ORIGINAL ARTICLE
Iran J Allergy Asthma Immunol
June 2017; 16(3):228-234.

Downregulation of Aquaporin3 in Systemic Sclerosis Dermal Fibroblasts

Bahman Yousefi[1,2], Mahdi Mahmoudi[1], Abdolfattah Sarafnejad[2], Elham Karimizadeh[1], Elham Farhadi[3], Ahmad Reza Jamshidi[1], Hoda Kavosi[1], Saeed Aslani[1], and Farhad Gharibdoost[1]

[1] Rheumatology Research Center, Tehran University of Medical Sciences, Tehran, Iran
[2] Department of Immunology, School of Public Health and Institute of Public Health Research, Tehran University of Medical Sciences, Tehran, Iran
[3] Hematology Department, School of Allied Medical Sciences, Iran University of Medical Sciences, Tehran, Iran

Received: 23 August 2016; Received in revised form: 13 November 2016; Accepted: 27 December 2016

ABSTRACT

Skin dryness and thickening are hallmarks of systemic sclerosis (SSc) disease. Aquaporins (AQPs) are plasma membrane proteins that transport glycerol and water, resulting in water retention and skin hydration. Expression of AQPs has been evaluated in human normal skin. However, expression of these proteins in SSc dermal fibroblasts has not yet been reported. The aim of this study was to assess the expression profile of AQPs in dermal fibroblasts of SSc patients.

Fibroblast cells were extracted from SSc and healthy skin biopsies and characterized using fibroblast surface protein antibody. To evaluate the mRNA expression of AQP1, 3, 5, 7, 9, and 10 in dermal fibroblasts, Real-time PCR was performed using SYBR Green Master mix. Immunoblotting was performed to confirm the results of Real-time PCR.

Our data demonstrated that only AQP1, AQP3, and AQP9 were expressed in human skin fibroblasts. Moreover, the expression of AQP3 mRNA and protein were significantly decreased in SSc dermal fibroblasts compared with healthy fibroblasts.

AQP3, which involves in skin hydration and wound healing through water and glycerol transmission, is downregulated in SSc fibroblasts. Based on previous studies and our results, it seems that SSc manifestations like skin dryness, abnormal wound healing, and fibrotic lesions may be related to downregulation of AQP3 in SSc fibroblasts. Therefore, induction of AQP3 expression can be a potential treatment to relieve SSc skin thickness in the future.

Keywords: Aquaporin; Fibroblast; Gene expression; Systemic sclerosis

Corresponding Authors: Mahdi Mahmoudi, PhD;
Rheumatology Research Center, Shariati Hospital, Kargar Ave., Tehran, Iran. Po.Box: 1411713137, Tele-Fax: (+98 21) 8822 0067, E-mail: mahmoudim@tums.ac.ir

Abdolfattah Sarafnejad, PhD;
Department of Immunology, School of Public Health and Institute of Public Health Research, Tehran University of Medical Sciences, Tehran, Iran. E-mail: sarrafnejad@tums.ac.ir

Published by Tehran University of Medical Sciences (http://ijaai.tums.ac.ir)

INTRODUCTION

Systemic sclerosis (SSc) is a disorder typically resulting in fibrosis of the skin and internal organs.[1] The pathogenesis of this disease is unclear and it includes inflammation, autoimmunity, and vascular damage.[2] The most prominent clinical manifestation of SSc is thickening of the skin and internal organs such as lungs, heart, and digestive system.[3, 4] Fibroblasts activation eventuates in abnormal synthesis and accumulation of extracellular matrix (ECM) in affected organs. Fibroblasts preserve the structural integrity of connective tissue, secreting fibrillar procollagens, and fibronectin. In addition, they regulate the turnover and composition of the ECM via highly specific proteases such as collagenase.[5]

