EVALUATION OF GENETIC ALTERATIONS OF IDH1, TP53 AND CASP9 GENES AS BIOMARKERS IN GLIOMA PATIENTS

PUA JING YIT

UNIVERSITI SAINS MALAYSIA

2022

EVALUATION OF GENETIC ALTERATIONS OF IDH1, TP53 AND CASP9 GENES AS BIOMARKERS IN GLIOMA PATIENTS

by

PUA JING YIT

Thesis submitted in fulfilment of the requirements
for the Degree of
Master of Science

NOVEMBER 2022

ACKNOWLEDGEMENT

First and foremost, I would like to express my heartfelt gratitude to the Almighty for making all of this possible through His blessings. I would also like to express my heartiest appreciation to my supervisor, Dr Mohd Nor Azim Bin Ab Patar for his guidance, motivation and support throughout my research and study. In addition, I would like to extend my appreciation to my co-supervisor, Prof. Dr Zamzuri Bin Idris and Assoc. Prof. Dr Abdul Aziz Bin Mohamed Yusoff for their advice. The research would not have been possible without the wise counsel of supervisors. A special thanks to my lab mates and the staff members of the Department of Neurosciences, School of Medical Sciences, Universiti Sains Malaysia, particularly Izzah Madihah Binti Rosli for their constantly assisting and supporting me during my research.

Furthermore, I would like to express my gratitude to the Department of Neurosciences, Department of Pathology and Central Research Laboratory, School of Medical Sciences, Universiti Sains Malaysia for providing the opportunities and facilities for the research. Not to mention, I would like to thank the Institute of Postgraduate Studies, Universiti Sains Malaysia for providing graduate student financial assistance (GRA-ASSIST) and the Ministry of Higher Education for funding this research through the Fundamental Research Grant Scheme (203.PPSP.6171240) to make this research possible. Last but not least, I would like to express my deepest gratitude to my family, who have always been there for me emotionally and financially.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS, ACRONYMS AND SYMBOLS

$(NH_4)_2SO_4$	Ammonium sulfate, molecular weight: 132.14 g/mol
µg	Microgram, 10^{-6} gram
µL	Microlitre, 10^{-6} litre
µM	Micromolar, 10^{-6} molar
2-HG	2-Hydroxyglutarate
ABC	Avidin-biotin complex
ACT	Adoptive cell transfer
AP	Anteroposterior
Apaf-1	Apoptotic protease activating factor-1
ATRX	Alpha-thalassemia/mental retardation, X-linked
bp	Basepair
C	Cys, Cysteine
℃	Degree Celcius
CASP9	Caspase-9
CC	Craniocaudal
CDK	Cyclin-dependent kinase
CI	Confidence interval
CIC	*Capicua* Transcriptional Repressor
cm^3	Cubic centimetre, 10^{-6} cubic meter
CNS	Central nervous system
Covid-19	Coronavirus disease 2019
CT	Computerized tomography
CTLA-4	Cytotoxic T lymphocyte antigen 4
DAB	3, 3'-diaminobenzidine
DAMPs	Damage-associated molecular patterns
dNTPs	Deoxynucleoside triphosphates
E	Glu, Glutamic acid
EGFR	Epidermal growth factor receptor
Ex	Exon
F	Phe, Phenylalanine

G	Gly, Glycine
GBM	Glioblastoma multiforme
G-CIMP	Glioma CpG island methylator phenotype
gDNA	Genomic DNA
GFAP	Glial fibrillary acidic protein
GSH	Glutathione
GvHD	Graft versus host disease
H	His, Histidine
HCl	Hydrochloric acid, molecular weight: 36.458 g/mol
ICD	Immunogenic cell death
IDH1	Isocitrate dehydrogenase 1
IDH1/2	Isocitrate dehydrogenase 1 and/or 2
IHC	Immunohistochemical
iPSCs	Induced pluripotent stem cells
K	Lys, Lysine
KCl	Potassium chloride, molecular weight: 74.5513 g/mol
L	Leu, Leucine
MAPK	Mitogen-activated protein kinase
mg	Milligram, 10^{-3} gram
MGMT	O-6-methylguanine-DNA methyltransferase
$MgSO_4$	Magnesium sulfate, molecular Weight: 120.366 g/mol
mL	Millilitre, 10^{-3} litre
mm	Millimetre, 10^{-3} meter
mM	Millimolar, 10^{-3} molar
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NF1	Neurofibromatosis type 1
ng	Nanogram, 10^{-9} gram
nm	Nanometer, 10^{-9} meter
OEM	Original Equipment Manufacturer

OV	Oncolytic virus
P	Pro, Proline
pAb	Polyclonal antibodies
PCR	Polymerase Chain Reaction
PD-1/PD-L1	Programmed Cell Death/ Programmed Cell Death Ligand 1
PI3K	Phosphatidylinositol 3 kinase
PTCH1	Protein patched homolog 1
PTEN	Phosphatase and tensin homolog
Q	Gln, Glutamine
R	Arg, Arginine
Rb1	Retinoblastoma 1
RELA	v-rel avian reticuloendotheliosis viral oncogene homolog A
S	Ser, Serine
SeV	Sendai Virus
SNP	Single Nucleotide Polymorphism
TAAs	Tumour-Associated Antigens
T-ARMS	Tetra-primer Amplification Refractory Mutation System
TERT	Telomerase reverse transcriptase
TP53	Tumor protein p53
TR	Transverse
U	Unit
V	Volt
V	Val, Valine
VEGF	Vascular endothelial growth factor
WHO	World Health Organization
X	Stop codon
Y	Tyr, Tyrosine
α-KG	Alpha-ketoglutarate
χ^2	Chi-Square test

