CHAPTER I

INTRODUCTION

1.1 STATEMENT AND SIGNIFICANCE OF THE PROBLEM

Gastric cancer is one of the most common malignant diseases worldwide. Gastric cancer is a disease of complex etiology involving infectious, dietary, environmental, and genetic factors that are intimately interconnected .The second half of the 20th century has seen a sharp worldwide decline in both the incidence and mortality of gastric cancer. Despite this, the condition remains the world's second leading cause of cancer mortality behind lung cancer. It has been estimated that there will have been more than 870,000 deaths from the disease in the year 2000, accounting for approximately 12% of all cancer deaths (1-3).

IL-1β is a pro-inflammatory cytokine with multiple biological effects, is a potent inhibitor of gastric acid secretion, and has been shown to be associated with enhanced IL-1 β production in vitro (4, 5). In patients with severe gastritis of the corpus, acid secretion is decreased markedly and the pH of gastric juice is increased. This decrease in acid secretion delays the elimination of bacterial toxins and products of inflammation, such as reactive oxygan radicals and nitrogen oxide, thereby increasing the concentration of these well-known mutagens in the stomach. Decreased acid secretion may also result in a reduction of vitamin C level in gastric juice (6, 7). An increased rate of cell turnover in inflamed mucosa further increases the opportunity for DNA damage. Point mutation at the IL-1β gene promoter serves as a risk factor for peptic cancer by enhancing IL-1 β production (8).

Gastric cancer and adenoma occur with atrophic gastritis, in which the serum pepsinogen (PG) I level and the PGI/PGII ratio are reduced. Because pepsinogen I is produced and excreted directly by chief cell, but pepsinogen II is expressed in many organs widely such as neck cells in the gastric fundus, the Brunner's gland in the proximal duodenum and prostate gland that why pepsinogen II are mostly stable in serum (9).

Therefore, In this study the simple method of Polymerase Chain Reaction PCR and Restriction Fragment length Polymorphism (RFLP) techniques were applied to detect the mutation at the IL-1 β gene promoter which there have been report in Northern Thai population to comparison with plasma pepsinogen. This newly modified method is simple and suitable for population screening and routine diagnosis.

1.2 LITERATURE REVIEW

1.2.1 Gastric cancer epidemiology and risk factor

Although gastric cancer rates have been declining in recent decades, it remains one of the most serious health burdens throughout the world. Understanding its causation is important for the primary and secondary prevention of disease. The highest death rates for many decades were registered in Japan, followed by northern Europe and the Andean populations of Latin America.

The second half of the 20th century has seen a sharp worldwide decline in both the incidence and mortality of gastric cancer. Despite this, the condition remains the world's second leading cause of cancer mortality behind lung cancer. It has been estimated that there will have been more than 870,000 deaths from the disease in the year 2000, accounting for approximately 12% of all cancer deaths(1, 2).

There is marked geographic variation in the incidence of gastric cancer. International Agency for Research on Cancer data for 1996 (Table 1), demonstrate age-standardized incidence rates in males ranging from $95.5/10^5$ in Yamagata, Japan, to $7.5/10^5$ in Whites in the United States. High-risk areas include Chiang Mai, Thailand and worldwide.

In the year 2000, Thailand had 2,018 cases of gastric cancer, for 1.07% of all cancer cases, with a male 1,181 cases and female 837 cases, but the mortality rate was very high, approximately 85.6%, and trends seem to increase if without prevention(3).

The incidence of gastric cancer rises progressively with age; with most patients being between the ages of 50 and 70 years at presentation. Gastric cancer has a higher male-to-female ratio, from 2:1 up to nearly 6:1. The incidence of gastric cancer varies according to several factors, such as age, sex, race and past history of *H.pylori* infection, gastric surgery, peptic ulcer disease, dietary factors and more(2).

Country, region	Male	Female	Ratio
Japan, Yamagata	95.5	40.1	2.4
Japan, Hiroshima	83.1	35.9	2.3
Korea, Kanwha	65.9	25.0	2.6
Japan, Osaka	65.0	27.3	2.4
Costa Rica	51.5	22.7	2.3
China, Shanghai	46.5	21.0	2.2
Italy, Florence	36.3	15.9	2.3
Columbia, Cali	33.3	19.3	1.7
Peru, Trujillo	31.1	20.1	1.5
Yugoslavia, Vojvodina	20.8	9.4	2.2
Hong Kong	19.4	9.5	2.0
Germany, Saarland	18.5	9.0	2.1
Italy, Genoa	17.6	8.3	2.1
United Kingdom	16.1	6.3	2.6
Spain, Granada	15.5	7.0	2.2
US, SEER[a] (Black)	14.5	5.9	2.5
Norway	13.6	6.4	2.1
Switzerland, Geneva	12.3	5.4	2.3
France, Bas Rhin	12.2	4.9	2.5
Australia, Victoria	11.7	4.9	2.4
Philippines, Manila	11.1	6.4	1.7
Canada	10.6	4.5	2.4
Australia, NSW	10.1	4.2	2.4
Singapore (Malay)	8.7	5.5	1.6
India, Bombay	7.7	3.8	2.0
Thailand, Chiang Mai	7.5	4.9	1.5
US, SEER* (White)	7.5	3.1	2.4
Highest/lowest ratio	12.7	12.9	

Table 1. International comparison of age-adjusted incidence rates (/100,000) of gastric cancer in selected countries (2).

1.2.2 Stomach and gastric cancer

Swallowed food is delivered from the esophagus to the cardiac region of the stomach. An imaginary horizontal line drawn through the cardiac region divides the stomach into an upper fundus and lower body; which together compose about two-thirds of the stomach. The distal portion of the stomach is called the pyloric region. The pyloric region begins in a somewhat widened area, the antrum, and ends at the pyloric sphincter. Contractions of the stomach churn the chime, mixing it more thoroughly with the gastric secretions. These contractions also push partially digested food from the antrum through the pyloric sphincter and into the first part of the small intestine (Figure 1).

The stomach is lined with simple columnar epithelium. The epithelium forms numerous tube-like gastric pits, which are the opening for the gastric glands. The epithelium cells of the stomach are of five types (Figure 2). The first type, surface mucous cells, which produce mucous, is on the surface and lines the gastric pit. The remaining four cell types are in the gastric glands. They are mucous neck cell, which produce mucus; parietal (oxyntic) cells, which produce hydrochloric acid and intrinsic factor; chief (zymogenic) cells, which produce pepsinogen; and endocrine cells, which produce regulatory hormones. The mucous neck cells are located near the openings of the glands; whereas the parietal, chief, and endocrine cells are interspersed in the deeper parts of the glands (10, 11).

The precancerous stages of the intestinal type present a very complex process, part of which results in a transformation of the normal mucosa into an intestinal type of mucosa (Figure 3). The morphological changes observed fall into three categories: inflammation, atrophy, and loss of cellular differentiation. The inflammatory changes (chronic gastritis) are more accentuated in younger individuals and become progressively less conspicuous with age, although persisting throughout most of the process. Because of atrophy, or gland loss, the gastric mucosa becomes practically devoid of its original gland. The loss of differentiation in reality appears to represent successive mutations (or similar changes in the genetic material of the cells), since the gastric epithelial cells disappear as such and are replaced by cells with intestinal phenotype; they lose some of their normal cytoplasmic secretions and gain autonomy, which eventually leads to uninhibited replication and invasion of the neighboring tissues (1).

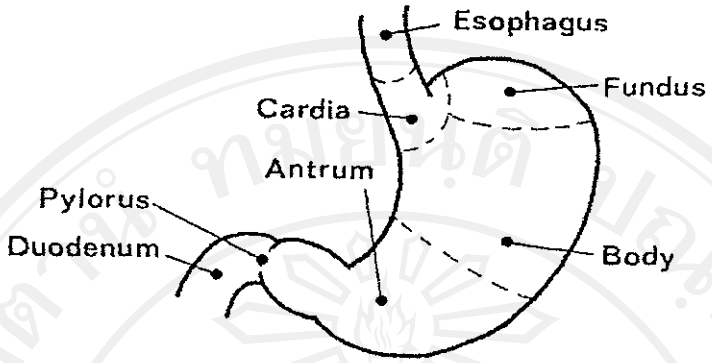

Figure 1. Primary regions and structures of the stomach.

