Original Article

Collagen Cross-Linking Therapy on Important Functional Genes Involved in Keratoconus Patients Using Protein-Protein Interactions (PPI) Network and Differential Expression Analysis

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Abstract

Background: Keratoconus (KC) is a progressive eye condition marked by the corneal protrusion, thinning, and scarring that adversely affects the ability to see and quality of life. A defective corneal Extracellular Matrix (ECM) assembly ultimately results in myopia, irregular astigmatism, and severe visual impairment. This condition is most commonly occurring in adolescence, this condition is one of the most commonly indicated clinical indications for corneal transplantation. In contrast to corneal transplantation, Corneal Collagen Cross-Linking (CXL) increases corneal stiffness without inflicting any invasive surgery.
Material and Methods: This study used RNA-Seq data to investigate which functional genes are regulated by CXL in keratoconus. The RNA-Seq expression matrix related to individuals with Keratoconus (KC) and patients treated with Corneal Collagen Cross-Linking (CXL) were obtained from the Gene Expression Omnibus (GEO) (GSE112155) database [1] at the National Center for Biotechnology Information (NCBI) were retrieved. Differential expression analysis and functional enrichment analysis were conducted. The hub genes were then identified with the Protein-Protein Interactions (PPI) network. Finally, Transcription Factor (TF) genes were identified that regulate these networks.
Results: We identified 126 genes in KC and CXL-treated patients that affect TF-mediated network adjustments that improve treatment. In addition, they may play a role in the pathogenesis of keratoconus. Visualization of the PPI networks enabled us to identify 63 highly connected or Hub genes that served an essential biological function in regulatory
Conclusion: networks.
Keywords: Keratoconus; PPI; Differential Expression Analysis; Transcription Factor; Systems biology.

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Introduction

Keratoconus (KC) is a progressive eye condition that causes the cornea to thin. This may lead to blurry vision, double view, nearsightedness, atypical astigmatism, and light sensitivity, all of which can significantly affect our quality of life [2]. In most cases, both eyes are affected. It is possible to see scarring or circle within the cornea when the cornea is more severely damaged [3]. defects in the assembly of the corneal Extracellular Matrix (ECM) is a leading cause of this condition [4].

A large number of clinical studies have been conducted, but the underlying pathobiology of KC remains unclear. The condition usually appears in adolescence and is considered one of the most commonly indicated clinical indications for corneal transplantation [5,6]. In addition, KC leads to an imbalance between collagen production and proteolysis. The crosslinking enzyme Lysyl Oxidase (LOX) activity and concentration in keratoconic corneas are significantly reduced [6,7].

The most popular ways to treat keratoconus are Epikeratophakia [8], Corneal ring implants [9], Radial keratotomy [10], Cross-linking [11], and corneal transplantation [12]. In contrast to corneal transplantation, Corneal Collagen Cross-Linking (CXL) is a relatively non-invasive procedure that increases corneal stiffness. The favored right-angled collagen fibril orientation of the normal human cornea is severely impaired in KC mechanically revealing a substantial reduction in corneal stiffness, contributing to a biomechanical unstable corneal environment.

CXL is now an established treatment for progressive ectasias like KC, and the US Food and Drug Administration (FDA) recently approved it as such. In summary, CXL utilizes the combined properties of ultraviolet A (UVA, 370 nm) and riboflavin, which act as photosensitizers for the induction of crosslinks between collagen fibrils [13]. In addition, a barrier prevents UVA ray penetration into underlying tissues. As a result of UV irradiation, the fluorescent molecule is excited to a triplet state, creating singlet oxygen and superoxide radicals. By strengthening collagen bonds and forming covalent bonds between neighboring collagen fibers, the radical products can then strengthen corneal stromal collagen bonds and increase resistance to enzymatic degradation [14,15].

Wollensak et al. introduced CXL in 2003, and it has since become a widely known, low invasive procedure that is highly successful and has low complication rates. A standard cross-linking technique called the Dresden protocol involves removing the corneal epithelium (epi-off) alone, followed by riboflavin and UV-A irradiation at 3 mW / cm2. CXL without epithelial debridement (epi-on) technique has also been attempted. This technique aims to reduce the risk of infection and postoperative pain associated with epi-off. The effectiveness of both techniques has been proven, however, long-term safety and adverse effects have yet to be determined [16].

Given that the exact mechanisms of this disease are not yet known, and on the other hand, this treatment controls the disease well, so the identification of genes that are regulated by this treatment, in addition to confirming the effectiveness of treatment, helps us to discover the mechanisms involved in the disease. In this study, an RNA-Seq study was used to investigate which functional genes are regulated by CXL in the keratoconus. genes that had completely different expressions were identified first, then by visualizing protein-protein interaction networks and ontology, major effective or hub and Transcription Factor (TF) genes were identified. Finally, the results of 126 TF and 63 Hub genes were

identified, many of which were involved in very important processes such as improving collagen regeneration, reducing tear(ing) production of the lacrimal glands, increasing endurance, and corneal diameter, and so on.

