CHAPTER II

LITERATURE REVIEWS

Cancer

Cancer is a major public health problem and the most commonly cause of death in many countries. Cancer is a group of disease that cells loss of growth control, differentiate to abnormal solid mass of cells or tumor and have abnormal homeostasis (27). Cancer is initially found in many organs, mostly in lung and other organs such as breast, prostate, and colon (28). The initial tumors are called primary tumors and often become life-threatening by obstructing vessels or organs (27, 29). The normal cells are induced to transform to be cancer cells via multiple steps. These steps result genetic alteration and lead to malignant transformation. There are six essential alterations of cancer cell physiology including: self-sufficiency in growth signals, resistance to antigrowth signals, overcoming programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis and tissue invasion, and metastasis which is the major cause of death in patients (30).

Lung cancer

Lung cancer was the most commonly diagnosed cancer in males and fourth most diagnosed cancer in females. But lung cancer was the first and second common cause of cancer death in males and females, respectively (31). In 2013, lung cancer was the second cancer types for the estimated new cases and first cancer types for the estimated deaths of cancer both in women and men (28). The most common cause of lung cancer is smoking. However there are non-smokers has been diagnosed with lung cancer, 1 in 5 women and 1 in 12 men. Other risk factors for

lung cancer are the exposure to several occupational and environmental carcinogens. Most of patients with lung cancer are diagnosed in an advanced stage with metastatic disease resulting high mortality rate (32).

Lung cancer is divided into two main subtypes based on cell histopathology including non-small cell and small cell lung cancers (33, 34).

Non-small cell lung cancer (NSCLC) is found mainly about 85–90% of all cases of lung cancer and this group is subclassified to adenocarcinoma, squamous cell cancer, and large cell cancer. Patients with NSCLC are mostly diagnosed in an advanced stage with metastatic disease. They have extremely poor prognosis with a median survival of about 8 months.

1) Adenocarcinoma is found about 35–40% of cases. These cancer cells arise from mucus glands or epithelial cells within or distal to the terminal bronchioles. Adenocarcinomas are usually found as peripheral nodules or masses.
2) Squamous cell carcinoma is found about 20% of cases. These cancer cells arise centrally from the bronchial epithelium.
3) Large cell carcinoma is a heterogeneous group of relatively undifferentiated cancers found only 3–5% of cases. Large cell carcinomas typically have rapid doubling times and an aggressive clinical course. They are found as central or peripheral masses.

Small cell lung cancer (SCLC) is found about 10–15% of all cases of lung cancer. SCLC is more aggressive and more responsive to chemotherapy and radiation than NSCLC. These cancer cells have the central origin at bronchus and infiltrate

submucosal lead to narrowing or obstruction of the bronchus without a discrete luminal mass.

Cancer metastasis

Cancer metastasis is the process that cancer cells spread to other sites in the body through systemic circulation and form secondary tumors. The main step of cancer metastasis is shown in Figure 1. Primary tumors are progressively grown. Tumor mass increases and consists of cancer cells that have genetic variation and different type of cells or tumor heterogeneity. If the tumor mass is to exceed 1–2 mm in diameter, extensive angiogenesis occurs. Cancer cells possess invasive properties, begin to detach from their extracellular matrix (ECM) and then invade from the primary tumor by releasing protease. There are two major pathways of dissemination and cell motility have been proposed including collective migration and single cell migration that mostly via EMT or via amoeboid transformation. Then the cancer cells intravasate into pre-existing and newly formed blood and lymph vessels. After that the cancer cells go through systemic circulation both single cells and aggregates. Detached cells are normally destroyed by anoikis process. Detached cells may interact with blood components in circulation. Then survived detached cells are trapped in a capillary bed of distant organs. These cells adhere and extravasate to blood vessel wall. Metastatic cancer cells can stay there for long time. Finally, these cancer cells form secondary tumor at distal site requiring ongoing ECM remodeling and angiogenesis. Importantly, these cells can produce additional metastases to other organs (35, 36).

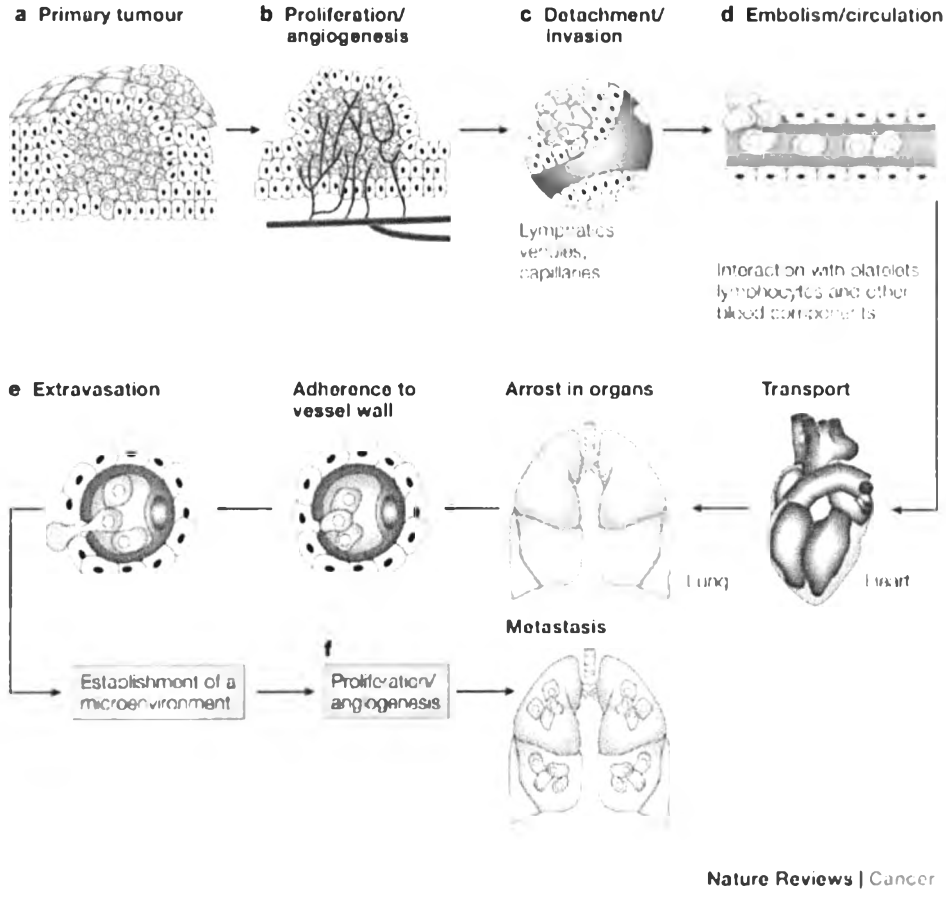

Figure 1 The main steps of metastatic cascade. Primary tumors that have genetic variation and different type of cells in tumor mass are proliferated and extensive angiogenesis occurs (a-b). Cancer cells begin to detach from their ECM and then invade from the primary tumor (c). After that the cancer cells that can survive in detached condition go through systemic circulation both single cells and aggregates. Cells are transported through systemic circulation and arrest in other organs (d). These cells adhere, extravasate to blood vessel wall and establish new microenvironment (e). Finally, these cells proliferate and new angiogenesis occurs. Importantly, these cells can produce additional metastases (f) (35).

Cell migration and invasion

In cancer metastasis, cancer cell migration and invasion are considered as critical steps. Cell migration is the movement of cells and cell invasion is the penetration of cells through tissue barriers such as basement membrane and interstitial stroma. There are many modulators of cell migration such as blood vessel and lymphatic endothelial cells, cancer-associated fibroblasts as well as chemokine gradient between tumor environment and blood vessel. The different of chemokine gradient between environments possess the effect to contribute secondary tumor at distal sites of metastatic cancer cells (37, 38). There are two main types of cell migration classified based on the morphology of migration patterns including single-cell migration and collective cell migration (Figure 2). In many tumors, both types of cell migration can be found with different degrees and combinations (39, 40).

Single cell migration is classified into three subtypes including mesenchymal, amoeboid and cell chains migration. This type of migration is an efficient mechanism for tumor-cell dissemination and metastasis because cells can migrate individually. For mesenchymal migration, their shapes are a fibroblast-like spindle morphology that is dependent on integrin mediated adhesion dynamics and the presence of high traction forces on both cell poles. For amoeboid migration, this movement uses less adhesion of cells that mostly found in stroma cells such as lymphocytes, neutrophils and some tumor cells. Movement is generated by cortical filamentous actin but lack of mature focal contacts, stress fibres and focalized proteolytic activity. Finally, chain migration occurs in non-neoplastic neural crest cells, myoblasts and melanomas. Cells form stream of cells after another in a strand-like fashion (37, 40, 41).

Collective migration is the movement of cells in groups that normally found in distinct cancer types, including high and intermediate differentiated types of lobular breast cancer, epithelial prostate cancer, large cell lung cancer, melanoma, rhabdomyosarcoma, and especially squamous cell carcinoma. Epithelial tumors commonly use collective migration mechanisms. In this type of migration, cells maintain cell-cell adhesion via the homotypic cell–cell interactions of cadherins that express on their membrane. In addition, these cells also communicate among cells. The movement uses pulling force of migratory leader cells on neighboring cells that adhere with cadherin interactions (37, 41, 42).

The step of cell movement is a complex process (Figure 3). Moving cells exhibit increased polarity with a defined front and rear of cells. At the front of the cell, the leading edge demonstrates a formation of a large, broad membrane protrusion, a flat ruffling of the cell membrane (lamellipodium) or thin and fingerlike protrusions (filopodia). These protrusions are made from the polymerization of actin at the membrane. These structures then form firmly attachment with the substratum and prevent the membrane from retracting. Membrane protrusion in the leading edge becomes anchored to the attachment site, called focal adhesion. This attachment with the substratum allows the cell to push forward. Then the body translocation of cell has been made, the bulk contents of the cell body are translocated forward. Due to the contraction of actin-myosin in cell, the nucleus and other organelles which embedded in the cytoskeleton in cytoplasm move forward as well. Finally, cell breaks the focal adhesions at the rear of the cell and the freed tail is brought forward (de-adhesion). The ability of a cell to move depends on a balance between the cytoskeleton-based mechanical forces and the resisting forces from cell

adhesions. Cells cannot move in such conditions, too strong attachment and no attachment with surface (43, 44).

