Higher Levels of the Anti-inflammatory Protein CC10 Are Associated with Improvement in Bronchial Dysplasia and Sputum Cytometric Assessment in Individuals at High Risk for Lung Cancer

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Abstract

Purpose: CC10, a 10-kDa anti-inflammatory protein secreted by bronchiolar Clara cells, is infrequently expressed in non–small cell lung cancer and its overexpression in non–small cell lung cancer cell lines results in a less malignant phenotype. Several lines of evidence have shown that bronchial dysplasia and sputum atypia are predictors of lung cancer. We investigated whether changes in CC10 expression correlate with regression of bronchial dysplasia and/or improvement in sputum abnormalities as measured by image cytometry.

Experimental Design: High-risk smokers enrolled in a chemoprevention trial underwent serial bronchoscopies with biopsies and bronchoalveolar lavage (BAL) collection, sputum assessment by image cytometry, and blood collection. CC10 was measured by competitive ELISA in BAL and plasma. Logistic regression analyses were done to determine the associations between CC10 levels and the improvement in bronchial dysplasia and sputum cytometric assessment.

Results: The net change in the BAL CC10 levels in subjects with improved bronchial lesions or improved sputum cytometry assessment was significantly higher than in those without improvement ($P < 0.05$). The odds ratio (95% confidence interval) associated with 1-unit increase in CC10 was 2.72 (1.31-5.64) for regression of dysplastic lesions and 2.94 (1.22-7.05) for improvement in sputum cytometry assessment after multivariate adjustment. Plasma CC10 was not significantly associated with either outcome.

Conclusions: Higher BAL CC10 levels are significantly correlated with regression of bronchial dysplasia and improvement in sputum cytometry assessment in smokers with high lung cancer risk. Whether CC10 levels can predict clinical outcomes among high-risk populations warrants further investigation.

Lung cancer is the leading cause of cancer death in both men and women in the United States and is a major public health problem worldwide (1, 2). Bronchial dysplasia and sputum atypia are considered precancerous lesions and are associated with increased risk for squamous carcinoma of the lung, suggesting that early intervention may prevent the development of invasive lung cancer (3–5). Interventions such as chemoprevention that use dietary or pharmaceutical agents to either reverse precancerous lesions or prevent the progression of precancerous lesions to invasive lung cancer hold great promise and could have a great effect in the reduction of lung cancer incidence and mortality (6–8). However, current early-phase clinical trials use invasive methodologies, such as serial bronchoscopies with biopsies, to sample the bronchial epithelium in high-risk yet healthy populations (7, 9). It is therefore important to develop methods that could non-invasively or semi-invasively determine the progression of precancerous lesions in the bronchial epithelium and evaluate the efficacy of chemopreventive agents.

The associations between chronic inflammation and lung cancer risk and progression have previously been shown in individuals with lung cancer as well as in individuals at high risk for lung cancer (3, 10–14). For example, higher levels of inflammatory markers, such as plasma C-reactive protein, are associated with progression of bronchial dysplasia in smokers (15). Given the importance of inflammation in lung carcinogenesis, in this study, we addressed whether alterations in the levels of an endogenous anti-inflammatory protein, CC10 (Clara cell 10-kDa protein), reflect the progression of precancerous lesions in the bronchial epithelium.

CC10 is a 10-kDa protein predominantly secreted by non-ciliated bronchiolar Clara cells, which are the progenitors for both normal and neoplastic lung epithelium and are involved in lung injury repair and xenobiotic metabolism (16, 17). Although

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its exact functions are poorly understood, CC10 seems to have a role in protecting the respiratory tract from oxidative stress and inflammation by inhibiting the expression and/or activity of proteins such as phospholipase A2, IFN-γ, and tumor necrosis factor-α (18). Measurable CC10 levels vary significantly when lung is exposed to a variety of insults, suggesting that it may be a serum biomarker for peripheral lung injury (19). CC10 levels are significantly lower in current smokers compared with healthy never or former smokers (19–22).

Several studies suggest that loss of CC10 may also be associated with lung carcinogenesis. CC10 is infrequently expressed in non–small cell lung cancer cell lines, and its overexpression antagonizes the growth of lung cancer cells *in vitro* by inhibiting anchorage-independent growth and invasion (23). CC10 knockout mice develop severe inflammation and subsequent pulmonary fibrosis at very low doses of bleomycin that do not cause fibrosis in wild-type littermates and also have significantly higher incidence of airway epithelial hyperplasia and lung adenomas after exposure to the tobacco-specific nitrosamine 4-(methylnitrosamino)-I-(3-pyridyl)-1-butanone compared with wild-type mice (24, 25).

Given the close association between CC10 and lung carcinogenesis and the ability to reliably measure CC10 in bronchoalveolar lavage (BAL) and plasma, in the current study, we investigated whether CC10 can be used as a noninvasive tool to classify and predict the progression of precancerous lesions in the bronchial epithelium. We show for the first time that CC10 levels in BAL are significantly associated with two different clinical outcomes associated with lung cancer risk, premalignant histologic changes in the bronchial epithelium and sputum cellular abnormalities reflected in abnormal DNA content, as measured by image cytometry.

