Underlying Genes and Molecular Mechanism of Keloids Investigated by Integrated Bioinformatics Analysis

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Research article

Keywords: Keloids, Integrated bioinformatics analysis, MicroRNAs, Transcription factors

DOI: https://doi.org/10.21203/rs.3.rs-148691/v1

Abstract

Background: We aimed to identify the overlapping differentially expressed genes (DEGs) of keloids distinguished from normal scar and normal skin and relevant underlying mechanism using integrated bioinformatics methods.

Methods: The expression profiles of 18 keloid samples, 7 normal skin and 5 normal scar, were obtained from the GSE7890, GSE44270, GSE92566, and GSE3189 datasets in the Gene Expression Omnibus database. DEGs were identified using the LIMMA package in R. Gene ontology (GO) functional enrichment analysis was performed using the R software. A DEG-associated protein–protein interaction (PPI) network was constructed using STRING and MCODE was used for module analysis of the PPI network. Moreover, the hub genes were verified by qRT-PCR. The predicted DEGs, their regulatory miRNA and TF regulation network was analyzed using miRnet.

Results: A total of 978 common DEGs were identified in the keloid samples. Genes with more than 45 interaction degrees, including neuropeptide Y (*NPY*), opioid receptor mu 1 (*OPRM1*), cholinergic receptor muscarinic 2 (*CHRM2*), and proopiomelanocortin (*POMC*), were found in the PPI network. Hsa-miR-335 and Sp1 as upstream-regulators regulated *CHRM2*, *NPY*, and *POMC*. Functional enrichment analysis revealed that hub genes were commonly enriched in the “G protein-coupled receptor signaling pathway” GO_BP term

Conclusion: Taken together, *CHRM2*, *NPY*, *POMC*, and *OPRM1* potentially have crucial roles in keloid disease. Furthermore, miR-335 and Sp1 are potential targets for preventing keloid formation.

Introduction:

Keloids are morbid and unique fibrotic disorders that result from various injuries that extend to the reticular dermis during wound healing [1]. The unaesthetic scarring occurrence is usually accompanied by concomitant increases in psychological and physical burden [2]. Different from the general scar, it possesses cancer-like properties such as aggressive unrestrained growth, a tendency for aggravation, and frequent recurrence [3]. Undoubtedly, alteration in gene and protein expressions have been demonstrated in keloid lesion compared with healthy skin. The genetic predisposition factor encompasses the entire pathological processes, especially ethnicity, where a slightly higher incidence of keloid occurrence is found in non-white races. Besides environmental factors and genetic predisposition, epigenetic modifications such as non-coding RNAs and transcription factors (TFs) also exert special effects in the regulation of gene expression in keloid formation. Studies on microRNA have proposed some effects on proliferation, apoptosis, extracellular matrix, and angiogenesis [4–6]. As for TFs, the function of STAT3, FOXM1, SFRP1, and YAP transcription factors have been verified in keloid [7–10]. However, there is still a lack of thorough knowledge of the keloid mechanism.

In this study, we merged the datasets of GSE7890, GSE44270, GSE92566, and GSE3189 via integrated bioinformatics methods to identify the core genes. Further, we predicted the potential regulatory network between DEGs and miRNAs based on this. This provides a reliable target and pathways for a better understanding of its molecular pathology and effective pharmaceutical therapy targeting.

Materials And Methods:

Data Resources

The mRNA expression profiles of GSE7890, GSE44270, GSE92566, GSE3189 (all homo sapiens) were acquired from Gene Expression Omnibus (GEO) database. Five normal scars and five keloid fibroblast samples were extracted from GSE7890. Three normal skin and nine keloid fibroblast samples were downloaded from GSE44270. Four keloid samples were derived from GSE92566. Other four normal skin samples were derived from GSE3189. Samples of both GSE7890 and GSE92566 datasets were located on GPL6244 (Affymetrix Human Gene 1.0 ST Array). Others belong to GPL570(Affymetrix Human Genome U133 Plus 2.0 Array) and GPL96(Affymetrix Human Genome U133A Array), respectively.

Data preparation and screening of DEGs

Downloading series matrix file and platform file for conversion into gene symbol from NCBI. Using R project for statistical computing (Version 4.0.0) that is inserted by both LIMMA (https://www.bioconductor.org/packages/release/bioc/html/limma.html) package to run a series of procedure, as followed ID conversion, merging, batch analysis for normalization and expression calculation. The final calculation of genes which were matched with multiple probes was the average of those probes value.

