

CHAPTER 2

Literature Review

2.1 Introduction

2.1.1 Oral cancer

At present, cancer is one of the most serious public concerns (1) and is still a serious life-threatening disease due to its morbidity and mortality (3-5). It creates a challenge for the medical team to seek a perfect treatment modality (3). Besides, cancer shows an increase in incidence in every continent (1, 6), at a rate greater than infectious diseases and other chronic non-infectious diseases, such as heart disease (6).

There are over 100 types of cancer, which are distinguished by sources of origin (1, 6-8). Two to five per cent of all cancers occur in the head and neck area, particularly occurring frequently in the oral cavity (5, 9). Oral and pharyngeal cancers are grouped together in the classification of International Classification of Diseases (ICD) and are considered to have the sixth highest incidence worldwide (1, 9-11) and the third most common cancer in developing countries (11). They include cancers of the tongue, lips, buccal mucosa, gingiva, hard palate, soft palate, floor of mouth, salivary glands, tonsils, nasopharynx, oropharynx, and laryngopharynx (3, 11-13). In addition, the most common type of oral and pharyngeal cancer is squamous cell carcinoma (1, 3, 10, 11, 13, 14), which is initially derived from one surface epithelial cell, lining the oral cavity and oropharynx (13). Other types, for example, non-squamous cell carcinomas, sarcomas, lymphomas, and melanomas, are rarely found (3). Thus, the term “oral cancer” can imply oral squamous cell carcinoma (OSCC).

Oral cancer is primarily found in the elderly diagnosed at the average age of sixty two (13). Nevertheless, its incidence has a tendency to increase

especially among younger people in many parts of the world with a high rate in northern Thailand (15). It is found more frequently in males than in females (3, 13). The region with the world's highest incidence is South Asia, particularly in India, Sri Lanka, and Pakistan (1). Other regions with high incidence are located in some parts of Western and Eastern Europe, Latin America and the Caribbean, and in Pacific regions (1). The distribution of oral cancer varies by age, ethnic group, culture and life style, and level of country development (3). However, globally the overall five-year survival rate for oral cancer is considerably lower than for other cancers and has not significantly changed during the last two decades (9, 11).

2.1.2 Risk factors and etiologies of oral cancer

Cancer etiologies are multifactorial (1) meaning that the paths that cells take on their way to turn cancerous are highly variable (8). Although there is a wide variety of the inductive agents, the scientists can discover the common etiologies of oral cancer (16) and currently many risk factors are already identified (13, 14, 17) (Table 1).

For OSCC, there are two major common risk factors, tobacco use and alcohol consumption. Tobacco use in the form of smoking and/or chewing is universally regarded as the greatest risk factor (3, 11, 13, 14). About eighty per cent of OSCC patients have a history of smoking and risk of developing OSCC increases with heavier daily use and longer duration of smoking (13). Not only do smoking forms of tobacco have a carcinogenic risk, but smokeless forms, including chewing tobacco, moist snuff, and dry snuff, are also implicated in oral cancer (13). Even though smokeless tobacco users are approximately twice as likely to develop oral cancer as those, who have never used such tobacco, the association between oral cancer and smokeless tobacco is not as strong as the relationship between oral cancer and tobacco smoking (13). The other major risk factor is alcohol consumption (11, 13, 18-20). Intake of alcohol remains high in many countries, particularly in Thailand (19). However, the mechanism of alcohol as an important etiologic agent for oral cancer is not well recognized (19, 20). Moreover, pure ethanol has never been shown to be carcinogenic in

laboratory experiments (19, 20). Thus, impurities in the alcohol may play a role in tumorigenesis.

Table 1 Risk factors/etiologies of oral cancer (14, 17)

- History of tobacco use (all forms)
- Alcohol consumption
- Betel quid (paan)
- Sexual history (multiple sex partners, unprotected sex, exposure to HPV)
- Infections (Candida, Syphilis, HPV)
- Exposure to ultraviolet light
- Exposure to ionizing radiation, arsenic or industrial chemicals
- Poor oral hygiene
- Low socioeconomic status
- Age > 40 years
- Mucosal diseases (iron deficiency associated with Plummer-Vinson Syndrome, lichen planus, lupus erythematosus)
- Immune system suppression

Besides tobacco and alcohol, betel quid has been used in some countries such as Thailand, Sri Lanka and Taiwan, and is considered to be a risk factor in oral cancer development (21, 22). A lot of research mentions synergistic outcomes of tobacco usage, alcohol consumption, and betel quid chewing (1, 10, 13, 19). Thus, reversal of betel quid use and smoking must be considered in therapeutic strategies for oral cancer (21).

Furthermore, exposure to sunlight ultraviolet and radiation, candidal and human papilloma viral infection, dietary problems and malnutrition, and immunosuppression are important risks, but less important than tobacco usage, alcohol consumption, and betel quid chewing (5, 10, 11, 13, 14). Long-term exposure to ultraviolet radiation is the primary causative factor in cancer of the lower lip vermillion, and radiation, such as x rays, is also risky (13). Nonetheless,

there is no association between oral cancer and the small amount of radiation generated by routine dental radiography (13). Viruses, particularly human papilloma virus (HPV), have recently been identified as a risk factor for development of OSCC (11, 13, 14, 18) and the subtype HPV-16 accounts for more than 90% of cases of HPV-positive squamous cell carcinoma (13, 14). Finally, a genetic predisposition has also been suggested due to the fact that the majority of the population exposed to these risk factors does not develop oral cancer, as well as the fact that sporadic cases of oral tumors occur in young adults and non-users of tobacco and alcohol (8, 11).

Taken together, the author can summarize that oral cancer etiology is multifactorial and many risk factors have been identified, including tobacco use, alcohol consumption, betel quid chewing, sunlight exposure and radiation, viral infection, and dietary problem.

Because cancer has a large variety of risk factors, this creates a continual question whether cancer progression has many sequences or not. Currently, it is accepted that carcinogenesis is a complex multistep process, called “multiple hit” (6, 8, 10, 11). This means that just one or a few alterations of gene expression is not adequate to directly or indirectly turn normal cell cancerous. Hence, accumulation of gene mutations must take a long period of time, at least a few decades. The progression demonstrates gradual alteration of the cell from hyperplasia to dysplasia, and eventually actual cancer, according to the level of gene alteration (8, 10).

2.1.3 Clinical characteristics of oral cancer

The most common site of oral cancer is the tongue, especially the lateral and ventral surfaces, whereas the occurrence on the dorsal surface is rare (13). Other common locations are the floor of the mouth, buccal mucosa, soft palate, and tonsils (13). The clinical signs and symptoms of OSCC vary between patient (13, 14). The most common clinical characteristic of OSCC is a non-healing sore or ulcer persisting longer than two weeks after removal of potential etiologic factors.

2.1.4 Behaviors and hallmarks of cancer

Under continuity of research on oncology, a large body of knowledge of the biological characteristics of cancer has been discovered. The characteristics of cancer can be defined in six hallmarks; 1) ability to generate their self-sufficient growth signals or respond to weak growth signals that are normally ignored by healthy cells; 2) insensitivity to antiproliferative signals; 3) resistance to cellular suicidal mechanisms that normally cause aberrant cells to die by apoptosis; 4) capacity for limitless replication; 5) ability to stimulate new blood vessel development in order to allow for tumor growth; and 6) capacity to invade tissues locally and later to metastasize distantly throughout the body (7, 8, 23, 24) (Figure 1). An additional characteristic of cancer cells is their potency to resist the therapeutic effects of radiation or hormonal treatment or chemotherapy. These six or seven characteristics are shared in common by perhaps all types of human cancers (8).

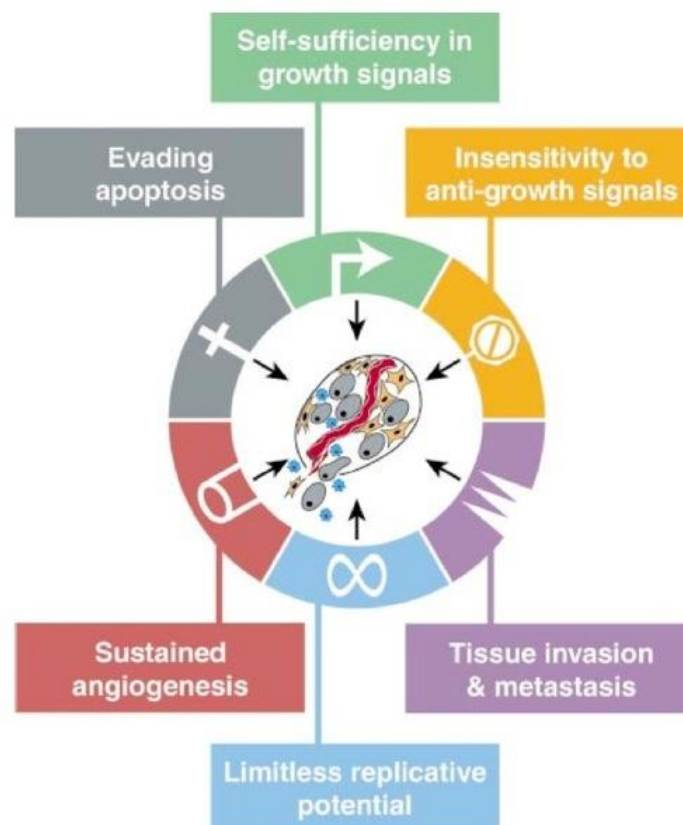


Figure 1 Hallmarks of cancer (8)

1) Self sufficiency in growth signals (8)

Progression through the mitotic cycle involves the successive formation, activation and subsequent inactivation of cyclin-dependent protein kinases (CDKs) (25). The kinases bind sequentially to a series of cyclins, which are responsible for differential activation of the kinase during the cell cycle (Figure 2). The G1 to S transition is thought to be controlled by CDKs containing D-type cyclins that phosphorylate the retinoblastoma protein, releasing E2F transcription factors. E2F is involved in the transcription of genes needed for the G1 to S transition. The G2 to M transition is carried by CDK complexes containing CycA and CycB cyclins. CDK complexes are kept in an inactive state by phosphorylation by the Wee1 kinase, and by interaction with inhibiting proteins (CKIs). At the G2 to M boundary activation of the kinase is brought about by release of the CKI protein, by positive phosphorylation (by CAK kinase), and by a still unidentified protein phosphatase (25).

A common characteristic of both benign and malignant tumors is the ability of a tumor cell to grow and divide beyond normal limits (26). Normal cells require mitogenic growth signals before moving from a quiescent (G_0) state into an active proliferative state (G1 to M phase) in the cell cycle (Figure 2) (8). These mitogenic growth signals are transmitted into the cell by signal transduction pathways and there are many regulatory network molecules controlling this progression (8, 23, 26). Independently from these mitogenic growth signals, the cancer cells can limitlessly proliferate upon activation of oncogenes by unclear mechanisms (8, 23, 26, 27).

2) Insensitivity to antiproliferative signals (8)

In normal cell cycle control, not only growth signals are critical for cell proliferation but counterparts of the signals, which are called antiproliferative signals, also play many roles. Examples of counterparts of the cell cycle progression are the inhibitors of cycle-dependent kinase or the cycle-dependent kinase inhibitor (CDKI or CKI) (Figure 2) (25).

In normal tissue, many antiproliferative signals work to maintain cellular quiescence and tissue homeostasis. Cancer cells must decrease these antiproliferative signals if they are to grow and proliferate (8, 26). For example, the reduced expression of CDKI and retinoblastoma protein (Rb) has been observed in various human cancers (8, 27).

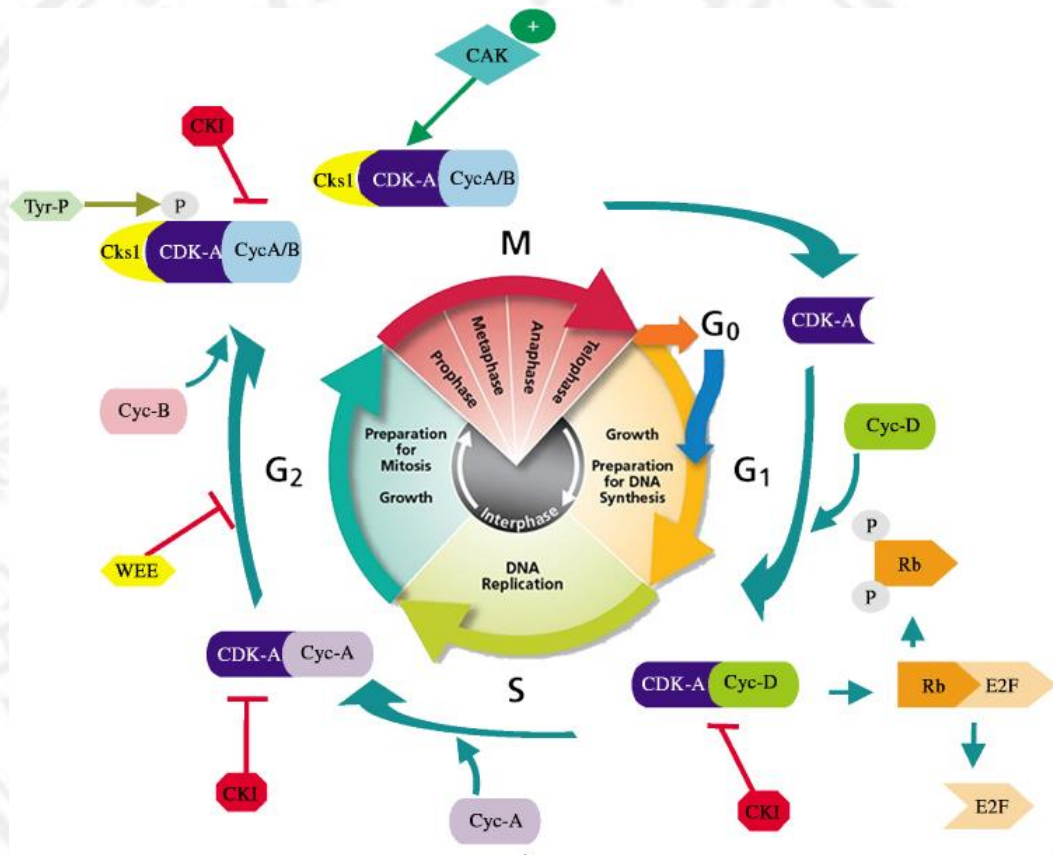


Figure 2 Stages of the cell cycle, and regulatory molecules (25)

3) Evasion of apoptosis (8)

The ability of cancer to increase the number of its cell population is not only determined by the increasing rate of cell growth and proliferation but also by the decreasing rate of cell attrition, or apoptosis, a type of programmed cell death (8). During cell division, which DNA opens and replicates, many processes can go wrong (23). Some cancer-causing stimuli can damage DNA seriously but the body has mechanisms to correct the mistake, including activation of a DNA repair system and/or induction of

apoptosis if the damage is very severe and beyond repair (23). It has been proven that apoptosis serves as a barrier to cancer development (8).

Conventionally, apoptosis occurs via two main pathways; (a) the death-receptor-induced extrinsic apoptotic pathway and (b) the mitochondrial-mediated intrinsic apoptotic pathway (8, 27-29) (Figure 3). Additionally, the third proposed mechanism of cell apoptosis is the apoptosis-inducing-factor (AIF) induced pathway, which is mediated by cysteine-aspartic proteases or cysteine-dependent aspartate-directed protease (caspase)-independent factors (29).

