A novel mutation in the \textit{NR2E3} gene associated with Goldmann-Favre syndrome and vasoproliferative tumor of the retina

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Purpose: Various autosomal recessive retinal dystrophies are reported to be associated with mutations in nuclear receptor subfamily 2, group E, member 3 (\textit{NR2E3}, also called PNR) gene. The present study proposed to understand the clinical and genetic characteristics of the family of a patient with an ocular phenotype consistent with Goldmann-Favre syndrome (GFS) and vasoproliferative tumors of the retina (VPTRs).

Methods: Twelve family members of the proband from three generations underwent complete ophthalmic examination, including best-corrected visual acuity with Snellen optotypes, tonometry, biomicroscopic examination, indirect ophthalmoscopy after pupillary dilatation, computerized perimetry, optical coherence tomography, fundus photography, intravenous fluorescein angiography, and electroretinography (ERG). All the study subjects underwent genetic analysis of the entire coding region of the \textit{NR2E3} gene with the bidirectional DNA sequencing approach. Hundred healthy individuals were screened for the variant.

Results: The phenotype of the proband had features of GFS with VPTRs. The tumors showed complete resolution with cryotherapy and transpupillary thermotherapy (TTT). Sequencing of the entire coding region of the \textit{NR2E3} gene in the proband revealed a novel homozygous c.1117 A>G variant that led to the amino acid change from aspartic acid to glycine at position 406 (p.D406G). This change was present in the homozygous state in affected family members and in the heterozygous state in unaffected family members, and was undetectable in the control subjects. The identified novel p.D406G homozygous mutation was at an evolutionarily highly conserved region and may possibly affect the protein function (Sorting Intolerant From Tolerant [SIFT] score = 0.00).

Conclusions: Patients with GFS may present with retinal VPTRs that respond to therapy with cryotherapy and TTT. Molecular genetic studies helped to identify a novel p.D406G mutation in the affected members, which will aid in confirming the diagnosis, for genetic counseling of family members and potentially provide some form of therapy for the affected patients.

Goldmann-Favre syndrome (GFS) is a vitreoretinal dystrophy that manifests with early onset of night blindness, atypical pigmentary dystrophy of the retina, degenerative changes in the vitreous humor, peripheral and, less often, central retinoschisis, lens opacities, and an enhanced S-cone response on electroretinography (ERG) [1]. Various autosomal recessive retinal dystrophies including enhanced S-cone syndrome (ESCS), GFS, and clumped pigmentary retinal degeneration have been described as associated with mutations in nuclear receptor subfamily 2, group E, member 3 (\textit{NR2E3} also called PNR [NCBI Reference Sequence: NM_014249.3]) gene [2–5]. The \textit{NR2E3} gene codes for a nuclear receptor that is specific to photoreceptors [2]. A common feature of these syndromes is a unique abnormality on ERG of absent rod activity and large S cone–mediated responses under photopic and scotopic conditions, known as the enhanced S-cone response [6].

Vasoproliferative tumors of the retina (VPTRs) are benign tumors of retinal vascular origin. These masses may be idiopathic or secondary to predisposing conditions such as intermediate uveitis, retinitis pigmentosa, ocular toxocariasis, Coats disease, chronic retinal detachment, and other traumatic or inflammatory diseases [7-10].

We report a patient with clinical features of GFS with secondary VPTRs successfully treated with a combination of transpupillary thermotherapy (TTT) and cryotherapy. The molecular genetic evaluation of the family revealed a novel mutation in the \textit{NR2E3} gene. To our knowledge, this is the first report of VPTRs in association with GFS, and the VPTRs successfully resolved with TTT and cryotherapy.
METHODS

The study was performed after receiving approval from the institutional ethics review board and in accordance with the Declaration of Helsinki. The nature of the study was conveyed, and informed consent was obtained from all study subjects. We certify that all applicable institutional and government regulations concerning the ethical use of human volunteers were followed during this research. The proband along with 11 family members from three generations were included in the study (Figure 1).

