CHAPTER III

MATERIALS AND METHODS

Specimens

The specimen used in this study was consisted of peripheral blood leukocytes from NPC patients and healthy blood donors.

Materials

1. Pipette tip : 10 μl, 1,000 μl (Elkay, USA)
2. Microcentrifuge tube : 0.2 ml, 0.5 ml, 1.5 ml (Bio-rad, Elkay, USA)
3. Polypropylene conical tube : 15 ml (Elkay, USA)
4. Beaker : 50 ml, 100 ml, 200ml, 500 ml, 1,000 ml (Pyrex)
5. Flask : 250 ml, 500 ml, 1,000 ml (Pyrex)
6. Reagent bottle : 100 ml, 250 ml, 500 ml, 1,000 ml (Duran, USA)
7. Cylinder : 25 ml, 50 ml, 100 ml, 250 ml, 500 ml, 1,000 ml (Witeg, Germany)
8. Glass pipette : 5 ml, 10 ml (Witeg, Germany)
9. Pipette rack (Autopack, USA)
10. Thermometer (Precision, Germany)
11. Parafilm (American National Can, USA)
12. Plastic wrap
13. Stirring-magnetic bar
14. Combs

15. Electrophoresis chamber set

Equipments

1. Automatic adjustable micropipette : P2 (0.1-2 μl), P10 (0.5-10 μl), P20 (5-20 μl), P100 (20-100 μl), P1000 (0.1-1 ml) (Gilson, France)
2. Pipette boy (Tecnomara, Switzerland)
3. Vortax (Scientific Industry, USA)
4. PH meter (Eutech Cybernatics)
5. Stirring hot plate (Bamstead/Thermolyne, USA)
6. Balance (Precisa, Switzerland)
7. Microcentrifuge (Fotodyne, USA)
8. DNA Thermal cycler 480 (Perkin Elmer, Cetus USA)
9. Thermal cycler (Touch Down, Hybraid USA)
10. Power supply model 250 (Gibco BRL, Scothland)
11. Horizon 11-14 (Gibco BRL, Scothland)
12. Incubator (Memmert)
13. Thermostat shaking-water bath (Heto, Denmark)
14. Spectronic spectrophotometers (Genesys5, Milon Roy USA)
15. UV Transilluminator (Fotodyne USA)
16. UV-absorbing face shield (Spectronic, USA)
17. Gel Doc 1000 (Bio-RAD)

18. Mitsubishi Video copy processor

19. Thermal paper

20. Refrigerator 4 ^{0}C (Misubishi, Japan)

21. Deep freeze -20 ^{0}C, -80 ^{0}C (Revco)

22. Water Purification equipment (Water pro Ps, Labconco USA)

23. Water bath

Reagent

1. General reagents

    1.1 Absolute ethanol (Merck)

    1.2 Agarose, molecular glade (Promega)

    1.3 Ammonium acetate (Merck)

    1.4 Boric acid (Merck)

    1.5 Bromphenol blue (Pharmacia)

    1.6 Disodium ethylenediamine tetracetic acid : EDTA (Merck)

    1.7 Ethidium bromide (Gibco BRL)

    1.8 Ficoll 400 (Pharmacia)

    1.9 Hydrochloric acid (Merck)

    1.10 Mineral oil (Sigma)

    1.11 Phenol (Sigma)

1.12 Chloroform (Merck)

1.13 Isoamyl alcohol (Merck)

1.14 Sodium chloride (Merck)

1.15 Sodium dodecyl sulfate (Sigma)

1.16 Sodium hydroxide (Merck)

1.17 Sucrose (BDH)

1.18 Tris base (USB)

1.19 Triton X-100 (Pharmacia)

1.20 100 base pair DNA ladder (Biolabs)

2. Reagents of PCR analysis

2.1 10X PCR buffer (500 mM KCl, 200 mM Tris-HCl pH 8.4) (GibcoBRL)

2.2 Magnesium chloride (GibcoBRL)

2.3 Deoxynucleotide triphosphates (dNTPs) (Promega)

2.4 Oligonucleotide primers (BSU)

2.5 *Taq* DNA polymerase (GibcoBRL)

2.6 Genomic DNA sample

3. Restriction Enzyme

3.1 *TaqI* (Biolabs)

3.2 *RsaI* (Biolabs)

3.3 *PvuII* (Biolabs)

