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ABERRANT METHYLATION
of *p16* and *MGMT* GENES
in LEBANESE SMOKERS

By

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fulfillment of the
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ABSTRACT

ABERRANT METHYLATION
of *p16* and *MGMT* Genes
in LEBANESE SMOKERS

By Carol Daoud

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Recent studies have demonstrated that aberrant methylation of *p16* (tumor suppressor gene) and / or *MGMT* gene (DNA repair enzyme) is detected in DNA samples from sputum in 100% of patients with squamous cell carcinoma as early as 3 years before clinical diagnosis. Moreover, the prevalence of these markers in sputum from cancer-free, high-risk subjects approximates lifetime risk for lung cancer. Knowing that the casual relationship between smoking and lung cancer is well established and that 90 percent of all lung cancer deaths can be attributed

to smoking, the goal of my research was to assess the methylation status of *p16* and *MGMT* genes in a controlled population of smokers versus a control population of non smokers.

Accordingly, sputum samples were collected randomly from 198 people; 102 smokers and 96 non-smokers in sterile specimen cups and were subjected to molecular genetic analysis. Aberrant promoter methylation of *p16* was seen in 73.33% of smokers above 40 years old, and in 8.7 % of smokers below 26 years old, where as methylation of *MGMT* was not detected at all. As for non smokers, methylation of *p16* gene was detected in 4 % of controls above 40 years old and non below 26 years old where as methylation of *MGMT* was detected in only one case.

The strong association seen between *p16* methylation and smokers seems to offer a potentially strong approach to population – based screening for the early detection of lung cancer, and possibly other forms of human cancer.

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Glossary

CT: Computed topography

AQC: Automated quantative cytometry

IARC: International agency for research on cancer

HPV: Human papilloma virus

AML: Acute myeloblastic leukemia

SCOTH: Scientific committee on Tabacco and health

SCC: Squamous cell carcinoma

DAP-K: Death associated protein kinase

MGMT: Methylguanine-DNA methyltransferase

ER: Estrogen receptor

MSP: Methylation-specific- PCR

Mo: Immortality checkpoint

Pu: Plutonium

DAP: Death-associated protein

BEC: Bronchial epithelial cells

NSCLC: Non small cell lung cancer

Chapter 1

Introduction

Lung cancer remains the largest cause of cancer death despite 50 years of antismoking efforts and advances in treatment (Jemal et al., 2003). The majority of lung cancers are inoperable at presentation, thereby limiting available treatment and potential cure (Fry et al., 1996). Patients with earlier stage disease have better survival rates but constitute a small proportion of presenting cases (Cortese et al., 1983).

Lung cancer will continue to be an enormous burdens as over 20% of the populations continue to smoke. In addition, former smokers remain at risk, as nearly half of newly diagnosed lung cancers occur in this group. The concept that earlier detection of lung cancer may improve the outcome of this disease has been a subject of controversy over the last 30 years (Tong et al., 1996).

In Lebanon, for example, lung cancer accounts for around 10-18 % of all male cancers and the percentage has recently decreased (although the number of cases per year has remained around 70-80 patients/year) despite a significant increase in the overall number of cancers/year. In females, the number of lung cancer is on the rise in terms of numbers and percentages as well, but accounts for only 4% of all female cancers (Mokdad et al., 2004).

Lung cancers has been closely linked to tobacco smoking with some of the highest rates seen in the Middle East, China, the Caribbean, South Africa, Zimbabwe and the Pacific. National incidence rates closely reflect the history of tobacco smoking. In countries with a long history of smoking about 90% of cases of lung cancer incidence and mortality reflect the maturity of the smoking epidemic in various countries (Macaron et al., 1997).

A study done in Lebanon revealed a smoking prevalence in the adult population in Lebanon of 53.6%. The percentage of ex-smokers was estimated to be 13.7%. By contrast, the reported prevalence of smoking in the USA in 1992 was 25.6% (Fiore, 1992), and has continued to decline since that year, emphasizing just how alarming is the Lebanese prevalence (Baddoura and Chidiac, 2001). The study showed smoking prevalence in Lebanon population varying with age, with the highest prevalence among the 30-40 years age group. The proportion of smokers decreased progressively after the age of 60 years, not necessarily because people quit smoking by that age, but more likely because many fewer smokers live to that age than do non-smokers. The decrease seen in Lebanon sample is less than that observed in France, and is considerably delayed in comparison to the USA where the proportion starts to decline around the age of 45 years (Grizeau et al., 1993 and Fiore, 1992). By comparison, the results obtained in the study are similar to those obtained from other developing countries in the early eighties: China 56% (1981 figures); Egypt 40% India 61% (1985); Indonesia 61% (1984); and Tunisia 58% (1984) (Bartstra, 1996).

Longer follow up is needed in Lebanon to determine whether these trends reflect awareness in the smoking epidemic. Certainly in women the trends is still increasing while in men many countries have reached this

knowledge. The age-standardized incidence of lung cancer in Lebanon in both males and females (18.7 and 5.1 per 100000 in 1988) is much less than that of countries such as the USA, UK, France (65-66 per 100000), and are closer to that of Israel and Algeria (28.5 and 14.5 per 100000). Yet, the main problem remains that most lung cancer patients whether in Lebanon or any other part in the world are not identified until their malignancy has reached an advanced stage. The concept that earlier detection of lung cancer may improve the outcome of this disease has been a subject of controversy over the last 30 years (Tong et al., 1996).

Results of previous screening studies using chest X-ray and sputum cytology contributed to the negative opinion regarding the benefit of lung cancer screening (Kubik et al., 1990). However, chest X-ray has been shown to fail to detect up to 77% of computed topography (CT) -detected cancers. The earlier screening studies were also underpowered to show a small benefit of screening using chest X-ray (Sone et al., 2000).

Rapid technological advances in multidetector spiral CT that scan the entire thorax at high resolution within 15 to 20 seconds have shown that CT detects lung cancer at an earlier stage and smaller size compared with chest X-ray. Unfortunately, improved sensitivity is associated with a higher false-positive rate. The frequency of noncalcified pulmonary nodules varies from 5 to 50%, with higher rates noted with thinner slices. Over 90% of these nodules are nonmalignant. In addition, CT scan is not sensitive for detection of superficial, pre-invasive / micro-invasive cancers in the central airways (Sone et al., 2000).

There is increasing interest in the use of sputum biomarkers such as immunohistochemical, methylation, and molecular markers as well as

automated quantitative cytometry (AQC) to detect gross genomic alterations present in early lung cancer.

Based on the information stated above, I have pursued, in my research, gene-specific aberrant promoter hypermethylation of *p16* and *MGMT* genes as one of the approaches to aid in the early diagnosis possibly of lung cancer especially that recent findings demonstrate that aberrant methylation can also be detected in the smoking-damaged bronchial epithelium from cancer-free heavy smokers, suggesting that aberrant methylation might be an ideal candidate biomarker for lung cancer risk assessment and monitoring of methylation can be reversed by demethylating agents resulting in gene re-expression. This concept is currently under investigation in clinical trial (Belinsky, 2004).

To sum up, this study comprised:

Observation of smoking habits in a controlled sample of the Lebanese population at the Lebanese American University campuses

Detection of aberrant promoter methylation of the *P16* and *MGMT* genes in smokers verses controls

Relationship of smoking to methylation and comparing results to similar studies in other countries

Chapter 2

Literature review

2.1- Smoking and cancer

Cancer is second only to heart disease as a cause of death and people are very frightened of it. But there are many cases of people being cured. The surest remedy is for a surgeon to remove the tumor before it starts spreading, but cancer can also be treated with drugs (chemotherapy) and radiation. Such treatment kills the tumor cells or at least stops them from dividing. For the treatment to be successful, it is important to discover the tumor as early as possible.

It is estimated that one in three people will develop cancer at some stage in their lives and that one in four will die from the disease (Rodenhuis, 1996). In 2000, there were 42,800 cancer deaths in the UK attributable to smoking: approximately a third of all cancer deaths. The UK Government has set a target (for England) to reduce the cancer death rate in people aged under 75 by 20% by 2010 (Merlo et al, 1994). The route to achieving this is set out in the National Cancer Plan, which includes targets to reduce smoking (Rodenhuis, 1996).

Cigarette smoking is an important cause of cancers of the lung, larynx (voice box), pharynx (throat), esophagus, bladder, kidney and pancreas. A recent review by the International Agency for Research on Cancer found that, in addition to these cancers, smoking is a cause of cancer of the nasal cavities and nasal sinuses, stomach liver, cervix and myeloid leukemia (Jones and Baylin, 2002).

