CHAPTER III

MATERIALS AND METHODS

1. Study group

Blood samples were taken randomly from 200 blood donors of Thai racial background, attending at the National Blood Centre, Thai Red Cross Society.

2. Genomic DNA preparation by QIAamp Blood Kits

Whole blood (200 µl) was pipetted into a 1.5-ml microfuge tube and 25 µl of protease and 200 µl buffer AL (both provided in the QIAamp blood extraction kit, QIAGEN, Hilden, Germany) were added to the sample. The sample was mixed immediately by vortexing for 15 seconds. The mixture was then incubated at 70 °C for 10 minutes. After incubation, 210 µl of ethanol (96-100%) was added to the sample, and it was mixed again by vortexing. The mixture was applied onto a QIAamp spin column and centrifuged at 8000 rpm for 1 minute. The QIAamp spin column was then sequentially washed twice by centrifugation with 500 µl of buffer AW. After a 1 minute incubation at room temperature, the genomic DNA were eluted with 200 µl of buffer AE preheated to 70 °C. The eluted DNA was stored at –70 °C until used.

3. Polymerase chain reaction (PCR) amplification

3.1 PCR for genotyping of the CCR2-64I polymorphism

Genomic DNA (2 µl) was used as the template for PCR amplification using primer CKR2_1A and CKR2_1Z [13] (Shown in table V) in a 50 µl PCR reaction mixture. The PCR conditions were as follows: 3 mM $MgCl_2$, 1x PCR buffer, 20µM dNTPs (Promega, USA), 15 µM of each primer , and 1.25 U *Taq* DNA polymerase (Promega, USA). After an initial denaturation at 95 °C for 2 min, the reactions were run for 40 cycles through a temperature profile of 95 °C for 30 seconds (denaturation),

55 °C for 30 seconds (annealing), and 72 °C for 30 seconds (extension). A final extension was performed at 72 °C for 7 minutes.

3.2 PCR for genotyping of the SDF1-3'A polymorphism

Genomic DNA (2 μl) was used as the template for PCR amplification using primer 3'UTRF and 3'UTRR[16] (Shown in table V) in a 50 μl PCR reaction mixture. The PCR conditions were as follows: 3 mM $MgCl_2$, 1x PCR buffer, 20μM dNTPs, 15 μM of each primer , and 1.25 U *Taq* DNA polymerase. After initial denaturation at 95 °C for 2 min, the reactions were run for 40 cycles through a temperature profile of 95 °C for 30 seconds (denaturation), 63 °C for 30 seconds (annealing), and 72 °C for 30 seconds (extension). A final extension was performed at 72 °C for 7 minutes.

4. Purification of PCR products by QIAquick PCR Purification Kit

A QIAquick spin column (QIAGEN, Hilden, Germany) was used to purify the PCR products and the manufacturer's reagents and protocol used throughout. Buffer PB was added in a ratio of 5:1 to the PCR reaction and mixed. The sample was applied to a QIAquick column and centrifuged at 13000 rpm for 1 minute. The column was washed by centrifugation with 750 ml Buffer PE and the purified PCR products were eluted with 16 μL distilled water (DW).

5. Restriction Fragment Length Polymorphism (RFLP) for detection CCR2-64I and SDF1-3'A polymorphism

After purification as above, the amplified fragments were digested by restriction endonucleases. For the CCR2-64I polymorphism, restriction conditions were as follows: 16 μl of purified PCR products, 2 μL of *BsaBI*,(New England Biolaboratories Inc., Beverly, MA) and 2 μL of 10x reaction buffer . The solution was incubated at 60 °C for 3 hours. And for the SDF1-3'A polymorphism, the restriction conditions were as follows: 16 μL of purified PCR products, 2 μL of *Msp I*,(New England Biolaboratories

Inc., Beverly, MA) and 2 μL of 10x reaction buffer. The mixture was incubated at 37 °C for 3 hours. After incubation period, both solutions were electrophoresed through 4% agarose gel.

6. Analysis of restricted endonucleases digestion products

A 4% agarose gel (GIBCO; Grand Island, N.Y. USA) in Tris-acetate (TAE) buffer was prepared containing 0.5 μg of ethidium bromide (Sigma, MO, USA) per mL. The pH of TAE buffer was pH 8.0. The 20 μL of restricted PCR products were electrophoretically separated by 75 volts until the bromphenol blue indicator in the loading buffer had migrated to half the length of the gel. The amplified products were then visualized on a short-wavelength of UV light transilluminator. Two DNA markers; 100 base pair DNA ladder (Promega, USA) and phiX174 DNA/Hae (Promega, USA) were used as a marker in the size estimate of the products for SDF1-3'A and CCR2-64I polymorphism, respectively. The genotypes were classified as wild type (w/w), heterozygous (mut/w), and homozygous (mut/mut) mutation by the observed digestion pattern (Shown in table 6).

