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ARTICLE

A comparison expression analysis of CXCR4, CXCL9 and Caspase-9 in dermal vascular endothelial cells between keloids and normal skin on chemotaxis and apoptosis

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ABSTRACT

This present study was designed to explore key biological characteristics and biomarkers associated with dermal vascular endothelial cells of keloids. GSE121618 dataset was downloaded in the Gene Expression Omnibus (GEO) Database, including the KECs group and NVECs group. Through GEO2R, we have screened the differentially expressed genes (DEGs) and performed gene ontology (GO), Gene Set Enrichment Analysis (GSEA), and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis. Then, we constructed a protein–protein interaction (PPI) network and analyzed hub genes via the Search Tool for the Retrieval of Interacting Genes (STRING) Online Database and Cytoscape software. Furthermore, experiments were performed to validate the expression of selected genes, including H&E staining, immunohistochemical staining, Western blot, and RT-qPCR. A total of 1040 DEGs were selected with GEO2R online tools. Most of the enriched pathways and processes focus on cell migration, tube development, chemotaxis, cell motility, and regulation of apoptosis. With the assistance of STRING and Cytoscape, hub genes were selected. In our validation experiments of RT-qPCR, the mRNA expression of selected genes has significant differences between different groups in tissue and cell experiments. As was shown in immunohistochemical staining, the proteins of CXCR4, CXCL9, and Caspase-9 had higher expression levels in tissue samples of the Keloid group than the Normal skin group. Western blot and RT-qPCR in dermal vascular endothelial cell experiments were consistent with the aforementioned results. This study has provided a deeper analysis of the pathogenesis of dermal vascular endothelial cells in keloids. Genes of CXCR4, CXCL9, and Caspase-9 may influence the processes of inflammatory responses and vascular endothelial cell apoptosis to exert crucial effects in the development of keloids.

Abbreviations: GEO: gene expression omnibus; DEGs: differentially expressed genes; KVECs: keloid vascular endothelial cells; NVECs: normal skin vascular endothelial cells; GO: gene ontology; KEGG: Kyoto encyclopedia of genes and genomes; PPI: protein protein interaction; BP: biological process; CC: cellular component; MF: molecular function; GSEA: gene set enrichment analysis; STRING: search tool for the retrieval of interacting genes; MCODE: molecular complex detection

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KEYWORDS
Keloid; differentially expressed genes; dermal vascular endothelial cells; inflammation; apoptosis

Background

The keloid is a fibroproliferative disease related to the excessive deposition of collagen in the extracellular matrix. The lesions of keloids often exceed the boundary of the initial wound and continue to invade the surrounding skin tissues, accompanied by a high recurrence rate [1]. In addition to causing symptoms such as itching, pain, and burning sensation, keloids also affect the patient's appearance, which can severely influence the patient's life quality. At present, there are many treatments for keloids, including surgery, radiotherapy, laser treatment, cryotherapy and corticosteroids treatment, however, the clinical effects are not quite satisfactory.

Previous studies on keloid cytology have mainly focused on the biological characteristics of fibroblasts, and research on dermal vascular endothelial cells is sorely lacking. In fact, both fibroblasts and vascular endothelial cells play an irreplaceable role in the healing process of wounds. The abnormal function of dermal vascular endothelial cells is closely related to the hypoxic state inside the keloid [2]. The hypoxic state within the tissue may affect the structure and function of endothelial cells through various signal pathways including Epithelial/Endothelial-mesenchymal transition (EMT/EnMT). Damaged dermal reticular layer and hypoxia will increase the permeability of blood vessels, with the induction of chemokines, more inflammatory cells will migrate to the extravascular area [2], aggravating the inflammatory status of the local region [3]. Chemokines play a vital role in inflammation, wound repair, angiogenesis and immune responses, which can control the directed migration of different types of cells [4]. Chemokines can also influence the tumor progression through several cell life activities, such as cell proliferation, migration, EMT/EnMT and apoptosis.

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Microarray data of GSE121618 was selected and downloaded from Gene Expression Omnibus (GEO) Database, which included a large amount of microarray and high-throughput gene expression data submitted by research institutions around the world [5]. Differentially expressed genes (DEGs) were identified between the keloid vascular endothelial cells (KVECs) group and NVECs group (normal skin vascular endothelial cells). Based on DGEs, gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was performed. Furthermore, the protein–protein interaction (PPI) network of DEGs was constructed with Search Tool for the Retrieval of Interacting Genes (STRING) Database and Cytoscape software, and CytoHubba plug-in of Cytoscape was applied for analyzing hub genes in PPI network. Furthermore, relevant experiments were performed to verify the expression of selected genes.

Materials and methods

Identification of DEGs

GEO2R (http://www.ncbi.nlm.nih.gov/geo/geo2r) is an online tool in GEO database designed for DEGs identification, based on GEOquery R package (for data reading and processing) and limma R package (for data analyzing) [6]. The DEGs between KVECs group (keloid vascular endothelial cells) and NVECs group (normal skin vascular endothelial cells) in GSE121618 dataset were screened by GEO2R ($|\log FC| > 1$ and p-value < 0.05) [7]. The data were obtained using the Agilent-072363 SurePrint G3 Human GE v3 8x60K Microarray.

Functional enrichment analysis

GO analysis performs functional enrichment analysis on one or more sets of genes to find the functional preference of the selected gene set, including enriched biological process (BP), cellular component (CC) and molecular function (MF) [8].

KEGG pathway enrichment analysis can systematically analyze the metabolic pathways of gene products in cells and the various functions of these gene products [9].

Gene Set Enrichment Analysis (GSEA) is used to evaluate the distribution trend of genes in a pre-defined gene set, to judge its contribution in phenotypic correlation ranking. Theoretically, GSEA is easier to cover the impact of subtle and coordinated changes on biological pathways.

To describe the DEGs' biological function, GO analysis was performed via ggplot2 package in R language. The bar graph and data table of KEGG pathway were collected from Enrichr. Furthermore, related pathways and molecular mechanisms in keloids patients were assessed by GSEA software. $p < 0.05$ was considered statistically significant.