Materials and Methods

Study population and specimen collection. The study population consisted of participants in a previously reported chemoprevention trial done at the British Columbia Cancer Agency that evaluated the efficacy and safety of inhaled budesonide in bronchial dysplasia regression (7). The participants were either current or former smokers aged 40 to 74 years with a smoking history of 30 or more pack-years (7). One hundred and one (98%) of the study participants (N = 103) were Caucasian. All participants had one or more sites of bronchial dysplasia (>1.2 mm in diameter) detected by autofluorescence bronchoscopy at enrollment. The study was approved by the Clinical Investigation Committee of the British Columbia Cancer Agency and the University of British Columbia.

Sputum, BAL, and blood samples were collected at both baseline and 6-month follow-up as described previously (7, 22). Briefly, participants underwent sputum collection with high-frequency chest wall oscillation with a ThAIRapy Vest (Advanced Respiratory, Inc.) combined with inhalation of 3% hypertonic saline for 12 min. Sputum samples that resulted from intermittent coughing during and 2 h after the process were collected, combined, and subjected to analyses.

Autofluorescence bronchoscopy was done using the LIFE-Lung device (Xillix Technologies Corp.) and BAL from the right upper lobe or left upper lobe was done using five 20-mL aliquots of normal saline. Fluid from the first wash was discarded, whereas the fluid from the subsequent 20-mL washes was collected until a total of 30 mL fluid was retrieved and immediately placed at 4°C. After differential cell counts, the fluid was centrifuged and the supernatant was stored at -83°C.

Measurement of CC10 levels in plasma and BAL. Quantitative CC10 levels at baseline and 6-month follow-up were measured in plasma and BAL using a competitive ELISA with a rabbit anti-human CC10 antibody (CC10 Sweden AB) as described previously (22, 26, 27). All samples were analyzed in duplicate. The sensitivity limit of the assay was 5 ng/mL. The coefficients of variation ranged from 10% to 20%. Duplicates that were not within a coefficient of variation of 20% were repeated.

Total protein concentration in BAL samples was measured by the bicinchoninic acid assay (Pierce). All determinations were made in duplicate. Duplicates that were not within a coefficient of variation of 20% were repeated. BAL CC10 measurements were normalized to the total protein concentration in BAL and were designated as BAL-P.

Outcome assessment. The outcomes of this study include the regression or progression of bronchial dysplasia and the improvement in sputum cytometry assessment after 6 months of follow-up compared with baseline, which were all dichotomized as "improved" and "nonimproved."

Bronchoscopies with biopsies, histologic review by two pathologists, and classification of the biopsies were described previously (7). All biopsy samples were classified into one of the seven classes according to the WHO criteria: normal, basal cell hyperplasia, metaplasia, mild/moderate/severe dysplasia, or carcinoma *in situ* (7, 28). Regression of individual lesions was defined as disappearance of dysplasia, whereas progression was defined as worsening by at least two histologic grades or development of new dysplastic lesions compared with the initial histologic classification done at enrollment (7). For this study, lesions that did not meet these criteria were considered as "stable." Individuals were considered as improved if all lesions regressed and none progressed [complete response (CR)] or if some but not all lesions regressed and none progressed [partial response (PR)]. Individuals were considered as nonimproved if one or more lesions progressed [progressive disease (PD)] or all lesions remained stable [no response (NR)].

High-resolution image cytometry (Cyto-Savant system, Perceptronix Medical, Inc.) measuring DNA content was done on at least 3,000 epithelial cells from each sputum sample at both the baseline and 6-month follow-up (7). A sputum sample was considered "abnormal" if at least five nondiploid cells with a DNA index >1.2 (compared with a DNA index of 1.0 in diploid cells) were identified in the sample (7). The threshold of using at least five nondiploid cells had a sensitivity of 94% for lung cancer detection at enrollment and 3.3-year mean follow-up and specificity of 38% at follow-up in a retrospective analysis of the Lung Health Study (7, 29). Individuals were classified as improved if the sputum assessment changed from abnormal at baseline to "normal" at the follow-up (CR) and as nonimproved if the sputum assessment changed from normal to abnormal (PD) or if the baseline abnormal assessment remained abnormal (NR).

Statistical analysis. In this study, 103 individuals were available for CC10 measurements. All 103 individuals had histologic assessment of bronchial dysplasia, whereas 99 individuals also had sputum cytometry results available.

Among the 103 participants with bronchial histology information, BAL CC10 levels were analyzed after normalization for the amount of protein in the BAL (BAL-P: ng/μg protein). Individuals were excluded from analysis of the association between CC10 BAL-P and dysplasia if they had very low baseline or follow-up BAL total protein levels (<0.1 μg/mL) that were considered to be measurement error (one for baseline and three for follow-up BAL CC10 levels) or if CC10 measurements were unavailable (one for baseline and three for follow-up). Altogether, 97 individuals had both the baseline and follow-up CC10 BAL-P measurements (101 had baseline and 97 had follow-up measurements, respectively) and were used for analysis. Additionally, 87 individuals had both the baseline and follow-up plasma CC10 measurements (93 had baseline and 90 had follow-up measurements) and were used in the analysis of the association between plasma CC10 and bronchial dysplasia.

