The first is cyclin B/CDK1 forming a positive feedback loop and a second signal is provided by a group of kinases, originally described in *Drosophila*, known as Polo kinases (Nigg, 1998).

Cyclin A-CDK1 and Cyclin B-CDK1 complexes trigger entry into M phase. Early in mitosis Cyclin B-CDK1 stimulates centrosome separation and chromosomal condensation. Later Cyclin B-CDK1 controls progression in mitosis by phosphorylation of proteins that control spindle assembly and phosphorylation of lamins results in breakdown of the nuclear envelope at the end of prophase and events leading to assembly of sister chromatids on the spindle. Both cyclin A/CDK1 and cyclin B/CDK1 promote microtubule formation from centrosomes (Alberts *et al*., 2002; Murray & Hunt, 1993; Nigg 1998). Also Cyclin B-CDK 1 complex activates Anaphase Promoting Complex (APC) which acts as a ubiquitin-protein ligase. APC causes destruction of securin, a protein that binds the sister chromatids together (figure 1.6). This event is mediated by the APC induced proteolysis of the protein securin. Securin binds to and inactivates a protein known as separase. Destruction of securin allows activation of separase, which is then free to cleave one of the subunits of the cohesin complex, thus allowing the sister chromatids to separate (Nasmyth *et al*., 2000; Morgan, 2007). Fully active APC appears to require activation by Cdc20, active cyclin B/CDK1, and completion of

correct metaphase alignment of chromatid pairs, although other, as yet unknown, factors may be important. Importantly, APC also promotes the destruction of Cyclin B thereby acting as a negative feedback loop for Cyclin B-CDK1 activity (figure 1.7).

As described above, Cyclin B/CDK1 complex is inactivated upon Cyclin B destruction by APC during metaphase. There are at least two checkpoints during mitosis. G2/M checkpoint controls mitotic entry. Damaged DNA or stalled DNA replication fork sends inhibitory signals that block mitotic entry by preventing activation of CDK1.

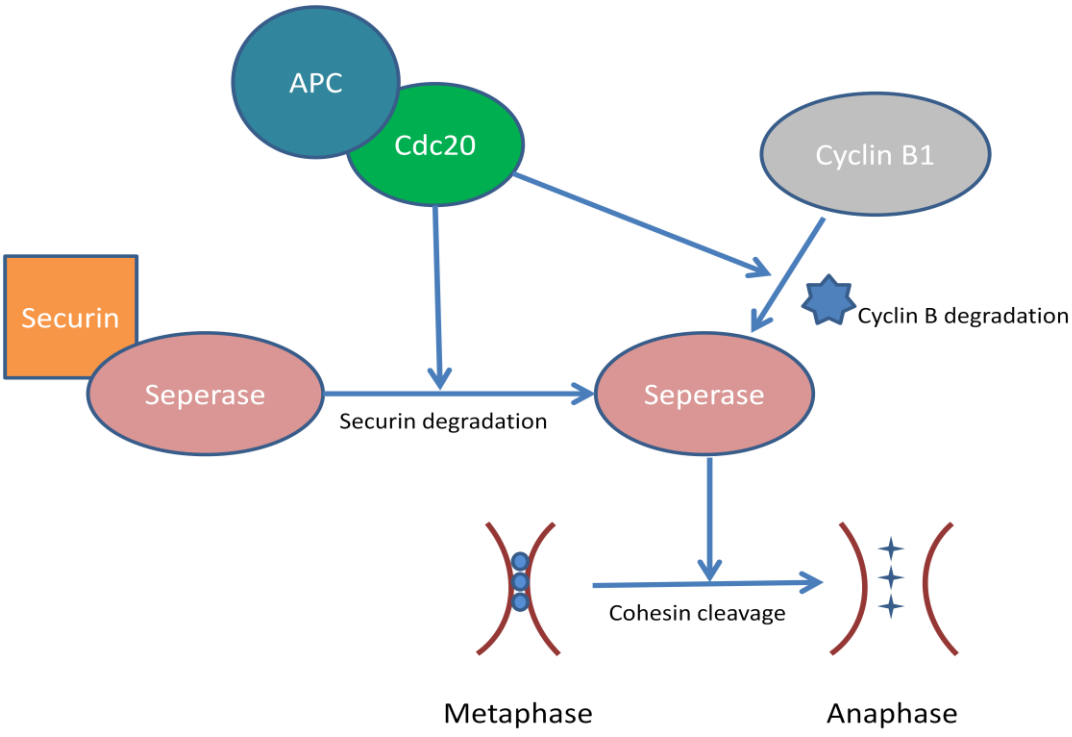


Figure 1.7: Regulation of sister chromatid separation by APC. APC mediates proteolysis of securin liberating seperase which in turn cleaves cohesion resulting in sister chromatid separation. APC-Cdc20 also mediates the degradation of Cyclin B which in turn activates Seperase.

Thus, early in prophase, progression into mitosis is tightly regulated by the integrity and replication status of the DNA and the activity of the Cdk1/cyclin B complex. The second checkpoint within mitosis is at the metaphase to anaphase transition and involves the APC. If sister chromatids are not attached to the spindle correctly, kinetochores send out inhibitory signals to the APC thereby preventing further progression of mitosis until correct spindle attachment has been achieved. Failure of these checkpoints may lead to mis-segregation of chromosomes with subsequent generation of daughter cells with chromosomal abnormalities and aneuploidy (Kaplan *et al*., 2001; Shichiri *et al*., 2002).

1:8) Markers of cell division cycle and their role in Cancer

Cancer is a heterogenous group of disease processes characterized by accumulation of genetic lesions which translate into increased activity of genes that drive cellular proliferation and reduced activity of genes that normally inhibit it. Markers of the cell division cycle can be used to estimate tumour cell populations in different phases of the cell cycle and this, in turn, may be important in predicting response to treatment because of varying sensitivity of cells in different phases of the cell cycle to adjuvant treatment (Terasima *et al*., 1963; Hama *et al*., 2003; Gravina *et al*., 2010) (figure 1.7).

Cells are most radiosensitive in G2 and M phase of the cell cycle. Cells in G1 are relatively resistant to radiotherapy whereas cells in S phase are the most radioresistant (Quitet *et al*., 1991; Tell *et al.,* 1998). Quitet *et al*. investigated two squamous cell lines and found the radioresistant cell line to contain twice the number of cells in S-phase in comparison with the radiosensitive line. On a similar note, Tell *et al.* reported that peripheral blood lymphocytes from patients with head and neck cancer who are non-responsive to radiotherapy had a higher level of cells in the S-phase compared with partial and complete responders.

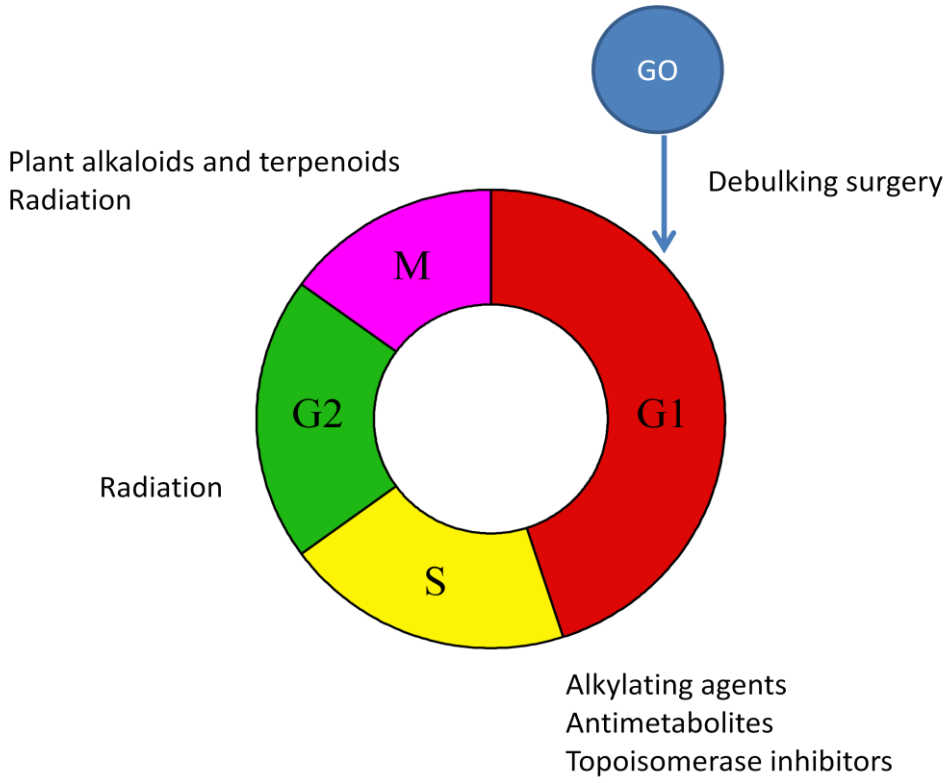


Figure 1.8: Cell cycle phases and sensitivities to various therapies at different phases of the cell cycle. Debulking surgery results in more of the remaining tumour cells entering the cell cycle due to better availability of substrates (oxygen and nutrients). Cells are more sensitive to radiotherapy in G2 and M phases. Chemotherapy agents work primarily during S or M phases of the cell cycle.

The markers of cell cycle phase distribution studied in this thesis include minichromosome maintenance protein-2 (Mcm-2), cyclin-A, geminin and Phosphorylated Histone H3 (PHH3), these markers have previously been used as surrogate markers of cell phase distribution in a variety of tumour types. The expression of these markers in gliomas is discussed separately in 1:9.

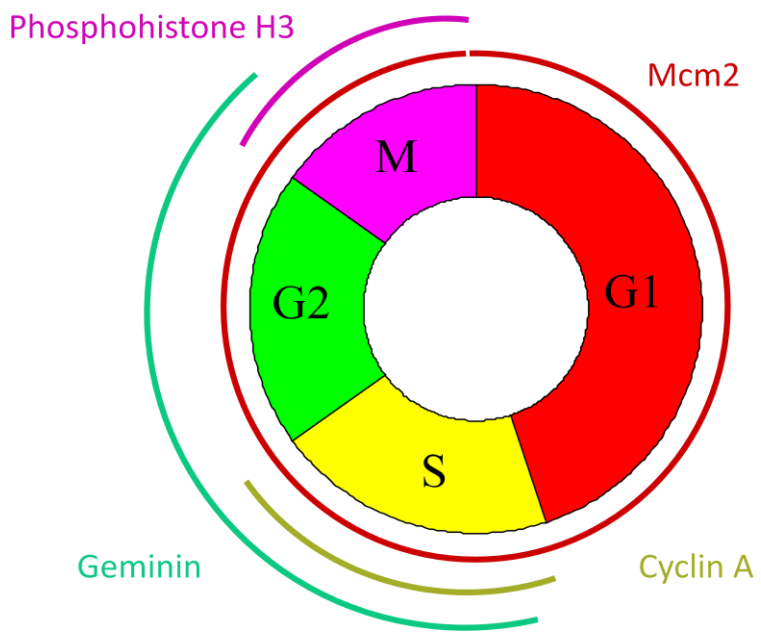


Figure 1.9: Schematic diagram demonstrating the distribution of Mcm2, Cyclin A, Geminin and PHH3 in the cell division cycle.

Mcm-2 is a 100kDa nuclear protein that is part of a multimeric heterohexamer complex comprising Mcm proteins 2-7 (Lei and Tye, 2001; Madine and Laskey, 2001; Kearsey and Labib, 1998). Mcm-2 is expressed abundantly throughout the cell cycle but is broken down rapidly on exiting the cycle or in quiescence (Kearsey and Labib, 1998; Maiorano *et al*., 1996; Musahl. *et al*., 1998) making it a reliable marker of cell cycle entry. Whilst Ki-67 is more a more widely used proliferative marker, Mcm2 offers superior detection of actively replicating cells as Ki-67 fails to detect cell in most of G1 phase. High Mcm2 expression has been shown to correlate with poor patient survival in diffuse large B-cell Lymphoma (Hou *et al*., 2011) and gastric carcinoma (Yang *et al.,* 2012), whilst a higher expression is associated with better prognosis in colorectal carcinoma (Zhao *et al*., 2011) and high grade sarcoma (Matsubara *et al.,* 2008).

Cyclin A, a 60kDa nuclear protein, acts as a surrogate marker of S-phase in mammalian eukaryotic cells that in astrocytomas is largely confined to S-phase (Scott *et al*., 2005). In some cell lines, expression of cyclin A is maximal in S-phase with low expression in G2-phase (Pines and Hunter, 1992). The expression pattern, therefore, tends to vary with the type of cell or cell line, with some lines showing low level of cyclin A expression into early metaphase (Pines and Hunter, 1992; Xouri *et al*., 2007). High cyclin A expression has been associated with poor prognosis in breast cancer in a study by Poikonen *et al.* however interestingly in this study, high Cyclin A was associated with a favourable response to anthracycline and antimetabolite chemotherapy although this did not impact on overall patient survival. This is thought to be because Cyclin A labels cells in S-phase of the cell cycle and this fraction of cells togther with cells in G2/M phase are considered to be more sensitive to the effects of chemotherapy. High cyclin A expression has also been reported to be associated with poor prognosis in pediatric embryonal brain tumours (Moschovi *et al*., 2011).

Geminin is a 25kDa protein whose nuclear expression is restricted to S-phase, G2-phase and the prophase and metaphase of mitosis (Madine and Laskey, 2001; Wohlschlegel *et al*., 2000; Wohlschlegel *et al*., 2002; Nishitani *et al*., 2001). High Geminin expression has been reported to be associated with poor prognosis in small cell lung cancer (Haruki *et al*., 2011), salivary gland carcinoma (Yamazaki M et al., 2010), colorectal cancer (Nishihara *et al*., 2009).

Phosphorylated Histone H3 (PHH3) protein, though not a cyclin, is a useful marker that is present throughout mitosis (Shibata and Ajiro, 1993). Phosphorylation at serine 10 of Histone H3 has been shown to correlate with chromatin condensation during mitosis and is a sensitive and specific marker of mitosis (Hendzel *et al*., 1997; Goto *et al*., 1999). PHH3

Labelling indices (LI) are known prognostic factors in tumours like breast cancer (Skaland *et al*, 2009), malignant melanoma (Ladstein *et al*, 2012) and meningiomas (Kim *et al*., 2007).

1:9) Cell Cycle phase distribution in Glioblastoma:

Expression of Mcm-2, cyclin A and geminin have been investigated previously in oligodendrogliomas (Wharton. *et al*., 2004) and astrocytomas (Scott *et al*., 2005; Hara *et al*., 2008; Shresta *et al*., 2007; Margraf *et al*., 2011) and, although all these studies have reported increased expression of these markers with increasing tumour grade, a correlation with patient prognosis and treatment response has not been reported in the literature for glioblastomas. Margraf *et al*. (2011) studied the expression of Mcm2 and PHH3 in pilocytic astrocytomas and found no significant association of either marker with prognosis. Wharton et al reported expression of Mcmc2 and Geminin in 55 cases of oligodendrogliomas (25 Grade II and 30 Grade III) and observed increased expression of Geminin with increasing tumour grade. Scott et al. analysed expression of multiple cell cycle markers in astrocytomas and found the expression of Mcm2, Cyclin A and PHH3 increased with increasing tumour grade but the expression of these markers did not correlate with patient survival. Of note is the study by Shresta *et al*. (2007) who investigated the expression of Geminin in 51 cases of high grade astrocytomas (19 anaplastic astrocytomas and 31 glioblastomas) and although they reported an improved survival with higher geminin LI, on further analysis, the prognostic significance was only observed in the whole group and in anaplastic astrocytomas but not in Glioblastomas. Interestingly, Coleman *et al*. (2006) found a positive association between PHH3 LI and survival in a study involving 103 patients with grade II and III astrocytomas. However, a recent study by Habberstad *et al*. (2011) involving twenty seven patients with anaplastic astrocytomas found no significant correlation between PHH3 LI and

overall survival on Kaplan Meier analysis, although the small cohort size may be an issue (Habberstad *et al*., 2011).

In this thesis, Mcm2, Cyclin A, Geminin and PHH3 expression in glioblastomas have been investigated and the expression correlated with patient survival and response to adjuvant treatment. It is possible that identification of a subpopulation of glioblastomas with a high replicative fraction may isolate a group of tumours that are more susceptible to therapies dependent upon active replication for their efficacy (Sallinen *et al.,* 1994). This in turn can provide clinicians with prognostic information and can aid in clinical decision making particularly in equivocal cases.

1:10) Apoptosis

Apoptosis or programmed cell death (Lockshin and Williams, 1965) type I (PCD I) is an important component of normal development and health of multicellular organism. Dysregulation of apoptosis is involved in various steps in the pathogenesis and progression of cancer, including increase in tumour size due to reduction in programmed cell death, accumulation of genetic instability and resistance to ischemia, chemotherapy and radiotherapy.

1:11) Pathways of apoptosis

Apoptosis is a tightly scripted death programme which results in activation of multiple groups of proteases and nucleases which breakdown various components of the cell including nuclear lamina, parts of cytoskeleton and DNA. The process is characterized by certain morphological features (Kerr *et al*., 1972). Upon induction of apoptosis, cells shrink due to breakdown of actin and laminins in the cytoskeleton. The nucleus condenses following breakdown of chromatin and often takes a "horse-shoe" appearance. The cells loosen from

neighbouring cells and eventually package themselves into a form by means of plasma membrane changes that allows for phagocytosis by macrophages, or allows the apoptotic cells to be engulfed by neighbouring cells or extruded from the tissue. The latter stages are characterized by formation of membrane blebs and blisters processes. Also characteristic but not universal, is the appearance of small vesicles called apoptotic bodies.

Apoptotic cell death is mediated through intracellular (osmotic stress, DNA damage) and extracellular cues (growth factor withdrawal, matrix detachment and direct cytokine mediated killing). Two main pathways of apoptosis are recognized. The extrinsic pathway is death receptor mediated whereas the intrinsic pathway is mediated via the mitochondria.

Both pathways involve a family of cysteine aspartate-specific proteases called caspases (Daniel and Korsmeyer, 2004). Caspases are synthesized as inactive zymogens or Pro-caspases and consist of a variable length pro-domain, a smaller p10 unit and a larger p20 unit. Caspase activation involves proteolysis at specific asparagines residues resulting in generation of an activated caspase which is a heterotetramer of two p10 and two p20 subunits. Apoptotic caspases are functionally divided into initiator caspases (Caspase 2, 8, 9 and 10) and effector or "executioner" caspases (Caspase 3, 6 and 7) (Adams, 2005).

Caspases are inactivated by Inhibitor of Apoptosis proteins (IAPs). These include XIAP, cIAP1, cIAP2, ILP2, ML-IAP, NIAP, SUVIVIN and BRUCE (Krastad and Chekenya, 2010). These proteins inhibit apoptosis by binding to and directly inhibiting Caspase 9 in the intrinsic pathway and also to downstreamj effector caspases 3 and 7. In addition they also promote caspase degradation by via ubiquitin-proteosome pathway. (Verhagen *et. al*., 2002; Srinivasula and Ashwell, 2008; Krastad and Chekenya, 2010).

1:12) Intrinsic pathway

The intrinsic pathway of apoptosis is triggered by a number of factors including UV or gamma radiation, growth factor withdrawal and chemotherapeutic drugs. The central event in this pathway is mitochondrial outer membrane permeabilization (MOMP). MOMP leads to release of multiple proteins from the mitochondrial intermembrane space (IMS) into the cytoplasm. MOMP results in release of pro-apoptotic factors from the IMS. These include cytochrome c, AIF (Apoptosis inducing factor), Smac/DIABLO, endonuclease G and omi/htra2. Cytochrome c in association with APAF-1 (Apoptotic protease activating factor-1) and dATP form Apoptosome. The apoptosome then activates caspase-9 which in turn leads to activation of effector caspases in particular caspase 3 and commits the cell to apoptotic death. Caspase 3 then cleaves the inhibitor of caspase-activated DNA (ICAD) activating CAD which then breaks DNA into fragments. Smac (Diablo) and HtrA2 (Omi) facilitate caspase activation by inhibiting inhibitors of apoptosis proteins, which are endogenous caspase inhibitors (Wolf and Green, 2002; Wang, 2001).

Members of Bcl-2 (B-cell lymphoma) protein family are important regulators in MOMP. The Bcl-2 family of proteins share sequence in 4 alpha-helical Bcl-2 homology (BH) regions, namely BH1, BH2, BH3 and BH4. The Bcl-2 proteins can be divided into two groups, pro-apoptotic and anti-apoptotic members

The pro-apoptotic members are subdivided into two main groups. Bax and Bak share BH1, BH2 and BH3 domains. Bid, Bim, Bik, Bad, Bmf, Noxa, Puma and Hrk are homologous in BH3 domain only (Tsujimoto, 2003; Danial *et al.*, 2004). The BH3 only proteins are further subdivided into activator pro-apoptotic members including Bid and Bim and sensitizer pro-apoptotic members including Bad, Bik, Bmf, Hrk, Noxa and Puma.

The anti-apoptotic members include Bcl-2, Bcl-xl, Bcl-w, Mcl-1, and Bfl-1 and these share homology in all BH1-BH4 domains.

Upon cytoskeleton or DNA damage or other apoptotic stimulation, BH3 only proteins including Bid and Bim are activated. Bid and Bim acting as death signal sensors then lead to oligomerization of Bax and Bak and antagonize the anti-apoptotic effects of Bcl-2, Bcl-xl and other anti-apoptotic members of Bcl-2 family. Bax and Bak then mediate MOMP by protein-conducting pore formation in mitochondrial outer membrane (Wei, 2001). The levels of pro- versus anti-apoptotic BCL-2 proteins play a critical role in regulating the apoptotic process (Lessene *et al*., 2008; Krastad and Chekenya, 2010).

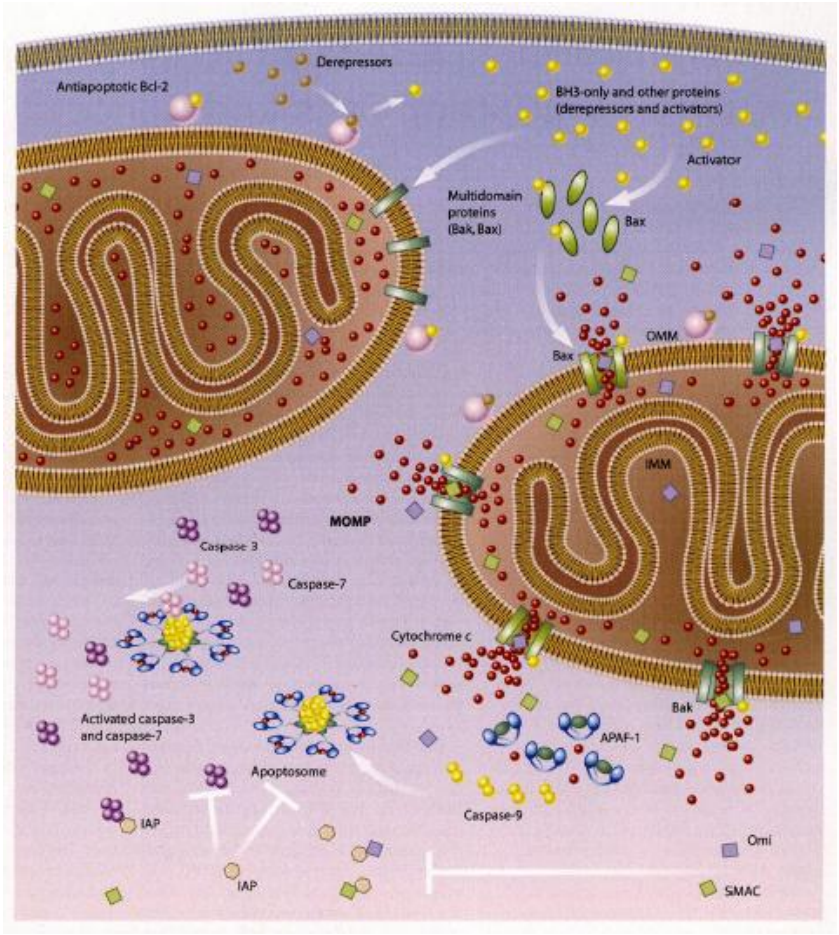


Figure 1.10: The mitochondrial pathway of apoptosis with involvement of different pro-apoptotic and anti-apoptotic proteins with the central event being the process of mitochondrial outer membrane permeabilisation (MOMP). (Spierings et al., 2005)

1:13) Extrinsic pathway

The extrinsic pathway of apoptosis begins with activation of certain death receptors e.g. Tumour necrosis factor receptor (TNFR) superfamily, by corresponding ligands e.g. Fas, TNF. These receptors are characterized by the presence of an intracellular death domain and upon appropriate stimulation undergo oligomerization. The activated receptors then form a death-inducing signalling complex (DISC) in association with a death domain containing adaptor molecule FADD (Fas associated death domain), procaspase 8 and the cellular FLICE-inhibitory proteins (FADD-like IL-1β-converting enzyme inhibitory protein (c-FLIP). The type of further response divides this pathway into two types. In type I cells, this leads to extensive activation of caspase 8, sufficient to activate downstream execution or effector caspases 3 and 7. These effector caspases subsequently target various cytoskeleton and nuclear substrate proteins, a step leading to culmination of apoptotic cell death.

In type II cells, extrinsic or death receptor mediated apoptosis leads to MOMP via the BH3 protein Bid. Activated Caspase 8 can cleave Bid to form truncated-Bid (tBid) which can activate Bax and Bak leading to MOMP leading to release of cytochrome C and Apoptosome formation followed by activation of caspase 9. Activated caspase 9 then leads to activation of effector caspases 3 and 7. This pathway leads to apoptotic signal amplification in cells characterized by low levels of DISC and active caspase 8 (Scaffidi *et al.*, 1998; Krastad and Chekenya, 2010).

1:14) Apoptosis and Glioblastoma

Dysregulation of apoptosis is a major contributor to the pathogenesis and progression of tumours in many ways. (Stenner-Liewen and Reed, 2003)

1. Failure of normal cell turnover leads to cell accumulation

2. Genetic instability and oncogene activation accumulates
3. Increased resistance to immune attack
4. Resistance to chemotherapy and radiotherapy
5. Resistance to hypoxia and angiogenesis based targeted therapies
6. Cellular survival in a detached state contributes to metastasis

Apoptotic pathways in glioblastomas are subject of ongoing research. Kuijlen *et al*. (2006) studied the expression of TRAIL (Tumour necrosis factor Related Apoptosis Inducing Ligand) and TRAIL receptors in a series of 62 patients with primary glioblastoma, and found an independent correlation between its expression and patient survival. Studies investigating Bcl-2 expression in glioblastomas have led to contrasting results both in terms of expression and correlation with patient prognosis. Strik *et al*. (1999) reported an up-regulation of Bcl-2, Bcl-xl and Mcl-1 and down regulation of Bax in recurrent glioblastomas independent of treatment (Strik *et al*., 1999). In contrast, Martin *et al*. (2002) reported increased Bcl-2 and Bax expression in low grade astrocytomas and low expression in glioblastoma when studied by immunohistochemistry and *vice versa* on immunoblotting, indicating that the proteins are expressed at different levels in the cell. The same study found no difference in Bcl-xl expression between low grade astrocytomas and glioblastomas. Overexpression of Bcl-2 or Bcl-xl in glioblastoma cell lines leads to resistance to apoptosis and has also been linked to increased tumour cell motility (Wick *et al*., 1998). This leads to enhancement of tumour cell migration and invasion by altering the expression of a set of metalloproteinases and their inhibitors (Wick *et al*., 1998, 2001, & 2004). A series of studies have reported no correlation between Bcl-2 expression and patient survival (Kraus *et al*. 2001; Shrik *et al*., 1999; Martin *et al*., 2001). Ruano *et al*. (2008) found that several apoptosis genes are dysregulated in glioblastoma and negative expression of Bax correlates with adverse clinical outcome. Carlton *et al*. (2002), reported that N-truncated form of Bax; Bax-Gamma correlated with

longer survival in patients with glioblastoma. From the above discussion, it can be inferred that higher expression of pro-apoptotic proteins (e.g., Bax and Bak) is likely to confer a survival advantage whereas an increased expression of anti-apoptotic proteins (e.g., Bcl-2 and Bcl-xl) is likely to be associated with poor prognosis.

Caspase 3 immunohistochemical expression has been reported to correlate positively with increased tumour grade and early recurrence in intracranial meningiomas (Konstantinidou *et al*., 2007). Similarly Caspase 3 immunohistochemical expression has been associated with increasing tumour grade in gliomas but not significantly correlated with patient survival (Kobayashi *et al*., 2007). While Caspase 3 is not strictly a "point of no return" on the route to apoptosis as further caspase activity can be inhibited by IAP, cIAP1 and Survivin (Salvesen and Duckett, 2002; Mellai and Schiffer, 2007), its place downstream in the apoptotic pathway makes it an indirect but effective marker of apoptosis (Mellai and Schiffer, 2007).

This thesis has analysed the immunohistochemical expression of four members of the Bcl2 family including Bcl2, Bcl-xl, Bax and Bak. These proteins play a major role in mitochondrial pathway of apoptosis and as discussed previously, have an important role in oncogenesis. The expression of final executioner caspase, Caspase 3, has been studied as an indirect marker of apoptosis.