Construction of PPI network

STRING Database is an online tool for searching PPI relationships [10]. In PPI network composed of DEGs, each node represents a specific protein, and the spiral structure inside the node represents that the structure of the protein was confirmed. Then, the PPI network was visualized through Cytoscape (a public bioinformatics software) [11]. MCODE (Molecular Complex Detection), an important plug-in of Cytoscape, was applied for discovering closely connected modules in the PPI network [12]. The most significant PPI module was screened and analyzed using MCODE, which helped us to investigate functional protein/gene interaction on deeper levels.

Hub genes analysis

Based on the PPI network that has been imported in Cytoscape and different topological algorithms in CytoHubba, we identified hub genes and related interaction networks, which displayed the connection status of these core nodes. The darker the node color was, the higher the score was. Top twenty hub genes were listed according to four topological algorithms, that is Maximal Clique Centrality (MCC), Maximum Neighborhood Component (MNC), Edge Percolated Component (EPC) and Degree. Meanwhile, hub genes were selected for further analysis using the GeneMANIA, a plug-in of Cytoscape which is committed to discovering the mutual relation of protein-protein, protein-gene, heredity, pathway and protein expression data. Furthermore, BiNGO and ClueGO (plugs-in of Cytoscape) were applied to analyzing biological processes, which were visualized in the form of interaction networks with some modifications.

Patients and samples management

This research was approved by the bioethical committee of Plastic Surgery Hospital and has the informed consent of all patients. Twenty patients were randomly assigned to different groups, who were selected from Plastic Surgery Hospital, Chinese Academy of Medical Sciences by randomization. Patients were counterbalanced into two groups: Keloid group, keloids samples from ten patients with identified keloids (no gender, age and scar site differences) and Normal skin group, normal skin samples from ten patients without obvious scars (no gender and age differences). The keloids were evaluated by professional plastic surgeons and samples of paraffin-embedded tissue were re-confirmed by pathological analysis. Keloids patients did not have any obvious infection or rupture on the surface of the lesion. Additionally, no regional or systemic treatments were applied with them.

Cell culture and isolation

The specimens were washed with phosphate buffer saline 2–3 times, and then were cut into 1 cm × 2cm pieces, respectively. Skin Dissociation Kit was applied to digesting with vascular endothelial cells for one hour according to manufacturer's instructions (Miltenyi Biotech, Bergish Gladbach, Germany), and vascular endothelial cells were cultured in DMEM (Dulbecco's Modified Eagle's medium, Thermo Fisher Scientific, USA) at 37 °C and 5% CO_2. Then, Miltenyi Biotech magnetic sorting system was applied to sort the isolated cells [7]. Two groups were set up in cell experiments: KC group (keloids vascular endothelial cells from keloids samples), NC group (normal skin vascular endothelial cells from normal skin samples).

RNA isolation and RT-qPCR

Total RNA was extracted with TRIzol™ Reagent (No. 15596026. PPS, Thermo Fisher Scientific), which is a monophasic solution of phenol, guanidine isothiocyanate and can facilitate the isolation of a variety of RNA species. All steps were in accordance with the manufacturer's instructions and performed at room temperature (20–25 °C). In this system, absorbance at 260 nm provides total nucleic acid content, while absorbance at 280 nm determines sample purity. Furthermore, the ProtoScript M-MuLV First Strand cDNA Synthesis Kit (New England Biolabs, USA) and the anchored oligod(T) primer [d(T)23VN] were applied in reverse transcription. The forward and reverse primers of CXCR4, CXCL9, Caspase-9, ADCY1, APLN, GRM2, APLNR, GALR2, TAS1R1, NPY2R, and GAPDH

Table 1. Sequences of primers used for quantitative real-time PCR.

Target gene	Forward	Reverse
CXCR4	5′-TAGAAATGGCCAGCCAGCAC-3′	5′-GAGCTTGCTTCAGTGGACCT-3′
CXCL9	5′-ATCTTCCTGGAGCAGTGTGG-3′	5′-AGTCCGGATCTAGGCAGGTT-3′
Caspase-9	5′-ATTCTCGAGACCATGGGAGTGCAGGTG-3′	5′-ATTGAATTCTTAGTCGAGTGCGTAGTCTGG-3′
ADCY1	5′-GCTGGAAGATGAGAATGAGA-3′	5′-TCGTGCCTCTGGATGTAA-3′
APLN	5′-TGCTCTGGCTCTCCTTGAC-3′	5′-GCCCATTCCTTGACCCTCT-3′
GRM2	5′-CTGTCTCTCTATCTCTCTGC-3′	5′-TGTGTGTGTGTAACATGATGG-3′
APLNR	5′-GTCCACCCCCTACTGGATTT-3′	5′-AGCAGGAACCCAGCTCAGTA-3′
GALR2	5′-CGATTGGGTGTTTGGCTCAC-3′	5′-CAGATACCTGTCCAGCGAGA-3′
TAS1R1	5′-TCACAGAGCGTGGACAGAAC-3′	5′-AGCACTTAGGTGGCAAGTGG-3′
NPY2R	5′-GGCTTTCCTCTCGGCCTTC-3′	5′-TGTCACGGACACCTCAGAGTG-3′
GAPDH	5′-GGGAAACTGCGGCGTGAT-3′	5′-AAAGGTGGAGGAGTGGGT-3′

are shown in Table 1, while GAPDH was used to normalize the expression of the selected genes.

Hematoxylin and eosin staining (H&E staining)

The specimens were fixed in formalin solution for 48 h. Then, they were embedded in paraffin, sectioned and set on slides, which were stained by hematoxylin and eosin successively.

Immunohistochemical staining

After the paraffin-embedded sections were dewaxed, we transferred the slides to 100% alcohol, 95% alcohol, 85% alcohol, 75% alcohol and ddH_2O, 3 min respectively, and then put them in hydrogen peroxide (3%) for 8 min to eliminate endogenous peroxidase activity. Then, antigen retrieval and incubation with goat serum were routinely conducted. Slides of different groups were incubated with anti-CXCR4 (1:100, Proteintech, Chicago, USA), anti-CXCL9 (1:100, Proteintech, Chicago, USA), anti-Caspase-9 (1:50, Proteintech, Chicago, USA), 4 °C overnight. Then secondary antibody was applied to label the primary antibody.

