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The role of SCCA1 in asthma related physiological events in the airway epithelium and the effect of promoter variants on asthma and gene function

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Protease inhibitor;
Squamous cell carcinoma antigen

Summary
Background: Even though the systemic level of SCCA1, a serine protease inhibitor, was shown to be elevated in asthma, its physiological role is unknown.
Objective: We sought to determine the effect of SCCA1 on apoptosis, cytokine expression and mucus production by A549 cells and define the effect of promoter variants on gene expression and association with asthma.
Methods: SCCA levels were measured by ELISA. Promoter variants were determined by direct sequencing. 442 asthmatic children and 191 controls were genotyped by RFLP. The functional effect of the polymorphisms was assessed in transient transfection experiments using reporter constructs. A transcription factor ELISA was used for differential binding of GATA proteins to the variant region. The effects of SCCA1 on cytokine synthesis, mucus production and apoptosis were determined in A549 cells transfected with SCCA1 pcDNA vector. MUC5AC expression in A549 cells was determined with RT-PCR.
Results: SCCA1 protein level was significantly higher in asthmatic children compared to healthy controls. Four polymorphisms SCCA1 promoter that were in linkage disequilibrium were associated with skin test positivity in asthmatic children and showed higher promoter activity and higher binding of GATA-2 and GATA-3 after IL-4 + IL-13 stimulation. IL-6, IL-8 levels were significantly higher in cells transfected with SCCA1 whereas RANTES increased only after IL-4 stimulation. Transfection of A549 cells with SCCA1 resulted in decreased MUC5AC expression and conferred protection against apoptosis.

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Conclusion: Our results showed that SCCA1 has diverse effects on many of the cellular events that characterize asthma and its role extends beyond protease inhibition.
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Introduction

The pathogenesis of asthma is incompletely understood. In addition to cytokines,[1,2] chemokines[3] and oxidants,[4] proteases acting through membrane receptors and the counteraction by anti-proteases may be important in the pathogenesis of asthma.[5-7]

Proteases can be exogenous such as allergens and various microorganisms or endogenous such as thrombin, plasmin, cathepsin G, elastase, tryptase and chymase.[6–11]

Proteases act through Protease-Activated Receptors (PARs) which are expressed on almost all cell types. In addition to their roles in coagulation and homeostasis, they stimulate inflammatory and resident cells in the airways,[12] disrupt tight junctions,[13] increase secretion of proinflammatory cytokines,[14] aggravate airway remodeling[15] and increase bronchial smooth muscle contraction[16] which make them critical for the inflammatory diseases of the airways.

To fight against the proteolytic injury, the human body is equipped with many protease inhibitors. They have important roles in the regulation of proteolytic events such as fibrinolysis and coagulation as well as apoptosis and inflammation.[17,18] Squamous cell carcinoma antigens (SCCAs) belong to the superfamily of high molecular weight serine and cysteine protease inhibitors. There are two tandemly arranged genes known as SCCA1 and SCCA2. SCCA1 and SCCA2 genes are also known as SERPINB3 and SERPINB4, respectively and both are located at 18q21.3. Although these two genes have high homology, they have distinct biological activities.[19] SCCA1 inhibits cysteine proteases such as cathepsin K, L, S and papain whereas SCCA2 inhibits serine proteases such as cathepsin G and human mast cell chymase.[20] Even though SCCAs were initially discovered as a marker of cervical carcinoma, its levels were subsequently found to be elevated not only in other carcinomas[21,22] but also in atopic dermatitis and psoriasis.[23,24]

A role for SCCA in asthma was initially suggested by a microarray analysis of human bronchial epithelial cell cultures after stimulation with IL-4 and IL-13. Subsequently its systemic levels were reported to be increased in asthma,[25] asthma exacerbation[25,26] and in patients with allergic rhinitis.[27]

Even though there is some evidence that SCCA may be an important molecule in asthma, its specific role, the mechanisms and the genetic associations remain to be elucidated. We have undertaken a comprehensive study to investigate the role of SCCA in asthma. In the genetic arm, we searched for single nucleotide polymorphisms (SNPs) that may influence the transcription of SCCA and performed association studies. Physiologically, we aimed to determine the effect of SCCA1 on apoptosis and mucus and cytokine production by bronchial epithelial cells.

Materials and methods

Children with asthma

The patients in the asthma group have been detailed previously.[28–30] Children aged 6–18 years who were diagnosed with asthma at the Pediatric Allergy and Asthma Unit of Hacettepe University, School of Medicine, Ankara, Turkey have been included in the study. Asthma diagnosis was confirmed according to GINA guidelines.[31] Skin testing was performed as detailed previously.[30]

Healthy children

Control group has been detailed previously[30] and was composed of Turkish school children who presented to the outpatient department of the same hospital. They presented for reasons such as minor trauma or for their regular follow-up. They responded negatively to an established and validated asthma questionnaire (ISAAC),[32] never had any diagnosis of asthma, and never had any history of wheezing. They all had normal pulmonary function tests. All children underwent skin prick testing and had their total IgE measured in serum.

Study procedures were approved by the Ethics Committee of Hacettepe University. All parents provided written informed consent for the study.

Serum levels of SCCA1 and SCCA2

Serum samples were concentrated five times by SpeedVac vacuum concentrator (Labconco, Kansas City, MO, USA). SCCA1 and SCCA2 levels were determined by ELISA (Fujirebio Diagnostic, Malvern, PA, USA).

