CHAPTER I

INTRODUCTION

1.1 STATEMENTS AND SIGNIFICANCE OF THE PROBLEM

Cancer of the ovary is the leading cause of death from gynecological malignancies. Nearly 95% of cases occur sporadically and, to date, no single causative factor has been identified. Ovarian cancer is the second most common malignancy of the female genital tract. This disease begins in, and is usually limited to, the peritoneal cavity. Without clearly definable symptoms, ovarian cancer often remains undetected until an advanced stage of disease, when prognosis is poor (Mayr and Diebold, 2003). However, little is known about the early pathologenic process of ovarian carcinogenesis or about premalignant lesions of the ovary, because more than half of ovarian cancer patients are diagnosed at the advanced stages.

Alterations in the cellular genome affecting the expression or function of genes controlling cell growth and differentiation are considered to be the main cause of cancer. Recent molecular studies of ovarian carcinoma have shown several genetic alterations may lead to uncontrolled proliferation by activating oncogenes or inactivating tumor suppressor genes. In the ovary, as in other human tumors, accumulation of genetic alterations may occur during malignant transformation of benign and/or borderline tumors to carcinomas.

Proto-oncogenes associated with signal transduction pathways have been identified to be involved in the pathogenesis of ovarian cancer, and may also be important targets for future molecular therapy. The most extensively studied oncogenes include cellular *ras* proto-oncogenes. The three members of the *ras* gene family, H-*ras*, K-*ras*, and N-*ras*, are the most common oncogenes associated with human neoplasia. These genes encode proteins with molecular weights of 21,000 (p21), which are located to the inner plasma membrane and demonstrate GTPase activity. These genes play an important role in cell proliferation and differentiation. The commonest mechanism for the activation of the *ras* family genes are point mutation which abolish the GTPase activity of Ras protein and thus, remain constitutively activated. Studies of mutations in ovarian cancer tissues revealed that mutations occurred mostly within codon 12 and codon 13 of K-*ras*.

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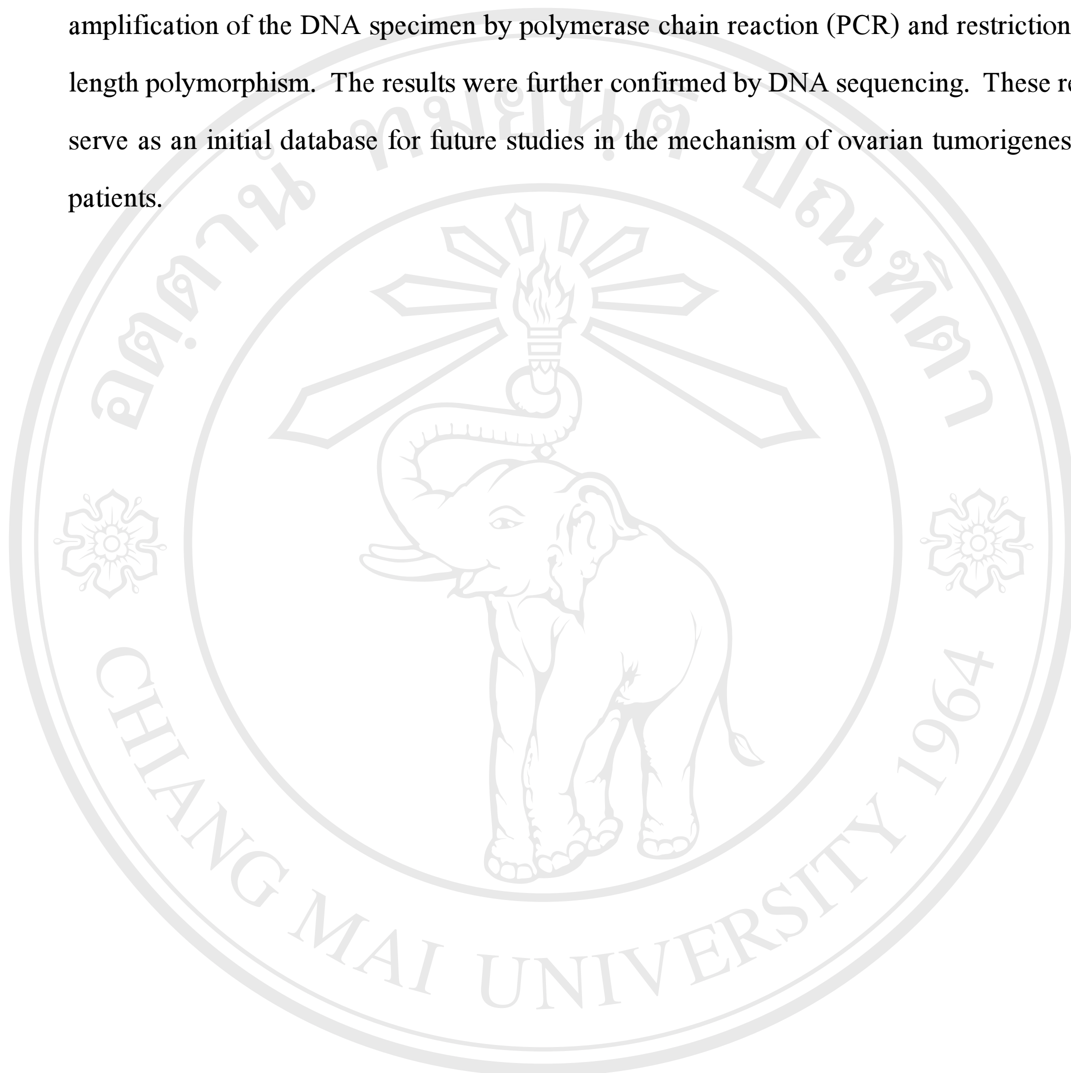
In this study, we investigated the prevalence of mutations at codon 12 and codon 13 of K-*ras* gene in ovarian cancer tissue specimens of Thai patients. The methods involved amplification of the DNA specimen by polymerase chain reaction (PCR) and restriction fragment length polymorphism. The results were further confirmed by DNA sequencing. These results will serve as an initial database for future studies in the mechanism of ovarian tumorigenesis in Thai patients.



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1.2 LITERATURE REVIEW

1.2.1 Ovarian cancer epidemiology and risk factor

Endocrine, environmental and genetic factors have been identified in epidemiologic studies to be important in ovarian cancer carcinogenesis. Ovarian cancer is a disease of the elderly population, and most patients are postmenopausal with a median age at diagnosis of approximately 60 years. Other factors associated with ovarian cancer risk include family history, multiparity, early menarche and late menopause. Multiparity, breast-feeding, prolonged use of oral contraceptives, and tubal ligation have all been shown to decrease the risk of ovarian cancer (Ozols, 1991).

The biologic events that lead to ovarian cancer remain unknown. Several factors such as hormonal, environmental, and genetic variables may play a role, although all women are at risk for developing this disease.

A risk factor is anything that increases an individual's likelihood of developing a disease. A number of specific risk factors are associated with epithelial ovarian cancer, although these factors are not linked with rare forms of ovarian cancer, such as germ cell tumors. Unfortunately, most ovarian cancers are not explained by any identifiable risk factors. There are several known risk factors for certain types of ovarian cancer.

A. Family history of ovarian cancer.

Ovarian cancer risk is higher among women whose close blood relatives (mother, sister, daughter) have this disease. The relatives can be from either the mother's or father's side of the family. There is a higher risk for ovarian cancer happened at an early age. These chances are linked to an increased risk of breast and colorectal cancer.

B. Age

The chances of developing ovarian cancer increase as a woman ages. The majority of ovarian cancers occur after menopause, which takes place in most women on or around age 51. Over 50% of all ovarian cancers occur in women over age 65.

C. Menstrual history, pregnancy history and infertility

There seems to be a relationship between the number of menstrual cycles that a woman has in her lifetime and her risk of ovarian cancer. That is, the risk of ovarian cancer is increased

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in women who began to menstruate before age 12 and/or experienced menopause after age 50. Also, never having completed a pregnancy (nulliparity) is a risk factor for the development of ovarian cancer, as is having a first child after age 30. In other words, women who have never been pregnant have a higher risk of ovarian cancer than those who have been pregnant. Multiple pregnancies have an increasingly protective effect. Similarly, women who take or have taken birth control pills have a 40% to 50% decreased risk of ovarian cancer. It is thought that the protective effects of pregnancy, birth control pills, and breast feeding are related to the suppression of ovulation that is, the fewer ovulatory cycles that a woman completes, the lower are her chances of developing ovarian cancer.

D. Fertility drugs

Women who have used ovulation-stimulating fertility medications have a slightly increased risk of ovarian cancer. The type of ovarian tumors most often associated with the use of fertility drugs are tumors of low malignant potential (LMP tumors).

E. High fat diet

Diets that are high in meat and animal fats have been linked to the development of ovarian cancer. Such diets are more common in industrialized countries, which have higher rates of ovarian cancer than undeveloped nations.

F. Talcum powder

Some research indicates that there is an increased risk of ovarian cancer among women who apply talcum powder to the genital area or sanitary napkins. Talcum has been implicated in ovarian cancer because it is an ingredient in many body powders and feminine hygiene products and, in the past, it was sometimes contaminated with asbestos, a known cancer-causing substance.

G. Acquired genetic mutations

Researchers have not yet been able to identify specific environmental factors that are responsible for the genetic mutations causing ovarian cancer. Such acquired genetic mutations, rather than inherited genetic mutations, make up the bulk of DNA defects that are associated with ovarian cancer. The identification of genetic changes may help to predict a woman's prognosis.

Ovarian cancers, like all cancers, may be caused by DNA mutations that alter oncogenes (genes that promote cancer cell division), tumor suppressor genes (cancer-preventing genes), or other genes.

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1.2.2 The human ovaries

The ovaries are a pair of female reproductive organs. They are located in shallow depressions, called ovarian fossae, one on each side of the uterus, in the lateral walls of the pelvic cavity. They are held loosely in place by peritoneal ligaments **(Figure 1)**. Each ovary is a solid, ovoid structure about the size and shape of an almond, about 3.5 cm in length, 2 cm wide, and 1 cm thick. The ovaries have two functions: they produce eggs and female hormones.

Each month, during the menstrual cycle, an egg is released from one ovary. The egg travels from the ovary through a fallopian tube to the uterus.

The ovaries are the main source of female hormones (estrogen and progesterone). These hormones control the development of female body characteristics, such as the breasts, body shape, and body hair. They also regulate the menstrual cycle and pregnancy.

