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Abstract

PURPOSE. Congenital stationary night blindness (CSNB) is a clinically and genetically heterogeneous retinal disease. Although electroretinographic (ERG) measurements can discriminate clinical subgroups, the identification of the underlying genetic defects has been complicated for CSNB because ofgenetic heterogeneity, the uncertainty about the mode of inheritance, and time-consuming and costly mutation scanning and direct sequencing approaches. METHODS. To overcome these challenges and to generate a time- and cost-efficient mutation screening tool, the authors developed a CSNB genotyping microarray with arrayed primer extension (APEX) technology. To cover as many mutations as possible, a comprehensive literature search was performed, and DNA samples from a cohort of patients with CSNB were first sequenced directly in known CSNB genes. Subsequently, oligonucleotides were designed representing 126 sequence variations in RHO, CABP4, CACNA1F, CACNA2D4, GNAT1,GRM6, NYX, PDE6B, and SAG and spotted on the chip. RESULTS. Direct sequencing of genes known to be associated with CSNB in the study cohort revealed 21 mutations (12 novel and 9 previously reported). The resultant microarray containing oligonucleotides, which allow to detect 126 known and novel mutations, was 100% effective in determining the expected sequence changes in all known samples assessed. In addition, investigation of 34 patients with CSNB who were previously not genotyped revealed sequence variants in 18%, of which 15% are thought to be disease-causing mutations. CONCLUSIONS. This relatively inexpensive first-pass genetic testing device for patients with a diagnosis of CSNB will improve molecular diagnostics and genetic counseling of patients and their families and gives the opportunity to analyze whether, for example, more progressive disorders such as cone or cone-rod dystrophies underlie the same gene defects.

Genotyping Microarray for CSNB-Associated Genes

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Purpose. Congenital stationary night blindness (CSNB) is a clinically and genetically heterogeneous retinal disease. Although electroretinographic (ERG) measurements can discriminate clinical subgroups, the identification of the underlying genetic defects has been complicated for CSNB because of genetic heterogeneity, the uncertainty about the mode of inheritance, and time-consuming and costly mutation scanning and direct sequencing approaches.

Methods. To overcome these challenges and to generate a time- and cost-efficient mutation screening tool, the authors developed a CSNB genotyping microarray with arrayed primer extension (APEX) technology. To cover as many mutations as possible, a comprehensive literature search was performed, and DNA samples from a cohort of patients with CSNB were first sequenced directly in known CSNB genes. Subsequently, oligonucleotides were designed representing 126 sequence variations in *RHO*, *CABP4*, *CACNA1F*, *CACNA2D4*, *GNAT1*, *GRM6*, *NYX*, *PDE6B*, and *SAG* and spotted on the chip.

Results. Direct sequencing of genes known to be associated with CSNB in the study cohort revealed 21 mutations (12 novel and 9 previously reported). The resultant microarray containing oligonucleotides, which allow to detect 126 known and novel mutations, was 100% effective in determining the expected sequence changes in all known samples assessed. In addition, investigation of 34 patients with CSNB who were previously not genotyped revealed sequence variants in 18%, of which 15% are thought to be disease-causing mutations.

Conclusions. This relatively inexpensive first-pass genetic testing device for patients with a diagnosis of CSNB will improve molecular diagnostics and genetic counseling of patients and their families and gives the opportunity to analyze whether, for example, more progressive disorders such as cone or cone–rod dystrophies underlie the same gene defects. (*Invest Ophthalmol Vis Sci.* 2009;50:5919–5926) DOI:10.1167/iovs.09-3548

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Congenital stationary night blindness (CSNB) is a clinically and genetically heterogeneous retinal disease. It can be associated with deficiency of vision under dim light conditions, nystagmus, refractive error, or retinal changes. Electroretinography is helpful in confirming and subclassifying the disorder. The disease can also be classified with respect to the gene defect. Mutations in genes involved in the phototransduction cascade (*GNAT1*, *PDE6B*, *RHO*, *RHOK*, and *SAG*) are among those that can lead to autosomal dominant CSNB. Although the phenotype of patients with mutations in *GNAT1*, *PDE6B*, or *RHO* may vary, the disease course seems to be stationary with primarily scotopic vision affected.[1] Mutations in *RHOK* and *SAG* lead to Oguchi disease,[2] which is a rare, autosomal recessive, nonprogressive congenital night blindness. It is characterized by a diffuse grayish white discoloration of the fundus that disappears after a long period of dark adaptation (Mizuo phenomenon).[3] Reduced rod function will improve after an extended period of dark adaptation. Mutations in genes involved

downstream of the phototransduction cascade can lead to either a complete or incomplete type of CSNB. Both types are characterized by an absent or severely reduced b-wave in the mixed ERG response, revealing a so-called electronegative ERG. The incomplete phenotype is characterized by a defect in the ON/OFF pathway and has been associated with mutations in genes (*CACNA1F*, *CABP4*, and *CACNA2D4*) that are essential for glutamate release from photoreceptors to the adjacent bipolar cells. Although mutations in *CACNA1F* are associated with X-linked recessive inheritance, mutations in *CABP4* and *CACNA2D4* are associated with an autosomal recessive trait. The complete CSNB phenotype is mainly associated with a defect in the ON pathway. It has been associated with a gene important for glutamate uptake (*GRM6*) and a gene of unknown function (*NYX*). Mutations in *GRM6* lead to autosomal recessive CSNB. Alterations in *NYX* lead to X-linked recessive CSNB (reviewed in Zeitz[4]). Although the disease course of CSNB has been described as nonprogressive, it may be progressive, at least in some patients carrying mutations in *CACNA1F*,[5–7] *CABP4*,[8] or *CACNA2D4*.[9] In summary, to date, 10 different genes have been associated with CSNB, with the majority (80%) of mutations identified in *CACNA1F* and *NYX*.[4]

