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Whole Genomic DNA Methylation Profiling of CpG Sites in Promoter Regions of Dorsal Root Ganglion in Diabetic Neuropathic Pain Mice

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Abstract

DNA methylation and demethylation play an important role in neuropathic pain. In general, DNA methylation of CpG sites in the promoter region impedes gene expression, whereas DNA demethylation contributes to gene expression. Here, we evaluated the methylation status of CpG sites in genomic DNA promoter regions in dorsal root ganglions (DRGs) of diabetic neuropathic pain (DNP) mice. In our research, streptozotocin (STZ) was intraperitoneally injected into mice to construct DNP models. The DNP mice showed higher fasting blood glucose (above 11.1 mmol/L), lower body weight, and mechanical allodynia than control mice. Whole-genome bisulfite sequencing (WGBS) revealed an altered methylation pattern in CpG sites in the DNA promoter regions in DRGs of DNP mice. The results showed 376 promoter regions with hypermethylated CpG sites and 336 promoter regions with hypomethylated CpG sites. In addition, our data indicated that altered DNA methylation occurs primarily on CpG sites in DNA promoter regions. The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis revealed that differentially methylated CpG sites annotated genes were involved in activities of the nervous and sensory systems. Enrichment analysis indicated that genes in these pathways contributed to diabetes or pain. In conclusion, our study enriched the role of DNA methylation in DNP.

Keywords Diabetic neuropathic pain · DNA methylation · Dorsal root ganglion · CpG

Abbreviations

DNP	Diabetic neuropathic pain
DRG	Dorsal root ganglion
KEGG	Kyoto Encyclopedia of Genes and Genomes
P2X3R	Purinergic P2X ligand-gated ion channel 3 receptor
PWT	Paw withdrawal threshold
STZ	Streptozotocin
WGBS	Whole-genome bisulfite sequencing

Introduction

According to the International Diabetes Federation data, 9.3% of adults aged 20 to 79 years—a staggering 463 million people—are living with diabetes, and in 2045, the number will increase to 700 million. About 25% of patients with diabetes mellitus suffer from diabetic neuropathic pain (DNP) (Jolivalt et al. 2016; Shillo et al. 2019; Sloan et al. 2018). As an intractable problem accompanied by diabetes mellitus, DNP causes a huge economic and psychological burden on patients. Although DNP has drawn increasing attention, the underlying mechanisms remain unclear. Available data suggest that the risk factors for the generation of DNP include glycemic burden, female gender, obesity, and genotype (variants of voltage-gated sodium channels [VGSC]) (Feldman et al. 2017; Rosenberger et al. 2020; Shillo et al. 2019). In addition, epigenetic regulation could play a vital role in DNP

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(Cheng et al. 2015; Ciccacci et al. 2020; Guo et al. 2019; Liu et al. 2018b).

Epigenetic regulation includes DNA modification, histone modification, chromatin remodeling, and non-coding RNAs. DNA modification primarily includes DNA methylation and demethylation. In the mammalian genome, DNA methylation refers to the transfer of a methyl group to the C5 position of cytosine to form 5-methylcytosine (5mC) by DNA methyltransferases (Li and Zhang 2014; Moore et al. 2013). DNA methylation can occur at several genetic locations, primarily including promoters, exons, and introns (Jones 2012; Rauscher et al. 2015). DNA methylation of promoters occurs especially on CpG sites, which impairs transcription factor binding and silences gene expression (Moore et al. 2013). Methylated DNA could be demethylated by ten-eleven translocation proteins (He et al. 2011; Kohli and Zhang 2013; Morgan et al. 2018), which can oxidize 5mC to 5-carboxylcytosine via intermediates 5-hydroxymethylcytosine and 5-formylcytosine and initiate the expression of specific genes. Hence, DNA methylation in the right place at the right time plays an important role in normal physiological functions. Altered DNA methylation may contribute to several disease processes (Ehrlich 2019; Liu et al. 2018a ; Luo et al. 2018).

