TELOMERASE REVERSE TRANSCRIPTASE PROMOTER MUTATIONS IN A COHORT OF ADULT GLIOMAS – CLINICOPATHOLOGICAL CORRELATES

A DISSERTATION SUBMITTED IN PART FULFILMENT OF THE REGULATION

FOR THE AWARD OF THE DEGREE OF M.D. PATHOLOGY BRANCH III.

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A dissertation submitted in part fulfillment of the regulation for the award of the degree of M.D. Pathology Branch III.

CERTIFICATE

This is to certify that this dissertation entitled “Telomerase reverse transcriptase promoter mutations in a cohort of adult gliomas –Clinicopathological correlates” is the bonafide work done by Dr. Shailaja Balakumar, in part fulfillment of the rules and regulations for the M.D. Branch III (Pathology) Degree Examination of Tamil Nadu Dr. M.G.R. Medical University, to be held in May 2018.

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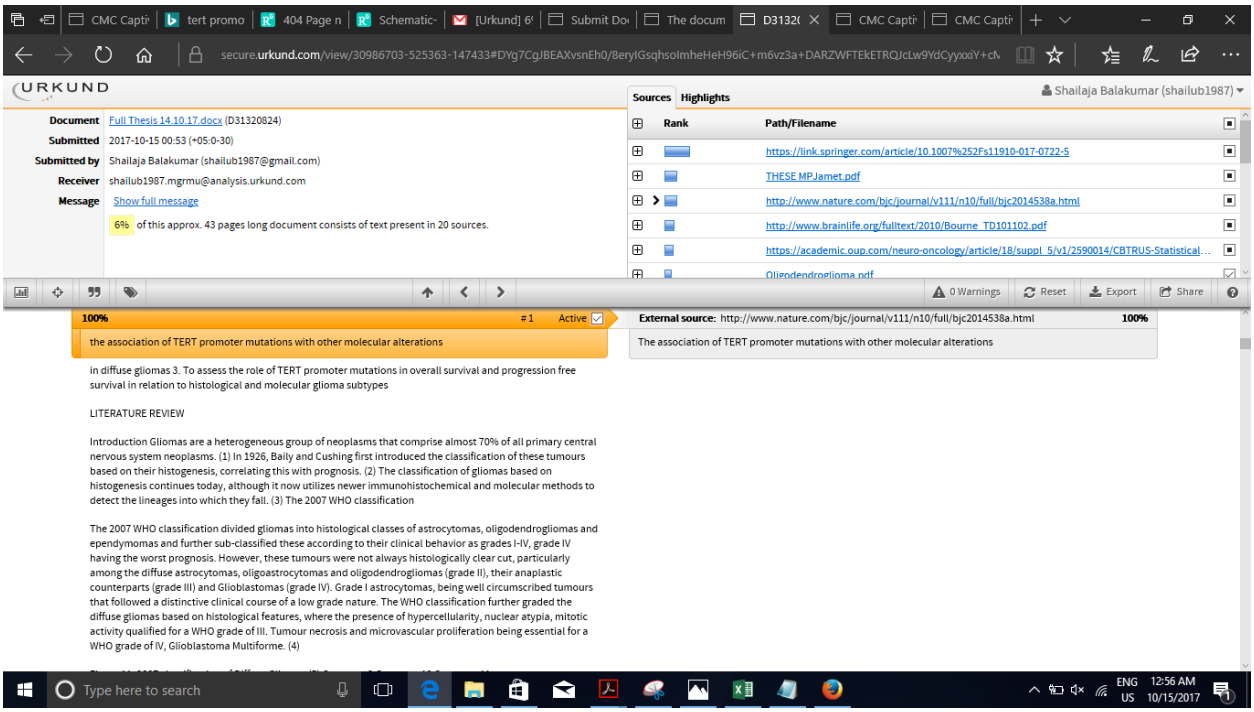
ANTIPLAGIARISM CERTIFICATE



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ABBRIEVATIONS

3DCRT – 3D conformational radiotherapy

ATRX – Alpha Thalassemia mental retardation X linked gene

αKG – Alpha ketaglutarate

CDK4 – Cyclin dependent kinase 4

CDKN2A – Cyclin dependent kinase inhibitor 2A

CIC – Capicua transcriptional repressor

CT – Computed tomography

DIPG – Diffuse intrinsic pontine glioma

DMG – Diffuse midline glioma

EGFR – Epidermal growth factor receptor

FISH – Fluorescence in situ hybridization

FUBP1 – Far upstream element binding protein 1

IDH – Isocitrate Dehydrogenase

IHC – Immunohistochemistry

MGMT –O6-methylguanine DNA methyltransferase

MRI – Magnetic resonance imaging

NADP – Nicotinamide adenine dinucleotide phosphate

NOS – Not otherwise specified

PCR – Polymerase chain reaction

PDGFRA – Platelet derived growth factor alpha

PTEN – Phosphate and Tensin Homolog gene

RB1 – Retinoblastoma 1 gene

TCA – Tricarboxylic Acid

TERT – Telomerase reverse transcriptase

WHO – World Health Organization

Contents

AIM

To determine the effect of Telomerase reverse transcriptase(TERT) promoter mutations on prognosis and survival in diffuse gliomas of the central nervous system.

OBJECTIVE

1. To determine the prevalence of TERT promoter mutations in diffuse gliomas of WHO grades II, III and IV.
2. To determine the association of TERT promoter mutations with other molecular alterations in diffuse gliomas.
3. To assess the role of TERT promoter mutations in overall survival and progression free survival in relation to histological and molecular glioma subtypes.

LITERATURE REVIEW

Introduction

Gliomas are a heterogeneous group of neoplasms that comprise almost 70% of all primary central nervous system neoplasms. (1) In 1926, Baily and Cushing first introduced the classification of these tumours based on their histogenesis, correlating this with prognosis.(2) The classification of gliomas based on histogenesis continues today, although it now utilizes newer immunohistochemical and molecular methods to detect the lineages into which they fall.(3)

The 2007 WHO classification

The 2007 WHO classification divided gliomas into histological classes of astrocytomas, oligodendrogliomas and ependymomas and further sub-classified these according to their clinical behavior as grades I-IV, grade IV having the worst prognosis. However, these tumours were not always histologically clear cut, particularly among the diffuse astrocytomas, oligoastrocytomas and oligodendrogliomas (grade II), their anaplastic counterparts (grade III) and Glioblastomas (grade IV). Grade I astrocytomas, being well circumscribed tumours that followed a distinctive clinical course of a low grade nature. The WHO classification further graded the diffuse gliomas based on histological features, where the presence of hypercellularity, nuclear atypia, mitotic activity qualified for a WHO grade of III. Tumour necrosis and microvascular proliferation being essential for a WHO grade of IV, Glioblastoma Multiforme.(4)

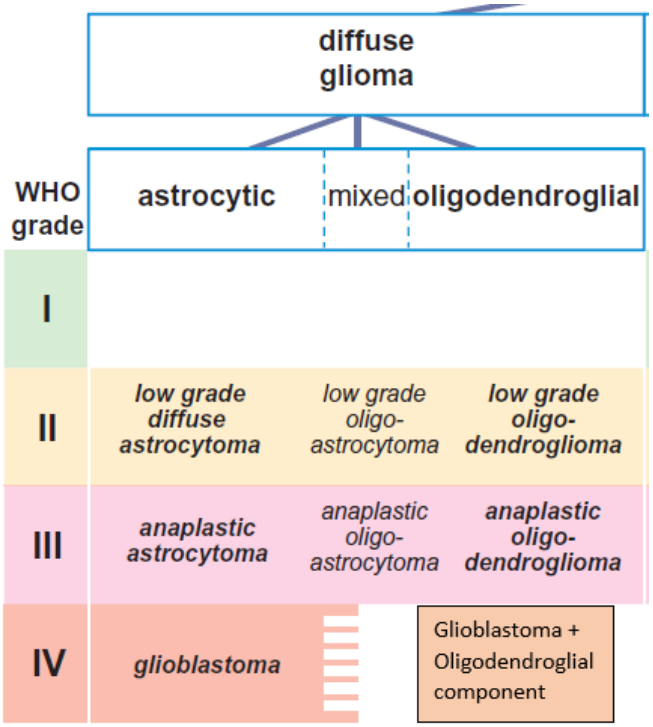

Figure 1: 2007 classification of Diffuse Gliomas*(5)*

Over the past decades, molecular testing eliminated much of the subjectivity in diagnosis of glioma subtypes.(6) Moreover, it was observed that several molecular subtypes were present under a single histological entity, which dictated prognosis and response to therapy better than a pure morphological classification. (3) These discoveries resulted in a sea change in the approach to classification of gliomas. The 2016 update of the WHO classification of CNS tumors relies on genetic parameters in addition to histological parameters to define many tumour entities.(7)

The subsequent section provides an overview of the prognostic and predictive molecular biomarkers in gliomas, as well as methods for molecular testing of these markers.

Isocitrate dehydrogenase mutations

The first major breakthrough in the re-classification of gliomas with a molecular outlook, was in 2008 with the discovery of IDH (Isocitrate dehydrogenase) 1 and 2 mutations, which have now been seen in 80% of diffuse gliomas, anaplastic gliomas and Glioblastomas.(8)

The Isocitrate dehydrogenase enzyme is an essential enzyme within the TCA or citric acid cycle, catalyzing the oxidative decarboxylation of isocitrate to alpha ketaglutarate, during which process, NAD+ is reduced NADH. There are 3 biologically relevant catalytic isoenzymes in eukaryotes, namely IDH-1, IDH-2 and IDH-3. These three enzymes are known to be relevant not only in the citric acid cycle, but also in protecting the cell against oxidative damage and as a source of NADPH for other reactions.(9)

These 3 isoforms of IDH are encoded by 5 different genes. IDH-3 is found in the mitochondria where it functions in the citric acid cycle. IDH-1is found in the cytosol and IDH-2 is found in the mitochondria, where they produce NADPH independent of the citric acid cycle.(10)

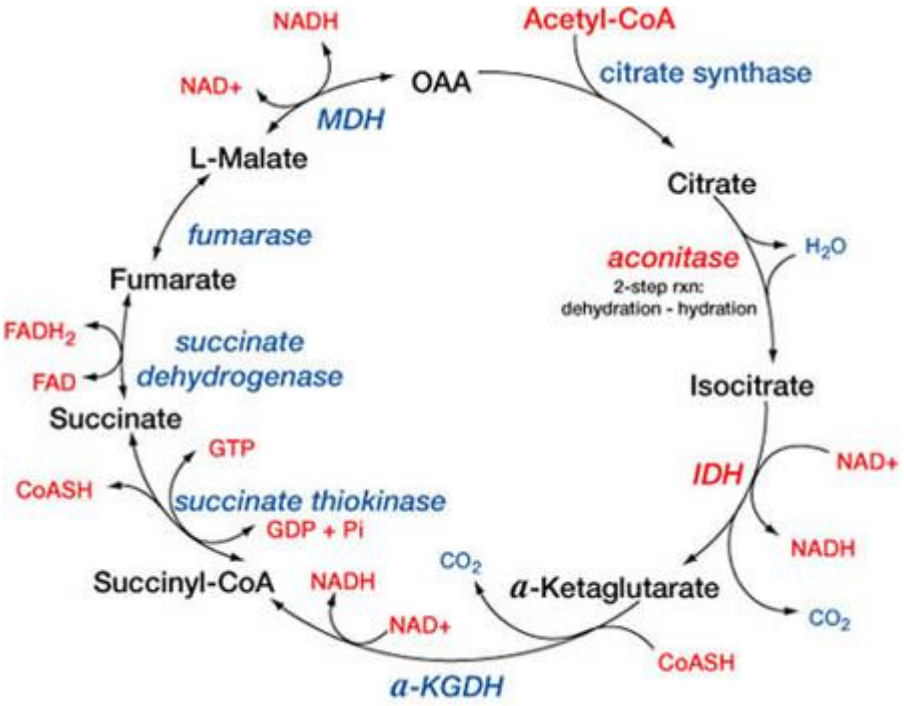

Figure 2 Citric Acid cycle*(11)*

This production of NADPH helps in making the cell less sensitive to oxidative stress.(12) The net effect of a mutation in the IDH gene therefore is a reduction in NADPH and Alpha ketaglutarate, making the cell more susceptible to oxidative damage. (13) In addition to the inability to produce alpha ketaglutarate, the mutant IDH enzyme produces increased levels of 2-hydroxyglutarate, which is an oncometabolite.(14)

All IDH mutations discovered in gliomas appear to fall into one of two categories – Either a missense mutation in IDH1 at Arginine 132(R132) or in IDH2 at Arginine 172. More than 85% of these mutations occur in IDH1.(15) IDH 3 mutations are not found in gliomas. It is believed that IDH mutations are drivers of oncogenesis and are amongst the first mutations to occur in tumourigenesis rather than a step along the neoplastic

evolution.(13) These mutations are also seen in various other tumours such as acute myeloid leukemia, cholangiocarcinoma, prostate and breast carcinomas.(16)(17) In addition, IDH 1 and IDH 2 mosaicism is also seen in syndromes with increased risk of gliomas, such as Ollier's disease and Mafucci syndrome. (18) IDH mutations are found generally to be highly associated with both TP53 mutations as well as the loss of 1p/19q, which are both lineage markers, and mutually exclusive in nature. (19) This suggests an early occurrence of IDH mutations in the neoplastic event, as well as implicating the IDH mutation in both astrocytic and oligodendroglial tumourigenesis.(20) IDH mutations are common in 70-80% of Grade II and Grade III infiltrating gliomas and are found in 100% of oligodendrogliomas and IDH-mutant Glioblastomas. Studies have shown that there is no significant impact of age or grade on the survival of the IDH mutant gliomas, and that in fact IDH mutant grade II, grade III and grade IV gliomas tend to behave much better than their non-mutant counterparts, known as IDH wild type (IDHwt).(21)(15) Therefore, although the IDH mutation does not help in classifying a glioma into a specific subtype, it is a strong marker of clinical behavior.

Detection of IDH1 and IDH2 mutations in gliomas was initially performed by direct PCR sequencing of the mutation, which till date remains the gold standard. However, a monoclonal antibody against IDH1 R132H is currently used in daily clinical practice as it allows the more common mutation to be detected with ease and at a lower cost .(22)

Moreover, the monoclonal antibody developed against IDH-1 R132H has been found to reliably stain even single mutated tumour cells within a biopsy. (22) Studies have shown

that there is a high concordance between antibody immunostaining for IDH1 R132H and genetic testing for IDH mutations. It has also been suggested that the antibody may be more reliable in detecting the mutation in smaller biopsies with a higher sensitivity than PCR analysis.(23) The concordance rates between the immunostaining and DNA sequencing are high, >88%.(24)More importantly however, false positives are rarely seen, and false negatives are even less common, making this both a sensitive and specific antibody in detection of the IDH1 mutation.(24)In tumours that are immunonegative, however, sequencing is required for detecting gene mutations in IDH1 codon 132 and IDH2 codon 172. Only if a tumor is immunonegative for IDH1 R132H and also lacks mutation on sequencing is it termed IDH wild type (IDHwt).

ATRX (Alpha Thalassemia Mental Retardation X linked gene) Mutations

The ATRX gene is located at Xq210.1. ATRX protein belongs to the SNF2 family of proteins, which are involved in chromatin remodeling, maintenance of telomere, nucleosome assembly and histone regulation.(25) Mutations in the ATRX gene are loss-of function mutations. ATRX also plays a role in regulation of telomere length.(26)
In 2012, ATRX mutations were detected in gliomas.(1) This loss of function mutation was seen to have a strong correlation with the astrocytic phenotype (60%), akin to TP53 mutation in previous studies.(27) The ATRX mutation was also shown to have a strong correlation with the TP53 mutation itself, making them both markers of an astrocytic lineage. (28). In addition, ATRX mutations were also found to have a strong association

with IDH mutations, occurring in a large proportion of astrocytic tumours as well as a subset of Glioblastomas harboring the IDH mutation. (29)This ATRX loss is likely to occur subsequent to the IDH mutation as many WHO grade II tumours had IDH mutations associated with retained ATRX expression while a larger proportion of WHO grade III/IV tumours with IDH mutation showed a loss of ATRX expression.(1) Furthermore, ATRX mutation is not seen in any oligodendroglial tumours, and seems to be mutually exclusive with 1p/19q co-deletion, the hallmark of oligodendrogliomas. (30) In the realm of prognosis, those with ATRX mutations are susceptible to agents that cause a double strand break in DNA, such as topoisomerase inhibitors, and are found to do better with adjuvant radiotherapy, demonstrating an increased progression free survival, and a longer overall survival.(31) In this regard they define not only a subset of tumours of astrocytic differentiation, but also a subset of tumours with superior prognosis, making them both lineage and prognosis determinants.(32)

The evaluation of a tumour for the presence of a mutation in the ATRX gene was carried out initially by both Sanger sequencing as well as by immunohistochemical staining for ATRX, where the mutant phenotype could be detected as loss of expression of the protein.

It was however found that loss of expression of ATRX protein on immunohistochemistry correlated well with the ATRX mutational loss by sequencing., barring a few discrepant results, which were attributed to missense mutations.(1) Routine molecular testing for ATRX mutation is therefore now done by immunohistochemistry alone.

As mentioned earlier, immunohistochemically, a sample is considered positive for ATRX mutation if the cells are immunonegative for ATRX protein. In such cases, the tumour nuclei show no staining in the presence of a positive staining of endothelial cells, native glial cells, cortical neurons and inflammatory cells, which serve as internal controls.(32)

TP53 mutations

TP53 mutations form an extensively studied area of interest involving oncogenesis of many tumours. Generally, these mutant p53 proteins are thought to promote the survival, proliferation and invasion of tumours.(33)

TP53 is an essential regulator of the cell cycle, forming a part of the tumour suppressor gene family. Loss of function in this tumour suppressor gene occurs early in the neoplastic evolution of astrocytomas, gathering additional genetic mutations with increase in the level of anaplasia or grade.(34)

TP53 mutations have been seen in approximately 50% of astrocytic tumours, as compared to only 10% of oligodendroglial tumours and was found to be almost mutually exclusive with 1p/19q co-deletion, strongly correlated with tumours of an astrocytic morphology and occurred in patients of a younger age group.(27)

Along with their close relationship to ATRX mutations, TP53 mutations also show a close relationship with the IDH mutation. The co-existence of these three mutations can be viewed as a molecular signature of astrocytomas.(35)

While the TP53 mutations were shown to have a close relationship with ATRX mutations, IDH mutations and astrocytic differentiation, it was also noted that the TP53 mutation in and of itself has been reported to have a conflicting relationship with prognosis and overall survival of the patient, with different studies seeing different outcomes.(36)

Like many other molecular alterations, DNA sequencing is the gold standard to detect the presence of a TP53 mutation in a particular sample. Furthermore, a mutation does not guarantee complete inactivity of the gene.(36) The overexpression of the p53 protein in itself has been used as an immunohistochemical marker that will serve as a surrogate for the detection of the mutation, with p53 protein accumulation seen as positive nuclear staining. It must be noted that while the presence of mutation and overexpression of p53 protein by immunohistochemistry do show a strong concordance, the nuclear accumulation cannot be taken as concrete proof of mutation in the Tp53 gene. Furthermore, the same cannot be said for null mutations in which the protein is absent and hence immunohistochemistry will not be informative on mutational status.(36)Immunohistochemistry for p53 and ATRX can serve as complementary markers for astrocytomas.

1p/19q Co-deletion

The presence of co-deletion of chromosome arms 1p and 19q in oligodendroglial tumours was first described in 1994, demonstrated using restriction fragment length polymorphism.(37) Subsequently it was proven that the loss of 1p and 19q resulted from a non-balanced translocation of t(1:19)(q10:p10) with a loss of one of the original chromosomes.(38)

The loss of these chromosome arms was seen to occur exclusively in tumours with an oligodendroglial histology, and subsequently went on to become a class defining molecular alteration, essential for the diagnosis of oligodendroglioma. The loss of either one of these chromosomal arms occurring separately did not seem to confer the same clinical behavior seen in those with a co-deletion of 1p and 19q.(39)

The relevance of 1p/19q co-deletion in clinical practice became apparent when this particular molecular alteration proved to be associated with not only an improved outcome in terms of survival, but also showed a higher sensitivity to chemotherapy. The survival benefit extended to include both a longer progression free survival and an overall survival. The enhanced sensitivity to chemotherapy was found to be of particular value in those tumours receiving adjuvant chemotherapy after radiotherapy.(40)

The detection of 1p and 19q co-deletion can be achieved in a few different ways, namely a loss of heterozygosity analysis by PCR, genomic hybridization and fluorescence in situ hybridization (FISH). FISH is the method used routinely in neuropathology laboratories

utilizing locus specific probes.(41) A co-deletion is said to have occurred if signals measured in 200 intact nuclei have >50% of nuclei showing one signal.(42)

MGMT Promoter methylation

The MGMT gene located at 10q26, encodes for an enzyme, O6-methylguanine DNA methyltransferase, that is essential to the repair of DNA, by removing the alkyl adducts from the O6 position of guanine. The enzyme ensures rapid repair of DNA, making a cell more resistant to chemotherapy of an alkylating or methylating nature.

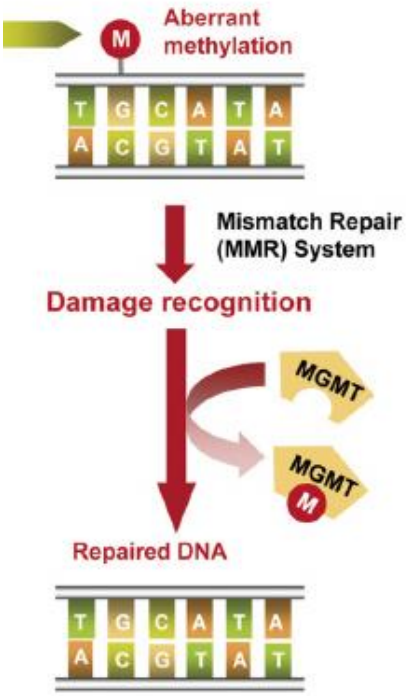

Figure 3 MGMT mechanism of action *(43)*

Epigenetic silencing by hypermethylation of the MGMT promoter gene renders the cell less capable of DNA repair and subsequently more sensitive to chemotherapeutic agents of an alkylating nature.(44)

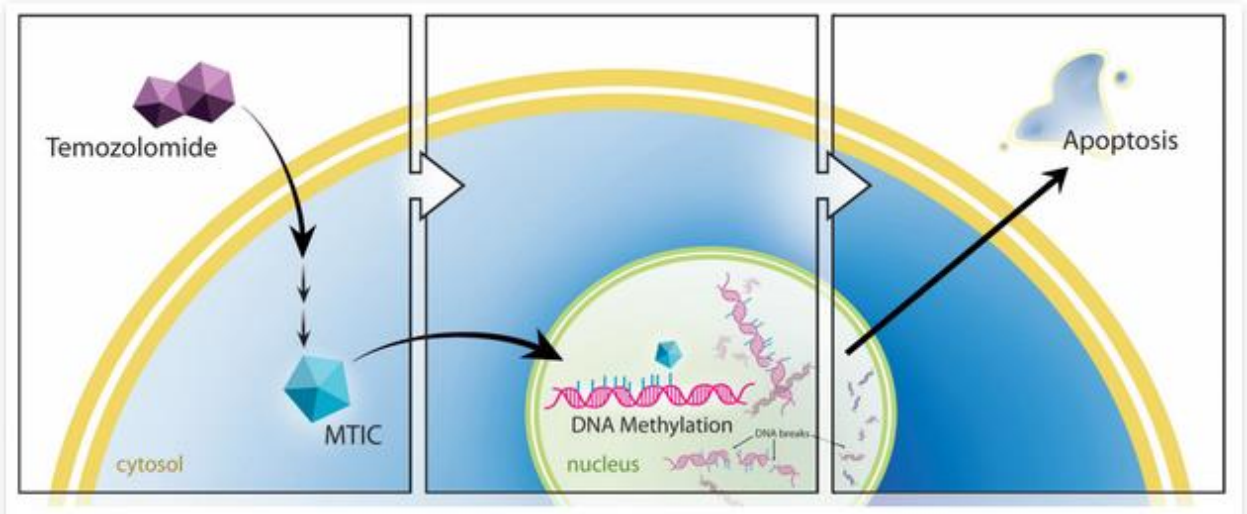

Figure 4 Mechanism of action of Alkylating agent Temozolomide*(45)*

While this explains the reason behind tumours with the MGMT promoter methylation, experiencing a better response to alkylating drugs, it was also found that tumours with MGMT promoter methylation seem to have a favorable prognosis even in the absence of chemotherapy with the said alkylating drugs. This has led to studies that have suggested that MGMT promoter methylation is an independent prognostic marker regardless of the type of treatment.(46)Some studies have suggested that the prognostic power of the MGMT methylation is derived from its close association with the IDH mutation. (47) However, it is definitely seen that the methylation status of the MGMT promoter gene is a predictive marker of chemotherapy response in IDH wild type tumours as well. (48)

CDKN2A/p16 Mutations

Cyclin dependent kinase inhibitor 2A (CDKN2A) gene is located on 9p21, the product of this gene being the p16 protein which is an inhibitor of the cell cycle. Frequent homozygous and hemizygous losses of this gene are seen in gliomas and glioblastomas, which causes both a dysregulation in apoptotic pathways, and promotes cell proliferation.(49) The frequency of these mutations is seen to be higher in higher grade gliomas, suggesting that this is one step on the pathway of malignant progression.(50) The loss of this gene is usually detected by FISH.

Prognostically, the loss of CDKN2A is associated with a worse prognosis, only if the tumour falls into an astrocytic lineage based on loss of ATRX by immunohistochemistry. The overall survival of astrocytic tumours with both ATRX mutation and CDKN2A loss is much poorer than an astrocytic tumour with no loss of ATRX on immunohistochemistry. The overall survival of oligodendroglial tumours does not seem to be significantly associated with the loss of CDKN2A gene.(51) Thus this marker may only provide a further prognostic stratification of tumours that have already been molecularly defined as astrocytic tumours.

CDK4 (Cyclin Dependent Kinase 4) Mutation

Cyclin dependent kinase 4 is one of the regulators of the cell cycle which, when bound to cyclin D1 causes phosphorylation of Rb1, which in turn then removes its negative effect

on the cell cycle and allows cell division to proceed.(52) Mutations in this gene occur in in the q arm of chromosome 12, and are seen in a subset of glioblastomas, amounting to ~11% of glioblastomas.(53) The protein is often overexpressed in other tumours such as malignant melanomas, sarcomas, breast cancer, colon cancer, lung cancer and ovarian cancers.(54)

While not as common as other mutations in gliomas, the significance of these mutations lies in the possibility of a therapeutic target. Ongoing studies on inhibitors of CDK4 are yet to conclude if there is a role for these drugs within the central nervous system, having already been approved for use in breast cancers.(55)

RB1 Gene Mutation

Most gliomas carry one or more of the more common genetic alterations such as IDH1, 1p/19q co-deletion, ATRX mutation and TP53 mutation. In the subset of gliomas that do not carry any of these more common mutations, the rarer mutations such as CDKN2A, CDK4 and Retinoblastoma 1 are seen. The Retinoblastoma tumour suppressor protein is encoded by the RB1 gene on 13q14-13q21 and is downstream to the CDK4 molecule in the cell cycle.(56) Mutations in this gene are seen more frequently in astrocytic than in oligodendroglial tumours.(57)

Overall, a mutation in this gene appears to carry a poorer prognosis for the patient, with a shorter overall survival as well as a shorter time to progression.(58,59)

In addition, the presence of the mutation in RB1 also has a role to play in the efficacy of targeted therapies towards CDK4. Being downstream in the cell cycle pathway, mutations in RB1 diminish the efficacy of inhibitors developed towards the upstream CDK4.(60)

CIC/FUBP1 Mutations

Oligodendrogliomas that are defined by the presence of IDH1 mutations and 1p/19q co-deletions commonly also shows mutations in the CIC gene located on 19q13 and the FUBP1 mutation on 1p31. Both of these mutations are strongly associated with the loss of 1p/19q which may play a role in their inactivation.(61) The CIC gene encodes a protein that is orthologous to the Drosophila melanogaster capicua gene and hence known as the capicua transcriptional repressor gene. This gene behaves as a suppressor of the FUBP1 (Far upstream element binding protein 1) which plays a role in signaling of the MYC pathway.(62)

Studies show that the loss of CIC and FUBP1 have a negative impact on prognosis, imparting a shorter time to recurrence in oligodendrogliomas. In addition, for those tumours carrying a mutation in CIC alone, the prognosis also appears to be worse.(63) Thus these genes, when lost identify a subset of oligodendrogliomas that are more aggressive and have a worse prognosis.

EGFR (Epidermal Growth Factor Receptor) gene Mutations

One of the most common mutations that occur in IDH wild type Glioblastomas is the gain of 7p, harbouring the Epidermal growth factor receptor (EGFR) gene. Approximately 40% of Glioblastomas carry this mutation.(64) The EGFR gene codes for a cell surface tyrosine kinase receptor that initiates the PI3K and MAPK pathways, which contribute to cell survival and proliferation. (65)The most common EGFR mutation is the EGFR vIII mutation which plays a significant role in gliomagenesis, increasing the survival of the mutant cells as well as adjacent cells through paracrine activity.(66)

The presence or absence of the EGFR mutation can be detected immunohistochemically with antibodies to EGFR vIII, or by PCR sequencing. However, this mutation appears to have no effect on the prognosis of the tumour.(67)

The value of detection of this mutation lies therefore in the ability to provide targeted therapy against the over amplified tyrosine kinase activity with anti-tyrosine kinase drugs such as gefotinib providing a valuable adjunct to therapy. (68)

PTEN (Phosphate and Tensin Homolog) gene Mutations

The other commonly seen mutation in IDH wild type Glioblastomas is the Phosphate and tensin homolog (PTEN) gene that is located on chromosome 10. This usually occurs as a loss of the 10q arm, most commonly co-existing with the EGFR mutation. The incidence of this mutation is high, 62-75%.(69)

The PTEN gene is a tumour suppressor gene which encodes a protein tyrosine phosphatase that has a negative regulatory effect on the PI3K and AKT pathways.(70)

PTEN mutations are seen more commonly in high grade gliomas, and less frequently in lower grade gliomas, suggesting that they are a key factor in malignant progression.(71)

The survival time in tumours with PTEN mutations is significantly reduced compared to those Glioblastomas that do not carry this mutation, with an average survival time being 67 months.(69)

PDGFRA (Platelet Derived Growth Factor Alpha) gene Mutations

Platelet derived growth factor alpha (PDGFRA)gene mutation is seen in 15% of gliomas. The normal function of this gene is regulation of proliferation of cells and differentiation of oligodendrocytes. When amplified, this results in excessive proliferation and angiogenesis.(72) The PDGFRA gene encodes for a cell surface receptor tyrosine kinase. Similar to EGFR, these mutations induce proliferation through paracrine mechanisms as well.(73)As a potential target for therapy the PDGFRA mutation is valuable, as studies have shown that the mutation confers an increasing sensitivity to radiotherapy if treated with a tyrosine kinase inhibitor such as imatinib mesylate.(74)

Haarlem consensus

Prior to the updated WHO classification of tumours in 2016, the International Society of Neuropathology met in Haarlem in 2014 and agreed upon broad guidelines to facilitate the upcoming reclassification. The recommendations included guidelines such as layered diagnoses – integrating histology, WHO grade and molecular information, separating pediatric gliomas from adult counterparts, identifying necessary molecular tests and entity defining molecular markers and defining narrow diagnostic entities to reduce the interobserver variability to a minimum.(7)

	Nomenclature
Layer 1	Integrated diagnosis (incorporating all tissue-based information)
Layer 2	Histological classification
Layer 3	WHO grade (reflecting natural history)
Layer 4	Molecular information

Figure 5 Sample of layered diagnosis for reporting*(3)*

The 2016 WHO classification

The 2016 WHO classification of glial neoplasms has shifted from the tradition of classifying based solely on morphological parameters and now employs a molecular basis for its classification as well. This serves to reduce the amount of intra-observer

variability, as well as clarify histogenesis in those tumours with divergent differentiation.(3)

With all of the molecular data uncovered in the last 20 years, morphology is no longer considered sufficient to detect the lineage or histogenesis of a tumour. Moreover, purely morphological classifications do not correlate with prognoses as much as was previously assumed.(3)

Diffuse astrocytic and oligodendroglial tumours	
Diffuse astrocytoma, IDH-mutant	9400/3
Gemistocytic astrocytoma, IDH-mutant	9411/3
Diffuse astrocytoma, IDH-wildtype	*9400/3*
Diffuse astrocytoma, NOS	9400/3
Anaplastic astrocytoma, IDH-mutant	9401/3
Anaplastic astrocytoma, IDH-wildtype	*9401/3*
Anaplastic astrocytoma, NOS	9401/3
Glioblastoma, IDH-wildtype	9440/3
Giant cell glioblastoma	9441/3
Gliosarcoma	9442/3
Epithelioid glioblastoma	*9440/3*
Glioblastoma, IDH-mutant	9445/3*
Glioblastoma, NOS	9440/3
Diffuse midline glioma, H3 K27M-mutant	9385/3*
Oligodendroglioma, IDH-mutant and 1p/19q-codeleted	9450/3
Oligodendroglioma, NOS	9450/3
Anaplastic oligodendroglioma, IDH-mutant and 1p/19q-codeleted	9451/3
Anaplastic oligodendroglioma, NOS	*9451/3*
Oligoastrocytoma, NOS	*9382/3*
Anaplastic oligoastrocytoma, NOS	*9382/3*

Figure 6 The 2016 WHO Classification of Gliomas*(75)*

In addition to integrating the molecular alterations into the final diagnosis in the updated classification, it has also been decided that where there is discordance between the morphology and genetic markers, the genotype has the final word in diagnosis.(76)

The term "Not otherwise specified (NOS)" can still be applied to a tumour in certain instances such as when genetic testing is unavailable, complete discordance between morphology and genetics or in cases when the basic morphology is uncertain.(7)

The essential points in the updated WHO 2016 classification of central nervous tumours are discussed below.

