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SCN9A Variants May be Implicated in Neuropathic Pain Associated With Diabetic Peripheral Neuropathy and Pain Severity

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Objectives: Previous studies have established the role of *SCN9A* in various pain conditions, including idiopathic small fiber neuropathy. In the present study, we interrogate the relationship between common and rare variants in *SCN9A* gene and chronic neuropathic pain associated with diabetic peripheral neuropathy.

Design: Using a cohort of 938 patients of European ancestry with chronic neuropathic pain associated with diabetic peripheral neuropathy enrolled in 6 clinical studies and 2 controls (POPRES, n = 2624 and Coriell, n = 1029), we examined the relationship between *SCN9A* variants and neuropathic pain in a case-control study using a 2-stage design. The exonic regions of *SCN9A* were sequenced in a subset of 244 patients with neuropathic pain, and the variants discovered were compared with POPRES control (stage 1). The top associated variants were followed up by genotyping in the entire case collection and Coriell controls restricting the analysis to the matching patients from the United States and Canada only (stage 2).

Results: Seven variants were found to be associated with neuropathic pain at the sequencing stage. Four variants (Asp1908Gly, Val991Leu/Met932Leu, and an intronic variant rs74449889) were confirmed by genotyping to occur at a higher frequency in cases than controls (odds ratios ~2.1 to 2.6, $P = 0.05$ to 0.009). Val991Leu/Met932Leu was also associated with the severity of pain as measured by pain score Numeric Rating Scale (NRS-11, $P = 0.047$). Val991Leu/Met932Leu variants were in complete linkage disequilibrium and previously shown to cause hyperexcitability in dorsal root ganglia neurons.

Conclusions: The association of *SCN9A* variants with neuropathic pain and pain severity suggests a role of *SCN9A* in the disease etiology of neuropathic pain.

Key Words: targeted deep sequencing, *SCN9A*, Nav1.7, neuropathic pain, diabetic painful neuropathy

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Neuropathic pain is initiated or caused by a primary lesion or intrinsic dysfunction in the nervous system (International Association for the Study of Pain) either within the peripheral or central nervous system. Many patients with peripheral neuropathy have injury to small-diameter myelinated and unmyelinated nerve fibers, either in isolation or concomitant with damage to the larger myelinated nerve fibers. Although there are many causes of neuropathic pain, diabetes mellitus is the most common cause, with ~50% to 60% of the patients developing neuropathy as a long-term complication and ~10% to 26% experiencing neuropathic pain.[1,2]

Neuropathic pain can be severe and difficult to treat. Several medications have shown efficacy in treating the pain of diabetic peripheral neuropathy (DPN), including tricyclic antidepressants, the serotonin noradrenaline reuptake inhibitor duloxetine,[3–5] anticonvulsants such as pregabalin and gabapentin,[6–9] and opioid analgesics such as oxycodone.[10] However, treatment for many patients with painful diabetic neuropathy is limited by modest efficacy and/or by significant side effects associated with these agents. Better understanding of the disease mechanism should lead to better therapeutic intervention. Previous genetic studies have implicated the roles of myelin protein zero, *TRPV1*, *TRPA1*, and *HTR2C*, yet refuted the role of *COMT* in neuropathic pain. Specifically, a novel Trp101X myelin protein zero mutation and Met315Ile *TRPV1* genotypes in females only have been associated with neuropathic pain.[11,12] Rs6318 (Cys23Ser) in the *HTR2C* gene has been linked to pain relief during treatment with escitalopram in patients with neuropathic pain.[13] Transient receptor potential ankyrin 1 (*TRPA1*) 710 G > A (rs920829, E179K) was associated with the presence of paradoxical heat

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sensation ($P = 0.03$), and transient receptor potential vanilloid 1 (*TRPV1*) 1911 A > G (rs8065080, I585V) with cold hypoalgesia ($P = 0.0035$) in neuropathic pain patients.[14] *COMT* (Val158Met) polymorphism in contrast was shown not to be associated with neuropathic pain in a Spanish population.[15]