1:15) Autophagy

The word autophagy derives from Greek words, "auto" oneself and "phagy" to eat. Autophagy refers to a cellular degradation pathway that involves the delivery of cytoplasmic substrates to the lysosomes. Three forms of autophagy are described (Klionsky 2005; Reggiori 2012). These include chaperone mediated autophagy, microautophagy and macroautophagy. These forms are involved in different physiological functions and also differ in the mode of delivery of cytoplasmic substrates.

In eukaryotic cells, macroautophagy (herein referred to as Autophagy) is the main regulatory degradation pathway and is involved in breakdown of long lived proteins and cellular organelles (Klionsky 2005; Reggiori 2012). Autophagy is a highly conserved multi-step pathway, in which cytoplasmic substrates sequestered inside double membrane vacuoles are delivered to lysosomes. This involves formation and expansion of an isolation membrane called phagophore, which then fuses to form a double membrane vesicle called an auotphagosome. The autophagosome then fuses with a lysosome to form an autophagolysosome, where the sequestered cytoplamic substrate is then catabolized by lysosomal enzymes (Klionsky and Ohsumi 1999; Klionsky 2005; Reggiori *et al*., 2012).

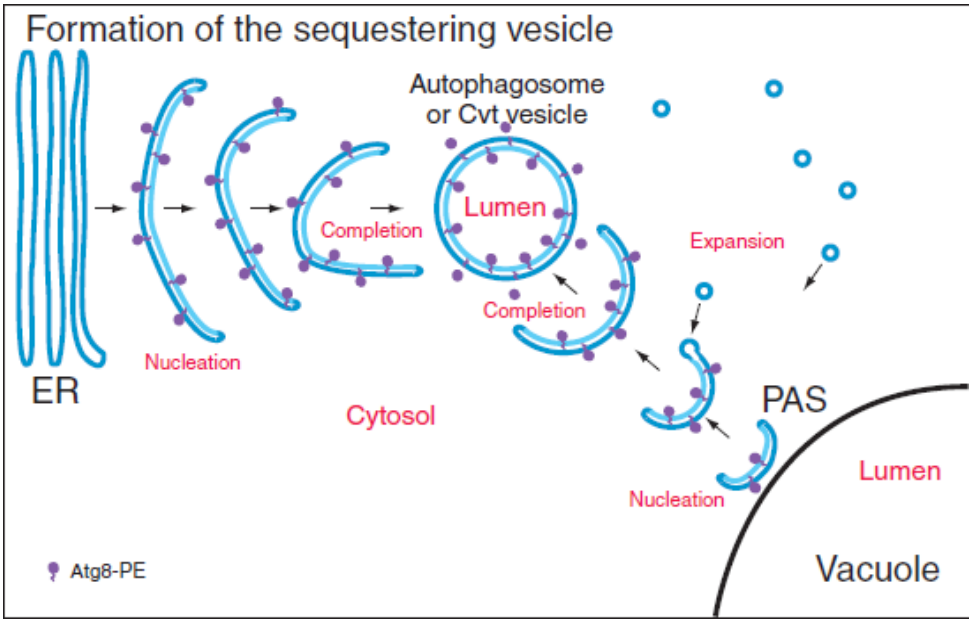


Figure 1.11: Schematic model for formation of the autophagosome or Cvt vesicle. In the model shown on the left, a membrane sheet from a pre-existing organelle such as Endoplasmic reticulum (ER) is induced to separate, undergo deformation and form a spherical shape that eventually seals. In this model, no additional membrane is needed for subsequent vesicle expansion. In the second model shown on the right, a portion of membrane forms the nucleus of the autophagosome or Cvt vesicle. In Yeast this nucleus is called Pre-autophagosomal structure (PAS). Additional membrane of unknown origin is then added to allow subsequent membrane expansion (Klionsky, 2005).

1:16) Autophagy Flux

Autophagy flux refers to the complete process of autophagy including the delivery of substrate to lysosomes and its subsequent breakdown and recycling. It is important to note here that autophagy is a dynamic, multistep process that can be positively and negatively modulated at several levels. Monitoring autophagy flux requires cautious interpretation. For example, an accumulation of autophagosomes could reflect either increased autophagosome formation due to increased autophagic activity or reduced turnover of autophagosomes. The latter can be due to defective autophagolysome formation or inefficient degradation of substrate (Reggiori *et al*., 2012).

1:17) Autophagy in cellular homeostasis

Autophagy is involved in protein and organelle turnover and bulk degradation in virtually all cells and is regulated in accordance with the intracellular energy and nutrient demands, structural remodelling and in degradation of accumulated toxic cytoplasmic aggregates. This makes it an important component of cellular homeostasis in basal conditions and an adaptive response in response to nutrient depletion, growth factor withdrawal, high energy demands and conditions of cellular stress, for example during infection, oxidative stress and protein aggregate accumulation (Shintani *et al*., 2004; Rubinsztein 2007). This degradation of proteins generates amino acids which can then be used for de novo synthesis of proteins or together with fatty acids used to maintain cellular Adenosine Triphosphate production. Autophagy is been implicated in limitation of DNA damage and chromosomal damage, however, the precise mechanism and the extent of its role is unclear and subject of ongoing research.

1:18) Autophagy in cell death

Autophagy under most circumstances is an important part of cellular adaptive pathway that promotes cell survival during periods of cellular stress. Paradoxically it is also considered to be an important contributor to non-apoptotic cell death or Programmed Cell Death Type II (PKD II) (Bursch *et al*., 2004). PKD II has a well defined phenotype characterized by abundance of autophagosomes and autolysosomes in the dying cell and differs from apoptotic cell death in that the dying cell is degraded by its own lysosomes rather than by phagocytosis. While Apoptosis or Programmed Cell Death type I is the main mechanism of cell death, certain stimuli or conditions seem to induce Autophagy or PKD II as the main mode of cellular demise. Also, certain stimuli can induce either apoptosis or autophagy in a mutually exclusive way, possibly due to different thresholds required for both processes (Maiuri et al., 2007).

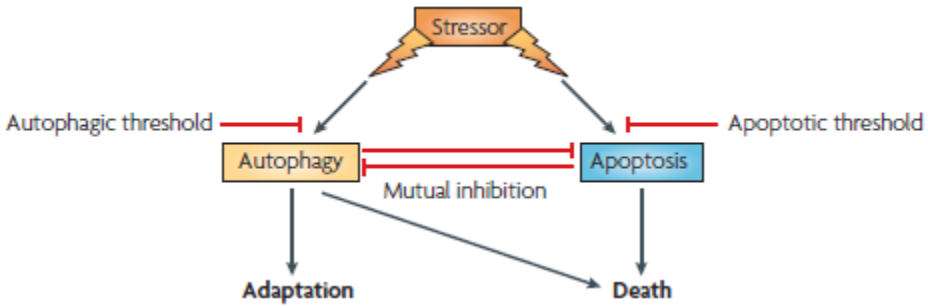


Figure 1.12: Autophagy and apoptosis can be induced by similar stressors in a context-dependent fashion. The exact mechanism of this process is not known however it could involve different sensitivity thresholds of the two processes or a degree of mutual inhibition between the two processes. Also in some cases a mixed phenotype can be detected. The prime function of autophagy remains adaptation to cellular stress but massive autophagy can result in cell death. (Maiuri et al., 2007)

1:19) Autophagy pathway

The process of autophagy is governed by a diverse set of protein encoded by Autophagy related genes (ATG). Most of these genes have been characterized and studied in Yeast and subsequent studies have identified highly conserved mammalian homologues. Thirty six ATG proteins have been identified thus far and 16 of these are essential for all autophagy related pathways. Upon induction, specific ATG proteins govern a tightly regulated hierarchal process (Itakura and Mizushima, 2010; Suzuki *et al*., 2007). This involves formation of the phagophore with subsequent expansion into an autophagosome (Xie and Klionsky, 2007; Yoshimori and Noda, 2008). The proteins are classified into four groups.

1. The Atg1/ULK complex
2. The phosphatidylinositol 3-kinase (PI3K) complex
3. The Atg9 trafficking system
4. 2 parallel ubiquitin like conjugation systems.

The Atg1/ULK complex comprises of Atg1, Atg 13 and Atg 17 in yeast with the mammalian equivalents being ULK1/2, Atg 13 and Atg 101. The complex has a central role in induction of autophagosome biosynthesis and is a terminal target of various signalling cascades including TOR, insulin, PKA and AMPK pathways (He and Klionsky, 2009). Activation of Atg1/ULK kinase induces and upregulates autophagy. ULK kinases (ULK 1, 2 and 3) are stimulated through phosphorylation and dephosphorylation modifications of various subunits of the Atg1/ULK complex. The PI3K complex in mammals exist in three forms: class I, II and III. Class I and III are involved in autophagy whereas the function of class II is currently unknown. Class I is involved in modulation of signalling cascades and Class III PI3K regulate organelle biosynthesis. Class III PI3K has three common components hVps34, p150 (Vps 15 in yeast) and Beclin 1 (Atg 6 in yeast). The fourth component can be either Atg 14L

or UVRAG (Atg 14 and Vps38 in yeast respectively) (Itakura *et al*., 2008; Liang *et al.*, 2006; Sun *et al*., 2008).

The Atg14L containing complexes work by directing the Class III PI3K complex I to phagophore to produce phosphatidyl-inositol-3-phosphate (PI3P) which in turn initiates recruitment of other Atg proteins. Atg14L exist on endoplasmic reticulum in all states of autophagy (Matsunaga *et al*., 2010). Upon induction of autophagy, Atg14L localizes to autophagosome membranes (Itakura *et al.*, 2010). Depletion of Atg14L reduces PIP3 production and impairs formation of autophagosome precursor proteins and inhibits autophagy.

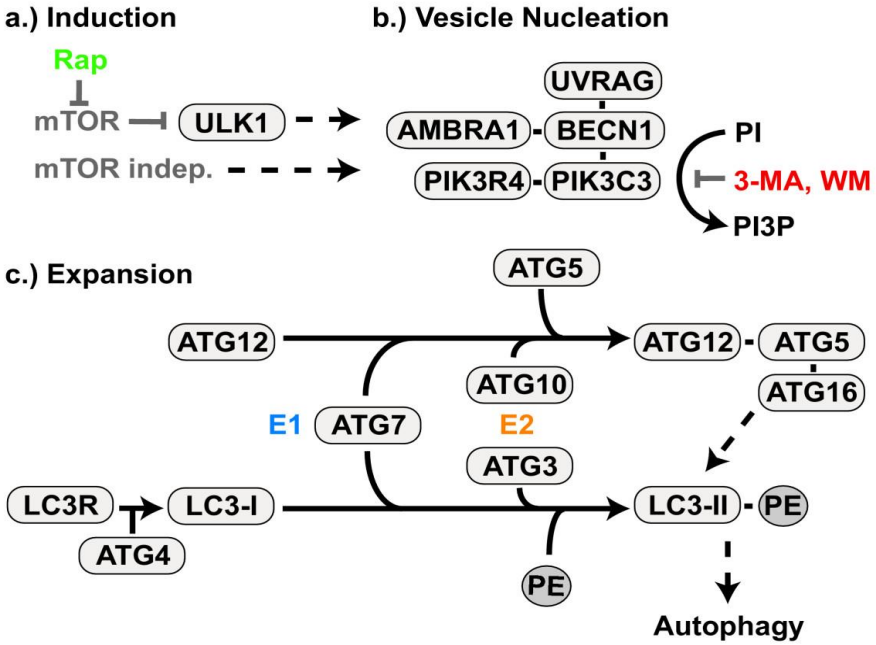


Figure 1.13: Autophagy pathway in mammals. a.) Autophagy can be induced via mTOR dependent or independent pathways which stimulate the nucleation and expansion of the phagophore/isolation membrane. b.) A multi-protein complex surrounding BECN1 with PI3K activity (mediated by PIK3C3) is important for the formation of the autophagosomal membrane. c.) Two ubiquitin-like modification systems are essential for mammalian autophagy; ATG12 is activated by ATG7 (E1 step), transferred to ATG10 (E2 step), conjugated to ATG5 and subsequently forms a complex with ATG16. This step is necessary early in autophagy for the formation of the phagophore or isolation membrane. MAP1LC3 (LC3) is cleaved by ATG4, activated by

ATG7 (E1 step), transferred to ATG3 (E2 step), and conjugated to the phospholipid phosphoethanolamine (PE). This form known as MAP1LC3-II (LC3-II), localizes to the autophagosome membrane and is subsequently degraded in the lysosome. ATG4 cleaves off a C-terminal arginine (R) to expose a glycine residue that is then being linked to PE. Rapamycin (Rap) inhibits mTOR and activates macroautophagy, while 3-methyladenin (3-MA) and wortmannin (WM) inhibit the PI3K activity and de-activate macroautophagy. (Jaeger and Wyss-Coray, 2009)

The UVRAG containing class III PI3K complexes interact primarily with the endosomal transport pathways. UVRAG initially associates with BAR-domain protein Bif-1 which may regulate mAtg9 trafficking from the trans-Golgi network (TGN) (Takahashi *et al*., 2007; Takahashi *et al*., 2011). UVRAG then promotes the fusion of autophagolysosomes with late endosomes and/or lysosomes by interacting with class C vps/HOPS protein complex. Lastly UVRAG-containing class III protein complex binds to Rubicon, a late endosomal/lysosomal protein that reduces hVps34 activity and thereby suppresses autophagosome maturation.

Of note, both Atg14L and UVRAG containing PI3K complexes interact through Beclin 1 with Ambra 1, which in turn tethers these protein complexes to the cytoskeleton via an interaction with dynein. Once autophagy is induced, ULK1 phosphorylates Ambra 1 thereby resulting in the release of Class III PI3K complexes from dynein (Bartolomeo *et al*., 2010; Fimia *et al*., 2007). PI3K class III complexes then trigger autophagosome formation. Ambra 1 thus constitutes the direct regulatory link between Atg1/ULK1 and the PI3K complexes (Bartolomeo *et al*., 2010).

Atg9 is a highly conserved transmembrane protein that is essential for autophagy. It is distributed to the phagophore assembly site (PAS) and multiple additional cytoplasmic tubulovesicular compartments derived from the Golgi (Noda 2000; Ohashi and Munro 2010; Reggiori *et al*., 2004). Atg9 is thought to act as membrane carrier providing lipid building

blocks for the expanding phagophore (Reggiori *et al*., 2004) and is one of the first factors to localize to PAS together with Atg1/ULK and PI3K complexes.

Two closely interconnected ubiquitin-like proteins Atg12 and Atg8/microtubule-associated protein (MAP1)-light chain 3 (LC3) form part of the core autophagy machinery (Ichimura, *et al*., 1998; Mizushima *et al*, 1998; Yang and Klionsky, 2010). Atg12 is conjugated to Atg5 through the activity of Atg7 (E1-like) and the Atg10 (E2-like) enzymes. The Atg12-Atg5 conjugate then interacts with Atg16 which then oligomerizes to form a large multimeric complex. Atg4 protease cleaves the C-terminus of Atg8/LC3 and generates a cytoplasmic LC3-I with a C-terminal glycine residue. LC3 I then conjugates to phosphatidylethanolamine (PE) in a reaction that requires Atg7 and the E2 like enzyme Atg3. This generates LC3 II, a lipidated form which is attached to both faces of the phagophore membrane. Once the autophagosome is formed, Atg4 removes LC3 II from the outer autophagosome surface.

The two systems are partially overlapping and closely interconnected. The multimeric Atg12-Atg5-Atg16 complex localizes to the phagophore and acts as an E3-like enzyme determining the site of Atg8/LC3 lipidation. On the other hand, Atg8/LC3 lipidation is essential for optimal function of the Atg12 conjugation system. There is also evidence to suggest that these two conjugation systems function together during expansion and closure of the phagophore.

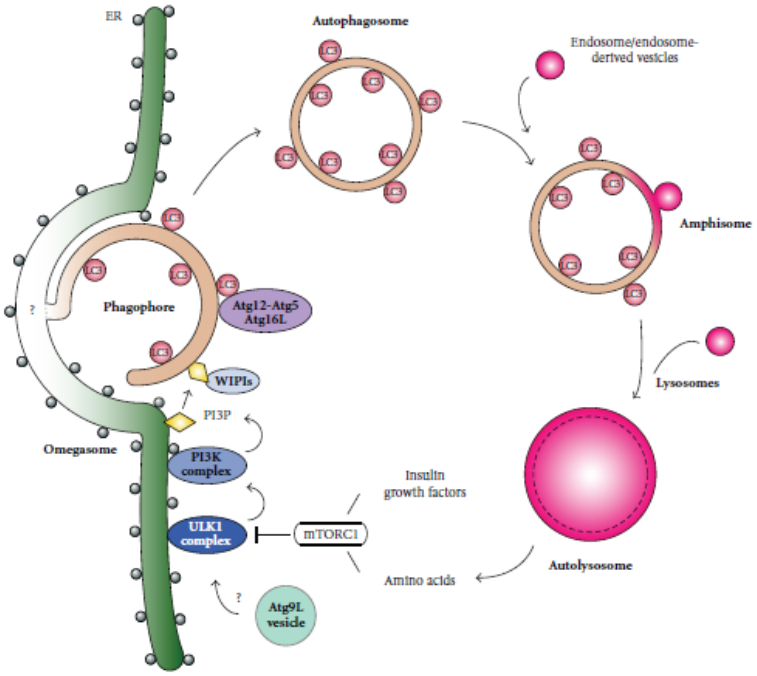


Figure 1.15: Atg proteins and their interaction to form autopgaosome in response to mTORC1 or other environmental cues. (Regiorri et al., 2012).

Recent evidence suggests that autophagy is a highly selective process relying upon specific cargo-recognizing autophagy receptors which connect the cargo to the autophagic membranes. Autophagy receptors are proteins capable of interacting with autophagosome cargo and the Atg8/LC3 family members through a specific (WxxL) sequence (Noda *et al.*, 2008), called LC3-interacting region (LIR) motif or LC3 recognizing sequence (LRS). The autophagy receptors interact with specific adaptors which in turn function as scaffolding proteins that bring the cargo-receptor complex in contact with the core Atg machinery and allows for specific sequestration of the substrate. Here, it is important to note that that the selective types of autophagy rely on the same molecular core machinery as non-selective (starvation-induced) bulk autophagy. Also it seems that the autophagy receptors and specificity receptors are not required for non selective autophagy (Reggior, *et al*., 2012).

1:20) AKT/mTOR pathway

An important regulator in the autophagy pathway is mTOR (mammalian target of Rapamycin) which directly phosphorylates Atg 13 thereby modulating its binding to Atg 1 and Atg 17. Inactivation of TOR leads to rapid dephosphorylation of Atg 13 which increases Atg1-Atg13-Atg17 complex formation which in turn stimulates Atg 1 kinase activity leading to induction of autophagy (Kamada *et al*., 2000; Yang, and Klionsky, 2009). Thus the activated PI3K/AKT/mTOR pathway negatively regulates autophagy. The activity of the PI3K/Akt and the mTOR pathway is often constitutively up regulated in tumours as a result of stimulation by growth receptors and mutations of the PTEN (Phosphatase and Tensin homologue deleted on chromosome ten) tumour suppressor gene. The tumour suppressor activity of PTEN is mainly implemented through its inhibitory effect on the PI3K-dependent activation of Akt signalling (McCubrey *et al*., 2006).

As discussed previously, class III PI3K plays a role in early stages of autophagosome formation. Class I PI3K activity inhibits autophagy and its effect is partly mediated via the mTOR pathway.

In addition, Beclin 1 which is a part of Class III PI3K complex, also binds Bcl-2 pro-apoptotic family members (Pattingre and Levine, 2006). It is interesting to note here that the interaction of Beclin 1 with class III PI3K stimulates autophagy and inhibits oncogenesis while its interaction with Bcl-2 inhibits autophagy and stimulates oncogenesis (Pattingre and Levine, 2006).

1:21) p62 and autophagy

One of the best studied mammalian autophagy receptors is p62/sequestosome 1 (SQSTM1). It binds ubiquitinated protein aggregates through an ubiquitin associated (UBA) domain and

to LC3 via LIR motif. This triggers aggregate formation through the oligomerization of p62 via its Phox and Bem1p (BP1) domain, thereby promoting specific autophagic degradation of ubiquitinated proteins. Ubiquitination of proteins and organelles serves as a signal for recognition by p62 which are then themselves degraded together with the associated substrate. P62 has been implicated in degradation of other substrates for example bacteria, viral capsid proteins, peroxisomes, damaged mitochondria and bacteriocidal precursor proteins. Suppression of autophagy leads to accumulation of p62 in large aggregates which are also positive for ubiquitin (Komatsu *et al*., 2007; Nezis. *et al.,* 2008). P62 inclusion bodies have been detected in neurodegenerative conditions, liver disorders and also cancer, including malignant gliomas (Zatloukal *et al*., 2002, Moscat and Diaz-Meco, 2009).

Recent evidence points to a much more central role of autophagy in tumour cell biology with influences on cell growth, survival and mitosis. It biochemically links nutrient sensing to signalling cascades that regulate inflammation and reactive oxygen species (ROS) levels, an important mechanism for tumour cell survival in conditions of autophagy defect (Moscat and Diaz-Meco, 2012). Also recent evidence points to a role of p62 in cell cycle transit by means of its interaction with CDK1 (Moscat and Diaz-Meco, 2012).

1:22) Autophagy in Glioma

Resistance to apoptosis is characteristic of many cancer cells (Viktorsson *et al*., 2005; Ricci and Zong, 2006). This has implications not only for tumourogenesis but also resistance to treatment including radiotherapy and chemotherapy (Okuda and Mak, 2004).

Recent evidence suggests that gliomas are resistant to apoptosis or programmed cell death type I but seem to be less resistant to autophagic cell death or programmed cell death type II. Indeed Temozolomide, the most successful chemotherapeutic agent in glioblastoma, seems to work by inducing autophagic cell death in glioma cells rather than apoptosis (Kanzawa *et al*.,

2004). Kanzawa and colleagues demonstrated this in glioblastoma cell lines by demonstrating the induction of autophagy, recruitment of LC3 to autophagosome membranes, inhibition of tumour cell vibility and induction of G2/M arrest in malignant glioma cells when treated with clinically achievable dose of Temozolomide. When autophagy was prevented in these cells at an early stage by PI3K inhibitor, LC3 localization to autophagosomes and antitumour effects of Temozolomide were suppressed.

Other agents that induce autophagic cell death in cancer cells include tamoxifen, rapamycin, adenoviruses, and gamma irradiation (Ito *et al*., 2005; Paglin *et al.,* 2001). Radiotherapy and Temozolomide are important in this context as they are the mainstay of adjuvant treatment for glioblastoma. Radiotherapy seems to induce autophagic cell death at least in glioma cell lines *in vitro* (Ito *et al.,* 2005). Temozolomide causes glioblastoma cells to undergo G2/M arrest and induce autophagic cell death (Kanzawa *et al*., 2004, Kanzawa *et al*., 2003). Part of Temozolomide activity also seems to be by induction of late apoptosis. This seems to be dependent on p53 and MGMT (methylated O^6-methylguanine-DNA methyltransferase promoter) status. Although contradictory, autophagy and apoptosis can be triggered by common upstream signals (LeFranc *et al.,* 2007). In certain circumstances apoptosis and autophagy can occur simultaneously whereas in other situations the cell switches between them in a mutually exclusive manner (LeFranc *et al*., 2007).

1:23) Measurement of autophagy flux:

Autophagy flux can be calculated by measuring the accumulation of autophagosomes as measured by electron microscopy image analysis, using green fluorescent protein (GFP) tag at the N-terminus, GFP-LC3, which is then reflected as an increase in punctuate dots under flouresecent microscope or western blot analysis of LC3 lipidation (conversion of LC3 I to LC3 II). In mammals the LC3 subfamily contains LC3A, LC3B, LC3B2 and LC3C. LC3 II

is the only protein marker that is reliably associated with completed autophagosomes and is, therefore, the most commonly used biomarker to calculate autophagy flux. LC3B immunohistochemical expression has been described previously in glioblastoma (Aoki *et al.*, 2008). This thesis aims to investigate autophagy flux in glioblastoma by analysing the immunohistochemical expression of both LC3B and p62 in glioblastomas.

1:24) Project aims:

Glioblastoma oncogenesis is an area of active research and whilst our understanding of this subject continues to progress, the development of effective therapies remains slow and patient prognosis remains poor. Markers of cell division cycle and apoptosis have been shown to be of prognostic and predictive value in other cancers, and the role of autophagy in oncogenesis is a subject of topical current research. This thesis aims to analyse markers of cell division cycle, apoptosis and autophagy flux in Glioblastoma and to identify prognostic and/or predictive markers that can be employed in everyday practice to stratify and support clinical decision making in the management of patients with Glioblastoma.

Chapter 2

Materials and Methods

Ethical approval and patient consent – Collection of patient data - Tissue collection - Fixation - Processing - Paraffin embedding - Section cutting - Preparation of tissue micro-array - Haematoxylin and Eosin (H&E) staining - Immunohistochemistry for markers of apoptosis, autophagy and cell cycle phase - Quantification of immunohistochemistry results - Statistical analysis.

2:1) Ethical Approval and patient consent:

Approval to conduct the study was sought from the Local Research Ethics Committee, Hull and East Yorkshire Hospitals NHS Trust[1]. This approval was initially obtained in 2002 to investigate the dysregulation of immune system in patients with intracranial tumours. A further approval was obtained in 2008 to include research on markers of apoptosis and cell cycle phase distribution. Patients were recruited following informed consent in accordance with the recommendations issued by the General Medical Council[2].

[1]PIS/Studygroup/Version4 12/05/2008. Ww/CJB/JEP/10.04.00/version1/informed consent/r&ddept/ HEYHT

[2]General Medical Council – Research: The role and responsibilities of doctors, 2002. http://www.gmc-uk.org/guidance/current/library/research.asp

2:2) Collection of patient data: The data was collected on:

- Patient demographics
    - Age
    - Sex
- Pre-operative Karnofsky performance score
- Date of diagnosis, taken as the date of first diagnostic biopsy
- Date of surgery
- Type of surgery (Based on surgeon's observations recorded in the operative note)
    - Image guided biopsy
    - Debulking
- Radiotherapy
- Chemotherapy
    - PCV (Procarbazine, Lomustine and Vincristine) chemotherapy
    - Temozolomide chemotherapy
- Date of death
- Patient survival in days, calculated from the date of diagnosis

The data was collected retrospectively for patients recruited to the study between 2002-2007, using hospital notes, Neuro-oncology Multidisciplinary team meeting records, outpatient clinic letters and the information on Patient Centre®[3]. Data was collected prospectively for patients recruited from June 2007 onwards. All data was collected on Microsoft® Excel spreadsheets and was stored in compliance with the Data protection Act 1998 [4].

[3]iSOFT PatientCentre 3.12.1102. Hull and East Yorkshire Hospitals NHS Trust intranet patient information software.

[4]Data Protection Act 1998. http://www.opsi.gov.uk/Acts/Acts1998/ukpga_19980029_en_1

2:3) Tissue collection:

Tumour tissue was obtained from recruited patients intra-operatively and sent to the Neuropathology Unit at Hull Royal Infirmary for further diagnostic work. Where possible, the tissue was sent fresh and upon receipt in the Neuropathology Unit, where feasible, approximately $1cm^2$ blocks of tumour tissue were snap frozen in a vial of liquid nitrogen and then transferred to a minus 75^oC freezer (New Brunswick Scientific freezer, Scientific Laboratory Supplies). The remainder of the tumour tissue was processed and embedded in paraffin wax as described later. The frozen and paraffin embedded tissue were stored in the diagnostic archives of the Neuropathology Unit, Hull Royal Infirmary. For the purpose of this research work, tissue blocks and stained diagnostic slides were removed and archived in accordance with the Human Tissue Act 2004[5]. Stained research slides produced from the above material were catalogued and stored in locked cabinets at the Neuropathology Unit, Hull Royal Infirmary.

2:4) Fixation:

Neutral buffered formalin[6] was used as for fixative for the biopsied tumour tissue. Depending on sample size, biopsies were fixed for 12-24 hours prior to processing and embedding.

[5]Human Tissue Act 2004. http://www.opsi.gov.uk/ACTS/acts2004/ukpga_20040030_en_1

[6]Neutral buffered Formalin: 40% formaldehyde 100ml, distilled water 900ml, sodium dihydrogen phosphate monohydrate 4g, disodium hydrogen phosphate anhydrous 6.5g.

2:5) Processing:

Tissue processing allows tissue fixed in water based fixatives to be impregnated by paraffin wax. The wax embedded tissue allows thin sections to be cut using a microtome. This process involves the following steps:

Dehydration: This step moves the fixative and water from the tissue and replaces them with dehydrating fluid, usually using a series of alcohols of increasing concentration.

Clearing: This step involves the replacement of dehydrating fluid with a fluid that is totally miscible with both the dehydrating fluid and the embedding medium. Chloroform is the agent used for clearing in our laboratory.

Impregnation: The step replaces the clearing agent with the embedding medium.

A typical automated 16 hour processing schedule, as used in our laboratory, is as follows: 10% formalin starting solution (60 minutes), 70% alcohol (60 minutes), 90% alcohol (60 minutes), Absolute Ethanol (4 cycles of 60, 90, 90 and 90 minutes), Chloroform (3 cycles of 60 minutes), Histowax (Leica) (3 cycles of 90, 60 and 60 minutes). Automated processing was performed using Leica TP1050 automated processing machine.