Diffuse versus Circumscribed Gliomas

The 2016 classification of CNS tumours, unlike the 2007 classification places an emphasis on the distinction between circumscribed gliomas and diffuse gliomas. This distinction made on light microscopy was essential, owing to genetic and prognostic differences between these two groups. It is necessary therefore to decide on circumscribed gliomas versus diffuse gliomas, in order to proceed with the appropriate genetic and molecular testing.(76)

These well circumscribed gliomas have a low proliferative potential and have been assigned as WHO grade I gliomas. The designation indicates that these tumours have a non-infiltrative growth which indicates that cure will be possible with surgical resection.

Diffuse gliomas include those of WHO grade II-III, where the growth is of an infiltrative type. These tumours, owing to their merging with the surrounding brain, tend to recur and require some adjuvant therapy. The level of anaplasia and malignant potential increases with increasing grade.(75)

Algorithm for glioma diagnosis

The figure given below is a summary of the various entities in diffuse gliomas in the updated 2016 WHO classification of central nervous system tumours. As can be seen the diagnosis of gliomas takes an algorithmic approach, based on molecular as well as histological parameters, and is further discussed below.

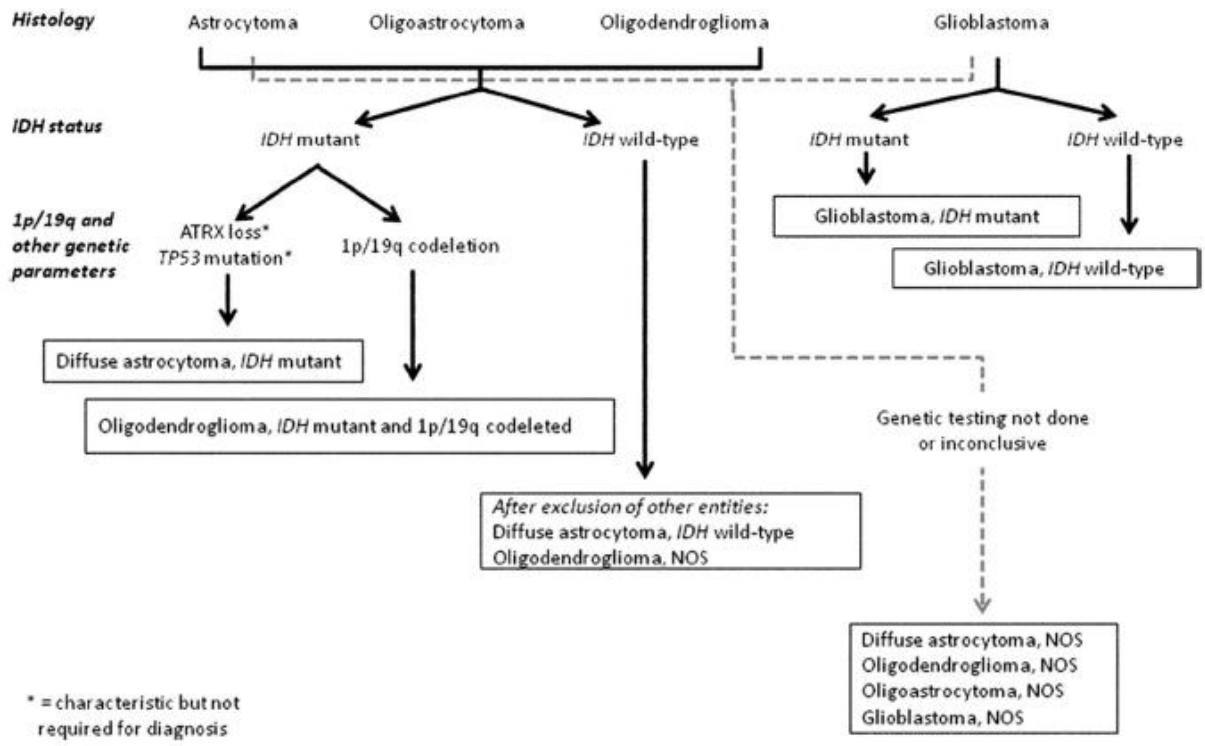

Figure 7 Algorithm for diagnosis of diffuse gliomas*(75)*

Diffuse astrocytomas and Oligodendroglial tumours

Following the WHO 2016 classification of CNS tumours, the diagnosis of a diffuse glioma, whether astrocytic or oligodendroglial, follows a similar algorithmic approach. Once a diffuse glial tumour is identified, the first step is to perform immunohistochemistry for IDH mutations. If the IDH mutation is negative, the tumour would likely go into an NOS category if oligodendroglial, or into IDH wild type category if it is an astrocytic neoplasm. It is necessary to perform PCR sequencing for IDH mutations for the tumour to be characterized as IDH negative. If the IDH mutation is positive, the 2nd step is immunohistochemistry for ATRX and p53 mutation. A loss of

ATRX indicates an astrocytic neoplasm and the tumour may be directly classified as such. If the ATRX is retained, FISH for 1p/19q is performed. If co-deletion of 1p/19q is detected by FISH, the tumour may be classified as oligodendroglial. In the case that there is no 1p/19q co-deletion, the tumour is then classified as an astrocytoma.(75)

Diffuse astrocytomas

The diffuse astrocytomas in the new WHO classification, have been classified as before into grade II (Diffuse Astrocytoma) and grade III (Anaplastic Astrocytoma) categories. However, the previous designations of grade II diffuse astrocytomas into diffuse fibrillary and protoplasmic astrocytomas has been removed, incorporating both into a single diagnostic entity of diffuse astrocytoma. Both grade II and grade III tumours, are further divided into IDH mutant, IDH wild type and NOS categories. The vast majority of astrocytomas, both of grade II and III, are of the IDH mutated type, and the diagnosis of an IDH wild type astrocytoma is rare. The third NOS category is usually reserved for those in which molecular testing has not been carried out or those that are negative with IDH immunohistochemistry but have not been tested for IDH mutation by sequencing(6)

It has been observed by some authors that the prognosis of WHO grade II IDH mutant diffuse astrocytomas and WHO grade III IDH mutant anaplastic astrocytomas are quite similar, both carrying a better prognosis than their wild type counterparts. However based on morphology these 2 entities are still categorized in separate grades.(77)

When all the molecular alterations are present, there is barely any room for doubt where diagnosis is concerned. However, the loss of ATRX or p53 overexpression is not required for the diagnosis of astrocytomas, in that there is a proportion of these tumours that do not express these molecular alterations. In the absence of these characteristic mutations, testing for 1p/19q chromosomal arm deletion by FISH must be performed. The presence of this co-deletion is an entity defining feature of oligodendrogliomas. Therefore, this then becomes the criteria for deciding between an astrocytic and oligodendroglial lineage. Co-deleted 1p/19q chromosomal arms will make the diagnosis of an oligodendroglioma, however the absence of this chromosomal loss will confirm an astrocytic lineage. An example of this is captured in the picture below.(6)

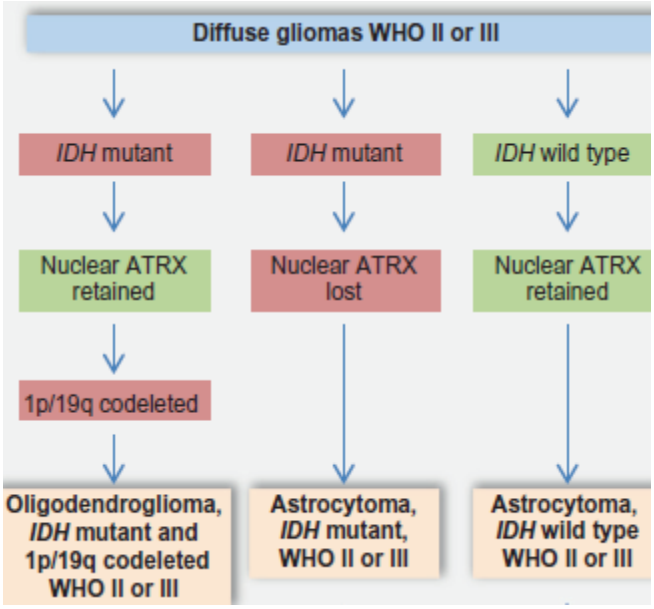

Figure 8 Molecular diagnosis of diffuse gliomas, grade II/III*(6)*

Although the entities of protoplasmic and fibrillary astrocytomas have been deleted from the WHO 2016 classification, the gemistocytic variant of diffuse astrocytomas has remained within the WHO classification as a variant of IDH mutant diffuse astrocytomas, WHO grade II. (76)

Oligodendrogliomas

The requirement to diagnose an oligodendroglioma now includes 3 components. The first is a classical histology of an oligodendroglial tumour. The second is an Isocitrate dehydrogenase(IDH) mutation, and the third is the co-deletion of 1p/19q chromosomal arms. The combination of IDH and 1p/19q co-deletion are considered an entity defining feature and the molecular signature of oligodendrogliomas. This signature applies to both the Grade II Oligodendrogliomas and the Grade III Anaplastic Oligodendrogliomas. In general, it is considered sufficient to detect an IDH mutation by testing for IDH1 R132H on immunohistochemistry. However, for that small subset of tumours that are negative for IDH mutations on immunohistochemistry, PCR sequencing is required for confirmation and to rule out a possible mutation in IDH2 R172H gene. This is expected to be necessary for <10% of all oligodendrogliomas. (76)

In the rare event that an Oligodendroglioma demonstrating classical histology and 1p/19q co-deletion is proven to be of an IDH wild type by PCR sequencing, the diagnosis of Ologodendroglioma, NOS is rendered, further emphasizing that a lack of IDH mutation is incompatible with a diagnosis of classical Oligodendroglioma. (3)

In the event that a glioma with oligodendroglial morphology does not show a co-deletion of the 1p/19q chromosomal arms, it cannot be classified as an oligodendroglioma, and needs must be placed within the astrocytic classification.

Oligoastrocytomas

Oligoastrocytoma was a category previously assigned to those tumours that featured a morphology that was both astrocytic and oligodendroglial, i.e. a mixed morphology. In the 2016 WHO classification, with the numerous molecular markers at our disposal, this category is strongly discouraged as a diagnosis.(3) Most oligodendrogliomas and astrocytomas in the current scenario do fall into definite lineages on a molecular basis. The presence of two distinct populations of cells, one showing an astrocytic molecular signature and another showing a definite oligodendroglial molecular signature is a rare event. The WHO 2016 classification has included two categories of Oligoastrocytoma, NOS, WHO grade II and Anaplastic oligoastrocytoma, NOS, WHO grade III. This NOS category is usually applied when there is an unavailability of molecular testing, and a provisional diagnosis is rendered based on morphology alone.(5)

A simplified way of looking at the classification of diffuse WHO grade II gliomas is seen in the figure given below, which holds true for the classification of grade III gliomas as well.

	Astrocytoma histology	Oligodendroglioma histology	Oligoastrocytoma or ambiguous histology
IDHmt, 1p19q-nondel, ATRX loss	Diffuse astrocytoma, IDHmt	Diffuse astrocytoma, IDHmt	Diffuse astrocytoma, IDHmt
IDHmt, 1p19q-codel, ATRX intact	Oligodendroglioma, IDHmt & 1p19q codel	Oligodendroglioma, IDHmt & 1p19q codel	Oligodendroglioma, IDHmt & 1p19q codel
IDHwt	Diffuse astrocytoma, IDHwt	Oligodendroglioma, NOS	Diffuse astrocytoma, IDHwt

Figure 9 Classification of grade II gliomas*(3)*

Grading

Diffuse gliomas in the 2016 update of the WHO classification of CNS neoplasms are graded from Grades II through IV.

Grade II tumours are those that have some amount of nuclear atypia. Grade III tumours are those that display mitotic activity and in general show malignant features including marked nuclear atypia. These tumours have a strong tendency to recur and progress, usually requiring adjuvant therapy.

Glioblastomas are Grade IV tumours that are frankly malignant featuring, mitoses, microvascular proliferation and necrosis including pseudopalisading necrosis. These tumors have an aggressive course.

In Astrocytic neoplasms, the presence of hypercellularity, nuclear atypia and mitotic activity make a glioma grade III. Additionally, tumour necrosis and microvascular proliferation are essential to classify a glioma into grade IV, Glioblastoma.

Oligodendroglial neoplasms on the other hand, allow for microvascular proliferation and necrosis as a feature of the grade III Anaplastic oligodendroglioma, provided the characteristic IDH mutations and 1p/19q co-deletions are present.(78)

Diffuse midline glioma H3 K27M-mutant

Pediatric gliomas in the past years have been extensively studied to look both for distinct mutations that would allow them to be differentiated from their adult counterparts, as well as for prognostic and predictive molecular alterations. However, despite the discovery of numerous mutations including MYB and BRAF mutations, entity defining mutations were not seen. (79,80) Most pediatric gliomas are therefore still grouped with adult gliomas across all categories in the WHO 2016 classification, with the exception of midline gliomas. .

It was found that diffuse midline gliomas, seen in children or young adults, showed a histone mutation K27M mutation in either H3F3A or HIST1H3B/C, with a role in chromatin remodeling. These tumors are now a newly identified entity in the WHO 2016 classification of central nervous system tumours.(81)

Mutations involving the H3 K27M have been identified in high grade tumours that are located primarily in midline areas such as the thalamus, pons, spinal cord and less commonly in the third ventricle, pineal region, cerebellum and hypothalamus. This includes the diffuse intrinsic pontine glioma(DIPG) and diffuse midline glioma (DMG). These tumours can present with a varied morphology including epithelioid cells, giant

cells, sarcomatous areas and primitive neuroectodermal tumour-like cells.(82)An interesting observation is that the H3 K27M mutation seems to be mutually exclusive with both EGFR and IDH1 mutations. Having said that, the H3 K27M mutation also appeared to consistently be associated with loss of ATRX on immunohistochemistry and p53 overexpression.(82)

Although commonly occurring in children, diffuse midline gliomas with H3 K27M mutations did occur in younger adults as well.(81,83)

This tumour is seen to be highly infiltrative and is always categorized as a high grade neoplasm, WHO grade IV. They are highly aggressive neoplasms with a poor prognosis with a median survival of 19.6 months.(83) It is important to bear in mind however, that should a midline diffuse glioma be negative for the H3 K27M mutation, the approach to diagnosis is the same as that of a diffuse astrocytoma or oligodendroglioma.(3)

Glioblastoma

In the 2007 WHO classification of central nervous system tumours, Glioblastoma, WHO grade IV was categorized into a few variants such as giant cell glioblastoma and gliosarcoma. Glioblastoma with an oligodendroglial component, small cell, and granular cell glioblastomas were patterns of the conventional glioblastoma based on the histomorphological features seen. Further glioblastomas could also be categorized as primary/denovo or secondary depending on whether a prior lower grade glioma could be identified, or on the basis of histology and molecular alterations .(75)

The diagnosis of a glioblastoma is still made on a histological basis, featuring a tumour with astrocytic differentiation and high grade features such as microvascular proliferation, necrosis, nuclear atypia and marked pleomorphism.(4) This category in the 2016 WHO classification of central nervous system tumours is further dichotomized on the basis of the IDH mutation into IDH mutant glioblastomas and IDH wild type glioblastomas, the former carrying a better prognosis

The majority of Glioblastomas are seen to arise de novo, i.e. without clinical or histological evidence of a prior lower grade glioma, and these are the glioblastomas that do not harbor the IDH mutation. On the contrary, glioblastomas which are secondary, i.e. with evidence of prior lower grade glioma, are seen frequently to harbor the IDH mutation. However, rather than classify the glioblastomas based on their origin, the 2016 classification divides on the basis of the IDH mutation, which makes prognostic sense.(84)The terms primary and secondary Glioblastoma are not in use any longer, and are rather classified as IDH wild type or IDH mutant Glioblastomas, more indicative of their pathogenesis and prognosis. A third category, Glioblastoma, NOS exists, in which IDH mutational analysis is not performed.

IDH1 immunohistochemistry, when negative, usually prompts the confirmation of absence of mutation by PCR sequencing. In glioblastomas occurring in patients >55 years of age, this is seen as unnecessary as the only the IDH1 mutation is ever seen in this age group. The confirmation of absence of IDH2 mutations is therefore not required. These tumours are considered IDH wild type.(3)

The subtypes of Glioblastomas, giant cell glioblastoma and gliosarcoma are now classified under Glioblastoma, IDH wild type category, along with a new variant – the Epithelioid glioblastoma. This new category of IDH wild type glioblastoma presents usually as discohesive sheets of cells that have either an epithelioid or rhabdoid morphology, and occurs in patients of a younger age group.(85) In approximately 50% of these tumours, a mutation in the BRAF V600E gene has been found, sparking interest in the potential treatment implications of this mutation.(86)

A new pattern of glioblastoma that has been added in this latest classification is Glioblastoma with a primitive neuronal component.(87) This pattern is usually seen to comprise of both nodules of primitive neuronal cells as well as a diffuse astrocytic component. The importance of this particular pattern is in its propensity to disseminate via cerebrospinal fluid seeding. Recognition of this pattern on histology could well prompt a thorough clinical examination for metastases through the cerebrospinal fluid.(88) This pattern develops from a lower grade glioma in approximately 25% of cases. Positivity for IDH mutation is usually seen on immunohistochemistry in both the astrocytic and neuronal components.(89)

Small cell and granular cell remain as patterns of Glioblastomas, both of which show a poorer prognosis than conventional morphologies.(75) Glioblastoma with an oligodendroglial component has been deleted since on genetic analysis these tumours are found to be either IDH-wild type glioblastomas, IDH-mutant glioblastomas or IDH mutant and 1p/19q-codeleted anaplastic oligodendrogliomas.(4)

The entity gliomatosis cerebri, a diffusely infiltrating astrocytic glioma, is also now no longer recognized in the new WHO classification. It is instead seen as a pattern of growth that commonly occurs in astrocytic and oligodendroglial tumours, including glioblastomas.(76)

Telomerase Reverse Transcriptase (TERT) promoter mutations

In recent years, TERT (telomerase reverse transcriptase) promoter mutations have been actively studied as mutations that are quite prevalent in gliomas and a host of other tumours, as well as being significantly associated with survival and prognosis, though the nature of this association depends in a large part upon the setting.(90)

To understand the importance of TERT promoter mutations in gliomas, it is first important to take a look at the role of telomeres in normal cellular regulation. In all living cells, the telomere length progressively shortens with each cell cycle, except in stem cells. With age and senescence, the telomere eventually shortens and the cell dies.(91) The enzymatic lengthening of telomeres by various methods is one of the ways in which cancer cells ensure their immortality.(92) The lengthening of telomeres produced by these enzymes, prevents the senescence of the cell, leading to an escape from natural death.(93)

In 2003, Hakin-Smith et al, published a study on the alternative lengthening of telomeres in Glioblastomas. They found that 25% of Glioblastomas had alternative lengthening, the

mechanism of which was not identified at that time. However, they did find that Glioblastomas with lengthened telomeres had a longer survival time when compared to those with normal telomeres, an association which was found to be a significant independent prognostic factor.(94)

A similar study in 2010 was done by McDonald et al which looked at the survival of Glioblastomas in the setting of alternative lengthening of telomeres. They found lengthened telomeres in 15% of Glioblastomas, assessed by FISH. They found a strong association with better survival outcomes in those tumours with lengthened telomeres as well.(95)

Boldrini et al subsequently showed that the increased length of telomeres was associated with an increased expression of hTERT mRNA in glial tumours, suggesting that the telomere length maintenance was secondary to an amplification of the TERT region. Interestingly in this study they also found that this increase in telomerase activity was associated with a worse overall and progression free survival.(96)

In 2013 Arita et al studied the role of TERT promoter mutations in gliomas, as well as their association with other commonly known molecular markers in gliomas. They found that these mutations were highest in Glioblastomas and Oligodendrogliomas, and comparatively low in Astrocytic neoplasms. They also found that TERT promoter mutations very frequently occurred in i) IDH wild type tumours, ii) Tumours with 1p/19q loss and IDH mutations and iii) Tumours harbouring EGFR mutations.(97)

Recent studies into the genetics of this telomere maintenance in tumour cells have led to the discovery of the TERT (Telomerase reverse transcriptase) gene with a point mutation in the promoter region. This mutation has been seen in many tumours including malignant melanomas, liver tumours, urothelial tumours and medulloblastomas.(98)

Tumours harboring this mutation are thus able to escape this eventual telomere shortening, ensuring continued indefinite proliferation.(99)

Studies done on human melanomas were able to isolate, through whole genome sequencing, three single nucleotide polymorphisms in the TERT promoter region. The two most common were single nucleotide cytosine to thymine mutations at positions 124bp and 146bp upstream from the start codon, denoted with C250T and C228T.(100)

Mutations in either of these regions of the TERT promoter region lead to an increase in the transcription of the TERT promoter gene via a new transcription binding site. This upregulation of the TERT promoter region being essential to lengthen telomeres and ensure the continued proliferation of the TERT promoter mutated cell lines(101)

The prevalence of these mutations in gliomas is by far the highest in Glioblastomas (75%). Oligodendroglial tumours also show a high frequency of mutations with 58% of WHO grade II and 64% of WHO grade III tumours displaying TERT promoter mutations.(97) Anaplastic astrocytic tumours, WHO grade III, showed a 40% frequency of the mutation, whilst WHO grade II tumours had mutations in only 7% of tumours.(102)

In a recent study published in India by Purkait et al in 2016, an attempt to prognostically stratify Glioblastomas with molecular markers was made. In this study it emerged that TERT promoter mutations and MGMT promoter methylation were independent prognostic determinants in IDH wild type Glioblastomas. The presence of TERT promoter mutations in isolation appeared to confer a worse prognosis while the presence of the MGMT promoter methylation in isolation imparted a good prognosis.(103)

Similarly, Rajmohan K S et al in 2016, studied a prognostic stratification based on IDH, 1p-19q co-deletion and MGMT methylation, only in Anaplastic Astrocytomas, WHO grade III tumours. They found that breaking Anaplastic astrocytomas into histo-molecular groups, did indeed aid in prognostication.(104)

Recently, attempts to sub classify gliomas according to molecular markers that would help determine clinical progression have been studied extensively. In 2015 Eckel-Passow et al published a study that identified three markers that would allow us to break gliomas into prognostically significant groups independent of grade. The three markers in this attempt to stratify are IDH mutations, co-deletion of 1p/19q chromosomal arms and TERT promoter mutations. Using these three alterations, tumours were sub classified into 5 groups that could classify most of the cases.

1) Triple positive – TERT promoter mutation, IDH mutation and 1p/19q co-deletion

2) TERT promoter mutation and IDH mutation (No deletion of 1p/19q)

3) IDH mutation only

4) Triple negative

5) TERT promoter mutation only

Gliomas with TERT promoter mutations and no other associated mutations were found to have by far the worst prognosis and usually occurred in grade IV gliomas. However, the presence of this isolated TERT promoter mutation in grade II/III gliomas defined a subset of clinically aggressive tumours that had a similar prognosis to grade IV.

TERT promoter mutations in conjunction with IDH mutations had an intermediate prognosis, with grade IV gliomas in this group having a poor survival and grade II/III having a good prognosis. TERT promoter mutation with associated IDH mutation and 1p19q co-deletions (triple positive) were seen to have the best prognosis.

The triple negative and IDH mutation only subgroups seemed to follow an intermediate course. All of these subgroups that were defined molecularly, appeared to show different ages of onset, and outcomes, suggesting that the pathogenetic mechanism behind each subgroup is likely to be different, justifying the breakup of gliomas into these subtypes. However, further studies correlating TERT promoter mutations to histological sub-types, molecular sub-types and prognosis are needed.(90)

Justification for this study

There have been a myriad new mutations and genetic alterations that have been discovered over the past few decades, and attempts to classify them into prognostic categories are ongoing. One substantial step has been the 2016 update of the WHO classification of central nervous system tumours which attempts to incorporate molecular alterations within the classification, making it more prognostically relevant. The prime aim of incorporating molecular markers is to prognosticate and predict clinical behavior better. If molecular stratification of gliomas can spell out a prognostic stratification as well, it is something that will be useful in clinical practice in a tangible manner.

Furthermore, there have been only a few studies analyzing prognostic stratification with TERT promoter mutation, 1p/19q codeletion and IDH mutation done in an Indian setting.

MATERIALS AND METHODS

This study was both a prospective and retrospective cohort study and was carried out in the Department of General Pathology, during the period 2015-2017.

All cases diagnosed as Diffuse astrocytomas, Anaplastic astrocytomas, Oligodendrogliomas, Anaplastic oligodendrogliomas, Oligoastrocytomas, Anaplastic Oligoastrocytomas and Glioblastomas between 1st January 2009 and 31st January 2012, were selected from the tumour bank register in the Neuropathology laboratory.

INCLUSION CRITERIA

- WHO grades II –IV gliomas diagnosed between 1st January 2009 to 31st January 2012
- Adequate follow-up information

EXCLUSION CRITERIA

- Paediatric gliomas
- WHO grade I gliomas
- Pilomyxoid astrocytoma, Pleomorphic Xanthoastrocytoma and Ependymoma
- Cases with no available paraffin blocks
- Insufficient tissue for molecular analysis
- Cases without follow-up information

- Cases seen as consultation from extramural centres

All gliomas of WHO grade II, III and IV diagnosed in the Department of General Pathology in the time period January 1st 2009 to January 2012 that had tissue in the tumour bank were traced in the registry. The corresponding paraffin blocks were retrieved from the archives of the General Pathology Department and assessed for adequacy of tumour tissue to perform immunohistochemistry and Fluorescence in situ hybridization (FISH). Tissue was retrieved for each of the cases from the tumor bank where they had been stored at minus 70-degree storage after snap freezing. The follow up information for each of these cases was ascertained from the Neurosurgery-I database, and cases with an adequate follow up period were included.

Paediatric gliomas were excluded, i.e. all the tumours occurring in <18 years of age were excluded from this study.

Ependymoma, Pilocytic Astrocytoma, Pleomorphic xanthoastrocytoma and Pilomyxoid astrocytoma were excluded from this study.

Those samples that had <30 ng of fresh frozen tissue were considered as having insufficient material for molecular analysis and DNA extraction.

Slides of each biopsy were reviewed. For those cases that had not had the complete panel of ancillary tests for arriving at a specific diagnosis, representative slides were selected and sections cut from their corresponding paraffin blocks. 2 to 3 micron thick sections

from these selected paraffin blocks were cut and mounted on Poly-L lysine coated for immunohistochemistry for IDH-1 mutation and ATRX mutation. Sections were mounted on silanized slides for FISH for 1p/19q co-deletion and. From the Neurosurgery I database, all cases with a follow-up period of less than three months were excluded, unless death had occurred within that time period.

Clinical details of the cases

The clinical details of these patients were obtained from the charts retrieved from the Medical Records Department. The clinical information that was retrieved included age, gender, size, presenting symptoms, MRI features, location of tumor, surgical treatment given, chemotherapy status, radiotherapy status, recurrence and death.

Histological details

The histological features of each of these cases was reviewed by the principal investigator. The cases were divided into those with an astrocytic morphology, those with an oligodendroglial morphology, and those with a mixed astrocytic and oligodendroglial morphology. In addition, the presence of microvascular proliferation and necrosis were noted as separate criteria. For those cases in which immunohistochemistry for Ki-67 proliferation index had been performed, that information was collected as well.

Diagnosis

The grading for each case was based on WHO criteria for grading. The final diagnosis for each case, was based on molecular criteria as elucidated by the WHO 2017 classification of CNS tumours, using expression pattern for IDH-1 and IDH-2 mutations, ATRX mutations and 1p/19q co-deletions.

Molecular details

The previous molecular data for each of these cases was collected from the pathology workstation and prior studies done in the Department of Pathology on a subset of the same cohort of cases. The molecular details that were collected were ATRX immunohistochemistry, IDH-1 immunohistochemistry, IDH-1 and IDH-2 mutation PCR data and FISH for 1p/19q co-deletion. For those in which the molecular information was not present, IDH-1 immunohistochemistry was performed for all cases. Prior to the advent of ATRX immunohistochemistry, gliomas with an oligodendroglial morphology had been analysed with FISH for 1p/19q co-deletion as part of the routine work-up. Those cases that were not studied with FISH were subjected to immunohistochemistry for ATRX mutation. ATRX immunohistochemistry was not done for all Glioblastoma cases. FISH for 1p/19q co-deletion was performed on all cases that showed a retained ATRX expression on immunohistochemistry. Details on the procedure for immunohistochemistry are as given in Appendix 1.

TERT Promoter mutation assessment

The status of the TERT promoter mutation was assessed by PCR amplification and DNA sequencing. Fresh frozen tissue was used for the DNA extraction.

DNA Extraction

Fresh frozen tissue was used to extract DNA for PCR amplification. For 24 of the 107 samples in this study, DNA extracts were available in the molecular pathology laboratory from a previous study. For the remaining 87 samples, DNA was extracted using the QIamp DNA mini kit (QIAGEN, India). Following extraction, 1.5 microlitres of the DNA samples were checked in terms of their quality and quantity using the Nanodrop (Nanodrop technologies, USA). Details of the procedure are as in Appendix 2.

Promoter Analysis

Fifty nanograms of the DNA sample was used in a Polymerase Chain Reaction (PCR) which included a high fidelity Taq polymerase (ThermoFisher, USA), PCR buffer and $MgCl_2$. This is in conjunction with primers designed to amplify 474 bp region of the promoter flanking hotspots of mutations found at positions 1295228 and 1295250 of chromosome 5. The details of the procedure for PCR and the primers are elucidated in Appendix 3.

Following PCR amplification of the desired promoter region, the amplified products were detected by the use of gel electrophoresis by using a 2% agarose gel. The image below

gives an example of gel electrophoresis, with a DNA ladder on the right and the single band representing the amplified PCR product. The PCR product was then sequenced in the sense and antisense direction using the Big Dye terminator sequencing reagent. Details of the sequencing procedure and product purification are in appendix 4.

