Genetic and mechanistic association of *TCF4*, *ZEB1*, *FEN1* and *MCT* genes with Fuchs' Endothelial Corneal Dystrophy

By

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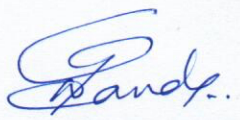

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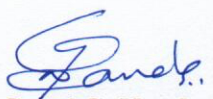

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DEDICATIONS

This thesis is dedicated to Maa, Baba and Utsav for their continuous motivation and belief in me.

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SYNOPSIS OF Ph.D THESIS

1. **Name of the Student:** GARGI GOURANGA NANDA

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SYNOPSIS

Decreased visual acuity, a common disorder of aging accompanied by progressive deterioration of corneal endothelial cells and formation of corneal guttae characterizes Fuchs Endothelial Corneal Dystrophy (FECD, MIM# 136800).[1] The inheritance pattern of this disease is regarded as both autosomal dominant and complex; although women seem to be the predominantly affected group.[1] Primary cause of the disease is unknown but clinical detection includes specular microscopic examination to ascertain endothelial cell loss and formation of bullae in the Descemet's membrane (DM) of the corneal endothelium.[2] This endothelial cell loss increases the cellular stress resulting in excrescences from the DM. The consequence of such thickened DM due to deposition of collagen, leads to failure of the primary function of the endothelial membrane, which is to maintain corneal deturgescence.[3] With 42,000 corneal transplantations taking place every year in Unites States[4] and FECD being the third most

frequent reason for corneal transplantations in India (16.6%) to treat the edematous cornea that is associated with FECD,[5] early stage FECD diagnosis and perceivability of the disease pathomechanism have become absolute necessity for the numerous patients undergoing refractive or cataract surgery every year.

Genetic contributions towards the pathogenicity of FECD have been reported and it differs according to different cohorts and FECD types. Unlike the more common late onset FECD type, early onset (EO) FECD is a rarer form and is reported in individuals as young as 15 years of age.[6] In case of EO-FECD, mutations in the gene encoding for the alpha 2 chain of collagen type VIII protein (*COL8A2*), secreted by the endothelial cells as the major component of DM, has so far been solely afflicted to cause this corneal disorder.[7,8] Attempts to provide evidences of genetic association of *COL8A2* with late onset (LO) FECD remained futile.[9] Abnormal deposition of various types of collagen in the DM of late onset FECD cornea has however been reported.[10] Linkage analysis in familial cases of late onset FECD have indicated mutations in genes with diverse functions such as, sodium borate transporter, member 11 (*SLCA411*), Zinc Finger E-Box Binding Homeobox 1 (*ZEB1*), Transcription factor 4 (*TCF4*), Clustrin, Lipoxygenase homology domains 1 (*LOXHD1*) and ATP/GTP binding protein-like 1 (*AGBL1*).[11, 12] Recent genome-wide association studies (GWAS) with large number of age/gender/ethnicity matched cohorts have identified *TCF4* as the strongest contributor of FECD.[13, 14] Other genes added to this multi-genic complex disorder through GWAS are, *KANK4* (KN motif- and ankyrin repeat domain-containing protein 4), *LAMC1* (laminin gamma-1) and *ATP1B1* (Na^+, K^+ transporting ATPase, beta-1 polypeptide.[14] Many other chromosomal loci are also involved in its pathogenesis like 13pTel-13q12.13, 18q21.2-q21.32, 5q33.1-q35.2 and 9p labeled as FCD1, FCD2, FCD3 and FCD4 respectively.[15-18]

The focus of this study is to understand the genetic markers associated with FECD occurrence in India and also to understand their molecular mechanism towards disease progression. For this, three previously associated genes; *TCF4*, *ZEB1* and *FEN1* were scanned for the genetic

variants that were significantly associated with FECD development in a sample Indian population. Various molecular and biochemical assays were also employed to understand their role in FECD pathogenesis. Finally, we also intended to understand the functional implications of the lactate transporter proteins, MCTs (mono-carboxylate transporters) under FECD conditions. For this, the following chapters were designed to achieve their respective objectives.

Chapters

1. Introduction
2. Materials and methods
3. Association of *TCF4* with FECD in Indian population
    - Genetic association of known polymorphisms in Indian cohort
4. Association of *ZEB1* with FECD
    - Genetic association through gene scan
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5. Association of DNA repair gene, *FEN1* with FECD
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6. MCT localization and expression in human corneal endothelial tissue
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Chapter 1. Introduction

1.1. Prevalence and predisposing risk factors of FECD

1.2. Interpreting the mechanism of FECD pathogenesis

1.3. Clinical interventions to correct an FECD eye

1.4. Hypothesis and Objectives

Chapter 2. Materials and Methods

Materials and methods employed in this study are adapted, modified and detailed in this thesis.

Chapter 3. Association of *TCF4* with FECD

3.1. Introduction

Genome wide association studies (GWAS) have reconfirmed association of *TCF4* single nucleotide polymorphism (SNP) rs613872 with FECD, first identified by Baratz et al.[13], among subjects from various ethnic backgrounds such as, Caucasian, European and Australian descent.[14-19] Thalamuthu *et al*., however indicated that two other polymorphisms (rs17089887 and rs17089925) and not the much evident rs613872 of *TCF4* gene were strongly associated with FECD among the Chinese cohort.[20] Apart from these SNP associations, *TCF4* has also been ascribed with trinucleotide repeat expansion (CTG18.1) among FECD affected individuals.[21] These studies reflect that the region surrounding rs613872 harbor potential disease causing elements for FECD. Studies comprising Indian late onset FECD subjects were very scant until the year 2014 and hence the genetic contributors for the disease specific to this region of the globe are veiled.[9] The aim of this study was to assess the genetic association of various *TCF4* polymorphisms, *i.e*., rs613872, rs17089887, rs17089925 and CTG18.1 allele among Indian FECD patients.

3.2. Results

Genetic association analysis using direct sequencing was done for *TCF4* intronic polymorphisms and FECD in 108 control and 44 patients of Indian origin within the age range of 38 to 81 years. After age and gender correction, we found that only one of the intronic *TCF4* SNPs, rs17089887, was significantly associated (*Genotype P value = 0.008*) under dominant model of inheritance in our population with "T" allele (*Allelic P value = 0.013*) imparting 2.073 (*95% CI = 1.18 - 3.62*) increased chances of acquiring FECD. We also observed that individuals with TT genotype for rs17089887 are at greater (65.9%) risk of acquiring FECD. The much-cited polymorphism, rs613872 show only a marginal association

with respect to genotype (*Df2, P value = 0.03*) but shows no association with respect to alleles *(Df1, P value = 0.246*).

The threshold limit of CTG trinucleotide repeats (>50) in *TCF4* gene assumed by Weiben and colleagues as a disease susceptibility marker for FECD was used in this study.[10] Agarose gel electrophoresis followed by direct sequencing was employed to efficiently detect at least fifty repeats of CTG18.1 allele. The frequency distribution of both the alleles of control and FECD populations illustrate that 15/44 (34%) FECD cases and 5/97 (5%) control individuals harbored these extended trinucleotide repeats (≥50). Out of these 15 patients, 14 individuals had at least one "T" allele of rs17089887 and the combined haplotype (T-X) shows significant association with the disease ($P = 2 \times 10^{-4}$).

3.3. Discussion

A genetic marker, be it a microsatellite with 2-, 3- or 4-base pairs of short tandem repeat pattern or a variation at a single DNA base pair position (SNP), is polymorphic if the variation is seen in more than 1% of a population. The associated marker can be a causal genetic variant where the change in the DNA sequence is itself contributing towards the disease trait or that the association reflects a nearby DNA element that is in LD with the polymorphic marker and is functionally responsible for the disease. The genetic markers associated in this study are rs17089887 and CTG18.1 allele. In context with the previously associated polymorphisms of *TCF4* gene in various populations, CTG18.1 allele remains consistently associated throughout. This suggests that CTG18.1 can be a putative causal variant for FECD.

Chapter 4. Association of *ZEB1* with FECD

4.1. Introduction

Riazuddin *et al*. associated *TCF8* alias *ZEB1* with late onset FECD, contributing upto 2% (7/384) of genetic load to develop FECD.[18] Few researchers have sketched a hypothesis that a deficiency in the DNA-binding properties of *ZEB1* may lead to late onset FECD conditions on the basis that COL8A2, along with other *COL4A* types, share the same consensus sequences in

the promoter region for *ZEB1* binding.[22] Therefore with this study we aimed at finding out the genetic variants or mutations of *ZEB1* gene that are associated with FECD individuals from Indian origin and also the role of *ZEB1* in regulating *COL8A2*.

4.2. Results

Bidirectional sequencing was carried out to scan the *ZEB1* gene for the presence of any mutations or variants that showed effective association with FECD in our Indian cohort. Previously associated or new mutations were not found in the exon-rich regions of *ZEB1*; however, the distribution of alleles of rs220057 in the 4th intron was biased among the preliminary sample of cases (n=30) and control (n=10) individuals. Upon validation with 63 cases and 124 controls, it was observed that the variant was significantly associated with FECD ($P = 0.016$, $Df = 1$). Other variants that were observed were found to be nonpathogenic. Bioinformatic analysis provided a 6bp region located downstream of CDS (+372) with maximum homogeneity score for consensus *ZEB1* DNA-binding element, which was further selected to check for *ZEB1* binding by electromobility shift assays (EMSA). When competitively challenged with 1000 fold unlabeled oligos or *ZEB1* specific antibody, prominent dissolution of specific protein:DNA complex was noted; indicating *ZEB1* specific binding to the biotin labeled oligos. These results indicate *ZEB1* as a genetic contributor for FECD as well as a probable regulator for *COL8A2* gene.

4.3. Discussion

The aim of this experiment was two-tier. Firstly, to identify genetic variants/mutations those were associated with FECD, and to analyze the regulatory role of *ZEB1* on the promoter region of *COL8A2*. With this study we report for the first time that a polymorphism in *ZEB1* gene, rs220057, shows significant association with FECD in our study population. Furthermore, identification of *ZEB1* binding sites in *COL8A2* gene essentially sheds light on the regulatory role of *ZEB1* in excessive collagen deposition on DM of late onset FECD

cornea. These results strengthen the association of *ZEB1* with FECD and partially help understand its role in disease progression.

Chapter 5. Association of DNA repair gene *FEN1* with FECD

5.1. Introduction

Flap endonuclease 1 (*FEN1*), is one of the DNA-damage repair genes, that has been recently associated with FECD in Polish population showing significant association with a *FEN1* polymorphism, c.4150G>T located at the 3'UTR of this gene.[23] Although functional effects of this polymorphism have been reported in various cancerous tissues, its effect on FECD disease background is scantly known.[24, 25] In this current study, we aimed to assess the genetic association of two polymorphisms, c.-69G>A (rs174538) and c.4150G>T (rs4246215), in *FEN1* gene among Indian population, and to identify the causal variant that contributes towards the oxidative damages incurred during disease progression.

5.2. Results

Restriction Fragment Length Polymorphism (RFLP) and direct DNA sequencing methods were employed to genotype the polymorphisms, c.-69G>A and c.4150G>T harbored in the 5' and 3' UTR regions of the *FEN1* gene respectively. It was observed that *FEN1* c.4150G>T showed significant association with FECD (Df2: P=0.001). 13% of the FECD individuals (8/62) were the carriers of c.4150TT genotype in comparison to a meager 2.64% (4/151) of the control population; rendering it as twice high a risk genotype to develop FECD (OR = 2.48, 95% CI = 1.45-4.25) than the rest of the genotypes. *FEN1* c.-69G>A variant on the other hand maintained its non-association with the disease unhindered by age or gender biases (P=0.41).

Endogenous DNA damage, expressed as Ln-OTM, was significantly higher in FECD cases (P= 0.0057, n=28) as compared to their unrelated controls (n= 24). We therefore analyzed for any correlation between the genotypes of the associated polymorphism (*FEN1* c.4150G>T) and DNA damage in FECD cases. It was observed that the *FEN1* c.4150TT FECD carriers (n=8) had significantly greater DNA damage than c.4150GT (P= 0.004, n=10) and 4150GG

(P= 0.037, n=10) FECD individuals. Most significant difference in endogenous DNA damage was observed between FECD carriers of c.4150TT genotype and their control counterpart with the same genotype (P<0.001).

5.3. Discussion

One of the most significant observations of the study was the heightened endogenous DNA damage in FECD cases as compared to the control group. This indicates that the PBMCs from patients are at higher oxidative stress that has allowed the accumulation of oxidative DNA damage, which remained unrepaired. Oxidative stress and deficiency in antioxidant defense system have been elaborately attributed as the etiology of FECD pathogenesis in numerous studies.[26,27] Our study identified that the 3'UTR polymorphism, *FEN1* c.4150G>T is strongly associated with increased DNA damage in subjects clinically diagnosed with FECD in Indian population. These results provide supportive evidence to confirm the genetic involvement of *FEN1* gene in the pathogenesis of FECD. However, it warrants further studies to dissect out the specific DNA damage and repair pathway genes that are de-regulated in FECD corneal tissues as compared to those of the control tissues.

Chapter 6: MCT localization in human corneal endothelial cells

6.1. Introduction

The aim of this chapter was to identify new candidates responsible for developing FECD. It has already been reported that mutations in solute carrier protein, *SLC4A11* cause late-onset FECD in Caucasian families.[28] This indicates that the failure of barrier function of corneal endothelial cells has a significant impact in the progression of FECD. Another SLC family transporter, *MCT1*, a monocarboxylate transporter, which is one of the key regulator for lactate concentration throughout the cornea and it's active functionality in corneal endothelium ensures corneal transparency and deturgescence. However, localization studies for *MCT1* in human corneal endothelial cells haven't been reported. We therefore intended to localize these

transporters, *MCT1*, 2, 3 and 4 in human corneal endothelial tissues and analyze their expression level in FECD as compared to control tissues.

6.2. Results

Non-transplantation grade, DM-endothelial sections post-trephining of whole human corneal tissues were used to study the localization of *MCT1*, 2, 3 and 4. Using antibodies specific to *MCT1*, 2, 3 or 4, immunohistochemical (IHC) analysis of the DM-endothelial sections were done. Positive localization was observed for *MCT1*, *MCT2* and *MCT4*. 3D localization tool of the Z-sections of the tissues showed that *MCT1* obtained basolateral localization, whereas *MCT2* and *MCT4* were localized in the apical regions and lateral regions respectively. *MCT3* failed to show any positive signal in the corneal endothelial tissue. RT-PCR analysis performed with whole thickness tissue of one FECD patient and 2 control individuals, showed 7.1 and 1.3 fold up-regulation of *MCT1* and *MCT4* respectively; whereas, *MCT2* was 0.4 fold down regulated. IHC of endothelial sections alone showed *MCT1*, *MCT4* and *MCT2* with 0.5, 0.3 and 0.7 fold decreased expression as compared to control tissues.

6.3. Discussion

Our study is in consensus with the MCT localization studies in rabbit eyes; such that, *MCT1*, *MCT2* and *MCT4* are expressed in corneal endothelium, but not *MCT3*.[29] However, spatial distribution differs in human than in rabbits. Human corneal endothelium shows dense *MCT1* localization in basolateral region, whereas rabbits have both *MCT1* and *MCT4* in the basolateral regions. *MCT2* localization in the apical region is similar in both human and rabbit endothelium. Due to limited availability of FECD tissues, multiple tissues could not be included for the analysis. However, this data suggests that in FECD condition, although the endothelial MCTs are downregulated, their increased expression in the epithelial layer shows an active compensatory mechanism to channel the monocarboxylates through cornea.

Chapter 7: Discussion

FECD is a sexually dimorphic disease with a dominance of females as the affected gender, which is reflected in the cases of this study group. With recent reports adding on more genes as contributors towards FECD, it is becoming even more difficult to understand its pathomechanism. Our study reports for the first time, the genetic scenario of a sample Indian population showing significant association of *TCF4, ZEB1 and FEN1* polymorphisms with FECD; thereby strengthening their role as major contributors globally. It also emphasizes on the pathomechanism involving DNA damage susceptibility associated with *FEN1* polymorphism, regulatory role of *ZEB1* over *COL8A2* and differential expression of *MCT1* across the cornea under FECD condition. These multi-genic components function in a composite way that consequently develops into FECD. Extensive trinucleotide repeats in *TCF4* transcript sequester the mRNA splice-machinery components and thereby make essential transcripts unavailable for their function;[30] this in turn puts the endothelial cells at a disadvantage where the DNA damage due to oxidative stress becomes difficult to repair.[31] This de-regulated chaotic condition aggravates the stress levels and the endothelial cells eventually succumb to apoptosis.[32] Remaining cells enlarge in size to compensate for the lost cells.[33] These deductions and hypothesis are based on the current study and published literatures. Further studies are needed to elaborate on the pathomechanism involving various risk factors so that early detection is feasible and risk-directed medications can be encouraged to replace corneal transplantation to treat FECD.

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Nanda GG, Das S, Alone DP. Genetic and functional association of ZEB1 with FECD.

Conference Proceedings:

1. **Nanda GG**, Malloji VK, Padhy B, Sujata S, Das S, Alone DP. Analysis of ZEB1 contribution towards the pathogenesis of Fuchs' endothelial corneal dystrophy. Euroscicon Event: Aging 2016; 21, London, UK.

ZEB1 contribution towards the pathogenesis of Fuchs' endothelial corneal dystrophy. Euroscicon Event: Aging 2016; 21, London, UK.

2. **Nanda GG**, Padhy B, Samal S, Das S, Alone DP. Genetic association of *TCF4* intronic polymorphisms, CTG18.1 and rs17089887 with Fuchs' endothelial corneal dystrophy in Indian population. International Symposium on Genetic Analysis: Translational and Developmental (iNSGTD) 2014,112, Burdwan.

Signature of the Student:

Date: 04/05/2017

Doctoral Committee:

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5	Dr. Sujata Das	Member, External		4/5/17

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Glossary of Abbreviations

8-OHdg	8-Oxo-2'-deoxyguanosine
ADD	additive model
AGBL1	ATP/GTP binding protein-like 1
ARE	antioxidant responsive element
ARNSHL	Autosomal recessive non-syndromic sensorineural hearing loss
ATP1B1	Na+, K+ transporting ATPase, beta-1 polypeptide
Bim	BCL2 Like 11
BS	Blocking solution
BSA	bovine serum albumin
BTR1	Bicarbonate transporter related protein 1
Cdk	Cyclin-dependent kinase
CDKN1A	Cyclin Dependent Kinase Inhibitor 1A
CDPD	Corneal dystrophy and perceptive deafness
CEn	Corneal Endothelial cells or corneal endothelium
CHB	Chinese Han population residing in Beijing
CHED	Congenital hereditary endothelial dystrophy
CHOP	C/EBP-homologous protein
CI	Confidence interval
CLU	Clusterin
COL4A1	Collagen Type IV Alpha 1 Chain
COL8A2	Collagen type VIII, alpha 2 chain
CTCF	Corrected total cell fluorescence

CTG	Cytosine-Thymine-Guanine
DAPI	4',6-diamidino-2-phenylindole
dbSNP	Database of single nucleotide polymorphism
Df	Degree of freedom
DJ-1	alias, PARK7 Parkinsonism Associated Deglycase
DLEK	Deep lamellar endothelial keratoplasty
DM	Descemet's membrane
DM1	Myotonic dystrophy type 1
DMEK	Descemet Membrane Endothelial Keratoplasty
DMEM	Dulbecco's Modified Eagle's Medium
DN	Double negative
DNA	Deoxyribonucleic acid
DOM	Dominant model
DSEAK	Descemet stripping (automated) endothelial keratoplasty
DSEK	Descemet's Stripping Endothelial Keratoplasty
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic
eIF2α	Eukaryotic initiation factor 2α
EMSA	Electromobility Shift Assay
EMT	Epithelial-mesenchymal transition
EO-FECD	Early onset Fuchs Endothelial Corneal Dystrophy
ER	Endoplasmic reticulum
ERK	Extracellular signal-related kinases

Ero1	Endoplasmic Reticulum Oxidoreductase
EST	Expressed sequence tags
FECD	Fuchs Endothelial Corneal Dystrophy
FS-DSEK	Femtosecond Descemet's Stripping Endothelial Keratoplasty
Gadd34	Growth Arrest And DNA Damage-Inducible 34
GIH	Gujarati Indians from Houston
GWAS	Genome wide association studies
H2O2	Hydrogen peroxide
HCEnCs	Human Corneal Endothelial Cells
HEK293	Human embryonic kidney cells 293
HLOD	Heterogeneity logarithm of the odds
HMOX-1	Heme oxygenase-1
HRP	horseradish peroxidase
IC3D	International Committee for Classification of Corneal Dystrophies
IHC	Immunohistochemistry
KANK4	KN motif- and ankyrin repeat domain-containing protein 4
Keap1	Kelch-like ECH-associated protein 1
LAMC1	Laminin gamma-1
LD	Linkage disequilibrium
LHON	Leber's hereditary optic neuropathy
LMP	low melting point
Ln-OTM	natural log of Olive tail moment
LO-FECD	Late onset Fuchs Endothelial Corneal Dystrophy

LOD	Logarithm of the odds
LOXHD1	Lipoxygenase homology domain 1
LSM	Laser scanning microscope
MAPK	Mitogen activated protein kinase
MBNL1	Muscleblind Like Splicing Regulator 1
MBNL2	Muscleblind Like Splicing Regulator 2
MCT	Monocarboxylate transporter
MEK	MAP-kinase
$MgCl_2$	Magnesium Chloride
MIM	Mendelian Inheritance in Man
MQ	Milli Q water
mRNA	messenger RNA
mtDNA	Mitochondrial DNA
NEB	New England Biolabs
NFW	Nuclease free water
NHE	Na(+)/H(+) Exchanger 1
NMP	normal melting point
Nrf2	Nuclear factor erythroid 2–related factor 2
OR	Odds ratio
PBS	Phosphate buffer saline
PBST	Phosphate buffer saline with triton X-100
PCI	Phenol: Choloroform: Isoamyl Alcohol
PCR	Polymerase Chain Reaction

PK	Penetrating Keratoplasty
PLK	posterior lamellar keratoplasty
pNBCe1	pancreatic type Na+-HCO3- cotransporter
PPCD	Posterior polymorphous corneal dystrophy
RBC	Red-blood cells
REC	recessive model
RFLP	restriction fragment length polymorphism
RNA	Ribonucleic acid
RNAi	inhibitory RNA
ROCK	Rho-kinase
ROS	Reactive Oxygen Species
RT	Room temperature
SAGE	Serial Analysis of Gene Expression
SD	Standard deviation
SIFT	Sorting Intolerant from Tolerant
SIPS	Stress induced premature senescence
SLC4A11	Solute Carrier 4
SNAI2	Snail Family Transcriptional Repressor 1
SNP	Single nucleotide polymorphism
SPSS	Statistical Package for the Social Sciences
STR	Short tandem repeats
TBE	Tris-borate EDTA
tbMO	translational blocking morpholino

TCF4	Transcription factor 4
TCF8	Transcription factor 8
TEM	Transmission electron microscopy
TGFβ1	Transforming growth factor beta 1
TNR	Trinucleotide Repeats
TP-PCR	Triplet repeat primed- polymerase chain reaction
Trb3	Tribbles Pseudokinase 3
Tris-HCl	Tris Hydrochloride
TUNEL	Terminal deoxy-nucleotide transferase dUTP nick end labelling
UPR	Unfolded protein response
UV	Ultraviolet
WT	Wild type
ZEB1	Zinc Finger E-Box Binding Homeobox 1

Chapter 1

A comprehensive knowledge about

Fuchs' Endothelial Corneal Dystrophy

1.0 FUCHS' ENDOTHELIAL CORNEAL DYSTROPHY

1.1 INTRODUCTION

Decreased visual acuity, a common disorder of aging accompanied by progressive deterioration of corneal endothelial cells and formation of corneal guttae characterizes Fuchs' Endothelial Corneal Dystrophy (FECD, MIM:136800). The inheritance pattern of this disease is regarded as both autosomal dominant and complex;[17, 34-36] although women seem to be the predominantly affected group.[2, 34, 35, 37] The primary cause of the disease is unknown but clinical detection includes specular microscopic examination to ascertain endothelial cell loss and formation of bullae in the Descemet's membrane (DM) of the corneal endothelium (**Figure 1.1**).[2, 38, 39] This endothelial cell loss increases cellular stress resulting in excrescences from the Descemet's membrane.[40, 41] Thickened DM[38, 42] due to abnormal deposition of collagen[42, 43] consequent into failure of primary function of the endothelial membrane, which is to maintain corneal deturgescence (**Figure 1.1**).[3] With 42,000 corneal transplantations taking place every year in the United States to treat the edematous cornea that is associated with FECD, the disease raises serious concern when corneal edema resurges after a cataract surgery.[4] FECD is also the third most frequent reason for corneal transplantation in India and the affected individuals have to overcome their socio-economic limitations to undergo any surgical interventions to treat the disease.[5] Therefore, early stage FECD diagnosis and perceivability of the disease pathomechanism have become absolute necessity for numerous patients undergoing refractive or cataract surgery every year.

Two kinds of FECD manifestations are reported so far, namely early onset (EO-FECD) and late onset FECD (LO-FECD) depending on the age of onset of the disease. EO-FECD is reported in individuals between the age bracket of 3 to 35 years of age, whereas LO-FECD is seen in individuals older than 35 years. There are distinct clinical differences also recorded for both

these types. [7, 10, 40, 44] Thicker DM (upto 35µm) with deeper, discontinuous corneal guttae in case of EO-FECD and comparatively less thickened DM (upto 17µm) with shallower, continuous guttae are representative of LO-FECD (**Figure 1.1**).[10] In comparison, the mean central thickness DM of normal elderly cornea measured *in vivo* under ultra-high resolution optical coherence tomography is 8-10µm.[45] EO-FECD is therefore considered the more severe form of disease. Its occurrence is rarer and inherited solely in individuals with familial predisposition; in contrast to the more frequent and sporadic inheritance pattern of LO-FECD.

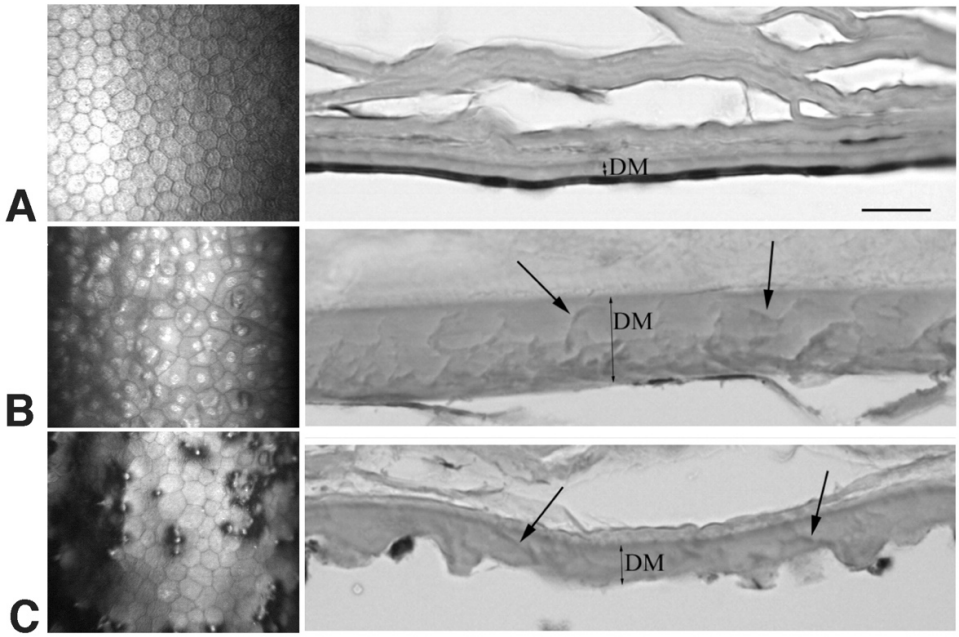


Figure 1.1: Comparative images of normal and FECD affected corneal endothelium. Specular microscopy images (first column) of healthy (A), early onset FECD (B) and late onset FECD (C) corneal endothelium shows excrescences on Descemet's membrane (DM) called guttae; the clinical hallmark of FECD. Adjoining column of images are bright field confocal micrographs highlighting the thickened DM in early (B) and late onset FECD (C) as compared to normal (A). Arrow marks indicate the wrinkle like structures in EO-FECD and refractile structures in LO-FECD formed due to aberrant collagen depositions; bar = 20µm. These images are adapted from Bennet 2006[46] and Gottsch *et al.* 2005.[7, 10]

1.2 Prevalence:

1.2.1 International Scenario:

Large-scale prevalence studies suggest high incidence of FECD in European countries as compared to other areas of the world. Studies done by Santo *et al.* in 1995 report extremely low incidence of this disease in Japanese population.[47] Kitigawa *et al.* (2002) carried out a comparative study between two Asians groups, i.e. Chinese Singaporeans (n=465) and Japanese (n= 266) comprising participants of age group ≥ 50 years by diagnosing endothelial dystrophies with slit lamp biomicroscopy and specular microscopy. They concluded that higher incidence of FECD was seen in Singaporeans (men 4.4% and females 8.5%) than the Japanese (men 1.5% and females 5.5%); with females dominating the affected group in both the ethnicities[48] The second study carried out in 2001 known as the "Reykjavik Eye Study" adopted similar techniques and suggested a higher FECD prevalence among Icelanders (n=846) than the Japanese or Singaporeans; by quantifying the sex ratio of affected individuals as 11% females and 7% males having prominent guttae.[49] Prevalence studies of FECD were also extended to Tangier Islands, Virginia, USA - a state with a population of 700 (as of 2011). This group used slit lamp biomicroscopy, retro-illumination photographs and genotyping to ascertain the disease category individuals amongst 156 participants. The investigation revealed maximum prevalence of 21% of the milder form of disease i.e., LO-FECD.[50] A familial study in USA revealed that 4% of the 228 relatives examined had corneal endothelial dystrophy with edema.[51] All the above studies with patients of LO-FECD declare female dominance for the affected individuals. The penetrance and sex ratio analysis of FECD remains unanswered because of the late onset nature of the disease and scarcity of full pedigrees of living patients with FECD.

1.2.2 Indian scenario:

Statistical data comparing the prevalence of FECD in India and other parts of the world remain elusive; although a study group from an eminent tertiary eye care center in South India conducted a frequency distribution and outcome analysis of patients requiring penetrating keratoplasty (PK) as a surgical intervention for relief from ocular dystrophies; and FECD accounted as the third most frequent (16.6%) reason for PK, preceded by the leading causes like congenital hereditary endothelial dystrophy (CHED, 34.8%) and macular dystrophy (29.3%).[5] Another group could recruit 80 FECD patients in the Tamil Nadu province (a state in South India) alone, for a genetic screening analysis.[9] Similar studies were carried out outside India, comprising Indians residing in a particular geographical region; such as Vithana *et al*. carried out genetic analysis with 89 FECD patients residing in Singapore, amongst which 25 were Indians.[28]

1.3 Risk Factors:

Recognizing the risk factors for FECD will be greatly beneficial for the patients and clinicians to avert or delay the advancement of disease. Effect of UV radiations to exacerbate the disease condition cannot be stated with concrete evidence; although Kitigawa *et al*. speculated a higher prevalence of FECD in Singapore (with tropical climate, located 1°N) relative to Japan (temperate climate, located 37°N) is most likely because of increased exposure to UV due to its proximity to the equator.[48] However, UV exposure studies carried out in Reykjavik, Iceland (temperate, located 64°08'N) do not ascertain towards collateral increase in corneal guttata formation and UV exposure. They performed a risk factor analysis and found that smoking 20 packs of cigarettes per year was linked (P= 0.013) with a two-fold increased risk, whereas higher bodyweight and BMI lowered the risk of developing corneal guttata.[49]

1.4 Genetics:

1.4.1 Chromosomal loci

At least five FECD susceptibility genomic loci 1p34.3-32, 13q12.11-q12.13, 18q21.2-q21.32, 5q33.1-q35.2 and 9p24.1-22.1 have been identified by extensive multigenerational familial linkage studies with an autosomal dominant nature of heritability.[15-19, 52] Two FECD-associated genes, collagen type VIII, alpha 2 chain (COL8A2) and Transcription factor 4 (TCF4) fall under the susceptibility loci 1p34.3-32 and 18q21.2-q21.32 respectively .[10, 15, 19] Concrete association of a particular gene in other three loci i.e. 13q12.11-q12.13, 5q33.1-q35.2 and 9p24.1-22.1 are yet to be deduced.[16, 17] On the basis of the order of discovery and manifestations as LO-FECD, these loci have been categorized by the International Committee for Classification of Corneal Dystrophies (IC3D) classification system as FECD1- 13pTel-13q12, FECD2- 18q21.2-21.32, FECD3- 5q33.1-q35.2, and FECD4- 9p24.1-22.1.[52] Magovern *et al*., (1979) identified the locus 1p34.3-32 in late 20th century and observed autosomal dominant nature of inheritance in the large family group under study.[34] But the pathogenic identification of the disease followed suit of a rare form of FECD i.e. EO-FECD, and is therefore not considered as a formal FECD loci. A significantly higher frequency of COL8A2 sequence alternations in FECD subjects compared with controls substantiated the contribution of this gene to the development EO-FECD through various studies.[7, 8, 17, 53-57] A comprehensive list of all the associated chromosomal loci are tabulated in **Table 1.1** (**Pages 18-22**).

