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Mutational spectrum of the *SLC4A11* gene in autosomal recessive congenital hereditary endothelial dystrophy

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Purpose: Autosomal recessive congenital hereditary endothelial dystrophy (AR-CHED or CHED2) is a bilateral corneal disorder manifesting at birth or in early childhood. CHED2 is caused by mutations in the sodium bicarbonate transporter-like solute carrier family 4 member 11 *(SLC4A11)* gene on chromosome 20p13. We screened 42 unrelated families with CHED2 in order to establish the spectrum of mutations in *SLC4A11* and to look for genotype-phenotype correlations.
Methods: Forty-two families (49 affected and 73 unaffected members) with recessive CHED were recruited according to predefined diagnostic criteria. Clinical data including age at onset and presentation, pre- and post-operative visual acuities, and presence of nystagmus were taken from patient records. Histopathologic parameters such as corneal thickness, Descemet membrane thickness, and endothelial cell counts were assessed on corneal sections. DNA from patients was screened for sequence changes by polymerase chain reaction (PCR)-amplification of coding regions of *SLC4A11* and single strand conformation polymorphism analysis followed by sequencing. Sequence changes found were tested in 50 unrelated normal controls.
Results: Twenty-seven different mutations were identified in 35 unrelated families, 19 of which were not previously reported. The mutations identified consisted of 13 missense, 5 nonsense, 7 deletions, 1 complex (deletion plus insertion) mutation, and 1 splice site mutation. Both mutant alleles were identified in 33 families and only one mutant allele in two families. No correlations were evident between clinical or histopathologic parameters and *SLC4A11* mutations.
Conclusions: These data add to the mutational repertoire of *SLC4A11* and establish the high degree of mutational heterogeneity in autosomal recessive CHED.

Congenital hereditary endothelial dystrophy (CHED) is a corneal disorder that results from degeneration and dysfunction of the endothelial cells. It manifests clinically as diffuse, bilateral corneal edema accompanied by corneal clouding with visual loss in the presence of an otherwise normal anterior segment. The corneal endothelium regulates corneal hydration by forming a barrier between the corneal stroma and the aqueous humor that limits the amount of water entering the stroma and by actively pumping out water from the stroma into the aqueous humor. An essential part of the pump mechanism is the Na/K ATPase-driven ion pump [1]. Loss of endothelial cell function results in excess water entering the stroma causing disruption of the collagen fibrils, light scattering, and opacification.

CHED has both autosomal dominant (locus CHED1; OMIM 121700) as well as autosomal recessive (locus CHED2; OMIM 217700) modes of transmission. Autosomal recessive CHED manifests at birth or within the first year of life and shows little or no progression. The autosomal dominant form of CHED generally presents itself later, but clinical manifestations of both dominant and recessive forms overlap [2]. Histological features of CHED-affected corneas include diffuse epithelial and stromal edema, defects in the Bowmans membrane, a degenerated corneal endothelium with multinucleated cells, and a thickened Descemet's membrane due to abnormal and accelerated secretion by the endothelial cells [3,4].

The locus for CHED2 maps to chromosome 20p13 as shown by studies on families from different populations [5-7]. Mutations in the sodium bicarbonate transporter-like solute carrier family 4 member 11 (*SLC4A11*) gene are responsible for CHED2 [8,9]. Mutations in *SLC4A11* are also responsible for Harboyan syndrome, a disorder involving congenital corneal endothelial dystrophy and perceptive deafness (CDPD) [10].

SLC4A11 (also known as BTR1 (bicarbonate transporter related protein-1), NaBC1) was identified and cloned on the basis of its homology with bicarbonate transporter proteins, and found to be widely expressed in several tissues [11]. The *SLC4* (solute carrier family 4) family of genes code for integral membrane proteins with 10-14 transmembrane segments. These proteins function as Cl-HCO_3 exchangers, Na/HCO_3 cotransporters, or Na^+-driven Cl-HCO_3 exchangers [12]. *SLC4A11* was subsequently recognized as the mammalian homolog of the borate transporter *BOR1* in *Arabidopsis* functioning as a Na+-coupled borate transporter that conducts Na+ and H+ in the absence of borate and as an electrogenic Na-borate cotransporter in presence of borate [13,14].