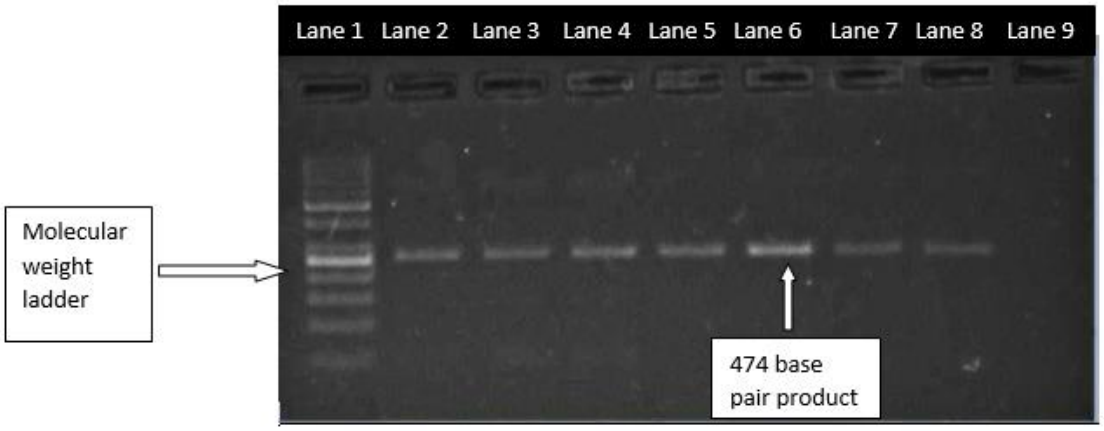

Figure 10 Agarose Gel Electrophoresis of TERT promoter amplification products, Lane 1 – Molecular weight ladder, Lanes 2-8 – Samples 1-7, Lane 9 – Negative test control

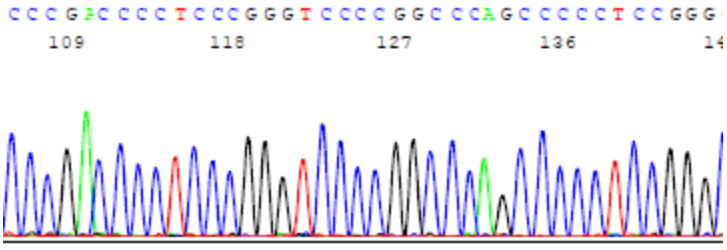

Figure 11 TERT Promoter Wild type Sequence

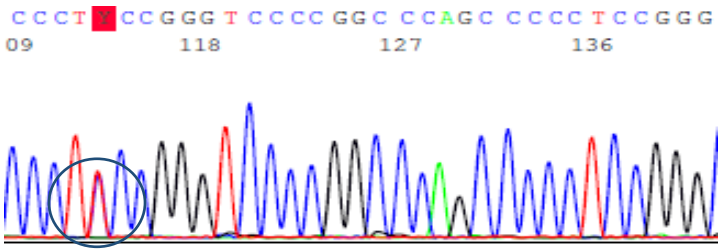

Figure 12 TERT promoter mutation at position 1295250

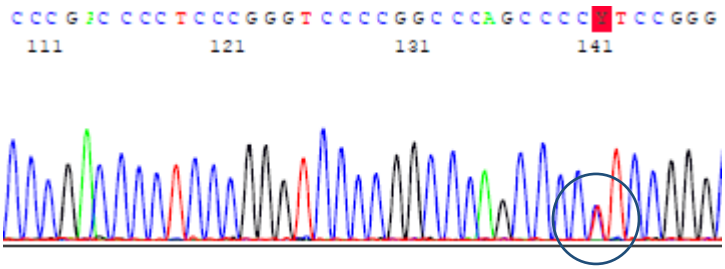

Figure 13 TERT Promoter mutation at 1295228

The sequences thus obtained were compared to a wild type sequence, and known mutations at the 1295228 and 1295250 loci were documented. These sequences were read by three independent investigators to eliminate possible bias.

Statistical Analysis

All study data was analyzed at first for descriptive statistics relating to demography and prevalence. Pearson's Chi square test was used to assess associations, using the SPSS software, where a P value of <0.05 was considered statistically significant. Survival analysis was performed by using the Kaplan Meier test.

RESULTS

A total of 145 cases of gliomas of WHO grades II-IV in adults reported within the time period January 2009 to January 2012 were selected based on presence of tissue in the tumour bank. The slides and paraffin blocks were retrieved from the archives of the General Pathology Department. Of these 145 cases, 22 cases were lost to follow up information and were hence excluded from the study. In a further 7 cases, archived blocks were missing from the files, and these too were excluded from the study. Of the remaining 116 cases, only 108 had adequate fresh frozen tissue for DNA extraction. One case was further excluded as the diagnosis given was that of Pleomorphic xanthoastrocytoma.

The clinical, morphological and immunohistochemical features were analysed for all 107 cases. Fifty-seven cases that had not been analysed for IDH mutational status were subjected to immunohistochemistry for IDH-1.

32 cases required ATRX immunohistochemistry in this cohort. 50 of the 107 samples already had FISH for 1p/19q co-deletion and PCR for IDH-1 and IDH-1 mutations done for an earlier study in the same department, the data of which was included in this study.

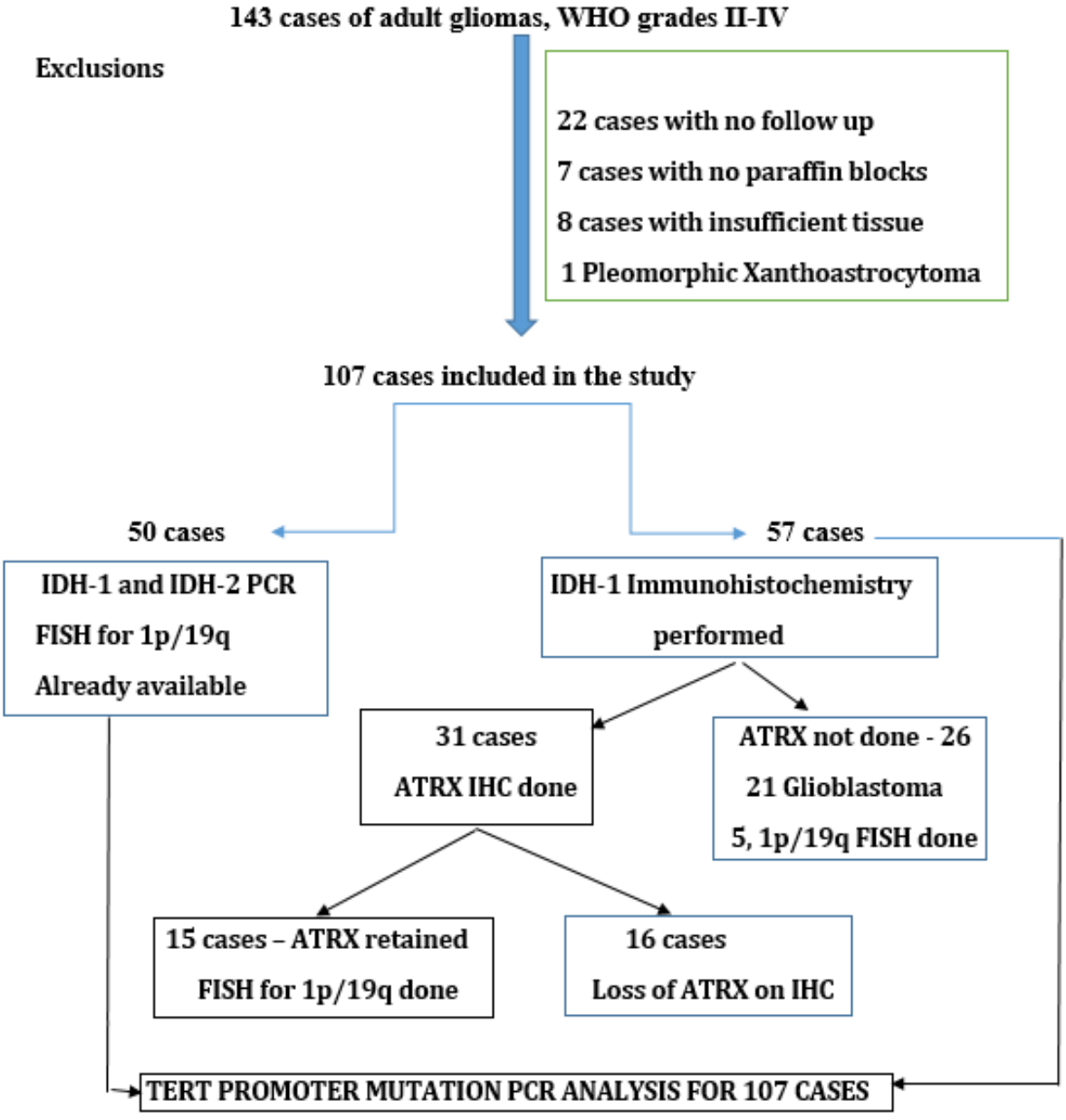

Figure 14 Selection of study cases and molecular test performed

Age

The median age of diagnosis for all gliomas was 36 years (+/- 11.88). The age range extended from 17 to a maximum of 68 years. The highest number of patients were present in the 31-40-year age group, totalling 40 out of a 107 cases. More than 75% of cases fell between the ages 21 to 50 years.

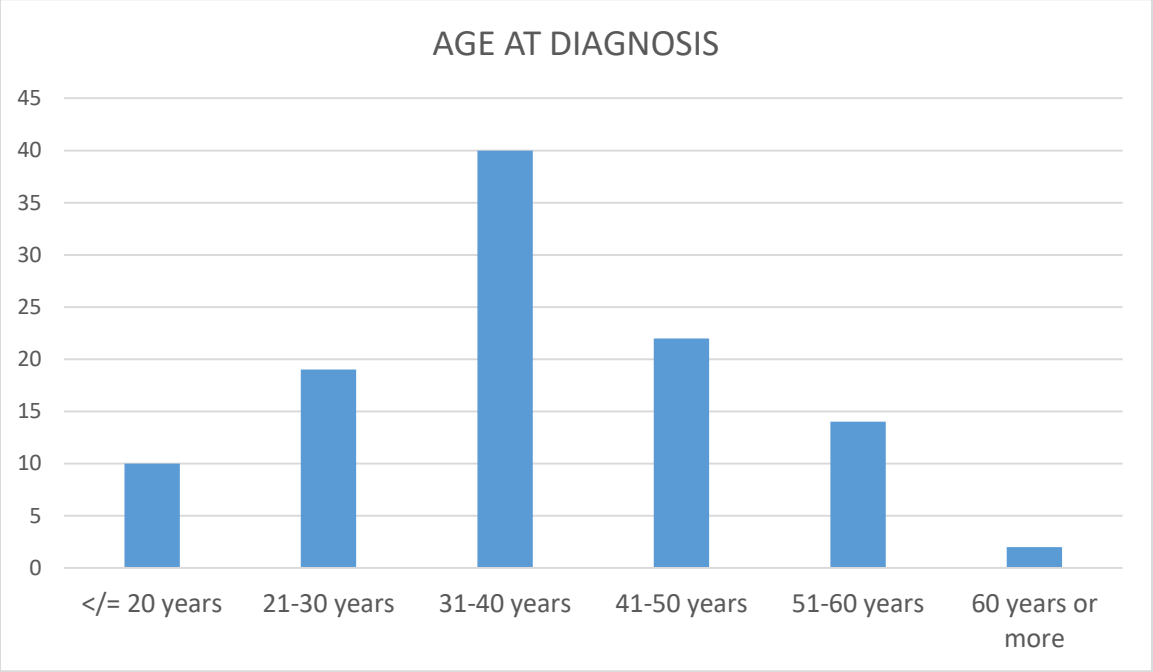

Figure 15 Age distribution of the 107 study cases of gliomas

Gender

The majority of patients in this study were male, with a male: female ratio of1.85.

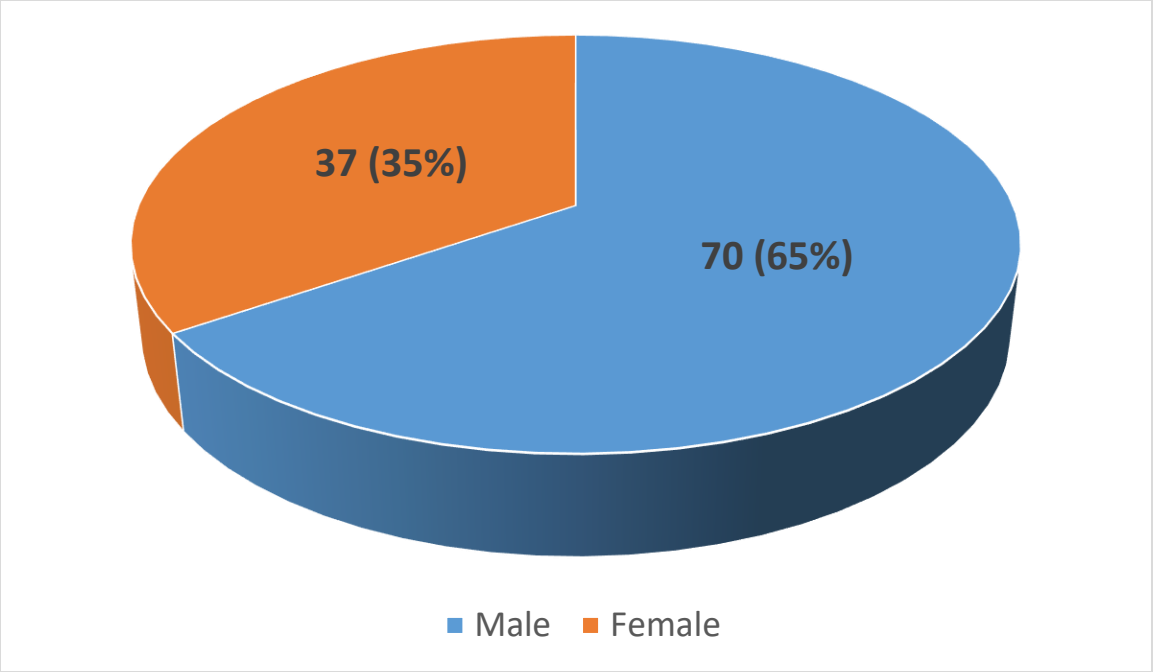

Figure 16 Gender distribution of the 107 study cases of glioma

Clinical Presentation

The most common clinical presentations in the study patients were found to be limited to 3 main categories, namely

1. Seizures
2. Focal neurological deficits
3. Signs and symptoms of raised intracranial tension

In this study it was noted that the most common clinical presentation was that of seizures, followed by raised intracranial tension and focal neurological deficits, in that order. Other presenting symptoms were not seen.

CLINICAL PRESENTATION	PERCENTAGE
Seizures	76%
Raised Intracranial Tension	39%
Focal Neurological deficits	35%

Table 1 Clinical presentation of 107 study cases of gliomas

The duration of symptoms in most cases was less than 6 months, with very few cases presenting with symptoms for more than one year, and even fewer for more than two years' duration.

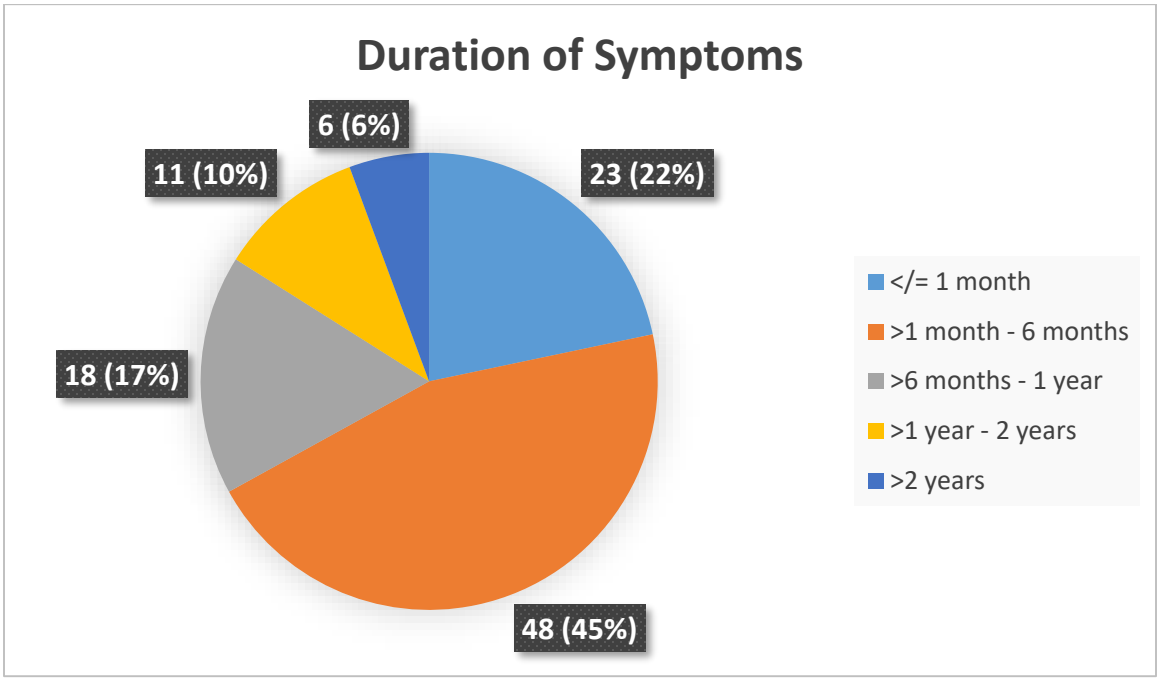

Figure 17 Duration of presenting symptoms in 107 study cases of gliomas

Radiology

All of the cases assessed had radiological investigation to localise site, determine size and margins, prior to surgery in the form of Magnetic Resonance Imaging (MRI) scans. In two out of the 107 study patients, an MRI scan was not done and the only imaging done prior to surgery was a Computed Tomography (CT) scan.

The predominant site of occurrence was the frontal lobe, accounting for almost half of the cases, followed by less frequent occurrences in the insula, temporal and parietal lobes.

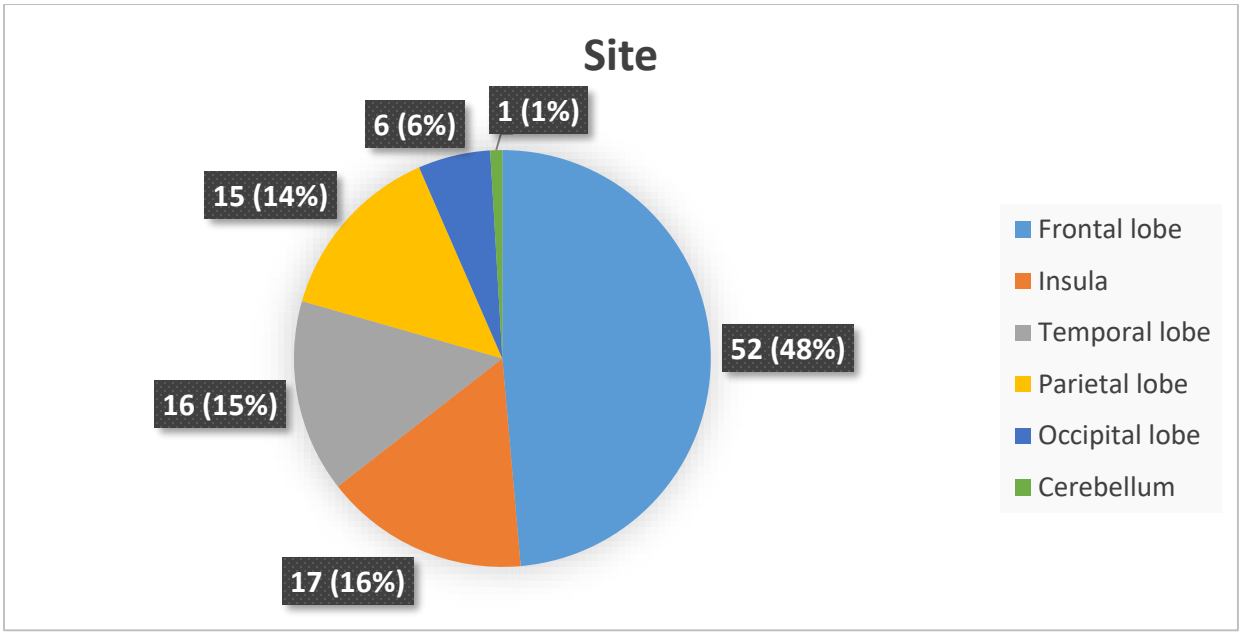

Figure 18. Site of gliomas in study group

Only 6% and 1% of cases occurred in the occipital lobe and cerebellum respectively. The size of the tumour was found very rarely to be <2cm in maximum dimension, with most cases falling between 2-5cm in maximum dimension.

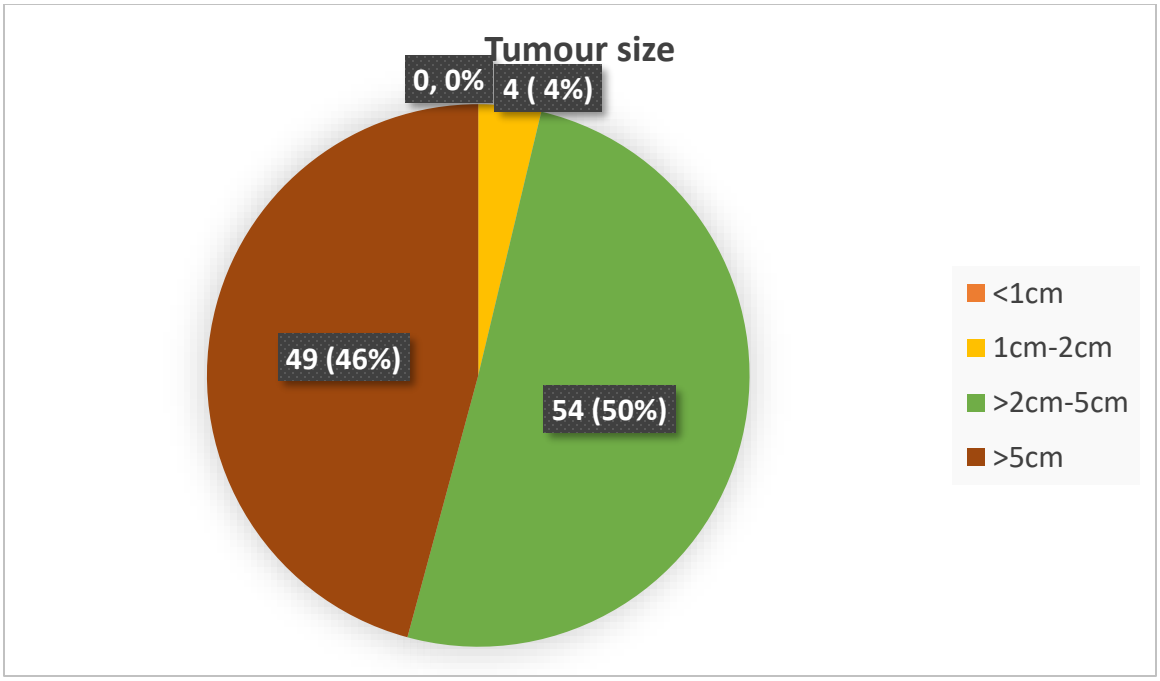

Figure 19 Size of tumours in 107 study cases of glioma

Right-sided tumours appeared to occur more frequently than left- sided tumours, 57% of tumours occurred on the right side, while 43% of tumours occurred on the left side.

In the 105 cases in which the MRI scan had been done, it was seen that the vast majority of tumours were hypointense in T1 weighted images, and hyperintense in T2 weighted images as can be seen in table 3.

	Hypointense	Hyperintense	Isointense	Heterogeneous
T1 weighted imaging	93%	2%	3%	2%
T2 weighted imaging	**3%**	**92%**	**1%**	**4%**

Table 2. T1 and T2 weighted imaging of 107 study cases of gliomas

The final parameter assessed by radiology was the margins; i.e. whether the margins were distinct or diffuse.

- Distinct margins – 27%
- Indistinct margins – 73%

Surgery

In 68% of cases a radical excision was possible and in 32% of cases, either a partial or subtotal excision was performed.

Adjuvant Therapy

Radiotherapy was given in nearly all cases, with or without chemotherapy and was either 3D conformational radiotherapy (3DCRT) or Cobalt 60 radiotherapy (Co60 RT). The amount of radiation given ranged from 50 to 60 Grays, with most patients receiving 54 Grays of radiation over 30 or more fractions.

Concurrent chemotherapy (Temozolomide at the time of radiotherapy for 1 month titrated to body weight) was given in 77 cases, amounting to 71.9% of the study patients.

22% of patients received concurrent chemotherapy with no subsequent adjuvant chemotherapy. This was primarily due to the patient's unwillingness for therapy, or in some instances because the tumour response to radiotherapy was optimal.

Adjuvant chemotherapy was given in 53 cases (50%). Adjuvant chemotherapy in this instance refers to oral Temozolomide chemotherapy titrated to body weight and given in 6 cycles of 5 days each. Only one case received Bevacizumab chemotherapy in addition to Temozolomide, due to treatment failure on Temozolomide chemotherapy alone. All of the cases receiving adjuvant therapy had also received concurrent chemotherapy with radiotherapy.

For three cases in the study sample, the adjuvant therapy received was unknown, as these patients had opted to undergo further treatment at another centre. In two cases, no adjuvant therapy (chemotherapy or radiotherapy), was given. In both these instances, adjuvant therapy was refused by the patient.

ADJUVANT THERAPY GIVEN	PERCENTAGE
Radiotherapy alone	23%
Radiotherapy with concurrent chemotherapy only	22%
Radiotherapy with concurrent and adjuvant chemotherapy	50%
No adjuvant therapy	2%
Unknown adjuvant therapy	3%

Table 3 Type of Adjuvant therapy given in 107 study cases of gliomas

The type of adjuvant therapy varied with the type of glioma diagnosed. 15 of 26 Diffuse Astrocytomas received radiotherapy alone, while among Anaplastic Astrocytoma,

Anaplastic Oligodendrogliomas and Glioblastomas, the most common adjuvant therapy given was Radiotherapy with adjuvant chemotherapy.

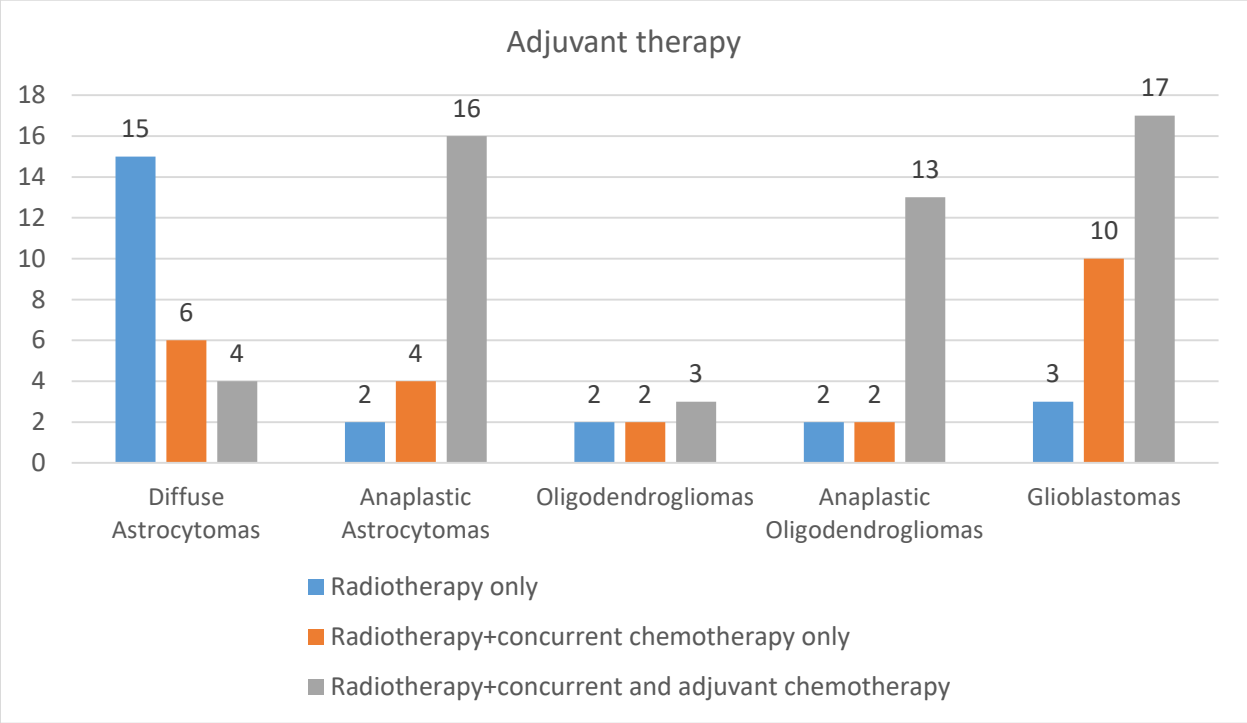

Figure 20Type of Adjuvant therapy across glioma groups

The recurrence and survival for each adjuvant therapy group is as given in the table below. This association was not statistically significant (p value=0.36).

Treatment	Recurrence	Death
Radiotherapy	37%	20%
Radiotherapy + Concurrent chemotherapy	45%	37%
Radiotherapy + Concurrent chemotherapy + Adjuvant chemotherapy	45%	28%

Table 4 Recurrence and death in categories of Adjuvant therapy

Primary/Recurrent

This study included both primary and recurrent tumours. The primary tumours comprised the larger portion of cases with numbers as follows

- Primary cases – 92% (98 cases)
- Recurrent cases – 8% (9 cases)

In total, nine cases were recurrent. In 3 cases, the diagnosis was that of a Glioblastoma, two cases each of Anaplastic Astrocytoma and Anaplastic Oligodendroglioma and 2 cases of Diffuse astrocytoma at the time of recurrence.

In the 7 cases which recurred as high grade tumours, there was a history of a prior lower grade glioma occurring in the same site at the time of first surgery. These 7 prior lower grade tumours included 2 Diffuse Astrocytomas WHO grade II, 4 Oligodendrogliomas WHO grade II and 1 Anaplastic Astrocytoma WHO grade III. Of the three glioblastomas seen at recurrence, one was previously an anaplastic astrocytoma, whilst 2 were oligodendroglioma grade II. The two cases of Diffuse Astrocytomas, had tumour of the same histological morphology and grade at the time of first surgery.

The details of the 9 recurrent cases are as given in the Table below:

Primary Tumour	**Recurrent Tumour**
Diffuse Astrocytoma, WHO grade II	Diffuse Astrocytoma, WHO grade II
Diffuse Astrocytoma, WHO grade II	Diffuse Astrocytoma, WHO grade II
Diffuse Astrocytoma, WHO grade II	Anaplastic Astrocytoma, WHO grade III

Diffuse Astrocytoma, WHO grade II	Anaplastic Astrocytoma, WHO grade III
Oligodendroglioma, WHO grade II	Anaplastic Oligodendroglioma, WHO grade III
Oligodendroglioma, WHO grade II	Anaplastic Oligodendroglioma, WHO grade III
Oligodendroglioma, WHO grade II	Glioblastoma, WHO grade IV
Oligodendroglioma, WHO grade II	Glioblastoma, WHO grade IV
Anaplastic Astrocytoma, WHO grade III	Glioblastoma, WHO grade IV

Table 5 Details of recurrent Gliomas

Morphology

The haematoxylin and eosin stained slides of all 107 cases were reviewed and the histological features such as morphology, microvascular proliferation and necrosis were noted. The cases were given a histological diagnosis and WHO grading based on their morphology as well as the Ki67 proliferation index where available.

