CHAPTER 1

REVIEW OF LITERATURE
Brain tumors

The human brain is made up of different types of cells, including the neurons and the glia. While neurons are the main functional cells of the brain, glia protect and nourish the neurons by making up the myelin sheath of neurons, absorbing excess neurotransmitters and also form the blood-brain barrier (BBB). Brain tumors result when one type of cell transforms from its normal characteristics and grows and multiplies in an abnormal way. They can be commonly classified as primary and secondary tumors (*Figure 1*) (1-3). Brain tumors that result from transformation and abnormal growth of brain cells are called primary brain tumors because they originate in the brain (4, 5). Usually they are named after the part of the brain or the type of brain cell from which they arise.

Classification of brain tumors

Primary brain tumors that originate from brain cells other than glia are of different types ([www.medcyclopaedia.com](http://www.medcyclopaedia.com), [www.medindia.net](http://www.medindia.net)):

**Medulloblastoma or primitive neuroectodermal tumor:** This tumor usually arises in the cerebellum. Rarely do these tumors spread outside the brain. It is the most common brain tumor in children.

**Meningioma:** This tumor arises in the meninges and grows slowly. Meningioma are benign and do not spread from their original site. Malignant meningiomas are rare.

**Schwannoma:** This tumor arises from the Schwann cells. These cells line the nerve in the inner ear that controls balance and hearing. The tumor is also called an acoustic neuroma. It occurs most often in adults. They are more common in people who have a genetic disease called neurofibromatosis type 2.

**Craniopharyngioma:** The tumor grows at the base of the brain, near the pituitary gland. This type of tumor most often occurs in children.

**Haemangioblastoma:** This is a rare type of tumor that develops from cells that line the blood vessels. They are benign and grow slowly.

**Pituitary tumors:** These types of tumors develop in the Pituitary gland. They are benign and are called pituitary adenomas.

**Germ cell tumor of the brain:** The tumor arises from a germ cell. Most germ cell tumors that arise in the brain occur in people younger than 30 years. The most common type of germ cell tumor of the brain is a germinoma.

**Pineal region tumor:** This rare brain tumor arises in or near the pineal gland. The pineal
gland is located between the cerebrum and the cerebellum. The most common tumors are germinomas, teratomas, pineocytomas and pineoblastomas.

**Gliomas**

Primary brain tumors that arise from the glia are termed gliomas. The term “glioma” was introduced by Rudolf Virchow in 1860 (6). Gliomas are classified, on the specific type of cell from which they originate, as follows:

**Astrocytoma:** The tumor arises from star-shaped glial cells called astrocytes. In adults, astrocytomas most often arise in the cerebrum (7). In children, they occur in the brain stem, the cerebrum, and the cerebellum. Glioblastoma multiforme (GBM or the type 4) is the most common astrocytoma.

**Brain stem glioma:** The tumor occurs in the lowest part of the brain. Brain stem gliomas most often are diagnosed in young children and middle-aged adults.

**Ependymoma:** The tumor arises from cells that line the ventricles or the central canal of the spinal cord. They are most commonly found in children and young adults.

**Oligodendroglioma:** This rare tumor arises from cells that make the fatty substance that covers and protects the nerves. These tumors usually occur in the cerebrum. They grow slowly and usually do not spread into surrounding brain tissue. They are most common in middle-aged adults.

The other classification of gliomas is that proposed by World Health Organization in based on their degree of malignancy identified by histological feature. This is a more widely used classification and includes the following grades:

**Grade I or pilocytic astrocytoma:** biologically benign, surgically cured

**Grade II or astrocytoma:** low grade malignancies that follow long clinical courses but not cureable by surgery. Oligodendrogliomas are also included as grade II. 70% of grade II gliomas transform into grade III and IV tumors. This is termed as “angiogenic switch” (8).

**Grade III or anaplastic astrocytoma:** malignant, lead to death within few years. Anaplastic oligoastrocytomas are also included as grade III.

**Grade IV or glioblastoma multiforme (GBM):** highly malignant, resistant to chemotherapy and lethal within 9-12 months.

Secondary brain tumors or metastatic tumor occur when cancer cells from other parts of the body, such as the lung, breast, skin, kidney, colon spread to the brain. These tumors
cells reach the brain via the blood-stream. Secondary tumors in the brain are far more common than primary brain tumors (4).

