ESTABLISHMENT OF GLIOMA CELL LINE FROM FRESHLY CLINICAL SAMPLE OF HUSM

by

NURHIDAYAH BINTI AB. RAHIM

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

December 2008

CERTIFICATE

This is to certify that the dissertation entitled "**Establishment of Glioma Cell Line from Freshly Clinical Sample of HUSM**" is the bonafide record of research work done by Miss Nurhidayah bt. Ab. Rahim during the period from July 2008 to October 2008 under my supervision.

Supervisor,

.....

Dr. Shaharum Shamsuddin
Lecturer,
School of Health Sciences,
Universiti Sains Malaysia,
Health Campus,
16150 Kubang Kerian,
Kelantan, Malaysia.

Date: 23/12/08

ACKNOWLEDGEMENTS

Alhamdulillah, all praises are only to Allah and may He shower His blessings and peace upon our beloved prophet, Muhammad s.a.w and on his family and companions. With the patient and earnestness, I can make serious efforts to do this research and have a great strength to complete it.

I also would like to express my sincere appreciation and gratitude to the following people for their help in fulfills this research.

First and foremost, to my parents, Ab. Rahim bin Ab. Wahid and Alimah binti Haji Omar that always understand my career as student and always give a support along with encouragement in all aspects of my field.

Dr. Shaharum bin Shamsuddin (now at Nanobiotix SA, Paris) my supervisor and Dr. Nik Norliza binti Nik Hassan, my Co-Supervisor that always help me to solve the problem that arise during this study. Special thank you for their guidance, encouragement and cooperation especially in the critical moments to complete this research.

Miss Siti Zawani binti Mohd. Ramli, research assistant who has been teaching me every single skills in doing the culture work as well as confirmatory technique and always giving me support and advise until I manage to complete this research successfully.

Also thanks to all the staffs and students of the Advance Molecular Biology Laboratory, PPSK who always care about virtue of my colleagues and me throughout our final year projects.

To all the surgeons and nurses at the neuroscience department, for their hardwork, permission and supplying me with a good quality glioma samples from brain surgical operation. Not to forget, to all the staff of pathology department that gives us great cooperation in evaluating the grade of our glioma samples.

To our kind sponsor, School of Health Science, USM who gave us the financial support especially the proper environment to perform this research and vital force to start this research until we were able to complete it successfully. Last but not least, to all my beloved friends for their support and help all this while.

Special thanks to all of you.

OBJECTIVES OF STUDY

The objective of this study is to establish the glioma cell line from the clinical sample that obtained from HUSM Operation Theater. The establishment of glioma cell line can be confirmed by using immunocytochemistry technique as well as western blot method. In addition, this study are carried out in order to evaluate whether or not cells in a particular sample express antigen in question and also to determine which subcellular compartment are expressing the antigen.

The establishments of glioma cell line were contributed for molecular and cellular study as well as to improve the treatment and therapy of glioma.

Table of Contents

LIST OF FIGURES AND TABLES

LIST OF SYMBOL AND ABBREVIATIONS

%	percentage
°C	degree Celcius
µg	microgram
µg/mL	microgram per miliLiter
µl	microliter
10X	10 times
1x	one time
4^{th}	Fourth
50^{th}	Fifty
6^{th}	sixth
AA	Anaplastic astrocytoma
Ab	Antibody
APS	Ammonium Persulfate
ASR	Age-Standardized Incidence
ATCC	American Type Culture Collection
BORIS	Brother of the Regulator of Imprinted Sites
BSC	Biosafety cabinet
CAPS	3-[Cyclohexylamino]-1-propanesulfonic acid
CBTRUS	Central Brain Tumor Registry of the United State
CI	Confident Interval
cm	Centimeter
Cm^2	Centimeter per square
CNS	Central nervous system

