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# The Role of Collagen VI α6 Chain Gene in Atopic Dermatitis

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**Background:** In a previous study, we carried out whole-exome sequencing to identify genetic variants associated with early onset atopic dermatitis (AD) in Koreans and found that collagen VI α6 chain (*COL6A6*) gene polymorphisms are associated. COL6A6 is one of the chains that makes up the triple helix of collagen VI, and little is known about its role in AD.
**Objective:** To identify how COL6A6 changes in AD and clarify its role.
**Methods:** Immunohistochemical staining for COL6A6 was performed on tissues of AD, other skin diseases, and healthy controls. Human keratinocytes and fibroblasts were exposed to inflammatory cytokines and cultured to evaluate changes in COL6A6 expression. *COL6A6* small interfering RNA (siRNA) was transfected into cells to identify the role of COL6A6.
**Results:** Total *COL6A6* mRNA was higher in AD than in controls. In AD tissues, COL6A6 mRNA decreased significantly in the epidermis compared to controls, whereas COL6A6 protein was increased in the dermis. In the cultured cells, COL6A6 mRNA was suppressed in the epidermis by interleukin (IL)-4 and IL-13, whereas COL6A6 protein was induced in the dermis. In the *COL6A6* siRNA-transfected keratinocyte, mRNA of *FLG*, *LOR*, and *CASP14* decreased compared to controls; in contrast, mRNA of *MMP1* increased.
**Conclusion:** The reduction of epidermal COL6A6 due to the genetic mutation can cause skin barrier damage and it can contributes to the early onset of AD. COL6A6 is induced by IL-4 and IL-13, and it may play a role in fibrotic remodeling and inflammatory processes, which are major features of AD.

**Keywords:** Atopic dermatitis, COL6A6, Collagen, Interleukin-4, Interleukin-13

## INTRODUCTION

Atopic dermatitis (AD) is a chronic relapsing inflammatory skin disease. Although the pathophysiology of AD is quite complex, it can be summarized as chronic inflammation caused by skin barrier impairment and immune dysregulation[1,2]. Both genetic and environmental factors are involved in these abnormalities. The most well-known genetic abnormality in AD is the *FLG* mutation; filaggrin deficiency is associated with early onset and severe AD, greater allergen sensitization, and increased susceptibility to infections[1-3]. However, in Koreans, the *FLG* mutation occurs only in 9.0% of the AD patients, which is much lower than in Irish (46%), Chinese (20.2%~31.4%), and Japanese (18.4%~26.7%) populations[4-7]. Therefore, we wanted to investigate if other genetic factors besides *FLG* are involved in early onset AD in Koreans. In our previous study, whole-exome sequencing was performed in three families with early onset AD. The results showed that family-specific collagen VI α6 chain (*COL6A6*) gene polymorphisms are associated with early onset AD in Koreans. Three variants of the *COL6A6* gene appeared in all three families, and their frequency was higher in AD patients than in controls in a population-based case-control study[8].

Collagen is an abundant, hard, insoluble, and fibrous protein present throughout the body, and mutations in collagen VI (COL6) cause myopathy[9,10]. Collagen types I and III comprise most of the skin collagen content in humans, but COL6 has not been extensively studied in dermatology[9,11]. In addi-

tion, it is known that collagen abnormalities in the skin cause some bullous disorders, but little is known about effect of collagen on AD. Therefore, we aimed to determine how COL6A6 changes in patients with AD and its role in AD.

## MATERIALS AND METHODS

### Study design

We aimed to determine whether the expression of COL6A6 was altered in patients with AD. Accordingly, two experiments were conducted. First, immunohistochemistry (IHC) staining was performed on skin biopsies form patients with AD, psoriasis, papular urticaria, and pityriasis rosea, as well as controls, to compare the expression of COL6A6 in each disease. Second, the *COL6A6* messenger RNA (mRNA) expression levels were compared by measuring fragments per kilobase of transcript per million (FPKM) in the tissues of AD patients and controls.

