CHAPTER 2

LITERATURE REVIEWS

2.1 Lung cancer in Chiang Mai

Lung cancer has been ranked the first cause of new malignancy and mortality rate in Chiang Mai since the population-based cancer registry from 1983. The incidence increased sharply with age in both genders, but the incidence in males was more common in the young and old-age groups than in females. During 1988-1991, the annual average age-adjusted incidence rates of the disease were 49.8 and 37.4 per 100,000 in males and females (Vatanasapt *et al.*, 1993; 1995). Furthermore, the incidence rate especially of female lung cancer in Chiang Mai was very high when compared to those in other Asian countries including China which were also recorded as the areas with high incidences of lung cancer (Parkin *et al.*, 1992). Although, the incline of the incidences in both genders have been observed as shown in Table 1, lung cancer has still been the most common malignancy and also the first cause of cancer death.

Table 1 The age-standardized incidence rates of new malignant cases and mortality rates of lung cancer in Chiang Mai

Year	Age-adjusted incidence rates (per 10^5 persons)		Mortality rates (per 10^5 persons)	
	males	females	males	females
1990	52.4	38.6	43.8	32.7
1991	46.7	37.7	37.7	33.3
1992	47.7	40.1	43.7	32.8
1993	44.7	33.5	38.5	33.1
1994	36.7	34.5	29.7	27.7
1995	42.5	29.7	30.4	24.8
1996	42.3	24.8	26.9	14.8
1997	30.6	25.8	23.6	17.2
1998	31.5	20.2	23.7	14.9
1999	23.8	19.9	19.7	15.9

Sources: Annual Reports from 1990-1998 of Cancer Registry, Maharaj Nakhon Chiang Mai Hospital, Chiang Mai University

The first etiology of lung cancer in Chiang Mai is cigarette smoking (Simarak *et al.*, 1977). Cigarette smoke contains more than 4,000 compounds, including mutagens and carcinogens, e.g., benzene, toluene, 1,3-butadiene and benzo *[a]* pyrene which are associated with an increase risk of lung cancer and other diseases. Simarak *et al.* (1977) studied about the causatives of oral cavity, pharynx/ larynx and lung

cancers in the north of Thailand. The result showed that cigarette smoking including "Khiyo", a long typical man-made cigarette, was associated with non-significant elevated risks in both genders. However, chewing of "Miang", fermented wild tea leaf, was associated with an increased risk of lung cancer only in females.

For the northern Thai hand-rolled cigarette, Khiyo is from shredded tobacco leaves and other flavorings such as tamarind shell and Koy-tree bark, wrapped with dry young banana leaf. A Khiyo contained tar in the range of 28.5 to 200.8 mg per piece (Simarak *et al.*, 1977). Besides, the cigarette smoke contained nicotine and carbon monoxide in the ranges of 0.19 to 5.77 mg and 3.1 to 9.8 mg per piece of cigarette which had suspected to generate the high incidence of lung cancer (Mitacek *et al.*, 1991).

Although cigarette smoking has been closely associated with the incidence of lung cancer in Chiang Mai, but the association was clear only in males, but unclear in females (Suttajit *et al.*, 1994). Thereafter, a collaboration research of Thai-Japan between Faculty of Medicine, Chiang Mai University and Saitama Cancer Research Institute, Japan, studied in many aspects, including diet, lifestyles, biochemical and molecular approaches to clarify the etiology of lung cancer especially among northern Thai women residing in Saraphi and Chom Thong districts, the areas with highest and lowest incidences (40.9 and 8.5 per 100,000) of lung cancer in Chiang Mai. There were no differences in rice and meat consumptions between these two populations. Nevertheless, females from Saraphi remarkably consumed less in both quality and variety of green or yellow fruits and confectionary than those from Chom Thong (Sone *et al.*, 1998).

In addition, the epidemiological studies of females residing in both areas were simultaneously characterized by Nakachi *et al.* (1999). The details of study consisted of the interview of lifestyles, the chemical analysis of drinking water, biochemical and serological analyses, urine mutagenicity test and collection and identification of fungi and bacteria in the air inside their houses. The result showed that there were not significantly different in the lifestyles between females residing in the two areas while smoking habit was. Moreover, it was surprised that females from Saraphi, the area with higher incidence of lung cancer, had a significant lower percentage of current-smokers and higher of passive-smokers than those from Chom Thong.

With regards to the serological analyses of vitamin B2 and B12 and folic acid in serum of both populations, the concentrations of these substances were still in normal range. However, the significantly higher concentrations of these substances related to high fruit and vegetable consumptions were observed in Chom Thong population.

The most remarkable difference between females residing in both areas was the history of chronic benign respiratory disease. The Saraphi population had the experience of chronic benign history more than those from Chom Thong. The extremely high concentrations of IgE was probably due to the parasite infection, were observed in sera of the two populations. But the increased IgE was associated with the history of benign respiratory disease and with cigarette smoking only in females from Saraphi. Furthermore, the high urine mutagenicity was also observed in females from Saraphi who had experienced on benign respiratory disease.

Moreover, the microorganism identification was determined in the air inside the houses of both populations. The higher in amount and size of fungi colonies, *Microsporum canis, Acremonium fulciforum* and *Fusarium soranii* which associated

with the history of the benign respiratory disease, were observed from the houses of Saraphi populations than those of Chom Thong.