After mentioned operation, the screening of DEGs was carried out via LIMMA package in R. Three groups, including A [keloid samples(n=18) from excision surgery], B [normal scar samples(n=5) from adults], C [normal skin samples(n=7) from elective plastic surgery and melanoma patients], were defined. To identify much more potential mRNA that are related with the pathogenesis of keloid, keloid groups(A) were compared with B, C groups, respectively. Then, we presented the common DEGs in the online Venn diagrams tool (http://bioinfogp.cnb.csic.es/tools/venny/index.html and http://bioinformatics.psb.ugent.be/webtools/Venn/), which were for subsequent analysis. The DEGs were obtained base on the filter condition of p-value□0.05 and |log2 fold change (FC)|□1, which were depicted by volcano plot.

Functional Enrichment Analysis

The Gene Ontology (GO) functional enrichment analysis of DEGs were accomplished by the cluster Profiler R package.

For detailed information of DEGs, we classified screening genes by biological process (BP), molecular function (MF), cellular component (GC) and the corresponding signaling pathway with the GO (gene ontology) analysis[11] and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway enrichment analysis[12], which displayed in ClueGO (Version2.5.5)plugin of Cytoscape (Version 3.7.2)[13].

PPI Network Analysis and Subnet Module Analysis

Applying with the official website (http://string-db.org/) of Search tool for the retrieval of interacting genes/proteins (STRING) acquired the interaction network among the DEGs-encoded proteins and the medium

confidence was required for ≥0.9[14]. The most significant PPIs were presented in Cytoscape software (version: 3.7.2, http://cytoscape.org/)[15] with MCODE application and acquired the most significant module under k-core>10 circumstance. The genes located in the same module tend to generate proteins that possess the same or analogous function, moreover, these proteins integrate into a module in a same biological role. Usage of the MCODE algorithm to calculate the score of each module. With the higher score of the module showed the more closed interactions and enrichments. For obtaining hub genes, PPI network were analyzed using R.

Prediction of hub-genes with miRNA and TF mutual regulating networks.

The miRnet, a visual network-based analysis, which could accomplish relationship of miRNA and TF with targets according to the microRNA target base(miRTarBase) and microRNA records(miRecords). After establishing regulation network, cytohubba was used to select the highest degree of miRNA and TF.

Quantitative real time polymerase chain reaction(RT-qPCR)

Total RNA was isolated and performed reverse transcription to acquire stable cDNA that was used to subsequent experiment. The expression of NPY, OPRM1, CHRM2, POMC was quantified by the $2^{(-\blacktriangle\blacktriangle Ct)}$ method.

Results:

Selection of DEGs

The DEGs from two sets were screened at $p<0.05$ and | log FC | >1 standard (Fig.1A, B). A total of 2714 genes were up-regulated and 926 were down-regulated genes as displayed in the volcano plot. The common genes screened from the two sets included 952 up-regulated genes and 26 down-regulated genes as displayed by the Venn tool (Fig.1C).

Functional Enrichment Analysis

The GO function (*p*-value<0.05) analysis (Fig.2,Table.1) revealed the most significant three terms that belong to BP, MF, and CC. For GO_BP process, the DEGs were mainly part of G protein-coupled receptor signaling pathway (*p*-value=7.43479E-24), nervous system process (*p*-value= 1.60029E-21), receptor ligand activity (*p*-value=1.48832E-20), receptor regulator activity (*p*-value=6.55E-20) and G protein-coupled receptor activity (*p*-value= 8.64E-20). The identified DEGs were significantly enriched in molecular function of the intrinsic component of plasma membrane (*p*-value= 3.89E-23), integral component of plasma membrane (*p*-value=1.48E-22), plasma membrane (*p*-value=1.63E-18), cell periphery (*p*-value=1.79E-13) and extracellular space (*p*-value=1.1E-11). In addition, their cellular composition were enriched in peptide receptor activity (*p*-value=5.12E-19), transmembrane signaling receptor activity (*p*-value=1.48E-16), neurotransmitter receptor

activity (*p*-value=2.03e-07), ammonium ion binding(*p*-value=3.61E-07), and serotonin receptor activity (*p*-value=9.32E-07).

Table 1
GO functional analysis for differentiated expression genes.

