ANALYSIS OF P27 AND CYCLIN D1 GENES IN GLIOMAS AND MENINGIOMAS USING MOLECULAR GENETIC, IMMUNOHISTOCHEMICAL AND IMMUNOGOLD ELECTRON MICROSCOPIC TECHNIQUES

by

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Dedication

In the name of Allah, The Most Gracious, The Most Merciful…

My heartfelt appreciation specially goes to my utmost beloved mom and dad (Wan Zainab Abu Bakar and Ahmad W. Nik), my lovely brothers and sisters, and my dearest fiancé (Zul Faizuddin Osman). I am grateful for their continuous enriching love, understanding and encouragement. Thank you for always be there for me.

I love you all.

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LIST OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AA215Gly	Aspartic acid to Glycine at codon 215
AA217Gly	Aspartic acid to Glycine at codon 217
bp	Base pair
BPH	Benign Prostatic Hyperplasia
C104T	C to T base substitution at codon 104
C223T	C to T base substitution at codon 223
CDK	Cyclin-dependent kinase
CDKI	Cyclin-dependent kinase inhibitor
CNS	Central Nervous System
c-onc	c-oncogene
CSGE	Confirmation Sensitive Gel Electrophoresis
Cyl-1	Cylicin-1
DHPLC	Denaturing High Performance Liquid Chromatography
DNA	Deoxyribonucleic Acid
dNTP	Deoxy Nucleotide Phosphatase
dsDNA	Double strand deoxyribonucleic acid
EDTA	Ethylenediaminotetra-acetate
EDTA	Ethylene Diamine Tetraacetic Acid
EGFR	Epidermal Growth Factor Receptor
G142A	G to A base substitution at codon 142
G242A	Guanine to Adenine at codon 242
GSK-3ß	Glycogen synthase-3beta
H_2O	Water
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
HER	Herstatin
HIF-1α	Hypoxia Inducible Factor 1 alpha
HRP	Horse Radish Peroxidase
kb	Kilo base
LOH	Loss of Heterozygoosity
Lys223Lys	Lysine to Lysine at codon 223
M	Mitosis phase of cell cycle
M	Molar
$MgCl_2$	Magnesium Cloride
ml	Milliliter
N	Normal
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NES	Nuclear Export Signal
nm	Nanometer
p	Significant value
PBS	Phosphate buffered saline
PCR	Polimerase Chain Reaction
PRAD	proline-rich attachment domain
pRb	Retinoblatoma protein???
Pro214Arg	Proline to Arginine at codon 214
RNA	Ribonucleic acid
rpm	Revolutions per minute
SNP	Single nucleotide polymorphism

SSCP	Single Strand Confirmation Polymorphism
ssDNA	Single strand deoxyribonucleic acid
STR	Short tandom repeat
T109G	T to G base substitution at codon 109
T119C	T to C base substitution at codon 119
T215C	T to C base substitution at codon 215
TBE	Tris Boric EDTA
TBS	Tris buffered saline
TDGS	Two-Dimensional Gene Scanning
TEAA	Triethyammonium acetate
TEM	Transmission Electron Microscope
UV	Ultraviolet
WHO	World Health Organization

ANALISIS GEN P27 DAN CYCLIN D1 DALAM GLIOMA DAN MENINGIOMA DENGAN MENGGUNAKAN TEKNIK GENETIK MOLEKUL, IMMUNOHISTOKIMIA DAN MIKROSKOPI ELEKTRON IMMUNOGOLD

ABSTRAK

Meningioma dan glioma merupakan dua jenis tumor otak yang paling kerap di laporkan di seluruh dunia. Kedua-dua jenis kanser ini mungkin berlaku akibat daripada gangguan pada kitaran sel yang normal yang dikawal rapi oleh gen p27 dan cyclin D1. Kajian ini dijalankan untuk menentukan status mutasi gen p27 dan cyclin D1, tahap pengeksresan protein dan lokasi kedua-dua protein tersebut melalui beberapa analisis termasuklah analisis genetik molekul, immunohistokimia, mikroskopi elektron immunogold. Analisis genetik molekul menunjukkan mutasi berlaku pada ekson 4 gen cyclin D1 tetapi tiada mutasi dilaporkan pada ekson 5 gen cyclin D1, dan ekson 1 dan 2 gen p27 yang turut dikaji. Lima mutasi yang berlainan telah dikesan dalam 2 sample glioma (8.0%) dan 3 sampel meningioma (11.5%). Penjujukan DNA yang dilakukan pada dua sampel glioma tersebut menunjukkan kehadiran mutasi tidak bererti akibat perubahan nukleotida C kepada T pada kodon 223 (Lys223Lys). Di dalam sample glioma yang pertama, kami juga menjumpai delesi nukleotida G pada kodon 214 yang mengakibatkan mutasi anjakan rangka (Pro214Arg). Selain itu, kami juga menjumpai mutasi salah erti pada sampel glioma yang kedua. Perubahan nukleotida T kepada C telah dijumpai pada kodon 215 dan 217 yang telah menyebabkan perubahan asid aspartik kepada Glysin pada dua lokasi yang berlainan. Penskrinan mutasi dalam kes meningioma telah menemui 3 mutasi tidak bererti dan 3 mutasi salah erti dalam 3 sampel yang berasingan. Dalam ketiga-tiga sampel, kami menjumpai 3 kes perubahan nukleotida C kepada T yang mengakibatkan mutasi tidak bererti pada kodon 223 (Lys223Lys). Dalam sampel kedua dan ketiga, kami menjumpai perubahan nukleotida T kepada C pada kodon 215 yang mengakibatkan mutasi salah erti (Asp215Gly). Satu lagi

mutasi salah erti telah dijumpai pada kodon 217 yang menyebabkan perubahan nukleotida T kepada C dalam sampel meningioma yang ketiga. Analisis imunohistokimia pada kumpulan sampel yang sama menunjukkan protein p27 mengalami peningkatan dalam pengekspresannya dalam semua kes termasuklah meningioma (82.6%), glioma gred rendah (80.0%) dan glioma gred tinggi (84.6%). Kami kemudiannya menjumpai peningkatan dalam pengekspresan protein cyclin D1 dalam kes meningioma (70.8%) dan pengurangan dalam kes glioma peringkat tinggi (76.9% kes adalah mengalami pengekspresan protein cyclin D1 yang rendah). Kami juga menjumpai kadar pengekspresan yang sama dalam kes glioma peringkat rendah di mana 50% daripada kesnya mengalami pengekspresan protein yang rendah dan 50% mengalami pengekspresan protein yang tinggi). Analisis mikroskopi elektron immunogold pada protein p27 dan cyclin D1 menunjukkan kehadiran kedua-dua protein pada sitoplasma dan nukleus sel. Analisis statistik tidak menunjukkan sebarang hubungkait yang signifikan antara kehadiran mutasi pada gen cyclin D1 dengan pengurangan dalam tahap pengekspresan proteinnya dala kedua-dua kes meningioma (p=0.616) dan glioma (p=0.905).