(http://www.uwgi.org/Images/Stomach/(7)%20St47.gif)

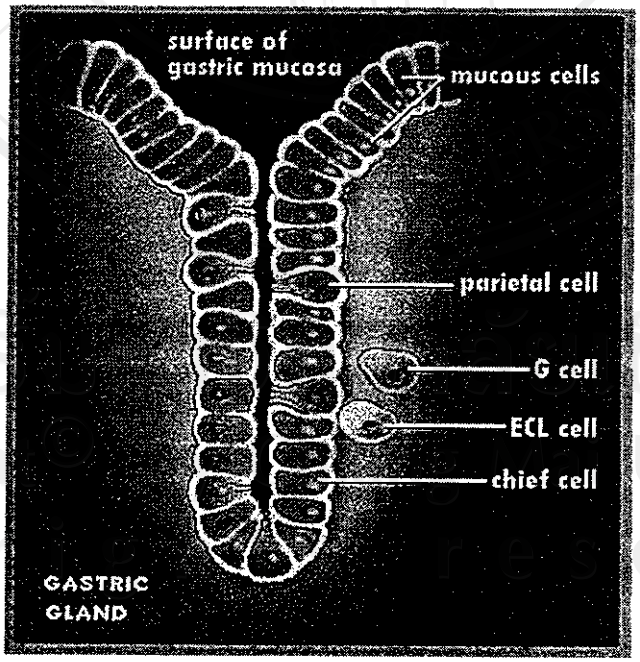

Figure 2. Gastric pits and gastric glands of the mucosa.

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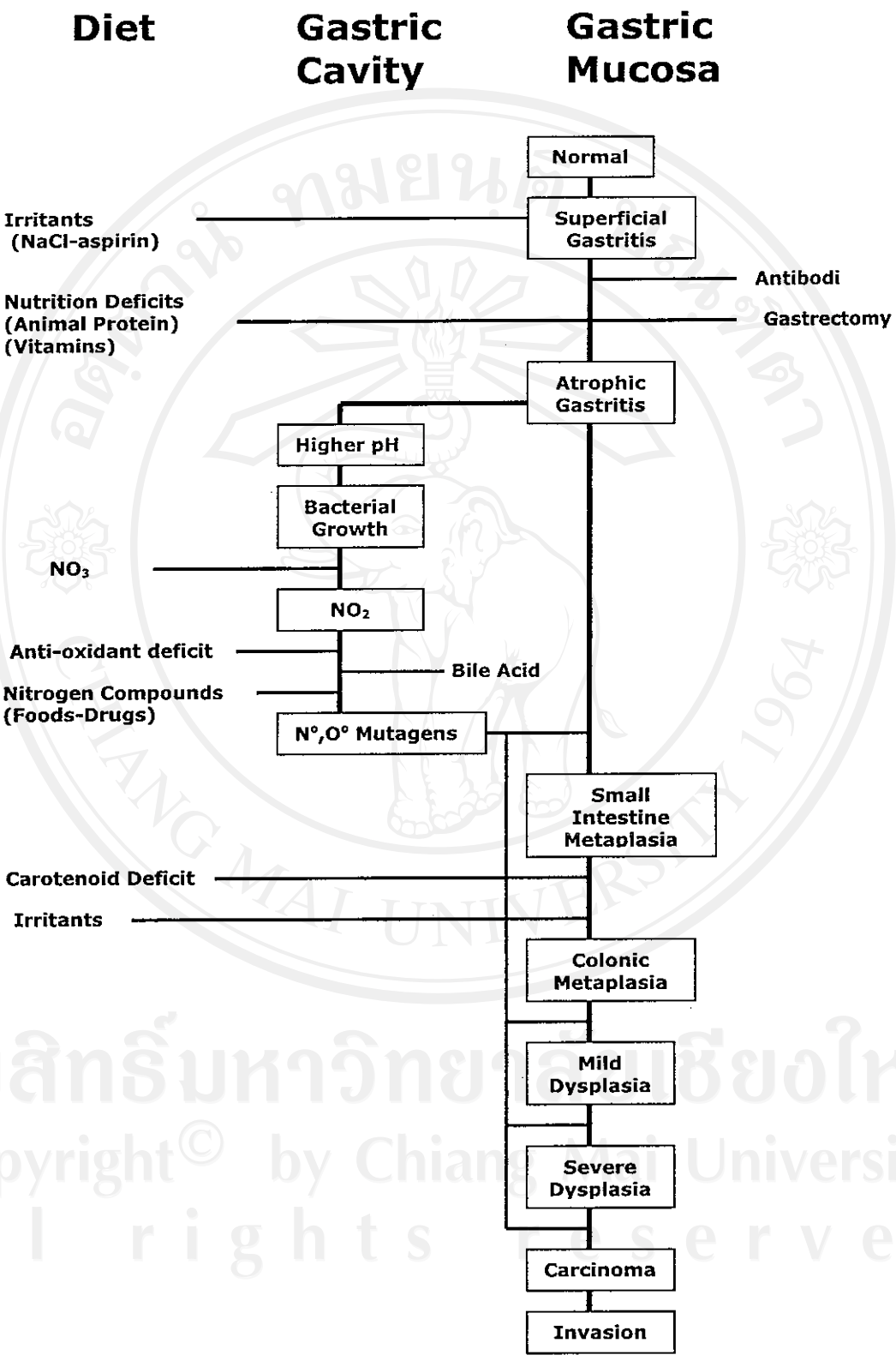


Figure 3. Hypothesis of gastric cancer etiology (1)

1.2.3 Pepsinogens: structure and function

Human gastric mucosa contains aspartic proteinase that can be separated electrophoretically on the basis of their physical properties into two major groups: Pepsinogen I (PGA, PGI); and Pepsinogen II (PGC, PGII). On the basis of IUB's Enzyme Nomenclature rules, proteases are designated and classified by code numbers: the first code refers to the class, the second to the sub-class, the third to the sub-sub-class (Table 2). Pepsinogen consists of a single polypeptide chain with molecular weight of approximately 42,000 Da, and it contains three disulphide bonds and has an isoelectric point of 3.7. Pepsinogens are synthesized as precursors containing a hydrophobic signal sequence of 15-16 amino acids at the N-terminal site serving a transport function. The signal sequence is lost during post-transcription processing. After appropriate physiological or external chemical stimuli, pepsinogens are secreted in the stomach lumen and when the pH drops below 5, secreted by the parietal cells. Activation starts with the protonation of a carboxylic acid, which causes an N-terminal peptide of 44-47 amino acids propart to emerge from the molecule. Limited proteolysis, that eliminates the propart, and the active site, which contains two aspartyl residues at positions 32 and 215, is formed. At a pH below 2.5 the propart is removed by the active centre of the same molecule, whereas above pH 2.5 an intermolecular, pepsin catalysed mechanism predominates. The resulting pepsin undergoes further conformational changes finally exposing a binding cleft that can accommodate a substrate sequence of approximately eight amino acids (Figure 4). Pepsinogen synthesis and secretion are regulated by positive and negative feed-back mechanisms. In the resting state pepsinogens are stored in granules, which inhibit further synthesis.

Pepsinogens are produced by the chief cells of the gastric body and fundus, and then secreted into the stomach lumen. PGC is also produced by the chief cells of the oxyntic gland areas; the mucosa neck cells in the gastric fundus; the cardiac gland; the pyloric gland cells in the gastric antrum; the Brunner's glands in the proximal duodenum; and the prostate gland. The different cellular origins of PGA and PGC are important because alteration in their serum concentration can be correlated with some histological gastric anomalies (9, 12).

Many previous studies have shown the serum pepsinogen levels are elevated in patients with gastric cancer, Samloff and his group showed an elevated serum PG I level ($\geq$130 μg/L), however associated with duodenal cancer more than gastric cancer, whereas an elevated PG II

level (≥30 μg/L) was associated with gastric cancer more than duodenal cancer (13-15). In addition a PG I/PG II ratio of < 4.0 was associated with chronic gastric more than duodenal cancer. In contrast, the PG I/PG II ratio was significantly lower in the gastric cancer in Hawaiian Japanese population, as shown by using radioimmunoassay (16). In the same way, As first report by Miki *et al.* study in Japanese subjects were found which the serum pepsinogen I level ≤70 μg/L and the PG I/PG II ratio ≤3.0 have high risk of gastric cancer and investigated by Yoshihara *et al.* were found to have gastric cancer and adenoma are related to atrophic gastritis, in which the serum pepsinogen I level (≤50 μg/L) and the PG I/PG II ratio (≤3.0) are reduced. They were measured by a modified radioimmunoassay method using Riabead Kits (17).

Recently, pepsinogen measurements have been used as an effective biochemical method for evaluating and monitoring patients with gastrointestinal diseases and for checking the effects of drug treatment. The level of PGA in the serum is always high in superficial gastritis, while in atrophic gastritis it is always low. In both cases the PGC level in the serum is high. In most gastrointestinal pathologies the ratio between the PGA/PGC decreases.

Aspartic proteinases	
Gastric protinases	
Pepsin A (or pepsin I, corresponding to zymogen PGA)	EC 3.4.23.1
Pepsin B (or cathepsin E, or slow-moving proteinase)	EC 3.4.23.2
Pepsin C (or fastricsin, or pepsin II, corresponding to zymogen PGC)	EC 3.4.23.3
Chymosin	EC 3.4.23.4
Cathepsin D	EC 3.4.23.5
Other proteinases	
Microbial aspartic proteinases (from fungi and HIV-1)	EC 3.1.23.6
Renin	EC 3.4.23.15

Table 2. Aspartic proteinase and code numbers designated on the (basis of IUB's Enzyme Nomenclature rules) (9).

