CHAPTER 1
INTRODUCTION

1.1 Statement and significance of problem

Cancer is a group of diseases characterized by uncontrolled cell division leading to growth of abnormal tissue. Worldwide, cancer is a leading cause of death in humans. In 2008, statistic estimation of the worldwide incidence and mortality about the top 22 cancer sites estimated by the International Agency for Research on Cancer (IARC) reported that the most commonly diagnosed cancers in males were lung, prostate, colorectal, and stomach, respectively. Furthermore, the most common causes of cancer death in males were lung, liver, stomach, and colorectal, respectively. On the other hand, the most commonly diagnosed cancers in females were breast, colorectal, cervix, and lung, respectively. Additionally, the most common causes of cancer death in females were breast, lung, colorectal, and cervix, respectively (1, 2).

Lung cancer is a type of cancer that involved in uncontrolled cell growth of lung tissues. The main types of lung cancer are small-cell lung cancer (SCLC), also called oat cell cancer, and non-small-cell lung cancer (NSCLC). Worldwide estimation in year 2008, lung cancer was the most commonly diagnosed cancer and causes of cancer death in males. However, it was the fourth most commonly diagnosed cancer and the second leading cause of cancer death in females (2). In Thailand, the incidence of lung cancer was the most common cancer in males and the fourth in females (after breast, cervix, and colorectal) (3, 4). Additionally, lung cancer was the most commonly diagnosed cancer in northern area of Thailand with 14.1% newly cases in year 2005 (5). In Maharaj Nakorn Chiang Mai Hospital, the incidence of lung cancer was the most common in both males and females with 19.8 and 23.7% of newly cases, respectively (5, 6). There are several well-known risk

factors in lung cancer development, including smoking, asbestos exposure, and exposure to radon. However, the most common cause of lung cancer is long-term exposure to tobacco smoke (2). The proportion of lung cancer cases due to tobacco smoking can be estimated by comparing observed incidence in different areas with that expected based on rates in non-smokers from several large cohort studies (7). About 80% of lung cancer in men and 50% of lung cancer in women were the consequence of tobacco smoking (8). The carcinogenic mechanisms of tobacco smoking are not well understood, but there are general principles that have emerged from intensive research provide the generally informed cancer scientist with a distillation of mechanistic information on the subject of tobacco smoke carcinogens and lung cancer as shown in Figure 1 (9).

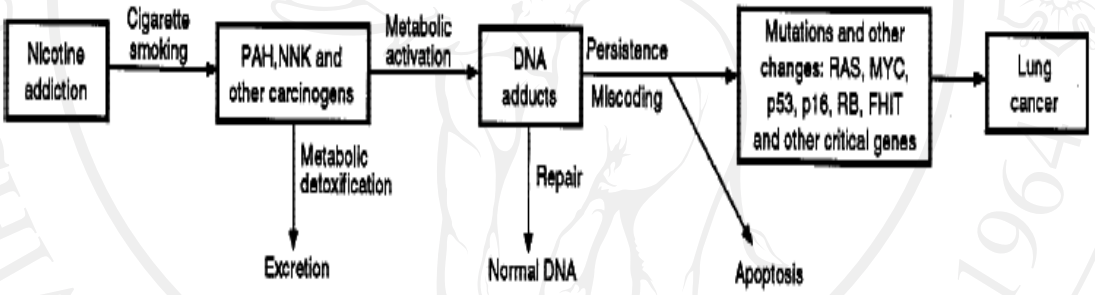


Figure 1 Scheme linking nicotine addiction and lung cancer via tobacco smoke carcinogens (9)

More than 60 carcinogens in tobacco smoke have been evaluated by the International Agency for Research on Cancer (IARC) (9-13). Among the multiple components of tobacco smoke, polycyclic aromatic hydrocarbons (PAHs) and the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) are the most carcinogenic molecules in tobacco smoke convincingly cause lung tumors in laboratory animals and humans (7, 9). The carcinogens such as NNK and PAHs require a group of enzymes to exert their carcinogenic effects by metabolic activation cascades. At the same time, these carcinogens and their metabolites are competitively detoxified by the same group of enzymes to excrete them out of the body. The balancing between the metabolic activation and detoxification differs among individuals and will affect cancer risk (11). After the metabolic activation

process, metabolites of carcinogen covalently bound to DNA, usually at guanine or adenine, and then leads to the formation of DNA adducts. If DNA adducts escape cellular repair mechanisms and persist, they may lead to miscoding and resulting in a permanent mutation. Cells contained the damaged DNA may be removed by apoptosis, or programmed cell death. If a permanent mutation occurs in a critical region of an oncogene or tumor suppressor gene, it can lead to activation of the oncogene or deactivation of the tumor suppressor gene. Multiple events of this mutation lead to aberrant cells with loss of normal growth control and ultimately lead to lung cancer (9, 14). NNK initially presents itself in the body as a pro-carcinogen and requires cellular metabolism and activation to induce lung tumorigenesis (12, 14, 15). Cytochrome P450 (CYP), a family of enzymes metabolizing a wide variety of xenobiotics, is responsible for the activation of NNK and plays a major role in NNK-induced lung tumorigenesis (16-19). α-Hydroxylation metabolites of NNK and NNAL (reductive metabolite of NNK) by P450s can react with DNA to form DNA adducts (9). Furthermore, NNK initiates a cascade of signaling pathways (ERK1/2, PKCα, PI3K/Akt, MAPK), resulting in uncontrolled cellular proliferation and tumorigenesis (14). Recently, scientific evidence has showed that NNK exposure induced hypermethylation on promoter of many tumor suppressor genes via the promotion of nuclear DNMT1 accumulation (20).

Colorectal cancer (colon cancer, large bowel cancer, or CRC) is a disease which consists of uncontrolled cell growth in the colon, rectum and appendix. Worldwide, the CRC was the third common type of cancer in men and the second in women. Furthermore, it was the fourth leading cause of cancer-related death in males and the third in females (2). The incidence rate of CRC in Thailand was the second in males after lung cancer, and the third in females after breast, and cervical cancer (5). In Chiang Mai, the CRC was the fourth most common type of cancer in both men and women (21). Although, the incidence rate of CRC in Thailand is low when compared with other types of cancer, but the number of cases in both sexes is rapidly increasing over the last 20 years due to the acquisition of Western lifestyle (21-24). Besides nutrition, genetic alterations and chronic inflammation are also involved in colorectal tumorigenesis. Sequential genetic alterations, such as the mutation of *APC* gene, mediate development of colon cancer (25-28). Mutations inactivating of the *APC*

(adenomatous polyposis coli) gene are found in approximately 80% of all human colon tumors and heterozygosity for such mutations produces an autosomal dominant colon cancer predisposition in humans and in murine models (25-27, 29). The *APC* gene produces a large multidomain APC protein, which downregulates the Wnt signaling pathway through the induction of β-catenin degradation. The loss of function of *APC* gene leads to accumulation of β-catenin in nucleus and resembles constitutively activation of Wnt signaling. Then, the accumulation of nuclear β-catenin activates transcription of genes such as *MYC*, *cyclin D* and other genes whose products promote cell proliferation and survival rather than differentiation (29, 30). The chronic inflammatory bowel diseases (IBD) are aetiological factors in the development of colorectal adenocarcinomas (31-33). Patients with longstanding extensive colitis, whether Crohn's disease (CD) or ulcerative colitis (UC), have a five to six times higher risk of developing colorectal cancer than the general population (31-35). In UC patients, several inflammation-associated genes such as *cyclooxygenase-2* (*COX-2*), and *nitric oxide synthase-2* (*NOS-2* or *iNOS*) are increased in inflamed mucosa and remain elevated in colonic neoplasms. The inflamed cells are setting of heightened epithelial cell turnover, mutagenic assault and sustained DNA damage caused by factors within an inflammatory cell-rich microenvironment, which appear to drive the carcinogenic process (36-38). The genetic alteration of *APC* gene is also involved in the colitis associated CRC as shown in Figure 2 (31-33).

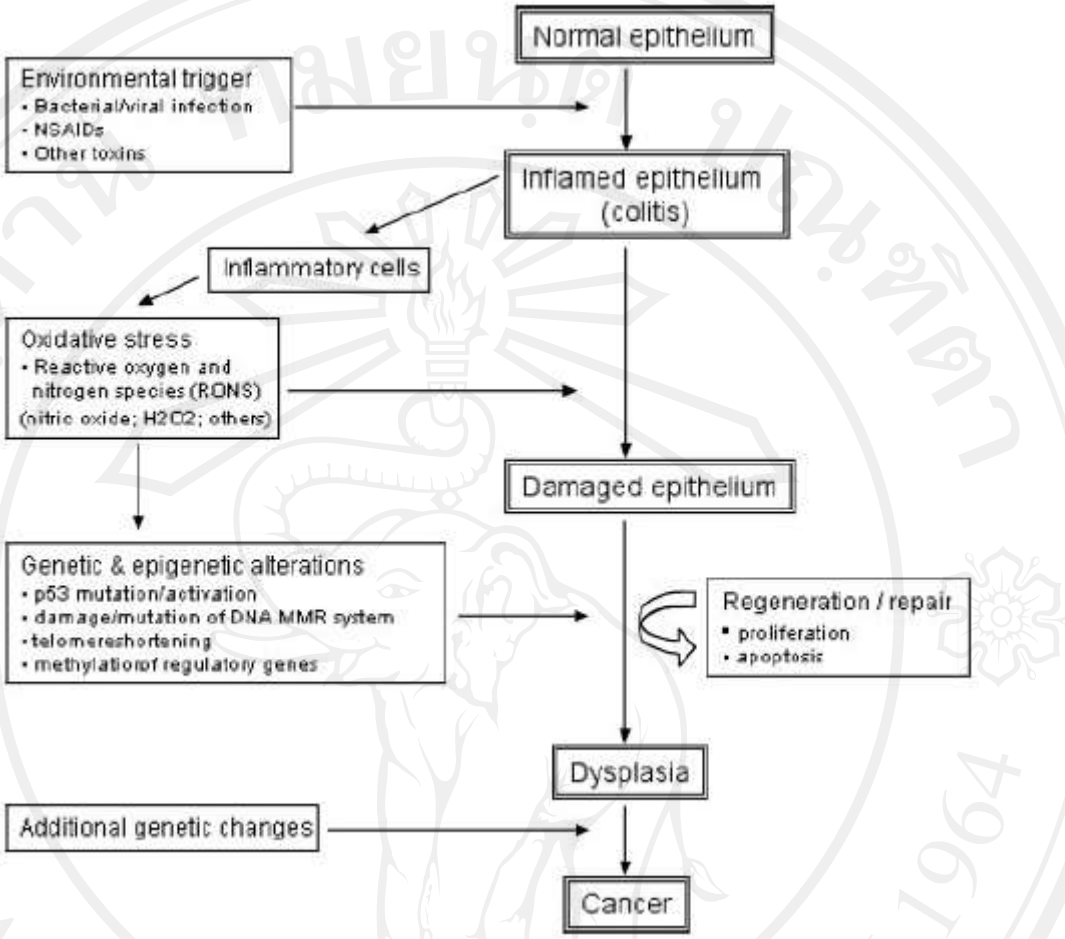


Figure 2 Proposed model of how inflammation associated with colitis promotes the development of colonic dysplasia and cancer (31)

One approach to restrain cancer incidence is a chemoprevention, which is the using of natural, synthetic, or biologic chemical agents to reverse, suppress, or prevent carcinogenic progression. Rice is one of the major cereal foods eaten as a staple food worldwide, especially in Asian countries. The candidate of rice products that have shown promising disease-preventing and health-related benefits in experimental research studies, including inositol hexaphosphate (IP_6 or phytate), ferulic acid, gamma-oryzanol, plant sterols, tocotrienols (39-41). Numerous *in vitro* and *in vivo* studies indicate that tocotrienols and phytic acid exhibit anticancer (42-46). Rice bran contains approximately 349 ppm of gamma-tocotrienol and Phytic acid represents about 1-2.5 mg/g in brown rice grains but their contents in rice are upon the genetic and environmental variation (47, 48). In addition, the Japanese fermented rice product named fermented brown rice and rice bran (FBRA), which is a processed

food prepared by fermented the brown rice and rice bran with *Aspergillus Oryzae*, had shown the chemopreventive effect against various types of cancer, including colon (49), stomach (50), bladder (51) and esophagus (52) in rodent models. In case of lung cancer and colorectal cancer, both can be effectively prevented by dietary agents. So, these two types of cancer are highly suitable for elucidating the underlying molecular mechanisms of cancer chemoprevention. The chemoprevention studies in NNK-induced lung tumorigenesis was focused on the modulation of the expression of genes and proteins involved in biotransformation (CYP enzymes; CYP2A13, CYP2A6), cell proliferation (PCNA), cell cycle regulation (Cyclin-D1), epigenetic mechanisms in DNA methylation (DNMT1), and apoptosis, while the chemoprevention studies in inflammation-related colorectal tumorigenesis was focused on the modulation of the expression of genes and proteins involved in inflammatory response (NF-κB, iNOS) (53, 54).

Because the chemoprevention is an important strategy for protection of cigarette smoke-induced lung cancer in human and a committee of the Institute of Medicine (IOM) recommended that animal models should be used to examine the pathogenic effects of tobacco smoke exposure and validate the biomarkers of exposure and biological effect. Therefore, the animal model of cigarette smoke-induced lung cancer could be critical for the evaluation of chemopreventive agents (55, 56). Limited research in this area has been performed in the A/J mouse model (57). The advantages of A/J mouse model is that it provides a relatively inexpensive way to induce lung tumors with cigarette smoke (A/J mice are highly susceptible to lung carcinogens) and lung tumors represented in NNK-treated A/J mice are similar in morphology, histology, and molecular characteristics to human adenocarcinoma, the most common type of human lung cancer (57-59).

To understand the correlation between inflammation and CRC, animal models of experimental colitis have been developed and are frequently used to evaluate new anti-inflammatory treatments for inflammatory bowel disease (IBD) (31). As the onset of inflammation is immediate and the procedure is relatively straightforward, chemically induced models of intestinal inflammation, such as the dextran sodium sulfate (DSS) model, belong to the most commonly used IBD animal models (60, 61). The mechanism by which DSS induces mucosal inflammation in the distal colon is

not fully understood, but most studies suggest that DSS exhibits a direct toxicity against colonic epithelial cells in the basal crypts and affects the integrity of the mucosal barrier. Mucosal surfaces are lined by epithelial cells, which establish a barrier between sometimes hostile external environments and the internal milieu. The defective barrier exposes lamina propria immune cells (macrophages and neutrophiles) to the continual presence of resident luminal bacteria, bacterial products (such as lipopolysaccharides: LPS), or dietary antigens, which perpetuates the inflammatory cascade. The chronic inflammation begins as an attempt of the body to remove injurious stimuli. However, the presence of inflammatory cascade for long time results in continuous tissue destruction and promotion and maintenance of carcinogenesis (62-67). The mouse provides an excellent *in vivo* system to show the model of human diseases and to test the therapeutic or chemopreventive agents. The $Apc^{Min/+}$ (multiple intestinal neoplasia: Min) has emerged as a powerful model of human intestinal tumour predisposition. This mouse carries a mutation at codon 850 and develops multiple small intestinal adenomas, in addition to a smaller number of colonic polyps (68-70). This lineage of mouse was established from an ethylnitrosourea-treated C57BL/6 male mouse. Its phenotype is an autosomal dominant trait and resembles of human familial adenomatous polyposis (FAP) syndrome, a type of CRC (68, 69). Colitis markedly accelerates the development of dysplasia and cancer in the $Apc^{Min/+}$ mice (71, 72). Additionally, DSS treatment led to colitis and contribute to the colonic neoplasms development in the $Apc^{Min/+}$ mice (73).

Taken together, the aims of this study are to investigate the chemopreventive effect for the rice product named fermented brown rice and rice bran (FBRA) against tobacco-derived nitrosamine (NNK)-induced lung tumorigenesis and inflammation-mediated colorectal tumorigenesis in *in vivo* model and to investigate the anti-tumorigenesis mechanism of the further rice products, gamma-tocotrienol (γ-T_3) and phytic acid (IP_6), on tobacco-derived nitrosamine (NNK)-treated lung cancer cell line A549 and lipopolysaccharides (LPS)-treated colorectal adenocarcinoma cell line SW480 in *in vitro* model.

1.2 Literature review

1.2.1 Carcinogenesis or tumorigenesis

Cancers are the most life-threatening health problems in the world (74). Carcinogenesis is a multi-step process by which normal cells are transformed into cancer cells. It is caused by mutations of the genetic material of normal cells, which impair the normal balance between proliferation and cell death. More than one mutation is necessary for carcinogenesis. The pathological observations in field carcinogenesis gave rise to the hypothesis of multistep carcinogenesis, which proposes that neoplastic changes evolve over a period of time due to the accumulation of somatic mutations in a single cell line, resulting in phenotypic progression from normal to hyperplastic to dysplastic, and finally, to fully malignant phenotypes (75). The carcinogenesis is classified into 3 different stages, including initiation, promotion, and progression. These stages of carcinogenesis are involved with a series of epigenetic and genetic alterations that affecting oncogenes and tumor suppressor genes. Initiation, the rapid and irreversible stage, involves direct DNA binding and damage by exogenous carcinogens. Promotion, which involves the epigenetic mechanisms, leads to premalignancy and is generally irreversible. Finally, progression stage, which involves the genetic mechanisms, is the period between premalignancy and the cancer and also generally irreversible (76-78). Inhibition of each stage of carcinogenesis by chemoprevention is a promising approach to restrain cancer incidence. The chemoprevention is the using of natural, synthetic, or biologic chemical agents to reverse, suppress, or prevent carcinogenic progression.

