European Review for Medical and Pharmacological Sciences 2014; 18: 2152-2159

Identification crucial genes in peripheral neuropathic pain induced by spared nerve injury

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Abstract. – AIM: Peripheral neuropathic pain (PNP) is a kind of neuropathic pain caused by damage or disease that affects the peripheral nervous system. This study aimed to investigate the molecular mechanism of PNP and identify therapy targets for treating PNP in a spared nerve injury (SNI) model.

MATERIALS AND METHODS: Gene expression data with accession number of GSE18803 were downloaded from Gene Expression Omnibus (GEO). This dataset included microarray data of four kinds of rat samples (adult rats with SNI, adult rats with sham injury, neonate rats with SNI, and neonate rats with sham injury). Differentially expressed genes (DEGs) were identified by using Limma software package, and further, Gene Ontology (GO) function and pathway analysis of DEGs were performed through the DAVID online tools. Protein-protein interaction (PPI) network of DEGs were constructed by STRING online database, and co-expression networks were constructed through Cytoscape.

RESULTS: Totally 111 DEGs which were specially differentially expressed in adult rats with SNI were identified. Functional enrichment analysis suggest the majority of DEGs were related with immune functions. By comparing the three lists of genes got from GO and KEGG enrichment analysis, PPI network, and co-expression network analysis, 15 crucial genes were identified.

CONCLUSIONS: Pathological nerve pain might be associated with immune dysfunctions and the 15 crucial genes might play an important role in the development of pathological nerve pain and have potential to be therapy targets.

Key Words:

Peripheral neuropathic pain, Microarray analysis, Co-expression, Protein-protein network.

Introduction

Peripheral neuropathic pain (PNP) is a kind of neuropathic pain caused by damage or disease that affects the peripheral nervous system. It is reported that neuropathic pain can be very difficult to treat with only about 40-60% of patients achieving partial relief[1]. At present, treatment of PNP mainly relies on drug, such as corticosteroids and traditional Chinese medicine. Besides, physical therapy is also a repair method, such as the very high frequency and electrical stimulation[2]. In addition, more and more researchers pay attention to gene therapy[3] and stem cell therapy[4-5] in recent years. However, it is a hot point and difficult problem in clinical research to find the suitable therapy targets to treat PNP.

PNP attribute to multiple etiological factors that initiate a number of diverse mechanisms operating at different sites and times and expressed both within, and across different disease states[6]. Therefore, a number of laboratory animal models were established to unraveling the mechanisms, such as the Bennett chronic constriction injury model, the Seltzer partial sciatic nerve ligation model, the Chung spinal nerve ligation model and the spared nerve injury (SNI) model[6-10]. Microarray expression profiling have been used to characterize the genes with altered expression in the dorsal horn of the adult rat spinal cord following peripheral nerve injury[11-13]. Early research found that genes with differential expression were mainly concentrated in the immune system in nerve injury of rats. Costigan et al[14] found that CD2, CD3, CD68 and interferon-γ (IFNγ) were upregulated in the dorsal horn after nerve injury in the adult rats. Weimin et al[15-16] studied the changes of gene expression after SNI of rats, and found the signal regulatory network in early Wallerian degeneration. Although, there are many reports use microarray to study the gene expression after nerve injury, the pathophysiological mechanism of SNI is still not fully clear.

Researchers found that, sciatic nerve injury of adult rats can cause pathological nerve pain, but of neonate rats cannot[17,18]. In this study, we downloaded microarray dataset of SNI samples

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of adult rats and neonate rats and performed a series of bioinformatics analyses to investigate the molecular mechanism of PNP. A number of crucial genes that might play important roles in development of PNP were identified. These genes might provide targets for treatment of pathological nerve pain caused by SNI.