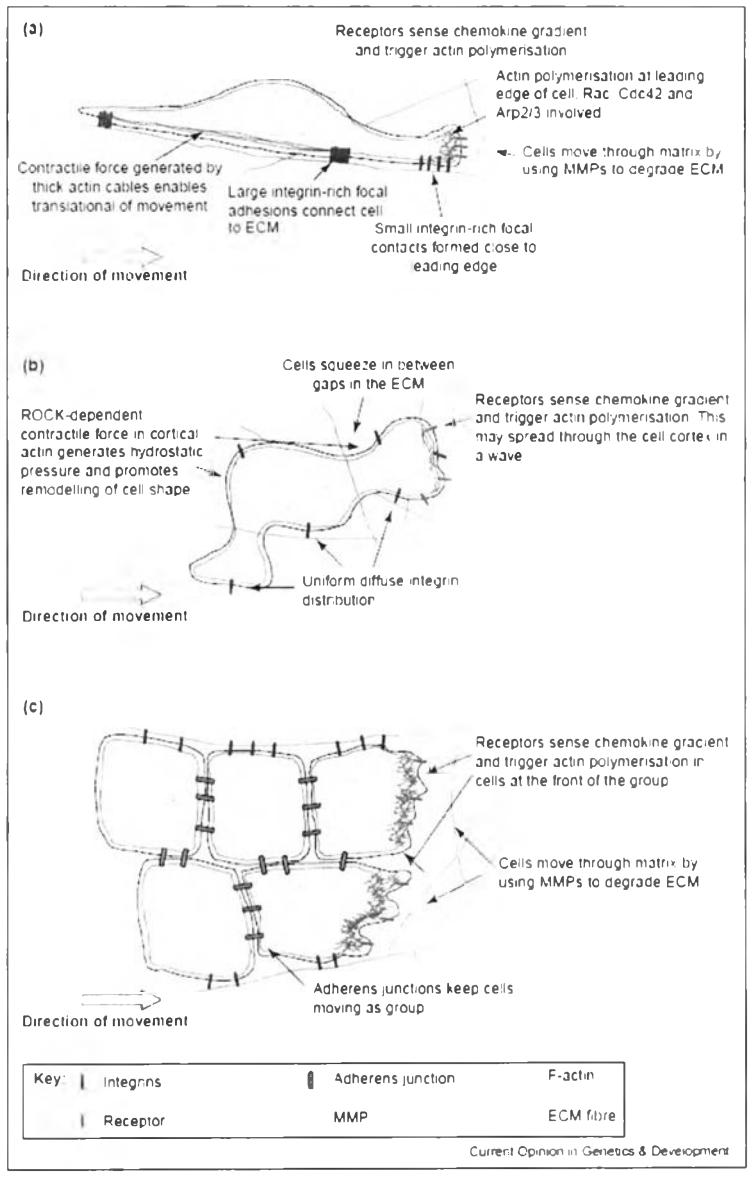

Figure 2 Mechanisms of cancer cell motility. For mesenchymal migration, the shapes of cells are fibroblast-like spindle morphology (a). For amoeboid migration, the shapes of cells are amorphous and cells rapidly changes in direction (b). For collective migration, cells form multicellular aggregates and move with stabilized cell-cell interactions (c) (41).

For cell invasion, cell recruit surface proteases to ECM contacts and focalize proteolysis before cell contraction. These enzymes cleave ECM components such as collagen, fibronectin and laminins. In addition, they also cleave pro-matrix metalloproteinases (pro-MMPs) to generate active soluble matrix metalloproteinases (MMPs), an ECM-degrading enzyme, as well as other collagenases that cleave native collagens into smaller fragments. Finally, the results from these enzyme actions facilitate cell movement through ECM (37).

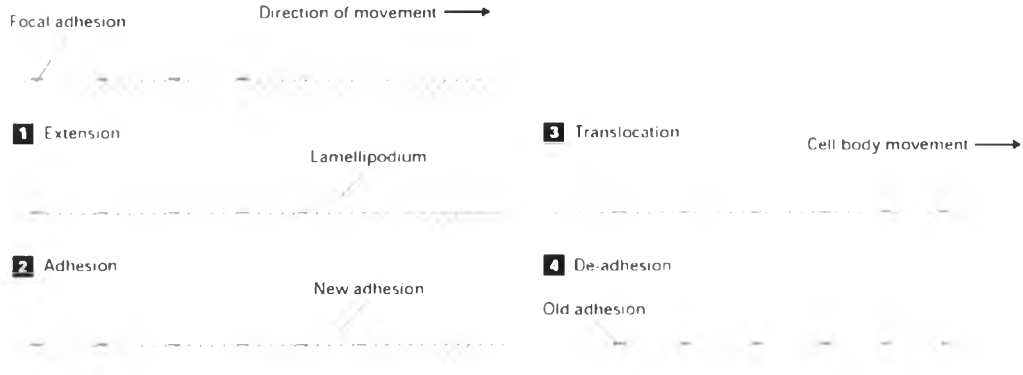

Figure 3 Steps in cell movement (1) At the beginning of movement, cell forms one or more lamellipodia from the leading edge of the cell. (2) Cell forms focal adhesions generated by the attachment of either lamellipodia or filopodia with the underlying surface. (3) Then cell body flows forward because of the contraction of cytoskeleton in cell. (4) The focal adhesion at tail of cell is detached and retracted into the cell body (43).

The role of migratory-related proteins

Cell migration occurs with the coordination of the dynamics of actin filaments and the formation and disassembly of cell adhesion sites (45). In addition, the speed of migration depend on the turnover rates of adhesion and de-adhesion events (37).

There are several migratory-related proteins that regulate these factors including focal adhesion kinase (FAK), signaling proteins such as Akt and Rho-family GTPase.

Cell adheres to ECM with integrin by the formation of focal contacts, a large integrin-based multiprotein complex, which connect together with cytoskeleton and regulate the action of several signaling proteins during the processes of cell adhesion and motility. Focal contacts consist of integrins, integrin-associated adaptor and signaling proteins. An important migratory regulator that found in focal contacts is focal adhesion kinase (FAK) that provides signaling and scaffolding functions at ECM-integrin junctions. When FAK is activated by various stimuli such as growth factors and clustering of integrins, FAK is phosphorylated. The phosphorylation of FAK is associated with the regulation of turnover of adhesion sites and also activates Rho-family GTPase such as Rac1 and RhoA via several intracellular signaling pathways. The activation of Rho-family GTPase proteins enables the polymerization, stabilization and disassembly of actin cytoskeletal structures in cell such as stress fibres, lamellipodia and filopodia. These processes result in membrane protrusion and cell polarization. Therefore the coordination of FAK phosphorylation and actin remodeling contribute to the migration of cell (Figure 4) (45, 46).

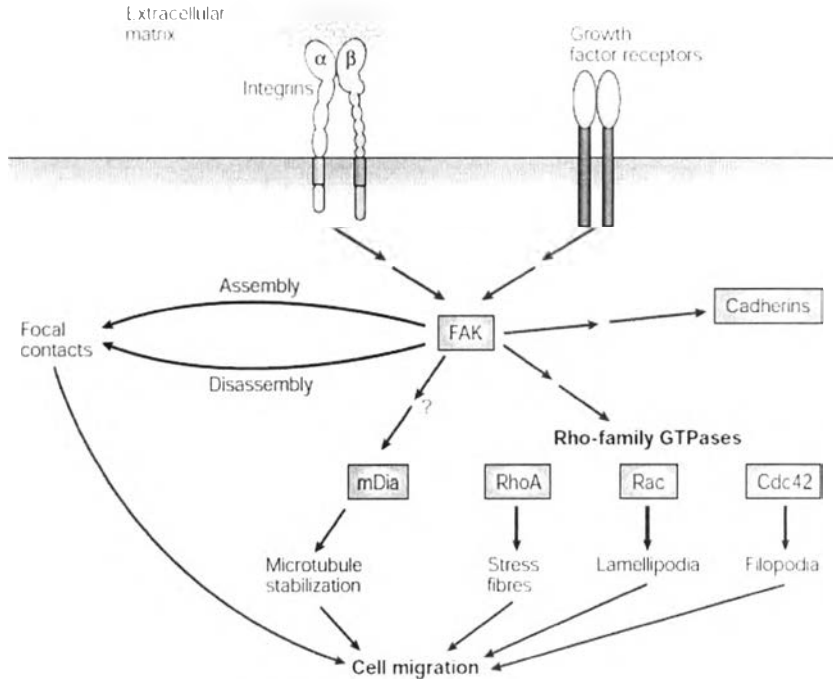

Figure 4 Focal adhesion kinase and signaling pathway in cell migration. There are several pathways to activate focal adhersion kinase (FAK) including growth factors and integrins during migration. FAK acts as an adaptor protein to recruit several migratory-related proteins that regulate an assembly and a disassembly of focal contacts. The activation of FAK by phosphorylation also mediate several downstream signaling that facilitate cell migration such as the Rho-family GTPases (RhoA, Rac and Cdc42) which are essential for the formation of stress fibres, lamellipodia and filopodia, respectively (45).

Focal adhesion kinase (FAK)

FAK is a 125-kDa nonreceptor protein tyrosine kinase enzyme localized on focal contact which is the area of cell attachment to ECM. It plays an important role in dynamic turnover of focal adhesion of cells and then modulates cell migration. FAK is activated by phosphorylation at least 6 tyrosine sites (Tyr-397, -407, -576, -577, -

861 and -925). FAK is activated by many types of stimuli including integrin-, growth factor- and G-protein-linked stimuli. Once cell receives motility-promoting stimuli, FAK is primary recruited to focal contacts resulting in autophosphorylation at Tyr397 of FAK. This autophosphorylation turns to Src-family protein tyrosine kinase recruitment at focal contact results in the phosphorylation of Tyr-576 and Tyr-577 in the FAK activation loop and then full catalytic FAK activation occurs. The formation of FAK-Src signaling complex enhances focal contact turnover and cell motility (47, 48). FAK plays as upstream of Rho-GTPase such as Rac1 and RhoA that control the formation and disassembly of actin cytoskeletal structures of cells resulting in promote increased cell motility (45, 48, 49). Moreover, it has been reported that FAK controls cell growth proliferation and survival signaling pathway as well as EMT process in cells (Figure 5) (46, 48).