7. Classification genotype

The primers CKR2_1A and CKR2_1Z, were designed to amplify a 128 bp fragment from nucleotides position 247 to 403 (reference sequence: GeneBank accession number U80923) of the human CCR2 gene. The amplified fragment contained one *Bsa BI* site at position 295 in the mutated CCR2-64I but not in wild type CCR2. Restriction digestion of this fragment with *Bsa BI* therefore resulted in either two fragments of 110 and 18 bp when amplified from a homozygous CCR2-64I individual or one fragment of 128 bp from a wild type CCR2 individual, while a heterozygous individual results in three bands of 128, 110 and 18 bp (Table VI, Figure II, and Figure IV).

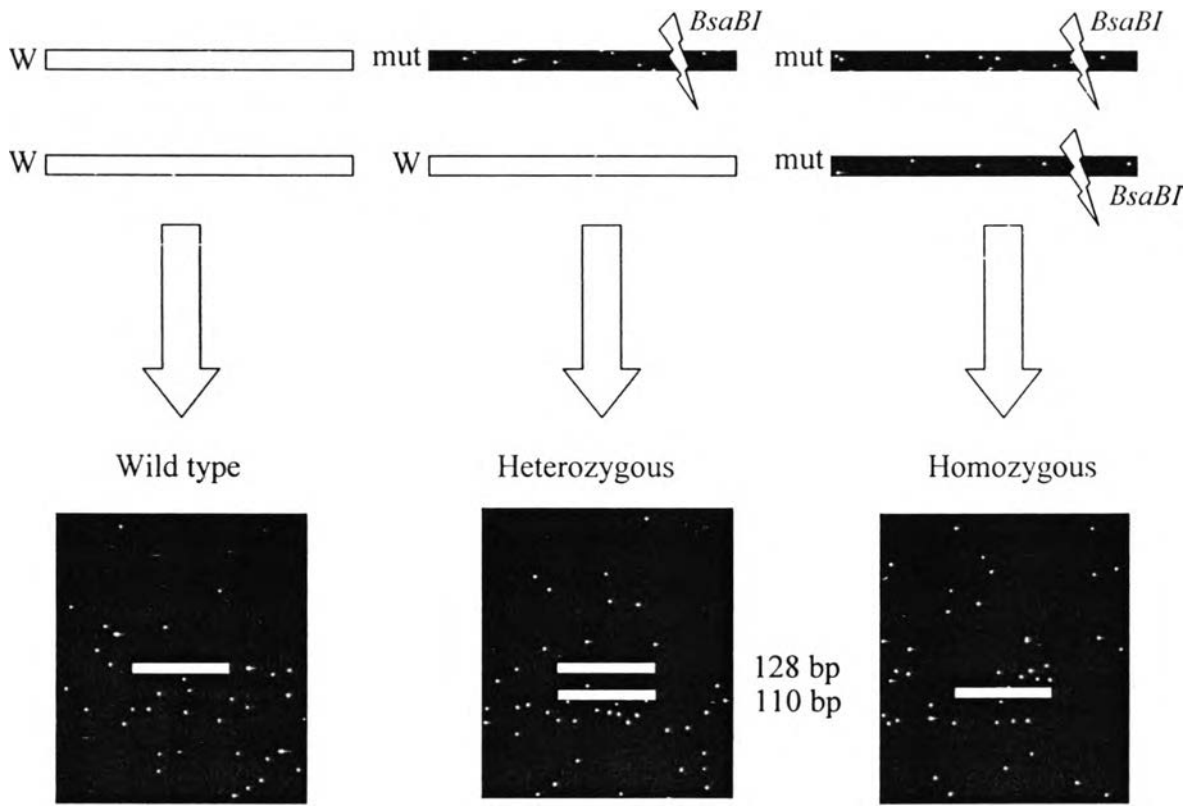

Figure II. Diagram for the *Bsa BI* digestion patterns of CCR2-641 in agarose gel electrophoresis

For SDF1-3'A genotyping, the designed primers, 3'UTRF and 3'UTRR, were designed to amplify a 302-bp fragemt from nucleotide position 777 to 1089 (reference sequence GeneBank accession number L36033) in the human SDF-1 gene. The amplified fragments contain one *Msp I* site at position 877 in wild type SDF1-3'A but not in mutant SDF1-3'A. Thus restriction digestion of this fragment with *Msp I* resulted in either two fragments of 202 and 100 bp when amplified from a wild type SDF-3'A individual or one fragment of 302 bp from a homozygous SDF1-3'A individual. Restriction digestion of PCR fragments from heterozygous SDF1-3'A individual resulted in three bands of 302, 202 and 100 bp, respectively (Table IV, Figure III and Figure VI).

