High myopia (HM) is defined as a spherical equivalent (SE) refractive error of at least \( \geq 6 \) diopters (D), or an axial length (AL) longer than 26 mm, or both [1,2]. The prevalence of myopia in children varies among ethnicities, with 53% in Singapore [3], 47% in China [4], 23% in the United Kingdom (UK) [5], and 31% in Australia [6]. Holden et al. predicted through meta-analyses that almost half of population in the world may become myopic by 2050, and 10% may develop HM [7]. Due to the severe ocular comorbidities, such as retinal detachment, glaucoma, macular degeneration, and cataract [8,9], HM has become the major cause of acquired blindness in East Asia [10]. Once the pathological changes are triggered, there is no effective method to efficiently stop or delay the development of HM.

Genetic factors play pivotal roles in the occurrence and development of high myopia. There is an arising number of studies elucidating the importance of genetic contribution [2]. Hitherto, there have been 25 MYP loci found by linkage analysis and exome sequencing studies [2]. Genome-wide association studies (GWAS) have detected more than 150 gene loci related with myopia [11-15]. Several causative genes have been identified and replicated across different populations, including seven autosomal dominant genes, \( ZNF644 \) [16] (Gene ID: 84146, OMIM: 614159), \( SCO2 \) [17] (Gene ID: 9997, OMIM: 604272), \( SLC39A5 \) [18] (Gene ID: 283375, OMIM: 608730), \( P4HA2 \) [19] (Gene ID: 8974, OMIM: 600608), \( BSG \) [20] (Gene ID: 682, OMIM: 109480), \( CCDC111 \) [21] (Gene ID: 201973, OMIM: 615421), \( CPSF1 \) [22] (Gene ID: 29894, OMIM: 606027); two autosomal recessive genes, \( LEPREL1 \) [23] (Gene ID: 20576, OMIM: 606000); two unclassified genetic loci, \( LEPRE2 \) and \( LEPRE3 \) [24] (Gene ID: 615146, OMIM: 61600001); and three non-coding RNAs, \( MIR503 \) [25] (Gene ID: 100086, OMIM: 608730), \( MIR616 \) [26] (Gene ID: 101004, OMIM: 608730), and \( MIR451 \) [27] (Gene ID: 102005, OMIM: 608730) [28].

We provide further evidence for the potential role of \( AGRN \) in HM inheritance and enlarged the current genetic spectrum of nonsyndromic HM by comprehensively screening the reported causal genes.
[23] (Gene ID: 55241, OMIM: 610341) and LRPAP1 [24] (Gene ID: 4043, OMIM: 104225); and two X-linked gene, OPNILW [25] (Gene ID: 5956, OMIM: 300822) and AR3 [26] (Gene ID: 407, OMIM: 301700). However, a large-scale screening manifested that only a handful of patients (<5%) harbored potential causal mutations in these genes [27], suggesting that other unidentified genes likely are responsible for HM as well. Kloss et al. analyzed 14 families of high myopia by utilizing whole exome sequencing (WES) and unveiled multiple genetic variants in the known MYP loci (e.g. AGRN (Gene ID: 375790, OMIM: 103320), EME1 (Gene ID: 146956, OMIM: 610885) and HOXA2 (Gene ID: 3199, OMIM: 604685) [28]. However, there is a lack of replicated cases to further prove the potential pathogenicity of these genes. Most recently, the AGRN gene was found to be involved in baseline refractive development through analysis of genetic networks regulating refractive ocular development in collaborative cross progenitor strain mice, further proving its role in myopia formation [29].

In this work, we performed WES and familial cosegregation in a two-generation family composed of an unaffected father and a mother and son pair with HM. An extremely rare mutation in AGRN (c.2627A>T, p.K876M) at MYP14 was identified in this family. Two other mutations in AGRN (c.4787C>T, p.P1596L/c.5056G>A, p.G1686S) were identified in two patients with sporadic HM, respectively. We then performed Sanger sequencing in the remaining patients with sporadic HM and unrelated patients, focusing on six reported causal genes. A total of eight heterozygous variants likely affecting the protein function were detected, enlarging the current genetic spectrum of nonsyndromic HM.

