Date of Initial IRB Submission:

Current Version Date: December 9th 2016

Protocol Number: OH98-C-N027

Title of Study:
DNA REPAIR, P53 AND APOPTOSIS PHENOTYPES IN LUNG CANCER

Principal Investigator: Bríd M Ryan, Ph.D., M.P.H.
Earl Stadtman Investigator/ Laboratory of Human Carcinogenesis
37 Convent Dr. Rm. 3060C
Bethesda, MD 20892-4258
301-496-5886 (voice) /301-496-0497 (fax)
ryanb@mail.nih.gov

Accountable Investigator: Curtis C. Harris, M.D.
Chief / Laboratory of Human Carcinogenesis / NCI-CCR
37 Convent Dr. Rm. 3068A
Bethesda, MD 20892-4258
301-496-2048 (voice) /301-496-0497 (fax)
harrisc@mail.nih.gov

Associate Investigators: Ana I. Robles, Ph.D.
Staff Scientist / Laboratory of Human Carcinogenesis / NCI-CCR
37 Convent Dr. Rm. 3060D
Bethesda, MD 20892-4258
301-496-1729 (voice) /301-496-0497 (fax)
roblesa@mail.nih.gov

Supporting Contract: NCI Contract #N02-RC-57700
Awarded to Dr Dean Mann (Univ. of Maryland, Baltimore, MD)
PI for "Resource Collection and Evaluation of Human Tissues and Cells from Donors with an Epidemiology Profile"

Study Location: Baltimore, MD and surrounding counties

Study Design: Case-Control with hospital- and population-based controls

PRECIS

Background. The Laboratory of Human Carcinogenesis is conducting an observational non-small cell lung cancer (NSCLC) case-control study in Baltimore, MD. This molecular epidemiology study was developed to test the reliability and validity of the mutagen sensitivity assay, where a case-control study is needed to assess the possibility of case bias. Importantly, this protocol establishes a resource that allows for the study of additional biomarkers and gene-environment interactions. Upon recruitment, cases and controls receive a structured, in person interview assessing prior medical and cancer history, use of tobacco and electronic cigarettes, alcohol use, current medications, occupational history, family medical history, menstrual history and estrogen use, recent nutritional supplements and caffeine intake, and socioeconomic status. Specimen collection consists of a one-time blood sample and/or mouthwash to collect cheek cells (oral cells), a one-time nasal swab collection and a one-time urine sample. In addition, cancer and surrounding non-cancer tissue that was surgically removed and not needed for diagnosis may be obtained for cases, as well as current medical information from medical records. Primary cell cultures may be established from available fresh tumor tissue. The phenotypic markers to be studied will assess proficiency of DNA repair in lymphocyte cultures exposed in vitro to radiation, bleomycin, benzo(a)pyrene-diol-epoxide by measuring induction of chromosomal aberrations, p53 and apoptosis. DNA from buffy coats or cheek cells will be used for analysis of germline variation in the form of Single Nucleotide Polymorphisms (SNPs) in genes involved in DNA repair, innate immunity, cell cycle control, angiogenesis, apoptosis, cytokines, nicotine addiction, inflammation, hormone metabolism and microRNA. Additionally, IRB approval was received in 2010 to include this study in a multi-institution genome-wide association study (GWAS) of lung cancer in African Americans. Tumors from cases will be evaluated for estrogen and progesterone receptors, somatic mutations, and gene expression. Urine, plasma, serum and tissue sample metabolomics will be analyzed by untargeted approach.

Objectives:

1. To determine if mutagen sensitivity, p53 induction, and apoptosis in cultured lymphocytes are predictive of lung cancer risk.
2. To determine the relationship between sex-steroid metabolism, estrogen exposure, and lung cancer risk.
3. To investigate and develop phenotypic or predictive markers of lung cancer risk and survival, based on mutagen sensitivity, polymorphic markers, gene expression, and metabolomics.
4. To investigate racial disparities associated with lung cancer risk and survival.
5. To examine the relationship between circulating cytokines with risk and survival of lung cancer and to establish the most robust method of cytokine detection.
6. To generate a more accurate measure of ancestry using ancestry informative marker analysis and to integrate this variable into our studies of health disparities
7. To conduct studies of metabolomics on serum, plasma and urine for the purposes of discovering novel markers of risk, diagnosis and prognosis. We will use ultraperformance liquid chromatography coupled to mass spectrometry (UPLS-MS) to search for small molecular weight endogenous metabolites that can classify cancer and predict outcome. This is a novel approach for biomarker discovery that also leverages the non-invasive process of biospecimen collection. Tumor and corresponding non tumor tissues from corresponding patients will also be tested using the same methods to extend the discovery of novel tumor metabolites. Further, metabolites of vitamin D will be examined on serum samples from lung cancer cases and controls to assess the relationship between circulating levels of Vitamin D metabolites with cancer risk and survival. This analysis will be coupled

with testing of Vitamin D pathway SNPs in corresponding patients to determine if certain SNPs are also associated with levels of vitamin D.

8. To evaluate biomarkers of cancer diagnosis and prognosis in circulating tumor DNA
9. To evaluate the microbiome (microbes) present in lung tissue using in situ hybridization of fixed tissues to be completed at Mayo Clinic by collaborators. The collaborators will receive no information on the samples other than their tissue of origin.
10. To collect data and biospecimens on patients that received low dose CT screening as part of their lung cancer diagnosis. This is for the purposes of investigating non-invasive biomarkers of lung cancer diagnosis and prognosis.
11. To culture lung cancer specific microbiome-bacteria from human lung cancers. This aim will involve an MTA with Dr Paul Owrin, pending IRB approval. He is an expert in culturing this microbial species from human tissues.

Eligibility:

- Histologically confirmed NSCLC diagnosed within the past 2 years (case).
- Frequency matched to cases according to age (5-year intervals), gender, and race (population-based control).
- Born in the United States, resident of the state of Maryland
- Subject Characteristics:
    - Speaks English well enough to be interviewed
    - Physically and mentally capable of performing the interview (i.e., must be able to hear the interviewer, mentally comprehend the interviewers questions, and verbally respond)
    - Has never been interviewed as a control for this study
    - Does not currently reside in an institution such as a prison, nursing home, or shelter
    - No history of cancer other than nonmelanoma skin cancer or carcinoma in situ of the cervix (population-based control)
    - Has a residential working phone within the home (population-based control)

Design:

- Case/Control; Observational
- Planned statistical analysis: Risk associations between the genotypes and cancer/survival will be assessed using unconditional logistic regression model, with covariate adjustment, as appropriate.
- Number of subjects to be enrolled: Target accrual is 5000 subjects, consisting of 450 cases/each gender in African Americans and 800 cases/each gender in Caucasians. An equal number of controls will be selected for each category of cases based on the combination of gender and race.
- For patients that undergo low dose CT screening at the participating hospitals in this protocol, we will enroll patients that have had a “positive” scan and are attending either UMMS or the VA hospital for further follow up.
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2 INTRODUCTION AND RATIONALE

According to the American Cancer Society (1), lung cancer is the leading cause of cancer-related death in men and women. The predominant cause of lung cancer is tobacco smoking, where over 90% of lung cancer patients report a smoking history (2). Only about 10% of heavy smokers suffer from lung cancer. Smoking cessation reduces the risk of lung cancer over a ten year period, but the risk never reaches the non-smoker level (3). Tobacco smoke constituents contain more than 100 mutagens and carcinogens. Exposure to tobacco leads to multiple somatic mutations, rapid cell proliferation and resistance to therapy. The suspected carcinogens in tobacco smoke include, but are not limited to, polycyclic aromatic hydrocarbons, *N*-nitrosamines (including tobacco specific nitrosamines) and maybe aromatic amines.

Studies of genetic susceptibility in lung cancer have primarily focused on carcinogen metabolic activation and detoxification. Specifically, several genetic polymorphisms have been implicated in lung cancer risk, although there are inconsistencies. These include glutathione-*S*-transferase M1 (*GSTM1*) (4-13), glutathione-*S*-transferase Pi (*GSTP*) (14-16), cytochrome P450 (CYP) 2D6 (17,18), *CYP1A1* (11,19-28), *CYP2E1* (29,30), and N-acetyltransferase 2 (*NAT2*) (31). There are certainly more genes that are suspected to be involved in lung cancer risk including *CYP2A6, CYP1B1, CYP3A4*, microsomal epoxide hydrolase (*MEH*), N–acetyltransferase 1 (*NAT1*), aryl hydrocarbon hydroxylase receptor (*AHR*), and others, although not all of these have demonstrated functional genetic polymorphisms (32-35). The presence of carcinogen-DNA adducts also can vary by genetic polymorphisms in carcinogen metabolizing genes such as *GSTM1*, *CYP2E1* and *CYP2D6* (36,37). Although less well studied because of statistical power limitations, gene-environment interactions for lung cancer risk also have been proposed, whereby genetic polymorphisms in carcinogen metabolizing genes modify the effects of carcinogen exposure on cancer risk, as well as DNA adduct levels (36-39).