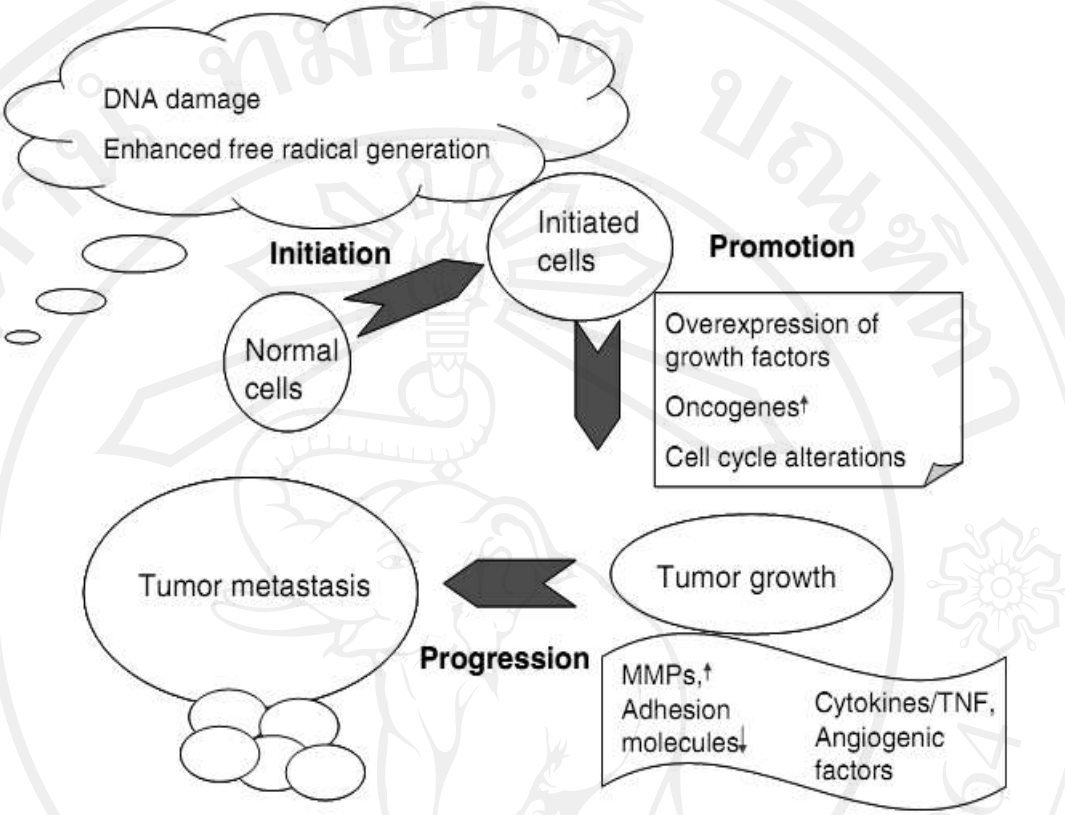


Figure 3 Scheme diagram of multi-step cancer progression process (79)

1.2.2 Nicotine-derived nitrosamine ketone (NNK)

The tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK or nicotine-derived nitrosamine ketone) is an aromatic compound with a molecular formula of $C_{10}H_{13}N_3O_2$ (MW = 207.2316). Although NNK have shown a significant relationship with lung tumorigenesis (80), NNK initially presents itself in the body as a procarcinogen (15, 81). Cytochrome P450 (CYP), a family of enzymes metabolizing a wide variety of xenobiotics, including drugs and carcinogens, plays central roles in the activation of many environmental chemicals to generate reactive intermediates (16). In *in vitro*, enzymes from the CYP2A subfamily efficiently catalyzed the metabolic activation of a number of nitrosamines, including NNK. However, the ability of each CYP2A enzyme to metabolize nitrosamines to their active forms was significantly different from others (17-19). Among of these CYP2A enzymes, CYP2A6 and CYP2A13, are plays a major role in NNK metabolism and activation in human (82-85). *CYP2A6* gene encodes a functional enzyme that is polymorphically expressed in the human liver accounting for about 1–

10% of total CYPs, and only trace amounts are found in extra-hepatic tissues (86). The CYP2A6 enzyme plays a crucial role in the metabolism of NNK (87) and a *CYP2A6* gene deletion-type polymorphism in Japanese male smokers had been shown to reduced lung cancer risk (82, 84). In mouse model, mouse Cyp2a5 enzyme is an enzyme in CYP family that closely related to the human CYP2A6 enzymes. Cyp2a5 enzyme catalyzed the α-hydroxylation of NNK and plays a key role in NNK activation in lung of A/J mouse (88, 89). The CYP2A13 enzyme is not expressed in the liver, but expressed predominantly in the human respiratory tract, including the nasal mucosa, trachea, and peripheral lung (90). CYP2A13 enzyme is believed to play an important role in the metabolic activation of NNK and *CYP2A13* gene polymorphisms with the missense variations decreased incidences of lung adenocarcinoma in smokers (83, 85). However, the study in *CYP2A6* and *CYP2A13* genes polymorphisms in Thai people has not been investigated. There are three primary pathways responsible for NNK activation: i) carbonyl reduction, ii) pyridine N-oxydation (both are reductive metabolism), and iii) α-hydroxylation (oxidative metabolism) (11, 14). NNK is metabolized to α-hydroxyNNKs by P450s and spontaneously decompose to diazonium ions and aldehydes. NNK can also undergo reduction to NNAL which is metabolized to α-hydroxyNNALs by P450s also results in diazonium ions (11, 12, 14).

1.2.2.1 The NNK-induced lung tumorigenesis

The diazonium ions, the metabolites of NNK, can easily bind to DNA (DNA adduct) and form methyl and pyridyloxobutyl adducts with genomic DNA, including O(6)-methylguanine, N7-methylguanine, and O(6)-[4-oxo-4-(3-pyridyl)butyl]guanine (11, 91, 92). If not repaired, these lesions could lead to mutations and the initiation of cancer. Several studies showed that the GC to AT mutation is observed in codon 12 of K-ras gene in the NNK-induced tumors and consistent with the formation of an O6-methylguanine (O6MG) adduct (92-95). It is well-known that ras activation plays a key role in the formation of spontaneous and chemically induced tumors. Therefore, these data support the involvement of NNK and other tobacco specific nitrosamines in mutagenesis and carcinogenesis. NNK exposure not only leads to gene mutation, but also induces hypermethylation of many tumor suppressor genes (20). NNK treatment induces nuclear DNMT1 accumulation by Akt pathway (20). Furthermore, NNK

activates a cascade of mitogen-activated protein kinase/extracellular signal regulated kinase 1 (MAPK/ERK1), phosphoinositide 3-kinase/Akt (PI3K/Akt), and nuclear factor-κB (NF-κB) pathway that resulting in uncontrolled cellular proliferation and tumorigenesis as shown in Figure 4 (14).

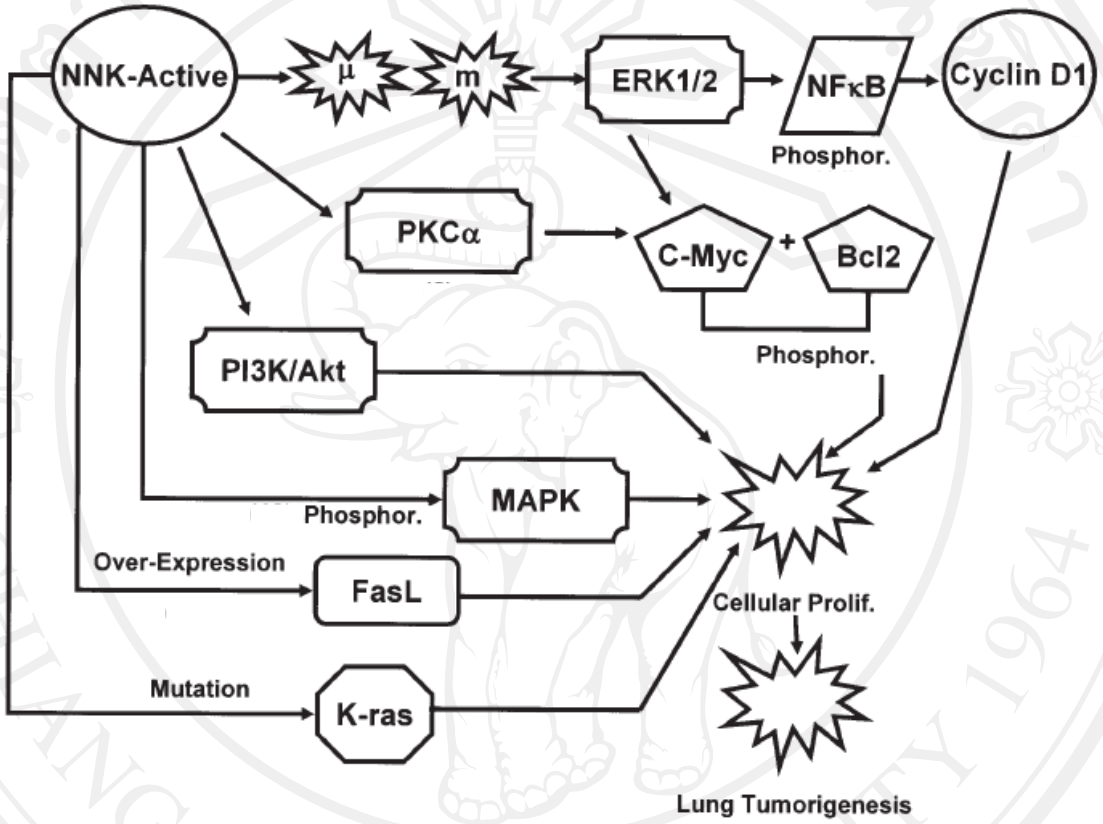


Figure 4 NNK-mediated activation of signaling pathways (14)

1.2.2.2 NNK-induced DNA methyltransferase 1 (DNMT1) nuclear accumulation

Epigenetic alterations are involved in several human diseases, including cancer. The cancer cells always undergo changes in 5′-methylcytosine distribution, which generated global DNA hypomethylation and region-specific hypermethylation of promoter CpG islands associated with tumor suppressor genes (TSGs) (96). Aberrant promoter hypermethylation of CpG islands associated with TSGs can lead to transcriptional silencing and result in tumorigenesis (97). DNMT1 is the most abundant DNA methyltransferase in mammalian cells, and responsible for both de novo and maintenance methylation of tumor suppressor genes. Alteration in expression level and activity of DNA methyltransferase 1 are involved in TSGs silencing and tumorigenesis. NNK induces activation of Akt pathway, promotes

GSK3β phosphorylation at Ser9 to form inactive GSK3β. The inactivated GSK3β subsequently attenuates the ability of βTrCP to degrade DNMT1 protein. In addition, the activation of Akt pathway induces interaction between phosphorylated hnRNP-U and βTrCP, which disrupts βTrCP/DNMT1 interaction and promotes the accumulation of DNMT1 in the nucleus as shown in Figure 5 (98).

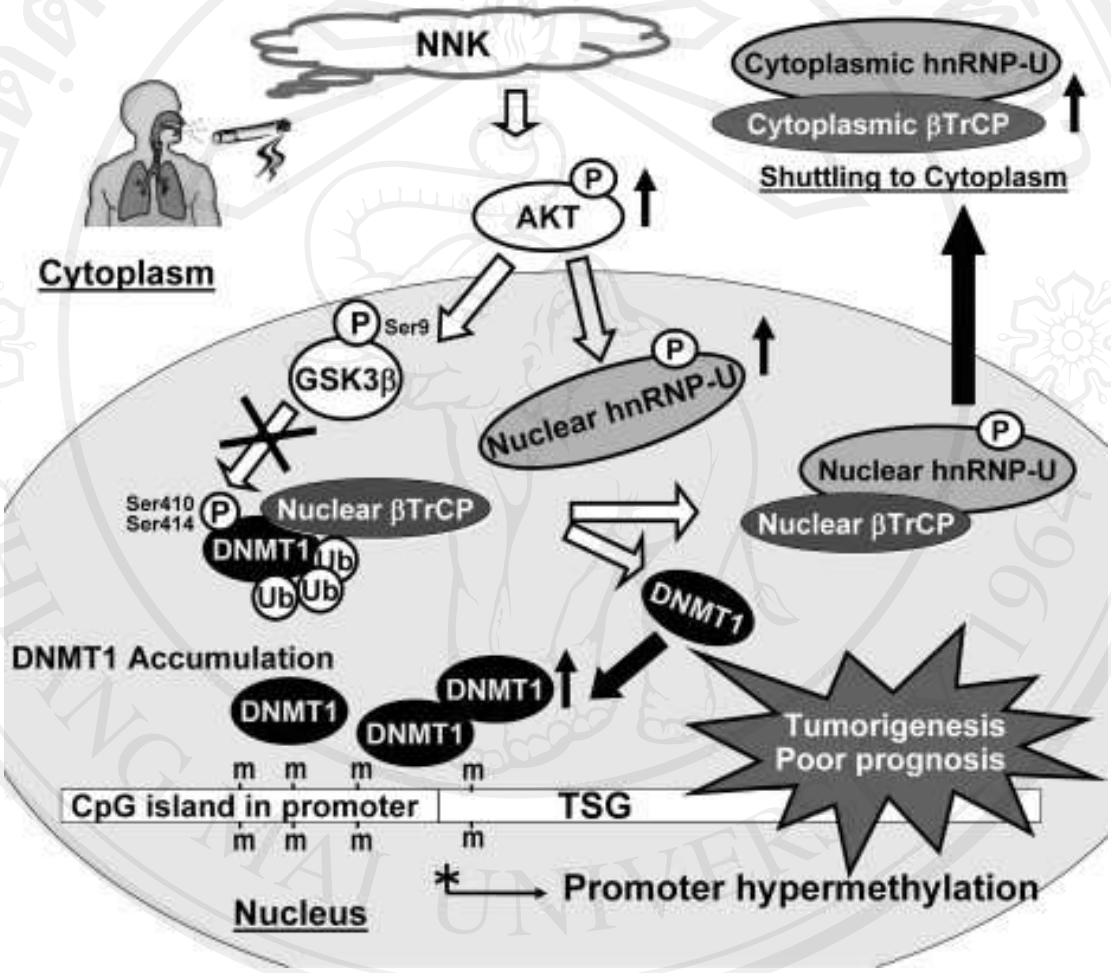


Figure 5 Possible mechenisms of NNK-induced DNMT1 nuclear accumulation (98)

1.2.2.3 Studies of NNK in animal models

Because the chemoprevention is an important strategy for protection of cigarette smoke-induced lung cancer in human and a committee of the Institute of Medicine (IOM) recommended that animal models should be used to examine the pathogenic effects of tobacco smoke exposure and validate the biomarkers of exposure and biological effect. Therefore, the animal model of cigarette smoke-induced lung cancer could be critical for the evaluation of chemopreventive agents

(55, 56). NNK has been reported that induced the pulmonary adenocarcinomas (PAC) in laboratory rodents, including rats, mice, hamsters, and ferrets, which therefore has been classified as a human lung carcinogen by the International Agency for Research on Cancer working group (80). Limited research in this area has been performed in the A/J mouse model (57). The advantages of A/J mouse model is that it provides a relatively inexpensive way to induce lung tumors with cigarette smoke because A/J mouse highly susceptible to lung carcinogens (99). Lung adenocarcinomas in mouse, including the A/J mouse strain, are morphologically similar to human lung adenocarcinomas. Furthermore, many of the signaling pathways with genetic and epigenetic alterations in oncogenes and tumor suppressor genes of carcinogens-induced lung adenocarcinomas in mouse are identical to those that are found in human lung cancer (100). Therefore, the A/J mouse is very useful mouse strain as a reliable in vivo model of lung carcinogenesis, which can be used for determining chemopreventive agents of lung carcinogenesis (99, 101).

1.2.3 The MAPK/ERK pathway and cancer cell growth

The mitogen-activated protein kinase/extracellular signal regulated kinase (MAPK/ERK) pathway transduces a large variety of external signals, leading to a wide range of cellular responses, including cell growth, proliferation, and apoptosis (102, 103). This signal starts when a growth factor binds to the receptor on the cell surface. The ligands binding to catalytic receptors induce phosphorylation of tyrosine residues within the receptors. The receptor's phosphotyrosines allow Ras protein, a class of small GTPase, releases its guanisine diphosphate (GDP) and binds guanisine triphosphate (GTP) (104, 105). Ras-GTP or active Ras activates the protein kinase activity of RAF kinase. Mutations in *Ras* genes result in Ras proteins that cannot hydrolyze GTP to GDP to inactivate the signaling process. Mutations of K-Ras protein occur frequently in many human cancers, including lung and colon (106). In transgenic mice, the mutation of K-Ras(G12D), but not N-Ras (G12D), activated the MAPK/ERK signaling and thereby promoted the proliferation of colonic epithelial cells (107). RAF kinase phosphorylates and activates MEK (MEK1 and MEK2). Then, MEK phosphorylates and activates extracellular-signal-regulated kinases (ERK1 and ERK2). Finally, the activated ERK1/2 stimulates the expression of cyclin D1, which responsible for cell cycle progression as shown in Figure 6 (108, 109).