**Figure 1: Classification of Brain tumors**

**Glioblastoma Multiforme (GBM)**

Type 4 or glioblastoma multiforme is most common and most aggressive (account for almost 50-60%) of adult primary brain tumors. The median survival of GBM patients is dismal and ranges between 9-12 months. GBMs have been reported to commonly overexpress oncogenes such as epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR) and consist of deletions or mutations in tumor-suppressor genes like p53 and PTEN (3, 4). These changes result in constitutive activation of signaling pathways that confer resistance to apoptosis, resulting in increased proliferation, growth, invasive and angiogenesis in the tumor cells. This leads to not only uncontrolled growth of the tumor cells but also the destruction of the surrounding normal tissue (4). Primary GBM presents in an acute *de novo* manner with no prior symptoms of low grade glioma. Secondary GBM derives consistently from lower grade astrocytomas by progressive transformation within 5-10y of diagnosis (*Figure 2*).
Figure 2: Chromosomal and genetic aberrations involved in the genesis of glioblastoma. Shown are the relationships between survival, pathobiology, and the molecular lesions that lead to the formation of primary (de novo) and secondary (progressive) glioblastomas. Although histologically indistinguishable, these grade IV gliomas occur in different age groups and present distinct genetic alterations affecting similar molecular pathways. For example, inactivation of p53 function occurs due to direct mutation in progressive GBMs or INK4aARF mutation/decrease in expression or MDM2 amplification in de novo GBMs. Similarly, activation of the PI3K pathway is achieved by several cooperative mechanisms, including EGFR amplification and mutation as well as PTEN mutation, although underexpression of PTEN in the absence of mutation is frequently seen as well. (OE) Overexpressed; (amp) amplified; (mut) mutated. Adapted from Furnari et al, Genes Dev. 2007 21: 2683-2710.

Symptoms:
GBM like most brain tumors commonly manifest symptoms such as headache, visual loss, seizures, vomiting, impaired memory, personality changes, impaired speech and paralysis on one side of the body.

Treatment:
The first attempt at a curative therapy of GBM is complete surgical resection, provided the location of the tumor is not close to critical areas that govern speech, movement,
breathing or thought processes. The complication in case of GBM is the ability of the tumor cells to move to a newer location within the brain and thus extend beyond apparent tumor boundaries. Given that complete surgical resection is not always achievable, adjuvant therapies play an important role in the treatment procedure. Surgical process is followed by intense chemotherapy and radiotherapy sessions. Nitrosurea derivatives constitute the main chemotherapeutic agents for adjuvant therapies that have entered in routine clinical treatment protocols. Nitrosurea constitute alkylating agents that induce strong DNA damage. The other common glioma drugs used in chemotherapy are temozolomide, procarbazine, lomustine and vincristine. These however are unable to cross the blood-brain barrier (BBB) in sufficient concentration. In more recent times, the combined administration of radiotherapy with temozolomide has been found to have a survival advantage over older therapies and increases the survival of patients by 2-2.5 months.

Molecular therapeutic strategies aiming at manipulation of skewed pathways are being vigorously pursued that can result in targeted killing of tumor cells and not the surrounding normal cells. Pathways involving Epidermal Growth Factor Receptor (EGFR), Platelet-derived Growth Factor (PDGFR), Mitogen-activated Protein Kinase (MAPK), Vascular Endothelial Growth Factor (VEGF), Phosphoinositide 3 kinase (PI3K/Akt) etc, are being extensively studied for developing monoclonal antibodies, immunotoxins, pathway inhibitors, anti-angiogenesis (interfere with growth of blood vessels that feed the tumor) and anti-invasive agents being designed for targeted molecular therapy of GBM. Most of these approaches are experimental or are in clinical trials (3).

**Tumor microenvironment and TAMs**

A relationship between tumors and inflammation has been observed as early as 1863 by Rudolf Virchow (9). The inflammatory infiltrate of both primary and secondary tumors consists of large numbers of host macrophages in the stromal compartment that forms the microenvironment of the tumor. The microenvironment is known to help maintain the cells’ quiescent state and preserve their potential to proliferate and differentiate (10). The “resident” tissue macrophages are termed as Tumor associated macrophages or TAMs and express a distinct phenotype that differs from that seen in nonmalignant tissues (11). Although few studies have shown a correlation between high TAM numbers and good prognosis, a good majority have linked them to reduced patient survival and poor
prognosis (12). Macrophages derived from inflamed tissues have the propensity to lyse tumor cells; however, tumor-derived molecules (such as IL-4, IL-10) reduce this ability (13, 14). The migration of macrophages to tumor sites results in a symbiotic relationship between the tumor and TAMs (Figure 3). The tumor cells sustain their survival while they are exposed to different microenvironmental signals that “program” them to perform functions that are required by the tumor cells (11, 15). There is a high correlation between increased TAM numbers and high vascular grades of many tumor types including breast carcinoma, malignant melanoma and glioma (16-18).

