Genome-wide analysis identified novel susceptible genes of restless legs syndrome in migraineurs

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Abstract

Background: Restless legs syndrome is a highly prevalent comorbidity of migraine; however, its genetic contributions remain unclear.

Objectives: To identify the genetic variants of restless legs syndrome in migraineurs and to investigate their potential pathogenic roles.

Methods: We conducted a two-stage genome-wide association study (GWAS) to identify susceptible genes for restless legs syndrome in 1,647 patients with migraine, including 264 with and 1,383 without restless legs syndrome, and also validated the association of lead variants in normal controls unaffected with restless legs syndrome (n = 1,053). We used morpholino translational knockdown (morphants), CRISPR/dCas9 transcriptional knockdown, transient CRISPR/Cas9 knockout (crispants) and gene rescue in one-cell stage embryos of zebrafish to study the function of the identified genes.

Results: We identified two novel susceptibility loci rs6021854 (in VSTM2L) and rs79823654 (in CCDC141) to be associated with restless legs syndrome in migraineurs, which remained significant when compared to normal controls. Two different morpholinos targeting vstm2l and ccdc141 in zebrafish demonstrated behavioural and cytochemical phenotypes relevant to restless legs syndrome, including hyperkinetic movements of pectoral fins and decreased number in dopaminergic amacrine cells. These phenotypes could be partially reversed with gene rescue, suggesting the specificity of translational knockdown. Transcriptional CRISPR/dCas9 knockdown and transient CRISPR/Cas9 knockout of vstm2l and ccdc141 replicated the findings observed in translationally knocked-down morphants.

Conclusions: Our GWAS and functional analysis suggest VSTM2L and CCDC141 are highly relevant to the pathogenesis of restless legs syndrome in migraineurs.

Keywords: Migraine, Restless legs syndrome, Genome-wide association study, Zebrafish, VSTM2L and CCDC141

Background

Migraine is a highly prevalent and disabling neurological disorder, which is comorbid with a variety of neuropsychiatric disorders, including an intriguing sensorimotor disease-restless legs syndrome (RLS) [1, 2]. Restless legs syndrome (RLS) is an intriguing sensorimotor disorder characterized by an urge to move legs, which
occurs mostly at night and disturbs sleep, being exacerbated by lying down with unpleasant sensations in legs, and can be temporarily relieved by voluntary leg movements [3]. Evidence has suggested complex associations between migraine and RLS. The prevalence of RLS in patients with migraine [1] could be up to seven times higher than that in the general population [4]. The severity of RLS in patients with migraine is worse than that of non-migraineurs [5], and the occurrence of RLS is more frequent in chronic compared with episodic migraineurs [6]. Moreover, RLS and migraine were found to have bidirectional trigger effects [7]. Yet, detailed mechanisms underlying comorbid RLS in migraineurs are unclear.

Both migraine and RLS are known to have high heritability, and genome-wide association studies (GWASs) have made substantial progress in identifying susceptibility genes for both diseases [8–17]. Dysfunctional dopaminergic neurotransmission and iron homeostasis have been proposed to be common mechanisms shared by RLS [18–20] and migraine [21, 22]; however, the genetic constituents contributing to RLS in migraineurs remain to be explored. We previously identified that a single-nucleotide polymorphism (SNP) rs2300478 at MEIS1, the gene responsible for iron homeostasis [23], increased the risk of RLS by 1.42-fold in migraine subjects via a candidate gene approach [24]. A recent small-scaled GWAS also suggested additional genes may contribute to RLS in migraineurs [25]. To further decipher the role of genetic variants in RLS in patients with migraine, we implemented a two-stage GWAS followed by in vivo functional analyses with zebrafish [26–28].

