Molecular profile of tumours with an Oligodendroglial morphology – Clinical Relevance

A Dissertation Submitted in Part Fulfillment of the rules and regulations for the M.D. Degree Branch III (Pathology) Examinations of The Tamil Nadu Dr. M.G.R. Medical University, Chennai to be held in April 2015

CERTIFICATE

This is to certify that this dissertation titled "***Molecular profile of tumours with an oligodendroglial morphology – clinical relevance***" is a bonafide work done by Dr Tulasi Geevar, in part fulfillment of rules and regulations for the M.D. Branch III (Pathology) Degree Examination of the Tamil Nadu Dr. M.G.R. Medical University, to be held in April 2015.

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ABBREVIATIONS

ATRX	Alpha thalassemia/mental retardation syndrome X
CFO	Classical for Oligodendroglioma
CGH	Comparative Genomic Hybridisation
CIC	homologue of Drosophila gene capicua
CIMP	CpG Island Methylation Phenotype
CNS	Central Nervous System
CT	Computed Tomography
DNA	Deoxyribonucleic acid
DNET	Dysembryoplastic neuroepithelial tumour
dNTPs	Deoxyribonucleotide triphosphate
EORTC	European Organisation for Research and Treatment of Cancer
FFPE	Formalin fixed paraffin embedded
FISH	Fluorescence in situ hybridisation
FUBP	Far Upstream Element Binding Protein
GBM	Glioblastoma multiforme
GBMO	Glioblastoma with oligodendroglial component
GFAP	Glial Fibrillary Acidic Protein
HG	Hydroxy glutarate
HIF-1	Hypoxia inducible factor
hpf	high power fields
ICMR	Indian Council of Medical Research
IDH	Isocitrate dehydrogenase
KPS	Karnofsky Performance Status

LOH	Loss of heterozygosity
MAP2	Microtubule associated protein 2
MDM2	Mouse double minute 2 homolog
MGMT	O-6-Methylguanine-DNA methyltransferase
MRI	Magnetic Resonance Imaging
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NCFO	Not classical for Oligodendroglioma
NCRP	National Cancer Registry Programme
NSC	Neuroectodermal stem cells
NTC	Non Template Control
OPC	Oligodendrocyte progenitor cells
OS	Overall survival
PCR	Polymerase chain reaction
PCV	Procarbazine, Lomustine and Vincristine
PFS	Progression free surival
PTEN	Phosphatase and tensin homologue
RT	Radiotherapy
RTOG	Radiation Therapy Oncology Group
SPSS	Statistical Package for Social Sciences
SEER	Surveillance, Epidemiology and End Results
TBS	TRIS Buffered Saline
TP53	Tumour protein 53
WHO	World Health Orgnisation
WT-1	Wilm' Tumour 1

α -KG	Alpha ketoglutarate
LSI	Locus specific identifier.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

TITLE : Molecular profile of tumours with oligodendroglial morphology - clinical relevance

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DEGREE AND SUBJECT : MD Pathology

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Aim: Characterization of the molecular profile of oligodendroglial tumors with relation to 1p/19q status and IDH mutation.

Objectives:

1. To determine the frequency of IDH1 and IDH2 mutations by polymerase chain reaction (PCR) sequencing and IDH1 mutations by immunohistochemistry among 50 cases of oligodendroglial tumours.
2. To calculate the performance indicators of IDH1 immunohistochemistry as a diagnostic marker.
3. To correlate 1p/19q status and IDH1/2 mutations with clinico-pathological variables and measures of outcome.

Methods: 50 oligodendroglial tumours diagnosed from January 2009 to January 2012 were included in the study. These included, 11 WHO grade II Oligodendroglioma, 15 WHO grade III Oliodendroglioma, 7 WHO grade II Oligoastrocytoma, 10 WHO grade III Oligoastrocytoma and 7 Glioblastoma with an oligodendroglial component. 1p/19q status was determined for all cases by Fluorescence in situ hybridization (FISH). Immunohistochemistry was performed for IDH1 mutations. Molecular analysis for IDH1 and IDH2 mutations was done by PCR.

Association between combined 1p/19q deletion, polysomy of chromosome 1/19 and IDH mutation with the various clinical, radiological and histopathological parameters was calculated using Pearson's Chi square test/Fishers exact test. Sensitivity and specificity of IDH1 immunohistochemistry was calculated taking PCR as the gold standard. Odds ratio and risk ratio was calculated for individual risk factors. Logistic regression model was used for multivariate analysis. A p value of ≤ 0.05 was considered significant.

Results: 1p/19q co-deletion and polysomy of chromosome 1/19 was seen in 46% (23/50) and 36% (18/50) of the cases respectively. 1p/19q co-deletion was significantly associated with pure oligodendroglial tumours (p = 0.0001), frontal lobe location and classical histology. Polysomy 1/19 was associated with mixed oligoastrocytic tumours (p=<0.00001). IDH1 and IDH2 mutations were seen in 86% and 6% of the cases respectively. The most common type of IDH1 mutation was of IDH1R132H (42 cases) type, followed by IDH1R132C (1 case). All 3 IDH2 mutations were of the IDH2R172H type.IDH1 immunohistochemistry was positive in 42 (84%) cases with IDH1R132H mutation and

negative in the remaining cases. The sensitivity and specificity of IDH1 immunohistochemistry was 91.3% and 100% respectively.

23 of the 46 cases with IDH mutation showed 1p/19q co-deletion and all 4 cases negative for IDH mutation were also negative for 1p/19q co-deletion.

On univariate analysis, necrosis, WHO grade and IDH mutation were found to have increased risk of recurrence and death. On multivariate analysis, utilizing the following variables, WHO grade (Grade IV vs. Grade II and III combined), classic histology, 1p/19q co-deletion and 1/19 polysomy, only WHO grade was found to be significantly associated with a poor PFS ($p = 0.022$).

Conclusion: IDH1/2 mutations were seen with a high frequency in this cohort of oligodendroglial neoplasms. IDH1 immunohistochemistry is a sensitive and specific marker for determining mutational status. There is a role for PCR for detecting IDH mutations in cases that are immunonegative for IDH1.

Keywords: Oligodendroglioma, Oligoastrocytoma, GBMO, Glioblastoma with oligodendroglial component, 1p/19q co deletion, polysomy 1/19, IDH1 immunohistochemistry, IDH1/2 mutation.

INTRODUCTION

Oligodendroglial tumours are one of most frequent primary parenchymal brain tumours, after astrocytic tumours. They include tumours which morphologically resemble oligodendroglial cells with or without an astrocytic component.

According to histological criteria established by the 4th edition of World Health Organization (WHO) Classification of Tumours of the Central Nervous System (CNS), oligodendroglial tumours can be subtyped into Oligodendroglioma (Grade II), Anaplastic Oligodendroglioma(Grade III), Oligoastrocytoma (Grade II), Anaplastic Oligoastrocytoma (Grade III) and Glioblastoma with oligodendroglial component (GBMO) (Grade IV).(1)

Oligodendrogliomas are more chemo sensitive compared to astrocytomas, showing a good response to the chemotherapy regimen using the combination of Procarbazine, Lomustine and Vincristine (PCV).(2,3)More recently, Temozolamide has replaced the aforementioned regimen due to its reduced cytotoxic effects.(4,5) Hence, it has become essential to differentiate oligodendrogliomas from astrocytomasto give the patients the benefit of adjuvant radiotherapy and/or chemotherapy.

Oligodendrogliomas showing classical histological features are not difficult to diagnose. The most commonly encountered problem is in diagnosing mixed oligoastrocytomas showing features of both oligodendroglioma and astrocytoma. Due to the lack of established criteria and highly subjective nature of diagnosis, there is high interobserver variability and low reproducibility in the diagnosis of these tumours.(6–9) Though

histologically ambiguous, these tumours are thought to be clonal neoplasms showing genetic features of either oligodendrogliomas or astrocytomas.(10) This highlights the possible pitfalls of misdiagnosing gliomas when based solely on morphological features.

Deletion of the short arm of chromosome 1 and long arm of chromosome 19 has been recognised as the typical molecular signature of oligodendrogliomas. This is thought to occur due to an unbalanced translocation between the short arm of chromosome 1 and long arm of chromosome 19 with subsequent loss of the derivative chromosome, der (1;19)(p10;q10).(11)

1p/19q deletion is a strong prognostic factor in patients receiving radiotherapy and/or chemotherapy. Studies suggest that 1p/19q deletion also has a role in predicting the response to treatment. (12–14)Hence, 1p/19q deletion can be used as a potential diagnostic, prognostic and predictive marker in oligodendroglial tumours.

Recently, it was found that majority of the gliomas harbor mutations in the Isocitrate Dehydrogenase (IDH) gene. Studies have shown that IDH mutation is associated with a better Progression free survival (PFS) and Overall survival (OS) in univariate and multivariate analysis. In 2009, Capper et al developed a monoclonal antibody targeting the mutant protein of IDH1R132H mutation(15).The high sensitivity and specificity of IDH1 immunohistochemistry makes it a useful and simple technique to determine the mutational status.

Besides the above factors, other variablescan also influence outcome in oligodendroglial tumours. These include clinical parameters like age, Karnofsky performance status (KPS), radiological features like homogenous signal intensity, tumour borders, site of tumour,

extent of surgical resection, presence of classical features of oligodendroglioma (CFO), MIB-1 proliferation index and other genetic alterations like O-6-methyl guanine methyltransferase (MGMT) promoter hypermethylation.

Progress in molecular diagnostics have identified various genetic markers which can aid in the classification and differential diagnosis of gliomas and also give additional information as to the outcome and response to treatment.

Current treatment decisions are mainly based on WHO grade despite the differencesin outcome withinthe glioma subgroups. A refined classification that incorporates genetic signatures is necessary and may become critical for management of these tumors in the future. Also, the detection of these novel mutations and chromosomal alterations open the possibility for the discovery of novel drugs targeting these changes and shed light on glioma tumorigenesis. This can lead to better therapeutic interventions and possible cure for these neoplasms.

In the light of the above findings, we sought to determine the 1p/19q status and frequency of IDH1/IDH2 mutations in a cohort of oligodendroglial tumours and correlate them with clinico-pathological features and measures of outcome.

AIMS AND OBJECTIVES

Aim: Characterization of the molecular profile of oligodendroglial tumors with relation to 1p/19q status and IDH mutation.

Objectives:

1. To determine the frequency of IDH1 and IDH2 mutations by PCR sequencing, and IDH1 mutations by immunohistochemistry among 50 cases of oligodendroglial tumours.
2. To calculate the performance indicators of IDH1 immunohistochemistry as a diagnostic marker.
3. To correlate 1p/19q status and IDH1/2 mutations with clinico-pathological variables and measures of outcome.

LITERATURE REVIEW

Introduction

Neoplasms of the central nervous system (CNS) may arise from neural elements within the brain, or may be metastasis from distant cancers. Gliomas, metastatic neoplasms, meningiomas, pituitary adenomas, and acoustic neuromas account for 95% of all intracranial tumours.(16)

According to the National Cancer Registry Programme (NCRP) of Indian Council of Medical Research (ICMR), the age adjusted annual incidence rates of brain cancers in India per 100,000 population ranged from 2.53 (Chennai registry) to 4.14 (Delhi registry) in males and 1.46 (Bhopal registry) to 2.66 (Delhi registry) in females.(17)According to Surveillance Epidemiolog, and End Results (SEER) data, the estimated incidence of primary invasive brain tumours in the United States is 6.36 cases per 100,000 population and the estimated mortality is 4.22 per 100,000 persons per year.(18)

Gliomas are one of the most common CNS neoplasms accounting for about 28% of all brain tumours and 80% of all malignant tumours(19). They are commonly seen in the subcortical surface and deep white matter of cerebral hemispheres. The term diffuse gliomas excludes WHO grade I tumours, like pilocytic astrocytoma which are well circumscribed and are biologically different.(20)

Gliomas can be divided into astrocytic, oligodendroglial and ependymal tumours depending on the similarities of their component cells to differentiated glial cells. Despite

the major advances in neuroimaging, histopathology is essential for definite diagnosis and typing of these tumours.

Grading is an important factor influencing the choice of treatment such as use of radiation or various chemotherapy regimens. Gliomas are graded by a four tier system, from grade I to grade IV based on the criteria established by World Health Organization (WHO)(1). Histological factors influencing grading are atypia, cellularity, endothelial proliferation, necrosis and mitosis. Grade I tumours are biologically distinct tumors and include Pilocytic astrocytomas, Subependymoma and Subependymal giant cell astrocytomas. These tumors are considered benign and have an excellent prognosis following complete excision. Grade II tumours are cellular tumors exhibiting nuclear pleomorphism, lacking mitotic activity and are infiltrative in nature with slow progression, tendency to recur and a risk of anaplastic transformation. Grade III tumours show histological features of malignancy like nuclear pleomorphism and high mitotic activity. These patients are additionally treated with radiation and/or chemotherapy. Grade IV are very aggressive tumours and are assigned to necrosis prone, mitotically active neoplasms with or without microvascular proliferation(1).

Cells of origin and differentiation

The cell of origin of gliomas remains enigmatic. It was postulated that oligodendrogliomas arise from oligodendrocytes and astrocytomas from astrocytes. If this were true, it would require mature brain cells to undergo cell division during adult life. Another possibility is

that oncogenic events occurred in still proliferating fetal cells or during glial proliferation in intrauterine and postnatal life.(21)

Work on animal models and primary gliomas have suggested that malignant gliomas possibly arise from progenitor cells. Neuroectodermal stem cells (NSCs) in adult brains can take diverse paths of differentiation, are migratory and have proliferative capacity. These features are intrinsic to glioma cells and possible characteristics of the neoplastic cell of origin. Glial progenitors, including oligodendrocyte progenitor cells (OPCs), neural stem cells and mature astrocytes are thought to be cells of origin for gliomas.(22) During normal brain development, the neural stem cells (NSCs) and multipotent progenitor cells in the proliferative zones of the sub ventricular zone give rise to lineage specific progenitor cells, which in turn generate neurons and glial cells of oligodendroglial or astrocytic lineage.

Factors that govern glioma differentiation and phenotype are still uncertain. A tumour stem cell arises either from a neural stem cell or from a mature glial cell. Activation of particular cellular pathways results in transformation of these tumour stem cells into tumours with phenotypic properties similar to oligodendrogliomas and astrocytomas. Different genetic events drive differentiation towards specific cell lineages, like TP53 mutations in astrocytic tumours and chromosome 1p/19q deletion in oligodendroglial tumours. (22)

Oligodendroglial tumours

Oligodendroglial tumours are diffusely infiltrating gliomas and represent the second most common primary intraparenchymal brain tumours in adults. These include tumours which

show morphology resembling normal oligodendroglial cells of the brain. The first description of an oligodendroglioma was published by Bailey and Cushing in 1926. (5) (23)In 1929, Bailey and Bucy provided a classic description of 13 oligodendrogliomas andpostulated a link between these tumors and oligodendrocytes based on the following 3 observations. a) both cell types have a uniform and round nuclei, (b) both show a swollen and clear cytoplasm following routine histological tissue preparation, and (c) on silver staining, both cells types show similar cell processes.(24)(23)

Oligodendroglial tumours, including oligodendrogliomas and mixed oligoastrocytomas constitute between 5% and 18% of all primary brain tumours.(25).

According to WHO, oligodendroglial tumours can be subdivided into Oligodendroglioma (Grade II), Anaplastic oligodendroglioma (Grade II), Oligoastrocytoma (Grade II), Anaplastic oligoastrocytoma (Grade III) and Glioblastoma with an oligodendroglial component (Grade IV).(1) Historically, several grading systems have been used to classify oligodendroglial tumours, such as Kernohan system, Smith grading system, St Anne-Mayo system and the three tiered modification of the Smith scheme. (21). However, currently, WHO grading is widely followed and studies have confirmed WHO grade as a significant predictor of survival(21).

These tumors arise preferentially in the cortex and white matter of the supratentorial brain with frontal lobe being the most common site, followed by temporal lobe. They are commonly seen in adults with a peak incidence between 30 to 50 years. There is a slight male predominance. Low grade tumours occur in a slightly younger age group, while higher grade tumors manifest 7-8 years later. The most common presenting symptom is

seizures, followed by headache, features of raised intracranial pressure and focal neurological deficits. These tumours have a tendency to involve the cortical grey white matter. This could probably account for the high incidence of seizures in these tumours.(1)

Until about 20 years ago, there was little significance in the distinction of oligodendroglial tumours from astrocytic tumours. There was large overlap in treatment regimens and prognostication was the sole reason to distinguish between these tumours.(26). In 1988, it was found that recurrent anaplastic oligodendrogliomas showed a dramatic response to chemotherapy using thePCV regimen.(3) In 1990, it was shown that newly diagnosed anaplastic oligodendrogliomas also responded to PCV treatment.(2) Several studies from various institutions have corroborated these findings.(27–30)However, the administration of PCV chemotherapy is associated with severe haemotological toxicity causing high morbidity. Because of its better toxicity profile, another alkylating drug, Temozolamide has largely replaced PCV chemotherapy. Studies have shown that oligodendroglial tumours also show good response to treatment with temozolamide.(4,5,31)

These clinical observations made it necessary to accurately distinguish oligodendroglial tumours as a separate entity. Although the diagnosis of classic cases of oligodendroglial tumour is straightforward, a significant proportion of the diffuse gliomas particularly, mixed glial tumours and higher grade tumours, show ambiguous histological features that make classification not only difficult but also subjective. At present, there is no single immunohistochemical marker that is specific for oligodendroglial tumours. These factors account for the large interobserver variability in the classification of glioma even amongst experienced neuropathologists.

Oligodendroglioma

According to WHO, Oligodendroglioma is defined as "*a diffusely infiltrating, well-differentiated glioma of adults, typically located in the cerebral hemispheres, composed of neoplastic cells morphologically resembling oligodendroglia and often harboring deletions of chromosomal arms 1p and 19q".*(1) Oligodendroglioma corresponds to WHO Grade II.

Adjusted annual incidence rate of oligodendrogliomas is 0.27 per 100,000 persons per year constituting about 1.2% of all brain tumours.(19)(1). The majority of oligodendrogliomas are seen in adults with a peak incidence is 40-45 years. Males are slightly more frequently affected than females. (1,32)

Neuroimaging: On Computed Tomography (CT) brain, oligodendrogliomas appears as hypo or isodense, well demarcatedlesions commonly located in the cortex and white matter. Calcifications are common. Magnetic Resonance Imaging (MRI) shows a well demarcated hypo intense lesion in T1 weighted images and a hyper intense lesion in T2 weighted images with little perifocal edema. Contrast enhancement is associated with a poor prognosis.(5, 6)

Location: Majority of the oligodendrogliomas arise in the cerebral hemispheres. Frontal lobe is the most common location and is involved in 50-65% of patients, followed by temporal, parietal and occipital lobe with decreasing frequencies.(21) Bilateral tumour spread and involvement of more than one cerebral lobe can be commonly seen.

Macroscopic appearance: The typical oligodendroglioma is a well-defined, greyish-pink and soft mass. Perifocal edema can be seen. The frequent presence of calcifications can impart a

a gritty texture to these tumours. Extensive mucoid degeneration in some tumours can appear as soft, gelatinous masses.

Microscopy: Oligodendrogliomas are diffusely infiltrating gliomas composed of a monomorphic population of cells with round to oval nuclei, evenly dispersed chromatin, small or inconspicuous nucleoli and characteristic perinuclear halos.(5, 6)

On smear preparations, cells have a thin rim of cytoplasm and sparse fibrillary eosinophilic cytoplasm. On routine formalin fixed paraffin sections, cellular swelling and retraction of the delicate cytoplasmic processes produces the hallmark perinuclear halos resulting in the characteristic 'fried egg' appearance of oligodendrogliomas. This artifact is not seen in smear preparations, frozen sections and paraffin sections made from frozen tissue and in these cases, the tumours cells show scant but distinct cytoplasm without perinuclear halos. The solid areas in the tumour show a paucity of fibrillary cytoplasm but the edge of these tumours can show entrapped background neuropil.(1)

Oligodendrogliomas show a dense network of delicate branching vasculature imparting the characteristic 'chicken wire' appearance. The vascular network can sometimes divide the tumour into lobules. Distinct nodules of hyper cellular areas can be seen in grade II tumours and is not per se a feature of anaplasia in the absence of increased mitotic activity. Calcifications and mucoid/cystic degeneration are other features. Tumor cells with signet-ring cell morphology have also been described in oligodendrogliomas.(21,32)

Some tumour cells show eccentric eosinophilic cytoplasm resembling small gemistocytes. The cytoplasm of these cells stains strongly for GFAP. These cells are called as

'minigemistocytes' or 'microgemistocytes'. Few other neoplastic cells are also seen and may have a rim of GFAP-positive intermediate filaments. These are called 'gliofibrillary oligodendrogliocytes'. Scattered reactive astrocytes can be seen in oligodendrogliomas particularly along the infiltrating edge. All these cells should not be mistaken for neoplastic astrocytes and care should be taken not to misdiagnose these tumours as oligoastrocytomas.(21)

Within the cortex, tumour cells tend to form 'secondary structures of Scherer'. These include perineuronal satellitosis, subpialcollections and perivascular aggregations. Although these secondary structures can be seen in other diffuse gliomas they tend to be more pronounced in oligodendrogliomas, perhaps because of their greater tendency for cortical grey matter infiltration.(21)

High cellularity, brisk mitotic activity (≥6 mitoses/10hpf), pleomorphism, microvascular proliferation or necrosis indicates progression to anaplastic oligodendroglioma. (33)(1)
Differential diagnosis: Tumours which can show oligodendroglia like morphology include small cell variant of glioblastoma, dysembryoplastic neuroepithelial tumour (DNET), neurocytoma and clear cell ependymoma.(1)(34)

Immunohistochemistry: There is no single distinct immunohistochemical marker that is diagnostic of oligodendroglioma. However, a panel of markers can be used to differentiate it from astrocytic tumours and other tumours showing an oligodendroglial morphology.

GFAP (Glial fibrillary acidic protein) expression is usually absent in the cytoplasm of oligodendroglial cells, however overlapping of GFAP-positive fibrillary neuropil

background can be seen which can be confused with astrocytic tumours. Minigemistocytes and gliofibrillary oligodendrocytes are usually positive for GFAP. Microtubule-associated protein 2 (MAP2), a protein linked to the neuronal cytoskeleton is strongly expressed in oligodendroglial tumours, but is also seen in 92% of astrocytomas and glioblastomas. A perinuclear 'capped' expression is seen in oligodendrogliomas, while in astrocytic tumours the elongated cell processes are also stained.Wilms' Tumour 1 (WT-1) immunostaining in oligodendrogliomas is either completely absent or restricted to single WT1-positive cells while it is strongly expressed in 83-92% of high grade astrocytic tumours. The presence of more than 50% WT1 staining rules out a diagnosis of oligodendroglioma and is more in favour of either an astrocytoma or oligoastrocytoma. Nogo-A expression is seen in 71% oligodendrogliomas and 24% glioblastomas, but is absent in astrocytomas.(20)
Oligodendrocyte specific transcription markers, Olig1 and Olig2 are strongly expressed in oligodendrogliomas and appeared to be promising markers, but studies have shown that they are also expressed in other glial neoplasms. (21)Positive IDH1-R132H reactivity is very common in oligodendroglial tumors. Hence, it can be used to differentiateoligodendrogliomas from other brain tumours such as clear cell ependymomas, DNET etc. (20)
Combined deletion of 1p and 19q is the hallmark of oligodendroglioma and is seen in about 80% of grade II tumours.(1)

Anaplastic oligodendroglioma

According to WHO, anaplastic oligodendroglioma is defined as "*an oligodendroglioma with focal or diffuse histological features of malignancy and a less favourable prognosis".*
Anaplastic oligodendroglioma corresponds to WHO histological Grade III.(1).

Adjusted annual incidence of anaplastic oligodendroglioma ranges from 0.07-0.11 per 1,00,000 persons per year constituting about 0.5% of all brain tumours.(19,35) In population based studies about 20-35% of oligodendrogliomas are considered anaplastic tumours.(1)

Anaplastic oligodendrogliomas are commonly seen in adults with a peak incidence between 45-50 years, which is on an average 7-8 years later than grade II tumours. Males are slightly more commonly affected than females.Frontal lobe is the most common site, followed by temporal lobe. Grossly, tumour resembles oligodendrogliomas. Areas of tumour necrosis may be seen.(1)(21)
On neuroimaging, anaplastic oligodendrogliomas show a heterogeneous pattern due to the variable presence of necrosis, calcifications, cystic degeneration and intra tumoural haemorrhages. Contrast enhancement is seen on CT and MRI imaging in majority of tumours.(21)

Microscopy: Similar to oligodendroglioma, cells show morphological features of oligodendroglial cells, like round nuclei, perinuclear halos and few cellular processes. Characteristic branching capillary vascular pattern and micro calcifications are frequently seen. Anaplastic features that are associated with malignancy include high cellularity,

increase in mitotic activity, cytological atypia, microvascular proliferation and necrosis, with or without pseudo palisading. (5) The presence of any one of the above histological features does not equate to anaplastic oligodendroglioma. Microvascular proliferation and brisk mitotic activity are of particular importance and they were found to be significantly associated with patient survival. Some authors prefer a cut off of 6 per 10 hpf to distinguish grade II and III oligodendrogliomas.(33) Since, number of mitotic figures is an important criteria in the diagnosis of anaplasia, caution is necessary in the distinction of mitotic figures from apoptotic/pyknotic nuclei. The proliferation index can be also be used to assess the level of proliferative activity. Several studies have suggested a MIB-1 labeling index of more than 5% to distinguish anaplastic oligodendrogliomas.(21) Gliofibrillary oligodendrocytes and minigemistocytes are more common in anaplastic oligodendrogliomas, but they do not have any prognostic significance.

Unlike anaplastic oligoastrocytomas, the presence of necrosis does not affect the prognosis of anaplastic oligodendrogliomas, as long as the tumour shows typical cytological and histological features of oligodendrogliomas. (36) Care should be taken to look for an astrocytic component in this scenario as this can alter the histological grade of these tumours. In contrast to high grade astrocytic tumours, the existence of grade IV high grade pure oligodendroglial neoplasms is controversial.(36)

Chromosome 1p and 19q deletions can be seen in up to two thirds of anaplastic oligodendrogliomas, which is less common than in its Grade II subtype where it is present in about 80% of the cases(1)

Oligoastrocytoma

According to WHO, Oligoastrocytoma is defined as *"a diffusely infiltrating glioma composed of a conspicuous mixture of two distinct neoplastic cell types morphologically resembling the tumour cells in oligodendroglioma and diffuse astrocytoma of WHO grade II".*
Oligoastrocytoma corresponds to WHO Grade II. (1)

The annual incidence is estimated as 0.1 per 100000.(35) Due to the subjective nature of judgments regarding just what qualifies as astrocytic versus oligodendroglial, there is wide variation in the incidence of oligoastrocytoma and it is one among the least reproducible diagnoses in surgical neuropathology. Mixed oligoastrocytomas accounted for about 9% of all glial tumors in the Norwegian Cancer Registry, while they formed only 3.3% of all glial in the Central Brain Tumor Registry of the United.(1) In a study of 155 tumours, diagnosed initially as oligoastrocytomas, the interobserver variability was great ranging from 9-80%.

Oligoastrocytomas are moderately cellular tumours that may contain micro calcifications or cystic degeneration, demonstrate minimal mitotic activity. Necrosis or endothelial proliferations are not present. The diagnosis requires recognition of distinct neoplastic astrocytic and oligodendroglial phenotypes. The tumor may be either biphasic, where the two components are clearly adjacent to each other ("compact"), or intermingled, when the components are diffusely intertwined ("diffuse").(1)

Oligoastrocytomas are heterogeneous tumours that have histological and molecular features that overlap between oligodendrogliomas and astrocytomas. But the distinction is often problematic because of the lack of clear discriminating criteria. Quantifying

oligodendroglial and astrocytic areas and applying threshold values is complicated by the problem of sampling bias. Also the reactive changes at the edges of the tumour can mimic neoplastic astrocytic proliferation. (25)Some authors have strict criteria and request a minimum of 50% neoplastic astrocytes, while others are satisfied with the presence of one single high power field of either astrocytic or oligodendroglial cells.(20). Recent European Organisation for Research and Treatment of Cancers (EORTC) and Radiation Therapy Oncology Group (RTOG) trials used the presence of 25% oligodendroglial elements for the diagnosis of a mixed tumor. (37).