1. Morphology of cells

Astrocytic morphology	32 cases (30%)
Oligodendroglial morphology	36 cases (34%)
Mixed – Astrocytic and Oligodendroglial	39 cases (36%)

Table 6 Morphology of study cases of gliomas

2. Microvascular proliferation – 39 cases (36%) showed microvascular proliferation, and was absent in the remaining 64%.
3. Necrosis – 34 cases (31%) showed evidence of necrosis.

Based on these parameters, a tentative diagnosis was made for all cases based on the histomorphology, prior to molecular studies.

There was an even distribution of tumours according to WHO grade, with a slight preponderance of WHO grade III tumours.

- WHO grade II – 31%
- WHO grade III – 38%
- WHO grade IV – 31%

Histological Diagnoses

Following slide review, the cases were given one of seven diagnoses, namely

a. Diffuse Astrocytoma, WHO grade II – 6%
b. Anaplastic Astrocytoma, WHO grade III – 10%
c. Oligodendroglioma, WHO grade II – 17%
d. Anaplastic Oligodendroglioma, WHO grade III – 16%
e. Oligoastrocytoma, WHO grade II – 8%
f. Anaplastic Oligoastrocytoma, WHO grade III – 12%
g. Glioblastoma, WHO grade IV – 31%

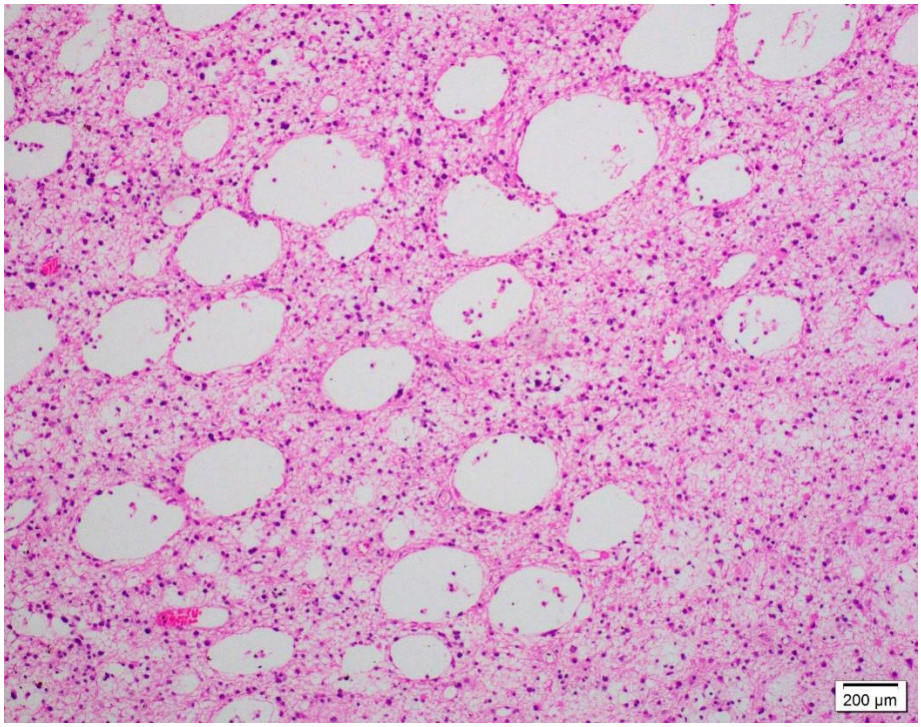

Figure 21 Diffuse Astrocytoma, WHO grade II with low cellularity and microcystic degeneration. (H&E x100)

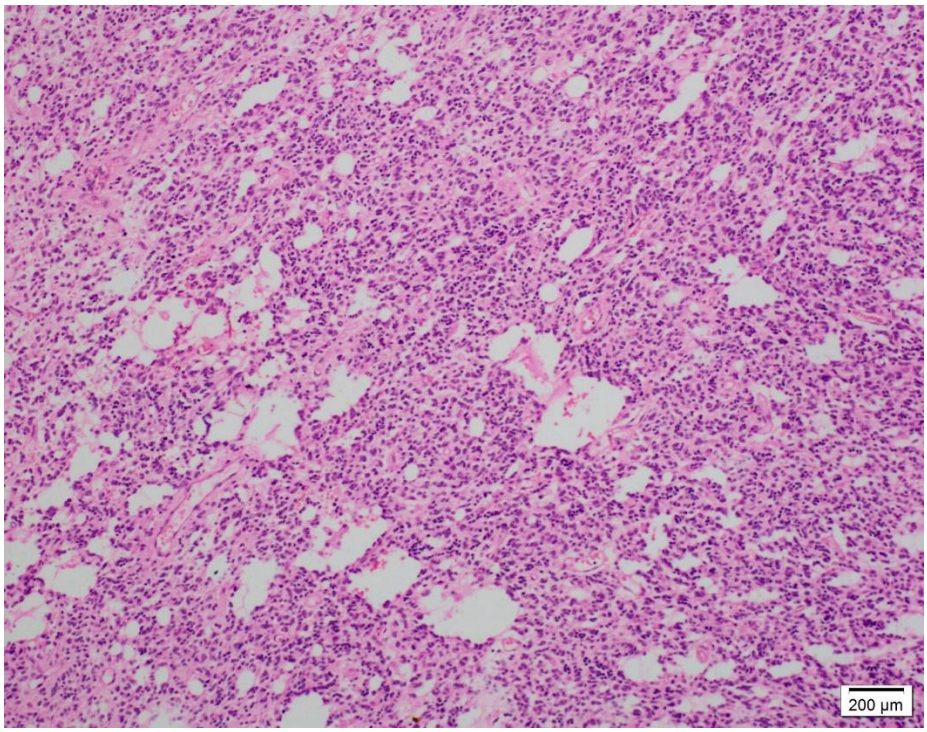

Figure 22 Anaplastic Astrocytoma, WHO grade III with hypercellularity and pleomorphism. (H&E x100)

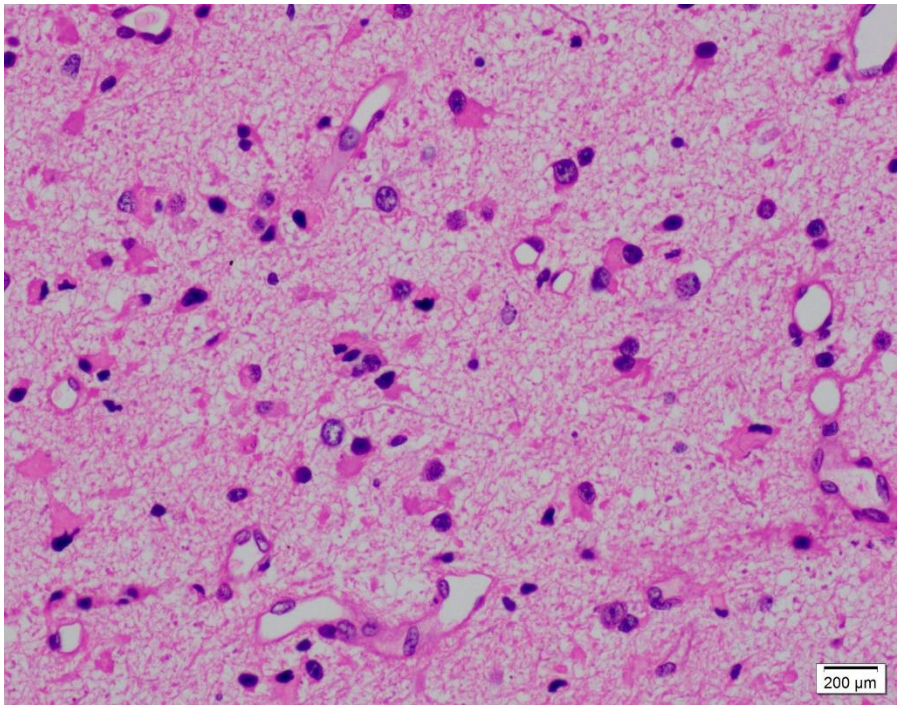

Figure 23 Diffuse Astrocytoma, WHO grade II with low cellularity. (H&E x400)

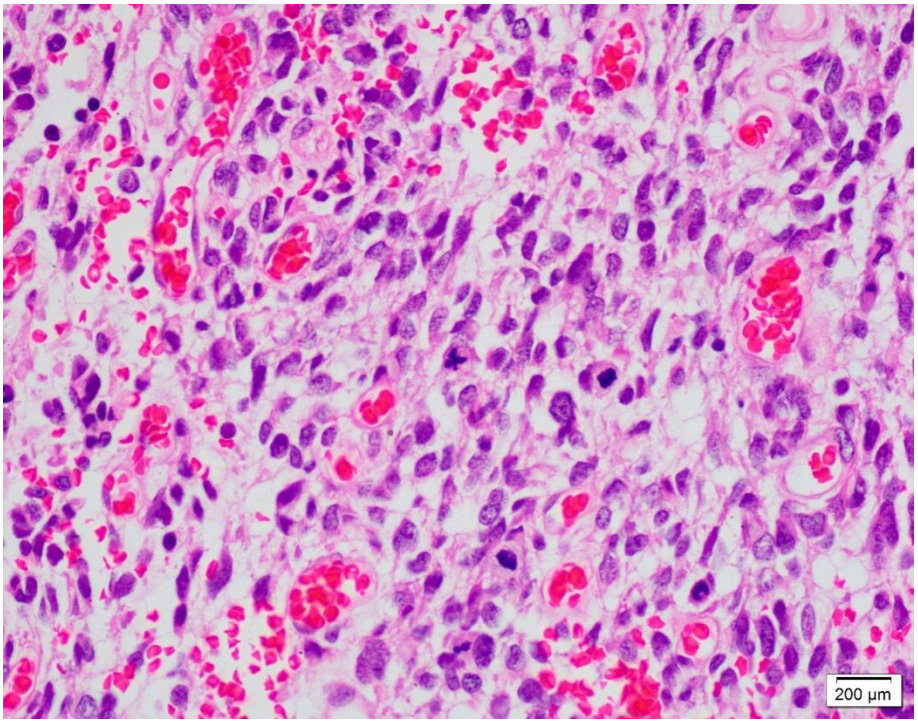

Figure 24 Anaplastic Astrocytoma, WHO grade III with mitotic activity. (H&E x400)

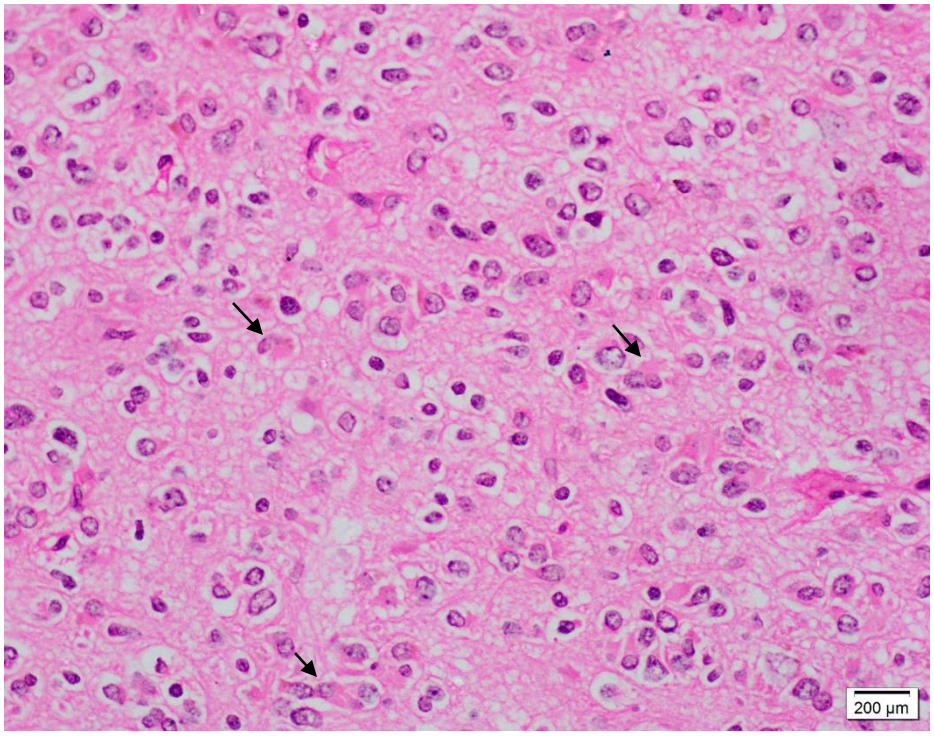

Figure 25 Oligodendroglioma, WHO grade II, (→ Minigemistocytes). (H&E x400)

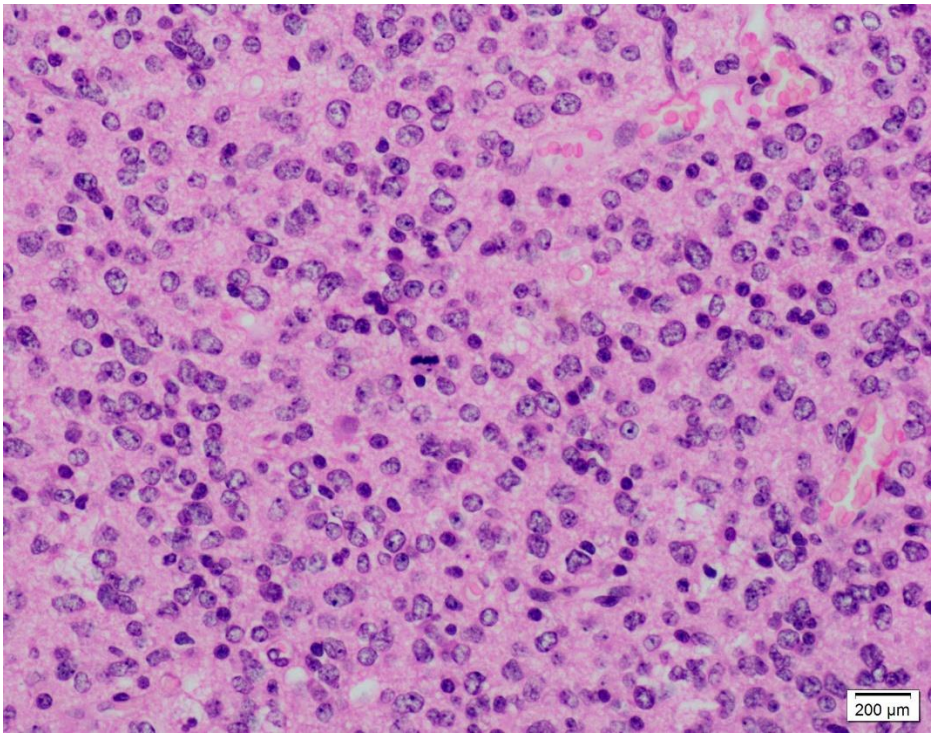

Figure 26 Anaplastic Oligodendroglioma, WHO grade III with increased cellularity and mitotic activity. (H&E x400)

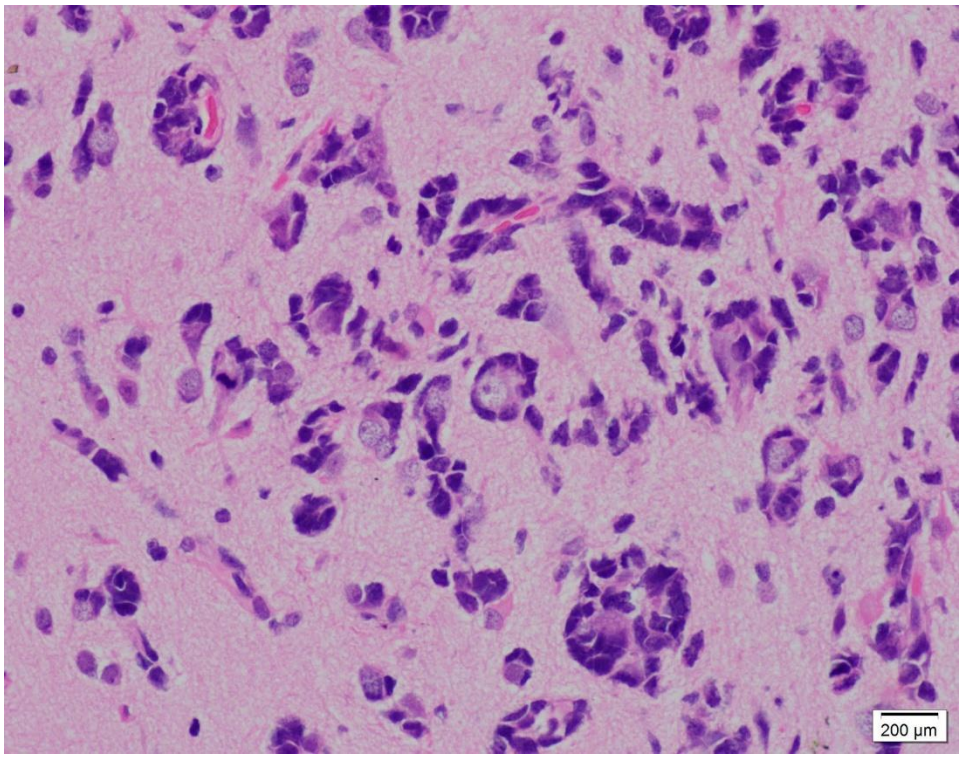

Figure 27 Anaplastic Oligodendroglioma, WHO grade III with perineuronal satellitosis. (H&E x400)

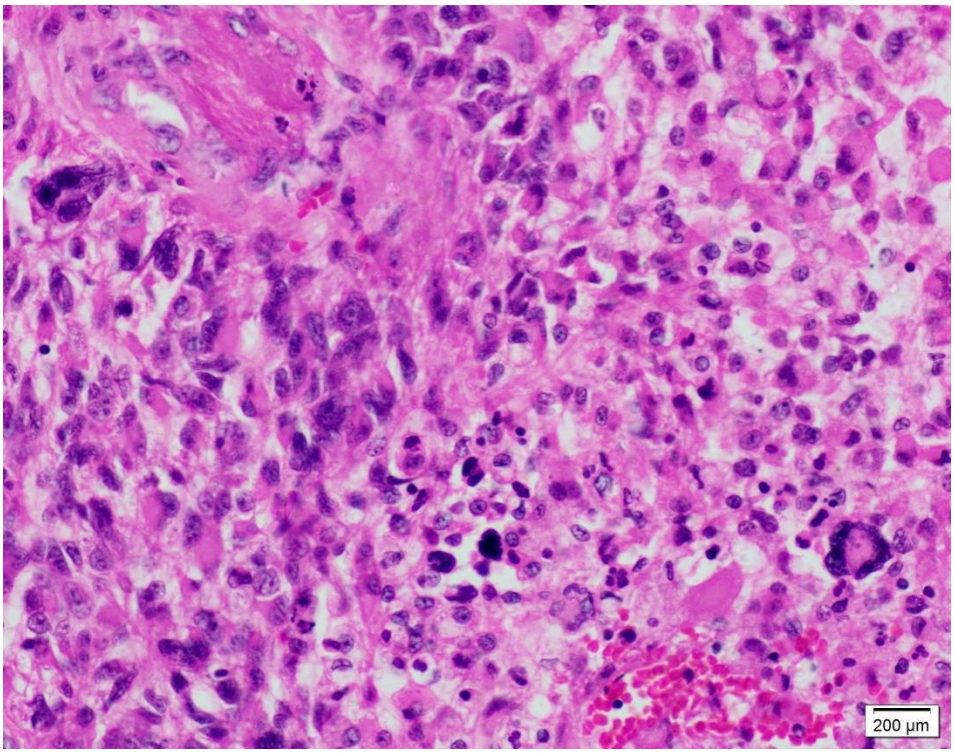

Figure 28 Glioblastoma, WHO grade IV with marked pleomorphism. (H&E x400)

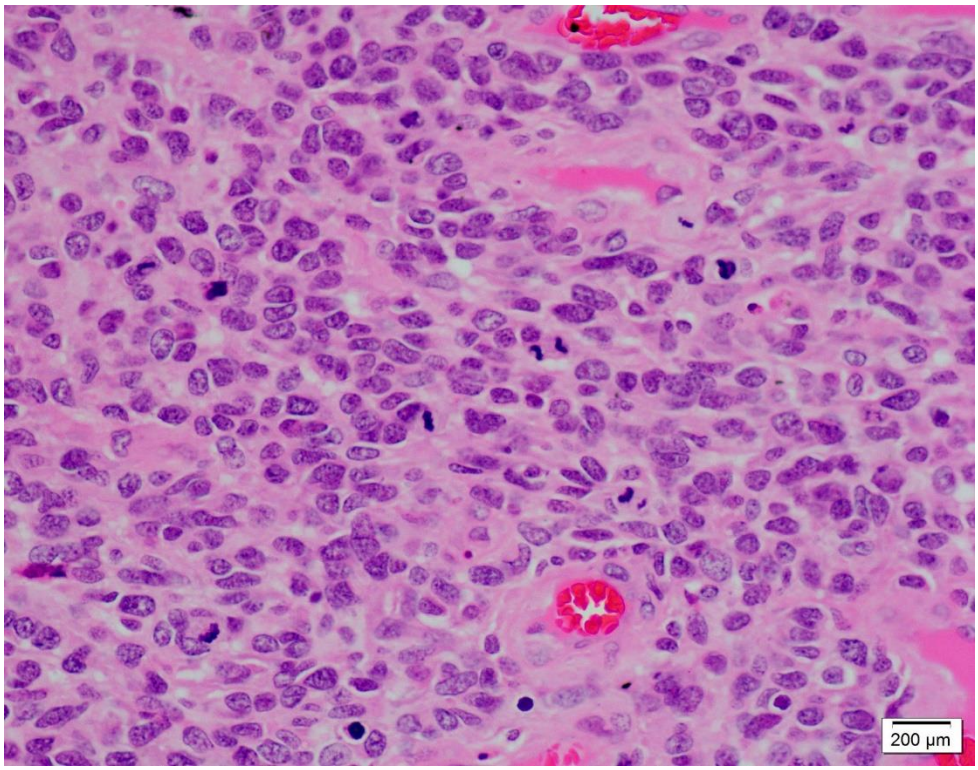

Figure 29 Glioblastoma, WHO grade IV with brisk mitotic activity. (H&E x400)

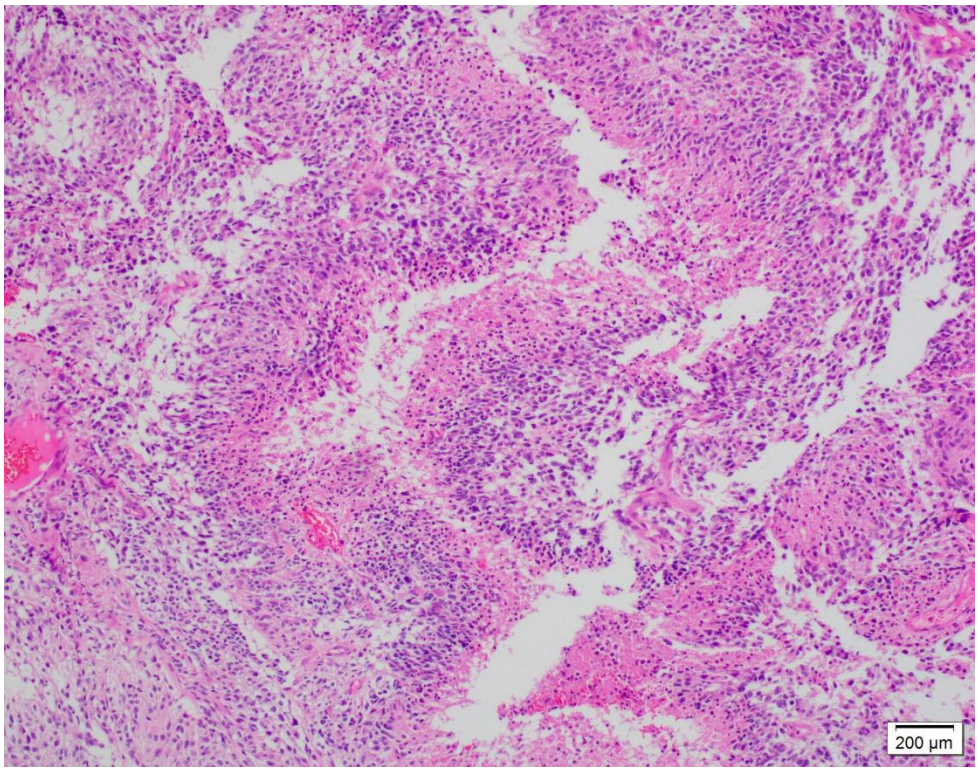

Figure 30 Glioblastoma, WHO grade IV with pseudopalisading necrosis. (H&E x100)

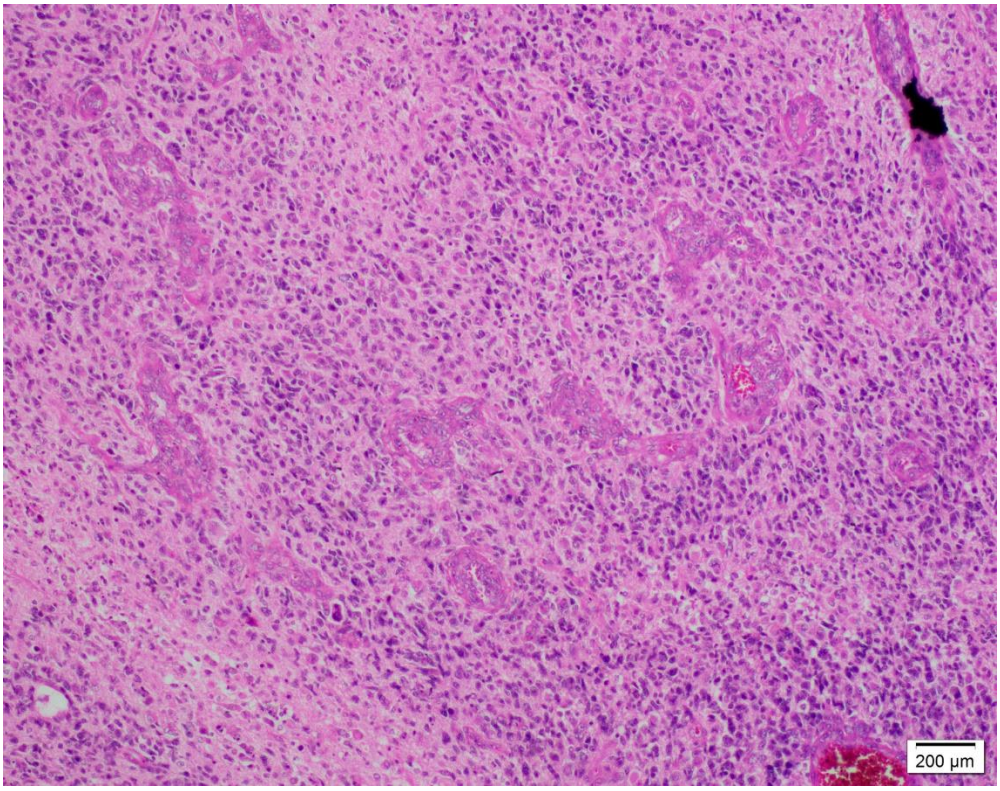

Figure 31 Glioblastoma, WHO grade IV with Microvascular proliferation. (H&E x100)

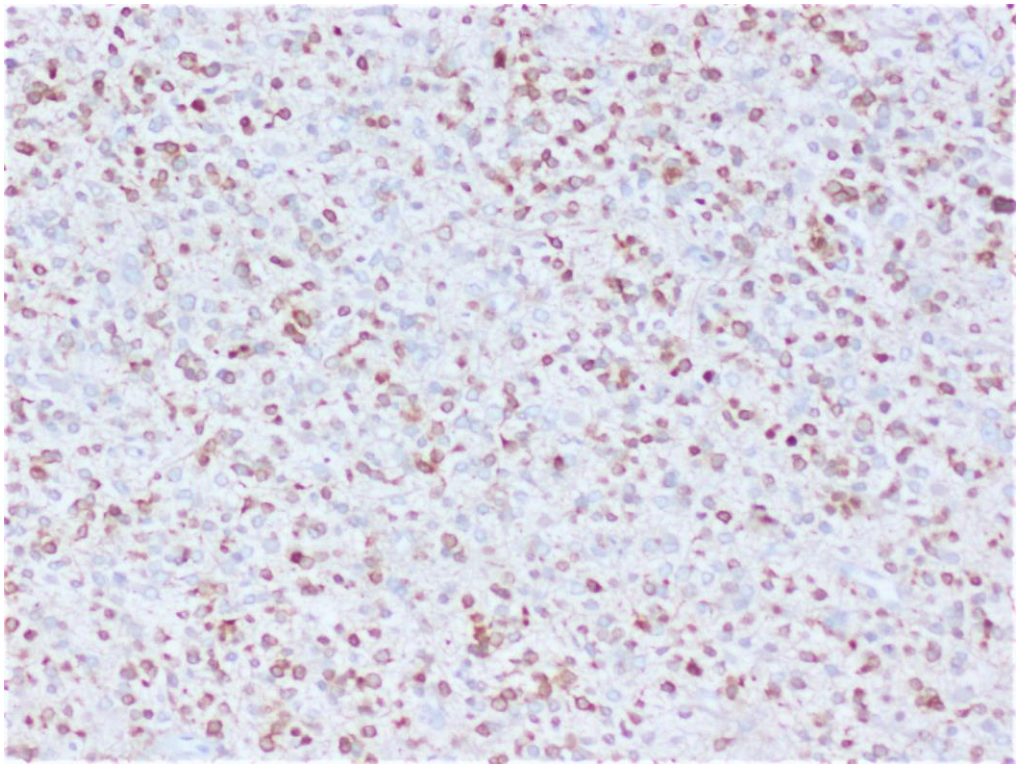

Figure 32 Positive IDH immunohistochemistry. (R132H IDH Antibody x100)

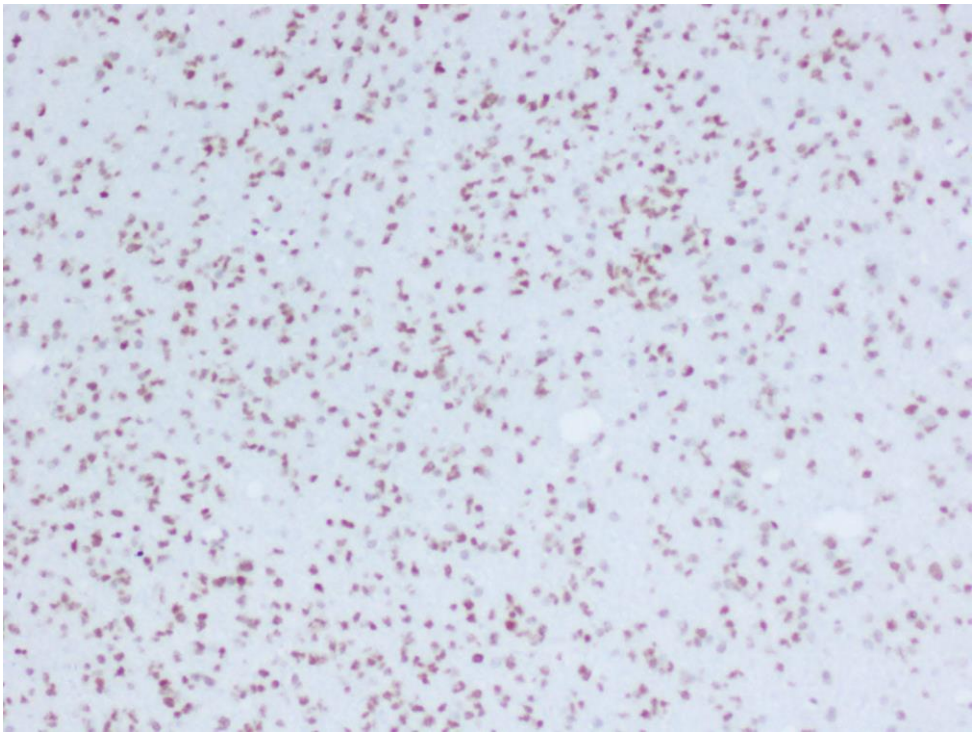

Figure 33ATRX Immunohistochemistry with retained expression of ATRX (IHC x100)

