INVESTIGATION OF CTCF GENE IN GLIOMA AND ITS CORRELATION WITH YB-1 GENE USING SYBR-GREEN I BASED REAL-TIME PCR

by

NAZIRAH BINTI ABD. KAHAR

Dissertation submitted in partial fulfillment of the requirements for the degree of Bachelor of Health Sciences (Biomedicine)

October 2009

CERTIFICATE

This is to certify that the dissertation entitled **'Investigation of CTCF Gene in Glioma and its correlation with YB-1 Gene using SYBR-Green I based Real-Time PCR'** is the bonafide record of research work done by Miss Nazirah binti Abd. Kahar (92737) during the period from July 2009 to October 2009 under my supervision.

ACKNOWLEDGEMENTS

In the Name of Allah, Most Gracious, Most Merciful

"Over the knowledgeable, is Allah the most knowledgeable"

All praises and gratitude is due to Allah, the Lord to whom every single creature in the heaven and the earth belongs to. Thank you Allah for giving me the strength and patient during this trying times. May peace and blessings be on the leader of all creation, the prophet Muhammad S.A.W, his family and companions.

I am grateful to my beloved mother, Mariam Othman had it not been due to her endless love, prays and encouragement, the completion of this thesis would not have been possible. I wish to express my deepest appreciation to Dr Nik Norliza Nik Hassan for her valuable supervision and thorough criticisms during the writing of this thesis. Special thanks also due to Dr. See Too Wei Cun, research project coordinator for his constant encouragements and assistances throughout this process. I would like also to acknowledge Dr. Shaharum Samsuddin for his kindness in providing normal sample. Without his cooperation and flexibility, this thesis would not have been possible.

Appreciation is extended to Mrs. Azizah, Scientific Officer of School of Health Sciences and all staff in Unit Kemudahan Makmal (UKM) for their cooperation in equipments preparation. My warmest gratitude goes to the members of the Forensic Science Lab, in particular, Kak Alia and Kak Syikin for their sincere assistance in many aspects of Real-Time experiment carried out in this study.

I would also like to acknowledge the valuable technical support, advices and discussion provided by the postgraduate students and members of the Molecular and Culture Lab, in particular, Bainah, Aini, Kak Wani and Ling that made this study possible. Finally, I am grateful to my beloved friends, Aisya Zulkifli, Wong Yee Lie, Kalaiselvi Manveeran, Khairun Nisa Ibrahim Asri and Khairul Nissa Saidun for their support, understanding and encouragement during this study period.

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LIST OF ABBREVIATIONS

CNS	Central Nervous System
µg	Microgram
µl	Microliter
µM	Micromolar
A	Adenine
bp	Base pair
C	Cytosine
CAT	Computerized Axial Tomographer
cDNA	Complementary deoxyribonucleic acid
Ct	Crossing threshold
CTCF	CCCTC-binding factor
DEPC	Diethypyrocarbonate
DNA	Deoxyribonucleic acid
dNTPs	Dioxynucleotide triphosphate
dsDNA	Double stranded deoxyribonucleic acid
EDTA	Ethylene diamine terta acetate
EtBr	Ethidium bromide
FRET	Fluorescent resonance energy transfer
G	Guanine
g	gram
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GUS	Beta-glucuronidase
L	Liter
M	molar

mg	milligram
min	minutes
ml	milliliter
mM	Millimolar
MRI	Magnetic Resonance Imager
mRNA	Messenger ribonucleic acid
NaOH	Sodium hydrogen
ng	nanogram
NTC	Non-template control
°C	degree celcius
PCR	Polymerase chain reaction
PET	Positron Emission Tomography
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT-PCR	Reverse transcriptase polymerase chain reaction
secs	Second
ssDNA	Single stranded deoxyribonucleic acid
T	Thymine
UV	Ultraviolet
V	volt
x g	Gravity
YB-1	Y-box protein 1
ZF	Zinc finger

ABSTRACT

A glioma brain tumor is a primary brain tumor that originates from the supportive cells of the brain, known as glial cells consists of astrocytes, oligodendrocytes and ependymal. Unlike neurons, these cells have the ability to divide and multiply. Though very rare, mostly they are malignant when occurs. These malignancies pose the greatest clinical problems due to difficulty in diagnosis. Even though scans are done, it only produces pictures that suggest a particular type of tumor. The definitive diagnosis is via sample biopsy of tumor examined under a microscope by neuropathologist. Reliable genetic markers are urgently needed to identify glioma patients to avoid invasive approach.

The first step in identifiying genetic marker is to analyze the gene expression in the patient sample in comparison to normal sample either its being up-regulated or down-regulated. Therefore, Total RNA is extracted and converted to cDNA and subsequently act as template in PCR providing Ct value by using ΔΔCt value determination. Comparative method of ΔΔCt provides the data of fold change which is useful to screen genes either being up-regulated or down-regulated in the patients in comparison to non-cancerous normal sample. However, the analysis expression of selected genes is unable to be accessed as the Ct value is unacceptable based on the follow-up assays for amplicon identification in PCR. Instead, the guidelines to optimize the result were obtained based on the experiences and flaws encountered during the experiment. Thus, guidelines reported here are very crucial to be followed by others who interested in gene expression experiment in the future so that good result can be produced.

ABSTRAK

Glioma adalah ketumbuhan otak primer yang berasal dari sel-sel penyokong otak, yang dipanggil sel-sel glial iaitu astrosit, oligodendrosit dan sel-sel ependymal umum yang merupakan komponen sel otak dan mempunyai keupayaan untuk membahagi dan membiak tidak seperti neuron. Ketumbuhan otak primer tersebut lebih jarang berlaku tetapi teruk apabila terjadi. Ketumbuhan ini menimbulkan masalah klinikal yang paling besar kerana kesukaran dalam proses diagnosis. Meskipun pengimbasan dilakukan, ia hanya menghasilkan foto yang menunjukkan jenis tertentu ketumbuhan. Diagnosis yang tepat ialah biopsi ketumbuhan sampel diperiksa di bawah mikroskop oleh ahli patologi saraf. Oleh kerana itu, penanda genetik yang boleh diharapkan diperlukan untuk mengenalpasti pesakit glioma daripada kaedah invasif yang telah diterapkan.

