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## Lung Cancer Chemoprevention with Celecoxib in Former Smokers

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### Abstract

Ample studies suggest that the cyclooxygenase-2 (COX-2)/prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) pathway plays a pivotal role in carcinogenesis and that COX-2 inhibition may help prevent lung cancer. Therefore, we conducted a randomized, double-blind, placebo-controlled trial of the COX-2 selective inhibitor celecoxib (400 mg bid for 6 months) in former-smokers (age ≥45, ≥30 pack-years of smoking, ≥1 year of sustained abstinence from smoking). We assessed the impact of celecoxib on cellular and molecular events associated with lung cancer pathogenesis; the primary endpoint was bronchial Ki-67 labeling index (Ki-67 LI). Of 137 randomized subjects, 101 completed both baseline and 6-month bronchoscopies and were evaluable for the primary endpoint analysis. The beneficial effect on Ki-67 LI was greater in the celecoxib arm (versus placebo) in a mixed-effects analysis ( $P = 0.0006$ ), and celecoxib significantly decreased Ki-67 LI by an average of 34%, whereas placebo increased Ki-67 LI by an average of 3.8% ( $P = 0.04$ ; t-test). Participants crossed over to the other study arm at 6 months. Therefore, at 12 months all remaining participants had received 6 months of celecoxib, and their decreases in Ki-67 LI correlated with a reduction and/or resolution of lung nodules on computed tomography. Celecoxib significantly reduced plasma c-reactive protein and interleukin-6 mRNA and protein and increased 15(S)-hydroxy-eicosatetraenoic acid levels in BAL samples. The baseline ratio of COX-2 to 15-hydroxyprostaglandin dehydrogenase mRNA in bronchoalveolar lavage (BAL) cells was a significant predictive marker of Ki-67 response to celecoxib ( $P = 0.002$ ). Our collective findings support the continued investigation of celecoxib for lung cancer chemoprevention in former smokers at a low risk of cardiovascular disease.

### Keywords

COX-2; 15-PGDH; PGE<sub>2</sub>; CRP; IL-6; 15-HETE

## Introduction

Lung cancer remains the major cause of cancer-related death in the world despite advances in lung-cancer therapy (1, 2). Smoking prevention and cessation are an essential component of the overall strategy for lung cancer prevention but have certain limitations. Smoking cessation does not eliminate the significant risk of former smokers for lung cancer, although this risk of course decreases over time with continued abstinence (3, 4). Therefore, chemoprevention is a highly sought-after approach to complement therapy and smoking prevention/cessation.

In addition to modulation of histopathology, markers causally linked to lung carcinogenesis, including Ki-67, have been studied as surrogate endpoint biomarkers (SEBs) in many chemoprevention trials. Ki-67 is a proliferation marker expressed in all phases of the cell cycle except in resting cells (5). Elevated Ki-67 labeling index (LI) has been reported to be an unfavorable prognostic factor in NSCLC (6). Abnormal epithelial proliferation is a hallmark of tumorigenesis and increased Ki-67 expression is seen in bronchial biopsies where premalignant changes are lacking (7), and so Ki-67 LI may reflect potential efficacy of antiproliferative chemopreventive agents such as celecoxib.

Ample preclinical data suggest that the cyclooxygenase-2 (COX-2)/prostaglandin E2 (PGE<sub>2</sub>) signaling pathway plays a pivotal role in the development of malignant cells (8). Due primarily to the action of COXs on the free arachidonic acid (AA) liberated from membrane phospholipids, overproduction of PGE<sub>2</sub>, which is predominantly generated by up-regulation of COX-2, is associated with a variety of carcinogenic mechanisms (9- 12). COX-2 expression has also been shown to be a poor prognostic indicator in non-small-cell lung cancer (NSCLC; ref. 13). Inhibition of COX-2 and thus of PGE<sub>2</sub> synthesis suppresses lung tumorigenesis in animal models (14, 15). Supporting the carcinogenic consequences of the targets COX-2 and PGE<sub>2</sub> and the anti-neoplastic effect of COX-2 inhibition in the lung, these data provide the rationale for examining the potential of the COX-2 selective inhibitor celecoxib for preventing bronchogenic carcinoma.

We previously reported our findings in a phase IIa, single-arm study of celecoxib as a chemopreventive agent for lung cancer in active smokers (16-18). Six months of celecoxib treatment significantly reduced the Ki-67 labeling index (Ki-67 LI) on bronchial epithelial biopsies serially obtained from 20 heavy active smokers (18). Celecoxib also inhibited the production of PGE<sub>2</sub> and IL-10 in the lung microenvironment of this cohort (16). Active smokers have fared poorly, however, in large-scale chemoprevention trials (most notably of combinations involving beta-carotene and/or retinoids), whereas former smokers have had neutral results or favorable trends in these trials (19, 20). Furthermore, former smokers with persistent histopathology or molecular (such as Ki-67) changes may be a higher-risk group than even active smokers since these changes frequently resolve with smoking cessation. Therefore, our encouraging phase IIa findings led us to design and conduct the current phase IIb randomized, double-blind, placebo-controlled trial in former smokers, with a primary endpoint of Ki-67 LI and secondary endpoints of other molecular markers, histology, and computed tomography (CT).

## Methods

The study was approved by the UCLA Institutional Review Board and conducted in full conformance with the FDA standards for human research as specified in 21 CFR 312. ClinicalTrials.gov identifier NCT00055978.