CO_2	Carbon dioxide
CR	Cancer incidence per 100 000 population
CTA	Cancer Testis Antigen
CTCF	CCCTC-binding factor
DAB	3,3' diaminobenzidine
DBTRG	Denver Brain Tumor Research Group
ddH_2O	Double distilled water
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPX	Distyrene plasticizer xylene
ECL	Enhanced Chemiluminescence Substrate
EDTA	Ethylene Diamine Tetra Acetic Acid
EGFR	epidermal growth factor receptor
FBS	Fetal Bovine Serum
FITC	Fluorescein isothiocyanate
FtsZ	protein encoded by the *ftsZ* gene
g/L	gram per Liter
GBM	Glioblastoma multiforme
GFAP	Glial Fibrillary Acidic Protein
HCl	Hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horseradish Peroxidase
HUSM	Hospital of University Science Malaysia
ICC	Immunocytochemistry

IgG	Immunoglobulin G
IHC	Immunohistochemistry
Inc.	Incorporated
INFORMM	Institute for Molecular Medicine Research
KCl	Potassium chloride
kD	Kilodalton
L	Liter
M	Molar
mg	milligram
mg/L	milligram per Liter
min	minute
mL	miliLiter
mM	miliMolar
mm	millimeter
MW	Molecular weight
Na	Sodium
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
NaOH	Sodium hydroxide
No.	Number
NP-40	Tergitol-type NP-40 (nonyl phenoxylpolyethoxylethanol)
OT	Operation theatre
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
Pen-strep	Penicillin streptomycin

PMN	Polymorphonuclear
PMSF	phenylmethanesulphonylfluoride or phenylmethylsulphonyl fluoride
PNET	Primitive neuroectodermal tumor
Psi	pound per square inch
PVDF	Polyvinylidene Fluoride
RBC	Red Blood Cell
RIPA	Radioimmunoassay Buffer
rpm	Revolutions per minute
RPMI 1640	Roswell Park Memorial Institute 1640
RT	Room Temperature
SDS PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SDS	Sodium Dodecyl Sulphate
ß-Tubulin	Beta tubulin
SVGp12	human brain astroglia
TBCE	Tubulin Folding cofactor E
TBS	Tris-buffered saline
TEMED	Tetramethylethylenediamine
Tris-Cl	Tris-Chloride
US	United State
V	Volt
v/v	volume per volume
WHO	World Health Organization

ABSTRACT

A human malignant continuous cell line, named as GBM IV was established from a patient diagnosed with recurrents glioblastoma multiforme which is grade IV glioma. This primary cell line has been serially subcultured until 6^{th} passage in the standard culture media that presenting the existence of various marker. The aim of this study is to assess the morphology of cultured glioblastoma multiforme cells as well as to apply the confirmatory technique such as immunocytochemistry and western blotting method in order to confirm the biological and immunological characteristic of gliomas. Immunocytochemistry finding indicate this cell line retain the expression of Glial Fibrillary Acid Protein (GFAP) despite some previous study show no GFAP are exist in established glioma cell line. However, our study suggests, to prove the existence of GFAP in glioma cell line; we need to culture and subculture until 50^{th} passage in where only the specific protein marker will be presented. Another technique used to confirm the establishment of glioma cell line is western blotting method which serves as total protein study in explaining the biology of tumor as well as to identify the protein that are very potential to cause human disease.

We believe that the information regarding the establishment of GBM IV cell line will give benefits to all research community especially who have some interest to cellular and molecular study of cancer with the main purpose to improve glioma's treatment.

