CHAPTER III

Materials and Methods

1. Specimens

The specimen used in this study were consisted of peripheral blood leukocytes, paraffin embedded tissues and serum.

2. Materials

2.1 Pipette tip : 10 μl, 100 μl,1,000 μl (Elkay,USA)

2.2 Microcentrifuge tube : 0.2 ml, 0.5ml, 1.5ml (Bio-rad Elkay,USA)

2.3 Beaker : 50 ml, 100 ml, 200 ml, 500 ml, 1,000 ml (Pyrex)

2.4 Flask : 250ml, 500ml, 1,000ml (Pyrex)

2.5 Reagent bottle : 100 ml, 250 ml, 500 ml, 1,000 ml (Duran, USA)

2.6 Cylinder : 25 ml, 50 ml, 100 ml, 250 ml, 500 ml, 1,000 ml (Witeg,Germany)

2.7 Glass Pipette : 5 ml, 10 ml (Witeg,Germany)

2.8 Microcentrifuge tube rack (USA/ Scientific plastics)

2.9 Thermometer (Precision, Germany)

2.10 Plastic wrap

2.11 Stirring-magnetic bar

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3. Equipments

3.1 Pipette boy (Tecnomara, Switzerland)

3.2 Vortex (Scientific Industry, USA)

3.3 pH meter (Eutech Cybernatics)

3.4 Stirring hot plate (Bamstead/Thermolyne, USA)

3.5 Balance (Precisa, Switzerland)

3.6 Microcentrifuge (Fotodyne, USA)

3.7 DNA Thermal cycler 480 (Perkin Elmer, Cetus USA)

3.8 Thermal cycler (Touch Down, Hybraid USA)

3.9 Power supply model 250 (Gibco BRL, Scothland)

3.10 Power poc 3000 (Bio-Rad)

3.11 Horizon 11-14 (Gibco BRL, Scothland)

3.12 Beta shield (C.B.S scientific. Co.)

3.13 Heat block (Bockel)

3.14 Incubator (Memmert)

3.15 Thermostat shaking-water bath (Heto, Denmark)

3.16 Spectronic spectrophotometers (Genesys5, Milon Roy USA)

3.17 UV Transilluminator (Fotodyne USA)

3.18 UV-absorbing face shield (Spectronic, USA)

3.19 Gel doc 1000 (Bio-RAD)

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

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

3.22 Water purification equipment (Water pro Ps, Labconco USA)

3.23 Water bath

3.24 Storm 840 and ImageQuaNT solfware (Molecular dynamics)

3.25 Gel star nucleic acid gel stain (Cambrex Bio Science)

4. Reagents

1 General reagent

1.1 Absolute ethanol (Merck)

1.2 Ammonium acetate (Merck)

1.3 Bisulfite (Merck)

1.4 Bromphenol blue (Pharmacia)

1.5 Chloroform (Merck)

1.6 Disodium ethylenediamine tetracetic acid : EDTA (Merck)

1.7 Ethidium bromide (Gibco BRL)

1.8 Hydrochloric acid (Merck)

1.9 Hydroquinone (Merck)

1.10 Isoamyl alcohol (Merck)

1.11 Isopropanol (Merck)

1.12 Mineral oil (Sigma)

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1.13 Phenol (Sigma)

1.14 Sodium chloride (Merck)

1.15 Sodium hydroxide (Merck)

1.16 Triton X-100 (Pharmacia)

1.17 Xylene (Merck)

1.18 10 base pair DNA ladder (Biolabs)

1.19 40%acrylamide/bis solution 19:1 (Bio-Rad)

1.20 Wizard DNA Clean-up System(Promega)

1.21 QIAamp DNA blood mini kit(QIAGEN)

2.Reagent of PCR

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

2.2 Magnesium chloride (GibcoBRL, Perkin Elmer)

2.3 Deoxynucleotide triphosphates (dNTPs) (Promega)

2.4 Oligonucleotide primers (BSU, GENSET) in appendix B

2.5 *Taq* Gold DNA polymerase (Perkin Elmer)

2.6 Genomic DNA sample

3.Restriction enzyme

3.1 *TagI* and buffer *TagI* (Fermentus)

3.2 *TasI* (Fermentus)

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Methods

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 $-20^{\circ}C$ 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 adds 900 μl lysis buffer2, 10 μl 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 50 μl. Mix vigorously for 15 seconds.