## Subject recruitment and screening

Participants were recruited through a combination of advertising strategies and serially screened by phone interviews, on-site evaluations, routine blood tests, serum cotinine, low-dose chest CT scans, and bronchoscopy as previously detailed (21). Qualifying subjects were former-smokers who had smoked at least 30 pack-years (pky, determined by multiplying the number of packs of cigarettes smoked per day by the number of years of smoking), quit smoking with abstinence for at least 1 year, and who had no evidence of major cardiovascular (CV), renal or hepatic abnormalities. They were required to have sufficient lung reserve to undergo bronchoscopy; be taking no contraindicated medications (e.g., coumadin, nonsteroidal anti-inflammatory drugs, or systemic corticosteroids); have no contraindications to celecoxib (e.g., pregnancy, peptic ulcer disease, sulfa allergy, prior CV events such as heart attack or stroke); and have had no cancer other than stage I NSCLC that had been treated with curative resection or non-melanomatous skin cancers post curative resections within the past 5 years. Persons who passed chest CT imaging without contraindicated findings were invited to undergo baseline bronchoscopy with both a white light and fluorescence examination followed by bronchoalveolar lavage and bronchial biopsies at predetermined sites (see below). Only participants who successfully completed bronchoscopy without evidence of cancer or other important findings were eligible for randomization. A complete list of inclusion and exclusion criteria and study schema is as previously described (21).

## Randomization and study protocol

According to the initial study design, eligible subjects were randomized in a 1:1 ratio to receive either six months of celecoxib (400 mg by mouth twice daily) or placebo followed by a crossover to the alternate therapy for a second 6-month period. Randomization was stratified by the presence of preneoplasia (defined as mild dysplasia or worse) on baseline bronchoscopy or prior stage I NSCLC. The study pharmacist was the only person not blinded. In person follow-up was scheduled every 3 months, which included history and physical examination, routine blood tests and serum cotinine. Follow-up bronchoscopies were performed twice, the first after 6 months, just before crossing over, and the second at one year. A follow up low dose CT was obtained after 12 months of treatment, prior to the 3<sup>rd</sup> bronchoscopy. Participants were also evaluated monthly, in-between 3-month visits, by phone or in-person (at their choice) to monitor their health status. Adverse events were monitored using the modified NCI common toxicity criteria scale and serial adverse reaction questionnaires.

## Protocol interruption and revision

On December 17, 2004, the study was voluntarily suspended following a report of unexpected and serious CV risk associated with the long-term use of celecoxib (22). After a 4-month hiatus, the study was reinstated with protocol amendments focused on minimizing CV risks. A Framingham 10-year-risk for coronary heart disease score of >10% was added as an exclusion criteria (web-based risk calculator: <http://hin.nhlbi.nih.gov/atpiii/calculator.asp?usertype=prof>) and those with findings of diffuse coronary calcifications on their low dose CT scan were also excluded. All subjects were re-consented with a new informed consent form detailing the newly-discovered CV risks associated with celecoxib. Due to this interruption, a significant number of subjects failed to return for and/or complete the crossover component of the study, which was ultimately eliminated from the protocol.

### Chest spiral CT scan

Low dose helical CT scans of the chest were performed on a 16-detector row scanner (Siemens Somatom Sensation 16; Siemens Healthcare; Forchheim, Germany). The chest was studied in a single volumetric sequence using 120 kVp, 50 mA, and contiguous image reconstruction of 3 mm slice thickness using the B30 and B50F reconstruction filters. All scans were interpreted by a single thoracic radiologist (DA). A positive CT result was defined in the presence of a noncalcified pulmonary nodule 4 mm or greater in diameter of solid, ground-glass, or part-solid attenuation. A nodule was considered benign if it contained benign calcification patterns. Follow up examinations were arranged in subjects with abnormal results. Lesions < 4 mm were reexamined at 12 months in keeping with the study protocol. Lesions  $\geq$  4 mm and < 10 mm were followed with repeat low dose helical CT scan in 6 months. After ensuring stability of the nodules, subjects who remained interested in the study were then continued with screening and randomized after passing all screening procedures. Abnormalities  $\geq$  10 mm were assessed on an individual basis for additional evaluation.

### Bronchoscopy and serial endobronchial biopsies and BAL

White light and fluorescence bronchoscopies (OncoLife, Novadaq Technologies, Inc.) were performed to rule out the presence of endobronchial cancer as previously described (23). Subjects were prepared with a combination of topical anesthesia and conscious sedation according to institutional guidelines. An Olympus BF20 fiberoptic bronchoscope (Olympus America Inc, center Valley, PA) was advanced into the airway and wedged into a subsegment of the right middle lobe. Four 60-ml aliquots of room temperature saline were serially lavaged and recovered by manual syringe suction. Recovered fluid was passed through a 100-micron sterile nylon filter (Becton Dickinson, San Jose, CA) to remove mucus and particulates, pooled, and centrifuged at  $300 \times g$  for 8 minutes at 4°C. The BAL fluid was then harvested, aliquoted and stored at -80°C until analyzed. Bronchial biopsies were obtained under fluorescence examination from areas with abnormal fluorescence, as well as at predetermined sites (main carina, carina between right upper lobe and bronchus intermedius, right middle lobe and right lower lobe, right lower lobe anterior and medial basal segment, lingua and upper division bronchus, left upper lobe and left lower lobe). Participants who were found to have bronchial dysplasia were informed of the finding and recommended to follow up with repeat bronchoscopy for surveillance at one year.

