CCDC132 is highly expressed in atopic dermatitis T cells

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Abstract. The analysis of genes preferentially expressed in the peripheral blood cells of atopic dermatitis patients may provide information on the molecular pathogenesis of the disease. We employed differential display PCR to clone a new gene (AB100163) with 99% homology to *coiled-coil domain containing 132, transcript variant 1 (CCDC132)* (NM_017667) (aliases, *FLJ20097, FLJ23581, KIAA1861* and *MGC176659*). Full-length *CCDC132* of approximately 4 kbp encodes mRNA expressed in almost all tissues, in particular brain tissue and skeletal muscle. A homologous gene has also been identified in mice. Using Western blot analysis, 111 kDa CCDC132 protein was detected in two human T-cell lines, MOLT-4 and Jurkat, and in the human cervical adenocarcinoma cell line HeLa. Quantitative RT-PCR revealed transcription levels of *CCDC132* in the T cells of atopic dermatitis patients to be higher than in those of normal individuals. This suggests that changes in *CCDC132* expression may be involved in the course of atopic dermatitis.

Introduction

Atopic dermatitis (AD), a chronic and relapsing inflammatory skin disease that often begins in infancy, is characterized by pruritus, elevated serum IgE levels and peripheral blood eosinophilia. A complex interplay between genes and environmental factors is likely involved in AD pathogenesis. A two-stage model of the pathogenesis of AD has been proposed, with T cells playing a critical role in both physiological and pathological immune responses. The analysis of genes differentially expressed between AD patients and normal individuals and between the different phases of AD may provide information on the molecular pathogenesis of the disease. To identify the genes involved in allergic diseases, we analyzed transcription profiles in peripheral blood immune cells from patients with allergic diseases, and found many genes that were differentially expressed between patients and healthy individuals (1,2). During differential display (DD) gene expression studies on peripheral blood CD3+ T cells from AD patients, we observed that the level of expression of a gene later designated *coiled-coil domain containing 132, transcript variant 1 (CCDC132)* was markedly higher in AD patients than in normal controls. However, the DD sequence showed homology to several genomic sequences. We overcame this problem by selecting the genomic sequence with the highest degree of homology and proving the actual existence of the predicted gene by RT-PCR. Based on this sequence, further analysis was carried out by EST clustering and with exon search software. This approach resulted in the cloning of an approximately 4-kbp full length cDNA (AB100163), which is 99% homologous to *CCDC132*. Hence, although the *CCDC132* DD sequence was homologous to the genomic sequence, we successfully cloned and detected the expression of transcript and protein encoded by the *CCDC132* gene.

Materials and methods

Study population. Patients with allergic disease and healthy individuals were recruited for participation in the study. Patient profiles and clinical parameters have previously been described in detail (1). Patients with AD were diagnosed according to the criteria of Hanifin (3). Diagnosis of atopic asthma and classification of asthma severity were based on the Asthma Prevention and Management Guidelines (4). Written informed consent to participate in the study was obtained from all participants. The study was conducted in accordance with the guidelines established by the National Research Institute for Child Health and Development.

Isolation of the CCDC132 gene by DD analysis. The methods for the DD analysis of human peripheral blood T cells from allergic patients have previously been described in detail (1). A slightly modified version of the fluorescent DD method

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Abbreviations: CCDC132, coiled-coil domain containing 132, transcript variant 1; AD, atopic dermatitis; DD, differential display; PBMC, peripheral blood mononuclear cells; TCR, T-cell receptor

Key words: CCDC132, atopic dermatitis, T cell

described by Ito *et al* was used in this study (5). Transcripts in CD3+ cells were analyzed by DD PCR using different primer sets in a combination of 3'-anchored oligo-dT primers and arbitrary decamer primers. The DNA fragment obtained by DD PCR was further elongated by PCR cloning using a kit based on the 5'-RACE method (Clontech). A human leukocyte cDNA (Clontech) was used as a template for PCR.

Cloning of the CCDC132 gene. Exon prediction was carried out using exon searching software (GENSCAN, GRAIL, Gene Finder or ER). The presence of the predicted exon sequence was confirmed by PCR using the forward primers 1153-143U17 (5'-GAAAAGCCCTCAAGAAA-3') and 1153-3'-207U18 (5'-GAGAACAGCCAAGTGACC-3'), and the reverse primers 1153-359L21 (5'-TTGTCTCTATACGCCTCTAAT-3') and 1153-3'-896L21 (5'-TTTTTCCAAGAAGTCGATAAG-3'), template cDNA synthesized from total RNA prepared from human peripheral blood-derived T cells and peripheral blood mononuclear cells (PBMC), as well as human peripheral blood leukocyte-derived poly (A) RNA (Fig. 1A).

