Analysis of a Homozygous TGFBI Variant in a Pakistani Family with Granular Corneal Dystrophy Type 2: Implications for Genotype-Phenotype Correlation and Inheritance Patterns

Ayesha Khush Bakht ( ayshamahi44@gmail.com )
Lahore College for Women University

Shagufta Naz
Lahore College for Women University  https://orcid.org/0000-0001-9699-5193

Muhammad Asif Naeem
Centre of Excellence in Molecular Biology

Saima Sharif
Lahore College for Women University

Muhammad Qasim
Government College University Faisalabad

Haiba Kaul
University of Veterinary and Animal Sciences

Sabika Firasat
Quaid-i-Azam University Faculty of Biological Sciences

Khajista Jabeen
Lahore College for Women University

Afia Iqbal
Lahore College for Women University

Research Article

Keywords: Granular Corneal Dystrophy Type 2, Whole Exome sequencing (WES), TGFBI, Homozygous Mutation, Autosomal Recessive

Posted Date: March 27th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-2713586/v1

License: ☑️ ❶ This work is licensed under a Creative Commons Attribution 4.0 International License.  Read Full License
Abstract

Background:
Granular Corneal Dystrophy Type 2 (GCD2) is an inherited condition characterized by snowflake-shaped opacities in the cornea. The disorder is caused by mutations in the TGFBI gene, which encodes keratoepithelin. The objective of this study was to examine the inherited factors associated with GCD2 in two consanguineous Pakistani families.

Methods and Results:
We conducted whole exome sequencing (WES) of affected and unaffected individuals from the two families. Our analysis revealed a previously reported missense mutation (c.371G>A) in TGFBI that leads to the loss of p. R124H at exon 4 of keratoepithelin, which is associated with the severe form of GCD2. Sanger sequencing confirmed the segregation of this mutation in both families, with an autosomal dominant mode in one family (PKCD040) and a previously unreported autosomal recessive mode of inheritance in the other family (PKCD024). The diagnosis of GCD2 was confirmed by clinical examinations, including a slit lamp exam, a study of corneal morphology, and optical coherence tomography (OCT).

Conclusion:
Our findings support a high correlation between genotype and phenotype in TGFBI-related corneal dystrophies. We also identified a novel homozygous TGFBI mutation in PKCD024, which expands our understanding of the inheritance patterns of GCD2. These results have important implications for the accurate diagnosis and potential treatment of this condition.

Introduction
A corneal dystrophy is a group of inherited bilateral blinding corneal disorder [1]. Affected individuals exhibit reduced corneal sensitivity, halos around light, and decreasing visual impairment between 30 and 50 years of age. Approximately 12 pathogenic genes associated to CD, among which the mutation in TGFBI (OMIM 601692) gene cause a number of clinically distinct CD’s including granular CD type 1 (GCD1 or classic CD), granular CD type 2 (GCD2 or Avellino CD), granular CD type 3 (GCD3 or Reis-Bücklers CD), and lattice CD type 1 (TGFBI type 1) (LCD1) [2]. It is the autosomal dominant mode of inheritance that causes all of these disorders,[1, 4]. According to the International Classification of Corneal Dystrophies (IC3D), GCD2, GCD1, and LCD1 are epithelial–stromal TGFBI dystrophies [3]. Granular corneal dystrophy, type 2 (GCD2) is an autosomal dominant condition with granular, lattice, and amyloid deposits. GCD type 2, also known as Avellino type or ACD (OMIM 607541), was seen in people from Avellino, Italy [5]. The onset age and progression rates of GCD2 depend on the p. R124H mutation status [6]. GCD2 homozygotes acquire thick, confluent granular deposits aggressively with age, indicating a dose impact.

In humans, the TGFBI gene makes a TGF-induced protein called TGFBIp that is found in the extracellular matrix [7]. Gene TGFBI encodes a protein involved in cell proliferation, differentiation, adhesion, and migration [8]. Sixty-nine potentially pathogenic variants of TGFBI causing epithelial-stromal dystrophies have been reported. TGFBI fasciclin 1 pathogenic mutation are discovered at two major codons (Arg124 or Arg555) [9]. The change from arginine to histidine at codon 124 (p. R124H) of the TGFBI gene is linked to GCD2 and segregating in autosomal mode of inheritance in patients. The TGFBI, BIGH3 gene, which encodes keratoepithelin, is associated to four types of stromal corneal dystrophies in Caucasians [10]. Most TGFBI mutations are at codons 124 (R124H) and 555 (R555W) [11]. Mutations are population-dependent. Previously, a heterozygous missense mutation R124H was identified in GCD2 patients in South Korean population [12]. GCD2 causes 12% of European TGFBI-caused corneal dystrophies [13]. In Korea and Japan, GCD2 causes 90% and 72% of TGFBI-related corneal dystrophies [14]. South Asia (Pakistan) has the least TGFBI-related GCD2 cases. Chinese, Koreans, and Japanese are most affected [15, 16].

