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Article

Mutational Analysis of the *SLC4A11* Gene in a (Filipino) Family with Congenital Hereditary Endothelial Dystrophy

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Abstract: Aim: The study aims to identify if mutations in the *SLC4A11* gene are present in Filipino families affected with congenital hereditary endothelial dystrophy (CHED). Methods: This is a family cohort study that investigated the genetic profile of a selected family in northern Luzon, Philippines, whose members were diagnosed with congenital hereditary endothelial dystrophy (CHED). A patient who was diagnosed with CHED prior to this study served as the proband for this family. A detailed family history was obtained and a complete ophthalmologic examination was performed on each of the family members. A total of six affected members and three unaffected members were included in this study. DNA was isolated from peripheral blood samples of the family members, polymerase chain reaction (PCR) was used to amplify the gene's entire coding region (19 exons and 2 putative promoter regions), and finally, the amplified regions were analyzed using DNA sequencing. Results: Consanguinity was not present in the family. Corneal haze was reported to have been present since birth or shortly thereafter in all the affected patients. Slit-lamp examination showed edematous corneas. Molecular studies of the *SLC4A11* gene revealed four novel homozygous point mutations variably presenting in the six affected members, as well as the three unaffected members. One unaffected family member (I-1) had a novel sense mutation absent in the other family members. All affected siblings showed little phenotypic variability. Conclusions: This is the first report that gives us a genetic profile of a northern Luzon family with members affected by CHED. This study supports earlier findings that mutations in the *SLC4A11* gene are not consistently the same among different ethnic groups worldwide, probably due to the disease's genetic heterogeneity. Our study documented five novel mutations, adding to the growing list of mutations probably responsible for acquiring the CHED phenotype. It is possible that there are more novel mutations waiting to be discovered in this hereditary disease. Screening for these specific mutations in other families may prove useful for genetic counseling, prenatal diagnosis, and the future development of gene therapy.

Keywords: congenital hereditary endothelial dystrophy; mutation; *SLC4A11* gene; cornea

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1. Introduction

Congenital hereditary endothelial dystrophy (CHED) is a condition characterized by bilateral, diffuse corneal clouding that impairs visual acuity. The condition is traditionally classified into two genetic forms: autosomal dominant (CHED1) and autosomal recessive (CHED2), although this classification has recently been modified [1]. The severity of the disorder varies, with the latter being more severe and usually more common, and many patients require penetrating keratoplasty to restore their vision.

The exact prevalence, sex distribution, and incidence of CHED are currently unknown, although some authors have reported an incidence of 3 per 100,000 births [2]. Most cases have been identified in children of consanguineous parents from Saudi Arabia, India, Pakistan, Myanmar (Burma), and Ireland [3].

CHED1 and CHED2 have been mapped to chromosome 20 at two distinct loci. The *SLC4A11* gene, located on chromosome 20p13, encodes a transmembrane protein that is involved in the transport of bicarbonate ions across cell membranes. This gene is expressed in various tissues, including the corneal endothelium, where it plays a critical role in maintaining corneal transparency. Mutations in *SLC4A11* have also been found to lead to Harboyan syndrome (characterized by CHED in association with sensorineural hearing loss), with little evidence to support a genetic difference between these phenotypes [4].

Over 60 different mutations in *SLC4A11* have been identified to date, and the functional consequences of these mutations vary depending on their location and severity. The primary abnormality is attributed to a dysfunctional endothelium, resulting in the thickening and opacification of the cornea [5]. How these mutations cause damage to endothelial cells is poorly understood, but is thought to be caused by either dysfunctional changes in protein structure, protein function, or both [6].

Mutational studies of *SLC4A11* have been performed mainly on patients from India [6], as well as from Korea [7] and China [8]. At present, no published data are available from the Philippines. This study aimed to determine the presence of mutations in the *SLC4A11* gene, characterize the mutations, and assess the role of these mutations in the causation of CHED2 in patients from northern Luzon.

2. Methodology

A patient who had been diagnosed with CHED and treated in our institution was selected as a proband for a family to be included in this study. The sample population included the proband's nuclear family and extended family. The study group included all subjects clinically diagnosed with CHED during screening. A separate study group included all unaffected subjects in the family being screened. Detailed family histories were collected and a pedigree chart was constructed to represent the subjects. Consanguinity was also noted based on marriage histories if present.

The study included the proband and their family members within one generation higher and lower. Family members who were not directly related to the proband by blood and those unaffected by the disease who were below 18 years old were excluded.