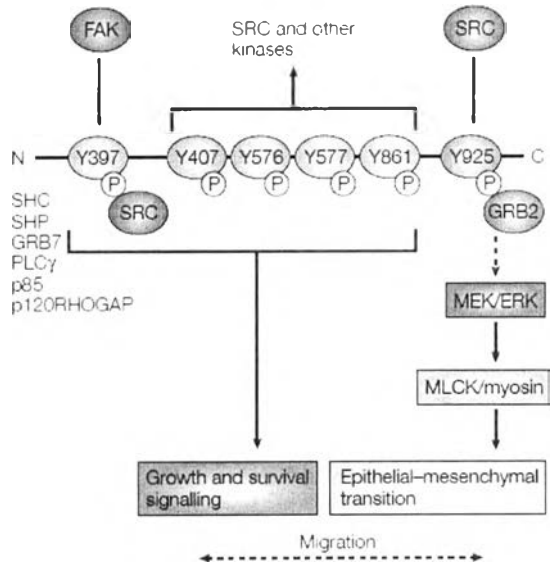

Figure 5 Focal adhesion kinase, phosphorylation sites and downstream signaling events. There are several phosphorylation sites on FAK protein resulting in different downstream signaling pathways (46).

There are many studies found the overexpression of FAK in cancer cells of various origins such as pancreatic, ovarian, cervical, osteosarcoma, kidney and lung. Increased FAK expression has been shown to be increased in invasive and metastatic tumors. In addition, FAK has been found to regulate proliferation, angiogenesis and survival in anchorage-independent condition of cancer cells. Importantly, the overexpression of FAK correlates with increased cancer aggressiveness resulting in increased cancer metastasis and reduced survival times of cancer patients (46, 48).

ATP dependent tyrosine kinase (Akt)

ATP dependent tyrosine kinase (Akt) is the serine/threonine protein kinase that plays an important role in the phosphoinositide 3-kinase (PI3K) pathway in cells. Akt/PI3K exhibits as a key regulator of many vital processes of cells including cell growth, proliferation, survival, metabolism as well as motility. An increased Akt expression was found in aggressive cancer cells. Akt consists of three isoforms including Akt1, Akt2 and Akt3 which has specific functions in distinct cell type. However, all three Akt isoforms are activated by similar mechanisms in PI3K signaling. Akt can play as an enhancer of cell motility in several cancer cells by regulation of actin organization, cell-to-cell adhesion and extracellular matrix degradation that facilitate cell invasion (50, 51).

PI3K is activated by growth factor receptor kinases (GFRKs), integrin, G-protein-coupled receptor (GPCR), and cytokine receptor (CR) signaling resulting in the phosphorylation of PI3K. Then activated PI3K converts phosphatidylinositol (4,5)-bisphosphate (PIP2) to phosphatidylinositol (3,4,5)-trisphosphate (PIP3). Akt which contains pleckstrin homology domain is recruited and binds to newly generated and enriched PIP3 at membrane and consequently activated. Once Akt translocates to

targeting site on membrane, it is rapidly phosphorylated on threonine 308 (T308) by phosphoinositide-dependent kinase-1 (PDK1) and on serine 473 (S473) by the mammalian target of rapamycin complex 2 (mTORC2) resulting in full Akt activation and then mediates downstream signaling pathways which control several cellular functions such as motility, survival and proliferation of cell (Figure 6). However, there are other pathways to regulate cell motility that are not depend on PI3K/Akt pathway (52).

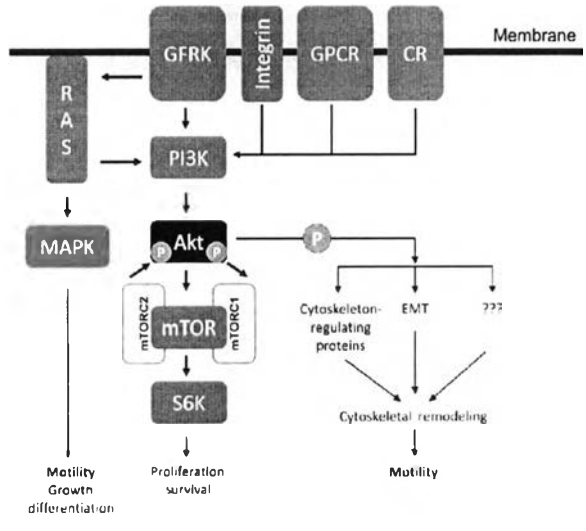

Figure 6 Phosphoinositide 3 kinase (PI3K)/Akt signaling pathway. The activation of growth factor receptor kinases (GFRKs), integrin, G-protein-coupled receptor (GPCR), and cytokine receptor (CR) lead to the phosphorylation of PI3K resulting in PI3K activation. Phosphorylated PI3K convert phosphatidylinositol (4,5)-bisphosphate (PIP2) to phosphatidylinositol (3,4,5)-trisphosphate (PIP3) and then promote the phosphorylation of Akt. Ras/MAPK signaling also activates PI3K activation. The activity of Akt requires mTOR complex action to regulate several cellular behaviors such as cell survival, cell proliferation and cell motility (52).

Akt has been reported as a downstream of FAK activation (49, 53). Activated AKt regulates reorganization of the cytoskeleton and mediates contraction of the cellular body that facilitates the migration of cell. The increase of Akt activation has been correlated with migration and invasion of cancer cells. Activated Akt promoted cell invasion and metastasis of breast and ovarian cancer cells through the upregulation of integrin signaling. In addition, knocking in Akt has been found to inhibit cancer cell migration and invasion resulting in decreased cancer metastasis both *in vitro* and *in vivo* study (52).

Rho-family GTPases

Rho-family GTPases are the downstream effectors of FAK/Akt signaling pathway and play an essential role to control cell migration and invasion. FAK can regulate the activation of these proteins through a direct interaction with, or phosphorylation of protein activators or inhibitors of Rho GTPases. The Rho GTPase cycle is shown in Figure 7. These proteins are activated by GTP binding and inactivated by GDP-binding (45, 53). The activation of Rho GTPases are regulated by three groups of proteins including guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs) and guanine nucleotide dissociation inhibitors (GDIs). GEFs stimulate the release of guanosine diphosphate (GDP) and then allow guanosine triphosphate (GTP) to bind to Rho GTPases resulting in the activation of the GTPase. GAPs are considered as negative regulators of Rho protein signaling pathways by enhancing intrinsic GTPase activity of Rho GTPases. Finally, GDIs are the proteins that bind to GDP-bound form of Rho GTPases and result in blocking of the GTPase cycle. Once Rho GTPases are activated, these proteins interact with cellular target proteins and initiate downstream signaling pathway that regulate actin reorganization (54).

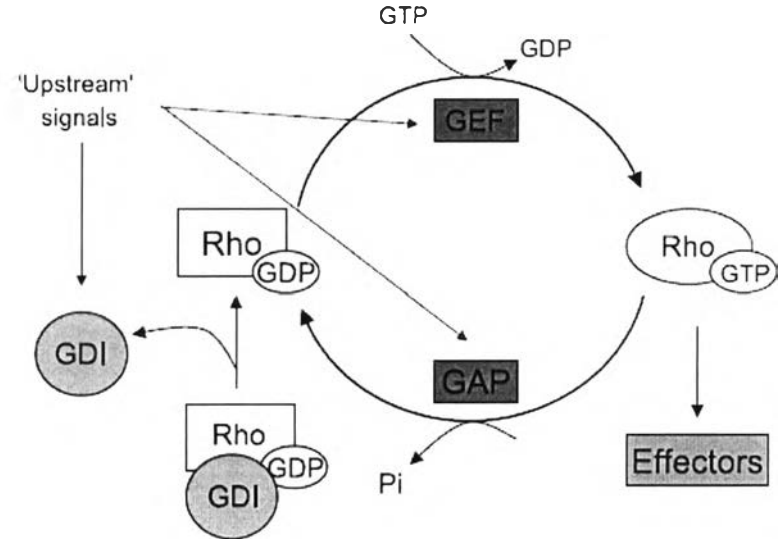

Figure 7 The Rho GTPase cycle. Rho GTPases are activated by GTP binding and inactivated by GDP binding. There are cycle regulators including guanine exchange factors (GEFs), GTPase activating proteins (GAPs) and guanine dissociation inhibitors (GDIs) (54).

The Rho-family GTPases consist of Rac1, RhoA and Cdc42. These proteins have functions to control the reorganization of actin cytoskeleton. RhoA primarily induces the formation of stress fibres and promotes actin-myosin contraction in the cell body and the rear. Its activity is enhanced in the initial cell-cell contact formation but downregulated in mature cell adhesion. Rac1 stimulates the formation of membrane protusion, lamellipodia and focal-complex formation through stimulation of actin polymerization and integrin adhesion complexes. Cdc42 promotes filopodia formation, cell rounding and the loss of actin stress fibres as well as the direction of cell movement (Figure 8) (45, 53, 54).

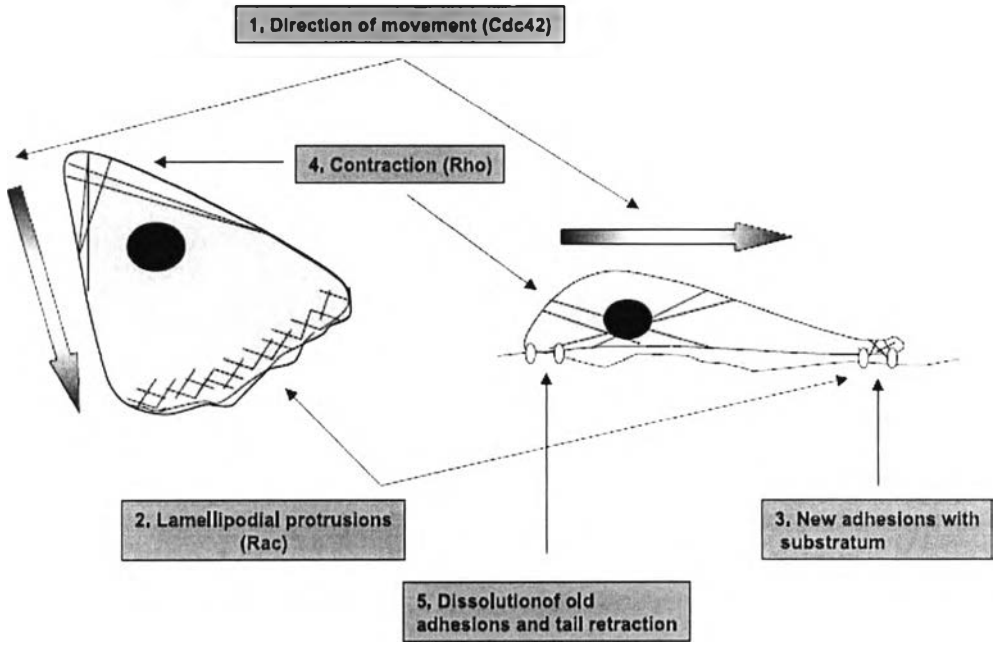

Figure 8 The functions of Rho GTPases in a migrating cell. The Rho-family GTPases including Rac1, RhoA and Cdc42 regulate several cytoskeletal modifications to facilitate cell migration. Cdc42 controls the direction of cell movement. Rac1 is essential to stimulate the formation of lamellipodia and focal-complex formation. RhoA induces the stability of cell-cell adhesion, stress fibres formation and promote actin-myosin contraction (54).