Among the 99 participants with sputum cytometry measurements, 6 participants were excluded because their assessments were normal at

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Table 1. Baseline characteristics of the study population according to improvement in histologic response or sputum cytometry assessment

Characteristics	Regression of bronchial dysplasia*			Improvement in sputum cytometry assessment †		
	Nonimproved (n = 60)	Improved (n = 43)	P ‡	Nonimproved (n = 65)	Improved (n = 28)	P ‡
Age (y)						
Mean (±SD)§	56.7 (±7.9)	56.3 (±7.6)	0.761	56.2 (±7.1)	57.5 (±8.7)	0.464
Sex, n (%)			0.215			0.907
Female	13 (21.7)	14 (32.6)		17 (26.2)	7 (25.0)	
Male	47 (78.3)	29 (67.4)		48 (73.9)	21 (75.0)	
Pack-years of smoking						
Mean (±SD)§	53.1 (±18.2)	46.3 (±16.0)	0.052	50.7 (±17.9)	50.2 (±17.3)	0.904
Smoking status			0.170			0.714
Current	50 (83.3)	31 (72.1)		51 (78.5)	21 (75.0)	
Former	10 (16.7)	12 (27.9)		14 (21.5)	7 (25.0)	
Treatment effect			0.907			0.511
Budesonide	30 (50.0)	22 (51.2)		35 (53.9)	13 (46.4)	
Placebo	30 (50.0)	21 (48.8)		30 (46.2)	15 (53.6)	

*The outcome was dichotomized as improved versus nonimproved. Individuals with "CR" or "PR" were classified as improved, and individuals with "PD" or "NR" were classified as nonimproved.
†The outcome was dichotomized as improved versus nonimproved. Individuals with responses from "abnormal" to "normal" (CR) were classified as improved, and individuals with responses from abnormal to abnormal (NR) or normal to abnormal (PD) were classified as nonimproved.
‡The P value was calculated with the use of two-sample Student's t test with equal variances for continuous variables and the Pearson χ^2 test for categorical variables, respectively.
§Values were expressed as mean ± SD.

baseline and remained unchanged at 6-month follow-up. Eighty-eight individuals had both the baseline and follow-up CC10 BAL-P measurements (91 had baseline and 88 had follow-up CC10 measurements after excluding individuals with very low level of baseline or follow-up BAL total protein levels as described above) and were used for the analysis of the association between CC10 BAL-P and sputum cytometry assessment. Eighty-two individuals had both the baseline and follow-up plasma CC10 measurements (88 with baseline and 83 individuals with follow-up measurements) and were used for the analysis of the association between plasma CC10 and sputum cytometry assessment.

The dependent variables of interest were dichotomously classified (improved versus nonimproved) clinical outcomes, including regression or progression of bronchial dysplasia and improvement in sputum cytometry assessment. The independent variables were CC10 levels (BAL-P or plasma CC10 levels at baseline, follow-up, or the change of CC10 between follow-up and baseline), age, sex, smoking status, pack-years of smoking, and budesonide treatment effect.

The distribution of the CC10 concentrations was strongly skewed to the right and was therefore transformed using the natural logarithmic scale (lnCC10) to reduce the influence of the extreme values and to improve normality before comparisons and regression analysis were done.

Baseline characteristics among those stratified by outcome status or exposure (CC10) levels were compared by the use of either a two-sample Student's t test for continuous variables or a Pearson χ^2 test for categorical variables. The lnCC10 levels according to outcome status were compared using the two-sample Student's t test. In addition, the nonparametric Kruskal-Wallis test was used to determine the differences in the distribution of the original nontransformed CC10 values.

Unconditional logistic regression analyses were done to determine the association between CC10 and regression or progression of the bronchial dysplasia or improvement in sputum cytometry assessment calculated as the odds ratio (OR) and the corresponding 95% confidence interval (95% CI). CC10 concentrations were examined both as continuous variables (log transformed) as well as by fourths with quartile cut points according to the distribution of the logarithm (base e) of CC10 among the nonimproved population, with the lowest level as the reference. Tests for trend were conducted by entering the CC10 value as a single ordinal variable into the model and the coefficient was evaluated using the Wald test. Multivariate regression analyses were done adjusting for age (continuous), sex, cigarette smoking status (former versus current), pack-year of smoking, treatment effect, and baseline CC10 levels. Stratification analyses by smoking status were also done to determine whether the associations were consistent between current and former smokers.

All statistical analyses were done using Stata statistical software version 8.2 (Stata Corp.). All P values were two sided and the level of statistical significance was set at $P < 0.05$.