Aquaporins (AQPs) are a family of small hydrophobic proteins with 30 KD molecular weight.[6] These highly expressed proteins are monomeric in the membrane and are comparable to the mineral ion channels.[7] To date, 13 members of this family (AQP0-AQP12) have been identified in the mammals. AQPs are water-selective channels that increase plasma membrane water permeability in secretory and absorptive cells.[8] The AQPs are located at strategic membrane sites in a variety of epithelial cells and most of their physiological functions have been well-defined in fluid absorption or secretion. [9] Moreover, it has been demonstrated that cells expressing AQPs on the plasma membrane show 5 to 50 times more water osmotic permeability.[6] Subfamilies of AQPs such as AQP3, AQP7, and AQP9 are aquaglyceroporins, which transport both water and glycerol.[10] Recent studies indicate that AQPs, like AQP3, are involved in the cell proliferation by cellular glycerol metabolism and biosynthesis dependent mechanism.[7] Therefore, the reduced glycerol results in reduced ATP synthesis and content, and impaired MAP kinase signaling, which in turn culminates in cell proliferation as well as AQP3 overexpression on plasma membrane.[11]Previous studies have shown that AQP3 is expressed on a variety of organs, such as skin, kidney, urinary tract, digestive tract, respiratory tract, eye, and brain.[10, 12]

Fibroblasts play an important role in the structural integrity of connective tissues such as skin and wound healing by migration, proliferation, secretion of ECM components, and synthesizing of highly specific proteases such as collagenase. [13] Some of the main complications of SSc such as defects in skin elasticity, hardness, thickening, and deformity in some parts of the body are due to higher production and secretion of extracellular matrix components.[14-16] In the normal skin physiology, AQPs transport both glycerol and water and, therefore, cause water retention and skin hydration.[17] In addition to abnormal wound healing and fibrotic lesions, it has been shown that water retention and skin hydration do not occur appropriately in SSc patients.[18]

The aim of this study was to investigate the mRNA expression of AQPs in SSc skin fibroblasts. Previous studies[19] have shown that AQP1, 3, 7, 9, and 10 are expressed in human skin. However, the specific skin cells, which expressed AQPs, have not yet been investigated. We evaluated the expression of AQPs (AQP1, 3, 5, 7, 9 and 10) mRNA in primary human dermal fibroblast cultures. Moreover, we compared the expression profile of AQPs in dermal fibroblast of SSc patients and healthy controls.

MATERIALS AND METHODS

Patients

Skin punch biopsies were obtained from the dorsal forearms of 20 diffuse cutaneous SSc (dcSSc) patients (16 females and 4 males) with a mean age of 45 years (25–54 years). All patients fulfilled the American College of Rheumatology Criteria (ACR) for classification and diagnosis of SSc.[20] Healthy skin punch biopsies were also obtained from the dorsal forearms of 20 age- and sex-matched healthy volunteers, who had no history of autoimmune disorders. SSc patients were chosen from those who had been referred to the outpatient clinic of Rheumatology Research Center, Shariati Hospital and Rheumatology Center of Iran. Informed consent forms were obtained from all participants prior to biopsy. This study was in accordance with the ethical standards of Ethics Committee of Tehran University of Medical Sciences (No.: IR.TUMS.REC.1396.2942).

Isolation of Dermal Fibroblasts from Skin Biopsies

Dermal fibroblast cells were extracted using enzymatic dissociation of each biopsy sample in Dispase II and Collagenase I (Roche, Mannheim, Germany), as established previously.[21, 22] Dermal fibroblast cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 100 U/mL penicillin, and

100 µg/mL streptomycin at 37ºC in an incubator with 5% CO2 and 95% humidity.

Characterization of Dermal Fibroblasts

Fibroblast cells were characterized using immunofluorescence assay. Briefly, Fibroblast cells were cultured in DMEM in 24-well plate. After 24 h, cells were washed twice with PBS, fixed with ice-cold methanol, and blocked with phosphate buffered saline containing Triton x-100 (PBST) and 1% bovine serum albumin (BSA). Then, cells were treated with anti-fibroblast surface protein antibody (Abcam, Cambridge, UK) in 1% BSA as primary antibody and sheep anti-mouse Ig (Human Ig Adsorbed)-fluorescein isothiocyanate (FITC) conjugate (Avicenna Research Institute, Iran) in 1% BSA as secondary antibody. Finally, cells were stained with 4',6-diamidino-2-phenylindole (DAPI) to visualize the nucleus and were observed under fluorescence microscopy.

