DETECTION OF ISOCITRATE DEHYDROGENASE 1 (IDH1), EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR), P53 AND C-ERBB2/HER2 BY IMMUNOHISTOCHEMISTRY METHOD IN ASTROCYTIC AND OLIGODENDROGLIAL TUMOURS

DR. NUSAIBAH BINTI AZMAN

DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE DEGREE OF MASTER OF PATHOLOGY
(ANATOMICAL PATHOLOGY)

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WHO	: World Health Organisation
GLOBOCAN	: Global Cancer Observatory
CBTRUS	: Central Brain Tumour Registry of the United States
MNCR	: Malaysia National Cancer Registry
ICP	: Intracranial pressure
CNS	: Central Nervous System
IDH	: Isocitrate Dehydrogenase
ATRX	: Alpha Thalassemia/Mental Retardation Syndrome X-linked
NOS	: Not otherwise specified
PXA	: Pleomorphic Xanthoastrocytoma
EGF	: Epidermal Growth Factor
MAPK	: Mitogen Activated Protein Kinase
EGFR	: Epidermal Growth Factor Receptor
Rb	: Retinoblastoma
PTEN	: Phosphatase and tensin homolog
NAD	: Nicotinamide adenine dinucleotide
NADH	: Nicotinamide adenine dinucleotide/hydrogenase
PCR	: Polymerase Chain Reaction
DNA	: Deoxyribonucleic acid
TCGA	: The Cancer Genome Atlas
LOH	: Loss of heterozygosity
MDM2	: Mouse double minute 2 homolog
ALT	: Alternative Lengthening of Telomeres
mTOR	: Mammalian target of rapamycin (mTOR)

TKI	: Tyrosine Kinase Inhibitors
FISH	: Fluorescence in situ hybridization
RT-PCR	: Reverse transcription polymerase chain reaction
IHC	: Immunohistochemistry
HUSM	: Hospital Universiti Sains Malaysia
LIS	: Laboratory Information System
HREC	: Human Research Ethics Committee
JEPeM	: Jawatankuasa Etika Penyelidikan (Manusia)
FFPE	: Formalin Fixed Paraffin Embedded
SPSS	: Statistical Package for Social Sciences
PCR-RLFP	: Polymerase Chain Reaction Restriction Fragment Length Polymorphism
CAR	: Chimeric Antigen Receptor
DLBCL	: Diffuse Large B cell Lymphoma
SD	: Standard Deviation
APJCP	: Asian Pacific Journal of Cancer Prevention
VEGF	: Vascular Endothelial Growth Factor
CDK	: Cyclin-dependent Kinase
CDKI	: Cyclin-dependent Kinase Inhibitor
RTK	: Receptor Tyrosine Kinase
GAP	: GTPase activating protein
H&E	: Hemoxylin and eosin
TBS	: Tris-buffered saline
DAB	: Diaminobenzidine

ABSTRAK

Pengenalan : Sedekad yang lalu, beberapa laluan molekular dalam pembentukan kanser otak telah ditemui. Ia melibatkan lebih daripada satu mutasi di peringkat molekular yang membentuk set-set yang unik. Ekspresi pada IDH1 telah menjadi penentu utama dalam diagnosa kanser otak berdasarkan Klasifikasi WHO 2016 bagi Kanser Otak. Ekpresi pada p53 pula dilihat pada kes-kes yang mempunyai mutasi pada protin IDH, terutama di dalam kes astrocytoma dan oligodendroglioma Gred II dan III, serta glioblastoma sekunder. Ekspresi pada EGFR dan c-erbB2/HER2 dianggarkan boleh dilihat pada gred glioma yang lebih tinggi, disebabkan oleh penyakit yang semakin merosot.

Metodologi : Kami telah menjalankan kajian keratan rentas secara retrospektif untuk menilai hubungan di antara expresi protin IDH1, EGFR, p53 dan c-erbB2 di dalam kes astrocytoma and oligodendroglioma dengan faktor klinikopatologinya di HUSM, Kota Bharu, Kelantan. Kajian ini mengkaji 61 arkib blok tisu parafin yang difiksasi dengan formalin bagi pesakit yang didiagnosa sebagai glioma. Kami melakukan ujian immunohistokimia menggunakan protin antibodi terhadap IDH1, EGFR, p53 dan c-erbB2/HER2. Ekspresi protin berkenaan telah dinilai secara mikroskopik. Hubungan di antara protin IDH1, EGFR, p53 dan c-erbB2 di dalam kes astrocytoma and oligodendroglioma dengan pembolehubah klinikopatologinya dianalisis secara statistik.

Keputusan : Kami melaporkan 61 kes glioma dengan 36 (59%) terdiri dari lelaki dan 25 (41%) adalah perempuan. Ekspresi protin IDH1 dilihat dalam 14 kes (23%). Ekspresi tinggi p53 didapati pada 26 kes (42.6%) manakala ekspresi tinggi EGFR didapati pada 34 kes (55.7%). Kes-kes glioblastoma menunjukkan ekspresi IDH1 dalam 2 kes (11.1%), EGFR dalam 14 kes (77.7%), p53 dalam 12 kes (66.7%) dan c-erbB2/HER2 dalam 1 kes (5.6%).

Terdapat perkaitan yang signifikasi di antara ekspresi protin IDH1, EGFR dan p53 dalam kes astrocytoma dan oligodendroglioma dengan jenis histologi dan kanser gred WHO.

Kesimpulan : Kajian kami mendapati bahawa terdapat hubungan yang ketara di antara ekspresi protin IDH1, EGFR dan p53 dalam kes astrocytoma dan oligodendroglioma dengan jenis histologi dan kanser gred WHO. Ujian immunohistokimia adalah ujian cepat, mudah ditafsirkan, senang didapati dan murah, yang dapat dijadikan alternatif kepada kajian molekular, terutama di pusat perubatan kecil yang tidak mempunyai kemudahan ujian molekular.

ABSTRACT

Background: In the last decade, several molecular pathways in gliomagenesis have been discovered, each involving a unique set of molecular alteration. Expression of IDH1 has become a major indicator in the latest 2016 WHO Classification of Central Nervous System Tumours. Expression of P53 is involved in the *IDH* mutant arm and is observed in astrocytoma and oligodendroglioma Grade II and III, and in secondary glioblastoma. Expression of EGFR and c-erbB2/HER2 were postulated to be expressed in higher grade glioma, as the disease progressed.

Materials and Methods: We conducted a retrospective cross-sectional study to evaluate the association of IDH1, EGFR, p53 and c-erbB2/HER protein expression in astrocytic and oligodendroglial tumour with its clinicopathological variable in HUSM, Kota Bharu, Kelantan. This study examined 61 archived formalin fixed paraffin-embedded tissue blocks of patients diagnosed with glioma. We performed immunohistochemistry test using antibodies protein of IDH1, EGFR, p53 and c-erbB2/HER2. The protein expressions were evaluated microscopically. The association between expression of the IDH1, p53, EGFR and c-erbB2/HER2 with the clinicopathology variables were statistically analysed.