Methods
Study participants and data collection
A two-stage case–control GWAS was implemented to identify susceptible genes for RLS in migraineurs by comparing the cases (i.e., migraineurs with RLS) with controls (i.e., migraineurs without RLS). The significant findings of the discovery cohort were validated in the replication cohort, and a combined analysis of both cohorts was employed to examine the significance of the validated SNPs. In addition, we also examined the significant SNPs in an independent normal control cohort unaffected with restless legs syndrome or migraine. Consecutive patients with migraine were enrolled in the headache clinic of Taipei Veterans General Hospital (TVGH). They filled out a structured questionnaire with questions regarding personal information, medical history, and headache history. Participants were interviewed and their questionnaires and medical records were reviewed simultaneously by board-certified neurologists specialized in headache diagnosis. Migraine was diagnosed according to the criteria proposed in the International Classification of Headache Disorders, 3rd edition [29]. Subjects with secondary headache disorders except for medication overuse headache were excluded. RLS was diagnosed based on the criteria proposed by the International RLS Study Group [30]. Subjects with ferritin < 50 ng/ml, anaemia, creatinine > 1.5 mg/dl, or pregnancy were eliminated to exclude secondary RLS. Subjects with any RLS symptom proposed in the criteria or periodic limb movements in sleep based on self-reported nocturnal leg jerks during sleep were excluded from the control groups.

Genotyping in the discovery cohort
We genotyped 642,832 SNPs using the Affymetrix Axiom Genome-Wide CHB 1 Array Plate, which has high coverage of genome-wide common variants for Han Chinese. SNP genotypes were called using the Axiom GT1 algorithm. Quality control (QC) criteria were applied to exclude SNPs if they (a) were monomorphic in both cases and controls, (b) had a total call rate of less than 95%, (c) had a minor allele frequency of less than 5% and a total call rate of less than 99%, or (d) showed significant \( P < 1 \times 10^{-8} \) deviation from Hardy–Weinberg equilibrium in controls. For sample filtering, arrays with generated genotypes for < 95% of the loci were excluded.

Heterozygosity of SNPs on the X-chromosome was used to verify the sex of the samples. PLINK version 1.09 [31] was used to identify samples with genetic relatedness, indicating that they were from the same individual (or monozygotic twins) or from first-, second- or third-degree relatives. These determinations were made based on evidence for cryptic relatedness from identity-by-descent status (pi-hat cut-off of 0.125).

Genotyping in the replication cohorts
We selected SNPs that were within 200 kb of a gene which contains at least two adjacent SNPs with a \( P \) value of \( < 1 \times 10^{-4} \). Single SNPs with a trend \( P \) value \( < 1 \times 10^{-4} \) but not within 200 kb of a gene were not chosen for replication because we aimed to explore known protein coding genes. Genotyping was performed in replication cohorts using the Sequenom MassARRAY iPLEX platform (Sequenom Inc., San Diego, CA, USA). Genotyping in both cohorts are services provided by the National Center for Genome Medicine (NCGM).

Imputation for the discovery case–control GWAS
We conducted a genotype imputation analysis in the discovery cohort using the 1000 Genomes Phase 3 reference data by implementing IMPUTE2 [32]. Well-imputed SNPs (info score > 0.4) were retained followed by systematic QC as described above.
Morpholino translational knockdown

Morpholino oligonucleotide can block translation by targeting the 5’ untranslated region (UTR) of mRNA or inhibit RNA splicing by targeting exon/intron junctions. We designed six 25-base morpholinos (Gene Tools, Philomath, OR) that target the 5’UTR or splicing junction of ccdc141 and vstm2l (Additional file 1).

CRISPR interference

CRISPR gRNAs were designed with Benchling and the cloning sequences are shown in Additional file 2. Oligonucleotides were annealed in a thermoblock at 95 °C for 5 min and cooled to room temperature. Annealed oligonucleotides were cloned into pT7-gRNA plasmid at BsmBI site and verified by sequencing. To make dCas9 mRNA, dead Cas9 plasmid [33] was linearized by XbaI enzyme and purified by Gel extraction kit (Qiagen, Hilden, Germany). mRNA was synthesized by mMESSAGE mMACHINE T3 kit (Life Technologies, Carlsbad, CA) and purified by RNeasy mini kit (Qiagen). To make gRNA mRNA, pT7-gRNA plasmid was linearized by BamHI enzyme and purified by Gel extraction kit. RNA probe was synthesized by in vitro transcription using a MEGAscript® T7 Transcription kit (Thermo Fisher Scientific, Waltham, MA) and purified by ethanol precipitation.