Langkah pertama untuk mengenal pasti penanda genetik adalah dengan menganalisis ekspresi gen dalam sampel pesakit dan membuat perbandingan dengan sampel yang normal, sama ada ia lebih ekspresi atau kurang ekspresi. Oleh kerana itu, RNA diekstraksi dan diubah menjadi cDNA yang kemudiannya bertindak sebagai acuan dalam PCR memberikan nilai Ct yang digunakan dalam penentuan nilai ΔΔCt. Kaedah perbandingan ΔΔCt menyediakan data ganda perubahan yang berguna untuk menapis gen sama ada lebih ekspresi atau kurang ekspresi dalam sampel pesakit berbanding sampel normal. Namun, analisis ekspresi gen yang dipilih tidak boleh digunakan kerana nilai Ct tidak dapat diterima berdasarkan pengujian lanjutan untuk pengenalan amplikon dalam PCR. Sebaliknya, panduan untuk mengoptimumkan keputusan diperolehi dengan berdasarkan pengalaman dan kesilapan yang dilalui sepanjang eksperimen ini. Panduan ini adalah langkah penting yang perlu diikuti bagi mereka yang berminat dalam eksperimen ekspresi gen di masa depan supaya keputusan yang baik dapat dihasilkan.

CHAPTER 1

INTRODUCTION

Central nervous system is a part of nervous system, consisting of the brain and the spinal cord which functioning to coordinate the activity of all parts of the bodies of multicellular organisms. The tumors of the central nervous system (CNS) are the second commonest form of cancer in children and the sixth commonest form in adult (Ironside *et al.*, 2002). Brain tumor is classified based on abnormal growth of a specific cell type. There are two main types of brain tumors which are those that start in the brain (primary) and those that spread from cancer somewhere else in the body (metastasis) (Kugler, 2008).

A glioma brain tumor is a primary brain tumor that originates from the supportive cells of the brain, called glial cells which are the commonest cellular component of the brain and have the ability to divide and multiply unlike neurons. The abnormal or rapid process of multiplication of three types of glial cells known as astrocytes, oligodendrocytes and ependymal cells will contribute to the formation of different gliomas which are astrocytomas, oligodendroglioma, ependymoma or mixed glioma such as oligoastrocytoma (US National Cancer Institute, 2009). Astrocytomas grow anywhere in the brain or spinal cord; oligodendrogliomas usually grow in the cerebrum (very rare, representing approximately 3% of all primary brain tumors) and ependymomas develop in the brain, in the lining of the ventricles. An advanced astrocytoma known as glioblastoma represent 23% of all primary brain tumors (Kugler, 2008).

Primary brain tumors happen less often but they are mostly malignant when occurred (Kugler, 2008). Tumors growing in the brain are difficult to diagnose (Kugler, 2008) because during a routine examination, the doctors cannot feel or see them since bones of the skull hide brain tumors (Segal, 2005). Therefore, scans are done in place of

conventional x-rays, which are not able to show tumors behind bone. Different types of imaging devices that commonly used to perform brain scans for both diagnosis and follow-up are the Computerized Axial Tomographer (CT or CAT) and the Magnetic Resonance Imager (MRI). Positron Emission Tomography (PET) is also available, but it tends to be used more for research than for routine diagnosis. Unfortunately, scans only produce pictures that suggest a particular type of tumor. Furthermore, only a biopsy sample of tumor examined under a microscope by neuropathologist can provide an exact diagnosis. If this type of examination is not possible due to patient's condition, an educated assumption is made based on available test results (Segal, 2005). Therefore, to identify glioma patients precisely and accurately without invasive method, reliable genetic markers are needed in the management of these problems.

The treatments of brain tumor are still being investigated, including modifying existing treatments as well as developing new ways. One type of treatment is external beam radiation, in which after a tumor is removed, a surgical balloon filled with liquid radiation is put in the cavity left by the tumor and over the next week, it radiates the tissue around it to kill off any remaining cancer cells. Unfortunately, this exposes healthy brain tissue to potentially damaging radiation. Another treatment is surgical removal of the tumor, if possible, followed by chemotherapy. One study also tried antioangiogenic drug such as Thalidomide with patients who had very serious gliomas that did not show any response to radiation and / or chemotherapies. A possible treatment also discovered by researchers which is the use of poliovirus to attack glioma because of its natural attraction to a chemical that is found on malignant gliomas (Kugler, 2008).

All of these treatments are difficult to go through, and pose risks to the patient. Unfortunately, many gliomas grow back even after treatment because of several reasons. Some drugs cannot get into the brain because of a special filtering mechanism in the body

called the blood-brain barrier. Furthermore, some tumors infiltrate the tissues around them with tiny projections. Many tumors have more than one kind of cell in them, so chemotherapy directed at one kind of cell in the tumor will not kill the other cells (Kugler, 2008). Therefore, identification of diagnostic and prognostic markers that identify glioma and it recurrence in patients would be of tremendous benefit to both patients and the healthcare system.

