Prospective analysis of DNA damage and repair markers of lung cancer risk from the Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Screening Trial

Alice J. Sigurdson1, Irene M. Jones1, Qingyi Wei2, Xifeng Wu2, Margaret R. Spitz2, Douglas A. Stram, Myron D. Gross3, Wen-Yi Huang4, Li-E Wang2, Jian Gu2, Cynthia B. Thomas1, Douglas J. Reding5, Richard B. Hayes4,6 and Neil E. Caporaso7

Introduction

Mutagen challenge assays were introduced in the early 1980s (1–4) and since then several hundred case-control study results have reported various measures of DNA damage or functional tests of DNA repair capacity (DRC) were associated with 2- to 10-fold increased cancer risk at several sites (reviewed in refs 5–10). All of those case-control studies shared the design limitation that these assays are unable to disentangle the host’s response to cancer and the postulated underlying genetic susceptibility. This limitation has been termed ‘reverse causation bias’. The reverse causation bias problem has been thoughtfully discussed in several reviews and editorials (5,8,9,11–13), with the suggested solution to conduct prospective or nested case-control studies with stored pre-diagnostic samples. A prospective study with assay determination on fresh (unfrozen) peripheral blood samples for a large cohort of subjects followed for cancer outcomes is prohibitively expensive because the assays are labor intensive. Nested studies using cryopreserved lymphocytes or blood may be promising (14,15) but laboratory cell culturing and other technical challenges of using thawed samples remain problematic (16). To our knowledge, one small mutagen sensitivity study followed cancer-free individuals with Barrett’s esophagus, finding a non-significantly 1.6-fold increased risk of esophageal carcinoma (17). Other supporting evidence that mutagen challenge assays measure inherent and tissue-specific cancer susceptibility include heritability and twin studies (reviewed in ref. 8), reports of similar findings of peripheral blood cells and target organ tissue (reviewed in refs 7,9–10), stability of the assay over time (reviewed in ref. 9) and in pre-and post-diagnosis samples (18) and case-only analyses for second tumors and recurrence risk (reviewed in refs 8,19). Despite this indirect evidence, prospectively designed studies are the only means to definitively determine whether DNA damage or mutagen challenge assays are an unbiased measure of underlying cancer predisposition.

We generated Epstein–Barr virus (EBV)-transformed lymphoblastoid cell lines (LCLs) from peripheral blood samples collected before diagnosis to analyze lung cancer risk using three separate assays that are considered to assess base excision (20), nucleotide excision (21) and double-strand break repair pathways (22), respectively: the alkaline Comet assay, the host cell reactivation (HCR) assay with the line Comet assay, the host cell reactivation (HCR) assay with the to assessed with the objective to measure the effect of periodic diagnostic screening on tumor and recurrence risk (reviewed in ref. 8). The development of peripheral blood assays for lung cancer has been thoughtfully discussed in several reviews and editorials (5,8,9,11–13), with the suggested solution to conduct prospective or nested case–control studies with stored pre-diagnostic samples. A prospective study with assay determination on fresh (unfrozen) peripheral blood samples for a large cohort of subjects followed for cancer outcomes is prohibitively expensive because the assays are labor intensive. Nested studies using cryopreserved lymphocytes or blood may be promising (14,15) but laboratory cell culturing and other technical challenges of using thawed samples remain problematic (16). To our knowledge, one small mutagen sensitivity study followed cancer-free individuals with Barrett’s esophagus, finding a non-significantly 1.6-fold increased risk of esophageal carcinoma (17). Other supporting evidence that mutagen challenge assays measure inherent and tissue-specific cancer susceptibility include heritability and twin studies (reviewed in ref. 8), reports of similar findings of peripheral blood cells and target organ tissue (reviewed in refs 7,9–10), stability of the assay over time (reviewed in ref. 9) and in pre-and post-diagnosis samples (18) and case-only analyses for second tumors and recurrence risk (reviewed in refs 8,19). Despite this indirect evidence, prospectively designed studies are the only means to definitively determine whether DNA damage or mutagen challenge assays are an unbiased measure of underlying cancer predisposition.