Materials and Methods

Microarray Data

Transcriptional profile of GSE18803 was downloaded from Gene Expression Omnibus (GEO) which was based on the platform of Affymetrix Rat Expression 230A Array. This dataset was deposited by Costigan et al[14]. In their study, adult male and 10 d old neonatal rats performed SNI surgery where the tibial and common peroneal branches of the sciatic nerve were ligated. Besides, the sciatic nerve was exposed but not ligated in sham-operated controls. Seven days later, total RNA of the L4 and L5 lumbar dorsal horns ipsilateral to injury were hybridized to the Affymetrix Rat $RAE_{230}A$ chip. For each condition, six biologically independent hybridizations were performed. Therefore, a total of 24 microarrays were obtained from GEO database, including 12 microarrays from adult rats (n=6 in SNI group and n=6 in sham-control group) and 12 microarray from neonate rats (n=6 in SNI group and n=6 in sham-control group).

Data Preprocessing

The original microarray data in CEL format was downloaded from GEO database, and read through the Affy package in R[19] and the microarray refSeq annotation file. The data were normalized according to Robust Multiarray Averaging (RMA) method and, then, expression level of mRNA was calculated. A total of 11190 mRNA expression levels were obtained through the above steps. Afterwards, the mRNAs that didn't express in all samples were removed according to the function of mas5calls in Affy package. Finally, expression levels of 8642 mRNA were obtained.

Selection of Differentially Expressed Genes

The dataset consists of four kinds of samples, adult rats with SNI, adult rats with sham injury, neonate rats with SNI, and neonate rats with sham injury. Differentially expressed genes (DEGs) between adult rats with SNI and adult rats with sham operation, as well as between neonate rats with SNI and neonate rats with sham operation, were identified by using Limma package[20], respectively. The p-value ≤ 0.05 and $|\log_2 \text{fold change}| > 0.41$ (i.e. the quotient of mRNA expression level between SNI samples and sham injured samples was less than 0.75 or greater than 1.33) were selected as cut-off criteria.

Functional Annotation and Pathway Analysis of DEGs

Database for Annotation, Visualization, and Integrated Discovery (DAVID) is a web-accessible program that integrates functional genomic annotations with intuitive graphical summaries. Lists of gene or protein identifiers are rapidly annotated and summarized according to shared categorical data for Gene Ontology (GO), protein domain, and biochemical pathway membership[21]. In order to make a thorough analysis of related pathway or biological processes in PNP which induced by SNI on the function level, GO and Pathway enrichment analysis on genes that were upregulated in adult rats with SNI were carried out through the DAVID online tools with threshold of p-value ≤ 0.05.

Analysis of Protein Interaction Networks

The Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database (http://string-db.org/) provides a global view of all the available interaction data, including lower-quality data and/or computational predictions. According to the relationship between known and predicted, it covered more than 1100 organisms in comprehensive protein networks[22]. In this study, protein-protein interaction (PPI) network of DEGs were constructed by STRING online database. The hub protein was selected according to the node degree, and the network were visualized by Cytoscape[23].

Co-expression Network Analysis

Gene co-expression network could comprehensively capture the relationships of individual genes perturbed by environments, and provide a powerful tool to gain new insights into the function of genes and mechanism of complex diseases[24-25]. The Pearson correlation coefficient (PCC) between DEGs were calculated. Then, gene pairs with PCC ≥ 0.9 were selected and constructed co-expression network through Cytoscape.

Results

Analysis of DEGs

By comparing microarray data of adult rats with SNI with that with sham operation, a total of 135 DEGs (1.5% of total) were identified, including 134 up-regulated genes and 1 down-regulated gene. Similarly, a total of 24 DEGs (0.26% of total) were identified to be up-regulated in neonate rats with SNI. Further comparison found that these 24 DEGs were included in the 135 DEGs of adult rats, i.e. these 24 genes were differentially expressed in both neonate and adult rats. Since SNI in neonate rats wouldn't cause pathological nerve pain, suggesting these 24 genes might be differentially expressed due merely to the natural aging process, and not closely related to SNI. The remaining 111 DEGs in adult rat with SNI were likely to be related with pathological nerve pain. Thus, the subsequent analysis was based on these 111 DEGs.