6. Incubate the tube(s) in $37^{\circ}C$ shaking water bath 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.)

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

<u>Isolation of plasma DNA</u>

1. Preparation of plasma

1.1 Centrifuge the blood sample at 1600 g for 10 minutes at room temperature.

1.2 Use a sterile pipette to transfer the plasma to a sterile 1.5 ml microcentrifuge tube, avoid taking out any cell.

1.3 Centrifuge the plasma at 15700 g for 10 minutes at room temperature.

1.4 Carefully pipette out the plasma without taking any of the cells.

1.5 Store the plasma in a 5 ml sterile round bottom tube and store it and 20 $^{\circ}$C before analysis.

2. Isolation of DNA from plasma by using the blood protocol of the QIAamp DNA Blood Minikit

2.1 Transfer 2x 400 µl plasma or 400 µl buffy coat into two 1.5 ml microcentrifuge tubes. Pipette 40 µl of QIAGEN protease into each tube. Vortex mix briefly for 10 seconds.

2.2 Add 400µl Buffer AL to each of the sample. Mix by pulse-vortexing for 1 min.

2.3 Incubate at 56 $^{\circ}$C for 10 minutes.

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2.4 Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid.

2.5 Add 400 μl of cold absolute ethanol to the sample, and mix again for 1 min. After mixing, briefly centrifuge the 1.5 ml microcentrifuge tube at room temperature to remove drops from the inside of the lid.

2.6 Carefully apply the mixture above (about 630 μl at one time) to the QIAamp spin column (in a 2ml collection tube) without wetting the rim, close the cap, and centrifuge at 15700 g for I min at room temperture.

2.7 Discard the liquid waste in the 2 ml collection tube and transfer the spin column to a clean 2 ml collection tube and repeat the above step.

2.8 Carefully open the QIA amp spin column and add 500 μl buffer AW1 without wetting the rim. Close the cap and centrifuge at 15700 g for 1 min Place the QIA amp spin column in a clean 2 ml collection tube, and discard the collection containing the filtrate.

2.9 Carefully open the QIA amp spin column and add 500 μl buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed for 3 min. Beware of any change of the possible buffer AW2 carryover. Discard the collection containing the filtrate.

2.10 Place the QIAamp spin column in a sterile 1.5 ml microcentrifuge tube. Carefully open the QIAamp spin column and add 50 μl sterile water

2.11 Incubate at room temperature for 1 min, and then centrifuge at 15700 g for 1min.

2.12 Label the sample and store the DNA solution at -20°C before further analysis

<u>DNA Preparation from paraffin-embedded section</u>

1. Wash paraffin slide with xylene twice 5 minutes.
2. Afterwards, Wash by absolute ethanol twice 5 minutes.
3. Microdisect cell in 200 µl lysis buffer.
4. Add 10% SDS 50 µl / lysis buffer 1 ml and 10 mg / µl PK 20 µl / lysis buffer 1 ml.
5. Incubate at 65 °C overnight.
6. Add 1 volume of phenol-chloroform-isoamyl alcohol shake vigorously for 5 minutes and centrifuge max speed 5 minutes.
7. Transfer the supernantant from each tube (contains DNA) to a new microcentrifuge tube.
8. Add 0.1 volume 3M sodium acetate, 2.5 volume absolute ethanol and 1 µl glycogen, mix by inversion.
9. Precipitate 2 hours at –20 °C centrifuge 15 minutes at 14,000 rpm wash with 70% ethanol and dry pellet.