Anoikis

Normally, cell survival signaling including PI3K/Akt and Ras-MAPK pathways is activated through several factors such as growth factors, cell-cell interactions and cell-ECM interactions as shown in Figure 9. Growth factors activate cell survival via growth factor receptor (GFR) activation. Cell-cell interactions are mediated by cadherin interaction resulting in activation of PI3K/Akt pathway and inhibition of pro-apoptotic protein functions. For cell-ECM interactions, cells use their specific cell surface receptors known as integrins to interact with ECM counterparts and these

interactions induce cell survival signaling as well as other cellular functions. The different integrin heterodimers (α and β-subunits) ligate to distinct ECM component specifically. Ligated integrins transduce survival signaling pathway whereas unligated integrins that found when cells are detached from ECM can transduce a pro-apoptotic cascade. This is the mechanism for preventing cells to survive in an inappropriate environment. (16, 20, 55).

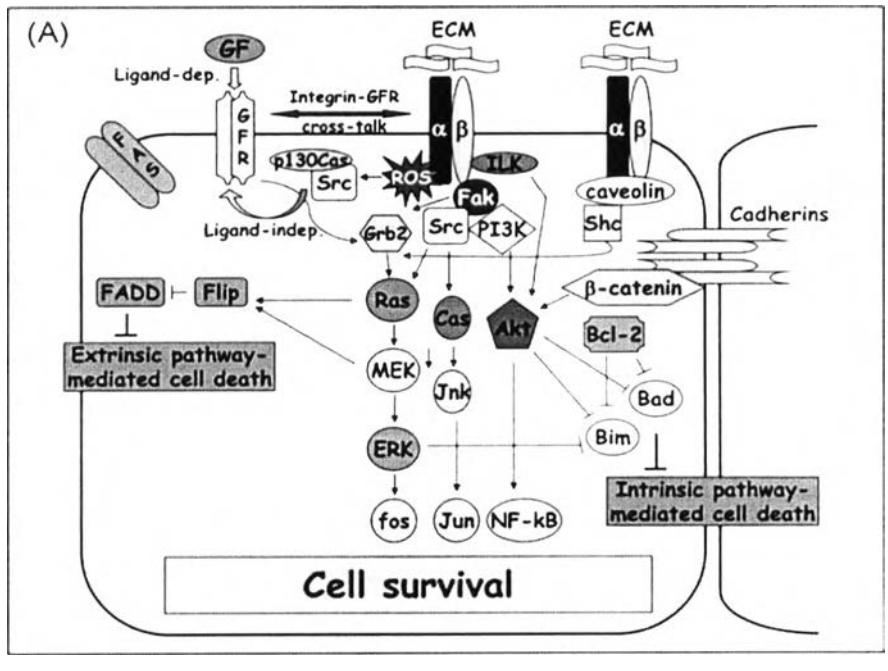

Figure 9 Survival signaling pathways include growth factor receptor (GFR) activation, intergrin-ECM engagement and cadherin mediated cell-cell interaction. These signals activate several pro-survival pathways including ERK, JNK and Akt resulting in the activation of fos, Jun and NF-kB transcription factors and the inhibition of pro-apoptotic proteins (20).

When cells are detached from ECM, there is a protective mechanism of the body to prevent these detached cells to form undesirable colonizing anywhere in the body. This mechanism is called "anoikis". Anoikis is a programmed cell death

that occurs when cells are detached from appropriate ECM and then cells undergo apoptosis via caspase activation. Anoikis is found in tissue homeostasis, development and disease such as cancer, cardiovascular disease and diabetes (16, 19).

Anoikis is found in several types of cells. The mechanism of anoikis can be classified as intrinsic and extrinsic pathways as shown in Figure 10. Intrinsic pathway is the main anoikis mechanism mediated by mitochondrial permeabilization which is the formation of oligomers in the outer mitochondrial membrane (OMM) creating channels within this membrane by the pro-apoptotic proteins of the Bcl-2 family including Bax and Bak. Intrinsic pathway is initiated by the pro-apoptotic BH3-only family of proteins. Especially, Bid and Bim play a major role as anoikis activators whereas other BH3-only family proteins are anoikis sensitizers. The sensitizer BH3-only proteins can bind to Bcl-2 which is an anti-apoptotic protein and then inactivate the anti-apoptotic functions of Bcl-2, thus there are free activator BH3- only proteins that able to induce Bax-Bak oligomer formation (20). When cells are detached from ECM and then integrins loss of engagement, Bid and Bim are activated and then interact with Bcl-XL at mitochondria to neutralize its pro-survival function. Then activated Bid and Bim rapidly promote the assembly of Bax-Bak oligomers within the OMM (19, 55). Then cytochrome C is released and form apoptosome with caspase-9 and apoptosis protease activating factor (APAF). Finally, this complex activates caspase-3 and cell undergoes apoptosis. In addition, loss of ECM contact also inhibits signal transductions including ERK and PI3K/Akt pathways resulting in the inhibition of Bim proteasomal degradation (19, 20).

Extrinsic pathway of anoikis is mediated by death receptors including Fas receptor and tumor necrosis factor-α receptor. Loss of contact with ECM induces the

expression of Fas Ligand (FasL) and its receptor. The activation of death receptors results in the assembly of a death-inducing signaling complex (DISC). Then DISC includes Fas-associated death domain protein (FADD) to form complex with several molecules of caspase-8 and then caspase-8 is activated. Activated caspase-8 proteolytically activates caspase-3 and -7 and cell then undergoes apoptosis. There are crosstalk between intrinsic pathway and extrinsic pathway of anoikis program because caspase-8 also promotes mitochondrial cytochrome c release and assembly of the apoptosome via Bid protein cleavage in its truncated form (t-Bid). In addition, the detachment-induced activation of caspase-8 is inhibited by Bcl-2 overexpression (19, 20).

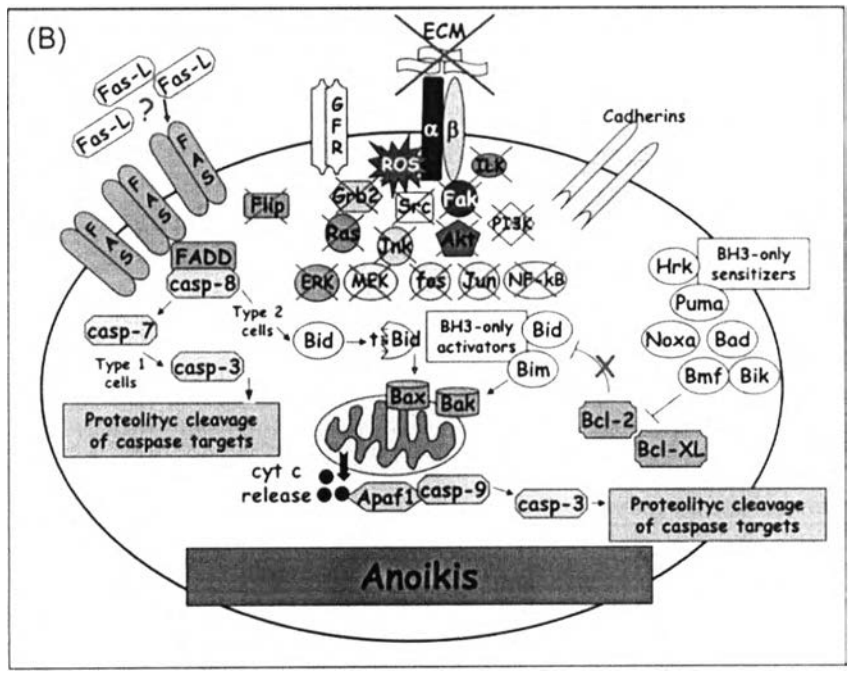

Figure 10 Anoikis signaling pathways. When cell are detached from ECM, cell survival signaling are inhibited. Detached cells undergo anoikis via intrinsic pathway and extrinsic pathway (20).

Anoikis resistance

In cancer metastasis, anoikis resistant cells survive after they are detached from primary site, go spread through circulation and then form secondary tumor at distal organ (Figure 11). So anoikis resistance is the critical mechanism of cancer metastasis (16). The mechanisms of anoikis resistance are not fully understood but there are many studies proposed the possible mechanisms of cells that promote survival pathways and inhibit apoptotic pathways when cells are detached. There are two main mechanism of cells to overcome anoikis after detachment including multicellular aggregation by E-cadherin and EMT (20).