Results: 61 cases of glioma cases with 36 (59%) males and 25 (41%) females. Positive protein expression of IDH1 were seen in 14 cases (23%). P53 was found to be highly expressed in 26 cases (42.6%), while EGFR was found to be highly expressed in 34 cases (55.7%). Glioblastoma cases expressed IDH1 in 2 cases (11.1%), EGFR in 14 cases (77.7%), p53 in 12 cases (66.7%) and c-erbB2/HER2 in 1 case (5.6%). There is significant association between expression of IDH1, p53 and EGFR in astrocytoma and oligodendroglial tumours with the histological types and WHO tumour grades.

Conclusion: Our study found significant association of protein expression of IDH1, EGFR, p53 in astrocytoma and oligodendroglial tumours with the histological types and WHO tumour grades. Immunohistochemistry is a quick, easy-to-interpret, widely available, and cost-effective test that can provide an alternative diagnostic method to molecular studies, particularly in small centres where molecular tests are not available.

CHAPTER 1: INTRODUCTION

1.1 Epidemiology

Primary brain tumours are defined as a heterogenous group of tumours arising from cells within the central nervous system, which can be further divided into glial and non-glial cells. Glial cells are supporting cells within central nervous system which include astrocytes, oligodendrocytes, microglial and ependymal cells. Tumours that develop from these cells are known as astrocytoma, oligodendroglioma, and ependymoma. Non-glial cells are cells other than glial cells, such as meningeal cells, lymphoid cells, choroid cells and pinealocytes. Examples of non-glial tumours are primary central nervous system lymphoma, meningioma, schwannoma, germinoma and medulloblastoma.

The tumours can be benign or malignant. Tumours are categorised from grade I to IV according to the most recent 2016 WHO Classification of the Central Nervous System Tumours. Grade I and II are classified as low grade and less aggressive, while Grade III and IV are classified as high grade and more aggressive. The use of histological grading across the tumour entities allows for the prediction of response to therapy and outcome.

Global Cancer Statistics 2020 (GLOBOCAN) reported an incidence rate of 3.9 per 100,000 in males and 3.0 per 100,000 in females for primary brain cancer. It also estimated 308,102 new cases (1.6% tumour burden) with 251, 329 deaths from primary brain tumours globally in 2020 (Sung *et al.*, 2021).

The Central Brain Tumor Registry of the United States (CBTRUS), the largest population-based registry, reported a higher rate, with average annual age-adjusted rate was 24.25 per 100,000 population between 2014 to 2018. Of all primary brain tumours, 29.1% were malignant. The most common malignant primary brain tumours in adults were

glioblastomas (49.1% of malignant tumours). The most common non-malignant tumours were meningiomas (54.5%) (Ostrom *et al.,* 2020).

In Malaysia, only 642 new cases (2.9%) of primary brain tumours reported from 2012-2016 in the latest Malaysia National Cancer Registry Report (MNCR). Although it was not one of the ten most common cancer in Malaysia, it was one of top ten cancers in Malay ethnic group. It was the 9th common cancer in Malay male (1.8 per 100,000) and 10th common cancer in Malay female (1.6 per 100,000). In general, primary brain tumours are low in incidence in Malaysia (Azizah *et al,* 2019).

The incidence of primary brain tumours varies with age, sex, and ethnic origin. Some malignant tumours such as glioblastoma, lymphomas, embryonal and germ cell tumors tend to occur in men, while meningiomas and pituitary tumors are more common in female (Lapointe *et al.*, 2018).

The common brain tumours in children aged 0-14 years were pilocytic astrocytoma (18.3%), while in adolescent and young adult aged 15-39 years, the most common histology was tumours of the pituitary. Glioblastoma incidence increased with age, with highest rate in individuals aged 75-84 years old (Ostrom *et al.*, 2020).

1.2 Clinical manifestation

Individuals with brain tumours may experience focal or generalized symptoms over a period of days to weeks, or months to years, depending on the tumour growth rate and location. Some cases were detected incidentally with no prior symptoms. The symptoms include seizures (50-80%), headaches (30%), and increased intracranial pressure (15%) (Lapointe *et al.*, 2018).

The most frequent primary brain tumour in children is pilocytic astrocytoma, which occurs in the infratentorial region. Unlike adults with glioma, these tumours rarely involve

the cerebral cortex. As a result, the most typical presentations in children are focal neurological impairments or non-localizing symptoms such macrocephaly, headache, and increased intracranial pressure due to mass effect or ventricular blockage. A study conducted in Austria discovered comparable results, with pilocytic astrocytoma patients presenting with headache (65.2%), visual abnormalities (34.8%), and high ICP (28.3%) when compared to seizures (8.7%) (Mair *et al.*, 2020).

1.3 WHO CNS Tumour Classification

The latest edition of the WHO Classification of the Central Nervous System Tumours (WHO CNS5) is the fifth edition and sixth version of the international standard for the classification of brain and spinal cord tumours, following previous editions in 1979, 1993, 2000, 2007, and 2016. It is an updated version of the fourth edition, which was published in 2016, and it incorporates recommendations from the Consortium to Inform Molecular and Practical Approaches to CNS Tumor Taxonomy (cIMPACT-NOW) (Louis *et al.*, 2021). However, the official published edition is not yet available, this dissertation follows the WHO Classification of the Central Nervous System Tumours 2016.

The 2016 WHO Classification represents a significant shift from the ancient traditional of histological-based diagnoses, with the inclusion of molecular characteristics into the classification of diffuse gliomas.

In this classification, diffuse glioma such as WHO Grade II and III astrocytomas, Grade II and III oligodendrogliomas, Grade II and III oligoastrocytomas, Grade IV glioblastomas and diffuse gliomas in children are further defined by their isocitrate dehydrogenase (*IDH*) status. Those individuals with IDH positive will be assigned as *IDH* mutant whereas, IDH negative cases are known as *IDH* wild-type. *IDH* mutant gliomas (other than Grade IV glioblastoma) are further tested for alpha thalassaemia/mental retardation, X-

linked (*ATRX*) loss, *TP53* mutation and 1p/19q deletion to differentiate diffuse astrocytoma and oligodendroglioma. Either loss of *ATRX* or *TP53* are features for diffuse astrocytoma, *IDH* mutant, while co-deletion of 1p/19q is synonym with oligodendroglioma as demonstrated in Figure 1.1 (Louis *et al.*, 2016).