Next, we aimed to determine the factors affecting the change in COL6A6 found in the epidermis of patients with AD. Changes in *COL6A6* mRNA expression were measured over time while culturing human keratinocytes with interleukin (IL)-4, IL-13, and thymic stromal lymphopoietin (TSLP) which are the representative cytokines involved in AD. Conversely, keratinocytes were also exposed to tumor necrosis factor (TNF)-α, which is involved in Th1 type immunity. To determine the effect of COL6A6 on the epidermis, several differentiation markers such as FLG and IVL were measured using real-time polymerase chain reaction (PCR) after transfection of *COL6A6* small interfering RNA (siRNA) into keratinocytes.

We also aimed to identify the factors affecting the change in COL6A6 found in the dermis of patients with AD. We measured changes in COL6A6 expression by western blot analysis over time while culturing human fibroblasts with IL-4, IL-13, TNF-α, and TSLP. To determine the effect of COL6A6 on the dermis, several protein levels were measured using real-time PCR after transfection of *COL6A6* siRNA into fibroblasts.

The present study protocol was reviewed and approved by the institutional review board of Chung-Ang University Hospital (IRB No. C2015258 [1716]). Informed consent was obtained from all participants when they were enrolled.

### Immunohistochemical analysis

This analysis included 7 control subjects, 7 AD patients, 7 were fixed in 4% formaldehyde for two days and embedded in paraffin. The section of blocks was cut into thickness of 4 μm musing a microtome (Leica RM2125, Leica Biosystems, Heidelberg, Germany). For hematoxylin and eosin (H&E), Sirius red staining the sections were deparaffinized rehydrated, and washed with distilled water to allow the hydrophilic solution to penetrate. After tissue watering, H&E staining was performed to observe liver sample histological changes. For IHC analysis, the sections were deparaffinized, rehydrated, cooked in antigen retrieval solution (Abcam, Cambridge, UK), and dipped in 3% hydrogen peroxide solution for 30 minutes. The primary antibody used was anti-COL6A6 (1:200, ab150926; Abcam) were then applied for 1 hour at room temperature, and the sections were incubated with secondary antibodies for 40 minutes. Immunoreactions were visualized with DAB staining, and the sections were counterstained with Mayer's hematoxylin. All data were normalized against the equivalent data in mice fed chow (control). Immunostaining was quantified using ImageJ software (ImageJ software, 1.52a; National Institutes of Health, Bethesda, MD, USA).

### RNA sequencing

This investigation included 6 control subjects and 7 patients with AD. RNA quality was assessed by analysis of ribosomal RNA band integrity using an Agilent RNA 6000 Nano kit (Agilent Technologies, Santa Clara, CA, USA). Ahead of complementary DNA (cDNA) library construction, 2 μg of total RNA and magnetic beads with Oligo (dT) were used to enrich poly (A) mRNA. The purified mRNAs were then disrupted into short fragments, and double-stranded cDNAs were immediately synthesized. The cDNAs were subjected to end-repair and, poly (A) addition, and were then connected with sequencing adapters using the TruSeq RNA sample prep Kit (Illumina, San Diego, CA, USA). Suitable fragments automatically purified using a BluePippin 2% agarose gel cassette (Sage Science, Beverly, MA, USA) were selected as templates for PCR amplification. The final library sizes and qualities were evaluated electrophoretically using an Agilent High Sensitivity DNA kit (Agilent Technologies), and the fragments were found to be between 350 and 450 bp. The library was sequenced using an Illumina HiSeq2500 sequencer (Illumina).

### Cell cultures

Human epidermal keratinocytes, neonatal (HEKn) were ac-

quired from Invitrogen (Carlsbad, CA, USA) and were grown in EpiLife medium supplemented with human keratinocyte growth supplement (HKGS, Cascade Biologics; Invitrogen). HEKn cells were incubated with recombinant human IL-4 (30 ng/ml), IL-13 (30 ng/ml), TNF-α (30 ng/ml), TSLP (10 ng/ml), and polyinosinic-polycytidylic acid (poly I:C; Calbiocham, Billerica, MA, USA) (4 μg/ml) for 0, 2, 6, 12, and 24 hours. Fibroblast cells were maintained in HyClone Dulbecco's midified Eagle's medium with 10% fetal bovine serum. Cell cultures were placed in an incubator with 5% $CO_2$ at 37°C.