8. Statistical Analysis

Genotype frequencies were calculated as

Wild type frequency $= (W/N)^2$

Heterozygous frequency $= 2 \times (H/N) \times (h/N)$

Homozygous frequency $= (H/N)^2$

Allele frequencies were calculated as

$$\frac{h+2H}{2N}$$

Where H was the number of homozygous mutation genotypes, h was the number of heterozygous mutation genotypes and N was the total number of the sample population. Conformity of these frequencies with Hardy-Weinberg equilibrium (HW) was tested by comparing genotype proportion in a 2 x 2 contingency tables with 2 degrees of freedom (SPSS for Windows release 7.5.1, SPSS Inc., 1989-1996).

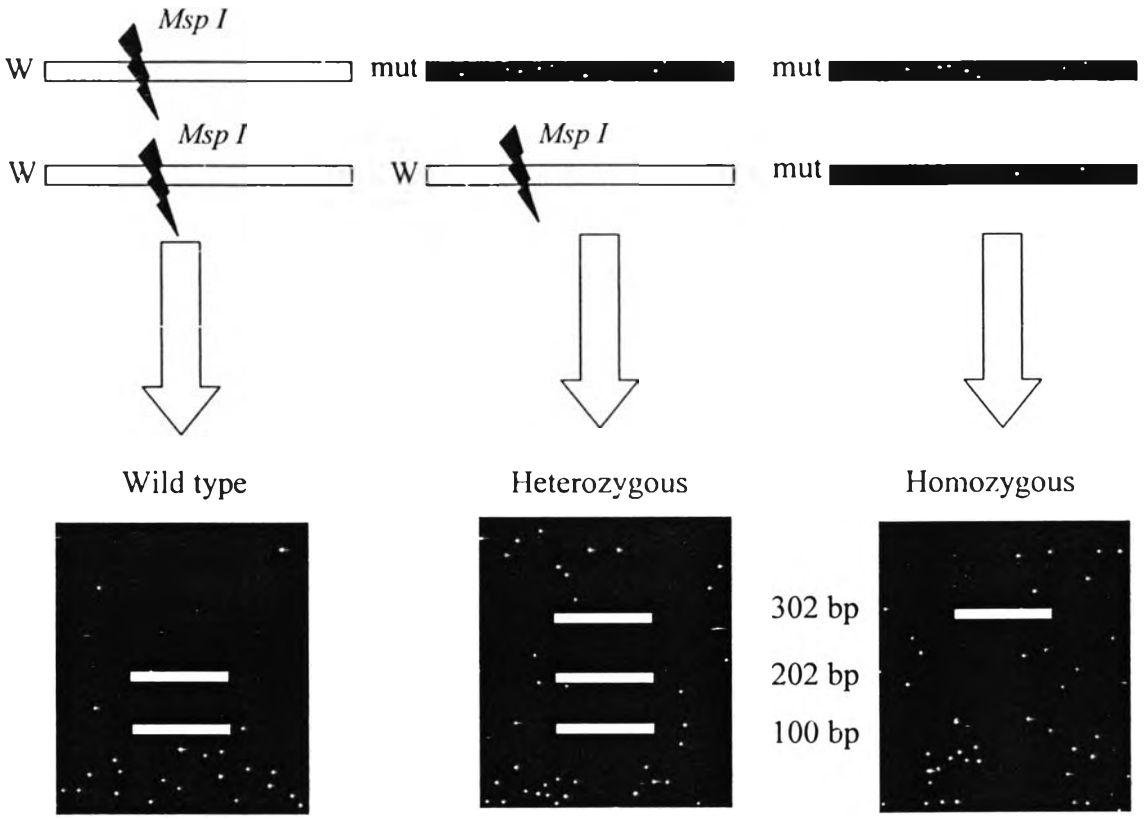

Figure III. Diagram for the *Msp I* digestion patterns of SDF1-3'A in agarose gel electrophoresis

Table V. Sequence of primer and size of PCR products

Target	Product size (bases)	Primer sequence
CCR2[13]	128	
CKR2_1A*		5'-TTGTGGGCAACATGaTGG-3'
CKR2_1Z		5'-GAGCCCACAATGGGAGAGTA-3'
SDF-1[16]	302	
3'UTRF		5'-CAGTCAACCTGGGCAAAGCC-3'
3'UTRR		5'-AGCTTTGGTCCTGAGAGTCC-3'

* The CKR2_A has a cytosine substituted for by adenine

Table VI. Size of the bands characteristic for different genotypes

	Genotype, bp		
Mutation	w/w	mut/w	mut/mut
CCR2-64I	128	128 , 110 , 18[a]	110 , 18[a]
SDF1-3'A	202 , 100	302 , 202 , 100	302

[a]The band is not visible in a 4% agarose gel electrophoresis