Methods

1. Sampling of Specimens

Venipuncture from 255 NPC patients at King Chulalongkorn Memorial Hospital and National Cancer Institute Thailand and 297 healthy blood donors obtained Blood samples. All subjects were interviewed and separated into three groups, Thai, Chinese and Thai-Chinese, based on their grandparents' ethnic origin. When their ancestors, including their great grandparents, originated from China, the patients were considered Chinese. When their ancestors originated from Thailand, the patients were considered Thai. Those subjects with mixed Thai / Chinese ancestors were defined as Thai-Chinese. There were 99 Thai, 98 Chinese and 100 Thai-Chinese in the control group. The NPC patients group included 136 Thai, 59 Chinese and 27 Thai-Chinese.

2. DNA Extraction

The extraction of DNA from peripheral blood leukocyte was performed as follow:

1. 5-10 ml. of whole blood is centrifuged for 10 minutes at 3,000 rpm.
2. Remove supernatant and collect buffy coat to a new polypropylene tube. Then add 10 volumes of cold lysis buffer1 (or 10 ml.), mix thoroughly and incubate at –20°C for 5 minutes.
3. Centrifuge for 8 minutes at 1,000 g, then remove supernatant.
4. Add 3 ml. Cold lysis buffer1, mix thoroughly and centrifuge for 8 minute at 1,000g,
5. Discard supernatant afterward add 900µl lysis buffer2, 10µl Proteinase K solution (20 mg Proteinase K in 1.0 ml. Of 1% SDS-

2mM EDTA, should be prepare 30 min before use.), and 10% SDS 50μl. Mix vigorously for 15 seconds.

6. Incubate the tube(s) in 37°C shaking waterbath overnight for complete digestion.

7. Add 1 ml. Phenol-chloroform-isoamyl alcohol shake vigorously for 15 seconds and centrifuge at 6,000 rpm for 5 minutes.

8. Transfer the supernatant from each tube (contains DNA) to a new microcentrifuge tube.

9. Add 0.5 volumes of 7.5 M CH_3COONH_4 and 1 volume of 100% ethanol mix by inversion. The DNA should immediately form a stringy precipitate. Recover the DNA by centrifugation at 14,000 rpm for 15 minutes. Then remove supernatant.

10. Rinse the pellet with 70% ethanol. Decant the ethanol and air-dried the pellet. (It is important to rinse well to remove any residual salt and phenol.)

11. Resuspend the digested DNA in 20-300μl of the double distilled water at 37°C until dissolved.

3. Calculation of DNA concentration

The reading at 260 nm is used for calculating the concentration of nucleic acid of the samples. An OD of 1 corresponds to approximately 50 μ l/ml for double-strand DNA. Therefore DNA concentration is calculated from the following:

DNA concentration = OD×50×dilution ratio (μg/ml)

4. Polymerase Chain Reaction (PCR) analysis

I. PCR for *CYP2E1* gene

1. PCR reaction for amplification of DNA is obtained by combining the following in a 20 µl reaction :

	Final concentration	Per reaction
10x PCR buffer	1X	2 µl
50mM $MgCl_2$	1.5 mM	0.6 µl
10mM dNTPs	0.125 mM	0.25 µl
10µM forward-primer	0.2 µM	0.4 µl
10µM reverse-primer	0.2 µM	0.4 µl
Taq DNA polymerase (5U/µl)	4U/100 µl	0.16 µl
DNA template (50 ng/µl)	-	2 µl
Sterile-deionized water to	-	20 µl

2. Centrifuge the reaction mixture briefly

3. PCR is performed in a Perkin – Elmer / DNA thermal Cycle 480.

The details of PCR cycles are:

-	Initiation :	95 ^{0}C	4 minutes
-	40 PCR cycle of : Denaturation	92 ^{0}C	1 minute
	Annealing	60 ^{0}C	1 minute

Extension	72 ^{0}C	2 minutes
- Final extension :	72 ^{0}C	7 minutes

II. PCR for *CR2* gene

1. PCR reaction for amplification of DNA is obtained by combining the following in a 20 µl reaction :

	Final concentration	Per reaction
10x PCR buffer	1X	2 µl
50mM $MgCl_2$	1.5 mM	0.6 µl
10mM dNTPs	0.2 mM	0.4 µl
10µM forward-primer	0.1 µM	0.2 µl
10µM reverse-primer	0.1 µM	0.2 µl
Taq DNA polymerase (5U/µl)	2U/100 µl	0.08 µl
DNA template (50 ng/µl)	-	2 µl
Sterile-deionized water to	-	20 µl

