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Original Article

Expression profiling of spinal genes in peripheral neuropathy model rats with type 2 diabetes mellitus

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Abstract: Pre-clinical diabetic peripheral neuropathy models mimicking the human condition are essential to elucidate the underlying mechanisms. Type 2 diabetic patients coping with peripheral neuropathy experience chronic pain. Spinal genes monitoring is thus required to clarify diabetic peripheral neuropathy mechanisms and refine treatments. Thus, in this study, we investigated the differentially expressed genes in the cervical spinal cord from diabetic neuropathic pain model (D group) and vehicle control (C Group) mice. Results from gene microarrays showed that 35 genes were significantly altered in cervical spinal cord of D group compared with C group. Among them, 25 genes were significantly up-regulated while the other 9 down-regulated. These differentially expressed genes were involved in inflammatory signaling, ion transport, protein phosphorylation, sensory perception, cell survival synaptic transmission, and synergistic regulation. These findings suggest that aberrant expressed genes may become new targets to study the pathogenesis of diabetic peripheral neuropathy and deserve further investigation for therapeutic interventions following pain modulation.

Keywords: Diabetic peripheral neuropathy, gene expression, spinal cord, pain modulation, microarray, rat

Introduction

The majority of patients with type 2 diabetes mellitus experience chronic peripheral neuropathy, which affects their quality of life [1-3]. It has been demonstrated that diabetic peripheral neuropathy with a high percentage experiencing dysfunction and aberrant pain involves in anatomical and pathophysiological changes in the nervous system [4]. As the combination of neuropathic and inflammatory pain [5-10], diabetic neuropathy's unique mechanical and neurochemical characteristics make it difficult for its therapeutics [11, 12]. The mechanisms that generate diabetic peripheral neuropathy are poorly understood, and currently available treatment for diabetic peripheral neuropathy is lacking despite its clinical importance.

Animal models mimicking the human condition with diabetic mellitus are required to respond to clinical realities in an attempt to elucidate the underlying mechanisms responsible for diabetic neuropathy. Rat models of painful diabetic neuropathy were developed to characterize systemic glucose changes or neural plasticity of CNS [13]. Nakajima et al reported that high-fat and high-sucrose (HF/HS) diets induced glucose intolerance and obesity [14]. It has been demonstrated that streptozotocin (STZ)-induced diabetic painful neuropathy is involved in nerve damage, neuropathic allodynia and hyperalgesia [13, 15]. It is known that the rat represents the most studied species in noxious stimuli paradigms [16, 17]. We therefore selected the combination of HF/HS diets and STZ injection to establish diabetic peripheral neuropathy rat model.

Identifying spinal gene expression patterns under normal and diabetic peripheral neuropathy condition is essential to understand spinal genetic and molecular mechanisms during the development of diabetic peripheral neuropathy. Several studies suggest that spinal gene expression profiling is involved in many pain states, e.g., spinal cord injury [18-20], paclitaxel-induced neuropathy [21], experimental neu-

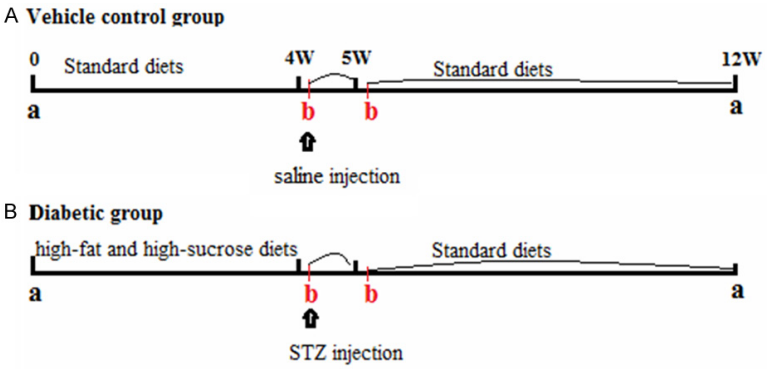

Figure 1. Experimental design of this study. A. Representing the assessment of mechanical pain sensitivity; B. Representing the collection of blood samples from the tail vein. Behavioral tests were performed by an experimenter who was blinded to the contents of the diets and injection.

rogenic bladder dysfunction [22]. Emerging evidence indicates that changes in spinal gene expression profiling including specific genes and several transcription factors contribute to pathophysiological alterations of secondary injury cascade following the genesis of pain [18, 23, 24]. However, there is no report whether the gene expression profiling in the spinal cord were affected during the development of diabetic peripheral neuropathy to date. Therefore, in the present study, we investigated the spinal gene expression profiling in rats with diabetic peripheral neuropathy using microarray analysis.