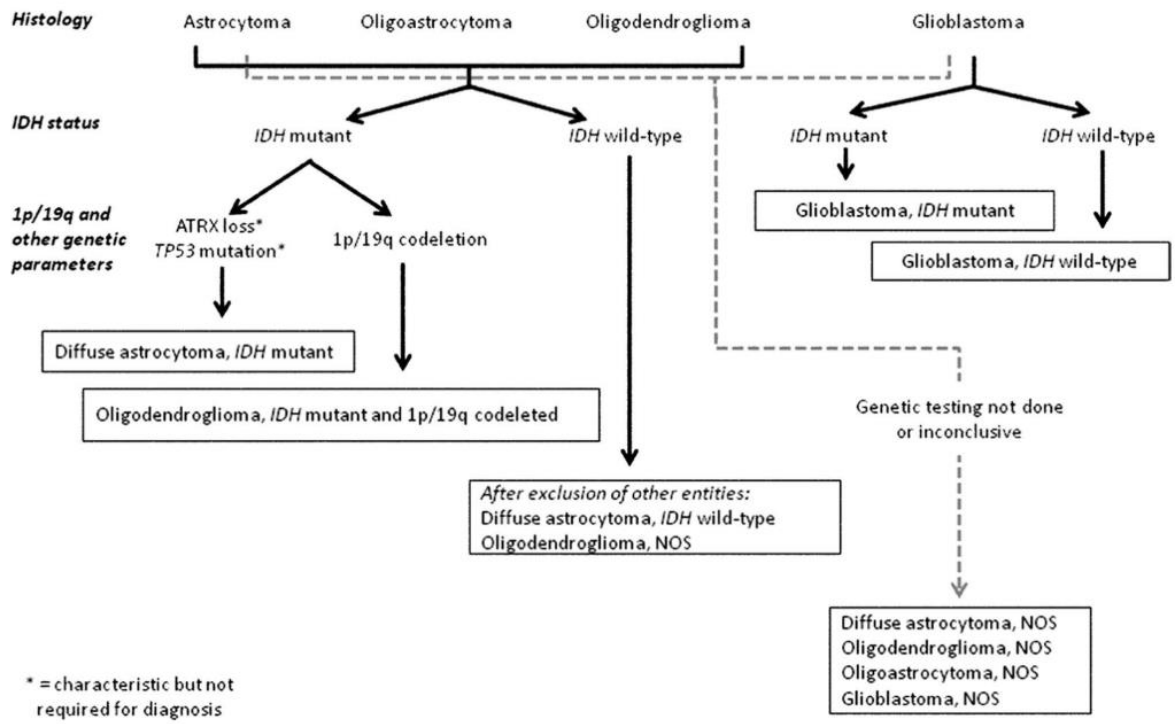

Figure 1.1 A simplified algorithm for classification of the diffuse gliomas based on histological and genetic features (Louis *et al.*, 2016).

In centres where molecular testing is not done, or inconclusive, the gliomas are classified according to the morphology and assigned as 'NOS' which means 'not otherwise specified'.

This new classification separates well-circumscribed astrocytomas (such as pilocytic astrocytoma, pleomorphic xanthroastrocytoma (PXAs), and subependymal giant cells astrocytoma) from diffuse gliomas, because they lack *IDH* gene mutation and have *BRAF*

mutation. Diffuse astrocytoma and oligodendroglioma are now more similar as they share *IDH* mutation status, despite their differences in morphology. In other words, the family tree of glioma has been re-drawn and classification is based on genotypes rather than phenotypes.

1.4 Glioma

Glioma is a tumour derived from the glial cells. It is the largest group of primary brain tumours that can be subdivided into distinct subgroups based on the clinical, histologic morphology and molecular features.

A well-circumscribed tumour such as pilocytic astrocytoma, ganglioglioma and pleomorphic xantroastrocytomas (PXAs) occurred more in children and young adolescent. These tumours are low grade and associated with more indolent behaviour.

Pilocytic astrocytoma arise in the infratentorial region, with preferred sites include the cerebellum and cerebral midline structures. Microscopically it has low to moderate cellularity and frequently has a biphasic pattern, which includes bipolar cells with Rosenthal fibres, and loose multipolar cells with microcysts. Most frequent genetic change is *BRAF* gene, resulting in oncogenic *BRAF* fusion proteins. Pilocytic astrocytoma largely behaves as of WHO grade I tumour.

Diffuse gliomas are infiltrative, with more aggressive clinical course. Some of them are not readily resected and may cause fatality. They may arise from bilateral cerebral hemispheres. These include astrocytomas, oligodendrogliomas and oligoastrocytomas, which further graded following 2016 WHO Classification grading from Grade II-III.

Diffuse astrocytomas are recognized by irregular, elongated, hyperchromatic nuclei with fibrillary background. Mitotic activity and degree of pleomorphism are used to differentiate diffuse astrocytoma, Grade II and anaplastic astrocytoma, Grade III. Mitotic activity is generally absent in diffuse astrocytoma, Grade II with Ki-67 proliferation index is

usually <4%. Anaplastic astrocytoma shows increase mitotic activity compared to the WHO Grade II, accompanied by distinct nuclear atypia and high cellularity. Necrosis and microvascular proliferation are absent in Grade II and Grade III astrocytoma. Prominent microvascular proliferation and/or necrosis is an essential diagnostic feature of glioblastoma, Grade IV in 2016 WHO Classification.

Diffuse oligodendrogliomas, WHO Grade II have uniform round nuclei with perinuclear halo (fried-egg appearance) and delicate branching (chicken-wire) vasculature in the background. Similarly, brisk mitotic activity, prominent microvascular proliferations and spontaneous necrosis are features of anaplasia, corresponding to WHO Grade III, Anaplastic oligodendroglioma.

1.5 Pathogenesis

Several studies have reported interobserver discrepancies and low reproducibility in the diagnosis of glioma, especially with oligodendroglial components (Eckel-Passow *et al.*, 2015). Factors that may contribute include inadequate biopsy tissue, ambiguous or mixed morphology, poor definition of degree of cellularity, and the quantity of mitoses in discriminating between Grade II and Grade III. The difference in subtyping and grading may cause undertreatment or overtreatment (Van Den Bent, 2010). It is thought that incorporation of molecular subtyping will help to aid pathologists in diagnostic classification and grading. Molecular classification also gives meaningful data in term of prognosis and targeted therapeutic treatments.

For years, we have understood the complex process of carcinogenesis, that involves multiple steps. These are 1) sustaining proliferative signaling, 2) evading growth suppressors, 3) resisting cell death, 4) enabling replicative immortality, 5) inducing angiogenesis and 6) activating invasion and enabling metastasis (Hanahan and Weinberg, 2011). Apart from these

cancer hallmarks, there is also underlying genetic instabilities and two new emerging concepts added to the list, which are reprogramming of energy metabolism and evading immune destruction (Hanahan and Weinberg, 2011).