Smoking cigarettes, pipes and cigars is a risk factor for all cancers associated with the larynx, oral cavity and esophagus. Over 90% of patients with oral cancer use tobacco by either smoking or chewing it. The risk for these cancers increases with the number of cigarettes smoked and those who smoke pipes or cigars experience a risk similar to that of cigarette smokers. In total, 3522 in the UK people suffered from oral cancer in 1993 (Merlo et al., 1994).

Heavy smokers have laryngeal cancer mortality risks 20 to 30 times greater than non-smokers. People who drink alcohol and smoke have a much higher risk of oral and pharyngeal (throat) cancers than those only using tobacco or alcohol. A US study revealed that among consumers of both products the risk of these cancers was increases more than 35-fold among those who smoked forty or more cigarettes a day and took more than four alcoholic drinks a day. It has been estimated that tobacco smoking and alcohol drinking account for about three quarters of all oral and pharyngeal cancers (Rodenhuis, 1996).

Tobacco smoking is the principal preventable risk factor for bladder cancer in both men and women. It is estimated that current smokers are two to five time more likely to develop bladder cancer than non-smokers. As for lung cancer, the risk is associated with both the dose and duration of smoking, while cessation of smoking reduces the risk (Jones and Baylin, 2002).

Some studies have demonstrated a link between both active and passive smoking and breast cancer. The IARC review concluded that most epidemiological studies have found that among women who had smoked for 40 years or longer the risk of breast cancer was 60% higher than that of women who had never smoked. Among those who smoked 20 cigarettes or more a day for 40 years, the risk rose to 83% (Jones and Baylin, 2002).

Cancer of the cervix has been found to be associated with cigarette smoking in many case-control studies. Until recently, scientists had been unable to decide whether the relationship was causal or due to confounding factors such as the number of sexual partners. A study in Sweden investigated whether environmental factors such as smoking, nutrition and oral contraceptive use were independent risk factors for cervical cancer and found that smoking was the second most significant environmental factor after human papilloma virus (HPV). The IARC review concludes that there is now sufficient evidence to establish a causal association of squamous-cell cervical carcinoma with smoking (Merlo et al., 1994).

Kidney cancer has consistently been found to be more common in smokers than in non-smokers and there is now sufficient evidence to show that smoking is a risk factor for the two principal types of kidney cancer. There is a dose-response relationship with increasing numbers of cigarettes per day and risk appears to drop after smoking cessation. Approximately, 24% of kidney cancer cases in men and 9% in women can be attributed to smoking (Jones and Baylin, 2002).

Smoking is causally associated with myeloid leukemia in adults but not to lymphoid leukemia. A French study on patients with newly diagnosed acute myeloid leukemia concluded that cigarette smoking had an adverse effect on survival in acute myeloblastic leukemia (AML), by shortening complete remission duration and subsequent survival (Merlo et al., 1994).

Tobacco smoking is a cause of cancer of the esophagus (gullet) and the risk increases with the number of cigarettes smoked and duration of smoking. The risk also remains elevated many years after smoking cessation (Rodenhuis, 1996).

Cancer of the pancreas is a rapidly fatal disease with a five-year survival rate of only 4%. Cigarette smoking is a strong and consistent predictor of pancreatic cancer although the risk diminishes to that of a non-smoker ten years, on average, after cessation. Risk of the disease is related to consumption and duration of smoking. Smokers have about twice as high a risk for this cancer as non-smokers (Sgerr, 1996).

Non-smokers are at risk of contracting lung cancer from exposure to other people's smoke. The UK's Scientific Committee on Tobacco and Health (SCOTH) found that the research findings were consistent with an increased risk of lung cancer in non-smokers of between 20% and 30% (Rodenhuis, 1996).

Subsequent review of the evidence by SCOTH in 2004 re-confirmed that the increased risk was in the order of 24%. This means that passive smoking causes several hundred-lung cancer deaths in non-smokers each year in the UK. The IARC review confirmed that the evidence is sufficient to conclude that involuntary smoking is a cause of lung cancer in never smokers. Lung cancer is the most common cancer in the world

with 1.2 million new cases diagnosed every year. Until recently, lung cancer was the most frequently occurring cancer in the UK; it has now been overtaken by breast cancer but it still accounts for 1 in 7 new cancer cases, that is, around 38,400 new patients annually (Merlo et al., 1994).

In Britain, one person every fifteen minutes is diagnosed with lung cancer. Lung cancer is the cancer most commonly associated with smoking: smoking causes around 90% of all lung cancers, either directly or through indirect exposure. Because of its poor prognosis, lung cancer is still the most common cause of cancer deaths in the UK, with around 33,600 deaths each year, accounting for 6% of all deaths and 22% of all cancer deaths in the UK (Jones and Baylin, 2002).

Nearly one in four of smokers die of lung cancer. The risk of dying from lung cancer increases with the number of cigarettes smoked per day, although duration of smoking is the strongest determinant of lung cancer in smokers. Smokers who start when they are young are at an increased risk of developing lung cancer (Merlo et al., 1994)).

Results of a study of ex-smokers with lung cancer found that those who started smoking before age 15 had twice as many cell mutations as those who started after age 20 (Rodenhuis, 1996).

A longitudinal study of British doctors by Doll and Peto (2000) examined the effects of prolonged cigarette smoking and prolonged cessation on mortality from lung cancer. They found that if people who have been smoking for many years stop, even well into middle age, they avoid most of their subsequent risk of lung cancer. Also, stopping smoking before middle age avoids more than 90% of the risk attributable to smoking (Merlo et al., 1994).

2.2- Epigenetics.

Epigenetics is the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequences. Epigenetics may even call for a new definition of gene, which includes sequence information, as well as range of possible epigenetic instructions. Epigenetics aims to describe the inheritance of information on the basis of expression in contrast to "genetics", which aims to describe the inheritance information on the basis of DNA sequence. During the last few years it has become clear that DNA methylation is an essential component of epigenetic phenomena such as genomic imprinting and x-chromosome inactivation. DNA methylation is a very effective mechanism to turn a gene off and alter gene expression of a cell. Thus, aberrant methylation patterns play a critical role in certain human diseases. Certain genes are inappropriately switched off in many cancers, due to inappropriate methylation. A change in the pattern of methylation is an early event in cancer development and progression. The pattern of which genes are methylated is disease specific. By reading these methylation patterns on the genome, the disease can be better identified, as well as the development of new and powerful strategies to fight disease (Xu et al., 1993).

2.3-DNA methylation

DNA methylation is one of several post-synthetic modifications that normal DNA goes through after each replication. Of the four types of base pairs only the Cytosine-Guanine (CG) base pair is methylated. Since DNA replication is semi-conservative, one strand of the new DNA is already methylated and the other strand remains to be methylated by an enzyme called a maintenance methylase. Furthermore, since not all CG base pairs in DNA are methylated the maintenance methylase enzyme must recognize the appropriate base pair to insure that the each piece of newly synthesized DNA is identical (Li et al., 1993). The following pictures (Figures 1, 2 and 3) illustrate these aspects of DNA methylation

Figure 1: A non methylated DNA sequence.

(Source: Li et al., 1993)

S-ADENOSYLMETHIONINE

Figure 2: Transfer of Methyl group to the DNA sequence

(Source: Li et al., 1993)

METHYLATED

Figure 3: A methylated DNA sequence.

(Source: Li et al., 1993)

2.3.1 Functions of Methylation

Methylation protects from endonucleases designed to destroy foreign DNA (mainly bacterial DNA). Endonucleases are enzymes that cut DNA at specific points (i.e. a CG base pair). However, if the base pair is methylated then the very specific restriction enzyme will not recognize the DNA. Since human DNA and bacterial DNA are methylated differently, but each in an extremely specific manner, this defense mechanism allows for only foreign DNA to be destroyed by the endonucleases (Li et al., 1993).

Methylation regulates gene expression. Experimental evidence has shown that in certain cells there are heavily methylated genes and these genes are not expressed. On the other hand cells that have non-methylated forms of these genes are expressed. An example of these is seen in housekeeping cells (cells that produce proteins used in "clean up" of cellular debris and dead organelles) in which cells with non-methylated genes for these cells continuously transcribe or produce the materials needed to make housekeeping cells. Also one of the X chromosomes in females is not expressed. A hypothesis for this phenomenon is linked to the heavy methylation of the inactive X chromosome (Xu et al., 1993).