A malignant tumor cannot grow beyond 2-3mm³ in size without angiogenesis that provides a route for nutrients and oxygen. Macrophages are multifunctional cells with the potential to perform diverse functions such as secretion of pro-angiogenic factors, cytokines and matrix-degrading enzymes. They produce factors such as interleukin-8, vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), transforming growth factor-α (TGF-α) and basic fibroblast growth factor (bFGF) (19-22). TAMs also secrete a variety of pro-inflammatory cytokines – Tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and enzymes - matrix metalloproteinases (MMP-2, MMP-7, MMP-9, MMP-12) (23). Angiogenin, a potent pro-angiogenic protein is expressed both by tumor cells and TAMs and is induced by pro-inflammatory cytokines such as TNF-α and IL-1β (11). TAMs contribute to tumor progression by synthesis of a serine protease known as Urokinase (uPA) that degrades extracellular matrix in tumors and thus promotes tumor angiogenesis, invasion and metastasis (24, 25). Lysosomal proteins called cathepsins expressed in TAMs have been implicated in invasion and metastasis processes. Two members cathepsin D and H have been directly linked to the aggressive nature of glioblastomas (11, 26, 27). Studies show that TAMs decrease the expression of E-cadherin that has been implicated in Epithelial-mesenchymal transition (EMT), a process by which epithelial cells separate from their neighbors and migrate to distal regions during development. Invasion of tumor cells has been proposed to follow a process similar to EMT (28, 29). The factors released by TAM into the microenvironment have been shown to upregulate certain genes in the tumor cells that induce angiogenesis, invasion, cytokine production, inflammation, adhesion, cell growth, cell cycle regulation, metabolism and other unknown functions. The up regulated genes include IL-6, IL-7R, IL-8, NF-κB, ICAM-1, MMP-9, MMP-1, VEGF-A, VEGF-C (16).
Figure 3: Potential pro-tumor effects of TAMs on cancer cells. The interaction between TAMs and cancer cells (Ca) may enhance cancer cell growth, invasion, metastasis and angiogenesis by stimulating cancer cells or TAMs to express multiple gene products that are involved in the regulation of tumor-associated angiogenesis, cell cycle, inflammation, signal transduction, invasion, and activities of protease and adhesion molecules. G0S2, G0/G1 switch gene 2; TIMP-1, matrix metalloproteinase tissue inhibitor-1; ICAM-1, intercellular adhesion molecule-1; IL-6 ST, interleukin-6 signal transducer; STC-1, stanniocalcin-1; PDGF, platelet-derived growth factor. Adapted from Shih et al, J. Cancer Mol. 2006 2(3):101-106.

Tumor cells thus interact with various components in the vicinity and result in the formation of complex tumor tissues. The study of tumor microenvironment and TAMs is therefore gaining greater relevance in improving the existing cancer therapy strategies. Cancer treatment strategies should aim at the elimination of not only the cancer cells but also their niche to avoid recurrence.

**Tumor Necrosis Factor -α (TNF-α)**

The crucial aspect of tumor microenvironment is the cytokine-mediated communication between the tumor and stromal cells. TNF-α is a member of TNF/TNFR cytokine super
family. The maintenance of immune system homeostasis, inflammation and host defense are the major functions of this cytokine (30). It is also involved in pathological processes such as chronic inflammation, autoimmunity and malignant disease. TNF-α is produced majorly by macrophages and also by a variety of other cells like fibroblasts, keratinocytes, tumor cells, Kupffer cells and astrocytes.

TNF-α is produced as 26KDa membrane-bound pro-peptide, that is cleaved by TNF-α-converting enzyme (TACE) to 17KDa soluble form. It binds as a homodimer to two distinct homotrimeric cell surface receptors – TNFR1 (p55) and TNFR2 (p75) (31, 32). Ligand binding to TNF receptors regulates at least four distinct pathways: a pro-apoptotic pathway that is induced by binding of caspase-8 to FADD; an anti-apoptotic program activated by binding of cIAP-1 to TRAF2; AP-1 activation via JNK-dependent kinase cascade and NF-κB activation by RIP (33, 34). TNF-α promotes motility and invasion of the tumor cells via induction of matrix metalloproteinases, by stimulating fibroblast and macrophage activity (35, 36). It has also been shown on colorectal cancer that TNF-α modulates the process of EMT (37). Thus TNF-α has conflicting roles in cancer – as both necrotic and growth promoting factor (30). Although it has powerful anti-cancer properties when administered at higher doses, it does not have the ability to kill most types of cancer cells in which the program of apoptosis has been turned off by NF-κB (38-40). It is now clear that TNF-α performs multiple activities that permit cell-cell communication and the outcome is determined by the concentration of the cytokine, cell type and the milieu (41).

