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—Impaired airway homeostasis in chronic obstructive pulmonary disease (COPD) could be partly related to club cell secretory protein (CCSP) deficiency. We hypothesize that *CCSP* G38A polymorphism is involved and aim to examine the influence of the *CCSP* G38A polymorphism on *CCSP* transcription levels and its regulatory mechanisms. *CCSP* genotype and CCSP levels in serum and sputum were assessed in 66 subjects with stable COPD included in a 1-yr observational study. Forty-nine of them had an exacerbation. In an in vitro study, the impact on the *CCSP* promoter of 38G wild-type or 38A variant was assessed. BEAS-2B cells were transfected by either the 38G or 38A construct, in the presence/absence of cigarette smoke extract (CSE) or lipopolysaccharides (LPS). Cotransfections with modulating transcription factors, p53 and Nkx2.1, identified by in silico analysis by using ConSite and TFSEARCH were conducted. A allele carrier COPD patients had lower serum and sputum CCSP levels, especially among active smokers, and a decreased body mass index, airflow obstruction, dyspnea, and exercise capacity (BODE) score. In vitro, baseline *CCSP* transcription levels were similar between the wild and variant constructs. CSE decreased more profoundly the *CCSP* transcription level of 38A transfected cells. The opposite effect was observed with p53 cotransfection. LPS stimulation induced *CCSP* repression in 38A promoter transfected cells. Cotransfection with Nkx2.1 significantly activated the *CCSP* promoters irrespective of the polymorphism. Circulating CCSP levels are associated with smoking and the *CCSP* G38A polymorphism. CSE, LPS, and the Nkx2.1 and p53 transcription factors modulated the *CCSP* promoter efficiency. The 38A polymorphism exaggerated the *CCSP* repression in response to p53 and CSE.

G38A polymorphism; CSE; CCSP; CC10; COPD

CHRONIC OBSTRUCTIVE PULMONARY DISEASE (COPD) is among the chronic diseases with the highest morbidity and mortality burdens worldwide. Cigarette smoking is one of the main risk factors of COPD, yet only 25% of smokers develop the disease (8, 19). Multiple genetic factors may influence the disease susceptibility effect of cigarette smoke (27, 35, 36).

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Club cell secretory protein (CCSP, CC10, international nomenclature SCGB1A1) belongs to the secretoglobin family, consisting of small disulphide bridge dimeric proteins. It is one of the most abundant proteins secreted by the respiratory epithelium (up to 5% of the total protein quantity recovered from a bronchoalveolar lavage) (2). Baseline serum CCSP level is a predictive biomarker of accelerated lung function decline in the ECLIPSE study (34) and a predictor of lung function and development of COPD across the lifespan (11). Notably, serum CCSP levels were reduced in COPD patients and also correlated to smoking status (20, 29).

CCSP is secreted by nonciliated epithelial club cells present in all airways, but more predominantly in the small airways. CCSP may protect against the pro-inflammatory response in the lung (10, 15, 17, 32). In human cells, exogenous CCSP supplementation decreased the epithelial release of interleukin (IL)-8 and mucin (MUC) 5AC (10, 32).

The *CCSP* gene is located on the human chromosome 11q12.3–13.1 and contains 18.108 bp comprising three exons and two introns. Elements that regulate *CCSP* expression in the lung have been extensively studied in mice and rats. It has been demonstrated that hepatocyte nuclear factor (HNF)-3α and -3β, Nkx2.1, and AP-1 are important for modulating basal *CCSP* transcription (7). However, less is known about transcription factors that regulate *CCSP* expression in humans. Both HNF-3α and -3β binding to the *CCSP* promoter seem to increase *CCSP* expression (18). Finally, *CCSP* knockout mice exposed to cigarette smoke developed a COPD-like phenotype reversible by exogenous CCSP supplementation (17).

Gene mutational screening detected an adenine to guanine substitution at position 38 (G38A), downstream from the exon 1 transcription initiation site (12), a region occupied by other genes involved in the regulation of inflammation. The G38A alteration described corresponds to c.-26G>A (GeneBank NM_003357.4) with single-nucleotide polymorphism (SNP) ID number rs3741240. The *CCSP* G38A polymorphism has been associated with increased susceptibility to inflammatory lung disorders, e.g., asthma, posttransplant obliterative bronchiolitis, acute respiratory distress syndrome, and sarcoidosis (4, 9, 16, 22, 23). This is supported by the genome-wide significant association between the G38A polymorphism and circulating CCSP levels in the ECLIPSE study (14). A negative correlation between the presence of the A allele and the level of *CCSP* mRNA expression in the induced sputum was also reported (32). Finally, donor *CCSP* G38A polymorphism was associated with decreased CCSP levels in the bronchoalveolar

lavage fluid early after lung transplantation (4). These observations support the therapeutic ambition and deserve research to better identify determinants of the transcription levels.