2:6) Paraffin wax embedding: Tissues were embedded in paraffin wax (Histowax, Leica) using a Leica EG1166 embedding station.

2:7) Section Cutting: Sections of 4μm thickness were cut using a cut using a rotary microtome (Leica RM2135). The sections were cut onto coated glass slides[7], dried on a hot plate and then transferred to a 37^{o}C incubator till they were ready to be stained.

2:8) Preparation of tissue micro-array:

The tumour tissue blocks and slides were obtained from the diagnostic archive and were reviewed to ensure that the diagnosis of Glioblastoma conformed to the WHO 2007 guidelines. The representative tissue was identified by the author together with Dr IS Scott and the tissue slides and blocks were marked. A tissue micro-array was then constructed by the author with a manual Tissue Micro-Arrayer[8] using a 6mm needle. This was performed at the Neuropathology Department, Queen's Medical Centre, Nottingham University Hospitals NHS Trust. Sections (4μm) were then cut off the tissue micro-array block using a rotary microtome as described before.

[7]Superfrost® Plus Slides, Menzel-Glaser, Menzel GMBH & Co KG, Braunschweig.

[8]Manual Tissue Arrayer 1, Beecher Instruments, Inc. USA. Supplied by Mitogen, UK.

2:9) Haematoxylin and Eosin (H&E) staining:

The sections from the tissue micro-array on coated slides were heated on a hotplate for a minimum of 15 minutes, dewaxed in xylene over 5 minutes and then rehydrated by taking through a series of alcohols. The slides were stained with Gill III Haematoxylin[9], rinsed in running tap water and differentiated with 1% acid alcohol[10]. The sections were then rinsed in running tap water and blued in ammonia solution. This was followed by counter stain with Eosin[11]. The sections were then mounted in DPX[12] (refractory index 1.52). The H&E stained tissue micro-array slides were then analyzed to re-confirm the presence of representative Glioblastoma tissue in the arrays.

[09]Gill III Haematoxylin solution, Surgipath Europe Ltd. Peterborough, UK.

[10]Eosin Solution: Prepare a 1% solution of eosin and add a crystal of phenol to inhibit mould formation. Filter prior to use.

[11]1% Acid alcohol: 1% hydrochloric acid, 70% alcohol.

[12]BDH DPX mountant (VWR International, Poole, UK. Cat. No. 360294H)

2:10) Immunohistochemistry for markers of cell cycle phase, apoptosis and autophagy:

Formalin fixed, paraffin-embedded tissue micro-array tissue sections on coated slides were stained using a technique standard in the Neuropathology Laboratory at Hull Royal Infirmary. The slides were heated on a hotplate for a minimum of 15 minutes, dewaxed in xylene over 5 minutes and then rehydrated by taking through a series of alcohols. The slides were rinsed in running tap water and placed in a solution of 3% hydrogen peroxide[12] in distilled water for 30 minutes to quench endogenous tissue peroxidase activity. The slides were then placed in a slide bath containing antigen retrieval citrate buffer solution[13] and antigen retrieval was carried out by heating the slide bath in a microwave oven at 800 Watt for 4 minutes and 150 Watts for 16 minutes. Following antigen retrieval, the slides were rinsed with running cold water. The slides were then loaded on cover plates, placed into Sequenza racks and rinsed in phosphate buffered saline[14] (Bio-stat Diagnostics system) for 7 minutes. 5% Normal goat serum[15] in phosphate buffered saline (PBS) was then applied to the slides for 20 minutes to block non-specific antibody binding sites. Primary monoclonal and polyclonal antibodies were prepared to appropriate dilutions in PBS and applied to the slides (Table 2:1). The slides were incubated with primary antibodies at room temperature for 60 minutes. Following incubation, the slides were rinsed with PBS for 7 minutes.

[12]3% Hydrogen peroxide solution: 3ml of H_2O_2 (30% w/v, Merck, Germany) in 100ml distilled water.

[13]Antigen retrieval citrate buffer concentrate. Preparation by adding 10ml of the buffer concentrate to 1000ml of distilled water, adjust pH to 6.0±0.1. (Cat. No. HDSO5, HD Supplies, Aylesbury, Bucks)

[14]Phosphate buffered saline (Lot number 450040, J T Baker, London UK).

[15]Normal Goat Serum (Dako): Preparation by diluting in phosphate buffered saline to make a 5% solution.

Secondary antibody (Dako REAL™ Envision™/HRP. Rabbit/Mouse ENV)[16] was then applied to the slides and the slides incubated for 30 minutes. The slides were rinsed with PBS for 7 minutes and this was followed by application of DAB+ Chromogen[17]. The slides were incubated with DAB for 5 minutes. The slides were then rinsed in PBS and Copper sulphate solution[18] was applied to the slides for 5 minutes following which the slides were rinsed again with PBS. Following this, the slides were removed from the Sequenza racks, rinsed in running tap water and counter stained in Gill III Haematoxylin for 30 seconds. The slides were then rinsed in running tap water, differentiated in 1% acid alcohol[19] and blued in ammonia solution. This was followed by dehydration of slides and mounting as described before. Details of individual primary antibodies, their suppliers, optimal concentrations and positive controls are given in the table 2:1. The immunohistochemical staining was performed by the author of the thesis.

[16]Dako REAL™ Envision™ Detection System, Peroxidase/DAB+, Rabbit/Mouse. Code K5007. The kit contains:

1. Dako REAL™ Envision/HRP Rabbit/Mouse (ENV). Dextran coupled with peroxidase molecules and goat secondary antibody molecules against rabbit and mouse immunoglobulins.
2. Dako REAL™ Substrate Buffer. Bufferred solution containing hydrogen peroxide and preservative.
3. Dako REAL™ DAB+ Chromogen. 3,3'-diaminobenzidine tetrahydrochloride in organic solvent.

[17]20µl of DAB+ Chromogen in 1000µl of substrate buffer.

[18]Copper sulphate solution: 4g hydrated copper II sulphate, 7.2g sodium chloride in 1000ml of distilled water.

Antibody Name	Cataloque No.	Description	Final Dilutions	Positive control
MCM-2 antibody CRCT2.1 (D1.9H5)	Abcam ab6153	Mouse monoclonal	1:150	Tonsil*
Geminin antibody	Abcam ab12147	Rabbit polyclonal	1:100	Tonsil*
Cyclin A antibody [6E6]	Abcam ab16726	Mouse monoclonal	1:50	Tonsil*
Histone H3 (phospho S10) antibody – Mitosis Marker	Abcam ab5176	Rabbit polyclonal	1:200	Tonsil*
Bcl-2 Clone 124	Dako	Mouse Monoclonal	1:50	Tonsil*
Bcl-xl Clone 2H12	Sigma Batch No. B9429-0.2mL	Mouse monoclonal	1:100	Hodgkin's Lymphoma
Bax antibody	Abcam ab10813	Rabbit polyclonal	1:500	Tonsil*
Bak antibody	Abcam ab32371	Rabbit monoclonal	1:200	Tonsil*
Active and pro Caspase 3 antibody	Abcam ab47131	Rabbit polyclonal	1:1500	Tonsil*
LC3b	Abcam ab51520	Mouse monoclonal	1:2000	Renal cell carcinoma
p62	BD transduction Lab 610833	Mouse monoclonal	1:50	Renal cell carcinoma

*The tonsil used as a positive control denotes to normal human tonsil tissue.

Table 2.1: Antibody details and dilutions.

2:11) Quantification of immunohistochemistry results:

The number of stained cells per high power field (X40 objective, magnification X400) were counted and a semi-quantitative labelling index was calculated for markers of cell cycle and apoptosis using the following formula.

Labelling index (LI) = Number of stained cells per high power field/Total number of cells per high power field.

At least 9 high power fields were counted for each case.

The staining of LC3B and p62 was calculated using a semi-quantitative staining score. Both cytoplasmic and nuclear staining was taken into account. The staining score was zero if negative and from 2-5 if positive, based on a sum of cytoplasmic staining intensity (1-3) and percentage of nuclear staining (1:less than 50%, 2:more than 50%). Staining score of 0-2 was considered as low and 3-5 as high protein expression.

The slides were reviewed independently by the author of the thesis, Dr IS Scott and Mrs Catherine Hills and the inter-observer variation was less than 5%.

2:12) Statistical analysis:

SPSS[20] version 20 software was used for Univariate statistical analysis on the labelling indices of markers of apoptosis and cell cycle phase. Cox regression and Kaplan Meier survival curves were then calculated to demonstrate the association between patient survival, response to treatment and markers of apoptosis and cell cycle phase.

[20]SPSS. Statistical Package for the Social Sciences. www.spss.com/s

Chapter 3

Results – Markers of cell division cycle

Cell cycle markers - Clinical data - Labelling Indices & Prognosis - Cell cycle markers in recurrent Tumours - Phosphohistone H3 (PHH3) - Discussion

3:1) Clinical data

A total of 66 patients were included in the study between 2007-2009. All patients underwent neurosurgical intervention in the form of either a biopsy or debulking surgery, and were further subdivided into two groups based on the administration of adjuvant treatment. In group 1 (n=50), all patients underwent surgery and radiotherapy with 24 of these patients also receiving temozolomide chemotherapy. Of note, 84% (n=42) of this group of patients underwent debulking surgery. Patients in group 2 (n=16) underwent surgery only, as adjuvant treatment, although planned, was not offered due to poor post-operative performance score. A similar percentage of patients in this group also underwent debulking surgery (87.5%, n=14). Table 1 gives the clinicopathologic details of patients in the two groups. It was observed that the two groups although not completely homogenous, have similar median age (61 years vs. 67 years) and similar percentage of patients undergoing debulking surgery (84% vs. 87.5%). This is important because as noted in chapter 1, age and debulking surgery are important prognostic factors in Glioblastoma.

There were three cases of recurrent tumour that were studied in greater detail. Two of these patients underwent debulking surgery, radiotherapy and temozolomide chemotherapy. The third patient underwent debulking surgery on three occasions and radiotherapy was administered after first surgery.

	Group 1	Group 2
N	50	16
Median age (yr)	61	67
Male : Female	33 : 17	7 : 10
Median Survival (days)	258	65
Debulking surgery (number of patients)	42	14
Preoperative median Karnofsky performance score	60	50
Image guided biopsy (number of patients)	8	2
Radiotherapy (number of patients)	50	0
Temozolomide chemotherapy (number of patients)	24	0

Table 3.1: Clinical data of patients in cell cycle marker study

3:2) Labelling Indices & Prognosis

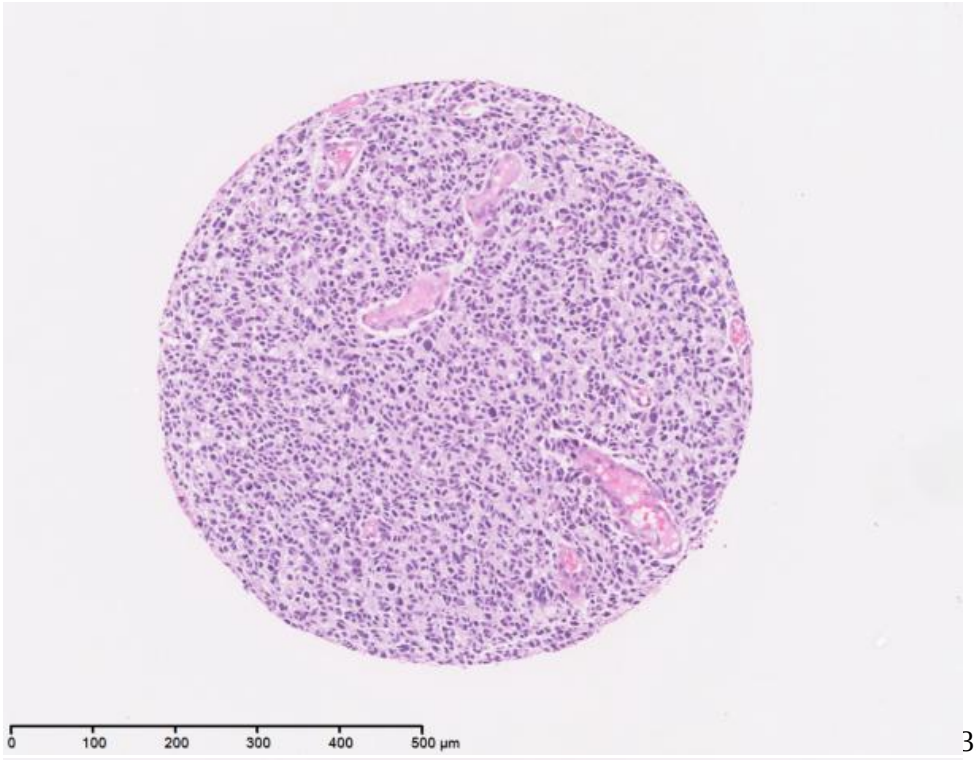
Representative array sections for each marker are shown in Figure 3.1. The median labelling indices of all three markers for group 1 and 2 are summarised in Table 3.2. The median Mcm-2 labelling index (LI) in group 1 was 36.7% Kaplan Meier analysis (Figure 3.2a) did not reveal any association of this factor with survival (Log Rank p= 0.522) although linear regression analysis did reveal a positive correlation with survival (p=0.0376). The median value of cyclin A LI in Group 1 was 4.2% and Kaplan Meier analysis (Figure 3.2b) showed a survival advantage for patients with a higher LI (Log Rank p=0.0063) and linear regression analysis showed a positive correlation with survival (p=0.004). The median level of geminin LI in Group 1 was 7.8%. Kaplan Meier analysis (Figure 3.2c) demonstrated a survival

advantage on patients with a higher geminin LI (Log Rank *p*=0.0046). Linear regression analysis showed a positive correlation between geminin LI and survival (p=0.0006). Survival correlations are shown in Figure 3.4.

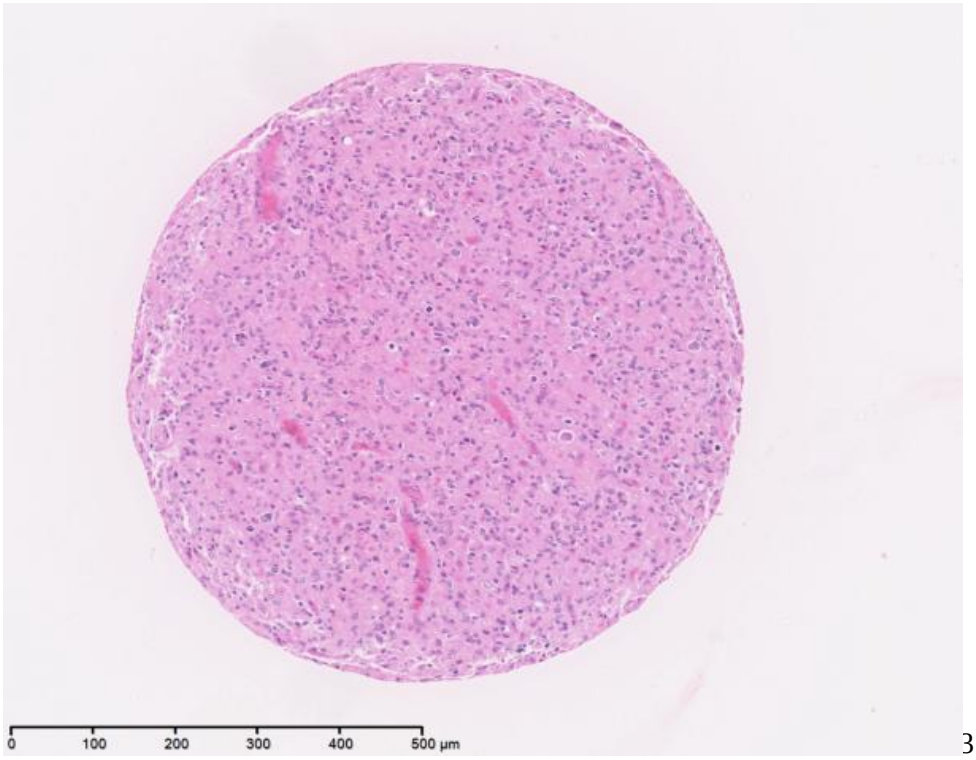
In group 2, there was no statistically significant relationship between patient survival and LI for Mcm-2, cyclin A and geminin (Figure 3.3a-c), although an association between high geminin expression and poor survival was observed (Log Rank p=0.1325). The survival correlation curves for Mcm2, Geminin and Cyclin A labelling indices in group I and 2 are illustrated in Figure 3.5.

	Group 1 (n = 50)	**Group 2 (n = 16)**
Mcm2	36.7 (22.9-51.8)	43.8 (33.5-51.1)
Cyclin A	4.2 (2.4-6.9)	4.8 (3.8-6.4)
Geminin	7.8 (5.8-10.5)	7.6 (5.9-9.1)

Table 3.2: Median Labelling indices (%) with interquartile range of cell cycle markers in group 1and Group 2



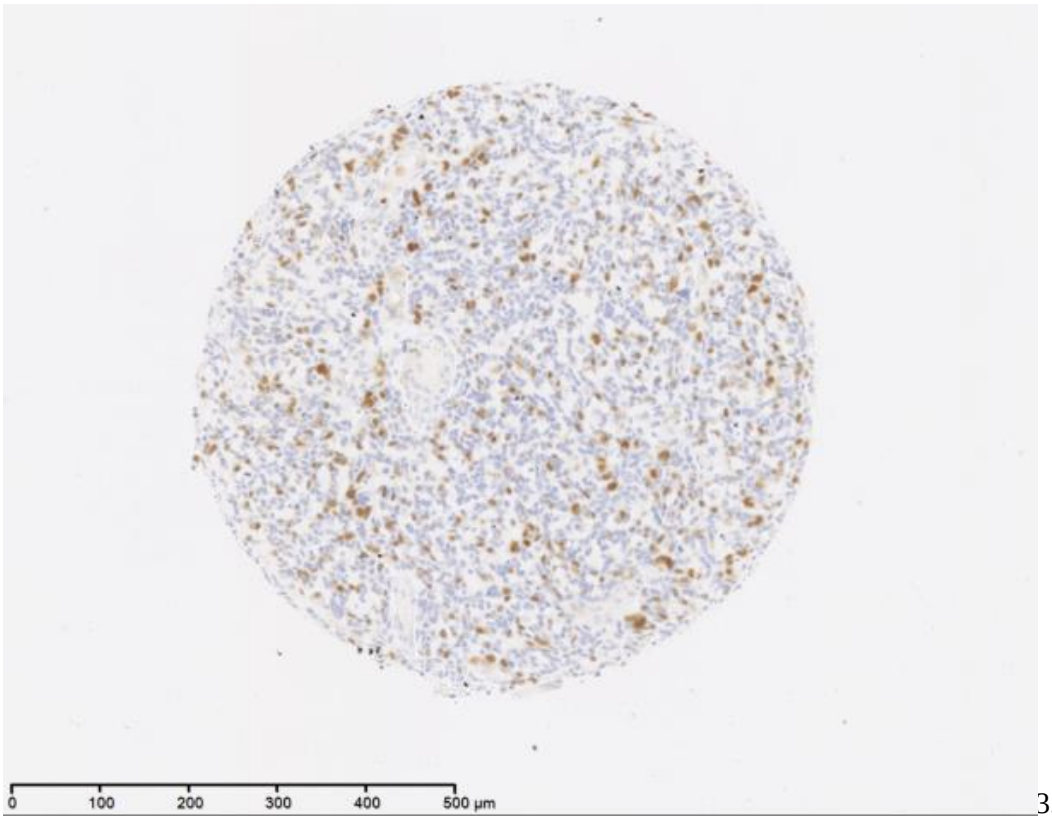
3.1a



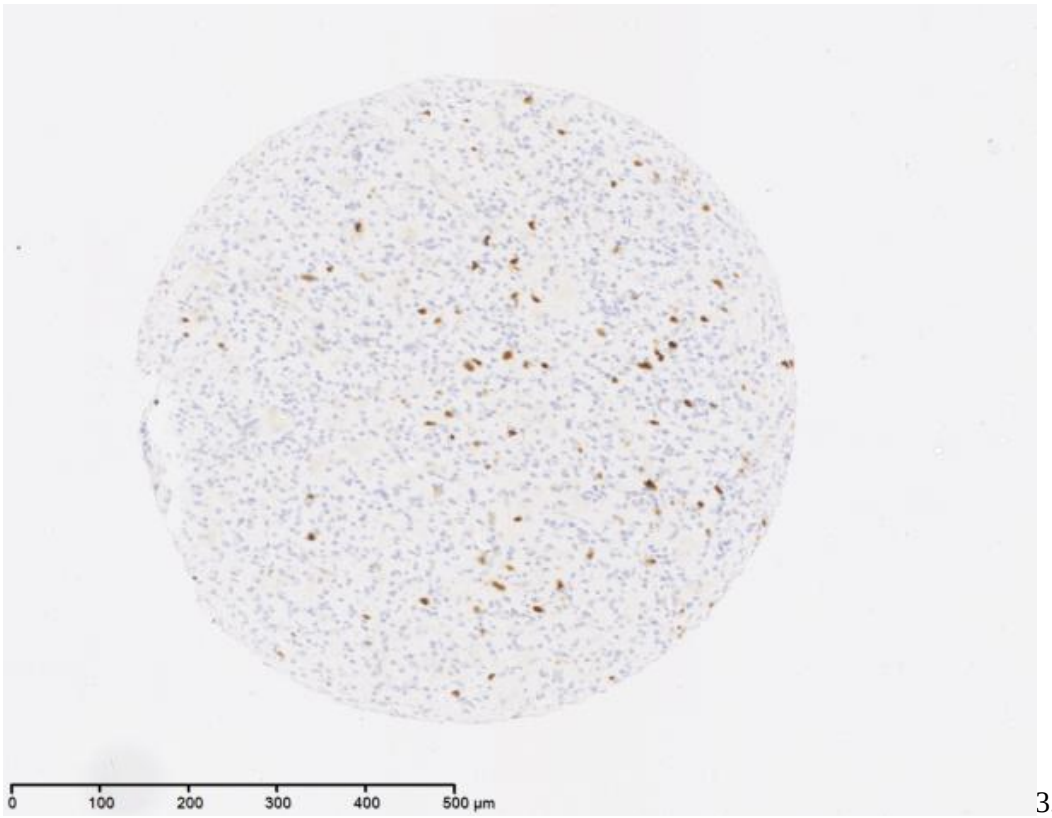
3.1b

Glioblastoma - H&E

Figure 3.1a-h: Representative tissue micro-array discs showing tumours in which there has been greater than median survival following adjuvant therapy (a, c, e, g) and tumours showing a poor response after adjuvant therapy (b, d, f, h). Adjacent levels in the array are shown to allow comparison of similar areas of the tumour. It can be seen that in tumours where there is a good response to adjuvant therapy, there is elevated cyclin A and Geminin expression indicating a greater proportion of cells actively progressing through the cell cycle. The total number of cells in cycle is also greater (Mcm-2 expression).



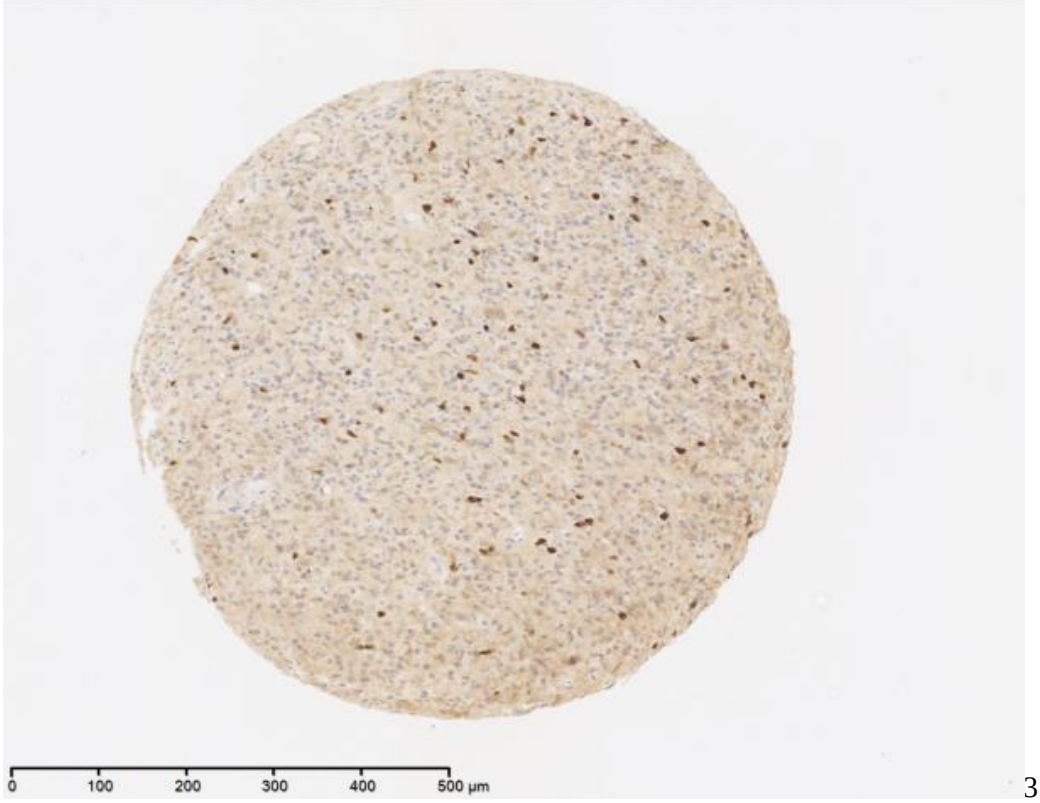
3.1c



3.1d

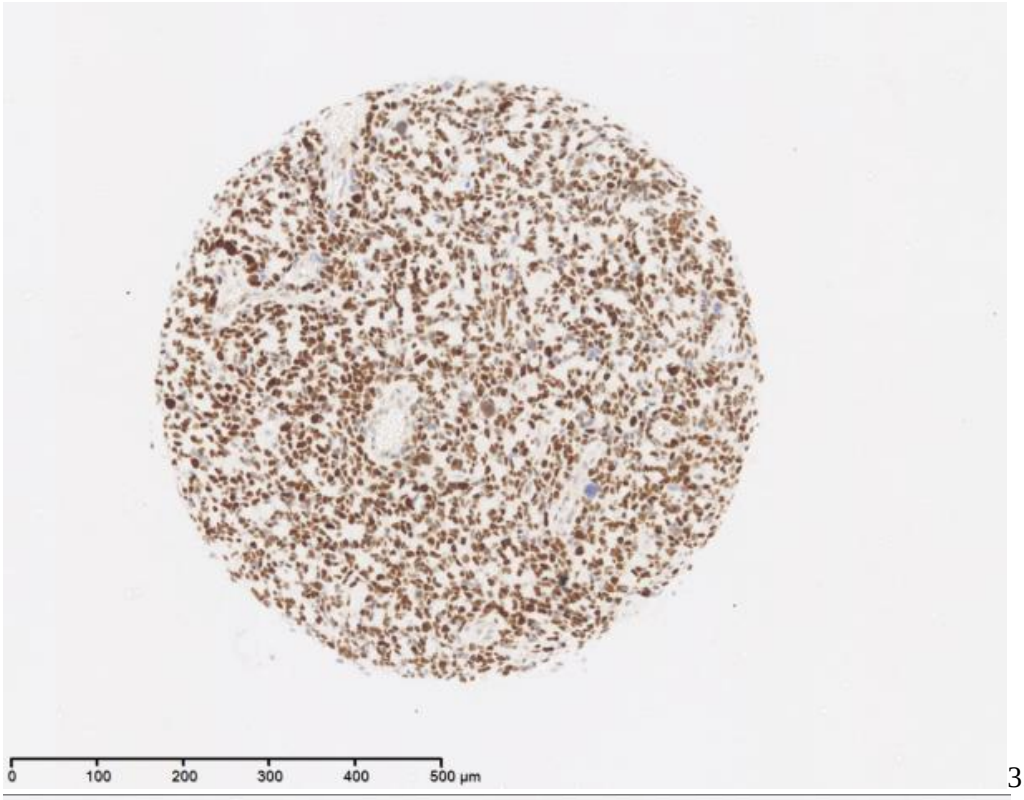
Glioblastoma - Cyclin A (S-phase)