Altered DNA methylation was also implicated in DNP (Zhang et al. 2015). In this research, the authors found demethylation of CpG sites in the *p2×3r* gene promoter region in the dorsal root ganglion (DRG) of DNP rats, along with an increased expression of purinergic P2X ligand-gated ion channel 3 receptor (P2X3R), annotated by the *p2×3r* gene. In type 2 diabetes (T2D), DNA methylation was associated with the progression of diabetic peripheral neuropathy (Guo et al. 2019). In their study, Kai Guo et al. found differentially methylated CpG sites between significant sural nerve regeneration and degeneration. These results indicated that DNA methylation and demethylation occurred during the progression of diabetes. Although there are reports about DNA methylation in DNP, the whole-genome DNA methylation profiling in DNP DRGs has not been reported.

In this study, we studied the changes in DNA methylation in genomic DNA promoter regions in DNP mice. We believe this study will provide a better understanding of the role of DNA methylation in DNP.

Materials and Methods

Animals. Male C57BL/6 wild-type mice (18–22 g) were purchased from Capital Medical University. Four mice were housed per cage in a temperature- and light-controlled room with a 12-h:12-h light: dark cycle. Mice were provided water and food ad libitum. All animal experimental procedures were approved by the experimental animal ethics committee of Capital Medical University.

Establishment of Type 1 Diabetics in Mice. Mice were fasted overnight and provided free access to water. Next, mice were administered an intraperitoneal injection of freshly prepared streptozotocin (STZ, 50 mg/kg/day; Sigma, S0130) for five consecutive days. Control mice received an intraperitoneal injection of citrate buffer (5 mL/kg/day) (Zheng et al. 2018) for five days. The fasting blood glucose (mmol/L) was detected at the fourth week using the blood sample collected from the tail vein using the ACCU-CHEK® Mobile blood glucose meter (Roche). When the blood glucose meter showed "high," the data were recorded as 33.3 mmol/L. Mice with fasting blood glucose over 11.1 mmol/L were considered diabetic and used for subsequent studies. In addition, the body weight of mice was recorded.

Paw Withdrawal Threshold. The 50% paw withdrawal threshold of hind paws was measured using calibrated von Frey hairs (Stoelting, Wood Dale, IL, USA). Briefly, mice were placed on a metal mesh floor and covered with transparent plastic cages. Before the test, mice were allowed to habituate for at least 30 min for three consecutive days. Eight von Frey hairs (0.02, 0.04, 0.07, 0.16, 0.40, 0.60, 1.00, 1.40 g) were selected (Zheng et al. 2017). Each test began with 0.16 g von Frey filament that was applied perpendicularly to the plantar surface in the center of either hind paw for 3 to 5 s. Responses were recorded as positive when mice showed paw withdrawal, flinching, or paw licking. In addition, 50% paw withdrawal threshold (PWT) was measured using the "up-and-down" method as previously described (Chaplan et al. 1994; Chen et al. 2018) and calculated using the following formula: $50\%\ \mathrm{PWT\ (g)} = 10^{X + \mathrm{kd}}/10^4$, where X is the value of the final von Frey hair used, k is the value measured from the pattern of positive/negative responses, and d is the average increment (in log units) between von Frey hairs used. If a mouse responded to the lowest von Frey hair, 0.02 g was recorded. If a mouse did not respond to the highest von Frey hair, 2.00 g was recorded.

Whole-Genome Bisulfite Sequencing. Harvested L3–L5 DRGs of mice and mixed DRGs from three mice to obtain sufficient tissue and considered as one sample. The control and DNP groups included three samples. Genomic DNA was extracted from DRGs using the Wizard Genomic DNA Purification Kit (Promega) and sequenced in Igenecode Technology (Beijing, China). The quality of extracted DNA was measured and evaluated. Only high-quality genomic DNA was used for the subsequent study. The genomic DNA was fragmented to about 200 bp by sonication. Bisulfite was used to convert the unmethylated cytosine to uracil, whereas methylated cytosine was unaffected. Finally, PCR amplification was performed. The libraries were quantified and sequenced on Illumina HiSeq. To better understand the function of differentially methylated CpG site annotated genes, Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis was performed (Kanehisa and Goto 2000). The KEGG database

is updated daily and is freely available (http://www.genome.ad.jp/kegg/). A hypergeometric test was used for enrichment analysis of the KEGG pathway, and $p < 0.05$ was considered significant.