### Real-time quantitative PCR

Total RNA was extracted from cells using RiboEx™ Trizol (GeneAll, Seoul, Korea), according to the manufacturer's instructions. RNA concentrations were measured using a Nanodrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and the purity was determined by measuring the A260/A280 ratio. cDNA was generated by reverse transcription, using 1 μg of purified RNA and the RevertAid First Strand cDNA Synthesis Kit (Applied Biosystems, Waltham, MA, USA; Thermo Fisher Scientific) and incubated for 1 hour at 42°C. Real-time quantitative PCR assays were performed using a QuantStudio 3 system (Applied Biosystems) using a PowerUp SYBR Green Master Mix (Applied Biosystems). All data were normalized to the housekeeping gene GAPDH. Relative quantitation was analyzed using the 2-ΔΔCt method according to the manufacturer's instructions.

### Small interfering RNA

HEKn and human fibroblast cells were plated at a density of $2\times10^5$ cells/35-mm tissue culture dish. After 20 hours and 70%~80% confluence, the cells were transfected with siRNAs in serum-free medium using Lipofectamine 3000 (Life Technologies, Carlsbad, CA, USA). Then, 5 μl of each siRNA stock solution (20 μM) and the PLUS reagent (10 μl) were mixed in Opti-MEM (85 μl; Invitrogen) in a small sterile tube. After immediate mixing and incubation at room temperature for 15 minutes, the Lipofectamine 3000 reagent (4 μl) in Opti-MEM (100 μl) was added, and the mixture was left at room temperature for 15 minutes. Then, 0.8 ml of HEKn and human fibroblast cells were added to generate the siRNA-lipid complex. The transfected HEKn and human fibroblast cells with the decreased level of COL6A6 were further identified by qRT-PCR.

### Statistical analysis

GraphPad Prism (v5; GraphPad Software, Inc., La Jo-lla, CA, USA) was used, and all data are expressed as mean±standard error of the mean. Differences in results were analyzed using the Mann–Whitney U test. *p*-values <0.05 were considered statistically significant.