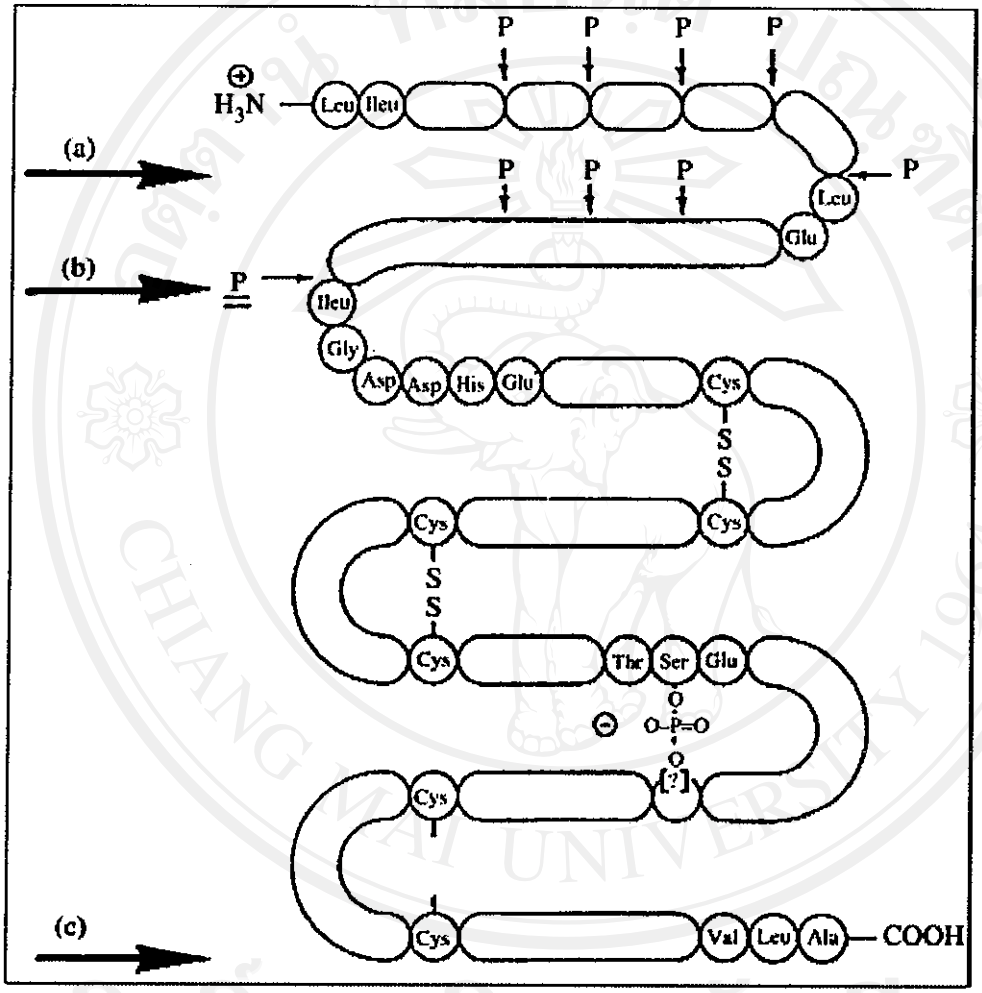


Figure 4. Diagram of the pepsinogen structure and of the major points of hydrolysis (small arrows and P) , which release peptides (large arrow, a) ; pepsin inhibitors (large arrow, b) ; and pepsin (large arrow, c) (9).

1.2.4 The role of Interleukin-1 in carcinogenesis and cell transformation

IL-1 is abundant in the microenvironment of tumors; it may be derived from stromal or immune cells or from the malignant cells themselves. IL-1 may affect growth and invasiveness of malignant cells, but may also promote the development of anti-tumor immune responses. The effects of IL-1 on the malignant process are possibly influenced by the amount of IL-1, the form of IL-1, the type of the malignant cells, the tumor's stage and the overall local network of cytokines and their receptors. We think that the expression of local small amounts of IL-1 in the vicinity of the tumor, at early stages of its development, can induce moderate or 'tamed' inflammatory responses that will subsequently activate specific immunity against the malignant cells (Figure 5). In contrast, large amounts of IL-1 will induce broad inflammation, due to its paracrine effects on stromal cells in the tumor's microenvironment, that will quickly potentiate invasiveness and metastasis, irrelevant of anti-tumor immunity that will not cope with the development of quickly growing and disseminating tumors. As to IL-1 of tumor cell origin, the sub-cellular localization of the IL-1 within the producing cell and its environment is crucial for determining its biological functions. Thus, the 'natural' membrane-associated form of IL-1α is immunostimulatory, by serving as an adhesion molecule that may promote interactions between IL-1-producing tumor cells and immune effecter cells that express IL-1Rs and thus, local and focused immune responses will develop at low levels of IL-1α expression level below those which are toxic to the host. In the producing cell, the active precursor of IL-1α may act as an intracellular messenger and may control, in intracrine or autocrine manners, homeostatic functions related to cell proliferation, cell cycle control, apoptosis or differentiation. The intracrine role of proIL-1α has not yet been thoroughly investigated in malignant cells. In contrast, secreted IL-1β, especially at high expression levels, has broad pro-inflammatory activities that promote invasiveness of tumor cells. It is currently unrealistic to use recombinant IL-1 in the treatment of cancer patients, due to its undesirable side-effects. The development of appropriate methods to selectively express the membrane-associated form of IL-1α in malignant cells, i.e. in tumor cell vaccines or vectors that will specifically harbor in the malignant cells, will enable the safe use of IL-1 in tumor immunotherapy. Such immunotherapeutic treatments can be combined with the concomitant neutralization of secreted IL-1 (mainly IL-1β) by IL-1Ra, the latter in order to inhibit tumor invasiveness. Membrane-associated IL-1α avidly binds to IL-1Rs

on immune cells and is much less accessible for neutralization by IL-1Ra, as compared to secreted IL-1β. The secreted form of IL-1, mainly IL-1β, is a central upstream molecule in regulation of tumor invasiveness, mainly acting to induce the expression of diverse pro inflammatory molecules; its neutralization has the potential to inhibit the generation of this downstream cascade of invasiveness-promoting factors. Thus, targeted local expression of IL-1α at tumor sites and systemic inhibition of IL-1β will synergistically act to eradicate residual disease and metastasis by potentiating the development of anti-tumor immunity and targeting tumor invasiveness, respectively (18).

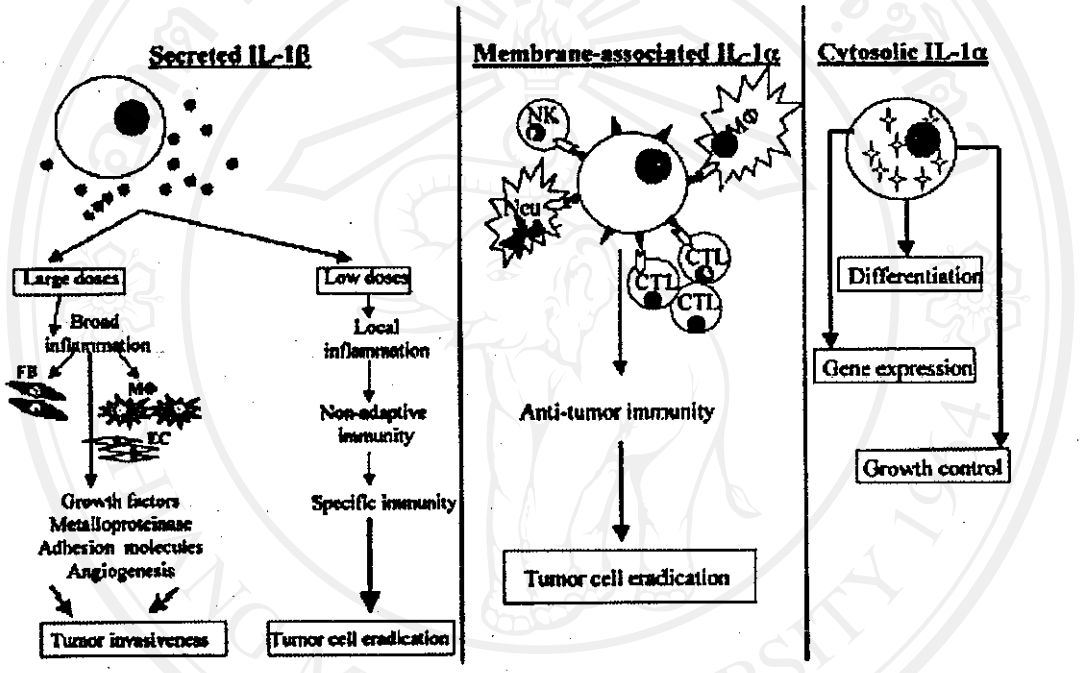


Figure 5. Patterns of expression of IL-1 in tumor cells and its possible consequences on malignancy patterns. The effects of IL-1 in different sub-cellular compartments of malignant cells are shown. In tumor cells which express the precursor of IL-1α, active cytosolic, and membrane-associated IL-1α can be detected. When calpain-dependent processing occurs, some of the cytokine can be secreted. In IL-1β expressing cells, pro-inflammative cytokine is secreted into the microenvironment only if processing through caspase-1 or other machanisms occurs. In primary cells, similar patterns of IL-1 expression are observed. For the sake of simplicity, activity of IL-1 in single sub-cellular compartments is demonstrated. The consequences of these patterns of IL-1 expression are also depicted (18).