Afshari *et al*. (2009) carried out a genome-wide linkage analysis using parametric two-point linkage analysis and nonparametric multipoint linkage analysis with many small families.[53] The linkage scan generated five regions with signals from chromosomes 1, 7, 15, 17 and X; which might carry potential candidate genes to add up to the pool of genes afflicted for FECD. The study group consisted of 22 multiplex families, which consisted mostly of small pedigrees and

therefore lacked a broader perspective to uncover other established linked regions of FECD. The results reflected both autosomal dominant and complex pattern of inheritance.[53] The following genes have been genetically associated with FECD.

1.4.2 *COL8A2*

COL8A2 falls under the susceptibility region, 1p34.3-32, which is adjacent to the linked chromosomal loci on 1p reported by Afshari *et al*.[53] This locus was first identified by linkage analysis of four multigenerational EO-FECD pedigrees.[55] Pathogenic mutations on the 703 amino-acid long α2 collagen type VIII encoding COL8A2 have been implicated with the rare form of FECD, EO-FECD and validated by various multigenerational familial studies. [7, 8, 17, 53-57] Strong candidate mutations, L450W and Q455K, which are positioned in the triple helical domain of α2, consequent upon structurally, altered DM.[7, 8, 55] FECD patients from English ethnicity showed R155Q, R304Q, and R434H missense mutations[7] out of which, R155Q along with T502M may not be pathogenic to cause FECD as they occurred both in Japanese control and patient populations as reported by Kobayashi *et al*.[56, 57] These mutations however failed to co-segregate with the American cohort of both LO- and EO-FECD familial cases.[54] Q455K mutation on the other hand, has been potentiated to be highly penetrant (100%) and pathogenic to cause EO-FECD in both English and Korean populations.[8, 55] Homozygous mutant (Q455K/Q455K) mouse knock-in model for EO-FECD demonstrate endothelial guttae, aberrant collagenous deposition and swollen ER indicating activated UPR and apoptosis.[58] On the basis of biological importance that Collagen type VIII alpha 2 is highly expressed in human cornea DM, the gene *COL8A2* is a favored candidate for FECD. Mutations and polymorphisms were not identified in LO-FECD cases, suggesting genetic involvement of COL8A2 to be exclusive for early onset cases (**Figure 1.2**).[9, 54] However, Gottsch *et al*., have elaborately compared the patterns of abnormal collagen distribution in DM of EO- and LO-FECD affected tissues.[10]

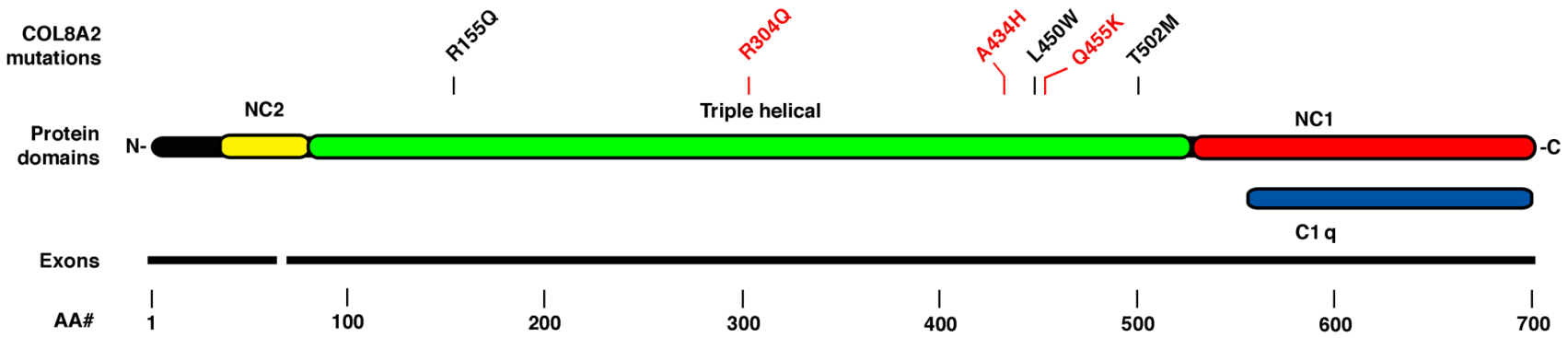


Figure 1.2: Mutations in COL8A2 gene associated with EO-FECD. Mutations marked in red are deemed pathogenic by Biswas *et al.*, 2001[55].

These reports suggest that although there isn't a genetic involvement, but a de-regulation of COL8A2 expression could consequent into LO-FECD.

1.4.3 *SLC4A11*

FECD has near resemblance in disease manifestations with other endothelial (posterior) corneal dystrophies like congenital hereditary endothelial dystrophy (CHED, MIM: 121700) and posterior polymorphous corneal dystrophy (PPCD, MIM:122000).[52, 59] Therefore various researchers hypothesize that some portion of clinical manifestations of these corneal dystrophies share different modifications of similar genes.[60, 61] SLC4A11 is a member of Solute Carrier 4 (SLC4) borate transporter family of proteins.[62] It is the only member that is reported for Na^+-coupled H^+ flux and OH^- transport, unlike the other borate transporter members of the same family.[63] Mutations in this protein are reportedly inherited in individuals affected with corneal dystrophies like CHED,[64] FECD [61] and a hearing disorder, Harboyan's syndrome.[60] Disease co-segregating missense mutations in SLC4A11 either cause malfunctioned transporter or retain mutant proteins in the ER thereby leading to insufficient efflux of water molecules and subsequent edematous cornea; a symptom common in CHED and FECD cases.[28, 64-70] Four likely loss-of-function mutations in SLC4A11 were uncovered by Riazuddin *et al.* after screening 86 sporadic Chinese and Indian descent patients; contributing upto 3-4 % of the genetic load to cause such sporadic cases of FECD (**Figure 1.3**).[28, 61] Recent evidences on methylation status of FECD affected cornea demonstrate hypermethylated promoter regions of SLC4A11, which corroborate with the Serial Analysis of Gene Expression (SAGE) data that indicate a downregulation of this transporter protein in FECD in comparison to healthy corneas.[71]

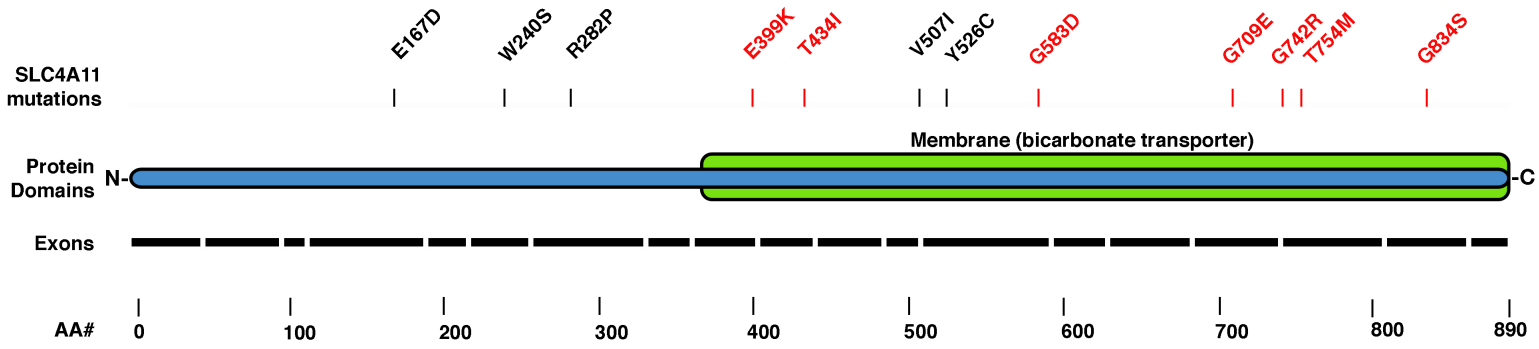


Figure 1.3: Mutations in SLC4A11 gene associated with FECD. Mutations in red are the loss of function mutations reported by Riazuddin *et al*. 2010.[61]

1.4.4 *ZEB1*

Similar to SLC4A11, mutations in ZEB1 (Zinc finger E- Box binding homeodomain) were also linked to closely related corneal dystrophies. Nonsense mutations or mutations resulting in premature termination of ZEB1 were associated with a severe form of corneal dystrophy- posterior polymorphous corneal dystrophy, type 3 (PPCD 3).[18, 22, 72, 73] But Mehta *et al.* studied this gene in Chinese population and associated it with the milder form of corneal dystrophy, i.e. LO-FECD, comprising of a missense (N696S) and a silent (D64D) mutation in exonic regions of *ZEB1*; both of which were identified as non-pathogenic through SIFT (Sorting Intolerant From Tolerant) and Polyphen analysis.[74] Riazuddin *et al.* later also identified five missense mutations in the American cohort of FECD probands; all of which identified as potentially pathogenic and sufficient to cause FECD.[18] One particular mutation, Q840P, was analyzed to be co-segregating with FECD4 locus at chromosome 9 in a multi-generational family, causing increased severity in 7/12 FECD affected individuals.[18] Nonsense mutations or mutations causing immature termination of ZEB1 were identified in PPCD[22, 75] in contrast to only missense mutations of ZEB1 in FECD cases (**Figure 1.4**).[18, 74, 76, 77] These studies suggest that PPCD and FECD could be allelic variants of a continuum of diseases in which genes interact to modulate the expressivity of the phenotype.[18]

1.4.5 *TCF4*

TCF4, that encodes for E2-2 protein – a group of E protein transcription factors known for cellular growth and differentiation, is the only gene to be extensively studied in FECD cases from varying ethnic backgrounds. Baratz *et al.* first identified its association with FECD by performing a genome-wide association study (GWAS) among subjects from European descent.[13] The group found four single nucleotide polymorphisms (SNPs) in the noncoding regions of the E2-2 encoding gene, strongly associated to FECD phenotype. Yi- Ju Li *et al.*

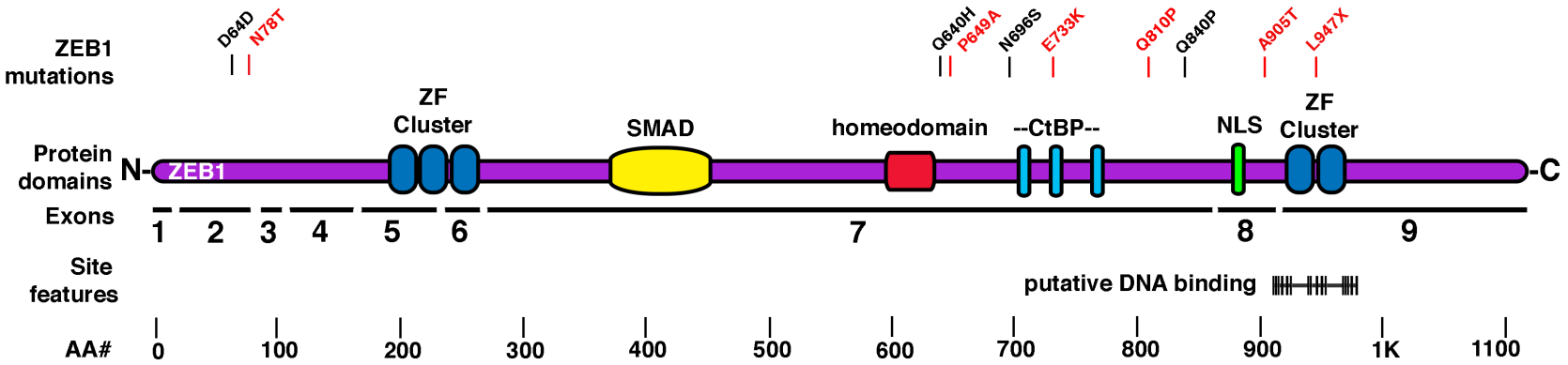


Figure 1.4: Mutations in ZEB1 gene associated with FECD. Mutations depicted in red are intolerant as identified by SIFT and PolyPhen analysis by Mehta *et al*., 2008[74], Riazuddin *et al*. 2010[18], and Gupta *et al*. 2015[76].

group concreted the association by conducting genome-wide linkage scan and association with 64 multiplex American families. They narrowed down the linkage region between 69.94cM to 85.29cM on Chromosome 18, which coincides with FECD2 locus. They also found, the intronic SNP, rs613872 identified by Baratz *et al*., to be proximal to the marker SNP, rs1145315 having peak multipoint heterogeneity LOD (HLOD) score of 2.5 on the linked locus.[19] Recent advances in this field proved major contributions of TCF4 polymorphism, rs613872, to be predominantly associated among populations in Iowa, USA.[78] These studies have thus consistently shown that the TCF4 variant, rs613872 confers strong association with FECD and is partially penetrant in European,[13] American[50, 78] and Australian[79, 80] ethnicity. For not being polymorphic at rs613872 among Chinese descent individuals, instead of rs613872, two other polymorphisms, rs17089887 and rs17089925 which are in close linkage disequilibrium (LD) with each other were found to be associated with FECD (**Figure 1.5**).[20] In subsequent studies, it was observed that a microsatellite region comprising of CTG (trinucleotide) repeats in the intronic region of TCF4 gene was abnormally expanded and segregated mostly among FECD patients. This genetic signature for FECD is most prominent in Caucasians with 80% penetrance,[21, 81] followed by Germans (77%)[82] and Asians (43% Chinese[83], 34% Indian[84]).

Reports suggest that post-translationally, these repeats sequester proteins involved in mRNA splicing machinery which consequent into RNA foci formation and accumulation of mis-spliced transcripts.[30, 85] Homozygous and heterozygous individuals with expanded repeats did not show any differences in severity on the grounds of comparison of Krachmer grade, Keratoplasty proportion or central corneal thickness (CCT).[86] It was also reported that CTG repeat length positively correlated with Krachmer severity grade for FECD.[86] However, it is worth noting that because the penetrance of this genetic signature is not 100% among FECD affected individuals and only a small number of healthy individuals (5%) [21, 81, 84, 86] harbour these expanded repeats,

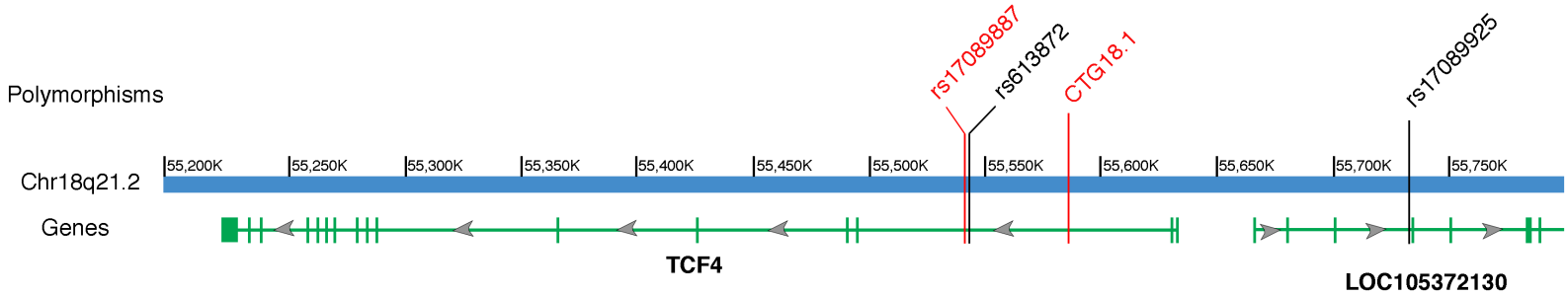


Figure 1.5: Polymorphisms in TCF4 gene associated with FECD. Polymorphisms in red are associated with FECD in India.[84] This figure is modified from https://www.ncbi.nlm.nih.gov/gene/6925) .

it can be stated that in conjunction with other factors yet unknown, the expanded repeats are sufficient to cause FECD but not absolutely necessary for the disease condition.

1.4.6 Other genes associated with FECD

Along with TCF4, association of Clusterin (CLU) and Transforming growth factor beta 1 (TGFβ1) with FECD in Caucasian Australian population was done by performing SNP analysis of these genes.[79] The result was supported by differential expression of TGFβ1 and CLU in FECD patients as identified by immunohistochemical (IHC) studies.[79] This study was carried out on the basis of proteomic analysis of TGFβIp and Clusterin done by Jurkunas *et al.* group that confirmed their overexpression in FECD cornea, claiming major contributions of these proteins in aberrant cell- extracellular matrix interactions seen in guttata of FECD patients.[87] Mutations in a glutamate decarboxylase gene, *ATP/GTP binding protein-like 1 (AGBL1)* were identified in Caucasian American cohort with a nonsense mutation (p.Arg1028*) resulting in nuclear localization of the truncated protein and a variant resulting in missense mutation p.Cys990Ser in the AGBL protein.[12] Biochemical analysis suggested specific binding of AGBL1 with TCF4 and not with ZEB1. However, the mutant AGBL1 variants showed led to dissociation with *TCF4*; suggesting a possible combined effect delivered by these two proeteins in the pathogenesis of FECD.[12]

Gottsch *et al*. enlisted the up-regulated and down-regulated genes in the corneal endothelium of FECD patients based on SAGE data analysis.[88] The expression studies elicited the role of secondary phenotypic manifestation of the disease, which arises due to activation of reactive oxygen species (ROS) pathway in the endothelial cells under stress. Decreased transcript level of anti-oxidant glutathione S-transferase-pi, nuclear ferritin, heat shock 70-kDa protein and increased level of nitrotyrosine, a ROS byproduct goes in accordance with the hypothesis. Other

downregulated transcripts picked up by SAGE included the bicarbonate transporter gene, Bicarbonate transporter related protein 1 (BTR1) alias SLC4A11, 26 known genes, 3 expressed sequence tags (ESTs) and 7 unknown at the time of study.[88] Amongst the upregulated ones included, serum amyloid A1 and A2, metallothionein, and apolipoprotein D.[88] Another study recently reported the methylation status of FECD affected corneal endothelial tissue and confirms the downregulation of membrane transport proteins on the grounds of hypermethylated signatures.[71] Both these studies corroborate on the fact that SLC4A11 is downregulated along with miR199b in FECD affected tissues, highlighting that failure of water and ion transport mechanism might be the leading cause of FECD pathogenesis.

A novel candidate gene, lipoxygenase homology domain 1 (LOXHD1) was enlisted in the repertoire of FECD afflicted genes, recently reported by Riazuddin *et al.*[36] The group reported 15 heterozygous missense mutations in LOXHD1 among the 200 sporadic cases they studied. LOXHD1 becomes a more fascinating candidate for FECD because it falls under the FECD2 locus and is recently linked to a progressive form of hearing degeneration - Autosomal recessive non-syndromic sensorineural hearing loss (ARNSHL).[89, 90] So far two FECD afflicted genes have been associated to hearing loss disabilities, namely SLC4A11[60, 69, 91, 92] and LOXHD1,[90, 93-95] both causing sensorineural hearing loss, although the pathomechanism of incursion is different. SLC4A11, which is expressed in the inner ear, is concomitant to Harboyan's Syndrome, a condition for corneal dystrophy and perceptive deafness (CDPD).[60] Of late, FECD has been statistically linked to hearing disability among the patient group (45.8%) in their late sixties as opposed to their age matched control (34.7%).[96] The heterogeneous nature of FECD inheritance makes it difficult to extrapolate the causal mechanism of pathogenesis and is therefore hypothesized that a sub-group of FECD could be implementing its incursion along with a second trait of hearing disability among the patients.[96]

In a recent report, Afshari and group conducted a GWAS with an enormous sample size of 1,404 FECD cases and 2,564 controls from European ancestry, and identified three novel loci, rs79742895 (KANK4, KN motif- and ankyrin repeat domain-containing protein 4), rs3768617 (LAMC1, laminin gamma-1) and rs1200114 (LINC00970/ATP1B1, Na^+, K^+ transporting ATPase, beta-1 polypeptide) linked with FECD.[14] These identified loci do not coincide with those previously reported and are novel genes (FECD 1-4) whose contribution to disease mechanism needs to be explored (**Table 1.1**). The associated genes are functionally involved in maintaining corneal detergence by regulating routine fluid transport, intercellular contact and tissue integrity. Improper functioning of these activities would lead to accumulation of FECD-like symptoms. This study has expanded the knowledge of various factors that can contribute to FECD pathogenesis and advocates continued search of novel therapeutics to disable the disease progression.

1.5: FECD- a mitochondrial disease?

The terminally differentiated human corneal endothelial cells are actively involved in pumping aqueous humor and solutes to maintain corneal hydration and transparency. This requires remarkably higher number of mitochondria in corneal endothelium than other ocular tissues. Initial reports of indicating FECD as a mitochondrial disease came from a particular case study carried out by Roger L. Albin in 1998 that reported a woman aged 48 years was diagnosed with symptoms like cardiac conduction defects, ataxia, hyperreflexia, sensorineural hearing loss and FECD.[97] Mitochondrial DNA analysis from lymphocytes reflected missense mutations in cytochrome b subunit of complex III at mt15257 (G to A, aspartate to asparagine) and ND1 subunit of complex I at mt4216 (T to C, tyrosine to histidine).[97] Mutations in these subunits which are reported to be implicated to Leber's hereditary optic neuropathy (LHON) [98, 99] and the peculiar symptoms similar to that of LHON,[100, 101] exhibited by the patient tempts to associate

Table 1.1 Comprehensive list of chromosomal loci and the genetic variations associated with FECD

Chromosomal loci	Genes associated	Gene Function	Study Design	Study Population	Sequence change	Reference	Pathogenicity
1p31.3	**KANK4**	KN Motif And Ankyrin Repeat Domains 4	GWAS	American[14]	rs79742895	Afshari *et al*. 2017	
1p34.3	**COL8A2**	major component of the basement membrane of the corneal endothelium	Familial linkage analysis	English[55]	R304Q	Biswas 2001	presumed pathogenic
			Familial linkage analysis	English[55], American[54]	A434H	Biswas 2001, Aldave 2006	presumed pathogenic
			Familial linkage analysis	English[55], Korean[8]	Q455K	Biswas 2001, Mok *et al*. 2009	presumed pathogenic
			Familial linkage analysis, Candidate gene study	English[55], Korean[8], Chinese[11], Japanese[56]	R155Q	Biswas 2001, Kobayashi *et al*. 2004, Mok *et al*. 2009, Tang *et al*. 2016	polymorphic, Pathogenic for UK
			Candidate gene study	Korean[8], Japanese[56]	Q455V	Mok *et al*. 2009, Kobayashi *et al*. 2004	pathogenic
			Candidate gene study	Korean[8], Japanese[56], Chinese[11]	T502M	Mok *et al*. 2009, Kobayashi *et al*. 2004, Tang *et*	polymorphic, non-pathogenic

						al. 2016	
			Candidate gene study	American[7]	L450W	Gottsch *et al*. 2005,	
1q24.2	**ATP1B1**	ATPase Na+/K+ Transporting Subunit Beta 1	GWAS	American[14]	rs1200114	Afshari *et al*. 2017	
1q25.3	**LAMC1**	Laminin Subunit Gamma 1	GWAS	American[14]	rs3768617	Afshari *et al*. 2017	
1q31.3	**CFH**	Complement factor H	GWAS	American[14]	rs2274700	Afshari *et al*. 2017	
5q33.1-q35.2	No genes associated yet		Familial linkage analysis	Caucasian American[17]		Riazuddin *et al*., 2009	
9p24.1-22.1	No genes associated yet		Familial linkage analysis	Caucasian American[18]		Riazuddin *et al*., 2010	
10p11.22	**ZEB1**	zinc finger transcription factor	Candidate gene study	Chinese[74]	N696S	Mehta *et al*., 2008	presumed pathogenic
			Candidate gene study	Chinese[74]	D64D	Mehta *et al*., 2008	tolerated
			Candidate gene study	American[18]	N78T	Riazuddin,2010	pathogenic

			Candidate gene study	American[18]	P649A	Riazuddin,2010	pathogenic
			Candidate gene study	American[18]	Q810P	Riazuddin,2010	pathogenic
			Candidate gene study	American[18]	A905T	Riazuddin,2010	pathogenic
			Candidate gene study	American[18], Indian[76]	Q840P,	Riazuddin,2010, Gupta *et al*. 2015	tolerated
			Candidate gene study	Indian[76]	E733K	Gupta *et al*., 2015	Damaging
			Candidate gene study	Indian[76]	L947X	Gupta *et al*., 2015	Damaging
			Candidate gene study	Indian[76]	A818V	Gupta *et al*., 2015	tolerated
			Candidate gene study	Indian[76]	S234S	Gupta *et al*., 2015	tolerated
13q12.11-q12.13	No genes associated yet		Familial linkage analysis	Caucasian American[16]		Sundin *et al*., 2006	
18q21.2-q21.32	TCF4	Transcription factor	Candidate gene studies and GWAS	Caucasian American[13, 14, 19], European[82, 102],	rs613872	Baratz *et al*. 2010, Li YJ *et al*. 2011, Nanda	

				Indian[76, 84]		*et al*. 2014, Gupta *et al*., 2015, Oldak *et al*. 2015, Afshari *et al*. 2017, Foja *et al*. 2017	
20p13	**SLC4A11**	electrogenic sodium-coupled borate cotransporter, sodium-mediated fluid transport	Candidate gene study	Singaporean[28], Chinese[28], Indian[28, 103]	E399K	Vithana *et al*., 2008, Soumittra *et al*, 2014	tolerated, pathogenic
			Candidate gene study	Singaporean[28], Chinese, Indian	G709E	Vithana *et al*., 2008	pathogenic
			Candidate gene study	Singaporean, Chinese, Indian[28]	T754M	Vithana *et al*., 2008	pathogenic
			Candidate gene study	Singaporean, Chinese, Indian[28]	c.99-100delTC	Vithana *et al*., 2008	pathogenic
			Candidate gene study	American[61]	E167D	Riazuddin *et al*., 2010	benign
			Candidate gene study	American[61]	R282P	Riazuddin *et al*., 2010	pathogenic
			Candidate gene study	American[61]	Y526C	Riazuddin *et al*., 2010	benign
			Candidate gene study	American[61]	G583D	Riazuddin *et al*., 2010	pathogenic
			Candidate gene	American[61]	G742R	Riazuddin *et al*.,	pathogenic

			study			2010	
			Candidate gene study	American[61]	G834S	Riazuddin *et al*., 2010	pathogenic
			Candidate gene study	Indian[103]	W240S	Soumittra *et al*, 2014	tolerated
			Candidate gene study	Indian[103]	T434I	Soumittra *et al*, 2014	damaging
			Candidate gene study	Indian[103]	V507I	Soumittra *et al*, 2014	tolerated

FECD as a mitochondrial disease. Another report suggested decreased cytochrome oxidase activity in decompensated corneal endothelium of FECD and predicted a decreased number of mitochondria and/or metabolic activity.[104] Supporting these lines of hypothesis, recent studies have shown that cultured corneal endothelial cells from FECD patients harbor decreased mitochondrial mass[105], increased copy number of mtDNA and an increased ratio of 4977 mtDNA deletion as compared to control cells.[106] These conditions consequently decreased the mitochondrial sensitivity and repair of H_2O_2 induced damages.[106] Downregulated mitochondrial ESTs identified in SAGE analysis of FECD cornea substantiates that an energy-dependent endothelial dysfunction can be a plausible mechanism for FECD.[88]

1.6 Interpreting the Mechanism:

FECD has been shown to have an autosomal dominant inheritance since its initial documentation by Ernst Fuchs in 1910.[1] But lately, researchers quarrying deep into the inheritance and pathomechanism, have come up with a complex pattern of its incursion. Current scenario depicts FECD as a heterogeneous disease with a peculiar trait of corneal guttata formation and excrescences from the DM occurring in the fourth to fifth decade of life. On further progression, the affected person acquires a decreased visual acuity due to endothelial cell loss and corneal edema that finally lead to painful epithelial bullae formation. Recently, it is brought into light that FECD is co-pathogenizing with hearing loss.[36, 60, 96] Deciphering the mechanism of disease progression behind these traits is becoming clearer but yet not absolute.

1.6.1 Corneal deturgescence

One of the characteristic traits of FECD is corneal edema that comes into effect as the disease progresses. This characteristic can be explained on the basis of barrier dysfunction of the endothelial cells.[107] Aqueous humor that baths the cornea contains solutes and nutrients to be

selectively transferred among various cell layers. Endothelial cells host a selective barrier allowing leakage of nutrients and channeling them to avascular cornea.[108] Corneal endothelium is essentially functional to maintain normal corneal hydration, thickness and transparency. Corneal stroma has a tendency to imbibe water which flows through the endothelium driven by the intraocular pressure. Thus acting like a hydrogel, corneal stroma has to strike a balance between water imbibing and pumping out to avoid stromal swelling. This regulation is maintained by swelling pressure isotherm.[109, 110] When this isotherm is disturbed, stroma gorges un-quantified amount of aqueous humor, thereby increasing the inter-collagen-fibrillar distance and leading to corneal opacity. A healthy stroma has a counter-active mechanism to deal with this uncontrolled swelling; as explained by Maurice *et al,*[111-113] the "pump leak system" of the endothelium. Presence of active ionic pumps housed in the endothelium; namely Na-K ATPase pumps, relatively leaks out aqueous humor when it senses the increased stromal pressure by lowering osmolarity at the basolateral endothelium.[111, 112] Deturgescence is disturbed if either the pumps or the barrier are dysfunctional which will lead to corneal endothelial cell loss and consequent edema of the cornea. McCartney *et al.* evidenced sharp decrease of Na-K ATPase pump site density on the corneal endothelium of the FECD patients in the late phases of disease progression as opposed to high density in the early phases of the disease.[114, 115] In concise, stressed corneal endothelium undergoes cell loss as a result of which it tries to compensate by increasing the density of pumps and barrier in the remaining cells to maintain the state of deturgescence. However, as the decay ensues, the remaining endothelial cells markedly reduces the pumps and barrier numbers resulting in corneal edema.

