CHAPTER III

MATERIALS AND METHODS

Materials

1. Specimens

The specimen used in this study were consisted of 4 group. 4 groups are cancer cell line,18 HNSCC microdissect cases,12 normal oral epithelial cases and 12 normal white blood cell cases. All details of cancer cell line and HNSCC microdissected group are describe in table 3.1

Table 3-1 : Anatomical localization of the tumors from which cell lines were derived and their clinical staging

Cell line	Disease	Origin	Cell type	Clinical stage
HN4[1]	Head and Neck squamous cell carcinomas	Base of tongue	Epithelial	T4N1M0
HN6	Head and Neck squamous cell carcinomas	Base of tongue	Epithelial	T3N2bM0
HN8[2]	Head and Neck squamous cell carcinomas	Lymph node	Epithelial	T3N2M0
HN12[1]	Head and Neck squamous cell carcinomas	Lymph node	Epithelial	T4N1M0
HN13	Head and Neck squamous cell carcinomas	Tongue	Epithelial	T2N2M0
HN17	Head and Neck squamous cell carcinomas	Neck dissect	Epithelial	T2N2M0
HN19	Head and Neck squamous cell carcinomas	Lymph node	Epithelial	T3N0M0
HN22[2]	Head and Neck squamous cell carcinomas	Epiglottis	Epithelial	T3N2M0
HN26	Head and Neck squamous cell carcinomas	Vocal cord	Epithelial	T2N2M0
HN30[3]	Head and Neck squamous cell carcinomas	Pharynx	Epithelial	T3N0M0
HN31[3]	Head and Neck squamous cell carcinomas	Lymph node	Epithelial	T3N0M0
KB	Head and Neck squamous cell carcinomas	Mouth	Epithelial	-
K-562	Chronic myelogenous leukemia	Bone marrow	-	-
Daudi	Burkitt's lymphoma	Peripheral blood	B-lymphoblast	-
Molt4	Acute lymphoblastic leukemia	-	T-lymphoblast	-
Jurkat	Acute T cell leukemia	-	T-lymphocyte	-
M1	Head and Neck squamous cell carcinomas	Larynx	Epithelial	-
M2	Head and Neck squamous cell carcinomas	Lip	Epithelial	-
M3	Head and Neck squamous cell carcinomas	Larynx	Epithelial	T4N1M0
M4	Head and Neck squamous cell carcinomas	Tongue	Epithelial	-
M5	Head and Neck squamous cell carcinomas	Tongue	Epithelial	T1N0M0
M6	Head and Neck squamous cell carcinomas	Larynx	Epithelial	T1N0M0
M7	Head and Neck squamous cell carcinomas	Larynx	Epithelial	T4N0M0
M8	Head and Neck squamous cell carcinomas	Lip	Epithelial	-

M9	Head and Neck squamous cell carcinomas	Larynx	Epithelial	T3N0Mx
M10	Head and Neck squamous cell carcinomas	Larynx	Epithelial	T2N1M0
M11	Head and Neck squamous cell carcinomas	Oral carvity	Epithelial	-
M12	Head and Neck squamous cell carcinomas	Larynx	Epithelial	T4N1M0
M13	Head and Neck squamous cell carcinomas	Larynx	Epithelial	T4N1M0
M14	Head and Neck squamous cell carcinomas	Lip	Epithelial	-
M15	Head and Neck squamous cell carcinomas	Lip	Epithelial	-
M16	Head and Neck squamous cell carcinomas	Mandible	Epithelial	T1N0M0
M17	Head and Neck squamous cell carcinomas	Floor of mouth	Epithelial	T4N2Mx
M18	Head and Neck squamous cell carcinomas	Larynx	Epithelial	T3N0M0

Superscripts indicate pairs of cell lines derived from the same patients. M48 is the mark of microdissected tissue group.