It is difficult in some cases to define the appropriate gene for mutation screening, because clinical data do not clearly identify the subtype or the mode of inheritance is not obvious (e.g., sporadic cases). In addition, disease-associated pathogenic variants identified in the 10 known genes so far have shown to be substantially heterogeneous with regard to clinical phenotypes. Currently, more than 100 different disease-associated variants have been identified in known CSNB genes.

To generate a time- and cost-efficient mutation-screening tool, we sought to develop a CSNB genotyping microarray. To cover as many mutations as possible on such a diagnostic tool, the DNA from our cohort of patients with CSNB was first sequenced in known CSNB-associated genes. These samples and those from previously characterized patients were also used to validate the microarray. The microarray was further tested on DNA samples of patients with CSNB of unknown genotype.

METHODS

Patients

The patients with CSNB involved in the study had the disease diagnosed in different centers in Europe and Canada (The Netherlands: Utrecht, Nijmegen, and Zeist; Belgium: Ghent; France: Paris and Montpellier; Germany: Freiburg, Erlangen, Giessen, Regensburg and Tübingen; Switzerland: Bern, Lausanne, and Basel; and Canada: Toronto). Research procedures were conducted in accordance with institutional guidelines and the Declaration of Helsinki. Before genetic testing, informed consent was obtained at each site from all patients, for diagnostic and/or research purposes, as appropriate.

Selection of Oligonucleotide Sequences to be Spotted on the Microarray

For the arrayed primer extension (APEX) microarray (Asper Biotech Ltd., Tartu, Estonia), 126 sequence variants were selected from multiple sources, including recent mutations identified in our laboratory and mutations or putative polymorphisms found in a comprehensive literature and database search.[4] DNA was extracted by standard methods (detailed information is available on request) and mutation analyses of *CABP4*, *CACNA1F*, *CACNA2D4*, *GRM6*, *NYX*, and *RHO* were performed as described recently.[8–11] Mutation analyses for *GNAT1* and *PDE6B* were performed by PCR-amplification of the 8 coding exons of *GNAT1* in 5 amplicons and the 22 coding exons of *PDE6B* in 20 fragments, by applying a polymerase enzyme (HotFire, Tartu, Estonia) and subsequently using direct sequencing (detailed conditions on request). Since none of our patients showed the typical fundus associated with Oguchi's disease, no sequence analysis of *SAG* and *RHOK* was performed.

Design of the CSNB Microarray: APEX Technology

We used APEX technology for designing a new microarray which is able to detect the 126 different CSNB-related variants. The assay is based on single-primer nucleotide extension,[12] and subsequently converted to an array format.[13] Detailed description of the methodology is available (Asper Biotech, Ltd., http://www.asperbio.com) and provided in the Supplementary Data, http://www.iovs.org/cgi/content/full/50/12/5919/DC1. In brief, 5′-modified (6-amino linker), sequence-specific oligonucleotides are arrayed on a glass slide. These oligonucleotides are designed with their 3′-end immediately adjacent to the variable site. PCR-prepared and -fragmented target nucleic acids are annealed to oligonucleotides on the slide, followed by sequence-specific extension of the 3′-ends of primers with fluorescence-labeled nucleotide analogues (ddNTPs) by DNA polymerase.[14] Reading of the incorporated fluorescence identifies the target sequence. APEX and PCR oligonucleotide primers were designed according to the wild-type gene sequences (ref. numbers.: *CABP4*, NM_145200; *CACNA1F*, AJ006216; *CACNA2D4*, NM_172364; *GNAT1*, NM_144499; *GRM6*, NM_000843; *NYX*, AJ278865; *PDE6B*, NM_000283; *RHO*, NM_000539; and *SAG*, NM_000541; provided in the public domain by the National Center for Biotechnology Information [NCBI], Bethesda, MD at http://www.snpper.chip.org, http://www.ncbi.nlm.nih.gov) for both sense and antisense strands.

Databases Used to Predict the Pathogenic Character of a Sequence Alteration

The following databases were used to evaluate the potential pathogenicity of sequence alterations: NCBI: http://www.ncbi.nlm.nih.gov/sites/entrez?db=snp&cmd=search&term/; Human Genome Browser: http://genome.brc.mcw.edu/cgi-bin/hgBlat/; GenCards: http://www.genecards.org/; PolyPhen (*Poly*morphism *Phen*otyping, http://genetics.bwh.harvard.edu/pph/, based on the information of sequence homologies and mapping of the affected amino acid to known 3-D protein structures[15,16]; and SIFT (Sorting Intolerant From Tolerant, http://blocks.fhcrc.org/sift/SIFT.html, Fred Hutchinson Cancer Center, Seattle, WA), which uses sequence homologies to predict whether an amino acid will affect protein function.[17] CSNB mutations are annotated according to the recommendation of the Human Genome Variation Society with nucleotide position +1 corresponding to the A of the translation initiation codon ATG in the cDNA nomenclature (http://www.hgvs.org/mutnomen).