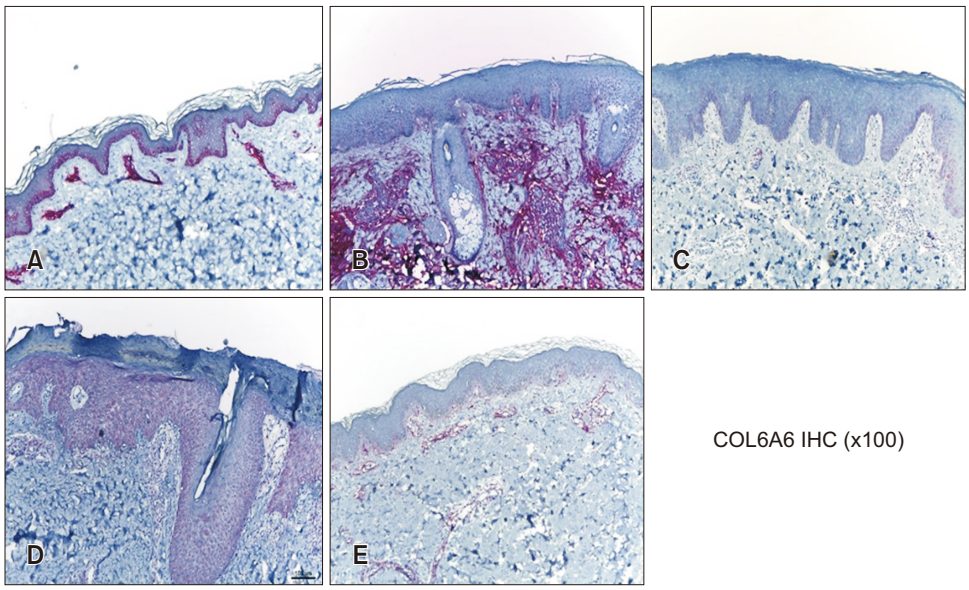


**Fig. 1.** (A) Immunohistochemical staining (IHC) of normal skin for COL6A6 protein expression (purple). (B) IHC of skin with atopic dermatitis shows reduced epidermal COL6A6 expression and increased dermal COL6A6 expression. (C) Skin with psoriasis shows reduced overall COL6A6 expression. (D) Skin with papular urticaria shows COL6A6 in all epidermal layers and reduced dermal COL6A6. (E) Skin with pityriasis rosea shows reduced epidermal COL6A6 expression. Scale bar 100 μm, magnification ×100. COL6A6: collagen VI α6 chain.

## RESULTS

### COL6A6 expression decreased in the epidermis of AD patients, while a significant increase was observed in the dermis

Seven healthy controls (age, 32.4±4.0 years), 7 patients with AD (age, 26.2±10.3 years), 7 psoriasis patients (age, 25.4±29.0 years), 1 papular urticaria patient (age, 28.0 years), and 3 patients with pityriasis rosea (age, 28.0±7.0 years) were enrolled for IHC analysis. COL6A6 was observed around the vessels of the papillary dermis and the basal layer of the epidermis in normal skin. COL6A6 expression was decreased in the epidermis of AD patients compared to that in the control group, while a significant increase and broad distribution were ob-

