CHAPTER II

RESEARCH DESIGN AND METHODS

2.1 RESEARCH DESIGN

DNA was isolated from blood samples using standard procedures with sodium dodecyl sulfate (SDS)-proteinase K digestion and cold ethanol extraction. To analyze the IL-1B promoter mutation, a fragment was Polymerase Chain Reaction (PCR) amplified to cover of -511 from the transcription start site of IL-1B that representing C-T base transition. The PCR products were then precipitation and use as the subject for restriction enzyme *Ava*I, which recognize the sequence C...PyCGPu...G, in order to screen out both normal control and gastric cancer patients. The samples that shown partially cut, expected to have homozygous C-T base transition or uncut were expected to have heterozygous C-T base transition. All PCR products and product of *Ava*I-cut were analyzed by using electrophoresis on 12% acrylamide gel and the DNA band was detected under UV light.

The plasma levels of pepsinogen I and II were analyzed in this study was carry out by modified radioimmunoassay method and the ratio of pepsinogen I to pepsinogen II (PG I/PG II) was calculated.

The method of each procedure was given in detail later in this chapter. The diagram below shows the whole procedure orderly.

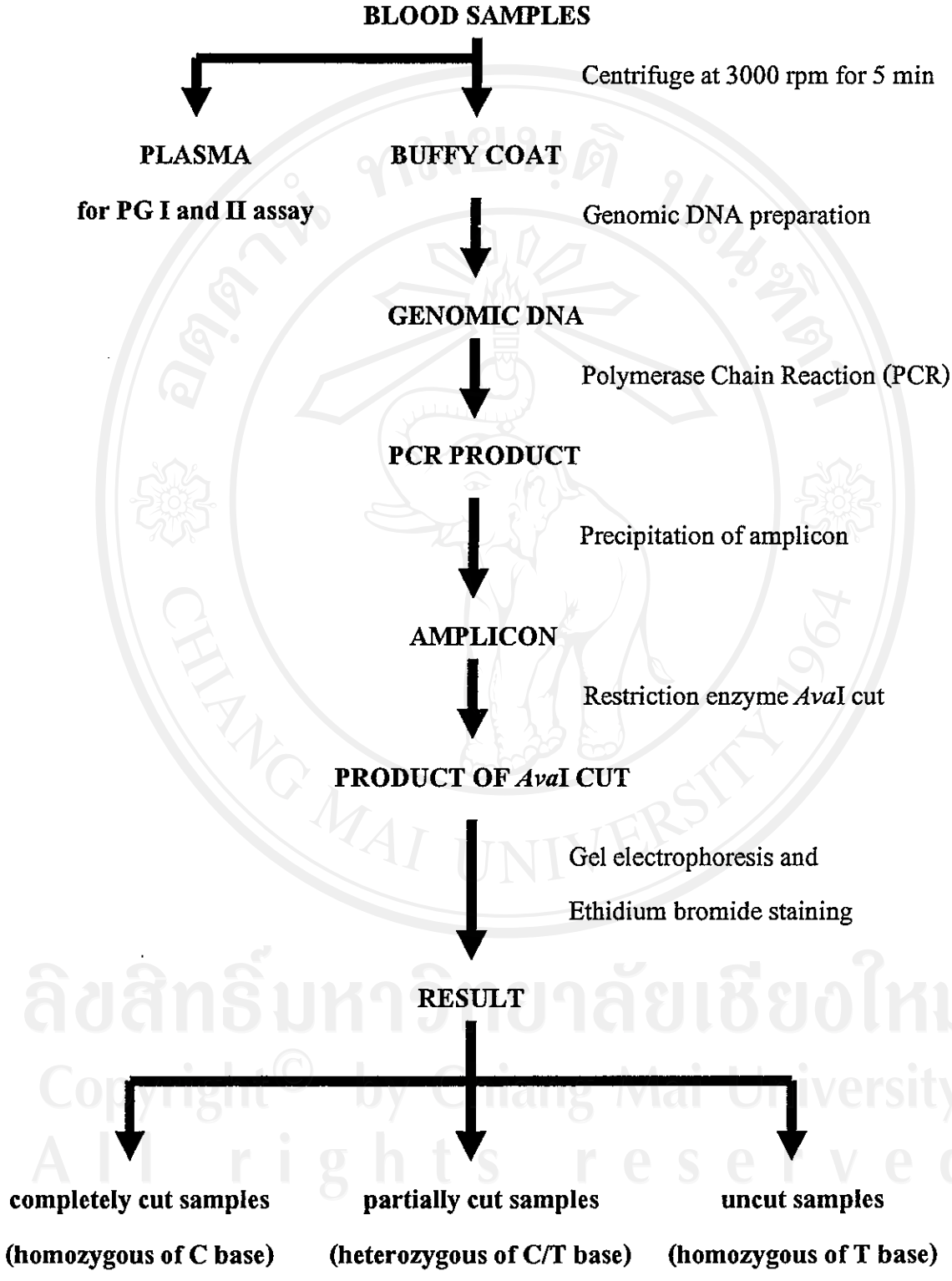

Figure 12. The whole scheme of interleukin-1β genotype determination from the blood sample.

2.2 METHODS

2.2.1 Blood samples

The peripheral venous bloods were collected from 41 consecutive patients with gastric cancer undergoing investigations prior to surgery, and from a control group of 89 selected people undergoing upper gastrointestinal endoscopies as part of routine investigation of their upper gastrointestinal symptoms, from Department of Surgery, Maharaj Nakorn Chiang Mai hospital, Chiang Mai University. None of the controls had any evidence of malignancies. All the subjects lived in northern Thailand. The 10 ml of whole bloods were mixed with 100 µl of 0.5 M Ethylenediaminetetra-acetic acid (EDTA) by inverting the tube for several times then kept in ice-bath until DNA extraction was performed. After the whole blood centrifugation step, the plasma was separated and kept at -20 °C for pepsinogens level screening.

