

ORIGINAL PAPER

Towards profiling the gene expression of fibroblasts from atopic dermatitis patients: human 8K complementary DNA microarray

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Clinical and Experimental Allergy

Summary

Background High-throughput technologies, including DNA-chip array, have been used to search for the genes that are dysregulated in human diseases. The atopic dermatitis (AD)-associated genes are gradually being reported; however, the differentially altered gene expression profiles of atopic fibroblasts have not been well elucidated.

Objective We wanted to gain more insights into AD and to find candidate genes, especially in regards to the role of fibroblasts in the pathogenesis of AD.

Methods cDNA microarray (8K) profiling of the primary cultured AD patients-derived fibroblasts was conducted by a pooling method of the recruited 22 normal controls, the 10 extrinsic type (ADe) patients and the 10 intrinsic type (ADi) patients. SAM analysis of the microarray results (2-fold cut-off) was conducted to select the candidate genes. Quantification by real-time PCRs confirmed the array data in the randomized paired samples (normal vs. ADe $n = 10$; normal vs. ADi $n = 10$).

Results We listed the 22 up-regulated and 95 down-regulated genes in the AD fibroblasts. Real-time PCR results showed that several genes such as hyaluronan synthase 2 (HAS2), TNF- α -induced protein 6 (TNFAIP6) and IL-8 were matched with the array results with statistical significance.

Conclusion These results suggest gene expression profiles that are associated with AD and this implied that fibroblasts may play important roles in the AD pathogenesis. We provided new insights into three candidate genes such as HAS2, TNFAIP6 and IL-8 with respect to their involvement in AD disease.

Keywords atopic dermatitis, cDNA microarray, HAS2, IL-8, pooling method, primary cultured fibroblasts, real-time PCR, TNFAIP6

Submitted 19 August 2005; revised 11 January 2006; accepted 27 January 2006

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Introduction

Atopic dermatitis (AD) is a chronic inflammatory skin disease that is accompanied with itchy dry skin and flexural dermatitis. The number of AD patients has increased with the world's modernization and industrialization, and AD patients have been experiencing severe quality of life issues. AD is a complex disease that is associated with environmental and genetic factors [1–3]. External allergens such as food and house dust mites and also non-allergic inducers such as *Staphylococcus aureus* colonization and irritants are known to be involved in AD. As AD is a systemic disorder, it simultaneously triggers asthma and allergic rhinitis as well as food allergies, and

the patients suffering with AD show elevated levels of serum IgE and increased counts of peripheral eosinophils [4]. Although most AD patients have high serum IgE levels, one subgroup of AD patients lacks the sensitization to aeroallergens or food allergens. Two types of AD have been defined, and they are the extrinsic and intrinsic types. The extrinsic type (ADe) is associated with IgE-mediated sensitization and it accounts for 70–80% of the AD patients, while the intrinsic type (ADi) is not involved with IgE-mediated sensitization and it is seen in 20–30% of AD patients. For the pathogenesis of AD, several cells such as T lymphocytes (Th1 and Th2), Langerhans cells (LCs), eosinophils and keratinocytes have been regarded as important as these cells are directly related with several

blood factors such as cytokines, chemokines and IgE, and they are also related with microbial infection [2, 5, 6].

Villagomez *et al.* [7] have recently found that the skin fibroblasts from AD patients expressed a higher level of inducible protein 10 (IP-10), a CXC chemokine subfamily, compared with the healthy controls. Moreover, atopic fibroblasts are more sensitive to TNF- α stimulation, and the IP-10 levels in the AD patient's plasma were higher than that in the normal control donors. They suggested that besides the keratinocytes, fibroblasts are also able to produce IP-10 and they are possibly involved in the pathogenesis of AD. It has been known that eotaxin is a key mediator in the development of tissue eosinophilia, and the fibroblasts are a major source of eotaxin. Huber *et al.* [8] have reported that the IKK-2/I κ B α /NF- κ B pathway plays a critical role for C-C chemokine eotaxin-1 receptor and the eotaxin-1 expression in fibroblasts; this suggests a critical link to the AD pathogenesis. We have recently profiled the differentially expressed protein patterns in AD fibroblasts vs. normal fibroblasts by using the proteomic approach [9]. As a result, we revealed that some proteins, including the post-translationally modified proteins, were thought to be involved in the pathogenesis of AD. Taken together, these previous experimental results as well as our recent proteomic study have implied that atopic fibroblasts are somehow importantly associated with the dermal inflammation and they are involved in AD disease. However, the role of dermal fibroblasts in AD patients has not been well elucidated. Therefore, we need more information regarding which genes are dysregulated in the atopic fibroblasts.

In this study, we tried to profile the AD-associated genes with using human 8K cDNA microarray and we suggest several important candidate genes that are associated with AD. Our recent proteomic investigation and the results from our present microarray suggest that fibroblasts also play an important role in the pathogenesis of AD. In this regard, this new study can enlarge our knowledge about AD and some of the genes that are distinctively altered in AD-derived fibroblasts.