The epidemiological study was concluded that cigarette smoking was inadequate to explain the high incidence of lung cancer in females residing in Saraphi. But the history of benign respiratory disease which possibly associated with the infection of fungi was likely to be involved in the etiology of female lung cancer in Saraphi district (Nakachi *et al.*, 1999).

Subsequently other possible environmental factors that might be related to lung cancer causation in Chiang Mai were investigated, for examples radon and polycyclic aromatic hydrocarbons (PAHs). Indoor radon is a radioactive gas which was ranked as a main cause of lung cancer in the United States. Mungmai *et al.* (2000) determined the concentrations of indoor radon in the randomly selected buildings in Chiang Mai province in 7 districts. The investigation showed that houses built from concrete had the higher indoor radon concentration than wooden houses and houses with the floor touching the ground also had the higher concentration than those on the higher posts. The average (mean ± SD.) concentrations ($Bq.m^{-3}$) of radon observed in 7 districts were 14.72 ± 8.31 (Saraphi), 18.32 ± 79.53 (Chom Thong), 13.27 ± 7.90 (San Pa Tong), 13.25 ±9.70 (Prao), 10.65 ± 5.07 (Mae Rim), 16.76 ± 10.30 (Mae Taeng) and 11.26 ± 9.26 (Mae Wang). However, these concentrations were lower than the safety range threshold of 148 $Bq.m^{-3}$, set by the US. Environmental Protection Agency.

Thereafter, Wiwatanadate *et al.* (2001) investigated the risk factors for lung cancer mainly from exposure to indoor radon in the houses of lung cancer patients in Saraphi comparing with the houses of healthy subjects who resided both in Saraphi

and Chom Thong using the activated charcoal method. The level of radon in the houses of lung cancer patients was the highest among the three groups. Moreover, the studies showed that lung cancer causative was related to multifactor. Neither cigarette smoking nor radon exposure alone was effective in increasing lung cancer risk. Nonetheless, the combination of both factors increased lung cancer risk, but only in Saraphi.

Another aspect associated with lung cancer in Chiang Mai city was the level of PAHs. The high levels of PAHs in Chiang Mai air were measured (Zhang, 1996). The mutagenicity test of airborne particulate matters, PM 2.5 and PM 10, was also investigated in the city of Chiang Mai (Vinitketkumnuen *et al.*, 2002). The concentrations of PM 2.5 and PM 10 were within the acceptable ranges in summer but very high in winter. The mutagenicity test from the PM extracts by chloroform showed the activities to *Salmonella typhimurium* strain TA100 either with or without metabolic activation. The excessive levels of the particulate matters in winter were suggested to be associated with the geographical of the district and the burning activity. Chiang Mai is located in a valley and the wind speed is low in winter causing the poor air ventilation. In addition, there have been a number of forest fires and burning activities after harvesting of the agricultural products, especially in winter.

Lung cancer has been a problem of the public health in Saraphi and Chom Thong. The crude incidence rate of lung cancer in both genders in Saraphi population was high as shown in Table 2. In addition, the trend of increasing incidence of the disease investigated during 1995 to 1999 was also observed in Chom Thong population. The healthy populations in these two districts being published to the same

environmental exposure as the lung cancer patients are probable being at risk of the disease. Therefore using some suitable biomarkers to monitor and assess their health risks should be appropriated.

Table 2 The crude incidence rates of lung cancer in Saraphi and Chom Thong districts

Crude incidence rates (per 10^5 persons)		**1995**	**1996**	**1997**	**1998**	**1999**
Saraphi district:	Males	65.1	95.0	44.3	52.0	44.0
	Females	54.7	44.3	36.3	30.7	61.4
	Both sexes	59.8	69.1	40.2	40.9	53.0
Chom Thong district:	Males	8.6	24.9	24.8	39.7	21.4
	Females	0.00	15.7	12.4	9.2	9.2
	Both sexes	4.3	20.3	18.6	24.4	15.3

Sources: unpublished data from the Chiang Mai Cancer Registry from 1995-1999

2.2 Biomarkers

Human can be exposed to the environment toxicants from air, water, soil and food including the occupational and residential ones. The toxic agent might interact at molecular level, biochemical or functional level or physiological of the cellular level. However, the degree of reaction to the exposures might vary from no effect,

some adverse effect with recovery and to high toxicity with morbidity (Kubiak *et al.*, 1999). Various methods have been used to determine the level of toxic agent or its metabolites in the cells, tissues, excrete or body fluid to assess the biological response in the exposed individual. Therefore, biomarker is mentioned for using in the broad sense of the measurement reflecting an interaction between a biological system and a potential hazard agent, which may be chemical, physical or biological agents (World Health Organization, 1993).

Biomarkers are identified in three classes, including the biomarker of exposure, biomarker of effect and biomarker of susceptibility (World Health Organization, 1993). Biomarker of exposure is a measure of an exogenous substance or its metabolite or the product of an interaction between a toxic agent and some target molecules or cells in the compartment within an organism. Biomarker of exposure will reflect the distribution of the chemicals or its metabolite throughout the body which can be tracked through various biological levels, i.e. tissues or cells. The biomarker of exposure can be developed to show the quantitative relationships between the markers under different exposure conditions or at different times after exposure. The examples of biomarkers of exposure are the use of haemoglobin, urinary and DNA adducts. The others genetic biomarkers such as DNA and protein adducts and cytogenetic methods, i.e. chromosome damage, micronuclei induction, sister chromatid exchange has been extensively used as biomarkers of exposure. Chromosomal aberration has widely used for quantification and assessment of the potential of the genotoxic agents since many human carcinogens can cause chromosome damages and the alterations of chromosomes either in number and structure have been investigated in cancer cells.