Ophthalmological and electrophysiological studies: All study subjects underwent a complete ophthalmic examination that included evaluation of best-corrected visual acuity (BCVA) with Snellen optotypes, Goldman applanation tonometry, biomicroscopic examination, indirect ophthalmoscopy after pupillary dilatation, computerized perimetry, optical coherence tomography (OCT; Zeiss Cirrus HD OCT –4000, Carl Zeiss meditec, Inc. Dublin, CA), fundus photography (TOPCON TRC 50 DX), and intravenous fluorescein angiography (IVFA; TOPCON TRC 50 DX, Tokyo, Japan). ERG (LKC Technologies, Gaithersburg, MD) was performed according to standard testing protocols recommended by the International Society for Clinical Electrophysiology of Vision (ISCEV).

Molecular genetic studies: A detailed family history and pedigree was constructed. One hundred unrelated ethnic- and age-matched control subjects were recruited for this study.

Sample collection and DNA preparation: Approximately 5 ml intravenous blood was collected in a anticoagulant (EDTA) coated tubes from all 12 study subjects and 100 Indian controls. Genomic DNA was prepared from peripheral blood leukocytes with salting-out method [11], by dehydration and precipitation of cellular proteins in a saturated sodium chloride solution. The isolated DNA will be dissolved in TE buffer (1 M Tris-pH 8.0; 0.5 M EDTA-pH 8.0) and stored at -20 °C until use.

Polymerase chain reaction and DNA sequencing: Eight sets of primers were used to amplify the entire coding region of NR2E3 gene [12]. The PCR products were gel eluted and column purified using an EZ-10 spin-column DNA gel extraction kit (Bio Basic, East Markham, Canada). A total of 20 μl master mix was prepared using 50–100 ng of genomic DNA, 1×PCR buffer, 200 μM of dNTPs (Medox Biotech India Pvt. Ltd, Chennai, India), 50% dimethyl sulphoxide (DMSO; Merck, Mumbai, India), 0.25 picomoles of each primer, and 1 unit of Taq DNA polymerase (Sigma, Saint Louis, MO), to perform PCR. The conditions followed were initial denaturation at 95 °C for 5 min, followed by 32 cycles (95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min) and final extension at 72 °C for 10 min. Bidirectional sequencing was performed using the ABI 3130 genetic analyzer (Applied Biosystems, Foster City, CA) with dye- termination chemistry.

Bioinformatics assessment: The evolutionary conservation of the identified NR2E3 mutation was checked using the clustalW multiple sequence alignment tool. Sorting Intolerant From Tolerant (SIFT) was used to assess the likely phenotypic
effect of the identified missense mutation. PolyPhen analysis was performed to calculate the probability of the identified mutation being deleterious toward disease pathogenesis.

RESULTS

Patient 1: The proband on presentation had a BCVA of perception of light in the right eye (RE) and 6/12 in the left eye (LE). Anterior segment examination of the RE showed severe corneal edema, neovascularization of the iris, posterior subcapsular cataract, and intraocular pressure (IOP) of 50 mmHg suggestive of neovascular glaucoma, and the LE showed a clear cornea and mild lens opacities due to posterior subcapsular cataract. The RE fundus details were not clearly visible through the hazy media due to corneal edema and lenticular opacity. However, peripheral retinal exudation and mass lesions similar to those in the LE could be seen hazily. The LE fundus examination showed vitreous degenerative changes, a healthy optic disc, and nummular pigment clumps at the level of the RPE in the midperiphery and along the vascular arcades, associated with diffuse RPE atrophy. The opaque, white dendritic appearance of the peripheral vessels was present throughout the midperiphery with mild arterial attenuation (Figure 2A). Three raised dome-shaped, yellowish-pink, vascular masses 4 DD in extent were noted in the peripheral retina in the superior, superotemporal, and inferotemporal quadrants. These masses were associated with subretinal exudation (Figure 2B,C). No dilated or tortuous feeder vessels were noted. IVFA showed rapid filling of the dye in the early phase, with the lesions becoming increasingly hyperfluorescent and leaking diffusely in the late phase. Telangiectatic and dilated vessels were observed within the tumor masses (Figure 3). Full-field ERG showed a severe decrease in the rod and cone responses in both eyes (BE). Based on the clinical, angiographic, and electrophysiological findings, a diagnosis of secondary VPTRs associated with retinal dystrophy was made. All the tumors were treated with transpupillary thermotherapy (TTT) in two sittings. Peripheral tumors were treated with cryotherapy with the triple freeze thaw technique. Complete regression of the vascular masses was noted over 6 months (Figure 2D), and the final BCVA was 6/9 in the LE. Notably, there was an absence of macular or peripheral retinal schisis in this patient.