Results

Baseline characteristics of participants according to disease status. The present study was based on a cancer prevention trial that enrolled 112 participants for 6 months of treatment with budesonide, an inhaled corticosteroid, or placebo to assess the effects on the regression of bronchial dysplasia (7). Pretreatment and posttreatment samples of plasma, BAL, or both were available for CC10 measurement from 103 participants with histologic response measurements and 93 participants with sputum cytometry assessment (43 and 60 participants were classified as improved and nonimproved for regression of bronchial dysplasia, respectively, and 28 and 65 were classified as improved and nonimproved for improvement in sputum cytometry assessment, respectively). There were no statistically significant differences between individuals stratified by improvement in bronchial dysplasia or sputum cytometry assessment with respect to age, sex, smoking status, and treatment effect, except for a marginally significant difference in average pack-years of smoking between individuals with regression of

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bronchial dysplasia compared with individuals without regression (P = 0.052; Table 1). Individuals with improvement in bronchial dysplasia seemed to have fewer pack-years of smoking than those without improvement [46.3 (±16.0) versus 53.1 (±18.2); Table 1]. In addition, there were no significant differences in demographics (including age, sex, pack-years of smoking, smoking status, and treatment effect) between individuals stratified by the baseline, follow-up, or the changes in BAL-P CC10 levels in quartiles (P > 0.05; data not shown).

BAL and plasma CC10 levels by improvement in clinical outcomes. The arithmetic mean concentrations of BAL or plasma CC10 and the comparison of the lnCC10 concentrations between individuals with or without improvement in clinical outcomes are shown in Table 2. For regression of bronchial dysplasia, although there was no statistically significant differences between the two groups in the baseline normalized BAL-P CC10, individuals with improvement in bronchial dysplasia had ~60% higher follow-up BAL-P CC10 levels than those without improvement and the difference was statistically significant [mean (±SD): 60.7 (±58.4) ng/μg protein for the improved and 36.8 (±37.6) ng/μg protein for the nonimproved; P = 0.007]. Moreover, the CC10 change between the follow-up and baseline was also significantly different between the two groups [17.9 (±32.5) and 1.5 (±33.6) ng/μg protein for improved and nonimproved, respectively; P = 0.020].

When sputum cytometry assessment was analyzed as the outcome, the net change in BAL-P CC10 was also significantly higher in individuals with improvement than in those without improvement [22.0 (±27.2) ng/μg protein for the improved and 5.2 (±36.5) ng/μg protein for the nonimproved; P = 0.018]. However, no significant difference was detected between the two groups in baseline or follow-up BAL-P CC10 levels (Table 2).

The relationship between plasma CC10 levels and these outcomes was also analyzed. In contrast to the results observed with BAL-P CC10, the baseline, follow-up, and the change in plasma CC10 levels were not significantly different between individuals with or without improvement in bronchial dysplasia or sputum cytometry assessment (P > 0.05; Table 2).

The associations between CC10 levels and regression or progression of bronchial dysplastic lesions or improvement in sputum cytometry assessment. The magnitude of the associations between CC10 levels and clinical outcomes, including the regression or progression of bronchial dysplasia or the improvement in sputum cytometry assessment, was first evaluated by univariate regression analyses and then by multivariate regression models after taking into account potential confounders, calculated as the OR and 95% CIs. CC10 was included in the model(s) as a continuous variable. As shown in Table 3, the OR (95% CI) of improvement in bronchial dysplasia (CR or PR versus PD or NR) associated with

Table 2. BAL and plasma CC10 levels in individuals with or without improvement in clinical outcomes

Clinical outcome		Baseline			Follow-up			Changes*		
		Improved	Nonimproved	P †	Improved	Nonimproved	P †	Improved	Nonimproved	P †
Bronchial dysplasia ‡										
BAL-P (ng/μg protein)§	Mean (±SD)‖	46.4 (±67.5)	40.4 (±53.1)	0.511	60.7 (±58.4)	36.8 (±37.6)	0.007	17.9 (±32.5)	1.5 (±33.6)	0.020
	Median	30.6	22.7	0.602	48.3	26.1	0.007	14.7	-0.3	0.003
	Range	(4.1-391.0)	(2.9-351.0)		(5.1-313.0)	(4.1-190.0)		(-78.0 to 105.6)	(-137.3 to 112.2)	
Plasma (ng/mL)	Mean (±SD)	46.0 (±33.7)	40.3 (±37.0)	0.298	38.1 (±21.5)	35.7 (±36.4)	0.177	-8.6 (±19.3)	-2.6 (±15.5)	0.687
	Median	35.0	29.5	0.163	32.5	24.0	0.084	-5.0	-3.0	0.211
	Range	(8.0-151.0)	(5.0-175.0)		(8.0-89.0)	(5.0-201.0)		(-62.0 to 18.0)	(-69.0 to 30.0)	
Sputum assessment¶										
BAL-P (ng/μg protein)§	Mean (±SD)	34.8 (±39.1)	47.2 (±69.3)	0.664	51.0 (±34.6)	47.6 (±55.0)	0.184	22.0 (±27.2)	5.2 (±36.5)	0.018
	Median	21.8	26.1	0.520	37.6	27.5	0.066	17.6	2.3	0.004
	Range	(7.2-195.0)	(2.9-391.0)		(5.0-137.7)	(4.1-313.0)		(-33.8 to 105.6)	(-137.3 to 112.2)	
Plasma (ng/mL)	Mean (±SD)	46.6 (±32.3)	40.3 (±33.9)	0.364	40.4 (±24.2)	34.8 (±33.0)	0.123	-5.8 (±17.3)	-4.6 (±16.0)	0.450
	Median	42.0	29.0	0.206	37.0	23.5	0.082	-2.5	-5.0	0.871
	Range	(9.0-143.0)	(6.0-175)		(8.0-117.0)	(6.0-201.0)		(-48.0 to 18.0)	(-62.0 to 30.0)	