We hypothesized that the G38A *CCSP* gene polymorphism may alter either the production of the protein and/or its activity. Thus, to better understand the molecular triggers involved, we obtained data from COPD patients and implemented an in vitro model allowing modulations of the *CCSP* promoter by using a luciferase reporter.

MATERIALS AND METHODS

Clinical Study Design

Sixty-six patients were enrolled in a 1-yr prospective observational study (CLARA study, EudraCT: 2009-A01200-57 approved by the ethics committee) to assess the potential of CCSP as a surrogate biomarker in COPD. Patients were included at steady state if they met the inclusion criteria (forced expiratory volume in 1 s and forced vital capacity < 0.7, smoking > 10 pack years, no other respiratory disease than COPD, optimally treated according to best standard of care, and aged 40 to 80 yr) and after signing the informed consent. One month after the inclusion visit (V1), demographic and functional data were recorded, and induced sputum and blood samples were obtained. Serum was obtained by centrifugation after venipuncture. Sputum was induced and processed as described previously (3). A separate specific informed consent form was obtained for genetic analysis. The body mass index, airflow obstruction, dyspnea, and exercise capacity (BODE) score was computed according to body mass index, forced expiratory volume in 1 s, dyspnea, and a 6-min walking test (6). Patients were asked to attend the clinic at the time of the first exacerbation (VX) and 1 mo later (VX+1). The final visit (V12) was planned 1 yr after V0. COPD prognosis was estimated based on the BODE score trend between V0 and V12 ("COPD progressors" were considered when the BODE score increased).

CCSP Genotype and CCSP Assay

G38A genotyping was performed after blood DNA extraction by using the restriction fragment length polymorphism method (24) to define G/G group and A/G + A/A group. CCSP were quantified in serum and induced sputum by ELISA by using human club cell protein (CC16) ELISA (Biovendor, Czech Republic), according to the manufacturer's protocol.

In Vitro Study

Computational analysis. CCSP minimal and distal promoters were determined by assessing the importance of promoter sequence conservation based on multiple sequence alignments of the sequence upstream the ATG of various species (human, rat, rabbit, horse, and mouse) by using BLAST software and based on existing literature (1, 7, 21, 25, 28). The importance of the G38A polymorphic site was assessed by evaluating its conservation through evolution by using ClustalW2 phylogeny software. Potential binding sites to the G38A locus were performed with ConSite. The threshold was designed at a score cutoff of 75.0% (highest: 100.0%) to optimize both sensitivity and specificity.

Table 2. *Subject baseline characteristics*

	G/G	A/A + A/G	*P* Value
N	32	34	NA
Age, yr	62.1 ± 11.1	55.4 ± 9.09	0.028*
Gender, *n* (%male)	25 (78)	23 (68)	0.413
Smoking history, pack years	43.8 ± 17.6	42.6 ± 21.2	0.566
Smoking status, *n* (% weaned >1 yr)	22 (69)	18 (53)	0.216
GOLD "2011" status, *n* (%I–II)	22 (69)	26 (79)	0.407
FEV_1, % th	60.6 ± 21.2	69.4 ± 21.2	0.102
FEV_1/FVC	0.53 ± 0.15	0.57 ± 0.16	0.324
BODE index	2.53 ± 2.44	2.55 ± 2.45	0.948
BODE trend at 1 yr, *n* (% increased index)	4 (12)	26 (76)	<0.0001*
Blood CCSP level, ng/ml	7.17 ± 4.18	3.77 ± 2.10	0.002*
Induced sputum CCSP level, ng/ml	3,466 ± 4,610	1,220 ± 1,599	0.013*

Data are expressed as means ± SD. *G/G vs. A/G and A/A patients comparison by Fisher or Mann-Whitney's test with $P < 0.05$ being significant. NA, not applicable; FEV_1, forced expiratory volume in 1 s; FVC, forced vital capacity.

Plasmid constructions. Expression plasmids containing either the wild-type 38G or the mutated 38A minimal (229bp) or distal (926bp) promoters downstream of the pGL3-basic plasmid were constructed to evaluate the transcription efficiency of the CCSP promoter. Based on computational analysis and the sequence of the CCSP gene, two constructs were generated from genomic DNA of NCI-H292, human pulmonary carcinoma cell lines, heterozygous for the G38A variant. PCR primers were designed to amplify a minimal promoter of 229 bp and a distal promoter of 926 bp using Primer3Plus. The PCR product was cloned into pGL3-basic vector (Promega) and sequenced. After verification of the PCR products, they were restricted with XhoI and NcoI endonucleases and then ligated by using heat shock protocol into the pGL3-basic vector downstream of the luciferase cDNA. Chemically competent *Escherichia coli* JM109 (Promega) were transformed. Positive clones were selected after verification conducted through DNA sequencing. The successfully constructed plasmids were named 229bp-38G, 229bp-38A, 926bp-38G, and 926bp-38A.

