Genetic Association Between an AACC Insertion in the 3′UTR of the Stratum Corneum Chymotryptic Enzyme Gene and Atopic Dermatitis

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Atopic dermatitis is a disease with an impaired skin barrier that affects 15%–20% of children. In the normal epidermis, the stratum corneum chymotryptic enzyme (SCCE) thought to play a central role in desquamation by cleaving proteins of the stratum corneum (e.g., corneodesmosin and plakoglobin). Genetic variations within the SCCE gene could be associated with dysregulation of SCCE activity leading to an abnormal skin barrier. We screened the SCCE gene for variations and performed a case–control study on 103 atopic dermatitis patients and 261 matched controls. 16 synonymous single nucleotide polymorphisms (SNPs) have been identified and a 4 bp (AACC) insertion has been found in the 3′UTR. We performed an association study of the SCCE AACC insertion in the 3′UTR, and found a significant trend between the AACC allele with the two insertions and disease in the overall data set [odds ratio (OR) = 2.31; p = 0.0007]. The AACC insertion in the SCCE gene may result in a change to SCCE activity within the skin barrier. These findings suggest that SCCE could have an important role in the development of atopic dermatitis.

Key words: atopic dermatitis/stratum corneum/SCCE
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Several genes have been associated and/or linked to increased IgE production ± atopic dermatitis, including IL-4 and the high-affinity IgE receptor (Cookson *et al*, 1989; Marsh *et al*, 1994). We have previously demonstrated that 30% of children with clinically defined atopic dermatitis have neither an elevated total nor specific IgE (Murphy *et al*, 1999). During the past fifty years, there have been several changes in our environment, including increased exposure to soap, detergents, and house dust mites (Williams, 1995; Cork *et al*, 2002). Washing with soap reduces the thickness and number of cell layers in the stratum corneum of normal individuals and those with atopic eczema. This effect is more pronounced in those with atopic dermatitis (Al-Jaberi *et al*, 1984; White *et al*, 1987). The demonstration that up to 30% of children with clinically defined atopic dermatitis are not immunologically atopic led us to hypothesize that changes in genes that regulate the barrier function of the skin would be very important in the development of atopic dermatitis (Cork *et al*, 2002). These genetic changes could render the skin barrier more vulnerable to breakdown by environmental agents such as soap, detergents, and house dust mite.

In the normal epidermis, the constant thickness of the stratum corneum is maintained by a balance between the proliferation of the keratinocytes in the basal layer and desquamation from the surface of the stratum corneum (Egelrud, 2000; Pierard *et al*, 2000). Corneodesmosomes are structures involved in cohesion between the corneocytes of the stratum corneum. Proteolysis of the corneodesmosomes is a crucial event prior to desquamation (Lundstrom and Egelrud, 1988; Egelrud and Lundstrom, 1990). Stratum corneum chymotryptic enzyme (SCCE) is a serine protease localized to the extracellular space of the stratum corneum and is specific for keratinizing cells undergoing desquamation (Egelrud and Lundstrom, 1991; Egelrud, 1993; Sondell *et al*, 1994; Ekholm & Egelrud, 1998). Apart from its tissue localization, SCCE has several properties and characteristics, including its pH and inhibitor profile of catalytic activity, matching the basic prerequisites for a crucial involvement in desquamation under *in vivo* conditions (Lundstrom & Egelrud, 1991; Egelrud *et al*, 1993; Franzke *et al*, 1996). Transgenic mice overexpressing human SCCE developed changes in their skin similar to those seen in chronic atopic dermatitis (Hansson *et al*, 2002). The overexpression of SCCE initially may lead to a premature breakdown of the epidermal barrier. This would allow the penetration of irritants and allergens, which triggers an inflammatory response, and subsequently a reactive hyperplasia. The SCCE gene is therefore an important candidate gene for atopic dermatitis. In this study, we screened the coding region of the SCCE gene for genetic variations and performed a disease case–control association study in patients with atopic dermatitis.

