

Gene expression profiling analysis of keloids with and without hydrocortisone treatment

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Abstract. The present study aimed to investigate the genetic effects of hydrocortisone (HC) treatment on keloids and screen medicines to be used in a combination therapy of keloids with HC. The dataset GSE7890 was downloaded from Gene Expression Omnibus. It contained data regarding 4 fibroblast samples from normal scar tissue and 5 samples from keloid tissue with HC treatment, as well as 5 samples from normal scar and 5 samples from keloids without HC treatment. Following the identification of differentially expressed genes (DEGs), the functions of these DEGs were analyzed by Gene Ontology (GO) and pathway enrichment analyses. Furthermore, adverse effects of HC were identified using WebGestalt. Additionally, candidate small molecule drugs associated with keloids were selected from a connectivity map database. A total of 166 and 41 DEGs, with and without HC treatment respectively, were only present in dermal fibroblasts from keloids (termed genesets A and B, respectively). A set of 26 DEGs was present following both treatments (geneset C). A number of DEGs in geneset B (*COL18A1* and *JAG1*) were associated with endothelial cell differentiation. However, in genesets A and C, certain genes (*CCNB1* and *CCNB2*) were involved in the cell cycle and p53 signaling pathways, and a number of genes (*IL1R1* and *COL1A1*) were associated with bone loss. Additionally, numerous small molecule drugs (including acemetacin) were associated with keloids. Thus, it has been determined that HC may treat keloids by targeting genes associated to endothelial cell differentiation (*COL18A1* and *JAG1*). However, HC has a number of adverse effects, including bone loss. Acemetacin may be applied in a combination therapy, along with HC, to treat keloids.

Introduction

Keloids are benign fibrotic tumors of the dermis that form during a prolonged wound healing process (1). Keloids exhibit aggressive dermal growth beyond the boundaries of the original margins of wounds, causing pain, pruritis and contractures. They are a cosmetic and psychological burden to patients (2).

The effect of one type of glucocorticoid, hydrocortisone (HC), on keloids has previously been investigated. HC is known to alter the chemistry and morphology of connective tissue cells and to hinder the production of intercellular substances, including collagenous fiber and glycosaminoglycan (3). It may also decrease the maximum density of keloid-derived fibroblasts (4), increase proline transport (5), lower prolyl hydroxylase activity, reduce the rate of collagen synthesis (6), enhance apoptosis rates (7) and diminish hyaluronan accumulation (8).

Microarray studies identify a broad spectrum of differentially regulated genes in biological samples under genomic sequencing, cloning, cDNA or PCR approaches (9). In 2008, Smith *et al* (10) conducted a bioinformatic analysis on RNA obtained from fibroblasts cultured from normal scars and keloids grown in the absence and presence of HC. The results indicated that there was elevated expression of a number of insulin-like growth factor (IGF)-binding and IGF-binding related proteins, in addition to decreased expression of a set of Wnt pathway inhibitors and numerous interleukin (IL)-1-inducible genes. Furthermore, it was observed that IGF binding protein (IGFBP)-3 and connective tissue growth factor (CTGF) were associated with the increase of fibroblast and collagen deposition and were overexpressed in keloid fibroblasts only in the presence of HC, suggesting that glucocorticoid resistance of HC is involved in the pathogenesis of keloids formation (10). However, the adverse effects of HC during its treatment on keloids and the drugs that may potentially be used to weaken or reverse these adverse effects, remain unknown.

The present study used the microarray data collected by Smith *et al* (10) to identify differentially expressed genes (DEGs) in fibroblasts cultured from keloids treated with or without HC. A Gene Ontology (GO) enrichment analysis was performed on DEGs, which were potentially associated with

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the positive efficacy of HC, and a pathway enrichment analysis for DEGs that may be associated with adverse effects of HC was completed. Furthermore, the adverse effects of HC were analyzed and small molecule drugs that may reduce the occurrence of adverse effects were screened from the connectivity map (CMAP) database. The aim of the present study was to elucidate the molecular mechanisms of HC treatment on keloids and provide novel information for the clinical treatment of this disease.

