CHAPTER III

MATERIALS AND METHODS

Subjects

The subjects of this study were ninety children with acute lymphoblastic leukemia in remission maintenance and off therapy at King Chulalongkorn Memorial hospital and Ramathibodi hospital (Table 2). Ethic approval of this study was obtained from both Ethics Committees of King Chulalongkorn Memorial hospital and Faculty of Pharmaceutical Sciences Chulalongkorn university. Informed consent was received from all participants. Exclusion criteria were hepatic and renal problems, and receiving blood transfusion.

Table.2 Characteristic of subjects

Hospital	Number	Gender	Age (year)	*Period of therapy
Chulalongkorn	53	M= 28	Average = 3-18	ϒoff = 8
			(10.46±4.67)	#maintenance = 20
		F = 25	Average = 1-15	off = 12
			(8.24±3.66)	maintenance = 13
Ramathibodi	37	M = 25	Average = 3-17	off = 7
			(9.0±4.46)	maintenance = 18
		F = 12	Average = 3-15	off = none
			(6.81±4.26)	maintenance = 12

*Period of therapy = treatment (#maintenance phase) or no treatment (ϒoff drug)

M = male; F = female

Solution preparation

1. 6-Mercaptopurine solution at concentration 18 milligram per milliliters (mg/ml)

   6-Mercaptopurine 180 mg was dissolved in 10 ml of Dimethylsulfoxide (DMSO) and divided into a small package of 1 ml which protected from light and kept at 4 °C.

2. Chelex-100 suspensions

   The chelex-100 was prepared by the addition of concentrated hydrochloric acid to the resin to a final pH of 1.5. The chelex was then rinsed 3 times with distilled water; 1 N NaOH was added until the pH of the slurry was 7.5; the suspension was washed 4 times with distilled water and was stored at 4°C in distilled water.

3. Potassium phosphate buffer pH 7.5 (Parida, 1997)

   - 0.2 M potassium phosphate solution was prepared by weighing 27.22 mg potassium phosphate monobasic and dissolved in one liter of distilled water.

   - 0.2 M Sodium hydroxide solution was prepared by weighing 8 g sodium hydroxide and dissolved in one liter distilled water.

   - 50 ml of potassium phosphate solution was mixed with 41.5 ml of sodium hydroxide solution was then adjusted to 200 ml with distilled water, and at 4 °C.

Chemicals

0.9% Normal saline solution

6-Mercaptopurine, 6-MP (Sigma, U.S.A.)

6-Methylmercaptopurine, 6-MMP (Sigma, U.S.A.)

Acetonitrile (HPLC grade)

Agarose gel (Gibco BRL)

Allopurinol (Sigma, U.S.A.)

Chelex-100 suspensions (Sigma, U.S.A.)

Deoxynucleoside triphosphates (dNTPs)

Dimethylsulfoxide, DMSO (Sigma, U.S.A.)

Distilled water

Dithiothreitol, DTT (Sigma, U.S.A.)

Ethylium bromide (Sigma, U.S.A.)

Ethyl acetate (AR grade)

Iced-cold water

Methanol (HPLC grade)

Nitrogen gas

O-phosphoric acid (Merck)

Potassium phosphate buffer saline

QIAamp® DNA Mini Kit

S-adenosyl-L-methionine, SAM (Sigma, U.S.A.)

Taq polymerase and mixture buffer (Fermantus)

Triethylamine, TE (AR grade)

Materials

Beaker

Bottle 1,000 ml

Centrifuge tube

Cylinder

Erlenmayer flask

PCR tube

Plaster pipet

Sample vial 2 ml and glass insert vial

Volumetric flask

Instruments

Automatic pipet (Pipetman Gilson®)

Column C_{18} (Alltech®) 250 mm. x 4.6 mm. with guard column

Digital balance (Mettler ®AJ 180)

Gel electrophoresis chamber (Bio-Rad®)

High Pressure Liquid Chromatography (Shimadzu®)/ LC-10AD pump/CBM-10A (communication bus module)/ SPD-10A(UV-VIS detector)/ SIL-10A(autoinjector)

High speed refrigerated centrifuge (Hitachi® Himac model CR20B3)

Hot air oven (Memmert®)

pH-meter (Beckman® Instruments, U.S.A.)