RESULTS

Design of the CSNB Genotyping Microarray

To date, more than 100 CSNB-associated mutations in 10 different genes have been identified (see review by Zeitz[4]). To cover as many mutations as possible on a genotyping microarray, we first sequenced the samples from our CSNB DNA cohort in known CSNB genes. We identified 21 different mutations (Supplementary Table S1; both Supplementary Tables are online at http://www.iovs.org/cgi/content/full/50/12/5919/DC1). These included two recently described mutations: a *RHO* mutation that co-segregates with an autosomal dominant CSNB-phenotype in a large Swiss family and an *NYX* deletion frequently occurring in Flemish patients with CSNB (Supplementary Table S1). In addition, seven known mutations in the two X-linked genes were identified: three in *NYX* and four in *CACNA1F* (Supplementary Table S1). To our knowledge, the remaining 12 mutations have not been described. Six of those were found in *NYX* and six in *CACNA1F* (Table 1, Supplementary Table S1). From those patients in whom ERG examinations

TABLE 1. Summary of Novel CSNB Causing Mutations in *NYX* and *CACNAIF*

Gene/Exon	Nucleotide Change	Effect
NYX		
3	c.65G>A	p.Trp22Stop
3	c.143G>A	p.Cys48Tyr
3	c.187G>T	p.Glu63Stop
3	c.518G>C	p.Arg173Pro
3	c.607C>T	p.Gln203Stop
3	c.1370_1387del18	p.Gln457_Ala463delinsPro
CACNAIF		
7	c.935delA	p.Asp312ThrfsX10
23	c.2797G>T	p.Asp933Tyr
28	c.3400G>A	p.Glu1134Lys
29	c.3471_3472delGC	p.Gln1157HisX25
38	c.4424T>C	p.Leu1475Pro
38	c.4466C>G	p.Pro1481A1a

Direct sequencing of DNA from patients revealed six novel *NYX* and six novel *CACNA1F* mutations.

discriminated the complete and incomplete form of CSNB, mutations in *NYX* were found to be associated in patients with the complete form, whereas mutations in *CACNA1F* were associated with incomplete CSNB. Some patients with *CACNA1F* mutations showed a slightly more progressive phenotype. Segregation of the respective mutation with the phenotype was shown for those patients for whom other family members were available for analysis (e.g., patient 715.01; Fig. 1). In addition, control DNA samples were investigated for novel mutations of uncertain pathogenicity (Supplementary Table S1).

These mutations, a large fraction of previously described mutations, and sequence variants with unknown pathogenic character were used to generate the genotyping microarray. Specific deletions or mutations that did not reveal a specific signal during the validation procedure were omitted. This method resulted in a genotyping microarray containing 126 sequence variants of nine different genes implicated in CSNB: 2 *CABP4*, 63 *CACNA1F*, 2 *CACNA2D*, 1 *GNAT1*, 12 *GRM6*, 37 *NYX*, 1 *PDE6B*, 4 *RHO*, and 4 *SAG* mutations. To facilitate the