*The change was defined as the difference in CC10 levels between follow-up and baseline values when calculating the differences in the arithmetic scale. The P value was calculated with the two-sample Student's t test using natural log-transformed CC10 values. [The changes were defined as the value of log-transformed follow-up CC10 minus the value of log-transformed baseline CC10 ($\ln CC10_{follow\text{-}up} - \ln CC10_{baseline}$)].

† The P value was calculated with the use of two-sample Student's t test with equal variances using the natural log-transformed CC10 levels (lnCC10). The median comparison was done using nonparametric Kruskal-Wallis test.

‡ The outcome was dichotomized as improved versus nonimproved. Individuals with CR or PR were classified as improved, and individuals with PD or NR were classified as nonimproved.

§ Only participants with total BAL protein concentration ≥0.1 μg/mL were included in the study.

‖ Values were expressed as mean ± SD.

¶ The outcome was dichotomized as improved versus nonimproved. Individuals with response from abnormal to normal (CR) were classified as improved, and individuals with responses from abnormal to abnormal (NR) or normal to abnormal (PD) were classified as nonimproved.

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Table 3. Unadjusted and multivariate-adjusted associations between various clinical outcomes and BAL or plasma CC10 levels

Clinical outcome	BAL-P (ng/μg protein)		Plasma (ng/mL)	
	Follow-up	Change	Follow-up	Change
Complete or partial regression of bronchial dysplasia*				
OR (95% CI) †	1.97 (1.18-3.28) ‡	1.96 (1.09-3.50) ‡	1.50 (0.83-2.72)	0.81 (0.30-2.21)
OR (95% CI)§	2.68 (1.36-5.28) ‡	2.68 (1.36-5.28) ‡	1.04 (0.35-3.05)	1.04 (0.35-3.05)
OR (95% CI)‖	2.72 (1.31-5.64) ‡	2.72 (1.31-5.64) ‡	0.95 (0.30-3.05)	0.95 (0.30-3.05)
Progression of bronchial dysplasia¶				
OR (95% CI) †	0.60 (0.37-0.98) ‡	0.62 (0.35-1.08)**	0.80 (0.45-1.44)	1.14 (0.42-3.08)
OR (95% CI)§	0.50 (0.26-0.93) ‡	0.50 (0.26-0.93) ‡	1.00 (0.35-2.89)	1.00 (0.35-2.89)
OR (95% CI)‖	0.51 (0.26-1.04)**	0.51 (0.26-1.04)**	1.05 (0.33-3.33)	1.05 (0.33-3.33)
Sputum assessment ††				
OR (95% CI) †	1.45 (0.84-2.50)	2.26 (1.12-4.54) ‡	1.72 (0.86-3.46)	1.54 (0.51-4.67)
OR (95% CI)§	2.51 (1.13-5.55) ‡	2.51 (1.13-5.55) ‡	2.07 (0.60-7.15)	2.07 (0.60-7.15)
OR (95% CI)‖	2.94 (1.22-7.05) ‡	2.94 (1.22-7.05) ‡	1.47 (0.40-5.46)	1.47 (0.40-5.46)

*The outcome was dichotomized as improved versus nonimproved. Individuals with CR or PR responses were classified as improved, and individuals with PD or NR responses were classified as nonimproved.
† Unadjusted.
‡ $P < 0.05$.
§ Adjusted for baseline CC10 levels only.
‖ Adjusted for age, sex, pack-years of smoking, smoking status, treatment effect, and baseline CC10 levels.
¶ The outcome was dichotomized as progressed versus nonprogressed. Individuals with PD response were classified as progressed, and individuals with CR, PR, or NR responses were classified as nonprogressed.
** $P < 0.1$ (marginally significant).
†† The outcome was dichotomized as improved versus nonimproved. Individuals with response from abnormal to normal (CR) were classified as improved, and individuals with responses from abnormal to abnormal (NR) or normal to abnormal (PD) were classified as nonimproved.

1-unit increase on the natural log scale was 2.68 (1.36-5.28; $P < 0.01$) for either the follow-up or the change in BAL-P CC10 levels after adjustment for baseline CC10 levels. Further adjustment for age, sex, pack-years of smoking, smoking status, and treatment effect did not significantly alter the results [2.72 (1.31-5.64); $P < 0.01$]. Plasma CC10 levels were not significantly associated with the regression of bronchial dysplasia, with or without multivariate adjustment (Table 3).