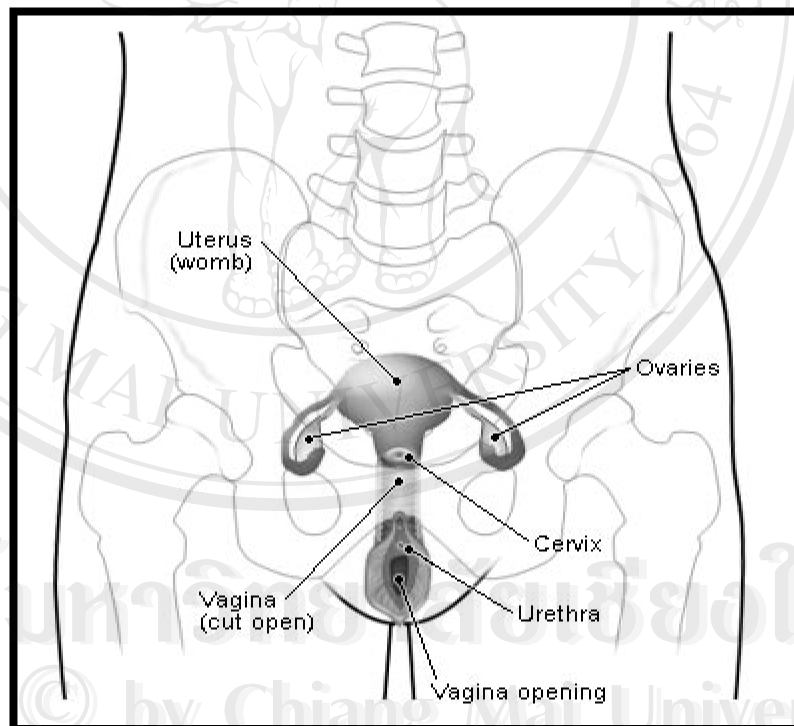

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Figure 1 The structure of female reproductive system. (http://www.medicinenet.com)

The ovaries are covered on the outside by a layer of simple cuboidal epithelium called germinal (ovarian) epithelium. This is actually the visceral peritoneum that envelops the ovaries.

Underneath this layer there is a dense connective tissue capsule, the tunica albuginea. The substance of the ovaries is distinctly divided into an outer cortex and an inner medulla. The cortex appears more dense and granular due to the presence of numerous ovarian follicles in various stages of development. Each of the follicles contains an oocyte, a female germ cell. The medulla is loose connective tissue with abundant blood vessels, lymphatic vessels, and nerve fibers.

1.2.3 Ovarian cancer classification

Cancer is a group of more than 100 different diseases. They all affect the body's basic unit, the cell. Cancer occurs when cells become abnormal and keep dividing and forming more cells without control or order. Ovarian cancer is cancer that begins in the cells that constitute the ovaries. Many types of tumors can start growing in the ovaries. Some are benign (noncancerous) and never spread beyond the ovary. Patients with these types of tumors can be treated successfully by surgically removing one ovary or the part of the ovary that contains the tumor. Other types of ovarian tumors are malignant and may spread to other parts of the body.

In general, ovarian tumors are named according to the kind of cells the tumor started from and whether the tumor is benign or malignant. There are 3 main types of ovarian tumors:

1. Epithelial ovarian cancer, which is the most common ovarian cancers, arises from cells lining or covering the ovaries.
2. Germ cells ovarian cancer. This type of ovarian cancer is uncommon. The germ cell cancers arise from the cells that are destined to form eggs within the ovaries.
3. Sex cord-stromal ovarian cancer. Cancers arise from the cells that hold the ovaries together and produce female hormones, estrogen and progesterone.

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Epithelial ovarian tumors

Epithelial ovarian tumors develop from the cells that cover the outer surface of the ovary. Most epithelial ovarian tumors are benign. There are several types of epithelial tumors, including serous adenomas, mucinous adenomas, endometrioid and Brenner tumors corresponding to different types of epithelia in the organs of the female reproductive tract. The epithelial tumors of each of categories are further subdivided into three subtypes: benign (adenoma), borderline (low malignant potential (LMP)) and malignant (carcinoma) to reflect their behavior

(Ishioka et al., 2003). Epithelial ovarian carcinomas account for 85 to 90% of all cancers of the ovaries (Berek, 2002).

Germ Cell Tumors

Ovarian germ cell tumors develop from the cells that produce the ova or eggs. Most germ cell tumors are benign, although some are malignant and may be life threatening. The most common germ cell malignancies are immature teratomas, dysgerminomas, and endodermal sinus tumors. Germ cell malignancies occur most often in teenagers and women in their twenties.

Sex cord-stromal Tumors

Sex cord-stromal tumors represent about 5% of all ovarian cancers. They are developing from cells of the sex cord or mesenchyme (connective tissue) that hold the ovary together and those that produce the female hormones, estrogen and progesterone. The most common types among this rare class of ovarian tumors are granulosa-theca tumors and Sertoli-Leydig cell tumors; other related tumors include lipid cell tumors and gynandroblastomas. These tumors are quite rare and are usually considered low-grade cancers, with approximately 70 percent presenting as stage I disease.

In addition to their classification by cell type, ovarian carcinomas are also further graded and staged. The grade is on a scale of 1, 2, or 3. Grade 1 ovarian carcinomas tend to have a better prognosis than Grade 3 ovarian carcinomas, which usually implies a worse outlook than Grade 1 ovarian carcinomas. The stage of the tumor can be ascertained after surgery, when it can be determined how far the tumor has spread from where it started in the ovary. Ovarian cancers are staged according to the FIGO system, a staging scheme developed by the International Federation of Gynecology and Obstetrics (Berek, 2002).

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1.2.4 Molecular genetic of ovarian cancer

Many studies have reported that cancer develops when the control system of cell growth and differentiation becomes imbalanced by environmental or genetic factors. Molecular genetic studies have identified several risk factors associated with the development of ovarian cancer.

Progression from normal tissue to invasive cancer takes place over a period of years and is driven by a series of accumulating genetic changes. There are three classes of genes commonly mutated in cancer: proto-oncogenes, tumor suppressor genes, and DNA repair genes. Proto-oncogenes are genes that encode proteins with important roles in cell growth and embryogenesis; however, when they are expressed in excessive amounts or in altered forms, they may induce uncontrolled cell proliferation. Tumor suppressor genes, formerly called anti-oncogenes, produce proteins involved in the negative control of cell growth. In contrast to oncogenes, both alleles of the tumor suppressor genes must be inactivated before the carcinogenic effect can take place. DNA repair genes are responsible for maintaining the integrity of the genome. DNA can be damaged by many factors. This damage is normally repaired quickly and efficiently. In some cancer cells, however, the DNA repair genes themselves are mutated, leading to a higher rate of mutagenesis. The increased rate at which oncogenes and tumor suppressor genes are mutated in these cells leads to increased tumorigenesis (Fujita et al., 2003).

It is thought that in the ovary, as in other tumors, the development of ovarian cancer is a multistep process that involves alterations in many oncogenes and tumor suppressor genes that have been thought to be involved in the uncontrolled proliferation of cells and progression of ovarian cancers.

Proto-oncogenes encode proteins that participate in growth stimulatory pathways in normal cells. Proto-oncogenes associated with signal transduction pathways have been identified to be involved in the pathogenesis of ovarian cancer, and may also be important targets for future molecular therapy. Activation of proto-oncogenes occurs by amplification, mutation, chromosomal translocation and/or rearrangement, or viral insertion promotes transformation. After activation of proto-oncogene has occurred, overexpression of their mRNA and oncoproteins induces various abnormal genetic cell changes, such as malignant transformation. In particular, activation of a human *ras* gene seems to be common in a variety of human tumor cell types.

In ovarian cancer, investigation of the biochemical genetic mechanism of tumor suppressor genes has been studied in details but studies on carcinogenesis by proto-oncogenes have, until recently, been few. Furthermore, little is known about the relationship between the clinicopathologic characteristics of ovarian cancer and the genetic changes in K-*ras* oncogene.

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1.2.5 The *ras* oncogenes

The members of the *ras* family proto-oncogenes are H-*ras*, K-*ras*, and N-*ra*s. They encode proteins with molecular weights of 21 kDa (p21), which are located on the inner surface of the plasma membrane and demonstrate GTPase activity. These genes play an important role in cell proliferation and differentiation. The normal function of *ras* is to interact with tyrosine kinase receptors and other proteins to activate cytoplasmic signal transduction pathways (Matias-Guiu and Prat, 1998).

Ras proteins are now well recognized for their essential function in transducing extracellular signals that regulate cell growth, survival, and differentiation. Although *ras* genes were originally identified in the mid-1960s as the transforming elements of the Harvey and Kirsten strains of rat sarcoma viruses, investigation of the biological properties of their protein products did not initiate until the early 1980s, when mutated alleles of cellular *ras* genes were identified as dominant proto-oncogenes in various types of human tumors. The mammalian *ras* gene family consists of three members: Harvey (H)-*ras*, Kirsten (K)-*ras* and Neuroblastoma (N)-*ras*, which are located on different chromosomes. The H-*ras* and K-*ras* genes are the cellular counterparts of the viral Harvey and Kirsten genes, respectively, and the N-*ras* gene is derived from a human neuroblastoma cell line.

H-*ras*, K-*ras*, and N-*ras* genes have been localized to chromosomes 11, 12 and 1, respectively, in humans. All three *ras* genes have a common structure with a 5' non-coding exon (exon I) and four coding exons (exons I–IV) while the introns of the genes differ widely in size and sequence. The K-*ras* gene has two alternative IV coding exons, thus encoding two proteins, K-*rasA* and K-*rasB*, with K-*rasB* being more abundant.

Various reports have been published concerning transcriptional regulation of the H-, K- and N-*ras* genes. The information demonstrates significant variations in the levels of expression of the three *ras* genes between tissues as well as during development. For example, in mice, the level of H-*ras* transcripts is highest in brain, muscle, and skin and is lowest in liver, K-*ras* transcripts are most abundant in gut, lung, and thymus and are rare in skin and skeletal muscle, and N-*ras* transcripts are most prevalent in testis and thymus. Differential expression of the three *ras* genes has also been observed during mouse prenatal development, with N-*ras* expression being

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highest at day 10 of gestation and K-*ras* expression being lowest toward the end of gestation. The simplest interpretation of these findings is that the three forms of Ras perform distinct cellular functions, with each function being biologically relevant in a particular tissue or cell type. Another line of investigation often considered as providing evidence in support of the differential function of the three *ras* genes concerns the analysis of *ras* mutation in human tumors. It is well documented that oncogenic forms of H-, K-, and N-*ras* are preferentially detected in certain tumor types. For example, more than 80% of pancreatic adenocarcinomas had a mutated K-*ras* gene, whereas in myeloid leukemia, the N-*ras* gene is most frequently mutated. However, in many tumor types, there is no absolute specificity for a mutated *ras* gene, and mutated forms of the three *ras* genes produce the same phenotype in in vitro transformation assays. Thus, differences in the function of Ras proteins, if they exist, cannot be the sole explanation for the bias in favor of particular *ras* mutations in certain tumor types. Taken together, while the conclusions drawn from gene expression studies have not answered definitively the question of functional divergence between *ras* genes, they certainly provide an interesting template for future work in this research area (Bar-Sagi, 2001).