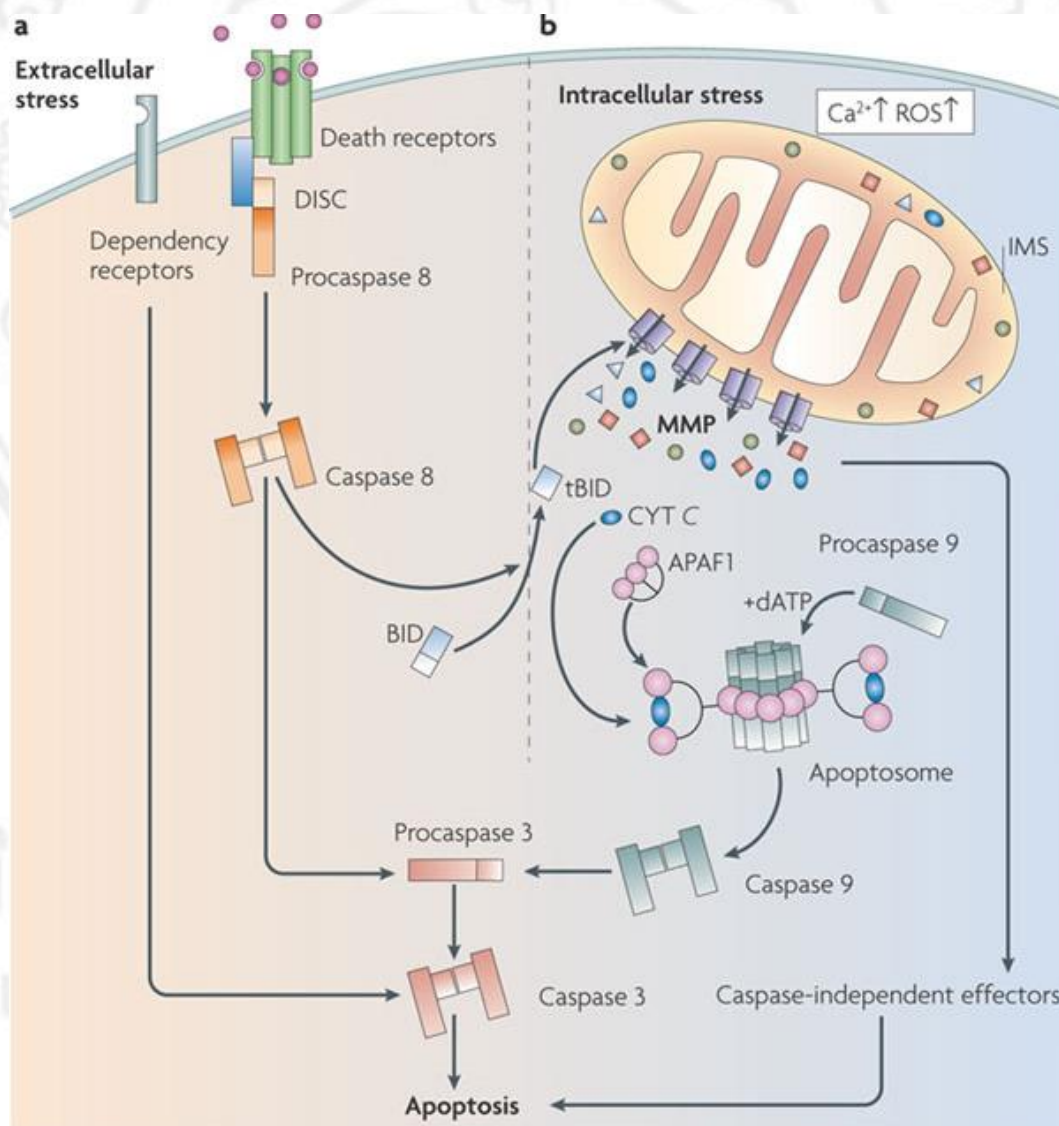


Figure 3 Apoptotic pathway (30)

For the mitochondrial-mediated intrinsic apoptotic pathway, mitochondrial membrane stability is determined by the Bcl-2 family, a family of proteins well-characterized as regulators of apoptosis (27). Whenever Bax, a proapoptotic subfamily of the Bcl-2 family, is activated or dephosphorylated by intracellular stress, mitochondrial instability results followed by the release of cytochrome C (Cyto C) that finally initiates caspase-9 and a downstream cascade of caspases leading to apoptosis (8, 27-29). On the other hand, extracellular signals can initiate apoptosis via the death-receptor inducing extrinsic apoptotic pathway (Figure 3). The extrinsic pathway is mediated by the activation of death-receptors, such as Fas and TNF receptors, resulting in initiation of caspase-8 and activation of its downstream, and eventually leading to apoptosis (8, 27-29).

By the two conventional apoptotic pathways, they are dependent on the ultimate effectors of apoptosis, termed cysteine aspartyl specific proteases or caspases, as gatekeeper of apoptosis (8); conversely, the AIF-induced pathway is another mechanism of apoptosis which does not use caspase, but is initiated by an effector molecule, AIF, released by the mitochondria whenever a cell is signaled to die (29). AIF migrates to the nucleus, passes through the membrane, binds to DNA, and triggers the characteristic chromatin condensation which is the hallmark of apoptosis, finally resulting in cell death (29).

Evidently, there are many survival mechanisms in malignant tumors (31). Developing resistance towards apoptosis, which is considered a hallmark of cancer, is one of the most important factors in the survival of malignant cells (23, 27, 29, 32). Thus, understanding of the mechanisms involved in the evasion of apoptosis plays an important role in perceiving cancer (29). Resistance to apoptosis can be acquired by cancer cells through a variety of strategies along apoptotic pathways (8) and the author wrote about the mechanism in another section in this literature review.

4) The capacity for limitless replication (8)

Generally, a normal cell cannot divide an infinite number of times — there is a kind of clock which keeps count, which has been discovered over the past decade (8, 23). During cell division, DNA is replicated by a protein called DNA polymerase (23). The result is double-strand DNA; one strand of which is the mother strand and the other is a new one; however, the DNA polymerase cannot replicate the last stretch of the mother DNA strand to which it binds (8, 23). This means that the end of each daughter DNA is shortened, compared to the original template, by about 100 nucleotides in each cell cycle (8, 23). This phenomenon is named “end replication.” The mentioned end of each chromosome is called the telomere, consisting of several thousand repeats of a short 6 base-pair sequence element (8, 23). Whenever the telomere is shortened too much due to a restricted number of replication times, the cell can no longer divide and becomes mortal by deterioration or by fusion with neighboring chromosomes (8). Nonetheless, the telomere can be maintained or elongated by telomerase, a reverse transcriptase enzyme (8). Although the body also contains cells that can divide eternally and are immortal, such as various types of stem cells (8, 23), in fully mature differentiated cells, expression of telomerase is suppressed and these cells cannot, therefore, divide infinitely (23). Terribly, cancer cells acquire the ability to elongate their telomeres and are not restricted to a certain number of replication, circumventing this limitation (8). At present, telomere maintenance is clearly a key component of the cancer’s capability for unlimited replication and this process is called “immortalization” (8, 23).

5) Sustainability of angiogenesis (8)

Before developing into a later stage of cancer or before invasion and metastasis, a malignant tumor requires additional supplies of nutrients and oxygen (8, 23, 26). For normal human tissue, the diffusion distance of oxygen is just a few hundred microns from the vessel (23). Then, when a tumor reaches 1-2 mm in diameter, the oxygen supply is insufficient for the

center of tumor mass due to the limited diffusion distance of oxygen or hypoxia (33). A characteristic, which needs to be acquired in order to resolve this cancer's problem, and is shared in common by most, and perhaps all, types of human tumors, is the capacity of cancer cells to induce neovascularization, or "angiogenesis" (8, 23, 26, 33).

Angiogenesis is the process by which new blood capillaries are generated from the preexisting vasculature (33). Tumors can activate angiogenesis by activation of angiogenesis inducers and inhibition of countervailing inhibitors, in order to supply the whole tumor mass and to develop its later stage of invasion (8).

6) Tissue invasion and metastasis (8)

The final hallmark of cancer that is responsible for the mortality of host in the severest stage of cancer is "metastasis" (34-36). Metastasis is an important negative prognostic indicator in many human cancers (34, 37), as 90% of patients die due to the cancer if metastasis is present (8). The word "metastasis" is derived from the Greek, "change in position," that refers pathologically to movement or migration of cancer cells to body parts distant from the primary site (34). Tumor metastasis is a clinical outcome that results from several sequential or complex multiple steps, similar to carcinogenesis, and depends on all of the other capabilities, not only angiogenesis, including: a) proliferation of cancer cells in the primary lesion; b) epithelial–mesenchymal transition (EMT); c) release of tumor cells from the primary tumor and invasion into vascular or lymphatic vessels; d) tumor cell migration in vessels; e) adherence of metastatic cells to endothelial cells in affected organ; f) extravasation of tumor cells out of blood vessel to specific target organ; g) mesenchymal–epithelial transition (MET) and survival in a new tissue environment where nutrients and space are not limited; and h) proliferation of tumor cells following variable periods of tumor latency (months to years) in order to form deleterious secondary tumors (8, 23, 34, 36-38).

EMT is a complex process in cancer biology (39) in which cancer cells switch from an epithelial phenotype to a mesenchymal phenotype (36, 39, 40). Normally, epithelium serves as the boundary between the external environment and the internal structure, and the epithelial barrier function is partly supported by firm cell-to-cell junctions, such as tight junctions and desmosomes (41). As a result of EMT, epithelial cells abandon their barrier function and characterize themselves as mesenchymal cells, leading to increased motility (39, 41). At the molecular level, cancer cells lose epithelial cell-to-cell junctions, and lose actin-cytoskeleton reorganization and epithelial cell markers, including cell adhesion proteins, such as E-cadherin and γ -catenin, but gain in the expression of mesenchymal markers such as vimentin, fibronectin, α -smooth muscle actin (SMA), and N-cadherin as well as increased activity of matrix metalloproteinases (MMPs), such as MMP-2, MMP-3, and MMP-9 (39, 40, 42). Even though EMT occurs physiologically in various steps of human morphogenesis and development, and in the healing process (13, 36, 40-42), it occurs pathologically in cancer progression (13, 36, 40, 42). Besides, EMT involves not only invasion and metastasis but also drug resistance (39).

In brief, the hallmarks of cancer are self-sufficiency in growth signals, insensitivity to antiproliferative signals, evasion of apoptosis, limitless replication potential or immortalization, sustainability of angiogenesis, and tissue invasion and metastasis.

2.1.5 Treatment modalities for oral cancer

Dental professionals may be the first individuals to suspect and identify potentially cancerous lesions before referring patients to oral and maxillofacial surgeons and oral medicine specialists (4). This early identification may be more critical than the treatment procedure. Although treatment modalities have improved significantly over the last few decades, the survival rate of patients with OSCC remains low (4, 12, 13, 43). The prognosis depends on the clinical stage of the tumor at the initial diagnosis. Staging is a classification system using the TNM

protocol, where T indicates the size of the primary tumor, N stands for involvement of local lymph nodes, and M refers to distant (below the clavicles) metastasis (4, 13). Once each of these factors is evaluated, a stage of I, II, III or IV is assigned to the cancer with the higher stages having the worse prognoses (13). The selection of treatment modality is roughly based on the location and stage of the tumor (13). Due to the heterogeneous nature of oral cancer, discovering a “silver bullet” for the treatment of oral cancer is very difficult (12). Consequently, continuous research is critical, to create a more effective treatment options for oral cancer (12).

At present, acknowledged treatment modalities for oral cancer consist of surgery, radiation, and chemotherapy, either individually or in combination (4, 13). Surgery may include a neck dissection if regional lymph node involvement is present or suspected (13). Both surgery and radiation have a long history, and general dentists are familiar with basic knowledge of these modalities but they result in many complications, such as deformity and osteoradionecrosis. Chemotherapy has become an area of great interest in conjunction with surgery (4). Its mechanism is to stop cancer cells from growing by inducing apoptosis (44, 45). Chemotherapy has many other pathways for killing cancer cells by focusing on the previously described hallmarks of cancer. Terribly, toxicities of chemotherapeutic agents, especially to quickly dividing normal cells, are frequently found and can be severe; therefore, chemotherapy may not be suitable for all patients (4, 32, 45). Accordingly, new therapeutic options must be developed with the goals of maximizing the desired effects on cancer cells while minimizing patient morbidity, and of improving survival and quality of life (13). “Targeting therapy” (sometimes referred to as targeted therapy) is a new cancer treatment modality, which blocks the growth of cancer cells by interfering with specific targeted molecules needed for carcinogenesis and tumor progression. It blocks cell-to-cell commutation or intracellular signaling, called “signal transduction pathway or signaling pathway,” not by induction of apoptosis as traditional chemotherapy (4). So, targeting therapy is a new hope and may be more effective than current treatments.

2.2 Signaling pathways and cancer progression

Prior to understanding the mechanism of targeting therapy, the knowledge of principle of signal transduction is required. The interplay between different cells and components has become increasingly well understood (46). Cell signals are commonly propagated by protein-protein interactions and are frequently controlled by protein phosphorylation (46). Protein phosphorylation is promoted by kinase and reversed by phosphatase (46). Importantly, regulation of protein phosphorylation is an important event in cellular function and gene expression (11). Phosphorylation occurs both extracellularly and intracellularly. The alterations of intracellular signals affect extracellular events; consequently, disruption of the normal intracellular signaling pathway can contribute to development of a variety of diseases, including malignancy (11, 26).

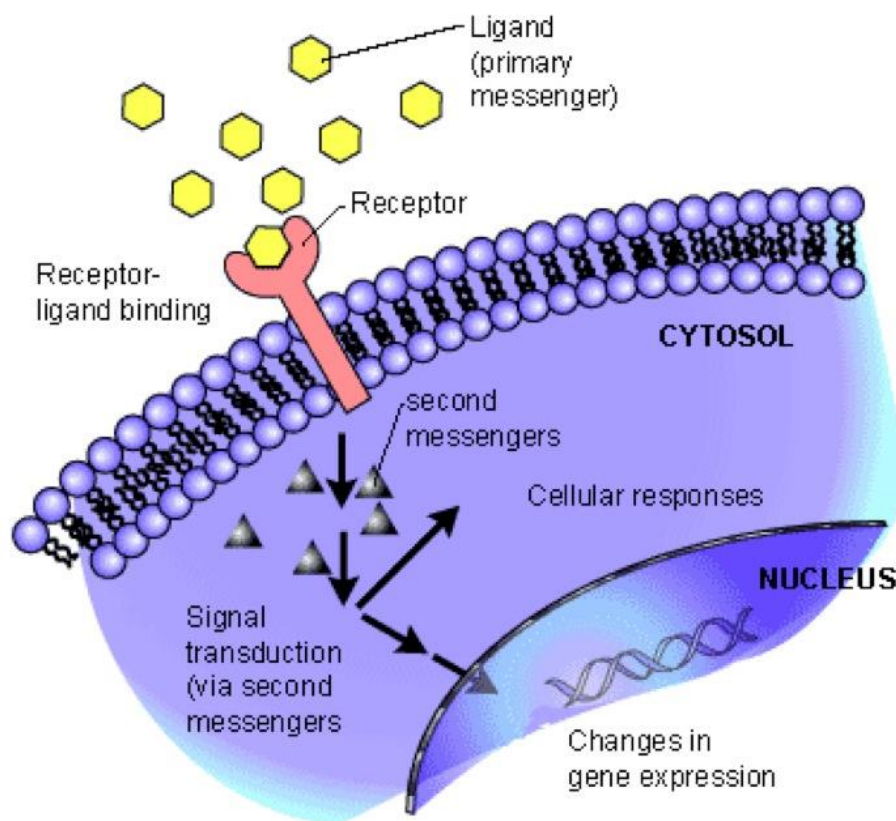


Figure 4 Signal transduction pathway (47)

As described by Wong *et al.* in 1996, molecules in signal transduction pathways are generally composed of extracellular ligands as a primary messenger, carrying a

message from other cells to communicate with the target cell (11) (Figure 4). The ligands, then, bind and activate their specific cell-surface receptors to initiate subsequent intracellular signaling (11). Following activation of the cell-surface receptor, intracellular signaling molecules, called second messengers, sequentially deliver continuous signal transduction (11). This step is usually complicated (31). Finally, the signaling molecule activates transcription factor in the nucleus, leading to a large range of cell functions (11).