Materials and methods

Affymetrix microarray data. The gene expression profile data of GSE7890 (10) was downloaded from the Gene Expression Omnibus (GEO; <http://www.ncbi.nlm.nih.gov/geo/>) database, which was based on the platform of GPL570 [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array (Affymetrix Inc., Santa Clara, CA, USA). A total of 19 primary cultures of dermal fibroblast samples were included in this dataset, including 4 samples from normal scar tissue and 5 samples from keloids treated with 1.5 μ M HC; as well as 5 samples from normal scar and 5 samples from keloids that did not undergo HC treatment.

Affymetrix CEL files and the probe annotation files were downloaded and the gene expression data of all samples were preprocessed via background correction, quantile normalization and probe summarization using the Affy software (version 1.30.0) package of Bioconductor (available at <http://www.bioconductor.org/packages/2.8/bioc/html/affy.html>) (11).

DEGs screening. Linear Models for Microarray Data package (version 3.22.7) (12) of Bioconductor (available at <http://www.bioconductor.org/packages/3.0/bioc/html/limma.html>) was used to identify genes that were differentially expressed in dermal fibroblasts from keloids and those from normal scars. The raw P-value was adjusted into False Discovery Rate (FDR) by the Bonferroni method (13) in a multtest package (version 2.20.0) of Bioconductor (<http://www.bioconductor.org/packages/2.14/bioc/html/multtest.html>). The \log_2 FC (fold change) >1 and FDR <0.05 were selected as the cut-off criteria.

Functional classification of DEGs. GO functional enrichment analysis of DEGs was performed to interpret their biological significance, via the Database for Annotation, Visualization and Integrated Discovery (<http://david.abcc.ncifcrf.gov/>) (14). P<0.01 was used as the cut-off criterion.

Pathway enrichment analysis of DEGs was performed using a Kegg Orthology Based Annotation System, version 2.0 (<http://kobas.cbi.pku.edu.cn>) (15). P<0.05 was used as the cut-off criterion.

Identification of potential diseases associated with DEGs. Potential diseases associated with the DEG genesets A and C were identified using the WEB-based GENE SeT AnaLysis Toolkit (WebGestalt; www.webgestalt.org) (16) with a cut-off criterion of P<0.05.

Prediction of small molecule drugs. CMAP database (www.connectivitymap.org/cmap) (17) was used to predict

candidate small molecule drugs targeting DEGs genesets A and C. An enrichment score between 0.9-1 was selected as the cut-off criterion. The closer to -1 the enrichment score was, the stronger the effect of the drug on the disease.

Results

Identification of DEGs. A total of 192 DEGs with HC treatment (50 upregulated and 142 downregulated) and 67 DEGs without HC treatment (15 upregulated and 52 downregulated) were screened from dermal fibroblasts in keloids and compared with normal scar tissue.

Among them, 166 DEGs were only present in the dermal fibroblasts from keloids treated with HC (geneset A), 41 DEGs were only present in dermal fibroblasts from keloids with no HC treatment (geneset B) and 26 DEGs were common in dermal fibroblasts from keloids with and without HC treatment (geneset C; Fig. 1).

Functional annotation of DEG. To investigate the potential molecular functions in keloid fibroblasts affected by HC, a GO functional enrichment analysis of the DEGs in geneset B, which were potentially associated with the positive efficacy of HC, was performed. According to the GO functional enrichment analysis, DEGs in geneset B were significantly enriched in 9 GO terms (all P<0.01), including endothelial cell differentiation (*COL18A1*, *HOXB5* and *JAG1*) and regulation of cell proliferation (*DLC1*, *COL18A1* and *JAG1*; Table I). Furthermore, 21.28% of DEGs were associated with the regulation of cell proliferation and 8.51% of DEGs with endothelial cell differentiation (Fig. 2).

To identify the potential dysregulation pathways associated with HC treatment and those that were unaffected by HC, a pathway enrichment analysis of the DEGs in geneset A (specifically, novel DEGs following HC treatment) and genes in geneset C (namely, genes that were not affected by HC), was performed. Based on this pathway enrichment analysis, the DEGs in genesets A and C were significantly enriched in two pathways: Cell cycle (*CCNB1*, *MAD2L1* and *BUB1*) and p53 signaling pathway (*CCNB1*, *RRM2* and *PERP*; Table II; P<0.05).

Potential diseases associated with DEGs in genesets A and C. To investigate the adverse effects of HC during its treatment on keloids, potential diseases associated with DEGs in genesets A and C were identified. It was determined that the newly added DEGs in dermal fibroblasts from keloids following HC treatment and genes that were not altered by HC were associated with two diseases: Bone loss (*IL1R1*, *HSPA2* and *COL1A1*) and osteoarthritis (*IL1R1*, *IGFBP7* and *COL1A1*; Table III).

