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Genomic analysis of 21 patients with corneal neuralgia after refractive surgery

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Abstract

Background: Refractive surgery, specifically laser-assisted in situ keratomileusis and photorefractive keratectomy, are widely applied procedures to treat myopia, hyperopia, and astigmatism. After surgery, a subgroup of cases suffers from persistent and intractable pain of obscure etiology, thought to be neuropathic. We aimed to investigate the contribution of genomic factors in the pathogenesis of these patients with corneal neuralgia.

Methods: We enrolled 21 cases (6 males and 15 females) from 20 unrelated families, who reported persistent pain (>3 months), after refractive surgery (20 laser-assisted in situ keratomileusis and 1 photorefractive keratectomy patients). Whole-exome sequencing and gene-based association test were performed.

Results: Whole-exome sequencing demonstrated low-frequency variants (allele frequency < 0.05) in electrogenisome-related ion channels and cornea-expressed collagens, most frequently in *SCN10A* (5 cases), *SCN9A* (4 cases), *TRPV1* (4 cases), *CACNA1H* and *CACNA2D2* (5 cases each), *COL5A1* (6 cases), *COL6A3* (5 cases), and *COL4A2* (4 cases). Two variants, p.K655R of *SCN9A* and p.Q85R of *TRPV1*, were previously characterized as gain-of-function. Gene-based association test assessing "damaging" missense variants against gnomAD exome database (non-Finnish European or global), identified a gene, *SLC9A3R1*, with statistically significant effect (odds ratio = 17.09 or 17.04; Bonferroni-corrected *P*-value < 0.05).

Conclusion: These findings in a small patient cohort did not identify a common gene/variant among most of these cases, as found in other disorders, for example small-fiber neuropathy. Further studies of these candidate genes/variants might enhance understanding of the role of genetic factors in the pathogenesis of corneal neuralgia.

Keywords: Pain, Cornea, Neuralgia, LASIK, Whole-exome sequencing

1. Introduction

Persistent corneal pain occurs in a subgroup of patients after refractive surgical procedures including laser-assisted in situ keratomileusis (LASIK) and photorefractive keratectomy (PRK). Although the pain is often described as dryness, tear production seems normal in these patients.[86,95] The syndrome has been linked to phantom pain after trauma,[4] and may be analogous to other persistent postoperative pain disorders.[61] This chronic postrefractive surgery corneal pain has been described as corneal neuralgia (CN),[83] neuropathic corneal pain,[41] and ocular neuropathic pain.[76] In this study, we refer to these patients as having CN.

Ectopic dorsal root ganglion (DRG) and trigeminal ganglion (TG) sensory neuron firing has been suggested to produce neuropathic pain after nerve injury,[12,21,92]; substantial evidence indicates that sodium channels can drive this ectopic impulse activity in animal models[20,94] and in humans.[9,11,19] Other channels, including *CACNA1A* and *TRPV1*, have also been linked to neuropathic pain.[10,70] Collagens play a key role in peripheral nerve development and the maintenance of normal nerve function during adulthood.[50,58] Function-altering collagen gene variants might be expected to impact the regeneration or function of corneal nerve fibers that are severed or damaged by refractive surgery.

Whole-exome sequencing (WES) provides the capability to find protein-altering variants from the ~20,000 coding genes within the human genome. In this study, we concentrated on

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uncommon gene variants within the electrogenisome (ensemble of genes that play major roles in tuning neuronal electrogenesis), which contribute to sensory neuron function and/or pain signals, and collagens, which are major structural elements within the cornea. We selected low-frequency missense variants in each gene, and performed a gene-based association test, comparing our case cohort with control populations.

2. Methods

2.1. Patient selection

We enrolled 21 cases (6 males and 15 females), who developed persistent (>3 months) corneal pain after LASIK/PRK surgery. Patients were recruited through posts "Do you have eye pain after LASIK?" to a Facebook group for CN patients, to a Facebook LASIK complications support group, and by email blast to The Dry Eye Zone mailing list (>18,000 members). Inclusion criteria for the phone questionnaire were history of LASIK or PRK with postoperative pain lasting >3 months, and U.S. residence. Clinical history and Ocular Surface Disease Index (OSDI) were ascertained by telephone questionnaire, assisted by a board-certified ophthalmologist. Exclusion criteria upon completion of the questionnaire included reported diagnosis or treatment of dry eye before surgery, any history of incisional refractive surgery or lid surgery, and OSDI score at time of questionnaire of </= 12. Qualifying patients provided informed consent and were sent instructions, a phlebotomy tube, and packaging materials to facilitate submission of a blood sample for WES. Cases 1 and 9 are brothers from the same pedigree. All 21 cases were American Caucasians. This study was approved by the Yale Human Investigation Committee.

2.2. Whole-exome sequencing and variant filtering

Whole blood samples were collected from 21 cases, and genomic DNA was extracted using NucleoSpin Tissue kit (Machery-Nagel, Duren, Germany). Exome library preparation and sequencing with Illumina NovaSeq6000 (101-bp paired-end) were performed by Yale Genome Analysis Center. Data processing and variant calling were as previously described.[23]

Variant call format files were processed for annotation and filtering using Ensembl Variant Effect Predictor (VEP, v97.2) tool and in-house R scripts. Frequency of all variants was checked against databases of Genome-Aggregation Database (gnomAD; global and non-Finnish European population), Exome Aggregation Consortium, 1000 genomes, NHLBI GO Exome-Sequencing Project (ESP), UK10K, and Yale whole-exome databases. PhastCons100way, SIFT_converted_rankscore, Polyphen2_HVAR_rankscore, and CADD_phred scores were annotated using the dbNSFP plugin.[62] Only protein-altering variants with high/moderate "IMPACT" were retained for further analysis.

A 4-step quality-based variant filtering was used: (1) sequencing read depth ≥ 10-fold; (2) variant allele frequency >20%; (3) genotype quality ≥ 20; and (4) passed GATK quality filters. Variants with allele frequency ≥ 0.05 in any of the annotated databases were removed.

2.3. Sanger sequencing

Genomic DNA was amplified using High-Fidelity AccuPrime Taq DNA Polymerase according to manufacturer's protocol (ThermoFisher Cat#12346-086). PCR amplicons were sequenced at the Yale Keck DNA Sequencing facility.