Data Analysis. Data are expressed as mean ± standard error of the mean (SEM). GraphPad Prism 7 software was used for statistical analysis. Unpaired Student's *t*-test was used for comparison between two groups (Fig. 1). A $p < 0.05$ was considered significant.

Results

Induction of DNP in Mice

To establish DNP in mice, diabetes was induced in male C57BL/6 wild-type mice by intraperitoneal injection of STZ for five days. After four weeks, mice with STZ treatment showed higher fasting blood glucose (mmol/L) when compared with control mice (Fig. 1a), and the values were 29.30 ± 1.64 mmol/L in STZ-treated mice and 8.58 ± 0.31 mmol/L in control mice ($n = 9$). STZ-treated mice were significantly lighter in weight (g) than control mice (Fig. 1b), with values 22.71 ± 0.65 g in STZ-treated mice and 27.99 ± 0.56 g in control mice. Frey hairs method used to test the sensitivity to mechanical stimuli revealed that STZ-treated mice showed reduced threshold (g) to mechanical stimulation in both left and right paws (Fig. 1c, d). The values were 0.19 ± 0.04 g in STZ-treated mice and 1.57 ± 0.17 g in control mice for left paws and 0.18 ± 0.05 g in STZ-treated mice versus 1.66 ± 0.15 g in control mice for right paws ($n = 9$). For all the aforementioned cases, the data are expressed as mean ± SEM, *** $p < 0.001$, and unpaired *t*-test. Therefore, STZ-treated mice showed DNP behavior.