GO/ID	Term	*P*-value	Count
GO_BP			
GO:0007186	G protein coupled receptor signaling pathway	7.43E-24	168
GO:0050877	Nervous system process	1.6E-21	172
GO:0048018	Receptor ligand activity	1.49E-20	87
GO:0030545	Receptor regulator activity	6.55E-20	91
GO:0004930	G protein coupled receptor activity	8.64E-20	113
GO_MF			
GO:0001653	Peptide receptor activity	5.12E-19	44
GO:0004888	Transmembrane signaling receptor activity	1.48E-16	149
GO:0030594	Neurotransmitter receptor activity	2.03E-07	30
GO:0070405	Ammonium receptor activity	3.61E-07	18
GO:0099589	Serotonin receptor activity	9.32E-07	11
GO_CC			
GO:0031226	Intrinsic component of plasma membrane	3.89E-23	200
GO:0005887	Integral component of plasma membrane	1.48E-22	192
GO:0044459	Cell periphery	1.63E-18	272
GO:0071944	Extracellular space	1.79E-13	419
GO:0005615	Plasma membrane part	1.1E-11	277

Analysis of PPI network and modules

PPI network of the DEGs was established via STRING and the degree among them was visualized from R. The network was with 390 nodes and 4426 edges. Top 10 genes with degree≥45 affiliated to PPI were found and including GNG3 (degree=92), GNG13 (degree=90), POMC (degree=69), ADCY8 (degree=66), NMUR1 (degree=64), SST (degree=49),CCR3(degree=48), GCG (degree=48), CHRM2 (degree=47), NPY (degree=46),OPRM1 (degree=46), PDYN (degree=46), and PYY (degree=46)(Fig.4A). Further, four functional subset modules mcode1 to mcode4 were screened under the MCODE SCORE≥3, K-core>20 and network nodes ≥4 criteria for the PPI network (Fig.3A). Specifically, 1 down-regulated nodes and all 111 down-regulated nodes formed the mcode1 and mcode4, respectively (Fig.3B). The mcode1, mcode2 and mcode4 contained only up-regulated nodes. In the mcode3, there were 11 up-regulated nodes besides 1 down-regulated node. The 12 nodes (CCR3, GNG3, GNG13, CHRM2, POMC, ADCY8, SST, PDYN, NPY, PYY, NMUR1, and OPRM1) of mcode1 and all genes of mcode2 were enriched in the “G protein-coupled receptor signaling pathway” (GO:0007186). All the genes of mcode3 and mcode4 participated in “extracellular space” (GO:0005615) and “intracellular part” of the GO_CC terms, respectively.

Identification of miRNA-target and miRNA-TF interaction network

Furthermore, we detected the possible correlated miRNA and TF through online analysis tool-miRnet for DEGs. The results revealed 2980 nodes, including 641 DEGs, 290 TFs, 2049 miRNAs (Fig.4B,4C). Among the miRNA and TF, hsa-mir-335-5p (degree=202) and SP1 (degree=45) had the highest degree. A total of 7556 edges were found in the PPI network and 6969 regulatory interactions were detected in miRNA that can interact with up-regulated DEGs (e.g.hsa-miR-335-5p-CHRM2, NPY, and POMC). A total of 587 regulatory interactions were found between miRNAs and down-regulated DEGs. A single TF (Sp1 Transcription Factor: Sp1) was predicted to target 44 up-regulated DEGs (e.g. OPRM1, NPY).

Quantitative real time polymerase chain reaction(qRT-PCR)

The mRNA levels of POMC,OPRM1,NPY,CHRM2 was significantly upregulated in our collected keloid samples(Fig.4D).

Discussion:

In this study, the common 978 DEGs(952 up-regulated and 26 down-regulated) in keloid samples were markedly differentiated from normal skin and normal scar samples. According to the analysis of the PPI network, the core genes were as followed: *GNG3, GNG13, POMC, ADCY8, NMUR1, SST, CCR3, GCG, CHRM2, NPY, OPRM1, PDYN* and *PYY.* Meanwhile, the miR-335-5p and Sp1 were identified as the main upstream factors, of which Sp1 was found to be associated with *NPY, OPRM1* and miR-335-5p modulated three central genes that were *NPY, POMC, CHRM2.* Except for PYY gene, these core genes were commonly enriched in G

protein-coupled receptor signaling pathway" in GO_BP terms. It is noticed that *NPY, POMC, CHRM2, OPRM1* all belong to the mocode1, which underlines the similarities of their functions.