However, the adverse effects in a biological system are not produced by the chemicals unless that the agents or their biotransformation products reach appropriate sites in the body at a concentration and for a length of time sufficient to produce the toxic appearance. Therefore, the exposure characterizations of the chemical in an individual, such as the source of the specific chemical, properties of the chemical, route to enter into the body, the concentration of the chemical, the duration and frequency of the exposure and site of toxicity and the information about susceptibility of the exposed individual are necessary to identify. Furthermore, the biological host characteristics, such as age, race, gender, health status and previous exposure to the same or other chemicals should be concerned.

Biomarker of effect is a measurable biochemical, physiological, behavioral or other alterations within an organism which can be possibly associated with an adverse outcome or disease. An example of biomarker of effect is haematological biomarkers, e.g. the inhibition of enzymes in the haem synthesis pathway has been used as a marker of effect of exposure to lead. The biomarkers of effect are approving for screening and/or identification of a toxic substance and characterization of the associated toxicity. Moreover, they are suitable for quantitative dose-response assessment. However, the inter-individual variations to the exposure and the suitable of accessible target tissues should be considered before measurement.

The other type of biomarker is biomarker of susceptibility which is an indicator of an inherent or acquired ability of an organism to respond to the challenge of exposure to a toxic substance. The activation or detoxification of the toxic substance in individuals might be different although the individuals were exposed to the same concentration of the chemical in the same environment because of the different rate of

enzyme activity. Therefore, the study of the genetic polymorphisms of the known genotypes which associate with metabolisms has been widely used to determine the linkage of susceptibility and the response to the adverse outcome for example, effect of glutathione *S*-transferase M1 polymorphisms on biomarkers of exposure and effect.

The main applications of biomarkers are recognized as providing data linking to the toxicity and mechanism of a chemical to assess the exposure and effect of the chemical and susceptibility of individuals. Moreover, they can be applied for the clinical diagnosis, monitoring purposes and health risk assessment. For the clinical approach, biomarkers can be used to confirm diagnosis of acute or chronic poisoning and applied to assess the efficiency of treatment and evaluate the prognosis of individual cases. The biomarkers can be used for the monitoring purpose to confirm the exposure of individuals in a population to a particular substance or exposure circumstance. It can be determined and applied on an individual basis or may be related to a population group. Population group "at risk" may be identified by deviations from normal of mean values determined by statistical analysis. The other application of biomarker is for the health risk assessment. The biomarker can provide the association between chemical exposures, internal dose of the chemical absorbed by individual over a period of time and adverse health outcome, and are estimated for the health risk (Au *et al.*, 2002).

2.3 Biomonitoring and health risk assessment

Biomonitoring has been used to evaluate exposure and risk for various environmental pollutants by means of biomarkers of exposure and biomarkers of effect (Černá *et al.*, 1997; Rössner *et al.*, 1998). It is the laboratory analysis of blood or other body fluid i.e. urine, serum, saliva, to identify the effect of certain chemicals present in the human body (Bickham *et al.*, 2000). The objective of the monitoring is to provide precise information on exposure for early warning and effective prevention of the health problems (Salama *et al.*, 1999). Many approaches and techniques have been developed for monitoring in human populations including the analysis of chemicals and metabolites or the analysis of genetic effects. Moreover, it is recommended to use more than one endpoint in biomonitoring study because each endpoint has its limitation (Hulka *et al.*, 1990).

Risk assessment is the process of the qualitative and quantitative of the data for estimating of the adverse health outcome (Tucker and Preston, 1996). The process for human health risk assessment is consisted of four major components consisting of the hazard identification, the exposure assessment, the dose-response assessment, and the risk characterization. The identification of hazard is performed to confirm that the chemical is capable cause of an adverse effect in humans. The epidemiological study and the *in vitro* short-term tests have been commonly used to identify the hazardous toxic substances. The exposure assessment is the process for measurement and identification and definition of the exposure that occurs or will occur in human population. The dose-response assessment is contrarily performed to establish the quantitative relationship between dose and effect in human while the risk

characterization is the final analysis of the data from all processes to assess the health risk in the individual or in the population.

However, it is necessary to select the suitable biomarker for the health assessment. The specificity and sensitivity of the measurement and other aspects including the laboratory measurement, quality assurance and control, validation and characteristics of biomarkers, and ethics and social should be considered. Briefly, specimens sampling should be non-invasive and the collection, handling and storage of them should be avoided of the contamination and deterioration. The measurement should be established and well documented of quality assurance and control, for example, it has been approved by The International Programme on Chemical Safety or World Health Organization (Albertini *et al.*, 2000). The biomarker should be able to define the interaction of the host biological system with the chemical of interest and it should be reproducible qualitatively and quantitatively with respect to time. Furthermore, the ethical and social implications are important aspects to concern. The participants should be comprehensively explained the details of the measurement in the study and they have the rights to consent or deny to participate in the study.

2.4 Genetic effect of environmental exposure in human population

Genetic effect is a widely approach for detection of biological effects from genotoxic exposure in human. The mutagenic chemicals can cause the damage of genetic material. The damage will stimulate the affected cells to respond which may

involve the repair of damage. Incorrect repair could certainly be the most causing health adverse outcome, or diseases (Bonassi and Au, 2002).