2.4. Transcriptome-level annotation

Gene expression profiles were obtained from published RNA sequencing data sets of human TG or adult corneal (AC) tissue.[13,38] Gene expression levels were estimated using mean values of normalized FPKM (Fragments-Per-Kilobase of gene per Million mapped reads) separately in each data set. Transcripts with FPKM >1 were retained, and grouped into 3 levels of expression, low (human_TG > 1 & < 10; human_AC >1 & < 4.7), moderate (human_TG ≥ 10 & < 100; human_AC ≥ 4.7 & < 15.9), and high (human_TG ≥ 100; human_AC ≥ 15.9).

2.5. Gene-based association test

Considering the homogenous ancestry of our CN cohort, we leveraged the non-Finnish European (NFE) exomes (n = 56,885) from gnomAD (v2.1) as control subjects, for gene-based association test against our 20 unrelated cases with CN (case 9 removed). Variants obtained from gnomAD were operated through identical annotation and filtering pipelines as the case cohort using VEP (v97.2).

Based on the estimated prevalence of CN after surgery, we introduced an MAF cutoff of 5% for case and control samples, as previously applied for low-frequency variant association studies.[39,67,72] Only qualified missense variants within targeted protein-coding genes (n = 19,363) were analyzed. Statistical genotypes of variants of cases were converted to discrete alternate allele counts, and tabulated with NFE data for each gene as previously described.[43] Odds ratio (OR) and 95% confidence interval were calculated; using two-sided Fisher exact test or χ^2 test, *P* values were calculated, followed by Bonferroni correction with p.adjust package, and would be considered significant if <0.05.

In addition to using all low-frequency missense variants, we adopted 2 diverse strategies to identify "damaging" missense variants: (1) PhastCons100way score >0.9; and (2) SIFT_converted_rankscore >0.39575, Polyphen2_HVAR_rankscore >0.47121, and CADD_phred ≥ 15. As an alternative analysis, we used the global exome from gnomAD (125,748 samples) (**Fig. 1**). Variant preparation and statistical analyses were performed within R studio.

3. Results

3.1. Clinical summary

The cases with LASIK (20 cases) or PRK (1 case) (3 with LASIK enhancement and 2 with PRK enhancement) underwent surgery at ages 26~62 years (40.7 ± 11.6 years). Corneal neuralgia emerged with variable latencies after surgery, within one week (11 cases), less than 3 months (5 cases), and after 3 months (2 cases). The majority reported bilateral CN (17/21). All patients had bilateral refractive surgery except patient C08. The OSDI score ranged from 12.5 to 100 (45.9 ± 21.7), with no significant difference between males (37.90 ± 22.37) and females (49.06 ± 21.30). Five cases had a history of migraine (C02, C07, C14, C16, and C21). Four cases had records of hypothyroidism (C06, C07, C10, and C11), and one had Hashimoto disease (C20). Five cases reported LASIK/PRK enhancement procedures in one or both eyes (**Table 1**).

We divided our patients into 3 groups, based on age at which they underwent LASIK/PRK surgery, consisting of young (<= 30 year old; 6 cases), middle (>30 and ≤ 50 year old; 10 cases), and old (>50 year old; 5 cases). We compared the OSDI scores among these groups, and the mean score of old group

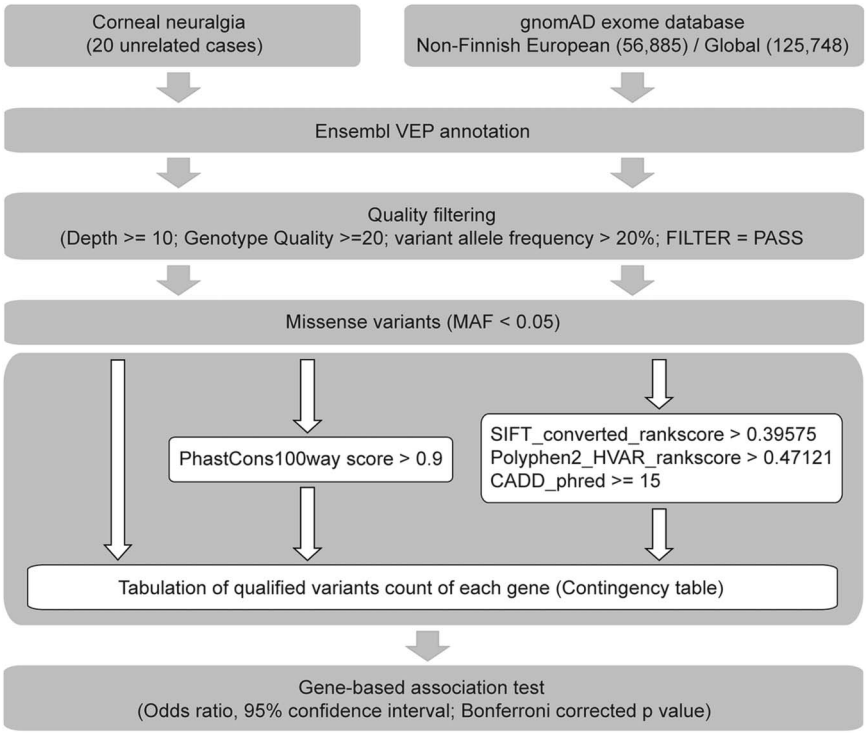

Figure 1. Gene-based association test workflow.

(38.82 ± 10.19) was lower than the young (50.37 ± 27.63) and middle-age (46.70 ± 23.04) groups; however, no significant difference was observed. The OSDI score of patients with and without migraine (62.04 ± 25.41 vs 40.82 ± 18.42) or thyroid disease (45.54 ± 30.97 vs 45.98 ± 19.24) showed no statistically significant difference. However, the mean OSDI score of cases with migraine was higher than that of cases without migraine history (**Fig. 2**). All these statistical analyses were performed using one-way ANOVA test in Origin (Version 2018; OriginLab Corporation, Northampton, MA), with *P*-value < 0.05 considered significant.

3.2. Whole-exome sequencing data analysis

All 21 WES were captured and sequenced at an average of 74-fold, and 98.4% of the target regions with mean coverage of 10 or higher. After quality and frequency filtering, we collected 12,584 low-frequency variants with "MODERATE/HIGH" impact annotated by VEP, consisting of missense variants (11,933; 94.83%), frameshift (328; 2.61%), stop-gained (179; 1.42%), splice-donor (66; 0.52%), splice-acceptor (45; 0.36%), start-lost (23; 0.18%), and stop-lost (10; 0.08%) (**Fig. 3**; supplementary Table 1, available at http://links.lww.com/PR9/A65).

