CHAPTER 3

MATERIALS AND METHODS

3.1 The studied subjects

The study has been approved by the Ethics Committee of Research Institute of Health Science, Chiang Mai University, Chiang Mai, Thailand. The studied subjects were the two healthy populations residing in Saraphi and Chom Thong, which were the areas with high and low incidences of lung cancer respectively.

Previous to the study, all subjects gave an informed consent to participate in the study. A questionnaire was used to collect the relevant information for the characterization of the studied populations and evaluation of confounding factors as shown in Appendix A. Each subject was interviewed about personal data, lifestyles and possible factors that might influence the genetic materials (confounding factors) for examples age range (grouping by 10 years interval), present pesticide exposure, burning activity, smoking habit, alcohol drinking and fermented tea leaf or betel nut chewing. Any persons with medical treatment and/or radiography within six months were excluded. Blood samplings were drawn using a 5 ml of heparinized syringe (500 unit/ml) from a subject by venipuncture for two times. The interval period between the blood samplings was approximately 8 months; the first sampling was for chromosomal aberration and micronucleus assays and the other for comet assay.

The total number of subjects participated in chromosomal aberration and micronucleus studies were 107 and 118 in the Saraphi and Chom Thong respectively. The subjects for DNA damage study detected by comet assay were the same subjects were the same groups as mentioned above but less in numbers, 91 and 94 subjects, because some of them denied to continue the participation.

The preparation of reagents used in the experiments of chromosomal aberration and micronucleus tests, and comet assay were shown in Appendix B and C respectively. The detailed method in each test was described as follow.

3.2 Chromosomal aberration assay

The method was performed as standard protocol of short term assay using human lymphocytes.

1) Eight drops (approximately 0.3 ml) of peripheral blood were added into 2.7 ml of PRMI 1640 medium containing 20% fetal calf serum and penicillin/streptomycin (Gibco, final concentrations at 100 unit/ml and 100 μg/ml respectively) in a labeled 15 ml centrifuge tube. Duplicated lymphocyte cultures were set up for each subject in the same medium and condition.

2) Phytohaemagglutinin (PHA) (Seromed, final concentration at 50 μg/ml) was added into each culture tube and the cultures were incubated in the 5% CO_2 incubator at 37°C for 48 h.

3) Half an hour prior to harvesting, colchicine (Sigma, final concentration at 1.25 μg/ ml) was added into each culture.

4) The cultures were centrifuged at 1,000 rpm for 10 min and discarded the supernatant.

5) Cells were mixed thoroughly and then 5 ml of Hank balanced salt solution was added.

6) Repeated step 4, then each culture was mixed again with 5 ml of pre-warmed 0.075 M KCl for two times.

7) The supernatant was discarded and the cells were thoroughly mixed. A drop-wise of freshly prepared chilled fixative solution, containing 3 part of methanol and one part of glacial acetic acid, was slowly added into the culture. When the sample turned dark in colour, approximately 4 ml of fixative was added more and mixed again.

8) The culture was centrifuged and the supernatant was discarded. Then the culture was washed twice more with fixative solution until the supernatant became clear.

9) The cell pellet was resuspended in a small volume of fixative and dropped on the clean slides and allowed drying.

10) The slides were checked for the quality of metaphases by phase-contrast microscope and stained with 5 % Giemsa in Weise buffer.

11) The 100 well-spread metaphases with 46±1 centromeres (50 metaphases from each culture) were evaluated by light microscope with 1,000X magnification. The types of structural chromosome aberration were classified as described in Appendix D. It is generally suggested that gaps should be recorded but their frequency should not be included in the totals for aberrations, since their significance and relationship to other "true" aberration types has not been clear (World Health Organization, 1985). Afterward, the percentage of aberrant cells (the number of metaphases with aberration in 100 metaphases) and the percentage of aberrations (the

number of aberrations in 100 metaphases) were determined. Moreover, the number of metaphases among 1,000 cells was counted to determine the mitotic index using the formula as follow.

$$\text{Mitotic index} = \frac{\text{Number of metaphases (in 1,000 cells)}}{\text{Number of cells (1,000 cells)}}$$

3.3 Micronucleus Test

The methods were performed according to the modified protocol of Vaglenov *et al.*, (1998).

