

Original article

A nonsynonymous substitution of cystatin A, a cysteine protease inhibitor of house dust mite protease, leads to decreased mRNA stability and shows a significant association with atopic dermatitis

Background: Cystatin A (*CSTA*) is a strong candidate for atopic dermatitis (AD) because it maps to AD susceptibility locus on chromosome 3q21 and it does inhibit Der p 1 and Der f 1, major house dust mite cysteine proteases and environmental triggers for AD and asthma.

Objective: To examine any association between polymorphisms in *CSTA* and AD and study the effect on the *CSTA* mRNA expression level.

Methods: We identified three polymorphisms and characterized the linkage disequilibrium mapping of the *CSTA* gene. All three *CSTA* polymorphisms were genotyped in 100 AD patients and 203 matched controls. Subsequently, we performed transfection-based RNA stability assays.

Results: We found a significant association between the *CSTA* +344C variant and AD [odds ratio (OR) = 1.91; $P = 0.024$]. When further 61 control samples were genotyped. The association with *CSTA* +344C allele was enhanced OR = 2.13; $P = 0.006$. To test whether the *CSTA* +344 affected the *CSTA* transcriptional activity, the decay rates of RNAs transcribed from the *CSTA* +344C and *CSTA* +344T variants were investigated. COS-7 cells were transfected with a pcDNA3.1-*CSTA* +344C or a pcDNA3.1-*CSTA* +344T construct and cultured in the presence or absence of actinomycin D. Real-time RT-PCR revealed that *CSTA* +344C mRNA is more than two times less stable than the *CSTA* +344T mRNA ($P < 0.001$).

Conclusion: These results suggest that the *CSTA* +344C allele associated with unstable mRNA could result in failing to protect the skin barrier in AD patients from both exogenous and endogenous proteases

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Atopic dermatitis (AD) is a chronic inflammatory skin disease associated with cutaneous hyperactivity to environmental triggers such as house dust mites, which are innocuous to normal individuals (1). A major contribution to this hyperactivity is changes in the cutaneous and systemic immune response (2). Of equal importance is the presence of a defective epidermal barrier in AD because this allows penetration of environmental triggers to the cutaneous immune system (3). The structural integrity of the epidermal barrier is maintained by corneodesmosomes, which contain adhesion proteins, such as corneodesmosin, desmoglein, desmocollin and plakoglobin.

Abbreviations: AD, atopic dermatitis; *CSTA*, Cystatin A; LD, linkage disequilibrium; OR, odds ratio; SNP, single nucleotide polymorphism.

During desquamation, corneodesmosin is cleaved by endogenous proteases, including serine proteases such as stratum corneum chymotryptic enzyme, stratum corneum tryptic enzyme and cysteine proteases such as cathepsins. These proteases are inhibited by protease inhibitors such as elafin, secretory leukocyte protease inhibitor, lympho-epithelial kazal-type 5 serine protease inhibitor and cystatin A (*CSTA*) (4). An increase in the levels of a protease and/or decreased level of a protease inhibitor could lead to premature desquamation of corneocytes and thinning of the stratum corneum. This permits the penetration of irritants and allergens triggering a flare of the AD (5).

The epidermal barrier can also be broken down by exogenous proteases such as the cysteine proteases Der p 1 and Der f 1, from house dust mites, which are present

on the surface of the skin (6). After these proteases have broken down and penetrated through the epidermis, they can induce a Th1–Th2 switch (7, 8). House dust mite are major allergens involved in the expression of the disease, with Der p 1 and Der f 1 being the allergens responsible for ~50% of the immunoglobulin E (IgE) antibodies against mite in AD serum (6). In the stratum corneum, *CSTA* is cross-linked to molecules, such as loricrin and transglutinases (9). Recently, it was shown that in addition of inhibiting the action of endogenous proteases, *CSTA* is a potent inhibitor of exogenous proteases such as Der p 1 and Der f 1 in cultured keratinocytes (10). In this way, the presence of *CSTA* in sweat provides an important natural barrier to exogenous proteases. This barrier may also provide protection against the action of other exogenous proteases such as those produced by *Staphylococcus aureus* which is another important environmental trigger in AD (11). On the other hand, *CSTA* is located in AD susceptibility region on chromosome 3q. In recent years, family-based analyses have revealed four susceptibility loci for AD on chromosomes 1q21, 3q21, 17q25 and 20p (12) but only the locus on chromosome 3q21 has been duplicated in an independent study (13). Cystatin A contains three exons of 111, 102 and 226 bp, respectively. Recently a novel genetic variant was discovered in the *CSTA* gene at position +344, namely *CSTA* SNP3 (14), which has a potential functional relevance because it gives an amino acid change from Threonine (T) to methionine (M) at position 96.