We screened 49 patients with CHED2 belonging to 42 families for mutations in the *SLC4A11* gene in order to identify the range of pathogenic mutations and to look for genotype-phenotype correlations.

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METHODS

Patients and controls: The study had the approval of the Institutional Review Board of the L. V. Prasad Eye Institute and conformed to the tenets of the declaration of Helsinki. Patients underwent complete ophthalmic examination and two ophthalmologists evaluated clinical parameters independently. Diagnosis of CHED was made in all cases by clinical and histopathological criteria. Affected individuals were diagnosed at ages ranging from <1 to 24 years. Inclusion criteria for patients recruited for the study were the presence of a cloudy cornea from birth to 10 years of age with increased corneal thickness and bilateral corneal edema, a normal corneal diameter, normal intraocular pressures, histopathological features as evaluated on corneal buttons, of thickening of the Descemet's membrane, and changes in endothelial cell count and/or morphology. Forty-nine patients and 73 unaffected family members from 42 unrelated families were enrolled in the study. Informed consent was obtained from all participants for clinical and molecular genetic studies. Genomic DNA was extracted from 4-8 ml of blood samples obtained from all the patients and the unaffected family members and from 50 unrelated normal controls of Indian origin who were free of corneal disease.

Mutational screening: The *SLC4A11* gene is 10.3 kb in length and consists of 19 exons which code for an 891 amino acid- residue protein. For amplification of the *SLC4A11* coding regions, 19 primer pairs were designed for amplifying exons and adjacent intronic regions. All polymerase chain reactions (PCR) were done (thermal cycler PTC 200; MJ Research, Watertown, MA) using 75 ng genomic DNA in 25 µl reaction containing 1X PCR buffer, 200 µM dNTPs, 0.5 µM of each primer, 4% dimethyl sulfoxide (DMSO), and 1 U *Taq* polymerase. Single strand conformation polymorphism (SSCP) was performed according to the protocol described previously [15].

Samples that showed variation in SSCP as compared with normal controls were subjected to direct sequencing of PCR products. PCR products were purified and sequenced bidirectionally. The sequences were compared with the published cDNA sequence of *SLC4A11* (GenBank NM_032034) and mutations identified were evaluated for their presence or absence in DNA samples from 50 unrelated normal control individuals using restriction enzymes where appropriate. For mutations without changes in restriction enzyme sites, SSCP or sequencing was used for screening of controls.

Clinical and histopathological features: Quantitative assessments of thickness of cornea, of Descemets membrane (DM), and endothelial cell counts were taken on PAS-stained corneal sections by image capture and analysis using the Axiovision digital imaging software (Axiovision AC Rel 4.5) from Carl-Zeiss AG (Hallbergmoos, Germany). For thickness of cornea/DM, thickness was measured at three points in the central cornea (magnification 4X for cornea, 100X for DM) and the average reading was taken for each specimen. Endothelial cell counts were done at a magnification 40X on one section from each patient. These values were compared with data on age-matched normal corneas. Normal corneas for different age groups such as 0-5 years, 5-10 years, 11-15 years, and 16-20 years were obtained from histopathology eyeball specimens of patients with retinoblastoma, uveal melanoma, malignant melanoma, and cryptococcal granulomatous lesions of choroid. Corneal thickness as measured in normal controls showed no significant difference in range between the age groups selected. Values obtained for normal thickness of the DM in different age groups were: 3.6 µ or less for 0-5 years; 4.3 µ or less for 6-10 years; 7.0 µ or less for 11-15 years; 8.7 µ or less for >16 years. Thickness of DM of CHED corneas was categorized into grades based on ranges of normal thickness for age-matched controls to facilitate correlations (N=normal; ±= borderline; 1+ to 4+=increasing thickness).