2. Centrifuge the reaction mixture briefly

3. PCR is performed in a Perkin – Elmer / DNA thermal Cycle 480.

The details of PCR cycles are:

- Initiation :	95 ^{0}C	4 minutes
- 35 PCR cycle of : Denaturation	95 ^{0}C	1 minute

Annealing	55 ^{0}C	1 minute
Extension	72 ^{0}C	1 minute
- Final extension :	72 ^{0}C	7 minutes

III. PCR for *pIgR* gene

1. PCR reaction for amplification of DNA is obtained by combining the following in a 20 µl reaction :

	Final concentration	Per reaction
10x PCR buffer	1X	2 µl
50mM $MgCl_2$	1.5 mM	0.6 µl
10mM dNTPs	0.2 mM	0.4 µl
10µM forward-primer	0.1 µM	0.2 µl
10µM reverse-primer	0.1 µM	0.2 µl
Taq DNA polymerase (5U/µl)	2U/100 µl	0.08 µl
DNA template (50 ng/µl)	-	2 µl
Sterile-deionized water to	-	20 µl

2. Centrifuge the reaction mixture briefly

3. PCR is performed in a Perkin – Elmer / DNA thermal Cycle 480.

The details of PCR cycles are:

- Initiation :	95 ^{0}C	4 minutes

- 40 PCR cycle of :	Denaturation	95 ^{0}C	1 minute
	Annealing	60 ^{0}C	1 minute
	Extension	72 ^{0}C	3 minutes
- Final extension :		72 ^{0}C	7 minutes

5. **Restriction Fragment Length Polymorphism (RFLP) assay**

The polymorphism study of entire three genes is determined by RFLP analysis. Each gene has specific restriction enzyme for identificated its polymorphism. There is *RsaI* restriction enzyme for *CYP2E1* gene, *TaqI* restriction enzyme for *CR2 gene* and *PvuII* restriction enzyme for *pIgR* gene. The procedures of this method are:

1. Make up mixture for restriction endonuclease digestion (total volume of 20 μl):

PCR product	5	μl
10X Buffer of restriction enzyme	2	μl
Restriction enzyme	0.2	μl
Sterile distilled water to	20	μl

2. Incubate the reaction mixture at 37 ^{0}C for overnight in waterbath

6. **Electrophoresis of the digested product by 2% agarose gel electrophoresis**

1. Load all of digested product (12μl) thoroughly mixed with 3 μl of 6X gel loading buffer, and concomitantly load 150 ηg of 100-bp DNA marker in an adjacent well as standard – size marker.

2. Run on 2% agarose gel electrophoresis in 1X TBE buffer.

3.After electrophoresis, visualize the digested product on an UV transilluminator and photograph with Gel doc 1000

7. Primer Design for DNA Amplification

Human genomic DNAs are used as templates for PCR amplifications from the genes of interest with their respective well-designed primers. The PCR products will be used for mutation screening by RFLP. Therefor, the well primer design should cover polymorphic site of those genes.

In *CYP2E1*gene, the previous study reported that polymorphism site located in the 5'-flanking region and nucleotide sequence of the primers for the PCR.[104] Hence, in this study, following the previous report uses the primers of this gene.

The *TaqI* polymorphic location of the *CR2* gene is reported near the exon1,2[93] but unknown specific site. Therefore, the primers of this gene are designed by using sequence database from GenBank and cover polymorphic region.

The *PvuII* site, located intron3, 156 nucleotides upstream of exon4, is the most likely candidate for the detected polymorphic site of the *pIgR* gene.[94] Owing to know specific position, forward-primer assigned on end exon3 and reverse-primer assigned on beginning exon4 were tested and showed polymorphic results.

8. Statistical Analysis

The relative risk [RR] was estimated by the odds ratio method, to determine the correlation between genotype of the three candidate genes and NPC development. In addition, the RR was used to estimate the association of the pattern of genetic inheritance of entire three genes and NPC phenotype. The 95% confidence interval [CI] was computed to

determine the statistical significance of the findings. The RR and 95%CI was calculated by using exact method from Epit info version6 program.