European Review for Medical and Pharmacological Sciences 2015; 19: 3332-3339

Bioinformatic analysis of the microarray gene expression profile in degenerative intervertebral disc cells exposed to TNF-α

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Abstract. – OBJECTIVE: We performed a bioinformatic analysis of the microarray data on the gene expression profiles of degenerative intervertebral disc cells after exposure to tumor necrosis factor-α (TNF-α) to uncover the key genes that were differentially expressed between cells with and without exposure, and to explore the related signaling pathways and interaction networks, providing clues for future investigations on the molecular mechanisms of disc degeneration.

MATERIALS AND METHODS: The microarray data for degenerative intervertebral disc cells after stimulation with TNF-α were downloaded from a public database, the GEO (Gene Expression Omnibus), in order to identify the genes that were differentially expressed between untreated degenerative disc cells and those stimulated with TNF-α, and then analyses of the gene ontology, signaling pathways and interaction networks for the differentially expressed genes were conducted using the DAVID, STRING and other online tools.

RESULTS: A total of 753 differentially expressed genes were found in the degenerative annulus fibrosus disci intervertebralis cells after stimulation with TNF-α, including 458 upregulated genes and 295 downregulated genes. The Gene Ontology annotation analysis showed that these differentially expressed genes were mainly associated with the extracellular matrix, damage reactions, inflammatory reactions, and the regulation of apoptosis. A signaling pathway analysis showed that these differentially expressed genes were mainly involved in the interactions of cytokines, apoptosis, NOD-like receptors, chemokines, and other signal transduction pathways. The interaction network analysis indicated that JUN, CCL3, ANHK and other genes may play key roles in intervertebral disc degeneration.

CONCLUSIONS: The bioinformatic analysis of the gene expression profiles of degenerative intervertebral disc cells stimulated with TNF-α showed that CCL3 and other genes may play a role in the development of the disc degeneration induced by inflammatory reactions. This suggests that bioinformatics methods can be used to identify potential therapeutic target genes, and to provide new insight into intervertebral disc degeneration.

Key Words:
Disc degeneration, TNF-α, Gene expression profiling, Bioinformatics.

Introduction

Disc degeneration refers to the aging and degeneration of various tissues in the intervertebral disc, which is thought to be the underlying pathological process of a group of degenerative diseases, such as a slipped disk, spinal stenosis, and spinal instability, which usually present clinically as neck pain and neurological symptoms that severely threaten human health and a normal life[1]. Disc degeneration is the result of many factors, and age, mechanical stress and lifestyle are generally considered to be its major causes, although these factors do not fully explain the occurrence and progression of disc degeneration[2]. Recent studies have found that abnormal lesions and imbalances in the microenvironment of the intervertebral disc, especially altered levels of inflammatory cytokines, such as IL-1β, TNF-α and other cytokines, are involved in the degeneration of

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discs, but the gene targets and molecular mechanisms of action are currently unknown[3,4]. In this study, differentially expressed genes and their features were identified by analyzing degenerative annulus fibrosus disci intervertebralis cells for changes in gene expression after stimulation with TNF-α. Several molecular markers indicating the induction of disc degeneration by inflammatory cytokines were found, providing a theoretical basis for further experimental studies.

Materials and Methods

Gene Expression Data

The microarray data obtained from the gene expression profiling for disc degeneration were downloaded from the GEO DataSets (http://www.ncbi.nlm.nih.gov/gds/) database: GSE41883. These data were provided by H.E. Gruber, who used the Affymetrix Human X3P Array gene chip for the analysis. The data consist of the results from eight gene chips, which included four degenerated disc tissue samples and four degenerative disc tissues subjected to additional treatment with TNF-α. The degenerative intervertebral disc cells were used as a control group, and were incubated for 14 days prior to extracting total RNA, which was amplified, subjected to RT, and hybridized with the Affymetrix Human X3P Array after fluoresce labeling. The degenerative intervertebral disc cells that were treated with TNF-α in the media were selected as the experimental group, and were similarly incubated for 14 days prior to the extraction of total RNA, amplification of the mRNA, RT, and hybridization with the Affymetrix Human X3P Array after fluoresce labeling.

Screening for Differentially Expressed Genes

The dataset GSE41883 file was downloaded from the NCBI GEO database, and the differentially expressed genes (DEGs) were screened using the R language[5] with the following data filtering settings: (1) the fold-change filtering was compared using the ratio of 2-fold the absolute value as a critical value; (2) the expression level filtering data were screened and compared using the log value of the fluorescence data for genes with a value of $p < 0.01$ from a paired *t*-test.

