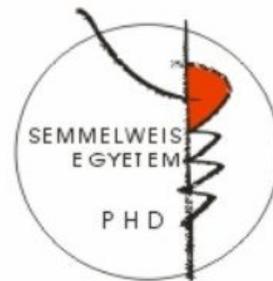


# Study of mutations and polymorphisms in ocular diseases

Ph.D. Theses

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## Introduction

The recent advances of Human Genom Project and extensive spread of molecular genetic techniques have impacted on all areas of clinical medicine including ophthalmology. The development of molecular biology has fundamentally altered our notions of disease etology and classification. Molecular genetic diagnosis is now possible for many inherited eye disorders and the identification of the genetic mutations causing eye disease improves greatly our understanding of pathomechanism. The investigation of polygenic or multifactorial disorders is also intriguing: to study the effect of genetic and environmental factors.

In my Ph.D. dissertation I summarize my work and results of one mendelian ocular disease and two multifactorial ocular disorders. We examined a multigeneration Danish family suffering from autosomal dominant congenital stationary night blindness in the Institute for Human Genetics at the University Hospital Hamburg-Eppendorf; the aim of this study was to identify the disease-causing mutation in the family and to investigate the mechanism. In the Department of Ophthalmology (Tömó Street) at Semmelweis University we studied two multifactorial disorders in the group of patients treated with photorefractive keratectomy (PRK): the genetic factors influencing persistent corneal haze development after PRK were analysed by mutational screening; and the steroid-induced ocular hypertension occurring during postoperative topical steroid treatment after PRK was examined by analyses of polymorphisms. The aim of our study was to enhance the safety of refractive surgical treatments and to decrease the prevalence and severity of potential complication of refractive surgery using feasible genetic screening and adequate postoperative therapy.

Autosomal dominant congenital stationary night blindness (adCSNB) caused by abnormal function of rod photoreceptors is a rare disorder. Fundus appearance, visual field and colour vision are usually normal, but patients have abnormal rod response on electroretinogram and abnormal curve of dark adaptometry. The first described family with adCSNB was found in 1838 in South France, by this time 135 night blind persons in 15 generations were known. Disease-causing mutation was identified in alpha-subunit of rod-specific G protein, transducin (*GNATI*) in 1996. A small number of unique missense mutations in the genes encoding various members of the rod phototransduction cascade, e.g. rhodopsin (*RHO*), cGMP phosphodiesterase  $\beta$ -subunit (*PDE6B*), and transducin  $\alpha$ -subunit (*GNATI*) have been reported to cause autosomal dominant CSNB. While the *RHO* and *PDE6B* mutations result in constitutively active proteins, the only known adCSNB-associated *GNATI* change (p.Gly38Asp) produces an inactive  $\alpha$ -transducin. In our study, we investigated the disease-causing mutation and how it leads to adCSNB in the Danish family.

Corneal wound healing after PRK shows high variability among patients. Corneal subepithelial haze is a rare complication after PRK, but it decreases the quality of vision especially in mesopic conditions. The most intriguing question is why subepithelial corneal haze develops in some cases and not in others. These differences may partly be explained by genetic predisposition, environmental effects, or other changes in the avascular wound healing mechanisms. Since this clinical condition resembles the lumican-null mouse phenotype, mutation analysis of lumican and keratocan genes, members of the family of small leucine-rich interstitial proteoglycans, was carried out to investigate whether germline genetic alterations have an effect on development of severe corneal haze in humans. We hypothesized that mutation of these genes may play a role in development of pathological subepithelial haze, because lumican and keratocan are necessary in collagen fibrillogenesis and in maintenance of corneal transparency. In humans, mutations of lumican gene were not examined yet. Mutations of keratocan genes were identified only in patients with cornea plana congenita.

Postoperative therapy after PRK routinely consists of administration of steroid eye drops, because glucocorticoids (GCs) have a dehydrating effect in the corneal stroma. GCs help maintain refractive stability and inhibit stromal scarring, rethickening, and consequent regression of refraction, although their molecular mechanism is not clearly known. Topical or systemic administration of GCs may produce a rise in intraocular pressure (IOP), but there is considerable variation in rate of occurrence of steroid – induced ocular hypertension and in severity of side effects among individuals. The normal population can be divided into three groups on the basis of steroid responsiveness: high responders, moderate-responders and non-responders. Variation in sensitivity to glucocorticoids observed in healthy population is influenced by genetic polymorphisms of the glucocorticoid receptor gene. N363S, ER22/23EK, and Bcl I have been previously described as glucocorticoid-sensitivity modulating polymorphisms. We investigated whether these variants may contribute to steroid-induced ocular hypertension and if they play a role as protective or risk factors during exogenous glucocorticoid administration.

