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Comparing *COL7A1* Gene Expression in Fibroblast Cells of Dystrophic Epidermolysis Bullosa Patients with Clinical Responses to Autologous Fibroblasts Transplantation

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Abstract

Objectives: This clinical research aimed to establish autologous fibroblasts transplantation as a possible treatment for patients with DEJ. The COL7A1 gene expression was also evaluated.

Methods: Six patients (3M and 3F), 4 with no recurrent wounds and 2 with recurrent wounds after surgery, and 15 healthy subjects were included in the study as controls. Quantitative real-time polymerase chain reaction (real-time PCR) analysis of the COL7A1 gene was performed using an oligonucleotide primer pair designed to amplify across the exon/exon junction.

Results: The COL7A1 expression level was down-regulated at exons 26-27, 47-48, 96-97, and 116-117 in all patients' fibroblasts compared with the healthy controls. However, the expression of the COL7A1 gene in the fibroblasts of the patients with a positive response to the treatment was not significantly changed compared with the patients with the poor response. (ClinicalTrials.gov NCT01908088)

Conclusion: In this study the mRNA expression levels of COL7A1 were significantly less in the patients when compared with healthy controls. However the COL7A1 expression after autologous fibroblasts transplantation was not different between the two groups of patients, and further examination is needed to elucidate the mechanism of the treatment.

Keywords: RDEB, COL7A1, gene expression, fibroblast, transplantation, clinical trial, dermal-epidermal junction

Introduction

Epidermolysis bullosa (EB) is a rare genetic skin disorder that results in fragility, easy blistering, and ulceration of the skin with painful and life-threatening complications. The disease has an incidence of 0.08–0.5 per million live births, occurs among all ethnicities, and is inherited either from one of the parents or both due to deficiency in several genes.[1-3] Recessive dystrophic EB (RDEB) is attributed to the bi-allelic loss-of-function mutations in COL7A1 (3p21.31), a gene expressed by skin keratinocytes and fibroblasts which encodes type VII collagen (C7).[4] C7 is the main component of anchoring fibrils (AFs) which ensures the adherence of the epidermis to the dermis within the basement membrane. Mutation in the COL7A1 gene alters the C7 structure, thereby compromising the integrity of the dermal-epidermal junction (DEJ), and causes blistering and tissue cleavage, which leads to extensive scarring.[5-9]

EB has four main types including simplex, junctional, dystrophic, and Kindler.[10,11] Recessive dystrophic epidermolysis bullosa (RDEB) is the most severe type of the disease. Children with RDEB are usually affected since birth and, in most cases, blisters virtually cover all the body and the patients who survive childhood frequently develop squamous cell carcinoma (SCC).[12,13] The blisters and ulcers on the hands and fingers of the patients suffering from RDEB cause hand deformities with pseudo-ductility and flexor contractures known as 'mitten hand'.[14]

Treatments for RDEB are Wound grafting, Allogeneic fibroblasts, Mesenchymal stromal cells, Bone marrow transplantation and Gene therapy.[15,16] Sometimes RDEB patients with mitten hand undergo hand reconstructive surgery to regain their hand function. Afterwards, the surgeon applies auto-graft skin or skin substitutes on the open wounds of the hand and fingers. To prevent further adhesion and facilitate satisfactory postoperative healing, fibroblast and/or keratinocyte transplantation could be applied to the wound. However, fibroblasts are easier to culture than keratinocytes and are better cells to target in planning cells.[17-19] The aim of this study was to investigate the responses of patients with RDEB to autologous fibroblasts transplantation as a possible treatment. The authors believe that the major effect of autologous fibroblasts transplantation is to increase COL7A1 mRNA levels. As a result of increased expression of COL7A1 we have greater deposition of the mutant type VII collagen at the DEJ and formation of rudimentary anchoring fibrils. The COL7A1 gene expression in the fibroblasts of the patients with RDEB was independently evaluated and the results were compared with the healthy controls. Furthermore, the COL7A1 gene expression in the patients with inappropriate response to autologous fibroblasts transplantation was evaluated and then compared with the patients with the appropriate response after 18 months.