RNA Extraction and Quantitative Real-time PCR

Fibroblast cells were washed with PBS and collected using trypsin for RNA extraction. Total RNA content was extracted using the high pure RNA isolation kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. From each sample, 1 µg RNA was reverse transcribed to cDNA using the transcriptor first-strand cDNA synthesis kit (Roche, Mannheim, Germany) according to the manufacturer's manuals. Primers were designed using Primer 3 online tool (http://primer3.ut.ee/). The primers sequences are presented in Table 1.

Quantitative Real-time PCR was performed using the StepOnePlus Real-time PCR (Applied Biosystems, Foster City, USA) and the SYBR Green gene expression master mix (Takara, Korea). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control to normalize the amount of total RNA in each sample. The purity of each amplified product was confirmed using melting curve and analyzed to ensure the identity of the specific PCR product. The comparative cycle threshold (Ct) method was used to calculate the relative quantification of gene expression. Then relative gene expression for each sample was calculated using the following equation: relative mRNA expression = $(2^{-\Delta Ct}) \times 10^3$ as previously described.[23]

Western Blotting

The cells were trypsinized, washed with cold PBS, and collected by centrifugation, and solubilized in radioimmunoprecipitation assay buffer (RIPA) [50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), and 0.5% sodium deoxycholate] containing a protease inhibitor cocktail (Roche, Mannheim, Germany) and 1 mM phenylmethylsulfonyl fluoride (PMSF) (Roche, Mannheim, Germany). The lysates were incubated for 30 min at 4˚C. Afterwards, debris of the lysate was collected by centrifugation at 14000g for 20 min at 4˚C.

Table 1. Target and housekeeping gene primers used for quantification of mRNA expression by Real-time PCR

Gene		Primer	Product Size (bp)
AQP1	F	5′-GTGGAGGAGGTGAAAGAAAGGG-3′	158
	R	5′-GGTGAGTCGGTGAGCAACTTTG-3′	
AQP3	F	5′- CCACTGGACCTTGCCCAAATAG -3′	156
	R	5′- CACACATACCTGCTGCCCATTC -3′	
AQP5	F	5′-GCCACCTTGTCGGAATCTACT-3′	169
	R	5′-CCTTTGATGATGGCCACACG-3′	
AQP7	F	5′-ACAGGTCTTCAGCAATGGGG-3′	132
	R	5′-GGGATGGTGGAGCCAATGAA-3′	
AQP9	F	5′-CTTCCAGTTCCCGCTATGCTAC-3′	76
	R	5′-CTGAATGCCACAATGTCCTCC-3′	
AQP10	F	5′-ACTGGGATGCTGATTGTGGG-3′	95
	R	5′-CCAGCCATTACCAGCACTGAA-3′	
GAPDH	F	5′-TGTGGGCATCAATGGATTTGG-3′	101
	R	5′-ACACCATGTATTCCGGGTCAAT-3′	

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; AQP, aquaporin

Protein concentrations were determined using Bradford's protein assay.

Equal amounts of proteins were denatured in sample buffer for 10 min at 70°C, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto polyvinylidene difluoride (PVDF) membranes (Roche, Mannheim, Germany). Nonspecific binding was blocked with 4% skim milk in tris-buffered saline tween (TBST) (20 mM Tris/HCl, 137 mM NaCl and 0.01% Tween 20, pH 7.4) for 1 h at room temperature. After blocking, the membranes were incubated with specific antibodies against AQP3 (Santa Cruz, CA, USA) (1:700), GAPDH (Santa Cruz, CA, USA) (1:1000) followed by incubation with horse radish peroxidase (HRP)-conjugated sheep anti-rabbit secondary antibody for 1 h at room temperature. The Enhanced chemiluminescence (ECL) prime detection reagent (Amersham, Buckinghamshire, UK) was utilized for visualizing the bands. The expression of GAPDH was used as an internal control for equal loading.