Western blot analysis

After endothelial cells were sorted and treated according to the experimental protocol [7], the cells were scraped off the petri dish and collected into a 15 ml centrifuge tube for centrifugation. Ten percent sodium dodecyl sulfate-polyacrylamide gel and four percent stacking gel were added with proper speed in the process of operation. After adding enough electrophoresis solution, samples were loaded respectively. Be careful not to add samples too quickly. Electrophoresis was performed at 90 V for 2 h. Equipped with electric transfer liquid, different materials were placed in accordance with the prescribed sequence. Samples were then stained with primary anti-CXCR4 (1:1000, Proteintech, Chicago, USA), anti-CXCL9 (1:1000, Abcam, Cambridge, UK), anti-Caspase-9 (1:500, Proteintech, Chicago, USA), anti-cleaved-Caspase-9 (1:500, Abcam, Cambridge, UK), or anti-β-actin (1:5000, Proteintech, Chicago, USA) antibody at 4 °C for 12 h. Secondary antibody was then applied in these samples and intensity detection was performed.

Statistical analysis

Based on one-way analysis of variance (ANOVA) and LSD-test, data analysis was applied with GraphPad Prism 8 in this study and was shown as mean ± SD (mean ± standard deviation). Statistical significance was set as $p < 0.05$.

Result

Identification of DEGs

Compared to NVECs group, 1040 DEGs in KVECs group were selected with GEO2R online tools, including 351 upregulated genes and 689 downregulated with the cutoff standard of p-value <0.05 and $|\log FC| > 1$.

Enrichment analysis for DEGs

Enriched results showed that the DEGs in the biological process of GO were mainly involved in cell migration, tube development, chemotaxis, cell motility and regulation of cell proliferation. Based on molecular function analysis of GO, the DEGs were markedly associated with signaling receptor binding, receptor regulator activity, extracellular matrix structural constituent, G protein-coupled receptor binding and cytokine activity. As for CC, the overlapping DEGs were remarkably related to an intrinsic component of the plasma membrane, an integral component of the plasma membrane, plasma membrane-bounded cell projection, extracellular matrix and plasma membrane region (Figure 1(A–C)).

In GSEA enrichment analysis, 133/177 gene sets are upregulated in the phenotype of KVECs group, and 99 gene sets are significant at FDR <25%. In the enriched results of gene sets, apoptosis in KEGG was in a high placing (Enrichment Score, 0.52119493, Figure 1(D)). Based on the dysfunction of dermal vascular endothelial cells in keloids, we analyzed and verified the expression of Caspase-9 via RT-qPCR, immunohistochemical staining and western blot, which was one of the differentially expressed genes.

The results of KEGG pathway reveal that DEGs are mainly involved in the apelin signaling pathway, long-term potentiation, calcium signaling pathway, inflammatory mediator regulation of TRP channels and GABAergic synapse (Figure 1(E,F)).

PPI network construction and hub genes analysis

STRING database was applied to predicting and analyzing the potential PPIs, genetic interaction, signal transduction pathways, cooccurrence and coexpression among DEGs (combined score >0.4). Then, the PPI network was established and constructed via Cytoscape, including 919 nodes and 3547 edges. In addition, the core network module was analyzed and extracted by MCODE, containing 10 nodes and 45 edges (Figure 2(A)). CytoHubba was used to identify hub genes, based on the contribution of hub nodes in the PPI network through different algorithm systems in Cytoscape. The top twenty nodes ranking in the PPI network calculated by the four topological analysis methods were shown (Table 2). Furthermore, ten hub genes