The sodium channel Nav1.7 encoded by *SCN9A* plays an important role in pain and nociception. Nav1.7 is preferentially expressed within the dorsal root ganglia, trigeminal ganglia, and sympathetic ganglion neurons and their fine-diameter axons, where it acts to amplify subthreshold stimuli, such as generator potentials in nociceptors. An important role for Nav1.7 in pain is supported by human genetic studies, where nonsense mutations cause a complete absence of pain, whereas gain-of-function mutations (single amino-acid substitutions) of *SCN9A* are linked to 3 pain syndromes: inherited erythromelalgia, paroxysmal extreme pain disorder, and idiopathic small nerve fiber neuropathy (I-SFN).[16–18] In a Dutch white patient cohort meeting strict criteria for I-SFN (ie, reduced intraepidermal nerve fiber density compared with age-adjusted and sex-adjusted normative values as confirmed by skin biopsy, plus abnormal QST, and no apparent cause), Faber et al[18] showed that a substantial proportion (28.6%; 8 of 28) of these I-SFN patients harbored heterozygous gain of function mutations (including p.M932L + p.V991L) in *SCN9A* and increased dorsal root ganglion neuron excitability, whereas none of these mutations was found in a control panel (DNA from 100 healthy white Dutch individuals). It was speculated that Nav1.7, $Na_v1.8$, and $Na_v1.9$ blockers may provide new treatment options to neuropathic pain.[19] In addition to I-SFN, missense gain-of-function mutations cause severe pain sensitivity in inherited erythermalgia[16] and paroxysmal extreme pain disorder[17]. In contrast, nonsense loss-of-function mutations in *SCN9A* cause a complete absence of pain sensation.[20–22] Furthermore, common variants in *SCN9A* are also implicated in pain conditions such as osteoarthritis (OA), sciatica, amputees with phantom pain, lumbar discectomy, and pancreatitis.[23] Specifically, genetic association study showed that pain perception was altered by a common variant rs6746030 (p.R1150W) in *SCN9A*, with the minor allele A being associated with increased pain scores compared with the major allele G in OA (n = 578, $P = 0.016$), amputees with phantom pain (Danish, n = 100, $P = 0.011$), lumbar discectomy (n = 179), pancreatitis (n = 205), and sciatica (Finnish, n = 195, $P = 0.039$), with a *P*-value of 0.0001 in the 5 cohorts combined (1277 patients in total).[23] Electrophysiological assessment by patch-clamp showed that the 2 rs6746030 alleles differed in the voltage-dependent slow inactivation ($P = 0.042$) in HEK293 cells, where the A allele was predicted to increase Nav1.7 activity. Rs6746030 was associated with an altered pain threshold in healthy females characterized by their responses to a diverse set of noxious stimuli (n = 186) and the effect was mediated through C-fiber activation. Finally, an intronic variant rs6754031 was shown to be associated with fibromyalgia and with Fibromyalgia Impact Questionnaire, although the study sample size was small (73 Mexican women with fibromyalgia and 48 age-matched women) and no multiplicity adjustment was applied.[24] Clearly, the emerging human genetic data strongly implicate Nav1.7 in a variety of painful conditions. Rather surprisingly, however, given the high clinical prevalence of diabetic neuropathy, very little is currently known regarding the role of this channel in pain associated with this condition. In this study, we aim to interrogate the relationship between common and rare variants in *SCN9A* gene and chronic neuropathic pain associated with DPN.

MATERIALS AND METHODS

Ethical Conduct of the Study

The clinical studies were conducted in accordance with the ethical principles set in the Declaration of Helsinki and are consistent with Good Clinical Practices and applicable regulatory requirements. Participation in the pharmacogenomic study was optional. All patients provided written informed consent before entering the study.

Patients and Clinical Assessments

Cases: Neuropathic Pain Associated With DPN

A full list of inclusion and exclusion criteria for patients enrolled into the 6 clinical studies (Table 1) could be found at clinicaltrials.gov or published manuscript. Briefly, patients enrolled into NCT00501202[25] and NCT00870454[25] must meet the following inclusion criteria: (1) have diabetes mellitus (type 1 or type 2); (2) have clinical evidence of diabetes-related peripheral neuropathy in the distal extremities for at least 6 months, or 1 to 5 years, respectively, before study entry. The symptoms must be attributable to sensorimotor neuropathy/DPN confirmed by history and detailed neurological examination; and (3) experienced lower extremity pain due to diabetic neuropathy on a nearly daily basis for the previous 3 months. For NCT00870454, patients are also required to have a mean daily average DPN pain score of ≥ 4 on NRS-11 during the baseline period. For NCT00993018,[26] patients must meet the following criteria: (1) DPN patients with pain (had to begin in the feet and with relatively symmetrical onset for