Rationale to study mutagen sensitivity, p53 and apoptosis: While most previous studies of inherited susceptibility have focused on carcinogen metabolism, certainly human variability for DNA repair and programmed cell death will also contribute to lung cancer risk. The p53 gene is involved in both DNA repair and apoptosis.

One method that can phenotypically characterize persons for DNA repair has been named the "mutagen sensitivity assay." Briefly, blood is collected for lymphocyte cultures. After the cultures are established, the lymphocytes are exposed to a clastogen, and then chromosomal aberrations are counted after giemsa staining. This method has been used for a variety of chemical clastogens and radiation. For example, this method is used to study the defect in ataxia telangectasia. For cancer risk in the general population, bleomycin has been widely used as the clastogen. (Bleomycin is a chemotherapeutic agent that does not require metabolic activation.) Here, individuals can be categorized as sensitive or resistant, depending on the number of chromosomal breaks. An increased number of breaks, which identifies the "sensitive individual" was associated with lung cancer risk in African Americans (40), and was positive for both adenocarcinoma and squamous cell cancer. There also was a positive interaction between smoking and mutagen sensitivity for cancer risk, although the number of cases was small after stratification. Notably, there was no relation between smoking and sensitivity. In addition to lung cancer, mutagen sensitivity has been associated with head and neck cancer in two separate studies (41,42), one of which was multi-institutional (42). Further, cases with multiple primary head and neck tumors had even higher levels (43,44). Smoking, age, alcohol use, gender and

stage of presentation were unrelated to increased sensitivity. Separately, using radiation rather than bleomycin, the number of chromatid breaks was associated with risk for malignant gliomas (45). The nature of the defect in persons with multiple primary malignancies of the head and neck, using radiation as the exposure, is thought to be a combination of increased initial levels of chromosomal damage and defective repair, which is a phenotype similar to defects in ataxiatelangectasia (46). Recent data has suggested that using a benzo(a)pyrene-diol-epoxide-based assay, in addition to bleomycin exposures, is more predictive of risk (M. Spitz, personal communication).

The mutagen sensitivity assay needs to be appropriately validated. The distribution of mean breaks per cell appears to be unimodal, ranging from less than 0.6 to more than 1.4, for 50 metaphases (42,44). But, the reproducibility of the assay (within laboratory on different days or within individual) has not been reported in large numbers of subjects. There is some data to indicate that scoring of 50 metaphases, rather than more, is sufficient (47). However, the validation and testing of reproducibility factors is problematic because the assay is performed with fresh blood, so that repeated testing of the same sample on different days is not possible. It is possible, however, to validate the assay under standard conditions using Epstein Barr virus immortalized lymphocytes, but this model probably over-represents the reproducibility. It also might be possible to cryopreserve the lymphocytes for repeated testing of aliquots, but the reliability of cryopreserving has not been tested. Equally important, while numerous studies suggest that this assay is predictive of different cancers (40-46), it remains possible that the results are related to case status (i.e., there are different results in cancer cases because the cancer affects the individual), and this assay has not been tested prospectively. Specifically, the presence of cancer might have affected a persons dietary intake of nutrients, overall nutritional status, the percentage of lymphocyte subpopulations, or other factors. It is known that stage, prior treatment with radiation, vitamin C intake and the presence of secondary cancer can affect the results (43,48,49), although age, gender, and alcohol use do not (48), but all of these variables have essentially only been tested once or twice. Separately, it is presumed that this assay reflects a heritable capacity for DNA repair, and while tested in breast cancer patients with radiation as the clastogen (50), it has never been reported in families without strong family histories of breast cancer. For this study however, a segregation analysis with a sufficient number of families is beyond our scope.

In order to validate this assay, we propose several studies that require an ongoing case-control study. These include the testing of individuals (normal and cancer cases) over time, both before and after treatment. Performing these assays in early stage cancer cases where surgery is the only treatment, a change in breaks per cell would suggest that the results were do to the presence of cancer. Repeated testing in persons before, during and after radiation would provide similar information, as well as the effects of radiation. Family members from persons in the general population would demonstrate the heritable component to this phenotypic assay. Other studies that are needed is the assessment of assay results by lymphocyte subtype and cryopreservation.

We also propose to develop a profile of phenotypes by testing different clastogens. Repair mechanisms for bleomcyin (which is radiomimetic), benzo(a)pyrene and *N*-nitrosamines are different. Whether any single clastogen, or a combination of them is more predictive of lung cancer remains to be determined. This study will establish a resource to validate genetic polymorphisms in DNA repair genes, as they are identified, in order to assess genotype-phenotype relationships. There will be a sufficient number of Caucasians and African

Americans to allow for the assessment of these relationships in both races.

An exciting component of this study will be the assessment of interindividual variation in p53 induction and apoptosis in response to chemical exposure (51). We hypothesize that persons with decreased p53 response or apoptosis will have an increased risk of lung cancer. We will focus on benzo(a)pyrene exposure. Previous studies in hamster tracheal epithelium (52), breast cell lines (53), and mouse skin (54) indicate that levels of p53 increase following benzo(a)pyrene exposure, and that this response correlates with DNA adduct levels. Should these studies indicate a wide interindividual variation (i.e., exceeding intra-individual variation by more than 10-fold), then p53 response genes can be studied as they relate to cell cycle control (51,55-57) or apoptosis (e.g., *BCL*-2 (58)). Importantly, this assay has not been tested in the population (i.e., on more than a few individuals), and so the variability and reproducibility is unknown. Using the population controls, we can define the extent of variation in the population, the extent of intra-individual variation, and assay reproducibility. If there is sufficient reliability and variation, we can assess our statistical power and compare these results with those from cancer cases.

Rationale for two control groups: Smoking is the predominant risk factor for lung cancer. Therefore, we have selected a smoking control group to maximize the efficiency for detecting the level of mutagen sensitivity in smokers. In a population-based study, the effects of metabolizing or DNA repair genes with a small relative risk might be obscured by the effects of smoking, and so difficult to detect even with statistical adjustment.. Overlapping distributions of smokers among cases and hospital-based controls will permit an efficient adjustment for residual confounding. There are two other advantages to hospital-based controls. The first is that it is easier to accrue heavier smokers through the hospital compared to the population. The second is the ability to have a control group with medical illnesses, in order to assess the effects of disease status (i.e., altered nutrition) on assay results. We will be able to examine controls with a variety of medical illnesses, compared to healthy persons, and assess the effected of specific organ disorders (i.e., renal insufficiency). The limitation for this control group is that some of these smokers will be coming to the hospital due to respiratory illnesses, and if these illnesses are also associated with the particular lung cancer markers of risk under study, then the frequencies will be the same in the cases and controls. However, the study of smokers in the control population shall permit the detection of such an effect.

Population-based controls provides the theoretical ability to extrapolate our results to the general population. It also will allow us to address an important deficiency in using only heavy smoking controls, namely that a particular risk factor in lung cancer also will be a risk factor for other smoking-related diseases. We could over sample the population controls, or match on smoking status, but this would prevent us from assessing concurrent medical problems. Also, because this study will include both hospital-based and population based controls, we will be able to produce data that is more easily comparable to other molecular epidemiology studies. The field currently uses both hospital- and population-based controls, which can provide conflicting results. But, it is not possible to know if differences in study results are due to study design, chance or other factors. Also, having both hospital- and population-based controls will allow us to study the effects of non-cancer medical problems on assay results.