Material and Methods

Gene Expression Dataset

The RNA-Seq expression matrix related to individuals with Keratoconus (KC) and patients treated with Corneal Collagen Cross-Linking(CXL) were obtained from the Gene Expression Omnibus (GEO) database [1] at the National Center for Biotechnology Information (NCBI) under the accession number of GSE112155. KC epithelial samples were obtained from ten eyes out of ten patients (five without treatment, and five with CXL). More details of the data can be found in the original paper [17]. Atopy and contact lens wear were only reported in a small proportion of the study participants; therefore, it was impossible to conduct a statistical analysis of their effects. Thus, we didn't include contact lens wear or atopy as covariance in this study.

DEG analysis

A differential expression test was conducted using DESeq2 (v1.30.0) [18] and edgeR (v3.32.1) [19]. DESeq2 was normalized using the median-of-ratios method. The distributions were estimated using Cox-Reid adjusted profile likelihoods and the Wald test was used to determine significance. The settings in DESeq2 automate the filtering of genes whose False Discovery Rate (FDR) are below a threshold calculated automatically (default of 0.1). Each gene was tested for differential expression using a negative binomial generalized linear model. Low count transcripts were excluded manually from edgeR analysis and only genes with a mean greater than 1 count per million were considered. A "Trimmed Mean of M-values" (TMM) method was used for matrix normalization [20]. Finally, an exact-test method was used to diagnose DEG. According to this study, in both methods (edgeR and DESeq2) FDR of 0.10 was used to adjust for multiple comparisons.

Functional Enrichment Analysis

To better understand the potential mechanisms of how CXL therapy effect keratoconus, all DEGs with overlap between DESeq2 and edgeR were enriched under the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways analysis using Enrichr online tool [21]. For this purpose, a set of valid Entrez gene symbols is placed in Enrichr's text-box and the results described above are made available. Only genes with FDR < 0.1 and the absolute value of the Log fold change > 2 were considered. Finally, the results of Functional Enrichment were illustrated using the ggplot2 package in R software.

PPI network construction and identification of hub and TF genes

Using "Search Tool for the Retrieval of Interacting Genes" (STRING) database and medium stringency settings (confidence level and interaction source set to 0.4 and experiments and database, respectively and also the rest of parameters set to default) was set and included all possible interactions [22], were created separate PPI networks for Up-regulated and Down-regulated genes, and assessing the significance of the connections between genes in each of the networks. Whether up-regulation or down-regulation is going on, is a hypothesis for further exploration

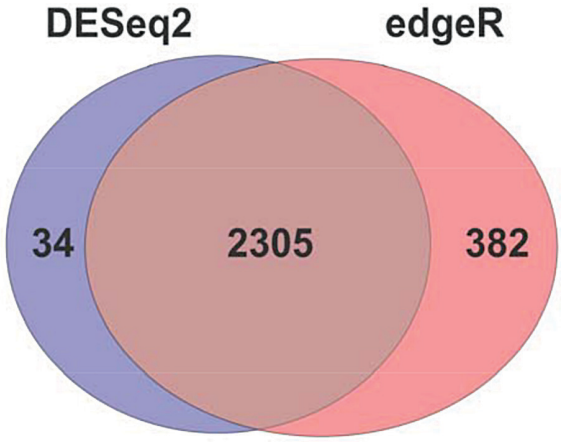

Figure 1: Venn diagram of common genes between two different methods of DEG analysis

in subsequent experiments. Genes with the highest degree of connectivity in the network are considered hub genes and are expected to exhibit higher biological significance than the other genes in the network. To detect TFs in each network, the genes of each network were aligned with human transcription factors extracted from the Human TFDB database (http://bioinfo.life.hust.edu.cn/HumanTFDB/). Finally, Cytoscape (version 3.8.2) [23] was used to visualize the network of the genes. Cytoscape is an open-source software platform for visualizing molecular interaction networks and biological pathways and integrating these networks with annotations, gene expression profiles, and other state data.

Results

DEG identification methods

Initially, only 18,358 protein-coding were selected from 46,312 transcripts. First, 2576 genes with an average expression < 1 were removed from the expression matrix, then the normalized matrix using the TMM method was formed (supplementary file S1 and Figure 2). According to the results of the edgeR package, 2687 genes were identified with an FDR < 0.01 and an absolute logFC value > 2. With the DESeq2 package, 2339 genes were identified with FDR < 0.01 and an absolute logFC value > 2 (Supplementary file S2). A Venn diagram for the identification of common genes was drawn in both methods (Figure 1 and Supplementary file S3).

Functional Enrichment Analysis

To understand the biological functions of the DEGs, all DEGs with overlap between DESeq2 and edgeR were investigated using the Gene Ontology (GO) and KEGG pathways analysis tool Enrichr online tool [21] (Supplementary file S4). GO Biological process and GO Cellular Component was illustrated in figure 3 and 4.

PPI network construction and identification of hub and TF genes

In general, there were 447 genes in the Up-regulated network, of which 5 hub and 22 TF genes were identified (Table 1). There were 1852 genes in the Down-regulated network, of which 57 hub genes and 104 TF genes were identified (Table 1). Both networks have significantly more interactions than expected (Table 2). The PPI networks were visualized using Cytoscape (version 3.8.2) [23] for each Up-regulated and Down-regulated network (Figure 5 and 6).