ABSTRAK

Sel kultur selanjar bagi kanser manusia yang berbahaya dinamakan sebagai GBM IV telah ditubuhkan daripada pesakit yang didiagnosiskan sebagai glioblastoma multiforme berulangan, gred IV. Sel kultur primer ini telah dikultur dan disubkulturkan sehingga fasa yang ke-6 di dalam media kultur piawai di mana sel kultur primer ini mempersembahkan kehadiran pelbagai penanda protein. Tujuan penyelidikan ini dilakukan adalah untuk mengkaji morfologi sel glioblastoma multiforme yang telah dikulturkan dan juga untuk mengaplikasikan teknik-teknik pengesahan seperti immunositokimia dan "western blotting" dengan tujuan untuk mengesahkan ciri-ciri biologi dan immunologi bagi glioma. Penemuan immunositokimia menunjukkan bahawa sel kultur ini menahan ekspresi protein asid fibrial glial (GFAP) walaupun sesetengah penyelidikan sebelum ini menunjukkan tiada kewujudan GFAP dalam sel kultur glioma yang dibangunkan. Walaubagaimanapun, penyelidikan kami mencadangkan, untuk membuktikan kewujudan GFAP di dalam sel kultur glioma, kami perlu melakukan pengkulturan dan pengsubkulturan sehingga fasa yang ke-50 di mana hanya spesifik penanda protein akan hadir. Kaedah lain yang digunakan untuk mengesahkan penubuhan sel kultur glioma adalah teknik "western blotting" yang berperanan sebagai penyelidikan keseluruhan protein di dalam langkah menerangkan biologi tumor dan juga mengenalpasti protein yang sangat berpotensi untuk menyebabkan penyakit di kalangan manusia.

Kami percaya bahawa maklumat berkenaan sel kultur primer ini akan memberi kebaikan kepada semua komuniti penyelidikan terutamanya kepada mereka yang berminat di dalam penyelidikan molekular dan selullar kanser dengan tujuan utama untuk meningkatkan rawatan bagi glioma.

1.0 INTRODUCTION AND LITERATURE REVIEW

The pathogenesis of tumor growth and invasion depends both on host factors and on properties of the tumor cells. These entities are involved during tumor progression. However, it is difficult to know what contribution the tumor cells or the host tissue have in the invasive process (Bjerkvig *et al.*, 1989). To understand these phenomena further, there is a need of a fundamental research on how gene regulation can contribute to the development of tumorigenesis. Establishment of a pure primary cell lines is indeed an important factor to start the whole experiment rolling.

1.1. Classification, grading and staging of Brain tumor

Brain tumor encompasses neoplasms that originate in the brain itself (primary brain tumors) or involve the brain as a metastatic site (secondary brain tumors). The primary brain tumors include tumors of the brain parenchyma, meninges, cranial nerves and other intracranial structures (the pituitary and pineal glands). Secondary brain tumors, which originate elsewhere in the body and metastasize to the intracranial compartment, are the most common types of brain tumors (Schiff and Batchelor, 2006).

Tumor classification is done by the pathologist microscopic examination. In concepts, brain tumor arises from different normal cells of the brain and spinal cord and the resulting neoplasms are designated accordingly (Burger and Cohen, 2004). Gliomas are primary Central Nervous System (CNS) tumors that are derived from glial cells, namely astrocytes, oligodendrocytes and ependymal cells (Moore and Psarros, 2005). Gliomas originate from glial cells, most often astrocytes. Sometimes the terms "astrocytoma" and

"glioma" are used interchangeably (St. Jude Children's Research Hospital, 2008). For glial tumors, tumor classification is achieved initially by assigning the lesion to one of these groups. The lesion is then placed among subsets of these groups. Thus, astrocytomas will be further subdivided since the term "astrocytoma" is non-specific as it includes different lesions of varying degrees of biological aggressiveness and potential cure. A common type of astrocytoma in children is "pilocytic astrocytoma," known for its slow rate of growth and, in many cases, surgical cure. Other forms of astrocytomas, e.g. the "fibrillary" types, infiltrate surrounding tissues and are more difficult to contain. A common additional class of brain tumors in children is the "embryonal" or "PNET" groups. The cerebellar medulloblastoma is the most common example (Burger and Cohen, 2004).

Astrocytomas are tumors that arise from brain cells called astrocytes. Astrocytomas are of two main types- high grade and low grade. High-grade tumors grow rapidly and can easily spread through the brain. Low-grade astrocytomas are usually localized and grow slowly over a long period of time. High-grade tumors are much more aggressive and require very intensive therapy. The majority of astrocytic tumors in children are low-grade, whereas the majority in adults is high-grade. These tumors can occur anywhere in the brain and spinal cord (St. Jude Children's Research Hospital, 2008).