3.3. Gene panel analyses

We identified variants of recurrent genes from multiple cases with CN (case number ≥ 3), including genes: (1) expressed in TG (human_TG > 1) and involved in electrogenesis in nociceptors, consisting of sodium, calcium, potassium, chloride, transient receptor potential (TRP), and gap junction channels[23]; and (2) collagens expressed in cornea (human_AC >1) that might affect axonal growth/regeneration/integrity. Because 5 cases had a history of migraine in our cohort, we also located variants in the genes responsible for familial hemiplegic migraine (*CACNA1A*, *ATP1A2*, and *SCN1A*).[82] Variants found in 3 or more cases are presented in **Table 2**.

3.3.1. Sodium channel genes

Gain-of-function mutations in *SCN9A* (encoding sodium channel Nav1.7) have been shown to increase excitability in DRG and TG neurons and have been linked to multiple pain disorders,[93] including inherited erythromelalgia, paroxysmal extreme pain disorder, and small-fiber neuropathy.[15,34,37,100] We detected low-frequency Nav1.7 variants p.P610T (C01, C09), p.K655R (C05), and p.V1428I (C19). p.P610T (frequency in gnomAD exome = 0.024) was previously described as a benign/likely benign polymorphism, but has not been electrophysiologically characterized.[57,69,78] However, p.K655R has been reported to shift activation in a depolarizing direction, and to accelerate recovery from inactivation.[28,80,101] p.V1428I was briefly described as a variant that does not cause biophysical abnormalities in Nav1.7.[52]

Increasing evidence has linked gain-of-function variants of *SCN10A* (encoding Nav1.8) to small-fiber neuropathy.[35,49,97] In this study, we found 4 low-frequency variants in Nav1.8, including p.I206M (C21), p.P1045T (C04, C11, C14), p.V1697I (C21), and p.R1847Q (C02). Although these variants have been reported in patients with pain syndromes or associated with inflammatory bowel disease (p.I206M),[40] cardiac conduction (p.P1045T),[44] or atrioventricular nodal reentrant tachycardia/chronic kidney disease (p.V1697I),[44,98] none of them has been functionally characterized. All variants in *SCN9A* and *SCN10A* were validated using Sanger sequencing.

Variants in *SCN7A* were found in C05 (p.R683Q), C19 (p.R1516K), and C20 (c.3712-2A>G). To date, *SCN7A* has not been found to be associated with any human disease; however, it is noteworthy that its RNA level in human TG neurons is much

Table 1

Clinical data of 21 cases with corneal neuralgia.

Case	Gender	Age at surgery (y)	Beginning of pain	Sides of pain	OSDI score	Years of surgery	LASIK/ PRK	Enhancement	Migraine	Thyroid disease	Other reported conditions
C01	Male	31	6 wk	B	12.5	2018	LASIK	No	−	−	/
C02	Male	47	Immediate	R to B	37.5	2015	LASIK	No	+	−	/
C03	Male	26	5 d	R	38.6	2016	LASIK	No	−	−	Marfan syndrome?
C04	Female	62	1 wk	R	31.8	2012	LASIK	No	−	−	Ulcerative colitis; gastroparesis
C05	Female	46	Immediate	L	30.5	2001	LASIK	No	−	−	Dental pain; hypertension
C06	Female	43	2 y	B	22.2	2005	LASIK	No	−	Hypothyroidism	Discoid lupus
C07	Female	30	1 mo	B	100.0	1999/2019	LASIK/ PRK	Yes	+	Hypothyroidism	Epstein–Barr virus infection
C08	Female	58	Immediate	R	56.8	2017	LASIK	Yes	−	−	LASIK in R eye only
C09	Male	26	Months	B	15.9	2018	LASIK	No	−	−	Brother of C01
C10	Female	55	Days	B	34.1	2002	LASIK	No	−	Postthyroidectomy	Heart attack
C11	Female	56	1 mo	B	35.0	2008	LASIK	No	−	Hypothyroidism	Breast cancer; hypertension; heart blockage
C12	Female	37	Immediate	B	42.5	2006	LASIK	No	−	−	/
C13	Male	27	2–3 wk	B	50.0	2006	LASIK	No	−	−	Postnephrectomy
C14	Female	30	Immediate	B	52.3	2018	LASIK	No	+	−	/
C15	Female	41	Weeks	B	62.5	2017	LASIK	No	−	−	Irritable bowel syndrome
C16	Female	25	Immediate	B	45.4	2013	LASIK	No	+	−	/
C17	Female	38	Immediate	B	36.4	1994/1994	PRK/ PRK	Yes	−	−	Asthma
C18	Male	33	Immediate	B	72.9	2013	LASIK	No	−	−	/
C19	Female	46	Immediate	B	75.0	2001	LASIK	No	−	−	/
C20	Female	52	Immediate	B	36.4	2008/2009	LASIK	Yes	−	Hashimoto disease	/
C21	Female	45	<1 y	L to B	75.0	2000/2001	LASIK	Yes	+	−	Diabetes; temporomandibular joint disorder

LASIK, laser-assisted in situ keratomileusis; PRK, photorefractive keratectomy.

higher (FPKM = 32.83) than *SCN9A* (FPKM = 12.1) and *SCN10A* (FPKM = 8.31).[38]

3.3.2. Transient receptor potential channel genes

We found a set of low-frequency variants in TG-expressed TRP channel genes from 2 families, *TRPV1* (4 cases, C03, C07, C11, C14) and *TRPM2* (3 cases, C06, C10, C19). Among these variants, p.Q85R in *TRPV1* was identified from 3 cases (C03, C11, and C14), whereas the other variants were detected only from singletons. Gain-of-function mutations of *TRPV1* have been associated with neuropathic pain.[10,29] TRPM2 expression in nociceptive neurons from TG has a role in peripheral Inflammation.[14] None of the variants in *TRPM2* were previously described or functionally analyzed, except p.A890V, which was reported in a patient with bipolar disorder.[59] In addition, we found 2 variants, p.D665N (C02) and p.V915M (C13), in the *TRPM8* gene.