2.1. Clinical Studies

The clinical examination primarily involved portable ophthalmic equipment, since the family resided in a remote region of the Philippines (Mayoyao, Ifugao). This included routine slit-lamp biomicroscopy and a tonopen to take the intraocular pressure. Ancillary diagnostics such as specular microscopy, ultrasonic pachymetry, and ultrasonography for posterior segment evaluation were offered to the family members who decided to visit our hospital. A diagnosis of CHED was made clinically on the basis of the following characteristics: the presence of mosaic corneal haze with corneal edema or an increased central corneal thickness (>0.7 mm in all cases), a normal horizontal corneal diameter (10–11 mm), and no evidence of congenital glaucoma (e.g., no buphthalmos, Haab's striae, or optic disc cupping). The affected family members formed the study group and a separate study group included all family members unaffected by the disease.

2.2. SLC4A11 Gene Mutation Screening Analysis

2.2.1. Sample Collection

Eight milliliters of peripheral blood were collected from the enrolled patients. Blood was then drawn into ethylenediamine tetraacetic acid (EDTA) tubes and processed for buffy coat separation. Each tube was centrifuged at 4000 rpm for 10 min at room temperature (25 °C). After centrifugation, the buffy coat layer was collected and placed in 1.5 mL microcentrifuge tubes. Each tube was centrifuged at 3000 rpm for 15 min at room temperature. Excess layer (plasma or RBC) was removed and the buffy coat was processed for the extraction of genomic DNA.

2.2.2. DNA Extraction

The collected buffy coat was processed for DNA extraction according to the QiaAmp DNA MiniKit ®((QIAGEN, Hilden, Germany). Briefly, 200 µL of the buffy coat was lysed using a lysis solution (Buffer AL) and proteinase K was added to degrade the proteins. Cells were incubated at 56 °C for 10 min or until complete lysis. To precipitate the isolated DNA, ethanol was added to each sample. Wash buffers (AW1 and AW2) were added separately to the spin columns to facilitate the removal of contaminants. To elute the purified genomic DNA (gDNA), Buffer AE was added to the spin columns. The DNA quality and quantity of the extracted eluent were assessed using q Nanodrop® v1000 spectrophotometer. A final working concentration of at least 50 ng/µL of gDNA was used for each sample.

2.2.3. Gene Amplification

DNA sequences of certain regions of the *SLC4A11* gene were amplified as described by Paliwal et al. [9], with some modifications, mainly in the annealing temperatures conditions for each exon (Table 1). In a 25 µL PCR reaction, 10 ng DNA, 1.5 mM $MgCl_2$, 0.25 mM of each dNTP, 10 pM of each primer, 0.7 units of Taq polymerase, and 1 × Q solution (Qiagen GmBH, Hilden, Germany) were mixed. The amplified samples were visualized on 2% agarose gel to determine their fragment length. The PCR products were purified and processed for DNA sequencing.

2.2.4. DNA Sequencing and Mutational Analysis

All PCR products were purified using NucleoSpin® Gel and PCR Clean-up. The purified PCR products were sent to 1st Base Sequencing ((JTC MedTech Hub, Singapore) in triplicates for DNA Sequencing Services Single Pass Reaction. Nucleotide sequences for the coding regions were compared with the reference nucleotide sequence of the *SLC4A11* gene on human chromosome 20 (NG_017072.1). The generated DNA sequences were analyzed using BioEdit, 4Peaks, and Seqotron software (2019 version) for alignment with the reference sequence and checked for mutations. Individuals who did not show *SLC4A11* coding region changes were screened for mutations in the putative promoter region, as well using their respective primers.

Table 1. Summary of forward and reverse primers that amplified certain exon regions for nucleotide sequence analysis. The expected product size and annealing temperatures used for each primer pair used for amplification are also presented. Reference gene sequence NG_017072.1 was used for succeeding comparison and analysis as well as exon designation to maintain consistency with NCBI website [9].