For cotransfection assays, the following expression vectors were used: pCMV6-XL4-Nkx2.1 and its control vector pCMV6-XL4, provided from OriGene, and pcDNA3.1-TP53 and its control vector pcDNA3.1, constructed from pC53-C1N, generously gifted by D. Reisman (University of South Carolina). The full length human TP53 cDNA was excised from the plasmid with ApaI and XhoI and ligated into the pcDNA3.1 expression vector. All the primers are listed on Table 1.

Cell culture and transfection assays. Human pulmonary epithelial BEAS-2B cell line obtained from ATCC was grown in DMEM 1640 completed with 5% fetal bovine serum (FBS), 1% ultroser G, 1% antibiotics, and 1% L-glutamine. They were seeded at a density of about 10,000 cells/100 μl of medium and plated in 96-well plates. After a culturing period of 28 h, cells were transfected with Fugene6 transfection reagent (Roche, France) according to the manufacturer's recommendations. For transient transfection, 72 ng of each CCSP constructs and 8 ng of internal control pRL-SV40 containing Renilla

Table 1. *List of mRNA primers*

Constructs	Forward	Reverse
229bp-CCSP	**CTCGAG**CAGTGGGGACAGAAACTGG	**CCATGG**CATGGTGGAGGAGGGGGAT
926bp-CCSP	**CTCGAG**GAGGCAAGAAAGGGGAGAA	**CCATGG**CATGGTGGAGGAGGGGGAT
P53	**CTCGAG**ATGGAGGAGCCGCAGTCAG	**GGGCCC**ATTTCTTCCTCTGTGCGCCG

Bold type indicates the restriction sites: *Xho*I, *Nco*I, and *Apa*I.

luciferase (Promega) were used. For cotransfection experiments, 40 ng of either pCMV6-XL4-Nkx2.1 or pCMV6-XL4 vectors and 10 ng of pcDNA3.1 or pCDNA3.1-P53 vectors were also transfected. After 24 h, cells were cultivated with or without cigarette smoke extract (CSE) at a different concentration and with or without LPS. Samples were harvested 48 h after transfection. The luciferase activity was evaluated with the Dual Luciferase Reporter Assay System (Promega, France). Luminescence measurements were performed on a Luminoskan Ascent luminometer (ThermoLabsystem, France). Firefly luciferase activity was normalized to Renilla luciferase activity. All luciferase activities represent at least four independent experiments with each construct tested in triplicate per experiment.

Cigarette smoke extract. CSE was prepared as previously described (10): the smoke of three commercial cigarettes (Marlboro) was withdrawn into a Pasteur pipette and bubbled into 30 ml of DMEM 1640 containing 5% FBS, 1% ultroser G, 1% antibiotics, and 1% L-glutamine. CSE was adjusted at pH 7.4 and sterilized through a 0.22-μm filter.

Statistical Analysis

Clinical data were expressed as means ± SD. Homozygous (G/G) patients were compared with heterozygous (G/A) and homozygous (A/A) patients by using a Mann-Whitney test for quantitative data and a Fisher test for qualitative data. Mixed effect models were built to test