The three human *ras* genes encode four highly related GTPases of 188 (K-*ras*B) or 189 (H-*ras*, K-*ras*A, and N-*ras*) amino acids in length. The A and B forms of K-*ras* are generated by an alternative splicing of the fourth exon of this gene, and the abundance of K-*ras*B transcripts is higher in comparison to that of K-*ras*A transcripts. All of the critical domains for GTPase function (including sequence motifs important for nucleotide binding and GTP hydrolysis) are present within the N-terminal 165 amino acids of Ras proteins. Based on primary sequence comparisons, Ras proteins can be viewed as consisting of three contiguous regions **(Figure 2)**. The first region encompasses the N-terminal 86 amino acids, which are 100% identical among the different Ras proteins. Within this region lies the Ras effector binding domain (amino acids 32 to 40), which is the critical interaction site with all known downstream targets of Ras. The next 80 amino acids define a second region where mammalian Ras proteins diverge only slightly from each other, exhibiting an 85% homology between any protein pair. The remaining C-terminal sequence, known as the hypervariable region, starts at amino acid 165 and shows no sequence similarity among Ras proteins except for a conserved CAAX motif (C, cysteine; A, aliphatic amino acid; X, methionine or serine) at the very C-terminal end, which is present in all Ras

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proteins and directs posttranslational processing. Thus, if a unique role for each Ras protein is to be determined by sequence divergence, the hypervariable region would most likely specify it (Ellis and Clark, 2000).

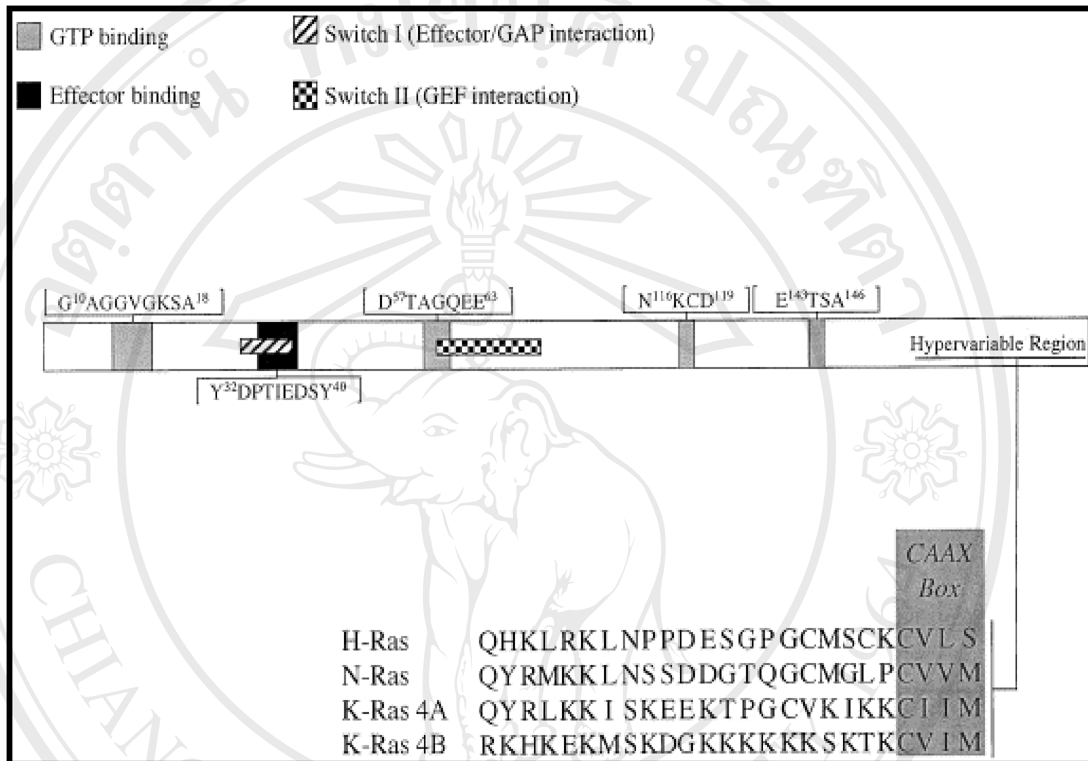

Figure 2 The structural motifs in the Ras protein sequence offer insight into its biological functions. Four epitopes are largely responsible for guanine nucleotide binding (Grey boxes): $\alpha-$ and $\beta-$ phosphate binding (residues 10–18), $\gamma-$phosphate binding (residues 57–63), and guanine ring binding (residues 116–119 and 143–146). Protein loops, switch I (Striped Box) and II (Checked Box), in the 3-dimensional structure Ras have different conformations in the GTP and GDP states. The sequence of Ras homologues diverges at the carboxyl terminal, Hypervariable Region. Within the Hypervariable Region, a CAAX Box exists that targets Ras proteins for post-translational lipid modification. (Ellis and Clark, 2000)

The polypeptide chain of Ras protein consists of six β-strands and five α-helices as shown in **Figure 3**. The structure of Ras-GTP and Ras-GDP complexes revealed that switching between the active and the inactive state is associated with a conformational change of two

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regions, designated as switch I (residues 30-38), overlapping with the core effector binding, and switch II.

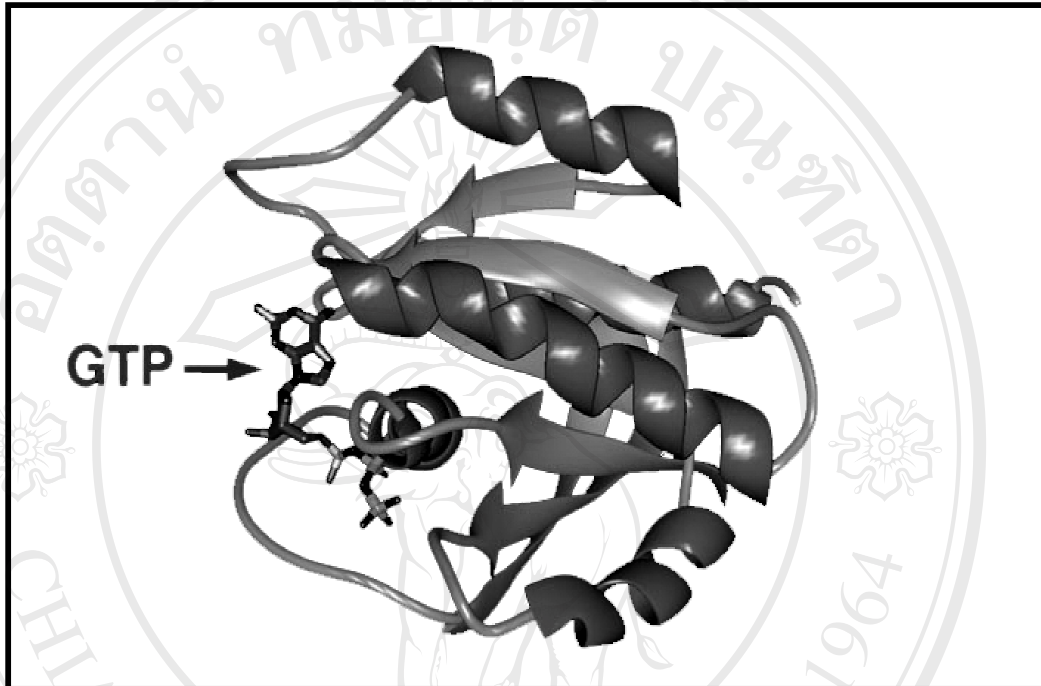

Figure 3 The structure of Ras protein. The polypeptide chain of Ras consists of six β-strands and five α-helices. (http://www.bmb.psu.edu/faculty/tan/lab/gallery/ras_ribbon1a.jpg)

1.2.6 The Ras signaling and the carcinogenesis process

Ras proteins are positioned on the inner face of the plasma membrane. Ras functions as a membrane-associated biologic switch that relays signals from ligand-stimulated receptors to cytoplasmatic signal cascades. Ras proteins control signal transduction pathways downstream form transiently activated these receptors include G-protein-coupled serpentine receptors, tyrosine kinase receptors (eg, platelet-derived growth factor receptor [PDGFR], epidermal growth factor receptor [EGFR]) and cytokine receptors that cause stimulation of associated nonreceptor tyrosine kinases (NRTKs). The Ras proteins function as guanosine triphosphate (GTP)/ guanosine diphosphate (GDP)-regulated switches that cycle between an active GTP-bound state and

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an inactive GDP-bound state. When bound to GDP, Ras is incapable of activating signal transduction pathways. In the GTP-bound state, however, Ras is fully competent to induce the activity of downstream effectors targets. This process is regulated by guanine nucleotide exchange factors (GEFs), which promote the formation of the active GTP-bound form of Ras, and GTPase-activating proteins (GAPs), which increase the rate of GTP hydrolysis, resulting in the formation of the inactive GDP-bound form of Ras, as shown in **Figure 4** (Ellis and Clark, 2000).