2.2.2 Genomic DNA Preparation

Genomic DNA preparation was modified from the method described by Miller et al.(1988). The first step was to separate nucleated cells from 10 ml of the whole blood. White blood cell, the major nucleated cells in blood, were kept from the buffy coat between plasma and packed red cells after centrifugation the whole blood at 3000 rpm for 5 minutes. Then they were washed with 10 ml of Red Cell Lysis buffer in order to get rid of the intact red blood cells. The mixture was subsequently centrifuged at 2,500 rpm for 10 minutes and the supernatant was discarded. The step was repeated once or twice to ensure the purity of the cells.

The pellet, which contained white blood cells, was then resuspending in 8 ml Nuclei Lysis buffer. After mixing gently with 0.5 ml of 10% Sodium Dodecyl sulfate (SDS), the cells were lysed and the mixture become viscous. The mixture was added with 0.5 ml Proteinase K solution and incubated in water bath at 56 °C overnight. The mixture was then taken another step of protein separation. The digested protein was separated by salting out with 5 M. NaCl at the ratio of 1 to 4 volumes of the mixture. To complete protein precipitation, the mixture was kept at 4 °C for 20 minutes. Then, it was centrifuge at 4 °C, 3000 rpm for 15 minutes. The clear supernatant was subsequently transfer to new tube and was added two volumes of ice-cold absolute ethanol. Invert the tube immediately and gently. DNA was precipitated and suspended in the solution. The white fluffy DNA was then hooked out, rinsed 3 times with 70% ethanol to wash the excess salt,

and kept dry at room temperature for 30 minutes. Finally, the DNA was dissolved in 0.5 ml of 10 mM Tris-base and 0.1 mM EDTA (TE) buffer and was ready to use for the experiment.