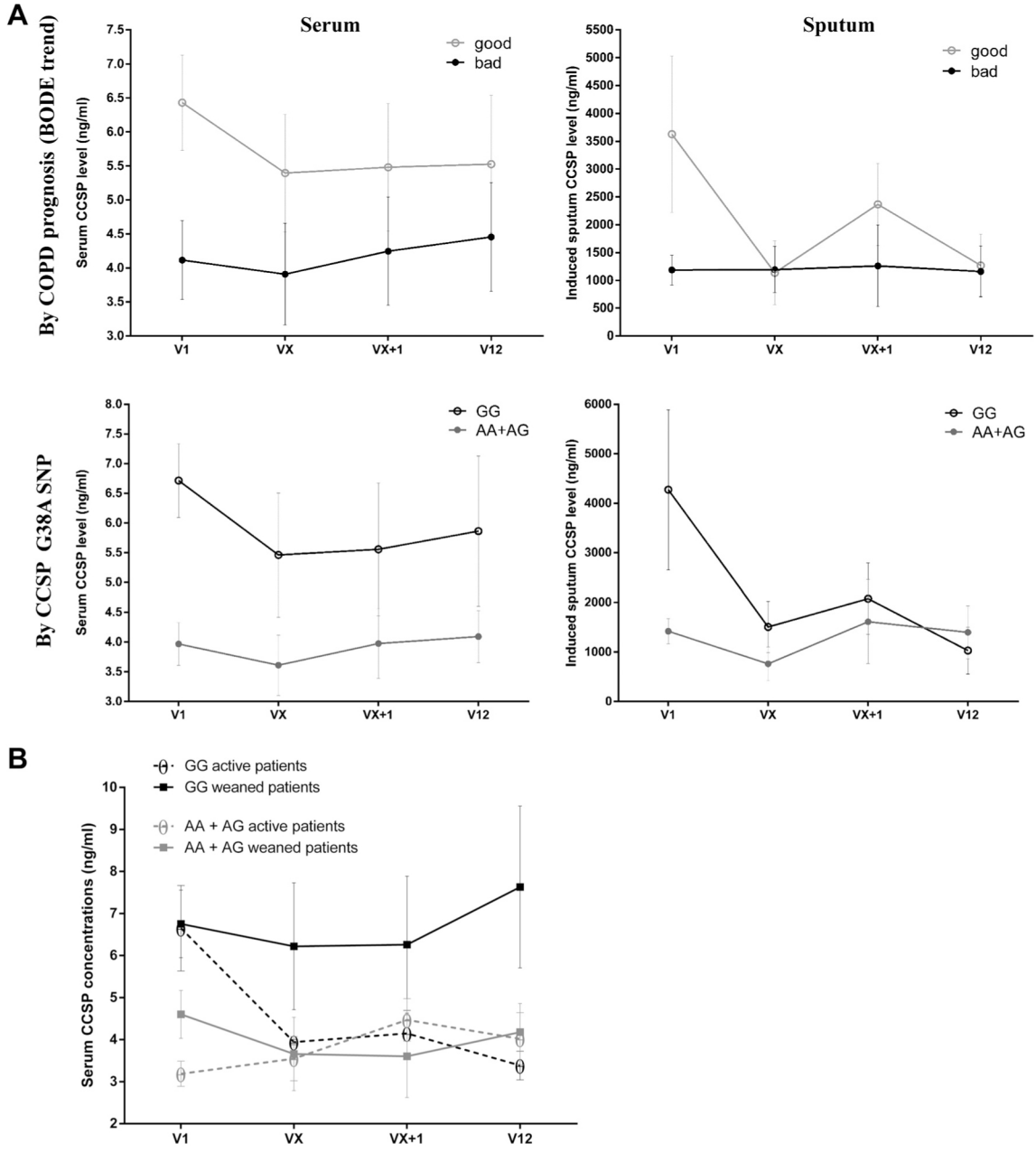

Fig. 1. Evolution of CCSP levels of 66 COPD patients during a 1-yr prospective observational study. *A*: at *top left*, evolution of serum CCSP levels depending on BODE prognosis. At *top right*, evolution of induced sputum CCSP levels depending on BODE prognosis. "COPD progressors" were considered when the BODE score increased ("bad," black line). At *bottom left*, evolution of serum CCSP levels depending on G38A polymorphism. *At* bottom *right*, evolution of induced sputum CCSP levels depending on G38A polymorphism (A allele carriers, gray line) *B*: evolution of serum CCSP concentrations depending on smoking status and G38A polymorphism (A allele carriers, gray line; still active smoking patients, dotted line). Serum CCSP levels were significantly affected by the combination of interactions between G38A polymorphism and the smoking status by using a mixed effect model ($P = 0.02$ for overall interaction).