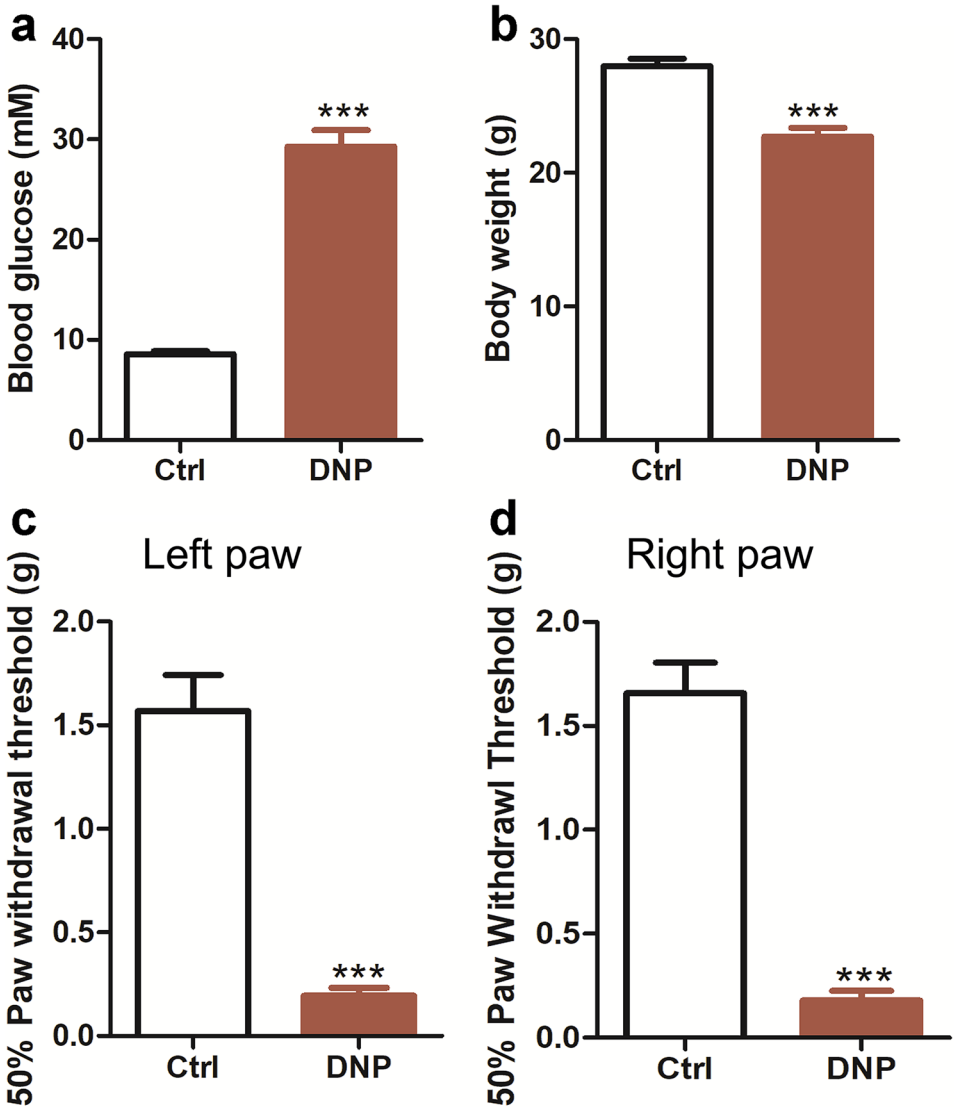

Fig. 1 Intraperitoneal injection of STZ to induce DNP in mice. **a** The level of fasting blood glucose in control (ctrl) and DNP mice. **b** The body weight of ctrl and DNP mice. **c** and **d** Mechanical allodynia in the left and right paws of DNP mice. All data are expressed as mean ± SEM. *** $p < 0.001$ when compared with ctrl, unpaired *t*-test

Distribution of Differentially Methylated Regions

The methylation of cytosine in different regions had varying effects on gene expression. The analysis of our data indicated that 386 promoter regions, 473 exon regions, and 8896 intron regions were hypermethylated in cytosines, whereas 347 promoter regions, 421 exon regions, and 7858 intron regions were hypomethylated in cytosines in STZ-induced DNP mice when compared with control mice (Fig. 2a). Further analysis of our data revealed that among 386 hypermethylated promoter regions, 376 promoter regions were CpG hypermethylated, and among 347 hypomethylated promoter regions, 336 promoter regions were CpG hypomethylated (Fig. 2b). These results indicate that altered DNA methylation in promoter regions primarily occurred in CpG sites. Based on these findings, we conclude that differentially methylated CpG sites play an important role in the regulation of gene expression.

Kyoto Encyclopedia of Genes and Genomes Enrichment Analysis of Differentially Methylated CpG Sites in Promoter Regions

To better understand the function of differentially methylated CpG sites in promoter regions, their annotated genes were analyzed by the KEGG pathway. We found annotated genes in the KEGG pathway that were involved in the sensory system, nervous system, neurodegenerative diseases, and energy metabolism (Fig. 3). Enrichment analysis showed that 13 pathways were significantly enriched ($p < 0.05$), including "starch and sugar metabolism" and "glycerophospholipid metabolism," which are involved in diabetes and diabetic peripheral neuropathy (Fig. 4). In addition, we noticed that the "asthma" pathway includes a gene (*IL-10*) related to pain (Fig. 4; Table 1) (Iwasa et al. 2019; Khan et al. 2015). The CpG sites in the *IL-10* gene promoter regions were hypermethylated in DNP mice, which may inhibit IL-10 expression and contribute to DNP. This finding was consistent with that reported in certain previous studies (Khan et al. 2015). The results indicated that altered DNA methylation occurs in genes associated with diabetes or pain in DRG of DNP mice.