Elute DNA with ddH2O, Then ready for preparing of DNA template in COBRA LINE-1

<u>Primer design for COBRA LINE-1</u>

Primers were designed to amplify the methylated and unmethylated allele equally. The primer design mention about the difference between methylated allele and unmethylated allele after standard sodium bisulfite treatment and PCR amplification results in the conversion of unmethylated cytosine residues to thymine and methylated cytosine residues to cytosine. This sequence conversion lead to the methylation-dependent creation of new restriction enzyme site. This is also dictated by the availability of restriction enzyme sites. Here general strategy:

1. Identify the region 5′UTR of LINE-1 / CpG island. Make a restriction map of the area (all enzymes). This is the unconverted map.
2. Copy sequence and paste in a text editor.
3. Convert all C to T except for CG. First convert all CG to XG. Then convert all C to T. Then convert all X to C. Make a restriction map of this converted sequence (Methylated map).

4. Convert all remaining C to T. Make a restriction map of this converted sequence(Unmethylated map)
5. Find restriction enzyme sites that are unique to the methylated map (not in the unconverted or unmethylated map). These are the best touse. If none is available, find restriction sites that are present in the methylated map but absent in the unmethylated map.

In this study use *TaqI* cutting TCGA sequence, to digest amplicon from methylated DNA and *TasI*, cutting AATT, for unmethylated DNA in which the last T was CpG nucleotide prior to DNA treatment (see primer sequence in appendix).

COBRA LINE-1-PCR

1. Preparing of DNA template
   - 1.1 Dilute DNA of each various tissue from paraffin section in 50 μl dH_2O
   - 1.2 Add 5.5 μl 2M NaOH (from fresh stock) and mix well
   - 1.3 Incubate at 37 °C for 10 minutes.
   - 1.4 Add 30 μl of the dilute hydroquinone (dilution1: 10 of 55 mg hydroquinone in 5 ml ddH2O), then vortex.
   - 1.5 Add 520 μl bisulfite (bisulfite 1.88 g in 5 ml ddH2O, bring pH to 5.0 with 5 drops of 19.5 M NaOH), then vortex.
   - 1.6 Incubate at 55 °C for 16 -18 hours.
   - 1.7 Desalt samples with the Wizard DNA Clean-up System,Promega by adding 1ml Wizard ™ resin to each tube and mix.
   - 1.8 Add to syringe attached to column anchored on the vacuum manifold and apply vacuum.
   - 1.9 Once drained, wash with 2 ml 80% isopropanol and apply vacuum.
   - 1.10 Once drained, elute DNA from column by adding 50 μl heated (50-70 °C) ddH2O and centrifuge 1 minute at maximum speed.
   - 1.11 Denature the sample with freshly prepared 5.5 μl 3mM NaOH and incubate at 37 °C for minute.

1.12 Neutralize by adding 5M-ammonium acetate and 2.3 volume of absolute ethanol.

1.13 Precipitate overnight at –20 °C centrifuge 15 minutes at 14,000 rpm wash with 70% ethanol and dry pellet.

1.14 Elute DNA with 10 μl TE buffer, then ready for PCR.

2. Reaction and condition

The PCR reaction was preformed in a total volume of 20 μl bisulfite treated DNA in 1X PCR buffer, 1 mM $MgCl_2$, 0.2 mM each of deoxynucleotide triphosphates (dNTPs). Each of primer pair was perfromed in optimal concentration 0.2 μM. In the multiplex PCR reaction, the initial denaturation step was 95 °C for 10 minutes then followed by 35 cycles of denaturation at 95 °C for 1 minute , annealing at 50 °C for 1 minute ,extension at 72 °C for 1 minute and a final extension at 72 °C for 7 minutes. Then, digested 5 μl of PCR product with 0.2 μl *TaqI*, 0.8 μl *TasI* and 1 μl buffer *TaqI* in total volume 10 μl. Incubate at 65 °C overnight. Afterward, separate PCR products by 12% acrylamide gel, electrophorase acrylamide gel in 1XTBE at 0.95 v/cm until dye front reaches the end of gel. Estimate size of digesting product by compare with 10 bp marker.

Data analysis

Quantitation is performed with a Molecular Dynamics Phosphorimager.

The percentage of hypomethylation in LINE-1

$$= \frac{\text{Intensity of } TasI}{\text{Intensity of } TasI + TaqI} \times 100$$

The difference of hypomethylation was determined by compared mean of % hypomethylation. Match cases used pair sample T-test (fig3-1) and unmatch cases (lymphoma,sex,age,serum and dysplatic polyps) used independent sample T-test (fig.3-2) .