2. Materials

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

2.2 serological pipette indiv. wrapped, sterile: 2 ml, 10 ml, 25 ml (CORNING)

2.3 Microcentrifuge tube : 0.2 ml, 0.5ml, 1.5ml (Axygen,USA)

2.4 Round culture dish : diameter 35mm, 100mm (CORNING)

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

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

2.7 T-flask : T-25, T-75 (CORNING)

2.8 Bottle Top Filter 150 mlCapacity, 50 mm Diameter Membrane (CORNING)

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

2.10Cylinder : 25 ml, 50 ml, 100 ml, 250 ml, 500 ml, 1,000 ml (Witeg,Germany)

2.11Glass Pipette : 5 ml, 10 ml (Witeg,Germany)

2.12Microcentrifuge tube rack (USA/ Scientific plastics)

2.13Thermometer (Precision, Germany)

2.14 Plastic wrap

2.15 Stirring-magnetic bar

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 Heat block (Bockel)

3.13 Incubator (Memmert)

3.14 Thermostat shaking-water bath (Heto, Denmark)

3.15 Spectronic spectrophotometers (Genesys5, Milon Roy USA)

3.16 UV Transilluminator (Fotodyne USA)

3.17 UV-absorbing face shield (Spectronic, USA)

3.18 Gel doc 1000 (Bio-RAD)

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

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

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

3.22 Water bath

3.23 Storm 840 and ImageQuaNT solfware (Molecular dynamics)

3.24 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 Ampicillin

1.4 Sodium bisulfite (Sigma)

1.5 Bromphenol blue (Pharmacia)

1.6 Chloroform (Merck)

1.7 Disodium ethylenediamine tetracetic acid : EDTA (Merck)

1.8 DMEM (Dulbecco's Modified Eagle's Media)

1.9 Ethidium bromide (Gibco BRL)

1.10 Hydrochloric acid (Merck)

1.11 Hydroquinone (Merck)

1.12 IPTG

1.13 Isoamyl alcohol (Merck)

1.14 Isopropanol (Merck)

1.15 Mineral oil (Sigma)

1.16 Phenol (Sigma)

1.17 RPMI-1640 media

1.18 Sodium chloride (Merck)

1.19 Sodium hydroxide (Merck)

1.20 Triton X-100 (Pharmacia)

1.21 TRIZOL Reagent (Invitrogen)

1.22 trypsin/EDTA

1.23 X-Gal

1.24 Xylene (Merck)

1.25 25 base pair DNA ladder (Biolabs)

1.26 40%acrylamide/bis solution 19:1 (Plusone)

1.27 Wizard DNA Clean-up System(Promega)

1.28 QIAamp DNA mini kit(QIAGEN)

1.29 cDNA synthesis kit (Promega)

2.Reagent of PCR

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

2.2 Magnesium chloride (QIAGEN)

2.3 Deoxynucleotide triphosphates (dNTPs) (Ependorf)

2.4 Oligonucleotide primers (BSU, GENSET) in appendix B

2.5 *Taq* hotstart DNA polymerase (QIAGEN)

2.6 DNA or cDNA sample

3.Restriction enzyme

3.1 *Taq I* and buffer *Taq I* (Fermentus)

3.2 *Tas I* (Fermentus)

Methods

Cell culture

Resuscitation of Frozen Cell Lines

1. Prepare the flasks as appropriate (information on technical data sheet). Label with cell line name, passage number and date.
2. Collect ampule of cells from liquid nitrogen storage wearing appropriate protective equipment and transfer to laboratory in a sealed container.
3. Still wearing protective clothing, remove ampule from container and place in a waterbath at an appropriate temperature for your cell line e.g. 37°C for mammalian cells. Submerge only the lower half of the ampule. Allow to thaw until a small amount of ice remains in the vial - usually 1-2 minutes. Transfer to class II safety cabinet.
4. Wipe the outside of the ampule with a tissue moistened (not excessively) with 70% alcohol hold tissue over ampule to loosen lid.
5. Slowly, dropwise, pipette cells into pre-warmed growth medium* to dilute out the DMSO (flasks prepared in Step 2).
6. Incubate at 37°C in 5% CO_2 in atmosphere.

7. Examine cells microscopically (phase contrast) after 24 hours and sub-culture as necessary

* Culture medium for all epithelial cell line are DMEM (Dulbecco's Modified Eagle's Media) and for all leukemic cell line are RPMI-1640 media.