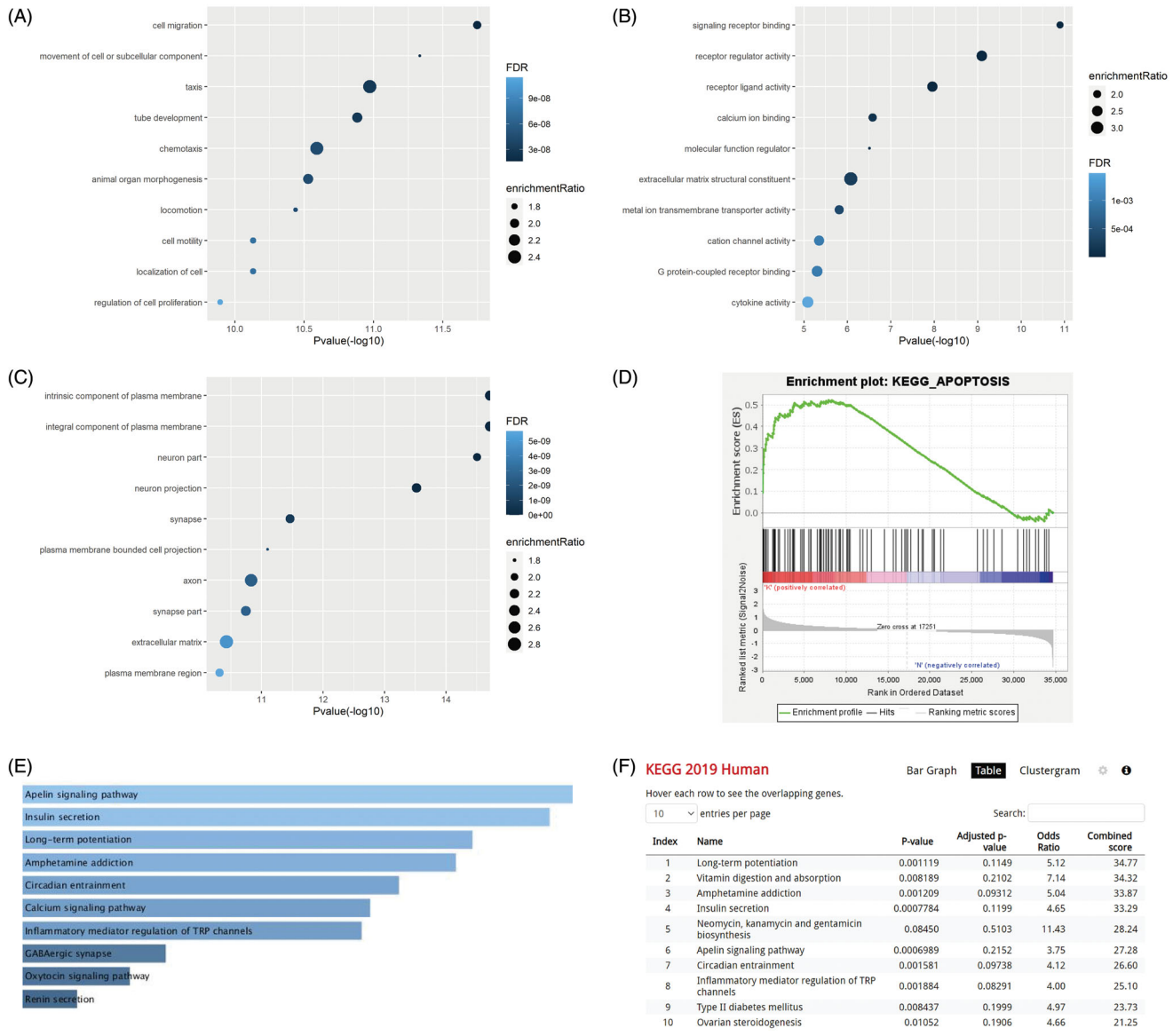

Figure 1. The results of GO, GSEA and KEGG pathway analysis were shown. (A–C) Enrichment results of biological process, molecular function, cellular component of GO analysis. (D) Results of GSEA. Apoptosis in KEGG was in a high placing (Enrichment Score = 0.52119493) and was selected for further analysis. (E,F) Pathway analysis of KEGG pathways 2019. We conducted the signaling pathway analysis with Enricher, which have shown the results of induction and integration of data from different levels and sources. The blue bars were ranked by *p*-value of each term. Additionally, the table data of KEGG pathway has also been shown in part F and was ranked by enrichment scores.

were selected for more comprehensive analysis by GeneMANIA, namely CXCR4, APLNR, CXCL9, GRM2, ADCY1, APLN, TAS1R1, GALR2, NPY2R and ADORA3 (Figure 2(B)). Biological processes analyzed by BiNGO and ClueGO were shown in Figure 2(C,D), mainly including chemotaxis, inflammatory response, response to wounding, cell migration and chemokine-mediated signaling pathway.

H&E staining analysis

H&E staining is widely used to evaluate the morphological characteristics of different tissues and the levels of inflammatory infiltration directly. As was shown in Figure 3, a larger amount of collagen fibers can be observed in tissues of the Keloid group, and deeper staining can be observed in the epidermis and dermis layer of keloids. Additionally, the capillaries in the dermis were more deeply stained in samples of the Keloid group, which represented a higher level of inflammation in dermal vascular endothelial cells.

Immunohistochemical staining analysis

After verifying differential expression levels in two different samples of selected genes screened by Cytoscape and GSEA software, we further verified the expression levels of CXCR4, CXCL9 and Caspase-9 by immunohistochemical staining studies, which were closely associated with chemotaxis, inflammatory responses and apoptosis.

The visual field with uniform coloring and light background was randomly selected and was evaluated by three pathologists. Compared with normal skin tissues, the factors of CXCR4, CXCL9 and Caspase-9 have higher expression levels in keloids tissues. The positively stained areas have been marked by red arrows in Figure 4 and the percentages of positively stained cells of epidermis and dermis layers were shown in Table 3. The expression