Small molecule drugs targeting DEGs in genesets A and C. To further investigate potential drugs that are able to weaken or eliminate the adverse effects of HC, small molecule drugs targeting DEGs in genesets A and C were identified. A total of 9 small molecule drugs were selected from the CMAP database, including acemetacin, scriptaid and alsterpaullone. Among them, acemetacin has a negative score and the lowest P-value (P=0.00002), indicating it had the strongest effect on the adverse effects of HC on gene expression (Table IV).

Table I. Enriched GO terms for differentially expressed genes in geneset B.

Term	Description	Count	P-value	Genes
GO:0045446	Endothelial cell differentiation	4	P<0.001 ^a	<i>COL18A1, HOXB5, JAG1, NR2F2</i>
GO:0042127	Regulation of cell proliferation	10	P<0.001 ^a	<i>DLC1, COL18A1, NCK2, TBX3, NKX3-1, PTN, JAG1, PPAP2A, CLEC11A, IGFBP5</i>
GO:0008285	Negative regulation of cell proliferation	6	0.001937 ^a	<i>DLC1, COL18A1, NCK2, NKX3-1, PPAP2A, IGFBP5</i>
GO:0060429	Epithelium development	5	0.002412 ^a	<i>DLC1, COL18A1, HOXB5, JAG1, NR2F2</i>
GO:0001568	Blood vessel development	5	0.003177 ^a	<i>COL18A1, TBX3, MMP19, JAG1, NR2F2</i>
GO:0001944	Vasculature development	5	0.003465 ^a	<i>COL18A1, TBX3, MMP19, JAG1, NR2F2</i>
GO:0030855	Epithelial cell differentiation	4	0.004833 ^a	<i>COL18A1, HOXB5, JAG1, NR2F2</i>
GO:0009952	Anterior/posterior pattern formation	4	0.005134 ^a	<i>TBX3, HOXB5, NKX3-1, NR2F2</i>
GO:0030334	Regulation of cell migration	4	0.008627 ^a	<i>DLC1, COL18A1, JAG1, IGFBP5</i>

^aP<0.01. Geneset B represents the differentially expressed genes specifically present in dermal fibroblasts from keloids without hydrocortisone treatment, specifically, genes that were not differentially expressed following HC treatment. GO, Gene Ontology.

Table II. Results of pathway enrichment analysis for differentially expressed genes in genesets A and C.

Term	Description	Count	P-value	Genes
hsa04110	Cell cycle	9	4.29E-05 ^a	<i>CCNB1, MAD2L1, CCNB2, DBF4, BUB1, TTK, CDC20, CDC25C, CCNA2</i>
hsa04115	p53 signaling pathway	5	0.005598 ^a	<i>CCNB1, CCNB2, RRM2, PERP, PTEN</i>

^aP<0.01. Geneset A represents the differentially expressed genes present in dermal fibroblasts from keloids with hydrocortisone treatment, specifically, novel differentially expressed genes following hydrocortisone treatment. Geneset C represents the differentially expressed genes that were not affected by hydrocortisone treatment.

Discussion

Keloids are benign fibrotic tumors of the dermis and are a cosmetic and psychological burden to patients (2). In the present study, 192 and 67 DEGs were screened from dermal fibroblasts in keloids with and without HC treatment, respectively, and compared with gene expression in normal scar tissue. According to the GO functional enrichment analysis for DEGs only present in dermal fibroblasts from keloids without HC treatment, namely those expressed normally following HC treatment, a set of genes (*COL18A1* and *JAG1*) were significantly associated with endothelial cell differentiation and regulation of cell proliferation (P<0.001).

