Neuropeptide Y: a potential theranostic biomarker for diabetic peripheral neuropathy in patients with type-2 diabetes

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Abstract

Background: Diabetic peripheral neuropathy (DPN), the most common microvascular complication of type-2 diabetes mellitus (T2DM), results in nontraumatic lower-limb amputations. When DPN is not detected early, disease progression is irreversible. Thus, biomarkers for diagnosing DPN are needed.

Methods: We analyzed three data sets of T2DM DPN: two for mouse models (GSE70852 and GSE34889) and one for a human model (GSE24290). We found common differentially expressed genes (DEGs) in the two mouse data sets and validated them in the human data set. To identify the phenotypic function of the DEGs, we overexpressed them in zebrafish embryos. Clinical information and serum samples of T2DM patients with and without DPN were obtained from the Korea Biobank Network. To assess the plausibility of DEGs as biomarkers of DPN, we performed an enzyme-linked immunosorbent assay.

Results: Among the DEGs, only NPY and SLPI were validated in the human data set. As npy is conserved in zebrafish, its mRNA was injected into zebrafish embryos, and it was observed that the branches of the central nervous system became thicker and the number of dendritic branches increased. Baseline characteristics between T2DM patients with and without DPN did not differ, except for the sex ratio. The mean serum NPY level was higher in T2DM patients with DPN than in those without DPN (p = 0.0328), whereas serum SLPI levels did not differ (p = 0.9651).

Conclusion: In the pathogenesis of DPN, NPY may play a protective role in the peripheral nervous system and may be useful as a biomarker for detecting T2DM DPN.

Keywords: biomarker, diabetic peripheral neuropathy, neuropeptide Y, type-2 diabetes mellitus, zebrafish

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Individuals with DPN complain of unbearable stabbing, burning, and tingling sensations in the limbs, in addition to depression, anxiety, and sleep deprivation. Moreover, sensory deficits and poor wound healing often result in foot ulceration and nontraumatic lower-limb amputations. Amputations in patients with diabetes affect their quality of life and are associated with substantially increased mortality, especially major amputations (5-year mortality rate of 44–68%, which is greater than those of prostate and breast cancer).

Early manifestations of DPN often go undiagnosed until the disease is well-developed, at which point they appear to be irreversible. Screening for early symptoms and signs of DPN is critical in clinical practice to improve patient prognosis and quality of life and reduce healthcare costs. The gold standard methods for detecting DPN are nerve conduction studies. However, these studies are often impractical to implement as they are labor-intensive, time-consuming, and costly. Because of the lack of treatments for nerve damage, preventing DPN is the best approach for improving patient prognosis; thus, it is essential to identify a biomarker for early detection of DPN.

Biomarkers provide precise diagnostic and predictive data and objectively identify pathogenic processes. A recent study reported that inflammation, oxidative stress, lipid and carbohydrate metabolism, axonogenesis, mitochondrion, and peroxisome proliferator–activated receptor signaling events are dysregulated in DPN and are important pathomechanisms in DPN development. Molecules associated with these pathways have been suggested as predictive biomarkers. However, traditional biomarker discovery processes have limitations, and few have been adopted in the clinic.

With the accumulation of gene expression data and the development of computational analyses, researchers, particularly in oncology, have begun investigating biomarkers for developing immunotherapeutic and other targeting agents to maximize the efficacy of treatments, while minimizing toxicity. The aim of this study was to identify a biomarker for DPN by gathering gene expression data and performing computational analysis, experimental validation in animals, and final validation using immunoaffinity assays.