RESULTS

All families had the recessive form of CHED as suggested by clinical examination and/or history of the disease in parents and siblings of the probands. Mutations were identified in 41 patients from 35 unrelated AR CHED families. We found 27 different mutations, which were absent in a control population of the same ethnic origin as determined by restriction enzyme digests or SSCP analysis on 100 chromosomes. Cosegregation of mutations with disease was verified in cases where family members were available (shown in Table 1). Out of the 27 mutations, there were seven deletions, one complex mutation (deletion plus insertion), five nonsense mutations, 13 missense mutations, and one substitution involving a splice site (Table 1). Twenty-three of thirty-five families with mutations were consanguineous.

Six deletions involved exons and were found in probands from 8 of 35 families. Four deletions are predicted to cause frameshifts (listed in Table 1). Two were inframe deletions; these were a four-amino acid deletion (p.Glu293_Glu296del) in CH-31 and a single amino acid deletion (p.Asp797del) in CH-50. Two families, CH-36 and CH-47, had an intronic deletion of 19 bp within intron 7 (c.996+26C_+44Cdel19bp) in the absence of any other changes detected. One complex mutation (deletion plus insertion) was found in family CH-12 (Table 1). Among the eight families with deletions, two families were non-consanguineous (CH-42, CH-50). In CH-42, a single heterozygous single base deletion leading to a frameshift was identified in the absence of a second mutation. The homozygous inframe deletion (p.Asp797del) in CH-50 was found to be heterozygous in both parents of the proband. More than one parent or unaffected relative was tested in four of six consanguineous families with deletions and they were found to be heterozygous for the mutations identified in probands. In two families (CH-5 and CH-31), only one parent was tested (Table 1) and found to be heterozygous.

Five nonsense mutations (Arg112X, Arg605X, Glu632X, Gln803X, and Arg875X) were found in nine probands and of these probands, affected individuals were apparently homozygous in five families all of which reported parental consanguinity (Table 1). Compound heterozygosity for nonsense and missense changes was seen in three families, all of which were non-consanguineous (CH-40, CH-30, CH-21). The compound heterozygous changes in these probands were tested for

Table 1. Details of families with autosomal recessive congenital hereditary endothelial dystrophy and mutations in *SLC4A11*