1.2.5 Interleukin -1 β and gastric cancer

In patients with severe gastritis of the corpus, acid secretion is decreased markedly and the pH of gastric juice is increased. This decrease in acid secretion delays the elimination of bacterial toxins and products of inflammation, such as reactive oxygen radicals and nitrogen oxide, thereby increasing the concentration of these well-known mutagens in the stomach. Decreased acid secretion may also result in increased production of carcinogenic N-nitroso compounds because of superinfection by other bacteria or a reduction in vitamin C levels in gastric juice. An increased rate of cell turnover in inflamed mucosa further increases the opportunity for DNA damage. Moreover, decreased gastric acid secretion permits the reflux of duodenal juice, including bile, into the stomach, which can induce intestinal metaplasia, a precancerous change (7).

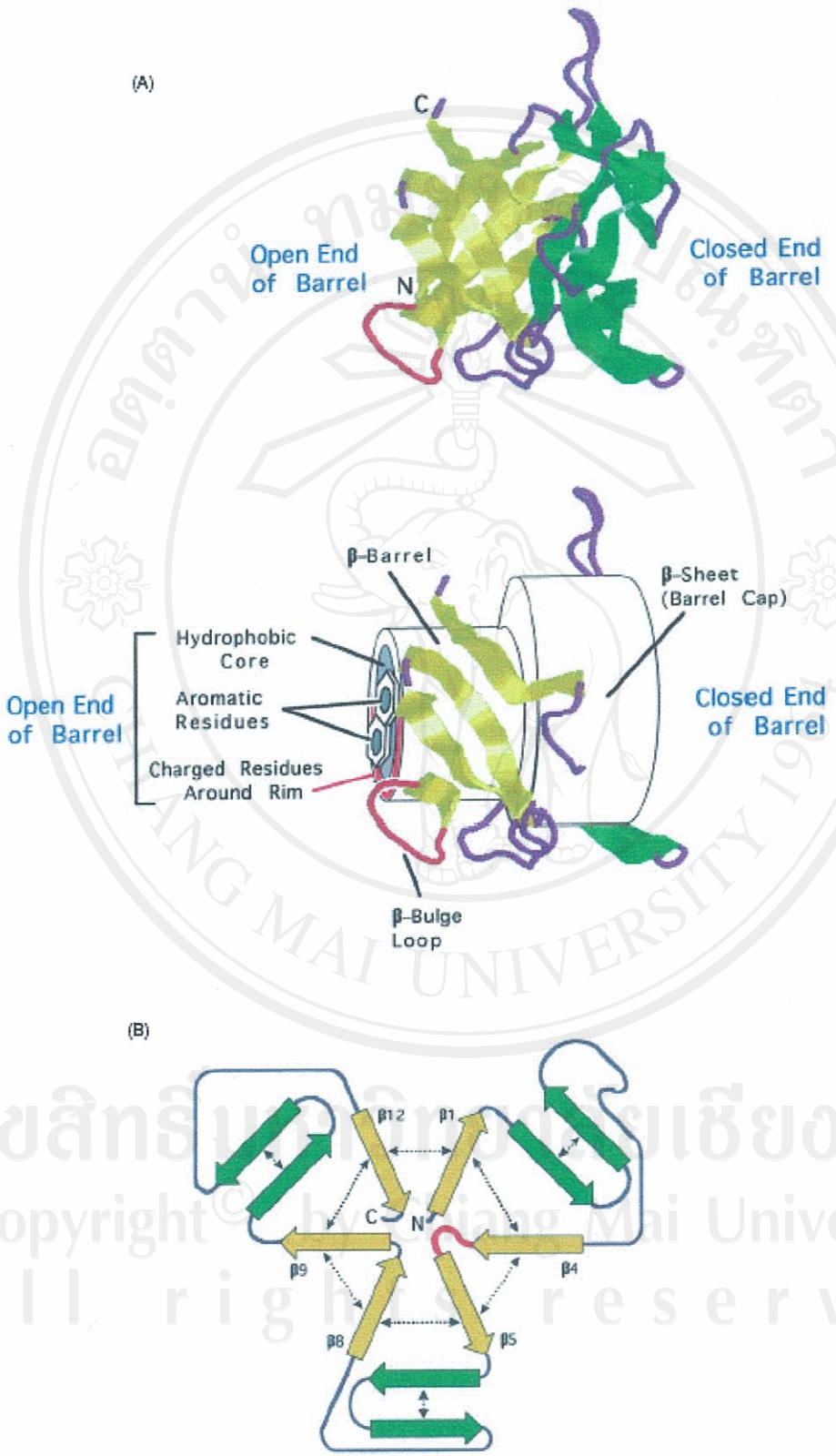
IL-1 β is upregulated in the presence of *H.pylori* and is important in initiating and amplifying the inflammatory response to this infection. IL-1β is also a potent inhibitor of gastric acid secretion; on molar basis it is estimated to be 100-fold more potent than proton pump inhibitors and 6,000-fold more potent than histamine antagonists. Three diallelic polymorphisms in IL-1β have been reported, all representing C-T base transitions, at position -511, -31 and +3954 base pair (bp) from the transcriptional start site. There are conflicting data regarding the functional effects to these polymorphisms on IL-1β production (4-6).

Subsequent studies of the association have been controversial. In the Portuguese population, IL-1B -511 T and IL-1RN*2 alleles were associated with increased risk of gastric cancer (19). However, in the Japanese and Korean population, the proposed association has not been confirmed (20). The discrepancy could reflect the genotypic differences in the study populations. According to the proposal by El-Omar et al., the gastric cancer risk genotypes IL-1B -31 C+ and IL-1RN*2/*2 were associated with decreased acid-secreting capacity (8). We presumed that it might be tested by analyzing the allelic frequencies in gastric cancer and duodenal ulcer subjects in a population. Because subjects with duodenal ulcer tend to have elevated acid-secreting capacity, the gastric cancer and duodenal ulcer groups would be expected to show different allelic frequencies, presumably in the opposite way compared to the controls.

1.2.6 Interleukin-1 structure and function

Interleukin -1 (IL-1) is a multifunctional primary inflammatory cytokine and has been implicated in mediating both acute and chronic pathological inflammatory diseases. That affects most cell types and results in numerous effects including: fever; increased hepatic acute phase response; increased metastases; angiogenesis; increased antibody and lymphokine production. It also causes the production of the other cytokines, cyclooxygenase2 (COX2) and nitric oxide. IL-1 was originally purified under a number of names corresponding to its different biological activities (endogenous pyrogen, Lymphocyte activating factor, thrombocyte activating factor, catabolin, amongst others). It was given the unifying term interleukin 1 in 1979 (21, 22).

Recent X-ray crystallographic structure studies on IL-1β, IL-1ra and the extracellular ligand-binding domain of IL-1RI (also called soluble IL-1RI or sIL-1RI) have demonstrated some insight into the extracellular requirements for ligand-induced signaling (23, 24). First, all three IL-1 molecules consist of 12 antiparallel β-strands assembled into two distinct, but associated, components. Six of the β-strands are arranged in a topologically closed and structurally cooperative β-barrel (Figure 6 (A), yellow strands). The remaining six strands associate to form a β-sheet that caps one end of the barrel (Figure 6 (A)), green strands. Figure 6 (B) shows a schematic representation of the molecule's secondary structure revealing the extensive hydrogen bonding (double-headed arrows) that cooperate to maintain protein conformation. The IL-1β agonist associates with the receptor in such a way that the uncapped end of the barrel, located where the amino and carboxyl ends of the molecule approach each other (which displays both hydrophobic and charged residues to the solvent), is presented to domain III of the receptor which has a structurally complementary nature. The association with this receptor domain effectively 'caps' the uncapped end of the ligand, resulting in 2,088 $^{\circ}$A of ligand surface being buried in the receptor (Figure 6 (C)) (25).