**Materials and methods**

**Microarray data**

We searched transcriptomics data from the Gene Expression Omnibus data set repository (GEO data set; https://www.ncbi.nlm.nih.gov/gds) using the search query “diabetes mellitus neuropathy.” We excluded data for animals with type-1 diabetes mellitus and samples from the central nervous system. We downloaded data sets using the getGEO function in GEO query R package. We analyzed three microarray data sets (two for mouse T2DM neuropathy and one for human T2DM neuropathy): GSE70852, GSE34889, and GSE24290. GSE70852 comprises microarray data for the dorsal root ganglia and sciatic nerve tissue of 26-week-old ob/ob mice and ob/+ mice. We analyzed 10 sciatic nerve tissue samples from 5 ob/+ mice and 5 ob/ob mice (M-GSE70852-26 W). GSE34889 comprises microarray data for sciatic nerves from 8- and 24-week-old BKS db/db mice and db/+ mice. We used 24-week-old db/db mice (n = 6) and db/+ mice (n = 7) for analysis (M-GSE34889-24 W). GSE24290 includes data for 35 human sural nerve tissues, including 18 nonprogressive diabetic neuropathy samples and 17 progressive diabetic neuropathy samples (H-GSE24290).

**In silico analyses**

A linear model for microarray analysis (LIMMA) R package from the Bioconductor project was applied to each GEO data set to identify significantly differentially expressed genes (DEGs). The basic statistic used in LIMMA for significance analysis is the moderated t-statistic. The p value of each DEG was calculated and then adjusted using the Benjamini–Hochberg (BH) adjustment method. In M-GSE78052-26 W and M-GSE34889-24 W, BH-adjusted p < 0.05 and |Log2 fold-change (FC)| > 1.5 were used to select notable DEGs from each data set. The Venn R package was used to identify overlapping DEGs in the two data sets. P < 0.05 was used to select notable DEGs from H-GSE24290.

**Maintenance of adult zebrafish and embryos**

Wild-type zebrafish AB and huc; mCherry transgenic zebrafish were maintained in an automatic circulation system (Genomic-Design, Daejeon, Korea) at 28.5°C. All experiments using...
zebrafish embryos were performed according to the guidelines of the Ulsan National Institute of Science and Technology Institutional Animal Care and Use Committee (approval number: UNISTIACUC-15-14, date: 2016-10-11). Zebrafish embryos were cultured using E3 solution in incubators at 28°C.16

In vitro transcription for mRNA injection
For overexpression experiments, full length was cloned into the pcs2p + vector. mRNA was synthesized using an mMESSAGE mMACHINE SP6 (Invitrogen, Carlsbad, CA, USA). The synthesized mRNA was injected into single-cell-stage zebrafish embryos. Because secretory leukocyte protease inhibitor (SLPI) is not evolutionally conserved in vertebrates, we did not synthesize slpi mRNA in zebrafish.

Live imaging of zebrafish embryos
Zebrafish embryos were anesthetized with tricaine for observation at 5 days after fertilization. Anesthetized embryos were observed using a Leica M165C microscope (Wetzlar, Germany).

Quantitative reverse-transcription polymerase chain reaction
Total RNA from zebrafish embryos was extracted using TRIZol reagent (Molecular Research Center, Cincinnati, OH, USA). Embryos were homogenized using a pestle and dissolved using TRIZol reagent. Total RNA was isolated, and cDNA was synthesized as previously described.16 A quantitative reverse-transcription polymerase chain reaction (qRT-PCR) was performed using PowerUP SYBR Green Master Mix (Thermo Fisher Scientific, Waltham, MA, USA). The expression level of the target gene was normalized to that of β-actin as an endogenous control.

Confocal imaging of transgenic zebrafish
To observe peripheral nervous system development in zebrafish embryos, hue; mCherry transgenic zebrafish were used. Transgenic zebrafish were anesthetized for observation using tricaine. Embryos were imaged using an LSM880 confocal microscope (Carl Zeiss, Oberkochen, Germany).

Statistical analysis of data from zebrafish experiments
Statistical analyses were performed using Student’s t-tests, and all experiments were performed in triplicate. The figures and graphs show the averages of three independent experiments. Error bars indicate the standard error mean. Statistical significance was set at p < 0.05.

