Modulation of Cystatin A Expression in Human Airway Epithelium Related to Genotype, Smoking, COPD, and Lung Cancer

Marcus W. Butler, Tomoya Fukui, Jacqueline Salit, Renat Shaykhiev, Jason G. Mezey, Neil R. Hackett, and Ronald G. Crystal

Abstract

The cathepsin inhibitor Cystatin A (CSTA) has antiapoptotic properties linked with neoplastic changes in squamous cell epithelium, where it has been proposed as a diagnostic and prognostic marker of lung cancer. Notably, cystatin A is upregulated in dysplastic epithelium, prompting us to hypothesize that it might be modulated in chronic obstructive pulmonary disease (COPD), a small airway epithelial (SAE) disorder that is a risk factor for non–small cell lung cancer (NSCLC) in a subset of smokers. Here we report that genetic variation, smoking, and COPD can all elevate levels of CSTA expression in lung small airway epithelia, with still further upregulation in squamous cell carcinoma (SCC), an NSCLC subtype. We examined SAE gene expression in 178 individuals, including healthy nonsmokers (n = 60), healthy smokers (n = 82), and COPD smokers (n = 36), with corresponding large airway epithelium (LAE) data included in a subset of subjects (n = 52). Blood DNA was genotyped by SNP microarray. Twelve SNPs upstream of the CSTA gene were found to associate with its expression in SAE. Levels were higher in COPD smokers than in healthy smokers, who, in turn, had higher levels than nonsmokers. CSTA gene expression in LAE was also smoking-responsive. Using publicly available NSCLC expression data we also found that CSTA was upregulated in SCC versus LAE and downregulated in adenocarcinoma versus smoke-exposed SAE. All phenotypes were associated with different proportional expression of CSTA to cathepsins. Our findings establish that genetic variability, smoking, and COPD all influence CSTA expression, as does SCC, supporting the concept that CSTA may make pivotal contributions to NSCLC pathogenesis in both early and late stages of disease development. Cancer Res; 71(7); 2572–81. ©2011 AACR.

Introduction

Cystatin A (CSTA; also referred to as stefin A, and acid cysteine proteinase inhibitor), a member of the class I or stefin subgroup of the 3 described cystatin families, is a 11 kDa single chain intracellular cysteine protease inhibitor capable of inhibiting papain and cathepsins B, H, and L, as well as the cysteine protease activity of the major house dust mite allergen Der p1 (1–3). CSTA has antiapoptotic properties (4–6) and cystatin A expression has been linked with neoplastic changes in squamous cell epithelium with cystatin levels paralleling the response to antitumor therapy (7–11). As with other members of the stefin family, cystatin A has been proposed as a marker and prognostic indicator of lung cancer (11, 12). In the lung, cystatin A expression is upregulated in dysplastic epithelium, and highly expressed in many lung cancers, especially squamous cell carcinoma (SCC; refs 7, 10, 12).

With this background, and based on the knowledge that the majority of lung cancers are derived from the small airway epithelium (SAE) of cigarette smokers (13–15), but that only a subset of smokers develop lung cancer (16, 17), we hypothesized that the expression of CSTA in the SAE is modulated by genetic variation and upregulated by smoking. Further, in the context that chronic obstructive pulmonary disease (COPD), a disease that starts in the small airways (13, 18–21), is a risk factor for lung cancer independent of smoking (22–26), we asked whether CSTA is upregulated in the SAE of smokers with COPD beyond that of smokers per se, and whether this upregulation is also dependent on genetic variation. In view of the fact that both smoking and COPD are stronger risk factors for SCC than for adenocarcinoma of the lung, and that most SCCs are derived from large airways (15, 27–30), we also examined CSTA expression in large airway epithelium (LAE) samples obtained in a subset of the study subjects, and compared our CSTA gene expression data to publicly available lung cancer microarray.
data derived using comparable methodologies (31), to see if 
*CSTA* is discordantly expressed in SCC and adenocarcinoma of 
the lung, relative to the SAE and LAE.