Subculture of Cell Lines

1. View cultures using an inverted microscope to assess the degree of confluency and confirm the absence of bacterial and fungal contaminants.
2. Remove spent medium.
3. Wash the cell monolayer with PBS without Ca_{2+}/Mg_{2+} using a volume equivalent to half the volume of culture medium. Repeat this wash step if the cells are known to adhere strongly.
4. Pipette trypsin/EDTA onto the washed cell monolayer using 1ml per $25cm_2$ of surface area. Rotate flask to cover the monolayer with trypsin. Decant the excess trypsin.
5. Return flask to the incubator and leave for 2-10 minutes.
6. Examine the cells using an inverted microscope to ensure that all the cells are detached and floating. The side of the flasks may be gently tapped to release any remaining attached cells.
7. Resuspend the cells in a small volume of fresh serum-containing medium to inactivate the trypsin. Remove 100-200uL and perform a cell count and the remains suspense cells are collect for DNA extraction later.
8. Transfer the required number of cells to a new labeled flask containing pre-warmed medium (refer to ECACC Cell Line Data Sheet for the required seeding density).
9. Incubate as appropriate for the cell line.
10. Repeat this process as demanded by the growth characteristics of the cell line.

DNA Extraction

The extraction of DNA from cell line, normal oral rinse epithelial and normal white blood cell 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. Add 1 ml ice cold PBS for wash cell. Centrifuge at 150g 5 minute.*
6. Discard supernatant afterward adds 900 µl 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.
7. Incubate the tube(s) in 37°C shaking water bath overnight for complete digestion.
8. Add 1 ml. Phenol-chloroform-isoamyl alcohol shake vigorously for 15 seconds and centrifuge at 6,000 rpm for 5 minutes.
9. Transfer the supernatant from each tube (contains DNA) to a new microcentrifuge tube.
10. 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.

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

* Step 5 is the start point of cell line and normal oral rinse epithelial DNA extraction.

RNA Extraction

1. HOMOGENIZATION : Lyse cells directly in a culture dish by adding 3 ml of TRIZOL Reagent to a T-75 flask, and passing the cell lysate several times through a pipette.

2. PHASE SEPARATION : Incubate the homogenized samples for 5 minutes at 15 to 30°C to permit the complete dissociation of nucleoprotein complexes. Add 0.2 ml of chloroform per 1 ml of TRIZOL Reagent. Cap sample tubes securely. Shake tubes vigorously by hand for 15 seconds and incubate them at 15 to 30°C for 2 to 3 minutes. Centrifuge the samples at no more than 12,000 × g for 15 minutes at 2 to 8°C. Following centrifugation, the mixture separates into a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase.

3. RNA PRECIPITATION : Transfer the aqueous phase to a fresh tube, and save the organic phase if isolation of DNA orprotein is desired. Precipitate the RNA from the aqueous phase by mixing with isopropyl alcohol. Use 0.5 ml of isopropyl alcohol per 1 ml of TRIZOL Reagent used for the initial homogenization. Incubate samples at 15 to 30°C for 10 minutes and centrifuge at no more than 12,000 × g for 10 minutes at 2 to 8°C. The RNA precipitate, often invisible before centrifugation, forms a gel-like pellet on the side and bottom of the tube.

4. RNA WASH : Remove the supernatant. Wash the RNA pellet once with 75% ethanol, adding at least 1 ml of 75% ethanol per 1 ml of TRIZOL Reagent used for the initial homogenization. Mix the sample by vortexing and centrifuge at no more than 7,500 × g for 5 minutes at 2 to 8°C.

5. REDISSOLVING THE RNA : At the end of the procedure, briefly dry the RNA pellet (air-dry for 5-10 minutes). Do not dry the RNA by centrifugation under vacuum. It is important not to let the RNA pellet dry completely as this will greatly decrease its solubility. Partially dissolved RNA samples have an A260/280 ratio < 1.6. Dissolve RNA in RNase-free water.

cDNA systhesis

1. Prepare the following reaction mixture in a tube on ice : Mix gently and spin down for 3-5sec. in a microcentrifuge.

   template RNA*: total RNA 5µg

   oligo(dT)18 primer (0.5µg/µl) 1µl

   DEPC-treated water to 12µl

2. Incubate the mixture at 70°C for 5min, chill on ice and collect drops by brief centrifugation.

3. Place the tube on ice and add the following components in the indicated order : Mix gently and collect drops by brief centrifugation.