Discussion

DNA methylation and demethylation are important regulators of gene expression and play a major role in pain (Chidambaran et al. 2017; Garriga et al. 2018; Gombert et al. 2020; Sun et al. 2015). Although certain reports indicate that under pain conditions, altered DNA methylation occurs in DRG and contributes to pain sensitivity (Garriga et al. 2018; Zhang et al. 2015), genomic DNA methylation status in DRG with DNP has not been reported. We examined the DNA methylation status in the DRG of DNP mice at four weeks using whole-genome bisulfite sequencing (WGBS). The results indicate that altered DNA methylation occurs primarily in intron regions; however, in the upstream

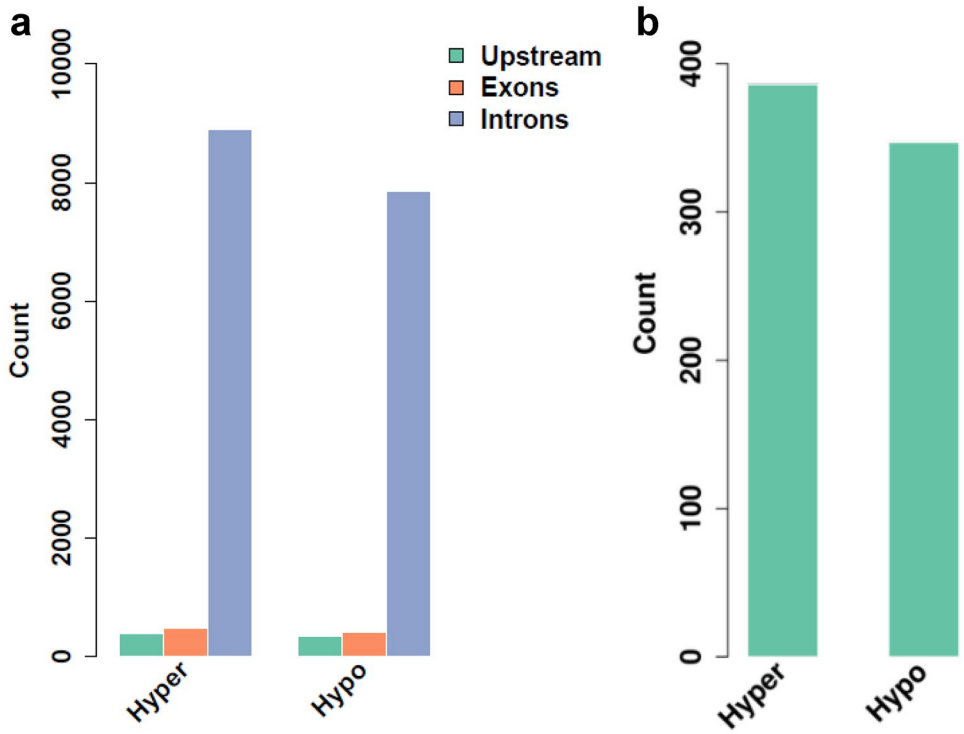

Fig. 2 Distribution of differentially methylated CpG sites. **a** The number of differentially methylated regions in DNP mice when compared with ctrl. **b** The number of promoter regions with differentially methylated CpG sites