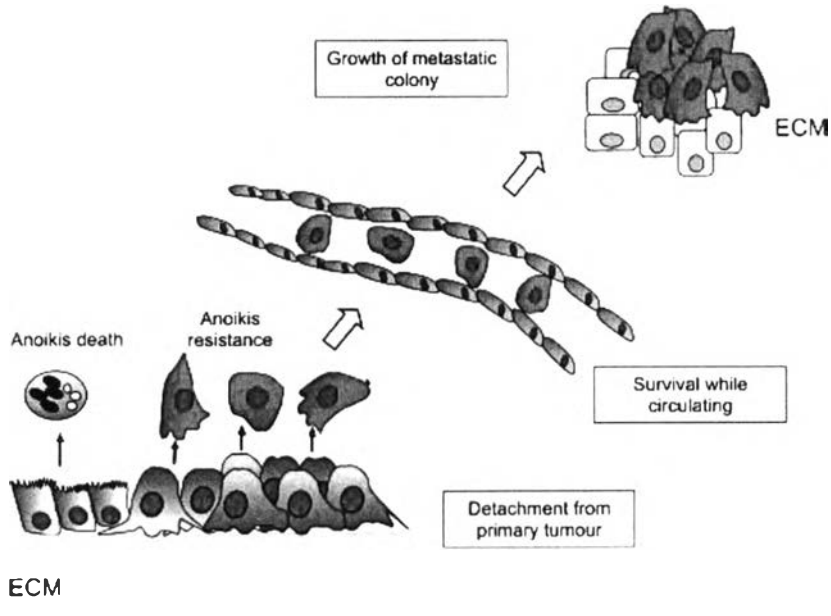

Figure 11 Anoikis resistance and cancer metastasis. Normally, when cells are detached from ECM, detached cells are dead by anoikis process. But aggressive cancer cells can overcome anoikis, survive in detached condition and then go through circulation to form secondary tumor at distal site (19).

The mechanism of anoikis resistance

Multicellular aggregation by E-cadherin

There are several studies found that cancer cells can overcome anoikis by increasing cell aggregation. This mechanism has been suggested as physiological mechanism to protect cells from anoikis. In this mechanism, cell-cell adhesion is mainly mediated by E-cadherin resulting in promote cell survival, cell proliferation as well as collective migration (20).

E-cadherin

E-cadherin is a member of type I classic cadherin and mainly found in epithelial cell membrane. Its domain structure is same as others (Figure 12). The function of E-cadherins is Ca^{2+} dependent and form homotypic homophilic binding between adjacent cells by using their extracellular domains. The intracellular domain of cadherins also interacts with the catenins family proteins including p-120, α and β-catenin which directly interact with actin filament by using their conserved cytoplasmic domains. E-cadherin regulates several epithelial cell phenotypes such as cell polarity, cell shape and cell motility. The expression of E-cadherin is reversible. E-cadherin expression can be downregulated via several pathways such as mutation, DNA methylation and transcription repressors such as snail, slug and twist. The increase of E-cadherin degradation can be caused by the competitively bind of N-cadherin to p120-catenin which is the stabilizer of E-cadherin (8).

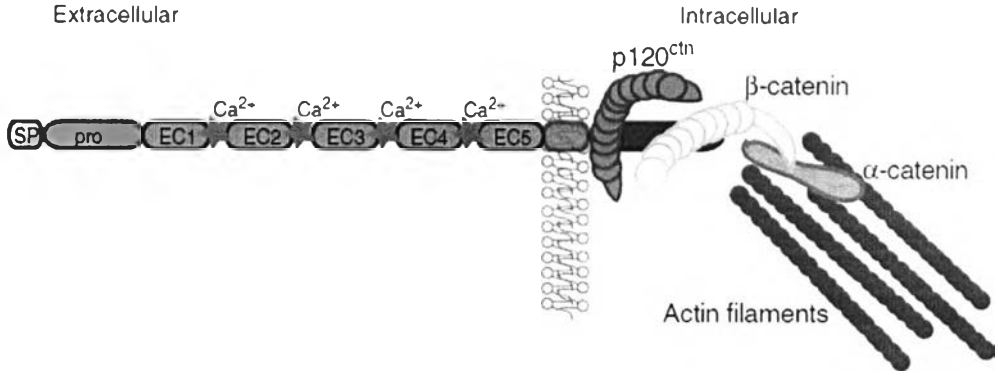

Figure 12 Cadherin domain structure. The domain structure of cadherin include extracellular domain which interacts with other cadherin of adjacent cells and intracellular domain which interacts with the catenins family proteins (8).

In this mechanism, cells use E-cadherin to form homotypic homophilic interactions with neighboring cells and form multicellular aggregation (56). Metastatic cancer cells possess ability to form homotypic aggregates and has metastatic potential more than non-metastatic cells (57). E-cadherin that mediates cell-cell aggregation also facilitates collective invasion of tumor cells as well as collective cell migration. E-cadherin interactions promote cell survival, cell proliferation as well as cell migration via ERK/MAPK and PI3K/AKT pathway, associated with ligand-independent activation of epidermal growth factor receptor (EGFR) as shown in Figure 13 (56).

Multicellular aggregation mediated by E-cadherin and anoikis resistance

There are many studies found that multicellular aggregation of cancer cells can resist to anoikis in both epithelial cells and nonepithelial cells. Lawlor et al. (2002) reported that anchorage-independent multi-cellular spheroids of Ewing tumor, which is sarcoma cells, activate ERK1/2 and the PI3K/Akt survival pathways resulting

in anoikis resistance in the suspension culture. However, cell proliferation of spheroidal cells was significantly lower than monolayer cells and more sensitive in serum starvation (58). Kang et al. (2007) showed the role of E-cadherin in anoikis resistance in Ewing tumor cells. E-cadherin cell-cell interaction leads to ErbB4 tyrosine kinase activation and then activate PI3K pathway resulting in anoikis suppression. In addition, E-cadherin interaction also increase chemotherapy resistance in tumor cells (59). For epithelial carcinoma cells, there was a study found that oral squamous epithelial cells form cell aggregation mediated by E-cadherin after detachment and this aggregates can survive and proliferate when compare with those of single cell culture (60). In addition, there was a study found that E-cadherin mediated cell-cell adhesion in oral squamous epithelial cells induce activation of EGFR and induced anoikis resistance through ERK/MAPK signaling pathway (61).

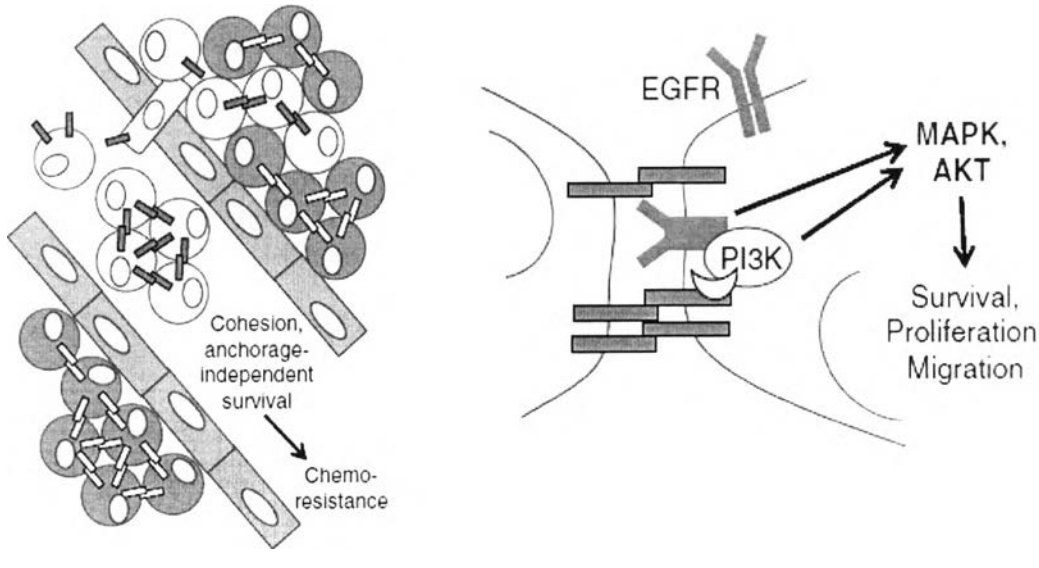

Figure 13 E-cadherin promotes anoikis resistance, cell proliferation and cell migration by mediate cell-cell aggregation of tumor cells (56).

Thus, these studies suggest the possible role of E-cadherin to protect cells from anoikis by maintenance of cell–cell contacts. E-cadherin transduces survival

signaling such as PI3K/Akt and ERK/MAPK signaling pathway. Several studies found that inhibition of E-cadherin function leads cells to anoikis. In addition, E-cadherin may associate with integrins and tyrosine kinase receptor activation and their signaling pathway for cell survival (20, 61, 62).

Epithelial-to-mesenchymal transition (EMT)

Another way of cancer cells to overcome anoikis is EMT (17). EMT is the reversible conversion of cell phenotypes from epithelial characteristics to mesenchymal characteristics. Epithelial cells have cell-cell junctions such as tight junctions, adheren junctions, desmosomes and gap junctions to form layers of cells. There are apical and basal sites on cell membrane which possess different properties. Each site contains different adhesion molecules such as cadherins, catenin and integrins. These adhesion molecules also transduce signaling to control cancer progression. Epithelial cells can move while cell-ECM interactions still remain. The phenotypes of mesenchymal cells are different from epithelial cells. Their shapes are irregular such as spindle-shaped and fibroblast-like morphology. They do not form a layer of cells and do not have the polarization on cell membrane. Cell-cell adhesions of mesenchymal cells are not as strong as those of epithelial cells leading to an increase of migration capability. The adhesion molecules and cytoskeleton of mesenchymal cells such as N-cadherin and vimentin, respectively, are different from those of epithelial cells (12, 63). In the beginning of EMT process, there is "cadherin switching" event, which consists of the downregulation of E-cadherin functions and the increasing in the N-cadherin expression (8). In EMT, cells switch the expression from E-cadherin to N-cadherin as well as from keratin-to vimentin-type intermediate filaments (14).

EMT is the rapid processes which cells lose many of their epithelial characteristics and acquire mesenchymal characteristics including cell-cell adhesion, cell polarity, cell architecture and behavior (14, 63). Cells that undergo EMT can be reversing by a mesenchymal-epithelial transition (MET), which is the conversion of mesenchymal characteristics to epithelial characteristics. MET is the process associated with kidney formation, somitogenesis, coelomic-cavity formation and also found before secondary tumor formation (63, 64).