### Histological scoring

Bronchial biopsies were fixed in Bayley's fixative and processed for routine hematoxylin-eosin stain as previously described (18). All biopsies were classified and scored by a single lung pathologist (MCF) without knowledge of the treatment assignment or time-point. Scores were recorded for each biopsy based on the most severe finding present according to a 7-point WHO scoring criteria (1 = normal; 2 = reserve cell hyperplasia; 3 = squamous metaplasia; 4 = mild dysplasia; 5 = moderate metaplasia; 6 = severe metaplasia; 7 = carcinoma *in situ*).

### Quantitation of Ki-67 expression

Bronchial biopsy sections were stained for immunohistology with a primary Ki-67 antibody (1:100 dilution, DAKO, Corp, Carpinteria, CA) and a diaminobenzidine detection reaction as previously detailed (18). Up to five high-magnification fields were examined until 400 bronchial epithelial cells were counted. Ki-67 was recorded as the percentage of bronchial cells demonstrating nuclear staining in all layers. A semi-quantitative method was also used to evaluate the intensity of staining by consensus of two dedicated readers (MCF and LG) using a 0-3 scoring system (0 being below the level of detection; 3 being intense staining).

When the intensity of staining was heterogeneous, the percentage of bronchial epithelial cells staining at each intensity level was recorded. The final Ki-67 score for each biopsy was generated based on a composite score of the percent of positive cells and intensity of staining. Negative controls using non-immune sera demonstrated no staining.

### Plasma biomarkers

As a measure of compliance, plasma celecoxib levels were determined on 180 samples randomly selected from 106 subjects (placebo first: 55; celecoxib first: 51) at either baseline, month 3, month 9 or in combination (with matched plasma sets for each subject, see supplemental materials). Paired plasma c-reactive protein (CRP) levels were also measured from baseline and 6-month samples using a commercial enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instruction. (Alpha Diagnostic International, Inc. San Antonio, TX).

### BAL fluid biomarkers

Paired sets of BAL fluid collected from baseline and 6-month bronchoscopies were analyzed for pathway-related biomarkers. For IL-6 analysis, BAL fluid samples were concentrated using Amicon Ultra-15 Centrifugal Filter Units with a Ultracel-3 membrane (Millipore, Billerica, MA). Total protein content was then determined using the Micro BCA protein assay Kit (Thermo Fisher Scientific Inc., Rockford, IL.). IL-6 protein levels were determined using an IL-6 enzyme immunoassay (EIA) kit according to the manufacturer's instruction (Cayman Chemical, Ann Arbor, MI) and reported as an IL-6/total protein ratio. 15(S)-hydroxy-eicosatetraenoic acid (15-HETE) concentrations were also measured using an EIA kit according to the manufacturer's protocol (Cayman Chemical, Ann Arbor, MI). To control for interprocedure variability associated with BAL, levels of albumin were also measured in BAL fluid using an albumin ELISA kit (Bethyl Laboratory, Montgomery, TX) and final 15-HETE concentrations reported as a 15-HETE/albumin ratio.

### BAL cell mRNA expression by real-time qPCR

Total RNA from freshly harvested BAL cell pellets was isolated using RNeasy miniprep kits according to the manufacturer's instructions (Qiagen, Valencia, CA.) and stored at  $-80^{\circ}\text{C}$ . Prior to performing the real-time polymerase chain reaction (qPCR), RNA concentrations were determined on a NanoDrop spectrophotometer and RNA quality on an Agilent 2100 BioAnalyzer with a RNA 6000 NanoChip. RNA samples were converted to first strand cDNA in a standard thermal cycler using a RT<sup>2</sup> First Strand Kit (SA Bioscience, Fredrick, MD). cDNA templates were mixed with RT<sup>2</sup> qPCR Master Mix (SA Bioscience) and aliquoted into 96 well plates containing pre-dispensed gene-specific primer sets (IL-6, COX-2, 15-PGDH and  $\beta$ -actin, SA Bioscience). Real time PCR was performed on the Biorad MyiQ cycler using a standard program per the manufacturer's instruction. The relative expression of genes was acquired and analyzed using the  $\Delta\Delta\text{C}_t$  method with SA Bioscience's web-based data analysis package.

### Statistical analyses

In order to reduce inter-assay and inter-subject variability, batch analyses were performed so that both baseline and follow-up samples from both treatment arms were assayed simultaneously. The only exception was for histopathology, where analysis was performed at the time of procurement in order to allow stratification during randomization. The impact of treatment assignment on the primary endpoint, modulation of the KI-67 LI, was analyzed using a mixed effects model focusing on the change in expression for each individual biopsy site. Potential baseline covariates (including age, gender and smoking history) were assessed individually and as interaction terms with treatment assignment. Additionally, a random



effects term was included to account for the multiple biopsies per subject. The aggregate mean change in Ki-67 LI for each subject from baseline to 6 months (the average change score for all paired biopsies for a given subject) was also determined. Differences in change scores between the two treatment arms were assessed by t-tests and ANOVA, with removal of the highest and lowest aggregate mean change score in each treatment group to compensate for outliers. Finally, we categorized subjects to either high or low baseline aggregate mean Ki-67 expression and compared their aggregate mean change in Ki-67 between treatment groups.

The following methods were utilized to analyze histopathology: changes in worst biopsy score (maximum score), dysplasia index (defined as the percentage of biopsies with a score of 4 (mild dysplasia) or worse, and the average of all biopsy scores. Changes in response to treatment were analyzed in two ways: 1) per subject, in which composite scores among biopsies were generated for each subject before and after treatment, by averaging the scores of all biopsies obtained from each subject at the same time point; 2) per biopsy, in which histology grading for each biopsy site was compared before and after treatment.