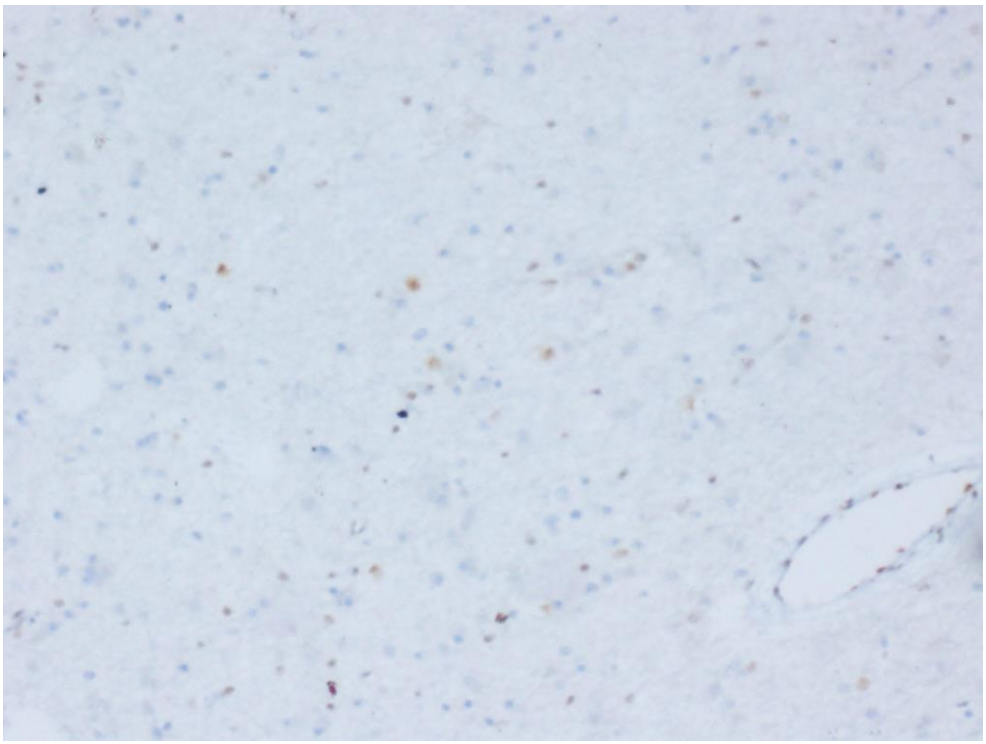

Figure 34 Loss of ATRX expression on Immunohistochemistry- note positivity in endothelial cells (internal control) (IHC x400)

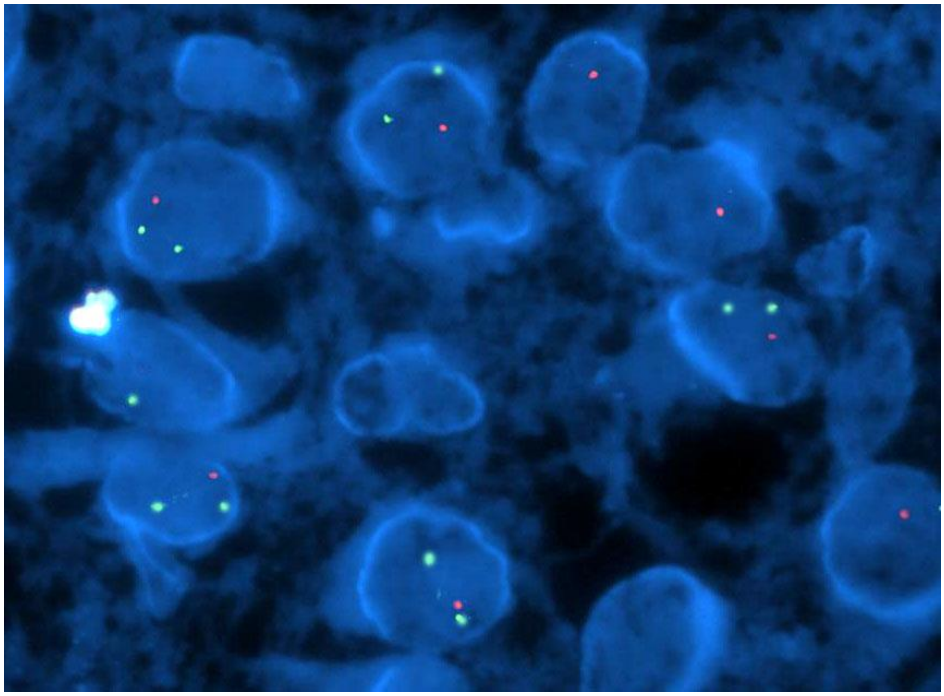

Figure 35 (A) 1p32 probe = red (1 signal per tumour nucleus), 1q42 = green (mostly 2 signals)

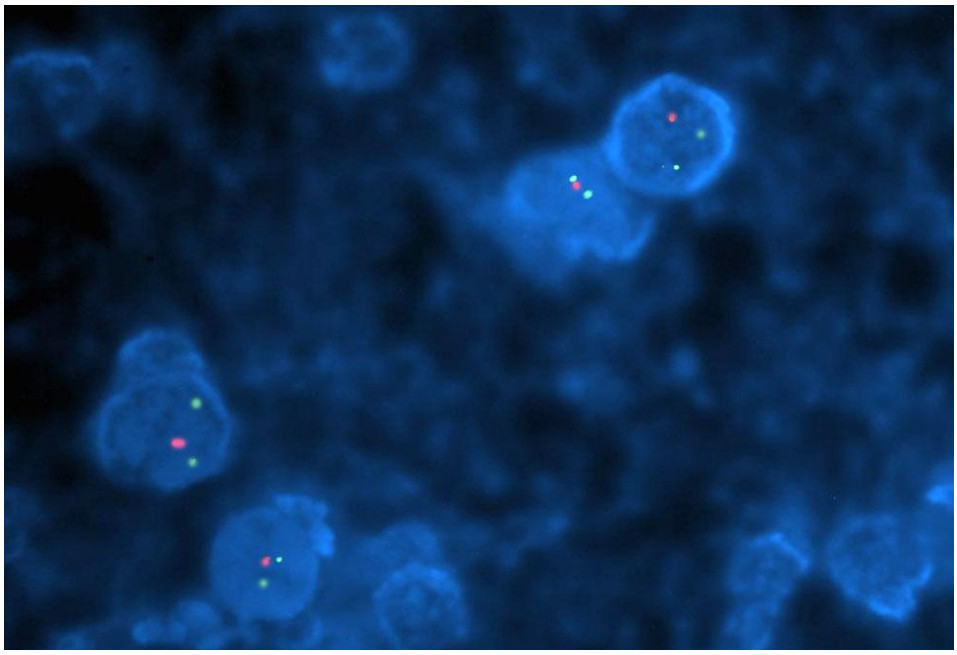

Figure 36 (B)19p13 = green (mostly 2 signals), 19q13 = red (1 signal).

Dual-colour fluorescence in situ hybridizations with combined 1p(A) and 19q(B) deletions. 4',6-Diamidino-2-phenylindole (DAPI) nuclear counterstain (blue).

The diagnoses of Oligoastrocytoma and Anaplastic Oligoastrocytoma are no longer used in the WHO classification of tumours where molecular testing is available. Those cases that were histomorphologically categorised as mixed tumours were classified into an astrocytic or oligodendroglial lineage based on molecular testing during this study.

Histological Diagnoses vs. Final Diagnoses

Once the molecular markers such as IDH mutational analysis, immunohistochemistry for ATRX, FISH for 1p/19q co-deletion, a final diagnosis on each case was made on a molecular basis. All of the cases histologically diagnosed as Oligoastrocytomas or Anaplastic Oligoastrocytomas were re-categorised under either an astrocytic lineage or oligodendroglial lineage. Depicted below in Fig 37. is a comparison between the histological and final diagnoses made.

Glioblastomas being primarily a histological diagnosis, remained the same in both categories.

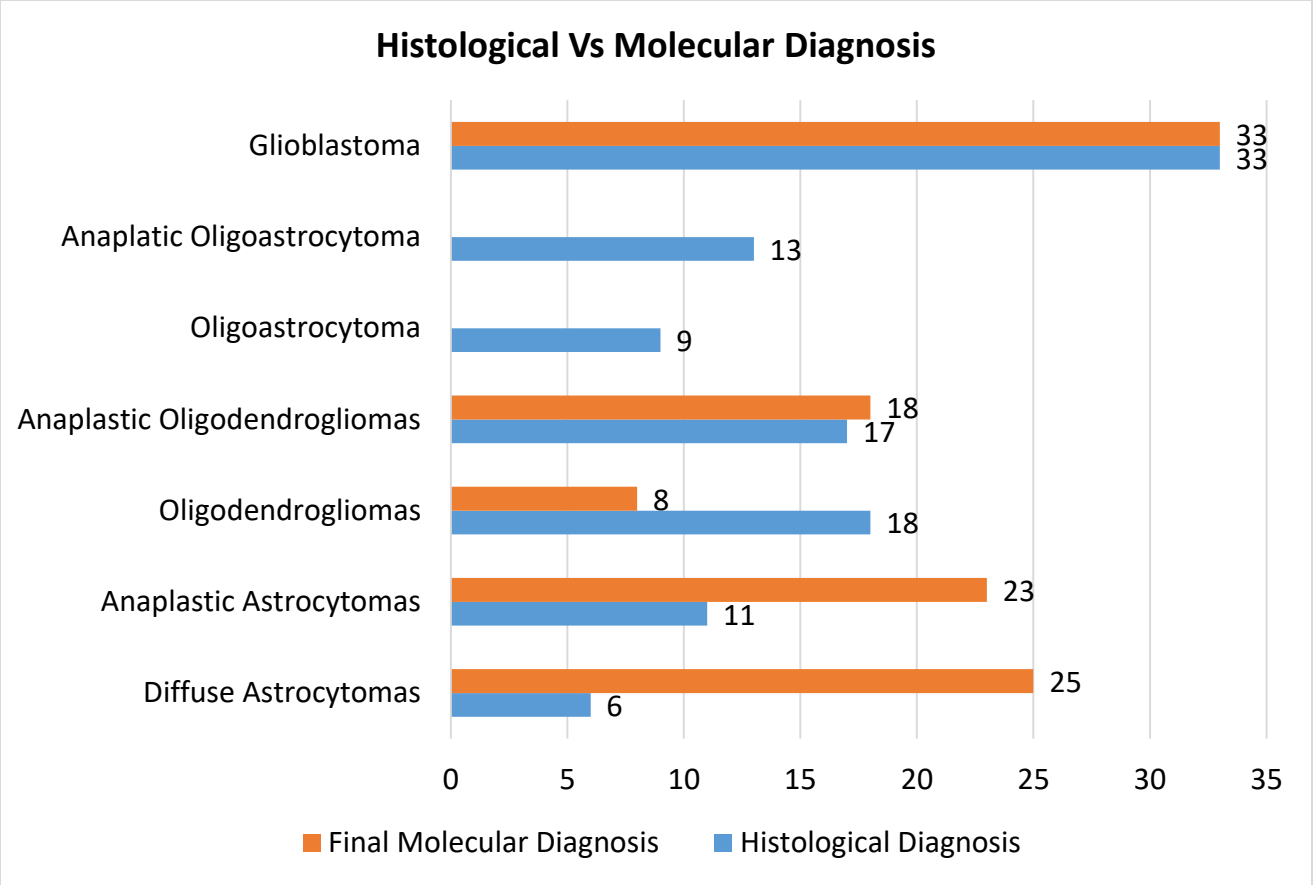

Figure 37 Histological and Molecular Diagnosis.

9 cases of Oligoastrocytomas, WHO grade II were all re-categorized as diffuse astrocytomas. Of the 14 cases diagnosed as Anaplastic Oligoastrocytomas on histology, 12 were re-categorized as Anaplastic Astrocytomas and 1 was re-categorized as an Anaplastic Oligodendroglioma following molecular work-up.

Although 18 cases were histologically diagnosed as Oligodendroglioma, WHO grade II, only 8 were molecularly proven, the remaining 10 being re-categorized as Diffuse Astrocytomas, WHO grade II based on molecular markers.

In effect, with all of these reclassifications, the number of Diffuse Astrocytomas, WHO grade II increased from 6 to 25 following molecular reclassification. Similarly, the number of Anaplastic Astrocytomas, WHO grade III increased from 11 to 23.

Histological Diagnosis	**Molecular reclassification**
9 Oligoastrocytomas	**9 Diffuse Astrocytomas**
13 Anaplastic Oligoastrocytomas	**12 Anaplastic Astrocytomas**
	1 Anaplastic Oligodendroglioma

Table 5 Molecular reclassification of Oligoastrocytomas and Anaplastic Oligoastrocytomas

IDH Mutations

Combining the IDH-1 mutations detected by immunohistochemistry and the IDH-1 and IDH-2 mutations detected by PCR, the total number of IDH mutant cases were 67 out of 107 cases (62%). The distribution of IDH mutations for each diagnosis is depicted in Fig 4 given below. As is evident, the majority of the IDH wild type cases are made up of WHO grade IV Glioblastomas. In contrast, the majority of all the WHO grade II and WHO grade III gliomas, both oligodendrogliomas and astrocytomas, are positive for an IDH-1 or IDH-2 mutation.

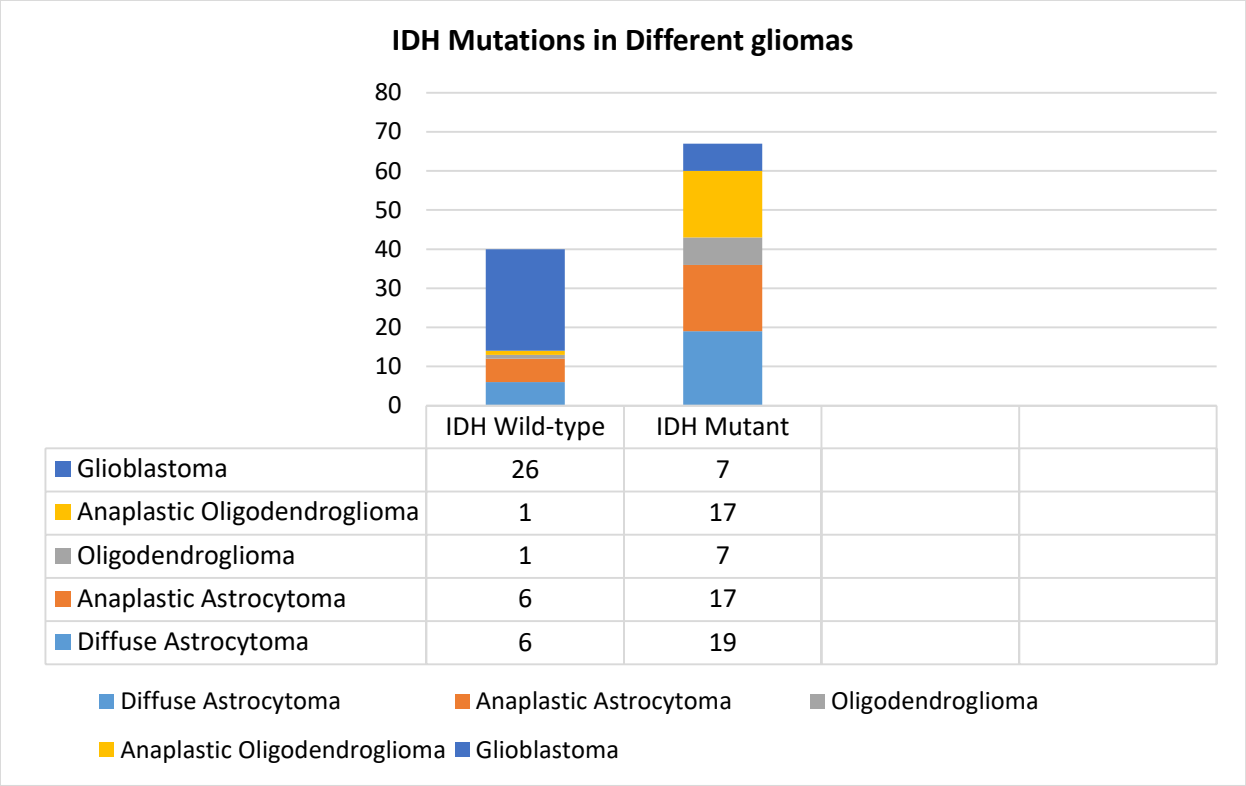

Figure 38 Distribution IDH mutations across glioma grades.

Only two cases of oligodendroglial lineage, 1 oligodendroglioma and 1 Anaplastic Oligodendroglioma, were found to lack the IDH mutation, despite carrying the co-deletion for 1p-19q detected by FISH. Six Diffuse Astrocytomas and 6 Anaplastic Astrocytomas were found to be negative for the IDH mutation by immunohistochemistry. PCR for IDH1 and IDH2 was not carried out to confirm the absence of mutation in any of these 8 cases, due to economic constraints.

ATRX Mutations

ATRX expression was lost in 35 (33%) cases, which included 19 cases from a cohort of 50 cases in a previous study and 16 from the 31 cases in this study requiring ATRX immunohistochemistry. ATRX was retained in 41 (38%) cases, which included 26 cases from a cohort of 50 cases in a previous study and 15 from the 31 cases on which ATRX immunohistochemistry was performed for this study.

Those cases with lost ATRX expression were designated astrocytomas, and those in which ATRX was retained underwent testing for 1p/19q co-deletion by FISH.

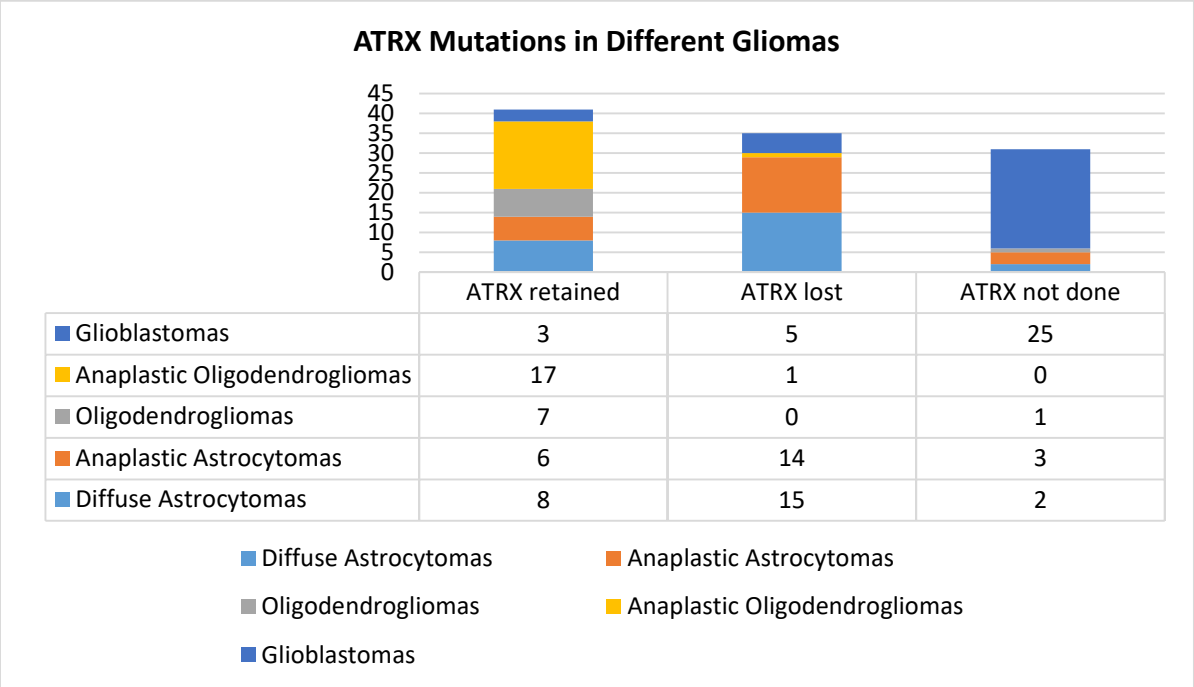

Figure 39Distribution of ATRX mutations across glioma groups

Of all the cases with retained ATRX expression on immunohistochemistry, 41 in total, 24 were given a diagnosis of Oligodendroglioma or Anaplastic Oligodendroglioma based on

1p/19q co-deletion testing by FISH. 14 did not have co-deletion of 1p and 19 q and hence remained within an astrocytic lineage.

1p/19q co-deletion

FISH for 1p-19q co-deletion was not performed on 37 cases, as these cases had loss of ATRX on immunohistochemistry, or were diagnosed Glioblastoma on histology. The remaining 70 cases underwent this testing, this included 50 cases from a previous study, as well as 20 cases in this current study. It was found that 26 cases carried a co-deletion of 1p/19q, which included 21 cases from the cohort of 50 cases from the previous study. This amounted to an overall frequency of 1p/19q co-deletion of 24% of all cases. The remaining 44 cases (29 from a previous cohort and 15 from the current study) were negative for 1p/19q co-deletion. One of the cases considered negative, carried a polysomy for 1p and 19q.

In keeping with the fact that the co-deletion of 1p/19q is a requirement for a diagnosis of Oligodendroglioma or Anaplastic Oligodendroglioma, the distribution of cases is as given in Fig. 6, with all Oligodendrogliomas carrying this co-deletion and all Astrocytomas and Glioblastomas negative for the 1p/19q co-deletion.

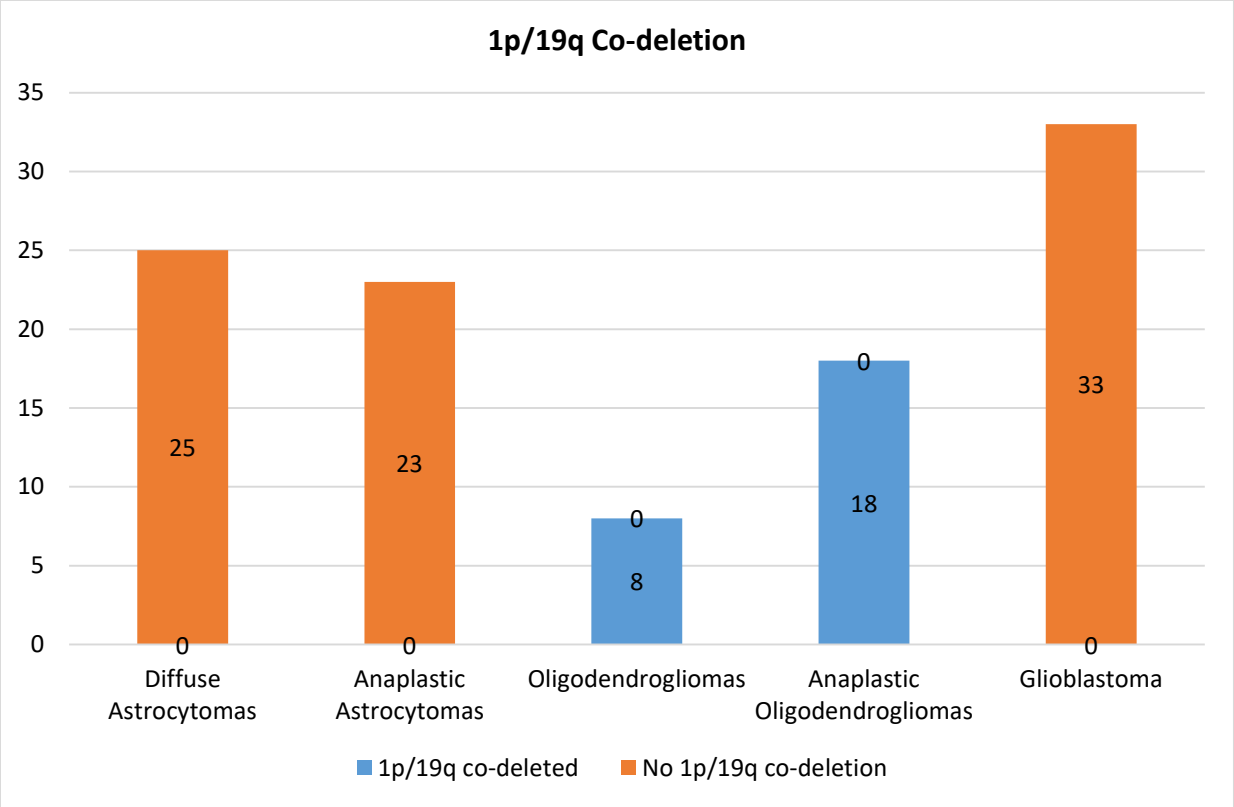

Figure 40 Distribution of 1p/19q co-deletion in the study cases

TERT Promoter Mutations

TERT promoter mutational analysis was done for all 107 cases and 51 of these cases showed a mutation in the TERT promoter region. There were 2 different loci in which this mutation occurred in positions 1295228 and 1295250 of chromosome 5. Of the 51 cases that showed TERT promoter mutations 41 cases showed mutations at position 1295250 and 11 cases showed mutations at position 1295228.

This mutation in the TERT promoter region was seen more commonly in Oligodendrogliomas, Anaplastic Oligodendrogliomas and Glioblastomas. Only two cases of the Oligodendroglial lineage were negative for TERT mutations. In contrast,

Astrocytic neoplasms were less commonly mutated at the TERT promoter region. Glioblastomas showed near equal prevalence of mutated and non-mutated cases.

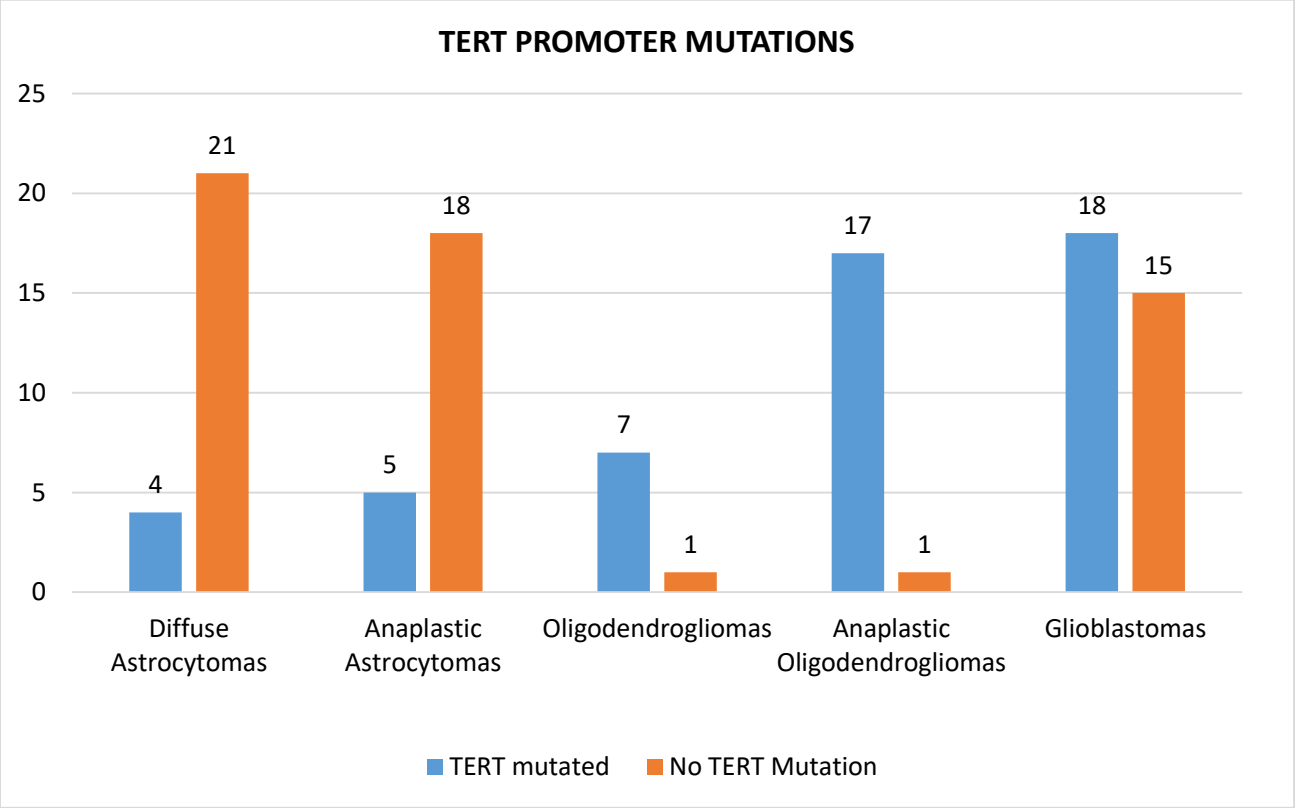

Figure 41 TERT promoter Mutations in each Glioma group

When considering the percentages of the TERT promoter mutations, the prevalence differs markedly among each glioma grade. The highest frequency of TERT promoter mutations was seen in Anaplastic Oligodendrogliomas (94%), followed by Oligodendrogliomas (87.5%). Glioblastomas carried TERT promoter mutations in 54% of cases. The TERT promoter mutation had the lowest frequency in Diffuse Astrocytomas (16%).

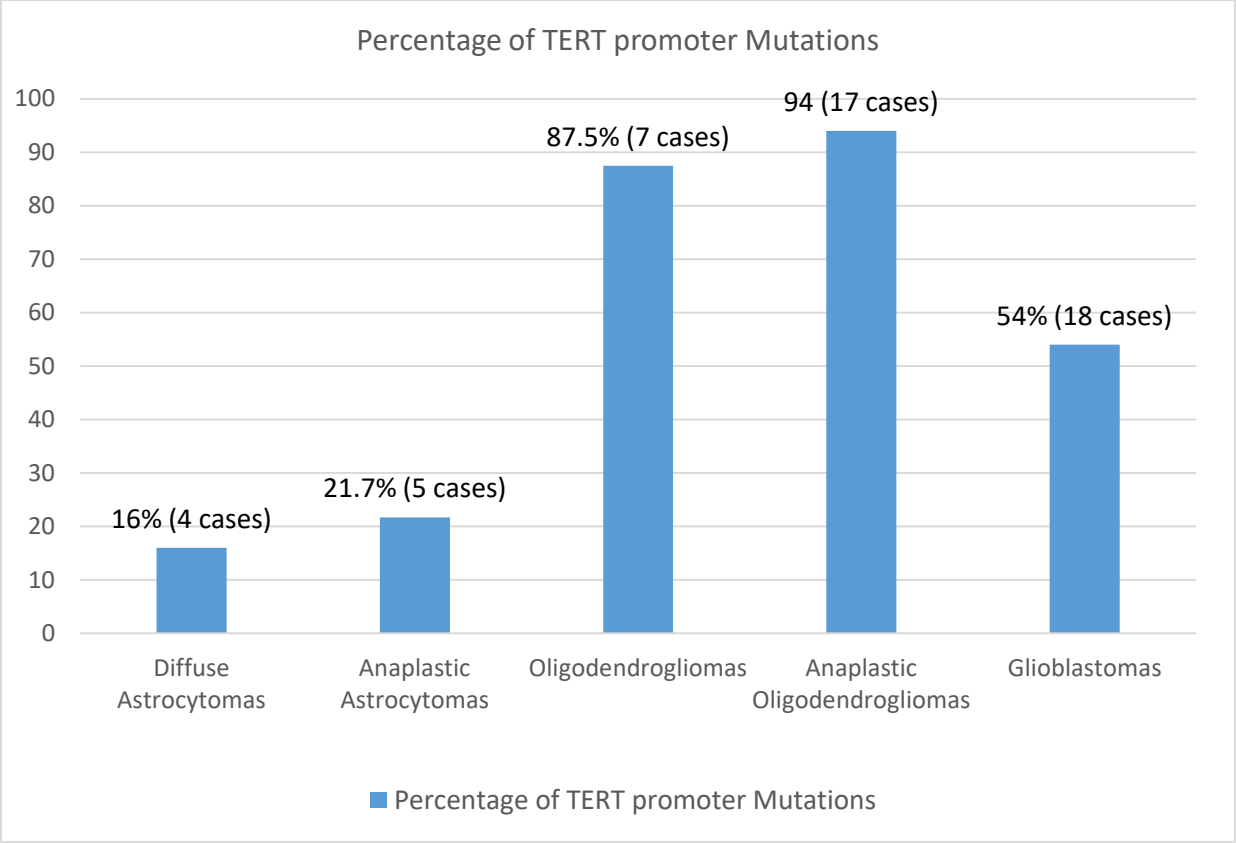

Figure 42 Frequency of TERT promoter mutations in each Glioma group

Recurrence and Survival

The mean follow-up period for all 107 cases in this study was 39.4 months, with a range from 3 months to 102 months and a median of 37 months. Within this follow up duration, the average progression free survival was 36 months and the average overall survival was 39 months.