1) Two parallel cultures were set up from a subject using the same medium and condition as described for chromosomal aberration assay but the cultures were incubated in the 5 % CO_2 incubator for 72 h.

2) At h 44 after PHA stimulation, Cytochalasin-B (Cyt-B) (Sigma, final concentration at 6 μg/ml) was added into a culture.

3) After adding Cyt-B for 28 h, cells were harvested. Briefly, the cultures were centrifuged at 1,000 rpm for 10 min. Then supernatant was discarded.

4) Cells in each culture were mixed thoroughly and washed with 5 ml of Hank balanced salt solution.

5) The culture was centrifuged then supernatant was discarded. Afterwards, cell pellet was resuspended in 5 ml of cold 0.075 M KCl for two times.

6) The cultures were centrifuged and the supernatant was discarded. Cell pellet was mixed thoroughly, then a drop-wise of freshly-prepared chilled fixative solution was slowly added into the culture as described in 3.2.

7) A 50 μl of formaldehyde was added in each culture and the cultures were kept in a refrigerator for 30 min.

8) Repeated step 5 twice more and removed the supernatant until the supernatant became clear.

9) The cell pellet was resuspended in a small volume of fixative and dropped on the clean slides and allowed drying.

10) The slides were stained with 5 % Giemsa in Weise buffer.

11) A thousand binucleated cells (500 from each culture) were evaluated for micronuclei as described in Appendix E. The total micronuclei in 1,000 binucleated cells and percentage of binucleated cells with micronuclei were recorded. Moreover, the numbers of cells with mono-, di-, tri-, tetra-nuclei in 1,000 cells were counted for calculating of the nuclear division index (NDI) using the formula of Eastmond and Tucker (1989) as follow.

$$\text{Nuclear division index} \quad \frac{[(N1)+2(N2)+3(N3)+4(N4)]}{N}$$

While (N1), (N2), (N3) and (N4) were the cells containing one, two, three and four nuclei respectively and N was the total cell count (1,000 cells).

3.4 Alkaline single cell gel electrophoresis (comet assay)

The methods were performed according to a modified protocol by Speit and Hartmann (1999). The experiment was divided into two parts, estimation of internal standard range and detection of DNA damage from the studied subjects. The detail of each measurement was described as follow.

3.4.1 Estimation of internal standard range

1) A 5 ml of peripheral blood sample was drawn from a healthy control subject from outside the Saraphi and Chom Thong population using heparinized syringe by venipuncture and was gently layered on the top of 15 ml centrifuge tube containing 5 ml of pre-warmed (37°C) Ficoll-Paque (Amersham Pharmacia).

2) Lymphocytes were isolated by centrifugation at 1,600 rpm for 25 min.

3) The lymphocyte layer was aspirated carefully and put into a new centrifuge tube. The cells were washed by using sterile RPMI medium without fetal calf serum and centrifuged at 1,000 rpm for 10 min for two times.

4) After washing, the supernatant was discarded then cryopreservation solution was added into the lymphocytes.

5) The lymphocytes were mixed thoroughly then the cell number was counted by using haemacytometer and the cell suspension with $1x10^6$ cells/ml was prepared.

6) For negative internal standard, a 10 µl of cell suspension was aliquoted into a 0.5 ml appendorf. Afterwards, lymphocytes were kept in a cryopreservation refrigerator at -80°C until used.

7) For positive internal standard, a 0.5 ml of cell suspension in 1.5 ml of appendorf was irradiated with 2 Gy of gamma radiation (Cobolt 60). After irradiation, the lymphocytes were placed on ice immediately for DNA repair prevention. Then, a 10 µl irradiated cells were aliquoted rapidly and cells were kept in a refrigerator at -80°C until used.