Abbreviation: SCCE, stratum corneum chymotryptic enzyme

Results

To investigate the presence of polymorphisms in the SCCE gene, we sequenced all 5 exons from both directions in 20 atopic dermatitis patients and 20 controls as described. We identified a total of 17 polymorphisms, 12 in the introns, and 5 in the exons (Table 1). The first two were in exon 1, the other three were in exon 5, with one being a 4 bp insertion in the 3′UTR. None of the exonic variants gave an amino acid change. Sequence alignments, however, confirmed the presence of a 4 bp insertion (AACC) in the 3′UTR of the SCCE gene of an atopic dermatitis patient (designated as AE2). This is illustrated in Fig 1*a*, where the insertion is shown as GGTT on the sequence of the chromatogram as we used the reverse primer to sequence that specific sample. For the purpose of clear illustration of the two-repeat allele, we present another chromatogram obtained from a control individual, where one can see the absence of the second repeat (AACC) shown as GGTT on the reverse sequence of the chromatogram (Fig 1*b*).

Based on the above discovery, a PCR-based assay was designed, using specific primers that discriminate between individuals that harbor the one-repeat (AACC) allele and those that harbor the two-repeat (AACCAACC) allele. The primers were designed such that they would be complementary to the DNA sequence, being exactly the same as each other but with the difference that one of them would miss the second repeat (AACC).

Based on the above optimization, we screened healthy controls and atopic dermatitis patients in order to elucidate the allelic distribution of the two distinct alleles of the SCCE gene.

Table 1. Summary of polymorphisms found in SCCE (the bp numbers are based on NCBI accession numbers AF166330 for genomic sequence and L33404 for cDNA)

Location in gene	Base no. (genomic/cDNA)	Nucleic acid change
Exon 1(5′UTR)	3230/09	G → A
Exon 1	3290/69	G → A
Intron 1	3310	A insertion
Intron 1	3346	G → A
Intron 1	3419	G → A
Intron 2	3879	G → A
Intron 2	3902	A → G
Intron 2	3908	G → A
Intron 3	4980	G → C
Intron 3	5004	C → T
Intron 3	5041	C → T
Intron 4	5340	G → A
Intron 4	5409	T → A
Intron 4	7327	A → C
3′UTR	7630/890	4 bp(AACC) insertion/deletion
3′UTR	7674/930	A → G
3′UTR	7687/943	A → C

The allelic distribution of the SCCE AACC/AACCAACC polymorphism in both atopic dermatitis and control groups is shown in Table 2. There was a significant increase in the frequency of rare allele (AACCAACC) in the patient group (0.56) compared with the control group (0.43). As a dose effect was evident for this polymorphism, the odds ratio (OR) of homozygotes for the rare allele was significantly greater than that of the heterozygous [OR 95% confidence interval (CI) 2.32 (1.31,4.11) and 1.01 (0.57,1.79), respectively]. Therefore, a χ^2 test for trend was carried out. A significant association was found between SCCE- AACCAACC variant and atopic dermatitis [OR = 2.31 (95% CI (1.42, 3.76)); p = 0.0007] (Table 2).

We then stratified patients according to their IgE level, elevated IgE ($IgE_{elevated}$), and non-elevated IgE ($IgE_{non\text{-}elevated}$), respectively. An elevated IgE was defined as raised two standard deviations above the age-adjusted mean. Interestingly, the OR increased in patients who did not have an elevated $IgE_{non\text{-}elevated}$ [(OR = 4.47 (1.49, 13.38)); p = 0.0039]. But the association was non-significant when patients with an elevated IgE ($IgE_{elevated}$) were analyzed separately.

Discussion

In this study, we identified 17 SNPs within the SCCE gene, 5 of which are located in the exons (see Table 1). We have demonstrated that homozygotes for the 3′UTR 4-bp insertion are more than two times more likely to develop atopic dermatitis than individuals with the common allele. It is important to emphasize that the control population of adults contained a significant proportion of individuals who would have had atopic dermatitis as a child (up to 15%), suggesting that the risk for developing atopic dermatitis reported here is underestimated.