***SLC4A11* GENE**		**Forward primer** (Location of the forward primer on *SLC4A11* Gene)	**Reverse Primer** (Location of the reverse complement of the reverse primer on *SLC4A11* Gene)	**Expected Product Size**	**Annealing Temperature**
Actual Exon Number Based on RefSeqGene on Chromosome 20 (NG_017072.1)	**Designated Exon Number by Paliwal et al. [9], 2010 according to mRNA and cDNA Sequences (NM_032034)**				
Exon 2	Exon 1	5′ CCTAGCAGATGGGCTAAGCA 3′ (6424–6443)	5′ GAGCAAAGCCACAGGACTCT 3′	327	60
Exon 3–4	Exon 2 and 3	5′ CGAGAGTGGGACAGTCCAG 3′ (9243–9261)	5′ CTCCCTGTTGAGTGCTCCT 3′	515	61
Exon 5–6	Exon 4 and 5	5′ TCCAGGAGCAGCTCAACAG 3′ (9774–9792)	5′ CAGCCCTCTTCTCCCAAGTT 3′	647	55
Exon 7	Exon 6	5′ CCAACCAACTTGGGAGAAGA 3′ (10434–10453)	5′ CCTTCAGAGGCCAGGACAT 3′	390	52
Exon 8–9	Exon 7 and 8	5′ AAAACCTGCTGCCAGTTCAT 3′ (12596–12615)	5′ CCTAGGAATGGGGGATGG 3′	551	57
Exon 10–11	Exon 9 and 10	5′ ACTGATGGTACGTGGCCTCT 3′ (13087–13106)	5′ CGTCCATGCGTAGAAGGAGT 3′	527	58
Exon 11	Exon 11 and 12	5′ TCTACATCCAGGGTGCAGTG 3′ (13499–13518)	5′ CGTCCATGCGTAGAAGGAGT 3′	115	58
Exon 14–15	Exon 13 and 14	5′ GAGCCCTTTCTCCCTGAGAT 3′ (14377–14396)	5′ GGTTGTAGCGGAACTTGCTC 3′	583	61
Exon 16–17	Exon 15 and 16	5′ CGGGAAATCGAGAGTGAGTT 3′ (14884–14903)	5′ CGTCTCCTTCACGTTCACAA 3′	633	54
Exon 18–19	Exon 17 and 18	5′ CTGGCCACATGGGACATAG 3′ (15454–15472)	5′ CTAGGCAGGACCCCTCCTC 3′	556	61
Exon 20	Exon 19	5′ CAGGAGGGGCTCCAGTCTA 3′ (16221–16239)	5′ CTGTCCCTTGCATTCCACTT 3′	692	61
	Putative Promoter Region 1	5′ GCCTTACTCACCCAATCTATGC 3′ (3921–3942)	5′ CCCTGTCTCCTCCTTTCGAC 3′	679	61
	Putative Promoter Region 2	5′ GGAGGAGGAGAAGGACTTGC 3′ (4534–4553)	5′ GCACACTCGCGCACTCAC 3′	532	61

3. Results

3.1. Clinical Examination and Demographics

One family was recruited during a visit to northern Luzon. The age of all the family members being screened ranged from 11 to 57 years (Table 2). Based on the pedigree (Figure 1) constructed for the affected family, the disease appeared to have an autosomal recessive transmission. The disease was only present in generation II, where six out of nine siblings (66.67%) were affected. In the family studied, both parents were unaffected and the presence of consanguinity was not noted.

Table 2. Summary of exon numbering and its corresponding locations in the *SLC4A11* gene. This is based on the reference sequence gene NG_017072.1 (NCBI).

Exon Number	Location	Exon Number	Location
Exon 1	5052–5147	Exon 11	13397–13510
Exon 2	6654–6698	Exon 12	13595–13727
Exon 3	9348–9500	Exon 13	13982–14055
Exon 4	9625–9674	Exon 14	14466–14718
Exon 5	9928–10159	Exon 15	14790–14896
Exon 6	10240–10321	Exon 16	14979–15147
Exon 7	10605–10728	Exon 17	15231–15404
Exon 8	12694–12912	Exon 18	15535–15730
Exon 9	13000–13093	Exon 19	15814–15983
Exon 10	13184–13309	Exon 20	16386–16820