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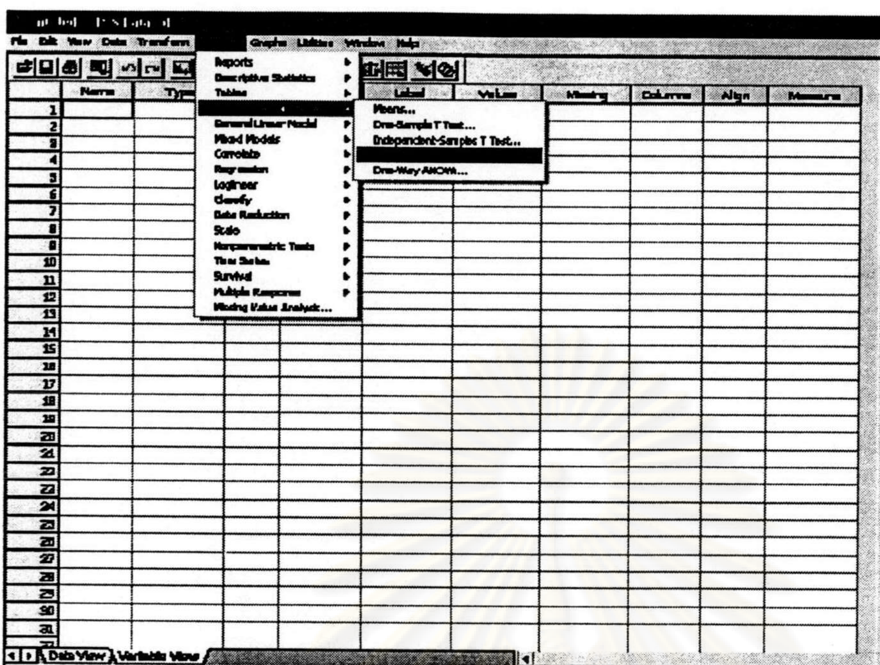

Figure 3-1 Pair sample T-test in SPSS program.

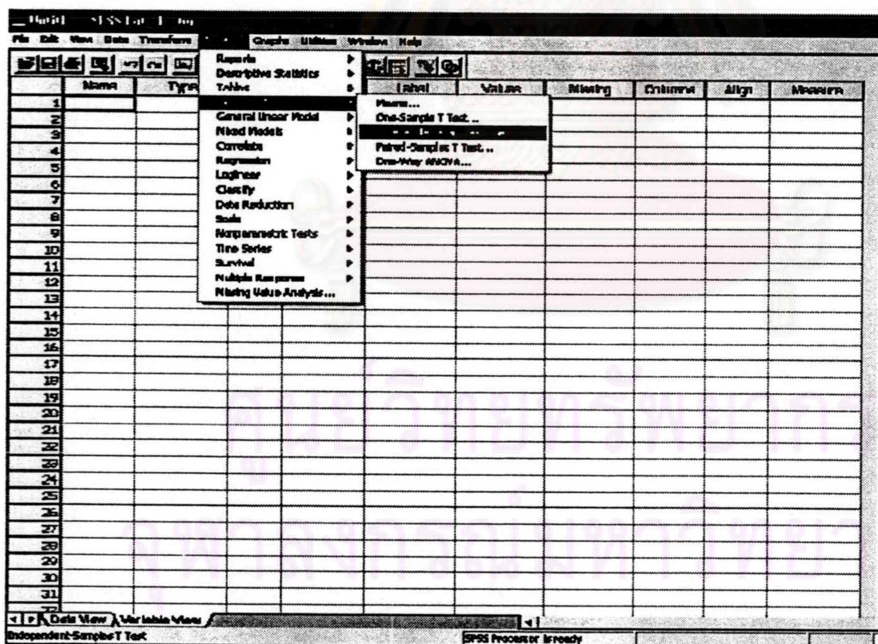

Figure 3-2 Independent sample T-test in SPSS program.