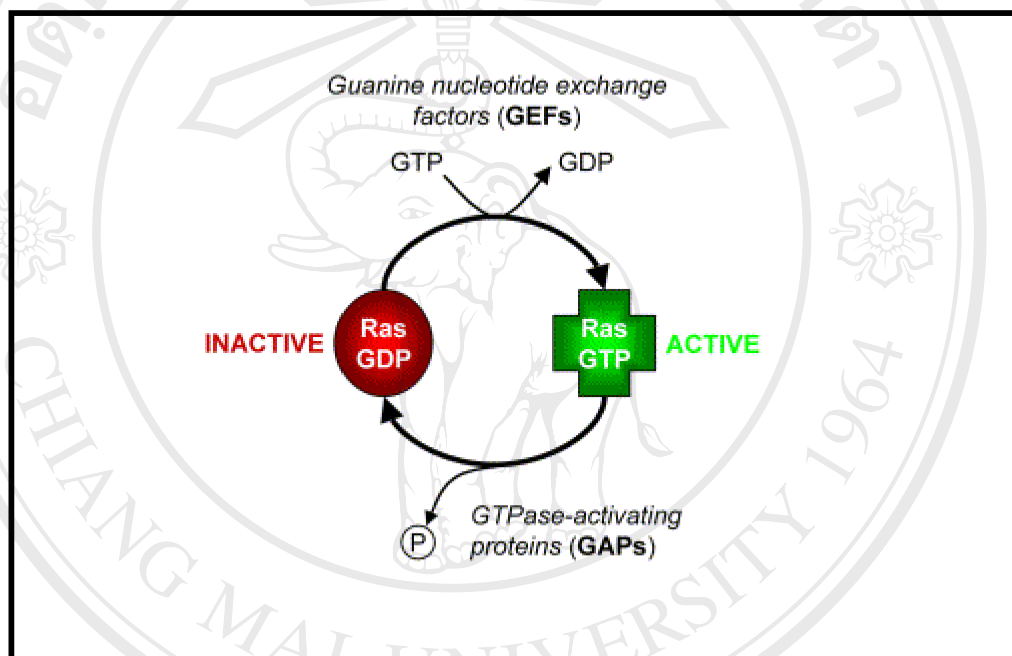

Figure 4 The Ras GTPase cycle. The Ras activation state depends on the action of GEFs and GAPs. Oncogenic Ras is locked in the GTP-bound state and is resistant to the action of GAPs. Many Ras effectors such as Raf, RalGDS and phosphoinositide 3-kinase bind to the active GTP-bound conformation to signal downstream.

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In quiescent cells, Ras exists in an inactive GDP-bound form at the plasma membrane. Upon stimulation of diverse cell surface receptors by extracellular signals such as growth factors and cytokines, Ras transiently converts to an active GTP-bound state. The best characterized pathways that involve Ras activation are stimulation of receptor tyrosine kinases, such as the

epidermal growth factor receptor (EGFR). Binding of EGF ligand to the receptor leads to the cytoplasmic tyrosine kinase domains become active, and this leads to the autophosphorylation of critical tyrosine residues. The phosphorylated tyrosine residues provide docking sites for the Src-homology-2 (SH2) domains of several proteins, including Grb2. After binding these sites, the Grb2 Src-homology-3 (SH3) domain associates with a proline stretch in Sos. This serves to localize Sos to the plasma membrane where Ras resides. Grb2 can also bind to tyrosine phosphorylated Shc proteins that then interact with the receptors through Shc SH2 domains. At the plasma membrane, Sos can release GDP from Ras, which allows the formation of active Ras-GTP complexes. (SH2 and SH3 refer to two highly conserved, noncatalytic domains often found in signaling molecules. SH2 domains function to bind phosphorylated tyrosine residues in a sequence-specific manner. This interaction is important in the assembly of signal transduction complexes. SH3 domains recognize proline-rich sequences, thereby signaling downstream targets. Molecules [such as Grb2] that lack a catalytic domain but contain SH domains are often referred to as "adaptors." Adaptors containing SH2 and SH3 domains are therefore important in the assembly of signal transduction complexes and in promoting downstream signaling).

In its GTP-bound state, Ras activates complex signal transduction cascades that are known to include Raf. Raf phosphorylates and thereby activates the kinase MEK, which in turn activates ERK1/2 members of the MAP-kinase family. ERK then translocates to the nucleus where it regulates the activity of downstream nuclear transcription factors and proto-oncogenes such as c-Jun, and c-Myc, which in turn regulate expression of diverse proteins and play a critical role in cell cycle and cellular proliferation. A second pathway that can be activated by Ras is the PI3K pathway, of which protein kinase B (PKB) is the main down-stream target. PKB plays a critical role in cell survival. Taken together, Ras can be viewed as a central switch in cell proliferation and cell survival. Deregulation of this switch results in growth signal autonomy, a crucial step in the process of cell transformation **(Figure 5).**

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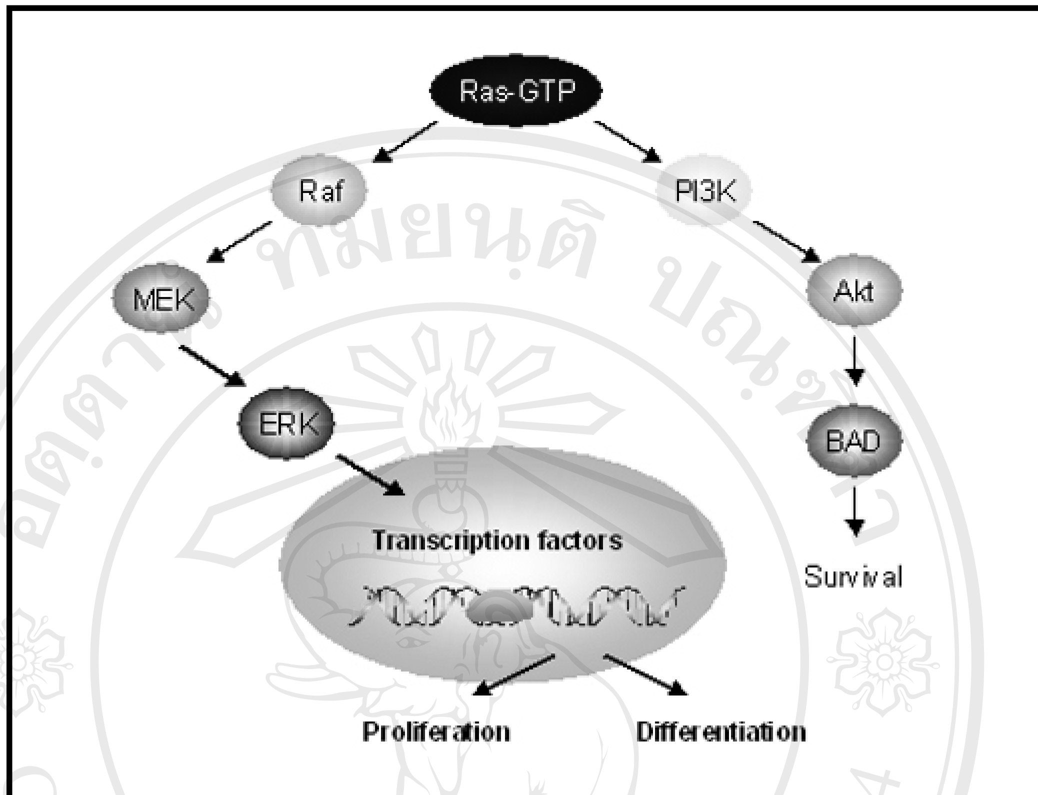

Figure 5 Schematic representation of the main Ras effector pathways.

In humans, about 30% of the tumors carry Ras mutations (Bos, 1989; Hanahan and Weinberg, 2000), of which K-*ras* is the most frequently mutated member. It has been well established that point mutations in members of the *ras* gene family can convert themselves into active oncogenes. Activation of *ras* oncogenes by point mutations has been suggested to play an important role in the multistep process of carcinogenesis. The most common *ras* mutations in tumors occur at sites critical for *ras* regulation. The most frequent *ras* alterations in human cancer are single point mutations at codons 12, 13, and 61, which completely abrogate the GAP-induced GTP hydrolysis of Ras, rendering them constitutively activated. These genes encode Mutant p21 produced by specific amino acid substitutions at this codons results in a loss of GTPase activity and activation of gene product, leading to increased cell proliferation and transforming cells into a malignant phenotype (Bos, 1989).

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Unlike normal Ras, oncogenic Ras remains constitutively in the active GTP-bound form. Thus, the transforming properties of oncogenic Ras are based on continuous activation of its down-stream effectors **(Figure 6)**.

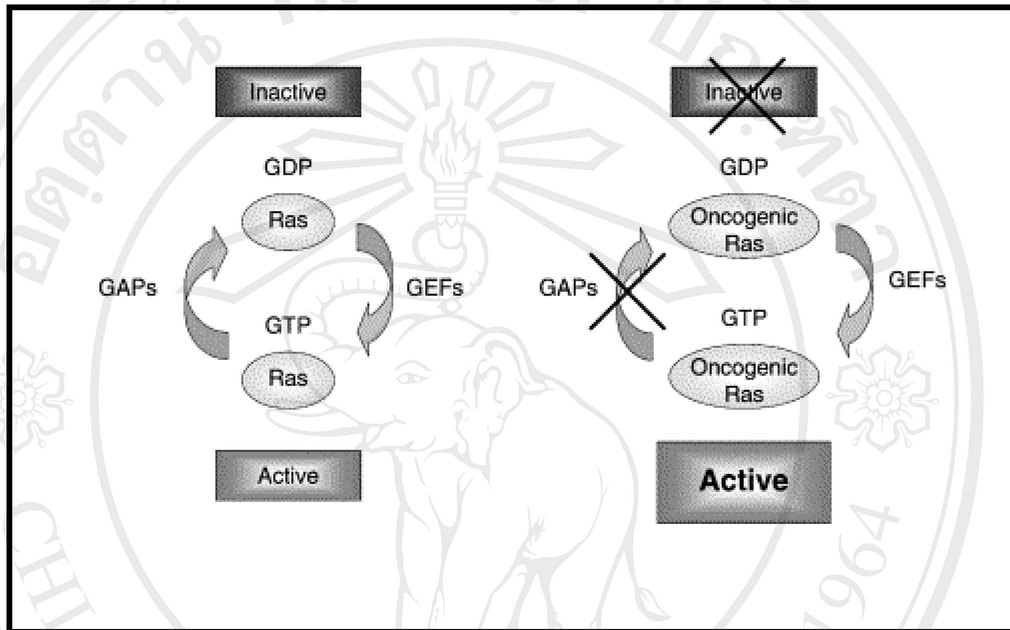

Figure 6 Regulation of Ras. Ras proteins are GTPases that cycle between an activated GTP-bound and an inactivated GDP-bound form. Guanine nucleotide exchange factors (GEFs) induce dissociation of GDP, which allows binding of GTP. GTPase-activating proteins trigger the hydrolysis of bound GTP. Oncogenic Ras remains in the active GTP-bound form, because the GAP-induced GTP hydrolysis is completely abrogated.