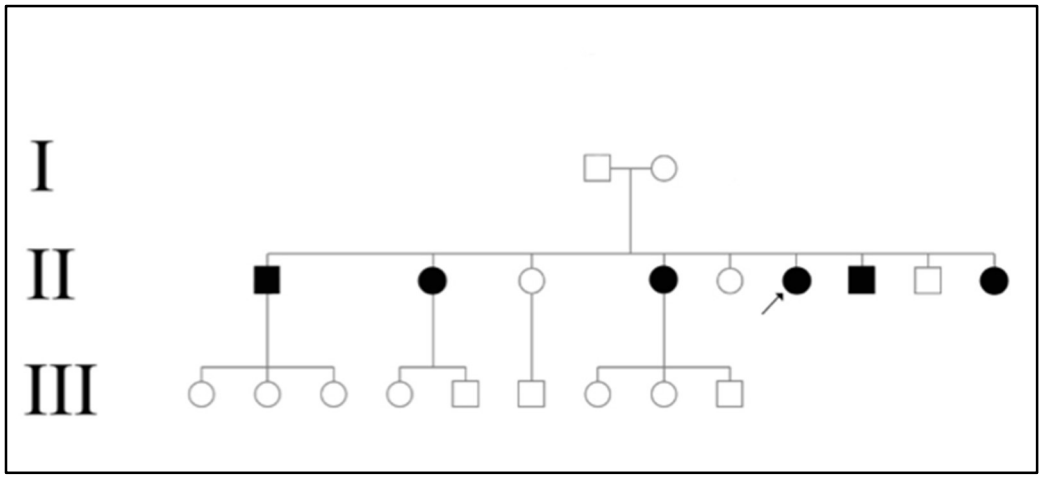

Figure 1. Pedigree showing the three generations (Labelled as I, II, and III) of the family being investigated. Male members are shown as squares while female members are shown as circles. Affected individuals are shown in black solid shapes. Proband is marked with an arrowhead.

A total of six family members were clinically diagnosed with CHED, with an age range from 11 to 37 years (Table 2). All of them presented with bilateral corneal opacity shortly after birth that gradually progressed towards early childhood (Figure 2). The average age of onset was noted to be 1 year after birth, while significant visual symptoms were first acknowledged at ages 6 to 7 years old. Corneal pachymetry revealed an increased corneal thickness (>0.7 mm) in two patients prior to undergoing a corneal transplant. There were no pachymetry data for the other four patients, however, slit-lamp examination revealed marked corneal edema bilaterally. The intraocular pressure was within the normal range (below 21 mmHg) for all patients. Best-corrected visual acuity (BCVA) among the surgically untreated CHED patients ranged from 20/640 to 20/800, while the BCVA of surgically treated CHED patients ranged from 20/30 to 20/70 when excluding eyes with complications. Eyes with BCVA of CHED patients with a failed graft ranged from 20/800

to hand movement. No other associated eye diseases were found during our examination. One patient (II-4) in family 1 reportedly had associated bilateral hearing loss, though this was only subjective, as there was no appropriate equipment available to properly document the associated symptoms.

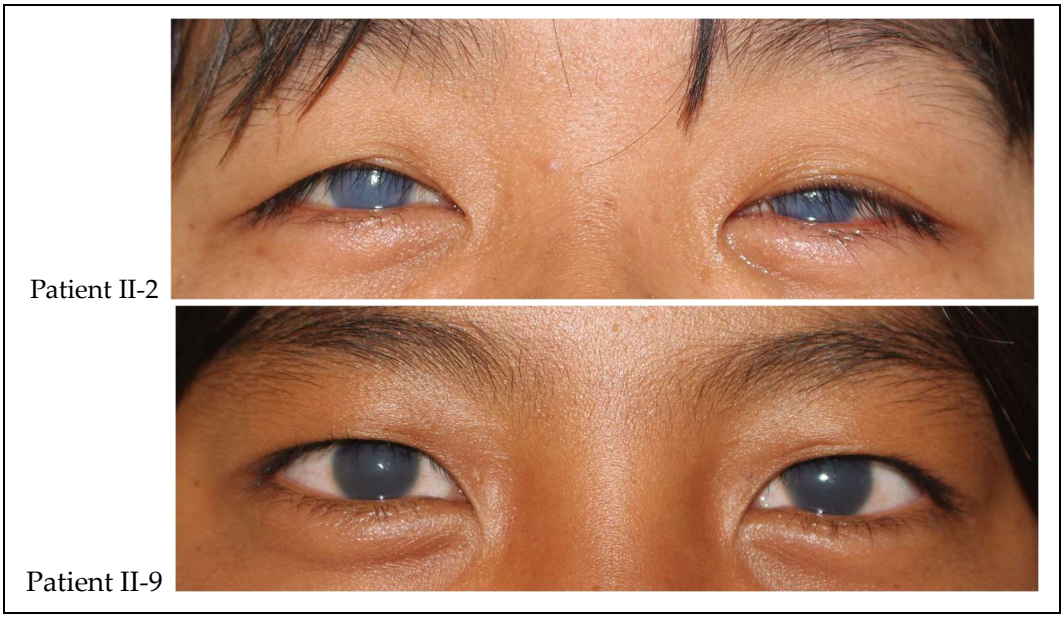

Figure 2. Representative photographs of the eyes of patient II-2 (**above**) and patient II-9 (**below**) showing (described eye).