EST clustering revealed the presence of a clustered sequence, the existence of which was confirmed by PCR using the primers 1153EST-F (5'-GGGTCATTTGTGTAGTGGCTCGG-3') and 1153EST-R (5'-CCTCCTCCAGCATTTCACTTAACCG-3').

A GT2 full-length testis cDNA library (Invitrogen) was screened by plaque hybridization with the GENE TRAPPERII System (Invitrogen) using a PCR-amplified probe (position 64-347 bp at *AB100163*; 284 bp in length).

Northern blot hybridization. For Northern blot screening of a variety of human and mouse tissues for *CCDC132* mRNA, commercially-available human and mouse MTN blot kits (Clontech) were used. Two fragments of *CCDC132* were amplified by PCR with the two pairs of primers, 1153EST-F and 1153EST-R, or with 1153LW2-1841U21 (5'-GAAACTCTA AAAAGCAGGAAG-3') and 1153R_2570L18 (5'-GGGTAT GCGAACTCTCTT-3') from a human T-cell cDNA library. The 649-bp and 764-bp fragments were labeled with ^{32}dCTP using a random primer labeling kit (Takara) and used as probes. Hybridization was carried out using ExpressHyb™ Hybridization Solution (Clontech) according to the manufacturer's protocol.

Cell culture. MOLT-4, Jurkat and HeLa cells were cultured in RPMI1640 medium containing 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 μg/ml).

Western blotting. MOLT-4, Jurkat and HeLa cells were lysed in M-PER (Thermo Scientific) containing protease inhibitor (Complete, Roche). Soluble lysates were resolved by 4-20% gradient SDS-polyacrylamide gel electrophoresis and subjected to immunoblot analysis using 0.5 μg/ml anti-CCDC132 monoclonal antibody (M01) (Abnova) and Anti-Mouse IgG, HRP-Linked Whole Ab Sheep (GE Healthcare).

Preparation of leukocyte cDNA samples. Leukocyte cDNA samples from five healthy volunteers were prepared as previously described in detail (6).

Real-time quantitative RT-PCR. Real-time RT-PCR for the quantitation of gene expression was performed using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems). Based on the ORF of *CCDC132*, primers and a dual-labeled fluorogenic probe (TaqMan Probe) were designed using the computer program Primer Express 1.0 (Applied Biosystems). The primer sequences were 5'-AAAGCCCTC AAGAAAGCCTCA-3' (1153-F2 forward), 5'-GGTCACTTG GCTGTTCTCGAA-3' (1153-R2 reverse) and the probe sequence was 5'-TGATCTTGGTGCCATAGAGAGTCTCCG G-3', located in the 5' region of *CCDC132*. The quantitative PCR reaction was performed using a TaqMan PCR Reagent Kit according to the manufacturer's protocol (Applied Biosystems). Sample cDNAs, equivalent to 5 ng of starting RNA, were used for each reaction in a 96-well PCR plate. The fragment that was amplified by PCR using the forward and reverse primers and the human leukocyte cDNA library used as a template was cloned into a plasmid. The resulting plasmid DNA was used to prepare absolute standards.

Levels of β-actin mRNA were used as an internal standard for each sample. β-actin was quantified in each freshly isolated sample as an absolute value. The copy number of β-actin was averaged for all such samples and the ratio of absolute to average β-actin values was used to normalize the copy number of the target sequence.