EMT can be classified as 3 types based on the biological setting (64). Each type has distinct biological setting and refers to very different functional consequences. Type I of EMT occur in the earliest stages of embryogenesis. Type II of EMT is associated with wound healing, tissue regeneration and organ fibrosis (65). Type III of EMT is associated with cancer progression and metastasis (Figure 14). EMT in cancer cells results in the increased resistance of anoikis and increased metastasis of cancer cells (12, 13). Primary epithelial cancer cells which have genetic and epigenetic changes undergo this type of EMT leading to lose of cell polarity and detach from basement membrane as well as neighboring cells. Then cells become isolated because their cell-cell adhesion molecules are downregulated such as E-cadherin and catenin. Their polarity and shape are also modified which enhance their migration capability and anoikis resistant (17). This type of EMT facilitates cancer cells to move through blood vessels and spread in systemic circulation. When cancer cells reach secondary site, they undergo MET to revert their phonotypes to be epithelial characteristics. There are many studies proposed EMT as the critical mechanism of facilitating the malignant phase of epithelial cancer cells (64). There is a study demonstrated that the alteration of EMT-related proteins can be used as a

prognostic marker to predict overall survival of patients with lung adenocarcinoma (10). Nowadays, there are several EMT markers that are used in clinic for prognostic of tumor progression and metastasis (13). In this study, we focus on type III of EMT which is involved in lung cancer progression.

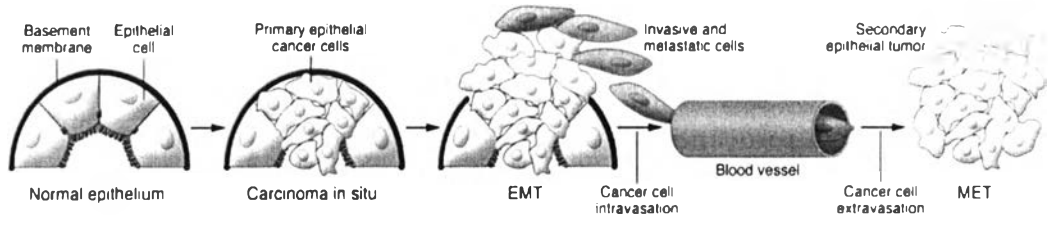

Figure 14 Type III of EMT. Primary epithelial cancer cells undergo EMT resulting in loss of cell polarity and detach from basement membrane as well as neighboring cells. EMT facilitates cancer cells to invade and then spread in systemic circulation. They undergo mesenchymal-epithelial transition (MET) at secondary site and then form secondary tumor (64).

EMT markers are the protein mainly found in mesenchymal cells such as N-cadherin and vimentin as well as transcription factors that repress the expression of E-cadherin such as snail and slug as shown in Figure 15.

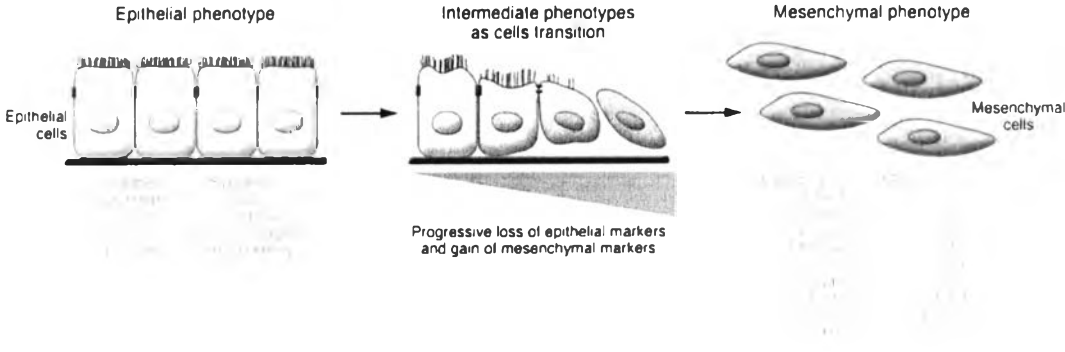

Figure 15 EMT process and EMT-related proteins. EMT is the reversible conversion of cell phenotypes from epithelial characteristics to mesenchymal characteristics

including cell-cell adhesion, cell polarity, cell architecture and behavior as well as their protein makers expression (64).

N-cadherin

N-cadherin is a member of type I cadherin and has the same domain structure of E-cadherin except sequence of homologous repeats at extracellular cadherin domain (EC). The adhesive function of N-cadherin is Ca^{2+} dependent mechanism which is similar to the function of E-cadherin. Cytoplasmic domain of N-cadherin interacts with p120-catenin, α-catenin and β-catenin, which are possible regulators of cadherin function and link to the cytoskeleton and several signaling pathways. N-cadherin possesses the ability to regulate the formation and remodeling of cell–cell adhesions and promoting cell survival and migration. N-cadherin is found in endothelial cells and mesenchymal cells such as neural cells and muscle cells. At firstly, N-cadherin is detectable during embryogenesis in type I of EMT. N-cadherin plays an important role in the development of several organs of embryos. In adult tissue, N-cadherin plays an important role to maintain adherence junction between cells and regulate several signaling pathways. However the signaling function of N-cadherin is complicated depending on the cell context (66, 67).

In type III of EMT, N-cadherin is upregulated in epithelial cell undergoing EMT and considered as the EMT marker. N-cadherin has been found to regulate several signaling pathways promoting cancer cell aggressiveness. N-cadherin can interact with FGFR and stabilizes the receptor on the cell surface, also enhances cell invasion and protects cell from apoptosis by suppressing apoptotic signals. N-cadherin also recruits PI3K and activates Akt resulting in promoting survival pathways and anoikis resistance. N-cadherin competitively binds to p-120 catenin which is E-cadherin stabilizer

resulting in increasing of E-cadherin degradation. The interaction between N-cadherin and catenins may directly affect cell adhesion and enhance cell migration. N-cadherin plays an important role to promote cell motility and invasion by increasing steady-state levels of active Rho-family GTPases including Rac1 and Cdc42 as well as crosstalking with integrins and growth factor receptors. The activation of these proteins influences cytoskeletal dynamics, both cell–cell and cell–ECM adhesions. In addition, N-cadherin facilitates the interactions with the endothelium that also express N-cadherin at extrajunction of cells as well as other mesenchymal cells (Figure 16) (8, 67).

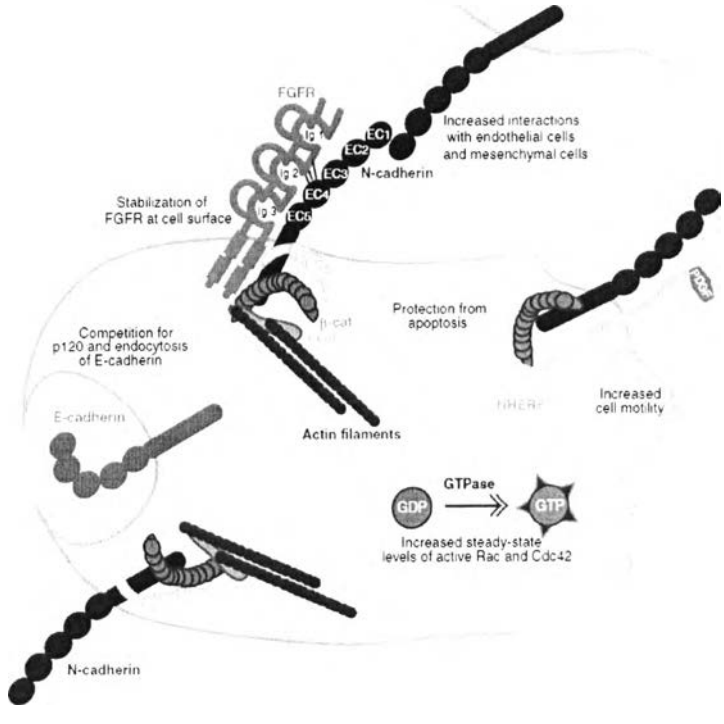

Figure 16 The role of N-cadherin in EMT process. N-cadherin promotes several cellular activities of cancer cells including cell survival, interactions with endothelial and mesenchymal cells, cell motility as well as the degradation of E-cadherin (8).

Vimentin

Vimentin is a 57-kDa protein, a member of type III intermediate filament protein. It is constitutively expressed in mesenchymal cells including endothelial cells lining blood vessels, renal tubular cells, macrophages, neutrophils, fibroblasts, and leukocytes. The regular function of vimentin is maintenance of the cytoarchitecture and tissue integrity. Vimentin also mediates several signaling pathways and cellular processes that are important in EMT and tumor progression. The overexpression of vimentin in cancer cells correlates with increased migratory and invasive ability of the cells. Vimentin has been considered to be a potential marker of EMT and an essential signaling protein involved in key cancer biological functions. In clinical practice, vimentin is considered as a clinical indicator to reflex the poor prognosis of cancer patient (Figure 17) (68-70).

Vimentin plays a role to regulate several intracellular signaling via the phosphorylation and protein interaction. The phosphorylation of vimentin by protein kinase A at serines 38 and 72 resulting in assembly dynamics of vimentin plays a critical role in cell attachment, motility, and cell signaling. Phosphorylated vimentin interacts with 14-3-3 proteins, the proteins involved in cell adhesion, cellular proliferation, and inhibition of tumorigenesis, resulting in an inactivation of 14-3-3 proteins. The vimentin/14-3-3 complex also prevents the formation of other 14-3-3 complexes and the dephosphorylation of proteins in the complex resulting in inhibition of anti-tumor activity in cells. In addition, vimentin interacts with phosphorylated ERK and MAPK and then protects it from dephosphorylation resulting in increased distance transport of activated ERK in cells. Vimentin was also reported as a downstream target of PI3K/AKT pathway resulting in increased cell motility and

cell invasion. The upregulation of vimentin also stabilized Scrib, a protein involved in cell migration, thereby enhancing migratory and invasive ability of cells. Furthermore the EMT inducing effect of slug and Ras was shown to be dependent on the up-regulation of vimentin. In addition, vimentin is essential for the formation and function of invadopodia and lamellipodia during cellular invasion and migration as well as the maintenance of cell polarity in migrating cells (68, 70).