Patient serum and data acquisition
Serum from T2DM patients diagnosed with and without DPN was obtained from the Korea Biobank Network. Serum from 20 T2DM patients diagnosed with DPN and 9 T2DM patients without DPN was obtained from Kangwon National University Hospital. Serum from five T2DM patients without DPN was obtained from the Gyeongsang National University Hospital. A nerve conduction study confirmed the diagnosis of DPN. Information on baseline characteristics including age, sex, HbA1c, and comorbidities, such as hypertension, coronary artery disease, cerebrovascular infarction, and cancer was also obtained. We examined the levels of NPY and SLPI in the patient serum to identify a serum biomarker for DPN. The Institutional Review Board of the Gachon University Gil Medical Center (approval id. GCIRB2020-044) approved this study.

Enzyme-linked immunosorbent assay
Serum NPY was measured by enzyme-linked immunosorbent assay (ELISA) (Cat. No. NBP2-76680, Novus Biologicals, Littleton, CO, USA). Standards or samples were added to each well, followed immediately by the addition of a biotinylated detection antibody and incubation for 45 min at 37°C. After washing, the horseradish peroxidase–conjugated NPY antibody was added and incubated for 30 min at 37°C. After washing, the substrate reagent was added to each well for 15 min at 37°C. Stop solution was added to each well, and the absorbance was read at 450 nm using a microplate reader (Spectra MAX M2e, Molecular Devices, San Jose, CA, USA).

We prepared an SLPI-targeted capture antibody for quantitative sandwich enzyme immunoassay (Cat. No. DY1274-05, R&D Systems, Minneapolis,
MN, USA) according to the manufacturers’ instructions. Standards or serum samples were added to each well and incubated for 2 h at room temperature. The plates were washed to remove any residual contaminants, and the detection antibody was added to each well. After aspiration/washing, the working solution of streptavidin-horseradish peroxidase was added and incubated for 20 min at room temperature. The plates were washed, and substrate solution was added per well for incubation for 20 min at room temperature.

Finally, stop solution was added to each well. The optical density was immediately determined using a microplate reader (Molecular Devices Spectra MAX M2e) at an absorbance of 450 nm.

Statistical analysis and graphical display of ELISA were performed using GraphPad Prism software (version 9.0.2, GraphPad, Inc., San Diego, CA, USA). Spearman’s rank correlation analysis was performed using R (version 3.6.0, Vienna, Austria).

Results

Identification of DEGs related to DPN
Using a threshold of $|\log_{2}FC| > 1.5$ and BH-adjusted $p < 0.05$, 65 and 608 DEGs were extracted from the expression profiles in the M-GSE70852-26 W and GSE M-34889-24 W data sets, respectively (Figure 1). Volcano plots show the distribution of these DEGs in the two data sets (Figure 2(a)). Subsequent to performing the integrated bioinformatics analysis, 33 consistent DEGs were identified from the two data sets. Among these, 27 were upregulated and 6 were downregulated. These genes were validated in H-GSE24290, and two genes were finally selected: NPY and SLPI. The expression levels of these two genes were significantly higher in sciatic nerves from DPN mice and sural nerves from patients with progressive DPN (Figure 2(b) and (c)).
Figure 2. DEGs in each GEO data set and expression of selected genes: (a) DEGs between two groups were identified via the LIMMA package and visualized using a volcano plot. Significantly expressed genes are represented as red dots. (b) Expression of NPY and (c) SLPI in the three data sets.

Overexpression of *npy* induced the development of the peripheral nerve system in zebrafish embryos

Zebrafish are commonly used as an *in vivo* model to investigate neurogenesis and nerve development, as most genes in this species are conserved and their physiological characteristics are similar to those of humans. Amino acid sequences were compared between human and zebrafish embryos to investigate the function of NPY and SLPI using zebrafish embryos. Although the amino acid sequence of SLPI was quite different between human and zebrafish embryos (data not shown), NPY is evolutionarily conserved in vertebrates. We found that 67% of residues were the same, and 11% of residues showed similar structures between humans and zebrafish (Supplemental Figure 1). These data suggest that NPY is conserved in vertebrates, and that zebrafish embryos can be used to investigate the function of NPY in the peripheral nervous system.