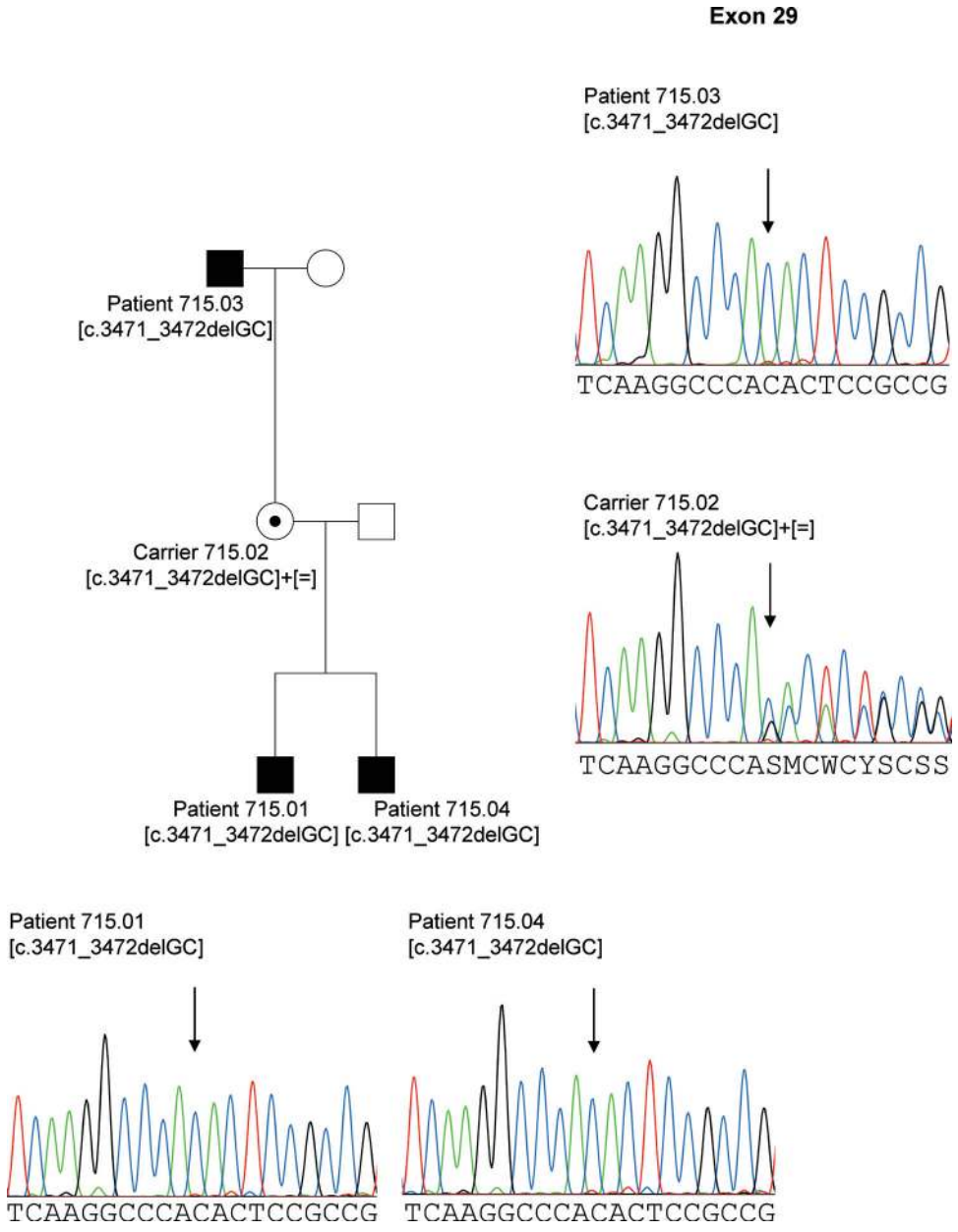

FIGURE 1. The electropherogram shows the novel dinucleotide deletion c.3471_3472delGC in exon 29 of *CACNA1F* in patient 715.01. The mother (715.02) was heterozygous for the deletion. The grandfather (715.03) and the brother (715.04) were hemizygous for this mutation. *Squares*: males; *circles*: females; *dots*: carriers; *filled symbols*: affected; *open symbols*: healthy.

TABLE 2. Detection of Known Mutations in Patients with the CSNB Genotype Microarray

Index	Phenotype	Gene	Exon Intron	Nucleotide change	Effect	Publication	Interpretation
CH2718	Incomplete CSNB	*CACNAIF*	Exon 7	c.945_947delCTT	p.Phe316del	21	Disease causing
D0706932	Incomplete CSNB	*CACNAIF*	Intron 21	c.2673+3G>A	Splice defect	18	SNP or modifier
27538	Incomplete CSNB	*CACNAIF*	Exon 24	c.2899C>T	p.Arg967Stop	27, 31	Disease causing
C1C00196	Incomplete CSNB	*CACNAIF*	Exon 25	c.3019G>A	p.Gly1007Arg	18	Disease causing
C1C00748	Incomplete CSNB	*CACNAIF*	Exon 33	c.3862C>T	p.Arg1288Stop	21, 33	Disease causing
825.01	Incomplete CSNB	*CACNAIF*	Exon 35	c.4091T>A	p.Leu1364His	27	Disease causing

interpretation of the outcome of such a microarray screening with CSNB patient samples with unknown genotype, we provide the original references in this study (Supplementary Table S2).

Validation of the CSNB Genotyping Microarray

The genotyping CSNB microarray was first validated with marked oligonucleotides, which served as positive internal controls. In addition, a negative control (with no DNA) was used to investigate the nonspecific background signal. To further test the capability of the microarray to detect sequence alterations, we screened 39 DNAs from patients with 37 known variants. All the expected variants were detected with 100% accuracy (Supplementary Table S2).

Screening Results in Previously Untested Patients with CSNB

To further evaluate the clinical validity of the CSNB array, we screened 34 additional patients with CSNB with unknown gene defect. Patients from different clinical centers were included in this study. This multicenter recruitment resulted in variability in the methods used to clinically assess patients included in the study. The screening, which was confirmed by direct sequencing, resulted in the detection of six sequence alterations in *CACNA1F*, of which five are thought to be disease causing (Table 2, Supplementary Table S2).

In summary, our screening detected sequence variants in 18% of these patients, of which 15% are thought to be pathogenic.

Rationale for Six CSNB Patients Screened on the Microarray Showing a Known *CACNA1F* Mutation

Patient 27538, had a c.2899C>T mutation in *CACNA1F*, which is predicted to lead to a premature stop codon at amino acid position 967 (p.Arg967Stop). His parents are consanguineous, and autosomal recessive inheritance was suspected. Because of the mutation identified in *CACNA1F* the assumed mode of inheritance was shown to be wrong. He is the only affected member of the family, and he shows a clear incomplete type of CSNB (Table 2).

The 12-year-old male patient CIC00196 with a c.3019G>A transition (p.Gly1007Arg) substitution in exon 25 of *CACNA1F* is a sporadic case. Mutation analysis in his father and mother did not show the mutation and thus the c.3019G>A transition represents a de novo mutation. Clinical data from this patient were suggestive of the incomplete type of CSNB (Table 2).

The 18-year old-male patient CIC00748 with a c.3862C>T transition in *CACNA1F* leading to a nonsense mutation (p.Arg1288Stop) in exon 33 mentioned having an affected cousin, indicative of an X-linked mode of inheritance. Again, clinical observations were suggestive of the incomplete type of CSNB (Table 2).