In terms of absolute numbers, it was seen that 48 cases (44%) had a recurrence, and 32 cases (30%) had died by the end of the study period. Overall survival curves for the entire study population are seen in the figures below.

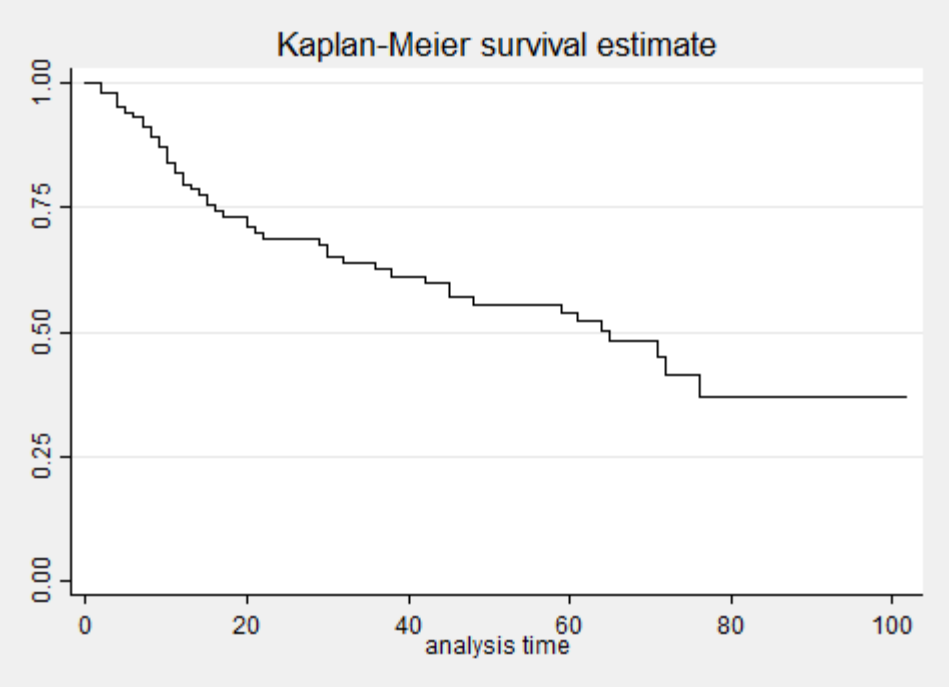

Figure 43 Progression free survival for the entire study population

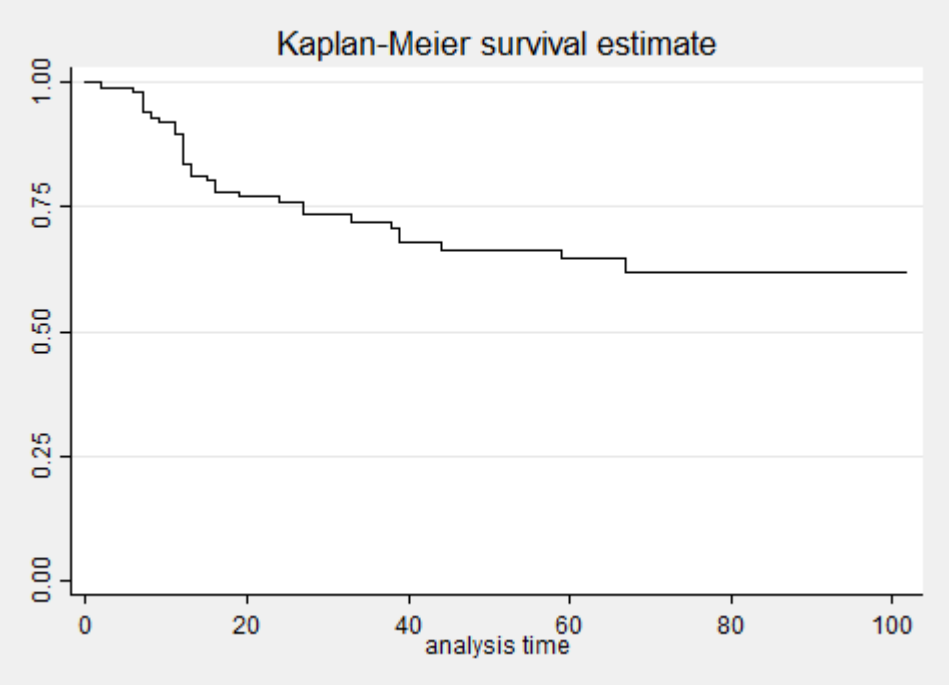

Figure 44 Overall survival for the entire study population

Survival Based On Individual Mutations

The association between survival (overall and progression free) with each of the molecular markers that we had studied was assessed individually for each mutation.

When IDH mutational status was compared with the overall and progression free survival of patients, it was seen that 51.2% of IDH wild type cases had died by the end of the study period as compared to only 17.9% of IDH mutant cases. This association was found to be significant with a p-value of <0.000. The average time to death was 47 months for IDH wild type, and 62 months for IDH mutant cases.

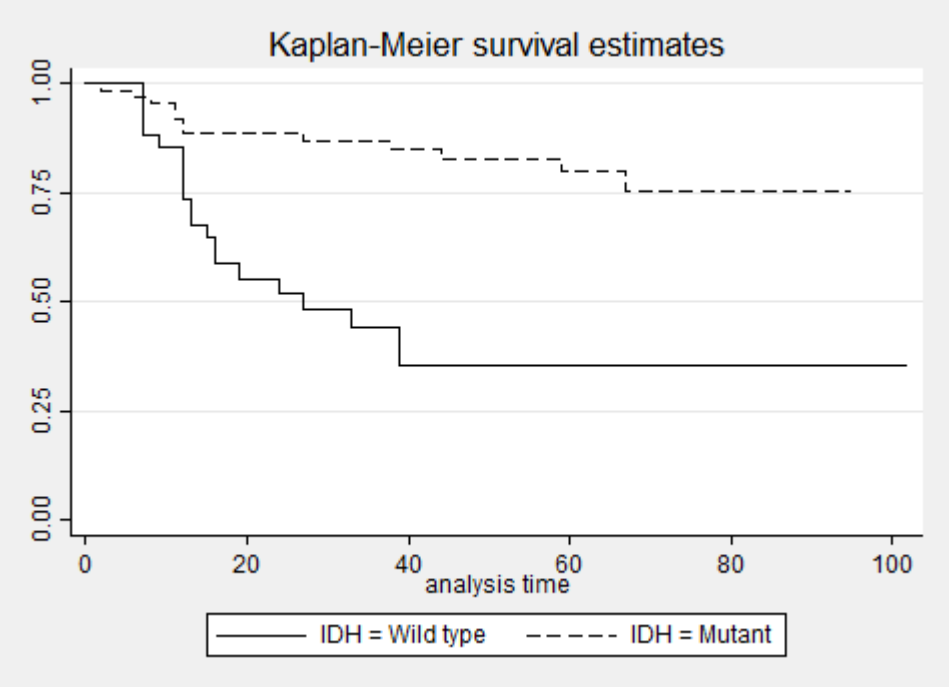

Figure 45 Overall Survival of IDH wild type and IDH mutant cases, p-value <0.000

The Hazard ratio was 4.8 for IDH wild type cases, associated with a worse overall survival, a finding which was significant with a p-value of <0.000.

Where progression free survival was concerned, the association of IDH mutation with a better progression free survival was also found to be significant with a p-value of 0.001. Twenty-three cases (57.5%)of the IDH wild type tumours recurred within the follow up period, as compared to only 25 cases (37.3%) of those with IDH mutation experiencing a recurrence. The average time to recurrence for IDH mutant cases was 67 months, in contrast to 42 months for IDH wild type cases. The Hazard ratio for IDH wild type cases to recur was 2.59, an association that was significant (p-value 0.0015).

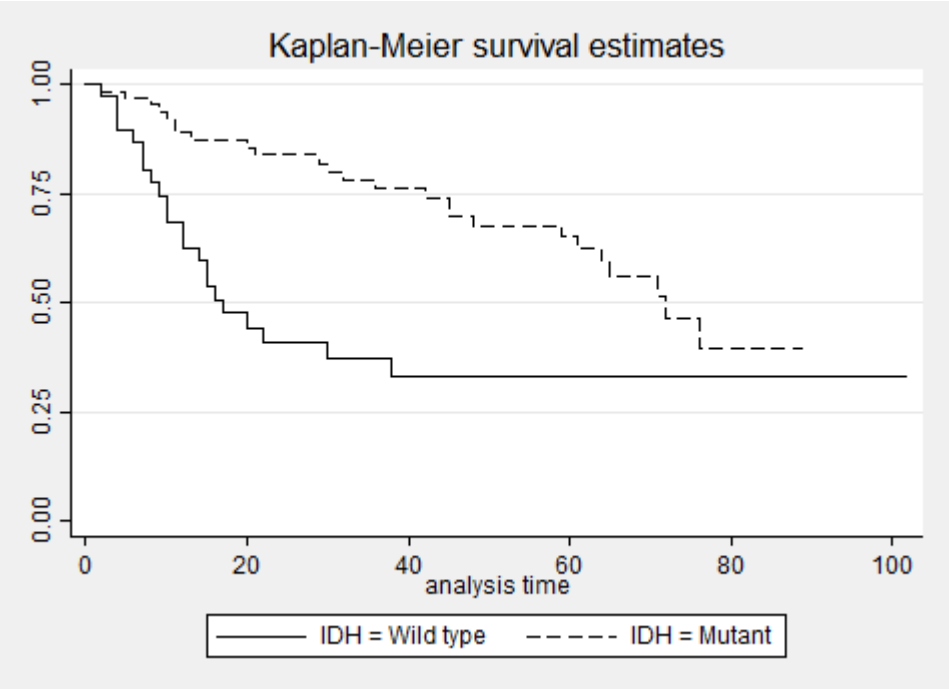

Figure 46 Progression free survival for IDH wild type and IDH mutant cases, p-value 0.001

Apart from IDH mutation, 1p/19q co-deletion was also found to be significantly associated with a better progression free and overall survival.

1p-19q co-deletion, a marker of oligodendroglial differentiation, was found to be consistently associated with better overall and progression free survival outcomes. Of those tumours with 1p19q co-deletions, 19.23% had a recurrence, with a mean time to recurrence of 71 months, and 7.69% had died by the end of the follow up, with a mean time to death of 87 months. In the group that did not harbor 1p/19q co-deletions, 53.75% had a recurrence (mean time to recurrence 51 months) and 37.97% had died by the end of follow-up (mean time to death 64 months). This association was found to be significant with a p-value of 0.006 for overall survival and 0.002 for progression free survival. Thus, there was a benefit to those harbouring 1p-19q co-deletions, as well as longer time to death or recurrence.

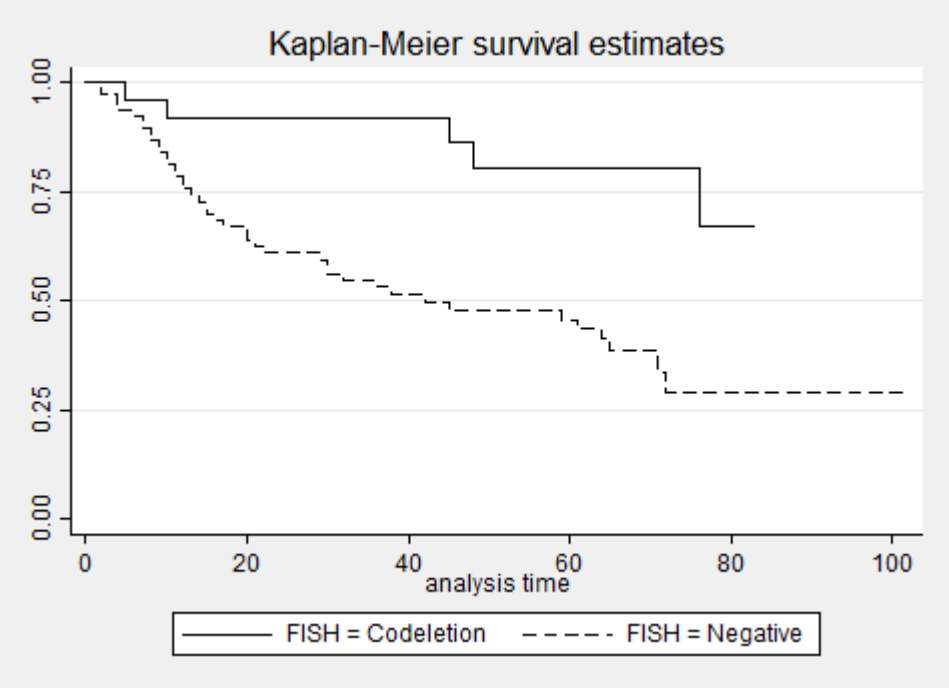

Figure 47 Progression free survival for 1p-19q co-deletion, p –value 0.002

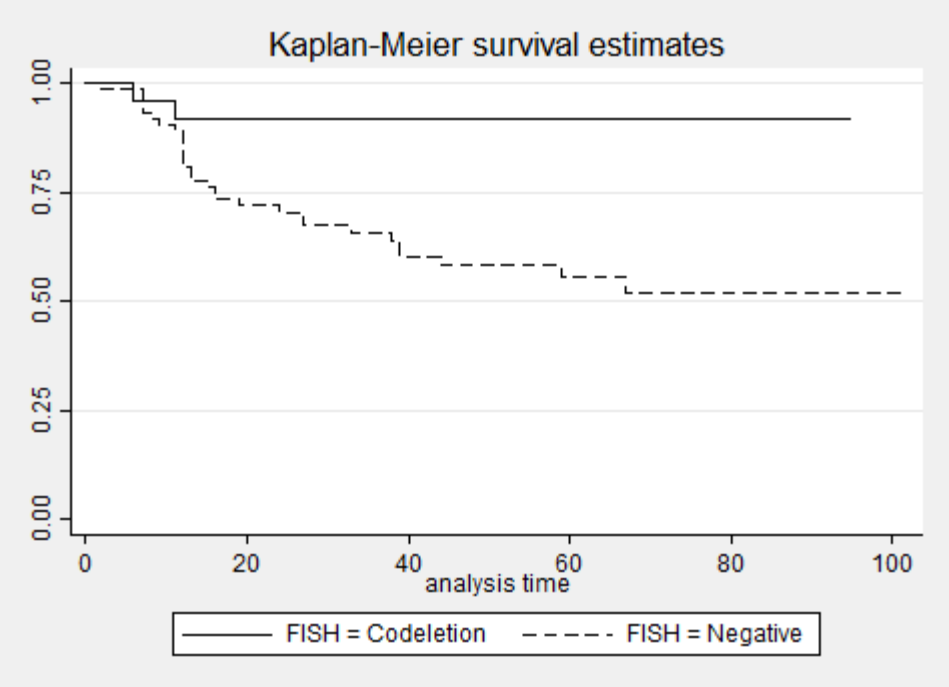

Figure 48 Overall Survival for 1p/19q co-deletion, p-value 0.006

It was found that those tumours that showed a loss of ATRX by immunohistochemistry also, had a higher chance and shorter time to recurrence. While 9.7% of those with retained ATRX had died by the end of the follow up, 20% of those with ATRX loss had died at the end of the follow up, this was however not found to be statistically significant (p=0.26). However, a statistically significant association was seen between the progression free survival and ATRX mutation. Only 19.5% of those with retained ATRX and 45.7% of those with loss of ATRX had a recurrence during the follow up period. The mean time to recurrence was 82 months for ATRX retained tumours, and 58 months in those with loss of ATRX (p-value of 0.0236).

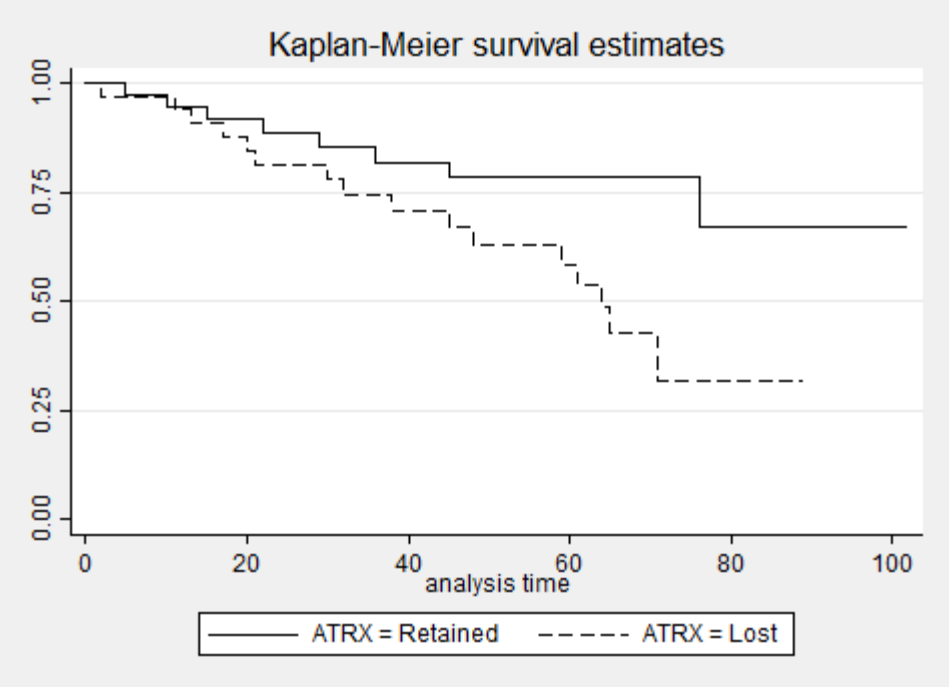

Figure 49 Progression free survival for ATRX mutations, p-value 0.024

Regarding TERT promoter mutations, the number of deaths occurring in those with TERT promoter mutations and in those without TERT promoter mutation were 30% and 30.3% respectively, and there was no statistically significant association found to overall survival (p=0.96). The average time to death was 67 months for tumours with TERT promoter mutations and 72 months for tumours without TERT promoter mutations. 39.2% of those with TERT promoter mutations, and 50% of those without TERT promoter mutations had a recurrence during the follow up period, however, this association was also not statistically significant (p=0.37). The average time to recurrence was 55 months for those tumours without TERT promoter mutations and 54 months for tumours with TERT promoter mutations.

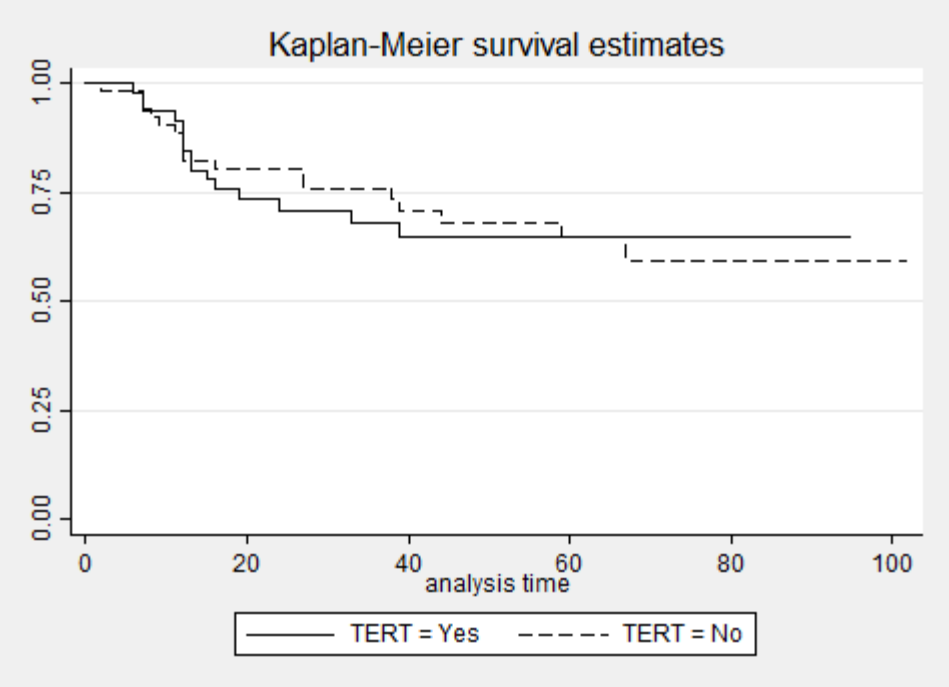

Figure 50 Overall survival for TERT promoter mutations, p value 0.96

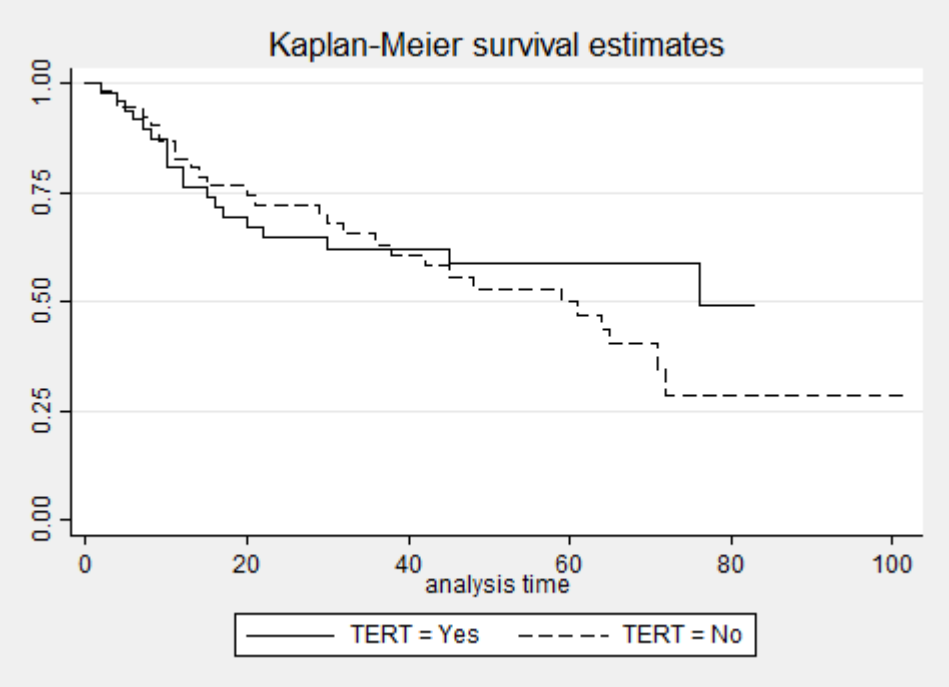

Figure 51 Progression free survival for TERT promoter mutations, p value 0.28

Survival with Molecular Sub-groups

In accordance with recent studies where gliomas were sub divided into molecular sub groups based on their mutational status for IDH, TERT promoter and 1p/19q co-deletion, the gliomas in this study were further divided into 5 molecular subgroups, namely

1. Triple positive –: Carrying IDH mutation, TERT promoter mutation and 1p/19q co-deletions
2. IDH + TERT –: Carrying IDH and TERT promoter mutations, negative for 1p/19q co-deletion
3. IDH only –: Carrying IDH mutation, negative for TERT promoter mutation and no 1p/19q co-deletion.
4. TERT only –: Carrying TERT promoter mutation, negative for IDH mutation and no 1p/19q co-deletion
5. Triple negative –: Negative for IDH, TERT promoter mutations and 1p/19q co-deletion

The number of cases in each of these molecular subgroups was assessed and the results are shown in Fig 52.

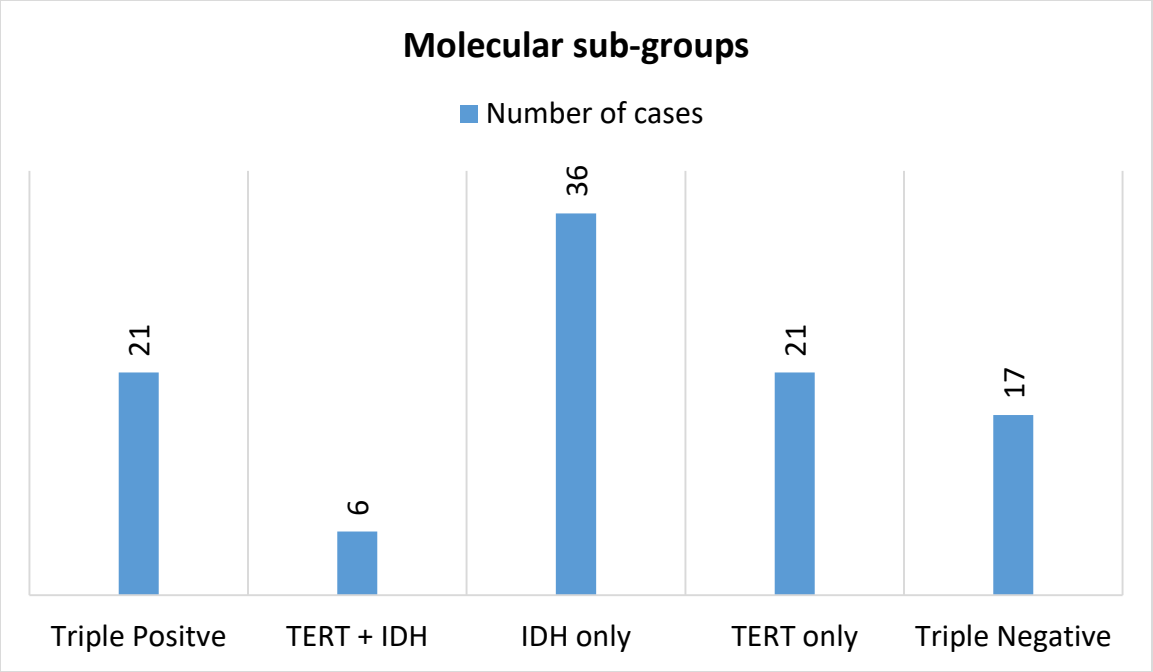

Figure 52 Molecular sub groups

Each of these molecular sub-groups were spread over the different diagnostic groups in a characteristic manner. As can be seen, the majority of 'IDH only' and 'IDH + TERT' sub-groups were seen in Diffuse or Anaplastic Astrocytomas. Triple positive gliomas were exclusively seen in Oligodendrogliomas and Anaplastic Oligodendrogliomas. 'TERT only' tumours were predominantly seen in Glioblastomas. (Fig 53)

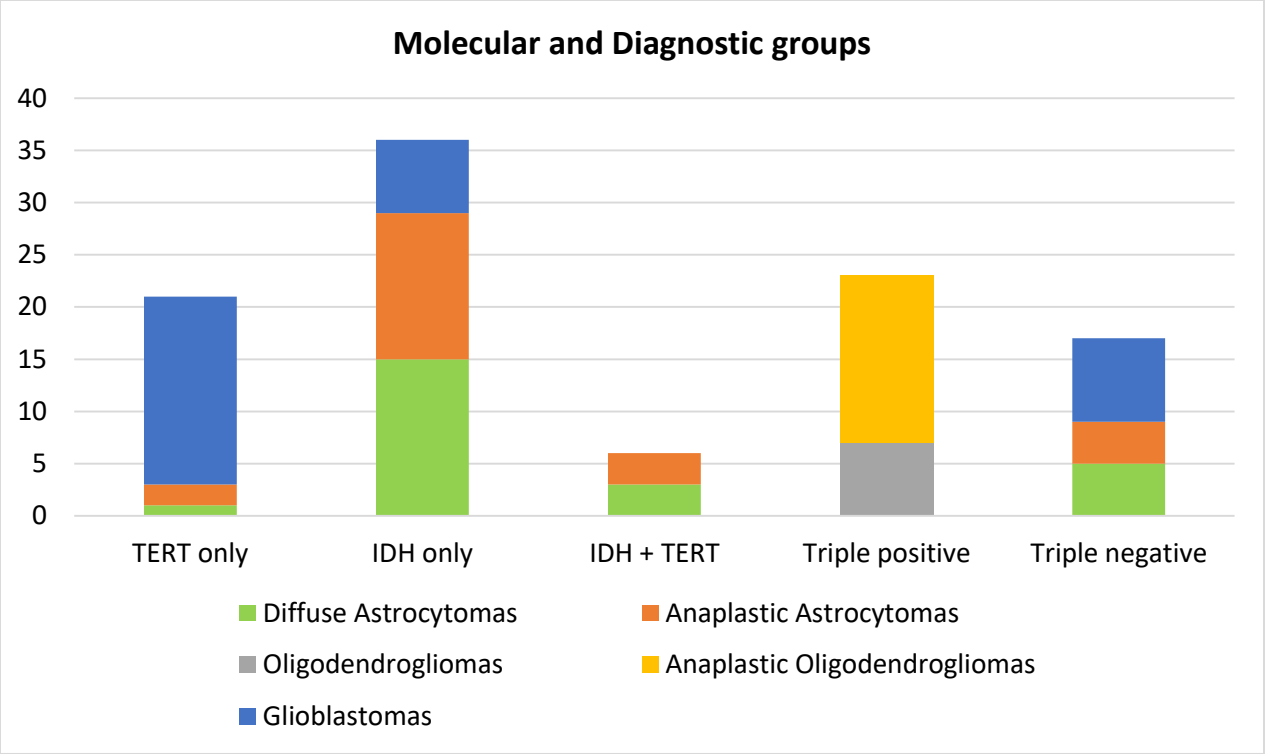

Figure 53 Distribution of Molecular sub-groups over diagnostic groups

Kaplan-Meier survival curves were drawn for each of these molecular sub-groups. However, the sub-groups 'TERT + IDH', contained only 6 cases in this study group. Consequently, addition of this sub-group did not yield significant results, the cases being too few for comparison. Hence, the sub-group 'TERT + IDH' was removed from the analysis.

The remaining four sub-groups were seen to have a significant association to the survival outcomes.

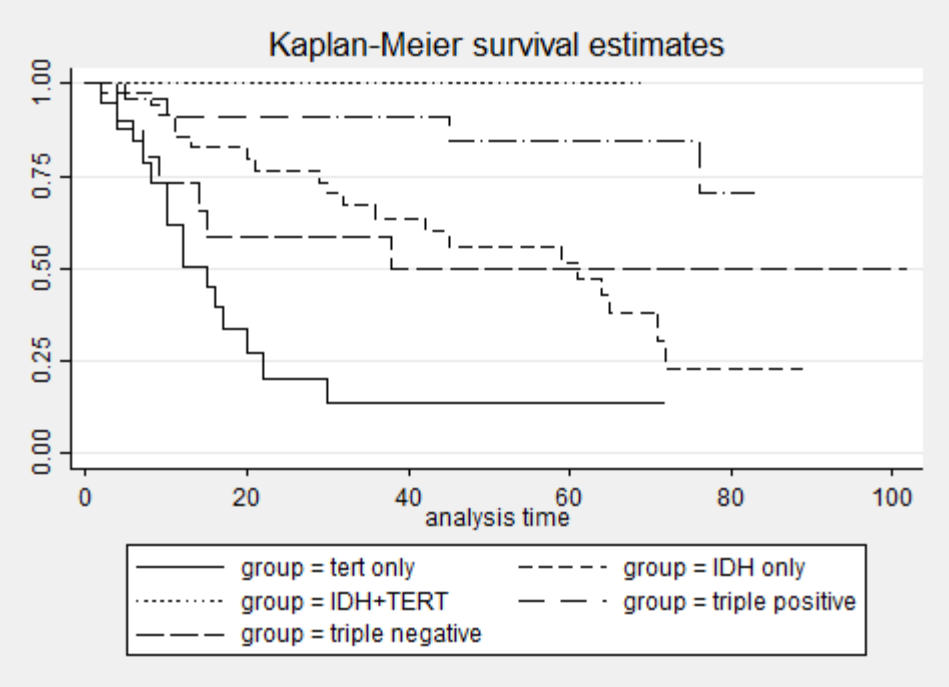

Figure 54 Progression free survival of Molecular Sub-groups, p-value<0.000

The best survival, after exclusion of the 'IDH + TERT' subgroup, was seen in the Triple Positive sub-group. The 'TERT only' sub-group had the worst survival outcomes by far. When considering the Triple negative and 'IDH only' sub-groups, it was seen that the triple negative sub-group had a better progression free survival (p-value <0.000) but a worse overall survival (p-value <0.000) when compared to the IDH only sub-group.