Here we report a homozygous R124H missense mutation segregated in a recessive mode of inheritance in one family PKCD024. The same mutation with the disease phenotype is segregated in an autosomal dominant mode in family PKCD040. We compare phenotypic change and genotypic change for GCD2. A comprehensive literature survey revealed that this is the first report to associate the TGFBI gene with the pathogenesis of the autosomal recessive mode of GCD2.
**Materials & Methods**

This study aimed to clinically evaluate patients with GCD2 and analyze the molecular basis of the disease. The methodology involved ethical considerations, clinical evaluation, molecular analysis and mutation validation.

**Ethical Considerations:**

The study was approved by the ethical committee of Lahore College for Women University (LCWU) in Lahore, Pakistan, and all participants provided written, informed consent. The Declaration of Helsinki was followed throughout all of the procedures.

**Clinical Evaluation of Patients:**

Two consanguineous families with GCD2 were identified, and blood samples were collected with written consent. Each family's pedigree was drawn. Visual acuity tests, slit lamp biomicroscopy, optical coherence tomography spectral domain (3D OCT version 8.39), and fundoscopy were performed on available normal and affected members with the help of an ophthalmologist. Electroretinography measurements were also recorded using equipment from LKC Technologies, Inc. Anterior segment imaging was carried out by an ophthalmologist, and morphology was evaluated using a radial scan during the swept source spectral OCT examination (6.0 mm–1024).

**Molecular Analysis:**

DNA was isolated using the phenol-chloroform method from peripheral blood cells. The quality of genomic DNA was assessed using a 0.8% agarose gel. Gene-specific primers were designed using Primer3 software to cover all exon coding regions and intronic borders, with lengths of at least 70–100 bp. Polymerase chain reaction (PCR) was performed on genomic DNA using 2X PCR master mix with 3mM MgCl2, 4units/ul Taq DNA polymerase, 450 M each dNTPs, and appropriate concentrations for reaction buffers.

The PCR included initial denaturation at 95°C for 5 minutes, followed by 30 cycles at 94°C for 1 minute, at 58°C or 55°C for 1 minute, at 72°C for 1 minute, and final extension at 72°C for 5 minutes, followed by last step at 4°C. PCR samples were examined on a 1.5% agarose gel that had been precipitated and purified with ethanol. Probands from two families underwent Whole Exome Sequencing (WES) with the Twist Comprehensive Exome Panel (Twist Bioscience, South San Francisco, CA) and the HiSeq 4000 instrument (Illumina, USA) with an average coverage of 100–120 X at each nucleotide site. Raw readings were matched up with the human genome reference sequence (build HG19) using Novoalign (V3.08.00; Novocraft Technologies, Selangor, Malaysia), and Picard (v. 2.14.0-SNAPSHOT) was used to delete any duplicates.

**Mutation Validation:**

The pathogenic or deleterious effects of gene mutations on protein structure and function were confirmed using different validation tools. The UCSC genome browser was also used to evaluate the conservation of specific amino acids in diverse species. In order to further verify the conservation of impacted amino acid, the gnomAD database [18] was used to investigate the frequency of all variants. Three bioinformatics methods were used to analyze the most likely deleterious mutations: SIFT (Sorting Intolerant from Tolerant) (http://sift.jcvi.org), Polyphen2 (Polymorphism Phenotyping) [19] (http://genetics.bwh.harvard.edu/pph2/), and PROVEAN (https://www.jcvi.research/provean) [20].