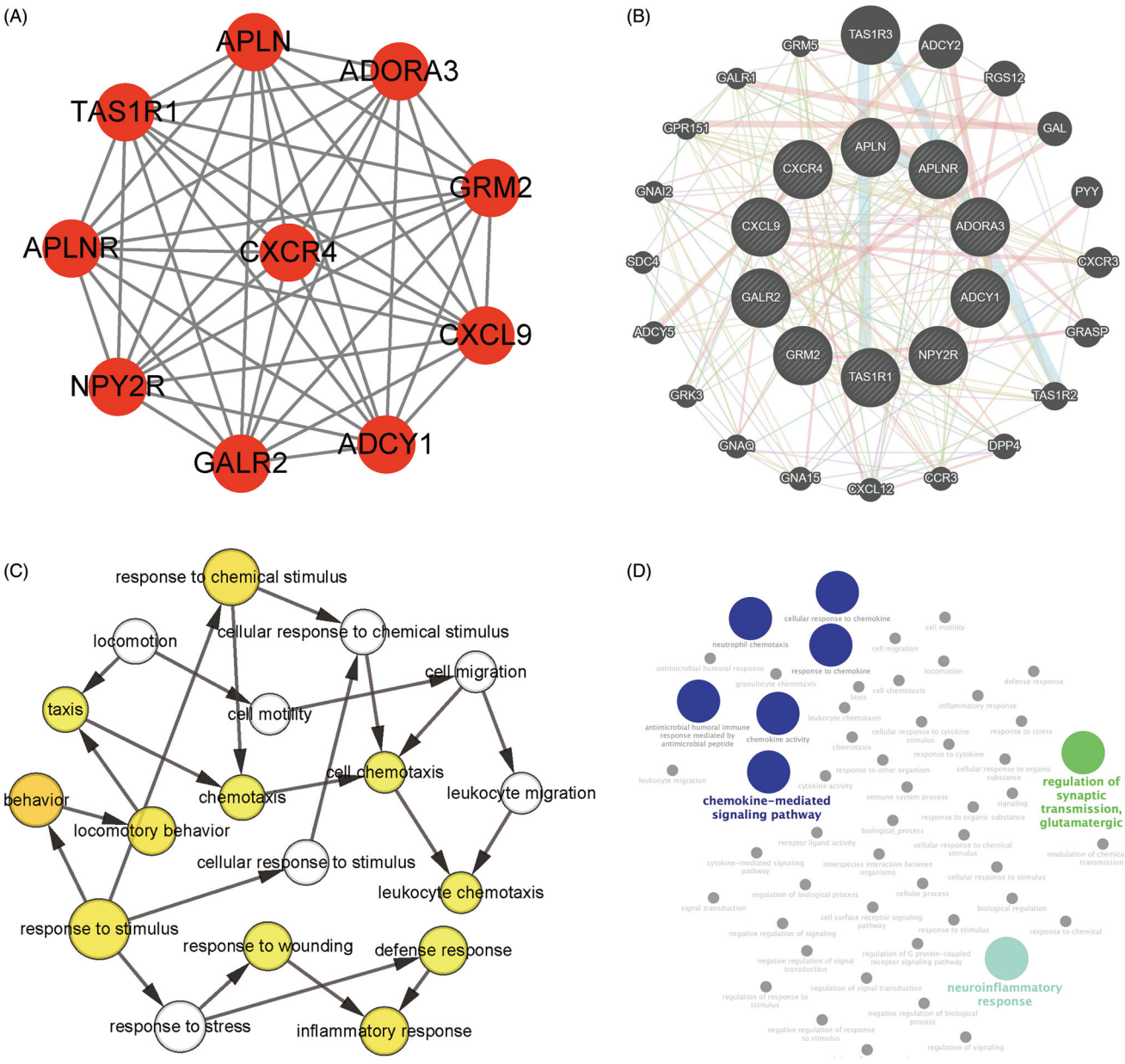

Figure 2. The results of MCODE module, GeneMANIA, BiNGO and ClueGO network. (A) Core network module with MCODE, which contained 10 nodes and 45 edges. (B) GeneMANIA analysis. GeneMANIA reports were submitted as supporting files. (C) Via the BiNGO plug-in, the results of core biological process were shown, mainly including inflammatory response, response to wounding, response to chemical stimulus and chemotaxis. (D) With ClueGO analysis, several biological processes were screened, including chemokine-mediated signaling pathway. The larger the circle was, the higher the enrichment degree was.

levels of CXCR4, CXCL9 and Caspase-9 were significantly different between the Keloid group than the Normal skin group.

The mRNAs expression of the selected genes

We examined and verified the expression of CXCR4, CXCL9, Caspase-9, ADCY1, APLN, GRM2, APLNR, GALR2, TAS1R1 and NPY2R in keloids and normal skin tissues by RT-qPCR. The mRNA expression levels of the selected genes in the Keloid group were significantly higher, compared with the Normal skin group, respectively (Figure 5).

The expression levels of the aforementioned genes in vascular endothelial cells of KC and NC groups were also detected by RT-qPCR. Compared with NC group, the mRNA expression levels were up-regulated in KC group (Figure 6(C)).

Expression of CXCR4, CXCL9, Caspase-9 and cleaved-Caspase-9 proteins

In KC and NC groups, the protein expression of CXCR4, CXCL9, Caspase-9 and cleaved-Caspase-9 was shown and analyzed (Figure 6(A,B)). The expression levels of CXCR4, CXCL9, Caspase-9 and cleaved-Caspase-9 were much higher in the KC group, relative to the NC group, which was in agreement with the immunohistochemistry studies and RT-PCR analysis.

Discussion

As a severe and complicated pathological scar, the occurrence and progression of keloids involve the interaction of fibroblasts, vascular endothelial cells, inflammatory cells and different kinds of cytokines [13]. However, the pathogenesis of keloids is still

unclear, and it may be closely related to gene expression, cell signaling pathways, collagen metabolism, cell proliferation and apoptosis [14]. Excessive proliferation of keloid fibroblasts and increased collagen synthesis can lead to rising cell metabolism and hypoxia status, which is closely associated with the pathogenesis of keloids [15]. Hypoxia and HIF-1α-rich microenvironment provide good conditions for keloid keratinized epithelial cells/dermal vascular endothelial cells to acquire fibroblast-like appearance through the EMT/EnMT process [16]. Obvious changes in hemodynamics of capillaries and severe hypoxia status in keloid tissues lead to increased secretion levels of chemokines and inflammatory cytokines in the keloid microenvironment, which is closely associated with the state of immune infiltration.

To some extent, keloids are an inflammatory disease involving the reticular dermis layer, and injuries of the reticular dermis and abnormal wound healing are major causes of the disease. Studies have shown that pro-inflammatory cytokines such as TNF-α (tumor necrosis factor-α), IL-1α (interleukin-1α), IL-1β (interleukin-1β) and IL-6 (interleukin-6) in keloid tissue are highly expressed, which could promote chronic inflammation and cause invasive growth of keloids [17]. As it is shown in the KEGG pathway analysis from Enrichr, inflammatory mediator regulation of TRP channels is significantly enriched in the KEGG pathway, including up-regulated DEGs of PRKCG, PRKCE, TRPV4, TRPV2, CALML4, ADCY1 and PRKACB. In this process, a large number of blood vessels are formed, while the functions are not complete. Moreover, due to the increase of blood vessel permeability and the induction of chemokines, a great number of inflammatory factors accumulate in the extracellular matrix, which is consistent with the high inflammation status shown by the HE staining results.

Current studies believe that different kinds of immune cells and leukocytes are drawn to the site of injury by chemokines and their receptors on the surface of target cells to participate in pathological processes such as inflammation, autoimmune diseases, tumors, and angiogenesis [18]. According to the enrichment results of GO, BiNGO and ClueGO in the biological process, chemotaxis is significantly enriched, which could be closely related to the pathogenesis of keloids endothelial cells. Therefore, our study aims to explore the differential expression of up-regulated chemokines between keloid vascular endothelial cells and normal skin vascular endothelial cells, including CXCL9 and CXCR4, which were also differentially expressed significantly in GSE121618 and verified in our experiments. CXCL9 is a kind of chemokine that responds to IFN-γ (interferon-γ) induction and participates in the migration and activation of inflammation-related cells [19]. As an important part of the CXCR3-CXCL9/10/11

Table 2. Hub genes of DEGs ranked in CytoHubba plugin of Cytoscape.

	Rank methods of CytoHubba			
Catelogy	MCC	MNC	Degree	EPC
Top 20 (Gene symbol)	**CXCR4**	CXCR4	CXCR4	ADCY1
	ADCY1	CXCL9	ADCY1	CXCR4
	CXCL9	APLN	CD79B	CXCL9
	ADORA3	ADORA3	ADORA3	ADORA3
	APLN	ADCY1	CXCL9	GRM2
	GRM2	TAS1R1	GRM2	NPY2R
	APLNR	GALR2	CEACAM5	TAS1R1
	TAS1R1	APLNR	APLN	APLN
	GALR2	GRM2	APLNR	APLNR
	NPY2R	NPY2R	PRKACB	GALR2
	CEACAM5	PRKACB	TAS1R1	PRKACB
	CD52	CACNA1C	GALR2	CACNA1C
	SPON1	KCNMA1	NPY2R	RYR2
	ADAMTSL4	CEACAM5	WDFY4	SPP1
	LY6H	ADAMTS3	SPP1	CALML4
	LY6K	THSD7A	CTSB	CD52
	ADAMTS3	SPON1	RYR2	CEACAM5
	THSD7A	ADAMTSL1	CACNA1C	CD79B
	ADAMTSL1	ADAMTSL4	IGFBP5	KCNMA1
	ADAMTSL2	ADAMTSL2	CD52	HIF1A

Four topological analysis methods: the maximal clique centrality (MCC), maximum neighborhood component (MNC), Degree and edge percolated component (EPC).
Bold gene symbols were the overlap genes screened by the four ranked methods in CytoHubba.