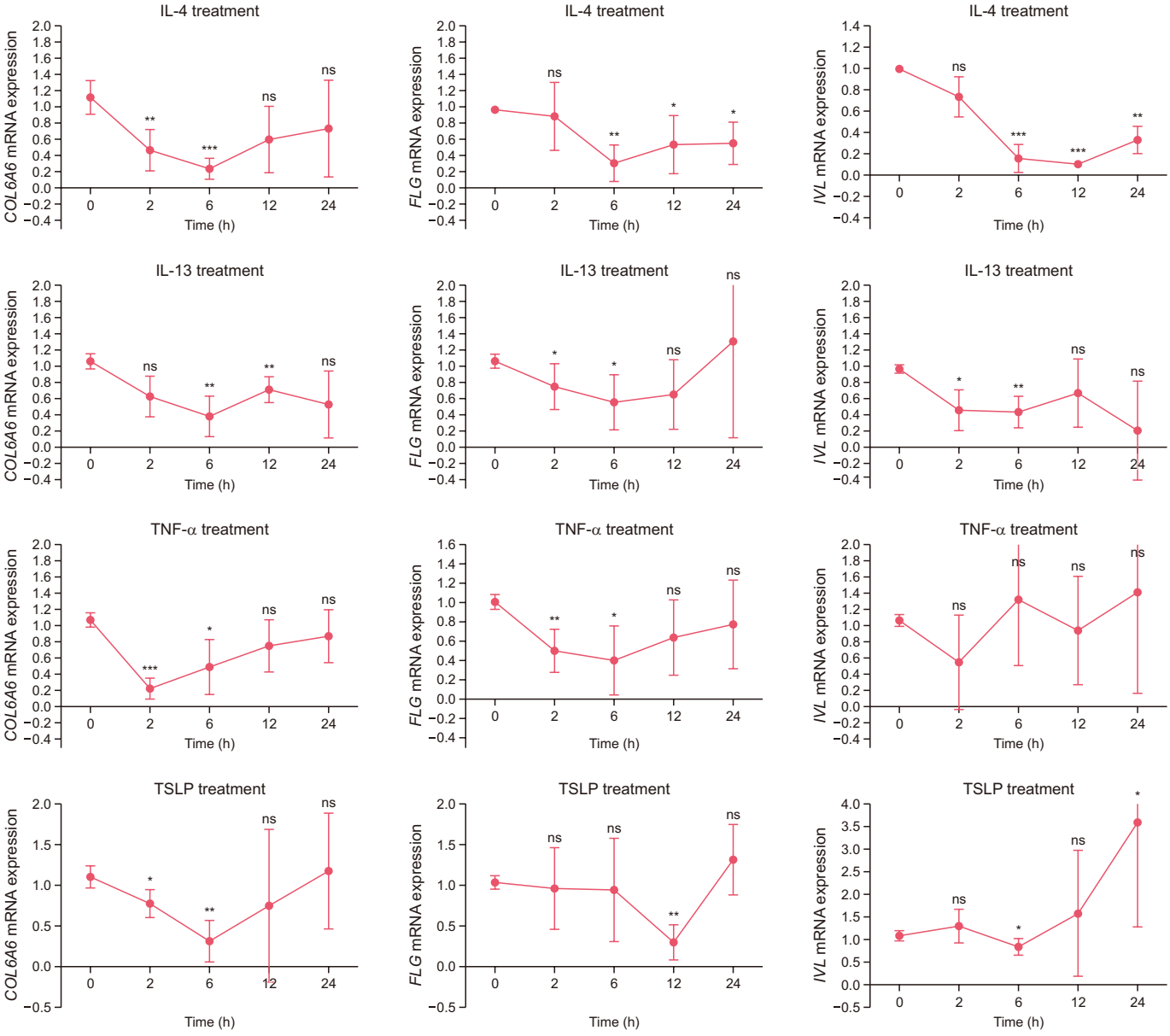


Fig. 2. COL6A6 mRNA expression in human keratinocytes exposed to various inflammatory cytokines. After IL-4 and IL-13 treatment, mRNA expression of *COL6A6*, *FLG*, and *IVL* was suppressed. After TNF-α treatment, mRNA expression of *COL6A6* and *FLG* was suppressed but mRNA expression of *IVL* was unchanged. After TSLP treatment, mRNA expression of *COL6A6* and *FLG* was suppressed, but the expression of *IVL* mRNA was increased by three times compared to the baseline. COL6A6: collagen VI α6 chain, IL-4: interleukin-4, IL-13: interleukin-13, FLG: filaggrin, IVL: involucrin, TNF-α: tumor necrosis factor-α, TSLP: thymic stromal lymphopoietin. All *p*-values less than *0.05, **0.01, or ***0.001 are summarized.

served in the dermis. IHC data from patients with psoriasis showed reduced overall COL6A6 expression. The sample from the patient with papular urticaria showed an increase in epidermal COL6A6 and a decrease in the amount of dermal COL6A6. Samples from patients with pityriasis rosea showed a decrease in the amount of epidermal COL6A6 (Fig. 1).

### Total mRNA expression of *COL6A6* increased in skin lesions of AD patients

RNA sequencing of *COL6A6* mRNA was performed on skin biopsies. Six healthy controls (age, 22.2±1.7 years) and 7 AD patients (age, 21.9±1.1 years) were enrolled to obtain skin samples. The FPKMs of the control group and AD patient group were 1.257 and 20.949, respectively, and the difference between the two groups was statistically significant ($p$=0.0225). Based on this data, the mRNA expression of *COL6A6* was 16.6 times higher in skin lesions of AD patients than in samples obtained from controls.

### *COL6A6* mRNA expression of human keratinocytes exposed to IL-4 and IL-13 was significantly suppressed

After IL-4 treatment, mRNA expression of *COL6A6*, *FLG*, and *IVL* was significantly suppressed by up to 80%, 60%, and 90%, respectively. After IL-13 treatment, the mRNA expression of *COL6A6*, *FLG*, and *IVL* was suppressed by up to 60%, 40%, and 60%, respectively. After TNF-α treatment, mRNA expression of *COL6A6* and *FLG* was significantly suppressed by up to 80% and 60%, respectively, while the expression of *IVL* was unchanged. After TSLP treatment, *COL6A6* and *FLG* mRNA expression was significantly suppressed by up to 60% and 70%, respectively, and the expression of *IVL* mRNA was increased by three times that of the baseline (Fig. 2).

### Real-time PCR results of *COL6A6* siRNA-transfected keratinocytes showed a reduced mRNA expression of *FLG*, *LOR*, and *CASP14*

In *COL6A6* siRNA-transfected keratinocytes, the mRNA expression of *FLG*, *LOR*, and *CASP14* was significantly suppressed compared to that of normal keratinocytes (Fig. 3).