3.4.2 Detection of DNA damage

Peripheral blood drawn from each studied subject in both Saraphi and Chom Thong populations was used for detection of DNA damage which determined from 3 types of blood samples, two slides each, as follow.

a) Peripheral blood (PB)

A 5 µl of peripheral blood was directly used for a slide preparation.

b) Stimulated peripheral blood (SPB)

Blood culture was set up by adding 300 µl of peripheral blood in 2.7 ml of RPMI 1640 medium as used for chromosomal and micronucleus analysis. Lymphocytes were stimulated for proliferation by adding PHA for 24 h in a 5% CO_2 incubator. After that, blood culture was centrifuged and supernatant was discarded. A 10 µl of cell suspension was used for a slide preparation.

c) Stimulated peripheral blood with the presence of DNA repair inhibitor, aphidicolin (SPB-APC)

Blood sample was prepared as described for SPB. At h 24, aphidicolin (final concentration at 15 µM/ml) was added and the culture. The culture was incubated for 2 hour more. Then, the culture was centrifuged and supernatant was discarded. Cell pellet was gently mixed for preparing the slides.

Both of internal standards (untreated and irradiated isolated lymphocytes of the control subject) and the three types of blood samples prepared from the studied subjects were used for slide preparation describe as follow.

1) A blood sample was mixed thoroughly with 120 µl of 1.5 % low melting temperature agarose (Sea Plaque® GTG) and then layered on a 0.5 % normal agarose (NEEO) coated slide.

2) The slide was covered with a cover slip, and then kept in a refrigerator for approximately 2-3 min for agarose hardening and DNA repair prevention.

3) The cover slip was gently removed and the slide was immersed into the cold freshly prepared lysis solution in a coupling jar, which was wrapped in aluminium foil for prevention of DNA damage from light, for at least 30 min at 4°C.

4) After lysis, the microscopic slides were placed into the electrophoresis unit in the electrophoresis buffer (pH>13) for 25 min for unwinding DNA and presenting of alkaline labile sites. In each electrophoresis session, 2 slides of untreated and irradiated isolated lymphocytes used as internal standard, were concurrently placed into the electrophoresis unit together with 10 coded microscopic slides from 1 or 2 studied subjects (6 slides from an individual). The electrophoresis run was numbered and the numbers were labeled on the slides both of the internal standards and of the subjects used for DNA damage detection.

5) The voltage and the current of a power supply were set to 25 V and 300 mA. The current could be adjustable by the level of buffer. An electrophoresis was run for 25 min on iced tray.

6) The slides were taken out of electrophoresis chamber and rinsed immediately with neutralization buffer (0.4 M Tris, pH 7.5) for three times with 5 min each. Afterwards, slides were rinsed with sterile water.

7) Slide dehydration was performed by dipping in absolute ethanol for 10 min.

8) The slides were allowed for air dry and kept in a dark box until analysis.

9) Before analysis, a 50 µl staining solution, ethidium bromide (final concentration at 20 µg/ml), was dropped on the slide and covered with a cover slip. Stained slides should be kept in a dark moist box before analysis.

10) The randomly 50 cells from each reference standards and subject were analyzed by Perceptive Image Analyzer Comet II, V 1.02e (Perceptive Instruments, Haverhill, UK). Three parameters including tail length, tail intensity and tail moment were used for detection of DNA damage from the cells as shown in Appendix F.

3.5 Statistical analysis

The statistical analysis was performed by STATVIEW software, SAS Institute Inc., Version 5. In this study, the data were ordinal scale and the distributions of the data were not normal. Therefore, non-parametric statistics were used for the analysis as follow.

1) The Mann-Whitney U Test was used to compare the average values (mean ±SD.) of the basal level of chromosome aberration, micronucleus frequency and DNA damage detected by comet assay between the two populations residing in the areas with high and low risk of lung cancer, Saraphi and Chom Thong, respectively.

2) The influence of confounding factors consisted of gender, age range, present pesticide exposure, smoking habit, alcohol drinking and fermented tea leaf or betel nut chewing, on the basal levels of chromosome aberration, micronucleus frequency and DNA damage detected by comet assay only from PB in the studied population were investigated using analysis of variance (ANOVA). In addition, any

confounding factor influencing the basal level of each endpoint was determined in details.

The analysis of variance was performed to investigate the influence of confounding factors. Differences were considered statistically significant at a P-value <0.05.

3.6 Health risk assessment

Besides the conclusion of the effects of each genetic test were evaluated between the studied populations residing in the areas expected to be at high and low risks of genetic damage, the combination of results from all genetic endpoints should be considered to assess the heath risk of the studied populations. Finally, the effective data would be informed to the subjects residing in both districts regarding their potentially genotoxic exposure in their environment for warning their adverse health outcome.