Because chest CT scans were obtained at baseline and at 1 year, all subjects with paired CT data had received celecoxib. As such, the analysis of chest CT scans was non-comparative. Changes in CT results before and after treatment were categorized as 1) unchanged, 2) nodules resolved or reduced in size, 3) nodules increased in size, and 4) development of new nodules. Categorized changes in nodules were correlated with changes in Ki-67 scoring, using a Fisher's exact test to evaluate the association between the changes in Ki-67 and the changes in nodules over time.

Descriptive statistics were used to evaluate patient characteristics and immunohistopathologic findings of the bronchial biopsy specimens using ANOVA and Pearson correlation. Patient characteristics include age, airflow obstruction, gender, pky, race, and family medical history of lung cancer

## Results

### Enrollment, baseline demographics and retention

We recruited former smokers from March 2003 until March 2008. The clinical phase of the study was closed on January 2009. A total of 4470 participants were prescreened over the telephone. Of this number, 323 (7.2%) were invited to on-site screening and 137 (3.1%) were randomized (68 to celecoxib, 69 to placebo), as previously described in detail (blinded recruitment data after accrual was completed; ref. 21). The unexpected, highly publicized data of others on the CV risk of celecoxib (22), reported for the first time at the mid-point of our study enrollment period, had a significant impact on recruitment and prevented our study from reaching the recruitment goal of 180 randomized participants (21). Participant demographics are summarized in Table 1). There were no significant differences in clinical characteristics between the two arms.

Seventy-four percent of participants (101/137) had evaluable and matched sets of bronchial biopsies for Ki-67 LI obtained at baseline and six months after assignment to a treatment arm. Thirty-five participants had treatment interruption, and 26 declined to continue on study because of concerns over increased CV risks with celecoxib. One participant was withdrawn from study because of probable gastrointestinal toxicity, and five were withdrawn by the investigators because of nonadherence. In addition, for technical reasons biopsy results were unevaluable for histopathology in 2 participants and for KI-67 LI in 4 participants. A complete accounting of subject recruitment, enrollment, and retention is summarized in Fig. 1.

## Adverse events

Forty-seven participants reported at least one adverse event (Supplementary Table S1); 31 participants reported grade 1 toxicity; 10 reported grade 2 toxicity; and 6 experienced grade 3 toxicity, with 4 of the grade 3 cases thought not to be study drug-related. All adverse events were reviewed in detail, and their relationship to study drug was adjudicated by the Executive Steering and Monitoring Committee. In general, 400 mg of celecoxib bid was well tolerated. One participant had a grade 1 and one had a grade 2 blood-pressure increase. The following serious adverse events were reported during follow-up of up to 3.5 years: 1 case of atypical chest pain and transient shortness of breath that required an emergency room visit; 4 myocardial events, 2 of which (including 1 on placebo) were reported during treatment phases and 2 of which were reported subsequent to treatment and during follow-up. Both latter cases were adjudicated as non-study-related. All cardiac events occurred in subjects with significant, baseline CV risk factors who were enrolled prior to amendment of the entry criteria for CV risk. No cardiac events were reported after protocol amendment. One subject developed black tarry stool associated with peptic ulcer disease during which time he had excessive alcohol consumption and was withdrawn from the study.

## Effect of celecoxib on Ki-67 expression and bronchial histopathology

All of the Ki-67 and bronchial histopathology results in this section involve the first six months of trial, as do the results in the following sections involving biomarkers in plasma and in BAL fluid and cells. Of the 101 subjects with evaluable Ki-67 LI, 52 (51%) received placebo and 49 (49%) received celecoxib. A strong and positive treatment effect was observed in favor of assignment to the celecoxib arm using a mixed effects model ( $P = 0.0006$ , Table 2). This analysis compared the change in Ki-67 expression in 245 biopsy pairs obtained from the placebo arm with 215 biopsy pairs obtained from the celecoxib arm. Baseline expression of Ki-67 and treatment assignment, were identified in the mixed effects model as significant correlates of the Ki-67 outcome ( $p < 0.0001$  and  $p = 0.0006$ , respectively, Table 2). In other words, the higher the baseline Ki-67 LI, the more likely a treatment response was observed.

When analyzed on a per-subject basis, the aggregate mean in Ki-67 score showed that placebo had little overall impact on Ki-67 (3.8% increase from baseline) while 6 months of celecoxib reduced the Ki-67 LI on average by 34% ( $P = 0.04$ , fig 2A). The magnitude of this response to celecoxib was similar to the 35% reduction reported from our pilot study in active smokers (18). Little difference in the change in Ki-67 was noted between treatment groups for those subjects with low baseline expression of Ki-67 (defined as  $< 2$ ). However, as the baseline expression of Ki-67 increased, increasingly larger differences between treatment groups were seen and the slopes of these relationships were significantly different between the two treatment arms ( $P = 0.002$ , fig 2B).

Of the 103 subjects with evaluable histopathology scores pre and post 6 months of treatment, 53 subjects received placebo (274 biopsy sites), 50 subjects received celecoxib (248 biopsy sites). The observed changes in histopathology scores were not statistically significant for either treatment arm. Whereas the change in maximum histopathology score was more notable in the celecoxib treatment arm, it was not statistically different from the change over time observed in the placebo arm (fig 3A).