### COL6A6 expression of fibroblasts exposed to IL-4 and IL-13 was significantly increased

Expression of COL6A6 gradually and significantly increased after 24 hours of IL-4 and IL-13 treatments. TSLP did not affect COL6A6 expression. COL6A6 expression was significantly suppressed at 24 hours after TNF-α treatment. To confirm these results, TNF-α was applied in a concentration-

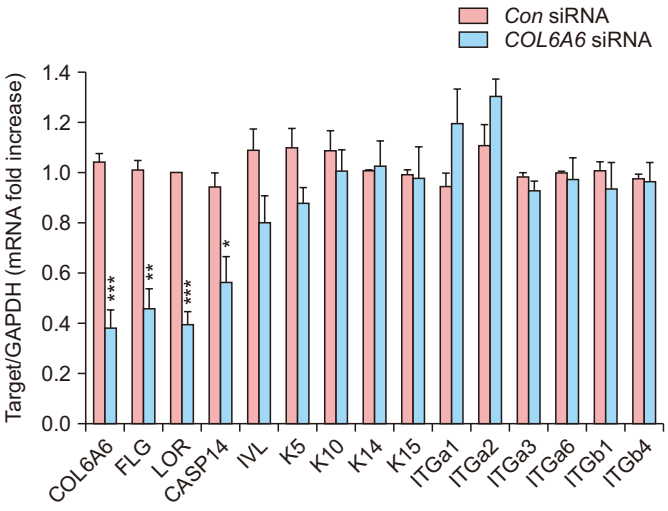


**Fig. 3.** Real-time PCR results of *COL6A6* siRNA-transfected keratinocytes. In *COL6A6* siRNA-transfected keratinocytes, expression of mRNA of *FLG*, *LOR*, and *CASP14* was significantly suppressed. COL6A6: collagen VI α6 chain, FLG: filaggrin, LOR: loricrin, CASP14: caspase 14, IVL: involucrin, K5: keratin 5, K10: keratin 10, K14: keratin 14, K15: keratin 15, ITGa1: integrin α1, ITGa2: integrin α2, ITGa3: integrin α3, ITGa6: integrin α6, ITGb1: integrin β1, ITGb4: integrin β4. All $p$-values less than *0.05, **0.01, or ***0.001 are summarized.

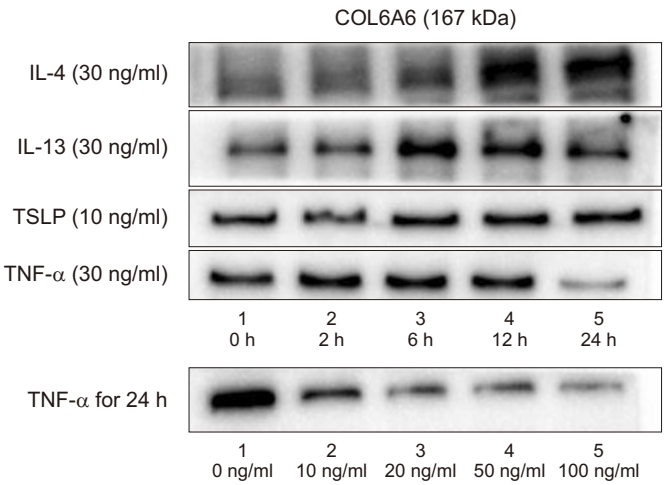


**Fig. 4.** COL6A6 expression in human fibroblasts exposed to various inflammatory cytokines. Expression of COL6A6 was increased for 24 hours with IL-4 and IL-13 treatment. TSLP did not affect COL6A6 expression. After TNF-α treatment, COL6A6 expression was suppressed in a concentration-dependent manner. COL6A6: collagen VI α6 chain, IL-4: interleukin 4, IL-13: interleukin 13, TSLP: thymic stromal lymphopoietin, TNF-α: tumor necrosis factor-α.