Matrix Metalloproteinases (MMPs)

The hallmark of human malignant gliomas is their invasiveness and vascularity. Tumor cells invade beyond the tumor mass and render them surgically incurable. The process of invasion and angiogenesis involves the degradation of the extra cellular matrix (ECMs) components by matrix metalloproteinases (MMP) family of proteolytic enzymes (42-44). MMPs are thus involved in a variety of physiological and pathological tissue remodeling processes, including wound healing, embryo implantation, tumor invasion, metastasis and angiogenesis. MMPs belong to the family of zinc-dependent, neutral endopeptidases that degrade most extra cellular matrix (ECM) components and are overexpressed in most cancers, leading to poor prognosis (42, 44). The localization of MMPs under physiological conditions dictates their biological function. Most MMPs localize to
specific areas that mediates interaction with surface receptors such as integrins and reduces the accessibility to endogenous inhibitors (42, 45). They are principally secreted proteins and their activity is controlled at the levels of gene transcription, zymogen activation and inhibition of active forms by tissue inhibitors of MMP (TIMP) (46). Of total 24 MMPs identified, five belong to stromelysin subclass (MMP-3, -10, -11, -7, and -26), two belong to the gelatinase subclass (MMP-2 and 9), four are collagenases (MMP-1, -8, -13 and -18), six are membrane type MMPs (MT-MMP1 to -6) and others belong to their subclasses (47). The general structure of MMPs shows three domains – the pro-peptide, the catalytic domain and the C-terminal region. MMPs are expressed in enzymatically inactive state. A mechanism called “cysteine switch” mediated by proteolytic removal of the pro-domain or chemical modification of the cysteine residue makes the enzyme proteolytically active (48). Their contribution to cancer invasion and metastasis was initially related to their capacity to degrade ECM components although recent studies reveal their multifunctional molecules with cell surface molecules, growth factors, growth factor binding proteins and cytokines/chemokines as substrates (42). The activity of MMPs in the pericellular space is controlled by specific inhibitors called Tissue inhibitors of metalloproteinases (TIMPs). All the four TIMP family members known, share structural features, with a molecular weight of ~21KDa (49). They bind to the zinc-binding catalytic site of the MMPs with a 1:1 molar ratio (50). The maintenance of a critical balance between the levels of activated MMP and free inhibitors determines the overall MMP activity; a disturbed balance affects the invasive process (44).

Studies show a high correlation between high levels of MMP-2 (Gelatinase-A) and MMP-9 (Gelatinase-B) that degrade gelatin and collagen type IV and mediate glioma invasion, migration and angiogenesis. MMP-2 and MMP-9 differ from other MMPs in their ability to interact with TIMP-2 and TIMP-1 respectively as proenzymes (23, 46, 47, 51). Studies showed that this interaction is necessary for the activation of MMPs. TIMP-1 and -2 are expressed in normal brain and in tumor tissues but are reported to be significantly lower in invasive glioblastomas (44, 52). Though both MMP-2 and -9 are overexpressed, the latter has more prominent role in neovascularization of gliomas. Most of MMP-9 protein was studied to be localized in tumor cells and faintly in blood vessels, while there was a pronounced expression of MMP-2 in both tumor cells and the surrounding blood vessels. Both proteins were observed to be absent in the surrounding normal brain tissue (53). There is a close interplay between the cytokines such as IL-1,
TNF-α, TGF-β, produced by the TAMs and the expression of MMP-9 in the invasion process of gliomas (47).

**Nuclear Factor- kappa B (NF-κB) pathway**

Nuclear Factor-kappa B (NF-κB) is a transcription factor family that was identified in 1986 by David Baltimore as a nuclear factor bound to an enhancer element of the immunoglobulin (Ig) κ light chain, believed to be specifically expressed in B cells. It is now clear that it is found in all cell types and governs the expression of large number of genes in response to infections, inflammation and stressful situations requiring rapid reprogramming of gene expression. The NF-κB family consists of five distinct members that form hetero- or homo dimers. The members are identified as NF-κB1 (p50 and its precursor p105), NF-κB2 (p52 and its precursor p100), c-Rel, RelA (p65) and RelB. NF-κB dimers are maintained in inactive form by sequestration in the cytoplasm by the inhibitory proteins, the IκBs that bind to the nuclear-localization sequence and prevent its entry into the nucleus. In response to pathogens or cytokines (TNF-α, IL-1β), LPS, UV light, the NF-κB dimer enter the nucleus, where it becomes potent transactivator (www.NF-kB.org) (54-56).

The activation of the p65/p50 dimer and its subsequent release into the nucleus has been well studied. The cytoplasmic complex of IκB kinase complex (IKK) phosphorylates IκBα on two serine residues (S32 and S36), making it a substrate for ubiquitination and proteolysis by 26S proteasome. The NF-κB dimer is released to enter the nucleus and stimulate gene expression. The subcellular signaling induced by the binding of cytokines such as TNF-α and IL-1β, activates the IKK complex made up of IKKα and IKKβ (catalytic subunits) and IKKγ (regulatory subunit) (57-61).