Materials and Methods

**Study subjects**

Healthy nonsmokers, healthy smokers, and smokers with 
COPD were assessed in the Weill Cornell National Institutes of 
Health Clinical and Translational Sciences Center and Depart-
ment of Genetic Medicine Clinical Research Facility using 
protocols approved by the Weill Cornell Medical College 
Institutional Review Board. Prior to inclusion in the study, 
each individual provided written informed consent. No 
abnormality was found in the nonsmokers (*n* = 60) or the 
healthy smokers (*n* = 82) following a standardized screening 
evaluation composed of a history, physical examination, 
complete blood count, serum chemistries, coagulation profile, liver 
function tests, urine studies, chest X-ray, EKG, and full lung 
function studies. The COPD smokers (*n* = 36) were diagnosed 
in accordance with standard GOLD criteria (32). Blood was 
collected for genotyping on a random subset of individuals 
(nonsmokers, *n* = 44; healthy smokers, *n* = 48; COPD smokers, 
*n* = 20). Fewer subjects with genotyped samples were available 
than subjects with SAE gene expression data because of a 
significantly longer temporal interest of our laboratory in the 
study of airway epithelium gene expression than in genomic 
studies. LAE samples were not given as high a priority as SAE 
samples when individuals were bronchoscopeed, reflecting the 
focus of our lab on SAE gene expression profiles, and as a consequence, LAE samples were available for a random subset of individuals who underwent bronchoscopy (healthy non-
smokers, *n* = 21; healthy smokers, *n* = 31). Urine nicotine and 
cotinine were measured, in conjunction with serum carbox-
ymphemoglobin, to verify the self-reported smoking status of 
each group. Detailed inclusion/exclusion criteria and char-
acteristics of the study populations can be found in the 
Supplementary Materials and Methods section.

**Collection of airway epithelium and assessment of gene 
expression**

Fiberoptic bronchoscopy was used to obtain small (and for a 
random subset, large) airway epithelial cells as previously 
described (33, 34). Further details of the airway epithelial 
sampling, preparation of cDNA, hybridization of labeled cRNA 
to Affymetrix HG-U133 Plus 2.0 arrays and confirmation by 
TaqMan (Applied Biosystems) real-time RT-PCR (reverse 
transcriptase PCR) can be found in the Supplementary Mate-
rials and Methods section. No cell lines were used in the 
conduct of this research; all samples used were freshly 
collected and immediately processed for RNA or protein.

**Correlation of genotypes and haplotypes with gene 
expression**

Genomic DNA was extracted from stored blood samples 
using the Autogen FX robotic system in accordance with 
Autogen’s protocols (Autogen). Preprinted bar-coded labels 
were affixed to sample containers to minimize sample 
mix-ups, and critical steps in sample processing were only 
undertaken when 2 technicians were present. The Affymetrix 
Human SNP Array 5.0 platform was used to assess genotype 
using the manufacturer’s protocols. The focus was on all 48 
SNPs found on the SNP array whose chromosomal location 
was within 100,000 base pairs either side of the *CSTA* gene. To 
avoid artifactual association of genotype with expression 
causely by SNPs with known minor allele frequency more 
than 5% situated within the target sequence of Affymetrix 
expression probe sets, the sequences of individual probes 
(obtained from NetAffx, Affymetrix) were cross-checked 
against the National Center for Biotechnology Information 
(NCBI) dbSNP build 129. Potential effects of copy number 
variation on associations of genotype with gene expression 
were assessed as outlined in the Supplementary Materials and 
Methods section.