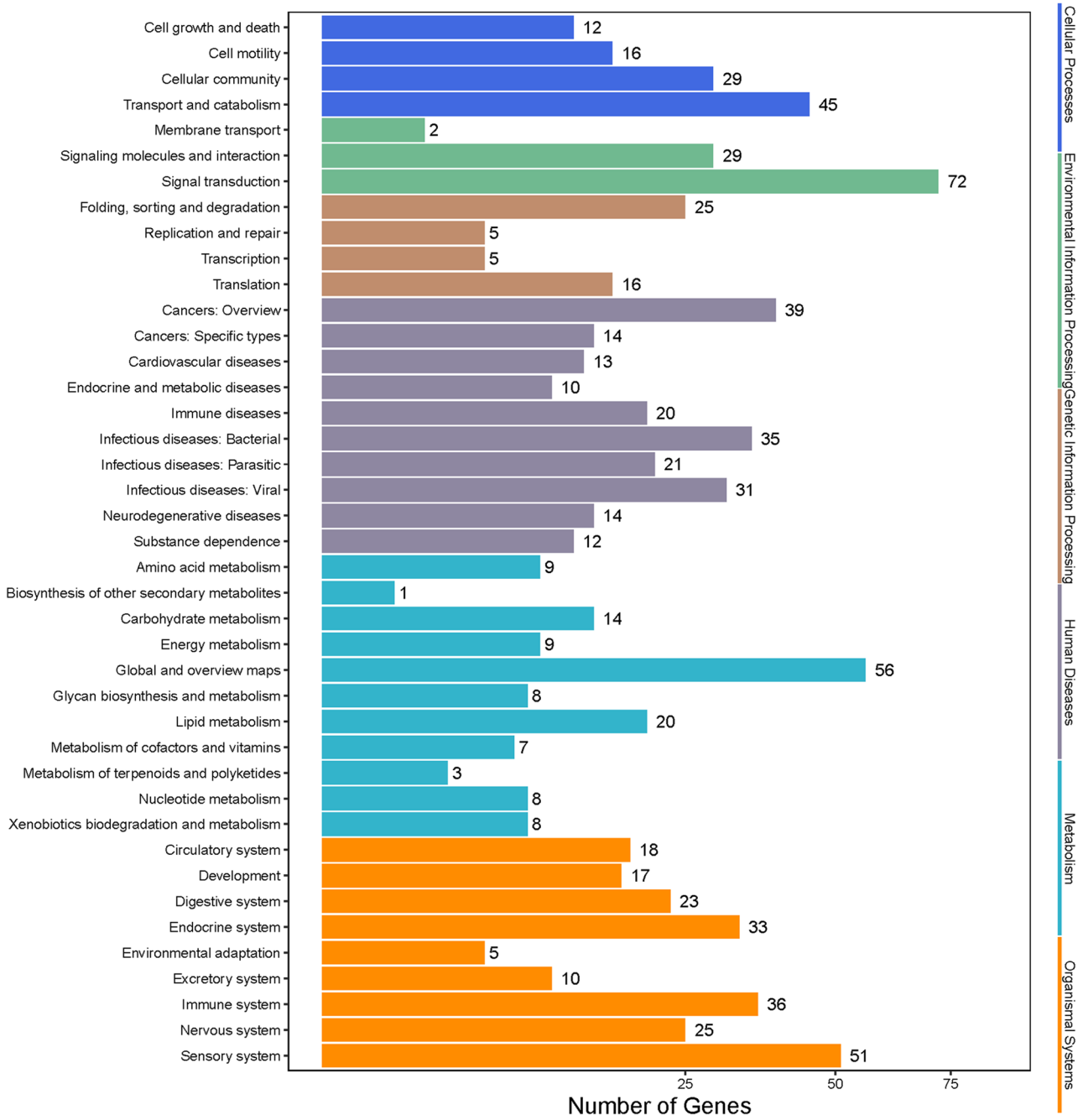

Fig. 3 KEGG pathway of differentially methylated CpG sites in the promoter regions annotated genes. Some annotated genes are involved in the sensory system, nervous system, neurodegenerative disease, and energy metabolism

regions, namely, promoter regions, altered DNA methylation preferentially occurs in CpG sites (Fig. 2). These findings are consistent with those reported earlier (Guo et al. 2019). We identified 712 promoter regions with differentially methylated CpG sites; among these, 376 were hypermethylated, and 336 were hypomethylated. DNA methylation at different sites had a varying effect on gene expression; for example, methylation in the promoter regions usually impeded gene transcription, whereas in intragenic regions increased the activity of the gene (Guo et al. 2019; Rideout III et al. 1990).