3.2. Mutation Analysis

Certain challenges were met during the PCR optimization, as the PCR conditions published by Paliwal et al. that were initially followed did not work in our laboratory. Furthermore, it was found that the primers used had not followed the conventional exon numbering being amplified in the whole *SLC4A11* gene. The exon numbering that they had followed was based on the exons present in a messenger RNA (mRNA) transcript and the complementary DNA sequence (NM_032034). Nevertheless, the primers used were able to amplify the sequences on both coding and non-coding regions of the *SLC4A11* gene. The corrected numbering of exons and the amplified coding and non-coding regions were adapted based on the *SLC4A11* reference gene sequence on chromosome 20 (NG_017072.1) available at the NCBI website, as shown in Table 1. The exact locations of the 20 exons spanning the *SLC4A11* gene are presented in Table 2.

A direct DNA sequence analysis showed five novel point mutations scattered in both coding and non-coding regions of the *SLC4A11* gene (Table 3): present in non-coding regions were 4291G>A (located before Exon 1), 6533G>C (located between Exon 1 and Exon 2), and 13165T>C (located between Exon 9 and 10); present in coding regions, specifically within Exon 6, were 10294G>C and 10307G>A (Figure 3). Both point mutations 4291G>A and 6533G>C were present in all the patients. The point mutation 13165T>C was absent in all patients except Patient I-1 and Patient II-8, who exhibited either the presence or absence of nucleotide changes suggesting heterozygosity.

Table 3. Clinical details of screened family members and mutations found in the *SLC4A11* gene.

Family 1 Generation No.	Age/Sex	CCT OD/OS	Age at PK	BCVA/OD	BCVA/OS	Complications	Position of Nucleotide Change in *SLC4A11* Gene			
							4291G>A (Before Exon 1)	6533G>C (Between Exon 1 and 2)	10294G>C 10307G>A (in Exon 6)	13165T>C (Between Exon 9 and 10)
I-1	57/M	-	-	20/30	20/30	-	Present	Present	Absent	Present
I-2	57/F	-	-	20/30	20/30	-	Present	Present	Present	Absent
II-1 *	37/M	-	-	20/640	20/800	-	Present	Present	Present	Absent
II-2 *	35/M	-	-	20/640	20/640	-	Present	Present	Present	Absent
II-4 *	31/F	-	-	20/800	20/800	-	Present	Present	Present	Absent
II-6 *	27/F	>0.7 mm OU	25 yrs.	20/800	20/70	Failed graft OD	Present	Present	Present	Absent
II-7 *	19/M	>0.7 mm OU	17 yrs.	20/30	HM	Failed graft OS	Present	Present	Present	Absent
II-8	16/M	-	-	20/20	20/20	-	Present	Present	Present	Either (Probably heterozygous)
II-9 *	11/F	-	-	20/800	20/800	-	Present	Present	Present	Absent

PK—Penetrating keratoplasty, F—Female, M—male, "-"—No data, CCT—Central corneal thickness, BCVA—Best-corrected visual acuity, OD—Right eye, OS—Left eye, OU—Both eyes, HM—Hand movement, and "*"—affected with CHED, yrs.—years.