In order to assess the potential role of polymorphisms in the *CSTA* gene and susceptibility to AD, we performed a case-control study on 100 AD patients and 203 matched controls. Additionally, we investigated the functional effect of a *CSTA* risk variant on the *CSTA* mRNA expression level.

Methods

Patients and controls

The 100 AD individuals who took part in this study were recruited from dermatology clinics in Sheffield, UK following approval from the local ethics committee. Each patient was individually examined by an experienced dermatologist (M.J.C) to confirm the diagnosis of AD and provided informed consent to testing. The clinical diagnosis of AD was made using the UK working party's diagnostic criteria for AD (15, 16). Briefly, an itchy skin condition (or parental report of scratching or rubbing by the child), plus three or more of the following: (i) history of flexural involvement, e.g. folds of elbows, behind the knees, fronts of ankles or around the neck (or cheeks in children under 4 years); (ii) a personal history of asthma or hay fever (or history of atopic disease in a first-degree relative in children under 4 years); (iii) a history of a generally dry skin in the last year; (iv) visible flexural dermatitis (or dermatitis involving the cheeks/forehead or outer limbs in children under 4 years); and (v) onset under the age of 2 years. Criteria use only clinical assessments and do not rely on IgE measurements. Morphology of the disease and family history of atopy has also been considered. Using the Pharmacia CAP system, total and specific serum IgE measurements

to common mixes of grasses, food, house dust mites and animals were made in all patients for whom there was total IgE data. The IgE level has been recorded for 30 atopy patients and IgE values in kU per litre BS 75/502 are as calculated by the Sheffield Supraregional Assay Service as shown in the protein reference unit handbook 1993. The radioallergosorbent test (RAST) score was used for specific IgE measurements. The specific IgE concentrations were measured in kU per litre (1 kU being equal to 2.4 ng IgE) (WHO-IgE standard 75/502) and expressed as a score (0–6, 0.35–100 kU per litre). Scores 0–1, which correspond to RAST score for specific IgE measurements of <0.35 and 0.35–0.7 units kU per litre, respectively, were classified as patients with normal IgE level. Patients with specific IgE >0.7 to >100 units kU/l were classified as patients with an elevated IgE level. An elevated total IgE was defined as a total serum IgE >2 SD above the age-adjusted mean; this was defined by the reference range from the Trent Regional Unit Immunology Laboratory at the Northern General Hospital, Sheffield. All individual patients with an elevated total IgE also had an elevation of at least one of the specific IgE groups. Seven patients did not have an elevated total IgE but did have an elevated nonspecific IgE level to one or more of the allergens in the four groups of animals, grasses, dust mite and foods tested (data not shown).

The control population was ethnically matched blood donors aged 18–60 from the North Trent region of the UK which is the same region from where the patients with atopic eczema were recruited (Caucasian, Northern English). The 203 samples were obtained from blood donors from the Trent Blood Transfusion service (Sheffield). The sex distribution of the control population was matched to that of the disease population. Patients were aged 34.8 ± 15.4 years, female : male ratio 1.13. Controls were aged 39.9 ± 14.9 years, female : male ratio 1.08. Genomic DNA was extracted from whole blood, obtained from the above individuals using standard protocols.