Sample number	Family number	Affected	Unaffected	Parental consanguinity	Mutation in cDNA	Mutation in protein	Exon	RFLP	Novel/ reported	Mutation type
1	CH-34	1	0	Y	c.140delA	Tyr47SerfsX69	2		N	Deletion
2	CH-42	1	2 (F,M)	N	c.[306delC]+[?]	Gly103ValfsX13	3	MspI (-)	N	Deletion
3	CH-2	2	2 (F,U)	Y	c.473_480del8 bp	Arg158GlnfsX4	4	ItaI (-)	[10]	Deletion
4	CH-5	1	1 (M)	Y	c.473_480del8 bp	Arg158GlnfsX4	4	ItaI (-)	[10]	Deletion
5	CH-19	2	2 (F,M)	Y	c.618_619delAG	Val208AlafsX38	5		N	Deletion
6	CH-45	1	4 (F,M,S)	Y	c.618_619delAG	Val208AlafsX38	5		N	Deletion
7	CH-31	1	1 (F)	Y	c.878_889del12	Glu293_Glu296del	7	EcoRI (-)	N	Deletion
8	CH-50	1	2 (F,M)	N	c.2389_2391delGAT	Asp797del	17	TaqI (-)	N	Deletion
9	CH-12	1	3 (F,M,S)	Y	c.1317_1322del6ins8	Leu440ValfsX6	10	HaeII (-)	N	Complex
10	CH-52	1	3 (F,M,S)	Y	c.334C>T	Arg112X	3		N	Nonsense
11	CH-40	1	4 (F,M, G,U)	N	c.[334C>T]+[2318C>T]	Arg112X+Pro773Leu	3+17	HapII(-)		Nonsense
12	CH-21	1	1 (F)	N	c.[334C>T]+[1751C>A]	Arg112X+Thr584Lys	13+3	AluI(+)	N	Nonsense
13	CH-63	1	0	Y	c.1813C>T	Arg605X	14		[8]	Nonsense
14	CH-65	1	0	Y	c.1813C>T	Arg605X	14		[8]	Nonsense
15	CH-28	1	0	N	c.[1813C>T]+[?]	Arg605X	14		[8]	Nonsense
16	CH-48	1	1 (G)	Y	c.1894G>T	Glu632X	14		N	Nonsense
17	CH-38	1	1 (F)	Y	c.2407C>T	Gln803X	17	MaeI (+)	N	Nonsense
18	CH-30	1	2 (F,M)	N	c.[2264G>A]+[2623C>T]	Arg755Gln+Arg875X	17+19		[8]	Nonsense
19	CH-3	2	6 (F,M,S,U)	Y	c.625C>T	Arg209Trp	5		N	Missense
20	CH-20	1	4 (F,M,S)	Y	c.625C>T	Arg209Trp	5		N	Missense
21	CH-1	1	2 (F,M)	Y	c.638C>T	Ser213Leu	5		N	Missense
22	CH-27	1	1 (U)	N	c.697C>T	Arg233Cys	6	BbvI (+)	N	Missense
23	CH-61	1	1 (M)	N	c.1253G>A	Gly418Asp	10	BveI (+)	N	Missense
24	CH-17	1	1 (F)	N	c.[1202C>A]+[1418T>G]	Thr401Lys+Leu473Arg	9+11	AccII (+)	N	Missense
25	CH-49	1	2 (F,M)	Y	c.1466C>T	Ser489Leu	12		[8]	Missense
26	CH-16	2	1 (M)	Y	c.1751C>A	Thr584Lys	13	AluI (+)	N	Missense
27	CH-11	1	3 (F,M,S)	N	c.2263C>T	Arg755Trp	17		[18]	Missense
28	CH-57	2	1 (F)	Y	c.2318C>T	Pro773Leu	17	HapII (-)	N	Missense
29	CH-8	1	1 (F)	N	c.2470G>A	Val824Met	18	BshNI (-)	[10]	Missense
30	CH-33	1	1 (M)	N	c.2470G>A	Val824Met	18	BshNI (-)	[10]	Missense
31	CH-58	2	1 (F)	Y	c.2605C>T	Arg869Cys	18		[8]	Missense
32	CH-32	1	1 (M)	Y	c.2605C>T	Arg869Cys	18		[8]	Missense
33	CH-36	1	2 (F,M)	Y	c.996+26C_+44Cdel19	Not known	IVS-7		N	Deletion
34	CH-47	1	1 (F)	Y	c.996+26C_+44Cdel19	Not known	IVS-7		N	Deletion
35	CH-53	1	1 (M)	Y	c.1091-1G>C	Not known	IVS-8	MaeI (-)	N	Splice site

Families with CHED2 in whom mutations were identified are shown with a number of affected and unaffected members available for study. Unaffected members such as the father (F), the mother (M), siblings (S), uncle (U), and grandparent (G) that were tested are shown in parentheses. Parental consanguinity is shown as present (Y) or absent (N). RFLPs show restriction enzyme sites created (+) or destroyed (-). In the Novel/Reported column, "N" indicates that the novel mutations and their numbers represent references of reported mutations. Mutation nomenclature is as per recommendations.

cosegregation in both parents as well as two other unaffected relatives in CH-40, in both unaffected parents in CH-30, and in one parent in CH-21. All parents tested had single heterozygous changes confirming that the two mutations found in each of the probands were in *trans*. In family CH-28 (Table 1), a single heterozygous change leading to mutation Arg605X was found and we did not identify the second mutant allele by the methods used.