Normally, the toxic substances potentially affect the genetic material both in somatic and germ cells (Bickham *et al.*, 2000). The genetic alterations in somatic cells may associate to disorder or disease especially cancer, but they are not transmitted to the offspring of the exposed population. While heritable mutation which occur in germ cells are potentially be passed to the offspring and leading to the birth defects. However, somatic mutation is commonly used in monitoring study because the numbers of somatic cells are easy to sampling and analysis.

The traditional approach in most genetic monitoring studies for developing strategies in risk assessment and disease prevention has been the use of human peripheral lymphocytes in blood because the blood contacts with whole body tissue and it is readily obtainable. Nevertheless, the relationship between the endpoint of biomarker and health outcome in blood cell has not been well concluded because some of the biological effects, e.g. cigarette smoking, which detected by cytogenetic biomarkers, such as chromosomal aberration and micronucleus test, were inconsistent (Au *et al.*, 1991; 1998). The use of non-blood cells might be available choice for more accurate prediction of the exposure and adverse outcome or disease, for example using buccal or nasal mucosal cells to assess the effect of airborne toxic substances and respiratory disease. However, the selection of non-or blood cells should be concerned about the exposure condition and the experimental design. The appropriate cell type should explain the dose-response to specific exposure conditions and they should be high sensitivity and specificity to provide the precise prediction on health effect.

Genetic damage can be analyzed at the molecular level, functional gene and chromosome levels either in somatic or in germ cells (World Health Organization, 1985). At the molecular level, DNA damage can be determined in the term of adduct formation, strand break and repair, or base sequence alterations. The molecular endpoint which widely used is Ames test in *Salmonella* bacteria (Albertini *et al.*, 2000). The gene function can be detected by alteration of cellular compositions by immunological method whereas the damage at the chromosome level can be detected by numerical and structural alterations.

The cytogenetic analyses of human peripheral lymphocytes are frequently used to document the early biological effect resulting from chemicals and are widely used as the biomarkers of exposure to mutagens and carcinogens (Legator and Au, 1994). The cytogenetic monitoring has been suggested as general tool for characterizing and quantifying adverse human health effects due to the highly association between cytogenetic alterations and carcinogenesis (Mitelman *et al.*, 1997; Tucker and Preston, 1996). Various endpoints such as classical chromosomal aberration analysis, micronuclei, sister chromatid exchanges and fluorescence *in situ* hybridization have been frequently used in this approach. It was reported that chromosomal aberration analysis is the most appropriate application for health assessment in a population exposed to ionizing radiation while sister chromatid exchanges is more preferable in those exposed to chemicals. Micronucleus test has been becoming used for detection both the numerical and structural alterations. However, the potential of health effect has been preferable performed by assessing the chromosomal aberration while the assessments done by sister chromatid exchanges and micronucleus test have not been extensively approved (World Health Organization, 1985). Furthermore, the other

detections of DNA damage using biochemical and electrophoresis assays such as DNA adduct, strand breaks, cross-linking and alkaline labile sites have been concurrently used with cytogenetic endpoints for monitoring and assessment the health risk (Kassie *et al.*, 2000).

In this study, the short-term assays with three genetic biomarkers including chromosomal aberration, micronucleus test and single cell gel electrophoresis (comet assay) were used to monitor the biological effect in two populations residing in the areas with the differences in the lung cancer incidences. The information on genetic damages will be used to estimate health risk for further prevention of health problems in the studied populations.

2.5 Chromosomal aberration

After, the analysis of induced chromosomal alteration was first studied in the irradiated pollen microspores (Sax, 1933) and the using of hypotonic solution was developed (Hsu, 1952), the high accuracy of the chromosome damage analysis was achieved. Thereafter, chromosome aberration in human was getting evaluated in clinical diagnosis and the alterations of chromosome structure were analyzed after exposed to the irradiation (Bender and Gooch, 1966). Consequently, a various approaches have begun to study the clastogenic activity of chemicals involving *in vivo* and *in vitro* exposure in animals and humans.

The alterations at chromosomal level can occur either in number or in structure which are called numerical and structural chromosomal aberrations. The structural

aberration can be performed in any cycling cell or in any non-cycling cell populations which have to be stimulated to enter the cell cycle by *in vitro* cultivation. The non- and cycling cell populations which are commonly used to investigate the aberrations are peripheral blood lymphocytes and bone marrow cells. Nevertheless, the detection of chromosome damage from lymphocytes has been mainly performed because these cells are easily to sampling, readily to grow in tissue culture and numerous number of cells in metaphase are available for analysis. Moreover, lymphocytes have circulated throughout the body and they have relatively long life. Therefore, lymphocytes are suggested to be used as the suitable cell type for estimation of the average exposure in whole body (Bogen, 1993).

Numerical chromosomal aberration refers to the chromosome number changing caused by a mistake during cell division, e.g. the damage of mitotic spindle and associated element, damage of chromosomal sub-structure, alteration of cellular physiology and mechanical disruption. The numerical alteration can be classified as aneuploid when cell contains a more (hyperploid) or a less (hypoploid) chromosome number than the normal complement and the alteration is classified as polyploidy when cell contains manifold of normal complement (Albertini *et al.*, 2000). Nevertheless, the evaluation of changing in chromosome structure in somatic cells is more widely used than the changing in number of the chromosome, because the consequence in the number alterations is not well elucidated and the technical artifacts might affect the variability of the evaluation (World Health Organization, 1985).