Rationale for selection of subjects by race: Although all races have an increased risk of lung cancer due to smoking, the risk of lung cancer by race appears to be different, even for the same

level of exposure to tobacco smoke. For example, studies at the American Health Foundation (59) indicated that there was a relative risk of 1.8 (95%C.I.=1.4-2.2) for African Americans compared to Caucasians. Risks for African Americans compared to Caucasians were higher at lower levels of smoke exposure. In a separate case-control study of different ethnicities (60), following adjustment for pack years, occupation, education and age, compared to Japanese, there was a 121%, 53% and 46% difference in risk for Hawaiian, Filipino, and Caucasian males. However, there has been little biological evidence to support an increased risk for African Americans. Preliminary data indicates that there is an increased level of *N*-Nitrosamines adducts in the lungs of African American smokers compared to Caucasian smokers (unpublished data), as well as an increase in urinary metabolites for tobacco-specific nitrosamines.

Rationale for selection of subjects by gender, and focus on sex-steroids and estrogen use: Recent epidemiological evidence indicates that women are at a relatively higher risk of lung cancer for a given degree of smoking. Within levels of smoking, a relative risk ranging from 1.7 to 2.9 has been reported (59,61,62) for women compared to men. The levels of risk did not appreciably differ by levels of smoking exposure. Corroborative laboratory data supports an increased risk for women. It has been found that DNA adducts in lung tissue of women are higher than men using a non-specific ^{32}P-postlabeling assay that tends to identify bulky adducts such as those from PAHs (36), and from a ^{32}P-postlabeling assay for *N*-nitrosamines (unpublished data). Also, while men have more p53 mutations overall, the level of G6T transversions are higher in women, suggesting a greater biological impact for smoking. Separately, the incidence of lung cancer types, i.e., Kreyberg I versus Kreyberg II, is different for men and women, where the latter predominates for women (59,63), and the difference is more pronounced for non-smoking women (64).

The reasons why women compared to men are more susceptible to tobacco is unknown. It is possible that risk increases due to circulating endogenous sex-steroids (estrogens and progesterone), or from estrogen use (oral contraceptives or estrogen replacement therapy [ERT]). The role of estrogens might relate to increased carcinogen metabolism through the induction of cytochrome P450s , or by causing DNA damage from estrogen metabolites or oxidative damage. Sex steroids also can play a role in lung cancer by enhancing cell proliferation; it has been demonstrated that lung cancer cells contain both estrogen and progesterone receptors, but that they are present in 66% of women and 33% in men. The relation of receptors to risk is unknown but could be explored in this study setting.

Rationale to study nicotine addiction: The amount of cigarette smoking, which is directly related to lung cancer risk, depends on a number of factors such as the need to maintain nicotine levels in the blood, avoidance of adverse stimuli, self-medication for depression or anxiety, satisfaction of the brain's reward pathways, cost of cigarettes, advertising, etc. The need to maintain nicotine levels appears to be very strong, as persons who try to consume less cigarettes tend to inhale more deeply, thereby maintaining overall exposure to tar but smoking fewer cigarettes (65,66). A number of smoking variables, such as age at initiation, years of smoking, and cigarettes per day are correlated, implicating a common risk factor, such as the need to satisfy an addiction (67). Nicotine has a "rewarding" property that serves to reinforce drug seeking behavior (68-70). Central nicotinic acetylcholine receptors are stimulated by nicotine and are upregulated and desensitized, simultaneously, by chronic exposure. These receptors

stimulate the secretion of dopamine into the neuronal synapse, which then stimulates postsynaptic dopamine receptors, thereby satisfying craving. Host susceptibilities may play a role in nicotine dependence, specifically metabolizing enzymes governing nicotine levels in the body and neurobehavioral factors relating to the reinforcing value of nicotine. The former might dictate the initial pharmacological reactions to nicotine and how much smoking is needed to maintain nicotine levels (68,69), while the latter may affect why people need to maintain nicotine levels. We have hypothesized that interindividual variation for dopamine pathways and the reward mechanism might lead to an increased risk of smoking. To examine this hypothesis, we have studied polymorphisms in genes that govern synaptic dopamine levels through active reuptake by the dopamine transporter and in dopamine receptors (71) and have recently shown that the risk of smoking is related to a genetic polymorphism in the dopamine reuptake transporter gene, and that there is an interaction with a dopamine D2 receptor polymorphism (Lerman, et al., unpublished data). Also, the presence of specific dopamine D4 receptor alleles have been associated with the risk of smoking in African Americans, as well as the ability to quit smoking following therapy (Shields, et al., unpublished data). These variables for smoking risk might also modulate the amount of smoke exposure, such as how deeply the cigarette is inhaled or how many puffs are actually obtained from an individual cigarette, which has been difficult to quantitate by history or observation. Thus, those genetic polymorphisms that are related to smoking addiction, may also increase lung cancer risk by affecting the biologically effective dose of cigarette smoke.

Rationale for studying patients undergoing low dose CT screening: In recent years, low dose CT screening has become the standard of care for lung cancer screening and early detection due to the 20% reduction in mortality demonstrated by the National Lung Screening Trial (NLST). With this advance, two key unmet needs have emerged. 1) Mechanisms to reduce the false positive rate, which currently stands at ~ 96% and 2) robust markers to prospectively predict which patients diagnosed with Stage IA or Stage IB lung cancer require adjuvant chemo and/or immuno-therapy. As part of our previous work with this protocol, we have developed a series of blood, tissue and urine based biomarkers that predict lung cancer risk, diagnosis and prognosis. We propose to extend these studies and to leverage this past work towards determining whether these markers can be used to address the two questions outlined above. Previously, some of the patients enrolled in this study protocol did receive LDCT screening as part of their diagnosis, but it was never recorded as part of the study. For the clinically important reasons outlined above, we wish to start documenting these data and to conduct nested biomarkers studies on this subset of patients.

Rationale for collecting nasal swabs

These biospecimens will supplement several of our ongoing hypotheses including assessment of the relationship between the microbiome with lung cancer and our work to identify biomarkers of diagnosis and prognosis. The collection of nasal swabs for these purposes is supported by recently published research regarding the field of injury model of lung cancer. Recent work has highlighted the scientific and clinical potential of collecting, analyzing and leveraging nasal swabs for cancer detection and monitoring. Similar to the smoking-associated field effect, the lung cancer-associated airway field of injury can be measured less invasively using gene-expression profiling of the nasal epithelium. Because early-stage lung cancers detected by CT

screening may have distinct biologic characteristics compared to those diagnosed in patients evaluated for clinical symptoms, we propose to develop an accurate biomarker integrating expression profiling of genes, microbiome and microRNA in order to distinguish early lung malignancies from benign lesions. The immediate clinical application of this research will allow clinicians to more accurately stratify patients to or from further diagnostic intervention. Ultimately, this test may also have utility for further stratifying lung cancer risk in patients eligible for screening prior to undergoing LDCT.

There is the potential that a participant could experience a nose bleed. However, this risk is minimal. If the participant blows their nose right after collection, they may notice a spec of blood or brown mucous on the tissue, but it should be clear in 2-5 minutes. To minimize this risk further, any participant with a bleeding disorder or who is taking blood thinning medications will not be asked to provide a nasal swab specimen. We will not collect nasal swabs from participants if they have used NSAIDS for 3 days (aspirin for 7 days). We will also not collect samples from individuals with active nasal allergy symptoms, cold/flu, infection in the nose, or recent nasal surgery (in past 6 weeks or so). In the unlikely chance that a nose bleed does occur, the team will carry silver nitrate sticks.

3 PREVIOUS STUDIES AT THE UNIVERSITY OF MARYLAND AND ASSOCIATED HOSPITALS

The first lung cancer case control study conducted by the University of Maryland (UMD) contractor began in 1986, using two sites – UMD and the Baltimore County Veterans Administration Hospital. A total of 96 cases and 92 controls were accrued. There were two control groups, consisting of heavy smokers with chronic obstructive pulmonary disease and persons with cancer other than lung cancer. The initial results was consistent with the hypothesis that debrisoquine phenotyping, a marker for *CYP2D6*, was predictive of lung cancer (18). Other markers have been studied too. We have measured 4-aminobiphenyl hemoglobin adducts (72), where a relationship to smoking was observed, but not to lung cancer. Genetic polymorphisms for HRAS was measured, where there was a small increased risk in persons with rare alleles (73). There were no associations found for *L-myc, p53, CYP1A1, GSTM1,* or *CYP2E1* genetic polymorphisms (24,29,30,74,75). However, statistical power was quite limited because less than 100 total subjects had available DNA. A second study is now ending which seeks to replicate the initial findings, although the study design has some modifications, so that participation rates could be greater. Specifically, dextromethorphan was used for CYP2D6 phenotyping rather than debrisoquine, and the "other cancer" control group was eliminated.