Transient and stable CRISPR/Cas9 knockout (KO)

The CRISPR/Cas9 KO is carried out by a non-for-profit service offered by the Taiwan Zebrafish Technology and Resource Center (TZTRC) according to previous reports. Briefly, together with the common tracrRNA and Cas9 protein, 4 gene-specific crRNAs (Additional file 3; Horizon, Waterbeach, UK), 2 for each gene, were injected into one-cell stage embryos separately [34]. Transient CRISPR/Cas9-injected embryos (crispants) have been demonstrated to largely phenocopy mutants [35]. The CRISPR/Cas9 activity detection and mutation screening were performed by high resolution melting analysis [36]. The stable KOs were confirmed by Sanger sequencing and maintained according to the standard operating protocol [37].

Tyrosine hydroxylase RNA in situ hybridization

Tyrosine hydroxylase (TH) is an enzyme responsible for the biosynthesis of dopamine precursors. The 3–5 dpf wild-type and injected embryos were used for in situ hybridization following previously established protocol [38]. The embryos were fixed in 4% fresh-made paraformaldehyde at 4 °C overnight and then treated with 3% H2O2 and 5% KOH for depigmentation. Embryos were washed and transferred into 100% methanol at -20 °C overnight. Digoxigenin-labelled antisense RNA probes were used for labelling to detect the distribution of dopaminergic cells, and then the embryos were mounted in glycerol for observation and photography.

Fin movement observation

We utilized a video system under normal laboratory lighting to observe pectoral fin movement and evaluate whether the injected embryos had hyperkinetic movements mimicking the “restlessness” and “urge to move the limbs” in patients with RLS. The 5 dpf embryos were used because pectoral fins and body organs are relatively well-developed. Embryos were mounted on glass slides covered with 1% low melting agar and put under a dissecting microscope to observe fin movements. One-to-three-minute videos were filmed by DFK 23UP031 USB Camera (The Imaging Source Asia Co., Taipei, Taiwan). Video Analysis Tools, After Effects and Tracker (Adobe, San Jose, CA), were used. The average flapping frequency (times/second) was acquired by catching the fin movement in x and time in y coordinates.

Quantitative RT-PCR (qRT-PCR)

Dechorionated 2 dpf embryos were collected and total RNA was extracted by RNAzol® RT reagent (Molecular Research Center, Inc.). cDNA was synthesized by SuperScript™ III Reverse Transcriptase kit (Thermo Fisher Scientific). The experiment was conducted by LightCycler® 480 Instrument II with SensiFAST™ SYBR® Hi-ROX kit (Bioline). Actin was used as an internal control in all triplicated experiments. The qPCR data was analysed by LightCycler® 480 software version 1.5.0.39.

Statistics

Association analyses were carried out by comparing allele/genotype frequencies between cases and controls using a single-point method: Cochran–Armitage trend test. The distribution of expected P values under the null hypothesis and genomic inflation value (λ) were calculated. The Manhattan and quantile–quantile (Q-Q) plots were created using the R package [39]. Genetic analyses were conducted using PLINK (version 1.09) [31]. Detection of possible population stratification was carried out by using principal component analysis (PCA) implemented in EIGENSTRAT to infer continuous axes of genetic variation. We adjusted for potential genetic heterogeneity by incorporating the first 10 PCs in the logistic regression tests of association with RLS. Joint analysis was conducted by combining data from the discovery and replication samples. In addition, we also examined the association of significant variants with migraine in an independent migraine case–control cohort. For studies
involving zebrafish, data are reported as the mean ± SD or median and interquartile range. Student’s t test was used for comparison of continuous variables; Mann–Whitney U test was used for comparisons of unpaired nonparametric variables. All calculated P-values were two-tailed, and statistical significance was defined as P-value less than 0.05. These analyses were performed using Graphpad Prism, version 7.00 (GraphPad Software, La Jolla, CA).