Function and Pathway Enrichment Analysis

The gene list of these 111 DEGs was submitted to the DAVID web site to carry out GO analysis under $p \leq 0.05$. The top 10 enriched GO terms were listed in Table I and we could find that most of them were related with immune system (6/10), such as immune response (20.5% DEGs were enriched with p = 3.64E-10), defense response (15.66%; p = 1.18E-06), leukocyte activation (12.05%; p = 4.79E-06), positive regulation of immune system process (12.05%; p = 2.3E-05). Besides of immune system, GO terms associated with cell activation were also found being perturbed, such as cell activation (15.66%; p = 1.73E-08), regulation of cell activation (10.84%; p = 1.62E-05). KEGG pathway analysis showed the similar results (Table II), with the most significant KEGG term of natural killer (NK) cell mediated cytotoxicity (9.64%; p = 2.86E-05). In addition, Fc gamma R-mediated phagocytosis (8.43%; p = 6.99E-04), Cytokine-cytokine receptor interaction (8.43%; p = 0.0015). There were 15 overlapped DEGs in the 9 KEGG pathways and 10 GO enriched terms, which might be related to pathological nerve pain caused by SNI: *CXCL13, LCP2, VAV1, PTPRC, FCGR2B, FCGR3A, CX3CR1, PYCARD, IL18, RAC2, IFI30, INPP5D, CCL2, PTPN6,* and *STAT3*.

Analysis of PPI Network

Firstly, the 111 DEGs were submitted to the STRING website to get the PPI data. Then, the PPIs with score lager than 0.4 were selected to construct PPI networks. A total of 103 pairs of PPI, containing 41 DEGs were extracted from STRING. By visualizing these interactions, these genes were mainly distributed in one PPI network (Figure 1). The top 20 degree hub nodes in the PPI network were as follows: *RAC2, CD4, CD68, PTPRC, CCL2, IL18, PTPN6, ARPC1B, STAT3, CD48, LCP2, CX3CR1, FCGR3A, IFI30, INPP5D, PLEK, PYCARD, ARHGDIB, CORO1A,* and *CYBA*. These genes (proteins) might play an important role in the progression of pathological nerve pain.

Analysis of co-expression Network

A total of 699 pairs of co-expression, involving 87 DEGs were filtered (Figure 2). The top 20 degree genes of co-expression network were as follows: *PTPRC, MX1, FCGR3A, UGT1A6, LY86, GRN, CP, TSPO, CD68, SKAP2, CD53,*

Table I. The top 10 enriched GO terms of functional enrichment analysis.

Category	Term	Count	%	*p* value
GOTERM_BP_FAT	GO:0006955~immune response	17	20.48192771	$3.64E^{-10}$
GOTERM_BP_FAT	GO:0001775~cell activation	13	15.6626506	$1.73E^{-08}$
GOTERM_BP_FAT	GO:0006952~defense response	13	15.6626506	$1.18E^{-06}$
GOTERM_BP_FAT	GO:0009611~response to wounding	15	18.07228916	$1.79E^{-06}$
GOTERM_BP_FAT	GO:0045321~leukocyte activation	10	12.04819277	$4.79E^{-06}$
GOTERM_BP_FAT	GO:0050865~regulation of cell activation	9	10.84337349	$1.62E^{-05}$
GOTERM_BP_FAT	GO:0002684~positive regulation of immune system process	10	12.04819277	$2.30E^{-05}$
GOTERM_BP_FAT	GO:0042127~regulation of cell proliferation	16	19.27710843	$8.45E^{-05}$
GOTERM_BP_FAT	GO:0002920~regulation of humoral immune response	4	4.819277108	$1.15E^{-04}$
GOTERM_BP_FAT	GO:0050867~positive regulation of cell activation	7	8.43373494	$2.01E^{-04}$

Table II. KEGG pathway enrichment analysis of differentially expressed genes.