CTCF is a widely expressed 11-zinc finger (ZF) transcription factor with highly versatile functions, localized to the nucleus and is ubiquitous and highly conserved (Chernukhin *et al.*, 2007) (Figure 1.1). CTCF is involved in different aspects of gene regulation including promoter activation (Vostrov & Quitschke, 1997) and repression (Fillippova *et al.*, 1996), hormone-responsive gene silencing (Burcin *et a*l., 1997), methylation-dependent chromatin insulation and genomic imprinting (Fillippova *et al.*, 2002). CTCF is also suggested as a tumor suppressor candidate because its involvement in regulating the expression of some genes that are directly implicated in cancer such as MYC, IGF2, p53, P27, p19/ARF and BRCA1 (Klenova *et al.*, 2002).

YB-1 was first named in 1988 to refer to transcription factors that bind to the Y-box of MHC class II promoters (Huang *et al.*, 2003). The human Y-box protein 1 (YB-1) is a member of the Y-box protein family or the cold-shock domain (CSD) protein super family, a class of protein involved in transcriptional and translational regulation of a wide range of genes (Kloks *et al.*, 2001). YB-1 also represents the most evolutionary conserved nucleic acid binding protein which performs a wide variety of cellular function including transcriptional and translational regulation, DNA repair, drug resistance and stress responses to extracellular signal (Huang *et al.*, 2003). As a result, YB-1 expression is associated with cell proliferation.

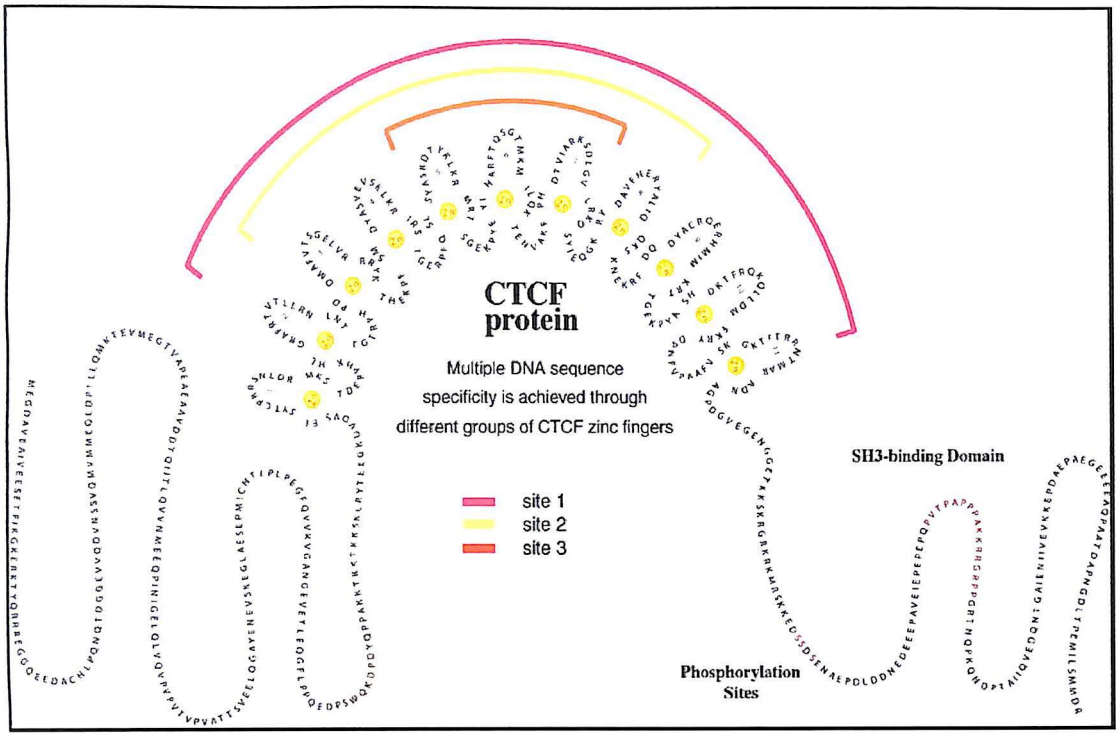

Figure 1.1 Structure of CTCF 11-zinc finger (ZF).

(Klenova *et al.*, 2002)

The general objective of this project is to determine the expression level of CTCF transcription factor in comparison to the normal glioma tissues using SYBR-Green I based Real-Time PCR that could be further develop as a panel of biomarkers which represent the various biological characteristics of the cancer. Specifically, this general research approach will be pursued by fulfilling the following research aims:

(i) To understand the role and correlation between expression level of CTCF gene and YB-1 gene in tumorigenesis.

(ii) To demonstrate SYBR-Green I based Real-Time PCR is a reliable method in enhancing the diagnosis of diseases by the pathologist.

(iii) To position CTCF gene in known signaling pathways.

(iv) To collect information towards providing treatment, prognostic, accuracy and decision making related to glioma cases in a better way than currently available.

CHAPTER 2

LITERATURE REVIEW

2.1 Brain cancer

2.1.1 Epidemiology

Cancer of the brain is a major epidemiological problem that continues to grow each year. In 2008, the American Cancer Society estimates there will be approximately 21,000 new primary brain cancer cases in the United States (America Cancer Statistic, 2008), while Central Brain Tumor Registry of the United States (CBTRUS) data estimated approximately 41,130 new cases of both malignant and benign brain tumors in 2004. Each year about 19,000 people in the United States are diagnosed with primary brain cancers. For every 100,000 people in the United States, the incidence rate of brain cancer for people under age 65 is 4.5 compared to 17.8 for persons 65 and older meanwhile the mortality rate from 1990 to 2002 decreased slightly; from 4.9 deaths to 4.4. The incidence and mortality rates for cancers that originate in the brain and central nervous system have remained relatively unchanged in the last decade (US National Cancer Institute, 2009). The risk of developing brain cancer increases with age and this may due to the improvements in the ability to diagnose brain tumors in elderly patients. The increased use of CT (computed tomography), MRI (magnetic resonance imaging) and stereotactic biopsy procedures (more precise methods for locating and diagnosing tumors) correlates with the increased incidence trends (US National Cancer Institute, 2009).

