Identification of differentially expressed genes in dorsal root ganglion in early diabetic rats

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Abstract: Objective To screen and identify differentially expressed genes in the dorsal root ganglion (DRG) in early experimental diabetic rats. **Methods** Diabetic model rats were induced by single intraperitoneal injection of streptozotocin (STZ). At the second week after STZ injection, the sensory nerve conduction velocities (SNCV) of sciatic nerve were measured as an indicator of neuropathy. The technique of silver-staining mRNA differential display polymerase chain reaction (DD-PCR) was used to detect the levels of differentially expressed genes in rat DRG. The cDNA fragments that displayed differentially were identified by reverse-hybridization, cloned and sequenced subsequently, and then confirmed by Northern blot. **Results** The SNCV in the diabetic model group [$n = 9$, (45.25 ± 10.38) m/s] reduced obviously compared with the control group [$n = 8$, (60.10 ± 11.92) m/s] ($P < 0.05$). Seven distinct cDNA clones, one was up-regulated gene and the others were down-regulated ones, were isolated by silver-staining mRNA differential display method and confirmed by Northern blot. According to the results of sequence alignment with GenBank data, majority of the clones had no significant sequence similarity to previously reported genes except only one that showed high homology to 6-pyruvoyl-tetrahydropterin synthase mRNA (accession No., BC059140), which had not been reported to relate to diabetic neuropathy. **Conclusion** These differentially expressed genes in the diabetic DRG may contribute to the pathogenesis of diabetic peripheral neuropathy.

Keywords: differential display polymerase chain reaction; silver staining; mRNA; dorsal root ganglion; diabetes; rat

1 Introduction

Diabetic peripheral neuropathy (DPN), a disorder of peripheral nerves is among the most common complications of diabetes mellitus. Intensive investigations showed that the pathogenesis of diabetic peripheral neuropathy were associated with hyperglycemia, which can increase flux through the polyol pathway and the hexosamine pathway, produce advanced glycation endproducts (AGEs), and elicit the activations of protein kinase C and polyADP-ribose polymerase (PARP). In addition, DPN were related to other disorders including progressive microvascular disease, deficiency of

neurotrophin support, disturbed neural regeneration capacity, oxidative stress, etc.^[1-4] However, the mechanisms involved in it have not been explained clearly.

Recently, the studies about mechanism of DPN are focused on the expression changes of DPN related genes at cellular and molecular levels. Diabetic peripheral neuropathy, which is mainly displayed as impairment of sensory nerve including hyperalgesia, allodynia, paresthesia and spontaneous pain, can result in injury of primary sensory neurons in the early phase. As we known, the functional changes of organs are frequently associated with gene expression changes in cells, and gene expression often changes prior to functional impairment. Therefore, studying changes of gene expression in the dorsal root ganglion (DRG) of early diabetic rats may be helpful to explore the pathogenesis and development of diabetic neuropathy.

In this study, streptozotocin (STZ) was used to produce the diabetic model rats. At the second week after the diabetic

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rats were prepared successfully, reduction of SNCV was selected as a functional marker of diabetic neuropathy, and the technique of silver-staining mRNA differential display polymerase chain reaction (DD-PCR) was used to screen the genes expressed differentially between STZ-induced early diabetes (2 weeks) group and control group in the DRG of rat. Then the isolated gene fragments were identified and confirmed to find out the genes related to DPN and to explore the potential molecular mechanisms of this disease.

2 Materials and methods

2.1 Animal grouping and diabetes model establishment A total of 24 adult male healthy SD rats (180-220 g, clean degree) were provided by the experiment animal center of Nantong University [certification number: SCXK(Su)200-9919]. The rats were raised under environment of normal temperature and humidity. Sixteen of the twenty-four rats were selected randomly for preparing diabetic model, and the rest eight were used as normal control group. According to the method described by Price SA *et al.*^[5], diabetic rats were induced by single intraperitoneal injection of STZ (Sigma, America, freshly dissolved in 0.1 mmol/L citrate buffer) at a dose of 60 mg/kg, which was administered in the morning after an overnight fast. Three days later, blood glucose was measured by the o-toluidine method, and only the rats with blood glucose higher than 16 mmol/L were used as diabetic model rats. The diabetic rats were group-housed with full access to food and water for 2 weeks, and then the blood glucose of rats was measured again to confirm the reliability and stability of the diabetic model.