Statistical Analysis

Data are expressed as the mean ± standard error of the mean (SEM). Statistical comparisons were performed using IBM SPSS v.22 (Armonk, NY, USA). The Mann–Whitney U test was used to evaluate the expression differences between SSc and normal fibroblasts. Density of bands was quantified using Image J 1.4.3.67 software (NIH Image, Bethesda, MD, USA). The GraphPad Prism version 6.00 (GraphPad Software, La Jolla, California, USA) was applied to illustrate data. *p*-values less than 0.05 were considered statistically significant.

RESULTS

Isolation of Dermal Fibroblasts

To ensure that there were no other contaminating cells, immunofluorescence staining of fibroblasts was performed. As shown in Figure 1, fibroblast phenotype of the cultured cells was confirmed by uptake of anti-fibroblast surface protein antibody. The proportion of positive cells indicated that>99% of the cells were fibroblasts.

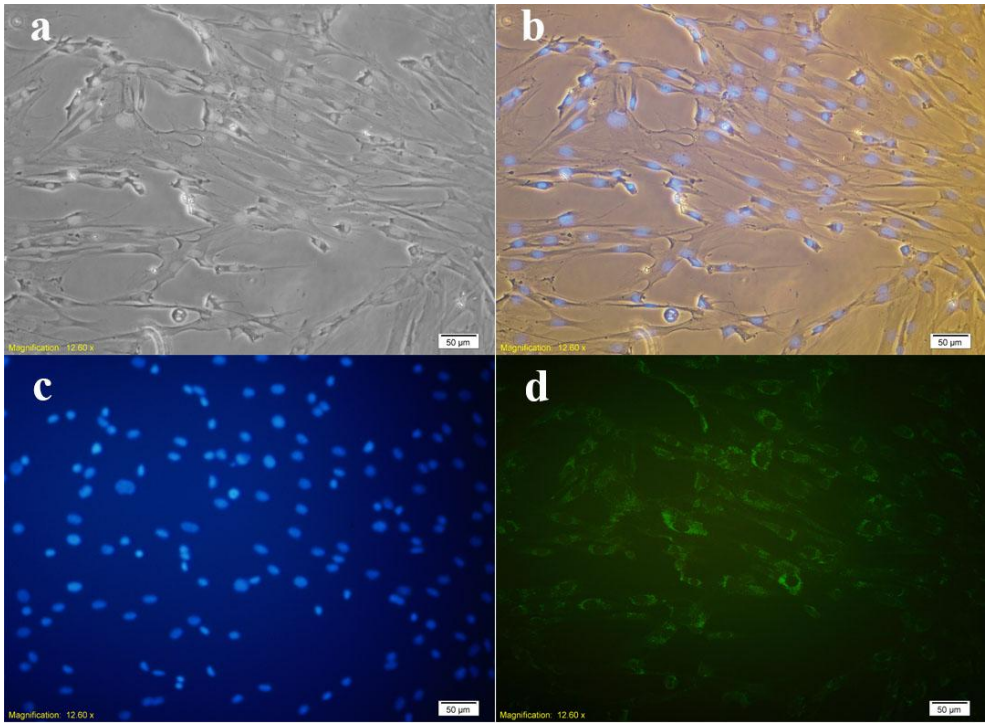

Figure 1. Characterization of fibroblast cells: a) Phase-contrast microscopy of dermal fibroblast cells in culture. b) DAPI stained and phase-contrast microscopy simultaneously. c) DAPI stained fibroblasts. d) Fibroblast cells stained with anti-fibroblasts surface protein antibody. Bars=50 µm.