There are at least three separate pathways for NF-κB activation (*Figure 4*). The canonical NF-κB pathway has been defined with respect to activation of RelA- NF-κB1 (p50) dimer in response to TNF-α, IL-1β and other pathogens. This signaling is typically triggered through TNFR (TNF receptor), IL-1R (IL-1 receptor) or TLR (Toll-like receptor). Signals mediated by IKKβ, result in degradation of IκBα and translocation of RelA-p50 dimer to the nucleus. This pathway is the first response to inflammation (62, 63).

The non-canonical or alternate pathway is characterized by the inducible phosphorylation of p100 by IKKα, leading to activation of RelB/p52 dimers. The upstream kinase that
activated IKKα has been identified as NIK (NF-κB-inducing kinase). The role of this pathway in inflammation is not very clear (64). However, it plays an important role in the generation of cell-mediated immunity (65-67).

The third is the atypical pathway and refers to DNA-damage-induced activation of p65/RelA, c-Rel and p50. Unlike the other pathways, the atypical pathway is independent of the IKK complex and relies on kinases such as MAPK p38 and casein kinase 2 (68).

**Figure 4: Activation of NF-κB proteins.** The NF-κB proteins can be activated by three distinct signaling pathways: (A) canonical or classical, (B) non-canonical or non-classical and (C) atypical. Key: extracellular - E, cytosolic - C and nuclear - N; black circles represent phosphorylated amino acids. Adapted from book chapter “The NF-κB Signaling Pathway in GBMs: Implications for Apoptotic and Inflammatory Responses and Exploitation for Therapy” by Laver et al, CNS Cancer, Cancer Drug Discovery and Development, 2009.

**NF-κB in cancer**

A role for NF-κB in cancer is supported from numerous reports showing that NF-κB is activated in a number of tumors. NF-κB (Rel A) activation has been documented in a variety of solid tumors including those of breast, prostate, melanoma, pancreatic cancer, lung adenocarcinomas, colorectal cancers and gliomas. There are several mechanisms by
which NF-κB causes oncogenesis (Figure 5). Suppression of apoptosis when NF-κB is activated by growth factors and cytokines in the tumor microenvironment is associated strongly with oncogenic potential (69-73). Upregulation of cyclin D1 gene by NF-κB is associated with enhanced transition from G1 to S phase. Cox-2, a protein involved in inflammation is upregulated by NF-κB and is a known promoter of angiogenesis. NF-κB is known to upregulate expression of cell adhesion molecules ICAM-1, VCAM, E-Selectin and proteases like MMP-2 and MMP-9, implicating it directly in metastasis and invasion (74-76). NF-κB positively regulates TNF-α gene expression and TNF-α in turn activates NF-κB. It has been speculated that the resistance of gliomas to existing therapies is due to activated NF-κB that plays a major role in growth and tumorigenesis of high grade gliomas (73, 77).

**Figure 5: NF-κB activation in GBM, its target genes, and the hallmarks of cancer.** NF-κB is constitutively activated in gliomas. While no single mechanism is likely responsible for this phenomenon, in gliomas, NF-κB may be inappropriately activated in numerous ways. These mechanisms include elevated immune cell filtration (TAM) and cytokine secretion (IL-1β and TNF-α), the absence (dashed lines) of intracellular negative regulators (ING4, PIAS3, ARF), or the presence of elevated levels of positive regulators of NF-κB (Pin1). Additionally, numerous
growth factors are elevated and/or their receptors are amplified and positively affect the PI3K pathway, which activates NF-κB. Once activated, NF-κB induces the expression of genes whose products regulate processes involved in cancer formation and progression. These processes include promoting cell growth, angiogenesis and cell migration and invasion, and inhibiting apoptosis. Adapted from book chapter “The NF-κB Signaling Pathway in GBMs: Implications for Apoptotic and Inflammatory Responses and Exploitation for Therapy” by Laver et al, CNS Cancer, Cancer Drug Discovery and Development, 2009.

NF-κB activity is much higher in GBM compared to the surrounding non-GBM tissue. The levels of NF-κB activation correspond with increasing tumor grade in astrocytic tumors (78). The activation of NF-κB is an inevitable consequence of the tumor microenvironment and the numerous proteins and pathways that are dysregulated in gliomas. The secretion of TNF-α and IL-1β by the TAMs leads to extended activation of NF-κB pathway, that remains unaffected by the synthesis of inhibitor, IκBα (79). Other factors that affect prolonged NF-κB activation in gliomas are the mutated ING4 gene (Inhibitor of growth4) that acts as negative NF-κB regulator and/or elevated levels of Pin1 protein that stabilizes p65 subunit (80). Also, elevated NF-κB activity induces expression of MDR1 (multi-drug resistance1) protein that renders gliomas particularly resistant to many chemotherapeutic agents (68).