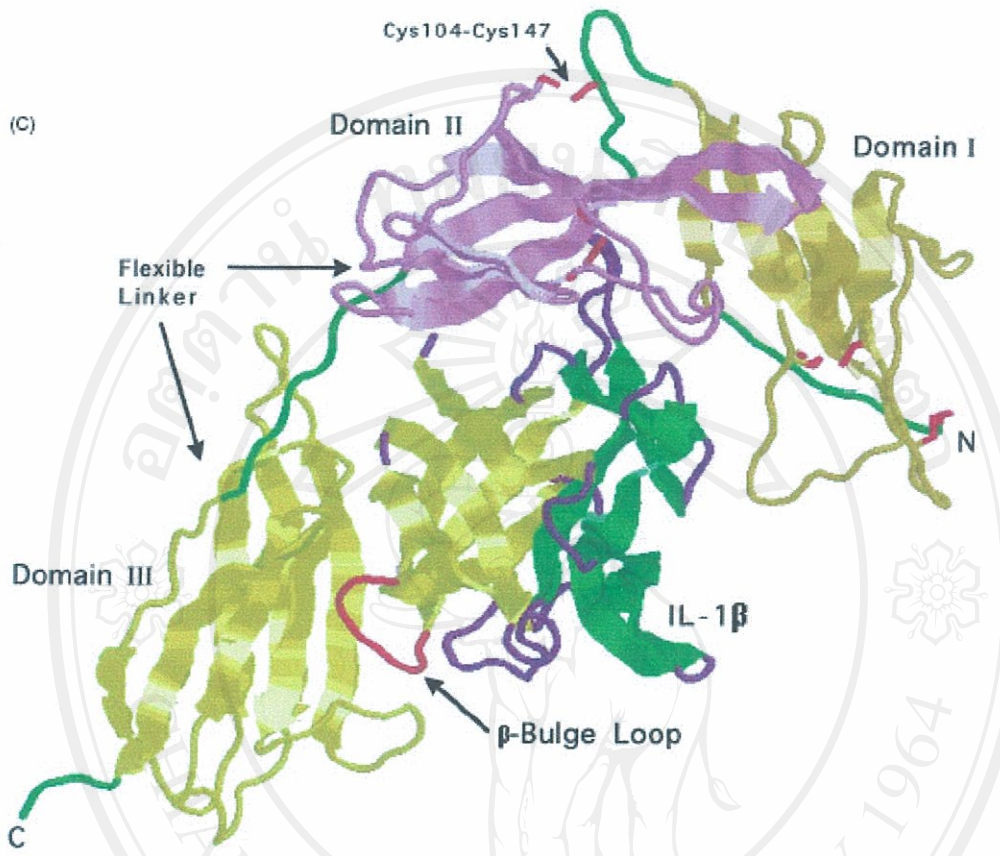


Figure 6. Structure of human IL-1β and the type I IL-1 receptor (IL-1RI). **A.** IL-1β consists of 12 anti-parallel β-strands. Six of the strands are arranged into a barrel which represents a topologically continuous, hydrogen-bonded (arrows), cylinder that is 'closed' at one end by a thick sheet consisting of the remaining six β-strands (green). The other end of the barrel (yellow) is 'open' exposing to the solvent the hydrophobic barrel core and a collection of aromatic and hydrophobic residues that are located on the barrel rim. A semicircular 'horseshoe' of charged residues is present on the rim along with a β-bulge loop located between strands 3 and 4. These various features are indicated on a schematic framework representative of the structure shape. **B.** Secondary structure schematic of IL-1β showing the relative orientation of the β-strands and the 3-fold pseudo-symmetry. **C.** The IL-1RI consists of three anti-parallel β-strand immunoglobulin like domains. The disulfide linkage that shortens the distance between domains I + II is indicated as is the flexible linker that connects domains II + III. The features and coloring scheme for the IL-1b ligand are the same as in **A** (25).

1.2.7 Interleukin-1 gene cluster

The classical IL-1-family genes IL1A, IL1B, and IL1RN were mapped by recombination analysis to the same chromosomal region in humans (26). Six novel genes encoding proteins with the interleukin (IL)-1 fold have been identified recently. The genes were shown to be clustered, and the maximum separation of the distal genes IL1A and IL1RN was estimated to be 430 kb by pulse field gel electrophoresis of restriction digests of human genomic DNA (27). The orientation of the three genes was determined by sequence analysis of physical clones and the cytogenetic map position of the cluster was found to be 2q13 (28). IL-18 is the fourth member of the IL-1 structural family resides on chromosome 11 (29). Acknowledging the existence of four previously known IL-1 family members, the new human genes have been named IL1F5, IL1F6, IL1F7, IL1F8, IL1F9, and IL1F10. Protein products are named in the style IL-1F7b (denoting the second described putative protein product of the gene IL1F7).

Previous study have mapped all of the IL-1 family cDNA sequences onto the genomic sequence (Figure 7), where the extent of the genes is shown with black rectangles. IL1A is the most centromeric gene and is transcribed towards the centromere, as is the adjacent gene, IL1B. The remaining genes, ending with IL1RN, the most telomeric member of the cluster, are transcribed towards the telomere, except IL1F8. The three last exons of each gene, which we have called common exons (CE) 1, CE2 and CE3, encode the IL-1-homologous domain and fall in compact regions within the sequence. CE1, CE2 and CE3 are indicated by vertical bars in Figure 7, but some cannot be resolved on the scale of Figure 7. Additional exons with little or no coding content extend the span of most of the genes considerably. The largest spans are IL1RN and IL1F8. In the latter case, the first reported noncoding exon is 20 kb telomeric of the rest of the gene. They have mapped previously identified polymorphisms in this region (30).

The program CpGplot was used to identify five potential CpG islands with $\geq$ 60% C+G content, $\geq$ 60% of the expected frequency of the CpG dinucleotide, and $\geq$ 300 nucleotides in length. With the exceptions of the first and the last two CpG-rich sequences, these regions are short and probably do not constitute "CpG islands". There are thus no CpG islands in the IL-1 cluster. They have attempted to locate the clusters of restriction sites that were used previously for physical mapping. CpG-rich sequences are labeled "CpGr" in Figure 7. Two are further labeled "Xrec" and "Zrec". These two regions contain the specific rare cutter restriction sites that were

identified previously, and so probably correspond to the cluster's flanks, as previously assigned (31).

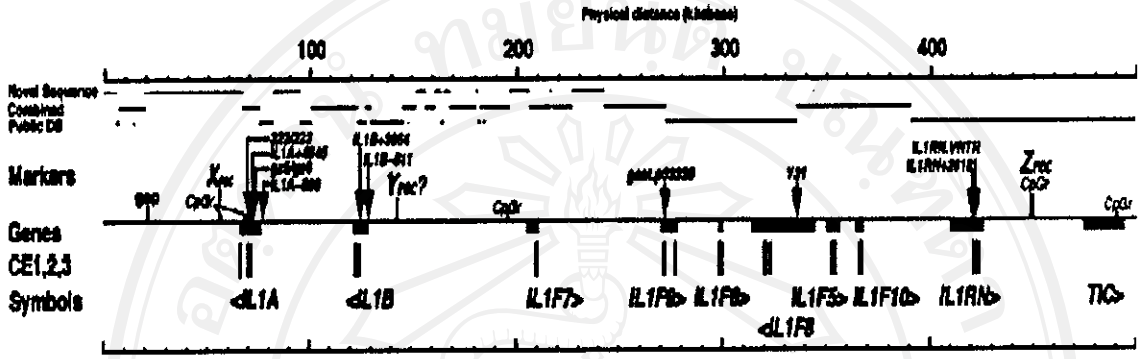


Figure 7. Map of the IL-1 gene cluster. Scale bars (in kb) are provided above and below the data to aid alignments. The sources of the data described are indicated by the top three lines. Novel sequence was determined entirely at Genome Therapeutics. Public DB indicates sequence taken from GenBank. Combined sequence was assembled from a combination of the two sources. Above the bar representing the contiguity, the positions of previously described polymorphic markers are indicated with labeled arrows. The single unfilled gap is also indicated. CpG-rich regions are indicated with CpGr. The probable sites of the rare cutter restriction enzyme sites clusters that were used in previous mapping are also marked as Xrec, Yrec?, and Zrec. The positions of the coding sequences for CE1, CE2, and CE3 are indicated by vertical bars. The gene symbols are followed or preceded by a chevron to indicate the direction of transcription (30).

1.2.8 Transcriptional regulation of Interleukin-1

The three members of the IL-1 family have been cloned. All bind both types of receptors. Type I (80 kD) IL-1 receptor (IL-1RI) transduces the IL-1 signal and is widely expressed (32). The type II receptor (67 kD; IL-1RII) is found on B cells, neutrophils and monocytes and preferentially binds IL-1β. It may act as a decoy inhibitor of IL-1. But only two, IL-1α and IL-1β, are agonistic. The third is the interleukin 1 receptor antagonist protein (IRAP). All share 20±25% amino acid homology. The structure of the three IL-1 gene promoters differ (Figure 8). The IL-1α promoter, unlike those of IL-1β and IL1ra, does not have a typical TATA box. All three promoters contain NFkB regulatory elements, but only IL-1α and IL-1β have binding sites for NF-IL6. The IL-1β and IL-1ra promoters have binding sites for AP1 (22).