The production of structural chromosome aberration is a complex cellular process. The mechanisms of chromosome breakage and rejoining are not completely understood. However, the structural aberrations have been classified as chromosome-

and chromatid-type aberrations. Chromosome-type aberration is aberration that involved the same locus of both sister chromatids in the same chromosome while chromatid-type aberrations involve one sister chromatid of any chromosome. Chromatid-type aberrations consist of chromatid break, exchange and intrachange while chromosome-type aberrations consist of chromosome break, acentric fragment, dicentric and ring chromosome (Palitti, 1998).

In addition, the structural chromosomal aberrations can be classified as unstable and stable aberrations depending on the ability to persist in dividing cell population. Unstable aberrations consist of ring, acentric fragment, and other asymmetrical rearrangements which will lead to cell death. Stable aberrations consist of balanced translocations, inversion, and other symmetrical aberrations which can be transmitted to progeny cells at division. The stable aberrations are more biologically effect than unstable aberrations and could be involved in carcinogenesis. However, they are difficult to quantify by conventional analysis without banding analysis (World Health Organization, 1985).

Chromosomal aberration assay has been proven to be a convenient test for somatic risk assessment. The frequency of chromosome damage in circulating lymphocytes was performed for prediction of health risk or future cancer onset in human exposed to the known mutagens/carcinogens (Bonassi *et al.*, 1995; 2000; Bolognesi *et al.*, 1997; Hagmar *et al.*, 1994; 1998). However, the association has not been restricted to cancer conclusion because carcinogenesis is multi-stage process and the effect of the chromosome damage and the mechanisms of the actions of human peripheral blood lymphocytes to the long-term exposure in individual are still

uncertain (Dulout *et al.*, 1996; Smerhovsky *et al.*, 2001; 2002; Weisburger and Williams, 1981).

Furthermore, chromosome aberration assay is the most extensively used and validated as a biomarker of early biological effect in population (Albertini *et al.*, 2000; Perera and Whyatt, 1994). It has certainly been used for detecting ionizing radiation exposure because the radiation directly induces chromosome-type aberrations, while the exposure to chemicals usually induced chromatid-type aberration. However, chromatid-type aberration can be converted into chromosome-type aberration such as translocation, which subsequently survives for a long time (Au, 1991). Cells with chromosomal aberration can lead to the development of cancer as indicated by the presence of chromosome deletions and translocations which were the major aberrations found in cancer cells (Mitelman *et al.*, 1997).

With regard to biomonitoring study, chromosomal aberrations were used for detecting the biological effect of known clastogenic agents in human population, for examples the chromosome analysis in the subjects exposed to arsenic (Dulout *et al.*, 1996; Gonsebatt *et al.*, 1997), 1,3-butadiene (Šrám *et al.*, 1998) and radon (Smerhovsky *et al.*, 2001; 2002). Furthermore, they were used to assess the impact of the mixtures of the toxic substances from the environment (Hüttner *et al.*, 1999; Klemans *et al.*, 1995; Lazutka *et al.*, 1999; Michalska *et al.*, 1999; Perera *et al.*, 1992) and occupational exposures, for example in workers exposed to X-rays (Iha and Sharma, 1991), benzene (Tompa *et al.*, 1994; Türkel and Egeli, 1996), coke oven (Kalina *et al.*, 1998), pesticides (De Ferrari *et al.*, 1991; Gómez-Arroyo *et al.*, 2000; Scarpato *et al.*, 1996), or in aircrews (Heimers *et al.*, 1995), pilots (Heimers, 2000), spray painters (Gajalaskshmi *et al.*, 2002; Pinto *et al.*, 2000). A large number of

human biomonitoring studies were based on data from the chromosomal aberration assay either alone or in combination with other biomarkers. Nevertheless, the significant differences in the basal levels of chromosomal aberrations between populations exposed to the complex substances could not be concluded in all studies (De Ferrari *et al.*, 1991; Pinto *et al.*, 2000).

In monitoring studies, however, not only the exposure to toxic substances can affect baseline of chromosome damage but other also exogenous and endogenous factors, such as age, gender, immunological and hormonal condition, genetic susceptibility, infection and medical treatment, could influence the damage (Ramsey *et al.*, 1995; Rössner *et al.*, 1998). The effects of age, gender, smoking habit and alcohol drinking in the studied populations have been commonly analyzed (Kasuba *et al.*, 1995; Landi *et al.*, 2000; Stephan and Pressl, 1999). Nevertheless, the influences of these factors have been inconclusive. For examples, age affected the frequency of chromosome damage in lymphocytes of individuals (Bolognesi *et al.*, 1997; King *et al.*, 1997) but some studies did not observe this effect (Bonassi *et al.*, 2000; Scarpato *et al.*, 1996). In addition, the differences of basal levels of chromosomal aberration between males and females were observed in some studies (Mahimkar, *et al.*, 2001; Scarpato *et al.*, 1996) while other studies did not find such observation (Bonassi *et al.*, 2000; Hüttner *et al.*, 1999).