Results
Association analysis
Demographic characteristics of participants including age and sex were not significantly different between cases (migraineurs with RLS) and controls (migraineurs without RLS) in the discovery (age: 38.7 ± 12.4 vs. 39.0 ± 12.5 yrs, P = 0.775; female: 87.0% vs. 78.9%, P = 0.056) or replication cohort (age: 40.4 ± 12.6 vs. 39.5 ± 11.9 yrs, P = 0.378; female: 82.6% vs. 76.6%, P = 0.132). In the discovery stage, we genotyped 115 migraine patients with RLS and 635 migraine patients without RLS using the Affymetrix Axiom Genome-Wide CHB 1 Array Plate (Fig. 1A). After applying stringent QC criteria, we obtained 590,468 (91.85%) SNPs with an average call rate of 99.6 ± 0.5%. The value of the genomic inflation factor was 1.000, suggesting that there was no evidence for population stratification (Fig. 1B). PCA based on genotype data from 590,468 SNPs with equal spacing across the human genome showed no outliers. In total, 81 SNPs showed significant (\( p < 10^{-6} \)) association signals with RLS. Four of the significant SNPs within or near (within 200 kb) genes were genotyped, and an additional 3 SNPs in the region were included for fine mapping in the replication cohort consisting of 149 migraine patients with RLS and 748 migraine patients without RLS (Additional file 4).

rs79823654 in CCDC141 and rs6021854 in VSTM2L are associated with risk of RLS in migraineurs
We identified two novel loci: rs79823654 in CCDC141 and rs6021854 in VSTM2L that were significantly associated with the risk of RLS in migraineurs in both discovery and replication cohorts (Table 1, Fig. 2). In the discovery dataset, rs6021854 and rs79823654 were the most significant SNP, which remained significant after adjustment for PC1–PC10 of population structure. The association between these two SNPs and RLS in migraineurs was further confirmed in the replication dataset with a similar genetic impact. Joint analysis of both cohorts demonstrated that both SNPs were associated with an increased risk of RLS in migraineurs (Table 1). By comparing these cases (i.e., migraineurs with RLS) with normal controls, these two variants remained significant (Table 1).

Association of RLS with SNPs within dopamine receptor or tyrosine hydroxylase genes
To gain insight on the potential association between dopamine and RLS, we also examined whether the SNPs in tyrosine hydroxylase (TH) or dopaminergic receptors (DRD1, DRD2, DRD3, DRD4, and DRD5) have different allele frequencies between patients with and without RLS. Among the 414 SNPs within these genes available in our imputation data, none have reached our pre-defined significance level (\( p < 1 \times 10^{-5} \)). Only 17 SNPs within DRD1, DRD2 or TH have shown borderline significance (1 × 10^{-4} < p < 5 × 10^{-4}) in association with RLS (see Additional file 5).

Expression pattern of ccdc141 and vstm2l in zebrafish
Expression of ccdc141 and vstm2l in 1–4 dpf embryos was shown in Additional file 6. Their expression patterns in zebrafish are similar to those of mice and rats [40, 41].

Morpholino translational knockdown of ccdc141 and vstm2l
Six morpholinos targeting the 5’ UTR and splicing junction on ccdc141 and vstm2l were injected into one-cell stage embryos. We checked the success rate of translational knockdown and compared the morphological and phenotypic differences between wild-type embryos and morphants (Additional file 7). Successful translational knockdown was observed in ccdc141 5’UTR (MO1) and vstm2l splicing (MO2) morphants, because the amacrine cell number can be restored by injecting ccdc141 mRNA in the former (also see below) and splicing morpholino caused a pre-terminated vstm2l transcript in the latter (Additional file 7). These morphants were selected for further evaluation.

Altered expression of th-positive cells in morphants
Because the pathogenesis of RLS is considered to be associated with dopaminergic neurotransmission, we compared the distribution of th-positive cells (most of which are dopaminergic) in wild-type and morphant embryos (Fig. 3A). While pretectum, retinal amacrine cells, DC1-6 neurons, and DC7 neurons are dopaminergic, locus coeruleus (LC) and medulla oblongata (MeO) neurons are noradrenergic [42, 43]. However, sympathetic superior cervical ganglion (SCG) [44, 45] neurons are mainly adrenergic, with a few cells exhibiting a cholinergic phenotype [46, 47]. We found that in ccdc141 5’UTR (MO1) morphants, the distribution of th was dispersed and the th-expressing amacrine cells were decreased; in vstm2l splicing (MO2) morphants, lower th expression in
pretectum, DC7 neurons and amacrine cells was observed. The distribution of \( \text{th} \) in SCG neurons was decreased and dispersed (Fig. 3A). In contrast, \( \text{th} \) expression in LC and MeO neurons did not change in both morphants (Fig. 3A). The morphants were further divided into groups according to their phenotypic severity before \( \text{th} \) in situ hybridization.