According to the World Health Organization (WHO), astrocytomas can be graded depending on their cellular characteristics; for example, Grade I (usually juvenile pilocytic type), Grade II (diffuse fibrillary, gemistocytic types), Grade III (anaplastic type), or Grade IV (Glioblastoma Multiforme) (Brain Tumor Society, 2004).

This tumor also can be subclassified clinicopathologically into low grade (astrocytomas, grade I and II), intermediate grade (anaplastic astrocytomas, grade III) gliomas and the most malignant form (glioblastoma, grade IV) and tumor progression from lower to higher grades is not uncommon (Onda *et al.*, 1999).

In general, astrocytomas are graded from I to IV depending upon the presence of pleomorphism, mitotic activity, endothelial proliferation and necrosis. Low grade astrocytoma often exhibit only one of these factors, whereas grade IV lesions (glioblastoma multiforme) usually exhibit all four. Cytogenetic abnormalities are also more prevalent in high grade astrocytomas than in low-grade astrocytomas, and this reflects nonrandom chromosomal changes that influence the biologic aggressiveness of the tumor. Common cytogenetic abnormalities of low grade astrocytoma include losses of chromosome 17p, 13q (Rb gene) and 22. Anaplastic astrocytoma (AA) is a grade III lesion that exhibits increased mitosis with markedly increased cellularity. AA is more common in males than females, is usually located in the frontal or temporal lobes and typically presents in the fifth decade of life (Moore and Psarros, 2005).

Malignant astrocytoma represents one of the most devastating tumors affecting children and the adults. Malignant astrocytomas usually composed of pleomorphic, hyperproliferative infiltrative astrocytes, with areas of necrosis, increased tumor angiogenesis and regions of blood brain barrier breakdown. These heterogeneous pathological characteristic pose major obstacles to the effective management of gliomas.

1.2 Incidence of brain tumor in world

Based on the World Health Organization (WHO) estimates that the incidence of primary malignant brain tumor is 3.7 per 100 000 per annum for males and 2.6 per 100 000 per annum for females. This represent an estimated 108 277 males and 81 305 females who were diagnosed with a primary malignant brain tumor in 2002, an overall total of 189 582 individuals. Central Brain Tumor Registry of the United State (CBTRUS) estimates that the incidence of malignant brain tumors may climb to 220 568 in 2010 (125 892 males and 94 676 females).

The estimated 2010 counts are based on anticipated changes in world population demographics and do not accounts for any changes in brain tumor incidence. Non malignant brain tumor are often as devastating as malignant brain tumors but were routinely collected by cancer surveillance organization in 2002. The CBTRUS has provided a preliminary estimate of 157 833 non malignant brain tumor per annum for 2002 (International Brain Tumor Alliance, 2007).

Table 1.1: Estimated Number of New Cases of Brain and Central Nervous System Tumor Diagnose World-Wide: Primary Malignant (GLOBOCAN 2002) and Non-Malignant (CBTRUS- Preliminary Estimate) (Source from International Brain Tumor Alliance, 2007).

	Count		
	Overall	Male	Female
Malignant	189 582	108 277	81 305
Non-malignant	157 833	58 851	98 982

1.3 Incidence of brain tumor in Malaysia

Table 1.2: Brain and Other Nervous System Cancer Incidence per 100 000 populations (CR) and Age-standardized incidence (ASR), by sex, Peninsular Malaysia 2003. (Source from Chye and Halimah, 2003).

Sex	No.	%	CR	ACR
Male	249	53.2	2.6	2.8
Female	219	46.8	2.3	2.5
Both	468	100	2.4	2.6

Table 1.3: Brain and Other Nervous System Age specific Cancer Incidence per 100 000 populations (CR), by sex, Peninsular Malaysia 2003. (Source from Chye and Halimah, 2003)

	Male			Female		
Age, year	No.	%	CR	No.	%	CR
0-9	33	13.3	1.5	32	14.6	1.5
10-19	38	15.3	1.9	31	14.2	1.6
20-29	34	13.7	2.2	26	11.9	1.7
30-39	43	17.3	3.2	36	16.4	2.7
40-49	33	13.3	2.9	26	11.9	2.3
50-59	26	10.4	3.5	36	16.4	5.0
60-69	29	11.6	7.3	22	10.0	5.3
70+	13	5.5	6	10	4.6	3.7

Figure 1.1: Brain and Other Nervous System Age specific Cancer Incidence per 100 000 populations (CR), by sex, Peninsular Malaysia 2003 (Source from Chye and Halimah, 2003).