Patient 2: The 43-year-old healthy elder brother of our proband had complained of defective night vision since childhood.
His BCVA was 6/6 BE. Anterior segment examination was normal. Dilated fundus examination showed a healthy optic disc and normal caliber of the retinal vessels. There was a mild diffuse RPE change with few pigment deposits in the midperipheral and peripheral retina. ERG showed decreased rod-specific responses, the response to a standard flash was delayed with low amplitude waveform under photopic and scotopic conditions, and the 30 Hz flicker response was delayed and of decreased amplitude. The responses to long duration stimulus using a blue flash with orange background (S-cone ERG response) showed an abnormally large delayed waveform typical of the enhanced S-cone response (Figure 4A,B). Ocular history and examination of the other ten members of the family were unremarkable.

The findings of the clinical, angiographic, and electrophysiological examinations suggested a possible diagnosis of GFS. Thus, full sequencing of the NR2E3 gene was undertaken in the proband. This revealed a novel, homozygous c.1117 A>G variant in exon 8 of the gene, a substitution mutation. This change leads to an amino acid change (aspartic acid to glycine) at position 406 of the gene. The p.D406G change was detected in the homozygous state in the proband’s brother (affected) and in the heterozygous state in eight unaffected family members (Figure 5; Table 1).

Screening of the NR2E3 gene in 100 unrelated Indian control samples of the same ethnic background did not show the p.D406G variant. The identified novel p.D406G homozygous mutation was evolutionarily highly conserved in different species (Figure 6). The SIFT score was 0.00, and the PolyPhen score was 0.998. These features suggest the pathologic nature of the identified genetic variation.

**DISCUSSION**

Patients with GFS typically present with early onset night blindness, atypical pigmentary retinal dystrophy, degenerative changes of the vitreous, peripheral or macular retinoschisis, lens opacities, and characteristic ERG abnormalities [1]. The presenting features and findings of the ophthalmic examination in the proband in this study led to the clinical diagnosis of GFS. Although ERG responses were severely reduced in the proband due to advanced disease, the proband’s brother displayed a hypersensitive response to blue light. The maximal response to short wavelengths, virtually no change in the response waveform to light and dark adaptation, some long- and middle-wavelength–sensitive cone dysfunction, and high subjective S-cone spectral sensitivity has been demonstrated in patients with GFS, and is termed the enhanced S-cone response [1,2].

The NR2E3 gene responsible for causing ESCS is located on chromosome 15q23 [12]. The gene coding region contains eight exons and spans a 7 kb region [13]. The gene contains the DNA-binding domain (DBD) at the N-terminus end and
Figure 4. Electrotetrotinography and S-Cone ERG findings in case 2. A: ERG findings in case 2. ERG shows severely reduced rod specific responses, response to standard flash was delayed with low amplitude waveform under photopic and scotopic conditions, the 30 Hz flicker was delayed and of lower amplitude. B: S-Cone ERG with blue flash and orange background showing abnormally large, delayed, simplified waveform as enhanced S cone ERG responses.