4 SPECIFIC AIMS

4.1 Primary Hypothesis

The study design will permit testing on a variety of genetic hypotheses, but the primary hypothesis is:

4.1.1 In cultured lymphocytes, we will see a difference for mutagen sensitivity, p53 induction and apoptosis, as a response to in vitro exposure to mutagens, which will be predictive of lung cancer risk.

4.2 Secondary Hypotheses

4.2.1 There will be a relationship between sex steroids (endogenous and exogenous) and estrogen and progesterone receptor positivity in lung tumors. The also will be a relationship between sex steroids and metabolism with lung cancer risk. These relationships will differ by histology.

4.2.2 Genetic polymorphisms relating to neuronal dopamine (dopamine D2 and D4 receptors and dopamine transporter gene), and nicotinic receptors will be predictive of smoking addiction in controls, may also be related to depression. For those genetic polymorphisms related to addiction, we postulate that there is an increased risk of lung cancer, after adjustment for smoking.

4.2.3 Genetic polymorphisms for carcinogen metabolism, DNA repair and in genes governing cell cycle control will be predictive of cancer risk. Also, gene-environment interactions will be explored in order to generate hypotheses that can be tested in larger studies.

4.2.4 Phenotypic and genetic markers of cancer risk will be different by gender and race.

5 METHODS

5.1 Feasibility Study

Prior to beginning the formal study, twenty Caucasian subjects (10 cases, 5 hospital controls and 5 population controls) and fifteen African American subjects (5 cases and 5 controls of each type) will be accrued in order to assess the feasibility of the study accrual and protocols for blood collection and culturing.

Blood from these individuals also will begin the validation and reproducibility testing of the mutagen sensitivity assay. These subjects, if they consent, will have blood collected at two week intervals for six weeks to assess the variability in the phenotyping assays. For the cases undergoing surgery, chemotherapy or radiation therapy, if the results indicate that the assays are not affected by these treatments, then the exclusion criteria might be loosened to include persons within six weeks of therapy, as appropriate. Having repeat blood draws will be optional for study subjects, however. The informed consent will require separate permission for recontacting confirmed by placing initials next to a check box.

During the feasibility phase, it also will be determined if cryopreservation will adversely affect the phenotyping results.

If major modifications are required to improve accrual or other aspects, then the feasibility study will be expanded.

Feasibility testing will be performed only at Johns Hopkins University.

5.2 Validation and Reproducibility Testing

For the first 50 subjects, assay results will be performed in duplicate. If cryopreservation is considered to be acceptable (i.e., has been found to provide the same results as fresh blood during the Feasibility Study), repeated testing of the cryopreserved samples, on a monthly basis, will continue for three months. Because of limited amounts of blood, we will initially only do the reproducibility testing with one clastogen, adding testing of the other clastogens at a later date if considered necessary. Concurrently, lymphocyte subset analysis will be performed to determine how different cases vary from controls. Lymphocytes also will be separated by subset, and the mutagen sensitivity assay will be applied, in order to determine if assay results differ by lymphocyte phenotype. After the first 50 subjects are accrued, we will break the code and examine the results separately for cases and controls in relation to questionnaire variables, in order to assess the effect of age, gender, race, smoking, alcohol, vitamin intake and caffeine use. The data also will be assessed in relation to subset ratios. Separately, we also will measure micronutrient levels in these fifty individuals to assess the relationship to mutagen sensitivity or cellular response assays. Depending on the variability of the study results for the mutagen sensitivity, p53 induction and apoptosis response, some assays might not be continued.

Following the evaluation of assay results from the first 50 individuals, the Validity and Reproducibility Testing phase may continue, in increments of 50 individuals, in order to test new hypotheses relating to validity and reproducibility. For example, if micronutrient levels are suggested to be related to assay results, then a food frequency questionnaire might be administered to the next 50 subjects. Separately, if there are suggested differences between cases and controls (one or both control groups), then additional experiments might be needed to elucidate such differences, where a greater proportion of collected blood might be used for the mutagen sensitivity assay.

5.3 Subject Selection

5.3.1 Case Subject Selection

All patients with a diagnosis of lung cancer are identified through daily visits to the hospital pathology department and operating room, and to daily visits to the oncology clinic, radiation therapy clinic, surgery clinic, tumor registry, internal medicine clinic, and primary care clinics. Multispecialty clinics for lung cancer and tumor board will be attended as they are conducted.

5.3.1.1 Inclusion Criteria

5.3.1.1.1 Diagnosis of non-small cell lung cancer made pathologically (with confirmation by a second pathologist).

5.3.1.1.2 Must reside in Baltimore city or contiguous metropolitan counties, Prince George's county or Anne Arundel county.

5.3.1.1.3 Have a residential working phone within their home.

5.3.1.1.4 Be born in the United States.

5.3.1.1.5 Speak English well enough to be interviewed.

5.3.1.1.6 Be physically and mentally capable of performing the interview (i.e., must be able to hear the interviewer, mentally comprehend the interviewers questions and verbally respond).

5.3.1.1.7 Never have been interviewed as a control for the study.

5.3.1.1.8 Consent by the physician from the clinic where the subject was identified, or listed as the treating physician by the tumor registry or surgical pathology report.

5.3.1.1.9 Report of a positive LDCT screen by a physician

5.3.1.2 Exclusion Criteria

5.3.1.2.1 More than 6 months after initial diagnosis.

5.3.1.2.2 Currently residing in an institution such as a prison, nursing home or shelter.

5.3.1.2.3 Severely ill subjects in the intensive care unit (after discharge from ICU, then can be reconsidered).

5.3.1.2.4 Subject is unable to give informed consent.

5.3.1.2.5 Known diagnosis of HIV, hepatitis B or C.

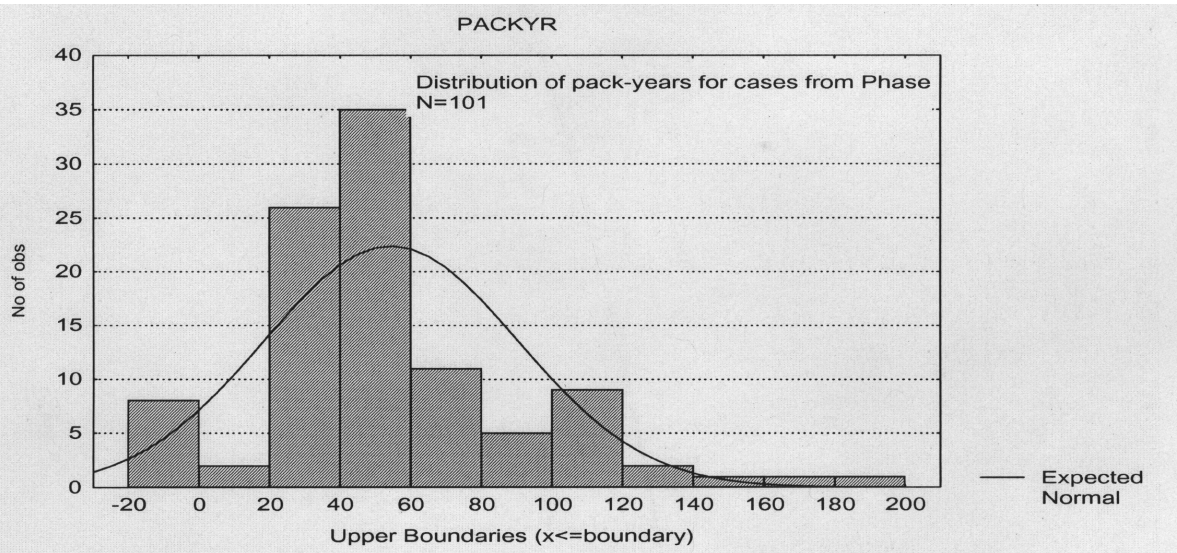

Figure 1

5.3.2 Hospital-Based Control Selection

Hospital-based controls will be frequency matched by age (5 year intervals), gender, race, smoking (5 categories based on pack years -- nonsmokers, 0.1-20, 21-40, 41-60, >60; ex-smokers will be included) and hospital. Recruitment for cases and controls will start concurrently, using the frequency distribution from the previous case-control study, but recruitment objectives will be assessed and identification altered to ensure a frequency matched study. See Figure 1 for distribution of cases by pack years from the initial study in 1986.

Subjects will be recruited through twice weekly visits to the internal medicine clinic, primary care clinic, pulmonary care clinic, and cardiology clinic.