2.4- Silencing of genes by promoter hypermethylation

The past two decades of research have seen a tremendous growth in defining gene target and pathways disrupted during the development of lung cancer. Some of the earliest findings identified activation of the *K-ras* oncogene and inactivation of the *p53* tumor suppressor gene by point mutation as predominant alterations in lung cancer etiology. In spite of

identifying dysfunction within these two important genes/pathways, many lung tumors were found to arise independently of alteration within these genes.). It became apparent through genome studies of loss of heterozygosity that many DNA regions exist that could contain tumor suppressor genes. One region on the short arm of chromosome 9p was found to contain the *CDKN2A (p16)* gene (Toyooka et al., 2001).

Mutations within the p16 coding sequence were commonly found in melanoma; however, low rates of mutation in other solid tumors such as lung suggested an alternative mechanism for the loss of function.

P16 was the first tumor suppressor gene found inactivated in lung cancer predominantly through aberrant hypermethylation of its 5' promoter region. The *p16* gene is inactivated by methylation at prevalence up to 60-70 % in adenocarcinomas and squamous cell carcinoma (SCC) of the lung, respectively. The targeting of *p16* for inactivation is likely due to the critical function of this gene in the cell, to inhibit cyclin dependent kinases that bind cyclin D1 and phosphorylate the retinoblastoma gene (Jones and Baylin, 2002).

Since the identification of aberrant promoter methylation as an inactivation mechanism for the *p16* gene, epigenetically mediated silencing of transcription has been associated with inactivation of >30 genes in lung cancer. Transcriptional silencing by CpG island hypermethylation affects genes involved in all aspects of cellular function such as cell-cycle control, differentiation and signaling, apoptosis and invasion. The commonality of gene promoter methylation in the etiology of lung cancer has led to investigate the role methylation plays in cancer initiation and whether exposure to defined carcinogens are associated with specific gene inactivation via methylation. The ability of pharmacologic agents to

reverse promoter hypermethylation also makes it an attractive target to pursue for prevention of lung cancer (Jones and Baylin, 2002).

2.5- Methylation as an initiating event in lung cancer

The fact that many genes controlling cell regulation are inactivated in lung cancer by promoter methylation suggests that a subset of these genes would likely be involved in initiation and others in progression.

A progression model similar to that developed for colon cancer is lacking for lung cancer, due to the difficulty in obtaining premalignant lesions from high-risk people without synchronous invasive tumors.

To overcome this obstacle studies on precursor lesions to adenocarcinoma and SCC were done in animal models of lung carcinogenesis and in human subjects, respectively (Belinsky, 2004).

2.6- *p16* Methylation

P16 is a tumor suppressor gene that plays an important role in inhibiting phosphorylation, in contrast to the growth factors such as RAS and MYC that encourage phosphorylation. So if silenced, cells will divide without control leading to tumor formation.

Initial studies were focused on the *p16* gene to see its methylation status. In the rat, 94% of adenocarcinomas induced by the tobacco-specific carcinogen four-methylnitrosamino-1- (3-pyridyl)-1-butanone (NNK) were hypermethylated at the *p16* promoter. This methylation change was also detected frequently in precursor lesions to the tumors-adenomas and hyperplastic lesions. The timing for inactivation of *p16* by methylation in SCC was recapitulated in human SCCs where the *p 16* gene was

coordinately methylated in 75% of carcinoma in situ lesions adjacent to SCCs containing this change (Belinsky, 2004) .

Moreover, in premalignant lesions obtained from persons without SCC, the frequency of this event increased during disease progression from basal cell hyperplasia (17%) to squamous cell metaplasia (24%) to carcinoma in situ (50%) (Belinsky, 2004).

Further studies substantiating that inactivation of *p 16* may be a very early event in lung cancer examined bronchial epithelial cells obtained through bronchoscopy from cancer-free smokers. Methylation of *p16* was detected in 25 of 137 biopsies (18%) that were classified histologically as normal, metaplastic or mildly dysplastic. In contrast, no methylation of *p 16* was seen in biopsies obtained from individuals who had never smoked. Also, observation was done whether in lung cancer cases an association existed between the methylation status of *p16* in the bronchial epithelium and the diagnosed tumor. Matched tumor tissue and bronchial epithelium (2-3 bronchial biopsies per case) obtained from lung lobes that did not contain the primary tumor were available from 18 cases of non-small cell lung cancer. An absolute concordance of being either methylated in the tumor and at least one bronchial epithelium site or unmethylated in the tumor and the bronchial epithelium was seen in 17 of 18 cases. The strong association seen between *p16* methylation in the bronchial epithelium and corresponding primary tumor substantiates the fact that inactivation of this gene, although not transforming by itself, is likely permissive for the acquisition of additional genetic and epigenetic changes leading to lung cancer (Belinsky, 2004).

2.7- Methylation of *MGMT* and other genes in lung cancer

MGMT gene encodes a DNA repair enzyme that carries a demethylation reaction. Cells can avoid most of the lethal methylating compounds that are powerful mutagens and carcinogens by reversing methylation of the O⁶ atom of DNA guanine. This reaction is carried out by the DNA repair enzyme, MGMT (O⁶ – methylguanine-DNA methyltransferase).

While p16 is clearly one of the earliest genes known to be altered through methylation in lung cancer, other genes are emerging that are likely inactivated during premalignancy. Methylation of the death associated protein kinase (DAP-K) and retinoic acid receptor 8 (RAR-B) genes were detected in approximately half of alveolar hyperplasia induced in mouse lungs exposed chronically to NNK (Vuillemonet et al., 2004).

Supporting the likelihood that DAP-K inactivation occurs at the time when premalignant changes can be observed histologically, only 3% of bronchial biopsies examined for p16 had methylation of DAP-K. The DNA repair gene, O⁶-methylguanine-DNA methyltransferase (*MGMT*), also appears to be inactivated after the *p16* gene. Only 3 of 40 (8%) biopsies with histology including normal, hyperplasia, metaplasia and dysplasia obtained from heavy smokers showed methylation of this gene (Zochbauer-Muller et al., 2001).

In addition, the prevalence for methylation of *MGMT* increased between stage I adenocarcinoma and stages II-IV. The timing for inactivation of the genes studied to date appears to coincide with their roles in cellular homeostasis. Disruption of cell cycle control (p16) would need to be one of the earliest events to allow for clonal expansion. Resistance to apoptosis (DAP-K) and loss of normal cell differentiation control (RAR-B) would further aid in the development of autonomous premalignant clones. The subsequent loss of DNA repair of alkyl adducts (*MGMT*) could lead to acquisition of somatic mutations in genes such as p53 whose inactivation occurs at more advanced stages of premalignancy (e.g. dysplasia, carcinoma in situ) (Wistuba et al., 1999).

2.8- Environmental exposures target genes for promoter hypermethylation

It is well established that specific DNA adducts are associated with specific somatic mutations in oncogenes and tumor suppressor genes. The best example is the *K-ras* gene where mutation profiles in codons (Belinsky et al., 1989) 12 and 61 have been strongly correlated with the promutagenic adducts generated from the metabolism of carcinogens such as NNK, benzo[a]pyrene and urethane (Belinsky et al., 1989).

In addition, hot spots for mutation in the p53 gene have also been linked to preferential binding of the diepoxide adduct generated through activation of benzo[a]pyrene (Denissenko et al., 1996).

In contrast, there have been no direct studies to determine the role of DNA damage in initiating gene-specific promoter hypermethylation. An examination was done on methylation of the estrogen receptor α (ER) CpG ER methylation was seen at a similar low prevalence (~20%) in lung tumors induced in humans by cigarette smoke or in rats and mice by NNK. Tumors in unexposed mice or never smokers showed a much higher prevalence for methylation of the ER gene (Denissenko et al., 1996).

The most striking differences were seen in rat lung tumors induced by exposure to X-ray or plutonium where the ER gene was methylated in 38 and 82% of tumors, respectively (Issa et al., 1996).

2.9- Methylation of *p16* in lung cancer patients of Chinese population

Moreover based on the study made in china on 2003 where promoter hypermethylation of the *p16* gene was investigated in 1 cases of tumor tissue, as well as in 136 circulating plasma and 95 sputum samples from Chinese patients with primary lung cancer, using a modified protocol of semi-nested methylation-specific- PCR (MSP)(Moula et al, 1996).

The results showed hypermethylated *p16* sequence in 80.2% of tumor tissues and frequencies of 75.7 and 74.7% in plasma and sputum specimens, respectively. Among the patients, 50 cases of matched plasma, sputum and tumor tissue from the same individual were analyzed. Of these, hypermethylation of the *p16* promoter was detected in 84.0% of the tumor tissues, with frequencies of 72.0 and 76.0% in the corresponding plasma and sputum, respectively. Notably, only patients whose tumor

tissue showed hypermethylation of *p16* exhibited the same aberrant methylation in their sputum and/or plasma (Moula et al., 1996).