3.1e

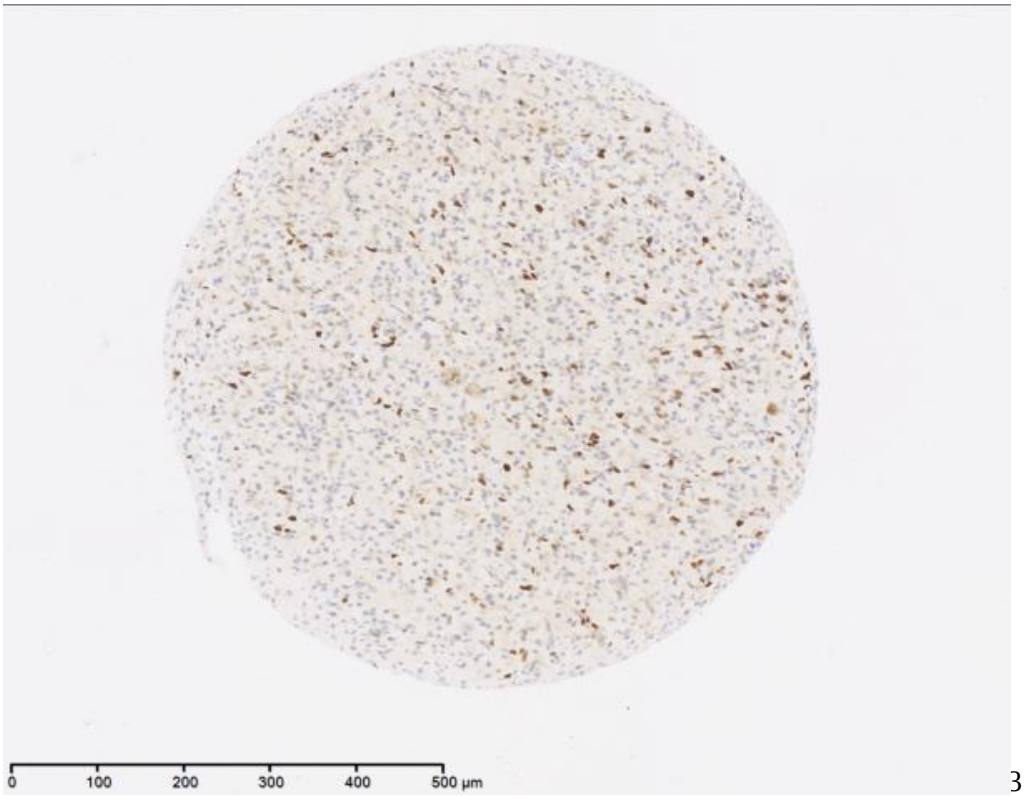


3.1f

Glioblastoma – Geminin (S-phase, G2-phase and Mitosis)

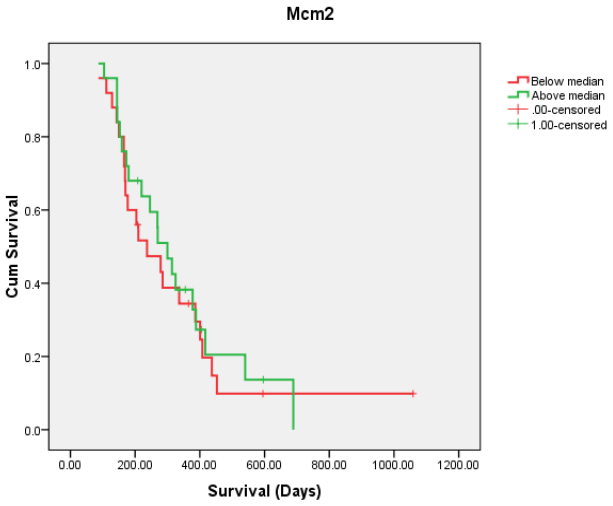


3.1g

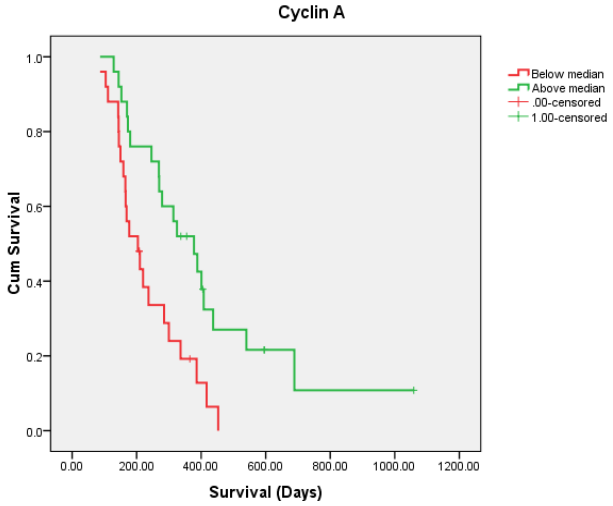


3.1h

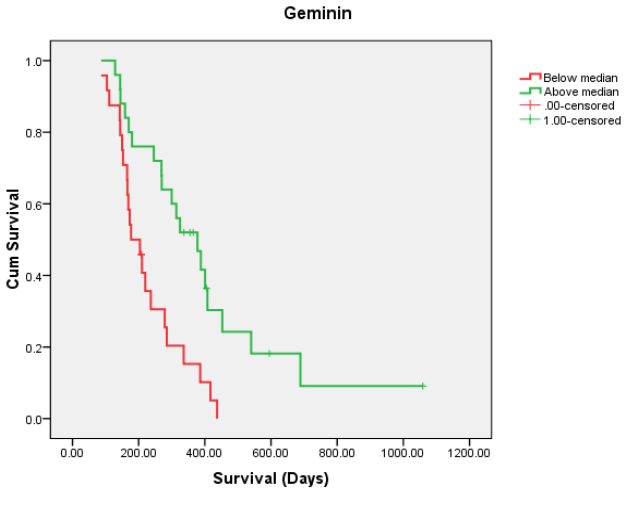
Glioblastoma - Mcm-2 (Cell Cycle Entry)



3.2a p=0.522

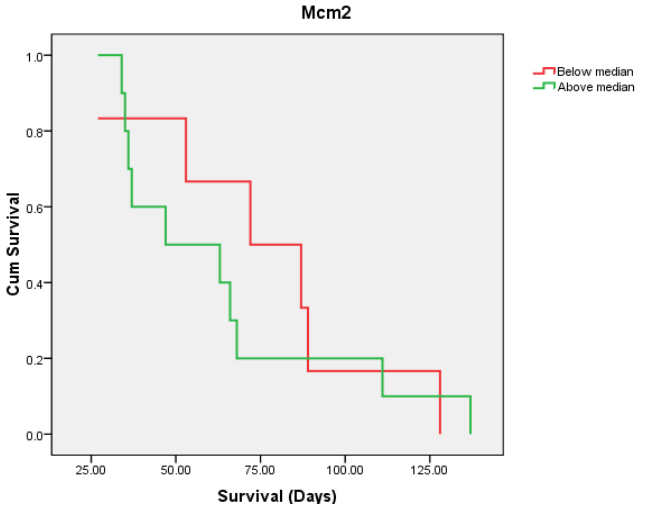


3.2b p=0.0063

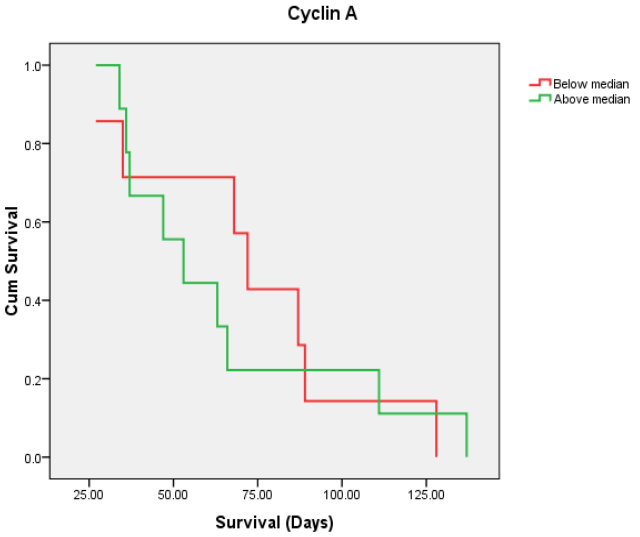


3.2c p=0.0046

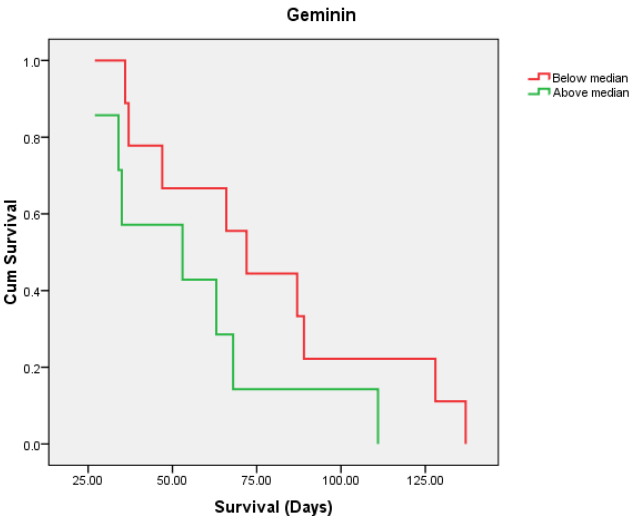
Figure 3.2a-c: Kaplan Meier survival curves for Mcm2, Cyclin A and Geminin in Group 1 (n=50). Kaplan-Meier curves calculated on the basis of marker LI being greater than (green) or less than (red) the median level.



3.3a p=0.6209

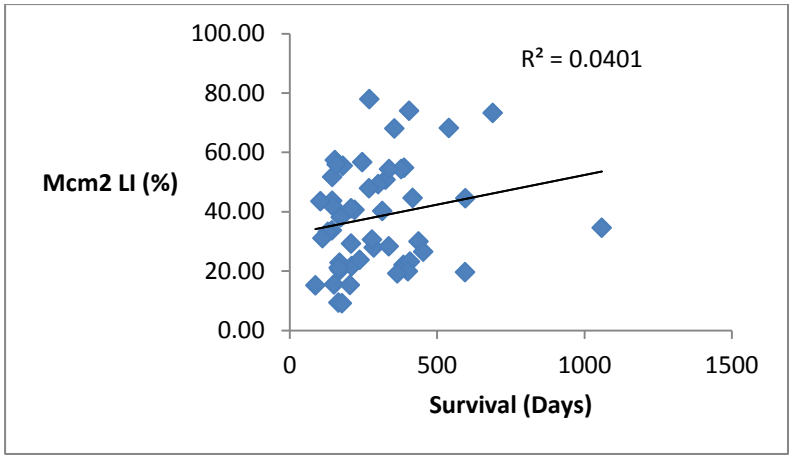


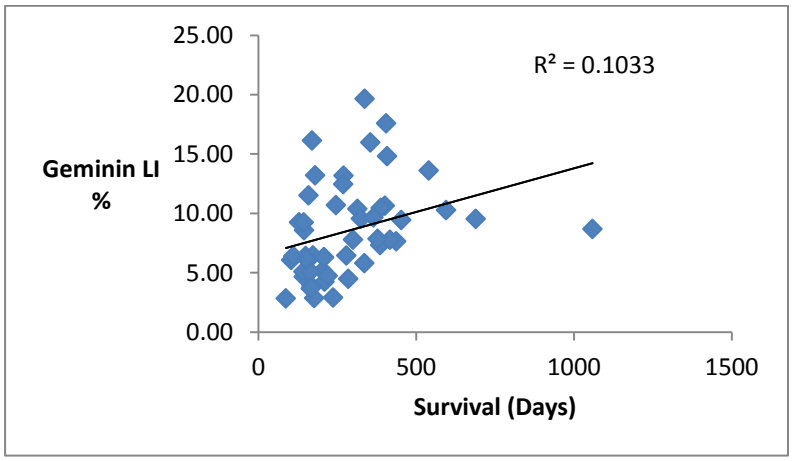
3.3b p=0.1325



3.3c p=0.7847

Figure 3.3a-c: Kaplan Meier survival curves for Mcm2, Cyclin A and Geminin in Group 2 (n=16). Kaplan-Meier curves calculated on the basis of marker LI being greater than (green) or less than (red) the median level.





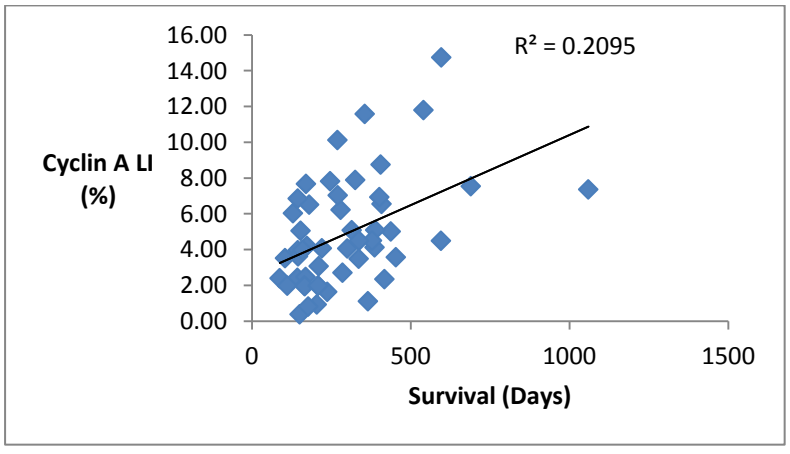
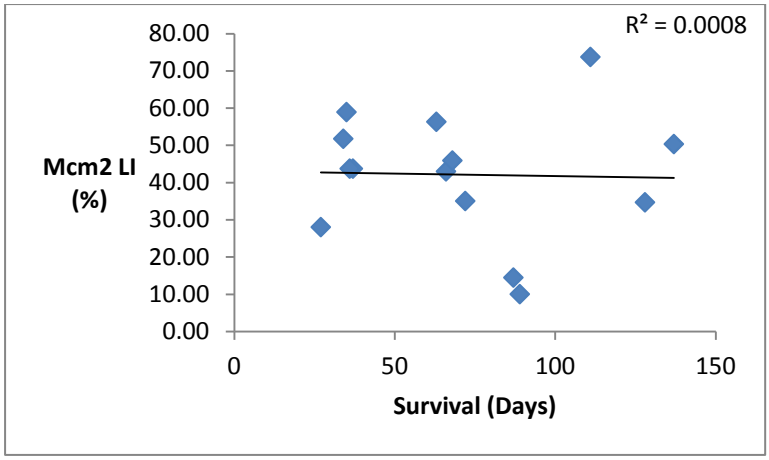
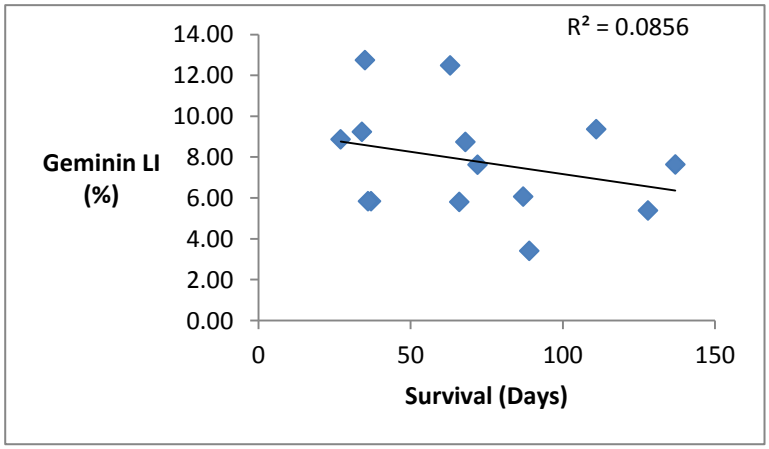


Figure 3.4: Correlation curves for Mcm2, Geminin and Cyclin A LIs in Group 1





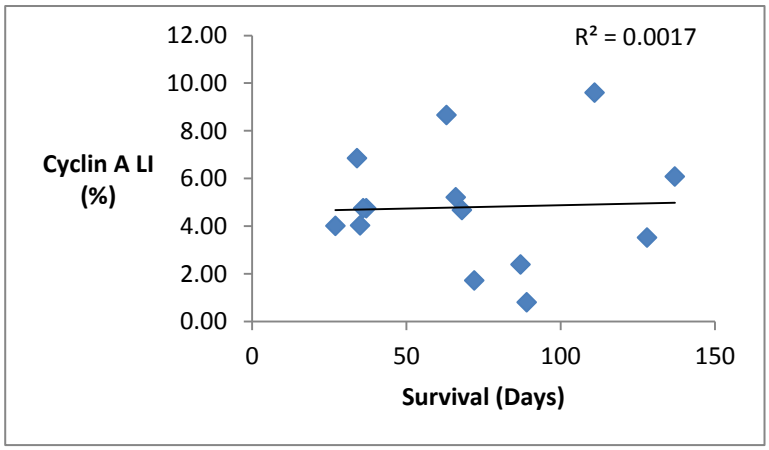


Figure 3.5: Correlation curves for Mcm2, Geminin and Cyclin A LIs in Group 2

3:3) Cell cycle markers in recurrent tumours

In the three patients with recurrent tumour, the LIs of Mcm2, Cyclin A and Geminin reduced by more than 50% after administration of adjuvant treatment (radiotherapy and temozolomide in 2 cases and radiotherapy alone in the third case). In the third case, the LIs of all three markers reduced after radiotherapy but these increased again by the time of third surgical procedure (figure 3.5).

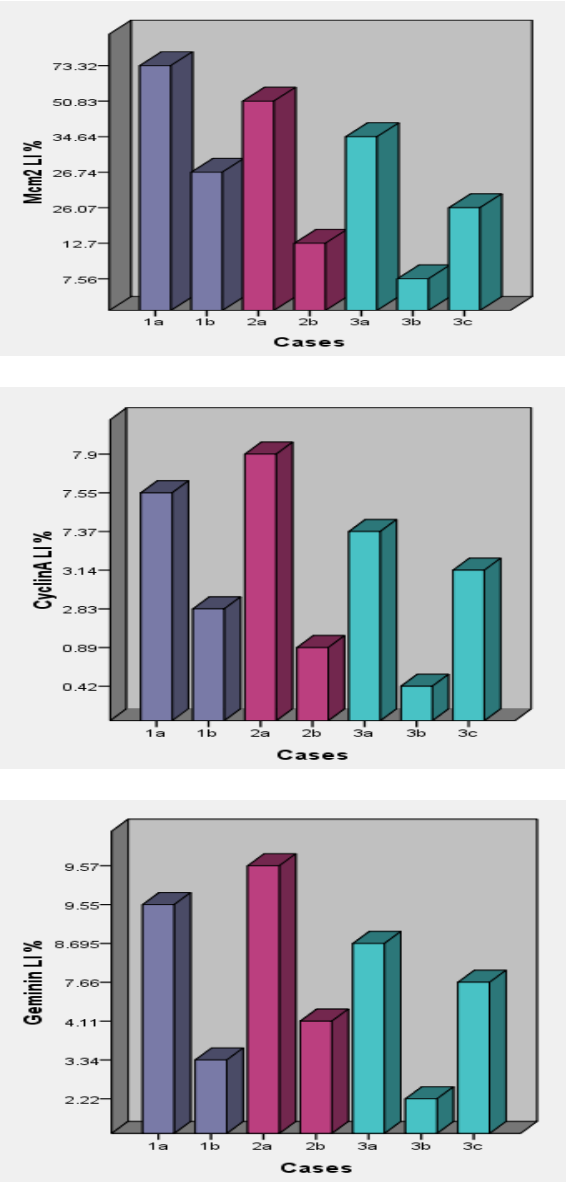


Figure 3.6: Mcm2, Cyclin A and Geminin labelling indices in three cases of recurrent Glioblastomas, (numbered 1, 2 and 3) and represented in three different colours. The three biopsies are represented by a, b and c for each case (Only case 3 had three biopsies).

3:4) Phosphohistone H3 (PHH3):

Phosphohistone H3 (PHH3) is a marker of mitosis. Analysis of PHH3 was performed in 30 patients due to lack of representative tumour tissue micro-array cores. A representative example of PHH3 immunohistochemical staining is shown in Figure 3.6. The median LI for pH3 was 8.3% with an IQR of 4.5-13.7%. On Kaplan Meier survival analysis, no significant correlation was observed between pH3 LI and patient survival (Log Rank p=0.819, figure 3.7). Linear regression also failed to show any significant relationship between PHH3 LI and patient survival (p=0.702, R^2=0.0057, figure 3.8). Here, it is important to note that the LIs of cyclin A and Geminin still correlated significantly with patient survival in these 30 patients (Log Rank p=0.004 and p=0.0006 respectively).

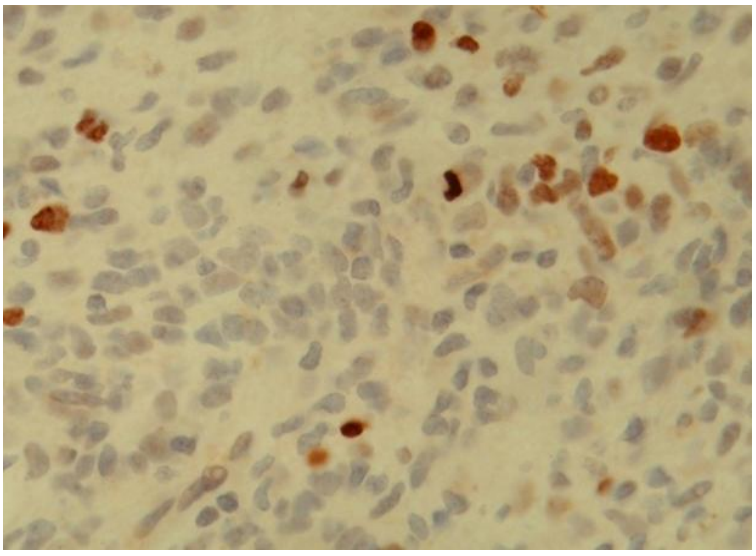


Figure 3.7: IHC staining of Glioblastoma tissue with PHH3 identifying mitotic cells – (Objective 40X)

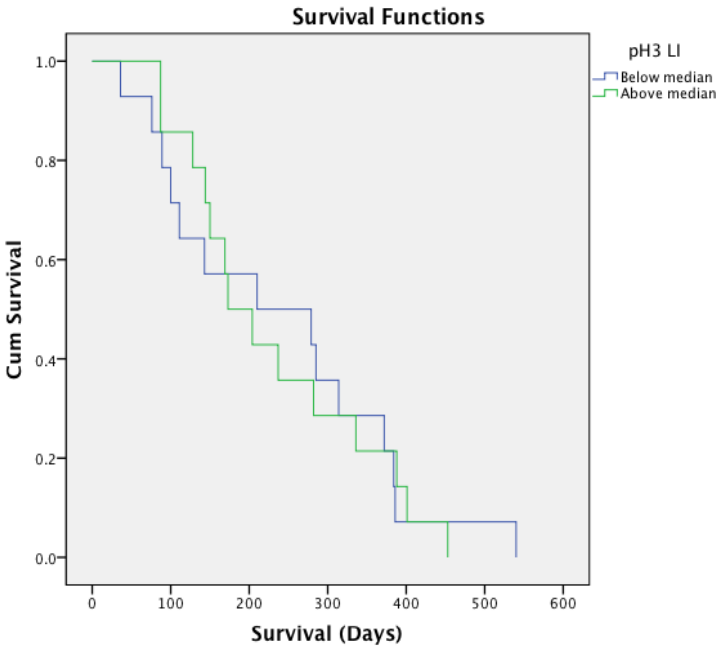


Figure 3.8: Kaplan Meier survival curve for PHH3. Kaplan-Meier curves calculated on the basis of marker expression being greater than (green) or less than (blue) the median level. Log rank p=0.819.

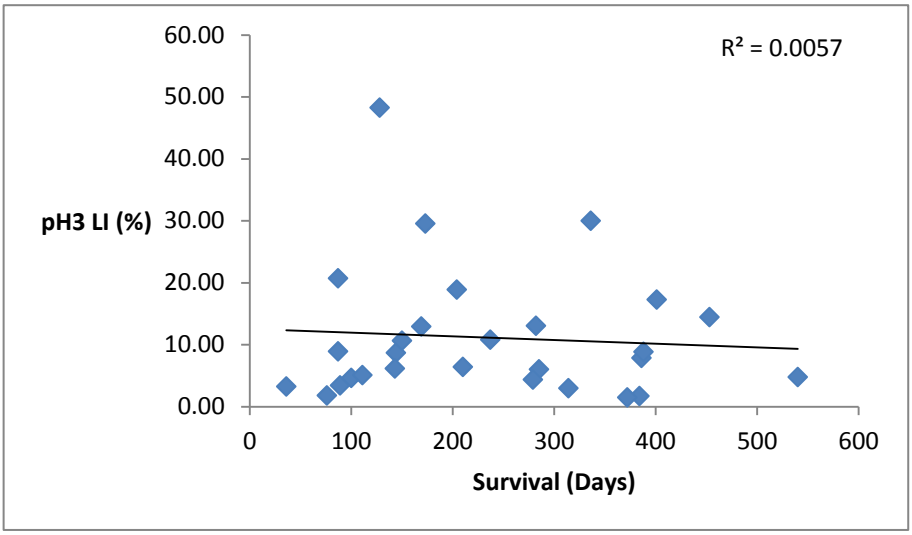


Figure 3.9: Survival correlation curve for PHH3 LI

3:5) Discussion:

Mcm-2 is a marker of cell cycle entry and is more inclusive than the widely used marker Ki67 in this regard because Ki67 fails to detect cells in the greater part of G1-phase (Scott *et al*., 2005). Mcm-2 expression increases with increasing tumour grade in oligodendrogliomas (Wharton *et al*., 2004) and astrocytomas (Scott *et al*., 2005) and correlates well with the Ki67 labelling index (Wharton *et al*.,2004; Scott *et al*., 2005). The expression of Ki-67 was therefore not studied in this thesis. A high level of expression of Mcm-2, representing a proliferating population of cells in glioblastoma is not unexpected as these are biologically aggressive tumours and thereby represent an important challenge in terms of treatment. No significant relationship between Mcm-2 expression and patient prognosis was identified however. This is possibly due to the fact that Mcm-2 is expressed by all cells that have entered cycle. It has been shown previously that a number of these cells will be stably arrested in the G1-phase of the cycle where they are not susceptible to adjuvant therapy (Scott *et al*. 2005). Thus, a high Mcm-2 LI may not correlate with increased susceptibility to adjuvant therapy if a large proportion of the cells detected by this marker are arrested in G1-phase. It is a well established fact that radiation sensitivity varies with different phases of the cell cycle with cells at the G1/S transition and prophase of Mitosis being the most sensitive (Hama *et al*., 2003; Tersima & Tolmach, 1963). These phases will be specifically detected by cells expressing cyclin A (S-phase) or geminin (S-phase through to Mitosis); markers that were found to correlate with survival following adjuvant therapy.

In mammalian cells, cyclin-A expression is limited to the S-phase of the cell division cycle where it forms a complex with cdk2 and is important in the initiation of DNA replication(Pines and Hunter, 1992). Cyclin-A expression has also been reported to increase with increasing tumour grade in astrocytomas (Scott et al., 2005) and higher expression has

been shown to correlate significantly with patient prognosis in low-grade astrocytomas but not in high grade astrocytomas (Shresta et al., 2007). Here, an elevated level of cyclin-A expression was associated with improved patient survival only in patients receiving adjuvant treatment. This suggests that an elevated S-phase fraction, as estimated by cyclin-A expression, predicts those tumours that are more sensitive to adjuvant treatment because they have an increased proportion of cells in S-phase, replicating DNA. In patients not receiving therapy, a high cyclin A LI may be detrimental to survival as the tumour is likely to grow more rapidly. Mammalian cells express nuclear geminin in S-phase, G2-phase and in mitosis until the stage where the nuclear membrane breaks down (Wohlschlegel *et al*. 2002). Increased geminin expression has been found in many types of human neoplasm including oral squamous cancer (Tamura *et al.*, 2010), colorectal cancer (Nishihara *et al*. 2009), breast cancer (Gonzales *et al*., 2004), oligodendrogliomas (Wharton *et al*. 2004) and astrocytomas (Shresta *et al*., 2007). High geminin expression has been demonstrated to correlate significantly with patient survival in anaplastic astrocytomas but not in glioblastomas (Shresta *et al*., 2007). Again, this study demonstrated a strong association between geminin expression and patient survival, but only in patients who received adjuvant treatment. In a similar way to that proposed for cyclin A, this correlation is likely to be because geminin is detecting actively proliferating cells; that population of tumour cells most sensitive to chemo/radiotherapeutic intervention. The failure of these markers to predict survival in patients not receiving adjuvant therapy implies that the effect being detected is related to therapy rather than features relating to the intrinsic biology of the tumour. Indeed, a high LI for cyclin A &/or geminin in untreated tumours confers an adverse prognosis due to rapid tumour growth. Previous studies have suggested that geminin in tumour cells is most often present in a non-mutated form with a low mutation frequency and there is no evidence for amplification of the gene in breast cancer (Gonzales *et al*., 2004). Thus it would appear that

the predictive properties of geminin expression are related to its normal biological function in cell cycle replication rather than a mutation or amplification causing loss of suppressor function; indeed, dysregulation of geminin during G1 may suppress tumour growth (Yoshida *et al*., 2004).

Traditionally, counts of mitotic figures have been used to identify grade in gliomas whereby increased number of mitotic figures suggests higher tumour grade and therefore poor prognosis. However, mitotic figures only detect a subpopulation of cells in mitosis i.e., those that have progressed beyond the formation of metaphase plate. Phosphohistone H3 (PHH3) is a surrogate marker of mitosis and overcomes this disadvantage by identifying cells in prophase; cells which are considered sensitive to effects of adjuvant treatment. Mitotic figure counts and PHH3 LIs are known prognostic factors in tumours like breast cancer (Skaland *et al.*, 2009) and malignant melanoma (Ladstein *et al.*, 2012). In this study, no significant correlation of PHH3 LI with patient survival was observed, both on Kaplan Meier analysis and linear regression. PHH3 analysis was however possible in only 30 patients due to lack of representative tissue sample and this could have impacted on the results due to the relatively small cohort size. It is important to note here that even in this cohort of 30 patients, Geminin and Cyclin A expression correlated positively with survival.