Figure 5. Chromatogram representing the p.Asp406Gly mutation in the NR2E3 gene. A: p.Asp406Gly mutation in the homozygous state. B: p.Asp406Gly mutation in the heterozygous state. C: Wild type. The underline marks the mutated codon. The arrow indicates the position of the mutation.
ligand-binding domain (LBD) at the C-terminus end. Most of the human NR2E3 variants and mutations are identified in these two domains [14]. In the present study, we identified a novel mutation in the LBD of the C-terminus end.

Thus far, nearly 50 NR2E3 gene variations and mutations have been reported in patients with various retinal degenerative disorders. Gerber et al. identified a p.R311Q mutation in the NR2E3 gene in the patients who may have ESCS [15]. Later, Haider et al. reported the same p.R311Q mutation with 44.8% frequency in patients with ESCS [12]. This same p.R311Q mutation in the NR2E3 gene was also reported with homozygosity in a patient with GFS [16]. In our study, we identified a novel p.D406G (c.1117A>G) mutation with homozygous state in the patient and in the affected sibling.

The variability of clinical features and the severity of retinal degeneration produced by NR2E3 mutations may often complicate the diagnosis. Patients with NR2E3 mutations may show characteristic features of GFS, as in our patient, or may have the ERG pattern typically seen in ESCS or clumped pigmentary retinal degeneration.

To our knowledge, the p.D406G homozygous mutation is novel. It was not seen in any of the control samples screened. The p.D406G homozygous mutation was also observed in the proband’s brother who has clinical features in a less severe form and did not show the presence of VPTRs. Both parents of the proband were heterozygous for the p.D406G mutation, which indicates that the parents were carriers for the disease and transferred the risk allele (G) to the proband. Out of the 12 family members who participated in this study, only two carry the normal allele (A). The remaining family members carry the risk allele with heterozygosity (A/G) that indicated they are carriers for the disease. A similar condition was reported by Haider et al., in the ESCS case. The p.M407K mutation was observed with the homozygous condition in the

| Individual ID | Age /Sex | Family member’s relation with the Proband | Disease state | Genotype | Genotype Status |
|---------------|---------|----------------------------------------|--------------|----------|----------------|
| 1–1           | 40/F    | Proband                                | Affected     | GG       | Affected homozygote |
| 1–2           | 41/F    | Sister                                 | Normal       | AG       | Carrier heterozygote |
| 1–3           | 48/M    | Husband                                | Normal       | AA       | Normal homozygous |
| 1–4           | 12/F    | Daughter                               | Normal       | AG       | Carrier heterozygote |
| 1–5           | 14/M    | Son                                    | Normal       | AG       | Carrier heterozygote |
| 1–6           | 75/M    | Father                                 | Normal       | AG       | Carrier heterozygote |
| 1–7           | 70/F    | Mother                                 | Normal       | AG       | Carrier heterozygote |
| 1–8           | 42/M    | Brother                                | Similar clinical features of proband | GG       | Affected homozygote |
| 1–9           | 9/F     | Brother’s daughter                     | Normal       | AG       | Carrier heterozygote |
| 1–10          | 9/F     | Brother’s daughter                     | Normal       | AG       | Carrier heterozygote |
| 1–11          | 16/F    | Sister’s daughter                      | Normal       | AA       | Normal homozygous |
| 1–12          | 13/F    | Sister’s daughter                      | Normal       | AG       | Carrier heterozygote |

The p.D406G change is detected in the homozygous state in the proband and proband’s brother and in the heterozygous state in eight unaffected family members.
patient, whereas two unaffected siblings carry the risk allele with heterozygous condition in their study [12].