   5x reaction buffer 4µl

   RiboLock™ Ribonuclease Inhibitor (20u/µl) 1µl

   10mM dNTP mix 2µl

4. Add RevertAid™ M-MuLV Reverse Transcriptase (200u/µl) 1µl. (Final volume 20 µl)

5. Incubate the mixture at 42°C for 60min (incubate at 25°C for 10min and fi nally at 42°C for 60min if the random hexamer primer is used). Stop the reaction by heating at 70°C for 10min. Chill on ice. The fi rst strand cDNA synthesized can be used directly for amplifi cation by PCR.

6. cDNA PCR amplification with following condition. The PCR reaction was preformed in a total volume of 20 µl cDNA in 1X PCR buffer, 1 mM MgCl2, 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 with GAPDH(cDNA quality control), 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 53 °C for 1 minute ,extension at 72 °C for 1 minute and a final extension at 72 °C for 7 minutes. Check with 2% agarose gel electrophoresis at 100volt constant, 1 hr.

DNA Preparation from paraffin-embedded section

a. Wash paraffin slide with xylene twice 5 minutes.
b. Afterwards, Wash by absolute ethanol twice 5 minutes.
c. Microdisect cell in 200 µl lysis buffer.
d. Add 10% SDS 50 µl / lysis buffer 1 ml and 10 mg/µl PK 20µl/lysis buffer 1 ml.
e. Incubate at 65 °C overnight.
f. Add 1 volume of phenol-chloroform-isoamyl alcohol shake vigorously for 5 minutes and centrifuge max speed 5 minutes.
g. Transfer the supernantant from each tube (contains DNA) to a new microcentrifuge tube.
h. Add 0.1 volume 3M sodium acetate, 2.5 volume absolute ethanol and 1 µ l glycogen, mix by inversion.
i. 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 inCOBRA LINE-1

LINE-1 specific location

Search for specific LINE-1 loci by blat LINE-1 (M80343). Total hit is 204 LINE-1s, 69 LINE-1 locate in genes and finally 17 loci were selected by intronic properties and technical limited. All data of LINE-1 and upstream unique sequence collected from www.genome.ucsc.edu/Blat (figure 3-1) , www.ncbi.nlm.nih.gov (figure 3-2) and L1BASE tool[80] (figure3-3) .