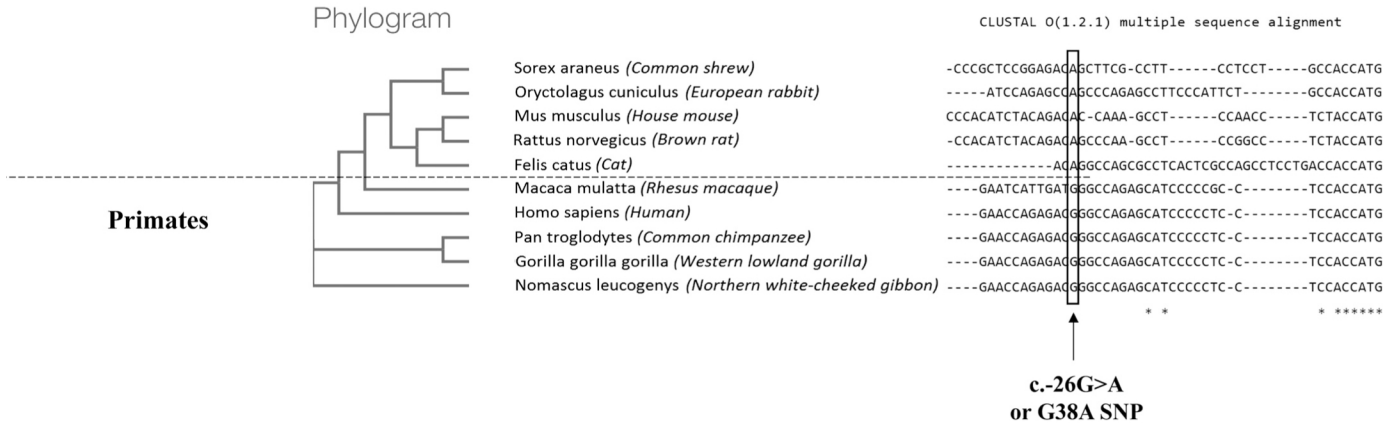

Fig. 2. In silico study. Dendrogram representing *CCSP* gene promoter in different animal species; the black arrow highlights the relevant polymorphic nucleotide: C.-26G>A or G38A SNP. The promoter of the *CCSP* gene was highly conserved throughout different species and especially across primates. Regions of similarity between biological sequences were matched with ClustalW2 phylogeny software.

differences of serum and sputum levels of the CCSP protein at different visits based on the G38A SNP and the BODE score trend. Transfection data were expressed as means ± SE. Paired comparisons were made using Wilcoxon matched pair tests. Data were considered statistically significant at $P < 0.05$. All graphical data and statistical analyses were generated with GraphPAD Prism software (Version 6.0).

RESULTS

Clinical Study

Subject baseline characteristics are displayed in Table 2. Patients had mild to moderate COPD, were mostly male, and had a similar cumulative smoking history. The G/G patient group was older than the A/G + A/A group. Serum and induced sputum CCSP levels were significantly higher in G/G patients compared with A/A + A/G patients ($P = 0.002$ and 0.013, respectively), suggesting that CCSP production was affected by the G38A polymorphism (Fig. 1*A*). COPD progressors defined by a worsening BODE score at 1 yr were more frequently A allele carriers ($P < 0.0001$) (Fig. 1*A*). Influences of the G38A polymorphism and smoking status on blood CCSP level are described in Fig. 1*B*. Serum CCSP levels were significantly affected by the interaction between G38A polymorphism and the smoking status ($P = 0.02$ for overall interaction) (Fig. 1*B*). The lowest CCSP levels were seen in A allele carriers still actively smoking.

In Silico Study: G38 Allele Conservation

To evaluate the importance of this polymorphic site, orthologous *CCSP* promoter regions from several mammalian species classically representing primates (rhesus macaque, human, common chimpanzee, western lowland gorilla, and northern white-cheeked gibbon), Carnivora (cat), Rodentia (house mouse, brown rat), Lagomorpha (European rabbit), and Soricomorpha (common shrew) were compared in silico by using ClustalW multiple *CCSP* sequence alignments. The human *CCSP* promoter was remarkably conserved (Fig. 2). A strict homology for the 38G allele was observed in the primates studied, whereas more genetically distant species carried the A allele.

In Vitro Study: CCSP Baseline

Transcriptional regulation of CCSP was assessed through a reporter assay by using different constructions. We tested four constructs, a minimal or a distal promoter, containing either the wild-type (WT) 38G, or the mutated 38A allele. Luciferase activity, reflecting basal activity of the *CCSP* promoter, indicated no significant difference between the WT and the 38A variant irrespective of the length of the constructions (Fig. 3).

In Vitro Study: Influence of CSE, LPS, and Transcription Factors on CCSP Promoter Activity

CSE influence was subsequently assessed. CSE repressed *CCSP* transcription. This repression was significantly more intense with the 38A variant compared with the 38G WT construction ($P = 0.02$) (Fig. 4*A*). LPS was tested to mimic

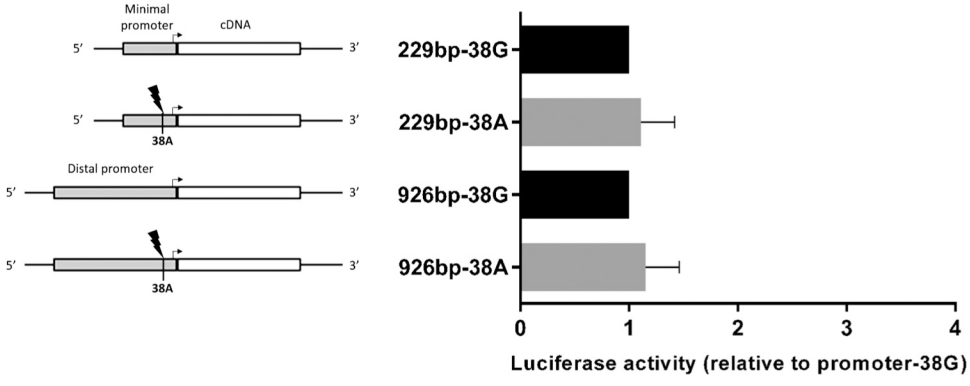

Fig. 3. In vitro study of *CCSP* baseline transcription in BEAS-2B transfected cells. *Left*: four constructions of the *CCSP* promoter mounted with a luciferase reporter sequence (A mutated vs. G WT allele, proximal, and distal promoters). *Right*: basal *CCSP* transcription levels according to different constructions and the G38A polymorphism. There was no significant difference between the WT and the 38A variant constructions.