Clinical data for patient CH2718, revealing the c.945_947delCTT (p.Phe316del) in exon 7 in *CACNA1F*, and of patient 825.01 with a c.4091T>A mutation in *CACNA1F* (p.Leu1364His), showed an incomplete type of CSNB (Table 2).

Patient D0706932, with the putative splice site mutation, c.2673+3G>A in intron 21 in *CACNA1F* represents a simplex male case showing clinically signs and symptoms of incomplete CSNB (Table 2). However, further investigation of this variant explained in the next paragraph showed that this variant is probably not disease causing.

In summary, all six patients with a C*ACNA1F* sequence alteration detected by our microarray showed an incomplete CSNB phenotype that is in accordance with this gene defect. Except for the predicted splice site mutation c.2673+3G>A in intron 21, the identified mutations can be considered to be disease causing.

Detection of Additional Variants in Patients with Known Genotype

The microarray screening revealed three additional *CACNA1F* variants in patients with known disease-associated sequence variations. These variants were verified by direct sequencing

TABLE 3A. Variants Detected by Screening Patients with Known Genotype or Unclear Pathogenic Character

Gene	Exon Intron	Nucleotide Change	Effect	Publication	Index	Interpretation
CACNAIF	Exon 13	c.1523G>A	p.Arg508Gln	27	5854, 2422	SNP or modifier
CACNAIF	Exon 16	c.2204A>C	p.Asn735Thr	8	13276	SNP or modifier, unclear
CACNAIF	Intron 21	c.2673+3G>A	Splice defect	18	MT, 446.1, DO706932	SNP or modifier
CACNAIF	Intron 24	c.2938+IG>A	Splice defect	21	1344.01 Female	SNP or modifier, unclear
GRM6	Exon 3	c.727G>T	p.Val243Phe	This study, 23	13154, 7330	SNP or modifier
GRM6	Exon 3	c.824G>A	p.Gly275Asp	This study	8798	Unclear
GRM6	Exon 8	c.2090A>T,	p.Gln697Lcu	This study	7699	Unclear
CACNA2D4	Exon 25	c.2452C>T	p.Arg818Cys	This study	Not tested	Unlcear

Based on the phenotype, functional analysis, or co-segregation studies, these variants are interpreted as nonpathogenic or of unclear pathogenicity.

TABLE 3B. Disease-Associated Genotypes of Patients with Second Variant Listed in Table 3A

Index	Phenotype	Gene	Exon Intron	Nucleotide Change	Effect	Publication	Interpretation
5854	CSNB	*NYX*	Exon 3	c.647A>G	p.Asn216Ser	This study, 22, 23	Disease causing
2422	CSNB	*NYX*	Exon 3	c.1040T>C	p.Leu347Pro	26	Disease causing
MT	Complete arCSNB	*GRM6*	Exon 6	c.1214T>C	p.Ile405Thr	20	Disease causing
446.01	Complete XlCSNB	*NYX*	Exon 3	c.518G>C	p.Arg173Pro	This study	Disease causing
13276	Incomplete arCSNB	*CABP4*	Exon 2	c.370C>T	p.Argl24Cys	7	Disease causing
			Exon 6	c.800_801delAG	p.Glu267ValfsX92		
7330	CSNB	*NYX*	Exon 3	c.607C>T	p.Gln203Stop	This study	Disease causing

Based on phenotype, functional analysis, or co-segregation studies, these mutations are interpreted as disease causing.

(Supplementary Table S2 and Table 3A) and described in more detail in the following sections.

Predicted Splice Site Mutation: c.2673+3G>A. Patient MT (a woman) showed a homozygous c.1214T>C transition (p.Ile405Thr) in *GRM6*, and patient 446.1 revealed a c.518G>C transversion (p.Arg173Pro) in *NYX* (Table 3B). In addition, both patients carried a known predicted splice site mutation (c.2673+3G>A)[18] in *CACNA1F* (Table 3A). Clinical examination including electroretinography of the female patient MT revealed autosomal recessive complete CSNB.[19] Functional analysis of the c.1214T>C transition in *GRM6* showed that the phenotype is due to the absence of the receptor on the cell surface.[20] The c.2673+3G>A change in *CACNA1F* was heterozygous in MT. These findings indicate that the *GRM6* mutation is the disease-causing mutation in this patient and not the *CACNA1F* variant (Tables 3A, 3B).

Clinical examination including electroretinography of patient 446.1 was consistent with complete XlCSNB. Mutation analysis in *NYX* identified a novel hemizygous c.518G>C transversion leading to a p.Arg173Pro substitution, which co-segregates with the phenotype (the affected brother and grandfather were hemizygous, whereas the mother was heterozygous). The association of the complete form of X-linked recessive CSNB with *NYX* mutations and co-segregation of the mutation in the family supports the hypothesis that the *NYX* mutation is indeed the disease-causing mutation and not the *CACNA1F* splice site mutation (Tables 3A, 3B).