3.3.3. Calcium channel genes

From 3 CN cases, we detected missense variants (p.R1161G and p.R2155C) in the *CACNA1A* gene. Although *CACNA1A* has been linked to familial hemiplegic migraine,[70] these variants have never been described, and none of these patients (C01/C09 and C12) had a history of migraine. Loss-of-function mutations of *CACNA1H* were found associated with a pediatric patient with chronic pain.[81] In our patients, we also detected 2 heterozygous missense variants of *CACNA1H* from C07 (p.R477H and p.V780F), as well as heterozygous variants 4 other cases, p.R506Q (C08), p.A555V (C10), p.T920M (C11), and p.A1705T (C12). Two variants, p.A555V and p.A1705T, have been reported in patients with epilepsy.[31,46] In addition, from 5 cases (C01/C09, C02, C03, and C06), the p.R75Q variant was detected in *CACNA2D2*, which encodes an alpha-2/ delta subunit of voltage-dependent calcium channel.

3.3.4. Potassium channel genes

KCNB1 (encoding potassium channel Kv2.1) and *KCNT1* (encoding sodium-activated potassium channel KNa1.1) have been linked to various epilepsy phenotypes.[3,47,84] We found 2 variants from *KCNB1*, consisting of p.T616S (C01, C09, and C13) and p.S857N (C13), and 3 variants from *KCNT1*, including p.G20A (C12), p.P1099S (C11), and p.A1130T (C02). In addition, we detected 3 variants in *KCNK6* (encoding 2-pore potassium channel, K2p6.1) and *KCNH6* (encoding Kv11.2) (**Table 2**).

3.3.5. Collagen genes

Collagens are crucial components in the corneal epithelium (type IV and VII),[87] nonregenerative Bowman layer (type I, III, and V),[96] and corneal stroma (type I, VI, V, III, XII, and XIV).[1] Collagen fibrils in stroma play a key role in peripheral nerve development and the maintenance of normal nerve function during adulthood.[50,58]

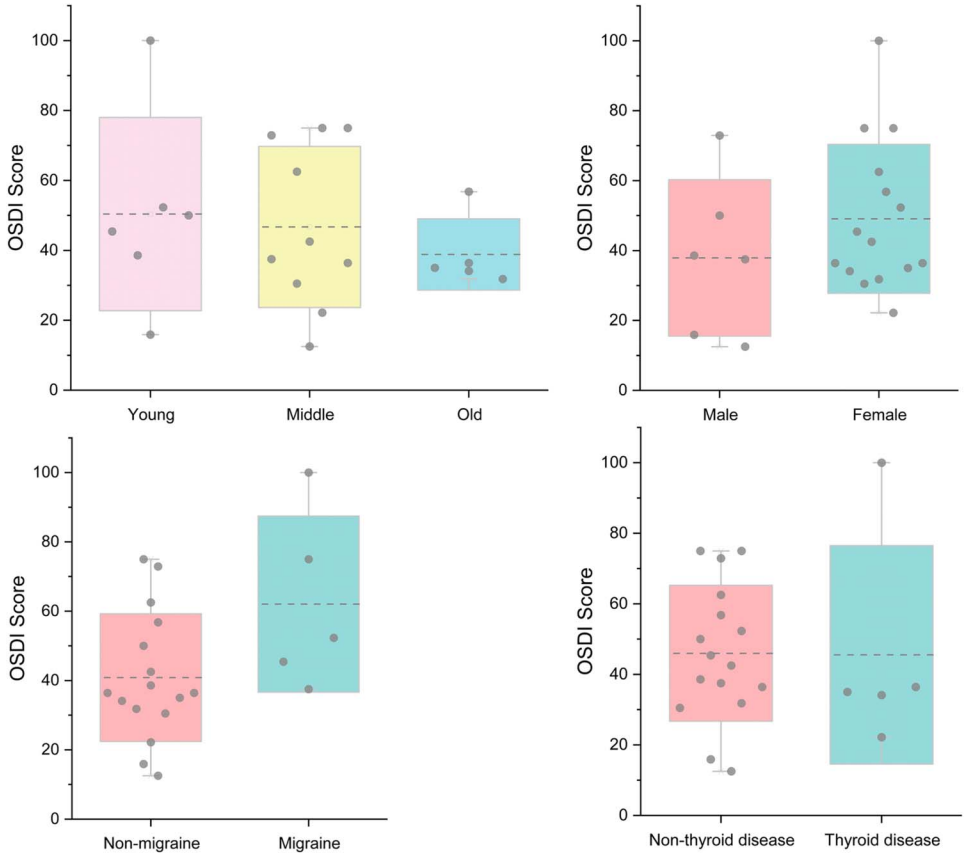

Figure 2. Comparison of OSDI scores (mean ± SD) among different groups of cases with corneal neuralgia after surgery. Although female cases and cases with migraine history have much higher OSDI scores than their comparator groups, no significant difference was observed. OSDI, Ocular Surface Disease Index.

RNA expression of these collagen genes was confirmed through human adult corneal transcriptome data.[13] Variants were found from fibril-forming collagens, *COL5A1* (C01, C12, C13, C14, C19, and C21), *COL5A2* (C12, C15, and C18), *COL5A3* (C08, C17, and C18), and *COL27A1* (C01, C16, and C21); fibril-associated collagens with interrupted triple helices, including *COL9A2* (C08, C11, and C16), and *COL12A1* (C09, C10, and C16); and networking collagens, including *COL4A2* (C08, C09, C18, and C21), *COL4A5* (C08, C10, and C14), *COL6A2* (C08, C11, and C13), and *COL6A3* (C07, C08, C12, C17, and C21). Two homozygous missense variants of *COL4A2* and *COL6A3* were identified in C21, and double variants from the same patient were found in *COL5A2* (C12 and C18) (**Table 3**).

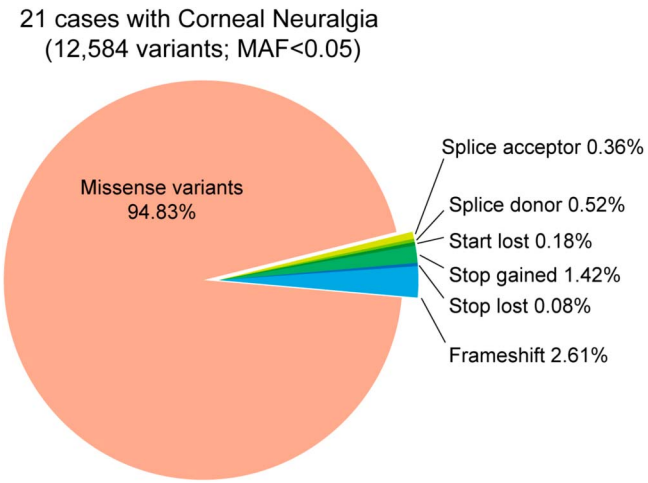

Figure 3. Proportion of 12,584 low-frequency variants from 21 cases with corneal neuralgia.