Materials and methods

Subjects

AD skin samples were collected from non-asthmatic atopic patients according to the method of our previous reports [9, 10]. Non-atopic control subjects who had neither a personal/family history nor any signs of atopic diseases were selected for the study. Punch biopsies of 5 mm diameter were individually obtained from the lesional skin of both the ADe and ADi patients. We recruited 10 ADe patients, 10 ADi patients and 22 normal specimen donors. The mean values for each group's total IgE level,

eosinophilia count, age and SCORAD score were 1546 U/mL, 670/ μ L, 26.6-year old and 54.34 for the ADe group and 105 U/mL, 252/ μ L, 27.1-year old and 44.45 for the ADi group, respectively. The gender of all samples was male. An informed consent was obtained from all the patients and the control individuals. This study was approved by the Samsung Biomedical Research Institute Medical Ethics Committee.

Cell culture

The specimens were briefly sterilized in 70% ethanol, minced and then treated with dispase for overnight at 4 °C. The dermis was separated and placed in a solution containing 0.05% trypsin and 0.025% ethylene diamine tetraacetic acid for 15 min at 37 °C. After vigorous vortexing, the cells were pelleted and re-suspended in DMEM containing 10% fetal bovine serum and 1% antibiotic-antimycotic (Gibco, Rockville, MD, USA). The cells were grown at 37 °C and 5% CO₂ in an incubator.

Complementary DNA microarray analysis

The total RNAs were individually isolated from the fibroblasts that were derived from the ADe patients ($n = 10$), the ADi patients ($n = 10$), and the normal donors ($n = 22$) with using an easy-BLUE™ kit (Intron, Seoul, Korea). Quality control for RNA sample was carried out by performing spectrophotometry and agarose gel electrophoresis. The individually prepared 22 normal RNA samples, 10 ADe RNA samples and 10 ADi RNA samples were correspondingly pooled at the same concentration to obtain three kinds of normal, ADe and ADi pooled samples, and we conducted further cDNA template synthesis and labelling for each of the pooled samples. cDNA labelling was conducted by performing reverse-transcription of total RNA in the presence of amino-allyl-dUTP, and this was followed by coupling of Cy3 dye for the normal control samples and coupling Cy5 dye for the AD samples. GenePloer Twinchip Human-8K (Digital Genomics, Seoul, Korea) was hybridized with a mixture of fluorescently labelled cDNAs from the control and AD samples at 42 °C for 16 h in the presence of hybridization solution that contained 25% formamide, 5 $\times$ SSC, 0.1% SDS, 25 μ g/mL Cot-1 DNA, 10% dextran sulphate, and then the slides were properly washed and dried. The slides were scanned with a Scanarray Lite (PerkinElmer Life Science, Billerica, MA, USA). We performed image analysis to obtain the gene expression ratios by using a GenePix Pro 3.0 (Axon Instruments, Union City, CA, USA). The logged gene expression ratios were normalized by LOWESS regression [11]. The eight sets of microarray data were analysed by using SAM with four sets of data being analysed for the ADe type and four sets of data being analysed for the ADi type, respectively, according to the

previous report of Tusher et al. [12]. The genes showing significant expression changes (> 2 -fold) were selected with a cut-off of 5% of the q -value. Our microarray data has been submitted to the Gene Expression Omnibus.

Real-time polymerase chain reaction

cDNA synthesis was carried out using M-MLV RT kit (Intron) according to the manufacturer's instructions with using the total RNAs extracted from 10 normal, 10 ADe and 10 ADi fibroblasts of the same donors who were recruited for the microarray study. RT-PCR was performed with an ABI PRISM[®] 7900 Sequence Detection System (Applied Biosystems, CA, USA). The reaction mixture for each gene contained 12.5 μ L of SYBR Green Master Mix (Applied Biosystems), 0.5 μ L cDNA and 0.5 μ L of 10 pmol of each primer in a final volume of 25 μ L. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was employed for the control quantification with using the same reaction mixture and conditions. The PCR amplification condition were as follows: 50 °C for 2 min and 95 °C for 10 min, and this was followed by 40 cycles at 95 °C for 30 s and then 60 °C for 30 s, and 1 cycle at 72 °C for 1 min. During the last steps, a temperature gradient between 95° and 60 °C was created for the analysis of the dissociation curves. The GAPDH primers were 5'-TGCACCACTGCTTAGC-3' for the forward primer and 5'-GGCATGGACTGTGGTCATGAG-3' for the reverse primer. Each of the 96-well plates contained serial dilutions of cDNA (0.25, 0.5 or 1 ng), and these were used to generate the relative standard curves for the target genes. Each sample, including the control, was tested in triplicate. The forward and reverse primers for hyaluronan synthase 2 (HAS2) were 5'-CTCCGGGAC-CACACAGAC-3' and 5'-TCAGGATACATAGAACTCT-CACA-3', respectively (the expected PCR product size was 86 bp); the forward and reverse primers for IL-8 were 5'-ACTGAGAGTGATTGAGAGTGGAC-3' and 5'-AACCTCTGCACCCAGTTTTC-3', respectively (the expected PCR product size was 112 bp); the forward and reverse primers for TNFAIP6 were 5'-GGCCATCTCGCAACTTACA-3' and 5'-CAGCACAGACATGAAATCCAA-3', respectively (the expected PCR product size was 64 bp); the forward and reverse primers for proteoglycan 1 (PRG1) were 5'-TACACAGATATCCAGGAAAAAAGG-3' and 5'-TGGATGTGTTTGTCTACAAGTATGA-3', respectively (the expected PCR product size was 125 bp); the forward and reverse primers for chemokine CXC motif ligand 12 (CXCL12) were 5'-TAGTGCTCCATGGCATA-3' and 5'-GATGTGTAATGGTCCCCCTG-3', respectively (the expected PCR product size was 138 bp); and the forward and reverse primers for lysyl oxidase-like 1 (LOXL1) were 5'-TGGATGAAAACCA CAGGGAT-3' and 5'-TTTATTGAGCCAGACCTGG-3', respectively (the expected PCR product size was 146 bp). Primers were designed by Lasergene software (DNASTAR, Inc., Madison, WI, USA). The primers were verified by a

single gel band of the expected size, DNA sequencing and the results from the dissociation curve analysis.

