SPANDIDOS PUBLICATIONS

MOLECULAR MEDICINE REPORTS 16: 3371-3378, 2017

Analysis of gene expression profile microarray data in complex regional pain syndrome

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Received April 6, 2016; Accepted March 16, 2017

DOI: 10.3892/mmr.2017.6950

Abstract. The aim of the present study was to predict key genes and proteins associated with complex regional pain syndrome (CRPS) using bioinformatics analysis. The gene expression profiling microarray data, GSE47603, which included peripheral blood samples from 4 patients with CRPS and 5 healthy controls, was obtained from the Gene Expression Omnibus (GEO) database. The differentially expressed genes (DEGs) in CRPS patients compared with healthy controls were identified using the GEO2R online tool. Functional enrichment analysis was then performed using The Database for Annotation Visualization and Integrated Discovery online tool. Protein-protein interaction (PPI) network analysis was subsequently performed using Search Tool for the Retrieval of Interaction Genes database and analyzed with Cytoscape software. A total of 257 DEGs were identified, including 243 upregulated genes and 14 downregulated ones. Genes in the human leukocyte antigen (HLA) family were most significantly differentially expressed. Enrichment analysis demonstrated that signaling pathways, including immune response, cell motion, adhesion and angiogenesis were associated with CRPS. PPI network analysis revealed that key genes, including early region 1A binding protein p300 (EP300), CREB-binding protein (CREBBP), signal transducer and activator of transcription (STAT)3, STAT5A and integrin α M were associated with CRPS. The results suggest that the immune response may therefore serve an important role in CRPS development. In addition, genes in the HLA family, such as HLA-DQB1 and HLA-DRB1, may present potential biomarkers for the diagnosis of CRPS. Furthermore, EP300, its paralog CREBBP, and the STAT family genes, STAT3 and STAT5 may be important in the development of CRPS.

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Key words: complex regional pain syndrome, differentially expressed genes, functional enrichment, protein-protein interaction network, module identification

Introduction

Complex regional pain syndrome (CRPS) is an uncommon chronic pain condition that develops following trauma. The features include limb pain, allodynia, hypersensitivity, motor abnormality and trophic alterations (1). Women are affected more commonly than men, and postmenopausal women demonstrate the highest risk (2). CRPS is classified into two subtypes depending on the presence of peripheral nerve injury. CRPS type I without peripheral nerve injury is more common than type II, which presents as peripheral nerve injury (3). In addition, CRPS is categorized into 'warm' or 'cold' CRPS depending on whether skin temperature is increased or decreased (4). Although CRPS is designated as an orphan disease in the general population, it affects ~4-7% patients experiencing limb fractures (5). CRPS is a burden on the healthcare system and society. A number of different therapeutic strategies have been applied to treat CRPS, including pharmacological, interventional and psychological methods; however, there evidence to support their efficiency of these methods is insufficient (5). The pathogenesis of CRPS is unclear (6), however several mechanisms have are thought to be involved, including inflammation, neurogenic inflammation and alterations of the central nerve system (6). Goebel *et al* (7) elucidated the association between inflammation, immune response and CRPS, and an autoimmune model was proposed. Oaklander *et al* (8) proposed that CRPS is associated with distal degeneration of small diameter peripheral axons. Barad *et al* (9) demonstrated that brain structure was involved in CRPS. However, little is known about how these mechanisms interact to lead to CRPS. Therefore, identifying key genes or signaling pathways involved in the development of CRPS may facilitate elucidation of the integrated mechanism of CRPS and enable the development of targeted therapies.

Genome-wide expression profiling of CPRS was recently performed by Jin *et al* (10). The authors collected and analyzed peripheral blood examples from healthy controls and patients with CRPS. Differentially expressed genes (DEGs)

were identified using microarray analysis, and out of those identified, 6 genes were selected for further confirmation by reverse transcription-quantitative polymerase chain reaction analysis. The authors focused on a significantly upregulated DEG (matrix metallopeptidase 9), thought to be associated with pain progression in CRPS. However, the gene/protein interaction networks involved in CRPS remain unknown, which is necessary to elucidate how inflammation, neurogenic inflammation and alterations of the central nervous system are involved in CRPS. Therefore, the authors of the present study constructed a protein-protein interaction network in order to identify the specific molecular interactions involved in CRPS.