2.2 Sensory nerve conduction velocity measurement On the second week after modeling, the rats in both normal group and diabetic group were anesthetized with 3% pentobarbital sodium. The sciatic nerves of rats were isolated and exposed, then the sensory nerve conduction velocities (SNCV) were detected according to the reference^[6]. The results are expressed as mean±SD. Differences between two groups were analyzed with *t*-test by SPSS 10.0 statistical software.

2.3 RNA extraction and quantification After the nerve conduction velocity was detected, DRG of rat was taken out and total RNA was extracted with TRIzol reagent (Bio Basic, Inc., Markham, Ontario, Canada). Total RNA was measured by the optical density at 260 nm, and the purity was estimated by the 260/280 (nm) ratio in a Biophotometer (Eppendorf AG,

Hamburg, German). The integrity of total RNA was observed by formaldehyde denaturing gel electrophoresis. Then total RNA was dealt with RNase-free DNase I (Worthington, Lakewood, NJ, USA) to remove genomic DNA.

2.4 Reverse transcription to synthesize the first strain of cDNA Total RNA was diluted to the concentration of 1 µg/µL. According to the manual for reverse transcription provided by Promega Corporation (USA), up to 2 µg of mRNA was used for reverse transcription in an 25-µL reaction system consisted of 1 µL RNA oligo (dT)15 primer (0.5 µg/µL), 1 µL RNA M-MLV reverse transcriptase (200 U/µL), 5 µL deoxynucleotide triphosphate-dNTP mix (2.5 mmol/L of each of dATP, dTTP, dCTP, and dGTP), and 1× RT buffer (10 mmol/L Tris-HCl pH 8.8, 50 mmol/L KCl, and 0.1% v/v Triton X-100). Reverse transcription was performed in an PTC-100 Peltier Thermal Cycler (MJ Research, Inc., Waltham MA, USA) at 37 °C for 1 h. The reverse transcriptase enzyme was inactivated by heating the reaction mix to 70 °C for 10 min and then placing it on ice for 3 min. Reverse transcribed samples (the first train of cDNA) were kept at -20 °C until for further use.

2.5 DD-PCR with silver staining DD-PCR was performed using the Delta Differential Display Kit (Clontech Corporation, USA). The PCR reaction system was 20 µL in total: cDNA 1.0 µL, 10 mmol/L mixed dNTPs 0.3 µL, 10× Taq DNA polymerase buffer 2.0 µL, 25 mmol/L MgCl₂ 1.2 µL, 10 µmol/L anchor and arbitrary primers 2.0 µL each, 2.0 units of Taq DNA polymerase (1 U/µL), and 11.5 µL sterile water. PCR was done as follows: predenaturation at 94 °C for 5 min; then 94 °C for 30 s, 40 °C for 5 min, 68 °C for 5 min, for 3 cycles; 94 °C for 30 s, 40 °C for 30 s, 68 °C for 2 min, for 5 cycles; 94 °C for 30 s, 60 °C for 30 s, 68 °C for 2 min, for 23 cycles; and finally extension at 72 °C for 10 min. The PCR-amplified products were isolated on 6% non-denaturing polyacrylamide gel (39: 1). Silver staining was done as follows: gel was fixed with 10% acetic acid for 10 min, washed with distilled water for 2 min for 3 times, then washed with 1% HNO₃ for 3 min, stained for 30 min using freshly prepared 0.1% AgNO₃ solution (with 0.02% formaldehyde), rinsed in distilled water for 30 s, and developed in 3% Na₂CO₃ (with 0.03% formaldehyde and 0.0004% sodium thiosulfate). The reaction was terminated with 3% acetic acid. Finally, photographs were taken and the gel was dried.