LIST OF APPENDICES

PENILAIAN TERHADAP PERUBAHAN GENETIK GEN IDH1, TP53 DAN CASP9 SEBAGAI BIOPENANDA DALAM PESAKIT GLIOMA

ABSTRAK

Mutasi titik panas *isocitrate dehydrogenase isoform 1* (IDH1) biasanya ditemui dalam glioma, terutamanya dalam glioma gred rendah dan glioblastoma secara sekunder. Kajian terdahulu menunjukkan mutasi IDH1 p.R132H (c.395G>A) diketahui berkait rapat dengan hasil klinikal. Laluan gen p53 dilaporkan berkait rapat dengan kebanyakan glioma. Laporan klinikal terkini menunjukkan bahawa pesakit glioma mempunyai mutasi germline gen CASP9 dan telah diperhatikan bahawa kehilangan CASP9 berlaku bersamaan dengan beberapa gen penindas tumor. Perkembangan glioma terutamanya dipengaruhi oleh beberapa perubahan genetik yang ketara. Walau bagaimanapun, hanya sebilangan kecil gen yang diperiksa secara mendalam. Gen TP53 dan IDH1 telah dicatatkan sebagai mempunyai mutasi yang paling kerap diperhatikan dalam pesakit glioma. Mutasi gen CASP9 yang diterangkan dalam laporan klinikal terkini telah mennunjukkan potensi gen mutaasi ini kepada perkembangan glioma. Salah satu daripada gen ini mungkin merupakan biopenanda prognostic bagi penyakit glioma. Untuk menambahbaik kaedah saringan awal mutasi gen titik panas IDH1, kajian ini membangunkan kaedah tetra primer amplifikasi sistem mutasi refraktori tindak balas rantai polimerase (T-ARMS PCR) sebagai kaedah ujian makmal yang lebih sensitive dan spesifik untuk mempercepatkan proses saringan awal penyakit glioma. Kajian ini turut mengenal pasti biopenanda baharu bagi pesakit glioma melalui pemeriksaan analisis perubahan genetik dalam semua ekson pengekodan dalam gen IDH1, TP53 dan CASP9. Sebanyak tiga puluh enam (n=36)

spesimen folikel rambut, darah, dan tisu glioma telah direkrut dan dianalisis menggunakan jujukan Sanger. Pengenalpastian perubahan genetik gen telah disahkan menggunakan ujian Chi-square dan Fisher's Exact, dan analisis bioinformatik daripada data sumber terbuka seperti The Cancer Genome Altas (TCGA) dan Chinese Glioma Genome Atlas (CGGA). Manakala untuk kajian pembangunan sistem T-ARMS PCR, sejumlah enam puluh satu (n=61) spesimen glioma telah dikumpulkan daripada pesakit dan diuji keboleupayaan T-ARMS PCR. Kajian ini mencadangkan bahawa *CASP9* p.Q221R (Ex 5 +32G>A) sebagai biopenanda prognostik baharu untuk pesakit glioma selain daripada biopenanda *IDH1* p.R132H. Kajian susulan telah dibuat melalui analisis secara separa-kuantitatif ekspresi protein CASP9 daripada tisu glioma dan menunjukkan perubahan signifikan dalam ekspresi protein CASP9 (p=0.024) antara pesakit glioma dengan mutasi dan tanpa mutasi *CASP9* p.Q221R. Kajian ini juga melaporkan kaedah T-ARMS PCR merupakan kaedah saringan awal yang sensitif, spesifik dan ketepatan pada 100% (95% CI: 87.94-100.00%), 93.94% (95% CI: 80.39-98.92%) dan 96.72 %, dengan skor F1 pada 0.966. Analisis *forest plot* digunakan untuk membandingkan kebolehupayaan T-ARMS PCR yang dibangunkan dalam kajian ini dengan kajian-kajian lain. Kesimpulannya, kajian ini menunjukkan *CASP9* p.Q221R sebagai biopenanda baharu untuk pesakit glioma di samping biopenanda *IDH1* p.R132H dan dihipotesiskan erkait rapat dengan kelangsungan hidup pesakit glioma. Prestasi ujian T-ARMS PCR menunjukkan bahawa teknologi ini setanding dengan ujian piawaian emas mutasi yang sedia ada dan berpotensi besar bagi saringan penyakit glioma secara praoperatif atau intraoperatif yang lebih murah, sensitif dan spesifik.

Kata kunci: Glioma, IDH1, TP53, CASP9 dan *T-ARMS PCR*

EVALUATION OF GENETIC ALTERATIONS OF IDH1, TP53 AND CASP9 GENES AS BIOMARKERS IN GLIOMA PATIENTS

ABSTRACT

Isocitrate dehydrogenase isoform 1 (*IDH1*) hotspot mutations are commonly found in gliomas, especially in low-grade gliomas and secondary glioblastoma. The *IDH1* p.R132H (c.395G>A) gathered a significant association with clinical outcomes in previous literature. The p53 pathway is usually dysregulated in most of the gliomas. Recent clinical reports showed that glioma patients have the germline mutation of the CASP9 gene and has been observed that the loss of CASP9 occurs in conjunction with several tumor-suppressor genes. The development of gliomas are primarily influenced by a number of significant genetic alterations. However, only a small number of genes were examined in depth. The TP53 and IDH1 genes were noted as having mutations most frequently observed in gliomas. CASP9 gene mutation described in recent clinical reports has illustrated their potential contribution to the gliomagenesis. One of these genes may be a predictive biomarker that may be applied in laboratory settings. To improve early mutation detection for IDH1 hotspot mutation, this study developed tetra primer amplification refractory mutation system polymerase chain reaction (T-ARMS PCR) assay as a sensitive and specific laboratory testing method to accelerate the process of early screening for glioma. This study identified a novel biomarker in glioma patients through the screening of genetic alteration analysis in all the coding exons in IDH1, TP53 and CASP9 genes. A total of thirty-six (n=36) specimens of hair follicles, blood, and glioma tissue were recruited and analysed using Sanger sequencing. Genetic alterations analysis of the genes of interest were confirmed using Chi-square test and Fisher's exact test, and bioinformatics analysis from open source

databases such as The Cancer Genome Altas (TCGA) and the Chinese Glioma Genome Atlas (CGGA). For the development of the T-ARMS PCR assay, a total of sixty-one (n=61) glioma specimens were collected from the patients to determine their clinical applicability. This study suggested *CASP9* p.Q221R (Ex 5 +32G>A) as a novel prognostic biomarker for glioma patients other than the current biomarker of *IDH1* p.R132H. A follow-up study was conducted through a semi-quantitative determination of CASP9 protein expression from the glioma tissue and showed a significant difference in the CASP9 protein expression (p=0.024) in the patient group with mutant *CASP9* p.Q221R as compared to the wildtype. This study also reported T-ARMS PCR assay is an early screening method with a sensitivity, specificity, and accuracy of 100% (95% CI: 87.94-100.00%), 93.94% (95% CI: 80.39-98.92%) and 96.72%, respectively, and the F1 score of 0.966. A forest plot analysis was used to compare the performance of the T-ARMS PCR assay developed in this study with other published assays. In short, this study demonstrated *CASP9* p.Q221R as a novel biomarker for glioma patients besides the current biomarker *IDH1* p.R132H and hypothesized to be associated with the survival of glioma patients. The performance of the T-ARMS PCR assay was comparable to the gold standard and could be adapted for preoperative or intraoperative diagnosis as a sensitive and specific screening tool.