Reverse transcriptase polymerase chain reaction

The cDNA templates were correspondingly used after conducting the real-time PCR. The primer sequences for the target genes were designed on the basis of the GENBANK accession sequences with using the Primer 3 program (<http://frodo.wi.mit.edu/>). The forward and reverse primers for HAS2 were 5'-AGAGCACTGGGACGAA GTGT-3' and 5'-ATGCACTGAACACACCCAAA-3', respectively (the expected PCR product size was 245 bp); the forward and reverse primers for IL-8 were 5'-CTGCGCC AACACAGAAATTA-3' and 5'-ATTGCATCTGGCAACCC TAC-3', respectively (the expected PCR product size was 238 bp); and the forward and reverse primers for TNFAIP6 were 5'-TCACATTTTCAGCCACTGCTC-3' and 5'-AGACCGT GCTTCTCTGTGGT-3', respectively (the expected PCR product size was 205 bp). The reaction mixture for the PCR of each gene contained a 1 μ L aliquot of the RT reaction, 1 μ L of each primer and 17 μ L of *i*-Master mix PCR solution (Intron). The final reaction volume was 20 μ L. PCR was conducted under the following conditions: (1) an initial denaturation step for all samples at a temperature of 94 °C for 2 min, (2) a denaturation step at 94 °C for 30 s, an annealing step at 60 °C for 20 s, and an extension step at 72 °C for 60 s for 30 cycles, and this was followed by a final extension step at 72 °C for 5 min. All the PCR products were confirmed by sequencing with the corresponded forward primers with using BigDye[™] Terminator Sequencing Kits (Applied Biosystems) according to the manufacturer's instructions.

Statistical analysis

The statistical significance between the normal control group and the two types of AD groups was analysed by using the Wilcoxon-rank sum test. A P (an associated probability)-value of less than 0.05 was considered significant.

Results

cDNA microarray (human 8K) profiling in the primary cultured AD patients-derived fibroblasts was conducted with using a pooling method, as is shown in the plots of M vs. A, Fig. 1. We performed eight sets of microarray analyses (four sets for the paired normal vs. ADe samples and four sets for the paired normal vs. ADi samples). The RNAs for the 22 normal subjects, the 10 ADe type samples and the 10 ADi-type AD samples were pooled in the procedures before conducting the chip-array. From these results, we listed the 22 up-regulated candidate genes and the 95 down-regulated candidate genes (Tables 1 and 2) in

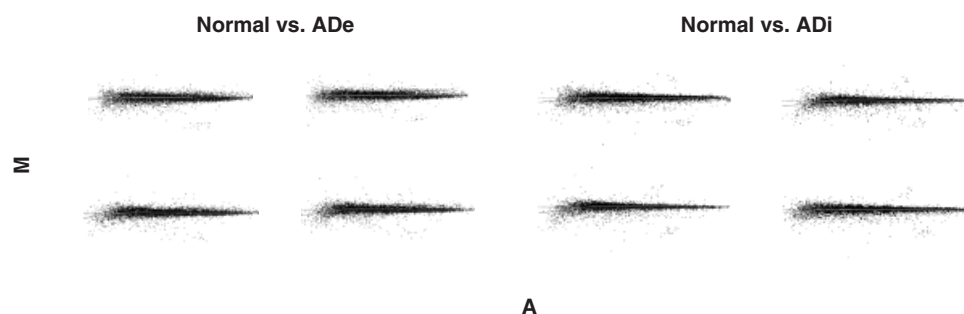


Fig. 1. Complementary DNA microarray results. Plot of M vs. A. Four sets of microarrays were performed for the paired normal vs. ADe samples were (left), and four sets of microarrays were performed for the paired normal vs. ADi samples (right). The pooling method for the 22 normal control samples, the 10 ADe samples and the 10 ADi type of AD samples was used to conduct the chip-array.

Table 1. List of up-regulated genes (more than 2-fold) in the AD group compare with the normal group