The predicted splice site mutation c.2673+3G>A in *CACNA1F* was first described in two patients from two independent families (T10, T26).[18] T10 was a simplex case and thus co-segregation analysis was not performed, whereas co-segregation was observed in the family of T26 (the affected brother was also hemizygous and the mother was a carrier). Because of co-segregation and the site of the variant in the consensus sequence of the splice donor site, the c.2673+3G>A was assumed to be pathogenic. However, now different databases (NCBI, Human Genome Browser) indicate that this substitution represents an SNP (rs41312124), although the frequency in different populations has not been defined. Taking into account the complete phenotype of our patients, as well as results of functional[20] and co-segregation analyses, we suggest that the c.2673+3G>A in *CACNA1F* reflects either a rare SNP or a variant modifying the phenotype of the patients (Table 3A).

Predicted Splice Site Mutation: c.2938+1G>A. Another known *CACNA1F* predicted splice site mutation (c.2938+1G>A)[21] was heterozygous in case 1344.01. This woman was clinically diagnosed with incomplete CSNB. Co-segregation analysis revealed that her unaffected sister was also heterozygous for the variation, her unaffected father was hemizygous for the variation, and her mother had two wild-type alleles (Fig. 2). These findings indicate that this sequence alteration did not co-segregate with the phenotype and thus, at least in this family, is not disease causing (Table 3A). Despite the fact that this sequence variant seems not to be disease causing the site is highly conserved and predicted to influence splicing. Splicing assays to be performed in the future will show the consequences of this sequence variant.

c.1523G>A Transition Leading to a p.Arg508Gln. Two patients, 5854 and 2422, showed a c.1523G>A transition causing a p.Arg508Gln substitution in *CACNA1F* (Table 3A), in addition to the already identified p.Asn216Ser (patient 5854) and p.Leu347Pro exchanges (patient 2422) in *NYX*, respectively (Table 3B). Re-evaluation of the clinical records of patient 5854 revealed no details about the CSNB phenotype. The mutation p.Asn216Ser in *NYX* has been described to be disease causing in two independent studies (three families).[22,23] In one of these studies co-segregation was shown in two affected family members.[22] Furthermore, the amino acid asparagine is highly conserved in a leucine-rich repeat.[23] These findings strongly argue for the fact that this sequence variant in *NYX* is indeed the disease-causing mutation (Table 3B).

Patient 2422 is a member of the large Dutch CSNB family that was used to link CSNB to *DXS228*, *MAOB*, and *NDP*.[24] Patients in this family showed clinical symptoms of night blindness, but it is also unclear whether they are affected by the incomplete or complete type of CSNB. Later, this linkage interval was refined to *DXS993* and *DXS228*. Subsequently, the *NYX* gene was identified in this region and shown to carry a mutation in this family (c.1040T>C; p.Leu347Pro) and in other patients.[22,25,26] *CACNA1F*, in contrast was mapped centromeric to *DXS2722* and *DXS255*. With respect to the haplotypes[24] of patient 2422, we suspect that all affected family members but also one unaffected male (III-5[24]) carry the p.Arg508Gln substitution in *CACNA1F* in addition to the *NYX* mutation. These findings suggest that, at least for this family the *NYX* mutation and not the amino acid substitution in *CACNA1F* is indeed disease causing. We cannot exclude the possibility that the *CACNA1F* sequence alteration modifies the phenotype (Tables 3A, 3B).

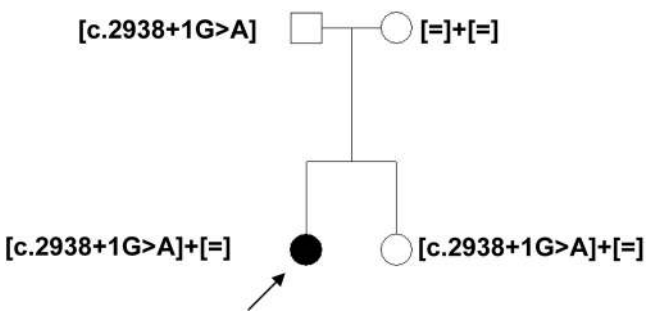

FIGURE 2. Segregation analysis of a heterozygous splice site mutation (c.2938+1G>A). The index patient (*arrow*) as well as the unaffected sister was heterozygous for the variation. The father was hemizygous for the variation, and the mother had two unaffected alleles.

The c.1523G>A transition leading to a p.Arg508Gln in *CACNA1F* itself was first described by Strom et al.[27] in two patients from two different families (03 and 06) (Table 3A). It was excluded in 120 control chromosomes analyzed by SSCP. The index patient of family 06 had a second substitution in *CACNA1F* (p.Leu849Pro) that was suggested to be non–disease causing as it "affected a non-conserved leucine." Hoda et al.[28] investigated the functional effect of p.Arg508Gln. They found no changes in the gating properties of the mutant channel subunit after heterologous expression in *Xenopus laevis* oocytes, but identified a temperature-dependent altered expression density of the Cav1.4 protein encoded by *CACNA1F*. It was thus theorized that the amount of expressed protein is critical for the correct function of the channel.[28] Different databases are available for use in investigating whether an identified variation is an SNP, based on allele frequency and evolutionary conservation (NCBI, UCSC Human Genome Browser and GeneCards). According to several databases, the sequence variation c.1523G>A (p.Arg508Gln) represents an SNP (rs34162630). Moreover, 294 samples have been investigated in populations from North America, Europe, East Asia, and West Africa and the A was found at a frequency of 0.25 (04.11.2008). Together, these findings indicate that the c.1532G>A transition in *CACNA1F* is either a polymorphism or a sequence alteration modifying the phenotype (Table 3A).