Identification of genetic variants of SCCA1 and SCCA2 genes

DNA was extracted from whole blood by standard techniques. Promoter region of SCCA1 and SCCA2 gene was defined according to the latest sequencing revealed by genome databases, Ensembl and NCBI.[33,34] To determine the polymorphisms in the promoter and 5′ UTR region of the transcript, DNA samples from 25 asthmatic patients and 25 healthy individuals were amplified by specific primers: SCCA1 forward: 5′-TGCTAAATGGAAGGACCACCA-3′, reverse: 5′-AGGTGGGCAGAGAGGCTA-3′; SCCA2 forward: 5′-TGCTAAACAGAAGGACCATTG-3′, reverse: 5′-AGGTG GGCAGAGA GGCTG-3′. Sequencing reaction was performed with Big Dye Terminator cycle sequencing kit (3.2 version) using ABI Prism 310 Sequence Detection System (Foster City, CA, USA). As the initial results suggested the presence of linkage disequilibrium among four SNPs in the SCCA1

promoter, 100 healthy and 100 asthmatic children were further sequenced to confirm or refute the observation.

Genotyping for promoter polymorphisms in SCCA1

Sequencing of 250 samples showed that four SNPs in the SCCA1 promoter were in perfect linkage disequilibrium. rs116864116 A/G variation was chosen to determine the genotype by restriction fragment length polymorphism (RFLP) analysis. A 549 base pair of the promoter region was amplified using the following primer pairs: forward 5′-AGACATACCTGAGATTAGGA-3′ and reverse 5′-TTTCTGTA-TAACAAGGAATA-3′. The amplicon was digested with *Alw26 I* (New England Biolabs, MA, USA). The products were fractionated on 2% agarose gel. Digestion occurred in the presence of the A allele and produced a 169 and 380 base pair products.

Genotyping for the promoter polymorphism in SCCA2

The genotyping of the SNP rs3760573 (G/T) in SCCA2 promoter was done by PCR-RFLP. However, since there was no enzyme recognition site, a restriction site for *Eco31I* was introduced using a primer containing mismatched bases adjacent to the SNP. Two sequential PCR reactions were performed. In first amplification, following SCCA2 specific primer pairs were used: forward 5′-AGGTGGGCAGA GAGGCTG-3′ and reverse 5′-TGCTAAACAGAAGGACCATTG-3′. In the second, nested PCR was carried out by using first reaction as a template with internal primers: forward 5′-TATGCTATAAGATACTGAAGTAC-3′; mismatch reverse primer 5′-CAGCTGTAAAACAAAAACGAGA-3′. The 249 bp amplicon from nested PCR was then subjected to *Eco31I* restriction endonuclease digestion (New England Biolabs). Digestion occurred with G allele and produced 22 and 227 base pairs.

Cell culture

A549 cells were purchased from DSMZ Company (DSMZ, Braunschweig, Germany). Cells were grown in 1640 RPMI (GIBCO BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin in 5% CO_2 atmosphere at 37 °C. IL-4 (Biosource, Camarillo, CA, USA), and IL-13 (CYTOLAB, Peprotech EC Ltd., London, UK) were used at a concentration of 25 ng/ml each in all experiment. All cell culture experiments were performed at least three times with three samples.

Transient transfection analysis with reporter constructs

Genomic DNA from individuals bearing four SNPs or wild type haplotypes was amplified to include 1000 base pairs upstream from the transcription start site of the SCCA1 promoter. Purified PCR products were cloned into pGL3 basic vector (Promega, Madison, WI, USA) between Kpn I and Xho I sites (New England Biolabs).

A549 cells were used for transfection assays. They were grown to 90% confluence in 24 well plates, transiently transfected with either promoter reporter constructs or empty vectors and co-transfected with pRL-TK vector containing Renilla Luciferase gene (Promega) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. A549 cells were treated with IL-4 or IL-13 or both. 48 h after transfection, cells were lysed with reporter lysis buffer and luciferase activity of each lysate was determined using Dual Luciferase Assay kit (Promega). Firefly luciferase activity was normalized to renilla luciferase activity to control for differences in transfection efficiency.

Transcription factor ELISA

Putative transcription factor binding sites were predicted using TRANSFAC-Biobase Biological Database software (Biobase Biological Database MA, USA). A549 epithelial cells were cultured in RPMI medium. Nuclear extracts were prepared from stimulated (IL-4 and IL-13, 25 ng/ml for 24 h) and unstimulated A549 cells as described.[35] Protein concentrations of the nuclear extracts were measured using Bradford Assay (Bio-Rad, Hercules, CA, USA). Double-stranded, 37 base pair biotin labeled oligonucleotides (IDT, Coralville, IA, USA) with and without the polymorphisms were synthesized: SCCA1 wild type primer 5′ ACCCCT**A** ATAAACCCATT**T**GGATCTCCTGAG**A**CTTATT 3′ and SCCA1 mutant type primer 5′ ACCCCT**G**TAAACCCAT**C**GGATCTCCT GAG**G**CTTATT 3′. Experiment was done according to the manufacturer's protocol with a little modification (Active Motif, Carlsbad, CA). Plates were coated with biotin labeled double stranded oligonucleotides with and without mutations followed by incubation with 5 μg of nuclear extracts for 1 h. The wells were then washed and incubated with GATA-1, GATA-2 or GATA-3 antibodies. The reaction was developed with an anti-IgG HRP conjugate and the colorimetric reaction was measured using a microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 450 nm. The experiment was performed in triplicate for each condition.

Cytokine measurements

A549 cells were seeded in 24 well culture dishes and 24 h later cells were transiently transfected with SCCA1 pcDNA full length expression vector (a kind gift by Dr. Sule Cataltepe, Brigham and Women's Hospital, Harvard Medical School, MA, USA) using lipofectamine 2000 reagent. After transfection, cells were stimulated with a combination of IL-4 and IL-13. 48 h after transfection, supernatants were collected and stored at −80 °C until measurement. Transfection efficiency was proven before each experiment.

The concentration of IL-1β, INF-γ, IL-6, IL-8, IL-10, IL-12 and RANTES were measured using ELISA (PIERCE, Thermo Fisher Scientific, Rockford USA).