3.4. Migraine-related genes

The trigeminal system has been implicated in migraine.[2] Among the 3 known migraine-related genes described above, we only detected variants from *CACNA1A* in 3 CN cases (**Table 2**).

3.5. Gene-based association test

From 20 unrelated cases with CN, after variant filtering pipeline, we obtained 11,452 low-frequency missense variants (94.87%). Likewise, 2,736,741 and 5,177,436 missense variants (91.76% and 91.53%) were selected from NFE and global gnomAD exome subjects, respectively (**Fig. 4A, B**).

After comparing all low-frequency missense variants between case cohort and NEF control, no gene yielded gene-phenotype association for CN (OR > 1) with statistical significance. However, computational predictions of variant effect can impact results by filtering out statistical noise introduced by benign variants.[43] We thus adopted 2 strategies to identify "damaging" missense variants. Using strategy 1, employing all variants with PhastCons100way score >0.9, no gene (OR >1) reached significant difference. With the most stringent strategy (all variants predicted to be damaging using SIFT, PolyPhen2, and CADD), we identified 2,818 "damaging" variants from 2,334 genes. Only one gene,

Table 2

Low-frequency variants detected in human TG-expressed electrogenisome-related genes (n ≥ 3).

Case	Location	Ref	Alt	Gene	Protein	hTG	gnomADE_AF	phastCons100	SIFT	Polyphen2	CADD
C05	2:167298015	C	T	*SCN7A*	R683Q	32.83	5.38e-3	1.000	0.91	0.88	33.00
C19	2:167262592	C	T	*SCN7A*	R1516K	32.83	3.76e-3	0.998	0.47	0.08	22.70
C20	2:167266447	T	C	*SCN7A*	c.3712-2	32.83	1.10e-2	/	/	/	/
C01,C09	2:167141109	G	T	*SCN9A*	P610T	12.10	2.40e-2	0.273	0.37	0.17	0.62
C16	2:167138296	T	C	*SCN9A*	K655R	12.10	1.95e-3	0.123	0.01	0.20	4.18
C04	2:167083160	C	T	*SCN9A*	V1428I	12.10	1.61e-3	1.000	0.68	0.04	13.43
C21	3:38805069	T	C	*SCN10A*	I206M	8.31	2.55e-2	0.000	0.56	0.19	15.03
C04,C11,C14	3:38766760	G	T	*SCN10A*	P1045T	8.31	1.55e-2	0.058	0.28	0.25	6.35
C21	3:38739622	C	T	*SCN10A*	V1697I	8.31	1.17e-2	0.000	0.17	0.12	5.44
C02	3:38739171	C	T	*SCN10A*	R1847Q	8.31	9.16e-5	0.984	0.48	0.30	25.40
C10	21:45815372	G	T	*TRPM2*	D624Y	4.13	1.32e-4	1.000	0.56	0.97	26.20
C19	21:45825799	C	T	*TRPM2*	A890V	4.13	2.06e-3	0.000	0.28	0.14	1.95
C06	21:45826486	G	A	*TRPM2*	V934I	4.13	1.11e-2	0.996	0.41	0.19	22.40
C03,C11,C14	17:3495391	T	C	*TRPV1*	Q85R	17.80	2.80e-2	0.000	0.22	0.08	0.02
C07	17:3481026	C	T	*TRPV1*	V527M	17.80	2.32e-5	0.050	0.42	0.54	26.60
C01,C09	19:13397389	T	C	*CACNA1A*	R1161G	5.67	/	1.000	0.15	0.20	23.50
C12	19:13320189	G	A	*CACNA1A*	R2155C	5.67	5.89e-5	1.000	0.42	0.51	25.60
C07	16:1251880	G	A	*CACNA1H*	R477H	3.60	2.57e-5	0.987	0.29	0.88	24.00
C08	16:1251967	G	A	*CACNA1H*	R506Q	3.60	2.06e-4	0.014	0.06	0.20	7.56
C10	16:1252114	C	T	*CACNA1H*	A555V	3.60	1.11e-2	0.000	0.09	0.04	0.83
C07	16:1254345	G	T	*CACNA1H*	V780F	3.60	3.42e-4	0.650	0.34	0.40	16.00
C11	16:1256259	C	T	*CACNA1H*	T920M	3.60	2.25e-3	1.000	0.01	0.09	13.50
C12	16:1265315	G	A	*CACNA1H*	A1705T	3.60	5.56e-3	1.000	0.37	0.77	26.80
C01,C02,C03,C06,C09	3:50513613	C	T	*CACNA2D2*	R75Q	2.56	1.81e-2	1.000	0.42	0.44	24.40
C01,C09,C13	20:47990250	G	C	*KCNB1*	T616S	4.56	1.72e-2	0.901	0.04	0.04	0.01
C13	20:47989527	C	T	*KCNB1*	S857N	4.56	1.02e-2	1.000	0.63	0.53	15.53
C20	17:61601584	A	G	*KCNH6*	Y54C	2.02	4.35e-3	1.000	0.91	0.92	26.00
C18	17:61611299	G	A	*KCNH6*	R243H	2.02	5.22e-4	1.000	0.50	0.77	23.90
C03	17:61611614	T	C	*KCNH6*	I348T	2.02	6.32e-4	1.000	0.91	0.97	25.10
C17,C19	19:38810700	G	A	*KCNK6*	R37Q	2.19	1.10e-2	0.979	0.40	0.44	28.50
C19	19:38810744	C	G	*KCNK6*	P52A	2.19	1.39e-2	1.000	0.30	0.24	15.85
C05	19:38817628	G	A	*KCNK6*	V240I	2.19	7.25e-3	1.000	0.41	0.36	27.10
C12	9:138594163	G	C	*KCNT1*	G20A	7.24	6.63e-3	0.000	0.13	0.01	9.64
C11	9:138678160	C	T	*KCNT1*	P1099S	7.24	4.32e-3	1.000	0.05	0.16	0.66
C02	9:138678253	G	A	*KCNT1*	A1130T	7.24	1.04e-2	0.474	0.55	0.24	1.77

/, no data; Alt, alteration; CADD, CADD_phred (damaging cutoff ≥ 15); gnomADE_AF, allele frequency in gnomAD exome database; hTG, RNA expression level (FKPM) of human trigeminal ganglion; Polyphen2, Polyphen2_HVAR_rankscore (damaging cutoff >0.47121); Ref, reference; SIFT, SIFT_converted_rankscore (damaging cutoff >0.39575).