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1.2.7 K-*ras* mutation and ovarian cancer

K-*ras* gene is the locus for the c-k-ras protooncogene, on chromosome 12, at 12p12.1, and is about 46,530 bp in length. It is also known as K-ras, ras2, Ki-ras, c-k-ras, K-ras2A, K-ras2B, K-ras4A, and K-ras4B. The K-*ras* gene was initially identified because it is the cellular proto-oncogenes from which the acutely transforming Kirsten murine sarcoma viruses derived their viral *ras* oncogenes. K-*ras* gene encodes for a 2.0 kb transcript which is highly conserved across species, and is translated into the p21-Ras protein. In humans, the most frequently mutations have been found at K-*ras* codons 12 and 13, and to a lesser extent, at codon 61. These mutations are single base pair substitutions, which lead to the change of one amino acid in the protein. Single point mutations at codons 12, 13, and 61 completely abrogate the GAP-induced GTP hydrolysis of Ras protein. Unlike normal Ras, oncogenic Ras remains constitutively in the active GTP-bound form. Thus, the transforming properties of oncogenic Ras are based on continuous activation of its downstream effectors, which may constitutive promote cell proliferation. The reason for the specificity of these mutations is not entirely clear. This location of mutation appears to confer higher change in the Ras protein three dimensional structure and Ras-GAP binding characteristics (Ellis and Clark, 2000).

In Ovarian cancer, K-*ras* mutations usually arise at codons 12 and 13, the hot spot of the gene itself (Cuatrecasas et al., 1998; Cuatrecasas et al., 1997), although mutations at codon 61 may also occur with substitution of the correspondent amino acid in the Ras protein. Normally, the mutations of K-*ras* gene in ovarian cancers were composed of several types of point mutations in the first and second positions of codon 12 and in the second position of codon 13 (Enomoto et al., 1991b; Mandai et al., 1998; Mok et al., 1993). There is only one case that a point mutations occurred on codon 61 (Teneriello et al., 1993).

K-*ras* mutations are more common in mucinous than in nonmucinous epithelial tumors. Enamoto et al. first detected K-*ras* mutations in 6 out of 8 (75%) mucinous tumors in comparison with 3 out of 22 (14%) nonmucinous neoplasms, and the mutations were always detected at codon 12 (Enomoto et al., 1991b). It has since been confirmed that K-*ras* mutations are characteristic of ovarian mucinous tumors and distinguish them from other histological types; the mutations may be detected in benign, borderline and malignant tumors with increasing frequencies (Cuatrecasas et al., 1997).

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The existing literature on this topic suggests that K-*ras* mutations are prevalent in ovarian cancers of mucinous histology but not in tumors of nonmucinous (Enomoto et al., 1991a; Ichikawa et al., 1994; Teneriello et al., 1993). K-*ras* mutations are also common in benign ovarian tumors of mucinous histology, and in borderline (low malignant potential) ovarian tumors of serous and mucinous histologies.

1.2.8 The principles of polymerase chain reaction

The polymerase chain reaction (PCR) is an *in vitro* enzymatic amplification of specific DNA sequences. PCR is a method used to generate billions of copies of genomic DNA within a very short time. Since Kerry Mullis at the Cetus Corporation introduced it in 1983, the technique has been modified for many uses and has essentially revolutionized molecular biology. The PCR is an important tool for many applications in almost all aspects of biomedicine. For example, it can be used to amplify a sample of DNA when there isn't enough to analyze, as a method of identifying a gene of interest, or to test for disease. PCR has become an important tool for medical diagnosis. This technique is used in genetic testing, to determine whether patients carry a genetic mutation that could be passed on to their children (e.g. the mutation that causes thalassemia) or to determine disease risk in patients themselves (e.g. a mutation in the proto oncogenes or tumor suppressor gene predisposes a man to cancer). PCR is used to amplify the gene, which is then sequenced to look for mutations.

In this technique, a DNA sample is incubated with DNA polymerase, a large quantity of the four nucleotides (A, T, G and C) and two oligonucleotide primer whose sequences flank the DNA segment of interest so that they provide a starting point for the extension of the DNA by a DNA polymerase to synthesize new complementary strands. Amplification is carried out in cycles. First, the double strands of the duplex DNA sample are separated by heat denaturation. The sample is cooled down, allowing the primers to anneal to their complementary segment on the DNA. Then, the sample is incubated at indicated temperature so that the DNA polymerase can extend the primers, directs the synthesis of the complementary strands of DNA **(Figure 7)**. The use of heat stable DNA polymerase eliminates the need to add fresh enzyme after each heat denaturation step. The high temperature stable DNA polymerase is the *Taq* DNA polymerase, named for the bacterium *Thermus aquaticus*, from which it was isolated. Hence, in the presence

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of sufficient quantities of primers and dNTPs, the PCR is carried out simple by cyclically varying the temperature. This cycle can be repeated 30 or more times. The double stranded products of the previous cycles become new templates for the next cycle such that in each round the amount of the specific target DNA flanked by the primes essentially double. In this way, one double strand of DNA becomes 2, 2 become 4, 4 become 8 and so on **(Figure 8)**. So, after 30 cycle of PCR amplification, the amount of the target DNA sequence increase up to a million fold with high specific. Taking into account the time it takes to change the temperature of the reaction, 1 million copies can be made in a short time.

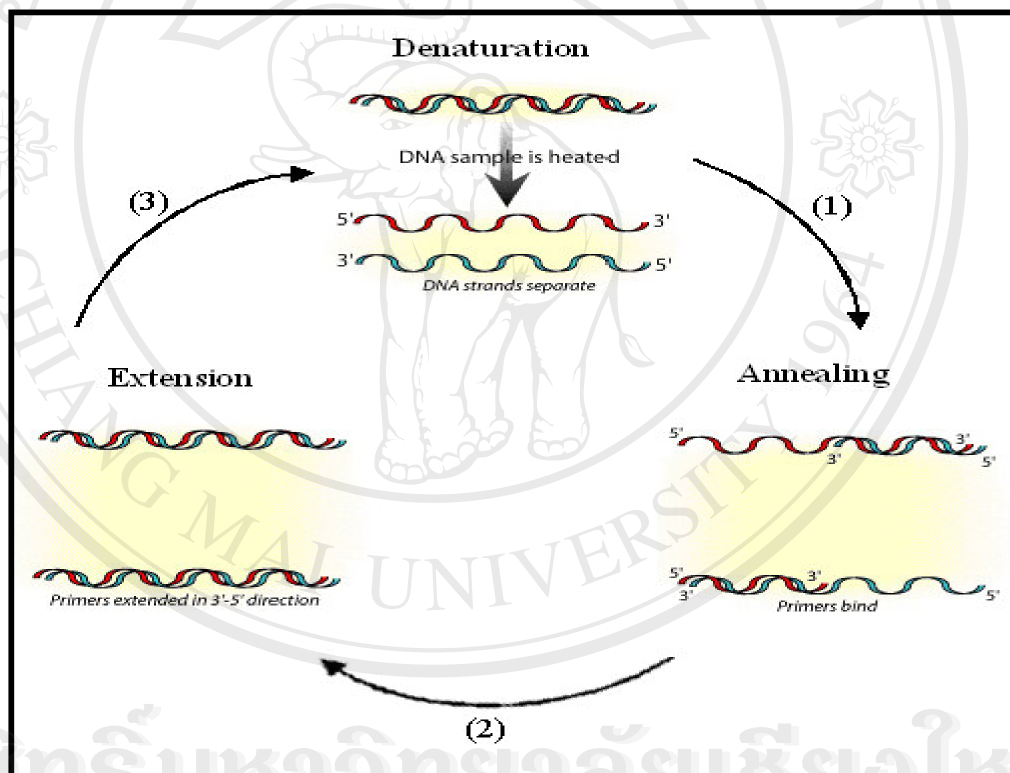

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Figure 7 The three steps in polymerase chain reaction.

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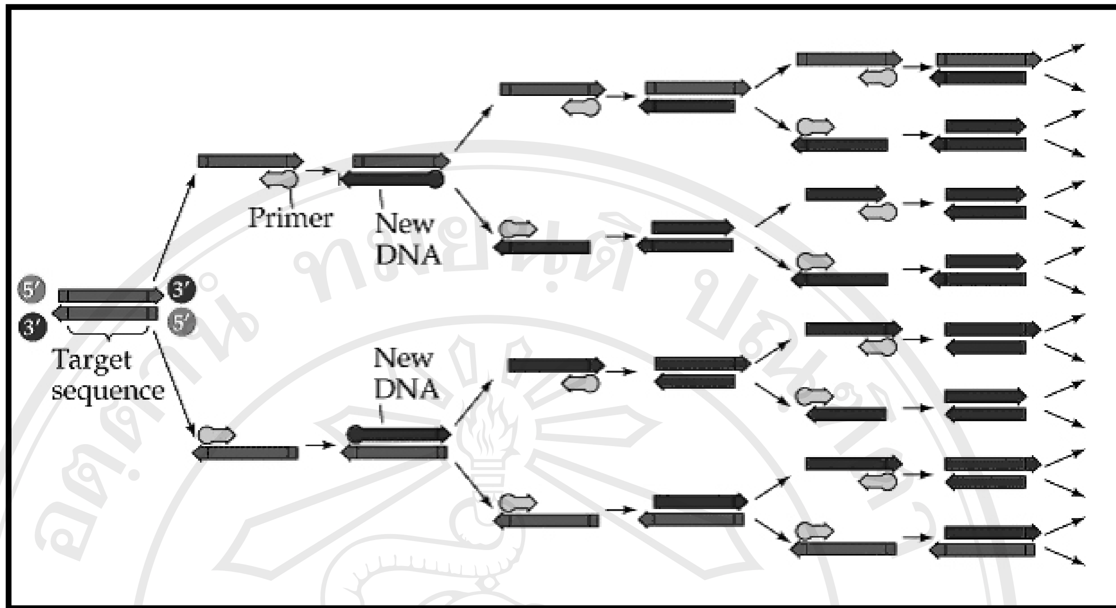

Figure 8 DNA amplification by polymerase chain reaction. PCR is used to amplify the amount of a particular DNA molecule in a sample. Primers complementary to particular regions of the DNA of interest are added to a sample along with the enzyme DNA polymerase. A complementary strand of DNA is built using the primer as the initial building block. The process can then be repeated for the resulting complementary strand resulting in the production of a copy of the original DNA present in the sample. With many cycles like this, DNA amplification can take place.

1.2.9 The standard PCR reaction

The standard PCR is typically done in a 50-100 µl volume and, in addition to the sample DNA, contains 50 mM KCl, 10 mM Tris-HCl (pH 8.4), 1.5 mM $MgCl_2$, 100 µg/ml gelatin, 0.25 µM of each primer, 200 pmol of each deoxynucleotide triphosphate, and 2.5 units of *Taq* DNA polymerase.