The expression of Mcm-2, cyclin-A and geminin in the three recurrent cases decreased after the administration of adjuvant treatment. This reinforces the above observations that the effects of adjuvant treatment is likely to be cell phase specific and the cell populations that are removed by treatment are those cells that are actively replicating. The cell population in G1 does not appear to be affected by treatment. In case 3, there was an increase in the expression of all three markers at the time of the third surgical procedure, suggesting that although radiotherapy was able to reduce the number of actively replicating cells at the time

of second surgery, the effect was not sustained, suggesting biological recovery of the tumour after the cessation of therapy. These data also provide a histological explanation for the common observation that the treatment effects, while effective in the initial stages, eventually fail to control the disease process.

In summary, the data show that high cyclin-A and geminin expression in glioblastomas was able to predict post-operative survival following adjuvant therapy by identifying those tumours with a high S-phase fraction or proliferating cell component. Cyclin A and geminin were superior Mcm-2 as markers of survival in these tumours, as Mcm2 also detect cells resident in G1-phase which are not actively proliferating and are thus contributing little to tumour growth. The incorporation of geminin into diagnostic panels for glioblastoma is therefore likely to assist oncologists in the selection of appropriate adjuvant chemo/radiotherapy especially where the decision is equivocal or complicated on other grounds.

Chapter 4

Results – Markers of apoptosis

Markers of Apoptosis – Discussion

4:1) Markers of Apoptosis:

Due to the limitation of available representative tumour tissue micro-array cores, markers of apoptosis could only be analyzed in 28 patients. The clinical details of these patients are summarized in table 4.1. A total of 5 apoptotic markers were used: Bcl2, Bcl-xl, Bak, Bax and Caspase 3. The representative examples of glioblastoma immunohistochemical staining are illustrated in Figures 4.1 a-e.

N	28
Median age (yr)	61
Male : Female	17 : 11
Median Survival (days)	258
Debulking surgery (number of patients)	25
Preoperative median Karnofsky performance score	70
Image guided biopsy (number of patients)	3
Radiotherapy (number of patients)	24
Temozolomide chemotherapy (number of patients)	None

Table 4.1: Clinical data in patients in apoptotic markers study

Bax staining was too diffuse to be considered as credible staining (figure 4.1c) and in spite of using antibodies from 2 different sources, different dilutions and different immunohistochemistry protocols, the staining was not deemed to be quantifiable. Bcl2, Bcl-xl, Bak and Caspase 3 produced specific cytoplasmic staining (figures 1b-1d) and the labelling indices were calculated as detailed in Table 4.2.

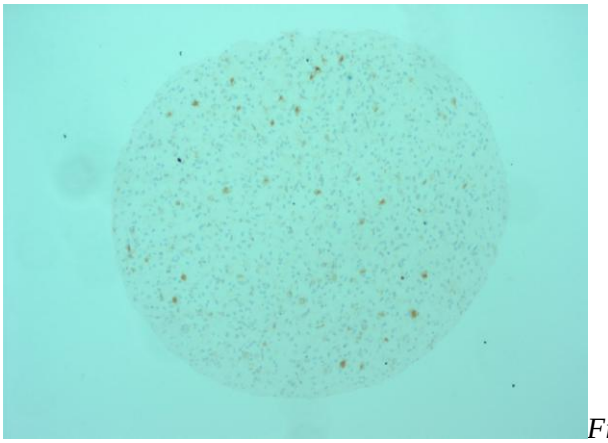


Fig 4.1a: Bcl2

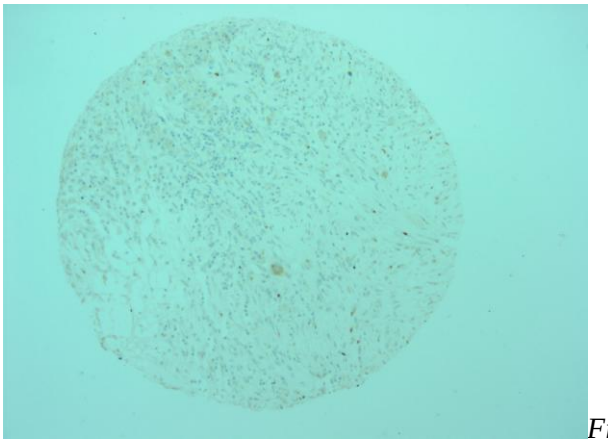


Fig4.1b: Bclxl

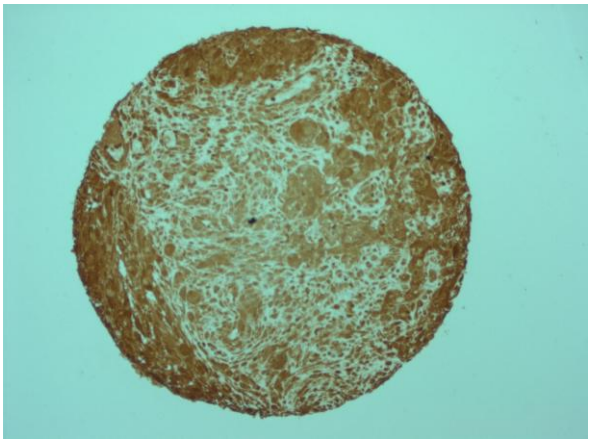


Fig 4.1c: Bax

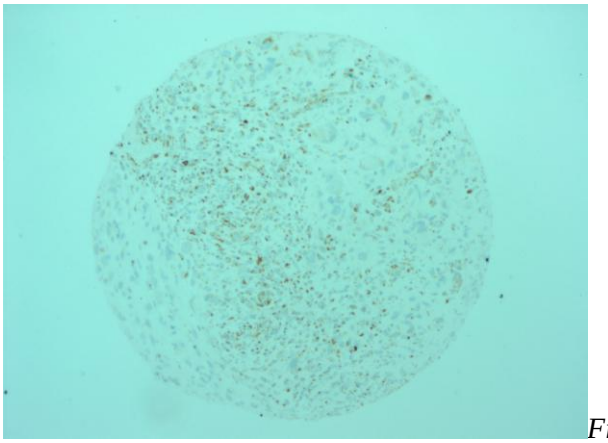


Fig 4.1d: Bak

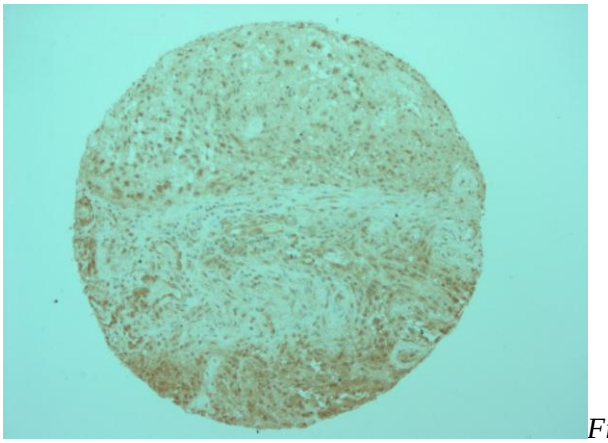


Fig 4.1e: Caspase 3

Figures 4.1a-e: Representative examples of Glioblastoma IHC staining with Bcl2, Bcl-xl, Bax, Bak and Caspase 3 (Objective 10X).

	Labelling Index % with interquartile range
Bcl2	8.1 (2.3-11.8)
Bcl-xl	1.84 (0.87-3.7)
Bak	16.1 (6.9-25.2)
Caspase 3	50.2 (34.56-72.9)

Table 4.2: Median Labelling indices (%) with interquartile range of apoptosis markers

The survival correlation curves of Bcl2, Bcl-xl, Bak and Caspase 3 labelling indices are shown in Figure 4.2. On Kaplan Meier survival analysis only, Bak LI correlated significantly with patient survival (Log rank *p*=0.0039, Figure 4.3c) with Bal LI values above median conferring a survival disadvantage. Linear regression Bcl2, Bcl-xl and Caspase 3 Labelling indices did not correlate with patient survival (Figure. 4.3a, 4.3b, 4.3d, table 4.3).

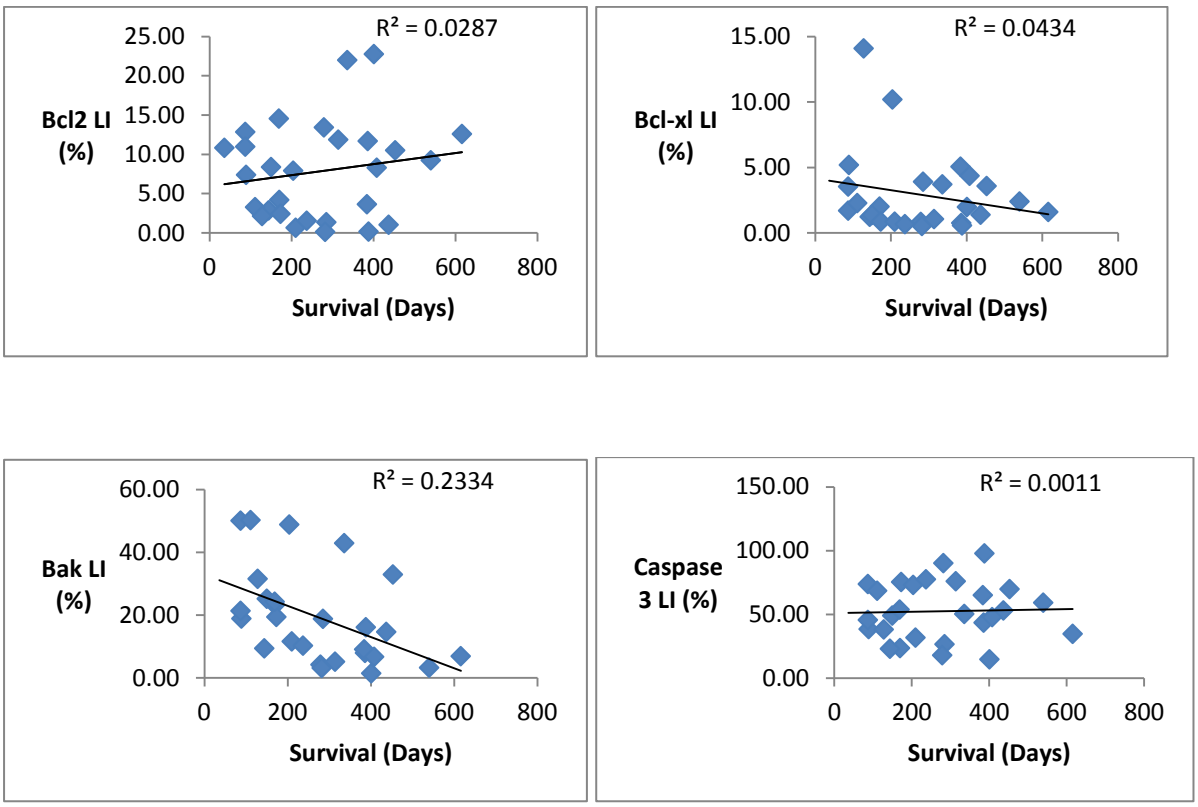
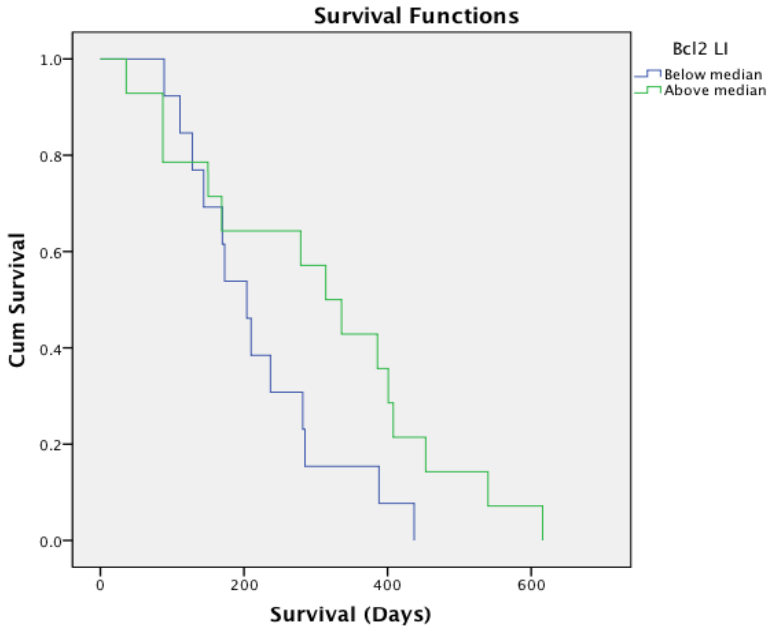
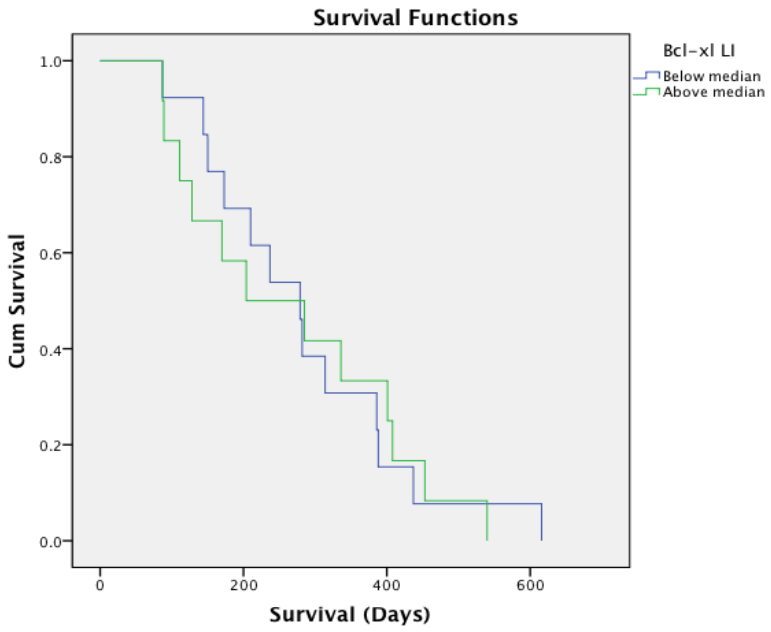


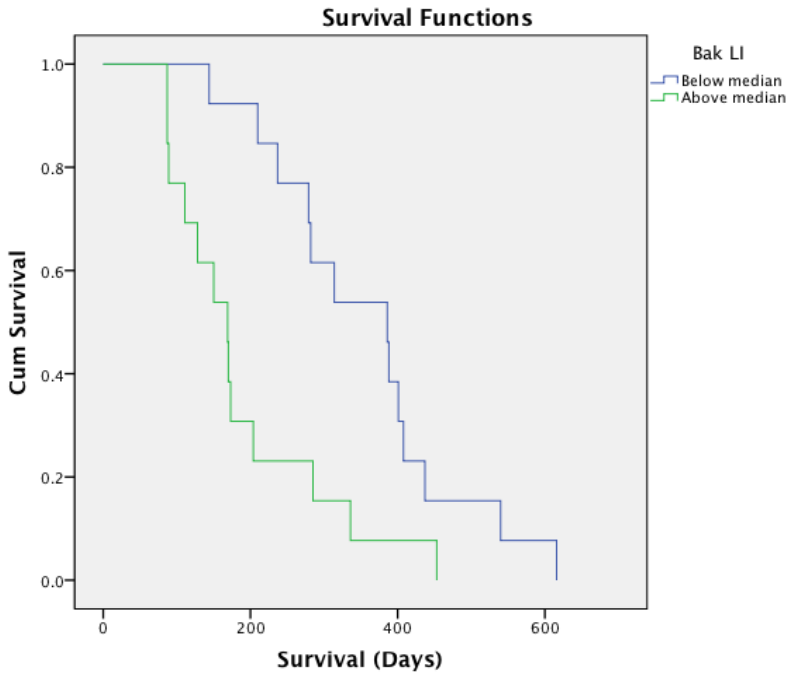
Figure 4.2: Survival correlation curves for Bcl2, Bcl-xl, Bak and Caspase 3 labelling indices


Survival Functions
Bcl2 LI
Below median
Above median
Cum Survival
1.0
0.8
0.6
0.4
0.2
0.0
0
200
400
600
Survival (Days)

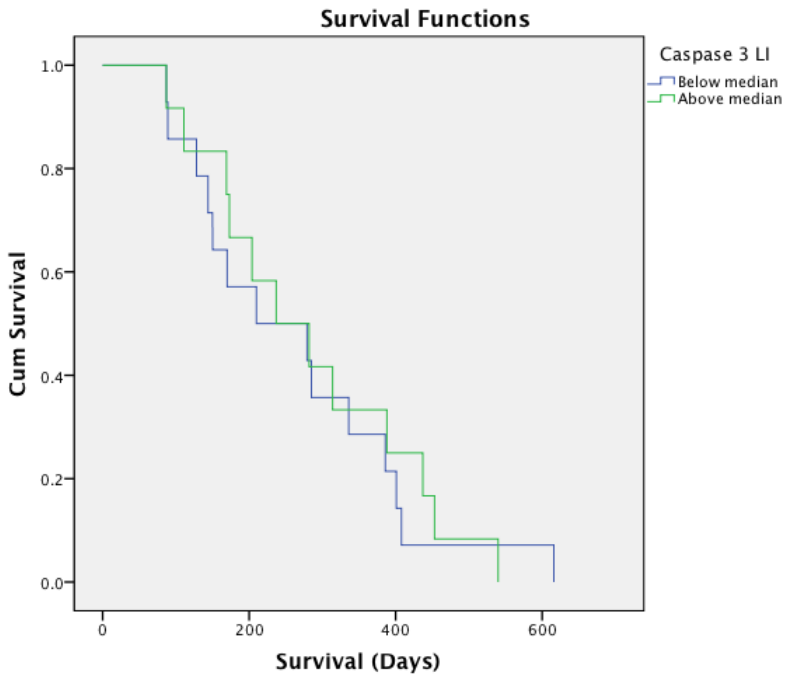
4.3a


Survival Functions
Bcl-xl LI
Below median
Above median
Cum Survival
1.0
0.8
0.6
0.4
0.2
0.0
0
200
400
600
Survival (Days)

4.3b



4.3c



4.3d

Figures 4.3a-d: Kaplan Meier survival curves for Bcl2, Bcl-xl, Bak and Caspase 3 LIs. Kaplan-Meier curves calculated on the basis of marker LI being greater than (green) or less than (blue) the median level.

	Log rank (p value)	Linear regression (p value)
Bcl2	0.166	0.389
Bcl-xl	0.876	0.307
Bak	0.0039	0.011
Caspase 3	0.596	0.869

Table 4.3: Log rank and linear regression p values for markers of apoptosis

4:2) Discussion:

The sample size for analysis of apoptosis was limited to 28 patients due to lack of representative tumour tissue. The labelling indices for two anti-apoptotic (Bcl-2, Bcl-xl) and two pro-apoptotic (Bax, Bak) members of the Bcl-2 protein family were analysed. Bcl-2 and Bcl-xl over-expression in glioma cell lines has been shown to be linked with increased tumour cell motility and resistance to apoptosis (Wick *et al*. 1998). Also, Bcl-2 inhibitor ABT-737 was shown recently to induce apoptosis in glioblastoma cells both *in vivo* and *in vitro* by inducing the release of BAX from its partner Bcl-2 (Tagscherer *et al*. 2008). Bcl-2 and Bcl-xl protein expression, however, had not been significantly correlated with survival by previous investigators (Martin *et al*., 2001; Kraus *et al*., 2001; Strik *et al*., 1999). In keeping with the existing literature, no significant correlation between Bcl-2 and Bcl-xl LIs with patient survival was observed in the current study. Whilst this could be due to the small sample size of the study, an important point in this regard is the observation made by Martin *et al*. (2002) who observed low expression of Bcl2 and Bax in glioblastoma with

immunohistochemistry but vice versa on immunoblotting suggesting a differential expression of these proteins in different cells.

Negative expression of pro-apoptotic Bax protein has been linked to adverse clinical outcome by Ruano *et al* (2008) in a DNA microarray study study on 20 Glioblastoma samples. Here problems were encountered with quantification of Bax immunohistochemical staining. This was in spite of trying two different antibodies from different sources and using different immunohistochemistry protocols. This could be due to the fact that Bax exists in different isoforms (Cartron *et al*., 2003) and current available antibodies are unable to differentiate between the different isoforms thereby producing a diffuse cytosolic staining which is difficult to quantify.

Bak is an important pro-apoptotic member of the Bcl-2 family that plays a role similar to Bax in the intrinsic pathway of apoptosis. Bak protein expression has been studied in glioblastoma tissue (Cartron *et al*., 2003) and glioblastoma cell lines (Cartron *et al*., 2003; Jin *et al*., 2006). Cartron and colleagues have described the severe impairment of the apoptotic pathways when glioblastoma cell lines were deficient in both Bax and Bak. There are however no studies which have correlated Bak protein expression with survival. The current study, a significant correlation of Bak expression with patient survival with higher expression of Bak correlating with poor prognosis, in spite of the small sample size was discovered. This result is contrary to what was expected as increased Bak expression has been previously shown to be associated with increased sensitivity to apoptosis - inducing therapies which in turn translates in to enhanced patient survival. The results will therefore have to be verified by studying the protein expression in a larger sample size and with other methods. These results may suggest a so far unknown role of Bak in glioblastoma oncogenesis and therefore needs to be investigated further.

Caspase 3 is the final effector caspase in the apoptosis pathway and its position as the last common step in apoptotic pathway makes it a useful indirect marker of apoptosis (Mellai *et al*., 2007). Increased immunohistochemical expression of caspase 3 has been associated with increasing tumour grade and early recurrence in meningiomas (Konstantinidou *et al.*, 2007). In gliomas, immunohistochemical analysis of caspase 3 expression increases with increasing tumour grade but no significant correlation with prognosis has been observed (Kobayashi et al., 2007). Here, in agreement with previous work no correlation of caspase 3 LI with patient survival was observed. Here it is important to note that Kobayashi *et al*. have used an antibody specific to cleaved caspase 3 in 21 patients with glioblastoma whereas the antibody used in this project identifies both the pro- and cleaved or activated form of caspase 3. Also important to note is that Vakkala *et al.* used an antibody similar to ours but use a staining score system rather than a labelling index, the former is generally considered to be a lot more subjective. Based on the results in this thesis, a study on larger number of patients with an antibody against the cleaved form of caspase 3 is recommended.

Chapter 5

Results – markers of autophagy flux

Markers of autophagy flux – Autophagy markers in Giant cell glioblastoma – Autophagy markers in recurrent glioblastoma - Discussion

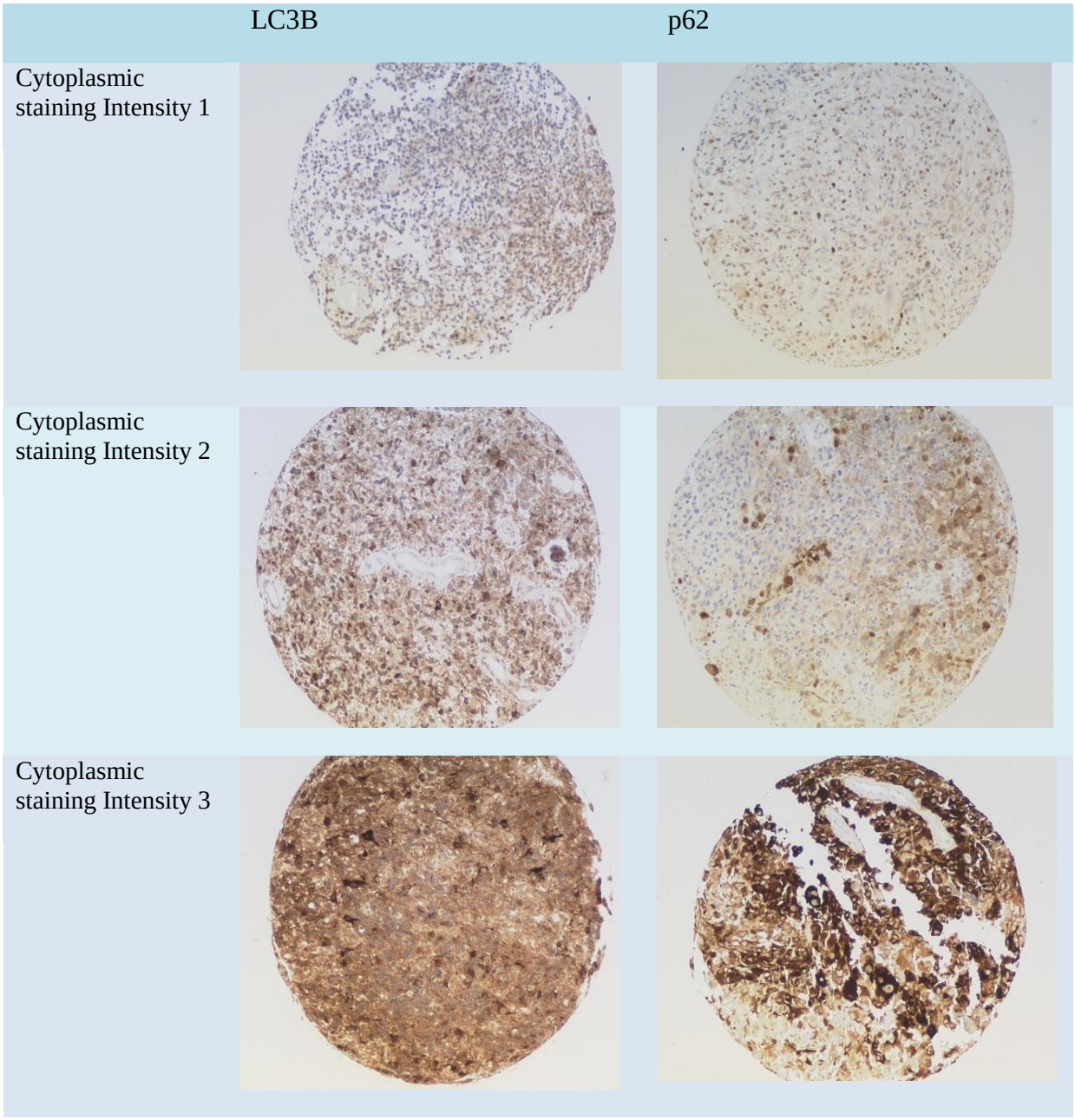
5:1) Markers of Autophagy

Immunohistochemical staining of autophagy flux markers, LC3b and p62, was analysed in 45 of the total 66 patients, due to lack of representative tumour tissue cores. Of the 45 patients, 39 patients underwent debulking surgery. All patients had cranial irradiation and 20 patients received temozolomide. The clinical details are summarized in Table 5.1.

N	45
Median age (yr)	61
Male : Female	30 : 15
Median Survival (days)	269
Debulking surgery (number of patients)	39
Preoperative median Karnofsky performance score	60
Image guided biopsy (number of patients)	6
Radiotherapy (number of patients)	45
Temozolomide chemotherapy (number of patients)	20

Table 5.1: Clinical data of patients in Autophagy markers study

Representative microarray immunohistochemical staining of LC3B and p62 is illustrated in Figure 5.1. LC3B and p62 staining was quantified as described in chapter 2 and figure 5.1 also demonstrates the representative samples for each criterion in the quantification method. LC3b and p62 have similar staining characteristics and a significant correlation was observed between LC3b and p62 staining scores (Linear regression p=0.001, R^2=0.22, Figure 5.2).