The 13 identified missense changes identified were Arg209Trp, Ser213Leu, Arg233Cys, Thr401Lys, Gly418Asp, Leu473Arg, Ser489Leu, Thr584Lys, Arg755Trp, Arg755Gln, Pro773Leu, Val824Met, and Arg869Cys (Table 1). Among the probands with missense mutations, 13 were apparently homozygous and four were compound heterozygous. Compound heterozygosity for missense+nonsense mutations was found in three families (CH-21, CH-30, and CH-40, discussed above). One proband (family CH-17) had two compound heterozygous missense changes (Thr401Lys+Leu473Arg). One unaffected parent of the proband from family CH-17 was tested and found heterozygous for one of these alleles indicating that the two mutations were in *trans*. In eight families with homozygous changes, only one unaffected parent or relative was tested and found to be heterozygous for the changes found in the probands (Table 1). In five families with homozygous missense changes, two or more unaffected relatives including both parents were tested for cosegregation of mutations.

A homozygous splice site mutation (c.1091-1G>C) was found in one affected from family CH-53, affecting the conserved splice acceptor dinucleotide AG at the intron 8-exon 9 junction.

No changes were found in seven families (four consanguineous, three non-consanguineous). Six of these families had a single affected member while one family had two affected (not shown).

Table 2. Mutations and phenotypic features of patients with autosomal recessive congenital hereditary endothelial dystrophy

Sample number	Family number	Mutation in SLC4A11	Age at onset (first graft)	Cornea	DM	Post-op VA (RE, LE)	Nystagmus
1	CH-34	Tyr47SerfsX69	At birth (20)	+	4+	20/125, 20/100	Y
2	CH-42	Gly103ValfsX13	NA (7)	+	1+	20/125, 20/200	Y
3	CH-2	Arg158GlnfsX4	At birth (9)	+	4+	20/60, 20/30	-
4	CH-5	Arg158GlnfsX4	NA (13)	+	±	20/40	-
5	CH-19	Val208AlafsX38	At birth (5)	+	2+	20/125	Y
6	CH-45	Val208AlafsX38	At birth (20)	+	1+	CF1.5M	Y
7	CH-31	Glu293_Glu296del	At birth (8)	+	N	20/25, 20/40	-
8	CH-50	Asp797del	At birth (6)	+	1+	20/50	-
9	CH-12	Leu440ValfsX6	At birth (9)	+	4+	20/70, 20/60	-
10	CH-52	Arg112X	At birth (-)	NA	NA	NA	-
11	CH-40	Arg112X+Pro773Leu	At birth (<1)	+	N	NA	-
12	CH-21	Arg112X+Thr584Lys	At birth (4)	+	1+	20/80	Y
13	CH-63	Arg605X	At birth (<1)	+	2+	NA	-
14	CH-65	Arg605X	At birth (4)	+	2+	20/40, 20/50	-
15	CH-28	Arg605X	At birth (9)	+	N	CF1M, 20/70	-
16	CH-48	Glu632X	At birth (12)	+	4+	20/80	-
17	CH-38	Gln803X	At birth (7)	+	4+	NA	-
18	CH-30	Arg755Gln+Arg875X	At birth (2)	+	2+	20/40	-
19	CH-3	Arg209Trp	NA (11)	+	4+	20/50, 20/40	-
20	CH-20	Arg209Trp	At birth (18)	+	4+	20/400	Y
21	CH-1	Ser213Leu	At birth (10)	+	2+	20/400, 20/125	Y
22	CH-27	Arg233Cys	At birth (6)	+	2+	20/50, 20/40	-
23	CH-61	Gly418Asp	At birth (<1)	+	N	NA	Y
24	CH-17	Thr401Lys+Leu473Arg	At birth (18)	+	2+	NA, 20/100	-
25	CH-49	Ser489Leu	At birth (<1)	+	±	FFL	-
26	CH-16	Thr584Lys	At birth (2)	+	±	20/100, 20/400	Y
27	CH-11	Arg755Trp	6 years (10)	+	2+	20/50, 20/80	-
28	CH-57	Pro773Leu	At birth (-)	NA	NA	NA	-
29	CH-8	Val824Met	At birth (9)	+	3+	20/30, 20/20	-
30	CH-33	Val824Met	NA (16)	+	N	20/50, NA	-
31	CH-58	Arg869Cys	At birth (5)	+	1-2+	20/400	Y
32	CH-32	Arg869Cys	NA (13)	+	1+	NA	
33	CH-36	c.996+26C_+44Cdel19bp	At birth (<1)	+	N	20/200, NA	Y
34	CH-47	c.996+26C_+44Cdel19bp	At birth (6)	+	N-1+	20/50, 20/100	
35	CH-53	c.1091-1G>C	NA (24)	+	N	20/200, 20/160	Y