TABLE 1. Demographic and Clinical Variables of Neuropathic Pain Cases

Clinical Study	NCT00501202	NCT00870454	NCT00993018	NCT00455520	NCT01041859	NCT01063868
No. patients (n)	77	167	56	346	263	29
Genetic Analysis	Sequencing + Genotyping		Genotyping Only			
Age (y) (mean [SD])	57.9 (10.4)	59.1 (8.8)	59.6 (9.7)	62.6 (10.1)	60.5 (10.4)	59.6 (11.4)
Sex						
Female (n [%])	27 (35.1)	60 (35.9)	22 (39.3)	127 (36.7)	96 (36.5)	9 (31.0)
Male (n [%])	50 (64.9)	107 (64.1)	34 (60.7)	219 (63.3)	167 (63.5)	20 (69.0)
Pain severity score (NRS)						
Mean (SD)	6.5 (1.3)	6.5 (1.5)	6.3 (1.3)	7.0 (1.4)	7.3 (1.3)	7.6 (1.5)
Median (range)	6.3 (5-10)	6.4 (3.7-10)	6.2 (3.6-8.7)	7 (3-10)	7.2 (4.8-10)	8 (4-10)

> 6 mo) due to bilateral peripheral neuropathy caused by type 1 or type 2 diabetes mellitus; and (2) patients with mean average pain intensity score of at least 5, but < 10, over 7 consecutive days on NRS-11. For NCT00455520,[27] NCT01041859, and NCT01063868, patients with type 1 or 2 diabetes mellitus must have a documented clinical diagnosis of painful DPN with symptoms and signs for at least 6 months, and pain present at the time of screening. In addition, for NCT01041859 and NCT01063868, diagnosis must include pain plus reduction or absence of pin sensibility and/or vibration sensibility on Total Neuropathy Score—Nurse examination in the lower and/or upper extremities at screening. For NCT01063868, patients must also have baseline score for average pain intensity in the previous 24 hours of ≥ 4 on NRS-11 at the beginning of the titration period. The summary demographic information on this cohort is shown in Table 1. Of 1216 patients, ~77% from 6 clinical studies were of European ancestry. The 244 patients of European ancestry from the first 2 studies, and the 887 subset of patients of European ancestry from either the United States or Canada from all 6 studies, were included in stage 1 (sequencing and genotyping) and stage 2 (genotyping only) of genetic analysis, respectively (See Supplemental Digital Content Tables 1 and 2, Supplemental Digital Content 1, http://links.lww.com/CJP/A149).

Controls

Population reference sample (POPRES) cohort: genotyping data generated through the targeted deep sequencing are available through the dbGaP (http://www.ncbi.nlm.nih.gov/projects/gap/cgi-bin/study.cgi?study_id = phs000145.v4.p2). The sequenced participants included 3381 healthy controls from 2 studies that were part of the POPRES project[28]: CoLaus (N = 2059) and LOLIPOP (N = 1322), among which 2624 participants of European ancestry were included in the stage 1 study as population controls. Samples most likely were not screened for neuropathic pain phenotype. This cohort was sequenced by Nelson et al,[29] for a total of 202 drug target genes including *SCN9A*. Over 93% of target bases were successfully sequenced at a median depth of 27 reads per site. Paired-end short reads were aligned with SOAP and variants were called using SOAPsnp. There were a total of 50,432 such candidate variant sites identified and 772 variants were within the *SCN9A* amplicon region.

Coriell cohort: DNA samples from plates NDPT079, NDPT093, NDPT094, NDPT095, NDPT096, NDPT098, and NDPT099 and 4 custom plates were ordered from Coriell Institute. Sample selection criteria included white from the United States or Canada to match the majority of the case population, age 54 years and above, family/medical history absent of autism, bipolar (manic-depressive), dementia, depression, schizophrenia, suicide/attempt (either absent or blank), and memory loss (either absent or blank). Samples most likely were not screened for neuropathic pain phenotype.