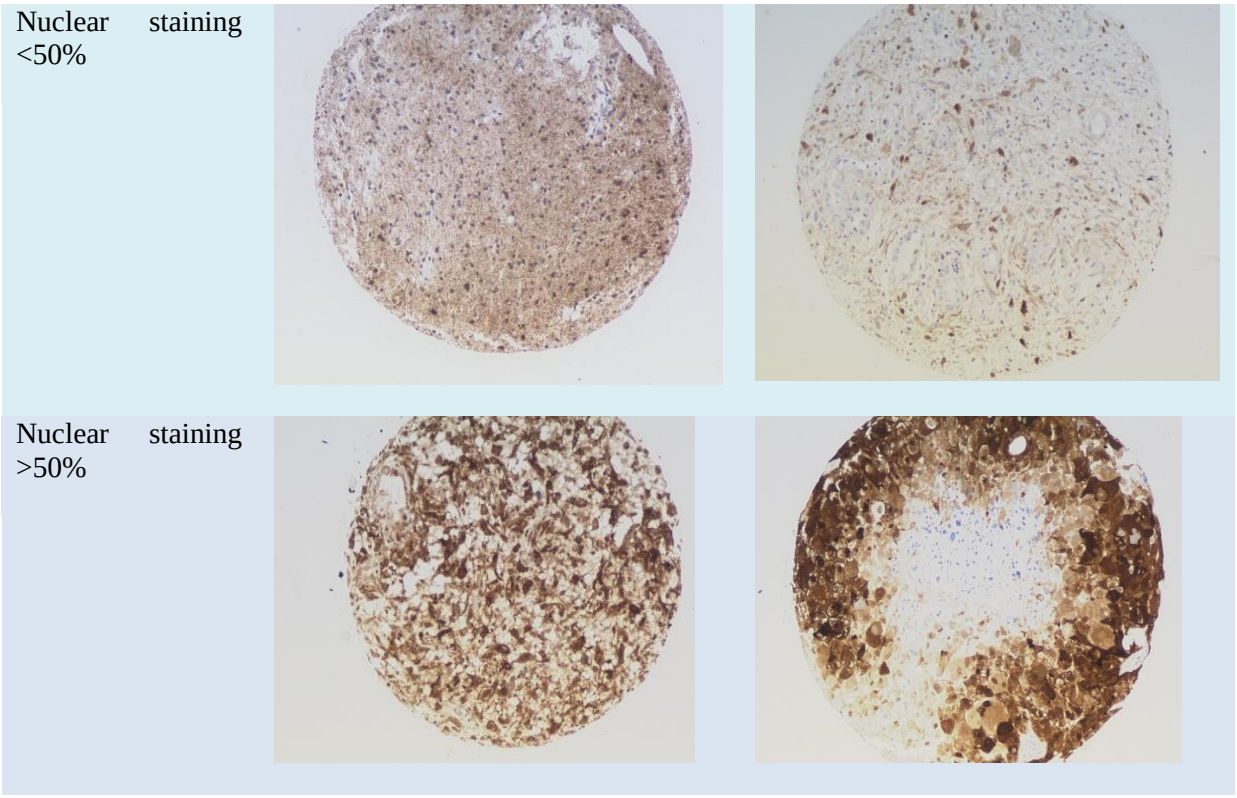


Figure 5.1: Immunohistochemical staining of LC3B and p62 IN Glioblastoma cores (Objective 10X). Illustrated are examples of each criteria used in calculating the staining score oh LC3B and p62 staining scores.

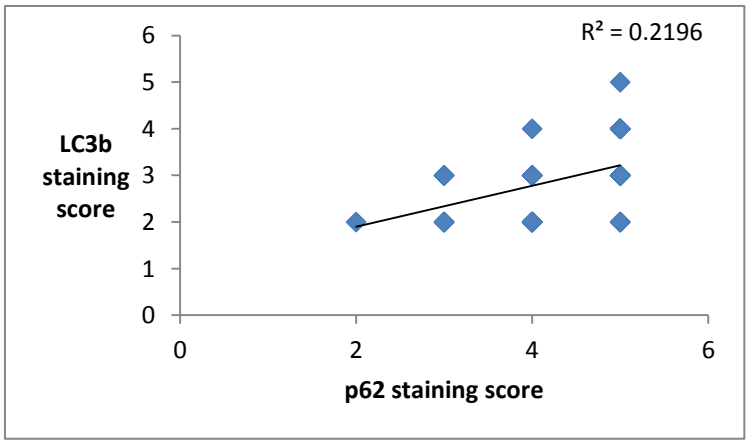


Figure 5.2: Correlation between p62 and LC3b staining scores. Linear regression p=0.001.

The survival correlation curves for the staining scores of both markers are shown in figure 5.3. Linear regression analysis did not show any significant relationship between p62 and LC3b staining scores with survival (p=0.29 and p=0.92 respectively). Kaplan Meier survival analysis revealed a significant negative relationship between raised p62 staining score and patient survival (Log rank p=0.017, figure 5.4a). LC3b staining score however did not correlate significantly with patient survival on Kaplan Meier analysis (Log rank p=0.68, Figure 5.4b).

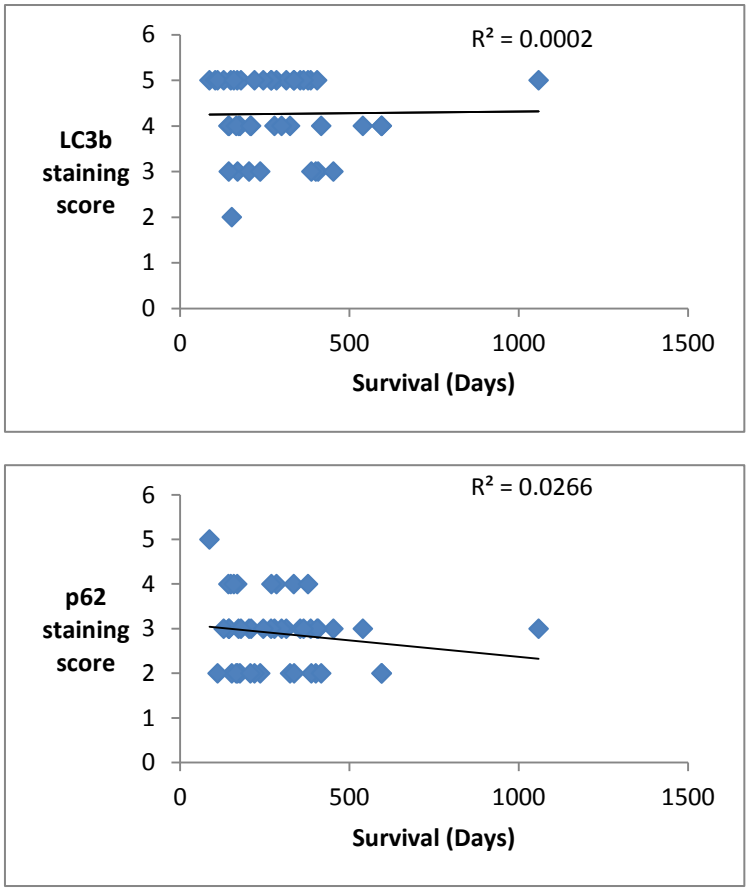


Figure 5.3: Survival correlation curves for LC3B and p62.

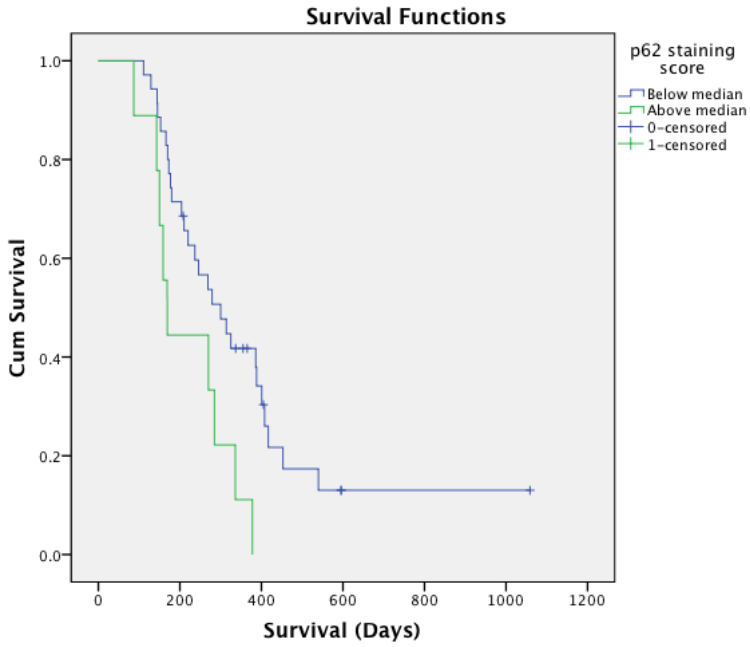


Fig 5.4a

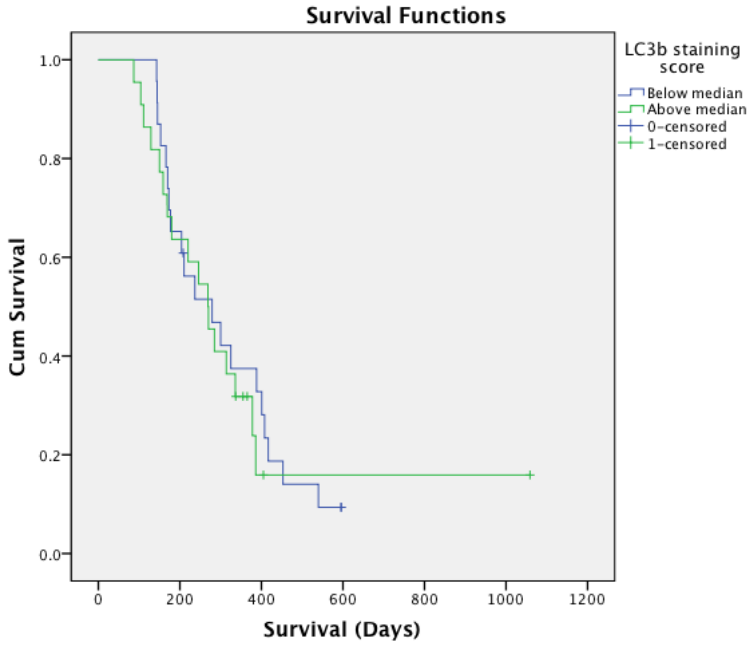


Fig. 5.4b

Figure 5.4: Kaplan Meier survival curves for LC3b and p62. Kaplan-Meier curves calculated on the basis of marker staining score being greater than (green) or less than (red) the median level.

5:2) Autophagy markers in Giant cell Glioblastoma

In this regard, an interesting finding was the distinct and heavy staining patterns of both LC3b and p62 observed in Giant cell glioblastomas as illustrated in Figure 5.5. Giant cell Glioblastomas have a worse prognosis as compared with the usual glioblastoma phenotype and the suggested survival disadvantage for higher p62 staining score would be in keeping with this.

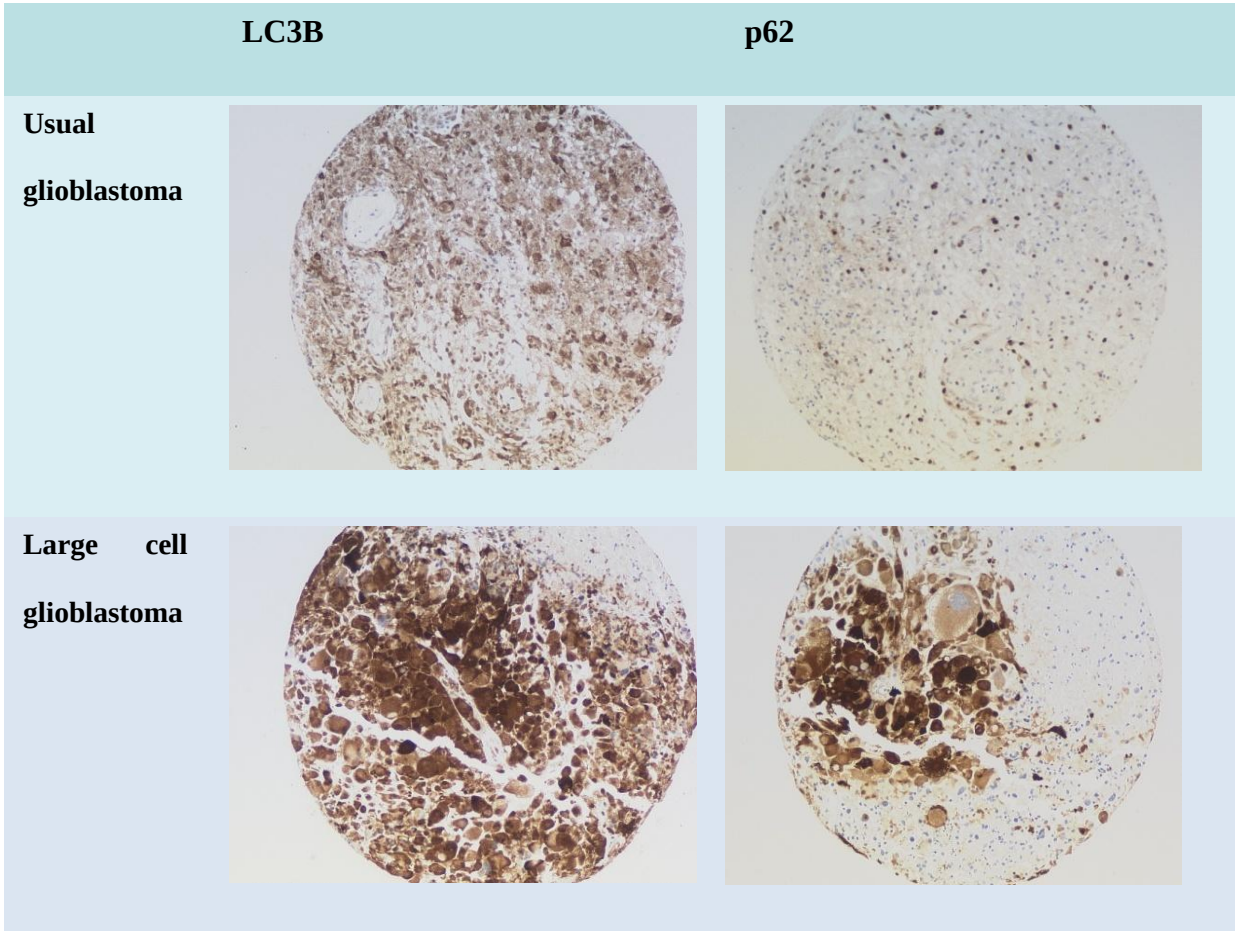


Figure 5.5: Staining patterns of LC3B and p62 in usual and Giant cell Glioblastoma

5:3) Autophagy markers in recurrent tumours:

Analysis of LC3B and p62 staining scores in the three cases of recurrent glioblastoma did not show any significant variation (Figures 5.6 and 5.7). As described in Chapter 3:1:1, case 1 and case 2 underwent debulking surgery, radiotherapy and temozolomide chemotherapy. Case 3 underwent debulking surgery on three occasions and radiotherapy was administered after first surgery.

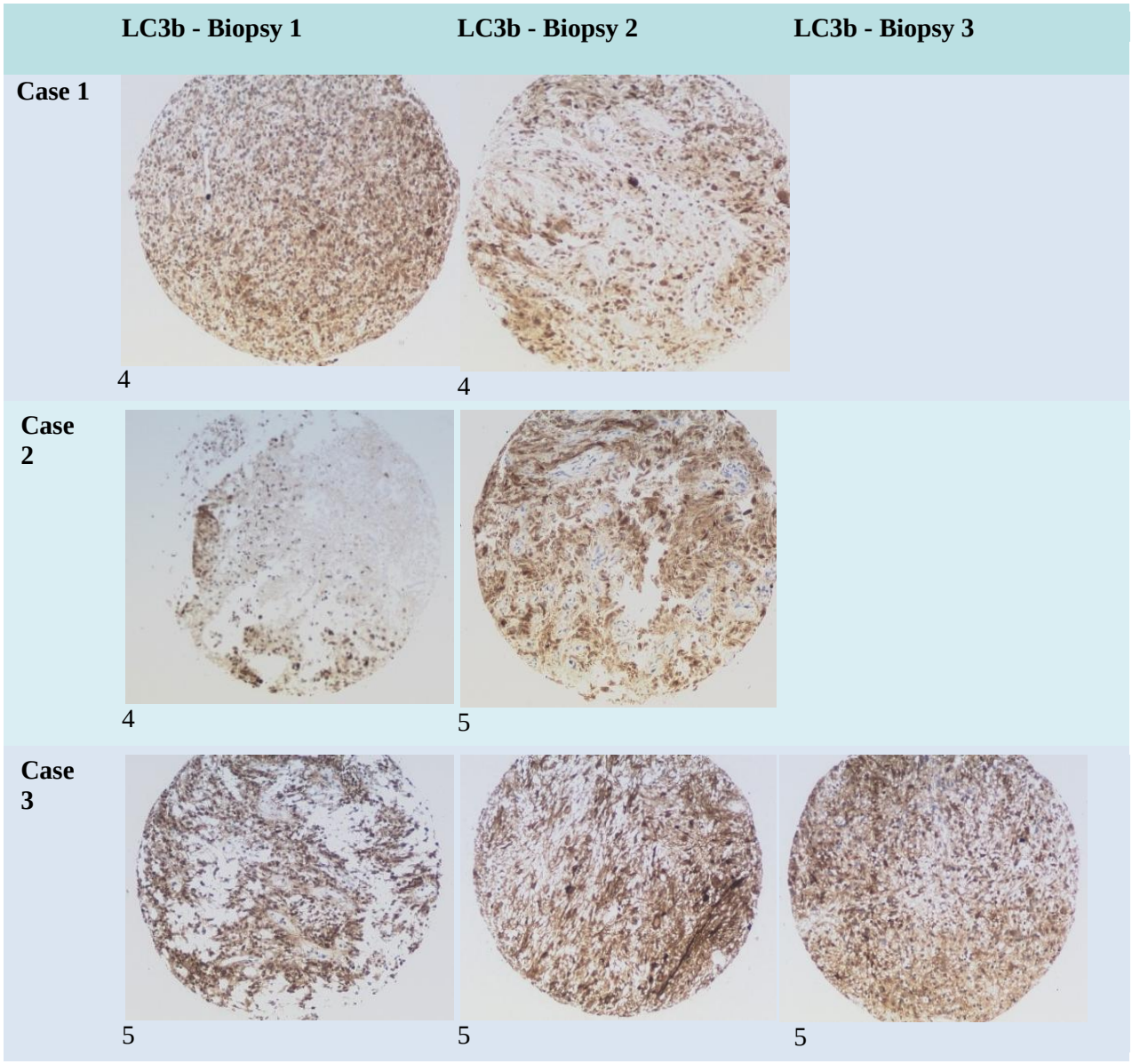


Figure 5.6: Representative staining examples and staining scores LC3b in three cases of recurrent glioblastoma.

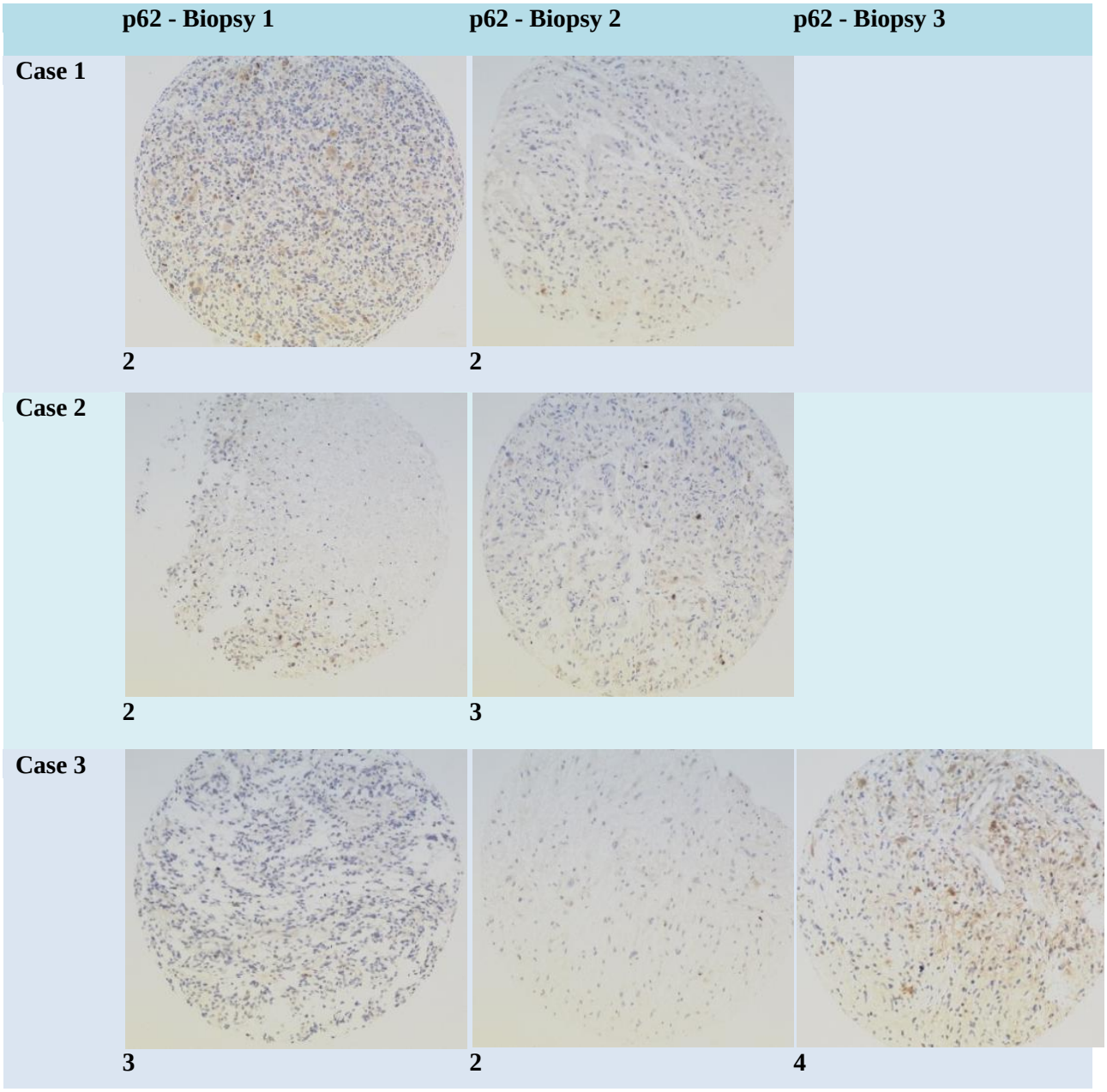


Figure 5.7: Representative staining examples and staining scores p62 in three cases of recurrent glioblastoma.

5:4) Discussion:

LC3b and p62 analysis was possible in only 45 patients. Here, LC3B staining was not significantly associated with patient survival by either linear regression or Kaplan Meier survival analysis. It is important to note here that the antibody used detects both cellular forms of LC3b i.e., LC3b I and LC3b II. Whilst LC3b I is cytosolic in distribution, LC3b II is associated with autophagosome membrane and autophagy induction leads to an increase in LC3b II. This probably explains the diffuse but punctate staining that was observed in our study. The two cellular forms of LC3b can be detected by immunoblotting but a commercially available antibody which differentiates between the two forms on immunohistochemistry is currently not available. To date, a study by Aoki *et al*. (2008) is the only previous study to have reported LC3b immunohistochemical analysis in glioblastomas. The study involved 65 patients with Glioblastoma and both immunoblotting and immunohistochemical analysis of LC3b was performed. Whilst they reported a significant relationship between LC3B and Karnofsky performance scale score, there was no significant relationship with survival. It is important to note here that Aoki *et al*. did not take into account the nuclear staining of LC3b and secondly the cytoplasmic staining was classified as weakly positive or strong positive. In this thesis, both nuclear and cytoplasmic staining were taken into account as nuclear presence of LC3b and LC3b shuttling between nucleoplasm and cytoplasm is a well known although poorly understood concept (Drake *et al*., 2010). Also, the thesis has employed a more quantitative method of analysing the staining, thereby reducing the extent of subjectivity in analysis. The study found no association between cytoplasmic staining scores and patient survival.

Interestingly a positive association between LC3b and p62 staining scores was observed. Similar immunohistochemical analysis in glioblastomas has not been reported before. This

observation is in keeping with the study by Shvets *et al*. (2008) who described how LC3 recruits p62 into autophagosomes, the role of this interaction requires further investigation.

A significant negative correlation between p62 staining and patient survival on Kaplan Meier survival analysis but not on linear regression was discovered. To the author's knowledge, this has not previously been reported in glioblastoma. Similar observations have however been made in non-small-cell lung cancer where higher levels of p62 are associated with poor prognosis (Inoue *et al*., 2012). Also p62 expression increases with increasing grade in breast Cancer (Rolland *et* al., 2007). The role of p62 in oncogenesis remains a subject of active current research (Puissant *et al*., 2012). p62 accumulation is not only an indication of autophagy blockade but is also associated with an amplification of pre-tumoural signalling with its presence at crossroads between multiple pro-oncogenic tumoural pathways (Puissant et al., 2012).

Another interesting observation is the high staining scores of LC3b and p62 observed in giant cell glioblastoma. This has not been reported before and probably contributes to the poor prognosis associated with these tumours as compared with the usual glioblastoma (Louis *et al*., 2007).

Analysis of three cases of recurrent Glioblastoma did not reveal any significant change in the staining scores of LC3b or p62. Although the number of cases is too small to draw definite conclusions, this observation suggests that adjuvant treatments including radiotherapy and temozolomide, do not affect the autophagy flux in the surviving tumour cells.

This analysis of immunohistochemical expression of LC3b and p62 suggests that autophagy plays an important role in glioblastoma oncogenesis and probably contributes to the high degree of resistance that these tumours exhibit towards available treatments. As described in chapter 1, immunohistochemical analysis of LC3b and p62 provides an indication and not the

exact state of autophagy flux and our results certainly encourage the need for further research in this field in Glioblastoma and other CNS tumours possibly with addition of more methods of protein quantification.

Chapter 6

Conclusions and future work

Improving patient survival remains a challenge in the management of Glioblastoma and in spite of advances in neuroradiology, image guided neurosurgery, neuropathology and neurooncology, prognosis remains poor. Age, performance score, gross total surgical resecttion, radiotherapy, temozolomide, MGMT status and IDH-1 are well described and established prognostic markers in glioblastoma. Whilst clinical experience and published data suggest that most patients with glioblastoma will be dead within fifteen months of diagnosis, in spite of maximal treatment, approximately 3-5% will survive much longer and seem to respond to the established treatments better than would be predicted (Krex *et al*., 2007) with isolated case reports of patient surviving up to 20 years after diagnosis (Sperduto *et al*., 2009). This clearly points to biological differences which, if identified, would help to predict treatment response and drive future research in to targeted therapy.

The analysis of complex pathways of cellular proliferation, differentiation, apoptosis, stress response, survival and DNA damage response are the subject of intense current research with an aim of identifying unique molecular signatures and biomarkers of prognostic and predictive significance in cancer. Identification of such markers will enable more focused targeting of existing therapies to patients who are likely to respond well to them. The aim of this thesis was to study the expression of a panel of cell division, autophagy and apoptosis markers in glioblastoma in an attempt to identify biomarkers prognostic and/or predictive significance. The markers used are readily available and can be applied to routine diagnostic immunohistochemistry (IHC) and neuropathology practice.

This study involved construction of tissue micro-array for IHC analysis of a panel of biomarkers. This not only proved cost effective in terms of antibody use but also allowed

standardised IHC staining as all representative glioblastoma tissue samples could be analysed on three slides which were processed together.

This study has its limitations. Firstly, the sample size was small which prevented the application of more advanced statistical analysis. Secondly, only a single semi-quantitative method of protein expression was used. The results will therefore need further validation by the use of another techniques particularly immunoblotting and also real time reverse transcription quantitative PCR (polymerase chain reaction) for mRNA expression of the studied markers. Thirdly, none of the patients in the study had characterization of MGMT and IDH-1 which are now known to be important prognostic markers. This was due to the fact that MGMT analysis was not in clinical use at Hull Royal Infirmary at the time of this study and IDH-1 was only reported as a prognostic marker in glioblastoma in 2009. Inspite of these limitations, this work has led to some interesting and significant results.

Whilst IHC expression of cell cycle markers has been studied previously in glioblastoma, a marker of prognostic significance has not been reported. This work has identified two cell cycle markers, Cyclin A and Geminin to be of prognostic significance in glioblastoma and the results also point to a significant predictive value.

The study also reconfirms the observation that the effects of adjuvant treatment is cell cycle phase specific and seems to target cell populations in S and G2 phase of the cell cycle; populations identifiable by cyclin A and geminin expression. The incorporation of cyclin A and Geminin in the neuropathology diagnostic panel would therefore provide neurooncology multidisciplinary teams with additional prognostic information that could aid in decision making and help to better target currently available adjuvant treatments.

Apotosis has been a subject of extensive research in cancer in general and glioblastoma in particular. The current study used five common, important, markers of apoptosis and showed

a negative correlation between Bak IHC expression and patient survival, in spite of the small sample size. To the author's knowledge, this correlation has not been reported previously in the literature. This observation however needs further investigation as it suggests a role of Bak in glioblastoma oncogenesis beyond its known pro-apoptotic function; such a function could have major implications for understanding key biological processes as well as prognostic value.

Autophagy flux and autophagic cell death is an extremely "hot topic" currently in cancer. IHC analysis of autophagy flux is difficult and this study is the first to analyse and quantify the IHC expression of two markers (LC3b and p62) of autophagy flux in gliblastoma. A key finding was the significant negative correlation between p62 expression and patient survival; this clearly points to autophagy flux playing a major role in glioblastoma onncogenesis. Whilst a recommendation for routine clinical use of p62 as a prognostic marker cannot be made at this stage, the study clearly identifies an area for future research and suggests a possible target for interventions.

Currently glioblastoma oncogenesis and targeted therapy is the subject of ongoing research (Westermark, 2012; Ohka *et al.*, 2012). The Cancer Genome Atlas (TCGA) project catalogues genomic abnormalities involved in the development of cancer using wide ranging techniques and its analysis has led to a recent description of glioblastoma subclassification in to four types based on gene expression, somatic mutations and DNA copy number (Cancer Genome Atlas Research Network, 2008; Verhaak *et al.*, 2010). These subtypes are characterized by PDGFRA, IDH1, EGFR and NF1 pathway abnormalities. This sub-classification will need to be considered in relation to other studies such as those conducted here as a focus on cell cycle and autophagy machinery is relevant as these pathways act as an integration point for information transduced through upstream pathways. The results of this

thesis are not only likely to be useful in every day clinical practice and management of patients with glioblastoma but they also provide material for further research into cell cycle and in particular autophagy machinery in an effort to identify prognostic and predictive biomarkers and targets for future therapies.

