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Molecular Analysis of Consanguineous Families Having X-Linked Congenital Stationary Night Blindness in Khyber Pakhtunkhwa, Pakistan

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Abstract: X-linked congenital stationary night blindness (XLCSNB) is a vision disorder characterized by reduced visual acuity, myopia (short-sightedness), strabismus, and nystagmus. This condition results from mutations affecting the transmission of visual signals in the retina. Genetic studies have identified the proximal short arm of chromosome X (Xp11.4) as the region harboring the candidate gene associated with XLCSNB. Electroretinogram (ERG), a diagnostic test, plays a critical role in differentiating XLCSNB categories by evaluating the electric responses of retinal, bipolar, and ganglion cells to light and dark stimuli. To locate the candidate gene, four microsatellite markers (*DXS574*, *DXS8012*, *DXS993*, and *DXS1201*) were designed near the proximity of the target region on Xp11.4. The gene was identified as the *NYX* gene, encoding nyctalopia, a 481-amino-acid protein, and a member of leucine-rich repeats (LRR). Nyctalopia plays a pivotal role in signal transmission between retinal and bipolar cells. The expression of the *NYX* gene is low in tissues such as muscles, the brain, the retina, and the testis. Following linkage analysis, specific primers were designed to amplify both exons of the *NYX* gene. Gel electrophoresis was used to observe DNA bands, and subsequent sequencing of the gene was performed to identify mutations associated with XLCSNB. Three mutations were identified in the *NYX* gene, all of which had been reported previously. These mutations, predominantly splicing mutations, are known to be pathogenic alterations. The *NYX* gene encodes 13 leucine-rich repeats (LRRs), and the identified missense mutations primarily affect these LRR sites, leading to impaired retinal signal transmission and disturbed eye function. To date, 42 different mutations in the *NYX* gene have been reported, with splicing mutations being the most common. This study successfully identified mutations in the *NYX* gene associated with XLCSNB. Although previously reported, the findings provide further insight into the molecular mechanisms underlying this disorder. Future research should focus on functional analyses of the *NYX* genes to better understand their role in regulating retinal cell interactions and signaling pathways.

Keywords: Myopia, Strabismus, Nystagmus, Electroretinography, *NYX* Gene, Leucine-Rich Repeat Proteins, Retinal Bipolar Cells, Pathogenic Variants