1.6.2 Early Onset (variant) Fuchs' Dystrophy

Clinical features more easily comprehend when it comes to COL8A2 mutations that are ascribed to cause a rare form of the disease, i.e. EO-FECD.[55] Collagen type VIII is a non-

fibrillar extracellular matrix material predominantly expressed in the corneal endothelium and forms homotrimers in the DM, consisting of either alpha 1 or alpha 2 chains of type VIII collagen.[116, 117] In an infant normal cornea, banded fibrils of the anterior zone of DM are the thickest and apparently maintain the thickness thereafter.[118] But in an endotheliopathic cornea, the DM secrets additional abnormal layers posterior to the normal banded layer which reflects as wart-like excrescences. Gottsch *et al*. carried out a comparative study to elucidate the histopathological changes in EO- and LO-FECD cornea in contrast to normal cornea.[10] Key revelations from the study included: a) both types of collagens, i.e. COL8A1 and COL8A2 were aberrantly deposited in the DM of diseased early onset L450W cornea, with an abnormal anterior banded region which shows comparatively heavy depositions of COL8A2; b) massive deposition of COL8A1 in the posterior fibrillar layer of LO-FECD cornea and c) presence of ~120nm periodic internal collagenous layer comprising of wide spaced collagen strips- a feature pathologically characteristic of FECD.[10] These findings press on the fact that EO-FECD incurs mutational changes in the structural component of DM, i.e. collagen, such that it probably leads to overtly thickened DM than the less thickened DM in case of LO-FECD. Implications of COL8A1 in LO-FECD has been tested in both familial and sporadic case group studies independently by Aldave *et al*. and Urquhart *et al*., respectively.[54, 117] Failure to find any associative pathogenic mutations in the familial study group and only two out of 102 unrelated FECD patients harboring nonpathogenic sequence variants in the gene of interest, compelled the researchers to outcast COL8A1 candidature as a genetic reason for classical LO-FECD.[54, 117] Although there are aberrant periodic depositions in both forms, LO-FECD must be tracing a parallel pathological pathway that concerns with factors other than collagen itself. These pathways incurred by the two forms of FECD somehow converge at a common point from which it delivers similar pathological consequences of the disease.

Studies of *col8A2* null mutant mouse proposed that *col8A2* is not essential for the DM formation during eye development; although it helps in vasculature, cellular differentiation, maintenance of corneal stability and assemblage of DM.[116, 119, 120] There are four protein domains namely, NC2 domain (29-76aa), Triple helical domain (77-536aa), NC1 domain (537-702aa) and C1q domain (566-702 aa). Given the importance to the fact that collagen VIII has roles in cellular differentiation and that genetic abnormality in this ECM material produce effects exclusively in cornea,[55] Gottsch *et al*. hypothesized that mutations like L450W and Q455K, which are positioned in the triple helical domain of α2 polypeptide of collagen VIII, may result in improper interaction of the protein with other ECM molecules resulting in structurally weak DM that extrude guttae- forming material.[7] These abnormal collagenous depositions were reportedly higher within the DM in the posterior collagenous zone which appears to contribute structurally to the guttae.[10] Proline in the position Y of the tri-peptide repeat G-X-Y is hydroxylated in most of the chains. Proline mutations residing in the triple helix region might show similar effects. One such familial FECD case identifies P678L as a pathogenic variant.[120] Missense mutations in the triple helical region lead to misfolding and accumulation of protein in the ER and consequently elevate the ER stress, leading to apoptosis.[121]

1.6.3 Late Onset (classical) Fuchs' Dystrophy

SLC4A11 is a sodium borate cotransporter, previously known as bicarbonate transporter-related protein-1 (BTR1), encoding gene which is thought to be essential for growth and terminal differentiation of neural crest cells during endothelium formation.[122] It mediates sodium-dependent transport of borate as well as flux of sodium and hydroxyl ions *in vitro.*[62, 123, 124] Borate functionally leads to phosphorylation of essential cell signaling kinases – MAP-kinase (MEK), extracellular signal-related kinases (ERK) and proteins of the mitogen activated

protein kinase (MAPK) cascade.[107] SLC4A11 has been previously linked to an autosomal recessive, heritable, bilateral corneal dystrophy- congenital hereditary endothelial dystrophy type 2 (CHED2) by conducting a familial study including CHED individuals from Asian ethnicity (Mayanmar, Pakistan, India and China).[64] CHED shares phenotypic similarity with FECD and is commonly associated with aging. Vithana *et al*. linked four heterozygous mutations of SLC4A11 [three missense mutations (E399K, G709E and T754M) and one deletion mutation (c.99-100delTC)] to LO-FECD.[28] These mutations fall under conserved residues of SLC4A11 and hence give deleterious results. Owing to information from the above studies, it was hypothesized that loss of function mutation in SLC4A11 lead to gradual accumulation of misfolded protein in ER which might augment chains of deleterious consequences downstream that finally lead to endothelial cell loss in cornea.[28, 64] SLC4A11 deletion mutants in immortalized human corneal endothelial cell lines (HCECs) showed drop in cell survivability with increased apoptosis and hence evidences the imperative role of its mutations in endotheliopathies.[125]

Reasons for corneal endothelial cell death still remain enigmatic, but researchers have tried to explain it on the basis of increase in cellular stress due to accumulation of intracellular aggregates and thereby resultant oxidative stress and apoptosis. Mutations in the FECD candidate genes, COL8A2 and SLC4A11 cause misfolding of the proteins which thereby lead to swollen RER; the downstream consequences of which are evidenced by increased oxidative stress response categorized by imbalance in oxidant-antioxidant levels, accumulation of oxidized DNA lesions and finally apoptosis.[121, 126] Because of this, FECD has become a popular target to study ER stress. Unfolded protein mediated apoptotic response has been believed to be root cause of FECD by many researchers.[121] The comparatively newer candidate gene ascribed to FECD- LOXHD1, follows similar suit as COL8A2 and SLC4A11. LOXHD1 that is usually

expressed in lower levels in corneal endothelium increases dramatically in FECD condition forming cytoplasmic aggregates which might confer long–term cytotoxicity leading to cell death.[36, 89, 90] Rare mutations that are reported on the surface of LOXHD1 by *in silico* analysis predict to be crucial for protein-protein interactions.[36]

Aberrations in the rate of transcription of certain regulatory genes that regulate the expression of endothelium-specific structural proteins or other FECD afflicted proteins may also help answer the pathomechanism of the disease. Researchers working with TCF8 as a candidate gene for FECD hypothesize it to contribute likewise to craft the disease condition. Riazuddin *et al*. associated TCF8 alias ZEB1 with LO-FECD, contributing upto 2% (7/384) of the genetic load of FECD.[18] Few groups have sketched a hypothesis that a deficiency in the DNA-binding properties of TCF8 may lead to LO-FECD conditions on the basis that COL8A2, along with other COL4A types, share the same consensus sequences in the promoter region for TCF8 binding.[22, 127] Loss of function of TCF8 is proposed to be the likely mechanism for FECD associated phenotype on the basis of *in vivo* studies where wild type (WT) human RNA of TCF8 rescues the morpholino (MO) effect against *tcf8* in zebrafish embryos (with severe defects in neurulation and gastrulation).[18] Missense mutations in conserved regions of TCF8 (649A, 840P, and 905G) gave phenotypes indistinguishable from that of translational blocking morpholino (tbMO)-injected zebrafish embryos indicating severity of these alleles. These complementation assays were based on the facts from earlier studies that gastrulation and neurulation defects in zebrafish are due to double negative (DN) *tcf8* RNA expression.[18] Although it is yet to be elucidated whether deficiency in the DNA binding properties of TCF8 leads to LO-FECD and that mutated TCF8 leads to aberrant levels of COL8A2.

FECD 2 associated gene, TCF4 is reported to upregulate E-box promoter binding transcription factor ZEB1.[13, 128] This leads to a convenient conclusion that inactivation of ZEB1, which is

implicated to cause FECD, might have altered expressions contributed by the TCF4 variants.[13, 19] Recent developments in FECD genetics have unveiled a potent genetic biomarker for FECD predisposition. It was reported that most of the FECD affected individuals from Caucasian, European and Asian ethnicities harbor expanded CTG repeats in the intronic region of TCF4 gene.[21, 81, 83, 84, 129] Downstream repercussions of these accumulated repeat expansions as post-transcriptional by-products is sequestration of splice-machinery proteins like MBNL1 and MBNL2,[85] which physically appears as RNA foci in FECD cells.[130] This further consequent into accumulation of mis-spliced transcripts in FECD affected cells. As these mis-splicing events coincide with those produced in myotonic dystrophy type 1 (DM1) conditions, FECD is now considered as a result of TNR-mediated RNA pathology (**Figure 1.6**). [85]

1.6.4 Apoptosis and oxidative stress response: A unifying pathogenic pathway for FECD

The distinct pathological features exhibited by FECD, such as, wart-like excrescences, edematous endothelium and heightened deposition of ECM by the endothelium has encouraged researchers to explore the contributions that oxidative stress response and apoptosis has to cater to the disease. Both these events have been predominantly evidenced to be playing a central role in FECD disease pathomechanism.[26, 33, 126, 131, 132] A hypothetical consolidated pathway responsible to drive the cells towards FECD condition can be chalked out based on recent advancements in elucidating the pathomechanism of the disease (**Figure 1.6**). During FECD condition, proteins like COL8A2, SLC4A11 and LOXHD1 have been independently afflicted in escalating the ER stress due to aberrant protein accumulation with consequent activation of unfolded protein response (UPR) pathway.[28, 133, 134] ER stress, oxidative response and apoptosis are closely related mechanisms acting through interconnected pathways. Raised ER stress leads to apoptotic cell death mediated through C/EBP-homologous protein (CHOP), which is a transcriptional activator of many apoptotic genes namely GADD34, ERO1, BIM, and *TRB3*.[135]

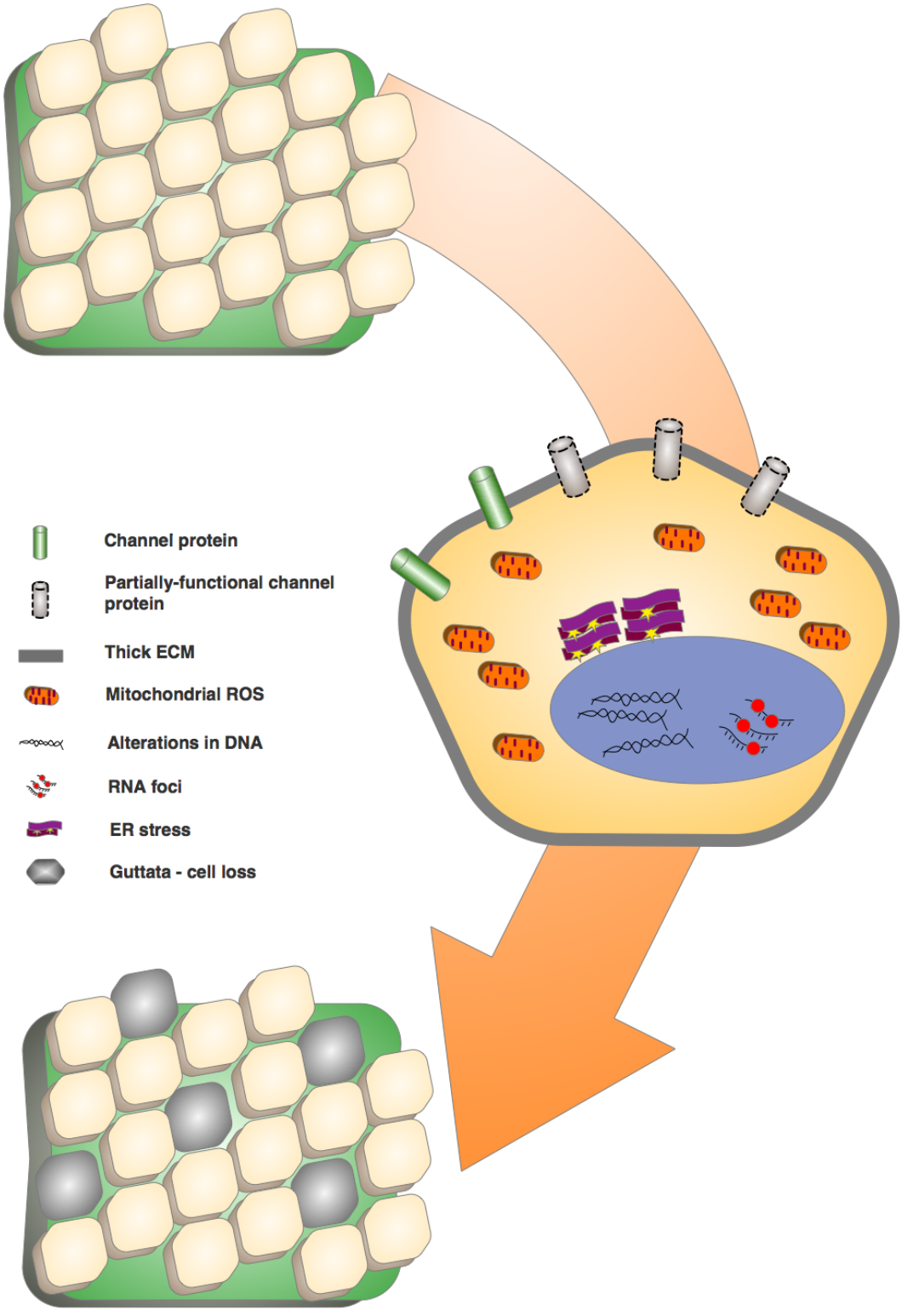


Figure 1.6: Pathomechanism of FECD.

Amongst these, GADD34 provides a reason to increase the oxidation of ER under stress conditions, by encoding a subunit of Phosphatase 1 which in turn dephosphorylates eukaryotic initiation factor 2α (eIF2α). Dephosphorylated eIF2α has been known to favour protein synthesis and oxidation of ER under ER stress conditions.[136, 137] Under such compromised conditions, protein misfolding leads to ROS production and elevated oxidative DNA damage as evident from higher than normal levels of oxidative DNA damage marker, 8-OHdg in FECD cells.[135, 138] Nuclear factor erythroid 2–related factor 2 (Nrf2) is an antioxidant responsive element (ARE) dependent transcription factor, which grossly promotes the synthesis of the antioxidant heme oxygenase-1 (HMOX-1), along with other antioxidants and is complexed with Keap1 (Kelch-like ECH-associated protein 1) in inactive form in the cytosol.[139, 140] Production of ROS triggers the antioxidant production pathway by inducing modifications in Nrf2-Keap1 complex in normal cells. In FECD cornea, Nrf2 and HMOX-1 have been reportedly downregulated, resulting in oxidant-antioxidant imbalance in the diseased endothelial cells.[139] Active Nrf2 dissociates from Nrf2-Keap1 complex in the cytoplasm on oxidative modification of the complex and is translocated to the nucleus with a transcriptional coactivator and stabilizer molecule- DJ-1 (also known as PARK7 Parkinsonism associated deglycase).[141] It has been proposed that oxidative stress conditions in FECD cells propels destabilization of DJ-1 by carbonylation and oxidation of cysteine at position 106.[135] This consequents into loss of biological activity of DJ-1 and thereby failed activation of antioxidant genes. Upregulated Keap1 in FECD endothelium imparts a strong binding of Nrf2 and its targeted degradation via cullin dependent pathway in the cytoplasm.[140] Moreover, recent reports with targeted deletion of SLC4A11 shows the essential role of this protein in NRF2 mediated antioxidant gene expression in HCEnC, whereas overexpression of SLC4A11 lead to increased cell viability and reduced ROS.[132] These studies suggest a definite impact of oxidative stress induced FECD

consequences which is a composite effect of various genes involved either genetically or physiologically.

ROS production as a result of UPR activation leads to extensive DNA damage that becomes irreparable for the cell and hence triggering the apoptotic machinery becomes imperative.[142] This is clearly reflected from the corneal endothelial cell loss as a consequence of disease progression. Works of several researchers evident the disturbed regulation of apoptotic pathway in FECD by the use of terminal deoxy-nucleotide transferase dUTP nick end labelling (TUNEL), nuclear labelling and transmission electron microscopy (TEM) visualization,[33, 143] DNA fragmentation assays,[144] oxidative stress susceptibility assays on normal and FECD corneal cell lines [138] and downregulated expression of anti-apoptotic cell defence genes as affirmed by SAGE analysis data.[88] The pathological route that the apoptotic corneal endothelial cell takes when affected with FECD, is not very well elucidated, although it hints towards a premature senescence of the diseased endothelial cells in both EO- and LO-FECD; supported by the fact that the growth-arrest protein, cyclin-dependent kinase (Cdk) inhibitor p21(CIP1/WAF1) (p21), encoded by the CDKN1A gene, is upregulated in an EO-FECD mouse model with *Col8a2* Q455K/Q455K knock-in mutation.[145] Co-stimulation of p21 and other cell cycle progression genes like Egr1, Ccnd1 and E2f1 leads to a state of hypertrophism which characterizes the bloated endothelial cell morphology in FECD.[146-149]

Hypertrophic growth arrest state occurs as a result of lack of compensatory ability of cell division to support the linear nature of the endothelium in lieu of the dying neighboring cells.[145] As opposed to replicative senescence observed in normal aging corneal endothelial (CE) cells in which irreversible growth arrest is witnessed due to telomere shortening; stress induced premature senescence (SIPS) is predominantly observed in FECD endothelial cells.[150-153] Oxidative DNA damage due to heightened ROS production ceases the proliferative capacity of

these cells and leads to premature senescence and p53 mediated apoptosis.[135, 138, 145] The same is also evident from the upregulated senescence-afflicted genes, i.e., clusterin, fibronectin, serpine and transgelin along with Cdkn1A and p53 in the transgenic knock-in mouse.[145] Overexpression of clusterin has been associated as one of the survival strategies of the cell during cytotoxic stress conditions and has been noted in both EO- and LO-FECD.[87, 154] Accumulation of clusterin and TGFβIp in the excrescences of the CE might have a role in scavenging aberrant ECM and promoting intercellular and cell-substratum adhesive interactions in order to maintain integrity in the disease stricken decaying tissue.[87, 155-157]

In concise, pathogenesis of FECD can be sketched to have a forked pathway. On one hand, regulatory genes like TCF4 and TCF8 alter the expression levels of proteins crucial to maintain homeostasis of the endothelium. To look into the alternate pathway, the structural proteins fail to fold into functional proteins and tend to accumulate in the ER. Dysregulation and aberrantly folded protein accumulation converge into a unifying pathway of disease progression that is active in both the forms of FECD, through UPR activation and subsequent oxidative stresses followed by apoptosis (**Figure: 1.6**).

1.7 Contemporary treatment procedures for FECD

FECD patients often complain of blurred vision in the mornings, difficulty to stare at bright lights or seeing halos around light, watery eyes and others show symptoms detected with corneal haze. Patients with FECD symptoms often feel the need to wear anti-reflective sun-glasses as they find the night lights too glazy to stare at. As these symptoms worsen at the sixth to seventh decade of life, corneal transplant becomes inevitable. Traditionally, Penetrating Keratoplasty (PK) was advised as the surgical method to graft donor's cornea on the diseased

one. The limiting visual acuity makes daily chores performance a challenge and this often leads to depression in adults.[158]

1.7.1 Surgical interventions

The leading cause for maximum number of corneal transplantation, FECD accounts as the reason for most frequent corneal endotheliopathy in the United States of America.[159] FECD constitutes 16.6% of total PKs performed for corneal dystrophies from a study reported from South India.[5, 160] This disorder has been frequently and traditionally addressed by penetrating keratoplasty (PK) [161]- a surgical method to graft donor's cornea on the diseased one which has been the gold standard for over a century. It has its own disadvantages postoperatively, such as wound leak, surface corneal incisions and sutures, persistent epithelial defect, long visual recovery, delayed healing of vertical stromal wounds, poor visual quality as a consequent of irregular astigmatism, suture trauma, infection, wound dehiscence and long-term use of topical steroids, unpredictable refractive error, ulceration, rejection and late wound rupture.[162, 163]

1.7.2 Contemporary techniques

If the surgical techniques could only displace the dysfunctional endothelium rather than full thickness corneal transplantation, most of the inherent disadvantages of PK can be addressed. Ophthalmologists, with this thought, commenced the beginning of the new era of endothelial keratoplasty; which includes several surgical modifications such as Posterior Lamellar Keratoplasty (PLK), Deep Lamellar Endothelial Keratoplasty (DLEK) and (femtosecond) Descemet Stripping (Automated) Endothelial Keratoplasty (DSEK/DSAEK/FS-DSEK) and, Descemet Membrane Endothelial Keratoplasty (DMEK).

In order to improve the visual acuity post operatively, Melles *et al*. designed a novel technique, PLK which left the recipient's anterior cornea untouched, minimized scarring at the surgical interface and enhanced wound healing.[164] Another technique was put forth in the year 2000 by Terry and Ousley; named as DLEK which addresses major goals of endothelial transplantation, such as, preservation of the corneal topography by avoiding corneal incisions and sutures, rendering a predictable and stable corneal power, minimizing injuries and infections and resolves edema of donor endothelium.[165] Therefore, DLEK has a considerable advancement over PK.[166-168]

Descemet stripping automated endothelial keratoplasty (DSAEK) method has been the treatment choice in the past decade for patients with endothelial failure. It has decreased rejection rate, intraoperative and postoperative complications, astigmatism, and faster visual recovery with more consistent results compared with PK. In DMEK, the Descemet's membrane along with endothelium is replaced. This technique has improved outcomes over DSAEK in rejection rates and visual recovery. However, it is not currently the technique of choice due to its learning curve.

1.7.3 Non-surgical interventions

In the recent year of 2013, Okumura *et al*. reported a game changing formula of Rho-kinase (ROCK) inhibitor (Y-27632) that when applied via eye-drops to monkeys with induced apoptosis of the corneal endothelium, could regenerate these terminally differentiated cells and restore vision.[169] It was reported that selective inhibitors of Rho-kinases, like Y-27632, jump-start the cell cycle in a tissue specific manner; the mechanism of action is however unclear. Subsequent trials to generate stable *in vitro* endothelial monolayers to be used as transplantation grafts have been pursued.[170-173] However, use of EDTA/trypsin to dislodge the intercellular

junctions and uplift the mitotic block,[174, 175] often resulted into triggering of epithelial-mesenchymal transition (EMT) of these HCEnCs.[176] This hindrance was averted by the use of ROCK inhibitors[177] or TGF-βblocker SB431542.[178] These reports suggested that EMT of mitotically blocked HCEnCs were due to activation of canonical Wnt signaling pathway.[179] Knockdown with p120 siRNA or p120-Kaiso siRNA (Kaiso is a transcriptional repressor that is released by nuclear translocation of p120 factor) resulted in activation of RhoA-ROCK signaling to unlock the mitotic block mediated in contact-inhibited HCEC monolayers; suggesting that p120-Kaiso signaling is involved in determining cell proliferation of HCEC.[180] These studies aim at replacing the traditional surgical techniques that depend on the availability of healthy donor cornea and post-operative side effects. Although most of these methods still need experimental improvements and successful animal trials, some of them, such as, ROCK inhibitor Y-27632 eye drops have successfully entered clinical trial stages.[169, 178]

1.8 Discussion

Fuchs' endothelial corneal dystrophy has been elaborately studied by various ophthalmologists around the world and many have put forward their surgical experiences. With advent of new technologies and disease specific management, surgical relief from a particular disease has come to an advanced level. Since it leaves scars and injuries surgical treatment of FECD additionally require a mental commitment apart from dealing with the disease. In such conditions, a genetic predisposition and early stage detection will open up many avenues to avert distressed consequences the disease has to offer at later stages of life. FECD is one such disease which is currently treated only with painful surgeries because of its heterogenous nature of pathogenesis. However, recent pharmacological advances have uncovered the regenerative property of ROCK inhibitor which has successfully reverted the FECD phenotype in monkeys and rabbits. [177]

Louttit *et al.* initiated a highly collaborative project called the 'FECD Multicentre study' with an intent to carry-out a high density genotyping of 322 FECD affected families to demarcate the susceptibility regions and genetic risk factors responsible for co-inheriting with the disease.[181] A recent study conducted a GWAS with 1,404 FECD and 2,564 control Caucasians from the FECD Multicentre study comprising of individuals from USA and Australia in order to identify novel genetic loci co-segregating with FECD.[14] They identified three novel loci in KANK4 (KN motif- and ankyrin repeat domain-containing protein 4, rs79742895), LAMC1 (laminin gamma-1; rs3768617) and LINC00970/ATP1B1 (Na^+, K^+ transporting ATPase, beta-1 polypeptide; rs1200114) genes that can provide new insights into the complex pathogenesis of FECD.[14]

Most of the literature discussed in this chapter comprises of genetic information biased to Caucasians from American and European decent. However, there have been extensive reports suggesting a great deal of impact imparted by ethnic diversities across the globe.[182-189] Several studies have witnessed differences in allele distribution for polymorphic disease markers.[20, 84, 185] These concerns encourage the researchers to carry out population specific genetic evaluations so as to gather ethnically rich information regarding a disease in Indian population. Additionally, such studies help substantiate the candidate genes that have an absolute contribution to a disease causation globally. However, the drawback of population specific studies can sometimes involve identification of such genetic markers that may not contribute significantly towards disease pathogenesis.[20]

With expanded trinucleotide repeats (TNR) co-segregating with FECD, this disease shares similar pathophysiology as those of other TNR-associated diseases like Huntington and Macular Dystrophy.[85, 130] These intronic expanded TNR are a component of post-transcriptional by-product which attracts the splice-machinery proteins like MBNL1 and MBNL2 and

consequently alter the effective concentration of correctly spliced transcripts. RNAseq transcriptome analysis performed with FECD and control tissue samples identified 18/24 transcriptional events that overlap with DM1 splicing changes.[85] However, there still remains the concern about the number of transcripts that are deregulated due to these changes. Similarly, studies are recommended to analyze whether current RNAi therapy that are in clinical trials to correct DM1 can also prove effective in case of FECD. Another transcriptional regulator, ZEB1 that has genetic association with FECD, has been shown to regulate transcription of COL4A1, which is one of the candidate for a similar corneal dystrophy, PPCD. However, it is yet to be seen whether COL8A2 is also transcriptionally regulated by ZEB1.

Apart from various genetic contributors, a complex disorder like FECD could be a resultant of various non-genetic factors such as UV radiation, topographical impact, smoking and dietary influences. Previous literatures indicate UV exposure and smoking as non-genetic factors predisposing to corneal guttata formation.[49, 190] Studies regarding the interaction of these factors with genetic risk variants that consequent into disease manifestation can unveil critical knowledge on FECD predisposition. It is also observed that expanded TNR stretches when present in upstream untranslated regions are hotspots for DNA methylation.[191] There are also reports of regulatory roles of introns. Provided the significant penetrance of TNR among various populations across the globe,[191, 192] it becomes essential to understand the functional implications of these accumulated repeats in the genome. It is therefore essential to follow whether genomic alterations causing non-ocular disease get associated with FECD. In-depth understanding of the disease is therefore warranted to help identify therapeutic targets to treat FECD without surgical intervention. The current plethora of information obtained for FECD pathogenesis has certainly broadened the understanding of several genetic and physiologic players. However, there still are many major unanswered questions (**Table 1.2**).

Table 1.2: Crucial lacunae in understanding FECD

Effect of collagen deposition on corneal functioning
Is the abnormally thickened DM altering the normal functioning of cornea?
Can there be a drug mediated clearance of abnormal collagen deposition in the thickened DM?
Pathology of expanded trinucleotide repeats in FECD
How are the expanded trinucleotide repeats affecting the overall expression profile of corneal endothelial tissue?
Can the repercussions incurred by expanded trinucleotide repeats be alleviated by RNAi based therapy?
Contribution of ZEB1 and FEN1 in FECD pathogenesis
Is ZEB1 involved in regulation of COL8A2 expression?
What is the extent of genetic load imparted by ZEB1 polymorphisms in FECD population?
What is the underlying mechanism of ZEB1 deregulation in FECD affected tissues?
Does the associated polymorphisms of FEN1 impart any functional role towards FECD pathogenesis?
Non-genetic players of FECD
Composite effects of UV exposure, smoking, geography and dietary factors in FECD pathogenesis.
What are the DNA-methylation alterations incurred due to FECD condition?
Non-ocular repercussions and therapies for FECD
Are the accumulated mutant proteins affecting non-ocular tissues?
Is there any drug mediated recovery available to alleviate the effects of expanded TCF4 repeats?
Are there any non-invasive biomarkers for FECD condition?
Are there any physiological changes in the overlying layers of FECD affected corneal endothelium?

A few of these lacunae have been addressed in this work in the subsequent chapters.

1. What is the status of TCF4, ZEB1 and FEN1 polymorphisms in Indian population?

2. Is there a transcriptional regulation of COL8A2 by ZEB1?

3. Does the associated polymorphism in FEN1 contribute towards the pathophysiology of FECD?

4. Could MCTs be involved in FECD disease?

Chapter 2

Materials and Methods

2.0 MATERIALS AND METHODS

This chapter elaborately describes the different methods employed in the following chapters. The source of materials commercially obtained has also been mentioned in round brackets.

2.1 Study participants recruitment

The Institutional Ethics Committee of National Institute of Science Education and Research and L. V. Prasad Eye Institute, reviewed and approved the study prior to the recruitment of subjects. All the study procedures followed the tenets of the Declaration of Helsinki.

2.1.1 Biological sample collection

All the control and FECD patient individuals recruited for this study at L. V. Prasad Eye Institute, a tertiary eye care institute in Bhubaneswar, are of Indian origin within the age range of 38 to 85 years (average age: 62.2 years including control and patients); after acquiring their written consent for enrollment in the study. Four milliliters of peripheral blood were extracted by a trained clinician at the eye care institute and stored at $4^{o}C$ in EDTA coated vacutainers in order to minimize blood clotting. For long time storage, the blood samples were stored at $-80^{o}C$.

Tissue samples from patients were collected immediately post operation in either RNA*later* (Invitrogen, USA) solution if it was intended for RNA extraction or in cornea transplantation media, McKarey-Kauffman (MK) Medium (L. V. Prasad Eye Institute, Hyderabad) if the tissue was to be used for immunohistochemical usages and stored at $4^{o}C$ till further use.

2.1.2 Inclusion-exclusion criteria

We obtained detailed reports concerning the medical and family history of all the participants after clinically screening them via slit lamp examination and categorizing on

modified Krachmer FECD grade scale of zero through six (grade 0 individuals exhibiting no central guttae; grade 1: upto 12 scattered central guttae; grade 2: ≥12 scattered central guttae; grade 3: 1-2 mm central guttae; grade 4: 2-5 mm of clustered central guttae; grade 5: 5 mm confluent central guttae without edema; grade 6: ≥5 mm confluent central guttae with edema).[51] The inclusion criteria for FECD patients involved individuals above 35 years of age exhibiting moderate to severe disease condition (grade 2 and above). Age-matched individuals with healthy cornea were enrolled as control for the study. In a few cases slit lamp examination was not possible in both the eyes/ one eye due to advanced disease, for these cases we ascertained the inclusion criteria by histopathological tests reporting thickened DM with nodular excrescence and sparse endothelium. Subjects not matching the age criteria and/or with inconclusive specular / histopathology reports were excluded from the study.

2.2 Genotyping of polymorphisms

Polymorphisms screened for association with FECD, were genotyped either by direct sequencing using Sanger's dideoxy method or by restriction fragment length polymorphism (RFLP).