SCN9A-targeted Resequencing and Variant Calling

SCN9A was one of the 202 drug targets extensively sequenced in a study consisting of 14,002 patients.[29] The populations sequenced included 2 population reference samples (n = 1322 and 2059) that was referred to as POPRES[28]) and 12 disease collections (n = 125 to 1125 cases, coronary artery disease, metabolic syndrome, multiple sclerosis, OA, rheumatoid arthritis, irritable bowel syndrome, epilepsy, Alzheimer disease, unipolar depression, bipolar disorder, schizophrenia, and chronic obstructive pulmonary disease). Neuropathic pain was not one of the disease cohorts sequenced, although some of the patients such as multiple sclerosis may indeed have neuropathic pain. Considering that there might be disease-specific variants to be uncovered, we embarked on a targeted deep sequencing of the *SCN9A* gene in a subset of 244 patients with chronic neuropathic pain enrolled in NCT00501202[25] (n = 77) and NCT00870454[25] (n = 167). It was estimated that sequencing ~200 samples would likely identify 98% of variants with 1% minor allele frequency (MAF).[30] *SCN9A*-targeted resequencing was performed using a TruSeq Custom Amplicon Kit designed to amplify the promoter and exon sequences of the human *SCN9A* gene. The targeted exon sequences are the coding regions plus 25 bp of flanking intron sequence, whereas the promoter included 1 kb 5′ of exon 1. The design targeted 11,146 bp and covered the entire promoter region, the exons, and part of the 3′ UTR that did not contain repetitive sequences. The amplicons extended past the target region and totaled 17,571 bp. Targeted resequencing was performed using paired-end 2 × 250 bp sequencing using Illumina MiSeq (Illumina Inc., San Diego, CA) by ACGT Inc. (Wheeling, IL). There were 4 runs of sequencing experiment in total. The average target coverage per run ranged from 1292× to 1918×. The regions that poorly amplified and had the lowest coverage were part of the promoter, part of the 3′ UTR, exon 9, and exon 24. The sequence reads were mapped to the *SCN9A* reference sequence and variants were called using GATK1.6.[31] Variant calls were filtered to remove those that failed any one of the filters, including low-variant frequency (variant frequency < 20% or > 80% for heterozygous sites), high-reference frequency (for homozygous alternative allele sites with reference frequency > 20%), low depth/coverage (depth < 10 for sites carrying homozygous reference or homozygous alternative alleles ([1/1 or 1/2 sites], alternative or reference allele count < 10 for heterozygous sites), low-sequence confidence (QUAL < 30 or FILTER = "LowQual," GQ < 30 for heterozygous sites and sites with homozygous alternative alleles 1/1 or 1/2), or an indel called in a region with a large number of repeats, and variants with a low score.

Genotyping

Genotyping of the entire case cohort and Coriell control cohort was carried out using polymerase chain reaction/ligase detection reaction (PCR/LDR). PCR was performed in a multiplex reaction as a 7-plex reaction. Genomic DNA (100 ng) was amplified in a 50 μL reaction mixture (0.8 U AmpliTaq Gold, 1× Gold Buffer, 3 mM $MgCl_2$, 0.4 mM dNTP mix, 0.2 μM of each primer) using the following cycling conditions: 1 cycle of 94°C for 4 minutes, 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 68°C for 1 minute, and 1 cycle of 68°C for 7 minutes. LDR was also performed in multiplex in 7-plex and 8-plex reactions using the PCR templates from the corresponding PCR multiplexes. LDR conditions are described elsewhere.[32] PCR primers, LDR primers, and synthetic templates are listed in Supplemental Digital Content Tables 3, 4, and 5 (Supplemental Digital Content 1, http://links.lww.com/CJP/A149), respectively.