Based on molecular studies, oligoastrocytoma has an intermediate position between astrocytomas and oligodendrogliomas. LOH (Loss of heterozygosity) 1p and LOH 19q are seen in 30-70% of oligoastrocytomas, genetically resembling oligodendrogliomas. Whereas, about 30% show mutations in TP53 gene or LOH 17p, suggesting a relation to diffuse astrocytomas.(25)

The original interpretation of two distinct patterns was that these mixed gliomas were 'collision' tumours. According to this hypothesis, two separate glial tumours arose side by side in a patient with a local propensity for glioma formation. This hypothesis is no longer acceptable in the light of recent molecular findings. On micro dissection, similar genetic alterations have been identified in oligodendroglial and astrocytic portions of the oligoastrocytomas, indicating a clonal origin. In accordance with these findings, the alternative hypothesis is that mixed gliomas are monoclonal tumours that show phenotypic heterogeneity at the morphological level. This might be because of regional variations in growth factors and signals that promote differentiation along different glial lineages.(21)

Anaplastic oligoastrocytoma

Anaplastic oligoastrocytoma is defined as "*An oligoastrocytoma with histological features of malignancy, such as increased cellularity, nuclear atypia, pleomorphism and increased mitotic activity.*" Anaplastic oligoastrocytoma corresponds to WHO grade III.(1)

Precise epidemiological data on the incidence of anaplastic oligoastrocytoma is not available. In a study of 987oligodendroglial and astrocytic tumours, only 11 (1.1%) were diagnosed as anaplastic oligoastrocytomas.(35). Another study of 1093 patients with newly-diagnosed high grade gliomas in adults included 215 (20%) anaplastic oligoastrocytoma patients .(1)(36)

Histologically, these tumors show features of anaplasia, including nuclear atypia, cellular pleomorphism, high cellularity, and increase in mitotic activity. Microvascular proliferation may be present. However, the grading is quite subjective and less reproducible than high grade astrocytomas with wide variability in survival rates.(5, 15)

Glioblastoma with oligodendroglial component

Glioblastoma multiforme witholigodendroglial component is a recent addition to the WHO classification. According to a study conducted by Miller et al(36) on high grade gliomas, the presence of necrosis in anaplastic oligoastrocytomas was associated with a poor prognosis. The median overall survival ofanaplastic oligoastrocytoma patients with necrosis (22.8 months) was significantly lower than patients with non- necrotic anaplastic oligoastrocytomas (86.9 months), but was better than conventional glioblastomas (9.8months). This justified the distinction of anaplastic oligoastrocytomas with necrosis as

a grade IV tumour. According to WHO 2007, these tumours are now classified as 'Glioblastoma with oligodendroglial component'.

These tumors follow an aggressive course but generally tend to have a better prognosis than classical glioblastoma multiforme (GBM). (1)(32)(38–41)However, few studies have shown that these tumours have a similar clinical outcome as that of classical GBMs. (42,43)(44) Some authors classify anaplastic oligoastrocytomas as glioblastoma only when the necrosis is present in the astrocytic component and not in the oligodendroglial component. (20)

Since it is a recent entity, the exact incidence of these tumours is uncertain. The reported frequency of GBMOs ranges from 4.2% to 20% among glioblastomas.(38,41,42,45,46) They are seen at a younger age when compared to classic glioblastomas.

1p/19q co deletion is seen in 3 to 29.6% of GBMOs which is slightly more common than classical glioblastomas. (38,40–44,47). These tumours represent a subgroup of glioblastoma associated with a high prevalence of IDH1 mutations (23.8% versus 4.4% in classical GBMs)(43)

Factors influencing prognosis in oligodendroglial tumours

There are various factors which have important prognostic significance in oligodendroglial tumours.

Age

Studies have shown that age of the patient is associated with prognosis and survival. Patients with age ≥ 40 years was independently associated with poor survival in multivariate analysis (48–50).

Clinical presentation

Initial presenting symptoms, like incidental detection of tumour and seizures had a better prognosis over other presenting symptoms like headache or other neurological symptoms.(50)Patients presenting with neurological deficits was associated with a poor survival.(48)

Karnofsky performance status (KPS)

The degree of disability and Karnofsky performance status (KPS) are relevant prognostic markers. Patients with a KPS score of 90 or 100 had a longer survival than patients with a KPS of 80.

Size

Large tumour size is associated with a bad prognosis probably owing to the difficulty in completely resecting the tumour.(51)(48)

Location

Many studies have shown that tumour location is an important predictor of overall survival. (25,51,52). Tumours located in the eloquent areas had a worse prognosis than those in the non -eloquent areas. An eloquent area is definedas any involving one or more of the following which contain the functional areas of the brain : internal capsule, basal ganglia, language cortex, sensory cortex, motor cortex, thalamus and hypothalamus.(51) It was found that tumours located in the frontal, parietal, and occipital lobes were significantly more likely to harbour 1p/19q deletions and were associated with a better prognosis that those in the temporal lobe.(25,53) It was found that oligoastrocytomas of non temporal origin had more 1p loss than those in temporal lobes and these tumours had a better prognosis, and behaved more closely to oligodendrogliomas. On the other hand,

oligoastrocytomas in the temporal lobe showed more frequency of TP53 mutations and behaved more aggressively similar to diffuse astrocytomas.(25)

Extent of tumour resection

Recent studies have shown that extent of tumour resection has a significant effect not only the rate of tumour progression and overall survival but also on the decrease in risk of anaplastic transformation.(51,54) Gross total resection of tumour is associated with a better prognosis. (50) However, gross total resection of tumour is sometimes difficult or impossible without serious neurological deficits especially when the tumour is located in eloquent cortical and subcortical functional pathways. Aggressive resections are more likely to be performed in patients with favourable tumour characteristics like non eloquent area, small size and localised mass.(51)(54)

Histology and WHO Grade

Currently, the WHO classification is widely followed and several studies have confirmed WHO grade as a significant predictor of survival. (21)Presence of oligodendroglial component is associated with a longer survival in low and high grade gliomas.(35)(36,55) Classical oligodendroglial histology is a strong predictor of clinical outcome.(56). Features considered classic for oligodendroglioma (CFO) are round/regular nuclei, cellular monomorphism, presence of hyper cellular tumour nodules, micro calcifications, chicken wire vasculature and micro cysts. Classical oligodendroglial histology is closely associated with 1p/19q status. It has also been found this histological feature is an independent prognostic factor and provides significant prognostic information in addition to 1p/19q status.(52).

1p/19q status

Two prospective studies conducted by RTOG and EORTC confirmed that 1p/19q co-deletion are significantly associated with a better prognosis in anaplastic oligodendroglial tumours and may have a better initial response to PCV chemotherapy and radiotherapy.(12)(14)The prognostic relevance of combined 1p and 19q deletion of low grade gliomas is more controversial. Majority of reports suggest that 1p/19q loss is associated with better prognosis in low grade oligodendroglial tumours.(11)

The pattern of 1p/19q loss also has prognostic relevance. The predominant pattern of 1p and 19q alterations among oligodendroglial tumours showed concurrent total 1p/19q loss, while astrocytic tumours involved partial deletions of one or both chromosomes. While total 1p loss is associated with a better prognosis, partial loss of 1p is associated with a poor prognosis.(57)(37) It is important to keep this in mind because FISH probes used to assess 1p loss are often located on 1p36.6 which also detects partial loss of 1p and thus may not be able to differentiate between complete and partial loss of 1p.(37)

MGMT promoter methylation

Recent studies have shown that MGMT promoter methylation have been documented in 60-90% of adult oligodendrogliomas and is suggested as one of the reasons for increased susceptibility of oligodendroglial tumors to chemotherapy and hence better prognosis.(21) The promoter of the *MGMT* gene has been found to be hypermethylated in various human cancers, including subsets of astrocytic gliomas and oligodendroglial tumors. O6 methyl guanine DNA methyltransferase (MGMT) is a DNA repair enzyme that may cause resistance to DNA alkylating agents commonly used in the treatment of gliomas. Methylation of the promoter of MGMT turns off gene transcription and thus reduces resistance.

IDH 1/2 mutations

Various studies have shown that IDH (Isocitrate dehydrogenase) mutations are associated with a younger age. It is well established that IDH1 mutations are a significant prognostic marker of favourable outcome in patients with glioblastoma and anaplastic glioma but its role in low grade gliomas needs to be established.(58)

Other genetic changes

Oligodendrogliomas of all grades with atypical histological features and high grade oligodendroglial tumors usually show chromosomal abnormalities commonly associated with astrocytic tumors. These include EGFR (Epidermal Growth Factor Receptor) amplification, 10q loss and PTEN (Phosphatase and tensin homologue) mutations. These tumours are associated with a poor prognosis.(37)

Chromosome 1p/19q deletion

The combined loss of genetic material from the short arm of chromosome 1 and long arm of chromosome 19 is considered as the molecular signature of oligodendroglial tumours.(1)

1p/19 q deletions are seen in approximately 80% of oligodendrogliomas, 50-60% of anaplastic oligodendrogliomas and 30-50% of oligoastrocytomas and anaplastic oligoastrocytomas.(1)

Astrocytic tumours rarely shows 1p/19q deletion(1). According to a study conducted at the Christian Medical College, Vellore on 100 gliomas it was found that 1p/19q deletion was

seen in 72.7% of oligodendrogliomas, 90.9% of anaplastic oligodendroglioma, 22.2% of mixed oligoastrocytomas and 42.9% of the anaplastic oligoastrocytomas.(59)

Early studies by Cairncross et al in the late 1980s and early 1990s showed that both recurrent and newly diagnosed anaplastic oligodendrogliomas have an improved chemotherapeutic response to the PCV regimen using combination of procarbazine, lomustine, and vincristine.(3)(2)Many independent studies from various institutions have substantiated these findings. (27–30)PCV regimen was long used as the chemotherapy of choice for both adjuvant therapy and also for treatment of recurrences. However, the administration of PCV chemotherapy is associated with severe haemotological toxicity causing high morbidity. Because of its better toxicity profile, another alkylating drug, Temozolamide has largely replaced PCV chemotherapy. Studies have shown that oligodendroglial tumours also show good response to treatment with temozolamide.(4,5,31)

In 1998, Cairncross et al first reported that 1p/19q deleted anaplastic oligodendrogliomas showed a better response to PCV chemotherapy and had longer survival times. (60)Three prospective randomized clinical trials demonstrated that 1p/19q deleted anaplastic glioma patients live longer on treatment with radiotherapy (RT) or chemotherapy with alkylating drugs or both.(14)(12)(13) 1p/19q co-deletion is thus both a prognostic and predictive marker.(61)

A North American Randomised Controlled study (Trial 9402) of the Radiation Therapy Oncology Group (RTOG) aimed to study the effect of early chemotherapy versus radiotherapy, and 1p/19q deletion on anaplastic oligodendroglial tumours. 289 patients

with anaplastic oligodendroglioma and anaplastic oligoastrocytomas were included and randomized to two arms. One arm received neoadjuvant PCV chemotherapy followed by radiotherapy, while the other arm received only radiotherapy.(14)(62) It was found that treatment with combined PCV chemotherapy and RT improved progression free survival but not overall survival when compared to patients who received only RT. 1p/19q status was determined by Fluorescence in situ hybridisation (FISH). 1p/19q co deletion was present in 48% of the patients. Frequency of co-deletion was more in pure oligodendroglial tumours than in mixed tumours (76% of anaplastic oligodendrogliomas while only 24% in anaplastic oligoastrocytoma showed co-deletion of 1p/19q). Patients with combined losses of 1p and 19q had a more favourable natural history, longer survival and better response to treatment. The better progression free survival seen in patients randomized to PCV and RT was statistically significant only in the 1p and 19q deleted subset of patients. This finding shows that 1p/19q co deletion is both a predictive and prognostic marker in anaplastic oligodendrogliomas and anaplastic oligoastrocytomas.

A similar prospective study conducted by EORTC included 368 patients with anaplastic oligodendroglioma or anaplastic oligoastrocytoma. Patients were randomized to radiotherapy or radiotherapy followed by PCV chemotherapy. In this study, only 25% of patients (80 patients) showed 1p/19q deletions. Patients with combined loss of 1p and 19q were found to be very sensitive to PCV chemotherapy, with almost all the patients responding to treatment. In the initial update of the study in 2006, it was found that adjuvant chemotherapy increases progression free survival but did not improve overall survival. The 2012 update,1p/19q codeleted tumours demonstrated a survival advantage for patients treated with early adjuvant PCV chemotherapy(63)(12).

Many studies have shown 1p/19q loss to be a strong predictor of long progression free survival and overall survival in grade III tumours whether they receive adjuvant therapy in the form of radiotherapy or chemotherapy in combination or alone. The long term follow up results from the EORTC trial shows an overall survival benefit in patients treated with combined treatment versus RT alone. But, patients with 1p/19q deleted tumours survived longer than patients with intact 1p/19q in both study arms patients. This suggests that the therapeutic response of 1p/19q deleted tumours also extends to radiotherapy and is not only restricted to chemotherapy .(63)(61)

Whilst the role of 1p/19 loss is well recognized in Grade III oligodendroglial tumors their role in prognosis is less well defined in grade II neoplasms. Most studies have shown that 1p/19q is an independent prognostic factor even in low grade oligodendroglial tumours. Also, imaging studies have shown that 1p/19q co deleted tumours have a slower growth rate in low grade tumours.(37)(64)

It is not clear whether 1p/19q deleted tumours represent a different genetic subtype that is more responsive to genotoxic treatment or whether these tumours generally take a less aggressive course regardless of the treatment modality. Generally, 1p/19q deleted tumours tend to have slower growth rates and are more responsive to treatment than tumours lacking this co-deletion. It is clear from all the aforementioned studies that 1p/19q co-deletion not only identifies a prognostically favourable subgroup of gliomas, it also predicts the outcome to treatment. It is therefore both a prognostic and predictor marker.(37)

These observations have made it necessary to accurately classify oligodendroglial tumours and distinguish them from astrocytic tumours since the prognosis and management are

different for both these tumours. Currently, the WHO classification of gliomas is based on traditional histopathological criteria which have a lot of limitations especially in the classification of high grade tumours and mixed astrocytic and oligodendroglial tumours. The lack of stringent morphological criteria accounts for the high interobserver variation in classification and grading of oligodendroglial tumours even among experienced neuropathologists. (6)Also, the prospect of beneficial chemotherapy has probably prompted pathologists to diagnose more oligodendroglial tumours so as not to deny any patient the possible benefits of chemotherapy. Hence, there is a great demand for an unambiguous classification of gliomas. Current studies show that assessment of molecular markers when combined with histology will make a more robust and prognostically significant classification.

It was recently demonstrated that the combined deletion of 1p and 19q is mediated by an unbalanced whole arm translocation between the short arm of chromosome 1 and the long arm of chromosome 19. This result in formation of two derivative chromosomes, one composed of 1q and 19p, and the other of 1p and 19q. Subsequent loss of the derivative chromosome, der(1;19)(p10;q10) results in the loss of both 1p and 19q. However, the exact breakpoint of translocation is not clear.(11)(65)(57)

According to a study by Jenkins et al, the prevalence of the translocation, t(1;19)(q10;p10) was found to be 44% in low grade oligodendrogliomas. They found that these tumours were independently associated with a better outcome. They also observed that 7 gliomas showed evidence of fusion but without deletion. This may be evidence that fusion is an early event and precedes translocation or deletion. The strong homology of the

centromeric regions of chromosome 1 and 19 suggests a centromeric or pericentromeric fusion as a possible mechanism for the translocation. Epigenetic alterations may contribute to the underlying centromeric instability in tumours which can predispose to translocations. This proposed mechanism is supported by the observation that combined deletions of 1p and 19q are highly correlated with hypermethylation of a large number of genes.(11) There is a strong association between 1p/19q co deletion and MGMT promoter methylation. It was observed in up to 80-90% of 1p/19q deleted tumours. MGMT hypermethylation is thought to be one of the possible explanations for the increased chemosensitivity of oligodendroglial tumours.(37)

The pattern of 1p and 19q deletion is also important and is different in oligodendrogliomas and astrocytomas. While combined loss of whole arm of chromosome 1p and 19q is specific and characteristic of oligodendrogliomas, partial interstitial deletions of 1p or 19q, commonly involving regions 1p36 or 19q13, is seen in astrocytic tumours. Only whole arm deletion of 1p and 19q is associated with a good prognosis. In a study of 363 astrocytic and oligodendroglial tumours, Vogazianou et al found that while total 1p/19q loss showed significantly better overall survival, patients with other 1p/19q status showed significantly shorter survival when compared to tumours with normal 1p/19q status. (57)

The most commonly used methods for detection of 1p/19q deletions in the laboratory are either FISH or loss of heterozygosity analysis (LOH) using microsatellite markers. These methods are technically simpler than array-CGH (Comparative Genomic Hybridisation). The major disadvantage of FISH or LOH is that they may not always be able to distinguish interstitial or partial deletions of chromosome 1p and/or 19q from total loss. Evaluation of

1p/19q status by FISH provides additional information of copy number assessment of polysomy, which is not possible by LOH analysis. The commonly used FISH probes are located on 1p36.32 and 19q13.32. These probes may also pick up smaller interstitial deletions which are commonly seen in astrocytic tumours and associated with a poor prognosis. Vogazianou et al identified 8 tumours with partial deletions of the 1p36/19q13 loci which would have been misinterpreted as having total 1p/19 loss. Thus, these cases would have been wrongly interpreted to have a better prognosis. The authors suggest using FISH probes from different loci to avoid this error in interpretation.(57)

Few cases of 1p/19q deletion also showed polysomy of chromosome 1 and 19. Polysomy is a risk factor of unfavourable outcome. They are more commonly found in mixed tumours than in pure oligodendroglial or astrocytic tumours and are found less frequently in low grade tumours.(66). Snuder et al showed that polysomy for chromosome 1 and 19 in anaplastic oligodendrogliomas with combined 1p/19q loss predicts earlier recurrence(67). He also found that increased MIB-1 labeling index was not associated with polysomy. Further studies have shown that irrespective of tumour grade, polysomy of chromosome 1 and/or chromosome 19 in 1p/19q co deleted tumours are associated with a shorter overall survival and progression free survival. (66,68,69)

Role of assessment of 1p/19q deletion in diagnosis

As 1p/19q deletions are quite specific for oligodendrogliomas, it can be used as a diagnostic marker in routine practice. There are a number of CNS tumours that show histological features mimicking oligodendrogliomas. (42) the most commonly encountered diagnostic difficulty is differentiating it from diffuse astrocytomas. Unlike

oligodendrogliomas, astrocytomas display more pleomorphism, irregular nuclei, fibrillary background and minimal halos. A subset of gliomas show features intermediate between the two often posing diagnostic challenges. Many of these tumours have been classified as mixed oligoastrocytomas. The large interobserver variability in the diagnosis of diffuse gliomas, especially in the diagnosis of mixed gliomas presents an opportunity for the use of 1p/19q status as a useful adjunct and diagnostic marker. Astrocytomas are commonly associated with other genetic alterations such as TP53 mutation, gains in chromosome 7 or loss of chromosome 10q. 1p/19q deletions are rare in astrocytomas.Small cell glioblastoma are cytological bland and monomorphous and can resemble high grade oligodendrogliomas. These tumors commonly show EGFR amplification.

Other diagnostic considerations include neurocytoma, dysembryonic neuroepithelial tumours (DNET), clear cell ependymoma and metastatic clear cell carcinoma. Extraventricular neurocytoma may be very difficult to differentiate from oligodendrogliomas. Neurocytomas are well defined, WHO grade II tumours and show rosettes or neuropil islands positive for synaptophysin. A significant number of nuclei are positive for Neu N and these tumors express synaptophysin. It has been found that oligodendrogliomas can also exhibit neurocytic differentiation. But, neurocytomas do not show 1p/19q loss. Another important differential is to distinguish an oligodendroglioma from a DNET. DNET is a WHO grade I tumour and does not require additional treatment following complete surgical resection. DNETs commonly present in children and show mucin rich nodules and floating neurons but are cytologically similar to oligodendrogliomas. These tumours are negative for 1p/19q deletion. On immunohistochemistry, the oligodendroglia like cells are positive for S-100 and neurons

are positive for NeuN and Synaptophysin. Clear cell ependymoma is another paediatric tumour which does not show 1p/19q deletion and can mimic oligodendrogliomas. Another important thing to note is that most of the paediatric oligodendrogliomas do not show 1p/19q loss and hence it may not be very useful as a diagnostic marker in this setting. But 1p/19q deletions, when present favours a diagnosis of oligodendroglioma.(1,70)

Classical oligodendroglial histology is a strong predictor of clinical outcome.(56). Features considered classic for oligodendroglioma (CFO) are round/regular nuclei, cellular monomorphism, presence of hypercellular tumour nodules, micro calcifications, chicken wire vasculature and microcysts. Studies have shown that the loss of 1p/19q is associated with tumours with a classic histology.(52,56) It is also known that classical histology is associated with a better prognosis.In one of the aforementioned study on 247 anaplastic oligodendroglial tumours, both classic oligodendroglial morphology and 1p/19q deletion were independently associated with improved PFS and OS on multivariate analysis (56).

1p/19q deletion status and location of tumor

There is association of tumour location with chromosome 1p/19q co deletion. Tumours located in the frontal, parietal and occipital lobes were more likely to harbor 1p/19 deletion than tumours in the temporal lobe, insula and diencephalon (25,56)(52)(71). Mueller et al found that oligodendrogliomas and oligoastrocytomas arising in non-temporal sites show increased frequency of 1p/19q deletion, while temporal oligoastrocytomas share genetic features of astrocytomas, like presence of TP53 mutations and absence of 1p/19q deletion.(25) Another study by Kouwenhoven et al on anaplastic oligodendroglial tumours showed that tumours with 1p/19q deletion were commonly seen in the frontal

lobe with a predominant pure oligodendroglial component, while tumours with EGFR amplifications and copy number alterations of chromosomes 7 or 10 were located outside the frontal lobe and showed a mixed oligoastrocytoma phenotype.(72)

Imaging and 1p/19q deletion

Imaging parameters also vary according to 1p/19q deletion status. Loss of 1p/19q is positively associated with mixed intensity signal on T1 and T2 images, indistinct borders on T1 images, paramagnetic effect (shortening on T1 and T2) and presence of calcifications.(73)

CIC and FUBP1 Mutations and 1p/19q deletion

Until recently, search for putative tumour suppressor genes on chromosomes 1 and 19 were largely unsuccessful. Recentexomic sequencing of oligodendrogliomas showed recurrent inactivating mutations in two tumor suppressor genes: homologue of Drosophila gene capicua (CIC) and Far Upstream Element Binding Protein (FUBP1) in 53% and 15% of oligodendroglioma respectively. (74)Since CIC is located on chromosome arm 19q and FUBP1 is located on chromosomal arms 1p, 1p/19q loss is thought to be a mechanism that inactivate CIC and FUBP1. CIC mutations were rare in other cancer types.(74)

Majority of CIC mutant oligodendrogliomas were clustered in exon 5 and exon 20. Exon 5 encoded a conserved DNA binding region, while exon 20 encoded a conserved domain lacking annotation in humans.

It was found that CIC mutations were closely associated with classic oligodendroglioma histology and therefore also associated with other oligodendroglioma related mutations in

IDH1/2 mutations and 1p/19q loss. It is suggested that the mutant CIC on the single retained 19q allele is linked to the pathogenesis of oligodendrogliomas with IDH mutation.

In a study by Yuchen Jiao et al, they found CIC mutations in 38% and 52% of grade II and II oligodendroglioma and only 6% and 9% of grade II and II of mixed oligoastrocytomas respectively. (75)Though allelic loss of regions of chromosome 19q and 1p harbouring CIC and FUBP1 are present in both oligodendrogliomas and oligoastrocytomas, CIC and FUBP1 mutations have been detected in oligodendrogliomas but are relatively rare in oligoastrocytomas.

IDH (Isocitrate Dehydrogenase) Mutation

Five genes, IDH1, IDH2, IDH3A, IDH3B and IDH3G encode for three isoenzymes of isocitrate dehydrogenase - IDH1, IDH2 and IDH3. Both IDH1 and IDH2 form homodimers, while IDH3 forms a heterotetramer which contains two alpha, one beta and one gamma subunit. The function of all isocitrate dehydrogenases is oxidative decarboxylation of isocitrate to alpha-ketoglutarate. During this enzymatic reaction, Nicotinamide adenine dinucleotide (NAD) in the case of IDH3 and Nicotinamide adenine dinucleotide phosphate (NADP) in the case of IDH1 and IDH2 acts as electron receptors and are converted to their reduced forms,NADH and NADPH respectively. IDH3 is seen in the mitochondria and is part of the Krebs cycle. IDH1 is seen in the cytosol and peroxisomes and takes part in lipid synthesis and cellular glucose sensing, while IDH2 is seen in the mitochondria where it maintains redox potential. Both IDH1 and IDH2 protect the cell against oxidative stress.(76–79)

The IDH1 gene is located on chromosome 2q33.3.Glioma specific mutations in IDH1 always affect the amino acid Arginine at position 132 of exon 4. The mutated sequence is located in an evolutionary highly conserved region and corresponds to the binding site for isocitrate. The most common type of mutation seen is IDH1 is R132H, where the amino acid Arginine is substituted by Histidine (codon CGT to CAT change). Other less frequent IDH1 mutations include substitution Arginine to Cysteine (R132C, codon CGT to TGT), Arginine to Glycine (R132G, codon CGT to GGT) and Arginine to Serine (R132S, codon CGT to AGT). A study by Hartman et al found that R132C was significantly associated with astrocytomas.(80) Interestingly, it was found that astrocytomas that developed in families with Li-Fraumeni syndrome contained the R132C mutations. Li- Fraumeni syndrome is a rare cancer predisposing disorder caused by mutations in TP53, which is also the commonly mutated gene in astrocytomas.(76,80)

IDH2 gene is located on chromosome 15q26.1. Mutations in IDH2 are seen at position 172 involving amino acid acid arginine, which is analogous to codon 132 in IDH1. The most common mutation involving IDH2 is R172K (Arginine to Lysine), followed by R172M and R172W. IDH2 mutations predominantly occur in oligodendroglial tumours.(80)

There are two types of mutations seen in cancer. Driver mutations cause and promote cancer, while passenger mutations occur concomitantly as a result of driver mutations. It was found that introduction of mutant IDH into normal cells causes increased colony formation, proliferation and inability of cells to differentiate. Also, somatic mosaicism for IDH1 or IDH2 causes enchondromatosis syndromes, Maffucci and Ollier's syndrome which

carry an increased risk of gliomas. These two evidences support the concept that IDH1 mutation is a key driver of oncogenesis of tumour formation.(76,81)

The exact mechanism of oncogenic tranformation is still controversial. Expression of IDH1 and IDH2 mutant protein expression decreases isocitrate dependent NADPH production. This suggested a tumour suppressor function of IDH mutations. Heterodimers are formed between mutant IDH allele and wild type allele within the same cell, thereby reducing the activity of wild type protein by a dominant negative inhibition.(76,79) One of the most striking features of IDH1/2 mutations is that the same amino acid residue (Arginine) is always mutated. In IDH, both R132 and R172 are highly conserved regions and create hydrophilic interactions that allow binding of isocitrate. The amino acids that substitute arginine are many which strongly suggest that it is the replacement of arginine and not the new amino acid which supports tumorigenesis.(78) Mutation of IDH1 changes substrate specificity and directionality of the enzyme. The mutant IDH1 protein converts alpha ketoglutarate (α-KG) to 2 hydroxy glutarate (2-HG) and not to isocitrate. These findings led to the hypothesis that IDH is an oncogene rather than a tumor suppressor gene and 2 hydroxy glutarate is an 'oncometabolite'(76,78)

Reduced levels of alpha KG in mutant IDH cells promote cellular accumulation of Hypoxia inducible factor (HIF-1α). Induction of HIF-1 target genes that have effect on angiogenesis, metabolism, apoptosis, cell motility, growth and differentiation. In turn, high levels of 2-HG inhibits the activity of α-KG dependent enzymes, histone demethylases and 5-methyl cytosine hydroxylase, thus bringing about genome wide hypermethylation. Thus, reduced

α-KG and increased levels of 2-HG contribute to tumorigenesis through epigenetic alterations.(76)(Fig 1)