Materials and methods

Animals care and use

SPF grade adult male Sprague-Dawley rats (180-220 g, 6-8 weeks of age) were used. Animals were housed in separated cages and the room was kept at controlled temperature (23 ± 1°C) and 50-60% humidity, under a 12-h light/12-h dark cycle and with free access to food and water *ad libitum*. All protocols of this study were approved by the Local Animal Care Committee and all experimental procedures were carried out in accordance with the guidelines of the National Institutes of Health on animal care and the ethical guidelines for investigation of experimental pain in conscious animal [25].

Diabetic neuropathic pain model and blood collection

Experimental design of this study was **Figure 1**. The rats were divided into two groups: vehicle control group (C group, n = 6) and diabetic group (D group, n = 6). Standard diets *ad libitum* were given to vehicle control groups, while high-fat and high-sucrose diets were given to diabetic groups of animals for a period of 4 weeks. Diets in diabetic groups were as described previously [26]. After 4 weeks, all rats had free access to water not diet for 12 h, and blood samples (120 μl) from the tail vein of two groups were collected. Following blood collection, diabetic groups were induced using a single intraperitoneal injection of STZ (Sigma Aldrich, St. Louis, MO, USA), 40 mg/kg body weight [15, 27, 28], whereas the animals in the control group were given only saline. STZ was dissolved in 3 mM citrate buffer (pH 4.5) immediately before injection. 1 week after STZ injection, blood samples from the tail vein were collected. Rats with blood glucose levels above 11.1 mmol/L were considered diabetic mellitus and used in this study. Standard diets were given to diabetic groups of animals for 8 weeks following STZ injection to allow for the development of neuropathic changes in diabetic rat [27, 29].

Behavioral tests by the assessment of mechanical pain sensitivity

The assessment of mechanical pain sensitivity was evaluated with a paw pressure analgesy meter (LE7306, Panlab Harvard) on the surface of the front paw as described previously [30, 31]. The marker of mechanical pain sensitivity was indicated by lifting of the paw or vocalized. The paw withdrawal threshold was determined using the up-down testing paradigm. The mechanical pain testing was duplicated at 10 min intervals in each paw and performed by an experimenter who was blinded to the injection.

RNA extraction and microarray procedures

After final behavioral test, rats were anesthetized with a mixture of ketamine and xylazine and decapitated, and total RNA of rat cervical spinal cord (C5-C8) was rapidly dissected and isolated according to the manufacturer's protocol [20]. RNA quantity was determined by TRIzol® reagent (Invitrogen, Carlsbad CA) and