Keywords: *Glioma*, *IDH1*, *TP53*, *CASP9* and *T-ARMS PCR*.

CHAPTER 1

INTRODUCTION

1.1 Research background

A brain tumour is devastating cancer that can be deadly and significantly affect the quality of life. Worldwide, it has reported the overall incidence rate of brain tumours is 10.82 per 100,000 person-year (95% CI: 8.63-13.56) and is expected to be increasing over the years (De Robles et al., 2015). In the Malaysian population, a report revealed the incidence rate to be 2.8 per 100,000 person-year with a cumulative rate of 0.3% (C. H. Goh et al., 2014). Interestingly, the number of cases was also varied by World Bank income groups, 6.29 (95% CI: 6.26-6.32) in high-income countries, howbeit 4.81 (95% CI: 4.77-4.86) in low-income countries (Bell et al., 2019).

In the past decades, the association between isocitrate dehydrogenase isoform 1 (*IDH1*) mutations and glioma tumours was reported, leading to the World Health Organization (WHO) revised the classification of tumours of the central nervous system by incorporating the mutation status of IDH1 as the key biomarker (Louis et al., 2016). IDH1 protein is believed to be the key enzyme, playing an important role in cellular metabolism, redox state regulation, DNA damage repair mechanism and epigenetic regulation (Cohen et al., 2013; Kaminska et al., 2019; A. Liu et al., 2016; Park and Turcan, 2019). The *IDH1* p.R132H (c.395G>A) mutation is suggested to lead to the neomorphic activity of 2-hydroxyglutarate (2-HG) and reduce or stop the reduced nicotinamide adenine dinucleotide phosphate (NADPH) production. Besides, several studies showed that IDH1 mutation was found in the vast majority of low-grade glioma (Komori, 2017) and secondary glioblastoma but almost absent in primary glioblastoma (Louis et al., 2016). However, whether IDH1 mutation is a germline

mutation remains controversial, although it is commonly recognized as a somatic mutation. Two recent reports (Blackburn et al., 2020; Kendroud et al., 2019) confirmed that IDH1 germline mutations were found in chondrogenic tumours and multiple gliomas, respectively. A clinical report (Ronellenfitsch et al., 2018) identified a novel CASP9 germline mutation that may increase the susceptibility to the development of brain tumours. Gliomas most frequently harbour alterations in TP53, and its germline mutation potential is debatable (Cotter et al., 2018; Muskens et al., 2020). These notions have inspired this study to determine a potential mutation associated with gliomas.

Single Nucleotide Polymorphims (SNPs) can be genotyped easily and affordably using the tetra-primer amplification refractory mutation system-polymerase chain (T-ARMS-PCR) reaction. T-ARMS PCR is conducted with four primers and is followed just by gel electrophoresis. The T-ARMS-PCR enables the simultaneous detection of both alleles (wild type and mutant) in one reaction, it is preferable to allele-specific PCR and RFLP. Comparing the T-ARMS PCR genotyping test to Taq polymerase-based assay, the T-ARMS PCR assay can be finished in 25-30 cycles. Besides, it is less affected by the annealing temperature and reaction mix.

1.2 Tumour of the central nervous system

1.2.1 The WHO classification of tumours of the central nervous system

Previously, the classification and grading of tumours of the central nervous system (CNS) have been based entirely on the histological characteristics of the brain tumour, according to their microscopic features, putative cells of origin and presumed levels of differentiation (Louis et al., 2016). The classification has primarily dependent on microscopic views of the haematoxylin and eosin-stained tissue sections, lineage-associated proteins and immunohistochemical staining, sometimes based on the ultrastructural characterisation of the tumour (Komori, 2017; Louis et al., 2016). The drawback of this classification method has led to a lot of confusion and argument among histopathologists.

In 2021, the World Health Organization (WHO) has updated the classification and grading of tumours of the CNS. The current update has introduced major changes that advance the role of molecular diagnostics (WHO Classification of Tumors of the Central Nervous System 2021, 5th edition) (Louis et al., 2021). The grading of the tumour of CNS does not have many changes in which it is mainly based on the cytological atypia, mitotic activity, microvascular proliferation and necrosis level (Bertero and Cassoni, 2019; Komori, 2017; Louis et al., 2016). However, the grading is written in Arabic numerals instead of Roman numerals to avoid typographical error (Louis et al., 2021). New tumour types and subtypes are included as some of which are based on innovative diagnostic techniques like DNA methylome analysis.

In a nutshell, there are several advantages brought by this updated classification. For instance, improvement in diagnostic accuracy and a more specialized patient treatment response are possible due to targeted therapies.

1.2.2 Gliomas

It has introduced major changes in the classification of gliomas based on their molecular markers in the WHO Classification of Tumors of the Central Nervous System 2016. Diffuse gliomas are categorized into several groups based on their growth pattern (astrocytoma, oligodendroglioma, ependymoma, oligoastrocytoma and glioblastoma), behaviour (grading) and IDH1 mutation status (IDH1-mutant type or IDH1-wildtype) (Bertero and Cassoni, 2019; Louis et al., 2016, 2021). In short, gliomas are types of CNS tumours that arise from the glial cells of the brain or of the spine. The Central Brain Tumor Registry of the United States (CBTRUS) 2017 Annual Statistical Report pointed gliomas are the second most commonly occurring type of CNS tumours (after meningioma) in the United States, accounting for 25-30% of many CNS tumours and about 80% of all malignant brain tumours (Kruchko et al., 2018; Ostrom et al., 2019). CNS tumours are the most common death-causing cancer in children (<15 years old) followed by leukaemia between 2010 and 2014 (Kruchko et al., 2018).