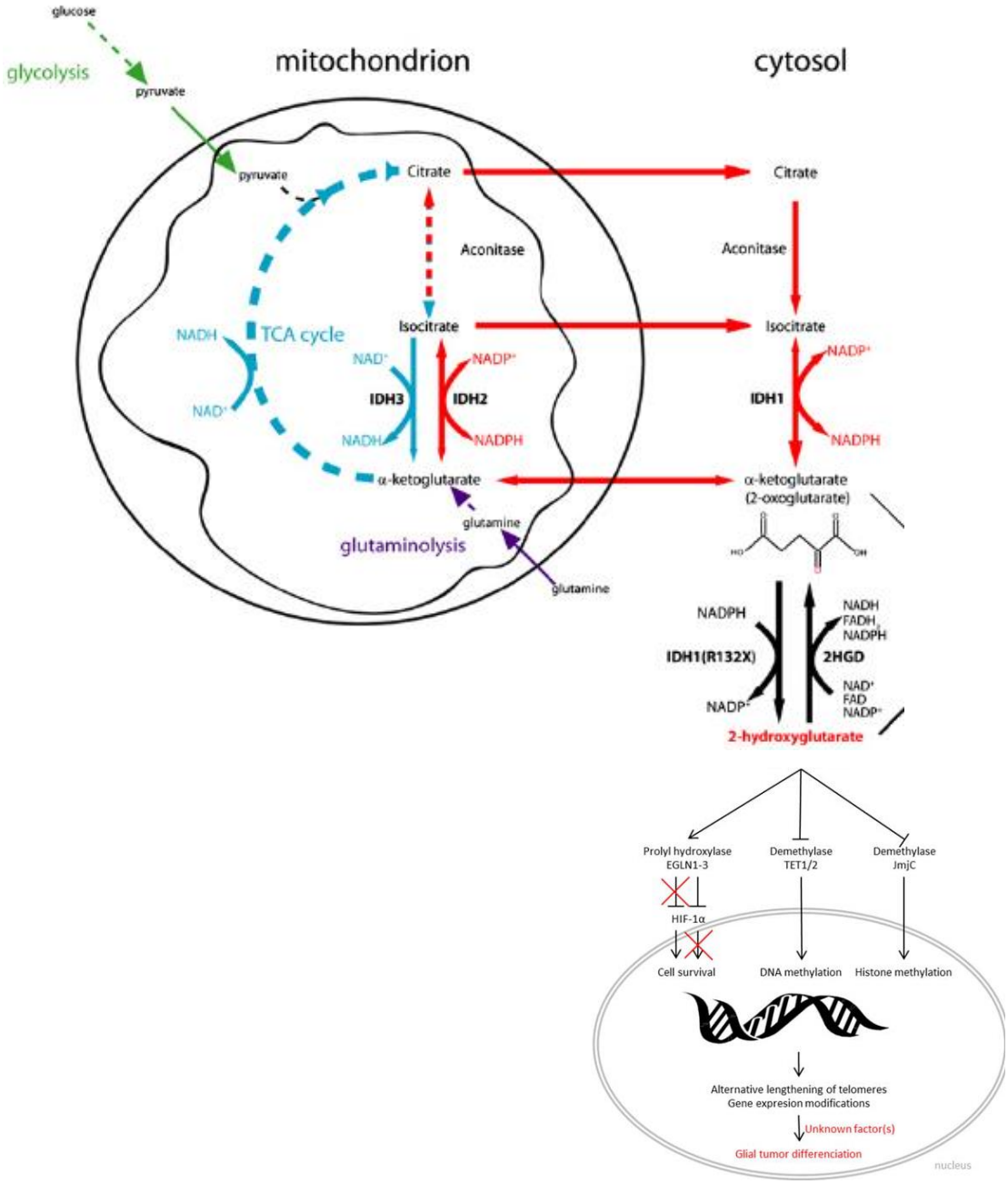


Figure 1. Effects of IDH1/2 mutations on metabolism in the cell(73,78)

IDH mutations and gliomas

Somatic mutations of IDH1 was initially detected in 2008 in 18 (12%) of 149 samples of glioblastoma multiforme. These mutations were commonly seen in in young patients that had progressed from low grade gliomas to secondary glioblastoma multiforme. Patients with these mutations were also associated with a longer survival.(82) These initial findings led to further studies which demonstrated mutations in genes encoding isocitrate dehydrogenase (IDH1) in most cases of diffuse astrocytoma, oligodendroglioma and oligoastrocytoma of WHO grade II and III with lower frequencies in genes encoding isocitrate dehydrogenase (IDH2).(80)

In a study of 1010 diffuse gliomas of grade II and III, it was found that IDH1 mutation was present in 70.9% of these tumours and IDH2 in 3.1% of the cases.(80) Several other studies also validate these findings with frequency of IDH1 mutations ranging from 60-80% in diffuse gliomas and secondary glioblastomas. (83)(84)(58)IDH1 mutations are rare in primary glioblastomas. The incidence of IDH1 mutations in astrocytic and oligodendroglial tumours is almost similar.(80)Few studies have reported a slight higher frequency in oligodendroglial tumours.(80,85)

In a study of 100 gliomas conducted at the All India Institute of Medical Sciences, India, IDH1 mutations were detected in 68.8% of grade II tumours and 85.7% of grade III tumours. 66.7% of secondary glioblastomas harboured this alteration as opposed to only 4.4% of primary glioblastomas. (58), 13 out of 14 (92.9%) anaplastic astrocytomas and 6 out of 48 (12.5%) GBMs. In this study, the frequency of IDH1 mutations in grade II and III astrocytomas was found to be higher than the globally reported numbers.(86)

Several studies suggest that IDH1 mutations are early events in the development of gliomas. (84,85,87)In a study by Watanabe et al (2009), analysis of multiple biopsies from the same patient did not reveal a single case in which an IDH1 mutation occurred after acquisition of a TP53 mutation (commonly associated with astrocytomas) or loss of 1p/19q, thus suggesting that IDH1 mutations are very early genetic events in glioma tumorigenesis. This suggests that astrocytoma, oligodendroglioma and oligoastrocytomas probably arise from common glial precursor cells carrying IDH1 mutations. Further loss of chromosome 1p/19q or acquisition of TP53 mutations may lead to oligodendroglial or astrocytic differentiation respectively. (84)

A study by Metellus et al showed that non mutated IDH tumours were associated with an older age, larger size (>6cm in size), infiltrative growth pattern on MRI and were frequently located in the insula.(88) In another study, IDH1 mutations were associated with frontal lobe location of tumour and inversely associated with necrosis.(89)

IDH 1 mutations are rare in pilocytic astrocytomas. This suggests that pilocytic astrocytomas have a different genetic etiology from that of diffuse gliomas. Pilocytic astrocytomas frequently harbor BRAF (B-Raf proto-oncogene) mutations. Hence, the combination of absent IDH1 mutation and presence of BRAF mutations distinguishes pilocytic astrocytomas from astrocytomas.(90) In a study by Sonada et al on Japanese glioma, IDH1 mutations were seen in 38% and 60% in gangliogliomas and anaplastic gangliogliomas.(91)IDH1 mutations are absent in other CNS tumours like ependymoma, DNET, schwannomas and meningiomas. Outside the CNS, IDH1 mutations have been reported in acute myelogenous leukemia, acute lymphoblastic leukemias cartilaginous

tumours, and rare cases of cholangiocarcinoma, prostate cancer, colon cancer and primary myelofibrosis. (77,92).

The genetic profile of primary and secondary glioblastomas differs significantly. Loss of heterozygosity 10p, EGFR amplification, MDM2 (Mouse double minute 2 homolog) amplification and PTEN mutations are typical of primary glioblastomas. While TP53 mutations, LOH 19q and LOH22q are more commonly seen in secondary glioblastomas. The relative high incidence of IDH1 mutations in secondary glioblastomas when compared to primary glioblastomas further suggests that these glioblastoma subtypes may have different origins. IDH1 mutation is the most specific molecular marker for secondary glioblastoma. (84)

IDH mutations are associated with a younger age. Several studies have shown that IDH mutation is an independent prognostic factor in glioblastomas and anaplastic gliomas. The prognostic role of IDH1 in low grade gliomas is controversial, though most of the studies have shown that IDH1 is prognostic even in this subset of tumours. Presence of IDH1 mutations do not predict outcome to adjuvant PCV chemotherapy.(13,81,85,89,93–95)

In a German Randomised controlled study (NOA-04 trial), Wick et al studied 318 anaplastic gliomas. Cases were randomized to three arms, 2:1:1 (A: B1:B2). Arm A received only radiotherapy, arm B1 received combination chemotherapy with procarbazine, lomustine and vincristine (PCV) and arm B2 received temozolamide. At unacceptable toxicity or progression, patients in arm A received either PCV or temozolamide, while patients in arms B1 and B2 received radiotherapy. They found no difference in survival time between the arms. But they found that IDH1/IDH2 mutations were strongly associated with a

favourable prognosis which was found to be more significant that MGMT promoter methylation and 1p/19q deletion.(13)

In another study by Takahashi et al, the author suggested IDH1 mutation to be a more reliable prognostic marker in anaplastic gliomas and glioblastomas. In contrast, MGMT methylation and 1p/19q co deletion, rather than IDH1 mutation status were more prognostic in anaplastic oligodendroglioma and anaplastic oligoastrocytoma.(81)

While most studies have shown IDH mutations to be a significant prognostic marker of favourable outcome in high and low grade gliomas, a study by Kim et al on a large number of low grade gliomas (360 cases) found that IDH1/2 mutations was not prognostic for survival in univariate and multivariate analysis. (83)

IDH1 mutations are strongly associated with molecular alterations such as 1p/19q co deletion and MGMT promoter methylation. They are inversely correlated with EGFR amplification, polysomy of chromosome 7 and loss of chromosome 10. (89)

IDH1 immunohistochemistry

Capper et al developed a monoclonal antibody targeting the IDH1-R-132H mutation. This antibody was shown to have high specificity and sensitivity in western blot and tissue sections from formalin fixed paraffin embedded tumor specimens. This antibody specifically identifies the substitution of amino acid arginine to histidine (R132H), which is the most common type of IDH mutation seen in >90% of the cases. It is useful for tumor classification, differential diagnosis of gliomas, detection of single infiltrating tumor cells

and characterization of the cellular role of mutant IDH1 protein.(15)Antibodies targeting other IDH1 and IDH2 mutations are also available.

IDH1 IHC can be used to distinguish astrocytoma/oligodendroglioma from pilocytic astrocytomas, DNETs or ependymoma and also to differentiate a neoplastic process from reactive gliosis.

Discovery of IDH mutations in glioma has opened up novel ideas of therapeutic approaches with possible druggable targets. These new avenues need to further evaluated.

Molecular expression profiling studies have proposed a new molecular classification of gliomas with four subgroups, neural, proneural, classic and mesenchymal based on specific genetic alterations(96). Secondary GBMs are virtually always proneural while primary GBM can be of any subtype. Mutations of IDH genes are closely linked to CIMP+ (CpG Island Methylation Phenotype), proneural subtype of glioblastomas. Majority of low grade gliomas have hypermethylated CpG islands throughout genome. This phenomenon is called glioma CpG island methylator phenotype. Turcan et al demonstrated that IDH mutation is the molecular basis of CIMP in gliomas. This highlights the interplay between genomic and epigenomic changes in human cancers.(76)

Conclusion

There are 3 proposed molecular pathways to the development of gliomas. (See Fig 2) First pathway starts with IDH mutation, followed by TP53 mutations/ATRX (Alpha thalassemia/mental retardation syndrome X) mutations resulting in formation of astrocytic tumours. These gliomas start as grade 2 diffuse astrocytomas with a G-CIMP phenotype and

then acquire further mutations resulting in progression to high grade tumours. Another pathway starts with IDH mutations, followed by 1p/19q deletion which is associated with mutations of CIC and FUBP1 genes. These changes result in formation of grade 2 oligodendrogliomas which later acquires other genetic alterations to become anaplastic oligodendrogliomas. The third pathway includes tumours with wild type IDH gene. They rapidly acquire multiple complex genetic alterations, like EGFR amplification and loss of PTEN to become glioblastomas.(76)

A molecular classification of gliomas provides distinct advantages, especially for oligoastrocytoma. The phenotypic heterogeneity of astroglial and oligodendroglial cell lineages and lack of reliable immunohistochemical markers makes it difficult to define diagnostic criteria in these mixed tumors. Poor characterization of these cell lineages causes considerable subjectivity in histological evaluation and marked interobserver variability. Despite their histological heterogeneity, oligoastrocytomas are genetically clonal neoplasms, usually showing either 1p/19q loss or TP53 mutations. Available evidence shows that oligoastrocytoma is unlikely to be a distinct entity, but arises from a common precursor cell with IDH1 mutation.

Currently, there are no significant differences in treatment of gliomas based on astrocytic or oligodendroglial differentiation, or based on molecular prognostic markers. Various molecular markers like 1p/19q deletion, IDH1/2 mutations and MGMT methylation have been found to be important prognostic factors that can predict response to treatment and overall survival in gliomas. This can help in personalized treatment for individual patients. Currently, treatment decisions are mainly based on WHO grade despite the fact that

outcomes vary significantly between glioma subgroups. In the near future, a refined classification that incorporates genetic signatures may become critical for management of these tumors.

Also, the detection of these novel mutations and chromosomal alterations opens the possibility for the discovery of novel drugs targeting these changes. This can lead to better therapeutic interventions and possible cure for theseneoplasms.

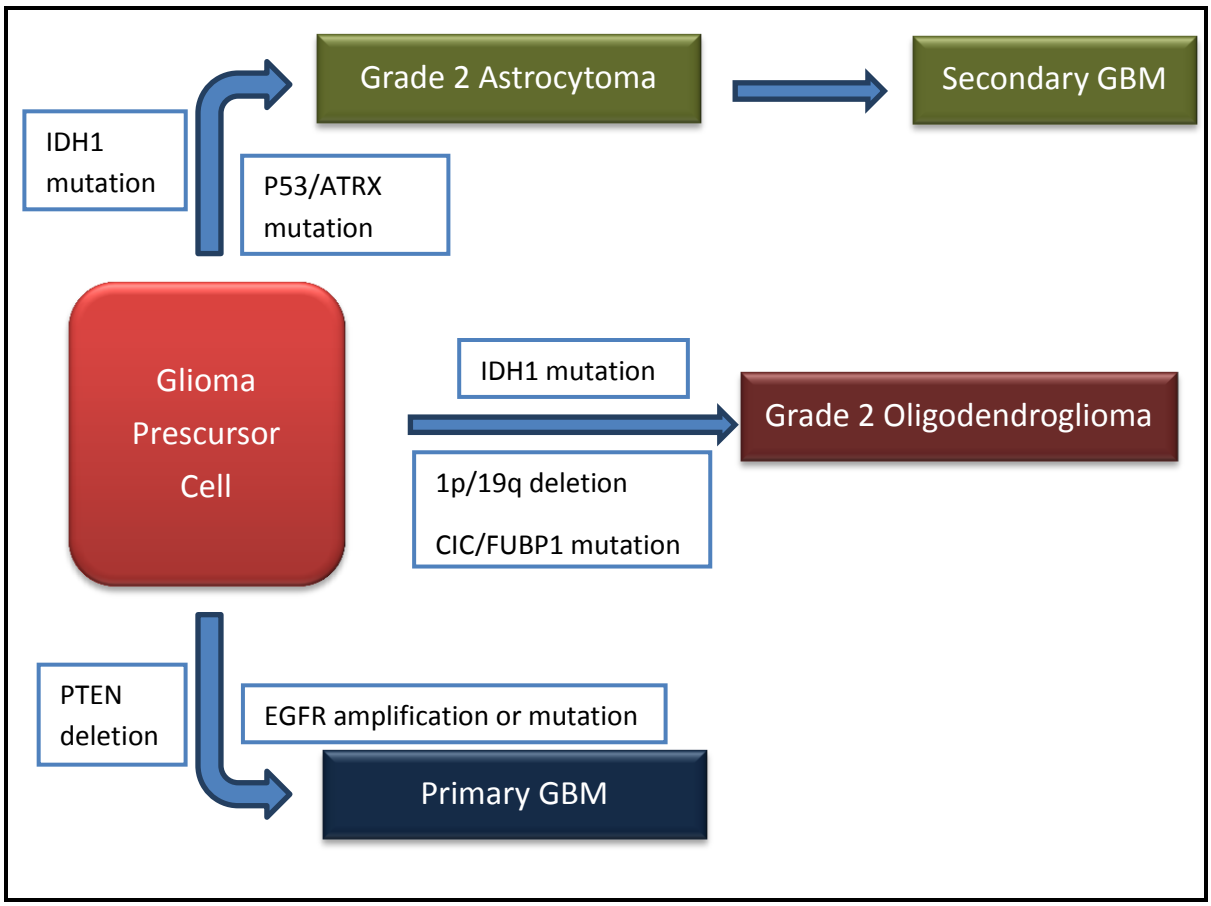


Figure 2. Schematic representation of the three possible pathways of gliomagenesis

MATERIALS AND METHODS

Tumour samples, histopathology and patient characteristics

This was a retrospective study in which 50 surgically resected cases of oligodendroglial tumours were obtained from the archives of the Department of General Pathology, Christian Medical College, Vellore from January 2009 to January 2012. Only cases with clinical follow up details and snap frozen fresh tumour tissue available in the tumor bank, were included in the study. Tumour types included were:

1. Oligodendroglioma, WHO Grade II

2. Anaplastic oligodendroglioma, WHO Grade III

3. Oligoastrocytoma, WHO Grade II

4. Anaplastic Oligoastrocytoma, WHO Grade III

5. Glioblastoma with an oligodendroglial component, WHO Grade IV.

Clinical and demographic details of the 50 patients such as age, gender, clinical presentation, duration of symptoms, and history of previous resection were obtained from the Department of Neurosurgery. Karnofsky performance scores (KPS) and clinical findings were also obtained from the aforementioned source.

Magnetic Resonance Imaging for site of tumour, laterality, size of tumour, borders, signal intensity on T1 and T2 was obtained from the Picture Archiving and Communication System (PACS).

Operative findings were noted for extent of tumor resection and classified as partial or total resection.

Follow up information was obtained from the Oracle based clinical work station, from the records of the Departments of Radiotherapy and Neurosurgery. Patients were followed up till July 2014.

Histopathology

The biopsy slides and paraffin blocks were retrieved from the archives. All cases were reviewed and the diagnosis confirmed. Detailed histopathological examination was performed as shown in the proforma (Appendix 1).

Classical features for oligodendroglioma like uniform round regular nuclei, well-defined nuclear contours, evenly dispersed chromatin, inconspicuous/small nucleoli, perinuclear halos and chickenwire vasculature were looked for in all neoplasms. Mitotic activity, necrosis and microvascular proliferation were utilized for grading. Tumours were classified as oligoastrocytoma if they had both astrocytic and oligodendroglial components with the oligodendroglial component constituting at least 25% of the tumour volume.

Arrangement of the two components as intimately mixed or present as discrete areas was noted. If necrosis and vascular proliferation were seen in the Oligoastrocytomas, the tumor was classified as a glioblastoma with an oligodendroglial component.

Data on the MIB-1 labeling index was obtained from the records.

Fluorescence in situ hybridization

Data on FISH for 1p and 19q was available for 28 of obtained from the records. For the remaining 22 cases FISH was performed and data provided by the FISH laboratory. The protocol followed is as given in the appendix. (Appendix 2) Presence of deletions and polysomies was noted.

FISH scoring was done usingVysis locus specific identifier (LSI) dual color probes localizing to 1p36/1q25; 19q13/19p13. The number of signals was counted in 200 cells. A ratio of 1p: 1q (or 19q: 19p) of 0.85-1.15 was considered normal with no deletion and a ratio of <0.75 was diagnosed as loss. The tumor was considered to have polysomy if ≥30% of nuclei showed three or more signals for 1q and 19p.(37)

Immunohistochemistry for IDH-1

For IDH1 immunohistochemistry, the monoclonal antibody against IDH1R132H was obtained from Dianova (Anti mouse antibody, clone H09).

Standardization of the dilution was done and optimal staining was done at 1:30 dilution.

Immunohistochemistry was then performed manually on Formalin-fixed paraffin-embedded (FFPE) tissue using the monoclonal antibody against IDH1R132H on all 50 cases using the protocol given below:

1. FFPE sections of thickness 5µm of tumour and control sections were floated onto poly-L-Lysine coated slides and incubated overnight at 37^0C.

2. Deparaffinise sections in xylene, followed by dehydration in graded alcohol (80%, 90% and 100%). Sections are then transferred to TRIS Buffered Saline (TBS) at pH 7.6

(Appendix 3)

3. Antigen retrieval was performed with heat treatment of de paraffinised sections by steam cooking in citrate buffer for 20 minutes. Rinse in TBS buffer, 2 washes for 5 minutes each.

4. Sections were then incubated with 1/5 dilution of normal human pooled serum.

5. Sections are drained and covered with 50ul of 1:30 dilution of mouse monoclonal primary antibody (2ul of primary antibody was diluted with 60ul of diluent) and incubated overnight.

6. Sections are rinsed in TBS buffer, 3 washes for 5 minutes each.

7. Indirect immunoenzyme labeling was done using a secondary antibody conjugate, biotin/streptavidin based detection system. Secondary antibody (1/200 dilution of biotinylated rabbit anti-mouse antibody) was added and incubated for 30 minutes.

8. Sections are rinsed in TBS buffer, 2 washes for 5 minutes each.

9. Endogenous peroxidase activity is blocked with 0.5% hydrogen peroxide in methanol for 30 minutes.

10. Sections are rinsed in TBS buffer, 3 washes for 5 minutes each.

11. Sections are drained and covered with 1/300 dilution of peroxidase conjugated Streptavidin and incubated for 30 minutes.

12. Sections are rinsed in TBS buffer, 3 washes for 5 minutes each.

13. Sections were developed with freshly prepared Diaminobenzidine solution in TBS/HCl containing hydrogen peroxide for 3-5 minutes.

14. Sections were counterstained with Harris haematoxylin for 10 seconds.

A known positive control slide was added to each batch. A strong cytoplasmic staining of tumour cells was considered positive.

DNA extraction, PCR amplification and DNA sequencing

Only fresh frozen tissue was used for DNA extraction. PCR amplification and sequencing of both IDH1 and IDH2 genes was done on 50 cases using specific primers to amplify the target region.

DNA extraction

Stored fresh frozen tissue of patients with oligodendroglial tumours was retrieved from the neuropathology laboratory. DNA extraction was carried out using the DNA tissue extraction kit from Qiagen using the protocol given below

1. 25mg of tissue was cut into small pieces and placed in a 2ml microcentriguge tube. 180 ml of ATL buffer was added.

2. 20 ul Proteinase K was added to the microcentrifuge tube, mixed by vortexing and incubated in a shaking waterbath at 56^0C overnight until the tissue was completely lysed.
3. 200ul of buffer AL was added to the sample and mixed by pulse vortexing for 15 seconds, followed by incubation at 70^0C for 10 minutes. Tubes were briefly centrifuged to remove drops from the lid.
4. 200 ul of 100% ethanol was added to the sample and mixed by pulse vortexing for 15seconds. After mixing, the 1.5ml microcentrifuge tube was centrifuged briefly to remove drops from the lid.
5. The mixture from step 4 (including the precipitate) was carefully transferred to the QIAamp spin column in a 2ml collecting tube. Cap is closed and centrifuged at 6000g (8000 rpm) for 1 minute. QIAamp spin column was then placed in a clean 2ml collecting tube and the filtrate was discarded.
6. 500 ul of buffer AW1 was added to QIAamp spin column without wetting the rim. Cap is closed and centrifuged at 6000g (8000 rpm) for 1 minute. QIAamp spin column was placed in a clean 2ml collection tube and the collecting tube containing the filtrate was discarded.
7. 500 ul of buffer AW2 was added to QIAamp spin column without wetting the rim, followed by centrifugation at 20000g (14000 rpm) for 3 minutes.
8. QIAamp spin column was placed in a clean 2ml collection tube and the collecting tube containing the filtrate was discarded. The spin column was centrifuged at full speed for 1 minute.

9. The spin column was placed in a clean 1.5ml microcentrifuge tube and the collecting tube containing the collection tube was discarded. 200ul of buffer AE was added to the spin column, followed by incubation at room temperature for 5 minutes. The spin column was centrifuged at 6000g (8000 rpm) for 1 minute.
10. 1.0µl of the DNA sample was used for quantification using the Nanodrop (NanoDrop technologies, USA) and the 260/280 ratio was determined. Measurements were repeated twice for confirmation.

If PCR was not carried out immediately, samples were stored at -70^{0}C and thawed only just before the PCR procedure.

PCR Amplification

The extracted DNA sample was used for determining IDH1 and IDH2 mutations. Exon 4 of the IDH1 gene was amplified with a polymerase chain reaction assay. A fragment of 248 bp length spanning the sequence encoding the catalytic domain of IDH1, including codon 132, was amplified using the sense primer IDH1F: CTCCTGATGAGAAGAGGGTTGT and the antisense primer IDH1R:TGGAAATTTCTGGGCCATG .(89)

A fragment of 227bp in length spanning the catalytic domain of IDH2 including codon 172 was amplified using the sense primer F 5' –TGGAACTATCCGGAACATCC 3' and the antisense primer R 5' – AGTCTGTGGCCTTGTACTGC 3'. Details of primers are summarised in the table below.

Mutation	No. of base pairs	Direction of sequence	Primer sequence	Exon	Chromosome
IDH1 mutation	248 bp	Forward	5' CTCCTGATGAGAAGAGGGTT 3'	4	2
		Reverse	3' TGGAAATTTCTGGGCCATG 5'		
IDH2 mutation	227 bp	Forward	5' TGGAACTATCCGGAACATCC 3'	4	15
		Reverse	3' AGTCTGTGGCCTTGTACTGC 5'		

The extracted DNA was amplified using the protocol given below. Fermantas Dream Taq polymerase was used. All reagents including buffer, magnesium chloride (MgCl2) and dNTPs used for PCR were from the same abovementioned source. IDH1 and IDH2 primers were obtained from Sigma Aldrich.

1. DNA sample and the reagents were thawed to room temperature before starting the reaction. Tubes were tapped and centrifuged to remove drops from the sides and lid.
2. All reactions were carried out in 25 µl volume. Master mix was prepared in 0.6ml PCR tubes by mixing 2.5µl buffer, 1.5µl MgCl2, 0.5µl dNTPs, 2µl of 20 picomoles of forward and reverse primers, 0.3µl of DNA Taq polymerase and 14.2 µl of distilled water. The tubes are nicely tapped and centrifuged to mix the reagents thoroughly.
3. 23 µl of the master mix was added to the respective 0.2ml PCR tubes.

4. The PCR tubes were then transferred to the amplification area and 2µl of optimally diluted DNA containing 50-80ng of DNA was added to the respective PCR tubes. A non template control (NTC) was also run with every batch of PCR reaction for both IDH1 and IDH2. 2µl of distilled water was added to the NTC tubes.
5. The PCR tubes were tapped and centrifuged before loading into Veriti thermal cycler (Applied Biosystems, USA). The following thermal cycling parameters were followed for all PCRs: initial denaturation at 95^0C for 8 min, followed by 38 cycles of denaturation at 95^0C for 45 sec, optimized annealing at 62^0C for IDH1 and 63^0C for IDH2 for 1 min and extension at 72^0C for 1 min. Final extension was carried out at 72^0C for 10 min.
6. PCR products were detected by gel electrophoresis on 2% agarose gel.
7. Once the PCR products are amplified, the product was cleaned to remove unwanted PCR fragments and unused reagents. The products were cleaned based on the principle of ultrafiltration. 15 µl of PCR product and 85ul of sterile water were applied onto the ultrafiltration membrane of the wells of the pre clean plates (Millipore/Merck, USA). 20 Hg pressure was applied for 10 minutes. 100µl of sterile water was then added into the well and 20 Hg of vacuum was applied for 10 minutes. The PCR products were eluted out with 30µl of sterile water.
8. Samples were run on 2% agarose gel to look for the bands of interest.

DNA Sequencing

1. Once good pre clean products were obtained on electrophoresis, sequencing reaction was set up to amplify the product by the dideoxy chain termination

method, using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, USA).

2. Sequencing PCR was carried at 8.2 µl volume. Master mix was prepared using 1 µl of buffer, 0.5 µl of RR mix, 5.1 µl of distilled water, 1.6 µl of 1 picomole of forward or reverse primers and 2 µl of optimally diluted pre clean product. The following thermal cycle was used for sequencing PCR: 25 cycles of denaturation at 96^0C for 15 sec, annealing at 50^0C for 20 sec and extension at 60^0C for 4 min.
3. Post clean up was done using membrane based ultrafiltration using injection solution (Millipore/Merck, USA). 10 µl of sequence product was mixed with 30 µl of injection solution. 20 Hg of pressure was applied for 10 min. Then 40 µl of injection solution was added and a vacuum of 20 Hg was applied for 10 minutes. Sample was eluted out with 30 µl of injection solution.
4. Samples were loaded onto 96 well plates and sequencing was performed with an automated DNA ABI 3130 Genetic analyzer. Mutational analysis was performed by comparing the sequence with the wild type and by looking for the presence of mutations at the respective codon.

Statistical Analysis

Statistical analysis was performed using “SPSS software” (Statistical Package for Social Sciences), Windows, version 16. Descriptive statistics for continuous variables was analysed using mean +/- SD (standard deviation) or median. Categorical data was described using frequencies and percentages. Association between combined 1p/19q deletion, polysomy of chromosome 1/19 and IDH mutation with the various clinical,

radiological and histopathological parameters was calculated using Pearson's Chi square test/Fishers exact test. Sensitivity and specificity of IDH1 immunohistochemistry was calculated taking PCR as the gold standard.

Progression free survival (PFS) was calculated from the date of surgery to either the date of first progression or date of last follow up. Overall survival (OS) was calculated from the date of surgery to the date of death or the date of last follow up. All patients who died were assumed to have had recurrence when radiological evidence of recurrence was not documented. For these cases, the date of death was taken as the date of recurrence to calculate the PFS time. Only primary tumours were included for survival analysis. All 8 recurrent tumours were excluded from the analysis. Kaplan Meier survival estimate was used to plot the survival curves for recurrence and the log rank test was used to determine the level of statistical significance. In univariate analysis, Odds ratio and Risk ratio was calculated for recurrence and death for possible risk factors and the significance level tested using 2 sided Fishers exact test. Multivariate analysis was done using Stata 11.2. Multivariate analysis was done using a logistic regression model. For all statistical analysis, a p value ≤ 0.05 was considered significant.