Efficiency of transfection

Efficiency of transfection with the SCCA1 expression vector was determined both by a PCR based method and western

blot. In the PCR based method, DNA extraction was carried out from transfected and untransfected cells and isolated samples were amplified by T7 forward primer specified for pcDNA vector and reverse primer of SCCA1 gene. Forward primer sequence is 5′ TAATACGACTCACTATAGGG 3′ and reverse primer is 5′ GGGATGAGAATCTGCCAT AG 3′.

In the western blot method, cells transfected and untransfected with SCCA1 pcDNA were lysed in buffer containing 0.5% Triton X-100, 100 mM NaCl, 1 mM EDTA, 20 mM Tris–HCl (pH 7.4) and protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN, USA). Total protein concentration was calculated by the Bradford method. Western blot analysis was performed as described previously.[36] Mouse anti human SCCA1 antibody (Abcam, Cambridge, MA, USA) was performed at 4 °C overnight at the 1/16,000 dilution and recombinant SCCA1 protein (Abnova, Walnut, CA, USA) was used as a positive control.

Measurement of MUC5AC expression

A549 cells were transfected with SCCA1 pcDNA vector. Transfected and untransfected cells were left unstimulated or stimulated with IL-4 and IL-13 (25 ng/ml) and neutrophil elastase (200 μM) for 24 h. Total RNA was isolated using RNeasy mini kit (Qiagen, Valencia, CA, USA). One microgram of total RNA was reverse transcribed using ImProm-II Reverse Transcription System (Promega, Madison, WI). MUC5AC gene was amplified as described before.[37] Intensity of amplification bands were measured by image analyzer (Syngene Gene Genius, Synoptics, Cambridge, UK) using Gene Tools Image Analysis Software 3.02.00 (Synoptics). Expression of MUC5AC gene in unstimulated samples was arbitrarily defined as 1 and expression index of other conditions calculated relative to unstimulated cells.

Measurement of cell viability with MTT assay

A549 cells were transfected with SCCA1 pcDNA expression vector. Cells were transferred to serum-free media and treated with 125, 250 or 500 μM H_2O_2 for 24 h. A modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) reduction assay was used to determine the cell viability.[38] Briefly, 65 μg of MTT (Sigma–Aldrich Corp, St Louis, MO) was added to each well and cells were incubated in 5% CO_2 for 4 h. Cells were lysed with 100 μl isopropanol and absorbance was measured at 550 and 630 nm.

Measurement of apoptosis by annexin V FITC/PI

Transfected A549 cells were treated with 125, 250 or 500 μM H_2O_2 for 24 h. After 24 h, cell pellets were obtained by trypsinization, washed with ice cold PBS twice and were resuspended in ice cold binding buffer at 1×10^6 cells/ml. 200 μl of cell suspension were stained with annexin FITC antibody and PI dye using ApopNexin™ Apoptosis Kit (Chemicon, MA, USA) and read by flow cytometry (Beckman Coulter, CA, USA). Annexin positive cells were considered as early, while annexin and PI double positive cells were considered as late apoptotic. Cells that stained positively with PI only were regarded as necrotic.

Eosinophils isolation, culture and RT-PCR of SCCA1

Eosinophils were isolated from blood of healthy volunteers using magnetic selection method (Dynaltech, Oslo, Norway).[29] 750×10^3/ml eosinophils were stimulated with GM-CSF (20 ng/ml), IL-4 (25 ng/ml), IL-5 (10 ng/ml), IL-13 (25 ng/ml) or their combination. 24 h after incubation at 37 °C in 5% CO_2, RNA was isolated and reverse transcribed as outlined above. Previously described SCCA1 specific primers[39] were used for RT-PCR reaction. Products were run on 2% agarose gel.

Statistical analyses

Statistical analyses were done with SPSS 15 for Windows (Chicago, IL, USA) and Prism 5 for Windows (GraphPad Software Inc., CA, USA). Comparison of quantitative variables was done with parametric or non-parametric tests depending on the normality of distribution. Chi square, Mann–Whitney *U* or Kruskal–Wallis test was used for comparison of categorical and numerical variables as appropriate. Repeated measures of ANOVA were used to compare cytokine levels, cell viability and apoptosis. A p value <0.05 was considered significant. In correcting for multiple comparisons, the p value was divided by the number of SNPs for the level of significance. We found that the four SNPs that we describe in the promoter region of the SCCA1 (see Results) are in perfect linkage disequilibrium. Therefore, we treated it as one single SNP and have done the adjustment for multiple comparisons for two SNPs: one in SCCA1 and one in SCCA2.

In order to adjust for the confounding factors, we used logistic regression for categorical variables and linear regression analysis for continuous variables. We examined 6 variables: age, gender, eosinophil count, skin test positivity, IgE level and SCCA1 genotype. A two-sided $p < 0.05$ was considered significant.

Results

Characteristics of the study population are summarized in Table 1. Asthma and atopy related phenotypes including FEV1, eosinophil counts, skin test positivity and IgE levels were significantly different between the two groups.

Serum levels of SCCA1 and SCCA2

SCCA1 levels were higher in children with asthma [0.88 ng/ml (0.57–1.28)] [median (interquartile range)] compared to controls [0.55 (0.41–0.83)] ($p = 0.011$, Mann Whitney *U* test) but there was no difference in SCCA2 levels (Fig. 1A and B). SCCA genotypes had no effect on serum protein levels (data not shown). There was no correlation between SCCA1 and SCCA2 levels and clinical parameters such as skin test positivity, IgE levels, eosinophil numbers, FEV1, and severity of asthma (data not shown).

Sequencing of SCCA1 and SCCA2 promoter regions

Of the 25 samples from each cases and controls which would allow us to detect any SNP with a frequency

Table 1 Characteristics of the study population.