We generated Epstein–Barr virus (EBV)-transformed lymphoblastoid cell lines (LCLs) from peripheral blood samples collected before diagnosis to analyze lung cancer risk using three separate assays that are considered to assess base excision (20), nucleotide excision (21) and double-strand break repair pathways (22), respectively: the alkaline Comet assay, the host cell reactivation (HCR) assay with the activated mutagen benzo[a]pyrene diol epoxide (BPDE) and the bleomycin mutagen sensitivity assay. Cryopreserved whole blood samples have been collected from > 50 000 participants in the Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Screening Trial and was the population base for the 117 incident lung cancers and 117 controls without lung cancer studied here.

Materials and methods

Study population and blood collection

PLCO study design and biospecimen collection methods have been published previously (22–24). In brief, the PLCO study is a randomized screening trial with the objective to measure the effect of periodic diagnostic screening on PLCO cancer incidence and mortality. The subjects in the trial are 154 938 men and women who were aged 55–74 years and were free of the studied cancers at time of entry into the study. In one arm, individuals were followed as they underwent usual care, whereas the other arm had additional screening tests for the cancers of interest as well as usual care. Blood samples were collected from subjects in the screening arm at prescribed intervals over the course of the trial including cryopreserved whole blood samples used in the present study. Maintenance of lymphocyte viability and successful EBV transformation, up to several years after collection, has been previously reported (23).

Cancer case and control selection

Cases were individuals with lung cancer diagnosed between 3 months and 6 years after whole blood collection and were not restricted by lung cancer.
histology. Controls without lung cancer were frequency matched to cases by gender, age at blood collection, calendar year of blood collection and smoking history (never, quit 10+ years ago and cigarettes/day ≤ 1 pack, quit 10+ years ago and cigarettes/day ≥ 1 pack, current smoker or quit <10 years ago and cigarettes/day ≤ 1 pack or current smoker or quit <10 years ago and cigarettes/day ≥ 1 pack). At the time of selection in mid-2005, 110 controls were cancer-free; 2 were diagnosed with colorectal cancer and 5 had other cancers. All participants gave informed consent. This study has been approved annually by the human subjects review boards of the National Cancer Institute and the individual institutions contributing to the PLCO trial. Studies conducted at Lawrence Livermore National Laboratory and The University of Texas M. D. Anderson Cancer Center were approved by their respective Institutional Review Boards.

Samples
The samples were collected between 1998 and 2002, with 63% obtained during 1998–99 and 90% during 1998–2000. The whole blood samples were sent to American Type Culture Collection (Manassass, VA) in September 2005. An LCL was prepared by EBV transformation of peripheral blood lymphocytes obtained from each subject. All stored samples were successfully transformed by July 2006 and each cell line was cryopreserved. Study samples were shipped in dry ice shippers to the study laboratories and tracked by a unique ID code. Laboratory investigators had no knowledge of case or control status, age, gender, ethnicity, smoking history or descriptive information for many of the samples. Each cell line sample was thawed and cultured in RPMI 1640 supplemented with 15% serum (Fetal Clone III, HyClone, Logan, Utah) and 2 mM glutamine prior to analysis. The period of culture prior to analysis varied among cell lines, from a few days to weeks, depending on the growth rate of the cell line, the proportion of viable cells measured by trypan blue dye exclusion (≥100 cells total were scored using a hemocytometer to obtain reported % dye-excluding, viable cells) and general timing for when experiments were performed. In general, ~73% of the cell lines grew and were evaluated within 2 weeks. For quality control assessment, four replicate samples of two individuals and duplicates from eight individuals were included in each shipment. Laboratory personnel were blinded to the identity of these replicate samples.

Measurement of DNA damage

Comet assay
The alkaline single-cell gel electrophoresis (Comet) assay quantitatively measures the amount of DNA single-strand breaks in individual cells. The assay reflects endogenous DNA damage and therefore high values are indicative of high repair capacity of LCLs is assumed to reflect the repair capacity of the donor in a cell is dependent on the ability of the host cell to repair the damage. The Comet assay was conceived and developed by T.C.Hsu in the early 1980s (1,2). The Comet assay reflects endogenous DNA damage and therefore high values are hypothesized to indicate increased cancer susceptibility.