**Data analysis and statistics for SAE expression and 
genomic data**

Gene expression analyses on all samples were performed 
using the Microarray Suite 5.0 (MAS5) software (Affymetrix). 
Publicly available data from Kuner and colleagues (31) for 40 
samples with lung adenocarcinoma and 18 subjects with SCC 
of the lung were downloaded from the NCBI Web site (acces-
sion number GSE10245), using the gene expression data from 
the only *CSTA* probe set 204971_at on the Affymetrix HG-U133 
Plus 2.0 chip, the same platform that was used on the SAE and 
LAE samples. The proportional relationship among *CSTA* and 
cathepsin gene expression was examined in all samples using 
the additional probe sets 200838_at (cathepsin B), 202295_s_at 
(cathepsin H), and 202087_s_at (cathepsin L). Gene expression 
data were normalized using GeneSpring version 7.2 software 
(Agilent Technologies) per chip and per gene across all 
samples. All microarray data have been deposited at the Gene 
Expression Omnibus (GEO) site (ref. 35; accession number 
GSE22047). Genomic data from the Affymetrix Human SNP 5.0 
were assessed using the BRLMM-P Analysis Tool (BAT) 2.0 
software (Affymetrix) to determine genotype for *cis*-SNPs in 
the vicinity of *CSTA* on chromosome 3. Associations between 
*CSTA* gene expression levels and genotype for the 48 SNPs 
located within 100 kb of the gene were performed using 
PLINK, with permutation testing used to control for the effect 
of genetic ancestry. Haplotype associations with expression 
were performed using PLINK and Haplovlew, with linear 
regression modeling (see the Supplementary Materials and 
Methods section).

**Results**

**Association of *CSTA* small airway epithelial gene 
expression with genomic variation**

The study population demographic findings and character-
istics of brushed SAE samples and microarray expression 
probe performance are detailed in the Supplementary Results 
section. Based on the hypothesis that genetic variability might 
modulate the level of *CSTA* expression in the SAE, we 
evaluated the correlation of *CSTA* gene expression with genotypes 
of *cis*-SNPs within 100 kbp either side of the *CSTA* gene using
within introns of the gene, displayed significant correlation of genotype with SAE expression levels of Cancer Research Butler et al. n expression and SNP data were available (healthy nonsmokers, from the subset of the study population for whom both gene paired airway gene expression data and blood DNA SNP data from the subset of the study population for whom both gene expression and SNP data were available (healthy nonsmokers, n = 44; healthy smokers, n = 48; and COPD smokers, n = 20). Ten SNPs located upstream of the CASA gene, and 2 more within introns of the CASA gene, displayed significant correlation of genotype with SAE expression levels of CASA (Table 1, Fig. 1A). For the total combined group of subjects (healthy nonsmokers, healthy smokers, and COPD smokers), the most significant correlation of the levels of CASA small airway gene expression was with the SNP rs16832956 (P < 5 × 10^-4). Nine of the 12 SNPs identified had P < 10^-2 in reference to the strength of their association with CASA SAE gene expression. The 4 SNPs most upstream of CASA were located within introns of the calcium-sensing receptor (CASSR) gene, but none had any relationship of genotype with CASR gene expression levels (P > 0.8, not shown).

These associations persisted at this significance level after assessing 10^3 permutations within clusters of similar genetic ancestry suggesting that genotype rather than genetic ancestry was the cause of the observed effect. For example, G_ genotypes of rs16832956, the SNP with the strongest genetic association, had low CASA expression, with a >1.4-fold ± 0.1-fold increase seen in CC genotypes (Fig. 1B, P < 1.6 × 10^-3). The SAE CASA gene expression levels were confirmed by TaqMan RT-PCR (Fig. 1C, r^2 = 0.67, P < 10^-4).

### Influence of ancestral background on genetic associations with CASA small airway epithelium gene expression

Genetic ancestry could be a potential confounding variable limiting the generalizability of observations made in relation to the modulation of CASA gene expression by genetic variability. However, the associations of rs16832956 genotypes with CASA expression levels were similar among the 2 major ancestral groupings (P < 0.03, both ancestral groups), with no significant difference in allele frequency among those of African American ancestry versus subjects of European ancestry (Fig. 2A, G allele frequency 0.19 in African American ancestral group, G allele frequency 0.09 in European ancestral group, P > 0.08). Haplotypes, comprising SNPs surrounding the CASA genomic location, were significantly associated with