Patients and Methods

Patients

Six patients with RDEB between the age range of 2 and 30 years old were selected. Previously, autologous fibroblasts

from patients had been seeded on the amniotic membrane. Fibroblasts were transplanted on the hands of the RDEB patients with mitten hands after hand reconstructive surgery. The patients were followed up for 18 months and it was revealed that wounds of some patients returned as soon as a few months after the surgery; however, wounds of the others remained intact for 18 months (Figures 1 and 2). The control group were 15 healthy individuals. The fibroblasts of these individuals with no personal or family history of EB were assessed as a control group.

If the recurrence time of deformity after hand surgery was less than or equal to 6 months, it was considered as an inappropriate treatment response. Diagnosis of RDEB patients was established based on the clinical symptoms and NGS test by a dermatologist. The clinical features of all the patients are shown in Table 1.

The study protocol was approved by Royan Institute's Ethics Board Committee in Medical Research. (ClinicalTrials.gov NCT01908088).

Gene Expression

Four patients with appropriate clinical response and two patients with a poor clinical response after the treatment were selected for assessment of COL7A1 gene expression.

RNA Extraction and cDNA Synthesis

Skin biopsies from healthy donors and EB patients were obtained and stored in liquid nitrogen for the investigation. Fibroblasts were extracted using an enzymatic method and cultured in the T75 flask in modified DMEM/F12 medium (Gibco-USA) supplemented with 10% fetal bovine serum (FBS) (Hyclone-USA) and 1% Glutamax (Gibco-USA). Total RNA was extracted from the cultured cells (at least 10^6 cells were used for each sample) using the Roche kit (Mannheim, Germany) with DNase I treatment according to the manufacturer's instructions. The quality and quantity of the extracted RNA were evaluated by Nano-drop UV–Vis spectrophotometer (Thermo, USA) and electrophoresis. cDNA was synthesized from 1 µg of mRNA using the Thermo Fisher cDNA synthesis kit (Waltham, MA, USA), followed by quantitative polymerase chain reaction (qPCR).

Primer Design

According to the ABI Biosystems StepOnePlus instrumentation, Beacon Designer software was used to design the primers. The default parameters of the software were set to be very limited. The most important parameters were the amplicon length, quality, and hotspot region for the dominant mutations of the primers. Exons and introns were relatively small and the default parameters for the amplicon lengths were set between 120 and 150 nucleotides. To overcome the problem of genomic DNA, the primers were designed from the exon–exon junctions (E-E-jns). Using Beacon Designer software, these primer sets had to be searched manually. The regions, in which mutations were more likely to occur, were selected. 5 sets of primers designed for 5 different regions of the COL7A1 gene: Exons 4-5 exons 26-27, exons 47-48, exons 96-97, and exons 116-117. The primers' positions and sequences are listed in Table 2. In addition, the β-actin housekeeping gene was measured in parallel to normalize the differences between the samples and operations.