1. Introduction

Congenital stationary night blindness (CSNB) is a rare genetic disorder affecting photoreceptors and their associated signal transmission pathways. CSNB is characterized by nyctalopia (difficulty seeing in dim or dark light), reduced visual acuity, strabismus, and myopia. This condition is congenital, non-progressive, and remains stable over time [1]. CSNB primarily arises from defects in rod phototransduction or errors in signal transmission from photoreceptors to downstream retinal cells. Electroretinography (ERG) is a diagnostic tool used to identify these functional abnormalities by assessing the electric response of retinal cells to light stimuli [2]. Two genes, such as *NYX* and *CACNA1F*, have been identified as the primary causative factors for X-linked CSNB (XCSNB), confirmed through positional cloning strategies. Mutations in these genes disrupt normal retinal signaling. Approximately 55% of XCSNB cases are attributed to mutations in the *CACNA1F* gene, while 45% are linked to mutations in the *NYX* gene [3][4]. The *NYX* gene encodes nyctalopin, a protein belonging to the leucine-rich repeat (LRR) family, which plays a pivotal role in transmitting signals between retinal photoreceptor cells and bipolar cells. Mutations in the *NYX* gene result in a loss of photo transmission, leading to the clinical manifestations of CSNB [5]. Mutations in the *CACNA1F* gene affect calcium (Ca^{2+}) channels, leading to disrupted Ca^{2+} influx at photoreceptor ribbon synapses. This disturbance impairs neurotransmitter release and disrupts normal visual signal transmission [6]. X-linked congenital stationary night blindness (XCSNB) is further classified into two subtypes: Type 1 CSNB and Type 2 CSNB, based on the functional assessment of rod pathways using electroretinography (ERG) [7]. In Type 1 CSNB, rod pathway function is severely diminished, resulting in the complete absence of oscillatory potentials and rod b-wave. In contrast, Type 2 CSNB is characterized by a reduced rod b-wave and the presence of a cone a-wave, indicating partial retention of cone and rod functionality [8]. Studies have shown that mutations in the *NYX* gene are associated with the complete form of XCSNB, while mutations in the *CACNA1F* gene are linked to the incomplete form of the disorder [9]. The inheritance pattern of XCSNB is primarily X-linked; however, cases of autosomal inheritance have also been reported, further complicating the genetic basis of the disease. Mutations in 17 specific genes have been identified as responsible for congenital stationary night blindness (CSNB), including *TRPM1* (MIM: 603576), *NYX* (MIM: 300278), *CACNA1F* (MIM: 300110), *GRK1* (MIM: 180381), *LRIT3* (MIM: 615004), *GPR179* (MIM: 614515), *GRM6* (MIM: 604096), *CABP4* (MIM: 608965), *GNAT1* (MIM: 139330), *SLC24A1* (MIM: 603617), *SAG* (MIM: 181031), *PDE6B* (MIM: 163500), *RDH5* (MIM: 136880), and *RHO* (MIM: 180380) [10]. These genes are primarily involved in the phototransduction cascade or the transmission of signals between photoreceptors and adjacent retinal cells, both of which are essential for normal vision. Autosomal recessive mutations in *LRIT3, TRPM1, GRM6*, and *GPR179* genes, as well as mutations in the *NYX* gene, are associated with the complete Schubert-Bornstein type of CSNB. In contrast, the incomplete form is caused by mutations in *CABP4* or *CACNA1F* genes [11]. *PDE6B* and *GNAT1* mutations are linked to Riggs-type CSNB, while mutations in RDH5 result in Fundus albipunctatus, a condition characterized by delayed dark adaptation. Additionally, mutations in *SAG* and *GRK1* genes are associated with Oguchi disease, a rare form of night blindness characterized by a unique golden-yellow appearance of the retina under ophthalmoscopic examination [11].

2. Methodology

A consanguineous family affected by X-linked congenital stationary night blindness (XCSNB) was recruited from Mardan, Khyber Pakhtunkhwa. Blood samples were collected from affected male individuals, their unaffected siblings, and their parents (**Figure**

1). The genetic relationships, health history, and inheritance patterns were deduced by constructing a detailed pedigree. All affected individuals were male, consistent with an X-linked inheritance pattern. The disorder was congenital and stationary, as it was present from birth without progression over time. Clinical characteristics observed in the affected individuals included myopia (short-sightedness), strabismus, and reduced visual acuity under dim or low-light conditions. Genomic DNA was extracted from blood samples using a commercially available extraction kit, followed by visualization on a horizontal agarose gel electrophoresis system to confirm DNA integrity. Specific polymorphic markers were selected to locate the candidate *NYX* gene, a known causative gene for XCSNB. Linkage analysis was performed to confirm the co-segregation of these markers with the disorder in the family. Upon identifying the candidate *NYX* gene, specific primers were designed to amplify its coding regions. The *NYX* gene was mapped to the X chromosome, specifically at Xp11.4, and consists of two exons. Both exons of the *NYX* gene were amplified using polymerase chain reaction (PCR). The sequence of the candidate gene, identified through linkage analysis, was selected using the UCSC Genome Browser (https://genome-asia.ucsc.edu/cgi-bin/hgGateway). Functional and expression data of the *NYX* gene were obtained from this resource, and additional gene prediction tools, such as SUSPECTS (http://www.genetics.med.ed.ac.uk/suspects), were used to scan for potential variants within the gene. All exon-intron junctions, splicing sites, and coding regions of the *NYX* gene were sequenced bi-directionally to identify the disease-causing variants. Primers for amplifying the gene regions were designed using Primer3 software (http://bioinfo.ut.ee/primer3) (**Table 1**). Sequence alignment and comparison were performed using SeqMan II software, enabling the identification of functional variations in the gene sequences of affected individuals. Control sequences were retrieved from the UCSC Human Genome Browser for comparison and documentation. Variants identified in the affected individuals were confirmed and validated across all family members, including unaffected individuals, to ensure the segregation of the mutation with the disorder.