The amplification can be conveniently perform in a DNA Thermal Cycler using the " Step Cycle" program set at denature at 95°C for 1 minute, anneal at the proper temperature for a total of 30 cycles. These conditions can be use to amplify a wide range of target sequence with excellent specificity. However, some PCR amplification may need the optimization for the best result.

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1.2.10 The optimization of PCR

As originally developed, the PCR process amplifies short (approximately 100–500 base pairs) segments of a longer DNA molecule (Saiki et al., 1985). A typical amplification reaction includes the target DNA, a thermostable DNA polymerase, two oligonucleotide primers, deoxynucleotide triphosphates (dNTPs), reaction buffer, magnesium and optional additives. The components of the reaction are mixed and placed in a thermal cycler, an automated instrument that 'cycles' the reaction through a predetermined series of specific temperatures and times. One cycle of amplification is defined by the series of temperature and time adjustments.

Each cycle of PCR after the first cycle theoretically doubles the amount of targeted template DNA sequence (amplimer). Therefore, ten cycles theoretically multiplies the amplimer by a factor of about one thousand; 20 cycles, by a factor of more than one million. Each cycle of PCR amplification consists of a defined number of reaction steps. The steps are designed using temperature and duration time to denature the template, anneal the two oligonucleotide primers and extend the new complementary DNA strands by polymerization. These steps can be optimized for each template and primer pair combination. Heating to 95°C or higher for 15–120 seconds denatures the target DNA. During denaturation, the two intertwined strands of DNA separate from one another, producing the necessary single stranded DNA (ssDNA) template for primer annealing and polymerization (extension) by a thermostable polymerase. To anneal the oligonucleotide primers, the temperature of the next step in the cycle is reduced to approximately 40–60°C. At this temperature, the oligonucleotide primers can anneal to the ssDNA strands and serve as primers for DNA synthesis by the polymerase. This step requires approximately 30–60 seconds. Finally, to extend from the primer-bound template DNA, the reaction temperature is raised to the optimum for most thermostable DNA polymerases, which is approximately 72–74°C. Extension of the primer by the thermostable polymerase requires approximately 1 minute per kb to be amplified. Extension completes one cycle, and the next cycle begins by returning the reaction to 95°C for denaturation. These conditions can be use to amplify a wide range of target sequence with excellent specificity. However, some PCR amplification may need the optimization, which is described below, for the best result.

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A. Template considerations

Successful amplification of the region of interest is dependent upon the amount and quality of the template DNA. Reagents commonly used to purify nucleic acids (e.g., salts, guanidine, proteases, organic solvents and SDS) are potent inhibitors of DNA polymerases. A final ethanol precipitation of the nucleic acid sample will eliminate most of the inhibitory agents. Spiking a control DNA fragment and the appropriate primer pair into the DNA preparation may be useful in verifying the purity of the DNA sample.

B. Primer design

PCR primers are usually 15-30 nucleotides in length and are designed to flank the region of interest. Longer primers provide higher specificity. The GC content of primers should be 40–60% and care should be taken to avoid sequences that might produce internal secondary structure. The 3'-ends of the primers should not be complementary to avoid the production of primer-dimers. Primer-dimers unnecessarily delete primers from the reaction and result in an unwanted polymerase reaction that competes with the desired reaction. Avoid three G or C nucleotides in a row near the 3'-end of the primer as this may result in nonspecific primer annealing, increasing the synthesis of undesirable reaction products. Ideally, both primers should have nearly identical melting temperatures (Tm); in this manner, the two primers should anneal roughly at the same temperature. In any case, the annealing temperature of the reaction is dependent upon the primer with the lowest Tm. Regardless of primer choice, the final concentration of the primer in the reaction must be optimized.

C. Magnesium concentration

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Magnesium concentration is a crucial factor affecting the performance of *Taq* DNA Polymerase. Since Mg^{2+} ions form complexes with dNTPs, primers and DNA templates, the optimal concentration of $MgCl_2$ has to be selected for each experiment. Reaction components, including template DNA, chelating agents present in the sample (e.g., EDTA or citrate), dNTPs and proteins, can affect the amount of free magnesium. In the absence of adequate free magnesium, *Taq* DNA Polymerase is inactive. Conversely, excess free Mg^{2+} ions reduces enzyme fidelity (Eckert and Kunkel, 1990) that result in a low yield of PCR product, and too many

increase the yield of nonspecific products (Ellsworth et al., 1993; Williams, 1989) and promote misincorporation. Lower Mg^{2+} concentrations are desirable when fidelity of DNA synthesis is critical. For these reasons, it is important to empirically determine the optimal $MgCl_2$ concentration for each reaction. The recommended range of $MgCl_2$ concentration is 1.5–3.0 mM, under the standard reaction conditions specified.

D. Enzyme concentration

Usually 1-2 units of *Taq* DNA Polymerase are used in 50µl of reaction mix. Higher *Taq* DNA Polymerase concentrations may cause synthesis of nonspecific products. However, if inhibitors are present in the reaction mix (e.g., if the template DNA used is not highly purified), higher amounts of *Taq* DNA Polymerase (2-3 units) may be necessary to obtain a better yield of amplification products. For most applications, enzyme will be in excess; the inclusion of more enzymes will not significantly increase product yield. Increased amounts of enzyme and excessively long extension times increase the likelihood of generating artifacts due to the intrinsic 5' to 3' exonuclease activity of *Taq* DNA Polymerase. Artifacts generally can be seen as smearing of bands in ethidium bromide stained agarose gels (Bell and DeMarini, 1991; Longley et al., 1990). The most frequent cause of excessive enzyme levels is pipetting error. Accurate dispensing of submicroliter (<1µl) volumes of enzyme solutions in 50% glycerol is nearly impossible. The use of reaction master mixes sufficient for the number of reactions being performed obviates this problem. A master mix increases the volumes of pipetted reagents and reduces pipetting errors.

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E. Deoxynucleotide triphosphates (dNTPs)

The concentration of each dNTP in the reaction mixture is usually 200 µM. It is very important to have equal concentrations of each dNTP (dATP, dCTP, dGTP, dTTP), as inaccuracy in the concentration of even a single dNTP dramatically increases the misincorporation level.

When maximum fidelity of the PCR process is crucial, the final dNTP concentration should be 50 µM to 250 µM, since the fidelity of DNA synthesis is maximal in this concentration range. In addition, the concentration of $MgCl_2$ should be selected empirically, starting from 1 mM

and increasing in 0.1 mM steps, until a sufficient yield of PCR product is obtained.

F. Denaturing of template

Denaturation is an initial heating to completely denature complex genome DNA. Then, primers can be annealing after cooling step. This step use high temperature to destroy hydrogen bond between leading and lagging strand of DNA. The denaturing step can be separated into 2 steps: initial denaturation and denaturation step. Initial denaturation is always run before the denaturation step of the first cycle. It's accomplished at 95°C for 5 minutes. Denaturation step during the PCR assay is usually accomplished at temperatures of 95°C for 30 seconds to 1 minute.

G. Primer annealing temperature

The length and base composition of the primers are a major consideration in determining the optimal temperature of the PCR amplification cycles. For primers with a high Tm, it may be advantageous to increase the annealing temperature. Higher temperatures minimize nonspecific primer annealing, increase the amount of specific product produced and reduce the amount of primer-dimer formation. Numerous formulas exist to determine the theoretical Tm of nucleic acids (Baldino et al., 1989; Rychlik et al., 1990), and these may serve as a starting point for annealing conditions. However, it is best to optimize the annealing conditions by performing the reaction at several temperatures starting approximately 5°C below the calculated Tm. The formula below can be used to estimate the melting temperature for any oligonucleotide:

The Wallace rule can be used to calculate the Tm for perfect duplexes 15-20 nucleotides in length in solvent of high ionic strength (e.g. 1 M NaCl);

$$\mathbf{Tm\ (in\ ^\circ C) = 2\ (A + T) + 4\ (G + C)}$$

where (A + T) = The sum of the A and T residues in the oligonucleotide

(G + C) = The sum of the A and T residues in the oligonucleotide

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H. Primer extension temperature

It's elongation step after primer annealing the complementary strand. The extension step is usually performed at 74°C that is the optimum temperature of the *Taq* DNA polymerase. The estimates of the extension time are approximately 1 minute for synthesis of PCR fragments up to 1 kb. When larger DNA fragments are amplified, the extending time is usually increased by 1 minute for each 1000 bp.

I. Number of PCR cycles

The number of PCR cycles depends on the amount of template DNA in the reaction mix and on the expected yield of the PCR product. For less than 10 copies of template DNA, 40 cycles should be performed. If the initial quantity of template DNA is higher, 25-35 cycles are usually sufficient. More cycles mean a greater yield of product. However, with increasing number of cycles the greater the probability of generating various artifacts also increase.

G. Final Extending Step

After the last cycle, the samples are usually incubated at 74°C for 5-15 minute to fill-in the protruding ends of newly synthesized PCR products. Also, during this step, the terminal transferase activity of *Taq* DNA Polymerase adds extra nucleotides to the 3'-ends of PCR products.

After 20–35 cycles, the amplified DNA fragments may then be characterized (e.g., for size or quantity) or it may be used in further experimental procedures (e.g., RFLP analysis or direct sequence).

Amplified DNA can then be isolated or separated on the basis of size by a process of electrophoresis, in which the fragments are drawn through a thin, flat gel by an electric potential that spans the length of the gel. The gel matrix impedes the larger DNA fragments to a greater degree than it does the smaller ones, and the fragments become distributed on the basis of size. At this point, DNA can be made visible by stained the gel with ethidium bromide, making it (the location of DNA on the gel) visualized when irradiated with ultraviolet light.

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1.2.11 The principles of restriction fragment length polymorphism

Approaches for DNA Typing are all based upon identification of polymorphic loci. Polymorphic loci are regions or points in the genetic structure that may vary from individual to individual. Polymorphism represented by the presence or absence of "restriction" sites, which are short sequences along the DNA that can be cut by commercially available "restriction enzymes". The length of the cut fragment depends on whether particular restriction sites are present or not (polymorphic). The presence and absence of fragments resulting from changes in recognition sites are used to identify species or populations.

The simplest form of mutation analysis using restriction enzymes involves the analysis of restriction fragment length polymorphisms (RFLPs). These RFLPs reflect the presence or absence of a particular restriction site within a PCR-amplified DNA sequence and are detected by digesting the PCR-amplified gene sequences with the relevant restriction enzymes. Differences in observed digestion patterns in tumors compared with the DNA from normal tissue indicate the presence of a mutation. In tumor tissue, restriction enzymes are most usefully employed to detect hotspot mutations when these hotspots fall within existing restriction sites. PCR products bearing mutations in existing restriction sites fail to be digested and are identified by gel electrophoresis (Jenkins et al., 2002).