RESULTS

Patient Characteristics

There were a total of 50 cases from January 2009 to January 2012 included in the present study. The patient demographics for these 50 cases are given in Table 1.

The median age of patients in our cohort was 39 years (Range 13 - 63years) (Fig 3). More than 2/3rd of patients in the study were males. The male female ratio was 2.3:1(Fig 4).

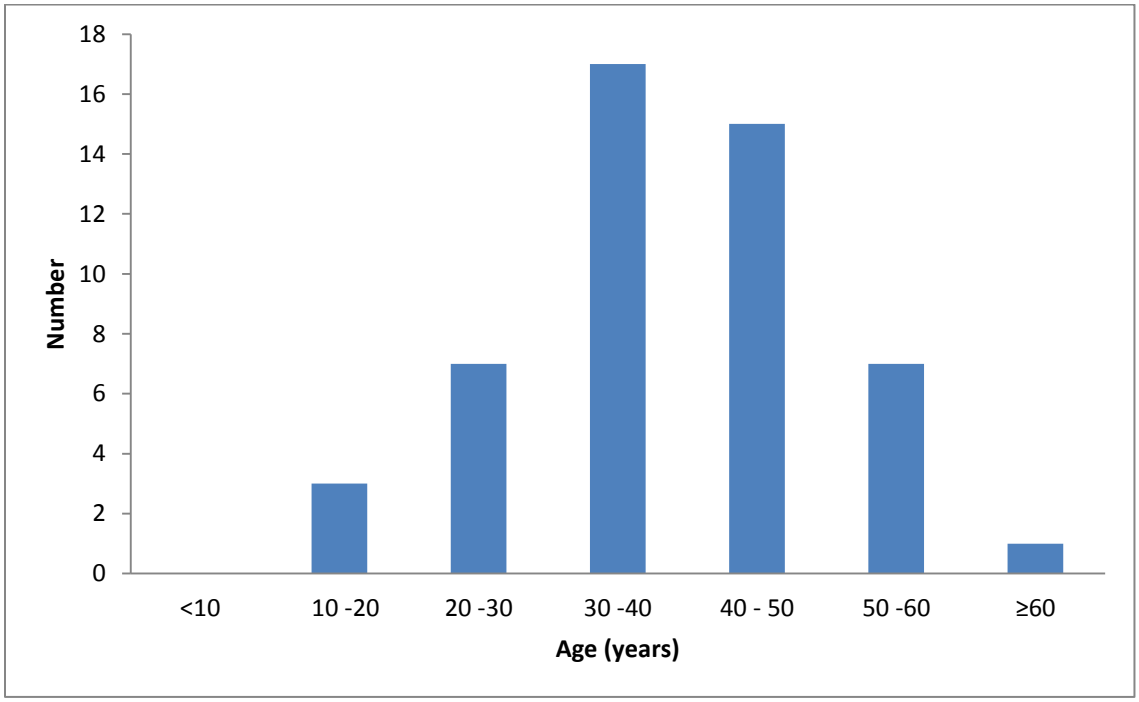


Figure 3. Age distribution of 50 cases in a cohort of oligodendroglial tumour.

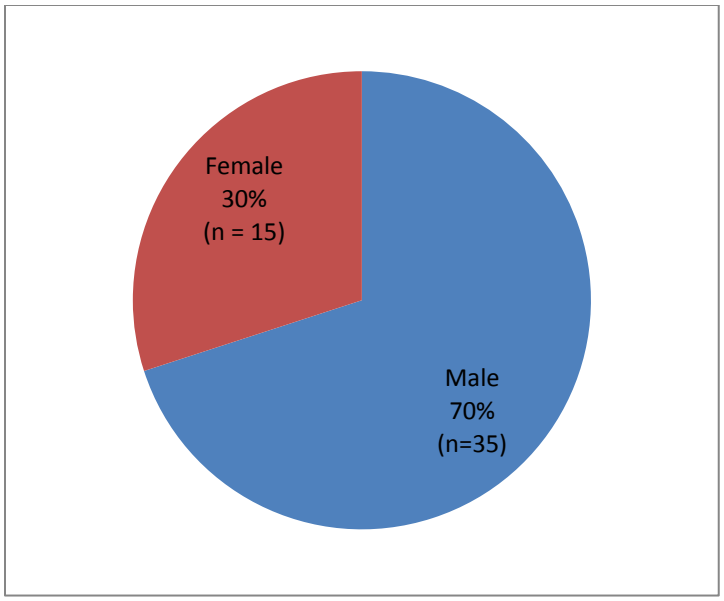


Figure4. Gender distribution of oligodendroglial tumours

The most common clinical presentation was seizures (71.4%), followed by focal neurological deficits (18.3%) and features of raised intracranial tension (10.2%).

Forty two of the 50 cases presented for the first time during the study period, whilst 8/50 were recurrent tumours. Of these 8, 4 patients had their previous tumor excised at this institution, while the other 4 cases presented to this institution for the first time during the study period and had their previous surgery elsewhere.

The mean KPS score of patients was 86.6 (Range 70-90).

Table1. Patient characteristics of all 50 oligodendroglial tumours.

Patient characteristics	Number
Sex	
Male	35
Female	15
Age (years)	
Median	39
Range	13-63 years
Clinical presentation	
Seizures	35
Focal neurological deficits (FND)	9
Features of intracranial pressure	5
Not available	1
Primary	42
Recurrent	8
KPS score	
Median	86.6
Range	70-90

Radiological findings

The radiological findings of the 50 cases are summarized in Table 2. Both right and left hemispheres were almost equally involved. 2 cases were bilateral. The most commonly

involved lobe was the frontal lobe (54%), followed by temporal lobe, parietal lobe and occipital lobe. 50% of the cases had a tumour size of more than 5 cm.

On MRI, 39 cases (78%) showed homogenous signal intensity, hypointense on T1 and hyperintense on T2, while the remaining cases showed mixed intensity signals on T1 and T2. 54% of the cases showed indistinct borders on imaging studies.(Fig 5&6)

Table 2 Radiological findings of 50 oligodendroglial tumours

Radiological findings	**Number**
Site	
Frontal lobe	27
Temporal lobe	18
Parietal lobe	4
Occipital lobe	1
Size	
≤2cm	2
2-5cm	23
>5cm	25
Borders	
Distinct	23
Indistinct	27
Tumour signal intensity	
Homogenous (T1 hypo T2 hyper)	39
Mixed signals	11

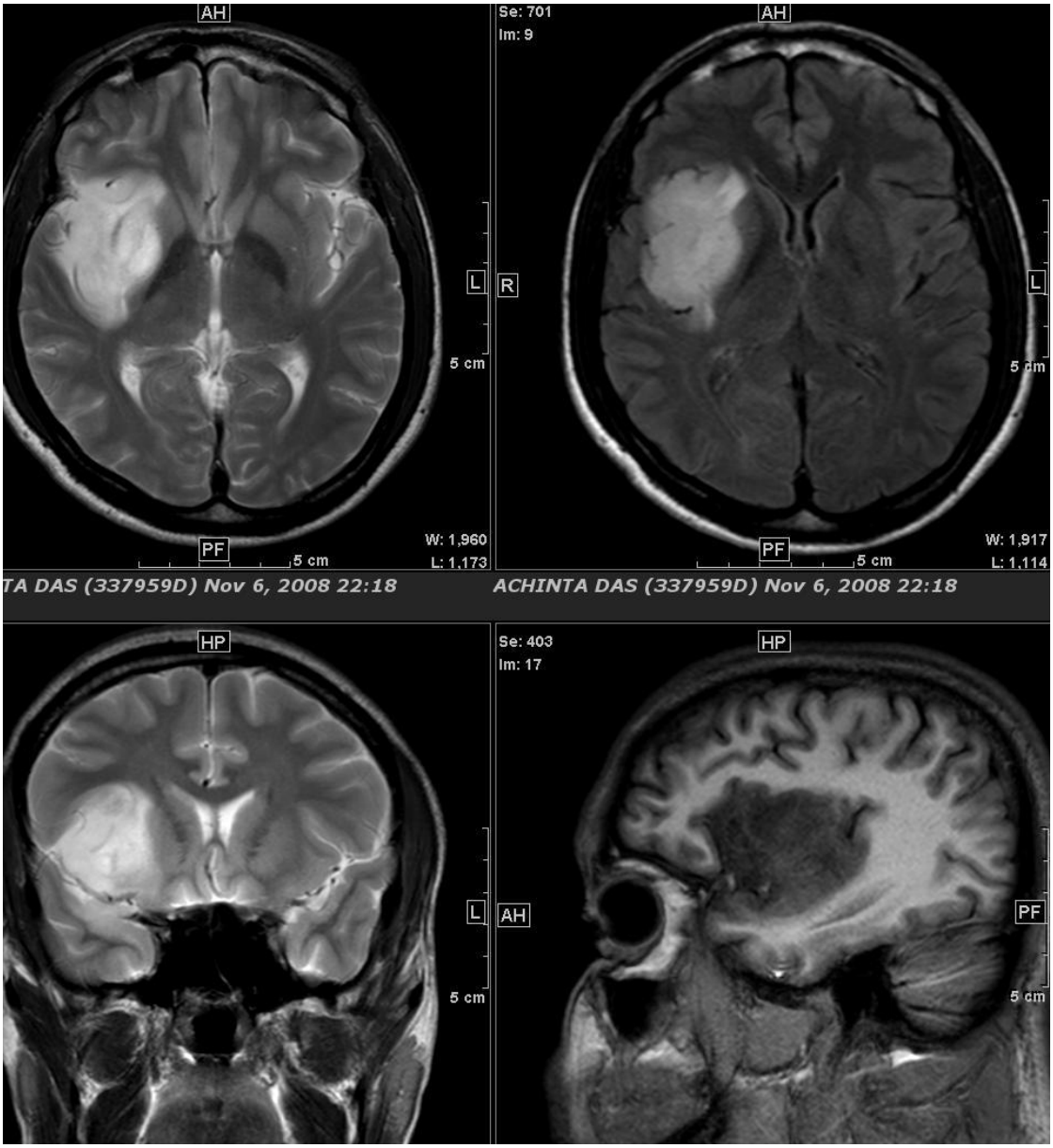


Figure 5. Magnetic resonance imaging in a patient with a right insula glioma displaying a distinct tumor-brain interface with perilesional edema on the T2 axial and coronal (a&c) images, T2 flair (b) and T2 sagittal images (d)

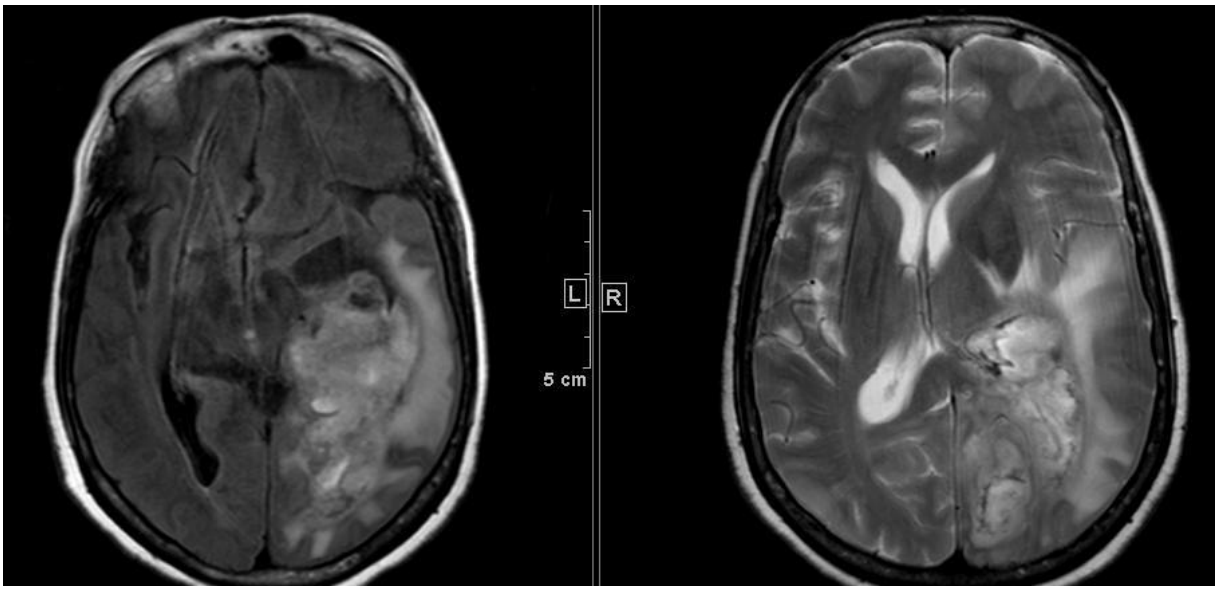


Figure 6. Magnetic resonance imaging in a patient with a left parietal glioma that has indistinct borders on the T2w and T2 flair axial images (a and b)There is marked perilesional edema.

Treatment details

21 out of the 50 patients had a partial or subtotal resection, while the remaining patients underwent a radical excision. (Table 8)

Histopathology

On histopathological examination using the WHO grading system, all the 50 cases were subtyped into different histological types as shown in Fig 7.

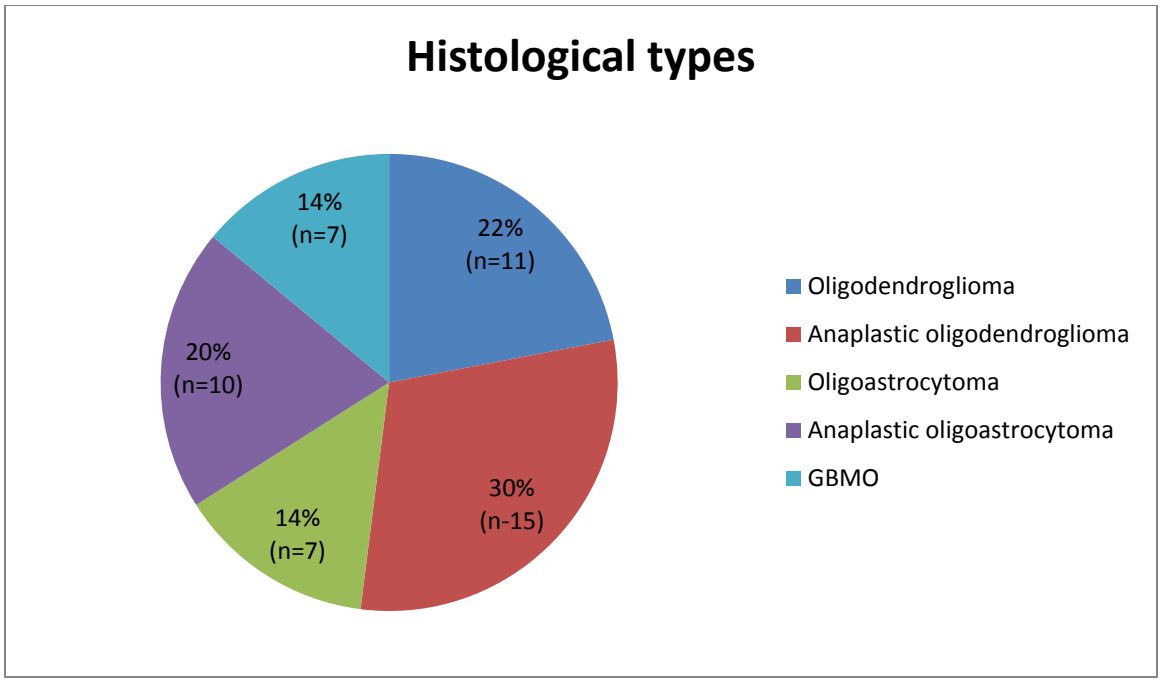


Figure 7. Distribution of 50 oligodendroglial tumours into histological subtypes

26 cases (52%) were pure oligodendroglialtumours, of which 11 were WHO grade II oligodendrogliomas and 15 were WHO grade III oligodendroglioma.

Oligodendrogliomas with classical histology showed uniform round regular nuclei, well-defined nuclear contours, evenly dispersed chromatin, inconspicuous/small nucleoli, perinuclear halos and chickenwire vasculature. (Fig 8&9) Minigemistocytes,

calcification,mucoid/microcystic degeneration were also seen (Fig 10-13) These tumours showed a tendency to form 'secondary structures of Scherer', like perineuronal and subpial satellitosis (Fig 14&15)

The anaplastic oligodendrogliomas showed in addition to the above features, high cellularity, increase in mitotic activity, cytological atypia, necrosis and microvascular proliferation (Fig 16-21). 3 out of the 15 anaplastic oligodendrogliomas showed necrosis.

Oligoastrocytomas had both neoplastic oligodendrocytes and neoplastic astrocytes. There were 17 cases (34%) of oligoastrocytomas, of which 7 cases were WHO grade II oligoastrocytoma (Fig 22) and 10 cases were WHO grade III anaplastic oligoastrocytoma. Anaplastic oligoastrocytomas showed the additional features of nuclear atypia, cellular pleomorphism, high cellularity, high mitotic activity and microvascular proliferation (Fig. 23)

In majority of the oligoastrocytomas (16 out of 17 cases), both the components were intermingled with each other ("diffuse" or "intermingled" variant), while in 1 case, distinct oligodendroglial and astrocytic areas were juxtaposed with each other ("compact" or "biphasic" variant). (Figs 24&25)

Glioblastoma with oligodendroglial component showed presence of both neoplastic oligodendroglial and astrocytic components together with microvascular proliferation and necrosis. (Fig 26&27)

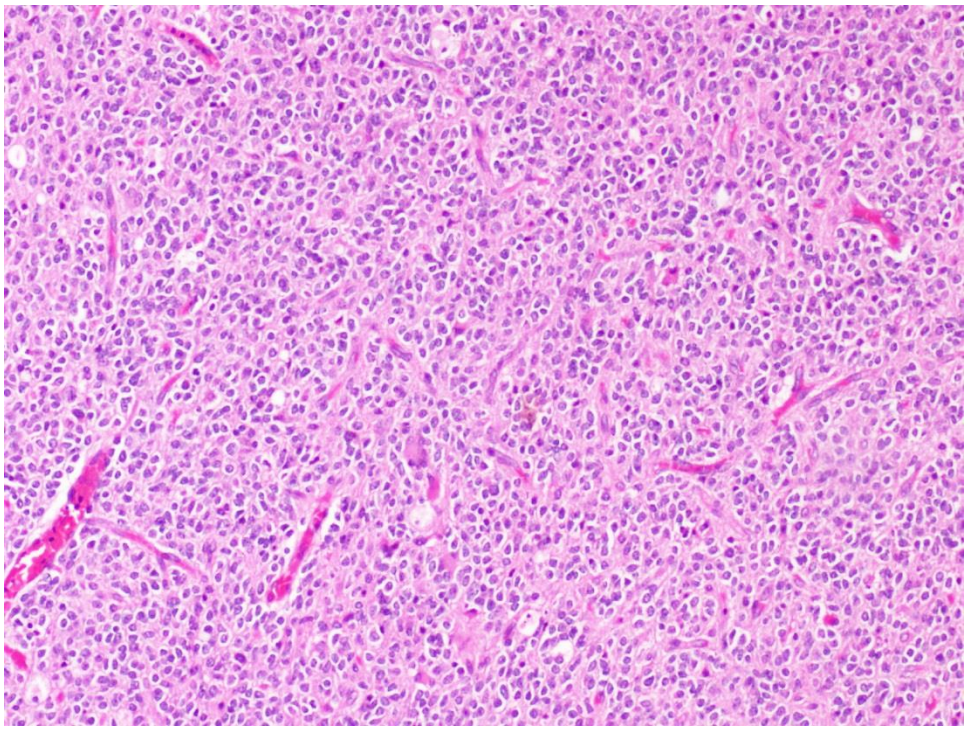


Figure 8.Oligodendroglioma with classical chicken wire vasculature (x 100)

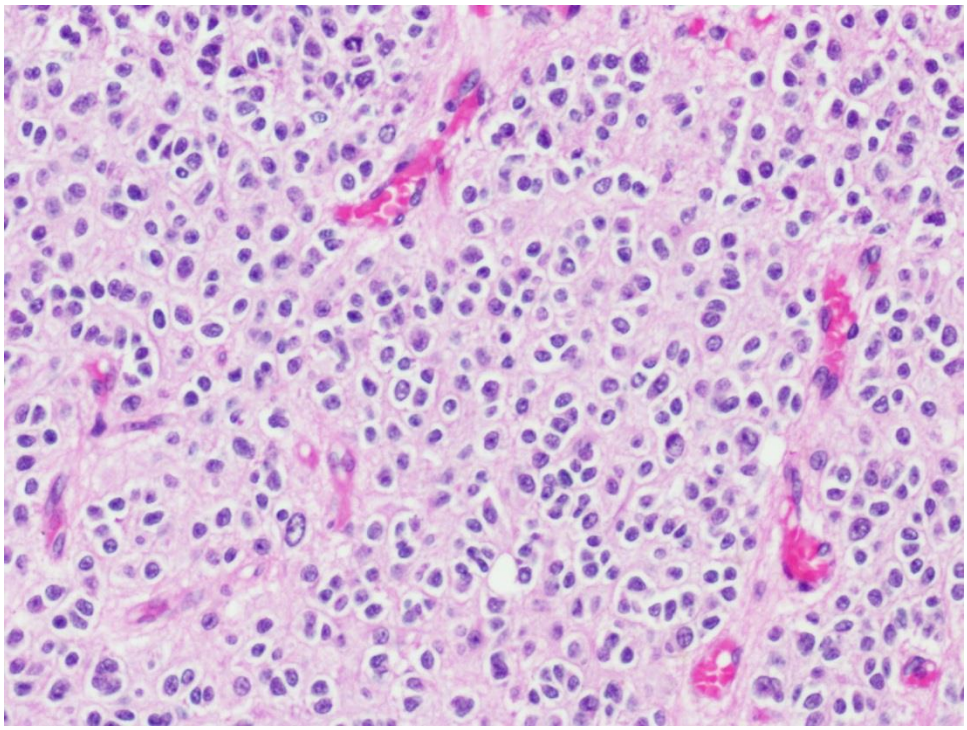


Figure 9.Oligodendroglioma with uniform, round, regular nuclei with perinuclear halos (x 200).

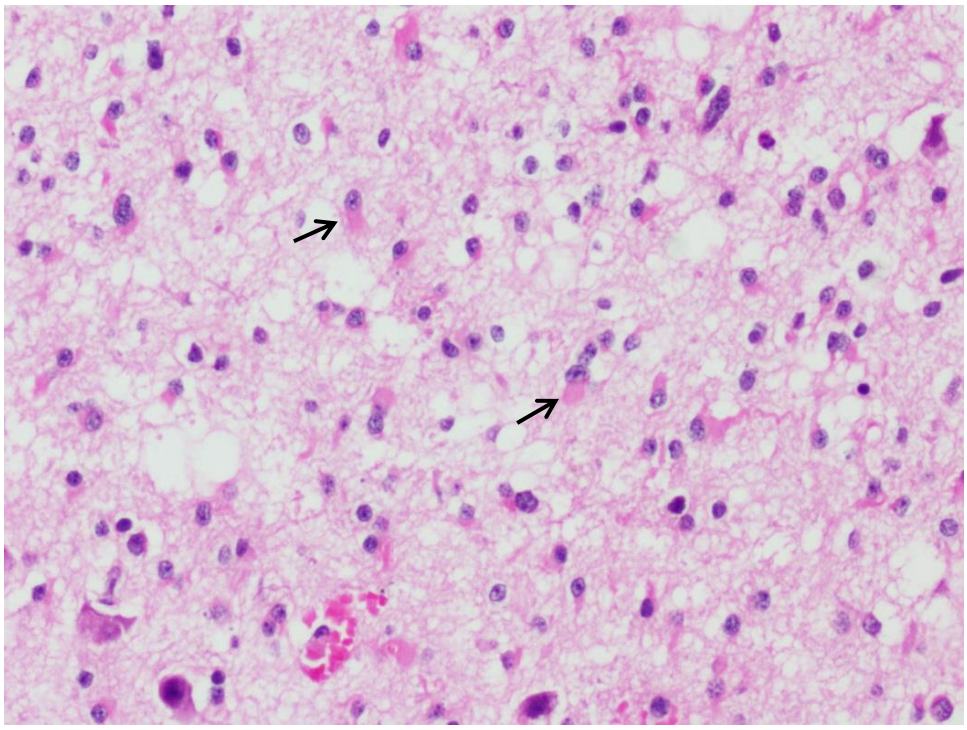


Figure 10 Minigemistocytes (arrows) in an oligodendroglioma (x 200)

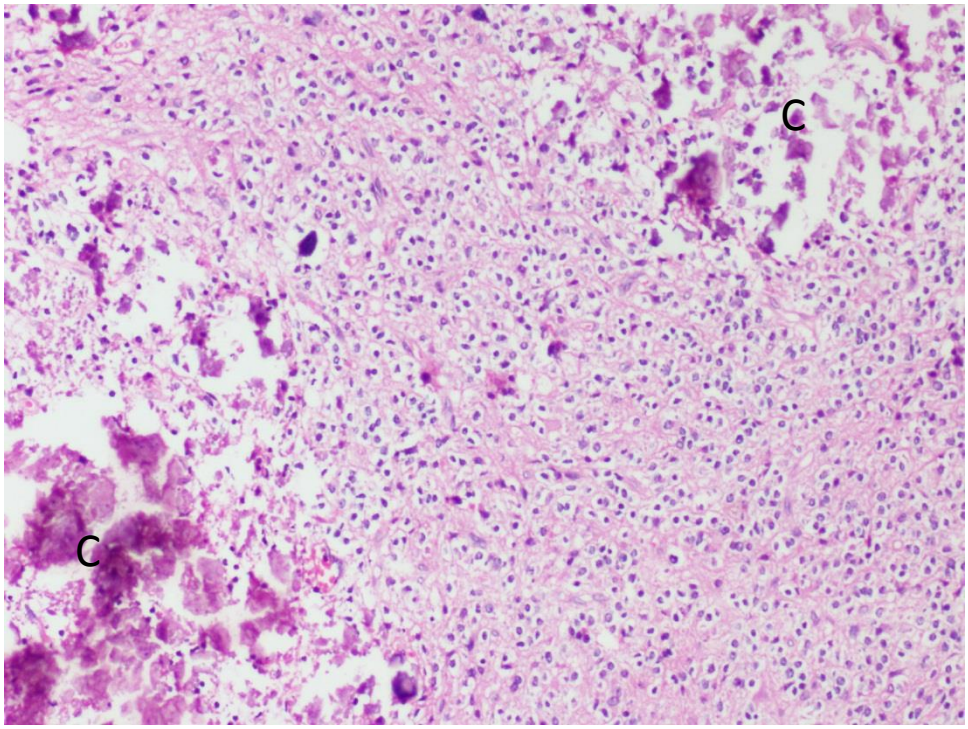


Figure 11. Calcification ('C') in an oligodendroglioma (x 100)

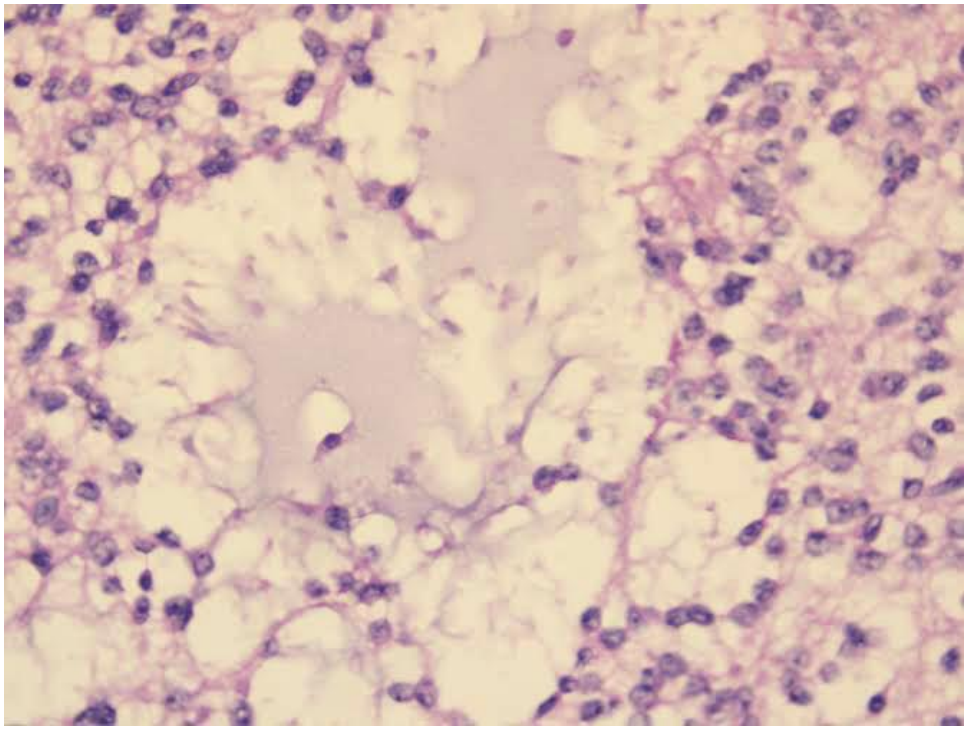


Figure 12. Mucin cyst in an oligodendroglioma (x 400)