**METHODS**

**Sample enrollment:** Consecutive patients who came to High Myopia Clinic of the Eye Hospital of Wenzhou Medical University were investigated from the end of 2019 to early 2020. In total, 103 Chinese individuals diagnosed with nonsyndromic HM, as well as 200 healthy controls were recruited for this study. Notably, the affected group included a pair of high myopic mother and son from a Han Chinese family, and 101 sporadic patients; while the unaffected father from the Han Chinese family was chosen as one member of the 200 controls. All participants were without any systemic disease and no age or gender bias was observed in this study group. A panel of ophthalmologists undertook detailed medical assessments on all the participants. The medical records of those who have received surgery were excluded from our study. All patients met the diagnostic criteria of a refractive error of at least -6.0 D, and an ocular axial length of at least 26 mm. The refractive error of controls for both eyes were within the range of -0.50 to +1.0 D. The study was approved by Ethics Committee of the Eye Hospital of Wenzhou Medical University and in accordance with tenets of the Declaration of Helsinki and the ARVO statement on human subjects. Written informed consents were signed by all participants or their statutory guardians. All members enrolled in this study donated about 10 ml of a peripheral blood sample. Peripheral blood was stored in EDTA- containing vacutainer tubes. 5 ml of a peripheral blood sample were kept at -80 °C freezer as a backup. DNA was extracted from leukocytes in peripheral blood by using the Blood DNA Mini Kit (Simgen, Hangzhou, China) [30].

**WES and analyses:** We performed WES on the mother and son pair as well as the unaffected father. The genomic DNA was sheared into 100 base pairs via the Exome Enrichment V5 Kit (Agilent Technologies, Palo Alto, CA) to generate the whole-exome region libraries, in agreement with the manufacturer’s protocol. A HiSeq 2000 sequencer (Illumina, San Diego, CA) was used to sequence whole exome–enriched DNA libraries. The variants, including single-nucleotide variants and insertion–deletion variants, were identified with GATK. Reads were mapped against UCSC hg19 via Burrows-Wheeler Aligner (BWA).

**Sanger sequencing:** Sanger sequencing was further used to verify the segregated variants in the family of three. We subsequently performed Sanger sequencing of the AGRN, SLC39A5, SCO2, P4HA2, BSG, ZNF644, and CPSF1 in 101 sporadic high myopia patients. PCR conditions consisted of three steps: an initial 10 min denaturation at 95 °C; 35 cycles at 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 60 s; a final 7 min elongation at 72 °C. PCR primer pairs spanning all coding exons, splicing sites, and untranslated gene region (UTRs) were designed in the online program Exon Primer. Sanger sequencing was used to detect potential causal variants by using the ABI 3730XL automated DNA sequencer (Thermo Fisher Scientific, Carlsbad, CA). In this work, we set up seven panels of ophthalmologists, each responsible for one gene enrolled in the study; 100% coverage was attained.

**Pathogenicity evaluation:** Mutations with a minor allele frequency (MAF) were assessed in Exome Aggregation Consortium (EXAC) and the Genome Aggregation Database (gnomAD). Pathogenicity was evaluated by SIFT, PolyPhen2 HDIV, PolyPhen2 HVAR, MutationTaster, and PROVEAN. Human Splicing Finder software was used to assess the effect of splicing mutations. Amino acid sequences of multiple species were obtained from the National Center for Biotechnology Information. Multiple sequence alignment and assessment of evolutionary conservation were visualized by
RESULTS

Clinical features: Two hundred and five eyes of 103 consecutive patients with HM were examined (except one eye that lacked refractive diopeters). Age of onset for all patients was the preschool years. Of the 103 patients in the study, 49 (47.6%) were male, and 54 (52.4%) were female. Their mean (± standard deviation, SD) examination age was 42.21 (±15.88) years of age. The mean (±SD) spherical refraction was −16.17 D (±5.690 D) for the right eye and −15.82 D (±6.070 D) for the left eye. The mean (±SD) AL was 28.84 mm (±2.500 mm) for the right eye and 28.89 mm (±2.640 mm) for the left eye. One hundred seventy-six phakic eyes (89.3%) had spherical equivalents greater than −10 D and were identified as extreme HM. No systemic disease was found in all participants.

