Stargardt Phenotype Associated With Two ELOVL4 Promoter Variants and ELOVL4 Downregulation: New Possible Perspective to Etiopathogenesis?

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Purpose. Stargardt disease (STGD) is the most common form of inherited juvenile macular degeneration. It is inherited as autosomal recessive trait (STGD1), although STGD3 and STGD4 are inherited as autosomal dominant inheritance pattern. STGD3 is caused by mutations in the elongation of very long-chain fatty acids-like 4 (ELOVL4) gene encoding for a very long-chain fatty acid elongase. Mutations lead to a truncated Elov4, lacking of a dlysine motif necessary for retention of transmembrane proteins in the endoplasmic reticulum. STGD occurs due to altered synthesis of very-long-chain polyunsaturated fatty acids (VLC-PUFA). Our work investigates the role of two variants in the ELOVL4 gene promoter region, c.-236 C>T (rs240307) and c.-90 G>C (rs62407622), identified in a patient with STGD in transconfiguration.

Methods. Their effects on ELOVL4 expression were examined by Dual-Luciferase Reporter assay.

Results. Rs62407622 and rs240307 variants caused 14% and 18% of expression reduction, respectively, compared with wild-type promoter. A very strong decreased gene expression was caused by coexistence of both variants.

Conclusions. A highly reduced activity of the ELOVL4 promoter was registered due to combination of two variants. Decrease of ELOVL4 enzymatic activity could lead to a deficiency of VLC-PUFA, essential components for rods function and longevity, which are among the parameters involved in the etiopathogenesis of STGD.

Keywords: Stargardt disease, macular degeneration, retinal degeneration, photoreceptors, VLC-PUFA

Stargardt disease (STGD) is a form of retinal dystrophy usually characterized by a progressive loss of central vision associated with irregular macular and perimacular yellow-white fundus flecks, and a so-called “beaten bronze” atrophic central macular lesion. Worldwide prevalence of STGD is estimated between 1/8000 and 1/10,000.1 Typical disease onset does not exceed the twentieth year of life, although symptoms can also appear during adulthood and as late as the seventh decade.2 Although disease severity and progression varies widely, STGD is usually characterized by loss of visual acuity, followed by wavy vision, blind spots, blurriness, impaired color vision, photophobia, and difficulty adapting in the dark.3

Today, three forms of STGD are known: STGD1, STGD3, and STGD4.4–6 STGD3 (Online Mendelian Inheritance in Man [OMIM] #600110) is a rare dominant form due to mutations in the elongation of very-long-chain fatty acids-like 4 (ELOVL4) gene on chromosome 6q16.3–5 ELOVL4 plays a fundamental role in the synthesis of very-long-chain polyunsaturated fatty acids (VLC-PUFA).6,7 VLC-PUFA make up a considerable part of phosphatidylcholine (PC) in the outer segment of both cell types of photoreceptors, suggesting a relevant role in the correct folding of disk rim and in cones and rods membrane fluidity.11 These functions, together with a close interaction with rhodopsin, strongly point to the possible involvement of ELOVL4 in phototransduction.12 Furthermore, recently, VLC-PUFA have also been found in conventional synapses and retina ribbon, probably being incorporated into vesicles containing glutamate, in rods terminals.13 So far only nine ELOVL4 variants are known: six single nucleotide and three indel/del mutations. Among these, four variants are in exon 6 and associated with the STGD3 form.14 According to this data, it would appear that Elov4 truncated protein loses the endoplasmic reticulum retention signal (KKXXX) and is mislocalized from the site of synthesis of VLC-PUFA. This condition leads to retina degeneration, due to: (1) production of toxic 3-keto-acylintermediates that imply cell death, and (2) reduced levels of VLC-PUFA and mislocalization of mutant protein, along with cellular stress, causing impairment of important cellular functions.5 It cannot be excluded that, in these situation, several chaperons, like HSP90, could help to solve the problem, as in other ocular diseases.15
Here, we report the case of a patient with dominant STGD in which we identified two ELOVL4 promoter variants, c.-236 C>T (rs6240307) and c.-90 G>C (rs62407622).

The effects of the single c.-90 G>C and c.-236 C>T variants, as well as two variants together ones (c.-90 G>C and c.-236 C>T) on gene expression and, consequently, on the onset of disease, were examined.