2.10- Plutonium targets the *p16* gene

Also a study approved by Institutional Review Boards in Ozyorsk, Russia and Albuquerque, New Mexico demonstrates that the elevated risk for radiogenic adenocarcinoma of the lung may, in part, be mediated by silencing tumor suppressor genes through promoter methylation (Belinsky et al., 2004).

The effect of targeting *p16* for inactivation on cancer risk is likely substantial because inactivation of this gene is an early event in lung cancer development and plays a critical role in immortalization by allowing cells to escape the immortality checkpoint 'MO'. The fact that the 3.5 - fold increase in risk for methylation of this gene for workers at the (Plutonium) plant approximated the previously reported 4- fold increased risk for adenocarcinoma in this group of workers establishes a link between Plutonium dose, *p 16* methylation and cancer risk.

As also seen in a limited number of female workers, six of eight who never smoked, it is apparent that Pu exposure alone can lead to inactivation of the *p 16* gene (Belinsky et al., 2004).

The strong association between methylation and α -rather than γ -radiation probably stems from the fact that α -particles deposit large amounts of energy (high LET) in small volumes along their paths, while rays deposit

relatively sparse amounts of energy (low LET). Estimation of DNA damage using *p53* expression indicates that to produce equivalent damage would require an absorbed dose of a low-LET emitter that is 10-fold higher than for α particles. Even accounting for differences in effective dose, in vitro exposure of peripheral blood lymphocytes to α particles resulted in more complex chromosome aberrations that were transmitted through cell division than seen with low LET X-ray (Sone et al., 1998)

This persistent DNA damage could be the common link as to how Pu exposure targets the *p16* gene for inactivation and increases overall the inactivation of genes by promoter hypermethylation in adenocarcinomas from the MAYAK (the first Russian nuclear enterprise establishment to manufacture plutonium) workers. A recent study detected and quantified stable intra-chromosomal rearrangements within chromosome 5 in lymphocytes of healthy former workers from the MAYAK facility. The yield of these aberrations was highly correlated with Pu dose to the bone marrow (Belinsky et al., 2004).

The extensive DNA damage suggests that radiation exposure to the lung epithelial cells from workers led to the accumulation of massive amounts of chromosomal damage that likely overwhelmed DNA repair pathways. DNA-dependent protein kinase is a serine-threonine kinase activated by double-strand breaks in DNA and may play a major role in non-homologous recombination and transcriptional control. The group demonstrates recently that reduced DNA protein kinase activity, which functionally diminished capacity to repair DNA damage, was associated with an increased risk for lung cancer¹. The disruption of replication timing because of chromosomal instability or loss of normal transcriptional control has been proposed as a mechanism through which normally unmethylated regions of DNA become aberrantly methylated.

Although exposure to Pu is not common, radon is a ubiquitous environmental carcinogen estimated to cause 10 000 cases of lung cancer annually in the US. The studies have highlighted a new mechanism for how radiation exposure could impact lung cancer risk by affecting not only a specific tumor suppressor gene, but also globally by epigenetic silencing of multiple key regulatory genes (Belinsky et al., 2004).

2.11- *p16* and *MGMT* methylation

Belinsky (2002) detected aberrant methylation of *p16* and / or *MGMT* in the Sputum of all tested lung cancer patient at the time of diagnosis. Moreover detection precedes clinical cancer was also observed using these two biomarkers, where 100% of squamous cell carcinoma patients (SCCs) were detected through the analysis of sputum collected at the time of clinical diagnosis. These findings offer new markers that should be tested in population-based screening and evaluated as potential targets to monitor in chemoprevention trials. Furthermore, in these individuals, sputum samples were positive in both patients in this cohort who developed SCC to date.

This biomarker approach should also aid the sensitivity and accuracy of other diagnostic approaches for lung cancer that are currently receiving much attention. For example, the imaging procedure spiral computerized tomography can detect very small peripheral lung cancers for which routine chest X-rays are negative (Sone et al., 1998).

However, in terms of the actual presence of lung cancer, many false-positive results are the rule, and actual cancer detection rates using spiral computerized tomography are currently 0.3-0.48% (Kaneko et al.,

1996). This scanning diagnostic approach might be enhanced by combining it with the determination of hypermethylation biomarkers in the sputum. Patients with stage I or -II disease have a 5-year cure rate of 60-80%, whereas stage III patients have a median survival of 13 months.

Lung cancer rates could be reduced through the development of a cost-effective screening approach. Key to this screening approach is the identification of markers of lung cancer that can be detected through sensitive and specific diagnostic assays. It is clear that mutation of the *K-ras* and *p53* genes and microsatellite instability constitute alterations important for lung carcinogenesis (Greenbalt et al., 1994).

However, a recent study examining the concordance of detecting these changes in broncho-alveolar lavage fluid from patients with early-stage lung cancer showed limited sensitivity. For example, microsatellite instability was detected in 46% of tumors but in only 14% of the corresponding lavage fluid. Similarly, 56% of tumors contained a *p53* mutation; however, this change was detected in 39% of the corresponding lavage fluid. Both of the genetic alterations are detected through assays that are either limiting in sensitivity (PCR for microsatellite instability) or labor intensive (plaque hybridization assay for *p53*) (Greenbalt et al., 1994).

The above study did examine methylation of *p16* using the original assay developed by Herman et al. (1996) that has sensitivity for detecting one methylated allele in a background of 1000 unmethylated alleles. The *p16* gene was the best marker, with 63% of the corresponding lavage fluids containing a methylated *p16* gene. One of the latest biomarkers proposed for noninvasive detection of cancer is mitochondrial DNA mutation.

Although it is fascinating that mitochondria within a cell accumulate identical cellular mutations, these mutations are dispersed across 16,000 bp and do not show any pattern within tumor types. In fact, across this length of DNA no two tumors of any type appear to contain identical mutations (Belinsky et al., 2002).

Furthermore, for lung cancer, mitochondrial mutation was seen in <50% of the 14 tumors examined. Thus, without prior knowledge of the mitochondrial mutation present in a given patient's tumor, an efficient screening system is not possible, and this precludes current use of this marker system for early cancer detection. The approach in contrast relies on assays that detect a very frequent and early DNA alteration in tumors that occurs in a genomic region identical for all patients. Thus, sputum analyses can be done in a cancer screening setting without knowledge of the methylation changes within the tumor.

The current improvement of the MSP procedure incorporates a nested, two-stage PCR approach, which is more sensitive (1 methylated allele in >50,000 unmethylated alleles). The improvement of the MSP procedure resulted in detecting the methylation of the *p16* and *MGMT* genes at frequencies higher than previously reported (Belinsky et al., 2002).

This higher frequency for methylation of *p16* actually corroborates immunohistochemical studies that have reported loss of *p16* protein in 60 - 80% of SCCs. Because of the inherent difficulties (e.g., DNA degradation) of assaying for methylation in DNA recovered from fixed of methylation. Previous studies have also shown a strong correlation between the loss of expression of both *p16* and *MGMT* and methylation (Belinsky et al., 2002).

This improvement almost assuredly also accounts for detecting at least one methylation marker in both the sputum and the corresponding tumor from every patient at the time of tumor diagnosis or within 3 years of tumor diagnosis. This data emphasize the functional implications that the loss of gene function associated with promoter hypermethylation has for early stages in lung cancer and other cancers. Hypermethylation – associated inactivation of *p16* is an early and frequent event in NSCLCs and other cancers. In experimental systems, such loss appears to act as a “gatekeeper” in permitting cells to escape through early steps of cellular immortalization (Belinsky et al., 2002).

2.12- Methylation of *p16*, *MGMT*, *DAP* and *RASSF1A*

A very important study done by Steven A. Belinsky on (2004) on aberrant promoter methylation of the *p16*, O⁶ – methylation-DNA methyltransferase (*MGMT*), death-associated protein (DAP) kinase, and Ras effector homologue (*RASSF1A*) genes in nonmalignant bronchial epithelial cells from current and former smokers in a of the *p16* gene and to a lesser extent, DAP kinase, occurs frequently in the bronchial epithelium of lung cancer cases and cancer-free controls, who smoked. These methylation changes persist after smoking cessation. They can also be detected within exfoliated cells.