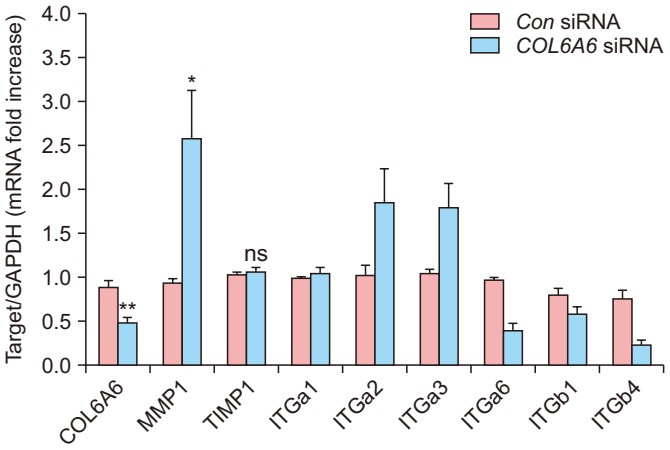


**Fig. 5.** Real-time PCR results of *COL6A6* siRNA-transfected fibroblasts. *COL6A6* siRNA-transfected fibroblasts showed a statistically significant increase in the production of *MMP1* mRNA. COL6A6: collagen VI α6 chain, MMP1: matrix metalloproteinase 1, TIMP1: tissue inhibitor of metalloproteinases, ITGa1: integrin α1, ITGa2: integrin α2, ITGa3: integrin α3, ITGa6: integrin α6, ITGb1: integrin β1, ITGb4: integrin β4. All *p*-values less than *0.05 or **0.01 are summarized.

dependent manner (0, 10, 20, 50, and 100 ng/ml) for 24 hours. The expression of COL6A6 was suppressed as the concentration of TNF-α increased (Fig. 4).

### Real-time PCR result of *COL6A6* siRNA-transfected fibroblasts showed a significant increase of *MMP1* mRNA

*COL6A6* siRNA-transfected fibroblasts showed a statistically significant increase in the production of *MMP1* mRNA compared to that of normal fibroblasts (Fig. 5).

## DISCUSSION

AD has a complex etiology. Although various mechanisms of AD have been studied, damage to the skin barrier, abnormal immune reactions, and inflammatory changes have been emphasized as important factors. As a result, little is known about involvement of the collagen. In particular, little is known about COL6 because the skin is mostly comprised of collagen types I and III[12].

COL6 is composed of a triple helix in which α1 and α2 chains are combined with α3, α4, α5, or α6[9,13]. Genes encoding α4, α5, and α6 chains are clustered together in chromosome 3q21[13]. In the skin, the α5 chain is mainly present in the papillary dermis, and the α6 chain is mainly present around the blood vessels, nerve fibers, hair follicles, hypodermis, and basement membrane[9,14].

As mentioned earlier, in previous studies, we identified that polymorphisms in the *COL6A6* gene are common in Korean families with early onset AD. There have been some other reports suggesting a connection between AD and COL6. First, 3q21, where the genes of the COL6 α4, α5, and α6 chains are located, is the major susceptibility locus of AD[15]. In addition, changes in *COL6A6* have been reported in AD-related transcriptome data studies[16]. Mutations in α1, α2, and α3 chains resulted in changes such as keloids, cigarette paper scars, dry skin, striae rubrae, and keratosis pilaris; in contrast, AD occurred in children with mutations in the α6 chain[9,11,17,18]. As such, there is some evidence that COL6A6 and AD are related, but no detailed studies have been conducted. This is the first full-scale study to date.

First, we wanted to know if there are any changes in COL6A6 in AD. And whether theses changes are characteristics that distinguishes it from other skin diseases. Therefore, IHC stain were performed after obtaining lesions of various skin diseases, and as a result, it was confirmed that a unique change in COL6A6 in AD appeared.