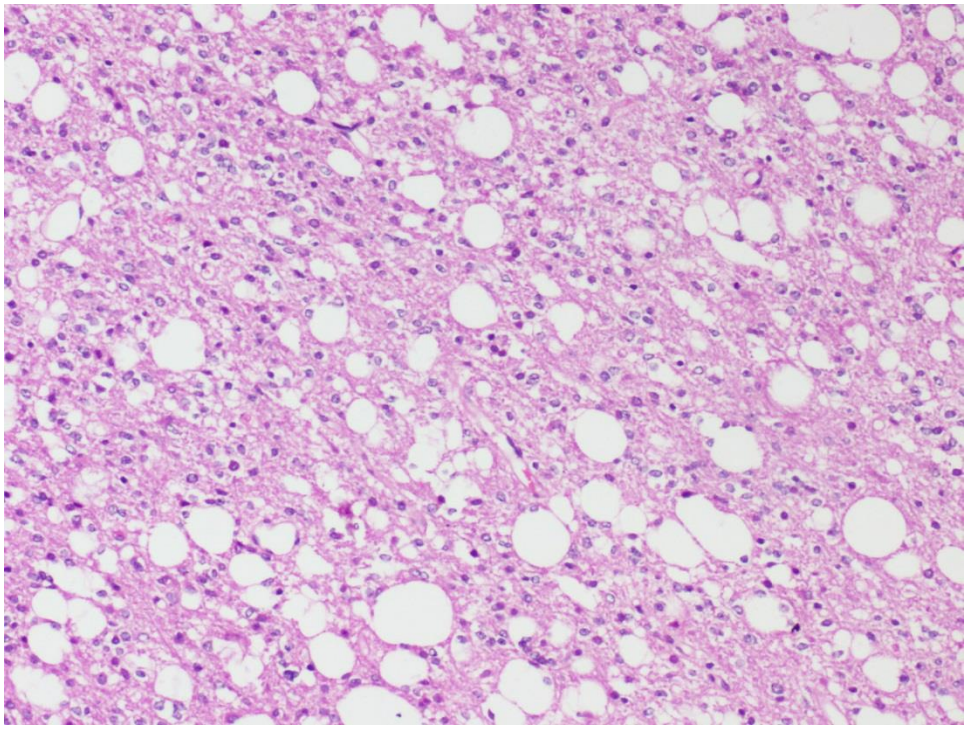


Figure 13.Microcystic change in an oligodendroglioma (x 100)

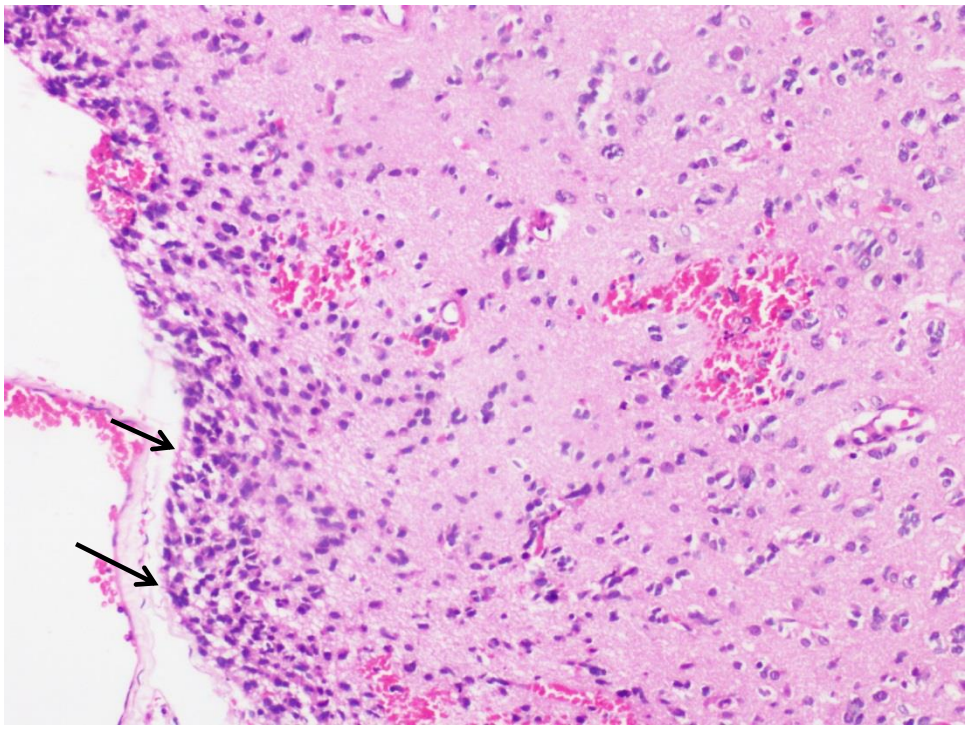


Figure 14. Subpial accumulation in an oligodendroglioma (arrows) (x 200)

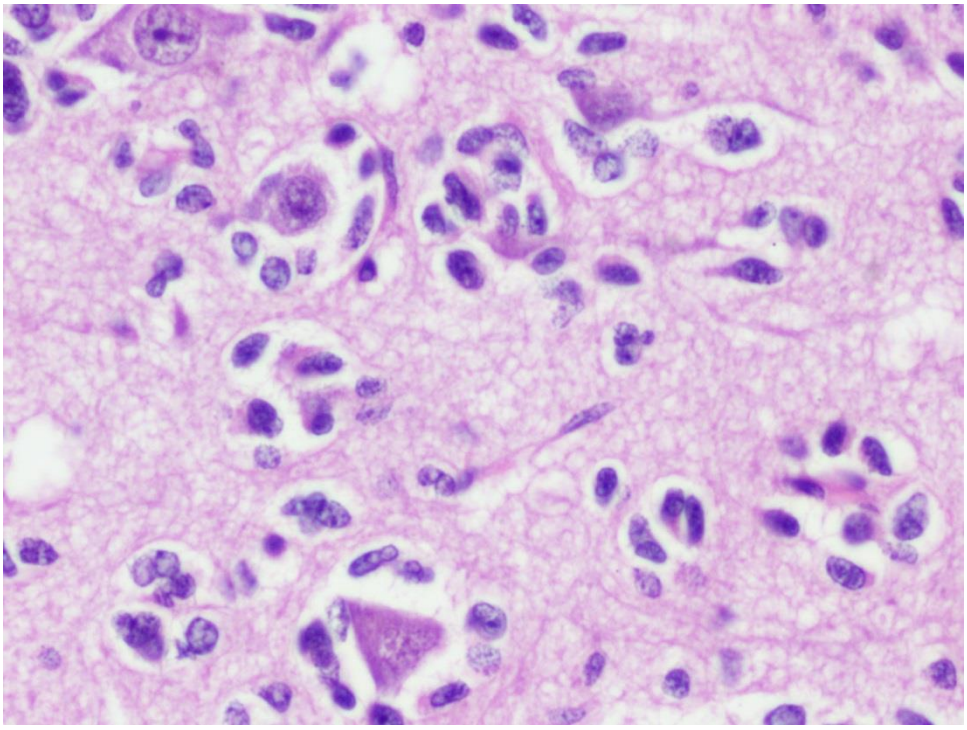


Figure 15.Perineuronal satellitosis in oligodendroglioma (x 400)

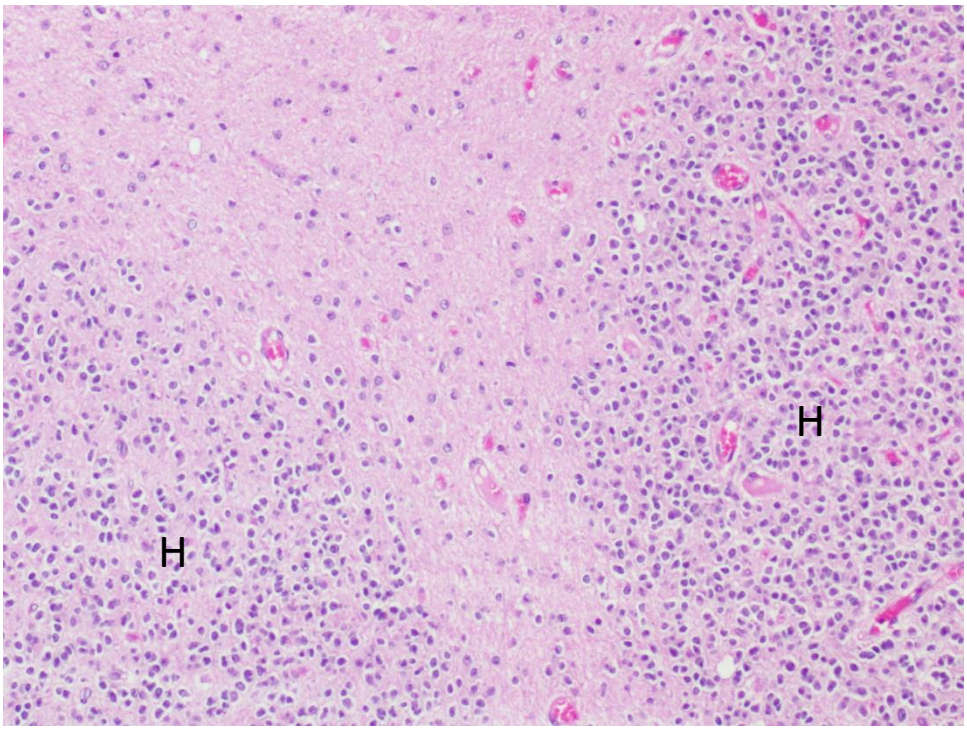


Figure 16. Nodules of hypercellularity ('H') in an anaplastic oligodendroglioma (x 100)

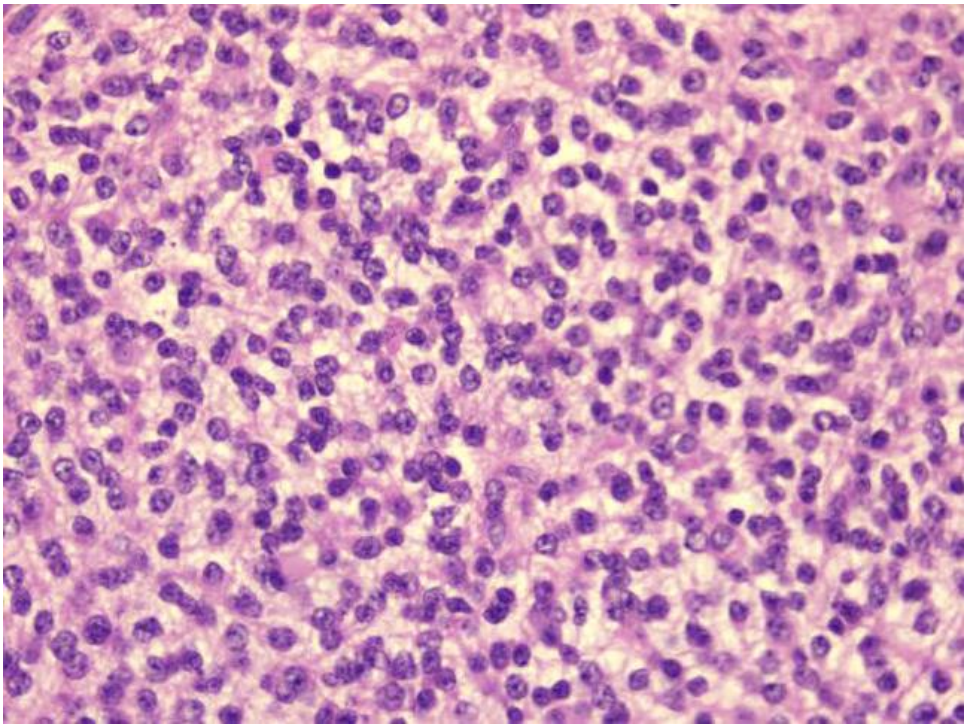


Figure 17.Hypercellular areas in an anaplastic oligodendroglioma (x 400)

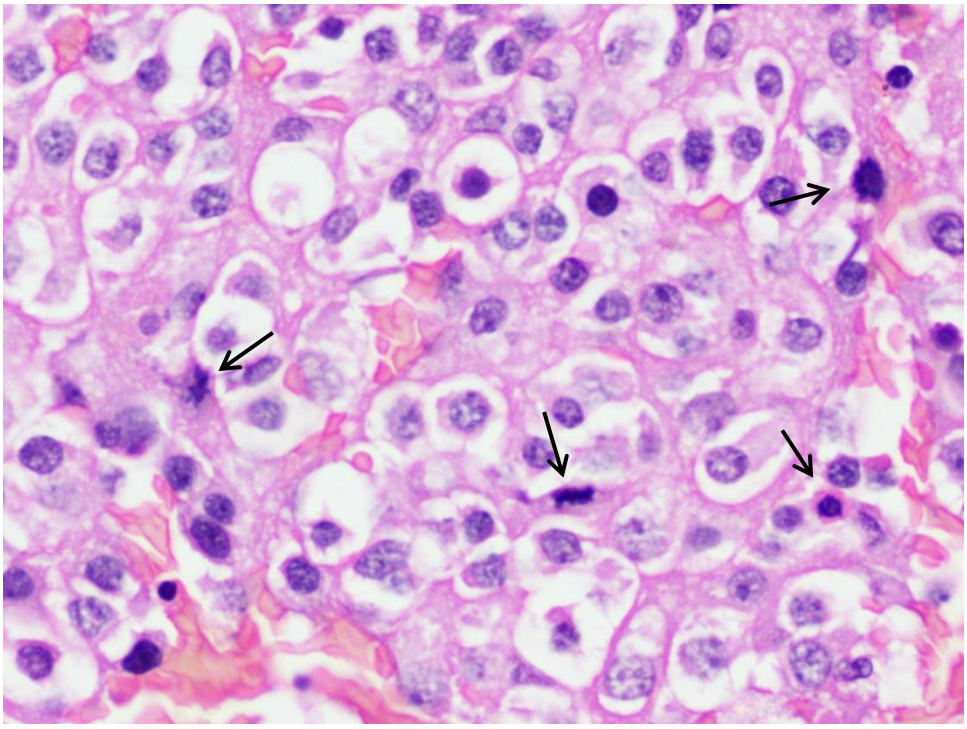


Figure18. Numerous mitotic figures (arrows) in an anaplastic oligodendroglioma (X 400)

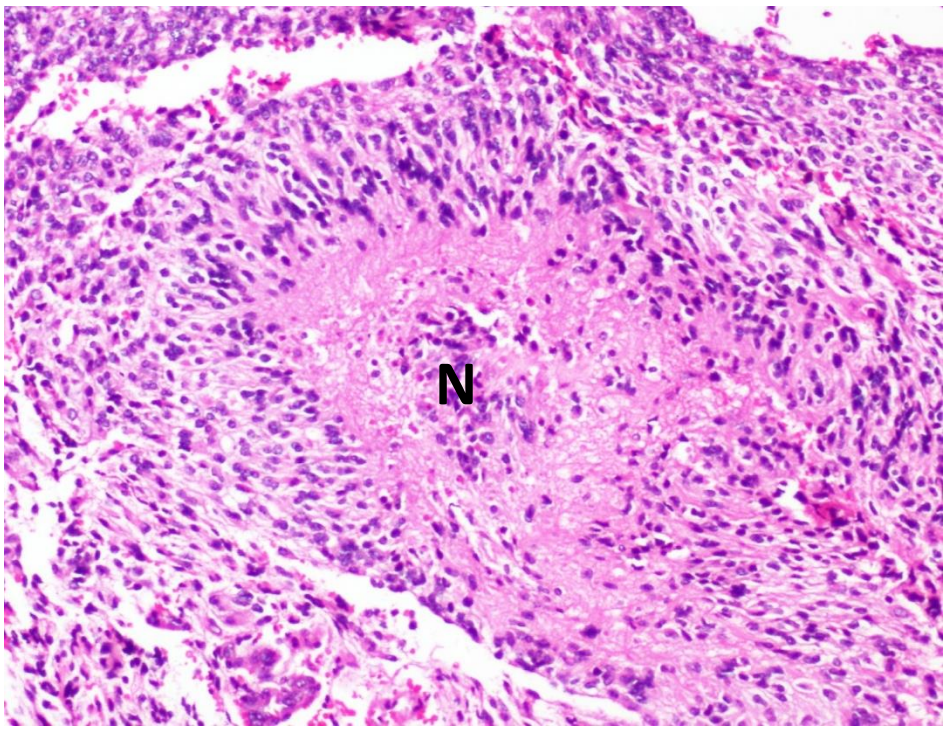


Figure 19.Pseudopalisading necrosis ('N') in an anaplastic oligodendrogliomas (X 200)

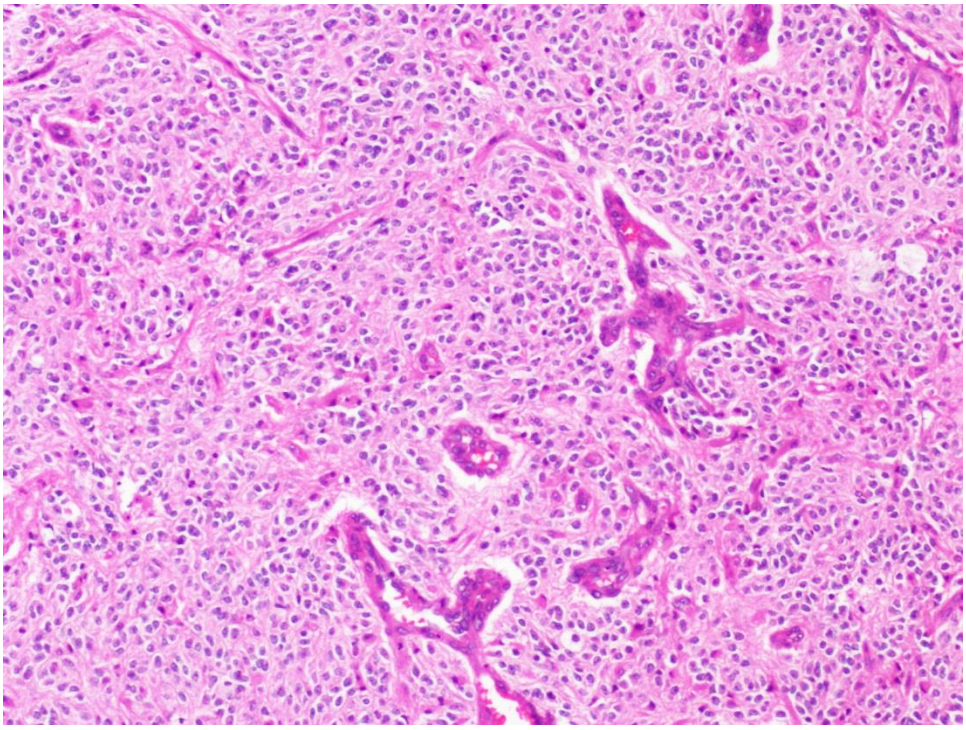


Figure 20.Low power view of microvascular proliferation in an anaplastic oligodendroglioma. (x 100)

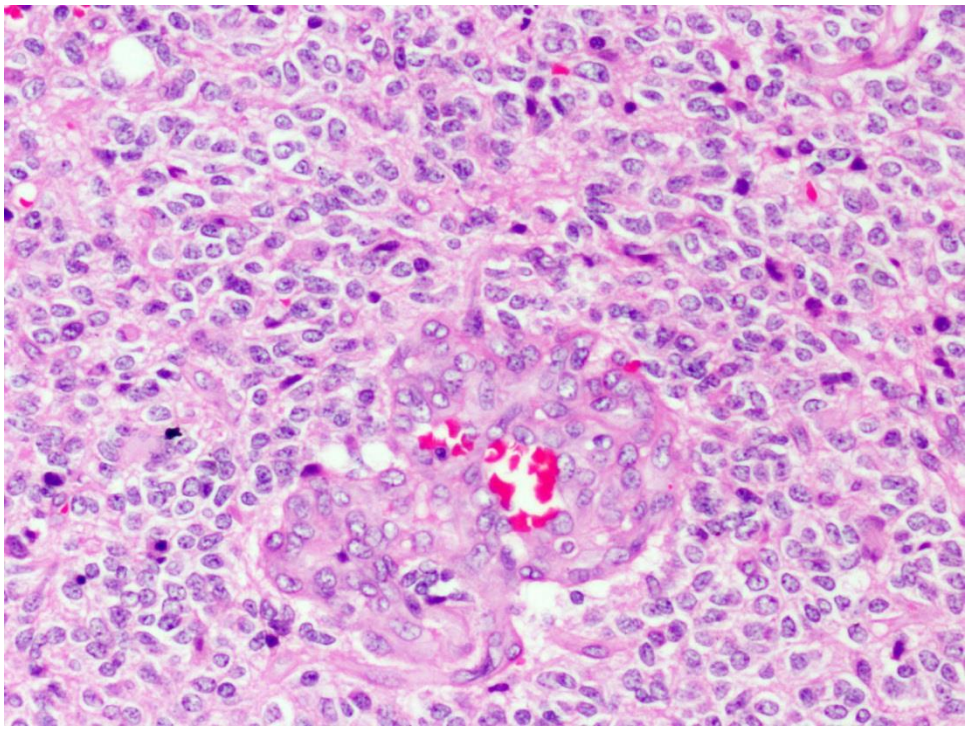


Figure 21. High power view of microvascular proliferation (x 200)

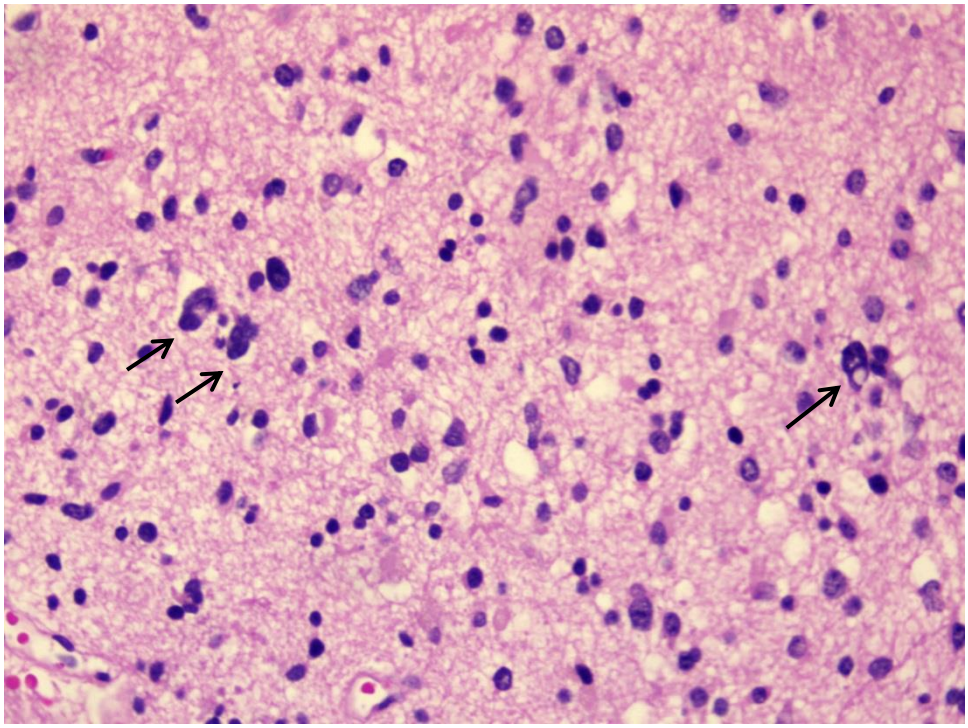


Figure 22 Oligoastrocytoma, WHO grade II; Oligodendroglial cells with round nuclei interspersed with pleomorphic astrocytes (arrows) (x 400)

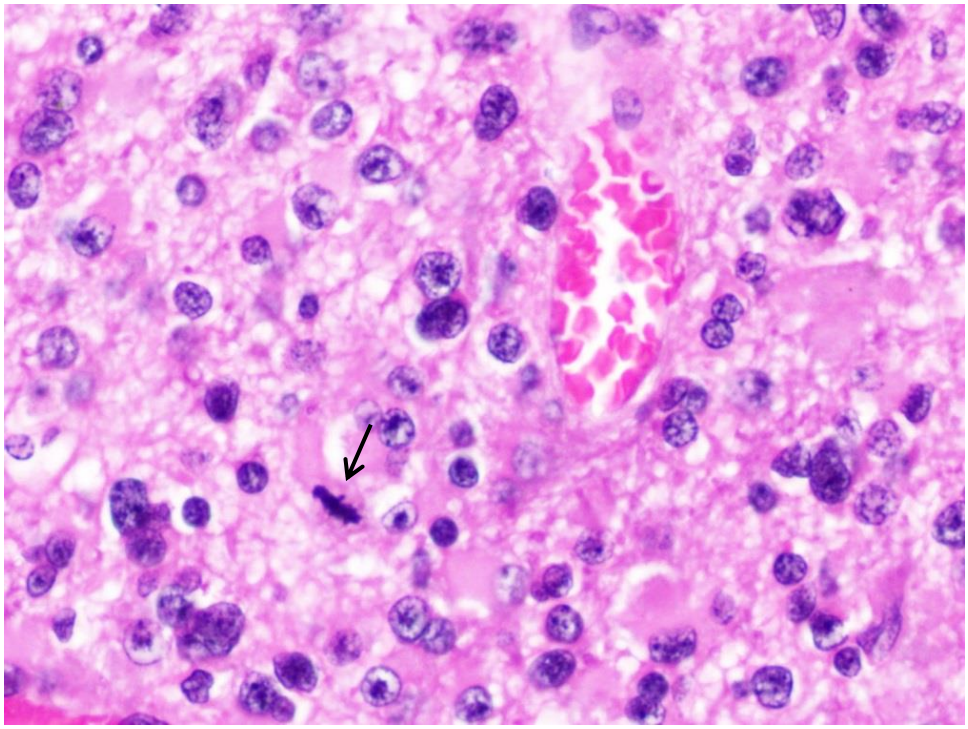


Figure 23 Presence of mitosis (arrow) in an anaplastic oligoastrocytoma (x 400)

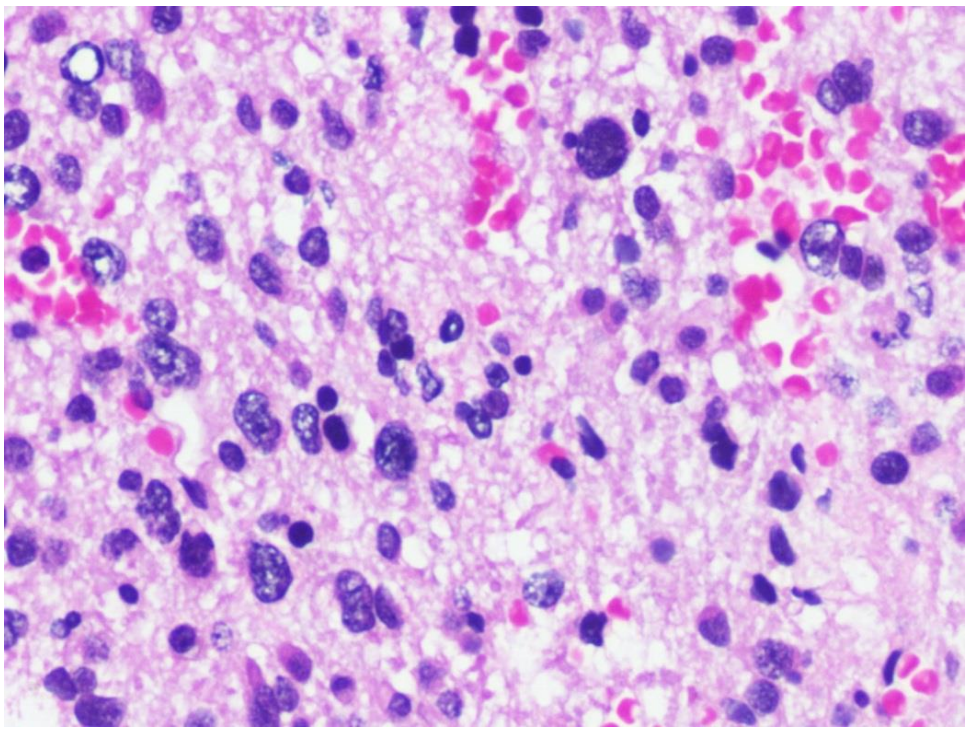


Figure 24.Oligoastrocytoma; Neoplastic oligodendrocytes and astrocytes arranged in a “diffuse” pattern (x 400)