Discussion

Keratoconus is the most common primary ectasia. A bilateral and asymmetric corneal degeneration results in corneal protrusion due to localized corneal thinning. In both the inferior temporal and the central corneas, corneal thinning occurs [2]. The progression of keratoconus usually occurs over the second to

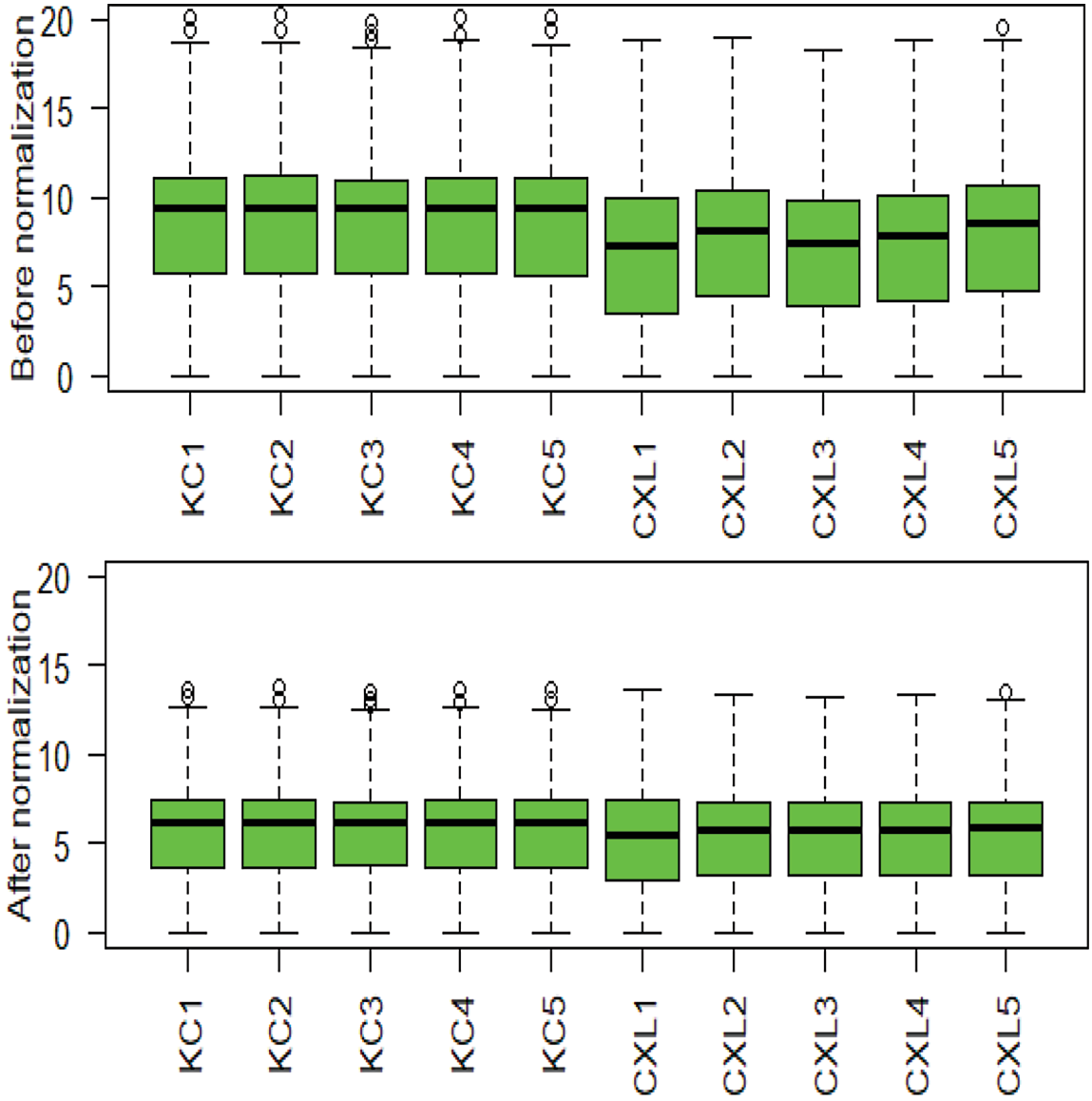

Figure 2: Pre- and post-normalized gene expression box plots. The horizontal axis represents the sample symbol, while the vertical axis represents gene expression levels. In a box plot, the black line represents the median value of gene expression

fifth decades of life, and it can eventually lead to intolerance to contact lenses and cornea transplantation in 10 % to 20 % of cases [24]. There are several new treatments for keratoconus and ectasia, including intrastromal corneal ring segment implantation [25,26], conductive keratoplasty [27], corneal transplantation [5,6], and Corneal Collagen Cross-Linking (CXL). Unlike corneal transplantation, CXL provides increased corneal stiffness without invasive surgical intervention [6,7]. This study used RNA-Seq data to investigate which functional genes are regulated by CXL in keratoconus. From the Gene Expression Omnibus (GEO) database at the National Center for Biotechnology Information (NCBI), we retrieved the RNA-Seq expression matrix related to individuals with Keratoconus (KC) and patients treated with CXL.