The concentration of DNA was estimated by spectrophotometric determination. An OD of 1 at wavelength of 260 nm corresponds to approximately 50 μg/ml for double-stranded DNA. The obtained DNA was diluted to 1% solution in TE buffer before measuring. The concentration of DNA was calculated by use the formula as below:

Concentration of DNA(μg/ml) = O.D. 260 x 50 x dilution factor

2.2.3 Polymerase Chain Reaction System

Primer Selection

For IL-1B, the cytogenetic map position of the cluster was found to be 2q13-21 consisted of six introns and seven exons as shown in figure12. To determined C-T base transition at positions -511 base pair from the transcriptional start site. There are using the two of specific primers were design for amplified genomic DNA and give a 155 bp of amplicon. The sequence of primer name SKF, representing forward primer position 4699-4718 at the promoter 5'- GCC ACT TGG GTA GTA ATC GA -3' were stocked at concentration 165 pmol/μl and the SKR, representing reverse primer 5'- AGG ATT TTC TTG GCC TGG CT -3' were stocked at concentration 166 pmol/μl, both primers were synthesized by Bio Basic Inc.

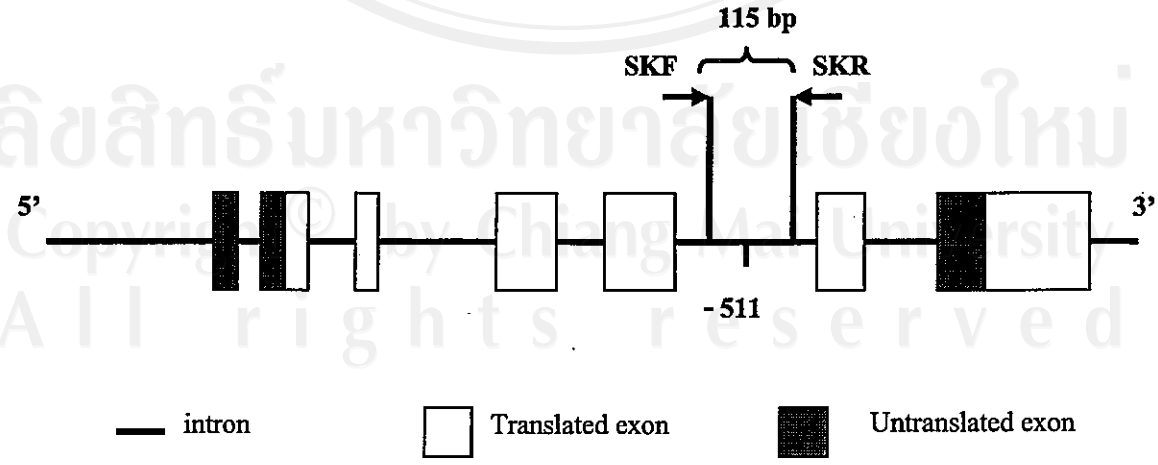

Figure 13. The location of primers for PCR to detection C-T base transition at positions -511 base pair from the transcriptional start site of interleukin-1β gene. SKF = forward primer, position 4699-4718 at the promoter, and SKR = reverse primer, position 4854-4873 at the promoter.

Figure 14. Normal IL-1 B seguence of intron 5, and the position of the primer in the IL-1 B gene. The sequences obtained from Gene Bank Locus HAS000002.