Bronchial epithelial cells (BEC) cultures were successfully established from 94% of the collected sites allowing the methylation state of the *p16* gene, *MGMT*, DAP kinase, and *RASSF1A* genes to be examined in 160 and 137 sites from cases and controls, respectively. Aberrant promoter methylation of the *p16* gene was seen in > 1 site from 44% of both cases and controls, and 14 and 15 % of cases and controls, respectively had 2 sites positive. Furthermore, 2 cases and 1 control had 3 sites positive, but

no subjects had methylation of *p 16* in all 4 BEC sites. The prevalence for methylation of the DAP kinase gene in bronchial epithelium was significantly less than observed for *p16*, irrespective of case control status. In marked contrast, methylation of the *MGMT* gene was seen in >75% of BEC sites from cases and controls. In addition, *MGMT* methylation was seen in 14 of 17 sites from the never smoker. This finding does not preclude the use of *MGMT* as a biomarker in sputum because the high prevalence for the changes seen in vitro occurs in a context quite distinct from that of the airway epithelium (Divine et al., 2004).

Moreover, smoking status had no association with the detection of methylation of either the *p16* or DAP kinase genes in the bronchial epithelium. Thus, promoter methylation was seen as frequently in BECs from current smokers as from former smokers. Specifically, for *p16* methylation was seen in a bronchial epithelial site from 43% of either current or former smokers that were cases or controls. In addition, none of the smoking variables categorized (duration, pack-years, and time quit) was associated with the detection of promoter methylation in the bronchial epithelium.

As for controls, abnormal sputum cytology was seen in 25% of them. Methylation of *p16* gene was detected in sputum from 23 of 66 controls. DAP kinase methylation was also seen in sputum from 16 controls, and 8 of these subjects were positive for methylation of the *p16*. *MGMT* methylation was seen in 9 controls. Four of these subjects were also positive for either *p16* or DAP kinase. In addition, 3 subjects positive for *MGMT* methylation also had methylation of both *p16* and DAP kinase in their sputum. Thus, 5 % of the control subjects were positive for three or

four of the methylation markers, respectively, in their sputum (Divine et al., 2004).

This finding may reflect the timing and role of these genes in the development of lung cancer. Previous studies have demonstrated that p16 methylation is detected at the earliest cytological stages of SCC and adenocarcinoma (Jones and Baylin, 2002).

Inactivation of P 16 has been proposed as an early step to immortalization by allowing cells to escape the immortality checkpoint "MO". The importance of p16 in the development of NSCLC is evident by the fact that it is inactivated by promoter methylation in 60-70% of SCCs (25) and < 48% of adenocarcinoma (Neville et al., 1995).

2.13-The Promising future

Although all studies above and previous work suggest that the presence of promoter methylation in sputum from cancer-free smokers conveys a higher risk for lung cancer, the necessity for obtaining additional genetic and epigenetic changes that promote tumor progression will ultimately impact on the time to clinical disease. Through gene discovery and evaluation of additional genes already identified that are inactivated- in lung cancer by promoter hypermethylation, a panel of workers should be developed whose inactivation occurs at different stages of malignant transformation (Divine et al., 2005). Conducting longitudinal studies with this panel of hypermethylation markers will determine whether more accurate risk models can be developed that incorporate time to tumor and the relationship to multiplicity of these biomarkers in sputum.

Chapter 3

Materials and Methods

3.1 - Sputum collection

Sampling was done according to a statistical design that recommended collecting 198 samples from a controlled population present in LAU campuses (Byblos and Beyrouth) and individuals should be aging under 25 and over 40.

Samples were collected after forced expectoration in sterile urine bags and were stored in freezer until extraction.

However that was theoretical design, in fact the sampling was done randomly and required consent of individual to participate in the study by answering the questionnaire about respiratory health seen in Appendix III. (Xu et al., 2001).

The questionnaire required answering 10 sections about age and gender then symptoms e.g. cough and phlegm or episodes of, wheezing, breathlessness, chest colds and chest illnesses, specific lung affection (bronchitis, pneumonia, hay fever, chronic bronchitis or asthma). And at the end of questionnaire they were asked to give a sputum sample which is usually difficult for a healthy individual and also female group.

3.2- DNA extraction from sputum (adapted from cell culture extraction) Hedrick lab protocols

Sputum samples (250 µl each) are put in an Eppendorf tube and 250 µl of lysis buffer added to each tube containing the sample. Lysis Buffer contains 10mM Tris-HCl pH 7.5 of 2M, 10mM EDTA of 0.5M, 10 mM NaCl of 5M, 0.5% (W/V) Sarkosyl-0.5 gm (N-Laurylsarcosine, Sigma # L-9150), 1 mg/ml Proteinase K (AB gene) add fresh each time.

Samples in lysis Buffer were incubated at 60⁰ C for 2 hours (in an Eppendorf Thermomixer Comfort).

Then remove and add 250 µl of the Phenol/Chloroform/Amyl solution (in a ratio of 25:25:1) to each tube and mix by tapping gently the tube ~ 1 min until an emulsion form. Centrifuge (Heraeus Sepatech Biofuge 13 R) at room temperature until phases are well separated. Transfer (pipet) the aqueous phase to a new tube. The phenol/chloroform/amyl extraction is repeated until no more protein phase (white thick phase between organic and aqueous phases) is seen at interface of aqueous and organic phases. Then after obtaining the aqueous phases add 250 µl of chloroform and repeat same procedure (mixing, centrifugation and removing aqueous two or three times to remove phenol traces).

When chloroform extraction end, 10 µl of 5M NaCl is added to each sample and mix well and add 2 volumes of ice cold ethanol and mix well solution. The ethanolic solution obtained is put on ice for 30 min. Centrifuge at 12,000 g for 10min at 0⁰ C or 4⁰ C and higher speed remove supernatant and be careful not to disturb its pellet.

Then half fill the tube with 70% Ethanol and spin for 2min at 4⁰ C. Suck off supernatant and leave tube open to air dry.

Finally dissolve DNA with 30ul of TE buffer by pipetting on the walls of tube dissolve at least 1 hour at 37⁰ C.

3.3 - Bisulfite Treatment

Bisulfite Modification

The CpGenomeTM DNA Modification Kit (Chemicon International) contains reagents required to perform a bisulfite modification on a DNA sample. As shown in Figure 1, in the bisulfite reaction, all unmethylated cytosines are deaminated and sulfonated, converting them to uracils, while 5-methylcytosines remain unaltered. Thus, the sequence of the treated DNA will differ depending on whether the DNA is originally methylated or unmethylated. Also, the initially complementary DNA strands will no longer be complementary after cytosine conversion. Primers for use in MSP can be designed to specifically amplify a bisulfite-sensitive, unmethylated strand or a bisulfite-resistant, methylated strand, based upon these chemically-induced differences.

Unmethylated DNA

ggg	gcg	gac	cgc	g
sodium bisulfite modification				
ggg	gug	gau	ugu	g

Methylated DNA

ggg	gc ^m g	gac	c ^m gc ^m	g
sodium bisulfite modification				
ggg	gc ^m g	gau	c ^m gc ^m	g

Figure 4: DNA treatment with Sodium bisulfite. (Source: Li et al., 1993)

3.3.1 - Modification Protocol

The process for conversion of cytosine to uracil using the CpGenome™ kit is as follows. Bases are first exposed by denaturing the DNA to its single stranded form using mild heat at an alkaline pH. Reagent I, containing a sodium salt of bisulfite ion (HSO₄) causes unmethylated cytosine to be sulfonated and hydrolytically de-aminated, yielding a uracil sulfonate intermediate. DNA is then bound to a micro-particulate carrier (Reagent III) in the presence of another salt (Reagent II), and is desalted by repeated centrifugation and re-suspension in 70% EtOH. The conversion to uracil is completed by alkaline desulfonation and desalting

is repeated in 90% EtOH. The DNA is finally eluted from the carrier by heating in TE Buffer.

3.3.2 - Reagent Preparation

3 M NaOH stock (freshly prepared prior to each use)

Dissolve 1 g of NaOH pellets in 8.3 mL of water.

20 mM NaOH /90% EtOH (freshly prepared prior to each use)

To prepare 1 ml of this solution, combine 900 μ L of 100% EtOH, 93.4 μ L, of H₂O and 6.6 μ L of 3 M NaOH.

Dissolve Reagent I (freshly prepared prior to each use)

Warm bottle to room temperature before opening. For each sample to be modified weigh 0.227 gm of DNA Modification Reagent I and add to 0.571 mL water. Adjust the pH to 5.0 with approximately 20 μ L of 3 M NaOH monitoring the pH with pH indicator paper (read out the pH immediately upon wetting with sample).