SLC9A3R1, yielded a statistically significant association with the CN (OR = 17.09; 95% confidence interval = 6.68–43.72; Bonferroni-corrected *P*-value ≈ 0.048) (**Fig. 4C**). Alternative analysis using global exomes yielded similar results (**Fig. 4D**) (Supplementary table 2, 3, available at http://links.lww.com/PR9/A65).

In *SLC9A3R1*, we found 3 low-frequency "damaging" variants from 5 CN patients: p.R180W (C08), p.R153Q (C13, C19, and C20), and p.E225K (C21).

4. Discussion

Neuropathic pain is defined by the International Association for the Study of Pain as pain caused by a lesion or disease of the somatosensory pathways in the peripheral and/or central nervous system.[7,30,51] Refractive surgery (in particular, LASIK) has been described as an associated risk factor for neuropathic corneal pain.[83] In this study, targeting a small cohort of cases with CN, unbiased WES analysis revealed candidate variants in gene panels of electrogenisome-related ion channels and collagens.

During PRK, the corneal epithelium is removed, and Bowman layer and anterior stroma along with nerves at the subbasal plexus and peripheral sensory processes are ablated. The remaining nerve endings are exposed until epithelium grows over the surgical wound (2–10 days).[66] Laser-assisted in situ keratomileusis uses a microkeratome (blade) or laser to create a flap in the anterior stroma, with subbasal and stromal nerves severed, but no exposure of nerve endings.[66] After LASIK, subbasal nerve

Table 3

Low-frequency variants detected in cornea-expressed collagen genes (n ≥ 3).

Case	Location	Ref	Alt	Gene	Protein	hAC	gnomADE_AF	phastCons100	SIFT	Polyphen2	CADD
C18	13:111143601	A	G	*COL4A2*	E1123G	1.20	9.10e-3	1.000	0.33	0.53	25.10
C08,C09	13:111156250	G	A	*COL4A2*	V1399I	1.20	3.00e-2	0.000	0.11	0.34	1.51
C21 (homo)	13:111156250	G	A	*COL4A2*	V1399I	1.20	3.00e-2	0.000	0.11	0.34	1.51
C08	X:107834411	C	A	*COL4A5*	A430D	13.71	4.65e-3	0.019	0.15	0.15	18.97
C10,C14	X:107844666	G	T	*COL4A5*	K664N	13.71	7.97e-3	0.023	0.15	0.06	8.49
C01	9:137593099	G	A	*COL5A1*	D192N	2.62	1.67e-2	0.024	0.07	0.12	17.88
C14,C19,C21	9:137642654	G	A	*COL5A1*	G530S	2.62	3.47e-2	1.000	0.15	0.88	24.90
C12	9:137648614	C	T	*COL5A1*	R611W	2.62	7.96e-5	1.000	0.91	0.82	35.00
C13	9:137726950	C	T	*COL5A1*	T1757M	2.62	1.20e-2	0.994	0.59	0.55	25.60
C15	2:189940142	T	G	*COL5A2*	M361L	7.11	1.84e-2	1.000	0.03	0.01	23.70
C12,C18	2:189931144	A	G	*COL5A2*	V512A	7.11	1.82e-2	0.990	0.01	0.34	22.80
C12,C18	2:189918622	G	A	*COL5A2*	P833L	7.11	1.70e-2	1.000	0.91	0.66	25.90
C18	19:10104458	G	A	*COL5A3*	R538W	6.00	4.37e-5	0.990	0.91	0.92	24.90
C08	19:10084460	A	G	*COL5A3*	V1195A	6.00	8.63e-3	0.001	0.35	0.18	25.50
C17	19:10076990	G	C	*COL5A3*	I1594M	6.00	2.18e-2	0.000	0.26	0.08	3.37
C13	21:47536717	G	A	*COL6A2*	D330N	21.29	3.32e-4	1.000	0.68	0.85	33.00
C08,C11	21:47546080	G	A	*COL6A2*	R784H	21.29	4.44e-3	1.000	0.13	0.14	21.30
C07,C17	2:238296306	G	C	*COL6A3*	L411V	7.82	4.00e-3	1.000	0.19	0.31	16.79
C12	2:238283429	C	T	*COL6A3*	G1102E	7.82	/	1.000	0.72	0.97	23.40
C08	2:238280504	C	T	*COL6A3*	E1386K	7.82	6.23e-3	0.402	0.33	0.60	23.80
C21 (homo)	2:238277596	G	A	*COL6A3*	R1504W	7.82	4.34e-4	0.989	0.59	0.92	24.20
C09	3:48618361	T	G	*COL7A1*	c.4936–2	95.97	/	/	/	/	/
C06	3:48621017	G	A	*COL7A1*	P1458L	95.97	1.99e-3	1.000	0.54	0.64	24.80
C05	3:48621037	C	A	*COL7A1*	E1451D	95.97	/	0.001	0.12	0.72	19.96
C11	1:40781270	C	G	*COL9A2*	G48R	3.09	1.94e-5	1.000	0.91	0.97	27.10
C08,C16	1:40775937	G	A	*COL9A2*	T246M	3.09	2.18e-2	0.802	0.19	0.04	17.52
C16	6:75898153	T	A	*COL12A1*	I308F	35.05	2.45e-4	1.000	0.68	0.53	23.90
C10	6:75875241	C	T	*COL12A1*	G989R	35.05	2.26e-3	0.993	0.63	0.72	23.20
C09	6:75843623	C	T	*COL12A1*	R1872H	35.05	1.60e-4	1.000	0.57	0.85	32.00
C01	9:116931664	C	T	*COL27A1*	S610L	2.12	4.03e-4	0.001	0.22	0.10	21.60
C21	9:117004489	C	T	*COL27A1*	P953L	2.12	1.41e-3	1.000	0.46	0.92	24.60
C16	9:117044843	C	T	*COL27A1*	T1293M	2.12	1.22e-2	1.000	0.14	0.27	25.00

/, no data; Alt, alteration; CADD, CADD_phred (damaging cutoff ≥ 15); gnomADE_AF, allele frequency in gnomAD exome database; hAC, RNA expression level (FKPM) of adult human cornea; Homo, homozygous; Polyphen2, Polyphen2_HVAR_rankscore (damaging cutoff >0.47121); Ref, reference; SIFT, SIFT_converted_rankscore (damaging cutoff >0.39575).

density takes more time to recover than after PRK and may never reach baseline levels.[32] We included both LASIK and PRK patients because both use laser ablation to alter corneal power, although combining these groups might reduce likelihood of detecting a genetic variant. The discordant type of enhancement procedure is a further confounding variable. Potential subjects having undergone incisional refractive surgery as primary procedure or enhancement were excluded from this study. Future studies could be limited to LASIK or PRK and further broken down by type of enhancement.