	Healthy controls	Children with asthma	p
	$n = 191$	$n = 442$	
Age (years)[a]	10.7 (8.1–13.1)	10.0 (8.1–12.2)	>0.05[b]
Gender			
Male (%)	92 (48.2)	240 (54.3)	>0.05[c]
Female (%)	99 (51.8)	202 (45.7)	
FEV1 (% predicted)[a]	101 (91–108)	93 (83–101)	<0.001[b]
Eosinophil count/mm^3[a]	146 (99–274)	260 (150–500)	<0.001[b]
Skin test positivity (%)	31 (16.2)	263 (59.5)	<0.001[c]
IgE (kU/L) [a]	35 (17–73)	141 (49–472)	<0.001[b]

[a] Median (interquartile range).
[b] Mann–Whitney *U* test.
[c] Chi-square test.

higher than 2% in either group, 24 were successfully completed.

We identified five SNPs in the promoter region of SCCA1; and one SNP at rs3760573 (G/T) in the SCCA2 promoter.

SCCA1 SNPs were coded as 1–5 depending on their distance from the transcription start site according to the most recent ensemble sequence [33]: (1) GAAa/gATG rs118005507, (2) CCTa/gATA rs7240852, (3) CATt/cGGA rs189633658 and (4) GAGa/gCTT rs116864116 (while this work was in progress this SNP was assigned an rs number in the Ensembl genome browser 65 but it does not exist in the current 67 version). According to the results of sequencing in 24 cases and controls, the four SNPs (1–4) looked in linkage disequilibrium. Therefore, in order confirm or refute, we sequenced further samples from 100 asthmatic and 100 healthy controls which showed that these four SNPs are in perfect linkage disequilibrium. LD score was calculated as 1 (LD 1) by R software for four SNPs in the promoter region of SCCA1. From these, rs116864116 A/G variation was chosen to determine the genotype. Hence, the AA genotype meant the presence of the following genotypes in other loci: AA at rs118005507, AA at rs7240852 and TT at rs189633658.

Polymorphism (5) rs60948708 GAGg/aGAG in SCCA1 promoter was found in only one child and was not pursued.

Genotyping for SNPs in the SCCA1 and SCCA2 promoters

There was no difference in the genotype frequencies between asthmatic individuals and healthy controls (Table 2). However, GG genotype at rs116864116 of the SCCA1 was associated with eosinophil count, skin test positivity and IgE in the asthmatic population (Table 3). Regression analysis has shown that SCCA1 genotype is independently associated only with skin test positivity OR = 1.427 95%CI (1.070–1.902, $p = 0.015$) but not with eosinophil counts or IgE levels. SCCA2 genotype was not associated with asthma or atopy phenotypes (data not shown).

Effects of promoter polymorphisms on SCCA1 gene expression

Even though the mutant genotype showed slightly higher promoter activity across all conditions, the difference became significant only after IL-4 and IL-13 stimulation (Fig. 2).

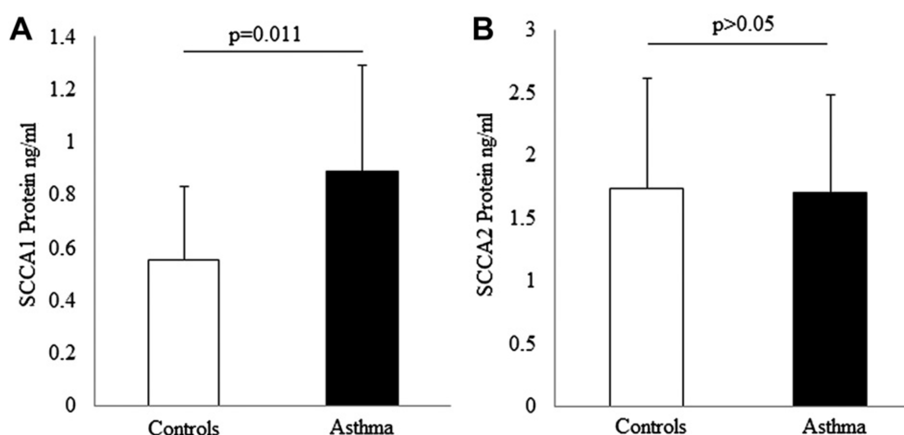

Figure 1 Serum levels of SCCA1 (A) and SCCA2 (B) for patients and controls. Error bars represent interquartile ranges.

Table 2 SCCA1 (rs116864116) and SCCA2 (rs3760573) genotypes.

	Healthy controls	Children with asthma	p
SCCA1 genotype			
	$n = 175$ (%)	$n = 430$ (%)	
AA	89 (50.9)	206 (47.9)	>0.05[a]
AG	69 (39.4)	180 (41.9)	
GG	17 (9.7)	44 (10.2)	
SCCA2 genotype			
	$n = 145$ (%)	$n = 177$ (%)	
GG	9 (6.2)	12 (6.8)	>0.05[a]
GT	55 (37.9)	78 (44.1)	
TT	81 (55.9)	87 (49.1)	

[a] Chi-square test.

Transcription factor ELISA

GATA-2 and GATA-3 transcription factors showed higher affinity to the mutant genotype. Stimulation with IL-4 + IL-13 basically caused decreased affinity for the wild genotype and thus resulted in widening of the difference observed under unstimulated conditions (Fig. 3). GATA-1 transcription factor did not show any affinity to either wild or the mutant genotype (data not shown).

Efficiency of transfection

Transfection efficiency was effectively determined using both a PCR based technique and Western Blot method (Supplemental Fig. 1A and B).

Cytokines

IL-1β, INF-γ, IL-10, IL-12 were not measurable in A549 cell culture system. IL-6 and IL-8 levels were significantly higher in cells transfected with SCCA1 under unstimulated conditions and after stimulation with IL-4 ($p < 0.05$), (Fig. 4A and B). IL-13 treatment with and without IL-4 abolished the increase in transfected cells. RANTES levels were significantly higher in the supernatants of SCCA1 transfected cells only after IL-4 stimulation ($p < 0.05$), (Fig. 4C).