In Malaysia, based on the report released by National Cancer Registry (National Cancer Registry Report, 2003), 468 of brain cancer cases were reported where number of men affected approximately similar to women. It accounted for 53.2% and 46.8% of all cases of cancer among males and females, respectively reported until 2003. The Malay has the highest risk of developing brain cancer among the ethnic groups. Age specific incidence for brain cancer followed an exponential fashion with a step rise from the age of 40 years but slightly reduced when reaching age of 70 years.

2.1.2 Anatomy, physiology and histology of brain

The human brain weighs about 3 pounds (1.36 kilograms) in an adult is a mass of pinkish-gray tissue, shiny, mushroom-shaped structure encased within the skull (US CBTRUS Statistical Report, 2004). The brain contains up to one trillion nerve cells involving approximately 100 billion nerve cells, called neurons and the remainders are the supporting neuroglia (US CBTRUS Statistical Report, 2004) in addition to blood vessels and secretory organs (National Cancer Registry Report, 2003). The anatomy of the brain is complex due its intricate structure and function. This amazing organ acts as a control center by receiving, interpreting, and directing sensory information throughout the body by three major divisions which are the forebrain, the midbrain, and the hindbrain (Sembulingam & Sembulingam, 2005).

The forebrain is responsible for a variety of functions including receiving and processing sensory information, thinking, perceiving, producing and understanding language, and controlling motor function. There are two major divisions of forebrain: the diencephalon and the telencephalon. The diencephalon contains structures such as the thalamus and hypothalamus which are responsible for such functions as motor control, relaying sensory information, and controlling autonomic functions. The telencephalon contains the largest

part of the brain, the cerebral cortex. Most of the actual information processing in the brain takes place in the cerebral cortex (Sembulingam & Sembulingam, 2005). .

The midbrain and the hindbrain together make up the brainstem. The midbrain is the portion of the brainstem that connects the hindbrain and the forebrain. This region of the brain is involved in auditory and visual responses as well as motor function. The hindbrain extends from the spinal cord and is composed of the metencephalon and myelencephalon. The metencephalon contains structures such as the pons and cerebellum. These regions assist in maintaining balance and equilibrium, movement coordination, and the conduction of sensory information. The myelencephalon is composed of the medulla oblongata which is responsible for controlling such autonomic functions as breathing, heart rate, and digestion (Sembulingam & Sembulingam, 2005). (Figure 2.1)

The brain consists of gray and white matter. Gray matter is the nerve tissue in the CNS composed of neuron cell bodies, neuroglia, and unmyelinated axons; white matter is the nerve tissue in the CNS composed chiefly of bundles of myelinated axons. The brain is protected by the skull and by three membranes called the meninges. The outermost membrane is known as the dura mater, the middle as the arachnoid, and the innermost as the pia mater. Also protecting the brain is cerebrospinal fluid, a liquid that circulates between the arachnoid and pia mater. Many arteries and veins on the surface of the brain penetrate inward. Glucose, oxygen, and certain ions pass easily from the blood into the brain; other substances, such as antibiotics, do not. Scientists believe capillary walls create a blood-brain barrier that protects the brain from a number of biochemical circulating in the blood (Sembulingam & Sembulingam, 2005). (Figure 2.2)

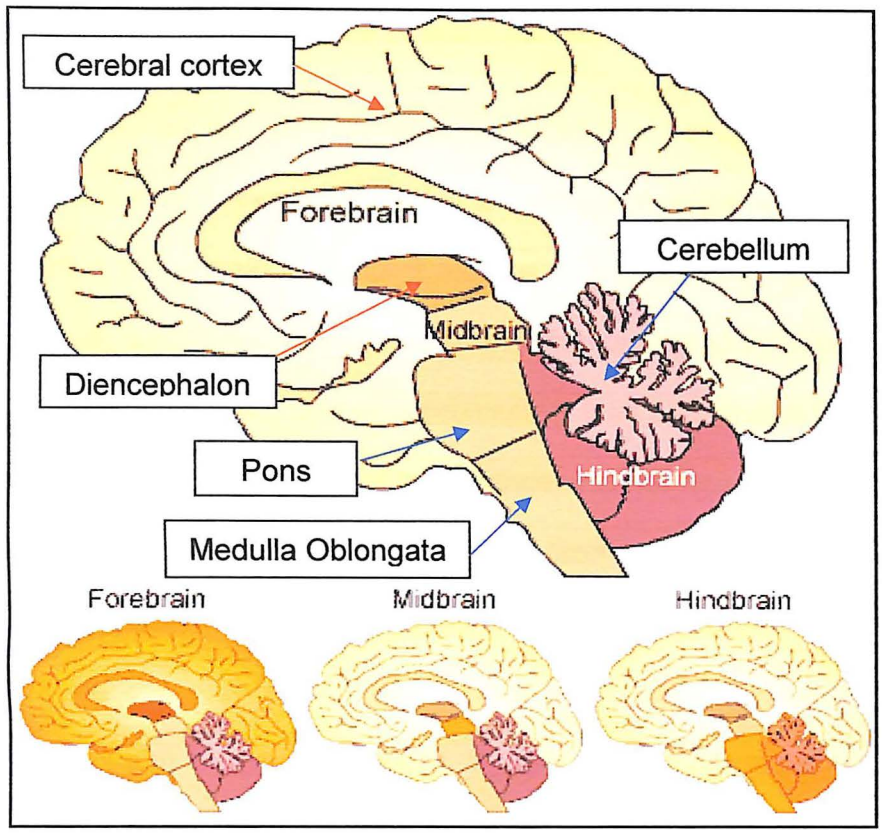

Figure 2.1 Parts of human brain.