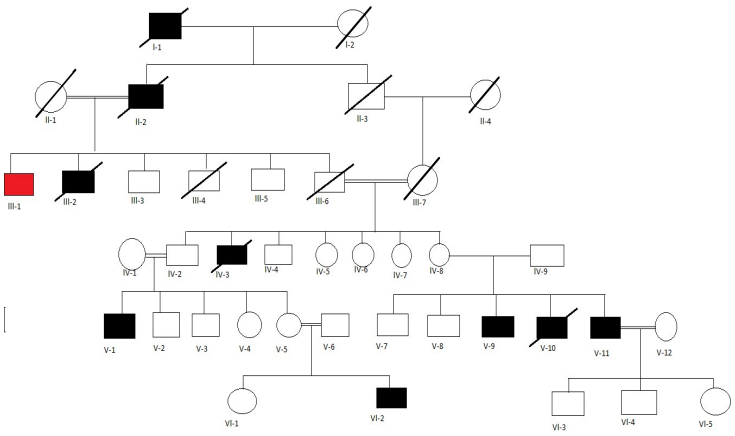

Figure 1

Figure 1. Pedigree analysis of the recruited family.

Table 1. List of primers designed for *NYX* gene

Exon No	Primers	Primer sequence	Tm (0C)	Amplicon size (bp)
1	Forward	CTCTCAAACCATTTTCAGCA	56.5°	589
	Reverse	ACTCTTTGGGCAGAAGCTC	57.2°	
2	Forward	TTTCTCTTTTCTCCTCCTTCC	56.8°	526
	Reverse	CGGAACAGGTTGTCGAAG	57.7°	
	Forward	CGCCGCCTAGACCTAGCA	62.6°	597
	Reverse	GTTGCGGAAGAGGAAGAGG	57.9°	
	Forward	CGCCTTCCAGAACCTCTC	58.5°	594
	Reverse	GCCCAGTTAAGCAAACTTTC	56.7°	
	Forward	GTCCGACAGCCTCTCCTC	58.4°	548
	Reverse	AAAGGACTTCGGTGTAATGG	56.7°	
	Forward	GCAAGGCCTGAATTAGAGAG	56.8°	574
	Reverse	CCTAATGGAATCATCCAAGC	57.1°	