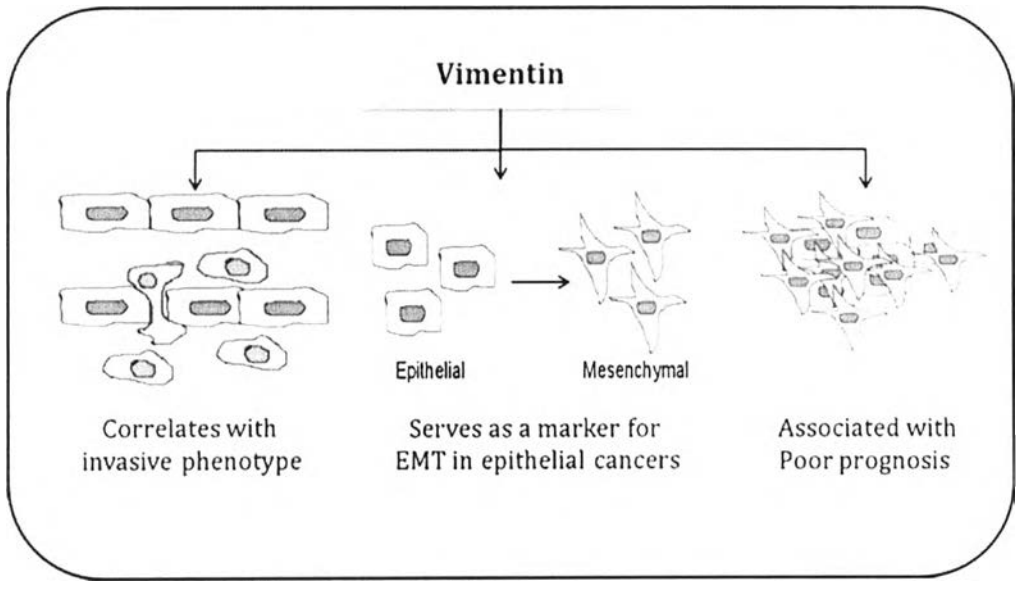

Figure 17 Vimentin's role in cancer. The expression of vimentin correlates with invasive phenotype of cancer cells. Vimentin is considered as a marker of EMT and indicates poor prognosis in cancer patients (70).

Snail and slug

Snail and slug, the members of Snail family of zinc-finger transcription factors, are EMT-activating transcription factors that modulate the process of EMT. Normally, EMT process is triggered by the orchestration of various EMT activating transcription factors including Snail proteins (snail and slug), ZEB proteins (ZEB1 and ZEB2) and basic helix-loop-helix (bHLH:E47 and twist). However, the underlying mechanisms of

different repressors to repress E-cadherin gene expression are still not fully understood. Snail and slug share the SNAG domain at N terminal region and zing finger cluster at C terminal region. These factors use C terminal region to bind to the E2-box DNA sequence in the promoter region of E-cadherin gene resulting in the recruitment of co-repressors and histone modification (deacetylation, methylation and demethylation) and the repression of E-cadherin expression. Snail seems to recognize E2-box with higher affinity than slug. The different effects of snail and slug on behavior of cancer cell have been found in various cancers such as breast cancer, colorectal cancer, ovarian cancer and lung cancer. In addition, the participation of repressors to silent E-cadherin expression is specific in different types of tumor and defined stages of tumor progression (71-73).

There are other target genes that beyond E-cadherin gene and involved in EMT process. Each repressors has the different subset of EMT-related target genes. Snail proteins also suppressed the expression of other epithelial molecules including cytokeratins, plakophilin, claudins, occludins, mucin1 and ZO proteins (71, 74). Snail directly binds cytokeratin and occludin gene as well as its own promoter while slug directly binds occludin promoter (71). Moreover, snail induced the expression of proteins involved in the mesenchymal and invasive phenotype of cell such as vimentin (74) and MMP (71). Snail and slug have been found to bind promoters of gene associated with cell survival and apoptosis such as p53, Bid, DFF40, PUMA and BRCA2. Furthermore snail and slug have been found to alter cell proliferation and cell survival. Cells undergoing EMT with up-regulation of snail possessed low cell proliferative ability by inducing cell cycle arrest. Snail and slug were reported to show the pro-survival action in human carcinoma cells under genotoxic stress (71).

The inducing signals of EMT

The signals that regulate EMT in cells include extracellular and intracellular stimuli. Extracellular signals can activate intracellular effector molecules, such as members of the small GTPase family and members of the Src tyrosine-kinase family, and then trigger EMT in cells. The types of extracellular stimuli are classified as biomolecule and xenobiotic compounds. The tumor microenvironment including the components of ECM and soluble growth factors, such as members of the TGF-β and FGF families, epidermal growth factor (EGF) and SF/HGF, trigger cells via receptor binding and activate transcription factors involved in EMT such as snail an slug which regulate the change of gene expression that lead to EMT (12, 63, 64). There are studies found that xenobiotic compounds can induce EMT in cancer cells such as celecoxib (75), indomethacin (76), gefitinib (77, 78), endosulfan (79) and hexavalent chromium (80) which induce EMT in human lung cancer cells A549. Heavy ions were also reported that they may trigger TGF-β1–induced EMT even at a low dose (81). Intrinsic stimuli of EMT are the mutation in signal transduction molecules. Although some factors like TGF-β can possibly induce EMT in cells, cells need to accumulate the particular gene mutations for unlock or maintain EMT (12, 13).

EMT and anoikis resistance

This mechanism has been suggested as pathophysiological mechanism to protect cancer cells from anoikis (20). There are several studies found that EMT promotes anoikis resistance in cancer cells. In 2002, Tran et al. studied the function of N-cadherin to anoikis resistance. They found that N-cadherin induced anoikis resistance via PI3K/Akt pathway and increased the expression of Bcl-2 which is the anti-apoptotic protein (82). Derksen et al. in 2006 investigated the role of E-cadherin

in breast oncogenesis by using mouse mammary model with E-cadherin mutation. They found that cells without E-cadherin can survive and proliferate in an anchorage-independent condition (83). In 2008, Onder et al. investigated the effect of E-cadherin loss in *ras*-transformed immortalized human mammary epithelial cells. Complete loss of E-cadherin induced cell to undergo EMT process. Their morphologies were changed to be fibroblast-like and the expression of EMT markers including N-cadherin, vimentin and twist were increased. These cells also resisted to anoikis in anchorage independent condition (9). Melanoma cell underwent EMT during cell development. N-cadherin expression induced anoikis resistance through Akt/PKB pathway activation and these cells underwent apoptosis when N-cadherin functions were blocked (84).

EMT and tumorigenicity of cells

Cells with EMT phenotype are able to grow as spheroids in suspension culture, called as tumorigenicity which is a hallmark of stem-like cells (85). EMT has been reported as a sufficient mechanism to generate a cancer cell population with stem cell characteristics which are the ability to self-renew, form tumor spheres, differentiate into heterogeneous populations of cancer cells, and seed new tumors in a xenotransplant system (15, 85, 86). Mani et al in 2008 found that the induction of EMT in nontumorigenic, immortalized mammary epithelial cells promotes increased population of stem-like cells. In addition, the levels of mRNAs encoding EMT markers such as N-cadherin and snail were increased in stem-like cells. This study provided evidence of a potential association between EMT and a cancer stem-like cell phenotype (15). The several EMT-inducing transcription factors also play a role to regulate stem-like properties such as snail and slug. Snail and slug were reported as

critical factors for ovarian cancer cells to acquire stem cell properties (87). In addition, Mani et al in 2008 found that snail was able to generate cells undergoing EMT with properties of stem cells (15).

EMT and cell migration

EMT is the transition process from a collective to a single-cell migration therefore it is considered as an important step in the invasive cascade of tumor cells (37). In EMT, N-cadherin plays an important role instead of E-cadherin and enhances the motility of cells because the interaction of N-cadherin is not as strong as E-cadherin interaction. N-cadherin also links Na^+/H^+ exchanger regulatory factor with platelet derived growth factor receptor via interactions with β-catenin in lamellipodia that enhance cell migration. N-cadherin also increases steady-state levels of activated Rac1 and Cdc42 which are the GTPases that regulate cell migration resulting in promoted cell migration (8). In addition, N-cadherin also promotes cell migration and invasion by modulating growth factor signaling and remodeling the actin cytoskeleton. N-cadherin forms the interaction with FGFR to stabilize FGFR at membrane. This event increases cell motility and MMP secretion (88, 89). In addition, snail and slug, transcription repressors of E-cadherin gene, also induce the expression MMP, a proteolytic enzyme to destruct the meshwork of basement membrane and is essential for cancer cell invasion. Loss of E-cadherin also leads to the accumulation of β-catenin in the cytoplasm. Then this protein enters into nucleus and interacts with transcription factors to stimulate the expression of a large number of genes involved in cell migration and invasion. In addition, β-catenin also regulates the signaling which is essential for filopodia formation and cancer cell invasion (39).

Circulating tumor cells (CTCs)

In cancer metastasis, cancer cells have to survive after detachment from primary tumor and spread through systemic circulation to form secondary tumor at distal sites. Once these cells are detached from primary site and circulate through bloodstream, these cells are called CTCs (Figure 18) (21, 26, 90). In clinical practice, CTCs have been considered to be a potential biomarker that reflects cancer aggressiveness of many types of cancers such as breast, prostate, colorectal, bladder, gastric, liver and lung cancers (22-26). The presence and quantity of CTCs in peripheral blood correlate with poor prognosis in cancer patients. CTCs are found in circulation as both single cells and multicellular aggregation (or circulating tumor microemboli). CTCs may involve coagulation in blood vessel via the expression and release of tissue factors. The characteristics of CTCs that found in cancer patients are highly heterogeneous resulting in a limit of detection (21, 22). The population of CTCs exhibit heterogeneous cell phenotypes including epithelial and mesenchymal phenotypes as well as a transitional state from epithelial to mesenchymal cells (22, 26, 91-93). However, once CTCs undergo EMT, their migration and invasion, angiogenesis and survival are potentiated resulting in more metastatic cancer cells. Moreover, EMT has been reported to induce cancer cells to exhibit stem-like properties (22, 25, 93). In addition, EMT increases ability of cells to release tissue factors resulting in promoted coagulation in blood vessel (21). CTCs which are highly aggressive additionally exhibit the ability to self-seeding to their original organs (94). Therefore, EMT is considered as critical mechanism to promote metastatic potential, anoikis resistance and invasive ability of CTCs (16, 22).