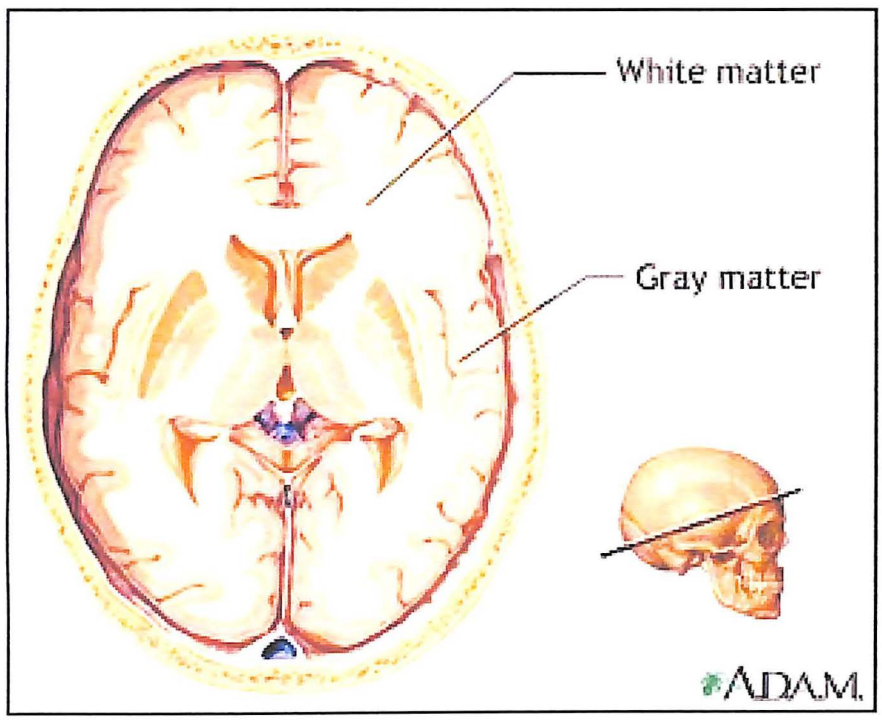

Figure 2.2 White and gray matter of brain.

2.1.3 Signs, symptoms and diagnosis of brain cancer

The symptoms of brain cancer often occur because of the damage to vital tissue and pressure on the brain as the cancer grow within limited space in the skull. Depending on their size and location in the brain, symptoms may be caused by swelling and a buildup of fluid around the tumor, a condition called edema and may be due to hydrocephalus, which occurs when the cancer blocks the flow of cerebrospinal fluid and causes a build-up in the ventricles. The most frequent symptoms of brain cancer include headaches that tend to be worse in the morning and ease during the day, seizures or convulsions, nausea or vomiting, weakness or loss of feeling in the arms or legs, stumbling or lack of coordination in walking, abnormal eye movements or changes in vision, drowsiness, changes in personality or memory and changes in speech. Symptoms may not appear in early stage of brain cancer if the growth is slow or these symptoms may be caused by other problems instead of brain cancer. Consequently, diagnostic tests can be performed to ensure the appearance of the symptoms are caused by the brain cancer and to determine if it is a primary or secondary one (US National Cancer Institute, 2009).

In the diagnosis of brain cancer, personal and family medical history will be asked, complete physical and neurological examination will be performed includes checks for alertness, muscle strength, coordination, reflexes and response to pain and examination to the eyes to look for swelling caused by a tumor pressing on the nerve that connects the eye and the brain. Depending on the results of the physical and neurologic examinations, doctor may request Computerized tomography (CT) or computerized axial tomography (CAT) scan or Magnetic resonance imaging (MR) or both (US National Cancer Institute, 2009). Furthermore, only a biopsy sample of tumor examined under a microscope by neuropathologist can provide an exact diagnosis. If this type of examination is not possible

due to patient's condition, an educated assumption is made based on available test results (Segal, 2005).

2.1.4 Types, staging and grading of brain cancer

In brief, brain tumors encompass neoplasms that originate in the brain itself (primary brain tumors) or involve the brain as a metastatic site (secondary brain tumors). Primary brain tumors include tumors of the brain parenchyma, meninges, cranial nerves, and other intracranial structures (the pituitary and pineal glands). Primary central nervous system lymphoma refers to non-Hodgkin lymphoma confined to the central nervous system (CNS). However, the site of origin of this type of tumor remains unknown. Secondary brain tumors, which originate elsewhere in the body and metastasize to the intracranial compartment, are the most common types of brain tumors. Although some entities are histologically and biologically benign, the nature of the brain often blurs the distinction between benign and malignant. A relatively small, slow-growing, mitotically inactive tumor with little or no metastatic potential may prove lethal if located in a region of the brain that renders it less than totally resectable.

Based on histophatologic classification, primary brain tumors are classified by light microscopy according to their predominant cell type and graded based upon the presence or absence of standard pathologic features. Historical attempts at developing a classification system for brain tumors date back to the 1830s. The German pathologist Rudolf Virchow first introduced the term "glioma" in 1860. Virchow was also the first to attempt a correlation of microscopic to macroscopic features of CNS tumors (Gonzales, 1995). Bailey and Cushing devised the first major classification system for brain tumors in 1926 and proposed that this type of cancer arose from primitive neuroectoderm. Their system consisted of fourteen different tumor types, each arising from a cell arrested at a

different stage of development and morphologically different from its normal counterpart. As examples, astrocytomas were tumors with the appearance of astrocytes, while oligodendrogliomas had the appearance of oligodendrocytes.