2.2.1 DNA extraction

The genomic DNA was extracted from the peripheral blood leucocytes (by DNA salting–out method) of the participants (controls and patients) and was maintained at 100ng/µl.[193] For this, 500-1000µl of human blood sample was taken in 1.5ml microfuge tube from the EDTA coated vacutainer tubes. It was centrifuged at 11000rpm for 5 minutes to pellet down the leucocytes. The supernatant containing mostly serum was discarded and 1000µl of RBC (Red-blood cells) lysis buffer (0.32M Sucrose, 1mM $MgCl_2$, 1% Triton X-100, 12mM Tris-HCl, pH adjusted to 7.6) was added. Pelleted cells were dislodged by inverting the

microfuge tubes for several times for not more than one minute and then centrifuged at 11000rpm for 5 minutes. To the retained pellet, an additional 200µl of RBC lysis buffer was added to it, mixed and re-centrifuge at 11000rpm for 5 minutes. Post centrifugation, lysed RBCs in the supernatant was discarded and only the pellet containing primarily leucocytes was processed further. The pellet was dissolved in 200µl of MQ water to remove the salts and impurities from the previous steps, by pipetting 2-3 times and centrifuged at 13500 rpm for 5 minutes. The supernatant was discarded and to this pellet, 80 μ l of Proteinase K buffer (20mM Tris-HCl, 4mM Na_2EDTA, 100mM NaCl, pH 7.4) was added along with 10µl of 10% SDS. Pipetting this mixture created saponated froth due to SDS which helped lyse the cell membrane and nuclear membrane. 100µl of pre-chilled 5M NaCl was added this solution in order to coalesce DNA molecules in presence of high salt concentration. Additionally, 200µl of MQ and 400µl of PCI solution (Tris saturated Phenol: Choloroform: Isoamyl Alcohol = 25:24:1, Sigma-Aldrich, USA) was also added. The tubes were mixed by inverting the tubes until the contents turn milky, and then centrifuged at 12000 rpm for 10 minutes. The aqueous layer containing genomic DNA was now taken out in fresh tubes and 1000µl of Pre-chilled absolute ethanol was added for its precipitation. Mixing was done gently by inverting the tubes very slowly 20 times which allowed the spool of DNA to aggregate. The tubes were further centrifuged at 13000 rpm for 10 minutes. The supernatant was discarded by draining off. The pellet containing the DNA spool was washed with 200µl of 70% ethanol and centrifuged at 13000rpm for 5 minutes. All centrifugation steps were carried out at room temperature (RT). The supernatant was discarded by draining off and the pellet was air dried for at least 3-4 hours until finally dissolving the DNA in 30µl of Tris-EDTA buffer (for each 500µl blood sample as starting material), pH 8.0 and leaving it at 56^{O}C for an hour to completely dissolve the DNA. The re-suspended DNA was quantified using NanoDrop 2000 (ThermoFisher Scientific, USA) and the quality of DNA was checked

on 1% agarose gel. DNA stocks were labelled, maintained at 100ng/µl and stored in -20ºC until further use.

2.2.2 Primer designing

Primers flanking the polymorphic site were designed using PrimerBLAST online tool and was manufactured at 25nmoles, under desalted conditions (IDT oligos, USA or GCC Biotech, India). The specifications of primers used during this study are listed as in **Table 2.1.**

Table 2.1: List of primers used for genotyping along with the annealing temperatures for product amplification.

Name	Sequence (5'-3')	Annealing temp.
CHAPTER 3		
TCF4 rs613872 F	CCCAGTAGGGTTGTGATGATGATG	60.9
TCF4 rs613872 R	CAGTTGGGAACACCCATTTGTCTG	
TCF4 rs17089987 F	TGGGCATAGAAGGCAAGAGAGA	60
TCF4 rs17089987 R	CAGGATTTCGTCTTTATCCACAGGC	
TCF4 rs17089925 F	TGGGTTGTGATGCTGTCTCAGTGT	60.3
TCF4 rs17089925 R	TTCCTGCTTCTGACCCTCCAATGT	
TCF4 CTG18.1 F	CAGATGAGTTTGGTGTAAGATG	61.1
TCF4 CTG18.1 R	ACAAGCAGAAAGGGGGCTGCAA	
CHAPTER 4		
ZEB1 EX1 F	CTTTTCCCTCGCCCCTCAA	56.5
ZEB1 EX1 R	TTCCCACTCCACTTTGCCG	
ZEB1 EX2 F	GTTACTCTCTCTCTGCCTTGATTT	57.9
ZEB1 EX2 R	CCTCCATGATTTATATGTGTTTGGG	
ZEB1 EX3 F	CTTCGTGAGTTTGGTAAGACTG	58.5
ZEB1 EX3 R	TGGGCAATTCAAAGCAGACC	
ZEB1 EX4 F	TACTATGGGAACCTGCTCTACT	58.3
ZEB1 EX4 R	CACACTCTGTACGCTGACTATT	
ZEB1 EX5 F	GAGCACAAAATTCCCCTTTAATCTG	59.2
ZEB1 EX5 R	GAGGCAACTCCCTTTACTACGG	
ZEB1 EX6 F	CAGTATTTGTGGATGCGGTTTC	59

ZEB1 EX6 R	ACTCCCTCTCATTGCCTCTA	
ZEB1 EX7A F	ACCGCTTGTTTAGGGAAATGAG	58.7
ZEB1 EX7A R	ACTTATGGGAGACACCAAACCA	
ZEB1 EX7B F	ACCCATAGTGGTTGCTTCAG	57.4
ZEB1 EX7B R	CCAGTAGCTGATGAAACAGAGG	
ZEB1 EX7C F	GCACTTGTCTTCTGTGTGATGATTG	58.4
ZEB1 EX7C R	GTGGTTGATCCCACTGGTAAA	
ZEB1 EX7D F	CACAAGAAGAGCCACAAGTAGA	57.9
ZEB1 EX7D R	CAGAAACCCAATTAGCGAGTATTTC	
ZEB1 EX8 F	GGAGGTTAAAGAACCCTGAAGT	54.3
ZEB1 EX8 R	AGTGAGCCGAGATTGAGATTG	
ZEB1 EX9A F	TGTAGCTCCCATTGACCTAATC	55.5
ZEB1 EX9A R	TTCAGACACTTGCTCACTACTC	
ZEB1 EX9B F	GCAGAGAATGAGGGAGAAGAAG	59
ZEB1 EX9B R	ATTGGATGAAGGCGGGTTAG	
ZEB1 EX9C F	AGTTCTGATTGCAGGGACTAAC	61.3
ZEB1 EX9C R	GTTCTACAGTCCAAGGCAAGTAT	
pGL4.23 RV3	CTAGCAAAATAGGCTGTCCC	60
pGL4.23 GL2	TGGAAGATGCCAAAAACATTAAG	
CHAPTER 5		
FEN1 rs174538 F	CTCTCGCCCTTAGAAATCGC	56.3
FEN1 rs174538 R	GGCAACCAGTCCCTCCAG	
FEN1 rs4246215 F	TATGTCAGGCTCAAACCAC	52.1
FEN1 rs4246215 R	CAGCCAGTAATCAGTCACAA	

2.2.3 PCR amplification

Polymerase chain reaction was used to amplify a particular genomic region. The PCR reaction mix comprising 100ng template DNA, 0.5µM of the flanking primers, 100mM dNTP mixture (GenetBio, India), 2.5µl of DMSO (Sigma-Aldrich, USA), ExPrimeTaq Buffer (GenetBio, India), 1.5 mM $MgCl_2$ and 1.0U ExPrimeTaq DNA polymerase (GenetBio, India) was constituted into 25µl reaction volume. These reactions were incubated at 94°C for 5 minutes followed by 35 cycles of 45 seconds of denaturation at

94°C, 45 seconds at annealing temperatures (**Table 2.1**), and extended for 45 seconds at 72°C and finally incubated at 72°C for 10 minutes of final extension. The amplicons were stored at 4°C until further use. These amplified products were either directly used for RFLP reaction or electrophoresed on 1% agarose gel at 150V and 200mA and subsequently eluted using QIAquick Gel Extraction Kit (QIAGEN, Germany). These eluents served as template for Sanger sequencing.

2.2.4 DNA Sequencing using Sanger's method

Sanger sequencing reaction was performed by employing Big Dye Terminator v.3.1 cycle sequencing kit (Applied Biosystems, Austin, TX78744, USA) following the manufacturer's protocol on an automated sequencing platform (3130xl genetic analyzer, Applied Biosystems, USA).

The eluted PCR amplicons were quantified and sequencing PCR was set up using Invitrogen BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) for 3130xl Genetic Analyzer (Applied Biosystems, USA). For each sequencing reaction, the components used were 0.25 μl of 2.5X ready reaction mix (0.1 μl of RR mix was used per 100bases), 2µl of dilution buffer (5X), upto 80ng of eluted DNA as template, 2µl of primer (2pmol) and nuclease free water (MP Biomedicals, USA) for a total reaction volume of 10µl. The thermal cycling sequencing PCR reaction was carried out for 25 cycles with each cycle having a denaturation step at 96^{O}C for 10 secs, annealing at 50ºC for 5 secs, extension at 60ºC for 4 minutes and kept at 4ºC until further use.

Each sequencing reaction products subsequently underwent cleanup steps before finally being sequenced on 3130xl Genetic Analyzer. Master Mix-I was constituted by adding 10µl nuclease free water and 2µl of 125mM EDTA per reaction. 12µl of master mix I was added to each sequencing reaction containing 10 μl of PCR product and the contents were mixed

properly. Master Mix-II was prepared by adding 2µl of 3M NaOAc (pH 4.6) and 50 μl of ethanol per reaction. 52µl of this master mix II was added to each reaction. The contents were mixed well and incubated at room temperature for 15 minutes. After incubation, the reaction mixture was centrifuged at a speed of 12000rpm for 20 minutes at room temperature. The pelleted sequencing products were washed by adding 250µl of 70% alcohol and centrifuged at 12000rpm for 10 minutes at room temperature. After decanting the supernatant, the pellet was dried in room temperature for 2 hours and resuspended in 15µl of Hi-Di formamide. These samples were transferred to a 96 well sequencing plate (ABI 1400, Applied Biosystems, USA), covered with septa, denatured at 95ºC for 5 mins and snap chilled on ice for 10 mins and finally proceeded for electrophoresis on 3130xl Genetic Analyzer. The sequencing read-out were obtained in .abi format and were analyzed using BioEdit v7.1 (Tom Hall, Ibis Biosciences, USA) software. Sequences obtained from 1000 Genomes database on NCBI was used to align using local alignment tool to either genotype the polymorphic site or scan for variations in the nucleotide sequence.

2.2.5 Restriction fragment length polymorphism for SNP genotyping

Restriction digestion was performed for a reaction mixture of 25µl where 15µl of PCR product was digested using the 1U of appropriate enzyme acquired from New England Biolabs (NEB, **Table 2.2**), by incubating at 37ºC for three hours in a water bath (Julabo GmbH, Germany). The digested products were electrophoresed alongside their respective PCR products on 3% agarose gels at 100V and 150mA. The gel picture of samples (after they had traversed three quarters on gel) was captured using a GelDoc XR system (BioRad, USA) and analyzed using the Quantity One software (BioRad, USA).

Table 2.2: List of restriction enzymes used in this study.

Name	Manufacturer Code (NEB)
BsmAI	R0529S
SalI	R3138S
KpnI	R3142S
XhoI	R0146S

2.3 Trinucleotide-repeat assessment

The region of interest encompassing the CTG18.1 allele (annotated as rs193922902, dbSNP) was amplified by polymerase chain reaction (PCR) using Invitrogen Platinum PCR Super Mix High Fidelity and a primer pair; employing previously reported reaction conditions. The first cue on hetero/homozygosity was obtained by checking the band intensity of the PCR products on 2% agarose gels. To confirm heterozygosity of the individuals for CTG18.1 allele, the PCR products were gel eluted from 1% agarose gels and sequenced bi-directionally using ABI 3130xl Genetic Analyzer by Sanger's method for both the alleles. To confirm homozygosity, the PCR products were purified using QIAGEN PCR purification kit (QIAGEN, Germany) followed by ExoSAP (USB Products Affymetrix, Inc., USA) treatment and directly utilized as sequencing template according to the manufacturer's protocol. Such two-tier verification confirms the number of individuals having at least more than fifty repeat units.

2.4 Statistical analysis

2.4.1 Population Analysis

To estimate the genetic power for the enrolled cases and control groups post hoc, G*Power v3.1.9.2 statistical power analysis software (University of Düsseldorf, Germany) was used. With the effect size of 0.5 (intermediate effect) and alpha error at 0.05, the genetic power of the sample size was estimated to be at 90.73%. The difference in age and gender distribution

across cases and controls were computed by carrying out Student t- test and Fisher's exact test, respectively.

2.4.2 Polymorphism Association tests:

Association of polymorphisms with FECD were analyzed by χ^2 test and binary logistic regression taking age and gender as covariates using SPSS 23.0 statistical software for MacOS (IBM SPSS, Inc., USA). We tested various models of genetic inheritance for each polymorphism using multiple logistic regression in SPSS: dominant (DOM, major allele homozygotes vs. heterozygotes+ minor allele homozygotes), recessive (REC, major allele homozygotes+ heterozygotes vs. minor allele homozygotes) and additive (ADD, mojor allele homozygotes vs minor allele homozygotes). Genotypic variables used for coding were 0, 1 and 2 with homozygous risk alleles for all the SNPs coded as 2; allele coding for CTG18.1 was adapted from Mootha and colleagues.[81] Linkage disequilibrium (LD) computation, LD plot generation and haplotype frequency calculation was done using Haploview 4.2 (Broad Institute). Association of polymorphisms below the threshold of 5% was considered significant for this study.

2.5 Electrophoretic Mobility Shift Assay (EMSA)

2.5.1 Oligomer designing and annealing

Four pairs of complementary 29-mer oligonucleotides, centered around ZEB1 binding sequence at +374 bases downstream of transcription start site of COL8A2 gene (RefSeq NG_016245.2) or a previously reported ZEB1 element on a scrambled sequence background were generated; both with and without biotin 5'-end labelling (GCC Biotech, India). Sequences of the oligomers are listed in **Table 2.3**. Complementary oligomers were re-dissolved in nuclease free water and annealed by incubating them at 95ºC for 5 mins, followed by step-cooling to room temperature and storage at -20ºC until further use.

Table 2.3: List of oligo sequences utilized during EMSA analysis.
F and R: complementary sequences of respective oligos, L: biotin labeled, UL: unlabeled, K: KpnI site, X: XhoI site, WT: Wild-type consensus sequence for ZEB1. All biotin labeled oligos are HPLC purified.

Name	Sequence (5'-3')
374 ZEB1 LF	TTAGCTGGCACAGGTGGTCTTCCAAGG
374 ZEB1 LR	CCTTGGAAGACCACCTGTGCCAGCTAA
374 ZEB1 ULF	TTAGCTGGCACAGGTGGTCTTCCAAGG
374 ZEB1 ULR	CCTTGGAAGACCACCTGTGCCAGCTAA
374 WT ULF	TTAGCTGGCACAGGTGTTCTTCCAAGGGGG
374 WT ULR	CCCCCTTGGAAGAACACCTGTGCCAGCTAA

2.5.2 Nuclear extract preparation and quantification

Nuclear protein lysate from Human Corneal Endothelial Cell line (HCEnC) was extracted by using NE-PER Nuclear and Cytoplasmic extraction reagents (ThermoFisher Scientific, USA). Nuclear protein extracts were quantitated using Bradford Reagent (Sigma-Aldrich, USA) and bovine serum albumin (BSA) for preparing protein standards (2.5, 5 and 10 mg/ml) in a 96-well system. Absorbance readings at 595nm region were recorded spectrophotometrically using Microplate Reader (BioRad, USA).

2.5.3 Binding reaction for EMSA

DNA-protein binding assays were carried out using 3µg of total nuclear extract and 50 fmol of biotinylated annealed oligonucleotides for each 20 μ l total reaction volume. Competitive EMSA was carried out using 2000-fold excess (100pmol) of unlabeled double-stranded oligonucleotides that were added 10 minutes prior to the addition of labelled double-stranded oligonucleotides which was incubated for 20 more minutes. For supershift assays, 2 μ g of ZEB1 antibody (sc-25388, Santa Cruz Biotechnology) were incubated with the final reaction mixture for an additional 60 minutes at RT before the addition of labelled oligos. Following is a tabulated description of a typical EMSA binding reaction (**Table 2.4**). All the

components of EMSA binding reaction and detection were taken from the LightShift Chemilumiscence EMSA kit (ThermoFisher Scientific, USA) and manufacturer's instructions were followed to carry out the experiment.

Table 2.4 Binding reaction formulation for a typical EMSA system

Components	Final Amount	Reactions			
		#1	#2	#3	#4
Ultrapure water					
10X Binding buffer	1X	2µl	2µl	2µl	2µl
50% Glycerol	2.5%	1µl	1µl	1µl	1µl
100mM $MgCl_2$	5mM	1µl	1µl	1µl	1µl
1 μ g/ μ l Poly (dI*dC)	50ng/ μ l	1µl	1µl	1µl	1µl
1% NP-40	0.05%	1µl	1µl	1µl	1µl
10pmol Unlabeled oligo	100pmol			10µl	
Nuclear extract	Upto 5 μ g				
Biotin End-Labelled oligo	50fmol	1µl	1µl	1µl	1µl
200 μ g/ml Antibody	2 μ g				10µl
Total Volume		20µl	20µl	20µl	20µl

2.5.4 Electrophoresis of binding reaction

Binding reaction is electrophoresed in a native polyacrylamide gel in 0.5X TBE (Tris-borate EDTA) buffer. For the current study, 6% polyacrylamide gel was prepared using 0.5X TBE. The casted gels were allowed for a 60-minutes pre-run at 100V and 100mA in order to flush out unpolymerized acrylic acid and any residual ammonium and persulfate ions. The electrophoresis tank is maintained at lower temperatures (4-15ºC) with the help of ice jacket in order to minimize heat development. Post pre-run, the binding reactions are loaded onto the wells using 5 μ l of 5X loading buffer for each 20 μ l binding reaction. Electrophoresis of

the binding reactions are performed at 100V and 100mA until 3/4th migration of bromophenol blue dye.

2.5.5 Electrophoretic transfer and crosslinking of binding reaction to nylon membrane

Transfer of the electrophoresed binding reaction from gel to 0.45 μ m positively charged nylon membranes (BrightStar Plus, Invitrogen, USA) are done using standard tank transfer mini gel (8 x 8 x 0.1cm) apparatus at 380mA (~100V) for 30 minutes. Post wet-transfer, the DNA:protein complexes on the nylon membrane were crosslinked at 120mJ/cm^2 using UVC 500-crosslinker (Amersham Biosciences, USA), exposed for 60 seconds. The crosslinked complexes are stored in RT until further use.

2.5.6 Chemiluminescent detection of biotin labelled DNA complexes

Crosslinked biotin labelled DNA complexes are visualized by using stabilized streptavidin horseradish peroxidase (HRP) conjugate as the blocking agent in 1:300 dilution with Blocking Buffer (component of LightShift Chemiluminescence EMSA kit, Thermo Scientific, USA). Following this, the membrane was washed 10 times for 5 minutes each in 1X wash buffer with gentle shaking. Membrane was then immersed into Substrate Equilibration Buffer for 30 minutes with gentle shaking. Substrate Working Solution was prepared by mixing Luminol and Stable Peroxide Solution in 1:1 volume and the membrane was completely covered with this admixture for 5 minutes without shaking. For this the membrane was placed onto a clean sheet of plastic wrap on a flat surface. After draining the excess Substrate Working Solution and avoiding drying of the membrane, it was wrapped in the plastic sheet and exposed to High Sensitivity Chemiluminescent detector (ChemiDoc XRS+ System, BioRad). Captured images were analyzed using Quantity One image analysis software (BioRad, USA).

2.6 Cell Culture

Human Corneal Endothelial Cell lines (HCEnC) was generated and kindly provided by Dr. Rajiv Mohan (University of Missouri). These cells were cultured in 1:1 Dulbecco's Modified Eagle's Medium (DMEM):F12 media (Invitrogen, USA) and 10% fetal bovine serum (US origin, Invitrogen) at 37^{O}C and 5% CO_2 under optimum humidity. The cells were sub-cultured twice a week to maintain them in an actively growing state. Upon reaching 70% confluency, the cells were trypsinized with 0.25% trypsin solution containing 0.038% EDTA and phenol red (HiMedia, India) to produce a single cell suspension. Suspended cells were either utilized for various downstream processes like sub-culturing, nuclear protein extract preparation or RNA extraction.

For reporter assay analysis, HEK293 cells were cultured in HiGlutaXL Dulbecco's Modified Eagle Medium, High Glucose (HiMedia AL007G, India) with 10% fetal bovine serum (HiMedia RM9952, India) and 1% penicillin and streptomycin (HiMedia A001, India), and maintained at 37ºC and 5% CO_2 under optimum humidity.

2.7 Luciferase reporter assay

Luciferase reporter assay was performed to check the regulatory activity of ZEB1 binding site present on the *COL8A2* promoter-proximal region, and to assess the target site for miRNA binding in the 3'UTR region of *FEN1* gene. For ZEB1 regulatory assay, pGL4.23 luciferase reporter vector with minimal promoter and pGL4.74 renilla vector were used (Promega, USA). Complementary oligos containing the ZEB1 binding site (ACAGGTG) present at +374 COL8A2 gene transcription start site (TSS) were designed either in concatenated double dose (2X) or triple dose (3X). Sequence details of these oligos are listed in **Table 2.5**. Equimolar amounts of these oligomers were dissolved in annealing buffer (100mM HEPES and 100mM potassium acetate) to obtain a final concentration of

40ng/µl of annealed DNA and incubated at 90^{o}C for 3minutes, followed by incubation at 37^{o}C for an hour. These oligos were either stored at -20^{o}C or cloned into KpnI-XhoI double digested pGL4.23 vector. Similarly, to assess the target site for miRNA binding, pMIR-report luciferase vector (Invitrogen) containing CMV promoter and pGL4.74 Renilla vector as reporter control plasmid. Target oligos (67bp) for mIR-1236 as identified through bioinformatics analysis on the 3'UTR of *FEN1* gene (**Table 2.5**), were synthesized and annealed as earlier. The annealed oligos were cloned into XhoI-NotI double digested pMIR-report vector at 3'UTR of the luciferase gene.

Table 2.5: Sequence details of the oligomers used for creating luciferase constructs. Pairs of complementary oligomers used for pGL4.23 vector includes KpnI (K) and XhoI (X) overhangs (denoted in lowercase) along with ZEB1 binding site (**ACAGGTG**). Oligomers designed for miRNA target analysis to be cloned into pMIR-report vector includes XhoI-NotI overhangs (denoted in lowercase), miRNA target site (in ***BOLD***) and position of c.4150G>T SNP (**G/T**).

Name	Sequence (5'-3')
2X 372 KX F	c**ACAGGTGACAGGTG**c
2X 372 KX R	tcgag**CACCTGTCACCTGT**ggtac
3X 372 KX F	c**ACAGGTGACAGGTGACAGGTG**c
3X 372 KX R	tcgag**CACCTGTCACCTGTCACCTGT**ggtac
WT1236/G	tcgagTGAAAGTGATAGATAGCAACAAGTTTTGGA***GAAGAGA***GAGGGAGATAAA AGGGGGA**G**ACAgc
WT1236/T	tcgagTGAAAGTGATAGATAGCAACAAGTTTTGGA***GAAGAGA***GAGGGAGATAAA AGGGGGA**T**ACAgc
MT1236/G	tcgagTGAAAGTGATAGATAGCAACAAGTTTTGGA***TTTTTTT***GAGGGAGATAAA AGGGGGA**G**ACAgc

In order to check the reporter activity, 10^5 HEK293 cells were seeded into each wells of 12-well plates. Upon 80% confluency, the cells were co-transfected with luciferase constructs (1µg of 2x372-pGl4.23 or 3x372-pGl4.23) along with renilla vector (pGL4.74, 10ng). Lipofectamine 2000 (Invitrogen, USA) along with serum free Opti-MEM (Invitrogen, USA) was used as the transfection medium. Empty pGL4.23 vector was used a negative control

and normalization of transfection efficiency was done by checking the Renilla reporter activity.

Similarly, 1µg of pMIR-1236 clones (pMIR-WT1236/G, pMIR-WT1236/T, or pMIR-MT1236) along with pGL4.74 vector (5ng) and hsa-miR-1236-3p mimic (10pmoles, Invitrogen) were co-transfected into 80% confluent HEK293 cells. Empty pMIR and renilla pGL4.74 vectors were used for negative control and normalization respectively.

Cell lysates were prepared 24-hour post transfection by using Dual-Luciferase® Reporter Assay System (Promega, USA). Reporter activities were measured with Varioskan® Flash Multimode reader (ThermoFisher Scientific, USA) following the manufacturer's instructions. Luciferase activity from the transfected cell-lysates were plotted after normalization with Renilla reporter activity and further analyzed. Each of the experiments was repeated independently with at least three replicates.

2.8 Bacterial culture and plasmid amplification

Vectors and vector constructs were amplified in *E.coli* DH5 α post chemical transformation and selected on Luria-Bertani (LB) agar plates supplemented with ampicillin (100mg/mL). Positive bacterial clones, identified by colony PCR using the vector specific primers (**Table 2.1**), were grown overnight in LB broth with ampicillin at 37ºC and 180rpm. Plasmids were extracted using Plasmid Miniprep Kit (QIAGEN, Germany).

2.9 Comet Assay

Each frozen whole blood sample (7µl) were mixed with 180µl of 0.6% low melting point (LMP) agarose in 1X PBS and applied as thin coating with the help of cover-slips onto 1% normal melting point (NMP) pre-coated, dry microscopic slides which were allowed to set at 4ºC for an hour. Comet Assay was performed with these slides following the protocol

described by Yang *et al.* 2014, with few modifications.[25] Alkaline electrophoresis (at 0.8V/cm) was performed for 20 minutes at 4ºC. The slides post washing, were stained in dark with 100 μl of 5:100 dilution of FastStart Universal SYBR Green Master-mix (Roche, Switzerland) in Tris-EDTA buffer for 2 hours in dark and at 4ºC, followed by water wash for 2-3 minutes and left to dry overnight at room temperature and observed under 10X fluorescent microscope (BX51, Olympus). Fifty individual fluorescent comet images per sample were analyzed using CASPLab v1.2.3beta2 comet analysis software (University of Wroclaw, Institute of Theoretical Physics, Poland). Endogenous DNA damage of the PBMC samples is expressed as the natural log of Olive tail moment (Ln-OTM) of the comets. Graphs were plotted on Graphpad Prism7 and significance was calculated using SPSS software. A representative figure depicting a processed comet image is shown in **Figure 2.1**.

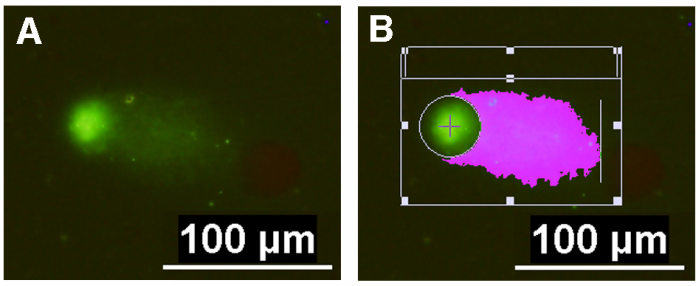


Figure 2.1: Representative image of a comet assay analysis on CASPLab platform.

2.10 Bioinformatic prediction of miRNA sites in 3'UTR of FEN1

In order to analyze the putative miRNA binding sites at the 3'UTR of FEN1, the most frequently used computational algorithms such as:

miRWalk 2.0 (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/index.html),

miRanda (http://www.microrna.org/microrna/home.do);

PicTar2 (http://www.pictar.org),

RNA22 (https://cm.jefferson.edu/rna22/)32,

TargetScan (http://www.targetscan.org);

FindTar3 (http://bio.sz.tsinghua.edu.cn) and

SegalLab (https://genie.weizmann.ac.il/index.html) were used.

SNPinfo database (http://snpinfo.niehs.nih.gov/) was also used to identify miRNA binding sites involved in the polymorphic site *FEN1* c.4150G > T.

2.11 RNA isolation from tissue samples

Non-processed corneal tissues were used for RNA extraction. Non-transplant grade control corneal tissues (not older than a week) were obtained from the Cornea Department of L. V. Prasad Eye Institute, Bhubaneswar in transplantation medium and transported on ice until extracted. The tissue samples were either trephined into 0.25mm endothelial layer with stroma or used as entire thickness corneal button for RNA extraction. In case of FECD cases, post-operative tissues were collected in RNA*later* solution (Invitrogen, USA). Each of these tissues were rinsed in nuclease free water (NFW) to remove transplantation medium / RNA*later* and followed the manufacturer's protocol to extract RNA (RNeasy Mini kit, QIAGEN). Cells were gently lysed with 350µl RLT buffer and mixed with an equal volume of 70% ethanol (in NFW). This solution was then passed through the RNeasy mini column at 13000rpm (10-15^{o}C). The column was then washed once with 750µl RW1 buffer and twice with 500µl RPE buffer to remove lipids, protein and DNA from the silica membrane. RNA was then eluted from the silica membrane with 20µl nuclease free water and kept on ice until quantified. RNA was quantification was done spectrophotometrically by Nanodrop 2000 (ThermoFisher Scientific, USA).

2.12 Reverse transcription and quantitative real time PCR to analyze expression of transcripts

Extracted and quantified RNA were immediately converted into cDNA using Verso cDNA Synthesis Kit (ThermoFisher Scientific, USA) and stored at -80ºC until further use. As the

total RNA extracted from FECD or control tissues were of limited quantity, entire eluted RNA was converted to cDNA by mixing it with anchored oligo dT and random hexamers in 3:1 ratio along with enzyme and buffer-mix into 20µl reaction volume. The cDNA synthesis was done at 45^{O}C for 30 minutes in a C100 Touch Thermal Cycler (BioRad, USA) followed by enzyme inactivation at 95^{O}C for 2 minutes. Following primers as listed in **Table 2.6** were used for quantitative gene expression analysis.

Table 2.6: List of Primer sequences employed during quantitative real-time PCR analysis.

Name	Sequence (5'-3')	NCBI Sequence Accession Number
MCT1 qRT F	TGCGTGGGTACTGGAACAAGC	NM_003051.3
MCT1 qRT R	TGCAGGTCAAATCCAAATATCGTT	
MCT2 qRT F	TGGTGATAGCAGGAGGCTTA	NM_001270623.1
MCT2 qRT R	AACCTAAACCTGTAATGAATCCCA	
MCT4 qRT F	TGGGGTCATCACGGGGTTG	NM_001206951
MCT4 qRT R	CGCTTGCTGAAGTAGCGGT	
GAPDH qRT F	GAAGTCAGGTGGAGCGAGG	NM_008084.3
GAPDH qRT R	GCCCAATACGACCAAATCAGAG	

qRT-PCR was performed using 8 μ M transcript-specific primers (**Table 2.6**, GCC Biotech, India) and FastStart Universal SYBR green mastermix (Roche, Switzerland) in ABI 7500 Real Time PCR System (Applied Biosystems, USA). Cycle threshold (Ct) values were exported in MS Excel and fold changes of the desired genes were calculated with respect to control after normalizing the values with internal control gene, GAPDH. The experiment was done with three technical replicates along with no-template-control. Primer specificity of the designed primers were confirmed from melt curve analysis.

2.13 Immunohistochemistry

Localization studies of Monocarboxylate transporters (MCTs) was done in non-transplant grade corneal tissues extracted (not over a week old) and submerged in corneal transplantation medium, maintained at 4ºC. Whole cornea was trephined (Hessburg-Barron Vacuum Trephine) and punched into a corneal button comprising of 0.25mm thick endothelial layer along with a portion of stroma. The endothelial corneal button was rinsed thrice in 1X PBS for 5 minutes to remove any residual corneal transplantation medium and fixed in freshly prepared 4% paraformaldehyde (Sigma-Aldrich, USA) in 1X PBS for 20 minutes. Three washes with 1X PBS were performed for 5minutes each, followed by PBST (1X PBS + 0.5% Triton-X) washes for 10minutes each, twice. 10% Blocking solution (BS) was prepared with normal horse serum (NHS) in PBST and the tissue samples were kept in BS for 40 minutes. The tissues were then subjected to primary antibody (**Table 2.7**) solution at a dilution of 1:100 in BS, kept at RT for an hour and followed by overnight incubation at 4ºC with gentle shaking. The tissue samples were rinsed off of unbound excess primary antibody with PBST washes, followed by incubation with respective secondary antibody at 1:250 dilution (in PBST) for 2 hours at RT. After washing off the excess secondary antibody with series of PBST and PBS washes, tissue samples were stained with 2.5µg/ml DAPI for 30 minutes and washed again with 1X PBS. Following this, the tissue samples were mounted on slides keeping the endothelial side up (convex side up) and applying sufficient mounting medium (ProLong Antifade Reagent, ThermoFisher) before putting the coverslip. The prepared slides were kept at RT for 2-3 hours before viewing under 63XLSM 780 Confocal microscope (Carl Zeiss, Germany). IHC staining was performed in 24-well culture plates and each corneal button were equally sectioned into four parts to be used for each MCT1 staining including negative control in duplicates. Each washing step comprised of three consecutive washes with gentle shaking (20rpm) over a nutating mixer (GyroMini Nutating Mixer, Labnet).