5.3.2.1 Inclusion Criteria

5.3.2.1.1 Stratified to frequency match cases by age (5 year intervals), gender, race, smoking (20 pack year intervals -- non-smokers, 0-20, 20-40, 40-60 and >60 and ex-smokers [>5 yrs]) and hospital.

5.3.2.1.2 Must reside in Baltimore city, contiguous metropolitan counties, Prince George's county or Anne Arundel county.

5.3.2.1.3 Have a residential working phone within their home.

5.3.2.1.4 Be born in the United States.

5.3.2.1.5 Speak English well enough to be interviewed.

5.3.2.1.6 Be physically and mentally capable of performing the interview (i.e., must be able to hear the interviewer, mentally comprehend the interviewers questions and verbally respond).

5.3.2.1.7 Never have been interviewed as a control for the study.

5.3.2.1.8 Physician consent by physician from clinic with subject is identified.

5.3.2.2 Exclusion Criteria

5.3.2.2.1 History of cancer other than non-melanotic skin cancer or in situ cervical cancer.

5.3.2.2.2 Currently residing in an institution such as a prison, nursing home or shelter.

5.3.2.2.3 Severely ill subjects in the intensive care unit (after discharge from ICU, then can be reconsidered).

5.3.2.2.4 Subject is unable to give informed consent.

5.3.2.2.5 Known diagnosis of HIV, hepatitis B or C.

5.3.3 Selection of Population-Based Controls

Population-based controls will be identified through the department of motor vehicles, and frequency matched by age (5 year intervals), gender, and race to cases. Recruitment for cases and controls will start concurrently, using the frequency distribution from the previous case-control study, but recruitment objectives will be assessed and identification altered to ensure a frequency matched study.

5.3.3.1 Inclusion Criteria:

5.3.3.1.1 Stratified to match cases by age (5 year intervals), gender, and race.

5.3.3.1.2 Must reside in Baltimore city or contiguous metropolitan counties.

5.3.3.1.3 Have a residential working phone within their home.

5.3.3.1.4 Be born in the United States.

5.3.3.1.5 Speak English well enough to be interviewed.

5.3.3.1.6 Be physically and mentally capable of performing the interview (i.e., must be able to hear the interviewer, mentally comprehend the interviewers questions and verbally respond).

5.3.3.1.7 Never have been interviewed as a control for the study.

5.3.3.2 Exclusion Criteria:

5.3.3.2.1 History of cancer other than non-melanotic skin cancer or in situ cervical cancer.

5.3.3.2.2 Currently residing in an institution such as a prison, nursing home or shelter.

5.3.3.2.3 Subjects unable to give informed consent.

5.4 Questionnaire

A questionnaire that is consistent with the previous lung cancer study, and with ongoing lung cancer case-control studies at MD Anderson and the lung cancer spore groups will be utilized, as well as the Women's Health Care Study, American Cancer Society II Nutrition Questionnaire and the Western New York Diet Study. There will be an assessment for prior medical and cancer history, tobacco use, alcohol use, current medications, occupational history, family medical history, menstrual history and estrogen use, and socioeconomic status. Additionally, the Fagerstrom index for nicotine dependence (FTND) and a modified version of the Horn-Waingrow Reasons for Smoking (RFS) Scale will be used. The FTND is a 6-item, self-report measure of nicotine dependence. Sample items include the number of cigarettes smoked in the past seven days and the average length of time from waking to smoking. Questions such as "time until first cigarette of the day" have been shown to be a highly reliable single items index of nicotine dependency. For the RFS, self-administered questions were selected that reflect a self-medication hypothesis relating to smoking for stimulation (4 items: e.g., "I get a definite lift and feel more alert when smoking") and smoking for negative affect regulation (3 items: e.g., "When I feel blue or want to take my mind off cares and worries, I smoke cigarettes"). Subjects will

be asked to rate the statements on a Likert scale "How much is each of the following characteristics of you: (0=not at all to 3=very much so).
Also, controls will receive the Center for Epidemiologic Studies Depression (CES-D) scale, in order to assess the interaction of depression with nicotine addiction. The CES-D is a 20 item Likert-style self-administered scale used to assess depressive symptomatology. This scale has high internal consistency and has been shown to correlate with clinical ratings of severity of depression.

5.5 Procedures

5.5.1 Advertisements and Flyers

Flyers will be placed in areas where cases or hospital-based controls might be accrued stating that there is an ongoing lung cancer study, and that they might be contacted to participate. If they are contacted, then this is because their physician has agreed to allow us to contact them. And that participation is voluntary, and that it will help us learn what causes lung cancer.

5.5.2 Procedures for Cases

Identify cases through resources including, but not limited to daily PM visits to the pathology departments (or phone calls) to identify all cases diagnosed that day with lung cancer. Daily AM visits to the pathology department and operating room. Daily visits to the oncology clinic, radiation therapy clinic, surgery clinic, tumor registry, internal medicine clinic, and primary care clinic. For these clinics, appointment logs and diagnoses will be reviewed from records, and practitioners will be queried. Tumor board and multispecialty lung cancer clinics will be attended as they are conducted. Cases will also be identified by daily visits to the nodule clinic at the participating intuitions to identify all patients referred to the clinic with positive low dose CT scans that day.

5.5.2.1 Interviewer obtains subject's name, location and physician name.

5.5.2.2 Interviewer screens medical record for eligibility. If ineligible, record age, gender, race, tobacco usage and family history and reason for ineligibility. Also record histological type of lung cancer.

5.5.2.3 Interviewer contacts physician, explains nature of study and receives verbal consent for participation. Interviewer records physician consent. Determine if there is a general consent form for participating for research purposes located in the chart. If physician refuses, interviewer records reason for refusal on eligibility record. If there is a general research consent form on the chart, then the interviewer

will record the subject's age, race, gender, family history, tobacco usage and lung cancer histology. If a consent form does not exist, then the Maryland tumor registry will be queried for age, race, gender, tobacco usage, stage and lung cancer histology. This information will be recorded anonymously.

5.5.2.4 Interviewer contacts case and obtains informed consent.

5.5.2.4.1 If subject refuses outright, then there is no further contact. This will be determined by asking if we can recontact the subject later (i.e., in one week before their treatment is to begin if that is the case, or after surgery if that is the case), either directly or through the physician. At this point, it should be stated to the subject that participation is voluntary, and refusal will have no impact upon them and their refusal will remain confidential. If subject agrees to be recontacted, but does not give consent after 2 additional requests, then record as a refusal. If physician agrees to contact subject, then recontact the subject after physician says to do so.

5.5.2.4.2 If subject agrees to participate, but fails to actually participate (i.e., fails to show up for their interview or does not agree to a particular time for the interview, they will be recontacted up to two more times.

5.5.2.4.3 If subject refuses (either outright or by repeatedly delaying consent- [failure to make commitment for 3 requests]), then no further contact is made. If there is a general research consent form on the chart, then the interviewer will record the subject's age, race, gender, family history, tobacco usage and lung cancer histology. If a consent form does not exist, then the Maryland tumor registry will be queried for age, race, gender, tobacco usage, stage and lung cancer histology. This information will be recorded anonymously.

5.5.2.5 Perform interview and complete questionnaire.

5.5.2.6 If family history is positive for a first degree relative with lung cancer, then ask permission to provide name, address and phone number to researchers at Johns Hopkins University, who will ask them to participate in a study of familial lung cancer. Provide name, address and number to Gloria Petersen. If the patient has not provided informed consent for the study (i.e., they are a refuser or ineligible), then this paragraph will not apply.

5.5.2.7 Collect up to 65 cc of blood -- 15cc in plain corvath tube,

two 15cc green top tubes and two 10cc in blue top tubes for separation by Leukoprep to collect lymphocytes. Immediately place on ice.

5.5.2.8 Collect urine (50ml).

5.5.2.9 Collect nasal swab

5.5.2.10 Provide reward of $50.00. $25.00 after completion of interview and $25.00 after providing blood, nasal swab and urine specimens.

5.5.2.11 Blood (separation of serum and blood clot; buffy coat, plasma and red blood cells; lymphocytes, red blood cells and plasma) and urine to be processed within 8 hours at the University of Maryland Department of Pathology. Four 2 cc aliquots of herparinized blood will be cryopreserved. The remainder of that green top tube (approximately 7 cc), will be sent to LHC within 24 hours for lymphocyte culture by messenger.