COL18A1 encodes the alpha chain of type XVIII collagen, which is an inhibitor of angiogenesis (18). It has demonstrated that collagen XVIII levels are downregulated in keloid patients compared with normal controls (19). Inhibition of collagen synthesis was observed following the addition of higher concentrations of HC (20). *JAG1* is one of Notch ligands from the Notch family (21). Increased expression of *JAG1* and Notch receptors has been observed in keloid fibroblasts and the inhibition of Notch signaling via *JAG1* knockdown led to the inhibition of proliferation, migration and invasion properties of keloid fibroblasts (22). Furthermore, the endothelial cells within blood vessels in keloids are somewhat rounded and projected into the lumen of the vessel (23); this has been

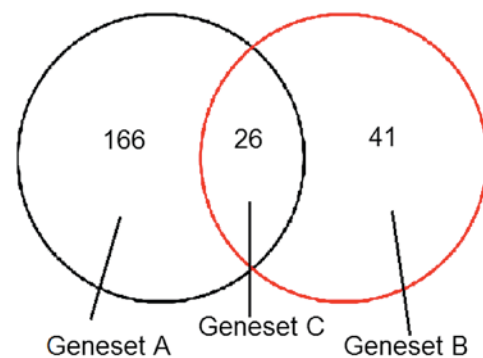


Figure 1. Venn diagram for the differentially expressed genes in dermal fibroblasts from keloids with and without hydrocortisone treatment, compared with normal scar tissue. Geneset A represents genes only present in dermal fibroblasts of keloids that have undergone HC treatment; geneset B represents genes only present in dermal fibroblasts of keloids without HC treatment; and geneset C represents genes present in dermal fibroblasts both with and without HC treatment. HC, hydrocortisone.

speculated as critical to facilitate the development and maintenance of keloids (24). Vascular endothelial growth factor, which promotes angiogenesis and enhances endothelial cell survival (25), is abundantly produced in keloids (26,27). As DEGs in geneset B were not present in the samples following HC treatment, these genes were considered to be potentially

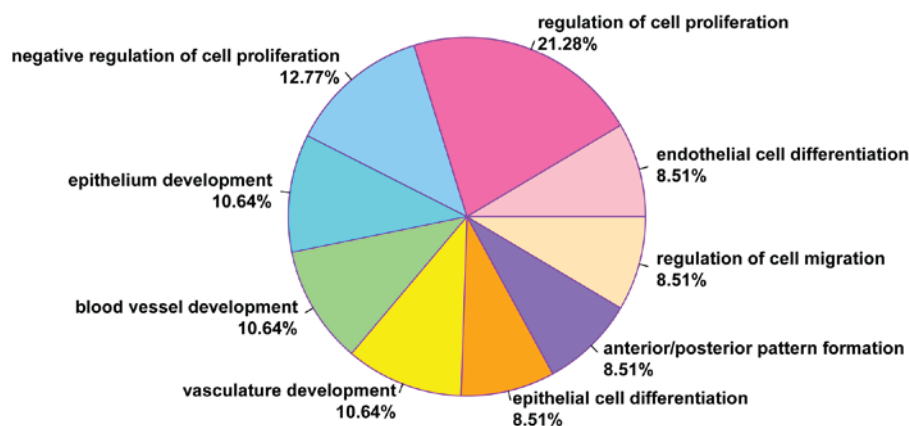


Figure 2. Pie Chart for Gene Ontology functional classification of genes only differentially expressed in dermal fibroblasts from keloids without HC treatment, compared with normal scar tissue. Genes presented in this fig. are differentially expressed in keloids without HC treatment, but expressed normally following HC treatment. HC, hydrocortisone.

Table III. Potential diseases associated with differentially expressed genes in genesets A and C.

Disease	P-value	Genes
Bone loss	0.002593 ^a	<i>IL1R1, HSPA2, COL1A1</i>
Osteoarthritis	0.033811 ^a	<i>IL1R1, IGFBP7, COL1A1, CALM1</i>

^aP<0.05. Geneset A represents the differentially expressed genes present in dermal fibroblasts from keloids with hydrocortisone treatment, specifically, novel differentially expressed genes following hydrocortisone treatment. Geneset C represents the differentially expressed genes that were not affected by hydrocortisone treatment.

associated with the positive efficacy of HC. Therefore, HC may serve a key role in the treatment of keloids by targeting genes associated with endothelial cell differentiation and regulation of cell proliferation (*COL18A1* and *JAG1*).