Table 2.7: List of antibodies used in this study.

Antibody Name	Type	Manufacturer Code	Raised in animal	Manufacturer
MCT1	Primary	sc-14916	Goat	Santa Cruz Biotechnology
MCT2	Primary	sc-50322	Rabbit	Santa Cruz Biotechnology
MCT3	Primary	sc-102019	Rabbit	Santa Cruz Biotechnology
MCT4	Primary	sc-376140	Mouse	Santa Cruz Biotechnology
Alexa Fluor 488 anti-goat IgG (H+L)	Secondary	A21467	chicken	Invitrogen Antibodies
Alexa Fluor 594 anti-goat IgG (H+L)	Secondary	A21468	chicken	Invitrogen Antibodies
Alexa Fluor 488 anti-mouse IgG (H+L)	Secondary	A21200	chicken	Invitrogen Antibodies
Alexa Fluor 594 anti-rabbit IgG (H+L)	Secondary	A21442	chicken	Invitrogen Antibodies

2.13.1 Image analysis

Images captured in confocal microscope were generated in .lsm file format and observed in Zeiss LSM File Browser software. To analyze the spatial localization, Z-section images were viewed using “ortho” and “cut” options to visualize distribution of signal at a single pane in comparison to whole thickness endothelial single cell layer. Generated images were exported in TIFF format and processed with Adobe Photoshop (CS6 for Mac OSX) to crop and rearrange the figures.

Chapter 3

Genetic association of *TCF4* with FECD in Indian population

3.0 TCF4 POLYMORPHISMS ARE GENETICALLY ASSOCIATED WITH FECD

3.1 INTRODUCTION

Multi-generational linkage studies have identified four chromosomal loci that are linked to FECD. One of the loci in long arm of chromosome 18 (18q21.2-21.32) was identified again as a significant locus during GWAS conducted by Baratz and group in the Caucasian American population.[13, 19] The polymorphism (rs613872) in chromosome 18, which was picked up as an associated marker SNP, falls in the intronic region of TCF4 gene.[13] Subsequent studies in various Caucasian populations have substantiated the strong association of this polymorphism with FECD.[11, 19, 21, 79, 81, 85] In a similar study, it has been also shown that Chinese individuals are not polymorphic at rs613872 position but show a strong association with two other polymorphisms, rs17089887 and rs17089925 in the intronic region of TCF4 gene.[20] Associated polymorphisms are genetic markers that hint at disease predisposition around the said locus. Apart from single nucleotide polymorphisms, Weiben *et al*., have identified an expansion in the microsatellite region, CTG18.1 allele of TCF4 intron to be associated with FECD in Caucasian American cohort.[21] The haplotype comprising risk alleles from the associated genetic markers, rs613872 and CTG18.1 allele, are reported to be significantly co-inherited among Caucasian FECD individuals.[21, 81] However, the genetic markers in TCF4 contributing towards FECD, differs in ethnically heterogenous individuals.[20, 21] With the lack of any study in Indian population, there was a great impetus to carry out this investigation in a sample Indian population.

Statistical data comparing the prevalence of FECD in India remain elusive. However, during a study conducted from an eminent tertiary eye care center in South India on frequency distribution and outcome analysis of patients requiring penetrating keratoplasty (PK) as a surgical intervention for relief from ocular dystrophies; FECD was accounted as the third most frequent (16.6%) reason for PK.[5] Studies comprising Indian late onset FECD subjects are very scant and hence the genetic contributors for the disease specific to this region of the

globe are veiled.[9] The aim of this study was to assess the genetic association of various *TCF4* polymorphisms*, i.e.*, rs613872, rs17089887, rs17089925 and CTG18.1 allele among Indian FECD patients.

3.2 RESULTS:

3.2.1 Demographics

Diagnosed with FECD, forty-four unrelated patients enrolled for this study, were recruited from two eastern provinces of India, i.e., Odisha and West Bengal. As is seen in earlier demographic studies,[20, 48, 49] FECD affected population in India replicates the fact that females are the most susceptible group for the disease (males = 40%, females = 59%). All the cases were within the age range of 38 to 80 years, with the mean age being 60 years. Not significantly distinct (*P= 0.168*) were the control group participants falling in the age range of 47 to 81 with a mean of 63 years. Males (58%) however slightly dominated the control population. Despite the gender bias, the study groups were not significantly distinct (*P= 0.084*, **Table 3.1**).

Table 3.1: Demographics of the study participants.

Variables	**Cases**	**Control**	***P* value**
Number of Individuals	44	108	
Number of females (%)	26 (59.09)	45 (44.11)	0.084
Mean Age (SD) in years	60 (9.24)	63 (8.52)	0.168

3.2.2 SNP rs17089887 is significantly associated with FECD in Indian population

Genetic association analysis was done for *TCF4* intronic polymorphisms and FECD. All the study participants have their genotype frequencies in Hardy-Weinberg equilibrium. The allele distribution and association results for all the SNPs are described in **Table 3.2**. After

age and sex correction, we found that only one of the intronic *TCF4* SNPs, rs17089887, is significantly associated (*Genotype P value = 0.008*) under dominant model of inheritance (*P value=0.019*) in our population with "T" allele (*Allelic P value = 0.013*) imparting 2.073 (*95% CI = 1.18 - 3.62*) increased chances of acquiring FECD (**Table 3.2**). We observed that individuals with TT genotype for rs17089887 are at 65.9% increased risk. Its significance remains even after carrying out 10,000 permutations (*P= 0.048*). Keeping type I error at 5% and degree of freedom as 1, the current study is at 75% power with effect size of 0.4. The SNP rs17089925 which was previously outlined to be associated in Chinese population, fails to be so in our population[20]. Whereas, the much-cited polymorphism, rs613872, show a marginal association with respect to genotype (*Df2, P value = 0.03*) but shows no association with respect to alleles *(Df1 P value = 0.246*).

Table 3.2: Association results of genotyped SNPs. Associated SNP is written in bold. Df = degree of freedom, OR = Odds Ratio, CI = Confidence interval. * Risk allele; *P* Value† = *P* values obtained from logistic regression under dominant (DOM, major allele homozygotes vs. heterozygotes+ minor allele homozygotes), recessive (REC, major allele homozygotes+ heterozygotes vs. minor allele homozygotes) and additive (ADD, major allele homozygotes vs minor allele homozygotes) models after correcting for age and gender; excluding 4 controls for which genotype data for any of the three SNPs were missing. *P* Value‡ = *P* values obtained after data were subjected to various tests such as, X^2 (Df2) = Chi squared test for difference between case and control genotypes; X^2 (Df1) = Chi squared test for difference between case and control alleles and 10,000 permutation tests for allele distribution.

SNP		Type	Control		FECD		Model	OR (95% CI)	*P* Value[†]	Tests	*P* Value[‡]
			n	%	n	%					
rs17089887	Genotype	TT	48	44	29	65	DOM	2.37 (1.14-4.93)	**0.01**	X^2 (Df2)	**0.008**
		TC	47	43	13	29	REC	0.37 (0.81-1.75)	0.19		
		CC	12	11	2	4	ADD	0.37 (0.10 -1.41)	0.12		
	Allele	T*	141	66	70	80		2.07 (0.81-1.93)	**0.01**	X^2 (Df1)	**0.016**
		C	71	33	17	19				10K permutation	0.048
rs613872	Genotype	TT	86	79	29	65	DOM	0.49 (0.22-1.07)	0.07	X^2 (Df2)	**0.03**
		TG	18	16	14	31	REC	1.65 (0.17-15.22)	0.65		
		GG	4	3	1	2	ADD	1.26 (0.21-7.48)	0.98		
	Allele	T	188	87	72	82		1.50 (0.50-1.88)	0.79	X^2 (Df1)	0.246
		G	26	12	15	17				10K permutation	0.552
rs17089925	Genotype	CC	57	53	26	59	DOM	1.26 (0.62-2.57)	0.51	X^2 (Df2)	0.35
		CT	42	39	16	36	REC	0.589 (0.12-2.89)	0.51		
		TT	8	7	2	4	ADD	0.63 (0.17-2.29)	0.71		
	Allele	C	154	72	68	77		1.37 (0.50-1.71)	0.24	X^2 (Df1)	0.405
		T	60	28	19	22				10K permutation	0.755

3.2.3 *TCF4* intronic CTG trinucleotide repeats shows significant association with FECD

The threshold limit of CTG trinucleotide repeats (>50) in *TCF4* gene assumed by Wieben and colleagues as a disease susceptibility marker for FECD was used in this study.[21] Agarose gel electrophoresis followed by direct sequencing was employed to efficiently detect at least fifty repeats of CTG18.1 allele (dbSNP rs193922902). Representative chromatograms depicting normal and extended repeats are as shown in **Figure 3.1**.

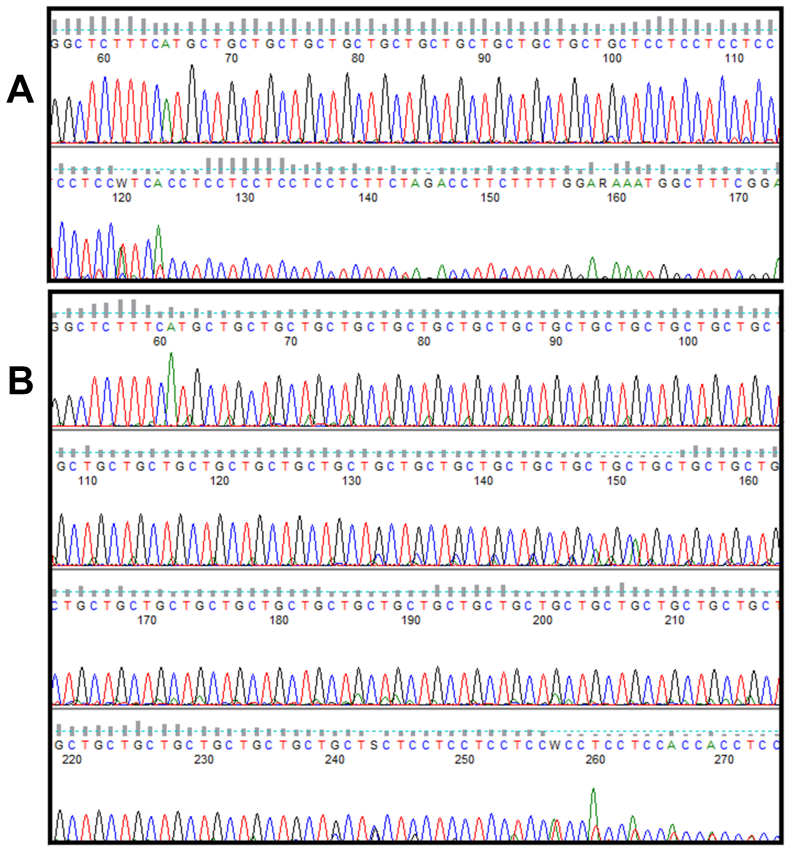


Figure 3.1: Sequence chromatogram of a control individual (A) with 12 repeats and an FECD patient (B) with 61 repeats.

The frequency distribution of both the alleles of control and FECD populations illustrate that 15/44 (34%) FECD cases and 5/97 (5%) control individuals harbored these extended trinucleotide repeats (≥50) (**Figure 3.2 and Table 3.3**). Out of these 15 patients, 14

individuals had at least one "T" allele of rs17089887 and the combined haplotype (T-X) shows significant association with the disease ($P=2 \times 10^{-4}$).

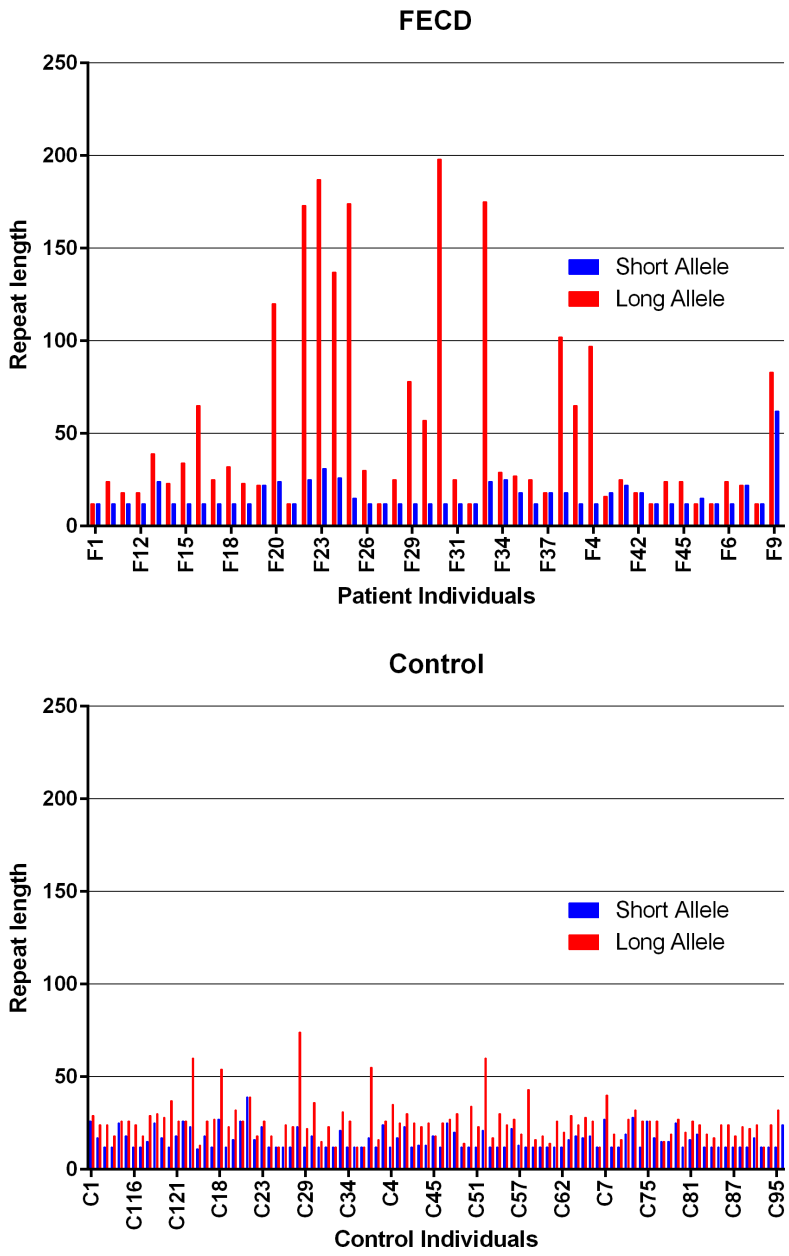


Figure 3.2: Frequency distribution of both the alleles of CTG18.1 in FECD and control population.

Table 3.3: Result of genotype and haplotype analysis of *TCF4* SNP rs17089887 and CTG18.1 variants in Indian FECD cohort.

CTG18.1	Cases (n=44)	Controls (n=97)	OR (95% CI)	P value
XX	1	0	6.7 (0.2-168)	0.24
XS	14	5	8.5 (2.8-25)	0.0001
SS	29	92	0.1 (0.03-0.3)	0.0001
rs17089887	**(n=44)**	**(n=107)**		
TT	29	48	2.37 (1.14-4.93)	0.013
TC	13	47	0.37 (0.81-1.75)	0.19
CC	2	12	0.37 (0.10 -1.41)	0.12
Haplotype				
T-S	0.676	0.644	0.71 (0.41 to 1.22)	0.5977
C-S	0.138	0.319	0.47 (0.23 to 0.96)	$1.2x10^{-3}$
T-X	0.131	0.024	6.92 (2.38 to 20.11)	$2x10^{-4}$
C-X	0.056	0.013	4.89 (1.19 to 20.07)	0.0303

3.2.4 Endothelial count is correlated with presence of extended TNR

Bilateral endothelial cell-count of every participant was noted and the minimum count was taken further for analysis. As reported earlier, individuals affected with FECD show significantly decreased (P<0.001) endothelial cell count as compared to control.[194-196] Furthermore, FECD cases with atleast single allele for expanded TNR (XX or XS) showed significantly decreased endothelial count than homozygous individuals with short repeats (P=0.004). This substantiates the prominent pathological effect imparted by expanded repeats which consequent into increased apoptosis of endothelial cells as reflected by the significantly decreased endothelial cell-count in FECD individuals. However, the same was not recapitulated among control individuals (P=0.44); which can be rationalized as smaller fraction of individuals (n=5) carrying expanded repeats. These results suggest confounding factors (yet unknown) responsible for FECD causation, including expanded TNR could be affecting endothelial cell count in these patients.

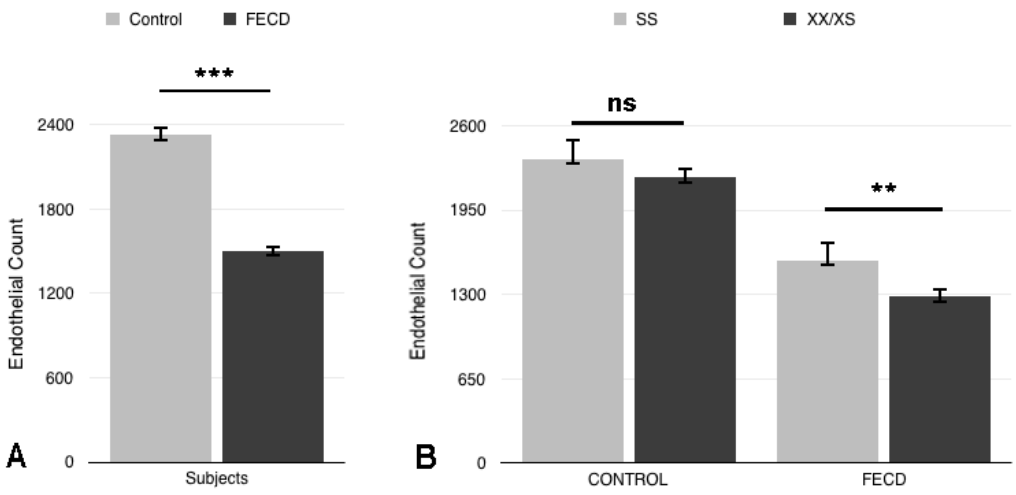


Figure 3.3: Comparison of Endothelial count with expanded repeat. (A) Mean of minimum endothelial count of FECD and control showed significant difference with P value <0.001. (B) Mean of minimum endothelial count of individuals with SS genotype (homozygous short repeats) in comparison to XS/XX individuals (with atleast one expanded repeats) showed significant difference in FECD cohort (P=0.004) but not in control population (P=0.44).

3.3 DISCUSSION

Employing keratoplasty procedures alone, 14,153 corneal transplantations were done solely for correcting endothelial cell failure associated with FECD in the year 2013 in the United States.[197] According to an earlier report conducted at a tertiary eye care center in South India in 2004, 24 (16.6%) out of the 144 corneal buttons of corneal dystrophy patients who underwent PK were tested positive for FECD.[5] Therefore, early stage FECD diagnosis and comprehension of the disease pathomechanism has become an absolute necessity for the rapidly rising number of patients undergoing keratoplasty every year globally. The present study attempts to elucidate the Indian scenario of FECD. However, the study participants gathered for this purpose exhibits a slight male dominance among the control population. As reported by Lewallan and colleagues, women in developing countries face sociocultural influences and financial barrier due to which, they have lesser access to treatments for visual impairment in comparison with men.[198] India being a developing country faces similar

hindrances that are reflected in the marginally skewed gender ratio of the control population. Despite the gender bias, we ensured that the study groups are not significantly distinct.

With increasing evidence of *TCF4* being a prominent genetic contributor for FECD and scarce studies to elaborate the Indian scenario, our study aimed to investigate the association of various polymorphisms of *TCF4* gene with patients suffering from FECD in the Indian cohort. We observed that rs17089887 and CTG18.1 show significant association with the disease, even after 10,000 permutation tests (**Table 3.2**). Unlike the Chinese cohort, the allele "T" of rs17089887 was found to be the risk allele for our population. These flip-flop associations can be reasoned as a consequence of difference in the Linkage disequilibrium (LD) structure of the gene in ethnically distinct groups.[199] When the LD structure of the region comprising these SNPs was compared between Chinese Han population residing in Beijing (CHB) and Gujarati Indians from Houston (GIH), we found it to be distinct in each group (**Figure 3.4**).

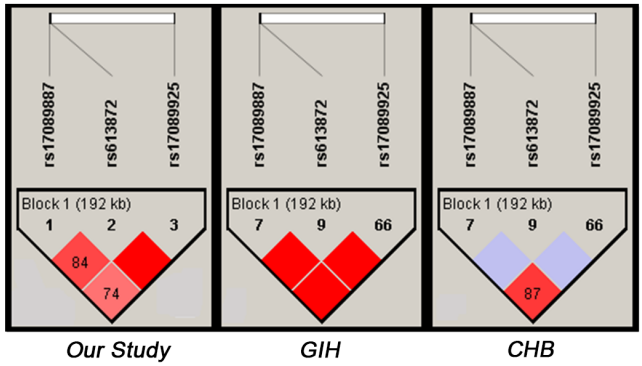


Figure 3.4: LD plot comparisons of three *TCF4* intronic SNPs in different ethnic groups. Situated at the third intron of *TCF4*, the LD plot of rs17089887, rs613872 and rs17089925 depicts high linkage disequilibrium amongst these SNPs in our study population and the GIH cohort; whereas only rs17089887 and rs17089925 are in LD in CHB population. The plot was constructed using Haploview 4.2 and the numbers in the diamonds represents the D' values.

All the three SNPs are in complete LD in GIH cohort, coinciding with the LD plot for this study; whereas in CHB, rs17089925 and rs17089887 is the only pair to be in LD with each

other. Indian FECD subjects do not show distinct association with rs613872 at the current power of the study; despite the fact that this population exhibits variation in polymorphism (T: 0.87, G: 0.12) unlike the Chinese population which is not polymorphic for this genomic position.[20] However, the genotypic frequency of this SNP shows marginal association with FECD (*P value = 0.03*); which explains that patient and control cohorts exhibit distinct distribution of genotypes such that individuals with genotype TT are at higher risk (65.9%) for contracting the disease. For rare diseases like FECD, the critical sample size sufficing the required genetic power (80%) is often difficult. Similar studies in future with a larger sample size and adequate power are required to improve such marginal associations.

A genetic marker, be it a microsatellite with two, three or four base pairs of short tandem repeat pattern or a variation at a single DNA base pair position (SNP), is polymorphic if the variation is seen in more than 1% of a population. Such polymorphic markers are said to be associated with a disease if there is a difference between the allele frequencies of unrelated diseased subjects (cases) and those without disease (controls). The association between the genotyped marker and the disease phenotype can be due to various reasons. The associated marker can be a causal genetic variant where the change in the DNA sequence is itself contributing towards the disease trait or that the association reflects a nearby DNA element that is in LD with the polymorphic marker and is functionally responsible for the disease.[185, 200] The association can also be a consequence of population stratification. The genetic markers associated in this study are rs17089887 and CTG18.1 allele. In context with the previously associated polymorphisms of *TCF4* gene in various populations, CTG18.1 allele remains consistently associated throughout. This suggests that CTG18.1 can be a putative causal variant for FECD, although it is subject to rigorous evaluation in order to avoid false assignment of causality.

With the help of direct sequencing, STR assay and genomic Southern blots, Wieben and colleagues were the first to report that repeat lengths arbitrarily assumed to be more than 50

units was associated with FECD in the Caucasian cohorts.[21] Using a combination of STR assay and TP-PCR, Mootha *et al.* also confirmed its strong association with the disease.[81] Such aberration in the repeat region, although present in almost 5% of normal population, is highly prevalent among FECD affected subjects. The techniques used in our study are PCR based amplification of the microsatellite region which consequence into a certain degree of differential amplification of size-variant alleles. The competitive nature of PCR favors amplification of shorter alleles over the extended ones.[201, 202] This technique therefore has a slight possibility of under-estimating the repeat lengths in a minority of individuals unlike the methodologies used in previous reports. Despite this under-estimation, the prevalence percentage (34%) obtained is still significantly associated ($P= 2x10^{-4}$) with FECD in our population. It was revealed through recent studies in neurodegenerative diseases like myotonic dystrophy and Huntington's disease that such unstable trinucleotide repeats can lead to selective cell death through a toxic gain-of-function mechanism by sequestering these RNA in ribonuclear foci.[203] Taking into account, the apoptotic nature of degenerative endothelial cells in FECD, some researchers have also hypothesized that these unstable intronic expansions might be responsible for incurring RNA stress in these cells and thereby causing apoptosis.[33, 203]

The haplotype (G-X) of rs613872 risk allele (G) and expanded CTG18.1 allele (represented as X) respectively, depicted significant ($P= 5.9 \times 10^{-19}$) association with the disease trait in Caucasians.[81] In this study, we replicate similar association with an Indian cohort; the expanded CTG repeats accompanying the disease trait along with the "T" allele of rs17089887; with the haplotype T-X being significantly associated ($P= 2x10^{-4}$). These SNPs, rs613872 and rs17089887, are situated very close to each other (2kb); and thereby share an appreciable LD between them (**Figure 3.4**). Combined signal of rs17089887 and CTG or rs613872 and CTG suggests the presence of significant disease-causing changes in the nearby regions of these alleles that are physically linked. Further studies catering to the

quest of mRNA profile of the various repertoire of *TCF4* RNA may solve its role in the involvement of disease progression.

Many subsequent studies have since been reported across the globe, substantiating the pathogenic role of CTG18.1 allele in FECD progression.[11, 82, 83, 85, 86, 129, 204-206] It is however interesting to note that in comparison to an average of 80% FECD affected Caucasians, only 34% of Asian FECD individuals harbor these expanded repeats. Thereby, indicating the presence of other confounding factors responsible to cause FECD in these parts of the globe. These expanded repeats are reported to accumulate in nucleus in the form of RNA foci, sequestering proteins involved in the splicing machinery, e.g., MBNL1 and MBNL2.[85, 130] This consequently result into multiple mis-splicing events in the endothelial cells. Most of these misspliced pre-mRNA transcripts coincide with those identified in DM1 affected tissues, suggesting similar pathological errors in diseases associated with expanded trinucleotide repeats.[85] The threshold for minimum repeat-length beyond which, it is rendered as a marker for FECD had not been demarcated until recently, when Gottsch *et al*., identified that individuals with monoallelic expansion of TNR require atleast more than 130 CTG repeats for inducing pathogenicity as compared to homozygous expanded-TNR individuals, for whom 40 repeats are sufficient for forming pathogenic nuclear foci.[129]

Previous studies have contributed to the fact that mutations and variations in *TCF4* have been afflicted for diseases like Pitt-Hopkins Syndrome,[207] Primary sclerosing cholangitis,[208] Schizophrenia[209-212] and FECD.[13, 21, 205] A basic helix-loop-helix transcription factor, *TCF4* encodes for a 667 amino acid protein called E2-2 protein that recognizes the Ephrussi-box (E-box) motif (CANNTG), globally recognized as immunoglobulin enhancers. It is a key regulator in epithelial-mesenchymal transition (EMT) that can be notably deduced from the fact that *TCF4* knockdown affects many genes involved in EMT regulation like *SNAI2*, *ZEB2* and genes involved in TGF-beta signaling pathway.[213] Variations in this gene have been associated with diverse tissue specific expression in different diseases;[214] probably

because of the diverse repertoire of *TCF4* isoforms transcribed in various tissues. Mutations in glutathione transferase gene, *ATP/GTP binding protein-like 1 (AGBL1*) that have been recently identified with FECD causation, lead to dissociation of *AGBL1* and *TCF4*. Thereby, suggesting a possible combined effect delivered by these two genes in the pathogenesis of FECD.[12]

With recent studies adding on more genes as contributors towards FECD, it is becoming even more difficult to understand its pathomechanism. Our study reports for the first time the genetic scenario of a sample Indian population showing significant association between *TCF4* polymorphisms and FECD; thereby strengthening its role as a major contributor globally.

Chapter 4

Genetic association and role of *ZEB1* in FECD progression

4.0 GENETIC ASSOCIATION AND ROLE OF ZEB1 IN FECD PROGRESSION

4.1 INTRODUCTION

In the previous chapter, we elaborated on how the expanded trinucleotide repeats in the transcription factor gene, TCF4 (Transcription factor 4) is responsible for incurring nuclear stress in FECD affected corneal endothelial cells. Unlike polymorphic variations in TCF4, researchers have studied the association of another transcription factor ZEB1 (Zinc finger E-Box binding homeodomain) through linkage analysis in FECD affected families and identified mutations that imparted 2% genetic load in causing the disease.[18] ZEB1 is a common genetic contributor for causing variants of corneal dystrophies like PPCD and FECD. Nonsense mutations or mutations resulting in premature termination of ZEB1 was associated with a severe form of corneal dystrophy- posterior polymorphous corneal dystrophy, type 3 (PPCD 3). [18, 22, 72, 73] But Mehta *et al*. studied this gene in Chinese population and associated it with the milder form of corneal dystrophy, i.e. LO-FECD, comprising of a missense (N696S) and a silent (D64D) mutation in exonic regions of *ZEB1*; both of which were identified as non-pathogenic through SIFT and Polyphen analysis.[74]

Riazuddin *et al.* later also identified five missense mutations in the American cohort of FECD probands; all of which identified as potentially pathogenic and sufficient to cause FECD based on morpholino studies in zebrafish embryos.[18] One particular mutation, Q840P was analyzed to be co-segregating with FECD4 locus at chromosome 9 in a multi-generational family, causing increased severity in 7/12 FECD affected individuals.[18] In contrast to nonsense mutations identified in PPCD,[22, 75] only missense mutations of ZEB1 were associated with FECD cases.[18, 74] The truncated ZEB1 transcripts resulted in improper nuclear localization of the encoded protein which indicates haploinsufficiency of ZEB1 could be responsible for PPCD.[77] Conversely, the missense mutations of ZEB1 reported in FECD cases did not significantly alter the nuclear localization or protein abundance.[77] These reports provide the impetus to analyze whether a plausible DNA-binding defect of ZEB1 is

in play for FECD. Although the direct functionality of ZEB1 in causing these corneal dystrophies are yet unclear, it can be suggested that PPCD and FECD could be allelic variants of a continuum of diseases in which genes interact to modulate the expressivity of the phenotype.[18]

From the previous studies, we understand that mutations in ZEB1 are identified to have genetic association with FECD.[11, 18, 74, 76, 77, 215] However, there isnt any particular mutation or variation that is common among various ethnic groups studied; which suggests there could be varying degree of genetic load contributed by this gene in different cohorts. Also, having a recurrent mutation or polymorphic marker in the affected population simplifies an early detection for the disease. With this impetus, we screened a sample Indian population to find the polymorphic markers or mutations that identifies ZEB1 association with FECD in Indian population.

Experimental validation for functional role of ZEB1 in human corneal endothelial cells reveal that the transcription factor directly binds to the COL4A3.[216] However, *in silico* promoter analysis of COL4A1, COL4A2, COL4A3, COL4A4, COL4A5, COL4A6 and COL8A2 showed possible ZEB1 binding site in all of these collagen subtypes except for COL4A4.[22] Abnormal deposition of collagen has been reported in EO- and LO-FECD, with COL8A2 showing significantly irregular deposition as compared to a healthy cornea.[10] An experimental validation of ZEB1 binding on COL8A2 promoter can extend the current understanding on the functional implications of ZEB1 in causing FECD. With these lacunae, our study design is two-tiered; scan the exons and adjacent intron boundaries of ZEB1 for common variants or mutations that segregate differentially in patients and control cohort and experimental validation for ZEB1 regulation over COL8A2 gene.

4.2 RESULTS

4.2.1 Mutational spectrum of ZEB1 in FECD

Coding and flanking non-coding sequences of ZEB1 (Refseq Gene: NG_017048.1) was scanned for mutations or variations in a sample Indian cohort comprising of 41 FECD cases and upto 30 control individuals with healthy cornea. We did not find any of the previously reported pathogenic mutations, Q810P and A905T, in our study cohort; except for one variation (rs7918614) causing a synonymous amino acid change (D64D) in the second exon of ZEB1 gene, which was observed in 26.47% FECD cases (9/34) and 33% of the controls (9/30), thereby rendering it as a polymorphic non-pathogenic nucleic acid variation (**Table 4.1**).