Gene name	Accession No.	Intensity*	q-value (%)	AD type
Keratin 14 (KRT14)	NM_000526	2.57	0.05	ADe
Proteoglycan 1 (PRG1)	J03223	1.86, 1.6	0.05, 0.16	ADe, ADi
Hyaluronan synthase 2 (HAS2)	AI049723	1.79, 1.41	0.05, 0.16	ADe, ADi
Cystatin A (CSTA)	AI680589	1.5	0.05	ADe
Chemokine CXC motif ligand 12 (CXCL12)	U19495	1.46	0.05	ADe
Caveolin 1 (CAV1)	AI878826	1.4	0.05	ADe
Carboxypeptidase (CPE)	X51405	1.35	0.05	ADe
Chondroitin sulfate proteoglycan 4 (CSPG4)	X96753	1.3	0.05	ADe
Collagen type 8 α -1 (COL8A1)	X57527	1.22	0.05	ADe
Ribosomal protein L29 (RPL29)	AL047869	1.2	0.05	ADe
Cadherin 13 (CDH13)	NM_001257	1.18, 1.01	0.05, 0.16	ADe, ADi
Lysyl oxidase-like 1 (LOXL1)	NM_005576	1.15	0.05	ADe
V-ets avian erythroblastosis virus e26 oncogene homolog 2 (ETS2)	NM_005239	1.14	0.05	ADe
Caveolin 2 (CAV2)	AI795995	1.13	0.05	ADe
Brain-derived neurotrophic factor (BDNF)	X60201	1.11, 1.05	0.05, 0.16	ADe, ADi
Selenoprotein W 1 (SEPW1)	U67171	1.08	0.05	ADe
Small proline-rich protein 1B (SPRR1B)	M19888	1.08	0.05	ADe
Cytochrome p450 family 51a (CYP51A1)	D55653	1.06	0.05	ADe
Cytochrome c oxidase subunit 8 (COX8A)	AI184515	1.04	0.05	ADe
Testis-specific protein Y-linked (TSPY)	M98525	1.03	0.05	ADe
Protein-tyrosine phosphatase receptor-type F polypeptide-interacting protein-binding protein 1 (PPFIBP1)	AA459628	1.03	0.05	ADe
Lectin C-type superfamily member 2 (CLECSF2)	X96719	1.31	0.16	ADi

*Data were calculated from $\log_2$ values; hence, 1.0 equals to 2-fold.

the AD fibroblasts by performing SAM with a 2-fold cut-off. The results of the gene expression patterns showed that the number of up-regulated genes was less than the number of down-regulated genes, and this indicated that the low level of gene products compared with the over-expressed gene products might well contribute the complexity of AD disease. The gene expression patterns between AD skin and psoriasis skins were previously compared by Nomura *et al.* [13]. In their report, several CC chemokines and CXC chemokines were suggested to be factors associated with AD or psoriasis. In our results, we found that CXCL12 showed an up-regulated pattern (Table 1) whereas CXCL6 and CXCL5 showed down-regulated

patterns in the AD fibroblasts (Table 2). It's interesting that the changes in CXCL12 and CXCL6 were observed only in the ADe samples, while the changes in CXCL5 were observed in both types of AD samples.

As the roles of the fibroblasts' chemokines and cytokines, as well as their related genes, in the pathogenesis of AD are unknown, we selected CXCL12, IL-8 and TNFAIP6 to discover their expression levels in the AD fibroblasts and to find new information regarding the dysregulation of these genes in the AD fibroblasts. In addition, we selected HAS2, PRG1 and LOXL1, which are associated with extracellular matrix (ECM) proteins: proteoglycan and hyaluronan are the components of the ECM and

Table 2. List of the down-regulated genes (more than 2-fold) in the AD group compared with the normal