The other confounding factor commonly analyzed is cigarette smoking. Some studies revealed that smokers had higher chromosomal aberration than non-smokers had (Anderson *et al.*, 1991; Au *et al.*, 1991; 1995; Scarpato *et al.*, 1996; Tompa *et al.*, 1994). Nevertheless, the others did not find any differences in chromosomal

aberration between these two groups (Gonsebatt *et al.*, 1997; Hüttner *et al.*, 1999; Kasuba *et al.*, 1995; Van Diemen *et al.*, 1995).

2.6 Micronucleus test

Heddle (1973) and Schmid (1975) reported an alternative and simpler approach to assess chromosomal damage *in vivo* by measuring micronuclei, also known as Howell-Jolly bodies in the polychomatic erythrocytes of bone marrow. After that, a cytokinesis-block micronucleus (CBMN) technique was developed for using as a sensitive and reliable method for measurement chromosome damage caused by genotoxic substances in lymphocytes (Fenech and Morley, 1985). The cells are recognized by their binucleated appearance after blocking cytokinesis using cytochalasin-B, an inhibitor of actin polymerization required for the formation of microfilament ring that constricts the cytoplasm between daughter nuclei during cytokinesis (Carter, 1967), allowing for the discrimination between cells which did not divide (momonucleated cells) and those which divided once (binucleated cells) or more (multinuclatated cells). The most important aspect of the CBMN method is the identification of the binucleated cells which allows a better precision because the data obtained are not confounded by altered cell division kinetics caused by cytotoxicity of agents tested or sub-optimal cell culture conditions. However, the assay can only be expressed in dividing eukaryotic cells. It cannot be used efficiently in non-dividing cell population or in dividing cell population which the kinetics of cell division is not well-understood or controlled (Fenech, 1993).

Micronucleus is expressed in dividing cell that contains either chromosome break lacking centromere (acentric fragment) and/or whole chromosome that is unable to move to spindle pole during mitosis. At telophase stage of cell cycle, the chromosome fragment is enveloped in a nuclear membrane and is morphologically indistinguishable from the main nuclei except for its much smaller size. Whole chromosome damages at the centromeric region or damage to kinetochore or spindle proteins may also result in a lagging chromosome at anaphase which subsequently gives rise to a micronucleus. Thus, micronucleus test is unique amongst cytogenetic tests in that it provides a reliable measure of both chromosome loss and breakage (Fenech, 1997).

The micronucleus assay is now applied for many purposes. In genotoxicity testing, micronucleus test has been used to assess the mutagenicity of chemicals and to identify potential carcinogens (Fenech, 2000; Kirsch-Volders *et al.*, 1997; Vanhauwaert *et al.*, 2001). Moreover, the assay is used for specific purposes such as the prediction of the radiosensitivity of tumours and the inter-individual variation in radiosensitivity for cancer risk (Scott *et al.*, 1998).

In addition, the micronucleus assay has also shown to be a reliable and sensitive biomarker for human biomonitoring study that being an adequate alternative to the *in vitro* chromosomal aberration test (Lucero *et al.*, 2000). The chromosomal aberration test is usually the most expensive and time consuming. It requires skill and considerable training for investigators to be fully competent to carry out this assay (Kirland, 1998) while the micronucleus test is simple and sensitive, easy to evaluate, less time consuming and requires less skill and experience. Moreover, the micronucleus assay has a high statistical power obtained from scoring larger number

of cells than are typically used for metaphase analysis. Nevertheless, the scoring of micronuclei was identified as an important factor to the experimental variables because there was no standard protocol for scoring the micronuclei (Brown *et al.*, 1997; Fenech, 1997). Until an international collaborative study on the use of the micronucleus technique was set up by Fenech *et al.* (1999) to develop a procedure for calibrating scorers and laboratories so that the results from different laboratories may be more comparable (Fenech *et al.*, 2003).

Recently, both micronucleus test alone and/or combination with chromosomal aberration or with other tests have been widely used in biomonitoring study. The assay is mainly applied to peripheral blood lymphocytes and to lesser extent in epithelial cells to compare chromosome damage between populations exposed to potential environmental mutagens (Surralles and Natarajan, 1997) such as 1,3-butadiene (Šrám *et al.*, 1998), lead (Vaglenov *et al.*, 1998), antineoplastic drugs (Maluf *et al.*, 2000), pesticides (Lucero *et al.*, 2000; Pastor *et al.*, 2001), air pollution (Köteles, *et al.*, 1993). In addition, the assay was applied to investigate the effect of occupational exposures and lifestyle factors, for examples in filling station attendants (Pitarque *et al.*, 1996a), airport personnel (Pitarque *et al.*, 1996b), fruit pickers (Davies *et al.*, 1998), workers at a phosphate fertilizer (Meng and Zhang, 1997), workers from laboratories of chemical analysis (Testa *et al.*, 2002), industrial radiographers (Sari-Monodier *et al.*, 2002). Moreover, the assay has been used for screening of chromosome damage in patients from medical treatment (Ballardin *et al.*, 2002).

With regard to the effects of the lifestyles and some biological factors, age and gender are the most important demographic variables influencing the MN frequency

in human lymphocytes (Barale *et al.*, 1998; Landi *et al.*, 2000). In fact, females and older subjects exhibit higher mean values of the MN frequency in comparison with males and younger persons. The MN frequency in females has been greater than that in males by a factor of 1.2 to 1.6 depending on the age group (Fenech, 1998). The association between advancing age and increasing of MN frequency has been due to aneuploidy process. The hypoploidy is more commonly observed than hyperploidy. The chromosomes most frequently lost are X and Y chromosomes in females and males respectively. The autosomes may be lost, but their loss should lead to cell death as they contain genes required for cell survival (Bukvic *et al.*, 2001, Jacob and Court Brown, 1961).