CSTA SNP genotyping

Genotyping for the *CSTA* gene (GenBank accession number: AB007774 and NM_005213) polymorphisms, T–190C, T+162C and C+344T were determined by PCR-restriction fragment length polymorphism analysis (PCR-RFLP) with mutated primers introducing a restriction site for RFLP analysis in dependence on the respective genotype. DNA from all available patients and controls was amplified by PCR carried out in a volume of 25 µl containing 100 ng of genomic DNA, 1 unit *Taq* polymerase (Qiagen Ltd, West Sussex, UK), 200 µM of each dNTP, x1 Qiagen buffer (10 mM Tris–HCl, 50 mM KCl, (NH₄)₂SO₄, 15 mM MgCl₂, pH 8.7), 0.6 µM of each primer. Samples were subjected to PCR amplification, in a 96-well Peltier thermal cycler (MJ Research, Waltham, MA, USA), using the following parameters: initial denaturation at 94°C for 3 min followed by 45 cycles of denaturation at 94°C for 30 s, annealing at 58–61°C for 30 s and extension at 72°C for 45 s, followed by a final extension step at 72°C for 10 min (Table 1). Restriction fragment length polymorphism analysis was performed in a volume of 30 µl containing 25 µl of the PCR product, 5U of the selected enzyme and the corresponding manufacturer's buffer at the appropriate temperature overnight (New England Biolabs, Beverly, MA, USA). Digestion fragments were resolved on 3% agarose gels, stained with ethidium bromide (Sigma; 5 µg/ml) and visualized on a UV transilluminator.

For the T–190C polymorphism, PCR using primers Pro-F. (5'-GAA-GACACATCCAGCCAAG-3') and Pro-Rmod (5'-GGAATTTG-GAAGAAAGAGGGGTTGGAGGATGAACC₋₁₈₈A-3'), created a *Bsr* I site by changing a T at position of –188 of the promoter

Table 1. Primers sequences and PCR conditions for *CSTA* variants at positions -190, +162 and +344

<i>CSTA</i> genetic variants	Forward primer 5'-3'	Reverse primer 5'-3'	Annealing temperature°C	MgCl ₂ mM
-190	GAAGACACATCCAGCCAAG	GGAATTTGGAAGAAAGAGGGTGGAGGATGAACCA	59	3
+162	GAAGAAAAACAAATGAGACAT	TGAGAGTCCACCACTTG	58	3
+344	GACCTGTGGCTCTCTCAC	GAACACTTTGGGTACATGCTGCTAAAAGCGC	60	2

sequence to G in the reverse primer. Restriction fragment length polymorphism analysis was performed after incubation with 5U of *Bsr* I at 65°C overnight. *Bsr* I does not cut the -190T allele (300 bp) and produces bands of 263 and 37 bp for -190C allele. In the case of T +162C polymorphism, PCR with primers Ex2-Fmod (5'-GAAGAAAAACAAATGAGACA₁₆₀T-3') and Ex2-R (5'-TGA-GAGTCCACCACTTG-3'), generated an *Nde* I restriction site by changing a T at position +160 of the exon 2 sequence to A in the forward primer. *Nde* I digestion resulted in the generation of two fragments of 214 and 21 bp for the +162T allele, as against a single 235 bp fragment with the +162C allele. For the C +344T polymorphism, PCR using primers Ex3-F (5'-GACCTGTGGCT-CTCTCAC-3') and Ex3-Rmod (5'-GAACACTTTGGGTACATG-CTGCTAAAAGCG₃₄₆C-3') created a *Bst*UI restriction enzyme site by changing the G at +346 on the *CSTA* sequence to a C on the reverse primer. *Bst*UI does not cut the 344T allele (228 bp) and produces bands of 198 and 30 bp for 344C. Positive and negative controls for the restriction enzyme digestion were included in each reaction. Each sample was genotyped twice to eliminate genotype errors. In addition, analysis was performed blindly with respect to case-control status. It should be mentioned here that control and patients samples were amplified in the same plate and genotype the same batch. There was a control for each genotype in each batch of enzymatic reaction. The additional 61 control samples were genotyped twice. They were controls for both amplification and genotype reactions.