Gene name	Accession No.	Intensity*	q-value (%)	AD type
Superoxide dismutase 2 (SOD2)	Y00472	- 3.01, - 2.8	0.05, 0.16	ADe, ADi
Interleukin 8 (IL8)	Y00787	- 2.7, - 4.93	0.05, 0.16	ADe, ADi
Chemokine CXC motif ligand 6 (CXCL6)	U81234	- 2.14	0.05	ADe
RNA-binding motif protein Y chromosome family 1 member a1 (RBM1A1)	X76059	- 2.07, - 1.7	0.05, 0.26	ADe, ADi
Apolipoprotein a (LPA)	X06290	- 2.06, - 1.81	0.05, 0.16	ADe, ADi
Plakophilin 2 (PKP2)	X97675	- 2.06, - 1.25	0.05, 4.83	ADe, ADi
Paired basic amino acid cleaving enzyme 4 (PACE4)	D87993	- 2.05, - 1.4	0.05, 0.16	ADe, ADi
Aldehyde dehydrogenase 1 family member a1 (ALDH1A1)	N92670	- 1.99, - 3.66	0.05, 0.16	ADe, ADi
Potassium channel subfamily K member 3 (KCNK3)	AI193606	- 1.93, - 1.36	0.05, 1.62	ADe, ADi
Solute carrier family 21 (organic anion transporter) member 3 (SLC21A3)	U21943	- 1.89, - 2.63	0.05, 0.16	ADe, ADi
V-ets avian erythroblastosis virus E26 oncogene related (ERG)	AI142842	- 1.84, - 2.1	0.05, 0.16	ADe, ADi
Tumor necrosis factor- α -induced protein 6 (TNFAIP6)	M31165	- 1.83, - 1.87	0.05, 0.16	ADe, ADi
Complement component C1r deficiency (C1R)	M14058	- 1.8, - 2.0	0.05, 0.16	ADe, ADi
Cochlin (COCH)	AF006740	- 1.79	0.05	ADe
FSH primary response rat homolog of 1 (FSHPRH1)	X97249	- 1.79, - 1.56	0.05, 0.16	ADe, ADi
Interferon-stimulated gene 20-kDa (ISG20)	X89773	- 1.78, - 1.31	0.05, 0.16	ADe, ADi
Killer cell immunoglobulin-like receptor two domains long cytoplasmic tail 3 (KIR2DL3)	U73395	- 1.76, - 1.41	0.05, 0.16	ADe, ADi
Matrix metalloproteinase 15 (MMP15)	Z48482	- 1.73, - 1.31	0.05, 0.16	ADe, ADi
Nuclear RNA export factor 1 (NXF1)	AJ132712	- 1.73, - 1.45	0.05; 0.16	ADe, ADi
E74-like factor 5 (ELF5)	AF049703	- 1.72, - 1.33	0.05, 0.85	ADe, ADi
Interferon-induced protein with tetratricopeptide repeats 1 (IFIT1)	M24594	- 1.72, - 1.95	0.05, 0.16	ADe, ADi
Complement component 3 (C3)	K02765	- 1.63, - 1.86	0.05, 0.16	ADe, ADi
Interleukin 6 (IL6)	M54894	- 1.63, - 1.46	0.05, 0.16	ADe, ADi
G protein-coupled receptor kinase 4 (GPRK4)	NM_005307	- 1.61	0.05	ADe
Cathepsin F (CTSF)	AA351201	- 1.6, - 1.24	0.05, 0.16	ADe, ADi
Bile acid CoA:amino acid <i>N</i> -acyltransferase (BAAT)	L34081	- 1.52, - 1.46	0.05; 0.16	ADe, ADi
Adenylate cyclase 9 (ADCY9)	AI248827	- 1.49, - 1.41	0.05, 0.16	ADe, ADi
Solute carrier family 22 member 1 (SLC22A1)	X98332	- 1.49	0.05	ADe
Protein phosphatase 2C magnesium-dependent catalytic subunit (PPM2C)	AI262149	- 1.48, - 1.52	0.05, 0.16	ADe, ADi
Chloride channel calcium activated 1 (CLCA1)	AI660957	- 1.45, - 1.2	0.05, 0.16	ADe, ADi
Fos-like antigen 2 (FOSL2)	AI088306	- 1.44, - 1.16	0.05, 0.16	ADe, ADi
Dipeptidyl peptidase 4 (DPP4)	X60708	- 1.42, - 1.11	0.05, 0.26	ADe, ADi
DNA methyltransferase 3b (DNMT3B)	NM_006892	- 1.41	0.05	ADe
Coronin 2a (CORO2A)	AI079558	- 1.41, - 1.13	0.05, 0.16	ADe, ADi
Supervillin (SVIL)	AI761936	- 1.41, - 1.72	0.18, 0.16	ADe, ADi
Lumican (LUM)	AL036211	- 1.32, - 1.52	0.05, 0.16	ADe, ADi
Meprin β subunit (MEP1B)	X81333	- 1.31	0.05	ADe
NADH-ubiquinone oxidoreductase 1 α subcomplex 10 (NDUFA10)	AI816028	- 1.30, - 1.19	0.05, 0.16	ADe, ADi
Oxysterol-binding protein-like protein 6 (OSBPL6)	AI732118	- 1.29, - 1.05	0.05, 0.16	ADe, ADi
Tweety drosophila homolog of 1 (TTYH1)	AI014634	- 1.27	0.05	ADe
BRCA1-associated ring domain 1 (BARD1)	U76638	- 1.27, - 1.18	0.05, 0.16	ADe, ADi
Matrix metalloproteinase 10 (MMP10)	X07820	- 1.24, - 1.3	0.05, 0.16	ADe, ADi
B7 homolog 4 (B7-H4)	AI684143	- 1.23	0.18	ADe
TRAF- and TNF receptor-associated protein (TTRAP)	NM_016614	- 1.22, - 1.07	0.05, 0.16	ADe, ADi
Semaphorin 4f (SEMA4F)	AB022317	- 1.20, - 1.06	0.05, 0.16	ADe, ADi
Pro-platelet basic protein (PPBP)	M54995	- 1.20	0.05	ADe
Suppressor of cytokine signaling 2 (SOCS2)	AF037989	- 1.18, - 1.02	0.05, 0.16	ADe, ADi
Flavin containing monooxygenase 1 (FMO1)	NM_002021	- 1.16, - 1.1	0.05, 0.16	ADe, ADi
MutS homolog (MSH5)	AF034759	- 1.15	0.05	ADe
N-acetylglucosamine-1-phosphodiester α -N-acetylglucosaminidase (NAGPA)	AF052111	- 1.14, - 1.18	0.05, 0.16	ADe, ADi
Dual adaptor of phosphotyrosine and 3-phosphoinositides 1 (DAPP1)	AA149868	- 1.14, - 1.07	0.05, 0.16	ADe, ADi
Rab3 GTPase-activating protein (RAB3GAP)	D31886	- 1.14	0.05	ADe
B-cell CLL/lymphoma 11a (BCL11A)	AA504591	- 1.13	0.05	ADe
Cholinergic receptor nicotinic delta polypeptide (CHRNA1)	X55019	- 1.12	0.05	ADe
Caspase 8 apoptosis-related cysteine protease (CASP8)	U60520	- 1.11, - 1.61	0.05, 0.16	ADe, ADi
Protein-tyrosine phosphatase receptor-type α (PTPRA)	AA701899	- 1.10	0.18	ADe

Table 2. Continued.