Human body functions are influenced by the differences between transcriptomic profiles among various cells and tissues. In the last few

Table 1: TFs detected in which Up-regulated and Down-regulated networks

	Up-regulated	Down-regulated
TF genes	ST18, SIM1, DRGX, EBF1, HMGA2, LIN28B, RFX4, PHOX2B, PAX3, PROX1, NR2E1, ZEB1, RORB, ZFPM2, RFX6, FOXB1, POU3F2, MYT1L, CREB5, POU4F1, MYRFL, HNF4G	PAX8, RARA, THAP4, PIAS4, HES4, TBX6, HES5, ZNF512B, ZMIZ2, ZNF282, TSC22D4, MEOX1, IRF3, ZNF513, MAFA, PITX1, ZNF696, MEIS3, THAP11, ZNF385A, ETV2, HMG20B, MYBL2, ZNF467, GLIS2, ZNF517, ZNF628, ZNF296, ZNF541, THAP7, BATF, ETV4, ZNF668, ESRRA, ZNF316, ZNF408, E4F1, ZNF865, ZNF707, ZNF205, SREBF1, SAMD11, ZNF787, SIX5, IRF7, ZNF579, ZNF687, GLI4, MBD3, BATF2, ATOH7, ZBTB48, ZBTB7B, ZNF500, ID3, ARID5A, ZNF213, ZNF653, THAP3, TFEB, ZNF219, ZNF446, MXD4, NR4A1, HSF4, REPIN1, NKX3-2, ZNF768, ZNF574, CIC, HES6, E2F1, HMX1, ZNF335, ZFPM1, CDIP1, ZNF423, CEBPA, ZNF524, NPAS1, KLF15, GLIS1, ZNF48, CSRNP1, HSF1, JUNB, NR2F6, ZBTB45, ZBTB17, NR1H2, ZNF324B, KLF16, CAMTA2, FOXD4L1, ZBTB16, SNAPC4, RELB, RFXANK, CAPN15, SLC2A4RG, RBCK1, CC2D1A, ZNF414, ZGPAT
Hub genes	ADCY8, CX3CR1, GRM7, KNG1, NRXN1	AKT1, APOE, BOP1, DDX49, EHMT2, FBXL19, H2AFX, H2BFS, HDAC5, HIST1H2AC, HIST1H2AD, HIST1H2AH, HIST1H2BH, HIST1H2BJ, HIST1H2BK, HIST1H2BL, HIST1H2BM, HIST1H2BN, HIST1H2BO, HIST1H3A, HIST1H3D, HIST1H3E, HIST1H4A, HIST1H4B, HIST1H4D, HIST1H4F, HIST1H4H, HIST1H4I, HIST1H4J, HIST1H4K, HIST1H4L, HIST2H2AA, HIST2H3D, HIST2H4B, HIST3H2A, HIST4H4, HRAS, KEAP1, MRPS2, NOTCH1, PLEC, POLR2E, POLR2L, PPP2R1A, RAC3, RPL13, RPL8, RPLP0, RPS14, RPS16, RPS5, RPS9, RUVBL2, STUB1, TCEB2, TPI1, A4GALT

Table 2: Analysis of Networks Stats

Name of networks	Number of nodes	Number of edges	Average node degree	avg. local clustering coefficient	Expected number of edges	PPI enrichment p-value
Up-regulated	447	857	3.83	0.394	387	1.00E-16
Down-regulated	1852	11874	12.8	0.286	9390	1.00E-16