Mutations in AGRN: WES was performed on the son (HP10), the mother (HP1033), and the unaffected father (HP1032). The mean read depth for WES was >30X, and the median coverage of the targeted regions reached >95%. The detailed workflow of the variant analyses is shown in Appendix 1. After a comparative analysis of the WES data and familial cosegregation, an extremely rare missense mutation of c.2627A>T (p.K876M) within exon 15 of AGRN was identified at MYP14 (chromosome 1p26; Figure 1A). AGRN encodes agrin, a large proteoglycan that has multiple isoforms which have varied functions. The variant c.2627A>T is within the EGF Lam domain of the AGRN protein (Figure 2A). The pathogenicity of c.2627A>T was predicted by all five bioinformatics tools (Table 1). Multiple orthologous sequence alignment showed that K876M was identified in a highly evolutionarily conserved region across varied species (Figure 3A). We then predicted the topological model via the Phyre2 server in automated mode. The variant K876M was found to generate a newly formed bond between residue 876 and residue 850 (Figure 4A). These findings suggest that the missense mutation c.2627A>T has great potential to influence the protein structure of AGRN. To the best of our knowledge, our work is the first to replicate the role of AGRN as a nonsyndromic myopia-causing gene in Chinese families with HM.

After we verified the variant c.2627A>T of AGRN in the family with Sanger sequencing, we performed Sanger sequencing of the AGRN exons and splicing junction sites in the remaining 101 patients with sporadic HM. Two missense variants (c.4787C>T p.P1596L / c.5056G>A p.G1686S) passed the strict filtering steps (Figure 1B,C). Neither occurred in the 200 controls. Both mutations were present in existing databases (EXAC, GnomAD) with extremely rare frequency. The pathogenicity of the two variants of AGRN were further predicted by different bioinformatics tools (Table 1), and both were presented as evolutionarily conserved (Figure 3B,C). Structural modeling of G1686S and P1596L in AGRN showed significant alterations in the structure, which probably influence the protein function (Figure 4B,C). Due to the lack of genetic information from the family members of these two patients, however, we could not confirm linkage of these variants with HM.

Mutations in SLC39A5, SCO2, P4HA2, and ZNF644: To further investigate the genetic basis of the remaining 99 patients with sporadic HM, we performed Sanger sequencing in all coding exons and splicing sites of four reported autosomal causal genes: SLC39A5, SCO2, P4HA2, and ZNF644. These genes have been ascertained as the causal genes and replicated in multiple studies of populations with HM across the world. Five extremely rare variants potentially affecting the protein function were identified in five patients. Neither the five variants nor variants similar to them (not exactly the same but at the same amino acid sites) occurred in the 200 controls.

Two novel frameshift variants (c.1350delC, p.V451Cfs*76 and c.1023_1024insA, p.P342Tfs*41) in SLC39A5 were detected (Figure 1D,E). The two frameshift variants were within the Zip domain of the SLC39A5 protein (Figure 2B) and predicted to stop the open reading frame at codon 526 and codon 382, respectively, thus generating two different prematurely truncated SLC39A5 proteins. Another frameshift variant (c.244_246delAAAG, p. K82del) was found in SCO2 and located in functional domain SCO1-SenC (Figure 1F, Figure 2C), which would markedly influence the function of the SCO2 protein.

Two heterozygous missense mutations were detected in two different genes: c.545A>G (p.Y182C) in P4HA2 and c.3266A>G (p.Y1089C) in ZNF644 (Figure 1G,J). Both variants were located in the coding region (Figure 2D,F) and...
displayed strong pathogenicity according to the computational analyses (Table 1). The two variants were highly evolutionarily conserved through assessment of multiple sequence alignment of polypeptides of different species (Figure 3D,F). The crystal structure modeling of Y182C in P4HA2 showed the absence of bonds between residue 177 tyrosine and residue 182 tyrosine, and between residue 219 glutamine and residue 182 tyrosine (Figure 4D). The crystal structure modeling of Y1089C in ZNF644 demonstrated the absence of a hydrogen bond between the mutated residue 1089 glutamic and residue 1090 tyrosine (Figure 4F).