Polymerase chain reaction ,PCR (Perkin®Elmer Gene Amp 2400)

Refrigerated centrifuge (ALC® 4237R)

Shaker water bath adjust temperature at 37°C. (Heto®)

Shaker water bath adjust temperature at 4°C. (Hetofrig®)

Sonicator (Elma®, Germany)

Vacuum pump (GE® motors)

Vortex mixture (Clay® adam, U.S.A.)

Water bath

Methods

1. Preparing thiopurine methyltransferase from red blood cell

Principle

Thiopurine methyltransferase (TPMT) activity presents in red blood cell (RBC) was correlated with TPMT in other human tissues. So in the present investigation, TPMT activity was measured in to represent the activity of TPMT in ALL children.

Procedure

Three milliliters (ml) of whole blood was collected by venipuncture into EDTA vacutainer tubes. Red blood cell (RBC), white blood cell and plasma were separated by centrifugation at 4°C, 3,000 rpm for 10 minutes. The plasma and buffy coat (white blood cell) were discarded. Aliquot of 1.8 ml, 0.9% normal saline solution was added to the RBC, and the RBC were gently resuspended. After centrifugation at 4°C, 3,000 rpm for 10 minutes (min), supernatant was discarded. The RBC were washed twice with 0.9% normal saline solution. Finally, volume of RBC was adjusted to 3 ml with 0.9% normal saline solution. The hematocrit of resuspended RBC was measured. After that, it was centrifugation at 4°C, 3,000 rpm for 10 min, supernatant was discarded. The RBC were lysed by adding iced-cold water and volume was adjusted to 6 ml. The RBC lysate was centrifuged at 4°C, 13,000x g for 10 min, supernatant was removed and used for enzyme assays. TPMT activity was either measured immediately or stored at -80°C, under these conditions, the enzyme is stable for several weeks.

One milliliters of water was added to the buffy coat for lysis RBC which remaining in part of buffy coat. After centrifugation at 6,000 x g for 2 min, supernatant was discarded and the buffy coat were washed. DNA was separated from buffy coat by QIAamp® DNA Mini Kit. DNA was measured quantitatively by UV absorbance detector and can be stored at -20°C until analysis.

2. Increased sensitivity of thiopurine methyltransferase activity from erythrocyte by inhibition of Hypoxanthine Guanine Phosphoribosyltransferase (HGPRT)

Principle

6-Mercaptopurine (6-MP) is a substrate in studying thiopurine methyltransferase activity from erythrocyte. 6-MP is converted by several metabolic pathways e.g. xanthine oxidase, hypoxanthine guanine phosphorobosyltransferase (HGPRT) and thiopurine methyltransferase (TPMT).

Chelex-100, the chelating resin of magnesium was used to inhibit hypoxanthine guanine phosphorobosyltransferase which was a magnesium dependent enzyme and also presented in the erythrocyte. Inhibition of competitive metabolic pathway of 6-MP by HGPRT would indirectly increase the activity of TPMT.

Procedure

900 µl of erythrocyte lysate was chelated by adding 100 µl of Chelex-100 resin, followed by gentle rotation for 1 h at 4°C and centrifugated at 4°C, 7,000x g for 10 min, supernatant was removed and kept at -20°C until analysis.

3. Thiopurine methyltransferase activity measurement

Principle

Thiopurine methyltransferase activity in erythrocyte has been firstly measured by a radiochemical assay but more recently, HPLC methods have been published. All these methods are based on the in vitro conversion of 6-Mercaptopurine (6-MP) to 6-methylmercaptopurine (6-MMP), using S-adenosyl-L-methionine (SAM) as the methyl donor. Advance of HPLC methods is lesser danger and cost compare to radiochemical assay.