The data indicates that the 'TERT only' sub-group had a 3.7 higher hazard for recurrence and a 2.8 higher hazard for death as compared to the triple negative sub-group.

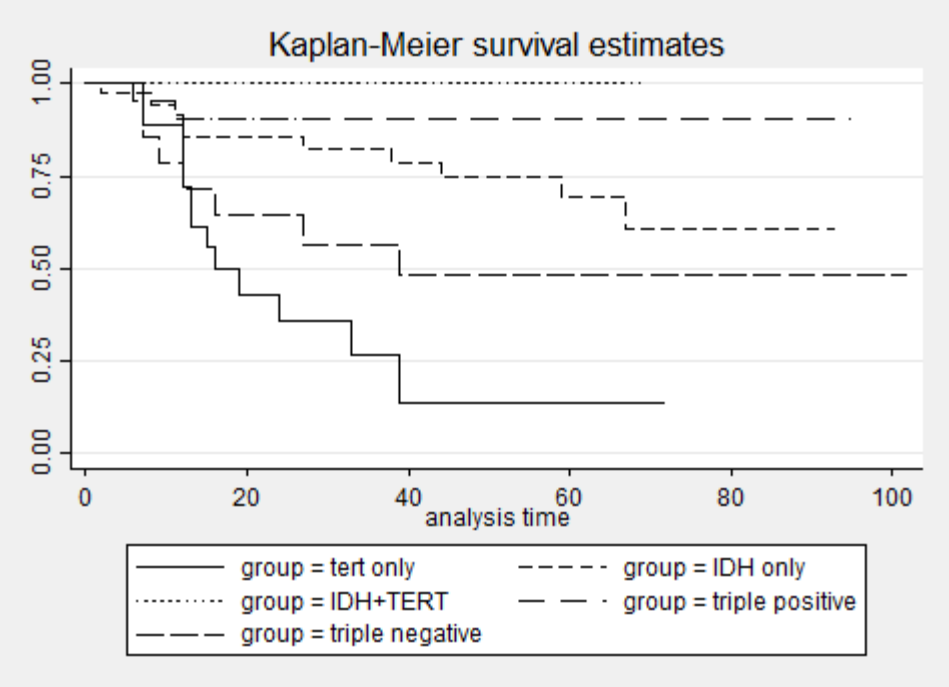

Figure 55 Overall survival of molecular sub-groups, p-value <0.000

DISCUSSION

Gliomas encompass many different tumours of varying grades with distinct pathways of oncogenesis and molecular drivers. Previously diffuse gliomas were classified by histological characteristics into tumours of astrocytic, oligodendroglial and mixed morphologies. These tumours were further then graded according to the degree of atypia, anaplasia, microvascular proliferation, mitoses and necrosis into WHO grades II-IV.(75) As molecular studies shed light on several mutations associated with these gliomas, it emerged that these molecular markers were closely associated with both the lineage from which these tumours were derived, as well as their prognosis. (105)

The 2016 WHO classification of CNS tumours shifted the classification of gliomas from a histological classification to one based on histology and molecular alterations. Significantly, IDH mutations, ATRX mutations and 1p/19q co-deletions became molecular alterations that were entity defining. IDH mutations and 1p/19q co-deletions became a requirement to make a diagnosis of an Oligodendroglioma. The loss of ATRX was indicative of an astrocytic lineage. IDH mutations were also used to classify glioma subtypes further into distinct entities with differing prognoses. The diagnosis of gliomas has now culminated in an algorithmic approach to glioma classification based on mutational studies.(3)

Telomerase reverse transcriptase (TERT) promoter mutation which occurred at a high frequency in gliomas was also seen to be associated with a prognosis, although not used to classify tumours per se.(106) With all of these molecular alterations being defined for

gliomas, it is of great clinical importance that these molecular markers give us greater clarity in understanding the survival and treatment outcomes of patients. As the paradigm has shifted to defining gliomas molecularly, so too in our study, we have attempted to study gliomas by reclassifying them by their molecular alterations.(107) A total of 107 cases of gliomas were included in this study, including tumours of astrocytic lineage, oligodendroglial lineage and Glioblastomas. We classified them both histologically and molecularly, looking for differences between the approaches. Further, we studied the individual association of each of these mutations with survival. We then reclassified all the gliomas in our study into molecular sub-groups to understand if this would allow us to better define glioma groups associated with prognosis.

Age

	Current Study	WHO Classification of CNS Tumours
Diffuse Astrocytomas	35 years	30-40 years
Anaplastic Astrocytomas	31 years	45 years
Oligodendrogliomas	34 years	35-45 years
Anaplastic Oligodendrogliomas	37 years	45-50 years
Glioblastomas	47 years	45-70 years

Table 7 Mean Age of Gliomas in Study Group and 2016 WHO Classification of CNS Tumours

The median age at diagnosis in this study for all tumours was 36 years, with the average age for each type of tumour as shown in Table 6.

The average age at presentation for the gliomas in our study were similar to that reported in the 2016 update of the WHO classification of CNS tumours, with the exception of Anaplastic Astrocytomas which showed a lower age at presentation.(75)

Gender

Across all the glioma types in this study there was a slight male preponderance, with 65% of cases occurring in men and 35% in women. This is a trend that is seen in most diffuse gliomas of WHO grades II through IV.(105)

Site and laterality

Gliomas usually occur preferentially in the cerebral hemispheres, more commonly in the frontal lobe, followed by the temporal and parietal lobes, with the occipital lobe being a rare site of presentation. The anatomical distribution does not appear to vary with respect to the histological diagnosis, being similar in gliomas of WHO grades II-IV.(108) In this study as well, the majority of tumours occurred in the frontal lobe (51%), followed by the temporal lobe (15%), parietal lobe (14%), occipital lobe (6%) and the cerebellum (1%).

Gliomas have not been shown to have a significant laterality in studies done thus far.(109) In our study, tumours occurred only slightly more frequently on the right side (57%) than the left side.

Size

The predominant size for most gliomas in this study, was between 2 and 5 centimetres (51%), closely followed by tumour size of >5cm (46%). Gliomas of less than 2cm were rare. Similar results are seen in other studies with gliomas usually ranging from 2.3 cm to 4.7 cm in size.(108)

Clinical Presentation

The most frequent presenting symptom in our study was seizures (76%) followed by symptoms of raised intracranial tension (39%) and/or focal neurological deficits (35%). Previous studies on the presenting symptoms of gliomas, concurred with the presentation as seizures and focal neurological deficits as common presenting symptoms. However, signs of raised intracranial tension have not been reported to be as common as that found in our study.(110)

Radiology

Gliomas of all grades are found to predominantly be hypointense on T1 weighted imaging, and hyperintense on T2 weighted imaging by MRI scans. The margins of both

lower and higher grade gliomas can be clearly demarcated or indistinct. However, the proportion of cased with indistinct margins increases with tumour grade, as does the presence of heterogeneity.(111) In keeping with this, 93% of tumours were T1 hypointense and T2 hyperintense, across all glioma subtypes. 73% of tumours had indistinct margins, of which more than half were found to be Glioblastomas. Given that most of the tumours dealt with in this study are WHO grade II and above, it stands to reason that the margins were indistinct/diffuse in a larger percentage of cases.

Histology

Morphology was assessed on haematoxylin and eosin sections and 30% of the tumours showed an astrocytic morphology and 34% had a characteristic oligodendroglial morphology. The remaining 36% of cases had a mixed oligodendroglial and astrocytic morphology, and were designated oligoastrocytomas according to the WHO 2007 classification. Recent studies following the WHO 2016 classification, which discourages the diagnosis of oligoastrocytomas, have shown that these tumours can be readily classified as astrocytic or oligodendroglial on the basis of molecular markers. In a study by Sahm et al, 43 cases of oligoastrocytomas were reclassified on a molecular basis. Of these 43 tumours, 31 were reclassified as Oligodendrogliomas and 12 as Astrocytomas.(112) In our study, we had 9 cases of histological Oligoastrocytomas and 13 cases of Anaplastic Oligoastrocytomas. On further molecular typing, the majority of these tumours were classified with an astrocytic genotype as depicted below.

Histological Diagnosis	Molecular reclassification
9 Oligoastrocytomas	9 Diffuse Astrocytomas
13 Anaplastic Astrocytomas	12 Anaplastic Astrocytomas
	1 Anaplastic Oligodendrogliomas

Table 8 Re-categorization of Oligoastrocytic tumours after Molecular work-up

Interestingly, following molecular categorisation, one oligodendroglial tumour was also reclassified as an astrocytic neoplasm based purely on the loss of ATRX on immunohistochemistry. The fact that most tumours with a mixed morphology were molecularly astrocytic, suggests that astrocytic tumours present with more histological heterogeneity.

Adjuvant therapy

50% of the cases in our study received radiotherapy with concurrent chemotherapy and 6 cycles adjuvant chemotherapy, 23% received radiotherapy alone and 22% received radiotherapy with concurrent chemotherapy alone.

In 2004 the European organisation for research and treatment of cancer (EORTC) conducted a randomised clinical trial where they broke patients into two groups, one that would receive standard radiotherapy and one that would receive radiotherapy with concomitant temozolomide chemotherapy and six cycles of adjuvant temozolomide chemotherapy. They found a survival benefit for the latter arm, both at 3 years and at five

years for tumours of WHO grades III and IV. This survival benefit was enhanced in those tumours with an MGMT promoter methylation as well.(113)

In the present study, radiotherapy alone was given predominantly in Diffuse Astrocytomas, WHO grade II. Radiotherapy with concurrent and adjuvant chemotherapy was given predominantly in Glioblastomas WHO grade IV, Anaplastic Astrocytomas and Anaplastic Oligodendrogliomas, WHO grade III. The correlation with recurrence and survival between the different types of treatment was however not statistically significant (p=0.36). The increasing number of recurrences and mortality with more aggressive treatments can be explained by the fact that the tumours that received more aggressive treatments were of a higher grade, with a poor prognosis at the outset.

IDH mutations

IDH mutations occur frequently in gliomas and are known to impart a better overall and progression free survival.(8) These mutations are seen in 80% of all diffuse gliomas of WHO grade II-IV.(114) In our study, IDH mutations were seen 62% of all tumours, with the largest proportion seen in the lower grade gliomas, i.e. WHO grades II and III. A total of 81% of WHO grade II and III tumours in the present study carried an IDH mutation, whereas only 21.2% of glioblastomas had IDH mutation. This is in keeping with studies that have shown that in Glioblastomas, IDH wild type is more frequent.(84)

In terms of survival data, a significant correlation was seen between the presence of an IDH mutation and better progression free survival (p-value 0.001) and overall survival

(p-value 0.0001). This is in keeping with studies done previously that show IDH mutations conferring a survival advantage to gliomas.(8)

ATRX mutations

ATRX mutations are markers of an astrocytic lineage.(30) Tumours that carry these mutations have been seen to have a much better prognosis in terms of survival and sensitivity to chemotherapy.(32) In keeping with the fact that ATRX loss is a marker of an astrocytic lineage, in our study we found that for those cases that carried 1p/19q co-deletion and had ATRX immunohistochemistry done, there was no loss of ATRX seen on immunohistochemistry. While the correlation to overall survival was not statistically significant in our study, there was a significant association between ATRX and progression free survival. Contrary to other studies that have shown a better survival in gliomas with an ATRX mutation,(32) the loss of ATRX on immunohistochemistry was associated with a shorter time to recurrence and lower progression free survival in the present study. Of the tumours with ATRX loss on immunohistochemistry, 45% had a recurrence, compared to 19.5% of patients with retained ATRX. The latter set of tumours had 1p/19q co-deletion. and were diagnosed as oligodendrogliomas. This would explain the longer PFS, as oligodendrogliomas are known to have a better prognosis than astrocytomas

1p/19q co-deletion

1p/19q co-deletions are now entity defining markers of oligodendroglial tumours.(75) These co-deletions also confer a better prognosis n.(39) In addition, 1p/19q co-deletion in tumours leads to a higher sensitivity to chemotherapy.(40) In concordance with previous studies we found that 1p/19q co-deletion was an independent prognostic factor for overall survival and progression free survival. Among the tumours without 1p/19q co-deletions, 53% recurred and 37% died. In tumours with 1p/19q co-deletions, only 19.2% recurred and 7.8% died. The latter group of tumours were also associated with an increased time to recurrence and death. This association was found to be statistically significant for progression free (p-value 0.002) and overall survival (p-value 0.006)

TERT promoter mutations

Studies have shown that the prevalence of TERT promoter mutations is higher in the higher grade gliomas with 75% of Glioblastomas harbouring this mutation, 53% of Anaplastic Oligodendrogliomas and 58% of Oligodendrogliomas.(97) In astrocytic tumours however, studies have shown that the prevalence is much lower, 40% in Anaplastic Astrocytomas and 7% of Diffuse Astrocytomas.(106) In our study mutations of the TERT promoter region were seen most frequently in oligodendroglial tumours, 87% of Oligodendrogliomas and 94% of Anaplastic Oligodendrogliomas. Amongst glioblastomas 54% harboured TERT promoter mutations, which is lower than that reported in literature. As with other studies the relative proportion of Astrocytomas with

TERT promoter mutations was the lowest, being 21.7% for Anaplastic Astrocytomas and 16% for Diffuse Astrocytomas

Although studies have shown that the TERT promoter mutation is an independent factor predicting a worse prognosis and survival(103), there was no statistical correlation seen in our study between TERT promoter mutations and survival.

Molecular subgroups

Similar to previous studies that attempted to stratify gliomas on the basis of molecular alterations into prognostic sub-groups we attempted to do the same in the present study. (REF) In 2015 a study by Eckel-passow et al brought to light glioma groups based on the mutational statuses of IDH, 1p/19q and the TERT promoter as a way to prognosticate.(107) We categorised all the study cases into one of five categories, similar to that done by Eckel-Passow et al :

1. Triple positive – Carrying IDH mutation, TERT promoter mutation and 1p/19q co-deletions
2. IDH + TERT – Carrying IDH and TERT promoter mutations, negative for 1p/19q co-deletion
3. IDH only – Carrying IDH mutation, negative for TERT promoter mutation and 1p/19q co0deletion.

4. TERT only – Carrying TERT promoter mutation, negative for IDH mutation and 1p/19q co-deletion
5. Triple negative – Negative for IDH, TERT promoter mutations and 1p/19q co-deletion

When divided into these subgroups, the previous study showed that tumours with IDH and TERT promoter mutations had the best survival, followed by triple positive tumour, tumours with IDH mutation only and Triple negative tumours. TERT promoter mutations when occurring by themselves, had the worst survival of all of the subgroups.(107)

In our study, the subgroup carrying IDH and TERT promoter mutations could not be analysed for correlation to survival as the numbers were small. In the remaining 4 subgroups, however, we found similar results. TERT promoter mutations when occurring alone had a significantly worse survival. The best prognosis by far was seen in the triple positive tumours, followed by IDH only and triple negative tumours. This association was statistically significant with a p-value of 0.00001.

The 2016 update of the WHO classification of CNS tumours alluded to TERT promoter mutations when discussing the genetic profile of gliomas. However, this was not incorporated into their algorithm for diagnosis as it is primarily a prognostic marker. WHO retained histology as the method for grading of gliomas. However, there have been observations regarding the prognostic similarity based on molecular profile despite differences in grade. The prime example being that of WHO grade II IDH mutant diffuse astrocytomas and WHO grade III IDH mutant anaplastic astrocytomas having similar

outcomes than their wild type counterparts which have a prognosis similar to glioblastoma. Given the fact that sub-classification using these molecular markers separates tumours into prognostically relevant categories, assessment of TERT promoter mutations together with assessment of IDH mutation and 1p/19q co-deletion may well be a step in the right direction as we move forward in this era of personalized medicine.

LIMITATIONS

1. For those tumours in which IDH-1 immunohistochemistry is negative, the recommended procedure is to perform IDH-1 and IDH-2 PCR to look for rarer mutations that are missed by immunohistochemistry. However, due to economic constraints, we were unable to confirm the absence of IDH mutation by PCR.
2. Due to the small number of cases with IDH and TERT promoter mutations in the study arm, these tumours could not be included in the analysis on survival.
3. Several cases did not have follow up information beyond a few years post surgery. If the lack of follow up is due to an adverse event, our data may show more biased and falsely favourable outcomes.
4. Several cases were excluded from the study due to missing paraffin blocks or inadequate frozen tissue.

CONCLUSION

- Gliomas had a mean age at diagnosis of 36 years.
- There was a slight male preponderance across all glioma groups.
- The most common site of occurrence was the frontal lobe, followed by the temporal lobe.
- The occipital lobe and cerebellum were rarely affected by diffuse gliomas
- Most gliomas were between 2cm and 5cm in size
- The most common clinical presentations in gliomas was seizures, followed by symptoms and signs of raised intracranial tension and focal neurological deficits.
- IDH mutations were seen in 62% of diffuse gliomas, and their presence was significantly associated with a better progression free and overall survival
- 1p/19q co-deletions were significantly associated with better overall and progression free survival.
- TERT promoter mutations were seen in 47.6% of diffuse gliomas, especially those of a higher grade
- Molecular subgrouping based on 1p/19q co-deletion, IDH mutation and TERT promoter mutation, showed a good stratification of patients in terms of overall and progression free survival.
- Triple positive tumours carried the best prognosis, followed by tumours with IDH mutations only.
- Tumours with TERT promoter mutations in isolation had the worst prognosis.

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APPENDIX

APPENDIX 1 Protocol for automated immunostaining

1. Paraffin embedded tissue sections were cut at 2-3µ thickness and floated in poly L lysine coated slides and baked at 60 degrees for 10-15 minutes.

2. These slides were then treated with 15% milk solution for 7 minutes to eliminate the hydrophobic effect and give positive charge to the slides.

3. Slide labels were bar coded and the labelled slides were loaded in Ventana Benchmark XT autostainer (a fully automated autostainer).

4. Individual protocols devised in the software attached to the machine for each marker.

5. Steps included in the protocol were as follows.
    a) Deparaffinisation
    b) Liquid coverslip application
    c) Heat induced antigen retrieval by treating with standard CC1 solution (pH patent for the company) for one hour at 90 degrees Celsius.

6. The primary antibody was added and incubated for 40 min at 37 degrees Celsius. (IDH-1 at 1:30 dilution, ATRX at 1:300 dilution)

7. The secondary antibody was added and incubated for 8 minutes.

8. Slides were counterstained with hematoxylin and incubated for 8 min, followed by incubation with bluing agent for 4 min.

APPENDIX 2 Extraction of DNA

1. 20 microlitre of proteinase and 100 microlitres of a digestion buffer (ATL) were added into an Eppendorf tube.
2. 30 nanograms of fresh frozen tumour tissue was added to a UV radiated mortar and pestle, along with 80 microlitres of digestion buffer, and ground finely.
3. The tissue was added to the proteinase in the Eppendorf tube.
4. Overnight water bath at 56 degrees Celsius.
5. 200 microlitres of isolation additive (AL buffer) is added and the mixture kept in a dry bath at 70 degrees for 10 minutes.
6. 200 microlitres of ethanol is added and centrifuged at 8000 rpm for 1 minute in a spin column
7. 500 microlitres of wash buffer 1 is added (AW1) and centrifuged at 8000rpm for 1 min.
8. 500 microlitres of wash buffer 2 (AW2) is added and centrifuged at 14,000 rpm for 3 minutes.
9. The resultant product is centrifuged again at 10,000 rpm for 1 minute.
10. The spin column is transferred to an Eppendorf tube and 80 microlitres of an elution buffer is added.
11. The elution buffer is allowed to stand for 1 minute and then centrifuged at 12,000rpm for 1 minute.
12. The quantity and quality of the extracted DNA is checked using a Nano-drop.

APPENDIX 3 PCR Assay for TERT Promoter mutation

PCR for TERT promoter mutation was done using the following reagents:

1. Platinum PCR SuperMix, High Fidelity, Invitrogen – Containing dNTPs, Buffer, Taq DNA polymerase and water – 22.5 microlitres
2. Forward Primer – 2 microlitres
3. Reverse Primer – 2 microlitres

Primers

Primers	**Sequence 5'-3'**
TERT F	**ACGAACGTGGCCAGCGGCAG**
TERT R	**CTGGCGTCCCTGCACCCTGG**

APPENDIX 4 – DNA Sequencing

Pre-cleanup: ExoSap-IT (USB, cat. No.78202)
2µl of Exo-Sap + 5 µl of PCR product mix by pipetting
Incubate in a thermal cycler
37^0C for 15 min
87^0C for 15min
15^0C

Sequencing reaction: Sanger's PCR mix is prepared using BigDye Terminator V1.1 (or) V3.1 Ready Reaction mix. This contains the ddNTPs, Taq with proof reading activity and all the additives required for a PCR.

Prepare 1pmole/uL primer from 10pmole primers.

	X1	x n+ 0.5
5 X Sequencing Buffer	1.0 µl	
BDT V.1.1 RR	0.5 µl	
H2O	6.0 µl	

1. Aliquot the pre-mix to all tubes (7.5ul) and add 1.6 ul of Primer (1pmole/ul) to appropriate tubes and 1-1.5 uL eluted PCR product
2. After adding the mix, primer and the sample, close the tubes. Mix by spin, vortex and spin again.
3. Thermal cycling with the BigDye sequencing program.

Post-cleanup:

1. Transfer the sequencing reaction products (10µL) to the wells of 96 well plate dedicated for cleanup.
2. Mix the HighPrep DTR beads by vortexing to fully re-suspend the particles.
3. Add 10µL of Highprep DTR beads and 40µL of 85% ethanol to each well.
4. Mix well by pipetting up and down around 10-12 times.
5. Keep the plate on the magnetic stand for 4-5 min or until the solution clears. This will attract the beads+DNA complex as a pellet to one side of the well as per the direction of magnet
6. With the plate still on magnet, discard the supernatant without disturbing the pellet.
7. Add 100µL of 85% ethanol to each well (Do not mix), wait for 1-2 minutes, and discard it.
8. Repeat the ethanol wash again to get total of 2 washes.
9. Ensure the ethanol traces are removed completely and dry the beads by incubating for 10 minutes at RT with the plate still on magnet.
10. After drying, add 40µL of elution buffer (0.1mM EDTA or Di-H_2O) to each well and remove the plate from magnet for elution.
11. Mix the beads in elution buffer by pipetting 20-25 times.
12. Incubate at room temperature for 5 minutes (outside the magnet)

13. Keep the plate again on to the magnetic stand for 5 minutes.
14. Now the clear supernatant contains the purified DNA. It can be directly transferred to the sequencing machine's loading plate (96 well plate dedicated for loading).

APPENDIX 5 Proforma

Serial number:

Biopsy number

Hospital number

Age

Gender

RADIOLOGY:

Site of Tumour

1. Frontal
2. Parietal
3. Temporal&insula
4. Occipital
5. Infratentorial - cerebellar
6. Brainstem
7. Spinal cord

Laterality: 1. Right2. Left 3. Bilateral

Size: 1 - < =1cm, 2 – 1 to 2cm, 3 – 2 to 5cm, 4 - >5cm.

T1w : 1 – hypointense; 2 – hyperintense; 3 – isointense; 4 – heterogenous

T2w : 1 – hypointense; 2 – hyperintense; 3 – isointense; 4 –heterogenous

Borders : 1 – distinct ; 2 -indistinct.

CLINICAL PRESENTATION :

Seizures1+ (Yes) 2- (No)

Focal neurological deficits 1+ (Yes) 2- (No)

Features of raised intracranial features 1+ (Yes) 2- (No)

DURATION OF SYMPTOMS

1. Less than/ equal to 1 month
2. 1-6 months
3. 6 months to one year.
4. 1 to 2 years
5. More than 2 years.

Primary/Recurrent tumours:

HISTOLOGICAL DIAGNOSIS

1. Diffuse fibrillary astrocytoma
2. Anaplastic astrocytoma
3. Oligodendrogliomas
4. Anaplastic oligodendroglioma
5. Oligoastrocytoma
6. Anaplastic oligoastrocytoma
7. Glioblastoma multiforme

MIB-1 labelling index:

Histology: 1 – Astrocytic, 2 – Oligodendroglial 3- Mixed

Necrosis: 0 – Absent, 1 – Present

Microvascular proliferation: 0 – Absent, 1 – Present.

MOLECULAR PARAMETERS:

FISH FOR 1P/19Q DELETION:

1-Positive 2-Negative 3- Polysomy 4- Not done

ATRX IHC

1-Retained 2- Negative 3 – Not done

IDH1 IHC (paraffin blocks)

1- (positive) 2- (Negative)

TERT promoter mutation

1-positive 2-Negative

If positive: 1- C250T 2-C228T

S**URGERY**

1. Radical excision
2. Partial excision
3. Biopsy

ADJUVANT THERAPY

1. Radiotherapy only
2. Radiotherapy with concurrent chemotherapy
3. Radiotherapy with adjuvant chemotherapy
4. Unknown
5. Not given

SURVIVAL

Date of surgery

Date of last follow up:

Date of last adjuvant therapy/duration:

Duration of follow up (months):

Recurrence: 1- (Yes) 2- (No)

Progression free survival (months):

Death 1- (Yes) 2- (No)

Overall survival (months):

APPENDIX 6 Institutional review board approval

OFFICE OF RESEARCH
INSTITUTIONAL REVIEW BOARD (IRB)
CHRISTIAN MEDICAL COLLEGE, VELLORE, INDIA

Dr. B.J. Prashantham, M.A., M.A., Dr. Min (Clinical)
Director, Christian Counseling Center,
Chairperson, Ethics Committee.

Dr. Alfred Job Daniel, D Ortho MS Ortho DNB Ortho.
Chairperson, Research Committee & Principal

Dr. Biju George, MBBS., MD., DM
Deputy Chairperson,
Secretary, Ethics Committee, IRB
Additional Vice-Principal (Research)

February 29, 2016

Dr. Shailaja Balakumar ,
PG Registrar,
Department of General Pathology,
Christian Medical College,
Vellore 632 004.

Sub: **Fluid Research grant project NEW PROPOSAL:**
TERT promoter mutations in a cohort of gliomas – clinico-pathological correlates Shailaja Balakumar (Employment Number: 29384), General Pathology, Dr Geeta Chacko, Employment number: 11713 , General Pathology, Dr Rekha Pai, Emp No: 30487, Molecular Pathology, Ms. Ramya Lakshmi, Emp No: 53306, Molecular pathology, Dr Bimal Patel, Emp No: 33508, General Pathology,Dr Ari Chacko, Emp No: 11790, Neurological Sciences

Ref: IRB Min No: 9778 [OBSERVE] dated 03.12.2015

Dear Dr. Shailaja Balakumar,

I enclose the following documents:-

1. Institutional Review Board approval 2. Agreement

Could you please sign the agreement and send it to Dr. Biju George, Addl. Vice Principal (Research), so that the grant money can be released.

With best wishes,

Dr. Biju George
Secretary (Ethics Committee)
Institutional Review Board.

Dr. BIJU GEORGE
MBBS., MD., DM.
SECRETARY - (ETHICS COMMITTEE)
Institutional Review Board,
Christian Medical College, Vellore - 632 002.

Cc: Dr Geeta Chacko, Dept. of General Pathology, CMC

Ethics Committee Blue, Office of Research, 1st Floor, Carman Block, Christian Medical College, Vellore, Tamil Nadu 632 002
Tel: 0416 – 2284294, 2284202 Fax: 0416 – 2262788, 2284481 E-mail: research@cmcvellore.ac.in

OFFICE OF RESEARCH
INSTITUTIONAL REVIEW BOARD (IRB)
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Ref: IRB Min No: 9778 [OBSERVE] dated 03.12.2015

Dear Dr. Shailaja Balakumar ,

The Institutional Review Board (Blue, Research and Ethics Committee) of the Christian Medical College, Vellore, reviewed and discussed your project titled "TERT promoter mutations in a cohort of gliomas – clinico-pathological correlates" on December 03rd 2015.

The Committee reviewed the following documents:

1. IRB Application format
2. Informed Consent Form
3. Cvs of Drs. Ari Chacko, Bimal Patel, Shailaja Balakumar , Geeta Chacko, Ms. Ramya Lakshmi, Rekha Pai
4. No. of documents 1 - 4

The following Institutional Review Board (Blue, Research & Ethics Committee) members were present at the meeting held on December 03rd 2015 in the CREST/SACN Conference Room, Christian Medical College, Bagayam, Vellore 632002.

Ethics Committee Blue, Office of Research, 1st Floor, Carman Block, Christian Medical College, Vellore, Tamil Nadu 632 002
Tel: 0416 – 2284294, 2284202 Fax: 0416 – 2262788, 2284481 E-mail: research@cmcvellore.ac.in

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Chairperson, Ethics Committee.

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Chairperson, Research Committee & Principal

Dr. Biju George, MBBS., MD., DM
Deputy Chairperson,
Secretary, Ethics Committee, IRB
Additional Vice-Principal (Research)

Name	Qualification	Designation	Affiliation
Dr. Nihal Thomas	MD, MNAMS, DNB(Endo), FRACP (Endo) FRCP(Edin) FRCP (Glasg)	Professor & Head, Endocrinology. CMC, Vellore	Internal, Clinician
Dr. RV. Shaji		Professor, Heamatology, CMC, Vellore	Internal, Basic Medical Scientist
Dr. Mathew Joseph	MBBS, MCH	Professor, Neurosurgery, CMC, Vellore	Internal, Clinician
Dr. Ranjith K Moorthy	MBBS, MCh	Professor, Neurological Sciences, CMC, Vellore	Internal, Clinician
Dr. Balamugesh	MBBS, MD(Int Med), DM, FCCP (USA)	Professor, Pulmonary Medicine, CMC, Vellore	Internal, Clinician
Dr. Visalakshi. J	MPH, PhD	Lecturer, Biostatistics, CMC, Vellore	Internal, Statistician
Dr. Rajesh Kannangai	MD, PhD.	Professor, Clinical Virology, CMC, Vellore	Internal, Clinician
Dr. Niranjan Thomas	DCH, MD, DNB (Paediatrics)	Professor, Neonatology, CMC, Vellore	Internal, Clinician
Mrs. Pattabiraman	BSc, DSSA	Social Worker, Vellore	External, Lay Person
Dr. B. J. Prashantham	MA(Counseling Psychology), MA (Theology), Dr. Min(Clinical Counselling)	Chairperson, Ethics Comm IRB. Director, Christian Counseling Centre, Vellore	External, Social Scientist
Mr. Samuel Abraham	MA, PGDBA, PGDPM, M. Phil, BL.	Sr. Legal Officer, CMC, Vellore	Internal, Legal Expert
Dr. Jayaprakash Muliyil	BSc, MBBS, MD, MPH, Dr PH (Epid), DMHC	Retired Professor, CMC, Vellore	External, Scientist &Epidem
Mrs. Emily Daniel	MSc Nursing	Professor, Medical Surgica Nursing, CMC, Vellore	Internal, Nurse

IRB Min No: 9778 [OBSERVE] dated 03.12.2015

Ethics Committee Blue, Office of Research, 1st Floor, Carman Block, Christian Medical College, Vellore, Tamil Nadu 632 002
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Chairperson, Research Committee & Principal

Dr. Biju George, MBBS., MD., DM
Deputy Chairperson,
Secretary, Ethics Committee, IRB
Additional Vice-Principal (Research)

Mrs. Sheela Durai	MSc Nursing	Professor, Medical Surgical Nursing, CMC, Vellore	Internal, Nurse
Mr. C. Sampath	BSc, BL	Advocate, Vellore	External, Legal Expert
Dr. Anuradha Rose	MBBS, MD, MHSC (Bioethics)	Associate Professor, Community Health, CMC, Vellore	Internal, Clinician

We approve the project to be conducted as presented.