5.5.2.12 Review pathology report to confirm diagnosis and collect pathological data.

5.5.2.13 Request and send slides to Dr. Borkowski for pathological confirmation. This should be done as soon as possible after enrollment into the study.

5.5.2.14 Request part of tumor block, and frozen tumor if available, for storage.

5.5.2.15 Ensure that Dr. Borkowski agrees with pathological diagnosis. Written confirmation of diagnosis by Dr. Borkowski.

5.5.2.16 If subject is recontacted at a later time for additional phlebotomy, than an additional $25.00 reward is given. If subject is contacted for additional questionnaire information, than no reward is given.

5.5.2.17 If subject refuses to give blood, DNA will be collected using a mouthwash procedure.

5.5.3 Procedures for hospital-based controls

Identify cases through resources including, but not limited by daily visits to the internal medicine clinic, primary care clinic, pulmonary care clinic, cardiology clinic, frequency-matched based on age (5 year interval), gender, race and smoking (20 pack year interval into 5 categories -- non-smoker, 0-20, 20-40, 40-60 and >60). For these clinics, appointment logs and diagnoses will be reviewed from records, and practitioners will be queried.

5.5.3.1 Interviewer obtains subject's name, location and physician name.

5.5.3.2 Interviewer screens medical record for eligibility. If ineligible, record age, gender, race, family history of lung

cancer, tobacco history and reason for ineligibility.

5.5.3.3 Interviewer contacts physician, explains nature of study and receives verbal consent for participation. Interviewer records physician consent. Determine if there is a general consent form for participating for research purposes located in the chart. If physician refuses, interviewer records reason for refusal on eligibility record. If there is a general research consent form on the chart, then the interviewer will record the subject's age, race, gender, family history, tobacco usage and lung cancer histology.

5.5.3.4 Interviewer contacts hospital-based control and obtains informed consent.

5.5.3.4.1 If subject refuses outright, then there is no further contact. This will be determined by asking if we can recontact the subject later in one month, either directly or through the physician. At this point, it should be stated to the subject that participation is voluntary, and refusal will have no impact upon them and their refusal will remain confidential. If subject agrees to be recontacted, but does not give consent after 2 additional requests, then record as a refusal. If physician agrees to contact subject, then recontact subject after physician says to do so.

5.5.3.4.2 If subject agrees to participate, but fails to actually participate (i.e., fails to show up for their interview or does not agree to a particular time for the interview, they will be recontacted up to two more times.

5.5.3.4.3 If subject refuses (either outright or by repeatedly delaying consent- [failure to make commitment for 3 requests]), then no further contact will be made.

5.5.3.5 Perform interview and complete questionnaire

5.5.3.6 If family history is positive for a first degree relative with lung cancer, then ask permission to provide name, address and phone number to researchers at Johns Hopkins University, who will ask them to participate in a study of familial lung cancer. Provide name, address and number to Gloria Petersen. If the patient has not provided informed consent for the study (i.e., they are a refuser or ineligible), then this paragraph will not apply.

5.5.3.7 Collect up to 65 cc of blood -- 15 cc in plain corvath tube, two 15 cc green top tubes and two 10 cc in blue top tubes for separation by Leukoprep to collect lymphocytes. Immediately place on ice.

5.5.3.8 Collect urine (50 ml).

5.5.3.9 Provide reward of $50.00. $25.00 after completion of interview and $25.00 after providing blood and urine specimens.

5.5.3.10 Blood (separation of serum and blood clot; buffy coat, plasma and red blood cells; lymphocytes, red blood cells and plasma) and urine to be processed within 8 hours at the University of Maryland Department of Pathology. Four 2 cc aliquots of herparinized blood will be cryopreserved. The remainder of that green top tube (approximately 7 cc), will be sent to LHC within 24 hours for lymphocyte culture by messenger.

5.5.3.11 If subject is recontacted at a later time for additional phlebotomy, than an additional $25.00 reward is given. If subject is contacted for additional questionnaire information, than no reward is given.

5.5.3.12 If subject refuses to give blood, DNA will be collected using a mouthwash procedure.

5.5.4 Procedures for Population-based controls

Identify subjects through Department of Motor Vehicle frequency-matched on age, race, and gender.

5.5.4.1 A letter is sent to the prospective subject notifying them of the study, the benefits of the study, how they were selected and to request participation. A return card will be enclosed that will allow subject to refuse to be contacted.

5.5.4.2 Interviewer contacts population-based control and obtains permission to come to house. Alternatively, provide additional reward if subject will come to UMD.

5.5.4.2.1 If subject refuses (either outright or by repeatedly delaying consent -- [failure to make commitment for 3 requests]) to participate in the full study, then ask permission for a five minute interview on the phone. If subject refuses, then no further contact is made. The interview will query age, gender, race, family history of lung cancer and smoking history. This information will be recorded anonymously.

5.5.4.3 In person, obtain informed consent. If subject refuses (either outright or by repeatedly delaying consent -- [failure to make commitment for 3 requests]), then no additional contact is made.

5.5.4.4 Perform interview and complete questionnaire.

5.5.4.5 If family history is positive for a first degree relative with lung cancer, then ask permission to provide name, address and phone number to researchers at Johns Hopkins University, who will ask them to participate in a study of

familial lung cancer. Provide name, address and number to Gloria Petersen. If the patient has not provided informed consent for the study (i.e., they are a refuser or ineligible), then this paragraph will not apply.

5.5.4.6 Collect up to 65cc of blood -- 15 cc in plain corvath tube, two 15 cc green top tubes and two 10 cc in blue top tubes for separation by Leukoprep to collect lymphocytes. Immediately place on ice.

5.5.4.7 Collect urine (50 ml urine).

5.5.4.8 Collect nasal swab

5.5.4.9 Provide reward of $50.00. $25.00 after completion of interview and $25.00 after providing blood and urine specimens.

5.5.4.10 Blood (separation of serum and blood clot; buffy coat, plasma and red blood cells; lymphocytes, red blood cells and plasma) and urine to be processed within 8 hours at the University of Maryland Department of Pathology. Four 2 cc aliquots of herparinized blood will be cryopreserved. The remainder of that green top tube (approximately 7 cc), will be sent to LHC within 24 hours for lymphocyte culture by messenger.

5.5.4.11 Subjects receive a self-administered nutritional survey or food frequency questionnaire.

5.5.4.12 If subject is recontacted at a later time for additional phlebotomy, than an additional $25.00 reward is given. If subject is contacted for additional questionnaire information, than no reward is given.

5.5.4.13 If subject refuses to give blood, DNA will be collected using a mouthwash procedure.

5.6 Re-contacting subjects for additional information or blood:

5.6.1 If known at time of entry into study, i.e., if they are part of the feasibility or validity phase, then request subjects permission to be recontacted for blood draw, and advise the subject of the schedule.

5.6.2 If it is determined at a latter date that more historical information, blood or urine is needed, then a letter is sent to the subject notifying them of the need for re-contacting, specifying whether information or blood is needed. Included will be a stamped envelope and form to be returned if the subject does not want to be contacted.

5.6.3 Two weeks later, the subject is called on the phone. If information is requested, it can be asked at that time.

5.6.4 If subject agrees, then additional phlebotomy can be done at the home or in the hospital, whichever is convenient.

5.7 Phlebotomy and collection procedures:

5.7.1 Observe universal precautions for prevention of transmission of blood borne pathogens.
5.7.2 Clean skin with alcohol wipe and wait to dry.
5.7.3 Obtain blood, filling tubes -- 1 plain corvath (15cc), 2 green top tube (15 cc), 2 blue top tubes (10cc).
5.7.4 Apply pressure and band-aid.
5.7.5 Place blood samples on ice.
5.7.6 Request urine sample in plain sterile container (50ml).

5.8 Processing of biological samples at UMD Department of Pathology:
5.8.1 Storage of urine at -70$_{o}$C in 10 ml aliquots.
5.8.2 Separation of serum from clots. Storage of both at -70$_{o}$C.
5.8.3 Cryopreserve four 2 ml of whole herparinized blood.
5.8.4 Send seven cc of herparinized whole blood to LHC within 24 hours for lymphocyte cultures by messenger.
5.8.5 Separation of buffy coat from red cells and plasma. Wash red blood cells according to protocol. Storage of all three at -70$_{o}$C.
5.8.6 Separation of lymphocytes from red blood cells and plasma. Wash red blood cells according to protocol. Cryopreservation of two 0.5 ml tubes of lymphocytes. Storage of all tubes at -70ºC.