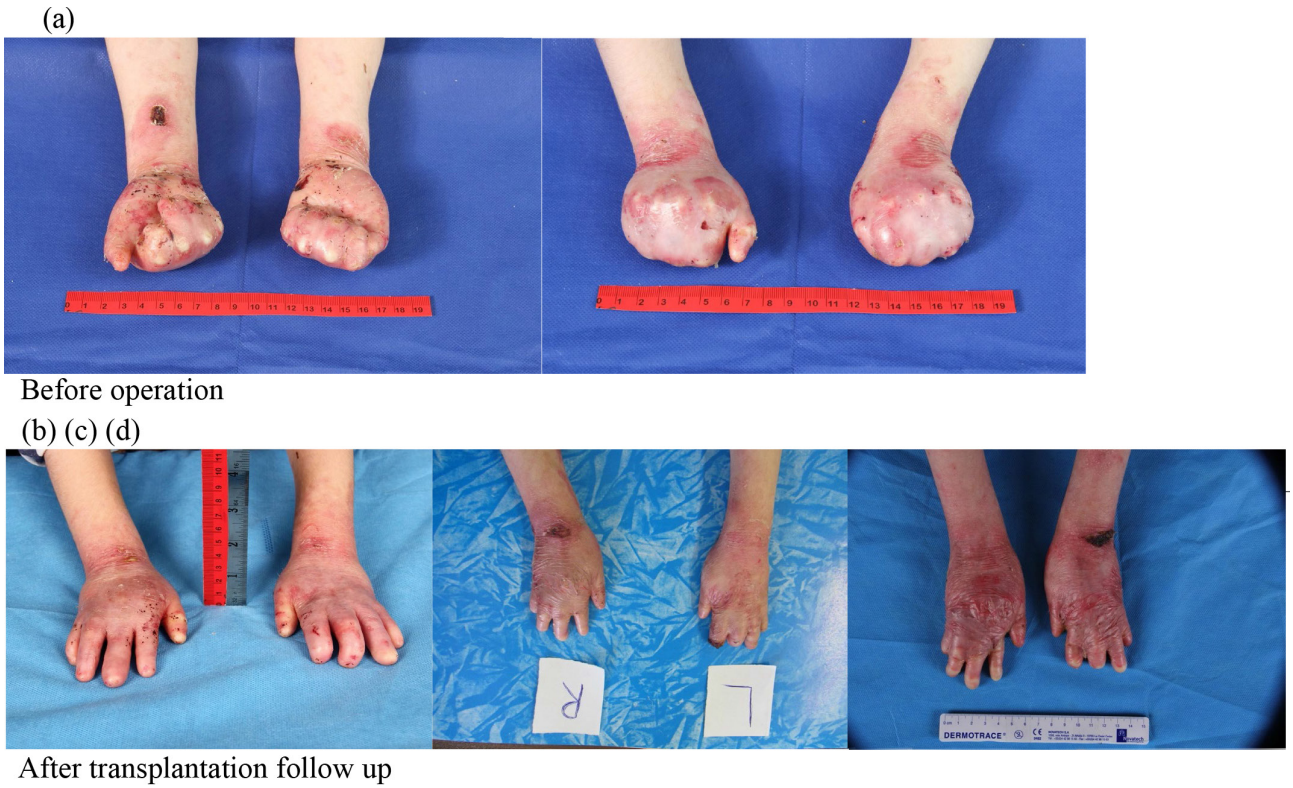

Fig. 1 **Successful autologous fibroblasts transplantation as a treatment for RDEB patients. (a) Hands of six-year-old boy (patient 1) before operation, (b) The grafted area remained epithelized without blistering and recurrent wounds, the grafted area after two (c), nine (d), and 15 months after the treatment.**