References:

Adams JM. 2003. Ways of dying: multiple pathways to apoptosis. Genes Dev. 17:2481-2495.

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K. & Walter, P. 2002. Molecular biology of the cell. Vol. 1. Garland Science: New York.

Aoki H, Kondo Y, Aldape K, Yamamoto A, Iwado E, Yokoyama T, Hollingsworth EF, Kobayashi R, Hess K, Shinojima N, Shingu T, Tamada Y, Li Zhang, Conrad C, Bogler O, Mills G, Sawaya R and Kondo S. 2008. Monitoring autophagy in glioblastoma with antibody against isoform B of human microtubule-associated protein 1 light chain 3. Autophagy 4(4): 467-475.

Barr JG, Grundy PL. 2012. The effects of the NICE Technology Appraisal 121 (Gliadel and temozolomide) on survival in high-grade glioma. Br J Neurosurg 26(6): 818-22.

Bartek J & Lucas J. 2001. Mammalian G1- and S-phase checkpoints in response to DNA damage. Curr Opin Cell Biol 13: 738-747.

Bartolomeo, S. Di, Corazzari, M., Nazio, F., et al. 2010. The dynamic interaction of AMBRA1 with the dynein motor complex regulates mammalian autophagy. Journal of Cell Biology 191(1): 155–168.

Batzdorf U, Malamud U. 1963. The problem of multicentric gliomas. J Neurosurg 20: 122-136.

Bell SP & Dutta A. DNA replication in eukaryotic cells. 2002. Ann. Rev Biochem 71: 333-374.

Bock HC, Puchner MJ, Lohman F, Schutze M, Koll S, Ketter R, Buchalla R, Rainov N, Kantelherdt SR, Rohde V, Giese A. 2010. First-line treatment of malignant glioma with carmustine implants followed by concomitant radiochemotherapy: a multicentre experience. Neurosurg Rev 33(4): 441-9.

Brada M, Stennning S, Gabe R, Thompson LC, Levy D, Rampling R, Erridge S, Saran F, Gattamaneni R, Hopskins K, Beall S, Collins VP, Lee SM. 2010. Temozolmide versus procarbazine, lomustine and vincristine in recurrent high grade glioma. J Clin Oncol 28(30): 4601-8.

Budirahardja Y and Gonczy P. 2009. Coupling the cell cycle to development. Development 136, 2861-2872.

Bursch, W., Ellinger, A., Gerner, C. and Schulte-Hermann, R. 2004. Autophagocytosis and programmed cell death. In Autophagy (ed. D. J. Klionsky), pp. 287-303. Georgetown, TX: Landes Bioscience.

Buschges, R., Weber, R.G., Actor, B., Lichter, P., Collins, V.P. & Reifenberger, G.

1999. Amplification and expression of cyclin D genes (CCND1, CCND2 and

CCND3) in human malignant gliomas. Brain Pathol, 9: 435-42; discussion 432-3.

Cancer Genome Atlas Research Network. 2008. Comprehensive genomic characterization defines human glioblastoma genes and core pathways. Nature 455: 1061–8.

Cartron P-F, Oliver L, Martin S, Moreau C, LeCabellec M-T, Jezequel P, Meflah K and Vallette FM. 2002. The expression of a new variant of the pro-apoptotic molecule Bax, Bax-gamma is correlated with an increased survival of glioblastoma multiforme patients. Hum Mol Genet 11: 675-687.

Colman H, Giannini C, Huang L, Gonzalez J, Hess K, Bruner J, Fuller G, Langford L, Pelloski C, Aaron J, Burger P, Aldape K. 2006. Assessment and prognostic significance of mitotic index using the mitosis marker phospho-histone H3 in low and intermediate-grade infiltrating astrocytomas. Am J Surg Pathol 30(5): 657-64.

Coverley D & Laskey RA. Regulation of eukaryotic DNA replication. 1994. Ann. Rev Biochem 63: 745-776.

Coverley, D., Laman, H. & Laskey, R.A. 2002. Distinct roles for cyclins E and A during DNA replication complex assembly and activation. Nat Cell Biol 4, 523-8.

Csikasz-Nagy A, Battogtokh D, Chen KC, Novak B and Tyson JJ. 2006. Analysis of a generic model of eukaryotic cell-cycle regulation. Biiophysical Journal 90: 4361-4379.

Danial, N.N., and Korsmeyer, S.J. 2004. Cell death: critical control points. Cell 116, 205-219.

Dea N, Fournier-Gosselin MP, Mathieu D, Goffaux P, Forthin D. 2012. Does extent of resection impact survival in patients bearing glioblastoma? Can J Neurol Sci 39(5): 632-637.

Dixit S, Hingorani M, Achawal S. & Scott IS. 2011. The sequential use of carmustine wafers (Gliadel) and post-operative radiotherapy with concomitant temozolomide followed by adjuvant temozolomide: a clinical review. Br J Neurosurg 25: 459-469.

Drake KR, Kang M, Kenworthy AK. 2010. Ucleocytoplasmic distribution and dynamics of the autophagosome marker EGFP-LC3. PLoS One 5(3): e9806.

Ehrmann J Jr, Rihakova P, Hlobilkova A, Kala M, Kolar Z. 2000. The expression of apoptosis-related proteins and the apoptotic rate in glial tumors of the brain. Neoplasma 47: 151–155.

Evans, T., Rosenthal, E.T., Yongblom, J., Distel, D. & Hunt, T. 1983. Cyclin: a protein specified by maternal mRNA in sea urchin eggs that is destroyed at each cleavage division. Cell 33: 389-396.

Feigl GC, Ritz R, Moraes M, Klein J, Ramina K, Gharabaghi A, Krischeck B, Danz S, Bornemann A, Liebsch M, Tatagiba MS. 2010. Resection of malignant brain tumors in eloquent cortical areas: a new multimodal approach combining 5-aminolevulinic acid and intraoperative monitoring. J Neurosurg 113(2): 352-7.

Filipini G, Falcone C, Boiardi A, Broggi G, Bruzzone MG, Caldiroli D, Farinia R, Farinotti M, Fariselli L, Finocchiaro G, Giombino S, Pollo B, Savoiardo M, Solero CL, Valsecchi M G; Brain Cancer Register of the Fondazione IRCCS (Istituto Ricovero e Cura a Carattere Scientifico) Istituto Neurologico Carlo Besta. 2007. Prognostic factors for survival in 676 consecutive patients with newly diagnosed primary glioblastoma. Neurooncol 10(1): 79-87.

Fimia, G. M., Stoykova, A., Romagnoli, A., et al. 2007. Ambra1 regulates autophagy and development of the nervous system. Nature 447(7148): 1121–1125.

Gerard C and Goldbetter A. 2009. Temporal self-organization of the cyclin/CDK network driving the mammalian cell cycle. PNAS 106(51): 21643-21648.

Gerard C and Goldbeter A. 2011. A skeleton model for the network of cycli-dependent kinases driving the mammalian cell cycle. Interface Focus 1: 24-35.

Gonzalez-Darder JM, Gonzalez-Lopex P, Talamantes F, Quilis V, Cortes V, Garcia-March G, Roldan P. 2010. Multimodal navigation in functional microsurgical resection of intrinsic brain tumours located in eloquent motor areas: role of tactography. Neurosurg Focus. 28(2)E5.

Gonzales MA, Tachibana KK, Chin SF, Callagy G, Madine MA, Vowler SLA, Pinder SE, Laskey RA, Coleman N. 2004. Geminin predicts adverse clinical outcome in breast cancer by reflecting cell-cycle progression. J Pathol 204(2): 121-30.

Gorlia T, van den Bent MJ, Hegi ME, Mirimanoff RO, Weller M, Cairncross JG, Eisenhauer E, Belanger K,Brandes AA, Allgeier A, Lacombe D, Stupp R. 2008. Nomograms for predicting survival of patients with newly diagnosed glioblastoma: prognostic factor analysis of EORTC and NCIC trial 26981-22981/CE.3. Lancet Oncol 9(1):29-38.

Goto H, Tomono Y, Ajiro K, Kosako H, Fujita M, Sakurai M, Okawa K, Iwamatsu A, Okigaki T, Takahashi T, Inagaki M. Identification of a novel phosphorylation site on histone H3 coupled with mitotic chromosome condensation. J Biol Chem 274(36):25543-9.

Gravina GL, Festuccia C, Marampon F, Povov VM, Pestell RG, Zani BM and Tombolini V. 2010. Biological rationale for the use of DNA methyltransferase inhibitors as new strategy for modulation of tumour response to chemotherapy and radiation. Molecular cancer 9: 305.

Habberstad AH, Gulati S and Torp SH. 2011. Evaluation of the proliferation markers Ki-67/MIB-1, mitosin, survivin, pHH3, and DNA topoisomerase IIa in human anaplastic astrocytomas - an immunohistochemical study. Diag Pathol 6: 43.

Hama S, Matsuura S, Tauchi H, Yamasaki F, Kijiwara F, Arita K, Yoshioka H, Heike Y, Mandai K & Kurisu K. 2003. p16 gene transfer increases cell killing with abnormal nucleation after ionising radiation in glioma cells. Br J Cancer 89: 1802-1811.

Hara A, Saegusa M, Ichinoe M & Okayasu I. 2008. Diagnostic and prognostic significance of cyclin A expression in low grade astrocytomas: comparison with astrogliosis and high grade tumours. J Clin Pathol 61: 287-92.

Harris S. L. and Levine, A. J. 2005. The p53 pathway: positive and negative feedback loops. Oncogene 24: 2899-2908.

Haruki T, Shomori K, Hamamoto Y, Taniguchi Y, Nkamura H, Ito H. Geminin expression in small lung adenocarcinomas: implication in prognostic significance. Lung Cancer 71(3): 356-362.

He C. and Klionsky D. J. 2009. Regulation mechanisms and signaling pathways of autophagy. Annual Review of Genetics 43: 67–93.

Hegi ME, Diserens A, Gorlia T, Hamou m, de Tribolet N, Weller M, Kros JM, Hainfellner JA, Mason W, Mariani L, Bromberg JEC, Hau P, Mirimanoff RO, Cairncross JG, Janzer RC and Roger Stupp. 2005. MGMT Gene Silencing and Benefit from Temozolomide in Glioblastoma. N Engl J Med 352: 997-1003.

Hendzel MJ, Wei Y, Mancini MA, Van Hooser A, Ranilli T, Brinkley BR, Bazett-Jones DP and Allis CD. 1997. Mitosis-specific phosphorylation of histone H3 initiates primarily within pericentromeric heterochromatin during G2 and spreads in an ordered fashion coincident with mitotic chromosome condensation. Chromosoma 106(6): 348-60.

Homma T, Fukushima T, Vaccarella S, Yonekawa Y, Di Patre PL, Franceschi S, Ohgaki H. 2006. Correlation among pathology, genotype, and patient outcomes in glioblastoma. J Neuropathol Exp Neurol 65: 846-854.

Hou Y, Wang HQ, Fu K, Zhang HL, Qian ZZ, Qiu LH, Li W, Zhou SY, LI LF, Hao XS. 2011. Expression of Cdc7 and mcm2 as a marker for proliferation and prognosis in diffuse large B cell lymphoma. Zhonghua Zhong Liu Za Zhi 33(12): 911-915.

Huang MC, Kubo O, Tajika Y, Takakura K. 1996. A clinic-immunohistochemical study of giant cell glioblastoma. Noshuyo Byori 13:11-16.

Hunter, T. & Pines, J. 1994. Cyclins and Cancer II: Cyclin D and CDK inhibitors come of age. Cell 79: 573-582.

Ichimura, Y., Kirisako, T., Takao, T., et al. 2000. A ubiquitin-like system mediates protein lipidation. Nature 408(6811): 488–492.

Inoue D, Suzuki T, Mitsuishi Y, Miki Y, Suzuki S, Sugawara S, Watanabe M, Sakrada A, Endo C, Uruno A, Sasano H, Nakagawa T, Satoh K, Tanaka N, Kubo H, Motohashi H, Yamamoto M. 2012. Cancer Science 103(4): 760-766.

Itakura E. and Mizushima N. 2010. Characterization of autophagosome formation site by a hierarchical analysis of mammalian Atg proteins. Autophagy 6(6): 764–776.

Itakura E, Kishi C, Inoue K and Mizushima N. 2008. Beclin 1 forms two distinct phosphatidylinositol 3-kinase complexes with mammalian Atg14 and UVRAG. Molecular Biology of the Cell 19(12): 5360–5372.

Ito H., Daido S., Kanzawa T., Kondo S. & Kondo Y. 2005. Radiation-induced autophagy is associated with LC3 and its inhibition sensitizes malignant glioma cells. Int J Oncol 26:1401–1410.

Ito H. et al. 2006. Autophagic cell death of malignant glioma cells induced by conditionally replicating adenovirus. Journal of National Cancer Institute 98(9): 625-636.

Jaeger PA, Wyss-Coray. 2009. All-you-can-eat: autophagy in neurodegeneration and neuroprotection. Molecular Neurodegeneration. 4:16. doi:10.1186/1750-1326-4-16.

Jin HO, Park IC, An S, Lee HC, Woo SH, Hong YJ, Lee SJ, Park MJ, Yoo DH, Rhee CH, Hong SI. 2006. Up-regulation of Bak and Bim via JNK downstream pathway in response to nitric oxide in human glioblastoma cells. J Cell Physiol 206(2): 477-86.

Kamada Y, Funakoshi T, Shintani T, Nagano K, Ohsumi M and Ohsumi Y. 2000. Tor-mediated induction of autophagy via an Apg1 protein kinase complex. Journal of Cell Biology 150(6): 1507–1513.

Kanzawa T, Bedwell J, Kondo Y et al. 2003. Inhibition of DNA repair for sensitizing resistant glioma cells to temozolomide. J Neurosurg 99:1047–1052.

Kanzawa T., Germano I. M., Komata T., Ito H., Kondo Y., Kondo S. 2004. Role of autophagy in temozolomide induced cytotoxicity for malignant glioma cells. Cell Death Differ 11: 448–457.

Kaplan KB, Burds AA., Swedlow JR., Bekir S.S., Sorger P.K. and Nathke I.S. 2001. A role for the Adenomatous Polyposis Coli protein in chromosome segregation. Nat Cell Biol 3: 429-32.

Kappelle AC, Postma TJ, Taphoorn MJB, Groeneveld GJ, Van Den Bent MJ, Van Groeningen CJ, Zonnenberg BA, Sneeuw KCA, Heimans JJ. 2001. PCV chemotherapy for recurrent glioblastoma multiforma. Neurology 56:118-120.

Kearsey SE & Labib K. MCM proteins: evolution, properties and role in DNA replication.1998. Biochem Biophys Acta 1398: 113-136.

Kerr JF, Wylie AH, and Currie AR. 1972. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 26: 239-257.

Kim YJ, Ketter R, Steudel WI and Feiden W. 2007. Prognostic significance of mitotic index using the mitosis marker anti-phosphohistone H3 in meningiomas. Am J Clin Pathol 128(1): 118-125.

Klionsky DJ. 2005. The molecular machinery of autophagy: unanswered questions. Journal of Cell Science 118: 7-18.

Klionsky DJ and Ohsumi Y. 1999. Vacuolar import of proteins and organelles from the cytoplasm. Annu. Rev Cell Dev Biol 15: 1-32.

Kobayashi T, Masumoto J, Tada T, Nomiyama T, Hongo K, Nakayama J. 2007. Prognostic Significance of the Immunohistochemical Staining of Cleaved Caspase-3, an Activated Form of Caspase-3 in Gliomas. Clin Cancer Res 13(13): 3868-3874.

Konstantinidou AE, Givalos N, Gakiopoulou H, Korkolopoulou P, Kotsiakis X, Boviatsis E, Agrogiannis G, Mahera H, Patsouris E. 2007. Caspase-3 immunohistochemical expression is a marker of apoptosis, increased grade and early recurrence in intracranial meningiomas. Apoptosis 12(4): 695-705.

Komatsu, M., Waguri, S., Koike, M. et al. 2007. Homeostatic levels of p62 control cytoplasmic inclusion body formation in autophagy-deficient mice. Cell 131(6):1149-1163.

Kraus JA, Wenghoefer M, Glesmann N, Mohr S, Beck M, Schmidt MC, Schder R, Berweiler U, Roggendorf W, Diete S, Dietzmann K, Heuser K, Muller B, Fimmers R, von Deimling A and Schlegel U. 2001. TP53 gene mutations, nuclear p53 accumulation, expression of Waf/p21, Bcl-2, and CD95 (APO-1/Fas) proteins are not prognostic factors in de novo glioblastoma multiforme. J Neuro-Oncol 52: 263-272.

Krex D, Klink B, Hartmann C, von Deimling A, Pietsch T, Simon M, Sabel M, Steinbach JP, Heese O, Reifenberger G, Weller M, Schackert G. 2007. German Glioma Network: Long-term survival with glioblastoma multiforme. Brain 130:2596-2606.

Kuijlen JMA, Mooij JJA, Platteel I, Hoving EW, Graaf WT, Span MM, Hollema H and Dunnen WFA. 2006. TRAIL-receptor expression is an independent prognostic factor for survival in patients with a primary glioblastoma multiforme. Journal of Neuro-oncology 87: 161-171.

Kyritsis AP, Bondy ML, Rao JS and Sioka C. 2010. Inherited predisposition to glioma. Neuro Oncol 12(1): 104-113.

Ladstein RG, Bachmann IM, Straume O, Akslen LA. 2012. Prognostic importance of mitotic marker phosphohistone H3 in cutnaeous nodular melanoma. J Invest Dermatol 132(4): 1247-52.

Lamborn KR, Chang SM, Prados MD. 2004. Prognostic factors for survival of patients with glioblastoma: recursive partitioning analysis. Neuro Oncol 6(3): 227-35.

Lechapt-Zalcman E, Levallet G, Dugue AE, Vital A, Diebold MD, Menei P, Colin P, Peruzzy P, Emery E, Bernaudin M, Chapon F, Guillamo JS. 2012. O(6) –methylguanine- DNA methyltransferase (MGMT) promoter methylation and low MGMT-encoded protein expression as prognostic markers in glioblastoma patients treated with biodegradable carmustine wafer implants after initial surgery followed by radiotherapy with concomitant and adjuvant temozolomide. Cancer 118(18):4545-54.

LeFranc, F., Facchini, V., Kiss, R. 2007. Proautophagic drugs: a novel means to combat apoptosis-resistant cancers, with special emphasis on glioblastomas. The Oncologist 12:1395–1403.

Lei, M. & Tye, B.K. (2001). Initiating DNA synthesis: from recruiting to activating the MCM complex. J Cell Sci 114: 1447-54.

Lessene G, Czabotar P.E, Colman P.M. 2008. BCL-2 family antagonists for cancer therapy. Nat Rev Drug Discov 7: 989-1000.

Levin VA, Wilson CB. 1976. Nitrosourea chemotherapy for primary malignant glioma. Cancer Treat Rep 60:719-724.

Levine A.J. 1997. P53, The cellular gatekeeper for growth and division. Cell 88: 323-331.

Liang C., Feng P., Ku B. et al. 2006. Autophagic and tumour suppressor activity of a novel Beclin1-binding protein UVRAG. Nature Cell Biology 8(7): 688–698.

Lockshin, R.A., and Williams, C.M. 1965. Programmed cell death-I. Cytology of degeneration in the intersegmental muscles of the pernyi silkmoth. J Insect Physiol 11: 123-133.

Loeb L. A. Mutator phenotype may be required for multistage carcinogenesis. 1991. Cancer Res 51: 3075-3079.

Louis D.N., Ohgaki H., Wiestler O. D., Cavenee W. K. (Eds). 2007. WHO Classification of Tumours of the Central Nervous System, 4th edition, IARC, Lyon.

Madine, M. & Laskey, R. (2001). Geminin bans replication licence. Nat Cell Biol 3:

E49-50.

Maiorano D, Van Assendelft GB & Kearsey SE. 1996. Fission yeast cdc21, a member of the MCM protein family, is required for onset of S-phase and is located in the nucleus throughout the cell cycle. EMBO-Journal 15: 861-872.

Maiuri F, Stella L, Benvenuiti D, Giamundo A, Pettinato G. 1990. Cerebral gliosarcomas: correlation with computed tomographic findings, surgical aspect, pathological features, and prognosis. Neurosurgery 26: 261-267.

Maiuri MC, Zalckvar E, Kimchi A, Kroemer G. 2007. Selfeating and self-killing: crosstalk between autophagy and apoptosis. Molecular cell biology 8: 741-752.

Maiuri MC, Criollo A and Kroemer G. 2010. Crosstalk between apoptosis and autophagy within Beclin1 interactome. EMBO 29: 515-516.

Margraf LR, Gargan L, Butt Y, Raghunathan N and Bowers DC. 2011. Proliferative and metabolic markers in incompletely excised pediatric pilocytc astrocytomas - an assessment of 3 new variables in predicting clinical outcome. Neuro Oncol 13(7): 767-774.

Martin S, Toquet C, Oliver L, Carton P, Perrin P, Melfah K, Cuillere P and Vallette F. M. 2001. Expression of bcl-2, bax and bcl-xl in human gliomas: A re-appraisal. Journal of Neuro-Oncology 52: 129-139.

Matsubara T, Eimoto T, Okabe M, Miyabe S, Fujiyoshi Y, Matsushita Y, Mizutani J, Yamada S, Otsuka T. 2008. Proliferation and apoptosis of tumour cells before and after neoadjuvant therapy for high-grade extremity sarcomas: divergent associations with tumour response and prognosis. Histopathology 52(6): 706-16.

Matsunaga, K., Morita, E., Saitoh, T., et al. 2010. Autophagy requires endoplasmic reticulum targeting of the PI3-kinase complex via Atg14L. Journal of Cell Biology 190(4): 511–521.

McCubrey, J. A., Steelman L. S., Abrams, S. L. et al. 2006. Roles of the RAF/MEK/ERK and PI3K/PTEN/AKT pathways in malignant transformation and drug resistance. Adv Enzyme Regul 46:249 –279.

McGirt MJ, Than KD, Weingart JD, Chaichana KL, Attenello FJ, Olivi A, Latterra J, Kelinberg LR, Grossman SA, Brem H Quinones-Hinojosa A. 2009. Gliadel (BCNU) wafer plus concomitant temozolomide therapy after primary resection of glioblastoma multiforme. J Neurosurg 110(3) 583-8.

Medical Research Council Brain Tumour Working Party. 2001. Randomized trial of procarbazine, lomustine and vincristine in the adjuvant treatment of high-grade astrocytomas: a Medical Research Council trial. J Clin Oncol 19:509-518.

Mellai M. and Schiffer D. 2007. Apoptosis in brain tumours: prognostic and therapeutic considerations. Anticancer Research 27: 437-448.

Mineo JF, Bordron A, Baronchi M, Ramirez C, Maurage CA, Blond S, Dam-Hieu P. 2007. Prognostic factors of survival time in patients with glioblastoma multiforme: a multivariate analysis of 340 patients. Acta Neurochir (Wien) 149: 245-253.

Mizushima, N., Noda T., Yoshimori, T., et al. 1998. A protein conjugation systemessential for autophagy. Nature 395(6700): 395–398.

Morgan D. O. 1997. Cyclin-dependent kinases:engines, clocks and microprocessors. Annu Rev Cell Dev Bio. 13: 261-291.

Morgan D. 2007. The cell cycle. New Science Press Ltd.

Moscat, J. and Diaz-Meco, M. T. 2009. p62 at crossroads of autophagy, apoptosis, and cancer. Cell 137(6):1001-1004.

Moscat, J and Diaz-Meco, M. T. 2012. p62: a versatile multitasker takes on cancer. *Trends in Biochemical Sciences* 37(6):230-236.

Moschovi M, Alexiou GA, Patereli A, Stefanaki K, Doussis-Anagnostopoulou I, Stofas A, Sfakianos G, Prodromou N. 2011. Prognostic significance of cyclin A and B1 in pediatric embryonal tumours. J Neurooncol 103(3): 699-704.

Murray AW & Hunt T. 1993. The Cell Cycle. Freeman, New York.

Musahl C, Holthoff HP, Lesch R & Knippers R. 1998. Stability of the replicative Mcm3 protein in proliferating and differentiating human cells. Exp Cell Res 241: 260-264.

Nasmyth K. Viewpoint: 1996. Putting the cell cycle in order. Science 274: 1643-1645.

Nasmyth, K., Peters, J.M. & Uhlmann, F. 2000. Splitting the chromosome: cutting the ties that bind sister chromatids. Science 288: 1379-85.

Nelson JS, Burchfiel CM, Fekedulegn D, Andrew ME. 2012. Potential risk factors for incident Glioblastoma Multiforme: the Honolulu Heart Program and Honolulu-Asia Aging Study. Journal of Neuroncol 109(2): 315-321.

Nigg, E.A. 1998. Polo-like kinases: positive regulators of cell division from start to finish. Curr Opin Cell Biol 10, 776-83.

Nishihara K, Shomori K, Tamura T, Fujioka S, Ogawa T, Ito H. 2009. Immunohistochemical expression of geminin in colorectal cancer: Implication of prognostic significance. Oncol Rep 21(5): 1189-1195.

Nishitani H, Taraviras S, Lygerou Z & Nishimoto T. 2001. The human licensing factor for DNA replication Cdt1 accumulates in G1 and is destabilised after initiation of S-phase. J Biol Chem 276: 44905-44911.

Nezis, I. P., Simonsen, A., Sagona, A.P. et al. 2008. Ref(2)P, the Drosophila melanogaster homologue of mammalian p62, is required for the formation of protein aggregates in adult Brain. Journal of Cell Biology 180(6):1065–1071.

Nobusawa S, Watanabe T, Kleihues P, Ohgaki H. 2009. IDH1 mutation as molecular signature and predictive factor of secondary glioblastomas. Clin Cancer Res 15(9): 6002-7.

Noda, T., Kim, J., Huang, W. P., et al. 2000. Apg9p/Cvt7p is an integral membrane protein required for transport vesicle formation in the Cvt and autophagy pathways. Journal of Cell Biology 148(3): 465–479.

Noda, N. N., Kumeta H., Nakatogawa H., et al. 2008. Structural basis of target recognition by Atg8/LC3 during selective autophagy. Genes to Cells 13(12):1211–1218.

Noel G, Schott R, Froelich S, Gaub MP, Boyer P, Fischer-Lokou D, Dufour P, Kehrli P, Maitrot D. 2012. Retrospective comparison of chemoradiotherapy followed by adjuvant chemotherapy, with and without prior gliodel implantation (carmustine) after initial surgery in patients with newly diagnosed high-grade gliomas. Int J Radiat Oncol Biol Phys 82(2): 749-55.

Nurse, P. 1990. Universal control mechanism regulating onset of M-phase. Nature 344: 503-508.

Nyberg KA, Michelson RJ, Putnam CW & Weinert TA. 2002. Toward maintaining the genome: DNA damage and replication checkpoints. Ann. Rev. Genetics 36: 617-656.

Ohashi, Y. and Munro, S. 2010. Membrane delivery to the yeast autophagosome from the golgi-endosomal system. Molecular Biology of the Cell 21(22)3998–4008.

Ohgaki H, Dessen P, Jourde B, Horstmann S, Nishikawa T, Di Patre PL, Burrkhard C, Schuler D, Probst-Hensch NM, Maiorka PC, Baeza N, Pisani P, Yonekawa Y, Yasargil MG, Lutolf UM, Keihues P. 2004. Genetic pathways to glioblastoma: a population-based study. Cancer Res 64(19): 6892-6899.

Ohgaki H and Kleihues P. 2005. Epidemiology and etiology of gliomas. Acta Neuropathol 109: 93-108.

Ohgaki H and Kleihues P. 2007. Genetic pathways to Primary and Secondary Glioblastoma. Am J Pathol 170(5): 1445-1453.

Ohka F, Natsume A, Wakabayashi T. 2012. Current trends in targeted therapies for glioblastoma multiforme. Neurol Res Int 2012: 878425.

Okada H, Mak TW. 2004. Pathways of apoptotic and non-apoptotic death in tumour cells. Nat Rev Cancer 4:592– 603.

Paglin, S. et al. 2001. A novel response of cancer cells to radiation involves autophagy and formation of acidic vesicles. Cancer Res 61: 439–444.

Pattingre, S., Levine, B. 2006. Bcl-2 inhibition of autophagy: A new route to cancer? Cancer Res 66:2885–2888.

Peiffer J, Keihues P. 1999. Hans-Joachim Scherer (1906-1945), pioneer in glioma research. Brain Pathol 9(2): 241-245.

Pines J & Hunter T. Cyclins A and B1 in the human cell cycle 1992. Ciba Found. Symp 170: 187-196.

Poikonen P, Sjostrom J, Amini RM, Villman K, Ahlgren J and Blomqvist C. 2005. Cyclin A as a marker for prognosis and chemotherapy response in advanced breast cancer. Br J Cancer 93: 515-519.

Puissant A, Fenouille N and Auberger P. 2012. When autophagy meets cancer through p62/SQSTM1. Am J Cancer Res 2(4): 397-413.