Functional Annotation for Differentially Expressed Genes

The DEGs were uploaded to the DAVID website (http://david.abcc. ncifcrf.gov/)[6], then the cell components, molecular functions and biological processes of the DEGs were analyzed using the gene function annotation online tools (Ver. 6.7)[7].

Related signaling pathways Analysis of the differentially expressed genes

Using the DAVID website, the relevant signaling pathways were also analyzed using the KEGG (Kyoto Encyclopedia of Genes and Genomes) database[8].

Interaction Relationship Analysis of the Differentially Expressed Genes

The DEGs were uploaded to the STRING 9.1 online analysis platform[9] (http://string.embl.de/), the protein-protein interaction map was obtained for the DEGs, and the key genes were identified.

Results

Screening for Differentially Expressed Genes

A total of 753 DEGs were identified by the screening, including 458 upregulated genes (Table I) and 295 downregulated genes (Table II), 151 and 103 of which were respectively upregulated and downregulated by at least 4-fold. All of other differentially expressed genes were distributed between 2-4-fold higher or lower than the level of the control. In the comprehensive analysis of the gene changes in degenerative intervertebral disc cells after the treatment with TNF-α, the differentially expressed genes with a value more than 2-fold of the fluorescence intensity ratio in this study were used as a target for the analysis in order to avoid missing any important genes.

GO Clustering Analysis

The GO functional annotation analysis of the DEGs showed: (1) the molecular functions of the altered genes were mainly related to cytokine activity, transcription factor binding and chemokine activity, (2) the cell components involved were mainly in the extracellular region and extracellular matrix, (3) the biological processes were mainly involved in the regulation of programmed cell death and the immune response (Table III).

Table I. The genes that were upregulated by a fold-change > 2 and with a value of p<0.01 (top 30).

Gene symbol	Gene ID	Fold-change	*p*-value
CSF2	1437	377.7078343	0.000260406
CCL20	6364	343.9234823	0.000235623
SERPINB2	5055	231.3459442	0.000116432
CCL3	6348	77.88543928	0.000212606
CXCL5	6374	72.39284857	0.001259708
IL1A	3552	68.9247972	0.000591508
EHF	26298	55.85116628	0.000116999
KYNU	8942	54.16792832	0.000703302
GPRC5B	51704	50.82115593	0.00064016
CLIC6	54102	47.70980235	0.001089245
CCL5	6352	44.57848093	0.006931507
CMPK2	129607	39.80179273	0.003041117
NR4A3	8013	38.53188035	0.002098614
SLC7A2	6542	38.13444715	0.000734087
IL1RN	3557	33.68247814	0.006043763
BST2	684	33.11027636	0.001469225
BCL2A1	597	31.94806265	0.000435286
LRRN3	54674	31.87994975	0.000152152
RSPO3	84870	31.84989823	0.00020939
IFI27	3429	31.5217443	0.000163913
C7orf69	80099	30.63592385	0.002786262
NEFM	4741	29.88524989	0.000268142
RSAD2	91543	27.24519656	0.006189876
MMP1	4312	25.5427857	0.000197198
NEURL3	93082	24.64979448	0.002519807
IL11	3589	24.15538468	0.003095441
IFI44L	10964	23.95316289	0.001343935
LIF	3976	23.58203688	0.000545704
CXCL2	2920	23.36617494	0.001727945
NOD2	64127	22.78769885	0.007366378

KEGG Signaling Pathway Analysis

In the KEGG signaling pathway analysis, the 753 DEGs were found to be mainly involved in the interactions of cytokines, apoptosis, NOD-like receptor signaling, chemokine interactions and other signal transduction pathways (Table IV).

Protein-protein Interaction Relationship

The protein-protein interaction relationship networks of the DEGs were generated using the STRING online tools, and the average degree of involvement for each node gene in the networks was respectively subjected to a statistical analysis, and the genes with a high average degree were considered to be "hub" genes. The hub genes identified in this study included JUN, CCL3 and ANHK (Figure 1).

Discussion

Degenerative disc disease is one of the major causes of low back pain. Although its exact pathogenesis remains unclear, it is generally considered to be the result of many factors [10]. The previous studies have found that a small quantity of underlying inflammatory factors were distributed in the normal disc tissues, however, compared with normal disc tissues, more or a larger number of inflammatory cy-

Table II. The genes that were downregulated with a fold-change < 0.5 and a value of p<0.01 (top 30).