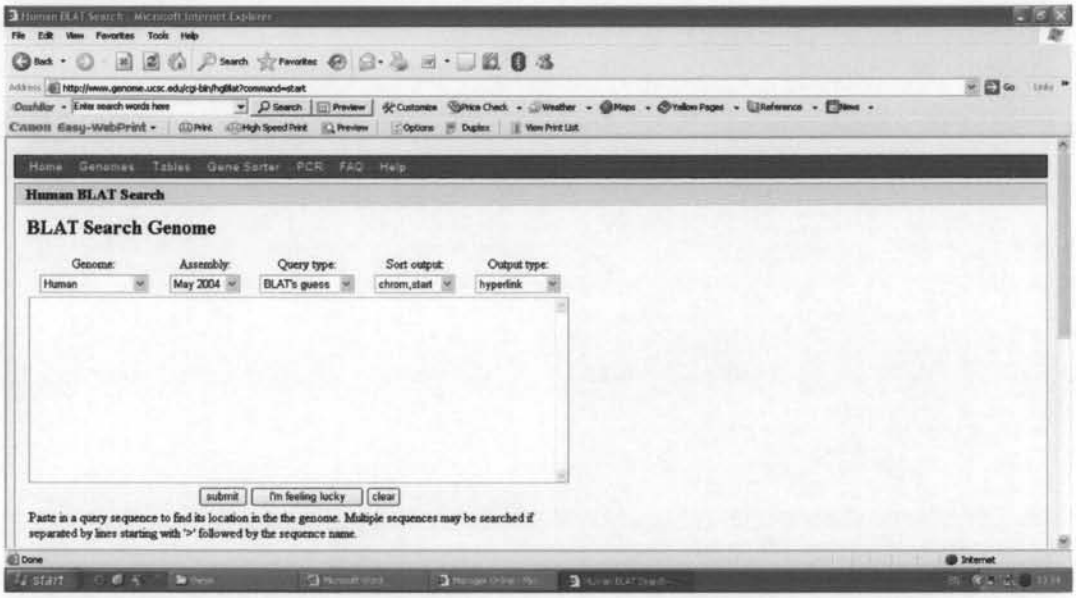

Figure 3-1 : Webpage of human blat search by www.genome.ucsc.edu/Blat.

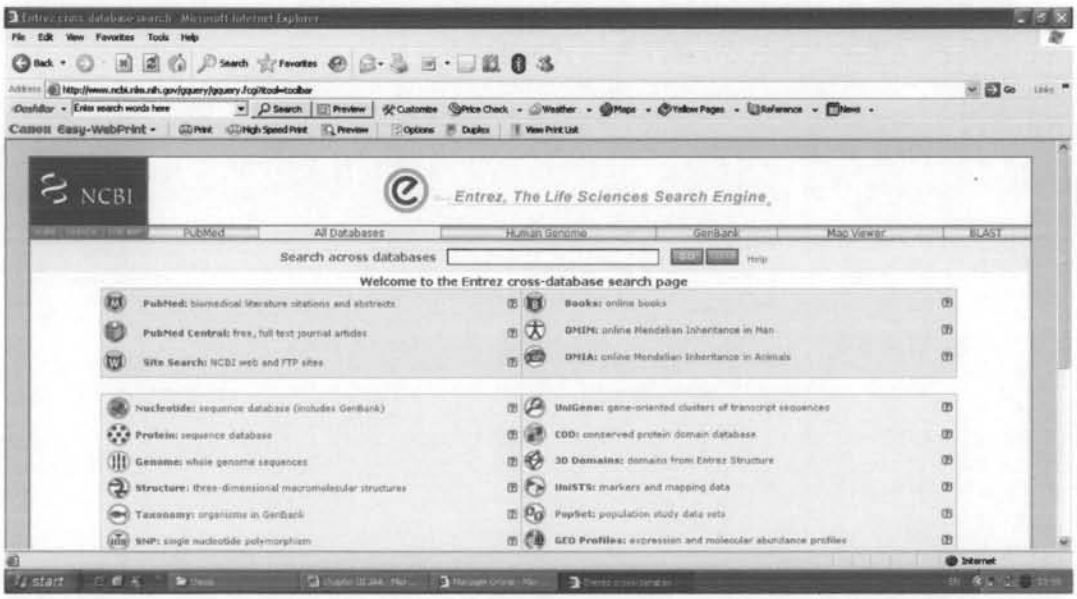

Figure 3-2 : Webpage of Entrez cross-database search by www.ncbi.nlm.nih.gov.