Next, dose-response analyses were done according to the fourths of the distribution of CC10 in the nonimproved group. The ORs of improved dysplastic lesions seemed to be associated with increased net change in BAL-P CC10 in a linear pattern ($P_{trend} < 0.05$; Table 4). Compared with individuals in the lowest quartile, the OR (95% CI) for improved lesions was 5.8 (1.35-24.90) for individuals in the fourth quartile after adjustment for baseline BAL-P CC10 and 6.55 (1.34-31.91) after further adjustment for other covariates, including age, sex, pack-years of smoking, smoking status, and treatment effects (Table 4). A similar pattern was observed for follow-up BAL-P CC10 [multivariate-adjusted OR (95% CI): 6.14 (1.33-28.26); $P < 0.05$; Table 4]. No clear pattern of association was observed between the baseline BAL-P CC10 and the improved dysplastic lesions.

Given the significant associations between CC10 and regression of bronchial dysplasia, we investigated whether CC10 could be used as a tool to correctly classify or predict the complete or partial regression of dysplastic lesions. The sensitivity was 38.1% and the specificity was 75.9% using the fourth quartile of the net change in BAL-P as a cutoff, which corresponds to a geometric mean ratio of 1.9 (follow-up versus baseline BAL-P CC10 level). Similarly, using the fourth quartile of the follow-up BAL-P CC10 as a cutoff (38.9 ng/μg protein), the sensitivity and specificity were 54.8% and 76.4%, respectively (data not shown).

Because the relative clinical benefits of partial versus complete histologic regression on disease progression in the bronchial epithelium are not known, we did additional outcome analyses. When comparing "progressed" (PD) versus "nonprogressed" (including CR, PR, and NR), the OR (95% CI) was 0.50 (0.26-0.93; $P < 0.05$) for either the follow-up or the change in BAL-P CC10 levels after adjustment for baseline CC10 and 0.51 (0.26-1.04; $P = 0.064$) after multivariate adjustment (Table 3). Furthermore, the OR of complete regression (CR) of bronchial dysplasia versus other histologic responses (including PR, PD, and NR) was also calculated. The baseline CC10-adjusted and the multivariate-adjusted ORs (95% CI) for either the follow-up or the change in the BAL-P CC10 were 2.29 (1.13-4.63; $P < 0.05$) and 2.25 (1.04-4.86; $P < 0.05$), respectively (data not shown).

In addition to studying the associations between CC10 and bronchial histology, we also analyzed the magnitude of the association between CC10 levels and the improvement in sputum cytometry assessment (CR versus PD or NR) by logistic regression analyses. The numbers of individuals with responses classified as CR, PD, and NR at 6-month follow-up were 28, 16, and 49, respectively. The baseline CC10-adjusted OR (95% CI) of improvement in sputum cytometry assessment associated with 1-unit increase on the natural log scale was 2.51 (1.13-5.55; $P < 0.05$) for either the follow-up BAL-P or the change in BAL-P CC10 levels. Further adjustment for age, sex, pack-years of smoking, smoking status, and treatment effect did not significantly change the estimate [2.94 (1.22-7.05); $P < 0.05$; Table 3]. Plasma CC10 levels were not significantly associated with improvement in sputum cytometry assessment (Table 3). Dose-response analyses also revealed that higher follow-up or the net change in BAL-P CC10 levels seemed to be associated with improvement in sputum cytometry assessment in a linear

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fashion ($P_{trend} < 0.01$). Multivariate-adjusted ORs (95% CI) for individuals in the third or fourth quartile of the BAL-P CC10 levels were significantly higher than those in the lowest quartile [9.19 (1.39-60.84) and 14.52 (1.82-115.76), respectively; data not shown]. Furthermore, the multivariate-adjusted OR (95% CI) for improvement was 5.59 (1.05-29.92) for individuals in the fourth quartile compared with the lowest quartile of the net change of BAL-P CC10 levels (data not shown). Individuals with CR or who remained normal at 6-month follow-up were compared with individuals who had PD or NR. The multivariate-adjusted estimate was 2.32 (1.06-5.06; $P < 0.05$) for the follow-up or the net change in BAL-P CC10 (data not shown). The OR of progression of sputum cytometry assessment was not assessed in this study due to the limited numbers of subjects in the progression categories ($n = 16$).

Finally, we examined the association between CC10 and improvement in both bronchial histology and sputum cytometry assessment. There was a statistically significant correlation between improvement in dysplasia and improvement in sputum cytometry assessment ($P = 0.036$). The OR (95% CI) of improvement in both variables associated with 1-unit increase on the natural log scale was 4.03 (1.40-11.62; $P = 0.01$) for either the follow-up or the change in BAL-P CC10 levels after adjustment for baseline CC10 levels (data not shown). Further adjustment for age, sex, pack-years of smoking, smoking status, and treatment effect did not significantly alter the results [5.13 (1.58-16.64); $P < 0.01$; data not shown].