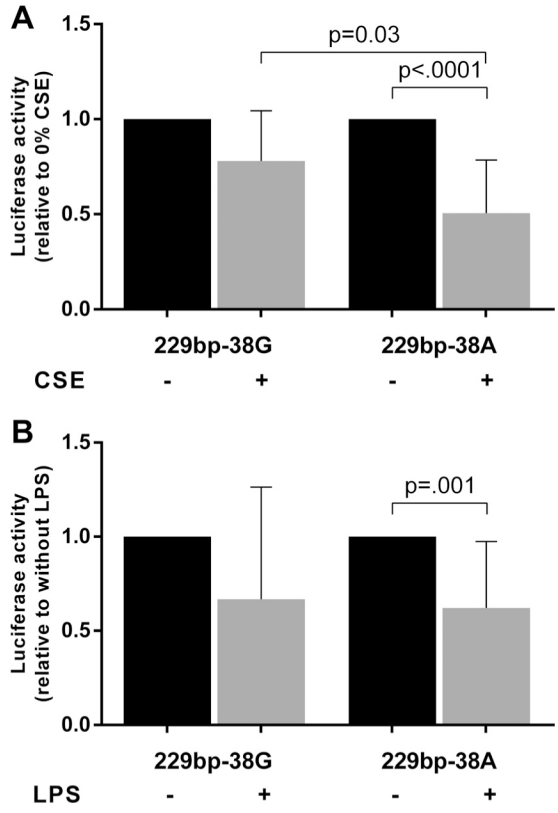

Fig. 4. In vitro study of the influence of CSE and LPS on *CCSP* promoter activity in BEAS-2B transfected cells. *A*: CSE repressed *CCSP* transcription. CSE repression was deeper with the A vs. G allele construction ($P = 0.03$, Wilcoxon matched pair tests). *B*: LPS, used as a model of exacerbation, repressed *CCSP* transcription. Repression was stronger in the A construction ($P = 0.001$, Wilcoxon matched pair tests).

exacerbation conditions. LPS also repressed *CCSP* transcription, significantly with the 38A variant construction ($P = 0.001$) (Fig. 4*B*). We obtained exactly the same results with the long construct promoters, and data were not represented for clarity.

ConSite in silico search identified two transcription factors which may bind differentially to the polymorphic site: Thing1-E47 and p53 (scores: 5.487 and 12.917, respectively) (Fig. 5*A*). Elsewhere, many *Nkx2.1* binding sites on the promoter were predicted by this analysis irrespective of the G38A polymorphism (Fig. 5*B*). BEAS-2B cells were cotransfected with the pCMV6-XL4-Nkx2.1 and its control empty vector pCMV6-XL4 with the different CCSP promoter constructs. Cotransfection with Nkx2.1 significantly increased *CCSP* transcription irrespective of the construction tested ($P < 0.001$ for each comparison, Fig. 5*C*). Subsequent cotransfection with the pcDNA3.1-P53 and its control empty vector pcDNA3.1 was then tested. P53 decreased the *CCSP* transcription level more intensely with the WT than with the mutated constructions ($P = 0.03$) (Fig. 5*D*). To evaluate whether CSE and p53 exerted synergistic effects, cotransfected cells with either pcDNA3.1-P53 and its control vector pcDNA3.1 with the different CCSP constructs were exposed to CSE. A synergistic effect of CSE and p53 was observed, but irrespective of the polymorphism (Fig. 6).

DISCUSSION

In this study, we observed that circulating levels of CCSP in COPD patients were influenced both by the G38A polymorphism located in the *CCSP* promoter and by active cigarette smoking. This SNP also affected the COPD prognosis by using the BODE score progression in a 1-yr prospective clinical observational study. We noted that the G allele, at the polymorphic site within the *CCSP* promoter, was highly conserved especially across primates. Thus in vitro experiments confirmed the deeper repression induced by CSE on *CCSP* transcription levels in A allele-carrying transfected cells. LPS globally repressed *CCSP* transcription in our transfection model. Transcription factor such as p53 was predicted in silico to bind the promoter differentially according to the *CCSP* G38A polymorphism. This was further confirmed in vitro where p53 repressed the *CCSP* transcription more in G than in A allele transfected cells. Finally, no synergistic effect of CSE and p53 was observed in our transfection model regarding the polymorphism.