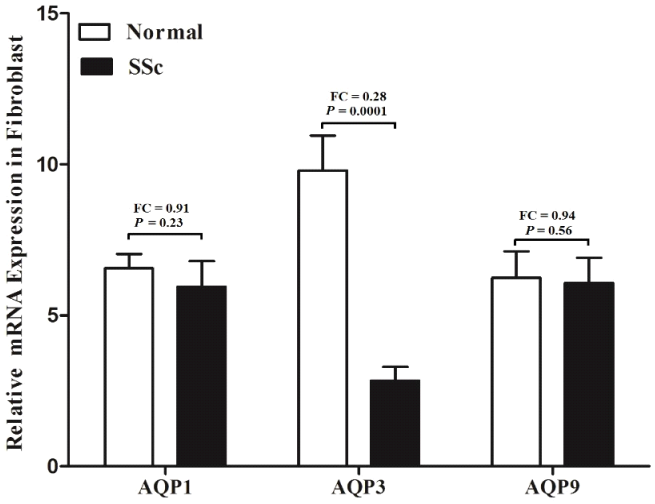

Figure 2. Expression of AQP1, AQP3, and AQP9 mRNA in SSc and healthy dermal fibroblasts

Expression of AQP1, AQP3, and AQP 9 in SSc dermal fibroblast (n=20) compared to healthy fibroblasts (n=20) were measured using SYBR Green Real-time PCR. The relative gene expression was calculated using $(2^{-\Delta Ct})\times 10^3$. (SSc: Systemic Sclerosis; FC: Fold Change)

Gene Expression of AQPs in Dermal Fibroblasts

We performed Quantitative Real-time PCR for AQP1, 3, 5, 7, 9, and 10 in dermal fibroblasts. Our results demonstrated that AQP1, AQP3, and AQP9, were expressed in dermal fibroblasts, but AQP5, AQP7, and AQP10 were not expressed in these cells (Figure 2). As shown in Figure 2, the mRNA expression of AQP3 was significantly downregulated (fold change=0.28; *p*=0.0001) in SSc fibroblasts compared to healthy fibroblasts. However, no significant difference was observed in the mRNA expression of AQP1 and AQP9 (fold change=0.91 and 0.94; *p*=0.23 and 0.56, respectively) in SSc fibroblasts in comparison to healthy fibroblasts.

Downregulation of AQP3 was Confirmed Using Western Blotting

To confirm the results obtained for AQP3 at mRNA level, we carried out immunoblotting. Consistent with the results achieved by Real-time PCR, the expression of AQP3 protein was also downregulated (fold change=0.35; *p*=2.85E-8) in SSc fibroblasts compared with healthy fibroblasts (Figure 3).

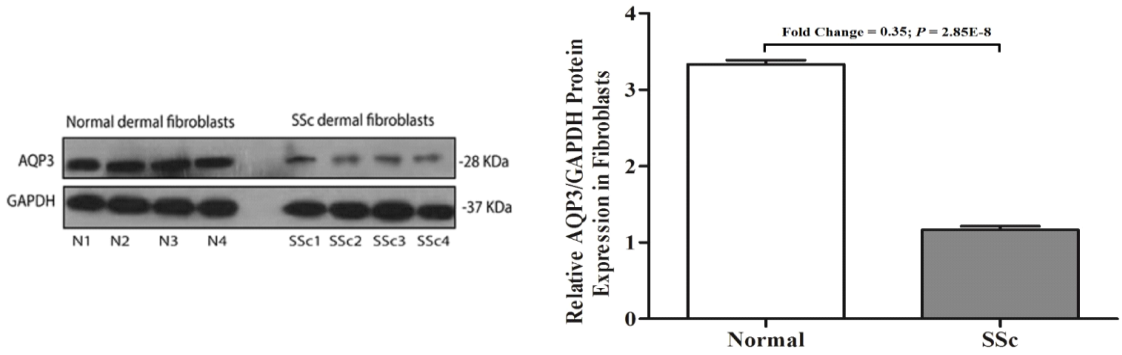

Figure 3. Expression of AQP3 protein in SSc and healthy dermal fibroblasts

Western blotting was performed using lysates of SSc (SSc1-SSc4) and healthy dermal fibroblasts (N1-N4). GAPDH antibody was used as control for equal loading.

(N: Normal; SSc: Systemic sclerosis; FC: Fold change).