A limitation of this study is the small number of patients studied and the potential for sampling bias related to our mode of recruitment. We do not know the overall number of patients who read our requests to participate, or who underwent LASIK/PRK without development of chronic pain. We cannot comment on the frequency of chronic pain after LASIK/PRK. A more rigorous study design would compare refractive surgery patients who develop persistent pain with those who do not. The PROWL (Patient-Reported Outcomes with Laser In Situ Keratomileusis) studies found that of participants with normal scores at baseline, about 28% had dry eye symptoms at 3 months, but only 4% to 6% developed moderate or severe symptoms by OSDI post-operatively during 6 or 3 months of follow-up.[33] Persistent postoperative pain or CN, per se, was not studied in the PROWL studies, but would be only a fraction of these dry eye cases. With this low incidence, a controlled study would entail resource requirements beyond those available to these investigators, and likely could not be undertaken unless candidate genes were previously identified. Nonetheless, our results provide some clinical and genomic perspectives. Previous reports have suggested higher incidence of symptoms of dry eye disease among female subjects, with significantly higher symptom scores and lower correlation between symptoms and signs as compared to males.[77,89] Females are represented in our cohort in >2.5:1

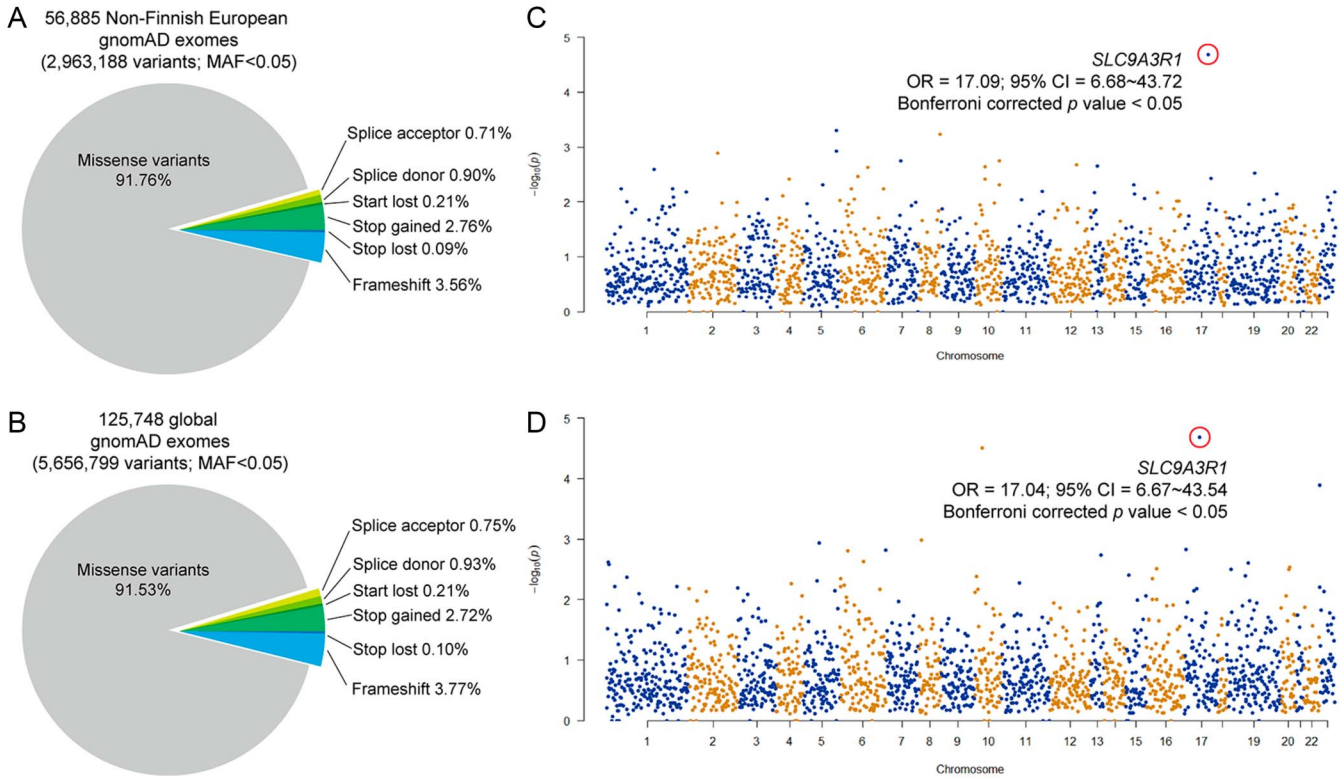

Figure 4. Proportion of low-frequency variants from non-Finnish European (NFE) and global gnomAD exomes, and their Manhattan plots exhibiting genes compared with 20 unrelated cases of corneal neuralgia using "damaging" missense variants. (A and B) Missense variants occupy 91.76% and 91.54% of all variants from NFE and global gnomAD exome, respectively. (C and D) Manhattan plots of all chromosomes shows only one gene, *SLC9A3R1* (odds ratio = 17.09 or 17.04; 95% confidence interval = 6.68–43.72 or 6.67–43.54), having a Bonferroni-corrected *P* value < 0.05.

ratio to males suggesting that there may be greater incidence, assuming the population undergoing refractive surgery is split equally between the sexes. The PROWL studies were 20% female in the military cohort and 54% female in the civilian cohort.[33] Others have found association of greater ocular pain severity with younger age, and with depression, anxiety, and migraine (all $P < 0.05$).[79,88] However, in our cohort, no statistical difference in OSDI scores was found between females and males, nor did we find any significant difference between cases with/without migraine or thyroid disease. Another limitation of our study is the use OSDI, which is not a validated pain assessment tool, as a metric for inclusion based on symptom severity. The NPSI-Eye,[36] not yet reported when enrollment for this study was begun, is likely to provide a useful instrument for future studies.