Gene symbol	Gene ID	Fold-change	*p*-value
COL15A1	1306	0.010723088	0.000982772
LSP1	4046	0.013069773	0.001756653
ADAMTS15	170689	0.023532077	0.008024998
TDO2	6999	0.027926186	0.000813597
SEPP1	6414	0.03067177	0.000587713
DBC1	1620	0.03253196	0.001952597
ADH1B	125	0.033173486	0.001587698
RIMS1	22999	0.035718202	0.003205944
LYVE1	10894	0.043692787	0.008312665
INMT	11185	0.044676307	0.001125112
OGN	4969	0.046562368	0.002216195
LOC158376	158376	0.051556371	0.002551702
STEAP4	79689	0.05404842	0.006601139
SNED1	25992	0.055345297	0.000246937
LAMP5	24141	0.056646825	0.007806893
SMOC2	64094	0.061171175	0.009472582
MYBPH	4608	0.066831236	0.004263321
OLFML2B	25903	0.070549146	0.000272391
KIAA0226L	80183	0.076443391	0.000331169
ASPN	54829	0.080645431	0.008932247
SLC40A1	30061	0.082586233	0.005812883
LDB2	9079	0.084300481	0.002467538
OLFML2A	169611	0.084970264	0.000869369
MAP1LC3C	440738	0.089293326	0.007414053
ID4	3400	0.095244693	0.002214093
CILP2	148113	0.098211327	0.001318023
ARPP21	10777	0.102054402	0.003080704
CLCA2	9635	0.103390795	0.005521843
SCRG1	11341	0.104524826	0.001504003
PDE5A	8654	0.108469477	0.001111892

Table III. The annotations for the GO cell functions.

Category	Term	Count	*p*-value	Fold enrichment	FDR
GOTERM_CC_FAT	extracellular region	74	6.6E-8	1.9	9.3E-5
GOTERM_CC_FAT	extracellular matrix	34	3.4E-6	2.5	4.6E-3
GOTERM_CC_FAT	proteinaceous extracellular matrix	32	5.2E-6	2.5	7.1E-3
GOTERM_MF_FAT	cytokine activity	23	1.0E-5	3	1.6E-2
GOTERM_MF_FAT	transcription factor binding	39	1.6E-4	1.9	2.5E-1
GOTERM_MF_FAT	chemokine activity	9	4.1E-4	4.9	6.2E-1
GOTERM_BP_FAT	regulation of programmed cell death	65	2.8E-7	2.0	4.9E-4
GOTERM_BP_FAT	immune response	58	2.9E-7	2.1	5.1E-4
GOTERM_BP_FAT	regulation of cell death	65	3.2E-7	1.9	5.7E-4

tokines could be found in the degenerative intervertebral disc tissues. The inflammatory cytokines involved in the synthesis and catabolism of the extracellular matrix of intervertebral discs can also induce apoptosis, and have been considered to be one of the main factors leading to disc degeneration[11]. However, the initiation of this process and the effects of these fac-

Table IV. The results of the KEGG pathway analysis.

Category	Term	Count	*p*-value	Fold enrichment	FDR
KEGG_PATHWAY	Cytokine-cytokine receptor interaction	28	1.3E-4	2.2	1.6E-1
KEGG_PATHWAY	Apoptosis	12	2.9E-3	2.8	3.4E0
KEGG_PATHWAY	NOD-like receptor signaling pathway	9	9.4E-3	3.0	1.1E1
KEGG_PATHWAY	Pantothenate and CoA biosynthesis	4	3.3E-2	5.5	3.3E1
KEGG_PATHWAY	Chemokine signaling pathway	16	3.6E-2	1.8	3.6E1