A grading system based upon histologic features was not incorporated into this classification. Until 1949, Kernohan and colleagues suggested that different histopathologic appearances do not represent separate tumor types but rather varying degrees of histologic differentiation (Tooth, 1912; Kernohan *et al.*, 1949). The Ringertz system in 1950 was based upon the notion that different brain cells give rise to different histologic types of brain tumors. In addition, Ringertz proposed that astrocytoma consisted of three grades: astrocytoma, astrocytoma with anaplastic foci, and glioblastoma. A tumor grading system (The St. Anne-Mayo system) published in 1981 was based upon the absence or presence of four criteria: nuclear atypia, mitoses, endothelial cell proliferation, and necrosis (Daumas-Duport & Szikla, 1981).

2.1.5 Treatment of brain cancer

Treatment for brain tumors is different for children and adults depending on a number of factors including the type, location and size of the tumor as well as general health. Briefly, brain tumors are treated with surgery, radiation therapy and chemotherapy involving the participation of neurosurgeons, medical oncologists, radiation oncologists, nurses, a dietitian and a social worker, who work together to provide the best possible care. The application of vaccine and injection of poliovirus for treating recurrent cancer of the central nervous system that occurs primarily in the brain, known as glioma is still investigated (US National Cancer Institute, 2009).

Surgery is the usual treatment for most brain tumors. An operation called craniotomy required a neurosurgeon makes an opening in the skull to remove a brain tumor. Whenever possible, the surgeon attempts to remove the entire tumor and if the tumor cannot be completely removed without damaging vital brain tissue, the doctor may remove as much of the tumor as possible. Partial removal helps to relieve symptoms by reducing pressure on the brain and reduces the amount of tumor to be treated by radiation therapy or chemotherapy. In some cases, which some tumors cannot be removed, the doctor may do only a biopsy where a small piece of the tumor is removed so that a pathologist can examine it under a microscope to determine the type of cells it contains. Consequently, this will helps the doctor to decide which the best treatment to apply on the patients (US National Cancer Institute, 2009).

Radiation therapy, also called radiotherapy, is the use of high-powered rays to damage tumor tissue that cannot be removed with surgery or to kill cancer cells that may remain after surgery or used when surgery is not possible. Radiation therapy may be given in two ways which is external radiation comes from a large machine or implant radiation therapy comes from radioactive material. Generally, external radiation treatments are given five days a week for several weeks depending on the type, size of the tumor and age. External radiation may be directed just to the tumor, the surrounding tissue or the entire brain from a large machine. In contrast, depending on the material used, the implant may be left in the brain for a short time or permanently. Implants lose a little radioactivity each day to kill the cancer cells. Therefore, the patient has to stay in the hospital for several days while the radiation is most active (US National Cancer Institute, 2009).

Chemotherapy is the use of one or combination of drugs to kill cancer cells usually administrated orally or by injection into a blood vessel or muscle. Intrathecal chemotherapy involves injecting the drugs into the cerebrospinal fluid. Chemotherapy is usually given in cycles where a treatment period is followed by a recovery period, then another treatment period and so on. Patients often not required staying in the hospital for treatment and most drugs can be given in the doctor's office or clinic. However, depending on the drugs used, the way they are given and the patient's general health, a short hospital stay may be necessary (US National Cancer Institute, 2009).

2.2 Real-Time Polymerase Chain Reaction (Real-Time PCR)

Polymerase Chain Reaction (PCR) is a molecular biology technique that provides extremely sensitive means of amplifying small amounts of nucleic acids fragment or genomic DNA, cDNA or RNA (Erlich *et al.,* 1991). Reverse transcriptase PCR (RT-PCR) is a technique to produce cDNA from ribonucleic acid (RNA) which can be easily adapted to measure the strength of gene expression when the amounts of available mRNA are limited or when the RNA of interest is expressed at very low levels (Sambrook & Russel, 2001). Among the modified RT-PCR techniques developed for quantification of target genes in the field of gene expression study is the Real-Time PCR (Bustin, 2000; Landeweert *et al.*, 2003; Mocellin *et al.*, 2003). Real-Time PCR permits a quick and sensitive quantification of DNA as well as transcriptional genes with minimal handling of the samples (Mocellin *et al.*, 2003). The concept of Real-Time PCR is the detection of PCR products as they accumulate during PCR thermal cycles rather than after the end of the reaction, using fluorescence detection systems (Higuchi *et al.*, 1992). The simplest and cheapest principle is based on the intercalation of double stranded DNA (dsDNA) using SYBR Green dyes (Higuchi *et al.*, 1992; Klein, 2002). However, specific and non-specific PCR products are detected by this system. Thus, the undesired side of reaction such as mispriming and

primer dimerisation will significantly affect the sensitivity of the Real-Time PCR detection (Bustin, 2002; Mocellin *et al.*, 2003).

The discovery of the Real-Time PCR technique as it is used today was made possible by two important findings. First, the *Taq* polymerase has, apart from its polymerase activity, a 5'-3' exonuclease activity (Holland *et al.*, 1991). Second, dual-labeled fluorogenic oligonucloetide probes have been created to emit a fluorescent signal only upon cleavage, based on the principle of fluorescence resonance energy transfer (FRET) (Cardullo *et al.*, 1988). There are major advantages of using Real-Time PCR compared to conventional semi-quantitative PCR. Firstly, the PCR progression reaction can be monitored after each cycle rather than at the end, thereby providing a much better quantification assay. Secondly, less DNA is required and thirdly, it is a non-radioactive assay and can be performed in approximately two hours.

Tremendous number of Real-Time PCR applications for gene expression studies have been reported in recent years proving that Real-Time PCR have become the method of choice for many kinds of research. Real-Time has been applied for *in vitro* transcription monitoring (Liu & Saint, 2002), direct detection of the effect of receptor signaling (Yuen *et al.*, 2002), gene expression studies in cancer (Bustin *et al.*, 2002; Bijwaard *et al.,* 2001, Mocellin *et al.*, 2003). In immunological aspect, Real-Time has been used to access T cell response *in vivo* (Stordeur *et al.,* 2002) and to quantify absolute gene expression levels of cytokines (Overbergh *et al.*, 2003; Whelan *et al.*, 2003).