---

### Table 1. Association of CASA genotypes with CASA gene expression in the small airway epithelium

<table>
<thead>
<tr>
<th>SNP identity</th>
<th>Ch. 3 location</th>
<th>Minor allele frequency</th>
<th>All subjects</th>
<th>Healthy nonsmokers</th>
<th>Healthy smokers</th>
<th>COPD smokers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>(n = 112)</td>
<td>(n = 44)</td>
<td>(n = 48)</td>
<td>(n = 20)</td>
</tr>
<tr>
<td>rs1354162</td>
<td>123,436,767</td>
<td>0.040</td>
<td>0.02</td>
<td>NS</td>
<td>NS</td>
<td>4.6 × 10^-3</td>
</tr>
<tr>
<td>rs7652858</td>
<td>123,480,795</td>
<td>0.125</td>
<td>3.6 × 10^-3</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>rs2134225</td>
<td>123,484,943</td>
<td>0.125</td>
<td>3.6 × 10^-3</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>rs16832956</td>
<td>123,500,198</td>
<td>0.161</td>
<td>4.5 × 10^-4</td>
<td>NS</td>
<td>3.4 × 10^-3</td>
<td>0.010</td>
</tr>
<tr>
<td>rs1402200</td>
<td>123,505,107</td>
<td>0.411</td>
<td>7.3 × 10^-3</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>rs5008830</td>
<td>123,513,152</td>
<td>0.089</td>
<td>1.0 × 10^-3</td>
<td>0.025</td>
<td>1.1 × 10^-5</td>
<td>0.038</td>
</tr>
<tr>
<td>rs2001548</td>
<td>123,515,479</td>
<td>0.101</td>
<td>1.7 × 10^-3</td>
<td>NS</td>
<td>3.4 × 10^-5</td>
<td>0.038</td>
</tr>
<tr>
<td>rs4678180</td>
<td>123,520,487</td>
<td>0.455</td>
<td>5.2 × 10^-3</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>rs9864290</td>
<td>123,522,752</td>
<td>0.451</td>
<td>7.8 × 10^-3</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>rs6803098</td>
<td>123,523,300</td>
<td>0.453</td>
<td>4.1 × 10^-3</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>rs9817571</td>
<td>123,538,670</td>
<td>0.099</td>
<td>0.03</td>
<td>NS</td>
<td>0.018</td>
<td>NS</td>
</tr>
<tr>
<td>rs9842752</td>
<td>123,539,752</td>
<td>0.076</td>
<td>0.03</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

Note: CASA gene expression levels in small airway epithelium as assessed by Affymetrix HGU133 Plus 2.0 microarray expression probe set 204971_at were correlated with Affymetrix Human SNP Array 5.0 cis-SNPs situated within 100 kbp of the CASA gene location on chromosome 3.3q21.

For analysis with the group of all subjects, P values represent Wald statistic following permutation analysis: after initial associations were identified, each association was further tested by performing 10^3 permutations within clusters of similar genetic ancestry, as defined by STRUCTURE, using PLINK software. For the subgroup analyses, t tests are shown.

Only those SNPs with P values for association of genotype with expression of <0.05 are shown. SNPs are listed in ascending order of location on chromosome 3.5’ to 3’, the first 4 SNPs listed are within introns of the upstream CASR gene, the next 6 are intergenic, and the last 2 listed SNPs are located within introns of CASA.

| Chr. | Chromosome. Locations refer to NCBI Human Genome Reference Genome Assembly, Build 36.3 |
| Minor allele frequencies observed in the study population. |
| All subjects with SAE gene expression data and genotype data. Healthy nonsmokers (n = 44), healthy smokers (n = 48), and COPD smokers (n = 20) combined. NS = not significant (P > 0.05). |
**Modulation of Small Airway Epithelial CSTA Gene Expression**

CSTA gene expression for specific ancestral subgroups examined separately (Fig. 2B and C), with the strongest association seen in the group of individuals of European ancestry for haplotype GAGGGACCCGCT (Fig. 2C, P < 0.008; see the Supplementary Results section and Supplementary Table II).