Kata kunci: meningioma; glioma; p27; cyclin D1; genetik molekul; imunohistokimia; mikroskopi elektron imunogold

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ABSTRACT

Meningiomas and gliomas are two most commonly reported brain tumor cases worldwide. These types of tumors might occur due to the disruption of the normal cell cycle which is highly controlled by p27 and cyclin D1 genes. This study was performed to determine the mutational status of p27 and cyclin D1, level of both protein expression and the localization of both proteins at ultrastructural level via analyses of molecular genetic, immunohistochemistry and immunogold electron microscopy respectively. The molecular genetic analysis revealed mutations in exon 4 of cyclin D1 gene but none was detected in other studied regions of exon 5 of cyclin D1 gene and exon 1 and 2 of p27 gene. Five different mutations were detected in 2 glioma (8.0%) and 3 meningioma (11.5%) samples. DNA sequencing for the two gliomas samples revealed the presence of non-sense mutation which resulted to the change of C to T nucleotide at codon 223 (Lys223Lys). In the first glioma sample, we also detected a G base deletion at codon 214 which caused a frameshift mutation (Pro214Arg). In addition to that, we also found two other missense mutations in the second glioma sample. T to C nucleotide changes were detected at codon 215 and codon 217 which caused aspartic acid to Glycine changes in two different loci. Screening of mutations in meningiomas cases revealed 3 cases of non-sense mutations and 3 cases of missense mutations in a total of 3 samples. In all 3 samples, we found 3 cases of C to T nucleotide change which resulted to non-sense mutations at codon 223 (Lys223Lys). In the second and third meningioma samples, we found an additional of T to C nucleotide changes at codon 215 which caused missense mutations (Asp215Gly). Another missense mutation was found at codon 217 which showed T to C nucleotide change in the third meningioma sample.

Immunohistochemistry analysis of the same group of samples revealed p27 protein overexpression in all cases including meningiomas (82.6%), low grades gliomas (80.0%) and high grades gliomas (84.6%). We subsequently found high level of cyclin D1 expression in meningiomas (70.8%), equal expression of cyclin D1 in low grades of gliomas (50% are low expressors and 50% are high expressors), and downregulation of the protein in higher grades of gliomas (76.9% were low expressors). Immunogold electron microscopy analysis of cyclin D1 and p27 proteins showed that both proteins were found to be localized at cytoplasm and nucleus of the cells. Our statistical analysis gave no significant correlation between the presence of cyclin D1 mutations with the downregulation of the protein in both meningiomas (p=0.616) and gliomas (p=0.905).

Keywords: meningiomas; gliomas; p27; cyclin D1; molecular genetics; immunohistochemistry; immunogold electron microscopy

CHAPTER I

LITERATURE REVIEW

1.1 INTRODUCTION

Cancer is a complex and unpredictable genetic disease which is referred to as an abnormal growth of cells. It is a multifaceted disease, which has long been regarded as a genetic disease (Cornelisse, 2003b). In 1914, the famous biologist Theodor Boveri postulated that abnormal distribution of chromosome could be the cause of cancer. Furthermore, he suggested that tumor could arise from a single abnormal cell (monoclonal origin), and predicted that specific chromosomal changes and genetic instability are important for tumor development (Cornelisse, 2003a). His opinion was extensively supported by many researchers, postulating that the original cause of cancer is by the accumulation of genetic alterations and consequently gene expression pattern changes (Evan and Vousden, 2001, Fingleton and Coussens, 2005, Garnis *et al.*, 2004, Zingde, 2001) which could allow them to grow outside their normal growth restraints (Garnis *et al.*, 2004). The accumulation of genetic aberrations are thought to drive the progression of normal cells through hyperplastic and dysplastic stages to invasive cancer and, finally, metastatic disease (Garnis *et al.*, 2004). Cancer cells posses the ability to invade and metastasize (Zingde, 2001), as well as to induce vascularization of the tumour in order to receive oxygen and nutrients (angiogenesis), and to suppress programmed cell death (apoptosis) (Garrett, 2001, Zingde, 2001).

Several essential steps are compulsory for normal cells to become cancer cells. Researches on experimental carcinogenesis in animals have shown that cancer

progression involves processes of initiation, promotion and progression, which are all involving various genetic alterations. Initiation step is an irreversible and not recognizable as a pathological entity. Promotion is a step which facilitates the expression of the initiated phenotype. Finally, progression is representing step for further phenotypic alterations in the initiated cells (Zingde, 2001).

The alterations which may occur during the three important steps of initiation, promotion and progression in cancer development may involve oncogenes and tumor suppressor genes. These two types of genes have been marked as genes responsible for the cancer phenotype (Zingde, 2001) which can deregulate cellular proliferation by loss of genes that normally check the cell growth (the tumor suppressor genes) and by the gain of functions of oncogenes that either promote cellular proliferation or prevent cell death (Perry, 2001). Oncogenes (from Greek *onkos*, tumor) can be defined as the activated forms of normal cellular genes whose protein products are involved in cell signaling governing cell survival, proliferation and differentiation (Perry, 2001). At present, over 100 oncogenes are now known and the listings of these are available at www.ncbi.nlm.gov/ncicgap/ (Zingde, 2001). Oncogenes were originally identified as sequences in the genome of animal tumor viruses that could transform normal cells into tumor cells. Through a huge breakthrough, there was an important discovery showing that similar sequences called proto-oncogenes, could be demonstrated in the DNA of normal cells. Proto-oncogenes are believed to have the ability to transform into becoming true oncogenes by mutations leading to abnormal activation (Cornelisse, 2003b) or if they are abnormally expressed in the cell (Melkoumian, 2001). Proto-oncogenes or c-oncogenes (cellular homologues of DNA, c-onc) are genes that have been identified as genes coding for components of the mitogenic signaling cascades and

growth control (Zingde, 2001) which normally involved in the regulation of cell proliferation (Melkoumian, 2001).

Tumor suppressor gene is a gene that reduces the probability that a cell in a multicellular organism will turn into a tumor cell. So far, more than 30 tumor suppressor genes have been identified (Cornelisse, 2003b, Zingde, 2001), and the majority of the genes are highly associated with the familial cancer syndrome. In contrast to oncogenes, which are mainly components of growth-signal transduction pathways, tumor suppressor gene products have quite diverging functions. Several of the genes' products control the DNA damage-repair signaling, and thus guard the integrity of our genome (Cornelisse, 2003b). An important criterion of tumor suppressor gene is the demonstration of loss-of-function or inactivating mutations in both copies of the gene. The reported mechanisms by which could abolish the normal functions of the tumor suppressor genes include loss of heterozygosity, methylation, cytogenetic aberrations, genetic mutations, gain of autoinhibitory function and polymorphism (Zingde, 2001).