Gene name	Accession No.	Intensity*	q-value (%)	AD type
Dynein heavy chain (DNAH17)	AJ000522	− 1.09, − 1.26	0.05, 0.16	ADe, ADi
Peroxisome proliferator-activated receptor-gamma (PPARG)	BC006811	− 1.09	0.05	ADe
Cartilage oligomeric matrix protein (COMP)	NM_000095	− 1.08, − 1.66	0.05, 0.16	ADe, ADi
Serine/arginine repetitive matrix protein 2 (SRRM2)	AB016092	− 1.08	0.05	ADe
Phospholipase D1 phosphatidylcholine-specific (PLD1)	U38545	− 1.08	0.05	ADe
Solute carrier family 30 member 4 (SLC30A4)	AF025409	− 1.07	0.05	ADe
Angiopoietin-like factor (CDT6)	NM_021146	− 1.07	0.05	ADe
Triggering receptor expressed on myeloid cells 2 (TREM2)	N50741	− 1.07	0.05	ADe
Iron-responsive element-binding protein 2 (IREB2)	AI565322	− 1.06	0.05	ADe
Diacylglycerol kinase β 90-kDa (DGKB)	AB018261	− 1.04	0.05	ADe
Keratocan (KERA)	NM_007035	− 1.04, − 1.43	0.12, 1.51	ADe, ADi
Thrombospondin 4 (THBS4)	Z19585	− 1.03	0.05	ADe
Protein 4.2 erythrocytic (EPB42)	M30646	− 1.03	0.05	ADe
Sperm-associated antigen 6 (SPAG6)	AL080136	− 1.03	0.05	ADe
NADH-ubiquinone oxidoreductase 1 β subcomplex 2 (NDUFB2)	AA146968	− 1.03	0.05	ADe
Chemokine CXC motif ligand 5 (CXCL5)	X78686	− 1.00, − 1.14	0.05, 0.16	ADe, ADi
Lymphoid nuclear protein (LAF4)	U34360	− 1.6	0.16	ADi
Retinol dehydrogenase 5 (RDH5)	U43559	− 1.6	0.8	ADi
Methylcrotonoyl-coenzyme A carboxylase 2 β (MCCC2)	BC014897	− 1.26	0.16	ADi
EGF-containing fibulin-like extracellular matrix protein 1 (EFEMP1)	U03877	− 1.23	0.16	ADi
Ceroid-lipofuscinosis neuronal 3 juvenile (CLN3)	NM_000086	− 1.22	0.16	ADi
Follistatin (FST)	NM_013409	− 1.2	0.16	ADi
Tenascin (TNC)	X56160	− 1.19	0.16	ADi
Hypoxia-inducible factor 1 α subunit (HIF1A)	U29165	− 1.15	0.16	ADi
IMP dehydrogenase type 1 (IMPDH1)	J05272	− 1.15	0.16	ADi
Major histocompatibility complex class 2 transactivator (MHC2TA)	U18288	− 1.14	0.16	ADi
Myxovirus resistance 2 mouse homolog of (MX2)	M33883	− 1.12	0.16	ADi
Aspartyl-tRNA synthetase (DARS)	J05032	− 1.12	0.16	ADi
Keratin hair acidic 1 (KRTHA1)	NM_002277	− 1.12	0.8	ADi
Protein kinase cAMP-dependent catalytic α (PRKACA)	X07767	− 1.11	0.16	ADi
Matrix metalloproteinase 1 (MMP1)	M13509	− 1.1	0.16	ADi
Prostaglandin-endoperoxide synthase 1 (PTGS1)	M59979	− 1.1	0.16	ADi
Hect domain and RCC1-like domain 1 (HERC1)	U50078	− 1.09	0.16	ADi
Prip-interacting protein with methyltransferase domain (NCOA6IP)	N34786	− 1.08	0.16	ADi
Cleavage stimulation factor 3-prime pre-RNA subunit 2 64-kDa (CSTF2)	M85085	− 1.02	0.16	ADi
Lipoyltransferase 1 (LIPT1)	NM_145197	− 1.02	1.28	ADi
Transforming growth factor β -induced 68-kDa (TGFB1)	NM_000358	− 1.01	0.16	ADi
Multiple endocrine neoplasia type 1 (MEN1)	AI082242	− 1.0	0.16	ADi
Thrombospondin 2 (THBS2)	L12350	− 1.0	0.16	ADi

*Data were calculated from $\log_2$ values; hence, − 1.0 equals to 2-fold.

LOXL1 is directly associated with the homeostasis of elastic fibre, which is a constituent in the formation of the ECM [14]. It is well known that ECM proteins play important roles in the pathogenesis of AD with respect to T-cell survival and keratinocytes apoptosis, and this resulted in the manifestation of eczema [15]. Quantification by real-time PCRs confirmed several sets of microarray data in the randomized pairing samples: the up-regulated genes such as HAS2, CXCL12, PRG1 and LOXL1, and the down-regulated genes such as TNFAIP6 and IL-8, Fig. 2. The overall patterns of quantification for the corresponding genes via real-time PCR were matched with the array results; however, the statistical results for the AD-fibro-

blasts listed in Table 3 showed that among these five genes, only HAS2, TNFAIP6 and IL-8 turned out to show significant differences between the normal control group and the AD sample groups.