**PI3K / Akt (PKB) pathway**

Phosphoinositide 3-kinase (PI3K) plays a crucial role in survival and a broad range of cellular functions in response to extracellular signals (81-83). PI3K phosphorylates and regulates a number of kinases, transcription factors and regulatory molecules (82). PI3K activity is required for growth factor-dependent survival of a wide variety of cultured cell types, and to block the apoptosis induced by toxic stimuli via downstream pathway activation (81, 84, 85). The effectors such as Mitogen-activated Protein Kinase (MAPK) cascade may sometimes act independently to promote survival (86). The product of PTEN antagonizes the effects of PI3K / Akt pathway; overexpression is sufficient to lower the basal phosphoinositide levels in cells. However, PTEN being mutated in most human malignancies, many components of the PI3K pathway have been found to be deregulated leading to extensive cellular transformation (82, 87). A number of targets of PI3K have been implicated in the suppression of apoptosis, among which Akt/PKB has
been found to be sufficient to block apoptosis induced by a number of death stimuli (Figure 6) (88).

![PI3K/Akt pathway diagram](image)

**Figure 6: The PI3K/Akt pathway.** GSK3 (glycogen synthase 3), p70S6K (ribosomal protein S6 kinase), BAD (Bcl-2, BclXL-antagonist causing cell death), IKK (IκB kinase), eNOS (endothelial nitric oxide synthase), mTOR (mammalian target of Rapamycin), 4E-BP (eukaryotic translation initiation factor 4E binding protein). Adapted from Paez and Sellers, Signal transduction in Cancer, Cancer treatment and research, 2004,115 (2):145-167.

The serine / threonine kinase Akt, also known as protein kinase B (PKB), is a critical node of signaling responsive to growth factors, cytokines and other stimuli. c-Akt is the cellular homolog of the transforming oncogene of the AKT8 retrovirus. A recombination event between viral gag sequences and the cellular Akt gene resulted in a fusion protein (82). There are three closely related isoforms of mammalian Akt family- Akt1 (PKβα), Akt2 (PKββ) and Akt3 (PKβγ) that are encoded by three different genes on chromosomes 14q32, 19q13 and 1q43 respectively. Though the three isoforms are widely expressed, Akt1 and Akt2 are primarily expressed in brain and testes, while the tissue distribution of Akt3 is more restricted (89, 90). All the isoforms are activated by a common mechanism and have similar structure. The central kinase domain has specificity
for serine or threonine residues in substrate proteins. The amino terminus includes a pleckstrin homology (PH) domain that promotes lipid-protein and/or protein-protein interactions (91, 92). The carboxy terminus includes a hydrophobic and proline-rich domain. The primary structure of Akt is conserved across evolution with the exception of the carboxy tail (81).

An important step in the activation of Akt requires that it binds to phospholipids and translocates from the cytoplasm to the inner surface of the plasma membrane. This relocalization brings Akt in proximity to regulatory kinases that phosphorylate and activate Akt. Akt is a phosphoprotein with four phosphorylated sites. Two of the sites, S124 and T450 are basally phosphorylated. In addition, there are two inducibly phosphorylated sites - T308 and S473 that are required for Akt activity. 3-phosphoinositide-dependent protein kinases (PDK) act as the secondary messengers at the inner cell membrane that activate Akt. PDK-1 activates many of the kinases downstream of PI3K. It has also been implicated as the kinase that phosphorylates Akt at T308 (93). A second kinase, PDK-2, is essential for phosphorylation at S473 and complete activation of Akt. The molecular identity of PDK-2 has been debated for several years. Many candidate S473 kinases have been proposed including PDK-1, integrin-linked kinase (ILK), Akt/PKB itself, DNA-dependent Protein Kinase (DNA-PK) and most recently mTORC2 complex (94).