First, we consider worth recalling that "G38A" is the right nomenclature for this polymorphism, but too often the literature still erroneously refers to it as "A38G." Nomenclature efforts have been proposed here to use the acknowledged symbol (international nomenclature recommended by the Human Genome Variation Society, http://www.hgvs.org/mutnomen). The present study aims to establish and understand the biological link between this polymorphism within the promoter and the transcription levels. The high level of conservation of the promoter site throughout different animal species improved our confidence in the biological potential of this polymorphism. Interestingly, phylogenetic tree analysis suggested that the G allele was privileged throughout primates segmentation.

Luciferase reporter is a well-established technique (26) satisfactorily reproducible throughout our assays. Nevertheless, this technique limits information to transcription, and confirmation at the protein level is required. Impact on stability of the *CCSP* mRNA is also neglected. Clinical observations could also result from posttranscriptional modifications impossible to address in the present study.

BEAS-2B cells were used for this study because they are of nontumoral bronchial origin and their endogenous CCSP production is nearly absent. This reduces the impact of the cell by itself and makes it a "pure" model for studying transfected CCSP promoters.

Nkx2.1 strongly activated the *CCSP* gene as reported before (5) irrespective of the SNP as predicted by our in silico analysis. This assay was tested as a control of our model. We gained confidence in our model when in vivo findings were reproduced, as CSE repression of the *CCSP* transcription level was deeper in A allele transfected cells. This was one further translational confirmation of previous reports from the longitudinal ECLIPSE study (20). Although p53 is a ubiquitous transcription factor critical in cancer, p53 deregulation was already reported in COPD (30–31). This protein encoded by the antioncogene may be influenced by the exposome mutagenic pressure. We think it may not represent a realistic therapeutic target for modulation. Another candidate transcription factor was identified by ConSite in silico analysis: "Thing1-E47." It is a basic helix-loop-helix transcription fac-