Materials and Methods

Clinical Data

The proband, a 42-year-old Caucasian man, came to our attention with a diagnosis of STGD, showing visual problems since young age. His visual acuity was 1.6/10 in the right eye and 2/10 in the left eye; his peripheral visual field was well represented, while the central one was almost absent. Moreover, he also showed an initial loss of color vision, photophobia, and a slow dark adaptation. Diagnosis was the result of the following evaluations: fundus analysis, fundus autofluorescence (FAF), infrared reflectance imaging (IR), optical coherence tomodography (OCT), visual field (VF), International Society for Clinical Electrophysiology of Vision (ISCEV) ERG, and pattern electoretinogram (PERG). Fundus examination revealed bilateral anatropic, rounded maculopathy with sharp edges, surrounded by pisciform flecks, confirmed by IR and FAF (Fig. 1). Furthermore, FAF showed mottled areas of hyperautofluorescence and hypoautofluorescence, corresponding to areas of lipofuscin accumulation and RPE atrophy, respectively. ERG revealed a generalized rods dysfunction with cones involvement (photopic and scotopic hypovolted ERG), with a delay in visual response (PERG with hypovolted P50 wave and increased latency; BiomedicaMangoni, Pisa, Italy; Fig. 2). VF showed central scotoma correlating with outer retinal subfoveal atrophy observed on FAF and OCT (Fig. 3). In details, OCT highlighted disruption of both inner and outer photoreceptor segment layers, combined with the loss of the inner segment–outer segment junction and thinning of other retina layers.

The patient's family, composed of father and mother, was evaluated by the same clinical and instrumental analyses and resulted healthy. Both parents did not manifest bilateral central visual loss, photophobia, color vision abnormalities, central scotomas, or slow dark adaptation. Moreover, they showed a visual acuity of 20/20, a normal visual field, and a clean fundus.

We screened all three known STGD causative genes (ABCA4, ELOVL4, and PROM1), and we found no associated or causative variants (HGMD Professional was the most important and updated database we considered; Qiagen Aarhus, Denmark), except those we analyzed in this paper.

In order to evaluate the variants effects on ELOVL4 expression, we performed a genetic analysis of the gene promoter through PCR and Sanger sequencing, followed by an in silico prediction and the functional Dual–Luciferase Reporter assay. The latter was essential to experimentally confirm the previously generated data.

Genotyping

DNA was extracted from leukocytes by using standard protocols. Amplification of regulatory regions of ELOVL4 gene was performed using primer pairs designed according to the published nucleotide sequence of GenBank (accession no. NG_009108.1; available upon request).

The PCR mix was prepared by adding 8 μg of genomic DNA to 50 μL reaction mixture containing a 0.2 μm concentration of each primer and 1 U MyTaq polymerase (Bioline, Aurogene Srl, Rome, Italy). PCR was carried out in the thermal cycler (Gene Amp PCR System 2700; PE Applied Biosystems, Foster City, CA, USA) under the following conditions: denaturation at 95°C for 15 seconds, annealing at 49.5°C for 15 seconds, and extension at 72°C for 10 seconds for 35 cycles, after an initial 1 minute denaturation at 95°C.

PCR products were sequenced by direct sequencing, using BigDye Terminator (ThermoFisher Scientific, Life Technologies, Monza, Italy) and Applied Biosystems 3500 Genetic Analyzer. The nucleotide number relative to variants identified in the promoter region of 805 bp was indicated in respect to the transcriptional start site of the reference sequence reported by the National Center for Biotechnology Information (NCBI) database. To name two polymorphisms we relied on Human Genome Variation Society (HGVS) nomenclature. Therefore, c.-90G>C (rs62407622) and c.-236C>T (rs2430307) indicated nucleotide substitutions at RNA level.

In Silico Analysis

In silico analysis was performed on the ELOVL4 promoter using two transcription factors (TF) prediction tools, individually and in pairs: BioBase TRANSFACTM Professional16 and Algene PROMO.17 These tools were used to identify potential TF binding sites in the region where the variants in the ELOVL4 promoter were found. TRANSFAC was set to use the profile matrix for vertebrates, with a cutoff to minimize false positives (minFP). This is defined as the score that gives 1% of hits in the used sequences relative to the number of hits received at the minimum false negative (minFN) cutoff (the score at which at least 90% of the positive test set are recognized, i.e., it equals a false negative rate of 10%). The false positive rate is estimated by applying the Match algorithm (BIOMBASE GmbH, Halchtersche, Wolfenbittel, Germany) to upstream sequences.

PROMO, instead, involves the dissimilarity threshold; a parameter that controls how similar a sequence must be to the matrix to be reported as a hit. It was set at 15% (85% similarity). Random expectation (RE) gives the number of expected occurrences of the match, in a random sequence of the same length as the query sequence, according to the dissimilarity index. Two models are considered: (1) equiprobability for the four nucleotides (RE equally), and (2) estimate the nucleotide probability as the nucleotide frequencies in the matrix for vertebrates, with a cutoff to minimize false positives.

Furthermore, Cytoscape software (The Cytoscape Consortium, New York, NY, USA) and its MCODE plug-in were used to analyze pathways between involved TFs, in order to predict possible interactions among them.