The importance of Real-Time PCR for cancer diagnostics has been reviewed by Bernard and Wittwer (2002) since current classification in surgical pathology for staging malignancies is primarily based on anatomic features (tumor-node-metastases) and histopathology (grading). The identification of differentially expressed genes by Real-Time PCR technique can eventually generate markers which can be used for future screening,

early diagnosis, staging and surveillance of brain cancer as well as accelerate the discovery of key biological process for therapeutic targeting and contribute towards providing better treatment, prognostic accuracy and improved decision making.

CHAPTER 3

MATERIALS AND METHODS

3.1 Clinical specimens

We used panel of 3 fresh samples tumor of glioma cases whereas for normal samples we used human brain cultured cell lines. The total RNA from tumor and normal sample was extracted using Tri Reagent and column spin technology, respectively.

3.2 Preparation of reagents and buffer

0.1% Diethylpyrocarbonate (DEPC) treated distilled water

One liter deionised water was added into 1 ml of DEPC solution (Amresco, USA). The mixture was stirred by using magnetic stirrer for 12 hrs to bring DEPC into solution. Afterward, the solution was autoclaved for 15 mins to remove any trace DEPC. The function of this solution is to be used in preparation of buffer and in the protocol of RNA extraction.

TAE buffer

For preparation of stock 50X, 1000 ml Tae buffer, 242 g Tris base was dissolving in 750 ml DEPC-treated water and added with 57.1 ml of glacial acid and 100 ml of 0.5 M Ethylene diamine terta acetate (EDTA) , pH 0.8. The solution was then adjusted to final volume of 1 L and pH of 8.5. The stock buffer was stored at room temperature. For preparation of 1X, 500 ml Tae buffer, DEPC-treated water was added into 10 ml of stock 50X Tae buffer up until final volume of 500 ml.

20 % Sodium Dodecly-sulfate (SDS)

This solution was prepared by dissolving 20 g of SDS (Amresco, USA) within 100 ml of DEPC-treated water and the mixture was heated at 68^0C with a magnetic stirrer. A few drop of hydrogen chloride (HCl) (Fisher Scientific, USA) was added to adjust pH to 7.2. The solution then adjusted to final volume of 1 L. Sterilization is not necessary. This stock solution was stored at room temperature and diluted into desired concentrations before use.

70% ethanol

This solution was prepared by adding 70 ml of absolute ethanol in 30 ml of DEPC-treated water. The function of this solution is to reduce the contamination during RNA extraction.

3.3 Preparation of glass wares, plastic ware and electrophoresis tanks

All glass wares were treated with 0.1% DEPC-treated water for overnight at room temperature and were autoclaved for 15 mins to remove any residual DEPC. However, the dust and hands may become the source of RNase contamination to the glass wares. Therefore, RNase remover was strongly recommended used before doing any RNA works. Electrophoresis tank was cleaned thoroughly with the detergent solution and DEPC-treated water. Then, the tank was rinsed with 70% ethanol in DEPC-treated water and allowed to dry.

3.4 RNA extraction

Quality of RNA extracted influence the reverse transcription of cDNA, which is the critical step in gene expression analysis. The successful production of cDNA relies on the non-degraded, pure and high integrity of RNA extracted. Therefore, to obtain those characteristic of RNA, the area surface of RNA extraction was first sprayed with RNase Zap to eliminate traces of RNases. For the normal sample, total RNA was extracted from human brain cultured cell lines by using column spin technology whereas TRI Reagent was used for the extraction of total RNA from the patient samples.

3.4.1 Extraction of tumor sample

Briefly, approximately 50 mg of fresh tissue samples were homogenized in 1 ml Tri Reagent using a pestle and the homogenate were incubated for 5-10 mins at room temperature (18-25 ^{0}C) to permit complete dissociation of nucleoprotein complexes. Subsequently, 200 µl of chloroform were added into the sample, vigorously shook and stored for 2 – 15 mins in room temperature to remove excess phenol of Tri Reagent. After incubation, the samples were centrifuged at 12, 000 rpm for 15 mins at 4^{0}C. Following centrifugation, the mixture was separated into a lower red, phenol-chloroform phase, interphase, and the colorless upper aqueous phase. RNA remained in the colorless aqueous phase whereas DNA and protein were in the interphase and organic phase, respectively.

Approximately 400 µl of the aqueous phase containing RNA was transferred into new 1.5 ml appendorf tube. The RNA was precipitated out from aqueous phase by mixing it with 500 µl isopropanol. The sample was then incubated at room temperature for 5-10 mins and pellet was obtained via centrifugation at 12,000 rpm for 8 min at 4^{0}C. The RNA pellet was washed with 1 ml of 75% ethanol and stored for 5-10 mins. Subsequently, the pellet

was centrifuged at 12, 000 rpm for 5 mins at 4^0C. At the end of the procedure, ethanol was removed and the RNA pellet was allowed to air-dry for about 15 mins. When the pellet completely dry, the RNA pellet was dissolved within 20 µl of free water and incubated at 55-60 ^{0}C for 10 mins. The integrity and purity of the resultant total RNA was assessed by gel electrophoresis and its yield determined by spectrophotometry (NanoQuant).

3.4.2 Extraction of normal sample

Approximately 1 X 10^7 of the human brain cultured cells grown in suspension was harvested in which the number was determined using hemacytometer. The appropriate number of cells was collected as pellet by centrifuging for 5 mins at 300 x g in a centrifuge and all supernatant was carefully removed by aspiration. The cell pellet was thoroughly loosened by flicking the tube and 350 µl of buffer RLT was added followed by vortexed to disrupt the cells. The lysate was passed at least 5 times through a blunt 20-gauge needle (0.9 mm diameter) fitted to a RNase-free syringe. Subsequently, 1 volume of 70% ethanol was added and mixed to homogenize lysate.