5.9 Research laboratory studies to test primary hypotheses:

5.9.1 Mutagen Sensitivity and Cellular Response
5.9.1.1 Culture of whole blood in the presence of phytohemogluttin using RPMI-1640 media supplemented with fetal bovine serum. Ten separate cultures will be established.
5.9.1.2 After 68 hours of culture, there cultures will be grouped, one set which will be treated with caffeine and the other without. Two cultures (the controls) will receive no additional treatment. The remaining cultures (8) will be treated with different clastogens, each one with and without caffeine. Two will receive bleomycin, benzo(a)pyrene-diol-epoxide, or *N*–methyl-nitrosurea for five hours. The last two will be radiated. Optimal duration and dose of exposures are currently being determined, using previously published doses as a guide.
5.9.1.3 Fix aliquot of cells onto 6 slides for later immunohistochemical staining.
5.9.1.4 Treat cultures with colcemid to arrest in metaphase.
5.9.1.5 Prepare cells and fix onto 4 slides.
5.9.1.6 Assessment of chromosomal aberrations by Giemsa staining, counting 50 metaphases.
5.9.1.7 Assessment of p53 induction by immunohistochemistry and by Western blot.
5.9.1.8 Assessment of apoptosis using DAPI.

5.10 Interviewer training and quality control:

The interviewers will receive a procedure manual. The will receive training in how to administer and properly complete the questionnaire, provide informed consent, how to identify eligible subjects, how to perform phlebotomy (observing Universal Precautions), and how to properly process the blood and urine samples for transport. Training will be conducted by the consulting epidemiologist and field supervisor.

Interviewers will first practice administering the questionnaire to office volunteers. During the feasibility phase of the study, and subsequently for newly hired interviewers, the interviews will then administer the questionnaire and draw blood on subjects at either UMD (or JHU for the interviewer at JHU) under the supervision of the epidemiologist and field supervisor, who will provide feedback after the interview.

During the main study phase, quality control will consist of data comparisons among the interviewers to determine the quantity and quality of information that they have gathered, by evaluating characteristics such as interview duration, number of interview problems reported, number of refusals, distribution of subject answers to "sensitive" questions (e.g., alcohol use, contraception), and number of missing and incomplete answers. In addition, a small random sample of subjects will be recontacted by the epidemiologist to inquire about the interview experience, to note any problems and confirm that the interview actually took place.

6 SAMPLE SIZE

6.1 Time for accrual -- 2 - 3 years.

6.2 Target Accrual Numbers:

Target Accrual Numbers of Cases and Controls[1]					
	Caucasian Males	**Caucasian Females**	**African American Males**	**African American Females**	**Total**
Cases	**322**	**322**	**179**	**179**	**1002**
Population Controls	**322**	**322**	**178**	**178**	**1000**
Hospital Controls	**321**	**321**	**178**	**178**	**998**
Total	**965**	**965**	**535**	**535**	**3000**
[1]Race will be identified by subject self-report.					

6.3 Known available cases from hospital tumor registries per year:

Known Available Cases by Hospital Per Year Calender Year 1995 or 1996					
Hospital	**Caucasian Males**	**Caucasian Females**	**African American Males**	**African American Females**	**Total**
UMD[1]	66	28	68	7	169
JHU	108	80	35	29	252
Harbor	44	45	9	3	101
Sinai	34	27	26	17	104
Liberty/Bon Secours	5	23	41	21	90
Total	257	203	179	77	716
[1]Includes VA patients too.					

With regards to numbers of patients that we hope to interview having undergone LDCT screening specifically, approximately 20-30 patients are referred to the hospital each month following an LDCT scan. Therefore, per year, we will attempt to consent 240-360 patients. Of these patients, approximately 30 will subsequently have a lung cancer diagnosis.

7 DATA ANALYSIS

7.1 Data Analysis

Data analysis for the primary hypothesis will assess the end point of lung cancer in relation to mutagen sensitivity and known risk factors for lung cancer. We will compare the lung cancer cases to the two control groups, separately and combined (if the data does not suggest heterogeneity for the two control groups). Lung cancer cases will be grouped together and then examined separately by race and gender. Mutagen sensitivity will be examined as a continuous variable and as a categorical variable (quartiles based upon distribution of sensitivity in the population based controls). We will also compare patients identified with a positive scan who subsequently received a lung cancer diagnosis to those who had a positive scan but did not receive a lung cancer diagnosis.

The data will be analyzed using the Statistical Analysis System. Initially, we will determine if there are variables that affect mutagen sensitivity, such as age, gender, race, smoking (lifetime and recent), caffeine use, vitamin use, alcohol, family history of lung cancer, and medications. This will be done separately for each of the study groups (cases and two control groups). Additionally, in cases it will be determined if mutagen sensitivity is related to lung cancer histology or stage, or weight changes.

The relationship of lung cancer to mutagen sensitivity will first be examined crudely using a student's T test (after transformation if needed) for the continuous variable, and odds ratios with 95% confidence intervals for the quartiles, using the least sensitive quartile as the reference group. Next, the data will be examined within racial, gender and smoking groups – never smokers, ex-smokers and current smokers.

Unconditional logistic regression will be used, where models will be examined separately for each control group. Lung cancer will be the dependent variable, and mutagen sensitivity, age, gender, race, and smoking will be the dependent variables. Additionally, if previous analyses suggest that there are variables that affect mutagen sensitivity such as caffeine use, alcohol, etc. then these also will be added to the model.

7.2 Power Calculations

The following provides power calculations for the primary hypothesis. The appendix contains additional calculations. These calculations assume a p value of 0.05 (two tail). For comparisons of means, power calculations are provided based on an assumed difference of 20% between cases and controls.

Mutagen Sensitivity as a continuous variable						
Study Group	Control Mean	Std. Dev.	Case Mean	N cases	Power (1:1 cases:controls)	Power (1:2 cases:controls)
Afr. Amer.	0.74	0.34	0.88	200	1.0	1.0
Caucasians	0.82	0.37	1.00	200	1.0	1.0
Total	0.78	0.35	0.94	400	1.0	1.0

Mutagen sensitivity as a categorical variable[1]				
Study Group	Freq. In Controls	N cases	Min. Detected OR (1:1 cases:controls)	Min. Detected OR (1:2 cases:controls)
Afr. Amer.	0.5	200	2.0	1.8
Caucasians	0.5	200	2.0	1.8
Total	0.5	400	1.6	1.5

[1]Phenotypes assuming that we will categorize subjects above and below median phenotype result. Therefore, all phenotypes (chromosome aberrations, apoptosis, p53) will have the same power.

8 LIMITATIONS

There are several limitations to this study that relate to both subject accrual and assay methodology. A frequent problem for case-control studies testing phenotypic or genetic markers is that there may be ethnic differences between cases and controls, yielding differences in genetic frequencies unrelated to case status. However, the alternative of restricting this study to a narrow ethnic or geographic strata is partially incorporated into this study. This study also attempts to reduce the problems with ethnic differences by assessing this in the questionnaire and matching by race. Cases could be rendered more similar to controls if we had added a selection criteria for controls that would match on census tract, however, this would not obviate this limitation, but would make the study substantially more difficult to perform. Also, the experience in Baltimore is that population control accrual is not skewed to one part of Baltimore (C. Loffredo, personal communication). Separately, because the population-based controls will be identified through the Department of Motor Vehicles, we might have limited case selection to those persons with valid drivers licenses or identification cards, but this would exclude some inner city and elderly subjects. It should be noted that the hospital-based controls do not have this exclusion criteria, so that in some ways this group might be more comparable to cases than the population-based controls. Finally, we would not expect that there would be a selection bias based on phenotype or genotype, as these factors are unknown to the subjects and there is no plausible relation to either behavior or other factors that might affect selection..

Another limitation relevant to our study and common to other case-control studies is that there will be differences between cases and controls due to disease status. We plan for a number of validity experiments to assess this (i.e., repeated testing, assessment by stage of disease, etc.). The use of two controls groups (one hospital-based with some non-cancer illness and the other population based and mostly healthy) also will help determine the magnitude of this limitation. After the first 50 subjects, we will assess the results of the assay and determine whether assay results vary by recent case factors (i.e., differences in lymphocyte subpopulations, nutrition, etc.), and make appropriate changes. Another limitation relates to limitations on rapid subject accrual. For cases, we expect to identify over 90% of the cases through hospital pathology and oncology departments. This will be verified through tumor registry records at 6 month intervals (there is no rapid-case ascertainment mechanism for the state of Maryland or within the hospitals). More importantly, in general we will, and the possible effects of treatment on phenotype results make this an absolute priority. Also, by allowing for accrual of subjects before or after surgery should allow more flexibility in at least a subset of subjects. We expect to contact more than 60% of subjects before treatment, but will confirm this during the feasibility study, and then confirm this at six month intervals by refusing refusal/exclusion and tumor registry data. Although, we will re-evaluate this 60% goal after the initial testing period.