Akt phosphorylates and regulates a variety of substrates involved in important cellular functions including cell growth and survival, glucose metabolism and protein translation (Figure 6). These targets include GSK3, Insulin receptor substrate-1, mTOR, eNOS, NF-κB and Forkhead transcription factors, BRCA1, p21<sup>161/waf1</sup>, phosphodiesterase-3B, BAD, caspase-9 and Raf protein kinase (81, 95). Akt consensus phosphorylation site is also a binding site for 14-3-3 proteins that bind phosphoproteins and retain them in the cytoplasm (96). BAD phosphorylation by Akt inhibits its proapoptotic effects by allowing binding to 14-3-3 proteins in the cytoplasm. Forkhead transcription factors are directly phosphorylated and negatively regulated through Akt-dependent phosphorylation on three conserved residues (97). Upon phosphorylation, Forkhead binds 14-3-3 proteins and remains inactive in the cytoplasm. Human caspase-9, associated with initiation of apoptosis, is phosphorylated and inhibited by Akt. In addition to inhibition of pro-apoptotic factors, Akt also activates the transcription of anti-apoptotic genes through the activation of NF-κB (98). More recently, mTOR and its target S6Kinase have been linked to Akt signaling. mTOR and S6K phosphorylate the eukaryotic translation initiation
factor 4E (eIF4E) binding proteins (4E-BPs) and positively modulate the translation initiation process (99).

**Akt in cancer**

Tumorigenesis is a multi-step process that leads to the acquisition of six common features of transformed cells such as self-sufficiency in growth signals, insensitivity to growth inhibitory signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis (100). Akt being a major survival pathway, there are several mechanisms that lead to deregulated Akt activity such as, inappropriate activation of PI3K, Akt gene amplification, Akt protein overexpression and more frequently loss of PTEN (101). Akt1 gene amplification has been found in gastric adenocarcinomas. Increased Akt1 kinase activity has been reported in prostate, ovary and breast carcinomas (102). Mutations in Akt2 have been documented to be more frequent than Akt1 or Akt3. Akt2 gene is amplified and overexpressed in primary ovarian carcinomas and pancreatic cancer and is closely associated with aggressive tumor phenotype (103). With respect to migration and invasion processes, Akt1 and Akt2 have been reported to have differential effects. Akt1 inhibits metastasis and migration while Akt2 promotes the same (104). Overexpression of Akt and increased Akt kinase activity has been reported in non-small cell lung cancer cells, astrocytoma and GBM (105-107). Signal transduction pathways downstream of EGFR such as PI3K/Akt have been found to be critical in mediating gliomagenesis, survival, proliferation, invasion and migration of gliomas (107). Akt and its targets such as S6K are known to suppress apoptosis, enhance survival and mediate radiation resistance in GBMs. The levels of phospho-Akt and phospho-S6K have been found to be elevated in GBMs compared to non-GBM counterparts (108). Moreover, the activation of Akt pathway has been shown to elevate low grade astrocytomas to grade 4 GBMs. Gliomas that either have inactivated PTEN (40%) or activated Akt (80%) have the propensity to take on the characteristics of a GBM (109). The primary reason for this could be that Akt activation may enable the tumor to surpass the hypoxia-induced limits on proliferation and/or suppress Bad-induced apoptosis (106). Recent reports have observed a population of glioma cells that is resistant to therapy and result in poor prognosis. This population has been termed as brain tumor stem cells (110). Akt pathway regulates the survival and resistance of these stem cells in the perivascular niche following radiation therapy (111).
**mTOR pathway**

In the 1970s a bacterial strain, *Streptomyces hygroscopicus*, was isolated from the soil of a Chilean island named Rapa Nui. This strain of bacteria secreted a potent anti-fungal macrolide that was named Rapamycin. Later Rapamycin was discovered to be a powerful immunosuppressant and anti-proliferative agent. In eukaryotic cells, Rapamycin binds a small protein receptor, FKBP12 (FK506 binding protein 12) and the complex in turn binds to a large protein resulting in growth arrest. This protein was named as Target of Rapamycin (TOR) and in mammalian cells as mammalian Target of Rapamycin or mTOR (112-114).

The enzyme mTOR (FRAP, RAPT, RAFT or SEP) is an evolutionarily conserved 298KDa atypical serine/threonine kinase of PIKK family. mTOR pathway acts downstream of Akt and integrates many cellular signals to control cell growth, proliferation and protein synthesis (99, 113). mTOR activation depends on several inputs, including nutrients (amino acids), energy (ATP) and growth factors such as insulin. mTOR forms two distinct multi-protein complexes – mTORC1 and mTORC2 - that integrate cellular signals and affect a wide variety of responses (*Figure 7*) (115, 116).

The **mTOR complex 1** (mTORC1) formed by mTOR with Raptor (*regulatory associated protein of mTOR*), mLST8 and PRAS40 regulates translation through its targets S6Kinase, 4E-BP1 and eIF-4E (116). Raptor is an essential but non-enzymatic subunit of mTORC1. Raptor has been proposed as scaffolding protein to recruit substrates for mTOR and is indispensible for phosphorylation of S6K and 4E-BP1 (114, 117). Amino acid withdrawal (nutrient deprivation) or treatment with Rapamycin enhances or reduces Raptor’s binding to mTOR respectively (116, 117). mLST8 is another mTORC1 subunit but it is not essential for the integrity or function of the complex (118, 119). Other subunits of mTORC1 such as Rheb and PRAS40 also regulate its activity depending on cellular GTP levels and Akt signaling respectively (120, 121).