Moreover, several reports described some other lifestyles affecting the MN frequencies, for examples cigarette smoking and alcohol drinking. Several reports showed that cigarette smoking elevated MN induction (Da Cruz *et al.*, 1994; Holmen *et al.*, 1995; Tomanin *et al.*, 1991) while other studies did not find any association (Migliore *et al.*, 1991; Perera *et al.*, 1992). Furthermore, the effect of alcohol drinking on the MN induction was also inconclusive since some studies found enhanced of MN frequency in people drinking alcohol (Maffei *et al.*, 2000; 2002) while others did not find the association (Pitarque *et al.*, 1996a; 1996b).

2.7 Single cell gel electrophoresis (comet assay)

Besides cytogenetic markers such as chromosome aberration (CAs) and cytokinesis-block micronucleus assay (CBMN) are among the extensive biomarkers

used for detection of early biological effect of genetic damages, comet assay is also the novel approach for the same purpose. The assay is used to visualize and measure DNA strand breaks in individual cells using microscopy. The assay works upon the principle that strand breakage of the supercoiled duplex DNA leads to the reduction of the size of the large molecule and these strands can be stretched out by electrophoresis. Cells are embedded in agarose-coated slide, immersed in lysis solution to remove lipids and proteins and unwound DNA with high salt. Nucleoids are formed, containing non-nucleosomal but still supercoiled DNA. Any breaks presented in the DNA cause the supercoiling to relax locally and loops of DNA are then free to extend toward the anode as a "comet tail". After electrophoresis, DNA is stained using a fluorescent dye, and viewed using a fluorescence microscope (Collins *et al.*, 1997).

The comet assay was first introduced by Ostling and Johanson in 1984. This was a neutral assay in which the lysis and electrophoresis were done under neutral conditions and stained with acridine orange. The image obtained looked like a "comet" with a distinct head, comprising of intact DNA, and a tail, consisting of damaged or broken pieces of DNA, hence the name "comet assay" was given. Under neutral condition, only double strand breaks are detected since DNA base pairing is not disrupted and thus discontinuities in single strand breaks can not be detected (McKelvey-Martin *et al.*, 1993).

Consequently, the assay was modified involving electrophoresis under highly alkaline conditions, pH>13 (Olive, 1989; Singh *et al.*, 1988). At this condition, DNA supercoils get relaxed and unwound, which are then pulled out during application of electric-current which made possible for the detection of single and double strand

breaks. But single strand breaks are not the most interesting of lesions. They are quickly repaired and are not regarded as a significant lethal or mutagenic lesion. Many genotoxic agents do not induce DNA strand breaks directly. They may create AP-sites, which are alkaline-labile and are probably converted to breaks while DNA is in the electrophoresis solution at high pH (Collin *et al.*, 1997). Therefore, not only single strand breaks (SSBs) and double strand breaks (DSBs), but also the alkali labile sites and DNA-crosslinking can be measured by this assay (Singh and Stephens, 1997).

Compared with other genotoxicity assays, comet assay has several advantages. It is a non-invasive technique that virtually any eukaryotic cell population is amenable to analysis. The method requires small numbers of cells per sample, approximately 10,000 cells of an individual and 50-100 cells per individual/treatment is sufficient for an experiment and for DNA damage measurement to give a robust statistics. The results are obtained in a few hours compared to conventional cytogenetic techniques which take a few days. The assay is easy and results in high sensitivity for detecting low levels of DNA damage (McKelvey-Martin *et al.*, 1993; Tice *et al.*, 2000). It was reported that the assay resolved break frequency up to a few hundred per cell. The size of DNA lower limit of single strand break (in Dalton) which can be detected by the test was $2x\ 10^9$ per break as shown comparison to other methods as reported by Singh (2000).

There are two main principles to determine the pattern of comet formation. The ability of DNA to migrate is a function of both size of the DNA and number of broken ends which may be attached to larger pieces of DNA but which can still migrate a short distance from the comet head. At low damage levels, stretching of attached

strands of DNA, rather than migration of individual pieces, is likely to occur. With increasing numbers of breaks, DNA pieces migrate freely into the tail of the comet, and at the extreme (apoptotic cell), the head and tail are well separated (Choucroun *et al.*, 2001; Fairbairn *et al.*, 1995).

With regard to the DNA damage measurement, the simplest of the technique is to score the comet empirically on the basis of damage extent. Visual scoring entails categorizing randomly selected comets according to relative tail intensity. The scorings are based on their appearance as either damaged or undamaged, or even with gradation into categories, for examples none, short, medium, long or from class 0 to class 4. Approximately 50-100 cells are classified according to individual class. The single overall rating for the slide can be obtained from the summation. The visual classes correspond very roughly to 20% intervals for the computer-assesses scores of percent DNA in tail (Collins *et al.*, 1997).