**Effect of smoking status and COPD on CSTA gene expression in the SAE**

Gene expression levels of CSTA in SAE were significantly higher in the group of healthy smokers (n = 82) compared with healthy nonsmokers (n = 60, P < 0.04, pairwise Student’s t test), but were even more upregulated in smokers with COPD (n = 36) compared with the healthy smokers (Fig. 3A, P < 10^-3, pairwise Student’s t test; P < 10^-4 by analysis of variance for all 3 groups). The association of CSTA expression with phenotype was confirmed by RT-PCR, and was explained neither by genetic ancestry nor by age, though there was evidence of a possible dose–response relationship (Fig. 3B–D; see the Supplementary Results section). The effect of genotype on SAE CSTA gene expression seen in the total genotyped study population, was also observed when healthy nonsmokers, healthy smokers, and COPD smokers were examined separately (see the Supplementary Results section, Supplementary Fig. 1A and B).

**Western analysis of cystatin A protein expression**

Whole-cell lysates of SAE from healthy nonsmokers, healthy smokers, and smokers with COPD were quantitatively assessed for cystatin A expression using Western analysis. Western analysis revealed increased CSTA protein expression in healthy smokers compared with nonsmokers, with even higher expression in smokers with COPD compared with the healthy smokers (Fig. 4A), which was confirmed quantitatively by densitometry (Fig. 4B, P < 0.04 by pairwise comparisons by Student’s t test; P < 10^-2 by analysis of variance for all 3 phenotypic groups).

**CSTA gene expression in LAE, SCC, and adenocarcinoma**

Most lung cancers, especially adenocarcinoma, are derived from the SAE, and yet it is well established that smoking and COPD are more strongly linked to SCC than to adenocarcinoma (14, 28, 30). SCCs tend to arise in the more central airways (15). A comparison of CSTA expression was made among SAE, LAE, and non–small cell lung cancer (NSCLC) specimens (see the Supplementary Results section for details of the study population demographics and characteristics of brushed LAE samples). CSTA expression levels in LAE of healthy nonsmokers were similar to the upregulated levels seen in the SAE of COPD smokers. LAE CSTA expression was smoke-responsive (Fig. 5A, P < 10^-3) and but further upregulated in SCC (Fig. 5A, P < 10^-2, all comparisons). In contrast, adenocarcinoma CSTA gene expression levels were significantly downregulated compared with all but the SAE of healthy nonsmokers (Fig. 5A; see the Supplementary Results section).

In view of the marked upregulation of CSTA observed in SCC subjects, a comparison was made of the ratio of CSTA

---

**Figure 1.** Genetic modulation of CSTA SAE gene expression. A, magnified view of the CSTA gene region, with direction of transcription (arrow). Below the gene, same scale, are the corresponding chromosomal locations of 48 cis-SNPs on the Affymetrix Human SNP Array 5.0 found within 100 kb either side of the gene. Shown are the correlations (Wald statistic) with microarray-assessed SAE CSTA gene expression in 112 healthy nonsmokers, healthy smokers, and smokers with COPD. B, microarray normalized average expression values of CSTA for genotypes of SNP rs16832956. Shown are data for all 112 genotyped subjects with CSTA SAE gene expression on the ordinate. G = data for the combined genotypes GG and CG. C, TaqMan RT-PCR confirmation of microarray CSTA gene expression levels in SAE in a random subset of healthy nonsmokers (n = 23), healthy smokers (n = 28), and COPD smokers (n = 13).

---

www.aacrjournals.org

Cancer Res; 71(7) April 1, 2011

Published OnlineFirst February 16, 2011; DOI: 10.1158/0008-5472.CAN-10-2046

Downloaded from cancerres.aacrjournals.org on May 2, 2017. © 2011 American Association for Cancer Research.
gene expression to expression levels of the 3 known cathepsin targets (B, H, and L) of \textit{CSTA} within individuals. These expression ratios differed significantly among most phenotypic and tissue groupings, except for adenocarcinoma versus nonsmoker SAE (see the Supplementary Results section, Fig. 5B–D).