Families and mutations listed in Table 1 are shown here with corresponding age at onset (given in years) and of first graft (in parentheses) and the thickness of cornea and Descemets membrane. "NA" indicates not available. Mutations for the last three patients listed are intronic and indicated in cDNA. Thickness of the Descemets membrane (DM) is graded as given in text. Corneal thickening is denoted by "+". For DM thickness, N indicates normal, ± indicates borderline thickening, and grades 1+ to 4+ represent increasing thickness as compared with corneas from age-matched normal controls. Visual acuities (VA) listed correspond to the best VA obtained after the first corneal graft. RE -right eye, LE-left eye, CF-counting fingers, FFL-fixing and following light. Nystagmus is shown as present (Y) or absent (-).

Clinical and histopathologic parameters of the patients were assessed and are shown in Table 2. Clinical parameters taken into account were age at onset, post-operative vision after the first graft, and presence of nystagmus. Histopathological parameters examined on the patients' original corneas were the thickness of cornea and of Descemets membrane and endothelial cell counts. Endothelial cell counts were found to be reduced significantly (about 10 fold) in all patients as compared with age-matched controls and are not detailed here. Corneal thickness as measured on formalin-fixed sections was not graded for severity due to the likelihood of artefactual changes in thickness during processing of the eyeballs. Thickness of Descemet's membrane in the CHED patients studied varied from 3 μm to 30 μm and was graded for extent of thickness taking into account the age at surgery and normal values for DM thickness for the particular age group (shown in Table 2). No apparent correlations were found between DM thickness or post-operative visual acuities (after first corneal graft) and the type or location of mutations in *SLC4A11* (Table 2). Better visual outcomes (as reflected by a post-operative visual acuity of 20/50 or better) showed no correlation with mutation type or location and were found in association with frameshift mutations (CH-2, CH-5), in-frame deletions (CH-32, CH-50), nonsense mutations (CH-65, CH-30), and missense mutations (CH-3, CH-27, CH-8, CH-33). The worse outcomes for visual acuities (VA of 20/100 or worse) were again not associated with types or locations of mutations. The duration of disease (time interval between the onset of disease and surgery), which may be expected to have an impact on the visual outcome, showed considerable variation in our series, ranging from <1 year to 18 years (Table 2). However, visual outcomes in this series did not necessarily correlate with duration of disease. Durations of disease in patients with poorer post-operative visual acuities (VA of 20/100 or more) ranged from <1 year (CH-16) to 18 years (CH-34, CH-20; see Table 2). It is possible that graft-related factors contributed to the final visual outcome. Nystagmus, which is an indicator of severe visual loss, was present in 12 out of 35 unrelated patients with mutations. Again these patients were heterogeneous with respect to types of mutations (Table 2). The age at onset of disease was similar in most cases, being at birth.

DISCUSSION

Screening of the *SLC4A11* gene identified 27 mutations (of which 19 mutations are previously unreported) in 35 unrelated patients with CHED2. Ten mutations are predicted to result in a premature termination of the encoded protein and may be expected to cause instability of the mutant mRNA or protein. Two of the six deletions found were in-frame deletions, one was a deletion of four amino acids (in CH-31) predicted to be in the NH_2-terminal cytoplasmic domain of the protein (Uniprot) and the other was a deletion of a single amino acid, Asp797 (in CH-50) predicted to be in the transmembrane domain 11/12 [11].