## Plasma biomarkers

We evaluated the effects of celecoxib treatment on plasma CRP levels obtained at the 6-month time point from randomly selected subjects in the placebo ( $n = 14$ ) and celecoxib ( $n = 14$ ) groups. Assignment to the placebo arm was associated with a significantly higher plasma CRP level at month 6 ( $p < 0.001$ , fig 3B).

### Biomarkers in BAL fluid and cells

Cells obtained from BAL are predominantly comprised of alveolar macrophages, which are the major effector cells in the lung. BAL cells provide a means to assess therapeutic effects of systemically administered drugs on the lung microenvironment. We found a significant reduction in IL-6 gene expression in the BAL cells from the celecoxib group compared with placebo ( $p < 0.05$ , fig. 3C). IL-6 protein in BAL fluid were also significantly decreased in response to celecoxib treatment ( $P < 0.05$ , fig. 3D).

We evaluated COX-2 and 15-PGDH gene expressions in pretreatment BAL cells from a subset of Ki-67 responders ( $n = 9$ ) and non-responders ( $n = 9$ ), with response defined as significant reduction of bronchial epithelial Ki-67 by celecoxib. The responders had a significantly higher (an average of 2.9 fold) COX-2 to 15-PGDH mRNA ratio (COX-2/15-PGDH) than that of the non-responders ( $p = 0.002$ , fig. 3E).

Six months of celecoxib treatment also significantly increased 15-HETE levels in BAL fluid ( $p < 0.05$ , fig. 3F,  $n = 14$  in each group).

### Lung nodules detected on chest CT

A total of 76 subjects had chest CT scans at baseline and at 12 months. Due to the original crossover design, all subjects who had completed 12 months of the study had received both placebo and celecoxib by the time of follow up CT scan. Forty-seven of these subjects (62%) had at least 1 noncalcified nodule detected at baseline. Twelve of these subjects (25%) had a reduction or resolution of nodules, 34 with stable nodules (72%) and 1 developed a new nodule (1.3%). The subject with a new nodule was noncompliant and reported never taking his celecoxib medications. When the categorical changes in nodules (reduced/resolved vs. unchanged) were correlated with categorical changes in Ki-67 score (decreased vs. unchanged or increased) using Fisher's exact test, there was a strong association between the decrease in Ki-67 and the reduction/resolution in the nodules over time ( $p = 0.008$ ).

### Discussion

This phase IIb study demonstrates that six months of celecoxib significantly reduced Ki-67 LI and favorably modulated a variety of secondary endpoints including plasma CRP, IL-6 expression (mRNA in BAL cells and protein levels in BAL fluid), and 15-HETE levels in BAL fluid in former smokers. It also showed that the baseline COX-2/15-PGDH ratio in BAL cells predicted Ki-67 LI response to celecoxib and that Ki-67 response was associated with the CT-measured response of lung nodules. Our collective findings provide further evidence that celecoxib is biologically active in the bronchial epithelium and capable of altering SEBs in addition to Ki-67 that are hypothesized to reflect the driving force of carcinogenesis in the lung, especially the microenvironment. They also have important implications for lung cancer prevention in former smokers, who remain at a significant risk of lung cancer for some time after quitting smoking (3, 4).

Key molecular and biochemical events occur before altered cellular morphology is apparent (24-26). Emerging data suggest that bronchial histologic response may not be sufficient to determine the efficacy chemoprevention agents (27). A central issue pertains to sampling with serial bronchial biopsies of the same site that may completely remove the premalignant lesions at baseline bronchoscopy (28). It is conceivable that complete lesion removal at baseline would lead to replacement with relatively normal tissue as the biopsy-site wound heals. Because premalignancy is expected to develop over a long period, it is highly probable that histopathology from serial biopsies of the same site at zero (complete lesion removal) and six months may not accurately reflect the driving force of carcinogenesis in



the lung. This hypothesis is consistent with our finding that the maximum histopathology score in the placebo group also decreased at six months. In view of all the caveats associated with repeated sampling of the bronchial epithelium, it is likely that only resolutions of advanced bronchial premalignancy will provide sufficient evidence to support continued development of a preventive agent. A further limitation of bronchial histopathology is that bronchial premalignancy is a precursor of squamous cell carcinoma, which is no longer the most common subtype of NSCLC. Therefore, the utility of bronchial histopathology as the primary SEB for lung cancer chemoprevention trials recently has been challenged.

As a hallmark of tumorigenesis and because increased in bronchial biopsies lacking histopathologic changes (7), the SEB Ki-67 LI in bronchial tissues may circumvent, to some extent, the potential problems associated with mechanical lesion removal when assessing chemopreventive agents such as celecoxib with antiproliferative properties. The notable, yet less dramatic (than in the celecoxib arm), decreases in Ki-67 over six months in the placebo arm with high baseline Ki-67 expression reinforce concerns over baseline lesion removal and subsequent sampling problems (Fig. 2B). Although these findings suggest that Ki-67 LI is subject to the same sampling issues as histopathology, they also suggest that it may be more sensitive and better than histopathology for indicating the effects of celecoxib on the overall driving force of carcinogenesis in the lung, perhaps because of the reduced time required to detect a significant change in Ki-67 compared with histopathology.