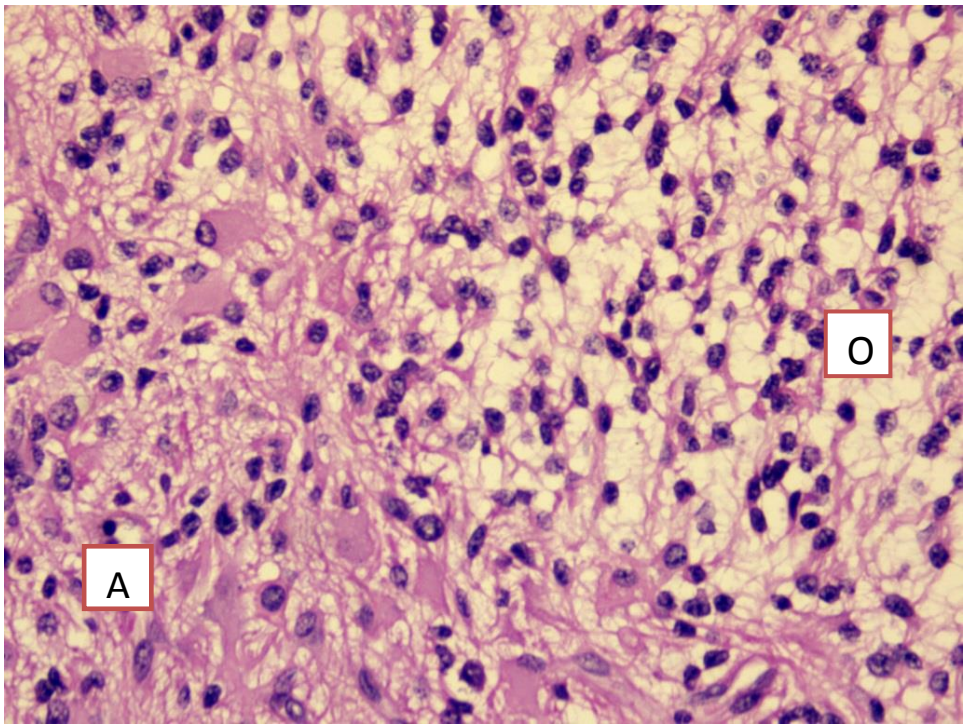


Figure 25. Oligoastrocytoma; Neoplastic oligodendroglial (O) and astrocytic components (A) juxtaposed to each other (“biphasic” pattern) (x 400)

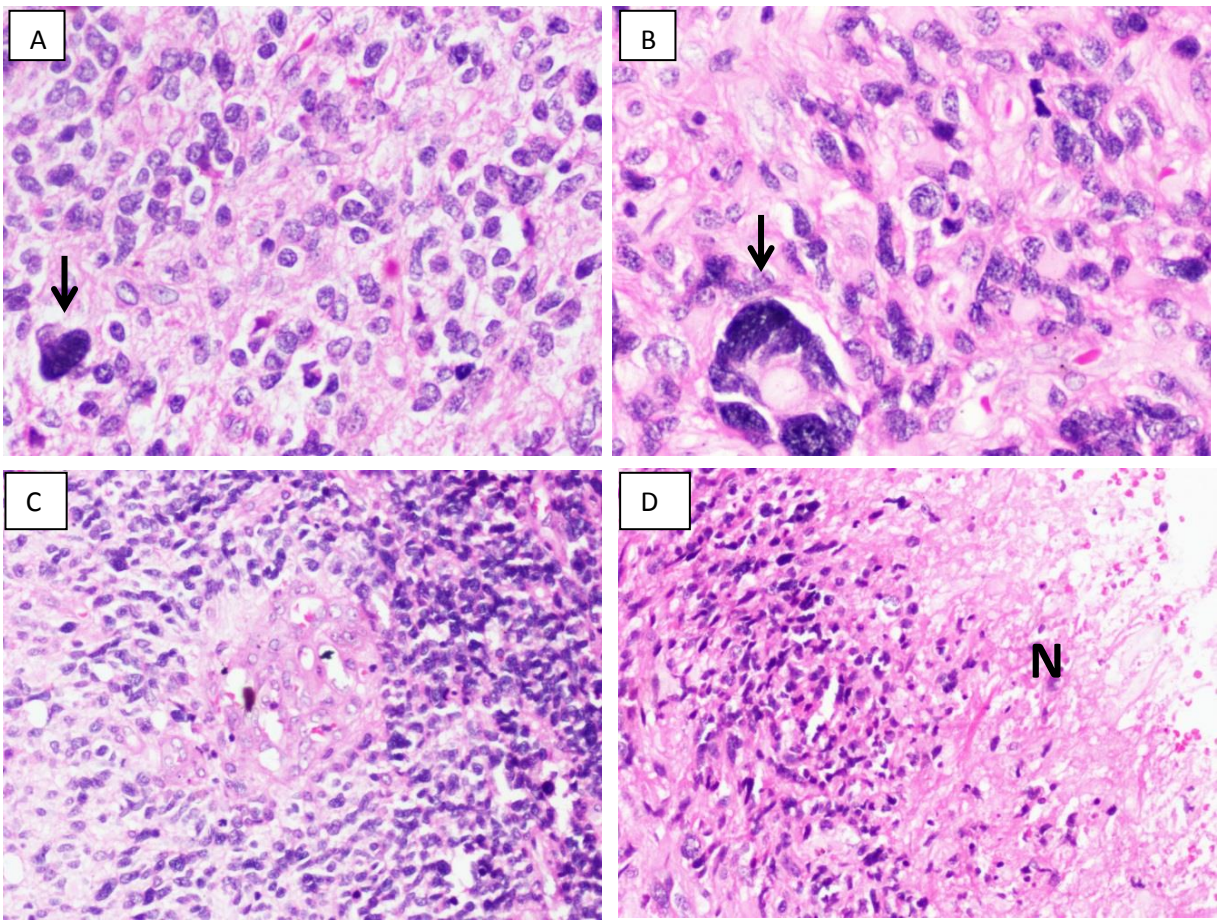
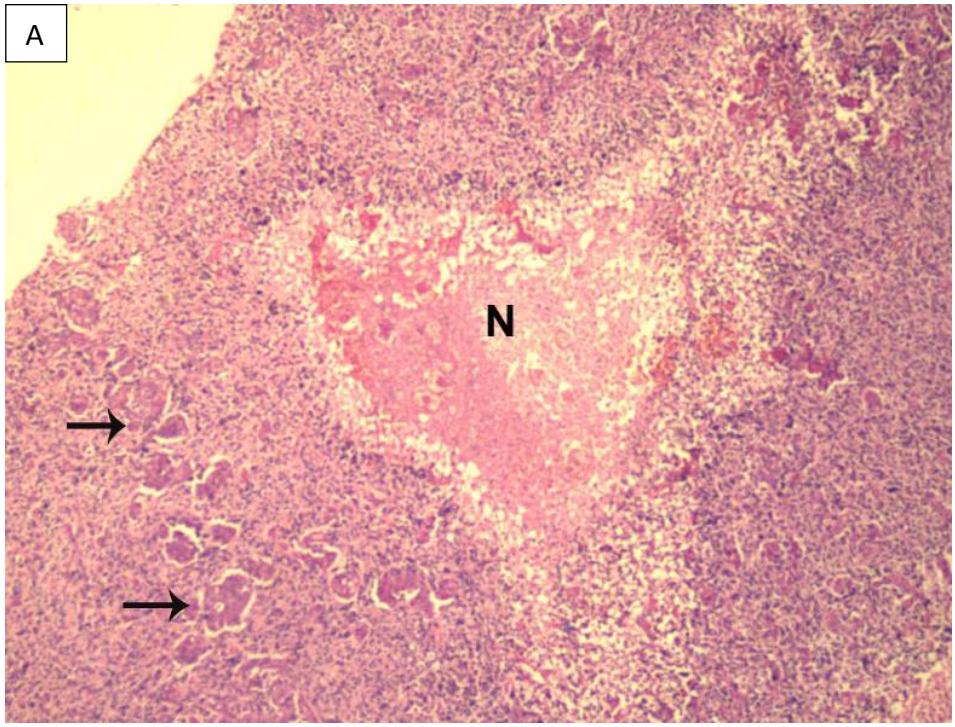


Figure 26. Glioblastoma with an oligodendroglial component. A) Oligodendroglial component with occasional pleomorphic astrocytes (arrow)(x 400) B) Astrocytic component with bizarre giant cells (arrow) (x 400) C) Microvascular proliferation (x 200), and D) Pseudo palisading necrosis (N) (x 200)



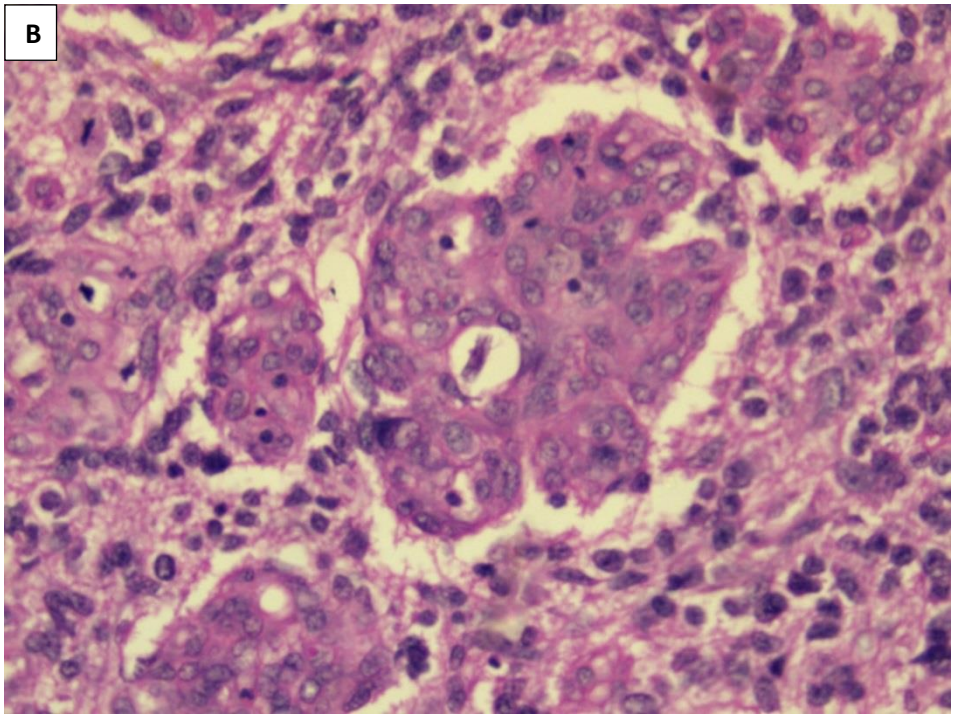


Figure 27. Glioblastoma with an oligodendroglial component with A) Pseudopalisading necrosis (N) and microvascular proliferation (arrow) (x 40) B) higher power view of microvascular proliferation (x 400)

The mean MIB-1 labeling index of Grade II and Grade III tumours was 5 and 18% respectively.Only one of the cases of GBMO had aMIB-1 labeling index done, which was 20%.

Taking all the histological subtypes into consideration, classical features for oligodendroglioma was seen in 38 out of the 50 cases. 88.8% (24 out of 27) tumours arising from frontal lobe showed classical histology, as opposed to 61% (14 out of 23) of tumours from other lobes. This difference was found to be statistically significant (p = 0.021) (Table 3).

Table 3. Distribution of tumours with classical histology in variouslobes

Site	Classical Histology (N=50)
Frontal lobe	24/27 (88.8%)
Temporal lobe	11/18 (61.1%)
Parietal lobe	2/4 (50%)
Occipital lobe	1/1 (100%)

Fluorescence In Situ Hybridisation (FISH) studies

Of the 50 cases, co-deletion of 1p/19q was detected in 23 cases (46%) by FISH. 1 case showed only 1p deletion and 4 cases showed only 19q deletion. Polysomies of chromosome 1/19 were seen in 18 cases (36%). The distribution of 1p/19q status among various subtypes is shown in Fig 28.

Table 4Alterations of chromosome 1 and 19 in 50 oligodendroglialtumours.

Chromosomal alterations	Number	Percentage
Combined 1p/19q deletion	23	46%
1p deletion only	1	2%
19q deletion only	4	8%
No deletion	22	44%
Polysomy 1/19	18	36%

Co-deletion of 1p/19q was seen in 73.1% (19/26) (Fig 29) of pure oligodendroglial tumours (WHO grade II and III), 11.8% (2/17) of mixed oligoastrocytomas (WHO grade II and III) and 28.6% (2/7) of grade IV GBMOs. 1p/19q co-deletion was significantly associated with pure oligodendroglial tumours(grade II&III) than mixed oligoastrocytic tumours (Grades II, III&IV). ($p = 0.0001$)

Among the pure oligodendroglial tumours with 1p/19q co-deletion 7 out of 11 (63.6%) cases were of WHO grade II and 12 out of 15 (80%) cases were WHO grade III tumours.

Among the mixed oligoastrocytomas, 1p/19q co-deletion was seen in none of the WHO grade II tumours and 2 out of 10 (20%) WHO grade III tumours.

Polysomy of chromosome 1/19 was seen in only 1/26 (3.8%) cases of pure oligodendroglial tumours and this was a WHO Grade III neoplasm. Amongst the mixed oligoastrocytic tumours polysomy of chromosome 1/19 was seen in 70.6% (12/17) of cases of which 7 were WHO Grade II and 5 were WHO Grade III. (Fig 30) 71.4% of WHO grade IV GBMOs showed polysomy of chromosomes 1/19. Polysomy of chromosome 1/19 was more commonly seen in mixed oligoastrocytic tumours than pure oligodendroglial tumours. (p = <0.00001)

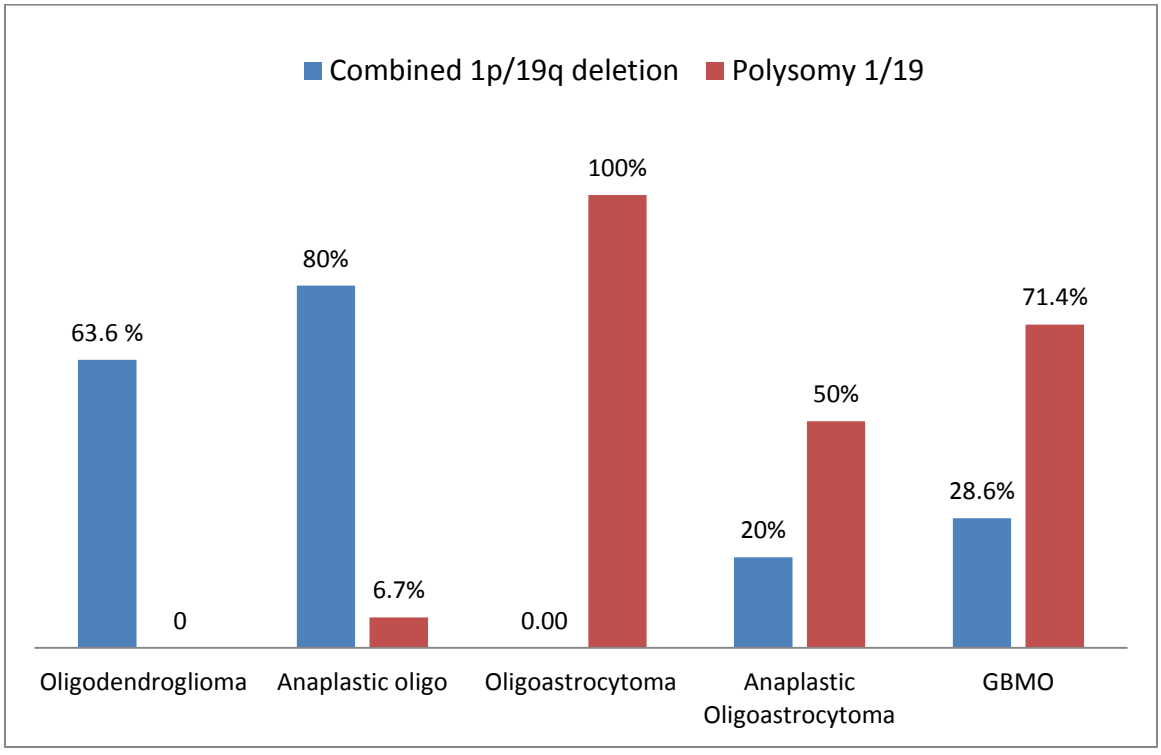


Figure 28. Distribution of combined 1p/19q deletion and polysomy 1/19 among the histological subtypes of oligodendroglialtumours.

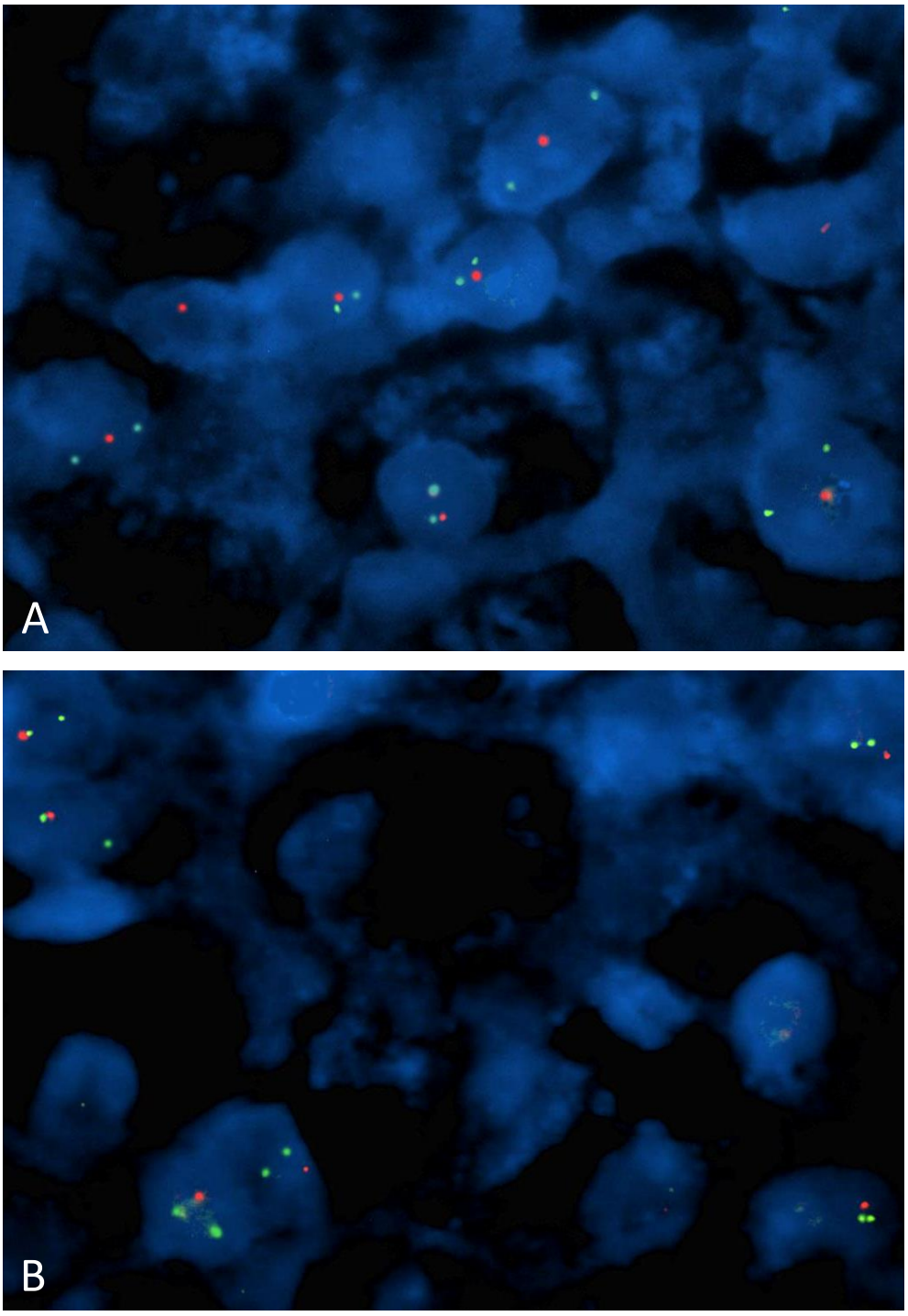


Figure 29. A case of anaplastic oligodendroglioma with co-deletion of 1p (A) and19q (B) (X10000)

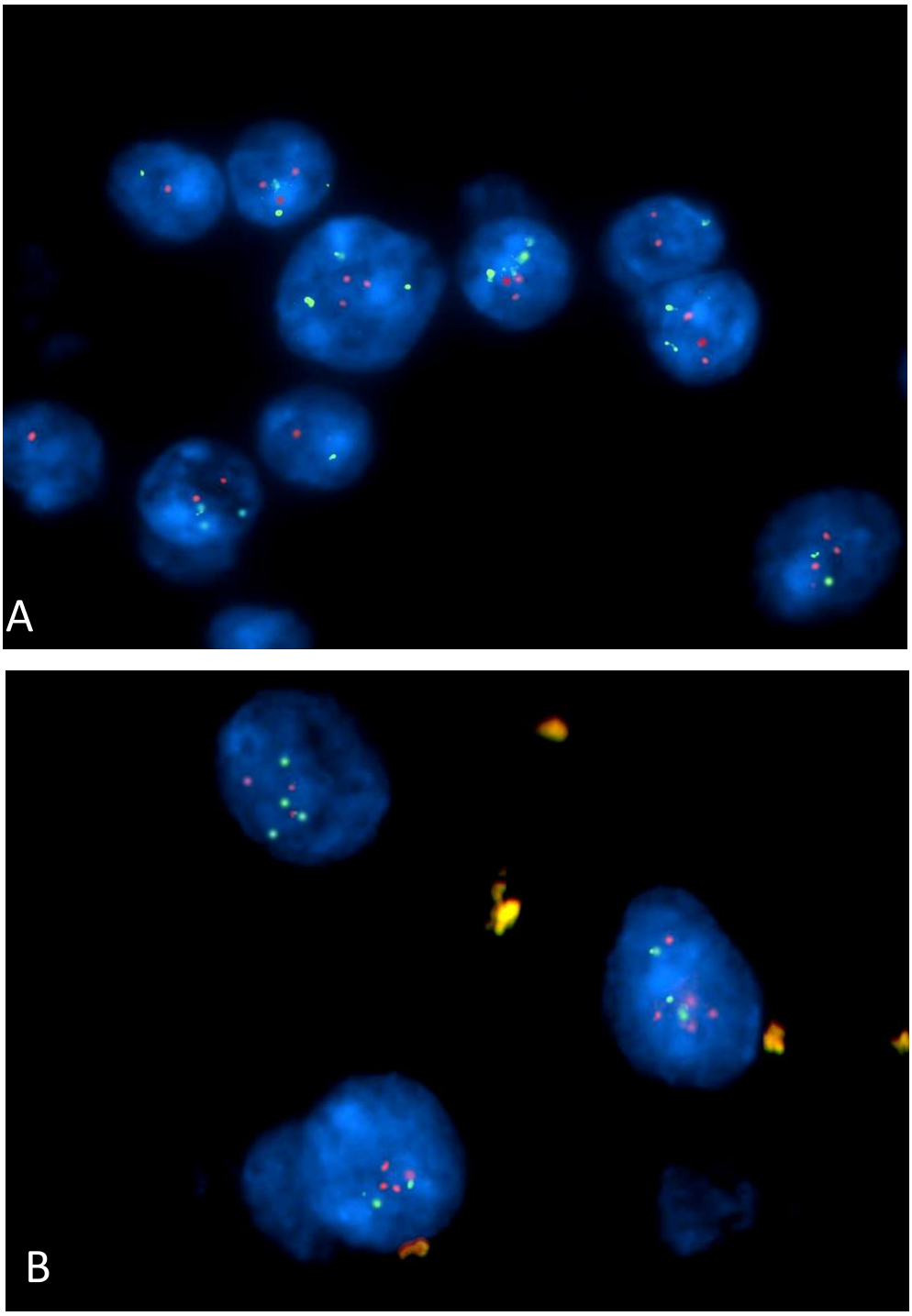


Figure 30. Acase of WHO grade II Oligoastrocytoma with polysomy of both chromosome 1 (A) and 19 (B)

Association of 1p/19q co- deletion with other variables

Various factors were shown to be associated with 1p/19q co deletion. (Table 5)

63% of tumours from frontal lobe showed 1p/19q codeletion, while only 26.1% of tumours from other lobes showed co-deletion ($p = 0.009$).

59.3% of tumours with indistinct borders on MRI showed presence of 1p/19q co-deletion as opposed to 30.4% of tumours with distinct borders.($p = 0.042$).

All tumours with 1p/19q co deletion showed classical features of oligodendroglioma.

($p = 0.0002$).

1p/19q co deletion was not associated with age, clinical presentation or tumour signal intensity (on MRI).

Table 5.Association of 1p/19q co deletion with various factors.

Variables	1p/19q co deletion (Percentage)	P value
Age		
<40 years	13/29 (44.8%)	0.845
≥40 years	10/21 (47.6%)	
Site		
Frontal lobe	17/27 (63%)	**0.009**
Others	6/23 (26.1%)	
Clinical presentation		
Seizures	17/35 (48.6%)	0.717
Others	6/14 (42.9%)	
Borders		
Distinct	7/23 (30.4%)	**0.042**
Indistinct	16/27 (59.3%)	
Histology		
Pure oligodendrogliomas	19/26 (73.1%)	**0.0001**
Oligoastrocytomas	2/17 (11.8%)	
GBMO	2/7 (28.6%)	
Classical Histology		
CFO	23/38 (60.5%)	**0.0002**
NCFO	0/12 (0%)	
IDH mutation		
Present	23/46 (50%)	0.054
Absent	0/4 (0%)	

Association of polysomies with other factors

There were various factors which were associated with polysomy of chromosomes 1/19 (Table 6). Tumours with polysomy of chromosomes 1/19 were less commonly seen in the frontal lobe than the other lobes. ($p = 0.028$). These tumours commonly showed a non classical histology when compared to tumours without polysomy ($p = 0.004$).

Polysomy 1/19 was not associated with age of the patient ($p = 0.126$).

Table 6. Factors associated with polysomy of chromosome 1/19.

Variables	Polysomy 1/19 (Percentage)	P value
Age		
<40 years	13/29 (44.8%)	0.126
≥40 years	5/21 (23.8%)	
Site		
Frontal lobe	6/27 (22.2%)	0.028
Others	12/23 (52.2%)	
Histology		
Pure oligodendrogliomas	1/26 (3.8%)	<0.00001
Oligoastrocytomas	12/17 (70.6 %)	
GBMO	5/7 (71.4%)	
Classical Histology		
CFO	9/38 (23.7%)	0.004
NCFO	9/12 (75%)	

IDH mutation by immunohistochemistry and PCR

Using PCR, IDH mutation was detected in all 43 cases of oligodendroglioma, anaplastic oligodendroglioma, oligoastrocytoma and anaplastic oligoastrocytoma, and in 3 out of 7 cases of glioblastomas with oligodendroglial component (Table 7 &Fig31). IDH mutations were absent in 4 cases. (Fig 32)

The most common type of IDH mutation was of the IDH1R132H (Arg →His) type which was seen in 84% (42/50) of the cases, which included all the 3 GBMOs with IDH mutation. Oneof the WHO grade II oligodendrogliomas showed IDH1R132C mutation (Arg → Cys). 3 cases showed IDH2 mutations, which were all of the R172H (Arg → His) type and all three cases were WHO grade III oligodendrogliomas.

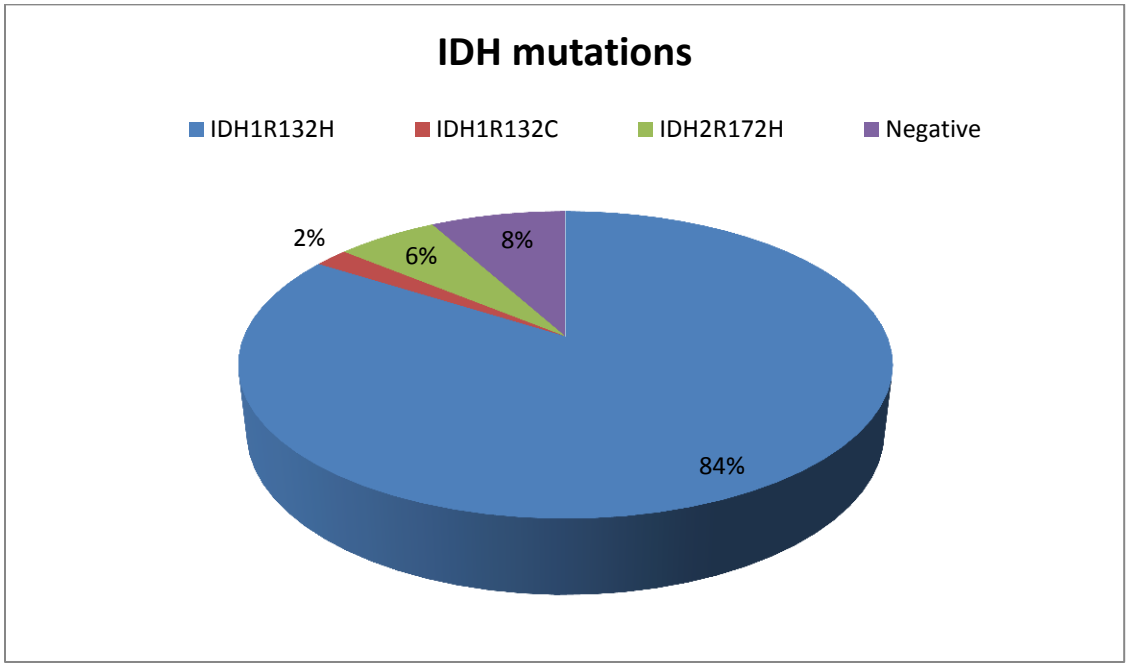
Immunohistochemistry with IDH1antibody was positive in 42 of the total 50 cases.These 42 cases were also found to have IDH1R132H type of IDH mutation (Fig 33). The 4 cases which showed other types of mutations (1 case of IDH1R132C and 3 cases of IDH2R172H) were negative for IDH1 immunohistochemistry.(Fig 34&35) The overall sensitivity and specificity of immunohistochemistry was therefore 91.3% and 100% respectively. There was 100% correlation between IDH1 mutationbyPCR and immunohistochemical expression of the specific mutant protein.