Table 4.1 Genetic variations in ZEB1 gene identified in FECD cohort with a discovery set of 41 FECD cases and 30 controls.

Nucleotide change	Amino acid change	Intron/Exon	No. of individuals with atleast one non-ancestral allele	
			Cases	Control
rs7918614	D64D	Exon 2	9/36	6/23
c.321+8G>A		Intron 2	1/40	0/30
rs41289011		Intron 2	1/41	0/30
rs578116396		Intron 3	1/41	0/30
rs220057		Intron 4	19/40	21/30
rs220060		Intron 5	5/38	3/24
rs141194628	T807A	Exon 7	1/39	0/25
	Q810P	Exon 7	0/40	0/23
	A905T	Exon 8	0/40	0/19
rs182893838		Intron 8	1/39	0/27

We also found a novel missense mutation T807A (NP_001167564.1) previously unreported, in the 7th exon of ZEB1 gene. The SIFT (0.48) and PolyPhen (0.0051) values suggest it to be

tolerated and benign respectively. Apart from these mutations, we also found other variations in the intronic (non-coding) region of the gene. Of particular interest are SNPs, rs220060 and rs220057. While our current study was underway, the former polymorphism (rs220060) was very recently associated with FECD in a report comprising North Indian population.[76] Both the polymorphisms, rs220060 and rs220057, surfaced again in a recent report that screened mutations in ZEB1 gene in a Chinese LO-FECD pedigree, but without any association with the disease.[11] After the preliminary scanning, we conducted a validation experiment comprising of 63 FECD patients (41 from the discovery set + 22 new samples) and 129 controls (30 from discovery set + 99 new samples) to test the association of these polymorphisms. It was observed that unlike rs220060, rs220057G>A shows a strong association with LO-FECD in our population comprising primarily of east Indian cohort (**Table 4.2**; MAF for cases= 0.61, MAF for Control = 0.45, P= 0.016).

Table 4.2: Validation set to analyse ZEB1 polymorphism association with FECD.
Significance is kept at 5% threshold and mentioned in bold. *: Risk allele.

SNP	Type	Control		FECD		P value (Test)	OR (95% CI)
		n	%	n	%		
rs220057							
Genotype	CC	53	41	17	27		0.53 (0.27-1.02)
	CT	36	28	15	23.8		0.80 (0.40-1.61)
	TT	40	31	31	49	**0.026** (χ^2, Df2)	2.15 (1.16-4)
Major Allele	C	142	55	49	38.88	**0.016** (χ^2, Df1)	1.92 (1.246 - 2.96)
Minor Allele	T*	116	45	77	61	**0.003** (10K permutations)	
rs220060							
Genotype	AA	50	87.8	101	86.2	NA (χ^2, Df2)	0.88 (0.35-2.22)
	AG	8	12.1	14	13.7		0.86 (0.34-2.20)
	GG	0	0	0	0		NA
Major Allele	G	8	6	14	6.8	0.9 (χ^2, Df1)	1.18 (0.38-3.64)
Minor Allele	A	108	93.9	115	93.1	0.9 (10K permutations)	

4.2.2 ZEB1 binds at the promoter proximal region of COL8A2 gene

Krafchak provided bioinformatic proof of ZEB1 (a ubiquitous transcription factor) binding to the promoter of various collagen genes, including COL4A2, COL4A3, COL4A5, COL4A1, COL8A2 and COL4A6.[22] However, experimental evidence is available for only COL4A3 which indicated ZEB1 mediated alterations could be responsible for PPCD progression.[216] ZEB1 recognizes bipartite E-boxes (CACCTG, CAGGTG) and/or Z-boxes (CAGGTA). With this preliminary information, we scanned the 1500bp region encompassing 1000bp 5'UTR and 500bp downstream of transcription start site (TSS) for ZEB1 core binding sequence, using TRANSFAC (geneXplain, Germany). We found only one site at 374 bases downstream of transcription start site (TSS) that had a core score = 1 and matrix score =1. This particular region also falls under DNaseI hypersensitivity region and has histone acetylation signatures as per ENCODE data (**Figure 4.1**). The first intron of COL8A2 also shows ChIP validated binding of various transcription factors as per the ENCODE data. This information provides impetus to experimentally analyze this region for ZEB1 binding.

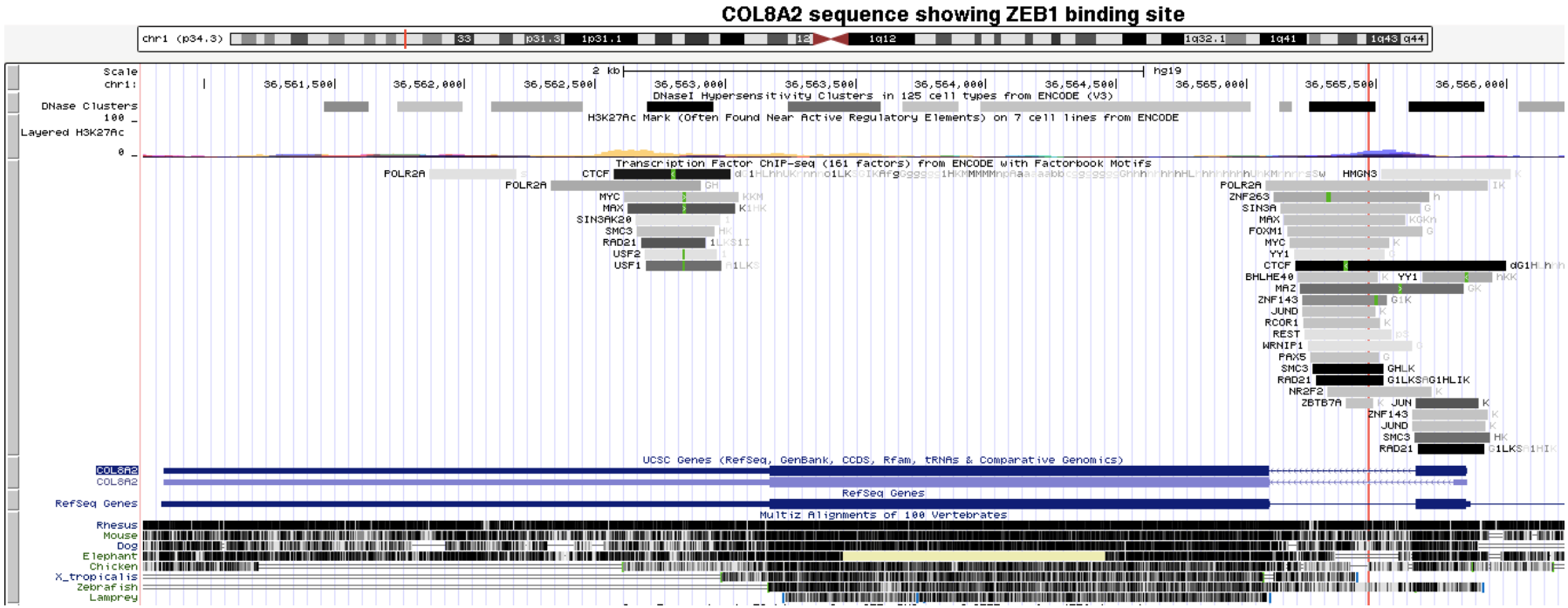


Figure 4.1: COL8A2 gene with tracks locating histone acetylation sites, DNaseI hypersensitivity region and Transcription Factors ChIP-seq data from ENCODE. Location of ZEB1 binding site in COL8A2 at +373 bases downstream of transcription start site is indicated in red vertical line (Adapted from UCSC genome browser).

The +374 sequence from the TSS was further analyzed for *in vitro* binding of ZEB1 using electrophoretic mobility shift assay (EMSA) or gel-shift assay. EMSA was performed using a 29bp oligo centered around the ZEB1 binding sequence in the first intron of COL8A2 gene and nuclear extract of cultured HCEnCs as a source for ZEB1 protein. Biotin labelled oligo, when competitively challenged with its unlabelled counterpart, showed disappearance of the DNA:Protein complex specific to this oligo sequence; suggesting this oligo is capable of forming DNA:protein complexes and thereby, might possess regulatory roles (**Figure 4.2**). However, we observed multiple bands of DNA:Proten complexes on lane 2 of **Figure 4.2**, which could be due multiple transcription factors targeting the E-box motif. Its specificity to ZEB1 binding was confirmed through competitive EMSA with unlabelled ZEB1 conserved sequence oligo and ZEB1 specific supershift assay, both of which showed disappearance of DNA:protein complex bands (**Figures 4.2 and 4.3**). This assay confirmed the involvement of ZEB1 binding to the +374 oligo sequence in the COL8A2 intronic sequence.

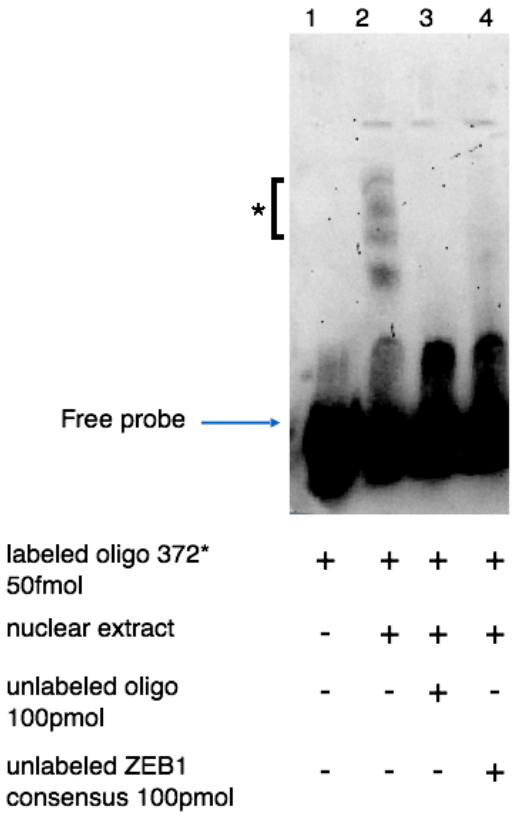


Figure 4.2: Competitive EMSA of ZEB1 binding on COL8A2 promoter proximal region. Competitive electrophoretic mobility shift assay was conducted with labeled and unlabeled +374 oligo containing the core binding element of ZEB1 using nuclear extract from HCEnC as the protein source. When challenged with 2000 fold excess of unlabeled ZEB1 consensus oligo, the DNA:protein complexes identifying the consensus oligo disappears in lane 4.

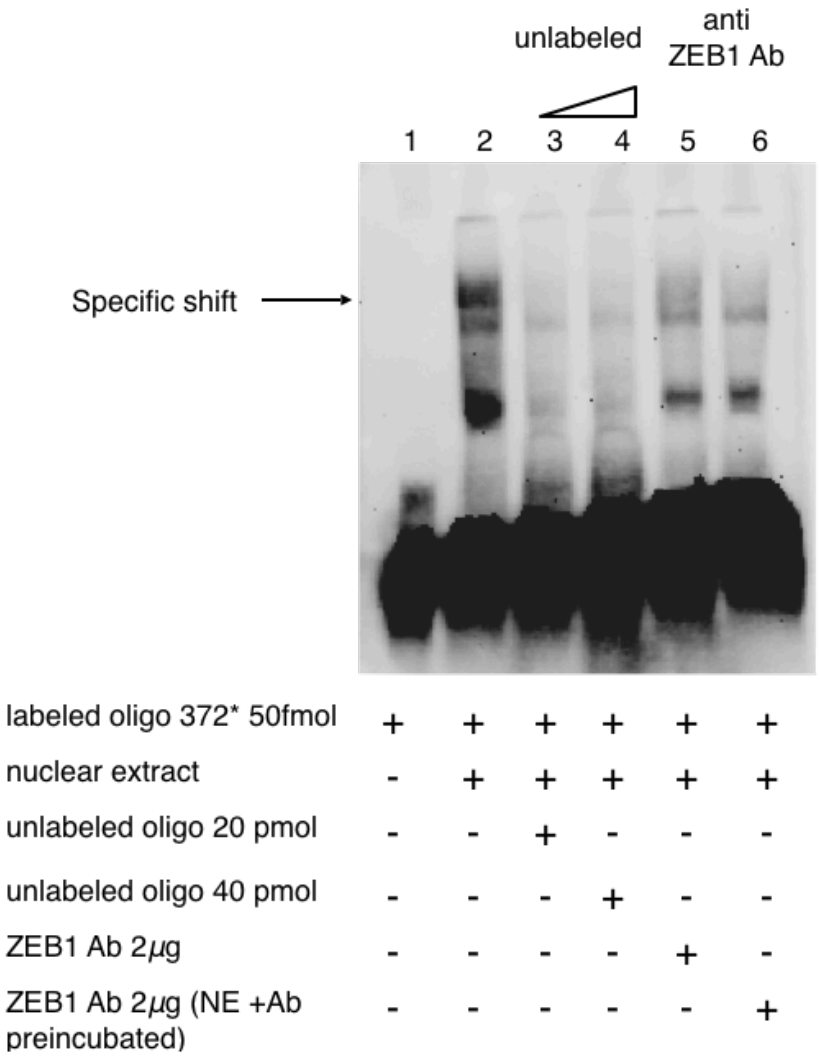


Figure 4.3: Antibody specific EMSA confirms ZEB1 binds to COL8A2 at 374 bases downstream of TSS. Increasing concentration of unlabeled oligo helps identify the specific DNA:protein complex formed on the +374 oligo (lanes 3 and 4). Challenging this reaction with ZEB1 specific antibody lead to disappearance of this specific shift (lanes 5 and 6).

4.2.3 ZEB1 confers a suppressive regulation over COL8A2

Gel-shift and supershift assays confirmed ZEB1 binding to the promoter proximal region of COL8A2. We therefore intended to check its regulatory activity by cloning the ZEB1 binding sequence (ACAGGTG) from +374 position of COL8A2 TSS into a minimal promoter luciferase vector, pGL4.23. In order to check enhanced reporter activity, 2x and 3x doses of the binding site was cloned upstream of the minimal promoter. Successful cloning of 1x374 could not be done because of smaller insert size (20bp). The transiently transfected HEK293 cells with 2x374-pGl4.23 and 3x374-pGL4.23 showed significantly (2x, P=0.009; 3x, P=0.01) suppressed reporter activity when compared with empty vector transfected cells as control (**Figure 4.4**). No significant difference in the reporter activities was observed between 2x374-pGl4.23 and 3x374-pGL4.23 constructs. This suggests that ZEB1 acts as a potent suppressor for COL8A2 transcript expression.

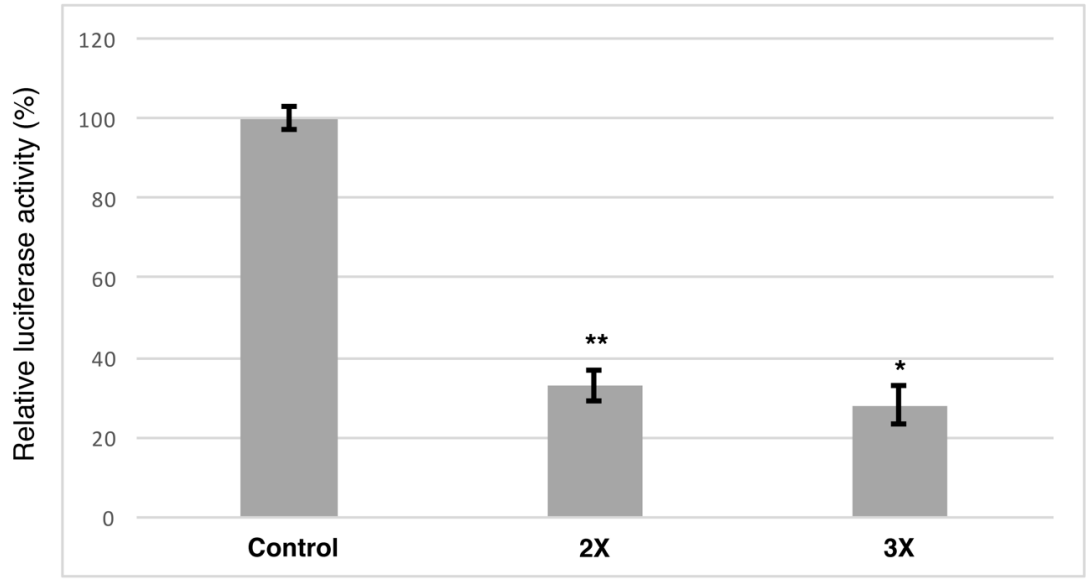


Figure 4.4: ZEB1 binding onto COL8A2 promoter proximal region cause significant suppression in transcript expression. Significance threshold at 5%, ** P<0.001, * P<0.05; Error bars represent standard error, SE: ±3.04-4.97.

4.3 DISCUSSION

Based on the recent findings that show involvement of overlapping genetic factors responsible to cause various endothelial dystrophies like CHED, PPCD and FECD, researchers speculate that these diseases are part of a continuum of corneal dystrophies that result into similar disease phenotypes.[18, 217, 218] Mutations in genes like SLC4A11 and LOXHD1 have been associated with corneal dystrophies like CHED and FECD, along with hearing loss disorder such as Harboyan syndrome or nonsyndromic hearing loss (**Table 4.3**).[9, 11, 28, 36, 60, 65, 91, 93-95, 103, 219, 220] These studies suggest a common pathomechanism responsible to cause hearing and ocular disorders. Similar to these cases, ZEB1 also is a common genetic factor to cause FECD and PPCD.[22, 72, 73, 221-225]

Table 4.3: List of genes genetically associated with various ocular and non-ocular disorders

Genes	Associated Diseases (References)
SLC4A11	CHED[60, 65, 91, 219] FECD[9, 28, 103, 220] Harboyan Syndrome[60, 91]
LOXHD1	FECD[11, 36] Nonsyndromic hearing loss[93-95]
ZEB1	PPCD[22, 72, 73, 221-225] FECD[11, 18, 182, 226]
COL8A2	PPCD[55, 216] FECD[7, 8, 55, 56, 182, 226, 227]

The current study focusses on the genetic variations and regulatory role of the candidate gene ZEB1 responsible to cause FECD. Previous literature has elaborated on the ZEB1 mutations accumulated in FECD affected families.[11, 18, 182, 226] Individual mutations identified through these studies were not replicated in other affected families, thereby

providing mutation information limited to solitary families. A population based study on the other hand enhances the scope for understanding the association of a gene with more generalized population of disease affected individuals. In the current study, we identified a particular polymorphism, rs220057 in the non-coding region of ZEB1 that co-segregates with FECD and it was observed that carriers of the risk genotype TT are at 49.2% increased risk [Odds ratio 2.15 (95% CI 1.16-4.00)] of developing FECD than control individuals. This polymorphism was also identified by Tang *et al.*, 2016 in a multigenerational Chinese family, but did not find any association with the disease because of the inherent drawback of a family based study that fails to recognize disease associated polymorphic variants.[11] ENCODE database search for this polymorphic site did not coincide with any transcription factor binding site. However, it was noted that this polymorphism falls within a 60bp MIR (mammalian-wide interspersed repeat) family of SINE sequence (**Figure 4.5**). Presence of MIR family of transposons in and around a gene has been associated with transcriptional regulation mediated by providing binding sites for transcription factors,[228-232] enhancers[230, 233-235] or miRNA transcripts.[236, 237] The said polymorphism (rs220057) also falls under a potential donor site (consensus score 73.33) for mRNA splice site.[238] It therefore warrants an in-depth scan of the gene to identify a disease-causing variant or decipher whether a SINE-mediated regulation[239, 240] is involved in corneal endothelial cells.

FECD exists in two variant forms, EO- and LO-FECD. Although there are subtle phenotypic differences,[7, 10, 40, 44] it has intrigued the researchers to find a common genetic factor that determines the age of onset for the disease in FECD affected individuals. So far, COL8A2 has been reported to be the sole genetic contributor for EO-FECD, and reports to search for its genetic involvement in late onset form resulted into non-association.[9, 54, 56] It is however important to note that abnormal deposition of COL8A2 gene product is seen in the DM of both EO- and LO-FECD affected tissues.[10, 44] Gottsch and group studied the deposition pattern of collagen type VIII in the EO- and LO-FECD affected tissues and reported

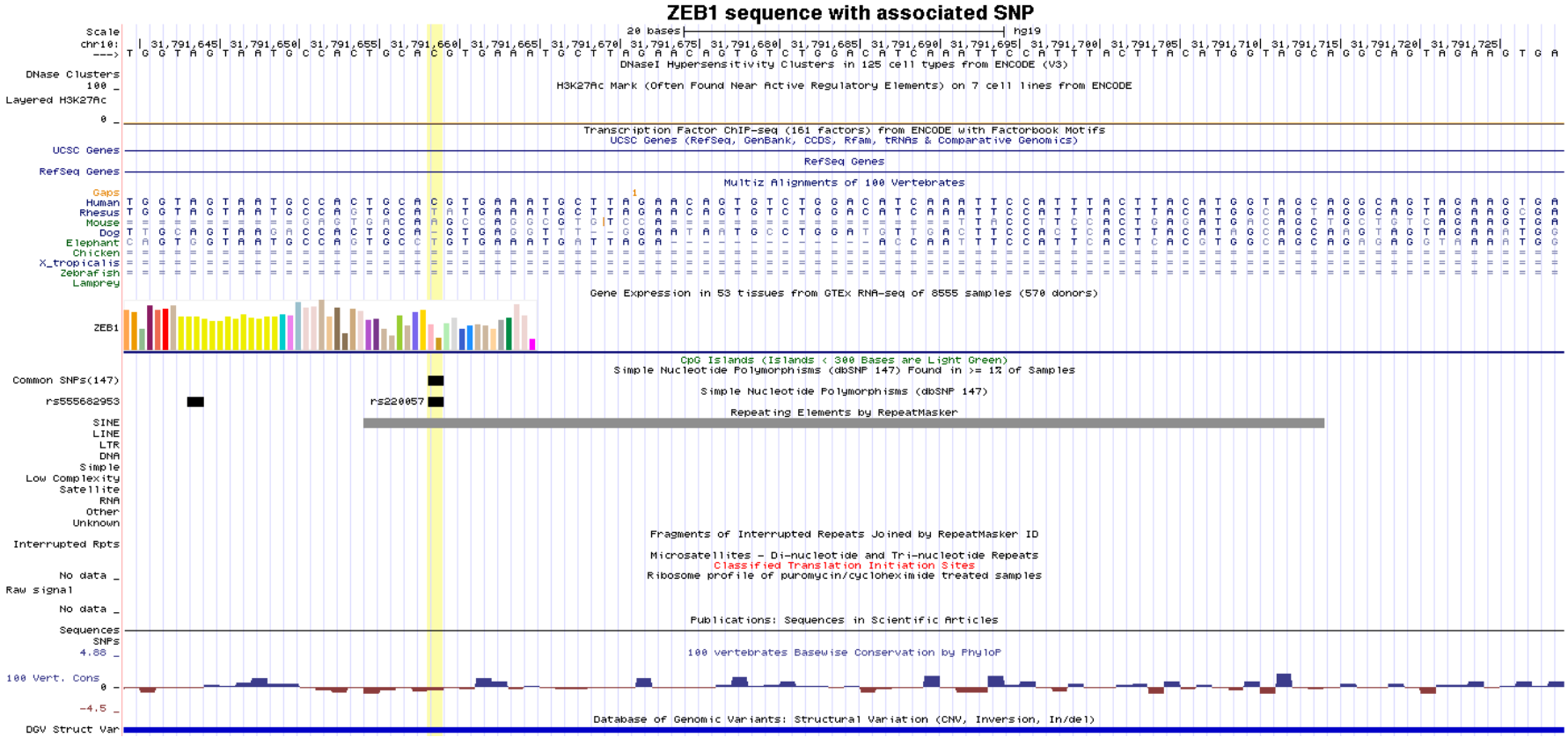


Figure 4.5: Polymorphism in ZEB1 associated with FECD sits within a mammalian-wide interspersed repeats (MIR) family of SINE sequence. Image was obtained from USCS genome browser.

differential distribution pattern of COL8A1 and COL8A2 in both the tissue types.[10] Key revelations from the study included: a) both types of collagens, *i.e.* COL8A1 and COL8A2 were aberrantly deposited in the anterior edge of DM, with an abnormal anterior banded region which shows comparatively heavy depositions of COL8A2, a feature common between both types of FECD tissues; b) massive deposition of COL8A1 in the posterior fibrous layer of LO-FECD cornea and c) presence of ~120nm periodic internal collagenous layer comprising of wide spaced collagen strips- a feature pathologically characteristic of FECD d) greatly increased deposition of collagen IV, fibronectin and laminin commonly observed in both EO- and LO-FECD tissues. It was reasoned that both forms of FECD progresses with abnormal basement membrane assembly, such that in case of EO-FECD, large amounts of disorganized collagen VIII presumably starts accumulating in-utero and continues through juvenile and adulthood.[10] The presence of common pathologic feature in EO-and LO-FECD gives rise to the similar deposition pattern of collagen VIII, however it remains to be seen if a regulatory defect is responsible for a thinner and delayed deposition in case of LO-FECD than its early onset counterpart. It was also noted that mutations in COL8A2[55] and ZEB1[224] are common genetic contributors for PPCD and FECD. One of the common mutation, ZEB1 (p.Gln640His) resulted in moderate downregulation of COL8A2 in cultured corneal keratocytes.[224] This along with reports from Krafchak *et al.* suggesting probable binding of ZEB1 in the COL8A2 promoter region provided impetus for the current study to experimentally validate the binding site of ZEB1 in the promoter region of COL8A2.[22] Studies in future to elucidate the regulatory player for COL8A1 expression can also further our current understanding of abnormal collagen deposition in FECD tissues.

The ZEB1 core binding sequence selected for this study resides in the first intron of COL8A2 (NM_005202), precisely 374 bases downstream of TSS. Our hypothesis of ZEB1 regulating COL8A2 expression is supported by the fact that this region has histone acetylation and DNaseI hypersensitivity signatures as reported in the ENCODE data. The

ChIP-Seq data from ENCODE also display several transcription factor-binding sites in this region (**Figure 4.1**). Our study reports for the first time about strong ZEB1 binding to this region of COL8A2, which thereby indicates a probable regulatory role of this transcription factor over collagen VIII expression. Moreover, reporter assay data further corroborates this hypothesis as it shows that the ZEB1 binding region at +374 of COL8A2 gene has a suppressor activity. Moreover, significant difference in reporter activity was not seen between 2x and 3x doses of the binding region which suggests that the suppression is not dose dependent.

ZEB1 expression is shown to be downregulated in FECD affected tissue with expanded TCF4-CTG repeats.[82] It is believed that these expanded repeats have a role in TCF4 mediated ZEB1 regulation.[82] However, the mechanism behind this is poorly understood. On the other hand, there is only one report suggesting a two-fold, but insignificant downregulation of COL8A2 in LO- FECD cells as compared to cultured control endothelial cells.[241] Moreover, Gottsch *et al.* have reported a detailed IHC labeling showing abnormal deposition of COL8A2 in the thickened LO-FECD DM.[10] Being a heterogeneous disease, FECD affected tissue samples show non-linear pattern of gene expression which lead to such discrepancies in these reports. It is therefore warranted to further analyze this relation by ZEB1-siRNA mediated reporter activity and check the expression of COL8A2 and ZEB1 transcripts in FECD affected cornea with increased sample size.

With genetic screening and reporter assays, this study extends our current knowledge regarding the function and contribution of ZEB1 gene in the progression of FECD. Although we did not document any pathogenic mutation in ZEB1 gene in our population, we found a novel genetic association of rs220057 with FECD and it can be said that ZEB1 acts by regulating the expression of COL8A2. It is yet to be seen how the different collagen subtypes are regulated to result into such peculiarly disorganized banded distribution in LO-FECD.

Chapter 5

Association of DNA repair gene *FEN1* with FECD

5.0 ASSOCIATION OF DNA REPAIR GENE FEN1 WITH FECD

5.1 INTRODUCTION

FECD is an autosomal dominant, bilateral, age related disorder, known since 1910 after its discovery by Ernst Fuchs.[1] Both genetic and environmental factors act as causative agents towards disease progression; with an increased predilection for females (2.5:1) and individuals over forty years of age.[48, 49, 51] FECD progresses gradually with age, such that the corneal endothelial layer undergoes polymegathism and decreased cell density.[242] The primary cause known so far indicates more than one reason; unfolded protein response[243], heightened oxidative stress,[126] DNA damage,[32] accumulation of misfolded proteins,[28] nuclear stress,[30, 204] apoptosis,[26] to name a few. These reasons spiral down to endothelial cell loss and thickening of DM, which consequence into obstructed passage of water, corneal edema and finally deteriorated visual acuity. FECD is clinically characterized by the presence of guttae in the DM, blurred vision, fluctuating eye sight, discomfort towards bright light, dry eyes and poor night vision. Corneal transplantation is considered as the sole alternative for restoring vision in FECD individuals.[244]

Linkage analysis of familial FECD cases have identified 4 chromosomal loci FECD1-4. Moreover, recent genome wide association studies with larger cohorts have identified mutations and polymorphisms in various genes like SLC4A11, ZEB1, TCF4, AGBL1 and LOXHD1.[12, 13, 15-18, 36, 61] Only TCF4 (transcription factor 4) has recently surfaced as a significant contributor towards the causation of late-onset FECD, globally.[21, 81, 84] Adding to the complexity of the pathogenesis of FECD, it has also been reported that the affected corneal endothelial cells are aggressively challenged with oxidative stress as the disease progresses.[126] Due to this, profound DNA damage and apoptosis eventually becomes inevitable. Flap endonuclease 1 (*FEN1*), is one of the DNA-damage repair genes, that has been recently associated with FECD in Polish population showing significant association with a *FEN1* polymorphism, rs4246215 (c.4150G>T) located at the 3'UTR of this gene.[23]

Although functional effects of this polymorphism have been reported in various cancerous tissues, its effect on FECD disease background is scantly known.[24, 25] In this current study, we aimed to assess the genetic association of two polymorphisms, rs174538 (c.-69G>A) and rs4246215 (c.4150G>T), in *FEN1* gene among Indian FECD population, and to identify the causal variant that contributes towards the oxidative damages incurred during disease progression.

5.2 RESULTS

5.2.1 Demographics of the study

The patient population (n=73) enrolled for this study were clinically diagnosed and documented for FECD from the eastern provinces of India. Females populated 67% of this group, which substantiates the fact that FECD is a female-biased disease reported worldwide. However, the number of females included in the control group was just 47% (**Table 5.1**), which creates a statistically significant difference in gender distribution between the two groups. For genetic association tests, both the uncorrected and age-gender corrected samples were used. The mean age of the cumulative study population is 62.7 years. Considering the essential age-sensitive bracket for FECD ranging from 40-85 years, individuals ranging from 43-80 years for patients and 42-85 years for control groups have been included in this study to keep the age distribution unbiased (P=0.09).

Endothelial counts of patients and controls were also included as a covariate during genetic analysis (**Table 5.1**). It substantiated the fact that FECD imposes significant ($P < 0.05$) endothelial cell loss in the patients. Diminished endothelial cell counts in FECD patients do not show any significant difference when segregated on the basis of *FEN1* c.-69G>A or c.4150G>T ($P > 0.05$).

Table 5.1: Demographics and clinical characteristics of the study population.