1.2.3 Astrocytomas, ependymomas and oligodendrogliomas

Gliomas are brain tumours arising from the glial lineage of the CNS cells, for instance, astrocytoma, ependymoma and oligodendroglioma from astrocyte, ependymocyte and oligodendrocyte, respectively. Gliomas in common, expressing glial markers such as glial fibrillary acidic protein (GFAP). The nomenclature of gliomas is based on the specific type of cell in which they share the histological features, but not necessarily from which they originate. The main gliomas will be further discussed below.

Astrocytomas are slightly more commonly occur in male adults than female adults and children (Bertero and Cassoni, 2019), except for pilocytic astrocytomas which are non-infiltrating neoplasms that are most commonly found in children and adolescents (Alattar et al., 2019). There are several circumscribed astrocytic gliomas, such as pleomorphic xanthoastrocytoma, subependymal giant cell astrocytoma, chordoid glioma and astroblastoma. Astrocytomas are proposed to be arising from the reactive astrocytes of the subventricular zone, neural progenitor cells of the frontal lobes and subventricular zone (Alattar et al., 2019). Histologically, a proliferation of well-differentiated astrocytes and mild nuclear atypia without microvascularization is categorized as diffuse astrocytoma (WHO grade 2), whilst a higher cell clustering, prominent nuclear atypia and increased proliferation without microvascularization are commonly known as anaplastic astrocytomas (WHO grade 3). It is reported that the median survival years after its diagnosis are approximately six to eight years and three to five years for diffuse astrocytomas and anaplastic astrocytomas, respectively (Alattar et al., 2019; Bertero and Cassoni, 2019). However, astrocytomas with IDH1-wildtype are a significantly poorer outcome, with a median of fewer than two years of survival time (Bertero and Cassoni, 2019).

Ependymomas are gliomas that arise from the ependymocytes or radial glial cells lining the ventricles and the central canal of the spinal cord. Although these tumours are weak in association with the IDH1 gene, ependymomas are characterized into subgroups based on the RELA gene fusion (Komori, 2017; Louis et al., 2016). Different variants of ependymoma are identified based on the site of origin (supratentorial, posterior fossa and spine) and the clinicopathological features. Ependymomas usually occur in the intracranial of children and the spinal of adults

(Bertero and Cassoni, 2019; Komori, 2017). The prognosis of ependymomas is variable, and poorer in children due to the tumour site (Bertero and Cassoni, 2019).

Oligodendrogliomas are the second most common type of gliomas, and they share similar characteristics as astrocytomas, which are also higher prevalent in male adults (Bertero and Cassoni, 2019). Oligodendrogliomas are commonly found in the white matter and the cerebral cortex of the frontal lobes which probably arise from oligodendroglial precursor cells (Komori, 2017). Classification of oligodendrogliomas requires information on the presence of IDH1-mutant and 1p/19q codeletion, usually associated with TERT alterations as well (Bertero and Cassoni, 2019; Komori, 2017; Louis et al., 2016). Oligodendrogliomas appear as infiltrating proliferative monomorphic cells with round nuclei and perinuclear haloes, similar to fried egg appearance. Further, a capillaries network and the absence of increased cell proliferation are common (Komori, 2017). High cellularity and severe nuclear atypia are usually found in anaplastic oligodendrogliomas. The clinical prognosis of oligodendrogliomas is greater than 10 years.

Astrocytoma, ependymoma and oligodendroglioma are the three main types of gliomas, and the clinical outcomes are variable across different gliomas. The categorization is based on their histological features and sub-categorized based on their molecular biomarkers, such as IDH1 mutation, 1p/19q codeletion or RELA fusion.

1.2.4 Glioblastoma multiforme

Glioblastoma multiforme (GBM) is the most invasive, aggressive and fatal form of glioma with only five per cent of patients with glioblastoma surviving for five years (Gittleman et al., 2017; Kruchko et al., 2018). GBM is graded as WHO grade 4

and sometimes also referred to as grade 4 astrocytomas as it can arise in the brain *de novo* (primary GBM, 90%) or develop from lower-grade astrocytoma (secondary GBM, 10%). Interestingly, IDH1-wildtype GBM is frequently found in temporal lobes and usually occurs in adolescents (60-70 years old) whereas IDH1-mutant GBM is usually found in frontal lobes (Bertero and Cassoni, 2019; Komori, 2017). Since the term GBM was first time mentioned in 1926, the definition of this tumour has changed substantially, especially when the molecular signature has become a key biomarker in defining these tumours. In the 5th edition of the WHO Classification of Tumors of the Central Nervous System (2021), GBM has been refined as WHO grade 4 glioblastomas, a diffused-type of astrocytic tumours and must be IDH1-wildtype and as WHO grade 4 astrocytomas with IDH1-mutant type (Louis et al., 2021). These tumours typically show a rapidly infiltrating growth and contralateral extension through the corpus callosum, histologically showing a poor differentiated cells population and highly cellular lesion (Komori, 2017). The median survival for patients with curative intent has doubled from 9 to 12 months in 2005 to 18 to 24 months in 2014 (Gittleman et al., 2017).

1.3 Gene alterations in gliomas

1.3.1 Important pathways and genetic alterations in glioma biology

Recent efforts at comprehensive genetic, transcriptomic and epigenetic profiling have resulted in an alternative approach to classifying gliomas. The characterization of gliomas has identified several common alterations and signalling pathways (Louis et al., 2021; Ludwig and Kornblum, 2017; Ohba et al., 2020; Velázquez Vega and Brat, 2018), including:

(i) IDH1 mutant astrocytic gliomas with frequent α-thalassemia/mental retardation syndrome X-linked (ATRX) and tumour protein p53 (TP53) gene mutations,

(ii) IDH1 mutant oligodendroglial gliomas with 1p/19q codeletion and frequent telomerase reverse transcriptase (TERT) promoter mutation or Drosophila homolog of capicua (CIC) gene mutation, as well as

(iii) IDH1-wildtype glioblastomas with frequent TERT promoter mutation, phosphatase and tensin homolog (PTEN) mutation and epidermal growth factor receptor (EGFR) amplification.

In glioma biology, the current understanding of the mechanism of gliomagenesis is reported to be altered in several signalling pathways: (i) growth factor receptor tyrosine kinases and their downstream signalling via the mitogen-activated protein kinase (MAPK) cascade or phosphatidyl inositol-3-kinase (PI3K) (Nicolas et al., 2019), (ii) loss of apoptosis via p53 (Ludwig and Kornblum, 2017), (iii) cell cycle regulation via cyclin-dependant kinases (CDK) and retinoblastoma 1 (Rb1) (Ludwig and Kornblum, 2017; Velázquez Vega and Brat, 2018), (iv) angiogenesis via vascular

endothelial growth factor (VEGF) (Griveau et al., 2018), and (v) invasion via Notch1 signalling pathway (Yi et al., 2019).