We still found fewer \( \text{th} \)-expressing amacrine cells in all groups among \( \text{ccdc141} \) MO1 morphants (Fig. 3B and D) and \( \text{vstm2l} \) MO2 morphants (Fig. 3C and D). The decrease of amacrine cells was partially rescued by co-injecting \( \text{ccdc141} \) mRNA into \( \text{ccdc141} \) morphants (Fig. 3F), suggesting that the phenotype is specific.

Fig. 1  A Manhattan plot for RLS association in patients with migraine. Manhattan plot of the discovery genome-wide association analysis of
115 cases and 635 controls. The x axis is chromosomal position, and the y axis is the significance (–\( \log_{10} P \)) of association derived from Cochran–Armitage trend tests. B Quantile–quantile plot of results from the Cochran-Mantel-Haenszel analysis. Red line represents the distribution of \( P \) values under the null hypothesis, given a study inflation factor (\( \lambda \)) of 1.000.
Table 1 Association results for restless legs syndrome in patients with migraine

| SNP        | Gene    | Chr | Position | Risk allele | stage | OR (95%CI) | P value       | The P value adjusted |
|------------|---------|-----|----------|-------------|-------|------------|---------------|----------------------|
| rs79823654 | CCDC141 | 2   | 179,839,018 A | 0.130 0.053 | 1 | 2.740 (1.715–4.377) | 1.05 × 10⁻³ | 2.51 × 10⁻³          |
| 2          | 0.101 0.061 | 1.642 (1.084–2.486) | 0.017 | 0.0179   |
| Joint      | 0.113 0.057 | 2.046 (1.501–2.788) | 3.27 × 10⁻⁶ | 5.81 × 10⁻⁶ |
| R1         | 0.113 0.066 | 1.857 (1.344–2.565) | 2.75 × 10⁻⁴ | 1.76 × 10⁻⁴ |
| rs6021854  | VSTM2L  | 20  | 36,545,927 A | 0.252 0.116 | 1 | 2.447 (1.738–3.446) | 8.63 × 10⁻⁸ | 4.69 × 10⁻⁷          |
| 2          | 0.182 0.136 | 1.421 (1.021–1.977) | 0.036 | 0.03598  |
| Joint      | 0.213 0.127 | 1.838 (1.451–2.328) | 2.73 × 10⁻⁷ | 4.63 × 10⁻⁷ |
| R1         | 0.213 0.154 | 1.504 (1.175–1.925) | 9.73 × 10⁻⁴ | 1.19 × 10⁻³ |

SNP single nucleotide polymorphism, Chr chromosome, OR odds ratio for risk allele, CI confidence interval, PC principal component; Stage 1 (GWAS) included 115 cases and 635 controls; Stage 2 (replication stage) included 149 cases and 748 controls; Joint: Combining stage 1 and 2; R1: combined cases (264 migraineurs with RLS) vs. 1,053 normal controls

P value is derived from trend test, the P value adjusted is derived from the logistic regression adjusted with age and sex; Risk allele, allele with higher frequency in cases compared to controls. All genomic information is from human genome build hg19

Table 1

RLS-relevant behavioural phenotypes in morphants
We observed hyperkinetic movements of pectoral fins in 5 dpf vstm2l MO2 morphants (Fig. 3G) (see Additional file 8 for video), resembling the core phenotypes, restlessness and urge to move the limbs, of RLS. The ccdc141 MO1 morphants also had a trend of hyperkinetic movements (Fig. 3H and Additional file 7).

Transcriptional knockdown of ccdc141 and vstm2l recapitulates findings in morphants
We then performed transcriptional genetic knockdown of ccdc141 and vstm2l by CRISPR interference (CRISPRi) [33]. Four CRISPRi gRNAs were designed for each gene. The gRNA was injected into one-cell stage embryos separately and its effect was measured by qPCR. The gRNAs of ccdc141 gRNA1, gRNA3, gRNA4 and vstm2l gRNA3 that can repress the expression of corresponding gene in homozygotes is approximately 0.5-fold examined by qRT-PCR were used to conduct the following experiments (Additional file 9). The embryos injected with ccdc141 gRNA4 caused reduced th-positivity amacrine cells and exhibited hyperkinetic movement compared with non-injected embryos (Additional file 10). The ccdc141 gRNA1 and vstm2l gRNA3 only caused decreased th-positive amacrine cells (Additional files 9 and 10), suggesting the possibility of different genetic thresholds for different phenotypes.