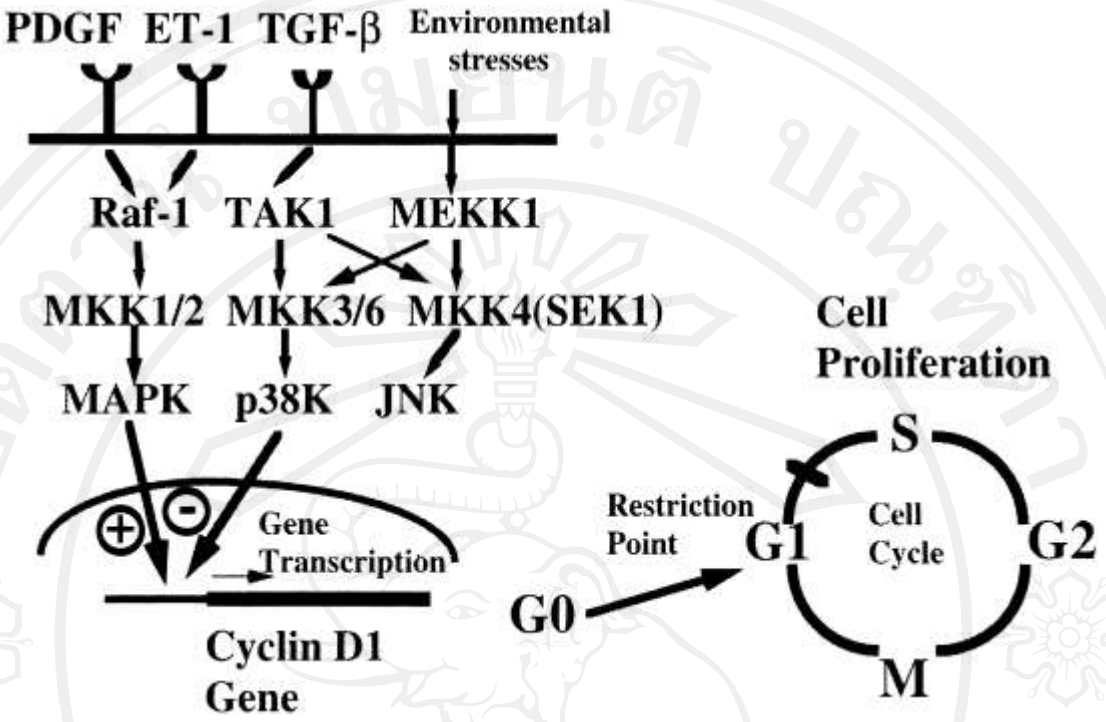


Figure 6 Possible interactions between MAPK pathways and the cell cycle (109)

1.2.4 The cell cycle

When an organism requires additional cells, either for growth or to replace those normally lost, cell division or proliferation is provided to produce the new ones. Somatic cells are generated by the division of existing cells in an orderly sequence of events, called the cell cycle. Cell cycle in most cells consists of four regulated processes, including cell growth (G1 phase), DNA replication (S phase), production of microtubules (G2 phase), and cell division or mitosis (M phase) as shown in Figure 7. G1 phase, also called the growth phase, located at the end of the previous M phase until the beginning of DNA synthesis. In this phase, proteins or enzymes that are required in S phase are synthesized. In S phase, the amount of DNA in the cell has effectively doubled. Then, microtubules which are required during the process of mitosis are produce in G2 phase. Finally, the chromosomes in nucleus are separate into two identical sets in two nuclei and generally followed immediately by cytokinesis, which divides the nuclei, cytoplasm, organelles and cell membrane into two cells containing roughly equal shares of these cellular components in M phase. Progression between all stages of the cell cycle is tightly regulated by a conserved regulatory apparatus in which coordinates the distinct phases of the cell cycle and also links the cell cycle with extracellular signals that control cell proliferation. Thus, the

integrity of the genome is completely maintained. Although, the cell cycle is usually a continuous process, there are the mature cells that no longer actively cycling or permanently in G1 phase. These specialized resting state of cells are called G0 phase. This phase is very common for the cells that are fully differentiated and occurs due to either specific anti-mitogenic signals or to the absence of proper mitogenic signaling. Some inactive or quiescent cells in G0 phase may re-entry the active phases of the cell cycle upon the proper stimulation (110, 111).

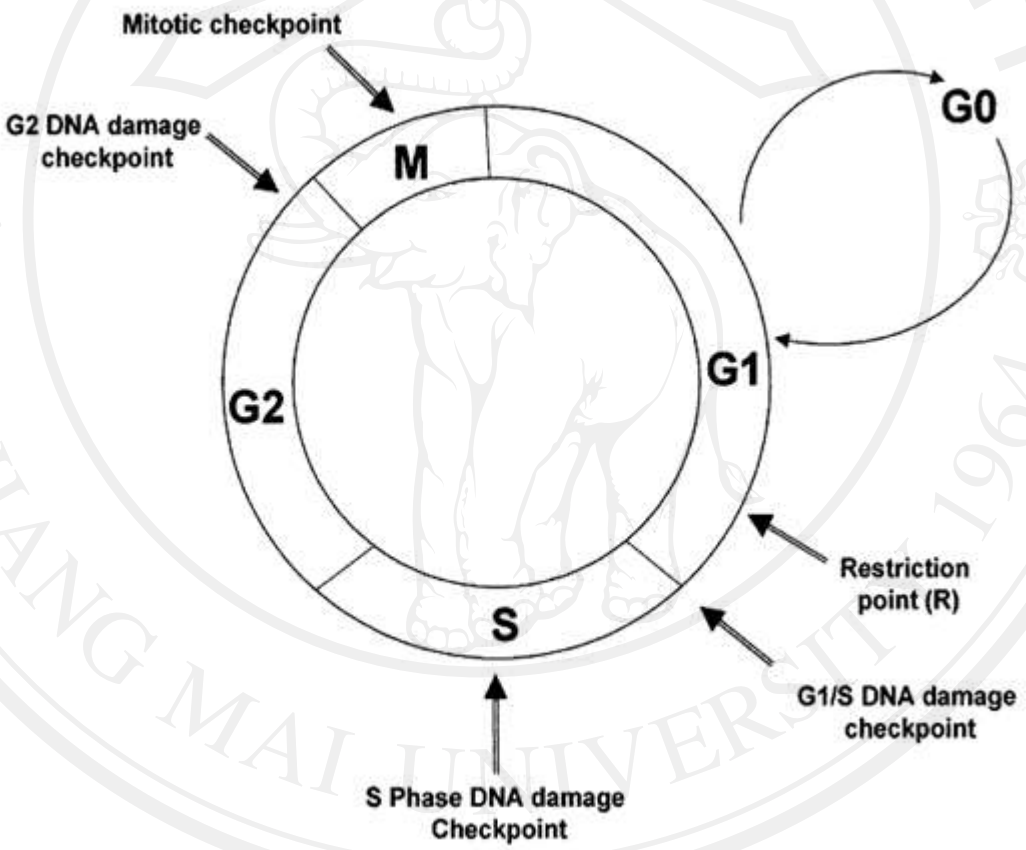


Figure 7 Checkpoints and stages of cell cycle (111)

1.2.4.1 The cell cycle checkpoints

The cell cycle proceeds by a defined sequence of events where late events depend upon completion of early events. Movement through each phase of the cell cycle is tightly regulated to ensure that critical events such as DNA replication and chromosome segregation are completed with high fidelity [110, 111]. Cell cycle checkpoints placed at critical points in the cell cycle to monitor the completion of these critical events and delay the progression to the next stage of the cell cycle if

necessary as shown in Figure 7 (111). The cell cycle checkpoints are the major mechanisms responsible for DNA damage, either by actively stopping the cell cycle or by induction of apoptosis. The first of these occurs at the G1/S phase transition, which is a major sensor of DNA damage. The cell may also arrest later in S phase due to incomplete DNA replication or again, damage to the DNA. The G2/M checkpoint is also an important sensor of DNA damage, which monitors the fidelity of DNA replication. This is followed by the spindle checkpoint, which is invoked during mitosis if a functional mitotic spindle has not been formed correctly (112, 113). Moreover, there are other 2 cell cycle checkpoints, restriction point and mitotic checkpoint. The restriction point (R) occurs between mid and late G1 and depends on external stimuli from growth factors. Progression through the restriction point requires continuous stimulation by mitogenic signals (e.g. growth factors) and a high rate of protein synthesis. Interruption of the mitogenic signals or moderate inhibition of protein synthesis leads to a rapid exit from the cell cycle to G0 in normal (untransformed) cells (114, 115). Signals from extracellular growth factors are transduced in a typical manner. This extracellular signaling must be maintained to support rapid protein synthesis, especially the accumulation of cyclinD protein. Cyclin D-bound cdk's 4 and 6 are activated by cdk-activating kinase and drive the cell towards the restriction point (116). Once beyond the R point, or point of no return, cells are committed to DNA synthesis and they no longer require the extracellular growth factors during the remainder of the cell cycle (117). Mitotic or spindle assembly checkpoint is a failsafe mechanism for the cell to ensure accurate chromosome segregation during mitosis. This checkpoint monitors the interaction between improperly connected kinetochores and spindle microtubules, and is maintained until kinetochores are properly attached to the spindle (118, 119). Misfuctions of this checkpoint can lead to chromosome missegregation, aneuploidy and even tumorigenesis (120-122).

1.2.4.2 The cell cycle regulators

Cell cycle regulators control cell cycle progression. The expression patterns of these proteins depend on the cell cycle phase (123). The core of the regulatory apparatus during the cell cycle progression is the cyclin-dependent kinase (Cdk). The cell cycle progression is driven by activation and inactivation of CDKs, which trigger

the transition to subsequent phases of the cycle. Although CDKs are present in constant amounts during the cell cycle, their kinase activities fluctuate depending upon available concentrations of cyclins. Certain cyclins form complexes with certain CDKs to stimulate the kinase activity of the CDK. The active cyclin-CDK complex catalyzes the phospholation of the substrate proteins on serine and threonine amino acid residues (124). For example, active CDK2 is responsible for S phase transition (G1 to S phase), while CDK1 activated the proteins involved in the initiation of mitosis. While the expression of the Cdks is usually constant throughout the cell cycle, the expression of each cyclin is rise and fall throughout the cell cycle due to its synthesis and degradation. Different cyclins are expressed to regulated specific phases of the cell cycle. For example, cyclin D is G1 regulator critical for cell progression through the restriction point. Furthermore, cyclin E and cyclin A are responsible for initiation of DNA synthesis in early S phase, and cyclin B regulates transition from G2 to M phase. In addition, the kinase activities of the active cyclin-CDK complexes can inhibit by cyclin-dependent kinase inhibitors (CKIs) as shown in Figure 8 (125).

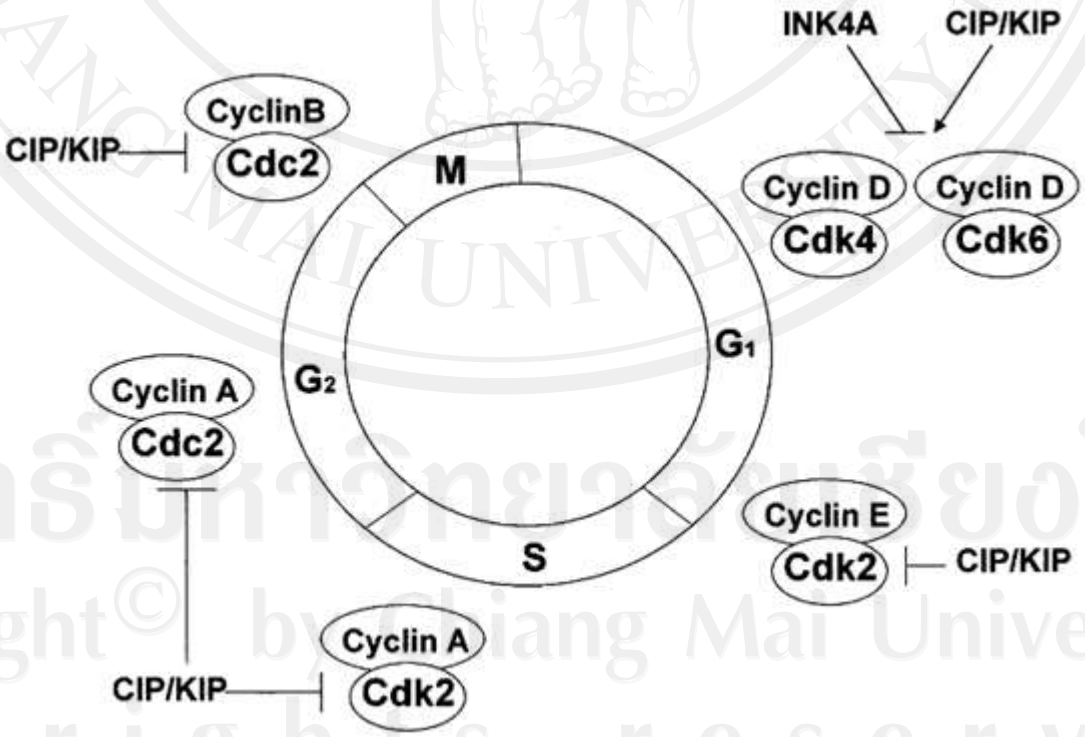


Figure 8 The cell cycle regulators (125)

The role of the Cdks is to control cell cycle progression through phosphorylation of proteins that function at a specific cell cycle stage. For example, retinoblastoma (Rb) protein, a key regulator of G1 progression, which possesses 16 potential sites of Cdk phosphorylation (126). In early stage of G1, Rb is found in a hypo-phosphorylated state and tightly binds to repress the activity of the transcription factors in E2F family, which are required for the expression of necessary proteins for S phase (127). During G1 phase, Rb is phosphorylated at the Cdk consensus sites to disrupt its interaction with the E2F proteins which allowing E2F dependent transcription to occur. This is necessary in order for the cell to pass through the restriction point in late G1. The phosphorylation of Rb at the Cdk consensus sites appears to be a sequential process initiated by association of Cdk4 and Cdk6 with one of three closely related cyclin subunits, D1, D2 and D3 as shown in Figure 9 (128).

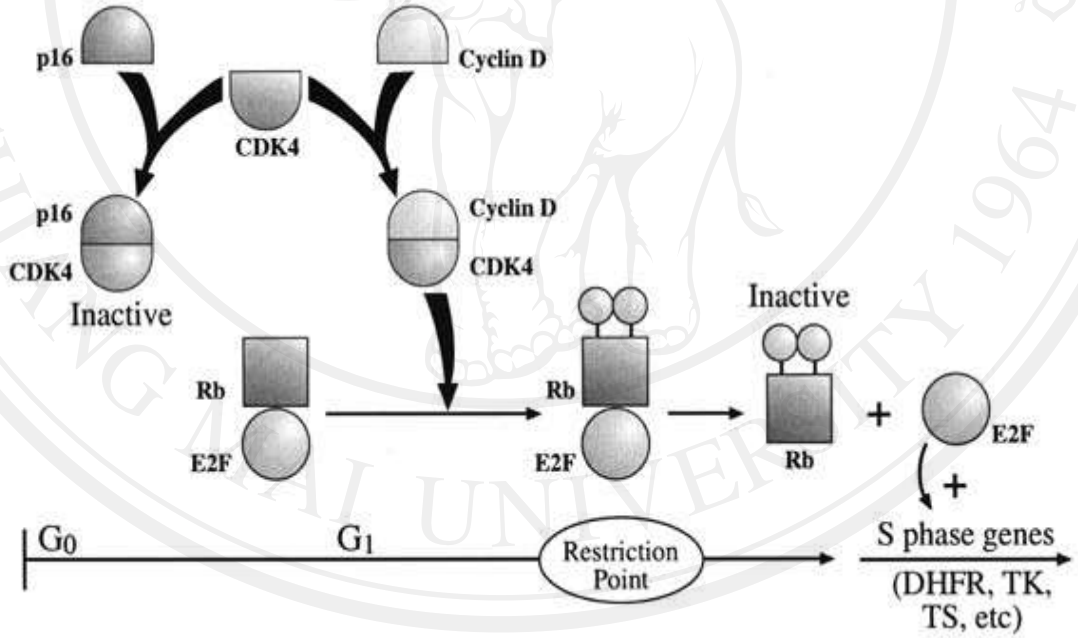


Figure 9 The kinase activity of CDK4 in the RB pathway (128)

Additionally, phosphorylation of RB by cyclin D–cyclin-dependent kinase 4 (CDK4) disrupt the complex of Rb with histone deacetylases (HDACs) and SWI/SNF, which represses transcription of cyclins E and A. After that, the complex releases HDACs, but RB remains associated with SWI/SNF, and allows the expression of cyclin E (129, 130). Expression of cyclin E concedes the formation of active Cdk2/cyclin E complexes which then continue the phosphorylation of Rb. This

leads to further disruption of the RB–E2F interaction results in transition from G1 into S phase (131). While Rb protein seems to be the primary substrate of cyclin D-Cdk4 and Cdk6 complexes, the Cdk2/cyclinE complex is known to phosphorylate several distinct types of proteins (132, 133). Accumulation of cyclin E during S phase leads to dissociation of SWI/SNF and Rb due to the hyper-phosphorylation of Rb protein. This dissociation allows the expression of cyclin A and entry into G2 phase as shown in Figure 10 (129). Transition from G2 into mitosis (M phase) requires the activity of the Cdk1 complexed with cyclin B, that has been shown to phosphorylate proteins regulated during mitosis (134-136).