The MAPK signalling pathway plays a significant part in many malignancies, including GBM. It has also been linked to several tumorigenic progressions, including migration, proliferation, and survival. According to reports, MAPK pathway signalling is essential for the co-activation of CREB, a crucial regulator of cyclin-D1 production in GBM cells, and cell proliferation (Nicolas et al., 2019). Glioma is frequently characterised by molecular changes in the PI3K signalling pathway, and numerous clinical trials that have been launched to target one or more players in this axis have so far produced disappointing results because of the low blood-brain-barrier permeability of some drugs or the occurrence of resistance/tolerance mechanisms. However, since PI3K is one of the crucial pathways that controls cell growth and survival in cancer biology, targeting it is still an important justification for creating treatments for gliomas. Patients with GBM who have the PI3K/Akt/mTOR pathway active also have a worse prognosis than those who do not have the pathway oncogenically activated (Nicolas et al., 2019). One of the most often dysregulated genes in cancer is TP53 (84% of GBM patients and 94% of GBM cell lines) and the p53-ARF-MDM2 pathway is dysregulated. Components of the p53 pathway that are dysregulated have been linked to GBM cell invasion, migration, proliferation, apoptosis evasion, and cancer cell stemness (Nicolas et al., 2019). The RB1 tumour suppressor is an important target for cancer treatment, according to recent findings. RB1 controls cell cycle progression and functions as the downstream target for CDK4/6 inhibitors now being used in therapeutic settings. However, recently identified RB1-pathway characteristics indicate novel therapeutic approaches to overcome resistance and advance precision medicine (Ludwig and Kornblum, 2017;

Velázquez Vega and Brat, 2018). Vascular endothelial growth factor (VEGF) plays a key role in glioblastoma angiogenesis, which is fueled by hypoxia-dependent and independent mechanisms and produces blood vessels with unique characteristics. Because of inefficient treatments, people with recurrent glioblastoma have poor outcomes (Griveau et al., 2018). In GBM cells, the Notch1 pathway and NF-B(p65) engage in a reciprocal regulatory loop; this axis is crucial for the development of GBM carcinogenesis. In glioma stem cells, where it suppresses differentiation and retains stem-like features, Notch signalling is very active, according to mounting data. This contributes to the development of glioblastoma tumours and the resistance of these tumours to standard medical therapy (Yi et al., 2019).

1.3.2 Role of IDH1 in gliomas

IDH1/2 gene alterations are one of the most clinically significant molecular markers in gliomas, serving as the diagnostic significance in astrocytomas, oligodendrogliomas and glioblastomas. Mutations in either IDH1 or IDH2 isoforms are found in approximately 80% of grade II and III gliomas and grade IV secondary glioblastoma, with *IDH1* p.R132H (c.395G>A) mutation being more common in most cases (Franceschi et al., 2021; Kaminska et al., 2019). In a clinical setting, the most commonly used technique for detecting the mutation is immunohistochemistry or Polymerase Chain Reaction with Sanger sequencing. If either approach fails, the glioma should be classified as IDH-wildtype.

The IDH1 mRNA is comprised of 1245 bp long consisting ten coding exons, that encode for 415 amino acids of an enzyme called isocitrate dehydrogenase isoform 1, which is not only found in the cytoplasm but also peroxisomes. IDH2 mutations are

much less common in glioma than IDH1 mutations. It has been reported that it is mutually exclusive with an IDH1 mutation (H. Y. Wang et al., 2016). Almost all the IDH1 mutations are heterozygous mutations, with the most commonly reported mutation involving a nucleotide substitution at position 395 (from G to A), resulting in an amino acid change at codon 132 (from glycine (R) to arginine (H)). Non-canonical IDH1 mutations are IDH1 single nucleotide polymorphisms that are relatively uncommon. For instance, *IDH1* R132C, R132L, R132S, R132G, R132V and R132P were described in several studies (Clark et al., 2016; W. C. Goh et al., 2020; Huang, 2019; Z. Liu et al., 2019). Intriguingly, the frequency of IDH1 mutations varied across the Asian population, with China (16.0 – 74.0%), Japan (7.8 – 31.2%), Korea (16.0 – 53.7%), India (18.7 – 56.0%), Indonesia (21.7%) and Malaysia (6.4 – 35.0%) having the highest frequency (S. Agarwal et al., 2013; W. C. Goh et al., 2020; Malueka et al., 2020; Myung et al., 2012; Ohno et al., 2016; H. Y. Wang et al., 2016; Yusoff et al., 2016).

For decades, IDH1 enzymes have been well-known to catalyse the oxidative decarboxylation process of the Kerb's cycle, oxidizing isocitrate into α-ketoglutarate (α-KG) while reducing its cofactors from $NADP^+$ to NADPH in a reversible manner. The IDH1 enzymes are not only involved in more than just oxidative decarboxylation but also play a role in mitochondrial oxidative phosphorylation, homeostasis of cellular redox status, glutamine metabolism, lipogenesis and glucose sensing (Alzial et al., 2021; Fantin et al., 2010; Huang, 2019; Miyata et al., 2019; Shi et al., 2015; Zhao et al., 2009).

The *IDH1* p.R132H mutation resulted in the production of an oncometabolite, R-2-hydroxyglutarate (2-HG), instead of α-KG, implying that the oxidative

decarboxylation Kreb's cycle and other cellular functions are disrupted (Clark et al., 2016; Huang, 2019; S. Li et al., 2019; Miyata et al., 2019). The imbalance of NADPH and $NADP^+$ leads to dysregulation of the reduced glutathione (GSH) pool, which further interrupted redox balance in cells (A. Liu et al., 2016). The notion was supported by Miyata *et al.* (2019) who found IDH1-mutant gliomas are significantly higher 2-HG levels than the IDH1-wildtype (Miyata et al., 2019). Further, a global hypermethylation landscape analysis showed that mutant-type glioma is also associated with epigenetic alterations, which is also known as the G-CIMP phenotype (Brennan et al., 2013). The survival rate of IDH1-mutant glioma patients is significantly higher compared to IDH1-wildtype glioma patients (Z. Liu et al., 2019; H. Y. Wang et al., 2016; Yusoff et al., 2016), suggesting this could be due to increased sensitivity towards chemotherapy (Alzial et al., 2021; Cohen et al., 2013; Myung et al., 2012; Ohno et al., 2016).