Unlike most cytokine promoters, IL-1β regulatory regions can be found distributed over several thousand basepairs upstream and few base pairs downstream from the transcriptional start site. IL-1β gene expression is regulated at different levels. Studies using a reporter gene transfected into human and mouse macrophage cell lines have shown sequences in the IL-1β promoter required for transcription. There are two independent enhancer regions, -2782 to -2729 and -2896 to -2846, that appear to act cooperatively. The latter contains a cAMP response element, whereas the former is a composite cAMP response element-NFIL-6 that is responsive to LPS. The 80 basepairs fragment (-2782 to -2729) is required for transcription and contains, in addition to a cAMP response element, an NFKB-like site. Activating protein-1 (AP1) sites also participate in endotoxin-induced IL-1β gene expression.

Proximal promoter elements between -131 and +14 have also been identified. Sequences in this region contain the binding sites for the novel nuclear factor NFβA, which appears to be similar to nuclear factors termed NFβ1 and NFβ2. This proximal promoter is required for maximal IL-1β gene expression. Recently, the nucleotide binding sequences of NFβA were found to be identical to those of transcription factor Spi-1/PU.1, a well-established NF in cells of myeloid and monocyte lineage. The requirement for Spi-1/PU.1 for IL-1β gene expression imparts tissue specificity because not all cells constitutively express this NF. Human blood monocytes, which constitutively express Spi-1/PU.1, are exquisitely sensitive to gene expression of IL-1β. Interestingly, the IL-1ra promoter contains the proximal Spi-1/PU.1 site which is also a highly sensitive site (21).

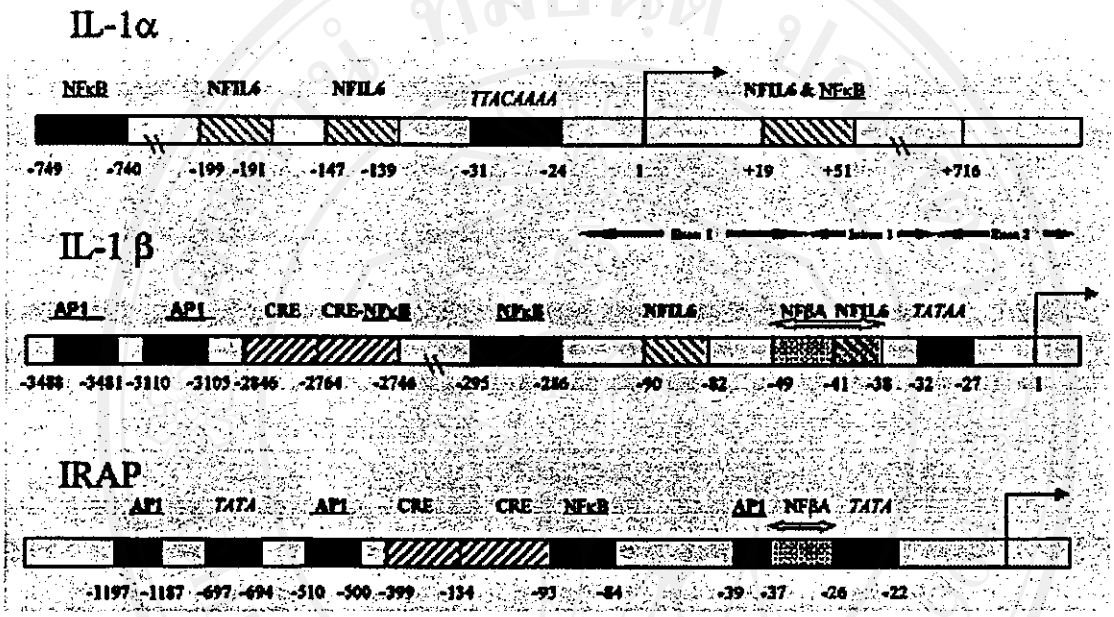


Figure 8. The IL1 gene promoters. Comparison of the regulatory elements of the IL-1α, IL-1β and IL-1ra promoters. The major transcription factor sites for NFkB, NF-IL6, AP1, cAMP response elements (CRE) that bind CREB, as well as the position of NFbA transcription factor binding elements, are shown with numbers below each colored box indicating the boundary of each region and the distance in base pairs from the transcriptional initiation site (shown as an arrow). TATA box sequences are italicized (22).

1.2.9 Production of Interleukin-1

Genes of the IL-1 family are located in the long arm of chromosome 2. Three gene products of this family have been thoroughly studied, two agonistic proteins, namely IL-1α and IL-1β, and one antagonistic protein, the IL-1 receptor (IL-1R) antagonist (IL-1Ra). IL-1Ra, which binds to IL-1Rs without transmitting an activation signal, represents a physiological inhibitor of pre-formed IL-1. IL-1 differs from most other cytokines by lack of a signal sequence, thus not passing through the endoplasmic reticulum (ER)–Golgi pathway; its mechanisms of secretion are not yet completely understood. IL-1Ra has a signal peptide and is secreted in the ER–Golgi exocytic pathway. Intracellular nonsecretable isoforms of the IL-1Ra (icIL-Ra), lacking a signal peptide, were also described; it was suggested that they neutralize the active intracellular precursor of IL-1α (proIL-1α) (Figure 9).

IL-1α and IL-1β are synthesized as precursors of 31 kDa that are further processed by proteases to their mature 17 kDa forms. IL-1β converting enzyme (ICE), or caspase-1, is a cysteine protease that cleaves the inactive precursor of IL-1β, while proIL-1α is processed by calpain. The participation of ICE in apoptosis is documented, however, apoptosis is probably not directly related to IL-1β processing and secretion (Figure 10).

Many cell types produce and secrete IL-1α, IL-1β, and IL-1Ra upon activation with microbes, microbial products, cytokines, and other environmental stimuli. Mononuclear cells secrete high levels of IL-1α and IL-1β, while nonphagocytic cells secrete IL-1 only a very limited amount, mainly IL-1β. As IL-1α is secreted to a much lesser extent than IL-1β, even in activated monocytes, it is not commonly detected in body fluids, except in severe inflammatory responses, in which case it is possibly released from necrotizing cells.

In their recombinant form, IL-1α and IL-1β bind to the same receptors and exert the same biological activities. In spite of extensive studies, no significant differences in the spectrum of activities of IL-1α and IL-1β could be detected when recombinant molecules were used in *in vitro* and *in vivo* experimental systems. In comparison to IL-1α, IL-1β expression/secretion is tightly controlled at the levels of transcription, mRNA stability, translation, processing, and secretion. Also, IL-1α and IL-1β differ dramatically in the sub-cellular compartments in which they are active. IL-1β is solely active as a secreted product, while IL-1α is mainly active as an intracellular precursor or as a membrane-associated form (23 kDa), but is only marginally active

in its secreted mature form, due to its limited secretion. The active membrane form of IL-1α is derived from myristoylation of proIL-1α, and it is anchored to the membrane via a mannose-like receptor. Ron N. Apte. and Elena Voronov hypothesized that the localization of the IL-1 molecules in context of the producing cell and its microenvironment dictates their biological functions. Thus, as will be shown below, membrane-associated IL-1α is immunostimulatory, while cytosolic proIL-1α may control homeostatic functions, such as gene expression, proliferation control, and differentiation. As to secreted IL-1 (mainly IL-1β), at low local doses it induces local inflammatory responses followed by activation of protective immunity, while at high doses broad inflammation accompanied by tissue-damage and tumor invasiveness are induced.

IL-1Rs, which belong to the immunoglobulin (Ig) supergene family, are abundantly expressed on many cell types. IL-1R of type I (IL-1RI) (80 kDa) is a signaling receptor, whereas the IL-1R of type II (IL-1RII) (68 kDa) serves as a decoy target, acting to reduce excessive amounts of IL-1. Following the binding of IL-1 to IL-1RI, a second chain, i.e. the IL-1R acceptor protein (IL-1RAcP), is recruited. This heterodimeric complex triggers IL-1 signal transduction that is initiated by activation of the IL-1 receptor-associated kinase (IRAK) and ultimately leads to activation of nuclear genes. On the contrary, IL-1RII and the IL-1Ra fail to form this heterodimeric complex with the IL-1AcP and to recruit IRAK.

Under physiological homeostasis, IL-1Ra, surface IL-1RtII and soluble IL-1Rs play a key role in limiting cell responsiveness to IL-1 (18).