Cell Culture

U373 MG (human, Caucasian, glioblastoma-astrocytoma) cells (Sigma-Aldrich, Milan, Italy) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 1 mg/mL ampicillin (Lonza, Amboise, France) at 37°C in a water-saturated atmosphere with 5% CO₂. The essential feature that led us to choose this cell line was the acquisition of infinite growth potential, which set the stage for multiplication of genetic variants with an ever increasing fitness for proliferation and spread.

We used a glioblastoma cells line to perform another dual luciferase assay, involving cerebral cavernous malformations.18 This cell line can be useful because the retina has a nervous derivation as glia. Despite this, it is not possible to exclude that the whole transcription factors set in glioblastoma cells could be quite different from that in retina cells. In order to clarify
At this point, we address some supporting evidence: almost all TFs coming from our in silico analysis result expressed both in glioblastoma cells and retina cells, as evidenced by DPRP database, based on expression and ChIP-Seq experimental data, and TiGER. Moreover, description of common TFs was reported among others also by Rheinbay et al. and Mysickova et al.

**Construction of the Reporter Gene Plasmids**

In order to highlight if the variants could affect ELOVL4 expression, the effects of two combined genotypes, c.-236T/c.-90C (T-C) and c.-236C/c.-90C (C-C), on promoter activity were studied. An 805 bp promoter sequence was amplified by PCR using genomic DNA from the proband and one donor, selectively carrying each haplotype, using the following primers: forward: 5′-AGATCTACATGCACCTTTCTCTTGTC-3′ and reverse: 5′-AAGCTTCACTACGTAGTAGGACAC-3′ under these conditions: 1 cycle of 95°C for 1 minute; 35 cycles of 95°C for 15 seconds, 49.5°C for 15 seconds, and 72°C for 10 seconds; and 1 cycle of 72°C for 7 minute.

Each PCR product, as well as pGL4.10 (luc2) was digested by BglII and HindIII (Promega Italia, Milan, Italy) and then purified (PureLink PCR Purification Kit; ThermoFisher Scientific, Life Technologies). Each promoter and pGL4.10 (luc2), digested and purified, was incubated overnight in appropriate concentrations, in order to execute ligation. The efficiency of promoter insertion upstream of the luciferase gene, cloned into the pGL4.10 (luc2), was verified by agarose gel electrophoresis.
FIGURE 1. Continued.
FIGURE 2. Proband’s ERG and PERG, compared with healthy control. PERG (A) The voltage of the P50 wave was abnormally decreased and the latency was increased. The scotopic (C) and photopic (E) ERGs showed abnormally low voltage. The combination of this data suggests an impairment of both rods and cones functions, compared with PERG (B), scotopic (D), and photopic (F) reference ERGs. The signal was amplified (gain 50,000), filtered (band pass, 1–100 Hz) and averaged with automatic rejection of artifacts by a BM 6000 unit. Analysis time was 250 msec. In healthy subjects, these peaks have the following implicit times: 35, 50, and 95 msec (N35, P50, N95).
FIGURE 3. OCT of proband (A, B), compared with that of a healthy control (C) and overt STDG3 patient (D). Proband’s right eye (A) depicts the extent of the transverse loss of the junction between the inner and outer segment of the photoreceptors in the foveal region. Furthermore, his left eye (B) showed abnormal pigmentation in the RPE layer, due to macular degeneration, as notable in the STDG3 patient (D). The integrity of all retina layers in a healthy subject is highlighted in (C).
Novel constructs were subcloned into *Escherichia coli* Top 10 cells (Life Technologies) and single colonies were miniprep. The correct sequence of all the clones was verified by DNA sequencing, using the Sanger method, and then selected for transient transfection.