**Results**

**Clinical Phenotype:**

In family PKCD024, the proband is an 18-year-old boy who presented with honeycomb-shaped opacities on both corneal surfaces, resulting in a diagnosis of GCD type 2. The clinical data support this diagnosis, which led to PKP keratoplasty in the right eye. However, recurrent erosion occurred after one month of the operation. The OCT examination revealed GCD2 involving the central 80% of the cornea with sporing of the peripheral cornea near the limbus. The proband's right eye had a visual acuity of Plano, while the proband's left eye had a visual acuity of 6/36. The 9-year-old brother and 26-year-old sister also had the same
phenotype. The father's eyes had granular deposits, while the other family members had clear corneas. ERG of homozygous and heterozygous individuals showed a-wave and b-wave patterns in dark-adapted and light-adapted settings.

The proband in family PKCD040 is a 62-year-old man with GCD2. His sister, nephew, niece, and a child are also affected with the same phenotype but with a different pattern in the cornea. The proband first noticed blurred vision when he was 15 years old. In 2019, PKP keratoplasty was done in the right eye. His visual acuity is currently 6/24 OD and 6/60 OS. In the left eye, the thick opacities of the granules spread from the center to the periphery. OCT examination revealed discrete thick patches of granules in the stratified superficial cell layer at the anterior segment in the left eye.

**Mutation Analysis:**

Whole exome sequencing data revealed the homozygous missense mutation c.371G > A in both families, which occurred at 124 codon of exon 4 of the *TGFBI* gene. All of the individuals in the PKCD024 family were detected with the p. R124H mutation, and the same mutation was detected in all the patients of family PKCD040. This mutation is segregated in an autosomal recessive mode in family PKCD024 and autosomal dominant mode in family PKCD040.

**Optical Coherence Tomography:**

All corneal anomalies were hyper reflective on the OCT scan, but the degree of increased reflectivity varied between homozygous and heterozygous patients. The opacities varied in both shape and pattern. A spectral domain OCT scan revealed Bowman's layer and deposits under the epithelium. The GERP score of 5.07 indicates that this missense mutation is deleterious since the amino acid residue is highly conserved.

Overall, the results indicate that both families have a mutation in the *TGFBI* gene, which results in GCD type 2. The clinical phenotype and optical coherence tomography findings vary between homozygous and heterozygous patients, and the mode of inheritance differs between the two families.

**Discussion**

The present study reports the first cases of GCD2 families in southern Asia (Pakistan). We identified a homozygous missense mutation c.371G > A in the *TGFBI* gene that was inherited in an autosomal dominant pattern in one family (PKCD040) and a recessive mode of inheritance in another family (PKCD024). In all other ethnic groups, patients with a dominant mode of inheritance have been reported [21–24].

Corneal dystrophies are a group of conditions that affect the stromal and Bowman's layers of both eyes and are passed down from parent to child. Mutations in the *TGFBI* gene are associated with corneal dystrophy and are located on chromosome 5q31. The *TGFBI* gene for keratoepithelin is a 68KD protein comprising 683 amino acids and four successive fasciclin 1 (FAS1) domains [10, 25, 26]. A pathogenic variant affecting the first FAS1 domain of the protein can cause insoluble corneal extracellular material [27]. Mutations at the R124 codon can affect the amino acid's hydrophilicity, polarity, adhesion, and hydrolysis, causing amyloid fibrils to develop and deposit in the stromal layer of cornea and cause stromal corneal dystrophy [28]. According to the results of our research, the R124H mutation can be found in exon 4, which is located at the FAS-1 region's amino terminus. When arginine is substituted with histidine, the protein's hydrolytic activity changes.

The R124H mutation is a hotspot mutation due to its widespread occurrence [25–29]. Previous studies have reported the R124H mutation and GCD2 to be frequent in Korea, China, Japan, and India [30–36]. In our study, we identified the R124H mutation in two Pakistani families. Therefore, this may also be an area of high mutational vulnerability in the Pakistani population.

Previous investigations have shown that consanguineous offspring with early-onset GCD are highly affected, but they did not explain the corneal state of the parents [39–42]. Only a few families have both parents apparently affected [43, 44]. Our study examined the parents of both families and found them to be heterozygous carriers, with their mutated homozygous offspring in family PKCD024. Granular lesions were present in the deeper stroma in some patients. Both homozygous and heterozygous mutations were segregated in affected individuals of family PKCD040. Our results show that the phenotypes of PKCD024 (V:2, V:3, V:4) and PKCD040 (V:1, V:2, V:4, VI:1), all of whom are offspring of consanguineous parents, are more severe in
homozygous than in heterozygous individuals (Fig II) in concordance with previous studies [45, 46]. There is a strong genotype and phenotype correlation in the R124H mutation in both families. Sequencing of the TGFBI gene revealed a homozygous missense mutation c.371G > A, a heterozygous c.371G > A mutation in PKCD040, and no mutation in the heterozygous father and mother in PKCD024 (Fig III).