In normal tissues, cellular homeostasis is maintained by controlling production and releases of growth factor signals within the cell cycle. Cancer cells may acquire proliferative signaling by producing their own growth factors or by interaction with normal cells within stroma to stimulate growth factors. For these signals to reach the cell, it needs to bind to growth factor receptor located on the cell membrane. Some of the receptors have intrinsic tyrosine kinase activity that only able ligate with extracellular proteins when activated by another intracellular bridging proteins. One of the best examples of oncoproteins that act as the growth factor receptor is overexpression of epidermal growth factor (EGF) family, particularly ERBB1 or also known as Epidermal Growth Factor Receptor (EGFR). ERBB2 (HER2) is also in EGF family and known to be amplified in breast cancers, as well as adenocarcinomas of stomach, lung, ovary, and salivary glands.

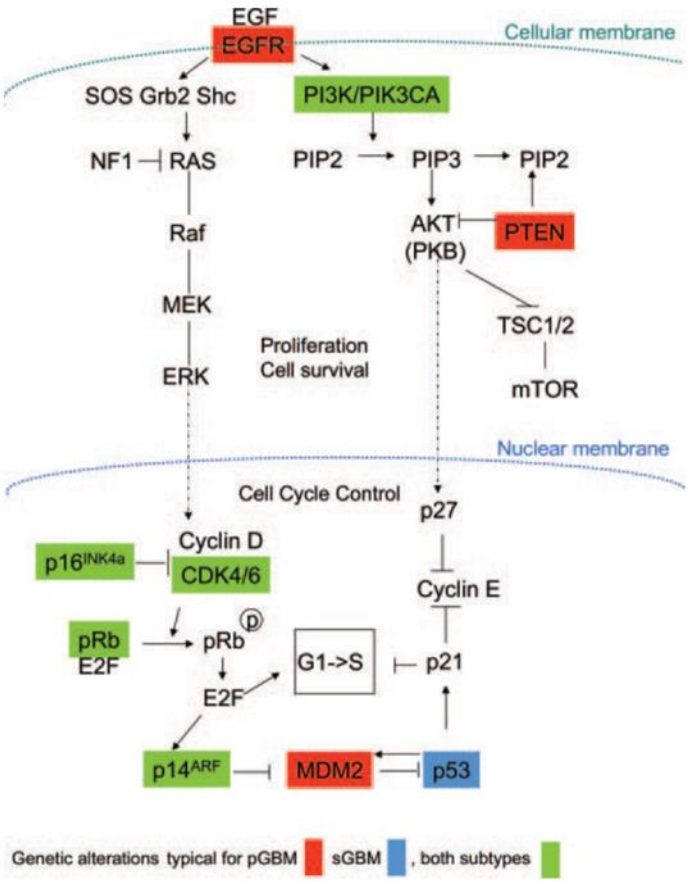

Figure 1.2 Major signaling pathways in pathogenesis of glioblastoma. (Ohgaki and Kleihues, 2007)

Once the growth factor receptor is activated, a cascade of activities occurs as illustrated in Figure 1.2. The cascade of activities includes activation of *RAS* oncogene which further stimulate specific pathways (e. MAP kinase pathway, PI3K/AKT pathway) that alter *MYC* protein to promote growth (Sigismund *et al.*, 2018). These are the first few steps of how cancer evolves and has become the subjects of many studies. Different cancer type has different activated oncogenes and tumor suppressor genes.

In glioma, studies have shown that cancer metabolism is induced by activated oncogenes (such as *EGFR*, *RAS* and *MYC*) and deregulation of tumour suppressor proteins (such as *TP53* and *Rb*) (Masui *et al.*, 2019).

There are also intracellular metabolic enzymes that are involved in carcinogenesis, that is *IDH1* or less commonly *IDH2* gene mutations.

1.6 IDH1 Expression

IDH1 mutation was first described by Parson and colleagues, who performed next generation sequencing technologies in 22 cases of glioblastoma multiforme. They found recurrent mutation of *IDH1* in 12% of glioblastoma patients (Parsons *et al.*, 2008).

IDH1 mutations are found frequently in diffuse astrocytic and oligodendroglial tumour (grade II and III), as well as in secondary glioblastoma. Secondary glioblastoma is a progression of disease from lower grade diffuse astrocytoma and anaplastic astrocytoma. It occurs in younger patient, located in frontal lobe and has a better prognosis compared to primary glioblastoma (Ohgaki and Kleihues, 2007).

Glial tumours with *IDH1* mutation are more likely to have mutation in *TP53* or co-deletion of 1p/19q and were less likely to have alteration in *PTEN, EGFR, CDKN2A* and *CDKN2B* which occur in primary glioblastoma. This suggest there is alternative pathway in the gliomagenesis. *IDH1* mutation is also associated with better prognosis in high grade glioma. *IDH1* mutation was not found in pilocytic astrocytoma, suggesting that these tumours are biologically unrelated to diffuse glioma (Yan *et al.*, 2009).

IDH catalyse the reversible carboxylation of isocitrate to α-ketoglutarate in the Krebs Cycle. This enzyme has 3 isoforms: IDH1, IDH2 and IDH3. IDH3 catalyse the next step of the citric acid cycle within the mitochondria, reducing NAD^+ to NADH in the process. IDH1 and IDH2 reduce $NADP^+$ to NADPH. IDH1 is the only isoform occurs within the cytoplasm. When mutation occurs, the enzyme develops preferential towards α-ketoglutarate instead of isocitrate, leading to accumulation of oncometabolite 2-hydroxyglutarate (2HG) within the cytoplasm, as demonstrated in Figure 1.3. The process uses NADPH to $NADP^+$ resulting

depletion of α-ketoglutarate and NADPH leading to dysfunction cellular processes (Turkalp *et al.*, 2014).