Mucus production

MUC5AC expression was significantly lower in transfected cells under all stimulation conditions (Fig. 5). In addition, stimulation with NE, IL-4 + IL-13 and their combination resulted in a progressive increase in MUC5AC transcription only in the untransfected cells (Fig. 5).

Cell viability and apoptosis

SCCA1 transfection partially prevented the increase in cell death and apoptosis induced by increasing doses of H_2O_2 (Fig. 6A–D).

SCCA1 expression in eosinophils

RNA from A549 cells transfected with SCCA1 vector was used as a positive control to validate the RT-PCR system. No SCCA1 transcription could be identified in the eosinophils regardless of the stimulation (data not shown).

Discussion

Our study shows that the systemic level of SCCA1 is increased in children with asthma and has profound effects on the airway epithelium other than protease inhibition which includes suppression of MUC5AC expression, increased cell viability, decreased apoptosis, and effects on the pro-inflammatory cytokines IL-6, IL-8 and RANTES. Furthermore, our results show that polymorphisms in the promoter region of SCCA1 are independently associated with skin test positivity.

The initial finding in our study was a difference in the SCCA1 serum protein levels between cases and controls. The genetic arm was designed to account for the observed difference in systemic protein levels. Therefore, we restricted our genotyping studies to the promoter region and did not cover the whole gene. Instead of using published databases, we preferred to use sequencing technology in our population for the determination of genetic variants for two reasons: the published databases may not detect some SNPs present in disease specific states and databases may not reflect population specific SNPs, which, in this case is Turkish population.

The results of our population study showed that only SCCA1 but not SCCA2 showed positive associations. Since

Table 3 The effect of SCCA1 genotypes on asthma and atopy phenotypes in asthmatic children.

	AA	AG	GG	p	p
	$n = 206$	$n = 180$	$n = 44$	Co-dominant	AA + AG vs GG
Eosinophil count/mm^3 [a]	295 (150–500)	202.5 (150–47)	400 (192–577)	0.034[b]	0.038[c]
Skin test positivity (%)	54.4	61.1	75.0	0.033[d]	0.025[d]
IgE (kU/l)[a]	136 (47–411)	122 (38–448)	248 (73–581)	>0.05[b]	0.021[c]

[a] Median (Interquartile range).
[b] Kruskal Wallis test.
[c] Mann–Whitney *U* test.
[d] Chi-square test.

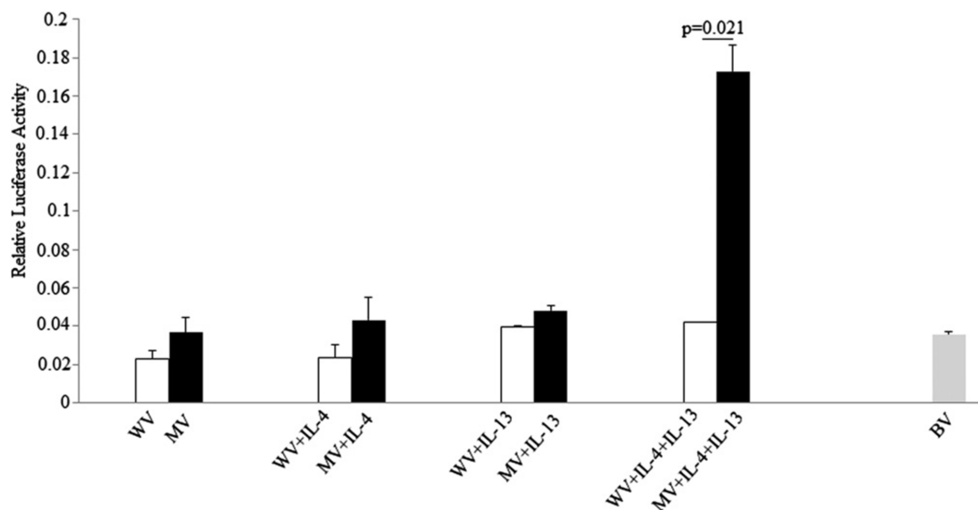

Figure 2 Promoter reporter assay: A549 cells were transiently transfected with wild type (WV) or mutant type (MV) reporter vector bearing a 1000 bp promoter region. Reporter activity was normalized by renilla luciferase pRL-TK vector. Open bars represent wild type and filled bars represent mutant type reporter vector. Basic vector (BV) was used as a control vector for experiment accuracy. Wild types vector bears A—A—T—A nucleotides and mutant type bears G—G—C—G nucleotides at rs118005507, rs7240852, rs189633658, rs116864116 respectively. Mean $\pm$ SEM, $N = 3$.

these two protease inhibitors have different substrate specificities and distinct biological activities,[19] it is not surprising that they have distinctive roles in the biology of airway diseases.

SCCA1 and SCCA2 are intracellular proteins. The source of the SCCA1 protein in plasma is not clear. Although it was initially suggested that the SCCA1 protein in biological fluids comes from either cell lysis or leakage,[40] Quarta et al. recently showed that the SCCA1 protein is actively secreted by transfected cells.[41] There is no membrane receptor described for the SCCA1 protein and the action of the extracellular SCCA1 protein on the other cells is unknown. SCCA proteins inhibit serine protease activity through irreversible suicide substrate-like mechanisms. In this mechanism serine protease recognizes the serine protease inhibitor from its reactive center loop (RCL). Hydroxyl group of serine residue located in the active site of serine protease attacks the RCL and forms a covalent bond between the active site and RCL. This interaction changes the conformation and deforms the active site of serine protease.[42]

Our study confirms previous studies that have reported increased systemic levels of the SCCA protein in atopic diseases such as asthma,[25] asthma exacerbation,[25,26] atopic dermatitis[24] and allergic rhinitis.[27] In our study, we could not find any association between SCCA protein levels and clinical parameters. It should be noted, however, that the levels of the protein is measured in the systemic circulation but not in the airways or airway tissue in this population composed of children. Therefore the results need to be confirmed locally in the airways.