To better understand the function of annotated genes with differentially methylated CpG sites, KEGG analysis was performed. As expected, the annotated genes were involved in the functions of nervous and sensory systems. Enrichment analysis of KEGG pathways revealed that "starch and sugar metabolism" and "glycerophospholipid metabolism"

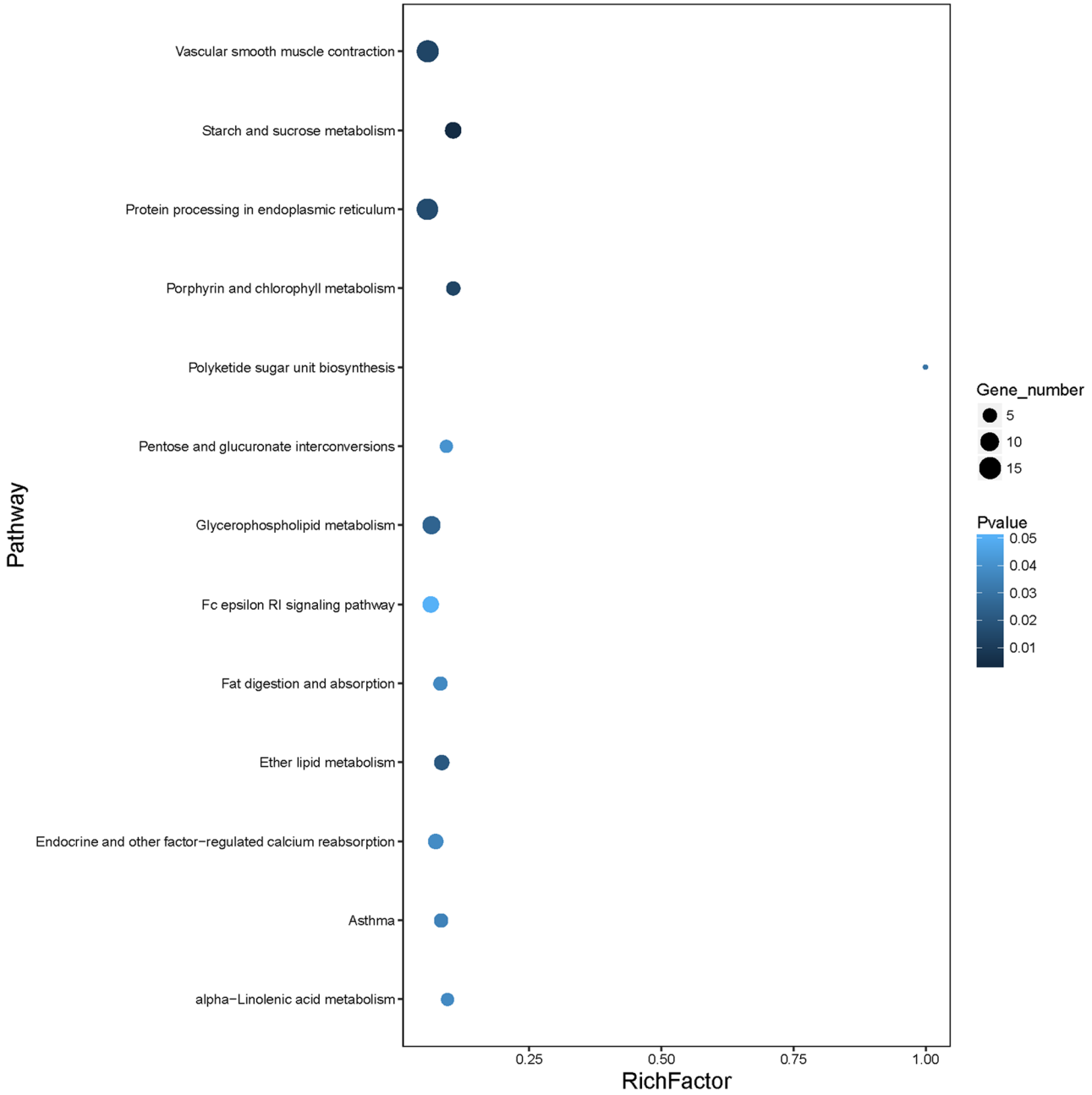

Fig. 4 Enrichment analysis of KEGG pathways and all 13 significantly enriched pathways are shown. The size of the dot means the number of genes in each pathway. The color means the *p*-value. Rich factor means the percentage of annotated genes to all genes belonging to the KEGG pathway. A hypergeometric test was used for enrichment analysis of the KEGG pathway. A $p < 0.05$ was considered significant