In the next step, we performed RT-PCR to qualitatively analyze the transcriptional changes of TNFAIP6, IL-8 and HAS2 in the AD-fibroblasts samples as compared with the normal subjects, Fig. 3. The RT-PCR results showed that the expression levels of IL-8 and TNFAIP6 were all conspicuously down-regulated in both types of AD patients (ADe $n = 5$; ADi $n = 5$) and HAS2 was up-regulated in both types of AD patients (ADe $n = 3$; ADi $n = 3$). Both the real-time and RT-PCR results consistently supported

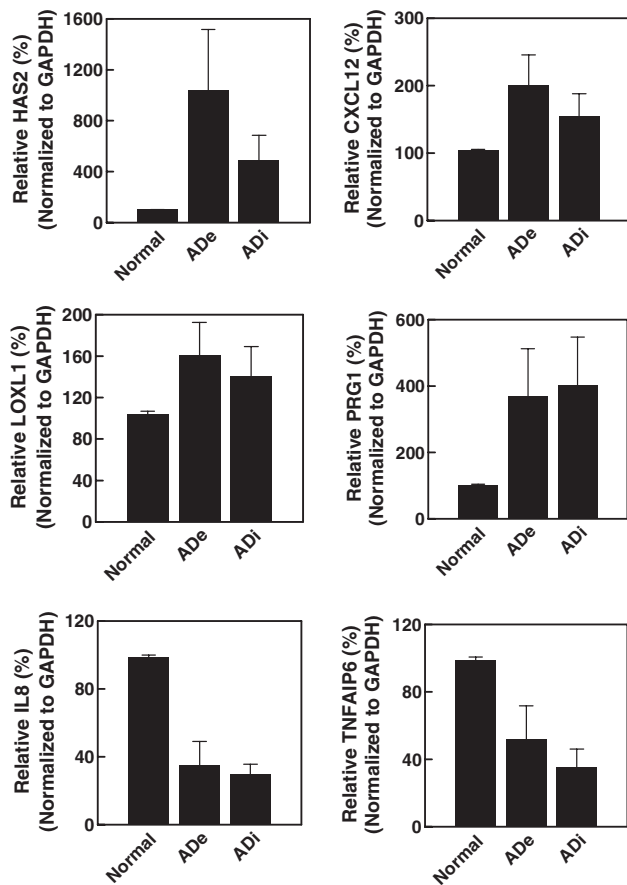


Fig. 2. Real-time polymerase chain reaction results of the several up- or down-regulated genes. cDNAs of the normal ($n = 10$), ADe ($n = 10$), and ADi ($n = 10$) samples were used as templates, respectively. Data are presented as means $\pm$ SEM. *P*-values for hyaluronan synthase 2 (HAS2), chemokine CXC motif ligand 12 (CXCL12), lysyl oxidase-like 1 (LOXL1), proteoglycan 1 (PRG1), IL-8 and TNF- α -induced protein 6 (TNFAIP6) in pairing normal vs. ADe were 0.001, 0.44, 0.16, 0.48, 0.02 and 0.01, respectively. *P*-values for HAS2, CXCL12, LOXL1, PRG1, IL-8 and TNFAIP6 in pairing normal vs. ADi were 0.001, 0.27, 0.53, 0.16, 0.001 and 0.001, respectively.

Table 3. Real-time polymerase chain reaction results

Gene name	Up- or down-regulated folds*		P-values†	
	Normal vs. ADe	Normal vs. ADi	Normal vs. ADe	Normal vs. ADi
HAS2	10.2	4.7	0.001	0.001
TNFAIP6	– 1.9	– 2.7	0.01	0.001
IL-8	– 2.8	– 3.3	0.02	0.001
CXCL12	1.9	1.4	0.44	0.27
PRG1	3.6	3.9	0.48	0.16
LOXL1	1.5	1.3	0.16	0.53

*Data were mean values (normal vs. ADe $n = 10$; normal vs. ADi $n = 10$). Randomized pairings between the normal cDNA and the ADe or ADi cDNA were made.

†The Wilcoxon-rank sum test was used to analyze the normal control group and the two types of AD groups.

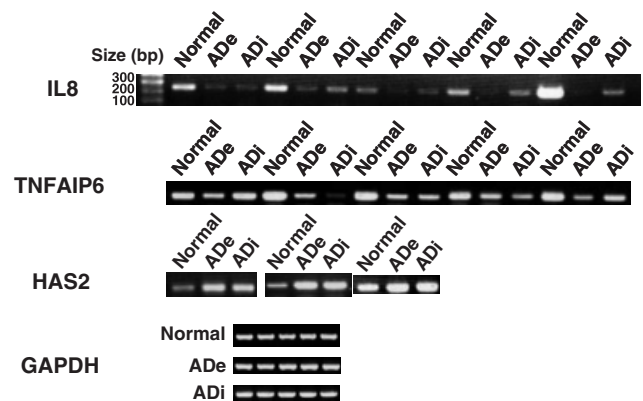


Fig. 3. RT-PCR results. IL-8, interleukin-8; TNFAIP6, TNF- α -induced protein 6; HAS2, hyaluronan synthase 2. Glyceraldehyde-3-phosphate dehydrogenase was used as the relative quality control. PCRs were conducted with randomized pairing.

the validity of the pooling method that was used in the cDNA microarray in this present study.