Genetic arm of our study has shown that asthmatic children carrying the G allele at rs116864116 in the promoter region of SCCA1 has higher frequency of skin test

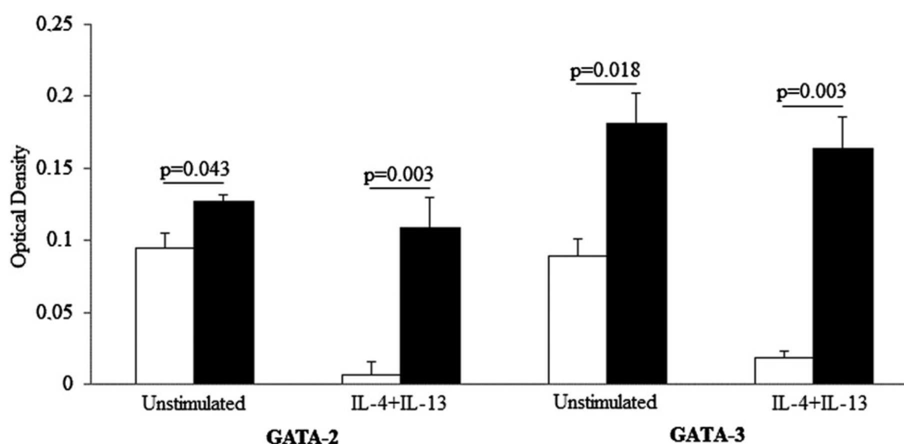

Figure 3 Transcription factor ELISA for GATA-2 and GATA-3 transcription factors. Open bars represent wild type probes and filled bars represent mutant probes. Wild types vector bears A—A—T—A nucleotides and mutant type bears G—G—C—G nucleotides at rs118005507, rs7240852, rs189633658, rs116864116 respectively. Mean $\pm$ SEM, $N = 3$.

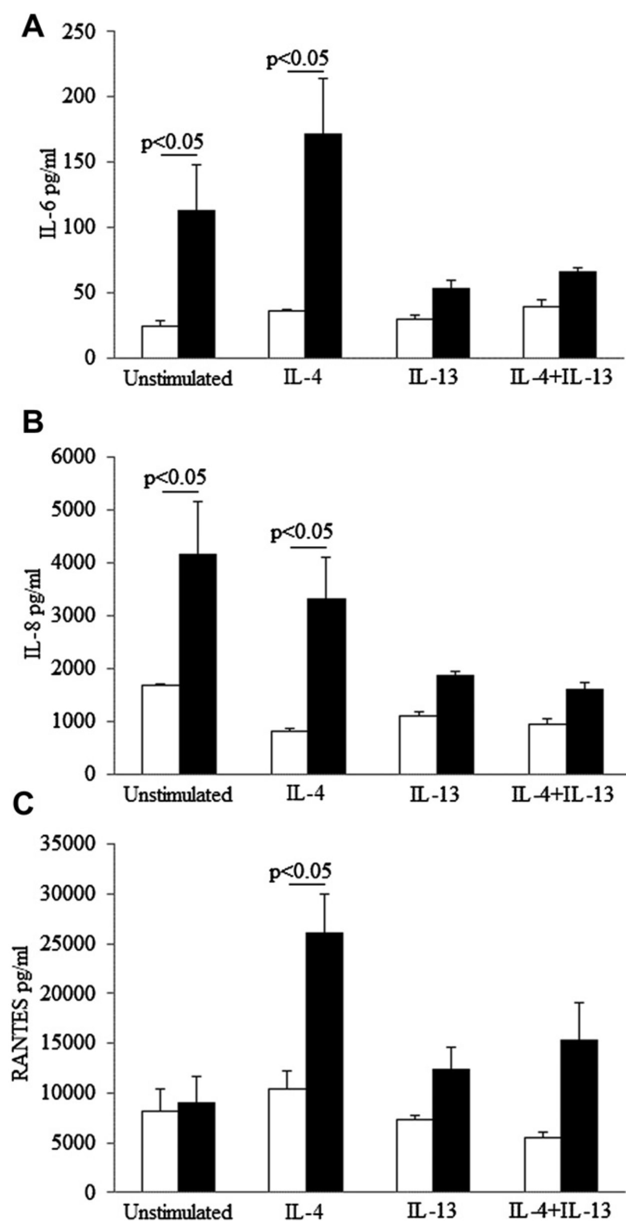

Figure 4 IL-6 (A), IL-8 (B) and RANTES (C) levels released from A549 cells transfected with empty (open bars) and SCCA1 vector (filled bars) (US = unstimulated cells, EV = empty vector, SCCA1 = SCCA1 expression vector). Mean ± SEM, $N = 3$.

positivity. Logistic regression analysis result further supported this observation and has shown that it is in fact independent of other possible confounding factors including IgE levels and eosinophil counts which may be associated with allergic diseases. Since previous studies have shown that systemic levels of SCCA are also elevated in allergic diseases other than asthma, taken together, these findings, including our results, suggest that the role of SCCA1 may not be disease specific but may be important in a variety of diseases that are related to atopy. On the other hand, serum levels of SCCA1 are not correlated with the genotypes in our study. However, since the most likely source of SCCAs in the extracellular fluid is cellular leakage related to cell disruption, the lack of a correlation may be due to the fact that the protein level in the extracellular compartment may not actually reflect the amount that is biologically active.