Bleomycin mutagen sensitivity assay
The bleomycin mutagen sensitivity assay was conceived and developed by T.C.Hsu in the early 1980s (1,2). The assay was designed to identify and measure indicators of genetic susceptibility based on quantifying the extent of chromosome breakage induced by the radiomimetic agent, bleomycin. Cultured LCL cells from subjects were treated with bleomycin (final concentration, 0.03 U/ml) (Blenoxane: Nippon Kayaku Co., Ltd, Tokyo, Japan). At 71 h, 0.04 µg/ml colcemid was added to induce mitotic arrest. At 72 h, the cells were harvested using conventional procedures. The cells were then treated with hypotonic 0.07 M KCI for 12 min, fixed, washed with freshly prepared Carnoy’s mixture (3:1 [v/v] methanol and acetic acid), air-dried and stained with Giemsa solution. A minimum of 50 well-spaced metaphases per sample were examined in each sample to determine the number of chromatin breaks (29). Gaps and attenuated regions were disregarded. Mutagen sensitivity was expressed as the average number of breaks per cell (breaks/cell). Higher values of breaks/cell are hypothesized to indicate increased cancer susceptibility.

Statistical analysis
Several statistical approaches were used to assess the quality of the assay results. To assess the possibility of laboratory drift over time, indices of central tendency, individual assay results and cell viability over various dates (thaw date, culture date, harvest date, electrophoresis date and experiment date) and batch number stratified by case and control status were plotted (scatter and box-and-whisker) and visually inspected. Time in days (weeks) between thawing cells and the harvesting of cells from culture or performing the assay was also evaluated. Although there was a high degree of heterogeneity in the assay measures from date to date, no clear trend was seen over time that would indicate problematic drift. Coefficients of variation (CVs) were calculated for the eight duplicate and the two sets of four replicate quality control samples according to Falk et al. (30) for which CVs of ≤15% are considered acceptable. Variation by age at blood collection, time since blood collection, gender, race, smoking status and the other host characteristics were also assessed in the aggregate and by case–control status.

We evaluated the geometric mean of tail length, tail DNA, CDM and OTM of 100 randomly selected cells per subject as a summary measure to reduce the influence of outliers. No data transformations were used for HCR DRC or breaks/cell outcomes. Quantile–quantile plots were visually inspected and ‘Kolmogorov–Smirnov tests’ conducted to assess assumptions of normality.

The association between the assay measures and cancer risk was evaluated by estimating odds ratios (ORs) and 95% confidence intervals (CIs) based on unconditional logistic regression. All of the assay measures (Comet tail DNA, tail length, CDM and OTM; DRC and bleomycin-induced chromatin breaks/cell) were divided into four categories based on the quartiles of the respective distributions in the control group. Other data categorizations including quintiles, tertiles and dichotomization at the median yielded essentially similar patterns. All models were initially adjusted for the matching variables: age in three categories (55–64, 65–69, ≥70 years), gender and smoking habits. Of these, age was the only factor to have even a modest impact on the logistic regression point estimates. Other potential confounders including race, education, lung cancer in a first-degree relative, history of emphysema or laboratory variables such as time between cell thawing and assay did not significantly change the point estimates (>10%), so none of these factors, other than age, were included in the final model. Tests for trend were adjusted for the matching variables and done in two ways: based on the underlying continuous variable and using the quartile-based categorical measure as a score test. All significance tests were two sided and α was set at 0.05. The Statistical Package for the Social Sciences version 16.0 (SPSS, Chicago, IL) was used for all analyses.

Results
CVs for the eight duplicates and the two sets of four replicate quality control samples are shown in Table I. All the CVs were
and lung cancer risk was slightly stronger when cases diagnosed within 0.04). The association between the bleomycin mutagen sensitivity assay versus lowest quartile, OR greatest numbers of chromatid breaks in the bleomycin assay (highest observed in some previous case–control studies using this assay, and lung cancer association was, however, less than the generally preclinical stage. Rather than a state induced in the host by the presence of tumor even at bleomycin assay reflects some component of cancer predisposition, assays were unchanged, but the relationship between the bleomycin 1 year of blood collection. The null results for the Comet and HCR cancers identified within a year of study entry could have been occult challenge assay were associated with increased risk of lung cancer. No that increased chromatid breaks/cell in the bleomycin mutagen chal-

Discussion

Our study is the first to prospectively evaluate three widely used in our study, the laboratory variation (CV) was greater for the bleomycin approximately ≤15% except for the bleomycin assay with a CV of 22% for the two sets of four replicates.