4119 gtaaatggaa acatcctggt ttccctgcct ggcctcctgg cagcttgcta attctccatg

4179 ttttaaacaa agtagaaagt taatttaagg caaatgatca acacaagtga aaaaaaatat

4249 taaaaaggaa tatacaaact ttggtcctag aaatggcaca tttgattgca ctggccagtg

4319 catttgttaa caggagtgtg accctgagaa attagacggc tcaagcactc ccagaccatg

4389 tccacccaag tctcttgggc atagtgcaat gtcaattctg ccacaatatg ggtcatttga

4459 tgacatgcct aactgcctgt gggttctctc ttcctgttgt tgaggctgaa acaagagtgc

4529 tggagcgata atgtgtccat cccctcccc agtcttcccc ccttgcccca acatccgtcc

4599 cacccaatgc caggtggttc cttgtaggga aattttaccg cccagcagga acttatatct

4669 ctccgctgta acgggcaaaa gtttcaagtg **cggtgaaccc atcattagct** gtggtgatct

4739 gcctggcatc gtgccacagt agccaaagcc tctgcacagg agtgtgggca actaaggctg

4809 ctgactttga aggacagcct cactcagggg gaagctattt gctctc**agcc aggccaagaa**

4879 **aatcct**gttt ctttggaatc gggtagtaag agtgatccca gggcctccaa ttgacactgc

4949 tgtgactgag gaagatcaaa atgagtgtct ctctttggag ccactttccc agctcagcct

4989 ctcctctccc agtttcttcc catgggctact tctctgttcc tgaaacagtt ctggtgcctg

5059 atttctggca gaagtacagc ttcacctctt tcctttcctt ccacattgat caagttgttc

5129 cgctcctgtg gatgggcaca ttgccagcca gtgacacaat ggcttccttc ctgtagtcta

5199 caccgacgcg ttaccttcca ccccgtggtg aagacctccg ttcctactgc tatgaggctc

5269 tgagaaaccc tcaggccttt gaggggaaac cctaaatcaa caaaatgacc ctgctattgt

5339 ctgtgagaag tcaagttatc ctgtgtctta ggccaaggaa cctcactgtg ggttcccaca

5409 gaggctacca aattacatgt atcctactca tggggcctag gggttggggt gaccctgcac

5479 tgctgtgtcc ctaaccacaa gacccccttc tttcttcag

Each amplification reaction was carried out in a 0.2 ml. microcentrifuge tube, each tube contained the PCR components as follow:

10 x buffer	5.0	µl
25 mM $MgCl_2$	5.0	µl
2 mM dNTPs	5.0	µl
Forward and Reverse primer solution (50 pmol each)	3.0	µl
genomic DNA solution (0.5-1 µg)	1.0	µl
Taq DNA polymerase (2.5 unit)	0.5	µl
Sterile deionized water to	50.0	µl

The reaction mixture above was taken to perform in a Mastercycle gradient (Eppendorf). The condition for denaturation, annealing and polymerization were as follow.

Cycle	Temperature (°C)	Time (min)
First cycle	94	5
2nd to 30th denaturing	94	1
annealing	64	1
extension	74	1
Final extension	74	7

The reaction mixture was soaked at 4°C until it was kept. The PCR products were then analyzed by 2% agarose gel eletrophoresis stained with ethidium bromide. The 100-bp DNA ladder was used as molecular-size standard.

2.2.4 The precipitation of Amplicon

The amplicon was partially purified before any step of restriction enzyme cut. First, the 5 M NaCl at the ratio of 1 to 5 volumes of the mixture was added in order to removed proteins from PCR condition solution. Then cold absolute ethanol at the ratio of 2.5 to 1 volumes of the mixture was dispensed in each tube, mixed throughly and kept at –20 °C overnight to ensure the precipitation of amplicon. The precipitated amplicon was recovered by centrifugation at 12,000

rpm for 30 minutes. The supernatant was removed and the pellet was 2-3 times rinsed with 70% ethanol to wash the excess salt, and kept dry at room temperature until it was completely dry. Then 10 µl of sterile deionized water was added to dissolve the pellet.

2.2.5 Agarose Gel Electrophoresis

The amplicons were check the size by 2% agarose gel electrophoresis. The 0.6 gm of agarose powder was boiled in 30 ml of 0.5X TBE buffer and the gel were poured into the mold when the solution cooled down to 55-60°C. The teeth of the comb were 5 mm-wide and 1 mm-thick was put in the melted gel to prepare the slots. After the gel was completely set (30-45 minutes at room temperature), carefully removed the comb and the sealing edge of the mold. Then 0.5X TBE buffer was poured into electrophoresis tank in the amount of buffer that could cover the gel to a depth of about 5 mm. Five microliters of amplicon was mixed with 2 µl of loading buffer and then were slowly loaded into the slots using and automatic micropipettor. Electrophoresis was carried out in at 100 V for 1 hour (8 V/cm). After electrophoresis, the gel was submerged in 0.5 µg/ml ethidium bromide solution for 25-30 minutes and then was destained in distilled water for 15-20 minutes for 3 times. The DNA bands were detected by UV light and documented by using a Bio-Rad gel doc 1000 system.