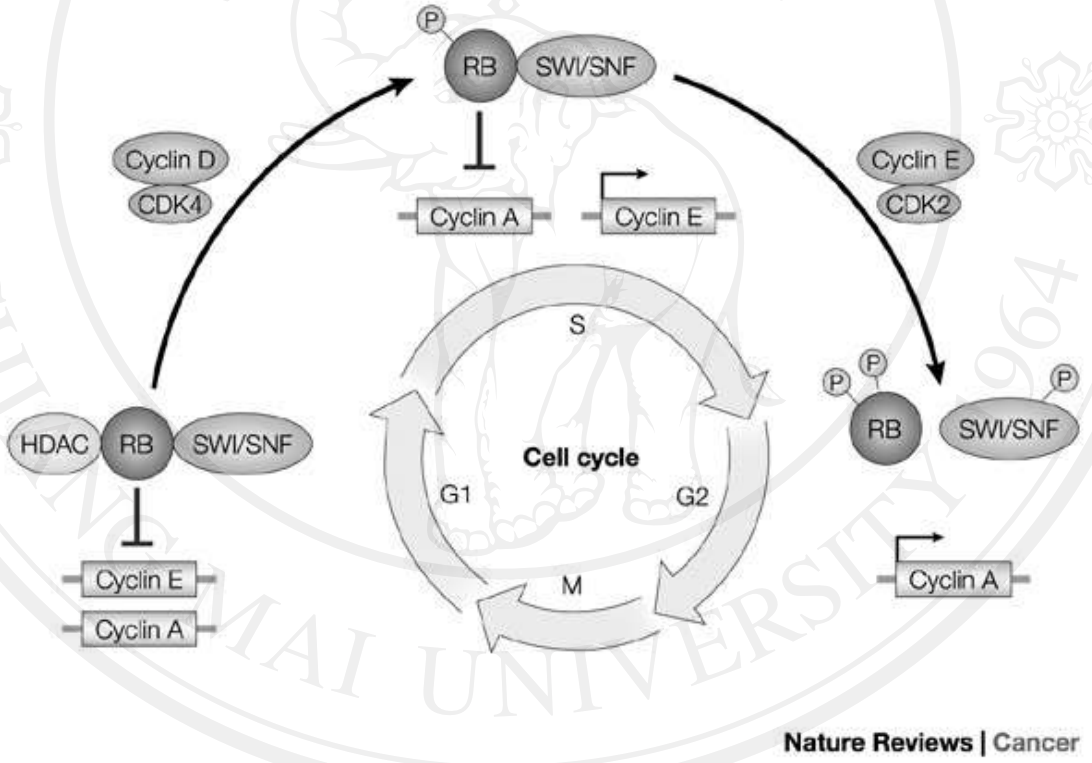


Figure 10 Retinoblastoma (RB) is bound to both SWI/SNF and histone deacetylase (HDAC) in G1 phase (129)

Proteolytic degradation of the cyclins occurs through ubiquitin-mediated proteolysis, a process whereby each protein is targeted to the 26S proteosome for degradation by the attachment of multiple ubiquitin molecules at one or more lysine residues, which is known as polyubiquitination (137) and involves a cascade of three enzymes, E1, E2 and E3 (138). Ubiquitin initially becomes attached to the E1 enzyme via an ATPdependent reaction. It is then transferred to one of 12 or so E2

enzymes and is finally added to one or more lysine residues on the target protein via the E3 ligase enzyme. There are two known types of E3 ligase, the SCF complex and the anaphase promoting complex (APC). Cyclins D1 and cyclin E, polyubiquitination is carried out through the SCF ubiquitin ligase system and is dependent on phosphorylation of a specific threonine residue on each protein (137). In contrast, cyclins A and B are polyubiquitinated by the APC (137). This is mediated by destruction box, a sequence motif present at the N-terminus of these and other cyclins, which acts as a signal for the timed degradation of these proteins (137).

Phosphorylation of the Cdk subunit can have both positive and negatives effects on its activity. Phosphorylation at a specific threonine residue towards the centre of the protein (T161 in Cdc2) is required for the Cdk/cyclin complex to have full activity (139). However, phosphorylation of a pair of adjacent threonine 14 (T14) and tyrosine 15 (Y15) residues at the N-terminus of the Cdk of Cdc2, inhibits Cdk activity even when it is phosphorylated at T161 (140). Phosphorylation on T14 of Cdc2 is performed by the Myt1 kinase, while Y15 is predominantly phosphorylated by the Wee1 kinase (141, 142). This allows the complex to be expressed during G2, but still inactive until entry into mitosis (143). Interestingly, Cdk2 has both the threonine and a tyrosine equivalent to T14 and Y15 of Cdk2, while the cyclin D-dependent kinases Cdk4 and Cdk6 only possess the tyrosine residue.

1.2.4.3 The cyclin-dependent kinase inhibitors (CKIs)

Two classes of these inhibitory proteins are recognized. First, INK4A family members inhibit D-type cyclins from associating with and activating CDK4 and CDK6. Second, CIP/KIP family members are potent inhibitors of CDK2 kinases. The first member of INK4 (Inhibitors of Cdk4) is $p16^{INK4A}$ (144). Recently, the other members of INK4 family called $p15^{INK4B}$, $p18^{INK4C}$ and $p19^{INK4D}$, which specifically inhibit the cyclin D-dependent kinases (Cdk4 and Cdk6), have subsequently been identified (145). In contrast, the CIP/KIP (Cdk Interacting Protein/Kinase Inhibitory Protein) family of proteins $p21^{CIP1}$, $p27^{KIP1}$ and $p57^{KIP2}$ can bind to a much broader range of Cdks that includes Cdk4, Cdk6, Cdk2 and Cdc2 as shown in Figure 8 (145). Recently, the CIP/KIP family not only were originally identified as Cdk inhibitors, but also has come to light that they may actually promote the activity of these Cdks by stabilizing the Cdk–cyclin subunit interaction, at least in the case of the cyclin D-

dependent kinases (146, 147). However, they still strongly inhibit Cdk2 activity. The 'sequestration model' of G1 Cdk/cyclin activation provides one possible explanation for their contrasting behavior towards the cyclin D-dependent kinases versus Cdk2 (148). The D-type cyclins are expressed and bind to Cdk4 and Cdk6. This assembly is promoted through stoichiometric association with CIP/KIP proteins, such that these complexes are still active and can initiate phosphorylation of Rb. A second function of the cyclin D-dependent kinases is that they consequently sequester the CIP/KIP proteins away from Cdk2/cyclin E, thus promoting Cdk2 kinase activity, which can then continue phosphorylation of Rb leading to E2F-dependent transcription.

1.2.4.4 The cell cycle and cancer

G1 phase

The G1 phase of the cell cycle is a critical time where extracellular signals both positive and negative are integrated into regulation of cell cycle progression. At the time the cell becomes committed to one round of cell division, this occurs until the restriction point. The cell cannot pass the restriction point and will instead enter the quiescent state (G0 phase), if it does not receive the correct cues during G1. At the molecular level, cyclin D-dependent kinases act as integrators of these extracellular signals. The expression of cyclin D1 can induced by both the MAPK and Phosphoinositide 3-kinase (PI3K) signalling pathways, to promote the transition from G1 to S phase (149, 150). Hence, their links with cancer are very strong. In cancer, the *Ras*, *PIK3CA* (encode the p110a subunit of PI3 kinase), and *PTEN* (encode the phosphatase and tensin homolog protein that inhibit the PI3K signaling) genes have been found to be mutated (151, 152). All these genetic alterations have the ability to cause the permanent activation of cyclin D dependent kinases, lead to inappropriate phosphorylation of Rb protein, and further deregulation of the restriction point. Cyclin D1 was first identified as the *BCL1* gene, found at the t (11; 14) translocation in mantle cell lymphoma and also as *PRAD1/CCND1* the gene at the inversion of part of chromosome 11, inv (11) (p15; q13) found in parathyroid adenoma (153). Amplification of the cyclin D1 at locus 11q13 has also been reported in several cancer types, including breast, lung and glioma. The deletion, mutation or expression silenced of tumour supressor gene CDKN2 encoding the $p16^{INK4A}$ protein is also found in multiple cancers (153). CDK4 amplification and CKI $p27^{KIP1}$ protein

reduction have been reported to indicate poor prognosis in both colon and breast cancer (153, 154). These genetic alterations have the ability to promote inappropriate phosphorylation and inactivation of Rb protein. In addition, mutation or deletion of the RB gene itself is a common occurrence in cancer and directly abrogating the requirement for cyclin D dependent kinase activity during G1 (153). In some tumor types, the genetic alterations on the $p16^{INK4A}$/cyclin D/pRb pathway were appeared to be a mutually exclusive behavior, such as the deletions or mutations in RB or CDKN2 encoding $p16^{INK4A}$ in lung cancer (153). This suggests that genetic alteration in one member of this pathway is a sufficient contribution to tumor progression.

G1/S transition

Activation of cellular pathways which induce cell cycle arrest at the G1/S transition is a primary response of the normal cell to double strand breaks in the DNA (DSBs). Thus, the cells contained DNA damage in G1 phase do not enter S phase. Induction of this arrest is a two-step process, a rapid initiation of the arrest and a following slow maintenance. At the present, two cellular events have been identified. The first event participate in early of the G1/S checkpoint by activation of cyclin D1 degradation process, which leads to a releasing $p21^{CIP1}$ from Cdk4 and further inhibit Cdk2 (155). The second is an increasing the inhibitory phosphorylation of Cdk2 at the site equivalent to tyrosine 15 of Cdc2 by degradation of the Cdc25A phosphatase, which dephosphorylates this site. The degradation of Cdc25A is induced by activation of Chk1, a serine/threonine checkpoint kinase (156).

TP53 gene plays a critical role on maintenance of the G1/S cell cycle checkpoint. Mutation or deletion of *TP53* gene founded in over half of all sporadic cancers making this genetic changes in *TP53* gene to be the most common defect in human cancer (157). The p53 protein, encoded from *TP53* gene, acts as a receiver of stress signals (including DNA damage) and induces the expression of proteins involved in the cell cycle arrest and apoptosis induction. One of these is the CIP/KIP family member, $p21^{CIP1}$, which binds to Cdk2/cyclin E causing cell cycle arrest at the G1/S transition (158). Blocking of p53 degradation is one of the ways in which upregulation of its expression is induced. Like the cyclins, p53 is degraded via ubiquitin-mediated proteolysis which initiated by the E3 ligase, Mdm2 (159). The activation of the cell cycle checkpoint serine/threonine kinase Chk2 (also known as

hCds1) is one route, which can abrogate degradation of p53 protein leading to its upregulation and induction of $p21^{CIP1}$. Chk2 is activated in response to DNA damage and phosphorylates serine 20 of p53 (160). Activation of Chk2 disrupts the interaction of p53 with Mdm2 and blocked the degradation of p53 protein (159). Mutations in *Chk2* gene have been identified in a subset of patients with Li-Fraumeni syndrome, a familial cancer phenotype usually associated with mutation of the *TP53* gene, which offering genetic evidence that p53 and Chk2 lay on the same pathway (161). Chk2 also activated through phosphorylation of threonine 68 by the ataxia telangiectasia mutated (ATM) protein kinase, a key player in activation of cell cycle checkpoints (162). The *ATM* gene is responsible for the autosomal recessive disorder Ataxia Telangiectasia (AT), caused by mutations of *ATM* gene. This gene is extremely sensitive to ionizing radiation which causes DSBs and a predisposition to certain forms of cancer. Moreover, ATM-deficient cells show reduction responses to genotoxic agents that cause DSBs, indicating the importance of defective cell cycle checkpoints in cancer (163).

S Phase

DNA damage during S phase also invokes a cell cycle checkpoint. In contrast to the G1/S checkpoint, the rate of DNA synthesis is slowed but not a complete arrest after exposure to DNA damaging agents. Instead, it appears to slow down the DNA replication and extend S phase (164). These suggested that the S phase DNA damage checkpoint does not actually stop replication in order to complete DNA repair but instead slows replication if damage has occurred (165). The protein kinase ATM, Nibrin, Mre11, and Rad50 have been shown to participate in this checkpoint of mammalian cells (166). Nibrin (also known as NBS1) is a novel DNA double strand break repair protein which is mutated in Nijmegen breakage syndrome (NBS). Mre11 is a protein which mutated in an AT-like disorder. In the cell, NBS1, Mre11, and Rad50 are formed a complex that can carry out ATP-dependent DNA unwinding and hairpin cleavage, which required for DNA repair (167). Phosphorylation of Nibrin by ATM provides a link between the checkpoint protein ATM and DNA repair (168).

Because, ionizing radiation exposure increased the levels of $p21^{CIP1}$ and inhibited the Cdks kinase activity. Therefore, the $p21^{CIP1}$ may also play a role in the S phase checkpoint. The $p21^{CIP1}$ has been reported to inhibit Cdk activity and slow

down the DNA replication (169). However, the S phase DNA damage checkpoint remained intact in $p21^{CIP1}$ knocked out cell line, suggested that $p21^{CIP1}$ is not essential for this checkpoint (170).

G2/M transition

Despite the G2/M DNA damage checkpoint appears in the late of G2 phase and involves with many proteins that participate in the G1/S checkpoint, its target is the Cdc2/cyclin B complex, which is required for progression from G2 to mitosis, not the Cdk2/cyclin E complex. As a consequence, the main function of this checkpoint is to maintain the Cdc2/cyclin B1 complex in an inactive state by upholding inhibitory phosphorylation on the T14 and Y15 residues of Cdc2 due to the blocking of Cdc25C phosphatase activity. ATM protein plays a role mediating phosphorylation and activation of the Chk1 and Chk2 checkpoint kinases (163, 171). These kinases can phosphorylate at serine 216 of Cdc25C, which promotes its association with 14–3–3 proteins and leads to Cdc25C sequestration in the cytoplasm. The sequestration of Cdc25C inhibits the dephosphorylation of the nuclear localised Cdc2/cyclin B. The p53 protein may also play a role in this checkpoint and sustain the G2 arrest. Upregulation of p53 at the G2 checkpoint leads to the activation of $p21^{CIP1}$, which can bind and inhibit the activity of Cdc2/cyclin B in the nucleus (172). Upregulation of p53 also induces the expression of 14–3–3s protein due to blocking the nuclear translocation of CDC2/cyclinB complex (173). Thus, p53 protein provides a double insurance policy to inhibit the Cdc2/cyclinB activity.

In terms of cancer, the relationship between many of these proteins and tumourigenesis has already been outlined but there are a few of exceptions. Although, linkage between Chk2 and cancer has been clearly made, the correlation between Chk1 and cancer is still unclear (161). It is difficult to determine whether this is because Chk1 mutations do not occur in tumors or whether they have yet to be discovered. However, inactivating mutations of $p21^{CIP1}$ have not been identified in tumors, the mutation of *TP53* gene, regulator of $p21^{CIP1}$, is founded in about 50% of human tumors (157). Interestingly, $p21^{CIP1}$ has recently been reported that its function can disrupt by phosphorylation process, which leads to the re-localization of $p21^{CIP1}$ from nucleus to the cytoplasm in breast cancers (174). This phosphorylation process is carried out by the proto-oncogene Akt, which overexpressed in Her2/neu

over-expressing breast cancers. This provides a novel mechanism in which the function of p21 can be removed without gene mutation in the tumors.

1.2.5 Cell death

Cell death is a part of normal development and maturation processes to maintain the homeostasis of multicellular organisms. The cell death is also the component of many response patterns of living cells to xenobiotic agents, such as microorganisms and chemicals, and to endogenous modulations, such as inflammation (175). The Nomenclature Committee on Cell Death (NCCD) suggests that a cell should be considered dead when it met with any of the following criteria. First, the cell has lost the integrity of the plasma membrane. Second, the cell including its nucleus has undergone complete fragmentation into discrete bodies (apoptotic bodies). Third, their corpses or fragments have been engulfed by an adjacent cell in *in vivo* (176, 177). There are several types of cell death (defined by morphological criteria), such as necrosis, apoptosis, autophagy, and mitotic catastrophe (176-178).

1.2.5.1 Necrosis or necrotic cell death

Necrosis is a morphologically characterized by gain in cell volume (oncosis), swelling of organelles, plasma membrane rupture and subsequent loss of intracellular contents (177). In comparison with apoptosis, in which the Bcl-2 family and caspases play key roles in the process, necrosis is induced by inhibition of cellular energy production, imbalance of intracellular calcium flux, generation of ROS, and activation of non-apoptotic proteases. These events often potentiate each other and synergize to cause necrosis (179).