2.6 Reamplification and purification The interest bands that expressed differentially in the gels were excised and

mashed, incubated at 70 °C for 30 min in 50 µL sterile water, and kept at -20 °C over night. The next day, the solution containing the bands was centrifuged, and 7 µL upper liquid was taken out for reamplification. The bands were reamplified by PCR in the presence of 2 mmol/L MgCl₂, dNTP mix (0.2 mmol/L of each dNTP), Taq DNA polymerase (2.5 U per reaction), and 1× PCR buffer [20 mmol/L Tris-HCl (pH 8.4), 50 mmol/L KCl] using target anchor and arbitrary primers (1 µmol/L), in a final volume of 50 µL. The PCR annealing temperature was 56 °C. Other conditions were the same as the prior amplification. Finally, the products were purified.

2.7 Dot blot, cloning, and sequence analysis The cDNA of normal rats was labeled as probe to hybridize with the purified bands according to the manual of Random primer Labeling Kit (Promega Corporation, USA). Then the bands of true positive were cloned using a TA cloning kit (pGEM-T vector system I, Promega Corporation, USA), and sequenced by BioAsia Biotechnology Co., Ltd (Shanghai, China). The obtained sequences were compared with GenBank available data using BLAST.

2.8 Northern blot Equivalent amounts of total RNA in the DRG of normal rats and diabetic rats were isolated by electrophoresis on a denaturing formaldehyde gel, and then the RNA was transferred to a nylon membrane. The differentially expressed bands were labeled as probe to hybridize with the RNA in the nylon membrane.

3 Results

3.1 Quantitative analysis of the experimental animals Of the sixteen rats for model preparation, nine rats were successfully induced to be diabetic, and the model was verified stable and reliable by re-measurement of blood glucose two weeks later. Nine rats in the diabetic model group and eight rats in the control group were involved in the result analysis.

3.2 Results of sensory nerve conduction velocity measurement The SNVC in the diabetic model group was significantly lower than that in the control group ($P < 0.05$, Table 1).

Table 1. Sensory nerve conduction velocities (SNCV) in the sciatic nerve of diabetic group and control group (mean±SD)

Group	Number of animals	SNCV (m/s)
Diabetic group	9	45.25±10.38*
Control group	8	60.10±11.92

* $P < 0.05$ vs control group

3.3 RNA integrity and purity Extracted RNAs of both groups were examined by electrophoresis on a denaturing formaldehyde agarose gel. On denaturing gels, total RNA typically gave bright 28S and 18S ribosomal RNA bands at approximately 4.5 kb and 1.9 kb, respectively, with an intensity ratio of about 2:1 (Fig. 1). Total RNA was measured by the optical density at 260 nm (A₂₆₀) and 280 nm (A₂₈₀), and the ratio of A₂₆₀/A₂₈₀ was higher than 1.8. These showed that the integrity and purity of the extracted total RNA were good.

3.4 Differential display A total of 16 sets, 4 arbitrary primers in combination with 4 anchor primers, were used to display the differentially expressed mRNA in the DRG between normal control group and diabetic model group. Out of more than 300 bands observed in the gel, 15 differential display bands were found reproducible. Figure 2 shows a part of the display result.

Fig. 1 Electrophoresis photograph of total RNA. The 18S and 28S ribosomal RNA bands were indicated. NG: normal control group; MG: diabetic model group.

Fig. 2 The result of a part of silver staining DD-PCR. The differentially expressed bands between control and diabetic groups were marked with arrows. M: DNA molecular weight marker (DL-2000 DNA Marker); 1 and 3: control group; 2 and 4: diabetic group.