**Discussion**

The cytosolic cysteine protease inhibitor cystatin A, coded for by the \textit{CSTA} gene, has attracted interest with a number of reports relating expression of \textit{CSTA} protein levels to neoplastic states in a variety of tissues including those of epithelial origin (7–12, 36). Immunohistochemical data have suggested that \textit{CSTA} is expressed at higher levels in dysplastic human bronchial epithelium, in SCC in particular, and less often in adenocarcinoma of the lung, compared with normal bronchial epithelium (11, 12). In light of the fact that COPD, a disease that arises in the small airways, is a risk factor for lung cancer independent of the risk attributable to smoking, but only occurs in a minority of smokers (16–20, 22–27, 29), we asked: does genetic variation modulate \textit{CSTA} gene expression levels in the human SAE; is the expression of \textit{CSTA} haplotypes, African American ancestry

- **Ancestry vs. genotype**
- **CSTA haplotypes, African American ancestry**
- **CSTA haplotypes, European ancestry**
CSTA in SAE influenced by both smoking and by COPD; and since smoking and COPD each have a propensity toward SCC rather than adenocarcinoma (28–30), is CSTA expression differentially influenced by SCC versus adenocarcinoma of the lung relative to SAE and LAE expression levels in the noncancerous samples? Using fiberoptic bronchoscopy, pure populations of SAE were obtained from 60 carefully phenotyped healthy nonsmokers, 82 healthy smokers, and 36 smokers with COPD, and for the majority, both SAE CSTA gene expression and corresponding blood CSTA genotypes were assessed by microarray. The data demonstrate that CSTA gene expression levels in the human SAE are modulated by 12 SNPs within the vicinity of the gene, a genetic association that is not confounded by genetic ancestry. There is an association of local cis-haplotypes with CSTA gene expression for 4 haplotypes in the case of subjects of African American ancestry and 1 haplotype in those of European ancestry. In addition, healthy smokers have higher CSTA gene expression levels than healthy nonsmokers, with even higher levels observed in smokers with COPD compared with the healthy smokers independent of pack-years, albeit with a suggestive evidence of a dose–response relationship for smokers. The microarray expression was confirmed at the transcript level by quantitative TaqMan RT-PCR and at the protein level by Western analysis. The genetic modulation of SAE CSTA gene expression was a consistent finding when the 3 phenotypic groups were examined separately. Finally, tumor tissues from individuals with SCC of the lung have the highest relative levels of CSTA gene expression, but tumor tissue from adenocarcinoma subjects have substantially lower levels of CSTA than in any of the other tissues and phenotypes examined (except for being similar to healthy nonsmokers). Interestingly, the phenotypic states examined (healthy, COPD, lung cancer) are each associated with significantly different proportional gene expression of CSTA to cathepsins B, H, and L. These observations are in keeping with the concept that the CSTA gene plays a role in the evolution of the bronchial epithelium of peripheral and central airways from normal to disease, and suggests a
complex interplay among genetics, smoking, COPD, and lung cancer histologic subtype in relation to CSTA gene expression. CSTA appears to have a more plausible connection to the specific evolution of healthy smoker and COPD smoker airway epithelium into SCC rather than into adenocarcinoma, in keeping with the known stronger relationship of the separate risk factors of smoking and COPD with SCC than with adenocarcinoma of the lung (27–30).