1.2.5.2 Apoptosis

The process of programmed cell death or apoptosis is considered a vital component of various processes including normal cell turnover, proper development and functioning of the immune system, hormone-dependent atrophy, embryonic development and chemical-induced cell death (180-183). Apoptosis is essential for successful embryonic development and maintains normal cellular homeostasis in adult organisms. Inappropriate apoptosis, either too little or too much, is a factor in many human conditions including neurodegenerative diseases, ischemic damage, autoimmune disorders and many types of cancer. In mammalian cells, apoptosis has

been divided into two major pathways, including the extrinsic pathway in which activated via death receptor activation, and the intrinsic pathway in which activated by stress-inducing stimuli (184, 185).

After receiving specific signals, a number of distinctive changes occur in the cell that undergoes apoptosis. During apoptosis, the controlled destruction within the cell is coordinated by the caspase family of cysteine proteases. Caspases or cysteine-dependent aspartate-directed proteases are typically activated at the early stages of apoptosis to catalyze or cleave the key cellular components, such as structural proteins in the cytoskeleton and nuclear proteins. The caspases can also activate other enzymes such as DNases and lead to the cleavage of DNA in nucleus (186, 187).

Morphology of apoptosis

Cell shrinkage and pyknosis are visible by light microscopy during the early process of apoptosis (188). With cell shrinkage, the cells are smaller in size due to the cleavage of lamins and actin filaments in the cell cytoskeleton. Then, the cytoplasm is dense and the organelles are more tightly packed. Pyknosis is the result of chromatin condensation due to the breakdown of chromatin in the nucleus and this is the most characteristic feature of apoptosis. On histologic examination with hematoxylin and eosin staining, the apoptotic cell appears as a round or oval mass with dark eosinophilic cytoplasm and dense purple nuclear chromatin fragments (186). After pyknosis, extensive plasma membrane blebbing occurs followed by karyorrhexis and separation of cell fragments into apoptotic bodies. Karyorrhexis is the destructive fragmentation of the nucleus of a dying cell whereby its chromatin is distributed irregularly throughout the cytoplasm (189). Apoptotic bodies consist of cytoplasm with tightly packed organelles with or without a nuclear fragment. The organelle integrity is still maintained and all of this is enclosed within an intact plasma membrane (177-179). These bodies are subsequently phagocytosed by macrophages, parenchymal cells, or neoplastic cells and degraded within phagolysosomes (176). In order to promote their phagocytosis by macrophages, apoptotic cells often undergo plasma membrane changes that alter the macrophage response. One such change is the translocation of phosphatidylserine from the inside of the cell to the outer surface.

Biochemical features of apoptosis

Apoptotic cells display several biochemical modifications such as protein cleavage, protein cross-linking, DNA breakdown, and phagocytic recognition (181). The activation of caspases cascade is responsible for the cleavage of important cellular substrates resulting in the classical biochemical and morphological changes associated with the apoptotic phenotype. The caspases are widely expressed as an inactive form called pro-enzyme in most cells. Once activation, activated upstream caspase catalyze other downstream pro-caspases to establish the proteolytic cascade. This cascade amplifies the apoptotic signaling pathway and thus leads to quick cell death. Caspase enzymes have proteolytic activity to cleave at aspartic acid residues of target proteins. However, each caspases have different specificities involving recognition of neighboring amino acids. Once caspase activation, the cell will be submitted to an irreversible commitment towards cell death. To date, more than ten major caspases have been identified and are broadly categorized into initiators (caspase-2,-8,-9,-10), effectors or executioners (caspase-3,-6,-7), and inflammatory caspases (caspase-1,-4,-5) (190, 191). In addition, extensive proteins cross-linking is another characteristic of apoptotic cells. This cross-linking is achieved through the expression and activation of tissue transglutaminase (192). DNA breakdown by Ca^{2+}- and Mg^{2+}- dependent endonucleases also occurs in apoptotic cells and result in around 180 to 200 base pairs of DNA fragments (193).

Another biochemical feature is the translocation of phosphatidylserine from inner layer to outer layer of the plasma membrane in which resulting in the early phagocytic recognition of apoptotic cells by adjacent cells (194).

Mechanisms of apoptosis

The mechanisms of apoptosis are highly complex, sophisticated, and involve with the energy-dependent cascades of molecular events. There are two main apoptotic pathways, including the extrinsic or death receptor pathway, and the intrinsic or mitochondrial pathway. However, the idea that extrinsic and intrinsic apoptotic pathways in certain cell types can link each other is now widely accepted (195-197). There is an additional pathway that involves T-cell mediated cytotoxicity and perforin-granzyme-dependent killing of the cell. The perforin/granzyme pathway can induce apoptosis through either granzyme B or granzyme A. The extrinsic,

intrinsic, and granzyme B pathways converge on the same execution pathway. The granzyme A pathway activates a parallel, caspase-independent cell death pathway via single stranded DNA damage (198).

Extrinsic pathway

The extrinsic pathway is triggered by the binding of an extracellular ligand to a death receptor on the plasma membrane. The death receptors are members of the tumor necrosis factor receptor (TNFR) gene superfamily, including TNFR-1, Fas/CD95, and the TRAIL receptors DR-4 and DR-5 (199). Members of the TNF receptor family share the similar cyteine-rich extracellular domains and have a cytoplasmic domain of about 80 amino acids called the death domain (DD). The death domain plays a critical role in transmitting the death signal from the cell surface to the intracellular signaling pathways (200). The FasL/FasR and TNF-α/TNFR1 models are best characterized with the sequences of events that define the extrinsic phase of apoptosis. In these models, there is clustering of receptors and binding with the homologous trimeric ligand. Upon ligand binding, cytplasmic adapter proteins are recruited which exhibit corresponding death domains that bind with the receptors. The binding of Fas ligand to Fas receptor results in the binding of the adapter protein FADD, while the binding of TNF ligand to TNF receptor allows the binding of the adapter protein TRADD with recruitment of FADD and RIP (201, 202). Stimulation of death receptors results in receptor aggregation and attraction of the adaptor molecule Fas-associated death domain (FADD), which recruits procaspase-8 (or procaspase-10) molecules through homologous death effector domains (DEDs). This assembly of proteins (receptor, FADD, and caspases) is termed the death-inducing signaling complex (DISC) (203). Recruitment of caspase-8/10 to the DISC leads to their autoproteolytic cleavage and further activation (179, 204). Activation of caspase-8 causes the execution phase of apoptosis. The activated caspase-8 initiates apoptosis by direct cleavage of downstream effector caspases (caspases 3, 6 and 7), which are responsible for the cleavage of important cellular substrates, including PARP. Finally, the cleavage of cellular substrates results in the classical biochemical and morphological changes associated with the apoptotic phenotype as shown in Figure 11. The extrinsic pathway of apoptosis can be inhibited by a protein called c-FLIP which binds to FADD and caspase-8, rendering them ineffective (205, 206).

Toso, another inhibitory protein has been shown to block Fas-induced apoptosis in T cells via inhibition of caspase-8 processing (207).

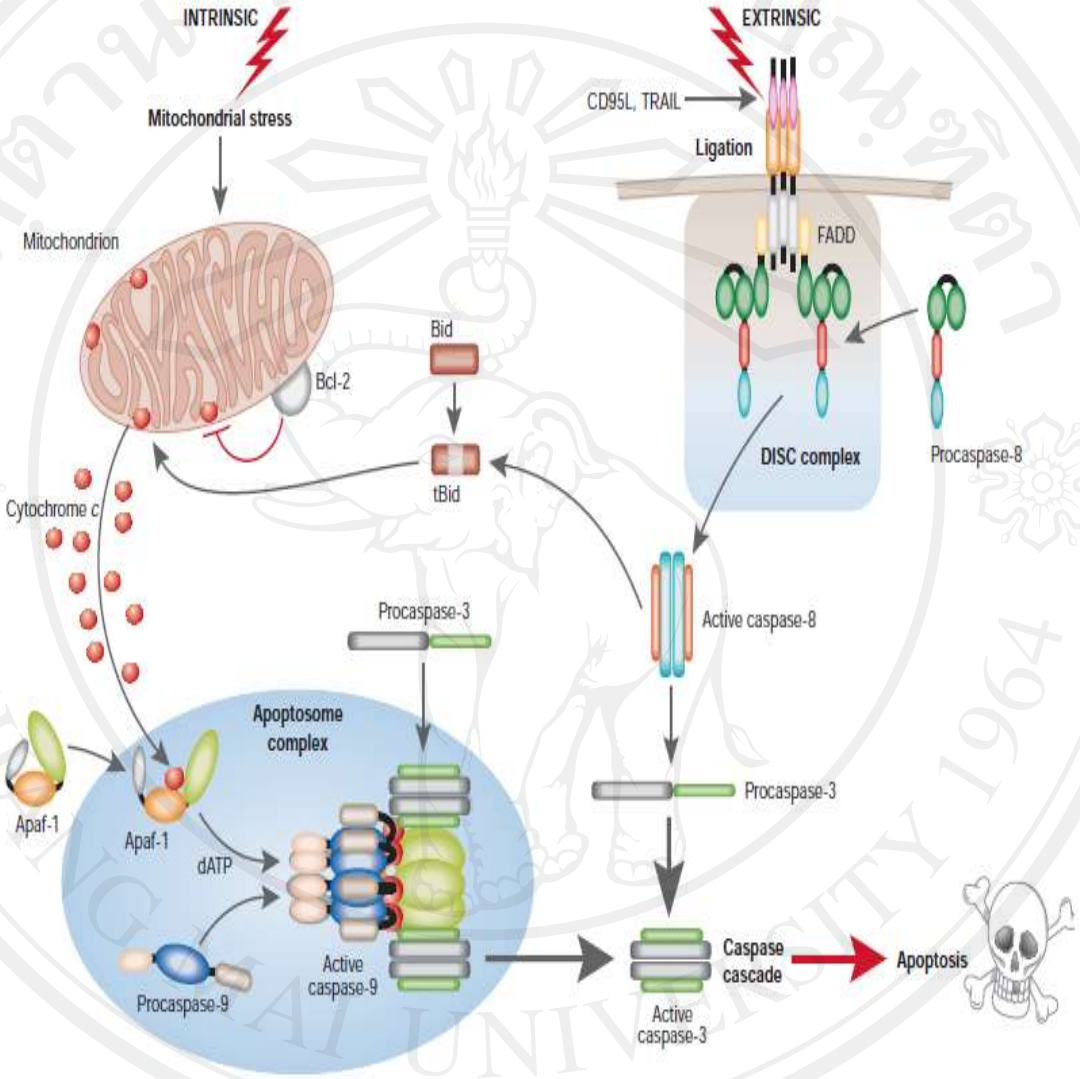


Figure 11 Apoptosis via the extrinsic and intrinsic pathways (187)

Intrinsic pathway

The intrinsic pathway is usually activated in response to intracellular stress signals, which include DNA damage and high levels of reactive oxygen species (ROS), as well as by viral infection and activation of oncogenes. The intracellular stress signals induced apoptosis via the perturbation of mitochondria and the ensuing release of proteins, such as cytochrome *c*, from the mitochondrial intermembrane space. The released cytochrome *c* binds to apoptotic protease-activating factor 1 (Apaf1), which results in formation of the Apaf1–caspase 9 apoptosome complex and

activation of the initiator caspase 9. The activated initiator caspases 9 activate the effector caspases 3, 6 and 7, which are responsible for the cleavage of important cellular substrates. Finally, the cleavage of cellular substrates results in the classical biochemical and morphological changes associated with the apoptotic phenotype as shown in figure 4 (179, 186, 187, 208, 209). During this phase of apoptosis, caspase 3 and caspase 7 recognize the DEVD (Asp-Glu-Val-Asp) motif in the nuclear localization signal of PARP-1 and cleave it into two fragments (~p89 and ~p24), which separates the DNA binding domain from the catalytic domain rendering PARP. The cleavage of PARP suppresses the PARP activity by inhibiting homoassociation and DNA binding of intact PARP-1, results the DNA fragmentation (210, 211). The release of cytochrome *c* from mitochondria is regulated by the Bcl-2 family of proteins via alteration of mitochondrial membrane permeability. The Bcl-2 family of proteins can be either pro-apoptotic or anti-apoptotic, the anti-apoptotic proteins include Bcl-2, Bcl-x, Bcl-XL, Bcl-XS, BAG, and the pro-apoptotic proteins include Bcl-10, Bax, Bak, Bid, Bad, Bim, Bik, and Blk. The intrinsic apoptotic pathway hinges on the balance of activity between pro- and anti-apoptotic members of the Bcl-2 family of proteins as shown in Figure 12 (178, 179, 186, 187, 208, 209, 212, 213).

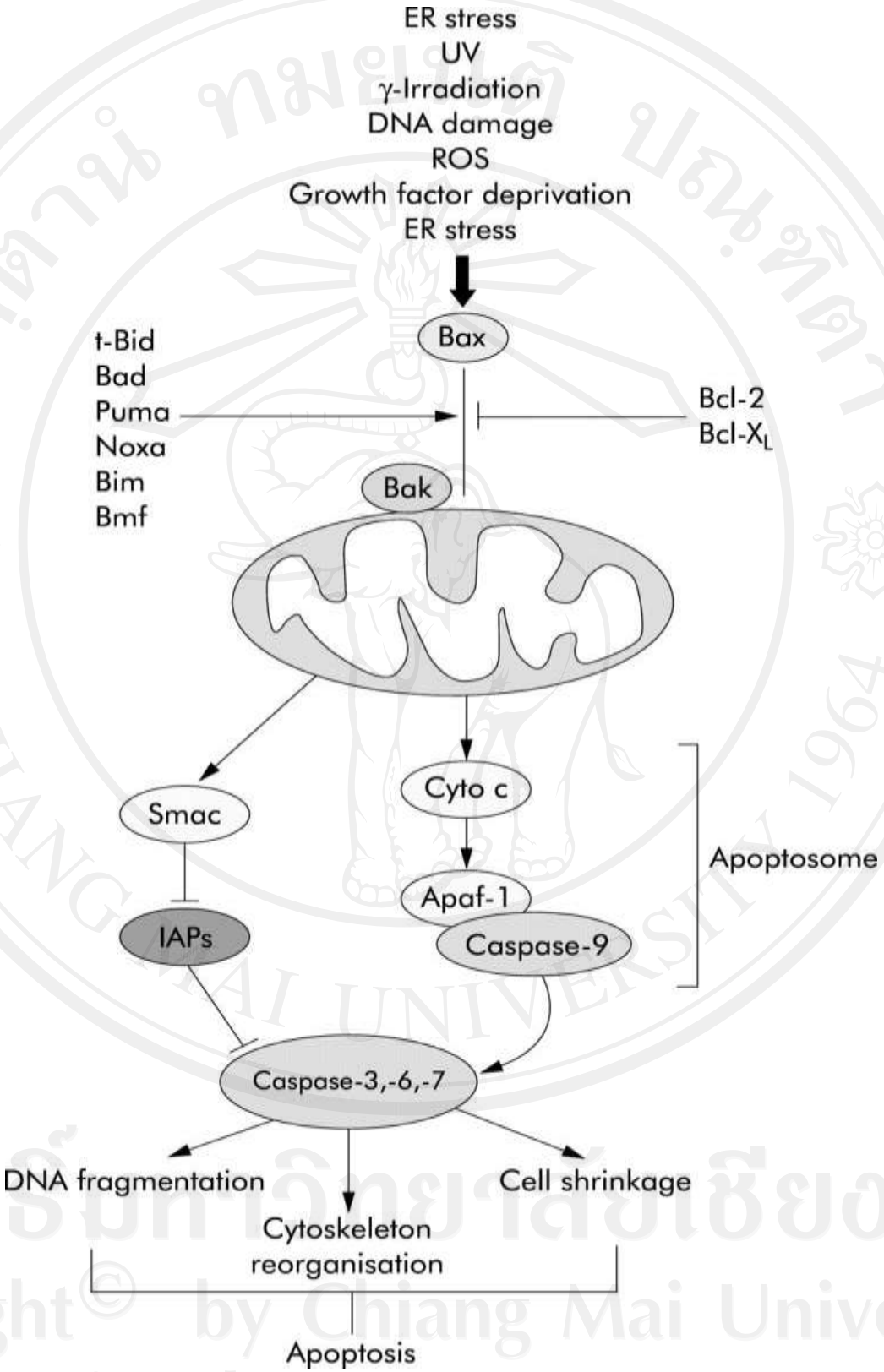


Figure 12 Mitochondria mediated (intrinsic) pathway of apoptosis (213)

Execution pathway

The extrinsic and intrinsic pathways both end at the point of the execution phase, which is considered as the final pathway of apoptosis. Cytoplasmic endo-nuclease, which degrades nuclear material, and proteases that degrade the nuclear and cytoskeletal proteins are activated by execution caspases. The executioner caspases, such as caspase-3, caspase-6, and caspase-7, cleaved various substrates, including cytokeratins, PARP, the plasma membrane cytoskeletal protein alpha fodrin, the nuclear protein NuMA and others. These cleavage ultimately cause the morphological and biochemical changes seen in apoptotic cells (214).