In the present study, the GSE47603 gene expression profiling microarray data deposited by Jin *et al* (10) was obtained and used to identify CRPS-associated genes in patients with CRPS. The microarray data was used to identify key DEGs associated with CRPS by employing comprehensive analysis methods in order to enrich the functions and signaling pathways of identified DEGs. In addition, a protein-protein interaction (PPI) network was constructed and analyzed to identify hub genes. The aim of the current study was to identify several key genes associated with the disease, and examine their potential function in the development of CRPS by expression profile analysis. The results may facilitate the identification of potential targets for the diagnosis and treatment of CRPS.

Materials and methods

Microarray data. The GSE47603 microarray data, deposited by Jin *et al* (10) was obtained from the Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo). This microarray used the GPL10558 platform (Illumina HumanHT-12 v4 expression beadchip; Illumina, Inc., San Diego, CA, USA). A total of 9 peripheral blood samples from 4 patients with CRPS and 5 healthy controls were included in the genome-wide expression profiling array, as described previously (10). Out of the patients with CRPS, 2 patients presented with CRPS type I and 2 patients presented with CRPS type II. Patients who received medication of CRPS were included; however, those with additional neurogenic disorders caused by this medication were excluded. The control subjects did not present with infectious disease, pain disorders and had not undergone recent surgery.

Data processing. Array data export, processing and analysis was performed using the Gene Expression Module (version, 1.9.0) of Illumina GenomeStudio software (version, 2011.1; Illumina, Inc.). Data were already quantile-normalized. Significant DEGs were identified by comparing profile data from patients with CRPS with that of control subjects using the online analysis tool, GEO2R (http://www.ncbi.nlm.nih.gov/geo/geo2r/, version R 3.2.3, Biobase 2.30.0, GEOquery 2.40.0, limma 3.26.8), as was accessible from the GEO website. This tool uses the linear models for microarray analysis package to identify DEGs and provides t-statistics and P-values. Benjamini and Hochberg's false discovery rate was applied to adjust the P-values. DEGs with adjusted P-values of <0.05 and fold change (FC) values of >1.5 were considered to be significant.

Gene ontology (GO) enrichment and Kyoto encyclopedia of genes and genomes (KEGG) pathway analysis. The GO database (http://geneontology.org) is a large-scale collection of genomic data divided into 3 categories according to biological process (BP), molecular function and cellular component. KEGG (http://www.genome/ad.jp/kegg/) is a pathway-associated database for gene classification. The Database for Annotation Visualization and Integrated Discovery (DAVID, https://david.ncifcrf.gov/) integrates biological data and analysis tools to provide systematic functional annotation for a large number of genes and proteins.

To analyze the putative functions of identified DEGs, GO annotation associated with BP and KEGG pathway enrichment analyses were performed using the online DAVID tool version 6.7 (11,12). A P-value of <0.05 and gene counts of >2 were considered to be significant.

PPI construction. The Search Tool for the Retrieval of Interaction Genes (STRING; http://string.embl.de/) database collects comprehensive information regarding predicted and experimental interactions between proteins in a given cell. In the present study, DEGs were mapped in the STRING database version, 10.0 (13) to construct a PPI network, which provides an improved understanding of the functional organization of the proteome. A combined score of >4 was set as the threshold. The PPI network was subsequently analyzed using Cytoscape (http://www.cytoscape.org/, version, 3.3.0) software. The degree of connectivity of each node in the PPI network was calculated, and the hub nodes were identified. Genes were clustered using the MCODE Cytoscape plugin (version, 1.4.1).

Results

DEG analysis. Using adjusted P-values of <0.05 and FC values of >1.5, a total of 257 DEGs were identified in blood samples from patients with CRPS when compared with the controls. This included 243 upregulated and 14 downregulated genes (Tables I and II). The most significantly upregulated and downregulated genes were human leukocyte antigen (HLA)-DRB1 and HLA-DQB1, respectively, which belong to the HLA family. An additional member of the HLA family, HLA-DRB4, was significantly downregulated in CRPS samples when compared with controls (Table II).