As aforementioned, disruption of the intracellular signaling pathway can contribute to the development of a variety of diseases, particularly malignant tumors. Nevertheless, there are many series of intracellular pathways. The alterations of these signaling pathways in each type of cancer are often not specific, but differ between individuals even with the same type of cancer (26). As a result, these pathways must be carefully assessed to identify valid targets to have therapeutic potential for cancer treatment (26). Many pieces of research over the last few decades have revealed a series of overactivated molecules shared by most, and perhaps all, types of human cancer, especially many molecules of the PI3K/Akt signaling pathway (8).

2.3 PI3K/Akt signaling pathway

2.3.1 Akt

Akt was originally discovered as a cellular homologue of a *gag* (a group specific antigen) gene of a viral oncogene named *v-AKT*, transduced by the acute transforming retrovirus, namely AKT-8, which was isolated from an AKR thymoma (a type of leukemia in mice) (33, 46, 48-56). The name Akt does not refer to its function. The name Akt has a long history. The “Ak” in Akt was a temporary classification name for a mouse strain, bred for experiments by Jacob Furth in 1928. Originally, mice from three different stocks, designated A, R, and S, were studied. Stock A was noted to yield many cancers, and inbred families of mice were further designated as strains by a second small letter, Aa, Ab, Ac to Ak. Later, he found that the Ak strain frequently developed spontaneous thymic lymphomas, and then, he added the letter “t” after “Ak” to create “AKT” to indicate Ak mice, developing “thymoma.” In 1977, a transforming retrovirus was isolated from the Ak mouse by Staal *et al.* as “t-8” (transforming clone from the

eighth culture) so that the retrovirus was termed “AKT-8” (57). Ten years later, in 1988, proviral DNA was cloned from the AKT-8 retrovirus, and its viral *gag* oncogene, which was associated with the development of thymoma in Ak mice, was discovered by Staal *et al.*, and named *v-AKT* (56). Human homologues of the *gag* gene of *v-AKT* were later discovered by Staal with gene amplification noted in a human gastric cancer; hence, the later identified human analogues were named accordingly “Akt” (58).

Akt is also known as protein kinase B (PKB) (28, 59) because the catalytic kinase domain of Akt shows high amino acid sequence identity with protein kinase A (PKA) and protein kinase C (PKC) (45, 50, 52, 54) so that Akt formerly was termed “Related to A and C Protein Kinase” (RAC-PK) (51, 54) (Figure 5). Akt belongs to the “AGC” superfamily of serine/threonine protein kinases together with PKA, Protein Kinase G or cGMP-dependent protein kinase (PKG), and PKC (45, 54). Moreover, Akt is a serine/threonine protein kinase (STK) that phosphorylates the OH group of serine or threonine, which have similar side chains (51). The main function of Akt, as a member of AGC serine/threonine superfamily, is to catalyze the phosphorylation of serine and threonine residues at R-X-R-Y-Z-(S/T)-(hydr) consensus phosphorylation sites in target proteins (where R is arginine, X is any amino acid, Y and Z are small residues other than glycine, and (hydr) is a bulky hydrophobic group) (24).



Figure 5 The structural identity of Akt2 (red) and PKA (blue) and the structural identity of full-length Akt2 and PKA (54).

After two decades of Akt identification, interest in Akt has increased enormously (52, 60). In 2004 alone, approximately 2000 articles were published pertaining to Akt (60). Evidently, Akt is a cardinal node in diverse signaling cascades, related to both normal cellular physiologies and various disease states (60). Akt has been identified as a key effector of the phosphoinositide 3-kinase (PI3K) signaling pathway (61-63), which regulates multiple cellular processes, such as cell growth, cellular energy metabolism, transformation, differentiation, and survival (28, 59, 61). Simultaneously, many works have focused on the intricacies of Akt signaling and its roles in cancer aggressiveness (60). Akt has also been extensively studied by many investigators in other areas, such as cardiovascular research, diabetes, and neuroscience (60).

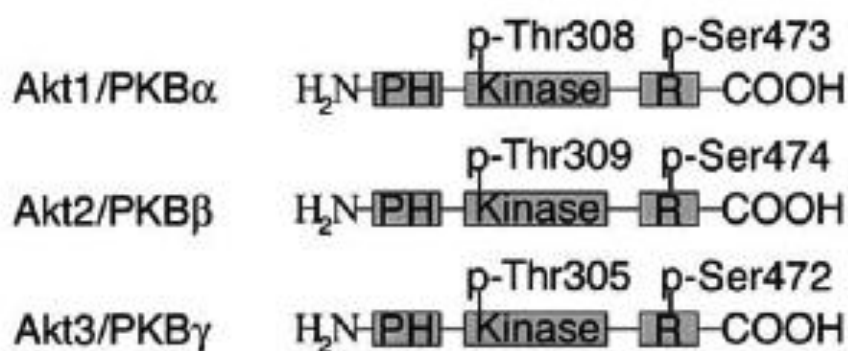


Figure 6 Domain structures of three Akt isoforms (45)

2.3.2 Isoforms of Akt

Mammalian, including human, cells express a few highly homologous Akt isoforms (52, 59, 60). At present, there are just three identified isoforms named Akt1, Akt2, and Akt3 (28, 51, 53, 54, 62, 64-66). As mentioned above, Akt is also known as protein kinase B (PKB); the Akt1, Akt2 and Akt3 proteins are also referred to PKB- α , PKB- β and PKB- γ , respectively (50, 51, 60). Each isoform is derived from distinct genes, which are located on chromosomes 14q32, 19q13, and 1q44, respectively (27, 33, 50, 65, 66). Despite distinct original chromosomes, Akt isoforms share over 80% amino acid sequence identity (50, 52, 61), with Akt2 and Akt3, respectively, showing 81% and 83% amino acid sequence identity with Akt1 (54) (Figure 6).

Although the Akt is abundantly expressed in mammalian cells, many bodies of evidence suggest that the isoform expression levels differ between tissues (24, 50, 54, 65), and specific functions of each Akt isoform appear to be complicated due to differential expression patterns and complex activation profiles (65). This means that Akt1 mRNA and protein are ubiquitously expressed in various tissues, as Akt2 is abundantly expressed in all tissues but predominantly in insulin target tissues such as fat cells, the liver, and skeletal muscle (54, 64, 66, 67). In contrast to Akt1 and Akt2 expression, Akt3 displays a more restricted tissue distribution and is found abundantly in the testis and neuronal tissue, such as brain (46, 53, 64, 66, 67).

Many studies have explored the roles of Akt in cellular behavior (64) and it is formerly assumed that Akt isoforms function both redundantly and non-redundantly (27, 53, 64, 66). But many current studies have clearly demonstrated that, rather than functioning in cellular signaling in a redundant manner, Akt isoforms have very distinct cellular functions in specific cell lineages (65, 67). The first hint that Akt isoforms function non-redundantly came when the Birnbaum laboratory generated Akt1, Akt2 and Akt3 knockout mice (53, 65). It was found that Akt1 null mice had growth retardation, Akt2 null mice developed insulin-resistant diabetes, and Akt3 null mice revealed reduced brain size (53, 54). Although close to 200 substrates of Akt have been identified in many cell types and tissues, only a small number of them has been evaluated for Akt isoform specificity (65). Specific substrates of Akt isoforms responsible for transducing distinct subsequent function clearly do exist such that the challenge is now to discover the subsets of these proteins that are exclusively regulated by Akt1, Akt2, or Akt3 (65), and especially the relationship between each Akt isoform and human diseases. Because of the differential tissue distribution and activation patterns of Akt isoforms, it is generally accepted that the Akt isoforms function non-redundantly in both physiologic and pathologic conditions (64, 67).

2.3.3 Structure of Akt

Even if Akt1, Akt2 and Akt3 are encoded by three separate genes, they exhibit greater than 80% homology at the protein level (33, 49, 67). Since Akt

active site residues are the same in all three isoforms, the structure of each isoform can be described simultaneously (45). The Akt isoforms share a common domain organization that consists of a pleckstrin homology domain (PH domain) at the amino terminus, a kinase catalytic domain (CD) in the center sequence, and a short regulatory region at the carboxyl terminus (45, 49, 54, 66, 67) (Figure 6). At the N-terminal region, the PH domain arranges approximately 100 amino acids (1st to 106th amino acids) and is found in many other proteins, such as phosphoinositide-dependent kinase-1 (PDK1) (24, 44). Pleckstrin is a protein found in platelets and its name is derived from “platelet and leukocyte C kinase substrate and the KSTR string of amino acids”. The center kinase domain, or the catalytic domain, which is very similar to those of PKA and PKC, stretches from the 148th to the 411th amino acids (24, 44). The kinase domain of Akt1 shares 91% and 88% of a sequence homology with Akt2 and Akt3, respectively (45). And, at the hydrophobic C-terminal region, the regulatory domain tails the Akt structure from the 412th to the 480th amino acids (24). Because of the similarity of the amino acid sequence in each Akt isoform, the sites of the threonine residue of the kinase domain and the serine residue of the regulatory domain of each Akt isoform are located near each other (Figure 6) (45). Essentially, phosphorylation at these two sites (the threonine residue of the kinase domain and the serine residue of the regulatory domain) is required to achieve maximal Akt activation (49, 50, 52-54) and phospho-serine-474 Akt is the major active form of Akt (62).

2.3.4 Activation of Akt and its upstream molecules

Full activation of Akt is a multistep process (46, 51) but the pathway leading to Akt activation is highly conserved across species (68) and all three Akt isoforms are activated by essentially identical mechanisms (53, 65) (Figure 7). This process is physiologically initiated by the binding of exogenous growth factors, such as platelet derived growth factor (PDGF), insulin, epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) and insulin-like growth factor 1 (IGF-1), to the extracellular moiety of their cognate cell surface growth factor receptor proteins (46, 65). The main upstream cell surface receptors in PI3K/Akt

pathway activation are receptor tyrosine kinases (RTKs) (69). Approximately 20 different RTK classes have been identified (27).

RTK class I (EGF receptor family or ErbB or HER or Neu family),
RTK class II (Insulin receptor family),
RTK class III (PDGF receptor family),
RTK class IV (FGF receptor family),
RTK class V (VEGF receptors family),
RTK class VI (HGF receptor family),
RTK class VII (Trk receptor family),
RTK class VIII (Eph receptor family),
RTK class IX (AXL receptor family),
RTK class X (LTK receptor family),
RTK class XI (TIE receptor family),
RTK class XII (ROR receptor family),
RTK class XIII (DDR receptor family),
RTK class XIV (RET receptor family),
RTK class XV (KLG receptor family),
RTK class XVI (RYK receptor family),
and RTK class XVII (MuSK receptor family)

Other groups of receptors are cytokine receptors, G-protein-coupled receptors (GPCR), B and T cell receptors, and integrins as extracellular matrix (ECM) receptors (27).

RTKs have four domains: the extracellular ligand-binding domain, the single transmembrane domain, the cytoplasmic tyrosine-kinase domain, and the regulatory domain. Normally, in the inactive form, most RTKs are single-subunit receptors, but activation of RTKs leads to their dimerization for autophosphorylation and subsequent phosphorylation of their downstream substrates.

After activation of the cell surface receptor, phosphatidyl inositol-3 kinase (PI3K), which is a heterodimeric enzyme in a family of lipid kinases, composed of 85-kD regulatory (p85) and 110-kD catalytic (p110) subunits,

afterwards acts as a second messenger (43, 50, 69). The p85 regulatory subunit of PI3K is recruited to phosphotyrosine-containing sequences within either the cytoplasmic domain of activated RTK or adapter molecules, such as insulin-receptor substrate-1 (IRS-1) and GRB2-associated-binding proteins 1 (GAB1) (50, 65). This binding co-localizes the p110 catalytic subunit of PI3K proximal to phosphatidyl inositol-4,5-bisphosphate (PIP2 or PI-4,5P2), which is a substrate of PI3K, and then phosphorylates PIP2 at the 3' position of the inositol head ring in order to convert PIP2 to phosphatidyl inositol-3,4,5-trisphosphate (PIP3) (24, 49, 50, 65, 70). PIP3, serving as a high-affinity binding site for the PH domain of Akt, effectively recruits Akt onto the inner leaflet of the plasma membrane (50, 65), which is the rate-limiting step in Akt activation (52). This binding between PIP3 and the PH domain of Akt induces a conformational change in Akt, which facilitates the phosphorylation of two critical residues (32, 48, 65). The threonine residue in the activation kinase domain is phosphorylated by phosphoinositide-dependent kinase-1 or PIP3-dependent kinase-1 (PDK-1), which is also recruited to membranes through a PH/PIP3 interaction (65). Simultaneously, phosphorylation of the carboxyl-terminal serine residue is mediated by the mammalian target of rapamycin (mTOR) kinase, comprising the target of rapamycin complex 2 (TORC2), although a number of other kinases may also function in specific settings (52, 65). Once fully phosphorylated, Akt is locked in the catalytically-competent conformation, loses the PIP3 requirement, and translocates to a variety of intracellular locations including the cytoplasm, mitochondria, and nucleus where it phosphorylates specific downstream substrates (65).