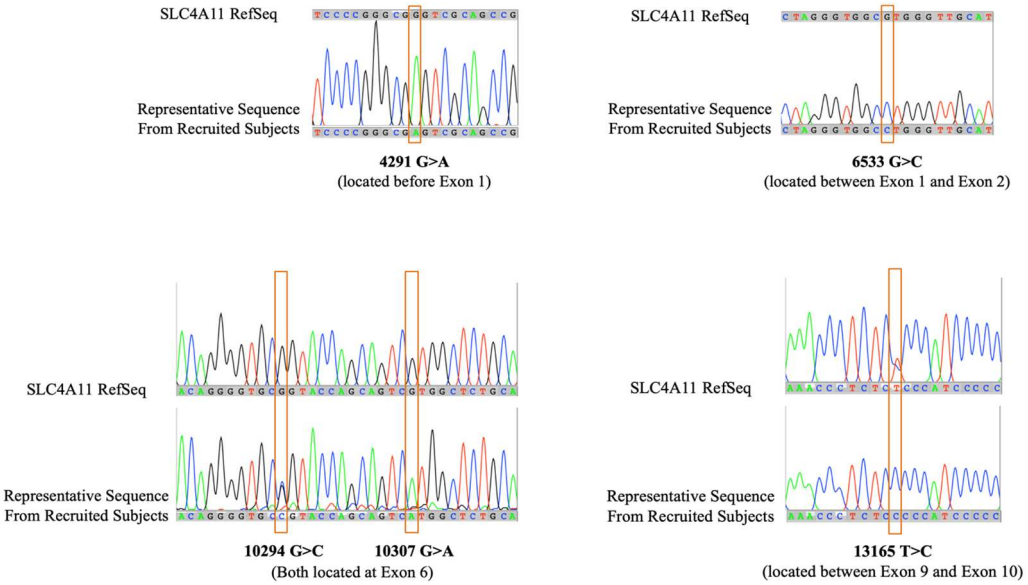

Figure 3. Representative electropherograms of amplified DNA sequences analyzed in comparison with representative *SLC4A11* reference sequence (Green: Adenine, Red: Thymine, Blue: Cytosine, Black: Guanine). Five point mutations were observed, mostly located outside the exon regions, except for 10294G>C and 10307G>A, which were both located in Exon 6. Electropherograms were generated using the consensus of triplicate sequences, viewed and analyzed using BioEdit, Seqotron, and 4 Peaks.

Interestingly, the two point mutations 10294G>C and 10307G>A, opposite to the 13165T>C mutation, were present in all patients except Patient I-1. Knowing that these mutations were located within Exon 6, mRNA transcripts of both normal and mutated forms were used to generate an amino acid sequence using the ExPASy tool. The resulting 5′ to 3′ Frame was used, as it showed the exact amino acid alignment with the reference amino acid sequence of the human sodium bicarbonate transporter-like protein 11 isoform 3 (NP_001167560.1). The 10307G>A point mutation did not result in a change in the translated amino acid (Figure 4).

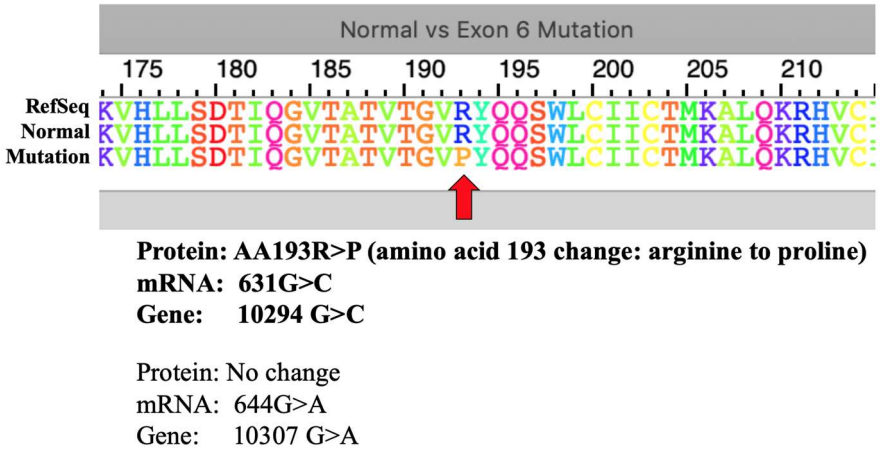

Figure 4. Amino acid sequence alignment of reference and from patient samples. Two point mutations, 10294 G>C and 10307 G>A, at Exon 6 had different effects on translated amino acid sequence. The 10294G>C is a missense mutation, resulting in a change in translated amino acid at location 193 (Red Arrow) to change from arginine (R) to proline (P), while the point mutation at 10307G>A did not result in an amino acid change. Amino acid sequence was generated via ExPASy using the reconstructed DNA sequences as input. Seqotron was used for multiple sequence alignment (Note: Amino acids are highlighted in different color schemes).

The 10294G>C point mutation was a missense mutation affecting the amino acid 193 to change from arginine to proline (aa193R>P). However, this missense mutation was predicted to be benign in nature using the Polyphen2 protein function prediction tool (Figure 5).