Linkage disequilibrium analysis

The standardized coefficient of disequilibrium (*D'*) between all combinations of single nucleotide polymorphism (SNP) pairs was assessed using the EMLD software (Huang and Anderson, University of Texas, TX, USA), which calculates pair-wise linkage disequilibrium (LD) based on SNP genotype data from unrelated individuals (<http://cge.mdanderson.org/~qhuang/software/pub.htm>; accessed 20 February 2007).

Statistical analysis

A chi-squared test for trend of *CSTA* +344C was performed. The critical value to assess the significance across these findings is 0.05.

Generation of *CSTA* constructs

The constructs used for the transfection-based RNA stability assays contained the whole *CSTA* cDNA sequence (GenBank accession no. NM_005213). Total RNA was isolated from a skin biopsy of a normal heterozygous individual that was obtained with the patient's informed consent, using the RNAeasy kit (Qiagen, Valencia, CA, USA), which was then reverse-transcribed using the GeneAmp Gold RNA PCR Reagent kit (Applied Biosystems, Foster City, CA, USA). The encoding region of *CSTA*, including its 3'UTR, was then amplified using the primers *CSTA*-cDNAf (5'-ACTTCCCTG-

TTCACCTTTGGTTCCAGCATCCTGTCCAGC-3') and *CSTA*-cDNAr (5'-GCTTCTTTATTGATGGTTATATTTATCAGCAA-GGATCATGACTCAGTAGC-3'). The resulting *CSTA* cDNA fragments containing either the 344T allele or the 344C allele, as confirmed by DNA sequencing, were inserted into the pcDNA3.1 expression vector under the control of the CMV promoter/enhancer (Invitrogen, Carlsbad, CA, USA). The plasmid sequences and orientation were confirmed by DNA sequencing and restriction enzyme analysis.

Cell culture and transfections

The COS-7 cell line (SV40 Tantigen-transformed CV1, ECACC 87021302, Cambridge, UK) was maintained in Dulbecco's modified Eagle's medium (Gibco, Paisley, UK), supplemented with 10% Foetal calf serum (Gibco), 100 U/ml penicillin and 100 mg/ml streptomycin (Gibco) and 2 mM L-Glutamine (Gibco). Cells were kept in a humidified 5% CO₂ atmosphere at 37°C. Cells at 80% confluency (seeded the day before at 4 × 10⁵ cells/well in a six well plate) were then transfected with the *CSTA* constructs using the Polyfect transfection reagent (Qiagen). Cells were then incubated for 48 h to allow for gene expression, followed by treatment with 10 mg/ml of actinomycin D, transcription inhibitor. The cells were then grown for further 24 h before being harvested. All transfection experiments were performed in duplicate.

Real-time RT-PCR

Total RNA was extracted from transfected cells, before and after treatment with actinomycin D, using the Trizol reagent (Life Technologies, Grand Island, NY, USA) according to the manufacturer's protocol. To avoid contamination with genomic DNA the RNA isolated from transfected cells were treated with DNase I, using the DNA-free™ kit (Ambion, Austin, TX, USA). Reverse transcription was performed using the GeneAmp Gold RNA PCR Reagent kit (Applied Biosystems) and random primers. In order to quantify the expression of each gene transcript the resulting cDNA were used as templates for real-time quantitative PCR with gene-specific primers for *CSTA* (*CSTA*-cDNAf, *CSTA*-cDNAr), Neof (5'-TGCTCCTGCCGAGAAAGTATCCATCATGGC-3') and Neo-r (5'-CGCCAAGCTCTTCAGCAATATCACGGGTAG-3') to amplify the neomycin cDNA used as an internal control and GAPDH-f (5'-GAAGGTGAAGGTCGGAGTC-3') and GAPDH-r (5'-GAGATGGTGATGGGATTTC-3') to amplify the GAPDH. Each PCR product was detected with the double-stranded DNA-binding dye SYBR Green? I (Applied Biosystems). All reactions for each gene or *CSTA* allelic variant were carried out in triplicate using the ABI 7900HT sequence detection system (Applied Biosystems). A no-template control, in which sterile water was replaced for template RNA was used in each experiment. This control was used to subtract any fluorescence that is not related to PCR amplification. Quantification was performed by relating threshold cycle (*C_T*) values for detection of each PCR product to standard curves generated

from a reference cDNA preparation. Then the average from the CSTA C_T values was obtained from each reaction and the no-template control value was subtracted. The resulting CSTA C_T values were then normalized to the ones corresponding to Neomycin, a gene present in the recombinant plasmid and used as an internal standard in these experiments. The GAPDH house-keeping gene was also used as an internal control for initial total RNA quantity.