Dissolve Reagent II

Warm bottle to room temperature before opening. Add 1 μ L, of β -mercaptoethanol to 20 mL of deionized water. Add 750 μ L of this solution to 1.35 g of DNA Modification II for each sample to be modified. Mix well to ensure complete dissolution. Excess reagent can be stored in a foil wrapped container at 15-25°C in the dark for up to 6 weeks.

3.3.3 - DNA Modification Procedure

In screwcap 1.5-2.0 ml, microcentrifuge tubes:

1. Add 7.0 μL 3M NaOH to 1.0 μg DNA in 100 μL , of water (10 ng/ μL) and mix.

Note: If the sample contains less than 1.0, μg of DNA, add 2 μL of DNA Modification reagent IV to the sample DNA and bring the total volume to 100 μL with water. Then add 7.0, μL 3M NaOH and mix.

2. Incubate DNA 10 minutes at 50°C (heat block or water bath).
3. Add 550 μL , of freshly prepared DNA Modification Reagent I and vortex.
4. Incubate at 50°C for 16 hours (overnight) in a heat block or water bath.

3.3.4 Initial Desalting

1. Resuspend DNA Modification III by vortexing vigorously. Draw suspension into and out of a 1 ml plastic pipette tip 10x to disperse any remaining clumps.
2. Add 5 μL of well-suspended DNA Modification Reagent III to the DNA solutions in the tubes.
3. Add 750 μL of DNA Modification Reagent II and mix briefly.
4. Incubate at room temperature for 5-10 minutes.

5. Spin for 10 seconds at 5,000 X g to pellet the DNA Reagent III. (A small white pellet should be present.) Discard supernatant.
6. Add 1.0 ml of 70% EtOH, vortex, and centrifuge for 10 seconds at 5,000 X g and discard supernatant. Perform this step for a total of 3 times.
7. After the supernatant from the third wash has been removed, centrifuge the tube at high speed for 2 minutes, and remove the remaining supernatant with a plastic pipette tip.

3.3.5 Completion of DNA Modification (Desulfonation), Second Desalting, and Elution

1. Add 50 μ L of the 20 mM NaOH/90% EtOH solution to the appropriate samples.
2. Vortex briefly to resuspend the pellet, and incubate at room temperature for 5 minutes.
3. Spin for 10 seconds at 5,000 X g to move all contents to tip of tube. Add 1.0 ml of 90% EtOH and vortex to wash the pellet. Spin again and remove the supernatant. Repeat this step one additional time.
4. After the supernatant from the second wash has been removed, centrifuge the sample at high speed for 3 minutes.
5. Remove ALL of the remaining supernatant with a plastic pipette tip. Allow the tube to dry for 10-20 minutes at room temperature (alcohol odor should diminish).
6. Add TE Buffer.

Note: The volume of TE to be added depends on the amount of starting DNA and on the required input concentration for the intended use. For example, if 25 μL of TE is added, the final concentration based on complete recovery of 1 μL DNA would be 40 ng/ μL . Vortex rapidly and forcefully until the pellet is completely resuspended. Manually flick or tap the contents to tube tip (but do not centrifuge).

7. Incubate the sample for 15 minutes at 50-60°C to elute the DNA.
8. Centrifuge at high speed for 2-3 minutes and transfer the sample (supernatant) to a new tube using a plastic pipette tip.
9. Proceed to MSP or sequencing, or store at -15°C to -25°C for up to 2 months, at -80°C for up to 6 months. Avoid repeated thawing and refreezing; store as aliquots. Do not store DNA in a self-defrosting freezer.
10. When removing thawed DNA from a tube for later use, avoid transfer of Reagent III to PCR reaction tubes. Always centrifuge the tube briefly first, to pellet any residual Reagent III solids.

3.4 MSP (Methylation Specific PCR)

3.4.1 – MSP materials

10xPCR buffer

MgCl₂ 25mM

DNTP 10mM each

Taq polymerase 5U/μl

Table 1: Primers for *p16* and *MGMT* for stage 1 PCR.

Primers	
p16 forward	5'-GAAGAAAGAGGAGGGGTTGG-3'
P16 Reverse	5'-CTACAAACCCTCTAC CCACC-3'
MGMT forward	5'-GGATATGTTG GGATAGTT-3'
MGMT Reverse	5'-CCAAAAAC-CCCAAACCC-3'

Table 2: Primers for *p16* and *MGMT* for stage 2 PCR.

Primers	
p16 unmethylated forward	5'-TTATTAGAGGGTGGGGTGGATTGT-3'
p16 unmethylated reverse	5'-CAACCCCAAACCACAACCATAA-3'
MGMT unmethylated forward	5'-TTT-GTGTTTTGATGTTTGTAGGTTTTTGT-3'
MGMT unmethylated reverse	5'-AACTCCACAC-TCTTCCAAAAACAAAACA-3'
p16 Methylated forward	5'-TTATTAGAGGGTGGGGCGGATCGC-3'
p16 Methylated reverse	5'-ACCCCGAACCGCGACCGTAA-3'
MGMT Methylated forward	5'-TTCGACGTTCGTAGGTTT-TCGC-3'
MGMT Methylated reverse	5'-GCACTCTTCCGAAAACGAAACG-3'

3.4.2 - MSP

The PCR primers are designed to specifically amplify the promoter regions of the gene of interest. If the sample DNA was originally unmethylated, an MSP reaction product will be detectable when using the primer set (labeled as 'U') designed to be complementary to the unmethylated DNA sequence. No product will be generated using a primer set (labeled as 'M') designed to be complementary to the derivative methylated DNA sequence. Conversely, an MSP product will be

generated only using the M primer set if the sample was originally methylated, and the U primers will not amplify such a template.

Methylation specific PCR permits sensitive detection of altered DNA. Because it is a PCR-based assay, it is extremely sensitive, facilitating the detection of low numbers of methylated alleles and the evaluation of DNA from small samples, including paraffin-embedded materials. MSP also allows examination of all CpG sites, not just those within sequences recognized by methylation sensitive restriction enzymes. Increasing the number of such sites that can be assessed allows rapid, fine mapping of methylation patterns throughout CpG regions. In addition, the bisulfite modification is ideally suited for analysis of CpG islands since it converts the majority of cytosines to uracils, making a region of the genome which is CG rich more easily amplified by PCR.

A nested, two-stage PCR approach was developed, which improved the sensitivity to detect methylated alleles. DNAs were subjected to bisulfite modification, and PCR was performed to amplify a 280-bp and 289-bp fragment of the *p16* and *MGMT* genes, respectively, including a portion of their CpG rich promoter region. The primers recognize the bisulfite-modified template but do not discriminate between methylated and unmethylated alleles. The stage – 1 PCR products were diluted 50-fold, and 5 ul was subjected to a stage-2 PCR in which primers specific to methylated or unmethylated template were used. Primer sequences used in the stage 1 amplification of the *p16* and *MGMT* genes are as follows: *p16*-Forward, 5' – GAAGAAAGAGGAGGGGTTGG – 3'; *p16*-Reverse, 5'- CTACAAACCCTCTAC CCACC – 3'; *MGMT*-Forward, 5'- GGATATGTTG GGATAGTT-3'; and *MGMT*-Reverse, 5'- CCAAAAAC-CCCAAACCC-3'. Taq Gold polymerase (Perkin-Elmer) in a 50ul volume was used in all of the PCRs. The PCR amplification

protocol for stage 1 was as follows: 95°C for 10 min, then denature at 95°C for 30 s, anneal at 60°C (*p16*) or 52°C (*MGMT*) for 30 s, extension at 72°C for 30 s for 40 cycles followed by a 10-min final extension. Primers used to selectively amplify unmethylated or methylated alleles of the *p16* and *MGMT* genes in the stage 2 PCR have been described previously. Annealing temperatures were increased to 70°C (*p16*) and 62°C (*MGMT*), and all of the cycling times were reduced to 15 s for a total of 40 cycles. Sputum never smokers for *p16* and *MGMT* methylation served as controls. All of the assays were conducted in at least duplicate.

Sputum samples that gave positive methylation products were also analyzed by a second method using restriction enzyme digestion of the resulting PCR product. Second-stage PCRs were performed in duplicate for each sample. The one of each sample pair was incubated with the restriction enzyme FNU4HI that cuts (G/CGGCG) at two sites within the amplified region of the methylated *p16* gene promoter. Thus, this restriction enzyme will only cut template that is methylated at the two CpG sites because the unmethylated cytosines would be modified by the bisulfite treatment to uracils. FNU4HI cuts at two different sites within the promoter. An identical approach was used for the *MGMT* gene using the TaqI and BstUI restriction enzymes.