We studied the 3′UTR AACC insertion because we believed that it could disturb the intercellular secondary structure of the SCCE mRNA. It has been shown previously that determinants of mRNA stability are frequently positioned in the 3′UTR of the gene and any mutation could affect the expression levels of the protein (Bilenoglu *et al*, 2002). The AACC insertion could increase the half-life of SCCE mRNA, leading to an increased enzymatic activity of SCCE. Interestingly, genetic variations and nucleotid acid insertion in the 3′UTR of genes encoding glycoprotein PC-1 and protein tyrosine phosphate 1B (PTP1B), encoding inhibitors of the insulin signaling pathway have shown that these variations stabilize PC-1 and PTP1B and increase insulin resistance (Frittitta *et al*, 2001; Di Paola *et al*, 2002). Subsequently, overexpression of SCCE would result in enhanced proteolysis of the corneodesmosomal proteins, producing a thin, defective epidermal barrier.

SCCE is capable of cleaving corneodesmosin (Cdsn) and plakoglobin *in vitro* (Simon *et al*, 2001). The Cdsn (52–56 kDa) is involved in cohesion between corneocytes and its proteolysis by SCCE leads to the production of different proteolysis fragments (48–46, 36–30 and 15 kDa) (Simon *et al*, 2001). This process takes place during differentiation

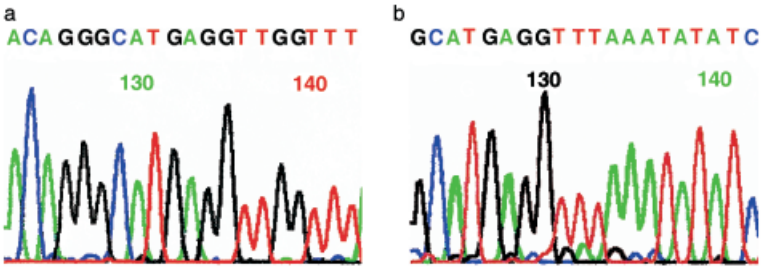

Figure 1
Chromatograms of a control and an atopic dermatitis 3′UTR sequences (*a*) Part of the chromatogram of the AE2 sequence (atopic dermatitis patient) that corresponds to exon 5 of the SCCE gene, from an atopic dermatitis patient. The 4 bp repeat is indicated by an arrow. (*b*) Part of the chromatogram of Poly9 (control) sequence where the second repeat (AACC) is absent. The 4 bp single repeat, indicated by an arrow, is shown as GGTT on the sequence of the chromatogram because we used the reverse primer to sequence the purified PCR product.

of keratinocytes and an increase of SCCE activity could enhance Cdsn proteolysis, and lead to an impaired epidermal barrier as observed in the skin of patients with atopic dermatitis.

We have previously reported that up to 30% of children with clinically defined atopic dermatitis do not have a raised non-specific or specific IgE, and are therefore not immunologically atopic (Murphy *et al*, 1999). We postulated that a primary genetically determined defect in the skin barrier could explain the development of atopic dermatitis in these children (Cork *et al*, 2002). Our findings of an association of an AACC insertion in the 3′UTR of the SCCE gene and atopic dermatitis support this hypothesis. The observation that the association of the SCCE AACC insertion was more significant in the patients who did not have an elevated IgE level is an important observation despite the small number of patients with recorded IgE in this study (n = 30). A defective epidermal barrier would allow the penetration of both irritants and allergens, which may induce the production of pro-inflammatory cytokines (e.g., TNF-α and IL-1α) within the skin (Archer, 2000). This could result in the development of eczematous lesions without the need for an IgE-mediated inflammation. Specific allergens such as DerP1 from house dust mites could also penetrate through a defective epidermal barrier triggering a transient Th1 to Th2 switch and specific IgE production. These IgE levels may remain elevated for months or years but may have returned to normal by the time IgE measurements were made. A primary defect in the skin barrier could therefore predispose to the development of eczematous lesions by two mechanisms.

SCCE has also been proposed to be a candidate for psoriasis and we have shown an increase in the amount of both pro-SCCE and SCCE in lesional psoriatic skin compared with non-lesional or normal skin (Simon *et al*, 2002). We therefore tested the association of the SCCE 3′UTR insertion in a psoriatic cohort (n = 100) and found no significant association between psoriasis and the SCCE 3′UTR insertion (data not shown). These findings suggest that SCCE is unlikely to be involved in psoriasis and that the overexpression of SCCE protein observed in lesional psoriatic skin could be the consequence of an unspecific response to hyperproliferation and inflammation in lesional skin.