The alterations of various oncogenes and tumor suppressor genes which control the normal cell cycle progression are suggested to be involved in the progression of many tumor types. The alteration may cause disruption of normal functions of the genes and lead to the uncontrolled proliferation of cancer cells (Garrett, 2001). However, Monceviciu-te-Eringiene (2005) (Eringiene, 2005) has suggested that genes alterations are playing the second important role in causing cancers. He proposed that the evolutionary resistance and its variability have an immense power to initially drive and control the progression of carcinogenesis. This resistance mechanism can be designated as physiological or natural, which requires resistance towards chemical,

physical and biological factors. The genetic changes on the other hand, will further help the cancer cells to survive under extreme condition.

1.1.1 Definition and category of brain tumors

Tumors of the central nervous system (CNS) are usually devastating because they often affect children, are difficult to treat, and frequently cause mental impairment or death. CNS tumors are reported as the second most common neoplasm in children, after leukemia (Levy, 2005).

A tumor is basically referred to as a mass or growth of abnormal cells. Tumors found in the brain typically are categorized as primary or secondary tumors. Primary brain tumors originate in the brain and can be cancerous (malignant) or non-cancerous (benign). Secondary tumors are tumors which spread to the brain from another site such as lung and breast cancer, which are the most common cause of brain metastases. Besides that, brain metastases are also found originating from unknown primary (10-15%), melanoma (10%) and gastrointestinal carcinoma (5%). This phenomenon usually occurs in 10-30% of all cancer patients (Kaye and Jr., 2001b).

Primary benign and primary malignant brain tumors are broadly classified by the tissue of origin. CNS tumors represent a diverse group of neoplasms of the brain and spine with varying histology (Levy, 2005). The major brain tumor histology groups are: tumors of neuroepithelial tissue, tumors of cranial and spinal nerves, tumors of the meninges, lymphomas and hematopoietic neoplasms, germ cell tumors and cysts, tumors of the sellar region, and local extensions from regional tumors (Doolittle, 2004).

Benign brain tumors are usually slower growing, easier to remove and less likely to recur than malignant brain tumors. Malignant brain tumors can grow rapidly, crowding or destroying nearby brain tissue. However, in a small number of cases even benign brain tumors can cause serious problems or be life threatening. Initial evaluation of suspected CNS tumor depends on the presenting illness, symptoms, and available resources at the health center, which provides the specific management for the patients (Doolittle, 2004).

Brain tumors are classified depending on the origin and seriousness of the tumor. To date, brain tumor groups can be referred to the World Health Organization (WHO) brain tumor classification. WHO recognizes 126 different types of central nervous system tumors of which some common types of primary tumors are astrocytomas, medulloblastomas, meningiomas and oligodendrogliomas.

These tumors are extremely diverse, biologically and in their response to treatment. Meningiomas are the most common non-malignant brain tumors in adults, and are curable with surgery. Glioblastomas, the most common malignant brain tumors in adults, are probably the most resistant of all cancers to treatment.

Treatment for central nervous system cancers includes surgery, radiation therapy and chemotherapy. These therapies may be delivered alone or in combination. Although neurosurgical techniques, radiation and chemotherapy have undergone various advances, brain tumors are still retaining their dismal prognosis and the patients are usually dying after certain period of time (Lam and Breakefield, 2001).

1.2 CENTRAL NERVOUS SYSTEM TUMORS

1.2.1 CENTRAL NERVOUS SYSTEM TUMORS CLASSIFICATION ACCORDING TO WORLD HEALTH ORGANIZATION (WHO)

The first edition of Central Nervous System (CNS) classification was published in 1979 which required almost a decade to be completed. The second edition of the tumors classification arose in 1993 after the introduction of immunohistochemistry which helped a lot in upgrading the system of classification. Improvement of the previous classification system has led to a new publication of the system which was done in 2000 and it will be used for the next 5 years. The Table 1.1 below shows the latest classification system of central nervous system tumors (Kleihues *et al.*, 2002).

Table 1.1: Classification of Tumors of the Nervous System.

Tumors of Neuroepithelial Tissue	
Astrocytic tumors	
Diffuse astrocytoma	9400/3[1]
Fibrillary astrocytoma	9420/3
Protoplasmic astrocytoma	9410/3
Geminocytic astrocytoma	9411/3
Anaplastic astrocytoma	9401/3
Glioblastoma	9440/3
Giant cell glioblastoma	9441/3
Gliosarcoma	9442/3
Pilocytic astrocytoma	9421/1
Pleomorphic xanthoastrocytoma	9424/3
Subependymal giant cell astrocytoma	9384/1
Oligodendroglial tumors	
Oligodendroglioma	9450/3
Anaplastic oligodendroglioma	9451/3
Mixed gliomas	
Oligoastrocytoma	9382/3
Anaplastic oligoastrocytoma	9382/3
Ependymal tumors	
Ependymoma	9391/3
Cellular	9391/3
Papillary	9393/3
Clear cell	9391/3
Tanycytic	9391/3
Anaplastic ependymoma	9392/3
Myxopapillary ependymoma	9394/1
Subependymoma	9383/1
Choroid plexus tumors	
Choroid plexus papilloma	9390/0
Choroid plexus carcinoma	9390/3
Glial tumors of uncertain origin	
Astroblastoma	9430/3
Gliomatosis cerebri	9381/3
Chordoid glioma of the 3rd ventricle	9444/1
Neuronal and mixed neuronal-glial tumors	
Gangliocytoma	9492/0
Dysplastic gangliocytoma of cerebellum (Lhermitte-Duclos)	9493/0
Dysembroplastic neuroepithelial tumor	9413/0
Gangglioglioma	9505/1
Anaplastic gnaggliogliomа	9505/3
Central neurocytoma	9506/1
Cerebellar liponeurocytoma	9506/1
Paraganglioma of the filum terminale	8680/1
Neuroblastic tumors	
Olfactory neuroblastoma (Aesthesioneuroblastoma)	9522/3
Olfactory neuroepitelioma	9523/3
Neuroblastoma of the adrenal gland and sympathetic nervous system	9500/3
Pineal parenchymal tumors	
Pineocytoma	9361/1
Pineoblastoma	9362/3
Pineal parenchymal tumor of intermediate differentiation	9362/3
Embryonal tumors	
Medulloepithelioma	9501/3
Ependymoblastoma	9392/3
Medulloblastoma	9470/3
Desmoplastic medulloblastoma	9471/3
Large cell medulloblastoma	9474/3
Medullomyoblastoma	9472/3
Melanotic medulloblastoma	9470/3
Supratentorial primitive neuroectodermal tumor	9473/3
Neuroblastoma	9500/3
Ganglioneuroblastoma	9490/3
Atypical teratoid/rhabdoid	9508/3
Tumors of Peripheral Nerves	
Schwannoma	
(Neurilemmoma, Neurinoma)	9560/0
Cellular	9560/0
Plexiform	9560/0
Melanotic	9560/0
Neurofibroma	9540/0
Plexiform	9550/0
Perineurioma	9571/0
Intraneural perineurioma	9571/0
Soft tissue perineurioma	9571/0
Malignant peripheral nerve sheath tumor (MPNST)	9540/3