Small airway epithelium, smoke-induced lung disease, and genetics

There is now a large body of evidence pointing to the SAE (defined as bronchi of ≥6 generations, <2 mm in diameter) as the earliest site of pathologic involvement in COPD, the primary site of airflow limitation in this disorder, and the site of development of most NSCLC (13–15, 18–21). Consistent with these observations in the major smoke-induced lung diseases, morphologic changes are found in small airways of asymptomatic smokers with normal lung function (37, 38). COPD is a relevant phenotype to study in the progression from healthy airway epithelium to lung cancer, an airway epithelial-derived disease, because COPD is itself an independent risk factor for the development of lung cancer, with a contribution to lung cancer risk that is separate from the risk attributable to cigarette smoking (22–26). Since only 15% to 20% of smokers will develop COPD, together with evidence of familial clustering in COPD and NSCLC and twin concordance for the risk for COPD (15–17, 22, 39–41), it is likely that genetic variation influences disease risk. The fact that SCC is more common than adenocarcinoma in smokers and COPD subjects also raises the question of the role of the LAE in the pathogenesis of these tumor subtypes. Although the current study was not designed to focus on LAE gene expression/genotype relationships, it is interesting to note that the smoke-responsive nature of CSTA expression in SAE was also observed in LAE. However, the levels of CSTA in healthy smoker LAE are still considerably less than in SCC subjects and taken together with the smoke-responsiveness, are therefore less likely to reflect mere constitutive expression of CSTA in a LAE-derived tumor. Although the observations of genetic modulation of CSTA gene expression and the upregulation of such expression in SAE from COPD smokers compared with healthy smokers is intriguing, the present study was not designed to address the question of whether or not CSTA expression is genetically associated with COPD. Testing this hypothesis would require the genotyping of a large number of subjects to have sufficient statistical power to reach a definitive conclusion.

Although the data show some evidence for a dose–response relationship between intensity of smoke exposure and CSTA gene expression, disease also influences the expression levels, as evidenced by the greater relative increase in CSTA levels in COPD and SCC versus healthy smokers compared with the smaller increase in healthy smokers versus nonsmokers, even though the latter comparison would have the greater difference in smoking exposure. Also, reciprocal effects of disease state and pack-years on CSTA gene expression in SAE were observed in the healthy smoker and COPD smoker age-defined subgroups.

Analysis of a published gene expression data set that used microarray technology to study lung cancer tissues did not show a significant effect of smoking status on CSTA gene expression (42). Others have noted that CSTA levels are higher in early-detected lung cancers compared with lung cancers presenting later in the disease course, despite higher pack-year values in the latter (43). These disparate observations highlight the need for further study to clarify if there is an effect of smoking-intensity on CSTA gene expression in lung airway epithelium and cancers derived from the airway epithelium. The observations suggest that CSTA expression is not uniformly smoke-responsive and is likely to be specific to cell populations. Controlling for the influence of age while examining pack-year consumption is challenging and would likely require large data sets, because more often than not, individuals with higher pack-year histories will also be older. From the data in the present study, it seems more likely that it is the intensity of smoking rather than age that exerts the greater influence on CSTA expression levels, as evidenced by the concordance among the magnitudes of pack-year difference and the difference in SAE CSTA levels in comparing the 2 smoking phenotypic groups (healthy smokers and COPD smokers) parsed by median age.
Cystatin A in lung disease

In the normal human airway epithelium, cystatin A is detected in the basal cells by immunohistochemistry, with more extensive staining in preneoplastic bronchial epithelium (12). Strong staining for cystatin A has also been observed in lung cancer tissue, especially SCC but also in adenosacarcoma and large cell carcinoma (11, 12). For SCC, cystatin A expression was less marked in less well-differentiated tumors, in which case lower cystatin A expression was also associated with tumor recurrence (12). Quantitative upregulation of CSTA protein has been noted in human lung cancer tissues versus control lung using ELISA, and univariate survival analyses showed that individuals with higher levels of cystatin A had a better survival probability, suggesting that CSTA is upregulated in lung tumors to counteract potentially harmful tumor-associated activity (11, 12). The present study confirms the previously published immunohistochemical data showing an upregulation of cystatin A in SCC versus normal airway epithelium, but also reveals a downregulation of CSTA in adenosacarcoma relative to levels in the healthy state, challenging data from another cohort that suggested an upregulation of cystatin A in some (but not in all) broncho-decic adenocarcinomas compared with normal airway epithelium (12).