**Transient Transfection and Promoter Assays**

Cells were first seeded in 96-well culture plates at a density of 2 × 10^4 cells per well. Then, a transient transfection was performed with following modality: (1) in 1/4 of wells (16), cells were cotransfected with 0.05 µg of the pGL4.10 [luc2] promoter construct containing the only c.-90 G>C variant, and with 0.05 µg of the pGL4.10 [luc2] promoter construct containing the *ELOVL4* wild-type promoter; (2) in a second 1/4 of wells (16), cells were cotransfected with 0.1 µg of the pGL4.10 [luc2] promoter construct containing only the c.-236 C>T variant, and with 0.05 µg of the pGL4.10 [luc2] promoter construct containing the *ELOVL4* wild-type promoter; (3) in another 1/4 of wells (16), cells were cotransfected with 0.05 µg of two pGL4.10 [luc2] promoter constructs containing, respectively, the c.-90 G>C and the c.-236 C>T variants; and (4) in the final quarter of wells (16), cells were transfected with 0.1 µg of the pGL4.10 [luc2] promoter construct containing the *ELOVL4* wild-type promoter. Finally, the remaining 32 wells were filled with not transfected cells (16) and with the luciferase substrate only (16). In each well, besides cells, the mixture included 0.2 µL of Lipofectamine 3000 Reagent (ThermoFisher Scientific, Waltham, MA, USA) and 0.2 µL of P3000 Reagent (ThermoFisher Scientific), in a serum-free medium and then incubated for 24 hours at 37°C in a humidified atmosphere of 5% CO₂ in air. After incubation, cells were washed twice with PBS and lysed by Passive Lysis Buffer (Promega). Luciferase activity was measured using Dual-Luciferase assay kit (Promega) and GloMax-Luminometer (Promega). Reporter construct activity was normalized by comparison with activity from the *Renilla* luciferase construct. Luciferase activities are representative of at least six independent experiments, with each construct tested 16 times per experiment.

**Population Screening**

Analyzed variants were screened in 500 unrelated healthy donors born and living in Messina for at least two generations,
Two Promoter Variants Associated With Stargardt Phenotype

constituting a heterogeneous group for age and sex, in order to assess their frequency in the same geographic site population in which the patients belonged.

Statistical Analysis

All data analyses were performed using the IBM SPSS 24 software for Macintosh (IBM SPSS Statistics for Macintosh; IBM Corp, Armonk, NY, USA). A 1-way ANOVA was performed to compare between the sample groups. All P values were Bonferroni’s corrected and considered significant if \( P < 0.05 \).

Ethical Statements

The study followed the tenets of the Declaration of Helsinki and was approved by the Scientific Ethics Committee of the Azienda Ospedaliero Universitaria-Policlinico “G. Martini” Messina. All family members and controls signed informed consent after explanation of the nature and possible consequences of the study.

Results

Genotyping of ELOVL4 promoter in the whole family components highlighted that the proband’s mother and father present an alternate heterozygosity for each variant in exam (Fig. 4), indicating that the probandinherited both variants in trans. The predictive analysis of the proband’s ELOVL4 promoter by TRANSEAC Professional evidenced the loss of one group of TF binding sites (ETF, ZF5, E2F-6, FBH1, HDAC2, and TFII250) in the double heterozygous genotype, due to the presence of c.-90 G>C for most of them. The exception was represented by FBH1 and the complement 666 through 679 binding sites for TFII250, whose loss was attributable to c.-236 C>T. A second group analysis, resulting from the combined predictions of TRANSFAC and PROMO, revealed the appearance of new possible binding sites of different TFs (CPB, BCL6B secondary motif, Spi-B, Pax-4, RXR-alpha, GKL, POLR3A, TFII-H, Pax-5, p53, SP1, and GRalpha), determined by c.-236 C>T for Spi-B, Pax-4 and RXR-alpha, and by c.-90 G>C for others (for further details see Table 1). This data suggest a probable transcription variation, due to the altered balance of TF binding properties. Furthermore, it is important to understand the relationship between the analyzed TFs, and how each one could influence the others. This was examined by Cytoscape pathway analysis, along with its MCODE plug-in, from which arose a 4-cluster division that highlighted a relevant network involving most of the TFs in exam (Table 2; Fig. 5). These data were confirmed by Dual-Luciferase Reporter Assay, involving the proband’s ELOVL4 promoter, compared in its wild form, versus both variants and c.-90 G>C and c.-236 C>T only samples. Results showed an expression reduction of approximately 14% in the c.-90 G>C sample and of approximately 18% in the c.-236 C>T one (compared with a healthy control), but a strong decrease (~97%) arose from the promoter carrying the combination of above variants (Fig. 6). The 1-way ANOVA, after Bonferroni’s correction, confirmed the statistical significance of analysis (\( P < 0.05 \)). Multiple comparison details are listed in Supplementary Materials.

These results take a particular value, considering that both analyzed variants showed a very low frequency distribution in Messina healthy population (c.-90 G>C; G frequency = 0.94; C frequency = 0.06; c. 236 C>T; C frequency = 0.95%; T frequency = 0.05%), in contrast to what reported for the European population (in the public domain, http://www.ensembl.org/Homo_sapiens/Variation/Population?db=core;\ r=6:79946869-79947869;v=rs62407622;vdb=variation;\vf=12792 675; in the public domain, http://www.ensembl.org/Homo_ sapiens/Variation/Population?db=core;r=6:79947015-79948015;\ v=rs240307;vdb=variation;\vf=129670).