This study confirmed the decrease in COL6A6 in the epidermis of AD patients. Exposure of keratinocytes to IL-4 and IL-13 suppressed the expression of *COL6A6*. The exact mechanism and significance of the results on AD require additional research, but there is a study that may be helpful for understanding: Söderhäll et al.[19] reported that the expression of collagen VI α5 chain (COL6A5) was reduced in the outer epidermis in AD patients. COL6A5 has a von Willebrand factor type A domain (vWA), is involved in the binding of proteins and ligands, and plays a role in keratinocyte cohesion[19]. Accordingly, it has been argued that when COL6A5 is reduced, the integrity of the epidermis decreases and the penetration of antigens through the skin increases[19,20]. *COL6A6* exists in the same genetic locus as *COL6A5*, and COL6A6 is collagen with vWA, an important component of the basal lamina. It is also involved in the binding of epithelial cells to fibronectin[9,21,22]. Therefore, it is possible that reduced COL6A6 has a similar effect as the reduction of COL6A5 and more research is needed on that.

Experiments using *COL6A6* siRNA-transfected keratinocytes showed the suppression of *FLG*, *LOR*, and *CASP14*

mRNA expression. Until now, the cause of reduction of filaggrin or loricrin in AD referred to *FLG* mutation, changes in skin pH, calcium gradient, and other factors[23]. However, considering the results of this study and some reports that coculture of keratinocytes with collagen promotes proliferation and differentiation of keratinocytes[24,25], it is possible that suppressed COL6A6 affects the expression of *FLG*, and *LOR*. In other words, the predominant TH2 cytokines in AD reduce the expression of COL6A6 in the epidermis, and the decrease in COL6A6 expression further decreases the expression of *FLG*, *LOR*, and *CASP14*, which are important factors in the skin barrier. Therefore, it can be interpreted that a baby born with a mutation of the *COL6A6* gene is in a condition similar to a baby born with a *FLG* gene mutation because the skin barrier is not strong[18]. Therefore, it is worth conducting a more detailed study.

IL-4 and IL-13, representative inflammatory cytokines in AD, are known to promote fibrosis[24]. IL-13 prevents fibroblasts from producing MMP, causing collagen to accumulate and promote the production of transforming growth factor β-1[26,27]. IL-4 has been reported to be involved in fibroblast proliferation and collagen production[27,28]. This study showed that the amount of COL6A6 in the dermis increased when fibroblasts were exposed to IL-4 and IL-13, and it can be considered that fibrotic cytokines promote COL6A6 production. These changes are not unique to skin fibroblasts. When conjunctival fibroblasts are exposed to Th2 cytokines, collagen production increases and the amount of MMP1 decreases[29].

The accumulated collagen in the dermis may primarily be a result of the disease, but it is questioned whether there is any further significance. Other studies investigated this matter, and the following studies provided insight.

Dendritic cells, which are important for immune reactions in the dermis, move across the extracellular matrix (ECM) and are, mainly composed of collagen1. Dendritic cell movement is influenced by the tissue origin of cells, the degree of maturity, and the three-dimensional structure of the ECM[30]. Because changes in collagen cause changes in the three-dimensional structure of the dermis, it can affect the migration of dendritic cells. When the migration of dendritic cells changes, the binding between antigen-presenting cells and T cells also changes. In other words, the time required for the two cells to cause an immune response becomes too short or too long[31]. Considering the results of the aforementioned study and the results of the present experiment, accumulated collagen is a result of AD, but there is a possibility that it may affect AD inflammation in a reversed manner.

The main limitation of this study was the small sample size. In addition, the expression of COL6A6 in the epidermis of AD patients was so low that protein expression could not be measured using western blot analysis. And when RNA sequencing of the entire lesion was initially performed, the epidermis and dermis were not separated. As the changes of COL6A6 in the epidermis and dermis are reversed, there is a limitation according to this.

In conclusion, AD patients with increased IL-4 and IL-13 levels showed reduced COL6A6 levels in the epidermis; *FLG*, *LOR*, and *CASP14* mRNA levels decreased accordingly. The increased amount of COL6A6 in the dermis coincided with the decreased expression of MMP1. In addition to confirming these specific changes of COL6A6 in AD, this study suggests topics that are worth researching, such as how changes in COL6A6 occur and how COL6A6 affects AD.

## CONFLICTS OF INTEREST

The authors have nothing to disclose.

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## DATA SHARING STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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