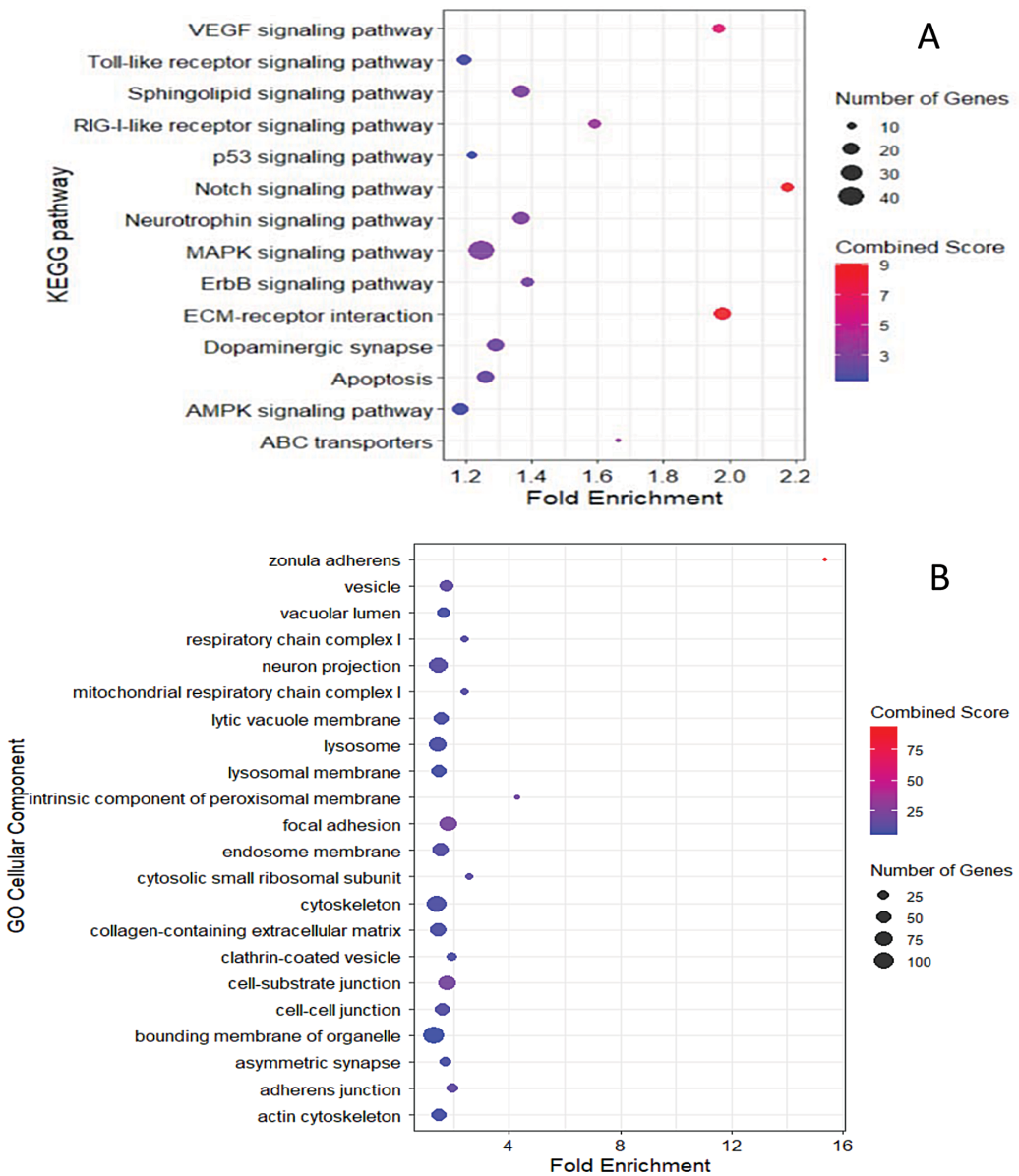

Figure 3: GO Cellular Component (A) and KEGG Pathway (B) of 2305 common genes (FDR < 0.1). Size and color of points represent -Log2 of FDR and the number of genes associated with each term, respectively. The fold enrichment measure is not shown for better visualization