Desmoplastic infantile astrocytoma/ganglioglioma	9412/1	Epithelioid	9540/3
MPSNT with divergent mesenchymal and/or epithelial differentiation	9540/3	Osteoma	9180/0
Melanotic	9540/3	Osteosarcoma	9180/3
Melanotic psammomatous	9540/3	Osteochondroma	9210/0
Tumors of the Meninges		Hemangioma	9120/0
Tumors of meningothelial cells		Epithelioid hemangioendothelioma	9133/1
Meningioma	9530/0	Hemangiopericytoma	9150/1
Meningothelial	9531/0	Angiosarcoma	9120/3
Fibrous (fibroblastic)	9532/0	Kaposi sarcoma	9140/3
Transitional (mixed)	9537/0	**Primary melanocytic lesions**	
Psammomatous	9533/0	Diffuse melanocytosis	8728/0
Angiomatous	9534/0	Diffuse melanocytosis	8728/0
Microcystic	9530/0	Malignant melanoma	8720/3
Secretory	9530/0	Meningeal melanomatosis	8728/3
Lymphoplasmacyte-rich	9530/0	**Tumors of uncertain histogenesis**	
Metaplastic	9530/0	Hemangioblastoma	9161/1
Clear cell	9538/1	**Lymphomas and Hemopoietic Neoplasms**	
Chordoid	9538/1	Malignant lymphomas	9590/3
Atypical	9539/1	Plasmacytoma	9731/3
Papillary	9538/3	Granulocytic sarcoma	9930/3
Rhabdoid	9538/3	**Germ Cell Tumors**	
Anaplastic meningioma	9530/3	Geminoma	9064/3
Mesenchymal, non-meningothelial tumors		Embryonal carcinoma	9070/3
Lipoma	8850/0	Yolk sac tumor	9071/3
Angiolipoma	8861/0	Choriosarcoma	9100/3
Hibernoma	8880/0	Teratoma	9080/1
Liposarcoma (intracranial)	8850/3	Mature	9080/0
Solitary fibrous tumor	8815/0	Immature	9080/3
Fibrosarcoma	8810/3	Teratoma with malignant transformation	9084/3
Malignant fibrous histiocytoma	8830/3	Mixed germ cell tumors	9085/3
Leiomyoma	8890/0	**Tumors of the Sellar Region**	
Leiomyosarcoma	8890/3	Craniopharyngioma	9350/0
Rhabdomyoma	8900/0	Adamantinomatous	9351/1
Rhabdomyosarcoma	8900/3	Papillary	9352/1
Chondroma	9220/0	Granular cell tumor	9582/0
Chondrosarcoma	9220/3		

Morphology code of the International Classification of Diseases for Oncology (ICD-O) and the Systematized Nomenclature of Medicine (SNOMED). Behaviour is coded /0 for benign tumors, /1 for low or uncertain malignant potential or borderline malignancy, /2 for in situ lesions and /3 for malignant tumors.

1.2.2 TYPES OF CENTRAL NERVOUS SYSTEM TUMORS

1.2.2.1 GLIOMAS

Gliomas were described as fast growing, poorly circumscribed lesions which diffusely infiltrated but did not destroy the brain parenchyma (Kaye and Jr., 2001a). It comprises of glioma grade I, grade II, grade III and grade IV including pilocytic astrocytoma (WHO grade I), Oligodendroglioma (WHO grade II), Anaplastic astrocytoma (WHO grade III) and Glioblastoma Multiforme (WHO grade IV).

1.2.2.1.1 ASTROCYTIC (GLIAL) TUMORS

Three main categories of astrocytic brain tumors are astrocytoma, anaplastic astrocytoma and glioblastoma multiform (Doolittle, 2004). Other sub groups which are also categorized as astrocytic tumors are diffuse astrocytoma, anaplastic astrocytoma, glioblastoma, pilocytic astrocytoma, pleomorphic xanthoastrocytoma and subependymal giant cell astrocytoma. They are differentially categorizied according to their clinical, histopatological, and genetic features (Kleihues *et al.*, 2002).

Among the three major categories of astrocytic tumors, anaplastic astrocytoma and glioblastoma multiform are the most common glial tumors. Anaplastic astrocytoma is graded as tumor grade 3 and glioblastoma multiform is graded as tumor grade 4 (Doolittle, 2004, Kaye and Jr., 2001b). Among all types of astrocytic tumors, glioblastoma is the most frequent brain tumor occurs in adults (Kaye and Jr., 2001b, RobertWechsler-Reya and Scott, 2001). Astrocytoma may be detected superficially

in the cerebral hemispheres, and may involve overlying meninges (Kaye and Jr., 2001b).

1.2.2.1.2 OLIGODENDROGLIAL TUMORS

Basically, oligodendroglial tumors can be divided into two main groups, which are oligodendroglioma and anaplastic oligodendroglioma (Kaye and Jr., 2001b). Oligodendrogliomas constitute about 5 to 10% of the primary CNS glial neoplasms which usually occur in the cerebral hemispheres and consist of oligodendroglial cells (Kaye and Jr., 2001b). Most are identified in adults in their fourth and fifth decades of life, but oligodendrogliomas can occur at any age.

1.2.2.1.3 EPENDYMAL TUMORS

Ependymoma occurs most commonly in fourth ventricle, and in that location, it may cause hydrocephalus. Besides that, ependymoma has also been found in spinal cord, filum terminale and infrequently in the third ventricle. Ependymal tumors can be classified into four categories which are ependymoma, anaplastic (malignant) ependymoma, myxopapillary ependymoma, and subependymoma (Kaye and Jr., 2001b). Previous documented cases of ependymal tumors showed no partiality of age or sex in the CNS (Kleihues *et al.*, 2002).

1.2.2.2 MEDULLOBLASTOMA

Medulloblastomas, are the most common primary brain tumors among children (Fomchenko and Holland, 2005). Normally, medulloblastomas arise in the

cerebellum and it occurs more frequently in males compared to females. There are a few genes which are closely correlated to the development of medulloblastomas, such as LOH on chromosome 17q, EGFR, HER2, HER3, HER4, and cyclin D2 genes.

1.2.2.3 MENINGIOMAS

Meningiomas arise from the meningiothelial cells at meninges, which is the outer layer of the brain including parasagittal/falcine, convexity, sphenoid ridge, suprasellar, posterior fossa, olfactory groove, middle fossa, tentorial and peritorcular (Kaye and Jr., 2001b). It may occur as benign, atypical or malignant tumors. Basically, meningiomas are slow-growing and are not associated with edema: however, they cause symptoms by compressing adjacent neural structures. Meningiomas encompass almost 20% of the central nervous system tumors (Doolittle, 2004) and they can be divided into four major sub-groups, which are tumor of meningiothelial cells, mesenchymal, non-meningiothelial tumors, primary melanocytic lesions, and tumors of uncertain origin (Kaye and Jr., 2001b). Meningiomas usually occur higher in patients older than 70 years old in both sexes. Only 1-2% of meningiomas are detected in children, suggesting that meningiomas are rarely found in children (Kaye and Jr., 2001b).