Discussion

The goal of the present study was to determine the association between CC10, an endogenous anti-inflammatory protein measured in blood and BAL fluid, and several clinical outcomes associated with bronchial premalignancy in individuals at high risk for lung cancer. Serial bronchoscopies with biopsies, as currently used in lung chemoprevention trials, are invasive and represent a barrier to accrual. To optimize chemoprevention trial efficiency and to monitor the progression of premalignant lesions in individuals at high risk for lung cancer, we asked whether CC10 can be used as a semi-invasive or noninvasive tool to assess the status of these lesions. BAL-P CC10 levels were found to be significantly associated with the regression and progression of bronchial dysplastic lesions as well as with improvement in sputum cytometry assessment. The ORs (95% CI) of regression of bronchial dysplasia, the progression of bronchial dysplasia, and the improvement in sputum cytometry assessment associated with 1-unit increase on the natural log scale of the changes in BAL-P CC10 levels were 2.72 (1.31-5.64), 0.51 (0.26-1.04), and 2.94 (1.22-7.05), respectively. Dose-response analyses also showed that the ORs of improvement in bronchial dysplasia for individuals in the fourth quartile of BAL-P CC10 levels (follow-up CC10 or net change in CC10) were approximately six times higher than those in the lowest quartile, adding to the consistent association between increases in CC10 levels and favorable clinical outcomes.

The reasons for the greater increase in BAL-P CC10 levels in individuals with improved lesions are not well understood. The increase is not due to the intervention in the trial because CC10 levels were not associated with budesonide treatment and budesonide was not shown to be effective in regressing bronchial dysplasia in this study (data not shown; ref. 7). Because dysplastic lesions represent an exceedingly small portion of bronchial surface area, it is highly unlikely that the

Table 4. Unadjusted and multivariate-adjusted associations between regression of bronchial dysplasia and BAL-P CC10 levels

CC10		Quartile				P_{trend}
		1st	2nd	3rd	4th	
Baseline BAL-P ($n = 101$)	lnCC10 (ng/μg protein)	<2.67	2.67-3.11	3.12-3.86	≥3.87	
	Improved (n)	11	6	20	6	
	Nonimproved (n)	14	15	15	14	
	OR (95% CI)*	1.0	0.51 (0.15-1.75)	1.70 (0.60-4.78)	0.55 (0.16-1.89)	
	OR (95% CI)†	1.0	0.41 (0.11-1.54)	1.91 (0.62-5.85)	0.53 (0.14-2.00)	0.994
Follow-up BAL-P ($n = 97$)	lnCC10 (ng/μg protein)	<2.65	2.65-3.25	3.26-3.65	≥3.66	
	Improved (n)	6	7	6	23	
	Nonimproved (n)	13	14	15	13	
	OR (95% CI)*	1.0	1.08 (0.29-4.08)	0.87 (0.22-3.35)	3.83 (1.17-12.51)‡	0.014
	OR (95% CI)§	1.0	1.08 (0.29-4.11)	1.03 (0.26-4.15)	5.61 (1.40-22.42)‡	0.014
	OR (95% CI)‖	1.0	1.09 (0.27-4.34)	1.13 (0.25-5.19)	6.14 (1.33-28.26)‡	0.015
Change in BAL-P ($n = 96$)	lnCC10 (ng/μg protein)	<-0.46	-0.46 to -0.03	-0.02 to 0.64	≥0.65	
	Improved (n)	4	6	16	16	
	Nonimproved (n)	12	15	14	13	
	OR (95% CI)*	1.0	1.20 (0.27-5.25)	3.43 (0.90-13.09)	3.69 (0.96-14.21)¶	0.018
	OR (95% CI)§	1.0	1.34 (0.30-5.99)	4.10 (1.04-16.17)‡	5.80 (1.35-24.90)‡	0.005
	OR (95% CI)‖	1.0	1.48 (0.31-7.09)	5.25 (1.21-22.73)‡	6.55 (1.34-31.91)‡	0.006

*Unadjusted.
†Adjusted for age, smoking status, sex, pack-years of smoking, and treatment effect.
‡$P < 0.05$, compared with the lowest quartile.
§Adjusted for baseline BAL CC10 levels (continuous).
‖Adjusted for baseline BAL CC10 levels (continuous), age, smoking status, sex, pack-years of smoking, and treatment effect.
¶$P = 0.057$, compared with the lowest quartile.

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increase in CC10 associated with the regression of dysplasia is directly due to replacement of abnormal (non-CC10 secreting) cells by normal (CC10 secreting) cells.

It is possible that transient smoking cessation in current smokers after enrollment in our study affected CC10 levels. Decreased CC10 levels have been documented in serum and BAL of current smokers compared with healthy nonsmokers by several investigators, and we have previously shown that CC10 levels increase in plasma and, to a lesser extent, in the BAL in long-term former smokers (19–22, 30–33). Transient smoking cessation has previously been shown to be associated with increased BAL CC10 levels at 3 to 9 months in a small clinical study with eight subjects (34). In fact, 16 of our 81 current smokers quit smoking after enrollment and sustained their "quit" status through the end of 6-month follow-up, whereas the remaining 65 current smokers increased, decreased, or maintained their level of smoking (data not shown). Although the numbers were small, current smokers who quit were found to be more likely to have improved lesions than "nonquitters" (relative risk, 2.9); ~81% of current smokers who quit smoking at baseline had improved lesions, whereas only 28% of nonquitters had improved lesions (data not shown). However, data about the timing after enrollment when current smokers quit or the extent of change in the amount of smoking were not available and thus we could not correct for these multiple variables that could potentially affect CC10 levels.