were significantly enriched. Similar to our results, Kai Guo et al., who performed KEGG pathway enrichment analysis, found that "glycerophospholipid metabolism" pathway was significantly enriched. However, they had compared significant sural nerve regeneration and degeneration in type 2 diabetes, whereas we compared healthy and DNP mice in DRG. Reduced sugar and starchy carbohydrate intake can reverse diabetic disorder (Feinmann 2016). In the "glycerophospholipid metabolism" pathway, glycerophospholipids are components of cell membranes and are involved in several neurological diseases, such as Alzheimer's disease, ischemia, and spinal cord trauma (Farooqui and Horrocks 2001; Frisardi et al. 2011). Another significantly enriched pathway that attracted our attention was the "asthma" pathway, including an annotated gene *IL-10*. Previous studies reported the involvement of IL-10 in pain.

Table 1 Differentially methylated CpG sites in promoter regions annotated genes in the "asthma" pathway

Genes	Gene IDs	Gene location	Type	Description	Methylation description
Epx	13,861	Chr11	Protein coding	Eosinophil peroxidase	Hypermethylated
Ms4a4c	64,380	Chr9	Protein coding	Membrane-spanning 4-domains, subfamily A, member 4C	Hypomethylated
IL10	16,153	Chr1	Protein coding	Interleukin 10	Hypermethylated
Fcer1g	14,127	Chr1	Protein coding	Fc receptor, IgE, high affinity I, gamma polypeptide	Hypermethylated
Fcer1a	14,125	Chr1	Protein coding	Fc receptor, IgE, high affinity I, alpha polypeptide	Hypermethylated

Changes in the IL-10 expression in different tissues under different pain conditions varied. For example, the expression of IL-10 in the paw (skin, muscle, and fascia) increased significantly in Complete Freund's Adjuvant-induced inflammatory pain (Martins et al. 2016). However, the levels of IL-10 decreased significantly in the affected side of DRGs in chronic constriction injury- and partial sciatic ligation-induced neuropathic pain (Khan et al. 2015). Further analysis of our data revealed that promoter regions of *IL-10* were hypermethylated, which could inhibit gene expression. These results suggest the involvement of decreased IL-10 expression in DRG in DNP mice, similar to that observed in chronic constriction injury and partial sciatic ligation neuropathic pain models. Although we found that only the *IL-10* gene was directly involved in pain, a report indicated that curcumin alleviated chronic pain induced-depression by modulating glycerophospholipid metabolism and ether lipid metabolism (Zhang et al. 2020). These findings suggested that genes involved in "glycerophospholipid metabolism" and "ether lipid metabolism," significantly enriched pathways in our research, might also be involved in pain.

Conclusions

We observed altered genomic DNA methylation status in DRGs of DNP mice. Certain genes were found to be related to diabetes, and others were involved in pain conditions. We believe that our work will further enrich the knowledge of epigenetic regulation in DNP and provide potential targets for DNP treatment.

Author Contribution All authors contributed to the study conception and design. Wen Chen, Fei Yang, and Weihua Cui prepared the manuscript. Wen Chen, Ting Lan, and Qingyu Sun conducted most of the experiments. Yurui Zhang, Danmin Shen, Tingting Hu, Jing Liu, Yingzi Chong, Peipei Wang, and Qian Li analyzed the data and commented on previous publications. All authors approved the final manuscript.

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Data Availability All data and materials described in the study are available from the corresponding author on reasonable request.

Declarations

Ethics Approval and Consent to Participate All animal procedures were approved by the experimental animal ethics committee of Capital Medical University and were performed in accordance with the National Institute of Health Guidelines for the Treatment Animals. The manuscript does not contain any study on human participants.

Consent for Publication All co-authors have agreed to the submission of the final manuscript.

Competing Interests The authors declare no competing interests.

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