Results

We have demonstrated the presence of three single nucleotide polymorphisms, T-190C, T+162C and C+344T, located in the promoter, exon 2 and exon 3 regions, respectively, of the CSTA gene. Linkage disequilibrium analysis was performed using the EMLD program and the resulting pair-wise D' values have demonstrated that the three CSTA polymorphisms are in moderate LD with each other ($0.47 < D' < 0.63$) (Table 2). We then analysed the CSTA polymorphisms in 100 unrelated AD patients and 203 matched controls. Genotype distribution for the CSTA polymorphisms at positions -190, +162 and +344 was in Hardy-Weinberg equilibrium in both control and patient groups. A significant association was found between the common allele, CSTA +344C variant and AD [Odds ratio (OR) = 1.91, 95% confidence interval (CI) = 1.06–3.46, $P = 0.024$], suggesting that the rare allele, CSTA +344T has a protective effect in the general population against risk of developing AD. When we analysed the distribution of two other CSTA genetic variants at positions T-190C and T+162C, located in the promoter and exon 2 of the CSTA gene respectively, no association between these variants and AD was detected (Table 3). Genotyping of 61 additional control samples, for the CSTA C+344T SNP, giving a total of 264 control samples vs 100 AD, revealed a much stronger association between the CSTA +344C variant and AD [OR = 2.13 (1.20–3.77); $P = 0.006$]. We did not find any significant association with the CSTA -190 or +162 when control sample size was increased (data not shown).

In order to test whether the CSTA +344 polymorphism affect mRNA stability, we compared the decay rate of RNAs transcribed from the CSTA +344C and +344T variants. COS7 cells were transfected with the two pcDNA3.1-CSTA constructs. Each construct carried a CSTA +344C or CSTA +344T and a neomycin gene which was used to normalize the two transfection experiments. Total RNA was isolated from the transfected cells, before and after treatment with actinomycin D. Following DNaseI treatment, real-time RT-PCR was performed for CSTA, Neomycin and GAPDH genes. The PCR bands were quantified and GAPDH (internal control of initial total RNA quantity) showed that the ratio of CSTA +344C/+344T, before and after actinomycin D, was equal to 1 (data not shown). Analysis of two independent transfection experiments showed that

Table 2. Pair-wise linkage disequilibrium between CSTA SNP's as estimated by D' values

	T-190C	T+162C
C+344T	0.47	0.41
T-190C	–	0.63
T+162C	–	–

CSTA, cystatin A; SNP, single nucleotide polymorphism.

$D' = 1$ corresponds to complete disequilibrium.

Table 3. Single-SNP association analysis of CSTA polymorphisms with AD

CSTA SNP	Genotype		Allelic frequency		$P (\chi^2)$	OR (95% CI)
	Control	AD cases	Control	AD cases		
T-190C	151/50/2	81/19/0	0.87	0.9	0.15	1.47 (0.81, 2.65)
T+162C	86/90/27	34/55/11	0.65	0.62	0.35	0.70 (0.43, 1.15)
C+344T	143/58/2	82/18/0	0.85	0.91	0.02	1.91 (1.06, 3.46)

AD, atopic dermatitis; OR, odds ratio; CI, confidence interval.

The three values in the genotype columns indicate the numbers of homozygotes with respect to the major allele, heterozygotes and homozygotes with respect to the minor allele, respectively. Allelic frequencies were calculated with respect to the major allele (significant values are shown in bold). Note that P -values reported here are chi squared test for trend.

the CSTA +344C allele produces significant higher quantity of mRNA compared with the CSTA +344T allele. However, the relative stability of the CSTA +344C variant was $0.01 \times$ Neo, whereas the relative stability of the CSTA +344T was $0.20 \times$ Neo, suggesting that CSTA +344C encodes an mRNA transcript that decays more rapidly than the one encoded by the +344T variant (unpaired t -test two-tailed $P < 0.001$) (Fig. 1). The CSTA +344C mRNA was ~ 2.2 times less stable than the CSTA +344T mRNA. A similar trend was found by semi-quantitative RT-PCR (data not shown).