Mutations in BSG and CPSF1: Knockdown animal models of BSG and CPSF1 genes have been established and provided convincing evidence of the roles of these genes in the development of myopia [20,22]. Three variants, c.415C>T (p.P139S) in BSG and c.1708C>T (p.R570C) and c.2252C>T (p.S751L) in CPSF1, were identified in this work (Figure 1H,J,K). The c.415C>T variant in the BSG mutation was novel and not present in existing databases (EXAC and gnomAD); the two CPSF1 variants were rare and present in existing databases (EXAC and gnomAD) with low frequency (Table 1). Further analysis through Human Splicing Finder showed that the c.415C>T in the BSG mutation potentially created a new exonic splicing silencer (ESS) site and was predicted to affect the splicing site. Structural modeling of P139S in the BSG gene showed significant alterations in the structure and the absence of the hydrogen bond between the mutated residue 1089 glutamic and residue 1090 tyrosine (Figure 4F).
543 arginine acid (Figure 4G). Structural modeling of S751L in the CPSF1 gene showed the absence of the bond between the mutated residue 751 serine and residue 750 aspartic acid (Figure 4H).

**DISCUSSION**

Myopia has become the most common ocular abnormality worldwide. The myopic changes occur in not only the optical media but also the ocular walls [30]. There exists an interplay of genetic factors with environmental stresses in myopia pathogenesis. Studies of two independent population-based
| Sample ID | Age | Sex | SE (diopter) | AL (mm) | Gene | Exon | Mutation | Status | Type | GnomAD | EXAC | SIFT | Mutation Taster | Polyphen2 | Polyphen2 | PROVEAN |
|-----------|-----|-----|-------------|--------|------|------|----------|--------|------|--------|------|------|----------------|-----------|-----------|---------|
| HP10      | 18  | M   | −10.50/−10.25 | 27.30/27.01 | AGRN | 15   | c.2627A>T (p.K876M) | Het    | Mis sense | 4.07983e−06 | 0.000008 | D(0.001) | DC(0.999) | PD(1.000) | PD(1.000) | D(−5.32) |
| HP1033    | 43  | F   | −6.00/−5.63   | 24.05/24.19 | AGRN | 15   | c.2627A>T (p.K876M) | Het    | Mis sense | 4.07983e−06 | 0.000008 | D(0.001) | DC(0.999) | PD(1.000) | PD(1.000) | D(−5.32) |
| HP1042    | 41  | M   | −6.13/−6.00   | 27.59/27.54 | AGRN | 27   | c.4787C>T (p.P1596L) | Het    | Mis sense | 8.4354e−06 | 0.000009 | D(0.004) | DC(0.999) | PD(0.999) | PD(0.985) | D(−5.97) |
| HP1021    | 17  | M   | −8.50/−8.50   | 27.46/27.50 | AGRN | 29   | c.5056G>A (p.G1686S) | Het    | Mis sense | 3.24275e−05 | 0.000017 | D(0.008) | DC(1.000) | PD(1) | PD(0.993) | D(−4.72) |
| WH167     | 32  | F   | −29.00/−30.00 | 36.79/35.84 | SLC39A5 | 6   | c.1023_1024insA (p.P342Tfs*41) | Het    | Frame-shift | Novel | Novel | - | DC(1.000) | - | - | - |
| S111      | 41  | F   | −16.50/−19.59 | 27.76/27.95 | SLC39A5 | 9   | c.1350delC (p.V451Cfs*76) | Het    | Frame-shift | Novel | Novel | - | DC(1.000) | - | - | - |
| H381      | 48  | F   | −21.75/−31.24 | 28.68/33.84 | SCO2 | 1    | c.244_246delAAG (p.K82del) | Het    | Frame-shift | 2.07952e−05 | 0.000017 | D(0.966) | - | - | - |
| M78       | 72  | F   | −22.38/NA     | 30.36/30.73 | P4HA2 | 5    | c.545A>G (p.Y182C) | Het    | Mis sense | 4.06095e−06 | 0.000008 | D(0.001) | DC(0.999) | PD(1.000) | PD(0.984) | N(−7.23) |
| M15       | 35  | F   | −6.75/−17.38  | 25.88/31.25 | BSG  | 2    | c.415C>T (p.P139S) | Het    | Mis sense/Splicing | Novel | Novel | D(0.001) | DC(0.999) | P(0.797) | B(0.102) | N(−0.04) |
| HP15      | 23  | F   | −10.50/−10.50 | 28.44/28.46 | ZNF644 | 4   | c.3266A>G (p.Y1089C) | Het    | Mis sense | 9.69368e−05 | 0.000255 | D(0.000) | DC(0.999) | PD(0.999) | PD(0.982) | N(−2.24) |
| WH126     | 86  | F   | −6.00/−0.75   | 25.36/22.48 | CPSF1 | 21   | c.2252C>T (p.S751L) | Het    | Mis sense | 3.67929e−05 | 0.000027 | D(0.007) | DC(1.000) | P(0.915) | B(0.348) | D(−3.61) |
| WH091     | 68  | F   | −27.75/−22.75 | 32.04/30.73 | CPSF1 | 17   | c.1708C>T (p.R570C) | Het    | Mis sense | 8.12222e−06 | 0.000008 | D(0.045) | DC(0.999) | P(0.758) | P(0.601) | N(−1.93) |