Genome-wide scans have identified linkage to atopic dermatitis on several chromosomes including 1q21, 17q25, 20p, and 3q21 (Lee *et al*, 2000; Cookson *et al*, 2001) but none of these loci contains the SCCE gene, which is located on 19q13.3. One explanation is that the effect of SCCE in atopic dermatitis is too small to be detected by linkage. Another explanation is that a high proportion of children with atopic dermatitis included in these studies had an elevated IgE level, which exceeded 70% in the Lee *et al*, 2000 study. The power to detect a locus that may contain the SCCE gene would be diluted since most of the association we reported here was with patients who did not have an elevated IgE level.

In conclusion, our findings indicate a statistically significant association between an SCCE AACCAACC insertion

Table 2. Allelic distribution of the SCCE AACC insertion in control and patient groups

	AACC	Heterozygotes	AACC AACC	Total
Control	**96**	**105**	**60**	**261**
	29	**32**	**42**	**103**
Atopic dermatitis	**OR (95% CI)**	**p-value**	**Analysis performed**	
OR_{HET}	1.01 (0.57, 1.79)	NS	—	
OR_{HOM}	2.32 (1.31, 4.11)	0.0037	—	
$OR_{OVERALL}$	2.31 (1.42, 3.76)	0.0007	Trend (recessive model)	
$OR_{OVERALL}$	1.48 (0.90, 2.44)	NS	Carriage (dominant model)	

Heterozygotes, homozygotes, and overall data set odds ratios (OR_{HET}, OR_{HOM} and $OR_{OVERALL}$ respectively) with 95% confidence intervals (CIs) for the SCCE 3′ UTR AACC insertion. Trend was carried out weighted by the number of putative disease susceptibility allele in each genotype group (recessive model). For the dominant model, a carriage of AACCAACC allele was carried out.
NS, non-significant.

and atopic dermatitis, which could play a central role in the development of the disease. The effect of SCCE could be more prominent in patients who do not have an elevated IgE level. This suggests that in these patients a defective skin barrier could be the primary event in the development of atopic dermatitis. Further studies of SCCE mRNA and/or protein, however, are required to strengthen the association between SCCE and atopic dermatitis and to provide further insights into the pathophysiological mechanisms of atopic dermatitis.

Materials and Methods

Patient and control individuals The 103 atopic dermatitis individuals who took part in this study were recruited from dermatology clinics in Sheffield and Nottingham, UK following approval from the local ethics committees. Each patient was individually examined by an experienced dermatologist (M.J.C, H.C.W, R.M) to confirm the diagnosis of atopic dermatitis and provided informed consent to testing. The clinical diagnosis of atopic dermatitis was made using the UK working party's diagnostic criteria for atopic dermatitis (Hanifin and Rajka, 1980; Williams *et al*, 1993, 1995). These 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. IgE level has been recorded for 30 atopy patients. IgE values in kU per liter BS 75/502 are as calculated by the Sheffield Supraregional Assay Service as shown in the protein reference unit (PRU) handbook 1993. The RAST score was used for specific IgE measurements. The specific IgE concentrations were measured in kU per liter (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 liter). Scores 0–1, which correspond to RAST score for specific IgE measurements of <0.35 and 0.35–0.7 units kU per liter, 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 greater than 2 standard deviations 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 non-specific IgE level to one or more of the allergens in the 4 groups of animals, grasses, dust mite, and foods tested (data not shown).

DNA from 261 healthy controls, ethnically matched to the disease population (Caucasian, Northern English), used in this study were obtained from blood donors from the Trent Blood Transfusion service (Sheffield). Genomic DNA was extracted from whole blood, obtained from the above individuals, using standard protocols and stored in 96-well microtiter plates, with each well containing 500 μL of DNA (100ng per μL).