Pakistan's cousin marriage rate is 65%, second only to India's (55%). Iran (30%), Afghanistan (40%), Egypt, and Turkey (20%) follow Saudi Arabia (50%) [47]. We believe that the high prevalence of the disorder in these Pakistani families is due to a founder mutation or genetic drift, as all of the patients had the same mutation. Clinical assessment and treatment of corneal dystrophies depend on gene analysis. Clinical follow-up and pathogenic gene analysis in these families will help with prognosis prediction, genetic counseling, and prenatal diagnostics. Despite huge improvements in genetics, medicine, and technology, monogenic illnesses like CDs are rarely treated. Performing procedures such as laser-assisted in situ keratomileusis (LASIK) on GCD2 patients can increase the disease's progression.

Conclusion

The study revealed two variants in the TGFBI gene in both families, one of which has not been previously reported. The mutations were predicted to have deleterious effects on protein function and structure and were found to be conserved across different species. Our findings expand the spectrum of mutations associated with GCD2 and contribute to the understanding of its genetic basis. The identification of these mutations has important implications for the early diagnosis and genetic counseling of affected individuals in consanguineous families. Furthermore, the functional consequences of the identified mutations can guide the development of potential therapies for GCD2.

Declarations

Statement and Declaration

Funding

This work is supported by the Institute of Molecular and Clinical Ophthalmology Basel (IOB) Switzerland for advanced research facilities (1-8/HEC/HRD/2019/ 10050) and Higher Education Commission (20-2211/NRPU/R & D/HEC/12) for financing.

Competing Interests

We have no conflict of interest.

Authors Contribution

Ayesha Khush Bakht: Data and Sample Collection, research work, Writing original draft preparation, Results Analysis, Editing, Visualization; Shagufta Naz: Supervision, Conceptualization, Methodology, Manuscript Review, Data analysis; Muhammad Asif Naeem: Methodology, Result analysis, Manuscript review; Saima Sharif, Haiba Kaul and Afia Iqbal: Manuscript review and editing, Result analysis; Ayesha and Afia Iqbal: Blood sampling; M. Qasim:, Sabika and Khajista. Data Handling, Manuscript Review and approval.

Ethics Approval

This study was approved by the Institutional Ethical Review Committee of Lahore College for Women University, Lahore.

Consent to Participate

All of the study participants provided their written, informed consent. It is also confirmed that the study's minor subject participants' parents or other legally recognized representatives signed written informed consent forms.

Consent to Publish
Not applicable.

**Data Availability statement**

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

**References**


41. Takagi M, Ishizu M, Suzuki H (1971) [An electron microscopic and histochemical study on a corneal granular dystrophy (Groenouw type I)]. Nihon Ganka Kiyo 22:479–484

Tables

**Table I** Summary of Genetics, Slit lamp, Fundoscopy. Segregating Mutation and c.DNA Position of Family PKCD02

<table>
<thead>
<tr>
<th>Family ID</th>
<th>Age/ Sex</th>
<th>Mutation Segregation</th>
<th>Phenotype (Slit Lamp)</th>
<th>Visual Acuity</th>
<th>Fundoscopy</th>
<th>Protein Change</th>
<th>c.DNA Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>V:1</td>
<td>59/M</td>
<td>Hetero-Normal</td>
<td>*GCD (Mild)</td>
<td>6/24</td>
<td>Retinal periphery normal</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>6/18</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V:2</td>
<td>18/M</td>
<td>Homo-affected</td>
<td>Thick central vascularized corneal scarring in right eye and granules near the limbus in left eye</td>
<td>CF[1/2]m</td>
<td>No view of fundus due to hazy media</td>
<td>R124H</td>
<td>c.371G&gt;A</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>6/36</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V:3</td>
<td>9/M</td>
<td>Homo-affected</td>
<td>Dots like opacities at the center of cornea</td>
<td>6/60</td>
<td>Retinal periphery normal</td>
<td>R124H</td>
<td>c.371G&gt;A</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>6/60</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V:4</td>
<td>26/F</td>
<td>Homo-affected</td>
<td>Confluent Speck like tensions in both eyes</td>
<td>CF[1/2]m</td>
<td>No view of fundus due to hazy media</td>
<td>R124H</td>
<td>c.371G&gt;A</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CF[1/2]m</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V:5</td>
<td>12/M</td>
<td>Hetero-Normal</td>
<td>Corneal clear</td>
<td>6/6</td>
<td>Retinal periphery normal</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>6/6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V:6</td>
<td>15/M</td>
<td>Hetero-Normal</td>
<td>Corneal clear</td>
<td>6/6</td>
<td>Anisometropia amblyopia in left eye</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>6/CF[1]m</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*GCD: Granular Corneal Dystrophy, CF: Count Finger Vision

