CHAPTER IV
MATERIALS AND METHODS

1. Materials

1.1 Instruments: All instruments used in this thesis were kindly provided by Department of Preclinical Science, Faculty of Medicine, Thammasat University (Table 10)

Table 10 Instruments for DNA extraction, DNA amplification and gel electrophoresis

Instrument	**Company**
Thermocycler	Perkin-Elmer (USA)
Horizontal gel electrophoresis	Bio-Rad Laboratories, Inc. (USA)
Power supply	Bio-Rad Laboratories, Inc. (USA)
Polaroid camera	Spectronics Corporation (USA)
UV transilluminator	Spectronics Corporation (USA)
High speed centrifuge	Sigma-Aldrich Pte., Ltd. (USA)
Heat box	Boekel Scientific (Germany)
Vortex mixer	Scientific Industries, Inc. (USA)
Low speed centrifuge	Qualitron, Inc. Ce. (USA)
Automatic pipette	Eppendorf (Germany)

1.2 Chemicals: All chemicals (except DNA ladder and dNTPs) are analytical grade (Table 11)

Table 11 Chemicals for DNA amplification and gel electrophoresis

Chemical	Source
Agarose	Sigma-Aldrich Pte., Ltd. (USA)
Boric acid	Merck (USA)
Bromophenol blue	Sigma-Aldrich Pte., Ltd. (USA)
Ethidium bromide	Sigma-Aldrich Pte., Ltd. (USA)
Ethylenediaminetetraacetic acid di-sodium salt (EDTA)	Merck (USA)
Tris base (hydroxymethyl aminomethane)	Sigma-Aldrich Pte., Ltd. (USA)
100 bp DNA ladder	New England Biolabs (USA)
dNTPs (dATP, dGTP, dCTP, dTTP)	Promega (USA)
Metaphor agarose	Cambrex (USA)

1.3 Enzymes: Restriction endonucleases were provided with their 10X reaction buffers. For *Taq* DNA polymerase, 10X PCR buffer and 25 mM $MgCl_2$ were included in the package (Table 12).

Table 12 Restriction endonucleases and thermophilic DNA polymerase

Enzyme	Source
*Bsm*AI	New England Biolabs (USA)
*Aci*I	New England Biolabs (USA)
Taq DNA polymerase	Promega (USA)

1.4 Primers

Oligonucleotide primers were synthesized by Bioservice Unit, National Science and Technology Development Agency (Table 13).

Table 13 Primers for DNA amplification

Primer	Sequence (5' to 3')	Product size	Reference
Human GSTP exon 5 primers		192 bp.	Menegon *et al*., 1998
hGSTP1	CTC TAT GGG AAG GAC CAG CAG GAG		
hGSTP2	CAA GCC ACC TGA GGG GTA AGG		
Human GSTP exon 6 primers		216 bp.	Menegon *et al*., 1998
hGSTP3	GTT GTG GGG AGC AAG CAG AGG		
hGSTP4	CAC AAT GAA GGT CTT GCC TCC C		
H. pylori glmM primers		294 bp.	Bickley *et al*., 1993
HPglmM 1	AAG CTT TTA GGG GTG TTA GGG GTT T		
HPglmM 2	AAG CTT ACT TTC TAA CAC TAA CGC		

1.5 DNA extraction kit

QIAamp DNA extraction kit was purchased from Qiagen (USA).

1.6 Specimens

There were two sources of genomic DNA in this study. For determination of gene and genotype frequencies of the GSTP in normal population, gDNA were extracted from peripheral blood from 100 Thai volunteers (50 male, 50 female; age 18-26 years, mean 20.29 ± 1.78). For study of an association between GSTP genotype and gastric lesion, gDNA were extracted from the gastric biopsied specimens obtained from 308 dyspeptic patients (146 male, 162 female; age 16-90 years, mean 46.89 ± 17.11 years) who came for gastroduodenal endoscopy in the Division of Surgery, Thammasat Hospital, from year 2000 to 2001. All cases had clinical dyspepsia. In each patient, endoscopic diagnosis was recorded and 3-4 biopsied specimens were taken from greater curvature of the antrum close to the pyloric ring. The use of these specimens for research was approved by the internal review board of the Faculty of Medicine, Thammasat University, Thailand.