Variables	Cases	Control	P Value
Number of Individuals	73	179	
Number of females (%)	43 (67.18)	72 (47.05)	0.001
Mean Age in years (SD)	61.08 (9.73)	63.32 (7.82)	0.09
Endothelial Count Mean (SD)	1476 (284.3)	2301 (349.1)	< 0.0001

5.2.2 Genetic association of the polymorphism *FEN1* c.4150G>T with FECD risk in Indian cohort

Restriction Fragment Length Polymorphism (RFLP) and direct DNA sequencing methods were employed to genotype the polymorphisms, c.-69G>A and c.4150G>T harbored in the 5' and 3' UTR regions of the *FEN1* gene, respectively (**Figure 5.1**). These polymorphisms were selected on the basis of their association with FECD as reported in the Polish study. Distributions of alleles in both the subject groups were under Hardy Weinberg equilibrium (HWE). Genotypic and allelic frequencies for *FEN1* c.-69G>A and *FEN1* c.4150G>T are as detailed in **Table 5.2**. Genetic analysis for both the polymorphisms was carried out with crude samples (that had significantly skewed gender distribution) and age-gender corrected samples. It was observed that *FEN1* c.4150G>T showed significant association with FECD (Df2: uncorrected P=0.001, corrected P=0.004) in both kinds of sample segregation. The said significance remained effective even after 10,000 simulations performed on Haploview software (uncorrected P=0.005, corrected P=0.002). 13% of the FECD individuals (8/62) were the carriers of c.4150TT genotype in comparison to a smaller percentage 2.64% (4/151) of the control population; rendering it a greater risk to obtain FECD (OR = 2.48, 95% CI = 1.45-4.25) than the rest of the genotypes. *FEN1* c.-69G>A variant on the other hand maintained its non-association with the disease unhindered by age or gender biases (uncorrected P=0.41, corrected P=0.32).

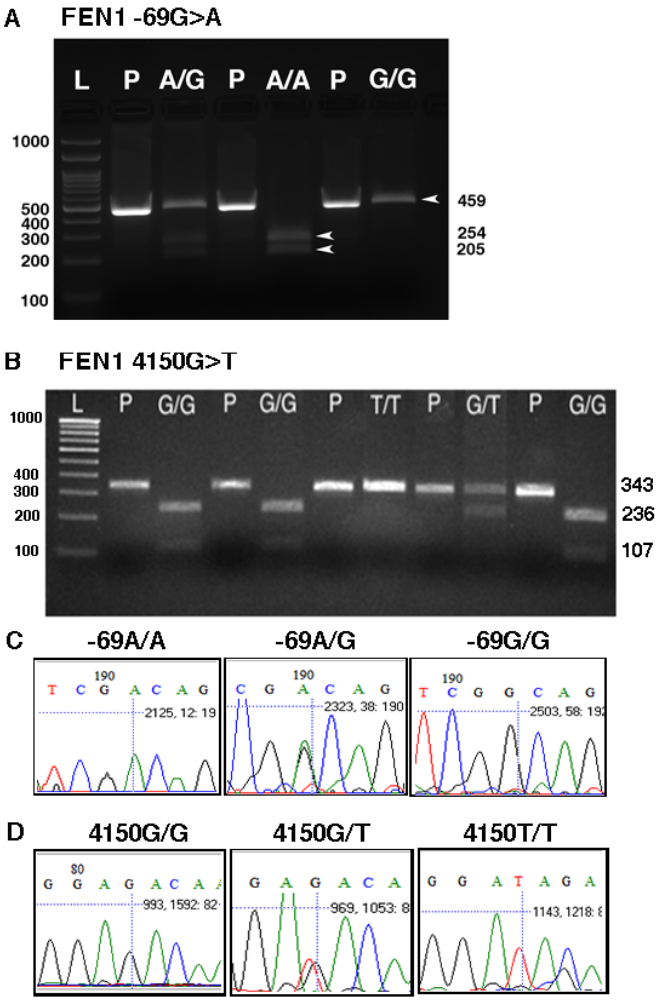


Figure 5.1: Restriction fragment length polymorphism was performed on PCR amplicons of the polymorphisms A) *FEN1* c.-69G>A (SalI) and B) *FEN1* c.4150G>T (BsmaAI). The digested products were separated on 2.5% agarose gels alongside their respective PCR products (P) and sized with the help of 100bp ladder (L).

Table 5.2: Genetic association of polymorphisms in *FEN1* gene with FECD. Allelic and genotypic distribution of SNPs, rs174538 and rs4246215 across patients and controls are tabulated. Polymorphism rs4246215 shows significant association with the disease.

* : Risk allele, OR : Odds Ratio, CI : Confidence interval

SNP	Type	Control		FECD		Uncorrected		Age - Gender Corrected	
						P value (Test)	OR (95% CI)	P value (Test)	OR (95% CI)
		n	%	n	%				
rs174538	GG	117	69.64	50	68.49		1.0		1.0
(c.-69G>A)	AG	50	29.76	22	30.13	0.82 (χ^2, Df2)	1.07 (0.59-1.94)	0.76 (χ^2, Df2)	1.04 (0.50-2.16)
	AA	1	0.59	1	1.36		NA		NA
Major Allele	G	284	84.52	122	83.56	0.70 (χ^2, Df1)	0.6 (0.31- 1.18)	0.32 (χ^2, Df1)	1.34 (0.72 - 2.48)
Minor Allele	A*	52	15.47	24	16.43	0.88 (10K permutations)		0.56 (10K permutations)	
rs4246215	GG	121	66.48	37	51.61		1.0		1.0
(c.4150G>T)	GT	57	31.31	24	35.48	**0.001 (χ^2, Df2)**	1.56 (0.82-2.94)	**0.004 (χ^2, Df2)**	1.27 (0.62-2.67)
	TT	4	0.02	9	12.90		6.50 (1.84-23.01		7.43 (1.91-28.96)
Major Allele	G	299	82.14	98	70	**0.003 (χ^2, Df1)**	2.48 (1.45-4.25)	**0.01 (χ^2, Df1)**	2.11 (1.26 - 3.54)
Minor Allele	T*	65	17.85	42	30	**0.005 (10K permutations)**		**0.002 (10K permutations)**	

5.2.3 Haplotype analysis of *FEN1* polymorphisms and FECD incidence

Haplotype analysis was carried out to understand if a particular combination of alleles when present in Linkage Disequilibrium (LD), could act as a genetic marker to predict the occurrence of a disease before the onset of its symptoms. Interestingly, in our study we observed that the haplotype "$G_{-69}T_{4150}$" of *FEN1* c.-69G>A (rs174538; SNP1) and c.4150G>T (rs4246215; SNP2), respectively show a strong association (P<0.001) with FECD (**Table 5.3**), unlike that reported by Wojcik *et al.,* where the $A_{-69}G_{4150}$ haplotype is associated with FECD occurrence in the Polish population (P=0.002). With an increased frequency in controls than in patients, haplotype $G_{-69}G_{4150}$ acts as protective haplotype (P=0.01) in our population.

Table 5.3: Haplotype analysis of *FEN1* polymorphisms, c.-69G>A (SNP1) and c.4150G>T (SNP2) and its association with FECD.

Haplotype	Cases	Control	OR (95%CI)	P value
$\mathbf{G_{-69}G_{4150}}$	0.88	0.96	0.25 (0.06-0.92)	0.01
$\mathbf{A_{-69}G_{4150}}$	0.28	0.30	0.86 (0.46-1.59)	0.38
$\mathbf{G_{-69}T_{4150}}$	0.47	0.33	**1.89 (1.06-3.37)**	$\mathbf{7x10^{-4}}$
$\mathbf{A_{-69}T_{4150}}$	0.29	0.25	1.29 (0.68-2.43)	0.40

5.2.4 Functional effect of *FEN1* gene in FECD disease background

In order to analyze the level of basal endogenous DNA damage, comet assay of peripheral blood leucocytes was performed in 28 FECD patients and 24 individuals with healthy cornea. For this assay whole blood was selected, considering the limited supply of post-operative corneal endothelial tissues from the study participants and genomic homogeneity between PBMCs and corneal cells.[25, 32] Generated comet images were analyzed based on genotypes and affected status of the individuals (**Figure 5.2**).

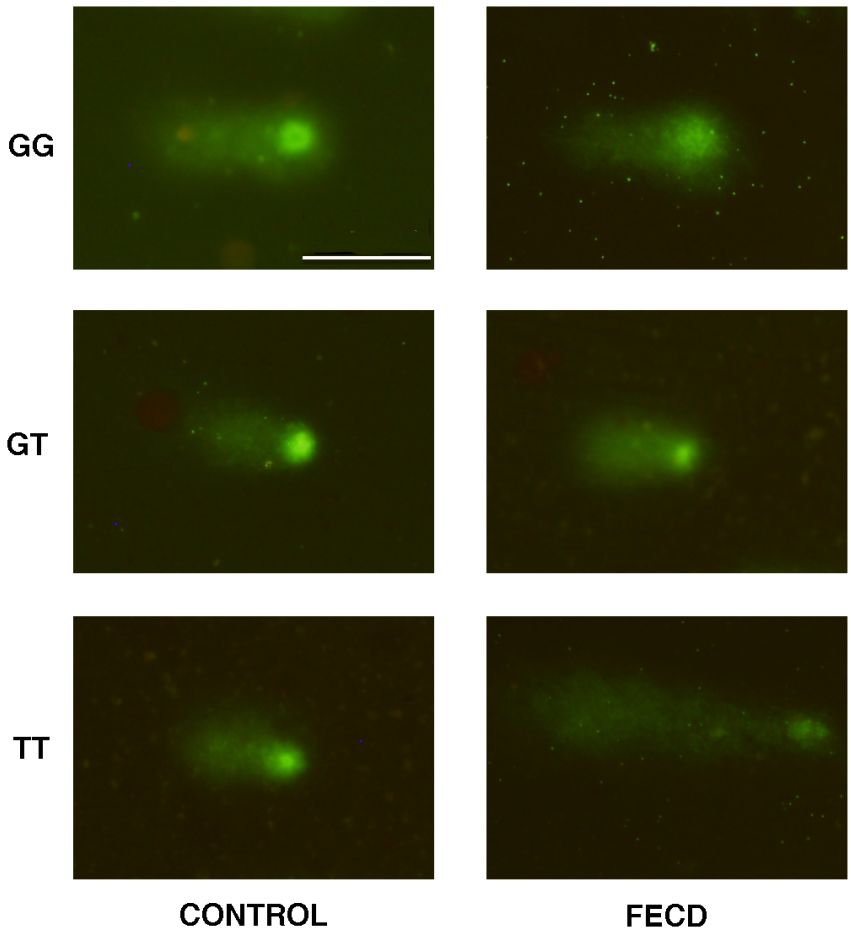


Figure 5.2: Representative images of comets from control and FECD individuals segregated as per rs4246215 genotypes (GG, GT and TT). The comets from peripheral blood leucocytes are stained with SYBR® green. Differences in tail lengths denote extent of DNA denaturation in these samples. Bar = 100μm.

Endogenous DNA damage, expressed as natural log of olive tail moment (Ln-OTM), was significantly higher in FECD cases (P= 0.0057, n=28) as compared to their unrelated controls (n= 24, **Figure 5.3A**). We therefore analyzed for any correlation between the genotypes of the associated polymorphism (*FEN1* c.4150G>T) and DNA damage in FECD cases (**Figure 5.3B**). It was observed that the *FEN1* c.4150TT FECD carriers (n=8) had significantly greater DNA damage than c.4150GT (P= 0.004, n=10) and 4150GG (P= 0.037, n=10) FECD individuals. Insignificant difference in DNA damage was observed when the samples (control + cases) were segregated on the basis of genotypes only (**Figure 5.3C**).

Most significant difference in endogenous DNA damage was observed between FECD carriers of c.4150TT genotype and their control counterpart with the same genotype (P<0.001, **Figure 5.3D**).

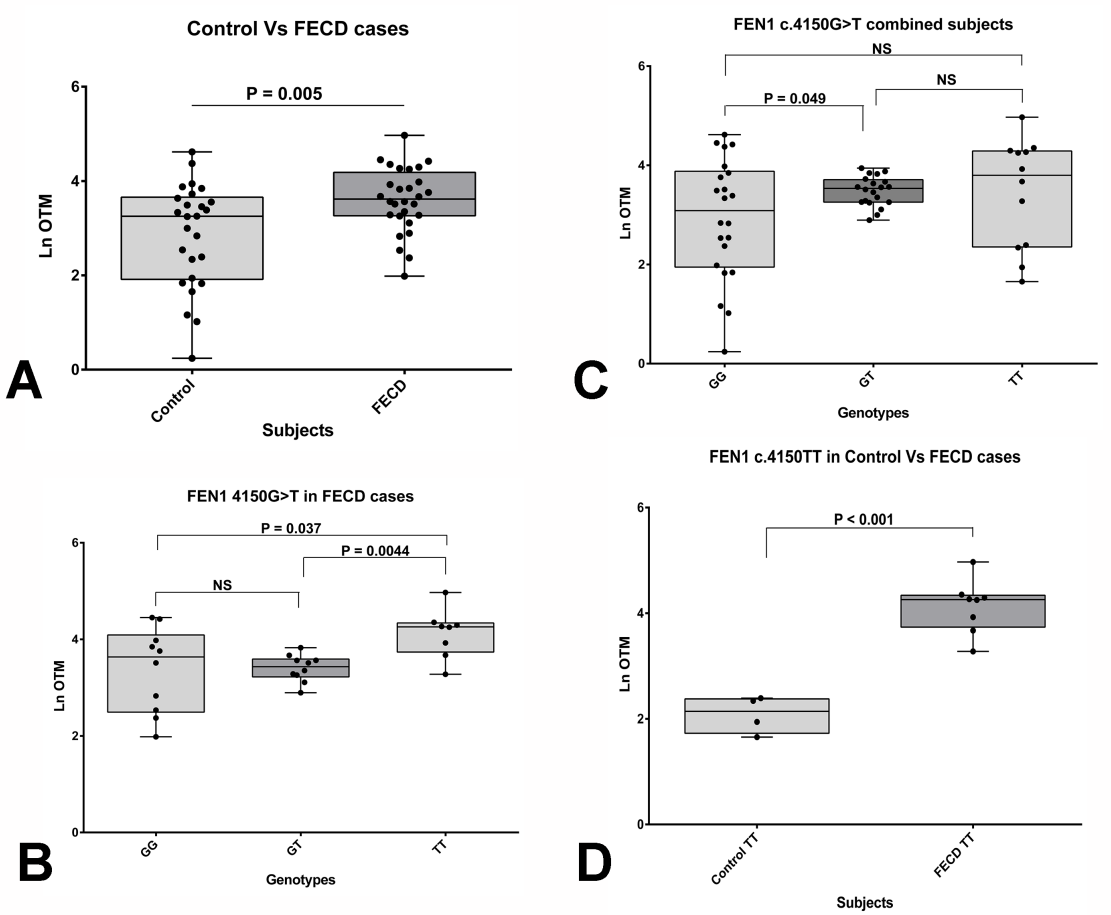


Figure 5.3: Ln-OTM values of the comets are plotted to compare the DNA damage in FECD cases against controls (A), FECD individuals segregated according to the genotypes of *FEN1* c.4150G>T polymorphism (B), Pooled samples segregated on the basis of genotypes (C) and carriers of *FEN1* c.4150TT genotype with FECD cases against control (D).

5.2.5 Putative miRNA targets for *FEN1* gene regulation

List of miRNA candidates having putative binding sites at the 3'UTR of *FEN1* gene were screened by the computational algorithms: miRWalk, miRanda, TargetScan, PicTar2, RNA22, FindTar and Segal Lab based on their alignment, energy and mirsvr scores (**Table 5.4**). About 274 miRNAs from TargetScan and nine miRNAs from MiRanda were obtained from initial screening. PicTar2 did not fetch any candidate miRNAs. Out of these, only eight candidates were common between minimum of five algorithm outputs. hsa-miR-1236-3p is the only miRNA that spans the associated *FEN1* c.4150G>T polymorphic site and was fetched from the three databases, MiRanda, TargetScan and SNPinfo. The binding site for hsa-miR-1236-3p includes 'G' allele from the polymorphic site and a seed region, GAAGAGA situated 19 bases upstream of it.

To validate this finding, we performed luciferase assay by transfecting HEK293 cells with constructs comprising of 60bp region from 3'UTR of *FEN1* gene, harboring target sites for hsa-miR1236-3p. Each of the three types of pMIR-constructs, WT1236/G, WT1236/T and MT1236/G, were co-transfected along with hsa-miR1236-3p mimic individually into HEK293 cells and percent luciferase activity was measured in comparison to cells transfected with empty vehicle (EV, pMIR-report). It was observed that presence of 'G' allele at c.4150G>T resulted in significantly reduced reporter activity (26.6%, $P = 0.008$) in comparison to 'T' allele or mutant constructs. This suggests that both the miRNA target sequence (GAAGAGA) and 'G' allele at c.4150G>T polymorphic site is required to for hsa-miR-1236-3p mediated downregulation of luciferase activity (**Figure 5.4**).

Table 5.4: Predicted miRNA targets on the 3'UTR of *FEN1* gene. EntrezID: 2237, RefseqID: NM_004111

miRNA	MIMATid	miRWalk	miRanda	Pictar2	RNA22	Targetscan	FindTar3	Segal Lab	SNPinfo
hsa-miR-532-3p	MIMAT0004780	1	1	0	1	1	1	1	
hsa-miR-515-5p	MIMAT0002826	1	1	0	1	1	1	1	
hsa-miR-548k	MIMAT0005882	1	1	0	1	1		1	
hsa-miR-519e-5p	MIMAT0002828	1	1	0	1	1	1		
hsa-miR-2116-3p	MIMAT0011161	1	1	0	1	1	1		
hsa-miR-610	MIMAT0003278	1	1	0	1	1		1	
hsa-miR-4297	MIMAT0016846	1	1	0	1	1	1		
hsa-miR-942-5p	MIMAT0004985	1	1	0	1	1	1		
hsa-miR-1236-3p	MIMAT0005591		1			1			1

1s and 0s indicate number of calls fetched for respective miRNA in various algorithms.

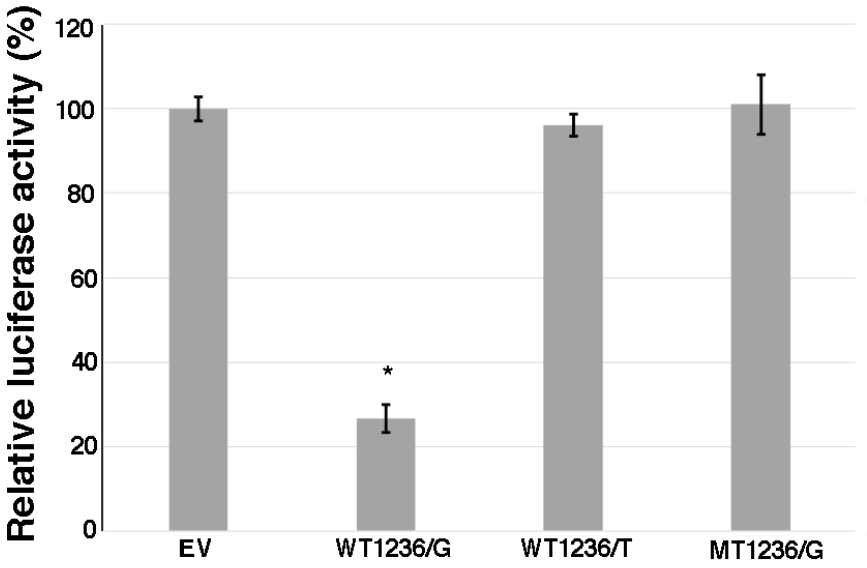


Figure 5.4: miRNA mediated reporter activity on 3'UTR regions of *FEN1* gene. Normalized luciferase activity in HEK293 cells transfected with hsa-miR-1236-3p mimic is shown to demonstrate the effect of allelic changes on luciferase activity. Trasnfected cells carrying contructs with 'G' allele exhibit reduced luciferase activity (26.6 ± 3.2) in presence of hsa-miR1236-3p as compared to those with 'T' allele (96.1 ± 2.5) or scrambled hsa-miR-1236-3p binding sequence with G allele at SNP position (MT1236/G; 101 ± 6.9). Luciferase acticity of cells transfected with empty pMIR-report vector (EV) were taken as control (100 ± 2.88). Error bars indicate standard error and * indicate *P* value = 0.008.

5.3 DISCUSSION

FECD is a sexually dimorphic disease with a dominance of females as the affected gender, which is reflected in the cases of the current study. However, in the control group, male participants are significantly more. As discussed in our previous reports,[84] developing countries like India suffer from skewed medical assistance for women due to socio-cultural and economic influences.[198] As a consequence of which, women who are unaffected with any severely decreased visual acuity are rare visitors in the eye care centers. Considering the impact of gender bias on genetic association studies, the data was re-analyzed with age and gender corrected case-control cohort and the association between *FEN1* c.4150G>T and

FECD remained unaffected. This suggests that the epigenetic differences due to sex discrimination might not play a major role with the association of this particular polymorphic site and FECD.

The current association study is in consensus with Wojcik *et al.,* which also demonstrates a significant genetic association of *FEN1* c.4150G>T and not c.-69G>A, with FECD. It also coincides with the risk genotype reported in their population (c.4150TT) and its likelihood to acquire FECD (OR_{Indian}= 2.11; OR_{Polish}= 2.26).[23] This indicates that *FEN1* contributes a significant genetic load towards the pathogenesis of FECD across the globe. This kind of information can aid the genetic counselors in predicting the chances of disease occurrence in a particular individual. An even sophisticated approach to perform such predictions with heightened efficacy is by performing haplotype analysis with a collection of alleles that are in LD with each other and provide association status for an individual with the disease of interest. In the current study, *FEN1* c.-69G>A and c.4150G>T are in considerable LD with each other (r^2= 0.34, D'= 0.71) which corroborates with the combined LD plot for South Asian and European populations (GIH+CEU) from 1000 Genomes Project Phase III database as shown in **Figure 5.5**. Individuals from both these populations show a strong LD between these two SNPs. However, the FECD-associated haplotypes for Polish ($A_{-69}G_{4150}$) and Indian ($G_{-69}T_{4150}$) populations are unparalleled to each other. Haplotype structures are sensitive to variant allele frequencies which differ between populations.[245] As can be seen from the allele distribution, only 15% variance of A allele (in rs174538) is observed in our population as compared to 33% in Polish, which affects the haplotype structure. It is therefore essential to carry out population specific studies. From our investigation, we report that individuals from Indian ethnicity that carry *FEN1* c.4150T allele or $G_{-69}T_{4150}$ haplotype are at significantly increased risk of acquiring FECD.

One of the most significant observations of the study was the heightened endogenous DNA damage in FECD cases as compared to the control group. This indicates that the PBMCs

from patients are at higher oxidative stress that has allowed the accumulation of oxidative DNA damage, which remained unrepaired.

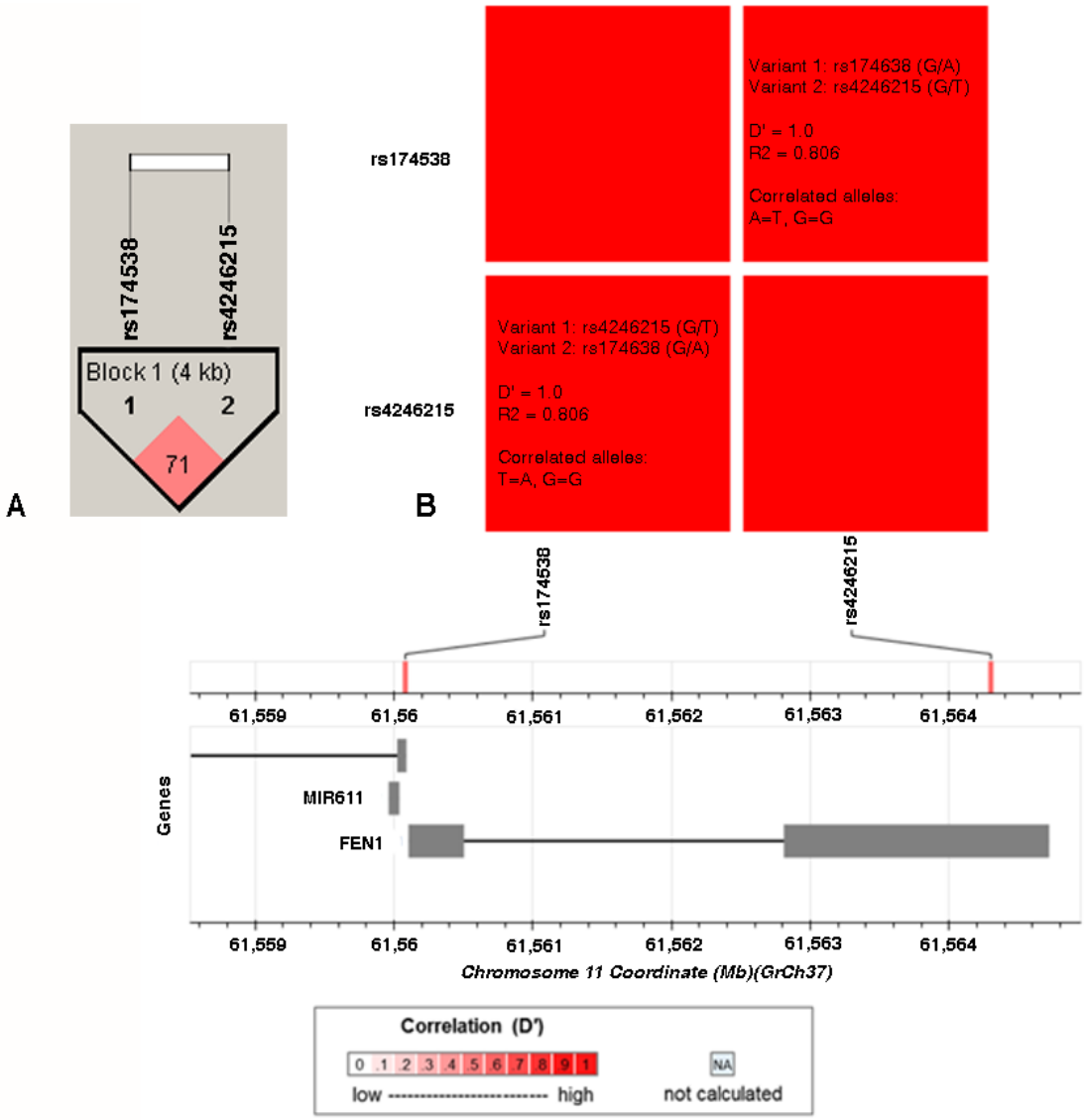


Figure 5.5: Linkage disequilibrium map for the region comprising FEN1 polymorphisms rs174538 (c.-69G>A) and rs4246215 (c.4150G>T). Individuals from our study show a considerable LD with a D' value of 0.71 and r2 = 0.39 (A). Combined LD plot of these polymorphisms as observed for CEU and GIH populations (B).[245]

Various environmental and genetic factors could be responsible for predisposing these individuals with higher levels of oxidative stress, which is more exaggerated in cornea. Oxidative stress and deficiency in antioxidant defense system have been elaborately

attributed as the etiology of FECD pathogenesis in numerous studies.[26, 27, 126, 246] DNA damage in the blood leucocytes of FECD patients and control individuals, due to induced oxidative insult was studied by P. Czarny *et al*., and they reported an insignificant difference in the extent of DNA damage between the two groups. However, the recovery of FECD leucocytes from stress-induced DNA damage was significantly lower than that of the controls; implying a faulty DNA repair mechanism under disease condition.[32] Later it was reported that the TT genotype of *FEN1* c.4150G>T was significantly associated with the increased incidence of FECD and Keratoconus in Polish population.[23] In extension to Czarny *et al*. and Wojcik *et al*. reports, our study imply that the homozygous "T" allele of *FEN1* c.4150G>T variation in conjunction with other factors responsible for FECD pathogenesis, contribute towards a compromised condition where the endogenous DNA damage is heightened; as can be seen in **Figure 5.2 and 5.3D**.

With the intent to identify whether the associated SNP confer any functional role, we performed several functional assays. Significantly heightened DNA damage and increased *FEN1* expression in individuals with homozygous risk allele, *FEN1* c.4150TT implies a genotype-phenotype correlation. The fact that there is significant DNA damage in FECD c.4150TT individuals than control c.4150TT individuals can be attributed to the disease background. This suggests that the risk genotype (*FEN1* c.4150TT) as well as other FECD-associated factors such as, elevated ER stress[132] and downregulated antioxidant response system[27, 247] responsible for increased oxidative stress, could work in conjunction to manifest this phenotype where DNA damage is significantly prominent. Supporting this line of hypothesis, is the recent literature that report the recovery of FECD leucocytes from stress-induced DNA damage was significantly lower than that of the controls; thus implying a faulty DNA repair mechanism is active under the disease condition.[32] Furthermore, our observation of elevated *FEN1* expression in c.4150TT carriers is in accordance with another study that reported decreased *FEN1* expression in c.4150G allele carriers diagnosed with

breast cancer.[24] This led us to hypothesize that a plausible mode of transcript regulation by the 3'UTR situated polymorphism, could be miRNA mediated. We therefore conducted a bioinformatics analysis of this region which fetched hsa-miR-1236-3p as the candidate miRNA, targeting the region comprising of *FEN1* c.4150G>T SNP. Reporter assay in HEK293 cells validated that hsa-miR-1236-3p bound specifically to 'G' allele, which corroborates with the mRNA expression data where GG/GT individuals show lower *FEN1* expression than c.4150TT individuals. Although it requires further validation through siRNA-mediated knockdown of miR-1236-3p and functional assays performed on corneal endothelial cells to strengthen the role of miRNA on *FEN1* expression, the current study provide initial report that factors associated with FECD act in conjunction with the risk genotype 'TT' to result in elevated levels of *FEN1* gene and increased accumulation of double stranded breaks.

These results provide fresh insight into the role of *FEN1* in FECD pathogenesis. Considering its pivotal role as a genome stabilization factor and in DNA damage repair during long-patch base-excision repair (BER), changes in *FEN1* gene may lead to increased susceptibility of DNA damage during oxidative injury. It has been established through previous studies that both c.-69G>A and c.4150G>T polymorphisms in *FEN1* were associated with deregulation of its transcript expression in lung- and breast cancer tissues.[24, 25] Genetic association analyses in patients diagnosed with hepatocellular carcinoma, esophageal cancer, gastric cancer, colorectal cancer and glioma have reported the homozygous c.4150T allele to limit cancer progression.[248-250] Also, significantly increased *FEN1* mRNA expression has been observed in carriers of c.4150T allele with lung[25] and gastrointestinal[249] cancers. Taken together, it suggests that although the c.4150T allele lowers the risk of developing cancer, in case of FECD, it increases the risk by elevating the *FEN1* expression. Validation through tissue-specific gene expression with availability of tissue samples would help further our understanding in future.

Chapter 6

MCTs as candidate gene for FECD

6.0 MCT AS CANDIDATE GENE FOR FECD

6.1 INTRODUCTION

Course of progression for FECD is varied and complex. One of the most popular pathomechanism known till date is failure of pump system of the corneal endothelial cells.[88, 114, 243, 251-254] Initial reports of genetic linkage analysis[61] suggested mutations in solute carrier protein, *SLC4A11* that resulted in retention of mutant proteins, consequent into insufficient efflux of water molecules and edematous cornea.[243, 254, 255] In a recent study that conducted a GWAS in Caucasian European decent individuals, a polymorphic marker, rs1200114 in ATP1B1 was identified.[14] As the name suggest, this ATPase is essential in maintaining the sodium-potassium electro-potential across the endothelial membrane. ATP1B1 was also reported to be significantly downregulated in FECD corneal endothelial tissues as compared to that of controls.[243] Based on this mode of pathomechanism, we aimed at checking if another SLC family transporter, MCT1, a monocarboxylate transporter, which is one of the key regulator for lactate concentration throughout the cornea[29, 256, 257] could be deregulated in FECD corneas. Active functionality of MCTs in corneal endothelium ensures corneal transparency and deturgescence.[29, 256, 257]

A detailed report of the transport function of these MCTs across rabbit corneal endothelium was provided by Bonano *et al.* in which they reported the presence of MCT1, 2 and MCT4 to be most populated across cornea and MCT3 was relatively low or absent.[29] Another study reported transcript levels of these MCTs in human corneal tissues.[258] However, protein localization studies for MCTs in human corneal endothelium haven't been reported. We therefore intended to localize these transporters, MCT1, 2, 3 and 4 in human corneal endothelial tissues and analyze their expression level in FECD as compared to control tissues.