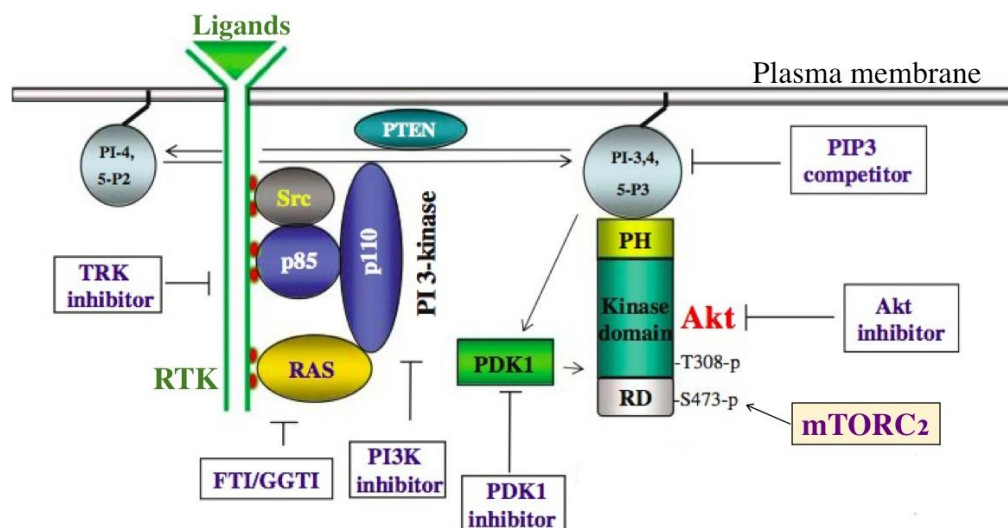


Figure 7 Regulation of Akt activation by the PI3K pathway and inhibitors (48)

PI3K, as an important second messenger in the PI3K/Akt signaling pathway, comprises eight mammalian isoforms that are grouped into three classes due to a variety of catalytic and regulatory subunits (32). Three catalytic subunits of the mammalian PI3Ks have been cloned and designated as p110 α , p110 β , and p110 γ sharing 42-58% amino acid sequence identity (32, 45), while five regulatory subunits have been cloned, termed as p85 α , p85 β , p50 α , p55 α , and p55 γ . Consequently, PI3K is divided into eight isoforms by combination of differential catalytic subunit and regulatory subunits, and then, is classified into three classes (24, 32, 45). PI3K class I is also divided into subclass IA and subclass IB (24, 45). Subclass IA is activated by receptors with RTKs, while subclass IB is activated by receptors coupled with G-proteins (44). Importantly, subclass IA is a major mediator of the function of the PI3K/Akt signaling pathway in oncogenesis (24).

In addition to RTK, PI3K, PIP3, PDK1, and mTORC2, many studies reveal other upstream molecules of Akt and its other regulators, such as PTEN, CCR7, Ras, and PHLPP. *Phosphatase and tensin homologue (PTEN)* is a tumor suppressor gene located on chromosome 10q23 (32, 49, 71). It is a major negative regulator of the PI3K/Akt pathway (60, 65) that antagonizes PI3K activity by dephosphorylation of PIP3 back to inactive PIP2, resulting in effective

termination of signal transduction (33, 65, 71) (Figure 7). A second important tumor suppressor in the PI3K/Akt pathway is the PH domain and leucine-rich protein phosphatase (PHLPP), a serine/threonine phosphatase that dephosphorylates Akt at the pSer473 residue of the regulatory domain (65). Dephosphorylation of the Akt regulatory domain by PHLPP does indicate some selectivity (65). The PHLPP1 isoform appears to dephosphorylate Akt2 and Akt3, whereas PHLPP2 targets Akt1 and Akt3 (65). With or without activation of RTK, Akt can also be activated by Ras via direct activation on the p110 catalytic subunit of PI3K (26, 59).

2.3.5 Downstream substrates of Akt

Once activated, Akt can phosphorylate many substrates to exert its functions (53, 72) and the downstream substrates are found increasingly (73). Currently, close to 200 Akt substrates have been uncovered, either by candidate screening approaches or by whole phospho-proteome mass spectrometry sequencing technologies (65). But only a few of them have been evaluated for isoform specificity (64). However, because of sharing similar protein structure, all Akt isoforms have been previously considered to possess identical or similar substrate specificity (67) but now the non-redundant functions of each Akt isoform are certainly proven. Some direct and indirect downstream examples of Akt are forkhead box class O (FOXO), BAD, caspase 9, inhibitor of kappa B kinase (IKK), murine double minute 2 (MDM2), forkhead homologue in rhabdomyosarcoma (FKHR), nuclear factor kappa B (NF- κ B), nitric oxide synthase (NOS), glycogen synthase kinase-3 (GSK3), mTORC1, cyclic-AMP response element-binding protein (CREB), and Cyclin D1 (33, 45, 46, 53) (Figure 8).

2.3.6 Physiologic functions of the PI3K/Akt signaling pathway

For decades, many studies have investigated the roles of Akt in cellular physiology (64) along with an increasing list of downstream substrates (45, 50, 73). Akt is a major signal transducer of the PI3K/Akt pathway in all cells and tissues and plays a critical role as a central control node in the maintenance of cellular processes (46, 52, 53, 72), including cell growth (size), proliferation

(number), survival and programmed cell death, transformation and differentiation, cell migration or motility, angiogenesis, genome stability, and various aspects of intermediary metabolism (33, 46, 49, 51, 60, 71, 73) (Figure 8).

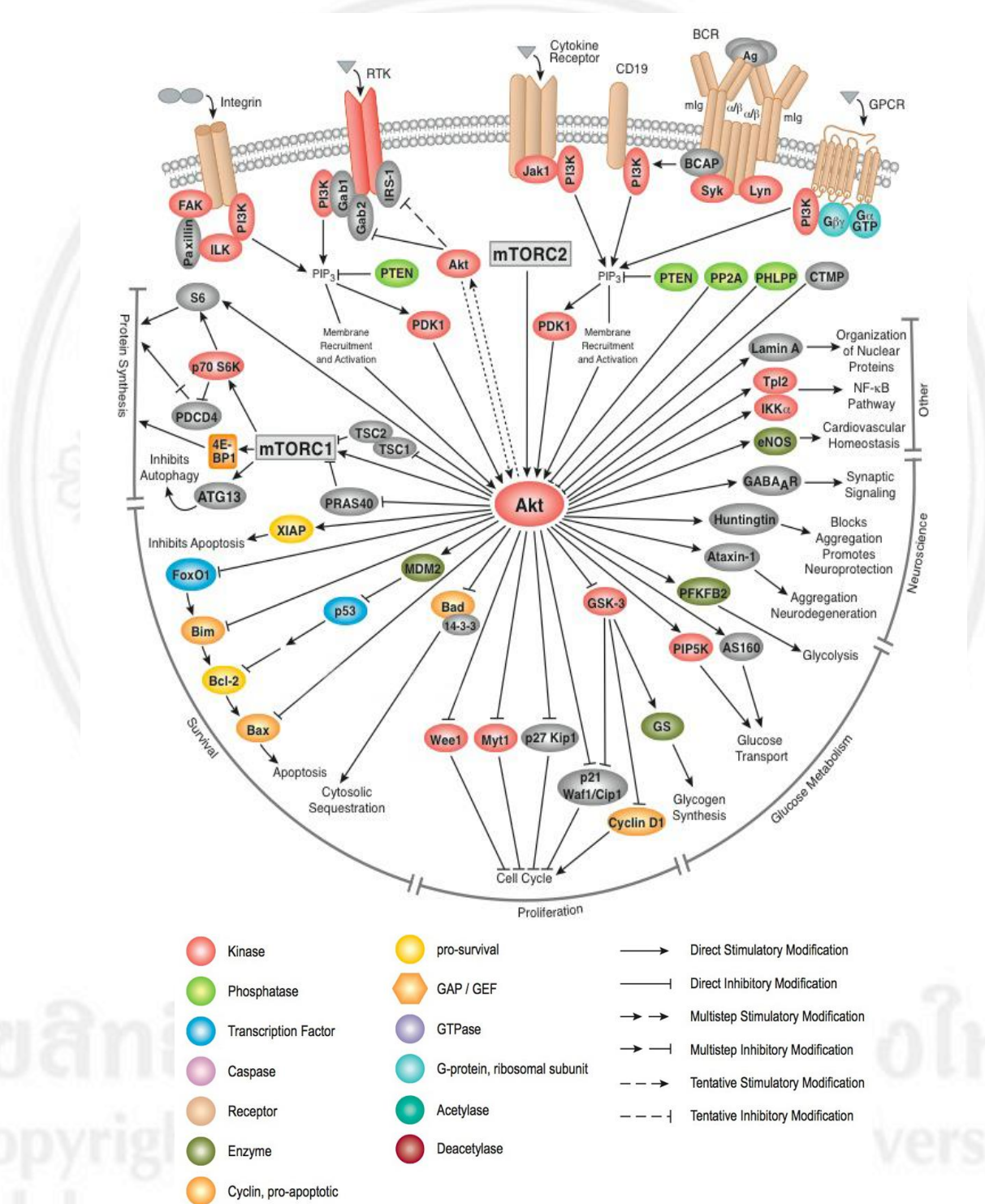


Figure 8 The PI3K/Akt signaling pathway and associated physiologic functions (74)

1) Cell growth and protein synthesis

To understand the mechanism by which Akt promotes protein synthesis and cell growth, mTOR should first be introduced. mTOR is a serine-threonine kinase (72) and its name implies that its inhibitor is rapamycin, a macrolide antibiotic (46, 64, 70). mTOR exists in two functionally distinct complexes in cells, namely mTORC1 and mTORC2 (70). mTORC1 is composed of mTOR, regulatory-associated protein of mTOR (Raptor), mammalian LST8 (mLST8) or G-protein β -subunit-like protein (G β L), proline-rich Akt substrate of 40 kDa (PRAS40), and DEPTOR (70) (Figure 9). PRAS40 and DEPTOR are mTOR inhibitors (70). mTORC1 is sensitive to inhibition by the macrolide antibiotic rapamycin (70). mTORC2 is composed of mTOR, rapamycin-insensitive companion of mTOR (Rictor), mammalian stress-activated protein kinase interacting protein 1 (mSin1), and mLST (G β L) (70) (Figure 9). Although originally reported to be insensitive to rapamycin, long-term treatment of mammalian cells with rapamycin indirectly inhibits mTORC2 (70). As above-mentioned, mTORC2 acts as an upstream regulator of Akt that phosphorylates Akt at the Ser473 regulatory domain. Interestingly, and conversely, mTORC1 is a downstream effector of Akt (32, 49, 59). So, a molecule mTOR can act as both substrate and activator of the Akt (70).

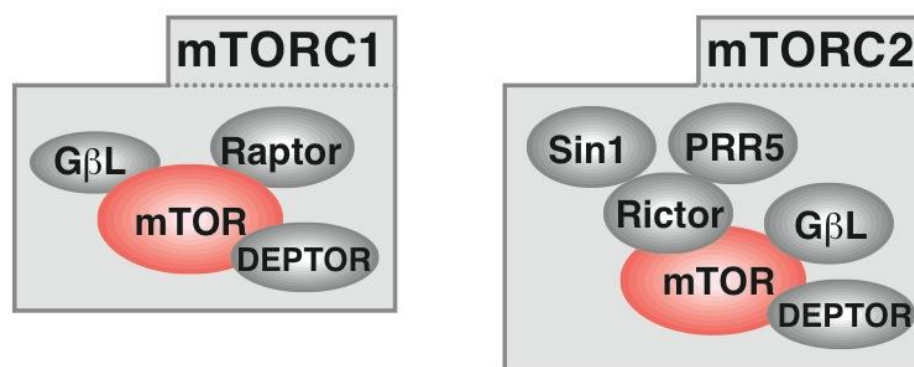


Figure 9 Compositions of mTORC1 and mTORC2 (74)

The multi-protein complex mTORC1, containing mTOR, regulates several intracellular functions including cell growth and inhibition

of autophagy (autophagocytosis) (32, 46, 52, 75). The activated Akt is both required and sufficient for mTORC1 activation (31, 46, 52). Akt can activate mTORC1 directly by either phosphorylation activation of mTOR or indirectly by phosphorylation inactivation of tuberous sclerosis complexes 1, and 2 (TSC1 and TSC2 which are also known as hamartin and tuberin, respectively) (49, 72, 75) and of PRAS40, an inhibitor of mTORC1 (74) (Figure 8). Activation of mTORC1 results in (1) phosphorylation of ribosomal protein S6 kinase (p70S6K) and then activation of the 40S ribosomal protein S6, by an unclear mechanism, which leads to increased messenger RNA translation; (2) phosphorylation of the 4E binding protein (4E-BP) resulting in the dissociation of eukaryotic initiation factor 4E (eIF4E) from the 4E-BP (49, 75); then, eIF4E functions to bind an mRNA cap and ultimately brings mRNA to the ribosome as a cap-dependent translation of messages (Figure 8) (71). mTOR, thereby, plays a major role in regulating ribosome biogenesis, mRNA translation, and protein synthesis (46, 49). Thus, it is evident that Akt plays many roles in cell growth and protein synthesis.

2) Cell proliferation

The PI3K/Akt pathway has been shown to be activated during the G1/S transition and to play a key role in cell cycle progression (49). Akt can promote cell proliferation by inactivation of cell cycle inhibitors such as p21, p27, FOXO, β -catenin, and GSK3; and by activation of cell cycle enhancers such as cyclin D1, D3, and E (44, 49, 71). First, the cyclin-dependent kinase inhibitor (CDKI) p21WAF1/CIP1 is directly phosphorylated by Akt, leading to cytoplasmic mislocalization of the CDKI --- retaining it within the cytoplasm --- and then cell cycle progression (27, 46, 49, 50, 65). Second, Akt directly phosphorylates p27KIP1 leading to p27 inactivation by a similar mechanism recently described for p21 (27, 46, 49, 50). Third, the forkhead transcription factors normally repress cyclin D1 expression, while enhancing expression of the cell cycle inhibitors (49). When forkhead transcription factors (such as FOXO1/3/4) are

phosphorylated and inactivated by Akt, they promote the cyclin D1 expression as well as inhibit the expression of p27KIP1 and p130Rb2 by preventing their ability to localize to the nucleus (46, 49). Fourth, Akt can activate MDM2, an important negative regulator of the p53 cell cycle regulator (44). MDM2, functioning as an E3 ubiquitin ligase, guides p53 to the cytoplasm, where p53 becomes degraded through the ubiquitin/proteasome-mediated process (44, 49, 76, 77). Finally, GSK3 β phosphorylates cyclin D1 and c-Myc, and promotes their degradation (49). When GSK3 β is phosphorylated by Akt, GSK3 β is inhibited, thereby contributing to the stabilization of cyclin D1 and c-Myc, which play distinct roles in cell cycle progression through the G1 phase (49, 50). Besides, there are other mechanisms by which Akt promotes cell proliferation such as via Myelin transcription factor 1 (Myt1) and Wee1 (74). Wee1/Myt1 kinase, an inhibitor of cell cycle within the G2 phase, normally phosphorylates and inhibits cyclin/CDK complex function but can be antagonized by cell division cycle 25 (CDC25) phosphatase (Figure 2). Akt can inactivate Myt1/Wee1 cell cycle inhibitor, leading to cell proliferation. Hence, it has been proven that Akt can promote cell proliferation.

3) Antiapoptosis and cell survival

Akt is one of the critical regulators of apoptosis (46, 55). Activation of Akt positively regulates antiapoptosis and cell survival (49, 75) both by phosphorylation inhibition of proapoptotic gene, such as *Bad*, *Bax*, *Bim*, and *FOXO*; and by phosphorylation upregulation of antiapoptotic genes such as *MDM2*, *I κ B kinase (IKK)*, *Bcl-2 family*, and *cyclic-AMP response element-binding protein (CREB)* (27, 49, 50, 55).