Functional enrichment analysis. The over-represented GO-BP terms of DEGs were primarily associated with antigen processing and presentation, the immune response process, the integrin-mediated signaling pathway, cell motion and adhesion, angiogenesis, and cell-substrate junction assembly (Table III). In addition, DEGs were enriched in KEGG pathways including viral myocarditis, systemic lupus erythematosus, asthma, allograft rejection, graft-vs.-host disease, type I diabetes mellitus, intestinal immune network for immunoglobulin A production, autoimmune thyroid disease, antigen processing and presentation and hematopoietic cell lineage (Table IV).

PPI analysis. The PPI network generated using the STRING database included 155 nodes and 400 edges (Fig. 1). Topological

Table I. Significantly upregulated differentially expressed genes (n=15).

Illumina ID	Adjusted P-value	logFC	Gene symbol
ILMN_1715169	0.0000060	3.8971524	HLA-DRB1
ILMN_1738075	0.0026403	1.3041826	CMIP
ILMN_1670130	0.0015978	1.2499989	ARID3A
ILMN_1722872	0.0033398	1.1720856	MYH9
ILMN_2371169	0.0026403	1.1696512	ZYX
ILMN_1696643	0.0026403	1.0804514	TLN1
ILMN_1760027	0.0026403	0.9688758	WAS
ILMN_1811823	0.0026403	0.9556934	MED25
ILMN_1663618	0.0026403	0.9497475	STAT3
ILMN_1800425	0.0026403	0.9133604	SLC9A1
ILMN_1777906	0.0030662	0.9120812	MAP7D1
ILMN_1725534	0.0026403	0.8451986	ACTN4
ILMN_1682930	0.0026403	0.8188014	SIPA1
ILMN_2275098	0.0026403	0.7684677	DTX2
ILMN_1764788	0.0026403	0.7212105	TNFRSF1B

Benjamini and Hochberg's false discovery rate was applied to adjust the P-value. FC, fold-change.

Table II. Significantly downregulated differentially expressed genes (n=14).

Illumina ID	Adjusted P-value	logFC	Gene symbol
ILMN_1661266	0.0151182	-2.4675689	HLA-DQB1
ILMN_2356991	0.0196116	-1.2897218	CD47
ILMN_1718766	0.0086679	-0.8003821	MT1F
ILMN_3241953	0.0341073	-0.7595469	GGACT
ILMN_1741133	0.0161371	-0.7282374	NME1
ILMN_1780368	0.0251894	-0.7091328	GPR18
ILMN_1676575	0.0251784	-0.6922376	IKZF1
ILMN_1712298	0.0169227	-0.6912831	ANKRD46
ILMN_1726460	0.0136377	-0.6765224	RPL14
ILMN_1715661	0.0211196	-0.6750304	TFAM
ILMN_1771333	0.0121527	-0.6515932	CD47
ILMN_1752592	0.0493698	-0.6494842	HLA-DRB4
ILMN_2326273	0.0241474	-0.6120625	CHI3L2
ILMN_1653026	0.0196116	-0.6098733	PLAC8

Benjamini and Hochberg's false discovery rate was applied to adjust the P-value. FC, fold-change.

structure analysis performed by Cytoscape software revealed the degree of genes in the PPI network. Nodes with a higher degree are more connected with other nodes, and they are considered to contribute to the stability of the network and are designated as hub nodes. In the present study, genes with a higher degree included adenovirus early region 1A binding protein p300 (EP300), CREB-binding protein (CREBBP), signal transducer and activator of transcription (STAT)3 and STAT5A of the STAT protein family, interleukin 8 (IL8) and integrin α M (ITGAM).

A total of 3 modules with a score of >4 were identified using the MCODE plugin (Fig. 2). Cluster 1 exhibited the highest score, and DEGs, such as EP300, CREBBP, STAT3, STAT5A and ITGAM were included. Enrichment analysis demonstrated that the GO-BP terms enriched by these genes were associated with regulation of transcription, biosynthesis, hormone stimuli responses and cell differentiation (Table V). DEGs in cluster 2 primarily included ITGAM, retinoic acid receptor α, integrin subunit α 2B and DNA-directed RNA polymerase II subunit RPB1 (Fig. 2), which were enriched

Table III. Most significant GO-biological process terms enriched by differentially expressed genes (n=15).