NSCLC can arise centrally in the proximal bronchial epithelium (predominantly squamous cell carcinoma) or peripherally in the distal bronchoalveolar respiratory epithelium (predominantly adenocarcinoma). Most published randomized, double-blind, placebo-controlled phase IIb lung cancer chemoprevention trials focused on evaluating SEBs in the central bronchi, with the notable exception of a phase IIb study of budesonide, which included chest CT scans as a secondary endpoint. This study showed a small but statistically significant decrease in the proportion of CT-detected nodules in the budesonide group (28), although a subsequent randomized trial designed to confirm this finding did not do so (29). Our present study included 12-month CT results as a secondary endpoint and found an apparent association between celecoxib and CT-detected reduction/resolution of lung nodules. This association is strengthened by the strong association of decreased bronchial Ki-67 LI (the primary endpoint) with the CT results. Although CT scans are limited to diagnosing precursor lesions in the peripheral lungs, our findings nonetheless suggest that oral celecoxib is biologically active in the respiratory epithelium, both centrally and peripherally.

Our trial also addresses the imperative of personalized medicine, or identifying the molecular characteristics of an individual predicting a favorable response to treatment. Of course, not all participants in the celecoxib arm had a primary-endpoint (Ki-67 LI) response, nor did all celecoxib-arm participants with elevated pretreatment Ki-67. To determine whether or not the expressions of key enzymes involved in the production (COX-2) and downstream metabolism (15-PGDH) of PGE<sub>2</sub> in BAL cells may predict responsiveness of bronchial Ki-67 to celecoxib, we compared the COX-2/15-PGDH ratio in BAL cells of Ki-67 responders to that of non-responders in the celecoxib group. This ratio indicates the balance between levels of COX-2 and 15-PGDH, which determines the ultimate level of PGE<sub>2</sub>, a likely contributor to the driving force of carcinogenesis in the lung, especially the microenvironment; this a balance also has been suggested to play a role in the responsiveness of an individual to COX-2 inhibition (30).

We found that the COX-2/15PGDH ratio on average was 2.9-fold higher in responders than in non-responders. To our knowledge, this is the first time the expression of key enzymes for a molecular target in pretreatment samples has been shown to correlate with treatment

response in a lung cancer chemoprevention trial. The implications of this finding are far-reaching. In people with higher COX-2/15PGDH, the COX-2/PGE<sub>2</sub> pathway likely plays a more dominant role in lung carcinogenesis. Therefore, the profile of COX-2/15-PGDH may allow the selection of patients who are more likely to benefit from celecoxib, or a personalized approach likely to enhance the efficacy and success of chemoprevention.

The acute-phase reactant CRP is often used as a marker of inflammation. It is becoming increasingly clear that chronic pulmonary diseases characterized by aberrant, persistent inflammatory pathways are associated with a greater risk of lung cancer, as are elevated CRP levels (31, 32). Therefore, measuring CRP may be useful in the assessment of response to chemoprevention. We found that six months of celecoxib treatment significantly lowered plasma CRP levels (versus the placebo group). IL-6 induces the production of CRP (33), and we found a significant reduction of IL-6 gene expression (in BAL cells) and IL-6 protein levels (in BAL fluid) in the celecoxib compared with the placebo group. IL-6 is a multifunctional cytokine that induces angiogenesis and modulates cell proliferation, differentiation, and apoptosis (34-36). Furthermore, COX-2 has been reported to induce IL-6 expression in NSCLC, and COX-2 induction of IL-6 may contribute to preinvasive carcinogenesis (37). These data suggest that reduced IL-6 likely reflects the mechanisms underlying the reduction of CRP.

In addition to being converted to prostanoids by COX, arachidonic acid is converted to leukotrienes (LTs) by 5-lipoxygenase (5-LOX) and to 15-HETE by 15-LOX. Preclinical data suggest that LOX signaling pathways also may play significant roles in lung cancer tumorigenesis. We therefore profiled the effects of celecoxib treatment on LTB<sub>4</sub> (data not shown) and 15-HETE in BAL fluid. Consistent with previous results (17), LTB<sub>4</sub> in BAL fluid was very low, barely at the level of detection by EIA, in our population of former smokers, whereas 15-HETE in BAL fluid was quite high, which is consistent with 15-HETE being the most abundant eicosanoid in the lungs. Six months of celecoxib treatment in our present study significantly increased 15-HETE levels in BAL fluid. 15-HETE is known to have pro-apoptotic and anti-inflammatory properties. Preclinical data show that 15-HETE is a ligand for the peroxisome proliferator-activated receptor gamma (PPAR-γ) nuclear receptor in inducing apoptosis in A549 NSCLC cancer cells (38). Therefore, our 15-HETE finding is novel and favorable, and since we did not see a significant increase of 15-LOX expression in BAL cells (data not shown), it likely results from the shunting of arachidonic acid toward 15-LOX pathways in the setting of COX-2 inhibition.

A recently reported phase IIb trial of celecoxib by Kim et al. found reduced Ki-67 expression at a high dose (400 mg bid) but not at a lower dose (200 mg bid) in the bronchial epithelium of current and former smokers; this effect was more pronounced in the subgroup of former smokers (39). Findings from our trial not only confirm the antiproliferative effects of high-dose celecoxib on the bronchial epithelium, indicating the importance of appropriate dosing to achieve antineoplastic effects, but also provide additional evidence for simultaneous, favorable modulations of a number of molecular SEBs. Collectively, these findings reflect the efficacy of COX-2 inhibition on the driving force of carcinogenesis in the lung, especially the microenvironment.