Category	Term	Count	%	*p* value
KEGG_ pathway	rno04650: Natural killer cell mediated cytotoxicity	8	9.638554217	2.86E^{-05}
KEGG_ pathway	rno04666: Fc gamma R-mediated phagocytosis	7	8.43373494	6.99E^{-04}
KEGG_ pathway	rno04060: Cytokine-cytokine receptor interaction	7	8.43373494	0.001514089
KEGG_ pathway	rno04662: B cell receptor signaling pathway	5	6.024096386	0.006115019
KEGG_pathway	rno04062: Chemokine signaling pathway	7	8.43373494	0.006778761
KEGG_ pathway	rno04660: T cell receptor signaling pathway	5	6.024096386	0.017899748
KEGG_ pathway	rno04612: Antigen processing and presentation	4	4.819277108	0.02461267
KEGG_ pathway	rno04664: Fc epsilon RI signaling pathway	4	4.819277108	0.043229769
KEGG_ pathway	rno04142: Lysosome	5	6.024096386	0.044248675

BIN2, SERPINB1A, APOBEC1, CXCL13, CD4, UGT1A1, UGT1A2, UGT1A3, and UGT1A5.

By comparing the three lists of DEGs in GO_KEGG enrichment analysis, PPI network, and co-expression network analysis (Figure 3), we found that, 2 genes existed in all three lists (*PTPRC* and *FCGR3A*), 10 DEGs appeared in both PPI network and GO_KEGG enrichment analysis (*RAC2, CCL2, IL18, PTPN6, STAT3, LCP2, CX3CR1, IFI30, INPP5D,* and *PYCARD*), 2 DEGs emerged in both PPI network and co-expression network (*CD68* and *CD4*), and 1 DEGs

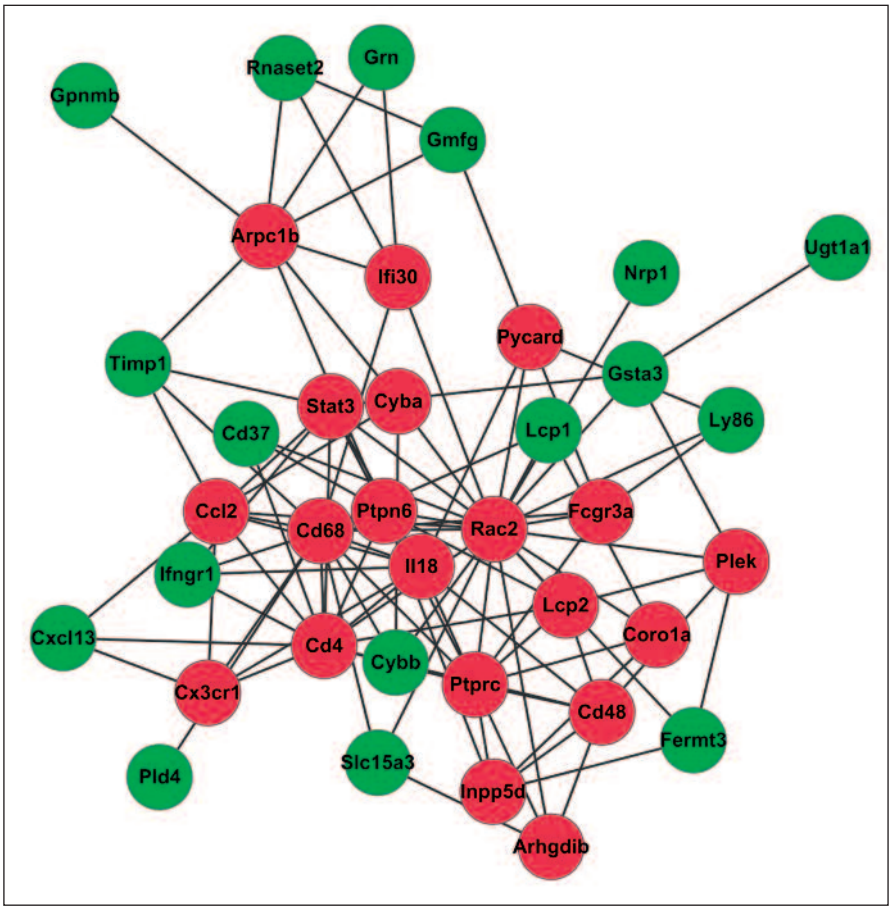

Figure 1. Protein-protein interaction network diagram of DEGs. Nodes in red color represent the top 20 degree genes; Nodes in green color represent the other genes.