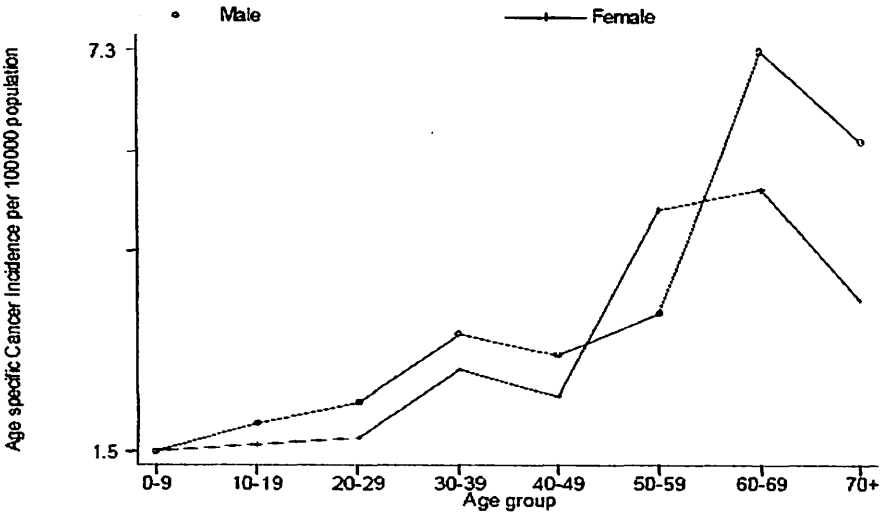


Table 1.4: Brain and Other Nervous System Cancer Incidence per 100,000 population (CR) and Age-standardized incidence (ASR), by ethnicity and sex, Peninsular Malaysia 2003. (Source from Chye and Halimah, 2003).

	Male				Female			
Ethic group	No.	%	CR	ASR	No.	%	CR	ASR
Malay	136	58.9	2.3	2.6	118	57.8	2.0	2.2
Chinese	79	34.2	3.0	3.0	71	34.8	2.8	2.8
Indian	16	6.9	1.8	2.3	15	7.4	1.7	1.8

Table 1.5: Brain and Other Nervous System Age specific Cancer Incidence per 100,000 population (CR), by ethnicity and sex, Peninsular Malaysia 2003. (Source from Chye and Halimah, 2003).

		Age groups, year								
		0-9	10-19	20-29	30-39	40-49	50-59	60-69	70+	CumR
Male	Malay	1.6	1.3	2.5	2.5	2.7	3.6	7.4	4.3	0.3
	Chinese	1.3	2.6	1.9	3.5	3.2	3.7	7	7.4	0.3
	Indian	0	0.6	0	2.9	3.4	2.9	9.5	12.1	0.3
Female	Malay	1.2	1.2	1.9	2.8	1.7	5.7	4.6	1.4	0.2
	Chinese	2.5	2.1	1.5	2	2.5	4.4	7.8	4.7	0.3
	Indian	0	1.2	0	2.9	3.3	2.9	0	15.3	0.2