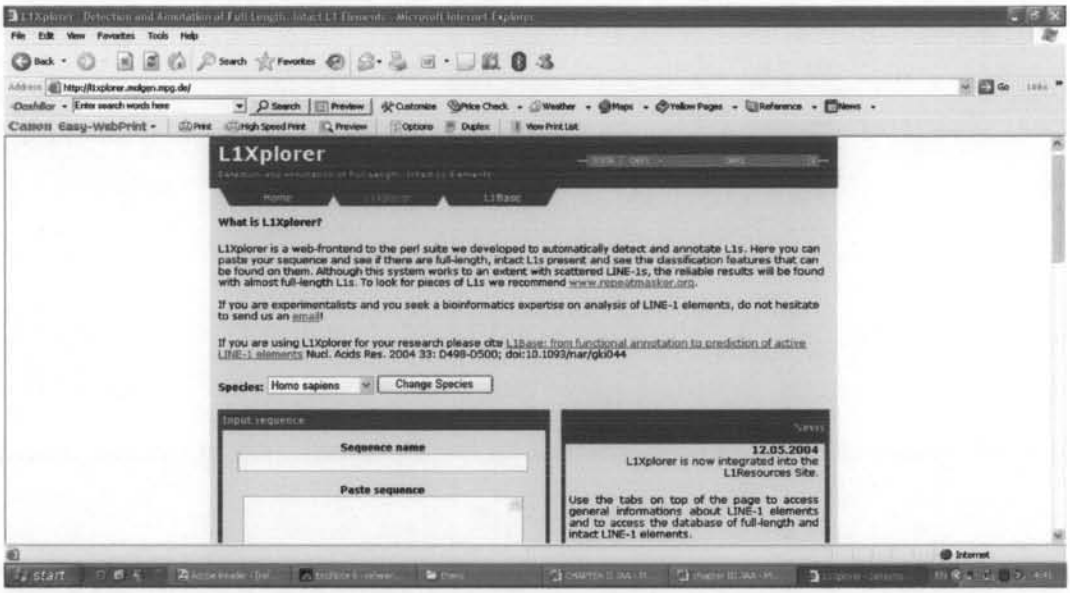

Figure 3-3 : Webpage of L1BASE database explorer tool.

Primer design for COBRA LINE-1

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:

- Identify the region 5′ upstream unique sequence of 5'UTR of LINE-1 / CpG island. Make a restriction map of the area (all enzymes). This is the unconverted map.
- Copy sequence and paste in a text editor.
- 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)
- Convert all remaining C to T. Make a restriction map of this converted sequence(Unmethylated map)
- 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 C).

<u>COBRA LINE-1 and COBRA unique to LINE-1 PCR</u>

A. Preparing of DNA template

a. DiluteDNA of each various tissue from paraffin section in 50 µl dH2O
b. Add 5.5 µl 2M NaOH (from fresh stock) and mix well
c. Incubate at 37 °C for 10 minutes.
d. Add 30 µl of the dilute hydroquinone (dilution1: 10 of 55 mg hydroquinone in 5 ml ddH2O), then vortex.
e. 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.
f. Incubate at 55 °C for 16-18 hours.
g. Desalt samples with the Wizard DNA Clean-up System,Promega by adding 1ml Wizard ™ resin to each tube and mix.
h. Add to syringe attached to column anchored on the vacuum manifold and apply vacuum.
i. Once drained, wash with 2 ml 80% isopropanol and apply vacuum.
j. Once drained, elute DNA from column by adding 50 µl heated (50-70 °C) ddH2O and centrifuge 1 minute at maximum speed.
k. Denature the sample with freshly prepared 5.5 µl 3mM NaOH and incubate at 37 °C for minute.
l. Neutralize by adding 5M-ammonium acetate and 2.3 volume of absolute ethanol.
m. Precipitate overnight at –20 °C centrifuge 15 minutes at 14,000 rpm wash with 70% ethanol and dry pellet.
n. Elute DNA with 10 µl TE buffer, then ready for PCR.

B. 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 53 °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, 0.8µ l TasI and 1 µl buffer TaqI in total volume 10 µl . Incubate at 65 °C overnight. Afterward, separate PCR products by 8% 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 25 bp marker.

PCR cloning

Ligations Using the pGEM®-T Easy Vectors and the 2X Rapid Ligation Buffer

1. Briefly centrifuge the pGEM®-T or pGEM®-T Easy Vector and Control Insert DNA tubes to collect contents at the bottom of the tubes.
2. Set up ligation reactions as described below.