The zebrafish ortholog of human NPY, *npy*, was overexpressed following mRNA injection. Embryos were injected with 100, 300, and 500 pg of *npy*-mRNA to determine the highest dose that did not change body length, brain size, heartbeat, and tail. Herein 500 pg/embryo was chosen as the experimental condition because this was the highest dose that did not cause phenotype changes (Figure 3(a)). After *npy*-mRNA injection, overexpression of *npy* was confirmed by qRT-PCR analysis (Figure 3(b)).

The effect of *npy* overexpression in the nervous system was investigated using the nerve-specific marker *huc*: mCherry and the vessel-specific marker *fli1*: GFP double-transgenic zebrafish. Although overexpression of *npy* did not affect vascular constitution, the signal of *huc*: mCherry changed. Compared to untreated control embryos, *npy*-mRNA-injected embryos showed a highly increased *huc*: mCherry signal in the central nervous system. In addition to the central nervous system, peripheral branches from the central nervous system became thicker and showed increased dendritic branching (Figure 3(c)). These data suggest that *npy* overexpression
induces central and peripheral nervous system development.

NPY level was higher in the serum of T2DM patients with DPN than in that of those without DPN

There were 20 T2DM patients with DPN and 14 T2DM patients without DPN. The mean ages of T2DM patients with and without DPN were 69.5 and 61.8 years, respectively ($p = 0.2475$). The number of males was higher in T2DM patients with DPN than in the group without DPN ($p = 0.0365$). The mean HbA1c and comorbidities, such as hypertension, coronary artery disease, cerebrovascular infarction, and all types of cancers, did not differ between the two groups (Supplemental Table 1).

In ELISA, the mean levels of NPY in the serum of T2DM patients with and without DPN were 2953 and 2882 pg/mL, respectively ($p = 0.0208$) (Figure 4(a)). The mean serum level of SLPI did not differ between the two groups ($p = 0.9651$) (Figure 4(c)). The areas under the ROC curves for NPY and SLPI were 0.718 ($p = 0.0328$) (Figure 4(b)) and 0.5464 ($p = 0.6492$), respectively (Figure 4(d)). Spearman’s rank correlation showed that NPY level was positively correlated with age in all T2DM patients ($\rho = 0.6, p < 0.001$) and T2DM patients with DPN ($\rho = 0.53, p = 0.0155$) (Supplemental Figure 2).

Discussion
We analyzed three microarray data sets acquired from GEO (two for mouse sciatic nerve with T2DM DPN and one for human sural nerve with DPN). Among the common DEGs from the two mouse data sets, only NPY and SLPI were validated in the human data set. Between NPY and SLPI, only NPY was conserved in zebrafish. When npy-mRNA was injected into zebrafish embryos, we observed an increased development of the peripheral and central nervous systems compared to that in untreated embryos. The serum level of NPY was higher in T2DM patients with DPN than in those without DPN. The level of NPY was positively correlated with age in all T2DM patients and T2DM patients with DPN. Despite the importance of DPN, current pharmacotherapies are limited to resolving symptoms rather than the disease mechanisms, as the precise pathogenic mechanisms of DPN are unknown. The known pathomechanisms of

![Figure 3](image-url). Overexpression of npy induced the development of peripheral nerve systems in zebrafish embryos: (a) lateral view of untreated embryos and npy-mRNA-injected embryos at 5 days after fertilization, (b) qRT-PCR analysis of npy using untreated embryos and npy-mRNA-injected embryos [***$p < 0.001$], and (c) lateral view of confocal imaging at 5 days of post-fertilization untreated embryos and npy-mRNA-injected embryos from flil: GFP; huc: mCherry transgenic zebrafish. White arrow indicates the peripheral nerve system in zebrafish embryos.
DPN include oxidative stress, inflammation, microvascular alterations, nerve degeneration, and regrowth. Oxidative stress plays a key role in the pathogenesis of DPN. Thus, biomarkers for systemic oxidative stress have been investigated.