Discussion

In this study we found a strong association between the common allele of CSTA +344C allele and AD. However, no significant association was detected between two neighbouring genetic variants CSTA -190 and CSTA +162 and AD. The moderate LD between these two markers and CSTA +344 could explain the absence of association between CSTA -190 and CSTA +162 and disease. Interestingly, the control population was not selected to exclude people with a previous history of atopic eczema and as a result approximately 20% of the control population would have had atopic eczema either previously or currently. In the majority this would have been a previous history of atopic eczema because it is predominantly a disease of young children and the prevalence falls from 20% in children to 2% of adults.

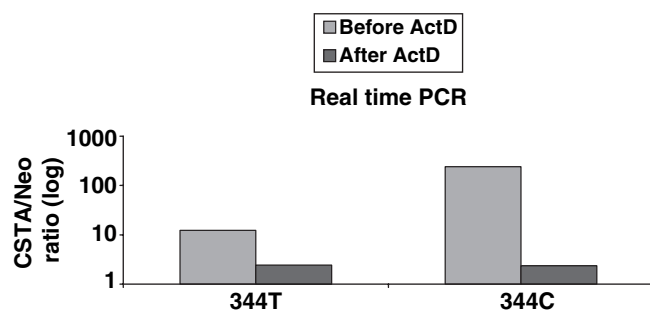


Figure 1. The C+344T nonsynonymous mutation in *CSTA* confers reduced mRNA stability as shown by a transfection-based RNA stability assay. Real-time RT-PCR analysis of the C+344T *CSTA* variants, before and after Actinomycin D treatment (*CSTA*, cystatin A; Neo, neomycin). GAPDH was used as an internal control of gene expression. Relative RNA stability of *CSTA* 344C and 344T variants, obtained by two independent transfection experiments. The RNA level of each *CSTA* variant was normalized with Neomycin and the relative ratio, before and after actinomycin D, is shown. There is a rapid decrease in the *CSTA*/Neo ratio following treatment with actinomycin D, suggesting that the risk 344C variant creates an unstable RNA transcript.

The individuals with atopic eczema within the control population would be likely to also have the *CSTA* +344C variant. This would result in a reduction in the difference in the frequency of the *CSTA* +344C variant between the disease and control population. Therefore, the association would be stronger than the one reported here. When the power for the study was calculated, we found that the power for 264 controls and 100 cases, OR = 2 and frequency of carrier = 0.3 was

62% and 82% at 1% and 5%, respectively, suggesting that larger sample size of control and patient groups is needed to increase the power of the study.

Real-time RT-PCR analysis of *CSTA* mRNA has shown that the *CSTA* +344C variant encodes an mRNA transcript that decays more rapidly than the one encoded by the +344T variant. Supposing that the translation efficiency is equal for both alleles, the *CSTA* +344C variant would therefore produce less protein and this would result in changes in the activity of skin barrier endogenous proteases (cathepsins B, L and H), exogenous house dust mite proteases (Der p 1 and Der f 1) and proteases derived from bacteria such as *Staphylococcus aureus*. The +344C variant in the *CSTA* protease inhibitor gene could therefore make a major contribution to the defective epidermal barrier in AD.

The integrity of the epidermal barrier is maintained by a balance between the levels of both endogenous and exogenous proteases and protease inhibitors. Cystatin A is a potent inhibitor of the endogenous cathepsins (B, H and L) within the epidermis and exogenous (Der p 1 and Der f 1) proteases from house dust mites. The Der p1 and Der f1 proteases can also induce a Th1–Th2 switching. The *CSTA* +344C variant may therefore represent an important contribution to both the defective epidermal barrier and Th2 switching in AD.

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