Procedure

3.1 Incubation conditions

Solutions of 6-MP in DMSO were prepared immediately prior to the incubation. Solutions of SAM, allopurinol and dithiothreitol in water were kept at -20°C. After slow thawing, 900 µl of erythrocyte lysate was chelated by adding 100 µl of Chelex-100 resin, followed by gentle rotation for 1 h at 4°C and centrifugated at 4°C, 7,000 x g for 10 min, supernatant was removed and kept at -20°C until analysis. Aliquots of 100 µl of supernatant were incubated with 25 µl potassium phosphate buffer (0.15 M, pH 7.5), 25 µl of a mixture containning SAM (final concentration 12 µM), allopurinol for inhibiting the xanthine oxidase pathway (final concentration 48µM), and dithiothreitol to protect the thiol group from oxidation (final concentration 1 mM), and 5 µl of 6-MP solution (final concentration 3.8 mM) in a final volume of 155 µl. The mixture was incubated for 1 h at 37 °C in a shaking water bath. For the extraction step, to each tube, added 7.0 ml of ethyl acetate. The tubes were mixed for 3 min, then centrifuged at 4°C, 3,000 rpm for 10 min. Five ml of the organic phase were removed and evaporated to dryness under nitrogen. The residue was dissolved in 200 µl of the mobile phase, transferred to a conical micro vial and placed in the autosampler for HPLC analysis and 50 µl was injected into the chromatograph.

3.2 HPLC conditions

Column: C_{18} (250mm. × 4mm × 5 µm) with guard column

Detector: UV wavelength 290 nm.

Flow rate: 1.5 ml/min

Mobile phase:

Water: Acetonitrile: Triethylamine = 90.7: 9.0 : 0.3 adjusted pH by O-phosphoric acid to 3.0 and added dithiothreitol (DTT) 0.1 g/L

4. Method Validation

Principle

Analytical method validation includes all of procedures recommended to demonstrate that a particular method for the quantitative measurement of an analyst in erythrocyte lysate is reliable and reproducible. The parameters essential to the validation include selectivity, linearity, recovery, within-run and between-run precision and accuracy, limit of quantification (LOQ) and limit of detection (LOD).

Procedure

4.1 Selectivity

Selectivity includes the ability to separate the analyst from degradation products, metabolites and co-administered drugs. Blank human erythrocyte lysate samples from six different human sources were evaluated to determine the presence of any interferences across the retention windows of 6-MMP.

4.2 Linearity

The linearity of an analytical method is its ability to elicit test results that are directly, or by a well defined mathematical transformation, proportional to the concentration of analyst in samples within a given range. Linearity can be expressed as a calibration curve which is the relationship between instrument response and known concentration of the analyst. A calibration curve should be prepared in the same biological matrix as the samples in the intended by spiking with known concentrations of the analyst.

The stock solution of 6-MMP in DMSO was prepared and appropriate volumes added to a 155 µl aliquots of blank erythrocyte lysate. The standard mixture of 6-MMP ranged 50-1000 ng/ml. Erythrocyte lysate standards were prepared and analyzed as described in section 3 under incubation condition. Peak area and concentrations of each analyst was

plotted and the relationship between these variables was explained by regression analysis.

4.3 Limit of quantification (LOQ) and Limit of detection (LOD)

Limit of detection (LOD) is the lowest concentration of an analyst in a sample that can be detected, but not necessary quantified under the stated conditions of the test.

Limit of Quantification (LOQ) is the lowest concentration of an analyst that can be determined with acceptable precision and accuracy under the stated conditions of the test.

Erythrocyte lysate standard containing 25 and 50 ng/ml of 6-MMP were prepared by serially spiking into 155 µl blank erythrocyte lysate. Standard were subjected to sample preparation and analyzed as described in section 3. The acceptable precision (%RSD) was 20% and accuracy (%Bias) was 20%.

4.4 Precision

The precision of a bioanalytical method is a measure of the random error and is defined as the agreement between replicate measurements of the same sample. Precision can be considered as having a within assay batch component or repeatability which defines the ability to repeat the same methodology with the same analyst, using the same reagents in a short interval of time, e.g. within a day. This is also known as intra-assay precision. The ability to repeat the same methodology under different conditions, e.g. change of analyst, reagents or equipment; or on subsequent occasions, e.g. loss several days or weeks, is covered by the between batch precision or reproducibility, also known as inter-assay precision.

- Intra-assay

Erythrocyte lysate samples spiked with 6-MMP at 50, 100 and 500ng/ml were prepared and analyzed as described in section 3.