   2X Rapid Ligation Buffer, T4 DNA Ligase 5µl

   pGEM®-T Easy Vector (50ng) 1µl

   PCR product 2µl

   T4 DNA Ligase (3 Weiss units/µl) 1µl

   deionized water to a final volume of 10µl
3. Mix the reactions by pipetting. Incubate the reactions 1 hour at room temperature. Alternatively, if the maximum number of transformants is required, incubate the reactions overnight at 4°C.
4. Prepare two LB/ampicillin/IPTG/X-Gal plates for each ligation reaction(Appendix A)

5. Centrifuge the tubes containing the ligation reactions to collect contents at the bottom of the tube. Add 2µl of each ligation reaction to a sterile polypropylene tubes (Falcon Cat.# 2059) on ice.
6. Remove tube(s) of frozen High Efficiency Competent Cells from storage and place in an ice bath until just thawed (about 5 minutes). Mix the cells by gently flicking the tube. Note: Avoid excessive pipetting, as the competent cells are extremely fragile.
7. Carefully transfer 100µl of cells into each tube prepared in Step 2 (100µl cells for determination of transformation efficiency).
8. Gently flick the tubes to mix and place them on ice for 20 minutes.
9. Heat-shock the cells for 45 seconds in a water bath at exactly 42°C (Do not shake).
10. Immediately return the tubes to ice for 2 minutes.
11. Add 900µl room temperature SOC medium to the tubes containing cells transformed with ligation reactions and 900µl to the tube containing cells transformed with uncut plasmid (LB broth may be substituted, but colony number may be lower).
12. Incubate for 1.5 hours at 37°C with shaking (~150rpm).
13. Plate 100µl of each transformation culture onto duplicate LB/ampicillin/ IPTG/X-Gal plates. For the transformation control, a 1:10 dilution with SOC medium is recommended for plating. If a higher number of colonies is desired, the cells may be pelleted by centrifugation at 1,000 × g for 10 minutes, resuspended in 200µl of SOC medium, and 100µl plated on each of two plates.
14. Incubate the plates overnight (16-24 hours) at 37°C. In our experience, if 100µl is plated approximately 100 colonies per plate are routinely seen when using competent cells that are 1 × 108cfu/µg DNA. Longer incubations or storage of plates at 4°C (after 37°C overnight incubation) may be used to facilitate blue color development. White colonies generally contain inserts; however, inserts may also be present in blue colonies.

15. Perform M13-PCR and COBRA unique to LINE-1 to check insertion status. Sequencing by BioService Unit, National Science and Technology Development Agency (NSTDA).

Data analysis

Band quantitation is performed with a Molecular Dynamics Phosphorimager.

$$\text{The percentage of hypomethylation in LINE-1} = \frac{\text{Intensity of TasI}}{\text{Intensity of TasI+TaqI}} \times 100$$

The difference of hypomethylation was determined by compared mean of % hypomethylation. All group compare by One-way ANOVA (figure 3-1) and unmatch cases (normal oral rinse epithelial and normal white blood cell) used independent t-test (figure 3-2). All graph and table create by SPSS program version 14, Adobe photoshop CS2 and Microsoft Excel 2003.