Genetic Association Analysis

At the end of sequencing stage, variants with MAF > 1% in either cases (sequencing cohort only) or POPRES controls were used in the allelic test where differences between

TABLE 2. Single Marker Case-Control Association Results

					Minor Allele Frequency					Genotype Frequency$_{case}$			Genotype Frequency$_{control}$			P_{HWD}	
dbSNP137	Ref	Alt	Feature	AA Pos*	Case	Control	P_{Fisher}	P_{perm}	OR_{Fisher}	Alt/ Alt	Ref/ Alt	Ref/ Ref	Alt/ Alt	Ref/ Alt	Ref/ Ref	Case	Control
887 cases, 1029 Coriell controls																	
rs74449889	G	A	Intron		0.010	0.004	0.028	0.064	2.6	0	18	869	0	8	1019	1	1
rs3750904	C	T	Non-synonymous	p.D1908G	0.018	0.008	8.96E-03	0.024	2.2	2	28	857	2	13	1012	0.030	0.002
rs4369876	A	C	Non-synonymous	p.V991L	0.011	0.005	0.047	0.106	2.1	0	20	867	1	9	1017	1	0.027
rs12478318	G	T	Non-synonymous	p.M932L	0.011	0.005	0.047	0.106	2.1	0	20	867	1	9	1017	1	0.027

*Amino acid position is in reference to NP_002968.1.

cases and controls in the frequency of the alleles were evaluated by either χ^2 test (for variants with MAF > 5%) or Fisher exact test (for variants with MAF < 5%) as implemented in PLINK (v1.07).[33] A multiple test correction (Bonferroni correction) was applied to correct for the number of variants tested. After genotyping confirmation of sequencing data and generating genotyping data for the entire case collection and Coriell controls, the allelic test in the entire collection of cases (restricting to patients from the United States and Canada using country as a surrogate for control matching) and controls (excluding POPRES) was repeated for the variants with confirmed genotype/association in the initial sequencing cohort and 10,000 permutations were performed using PLINK–mperm option to correct for the number of tests in the full cohort, where permutation *P*-values are calculated as $(R + 1)/(N + 1)$, where R is the number of times the permuted test is greater than the observed test, and N is the number of permutations. For rare variants, rare variants with MAFs < 1% in cases and POPRES controls were tested for "unusual distribution" using C(α),[34] as implemented in variant tools[35] (v1.0.6), using 3 variant set definitions. C(α) tests the hypothesis of rare variant disease association under the assumption that rare variants observed in cases and controls are a mixture of phenotypically deleterious, protective, and neutral variants. Instead of using a cumulative dosage (or "burden")-based summary statistic over a gene region, it directly contrasts the observed and expected distribution of minor alleles in cases and controls at each locus as an evidence of "unusual distribution," and combines evidence from multiple loci to formulate the C(α) test statistics.

RESULTS

Using a cohort of 938 patients of European ancestry with chronic neuropathic pain associated with DPN enrolled in 6 clinical studies and 2 population control cohorts (POPRES, n = 2624 and Coriell, n = 1029), we examined the relationship between both common and rare variants in *SCN9A* and chronic neuropathic pain associated with DPN in a 2-stage case-control study. In stage 1, a targeted resequencing of the *SCN9A* gene in a subset of 244 patients with chronic neuropathic pain enrolled in NCT00501202[25] (n = 77) and NCT00870454[25] (n = 167), a total of 170 variants were discovered as candidate variant sites. Nelson and colleagues observed a total of 772 variants within the *SCN9A* amplicon region identified from a total of 14,002 patients, some of which are observed in the POPRES controls of European ancestry. Of the 170 variants identified in painful DPN cases, ~62 overlapped with POPRES variants, whereas ~107 variants were not detected in POPRES (55 of them were in dbSNP Build 137 and 52 of them were not). Among these, 187 variants were polymorphic in either cases or controls, and 21 of them had a MAF > 1% and were included in single-marker Fisher exact test. A total of 21 SNPs with MAF > 1% in either cases or controls (multiplicity-adjusted raw *P*-value threshold ~0.00238) were tested, and 7 variants exhibited *P*-value < 0.00238 and were followed up in the genotyping experiment.