For example, Akt activates Bcl-2 and Bcl-xL, antiapoptotic proteins in the Bcl-2 family (52). Consequently, mitochondrial membrane integrity is stabilized by Bcl, resulting in inhibition of cytochrome C release from mitochondria, which is an important process in the mitochondrial-mediated intrinsic apoptotic pathway (27, 28, 52) (Figure 3). Cell survival is also promoted by Akt phosphorylation of Bad (27, 43). Bad is a

proapoptotic member of the Bcl-2 family and is identified as an intersection point of proapoptotic and antiapoptotic regulatory cascades (27, 43, 61). The non-phosphorylated form of Bad promotes apoptosis by forming a non-functional heterodimer with Bcl-2 or other antiapoptotic members of the Bcl-2 family, such as Bcl-xL and antagonizes their antiapoptotic activity (24). Bad can be phosphorylated at Ser136 by Akt, resulting in dissociation of the complex and restoring the antiapoptotic function of Bcl-xL (27, 43, 61).

Furthermore, Akt inhibits apoptosis, not only via an intrinsic apoptotic pathway, but also via an extrinsic apoptotic pathway. Generally, the forkhead family of transcription factors induces the expression of proapoptotic factors, such as Fas ligand (44). Akt phosphorylation inactivation of FOXO causes decreased FOXO expression of Fas ligands and/or tumor necrosis factor-related apoptosis-inducing ligands (TRAILs), resulting in signaling discontinuation of the extrinsic apoptotic pathway (27). However, Akt-phosphorylated inactive FOXO can also promote cell survival via an intrinsic apoptotic pathway by upregulation of the *Bcl-2* antiapoptotic gene (Figure 3). For the other example, *MDM2* is an antiapoptotic gene upregulated by Akt phosphorylation (66). Akt phosphorylates at serine 166 and 186 of MDM2, a negative regulator of the p53 tumor suppressor protein, causing that phosphorylated MDM2 to translocate more efficiently to the nucleus, where it functions for p53 degradation, in order to decrease apoptosis (24, 66).

In brief, Akt can promote cell survival by downregulation of proapoptotic genes and by upregulation of antiapoptotic genes. Moreover, Akt can interrupt both intrinsic and extrinsic apoptotic pathways.

4) Glucose metabolism

Some studies found that mammalian cells with hyperactive Akt accumulated ATP whereas cells with reduced Akt abundance or activity exhibited markedly reduced ATP content (52). At present, it is accepted that Akt plays an important role in metabolism (27, 46, 52). In response to

insulin stimulation, Akt affects glucose and amino-acid metabolism, including glycogen synthesis, cellular energy metabolism, and glycolysis via phosphorylation of GSK3 (27, 46, 49, 52) (Figure 8). Furthermore, the other downstream effectors of Akt that are critical regulators of glucose metabolism and energy sensor mechanisms include 6-phosphofructo-2-kinase (PFK-2), mTOR, and GLUT transporters (24, 46). Akt substrate of 160 kDa (AS160), which is a protein that modulates glucose transport in insulin-responsive cells and tissues through the GLUT4 transporter, appears to be regulated primarily by Akt2 (65). So Akt plays many roles in glucose metabolism.

5) Neuroscience (74)

Akt plays several roles in the generation of nerve diseases, such as Alzheimer's and Huntington's diseases. Some Akt targets in neuroscience are Huntington protein, Ataxin-1, GSK3 β , and Gamma amino-butyric acid A (GABA_A) receptor. Huntington's disease is caused by the accumulation of poly-glutamine repeats within Huntington protein. Akt phosphorylates Huntington protein at Ser421 resulting in reduction of poly-glutamine repeats, and then, reduction of occurrence of Huntington's disease. GABA_A receptor is ion channel. Phosphorylation of GABA_A receptor by Akt increases a number of GABA_A receptors, expressing on the cell surface, and increases some synaptic signals; then, modification of nerve diseases.

6) Others

Akt has other physiologic functions such as cardiovascular homeostasis (74). One downstream effector of Akt in cardiovascular homeostasis is endothelial nitric oxide synthase (eNOS) (46, 74), phosphorylated by Akt at Ser1177 of eNOS (74). eNOS, responsible for the production of nitric oxide, is a key regulator of blood pressure control, vascular remodeling, and angiogenesis (46, 74). In angiogenetic process, Akt phosphorylates endothelial differentiation sphingolipid G-protein-

coupled receptor 1 (EDG-1), required for the activation of endothelial cell migration (46).

In summary, Akt acts as a central node in various cellular physiologic functions, including protein synthesis and cell growth, cell survival (antiapoptosis), cell proliferation, glucose metabolism, and others. Thus, deregulation of the PI3K/Akt signaling pathway may be responsible for diseases, particularly malignant tumors.

2.4 PI3K/Akt signaling pathway and cancer

2.4.1 PI3K/Akt signaling pathway and cancer biology

For many decades of oncologic research, it has been clear that there are many insufficiently explainable questions on cancer biology and they cannot be explained in terms of separate linear molecular pathways alone (16). Akt is not only involved with normal cellular physiology as above mentioned but is also associated with various disease states, including a number of human malignancies (54, 60). Interest in the role of Akt in cancer has enormously increased and the molecular mechanisms of the PI3K/Akt signaling pathway in cancer cells have been extensively studied as a major focus of modern cancer research (24, 45, 71).

Many previous studies obviously indicated that dysregulation of the PI3K/Akt pathway played a pivotal role in many human malignancies including squamous cell carcinoma of the head and neck (SSCHN) (46, 49, 59, 65, 78). Various oncoproteins and tumor suppressors both upstream and downstream, which intersect the Akt signal transduction pathway, are activated or inactivated, by multiple mechanisms (51, 52, 60, 69, 71). Consequently, aberrant overactivation of PI3K/Akt signaling is today considered a hallmark of cancer (27, 60, 71). Numerous cancer-associated characteristics including cancer transformation, protein synthesis and cell growth, antiapoptosis, cell proliferation, immortalization, angiogenesis, invasion and metastasis, metabolic reprogramming, and resistance to radio-, chemo-, and hormonal therapies are involved (46, 53, 65, 79).

Importantly, malignant characteristics are likely regulated by specific Akt isoforms (65). Recent studies suggested that Akt2 overexpression is a frequent event in human cancers and is also sufficient to replicate the invasive phenotype, whereas Akt1 amplification and mutation are rarely found (51, 71). High-level amplification of Akt3 was not described in human cancers, although a small increase in Akt3 copy number was found in a subset of tumors (71). Nevertheless, the relationship between specific Akt isoforms and cancer behavior is currently not truly understood (65).

Due to the roles of Akt as a central node of cancer biology, the PI3K/Akt signaling pathway has become an attractive pathway for cancer therapeutic intervention, according to the concept of targeting therapy as aforementioned (24, 51, 59, 60, 65, 68, 71, 73).

2.4.2 PI3K/Akt signaling pathway and hallmarks of cancer

Many previous studies have shown that inappropriate stimulation of the PI3K/Akt signaling pathway contributes malignant aggressiveness by promotion of all hallmarks of cancer in several tumor types (24, 32, 52, 71).

1) Cell overgrowth, cell cycle dysregulation, and cell survival

Due to the concept that Akt regulates physiologic cell growth, Akt overexpression can lead to cell overgrowth (24, 71) (Figure 8). Cell cycle regulation and programmed cell death also can be dysregulated by aberration of Akt activity, causing cell overproliferation and cell survival, considered hallmarks of cancer, as previously described in recent section.

2) Limitless replication

Akt phosphorylates telomerase reverse transcriptase (TERT), which is a catalytic subunit of the enzyme telomerase and responsible for increasing activity of telomerase, an enzyme frequently expressed in tumors and believed to promote one mechanism by which cancer cells maintain their telomeres and circumvent replicative senescence. Thereby, Akt can

contribute to the limitless proliferative capacity or immortalization of cancer cells (24, 46, 71).

3) Angiogenesis

Hypoxia and shortage of nutrients are problems for tumor mass expansion (24). Angiogenesis is a means to solve the problems (24, 33). Recently, it was found that Akt plays an important role in regulating normal vascularization and pathological angiogenesis (33, 49).

Angiogenesis is regulated by the increase of angiogenesis inducers or the decrease of angiogenesis inhibitors (33). Vascular endothelial growth factor (VEGF) is a major angiogenesis inducer associated with tumor angiogenesis and growth in numerous solid malignancies (33, 53). PI3K/Akt signaling can regulate VEGF expression by many mechanisms: 1) Ras expression was showed to increase VEGF expression and PI3K was a downstream target of Ras to mediate Ras-induced VEGF expression; 2) insulin-induced VEGF expression through PI3K/Akt signaling; and 3) hepatocyte growth factor induced VEGF expression through Akt activation (33). Forced expression of PI3K or Akt alone directly increases VEGF mRNA expression, suggesting that PI3K and Akt are sufficient to regulate VEGF expression for endothelial cell migration and angiogenesis (24, 33, 46, 49, 53). Furthermore, Akt and VEGF may form an autocrine loop in the cells for regulating tumor growth and angiogenesis (33) (Figure 10). A secondary angiogenesis key regulator of the response to hypoxia is the transcription factor hypoxia inducible factor-1 (HIF-1 α) (24, 33). Activation of Akt may lead, via mTOR, to the stabilization of HIF-1 α and consequently, to increased production of VEGF (24, 49) (Figure 10).

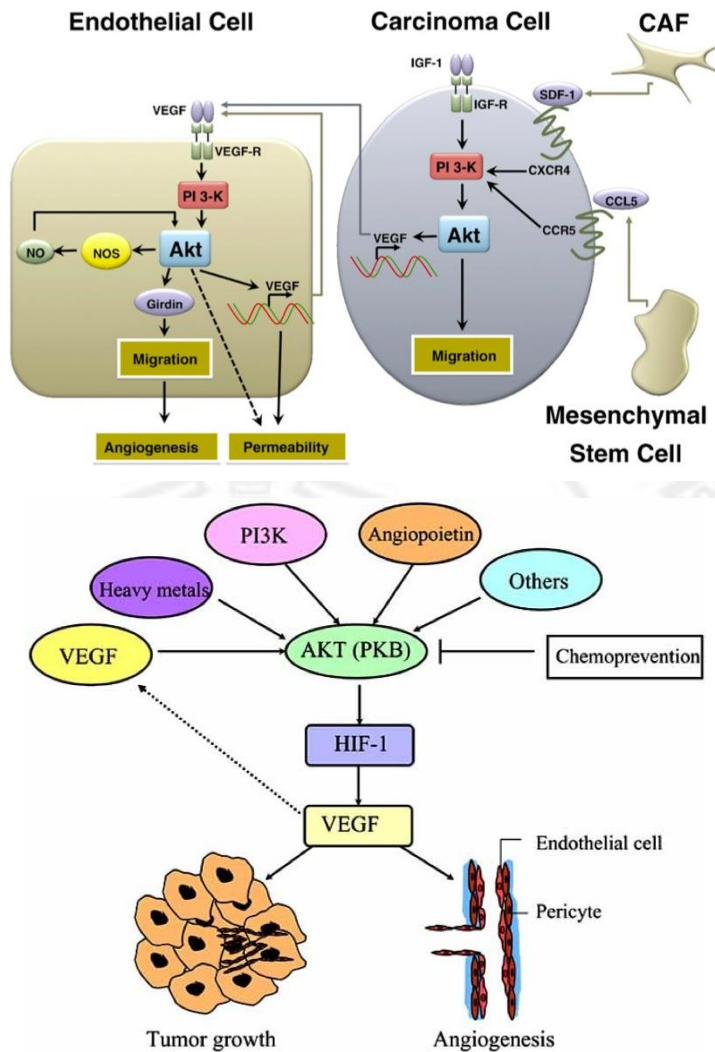


Figure 10 Paracrine and autocrine signaling mechanisms of Akt-mediated tumor-stromal interactions (upper) and the outline of Akt signaling in regulation of tumor growth and angiogenesis (lower) (33)

Similar to VEGF, Akt phosphorylates and activates eNOS to produce nitric oxide (NO) for nitric oxide dependent angiogenesis (24, 49, 53). Other examples of proangiogenesis inducers are calcium-activated neutral protease, or calpain (CAPN6) (49) and angiopoietin-1, which is a growth factor required for the formation of mature blood vessels, and is a ligand of the RTK class XI TIE (24, 80). Although the roles of individual Akt isoforms in angiogenesis have not been explored, studies using Akt-isoform-deficient mice have shown that Akt1, but not Akt2, is critical for VEGF-mediated angiogenesis (53).

4) Tissue invasion and metastasis

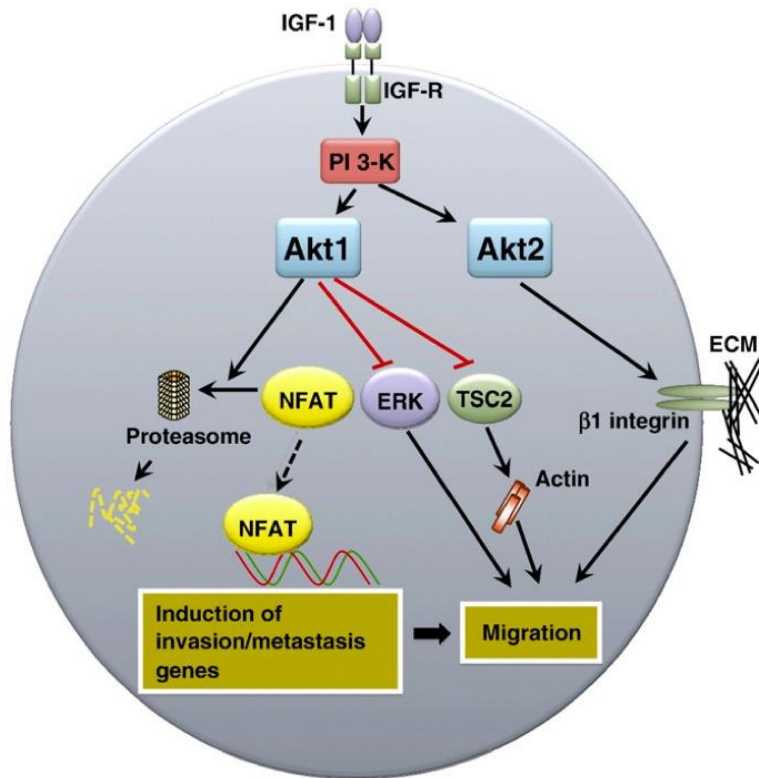


Figure 11 Mechanisms of carcinoma cell migration mediated by Akt

Cancer cells respond to IGF-1, leading to the activation of PI3-K and, in turn, phosphorylation and activation of Akt1 and Akt2. Akt1 can function to suppress carcinoma cell migration by various redundant mechanisms, inducing degradation of NFAT and the attenuation of pro-invasion and metastasis genes. Akt1 can also suppress ERK and TSC2 activity, leading to the suppression of migration. In contrast, Akt2 functions to enhance carcinoma cell migration, in part by upregulating $\beta 1$ integrins and other yet-to-be-determined mechanisms. (53, 65)

A recent study has shown that the knockdown of Akt by antisense or siRNA significantly reduced tumor growth and invasiveness (48). Accordingly, Akt activation has also been shown to contribute to tumor invasion and metastasis (65, 71) by promoting the secretion of matrix metalloproteinases (MMP), especially MMP-9 (40, 46, 71), and the induction of epithelial-mesenchymal transition (EMT) (37, 40, 46, 65, 71). Additionally, AMP-activated protein kinase-related kinase 5 (ARK5), a

member of the adenosine monophosphate-activated protein kinase (AMPK) family, is activated by Akt; and then, plays a role in cancer invasion and metastasis (46). Some *in vivo* and *in vitro* studies have provided evidence of the non-redundant effects of Akt isoforms on invasion and metastasis (65). It can be summarized that in many cancer types, while Akt1 can block migration, Akt2 actually enhances this phenomenon in the same cells (Figure 11) (65).