Validated SEBs, probably in combinations, for early-phase trials that could amplify the progress of lung cancer chemoprevention are a messy problem beyond the scope of this discussion (40). Nevertheless, our collective biomarker data provide strong evidence for the potential efficacy of celecoxib for lung cancer prevention, particularly in former smokers. We have identified two potential predictive biomarkers for personalizing future lung-cancer chemoprevention trials of celecoxib. The mixed effects-model analysis clearly demonstrates that the higher the baseline Ki-67 LI, the more likely a treatment response. A higher

COX-2/15-PGDH ratio in BAL cells predicted a Ki-67 response. These data suggest that restricting inclusion to people with elevated baseline bronchial Ki-67 LI and BAL-cell COX-2/15-PGDH would be a more-focused, personalized direction for future lung cancer prevention with celecoxib. Both markers were associated with drug sensitivity and both likely signify a stronger driving force of carcinogenesis in the lung, including the microenvironment, and a higher lung cancer risk—Ki-67 because of its association with aberrant-cell proliferation, COX-2/15-PGDH because of its reflection of increased PGE<sub>2</sub>. We did not measure urine PGE<sub>2</sub> levels, and whether or not urine PGE<sub>2</sub> will be a suitable SEB for lung cancer chemoprevention is an important open question remaining to be elucidated. Although by no means conclusive, our findings on Ki-67 and other SEBs provide supporting evidence for the continued investigation of celecoxib for lung cancer chemoprevention in patients with appropriate profiles of low CV risk and provide new molecular clues toward the development of practical, personalized paradigms for lung cancer chemoprevention.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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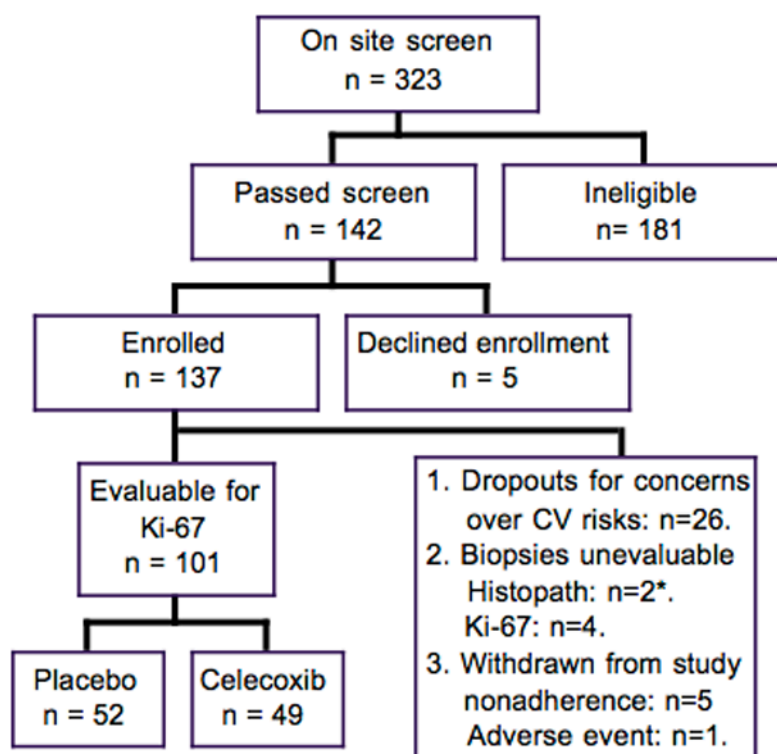
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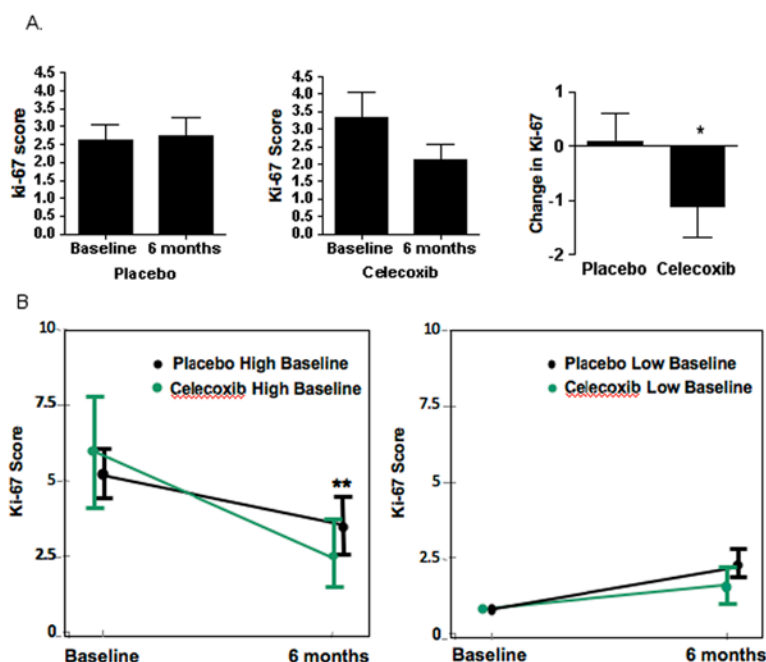
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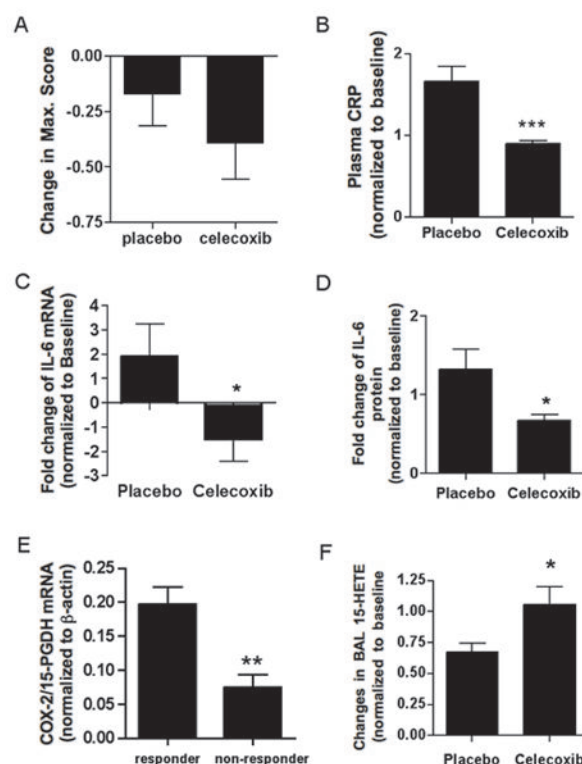
**Figure 1.**