GO ID	Biological process	Count	P-value
GO:0007229	Integrin-mediated signaling pathway	9	5.01×10^{-6}
GO:0007010	Cytoskeleton organization	20	9.05×10^{-6}
GO:0030036	Actin cytoskeleton organization	14	1.45×10^{-5}
GO:0030029	Actin filament-based process	14	2.88×10^{-5}
GO:0006955	Immune response	24	7.43×10^{-5}
GO:0006952	Defense response	22	1.12×10^{-4}
GO:0022604	Regulation of cell morphogenesis	9	4.51×10^{-4}
GO:0007242	Intracellular signaling cascade	33	4.52×10^{-4}
GO:0045321	Leukocyte activation	12	5.23×10^{-4}
GO:0045596	Negative regulation of cell differentiation	11	8.13×10^{-4}
GO:0008360	Regulation of cell shape	6	8.53×10^{-4}
GO:0001775	Cell activation	12	2.06×10^{-3}
GO:0006928	Cell motion	16	2.36×10^{-3}
GO:0035023	Regulation of Rho protein signal transduction	7	2.42×10^{-3}
GO:0051056	Regulation of small GTPase mediated signal transduction	11	2.56×10^{-3}

GO, Gene Ontology; GTP, guanosine triphosphate.

Table IV. Most significant KEGG terms enriched by differentially expressed genes (n=15).

KEGG ID	KEGG term	Count	P-value
hsa04810	Regulation of actin cytoskeleton	14	9.87×10^{-5}
hsa04670	Leukocyte transendothelial migration	9	1.13×10^{-3}
hsa04666	Fc-ε R-mediated phagocytosis	8	1.43×10^{-3}
hsa04520	Adherens junction	7	2.38×10^{-3}
hsa05221	Acute myeloid leukemia	6	3.57×10^{-3}
hsa05416	Viral myocarditis	6	8.45×10^{-3}
hsa04330	Notch signaling pathway	5	9.52×10^{-3}
hsa05200	Pathways in cancer	13	1.32×10^{-2}
hsa04142	Lysosome	7	1.78×10^{-2}
hsa04640	Hematopoietic cell lineage	6	1.84×10^{-2}
hsa04630	Jak-STAT signaling pathway	8	2.03×10^{-2}
hsa04510	Focal adhesion	9	2.66×10^{-2}
hsa05211	Renal cell carcinoma	5	3.59×10^{-2}
hsa04144	Endocytosis	8	4.57×10^{-2}
hsa04062	Chemokine signaling pathway	8	4.91×10^{-2}

KEGG, Kyoto Encyclopedia of Genes and Genomes.

in GO-BP terms associated with cell adhesion and apoptosis (Table V). DEGs in cluster 3 included C5 anaphylatoxin chemotactic receptor 1, GPR18, hydroxycarboxylic acid receptor (HCAR) 2 and HCAR3 (Fig. 2), which were enriched in the G-protein signaling pathway (Table V).

Discussion

In the present study, a total of 257 DEGs were identified in blood samples from patients with CRPS when compared with healthy controls. Pathway enrichment analysis demonstrated that the identified DEGs were primarily enriched in immune response, cell motion, adhesion and angiogenesis signaling pathways. In the PPI network, EP300 displayed the highest degree and was present in cluster 1. Additional DEGs in cluster 1 with relatively high degrees were STAT3, CREBBP, STAT5A, ITGAM, notch homolog 1, CCAAT/enhancer binding protein β and mitogen-activated protein kinase 3.

Goebel *et al* (14) demonstrated that intravenous immunoglobulin (IVIG) is effective for the reduction of pain in patients