1.2.2.4 Other types of CNS tumors

Other types of CNS tumors include Germ Cell Tumors and Tumors of the Sellar Region which are included in the Table 1.1.

According to the National Cancer Registry, Malaysia (Chye and Yahaya, 2003), brain tumors are the second most frequent reported cases after leukemia in Malaysia. The tumors are more frequently reported in male as compared to women. Brain tumor patients of age between 30-39 years old (Mouriaux *et al.*) seems to render the highest percentage with 17.3%, while patients of age 30-39 and 50-59 years old (female) are the second and third most reported cases with 16.4% respectively. Brain tumors are reported most among Indian male patients with 31.4% and Chinese female patients with 27.5% (Chye and Yahaya, 2003).

Among all types of brain tumors, glioma is the most common type found in adults (Park *et al.*, 2004, Tyynela, 2006). Malignant gliomas have been treated with the standard treatment of tumor resection followed by radiation therapy with or without adjuvant chemotherapy. Even though advances in neurosurgical techniques, radiation therapy and drug deliveries have emerged, the prognosis of the patients remain poor (Lam and Breakefield, 2001, Tyynela, 2006). Besides gliomas, meningiomas are also categorized as one of the most reported central nervous tumor, accounting for 26% of primary brain tumors (Watson *et al.*, 2002). Majority of meningioma cases also shows aggressive tumors features and caused mortality of the patients (Mihaila *et al.*, 2003). Treatments for meningiomas are usually limited to surgical intervention and radiation (Watson *et al.*, 2002). Molecular genetics and histopatological studies on meningiomas are very limited so far, as compared to other malignant brain tumors such as gliomas. In this study, we are focusing on both meningiomas and gliomas for the molecular genetics and histopatological studies to understand the role of cell cycle genes and their protein in brain tumor development and to gain valuable information which might further help in developing new treatments modalities for meningiomas and gliomas.

1.3 CELL CYCLE

Cell division cycle serves as the important mechanism which allows cell grows and duplicates by replicating its DNA and then divides to form two daughter cells. Cell cycle process comprises of four distinct chronological phases, which are G1, S, G2 and M phases (Figure 1.1) (Garrett, 2001). The sequence begins with a silent gap period, G1, followed by a period during which new DNA is synthesized in a semi conserved fashion, called the S phase or period. Following that is another gap period, G2, then the M phase, during which the actual cell division/mitosis takes place.

The activities of cell cycle depend mostly on the sequential series of checkpoints which lie amongst the four significant phases, ensuring that the conditions are suitable for the proper function of that cell and for DNA replication and cytokinesis (Humphrey and Brooks, 2005). Each check point is made up of sensor mechanism that detects aberrant or incomplete cell cycle events such as DNA damage, signal transduction pathway which carries the signal from the sensor to the third component and the effectors that can invoke a cell cycle arrest until the problem has been resolute. The first checkpoint occurs at G1/S phase transition and is the major sensor of DNA damage. Other checkpoints involve in cell cycle are G2/M phase transition and spindle checkpoint, which may arrest the cell cycle progression due to DNA damage and incomplete formation of functional mitotic spindle, respectively (Garrett, 2001). The mechanisms involved in cell division must be precise. Any error, whether in the replication of DNA or the distribution of the genetic complement to the daughter cells, would have severe impact to the organism. Disruption of cell cycle checkpoints may lead to cellular proliferation, increase genomic instability and ultimately cancer development (Cheung *et al.*, 2001, Garrett, 2001).

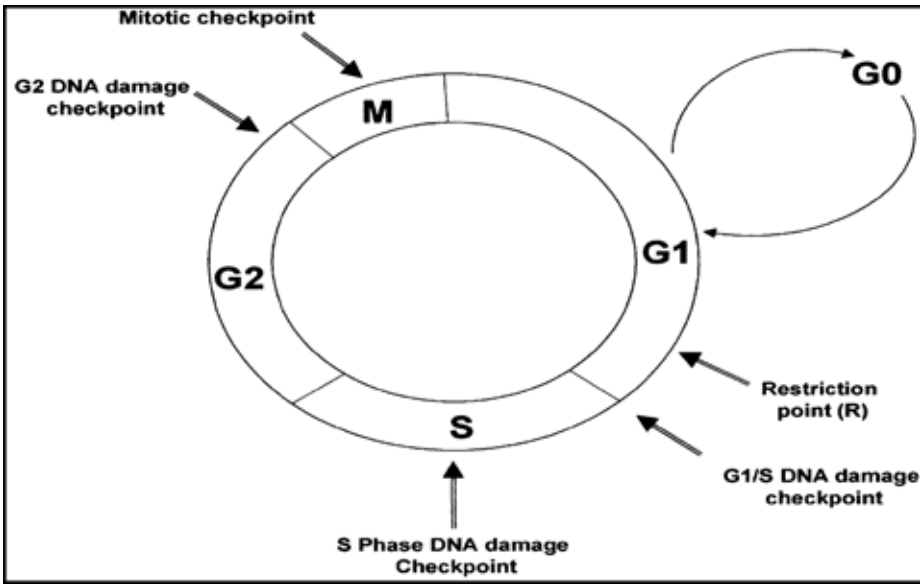

Figure 1.1: Checkpoints and cell cycle.

The cell cycle progression is tightly controlled by three families of key cell cycle regulatory proteins: cyclins and their catalytic subunits known as cyclin-dependent kinase (CDK), and cyclin-dependent kinase inhibitor (CDKI), (Cheung *et al.*, 2001, Humphrey and Brooks, 2005) which themselves are regulated by phosphorylation and dephosphorylation events (Cheung *et al.*, 2001, Humphrey and Brooks, 2005). Various CDKs, cyclins and CDKIs are involved in regulating cell cycle progression and different types of components control different stages of the cycle.

Cyclin Dependent Kinases (CDKs) are the engines driving the proliferative cycle, phosphorylating substrate to promote progression through cell cycle transitions, maintaining checkpoints functions and ensuring overall integrity of the process (Nho and Sheaff, 2003). The CDKs are activated once they are forming complexes with a cyclin regulatory subunit (Garrett, 2001). In mammalian cells, different CDKs are active and required at different phases of the cell cycle. The cyclin levels vary dramatically through the cell cycle as a consequence of changes in transcription and ubiquitin-mediated degradation. Whilst the expression level of the CDKs subunit is generally constant throughout the cell cycle, the expression of each cyclin (of which there is a whole family) tends to be cell cycle dependent so that a specific CDKs will have full activity when its cyclin partner is expressed. The cyclins and CDKs activities are negatively regulated by association with various CDKIs (Garrett, 2001, Spirin *et al.*, 1996).