- Inter-assay

The inter-assay was evaluated over three days with five replicates of erythrocyte lysate samples being prepared in the same manner as those described in intra-assay.

The precision is expressed as the percentage relative standard deviation (%RSD) of the replicate measurements. The %RSD value should be within ± 15% except at LOQ, where it should not deviate by more than ± 20%.

$$\% \text{ RSD} = (\text{standard deviation/mean}) \times 100$$

4.5 Accuracy

The accuracy of an analytical method is the closeness of mean test results obtained by the method to the true value of the analyst. The amount of analyst added and found in spiked erythrocyte lysate sample obtained from section 4.4 were used to calculate the accuracy of the developed method. Accuracy is best reported as percentage bias which is calculated from the expression:

$$\%\text{Bias} = [(\text{measured value- true value})/\text{true value}] \times 100$$

The accuracy of the method should be within ±15% at all concentrations.

4.6 Recovery

The recovery of an analyst in an assay is the detector response obtained from an amount of the analyst added to and extracted from erythrocyte lysate, compared to the detector response obtained for the true concentration of the pure standard. Recovery relates to the extraction efficiency of an analytical method within the limits of variability.

Set A : Five replicates of erythrolysate samples spiked with 6-MMP at 100, 250 and 500 ng/ml were prepared and carried out the entire procedure as described in section 3.

Set B : Five replicates of erythrolysate samples were prepared and extracted with the 7 ml of ethyl acetrate. The tubes were mixed for 3 min,

then centrifuged at 4°C, 3,000 rpm for 10 min. Five ml of the organic phase were removed and evaporated to dryness under nitrogen. The residue was dissolved in 200 µl of the mobile phase, transferred to a conical micro vial and placed in the autosample for HPLC analysis and 50 µl was injected into the chromatograph.

Extraction efficiency was calculated by comparing peak area obtained from spiked erythrolysate standard, set A with those obtained from set B. Values for absolute recovery of method not less than 50, 80 and 90% have all been used as numerical acceptance limits.

$$\text{Absolute recovery} = \frac{\text{response of analyte spiked into matrix (processed)}}{\text{response of analyte of pure standard (processed)}} \times 100$$

5. Detection of TPMT variants by Polymerase Chain Reaction (PCR)

Principle

The polymerase chain reaction (PCR) is an in vitro technique which allows the amplification of a specific deoxyribonucleic acid (DNA) region, that lies between two regions of known DNA sequence. PCR amplification of DNA is achieved by using oligonucleotide primers, also known as amplimers. These are short, single-stranded DNA molecules which are complementary to the ends of a defined sequence of DNA template. The primers are extended on single-stranded denatured DNA (template) by a DNA polymerase, in the presence of deoxynucleoside triphosphates (dNTPs) under suitable reaction conditions. This results in the synthesis of new DNA strands complementary to the template strands. These strands exist at this stage as double-stranded DNA molecules. Strand synthesis can be repeated by heat denaturation of the double-stranded DNA, annealing of primers by cooling the mixture and primer extension by DNA polymerase at a temperature suitable for the enzyme reaction. Each repetition of strand synthesis comprises a cycle of amplification. Each new DNA strand synthesized becomes a template for any further cycle of amplification and so the amplified target DNA sequence is selectively amplified cycle after cycle. (figure 10) The product of PCR is specifically cut by restriction enzyme that tailored with site of recognize. The final product is detected by gel electrophoresis method (Newton and Graham, 1997).