## Aims

The aims of our studies were the following:

- 1. a,** To identify the gene from the known autosomal dominant retinitis pigmentosa and adCSNB genes carrying the disease-causing mutation in the multigeneration Danish family with congenital stationary night blindness.
- b,** To investigate the functional role of novel mutation in the mechanism of autosomal dominant congenital stationary night blindness.
- 2.1. a,** To prove the hypothetical role of germline mutations of the lumican gene in mechanism of persistent corneal subepithelial haze development in PRK treated patients.
- b,** To examine the role of germline mutations of the keratocan gene, as a further candidate gene, in mechanism of persistent corneal subepithelial haze development in PRK treated patients.
- 2.2 a,** To study the allele frequency of the polymorphisms N363S, Bcl I and ER22/23EK of glucocorticoid receptor gene in our group of PRK treated patients.
- b,** To investigate the association between the steroid-induced ocular hypertension and the polymorphisms N363S, Bcl I and ER22/23EK of glucocorticoid receptor gene in the group of PRK treated patients who were postoperatively administered with topical fluorometholone (0.1%).
- c,** To investigate the association between the steroid-induced ocular hypertension and the polymorphisms N363S, Bcl I and ER22/23EK of glucocorticoid receptor gene in the group of PRK treated patients who were postoperatively administered with topical prednisolone acetate (0.5%).

## Methods

1. The Danish pedigree studied here consists of 9 family members presenting with typical symptoms of CSNB in three generations suggesting an autosomal dominant trait. In addition to standard ophthalmologic examination, dynamic visual field measurement with a Goldmann apparatus, colour vision testing, dark adaptometry (Goldmann-Weekers) and full-field ERG were performed at the National Eye Clinic, Hellerup, Denmark. The further examinations were carried out in the Institute of Human Genetics at University Hospital Hamburg-Eppendorf, Germany.

**Linkage analysis:** fluorescence-tagged microsatellite markers were analysed on an ABI PRISM 310 automated DNA sequencer. We evaluated the obtained data using MLINK software.

**Mutational analysis** was performed using standard PCR protocol and automated bidirectional sequencing. Verification of the found mutation and cosegregation analysis were performed by digestion with Tse I restriction enzyme. We examined 104 control chromosomes and all family members.

**Molecular modelling** was performed using the SWISS-Model server at <http://swissmodel.expasy.org>. Modelling was based on the known transducin/G $\alpha$ i-chimera structure published previously. Structures were visualized using the PDBviewer software.

- 2.1 A clinical and a genetic study was carried out in the group of patients, who were treated with excimer laser in the period 1999-2004 in the Department of Ophthalmology at the Semmelweis University. We examined ten patients who had a severe corneal haze graded 1.0-2.0 according to Hanna's scale one year after PRK, seven males and three females with persistent subepithelial corneal haze participated in the study.

**Clinical examinations:** routine ophthalmological examination, and in vivo confocal microscopy examination was also performed to study corneal structure and endothelial cells. Severity of corneal haze was evaluated by slit-lamp biomicroscopy according to Hanna's scale.

**Genetic examinations:** Genomic DNA was isolated, then we amplified the promoter region and the entire protein coding regions of the candidate genes. Mutations and polymorphisms of the lumican gene were analysed by direct sequencing. PCR-based mutational analysis of the keratocan gene was performed using a sensitive mutational pre-screening method, the temperature gradient gel electrophoresis (TGGE, Temperature Gradient Gel Electrophoresis) and in cases of positive samples we performed bidirectional direct sequencing.

**2.2** We examined 102 patients, who underwent photorefractive keratectomy in the Department of Ophthalmology at the Semmelweis University, and received topical steroids (either fluorometholone 0.1% or prednisolone acetate 0.5% alone or combined) as part of postoperative therapy. The choice of steroid depended on course of wound healing and regression of refraction.

**Clinical examinations:** routine eye examination, corneal thickness measurement, and Goldmann applanation tonometry was carried out. Variations in intraocular pressure (IOP) levels in response to steroid therapy were observed. We separately analyzed data from three groups of patients: those who received fluorometholone only (analysis A1, 132 eyes of 73 patients); those who were initially given fluorometholone then later switched to prednisolone acetate (analysis A2, 42 eyes of 26 patients); and those who received prednisolone acetate only (analysis B, 76 eyes of 38 patients).

**Genetic examinations:** To genotype DNA, allele-specific PCR amplification was applied for the N363S polymorphism, and PCR-based restriction fragment length polymorphism analysis was performed to examine the Bcl I and the ER22/23EK polymorphisms.

**Statistical analysis:** Analysis of covariance with forward stepwise variable selection model building was carried out. Analysis was carried out with the STATISTICA 5.0 software package.

## Results

1. Using microsatellite markers we genotyped all family members. For the **linkage** relationships *D3S1289*, the locus close to *GNAT1* gave positive lod scores of 1.81 at  $\theta=0.00$  using two point linkage analysis. Direct sequencing of all PCR-amplified exons and flanking intronic regions of *GNAT1* detected a heterozygous C to G change of the first nucleotide (c.598C>G) in codon 200 in exon 6, in the Switch 2 domain of  $\alpha$ -transducin. The **mutation** results in loss of a *TseI* site, and restriction enzyme analysis of the family showed that the c.598C>G mutation was present in all affected family members in heterozygous state. Two-point linkage analysis for disease locus vs. C>G produced a lod score of 3.01 at  $\theta=0.00$  assuming complete penetrance and a frequency of 0.0001 for the mutant allele. The c.598C>G change was absent from 104 alleles of unaffected and unrelated controls.