Caspase-3 is considered to be the most important of the executioner caspases. Caspase-3 is activated by any of the initiator caspases, such as caspase-8, caspase-9, or caspase-10. Activated caspase-3 has been reported to cleave an inhibitor of caspase activated DNase (ICAD) protein to release caspase-activated DNase (CAD) protein from the CAD-ICAD complex. Then, the CAD degrades chromosomal DNA within the nuclei and causes chromatin condensation in apoptotic cells as shown in Figure 13 (215, 216). Caspase-3 has also been reported to induce cytoskeletal re-organization and disintegration of the cell into apoptotic bodies. An actin binding protein, Gelsolin, has been identified as one of the key substrates of activated caspase-3. Gelsolin acts as a nucleus for actin polymerization and also bind to phosphatidylinositol biphosphate to establish the linking actin organization and signal transduction. Activated caspase-3 cleaves gelsolin and its cleaved fragments, and also cleaves actin filaments in a calcium independent manner. These cause the disruption of cytoskeleton, intracellular transport, cell division, and signal transduction (217).

Phagocytic uptake of apoptotic cells is the last event of apoptosis process. Phospholipid asymmetry and externalization of phosphatidylserine (PS) on the surface of apoptotic cells and their fragments is the hallmark of this phase. The mechanism of PS translocation to the outer leaflet of the cell during apoptosis has been associated with the loss of aminophospholipid translocase activity and nonspecific flip-flop of phospholipids in various classes (194). Fas, caspase-8, and caspase-3 have been indicated to involve in the regulation of PS externalization on oxidatively stressed erythrocytes. However, caspase-independent phosphatidylserine

exposure also occurs during apoptosis of primary T lymphocytes (218, 219). The appearance of PS on the outer leaflet of apoptotic cells further facilitates non-inflammatory phagocytic recognition, allowing apoptotic cells to uptake and disposal (220). This process of early and efficient uptake with no release of cellular constituents, results in essentially non-inflammatory response.

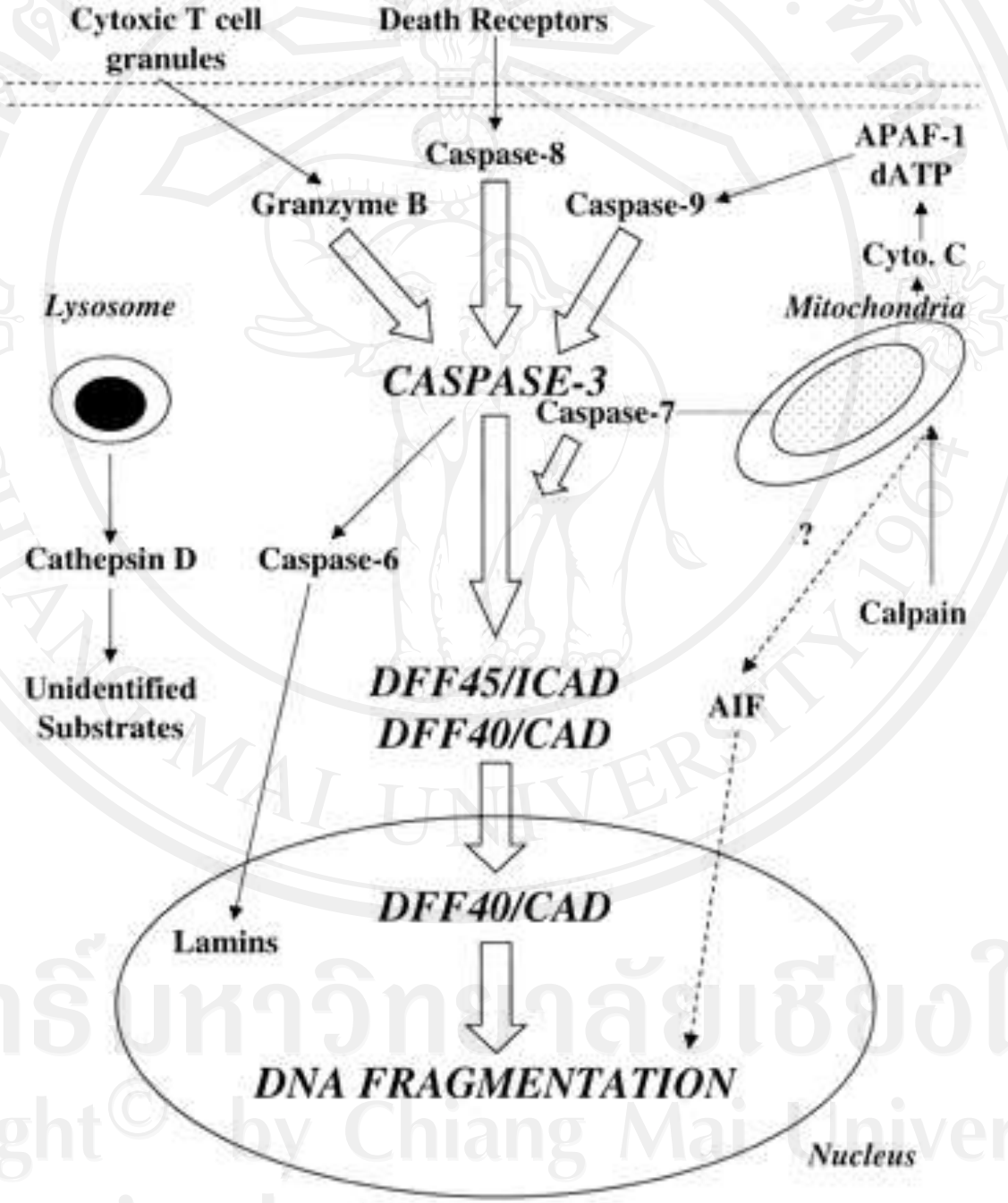


Figure 13 A model of apoptotic DNA fragmentation (216)

Apoptosis and cancer development

Abnormalities in the regulation of cell death, either insufficient or excessive apoptosis have involved in the development of various diseases such as cancer, autoimmune lymphoproliferative syndrome, AIDS, ischemia, and neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, Huntington's disease, and Amyotrophic Lateral Sclerosis.

Cancer is an significant disease which the cell cycle regulation are dysfunctional, either overproliferation of cells and/or decreasing removal of uncontrolled cells (221). During carcinogenesis, the suppression of apoptosis plays an important role in the cancer development and progression (222). To date, many molecular mechanisms have been identified to involve in the suppression of apoptosis in tumor cells. Tumor cells can acquire apoptosis resistance by the upregulation of anti-apoptotic proteins such as Bcl-2, and/or by the downregulation or mutation of pro-apoptotic proteins such as Bax. The expression of both Bcl-2 and Bax is regulated by the p53 tumor suppressor protein (223). Overexpression of Bcl-2 was identified in certain forms of human B cell lymphoma, suggested that failure of cell death contributes to cancer development (224). Evasion of immune surveillance is also a channel of tumor cells to avoid the apoptosis (225). Certain immune cells such as T cells, and natural killer cells, usually destroy tumor cells by the death-receptor pathway or the perforin/granzyme B pathway. Some tumor cells evade the immune-induced destruction by reducing the response of the death receptor pathway to Fas ligands, which produced by T cells. This reduction arise from various events in tumor cells, including down-regulation of the Fas receptor, expression of non-functioning Fas receptor, or hypersecretion of a soluble Fas receptor to sequester the Fas ligand (226, 227). Moreover, some tumor cells are capable to express the Fas ligand on their surface, which results in apoptotic depletion of activated tumor infiltrating lymphocytes (228).

The cell signaling alterations can lead to dysregulation of apoptosis and result in cancer development. In human tumorigenesis, the *TP53* tumor suppressor gene is the most widely mutated gene (229). This gene encodes p53 protein, a transcription factor that regulates the cell cycle. Once the DNA has sustained damage, p53 protein holds the cell cycle at the G1/S regulation checkpoint, activates DNA repair proteins,

and induced the cell to undergo apoptosis if the damage cannot repaired (230). Mutation of *TP53* gene disrupts these functions of p53, and further leads to tumorigenesis due to reducing tumor suppression. The mutation of *TP53* gene is occurred by radiation, various chemicals, and viruses such as the Human papillomavirus (HPV). People who inherit only one functional copy of this gene will most likely develop Li–Fraumeni syndrome, which is characterized by the development of tumors in early adulthood (231, 232). The *ataxia telangiectasia-mutated* or *ATM* gene has also been shown to be involved in tumorigenesis. ATM protein is activated via ionizing radiation damage of DNA, and further phosphorylates p53 protein. This phosphorylation of p53 stimulates the DNA repair and blocks the cell cycle progression (233). On the other hand, other cell signaling pathways such as PI3K/Akt, and MAPK/ERK pathways, can also be involved in tumor development. In tumor cells, up-regulation of the PI3K/Akt pathway renders them independent of survival signals (234).

1.2.6 Inflammation

Inflammation is a response of a tissue to harmful stimuli, such as pathogens, damaged cells, or irritants. Inflammation is a protective attempt by the organism to remove the injurious stimuli and to initiate the healing process. Inflammation can be classified as acute or as chronic. Acute inflammation is the initial response of the body to harmful stimuli. This type of inflammation is achieved by the increased movement of plasma and leukocytes, especially granulocytes from the blood into the injured tissues due to the secretion of chemoattractants. Chronic or Prolonged inflammation, other type of inflammation, leads to a progressive shift in the type of cells present at the site of inflammation and is characterized by simultaneous destruction and healing of the tissue from the inflammatory process (235-237). Besides the repairing of tissue injury, the chronic inflammation can also be involved with various diseases, such as hay fever, periodontitis, atherosclerosis, rheumatoid arthritis, and cancer (238).

1.2.6.1 Inflammation and colorectal cancer

Inflammatory bowel diseases (IBD) are complex disorder, characterized by chronic, relapsing inflammation of gastrointestinal tract. There are two main types of IBD, Ulcerative colitis (UC) and Crohn disease (CD), which both increase the risk of

colorectal cancer in patients with chronic colitis. The genetic, immune, and environmental factors involve in the progression of IBD (21-24). In UC patients, several inflammation-associated genes such as *cyclooxygenase-2* (*COX-2*), and *nitric oxide synthase-2* (*NOS-2* or *iNOS*) are increased in inflamed mucosa and remain elevated in colonic neoplasms. The inflamed cells are setting of heightened epithelial cell turnover, mutagenic assault and sustained DNA damage caused by factors within an inflammatory cell-rich microenvironment, which appear to drive the carcinogenic process (36-38). Cancers in the setting of IBD are believed to occur by a progression from no dysplasia to indefinite dysplasia to low-grade dysplasia (LGD) to high-grade dysplasia (HGD) to carcinoma in association with a consequence of sequential episodes of somatic genetic mutation including the loss of function of *APC*, *p53* and *K-ras* gene as shown in Figure 14 (23, 31-35, 239-244). Loss of function of *APC* gene leads to the accumulation of β–catenin and increases the β-catenin-mediated Wnt signaling pathway (the constitutively activation of Wnt signaling). Then, the constitutively activation of Wnt signaling induces the transcription of many target genes that involved in cell proliferation and differentiation, including *MYC*, *cyclooxygenase-2* (*COX-2*), *hiNOS*, and *cyclin D1* (*CCND1*) (245-249).

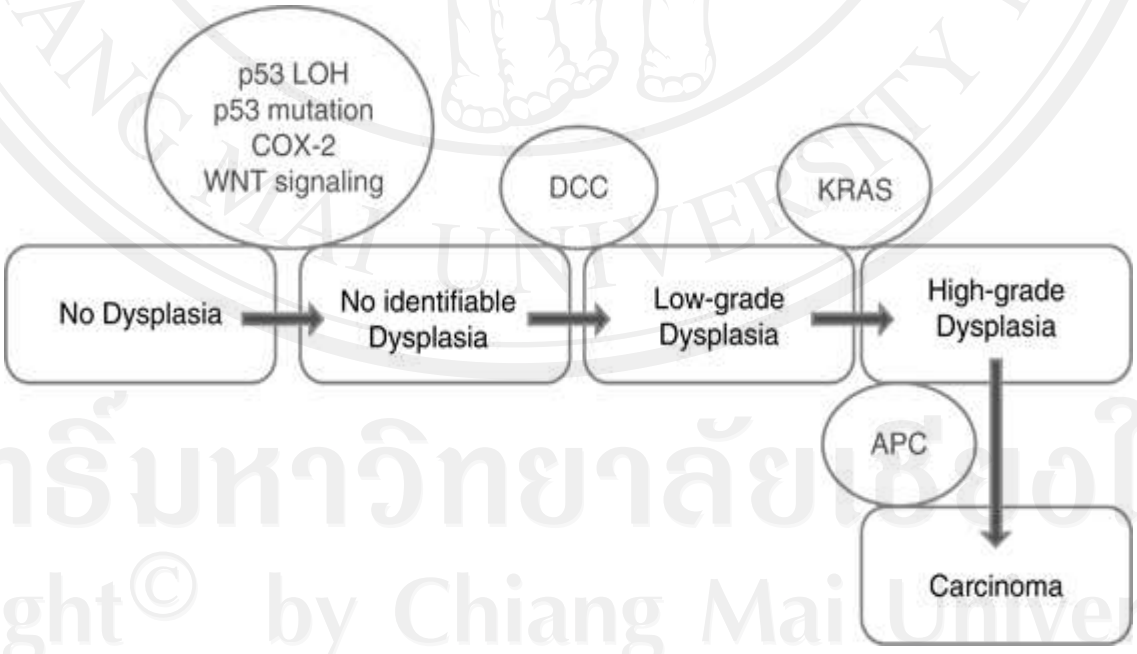


Figure 14 Colitis-associated colon cancer (244)

1.2.6.2 Animal models to study the inflammation-related colorectal cancer

To understand the correlation between inflammation and CRC, animal models of experimental colitis have been developed and are frequently used to evaluate new anti-inflammatory treatments for IBD (31). As the onset of inflammation is immediate and the procedure is relatively straightforward, chemically induced models of intestinal inflammation, such as the dextran sodium sulfate (DSS) model, belong to the most commonly used IBD animal models (60, 61). The mechanism by which DSS induces mucosal inflammation in the distal colon is not fully understood, but most studies suggest that DSS exhibits a direct toxicity against colonic epithelial cells in the basal crypts and affects the integrity of the mucosal barrier. Mucosal surfaces are lined by epithelial cells, which establish a barrier between sometimes hostile external environments and the internal milieu. The defective barrier exposes lamina propria immune cells (macrophages and neutrophils) to the continual presence of resident luminal bacteria, bacterial products (such as lipopolysaccharides: LPS), or dietary antigens, which perpetuates the inflammatory cascade. The chronic inflammation begins as an attempt of the body to remove injurious stimuli. However, the presence of inflammatory cascade for long time results in continuous tissue destruction and promotion and maintenance of carcinogenesis (62-67). The mouse provides an excellent *in vivo* system to show the model of human diseases and to test the therapeutic or chemopreventive agents. *Apc*$^{Min/+}$ mice, contain a point mutation in the *APC* gene (Min; multiple intestinal neoplasia), has emerged as a powerful model of human intestinal tumour predisposition. This mouse carries a mutation at codon 850 of *Apc* gene and develops multiple small intestinal adenomas, in addition to a smaller number of colonic polyps. The *Apc* gene encodes adenomatous polyposis coli or Apc protein (68-70). This lineage of mouse was established from an ethylnitrosourea-treated C57BL/6 male mouse. Its phenotype is an autosomal dominant trait and resembles of human familial adenomatous polyposis (FAP) syndrome, a type of CRC (68, 69). Colitis markedly accelerates the development of dysplasia and cancer in the *Apc*$^{Min/+}$ mice (71, 72). Additionally, DSS treatment led to colitis and contribute to the colonic neoplasms development in the *Apc*$^{Min/+}$ mice (73).

1.2.6.3 Wnt signaling and colorectal cancer

Wnt signaling participates in multiple developmental events, inclulding embryogenesis, adult tissue homeostasis, and also during tumorigenesis. The Wnt signals are pleiotropic, with effects that include mitogenic stimulation, cell fate specification, and differentiation (250-252). At the beginning of Wnt Signaling, Wnt ligand binds to frizzled (seven-transmembranes receptors)/low density lipoprotein receptor-related protein (LRP, co-receptors of Wnt ligands) complex at the cell surface. The binding of Wnt glycoprotein and its receptor complex transduce a signal to Dishevelled (Dsh). Then, the disheveled interact with APC, and axin in the multiprotein complex of β-catenin-Axin-adenomatous polyposis coli (APC)-glycogen synthase kinase (GSK)-3β. The interaction of disheveled with the multiprotein complex inhibits the activity of GSK-3β, which is enzymes for phosphorylation of β-catenin, and prevents the degradation of β-catenin proteins. Stabilized β-catenin then accumulates and translocates to the nucleus where it binds to members of the lymphoid-enhancer-factor/T-cell factor (LEF/TCF) family of HMG-box transcription factors. This canonical Wnt signaling (β-catenin-mediated Wnt signaling) induces the transcription of many target genes, including *MYC*, *cyclooxygenase-2* (*COX-2*), *hiNOS*, and *cyclin D1* (*CCND1*) (245-249). The canonical Wnt signaling can be blocked by soluble Fz-related proteins (sFRPs, FzBs), which extracellularly bind to Wnt ligands (absence of a Wnt ligand) and by proteins of the dickkopf (dkk) family, which bind to the LRP surface molecule (253, 254). In the absence of Wnt signaling, the multiprotein complex of GSK-3β and the scaffolding proteins APC, Axin1, and Axin2 is formed and regulates the intracellular levels of β-catenin by the phosphorylation of β-catenin at serine and threonine residues. Finally the phosphorylated β-catenin will be the target for ubiquitination and proteolytic degradation as shown in Figure 15. The mutation of *APC* gene commonly results in the accumulation of β-catenin protein and resembles constitutively active Wnt signaling (25-30). The vast majority of human APC mutations occur in a region known as the mutation cluster region (mcr) that starts around codon 1300. For example, the human colon cancer cell line DLD-1 harbors a truncation in *APC* gene at codon 1427 and SW-480 cells is truncated at codon 1337 (255).