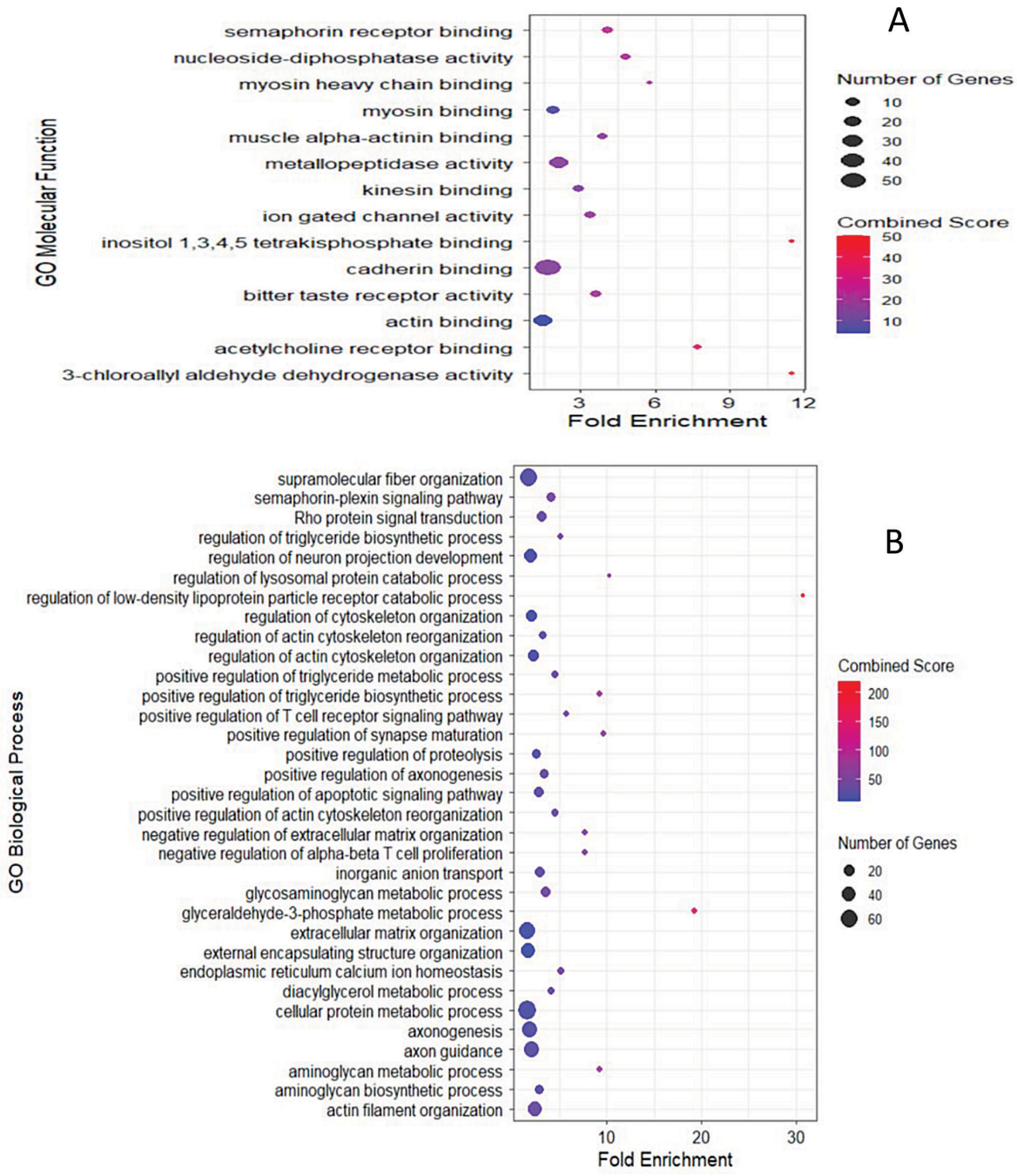

Figure 4: GO Biological process (A) and GO Molecular Functions (B) of 2305 common genes (FDR < 0.1). Size and color of points represent -Log2 of FDR and the number of genes associated with each term, respectively. The fold enrichment measure is not shown for better visualization

years, many transcriptome studies have been done to better understand how changes in gene expression can lead to different phenotypes, such as Keratoconus (KC) [28,29]. Transcriptomic analysis of RNA-Seq-based transcriptome data of human corneas indicated that downregulation of collagen and other genes coding for extracellular matrix components may be involved in KC etiology since it can impair the mechanical stability of affected