3.5 Dot blot and sequence analysis Among the fifteen differential display bands in the gel, seven cDNAs were identified using dot blot as indicated in Fig. 3. Then, the seven cDNAs were cloned and sequenced. The length of these cDNA fragments ranged from 60 bp to 311 bp. One of them was up-regulated and the others were down-regulated in the diabetic DRG. By comparing the sequences of these clones with GenBank data, we found that majority of the clones had no significant sequence similarity to previously reported genes. Only one of the clones named Fragment-1 showed high homology to 6-pyruvoyl-tetrahydropterin synthase gene (accession No. BC059140), which had not been reported to relate to diabetic neuropathy. The sequence of Fragment-1 is presented as follows: ATT AAC CCT CAC TAA ATG CTG GGG AGA GGC TTG AGG GAA TGA AGT AAT CAA GCG GGT TGG GGA TTT GGC TCA GTG GTA GAG CGC TTA CCT AGG AAG CGC AAG GCC CTG GGT TCG GTC CCC AGC

Fig. 3 Dot blot identifying the differential cDNA fragments. The arrow indicated the hybridization signal of house keeping gene β -actin (as positive control).

Fig. 4 Northern blots confirming the differential display band Fragment-1. Position of the 18S ribosomal RNA was indicated. NG: normal control group, MG: diabetic model group.

TCC GTAAAAAAA AA.

3.6 Northern blot The cDNA band named Fragment-1 and house keeping gene β -actin were labeled as probes to hybridize with the RNAs in the DRGs of normal control group and diabetic model group, respectively. Beta-actin was used as the criterion for evaluating the quantity of total RNA. As shown in Fig. 4, the hybridized signal of β -actin had no obvious difference between the two groups, but the hybridized signal of Fragment-1 was stronger in the diabetic group than that in the normal control group, which was in accordance with the differential display result. The full length of the Fragment-1 mRNA was estimated about 1.2 kb.

4 Discussion

DPN is the most common of various peripheral nerve disorders associated with diabetes. The symptoms are mainly displayed as impairment of sensory nerve including hyperalgesia, allodynia, paresthesia and spontaneous pain, and as motor or autonomic nerve disorders. At the early stage of diabetes, the symptoms of DPN may be not obvious, but the nerve function has been injured, with the proofs provided by electrophysiological changes of nerve, including the reductions of both SNCV and motor nerve conduction velocity (MNCV). Especially, the reduction of SNCV can be the basis for diagnosis of sub-clinical DPN^[7-9].

In this study, at the early stage (2 weeks) of induced experimental diabetes, SNCV reduced compared with that in control, which was in accordance with previous study^[6]. In fact, there was no significant change in the SNCV on the next week after STZ-induced diabetes in our preliminary test (data not shown), which was in accordance with the result as described previously^[5]. This indicated that the function of sensory nerve was impaired in the early phase of diabetes (between 1 and 2 weeks). Many investigations have verified that hyperglycemia is the major factor for initiation of DPN. The abnormality of glucose metabolism induced by hyperglycemia can lead to increased risks of atherosclerosis, neurological and microvascular complications, and impairment of neurotrophic factors, which were critical reasons for disorders of peripheral nerves and ganglia^[1,2]. However, the early gene response for DPN in nerves is still unclear.

Price SA *et al.*^[5] explored the expression changes of genes related to DPN in week 1, 4 and 8 after STZ-induced diabetes by using the technique of Affymetrix oligonucle-

otide microarrays. They found that many known genes expressed differentially in diabetic DRG, which may be responsible for the functional deficits. Nevertheless, they did not investigate the changes in week 2. In the present study, the reduction of SNCV happened in week 2. Gene changes in this stage should be obvious and could reflect the early molecular response to the injury of nerve function. Therefore, we selected week 2 as the time point for observation to study gene expression changes in the diabetic DRG.

DD-PCR enables researchers to identify the RNAs expressed in one RNA population but missed in another. Identification of such "differentially expressed" RNAs is a powerful tool for studying the changes of gene expression during courses of differentiation, cell cycle, carcinogenesis, inductive events, and other biological phenomena. The traditional DD-PCR, which uses isotopes to detect and develop differentially displayed bands, will bring on radioactive pollution and make the procedure complicated even though it has high sensitivity. To avoid this disadvantage, silver staining is used to develop the image of differentially expressed bands.