Our study also provides some mechanistic explanation regarding the effect of the polymorphisms in the promoter region. The presence of the G allele at rs116864116 confers higher promoter activity after stimulation with IL-4 and IL-13, two cytokines that are known to be elevated in allergic diseases, thus providing another linkage to the connection between SCCA1 and atopic diseases. This difference in the promoter activity may be due to the altered binding affinity of the transcription factors GATA-2 and GATA-3. The higher binding affinity of the mutant genotype for both GATA-2 and GATA-3 may also account for the increased promoter activity in the mutant reporter constructs. Both GATA-2 and GATA-3 are expressed in epithelial cells.[43,44] GATA-3 is an important activator IL-4, IL-5 and IL-13 and inhibitor of interferon γ.[44] This *in-vitro* observation can provide an explanation for the consistent association that we observed between the mutant genotype of the SCCA1 with the atopic indices such as positive skin testing and high IgE. Even though there is a clear effect of IL-4 and IL-13 on the mutant variant that can account for the *in-vivo* observations, our results do not provide an explanation for the observed decrease in the binding affinity of GATA-2 and GATA-3 to the wild type allele after IL-4 and IL-13 stimulation. One possible explanation may be the presence of another trans-acting element preventing the binding of GATA transcription factors to promoter region by binding this sequence itself. The possibility that the variants in other parts of the cloned region may be responsible for the observed difference cannot be excluded.

SCCA1 induced the production IL-6 and IL-8 and RANTES from the airway epithelium which are activators, attractants and growth factors for B and T cells, neutrophils, basophils and eosinophils[45–50] .Our results suggest that SCCA1 can activate almost all cell types that underlie the inflammation in asthma by increasing the secretion of IL-6, IL-8 and RANTES from airway epithelium. Interestingly, SCCA1 induced increase in these cytokines seems to be abolished by IL-13 even in the presence of IL-4. IL-4 and IL-13 have overlapping functions but their receptor interactions are different.[51] IL-4 and IL-13 receptor complex comprise of four types receptor chains: IL-13Rα1 and 2, IL-4Rα, and γ together they produce three types of receptor complexes one of which is common for both cytokines.[52] Although IL-13Rα2 chain is accepted as decoy receptor and regulates IL-13 level, it is also a negative regulator for IL-4 signaling.[53] Depending on the receptor complex, IL-13 cytokine may compete with IL-4 for binding to receptor.[54] Therefore, the suppression of IL-6 and IL-8 in the presence of SCCA1 and IL-13 may be due to a competition for the receptor and a decrease in the signaling of the IL-4 pathway.

SCCA1 acts differently than other protease inhibitors in this regard. For example, while non-covalent serine protease inhibitor benzamidine decreased IL-6, covalent serine protease inhibitors forskolin, dibutyryl cAMP and 4-(2-aminoethyl), benzene-sulfonyl fluoride increased IL-6 level in the cell culture supernatants.[55] We have recently shown that SPINK5, a serine protease inhibitor, induces not