The pathway enrichment analysis for the DEGs in genesets A and C revealed that certain DEGs (*CCNB1* and *CCNB2*) were significantly enriched in the cell cycle and p53 signaling pathways (P<0.05). Keloids are characterized by aggressive dermal growth beyond the boundaries of the original margins of a wound (2), which is dependent on the ectopic cell cycle. A previous study demonstrated that mRNAs associated with cell cycle suppression, such as cyclin B1, were detected in the bottom region of keloids (28). Furthermore, *CCNB1* gene expression is reduced by low-dose 5-fluorouracil in treated keloid fibroblasts (29). The p53 tumor suppressor acts as a transcription factor and has a central function in controlling apoptosis (30). p53 levels are higher in keloids compared with normal scar tissues (31). Therefore, the current study demonstrated that *CCNB1* and *CCNB2* are involved in the cell cycle and p53 signaling pathways may be essential in the development of keloids. However, the current results indicated that the expression of these genes was altered following HC treatment, and did not change in the samples without HC treatment. Thus, it is speculated that the alterations of *CCNB1* and *CCNB2* may contribute to keloids via dysregulation of cell cycle and p53 signaling pathways.

Table IV. Small molecule drugs potentially used to treat the diseases associated with differentially expressed genes in genesets A and C.

Small molecule drug	Enrichment	P-value
Acemetacin	-0.925	0.00002 ^a
Scriptaid	0.912	0.00142 ^a
Alsterpallone	0.923	0.00094 ^a
Mycophenolic acid	0.957	0.0001 ^a
MG-262	0.962	0.00008 ^a
Trifluridine	0.975	<0.001
Camptothecin	0.975	0.00004 ^a
Cicopirox	0.987	<0.001
MS-275	0.998	0.00002 ^a

^aP<0.05. Geneset A represents the differentially expressed genes present in dermal fibroblasts from keloid with hydrocortisone treatment, specifically, novel differentially expressed genes following hydrocortisone treatment. Geneset C represents the differentially expressed genes that are not affected by hydrocortisone. The closer to -1 the enrichment score is, the stronger the effect of the drug on the diseases.

A number of diseases, such as bone loss, were predicted to be associated with DEGs in keloid fibroblasts treated with HC (*IL1R1* and *COL1A1*). Bone loss is one of the most devastating adverse effects of glucocorticoids as it results in the inhibition of calcium transport and the impairment of osteoblast function (32). A previous study has reported that long-term treatment of secondary hypocortisolism with a high replacement dose of hydrocortisone (30 mg/day) induces bone loss (33). *IL1R1* encodes the type I interleukin 1 receptor. It has been demonstrated that IL-1 production may accelerate bone loss in postmenopausal women (34). The interleukin-1 receptor antagonist (IL-1ra) may decrease bone loss and bone resorption in ovariectomized rats (35) and it has been reported that a gene polymorphism of *IL-1ra* is associated with bone mineral density and osteoporosis in postmenopausal women (36). Additionally, it has been

demonstrated that *COL1A1* (encoding collagen, type I, alpha 1) Sp1 alleles are associated with a modest reduction in bone mineral density and a markedly increased risk of osteoporotic fracture, particularly vertebral fracture (37). Previously, it was demonstrated that the crosslinking process of collagen is important in bone strength, osteogenesis imperfecta and osteoporosis (38). Therefore, the present study hypothesized that HC may contribute to bone loss during treatment with keloids, through certain genes including *ILIR1* and *COL1A1*.

Although HC has been successfully used to treat keloids, it also induces certain adverse effects, such as arthralgia (39). In the present study, 9 small molecule drugs were identified by the CMAP database and acemetacin was predicted to be the most effective drug at reducing the adverse effects of HC. It has previously been demonstrated that acemetacin may treat arthralgia effectively (40). Therefore, combining acemetacin with HC may be a novel therapeutic method of treating keloids, thus reducing the adverse effects of HC treatment.

Despite the aforementioned results, there were a number of limitations in the present study. Confirmation by experiment of these predictions is necessary. Future *in vivo* studies are required to detect the expression of genes associated with endothelial cell differentiation and regulation of cell proliferation. Furthermore, the efficacy of acemetacin-HC combination treatment compared with HC treatment alone requires evaluation.

In conclusion, the present study suggests that HC treats keloids effectively by targeting *COL18A1* and *JAG1*, which are associated with endothelial cell differentiation. However, the cell cycle and p53 signaling pathways in keloids do not return to normal following treatment with HC. A number of adverse effects such as bone loss, which involves *ILIR1* and *COL1A1*, may occur during the therapeutic process of HC treatment. Additionally, it was predicted that acemetacin may be used in combination with HC to treat keloids, although further studies are required to demonstrate its efficacy. These findings may contribute to the understanding of mechanisms involved in HC treatment on keloids and provide candidates for subsequent validation and further study.

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