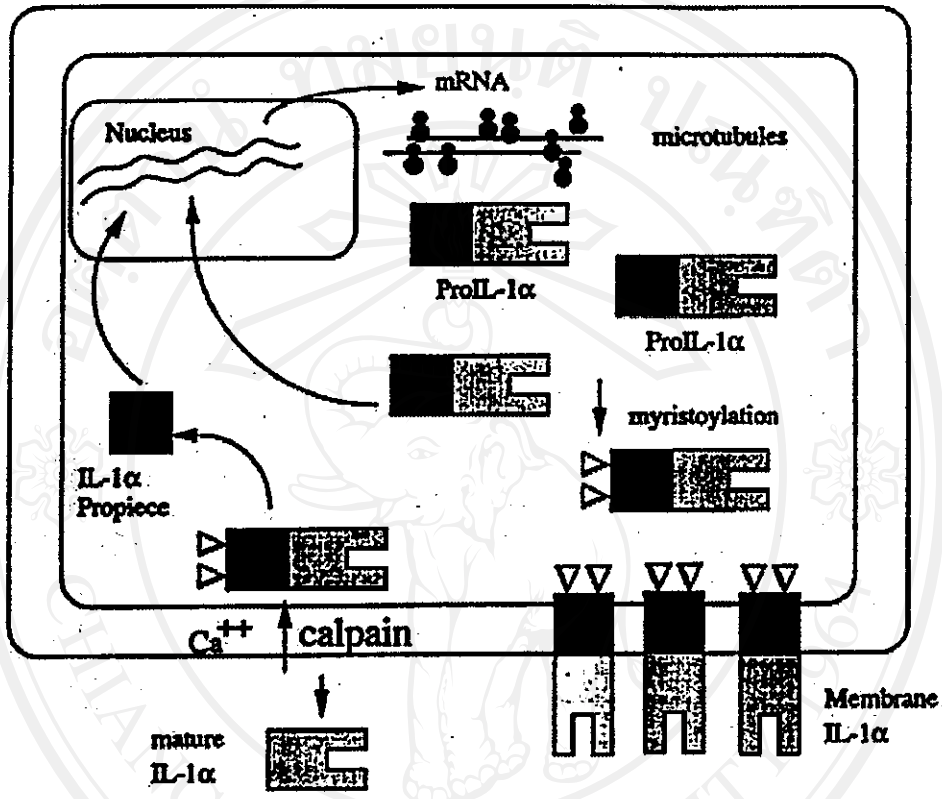


Figure 9. Monocyte producing IL-1α mRNA coding for proIL-1α is translated in association with microtubules. ProIL-1α remains in the cytosol, where it is myristoylated. Myristoylated proIL-1α is translocated to the cell membrane where it can be anchored to the cell membrane or to a putative cell-surface lectin. Myristoylated proIL-1α can also be cleaved into a mature form by the cysteine protease, calpain, which requires calcium for activation. After cleavage, 17-kD Il-1a is released into the extracellular component. ProIL-1α can also "leak" from dying cell. Intracellularly, either proIL-1α or the 16-kD Il-1a propiece (amino acids 1-115), which is liberated by the calpain cleavage step, can bind to nuclear DNA (21).

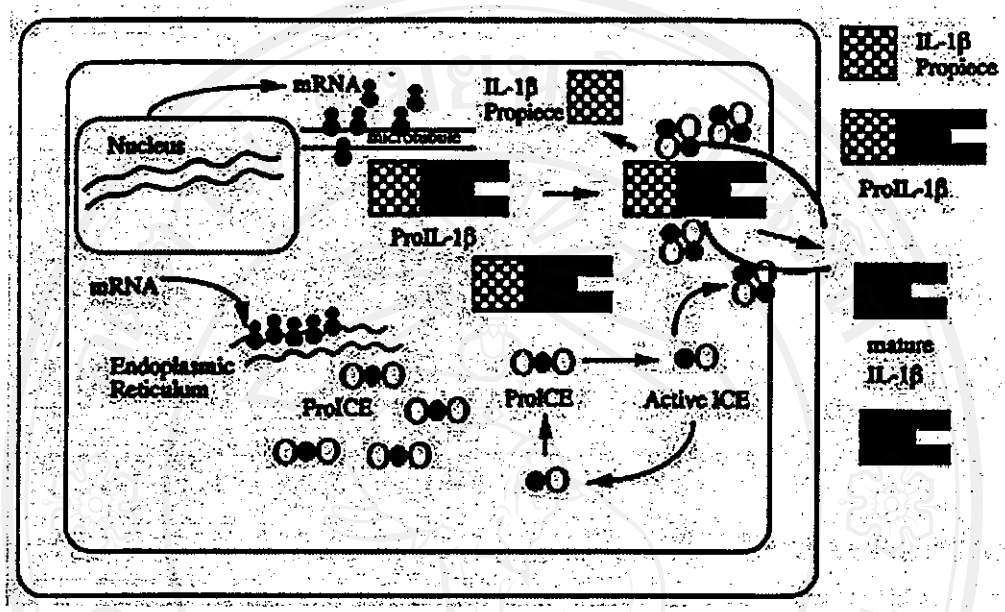


Figure 10. Human blood monocyte producing IL-1β mRNA coding for proIL-1β is translated on polysomes in the cytosol and associated with microtubules. ProIL-1β remains cytosolic until cleaved by the ICE. Before cleavage, proIL-1β may be myristoylated to facilitate localization to the cell membrane. ICE is translated in the cytoplasmic reticulum as an inactive precursor (proICE), remains cytosolic, and requires two internal cleavage steps (also by ICE) to form the enzymeaticlly active heterodimer. Two heterodimers form a tetramer in association with two molecules of proIL-1β and cleavage occurs. Active ICE is found predominantly on the inner surface of the cell membrane. After cleavage, 17-kD IL-1β is secreted into the extracellular component through a putative membrane channel. The 16-kD IL-1β propiece (amino acids 1-116) can be found both inside and outside the cell. A small amount of proIL-1β can be transported into the extracellular space from intact cells, presumably using the same channel; however, when ICE activity is inhibited, increased proIl-1b is found in the extracellular component, where it can bind to soluble type II IL-1R (21).

1.2.10 Polymerase Chain Reaction (PCR) Technology and protocol

PCR is a wonderful technology for amplifying DNA. It allows researchers to take a specific region of DNA on the chromosome and through the use of primers, copy back and forth, only a particular desired segment, making two, then four, then eight, then sixteen, and so on, up to millions of copies. It is possible to start from the DNA segment of a single cell and produce enough of it for use in DNA typing or fingerprinting.

PCR relies on the ability of DNA-copying enzymes to remain stable at high temperatures. The process was sparked by a high temperature bacterium (*Thermus aquaticus*) inhabiting the hot springs of Yellowstone National Park. In nature, most organisms copy their DNA in the same way. PCR mimics this process, only it does it in a test tube. A PCR vial contains all the necessary components for DNA duplication: a DNA segment, a large quantity of the four nucleotides (A,T,G & C), large quantities of the primer sequence (DNA duplication cannot take place without first a short sequence of nucleotides to "prime" the process or get it started), and DNA polymerase (enzymes that make a copy of all the DNA in each chromosome). The polymerase (enzyme) is the "Taq" polymerase, named for the bacterium *Thermus aquaticus*, from which it was isolated.

The three parts of the polymerase chain reaction are carried out in the same vial, but at different temperatures. The first part of the process separates the two DNA chains in the double helix by heating the vial to 90-95 degrees centigrade for 30 seconds. But the primers cannot bind to the DNA strands at such high temperature, so the vial is cooled to 55 °C. At this temperature, the primers bind or "anneal" to the ends of the DNA strands, taking about 20 seconds. The final step of the reaction is to make a complete copy of the templates. Taq polymerase works best at around 75 °C (the temperature of the hot springs where the bacterium was found), hence the temperature of the vial is raised. The Taq polymerase begins adding nucleotides to the primer and eventually makes a complementary copy of the template. (If the template contains an A nucleotide, the enzyme adds on a T nucleotide to the primer. If the template contains a G, it adds a C to the new chain, and so on to the end of the DNA strand). This completes one PCR cycle.

The three steps in polymerase chain reaction (separation of the strands, annealing the primer to the template, and the synthesis of new strands) will take less than two minutes and the process can be carried out in the same vial. At the end of a cycle, each piece of DNA in the vial

has been duplicated. This cycle can be repeated 30 or more times. Each newly synthesized DNA piece can act as a new template. So, after 30 cycles, about a million copies of a single piece of DNA can be produced. Taking into account the time it takes to change the temperature of the reaction vial, 1 million copies can be made in about three hours (Figure 11).

Amplified DNA fragments 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 bathing the gel in chemicals, making it (the location of DNA on the gel) intensely fluorescent when irradiated with ultraviolet light. By performing this process over and over for different DNA fragments and digitally scanning the information for storage, a species library of DNA sequence locations is generated. Variation or uniqueness of sequence locations serve as markers of economic importance once statistically associated with frequency of observed (desirable) traits.