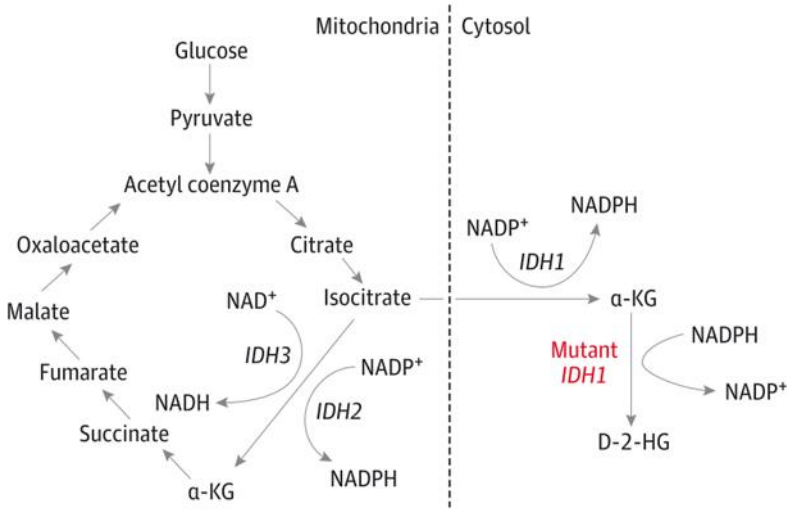

Figure 1.3 IDH Pathway in Physiology and Disease. (Turkalp *et al.*, 2014)

The most frequent *IDH1* mutation is R132, which occur at codon 132, and lead to exchange of arginine to histidine. It accounts for more than 90% of all *IDH* mutations in diffuse gliomas. *IDH2* mutation occur at analogous amino acid R172 and less common than *IDH1* (Appin and Brat, 2015). Immunohistochemistry for the *IDH1* mutant protein can detect positive cells and has comparable sensitivity to PCR or next generation sequencing (Loussouarn *et al.*, 2012). Since immunohistochemistry method is less expensive that other methods, it is widely used as a diagnostic marker.

IDH1 immunohistochemistry is highly sensitive and specific, it can be used to distinguish anaplastic astrocytoma from primary glioblastoma, as well as diffuse astrocytoma from pilocytic astrocytoma. IDH1 immunohistochemistry can also be used to distinguish between reactive gliosis and the infiltrating edge of diffuse astrocytoma (Capper *et al.*, 2010).

1.7 P53 Expression

The *TP53* gene is located on human chromosome 17p13.1. It encodes P53 protein which is responsible for maintaining cellular homeostasis (Sui *et al.*, 2011). In normal tissue, P53 protein acts like "Guardian of the Genome" by preventing neoplastic transformation using three mechanisms: Activation of cell cycle arrest (termed quiescence), induction of permanent cell cycle arrest (termed senescence) or triggering programmed cell death (termed apoptosis).

The P53 protein will be activated by multiple stresses such as hypoxia, aberrant growth signals, oncogene activation and DNA damage. Activation of P53 protein will cause cell cycle arrest at G1 phase, allowing time to "repair" the DNA damage. If the DNA damage cannot be repaired, it will undergo senescence or apoptosis (Zhang *et al.*, 2018).

Mutations in *TP53* gene will cause major cell damage, as there will be instability in maintaining the regular cell cycle regulation. Failure for the cell cycle arrest and DNA repair will cause uncontrolled proliferation of tumour cells. The mutation also may cause less sensitivity to cell apoptosis. *TP53* mutation is the most common mutation in all types of cancers. In The Cancer Genome Atlas (TCGA) Glioblastoma Multiforme study, *TP53* was found to be deregulated in 85% of tumours, with 28% were found in lower grade gliomas. More than 90% of *TP53* mutations were related with *IDH* mutations (Brennan *et al.*, 2013).

In the early years of IDH1 discovery, most research were done on two subtypes of glioblastoma, primary and secondary glioblastoma. It was noted that both glioblastoma subtypes show frequent loss of heterozygosity (LOH) 10q but differ in respect of other genetic alterations such as LOH 10p, *EGFR* amplification, *MDM2* amplification and *PTEN*, which are more common in primary glioblastoma. *TP53* mutation, LOH 19q and LOH 22q are more frequent in secondary glioblastoma (Watanabe *et al.*, 2009). This is consistent with

alternative pathway of gliomagenesis which has a different set of genetic alterations, shown in Figure 1.4.

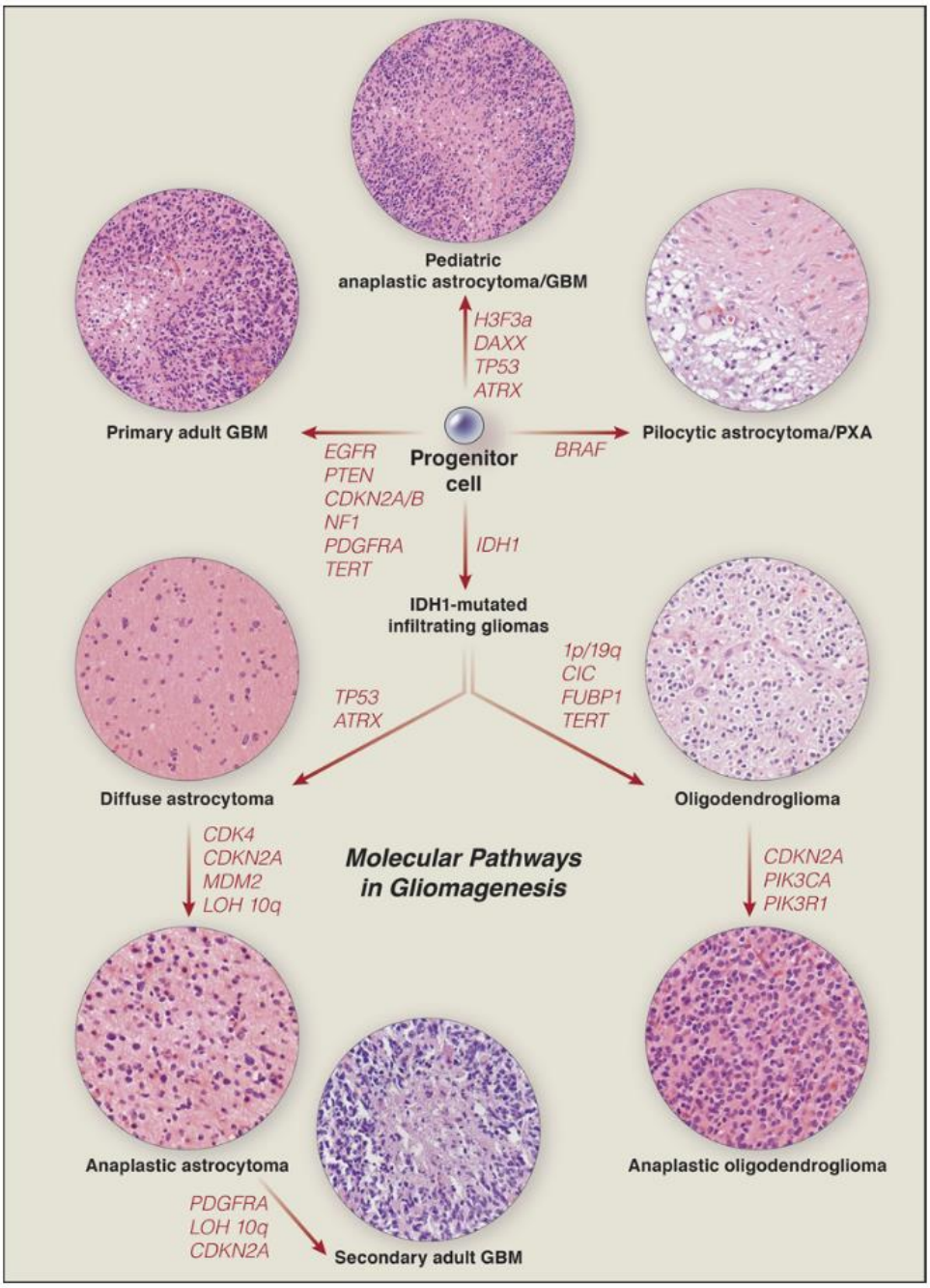

Figure 1.4 Overview of altered genes that characterized distinct forms of glioma. GBM, glioblastoma; PXA, pleomorphic xanthoastrocytoma (Appin and Brat, 2015).