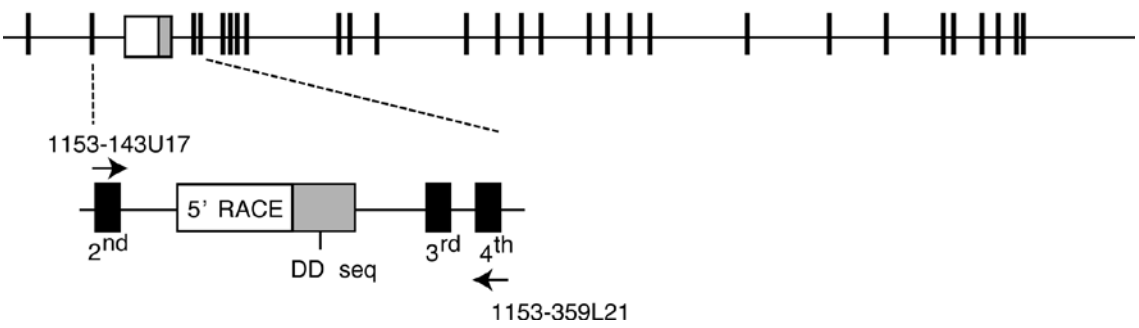
Statistical analysis. The Wilcoxon rank-sum test was used for comparisons between the normal controls and patient groups. When making multiple comparisons between groups, significant intergroup variability was first established using the Kruskal-Wallis test. The Tukey method was then used for intergroup comparisons. Probability values of $P<0.05$ were accepted as significant. SAS system software (SAS) was used throughout the analyses.

Results

Isolation of the DNA fragment by DD analysis and cloning of CCDC132. Peripheral blood CD3+ cells were prepared from 59 subjects (12 healthy volunteers, 23 AD patients and 24 asthmatic patients). DD PCR identified a 184-bp DNA fragment amplified using an anchor primer 5'-GT_{15}A-3' and an arbitrary primer 5'-TGACCTGAGT-3' as being expressed at a higher level in allergic patients than in normal individuals. To confirm the results of the DD PCR, the transcription level of the 184-bp DNA fragment in the same RNA samples was examined by quantitative RT-PCR, and was confirmed to be expressed at a significantly higher level in allergic patients than in the healthy controls ($P<0.05$, data not shown).

The 184-bp DNA fragment was further elongated to obtain a DNA fragment of 2.0 kbp. However, a BLAST search on a public database revealed that the genomic sequence (AC002453) showed the greatest homology to a 2.0 kbp sequence. Therefore, for the genomic sequence (AC002453), exon prediction was carried out, and a putative gene was found. The 2.0-kbp sequence was identified as being in the intron region of the putative gene. The presence of the predicted exon sequence (199 bp) was confirmed by PCR using 1153-143U17 and 1153-359L21 as the forward and reverse primers, respectively (Fig. 1A). The sequence was shown to be part of the gene and consisted of the 2nd, 3rd and 4th exons in Fig. 1A.

A



B

MQKIKSLMTRQGLKSPQESLSDLGAIESLRVPGKEEFRELREQPSDPQAEQELINSIEQVYFSVDSFDIVKYEL
EKLPP***VLNLQELEAYRDKLKQQQAAVSKKVADLILEKQ*** PAYVKELERVTSLQTGLQLAAVICTNGRRHLNIA
KEGFTQASLGLLANQRKRQLLIGLLKSLRTIKTLQRTDVRLSEMLEEEDYPGAIQLCLECQKAASTF***KHYSCI***
SELNSKLQDTLEQIEEQLDVALSKI CKNFDINHYTKVQQ**AYRLLGKT** QTAMDQLHMHFTQAIHNTVFQVV
LGYVELCAGNTDTKFQKLQYKDLCTHVTPDSYIPCLADLCKALWEVMLSYYRTMEWHEKHDNEDTASASE
GSNMIGTEETNFDRGYIKKKLEHGLTRIWQDVQLKVKTYLLGTDLSIFKYDDFIFVLDIISRLMQVGEEFCGS
KSEVLQESIRKQSVNYFKNYHRTRLDELRMFLENETWELCPVKSNFSILQLHEFKFMEQSRSPSVSPSKQPV
STSSKTVTLFEQYCSGGNPFEIQANHKDEETEDVLASNGYESDEQEKSAYQEYDSDSDVPEELKRDYVDEQT
GDGPVKSVSRETLKSRKKSDYSLNKVNAPILTNTTLNVIRLVGKYMQMMNILKPIAFDVIHFMSQLFDYYLY
AIYTFFGRNDSLESTGLGLSSSRLRTTLNRIQESLIDLEVSADPTATLTAAEERKEKVPSPHLSHLVVLTSGDT
LYGLAERVVATESLVFLAEQFEFLQPHLDAVMPAVKKPFLQQFYSQTVSTASELRKPIYWIVAGKALDYEQ
MLLLMANVKWDVKEIMSQHNIYVDALLKEFEQFNRRLNEVSKRVRIPLPVSNILWEHCIRLANRTIVEGYAN
VKKCSNEGRALMQLDFQQFLMKLEKLTDIRPIPDKEFVETYIKAYYLTENDMERWIKEHREYSTKQLTNLVN
VCLGSHINKKARQKLLAAIDDIDRPKR