DISCUSSION

SSc is an autoimmune disorder characterized by fibrosis in skin and internal organs.[14] Fibrosis is generated by delayed wound healing and excessive synthesis of extracellular matrix components in the affected organs.[24] Wound healing and regeneration of the skin is a multi-step process with the involvement of several cell types, including epidermal keratinocytes, fibroblasts, endothelial cells, and peripheral nerve cells.[25] Activation of fibroblast population is a central event in the initiation and progression of fibrosis in SSc.[14]

AQPs promote wound healing and tissue regeneration through water and glycerol transportation.[11] In addition, AQP3 involves in skin hydration, cell growth, proliferation, and migration.[13, 17] A previous study demonstrated that AQP3 null mice displayed relatively dry skin, reduced skin elasticity, and delayed recovery of the barrier function compared with wild-type mice.[12]

Further studies showed that epidermal growth factor (EGF) binds to epidermal growth factor receptor (EGFR) and induces AQP3 expression via phosphatidylinositol-3 kinase (PI3K) and extracellular signal-regulated kinases (ERK) pathway in human skin fibroblasts.[13] The upregulation of AQP3 by EGF/EGFR signaling plays an important role in wound healing through facilitating of cell migration.[26-29] Other studies revealed that switched off AQP3 eventuated in a reduced expression of matrix metalloproteinases (MMPs), which are among the key regulatory factors in formation and degradation of ECM in both physiological and pathological processes like fibrosis.[30] Our results demonstrated that AQP5, AQP7, and AQP10 were not expressed in human dermal fibroblasts. However, AQP1, AQP3, and AQP9 were expressed in SSc and healthy skin fibroblasts. Additionally, we demonstrated for the first time that the expression of AQP3, at both mRNA and protein levels, was significantly downregulated in SSc skin fibroblasts in comparison to healthy controls.

In conclusion, our study demonstrated that AQP3 was downregulated in SSc dermal fibroblasts compared with healthy dermal fibroblasts. Considering the fact that AQPs regulate glycerol content in tissues through water and glycerol transportation, it seems that loss of elasticity and excessive skin dryness may be associated with reduced expression of AQP3 in SSc fibroblasts. Therefore, induction of AQP3 expression can be a potential treatment for relieving the thickness and dryness of SSc skin.

ACKNOWLEDGEMENTS

This study was funded by Deputy of Research, Tehran University of Medical Sciences (Grant Number: 93-02-41-26560).

REFERENCES

1. Varga J, Abraham D. Systemic sclerosis: a prototypic multisystem fibrotic disorder. J Clin Invest 2007; 117(3):557-67.
2. Balbir-Gurman A, Braun-Moscovici Y. Scleroderma - new aspects in pathogenesis and treatment. Best Pract Res Clin Rheumatol 2012; 26(1):13-24.
3. Korn JH. Immunologic aspects of scleroderma. Curr Opin Rheumatol 1989; 1(4):479-84.
4. Mauch C, Kreig T. Fibroblast-matrix interactions and their role in the pathogenesis of fibrosis. *Rheum Dis Clin North Am;* 16(1):93-107.
5. Abraham DJ, Krieg T, Distler J, Distler O. Overview of pathogenesis of systemic sclerosis. Rheumatology (Oxford) 2009; (48 Suppl 3):iii3-7.
6. Verkman AS, Mitra AK. Structure and function of aquaporin water channels. Am J Physiol Renal Physiol 2000; 278(1):F13-28.
7. Hara-Chikuma M, Verkman AS. Aquaporin-3 functions as a glycerol transporter in mammalian skin. Biol Cell 2005; 97(7):479-86.
8. Verkman AS. More than just water channels: unexpected cellular roles of aquaporins. J Cell Sci 2005; 118(Pt 15):3225-32.
9. Brown D, Katsura T, Kawashima M, Verkman AS, Sabolic I. Cellular distribution of the aquaporins: a family of water channel proteins. Histochem Cell Biol 1995; 104(1):1-9.
10. Verkman AS. Aquaporins at a glance. J Cell Sci 2011; 124(Pt 13):2107-12.
11. Verkman AS. Aquaporins: translating bench research to human disease. J Exp Biol 2009; 212(Pt 11):1707-15.
12. Hara-Chikuma M, Verkman AS. Roles of aquaporin-3 in the epidermis. J Invest Dermatol 2008; 128(9):2145-51.
13. Cao C, Sun Y, Healey S, Bi Z, Hu G, Wan S, et al. EGFR-mediated expression of aquaporin-3 is involved in human skin fibroblast migration. Biochem J 2006;