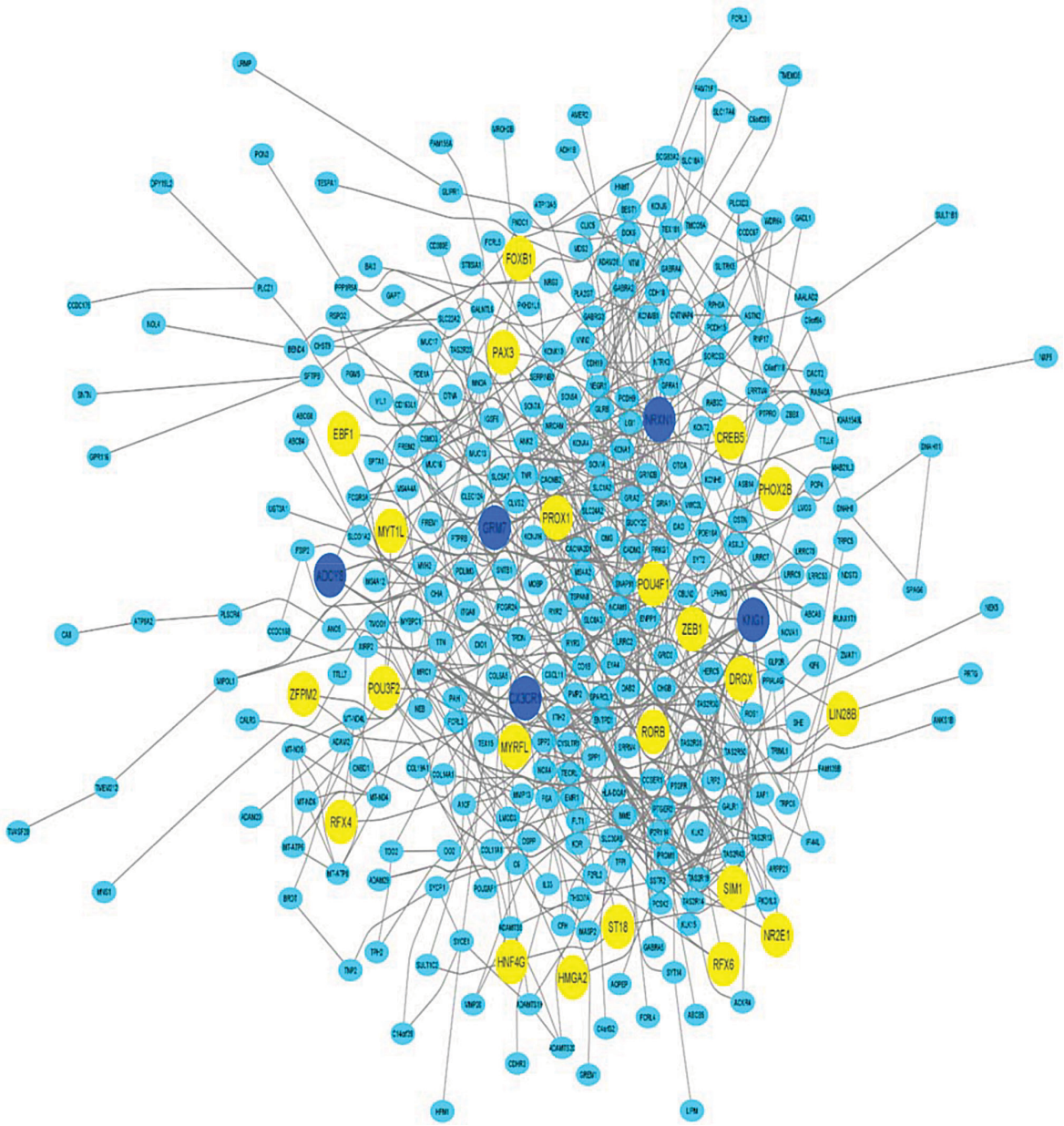

Figure 5: PPI network of Up-regulated genes, dark blue indicates hub genes, and yellow indicates TF genes

tissues [29]. Previous research suggested that KC corneal collagen levels may be lower because of downregulation of the Connective Tissue Growth Factor (CTGF) and its modulators, proteins involved in TGF- β, Hippo, and Wnt signaling pathways [29]. Transforming Growth Factor-Beta (TGF-β) isoforms TGFB1, TGFB2, and TGFB3 influence extracellular matrix composition through their binding to their receptor ligands (TGFBR1 and TGFBR2). It is known that TGFB1 and TGFB2 stimulate a profibrotic response in the cornea, while TGFB3 has an antifibrotic effect [14]. The TGF- β signaling pathway stimulates CTGF synthesis downstream [30]. Consequently, corneal cells are activated, increasing the amount of collagen and other extracellular matrix components in the cornea. The TGFB1 and TGFBR2 genes

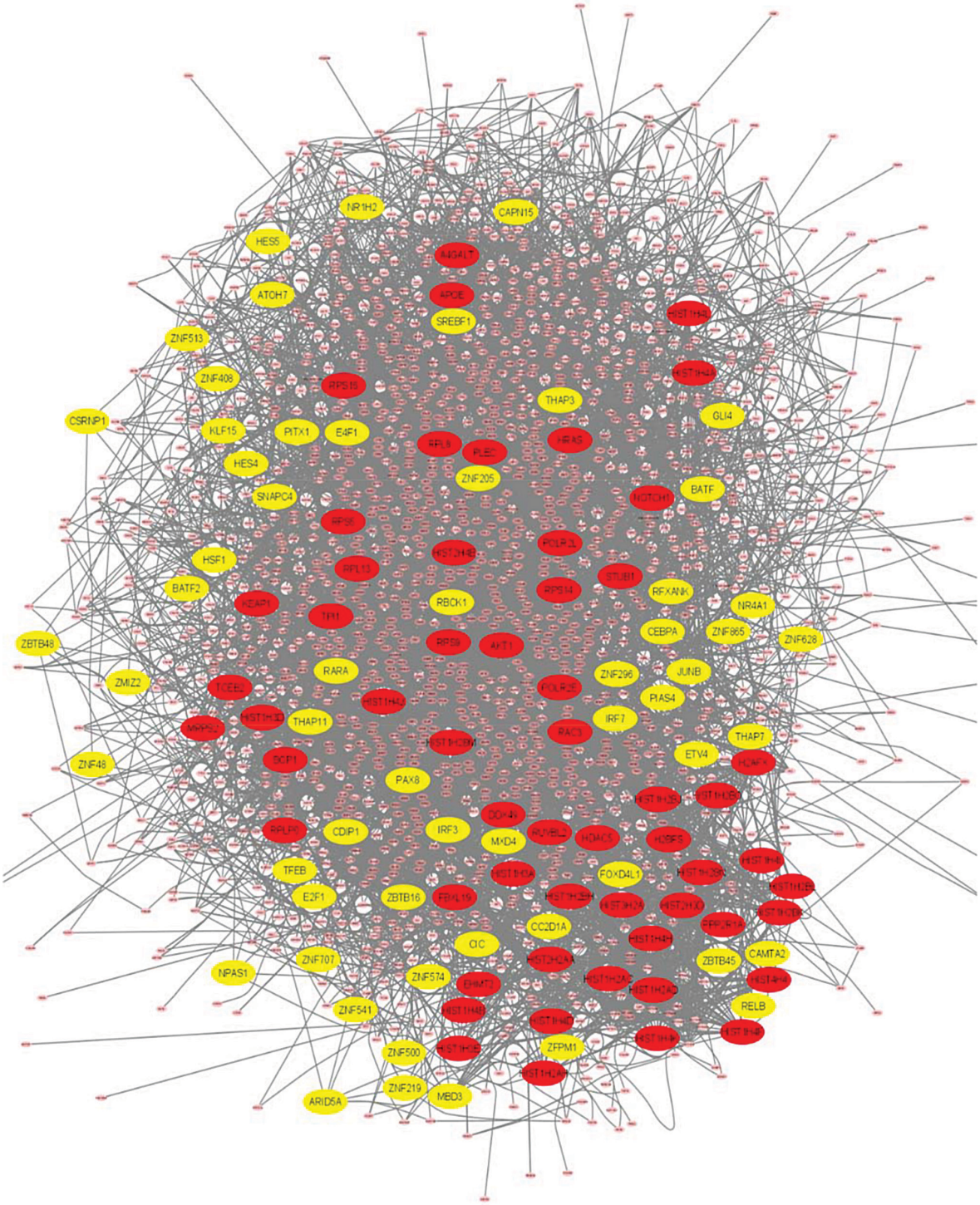

Figure 6: PPI network of Down-regulated genes, dark red indicates hub genes, and yellow indicates TF genes

were DEG in this study (Supplementary file S2) confirming may be an effect of TGF-β signaling alteration in Corneal Collagen Cross-Linking (CXL) therapy [29].