Out of the 7 GBMOs, 4 cases were negative for both IDH1 and IDH2 mutations by PCR. These cases were also negative for IDH1 immunohistochemistry. Both cases of secondary GBMO and 1 of 5 cases of primary GBMO (20%) were positive for IDH 1mutation.

Table 7.Distribution of IDH mutations in various histological subtypes by PCR.

Histological subtype	Number	Percentage
Oligodendroglioma	11	100
Anaplastic oligodendroglioma	15	100
Oligoastrocytoma	7	100
Anaplastic oligoastrocytoma	10	100
GBMO	7	42.8
Primary	1/5	20
Secondary	2/2	100

Figure 31. Types of IDH mutations



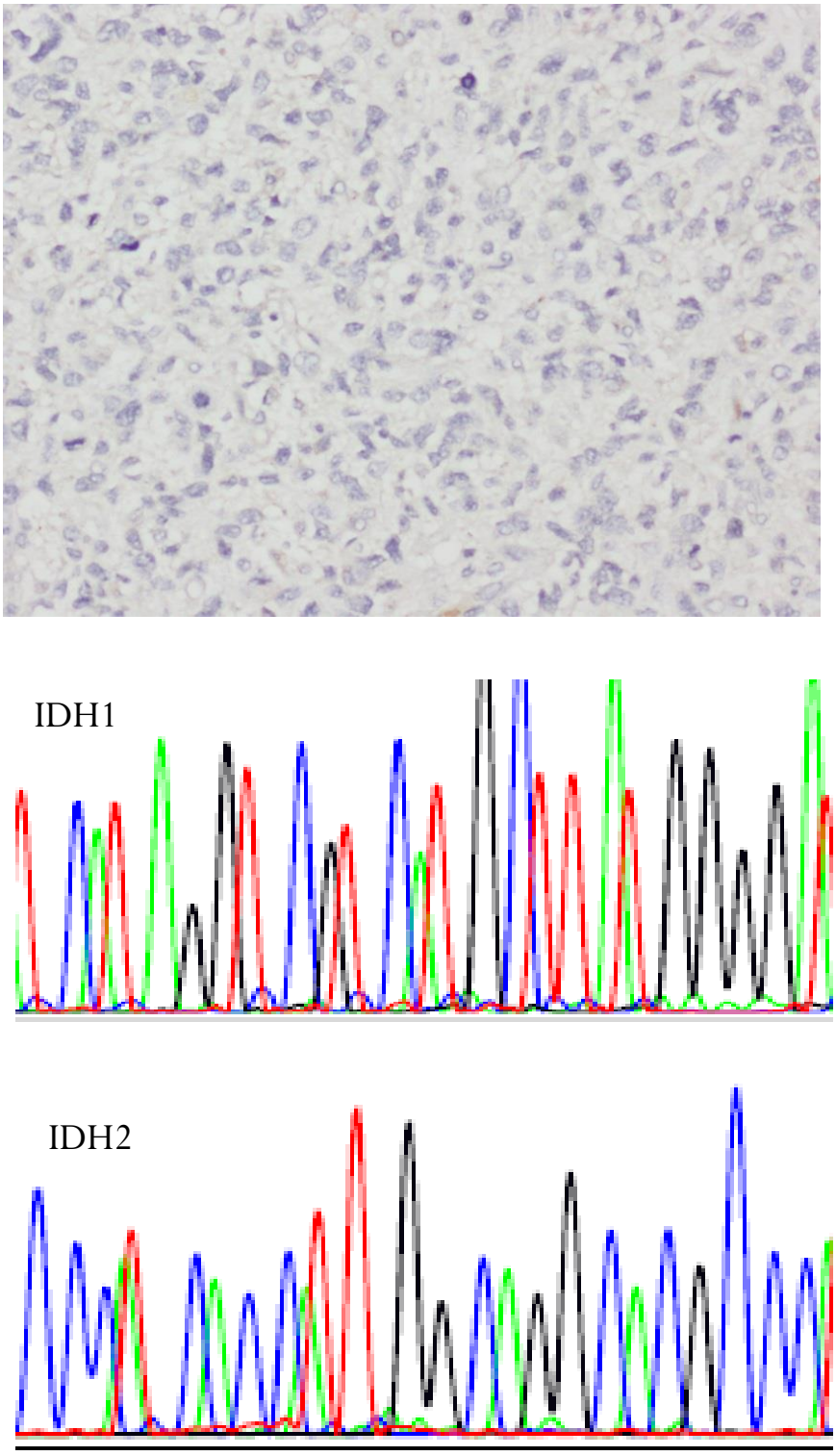


Figure 32. A case of Glioblastoma with oligodendroglial component showing negative immunohistochemical staining for IDH1 IHC (Streptavidin Biotin Peroxidase x200) with the corresponding wild type IDH1 and IDH2 on PCR.

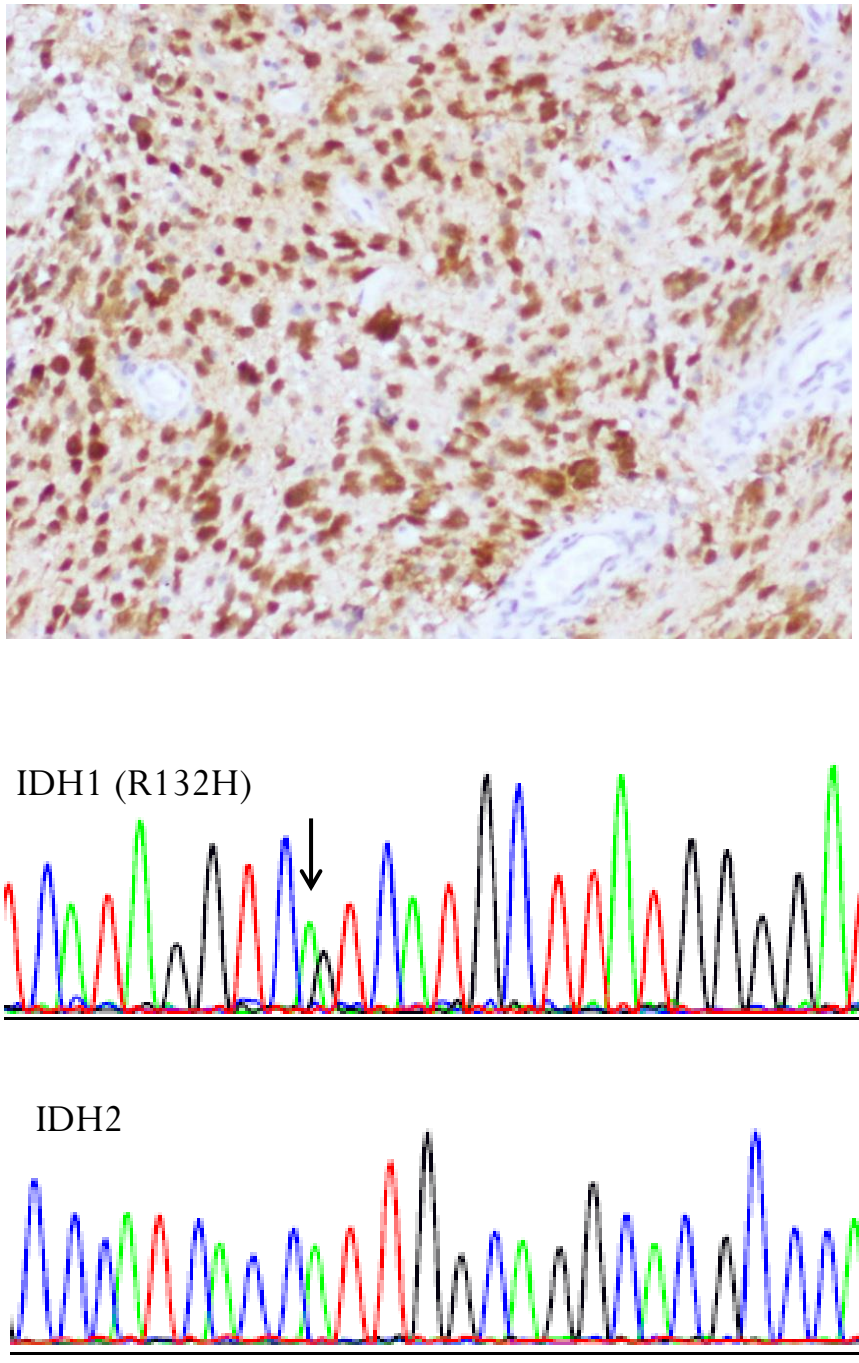


Figure 33. A case of Secondary glioblastoma with oligodendroglial component showing positive cytoplasmic staining of IDH1 IHC(Streptavidin Biotin Peroxidase x200) and corresponding PCR sequences showing IDH1R132H type of mutation (arrow) and wild type IDH2.

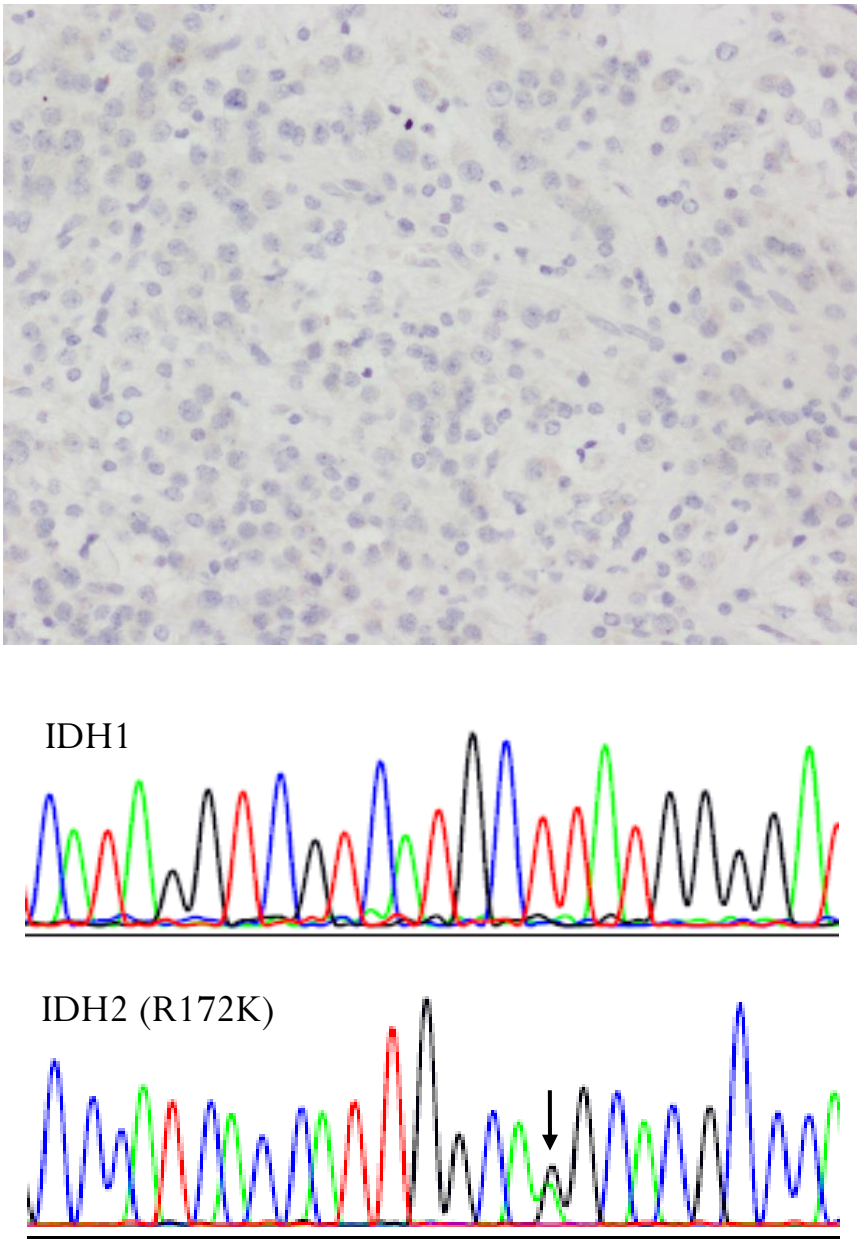


Figure 34. A case of anaplastic oligodendroglioma showing negativeimmunostaining for IDH1 IHC(Streptavidin Biotin Peroxidase x200)and corresponding PCR sequences showing wild type IDH1 and IDH2R172K type of mutation (arrow)

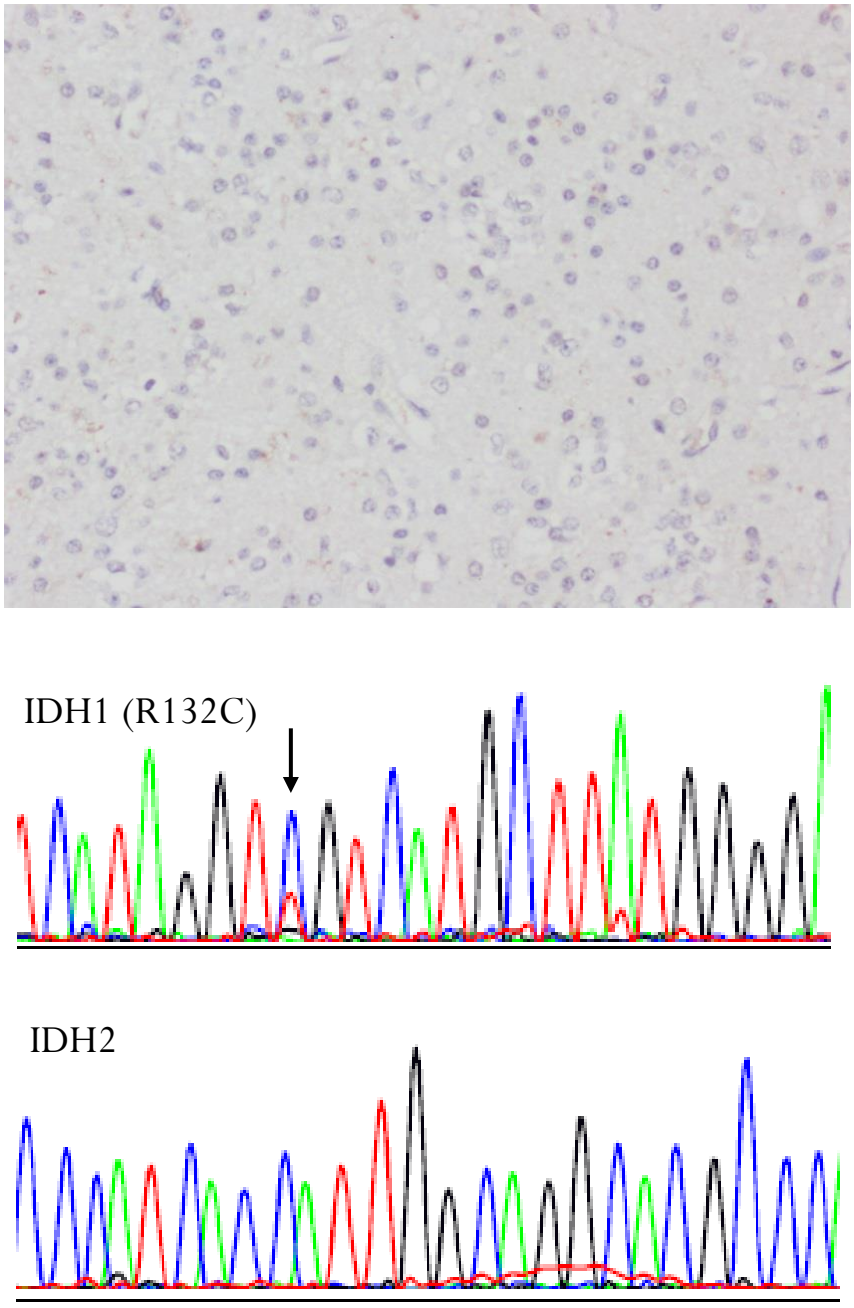


Figure 35. A case of oligodendroglioma showing negative staining for IDH1 IHC (Streptavidin Biotin Peroxidase x200)and corresponding PCR sequences showing IDH1R132C type of mutation (arrow) and wild type IDH2.

23 of 46 (50%) of tumours with IDH mutations showed combined 1p/19q deletion. All the 4 cases that were negative for IDH mutations were also negative for 1p/19q co-deletion, though this did not reach statistical significance (p = 0.054). (Table 5)

Adjuvant therapy

Adjuvant treatment was given in the form of radiotherapy only or combination of radiotherapy and chemotherapy with temozolamide. Majority of the patients (72%) received combination of radiotherapy and chemotherapy. 10 patients (20%) received only radiotherapy. One patient deferred treatment.

Table 8. Treatment details of 50 oligodendroglial tumours.

Treatment details	Number	Percentage
Extent of resection		
Complete/radical	29	58
Partial	21	42
Adjuvant treatment		
Radiotherapy only	10	20
Radiotherapy + Temozolamide	36	72
Deferred treatment	1	2
Not available	3	6

Survival analysis

Follow up information was available for all 50 cases. The duration of follow up ranged from 2 months to 66 months (Median 34 months).

From the cohort of 50 cases, 8 cases were recurrent tumours at the time of presentation. These 8 cases were excluded from survival analysis as the time to recurrence and time to death in these cases would not be comparable to that of the primary tumours. The 8 cases that were excluded were 1 oligodendroglioma, 1 anaplastic oligodendroglioma, 1 oligoastrocytoma, 3 cases of anaplastic oligoastrocytoma and 2 cases of GBMOs. 6 of these patients had another recurrence and 2 patients died during the period of follow up.

10 out of 42 patients had recurrence and 7 patients were dead at the end of follow up period.Of the 10 recurrent tumours there were 3 cases of WHO grade III oligodendroglioma, 2 cases of WHO grade II oligoastrocytoma, 1 case of WHO grade III oligoastrocytoma and 4 cases of grade IV GBMO. Of the patients who died during the follow-up period, there were 2 cases of anaplastic oligodendroglioma, 1 case of anaplastic oligoastrocytoma and 4 cases of GBMO.

8 of the 10 patients with recurrence and 5 of the 7 patients who died had undergone radical surgical excision of tumour.

The total analysis time of follow up was 1514 days on 42 subjects with a median duration of follow up of 36 months. The median survival time was 20.3 months for Grade IV tumours. The median survival time was not reached for grade II and III tumours.

Using Kaplan Meier survival estimates, on comparing the pure oligodendroglial neoplasms with those that also had an astrocytic component, pure oligodendroglialtumours had a better PFS (p = 0.075 (Fig 36)This was however not statistically significant.

Using Kaplan Meier survival estimates, combined WHO grade II and III was compared with WHO grade IVtumours. WHO grade IV showed a significantly worse PFS than tumours with WHO grade II and III (p = <0.0001) (Fig 37).

The 2 sided Fishers' exact test was used to calculate the Risk ratio and Odds ratio for the risk factors of recurrence and death (Table 9&10).

In univariate analysis, necrosis, WHO grade and IDH mutations were found to be associated with a higher risk of recurrence. Patients with tumour necrosis had a 5 times higher risk of recurrence than tumourswithout necrosis (p = 0.005) and patients with a WHO grade IV tumour had nearly 5 times more risk of recurrence than patients with WHO grade II or III tumours (p = 0.0082). Patients with IDH1/2 mutations had nearly 4 times increased risk of recurrence than patients with no mutation (p = 0.03).

In univariate analysis, necrosis, microvascular proliferation, WHO grade (Grade IV vs Grade II&III) and IDH mutations were found to be associated with a significantly increased risk of death. Tumors with necrosis resulted in a 8 times higher risk of death than tumours without necrosis (p = 0.0005), presence of microvascular proliferation showed a 5 times higher risk of death (p = 0.03) and tumours of WHO grade IV resulted in an almost10 times higher of risk of death than patients with WHO grade II or III tumours (p = 0.0015). Presence of IDH mutations was associated with more than 9 times higher risk of death (p = 0.011).

The hazard ratio (HR) of necrosis was found to be significantly higher in tumours with necrosis than tumours without necrosis in a Cox regression model even after adjusting for WHO grade and microvascular proliferation. (HR = 27.2, p =0.037)

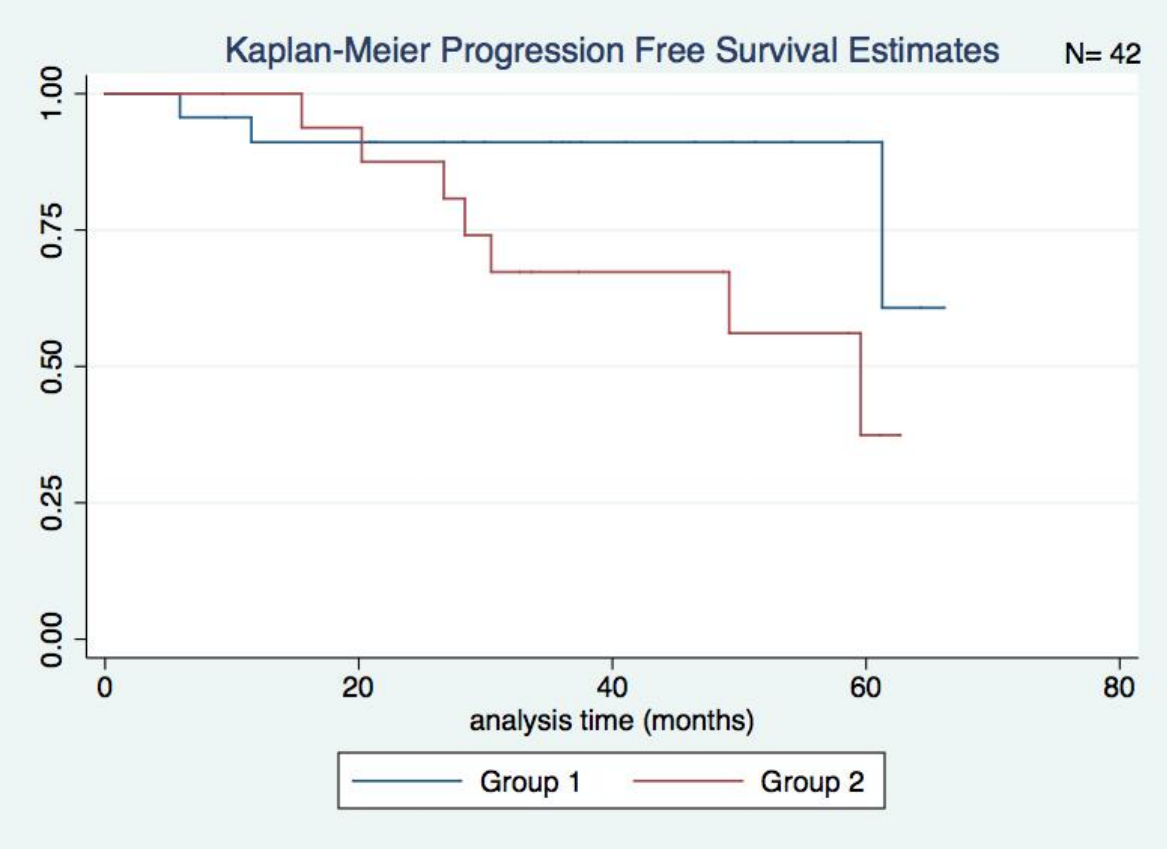


Figure 36. Kaplan-Meier Progression Free Survival Estimates for recurrence in 42 primary oligodendroglial neoplasms, comparing pure oligodendroglial tumours (Group 1) with tumours with both oligodendroglial and astrocytic components (Group 2) (p = 0.075)

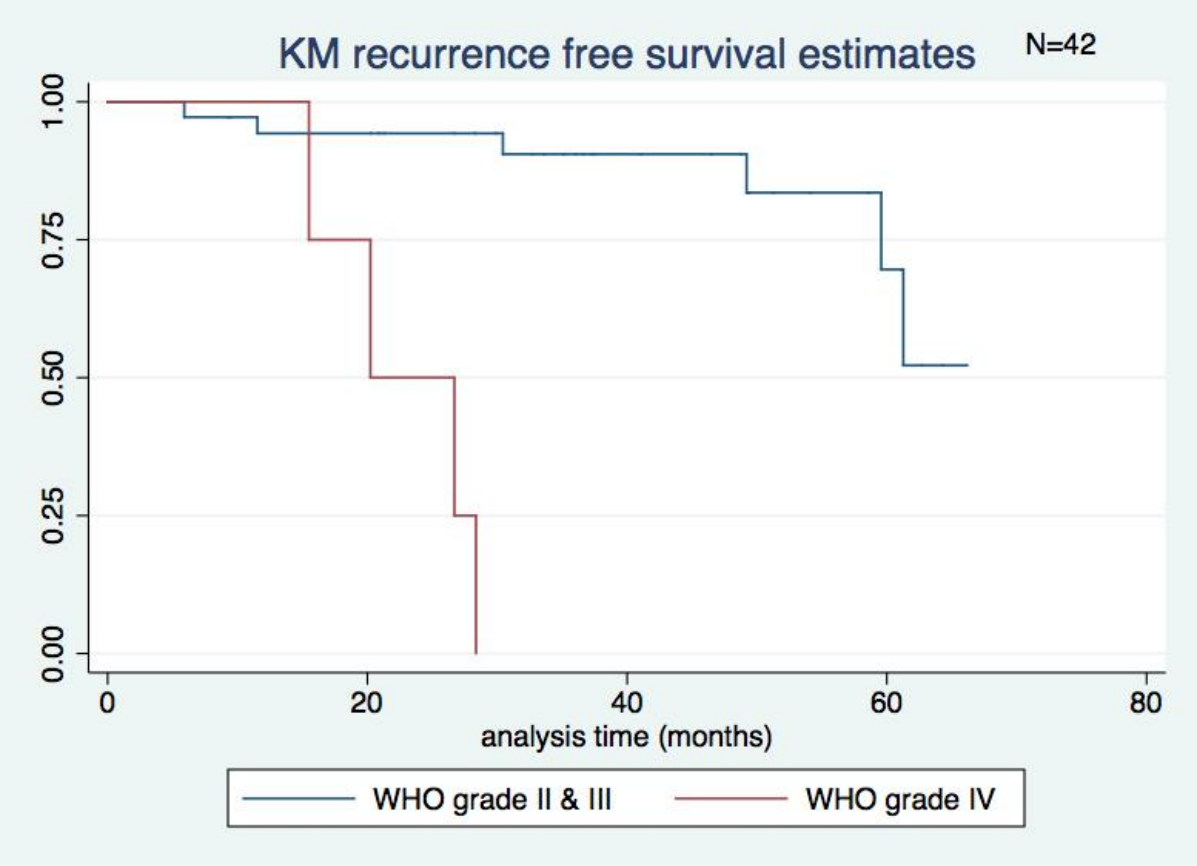


Figure 37. Kaplan-Meier Progression Free Survival Estimates for recurrence in 42 primary oligodendroglial neoplasms, comparing tumors that were WHO grade IV against combined WHO grade II&III tumors. (p = <0.0001)

Table 9.Odds ratio and risk ratio for recurrence for the following risk factors among the 42 primary oligodendroglial tumours.

Risk Factor	Risk ratio	Odds ratio	P value
Age ≥40 years	1	1	1.00
Temporal lobe lesion	1.48	1.7	0.7
KPS scores <90	0.92	0.89	1.00
Partial surgical excision	0.33	0.25	0.15
WHO grade IV (vs WHO grade II & III)	**4.9**	**20.6**	**0.0082**
Absence of classical Histology	1.25	1.35	1.00
Necrosis	**5**	**15**	**0.005**
Microvascular proliferation	3	4.5	0.06
Absence of combined 1p/19q deletion	1.5	1.7	0.72
Absence of 1p/19q deletion	2	2.5	0.28
Polysomy 1/19	2	2.55	0.26
Absence of IDH1/2 mutations	**4.07**	**13.2**	**0.03**

Table 10.Odds ratio and risk ratio for death for the following risk factors among the 42 primary oligodendroglial tumours

Risk Factors	Risk ratio	Odds ratio	P value
Age ≥40 years	2.5	2.97	0.41
Temporal lobe lesion	0.9	0.87	1.00
KPS scores <90	1.47	1.6	0.64
Partial surgical excision	0.53	0.475	0.68
WHO grade IV (vs WHO grade II&III)	**9.86**	**45.33**	**0.0015**
Absence of classical Histology	0.83	0.80	1.00
Necrosis	**12.5**	**41.25**	**0.0005**
Microvascular proliferation	**5**	**7.22**	**0.03**
Absence of combined 1p/19q deletion	1.33	1.41	1.00
Absence of 1p/19q deletion	1.78	2	0.44
Polysomy 1/19	1.5	1.63	0.67
Absence of IDH1/2 mutations	**7.125**	**25.5**	**0.011**

In multivariate analysis using the logistic regression model, we decided to include the following co-variables, WHO grade (grade IV vs combined grade II&III), absence of 1p/19q co-deletion, absence of classical histology and polysomy of chromosome 1/19 into the logistic regression model and found that only WHO grade was significantly associated with an increased risk of recurrence. (Table 11) Patients with WHO grade IV tumours had nearly 20 times Odds of recurrencethan patients with WHO grade II/III (p = 0.022). Although IDH mutation was significant in univariate analysis, we could not include IDH mutations in a multivariate analysis because of the small number of negative cases and this made the model unstable.