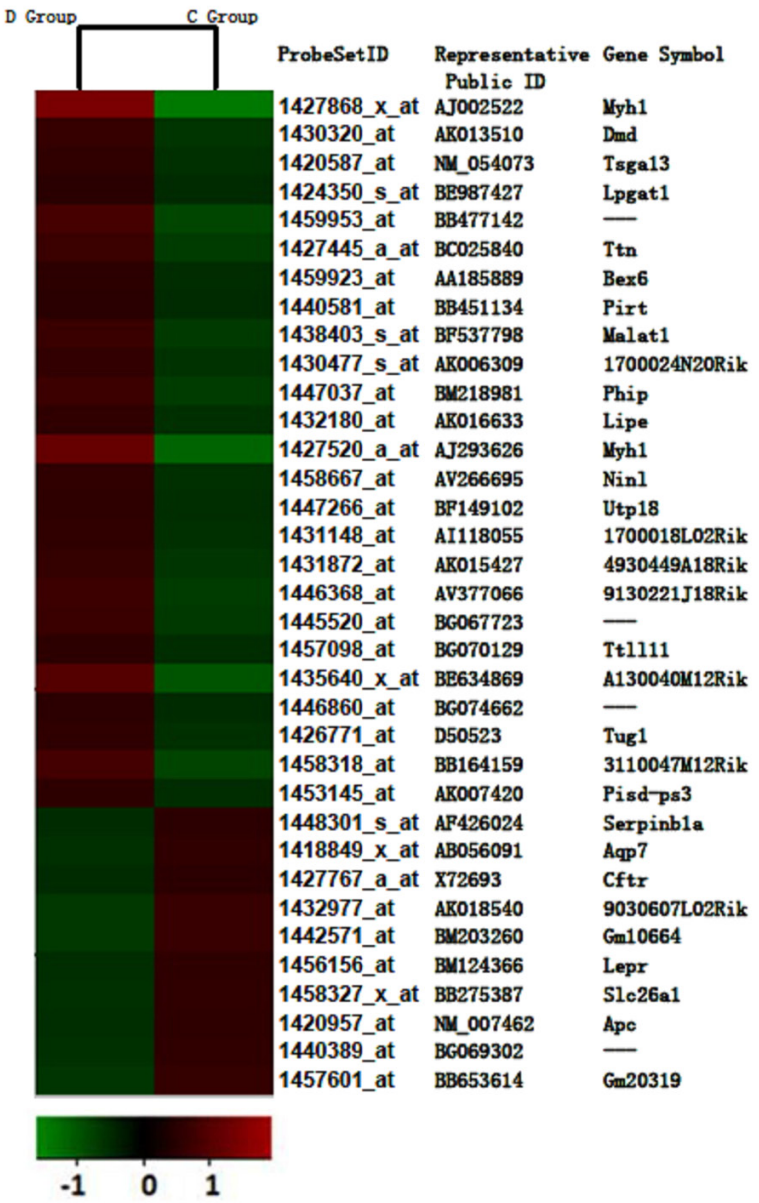

Figure 2. Map showed significant expressional changes of 35 genes in peripheral neuropathy model rats with type 2 diabetes mellitus (D group, n = 6) as compared with vehicle control (C group, n = 6).

RNA integrity was verified by gel electrophoresis. RNA samples were performed by Ambion mirVana miRNA Isolation Kit for purity and concentration. Gene expression profiling was performed using the Affymetrix Mouse Genome 430 2.0 Array platform (CapitalBio, Beijing, China) as described previously [32].

Statistical analysis

All data are presented as mean ± standard error (SE). Statistical significance was determined using the Student's *t* test. Values of $P < 0.05$ were considered to be statistically significant.

Results

Changes in blood glucose level and mechanical pain sensitivity in rats

After high-fat and high-sucrose diets for 4 weeks, diabetic groups of animals showed the signs of obesity including weight gain. 1 week following STZ injection, rats in D group exhibited significantly increased blood glucose level (25.60 ± 4.11 mmol/L) when compared to C group (4.30 ± 0.47 mmol/L; $P < 0.01$). Within 8 weeks after STZ injection, rats showed weight loss and polydipsia in D group.

Mechanical withdrawal threshold were found to be 79 ± 28.69 g and 41 ± 20.82 g in vehicle control and diabetic rat, respectively, which were significantly different from each other ($P < 0.05$, n = 6), suggesting that mechanical withdrawal threshold developed in diabetic rat by mechanical pain sensitivity test.

Spinal expression profiling in rat with diabetic peripheral neuropathy

The 35 differentially expressed genes were converted into a map file to show distinguishable gene expression profiling samples (**Figure 2**). Results from spinal gene microarrays showed that 35 genes were significantly altered in cervical spinal cord of D group ($P < 0.05$) compared with C group. Among them, 25 genes were significantly up-regulated while the other 10 down-regulated.

To functionally investigate a possible link between the changes of spinal gene expression patterns and the development of diabetic peripheral neuropathy, the relative gene expres-