In this study, the technique of DD-PCR with silver staining was established. Seven distinct cDNA bands were isolated and identified, subsequently cloned and sequenced, finally detected by DD-PCR and verified by Northern blot. By comparing the sequences of these clones with GenBank data, we found that the up-regulated clone named Fragment-1 in the diabetic DRG had high homology to 6-pyruvoyl-tetrahydropterin synthase mRNA (accession No. BC059140), which had not been reported to relate to diabetic neuropathy.

As references reported, the distribution of 6-pyruvoyl-tetrahydropterin synthase mRNA is relatively homogeneous and most abundant in the adrenal gland, kidney, pineal gland and various brain regions including the cerebellum, substantia nigra and locus coeruleus^[10]. 6-Pyruvoyl-tetrahydropterin synthase catalyzes the second step in the biosynthesis of tetrahydrobiopterin (BH4), which is an essential cofactor for the three nitric oxide synthase (NOS) isoforms and for the aromatic amino acid hydroxylases. BH4 starvation can affect nitric oxide, catecholaminergic and serotonergic systems to different extents, and lead to neurological and neuropsychiatric disorders^[11,12]. Human inducible NOS (iNOS) subunits dimerize to form an active enzyme. BH4 plays a critical role in promoting the dimerization in intact cells. This reveals

a post-translational mechanism by which intracellular BH4 can regulate iNOS expression^[13]. Augment of iNOS system leads to the abnormal increase of nitric oxide, which possibly has toxicity to ganglion and may participate in the injury of nerve function and neuropathic pain^[14,15]. Elevation of iNOS is also related to inflammation, which has been implicated as a secondary mechanism for ischemia-induced neuronal injury^[16], and inhibition of NOS activity in the patients with acute and chronic pain could decrease the hyperalgesia^[17]. We had reported that the mRNA level of iNOS increased in early diabetic DRG (2 weeks) compared with that in control^[18], but it was not reported previously that 6-pyruvoyl-tetrahydropterin synthase was associated with diabetic neuropathy. From the results in the present study, we inferred that Fragment-1 might be the homologous gene of 6-pyruvoyl-tetrahydropterin synthase gene in the DRG, and the enhancement of Fragment-1 expression in early diabetic DRG might result in elevation of BH4, which would facilitate the activation of iNOS and increase the mRNA level of iNOS at the same time.

Therefore, the changes of gene expression of Fragment-1 may participate in the initial step of generation and development of diabetic peripheral neuropathy. However, all of these described above need to be confirmed and verified by further study. These possibly provide a new clue for studying the pathogenesis of DPN in the future. Furthermore, other differential display clones, which did not show significant sequence similarity to previously reported genes, also may contribute to the pathogenesis of DPN, but it needs further and more investigations.

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糖尿病早期大鼠背根神经节中的差异表达基因

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摘要: **目的** 研究糖尿病早期大鼠背根神经节(dorsal root ganglion, DRG)中基因的差异表达, 探索糖尿病神经病变相关基因。**方法** 一次性腹腔注射链脲菌素(streptozotocin, STZ)制备糖尿病模型大鼠,于造模成功后第2周,测量其感觉神经传导速度(sensory nerve conduction velocities, SNCV),然后运用银染差异显示逆转录聚合酶链反应法(differential display polymerase chain reaction, DD-PCR)从糖尿病模型组与对照组大鼠的DRG中获得两者差异表达的cDNA片段,并进行反杂交筛选、克隆测序、DNA序列检索分析与Northern印迹验证。**结果** 糖尿病模型大鼠建模2周时,其SNCV与对照组相比显著下降($P < 0.05$)。银染差异显示并经克隆获得7个差异片段,一个为上调基因,其余为下调基因。经GenBank查询,仅其中的上调基因的cDNA序列与6-丙酮酰四氢喋呤合酶基因(6-pyruvoyl-tetrahydropterin synthase, 序列号BC059140)高度同源,其它差异表达片段未检出与已知基因有同源性。**结论** 这些在糖尿病早期背根神经节中差异表达的基因可能参与了糖尿病神经病变的发生发展。

关键词: 差异显示聚合酶链反应; 银染; 信使RNA; 背根神经节; 糖尿病; 大鼠