The *IDH1* p.R132H mutation was found associated with the early event of gliomagenesis (Huang, 2019; Yusoff et al., 2016). Besides, Kamiska *et al.* (2019) asserted that IDH1 mutation persists throughout the progression of glioma (Kaminska et al., 2019). This notion was supported by Liu *et al.* (2019) as the mutations lead to epigenetic alterations and can respond to hypoxia (Z. Liu et al., 2019). There is also rarely observed IDH1 mutation in primary glioblastoma and WHO grade I gliomas, suggesting the idea of IDH1 mutation is not a driver of tumour initiation in this glioblastoma subtypes (Huang, 2019; A. Liu et al., 2016). It is hypothesized that IDH1 mutation is one of the major drivers for gliomagenesis, especially for secondary glioblastoma, but not the only oncogenic driver for gliomagenesis.

In a nutshell, IDH1 mutation is a key biomarker to aid clinicians in their treatment and patient management plan. IDH1-mutant has a better prognosis, howbeit the prevalence rate is mostly less than half, meaning most cases are harbouring IDH1-wildtype, which has a poorer prognosis. Thus, more biomedical research should be carried out to model and find the ultimate cure to treat gliomas.

1.3.3 TP53 and CASP9 mutation in gliomas

The p53 pathway is frequently deregulated in glioblastoma. The Cancer Genome Atlas (2018) reported that the p53 pathway has been shown much deregulated in over 80% of gliomas and up to 90% of glioblastoma cell lines (Y. Zhang et al., 2018). The TP53 gene mutations are one of the most common biomarkers found associated with almost all cancers (Cohen et al., 2013; Huang, 2019). Studies showed that TP53 mutations were more commonly found in certain subtypes of gliomas, such as diffuse astrocytomas, anaplastic astrocytomas and secondary glioblastomas, whereas oligodendrogliomas or anaplastic oligodendrogliomas were found less common (Huang, 2019). Interestingly, most of these gliomas harbouring TP53 mutation demonstrated a strong association with IDH1-mutant (Huang, 2019). In addition, these tumours rarely demonstrated 1p/19q codeletion compared to oligodendrogliomas virtually all demonstrated 1p/19q co-deletion (Cohen et al., 2013). Bertero and Cassoni (2019) suggested that 1p/19q codeletion that results in the complete deletion of both the 1p and 19q chromosomal arms is associated with increased sensitivity towards current alkylating chemotherapy drugs such as temozolomide but not in the case of partial deletion of 1p or 19q chromosomal arms (Bertero and Cassoni, 2019). In short, it is believed that IDH1 mutations are commonly

associated with TP53 mutations and 1p/19q codeletion, although TP53 mutations and 1p/19q codeletion are almost mutually exclusive.

It is reported that most brain tumours are somatic mutations, even if germline mutations. They were rarely reported, mainly because most the glioma is sporadic and with no known predisposing germline variants (Nordfors et al., 2018). CASP9 gene mutation has recently been reported as a germline mutation found in glioma patients (Ronellenfitsch et al., 2018). It was reported that CASP9 deletion together with multiple tumour suppressor genes such as TP53, NF1, PTEN and PTCH1 may, therefore, induce gliomagenesis (Zuckermann et al., 2015). Ronellenfitsch and his colleagues (2018) asserted that even though the CASP9 germline mutation was reported, CASP9 mutation alone may not be sufficient for the development of brain tumours (Ronellenfitsch et al., 2018).

In closing, these three genes (IDH1, TP53, CASP9) are the potential gene mutations associated with gliomagenesis, motivating this study to detect its genetic alterations and perhaps also in their germline. Understanding the mutations that lead to gliomagenesis allows the researcher to create new and specific drugs with more tailored and personalized-therapy approaches.

1.4 Diagnostics and treatment approaches for gliomas

1.4.1 Current diagnostic method for detecting IDH1 mutation in gliomas

There are several diagnostic tests for glioma, including medical history, family history, medical imaging examination, tissue biopsy and neurological examination. However, none of these could give a conclusive diagnosis. An imaging examination of the brain such as magnetic resonance imaging (MRI) and computerized tomography (CT) scan is an elementary diagnostic test for glioma as they only provide a structural view of the brain. Thus, tissue biopsy and histological examination are necessary to provide a piece of more accurate and useful information.

In molecular diagnostics, immunohistochemistry (IHC) and Sanger sequencing are the most commonly used methods for the detection of *IDH1* p.R132H mutation in glioma patients. Both the IHC and Sanger sequencing technologies have flaws. If the tumour samples are not of good quality, IHC is likely to miss the detection. It also has low sensitivity when compared to sequence-based techniques (Capper et al., 2010; Suzuki et al., 2017). Although Sanger sequencing is considered the gold standard for a confirmatory test for IDH1 mutations, it is laborious and requires specialised staff, which can limit its applicability. Both procedures take a considerable time to arrive at a conclusive diagnosis.

1.4.2 Treatment for gliomas

Gliomas are typically treated with surgery, radiation, or chemotherapy. The current standard of care for treating gliomas is surgery or a combination of chemotherapy and radiation therapy. The chemotherapy drug used most often to treat gliomas is an oral pill, temozolomide. Despite the mild to severe side effects of the

treatment, close monitoring of patients is necessary to determine whether the glioma has recurred. Unfortunately, recurrence in these cases is extremely likely. Immunotherapy and stem-cell-based therapy have recently provided a promising outcome, however, with significant drawbacks such as unspecific or off-target tumour sites, resulting in "bystander" killing (Daga et al., 2007), limited efficacy (Daga et al., 2007; Hotchkiss and Sampson, 2021), graft-versus-host disease (GvHD) (Singh et al., 2016), therapy resistance (Jackson et al., 2019), and tumour recurrence (Jackson et al., 2019; Kong et al., 2018).