Table 11.Multivariate analysis using logistic regression model on 42 oligodendroglial neoplasms for PFS.

Risk factor	Odds ratio	P value
WHO grade (IV vs II&III)	20.17	0.022
Absence of classical histology	1.33	0.818
Absence of 1p/19q co-deletion	0.88	0.903
Polysomy 1/19	1.13	0.907

DISCUSSION

Oligodendroglial tumours are the one of the most common primary parenchymal brain tumours in adults after astrocytic tumours. These tumours include a spectrum of diffusely infiltrating gliomas morphologically resembling normal oligodendroglial cells of the brain. The first description of oligodendroglioma as a distinct entity was published by Bailey and Cushing in 1926. (23)

They can be classified into five types according to the WHO classification of Tumours of the Central Nervous System.(1) These include WHO grade II Oligodendroglioma, WHO grade III Oligodendroglioma, WHO grade II Oligoastrocytoma, WHO grade III Oligoastrocytoma and WHO grade IV Glioblastoma with an oligodendroglial component.(1)

Pure oligodendrogliomas have characteristic histological features like monomorphic round, regular nuclei, well defined nuclear outlines, evenly dispersed chromatin, small/inconspicuous nucleoli, perinuclear halos and chicken wire calcifications. Anaplastic or WHO grade III oligodendogliomas show in addition to the above features, high cellularity, nuclear atypia, increase in mitotic activity, necrosis or microvascular proliferation.(1)

Mixed oligoastrocytomas have a conspicuous mixture of both neoplastic oligodendrocytes and neoplastic astrocytes within the same tumour. Anaplastic or WHO grade III oligoastrocytomas show other features like increased cellularity, pleomorphism, nuclear atypia and increase in mitotic activity(1). Glioblastoma with an oligodendroglial component is a WHO grade IV tumour and is a recent addition to the WHO classification.

According to a study by Miller et al, presence of necrosis in an anaplastic oligoastrocytoma was significantly associated with a shorter survival time, thus justifying the separation of anaplastic oligoastrocytomas with necrosis into Glioblastoma with an oligodendroglial component.(36)

Although the diagnosis of classic examples oligodendroglial tumours is straightforward, a significant proportion of these tumours, particularly among the high grade gliomas and mixed oligoastrocytic tumours, can pose diagnostic difficulties. Due to different diagnostic approaches and subjective morphologic criteria, there has been considerable interobserver variability and low diagnostic reproducibility in the classification of mixed oligoastrocytic tumours even among experienced neuropathologists.(6–9)

Hence, histological classification and grading of these tumours needs to be supplemented by more objective molecular markers that can aid in the diagnosis and predict response to chemotherapy and overall survival.

Chromosome 1p/19q deletion has been recognized as a typical molecular signature of oligodendroglial tumours. The frequency of 1p/19q deletion varies from approximately 80% in oligodendrogliomas, 50-60% in anaplastic oligodendrogliomas and 30-50% in oligoastrocytomas and anaplastic oligoastrocytomas.(1) In 1998, Cairncross et al showed that 1p/19q co-deleted anaplastic oligodendrogliomas showed better response to chemotherapy and was significantly associated with longer overall survival.(60) Further studies have validated and confirmed these findings.(12–14) Hence, combined 1p/19q deletion can be used as a predictive and prognostic marker in oligodendroglial tumours.

Parsons et al in 2006 first demonstrated the presence of IDH1 (Isocitrate Dehydrogenase) mutations in 12% of glioblastomas.(82) The mutation was more common in secondary glioblastomas. These initial findings led to further studies which showed that IDH1 mutations were seen in most cases of WHO grade II and III diffuse astrocytomas, oligodendrogliomas and oligoastrocyomas and lower frequencies of IDH2 mutations.(79,80,83–87,89,93,95,97,98)

IDH mutations are thought to be early genetic events in glioma tumorigenesis. It is suggested that astrocytoma, oligodendroglioma and oligoastrocytomas probably arise from common glial precursor cells carrying IDH1 mutations. Further loss of chromosome 1p/19q or acquisition of TP53 mutations may lead to oligodendroglial or astrocytic differentiation respectively.(84,96) Studies have shown that IDH mutations are associated with a better prognosis..(13,61,81,85,89,91,93,94,99) IDH mutations are strongly associated with other molecular markers like 1p/19q deletion and MGMT methylation.(89)

The peak incidence of oligodendroglial tumours is in the 4^{th} to 5^{th} decade.(1,21,35,100) In our study also, the median age was 39 years. According to literature, males are slightly more commonly affected than females(1)In our study, males were much more commonly affected with a male female ratio of 2.3:1. As our cohort was selected based on availability of frozen tissue and as they were not consecutive cases, this may not be a true representation.

The most common clinical presentation in our study was seizures, followed by focal neurological deficits and features of raised intracranial tension. Seizures are reported as the most common mode of presentation in other studies as well.(1,21,101,102)

The most common site of tumour in our subset of patients was the frontal lobe, followed by temporal lobe, parietal lobe and the occipital lobe in decreasing order of frequency as was seen in other studies.(1,56,101) Moreover, tumours showing classical features of oligodendroglioma were more commonly seen in the frontal lobe than tumours with non classical histology (p= 0.021). In a study by Giannini et al on 247 anaplastic oligodendroglial tumours, tumours with CFO were more frequently seen in the frontal lobe (56). A similar study conducted at our institution on a cohort of glial neoplasms also showed similar results(59)

Fluoresence In Situ Hybridisation

Combined 1p/19q deletion was seen in 73.1% of pure oligodendroglial tumours, which is similar to results from other studies where the frequency of 1p/19q deletion in pure oligodendrogliomas ranges from 60-80%.(1) In contrast to other studies, the frequency of combined 1p/19q deletion was more in WHO grade III oligodendroglioma (80%) than WHO grade II oligodendroglioma (63.6%). However, in our cohort of cases, the number of Grade III oligodendrogliomas was more than grade II tumours. The mode of selection of cases in our study may also explain the discrepancy in the results.

The percentage of combined 1p/19q deletion among oligoastrocytomas was 11.6%. Although, severalstudies have found frequencies of 1p/19q deletion in oligoastrocytomas between 30-50%, there have been other reports which are to the contrary.(25,35,71,83,100) In a study by Fuller et al on 90 mixed oligoastrocytomas, 1p/19q co-deletion was seen only in 9% of the cases.(7) In over 2500 gliomas, including 1006 oligoastrocytomas, tested at the Washington University spanning over a decade, 1p/19q co deletion was seen in only

17% of oligoastrocytomas. (103)These variations in frequency in 1p/19q status among the mixed oligoastrocytomas reflect the low reproducibility and highly subjective nature of diagnosis of these ambiguous gliomas.

The percentage of combined 1p/19q deletion in GBMOs in our cohort was 28.6%. According to studies the frequency of combined 1p/19q deletion in GBMOs ranges from 3 to 29.6%, which is similar to that seen in our study.(38,40–44,47) However, in a study by He et al on 25 GBMOs, the reported frequency of 1p loss and 19q loss was as high as 40% and 60% respectively.(43)

In a study by Mc Donald et al on 131 anaplastic oligodendroglial tumours, it was observed that only the most classic oligodendroglial tumours, as scored by 4 of 5 experienced neuropathologists exhibited favourable survival.(52)Few studies have shown that classical oligodendroglial morphology was associated with better PFS and OS on univariate and multivariate analysis.(52,56,104,105) In the present study, combined 1p/19q deletion was significantly associated with classical features of oligodendroglioma (CFO) ($p = 0.0002$). Twenty three of 38 cases (60.5%) with classical histology showed 1p/19q co-deletion and none of the tumours with non classical histology showed deletion. In a study by Mueller et al, 1p/19q loss was seen in 19 of 22 cases of classic oligodendroglioma, while only 6 of 22 non classic tumours showed this pattern.(25) Since, classical histology is in itself a predictor of survival, attention to histological detail and strict criteria for oligodendroglial morphology may provide prognostic information comparable to 1p/19q analysis. Hence, classical histology may be used as a surrogate marker for 1p/19q status in centres without access to molecular genetic testing. However, it is important to note that although classical

histology is significantly associated with 1p/19q co deletion, the correlation is not absolute. Few tumours with classical histology are intact for 1p/19q, while a proportion of tumours with non classical histology show 1p/19q co-deletion.

Majority of the tumours (63%) arising from the frontal lobe showed combined 1p/19q deletion, while only 23.1% of tumours from other lobes showed deletion ($p = 0.009$). Others studies have also shown that tumours located in the frontal, parietal and occipital lobes were more likely to harbor 1p/19 deletion than tumours in the temporal lobe, insula and diencephalon.(25,52,53,72,104)

Similar to other studies, we found a positive correlation of 1p/19q co-deletion with indistinct borders as seen on imaging. In a study by Megyesi et al, 1p/19q deletion was associated with indistinct borders on T1 images. Hence indistinct borders or margins may be the first identifier of tumours with a good prognosis.(73)

Polysomy of chromosome 1/19 was more commonly seen in mixed oligoastrocytic tumours than pure oligodendroglial tumours ($p = <0.0001$). A study by Jiang et al on 584 gliomas showed that polysomy of chromosome 1/19 was more common in mixed gliomas than pure oligodendrogliomas or astrocytomas.(66) Studies have shown that polysomy of both chromosomes 1 and 19 in 1p/19q co-deleted tumours predicts earlier recurrence in oligodendroglial tumours including GBMOs.(66–69). This association could not be analysed in our cohort because of the small number of cases with both 1/19 polysomies and combined 1p/19q deletion.

Polysomy of chromosome 1/19 was negatively associated with frontal lobe tumours and classical histology ($p = 0.028$ and $p = 0.004$ respectively).

IDH mutations

IDH mutations were detected in all 43 cases of WHO grade II oligodendroglioma, WHO grade III oligodendroglioma, WHO grade II oligoastrocytoma, WHO grade III oligoastrocytoma and 3 of 7 cases (42.8%) of grade IV GBMOs. IDH1 mutations were more frequent than IDH2 mutations and were present in 86% and 6% of the tumours respectively.

The reported frequencies of IDH mutations in astrocytomas, oligodendrogliomas and oligoastrocytomas range from 50 – 80%. (87,91,93,97) In a study by Jiao et al, the frequency of IDH1 mutations was found to be 86% in grade II oligodendroglioma, 96% in grade III oligodendroglioma, 100% in grade II oligoastrocytoma and 95% in grade III oligoastrocytoma which is comparable to high frequencies seen in our study.(75) Another study by Yan et al also found high frequencies of IDH1/IDH2 mutations in oligodendroglial (84% and 94% of grade II and III oligodendrogliomas) and oligoastrocytic tumours (100% of grade II and III oligoastrocytomas).(85) In an Indian study by Thota et al conducted at a centre in South India, the frequencies of IDH1 mutations were found to be 100% in diffuse astrocytomas, 92.9% of anaplastic astrocytomas and 83.3% of secondary glioblastomas. However, IDH mutations in oligodendrogliomas were not described in the above study.(86)

IDH1 mutations were seen in 42.8% of the GBMOs. In the present study, both cases of secondary GBMOs and 20% (1 out of 5 cases) of primary GBMOs harbored IDH mutations. Several studies have shown that IDH1 mutations are more common in secondary GBMs (50-88%), and are infrequently seen in primary GBMs (3-12%). (79)The frequency of IDH1 mutation is higher in GBMOs than classical GBMs with a reported frequency of 3% to

35%(38,41–43,93) There were only 7 cases of GBMOs in our study and hence our data cannot be used to comment on true prevalence of IDH mutations in GBMO. Although, similar to other reports we too observed IDH mutations more frequently in secondary GBMO.

The most common type of mutation in IDH1 mutation was of the IDH1R132H type (42 cases), followed by IDH1R132C (1 case). All 3 positive cases of IDH2 mutation were of the IDH2172K type. According to a study by Hartmann et al on 1010 diffuse gliomas, the most common IDH1 mutation was of the R132H type (92.7%), followed by R132C, R132S, R132G and R132L in decreasing frequencies. They found significant association of R132C mutation with astrocytomas.(80) In our study, the one case which showed the R132C mutation was a WHO grade II oligodendroglioma. Studies have shown a positive association between IDH2 mutations with an oligodendroglial component.(80) We found that all the 3 IDH2 mutations were seen in WHO grade III oligodendroglioma. IDH1 and IDH2 mutations were mutually exclusive as seen in literature.(80) None of the cases showed both IDH1 and IDH2 mutations.

We also analysed the usefulness of IDH1 immunohistochemistry in detecting the presence of IDH mutations in FFPE section. Immunohistochemistry with IDH1antibody was positive in 42 of the total 50 cases. These 42 cases were also found to have IDH1R132H type of IDH mutation by PCR. The 4 cases which showed other types of mutations (1 case of IDH1R132C and 3 cases of IDH2R172H) were negative for IDH1 immunohistochemistry. The overall sensitivity and specificity of immunohistochemistry for detection of IDH mutation was therefore 91.3% and 100% respectively. IDH1 antibody is a mouse

monoclonal antibody targeting the IDH1-R132H mutation.(15) Hence, there was 100% correlation between IDH1 mutation status by PCR and immunohistochemical expression of the specific mutant protein. The high sensitivity and specificity of IDH1 immunohistochemistry highlights the usefulness of a simple and cost effective laboratory technique in assessing IDH1 mutational status. IDH1 immunohistochemistry can be used a potential diagnostic marker to differentiate between tumour cells from reactive gliosis, and astrocytomas/oligodendrogliomas from pilocytic astrocytomas, ependymomas and glioneuronal tumours. It can be also used as a prognostic marker in diffuse gliomas.(13,85,89,93)As observed in our study, one potential pitfall is the inability of the IDH1 antibody to pick up tumours showing other types IDH1 mutation or IDH2 mutations which are negative on immunohistochemistry. Hence, a subgroup of gliomas with a favourable prognosis can be missed if only immunohistochemistry is used to detect the mutational status. PCR for IDH mutations therefore has a role in those cases that are negative for IHC.

Survival Analysis

In our study, tumours with a pure oligodendroglial component (Grade II and III) were compared with tumours containing both oligodendroglial and astrocytic components (Grade II, III and IV) for the risk of recurrence in 42 cases using the Kaplan Meier survival estimates. Tumours with pure oligodendroglial tumours were found to have better prognosis, though this did not reach statistical significance. ($p = 0.075$). A study by Miller et al on 1093 high grade gliomas showed that anaplastic oligodendrogliomas showed a significantly better overall survival than tumours with either anaplastic oligoastrocytoma

(WHO grade III and IV) or anaplastic astrocytoma (WHO grade III).(36) Kowenhoven et al in their study on 368 anaplastic oligodendroglial tumours also showed that survival of anaplastic oligodendrogliomas is better than anaplastic oligoastrocytomas.(72) According to a population based study in Switzerland, presence of oligodendroglial component is associated with a longer survival in low grade gliomas. Patients with oligodendrogliomas (median survival times 11.6 years) survived longer than patients with oligoastrocytomas (median survival time 6.6 years).(35) However, in another population based study, there was no difference in survival between WHO grade II oligodendroglioma and oligoastrocytoma.(100)

Miller et al showed that Grade IV GBMOs (necrotic oligoastrocytomas) have a significantly worse median overall survival than patients with WHO grade III gliomas.(36) In our study as well, using Kaplan Meier survival estimates for recurrence, WHO grade IV tumours were found to have a significantly worse PFS time than combined WHO grade II and III tumours. We combined grade II and III together as there was no significant difference between grade II and III tumours in terms of PFS and OS.

According to population based statistics, the median overall survival time for Grade II tumours ranges from 3.5 years to more than 10 years and for Grade III tumours ranges from 3 to 5 years.(1) The median survival was not reached for grade II and III tumours in our subset of patients. Hence, our cases need to be followed up for a longer duration of time for a reliable comparison between WHO grade II and III tumours.

The median overall survival for GBMOs in our study was 20.3 months which is comparable to other published studies.(36,38,41,43,45)

On univariate analysis, necrosis, WHO grade and IDH mutations were found to be significantly associated with higher risk of recurrence. Tumours with necrosis were 5 times more at risk of recurrence than tumours without necrosis (p =0.005). WHO grade IV tumours were 5 times more at risk of recurrence than WHO grade II and III tumours. (p = 0.0082).Absence of IDH mutations was associated with 4 times increased risk of recurrence (p = 0.03)

On univariate analysis for overall survival, necrosis, microvascular proliferation, WHO grade and IDH mutations were found to be associated with a higher risk of death. WHO grade IV tumours had almost 10 times increased risk of death than WHO grade II and III tumours. Tumours with necrosis were 12.5 times more at risk of death, and tumours with microvascular proliferation were 5 times more at risk than tumours without necrosis and microvascular proliferation respectively.Absence of IDH mutations was associated with 7 times increased risk of death (p = 0.01)

The hazard ratio of necrosis (HR = 27.2) for recurrence was found to be significantly higher than tumours without necrosis on a Cox regression model even after adjusting for WHO grade and microvascular proliferation (p = 0.037). However, we preferred a logistic regression analysis model for multivariate analysis of other variables because of the small number of cases in our study and the short duration of follow up.

Miller et al argued that there was no difference in survival between Grade III oligodendrogliomas with and without necrosis(36)Kouwenhoven et al found that necrosis was prognostically significant in anaplastic oligodendrogliomas on univariate and multivariate analysis in their study on 368 anaplastic oligodendroglial tumours. (72)In our

cohort, one of the 2 patients of anaplastic oligodendroglioma with necrosis had a recurrence and died within 11 months of diagnosis. The association of necrosis with recurrence probably suggests that presence of necrosis is significant even in WHO grade III oligodendrogliomas.As the number of cases in our study was too small, we feel that a larger number of cases need to be analysed before a definite conclusion can be made.

On multivariate analysis using a logistic regression model, only WHO grade was found to be a predictor of recurrence when compared with other variables like classical histology, 1p/19q status and polysomy of chromosome 1/19. Though necrosis and microvascular proliferation were found to be significant in univariate analysis, we did not add these variables in the multivariate model as necrosis and microvascular proliferation are criteria used for grading of a grade IV neoplasm.

As opposed to other studies, we did not find a significant association of classical histology and 1p/19q co-deletion with recurrence. Since the association of classical histology and 1p/19q co-deletion is more established in Grade II and III oligodendroglial tumours, and the median PFS and OS time was not reached for Grade II and III tumours in our study, a longer duration of follow up is required before commenting on the prognostic significance of these variables.

Though absence of IDH mutation was found to be significant in univariate analysis for risk of recurrence and death, we could not add this variable in a multivariate model because of the small number of negative cases. Hence, a meaningful assessment of IDH as a prognostic marker could not be made.

In this study of 50 cases with oligodendroglial neoplasms, IDH mutations were found to have a high prevalence.

In conclusion, as both IDH mutation and 1p/19q co-deletion are considered good prognostic variables and IDH mutations were more frequently observed than 1p/19q deletions, there is a role to assess for IDH mutations in oligodendroglial tumours.

Studies on a larger cohort with longer follow up are required to draw meaningful conclusions.

CONCLUSION

- 1p/19q co-deletion was seen in 46% of the oligodendroglial tumours.
- 1p/19q co-deletion was more commonly seen inpure oligodendroglial tumours and frontal lobe tumours.
- 1p/19q co-deletion was significantly associated with classical histology. Classical histology may be used as a surrogate marker for 1p/19q status in centres where genetic testing is not readily available.
- Polysomy of chromosome 1/19 was present in 18/50 (36%) of the oligodendroglial tumours.
- Polysomy of chromosome 1/19 was commonly seen in mixed oligoastrocytic tumours. Presence of polysomy of 1/19 may be used to distinguish mixed oligoastrocytic tumours from pure oligodendroglial tumours.
- Polysomy of chromosome 1/19 were negatively associated with frontal lobe location and classical histology..
- IDH1 and IDH2 mutations were seen in 86% and 6% of the cases respectively.
- The most common type of IDH1 mutation was IDH1R132H (42 cases), followed by IDH1R132C (1 case). All 3 IDH2 mutations were of the IDH2R172H type.
- IDH1 immunohistochemistry was seen in 42 (84%) cases with a sensitivity and specificity of 91.3% and 100% respectively.
- IDH1 immunohistochemistry can be used as a simple and inexpensive laboratory test to determine IDH mutational status in places where genetic testing is not readily available.

- IDH1 immunohistochemistry can be supplemented by IDH PCR in all cases that are negative by immunohistochemistry.
- 50% (23/46) of the cases with IDH mutation also showed 1p/19q co-deletion and all cases negative for IDH mutation were also negative for 1p/19q deletion.
- Determination of IDH mutational status is imperative in addition to assessement 1p/19q status in order to identify patients with better prognosis.
- On univariate analysis, necrosis, WHO grade and IDH1/2 mutation were significantly associated with higher risk of recurrence and death. Microvascular proliferation was associated with a higher risk of death but not of recurrence.
- On multivariate analysis only WHO grade was associated with an increased risk of recurrence.

LIMITATIONS

- Our sample size was limited by financial constraints. A study on a larger cohort of patients is necessary to draw meaningful conclusions.

- The median survival time was not reached for Grade II and III tumours in our study. Hence, a longer duration of follow up is needed before commenting on the significance of prognostic variables like 1p/19q and IDH mutastion.

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APPENDIX 1

PROFORMA

Serial number:

Biopsy number

Hospital number

Age

Gender

RADIOLOGY:

Site of Tumour

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

Laterality: 1.Right 2.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 - indisitnct.

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.

KPS score:

Primary/Recurrent tumours:

HISTOLOGICAL DIAGNOSIS

1. Oligodendroglioms
2. Anaplastic oligodendroglioma
3. Oligoastrocytoma
4. Anaplaticoligoastrocytoma
5. Glioblastoma multiforme with oligodendroglial component.

MIB-1 labelling index:

Classical Histology: 1 – classical, 0 - Present

Intermingled/separate (For mixed tumours): 1 – intermingled, 2 – separate

Necrosis: 0 – Absent, 1 – Present

Microvascular proliferation: 0 – Absent, 1 – Present.

MOLECULAR PARAMETERS:

FISH FOR 1P/19Q DELETION

1p deletion 1+ (Yes) 2- (No)

19q deletion 1+ (Yes) 2- (No)

1p polysomy 1+ (Yes) 2- (No)

19q polysomy 1+ (Yes) 2- (No)

IDH1 IHC (paraffin blocks)

1+ (positive) 2- (Negative)

IDH MUTATION (fresh tissue)

IDH1 1+ (positive) 2- (Negative)

IDH2 1+ (positive) 2- (Negative)

Type of mutation (if present)

SURGERY

1. Radical excision
2. Partial excision
3. Biopsy

ADJUVANT THERAPY

1. Chemotherapy
2. Chemotherapy with Temozolamide
3. Radiotherapy
4. Radiotherapy with Temozolamide

SURVIVAL

Date of surgery

Date of last follow up:

Duration of follow up (months):

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

Progression free survival

Death 1+ (Yes) 2- (No)

Overall survivial

APPENDIX 2

Procedure for FISH

1. Paraffin embedded tissue was cut at 3 um thickness.
2. The slides were coated by sialinization method.
3. Incubation
    - The tissue block cut at 3um was floated onto a coated slide in a water bath at 40 C.
    - Slides were incubated overnight at 37 C.
    - Next day morning the slides were transferred to an incubator and kept at 56 C for 2 hours.
    - The area of interest was marked with a diamond pencil
4. Deparaffinising
    - Deparaffinising was done with xylene. Three changes, each lasting 10 minutes were made.
    - The slides were dehydrated with 100% isopropanol by making these changes, each lasting 3 minutes.
    - The slides were air dried for 2-5 minutes.
5. Slide pretreatment

    Note: In the beginning of the procedure two water baths were switched on, one at 80 C and another at 37 C.
    - A coplin jar with pretreatment solution (received as a part of the FISH kit) was kept in the 80 C water bath until its temperature reached 80 C.
    - The slides were immersed in pretreatment solution at 80 C for 10 minutes. (A maximum of 6 slides were processed at once by placing 2 slides back to back in the coplin jar slot. The end slides were kept singly with the tissue section facing the centre of the jar).
    - The slides were immersed in purified water for 3 minutes following which extra water was blotted off the slide edges using paper towels.
    - Protease solution was prepared by thoroughly mixing protease powder (received as a part of the FISH kit and stored in a freezer at -20 C) with protease buffer in a coplin jar. The coplin jar was placed in a water bath and allowed to attain a temperature of 37 C.
    - Slides were immersed in the protease containing coplin jar after it had reached a temperature of 37 C for 15 minutes.
    - Slides were then immersed in purified water for 3 minutes.
    - After removing the slides from the jar of purified water, they were air dried for 2-5 minutes
6. Hybridisation was done by codenaturation on the Abbot Molecular ThermoBrite hybridization unit.

- Dehydration was performed by serially immersing the slides in 70%, 85% and 100% ethanol for 1 minute each.
- A strip of paper towel was moistened with water and placed in the channel along the heating surface of the hybridisation unit.
- The denaturation temperature was set to 90 C with a melt tine of 13 minutes. The hybridisation temperature was set to 37 C and hybridisation time to 16 hours overnight.
- 10 microlitres of FISH probe (Spectrum Orange flurophore labeled probe against telomeric region of 14q) was applied to the slide and coverslip was placed immediately.
- The coverslip was sealed with rubber cement and co-hybridisationprogramme was initialised.

7. Washing
    - After overnight hybridization the rubber cement is removed from the slides.
    - 50 ml of 2XSSC/0.3% NP40 (commercially available wash solution) was placed at 73 C water bath in a coplin jar.
    - The slides are washed in 2XSSC/0.3% NP40 at room temperature and allowed to stand for 2-5 minutes till the coverslips floated off the slides.
    - The slides were then immersed in pre-warmed 2XSSC/0.3% NP40 at 73 C and agitated for 1-3 seconds.
    - Finally the slides were again agitated in 2XSSC/0.3% NP40 at room temperature for 1-3 seconds and kept in the same solution for 5 seconds to 1 minute.
8. Visualisation
    - The slides were air dried in darkness.
    - 10 microlitres of DAPI (4’6-diamino-2-phenylindole) counterstain was applied to the tissue section and coverslip was applied.
    - The slides were viewed using Spectrum orange filter under Olympus BX51 fluorescence microscope.

APPENDIX 3

Preparation of TRIS Borate EDTA Buffer

1. Preparation of Stock Solution of 0.5M EDTA
    a. Dissolve 93.05g of EDTA disodium salt in 400 ml of deionized water.
    b. Adjust pH to 8.0 with Sodium hydroxide for the EDTA salt to completely dissolve.
    c. Top up the solution with deionized water to 500ml.

2. Preparation of Concentrated (5x) Stock Solution of TBE
    a. Dissolve 54g of TRIS-HCl and 27.5g of boric acid in 900 ml of deionized water.
    b. Add 200ml of 0.5M EDTA (pH 8.0)
    c. Adjust to final volume of 1 litre with deionized water.
    d. Store at room temperature in glass bottles.

3. Preparation of Working (1x) Solution of TBE
    a. Dilute 50ml of 5x TBE stock solution with 200ml of deionized water (1:5 dilution).
    b. Use for agarose electrophoresis.

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July 25, 2013

Dr. Tulasi Geevar
Department of Pathology
Christian Medical College
Vellore 632 002

Sub: **FLUID Research grant project NEW PROPOSAL:**
Molecular profile of tumours with oligodendroglial morphology – clinical relevance.
Dr. Tulasi Geevar, Pathology, Dr Geeta Chacko, Dr Rekha Pai, Pathology,
Dr Ari G Chacko, Neurosurgery, Dr Subhashini John, Dr Rajesh B Radiotherapy.

Ref: IRB Min. No. 8246 [OBSERVE] dated 19.03.2013

Dear Dr. Tulasi Geevar,

I enclose the following documents:-

1. Institutional Review Board approval
2. Agreement

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

With best wishes,

Dr. Nihal Thomas
Secretary (Ethics Committee)
Institutional Review Board

Dr Nihal Thomas
MBBS MD MNAMS DNB (Endo) FRACP(Endo) FRCP(Edin)
Secretary (Ethics Committee)
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CC: Dr. Geeta Chacko, Department of Pathology, CMC.

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