Obviously, as restriction enzyme sites cover less than 50% of the genome, not all DNA sequences are available for study with such enzymes. However, given that over 200 restriction enzymes with different recognition sequences exist, many hotspot codons in genes implicated in tumor progression are available for study. Similar methods may be employed to study mutation in any of the several hundred cancer related genes. The sequencing of the mutated PCR products identified through RFLP analysis allows identification of the exact position and type of mutation induced.

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1.2.12 The principles of amplified created restriction site

To overcome the lack of restriction sites at interested codons of tumor related genes and to harness the ease of restriction enzyme mediated mutation analysis, methods have arisen to introduce restriction sites artificially where required. These facilitate mutation analysis with restriction enzymes at practically any sequence. **Figure 9** show the methodology employed in

the case of the K-*ras* gene. In this method, one PCR primer is designed to create an artificial enzyme *BstN*I restriction site in the hotspot codon 12 of the K-*ras* gene, by annealing a mismatched primer at codon 11. Normal K-*ras* DNA amplified with this primer will subsequently digest with the *BstN*I restriction enzyme owing to the creation of this artificial site. However, if mutations are induced at codon 12, this artificially created restriction site is lost and the PCR products fail to digest with enzyme *BstN*I. Undigested PCR products are subsequently sequenced to ascertain the mutations therein. Given that K-*ras* mutations causing tumor formation occur at a small number of hotspots (more than 80% occur at codon 12), screening for such mutations is feasible in tumor tissues. This artificial restriction site methodology can be extended to study just about any codon (of any gene) for the presence of known mutation hotspots by using mismatched primers and one of the commercially available restriction enzymes (Jenkins et al., 2002).

	9	10	11	12	13
Normal primer	GTT	GGA	GCT	GGT	GGC
Template strand	CAA	CCT	CGA	CCA	CCG
	9	10	11	12	13
Mismatch primer	GTT	GGA	<u>C</u>CT	GGT	GGC
Templated strand	CAA	CCT	CGA	CCA	CCG

Figure 9 Amplified created restriction site analysis at codon 12 of the K-*ras* gene. A mismatched primer introduces a G to C alteration in codon 11, producing a *BstN*I (CCTGG) site at codon 12. Normal DNA amplified by polymerase chain reaction (PCR) with this mismatched primer will digest with *BstN*I after PCR. This *BstN*I site is lost if mutations are present at codon 12 (Jenkins et al., 2002).

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1.2.13 The principles of DNA Sequencing

The process of determining the order of the nucleotide bases along a DNA strand is called sequencing. DNA sequencing enables us to perform a thorough analysis of DNA because it provides us with the most basic information of all the sequence of nucleotides. With this knowledge, for example, we can locate regulatory and gene sequences, make comparisons between homologous genes across species and identify mutations. Scientists recognized that this could potentially be a very powerful tool, and so there was competition to create a method that would sequence DNA. In 1977, twenty-four years after the discovery of the structure of DNA, an American scientist and an English scientist to do exactly this independently developed two methods. Two separate methods for sequencing DNA were developed: the English scientist, lead by Sanger, designed a procedure similar to the natural process of DNA replication “the chain termination method” and the Americans scientist, lead by Maxam and Gilbert, used a chemical cleavage protocol “the chemical degradation method”. Even though both scientists shared the 1980 Nobel Prize, Sanger’s method became the standard because of its practicality.

Sanger’s method, which is also referred to as dideoxy sequencing or chain termination, is based on the use of: dideoxy nucleotides (ddNTPs) in addition to the normal nucleotides (NTPs) found in DNA **(Figure 10)**. Dideoxynucleotides are essentially the same as nucleotides except they contain a hydrogen group on the 3’ carbon of the deoxyribose moiety instead of a hydroxyl group (OH). These modified nucleotides, when the ddNTPs molecules was incorporated normally into a growing DNA chain through their 5’ triphosphate groups, the absence of the hydrogen group on the 3’ carbon of the deoxyribose moiety prevent the formation of a phosphodiester bond with the next incoming deoxynucleotide triphosphates (dNTPs) **(Figure 11)**. When a small amount of a specific dideoxynucleotide triphosphates (ddNTP) is included along with the four dNTPs normally requires in the reaction mixture for DNA synthesis by DNA polymerase, the products are a series of chains that are specifically terminated at the dideoxy residue.

Chain termination sequencing involves the synthesis of new strands of DNA complementary to a single-stranded template, is first prepared as a single-stranded DNA. Next, a short oligonucleotide is annealed to the same position on each template strand. The oligonucleotide acts as a primer for the synthesis of a new DNA strand that will be complimentary

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to the template DNA. The template DNA is supplied with a mixture of all four deoxynucleotides (dGTP, dATP, dCTP, dTTP), four dideoxynucleotides (ddGTP, ddATP, ddCTP, and ddTTP)-each labeled with a different color fluorescent tag, a primer tagged with a mildly radioactive molecule or a light-emitting chemical and DNA polymerase. After the first deoxynucleotide is added to the growing complementary sequence, DNA polymerase inserts a dideoxynucleotide along the template and continues to add base after base. The chain elongation proceeds continues until a dideoxynucleotide is added, blocking further elongation. This is because dideoxynucleotides are missing a special group of molecules, called a 3'-hydroxyl group, needed to form a connection with the next nucleotide. Only a small amount of a dideoxynucleotide is added to each reaction, allowing different reactions to proceed for various lengths of time, until, by chance, DNA polymerase inserts a dideoxynucleotide, terminating the reaction. Therefore, the result is a new set of DNA chains all of different lengths. The fragments are then separated by size using gel electrophoresis. As each labeled DNA fragment passes a detector at the bottom of the gel, the color is recorded. (Speed et al., 1992).

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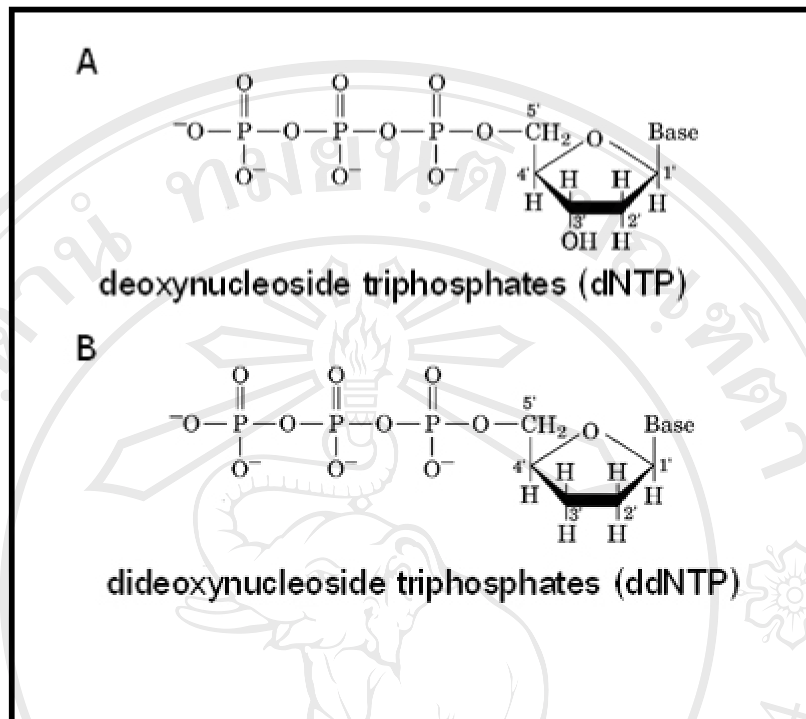

Figure 10 The structure of deoxynucleoside triphosphates (dNTP) and dideoxynucleoside triphosphates (ddNTP). **(A)** The structure of normal deoxynucleoside triphosphates (dNTP) with the on the 3’ carbon of the deoxyribose moiety. **(B)** The structure of dideoxynucleoside triphosphates (ddNTP), which lacks a 3’ hydrogen residue.

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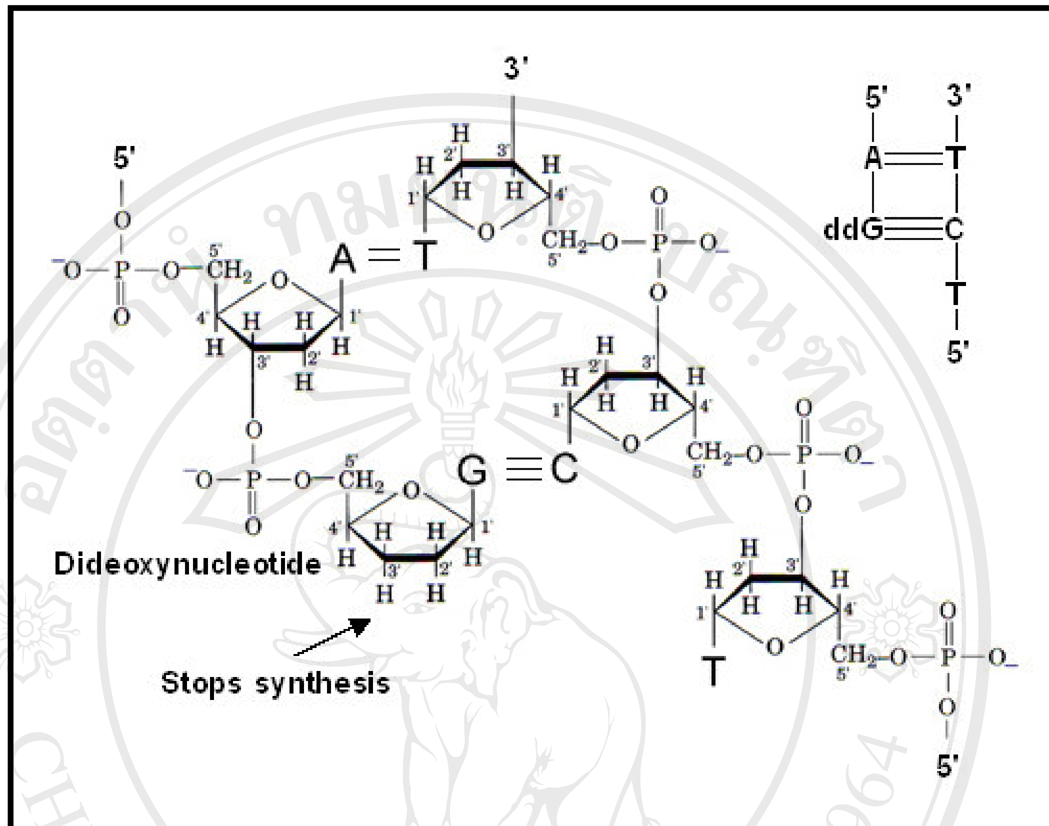

Figure 11 The mechanism of ddNTP terminate the chain elongation proceed. The ddNTP can be incorporated into the growing chain by DNA polymerase, where its acts as a terminator because it lacks the 3’ hydroxy group required for formation of further 5’ to 3’ phosphodiester bond.