5) Resistance to radiation, chemotherapy, and hormonal therapy

The activation of the PI3K/Akt pathway has been shown to contribute to tumor resistance to various conventional therapies, including chemotherapy, radiation, and hormonal therapy (32, 49, 50, 60, 62). Although previous studies have suggested that overexpression of Akt and its upstream regulators increase treatment resistance, the clear underlying mechanism is not fully understood (72). Because the mechanism of the treatment modalities is to promote cancer cell death or apoptosis, it is suggested that activation of the Akt/mTOR pathway can promote cell survival and antiapoptosis to resist the effect of these treatment modalities (71, 72). Shtibans *et al.*, in 2008, reported that immunohistochemical staining for phosphorylated Akt (pAkt) was significantly more frequent in radiation-resistant groups than in radiation-sensitive groups (46). Similarly, Akt activation induces hormonal resistance, irrespective of the kind of administered endocrine agents (63).

Chemoresistance is a major obstacle to successful cancer therapy (71). Akt has been shown to play a significant role in the therapeutic resistance of tumor cells, because it works against apoptotic mechanisms to promote cell survival (71, 72). For example, either constitutive Akt1 activity or *Akt2* gene amplification has an important role in the protection of cells from paclitaxel-induced apoptosis (28, 71) and PI3K/Akt inhibition increases paclitaxel efficiency in *in vivo* and *in vitro* ovarian cancer models (44). Akt may counteract not only paclitaxel efficiency, but also cisplatin-induced apoptosis, leading to cisplatin resistance (72). Recently, many

signaling molecules have been found to be involved in drug resistance, such as PTEN, Akt, mTOR, NF- κ B, EGFR, fibroblast growth factor receptor (FGFR), Raf/MEK/ERK, mitogen-activated protein kinase (MAPK), insulin-like growth factor (IGF), and the Notch signaling pathway (39). Furthermore, evaluation of Akt activity may be a prognostic marker for response to radio-, chemo-, and endocrine therapies (46, 63).

Collectively, Akt overexpression can amazingly promote several cancer behaviors, including cell overgrowth and protein synthesis, cell cycle dysregulation, antiapoptosis and cell survival, limitless replication, sustained angiogenesis, tissue invasion and metastasis, and resistance to radiation, chemo and hormonal therapies.

2.4.3 Aberration of PI3K/Akt pathway and various cancer types

For the last few decades, many worldwide research teams have been interested in the association between Akt aberration and cancer biology (52, 60). In 2004 alone, approximately 2000 articles were published pertaining to Akt (60). Not only cancer of the head and neck, but also all cancer types have been studied.

- 1) In central nervous system neoplasms, increased Akt protein expression and activity have been detected. PTEN is frequently lost in brain tumors (46, 53, 69).
- 2) Adenocarcinoma of the endometrium is also associated with mutations or deletions of the tumor suppressor PTEN and increased levels of pAkt (46, 59, 81).
- 3) Noske *et al.*, in 2007, observed increased Akt expression in 58% of primary ovarian carcinomas in comparison with normal ovaries by immunohistochemistry (68). Many other studies reported on this possible association (49, 54, 59). Increased activation of Akt is noted in ovarian cancer, which exerts antiapoptotic effect, antagonized cell cycle arrest, modulated angiogenesis, cisplatin resistance, and mediated mRNA translation through mTOR signaling (59, 72). PTEN mutation is believed

to be a mechanism for constitutive activation of the Akt pathway (81). Actually, PTEN mutations are frequently identified in many other malignancies, including malignant melanoma, glioblastoma, prostate and breast cancers (81). The isoforms Akt1 and Akt2 are expressed at the mRNA level in all ovarian cell lines (61, 66, 68), whereas no relevant Akt3 mRNA levels has been detected by conventional and quantitative RT-PCR (68). The elevation of Akt2 occurs in 10–20% of this cancer type (66, 71).

- 4) In breast cancer, the increased amplification of wild-type Akt protein is also detected (49, 53, 54, 82). In contrast to ovarian cancer, breast cancer cells show overexpression of whole Akt isoforms, including Akt3 (28, 33, 53, 66, 82). Akt2 has been shown to enhance the migratory and invasive characteristics of breast carcinoma cells; moreover, Akt2, but not Akt1 or Akt3, has been shown to upregulate α 1-integrins, and to promote adhesion and invasion of breast cancer cells *in vitro*, and also metastasis *in vivo* (53).
- 5) *Akt2* gene amplification is found in 10–20% of pancreas cancers (66).
- 6) Other studied types of cancer associated with Akt irregularity are cancers of the prostate (46, 49, 69), kidney (46), colon (46, 71), lung (46, 75), thyroid (46, 49), and blood components (46, 49), glioblastoma (49), and hereditary cancer syndromes (such as neurofibromatosis 1 and 2, tuberous sclerosis 1 and 2, von-Hippel–Lindau disease, nevoid basal cell carcinoma syndrome, Cowden disease, Peutz-Jeghers syndrome, familial adenomatous polyposis, and juvenile polyposis) (24, 71).
- 7) Importantly, Akt is also significantly phosphorylated at both the kinase domain and the regulatory domain in human head and neck squamous carcinoma specimens and other head and neck non-squamous carcinoma cell lines (46, 78), in which Akt is activated about 17% more in fresh tumor samples than in their normal tissues (45). The consequences are to promote tumor cell proliferation (78), survival (78), invasion and

metastasis (71), and radiation resistance (46). Detectability of pAkt, moreover, is significantly correlated with lymph node metastasis and TNM stage (46).

2.4.4 Akt and its upstream and downstream proteins as markers of cancers

When detecting suspicious lesions, healthcare providers should note that malignancies may be involved, and should use the appropriate detection methods (3). Although the eye is a valuable tool in the performance of an oral examination, it is not the sole tool available for identifying early SCC (14). Moreover, the development of novel anticancer drugs remains a slow, costly, and inefficient process, and only a small proportion of agents are proven effective in cancer treatment (51). Thus, one of the crucial elements to improve the success rate of cancer treatment is to develop valuable tools for cancer diagnosis and prognostication in a wide spectrum of common and uncommon malignancies (46, 51).

Studies have used pAkt immunohistochemistry as a prognostic marker for many types of cancer, including squamous carcinoma of the cervix and of the head and neck; adenocarcinoma of the endometrium, ovary, breast, prostate, kidney, colon and pancreas; carcinomas of lung and thyroid; and hematopoietic, soft tissue, and central nervous system neoplasms (46). They found that pAkt correlated with worse treatment outcome (46) such as radiotolerance (46, 83), hormonal therapeutic resistance (63), and less disease-free survival and more relapse (46, 53, 63). In the addition, pAkt can be detected in preneoplastic lesions (46). It may become a tool available for identifying early cancer and will become perfect if the biomarker can detect all malignancies in the premalignant phase (for early detection), the intra-curative period (for treatment monitoring), and the posttherapeutic follow-up (to evaluate treatment outcome and to sentinel recurrence). Besides, it is interesting that ability to detect individual molecular abnormality, particularly in PI3K/Akt pathway, can lead to proper personalized medicine in the future (51, 83).

If biomarkers are successfully developed, they may also be utilized for selecting the optimal dose and schedule of drug administration and for designing rational

drug combinations, at least to reduce the adverse effects and to evaluate treatment proficiency (51). A traditional way to assess efficacy of therapy is represented by immunohistochemistry analysis and immuno-blotting, using phospho-specific antibodies to detect the status of activation of specific markers. However, this approach has limitations, such as difficulties in getting quantitative immunohistochemistry analysis and in obtaining cancer specimens after treatment (32). For one already successful example, fluorodeoxyglucose (FDG)-positron emission tomography (FDG-PET), which is non-invasive imaging technique has already been used to monitor PI3K-inhibiting-drug usage (32). Strategies involving horizontal and vertical blockade of the pathway, as well as the use of biomarkers to select appropriate patients, and to provide proof of target modulation are also highlighted (51).

2.5 Blockage of aberrant signaling pathway as cancer treatment

Over the past decades, many investigations have revealed a number of signaling pathway that connects with each other as complex networks. The complex nature of these networks continues to be a challenge for the cancer biologist (26). Proper analysis of all of these signaling systems cannot only aid in understanding the behaviors of malignancies, but can also lead to innovation of successful therapies (26, 29). However, there are many mediators in the pathways that affect both cellular physiology and pathology (51, 79). In the concept of targeting therapy, precise intervention that dominantly targets signaling molecules critical to the pathogenesis and progression of cancer is needed (78). Intervention in the pathway involves both horizontal blockade, which uses targeting agents to inhibit two or more signaling pathways, and vertical blockade that aims to inhibit one or more agents in a specific pathway (51). Research on blockade of cancer-associated signaling pathways, especially the PI3K/Akt pathway, should continue.

2.6 Inhibition of the PI3K/Akt pathway for cancer treatment

Perturbations of the Akt pathway appear to occur in many human cancers, as described above (50, 53, 59, 60, 65). Because molecular targeting of tumor-specific signaling pathways is an interesting strategy for discovering and developing novel and potent anticancer drugs, the evolution of our knowledge of the PI3K/Akt pathway

should serve as an important guide in the design of future anticancer agents with maximal efficacy and minimal side effects (27, 70). So, inhibition of the pathway seems a powerful approach to induce anti-tumor activity and becomes an attractive potential candidate in combination with currently used treatment modalities (27, 53, 70, 81). Recent studies have shown that some natural products from daily diets have anticancer effects and this implies that some natural compounds in daily diets may inhibit Akt activation in cancer cells (33). A 270,000-compound library was screened for inhibitors of full-length Akt1, Akt2, and Akt3 (51). However, as an initial step toward inhibitor design, biologists must determine Akt expression behaviors (45).

Inhibitors of the upstream and downstream molecular targets of the PI3K/Akt pathway are discussed in the context of using the inhibitors to block this pathway for targeting cancer therapy (45). According to classification of Chen *et al.* in 2005, four representative targets are discussed here: a) the upstream mediators of the PI3K/Akt, b) PI3K, c) Akt, and d) the downstream effectors of PI3K/Akt (45).

2.6.1 Four representative targets

1) The upstream mediators

Many tumors have abnormal RTK activity caused by either mutation or overexpression of the receptors or ligands, resulting in PI3K/Akt signaling-dependent cancer progression (45, 49). In order to inhibit abnormal RTK activity, usage of antagonists and inhibitors of RTKs and their ligands is needed. Tyrosine kinase inhibitors are a subset of compounds that have reached the most advanced stage of development (26, 36). Furthermore, other components of the upstream signaling pathways seem good therapeutic targets (49). Examples of inhibitors are shown in Table 3 (26, 44, 45). Most of them can be divided into two main groups. One group is small-molecule tyrosine kinase inhibitors, such as gefitinib and erlotinib, which target EGFR. The other group is humanized antibodies against extracellular domains of receptors, such as cetuximab, which targets EGFR, and trastuzumab, which targets HER2 (49, 50).

Table 2 Examples of new drugs targeting upstream PI3K/Akt related molecules (44)

Target	Drugs	Class	Development stage
EGFR	•IMC-C225 cetuximab (Erbix; Imclone) •ABX-EGF (Abgenix) •EMD 72000 (Merck KgaA Darmstadt) •RH3 (York Medical Bioscience Inc.) •MDX-447 (Medarex/Merck KgaA) •ZD1839 gefitinib (Iressa; AstraZeneca) •OSI-774 erlotinib (Tarceva; OSI-Pharma.) •PKI116 (Novartis) •CI-1033/PD183805 (Pfizer) •EKB-569 (Wyeth–Ayerst) •GW2016/572016 (GlaxoSmithKline)	•Antibody •Antibody •Antibody •Antibody •Antibody •Kinase inhibitor •Kinase inhibitor •Kinase inhibitor •Kinase inhibitor •Kinase inhibitor •Kinase inhibitor	•Phase III •Phase II •Phase I •Phase II •Phase I •Phase III •Phase III •Phase II •Phase I •Phase I •Phase I
HER-2/Neu	•Trastuzumab (Herceptin; Genentech) •MDX-210 (Medarex/Novartis) •2C4 (Genentech) •17-AAG (Kosan)	•Antibody •Antibody •Antibody •HSP90 inhibitor	•Registered •Phase I •Phase I •Phase I
PDGFR	•Imatinib (STI571/Gleevec; Novartis)	•Kinase inhibitor	•Registered
PDK1	•UCN-01 (Kyowa Hakko Kogyo)	•Staurosporine analogue	•Phase I/II

Table 2 Examples of new drugs targeting upstream PI3K/Akt related molecules (44) (Cont.)

Target	Drugs	Class	Development stage
Ras	•ISIS 2503 (Isis Pharmaceuticals) •R115777 (Johnson and Johnson) •SCH66336 (Schering-Plough) •BMS214662 (Bristol-Myers Squibb)	•Antisense oligonucleotide •Farnesyl transferase inhibitor •Farnesyl transferase inhibitor •Farnesyl transferase inhibito	•Phase II •Phase II/III •Phase II •Phase I

2) PI3K

Phosphoinositide 3-kinase or PI3K is activated in a variety of different human cancers, and its inhibitors are under active development (69). In 1992, the first evidence was provided for a compound, Quercetin, having an inhibitory activity on PI3K. One year later (1993), Wortmannin, a compound from a metabolite of a fungus, was identified as a potent PI3K inhibitor (32). Then, immediately after this, another compound, LY294002, was described as the first synthetic PI3K inhibitor, although less potent than Wortmannin (32). The two classic PI3K inhibitors, Wortmannin and LY294002, have been widely used for more than a decade as research tools in both *in vitro* and *in vivo* models to elucidate the role of PI3K/Akt/mTOR signaling in various tumor cells (27, 45, 49).

In detail, Wortmannin is a furanosteroid (a class of steroids that have an extra-fused furan ring) fungal metabolite that was first isolated from a fungus, *Penicillium funiculosum* or *Talaromyces (Penicillium) wortmannii* (32, 45, 50) (Figure 12). It irreversibly inhibits PI3K by covalent modification of conserved Lys802 residue in the ATP binding site of the catalytic subunit p110 (45, 48). However, two disadvantages of the use of

Wortmannin are its solubility, which may limit its use in clinical trials (44); and its high hepatotoxicity (27, 51).

For better solubility, less cytotoxicity, and a better therapeutic index, many Wortmannin derivatives have been developed, two of which are PWT-458 (pegylated-17-hydroxy-wortmannin) and PX-866 (acetic acid (1S, 4E, 10R, 11R, 13S, 14R)-[4-diallylaminomethylene-6-hydroxy-1-methoxymethyl-10, 13 -dimethyl-3,7,17-trioxo-1, 3, 4, 7, 10, 11, 12, 13, 14, 15, 16, 17 -dodecahedron-2-oxa-cyclopenta [a] phenanthren-11-yl ester) (45, 50, 51).