Current immunotherapy treatments aim to generate immune responses against gliomas. Active immunotherapy uses a tumour vaccine to activate the host's immune system, whereas passive immunotherapy uses adoptive cell transfer (ACT) to evoke immune effector capabilities. However, clinical experience with immunotherapy for glioma is limited and the outcomes were rather disappointing. Despite the success in other malignancies, immune checkpoint inhibition of PD-1/PD-L1 and CTLA-4 has failed miserably in GBM so far. Besides, genetically engineered stem cells are used to increase the expression of prodrug-converting enzymes or to produce tumour-killing bioproducts (Kimura et al., 2019; Mirzaei et al., 2018). Because of their ability to tumour-homing and transverse the blood-brain barrier, stem cells can produce antibodies and act as a delivery courier (Bexell et al., 2013). Oncolytic viruses (OV) are genetically engineered viruses that replicate selectively within target tumour cells and induce immunogenic cell death (ICD), allowing tumour-associated antigens (TAAs) and damage-associated molecular patterns (DAMPs) to be released, and then recognized by immune cells. OV also can be altered to induce cell death, stimulate an immunogenic anti-tumour response, or deliver therapeutic transgenes. To date, most of the trials did not show promising therapeutic benefits.

1.5 T-ARMS PCR

Tetra primer amplification refractory mutation system polymerase chain reaction (T-ARMS PCR) is a fast, sensitive, specific, and cost-effective molecular-based technique for detecting single nucleotide mutation. The principle of T-ARMS PCR is based on the modification of ARMS PCR by adding a strong mismatch destabilising nucleotide at the -3 position of the 3' end of each allele-specific primer, as well as an additional set of control primers that are present on both alleles and nonallele-specific. The strength of mismatch destabilization was reported by Liu *et al* (J. Liu et al., 2012). An overview of the workflow of T-ARMS PCR in detecting *IDH1* p.R132H (c.395G>A) mutation in glioma patients is illustrated in Figure 1.1. In T-ARMS PCR, a single PCR reaction using two control primers and two allele-specific primers can simultaneously amplify both mutant and normal alleles, as well as a common DNA fragment. The control primers amplify a larger fragment of the target gene, irrespective of its genotype in a single step reaction whereas each allele-specific primer pairs with either one of the control primers to generate a smaller allele-specific fragment of varying product size (Medrano and De Oliveira, 2014). Two different sizes of allele-specific fragments could be easily distinguished on gel electrophoresis as homozygous or heterozygous.

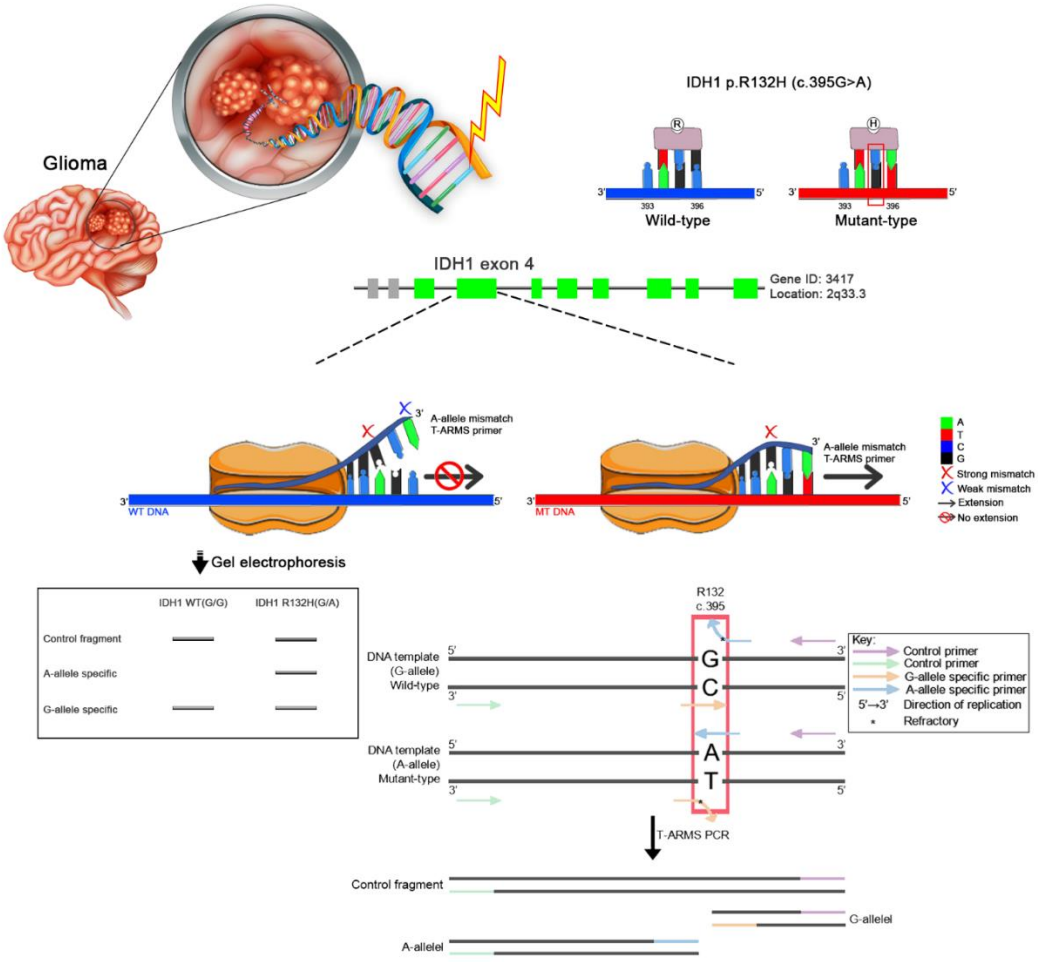

Figure 1.1 An overview of the workflow of T-ARMS PCR in detecting *IDH1* p.R132H (c.395G>A) mutation in glioma patients.

1.6 Problem statement

Several important genetic defects associated with gliomagenesis have been identified as key drivers for the initiation and progression of gliomas. Nonetheless, not many genes were thoroughly investigated. Gliomas were reported as most commonly found to have mutations in the TP53 and IDH1 genes. Together, a novel mutation of the CASP9 gene reported in the clinical reports (Ozdogan et al., 2017; Ronellenfitsch et al., 2018) has picked our interest in determining their role in the formation of glioma. The study hypothesizes that one of these genes can be a predictive biomarker that could be potentially used in laboratory settings.