CONSORT flow diagram of subject accrual into the trial. \*Of the 103 subjects with evaluable histopathology scores pre and post 6 months of treatment, 53 subjects received placebo and 50 subjects received celecoxib (data not shown).



**Figure 2.**

A). Six months of celecoxib treatment significantly reduced the aggregate mean Ki-67 LI by an average of 34%, whereas the placebo group had an average increase of 3.8 % (\*  $P$ , 0.04,  $t$  test). B). Aggregate mean Ki-67 LI between groups stratified by baseline expression. The high baseline group is comprised of those subjects with Ki-67 Li  $\geq 2$  ( $n = 19$  in the placebo arm, 24 in the celecoxib arm), The low baseline group is comprised of subjects with Ki-67 Li  $< 2$  ( $n = 33$  in the placebo arm, 25 in the celecoxib arm). Little difference in the change in Ki-67 was noted between treatment groups for those subjects with low baseline expression of Ki-67. However, as the baseline expression of Ki-67 increased, increasingly larger differences between treatment groups were seen and the slopes of these relationships were significantly different between the two treatment arms (\*\* $P = 0.002$ ,  $t$ -test).



**Figure 3.**

A). The maximum histopathology scores decreased in both placebo and celecoxib treatment groups with more notable (albeit not statistically significant) decreases in the treatment group. These findings are confounded by the sampling issue associated with biopsy (see text). B). CRP has been used as a marker of inflammation and may be useful in determining disease progression or the effectiveness of treatment. We evaluated the effects of celecoxib treatment on plasma CRP levels before and after 6 months of treatment from randomly selected subjects in the placebo ( $n = 14$ ) and celecoxib ( $n = 14$ ) groups. We found that the placebo group had significantly higher plasma CRP levels after months 6 than the celecoxib treated group compared to baseline (\*\* $p < 0.001$ ). C). The production of CRP is induced by IL-6. We found a significant reduction of IL-6 gene expression in the celecoxib group compared with placebo (\* $p < 0.05$ ). D). In keeping with the downregulation of IL-6 gene expression, IL-6 protein levels in BAL fluid were also significantly decreased in response to celecoxib treatment (\* $P < 0.05$ ). The decrease in IL-6 production likely explains the mechanism underlying the reduction of CRP. E). The balance of COX-2 and 15-PGDH has been suggested to play a role in the responsiveness of an individual to COX-2 inhibition. Comparisons of the COX-2/15-PGDH gene expressions in pretreatment BAL cells from a set of Ki-67 responders ( $n = 9$ ) vs. non-responders ( $n = 9$ ) show that the responders had a significantly higher COX-2/15-PGDH than that of the non-responders (\*\* $p = 0.002$ ). F). 15-HETE is the most abundant eicosanoid in the lungs. Six months of celecoxib treatment significantly increased 15-HETE levels in BAL fluid in comparison with placebo (\* $p < 0.05$ ,  $n = 14$  in each group). This is likely due to shunting of arachidonic acid precursor toward 15-LOX pathways in the setting of COX-2 inhibition.

**Table 1**

Background characteristics of randomized subjects.

Demographic Variables	Placebo (n = 68)	Celecoxib (n = 69)
<b>Age</b> (Mean±SD)	55.8 ± 7.6	55.7±15
<b>Ethnicity</b> n(%)		
Asian	3 (4.4%)	2 (2.9%)
Black	7 (10%)	5 (7.2%)
Caucasian	53 (78%)	60 (87%)
Hispanic	5 (7.3%)	2 (2.9%)
<b>Gender</b>		
Male	40 (59%)	38 (55%)
Female	28 (41%)	31 (45%)
<b>Pack-years</b> (Mean ± SD)	44.8 ± 15	42.5 ± 13.7
<b>FMH of Lung Cancer</b> n%	14 (21%)	20 (29%)
<b>FMH of Other Cancer</b> n%	34 (50%)	38 (55%)
<b>Emphysema on CT</b> * n% *	19 (28%)	13 (19%)
<b>Airflow obstruction</b> ‡ n%	14 (21%)	14 (20%)
<b>Prior stage I NSCLC</b>	3 (4.4%)	1(1.4%)
<b>Presence of bronchial dysplasia</b>	3 (4.4%)	3(4.3%)
<b>Presence of squamous metaplasia</b>	15 (22%)	22 (32%)

Abbreviations: FMH, Family medical history; NSCLC, non-small cell lung cancer

\* Presence of emphysema on chest CT scans.

‡ FEV1/FVC &lt;70.

**Table 2**

Mixed Effects Model for change in Ki-67

	Coefficient	Standard Error	p-value
Baseline Ki-67	-0.83	0.05	<0.0001
Treatment Group	0.09	0.55	0.88
Baseline Ki-67 by Treatment Group	0.24	0.07	0.0006