400(2):225-34.
14. Jinnin M. Mechanisms of skin fibrosis in systemic sclerosis. J Dermatol 2010; 37(1):11-25.
15. LeRoy EC. Increased collagen synthesis by scleroderma skin fibroblasts in vitro: a possible defect in the regulation or activation of the scleroderma fibroblast. J Clin Invest 1974; 54(4):880-9.
16. Moinzadeh P, Denton CP, Abraham D, Ong V, Hunzelmann N, Eckes B, et al. Biomarkers for skin involvement and fibrotic activity in scleroderma. J Eur Acad Dermatol Venereol 2012; 26(3):267-76.
17. Fluhr JW, Mao-Qiang M, Brown BE, Wertz PW, Crumrine D, Sundberg JP, et al. Glycerol regulates stratum corneum hydration in sebaceous gland deficient (asebia) mice. J Invest Dermatol 2003; 120(5):728-37.
18. Muratore M, Quarta L, Raho L, Costanza D, Frisenda S, Calcagnile F, et al. Management of cutaneous discomfort in patients with scleroderma: a clinical trial. Reumatismo 2013; 65(5):240-7.
19. Boury-Jamot M, Sougrat R, Tailhardat M, Le Varlet B, Bonte F, Dumas M, et al. Expression and function of aquaporins in human skin: Is aquaporin-3 just a glycerol transporter? Biochim Biophys Acta 2006; 1758(8):1034-42.
20. LeRoy EC, Black C, Fleischmajer R, Jablonska S, Krieg T, Medsger TA, Jr, et al. Scleroderma (systemic sclerosis): classification, subsets and pathogenesis. J Rheumatol 1988; 15(2):202-5.
21. Takashima A. Establishment of fibroblast cultures. Curr Protoc Cell Biol 2001; Chapter 2:Unit 2 1.
22. Loder RM, Martin GM. Establishment of human skin fibroblast cultures with an automatic wedge biopsy instrument. J Invest Dermatol 1966; 47(6):603-5.
23. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc 2008; 3(6):1101-8.
24. Bou-Gharios G, Osman J, Black C, Olsen I. Excess matrix accumulation in scleroderma is caused partly by differential regulation of stromelysin and TIMP-1 synthesis. Clin Chim Acta 1994; 231(1):69-78.
25. Hara-Chikuma M, Verkman AS. Aquaporin-3 facilitates epidermal cell migration and proliferation during wound healing. J Mol Med (Berl) 2008; 86(2):221-31.
26. Matthay MA, Thiery JP, Lafont F, Stampfer F, Boyer B. Transient effect of epidermal growth factor on the motility of an immortalized mammary epithelial cell line. J Cell Sci 1993; 106 (Pt 3):869-78.
27. Fujii K, Dousaka-Nakajima N, Imamura S. Epidermal growth factor enhancement of HSC-1 human cutaneous squamous carcinoma cell adhesion and migration on type I collagen involves selective up-regulation of alpha 2 beta 1 integrin expression. Exp Cell Res 1995; 216(1):261-72.
28. Chen JD, Kim JP, Zhang K, Sarret Y, Wynn KC, Kramer RH, et al. Epidermal growth factor (EGF) promotes human keratinocyte locomotion on collagen by increasing the alpha 2 integrin subunit. Exp Cell Res 1993; 209(2):216-23.
29. Blay J, Brown KD. Epidermal growth factor promotes the chemotactic migration of cultured rat intestinal epithelial cells. J Cell Physiol 1985; 124(1):107-12.
30. Xu H, Xu Y, Zhang W, Shen L, Yang L, Xu Z. Aquaporin-3 positively regulates matrix metalloproteinases via PI3K/AKT signal pathway in human gastric carcinoma SGC7901 cells. J Exp Clin Cancer Res 2011; 30(1):86.