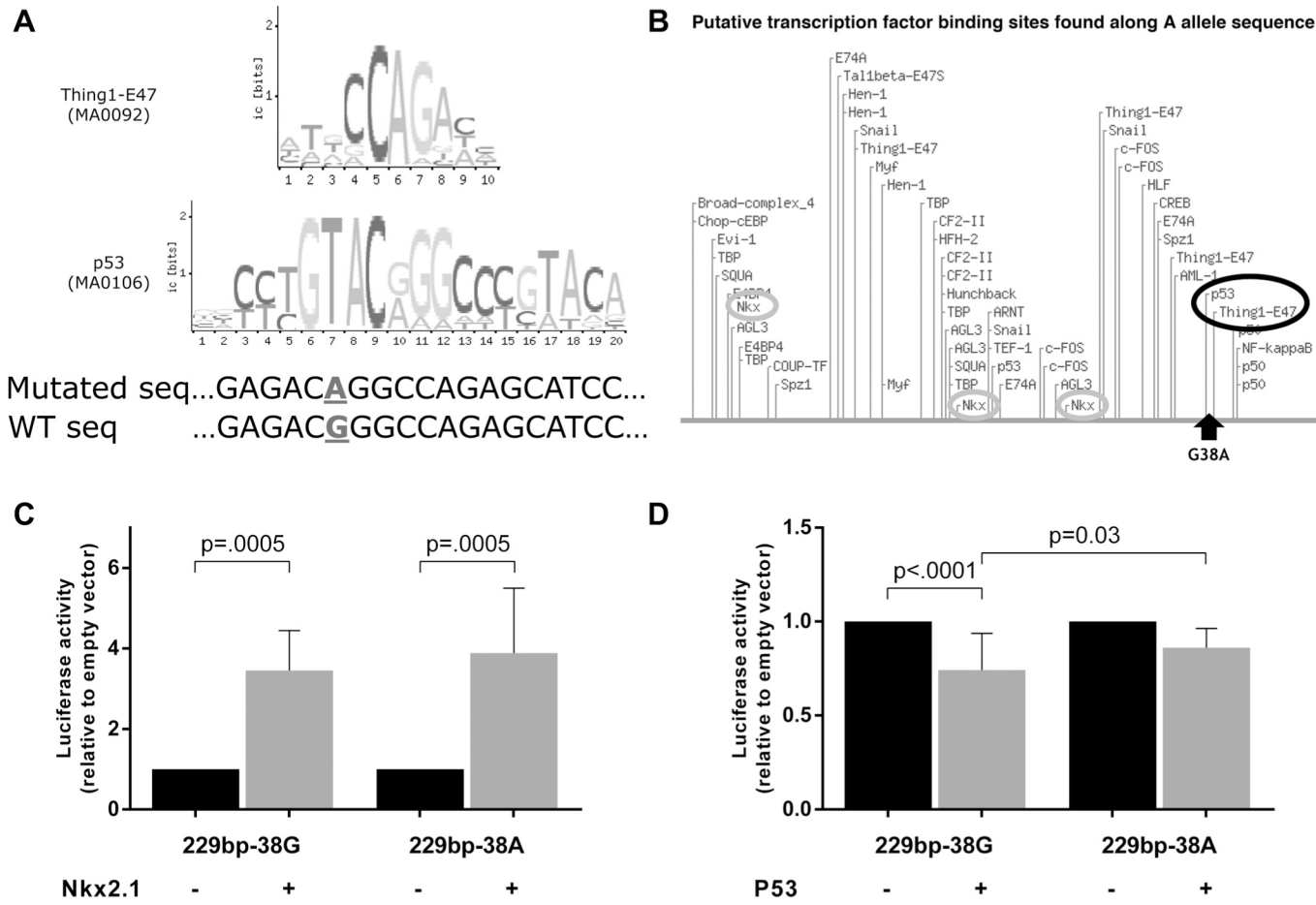

Fig. 5. In vitro study of the influence of transcription factors on *CCSP* promoter activity in BEAS-2B transfected cells. *A*: modification of binding sites induced by the G38A polymorphism according to ConSite. The threshold was designed at 75.0 point (highest: 100.0) to optimize sensitivity and specificity. Two transcription factors potentially bind to the polymorphic site: Thing1-E47 and p53. *B*: in-depth in silico analyzes (consite.genereg.net) revealed many Nkx binding sites (surrounded by gray) around the polymorphic site (transcription factors that potentially bind to the SNP surrounded by black). *C*: Nkx2.1 strongly increased the *CCSP* transcription irrespective of the G38A polymorphism. *D*: p53 repressed *CCSP* transcription. Deeper repression was noted in G allele constructions ($P = 0.03$, Wilcoxon matched pair tests).

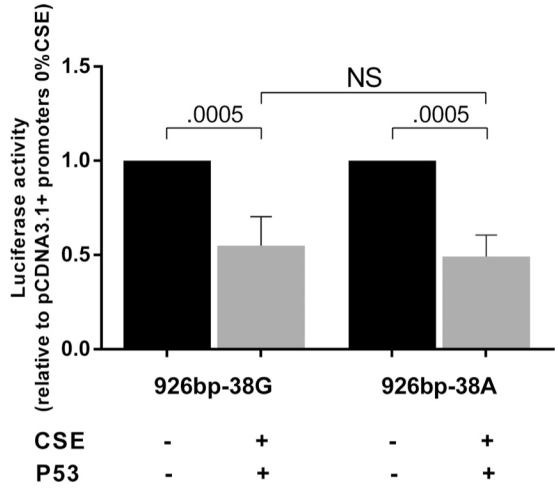

Fig. 6. In vitro study of the synergistic effect of CSE and p53 on *CCSP* promoter activity in BEAS-2B transfected cells. Absence of differential synergistic effect of CSE and p53 cotransfection in *CCSP* transcription levels according to the G38A polymorphism.

tor, but its link with pulmonary disease is unclear. Moreover, extensive research failed to clearly identify a validated structure. Subsequently, it was not tested in our model.

In COPD, exacerbations highly affect the history of the disease (13). Because CCSP may play a protective role by maintaining airway homeostasis (15), exacerbations were events of special interest in our longitudinal study. We found no clear influence of the polymorphism in this period and at recovery. This observation might be cautiously appreciated because of a clear lack of power due to small effectives. In vivo, there seems to be no difference regarding the exacerbation. Yet, we showed that LPS, a model of bacteria-induced exacerbations, provoked a stronger *CCSP* repression with the A allele constructions.

Finally, our in vivo study confirmed that circulating CCSP levels are associated with smoking and the G38A polymorphism. Our in vitro study supports those findings, as CSE repressed the *CCSP* transcription of the A allele transfected cells more intensely than the WT cells.

In conclusion, cigarette smoke is a confirmed repressor of *CCSP* transcription amplified by the G38A polymorphism. We demonstrated the potential involvement of a gene-environment

interaction in airway susceptibility. Surveying active smokers for the presence of low CCSP levels may be warranted.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

L.K., J.V., A. Bergougnoux, A.-S.G., J.B., A. Pommier, and A. Petit performed experiments; L.K., N.M., M.T.-C., and A. Bourdin analyzed data; L.K., I.V., M.T.-C., and A. Bourdin interpreted results of experiments; L.K., N.M., and A. Bourdin prepared figures; L.K. drafted manuscript; L.K., N.M., I.V., M.T.-C., and A. Bourdin edited and revised manuscript; L.K., J.V., A. Bergougnoux, A.-S.G., J.B., A. Pommier, A. Petit, N.M., I.V., M.T.-C., and A. Bourdin approved final version of manuscript; I.V., M.T.-C., and A. Bourdin conception and design of research.

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