Discussion

ELOVL4 encodes a member of the elongase family, expressed in retina, brain, skin, and sperm, involved in the elongation of very long-chain fatty acids. Although little is known about the role of this protein, data report that the contribution of the enzyme is to be found in the initial rate of VLC-PUFA production and condensation reactions between a fatty acyl-CoA and malonyl-CoA. The role of VLC-PUFA is fundamental. The most reliable hypothesis argues that the VLC-PUFA acyl chain may cover the entire bilayer, representing a flexible hinge at the rim site where the curvature of photoreceptor disk membranes is the greatest. At rim level, alteration of ELOVL4 could impair the turnover of photoreceptor disk membranes, due to a modified balance of fatty acid precursors. The direct consequence of this variation leads to an abnormal accumulation of lipofuscin granules, observed in the RPE of mutant retinas that may impair retinoic acid trafficking between RPE and photoreceptors.

Recent experiments have shown a reduction in rods ERG oscillatory potentials and scotopic threshold responses in ELOVL4 KO mice, and presented biomorphologic evidence that the ERG changes are correlated with reduced VLC-PUFA and synaptic architecture. It may affect vesicle tethering or recycling pathways, as well as glutamate release mechanisms, due to VLC-PUFA interaction with synaptic proteins that mediate endo/exocytic activity or that were localized to the synaptic ribbon in photoreceptor terminals.

It is known that ELOVL4 mutations, alone or with PROM1 mutations, could cause enzyme activity loss and, subsequently, the onset of the dominant form of STGD.

Analysis of ELOVL4 gene sequence in our patient affected by STGD permitted us to identify two variants, transconfigured on the gene promoter. We demonstrated that the coexistence of two variants determined the down regulation of gene transcription. To be precise, the Dual-Luciferase Reporter assay highlighted the down regulation of ELOVL4 transcription by 97% in the patient’s sample (c.-90 G>C and c.-236 C>T). The possible protein elonging activity loss could lead to several consequences:

1. Compromising the integrity of photoreceptor membrane compartments, such as Golgi, or even the retinal pigment epithelium;
2. Causing a corresponding reduction of rhodopsin levels within outer segment disk membranes, or the production of abnormal hetero-oligomers with its membrane proteins, which may lead to alterations in membrane ultrastructure or biochemistry; and
3. Altering VLC-PUFA direct signaling and possible alteration of the rim site where the curvature of photoreceptor disk membranes is greater or the greatest. It was proposed that lipid molecules, such as docosahexaenoic (DHA), eicosapentaenoic, and arachidonic acids could activate specific receptors or modulate transient receptor potential cation channel activity. The latter task is enforced by the presence of VLC-PUFAs also in ribbon synapses, as well as the smaller conventional synapses in the retina.

Results showed that many TF binding sites were altered in the ELOVL4 promoter, both for activators (ETF, FBH1, HDAC2, TAPII250) and for repressors (Spi-B, Pax-4, POLR3A, TFII-H, Pax-5, P53, SP1, and GRalpha), determined by c.-236 C>T for Spi-B, Pax-4 and RXR-alpha, and by c.-90 G>C for others (for further details see Table 1). These results take a particular value, considering that both analyzed variants showed a very low frequency distribution in Messina healthy population (c.-90 G>C; G frequency = 0.94; C frequency = 0.06; c. 236 C>T; C frequency = 0.95%; T frequency = 0.05%), in contrast to what reported for the European population (in the public domain, http://www.ensembl.org/Homo_sapiens/Variation/Population?db=core;r=6:79946869-79947869;v=rs62407622;vdb=variation;vf=12792 675; in the public domain, http://www.ensembl.org/Homo_ sapiens/Variation/Population?db=core;r=6:79947015-79948015;\ v=rs240307;vdb=variation;\vf=129670).
### TABLE 1. BioBase and TRANSFAC TFs Binding Sites Prediction