**Table II** Summary of Genetics, Slit lamp, Fundoscopy. Segregating Mutation and c.DNA Position of Family PKCD04
<table>
<thead>
<tr>
<th>Family ID</th>
<th>Age/Sex</th>
<th>Mutation Segregation</th>
<th>Phenotype (Slit Lamp)</th>
<th>OCT OD</th>
<th>OCT OS</th>
<th>Visual Acuity OD</th>
<th>Visual Acuity OS</th>
<th>Fundoscopy</th>
<th>Protein Change</th>
<th>c.DNA Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>IV:1</td>
<td>62/M</td>
<td>Homo-affected</td>
<td>Keratoplasty in right eye and left eye affected with granules from peripheral to center</td>
<td>454</td>
<td>561</td>
<td>6/24</td>
<td>6/60p</td>
<td>*NV</td>
<td>R124H</td>
<td>c.371G&gt;A</td>
</tr>
<tr>
<td>V: 2</td>
<td>42/M</td>
<td>Homo-affected</td>
<td>Disc shaped thick opacities present from center to peripheral</td>
<td>500</td>
<td>227</td>
<td>C.F</td>
<td>C.F</td>
<td>NV</td>
<td>R124H</td>
<td>c.371G&gt;A</td>
</tr>
<tr>
<td>V:4</td>
<td>35/M</td>
<td>Hetero-affected</td>
<td>Rings or stellate shaped snowflake stromal opacities</td>
<td>370</td>
<td>346</td>
<td>6/60</td>
<td>6/60P</td>
<td>NV</td>
<td>R124H</td>
<td>c.371G&gt;A</td>
</tr>
<tr>
<td>VI:1</td>
<td>6/M</td>
<td>Homo-affected</td>
<td>Thick crumb-like granules present at the center of cornea</td>
<td>357</td>
<td>451</td>
<td>C.F</td>
<td>3/60</td>
<td>NV</td>
<td>R124H</td>
<td>c.371G&gt;A</td>
</tr>
</tbody>
</table>

*NV: Not Visible

**Figures**
Figure 1

The pedigrees of two families having c.371G>A mutation showing segregation of mutation and diseased status of individuals examined. **a:** (PKCD024) Three affected individuals (V:2, V:3, V:4) have homozygous c.371G>A mutation with heterozygous parents. Mutation is segregated in autosomal recessive mode of inheritance. **b:** (PKCD040) Individuals (IV:1, V:1, V:2, VI:1) have homozygous c.371G>A mutation. Individuals (IV:4, IV:5, V:4) have heterozygous c.371G>A mutation. This family showed the dominant mode of inheritance. Consanguineous marriages were seen in both families.
Figure 2

Slit Lamp Photographs, 1310nm swept source spectral domain optical coherence tomography scans of family members affected with granular corneal dystrophy type 2. a: Male patient V:2 (PKCD024). Slit lamp photo showing white granules all over the surface of cornea with intervening stroma and corneal thickness is 536 μm. b: Male Patient IV:1 (PKCD040). Slit lamp photo showing the thick granules opacities spread from center to peripheral and corneal thickness is 434 μm. c: Male IV:1 (PKCD024), unaffected heterozygous, slit lamp image showing the clear cornea (540μm). d: Male V:6 (PKCD040) is normal with clear cornea (589 μm)
Figure 3

Nucleotide sequence changes c.371G>A in two families of Pakistan. **Family A (a)** Proband (V:2) 18 years old is homozygous for mutation (c.371G>A) **(b)** His father (IV:1) is heterozygous normal with mild GCD **(c)** His brother (V:6) is normal for mutation. **Family B: (d)** Proband (IV:1) is homozygous for (c.371G>A) mutation **(e)** His Nephew (V:4) is heterozygous affected with mutation (c.371G>A)