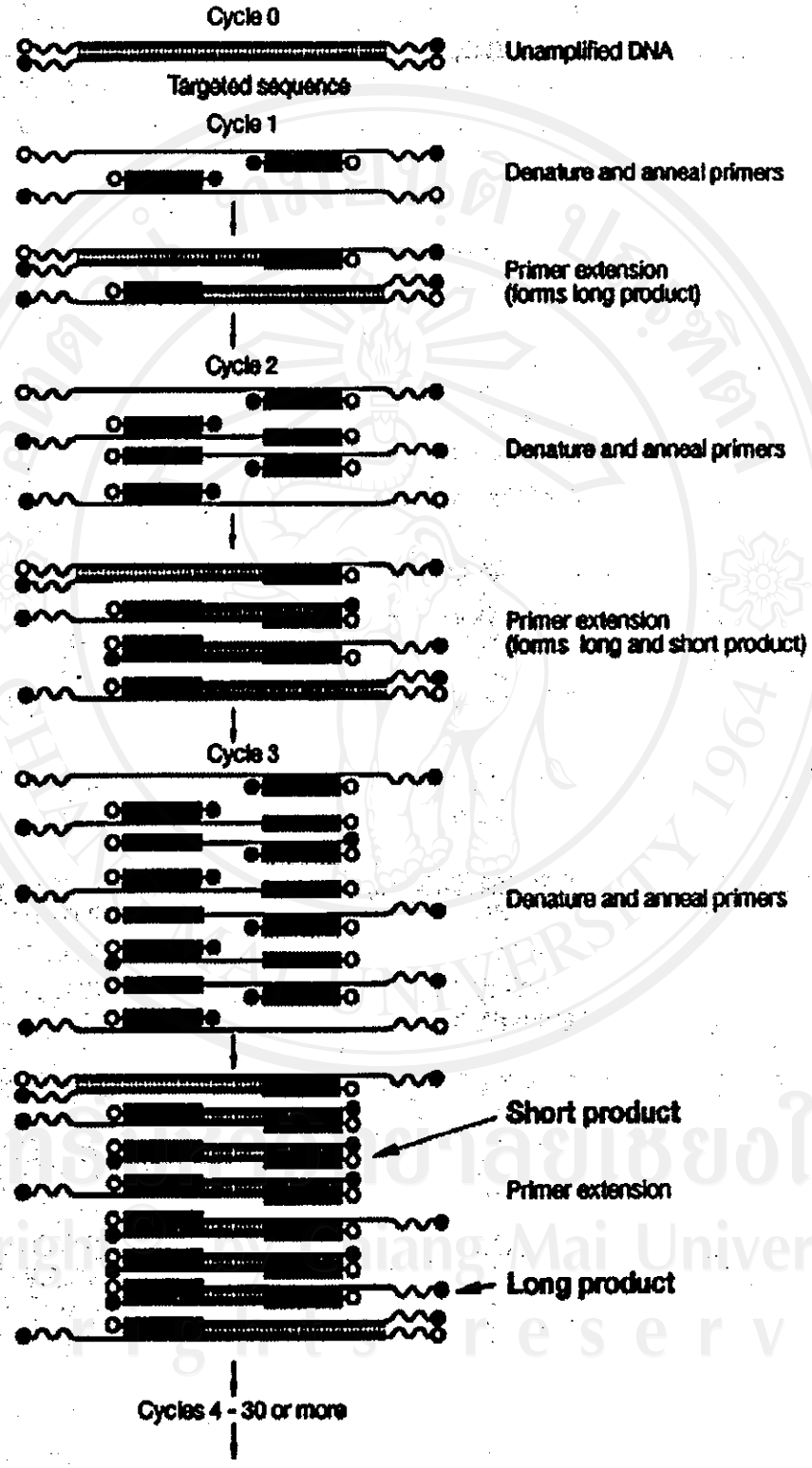


Figure 11. Polymerase chain reaction amplification cycles. Symbols: circle = 5' or phosphate end, dot = 3' or hydroxyl end of polynucleotide sequence.

1.2.11 Optimization of PCR

As originally developed, the PCR process amplifies short (approximately 100–500bp) segments of a longer DNA molecule (33). 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 sequence (amplicon). Therefore, ten cycles theoretically multiplies the amplicon by a factor of about one thousand; 20 cycles, by a factor of more than one million. PCR amplification can be completed in as little as 2 hours. 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. The target DNA is denatured by heating to 95°C or higher for 15–120 seconds. 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. After 20–40 cycles, the amplified nucleic acid may then be analyzed (e.g., for size, quantity or sequence) or it may be used in further experimental procedures (e.g., cloning or mutagenesis).

A. Magnesium Concentration

Magnesium concentration is a crucial factor affecting the performance of Taq DNA Polymerase. 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, TaqDNA Polymerase is inactive. Conversely, excess free magnesium reduces enzyme fidelity (34) and may increase the level of nonspecific amplification (35, 36). For these reasons, it is important to empirically determine the optimal $MgCl_2$ concentration for each reaction. This is accomplished by preparing a series of reactions containing 1.5–3.0 mM Mg^{2+}, in increments of 0.5 mM, by adding 3, 4, 5 and 6 µl of a 25 mM $MgCl_2$ stock to 50 µl reactions.

B. Enzyme Concentration

For most applications, enzyme will be in excess; the inclusion of more enzyme 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 TaqDNA Polymerase. Artifacts generally can be seen as smearing of bands in ethidium bromide stained agarose gels (37, 38). 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.

C. Primer Design

PCR primers generally range in length from 15–30 bases and are designed to flank the region of interest. Primers should contain 40–60% (G + C) 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.

D. 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.

E. Hot Start PCR

Certain unwanted amplifications can occur in PCR, and these usually begin at room temperature once all reaction components are mixed. These unwanted reactions, such as nonspecific amplification and primer-dimer formation, can be avoided or reduced by "hot start" amplification. In general, hot start techniques limit the availability of one necessary reaction component until a temperature >60°C is reached. This can be done manually by the addition of the critical component once the reaction mixture reaches the higher temperature. However, this is tedious and can increase the chances of introducing contaminants into the reaction. Other approaches include using an antibody to inactivate the polymerase at lower temperatures. At higher temperatures, the antibody is denatured and binding is reversed, releasing a functional polymerase (39).

F. Primer Annealing Temperature

The sequences 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 (40, 41), 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:

Tm = 81.5 + 16.6 (log10[Na+]) + 0.41 (%G+C) – 675/n

where $[Na^+]$ is the molar concentration of monovalent cations and n = number of bases in the oligonucleotide.

G. Extension Temperature

During the extension step, allow approximately 1 minute for every 1kb to be amplified (minimum extension time of 1 minute). Generally, 25–40 cycles are sufficient for most reactions.

1.2.12 Restriction Fragment Length Polymorphism (RFLP)

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. This variation is basically differences in the number of repeats (of nucleotides) in the same location. However, polymorphism may be relative in that the loci or gene may be variable in one strain but not another, or in one individual (heterozygous) but not in another (homozygous).

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. This is the oldest DNA-based method for finding polymorphic loci, (which are difficult to find using this methodology), and the analysis may be awkward.

Restriction Fragment Length Polymorphism (RFLP) is a technique in which organisms may be differentiated by analysis of patterns derived from cleavage of their DNA. If two organisms differ in the distance between sites of cleavage of a particular restriction endonuclease, the length of the fragments produced will differ when the DNA is digested with a restriction enzyme. The similarity of the patterns generated can be used to differentiate species (and even strains) from one another.

1.2.13 Radioimmunoassay

Radioimmunoassay (RIA) is a technique that traditionally has been used for detection of trace amounts in the nanomolar and picomolar range of various substances with the help of radioactively labeled molecules. Several other radioactive labels including ^{125}I, ^{3}H, ^{35}S, ^{32}P and ^{14}C are used to label biological substance. In the medical clinic, commercially available RIA tests are used daily to determine the concentration of circulating biological substances. Radioimmunoassays are based on the reaction between an antibody and an antigen whose concentration has to be quantified. There are several ways to quantify the antigen concentration but the most frequently used method is the indirect assay. In this assay a known quantity of radioactively labeled antigen is mixed with a dilution series of unlabelled antigen. The dilution series is brought to reaction with a fixed amount of antibody specific against the antigen. Since unlabelled and radioactively labeled antigens compete with each other for the antibody binding sites, a high concentration of unlabelled antigen will result in little radioactive antigen bound to the antibody and vice versa. After a fixed time, a second antibody directed against the first antibody is used which leads to the formation of large complexes which upon centrifugation are counted with a radioactive counter. This fraction contains the unlabelled antigens and the radioactive antigen which has bound to the specific antibody, while the supernatant in the centrifugate contains the unbound antigen. The serially diluted probes yield points on a curve relating radioactive counts to the concentration of unlabelled antigen: a so-called (unlabelled) reference curve. Using this reference curve, an unknown quantity of antigen in a probe can be quantified by performing the same reactions with first specific, then unspecific antibody and a fixed amount of radioactive antigen. Identification of the radioactive counts in the centrifugate and use of the reference curve yields the unknown antigen concentration.

1.3 OBJECTIVES

1. To determine the association between IL-1β gene mutation with pepsinogen level of gastric cancer in northern Thai population.

2. To examine the correlation between pepsinogen level and risk for peptic cancer in northern Thai population.

3. To determine the association between IL-1β gene mutation and risk of gastric cancer in northern Thai population.