Recently, Raap et al. [16] reported that the expression of brain-derived neurotrophic factor (BDNF) is up-regulated in the peripheral blood eosinophils of AD patients and they suggested a functional role of BDNF and the systemic involvement of neurotrophic factors in chronic inflammatory AD disease. We also found that BDNF was significantly up-regulated in the fibroblasts of AD patients, Table 1. Our result supported that BDNF could have an important function not only for blood eosinophils, but also in the local dermal tissues of AD patients. In addition, we recently submitted our proteomic results regarding ALDH1A1 down-regulation patterns that were detected at the range of pH, from 6.0 to 9.0, from the cultured fibroblast of AD patients, and that data supported the present microarray data of the down-regulated patterns in Table 2. ALDH1A1 can also be an important candidate gene in the pathogenesis of AD. Therefore, the candidate genes listed in the tables of this study may provide important information to piece together the complex nature of AD disease.

Discussion

In our experiments using real-time PCR, we suggested that three candidate genes (IL-8, TNFAIP6, HAS2) are important in the AD-fibroblasts, and these three genes showed statistical significance in both type of the patient-derived samples. Regarding IL-8, its level was increased in the allergen-stimulated samples, which suggests a specific release of IL-8 by the leucocytes in response to *in vitro* antigen stimulation [17]. Significantly higher IL-8 concentrations were detected in the severe AD patients rather than in the mild or moderate AD patients [18] and most of

the patients with AD showed an up-regulated IL-8 protein expression pattern in the eosinophils, but not in the neutrophils [19]. In our observations (the microarray, and the real-time and RT-PCR data), IL-8 was significantly down-regulated in the AD-fibroblasts. The skin lesions of AD patients are occasionally infiltrated by T-cells, eosinophils and antigen-presenting LC, and the production of cytokines showed conflicting results. These conflicting results may have come from the differences in the age of the patients, the duration of the skin lesions, the isolated sites of the target cells and the cell culture methods. The low level of IL-8 in the fibroblasts might play a different role, compared with the eosinophils and leucocytes, in the pathogenesis of AD. The discrepancy of the IL-8 expression levels between the cell types can suggest new insight into AD disease when further studies are conducted.

TNFAIP6 (gene alias: TSG6) is known to be a member of the hyaluronan-binding protein family that contains a hyaluronan-binding domain. The inflammatory cytokines such as TNF- α and IL-1 can induce TNFAIP6 gene expression [20]. Milner and Day [21] suggested that TNFAIP6 has an anti-inflammatory effect and it can be a component of a negative feedback loop that is capable of down-regulating the inflammatory response. In addition, TNFAIP6 activates inter- α -inhibitor (I α I) by forming a stable complex, and I α I, as an anti-inflammatory agent, is important in the protease network that is related with inflammation [22]. In the present study, TNFAIP6 was consistently detected to be down-regulated on the results of the microarray, real-time PCR and RT-PCR. Taken together, the down-regulated expression patterns of TNFAIP6 in the AD patients strongly imply that the inflammatory severity of AD can be associated with the low level of TNFAIP6. Therefore, activation of TNFAIP6 might be a new target for the treatment of AD.

Hyaluronan is a high molecular weight polysaccharide (glycosaminoglycan) that is synthesized by hyaluronan synthase (HAS1, -2 and -3) in a wide variety of organisms and it is known to be a core component of the ECM. Depolymerized fragments of hyaluronan initiate an inflammatory response during tissue injury [23]. CD44 is the hyaluronan receptor that plays a role in the binding, uptake and degradation of hyaluronan [24]. HAS2 is a member of the hyaluronan synthase gene family and it has significant homology to human and murine hyaluronan synthase. Recent studies have shown that human HAS2 was significantly up-regulated in respond to retinoic acid and epidermal growth factor in HaCaT human immortal keratinocytes [25]. HAS2 was induced by vasodilatory prostaglandins in human vascular smooth muscle cells and the up-regulation of HAS2 was thought to contribute to the accumulation of hyaluronan during human atherosclerosis [26]. Taken together, the up-regulation of HAS2 during the AD inflammatory process is

comprehensible and our results support the up-regulated patterns that have been observed in AD-fibroblasts.

In respect to the complexity of the AD pathogenesis where many factors are associated with AD and various triggers are known to exist among different patient, our data suggest that human dermal fibroblasts may actively participate in AD disease along with the other types of cells. However, some of the genes that are observed in the primary cultured fibroblasts may not be representative of the *in vivo* changes, and the differences between the mRNA levels and the protein expression levels limit our understanding with regard to their exact roles in AD.

In conclusion, we suggest several points: (i) the down-regulation of IL-8 and TNFAIP6 can be associated with AD without any patient-to-patient variations, and this was confirmed at the transcriptional level; (ii) up-regulation of HAS2 is associated with the pathogenesis of AD, and this was putatively coupled with the hyaluronan contents in the dermis of AD patients; (iii) the microarray data suggested that several ECM component proteins have important contributions to AD pathogenesis; (iv) microarray techniques and adapting the pooling method can enhance the validity of probing the critical AD-associated factors.

Acknowledgements

This work was supported by a grant (01-PJ3-PG6-01GN12-0001) of the 2001 Good Health R&D Project, Ministry of Health and Welfare, Republic of Korea. We especially want to thank Jong-Pil Youn for his excellent technical support for the real-time PCR.

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