2.2.6 Restriction Enzyme *Ava* I Cut

The total 15 µl *Ava*I reaction mixture was contained 2 µl of partially purified amplicon, 2.5 units of restriction enzyme *Ava*I (GIBCOBRL), 1.5 µl of 10X buffer and sufficient amount of sterile deionized water Figure 15.The reaction mixture was incubated at 37 °C overnight for completely cut before analyzing with 12% acrylamide gel electrophoresis.

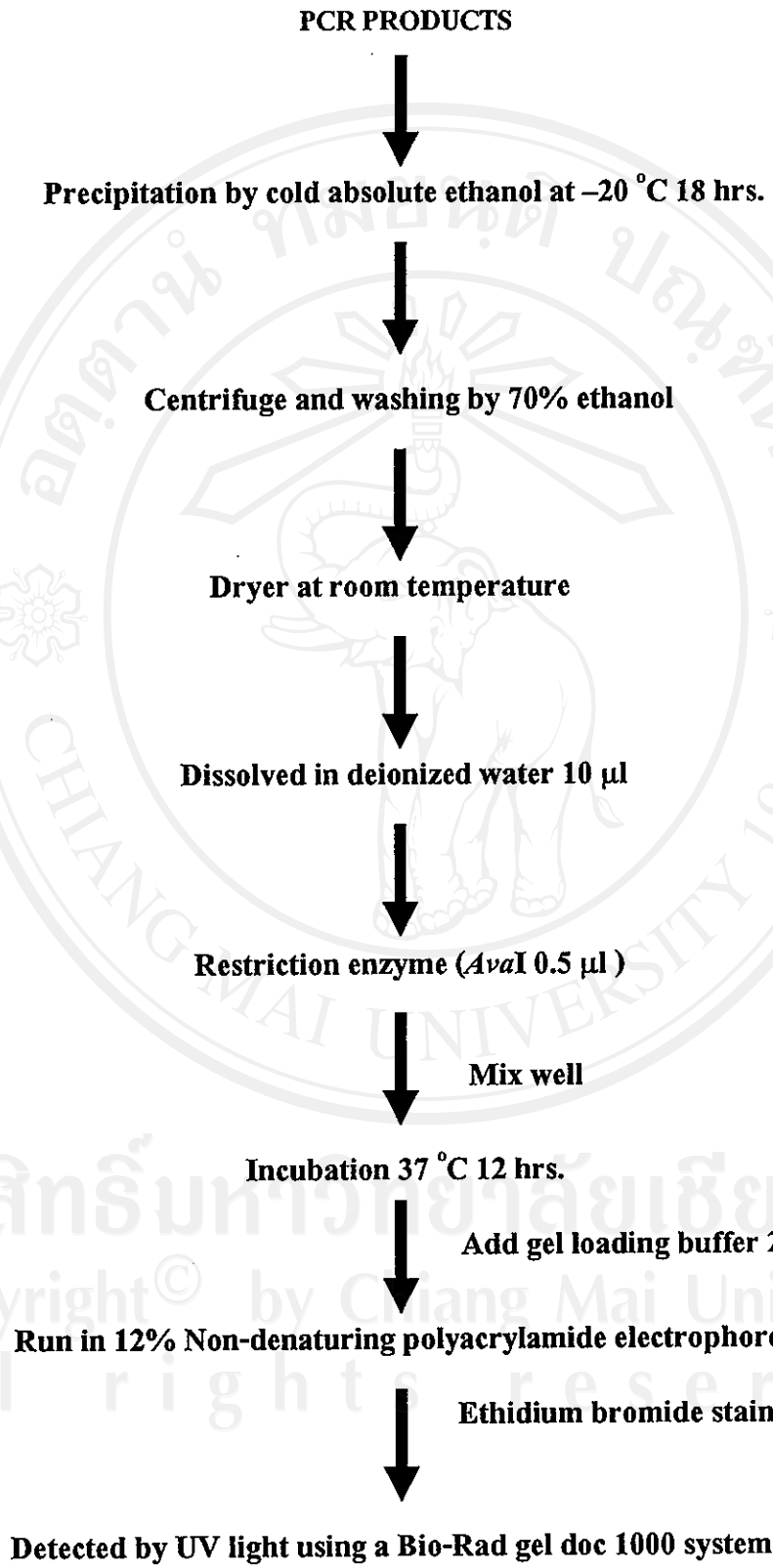

Figure 15. The scheme of restriction fragment length polymorphism (RFLP) procedure.