3.5 Gel Electrophoresis

3.5.1 - Gel Electrophoresis Materials

2.5% Agarose

0.5 x T B E

10 ml of Ethidium Bromide

3.5.2 - Gel Preparation

2.5 g Agarose are put in 100 ml of 0.5 TBE.

The mixture is put in microwave till no bubble or white layer is seen (Put in microwave around 3 times, each time ~1 minute)

Then put mixture on stirrer at low speed to cool, add 10 ml of Ethidium Bromide and then keep to cool.

3.5.3 - Pouring of Gel.

Pour the gel and keep it for 45 minutes to solidify

3.5.4 Loading in wells

Load samples of 5 μ l in each well and run the samples on 110 V for one hour and then stop current.

Chapter 4

Results

4.1-Prevalence of smoking:

The best validated method for assessing human exposure and predicting lung cancer is to ask subjects about their tobacco smoking history. Lifetime exposure can be estimated by calculating pack-years smoked (average packs smoked per day over a lifetime multiplied by number of years smoked) or cumulative tar exposure. Thus, a smoking history approximates the level of exposure of an individual.

My sample included 198 individuals (105males and 93females). From the 198 individuals, 108 were less than 25 years old where as 90 were above 40 years) .

The smokers accounted for 51.52 percent of the total sample versus nonsmokers (48.48%). 58.52% of smokers were males versus 41.18% of smokers being females (Figure 5).

Figure 5: Percentage of individuals less than 25 and those above 40 years; males (M) versus females (F), percentage of smokers (S) versus non-smokers (NS)

Moreover, 57.89 percent of smokers less than age 25 were males versus 42.11 percent females (table 3).

Table 3: Percentage of male and female smokers less than the age of 25 years

	Males	Females	MGMT-UV	MGMT-M	p16-UM	p16-M
Smokers n=57	57.89	42.11	100	0	91.23	8.77
Non smokers n=51	49.02	50.98	98.04	1.96	94.12	5.88

As for those above 40 years old, 60% of smokers were males versus 40% of smokers females.(table 4)

Table 4: Percentage of male and female smokers greater than the age of 40 years

	Males	Females	MGMT-UV	MGMT-M	p16-UM	p16-M
Smokers n=45	60	40	100	0	26.67	73.33
Non smokers n=45	44.44	55.56	100	0	95.56	4.44

4.2-Smoking Behavior:

The mean age at starting smoking was 17 years.90% had started before age 25 years .The average duration of smoking was approximately 27 years for those above 40 years old. Among ex-smokers, the average

duration of smoking was approximately 10 years, and average duration of quitting was approximately 5 years.

Cigarettes were the most common form of tobacco consumption, reported by 96% of participants. Other forms of tobacco consumption included shisha (25% of respondents), cigars (5%) and pipe tobacco (<1%).

Of those who smoked the shisha 80% were below the age of 25 and 20 % of them did so daily.

The average number of cigarettes smoked per day was 21, although around 15% smoked 40 cigarettes per day.

The daily number of cigarettes smoked also varied by sex. Male smoked an average of 27 cigarettes per day while females 15 cigarettes per day even though some exceptions were seen in women above 40 years that were very heavy smokers.

4.3-Detection of aberrant methylation of *p16* and / or *MGMT*:

From the 198 samples, 197 showed unmethylated *MGMT*, only one sample showed methylation of *MGMT* and it was for a nonsmoker.

As for *p16*, 155 samples were unmethylated while 43 were methylated.

4.4-Smokers category:

In the smokers' category, 100 percent of the *MGMT* gene was unmethylated compared to 62.75% of the *p16* gene, while the other 37.25% showed methylation (figure 6 and 9).

Figure 6: Methylation analysis in total population studied: blue shows the percentages in smokers while red in nonsmokers. (UM) is for unmethylated and (M) is for methylated

Figure 7: Methylation status of methylated and unmethylated *p16* and *MGMT* genes in individuals above 40 years old. Blue is for smokers, red is for non-smokers

It is interesting to note that most methylation was seen among smokers greater than 40 years, where 73.33% of them had the *p16* methylated (Figure 7 and 8).

As for those less than 25 years, a lower percentage was observed, where 8.77% of the smokers had the *p16* methylated.

Fig 8: MSP analysis for *p16* methylation in smokers. Bisulfite-modified DNA was amplified using MSP primer specific to a CpG rich portion of the *p16* promoter

Fig 9: MSP analysis for *MGMT* in smokers, example of no methylation

4.5-Nonsmoker category:

In the nonsmoker category, only one sample showed methylation of *MGMT* and it was for an individual whose age is less than 25 years and this individual showed no methylation of the *p16* gene.

For the cases of nonsmokers, 94.79% of them showed unmethylated *p16* while only 5.21% were methylated (figure 10).

Fig 10: MSP analysis for *p16* where no methylation was seen and it is in the nonsmoker category.

Chapter 5

Discussion:

This study revealed a smoking prevalence in the adult population in Lebanon. It is important to note that during collection, we faced no problem in collecting samples for smokers since most of the people we encountered were smokers especially those less than 25 years. The difficulty was in collecting samples for nonsmokers especially females under 25 years.

Our study showed smoking prevalence in our population varying with sex, where the male number was more than the female (58.52% male). This is related maybe to the fact that our society is a patriarchal society where females are still in a way, conservatives compared to males. This study is similar to other studies too.

Intensity of smoking was also comparatively high in our population. The average daily number of cigarettes smoked daily was 21, compared to 15 in France (Spinella and Dmitrovsky, 2000).

The average number of years of smoking in our sample of smokers above 40 years was 27 years.

Shisha, a particularity of oriental populations, is relatively common in Lebanon. In our sample, the percentage of individuals who reported

smoking the shisha was 25% where 80% were below the age of 25 and 20% of them did so daily. This proportion is very important since it has been shown that the urine nicotine level after two shisha is equivalent to that after 30 cigarettes (Collishaw and Lopez, 1996).

Smoking in Lebanon is socially entrenched with cigarettes commonly offered during many types of social occasions, both celebratory and sorrowful. Our study confirms the ready acceptance of smoking in our society, where smokers encouraging others to smoke and reporting that they like to offer and receive cigarettes. Furthermore, we have noticed that 55% of nonsmokers were accepting of others' smoking habits now, the only known way to reduce lung cancer risk in smokers Till is complete cessation, but in our study. We realized that 83% of smokers were unwilling or unable to quit. The way people smoke and the way they respond to carcinogen exposure are both highly variable, from individual to another. Biomarkers, in this case, will be promising in detecting lung cancer ahead of time. Studies of sputum samples as mentioned before in literature review have yielded promising evidence that lung cancer can be detected or its development predicted up to three years before clinical detection of tumors in persons who smoke.

The result of this study showed aberrant promoter methylation of the p16 gene in the sputum of smokers, where 37.25% showed methylation compared to 5.21% in nonsmokers. This is a clear indication that smoking does play a role in methylating the *p16* gene. Moreover, a stronger evidence supporting the fact that smoking might be a cause of methylation ,we notice that 73.33% of samples being methylated are for smokers above 40 years old which means that they have been smoking for at least the last 20 years ,while new beginners had a low percentage of methylation (8.7%). So to be more precise, our study clearly

demonstrates the high relation between the duration of smoking and the number of packs/year smoked to *p16* methylation.

If these results are to be compared to other studies, we can see a lot of similarity. Kersting et al. (2000) analyzed the frequency of *p16* methylation in ex-foliate material from lung cancer patients. *p16* methylation was detected in 35% of sputum samples, in 22% of bronchial lavage samples, and in 16% of bronchial brushings from patients with lung cancer, concluding that *p16* could be a good biomarker for early detection of lung cancer.

As for Belinsky et al., (1998) they were the first to determine the timing of *p16* methylation in an animal model of lung carcinogenesis and in human squamous cell carcinomas. In the rat, *p16* methylation was frequently detected in precursor lesions to lung tumors. In humans, *p16* methylation was found in 75% of carcinoma in situ lesions adjacent to squamous cell carcinomas harboring this change. Interestingly, the frequency of *p16* methylation increased during disease progression from basal cell hyperplasia to squamous metaplasia to carcinoma in situ. Moreover, the authors were able to detect *p16* methylation in sputum samples from cancer-free individuals at high risk for lung cancer proving that *p16* is a potential biomarker for early detection of lung cancer.

In addition to that, in one of their studies made *p16* was seen in sputum samples from 3 out of 7 (43%) cancer patients and 5 out of 26 (19%) cancer-free smokers, with no methylation seen in controls.