9 HUMAN SUBJECTS

Prior to enrolling subjects, this protocol will need to be approved by the Institutional Review Board of the National Institutes of Health, followed by the IRB's at the University of Maryland, John's Hopkins University and all other hospitals where recruitment will occur.

Written informed consents from the study subjects will be required for participation in the study. The informed consent will allow for us to obtain biological samples, perform genetic testing, and recontact subjects if necessary. It will state that individual results will not be provided to the participants, but overall study results and progress, as they are obtained, can be provided at the subjects request.

Study subject's confidentiality will be maintained at all times. Subjects will be assigned unique study numbers. These unique study numbers will be linked to the subject's identifier information in a database and on the hard copy of the Identifier Sheet. This information will be secured at the University of Maryland. The database will require at least 2 levels of security (i.e., passwords), which will allow only authorized individuals to access the information. A log will automatically who accesses the information and what was accessed. The Identifier Sheets from the questionnaire will be physically separated from the questionnaire, and stored in a locked cabinet. The questionnaires will retain only the unique study number. Biological samples will be labeled with the unique study number and no other identifier information. No identifier information that can be linked to study results or other data will leave the University of Maryland premises.

Identifier information for non-participants (refusers and ineligibles) will recorded in order to avoid recontact. This information will be stored in a database with at least 2 levels of security (i.e., passwords), which will allow only authorized individuals to access the information. A log will automatically who accesses the information and what was accessed. Unique study numbers for non-participants also will be assigned. This number will be used for tracking reasons for non-participation and available demographic information.

Two databases will be maintained. The first will include the Contact Database and will include identifier information. It will record if subjects refused, were ineligible, or are participants. If participants, it will record when the interview will occur, the outcome, and track sample handling. There will be a calender program that will identify when subjects are to be recontacted, and how many times they have been contacted. It will record physician contacts. For refusers and ineligibles, it will record that their data was entered into the Refusal and Ineligible Database. This data has been obtained from the Maryland tumor registry for cases, by prior consent from the medical record when available for cases and hospital controls, and from a brief telephone interview from population controls. The Refusal and Ineligible Database will record data for age, gender, race, smoking history, family history, lung cancer histology and why subject was ineligible or refused. This database will be anonymous and will not contain identifier information or their unique numbers.

10 TIME SCHEDULE

Month 1-- Review by Technical Evaluation of Protocols and Questionnaire Committees, DCEG.
Month 2 -- Review by NIH IRB.
Produce interviewer handbooks.
Begin recruitment of interviewers.
Month 3 -- Review by Johns Hopkins and University of Maryland IRBs
Train interviewers.
Month 4 -- Begin Feasibility testing at Johns Hopkins University.
Month 5 – Continue Feasibility Study.
Month 6 – Review and revise protocol if needed.
Resubmit to IRBs if needed.
Month 7 – Begin Accrual at all sites.
Month 9 – Analyze data after accrual of 50 controls to assess variables that relate to mutagen sensitivity, in order to determine if the procedure can simplified (i.e., eliminate an exposure), or if there are areas of the questionnaire need to be expanded.
Continue accrual at projected rate of 17 cases, 17 smoking controls and 17 population controls per month.
Month 17 – Analyze data to assess variables that relate to mutagen sensitivity, in order to determine if the procedure can simplified (i.e., eliminate an exposure), or if there are areas of the questionnaire need to be expanded.
Month 31 – Close enrollment if target accruals are met.

11 BUDGET – Annualized

Field collection budget will be paid for from LHC contract funds

Title	FTE	Rate/FTE	Subtotals	Total
Epidemiologist (Senior)	0.05	$125,000.00	$6,250.00	
Epidemiologist (Junior)	0.20	$125,000.00	$25,000.00	
Nurse Coordinator	1.00	$40,000.00	$40,000.00	
Interviewers	3.50	$27,000.00	$94,500.00	
Telephone Recruiter	1.00	$26,000.00	$26,000.00	
Collection supervisor	0.50	$29,000.00	$14,500.00	
Collection Assistant	0.50	$20,000.00	$10,000.00	

Total Labor:	$216,250.00	
Fringe Benefits (26%):	$56,225.00	
Total Labor and Fringe Benefits:		$272,475.00
Supplies and Services		
Tissue Collection	$12,000.00	
Transportation of specimens	$8,000.00	
Communications	$3,000.00	
Office Supplies and Services	$2,000.00	
Data Support	$10,000.00	
Driver	$4,600.00	
Participant Fees	$15,000.00	
Mailings	$5,000.00	
Total Supplies and Services	$59,600.00	
Total Labor, Fringe and Supplies and Services		$332,075.00
Indirect costs (48%)		$159,396.00
Total Annual Cost Year 1		$491,471.00

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16 CHECK LIST FOR INCLUSION AND EXCLUSION CRITERIA

CHECK LIST
INCLUSION AND EXCLUSION CRITERIA
LUNG CANCER CASES

NAME_____ Date: _____

DATE OF BIRTH _____

ID# (Hosp. Accession, SS# or DMV #)_____

Yes	No	Criteria (ALL MUST BE CHECKED)
		Diagnosis of non-small cell lung cancer made pathologically at a local hospital pathology department (and sample available for confirmation by a UMD pathologist).
		Resides in Baltimore city or contiguous metropolitan counties, Prince George's county or Anne Arundel county.
		Has a residential working phone within their home.
		Born in the United States.
		Speaks English well enough to be interviewed.
		Is physically and mentally capable of performing the interview.
		Has not been interviewed as a control for the study.
		Is not residing in an institution such as a prison, nursing home or shelter
		Is not severely ill in the intensive care unit (after discharge from ICU, then can be reconsidered
		Is not known diagnosis of HIV, hepatitis B or C.
		Consent by the physician from the clinic where the subject was identified, or listed as the treating physician by the tumor registry or surgical pathology report.
		Subject provides informed consent and signs form. _____**Unwilling** _____**Unavailable**

CHECK LIST
INCLUSION AND EXCLUSION CRITERIA
HOSPITAL-BASED CONTROLS

NAME_____ Date: _____

DATE OF BIRTH _____

ID# (Hosp. Accession, SS# or DMV #)_____

Yes	No	Criteria (ALL MUST BE CHECKED)
		Does not have a history of cancer other than non-melanotic skin cancer or in situ cervical cancer.
		Fits criteria for frequency matching.
		Resides in Baltimore city or contiguous metropolitan counties, Prince George's county or Anne Arundel county.
		Has a residential working phone within their home.
		Born in the United States.
		Speaks English well enough to be interviewed.
		Is physically and mentally capable of performing the interview.
		Has not been interviewed as a control for the study.
		Is not residing in an institution such as a prison, nursing home or shelter
		Is not severely ill in the intensive care unit (after discharge from ICU, then can be reconsidered
		Is not known diagnosis of HIV, hepatitis B or C.
		Consent by the physician from the clinic where the subject was identified, or listed as the treating physician by the tumor registry or surgical pathology report.
		Subject provides informed consent and signs form. _____**Unwilling** _____**Unavailable**

CHECK LIST
INCLUSION AND EXCLUSION CRITERIA
POPULATION-BASED CONTROLS

NAME_____ Date: _____

DATE OF BIRTH _____

ID# (Hosp. Accession, SS# or DMV #)_____

Yes	No	Criteria (ALL MUST BE CHECKED)
		Does not have a history of cancer other than non-melanotic skin cancer or in situ cervical cancer.
		Fits criteria for frequency matching.
		Resides in Baltimore city or contiguous metropolitan counties, Prince George's county or Anne Arundel county.
		Has a residential working phone within their home.
		Born in the United States.
		Speaks English well enough to be interviewed.
		Is physically and mentally capable of performing the interview.
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		Is not residing in an institution such as a prison, nursing home or shelter
		Consent by the physician from the clinic where the subject was identified, or listed as the treating physician by the tumor registry or surgical pathology report.
		Subject provides informed consent and signs form. _____**Unwilling** _____**Unavailable**