Briefly, five major classes of mammalian cyclins (termed as A-E) have been described. All the five major classes of cyclins are playing important roles in various stages of cell cycle. For example, cyclin C, D1-3, and E reach their peak of synthesis and activity during the G1 phase and apparently regulate the transition from G1 to S phase. On the other hand, cyclins A and B1-2 achieve their maximal level later in the cycle, during S

and G2 phases, and are regarded as regulators of the transition to mitosis. It is postulated that the G1/S transition is regulated by complexes of cyclin E/CDK2 and cyclin D/CDK4 or CDK6, while cyclin E-CDK2 controls entry into S phase. Cyclin A-CDK2 units effect their regulation through S phase and cyclin B-CDC2 (also known as CDK1) control entry into mitosis. The kinase activity of these CDKs requires association between a catalytic subunit (the CDK) and a regulatory subunit (the cyclin) (Cordon, 1995).

In an active cell cycle processes, each cyclin binds to the corresponding CDK and activates its catalytic subunit. Activated CDK then phosphorylates another important of cell cycle regulator of G1 progression, the retinoblastoma protein, pRb which has 16 potential sites of CDK phosphorylation. Once the pRb becomes phosphorylated at the CDK consensus sites, this will disrupt the interaction between pRb proteins with the E2F proteins, allowing the E2F dependent transcription to occur. This is required in order for the cell to pass through the restriction site late in G1. During G1 phase, Cyclin D1 is expressed throughout G1 phase but its peak occurs during mid G1. The main function of cyclin D1 is to activate CDK4/6 catalytic subunits to induce initial phosphorylation of pRb. Cyclins E and A begin to rise later in G1. They are responsible for activation of CDK2 to maintain pRb in its hyperphosphorylated state during the late G1 and ensure G1 to S progression. Figure 1.2 shows a schematic diagram of the temporal relationship between different cyclin/CDK complexes and cell cycle phases. Figure 1.3 shows the schematic representation of pRb phosphorylation by cyclin/CDK complexes and E2F release during G1 to S progression.

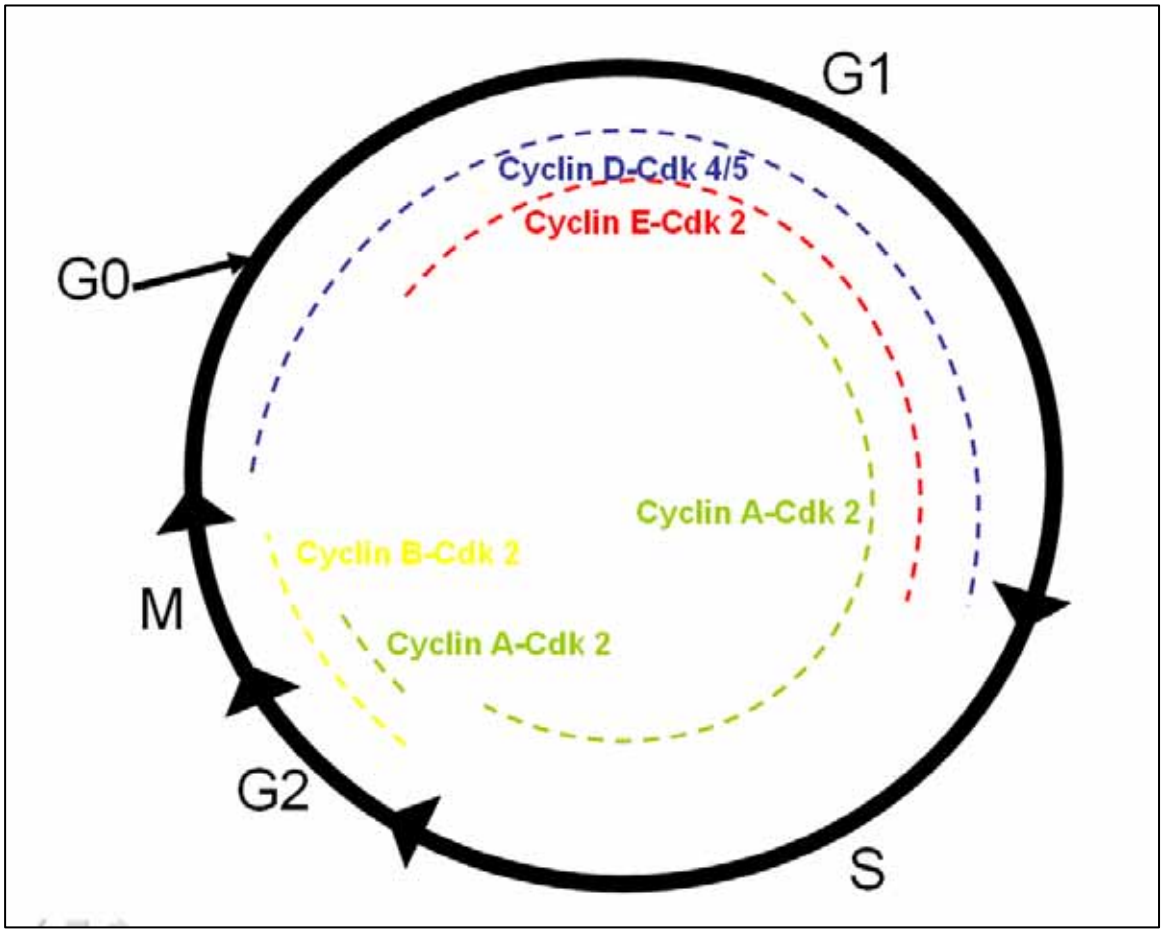

Figure 1.2: A schematic diagram showing the temporal relationship between different cyclin/CDK complexes and cell cycle phases.