Baseline and other characteristics for lung cancer cases and controls are presented in Table II. The case and control groups did not differ significantly in any of the matching or demographic variables, although cases tended to have a somewhat lower level of education than controls. Calendar time between blood collection and case diagnoses was fairly evenly distributed and 79.5% of cases occurred a year or more after blood donation. The means of all the individual assay measures by case and control status for the demographic variables, calendar time between blood draw and diagnosis, family history of lung cancer, family history of any cancer, history of emphysema and lung cancer histology did not significantly differ across categories except that among controls, Comet tail DNA tended to increase with age and the HCR assay DRC tended to decrease with increasing age (supplementary Table 1 is available at Carcinogenesis Online).

Lung cancer risks adjusted for age, gender and smoking history are shown in Table III. No statistically significant associations with lung cancer were found for the Comet or the DRC assays. However, statistically significantly increased lung cancer ORs for the bleomycin assay were observed for increasing quartiles of chromatid breaks/cell relative to the lowest quartile (OR = 1.2, 95% CI: 0.5–2.5; OR = 1.4, 95% CI: 0.7–3.1; OR = 2.1, 95% CI: 1.0–4.4, respectively, \( P_{\text{trend}} = 0.04 \)). The association between the bleomycin mutagen sensitivity assay and lung cancer risk was slightly stronger when cases diagnosed within a year of blood collection were excluded (\( P_{\text{trend}} = 0.02 \)) and there were no changes in the associations for the other assays when these cases were excluded (data not shown).

Table I. CVs for blinded quality controls samples included in shipments to each laboratory

<table>
<thead>
<tr>
<th>Assay name</th>
<th>Eight duplicates (%)</th>
<th>Two sets of four replicates (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>% DNA in Comet tail</td>
<td>8.0</td>
<td>11.8</td>
</tr>
<tr>
<td>Comet tail length</td>
<td>7.1</td>
<td>13.7</td>
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<tr>
<td>CDM</td>
<td>5.2</td>
<td>6.8</td>
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<tr>
<td>OTM</td>
<td>8.4</td>
<td>15.4</td>
</tr>
<tr>
<td>DRC</td>
<td>5.2</td>
<td>8.4</td>
</tr>
<tr>
<td>Bleomycin sensitivity</td>
<td>15.1</td>
<td>22.6</td>
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</table>

The alkaline Comet assay was performed on unchallenged cells and measured endogenous levels of DNA damage. The DRC measure used the HCR assay with the mutagen BPDE. The mutagen sensitivity assay used the mutagen bleomycin and measured the number of chromatid breaks per cell.

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Discussion

Our study is the first to prospectively evaluate three widely used mutagen sensitivity assays in relation to lung cancer risk. We showed that increased chromatid breaks/cell in the bleomycin mutagen challenge assay were associated with increased risk of lung cancer. No lung cancer associations were found for the four Comet assays or the HCR assay using BPDE as the test mutagen. Because some lung cancer associations were found for the four Comet assays or the DRC assay, we excluded cases diagnosed with lung cancer within 1 year of study entry. The null results for the Comet and HCR assays were unchanged, but the relationship between the bleomycin assay and lung cancer risk was slightly strengthened. We interpreted the strengthened association as supporting the contention that the bleomycin assay reflects some component of cancer predisposition, rather than a state induced in the host by the presence of tumor even at a preclinical stage.

In our study, lung cancer risks rose to ~2-fold for those with the greatest numbers of chromatid breaks in the bleomycin assay (highest versus lowest quartile, OR = 2.1). The magnitude of the bleomycin and lung cancer association was, however, less than the generally observed in some previous case–control studies using this assay, where risks up to 10-fold were reported (reviewed in refs 5–10). In our study, the laboratory variation (CV) was greater for the bleomycin assay than for the Comet and DRC assays that are more mechanized, relying less on reader interpretation. As reader variability introduces a level of error in bleomycin assay scoring, it is possible that the lung cancer risks observed in our study underestimate the true risks,
OTM

CDM

bleomycin mutagen sensitivity (breaks/cell)

Bleomycin mutagen sensitivity (breaks/cell)

OTM

CDM

Bleomycin mutagen sensitivity (breaks/cell)

Bleomycin mutagen sensitivity (breaks/cell)

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Prospective analysis of DNA damage and repair markers


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