Table 1. List of genes which were differentially expressed in C and D group

Representative Public ID	Ratio	Gene Symbol	Gene Title	Molecular Function
NM_054073	2.1822↑	Tsga13	Testis specific gene A13	Most types of human carcinoma tissues displayed reduced expression of TSGA13 [33]
BE987427	2.0097↑	Lpgat1	Lysophosphatidylglycerol acyltransferase 1	An endoplasmic reticulum-associated lysophosphatidylglycerol acyltransferase [34]
D50523	2.2174↑	Tug1	Taurine upregulated gene 1	A growth regulator [35]
BC025840	2.6287↑	Ttn	Titin	A critical determinant of myofibril elasticity and sarcomere structure [36]
AJ293626	5.3097↑	Myh1	Myosin, heavy polypeptide 1, skeletal muscle, adult	DNA repair and DNA damage-induced checkpoint activation [37]
AJ002522	7.3411↑	Myh1	Myosin, heavy polypeptide 1, skeletal muscle, adult	DNA repair and DNA damage-induced checkpoint activation [37]
AK013510	2.403↑	Dmd	Dystrophin, muscular dystrophy	The dystrophin gene [38, 39]
BF537798	2.617↑	Malat1	Metastasis associated lung adenocarcinoma transcript 1 (non-coding RNA)	Cell grow, tumor metastasis [40, 41]
AA185889	2.0916↑	Bex3	Brain expressed gene 3	Neuronal development [42, 43]
AK006309	2.2967↑	1700024N20Rik	RIKEN cDNA 1700024N20Rik gene	
AI118055	2.2053	1700018L02Rik	RIKEN cDNA 1700018L02 gene	
AK015427	2.3672↑	4930449A18Rik	RIKEN cDNA 4930449A18 gene	
AK016633	2.2121↑	Lipe	Lipase, hormone sensitive	The regulation of gene expression and steroid hormone synthesis [44]
BE634869	3.9919↑	A130040M12Rik	RIKEN cDNA A130040M12 gene	
BB335888	2.0099↑	Scai	Suppressor of cancer cell invasion	Cell migration and invasion [45, 46]
BG067723	2.5656↑	---	---	
AV377066	2.7021↑	9130221J18Rik	RIKEN cDNA 9130221J18 gene	
BG074662	2.033↑	---	---	
BM218981	2.6962↑	Phip	Pleckstrin homology domain interacting protein	Cell migration and invasion [47]
BF149102	2.1519↑	Utp18	UTP18, small subunit (SSU) processome component, homolog (yeast)	Ribosome synthesis [48]
AK007420	2.1012↑	Pisd-ps3	Phosphatidylserine decarboxylase, pseudogene 3	
BG070129	2.1236↑	Ttll11	Tubulin tyrosine ligase-like family, member 11	Posttranslational modification and chromosome ploidy [49]
BB164159	3.0798↑	3110047M12Rik	RIKEN cDNA 3110047M12 gene	
AV266695	2.185↑	Ninl	ninein-like	Cell cycle and protein degradation [50]
BB477142	3.1207↑	---	---	
AB056091	0.4564↓	Aqp7	Aquaporin 7	Water channel expression and water/solute homeostasis [51]
NM_007462	0.4663↓	Apc	Adenomatosis polyposis coli	Genetic pathway [52]
X72693	0.492↓	Cftr	Cystic fibrosis transmembrane conductance regulator	Chloride-channel activity [53, 54]
AF426024	0.4927↓	Serpinb1a	Serine (or cysteine) peptidase inhibitor, clade B, member 1a	Cell survival and synergistic regulation [55, 56]
AK018540	0.3973↓	9030607L02Rik	RIKEN cDNA 9030607L02 gene	
BG069302	0.4607↓	---	---	
BM203260	0.3933↓	Gm10664	Predicted gene 10664	
BM124366	0.4669↓	Lepr	Leptin receptor	Nociceptive behavior and energy homeostasis and glucose metabolism [57-59]
BB653614	0.425↓	Gm20319	Predicted gene, 20319	
BB275387	0.4584↓	Slc26a1	Solute carrier family 26 (sulfate transporter), member 1	Encoding the sulfate anion transporter 1 (SAT1) protein [60, 61]

Ratio: indicating the fold change revealed by microarray analysis; ↑ and ↓ indicating the up- and down-regulation of gene expression.

sion of spinal cord between control and diabetic rat was analyzed using microarrays. The microarray based experiments identified 25 up-regulated and 10 down-regulated genes at least 2.0-fold in diabetic samples (shown in **Table 1**).

Compared to C group, expression in D group was increased on average by 2.0- to 7.3-fold, but decreased by 0.05-to 0.49-fold (shown in **Table 1**). The *P* values for these 35 genes were less than 0.05 in spinal tissue of D group compared with control tissue of C group. Based on their biological function, these differentially expressed genes were involved in inflammatory signaling, ion transport, protein phosphorylation, sensory perception, cell survival synaptic transmission, and synergistic regulation (**Table 1**).

Discussion

In the present study, we used the combination of HF/HS diets and STZ injection to duplicate the rat experimental model of diabetic peripheral neuropathy. The blood glucose concentrations of diabetic rat significantly increased after HF/HS diets and STZ injection. Furthermore, compared with control rats, the paw withdrawal thresholds in diabetic rats were significantly reduced. These founding confirm that we successfully establish diabetic peripheral neuropathy rat model.