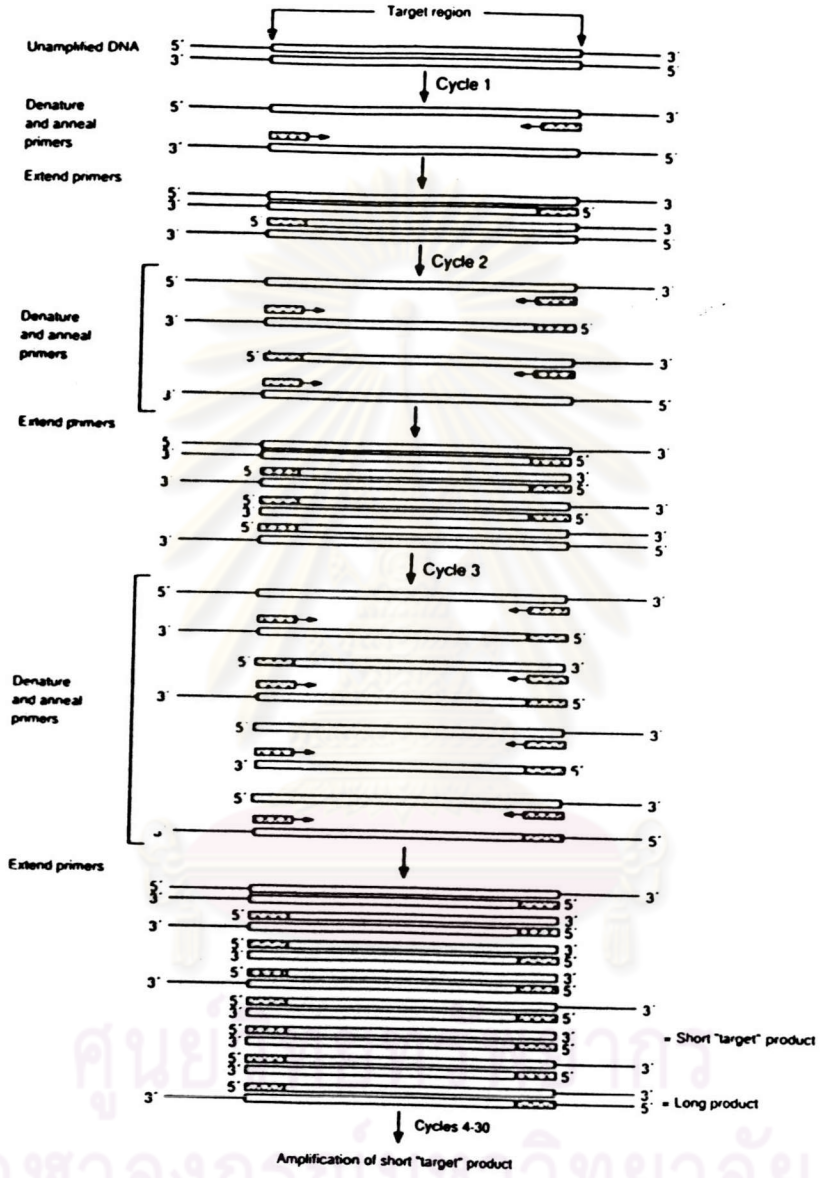

Figure.10 The polymerase chain reaction. PCR is a cycling process; with each cycle the number of DNA targets doubles. The stands in the targeted DNA are separated by thermal denaturation and then cooled to allow primers to anneal specifically to the target region. DNA polymerase is then used to extend the primers in the presence of the four dNTPs and suitable buffer. In this way duplicates of the original target region are produced and this 'cycle' is normally repeated for 20-40 cycles. The short 'target' products, which increase exponentially after the fourth cycle, and the long products, which increase linearly, are shown. From Newton and Graham, 1997.

Procedure

- Detection of *G238C*

To detect the *G238C* mutation, we used a PCR assay, using 0.1 μM of primers P2W (5′-GTATGATTTTATGCAGGTTTG-3′) or P2M (5′-GTATGATTTTATGCAGGTTTC-3′) with 0.1 μM of primer P2C (5′-TAAATAGGAACCATCGGACAC-3′). Genomic DNA, 500 ng, was amplified. We used 5 μl of 10xbuffer, 2.0 mM $MgCl_2$, 200 μM dNTPs and 1 unit Taq polymerase. The total volume of reaction was 50 μl. The PCR amplification was done for 30 cycles consisting of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 30 sec. Unpurified PCR products were analyzed by electrophoresis in 1.5% agarose gel stained with ethidium bromide. A DNA fragment was amplified with P2M and P2C primers when either C238 (variant) or G238 (wild type) was present (figure 11).