700 µl of sample, including any precipitation that might have formed was transferred to an RNeasy spin column placed in a 2 ml collection tube and subsequently centrifuged for 15 secs at ≥ 8000 x g (≥10, 000rpm). The flow-through was then discarded. The sample was added with 700 µl Buffer RW1 to the RNeasy spin column and subsequently centrifuged at same time duration and speed to wash the spin column membrane. The flow-through was then discarded. The sample was added with 500 µl Buffer RPE to the RNeasy spin column and subsequently centrifuged at same time duration and speed to wash the spin column membrane. The flow-through was then discarded. After that, an equal volume of Buffer RPE was added and centrifuged for 2 mins with the same speed as before. The RNeasy spin column was placed in a new 2 ml collection tube and the old collection tube with the flow-through was discarded. Subsequently, the column was centrifuged at full speed for 1

min. The RNeasy spin column was placed in a new 1.5 ml collection tube and 30 µl of RNase-free water was directly added to the spin column membrane followed by centrifugation for 1 min at ≥ 8000 x g (≥10, 000rpm) to elute RNA. The spin column membrane was discarded and the integrity and purity of the resultant total RNA in 1.5 ml collection tube was assessed by gel electrophoresis and its yield determined by spectrophotometry (NanoQuant).

3.4.3 Determination of total RNA purity and concentration

The purity and concentration of the RNA was determined by measuring the ratio of 260 nm (A_{260}) and 280 nm (A_{280}) using a spectrophotometer. The ratio of the reading at 260 nm and 280 nm (A_{260}/A_{280}) provides an estimation of the purity of RNA with respect to the contaminants that absorb in the UV such as protein. The range of the ratio of 260 nm (A_{260}) and 280 nm (A_{280}) indicating the purity of RNA obtained without contaminants is 1.8-2.0.

3.4.4 Determination of total RNA integrity

The integrity and size distribution of total RNA was determined by using 1.0% agarose gel. In brief, 1.0% agarose gel was prepared by adding 0.5 g of agarose to 50 ml of 1 x Tae buffer in 100 ml conical flask and placed in microwave oven to melt the agarose. Ocassionally, during the heating, the bottle was taken out to mix the solution several times. Heating was continued until all agarose was totally dissolved. The bottle was allowed to cool approximately at 50°C-60°C under running tap water with continuous gentle mixing. Subsequently, 1 µl of 10 mg/ml EtBr solution was added into agarose and mixed gently. The agarose was then poured into a clean and dry gel platform placed in a horizontal electrophoresis set. A suitable comb was inserted into its slot in the tray. The gel was allowed to set for approximately 30 min at room temperature before samples can be

loaded. Subsequently, 1 µL of each of the diluted RNA sample was mixed with 1 µL of RNA loading buffer. Electrophoresis was carried out in 1 x Tae buffer at a constant voltage of 80 V for 45 mins. After electrophoresis, the RNA bands were visualized under UV transluminator and photographed.

3.5 Reverse Transcription of cDNA synthesis

The samples proceeding to the conversion of RNA to cDNA by reverse transcription process. The materials involve are buffer reverse transcriptase (RT), dNTP mix, oligo-dT primer, RNase inhibitor, omniscript reverse transcriptase, RNase free water and template which were added into new tube respectively. The mixing of the materials was done in the ice box and then, the tube was incubated for 1 hr at room temperature. After that, the purity and concentration of cDNA was determined by the same method in RNA purity and integrity determination. Qiagen cDNA synthesis kit was used and the protocol was as described by the manufacturer. The preparation of the Real-Time PCR reaction was performed as described by the manufacturer, as shown in Table 3.1. Master Mix 1 and 2 were mixed together and were incubated at 37°C for 1hr and subsequently kept at -20°C for Real-Time PCR.

3.6 Real-Time PCR

Real-Time PCR was performed using the Applied Biosystems 7500 along with the Quantifast SYBR-Green PCR Kit which has been developed for use in a two-step cycling protocol, with a denaturation step at 95^0C and a combined annealing/extension step at 60^0C. Final reactions volumes of 25 µl contained 12.5 µl of Quantifast SYBR-Green PCR Master Mix, 0.3 µM forward primer, 0.3 µM reverse primer, and 20 ng/µl of cDNA template and RNase-free water.

Primers were based on CTCF and YB-1 gene sequence. Below are the sequences used in the experiment:

Forward CTCF primer (5′-AATACCATGGCAAGACATGC-3′)

Reverse CTCF primer (5′-CACTGTCAGAGGAATCTTCT-3′)

Forward YB-1 primer (5′- CTCGCCAAAGACAGCCTAGA -3′)

Reverse YB-1 primer (5′- GTCTGCGTCGGTAATTGAAGTT -3′)

Primers were designed to exactly match the expected cDNA sequences derived from the target mRNAs. Forward and reverse primer of CTCF was derived from full sequence of mRNA of CTCF gene (Appendix 1) with accession no: NM_006565 meanwhile forward and reverse primer of YB-1 was derived from full sequence mRNA of YB-1 gene (Appendix 2) with accession no: NM_004559.

Reagent	Volume (µL)
Master mix 1	
RT plus buffer	2.0
dNTP mix	2.0
Oligo dT	2.0
RNase Inhibitor	1.0
Reverse Transcriptase enzyme	1.0
Master mix 2	
RNase-free water	variable
Total RNA (sample)	variable
Total vol. requirement	**20.0**

Table 3.1 Preparation of master mix for cDNA synthesis.