Table 1. Potential causative variants uncovered in 103 Chinese patients with nonsyndromic HM.

Abbreviations: Het, heterozygous; SE, spherical equivalent; AL, axial length; OD, right eye; OS, left eye; M, male; F, female; NA, not available; PD, probably damaging; DC, disease causing; D, deleterious; B, benign; P, possibly damaging; N, neutral
cohorts comprising 5,256 and 3,938 individuals of European descent revealed the interactive effect between genetic predisposition and education [31]. The combined effect of these two factors on the risk of developing myopia is much higher than their additive effect. A similar interactive effect was also reported for axial length [13]. Notably, for people with low-frequency variants of a myopia susceptibility gene identified by Tkatchenko et al., time spent reading was associated with differential degree of refractive error [32]. Moreover, a recent study provided evidence of gene–environment interactions for 88% (128) of 146 refractive error-associated variants tested [33]. In one meta-analysis, heritability was estimated at 0.71 for refractive error [34], indicating that myopic changes were due more to genes than to environments.

The AGRN gene was shown to interact with EGR1, which was previously implicated in refractive eye development, and to regulate synaptic physiology in the retina [35,36]. In this study, an extremely rare variant (c.2627A>T, p.K876M) in AGRN was found in a family with HM. We also found two other rare variants (c.4787C>T and c.5056G>A) in the coding sequence of AGRN from two patients with sporadic HM. AGRN was located in the MYP14 locus, which was mapped with HM in Ashkenazi Jewish families [37]. Kloss et al. first reported that a heterozygous mutation (c.1304C>T, p.T435M) in AGRN was detected in a Danish family with HM that appeared to demonstrate autosomal dominant (AD) inheritance transmission [23]. Homozygous or compound heterozygous mutations in AGRN were previously reported to be associated with congenital myasthenia syndrome (CMS), which is characterized as fatigable muscle weakness [38]. AGRN encodes agrin, a large protein that is responsible for the integrity of neuromuscular transmission. The defect of neuromuscular transmission may lead to disorders of the nervous system or muscle, including ciliary muscle and extraocular muscles [39]. A previous study found a 17-month-old boy with CMS who harbored a homozygous mutation in AGRN exhibited ophthalmoplegia and ptosis [40]. Ptosis may develop into myopia [41]. Myopic staphylomata were reported to be related to the path of the lateral rectus and defect of the lateral rectus to the superior rectus band ligament [42]. The ciliary muscle was also thought to be associated with the development of refractive error [43]. The present study is the first to replicate the role of AGRN in HM and suggest AGRN as a possibly HM-causing gene in a Chinese population. The pathogenic mechanism of HM based on the AGRN variation is still obscure, and more efforts are needed to explain the role of AGRN in the pathogenesis of HM.