Atsuhiro Kanda et al. showed that the NR2E3 sequence is highly conserved during evolution and the residue p.M407K was conserved in all NR2E3 orthologs [14]. The identified p.D406G mutation in the proband was also highly conserved during evolution (Figure 6). The SIFT score (0.00) showed that this mutation affected the protein function. The PolyPhen analysis also predicted that this mutation probably damaging the protein function with a score of 0.998. The amino acid aspartic acid/aspartate that is acidic polar and has been changed by the substitution of a nonpolar amino acid glycine at the 406 conserved position might have altered the NR2E3 protein structure and led to the disease occurrence in this case. The SIFT and PolyPhen scores support the deleterious nature of the p.D406G mutation. Further functional studies are required to confirm the pathogenesis of the p.D406G mutation.

VPTRs may be primary or secondary. In the largest review of 295 patients with 334 VPTRs by Shields et al. in 2012, 80% were idiopathic, and 20% were secondary. The most common preexisting ocular disease included retinitis pigmentosa (22%), pars planitis (21%), Coats disease (16%), previous retinal detachment surgery (12%), idiopathic peripheral retinal vasculitis (6%), and familial exudative vitreoretinopathy (4%). Other retinal lesions that might predispose to the development of VPTRs include toxoplasmic retinitis, toxocariasis, and traumatic choroidopathy [8]. A literature review showed no previous documentation of VPTRs in patients with GFS.

Primary tumors tend to be solitary, small, and located near the inferotemporal portion of the fundus. Secondary VPTRs are more often multifocal, bilateral, and believed to be a reactive vascular response to various ocular insults. The vascularized retinal nodules may threaten vision due to retinal exudation, macular edema, intraretinal or vitreous hemorrhage, and formation of epiretinal membranes [8]. These benign but vision-threatening tumors have been shown to respond to various treatment modalities such as cryotherapy, TTT, brachytherapy, and tumor resection [9]. The proband lost vision in her RE due to neovascular glaucoma (NVG) secondary to VPTRs. The tumors in the LE were treated with TTT (posterior tumors) and cryotherapy with the triple freeze thaw technique (extreme peripheral tumors). Complete regression of the vascular masses in our patient was observed within 6 months.

Retinoschisis, a feature of GFS, was not seen in our patient. However, the distinctive ophthalmoscopic feature is nummular pigmentary deposition at the level of the RPE, usually located in the midperiphery and often associated with RPE atrophy. This characteristic pigmentary clumping was noted in our proband, along with degenerative vitreous changes, lenticular opacity, and the abnormal ERG pattern. In our patient, opaque white dentritic retinal vessel changes were present throughout the midperiphery extending anteriorly between the equator and ora serrata. Since the fundus features vary among patients, Fishman et al. concluded that a diagnosis of GFS should be considered in patients presenting with an early history of poor night vision, bilateral atypical pigmentary changes in the retina, and degenerative changes in the vitreous humor. Additional diagnostic findings include retinoschisis, opaque dentritic retinal vessels, diffuse leakage from retinal capillaries, and cystoid macular edema [1]. Molecular genetic testing is essential for establishing the correct diagnosis in patients with NR2E3 mutations because of the variable phenotype associated with these degenerations. Further research may shed light on the association between the genetic mutation seen in our family with the observed phenotype.

In conclusion, we have described a heretofore unreported association of retinal VPTRs in GFS. The tumors regressed with standard treatment modalities. Detection of a novel p.D406G mutation in the NR2E3 gene helped to confirm the diagnosis. Genetic testing also helped in detecting the presymptomatic carrier and thus proved to be of immense value in informed genetic counseling of the family members and would potentially provide some form of therapy for the affected patients.

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REFERENCES
1. Fishman GA, Jampol L, Goldberg M. Diagnostic features of the Favre - Goldmann syndrome. Br J Ophthalmol 1976; 60:345-[PMID: 1085161].
2. Pachydaki SI, Klaver CC, Barbazetto IA, Roy MS, Allikmets R, Yannuzzi LA. Phenotypic Features of Patients With NR2E3 Mutations. Arch Ophthalmol 2009; 127:71-5. [PMID: 19139342].
3. Sharon D, Sandberg MA, Caruso RC, Berson EL, Dryja TP. Shared Mutations in NR2E3 in Enhanced S-cone Syndrome, Goldmann-Favre Syndrome, and Many Cases of Clumped Pigmentary Retinal Degeneration. Arch Ophthalmol 2003; 121:1316-23. [PMID: 12963616].
4. Udar N, Small K, Chalukya M, Garcia RS, Marmor M. Developmental or degenerative - NR2E3 gene mutations in two patients with enhanced S cone syndrome. Mol Vis 2011; 17:519-25. [PMID: 21364904].