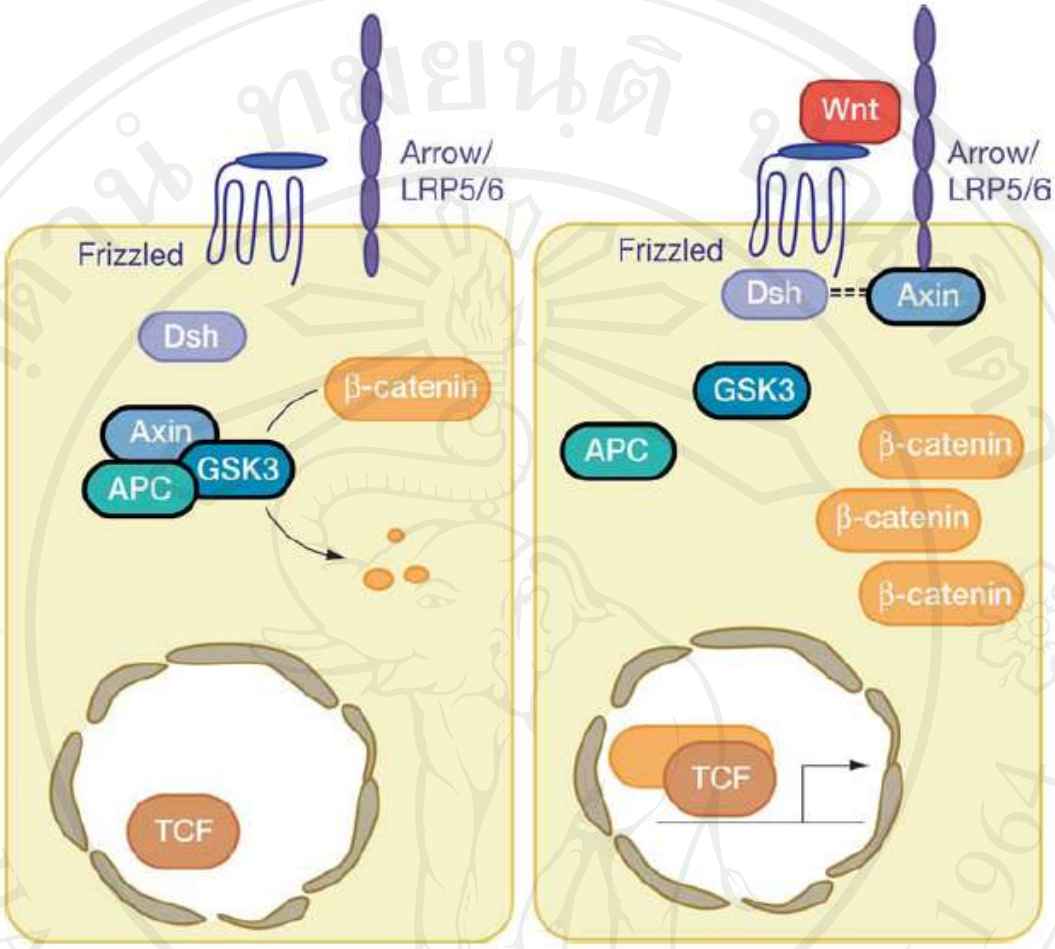


Figure 15 The canonical Wnt signaling (252). Left; the Wnt signaling in the absence of Wnt ligand, and Right; the Wnt signaling in the presence of Wnt ligand

1.2.7 Cancer chemoprevention

Cancer chemoprevention is defined as the use of natural, synthetic, or biologic chemical agents to reverse, suppress, or prevent carcinogenic progression to invasive cancer as shown in Figure 16. Strategically targeted cancer chemopreventions are emerging from enormous efforts spent investigating basic signaling mechanisms involved in carcinogens activation, cell growth, and cell death pathways (75, 256). Numerous phytochemicals derived from edible plants have been reported to interfere with a specific stage of the carcinogenic process. Many mechanisms such as the cell cycle arrest, and apoptosis induction, have been shown to account for the anti-carcinogenic actions of dietary constituents. Therefore, dietary phytochemicals are

considered to be promising chemopreventive or chemotherapeutic agents due to its (257, 258).

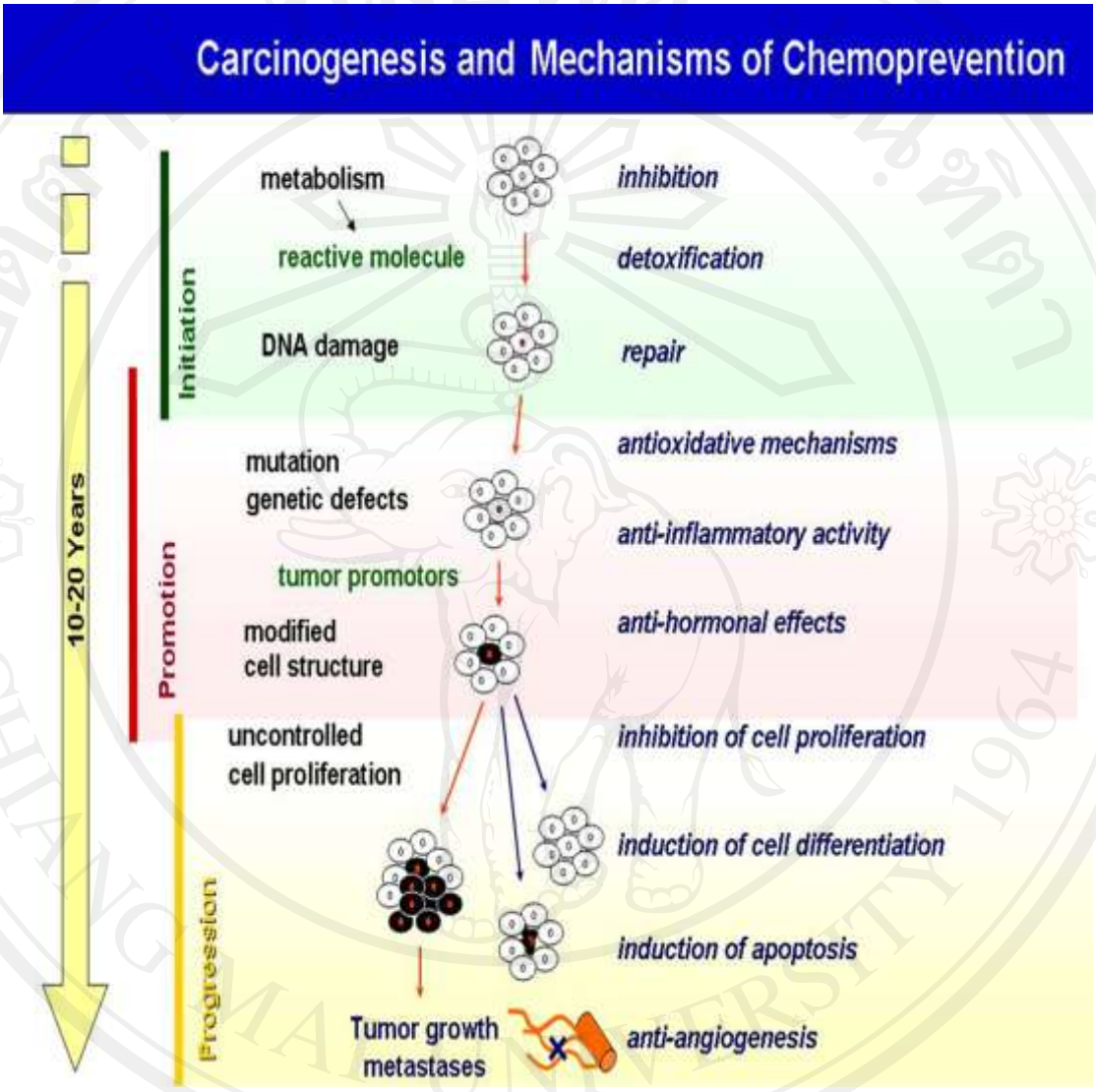


Figure 16 Carcinogenesis and mechanisms of chemoprevention

Dietary chemopreventive compounds offer great potential in the fight against cancer by inhibiting the carcinogenesis process through the regulation of cell defensive and cell-death machineries. Various research studies have demonstrated that dietary agents may be used alone or in combination with conventional chemotherapeutic agents to prevent the occurrence and spread of cancer. Consumption of fruits, vegetables, and whole grains may reduce the risk of cancer in some individuals. The rationale for selecting these compounds is that they are present in large amounts in the dietary substances and have been shown to exhibit

chemopreventive and/or chemotherapeutic effects against cancers with less side-effect (257, 259). Regulation of cell cycle progression in cancer cells is considered to be a potentially effective machination for the control of tumor growth (260, 261). The molecular analyses of human cancers have revealed that cell cycle regulators frequently often display abnormalities in their genes in most common malignancies (262, 263). This process offers several potential targets for chemopreventive agents (257, 264, 265). Moreover, accumulating evidence clearly indicates that apoptosis is a critical molecular target for dietary bioactive agents for chemoprevention of cancer (266, 267). The understanding of the critical events associated with carcinogenesis provides the opportunity for dietary intervention to prevent cancer development through induction of apoptosis. It is encouraging that dietary agents can directly and indirectly influence most, if not all of the various targets of apoptosis. Additionally, many of dietary chemopreventive agents appear to exhibit some degree of selectivity for neoplastic cells more than the surrounding normal cells. The administration of chemopreventive agents as an adjunct to radiation therapy or chemotherapy may improve their efficacy by increasing tumor sensitivity, and decreasing toxicity.

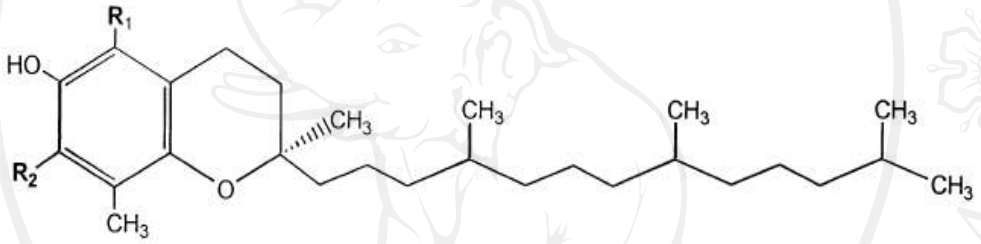
1.2.8 Fermented brown rice and rice bran (FBRA)

FBRA is a processed food, which prepares by fermention of brown rice and rice bran (from rice in Binomial name: *Oryza sativa*, subspecies: *Japonica*) with a filamentous fungus, *Aspergillus Oryzae*. The FBRA is a fermented rice product, which is manufacturing by Genmai Koso Co., Ltd. (Sapporo, Japan). The manufacturing process of FBRA begins with fermentation of steamed of brown rice and rice bran by *Aspergillus Oryzae* for 18-24 hours. Subsequently, second fermentation was continued for additional 12-24 hours for aging purpose. Fermented product was then dried and powdered. The nutritional and sanitary advantage of fermentation has been recognized, but the molecular mechanisms are still unknown. Furthermore, the chemopreventive effect of FBRA has demonstrates in the chemicals-induced tumorigenesis of many organs, such as colon, stomach, bladder and esophagus in the *in vivo* model (50-52, 268-270).

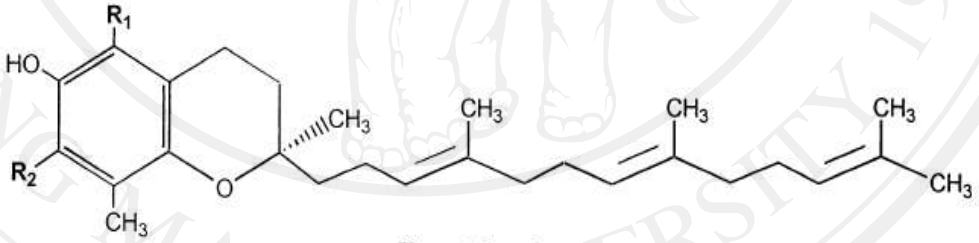
1.2.9 Gamma-tocotrienol (γ-T_3)

The compounds of natural vitamin E are divided into two subgroups, tocopherols and tocotrienols. Both subgroups have four isoform, including α-, β-, γ-

and δ-isoforms. The structural difference between tocopherols and tocotrienols is that tocopherols have a saturated phytyl chain, and tocotrienols have an unsaturated phytyl chain as shown in figure 17 (48, 271, 272). The natural sources of tocopherols and tocotrienols are difference. The tocopherols are components of nuts and common vegetable oils, but tocotrienols are primarily derived from oat, wheat germ, barley, rye, rice bran and palm oil (48, 273). In rice bran, the content of γ-tocotrienol is approximately 349 ppm, but the content of γ-tocotrienol in rice is upon the genetic and environmental variation (48). The γ-tocotrienols exhibit anti-proliferative effects, anti-cancer activities (apoptosis induction), anti-oxidant activities, and anti-inflammation activities (39, 42, 48, 271-281).



Tocopherol



Tocotrienol

	R_1	R_2
α-Tocotrienol/ Tocopherol	CH_3	CH_3
β- Tocotrienol/Tocopherol	CH_3	H
γ- Tocotrienol/Tocopherol	H	CH_3
δ– Tocotrienol/Tocopherol	H	H

Figure 17 Structures of various homologs of tocopherol and tocotrienol (272)

1.2.10 Inositol hexaphosphate (IP_6) or phytate

Inositol hexaphosphate (IP_6) or phytic acid (also called phytate) is a naturally occurring polyphosphorylated carbohydrate as shown in Figure 18. The IP_6 is found in substantial amounts in whole grains, cereals, legumes, nuts, and seeds, and is the primary energy source for the germinating plant (282, 283). The IP_6 and its lower phosphorylated forms are also found in most mammalian cells, where they assist in regulating a variety of important cellular functions (283, 284). Phytic acid represents about 1-2.5 mg/g in brown rice grains but their contents in rice are upon the genetic and environmental variation (47). The IP_6 exhibits anti-cancer activities, anti-oxidant activities, and anti-inflammation activities (43, 45, 284-290).

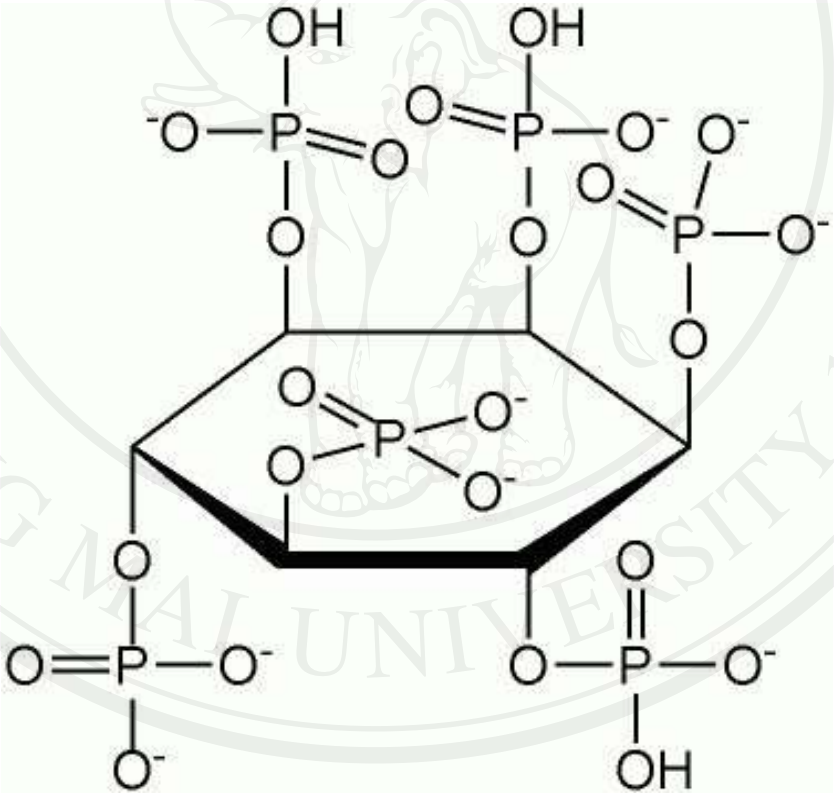


Figure 18 Chemical structure of phytate (284)

1.3 Objectives

1. To investigate the chemopreventive effect of fermented brown rice and rice bran (FBRA) against tobacco-derived nitrosamine (NNK)-induced lung tumorigenesis in *in vivo* model
2. To investigate the anti-tumorigenesis mechanism of gamma-tocotrienol (γ-T_3) and phytic acid (IP_6) on tobacco-derived nitrosamine (NNK)-treated lung cancer cell line A549 in *in vitro* model
3. To investigate the chemopreventive effect of fermented brown rice and rice bran (FBRA) against inflammation-mediated colorectal tumorigenesis in *in vivo* model
4. To investigate the anti-tumorigenesis mechanism of gamma-tocotrienol (γ-T_3) and phytic acid (IP_6) on lipopolysaccharides (LPS)-treated colorectal cancer cell line SW480 in *in vitro* model

1.4 Scopes of study

To investigate the chemopreventive effects of rice products on the lung and colorectal tumorigenesis, 4 phases of studies are provided as following:

Phase 1: The chemopreventive effect of fermented brown rice and rice bran (FBRA) against tobacco-derived nitrosamine (NNK)-induced lung tumorigenesis in *in vivo* model as shown in Figure 19.