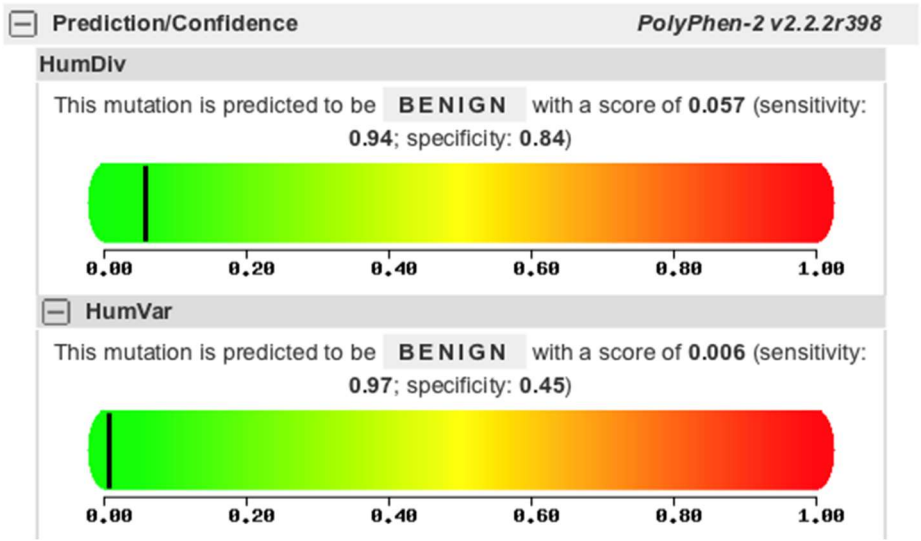

Figure 5. Results of prediction/confidence on the effect of the change in the translated amino acid 193 from arginine to proline due missense mutation 10294G>C. The missense mutation was predicted to be "BENIGN" on both HumDiv and HumVar. According to the PolyPhen2 website (http://genetics.bwh.harvard.edu/pph2/dokuwiki/overview, accessed on 15 July 2020) "HumDiv was compiled from all damaging alleles with known effects on the molecular function causing human Mendelian diseases, present in the UniProtKB database, together with differences between human proteins and their closely related mammalian homologs, assumed to be non-damaging; HumVar consisted of all human disease-causing mutations from UniProtKB, together with common human nsSNPs (MAF > 1%) without annotated involvement in disease, which were treated as non-damaging".

4. Discussion

The *SLC4A11* gene has been extensively analyzed by numerous studies. This gene codes for BTR1 (Bicarbonate Transporter-Related protein-1), which functions as a sodium borate co-transporter (NaBC1) and plays a role in the activation of the mitogen-activated protein kinase pathway [9]. Various mutations scattered across the gene have been implicated in a few ophthalmologic conditions, mainly involving the corneal endothelium. CHED, Harboyan syndrome, and Fuch's endothelial corneal dystrophy (FECD) all share similar mutations all involving the same gene, suggesting that these conditions are part of a broader disease spectrum. This shared disease feature highlights the critical role of the *SLC4A11* protein in the proper development and differentiation of the corneal endothelium, which may explain how mutations in the same gene can contribute to the pathogenesis of both congenital and late-onset diseases [10–14].

Moreover, the characteristic abnormal posterior non-banded zone of the Descemet's membrane, which represents an abnormal function of the corneal endothelium in CHED and FECD, underlies the importance of the *SLC4A11* protein for the proper development and differentiation of the corneal endothelium and may explain how the same gene can be involved in the pathogenesis of CHED [9]. This observed endothelial pathology can impair the function of the "corneal pump", leading to water accumulation in the corneal stroma, which presents as bilateral corneal edema and is characteristic of CHED (Figure 2).

The family that participated in the study was located in Mayoyao, Ifugao, in the northern Luzon region. During the visit to the area, it was discovered that many locals had CHED. Although they were not included in our study, it is worth mentioning that CHED

may have a geographic predilection for this area. One possibility is that a founder effect or mutation is responsible for causing the disease to cluster among individuals within the region. It is possible that one common ancestor passed down the CHED mutations to subsequent generations. Despite our efforts to construct a detailed family history, no consanguinity was reported during the interview. However, this may not have been the case, since we only managed to extract data from three generations. Additionally, as mentioned in our results, an interesting feature of the family studied was the expressivity of the disease, as if it were dominantly inherited. Our pedigree chart, however, shows the disease to have an autosomal recessive transmission.

This pilot study was conducted to analyze the genetic causes of CHED specifically among Filipinos. We recommend further extending our findings by obtaining more epidemiological data, which are currently lacking, and conducting a histopathologic study and ultrastructural analysis of the corneas of CHED patients, if possible. The mutations that may be present in this specific population have yet to be analyzed for their specific gene products and their ultimate role, whether they are pathogenic or not.

4.1. Potential Functional Significance of Each Mutation

The only missense mutation found in this study, 10294G>C, which resulted in a change in the 193rd amino acid translation from arginine to proline, was predicted to have a benign effect in the population. In addition, the presence of this point mutation together with 10307G>A, both located at Exon 6, varied among patients and was specifically present in all patients except Patient I-1, despite the observed similar phenotype. The reverse was true for the point mutation 13165T>C, which was absent in all patients but present in Patient I-1. It is possible that there was compensation between the variations in the presence of these point mutations resulting in similar observed phenotypes, but this could not be confirmed by this study, since they were not within the coding regions for analysis using ExPASy and PolyPhen2. Still, the frequency of these mutations in related patients suggests a unique marker for the CHED population in the Philippines setting, including all other mutations found in this study.