2.2.7 **Polyacrylamide Gel Electrophoresis**

The product of *Ava*I-cut were analyzed by 12% Polyacrylamide Gel electrophoresis. Model Mighty Small II SE250/SE260 (Hoefer) were used. Acrylamide is a neurotoxin and should be handled with caution. Wear gloves at all times when handling acrylamide and be careful to avoid spills. First, clean the multiple gel caster and place flat on the bench top in front of you. Place the rubber gasket in its groove without stretching it and lubricate with a thin layer of the Cello-seal provided by Hoefer. Second, build the gel casting units by carefully placing and seating components in the following order from the bottom up: waxed paper, notched alumina plate, T-shaped spacers (0.75 or 1.0 mm), glass plate, waxed paper, etc. Approximately 5 complete 0.75 mm gels can be cast at one time with one or two additional glass plates needed to fill extra space. After that, place the top cover on the multiple gel caster and apply red spring clamps to side grooves, ensuring adequate sealing. Be sure that the port at the bottom of the front plate has a small piece of rubber tubing on it and is clamped off. Then mix the ingredients for 8 ml of acrylamide solution (3.2 ml of 30% polyacrylamide solution; 3.2 ml of deionized water and 1.6 ml of 5x TBE) in a clean beaker, as detailed in the recipe below for a 10% polyacrylamide gel. Add the TEMED with thorough mixing just before pouring the gels. Then carefully pour the acrylamide evenly into the gel casting units in the multiple gel caster until the multiple gel caster is almost overflowing. Finally insert the appropriate sized comb (5 mm-wide and 0.75 mm-thick) into each gel casting unit, and allow the acrylamide to polymerize for at least 1 hour. After complete polymerization, the gels may be wrapped in cellophane and stored at 4 °C.

Attached the gel to the electrophoresis tank. Then 1X TBE buffer was poured into electrophoresis tank in the amount of buffer that could cover the tank and checked the gel plate was submerged. Whole *Ava*I-cutting PCR product was mixed with 3 µl of loading buffer and then were slowly loaded into the slots using and automatic micropipettor. Electrophoresis was carried out in at 55 V for 4 hours (8 V/cm). After electrophoresis, the gel was submerged in 0.5 µg/ml ethidium bromide solution for 25-30 minutes and then was destained in distilled water for 15-20 minutes for 3 times. The DNA bands were visualized the fragmented DNA under UV light and documented by using a Bio-Rad gel doc 1000 system.

2.2.8 Radioimmunoassay for Plasma Pepsinogen

Pepsinogen values were measured using modified radioimmunoassay provided from Dr. Takeshi Matsuhisa, Tama-Nakayama center, Nippon Medical School. The plasma sample was mixed with the pepsinogen antibody bead and the tracer (^{125}I-lebelled pepsinogen antibody). The reagents were agitated for 3 hrs at room temperature, and wash with distilled water. The radioactivity of the beads was then count. These method was applied from competitive binding double antibody radioimmunoassay as previously described by Samloff IM(42).

2.2.9 Statistical Analysis

The serum pepsinogen levels content for benign gastritis groups and gastric cancer groups were expressed as median with interquartile ranges. The effect of the IL-1B -511 on risk of genetic cancer were expressed as odd ratios (OR) with 95% confidence intervals (95%CI). Differences in serum pepsinogens among different IL-1B -511 genotype groups or among benign gastritis and gastric cancer examined by Kruskal-Wallis test, followed by Man-Whitney *U* test. All *p* values were 2-sided, and only values < 0.05 were considered statistically significant.