Kindly provide the total number of patients enrolled in your study and the total number of withdrawals for the study entitled: "TERT promoter mutations in a cohort of gliomas – clinico-pathological correlates " on a monthly basis. Please send copies of this to the Research Office (research@cmcvellore.ac.in)

Fluid Grant Allocation:

A sum of 1,47,000/- INR (Rupees One Lakh Forty Seven Thousand Only) will be granted for 3 years. 50,000/- INR (Rupees Fifty Thousand only) will be granted for 12 months as an Ist Installment. The rest of the amount will be released at the end of the first year as 2 nd Installment

Yours sincerely

Dr. Biju George
Secretary (Ethics Committee)
Institutional Review Board

Dr. BIJU GEORGE
MBBS., MD., DM.
SECRETARY - (ETHICS COMMITTEE)
Institutional Review Board,
Christian Medical College, Vellore - 632 002.

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APPENDIX 6 Thesis Data

SNo	BNO	HNO	Age	Sex	Unit	SITE	Side	Size	T1w	T2w	Borders	Seizures	FND	ICT	Duration	Sx	Primary	HISDIAG	WHO	HISTO	Nec	Microvasc
1	1759/09	386966D	25	2	NS1	6	2	3	1	2	2	2	1	1	2	1	1	1	1	1	0	0
2	2849/09	351542D	27	1	NS1	1	1	2	1	2	2	1	2	2	1	1	1	1	1	1	0	0
3	5002/09	388696D	25	1	NS1	1	2	4	1	2	2	1	2	2	2	2	1	2	2	1	0	0
4	9477/09	418066D	53	2	NS1	1	1	3	3	2	2	1	2	1	5	1	1	8	3	2	1	1
5	10331/09	417808	51	1	NS1	1	2	3	1	2	1	1	1	2	2	1	1	7	3	1	1	1
6	12592/09	441099D	32	2	NS1	1	2	3	1	2	2	2	2	1	1	1	1	8	3	3	1	1
7	13458/09	569739B	34	1	NS1	1	1	3	1	2	2	2	1	2	1	2	2	8	3	3	1	1
8	19257/09	475843D	48	2	NS1	2	1	3			2	2	1	1	2	1	1	7	3	1	1	1
9	22769/09	494844D	32	1	NS1	5	2	3	1	2	2	1	2	2	2	1	1	7	3	1	1	1
10	24306/09	500109D	32	2	NS1	1	2	4	1	2	2	1	2	2	1	1	1	2	2	1	0	0
11	24518/09	503236D	31	1	NS1	1	2	3	1	2	1	1	2	2	1	1	1	5	1	3	0	0
12	27708/09	527637D	35	1	NS1	3	1	4	1	2	2	2	1	1	1	1	1	7	3	1	1	1
13	29194/09	534447D	36	2	NS1	1	1	3	1	2	2	2	2	1	5	2	1	2	2	1	0	0
14	31851/09	543468D	46	1	NS1	1	1	3	1	2	2	2	2	1	1	1	1	7	3	1	1	1
15	32707/09	640782C	45	1	NS1	1	2	3	1	2	2	1	2	2	1	1	1	2	2	1	0	0
16	32930/09	864738C	27	2	NS1	1	1	4	1	2	2	1	2	1	2	1	1	2	2	1	0	0
17	33569/09	520216D	31	1	NS1	1	2	3	1	2	2	1	2	2	5	1	1	1	1	1	0	0
18	39307/09	580229D	39	2	NS1	1	1	3	1	2	2	1	2	1	2	1	1	6	2	3	0	0
19	40170/09	588534D	39	2	NS1	1	2	4	1	2	2	1	2	2	1	1	1	4	2	2	0	0
20	1203/10	602703D	38	2	NS1	4	1	4	1	2	1	1	1	2	3	2	1	1	1	1	0	0
21	2934/10	612228D	17	1	NS1	4	2	4	1	2	2	2	1	1	1	2	1	6	2	3	0	0
22	3807/10	620877D	26	1	NS1	2	2	3	1	2	1	1	1	2	4	1	1	2	2	1	0	0
24	6094/10	610846D	37	1	NS1	1	1	3	1	2	2	1	2	2	3	1	1	5	1	3	0	0
24	10719/10	545994D	28	2	NS1	1	1	3	2	1	2	2	1	2	3	1	1	7	3	1	1	1
25	11434/10	631602D	12	2	NS1	3	2	2	1	2	2	1	2	2	2	1	1	3	1	2	0	0
26	11715/10	632321D	29	1	NS1	1	2	3	1	2	2	1	2	2	4	1	1	3	1	2	0	0

MIB	IDH	ATRX	FISH	TERT	Locus	DIAGNOSIS	rx	RT	Chemo	Adjuv	Sur	FU	FUDUR	REC	PFS	OS	Death
5%	1	1	3	2		1	2	3DCRT	Nil	02-06-2009	16-01-2009	10-07-2017	102	2	102	102	2
<1%	1	1	3	2		1	2	3DCRT	Nil	18-02-2009	27-01-2009	21-05-2009	4	2	4	4	2
5%	1	2	4	2		2	2	Cobalt 60	Nil	17-04-2009	12-02-2009	31-03-2010	25	2	25	25	2
10%	2	3	4	2		7	3	3DCRT	TMZ 1 cycle	15-07-2009	24-03-2009	02-11-2009	8	1	8	8	1
Not done	1	3	4	1	1	7	1	3DCRT	TMZ	12-06-2009	31-03-2009	01-03-2010	12	1	12	12	1
Not done	1	3	4	2		7	4	Nil	Nil		21-04-2009	10-11-2009	7	1	7	7	1
Not done	1	3	4	1	1	7	1	3DCRT	TMZ	13-07-2009	28-04-2009	01-11-2009	4	1	4	7	1
Not done	1	3	4	1	2	7	3	3DCRT	TMZ 4 cycles	16-01-2010	17-06-2009	16-06-2010	12	1	6	12	1
Not done	1	3	4	1	1	7	1	3DCRT	TMZ	21-09-2009	16-07-2009	01-02-2010	7	1	7	7	1
8%	1	2	4	2		2	3	3DCRT	TMZ 6 cycles	01-03-2010	28-07-2009	16-08-2016	85	2	85	85	2
5%	1	3	3	1	1	1	1	3DCRT 55Gy	TMZ	07-10-2009	29-07-2009	30-07-2015	72	2	72	72	2
Not done	1	3	4	2		7	3	3DCRT	unknown	25-11-2009	25-08-2009	01-06-2010	12	1	9	12	1
8%	2	3	3	2		2	3	3DCRT 55Gy	TMZ 6 cycles	02-05-2010	07-09-2009	11-05-2013	44	1	42	44	1
Not done	1	3	4	1	1	7	3	3DCRT	TMZ 6 cycles	19-07-2010	29-09-2009	02-02-2011	17	1	16	17	2
8%	2	3	3	2		2	3	3DCRT 54Gy	TMZ 6 cycles	08-06-2010	06-10-2009	10-07-2017	93	1	72	93	2
10-12%	2	3	3	2		2	2	3DCRT	Nil	07-12-2010	07-10-2009	17-01-2011	15	2	15	15	2
2%	2	2	4	2		1	3	3DCRT 55Gy	TMZ 9 cycles	30-09-2010	13-10-2009	05-01-2017	89	2	89	89	2
7%	2	1	1	1	1	4	3	3DCRT 55Gy	TMZ 6 cycles	01-08-2010	30-11-2009	19-07-2016	81	2	81	81	2
7%	2	1	1	1	2	4	3	3DCRT 55Gy	TMZ 6 cycles	15-05-2010	08-12-2009	15-05-2010	6	2	6	6	2
3%	2	1	3	2		1	2	3DCRT 54Gy	Nil	19-03-2010	13-01-2010	13-07-2015	66	2	66	66	2
20%	1	2	4	1	1	2	1	3DCRT 54Gy	TMZ	24-03-2010	29-03-2010	21-05-2010	4	2	4	4	2
18%	2	2	4	2		2	3	3DCRT 55Gy	TMZ 6 cycles	01-12-2010	05-02-2010	12-06-2015	64	2	64	64	2
Not done	1	2	4	2		1	2	3DCRT 54Gy	Nil	26-04-2010	25-02-2010	12-06-2013	39	1	38	39	1
Not done	1	3	4	2		7	1	3DCRT	TMZ	23-04-2010	05-04-2010	01-11-2010	7	1	4	7	1
1%	1	3	1	2		3	1	3DCRT 50Gy	TMZ	12-07-2010	09-04-2010	01-10-2011	18	2	18	18	2
1%	1	1	3	2		1	2	3DCRT 55Gy	Nil	23-06-2010	12-04-2010	06-12-2015	68	2	68	68	2

27	16175/10	652570D	55	2	NS1	2	1	4	1	2	2	2	1	1	2	2	1	8	3	2	1	1
28	16968/10	639709D	15	1	NS1	3	1	4	1	1	1	1	2	2	3	2	1	2	2	1	0	0
29	23319/10	726650D	57	1	NS1	3	1	3	1	2	1	1	1	2	3	2	1	7	3	1	1	1
30	27482/10	732748D	36	1	NS1	1	1	3	1	2	2	1	2	1	2	2	1	3	1	3	0	0
31	31484/10	772880D	44	2	NS1	3	2	3	1	2	2	1	2	2	1	2	1	3	1	2	0	0
32	33846/10	727132D	34	1	NS1	1	1	4	1	2	2	1	2	2	5	2	1	4	2	2	1	0
33	35600/10	800421D	36	2	NS1	2	2	3	1	2	2	1	2	1	2	1	1	8	3	3	1	1
34	36106/10	810144D	52	2	NS1	3	2	4	1	2	2	2	1	1	1	2	1	7	3	1	1	1
35	38687/10	813293D	29	2	NS1	1	1	3	1	2	2	1	2	2	2	1	1	4	2	2	0	1
36	1465/11	854704D	49	1	NS1	5	2	4	1	2	1	2	1	1	2	1	1	8	3	3	1	1
37	1703/11	862993D	68	1	NS1	3	1	3	4	2	2	1	2	2	2	1	1	7	3	1	0	1
38	1847/11	845933D	20	2	NS1	3	2	4	1	2	2	1	2	1	2	2	1	1	1	1	0	0
39	4562/11	727112D	24	2	NS1	5	2	3	1	2	1	1	2	2	3	1	1	2	2	1	0	0
40	8540/11	893588D	18	1	NS1	1	2	4	1	2	2	2	1	1	3	1	1	6	2	3	0	0
41	11594/11	579291C	40	2	NS1	2	1	3	1	2	1	2	2	1	1	2	1	7	3	1	1	1
42	12100/11	329101C	60	1	NS1	2	1	4	1	2	2	2	1	1	1	1	1	7	3	1	1	1
43	12207/11	925535D	58	1	NS1	2	1	3	1	2	2	1	2	2	2	1	1	7	3	1	1	1
44	12646/11	9044468D	30	1	NS1	4	1	4	1	2	1	1	2	1	2	2	1	2	2	3	0	0
45	16478/11	935320D	32	2	NS1	1	1	3	1	2	2	2	1	1	4	1	1	7	3	1	1	1
46	17745/11	877153D	32	1	NS1	4	1	4	1	2	2	1	1	2	3	2	1	6	2	3	0	0
47	18032/11	920959D	39	1	NS1	4	1	4	1	2	1	1	1	2	2	1	1	2	2	1	0	0
48	18353/11	954015D	44	1	NS1	1	1	3	1	2	1	1	1	1	5	2	1	8	3	3	0	1
49	21534/11	981795D	45	1	NS1	1	1	3	1	2	2	2	1	1	1	2	1	7	3	1	1	1
50	22905/11	930255D	19	1	NS1	2	1	4	1	2	2	1	2	2	3	1	1	2	2	1	0	0
51	25556/11	994064D	49	1	NS1	5	1	4	1	2	2	2	1	1	2	1	1	8	3	3	1	1
52	27865/11	969877D	22	1	NS1	4	1	4	1	2	1	1	2	2	3	2	1	1	1	3	0	0
53	32744/11	991145D	35	1	NS1	2	2	3	1	2	2	1	1	2	4	1	1	5	1	3	0	0

Not done	1	3	4	1	1	7	1	3DCRT	TMZ		17-05-2010	02-05-2012	24	1	2	24	1
13%	1	1	3	2		2	3	3DCRT 55Gy	TMZ 6 cycles	20-02-2011	25-05-2010	22-12-2015	67	2	67	67	2
Not done	1	3	4	1	1	7	4	Nil	Nil		15-07-2010	13-10-2011	15	1	10	15	1
4%	2	1	3	1	1	1	2	3DCRT 54Gy	Nil	16-10-2010	19-08-2010	30-05-2016	69	2	69	69	2
Not done	2	3	2	2		1	2	3DCRT 54 Gy	Nil	06-12-2010	22-09-2010	18-12-2010	3	2	3	3	2
20-30%	1	1	1	1	1	4	3	3DCRT 55Gy	TMZ 6 cycles	01-10-2011	12-10-2010	28-06-2016	68	2	68	68	2
Not done	1	3	4	1	2	7	3	3DCRT 60Gy	TMZ 4 cycles	01-05-2012	26-10-2010	01-01-2014	39	1	20	39	1
Not done	1	3	4	2		7	3	3DCRT	TMZ 3 cycles	11-01-2011	31-10-2010	01-01-2013	27	1	15	27	1
12%	2	1	1	1	1	4	3	Co60 59Gy	TMZ 6 cycles	01-08-2011	23-11-2010	15-01-2015	50	2	50	50	2
Not done	1	3	4	1	1	7	3	3DCRT 55Gy	TMZ 6 cycles	01-09-2011	01-01-2011	04-02-2012	13	1	10	13	1
Not done	1	3	4	1	1	7	1	3DCRT 56Gy	TMZ	21-03-2011	18-01-2011	09-04-2012	13	1	12	13	1
6%	1	2	4	2		1	1	3DCRT 60Gy	TMZ	08-03-2011	18-01-2011	06-12-2015	59	2	59	59	2
Not done	1	2	4	2		2	3	Co60 54Gy	TMZ 6 cycles	21-10-2011	11-02-2011	05-12-2015	58	2	58	58	2
18%	1	1	3	1	2	2	3	Co60 55Gy	TMZ 6 cycles	01-01-2012	18-03-2011	22-09-2012	18	2	18	18	2
Not done	1	3	4	1	2	7					12-04-2011	29-01-2012	9	1			
40%	1	3	4	1	1	7	3	3DCRT 58Gy	TMZ C6 and BVZ	01-12-2011	17-04-2011	06-04-2012	12	1	8	12	1
36%	1	3	4	1	2	7	3	3DCRT 60Gy	TMZ 6 cycles	30-07-2011	19-04-2011	16-12-2013	33	1	30	33	1
7%	2	2	4	2		2	3	3DCRT 54Gy	TMZ 6 cycles	01-03-2012	21-04-2011	30-09-2014	41	2	41	41	2
Not done	1	3	4	2		7	3	3DCRT 58Gy	TMZ 2 cycles	10-10-2011	26-05-2011	02-11-2011	6	2	6	6	2
12%	2	2	4	2		2	1	3DCRT 54Gy	TMZ	27-12-2012	07-06-2011	10-03-2012	10	2	10	10	2
10%	2	1	3	2		2	3	3DCRT 56Gy	TMZ 6 cycles	01-02-2012	09-06-2011	20-06-2017	72	2	72	72	2
Not done	2	3	4	2		7	3	3DCRT 58Gy	TMZ 6 cycles	01-04-2012	11-06-2011	01-06-2012	12	1	11	12	1
Not done	1	3	4	2		7	2	3DCRT 55Gy	Nil	02-09-2011	08-07-2011	01-03-2012	9	1	4	9	1
6%	2	1	3	2		2	3	3DCRT 59Gy	TMZ 6 cycles	01-06-2012	21-07-2011	19-09-2014	38	1	36	38	1
Not done	2	3	4	2		7	2	Co60 59Gy	Nil	19-10-2011	11-08-2011	01-07-2012	11	1	9	11	1
5-6%	2	2	4	2		1	2	Co60 54Gy	Nil	07-11-2011	01-09-2011	21-03-2015	42	2	42	42	2
3%	2	1	3	2		1	2	Co60 54Gy	Nil	03-01-2012	04-10-2011	14-04-2014	30	1	29	30	2

55	35662/11	068946F	29	2	NS1	3	2	3	1	2	2	2	2	1	1	2	1	7	3	1	1	1
55	36392/11	064526F	42	1	NS1	2	1	4	1	2	1	2	1	1	1	1	1	7	3	1	1	0
56	36789/11	050563F	26	1	NS1	1	1	4	1	2	1	1	2	1	2	1	1	5	1	3	0	0
57	37539/11	054146F	34	1	NS1	3	2	3	1	4	2	1	1	2	2	2	1	7	3	1	1	1
58	4099/09	337959D	33	1	NS1	4	1	4	1	2	2	1	2	2	2	1	1	4	2	2	0	0
59	4447/09	402623D	56	1	NS1	1	1	3	1	2	2	1	2	1	2	1	1	6	2	3	0	0
60	10707/09	397021D	40	2	NS1	2	2	3	1	2	2	1	2	2	2	1	1	4	2	2	0	1
61	16119/09	444718D	35	1	NS1	1	2	3	4	2	2	1	2	1	3	2	1	6	2	3	0	0
62	16497/09	458658D	59	2	NS1	5	2	4	1	4	2	2	1	1	2	1	1	8	3	3	1	1
63	18427/09	475432D	17	2	NS1	3	2	3	1	3	1	1	2	1	2	1	1	8	3	3	1	1
64	21193/09	485471D	13	1	NS1	1	1	3	1	2	2	1	2	2	3	1	1	4	2	2	0	1
65	21743/09	491133D	42	1	NS1	2	1	3	1	2	1	1	1	2	1	1	1	5	1	3	0	1
66	27862/09	501513D	47	1	NS1	1	2	3	1	2	2	1	2	2	1	1	1	4	2	2	0	0
67	30477/09	537617D	27	1	NS1	1	2	3	1	2	2	1	2	2	1	2	1	3	1	2	0	1
68	31030/09	541051D	45	2	NS1	1	2	4	1	2	2	2	2	1	4	2	1	6	2	3	0	0
69	34214/09	233834C	37	2	NS1	2	2	2	2	1	2	2	1	2	4	1	2	6	2	3	0	0
70	34282/09	002863C	48	2	NS1	1	2	4	1	2	1	2	1	2	2	2	2	4	2	2	1	0
71	38669/09	584551D	63	2	NS1	3	2	3	1	2	2	2	1	1	2	1	1	8	3	3	1	1
72	2936/10	615217D	42	1	NS1	1	1	4	1	2	2	1	2	2	2	1	1	8	3	3	1	1
73	3562/10	616948D	52	1	NS1	4	1	3	1	2	2	1	2	2	2	2	1	3	1	2	0	0
74	3704/10	622646D	48	2	NS1	4	1	4	1	2	2	1	2	1	3	2	1	3	1	2	0	0
75	13885/10	659086D	36	1	NS1	4	2	4	1	2	2	1	1	1	3	2	1	3	1	2	0	0
76	19479/10	375440C	38	1	NS1	3	1	3	1	2	2	1	2	2	2	1	2	6	2	3	0	0
77	19935/10	145326D	54	1	NS1	3	1	2	1	2	2	2	1	2	2	1	2	3	1	2	0	0
78	20071/10	690897D	17	2	NS1	4	1	4	1	2	1	1	2	2	2	2	1	8	3	3	0	1
79	20615/10	656411D	42	1	NS1	1	1	4	1	2	2	1	2	2	3	2	1	4	2	2	0	0
80	21358/10	725236D	28	1	NS1	1	1	4	3	2	2	1	2	2	3	2	1	6	2	3	0	0

Not done	1	3	4	2		7	1	Co60 56Gy	TMZ	07-01-2012	05-11-2011	07-02-2012	3	2	3	3	2
10%	1	3	4	2		7	3	Co60 60Gy	TMZ 6 cycles	13-07-2012	11-11-2011	21-03-2013	16	1	14	16	1
5%	2	1	3	2		1	2	unknown	Nil		15-11-2011	04-12-2014	37	2	37	37	2
8%	1	3	4	1	2	7	3	3DCRT 59Gy	TMZ 6 cycles	24-01-2012	22-11-2011	02-12-2014	37	2	37	37	2
31%	2	1	1	1	1	4	3	3DCRT 54Gy	TMZ 6 cycles	05-01-2010	06-02-2009	15-07-2014	77	2	77	77	2
16%	2	2	3	2		2	3	Co60 55Gy	TMZ 3 cycles	28-07-2009	09-02-2009	01-01-2014	59	1	59	59	1
3-4%	2	1	1	1	1	4	3	3DCRT 55Gy	TMZ 6 cycles	01-01-2010	03-04-2009	24-10-2015	78	2	78	78	2
7%	2	1	1	1	1	4	1	Co60 55Gy	TMZ	03-08-2009	21-05-2009	13-04-2015	71	2	71	71	2
Not done	1	1	3	1	1	7	1	3DCRT 54Gy	TMZ	04-08-2009	26-05-2009	01-09-2009	4	2	4	4	2
Not done	1	2	3	1	2	7	1	3DCRT 54Gy	TMZ	01-08-2009	03-06-2009	31-01-2011	19	1	17	19	1
6%	2	1	1	1	1	4	3	3DCRT 57Gy	TMZ 6 cycles	01-06-2010	03-07-2009	24-10-2015	75	1	45	75	2
2%	2	2	3	2		1	2	3DCRT 54Gy	Nil	08-09-2009	08-07-2009	18-01-2017	90	1	71	90	2
6%	2	1	1	1	1	4	2	3DCRT 55Gy	Nil	03-11-2009	28-08-2009	19-02-2009	6	1	5	6	1
7%	2	1	1	1	2	3	3	3DCRT 54Gy	TMZ 6 cycles	01-06-2010	17-09-2009	03-08-2017	95	1	76	95	2
8%	2	1	1	1	1	4	3	Co60 55Gy	TMZ 6 cycles	21-08-2010	22-09-2009	29-08-2016	83	2	83	83	2
6%	2	2	3	2		2	3	C060 55Gy	TMZ 6 cycles	25-09-2010	19-10-2009	24-10-2015	72	2	72	72	2
60%	2	1	1	1	1	4	3	3DCRT 56Gy	TMZ 6 cycles	28-08-2010	20-10-2009	13-04-2015	54	2	54	54	2
Not done	1	1	3	1	1	7	1	Unknown	Unknown		24-11-2009	04-03-2011	16	1	15	16	1
Not done	2	2	3	2		7	3	3DCRT 64Gy	TMZ 6 cycles	07-04-2010	29-01-2010	09-04-2012	27	1	20	27	1
2%	2	2	3	2		1	1	3DCRT	TMZ	01-04-2010	02-02-2010	12-09-2015	67	2	67	67	1
2%	2	1	1	1	1	3	1	Co60 55Gy	TMZ	14-04-2010	04-02-2010	24-10-2011	20	2	20	20	2
5%	2	2	3	2		1	1	Co60 55Gy	TMZ	02-08-2010	29-04-2010	24-10-2015	66	1	65	66	2
18%	2	2	1	2		4	3	3DCRT 55Gy	TMZ 6 cycles	19-11-2010	14-06-2010	24-07-2015	61	1	48	61	2
2%	2	2	3	2		1	1	3DCRT	TMZ	02-08-2010	01-06-2010	14-07-2015	61	1	13	61	2
50-60%	2	2	3	2		7	3	3DCRT	TMZ 6 cycles	01-03-2011	28-06-2010	01-10-2015	64	1	64	64	2
15%	2	1	1	1	1	4	3	Co60 55Gy	TMZ 6 cycles	01-03-2011	23-06-2010	24-10-2015	64	2	64	64	2
15%	2	2	3	2		2	6	Unknown	Unknown		30-06-2010	28-02-2012	20	2	20	20	2

81	22935/10	710645D	58	2	NS1	1	2	3	1	2	1	1	2	2	2	1	1	6	2	3	0	0
82	23024/10	689655D	31	1	NS1	4	1	4	1	2	1	1	2	2	2	1	1	5	1	3	0	0
83	23377/10	730834D	33	1	NS1	3	1	4	1	2	2	2	2	1	2	1	1	3	1	2	0	0
84	25055/10	974698C	39	2	NS1	3	1	3	1	2	2	2	2	1	2	1	2	8	3	3	1	1
85	27605/10	735334D	41	1	NS1	1	1	3	1	2	1	1	2	2	2	1	1	3	1	2	0	0
86	30754/10	772840D	34	1	NS1	1	1	3	1	2	2	1	2	2	2	2	1	4	2	2	0	0
87	33130/10	763174D	31	1	NS1	1	2	3	1	2	2	1	1	2	1	2	1	4	2	2	0	0
88	33687/10	755555D	29	1	NS1	1	2	3	1	2	1	1	2	2	2	1	1	3	1	2	0	0
89	38818/10	808055D	59	2	NS1	1	2	3	1	2	1	1	2	2	1	1	1	4	2	2	0	0
90	526/11	846131D	21	1	NS1	1	2	4	1	4	2	2	2	1	2	2	1	4	2	2	1	1
91	3282/11	871389D	44	2	NS1	5	2	4	1	2	2	1	2	2	2	1	1	8	3	3	1	1
92	3740/11	808433D	39	1	NS1	1	2	3	1	2	2	1	2	2	3	2	1	3	1	2	0	0
93	4987/11	816558D	55	1	NS1	1	1	4	1	2	2	1	2	2	5	1	1	3	1	2	0	0
94	6606/11	847861D	47	1	NS1	1	1	4	1	2	2	1	2	2	2	1	1	4	2	2	1	1
95	6682/11	841983D	47	1	NS1	1	2	4	1	2	2	1	2	2	4	1	1	4	2	2	0	0
96	14540/11	926437D	48	2	NS1	4	2	4	1	2	2	1	2	2	3	2	1	3	1	2	0	0
97	23895/11	959143D	34	1	NS1	1	2	4	1	2	1	1	2	2	4	2	1	4	2	2	0	0
98	26504/11	983371D	47	2	NS1	4	2	4	1	2	2	1	2	2	5	1	1	3	1	2	0	0
99	27110/11	954251D	30	1	NS1	1	1	4	1	2	2	1	2	2	2	1	1	3	1	2	0	0
100	32599/11	772849D	39	1	NS1	1	1	3	1	2	1	1	2	2	4	1	1	5	1	3	0	0
101	34681/11	047776F	32	1	NS1	4	1	3	1	2	2	1	2	2	2	1	1	6	2	3	0	0
102	35796/11	024702F	40	1	NS1	2	1	4	1	2	1	2	1	2	2	1	1	5	1	3	0	0
103	37110/11	032677F	28	1	NS1	4	1	4	1	2	2	1	2	2	4	2	2	5	1	3	0	0
104	37683/11	047318F	39	1	NS1	1	1	4	1	2	2	1	2	2	4	1	1	4	2	2	0	0
105	40382/11	091611F	23	1	NS1	2	1	3	1	4	1	2	1	1	1	1	2	6	2	3	0	0
106	1123/12	899496C	39	1	NS1	4	1	4	1	2	1	1	1	2	2	2	2	8	3	3	1	1
107	3259/12	115325F	29	1	NS1	1	1	4	3	2	2	1	2	2	2	1	1	3	1	2	0	0

11%	2	2	3	2		2	1	3DCRT 58Gy	TMZ	27-09-2010	15-07-2010	25-09-2014	50	2	50	50	2
8%	2	2	3	2		1	2	Co60 55Gy	nil	11-09-2010	14-07-2010	12-01-2013	30	1	30	30	2
11%	2	2	3	2		1	3	3DCRT 54Gy	TMZ 6 cycles	05-02-2011	16-07-2010	15-08-2014	49	1	45	49	2
Not done	2	2	3	2		7	2	3DCRT	Nil	25-08-2010	29-07-2010	09-09-2010	2	1	2	2	1
1-2%	2	2	3	1	1	1	1	3DCRT 60Gy	TMZ	18-10-2010	20-08-2010	28-12-2015	64	2	64	64	2
15%	2	1	1	1	1	4	2	Co60 54Gy	Nil	18-11-2010	15-09-2010	11-01-2013	28	2	28	28	2
20%	2	2	3	1	2	2	3	3DCRT 55Gy	TMZ 6 cycles	01-07-2011	06-10-2010	18-05-2016	67	2	67	67	2
10%	2	1	1	1	1	3	3	Co60 54Gy	TMZ 6 cycles	01-07-2011	11-10-2010	24-10-2015	60	2	60	60	2
10%	2	1	1	1	1	4	1	3DCRT 54Gy	TMZ	31-01-2011	24-11-2010	11-04-2011	5	2	5	5	2
40%	2	1	1	1	2	4	6	Unknown	Unknown		06-01-2011	18-12-2011	11	1	10	11	1
20%	1	1	3	1	1	7	3	3DCRT 60Gy	TMZ 6 cycles	20-09-2011	31-01-2011	31-05-2013	28	1	22	28	2
5%	2	2	3	2		1	2	3DCRT 54Gy	Nil	12-04-2011	03-02-2011	18-05-2017	75	1	61	75	2
7%	2	1	1	1	1	3	6	Unknown	Unknown		14-02-2011	14-11-2012	21	2	21	21	2
20%	2	1	1	1	1	4	3	3DCRT 59Gy	TMZ 6 cycles	01-04-2012	01-03-2011	26-07-2017	77	2	77	77	2
40%	2	1	3	1	1	2	3	Co60 55Gy	TMZ 6 cycles	11-12-2011	01-03-2011	15-04-2015	49	2	49	49	2
7%	2	1	1	1	1	3	3	3DCRT 54Gy	TMZ 6 cycles	01-01-2012	10-05-2011	18-07-2013	26	2	26	26	2
19%	2	1	3	1	1	2	3	Co60 59Gy	TMZ 6 cycles	11-05-2012	30-07-2011	30-08-2016	61	2	61	61	2
8%	2	1	3	1	1	1	3	Co60 55Gy	TMZ 4 cycles	16-02-2012	22-08-2011	02-06-2012	10	2	10	10	2
5%	2	1	1	1	1	3	2	Co60 54Gy	Nil	20-10-2011	26-08-2011	24-10-2017	50	2	50	50	2
3%	2	2	3	2		1	2	3DCRT 54Gy	Nil	16-12-2011	11-10-2011	05-06-2017	68	2	68	68	2
8%	2	2	3	2		2	3	Co60 55Gy	TMZ 6 cycles	22-07-2012	28-10-2011	01-08-2012	10	2	10	10	2
4%	2	2	3	2		1	2	Co60 54Gy	Nil	05-01-2012	07-11-2011	15-07-2014	32	2	32	32	2
4%	2	2	3	2		1	3	Co60 54Gy	TMZ 4 cycles	01-08-2014	21-11-2011	06-07-2016	56	1	21	56	2
14%	2	1	1	1	1	4	3	3DCRT 59Gy	TMZ 3 cycles	29-05-2012	23-11-2011	23-05-2016	54	2	54	54	2
8%	2	2	3	2		2	1	Co60 59Gy	TMZ	01-03-2012	15-12-2011	01-08-2015	44	1	32	44	2
Not done	2	2	3	2		7	3	3DCRT 50Gy	TMZ 3 cycles	11-07-2012	11-01-2012	20-01-2013	12	1	11	12	1
5%	2	1	1	1	1	3	2	Co60 54Gy	Nil	23-03-2012	30-01-2012	19-05-2014	27	2	27	27	2