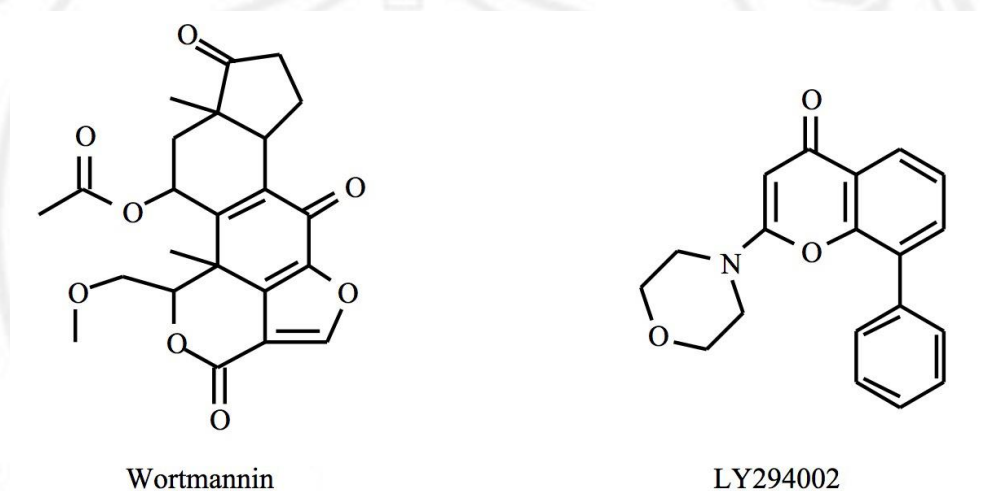


Figure 12 Chemical structures of Wortmannin and LY294002 (45)

Another classic PI3K inhibitor is LY294002 (Figure 12). LY294002 (2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one) is a water-insoluble flavonoid derivative and is the first synthetic specific inhibitor of PI3K (44, 45). LY294002 inhibits PI3K by reversible binding to ATP site of PI3K (44, 45). Look alike Wortmannin, the high cytotoxicity of LY294002 precludes its clinical application (27). Other inhibitors are shown in Table 4.

Table 3 Examples of PI3K inhibitors in clinical trials (51)

Drug	Sponsor	Phase	Schedule (route)	Target
•SF1126	•Semaphore	I	•Twice a week every 4 weeks (IV)	•Pan PI3K, mTOR, DNA-PK
•XL765	•Exelixis Inc.	I	•Twice daily (oral)	•Class I PI3K, mTOR
•XL147	•Exelixis Inc.	I	•21 days/7 days off and daily (oral)	•Class I PI3K
•XL147 and erlotinib	•Exelixis Inc.	I	•XL147 21 days/7 days off + erlotinib daily (oral)	•Class I PI3K, EGFR
•GDC-0941	•Genentech	I	•Daily (oral)	•Class I PI3K
•BGT226	•Novartis	I/II	•Daily (oral)	•Class I PI3K, mTOR
•BEZ235	•Novartis	I/II	•Daily (oral)	•Class I PI3K, mTOR

3) Akt

Akt is an attractive therapeutic target in cancer cells (73). In the past, very few small-molecule inhibitors of Akt have been reported. However, in recent years, the rate of discovery of new inhibitors has been drastically accelerated (45, 46). Rational design of Akt inhibitors is generally based on targeting three regions of this enzyme: the PH domain, the ATP binding pocket in the kinase domain, and the regulatory domain (45). Moreover, since each Akt isoform overexpresses differently in different cancers, Akt-specific-isoform inhibitors must be developed (45). These first generation inhibitors displaying some selectivity toward Akt1, Akt2, or both Akt1 and Akt2 (both *in vitro* and *in vivo*) have been named Akti-1, Akti-2 and Akti-1/2, respectively (48, 65) (Figure 13).

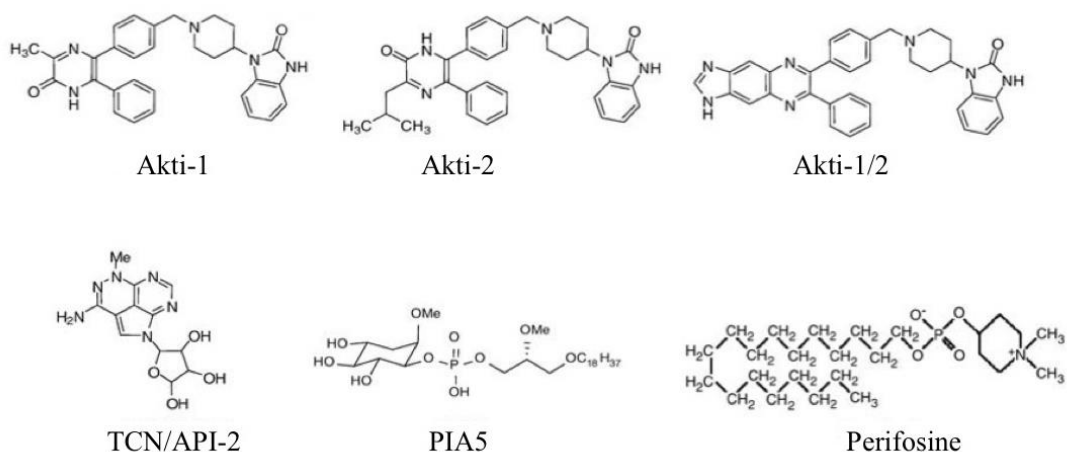


Figure 13 Compound structures of some Akt inhibitors (48)

Some other examples of Akt inhibitors are MK-2206 (a pan-Akt inhibitor), CMEP, API-59CJ-OMe, deguelin, KP372-1, perifosine, D-3-deoxy-phosphatidyl-myo-inositol PX-316, API-2/TCN (tricitriline phosphate monohydrate, VD002), GSK690693, XL418, and phosphatidyl inositol ether lipid analogues (PIAs) (32, 46, 51, 62, 70, 78, 81) (Table 5).

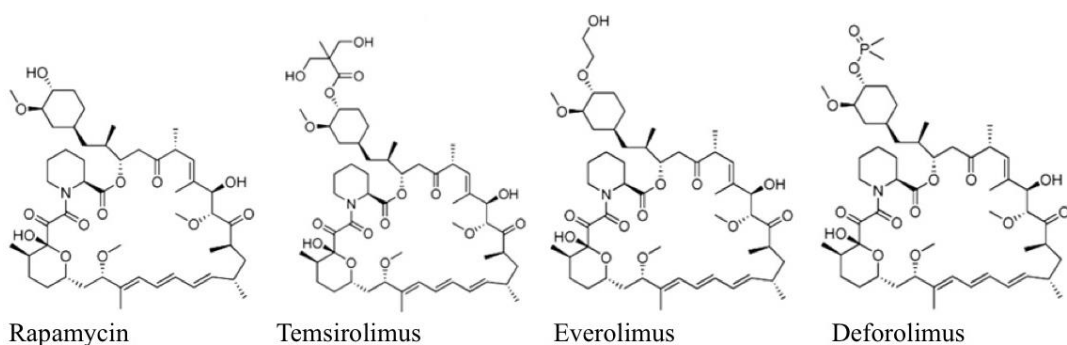


Figure 14 Compound structures of rapamycin and its derivatives (51)

Table 4 Examples of Akt inhibitors in clinical trials (51)

Drug	Sponsor	Phase	Akt Target	Patient population	Status
•GSK690693	•Glaxo-SmithKline	I	•Pan-Akt	•Advanced solid tumors	•Actively recruiting
•API-2/TCN	•violet	I	•Activation	•Phosphorylated-Akt positive (IHC) advanced solid tumors	•Actively recruiting
•API-2/TCN	•violet	I	•Activation	•Advanced hematological malignancies	•Actively recruiting
•XL418	•Exelixis Inc.	I	•AKT and p70S6K	•Advanced solid tumors	•Enrollment suspended because of low drug exposure
•Perifosine	•AOI Pharma, Inc. /Keryx Biopharma.	II	•Translocation	•Metastatic HNSCC	•Ongoing but not recruiting
		II		•Metastatic breast cancer	•Completed
		II		•RCC (post-sorafenib/sunitinib)	•Actively recruiting
		II		•Advanced solid tumors	•Ongoing but not recruiting
		II		•Malignant glioma	•Actively recruiting
		II		•Sarcoma	•Completed
		II		•Refractory and relapsed leukemia	•Actively recruiting

4) The downstream mediators

Although pathway inhibition involves both upstream regulators and downstream effectors, it is possible that a drug that targets a downstream effector might render fewer side effects because a downstream target usually controls fewer molecular events than an upstream mediator does (45, 50). Of many molecules in PI3K/Akt pathway, the most studied target is mTOR (27, 32). Just four drugs that target mTOR have been explored for clinical use: rapamycin, temsirolimus, everolimus and deforolimus (27, 50, 51) (Figure 14). The last three drugs, namely those tailed with “-limus,” are derivatives of rapamycin (45, 49, 59).

Rapamycin and its derivatives inhibit mTOR by binding a member of the ubiquitous immunophilin family of FK-506-binding proteins (FKBP-12, currently named Peptidyl-prolyl cis-trans isomerase FKBP1A). Even though these mTOR inhibitors have been extensively studied, much is unknown about mTOR and its inhibition (51) (Table 6) . In addition, other downstream molecules, which are less interesting and less studied than mTOR, are CAPN6 (64) and FOXOs (27).

Table 5 Summary of mTOR inhibitors in clinical trials (51)

mTOR inhibitor	Tumor types	Study phase
Rapamycin (sirolimus, Rapamune; Wyeth)	Not listed in view of large number of clinical trials (see www.clinicaltrials.gov)	
Temsirolimus (CCI-779; Torisel, Wyeth Pharmaceuticals)	Advanced solid tumors (I), prostate (I/II), breast, NSCLC, small cell lung cancer, endometrial, B-cell lymphoma, chronic lymphocytic leukemia, pancreatic cancer, glioblastoma multiforme, islet cell carcinoma, GIST, sarcoma, melanoma, mantle cell lymphoma (II, III), renal cell carcinoma (RCC), ovarian, head and neck SCC, neuroendocrine, multiple myeloma	Phase II unless otherwise stated

Table 5 Summary of mTOR inhibitors in clinical trials (51) (Cont.)

mTOR inhibitor	Tumor types	Study phase
Everolimus (RAD001, Novartis)	NSCLC, small cell lung cancer, endometrial cancer, colorectal cancer (CRC), melanoma, islet cell carcinoma (II, III), AML, hepatocellular carcinoma (I–II), pancreatic, glioblastoma multiforme, prostate, neuroendocrine (III), breast, RCC, NHL, gastric, hematological malignancies, melanoma	Phase II unless otherwise stated
Deforolimus (AP23573, ARIAD Pharmaceuticals, Inc. and Merck & Co., Inc.)	Advanced solid tumors (I), endometrial cancer, taxane resistant CRPC, hematological malignancies, advanced sarcoma (II, III versus placebo), glioma (I)	Phase II

2.6.2 Usage of the inhibitors in combination with conventional treatments for cancer treatment

It has been postulated that inhibition of Akt alone or in combination with conventional chemotherapeutics will reduce the apoptotic threshold and preferentially kill cancer cells (48). It is still not clear whether targeting multiple targets of the same signaling pathway at the same time will reach a better clinical outcome. Nonetheless, such a multiple-target inhibition approach may have some advantages over a single target approach in terms of therapeutic index because of synergistic effects observed in preclinical studies (45). The combination of RTK inhibitors with other inhibitors of the PI3K/Akt signaling axis has been shown to be beneficial for overcoming RTK-inhibitor resistant cancer cells (45). Moreover, the inhibitors of the PI3K/Akt signaling axis can be combined with currently-used conventional chemo- and/or radiation therapies (49, 81). These combinations may lead to an improvement of treatment outcome (49) by reducing the side effects of

conventional therapies while enhancing antitumor efficacy and/or overcoming resistance to chemotherapy, radiation therapy, and hormone therapy (44, 49, 71).

For example, rapalogs, RAD-001 and CCI-779 have been successfully combined *in vivo* and *in vitro* with cisplatin, gemcitabine and camptothecin (32). In addition, rapamycin and RAD-001 are also potent radiosensitizers, indicating that combined inhibition of mTOR with chemotherapy or radiotherapy is a promising approach in cancer treatment (32). Currently, many worldwide research laboratories are working to create successful combinations of anticancer treatment (Table 7). In future, combinations of conventional treatment modalities with PI3K/Akt pathway inhibitors may possibly be used clinically and effectively (49).

Table 6 Examples of studied combination treatments (51)

PI3K/Akt inhibitor	Combination agent	Tumor types	Phase
Perifosine	•paclitaxel	•Advanced solid tumors	I
	•gemcitabine	•Advanced solid tumors	I
	•UCN-01	•Relapsed or refractory acute leukemia, chronic myelogenous leukemia or high risk myelodysplastic syndromes	I
Temsirrolimus	•Hormone therapy	•Prostate cancer	I
	•Carboplatin and paclitaxel	•Advanced solid tumors	I
	•Imatinib mesylate	•CML	I
Everolimus	•Cisplatin	•Head and neck SCC	I
	•Docetaxel	•Breast cancer, NSCLC, CRPC	I-II
	•Imatinib mesylate	•CML, RCC, melanoma, GIST	I-II
Deforolimus	•Doxorubicin	•Soft tissue sarcoma	I

2.6.3 Tumor-specific Akt isoform and personalized medicine

Although downregulation of the PI3K/Akt pathway should provide antitumor effects against cancers, undesirable side effects are possible because of the role of PI3K/Akt in normal physiology; thus, understanding the balance of effects on tumor versus normal cells will be important for maximizing therapeutic benefit and minimizing toxicities (49, 51). At present, several types of Akt inhibitors have been investigated; nevertheless, the use of these inhibitors is limited either by high toxicity or by low bioavailability and stability *in vivo* (27, 70). Some studies have implied that the specificity for the inhibition of Akt signaling may prevent toxicity to normal cells because each isoform appears to have separate functions in normal cells (45, 71) but Akt1 and Akt2 chemical inhibitors with good selectivity have not proven to be successful in clinical trials due to their toxicity (53); moreover, at this time, there is no reports of specific Akt inhibitor that have been shown to be both active against tumors *in vivo* and devoid of major toxicities to normal tissues (24). Therefore, it is very important to be aware of the potential toxicity to normal cells, and the domination of Akt isoforms in each cancer or in each individual is also very important (51).

Therapeutic advances to date have been achieved by performing large randomized clinical trials (83). The problem is that these trials were designed to determine the best therapeutic approach for the median population, not for a specific individual (83). Different gene alterations are present in different cancer types and the progression of each cancer is often unique to each patient (26, 48). Hence, it is likely that each combinational therapy must be tailor-made to the individual patient; in addition, prospective therapies must be tumor cell-specific and easy to administer (26, 45). For example, an Akt1 inhibitor may be beneficial in fibrosarcomas to inhibit tumor growth and metastasis as well as stromal induction; however, Akt1 inhibition may not be recommended and may be contraindicated in patients with breast cancer, since it may promote invasive migration to metastasis (53). Since the specific isoform aberration should be determined in each cancer field and individual patients, it is fundamental to design

more effective therapeutics for each cancer treatment by using the concept of personalized medicine (51, 53).

2.7 Relation of Akt isoforms and oral cancer

Although each isoform has non-redundant functions, and each cancer type may express different Akt isoforms, many reports have revealed that only Akt2 is frequently amplified and overexpressed in human cancers, whereas Akt1 amplifications and mutations are rarely found (48-51). Akt2 overexpression has been reported in cancers of the ovary, pancreas, breast, stomach, colon, and, particularly, cancer of the head and neck (2, 24, 33, 49, 71). Furthermore, it is possible that increased Akt2 expression may occur in levels of mRNA and/or protein (48, 71).