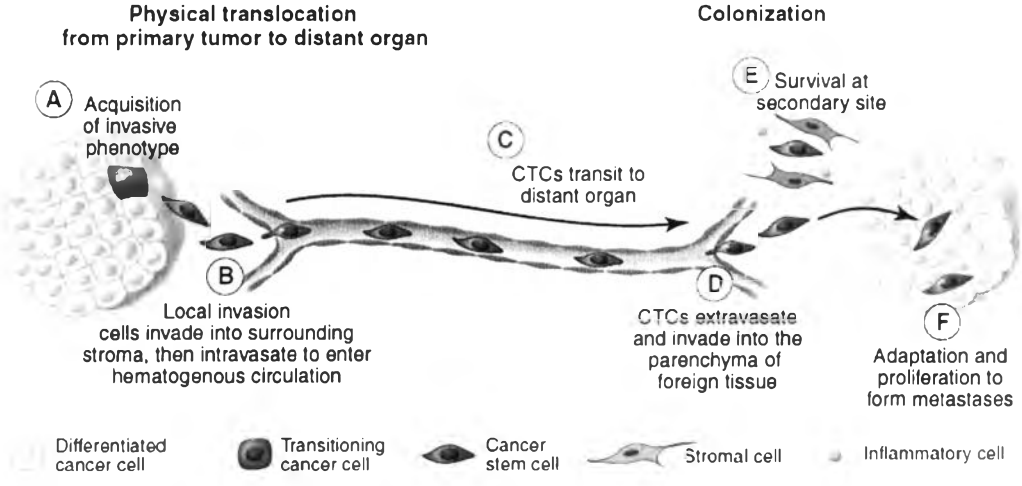

Figure 18 The role of CTCs in metastatic cascade. Anoikis resistant cancer cells circulating through the systemic circulation are celled CTCs. At the distant site, CTCs extravasate and invade into the microenvironment of the foreign tissue. Then cancer cells must be able to overcome the innate immune response and also survive as a single cell (or as a small cluster of cells). Finally, the cancer cell must be able to adapt and proliferate in the new microenvironment and form metastases (90).

Triclosan (TCS)

TCS is a synthetic board spectrum antibacterial ingredient and lipid soluble compound. Its chemical name is 2,4,4'-trichloro-2'-hydroxydiphenyl ether (Figure 19) and its molecular weight is 289.54. Its antimicrobial mechanism is bacterial cell membranes intercalating without causing leakage of intracellular components resulting in cell death. TCS also inhibits enoyl reductase activity lead to inhibition of fatty acid synthesis. TCS has been widely used in personal care products since 1968 such as toothpastes, mouthwashes, antibacterial soaps (bars and liquids),

deodorants, deodorant soaps (bars and liquids), cosmetic and antiseptic products, and even in the textiles. TCS also has been used in medical devices and hospital to control the spread of methicillin-resistant *Staphylococcus aureus*. TCS is regulated by both the US Food and Drug Administration (FDA) and US Environmental Protection Agency (EPA). It was approved by European Community Cosmetic Directive in 1980 and the US FDA in 1990 to be used in health care products at level up to 0.3% (2, 3).

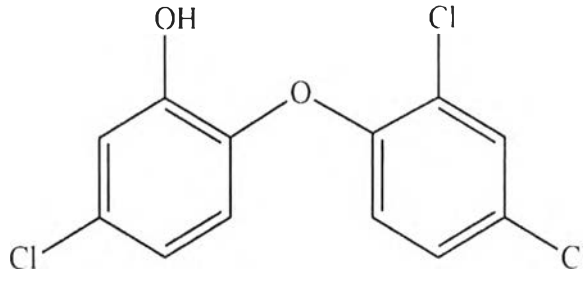

Figure 19 The chemical structure of triclosan (TCS) (3)

TCS is readily and completely absorbed following oral administration and less than 10% following dermal application. Maximum plasma concentration is reached at 4-6 h after oral administration. From the studies in rodent, distribution of TCS was a two-compartment open-system model. It rapidly distributed from blood to tissues after oral administration. Plasma protein binding of TCS is greater than 95% in human and mice with noncovalent binding. In human, most of TCS is metabolized by glucuronide conjugation and minor pathways of TCS metabolism are sulfate conjugation and CYP450s (Figure 20) (3).

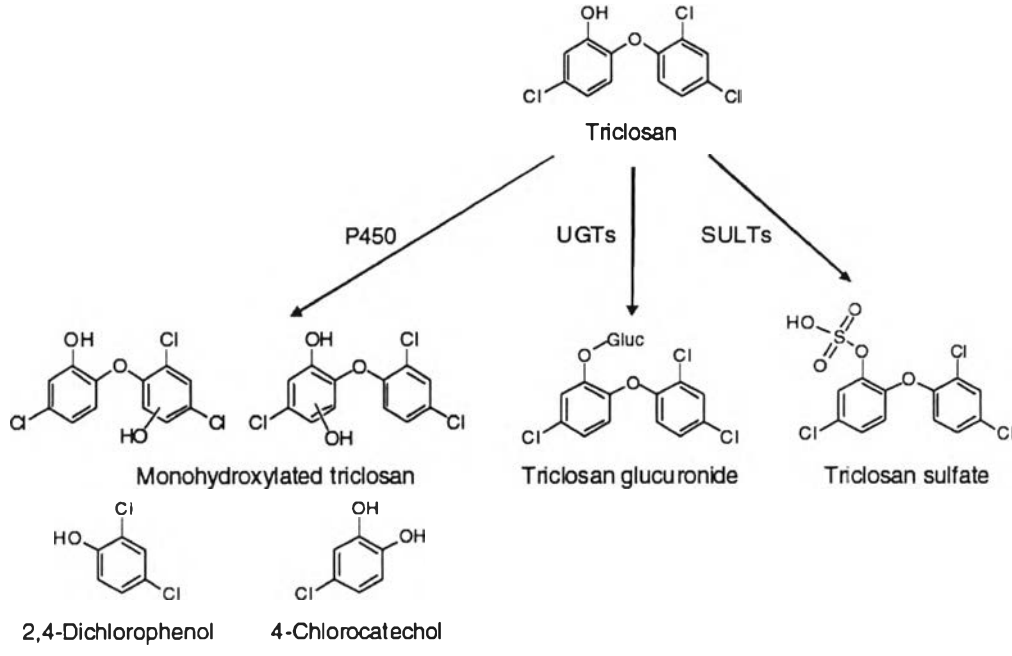

Figure 20 The metabolic pathways of triclosan (TCS) in human (3)

TCS is mainly excreted in urine and its half-life is about 10-20 h. TCS has been identified in urine, plasma, and breast milk of humans. The level of TCS in the body is very wide range because the levels of TCS depend on the individual daily intake of TCS, the concentration of TCS in products and the route and frequency of administration (Table 1) (2-4). In 2006, Sandborgh-Englund et al. studied the pharmacokinetic of TCS after oral administration in humans. They found that there were three forms of TCS found in human plasma including unconjugated form, glucuronide conjugated form and sulfate conjugated form. The level of unconjugated TCS in human plasma increased parallel to total concentration of TCS and constituted about 30-35% of total level during the first 6 h after oral administration. After 6 h, the level of unconjugated TCS was below the limit of detection (5).

Table 1 The reported concentrations of TCS that relevant to concentrations found in animals, human and environment (4)

The concentrations of total TCS	Reported concentrations
0.002 μg/ml	High range of TCS concentrations observed in the plasma of bottlenose dolphins
0.02 μg/ml	Low concentration of TCS observed in human plasma
2 μg/ml	Low concentration detected in Charleston Harbor water samples
20 μg/ml	High concentration detected in Charleston Harbor water samples, low concentration observed in human breast milk and high concentration in human plasma of exposed individuals
200 μg/ml	Concentration in Charleston, SC area wastewater treatment plant effluent
2000 μg/ml	Concentration in Charleston, SC area wastewater treatment plant influent

Toxic effects of TCS have been studied including subacute, subchronic, and chronic administration via oral, dermal and inhalation route in several animal models. Adverse effects were seen in the liver of some species such as changes in liver weight or liver enzymes, increases in hypertrophy without alterations in histopathology that may be the results from adaptive responses. TCS was also found to decrease circulating thyroxine (T4) and triiodothyronine (T3) levels with dose-dependent manner in rodents after 4 days of treatment in female rats and after 31 days of treatment in male rats. However these effects are not found in other animal models both in subchronic and chronic studies. TCS was also conducted in

carcinogenicity studies in several animal models. It possessed liver toxicity and produced liver tumors only in mice models of both male and female after 18 months of treatment. The mechanism of tumor induction effects was PPARα agonist effects of TCS. TCS induced the expression of PPARα -specific target genes resulting in liver hypertrophy and the increase of proliferation rate of hepatic cells leading to increases in hepatic tumors in mice. However PPARα -induced effects of TCS were not relevant in human. There are several studies about the safety of TCS in human and the results showed that TCS can be used safely in human and no chronic effects were shown in workers in TCS production and consumer products containing TCS manufacture (2).

Although TCS does not possess serious toxic effects in several studies both in animal and human experiments, there are recent studies found the biological effects of TCS that do not relate to its antibacterial properties. TCS was a slow binding inhibitor of human type I fatty acid synthase and partial inhibitor of enoyl-reductase activity type I fatty acid synthase that promote cytotoxic effect on MCF-7 cells and SK-BR-3 human breast cancer cells (95). TCS exhibited endocrine disruption effects, intrinsic estrogenic and androgenic activity in MCF-7 cells. It displaced [^{3}H]oestradiol from estrogen receptors (ER) and [^{3}H]testosterone from binding to the ligand binding domain of the rat androgen receptor (AR). In addition, TCS also increased MCF-7 cell growth over 21 days for exposure (96). These studies raise the question about the safety of TCS and the US FDA recently initiated a review of TCS in consumer products (97). Ma et al. (2013) investigated the effect of TCS on global DNA methylation (GDM) which is the total level of 5-methyl cytosine in the genome in HepG2 cells. The results showed that treatment of TCS for 24 h reduce the levels of

GDM and down-regulated the methylated DNA-binding domain 2 (MBD2), MBD3, and MeCP2 gene expression by increasing 8-hydroxy-2-deoxyguanosine (8-OHdG) levels and inhibiting the mammalian DNA methyltransferases 1 (DNMT1) activity which is the enzyme that is directly responsible for methyl group transferring to cytosines in the genome (98). DNA hypomethylation is considered as global hallmark of cancer and might lead to activation of prometastatic genes in cancer (99, 100). DNA hypomethylation of specifying transcription factors in cancer cells possibly lead to EMT and increase the risk of distant metastasis (101). Thus, TCS induced DNA hypomethylation raise a question whether TCS can promote cancer metastasis in the carcinogenesis safety aspect of this compound.