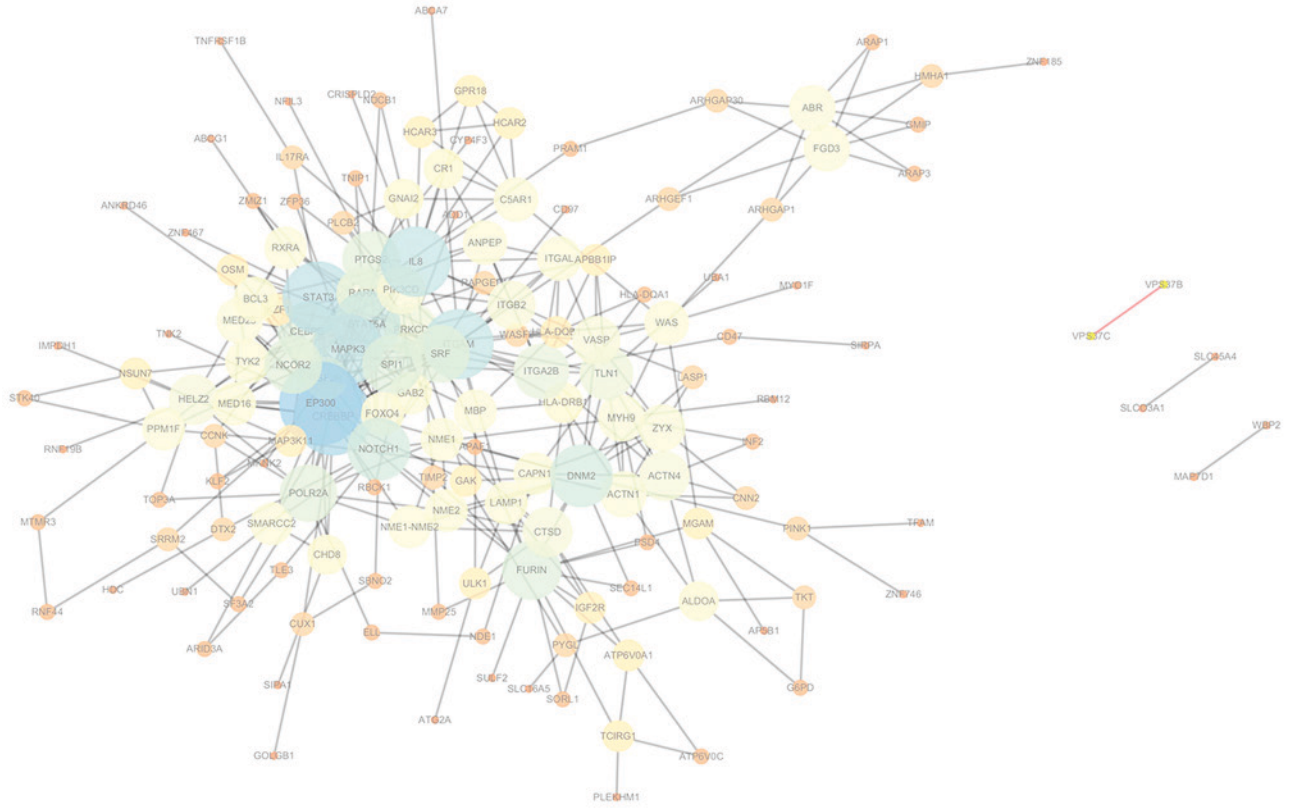

Figure 1. Protein-protein interaction network of differentially expressed genes. Degree is used to describe the number of a node's connection with other nodes. Node size and color represent the degree of proteins. Larger size with a blue color indicates nodes with larger degree, whereas a smaller size with a red color indicates a lower degree.