Interestingly, both point mutations 4291G>A and 6533G>C were consistently present in all patients. These mutations, despite not being exactly within Exon 1 or the coding region sequence included in an mRNA sequence, were located near Exon 1 and 2, and could possibly have had an indirect influence on Exon 1 and/or Exon 2 at the transcription level process. We recommend isolating and sequencing an mRNA transcript from the samples. As stated, there could be a pathologic process that takes place at the level of transcription that could contribute to the disease phenotype. Analyzing the mRNA levels, as well as their nucleotide sequences, could provide an explanation as to why the patients exhibited CHED phenotypes despite detecting most point mutations in non-coding regions. In this line of thought, a different protein at the transcription level might also be involved in disease progression. If mRNA transcription is not found to be defective, then it is possible that post-translational processing could be involved in rendering proteins defective.

A brief summary of the five mutations is mentioned below:

Before Exon 1, (Novel Mutation) 4291G>A: The mutation located before Exon 1 is a novel finding. Further investigations, including reporter assays to assess potential effects on gene expression, are recommended.
Between Exon 1 and 2, 6533G>C: This mutation has been associated with the CHED phenotype [15]. Subsequent experiments, particularly reporter assays, can elucidate its impact on gene expression.
Exon 6, 10294G>C and 10307G>A: Two mutations were identified in Exon 6. The literature suggests that mutations in this region may also contribute to the CHED phenotype [15]. However, the Polyphen2 predictions were benign. Functional assays, especially electrophysiological studies, are warranted for validation.
Between Exon 9 and 10, 13165T>C: This mutation, located between Exon 9 and 10, has been associated with the CHED phenotype [15]. Functional assays, including reporter

assays, can provide further insights into its impact. Notably, it was absent in all patients, but present in Patient I-1.

To date, 74 mutations in 17 of the 19 coding exons of *SLC4A11* have been identified, although 32 of the 136 (23.5%) pedigrees screened to date have not demonstrated coding region mutations in *SLC4A11* [15]. The majority of these mutations have been missense mutations involving a base substitution. However, mutations possibly involving Exon 1 have not been documented. Our first documented novel mutations flanking this exon support the genetic heterogeneity of the disease among races. The other exons have been previously known to harbor mutations responsible for the CHED phenotype. This study adds to the growing list of point mutations not only in coding regions, but also in non-coding regions. The full list of mutations can be found in the various online databases available. Recognizing the evolving nature of genetic research, new or duplicate studies may reveal similar findings. The novelty status of certain mutations should be interpreted cautiously, and staying informed about the latest research is advised.

4.2. Limitations of Polyphen Tool and Recommendations

In this study, we used the Polyphen2 tool to predict the potential functional impact of mutations in the *SLC4A11* gene. However, it is important to note that the predictions made by this tool have limitations, particularly when applied to missense variants, as false positive and false negative predictions are common. Therefore, to provide direct evidence of the impact of these mutations on protein function, further experiments are needed.

For non-coding region mutations, we recommend the use of reporter assays to measure the impact of the variant on gene expression. For coding region mutations, we recommend using electrophysiological assays to directly measure the transport activity of the *SLC4A11* protein, which is the primary function of the protein. However, expression assays and functional complementation assays can also provide important information on the impact of coding region mutations on protein expression and stability, and on the ability of mutant proteins to restore normal function, respectively [16–18].

The choice of assay will ultimately depend on the specific research question being asked and the available resources. Overall, our results highlight the importance of using multiple lines of evidence to determine the functional impact of mutations in the *SLC4A11* gene.

Considering that this is a preliminary report on the genetic profile of Filipino patients affected with CHED, we are limited by the fact that we managed to obtain samples from only one family. A larger sample size and appropriate statistical analyses are therefore encouraged whenever possible Though our findings may likely be applicable to CHED patients in the northern Luzon region, it is recommended to obtain samples from other unrelated families affected with CHED elsewhere to verify if these mutations are consistently present as a Filipino variant. The goal of ultimately identifying all potential mutations responsible for the CHED phenotype in this specific racial group is to develop a panel to facilitate rapid prenatal diagnosis and aid families in genetic counseling. Finally, the potential for the development of gene therapy for CHED remains on the horizon.

5. Conclusions

This is the first report that gives us a genetic profile of a northern Luzon family with members affected with CHED. It is also evident that not all mutations are consistently present among different races, probably due to the disease's genetic heterogeneity. Our study documented five novel mutations, adding to the growing list of mutations responsible for acquiring the CHED phenotype. It is possible that there are yet more novel mutations to be discovered causing the same disease. Screening for these specific mutations in other families may prove useful for genetic counseling, prenatal diagnosis, and the future development of gene therapy.

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