A large population-based Cooperative Health Research study was conducted in Augsburg, F4, and included a large proportion of individuals with prediabetes and T2DM. This study showed that myeloperoxidase was positively associated with DPN and that baseline superoxide dismutase levels were related to the incidence of DPN. Methylglyoxal is a highly reactive metabolite formed during pyruvate metabolism. High baseline blood methylglyoxal was significantly associated with DPN risk in a Danish cohort. Cross-sectional studies revealed a positive correlation between proinflammatory cytokines (interleukin-6 and tumor necrosis factor α), adipokines

Figure 4. Results of ELISA in type-2 diabetes patients: (a) serum level of NPY in type-2 diabetes patients with diabetic PN and without diabetic PN. (b) serum level of SLPI in type-2 diabetes patients with diabetic PN and without diabetic PN, (c) receiver operating characteristic curves for NPY, and (d) SLPI.
(adiponectin and leptin), and inflammation-related proteins (osteoprotegerin) and DPN development.\textsuperscript{22,23} ICAM-1 is an endothelial- and leukocyte-associated transmembrane protein reported to be positively associated with DPN.\textsuperscript{24}

Biomarkers are a cornerstone of medical diagnostics and are used in various applications, such as disease diagnosis, prognosis, and patient stratification. An array of molecular pathways involved in DPN pathogenesis has been reported; however, there are no disease-targeted treatments or biomarkers for predicting the onset or progression of DPN. In this context, hypothesis-free approaches involving multiomics technologies have been employed to identify biomarkers associated with pathways involved in DPN. Hur and colleagues\textsuperscript{13} examined gene expression profiles in sural nerve samples from two groups of patients with progressing or nonprogressing DPN. A literature-derived co-citation network of the DEGs revealed five subnetworks centered on apolipoprotein E, c-Jun, leptin, serpin peptidase inhibitor E type 1, and peroxisome proliferator-activated receptor-gamma. Ridge regression models, including 14 DEGs, correctly identified the progression status of 92% of the patients with DPN.

In the clinic, peripheral nerve biopsies, such as for the sural nerve, are rarely performed; thus, human tissue sources in omics studies are limited. McGregor and colleagues\textsuperscript{25} used a systems biology approach and demonstrated a highly conserved pathway across human and murine DPN models. Hinder and colleagues\textsuperscript{26} examined gene expression changes in the sciatic nerve and dorsal root ganglia of \textit{db/db} diabetic mice at 8, 16, and 24 weeks. They observed that multiple immunomodulators were upregulated throughout DPN and suggested that metalloproteinase 12 was notably prevalent throughout DPN progression.

We identified \textit{NPY} and \textit{SLPI} as commonly expressed DEGs in the peripheral nerve tissues of a mouse model of T2DM DPN and T2DM with DPN. Injection of \textit{npy}-mRNA into zebrafish embryos promoted peripheral and central nerve growth. NPY is a 36-amino acid peptide containing tyrosine residues and is a highly conserved endogenous peptide in all mammal central and peripheral nervous systems.\textsuperscript{27}

Several studies have investigated the function of NPY and its receptors in regulating food intake, blood pressure, seizure activity, anxiety, depression, and the pathogenesis of neurodegenerative disorders.\textsuperscript{28,29} A growing body of research has suggested that NPY and its receptors influence brain activity and drive feeding behaviors; research in this area is relatively young. Howell and colleagues\textsuperscript{30} found that NPY promoted dentate gyrus neural precursor cell proliferation. They also showed that NPY1 receptor knockout mice exhibited reduced proliferation of precursor cells and the generation of immature neuroblasts in the dentate gyrus compared to wild-type mice.