3. Results

Electroretinography (ERG) analysis of the affected individuals revealed significant impairment in the rod pathway, which is responsible for vision under low-light conditions, with a marked reduction in rod function. Additionally, cone function, essential for daylight vision, was also affected (**Table 2**). Genomic DNA was extracted using the Thermo Scientific DNA Extraction Kit. The DNA concentration and purity were assessed using a NanoDrop spectrophotometer, yielding a ratio between 1.8 and 2.1 ng/μL, indicating high-quality DNA. Extracted DNA was visualized on horizontal agarose gel electrophoresis to confirm integrity (**Figure 2**). To identify the *NYX* gene, four microsatellite markers (*DXS574*, *DXS8012*, *DXS993*, and *DXS1201*) were selected and located close to the candidate gene. These markers were resolved on vertical polyacrylamide gel electrophoresis using the Elite 300 Plus Apparatus under conditions of 100 volts for 3 hours. Clear bands were visualized under UV light using a gel documentation system, confirming the linkage of these markers to the *NYX* gene. After linkage analysis confirmed the presence of the *NYX* gene, it was mapped to the X chromosome (Xp11.4) with two exons. Specific primers were designed for amplification of both exons. The first exon was amplified using a single primer set, whereas five primer sets were required to amplify exon 2 due to its length and complexity (**Figure 2**). PCR amplification successfully produced clear and specific bands for both exons. Both exons of the *NYX* gene were analyzed in affected individuals, their siblings, and their parents. DNA sequence data were processed and analyzed using the software BioEdit, and the results were compared with the reference sequence of the *NYX* gene available in the publicly accessible GenBank database (Accession Number: ENST00000342595.2). Sequence analysis revealed three pathogenic mutations in the *NYX* gene: Splicing Mutation: A G>C substitution at the +1 position of exon 2, located within a highly conserved splice donor site. This mutation is predicted to disrupt RNA splicing, leading to aberrant transcript processing. Missense Mutation (p.Ile101Thr): A substitution at position c.302 in exon 2, replacing isoleucine with threonine at codon 101. This amino acid change is likely to affect the protein's structure or function. Missense Mutation in the Seventh LRR Domain: A substitution leading to the replacement of asparagine with serine (p.Asn216Ser) at the 216th amino acid position. This mutation is localized in the seventh leucine-rich repeat (LRR) domain of nyctalopia, a region critical for its functional role in signal transmission between retinal and bipolar cells. These mutations are

consistent with the clinical phenotype observed in the affected individuals and highlight the critical role of the *NYX* gene in maintaining normal visual function.

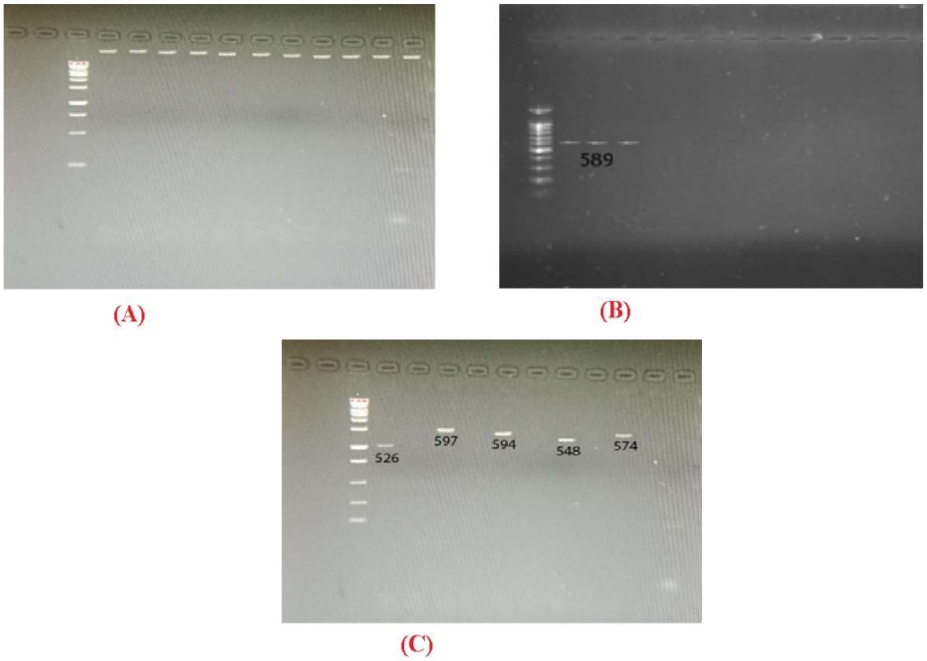

Figure 2: (A) The figure shows the DNA on agarose gel runs for 40 mins at 90 volts. **(B)** DNA bands of exon 1 of *NYX* gene having a size of 589 bp. **(C)** DNA bands of exon 2 of *NYX* gene size 526, 548, 574, 594, and 597 bp using 5 different primers.

Table 2. Shows the ERG report of an affected individual.