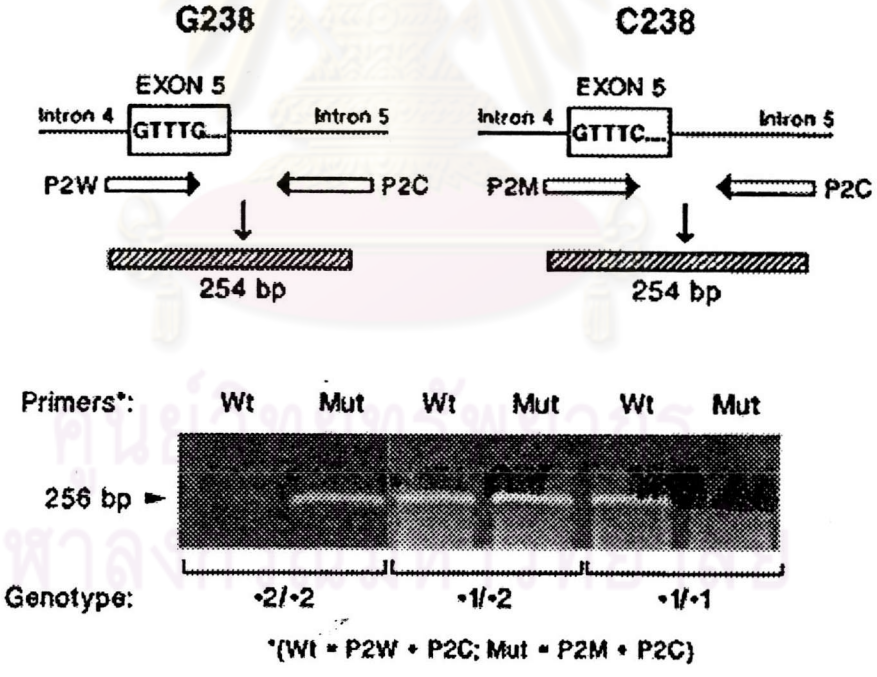

Figure.11 PCR product of G238C; Wt = wild-type, Mut =mutant.

- Detection of *G460A*

To detect the *G460A* mutation, we used a PCR assay, using 0.5 μM of primer P460F (5′-ATAACAGAGTGGGGAGGCTGC-3′) and P460R (5′-CTAGAACCCAGAAAAAGTATAG-3′). Genomic DNA, 500 ng, was amplified. We used 5 μl of 10x buffer, 1.5 mM $MgCl_2$, 200 μM dNTPs, and 1 unit Taq polymerase. The total volume of reaction was 50 μl. The PCR amplification was done for 30 cycles consisting of denaturation at 94°C for 30 sec, annealing at 63 °C for 30 sec, and extension at 72°C for 30 sec. The PCR product was digested with *Mwo* I (New England Biolabs) for 1 hr at 60°C. Digested products were analyzed using 1.5% agarose gel electrophoresis. *Mwo* I digestion of wild-type DNA yielded fragment of 267 and 98 base pairs, whereas DNA containing the G460A mutation was not digested and yielded an uncleaved fragment of 365 base pairs (figure 12).