Figure 1. Cloning of the *CCDC132* gene. (A) Genome view of the *CCDC132* gene. *CCDC132* is located on chromosome 7q21.3 and consists of 28 exons (closed boxes). The DD sequence (shaded box) is located in an intron between the 2nd and 3rd exons. We first cloned the 2nd, 3rd and 4th exons predicted by gene prediction software using PCR with 1153-143U17 (forward) and 1153-359L21 (reverse). (B) The CCDC132 964 amino acid sequence was deduced from the cDNA sequence. CCDC132 has an ATP_GTP binding-site motif A at position 259-266 aa (underlined) and two coiled-coil sequences at positions 80-112 and 214-244 (italics).

From the public databases, a 524-bp EST sequence (AI793062) identical to the 199-bp sequence was found. EST clustering revealed the presence of a clustered sequence, the existence of which was confirmed by PCR. A 601-bp PCR amplified fragment was obtained and found to be identical to the clustered sequence.

Exon analysis of the genomic sequence (AC002453) was pursued based on the 601-bp sequence. For the hypothetical exon sequence predicted to be downstream of the 601-bp sequence, PCR was performed with 1153-3'-207U18 and 1153-3'-896L21 primers. This PCR-amplified 636-bp sequence contained the predicted exon sequence. By assembling the previously clustered 601-bp sequence with the 636-bp sequence obtained here, the 824-bp sequence of the gene as a whole was obtained.

The 3596-bp full-length cDNA clone was identified by plaque hybridization screening using a PCR-amplified probe (position 64-347 bp in AB100163; 284 bp in length). A BLAST search on a public database revealed that 3596 bp of the cloned cDNA (AB100163) had 99% homology to the cDNA of *CCDC132* (NM_017667). The *CCDC132* gene was 3625 bp long, with an ORF region at 129-3023 bp, corresponding to a region at 86-2980 bp of the cloned cDNA (AB100163). With the exception of this nucleotide sequence, no other sequence with a known function was found to be homologous to the amino acid sequence encoded by *CCDC132*. Therefore, *CCDC132* was considered to encode a 964 aa protein with no functional motif, except an ATP_GTP binding site in the 259-266 aa region and coiled-coil sequences at 80-112 aa and 214-244 aa (Fig. 1B). These may play a role in interactions between proteins. In mice, the human *CCDC132* homolog is *BCL2 like protein* (NM_024260). There is 94% homology between the two sequences.

Expression of CCDC132 in tissues. A *CCDC132* ~4.0 kbp message was detected in various tissues, including immune-related tissues and cancer cell lines (Fig. 2A). *CCDC132* mRNA was detected using not only the 57-705-bp probe, but also a 1887-2650-bp probe (data not shown), and was identical in size to the cloned *CCDC132* cDNA. Although *CCDC132* has been reported to have a 1216-bp 3' truncated isoform, *CCDC132*, *transcript variant 2* (NM_024553), this was not detected in our study. Furthermore, when mouse mRNA blots were hybridized with the human *CCDC132* 1887-2650-bp probe, we detected ~4.0-kbp mRNA in various tissues (Fig. 2B). Although in human subjects abundant *CCDC132* expression was mainly observed in brain tissue and skeletal muscle, in mice it was predominantly expressed in the heart, brain and kidney.

Identification of endogenous CCDC132 protein in human T-cell lines. Using Western blot analysis, CCDC132 protein