Transient knockout of ccdc141 and vstm2l recapitulates the findings in knocked-down embryos
Transient CRISPR KO cause phenotypes in crispants indistinguishable to those of loss-of-function mutants [35, 48]. We, therefore, further used CRISPR/Cas9 to transiently knock out ccdc141 and vstm2l and aimed to generate stable KO lines. Two sets of crRNAs were used to target exons 1 and 2 of each gene (Additional file 3). In the exon 1 crispants of two genes, the number of th-expressing amacrine cells in the eyes is decreased (Fig. 4A) and the movement of pectoral fins is hyperkinetic (Fig. 4B). The exon 2 crispants had similar phenotypes (Fig. 4C and D). These results repeat the conclusion obtained from translational and transcriptional knockdowns.

We can only identify stable KO lines from the offspring of exon 2 CRISPR/Cas9-injected F0: three ccdc141 lines and two vstm2l lines (Additional file 11). Unexpectedly, F2 embryos from two examined KO lines showed neither decreased th-expressing amacrine cells nor hyperkinetic fin movement (Additional file 12). Interestingly, the expression of corresponding gene in homozygotes is diminished (Additional file 12).

Discussion
By using a two-stage GWAS, we identified two novel susceptibility genes, VSTM2L and CCDC141, accountable for an increased risk of RLS in patients with migraine. These two genes were highly expressed in the central nervous system (CNS) among species. Inhibiting expression of these two genes at the transcriptional or translational level resulted in morphological changes involving fin development, decreased number of dopaminergic neurons, and hyperkinetic movements of pectoral fins in zebrafish, compatible with the clinical symptoms and putative pathogenic pathways of RLS. Gene rescue reversed the phenotypes of the morphants, which further supports that these findings are not due to non-specific toxic effects from morpholino
Fig. 2 Regional plots of association signals. Regional plots for two newly identified loci associated with risk of restless legs syndrome in patients with migraine. Each regional plot shows the chromosomal position (GRCh37/hg19) of SNPs in the specific region against $-\log_{10} P$ values from association results of genotyped and imputed SNPs in stage 1 GWAS samples and stage 2 replication samples.
and augmented the functional roles of these two genes in RLS pathogenesis. Our data confirmed the crucial role of VSTM2L and CCDC141 in RLS in patients with migraine; however, pre-existing information regarding these two genes is scarce. VSTM2L, short for V-set and transmembrane domain containing 2 like, was previously known as C20orf102. The protein encoded by VSTM2L has an exquisitely CNS-specific expression and is known to be a secreted antagonist of a neuroprotective mitochondrial peptide Humarin [40]. CCDC141 (short for coiled-coil domain containing 141), also named CAMDI after coiled-coil protein associated with myosin II and DISC1 (disrupted in schizophrenia 1), is known to affect neuronal development by impairing radial migration through DISC1 and myosin II-mediated centrosome positioning [41]. How these known functions of VSTM2L and CCDC141 contribute to RLS is unclear, but our data indicate that it might be mediated through affecting the development and distribution of dopaminergic neurons. The A11 dopaminergic nucleus of the dorsal-posterior hypothalamus has been considered to be important in the pathogenesis of RLS [19] and migraine [21] in rodent models. In zebrafish, we also demonstrated that inhibition of the expression of vstm2l and ccdc141 could affect the distribution of dopaminergic cells in the CNS. Though the th expression level of DC2,4–6 (A-11 type, the rodent A11) is dispersed and the th-expressing amacrine cells (red arrows) are decreased; in vstm2l splicing (MO2) morphants, lower th expression in dorsal pretectum (red asterisk), DC7 neurons (red square) and amacrine cells was observed; and the distribution of DC7 neurons (red square) and amacrine cells was observed; and the distribution of in sympathetic superior cervical ganglion (SCG, green rectangle) is decreased and dispersed. Of note, the distribution of DC2,4–6 neurons seems dispersed in morphants. Nevertheless, we could not exclude the possibility that it was due to morphological changes. Interestingly, the th expression of A11-type dopaminergic neurons, LC and MeO neurons with far-ranging projections is not affected, while that of DC7 neurons and retinal amacrine cells projecting exclusively locally or to adjacent brain regions is decreased [42]. Evolutionarily, there is no direct zebrafish counterpart of mammalian substantia nigra/ventral tegmental area dopaminergic neurons. A trans-species comparison of the A11-type and other dopaminergic systems, which are also less well studied in mammals [43], and behavioral phenotypes need to be examined.