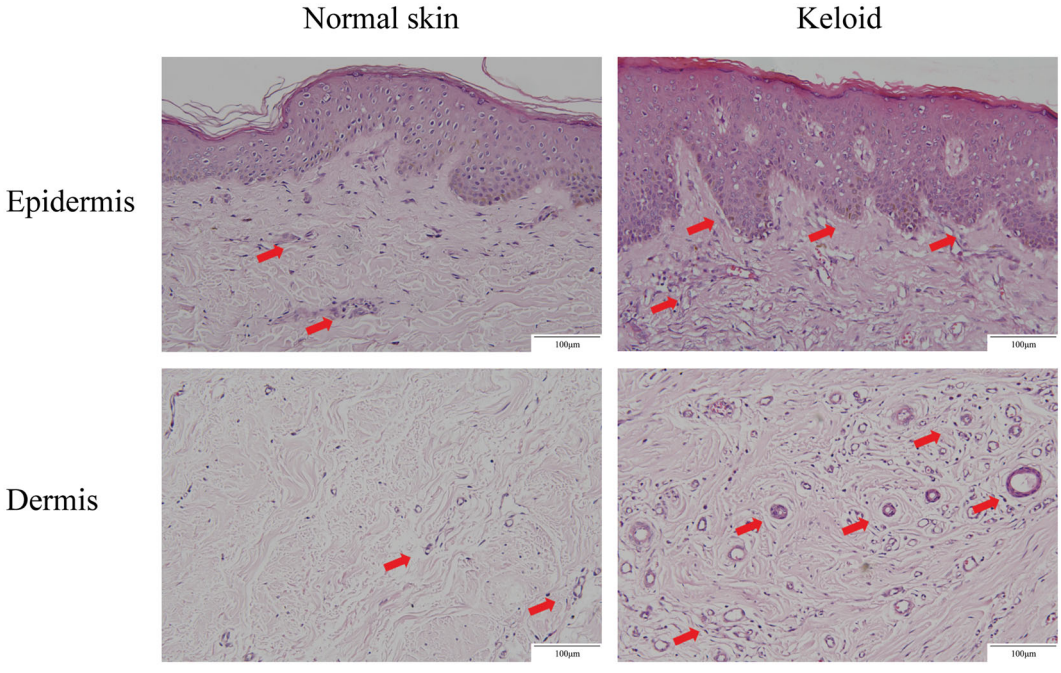

Figure 3. The results of H&E staining. The number of infiltrated cells (red arrow) was much higher in the Keloid group than in the Normal skin group. A larger amount of collagen fibers can be observed in tissues of the Keloid group, and deeper staining can be observed in the epidermis and dermis layer of keloids tissue. The capillaries in the dermis were also more deeply stained in samples of the Keloid group. Normal skin group = samples from patients without obvious scarring, Keloid group = samples from patients with keloids.

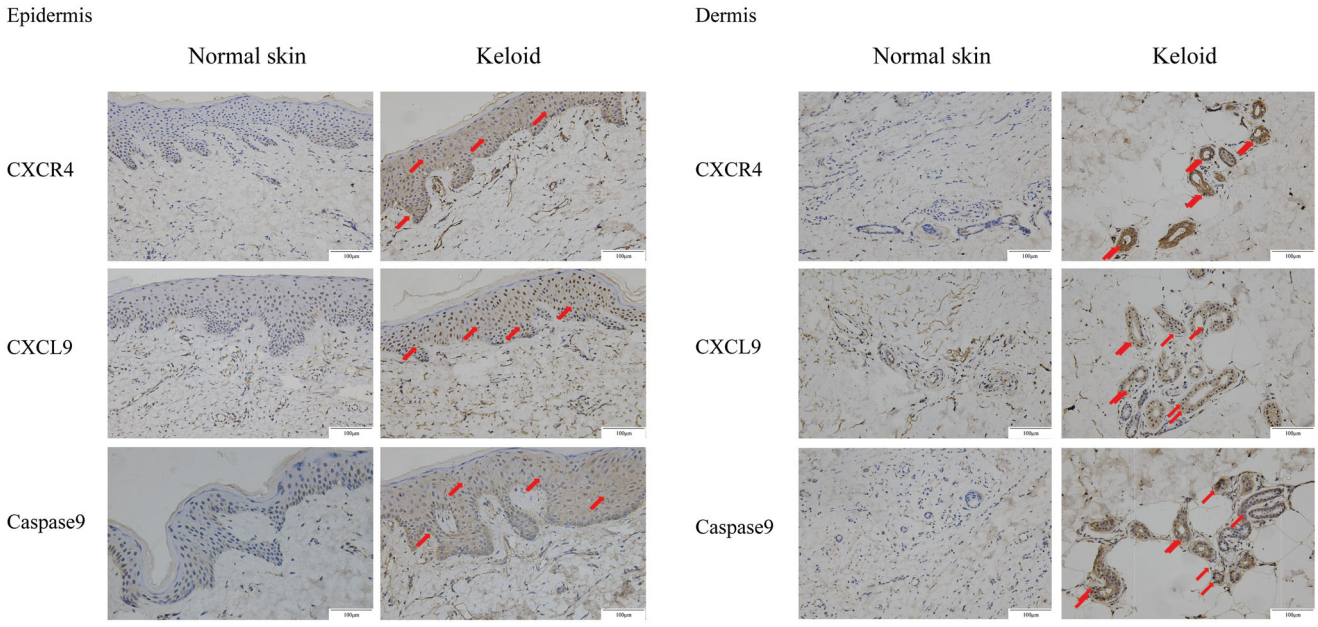

Figure 4. The results of immunohistochemical studies for CXCR4, CXCL9 and Caspase-9 in the two groups. The shade of the brown color is corresponding to the protein levels and the positive stained areas have been marked by red arrows. The levels of CXCR4, CXCL9 and Caspase-9 were over expressed in the Keloid group both in epidermis and dermis layers relative to the Normal skin group.