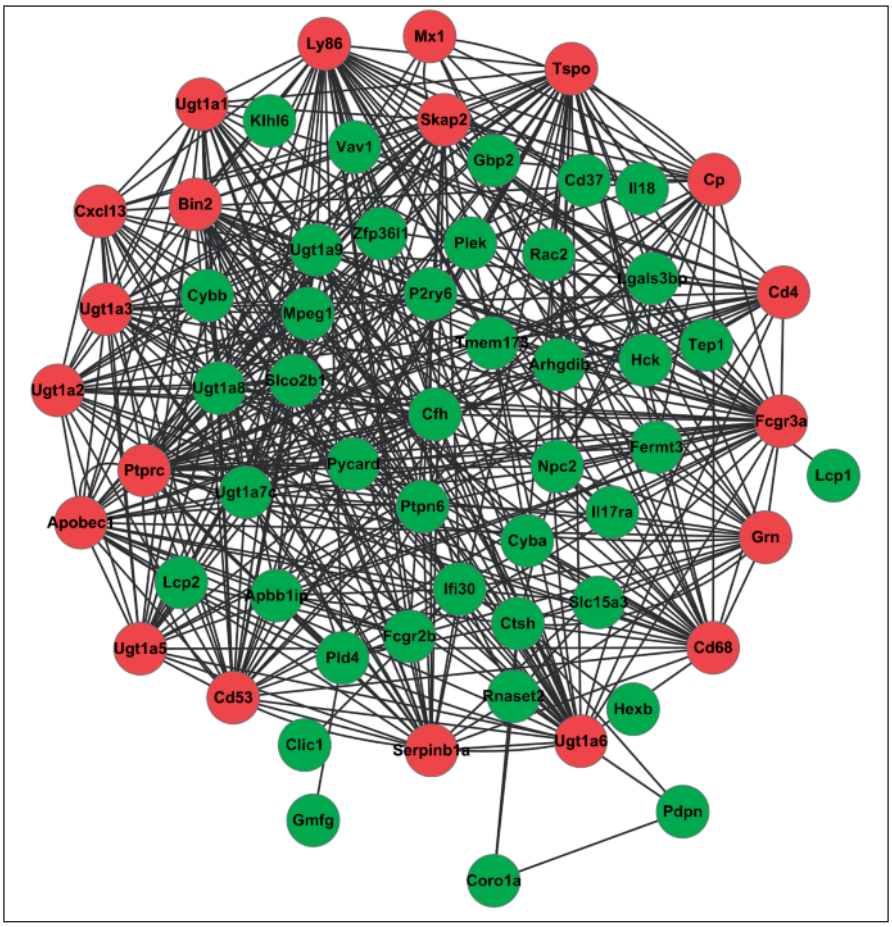

Figure 2. Co-expression network diagram of DEGs. Nodes in red color represent the top 20 degree genes; Nodes in green color represent the other genes.

shared by co-expression network and GO and KEGG enrichment analysis (*CXCL13*). These overlapped DEGs might play more important roles in causing of pathological nerve pain.

Discussion

Researchers has showed that sciatic nerve injury of adult rats would cause pathological nerve pain, but of neonate wouldn't[17-18]. Study on DEGs between adult rats and neonate with SNI might be helpful on finding related genes of pathological nerve pain caused by SNI. In this study, we downloaded the transcriptional profile of GSE18803 from GEO, and identified 111 DEGs particularly expressed in adult rats with SNI. We hypothesized that they were likely to be related with pathological nerve pain. GO and KEGG enrichment analysis, PPI network analysis, and co-expression network analysis were carried out to the 111 DEGs, and some interesting results were discovered.

By performing GO enrichment analysis, the DEGs were found to be related with GO terms of immune response. KEGG pathway analysis showed the similar results (Tables I and II). Our results were consistent with Costigan et al[14], and suggest that infiltration and signal transmission of T cells in the dorsal spinal cord of adult rats is one of the main reasons to cause pathological nerve pain. Besides, enrichment of DEGs were also found in other immune related pathways, such as B cell receptor signaling pathway and natural killer cell mediated cytotoxicity. NK cells are part of the innate immune system, and they act like cytotoxic T-cells to destroy any cell not displaying a MHC class I tag. The involvement of T cells in PNP was proposed initially after the identification of both NK cells and T cells at the site of nerve injury in several rodent models[26].