**Molecular modeling** data suggest that the mutation p.Gln200Glu is most likely to interfere with the GTPase activity of the transducin  $\alpha$ -subunit causing a constitutive activation of the visual cascade.

- 2.1 Clinical examinations:** The most severe corneal haze was grade 2.0 on Hanna's scale one year after PRK. There was no significant difference in endothelial cell density of PRK-treated hazy corneas compared to healthy control corneas.

**Genetic examinations:** we optimized the PCR method; amplicons of exons and the flanking intronic regions of the lumican gene were analyzed by DNA sequencing. None of these patients and the control individuals had any germline genetic alterations in the 5'-UTR sequences in exons 1 and 2, in the coding region of the lumican gene in exons 2 and 3, and in the initial part of the 3'-UTR sequence in exon 3. We optimized the pre-screening TGGE method to screen the three amplicons of the keratocan gene. An unusual TGGE pattern was detected in one control individual, which was identified after DNA sequencing as an intronic heterozygous G>T nucleotide change. No other genetic alterations were detected in the keratocan gene.

- 2.2 Clinical examinations:** A switch from fluorometholone 0.1% to prednisolone acetate 0.5% therapy was necessary in 37% of the patients after a median fluorometholone 0.1% treatment period of three months.

**Results of analyses A1 and A2:** The mean highest IOP during the steroid administration was in analysis A1 and A2 18.0 mmHg (range: 11-36 mmHg), 24.8 mmHg (range: 14-44 mmHg), respectively. No significant predictors of the maximal IOP during follow-up were identified. Thus we could not prove the effect of the polymorphisms or clinical factors studied on the maximal IOP in the A1 and A2 analyses.

**Results of analysis B:** Follow-up started at the first usage of prednisolone acetate 0.5%. The mean highest IOP during the prednisolone acetate 0.5% therapy was 26.6 mmHg (range: 14-44 mmHg). After the variable selection, only N363S remained in the model, with the coefficient of 9.1 mmHg (SE=4.1 mmHg, p=0.03), which corresponds to a confidence interval of +1.0 to +17.3 mmHg. The other variables did not have a significant effect.

**Genotyping:** Allele frequencies found in our study population are in line with allele frequencies found previously in Caucasian population. Homozygotes carrying the polymorphic alleles were not found in our study population neither in the case of N363S polymorphism nor in the case of ER22/23EK polymorphisms.

## Conclusions

- 1. a,** Linkage analysis was carried out to find the candidate *GNATI* gene of autosomal dominant retinitis pigmentosa and adCSNB genes. We optimized the PCR for the mutational analysis using self-designed primers. In a multigeneration Danish family with adCSNB a novel heterozygous missense mutation (c.598C>G) in exon 6 of *GNATI* gene was identified that should result in the p.Gln200Glu substitution in the evolutionarily highly conserved Switch 2 region of  $\alpha$ -transducin.
  - b,** Computer modeling based on the known crystal structure of transducin suggests that the mutant protein exhibits impaired GTPase activity due to conformational changes in evolutionarily highly conserved Switch 2 region of  $\alpha$ -transducin, thereby leading to constitutive activation of phototransduction. We identified the first known constitutively activating mutation in the *GNATI* gene.
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- 2.1 a,** We foremost studied mutations of lumican gene using self-elaborated method in humans. In the lumican gene no genetic alteration was found in PRK treated patients with persistent corneal haze, thus there was no evidence that germline mutation of lumican gene participate in the aetiology of subepithelial corneal haze in PRK treated patients.
  - b,** We optimized the PCR and the pre-screening method based on heteroduplex analysis to examine the keratocan gene. No germline mutation was found in the keratocan gene among patients with persistent corneal subepithelial haze, as a result of mutational analysis. Thus there was no evidence that germline mutation of keratocan gene participate in the aetiology of subepithelial corneal haze in PRK treated patients.
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- 2.2 a,** We foremost examined the allele frequency of the polymorphisms N363S, Bcl I and ER22/23EK of glucocorticoid receptor gene in group of ophthalmologic patients. The allele frequencies found in our study group were in line with previously described data in the literature.
  - b,** We first studied the association between the functional polymorphisms N363S, Bcl I and ER22/23EK of glucocorticoid receptor gene and the steroid-induced ocular hypertension. No significant associations were found between the polymorphisms N363S, Bcl I and ER22/23EK and the steroid-induced ocular hypertension in PRK treated patients who were postoperatively administered with topical fluorometholone (0.1%).

- c, We foremost described the significant association between the polymorphism N363S of glucocorticoid receptor gene and the steroid-induced ocular hypertension in PRK treated patients who were postoperatively administered with topical prednisolone acetate (0.5%). No significant associations were found between the variants Bcl I and ER22/23EK and the steroid-induced ocular hypertension in PRK treated patients who were postoperatively administered with topical prednisolone acetate (0.5%).

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