Table 3. Percentage of immunohistochemical staining positive cells in all groups.

		Percentage of immunohistochemical staining positive cells		
Factor		Normal skin	Keloid	*p*-Value
CXCR4	Epidermis	0.1984 ± 0.1957	0.6659 ± 0.2635	Keloid vs. Normal skin, $p < 0.05$
	Dermis	0.2387 ± 0.1142	0.8862 ± 0.1076	Keloid vs. Normal skin, $p < 0.05$
CXCL9	Epidermis	0.2472 ± 0.2560	0.7324 ± 0.1982	Keloid vs. Normal skin, $p < 0.05$
	Dermis	0.1799 ± 0.0744	0.9231 ± 0.1651	Keloid vs. Normal skin, $p < 0.05$
Caspase-9	Epidermis	0.1649 ± 0.2937	0.7692 ± 0.2954	Keloid vs. Normal skin, $p < 0.05$
	Dermis	0.3405 ± 0.1758	0.8189 ± 0.2036	Keloid vs. Normal skin, $p < 0.05$

Values are mean ± SD.
Percentages of positive cells in immunohistochemical staining (brown staining area) are shown in this table. Compared with Normal skin group, the percentages of CXCR4, CXCL9 and Caspase-9 in Keloid group were higher both in the epidermis and dermis layers.

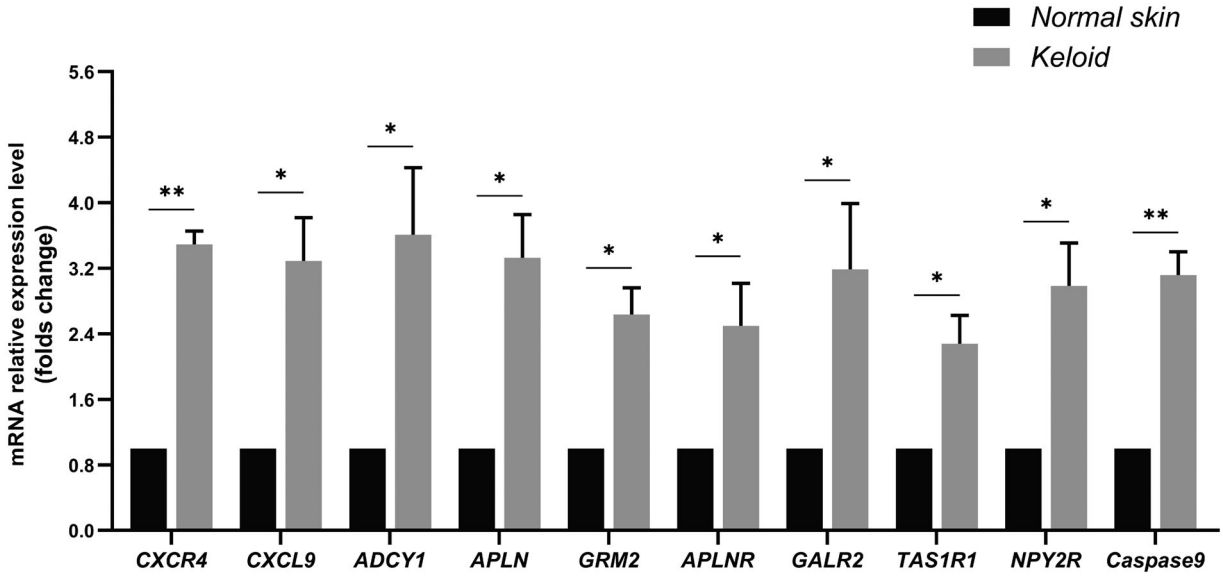

Figure 5. Levels of the CXCR4, CXCL9, Caspase-9, ADCY1, APLN, GRM2, APLNR, GALR2, TAS1R1 and NPY2R mRNAs in the Keloid and Normal skin groups. The expression levels of CXCR4, CXCL9, Caspase-9, ADCY1, APLN, GRM2, APLNR, GALR2, TAS1R1 and NPY2R were examined and verified in keloids and normal skin tissues. The aforementioned genes of samples in the Keloid group have higher expression levels than the Normal skin group (*$p < 0.05$; **$p < 0.01$).