Putative Polymorphisms and/or Disease-Modifying Sequence Variations on the CSNB Microarray

In addition to the putative polymorphisms mentioned herein, other sequence variants, probably also representing SNPs or modifiers, can be detected with the CSNB genotyping microarray. A *CACNA1F* mutation c.2204A>C (p.Asn735Thr) in exon 16 was has been identified in a patient showing compound heterozygous mutations in the *CABP4* gene. Since his unaffected brother showed this substitution also, the sequence variant was classified as a rare polymorphism or modifier[8] (Tables 3A, B).

Furthermore, three different *GRM6* sequence variants were detected by applying the CSNB microarray (Table 3A): A heterozygous c.727G>T transversion (p.Val243Phe) in exon 3 was originally detected by direct sequencing of *GRM6* in a patient with CSNB, in whom the ERG data did not discriminate between the complete and incomplete form (patient 13154, Tübingen, Germany; CZ, EZ, BW, SK, WB, unpublished data, 2008). Because of the lack of DNA samples of family members, co-segregation could not be performed. A second mutation was not identified. We also detected this variant in another patient from Tübingen (7330) showing a nonsense mutation in *NYX* (p.Gln203Stop). The database GeneCards annotates this *GRM6* variant as a rare SNP (rs17078894). An investigation of 172 Europeans revealed an allele frequency of G: 0.99, T: 0.01 (November 4, 2008). Two of 178 control alleles analyzed by Dryja et al.[29] showed the same variant, suggesting that the *GRM6* variant is not disease causing. In patient 8798, a previously unreported c.824G>A nucleotide exchange (p.Gly275Asp) was identified in exon 3 of *GRM6*. Direct sequencing of the coding exons and flanking intronic regions revealed no second mutation, and thus it is not clear whether the c.824G>A exchange is pathogenic. A second mutation may represent a deletion of one or more exons, which would not be detected by direct sequencing. The c.824G>A nucleotide exchange was neither published nor predicted as an SNP in the available databases. Family members were not available for co-segregation analyses. Two bioinformatic algorithms, Polyphen and SIFT, were applied to predict the pathogenic character of this substitution: Polyphen classified this variant as probably damaging, whereas in SIFT, it was considered to be benign. A previously unreported third heterozygous *GRM6* sequence variant was found in exon 8 (c.2090A>T, p.Gln697Leu) in a female patient (7699). Again, due to the absence of a second mutation, the pathogenic character of the c.2090A>T substitution is not clear. It was neither published nor predicted as an SNP in the databases available and listed herein. Polyphen and SIFT predicted this variant as probably damaging. However, because of the absence of a second mutation, it is not clear whether this substitution in *GRM6* is indeed disease causing. Similarly, when we screened *CACNA2D4* for sequence alterations in our CSNB cohort, we detected a heterozygous c.2452C>T transition (p.Arg818Cys) in exon 25 in a patient with incomplete CSNB. A second mutation was not detected, and thus the pathogenic character remains to be unresolved. It was neither published nor predicted as an SNP in available databases. Polyphen and SIFT predicted this variant as probably damaging (Table 3A). Functional studies are needed to determine whether these sequence variations are pathogenic.

Summary of CSNB Microarray

In total of 126 sequence variants can be detected by the CSNB microarray. Based on the literature and our own validation of some cases, 118 of those are disease causing, whereas 8 of them are of uncertain pathogenic character, representing SNPs or modifiers (Table 3B, Supplementary Table S2). The microarray was 100% effective in detecting known variants and revealed a sequence variant in 18%, of which 15% are thought to be disease causing in DNA samples with previously unknown genotype.

Discussion

In this study we established a mutation detection tool for CSNB, which overcomes costly, low-sensitivity, and time-consuming prescreening methods such as SSCP and DHPLC. Although direct sequencing is the gold standard for genetic testing, genetic heterogeneity and large genes containing more than 30 exons remain labor intensive to investigate. The advantage of a CSNB microarray is that this method neither depends on large family pedigrees with more than one patient affected nor on a precise clinical discrimination of the different subforms of CSNB (e.g., incomplete versus complete CSNB; Table 4).

Initially, mutation analysis in our CSNB cohort was performed by direct sequencing to cover as many mutations as possible on this microarray. By doing so, 21 mutations were identified, including 2 recently published and 7 that had been described earlier. These studies indicated that at least 33% of patients with CSNB carry a known mutation in one of the known CSNB-associated genes. These findings led to the assumption that a CSNB microarray is a valuable diagnostic tool for new patients with CSNB. In total of 126 sequence variants can be detected by the CSNB microarray. Based on the literature and our own validations 118 of those are disease causing, whereas 8 of them are of uncertain pathogenic character, representing SNPs or modifiers. The microarray was 100% effective in detecting known variants, and 37 known variations in 39 DNA samples were reliably detected from both strands. By applying DNA samples from a CSNB cohort with unknown gene defect, the chip revealed a sequence variant in 18%, of which 15% are thought to be pathogenic. The detection rate may change in the future, when more laboratories are aware of such an array and will analyze their CSNB cases with this relatively inexpensive screening method. At this time our cohort of patients with CSNB with unknown gene defect was small ($n = 34$). The remaining mutations not detectable by the