Whereas all of the functions of CSTA are not clear, other than its known cysteine protease inhibitory effects, there is some evidence that it inhibits apoptosis in the presence of stimuli such as UVB radiation and viruses (4–6). Although deficiency states have been described arising from mutations in the genes that code for cystatins B and C, resulting in phenotypes of progressive myoclonus epilepsy and hereditary cystatin C amyloid angiopathy respectively, to date no functional mutations have been reported for CSTA in humans (44, 45).

The putative role of CSTA as a tumor suppressor is supported by observations in other tissues, including breast, prostate, and esophageal tumors, with evidence that cystatin A can inhibit tumor cell growth, angiogenesis, invasion, and metastasis (9, 36, 46). The observations in the present study that CSTA is downregulated in adenosacarcoma of the lung with evidence of less-well–opposed cysteine protease activity versus other smoke-exposed phenotypes are compatible with a tumor suppressor role in adenosacarcoma, but with a different function in the progression of some smokers to COPD and SCC, perhaps reflecting an “excess” of cystatin relative to its cognate proteases, the cathepsins. In contrast with these observations, a homozygous mutant mouse model involving chromosomal deletion of csta, the murine homolog of human CSTA, together with 3 other genes, showed no

Figure 5. Comparison of CSTA gene expression in SAE, LAE, and NSCLC. A, normalized average microarray CSTA gene expression levels are shown on the ordinate as box and whisker plots with median, interquartile range, and range for each of the groups depicted on the abscissa. B, ratio of microarray-determined gene expression level of CSTA to cathepsin B within individual subjects, for each of the 7 indicated phenotypic groups, including data from NSCLC. C, the ratio of microarray-derived SAE, LAE, and tumor CSTA levels to corresponding cathepsin H gene expression levels. D, shown on the ordinate is the same analysis as in B and C but with cathepsin L as the denominator. Error bars in B, C, and D represent standard error. P values are from ANOVA. HNS, healthy nonsmoker; HS, healthy smoker; COPD, smokers with chronic obstructive pulmonary disease; SAE, small airway epithelium; AdCa, adenocarcinoma of the lung; SCC, squamous cell carcinoma of the lung.

CSTA and Cathepsin B

CSTA and Cathepsin H

CSTA and Cathepsin L
phenotypic abnormality, the animals were not overtly susceptible to spontaneous or irradiation-induced tumor formation, and had evidence of compensatory gene expression of genes phylogenetically related to Csta (47).

Based on reports that cystatin A is upregulated in dysplastic airway epithelium and in lung cancer, the findings of the present study, which shows that the regulation of CSTA expression in SAE is influenced by genetic variability, smoking, and COPD, and that CSTA is differentially expressed by lung cancer histologic subtypes, suggests that each of these factors should be controlled for when considering the use of CSTA as a marker related to the pathogenesis of lung cancer. The progressive rise in CSTA expression observed in disease states (i.e., COPD and SCC) does not appear to be offset by a corresponding rise in cognate cathepsin levels in the same tissue from any given individual, an observation that may have implications for disease pathogenesis if intracellular cysteine protease-anti-protease homeostasis is relevant, particularly in genetically predisposed smokers.

Disclosure of Potential Conflicts of Interest

There are no potential conflicts of interest to disclose.

Acknowledgments

We thank N. Mohamed and T. Benios for help in preparing the manuscript.

Grant Support

These studies were supported, in part, by RO1 HL074326, PS0 HL084936, and UL1 RR024996.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received June 8, 2010; revised January 4, 2011; accepted February 2, 2011; published OnlineFirst February 16, 2011.

References


Modulation of Cystatin A Expression in Human Airway Epithelium Related to Genotype, Smoking, COPD, and Lung Cancer

Marcus W. Butler, Tomoya Fukui, Jacqueline Salit, et al.

Cancer Res 2011;71:2572-2581. Published OnlineFirst February 16, 2011.