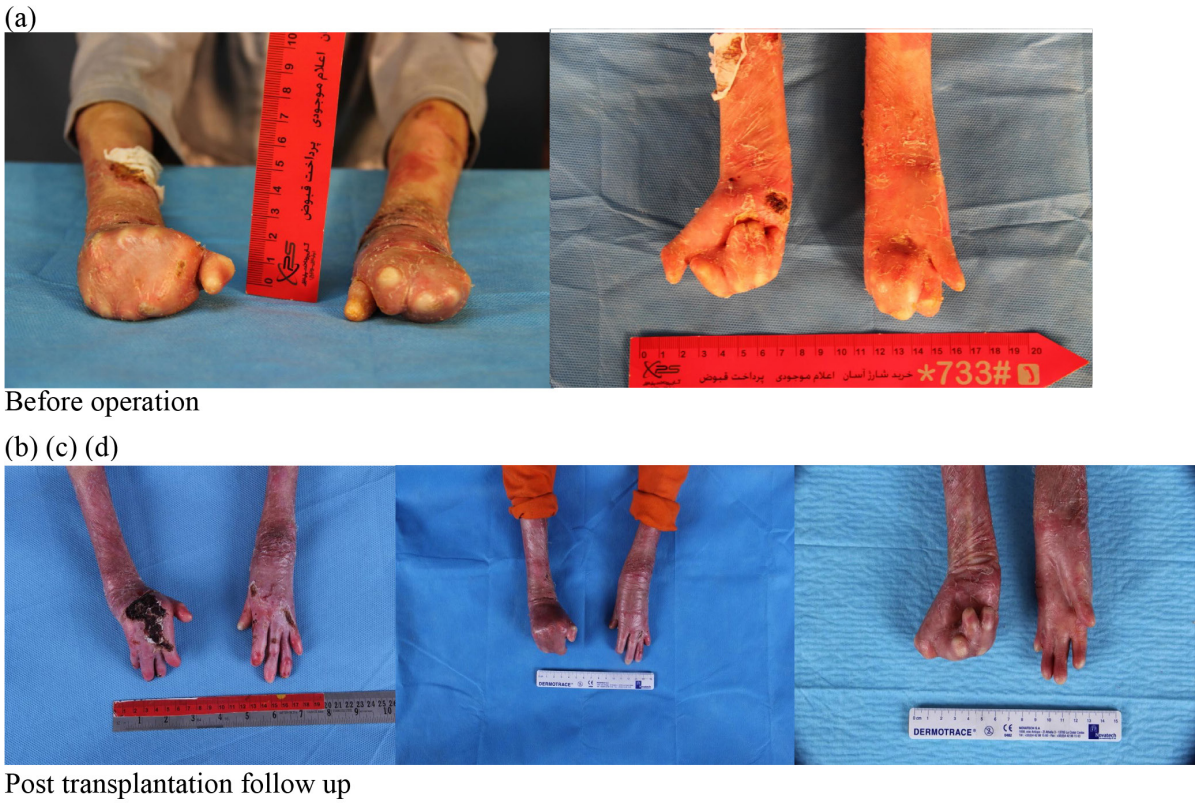

Fig. 2 **Unsuccessful autologous fibroblasts transplantation. (a) Hands of patient 3 before operation, (b) The grafted area with blistering and recurrent wounds 2 months after the treatment, (c) The grafted area 9 months after the treatment, (d) The grafted area 15 months after the treatment.**

Table 1. **Characteristics of RDEB patients**

Patient	Age (years)/Sex	Distribution of disease (%)	Parents affected	Familial marriage	GI surgery	Previous hand surgery	Recurrence of deformity (month)
01	6/M	25	No	Yes	No	Yes	9
02	17/F	30	No	Yes	Yes	Yes	12
03	7/F	20	No	Yes	No	No	6
04	9/M	15	No	Yes	No	No	18
05	15/M	40	No	Yes	Yes	No	18
06	7/M	50	No	Yes	No	Yes	5

M: Male; F: Female; GI: Gastrointestinal.

Table 2. **Forward and reverse primers for B-ACTIN and Col7A1 genes, with temperature melting at 56°C**

Gene	Forward primers	Reverse primers
B-ACTIN	TGAAGATCAAGATCATTG	TAACGCACTAAGTCATAA
Col7A1 (exon4,5)	CTATTTGCTGTGGGGATC	AAGATGCTGAAGTCATTGA
Col7A1 (exon26,27)	GTCACAGCTCACAGATAC	CCACATTAAGCCCAGAAG
Col7A1 (exon47,48)	CAAAGGAGAAAAGGGAGATG	TCTCCAGGAAGAAACCAAG
Col7A1 (exon96,97)	AAAGGAGACAAGGGAGAC	CTTGTCACCCTTTAGTCC
Col7A1 (exon116,117)	GATAGTGATGACCCTGT	GCCACCATAGACAAAAGG

Real-time Polymerase Chain Reaction

The expression level of COL7A1 in fibroblasts of 6 patients with DEB and 15 healthy controls was measured by qPCR. For qPCR, cDNA was added to the qPCR MasterMix (ROX) and SYBR Green. All the reaction mixtures were amplified using an ABI Biosystems StepOnePlus real-time PCR system according to the following steps: Pre-denaturing step at 95˚C for 30 seconds, 40 cycles of denaturing step in 95˚C for 15 seconds, 40 cycles of annealing step in 56˚C for 30 seconds, 40

cycles of extending step in 72˚C for 30 seconds, and finally the melting curve step in 95˚C for 15 seconds. Relative expression levels were determined using the threshold cycle (Ct) numbers or values.

Data Analysis

The relative quantitative real-time PCR technique was computed and used for statistical analysis. The efficiency of the primers of the target and housekeeping genes was calculated using Linreg PCR software (AMC, Amsterdam). The cycle threshold (Ct) values were analyzed using REST software (REST© 2009, Qiagen, Germany) based on the Pfaffl method.[20] This software is specifically designed for molecular biology applications and compares two or more groups or conditions using Ct values (Ct) in the control group for multiple references and target genes. The target gene level was normalized to the *ACTB* housekeeping gene in the same sample. Each sample was measured in triplicate. Moreover, the melting curves and amplification plot for each PCR product were analyzed to ensure the specificity of the amplification product. Moreover, the standard curve revealed that the efficiency of the reactions for *ACTB* and COL7A1 was 1.86 and 1.97, respectively. The maximum PCR efficiency was 2 and the minimum was 1. The data were performed by using a one-way analysis of variance (ANOVA). Data represent the mean ± SD of three replicates.