Yan and colleagues reported *TP53* mutations were more common in diffuse astrocytoma (74%), anaplastic astrocytoma (65%) and secondary glioblastoma (62%) than in

diffuse oligodendroglioma (16%) or anaplastic oligodendroglioma (9%) ($p<0.001$) (Yan *et al.*, 2009).

Majority of grade II and grade III astrocytoma, as well as secondary glioblastoma have both mutation of *IDH1* and *TP53*. Watanabe and colleagues found 63% of low grade astrocytoma patients had both *IDH1* and *TP53* mutations, indicating a significant association ($p=0.015$) (Watanabe *et al.*, 2009).

Apart from that, *IDH* and *TP53* mutations are strongly associated with alteration in *ATRX* gene, a gene that produces a protein that function in chromatin remodelling. Previous study reported frequent *ATRX* mutations in the grade II and III astrocytoma and oligoastrocytoma (67% to 73%) and secondary glioblastoma (57%) but were uncommon in primary glioblastoma (4%) and oligodendroglial tumours (20%). There were also association with the alternative lengthening of telomeres (ALT) phenotype. Telomere is a region at the end of chromosome that is responsible to protect the chromosomal DNA from progressive degradation. Lengthening of telomeres *ATRX* alteration and 1p/19q loss a rarely occurred ($p<0.001$) (Jiao *et al*, 2012).

1.8 EGFR Expression

Epidermal growth factor receptor (EGFR) is a transmembrane glycoprotein that binds to epidermal growth factors (EGF) and several other ligands. It is a member of the ErbB family of receptors which consists of 4 related tyrosine kinases: EGFR (ErbB-1), HER2/neu (ErbB-2), Her 3 (ErbB-3) and Her 4 (ErbB-4). Activation of EGFR stimulates tyrosine kinase activity, resulting in a cascade of downstream signaling events involving the RAS/RAF/MAPK pathway or the PI3K/AKT/mTOR pathway intracellularly, which causes increase DNA synthesis and cell proliferation. *PTEN* deletion is also frequently noted in primary glioblastoma (Saadeh *et al.*, 2018).

Alteration in *EGFR* gene is involved in the development and progression of several human malignancies, such as non-small-cell lung, gastric, pancreatic and nasopharyngeal carcinomas (Li *et al.*, 2018). Clinical trials are being conducted to investigate EGFR-targeted therapy using EGFR Tyrosine Kinase Inhibitors (TKI) to block the aberrant EGFR signaling (Wilson *et al.*, 2014).

Many *EGFR* modifications in gliomas has been reported in the literature, some are specific to glioblastoma. *EGFR* amplification was seen in grade II (0-4%), grade III (0-33%) and grade IV (34-64%) astrocytoma (Wilson *et al.*, 2014). Approximately 30-40% primary glioblastoma show amplification of *EGFR* (Appin and Brat, 2015).

There are several ways for detecting *EGFR* alterations in tumour tissue. These include fluorescence in situ hybridization (FISH), real-time reverse transcription-polymerase chain reaction (RT-PCR) and immunohistochemistry (IHC). It was noted that methods for assessing *EGFR* include mRNA expression (RT-PCR and RNAseq) and copy number (WES), but not protein expression IHC, that can be used as surrogate for *EGFR* amplification in glioblastoma (Lassman *et al.*, 2019).

Although it has low specificity to detect amplification compared to FISH method, IHC is a broadly used and cost-effective. IHC has been used in several research to evaluate EGFR expression. A study showed that *EGFR* mutations were found in 49% of glioblastomas, and there was significant concordance between IHC and FISH (96%) and IHC and RT-PCR (98%) (Faulkner *et al.*, 2015). Another research also used IHC and reported 70% low EGFR expression (0 or 1+) and 30% high EGFR expression (2+ or 3+) in brain tumours (Bhalala *et al.*, 2019).

1.9 C-ERB B2/HER2 Expression

C-erbB2/HER2 is an oncogene, known for its prognostic and predictive factor in multiple human cancer, especially in breast cancer. It belongs to the ErbB/HER family, a receptor tyrosine kinase family which includes EGFR (c-erbB1/HER1), c-erbB2/HER2, c-erbB3/HER3 and c-erbB4/HER4. The cerbB2/HER2 is located on chromosome 17q21 and encodes an 185kD transmembranous receptor protein. Protein kinases have a prominent role in cellular biology, including regulation of apoptosis, cell cycle progression, cellular adhesion and angiogenesis. Overexpression of the protein through gene amplification or transcriptional deregulation transformed it the into oncogene and contributed to the development of cancer (Roskoski, 2014).

The study of *C-erbB2/HER2* mutation in glioblastoma increases following the discovery of *EGFR* gene amplification and overexpression in glioblastoma, which belongs to the same family group. Other members of the EGFR family have been shown to be expressed in glioblastomas, but at a lower level (Torp *et al.*, 2006). According to Waage and colleagues, the clinical significance of *c-erbB2/HER2* in most brain tumour is uncertain. Most studies in glioma reported expression of c-erbB2/HER2 in positive brain tumour ranging from 0 to 90% (Waage *et al.*, 2013).

This disparity in results could be due to antibody dependence, as demonstrated by another study in which three distinct antibodies (CB11, 5A2, 3B5) yielded varying levels of positive ranging from 45% to 100%. To achieve the best results, it is advised to utilize a commercial antibody (CB11) as it showed statistically significant poor prognostic factor in patients with anaplastic astrocytoma ($p=0.004$) (Gulati *et al.*, 2010).

Waage *et al* also compared various research and discovered that the distribution of immunoreactive cells in glioma is defined as diffuse or focal, with both cytoplasmic and

membranous patterns being observed. In contrast, only membranous staining is approved in breast cancer (Waage *et al.*, 2013).

Another study on primary malignant brain tumours in Iran that used the antibody c-erbB2 from Dako (Clone Code A0485). Tumour cells with cytoplasmic membranes were considered as c-erbB2 positive. The percentage and intensity of staining were graded from 0 to 3+, as follows: No staining (0), low intensity and incomplete membrane staining in less than 10% of cells (1+), low intensity and complete membrane staining in more than 10% of cell (2+), and high intensity and complete membrane staining in more than 10% of cells (3+) (Ramezani *et al.*, 2018).