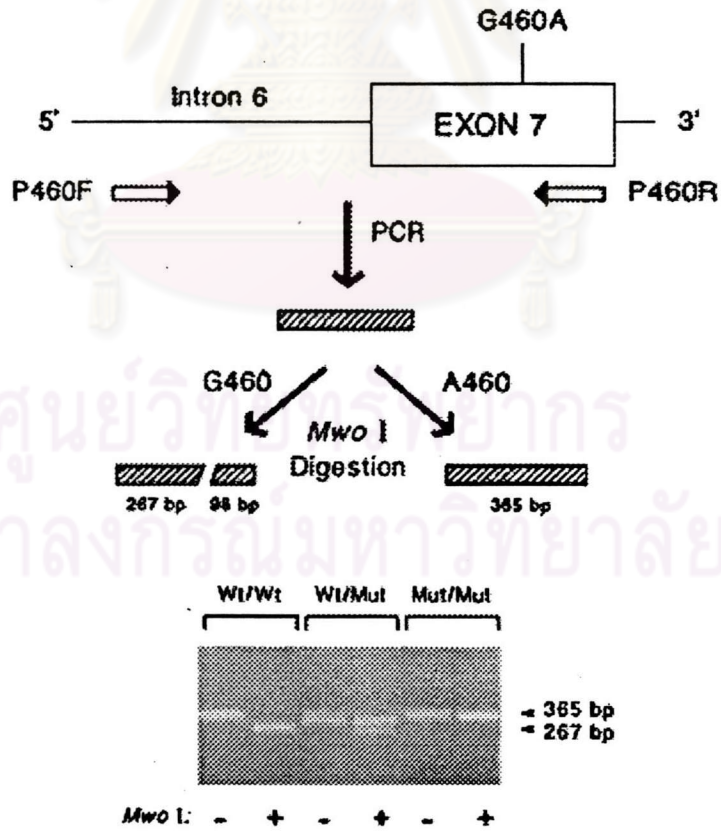

Figure.12 PCR product of G460A; Wt = wild-type, Mut =mutant

- Detection of *AA719G*

To detect the *A719G* mutation, we used a PCR assay, using 0.4 μM of primer P719R (5′-TGTTGGGATTACAGGTGTGAGCCAC-3′) and P719F (5′-CAGGCTTTAGCATAATTTTCAATTCCTC-3′). Genomic DNA 500 ng was amplified. We used 5 μL of 10x buffer, 2 mM $MgCl_2$, 200 μM DNTPs, and 1 unit Taq polymerase. The total volume of reaction was 50 μl. The PCR amplification was done for 30 cycles consisting of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 30 sec. The PCR product was digested with *Acc* I (New England Biolabs) for 2 hr at 37°C, and analyzed using 1.5% agarose gel electrophoresis. The *A719G* mutation introduces an *Acc* I restriction site in the amplified fragment and yields fragments of 207 and 86 base pairs. Wild-type DNA yields an uncleaved fragment of 293 base pairs (figure 13).

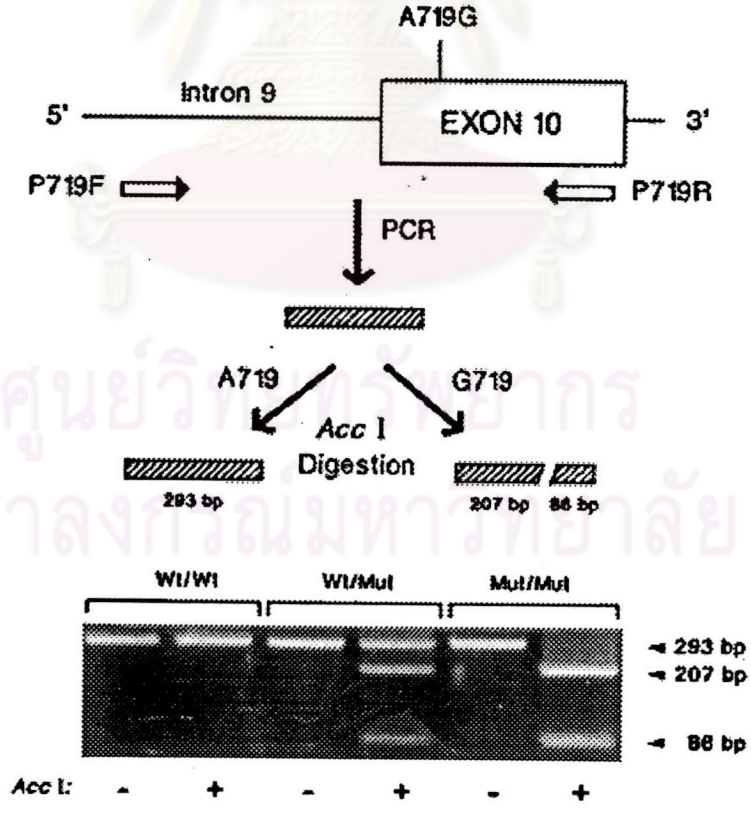

Figure.13 PCR product of A719G; Wt = wild-type, Mut =mutant.

6. Statistical Methods

TPMT activity is expressed in nanomoles of 6-MMP formed per milliliter of packed red blood cells per hour (nmol/h/ml PRBC). Results are expressed as mean ± standard deviation and probit analysis. Variations of TPMT mutant alleles were expressed with percentage. Data comparison was tested by t-test.