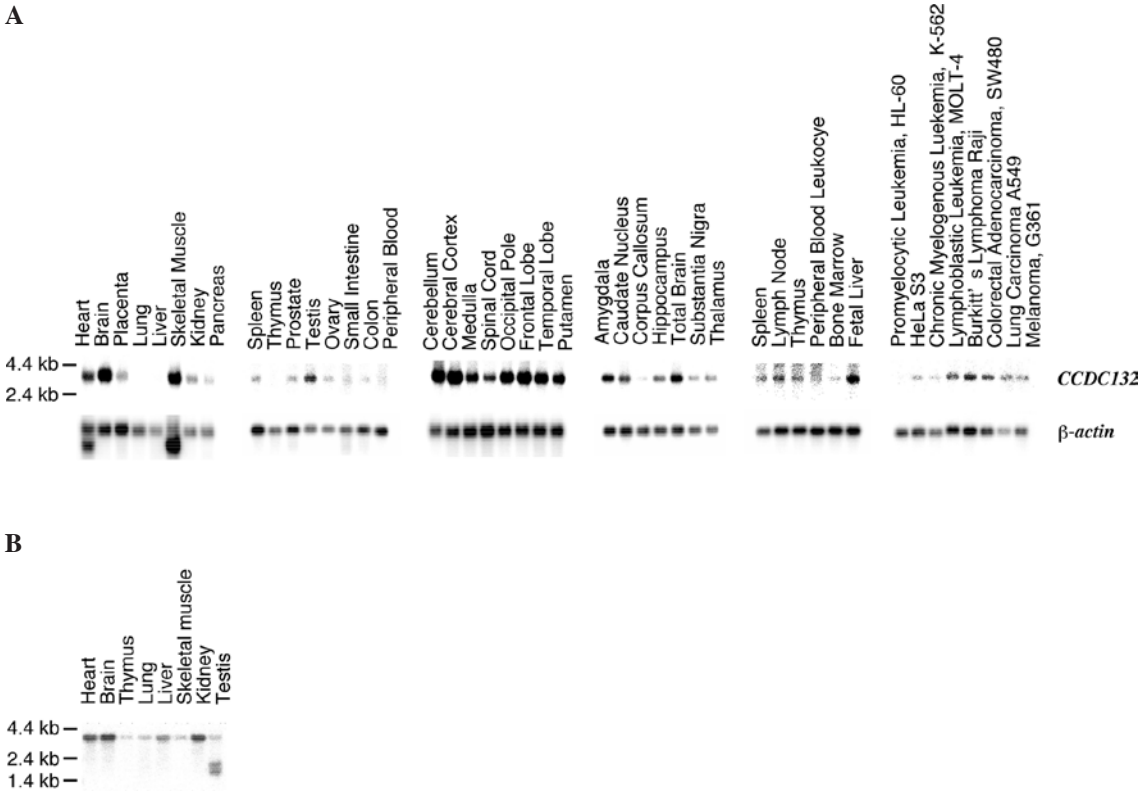


Figure 2. Expression of *CCDC132* mRNA in various human and mouse tissues or cell lines. RNA kb molecular size markers are shown at left. (A) A human multiple tissue Northern blot was probed with a fragment of the *CCDC132* gene (57-705 bp). *CCDC132* mRNA was expressed in most of the human tissues, particularly in brain tissue and skeletal muscle. The β-actin probe was hybridized to the same blot for comparison and is shown at the bottom of each blot. (B) A mouse multiple tissue Northern blot was probed with a fragment of the *CCDC132* gene (1887-2650 bp). The mouse homologue of *CCDC132* mRNA was almost the same size as human *CCDC132* and was also expressed in most mouse tissues, particularly in the heart, brain and kidney.

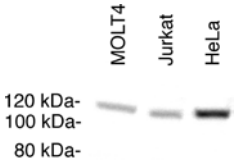


Figure 3. Expression of CCDC132 protein in human T-cell lines. Protein kDa molecular size markers are shown at left. MOLT-4, Jurkat and HeLa cell lysates (50 μg protein each) were loaded onto a 4-20% SDS-PAGE. Mouse monoclonal antibody to CCDC132 was used at 0.5 μg/ml.

was detected not only in the human T-cell lines MOLT-4 and Jurkat, but also in the human cervical adenocarcinoma cell line HeLa (Fig. 3). Predictably with a primary amino acid sequence, the protein size was almost 111 kDa.

High transcription levels of CCDC132 in T cells from AD patients. To reconfirm the results of differential expression analysis of the 184-bp DNA fragment, the transcription