Previous GWASs have identified six RLS risk loci (MEIS1, BTBD9, MAP2K5, PTPRD, TOX3, and an intergenic region on chromosome 2p14) [14–17]; however, only MEIS1 has been found to be associated with RLS in patients with migraine via candidate gene approach [24]. Hence, susceptibility genes for RLS in migraineurs might not be completely the same as those for RLS in general population. None of the above genes were identified associated with risks of RLS in migraineurs in this study. Whether CCDC141 and VSTM2L also contribute to the risk of RLS in general population remains to be explored.

We have used translational knockdowns (morphants), transcriptional knockdowns, and transient knockouts (crispants) in the zebrafish system to examine the functional relationship of CCDC141 and VSTM2L to the symptoms of RLS and migraine and obtained relatively consistent results. The stable ccdc141 and vstm2l KO lines did not show a decrease in th-expressing cells or a hyperkinetic movement in pectoral fin and basically behaved like wildtype embryos. Though unexpectedly, some similar cases have been reported in zebrafish, such as egfl7 and slc25a46 [33, 48]. The mechanism of genetic compensation for egfl7 has been shown to be transcriptional adaptation that is triggered by degradation of the mutated mRNA through nonsense-mediated mRNA decay (NMD) to upregulate sequence-similar genes.
that thereby enable functional compensation [49, 50]. However, the mechanism for \textit{slc25a46} is currently unknown [48]. The expression of \textit{ccdc141} and \textit{vstm2l} in corresponding KO mutants is decreased (Additional file 12), suggesting a transcriptional adaptation caused by NMD [48, 50]. To overcome the genetic compensation and examine the phenotypes in adult animals, different animal models may help. For example, various mouse \textit{Slc25a46} mutants exhibit a spectrum of
disorders similar to those in patients with recessive loss of SLC25A46 function [51–53].

Our study has several implications. First, although the true biological significance of the genes identified from GWAS for complex disorders is often questioned, our findings provide evidence to support the functional roles of the identified genes which is consistent with the prevailing theories of RLS pathogenesis. Of note, the function of CCDC141 and VSTM2L has not been fully elucidated. Further studies for these two genes might provide novel mechanisms of RLS, particularly in patients with migraine. Second, only one previous study had employed zebrafish to evaluate the function of Meis1 gene; however, the study investigated only hindbrain development [54], without phenotypic studies to simulate RLS. Our study further demonstrated the utility of zebrafish to model the behavioural phenotypes of RLS in humans. Spreading depression (or depolarization) (SD) could be used as a preclinical model for migraine study, particularly migraine with aura [55]. A recent paper has established the method to measure SD in the adult zebrafish tectum [56]; therefore, it can be used to examine the “migraine-like” phenotype in the corresponding adult zebrafish mutants in the future. With accurate diagnoses and strict criteria for the patient recruitment, we obtained significant signals with a limited sample size. However, only common variants were included from the GWAS results in this study. Further investigations are required to look at rare variants with fine mappings. Moreover, we focused on SNPs located in or near a gene in the replication analysis for reasons stated in Methods. The possibility that SNPs not mapped to a gene have roles in pathogenesis remains to be examined. Finally, our findings provide biological insights on the ample clinical evidence supporting the RLS-migraine comorbidity, which may support the implement of a detailed questionnaire about sleep disorder and restless legs symptoms in patients with frequent migraine in clinical practice. For those with symptoms with RLS, testing for iron, ferritin or other secondary causes of RLS may be mandatory. Moreover, it may be appropriate to treat RLS with dopaminergic D2 agonist in patients with migraine, which may be beneficial for both RLS symptoms and migraine in these patients [57].
Conclusions
To conclude, our study suggests that CCDC141 and VSTM2L are associated with increased risks of RLS in patients with migraine. Interference of these two genes, as explored in zebrafish, leads to RLS-like phenotypes which might be related to dysregulated dopaminergic neurotransmission.