To confirm the genotype accuracy, we first genotyped the same cases from the sequencing cohort (stage 1), the concordance rate between sequencing and genotyping results was >99.2%. However, 4 variants remained significant after redoing single-marker case-control association analysis in the same cohort of 244 cases and 2624 POPRES controls. The other 3 variants were no longer significantly associated with neuropathic pain due to sequencing/variant call errors (n = 2, variant frequencies as defined by the number of reads supporting alternative alleles divided by the total number of reads were between 20% and 30% and passed the filter criteria as described earlier), or due to excessive missing data in sequencing stage (n = 1). To increase the case sample size and use controls better matched to cases, we next genotyped additional cases from 4 additional clinical studies (NCT00993018,[26] NCT00455520,[27] NCT01041859, and NCT01063868) and Coriell controls from the United States and Canada (See Supplemental Digital Content Table 2, Supplemental Digital Content 1, http://links.lww.com/CJP/A149). The 4 variants' association statistics in the full cohort are listed in Table 2. Although p.M932L and p.V991L are in complete linkage disequilibrium (Fig. 1), they are in partial linkage disequilibrium with p.D1908G and the intronic variant rs74449889. Using the genotyped cohorts of 887 cases and 1029 controls of European ancestry from the United States and Canada, the association *P*-values for p.D1908G, p.M932L/p.V991L, and rs74449889 were 0.009, 0.05, and 0.03, respectively (permutation *P*-values were 0.02, 0.06, and 0.10, respectively; odds ratios between 2.1 and 2.6, the minor alleles occurred at a higher frequency in cases than controls). Although there was a slight deviation from Hardy-Weinberg Equilibrium for p.M932L and p.V991L in controls, these 2 variants were independently genotyped and 100% concordant suggested that it is unlikely for this deviation to be due to a genotyping error.

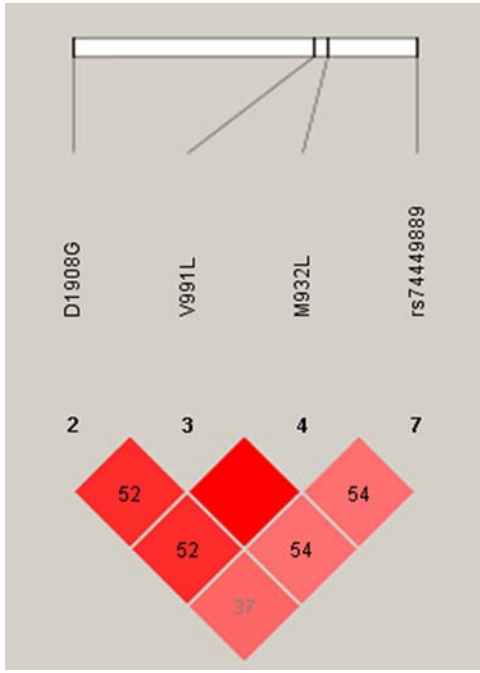

FIGURE 1. LD relationship among *SCN9A* variants (LD color scheme is standard D′/LOD, LD values are R^2).

Slight deviation from the Hardy-Weinberg Equilibrium for p.D1908G in both cases and controls were also observed; however, a visual inspection of the multiplex PCR-LDR assay trace files (Supplemental Digital Content Figure 1, Supplemental Digital Content 1, http://links.lww.com/CJP/A149) suggested that the genotyping assay was robust. We further tested whether these 4 variants were associated with the severity of pain associated with DPN as measured by the NRS-11, which is an 11-point scale for patient self-reporting of pain. For the p.M932L/p.V991L variant, the patients carrying the minor variant L_{932}/L_{991} experienced in average 0.6 point higher in pain score than patients carrying homozygote M_{932}/V_{991} (Table 3).

Our findings also suggest an apparent differential distribution of rare variants between cases and controls in the sequencing cohort. All 3 variant sets showed significant rare variant association, suggesting that the observed distribution of minor alleles in cases and controls has an "unusual distribution" (Table 4). Although interesting, these findings should be interpreted with caution for several reasons. In silico prediction of the functional consequences of amino-acid substitutions has low accuracy. For example, both p.M932L and p.V991L variants were predicted to be benign mutations, yet displayed functional consequence in channel characterization assays. In addition, most of these rare variants were not confirmed by genotyping in both the case and control cohorts, although the overall concordance rate between sequencing and genotyping results was >99.2%, based on the confirmation of 7 variants in the first 2 clinical studies.