The mechanisms linking increased CC10 expression with smoking cessation are not well understood. CC10 expression is regulated by a variety of cytokines, such as IFN-γ and tumor necrosis factor-α (35, 36). Cigarette smoke exposure suppresses IFN-γ and tumor necrosis factor-α production by peripheral blood monocytes (37), providing a potential mechanistic link that deserves investigation. Conversely, CC10 inhibits the production and biological activity of IFN-γ *in vitro* and suppresses the T_H2 cytokine response and the levels of tumor necrosis factor-α, interleukin-8, and myeloperoxidase *in vivo* (38–40). It is thus tempting to hypothesize that the reduction in inflammation that accompanies smoking cessation may be related to increased CC10 levels and that the association between increased CC10 and improvement in bronchial premalignancy may be related to the suppression of inflammatory mediators by CC10. Further studies will be needed to explore this hypothesis.

Additionally, other health and lifestyle factors, which may or may not track with smoking cessation, may also potentially contribute to changes in CC10 levels during the 6-month follow-up. Age, gender, and renal function are all known to affect CC10 levels, although these variables did not change sufficiently in a 6-month period to account for the observed changes (30, 31, 33, 41).

Although BAL-P CC10 levels were associated significantly with clinical outcomes, plasma CC10 levels were not associated with either the regression of bronchial dysplasia or the improvement in sputum cytometry assessment. Stratification analyses by smoking status did not detect a significant relationship between baseline, follow-up, and net change in plasma CC10 levels and regression of bronchial dysplasia in current smokers (data not shown). This negative finding needs to be confirmed in other large studies. Nevertheless, it was not surprising that the CC10 levels in the fluid adjacent to the bronchial epithelium, in the BAL, might better reflect the local histologic changes in the lung environment than the remotely located blood CC10 levels. Furthermore, because blood CC10 levels are also affected by renal function and genitourinary sources of CC10, blood is less likely to accurately reflect the lung microenvironment (41).

Although BAL is not as invasive as bronchial biopsy, it is, nevertheless, too invasive to meet our goal of identifying a noninvasive biomarker set to measure outcomes in chemoprevention trials. The relative low sensitivity (38-55%) and specificity (~75%) of BAL-P CC10 as a classifier suggest that CC10 levels are not useful as a single clinical measure to classify or to predict clinical outcomes. It is likely that a panel of biomarkers that are involved in carcinogenesis, which may include CC10, will be needed to replace invasive bronchial biopsies for assessing outcomes in chemoprevention clinical trials.

Our study has several important strengths. It is the first report documenting the association between CC10 levels and various clinical outcomes associated with premalignant disease in a population at high risk for lung cancer. Although multiple epidemiologic studies have documented the associations between lung cancer and inflammation (10–14), only one recent study has addressed premalignant disease clinical outcomes by showing that higher blood levels of C-reactive protein, an acute-phase protein and a marker for inflammation, are associated with the progression of bronchial dysplasia in smokers at high risk for lung cancer (15). The relationship between endogenous proteins that are actually involved in the modulation of inflammation and clinical outcomes are only now beginning to be studied. Our report therefore strengthens the link between inflammation and premalignant disease by showing the association between CC10 levels and improvement in two different indicators of premalignancy (bronchial dysplasia and sputum cell DNA content as assessed by image cytometry). The consistency of the data between the different clinical outcomes measured in diverse clinical specimens, such as sputum and the bronchial epithelium, suggests that the associations are, indeed, real. Finally, an additional strength of our study is that it is the largest study with sequential BAL and plasma CC10 samples, showing a wide range of values in people at risk for lung cancer.

Certain limitations to the current study deserve consideration. First, there were limited numbers of participants in the study, especially former smokers. As such, the study had limited power and precision in determining the associations. For example, we were not able to detect a significant association between plasma CC10 levels and clinical outcomes, which may either be due to a true lack of association or to insufficient power to detect small associations. Although the associations between follow-up or change in BAL-P CC10 and complete or partial regression of bronchial dysplasia were significant in current smokers [multivariate-adjusted OR (95% CI): 2.93 (1.28-6.69); $P = 0.011$; data not shown], the association was not detected in former smokers, perhaps due to the small sample size ($n = 21$). Second, the study had relatively short follow-up time and therefore is not informative about long-term changes in the bronchial epithelium. Third, our findings may have limited generalizability to the general population. Due to the design of the chemoprevention trial, only current or former smokers with bronchial dysplasia were included in the trial. Smokers with bronchial dysplasia are generally thought to

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be at higher risk for subsequent lung cancer than smokers without dysplasia. Subsequent studies will be needed to determine if the results can be extrapolated to the general population without dysplasia or without abnormalities on sputum cytometry analysis. Finally, we were not able to control for other potential confounders, such as other concomitant pulmonary pathology.

In summary, the results from this study showed that higher CC10 levels are significantly associated with regression of bronchial dysplasia and improvement in sputum cytometry assessment in smokers at high risk for lung cancer. Whether CC10 levels, in combination with other inflammatory markers, can predict clinical outcomes and the response to chemopreventive agents in the bronchial epithelium warrants future investigations.

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