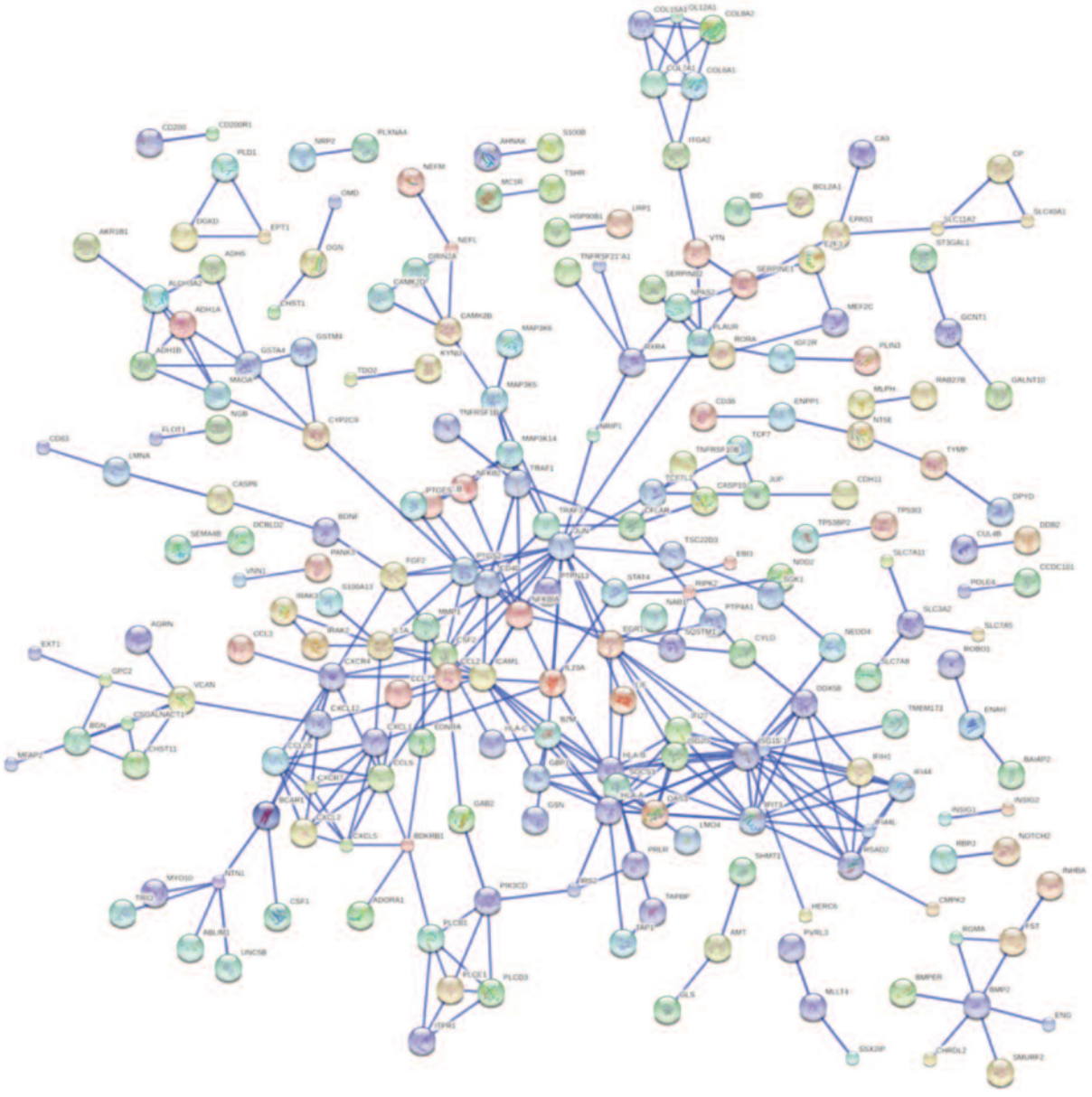

Figure 1. The protein-protein interactions.

tors on disc degeneration caused by inflammatory reactions remain unclear. *In vitro* and *in vivo* studies have demonstrated that early gene therapy may provide a possibility to delay, prevent or even reverse disc degeneration, but selecting a valid target gene and a suitable carrier

are obstacles limiting the application of this strategy[12]. Thus, the identification of the key genes associated with disc degeneration may not only reveal the molecular mechanism(s) underlying the occurrence and progression of disc degeneration, but can also provide targets for further delaying or reversing degeneration.

Early studies identified a number of molecular markers of disc degeneration. For example, Gruber et al[13] found that aspirin caused changes in gene expression in the degenerative disc cells. Zhang et al[14] found that the expression of ADAMTS-7 was significantly increased in the degenerative cartilage endplate cells compared to non-degenerative cells of the intervertebral disc. Kalb et al[15] found that inflammatory cytokines, such as IL-1, IL-6 and TNF-α, play an important role in the development of disc degeneration. Moreover, some investigators have studied the gene expression profiles of the intervertebral disc using clinically excised degenerative intervertebral disc tissue or rat intervertebral disc as the subjects of the study, seeking to identify degeneration-related genes by comparing the changes in gene expression profiles between normal and degenerative discs. Many related genetic changes associated with the occurrence of disc degeneration have been found, but the causality or intermediate links among each variant gene in terms of degeneration have remained unclear, and experts in the field have been unable to obtain useful information based on manual or experimental searches or verification. In the current age of information development and bioinformatics, how useful information about genes of interest and the meaning of the findings from mass information can be obtained has become a hot topic.