DISCUSSION

The findings of the genetic association between p.M932L/p.V991L and neuropathic pain associated with DPN as well as pain severity are consistent with the observation of p.M932L/p.V991L in I-SFN population and the characterization of p.M932L/p.V991L mutant channels by Faber et al[18] where it was demonstrated that the mutant channel enhanced the generation of resurgent currents and hyperexcitability. Persson et al[36] also showed a trend toward reduced (~7%) neuritic length in dorsal root ganglia neurons expressing the M932L/V991L mutant channel, although this finding did not reach the level of statistical significance. Whereas p.M932L may be located at the S6 of domain II and p.V991L is located within the L2 cytoplasmic loop between domain II and III, p.D1908G is likely located at the cytoplasmic C-terminal domain. It appears that the p.D1908G mutant channel has not yet been characterized in detail, and future characterization of this mutation and the intronic variant is warranted.

Rs6746030 (p.R1150W) was not significantly associated with neuropathic pain in the sequencing cohort and was not followed up. Retrospectively, this variant should have been followed up, as the initial sample size might be too small and could have failed to result in a significant association. There were also a few caveats with this initial analysis. The case and control samples were assayed using different platforms (different depth, different alignment, and variant calling algorithms). In addition, the POPRES control samples and the case samples were not ideally matched genetically, with the majority of the case samples but not the POPRES samples originating from the United States or Canada. These problems have been addressed by the genotyping stage with the matching controls. However, we cannot rule out a false-negative association at the sequencing stage, resulting in a variant of interest not being followed up.

Although individual uncommon variants (MAF < 5%) were confirmed to carry different allele frequency in patients of European ancestry using country as surrogate for case-control matching, the genetic effect sizes of these uncommon variants are not large. Furthermore, both p.M932L/p.V991L and p.D1908G have higher frequency in Asian population (5% to 10%, specifically based on the HapMap samples) than in European population. It is unclear, however, whether race is a risk factor for neuropathic pain associated with DPN though. The differences in case ascertainment tool used and presentation of prevalence rates make it difficult to compare prevalence rates across studies and draw conclusion on the impact of race. Furthermore, most studies only examined the prevalence rate for peripheral neuropathy.

TABLE 3. Single Variant Linear Regression With Baseline Pain Score (n = 924)

Variant	AA Change	A_1	β	*P*	n_{11} (%)	n_{12} (%)	n_{22} (%)	$Mean_{11}$ (SD)	$Mean_{12}$ (SD)	$Mean_{22}$ (SD)
rs4369876	V991L	A	0.6	0.05	0 (0.0)	20 (2.3)	855 (97.7)	NA	7.6 (1.5)	7.0 (1.4)
rs12478318	M932L	G	0.6	0.05	0 (0.0)	20 (2.3)	855 (97.7)	NA	7.6 (1.5)	7.0 (1.4)

A_1 is the minor allele, n_{11}, n_{12}, and n_{22} represents the sample size for rare homozygote, heterozygote, and common homozygote genotype group.

TABLE 4. Rare Variant Set-based Association Analysis

Rare Variant Set (MAF < 0.01)	Sample Size	No. Variants	Total MAC	Statistics	*P*
C(α)					
Deleterious mutation (AVSIFT≤0.05)	2869	53	191	24.5	0.003
Nonsynonymous, stopgain	2869	78	407	793.8	0.000
Nonsynonymous, stopgain, splicing, UTR	2869	106	501	789.3	0.000

Strengths and Limitations of This Study

We systematically screened for putative functional variants in *SCN9A* gene using a targeted deep sequencing approach in the neuropathic pain population and confirmed some of the discovered variants and association in a large matching case and control population. Despite the power for uncommon variants being low for single-marker analysis, we have identified a few variants passing or close to passing permutation *P*-value threshold of 0.05 (family-wise error rate). Future replication to confirm the observed association is warranted. Only 7 variants were followed up by orthogonal genotyping approach. The lack of independent analytical validation of rare variant accuracy weakens the set-based association analysis.

CONCLUSIONS

Taken together, our study, using both common and rare variant approaches, suggested a possible role for uncommon and rare variants of *SCN9A* in the disease etiology of neuropathic pain associated with DPN. Uncommon and rare variants were associated with neuropathic pain and pain severity score. These findings may facilitate the evaluation of Nav1.7 blockers in this pain population, although a specific genetically defined subpopulation cannot be easily identified due to allelic heterogeneity.

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