Potential pathologic mutations were also identified in SLC39A5, SCO2, P4HA2, BSG, ZNF644, and CPSF1. We summarize the reported mutations of the seven autosomal dominant genes from populations with HM worldwide. SLC39A5 and ZNF644 contributed most of the reported mutations (Figure 5A). Previously, mutations in ZNF644 have
been found in Chinese, African American, and Caucasian subjects [2]. However, we found only a previously reported mutation in ZNF644. SLC39A5 was involved with the bone morphogenetic protein/transforming growth factor-β (BMP/TGF-β) pathway. This pathway is responsible for modulating the extracellular matrix (ECM) of the sclera, which is thought to be one of the explanations of HM pathomechanism [18,44].

Two frameshift mutations were identified in the SLC39A5 gene in this study. A frameshift mutation was identified in SCO2 (cytochrome c oxidase assembly protein). Previous exome sequencing and Sanger sequencing has uncovered ten heterozygous mutations in SCO2 [2]. A mutation was identified in the BSG gene in this work. Previously, Jin et al. first generated Bsg knockin mice presenting the typical the HM

Figure 4. Simulated three-dimensional crystal structures of proteins. Predicted crystal structures of wild-type (left) and mutant (right) proteins. The wild-type (left) and mutant (right) residues are green, while the residues that bind with them are yellow (A–H).
A missense mutation was identified in P4HA2, which is critical in the stabilization of collagen formation. Disruption of the function of the P4HA4 protein may generate unstable collagen polypeptide chains, which may lead to sclera inclined to elongation because collagen is a vital part of the ECM of the sclera [45, 46]. Two missense mutation were identified in CPSF1, which is highly expressed in human ocular tissues and related to retinal ganglion cell (RGC) axonal growth in zebrafish [22]. Ouyang et al. detected six rare heterozygous loss-of-function variants in CPSF1, and this study further expands the mutational pool of mutations in CPSF1 of HM.

There is a large spectrum of variants for nonsyndromic HM. Seventy-seven mutations have been identified in AGRN, ZNF644, SCO2, SLC39A5, P4HA2, BSG, and CPSF1, including 34 novel variants and 43 rare variants. Approximately four fifths of patients harbor missense variants. After collecting data from GnomAD database, we depicted a picture to show the differences in allele frequency of 43 rare variants between East Asians, Americans, and Europeans (Figure 5B). Europeans have a lower allele frequency of all mutations. Americans have higher allele frequencies in mutations in SCO2 and ZNF644. East Asians have higher allele frequencies in ZNF644, SLC39A5, and BSG. Given that a few patients with nonsyndromic HM may be mistaken as healthy and enrolled in public databases, the real allele frequencies of known mutations in cohorts with nonsyndromic HM may be higher. ZNF644 contributed the most mutations to the genetic spectrum of nonsyndromic HM. Variants in ZNF644 are frequently found in Europeans, and even more frequently in East Asians and Americans. It is wise to give ZNF644 priority during genetic screening of nonsyndromic HM cases. The prevalence of myopia varies in different areas, and is especially high in East Asians. These studies provide preliminary data implying relatively unique genetic backgrounds of different population and geographic areas. More studies are needed to further elucidate founder effects associated with ethnicity and further dissect the geographic or regional differences. In conclusion, we provided additional evidence for the potential role of AGRN in HM inheritance and enlarged the current genetic spectrum of nonsyndromic HM by comprehensively screening the reported causal genes.

APPENDIX 1. THE WORKFLOW DIAGRAM OF VALIDATION.

To access the data, click or select the words “Appendix 1.”

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