R – Late G1 restriction site

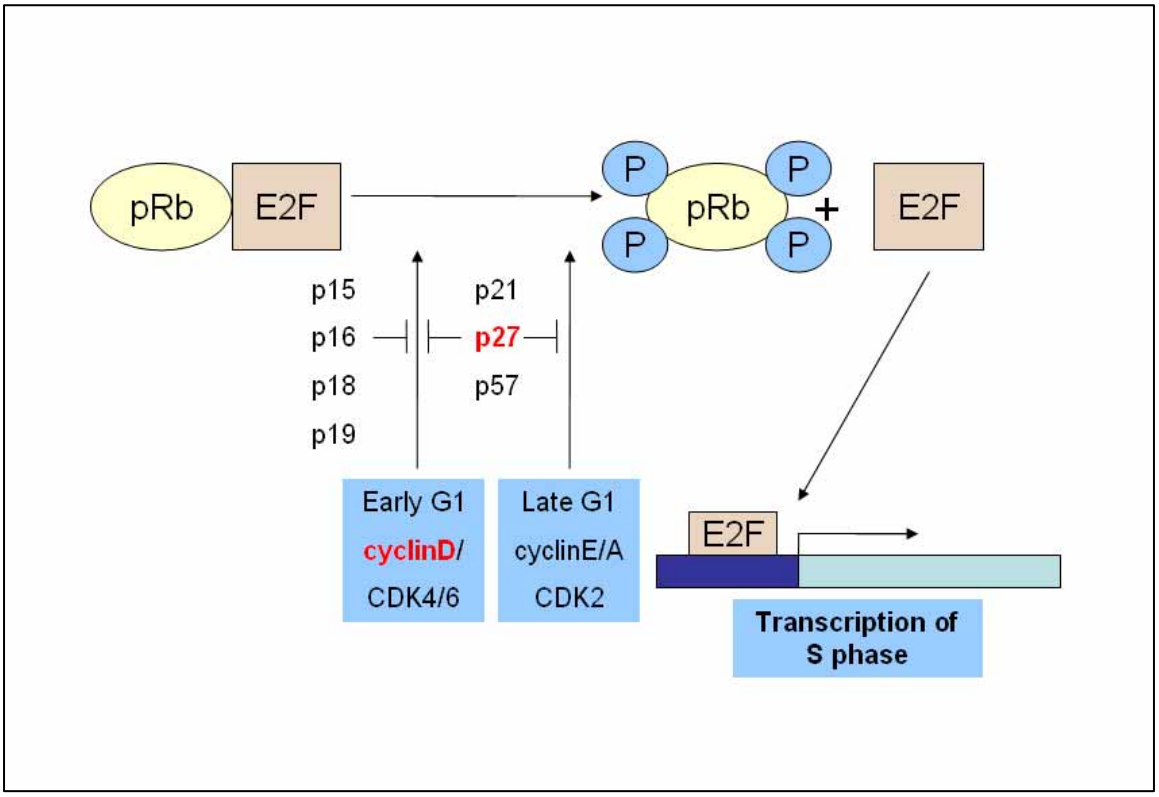

Figure 1.3: Schematic representation of pRb phosphorylation by cyclin/CDK complexes and E2F release during G1 to S progression.

As cells progress into S phase, cyclin A is expressed and becomes the primary cyclin associated with CDK2. This switching of cyclin partner allows CDK2 to also switch substrate specificity so that it can now target to a new set of proteins during S phase,

including CDC6 and E2F. CDC6 is a protein required for initiation of replication which when phosphorylated by CDK2/cyclin A, will relocalize from the nucleus to the cytoplasm. Progression from G2 into mitosis requires the activity of the CDK, CDC2 (also known as CDK1) complexed with cyclin B, which has been shown to phosphorylate proteins regulated during mitosis. Figure 1.4 shows the cyclins and CDKs which are required in cell cycle.

Interestingly, the kinase activity of CDKs can be inhibited by various kinase inhibitors (Fredersdorf *et al.*, 1997). There are two classes of CDKIs which are negatively regulating the CDK activities. They are categorized based on their structural and functional homologies. One class is known as INK4-proteins which includes p16/INK4A, p15/INK4B, p18/INK4C and p19/INK4D. The other group is so-called CIP/KIP group, which includes p21/WAF1, p21/Kip1 and p57/Kip2 (Spirin *et al.*, 1996).

Cyclin-dependent kinase inhibitors (CDKIs) are generally regulating Cyclin-CDK complexes during cell cycle progression. These proteins (CDKIs) are grouped into two families based on their structural functional properties. The INK4 group has four anykrin repeats and form complexes with CDK4 and/or CDK6 and the D-type cyclins. They have functional activities that are dependent on the presence of a normal retinoblastoma protein (Lloyd *et al.*, 1999). Maximal expression of the INK4 proteins occurs during the middle of the S phase in proliferating cells. Among all types, both p15 and p16 show a high frequency of gene deletions in various human cancer types, suggesting that these genes may function as tumor suppressors.

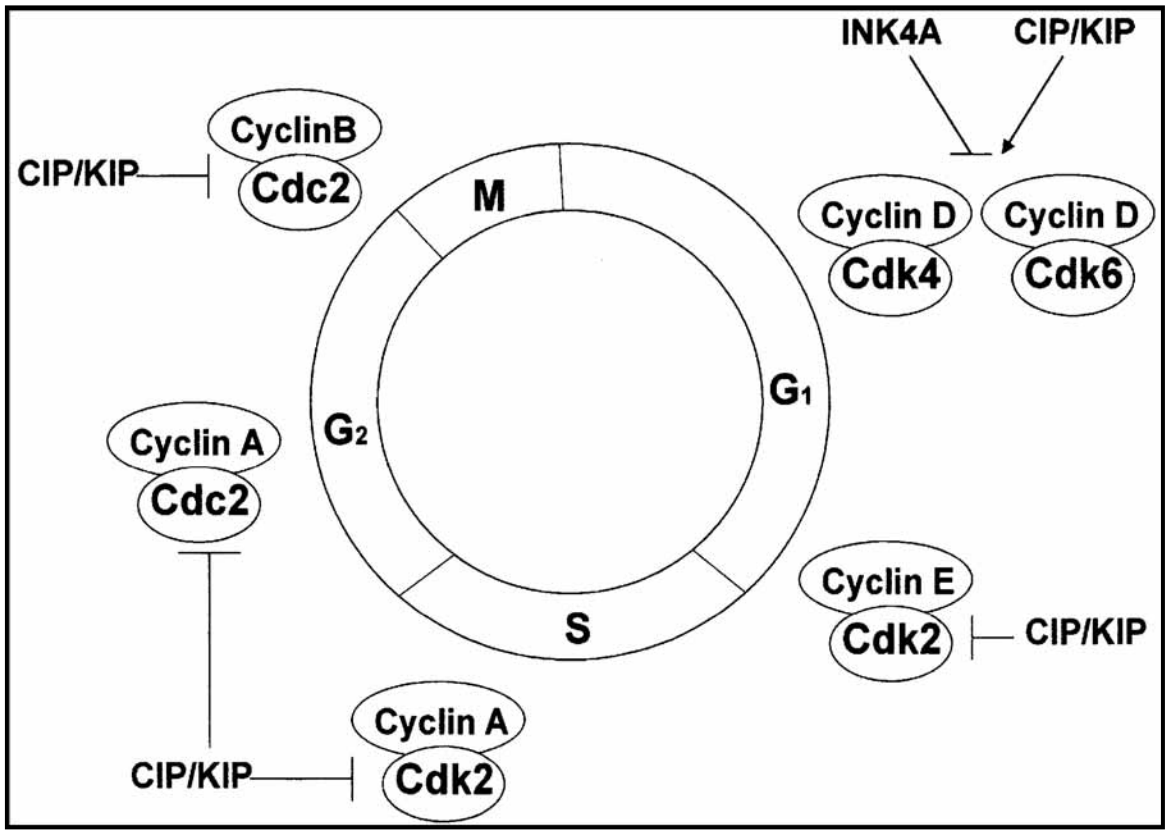

Figure 1.4: Cyclins, CDKs and the cell cycle.