SNP detection The exon–intron structure of SCCE was retrieved from the NCBI database (accession number; AF166330). To facilitate polymorphism analysis, we amplified all 5 exons of the SCCE gene from 20 atopic dermatitis individuals and 20 controls in separate polymerase chain reaction products, using the following SCCE-specific primers: exon 1—forward 5′GAATGCTTTCTCC-TTCTCAGCTC3′, reverse 5′CGCAT CTCATCTGGGGAAC3′, 60°C; exon 2—forward 5′TGTCTCTGTCCATCTCTGACTCTGGGA3′, reverse 5′ACTGCCCTTCCACCCCCATAG3′, 62°C; exon 3—forward 5′TGCACACCCTCTCTGTCACAG3′, reverse 5′TGTTGTTCAGGCT-CCTGGTTCC3′, 61°C; exon 4—forward 5′AGCCTGAACAACA-GCCCT TCTG3′, reverse 5′GAGAATGAGGTGGAAAAAGCTGAG3′, 60°C; and exon 5—forward 5′TCCCTAGCACT AGCTCTCCCA-TTAG3′, 5′GAACGTCCAGTTCCAGTGTTTGAG3′, 57°C at 2mM $MgCl_2$ for all the exons. Amplification was achieved using Pfx polymerase (Gibco, Paisley, UK), using 100 ng control or patient DNA as template, in a 20 μL reaction in a 96-well Peltier thermal cycler (MJ Research, Waltham, Massachusetts), under standard conditions. Amplified products were run on a 1.5% agarose gel and the desired bands were excised and purified using glasswool tubes (Sigma). The purified DNA was sequenced using an ABI Prism 377 DNA sequencer (Applied Biosystems, Foster City, California). To detect possible polymorphisms, the resulting sequences were compared with each other and with the corresponding sequence of SCCE, using CLUSTALW (EBI; www.ebi.ac.uk/CLUSTALW).

Genotyping For the allelic discrimination of the 4 bp (AACC) insertion, two different primers were designed, one (I/DR1: 5′GGTTTATCAACAGGGCAT GAGGTTTAAAT3′, 61°C) composed of one AACC repeat and the other (I/DR2: 5′GGTTTATCA-ACAGGGCATGAGGTTGGTT3′, 60°C) with two AACC repeats. The following PCR conditions were used: 2 μl Pfx polymerase buffer ($\times$ 1) (Gibco; $\times$ 10), 1.0 μL $MgSO_4$ (2.5 mM) (Gibco; 50 mM), 1.6 μL dNTPs (10 mM) (Promega, Southampton, UK), 2 μL PCR enhancer solution ($\times$ 1) (Gibco; $\times$ 10), 0.06μl Pfx polymerase (Gibco; 250 U), 1 μL primer F 5′CACTAGCTCTCCCATTAGTCCCC3′ (1.5 μM), 1 μL primer R (I/DR1 or I/DR2) (1.5 μM), 1 μL DNA template (100 ng per μL), and 10.34 μL of sterile H_2O, in a 96-well Peltier thermal cycler (MJ Research), under the following conditions: 98°C for 5 min (1 cycle), 97°C for 1 min, 60–61°C for 30 s, 72°C for 1 min (35 cycles), and 74°C for 5 min and 4°C hold. Three hundred and sixty-four samples including 103 atopic dermatitis patients and 261 controls have been genotyped for the SCCE 3′UTR insertion. Possible genotyping errors have been considered by re-typing 20% of the patients and control samples. We found zero error.

Statistical analysis Disease and control groups were compared using 2×3 tables. In the control group, the allelic distribution of SCCE AACC allele/AACCAACC allele polymorphism was in Hardy-Weinberg equilibrium. To investigate the possibility of a dose effect, the odd ratios (ORs) for the heterozygotes and homozygotes were calculated separately by comparing their risk with that for individuals homozygous for the alternative allele. A dose effect was evident because the ORs for the individual homozygous for the rare allele (AACCAACC) were greater than individual heterozygous for the same allele. Therefore, a χ^2 analysis for trend was carried out, weighted by the number of putative susceptibility alleles in each genotype group and Fisher's exact p-value was calculated.

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