Emerging evidence for the involvement of leptin receptor, energy homeostasis and glucose metabolism in pain perception has established [58, 62-69]. The result of our study revealed that leptin receptor was involved in the pain perception in diabetic peripheral neuropathy, and we found that three genes which were related to energy homeostasis and glucose metabolism were regulated in D group. Several lines of evidence show that leptin signaling may be involves in nociceptive behavior induced by nerve injury [58, 62]. Maeda et al showed that partial sciatic nerve ligation (PSL) increased leptin expression in adipocytes, and macrophages recruited to the perineurium of the injured sciatic nerve expressed the leptin receptor [70], suggesting that leptin associated with primary afferent neurons may be linked to the development of neuropathic pain through adipokine secretion. Our results revealed leptin receptor downregulation in spinal cord of rats with diabetes mellitus, suggesting that there exists a critical role for spinal leptin receptor in the pathogenesis of diabetic peripheral neuropathy.

Our findings suggested that these differentially expressed genes were involved in inflammatory signaling, cell survival synaptic transmission, and synergistic regulation. Traurig et al reported that LPGAT1 belongs to a large family of acyltransferases, which are involved in a variety of biological processes including pathways that regulate energy homeostasis and body weight [71]. Huang et al reported that taurine up-regulated gene 1 (TUG1), a 7.1-kb lncRNA, recruiting and binding to polycomb repressive complex 2 (PRC2), is found to be disregulated in non-small cell lung carcinoma (NSCLC) and esophageal squamous cell carcinoma (ESCC) [35]. Okugawa et al indicated that the long non-coding RNAs (lncRNAs) metastasis-associated lung adenocarcinoma transcript 1 (Malat1) served as an important role in tumor development and progression [40, 72]. Cadar reported that Titin (Tin) is the largest known protein and a critical determinant of myofibril elasticity and sarcomere structure in striated muscle. Accumulating evidence that mRNA transcripts are post-transcriptionally regulated by specific motifs located in the flanking untranslated regions (UTRs) led us to consider the role of titin 5'-UTR in regulating its translational efficiency [36]. A discovery of zheng et al broadened the mutation spectrum of the Tin gene associated with limb-girdle muscular dystrophies (LGMD) 2J, a highly heterogeneous group of genetic myopathies characterized by progressive proximal pelvic and/or shoulder girdle muscle weakness [73].

It's known that MutY is the highly conserved DNA glycosylase which excises adenine paired with the oxidative lesion 8-oxo-7,8-dihydroguanine [74, 75], implicating in DNA replication, repair of oxidative DNA damage, and checkpoint signaling. As a MutY homologue (MutYH), Myh1 plays an important role in DNA repair and DNA damage-induced checkpoint activation [37, 75]. Recent studies have shown that that the Wnt/β-catenin signaling plays an important role in the development of neuropathic pain [76-79]. Chen et al reported that nerve injury caused expression of WNTs and activation of WNT/frizzled/β-catenin signaling, and spinal

blockade of WNT signaling pathways inhibited neuropathic pain [77]. A study from Chen et al indicated that SCAI downregualtion activated the Wnt/β-catenin signaling [46]. Our results provided the first demonstration of spinal SCAI upregulation underlying the pathogenesis of diabetic peripheral neuropathy, thereby, its upregulation contributed to reduce the development of diabetic peripheral neuropathy by inhibiting the Wnt/β-catenin pathway, suggesting that targeting the SCAI signaling may be an effective approach for treating diabetic peripheral neuropathy. Aquaporins (Aqps) are the pore-forming protein family transporting water molecules and small solutes across biological membranes [51, 80]. Ricanek et al reported that Aqp 3 and 7 expression is significantly reduced in patients with inflammatory bowel disease, suggesting that there is a link between gut inflammation and Aqps signaling [51]. Although Aqps have been reported to involve in DRG axonal growth and modulate the sensing of certain types of pain [81, 82], their impact on peripheral neuropathy following diabetic mellitus is not clear. Our results demonstrated spinal Aqp 7 downregulation in diabetic peripheral neuropathy, suggesting that Aqp 7 may play a significant role in the pathophysiology of inflammatory peripheral neuropathy.

Conclusion

Despite tremendous research effort in the spinal field, our current understanding of the spinal molecular mechanisms underlying diabetic peripheral neuropathy is still incomplete. Our data provided a global view of the spinal differentially expressed genes in peripheral neuropathy model rats with type 2 diabetes mellitus. The differential changes of these genes may involve in inflammatory signaling, ion transport, protein phosphorylation, sensory perception, cell survival synaptic transmission, and synergistic regulation. These genetic differences contribute to elucidating the mechanism of diabetic peripheral neuropathy and may be new targets for developing therapeutic interventions following pain modulation.

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Disclosure of conflict of interest

None.

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