Furthermore, NPY treatment promoted the differentiation of newly generated stem cells toward hippocampal progenitors.\textsuperscript{31} Studies have shown that peripheral nerve injury causes an increase in NPY expression in adult dorsal root ganglion neurons in mice and rats.\textsuperscript{32,33} It appears to mediate antinociceptive actions via the NPY1 and NPY2 receptors in sciatic nerve injury models of neuropathic pain and plantar incision models of postoperative pain.\textsuperscript{34,35}

However, the roles of NPY in central nervous system neurogenesis and antinociception and its functions in the peripheral nervous system have not been widely examined. We found one study showing that in the peripheral nervous system, NPY promotes angiogenesis through the NPY2 and NPY5 receptors during ischemic revascularization and wound healing.\textsuperscript{36} Matyal and colleagues proved that plasma NPY levels were increased in patients with diabetes and nondiabetic subjects undergoing cardiac surgery under cardiopulmonary bypass.\textsuperscript{37} Atrial tissue NPY mRNA levels were lower in patients with diabetes than in nondiabetic patients. They suggested that the downregulation of NPY observed in patients with diabetes may decrease angiogenesis, dysregulate apoptosis, and increase vascular smooth muscle proliferation, resulting in coronary disease. They also explained that the discrepancy between the levels of NPY in the serum and atrial tissue is a compensatory increase in extra neuronal sources of NPY, such as smooth muscle cells, endothelial cells, and pancreatic acinar cells.

We compared the serum levels of NPY between patients with T2DM DPN and without T2DM DPN and showed that the serum level of NPY was increased in patients with T2DM DPN. As
the AUC of NPY was 0.7179, it may not suffice as a sole biomarker for detecting DPN in T2DM patients. Moreover, early-stage DPN is usually asymptomatic, leading to a delay in diagnosis and poor prognosis. If the serum level of NPY is serially investigated for T2DM patients and is revealed to be useful for detecting DPN in the early stage, it could be used in combination with current diagnostic tools and could impact T2DM patients’ prognosis. For this purpose, further prospective study should be performed on a larger cohort of T2DM patients.

The consistency of mRNA expression and serum levels of NPY in T2DM patients with DPN suggests that NPY is a key biomarker for detecting T2DM DPN. Based on the phenotypic changes observed in zebrafish overexpressing NPY, NPY may affect nerve regeneration in the peripheral nervous system.

This study had some limitations. Our clinical samples for the ELISA were relatively small. When we requested for serum samples from T2DM patients with DPN and without DPN from the Korea Biobank, only samples from 20 T2DM patients with DPN and 14 T2DM patients without DPN were available. Even though our sample size was not adequate (low statistical power) to reach a definite conclusion, baseline characteristics and comorbidities were not different between the two groups. Thus, our results could provide insights into the possibility of using NPY as a biomarker for DPN. In addition, we did not determine the molecular mechanism of NPY in T2DM DPN. However, our study suggests that NPY is mechanistically involved in nerve regeneration and can be used as a biomarker of neuropathy in patients with T2DM DPN.

**Conclusion**

We overexpressed NPY in T2DM DPN mouse models and T2DM patients with DPN. Following overexpression of npy in zebrafish, outgrowth of peripheral nerve and central nerve was observed. Finally, we found that NPY can function as a serum diagnostic marker for T2DM patients with DPN.

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**Author contributions**

N.R.C. and Y.H.K. made substantial contributions to the conception and design. D.S.K., K.M., and Y.L. collected data, and N.R.C. and Y.Y. performed statistical analysis. N.R.C. and H.S.N. analyzed and interpreted the data. C.-K.O. produced data using zebrafish. N.R.C. and C.-K.O. wrote the manuscript. D.S.K., H.S.N., and Y.H.K. made critical revision.

**Ethical statement**

The Institutional Review Board of the Gachon University Gil Medical Center (approval id. GCIRB2020-044) approved this study. The acquisition of informed consent was waived by the Institutional Review Board of the Gachon University Gil Medical Center as only unidentified information was collected. All experiments using zebrafish embryos were performed according to the guidelines of the Ulsan National Institute of Science and Technology Institutional Animal Care and Use Committee (approval number: UNISTIACUC-15-14, date: 2016-10-11).

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**Conflict of interest statement**

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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**Availability of data and materials**

The data that support the findings of this study are available from the corresponding author upon reasonable request.
Supplemental material
Supplemental material for this article is available online.

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