Results

COL7A1 is less expressed in the *patients' fibroblasts* than that of healthy controls:

Expression of the COL7A1 gene (Figure 3) using the primer target exons 4-5 showed no significant difference between healthy volunteers and the patients group. However, the expression decreased significantly when using the other designed primers: Exons, 26-27, 47-48, 96-97, and 116-117.

COL7A1 expression in the patients with poor response did not change significantly compared to patients with the appropriate response.

Expression of the COL7A1 gene in fibroblasts of the patients with appropriate response demonstrated no difference compared with that of the patients with poor response; So Differences in response to treatment can be traced to other factors such as differences in patients 'mutations, mutation quality, protein modelling, and patients' clinical and physiological conditions (Figure 4).

Discussion

EB is a blistering disorder caused by at least 18 variable gene mutations.[20] Identification of specific mutations in patients with RDEB, as well as other heritable disorders, has several advantages for the diagnosis and prognostication of the disease. Several studies have provided evidence for the important role of the COL7A1 gene in RDEB.[2,21,22] COL7A1 is essential for promoting the attachment of the epidermis to the dermis. Its dysfunction may lead to the generalized mucosal and cutaneous blistering associated with severe deformities.[23,24]

Over the past few years, significant progress has been made in preclinical studies aiming at developing new treatments for RDEB patients using Various gene- and cell-based therapies. There are two major approaches to introducing the expression of intact COL7A1.

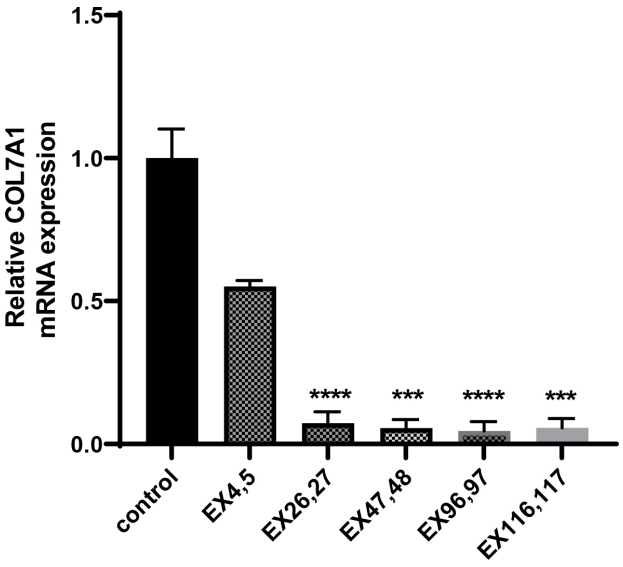

Fig. 3 **The outcome of the gene expression of Col7A1. Col7A1 (exon4, 5) expression change was not significant in the patient group compared to the control group ($P > 0.05$). Col7A1 (exon26, 27) is down-regulated in patient group in comparison to control group (****$P < 0001$). Col7A1 (exon47, 48) is down-regulated in patient group in comparison to control group (****$P < 001$). Col7A1 (exon96, 97) is downregulated in patient group in comparison to control group (****$P < 0001$). Col7A1 (exon116, 117) is down-regulated in patient group in comparison to control group (****$P < 001$).The data were performed by using one-way analysis of variance (ANOVA). Data represent the mean ± SD of three replicates. ***$P < 0.001$ compared with control?**