At last, 15 genes were found overlapped in two or three gene lists in this study, and these overlapping genes involve immune and inflammatory responses, macrophage cell activation, antigen processing presenting, migration of neu-

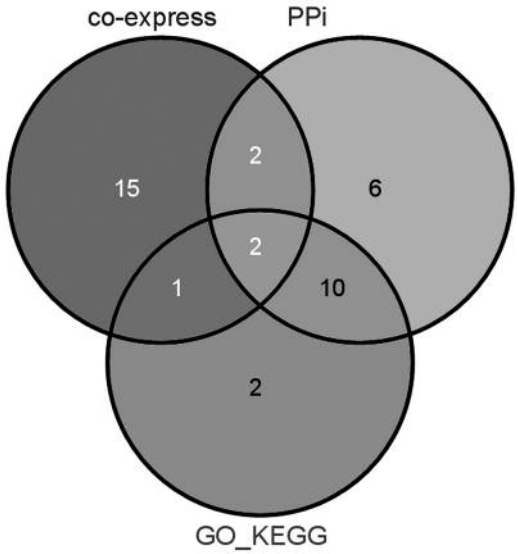

Figure 3. Venn diagram of the three lists of genes.

rons cells. Cytokine-cytokine receptor interaction, and chemokine signaling pathway were also significantly enriched. Several proinflammatory cytokines and chemokines have been implicated in altered nociceptive processing[27]. Research showed that interferon-γ (IFNγ) signaling is required for full expression of adult neuropathic hypersensitivity[14]. Chemokine (C-C motif) ligand 2 (CCL2) which is upregulated by IFNγ involves in IFNγ signaling pathway. Expression of CCL2 in neurons surface and its combination with receptor as a kind of immune activation mechanism may be involved in the regulation of sensitivity of pain signal transduction under pathological nerve pain. CX3CR1 and CXCL13 were also involved in pain transduction pathway[28-31].

We found *PTPRC* was differentially expressed in all three gene lists, indicating they may play important role in the causing of pathological nerve pain after SNI. Protein tyrosine phosphatase, receptor type, C (PTPRC) is a member of the protein tyrosine phosphatase (PTP) family. It is the leukocyte common antigen located in white blood cell surface. It is mainly involved in the regulation of signal transduction, development of lymphocyte, and activation of T cell[32]. Activation of T cells was thought to be an important reason for pathological pain after SNI. Another gene overlapped in all three gene lists was FCGR3A (Fc gamma receptor III A). FCGR3A is expressed on NK cells as an integral membrane glycoprotein anchored through a transmembrane peptide. FCGR3A polymorphisms affect the affinity of macrophages and NK cells to bind IgG1. Differential expression of FCGR3A was also observed in a previous study which was involved in peripheral nerve injury[33]. Pathological nerve pain is also related with some cytokines such as IL-18, which is a new pro-inflammatory cytokine with complex multi-functions. Through IL-1 and TNF-α gene expression and synthesis which were induced by IL-18 receptor complex, it can enhance the Th1 cells and NK cells express Fas ligand to mediate cytotoxicity[34]. Low concentration of TNF-α can induce apoptosis including polymorphonuclear neutrophils (PMN), and concentration which is over a certain threshold can inhibit neutrophil apoptosis. Studies showed that neutrophil apoptosis delay or disorder was related with inflammatory reaction and the secondary tissue damage[35]. Apoptosis of neutrophils decrease will lead to excessive local tissue damage.

Conclusions

In this study, a total of 111 DEGs were identified to be related with pathological nerve pain induced by SNI. Among them, 15 crucial genes were further identified by comparing GO and KEGG enrichment analysis, PPI network, and co-expression network analysis. These genes might play an important role in the development of pathological nerve pain and might be served as therapy targets.

Acknowledgements

This study was supported by grant from Traditional Chinese Medicine Research Project of Chongqing Health Bureau (2009-1-10).

Disclosure Statement

The authors have no conflicts of interest to declare.

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