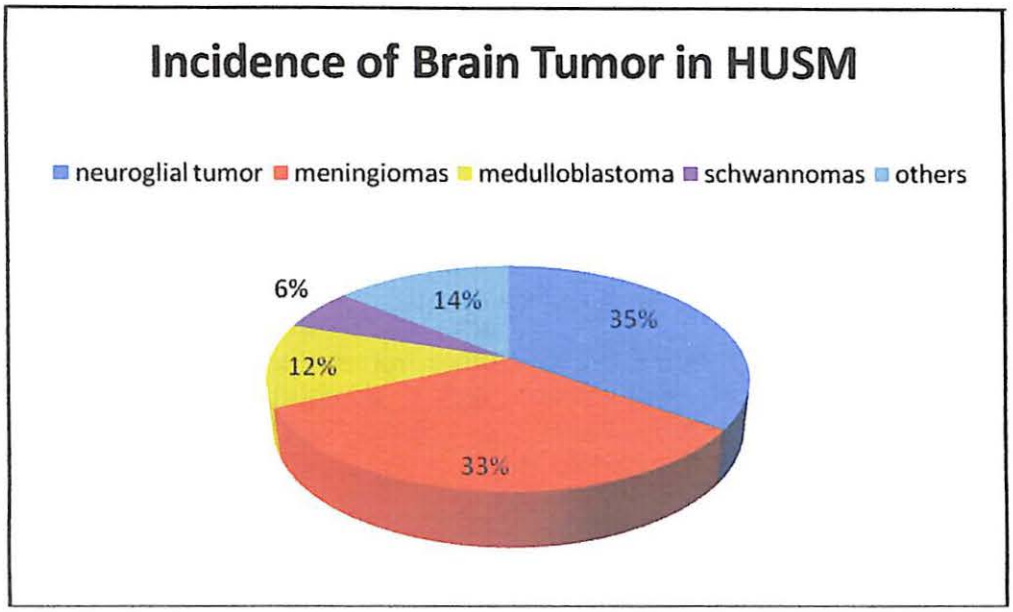
A total of 21,464 cancer cases were diagnosed among Malaysians in Peninsular Malaysia in the year 2003, comprising 9,400 males and 12,064 females. Cancer occurred at all ages. The median age at diagnosis for cancer in Malaysian males was 59 years and 53 years for Malaysian females.

There is variation of brain tumor cancer incidence rate between the different ethnic groups. The crude incidence rate for brain tumor cancers in Malay male and females were 58.9 and 57.8 per 100,000 populations respectively; for Chinese male and females 34.2 and 34.8 per 100,000 population respectively; and for the Indian male and females 6.9 and 7.4 per 100,000 population respectively (Chye and Halimah, 2003).

1.4 Incidence of brain tumor in HUSM

The classification of brain tumor was based on the recent World Health Organization classification. The brain tumor that reported in the Hospital of University Science Malaysia (HUSM) was low (0.4 per 100 000) population), with no significant gender preponderance. Neuroglial tumor (35%) was the most common brain tumors followed by meningiomas (33%), medulloblastomas (12%) and schwannomas (6%). The incidence of brain tumor with a size less than 4 cm in diameter and less than 30 cc in volume was 68.2% and 58.7% respectively. Primary brain tumors were more common in male and female except meningiomas and nerve sheath tumors, which were more common in the female. The incidence rate by age was low in the teens; this rose steadily to a peak in the fourth decade and then declined somewhat after the age of 60. Headache, vomiting, papilloedema and cranial nerve deficits were the common presenting features (Mohd R. Yusoff *et al.*, 1998)

Figure 1.2: Incidence of brain tumor in HUSM



1.5 Incidence of Glioma by anatomic location

The anatomic location of a glioma influences prognosis and treatment options. The gliomas were located in the frontal lobe in 40% of the cases, temporal in 29%, parietal in 14% and occipital lobe in 3% with 14% in the deeper structure. The difference in distribution between lobes remained after adjustment for their tissue volume: the tumor: volume ratio was 4.5 for the frontal, 4.8 for temporal and 2.3 for parietal relative to the occipital lobe. The area with the densest occurrence was the anterior subcortical brain.

The crude incidence rate of gliomas (per 100,000) was 1.68 (95% CI, 1.36–2.00) for the frontal lobe, 1.21 (95% CI, 0.94–1.48) for the temporal lobe, 0.58 (95% CI, 0.39–0.77) for the parietal lobe, and 0.13 (95% CI, 0.04–0.21) for the occipital lobe. Glioma were located more frequently in the right hemisphere (51%) than in the left (40%) while 9% of glioma were in the center of the brain (Larjavaara *et al.*, 2007).