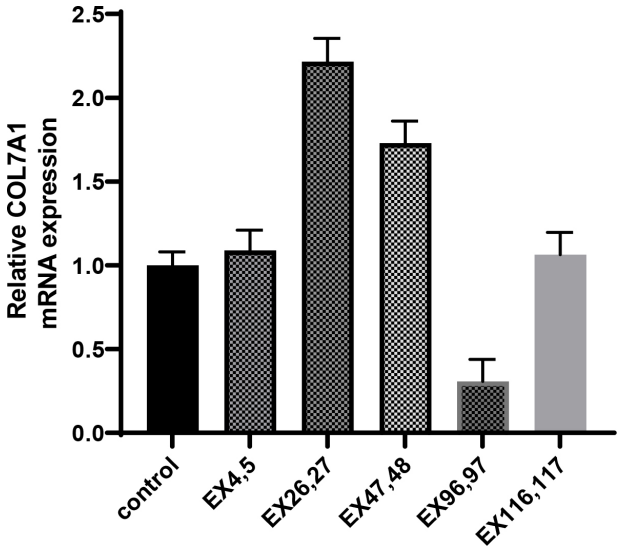

Fig. 4 **The results of COL7A1 expression in the patients with poor response compared to patients with appropriate response. COL7A1 expression in the patients with poor response did not change significantly compared to patients with appropriate response ($P > 0.05$). The data were performed by using one-way analysis of variance (ANOVA). Data represent the mean ± SD of three replicates.**

One is *ex vivo* gene therapy with retrovirus vectors transferring the full-length COL7A1 cDNA into epidermal stem cells or fibroblasts based on autologous transplantation of epidermal grafts or intradermal injection, respectively.[25,26]

Matsumura W. et al.[27] have been used cultured epidermal autografts (CEAs) from clinically normal skin for RDEB. The grafted area remained epithelized for more than 16 years without blistering. COL7 expression increased in the basement membrane zone (BMZ) of the grafted area than in the affected (untreated) area. They reported (CEA) as a potentially well-tolerated treatment for RDEB patients.

Quantitative data analysis indicated that the expression of COL7A1 in DEB patients was significantly downregulated compared with the control group ($P < 0.05$). It has been estimated there were about 200 families with DEB in Iran. They examined 152 families with DEB.[28] COL7A1 mutations were found in 95 of these patients (96.9%) and 104 distinct mutations were identified.

Here, the gene expression profiling of autologous dermal fibroblasts was shown in the RDEB patients with the inappropriate response and the RDEB patients with an appropriate response to the autologous fibroblasts transplant after 5 years. Similar to the present investigation. The potential clinical benefits of intradermal injections of allogeneic fibroblasts has been studied in five patients with RDEB. No adverse effects were observed.[19,29] Injections of allogeneic fibroblasts led to less dermal-epidermal blistering and the increased type VII collagen expression at the DEJ. The mutant COL7A1 gene expression in the recipients was increased 3 months after the intradermal injection of allogeneic fibroblasts. They believed that in RDEB patients, the main cause of the increase in type VII collagen was the increased expression of the COL7A1 gene.

It has been showed that intradermal injections of genetically corrected patient-derived fibroblasts have positive effects on the treatment of patients.[30] He demonstrated that a single *in vivo* intradermal injection of 3×10^6 fibroblasts is efficient to restore C7 expression and anchoring fibril formation at the dermal-epidermal junction. Injected fibroblasts are detectable in the injected area 8 weeks after a single injection and dermal-epidermal adherence had been improved. The test on the dissemination potential of intradermally injected fibroblasts by PCR for analyzing COL7A1 gene sequence and COL7A1 sequence were detected in injected skin samples as they expected.

The application of gene reframing therapy for RDEB fibroblast with CRISPR/Cas9 is widely used for gene editing.[31] qRT-PCR analysis showed that COL7A1 expression of treated primary RDEB fibroblasts was higher than that of non-treated primary RDEB fibroblasts. They also intradermally injected reframed immortalized RDEB fibroblasts and normal fibroblasts into the back skin of NOD/ShiJic-*SCID* mice. Two weeks after injection, human COL7(hCOL7) was detected along the dermal-epidermal junction, suggesting that RDEB fibroblasts can express COL7 protein after gene reframing therapy.[32-34]

In the present study, a comparison of the COL7A1 gene expression between the two groups after this treatment revealed no significant up- and/or down-regulation ($P > 0.05$); however, the decreased expression of COL7A1 was observed. The COL7A1 secreted from fibroblasts was more likely to be degraded in the wound bed. This is the first study that examined the COL7A1 expression of RDEB patients after autologous fibroblasts transplantation.

Conclusion

This preliminary study showed that the mRNA expression levels of COL7A1 were significantly less in the patients with RDEB compared with a healthy volunteer with match age and sex. The COL7A1 expression after transplantation of the fibroblasts did not significantly change in the patients with poor response compared with the patients with positive response and further studies are needed to elucidate the mechanism of the treatment.

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Conflict of Interest

None.

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