Iamaroon and Krisanaprakornkit in 2009 analyzed mRNA expression of each Akt isoform from five oral cancer cell lines (HN008, HN5, HN6, HN15, Detroit) and three cell lines of normal human oral keratinocytes (HOK #1-#3) by RT-PCR (2). The results showed that mRNA expression of Akt1 and Akt2 in five oral cancer cell lines did not clearly differ from that in normal oral keratinocytes, whereas Akt3 mRNA was expressed at much lower levels than were Akt1 and Akt2 mRNAs, implying that Akt3 protein is minimally expressed in OSCC cell lines (Figure 15). GAPDH mRNA, as a control, was equally expressed among the different samples.

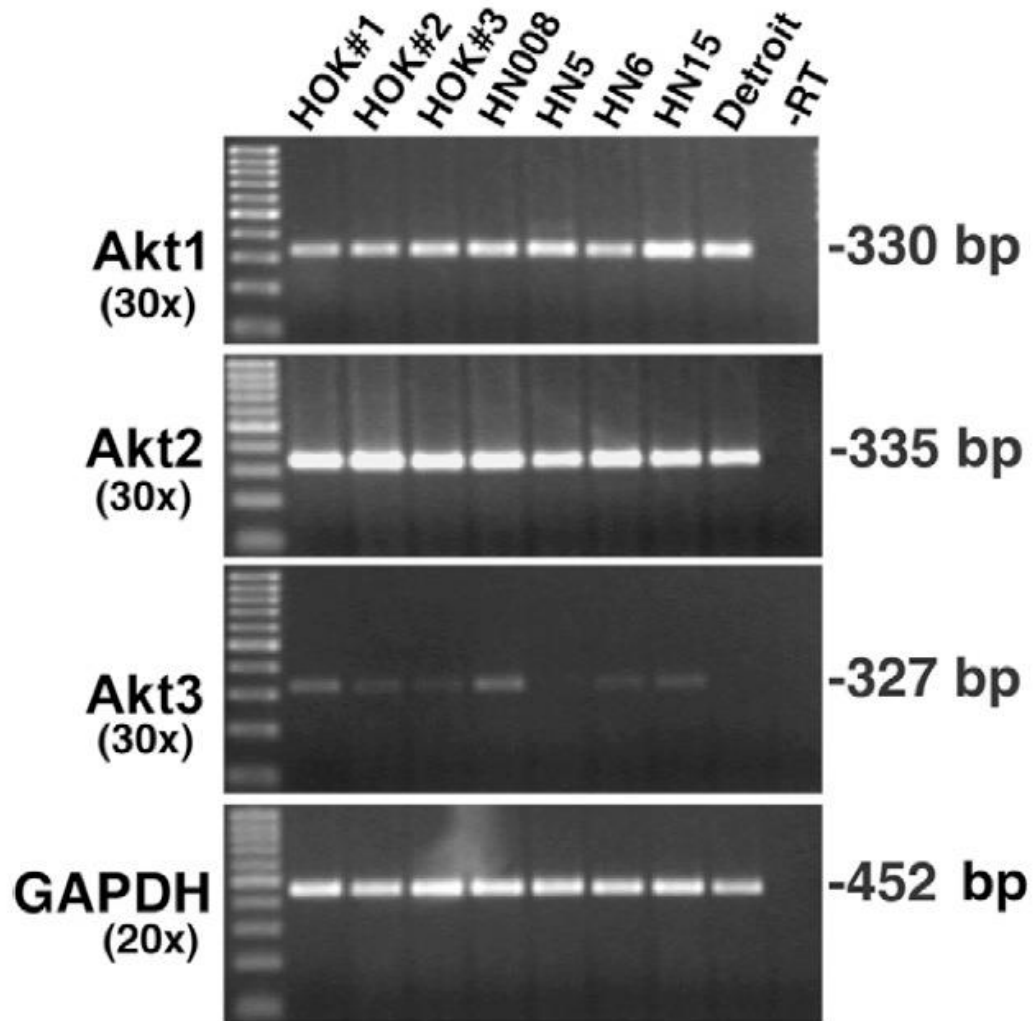


Figure 15 RT-PCR analysis of mRNA expression for each Akt isoform from five oral cancer cell lines compared with three cell lines of normal human oral keratinocytes; 30x = 30 cycles; 20x = 20 cycles of PCR (2)

Furthermore, Iamaroon and Krisanaprakornkit investigated the expression of Akt1 and Akt2 proteins, and the phosphorylated form of Akt (pAkt1/2/3) in whole cell lysates by Western blot analysis (2). The results clearly showed upregulation of Akt2 and pAkt1/2/3 proteins in all five oral cancer-derived cell lines, whereas Akt1 protein was constitutively expressed in three cell lines of normal oral keratinocytes (Figure 16). GAPDH protein was equally expressed among all samples.

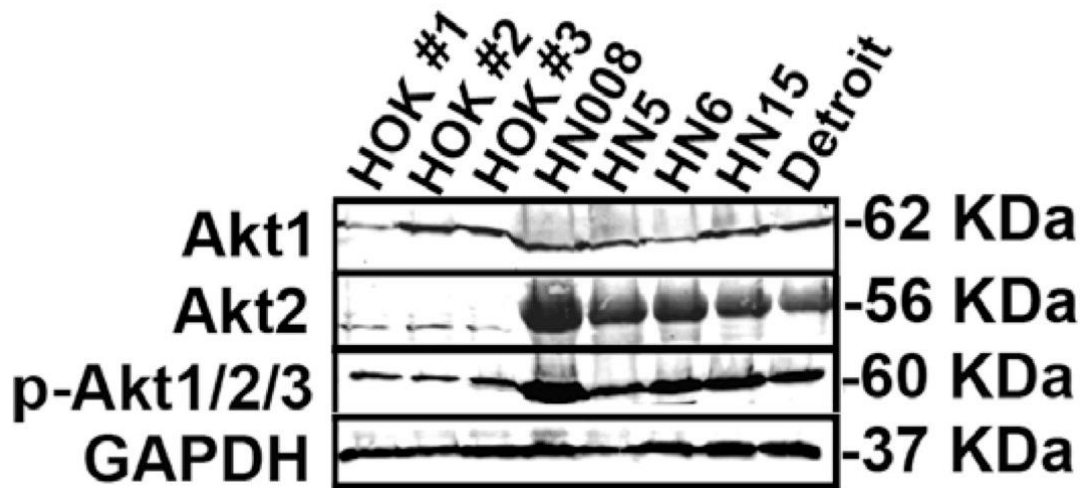


Figure 16 Western blot analysis of expression for Akt1 and Akt2 proteins from five oral cancer cell lines compared with three cell lines of normal human oral keratinocytes (2)

Interestingly, the phosphorylated form of Akt1/2/3 was upregulated in five oral cancer cell lines, implying that the phosphorylated form of Akt2 is predominant in oral cancer cell lines due to constitutive Akt1 and minimal Akt3 expressions.

Therefore, Akt2 was found to be overexpressed in OSCC cells at the translational level but not overexpressed at the transcriptional level. These results bring out the question of why Akt2 protein continued to be expressed at a high level in comparison with normal epithelial cells, whereas low and/or constant levels of Akt2 mRNA were seen. It is possible that translational and/or post-translational modification of Akt2 takes place during gene regulations in the concept of the central dogma of molecular biology (CDMB)

2.8 Central dogma of molecular biology and gene regulation

The central dogma of molecular biology was first stated by Francis Crick in 1958 in a published first draft and then restated in a journal published in 1970 (23, 84, 85). The direction of the flow of information from DNA to protein through an intermediate molecule (mRNA) is the backbone of the central dogma (23) (Figure 17). According to Kroll *et al.* in 2010 (23), the information transferred from DNA to protein begins with transcription. An enzyme RNA polymerase (RNAP) reads out the gene corresponding to the protein that the cell intends to produce. To do so, the double helix

of DNA opens and unwinds at the appropriate genomic position and then splits into a coding strand and a template strand. While traversing the DNA template strand, RNAP simultaneously creates a copy of RNA strand. This new product, which is called ribonucleic acid (RNA), is very similar to DNA except for a few important details: RNA is normally a single strand, containing ribose instead of deoxyribose, and the base uracil (U) is present instead of thymine (T). The transcribed RNA undergoes several processing steps – the most important is called splicing – the introns are cut out and the exons are joined to form messenger RNA (mRNA). After mRNA has been transported out of the nucleus, protein biosynthesis begins. mRNA carries the coded information to the ribosomes which are huge complexes of RNA and proteins; many ribosomes run along the mRNA, read the information on it and use it to synthesize chains of amino acids, and proteins. This process is called translation (23).

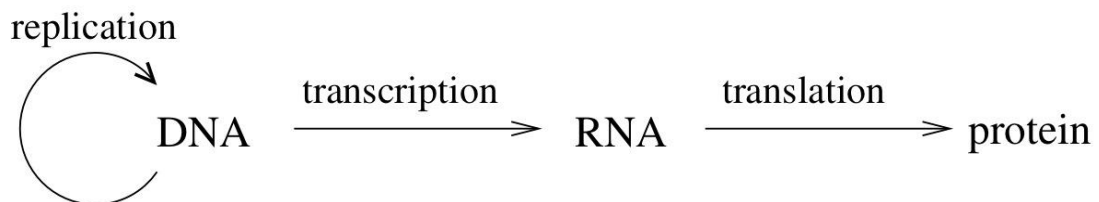


Figure 17 A skeleton of central dogma of molecular biology (23, 85)

There are more than 30,000 genes in human. Different genes are expressed in each cell at a different level and time (23). Importantly, the modification (or gene regulation) can occur in four steps (Figure18);

- a) transcriptional control (DNA to RNA);
- b) post-transcriptional control (immature RNA to mature stable RNA), such as RNA processing, RNA transport, and RNA degradation;
- c) translational control (RNA to protein);
- d) post-translational control, which occurs in the transition of immature protein to mature protein, and can appear in many mechanisms, including by addition of a carbohydrate group (carboxylation), a lipid group, a chemical group (such as phosphorylation, methylation, hydroxylation, sulfation, farnesylation, acetylation, and ubiquitination), and by cleavage of some amino acids from polypeptide chains.

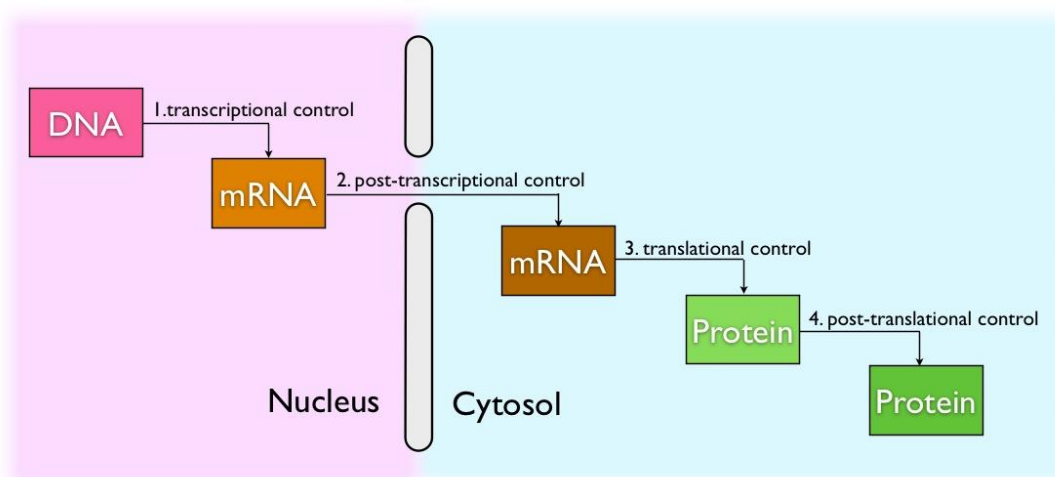


Figure 18 Regulation of gene expression

Many antibiotics encompass an important role in the field of protein synthesis or translation control, since they are able to inhibit specific steps of this process. Nevertheless, their mode of action at the molecular level is not yet fully understood, notwithstanding wide usage (86). Cycloheximide or anisomycin has a very wide spectrum. It is used as a fungicide and is also toxic for algae, protozoa, higher plants, and animals (86). Generally cycloheximide is the generic name for the compound B-[2-(3,5-di-methyl-2-oxo-cyclohexyl)-2-hydroxyethyl]-glutarimide or actidione; its crude formula is $C_{15}H_{23}NO_4$ (molecular weight: 281.36 gmol^{-1}) (86) (Figure 19). It is a natural substance isolated from cultures of *Streptomyces griseus*, which can also lead to the production of both cycloheximide and streptomycin antibiotics (86). Importantly, cycloheximide (CHI) is one of the most interesting and well-known protein synthesis inhibitors, which is widely used in many fields of research (86-88).

Cycloheximide inhibits protein synthesis through the ribosomal system in cytoplasm but leaves the mitochondrial protein synthesis unaffected (86). However, a complete understanding of the mechanism underlying cycloheximide activity is still incomplete (86). Most likely, this antibiotic irreversibly inhibits translation at the elongation and termination phases by provoking the accumulation of ribosome subunits on mRNA, and forms heavy polyribosomes in which the protein synthesis is disturbed (86, 88).

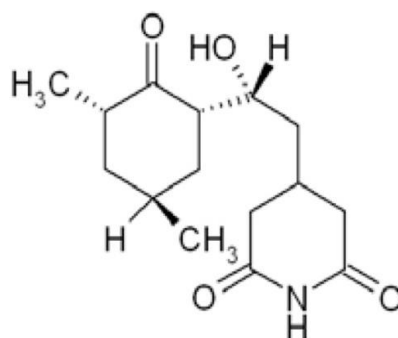


Figure 19 Chemical structure of cycloheximide (86)

In summary, cancer is one of the most complex and important diseases. Of all cancers, oral cancer or OSCC is an important worldwide public health problem. Many risks and etiologies of oral cancer were discovered; hence, carcinogenesis is complex multistep process. The hallmarks of cancer involve self sufficiency in growth signals, insensitivity to antiproliferative signals, antiapoptosis, immortalization, sustained angiogenesis, and tissue invasion and metastasis. Importantly, the basis of hallmarks of cancer is associated with alteration or aberration of signal transduction in some signaling pathways. The PI3K/Akt signaling pathway is now the most attractive one, which plays many roles in both cellular physiologies and all hallmarks of cancer of various malignancies, including oral cancer. Even though treatment of oral cancer, including surgery and radiation, has been developed for many decades, the survival rate of patient with oral cancer remains low. Targeting therapy is a new treatment modality which specifically focuses on specific aberrant molecules in any signal transduction pathway, especially the PI3K/Akt signaling pathway. Many inhibitors of the PI3K/Akt signaling pathway have been investigated in order to intercept the cancer progression. However, Akt has three isoforms that express differently in different cancers. Treatment of cancer by inhibition of Akt requires knowledge of the characteristics of expression of Akt isoforms in each cancer; otherwise inhibition of Akt becomes toxic to normal cells due to inhibition of other physiologic functions. A previous study found that Akt2 overexpresses only at the protein level, but not at the mRNA level (2). Consequently, in this thesis, the author wishes to confirm the Akt expression phenomenon and further prove whether or not Akt has translational and/or post-translational modification in oral cancer cells.