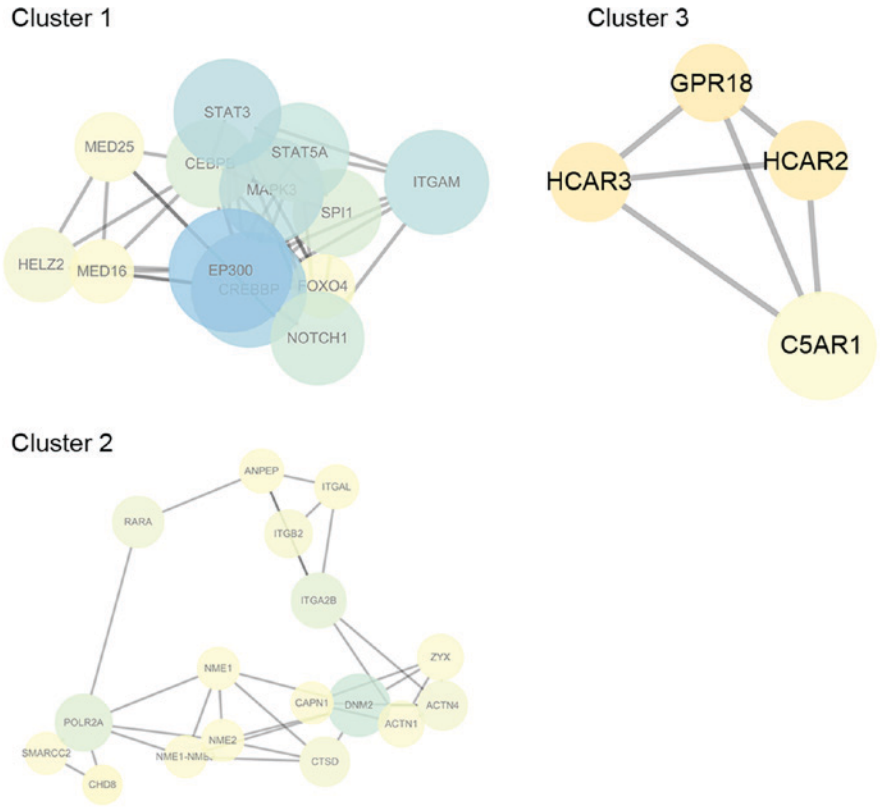

Figure 2. Significant modules in the protein-protein interaction network. Degree is used to describe the number of a node's connection with other nodes. Node size and color represent the degree of proteins. Larger size with a blue color indicates nodes with larger degree, while smaller size with a red color indicates a lower degree. The score calculated by MCODE plugin in the process of module identification to evaluate the size and density of the subgroup for clusters 1, 2 and 3 were 8.3, 4.3 and 4.0, respectively. A larger and denser module had a higher score.

Table V. Significant GO-biological process terms enriched by differentially expressed genes in clusters.

A, Cluster 1

GO ID	Biological process	Count	P-value
GO:0045941	Positive regulation of transcription	8	6.02×10^{-8}
GO:0010628	Positive regulation of gene expression	8	7.38×10^{-8}
GO:0045935	Positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	8	1.21×10^{-7}
GO:0051173	Positive regulation of nitrogen compound metabolic process	8	1.50×10^{-7}
GO:0010557	Positive regulation of macromolecule biosynthetic process	8	1.66×10^{-7}
GO:0031328	Positive regulation of cellular biosynthetic process	8	2.29×10^{-7}
GO:0009891	Positive regulation of biosynthetic process	8	2.52×10^{-7}
GO:0006357	Regulation of transcription from RNA polymerase II promoter	8	3.43×10^{-7}
GO:0045893	Positive regulation of transcription, DNA-dependent	7	7.40×10^{-7}
GO:0051254	Positive regulation of RNA metabolic process	7	7.77×10^{-7}
GO:0010604	Positive regulation of macromolecule metabolic process	8	1.05×10^{-6}
GO:0006350	Transcription	10	2.11×10^{-6}
GO:0045944	Positive regulation of transcription from RNA polymerase II promoter	6	6.09×10^{-6}
GO:0006355	Regulation of transcription, DNA-dependent	9	9.79×10^{-6}
GO:0051252	Regulation of RNA metabolic process	9	1.16×10^{-5}

B, Cluster 2

GO ID	Biological process	Count	P-value
GO:0007155	Cell adhesion	6	4.95×10^{-4}
GO:0022610	Biological adhesion	6	4.98×10^{-4}
GO:0007229	Integrin-mediated signaling pathway	3	2.31×10^{-3}
GO:0007160	Cell-matrix adhesion	3	3.70×10^{-3}
GO:0031589	Cell-substrate adhesion	3	4.47×10^{-3}
GO:0042981	Regulation of apoptosis	5	7.67×10^{-3}
GO:0043067	Regulation of programmed cell death	5	7.94×10^{-3}
GO:0010941	Regulation of cell death	5	8.05×10^{-3}
GO:0051017	Actin filament bundle formation	2	2.15×10^{-2}
GO:0007159	Leukocyte adhesion	2	2.86×10^{-2}
GO:0006338	Chromatin remodeling	2	5.64×10^{-2}
GO:0051271	Negative regulation of cell motion	2	6.33×10^{-2}
GO:0007015	Actin filament organization	2	7.20×10^{-2}