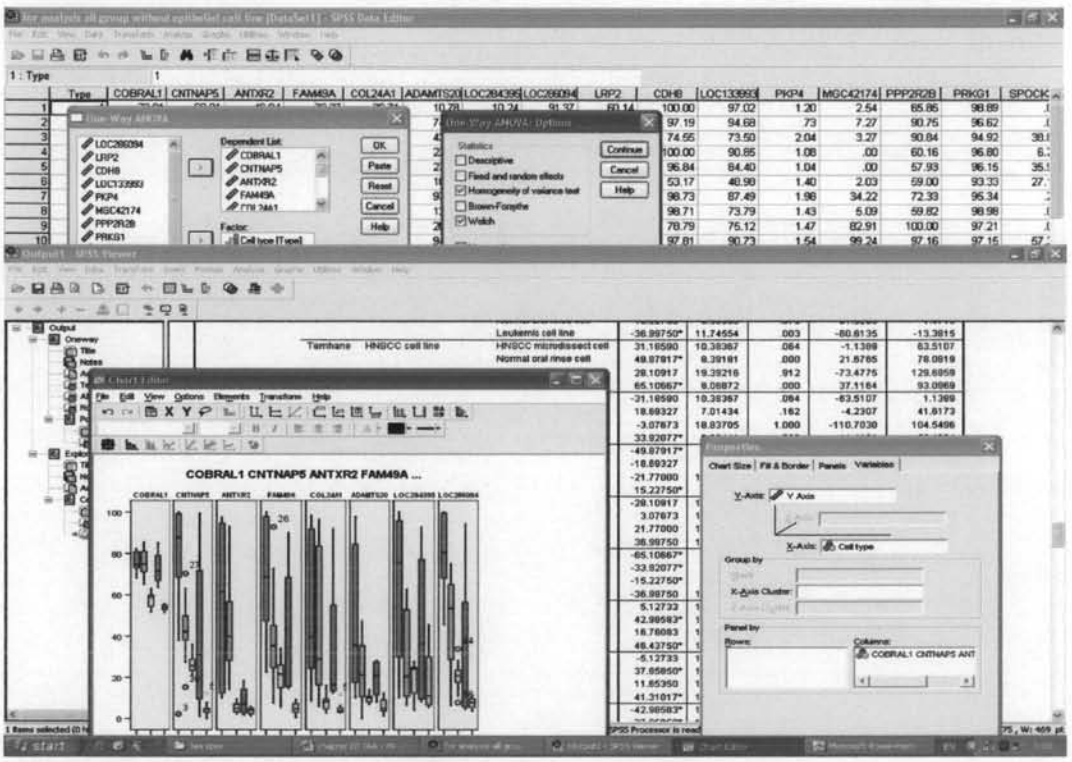

Figure 3-4 One-way ANOVA by SPSS program version 14.

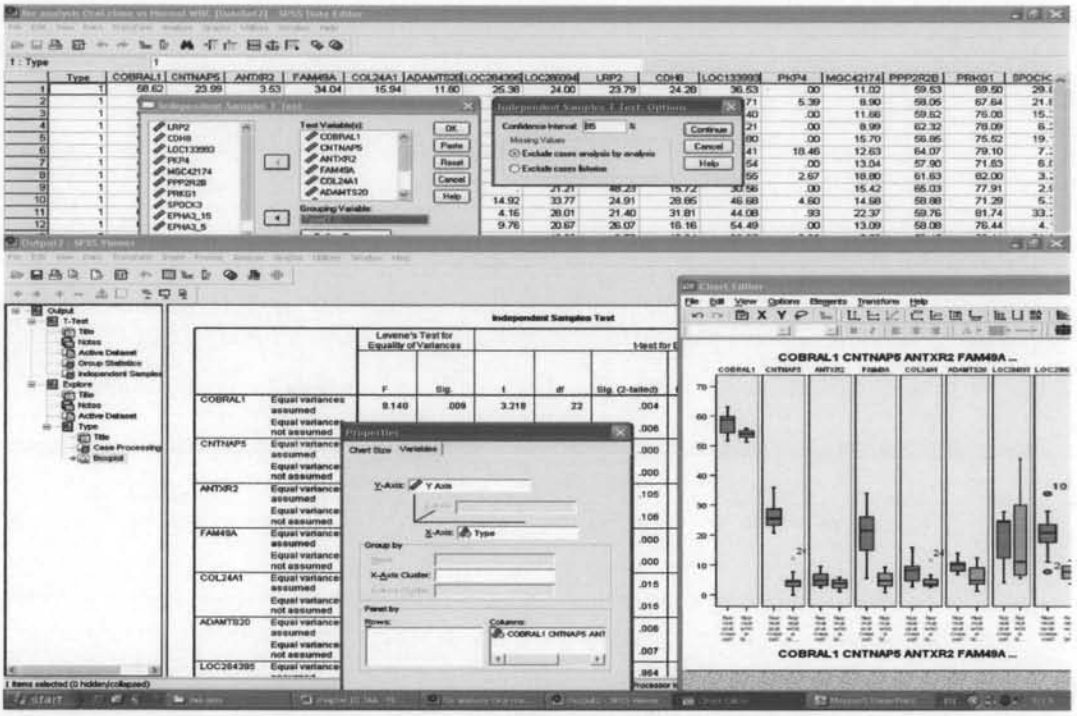

Figure 3-5 Independent-sample T-test by SPSS program version 14.