The **mTOR complex 2** (mTORC2) formed by the association of mTOR with Rictor (*Rapamycin-insensitive companion of mTOR*), mLST8 and mSIN1 is important in the regulation of actin cytoskeleton (122, 123) and phosphorylation of Akt (PKB) at the S473 residue (94). Rictor (200KDa) was the first identified subunit of this complex. The interaction between Rictor and mTOR is neither affected by Rapamycin nor by nutrient levels. Thus the physical structure and the physiological functions of mTORC2 are distinct from mTORC1. Rictor is essential for mTORC2 complex formation and its biological function. Rictor knockdown studies have revealed its role in regulation of actin
cytoskeleton through Rho-GTPase family proteins and PKC-α (113). More importantly, mTORC2 has been speculated to function as the PDK2 that phosphorylates Akt at S473, knockdown of Rictor blocked this effect (94, 124). The other subunits of mTORC2, Sin1 and mLST8 are essential for the function and assembly of the complex respectively (118, 125). Recent reports provide evidence that prolonged exposure to Rapamycin affects the phosphorylation of Akt (PKB) at S473 residue and thus affects mTORC2 which was earlier considered Rapamycin-insensitive (126, 127). Rictor contributes to cell cycle progression (128), cell survival and migration (129) and expression of transcription factors including HIF-2α (130).

**Figure 7: Model of the mTOR Signaling Network in Mammalian Cells** The mTOR signaling network consists of two major branches, each mediated by a specific mTOR complex (mTORC). Rapamycin-sensitive mTORC1 controls several pathways that collectively determine the mass (size) of the cell. Rapamycin-insensitive mTORC2 controls the actin cytoskeleton and thereby determines the shape of the cell. mTORC1 and possibly mTORC2 respond to growth factors (insulin/IGF), energy status of the cell, nutrients (amino acids), and stress. mTORC1 (and likely mTORC2) are multimeric, although are drawn as monomers. Adapted from Wullschleger et al, Cell, 2006,124:471-484.
mTOR in cancer

In recent years many malignancies have been identified to have hyper active mTOR pathway, particularly the cancers with elevated PI3K signaling or harboring PTEN mutations (131). mTOR lies downstream of PI3K/Akt pathway and is many cases activated by Akt. Aberrant activation of Akt upregulated mTOR growth signaling (114). The PI3K/Akt/mTOR pathway is found to be activated in 30-50% of prostate cancer, 30-60% of malignant gliomas, 30-50% of endometrial carcinoma, >50% of melanoma, >30% of renal cell carcinoma and ~10% of breast cancer (132). Elevated mTORC1 activity has been reported as the common underlying cause in many hamartoma syndromes. Upregulation of mTORC1 activity provides the cells with growth advantages in multiple biological processes including proliferation and survival. These cells are disorganized from the tissue and form benign tumors (133). In cancers resulting from elevated PI3K/Akt activity, the activation of mTORC1 is also upregulated. In many such cases Rapamycin and its analogs have been found to be effective in clinical trials. However, certain factors like intrinsic levels of proteins like c-myc and S6 Kinase have to be considered in the tumor cells for Rapamycin-based therapy to be effective (132).

Rictor in cancer

The activation of mTOR signaling pathway in response to insulin and nutrients has been studied widely. Its role in cancer progression, in response to growth factors is mostly studied with respect to the mTORC1-mediated pathway (134-139). The role of mTORC2 and its component proteins is an area that has become important in cancer progression only in recent years. Most of mTORC2 involve the complex subunit – Rictor. Hence the study of Rictor subunit in cancer progression is important to determine the role of mTORC2 in cancer. Rictor has been reported to be important for proliferation and anchorage dependent growth in breast (MCF7) and prostate cancer cells (PC3) (128). Rictor was observed to form a complex with Integrin-Linked Kinase (ILK) in breast cancer cells and rendered them resistant to cell death by mTOR inhibitors (140). Studies on cervical carcinoma (HeLa) cells showed the ability of Rictor to control the apoptosis and cell growth by regulating the activity of PKC-α (141). Rictor is reported to activate signaling by insulin-like growth factor-1 receptor and induce cell motility in gastric and pancreatic cancer cells (142). Increased activity of mTORC2 has been reported in gliomas and Rictor has been studied to promote the cell motility in these studies (143). The studies thus indicated that targeted inhibition of only mTORC1 pathway is effective.
but not sufficient to control the cancer growth and proliferation and have suggested that mTORC2-mediated pathway should also be explored as a target to control tumor growth.