Preusser M, de Ribaupierre S, Wohrer A, Erridge SC, Hegi M, Weller M and Stupp R. 2011. Current concepts and management of glioblastoma. Annals of Neurology 70(1): 9-21.

Quitet CA, Weichselbaum RR, Gradina DJ. 1991. Variation in radiation sensitivity during the cell cycle of two human sauamous cell carcinomas. Int J Radia Oncol Bio Phys 20(4): 733-738.

Rolland P, Madjd Z, Durrant L, Ellis IO, Layfield R and Spendlove I. 2007. The ubiquitin-binding protein p62 is expressed in breast cancers showing features of aggressive disease. Endocr Relat Cancer 14: 73-80.

Reggiori F., Komatsu M., Finley K., Simonsen A. 2012. Selective types of autophagy. International Journal of Cell Biology Volume 2012, Article ID 219625, 18 pages.

Reggiori, F., Tucker, K. A., Stromhaug, P. E. and Klionsky, P. E. 2004. The Atg1-Atg13 complex regulates Atg9 and Atg23 retrieval transport from the pre-autophagosomal structure. Developmental Cell 6(1): 79–90.

Ricci MS, Zong WX. 2006. Chemotherapeutic approaches for targeting cell death pathways. The Oncologist 11:342–357.

Ruano Y, Mollejo M, Camacho FI, de Lope Rodriguez A, Fiano C, Ribalta T, Martinez P, Hernandez-Moneo JL, Melendez B. 2008. Identification of survival related genes of the phosphatidylinositol 3'-kinase signaling pathway in glioblastoma multiforme**.** Cancer 112:1575-1584.

Rubinsztein, D. C., Gestwicki, J. E., Murphy, L. O. & Klionsky, D. J. 2007. Potential therapeutic applications of autophagy. Nature Rev Drug Discov 6: 304–312.

Russell DS, Rubinstein LJ. 1989. Pathology of tumours of the Nervous system. Edward Arnold: London.

Sanai N, Berger MS. 2008. Glioma extent of resection and its impact on patient outcome. Neurosurgery 62(4): 753-764.

Sallinen PK, Haapasalo HK, Visakorpi T, Helen PT, Rantala IS, Isola JJ & Helin HJ. 1994. Prognostication of astrocytoma patient survival by Ki67 (Mib-1), PCNA, and S-phase fraction using archival paraffin-embedded samples. J Pathol 174: 275-282.

Salvesen GS and Duckett CS. 2002. IAP proteins: blocking the road to death's door. Nat Rev Mol Cell Biol 3: 401-410.

Sawa, H., Ohshima, T.A., Ukita, H., Murakami, H., Chiba, Y., Kamada, H., Hara, M. & Saito, I. 1998. Alternatively spliced forms of cyclin D1 modulate entry into the cell cycle in an inverse manner. Oncogene 16: 1701-12.

Scaffidi, C., Fulda, S., Srinivasan, A., Friesen, C., Li, F., Tomaselli, K. J., Debatin, K. M., Krammer, P. H., and Peter, M. E. (1998) Two CD95 (APO-1/Fas) signalling pathways. EMBO J 17: 1675–1687.

Schmidt F, Fischer J, Herrlinger U, Dietz K, Dichgans J, Weller M. 2006. PCV chemotherapy for recurrent glioblastoma. Neurology 66(4): 587-589.

Scott IS, Morris LS, Rushbrook SM, Bird K, Vowler SL, Burnett NG & Coleman N. 2005. Immunohistochemical estimation of cell cycle entry and phase distribution in astrocytomas: applications in diagnostic neuropathology. Neuropathol. Appl. Neurobiol 31: 455-466.

Shibata, K. & Ajiro, K. 1993. Cell cycle dependent suppressive effect of histone H1 on mitosis specific H3 phosphorylation. J. Biol. Chem 268: 18431-18434.

Shichiri, M., Yoshinaga, K., Hisatomi, H., Sugihara, K. & Hirata, Y. 2002. Genetic and epigenetic inactivation of mitotic checkpoint genes hBUB1 and hBUBR1 and their relationship to survival. Cancer Res 62: 13-7.

Shinojima N, Ohta K, Yano S, Nakamura H, Yano S, Makino K, Tsuiki H, Tada K, Kuratsu J, Ishimaru Y, Ushio Y. 2004. The influence of sex and the presence of giant cells on postoperative long-term survival in adult patients with supratentorial glioblastoma multiforme. J Neurosurg 101: 219-226.

Shintani, T. & Klionsky, D. J. 2004. Autophagy in health and disease: a double-edged sword. Science 306: 990–995.

Shresta P, Saito T, Hama S, Arifin MT, Kajiwara Y, Yamasaki F, Hidaka T, Sugiyama K & Kurisu K. 2007. Geminin: a good prognostic factor in high grade astrocytic brain tumours. Cancer 109: 949-56.

Shvets E, Fass E, Scherz-Shouval, Elazar Z. 2008. The N-terminus and Phe52 residue of LC3 recruit p62/SQSTM1 into autophagosomes. Journal of Cell Science 121: 2685-2695.

Skaland I, Janssen EA, Gudlaugsson E, Hui Ru Guo L, Baak JP. 2009. The prognostic value of the proliferation marker phosphohistone H3 (PPH3) in luminal, basal-like and triple negative phenotype invasive lymph node-negative breast cancer. Cell Oncol 31(4): 261-71.

Sperduto CM, Chakravarti A, Aldape K, Burger P, Papermaster GB, Sperduto P. 2009. Twenty-year survival in Glioblastoma: A Case report and molecular profile. Int J Radiat Oncol Biol Phys 75: 1162-1165.

Spierings D, Mcstay G, Saleh M, Bender C, Chipuk J, Maurer U, Green D. 2005. Connected to death: The (unexpurgated) mitochondrial pathway of apoptosis. Science 310(5745) :66-67.

Srinivasula SM and Ashwell JD. 2008. IAPs: what's in a name? Mol Cell 30:123-135.

Stenner-Liewen F. And Reed J.C. 2003. Apoptosis and Cancer: Basic mechanisms and therapeutic opportunities in postgenomic era. Cancer Research 63, 263-268.

Stillman B. 1996. Cell cycle control of DNA replication. Science 274: 1659-1663.

Strik H, Deininger M, Streffer J, Grote E, Wickboldt J, Dichgans J, Weller M, Meyermann R. 1999. BCL-2 family protein expression in initial and recurrent glioblastomas: modulation by radiochemotherapy. J Neurol Neurosurg Psychiatry 67:763-768.

Stummer W, Pichmeier U, Meinel T, Wiestler OD, Zanella F, Reulen HJ; ALA-Glioma Study Group. 2006. Flourescence-guided surgery with 5-aminolevulinic acid for resection of malignant glioma: a randomised controlled multicentre phase III trial. Lancet Oncol 7(5): 392-401. Stummer W, Reulen HJ, Meinel T, Pichlmeier U, Schumacher W, Tonn JC, Rohde V Oppel F, Turowski B, Woiciechowsky C, Franz K, Pietsch T; ALA-Glioma study group. 2008. xtenet of resection and survival in glioblastoma multiforme: identification of and adjustment for bias. Neurosurgery 62(3): 564-76. Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJ, Belanger K, Brandes AA, Marosi C, Bogdahn U, Curschmann J, Janzer RC, Ludwin SK, Gorlia T, Allgeier A, Lacombe D, Cairncross JG, Eisenhauer E, Mirimanoff RO; European Organisation for Research and Treatment of Cancer Brain Tumor and Radiotherapy Groups; National Cancer Institute of Canada Clinical Trials

Group. 2005. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N Engl J Med 352: 987-96.

Stupp R, Hegi ME, Mason WP, van den Bent MJ, Taphoorn MJ, Janzer RC, Ludwin SK, Allgeier A, Fisher B,Belanger K, Hau P, Brandes AA, Gijtenbeek J, Marosi C, Vecht CJ, Mokhtari K, Wesseling P, Villa S,Eisenhauer E, Gorlia T, Weller M, Lacombe D, Cairncross JG, Mirimanoff RO; European Organisation for Research and Treatment of Cancer Brain Tumour and Radiation Oncology Groups; National Cancer Institute of Canada Clinical Trials Group. **2009.** Effects of radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on survival in glioblastoma in a randomised phase III study: 5-year analysis of the EORTC-NCIC trial. Lancet Oncol 10(5): 459-66.

Sun Q., Fan W., Chen K., Ding X., Chen S. and Zhong Q. 2008. Identification of Barkor as a mammalian autophagy-specific factor for Beclin 1 and class III phosphatidylinositol 3-Kinase. Proceedings of the National Academy of Sciences of the United States of America 105(49): 19211–19216.

Suzuki K., Kubota Y., Sekito T. and Ohsumi Y. Hierarchy of Atg proteins in pre-autophagosomal structure organization. Genes to Cells 12(2): 209–218, 2007.

Tagscherer KE, Fassl A, Campos B, Farhadi M, Kraemer A, Bock BC, Macher-Goeppinger S, Radlwimmer B, Wiestler OD, Herold-Mende C, Roth W. 2008. Apoptosis-based treatment of glioblastomas with ABT-737, a novel small molecule inhibitor of Bcl-2 family proteins. Oncogene 27:6646–6656.

Takahashi Y., Coppola D., Matsushita N., et al. 2007. Bif-1 interacts with Beclin 1 through UVRAG and regulates autophagy and tumorigenesis. Nature Cell Biology 9(10):1142–1151.

Takahashi, Y., Meyerkord C. L., Hori, T., et al. 2011. Bif-1 regulates Atg9 trafficking by mediating the fission of Golgimembranes during autophagy. Autophagy 7(1):61–73.

Tamura T, Shomori K, Haruki T, Nosaka K, Hamamoto Y, Shiomi T, Ryoke K, Ito H. 2010. Minichromosome maintenance protein-7 and Geminin are reliable prognostic markers in patients with oral squamous cell carcinoma: immunohistochemical study. J Oral Pathol Med 39(4): 328-34.

Tell R, Heiden T, Granath F, Borg AL, Skog S, Lewensohn R. 1998. Comparison between radiation-induced cell cycle delay in lymphocytes and radiotherapy response in head and neck cancer. British J Cancer 77(4): 643-649.

Terasima T & Tolmach LJ. Variations in several responses of HeLa cells to x-irradiation during the division cycle. 1963. Biophys J 3: 11-33.

Tsijimoto, Y. 2003. Cell death regulation by Bcl-2 protein family in the mitochondria. J. Cell. Physiol 195: 158-167.

Verhaak RG, Hoadley KA, Purdom E, Wang V, Qi Y, Wilkerson MD, et al. 2010. Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. Cancer Cell 17: 98–110.

Verhagen AM, Coulson EJ and Vaux DL. 2001. Inhibitors of apoptosis proteins and their inhibitors: IAPs and other BIRPs. Genome Biol 2, Reviews 3009.

Viktorsson K, Lewensohn R, Zhivotovsky B. 2005. Apoptotic pathways and therapy resistance in human malignancies. Adv Cancer Res 94:143–196.

Wang, X. 2001. The expanding role of mitochondria in apoptosis. Genes Dev. 15, 2922-2933.

Wei MC, Zong WX, Cheng EH, Lindsten T, Panoutsakopoulou V, Ross AJ, Roth KA, MacGregor GR,Thompson CB, Korsmeyer SJ. 2001. Proapoptotiv BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. Science 292 (5517): 727-30.

Westermark B. 2010. Glioblastoma-a moving target. Ups J Med Sci 117(2): 251-256.

Westphal M, Ram Z, Riddle V, Hilt D, Bortey E; Executive Committee of the Gliadel Study Group. 2006. Gliadel wafer in initial surgery for malignant glkioma: long-term follow-up of a multicentre controlled trial. Acta Neurochir (Wein) 148(3): 269-75.

Wharton SB, Hibberd S, Eward KL, Crimmins D, Jellinek DA, Levy D, Stoeber K & Williams GH. 2004. DNA replication licencing and cell cycle kinetics of oligodendroglial tumours. Br J Cancer 91: 262-269.

Wick W, Wagner S, Kerkau S, Dichgans J, Tonn JC, Weller M. 1998. BCL-2 promotes migration and invasiveness of human glioma cells. FEBS Lett 440:419-424.

Wick, W., Grimmel, C., Wild-Bode, C., Platten, M., Arpin, M., and Weller, M. 2001. Ezrin-dependent promotion of glioma cell clonogenicity, motility, and invasion mediated by BCL-2 and transforming growth factor-_2. J Neurosci 21: 3360–3368.

Wick, W., Wild-Bode, C., Frank, B., and Weller, M. 2004. BCL-2-induced glioma cell invasiveness depends on furin-like proteases. J Neurochem 91: 1275–1283.

Wohlschlegel JA, Dwyer BT, Dhar SK, Cvetic C, Walter JC & Dutta A. 2000. Inhibition of eukaryotic DNA replication by geminin binding to Cdt1. Science 290: 2309-2312.

Wohlschlegel JA, Kutok JL, Weng AP & Dutta A. 2002. Expression of geminin as a marker of cell proliferation in normal tissues and malignancies. Am J Pathol 161: 267-273.

Wolf, B.B., and Green, D.R. 2002. Apoptosis: letting slip the dogs of war. Curr Biol 12: R177-R179.

Xie Z. and Klionsky D. J. 2007. Autophagosome formation: core machinery and adaptations. Nature Cell Biology 9(10): 1102–1109.

Xouri G, Dimaki M, Bastiaens PIH & Lygerou Z. 2007. Cdt1 interactions in the licencing process: a model for dynamic spatiotemporal control of licensing. Cell Cycle 6: 1549-1552.

Yamazaki M, Fujii S, Murata Y, Hayashi R, Ochiai A. 2010. High expression level of geminin predicts poor clinical outcome in salivary gland carcinoma. Histopathology 56(7): 883-892.

Yang C, Wen Y, Li H, Zhang D, Zhang N, Shi X, Jiang B, Ma X, Yang P, Tang H, Peng Z, Yang Y. 2012. Overexpression of minichromosome maintenance 2 predicts poor prognosis in patients with gastric cancer. Oncol Rep. 27(1): 135-142.

Yang Z. and Klionsky D. J. 2009. An overview of the molecular mechanism of autophagy. Current Topics inMicrobiology and Immunology 335(1): 1–32.

Yang Z. and Klionsky, D. J. 2010. Mammalian autophagy: core molecular machinery and signaling regulation. Current Opinion in Cell Biology 22(2): 124–131.

Yoshida K, Oyaizu N, Dutta A, Inoue I. 2004. The destruction box of human Geminin is crictical for proliferation and tumour growth in human colon cancer cells. Oncogene 23(1): 58-70.

Yoshimori T. and Noda T. Toward unraveling membrane biogenesis in mammalian autophagy. 2008. Current Opinion in Cell Biology, 20(4): 401–407.

Zatloukal, K., Stumptner, C., Fuchsbichler, A., et al. 2002. p62 is a common component of cytoplasmic inclusions in protein aggregation diseases. American Journal of Pathology 160(1):255–263.

Zhao DB, Chandler I, Chen HC, Pan HC, Popat S, Shao YF and Houlston RS. 2011. Mismatch repair, minichromosome maintenance complex component 2, cyclin A, and transforming growth factor β receptor type II as prognostic factors for colorectal cancer: results of a 10-year prospective study using tissue microarray analysis. Chin Med J. 124(4): 483-490.

Table 1: Group 1 cell cycle markers

No.	Sex	Date of Diagnosis	Survival (days)	Age at Diagnosis (years)	Procedure 1-Debulk, 2-Bx	Geminin %	MCM-2 %	Cyclin A %	Karnovsky Score	Radiotherapy	PCV Chemo-therapy	Temozolomide
1	F	20/11/2002	170	53	1	16.15	20.40	7.68	80	Yes	No	No
2	F	01/02/2003	144	61	1	4.68	43.73	3.97	70	Yes	No	No
3	M	13/02/2003	285	55	1	4.50	27.99	2.72	60	Yes	No	No
4	M	02/07/2003	386	58	1	7.34	22.13	4.13	70	Yes	No	No
5	M	21/07/2003	150	63	1	6.42	15.56	0.39	70	Yes	No	No
6	M	31/07/2003	437	50	1	7.66	30.04	5.02	90	Yes	Yes	No
7	F	22/10/2003	173	74	1	6.47	38.18	4.21	60	Yes	No	No
8	M	19/11/2003	408	47	1	14.83	23.24	6.56	80	Yes	Yes	No
9	M	27/11/2003	401	56	1	10.66	19.99	6.94	90	Yes	No	No
10	F	01/12/2003	540	54	1	13.63	68.25	11.81	70	Yes	No	No
11	M	02/12/2003	204	59	1	5.18	15.38	0.94	70	Yes	No	No
12	F	22/12/2003	143	69	1	5.11	33.78	2.43	60	Yes	No	No
13	F	15/01/2004	453	60	2	9.46	26.59	3.58	80	Yes	No	No
14	M	04/02/2004	279	63	1	6.47	30.66	6.23	70	Yes	No	No
15	M	13/09/2004	336	64	1	5.82	28.40	3.50	60	Yes	No	No
16	M	05/10/2004	87	61	1	2.85	15.26	2.41	70	Yes	No	No
17	M	24/01/2005	210	43	1	4.26	21.72	3.08	70	Yes	No	No
18	F	09/02/2005	169	79	1	3.88	22.90	2.48	50	Yes	No	No
19	M	18/05/2005	314	81	2	10.39	40.29	5.09	70	Yes	No	No
20	F	19/05/2005	237	58	1	2.93	23.85	1.65	60	Yes	No	No
21	M	12/08/2005	388	64	2	10.47	54.86	5.10	60	Yes	No	No
22	F	13/09/2006	689	40	1	9.55	73.32	7.55	80	Yes	No	Yes
23	M	05/07/2007	165	29	2	5.11	9.45	2.00	60	Yes	No	Yes
24	F	15/08/2007	417	66	1	7.79	44.69	2.36	60	Yes	No	No

25	M	28/08/2007	177	69	1	2.88	9.22	0.83	60	Yes	No	No
26	F	11/09/2007	325	38	1	9.57	50.83	7.90	60	Yes	No	Yes
27	M	04/10/2007	300	64	1	7.80	49.33	4.07	50	Yes	No	No
28	M	15/11/2007	270	56	1	13.19	77.98	7.04	60	Yes	No	No
29	M	28/11/2007	378	57	1	7.87	54.40	4.51	60	Yes	No	Yes
30	F	30/01/2008	145	58	1	8.60	42.03	3.63	60	Yes	No	Yes
31	M	03/03/2008	129	61	1	9.26	33.25	6.04	50	Yes	No	Yes
32	M	03/03/2008	269	61	1	12.48	47.95	10.13	60	Yes	No	Yes
33	M	07/03/2008	180	65	1	13.22	55.52	6.52	60	Yes	No	Yes
34	M	25/03/2008	246	65	1	10.71	56.75	7.83	60	Yes	No	Yes
35	M	31/03/2008	220	64	1	4.76	40.67	4.08	60	Yes	No	Yes
36	M	28/04/2008	104	56	2	6.09	43.59	3.53	60	Yes	No	Yes
37	M	30/04/2008	144	61	2	9.25	51.78	6.86	60	Yes	No	Yes
38	M	17/06/2008	153	67	2	6.16	57.45	5.06	60	Yes	No	Yes
39	M	30/06/2008	111	62	2	6.41	31.14	1.99	50	Yes	No	Yes
40	M	02/07/2008	159	69	1	11.53	55.97	3.99	60	Yes	No	Yes
41	M	05/08/2008	166	72	1	3.69	21.15	1.96	60	Yes	No	Yes
42	M	25/02/2006	1059	19	1	8.70	34.64	7.37	70	Yes	Yes	No
43	M	06/08/2007	596	28	1	-	44.59	14.75	80	Yes	No	Yes
44	M	07/08/2007	595	46	1	10.29	19.69	4.50	60	Yes	No	Yes
45	F	15/02/2008	405	70	1	17.60	74.04	8.76	60	Yes	No	Yes
46	F	06/04/2008	355	60	1	15.99	68.09	11.59	70	Yes	Yes	Yes
47	F	27/03/2008	365	54	1	9.67	19.27	1.12	60	Yes	No	Yes
48	F	24/04/2008	337	36	1	19.67	54.41	4.50	70	Yes	No	Yes
49	M	01/09/2008	208	50	1	6.33	29.31	1.98	60	Yes	No	Yes
50	F	01/09/2008	208	62	1	6.29	41.21	2.00	60	Yes	No	Yes

Table 2: Group 2 cell cycle markers

No.	Sex	Date of Diagnosis	Survival (days)	Age at Diagnosis (years)	Procedure 1-Debulk, 2-Bx	Geminin LI %	MCM-2 LI %	Cyclin A LI %	Karnovsky Score	Radio-therapy	PCV Chemo-therapy	Temozolomide
1	F	29/12/2003	53	70	2	8.60	32.34	6.655	50	No	No	No
2	F	13/03/2004	47	50	2	7.61	47.24	5.25	50	No	No	No
3	M	25/06/2003	89	73	1	3.43	10.04	0.82	60	No	No	No
4	F	13/10/2003	87	62	1	6.08	14.49	2.40	50	No	No	No
5	M	27/04/2005	111	64	1	9.38	73.75	9.61	60	No	No	No
6	F	04/08/2005	37	75	1	5.86	43.76	4.76	50	No	No	No
7	F	13/10/2005	36	75	1	5.86	43.76	4.76	50	No	No	No
8	F	13/12/2005	128	77	1	5.40	34.71	3.53	60	No	No	No
9	M	31/07/2007	27	74	1	8.88	28.05	4.02	40	No	No	No
10	M	09/08/2007	66	63	1	5.82	43.02	5.22	60	No	No	No
11	F	07/01/2008	63	73	1	12.50	56.34	8.67	60	No	No	No
12	F	29/04/2008	34	51	1	9.25	51.78	6.86	20	No	No	No
13	F	27/05/2008	68	70	1	8.76	45.93	4.69	50	No	No	No
14	M	24/06/2008	35	52	1	12.76	58.95	4.04	50	No	No	No
15	M	18/09/2008	137	63	1	7.65	50.36	6.09	40	No	No	No
16	F	21/10/2008	72	61	1	7.64	35.06	1.73	50	No	No	No

Table 3: Markers of apoptosis

No.	Sex	Date of Diagnosis	Survival (days)	Age at Diagnosis (years)	Procedure 1-Debulk, 2-Biopsy	Bcl-2 LI %	Bcl-xl LI %	Bak LI %	Caspase 3 LI %	Karnovsky Score	Radio-therapy	PCV Chemo-therapy	Temozolomide
1	F	20/11/2002	170	53	1	4.20	2.01	23.22	23.39	80	Yes	No	No
2	M	23/01/2003	616	64	1	12.59	1.60	6.91	34.56	70	Yes	No	No
3	F	01/02/2003	144	61	1	2.87	1.23	9.41	22.88	70	Yes	No	No
4	M	13/02/2003	285	55	1	1.36	3.91	18.81	26.42	60	Yes	No	No
5	M	25/06/2003	89	73	1	7.39	5.19	18.89	38.31	60	No	No	No
6	M	02/07/2003	386	58	1	11.69	0.76	7.96	43.36	70	Yes	No	No
7	M	21/07/2003	150	63	1	8.38	1.37	25.20	48.90	70	Yes	No	No
8	M	31/07/2003	437	50	1	1.04	1.39	14.64	52.92	90	Yes	Yes	No
9	F	13/10/2003	87	62	1	12.85	1.70	21.35	45.48	50	No	No	No
10	M	16/10/2003	384	56	1	3.65	5.06	9.09	64.95	90	Yes	No	No
11	F	22/10/2003	173	74	1	2.43	0.87	19.41	75.26	60	Yes	No	No
12	M	19/11/2003	408	47	1	8.30	4.36	6.71	47.69	80	Yes	Yes	No
13	M	27/11/2003	401	56	1	22.76	1.98	1.46	14.56	90	Yes	No	No
14	F	01/12/2003	540	54	1	9.24	2.40	3.26	59.05	70	Yes	No	No
15	M	02/12/2003	204	59	1	7.92	10.19	48.83	72.97	70	Yes	No	No
16	F	15/01/2004	453	60	2	10.51	3.58	32.92	69.69	80	Yes	No	No
17	M	04/02/2004	279	63	1	13.43	0.84	4.21	17.79	70	Yes	No	No
18	F	24/02/2004	282	59	1	0.14	0.53	3.17	90.06	60	Yes	No	No
19	M	13/09/2004	336	64	1	21.99	3.71	42.90	50.18	60	Yes	No	No
20	M	05/10/2004	87	61	1	10.98	3.54	50.06	73.68	70	Yes	No	No
21	M	24/01/2005	210	43	1	0.66	0.86	11.62	31.62	70	Yes	No	No
22	F	09/02/2005	169	79	1	14.55	-	24.26	53.63	50	Yes	No	No
23	M	27/04/2005	111	64	1	3.28	2.28	50.24	68.40	60	Yes	No	No
24	M	18/05/2005	314	81	2	11.87	1.06	5.16	75.85	70	Yes	No	No
25	F	19/05/2005	237	58	1	1.55	0.67	10.27	77.39	60	Yes	No	No

26	M	12/08/2005	388	64	2	0.17	0.57	16.13	97.72	60	Yes	No	No
27	F	13/10/2005	36	75	1	10.83	-	-	-	50	No	No	No
28	F	13/12/2005	128	77	1	2.15	14.09	31.54	38.02	60	No	No	No

Table 4: Markers of autophagy

No.	Sex	Date of Diagnosis	Survival (days)	Age at Diagnosis (years)	Procedure 1-Debulking, 2-Biopsy	LC3B staining score	p62 staining score	Karnovsky Score	Radio-therapy	PCV Chemo-therapy	Temozolomide
1	F	20/11/2002	170	53	1	3	2	80	Yes	No	No
	F	01/02/2003	144	61	1	3	3	70	Yes	No	No
3	M	13/02/2003	285	55	1	5	4	60	Yes	No	No
4	M	02/07/2003	386	58	1	5	3	70	Yes	No	No
5	M	21/07/2003	150	63	1	5	4	70	Yes	No	No
6	F	22/10/2003	173	74	1	4	3	60	Yes	No	No
7	M	19/11/2003	408	47	1	3	3	80	Yes	Yes	No
8	M	27/11/2003	401	56	1	3	2	90	Yes	No	No
9	F	01/12/2003	540	54	1	4	3	70	Yes	No	No
10	M	02/12/2003	204	59	1	3	3	70	Yes	No	No
11	F	22/12/2003	143	69	1	4	4	60	Yes	No	No
12	F	15/01/2004	453	60	2	3	3	80	Yes	No	No
13	M	04/02/2004	279	63	1	4	3	70	Yes	No	No
14	M	13/09/2004	336	64	1	5	4	60	Yes	No	No
15	M	05/10/2004	87	61	1	5	5	70	Yes	No	No
16	M	24/01/2005	210	43	1	4	3	70	Yes	No	No
17	F	09/02/2005	169	79	1	5	4	50	Yes	No	No
18	M	18/05/2005	314	81	2	5	3	70	Yes	No	No
19	F	19/05/2005	237	58	1	3	2	60	Yes	No	No
20	M	12/08/2005	388	64	2	3	2	60	Yes	No	No
21	F	15/08/2007	417	66	1	4	2	60	Yes	No	No
22	M	28/08/2007	177	69	1	4	2	60	Yes	No	No
23	F	11/09/2007	325	38	1	4	2	60	Yes	No	Yes
24	M	04/10/2007	300	64	1	4	3	50	Yes	No	No

25	M	15/11/2007	270	56	1	5	4	60	Yes	No	No
26	M	28/11/2007	378	57	1	5	4	60	Yes	No	Yes
27	F	30/01/2008	145	58	1	4	3	60	Yes	No	Yes
28	M	03/03/2008	129	61	1	5	3	50	Yes	No	Yes
29	M	03/03/2008	269	61	1	5	3	60	Yes	No	Yes
30	M	07/03/2008	180	65	1	5	3	60	Yes	No	Yes
31	M	25/03/2008	246	65	1	5	3	60	Yes	No	Yes
32	M	31/03/2008	220	64	1	5	2	60	Yes	No	Yes
33	M	28/04/2008	104	56	2	5		60	Yes	No	Yes
34	M	17/06/2008	153	67	2	2	2	60	Yes	No	Yes
35	M	30/06/2008	111	62	2	5	2	50	Yes	No	Yes
36	M	02/07/2008	159	69	1	5	4	60	Yes	No	Yes
37	M	05/08/2008	166	72	1	4	2	60	Yes	No	Yes
38	M	25/02/2006	1059	19	1	5	3	70	Yes	Yes	No
39	M	06/08/2007	596	28	1	4	2	80	Yes	No	Yes
40	M	07/08/2007	595	46	1	4	2	60	Yes	No	Yes
41	F	15/02/2008	405	70	1	5	3	60	Yes	No	Yes
42	F	06/04/2008	355	60	1	5	3	70	Yes	Yes	Yes
43	F	27/03/2008	365	54	1	5	3	60	Yes	No	Yes
44	F	24/04/2008	337	36	1	5	2	70	Yes	No	Yes
45	M	01/09/2008	208	50	1	4	2	60	Yes	No	Yes