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<th>Factor Name</th>
<th>Region</th>
<th>Strand</th>
<th>Match Sequence</th>
<th>Core Similarity Matrix Similarity Matrix ID WT</th>
<th>c.-236 C&gt;T c.-90 G&gt;C c.-26 C&gt;T Reference (PMID)</th>
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<td>1 VS ETF_Q6</td>
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<td>0.896 VS ZF5_01</td>
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<td>0.625</td>
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</tr>
<tr>
<td>HDAC2</td>
<td>811.819 plus</td>
<td>CGCTGcc</td>
<td>0.829</td>
<td>0.82 VS HDAC2_04</td>
<td>✓ ✗ 26129908; 25940751; 2696608</td>
</tr>
<tr>
<td>TAFII250</td>
<td>666.679 minus</td>
<td>CTCCTcctctcg</td>
<td>0.807</td>
<td>0.84 VS TAFI_05</td>
<td>✓ ✗ 18117127</td>
</tr>
<tr>
<td>ETF</td>
<td>807.813 minus</td>
<td>CGGCGGC</td>
<td>1</td>
<td>1 VS ETF_Q6</td>
<td>✓ ✗ 2768275</td>
</tr>
<tr>
<td>ZF5</td>
<td>810.817 minus</td>
<td>cggCTGGC</td>
<td>0.844</td>
<td>0.896 VS ZF5_01</td>
<td>✓ ✗ 17714511</td>
</tr>
<tr>
<td>E2F-6</td>
<td>811.819 minus</td>
<td>cgcctGGGCC</td>
<td>0.69</td>
<td>0.787 VS E2F-6_02</td>
<td>✓ ✗ 9501179</td>
</tr>
<tr>
<td>FBII</td>
<td>660.668 minus</td>
<td>ggGCTGctt</td>
<td>0.625</td>
<td>0.79 VS ZBTB7A_02</td>
<td>✓ ✗ 19853566; 15917220</td>
</tr>
<tr>
<td>HDAC2</td>
<td>811.819 plus</td>
<td>CGCTGcc</td>
<td>0.829</td>
<td>0.82 VS HDAC2_04</td>
<td>✓ ✗ 26129908; 25940751; 2696608</td>
</tr>
<tr>
<td>TAFII250</td>
<td>666.679 minus</td>
<td>CTCCTcctctcg</td>
<td>0.807</td>
<td>0.84 VS TAFI_05</td>
<td>✓ ✗ 18117127</td>
</tr>
</tbody>
</table>

**Allgene PROMO**

<table>
<thead>
<tr>
<th>Factor Name</th>
<th>Region</th>
<th>Strand</th>
<th>Match Sequence</th>
<th>Dissimilarity</th>
<th>RE Equally</th>
<th>RE Query</th>
<th>WT</th>
<th>c.-236 C&gt;T c.-90 G&gt;C c.-26 C&gt;T Reference (PMID)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TFIIH</td>
<td>664.669 plus</td>
<td>TTCTCC</td>
<td>11.357888</td>
<td>1.24512</td>
<td>1.92792</td>
<td>✓</td>
<td>✓</td>
<td>17529664</td>
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<td>PAX5</td>
<td>805.811 plus</td>
<td>GCGGCCCC</td>
<td>9.552105</td>
<td>0.74707</td>
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<td>✓</td>
<td>22278146</td>
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<tr>
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<td>GCGGCCCC</td>
<td>6.188498</td>
<td>0.31128</td>
<td>0.9106</td>
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<td>✓</td>
<td>26491919; 25407019</td>
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<td>SPI1</td>
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<td>CCTGCC</td>
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<td>3.985438</td>
<td>5.91804</td>
<td>✓</td>
<td>✓</td>
<td>25211818</td>
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</table>

Factor name, binding factor name, represented by the matrix; Region, position of the matrix match (putative binding site) within the analyzed sequence; Strand (plus/minus), the strand on which the putative site was found depends on the orientation in which the matrix is given in TRANSFAC; Match sequence, capital letters indicate the positions in the sequence that match with the core sequence of the matrix, while the lower case letters refer to positions that match to the remaining part of the matrix; Core similarity, the core similarity score for the matrix match (the matrix core is defined as the five consecutive most conserved nucleotides within the matrix); Matrix similarity, the matrix similarity score for the matrix match. The Match score can vary from 0 (lowest similarity) to 1 (highest similarity) of the match to the matrix. Only those matches are listed in the result, for which the core and matrix similarity are higher than the chosen cutoffs; Matrix ID, identifier for the matrix with which the putative binding site was found; Dissimilarity, the dissimilarity threshold controls how similar a sequence must be to the matrix to be reported as a hit. It was set at 15% (85% similarity). Random expectation (RE), gives the number of expected occurrences of the match in a random sequence of the same length as the query sequence according to the dissimilarity index. Two models are considered: (1) Equiprobability for the 4 nucleotides (RE equally), and (2) Estimate the nucleotide probability as the nucleotide frequencies in the query sequence (RE query). Results from these two software analyses highlight the appearance or disappearance of specific TFs binding sites in the presence of no analyzed mutations (wild-type column), both examined ones (c.-236 C>T, c.-90 G>C column), and single separated variants (respectively, c.-90 G>C column, and c.-236 C>T column).
alpha\(^{50,51}\) and repressors (ZF5\(^{52}\), EZF-6\(^{53}\), CPBP\(^{54-56}\), BCL6B\(^{57}\), GKL\(^{58-60}\), and RXR-alpha\(^{61-64}\)).