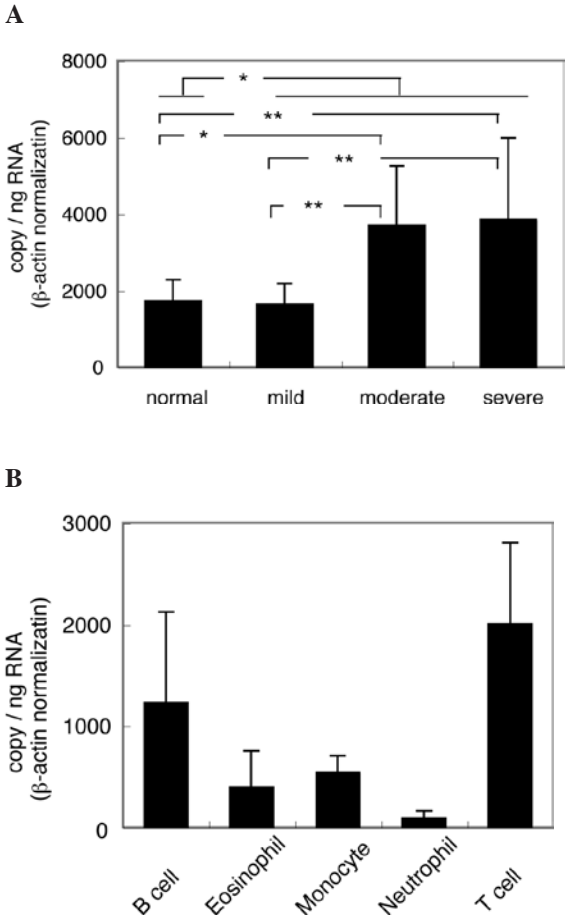


Figure 4. Transcription levels of *CCDC132* in peripheral blood leukocytes. (A) *CCDC132* transcription levels in CD3+ cells from healthy controls and AD patients with mild, moderate or severe disease. *CCDC132* gene expression levels were significantly higher in T cells from AD patients than in those from healthy controls. *P<0.05, **P<0.01. Results are presented as the mean ± SD. (B) *CCDC132* transcription levels were examined in subsets of PBMC cells from five healthy individuals. The *CCDC132* gene was most highly expressed in peripheral blood T cells. Results are presented as the mean ± SD.

levels of *CCDC132* in the CD3+ cells from AD patients were measured by quantitative RT-PCR using primers and a probe from the ORF region. As the cDNA samples used for the DD study were no longer available, transcription levels were measured in another set of cDNA samples that had been prepared previously and independently of the DD study. The set consisted of individual cDNAs from the CD3+ cells of 10 healthy volunteers and 30 AD patients (1). Expression of *CCDC132* mRNA was significantly increased in T cells from a group of 30 AD patients with all levels of disease severity compared to T cells from healthy controls (P=0.028). *CCDC132* mRNA expression was also significantly increased in T cells from patients with moderate or severe AD compared to T cells from normal individuals and from patients with only mild AD. These results reveal that *CCDC132* gene expression is increased in AD patients (Fig. 4A).

CCDC132 transcription levels in different subsets of peripheral blood leukocytes were examined by quantitative RT-PCR. The *CCDC132* transcript was found to be highest in T cells (Fig. 4B). We also investigated T-cell subsets, but *CCDC132* mRNA was expressed at the almost same level in Th1 compared to Th2, and in CD8+, CD4+, CD4+ CD45RA+ and CD4+ CD45RO+ compared (data not shown).

Discussion

The *CCDC132* gene was identified on the basis of higher expression in the T cells of AD patients compared to normal controls. Because the DD sequence was located in the intron region of the predicted gene, we used bioinformatics methods to clone a whole new gene (AB100163), which had 99% homology to *CCDC132* (NM_017667). *CCDC132* is believed to encode a 964 aa protein with no functional motif, except an ATP_GTP binding site in the 259-266 aa region and coiled-coil sequences at 80-112 aa and 214-244 aa. The function of *CCDC132* is unknown; however, our results suggest for the first time that it encodes a protein involved in allergic pathogenesis.

CCDC132 gene expression levels were significantly higher in T cells from AD patients compared to normal healthy controls. *CCDC132* mRNA expression was also significantly increased in T cells from patients with moderate or severe AD compared to T cells from patients with mild AD. These results indicate that the product of the *CCDC132* gene may serve as a marker of allergic irritation and the severity of symptoms. T cells play a key role in the immune system. In allergic reactions, they are activated by Ag presented by dendritic cells, and are guided to inflammatory sites by adhesion molecules, cytokines and chemokines (7). As a result, high *CCDC132* expression in AD patients indicates that, during TCR-mediated T-cell activation, proliferation and migration, *CCDC132* may be among the proteins that promote or suppress the allergic reaction.

In conclusion, the present study demonstrated that *CCDC132* gene expression is significantly higher in T cells from AD patients than in those from normal healthy control subjects.

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