In the keloid microenvironment, the mechano-physiological conditions are important, they are involved in mechanical tension and inflammatory tension and a vicious circle was formed in between. The mechanical information needs to be converted into a biological signal through the cell membrane mechanoreceptors. Besides integrins, there are non-integrins for accomplishing the transformation process such as G-protein coupled receptors [16]. In the present study, both *OPRM1* and *CHRM2* belong to a superfamily of the G-protein-coupled receptors, inferring their latent correlation with the mechanical tension. Moreover, these receptors were also involved in growth and progression of tumor [17, 18]. In the view of the abnormal proliferation of keloid fibroblasts is the major reason for keloid formation, the similar role of receptor genes is speculated in keloid.

Altered balance between proangiogenic and antiangiogenic is required for tumor growth beyond a certain size. Vascular endothelial growth factor (VEGF), periostin, and endostatin have been shown to be related to new vessel formation in keloid lesions [19, 20]. We found that *NPY*, which encodes another direct angiogenic stimulator, was up-regulated in the keloid samples in our study. *NPY*-stimulated *VEGF* production and secretion was found to contribute highly to angiogenesis activity in human breast cancer [21] and *NPY* also was identified as a promoter of prostate and breast cancer, affecting the proliferation and migration of cells[22, 23]. Furthermore, *NPY* was shown to participate in fibrogenesis of hepatic stellated cells, thereby contributing to hepatic cancer [24].

The deregulation of extracellular matrix deposition is one of the pathological processes in keloids. *POMC* mRNA was detected in keloid-derived fibroblast, which is in agreement with our results [25]. As the precursor of various active peptides, *POMC*-derived products were found to have biological roles under cytokine stimulation in the regulation of extracellular matrix deposition and inflammation[26],suggesting their potential functions during the development of keloids.

Among the 2049 predicted miRNAs, miR-335 had the highest degrees and targeted DEGs in the regulation network. MiR-335 has been verified as both a tumor suppressor and tumor promoter in various cancers[27]. One of the inhibition mechanisms of miR-335 is the activation of tumor suppressor p53 by alerting Rb1 to repress cell proliferation [28], and the antagonistic effect of miR-335 on miR-21 was shown to be mainly a pro-tumorigenic mechanism [29]. miR-21 involvement in keloids has also been reported[30], suggesting that miR-335 is involved in the underlying mechanism and neuropeptides, including NPY, POMC, and CHRM2, are involved downstream. This axis mechanism requires further validation. SP1 is a well-known TF that is involved in keloid pathogenesis mainly by regulating the extracellular matrix process of keloids[31]. However, a more detailed understanding of the underlying mechanism is required. We found that Sp1 formed an interaction network with NPY and *OPRM1* in the TF–DEG network.

Conclusion:

Taken in combination,the common 978 DEGs (952 up-regulated DEGs and 26 down-regulated DEGs) were confirmed by comparing keloid samples with normal skins samples and normal scars samples. As for screened core genes from PPI network, especially for *NPY, POMC, CHRM2, OPRM1* that was supported by RT-

qPCR experimental validation. Aimed at four genes, we established a interaction network to identify the up-regulators including miRNA and TFs, moreover, GO functional enrichment analysis provided clues to speculated the “G protein-coupled receptor signaling pathway” GO_BP is highly related with the molecular of keloids development. Furthermore, certain observation should be made by experimental validation.

Abbrevations:

DEGs Differentially Expressed Genes

KEGG Kyoto Encyclopedia of Genes and Genomes

PPI Protein–Protein Interaction

GO Gene Ontology

miRNA microRNA

NPY Neuropeptide Y

OPRM1 Opioid Receptor mu 1

CHRM2 Cholinergic Receptor Muscarinic 2

POMC Proopiomelanocortin

TF Transcription Factors

VEGF Vascular endothelial growth factor

Sp1 Specificity Factor 1

Declarations

Acknowledgement

Not applicable

Authors’ contributions

LI chuying conceptualized the study; JIN meitong and LUO yinli designed the methodology; LI chuying wrote the paper; JIN zhehu and PI longquan supervised and gave suggestions on the manuscript; PI longuqan substantively revised the manuscript. All authors have read and approved the final manuscript.

Funding:

This research is supported by the Natural Science Foundation of China (Grant No. 81960561).

Ethics approval and consent to participate:

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets generated and/or analysed during the current study are available in the NCBI repository https://www.ncbi.nlm.nih.gov/gds/.

Competing interests

The authors declare no competing of interest, financial or otherwise.

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