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s10194-022-01409-9.

Additional file 1. Morpholino sequences. Supplementary Table 1. describing morpholino sequences used in this study.

Additional file 2. Primer sequences for sgRNA cloning. Supplementary Table 2. including the Primer sequences for sgRNA cloning.

Additional file 3. Protospacer for making crRNA. Supplementary Table 3. describing the protospacer for making crRNA.

Additional file 4. SNPs selected for association studies for restless legs syndrome in patients with migraine. Supplementary Table 4. detailing the SNPs selected for association studies for restless legs syndrome in patients with migraine.

Additional file 5. Association of RLS with SNPs within dopamine receptor or tyrosine hydroxylase genes. Supplementary Table 5. describing the SNPs within genes of dopamine receptor or tyrosine hydroxylase with borderline significant association with restless legs syndrome in patients with migraine.

Additional file 6. Expression pattern of ccdc141 and vstm2l in 1–4 dpf embryos of zebrafish. Supplementary Figure 1. in situ hybridization was conducted with ccdc141 and vstm2l antisense RNA probes on wild-type embryos.

Additional file 7. Summary of morpholino (MO) results. Supplementary Table 6. showing the summary of MO results.

Additional file 8. Hyperkinetic movements of pectoral fins in vstm2l morphants in comparison with that of wild-type. A video showing that the vstm2l morphants (right) having a higher fin flapping frequency than that of wild-type (left).

Additional file 9. Summary of CRISPR/dCas9 results. Supplementary Table 7. showing the summary of CRISPR/dCas9 results.

Additional file 10. Gene expression of targeted genes, th expression and fin movement of ccdc141 and vstm2l CRISPR‑injected embryos. Supplementary Figure 2. (A) ccdc141 CRISPR‑injected embryos showed (Aa) increased gene expression level, (Ab) reduced th-positive amacrine cells and (Ac) hyperkinetic movements compared with non-injected embryos (AB).

Additional file 11. Stable KO lines and corresponding genotyping methods. Supplementary Table 8. detailing the stable KO lines and corresponding genotyping methods.

Additional file 12. The analysis of stable F2 ccdc141E12 −4 bp and vstm2lE2 −8 bp knocked-out embryos. Supplementary Figure 3. The number of th-expressing amacrine cells in the homozygous mutants showed no statistically significant difference, compared with respective sibling controls, including 4 dpf (A) ccdc141E12 −4 bp and (B) vstm2lE2 −8 bp embryos.

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Authors’ contributions
SJC and SJW had full access to all of the data in the study and take the responsibility for the integrity of the data and the accuracy of the data analysis. SJW, JLF, CSF, SPC, MYC, YJJ, and LSK were involved in the study conception and design. SJW, JLF, SPC, and YFW recruited the patients; CSF, MYC, CLH, and SPC were responsible for acquisition, analysis, and interpretation of QWAS data; YJJ, HYH, and KCC were responsible for the zebrafish studies. YJJ and SPC were responsible for manuscript drafting. YJJ, CSF, JLF, MYC, HYH, KCC, YFW, CLH, LSK, SPC, and SJW contributed to critical revision of the manuscript for important intellectual content. All authors provided the final approval of the version to be published.

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Availability of data and materials
The details of zebrafish experiments were provided in the Additional files. The other supporting data are available from the corresponding authors upon reasonable request.

Declarations
Ethics approval and consent to participate
The human study was approved by the Institutional Review Boards of TVGH, Taiwan (TVGH-IRB-2011–11–002QA & 2013–11–001AC). Written informed consent was obtained from each participant after full explanation of the study objectives and procedures. All clinical investigations were conducted according to the principles expressed in the Declaration of Helsinki. All collected information was de-identified before statistical analysis. The zebrafish study was approved by the Institutional Animal Care and Use Committee of National Health Research Institutes (NHRRI) (NHRRI-IACUC-105101-A and NHRRI-IACUC-108037) and TVGH (TVGH-IACUC-2017–002), Taiwan. The corresponding authors had full access to all of the data in the study and had full responsibility for the decision to submit for publication.

Consent for publication
Not applicable.

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

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