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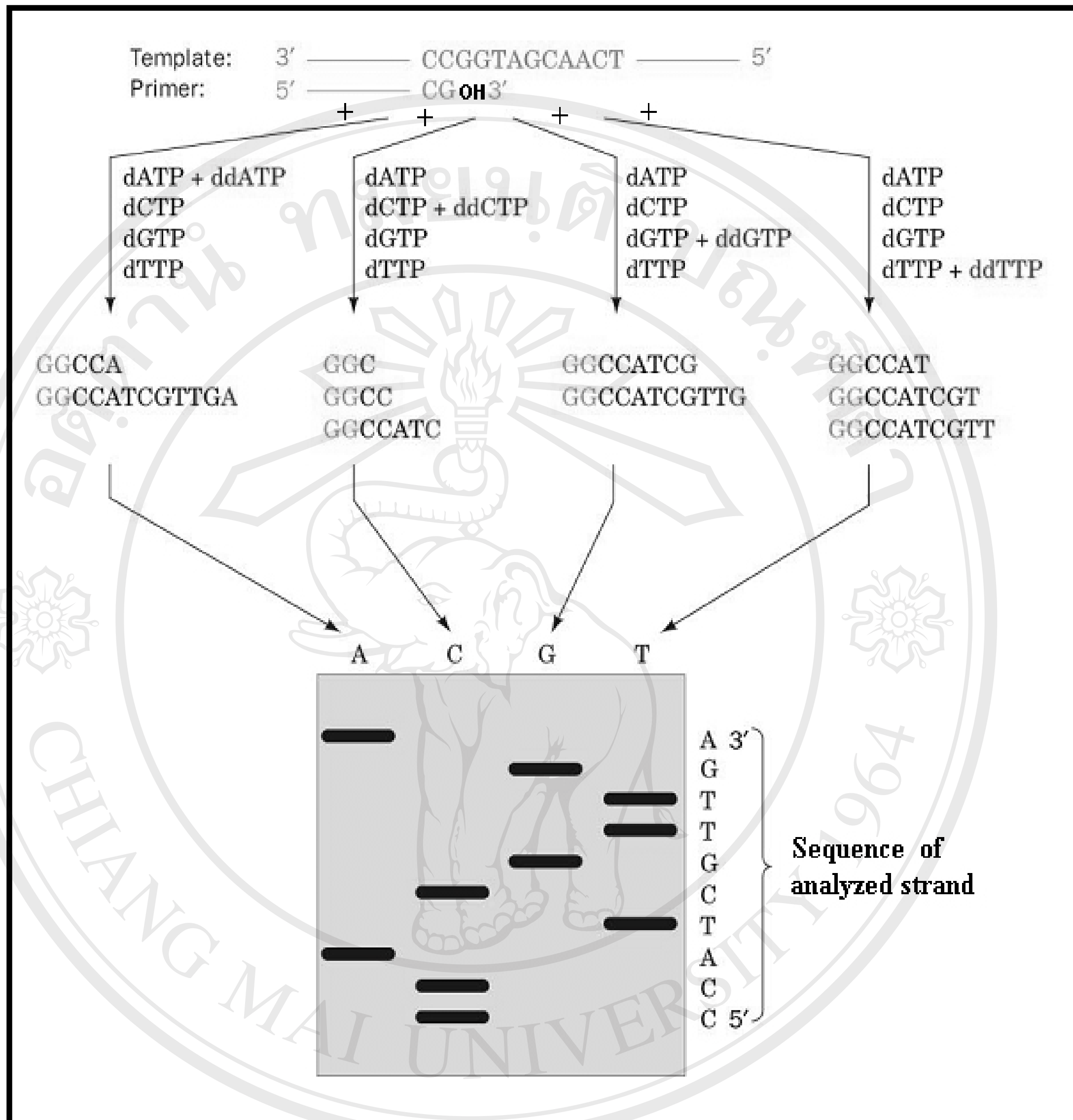

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Figure 12 DNA sequencing with dideoxynucleotides chain termination (Sanger method). The template DNA is supplied with a mixture of all four deoxynucleotides (dGTP, dATP, dCTP, dTTP), four dideoxynucleotides (ddGTP, ddATP, ddCTP, and ddTTP, a labeled primer and DNA polymerase. DNA synthesis occurs until terminated by the incorporation of ddNTP. The result is a new set of DNA chains all of different lengths. The fragments are then separated by size using gel electrophoresis. The DNA sequence is then reconstructed from the pattern of bands representing each nucleotide sequence.

1.2.15 The principles of automated DNA sequencing

Automated DNA sequencing runs on the same principle as the dideoxynucleotides chain termination (Sanger method). The method of dideoxy DNA sequencing has been improved by the use of fluorescent ddNTPs. The automated sequencing use a different colored fluorescent dyes label attached to each of the four dideoxy nucleotides (dGTP, dATP, dCTP, and dTTP), that become incorporated at the 3' end of the products of sequencing reaction, Each of the four ddNTPs is labeled with a different dye liked to the nitrogenous base via a linker. The attached dyes are energy transfer dyes and consist of a fluorescein energy donor dye linked to an energy acceptor dichlororhodamine dye. All four chain extension reactions can then be carried out with the same primer in one reaction and are separated in the same lane on the gel. These dye-labeled fragments are loaded onto the sequencers and during electrophoresis, migrate either through the polyacrylamide gel, which are separated based on their size, past a laser and detector system **(Figure 13)**. This laser excites the fluorescent dyes attached to the fragments and they then emit light at a wavelength specific for each dye. As each labeled DNA fragment passes a detector at the bottom of the gel, the color of fluorescence that indicates the ddNTPs incorporated into each fragment is recorded and the sequence is reconstructed from the pattern of colors representing each nucleotide in the sequence.

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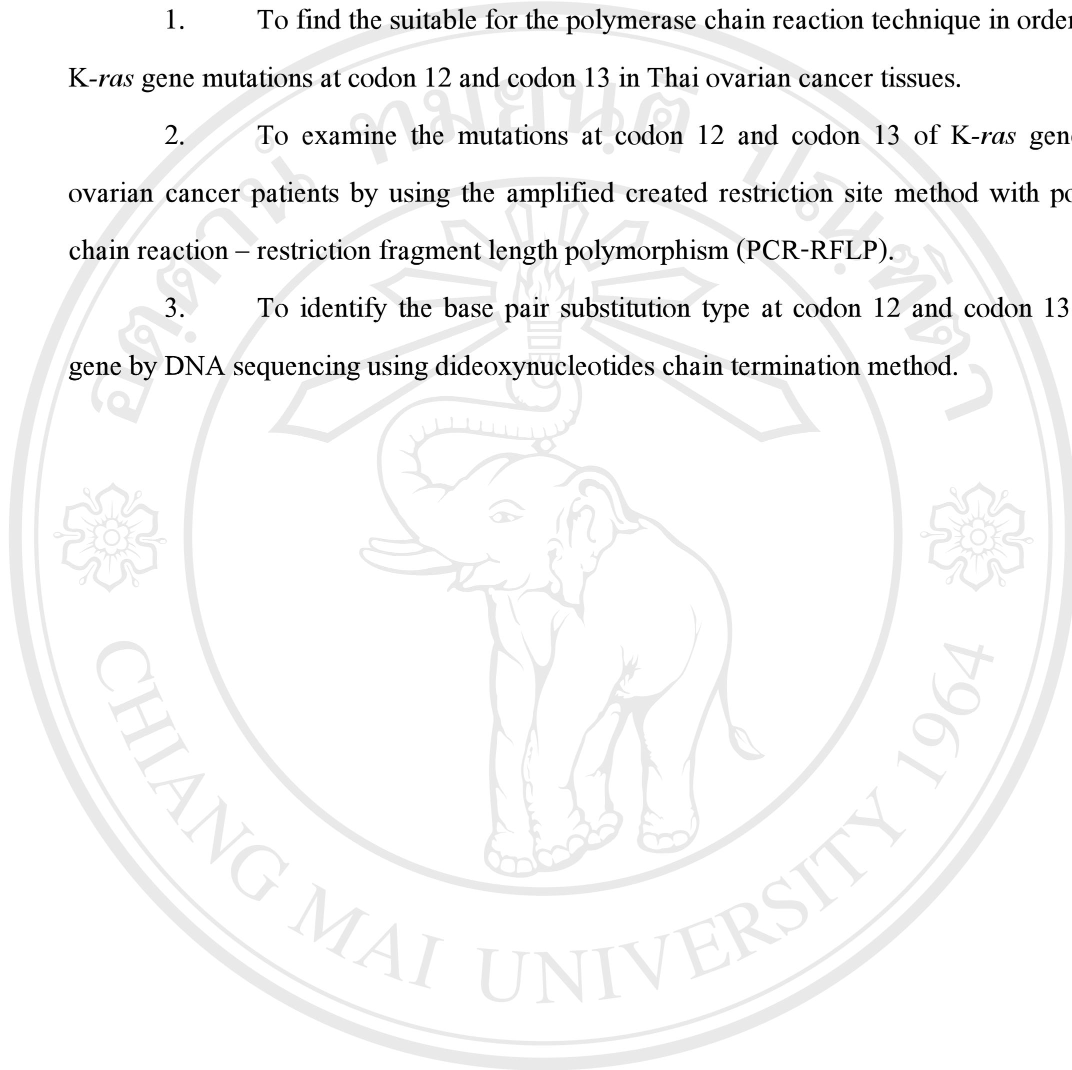
Figure 13 The schematic representation of the automated sequencing method.

1.3 OBJECTIVES

1. To find the suitable for the polymerase chain reaction technique in order to detect K-*ras* gene mutations at codon 12 and codon 13 in Thai ovarian cancer tissues.

2. To examine the mutations at codon 12 and codon 13 of K-*ras* gene in Thai ovarian cancer patients by using the amplified created restriction site method with polymerase chain reaction – restriction fragment length polymorphism (PCR-RFLP).

3. To identify the base pair substitution type at codon 12 and codon 13 of K-*ras* gene by DNA sequencing using dideoxynucleotides chain termination method.



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