The second family of inhibitors is composed of Cip/Kip family, includes p21/WAFI/CIP1 (p21), p27/Kip1 (p27) and p57/Kip2 (p57). Proteins of this family act by inhibiting kinase activities of pre-activated G_1 cyclin E-CDK2, cyclin D-CDK4/6, and other cyclins (Lloyd *et al.*, 1999). These proteins are not specific for a particular phase (Coqueret, 2003) and they are designated as universal CDKIs because they interact with various CDK complexes, with cyclins A, E, D1, D2, and D3 and CDKs. Overexpression of the Kip proteins leads to cell cycle arrest. Members of the kip proteins share a great deal of homology. p27 protein has a 42% amino acid homology with p21 and a 47% homology with p57 at the amino-terminal domain, the region that mediates inhibition of CDK. Kip proteins all have a nuclear localization signal at their carboxyl-terminal domain. Unlike the INK4 family, which inhibits CDK4/6 only, the Cip/Kip inhibitors can also target CDK2 in complexes (Lloyd *et al.*, 1999).

1.3.1 P27 GENE

p27 (kip1/p27kip1) is one of the cyclin-dependent kinase inhibitors (CDKI), which also plays important roles as a negative regulator in cell cycles (Coqueret, 2003, Keles *et al.*, 2003). P27 is encoded by CDKN1B gene, which is located on chromosome 12q13 at the junction of 12p12-12p13.1. The gene consists of three exons and encoded for 4.99 kb of DNA.

p27 is a CKI specific for CDK2 and normally acts at the G1 stage (Coqueret, 2003, Nakayama *et al.*, 1996, Nho and Sheaff, 2003). p27 protein has separate binding sites for different cyclins and CDK subunits, explaining how this protein and other Kip/Cip inhibitors can bind isolated subunits.

p27 was first identified as a CKI due to its ability to block the activity of cyclin E/CDK2 and cyclin A/CDK2 in cells arrested in G1 by TGF-ß, lovastatin and contact inhibition (Kudo *et al.*, 2005). The association of p27 with CDK-4 cyclin D or with CDK2-cyclin E complexes blocks phosporylation of CDK4 on Thr 172 and CDK2 on Thr 160 via CDK activation kinases. p27 can be induced by cyclic AMP and other negative regulators of the cell cycle and can be down-regulated by interleukin 2 (Lloyd *et al.*, 1999).

p27 is generally viewed as an integral component of the cell cycle machinery controlling the cell's proliferative capacity. The levels of p27 protein increased in quiescent cells and rapidly decreased after stimulation with mitogens or as cells reenter the cell cycle (Clurman and Porter, 1998, Lloyd *et al.*, 1999). p27 can directly binds to the cyclin-CDK complexes and inhibit their enzymatic activities (Coqueret, 2003, Lloyd *et al.*, 1999). This will disrupt the progression of G1 to S phase and leads to apoptosis of the cells (Kang *et al.*, 2002). In addition to its role as a CDKI, p27 is a putative tumor suppressor gene, regulator of drug resistance in solid tumors, and a promoter of apoptosis (Lloyd *et al.*, 1999).

There are two findings which contribute to the indication of p27 as a putative tumor suppressor gene. The first evidence showed that *p27*-knockout mice developed multiorgan hyperplasia and parathyroid tumor, and *p27* haplo-insufficient mice have become more sensitive to tumor development induced by radiation or chemical carcinogens. Furthermore, adenoviral gene transfer of p27 into breast cancer cell lines has shown successful results in arresting the cell cycle progression and apoptosis. They have also demonstrated that the transduction of *p27* via an adenoviral vector into human lung cancer cell lines induces cell growth suppression via G_1-S arrest (Park *et al.*, 2004).

1.3.1.1 Regulation of p27 expression

In most of the cellular system, p27 levels are significantly regulated at the level of both protein translation and protein stability (Lloyd *et al.*, 1999, Philipp-Staheli *et al.*, 2001). In relation to that, it has been proposed that p27 half-life is much longer in quiescent cells compared to proliferating cells. There are three mechanisms by which the levels of p27 are controlled, ie. ubiquitination/phosphorylation and methylation (Lloyd *et al.*, 1999).

Ubiquitination and/or proteosome activities are the believed procedures which control the p27 protein degradation (Figure 1.5) (Lloyd *et al.*, 1999). Enhancement of the proteasomal degradation may result to the decrement of p27 levels (Nho and Sheaff, 2003). Enzymes E1, E2 and E3 are 3 important enzymes which involve in the ubiquitination process, targeting on the ubiqutin-target protein complex specifically. These three enzymes are targeting on ubiquitin molecules, internal cystein residue and internal systemic residue which involve in ubiquitination processes. Another mechanism which has been shown to contribute in ubiquitination processes is phosphorylation (Lloyd *et al.*, 1999). p27 phosphorylation is actively taking place during S and G2 phase in cell cycle progression (Lloyd *et al.*, 1999, Sa and Stacey, 2004). Increasing evidences indicate that p27 must be initially phosphorylated by cyclin E/CDK2 on Thr187. The p27 phosphorylation occurs on a conserved carboxyl-terminal CDK target site before being degraded by the proteasome (Lloyd *et al.*, 1999, Nho and Sheaff, 2003, Sa and Stacey, 2004).

Another important mechanism which regulates the p27 levels in cells is DNA methylation (Lloyd *et al.*, 1999, Nakatsuka *et al.*, 2003). p27 methylation has been observed in the lymphoid malignancies suggesting that the mechanism might contribute to the loss of

p27 expression which give the cell proliferative advantage (Nakatsuka *et al.*, 2003). However, the ultimate importance of methylation of p27 gene in regulating its function is not known (Lloyd *et al.*, 1999).

1.3.1.2 p27 and cancer

Since the last few years, many researches have been carried out concerning the importance of p27 expression in many types of tumors. p27 has met the three criteria which validate it to be a prognostic marker and/or diagnostic marker. According to Steeg and Abrams (Steeg and Abram, 1997), as a new prognostic marker, it must provides information independent of and better than conventional pathological criteria, it should also gives information that can alter treatment decisions and the studies with the marker are reproducible (Lloyd *et al.*, 1999).

The level of p27 protein expression is the most crucial component which has been studied. In many of the reported studies, p27 protein expression often showed decrement in more aggressive tumors, such as brain tumors (Park *et al.*, 2004), breast carcinoma, pituitary tumors, oral squamous cell carcinoma and lung non-small cell carcinoma (Kudo *et al.*, 2005, Lloyd *et al.*, 1999). In the studies done by Fuse *at al.* (Fuse *et al.*, 2000) and Kirla *et al.* (Kirla *et al.*, 2003) showed that decrement of p27 protein expression occurred in higher grades of brain tumors.

Downregulation of p27 protein expression is believed to contribute in cancer progression by upregulating cyclin-CDK activities, which drive inappropriate cell cycle progression and lead to uncontrolled proliferation of cancer cells (Nho and Sheaff, 2003).