C, Cluster 3

GO ID	Biological process	Count	P-value
GO:0007186	G-protein coupled receptor protein signaling pathway	2	8.30×10^{-2}

GO, Gene Ontology.

with CRPS, which suggests that the immune response may be important in the development of CRPS. Subsequent studies indicated that neuroautoimmunity and neurogenic inflammation contribute to CRPS. Cooper *et al* (15) demonstrated that infiltrating leukocytes reacted to autoantibodies that bind to autoantigens located on the surface of neuronal and glial cell targets, and were closely associated with neuroautoimmune responses. In addition, the development of autoantibodies against the β_2 adrenergic receptor and muscarinic-2 receptor were identified in patients with CRPS (16).

HLA-DQB1, HLA-DRB4 and HLA-DRB1, which belong to class II of the HLA family, are expressed by a subgroup of immune cells, including B cells, activated T cells and macrophages (17). Kemler *et al* (18) discovered increased expression of HLA-DQB1 among CRPS patients. In addition, a previous study demonstrated that HLA is associated with CRPS whereby two members of the HLA family (HLA-DQ8 and HLA-B62) were observed to be differentially expressed in different subtypes of CRPS (19). Furthermore, genes in class I of the HLA family were observed to be associated with the development of CRPS (20). HLA-DR2 and HLA-DR13 have been reported to be associated with CRPS (21,22). However, studies in HLA-DRB4 and DRB1 have not focused on their roles in CRPS. Therefore, further research to determine how HLA may be involved in CRPS may provide additional important information. Changes in the expression levels of the HLA family indicates that CRPS may be associated with inflammation regulation (19). Additionally, CRPS may be important to genes closely associated with HLA instead of HLA genes themselves (20).

EP300 and its paralog CREBBP are transcriptional coactivators that regulate gene transcription by connecting DNA-binding and transcription factors, relaxing chromatin through its intrinsic histone acetyltransferase activity and modifying specific transcription factors (23). EP300 and CREBBP have been implicated in a large number of diseases. Lunning *et al* (24) demonstrated that mutations in EP300 and CREBBP are present in germinal center B-cell lymphomas. Kishimoto *et al* (25) demonstrated that EP300 and CREBBP may be involved in the pathogenesis of follicular lymphoma. Notably, Seltzer *et al* (26) indicated that EP300 and CREBBP were associated with postnatal microcephaly. That EP300 and CREBBP have a high degree in the PPI network found by our study may add to the myriad of roles of EP300 and CREBBP in disease.

STAT3 and STAT5 belong to the STAT protein family, which was first discovered as part of the cytokine signaling pathway (27). These proteins demonstrate a significant effect on the immune response and in the process of oncogenesis (27). STAT3A serves multiple roles in cytokine signaling pathways (27). STAT3-knock out mice were incapable of completing gastrulation (28), and conditional STAT3-knock out promotes apoptosis (29). In addition, STAT3 is involved in the differentiation of $CD4^+$ cells to Th17 cells, which protect against invading bacteria and fungi and are involved in the autoimmune response (30,31). Furthermore, STAT3 is a recognized oncogene (32). Similarly, STAT5 serves an important role in the differentiation of $CD4^+$ cells into Treg cells, which are involved in reducing the immune response and protecting against autoimmune disease (31,33). STAT5 expression deficiency was found to closely associated with tumorigenesis by changing the function of natural killer cells to tumor promotion (34). This implies that STAT may be the potential therapeutic target for CRPS (31).

In conclusion, the results of the present study provided additional evidence in support of the hypothesis that neuroautoimmunity is an important factor for the pathogenesis of CRPS. And significant genes such as HLA-DQB1, HLA-DRB4, HLA-DRB1 from the HLA family, EP300 and its paralog CREBBP, STAT3 and STAT5 from the STAT family were identified by differentially expressed gene and topological analyses, and module identification of the PPI network constructed from these genes. However, further experiments such as western blotting are required to confirm the changes of expression levels of these genes. Additionally, to fully elucidate the mechanism behind how change in the expression levels of these genes lead to CRPS, further studies are required to determine, which pathways contribute to CRPS. Due to the small sample size of the present study the current findings are limited. Therefore, pooling CRPS samples from different sources in future investigations will increase the sample size and confirm the current findings.

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