Hence, this study aims at contributing more data for beneficiary study of c-erbB2/HER2 in glioma.

1.10 Rationale of study

In this study, we would like to determine the association between these protein expressions of IDH1, p53, EGFR and c-erbB2/HER2 in glioma cases, particularly astrocytoma and oligodendroglial tumour using immunohistochemical stain. We used immunohistochemical staining technique as this method is widely available and cost-effective.

CHAPTER 2: OBJECTIVES OF THE STUDY

2.1 General objective

To study the association of IDH1, p53, EGFR and c-erbB2 expressions in astrocytic and oligodendroglial tumours with the clinicopathological variable in HUSM, Kota Bharu, Kelantan.

2.2 Specific objectives

1. To describe the demographic and clinicopathological data of astrocytic and oligodendroglial tumours in HUSM, Kota Bharu, Kelantan.

2. To determine the expression (IDH1, EGFR, p53 and C-erbB2/Her2 using immunohistochemical staining in astrocytic and oligodendroglial tumours.

3. To determine the association of protein expression of IDH1, EGFR, p53 and C-erbB2/Her2 with the clinicopathological data.

2.3 Research hypothesis

There is a significant association between protein expressions of IDH1, EGFR, p53 and c-erbB2/HER2 in astrocytic and oligodendroglial tumours with the clinicopathological parameters.

CHAPTER 3: MANUSCRIPT

3.1 Title

Detection of Isocitrate Dehydrogenase 1 (IDH1), Epidermal Growth Factor Receptor (EGFR), P53 and C-erbB2/HER2 by Immunohistochemistry Method in Astrocytic and Oligodendroglial Tumours

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Running title:

IDH1, EGFR, P53 and C-erbB2/HER2 Expression in Astrocytic and Oligodendroglial Tumours

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3.2 Abstract

Background: There are several molecular pathways in gliomagenesis that have been discovered, involving unique sets of molecular alterations. Expression of IDH1 has become a major indicator in the 2016 WHO Classification of Central Nervous System Tumours. Expression of P53 is involved in the *IDH* mutant arm and seen in astrocytoma and oligodendroglioma Grade II and III, including secondary glioblastoma. Expressions of EGFR and c-erbB2/HER2 are postulated to be expressed in higher grade glioma, as the disease progressed.

Materials and Methods: This was a retrospective cross-sectional study to evaluate the association of IDH1, EGFR, p53 and c-erbB2/HER2 protein expressions in astrocytic and oligodendroglial tumours with the clinicopathological variables in HUSM, Kota Bharu, Kelantan, Malaysia. This study examined 61 archived formalin fixed paraffin-embedded tissue blocks of patients diagnosed with glioma. We performed immunohistochemistry test using antibody protein of IDH1, EGFR, p53 and c-erbB2/HER2. The protein expressions were evaluated microscopically. The association between these proteins with the clinicopathology variables were statistically analysed.

Results: 61 cases of glioma cases with 36 (59%) males and 25 (41%) females. Positive protein expression of IDH1 in 14 cases (23%). P53 expressed in 26 cases (42.6%), and EGFR expressed in 34 cases (55.7%). Glioblastoma cases expressed IDH1 in 2 cases (11.1%), EGFR in 14 cases (77.7%), p53 in 12 cases (66.7%) and c-erbB2 in 1 case (5.6%). There is significant association between expression of IDH1, p53 and EGFR in astrocytoma and oligodendroglial tumours with the histological types and WHO tumour grades.

Conclusion: Our study found significant association of protein expression of IDH1, EGFR, p53 in astrocytoma and oligodendroglial tumours with the histological types and WHO

tumour grades. Immunohistochemistry is a quick, easy-to-interpret, widely available, and cost-effective test that can provide an alternative diagnostic method to molecular studies.

Keywords: IDH1, p53, EGFR. C-erbB2/HER2, astrocytoma, oligodendroglioma

3.3 Introduction

Primary brain tumours are defined as a heterogenous group of tumours arising from cells within the central nervous system, which can be further divided into glial and non-glial cells. Gliomas are tumour that arised from the glial cells, which include diffuse astrocytoma, oligodendroglioma, glioblastoma and pilocytic astrocytoma. The diagnosis is based on clinical evaluation, neuroimaging and histopathological assessment.

The tumours can be benign and malignant, defined by the clinical behaviour and histomorphology. It can be graded using WHO Classification Grade I to IV. Latest edition of the WHO Classification of Tumors of the Central Nervous System (WHO CNS5) was the 5th edition published in 2021. It was an updated version of the fourth edition, which was published in 2016 (Louis *et al.,* 2021). The 2016 WHO classification represents a significant shift from the ancient tradition of histological-based diagnosis, with the inclusion of molecular characteristics into the classification of diffuse gliomas. It is thought that incorporation of molecular subtyping will help to aid pathologists in cases with limited tissue biopsy and ambiguous or mixed morphologies. Molecular classification also gives meaningful data in term of prognosis and targeted therapeutic treatments (Louis *et al.*, 2016).

The incidence of primary brain cancer in all populations worldwide is approximately 3.9 per 100,000 in male and 3.0 per 100,000 in female with new cases reported in 2020 was 308,102 (1.6% tumour burden) (Sung *et al.*, 2021). In Malaysia, the incidence is slightly lower, 1.8 per 100,000 in male and 1.6 per 100,000 in female, with reported 642 new cases

(2.9%) from 2012 to 2016 (Azizah *et al.*, 2019). In Malaysia, the incidence has been increasing in both adults and children (Dzali *et al.*, 2017). As a result, it is prudent to conduct research on the subject for diagnostic, prognostic and therapeutic purposes.

The most common malignant primary brain tumours in adults are glioblastomas (49.1%) while the most common benign tumours are meningiomas (54.5%). Pilocytic astrocytoma is common in children aged 0-14 years old, while tumours of pituitary are common in adolescent and young adults aged 15-39 years old. Glioblastoma was noted to increase with age, highest in patients aged 75-84 years old (Ostrom *et al.*, 2020).

Patients may be presented with variable symptoms depending on the tumour growth rate and location. These includes seizures (50-80%), headaches (30%) and increased intracranial pressure (15%) (Lapointe *et al.*, 2018). Children with pilocytic astrocytoma had a higher rate of headache (65.2%), visual problems (34.8%), and high intracranial pressure (28.3%) than seizure (8.7%), since it involves less cerebral hemispheres (Mair *et al.*, 2020).