TABLE 4. Advantages and Disadvantages of the CSNB Microarray

Advantage	Disadvantage
Robust	Detects only known variants
Can be updated regularly with new mutations	Detection rate at the moment only 15%–18%, new mutations can be added only after direct sequencing of CSNB genes
Validated with patients from different ethnic backgrounds	Number of patients at the moment low (39 DNA samples with known mutation, 34 with unknown genotype in which 6 showed a mutation on microarray screening)
Inexpensive	If no mutation is detected CSNB genes need to be sequenced directly
Simplex cases can be used	To confirm pathogenic character of mutation larger family still advantageous for cosegregation studies
Mutation detection does not depend on precise clinical discrimination	
Exclusion of CSNB mutation to use DNA for linkage or candidate gene approaches to identify new genes	
Prescreening method for diagnostics	Sequence validation required
Each sequence variant on the microarray can be followed up by the given reference	Needs careful interpretation and validation of the original reference

chip may be identified by direct sequencing of known CSNB genes or in novel genes underlying this disorder. These data will be then used to update the chip and will result in a higher detection rate in the future.

Nevertheless, these initial studies already suggest that the CSNB microarray is an efficient first-pass screening to detect known variants. It is especially useful for simplex cases, in patients in whom the mode of inheritance is unclear and in whom the ophthalmic examinations do not discriminate between the incomplete and the complete forms of CSNB. It can also be used to exclude CSNB cases of known mutations to furthermore use these samples to identify novel genes underlying CSNB by candidate gene approaches and in larger families by linkage analysis. Certainly, one must be aware that novel mutations in the known genes are missed by this strategy (Table 4).

Of note, our preliminary screening of CSNB patients with unknown gene defects revealed six *CACNA1F* variants (five pathogenic and one probable polymorphism or modifier). *CACNA1F* consists of 48 coding exons, and thus direct sequencing, although it is the gold standard, is still time consuming and costly compared with chip analysis. Therefore, we suggest that the chip is particularly useful for patients with the incomplete form of CSNB and in particular for patients with X-linked inheritance. Taken into account that the autosomal recessive genes *CABP4*, *CACNA2D4*, and *GRM6* have been only recently associated with CSNB, only a few mutations have been discovered in these genes. Thus, in the near future comprehensive analysis of these genes in families with an autosomal recessive inheritance may be more successful in identifying additional disease-causing mutations. The corresponding oligonucleotides of the newly identified mutations will then be added on the array and will make this tool also more attractive for recessive forms.

Furthermore, the CSNB microarray may also be used as a prescreening method for patients with more progressive retinal disorders including cone- or cone-rod dystrophies. Mutations in *CACNA1F*, *CABP4*, as well as *CACNA2D4* have been identified in patients initially diagnosed with nonprogressive CSNB. However, in some cases the phenotype turned out to be more progressive than originally believed. The diagnosis is often based on a first examination by ERG, revealing the typical electronegative ERG that is associated with CSNB.[30] Several cases with *CACNA1F* mutations have been reported in which either the same or different mutations lead to different phenotypic manifestations varying from classical incomplete CSNB to retinal and optic atrophy with a clinical progressive course of visual dysfunction and to X-linked cone–rod dystrophy.[4–7,31]

Similar phenotypic variations have been reported in patients carrying *CABP4* mutations. Two male patients from the same family with the same homozygous frameshift mutation developed either incomplete CSNB or a more progressive form associated with a decrease in visual acuity and photophobia, respectively.[8] Of interest, just recently a novel homozygous mutation in *CABP4* (p.Arg216Stop) was described leading to a congenital cone–rod synaptic disorder. The Dutch sib pair carrying this novel mutation showed reduced visual acuity, photophobia, and abnormal color vision, without symptoms of night blindness. Clinical presentations and ERG measurements displayed a predominant cone dysfunction.[32]

Mutations in *CACNA2D4* have been identified in a patient with the full-field ERG results suggestive of incomplete CSNB. However, the patient showed a mild form of cone dystrophy with a progressive decrease in visual acuity.[9] These studies indicate that the initial clinical diagnosis, especially for incomplete CSNB, must be validated over a certain time period. In some cases, the disease course turns out to be progressive rather than stationary or can even result in another severe retinal disease.

Thus, the CSNB microarray can also be considered for patients showing a more progressive form than the classic incomplete CSNB. It would also be interesting to investigate whether *CACNA1F* plays an important role in patients showing cone or cone–rod dystrophies.

As in our case, screening patients with known disease-associated sequence variations on such a microarray can also reveal unexpected findings. The chip outcome should always be compared to the respective references presented herein. In addition, for diagnostic purposes, it is mandatory to validate the outcome by direct sequencing and to perform co-segregation analysis if family members are available and, for autosomal recessive conditions, to screen the whole gene in case of identification of only one disease allele by the chip.

In conclusion, the microarray presented herein offers a prescreening tool for CSNB diagnostics. It is not only a cost-efficient method of screening patients with the different forms of CSNB but can also be used to test the hypothesis that *CACNA1F* plays an important role in more progressive retinal disorders like cone- or cone–rod dystrophies. Furthermore, as new mutations are identified, updated versions of the microarray will be generated in regular time intervals. The detailed information concerning the origin and clinical context of the

mutations described herein will help to better interpret the results of chip screening.

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