P16 methylation in sputum samples from cancer-free chronic smokers has also been described by Kersting et al. (2000) (similar to my work done).

Four of 25 (16%) sputum samples were *p16* methylated. In addition, *p16* methylation was found in 12% of bronchial lavage samples and in 8% of bronchial brushings, concluding that *p16* is a potential biomarker for individuals at a high risk of developing lung cancer.

Palmisano et al. (2000) investigates whether lung cancer could be predicted by detection of methylation in sputum samples. Using a highly sensitive PCR approach to detect methylated DNA sequences, the authors reported that methylation of *p16* and/or *MGMT* could be detected in DNA from sputum in 100% of patients with squamous cell lung carcinomas up to 3 years before clinical diagnosis. In addition, the authors described that the prevalence of these markers in sputum from cancer-free, high-risk subjects approximated lifetime risk for lung cancer. He also detected that *p16* was methylated in 18 of 123 (15%) individuals in sputum from cancer free, tobacco and or radon-exposed individuals (Palmisano et al., 2000).

Also, methylation of *p16* was detected in bronchial brush samples from former cigarette smokers in 17% of the samples, respectively (Pulling et al., 2004).

Kulkarni et al. (2004) have recently reported frequent methylation of the *p16* and *MGMT* genes (48%) in primary tumor and adjacent mucosa in Indian patients with smoking history indicating that methylation at these two genes reflects field epigenetic instability.

All these results including mine, demonstrate that methylation of *p16* can be an early event in the pathogenesis of lung cancer and might be a useful biomarker for lung cancer risk assessment, noting that methylation of *p16* is significantly associated with pack-years smoked and duration of smoking.

As for nons smokers (controls), we had a very low percentage of methylation of *p16* gene. The methylation was seen in five individuals only. Surprisingly, out of this five, it is interesting to note that one had a history of lung cancer in his family where his grandmother died from lung cancer at the age of 55 years old. A study made by Soria (2002) showed that *p16* methylation was higher in former smokers with a prior history of cancer than in those without a prior history. The other two were ex-smokers. Although cessation of smoking plays a role in decreasing lung cancer risk, a study has shown that cumulative risk for lung cancer by age 75 for a person who quits smoking at age 50 is still six times greater than a never smoker (Peto et al., 2000). This is an additional support confirming how strongly is smoking associated to *p16* methylation and how its effect lasts even when cessation. But till now no molecular mechanism pathway has been seen or analyzed that relates tobacco to the methylation process.

As for *MGMT* gene, my results does not fully support the results of most studies done before because no methylation was detected in any sample of mine except for one, but we can not say that they contradict them since most studies done before were on patients with lung cancer. For example, in a study done by Palmisano (2000), *MGMT* was seen in sputum from 43 % of persons with confirmed SCC but only in 3-6% of cancer free controls (Viramani et al., 2000), which is in a way close to my results.

Toyooka et al. (2001) analyzed the pattern of methylation of seven different genes (*APC*, *CDH13*, *GSTP1*, *MGMT*, *RAR β* , *CDH1*, and *RASSF1A*) in 198 lung tumors consisting of SCLC_s, NSCLC_s, and bronchial carcinoids. The profiles of methylated genes in the neuro-endocrine tumors (SCLC_s and carcinoids) were very different from that of NSCLC_s. While the overall pattern of methylation of carcinoids was similar to that of SCLC_s, carcinoids had lower frequencies of methylation for some of the genes tested.

For example, in his study he found that 16% of smokers had *MGMT* methylated but this same percent was also seen in patients with small cell lung carcinoma (SCLCs). Perhaps those smokers in his study are at a very high risk to develop lung cancer, which is perhaps not yet the case in my sample of smokers, this is why my samples showed no methylation at all. Moreover, in a study done by Palmisano and Belinsky (2000) *MGMT* was not present in two sputum samples collected 3 years before clinical diagnosis could be related to the timing for inactivation of this gene in those tumors. Similarly, *MGMT* methylation was not seen in the sputum sample collected 15 months before clinical diagnosis but was present at 9 months before clinical diagnosis.

Belinsky (2005) found that methylation of *p16* has been detected in ras bronchial epithelium from chronic smokers. Inactivation of the death associated protein kinase (DAPK) and retinoic acid receptor (*RARB2*) genes occurs at the stage of alveolar hyperplasia, while inactivation of the effector homolog 1 (*RASSF1A*) gene appears to be an even later event.

Inactivation of the DNA repair gene, *MGMT*, appears to occur closer to the development of a malignant lung cancer.

Topaloglu (2000) in his study on detection of promoter hypermethylation of multiple genes in patients with lung cancer noticed that in the controls *MGMT* methylation did not exist at all while other genes such as *GSTP1*, *p16*, *ARF* genes had methylation but at very low levels.

As a conclusion, it seems from above studies including mine, that *MGMT* is not easily methylated as *p16* gene is. It is more common to see methylation of *p16* than *MGMT* in sputum samples of smokers.

So none of the smoking variable categorized (duration, pack-years, and time quit) was associated with the detection of promoter methylation of *MGMT*, in contrast to *p16* gene results.

For *MGMT* gene, it seems that this gene is a resistant gene where methylation takes time to occur, and perhaps, its methylation is a dangerous alarm that precautions should be taken as soon as possible, and that a person having *p16* and *MGMT* methylated is at high risk to develop lung cancer soon.

Methylation of certain genes is frequently observed in primary lung carcinomas and can also be detected in the sputum of heavy smokers. These findings open a wide spectrum for potential clinical applications of detecting methylation. First of all, detecting methylation in primary lung carcinomas may influence treatment strategies for lung cancer patients. Finding genes of prognostic impact methylated in tumors could allow one to "customize" therapy for those lung cancer patients. Those patients potentially might benefit from a more aggressive treatment strategy or the use of demethylating agents.

Secondly, detection of methylation in the bronchial epithelium of heavy smokers could be used in a population with greater risk for lung cancer to assess the "individual" risk. Determining the methylation status of several genes and performing a "methylation profile" could allow the identification of individuals with a very high risk. This approach is currently being tested in clinical trials. In addition, this subgroup of people could then undergo other, but more costly, procedures for early detection of lung cancer, such as spiral computerized tomography scans, and would also be ideal candidates for chemoprevention trials (Malkinson 2001).

For this approach, it is important that sputum samples can be obtained in a noninvasive, easy, fast, and inexpensive way. Moreover, the frequency of methylation in sputum samples seems to be comparable with the frequency in bronchial brushings, which can be obtained only by bronchoscopy. Thus sputum samples are ideal for this kind of study.

Finally, one of the most important findings is that methylation can be reversed *in vitro*. Several studies demonstrated that gene expression could be restored after treatment of cells with demethylating agents, such as 5-AZA-CdR. Although it is too early to make any conclusions about the effect and possible side effects of these drugs in lung cancer patients, this is a very promising concept and needs to be tested in clinical trials with monitoring of methylated markers (Belinsky et al., 2003).

Chapter 6

Summary and conclusions

Finding from this study reports the high prevalence of smoking in Lebanese society and particularly in men more than women.

This study also strongly supports the implementation of longitudinal studies in subjects at high risk for developing lung cancer where aberrant methylation of *p16* was seen in 73.33% of heavy smokers (above 40 years old). This demonstrates that methylation of *p16* can be an early event in the pathogenesis of lung cancer and might be a useful biomarker for lung cancer risk assessment, noting that methylation of *p16* is significantly associated with pack-years smoked and duration of smoking.

But this study emphasizes the need for the development of additional hypermethylation markers for lung cancer assessment especially that *MGMT* methylation was not detected in this study in the smokers and nonsmokers category, predicting that this gene might be a resistant gene where methylation takes time to occur, and perhaps, its methylation is a dangerous alarm that precautions should be taken as soon as possible, and that a person having *p16* and *MGMT* methylated is at high risk to develop lung cancer soon.

Thus, all studies done with these methylation markers should facilitate the development of more accurate risk models to incorporate time to tumor and the relationship to multiplicity of biomarkers in sputum.

We hope that new methods of screening for lung cancer will be developed and refined soon. Even a small decrease in lung cancer mortality from screening would save thousands of lives each year. Moreover, the fact that methylation can be reversed in vitro and the synergistic effect of the demethylating agent 5-AZA-Cdr with the histone deacetylase inhibitor TSA in vitro raise hope for new treatment strategies for lung cancer patients. If this concept works in cancer patients, then an essential step toward prevention and improved treatment of lung cancer patients could be taken.

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