Phase 2: The anti-tumorigenesis mechanism of gamma-tocotrienol (γ-T_3) and phytic acid (IP_6) on NNK-treated lung cancer cell line A549 in *in vitro* model as shown in Figure 20.

Phase 3: The chemopreventive effect of fermented brown rice and rice bran (FBRA) against inflammation-mediated colorectal tumorigenesis in *in vivo* model as shown in Figure 21.

Phase 4: The anti-tumorigenesis mechanism of gamma-tocotrienol (γ-T_3) and phytic acid (IP_6) on lipopolysaccharides (LPS)-treated colorectal cancer cell line SW480 in *in vitro* model as shown in Figure 22.

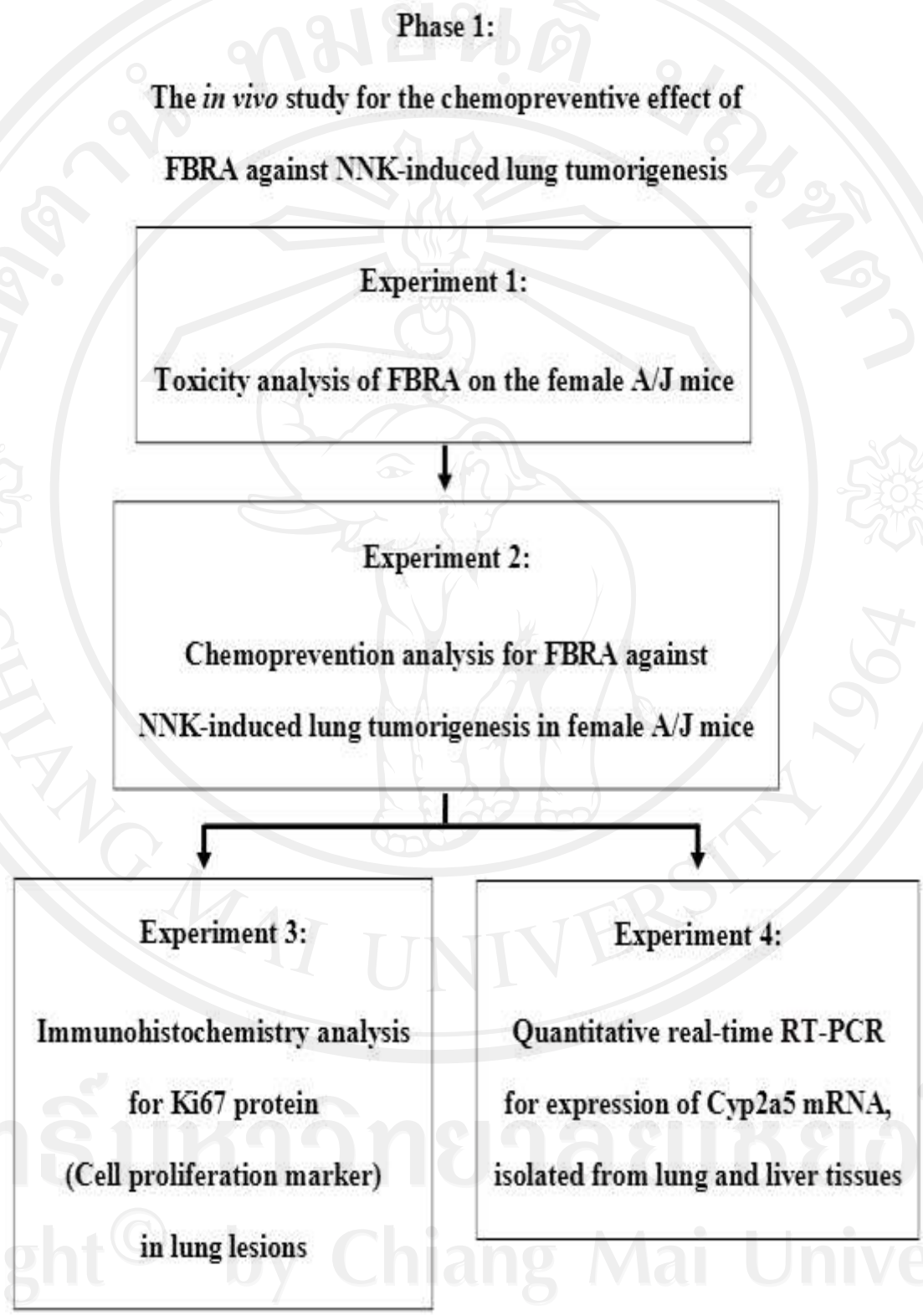


Figure 19 Scope of study in Phase 1

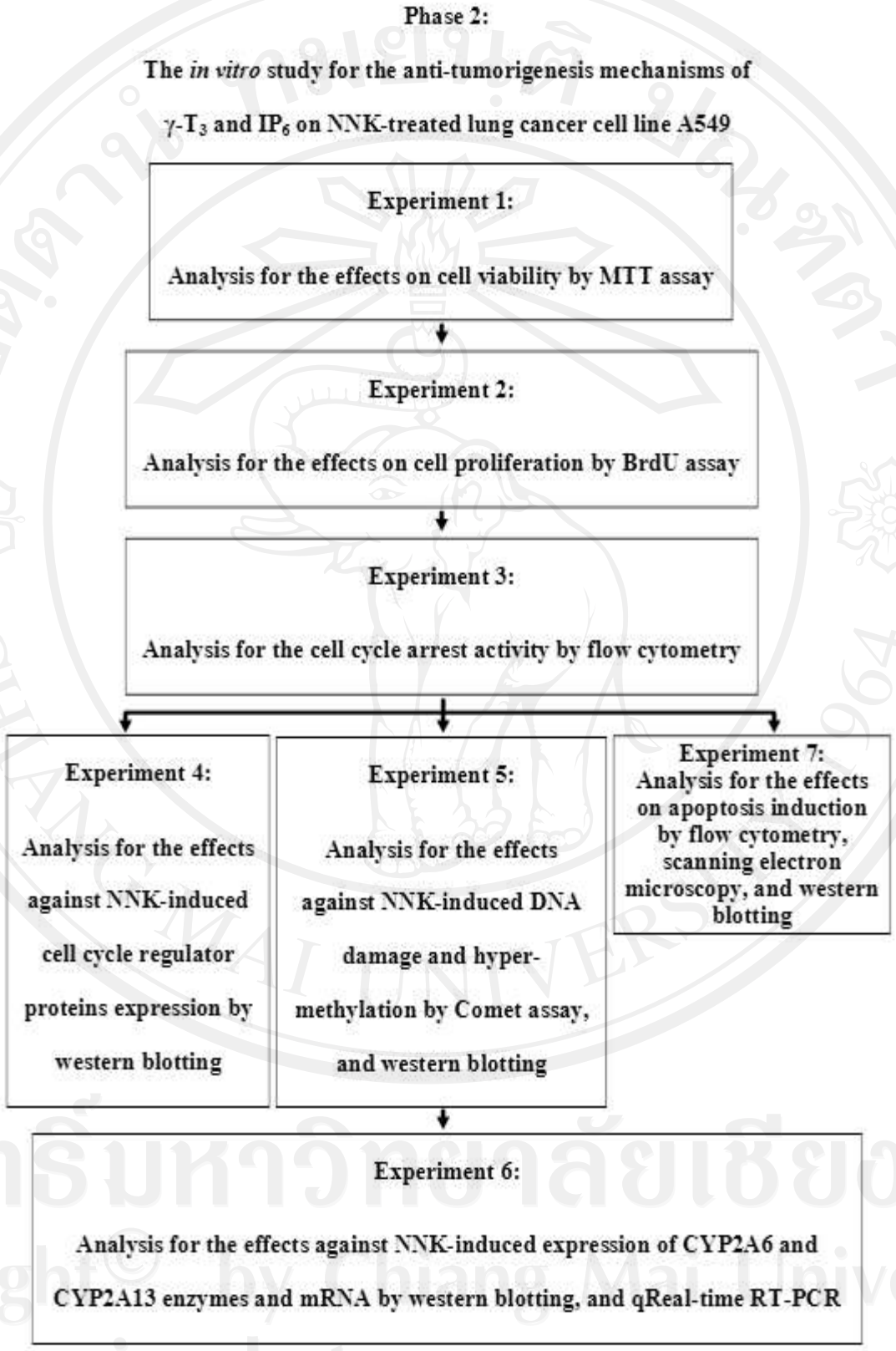


Figure 20 Scope of study in Phase 2

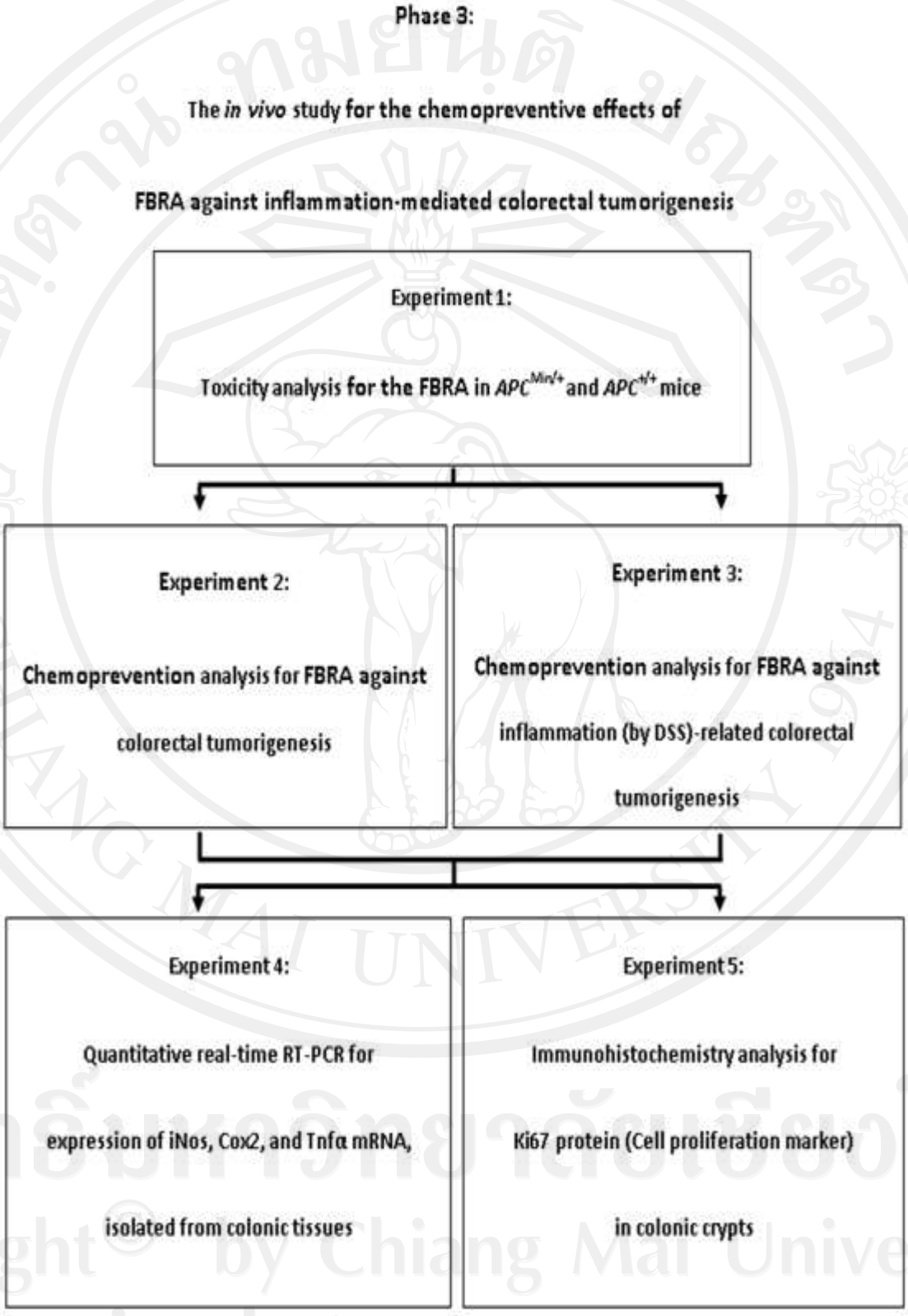


Figure 21 Scope of study in Phase 3

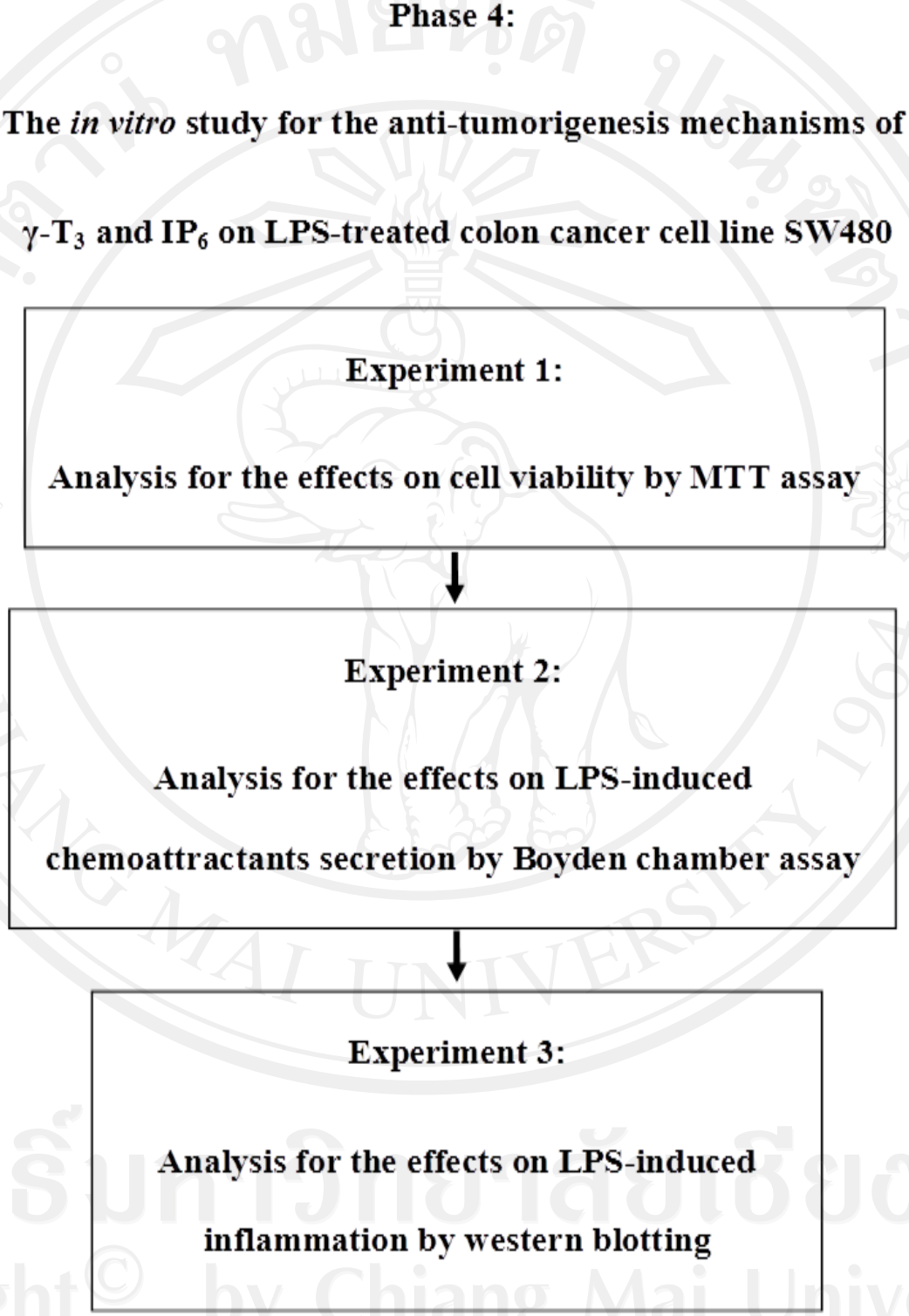


Figure 22 Scope of study in Phase 4