In the human corneal stroma, TGFBI is an important component which is responsible for cell adhesion and migration through its interaction with collagen and other

extracellular matrix elements [31]. Variants of TGFBI are frequently found in patients with corneal dystrophies and KC [32]. The level of TGFBI in KC tissues has also been found previously to be significantly regulated when compared to controls [33], in most transcriptomic or proteomic studies TGFBI levels were decreased. Expression of this gene was significant in this study (Supplementary file S2), which may be due to CXL therapy.

Gene ZNF469 has been linked to central corneal thickness, which is abnormal in KC or corneal dystrophies and is most discussed in KC. The protein encoded by ZNF469 regulates extracellular matrix production and remodeling and participate in collagen homeostasis in the human cornea [34]. Therefore, the difference in the observed expression level of this gene in the treated individuals against KCs can be strongly related to the effectiveness of CXL.

With regard to the genes with decreased expression in CXL therapy, TGFBI, which has been discussed above, and SLC4A11 are the most interesting candidates. The sodium bicarbonate transporter-like protein 11 (SLC4A11) operates via a Na+-dependent borate co-transport mechanism, which triggers mitogen-activated protein kinase-dependent cell growth and proliferation [35,36]. The expression of these genes has been reported to increase in KC [29], although in this study their expression was decreased (Supplementary file S2).

One of the most interesting findings in this study is the proteins associated with the lacrimal glands. According to recent observations, the most important lacrimal gland proteins associated with this disease include Clusterin (CLU) and Pre-pro-megakaryocyte-enhancing factor (MSLN), Lactotransferrin (LTF), Kininogen-1 (KNG1), Peroxiredoxin-1 (PRDX1), Alpha-crystalline B chain (CRYAB), and Alpha-2-glycoprotein 1 Zinc (AZGP1). Ghosh et al. [37] registered these genes in 2013 as highly biomarkers related to KC. After CXL therapy, the expression of these genes was significant than in the KC group (Supplementary file S2), which means the treatment was very effective and could reduce disease-related tears.

In both the Up-regulated and the Down-regulated networks, protein-protein interactions are significantly higher than expected (Table 2). This means that proteins exhibit more interactions than would be expected from a random collection of proteins of the same size from the genome. This enrichment indicates that proteins are at least biologically bound together (Figure 5 and 6). This is due to TFs that regulate these proteins. Consequently, such adjustments show that CXL therapy effectively regulates genes, and the differences in expression after treatment are related to it.

Innovation in systems biology has brought us a variety of computational tools and analytical means. This approach successfully helps researchers to investigate molecular mechanisms of inhibition and activation of the genes and transcription factors, predict drug-target interactions, and detect novel molecular biomarkers of phenotypes [38-41]. Many RNA-RNA, RNA-miRNA, and RNA-Lnc-RNA interaction beside other macromolecules interactions in the cells; have been detected in the light of systems biology method [42-45]. Proposing of the novel panels for early detection of diseases, drug discovery, analyzing signaling networks and metabolomics pathways, and repurposing drugs are attractive subjects in systems biology studies [45-49]. Our study, besides the same ones, shows the potential of these techniques in ophthalmology and

should be more considered by the researchers. However, the shortage of online data is the main obstacle in the application of the high throughputs approach to ophthalmic.

Conclusion

As a result of our study, we identified 126 genes between KC and CXL-treated patients that are involved in TF-mediated regulatory network adjustments that improve disease treatment. They may also play an important role in the pathogenesis of keratoconus. PPI networks visualization enabled us to identify 63 highly connected or Hub genes that played an essential biological role such as improving collagen regeneration, reducing tearing of the lacrimal glands, increasing endurance and corneal diameter, and so on in regulatory networks. However, for better understanding, further analysis and experimentally validation of the candidate genes described here are needed. Finally, it can be concluded that CXL therapy is an excellent alternative to corneal transplantation.

Acknowledgments

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Supplementary Material

FILE S1 | The normalized gene expression matrix of all samples in KC and CXL groups.
FILE S2 | The complete list of DEGs detected by edgeR or DESeq2.
FILE S3 | The complete list of overlapped and specific DEGs between DESeq2 and edgeR.
FILE S4 | The complete list of the functional enrichment analysis of the identified overlapped GEGs.

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Footnotes and Financial Disclosures

Conflict of interest

The authors have no conflict of interest with the subject matter of the present manuscript.