Eight missense changes found in this study were not reported earlier (Arg209Trp, Ser213Leu, Arg233Cys, Thr401Lys, Gly418Asp, Leu473Arg, Thr584Lys, Pro773Leu). Multiple sequence alignment of members of the *SLC4* family from different species to examine the degree of evolutionary conservation of these residues showed that the mutated residues identified are well conserved except for Arg209 and Ser213 (not shown). These two residues are predicted to lie within the NH_2-terminal cytoplasmic domain of the protein (Uniprot). Both missense changes, Arg209Trp and Ser213Leu, involve non-conservative substitutions and may be speculated to be unfavorable to the structure of the protein. The Blosum-80 score for substitution of Arg to Trp is -5 and that for serine to leucine is -4, suggesting that these substitutions are unlikely to occur. The high degree of conservation of the residues mutated and the nonconservative nature of the substitutions involved added support to the conclusion that these missense changes are likely to be pathogenic mutations rather than rare polymorphisms. Cosegregation analyses were not complete in families where only one parent or relative was available. In such cases, the observed homozygosity for mutations especially in non-consanguineous families may represent either true homozygosity or compound heterozygosity for a missense mutation and a large deletion or non-amplification of the second allele.

An intronic deletion of 19 bp (+26 to +44) in intron 7 was found in patients of two families (CH-36 and CH-47, Table 1). It was absent in 100 normal chromosomes. Splice enhancers both within exons as well as introns are known to regulate splicing of mRNA. A class of *cis* elements (intronic splice enhancers, ISEs) with G-repeat sequences regulates splice site selection of small introns of vertebrate genes [16]. Mutations of these ISEs, located 28-45 nucleotides (nt) into IVS3 (92 nt long) of the human GH-1 gene, have been reported to cause skipping of the adjacent exon and result in autosomal dominant form of isolated growth hormone deficiency (IGHD-II) [17]. The intronic deletion identified in the present study is in intron 7, which is a small intron of 87 base pairs and contains G-repeats within the deleted sequence. We hypothesize that sequences affected by the deletion in intron 7 of *SLC4A11* function in mRNA splicing and the deletion may therefore disrupt splicing.

Among the 35 families with identified mutations, 29 families had apparent homozygous changes and 23 of these were consanguineous. Four families had compound heterozygous changes and two families had single heterozygous changes within the coding region of *SLC4A11*. The simplest explanation for the detection of a single mutant allele in two families (CH-28 and CH-42) and no mutations in the coding region of *SLC4A11* in seven families is failure of the SSCP method to detect all changes. Data obtained so far suggest that recessive CHED is genetically homogeneous and that mutations in SLC4A11 are responsible for the phenotype [8,9,18]. It is unlikely that these cases represent dominant CHED since family history did not show affected members in previous generations. However, the possibility of locus heterogeneity for recessive CHED cannot be ruled out altogether at present.

This study adds 19 mutations to the 34 different mutations in *SLC4A11* reported to date [8-10,18,19] with a total of 53 mutations in this gene in 76 families including those who

participated in this study. Recurrent mutations found in three or more families identified from various studies so far include Arg112X (present study, Table 1), p. Arg158GlnfsX4 (this study, [10]), and Arg605X (this study, [8,9]).

Phenotypes of CHED patients in our study showed variations in the extent of the thickening of DM, in the presence of nystagmus, and in post-operative visual acuities. None of the variations could be correlated with types or distribution of mutations in *SLC4A11*. Although we cannot at present rule out that some of the CHED patients included here may actually have CDPD, the identification of mutations in CDPD/Harboyan syndrome that are essentially of a similar nature and overlap with those in CHED2 [10] suggests that there may be other factors modifying the effect of *SLC4A11* in a tissue-specific manner.

In summary, data from this and our earlier study [9] describing *SLC4A11* mutations in 47 families of Indian origin demonstrate a high degree of mutational heterogeneity for the CHED2 phenotype in this population. No correlations could be found between clinical or histopathological features of the disease and *SLC4A11* mutations.

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