Field	Details
Institute	Armed Forces Institute of Ophthalmology
Report Type	ERG Report
Patient Name	ABC
Date	25-11-15
Photopic (Cone) Response	Cone response is depressed in both eyes
Scotopic (Rod) Response	Rod's response is also depression in both eyes
Conclusion	Depressed ERG in both eyes

4. Discussion

A consanguineous family with X-linked congenital stationary night blindness (X-CSNB) was recruited from Khyber Pakhtunkhwa, Pakistan. X-CSNB is a rare genetic disorder that primarily affects photoreceptors in the retina. It is characterized by nyctalopia (difficulty seeing in dim or dark light), reduced visual acuity, strabismus, and myopia. The condition is congenital and remains stable over time [1]. The disorder arises from defects in rod transduction or errors in signal transmission between photoreceptors and the retina. Electroretinography (ERG) is a diagnostic tool that can identify faulty rod transduction or impaired photoreceptor signal transmission. The inheritance pattern of CSNB

can be either X-linked or autosomal, and mutations in 17 specific genes have been identified as causative factors. Among these, the *NYX* and *CACNA1F* genes are considered primary contributors to X-CSNB. Mutations in these genes disrupt critical processes involved in visual signal transduction and retinal function. To investigate the genetic basis of X-linked congenital stationary night blindness (X-CSNB), blood samples were collected from the affected family members. Genomic DNA was extracted, and its quantity and quality were evaluated using a NanoDrop spectrophotometer, confirming high-quality DNA. Four microsatellite markers (DXS574, DXS8012, DXS993, and DXS1201) were designed near the *NYX* gene to perform linkage analysis, which confirmed the association of the *NYX* gene with the disorder. Subsequent sequencing of the *NYX* gene revealed multiple pathogenic mutations. A splicing mutation at position +1 was identified, disrupting RNA splicing and causing abnormal transcript processing. Additionally, a missense mutation at position c.302 resulted in substituting isoleucine with threonine (p.Ile101Thr), potentially altering the protein's structure and function. Another missense mutation in the seventh leucine-rich repeat (LRR) domain caused the substitution of asparagine with serine at amino acid position 216, leading to a truncated protein. These mutations collectively impair the functional role of nyctalopin, disrupting signal transmission between retinal and bipolar cells, and thereby contributing to the pathogenesis of X-CSNB. To date, 42 distinct mutations have been reported in the *NYX* gene. Of these, most (59%) are missense mutations, while deletions and insertions account for 19%, and frameshift and splicing mutations represent 2%. These mutations are predominantly pathogenic rather than benign polymorphisms. The *NYX* gene encodes the nyctalopin protein, which comprises 13 leucine-rich repeats (LRRs), functioning as extracellular membrane proteins. The reported missense mutations frequently occur within these LRR regions. These LRRs are pivotal in signal transmission between the retina and the brain. Mutations in these regions disrupt this transmission, leading to impaired visual function and abnormalities in eye physiology.

5. Conclusions

X-linked congenital stationary night blindness (X-CSNB) is a genetic disorder predominantly affecting males due to its X-linked inheritance pattern. This condition is characterized by impaired vision in low-light or night-time conditions, accompanied by associated abnormalities such as myopia, nystagmus, and strabismus. In this study, electroretinography (ERG) examinations revealed that the photoreceptor cells (rods and cones) in affected individuals exhibited dysfunctional activity. Genetic analysis further identified mutations in the *NYX* gene as the primary cause of this disorder. These findings emphasize the pivotal role of *NYX* gene mutations in the pathogenesis of X-CSNB and contribute to a deeper understanding of its molecular basis.

Author Contributions

Faiza Mazhar conducted experiments and contributed to data analysis and manuscript drafting. Sajeela Akbar supervised the study and finalized the manuscript. Sadaf Gohar, Syed Sohail Shah, Aizaz Ali, and Saqib Ullah assisted with genetic analysis, data validation, and technical support.

Conflicts of Interest

The authors declare no conflicts of interest.

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