2. Methods

2.1 Reagent preparation

Preparation of general solutions were carried out according to a standard protocol (Sambrook and Russell, 2001)

2.2 DNA extraction

Genomic DNA samples were extracted from either peripheral leucocytes or gastric biopsies using QIAamp DNA Mini kit (Qiagen, USA) according to the manufacturer's recommendation. The procedure was briefly described below. The ALT lysis buffer and proteinase K solution were added into a sample tube and mixed well by vortex mixer. The reaction was incubated at 56^{o}C until the sample was completely lysed (about 3 hr). Then the AL buffer was added and further incubated at 70^{o}C for 10 min to stop the reaction of proteinase K. Absolute ethanol was added into this solution, mixed well and then transferred into a QIAamp spin column to bind DNA to the resin. Next step, the bound DNA was washed using AW1 and AW2 washing buffers by centrifugation. In the final step, the DNA was eluted from the spin column using the AE elution buffer. The isolated DNA was stored at -20^{o}C until use.

2.3 DNA amplification of exons 5 and 6 of the *GSTP* gene

Exons 5 and 6 of the *GSTP* gene were amplified in a final volume of 50 µl reaction that consists of 50-100 ng DNA template, PCR buffer (50 mM KCl, 20 mM Tris-HCl pH 8.0, 0.05% Tween 20), 1.5 mM $MgCl_2$, 60 µM dNTPs, 0.2 µM each primer and 1.25 units *Taq* DNA polymerase. The PCR conditions for amplification of both exons were 95^{o}C; 40 sec, 65^{o}C; 40 sec, 72 ^{o}C; 40 sec (40 cycles). The primers of exon 5 of the *GSTP* gene were 5'-CTCTATGGGAAGGACCAGCAGGAG-3'and 5'-CAAGCCACCTGAGGGGTAAGG-3' and the primers of exon 6 were 5'-GTTGTGGGGAGCAAGCAGAGG-3' and 5'-CACAATGAAGGTCTTGCCTCCC-3' (Bickley *et al*., 1993; Lu *et al*., 1999). Detection of the GSTP PCR product was carried out by 1.5% agarose gel electrophoresis in 1X TBE buffer (90 mM Tris-borate, 2 mM EDTA, pH 8.0).

2.4 GSTP genotyping

PCR-restriction fragment length polymorphism (PCR-RFLP) was performed to investigate the GSTP genotype. For exon 5, after the DNA amplification process, the PCR product (~20 µl) was digested with 5 units of *Bsm*AI restriction endonuclease in a total volume of 25 µl. The *Bsm*AI enzyme recognizes a specific nucleotide sequence that was shown in Table 14. The digested reaction was incubated at 55^{o}C for 3 hr and then the digested product was separated by electrophoresis on an ethidium bromide containing 3% Metaphor agarose gel in 1X TBE buffer, at 50 volts for 2.5 hr.

In similar manner, determination of GSTP exon 6 genotype was also investigated by PCR-RFLP technique. The *Aci*I endonuclease was used to digest the amplified exon 6 at 37^{o}C and the digested products were subjected to 3% Metaphor agarose gel as described above.

Table 14 The recognition site of restriction endonucleases used in this study

Restriction endonuclease	**Recognized site**
*Bsm*AI	▼ 5'…GTCTC$(N)_1$…3' 3'…CAGAG$(N)_5$…5' ▲
*Aci*I	▼ 5'…CCGC…3' 3'…GGCG…5' ▲

2.5 *Helicobacter pylori* detection by DNA amplification of the bacterial *phosphoglucosamine mutase* (*hpglm*M) gene

To detect *H. pylori* in the gastric biopsied sample, a PCR was performed using a pair of primers bound specifically to *H. pylori ure*C gene (5'-AAGCTTTTAGGGGTGTTAGGGGTTT-3' and 5'-AAGCTTACTTTCTAACACTAACGC-3') that was renamed the *phosphoglucosamine mutase* (*glm*M) gene (De Reuse *et al*., 1997). The primers for the exon 5 of human pi class glutathione S-transferase (GSTP) were also added in the reaction as an internal control. The 50 µl PCR reaction consists of 50-100 ng DNA, PCR buffer (50 mM KCl, 20 mM Tris-HCl pH 8.0, 0.05% Tween 20), 1.5 mM $MgCl_2$, 60 µM dNTPs, 0.2 µM each primer and 1.25 units *Taq* DNA polymerase. The PCR condition was 95^{o}C; 40 sec, 55^{o}C; 40 sec, 72^{o}C; 40 sec (45 cycles). Detection of the PCR product was done by 1.5% agarose gel electrophoresis in 1X TBE buffer, at 100 volts for 45 min.

2.6 Statistical analysis

Data was analyzed using the computer software SPSS for windows (version 13). Chi-square test was used to test the significant difference of the GSTP genotype frequencies between observed and expected values. In order to test the association between the GSTP genotype and gastric pathology, the relative risk of the genotype was evaluated by the odds ratios (OR), 95% confidence intervals (CIs). The OR was defined as the odds of case (patients having the susceptible genotype) divided by odds of controls (individuals having the susceptible genotype). The limitation of statistical significance was set at $p < 0.05$.