Several molecular pathways in gliomagenesis have been discovered in the last decade involving different sets of molecular alterations. Isocitrate dehydrogenase 1 (IDH1) has emerged as a major stratifier of infiltrating glioma, distinguishing between *IDH* mutant and *IDH* wild-type groups with prognostic importance (Appin and Brat, 2015). *IDH1* mutations are found frequently in diffuse astrocytic and oligodendroglial tumours (grade II and grade III) and in secondary glioblastoma. *IDH1* mutations are thought to be an early mutation in gliomagenesis, occurring before genetic modifications that promote specific differentiation, such as *TP53* and *ATRX* mutations in astrocytomas or 1p/19q codeletion in oligodendrogliomas (Watanabe *et al.*, 2009). Patients with *IDH* mutations have better prognosis.

Primary glioblastoma, on the other hand, was thought to have a different gliomagenesis pathway due to the lack of *IDH* mutation. Grade IV *IDH* wild-type also show

other molecular alterations such as *EGFR, PTEN, NF1, RB1, CDKN2A* and *CDKN2B* (Appin and Brat, 2015). *EGFR* mutations has been described in literature and have been observed in grade II (0-4%), grade III (0-33%) and grade IV (34-64%) astrocytoma (Wilson *et al.*, 2014).

The study of *c-erbB2/HER2* mutation in glioblastoma has increased following the discovery of *EGFR* gene amplification and overexpression in glioblastoma, which belongs to the same ErbB/HER family group. It is a receptor tyrosine kinase family which includes EGFR (c-erbB1/HER1), c-erbB2/HER2, c-erbB3/HER3 and c-erbB4/HER4 (Saadeh *et al.*, 2018). C-erbB2/HER2 is an oncogene and serves as a significant diagnostic and prognostic indicator in multiple human cancers, especially in breast cancer, but its exact role in glioma is largely uncertain (Mei *et al.*, 2021). Most studies reported expression of c-erbB2/HER2 in primary brain tumour ranging from 0 to 90% (Waage *et al.*, 2013).

Most of the research were conducted in the Western countries, which may not apply to our local settings. There are only few studies on brain tumours in Malaysia (Goh *et al.*, 2020; Yusoff *et al.*, 2016) use methods other than immunohistochemistry. Previous studies have shown concordance of result between immunohistochemistry and DNA sequencing with 94% sensitivity and 100% specificity (Capper *et al.*, 2010). Immunohistochemistry method can provide an alternative diagnostic method to molecular studies, particularly in small centres where molecular tests are not offered or available. We would like to determine the association between protein expression using the IHC technique of IDH1, EGFR, p53 and c-erbB2/HER2 in astrocytoma and oligondendroglial tumours.

3.4 Materials and Methods

This is a single centre retrospective cross-sectional study which included 61 cases of glioma cases diagnosed in Hospital Universiti Sains Malaysia (HUSM), Kubang Kerian between January 2009 and December 2019. The study protocol was approved by Human Research Ethics Committee (HREC), Universiti Sains Malaysia (USM/JEPeM/19020157).

The tissue blocks were selected after fulfilling the inclusion and exclusion criteria. Due to limited time of study and other unforeseen circumstances, only 61 cases of glioma included in this study using convenient sampling method. Cases excluded from this study were those with unavailable or missing paraffinized tissue blocks, inadequate tumour tissue and indeterminate diagnosis. The demographic data were retrieved from Lab Information System (LIS) in HUSM.

Selected formalin-fixed, paraffin embedded (FFPE) tissue blocks were cut at 2µm to 5µm thickness for staining of IDH1, p53, EGFR and c-erbB2/HER2 in pathology laboratory Hospital Universiti Sains Malaysia (HUSM). Sections were deparaffinized, followed by epitope retrieval using EnVision Flex Target retrieval solution, high pH. All the slides were subsequently placed in Squenza immunostainer incubated at 30 minutes (except EGFR for 40 minutes) at room temperature with Anti-IDH1 R132H/DIA-H09 (Clone H09 from Dianova), Anti-EGFR (phospho Y1068 antibody [EP774Y] from ABCAM), Monoclonal Mouse Anti-Human p53 Protein (Clone DO-7 from DAKO) and Polyclonal Rabbit Anti-Human c-erbB-2 Oncoprotein (Code A0485 from DAKO), which were diluted to 1:100 (IDH1), 1:200 (EGFR), 1:100 (p53) and 1:400 (c-erbB2) respectively. Localization of the antigen-antibody was achieved using labeled polymer, EnVFLEX/HRP. The complex was visualized using Substrate Chromogen (Envision FLEX Substrate Working Solution). Diffuse astrocytoma, colon adenocarcinoma, cervical cancer and breast cancer tissue served as external positive controls for IDH1, p53, EGFR and c-erbB2/HER2 respectively.

The expression of IDH1 was determined by assessing the proportion of positively stained cytoplasm/weak nuclear of tumour cells. Cases with ≥ 10% cells were rated as positive, and cases with <10% cells were rated as negative (Takano *et al.*, 2011). The immunohistochemistry scoring for p53 and EGFR were based on previous literature (Bhalala *et al.*, 2019). The scores were based on the staining of cytoplasmic and/or membranous

distribution (EGFR) and nuclear distribution (p53), as well as percentage of positive tumour cells. The staining is scored 0 (no staining), 1+ (staining in <10% of cells), 2+ (staining in 10% to 50% of cells, and 3+ (staining in >50% of cells).

For statistical analysis, 0 and 1+ grouped as low expression while 2+ and 3+ grouped as high expression. The expression of c-erbB2/HER2 was determined by percentage and intensity of staining, graded from 0 to 3+, as follows, 0 (no staining), 1+ (low intensity and incomplete membrane staining in >10% of cells), 2+ (low intensity and complete staining in >10% cells) and 3+ (high intensity and complete membrane staining >10% of cells) (Ramezani *et al.*, 2018). The staining evaluation was performed under high power magnification (400x).

Statistical analysis was performed using the SPSS version 25.0. Descriptive analysis was used for frequency distributions of categorical variables and median distributions of continuous variable. The association IDH1, p53, EGFR and c-erbB2/HER2 protein expression with the clinicopathological variable was analyzed using the Pearson Chi Square test or Fisher's Exact test. Level of significance in this study was set as p value <0.05.

3.5 Results

Clinicopathological data

There were 61 patients, 36 (59%) male and 25 (41%) females. The age ranged from 8- to 67-year-old, with the mean age of the patients was 38.84 years old (Table 3.1). The most common age group was older adults (40-70 years old), with 34 cases (55.7%). Age groups was based on previous study (Ostrom *et al.*, 2020). All cases were Malay ethnicity.

There were 7 histological types of primary brain tumour included in this study according to the 2016 WHO Classification of Central Nervous System Tumours. The most common histological subtypes were glioblastoma, 18 cases (29.5%), anaplastic astrocytoma,