The occurrence of disc degeneration is a process in which a variety of pathological changes are produced by multiple factors on multiple genes through various pathways. In the present study, we focused on inflammation as the primary etiological factor impacting disc degeneration, and TNF-α was selected as a representative inflammatory cytokine. We therefore obtained gene expression profiling data from degenerative intervertebral disc cells with and without treatment with TNF-α. The related key genes that were affected during the disc degeneration caused by inflammatory cytokine exposure were analyzed using a bioinformatic analysis, and the functions, signaling pathways and interaction networks of these genes were studied.

The analysis showed that that there were 753 differentially expressed genes in the degenerative intervertebral disc after treatment with TNF-α, including 458 upregulated genes and 295 downregulated genes, compared to the control (unstimulated) degenerative intervertebral disc cells. The present study showed that the expression of matrix metalloproteinase-1 (MMP-1) was increased by 25.5-fold, NF-κB (nuclear factor kappa B) was increased by 9.3-fold and ADAMTS6 (a disintegrin and metalloproteinase with thrombospondin motifs) was increased by 3.57-fold. Cho et al[16] confirmed in their experiments that cytokines such as IL-1β and TNF-α can increase the expression of MMP-1 to cause the degradation of the extracellular matrix of intervertebral discs. Compared to normal disc tissues, those with activation of the NF-κB signaling pathway may exhibit activation of related transcription factors that promote the secretion of inflammatory cytokines (IL-1β, TNF-α, and IL-6) and effectors (ADAMTS, MMPs)[17]. These experimental results are consistent with the results of our analysis.

The present study found that CCL3 gene was upregulated 77.9-fold. It was also revealed from the GO and KEGG analyses that CCL3-related genes are involved in the immune and inflammatory responses, and are downstream of the MAPK, NF-κB and C/EBPβ signaling pathways. Wang et al[18] reported that inflammatory cytokines, including IL-1β and TNF-α, can regulate the expression of the CCL3 gene through the activation of the MAPK and NF-κB signaling pathways, and are involved in the disc degeneration. The CCL3 gene can promote macrophage recruitment through CCR1 to produce an inflammatory cascade, which may be a new idea to explain the role of chronic inflammatory reactions in disc degeneration.

In the downregulated genes, ANKH was downregulated by 3.2-fold. The ANHK gene encodes a transmembrane protein, and studies have shown that it can transport intracellular inorganic pyrophosphate (PPi) to the extracellular region, and the PPi concentration is known to have effects on calcification. Ho et al[19] found that, in ANKH gene-deficient mice, the joints of the limbs and spinal column gradually developed tetany, and ossification can occur in the spinal ligaments and intervertebral discs. However, after the transduction of the wild-type ANKH gene into the animals with ANKH gene mutations, the ANKH protein function could be

recovered, and the abnormal calcification of the bones and joints could be reduced or eliminated. Xu et al[20] found that degenerative cartilage endplates and calcified nodules appeared in patients with cervical osteoarthritis, and the expression of ANKH was significantly decreased in these patients, suggesting that decreased ANKH gene expression may induce calcification of the cartilage endplate to accelerate disc degeneration in humans. These findings suggest that ANKH may provide a novel target for the treatment of intervertebral disc degeneration.

Conclusions

In this study, bioinformatics methods were used to analyze the microarray data of the gene expression profiles associated with disc degeneration, and the study found that the damage response, inflammatory reactions, regulation of apoptosis and other factors are closely associated with the degeneration of the intervertebral disc. The findings also indicate that CCL3 and ANKH, in addition to other genes, may play a role in the occurrence and development of disc degeneration through their participation in the inflammatory responses, while the specific mechanism(s) require further experimental verification.

Acknowledgements

This research was supported by the Key Laboratory of Cell Differentiation and Apoptosis of National Ministry of Education Shanghai Jiao Tong University School of Medicine (SJTU-SM), Shanghai, China. We gratefully acknowledge the technical assistance provided by Xin-yi Liu.

Conflict of Interest

The authors claim no conflicts of interest in relation to this study or the publication of the paper.

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