We can speculate that, as emerges from Cytoscape pathway analysis, the \textit{ELOVL4} expression reduction in the proband could derive from a complex balance of all TFs, most of which could present a mutual influence in determining the final effect. Focusing on TFs binding sites formed as a result of the presence of both examined variants, SP1 probably represents...
Table 2. Cytoscape Pathway Analysis of TFs Involved Into ELOVL4 Promoter Integrity

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Annotations (GO ID)</th>
<th>Ensembl Protein ID</th>
<th>Entrez Gene ID</th>
<th>RefSeq mRNA ID</th>
<th>UniProt ID</th>
<th>Score</th>
<th>Log Score</th>
<th>MCODE Node_Status</th>
<th>MCODE Cluster</th>
<th>MCODE Score</th>
</tr>
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<tbody>
<tr>
<td>KLF4</td>
<td>GO:0048598</td>
<td>GO:0001159</td>
<td>GO:0000976</td>
<td>GO:0000790</td>
<td>ENSP00000404922</td>
<td>9314</td>
<td>NM_004235</td>
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<td>−0.426</td>
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<td>KLF6</td>
<td>GO:0003609</td>
<td>GO:0003790</td>
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<td>NM_001300</td>
<td>Q9N62</td>
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<tr>
<td>E2F6</td>
<td>GO:0003677</td>
<td>GO:0003700</td>
<td>GO:000571</td>
<td>GO:0005515</td>
<td>ENSP00000466315</td>
<td>1876</td>
<td>NM_212540</td>
<td>Q7A661</td>
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</tr>
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<td>GTF2I</td>
<td>GO:0006667</td>
<td>GO:0006525</td>
<td>ENSP00000460070</td>
<td>2969</td>
<td>NM_0536001</td>
<td>P73847</td>
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<td>−0.577</td>
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<td>Cluster 2</td>
</tr>
<tr>
<td>TAF1</td>
<td>GO:0006670</td>
<td>GO:000975</td>
<td>GO:000571</td>
<td>GO:0004565</td>
<td>ENSP00000424526</td>
<td>6872</td>
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<td>RXRA</td>
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<td>GO:0006671</td>
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<td>GO:0000785</td>
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<td>NM_002957</td>
<td>RXRA_HUMAN</td>
<td>0.616</td>
<td>−0.484</td>
</tr>
<tr>
<td>ZBTB7A</td>
<td>GO:0000978</td>
<td>GO:0001078</td>
<td>GO:0006677</td>
<td>GO:0003700</td>
<td>ENSP00000471865</td>
<td>5154</td>
<td>NM_015898</td>
<td>ZBT7A_HUMAN</td>
<td>0.770</td>
<td>−0.262</td>
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<tr>
<td>HDAC2</td>
<td>GO:0004958</td>
<td>GO:0004585</td>
<td>GO:0001085</td>
<td>ENSP00000450432</td>
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<td>NM_000827</td>
<td>Q92669</td>
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<tr>
<td>PAX5</td>
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<tr>
<td>NR1C1</td>
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<td>GO:0006352</td>
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<tr>
<td>SP1</td>
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<td>GO:0004555</td>
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<td>NM_00176761</td>
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<tr>
<td>TPS3</td>
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<td>−0.603</td>
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<td>SPI1</td>
<td>GO:0006679</td>
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<td>PAX4</td>
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<td>GO:0001206</td>
<td>GO:0006677</td>
<td>GO:0003690</td>
<td>ENSP00000473584</td>
<td>5078</td>
<td>NM_006193</td>
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<tr>
<td>ZBTB14</td>
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<td>GO:0005870</td>
<td>GO:0005515</td>
<td>ENSP00000463555</td>
<td>7541</td>
<td>NM_004090</td>
<td>ZBT14_HUMAN</td>
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<tr>
<td>TFAP2A</td>
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<td>GO:0001159</td>
<td>GO:0000976</td>
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<td>ENSP00000420568</td>
<td>7020</td>
<td>NM_009320</td>
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<tr>
<td>PCLR3A</td>
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<td>TED4</td>
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<td>0.817</td>
<td>−0.203</td>
<td>Clustered</td>
<td>/</td>
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</tbody>
</table>

Gene name, name of the gene that encodes for one of involved transcription factor. Annotation (GO ID), ID associated to gene ontology. The GO classifies gene functions along three aspects: (1) molecular function, (2) cellular component, and (3) biological process; Ensembl protein ID, protein ID for Ensembl; Entrez gene ID, gene ID for Entrez; RefSeq mRNA ID, mRNA ID for NCBI Reference Sequence Database; UniProt ID, UniProt protein ID score. Prior to construction, the selected networks are each assigned a weight by the GeneMANIA algorithm. The query genes are assigned a label value of 1, while all other genes are 0. Label propagation is then applied to the entire network and the resulting labels are saved as the score attribute, used to rank the genes. The score assigned to each gene reflects how often paths starting at a given gene node end up in one of the query nodes and how long and how heavily weighted those paths are. This score indicates the relevance of each gene to the original list based on the selected networks. Higher scores indicate genes that are more likely to be functionally related. We also chose not to add any other genes coming from pathway analysis to visualize only how the members of our list are connected; Log score, logarithmic scale applied to score values; MCODE cluster, indicates the number of cluster analyzed genes belong to; MCODE Node Status, no evidence if query gene is part of a cluster; Seed status represents the highest scoring node in the cluster; MCODE Score defines the weight value that permit a gene to be clustered; Unclustered genes typically show the lowest value.