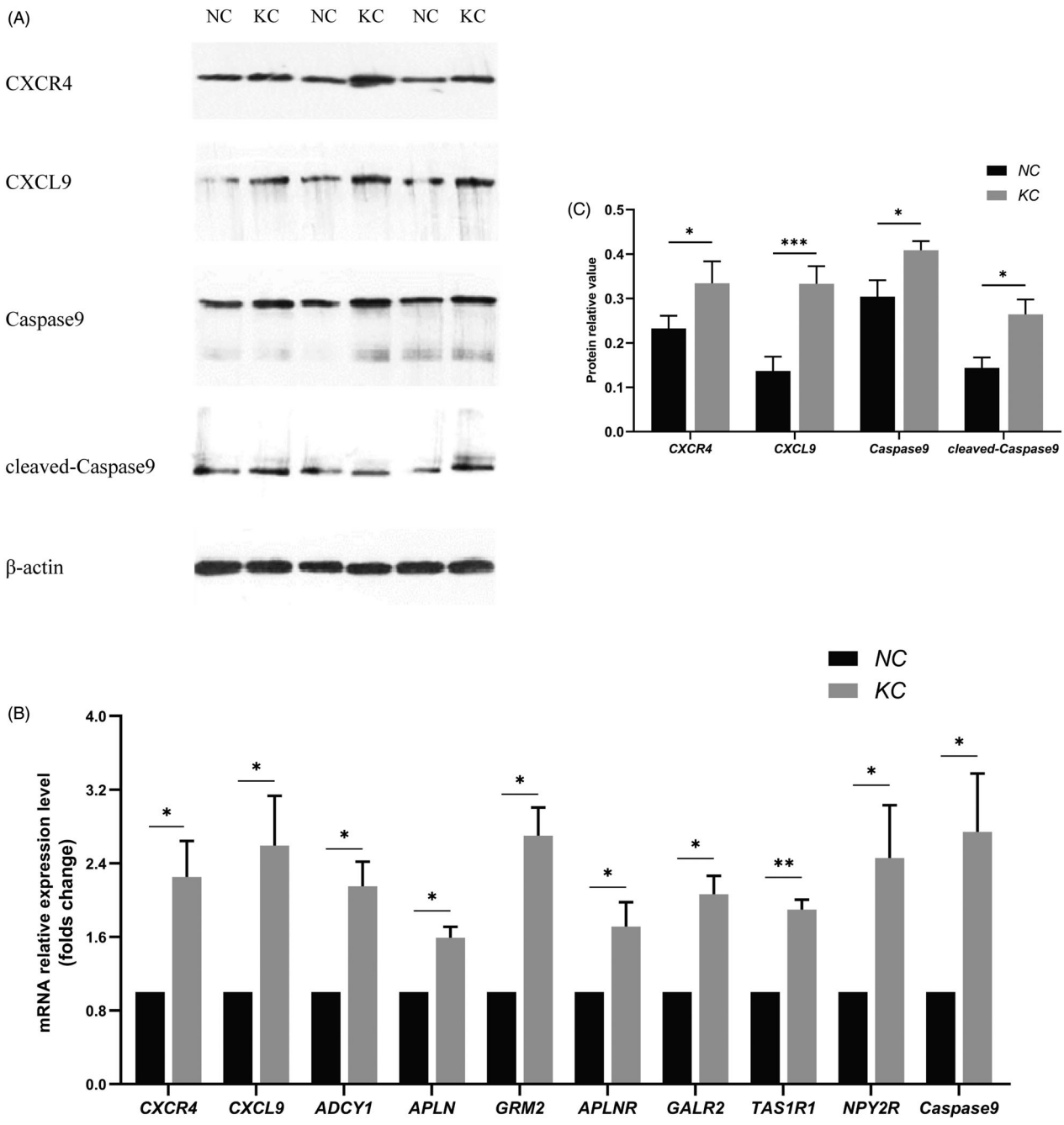

Figure 6. Expression of CXCR4, CXCL9, Caspase-9 and cleaved-Caspase-9 proteins and the mRNA expression levels of selected genes. (A, B) In the KC group, the expression levels of CXCR4, CXCL9, Caspase-9 and cleaved-Caspase-9 were much higher than NC group. (C) Compared with the NC group, mRNA expression levels of the selected genes were up-regulated in the KC group. KC group = keloids vascular endothelial cells from keloids samples, NC group = normal skin vascular endothelial cells from normal skin samples ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

axis, CXCL9 coordinates the positioning and function of lymphocytes in time and space [20]. Furthermore, studies have found hypoxic environment will stimulate the production of various angiogenic factors, including VEGF (vascular endothelial growth factor), PDGF (platelet-derived growth factor), TGF-β (transforming growth factor β) and chemokines, promoting the repair and regeneration of blood vessels [21]. CXCR4 is a specific chemokine receptor for SDF-1 (stromal cell-derived factor-1) and an important chemotactic factor for the pathogenesis of chronic inflammation [22]. It has been found that SDF-1/CXCR4 biological axis plays an indispensable role in the preservation and repair of injured organs or tissues such as skeletal muscle injury, myocardial infarction and stroke. However, excessive expression of chemokines may lead to the accumulation of non-specific inflammatory cells and cause exaggerated inflammation responses [23]. Chemokine receptors are the most promising drug targets that regulate inflammatory diseases. Therefore, clinical trials involving antagonists of chemokine receptors for the treatment of inflammation have already begun.

Additionally, hypoxia aggravates dermal vascular endothelial cell dysfunction and apoptosis, which has been proven in GSEA analysis and relevant experiments. Furthermore, the apelin signaling pathway was significantly enriched in KEGG enrichment analysis (p-value < 0.001), which involves RYR2, PRKCE, APLNR, SPP1,

CALML4, ADCY1, PRKACB, PIK3CG and APLN. Apelin is an endogenous peptide capable of binding G-protein-coupled receptors, which is implicated in different key physiological processes such as angiogenesis, cardiovascular functions and apoptosis. Caspase (cysteinyl aspartate specific proteinase) plays an essential role in the process of cell apoptosis. In microarray data of GSE121618, Caspase-9, which acts as an inducer of apoptosis, was over-expressed in keloid vascular endothelial cells, with more than double the amount in samples of normal skin vascular endothelial cells [7]. After receiving upstream signals from internal or external stimuli, Caspase-9 activates downstream signaling targets of Caspase-3, -6 and -7, and triggers subsequent cascade reactions to cause irreversible cell death [24]. Additionally, immunohistochemical staining showed that the positive staining of Caspase-9 was significantly higher in the dermal vascular area of keloids than normal skin, indicating that endothelial cells in keloids were likely to have a high level of apoptosis, which was closely related to endothelial dysfunction. Western blot and RT-qPCR results were consistent with the immunohistochemical studies.

Studies have shown that the high levels of inflammation in keloid tissue is closely related to apoptosis [25], but previous research has mostly focused on fibroblasts, and studies on dermal vascular endothelial cells are relatively rare. Based on the data set of GSE121618, we have conducted a more in-depth study of chemotaxis, inflammation and apoptosis of keloid tissue. The results of this study revealed that the microenvironment of endothelial cells in keloids was accompanied by a high level of apoptosis in addition to the high inflammatory status based on the bioinformatics results and validation experiments. However, as mentioned before, the hypoxia state within the keloids tissue will promote the proliferation of endothelial cells, and the relationship between proliferation and apoptosis of keloid dermal vascular endothelial cells must be in a complicated and dynamic equilibrium status. According to these studies, we speculated that the hyperinflammatory state and the increased levels of chemokines, including CXCR4 and CXCL9, led to the inevitable dysfunction of dermal vascular endothelial cells, as well as the increased levels of apoptosis. The limitation of our research is that systematic study of the proliferation level of keloid vascular endothelial cells and analysis of the balance between proliferation and apoptosis is lacking. Therefore, the interactive relationship of proliferation and apoptosis on the dermal endothelial cells is of great significance and further experiments would be carried out.

Disclosure statement

All authors declare no potential conflicts of interest with respect to the research, authorship and publication of this article. The datasets analyzed during this study are available in the Gene Expression Omnibus database [GSE121618] (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE121618).
Requests for material should be made to the corresponding authors.

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