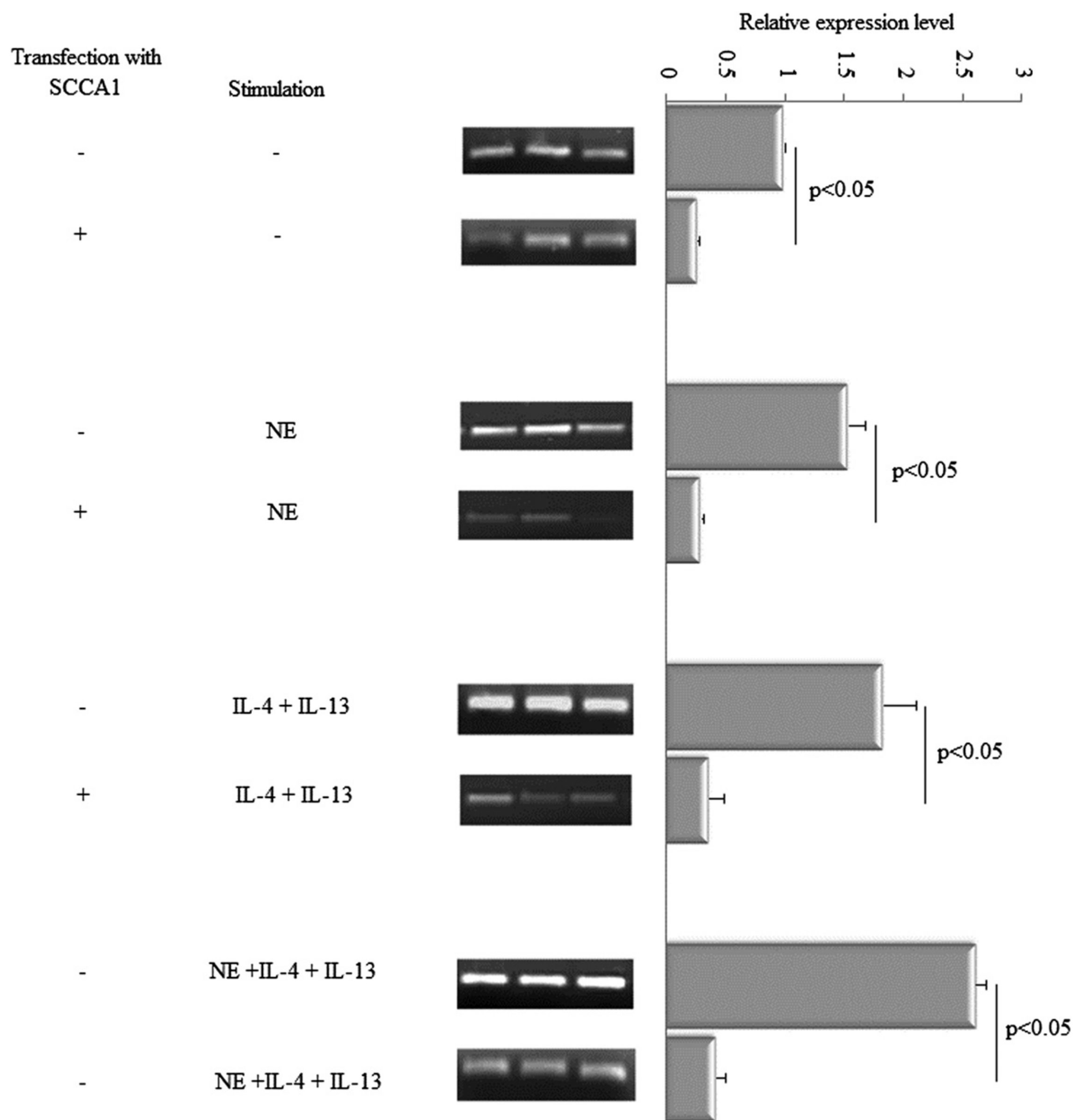

Figure 5 RT-PCR results for MUC5AC. Columns represent the band densities of MUC5AC gene/Cyclophilin (NE = Neutrophil elastase). Mean ± SEM, $N = 3$.

only IL-6 and IL-8 like SCCA1 but also RANTES.[37] It seems that the effects of protease inhibitors are independent from their protease related functions and can vary from one molecule to another.

Our results showed that SCCA1 suppresses mucus production, which is an important factor in the morbidity and mortality of asthma,[56] from the airway epithelium at the transcriptional level. Thus, the effect of SCCA1 on mucus production seems to be a protective one in asthma. This is in contrast to our previous observation that SPINK5 had no effect on mucus production by the airway cells.[37]

Supporting the previous observations,[57,58] our results suggest SCCA1 protein protects the cells from cell death induced by H_2O_2 by decreasing apoptosis. Attenuation of apoptosis through SCCA1 can occur through its protease inhibition properties in the upstream of caspase-3 pathway[57]; or it can occur by a c-Jun NH2-terminal kinase-1 related pathway which is independent from protease inhibitor activity.[58]

Taken together, the results of our study suggest that it is very difficult to assign SCCA either a protective or pro-pathogenic role in asthma. While certain effects such as up regulation of IL-6, IL-8 and RANTES by SCCA1 over-expression appear to be pro inflammatory in asthma, other effects such as protection against apoptosis and reduction in mucus production appear to be protective in asthma. Hence SCCA1 might be regulative effects on asthmatic complications. Alternatively SCCA1 upregulation may well be a protective mechanism in asthmatic to lung epithelial from repeated insults.

It should be noted that the physiological part of our study was done using A549 cell line which is not an excellent model to reflect the real biological events that in chronic airway diseases but are commonly used in *in-vitro*

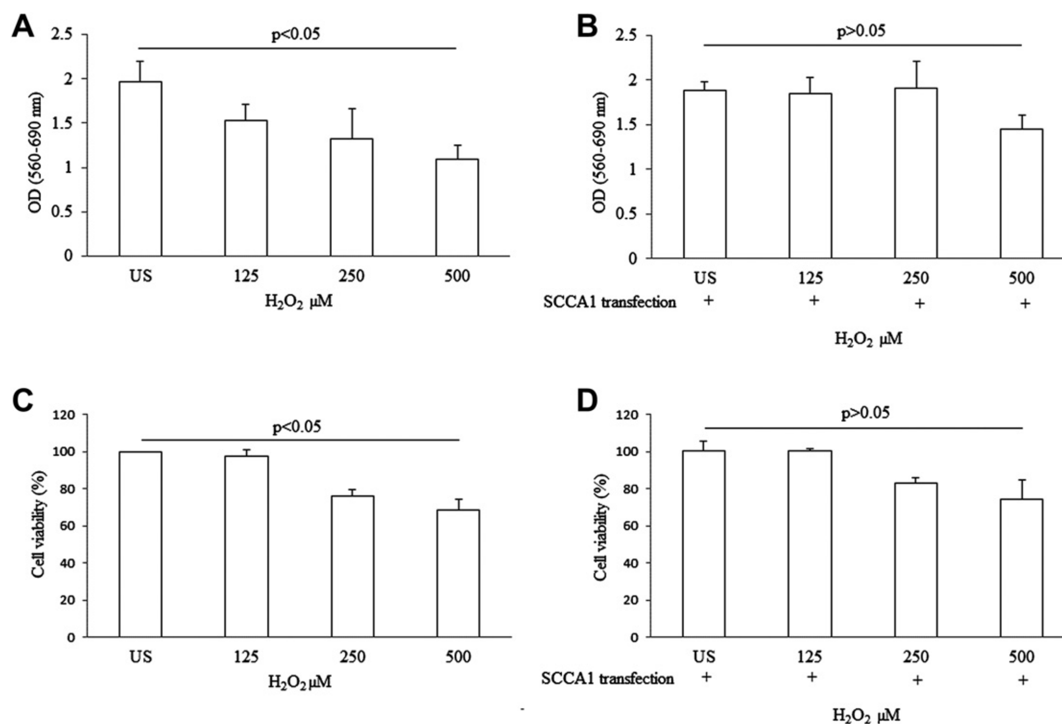

Figure 6 MTT assay (A and B) and Annexin V/PI staining (C and D) after stimulation with increasing doses of H_2O_2. Cells are either transfected with an empty (A and C) or with SCCA1 vector (B and D). US = unstimulated cells, SCCA1 = SCCA1 expression vector. Mean ± SEM, $N = 3$.

studies. Therefore, the results need to be verified with primary epithelial cells from asthmatic patients.

To conclude, our study shows that SCCA1 may be an important mediator in asthma and its actions on the airway epithelium are not limited to its protease inhibition properties. It can be protective or inflammatory depending on extra and intra-cellular signals and may result in quite diverse physiological outcomes.

Conflict of interest statement

The authors have no conflict of interest to declare.

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Appendix A. Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.rmed.2012.11.003.

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