While molecular diagnostics are highly sensitive and specific, they are typically more expensive than conventional methods, thus constraining their application. Immunohistochemistry and PCR with Sanger sequencing, the most commonly used methods, take longer to complete and are ineffective for preoperative and intraoperative diagnosis. In that respect, this study aims to develop a sensitive and specific molecular assay for the genotyping of glioma patients in the future, by employing the tetra primer amplification refractory mutation system polymerase chain reaction (T-ARMS PCR) technique.

To the best of our knowledge, no patient-derived glioma model has been reported to have the *IDH1* p.R132H mutation because the mutation is resistant to cellular reprogramming (Z. Liu et al., 2019). To model glioma with *IDH1* p.R132H mutation, genetically modification using gene editing (Köpp et al., 2019) or an inducible system (Rosiak-Stec et al., 2020) is necessary. On the other hand, a novel oncogenic alteration is demanded to model glioma for a better understanding of the disease and drug discovery.

1.7 Motivation of the study

Gliomas are one of the most common types of brain tumours that can impair brain function and can be fatal. Not only that, but a fiscal burden in terms of socioeconomics and wellbeing could halt the progress of the national development plan. Furthermore, the Covid-19 public health emergency threatens to reverse years of momentum in brain cancer research. Despite the fact that federally funded cancer research has resulted in significant advances in cancer prevention, detection, diagnosis, treatment, and patient quality of life, there are thousands of cancer survivors living in Malaysia today. This study will establish a precedent if one can identify missense mutations in the IDH1, TP53 and CASP9 genes of glioma patients. Future studies not only can detect the mutation status in a short period of time but also manipulate it *in vitro* by correcting the missense mutated gene using the current gene-editing technique of CRISPR-Cas9. This will be benefiting glioma patients in the future. Furthermore, targeting mutated sites or cell populations can be used to select potential chemotherapy or immunotherapy drugs, drug cocktails, or even personalised medicine. Overall, the nation needs additional funding on top of a robust annual increase in baseline budgets to offset pandemic-related disruptions and re-establish the nation's biomedical research, particularly brain cancer research.

1.8 Aim and objectives

The research aims to evaluate the genetic alterations of IDH1, TP53 and CASP9 genes as biomarkers in glioma patients. As a result, this study aims to achieve this goal through the following objectives:

- **Objective 1: To identify the genetic alterations in IDH1, TP53 and CASP9 genes in glioma patients.**

  Screening of all the exons in IDH1, TP53 and CASP9 genes from the paired samples of the hair follicle, blood, and tumour to identify the genetic alterations.

- **Objective 2: To investigate the association between genetic alterations and glioma.**

  Suggest a genetic alteration from the screening results as biomarkers in precedence that the association between the genetic alteration and glioma is significant.

- **Objective 3: To develop and validate the sensitivity and specificity of T-ARMS PCR for detecting *IDH1* p.R132H mutation in glioma patients.**

  Develop, optimize and validate the clinical applicability of T-ARMS for detecting *IDH1* p.R132H mutation in glioma patients.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 General instruments

The general instruments used in this study are listed in Table 2.1.

2.1.2 General consumables

The general consumables used in this study are listed in Table 2.2.

2.1.3 General reagents

The general reagents used in this study are listed in Table 2.3.

2.1.4 Commercial kits

The commercial kits used in this study are listed in Table 2.4.

2.1.5 Nucleotides

The nucleotides used in this study are listed in Table 2.5.

2.1.6 Software

The software used in this study is listed in Table 2.6.

Table 2.1 List of general instruments

Instrument	Manufacturer (Country)
Analytical balance XB 220A	Precisa (Switzerland)
Benchtop pH meter Phs-25cw	Biobase (China)
Dry bath incubator	Major Science (USA)
Eppendorf Research® plus micro pipettor	Eppendorf (USA)
FluorChem M image analyser	Protein Simple (USA)
HistoCore MULTICUT Semi-Automated Rotary Microtome	Leica Biosystem (USA)
Laminar flow hood	Erla (Malaysia)
LED transilluminator	MaestroGen (Taiwan R.O.C)
Magnetic stirrer MK 418	Nüve (Turkey)
Mastercycler® nexus	Eppendorf (USA)
Microwave oven	Electrolux (Malaysia)
NanoDrop™ 2000 spectrophotometers	Thermo Fisher Scientific (USA)
Olympus BX41 Phase Contrast & Darkfield Microscope integrated with Olympus XC50 digital camera	Olympus (Japan)
Power PAC basic	Bio-Rad (USA)
Profuge 6k mini centrifuge	On Wing Tat Co. Ltd (Hong Kong)
Refrigerated centrifuge 5417R	Eppendorf (USA)
Scotsman AF 124 Ice Flaker	Scotsman (USK
Sprout® Mini Centrifuge	Heathrow Scientific (USA)
Sub-Cell GT cell 1704401	Bio-Rad (USA)
SureCycler 8800	Agilent Technologies (USA)
TES Valida embedding system	MEDITE (Germany)
Tissue processor TP1020	Leica Biosystem (USA)
Vortex mixer	Stuart (UK)
VWR Signature™ Single-Channel Pipettors	Avantor (USA)
Water bath for paraffin sections HI1210	Leica Biosystem (USA)

Table 2.2 List of general consumables

Consumable	Catalogue No.	Manufacturer (Country)
Conical tube, 15 mL	50015	SPL Life Sciences (Korea)
Conical tube, 50 mL	50050	SPL Life Sciences (Korea)
Coverslip	7201	OEM (China)
Leica Classic Series High-profile Disposable Microtome Blades	3802110	Leica Biosystem (USA)
Microcentrifuge tube, 1.5 mL	SGB003	Bio-Seen (China)
PCR tube, 0.2 mL	HX-B01	Accumax (India)
Petri dish, 100mm	10100	SPL Life Sciences (Korea)
Pipette tip, 10 µL	SGA001	Bio-Seen (China)
Pipette tip, 1000 µL	319.1000B	Socorex (Switzerland)
Pipette tip, 200 µL	328.0200B	Socorex (Switzerland)
Polysine™ Coated Microscope Slides	26414-1	Polysciences (USA)
Sterile specimen container, 60 mL	8014L	LabChem (Malaysia)
Strip Embedding Cassette with removable lid	N1414001	OEM (China)