Pathways analysis by Cytoscape and its plugins GeneMANIA and MCODE revealed a strict interaction between all TFs whose binding sites presence/absence is determined by examined variants.
the key node around which other factors determine the overall ELOVL4 protein under expression. As literature evidences, SP1 should be enhanced by RXR-alpha activity, but this positive status should be repressed or seized by interaction with TFII-I, TP53, and KLF6, the latter stimulated by KLF4. The direct consequences of SP1 inhibition could be related to a decrease of connected PAX-5, in turn influencing PAX-4. Although there is no solid proof, in literature, of a possible direct interaction, we can hypothesize, basing on previous assumptions, that TFII-I, usually acting as a repressor, could contribute to the inhibition of SP1, as well as the possibility that SP1 impairment could reflect on POLR3A, reducing its activity.

Due to the unavailability of data on involved TF interactions, analysis on TF binding sites lost in the patient is not clear. We can only speculate that, because HDAC2 usually acts as a repressor, with FBI-I as corepressor, both could downregulate another two inhibitors, ZF5 and E2F-6. This situation leaves most of the transcriptional activity to TAFII250, which functions as an activator, and which presents many binding sites in wild-type genotype.

**CONCLUSIONS**

A reduction of Elovl4 enzymatic function in the endoplasmic reticulum (ER) could result in a deficiency of VLC-PUFA,

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>c.-90 G&gt;C</th>
<th>c.-236 C&gt;T</th>
<th>c.-236 C&gt;T c.-90 G&gt;C</th>
<th>Control</th>
<th>Not Transf. Cells.</th>
<th>Substrate only</th>
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<tr>
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<td>2,040</td>
<td>0,810</td>
<td>2,502</td>
<td>0,773</td>
<td>0,772</td>
</tr>
<tr>
<td>SD</td>
<td>0,045</td>
<td>0,040</td>
<td>0,063</td>
<td>0,049</td>
<td>0,046</td>
<td>0,010</td>
</tr>
<tr>
<td>SEM</td>
<td>0,011</td>
<td>0,010</td>
<td>0,016</td>
<td>0,012</td>
<td>0,011</td>
<td>0,002</td>
</tr>
</tbody>
</table>

Figure 6. Luciferase assay results. The histogram shows the means coming from luciferase ratios between Firefly and Renilla bioluminescence measurements (in relative luminescence units - LRU) for each sample. As reported, ANOVA test resulted significant ($P = 1.1391E^{-109}$), also for multiple comparisons (highlighted with pairwise lines on bars and asterisk). The presence of only the c.-90 G>C or the c. 236 C>T variant determines an ELOVL4 expression reduction, respectively, of approximately 14% or 18%, compared with the healthy control, while the heterozygous condition for both examined variants drastically lowers it (~97%).

[Diagram: Error Bars: +/- 1 SD]
which may be required for the construction, function, and maintenance of healthy OS or other photoreceptor membranes; hence, the absence of sufficient quantities eventually results in retinal degeneration. The results presented here reported reduced Elovl4 enzyme activity, fundamental in VLC-PUFA synthesis, vital for rod function and rod longevity, parameters that are involved in the etiopathogenesis of STGD.

We speculate that an altered balance of TF binding sites, due to the presence of c.-90 G>C and c.-236 C>T, and the possible interaction of involved transcription factors, could determine an overall prevalence of repressive activity rather than enhancing activity, resulting in a downregulation of ELOVL4 expression, as functionally demonstrated by the dual-luciferase reporter assay.

Even if the in vitro experiments demonstrate an expression reduction of ELOVL4 promoter due to transconfiguration of analyzed variants, we cannot assert with certainty that the same effect, in vivo, is limited to both variants’ presence. For example, cells are treated outside their normal ‘microenvironment’ (no surrounding tissues, no blood supply, no normal supply of nutrients, etc.), and we cannot exclude the involvement of other factors into the altered expression of ELOVL4. Moreover, further experiments (e.g., ChIP-sequencing of involved TFs) will be needed to confirm, or not, the role of single transcription factors and reciprocal interactions, involved in ELOVL4 downregulation.

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References