Dominant gain-of-function mutations of Nav1.7 can produce hyperpolarizing shifts in channel activation and cause sensory neuronal hyperexcitability.[24,25] Nav1.8 mutations can hyperpolarize activation, accelerate recovery from fast-inactivation, and increase sensory neuron action potential firing frequency.[35] Nav1.7 and Nav1.8 are expressed, at both RNA and protein levels, in DRG and TG neurons.[27,38,85] The p.I234T mutation of Nav1.7 was reported in a patient with a complex syndrome that includes episodic pain together with bilateral congenital corneal anesthesia.[55] Our recent study found a Nav1.8 gain-of-function mutation (p.A1304T) in a patient with familial trigeminal neuralgia.[23] Thus, it is reasonable to suggest that, as in painful peripheral neuropathy,[34,35] gain-of-function variants in sodium channels may contribute to CN.

Transient receptor potential channels are important in conveying pain signals due to thermal stimuli and mechanical irritants through corneal nociceptors[6] and are differentially expressed in various corneal layers and corneal afferent nerves.[64,74] Thus, variants in TRP channels could contribute to the pathophysiology of corneal pain in our cohort. The p.Q85R variant of *TRPV1* (frequency in gnomAD exome = 0.028), identified in 3 cases in our cohort, is located at the N-terminal domain, and has been shown to cause a significant increase of Ca^{2+} influx compared to wild-type channels, and thus is considered as having a gain-of-function impact.[91] We also found 2 variants, p.D665N and p.V915M, in the *TRPM8* gene. This channel is widely expressed within cornea layers and is activated by cold temperature and cooling agents.[99] It has been reported that TRPM8 activation produces analgesia,[73] but TRPM8 inhibition has also been found crucial to reduce acute and chronic pain.[16,17] Effect of the variants on the functional properties of the TRPM8 channel are not known, and thus their contribution to the pain phenotype in the 2 carriers awaits further studies. Expanding understanding of TRP channels involved in ocular itch and pain[48] and of how injury and inflammation can induce changes in ocular pain pathways[5] likely add to our understanding of CN in the future.

Other receptors and ion channels have been linked to pain. Some mutations in *CACNA1H* have been found to produce gain-of-function through hyperpolarizing activation or slowing inactivation, which can generate a greater calcium influx and increased neuronal excitability.[54,71] A p.R75Q variant of *CACNA2D2* was detected in 5 cases of CN; this gene has been implicated in neuropathic pain and opioid sensitivity.[75] Potassium channel variants can alter ion selectivity and gating, and cause a tonic inward cation conductance (KCNB1),[84] leading to depolarization and increased neuronal excitability (KCNK6)[56]; some mutations increase maximal current size and shift activation threshold toward less depolarized potentials (KCNT1).[3,26] These

findings suggest a contribution of these electrogenisome-related genes in neuropathic pain, and we speculate that some mutations in these channels could possibly trigger CN after nerve injury caused by refractive surgery.

An association has been discovered between a collagen gene, *COL6A5*, and neuropathic itch.[63] Pain and itch can activate common pathways in the dorsal horn,[22,60] although a recent study suggests that separate pain and itch pathways emanate from the cornea and conjunctiva, respectively.[48] Multiple pain-related genes, such as *SCN9A* and itch-TRP channels, have been linked to itch.[18,68] We studied the variants in cornea-expressed collagen genes; notably, 7 *COL5A1/2* variants were detected from 9/21 cases with CN, including 3 cases with p.G530S. *COL5A1/2* genes have been linked to Ehlers–Danlos syndrome, a connective tissue disorder characterized by laxity and fragility of soft connective tissues including cornea.[42,65] Together with *COL4A1/2*, which have been linked to migraine with/without aura,[82] the contribution of collagen genes and variants in corneal nerve fiber regeneration and CN requires further study.

Although individual-level data are inaccessible, it has been suggested that genomic variants from population databases, such as Exome Aggregation Consortium and gnomAD, can be used as control samples for association analyses.[43,45] We are aware that varied exome capture technologies, sequencing platforms, and data processing pipelines would affect the results. With a stringent filtering strategy, 3 prediction tools were enrolled for selecting "damaging" missense variants. This step is imperfect at distinguishing pathogenic from benign variants, which might affect the power to detect certain genes. However, using this gene-based association test, compared with both NFE and global exomes from gnomAD, we identified an *SLC9A3R1* gene as being statistically associated with the CN phenotype. Considering the small cohort size and borderline *P*-value (≈0.048), the statistical power was limited. The moderate RNA expression level of *SLC9A3R1* (FPKM = 21.11 in human TG) indicates a potential functional role in the trigeminal nervous system.[38] This gene encodes a sodium/hydrogen exchanger regulatory cofactor, NHERF1 (also known as PDZK3) that recruits signaling proteins, cellular receptors, ion transporters, and other proteins to the plasma membrane of epithelia and other cell types.[90] Among 3 variants of *SLC9A3R1*, p.R153Q and p.E225K have been reported from patients with hypophosphatemia and recurrent nephrolithiasis,[53] although other studies and ClinVar have challenged the pathogenicity of these variants. Recently, structural analysis revealed significant differences in the NHERF1 protein harboring these mutants, compared to the wild-type protein.[8] Further functional analysis of these variants, particularly in sensory neurons, might be informative.

The pathogenesis of CN remains unclear. Based on a small cohort of patients with CN, we investigated the possible genetic etiological factors through unbiased WES analysis. Although no universal variants or genes were identified from these patients, we detected multiple variants in pain-related gene panels and in corneal collagen genes and found an associated gene using gene-based association test. Our results underscore the need for further investigation of the pathophysiological role of these variants. Multiple variants would be in keeping with the insights of Belmonte[5] on the complexity of neuronal and inflammatory interactions at the ocular surface, in the TG, and centrally. We suggest that the trigeminal system is particularly susceptible, when there is injury, to changes that can contribute to chronic pain. Our findings support the idea that CN is a multifactorial disorder, in which genetic factors might contribute, at least partially to the persistent and intractable postoperative pain phenotype. More stringent selection criteria, including use of a validated instrument for CN, and a larger case cohort may reveal statistically stronger and more informative results.

Disclosures

The authors have no conflicts of interest to declare.

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Appendix A. Supplemental digital content

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