5. Bandah D, Merin S, Ashhab M, Banin E, Sharon D. The Spectrum of Retinal Diseases Caused by NR2E3 Mutations in Israeli and Palestinian patients. Arch Ophthalmol 2009; 127:297-302. [PMID: 19273793].

6. Chavala SH, Sari A, Lewis H, Simpson E, Hagstrom SA, Traboulsi EI. An Arg311Gln NR2E3 mutation in a family with classic Goldmann – Favre syndrome. Br J Ophthalmol 2005; 89:1065-6. [PMID: 16024868].

7. Shields CL, Shields JA, Barrett J, Potter P. Vasoproliferative tumors of the ocular fundus. Arch Ophthalmol 1995; 113:615-23. [PMID: 7748132].

8. Shields CL, Kaliki S, Al-Dahmash S, Rojanaporn D, Shukla SY, Reilly B, Shields JA. Retinal vasoproliferative tumors: comparative clinical features of primary Vs secondary tumors in 334 cases. JAMA Ophthalmol 2013; 131:328-34. [PMID: 23494037].

9. Heimann H, Bornfeld N, Vij O, Coupland SE, Bechrakis NE, Killner U, Foerster MH. Vasoproliferative tumours of the retina. Br J Ophthalmol 2000; 84:1162-9. [PMID: 11004104].

10. Rennie IG. Retinal vasoproliferative tumours. Eye (Lond) 2010; 24:468-71. [PMID: 20075974].

11. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 1988; 16:1215. [PMID: 3344216].

12. Haider NB, Jacobson SG, Cideciyan AV, Swiderski R, Streb LM, Seary C, Beck G, Hockey R, Hanna DB, Gorman S, Duhl D, Carmi R, Bennett J, Weleber RG, Fishman GA, Wright AF, Stone EM, Sheffield VC. Mutation of a nuclear receptor gene, NR2E3, causes enhanced S cone syndrome, a disorder of retinal cell fate. Nat Genet 2000; 24:127-31. [PMID: 10655056].

13. Kobayashi M, Takezawa S, Hara K, Yu RT, Umesono Y, Agata K, Taniwaki M, Yasuda K, Umesono K. Identification of a photoreceptor cell-specific nuclear receptor. Proc Natl Acad Sci USA 1999; 96:4814-9. [PMID: 10220376].

14. Kanda A, Anand S. A comprehensive analysis of sequence variants and putative disease –causing mutations in photoreceptor - specific nuclear receptor NR2E3. Mol Vis 2009; 15:2174-84. [PMID: 19898638].

15. Gerber S, Rozet JM, Takezawa SI, dos Santos LC, Lopes L, Griebouval O, Penet C, Perrault I, Dueroq D, Souied E, Jeanpierre M, Romana S, Frézal J, Ferraz F, Yu-Umesono R, Munnich A, Kaplan J. The photoreceptor cell-specific nuclear receptor gene (PNR) accounts for retinitis pigmentosa in the Crypto-Jews from Portugal (Marranos), survivors from the Spanish Inquisition. Hum Genet 2000; 107:276-84. [PMID: 11071390].

16. Bernal S, Solans T, Gamundi MJ, Hernan I, de Jorge L, Carballo M, Navarro R, Tizzano E, Ayuso C, Baiget M. Analysis of the involvement of the NR2E3 gene in autosomal recessive retinal dystrophies. Clin Genet 2008; 73:360-6. [PMID: 18294254].