However, a flexible approach for collecting data has been done by computerized image analysis (Fairbairn *et al.*, 1995; Tice *et al.*, 2000). Tail length, tail intensity and tail moment have been widely used as the parameters to classify the DNA damages (Kobayashi *et al.*, 1995). Tail length is the most commonly used as a parameter for measurement of the length of DNA migration, usually presented in micrometre. The parameter relates directly to fragment size of DNA and would be expected to be proportional to the extent of DNA damage (Rojas *et al.*, 1999). Nevertheless, under neutral or pH 12.3 conditions, tail length initially increases with damage but reaches a maximum while the percentages of migrated DNA continue to increase (Fairbairn *et al.*, 1995). However, this limitation is not a characteristic of the assay with pH>13,

where tail length has been reported to be the best parameter for this version of the assay (Tice, 1995; Vijayalaxmi *et al.*, 1992).

While tail intensity is a parameter based on the relative amount of migrated DNA, presented either as the percentage of migrated DNA (Olive *et al*, 1990) or as the ratio of DNA in the tail and in the head (Müller *et al.*, 1994). This parameter assumes signal linearity for quantifying the amount of DNA ranging over multiple orders of magnitude and that the staining efficiency of the fluorescent dye is identical for migrated and non-migrated DNA.

In addition, an increasingly popular parameter used for the comet evaluation is tail moment which was introduced by Olive *et al.* (1990). This parameter can distinguish the information on the relationship between tail length and percentage of migrated DNA or that between tail length and tail intensity. Such information may provide insight into agent-specific differences in the intragenomic distribution of DNA damage within a cell. However, tail moment has not been approved as the most appropriate parameter. The data contained DNA damage detected by all parameters, tail length, tail intensity and tail moment, should be provided when the damage is analyzed (Tice *et al.*, 2000).

Comet assay under alkaline condition is the most common method for detection of DNA damage. The applications of the assay were used in several purposes. Comet assay has been widely used to investigate cellular responses to DNA damage and in *in vitro* studies for genotoxicity. Many data were published in the last few years because of the high sensitivity and specificity to detect the genotoxic effects (Fairbairn *et al.*, 1995; Rojas *et al.*, 1999; Tice *et al.*, 2000). Moreover, the assay has been usefully applied to screen lymphocyte samples from human population for their susceptibility

to oxidative damage induced by ionizing radiation (Collins *et al.*, 1996; Duthie *et al.*, 2002; Green *et al.*, 1992; Hartmann *et al.*, 1994; Tice *et al.*, 1990). In addition, the assay has becoming used in DNA repair study. DNA repair capacity was detected by measured the removal of DNA damage in cells after the treatment (Gedix *et al.*, 1992; Speit and Hartmann, 1999). The assay has also been used in the biochemical dissection of excision repair of DNA (Mayer *et al.*, 2002).

Most of epidemiology studies published on the comet assays either using resting or PHA stimulated lymphocytes. Circulation lymphocytes are usually resting in G_0 of the cell cycle but they can be efficiently stimulated by mitogens (e.g. phytohaemagglutinin, PHA) to divide *in vitro*. The higher extent of background DNA migration in PHA simulated cells versus quiescent cells has already been reported (Mayer *et al.*, 2002). During the proliferation, the excision repair will be processed to fix the damaged cells. Thus, the increasing of DNA damage will be investigated associating with the accumulation of incomplete excision repair site by the presence of aphidicolin, DNA repair inhibitor, in the culture (McKelvey-Martin *et al.*, 1993; Tice, 1995).

Recently, the comet assay has been introduced as a useful technique for human biomonitoring study (Collins *et al.*, 1997; Kassie *et al.*, 2000; Olive *et al.*, 1992; Poli *et al.*, 1999), especially in occupational, lifestyle and environmental exposure studies, for examples exposure to known toxic agents as aluminum (Crebelli *et al.*, 2002), anaesthetic gases (Sardas *et al.*, 1998), benzene (Andreoli *et al.*, 1997), pesticides (Lebailly *et al.*, 1998), radiation (Wojewódzka *et al.*, 1998), styrene (Somorovská *et al.*, 1999a). Moreover, the DNA damage was determined from workers exposed to

the combination of hazardous reagents from environmental pollutant at a waste disposal site (Hartmann *et al.*, 1998) or in rubber factory (Somorovská *et al.*, 1999b).

In biomonitoring studies, however, samples are collected over an extended time period. Slides are prepared and the experiments are run in shift of electrophoresis. Therefore, it is necessary to include the referent standard within each run of electrophoresis to compare measurements from the different electrophoresis runs for minimizing the experimental variations when large numbers of samples are needed to be analysis (De Boeck *et al.*, 2000). Although, the use of referent standard was recommended in human biomonitoring study as in IPCS guidelines (Albertini *et al.*, 2000), however, most of published studies have not considered about it.

With regard to the confounding factor effect on the comet result, a report showed that gender influenced DNA damage. Males had the higher baseline of DNA damage than females (Bajpoayee *et al.*, 2002). Cigarette smoking has also been a factor commonly analyzed. However, the contrary effects of smoking have been reported. Some studies showed the significant increase in DNA damage in smokers (Betti *et al.*, 1994; 1995, Dhawan *et al.*, 2001; Sardas *et al.*, 1998) whereas the other studies did not find any differences on the DNA damage between smokers and non-smokers (Binkova *et al.*, 1996; Friis *et al.*, 1997; Sram *et al.*, 1998; Valverde *et al.*, 1997). Furthermore, it was shown that the inter-individual variations such as, DNA susceptibility to DNA damage (Klaude *et al.*, 1996; Wojewódzka *et al.*, 1998) and to DNA repair (Berwick and Vineis, 2000; Mayer *et al.*, 2002), influenced the base line of DNA damage between individuals.