6.2 RESULTS

6.2.1 Spatial localization of MCTs in human corneal endothelial tissues

For localization purpose, non-transplant grade human corneal tissues were used. These tissues were surgically trephined so that only a thin 3µm layer of endothelium along with stroma was further processed for immunohistochemical detection of MCT1-4. Corneal endothelial cells appear as closely apposed hexagonal cells under confocal microscope. The tissues from healthy donors were stained with primary antibodies against MCT1, MCT2, MCT3 and MCT4. These transporters mostly populated the cell membrane as expected, and were also present in the cytoplasmic region. To analyze their spatial distribution, collapsed Z-stack of the high-resolution images were viewed using Zeiss LSM file browser. MCT1 is mostly populated along the basement membrane, whereas MCT2 and MCT4 acquire apical and lateral locations respectively (**Figure 6.1**).

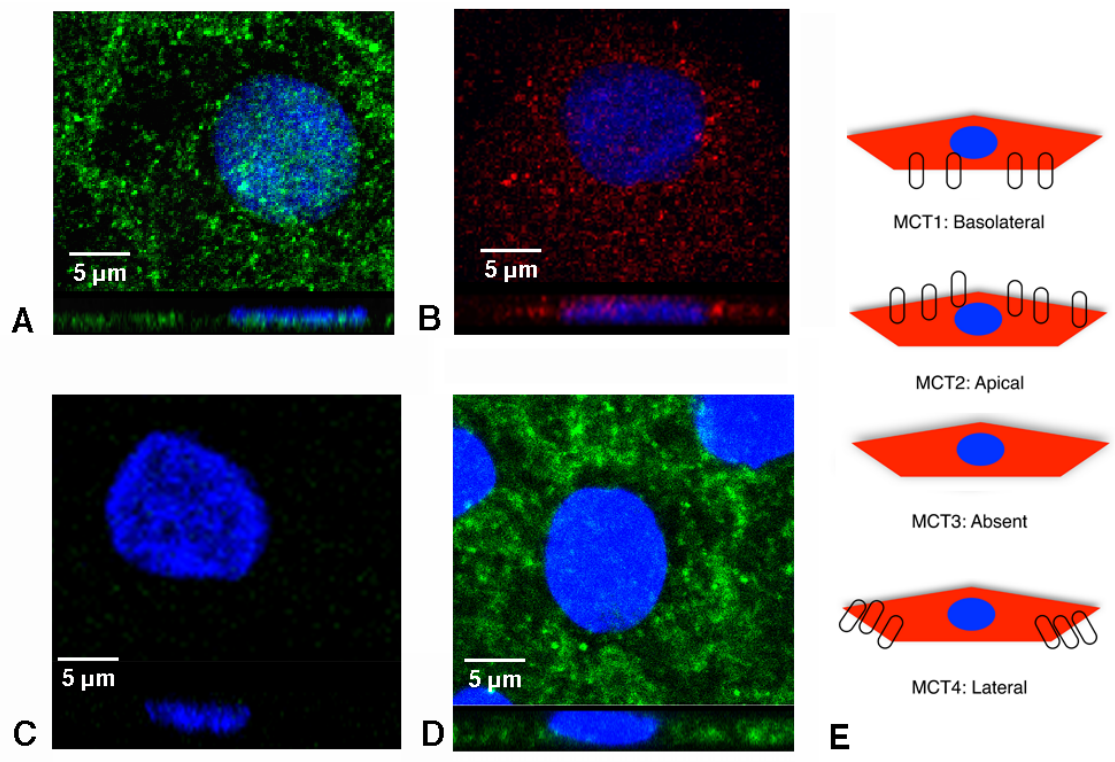


Figure: 6.1 Localization of Monocarboxylates in human corneal endothelial tissue. Each panel represents one Z-section of a high definition confocal image and the lower panes represent ortho section of the Z-stack image in Y plane. (A) MCT1 is located along the

basement membrane as visualized through green channel, (B) MCT2 localization is seen in red channel and is apical in nature, (C) MCT3 is absent in human corneal endothelial tissue, (D) MCT4 as seen in green channel is laterally located, (E) Schematic representation of MCT localization in human corneal endothelial cells.

These localization studies correlate with that of the rabbit corneal tissues with the exception that MCT4 in rabbits are basally located unlike the more lateral localization in case of human corneal endothelial tissue[29]. Beside these differences, MCT3 is absent in corneal endothelium of both the species.

6.2.2 Immunohistochemical comparison of MCT expression in control and FECD corneal endothelium

Due to unavailability of fresh post-operative samples, paraffin transverse sections comprising of endothelial layer and DM from cases and controls were challenged with MCT1, MCT2 and MCT4 antibodies to analyze their expression difference in both the tissue types. IHC analysis showed a clearly thickened Descemet's membrane (DM) and significantly reduced number of endothelial cells (**Figure 6.2**).

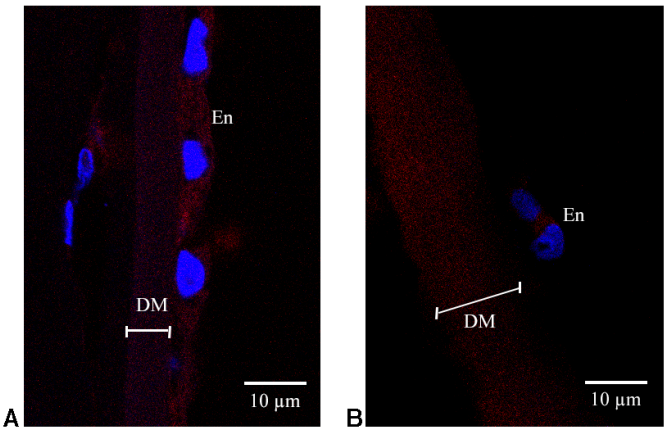


Figure 6.2 Comparative paraffin sections from (A) control cornea and (B) FECD cornea depicting significantly decreased endothelial cells and thickened DM.

Corrected total cell fluorescence (CTCF) values were calculated using Image J software and the below formula.[259, 260] CTCF = Integrated Density – (Area of selected cell x Mean fluorescence of background readings). In **Figures 6.3** the expression of these MCTs as proteins in FECD tissues that are individually normalized with their control counterparts has been illustrated. Paraffin sections of MCT1 and MCT2 were analyzed whereas MCT4 expression was analyzed in post-operative DSEK samples (**Figure 6.4**). The analysis of these sections showed a significant downregulation of MCT1 in comparison to MCT2 and MCT4; the same is represented in **Figure 6.3**.

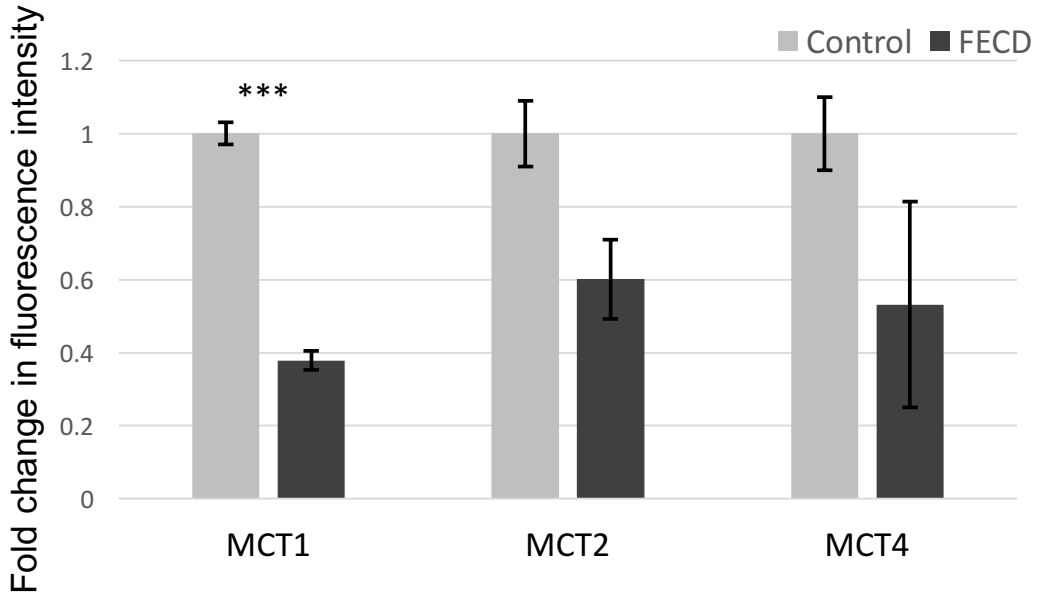
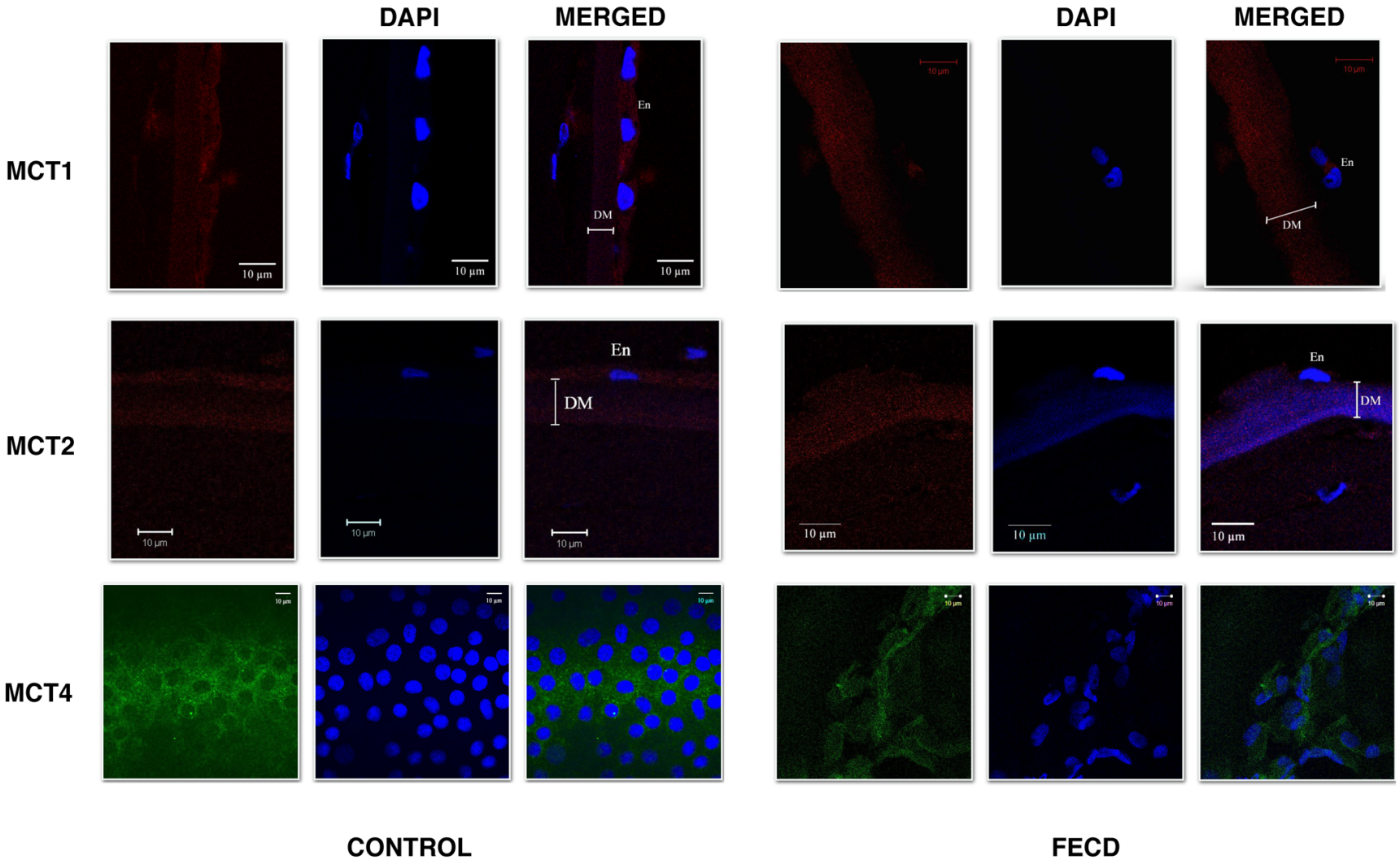


Figure 6.3: Difference in protein expression of MCT1, MCT2 and MCT4 in control versus FECD affected corneal endothelium. Paraffin sections from FECD (n=2) and control (n=3) tissues were analyzed for MCT1 and MCT2. For MCT4 immunohistochemical analysis fresh tissue samples after DSEK (n=2) and trephined control samples (n=3) were used. Each protein localization analysis was normalized individually. ns: non-significant, *** : P<0.005.

Figure 6.4: Localization of MCTs in Control and FECD tissues. Expression of MCT1 (first row) and MCT2 (second row) are observed in paraffin sections, whereas MCT4 (third row) staining is done in DSEK tissues from control and patients. Scale bar: 10 μ m. (**Page 111**) →


DAPI
MERGED
DAPI
MERGED
MCT1
MCT2
MCT4
En
DM
10 µm
CONTROL
FECD

6.2.3 Differences in transcript expression of MCTs in control and FECD corneal tissue types

In order to check the differences in MCT expression in transcript level, mRNA was extracted from Penetrating Keratoplasty (PK) tissues from FECD and whole thickness non-transplant grade control cornea. mRNA expression in FECD tissues were normalized with the control tissue expression. RT-PCR analysis of MCT1, MCT2 and MCT4 in these tissues showed 7.1-fold increase in MCT1 expression, as compared to 0.4 and 0.08-fold differences in MCT2 and MCT4 transcripts respectively. Due to unavailability of patient tissues, analysis was done with one PK tissue and three age-matched control tissues. The difference remained unchanged when compared with three control tissues (**Figure 6.5**). This data suggests MCT1 is overexpressed across the various layers of cornea in order to compensate the decreased lactate flux delivered by lesser expressed MCT1 in the endothelial layer. However, this data needs to be reproduced with increased patient sample size.

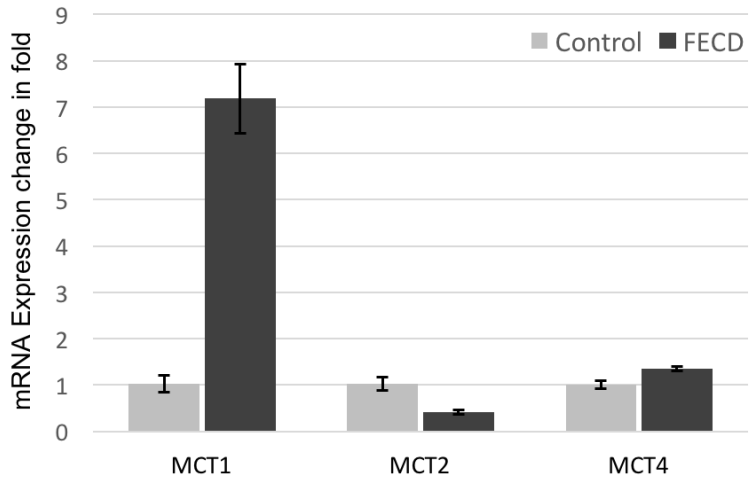


Figure 6.5: Difference in transcript level of MCT1, MCT2 and MCT4 in whole thickness control corneal tissues (n=3) and one PK tissue from an FECD patient. Test for significance could not be calculated because of insufficient sample size. Error bars denote standard error.

.6.3 DISCUSSION

Monocarboxylate transporters are ubiquitous lactate coupled proton flux channel proteins essential in maintaining the intracellular pH in highly glycolytic cells such as corneal epithelial and stromal keratocytes.[261, 262] The localization of these MCTs around the corneal endothelium is strategic and based upon their affinity towards L-lactate molecules. Bonnano *et al.*, studied the kinetic properties of these MCTs in bovine corneal endothelium (BCE) and reported that MCT2 has a higher affinity towards L-lactate (K_m = 0.7mM) than MCT1 (K_m = 3 to 5mM) or MCT4 (K_m = 28mM).[257] Similar to BCE, the basolateral distribution of MCT1 in HCEnC is the first line of transporters that channels the lactate molecules generated in bulk by the corneal epithelium and stromal keratocytes and effluxed through MCT2, present in apical layer, into the anterior chamber of eye. Decreased MCT1 transporters in the remaining endothelial cells of FECD affected cornea will lead to lactate buildup in the entire thickness of cornea which consequent into acidosis, loss of corneal transparency and edema; all of which are symptoms of a severe grade FECD condition (**Figure 6.6**). Based on the current findings it could be reasoned that the epithelial cells and stromal keratocytes express more MCT1 transcripts in order to compensate for the lack of efficient lactate flux into the endothelium due to decreased membrane density of MCT1 transporters in FECD endothelium. However, it is essential to check the expression of MCT1 in the epithelium and stroma of FECD patients to verify this hypothesis.

It is worthy to note that the tissue sections used during IHC analysis and RT-PCR are from patients suffering from late stage FECD, as the tissue sections were obtained post PK procedure, which is performed only on such cases.[263, 264] It is also for this reason that in our study, we have more blood samples than tissue samples as clinicians do not suggest a surgical intervention until after a development of Krachmer grade 4 or above where the central corneal guttae confluence is greater than 5mm or have edematous stroma or epithelium.[51, 244]

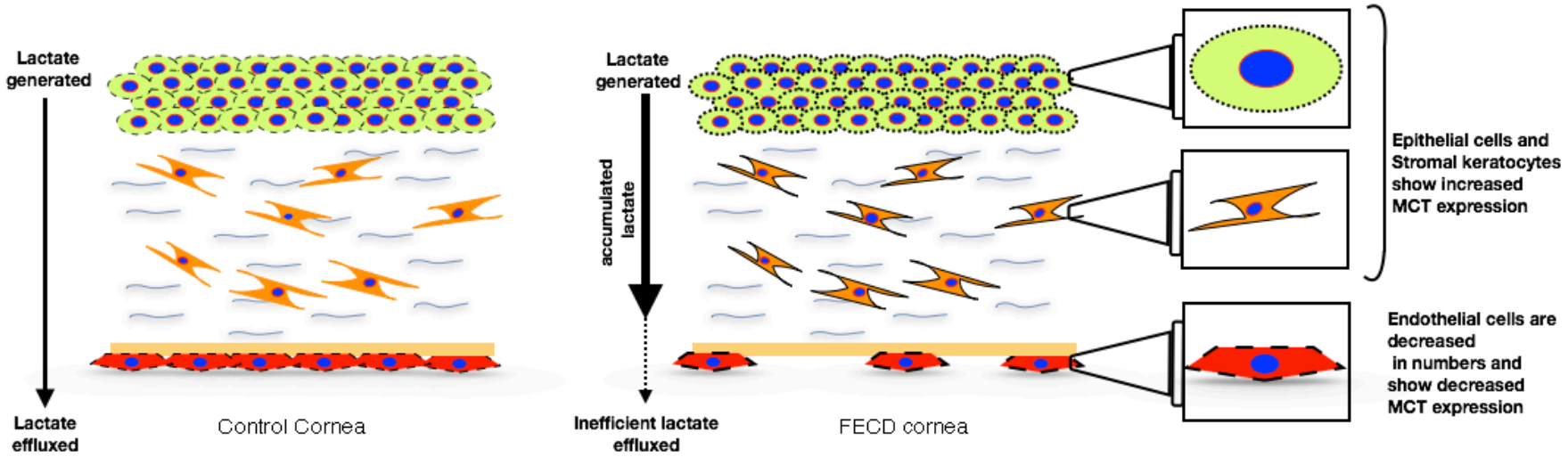


Figure 6.6: Proposed model for endothelial pump failure affects overlying layers of cornea. Monocarboxylate transporters (depicted as dashed lines on cells) show differential expression pattern across the FECD affected cornea. In contrast to normal cornea, epithelium (green cells) and stroma (keratocytes, orange cells) of FECD cornea show increased MCT1 expression. Endothelial layer on the other hand has depleted number of endothelial cells which cumulatively show decreased MCT1 expression as compared to that of control. This can lead to inefficient lactate efflux, resulting into stromal acidosis.

Our study is in consensus with a very recent similar study where researchers characterized the expression levels of MCT1, 2 and 4 along with other carrier pump proteins such as SLC4A11, ATP1B1, pNBCe1, NHE, in FECD corneal endothelium (CEn) in comparison to control CEn.[243] They too reported a significantly decreased *MCT1* and *MCT4* transcripts in FECD tissues.[243] However, they did not find a compensatory increase of other ion-transporters like SLC4A11, pNBC and NHE in the surviving endothelial cells to channel the accumulated proton. Our work extends the information regarding the localization of MCTs in human corneal endothelial layer and also hint towards a mechanism in which FECD condition could be a result of not only a metabolic failure of endothelial layer, but a composite effect contributed by the overlying layers of corneal endothelium. With further studies verifying the contribution of epithelium and stromal keratocytes, the pathomechanism of FECD can be better understood.

Chapter 7

Discussion

7.0 DISCUSSION

7.1 Summary

Untreated Fuchs Endothelial Corneal Dystrophy (FECD) can deteriorate visual acuity to an extent that renders the sufferer visually impaired. About 285 million individuals are struggling with visual impairment and 90% of these individuals belong to financially challenged settings (WHO fact sheet, August 2014).[265] Most of these visually impaired (65%) are people aged above 50 years, which although comprises 20% of the world population, it impacts the countries populated with senile citizens.[265] Corneal diseases have affected about 6.6 million people with vision loss and blindness in India (National Programme for Control of Blindness 2015-16)[266] and about 8.1% of these have corneal dystrophies.[267] Being the third most frequent reason for corneal transplantation, FECD affected individuals in India have to overcome their socio-economic limitations to undergo any surgical interventions to treat the disease.[5] Recent studies have elaborated on various molecular pathological aspects of this complex disorder, which includes oxidative stress,[26, 106, 126, 132, 243, 247, 268-270] DNA damage,[32, 106, 126, 131, 268] RNA stress[86, 129, 130] and malfunctioning of channel proteins.[103, 132, 243, 254] However, there are many unanswered questions which need to be addressed in order to improve current treatment procedures. With this impetus, the present study aims at determining the contribution of various genetic players in Indian FECD patients and their mechanistic role in disease progression. This study intends to provide ethnically rich information about these genetic contributors as there are enormous reports that suggest disease associated genetic markers/players can have population-specific varying penetrance.[182-184, 186-189, 271]

With increasing evidence of *TCF4* (Transcription factor 4) being a prominent genetic contributor for FECD[19-21, 76, 79, 129, 130, 205, 206] and scarce studies to elaborate the Indian scenario, our study aimed to investigate the association of various polymorphisms in *TCF4* gene, rs613872, rs17089887, rs17089925 and CTG18.1 allele, with patients suffering from

FECD in Indian cohort.[84] After age and sex correction, we found that only one of the intronic *TCF4* SNPs, rs17089887, is significantly associated under dominant model of inheritance in our population with "T" allele imparting 2.073 (*95% CI = 1.18 - 3.62*) times increased chances of acquiring FECD. We observed that individuals with TT genotype for rs17089887 are at 65.9% increased risk. The Caucasian associated TCF4 rs613872 show genotypic association with FECD in our report (**Table 3.2**). However, we did not observe an association with respect to rs17089925 which were previously reported to co-segregate with FECD in Chinese[20] and Caucasian[11, 19, 79] cohorts respectively. Although the power of the study at the time was, 75% with a sample size of 44 patients and 108 control individuals, validation of these associations with an increased sample size comprising of participants from different regions of India should be encouraged. The trinucleotide expansion in the fourth intron is polymorphic in our population and expansions above 50 repeats show a 34% penetrance in FECD population as opposed to 5% in controls. These SNPs, rs613872 and rs17089887, are situated very close to each other (2kb); and thereby share an appreciable LD between them (**Figure 3.4**). Combined signal of rs17089887 and CTG or rs613872 and CTG suggests the presence of significant disease-causing changes in nearby regions of these alleles that are physically linked. Recent advances reveal inefficient post-transcriptional splice events as a prognosis for FECD causation.[30, 85, 130] It was reported that the expanded repeats, as transcriptional byproduct, enhanced sequestration of MBNL1 protein, which is a component of splice-machinery. This in the downstream led to accumulation of mis-spliced transcripts in the FECD affected corneal endothelial cells.[30] However, it is yet unclear of the various repertoire of *TCF4* transcripts generated due to these repeats and the downstream deregulation of various genes that may ensue.

Caucasian based familial genetic studies to screen mutations in ZEB1 gene have reported its involvement in PPCD[22, 72, 73, 221-225] and FECD.[11, 18, 182, 226] The current study also focuses on scanning the ZEB1 gene to identify polymorphisms or mutations that can help understand

the association of this gene with more generalized population of disease-affected individuals and avoid statistical clustering of unmeasurable data involved in family-based studies. Apart from various non-pathogenic genetic variants, we identified a particular polymorphism, rs220057 in the non-coding region of ZEB1 that co-segregates with FECD and can be utilized for disease prediction early on along with other SNPs (**Table 4.2**). The other facet of the study focuses on discovering a regulatory role of ZEB1 in the expression of COL8A2, a prominent component of aberrant ECM deposition in LO-FECD. DNA binding assays reveal the initial reports of ZEB1 binding to the first intronic region of COL8A2, which has histone acetylation and DNaseI regulatory signatures (**Figures 4.1, 4.2 and 4.3**). *In vitro* reporter assay corroborates the same and represent ZEB1 as a potent suppressor for COL8A2 expression (**Figure 4.4**).

Oxidative stress and deficiency in antioxidant defense system have been elaborately attributed as the etiology of FECD pathogenesis in numerous studies.[26, 27, 126, 246] In the current study, we assessed the genetic contribution of FEN1 gene in Indian FECD cohort. Genetic analysis of two polymorphisms, rs174538 (c.-69G>A) and rs4246215 (c.4150G>T) harbored in the 5' and 3' UTR regions of *FEN1* gene respectively was carried out in a sample Indian population which demonstrated that carriers of homozygous risk allele "T" of rs4246215 (c.4150G>T) polymorphism were at higher risk of developing FECD (**Table 5.2**). Basal endogenous DNA damage was reportedly high in these individuals (FEN1 c.4150TT) (**Figures 5.2 and 5.3**). We therefore scanned the 3'UTR of FEN1 for miRNA targets using data from various bioinformatics repository and identified hsa-miR-125a-3p and hsa-miR-1236 as putative candidates for miRNA-mediated regulation of FEN1 expression (**Table 5.4**).

Based on the mode of pathomechanism where failure of pump system has been associated with FECD,[243, 254] we intended to assess the involvement of another family of transporter proteins, Monocarboxylate transporters (MCTs) by analyzing their differential expression in

control versus FECD cornea. Before doing so, we carried out an immunohistochemical (IHC) localization study for these MCTs in the human corneal endothelium which was not documented previously. We report that MCT1 is mostly populated along the basement membrane, whereas MCT2 and MCT4 acquire apical and lateral locations respectively. MCT3 is absent in human corneal endothelium (**Figure 6.1**). Following this, we did a comparative study on the distribution differences of these MCTs both in protein and mRNA levels in control versus FECD tissues and observed that mRNA levels of MCT1 is significantly overexpressed in the FECD-affected whole thickness cornea than that in healthy cornea (**Figure 6.5**). In contrast, IHC comparisons of these MCTs indicate a significantly decreased MCT1 in endothelial cells of FECD as compared to healthy endothelium (**Figures 6.3 and 6.4**). These results suggest a failure in pump system of FECD affected cornea which resonate not only a metabolic failure of endothelial layer, but also a composite effect contributed by the overlying layers of the corneal endothelium (**Figure 6.6**). A validation of these results with increased sample size is however essential.

7.2 Conclusion

We report that the individuals with haplotype "T-X" of rs17089887 risk allele "T" and expanded CTG18.1 allele (represented as X) respectively show increased susceptibility to acquire FECD. It is however interesting to note that in comparison to an average of 80% FECD affected Caucasians[21, 130], only 34% of Asian FECD individuals harbor these expanded repeats in TCF4 gene. Thereby, indicating the presence of other confounding factors responsible to cause FECD in these parts of the globe.

With genetic screening of ZEB1 coding region in FECD population, this study has identified a novel polymorphic association in the intronic region, rs220057 that co-segregates with the disease. Although we did not find any transcription binding at the polymorphic site, it was noted that this polymorphism falls within a SINE sequence. This study suggests a significant

contribution of ZEB1 gene in the pathogenesis of FECD and warrants an in-depth scan of the gene to identify a disease-causing variant or decipher whether a SINE-mediated ZEB1 regulation is involved in corneal endothelial cells. Binding of ZEB1 to COL8A2 thereby showing a suppressive activity through luciferase assay also hints at a regulatory role involved in the expression of COL8A2 which in extension could result in over-accumulation of this collagen in the DM of corneal endothelium.

Our study identified that the 3'UTR polymorphism, FEN1 rs4246215 (c.4150G>T) is strongly associated with increased DNA damage in subjects clinically diagnosed with FECD in Indian population. The study also provides initial insights into the fact that the FECD associated risk allele, FEN1 c.4150T, shows significant physiological changes. DNA damage in the blood leucocytes of FECD patients and control individuals, due to induced oxidative insult was studied by P. Czarny *et al.*, and they reported that the recovery of FECD leucocytes from stress-induced DNA damage was significantly lower than that of the controls; implying a faulty DNA repair mechanism under disease condition.[32] Later on, it was also reported that the TT genotype of *FEN1* c.4150G>T was significantly associated with the increased incidence of FECD and Keratoconus in Polish population.[23] In extension to Czarny *et al.* and Wojcik *et al.* reports, our study imply that the homozygous "T" allele of *FEN1* c.4150G>T variation in conjunction with other factors responsible for FECD pathogenesis, contribute towards a compromised condition where the endogenous DNA damage is heightened. These results provide supportive evidence to confirm the genetic involvement of *FEN1* gene in the pathogenesis of FECD. However, it warrants further studies to dissect out the specific DNA damage and repair pathway genes that are de-regulated in FECD corneal tissues as compared to those of the control tissues.

MCTs are ubiquitous lactate coupled proton flux channel proteins essential in maintaining the intracellular pH in highly glycolytic cells such as corneal epithelial and stromal keratocytes.[29, 257, 272, 273] The localization of these MCTs around the corneal endothelium is

strategic and based upon the affinity towards L-lactate molecules. Decreased MCT1 transporters in the remaining endothelial cells of FECD affected cornea will lead to lactate buildup in the entire thickness of cornea which consequent into acidosis, loss of corneal transparency and edema; all of which are symptoms of a severe grade FECD condition. Based on the current findings it could be reasoned that the epithelial cells and stromal keratocytes might express more MCT1 transcripts in order to compensate for the lack of efficient lactate flux into the endothelium due to decreased membrane density of MCT1 transporters. Our work extends the information regarding the localization of MCTs in human corneal endothelial layer and also hint towards a mechanism in which FECD condition could be a result of not only a metabolic failure of endothelial layer, but also a composite effect contributed by the overlying layers of the corneal endothelium. With further studies verifying the contribution of epithelium and stromal keratocytes, the pathomechanism of FECD can be better understood.

This study in its entirety represents the plethora of alterations in the genetic makeup of the Indian individuals suffering from FECD. This is the first step towards our goal in developing a genetic biomarker in which by studying a handful of genetic variations one can predict the disease early in the life of an FECD patient and will aid in understanding their population specific genetic contribution for the disease. The study also attempts to highlight the probable course of action pursued by these genetic contributors in the pathogenesis of FECD. We aim to carry out in-depth analysis of each gene discussed in this study further in near future to elaborate their molecular involvement in disease pathogenesis.

7.2 Key findings from the study

- Expansion of TCF4 repeats in the fourth intron show only 34% penetrance in Indian population as opposed to 80% in Caucasians to cause FECD.

- ZEB1 shows a prominent binding at the first intron of COL8A2 and thereby imparts a suppressive activity suggesting probable regulatory mechanism involved.
- FEN1 works on the lines of oxidative DNA damage and the associated risk allele *FEN1* c.4150T along with other confounding factors are involved in bringing out the heightened DNA damage in homozygous carriers of the polymorphism.
- MCT1 is differentially expressed in endothelium and entire thickness cornea of FECD versus control tissues suggesting a composite metabolic failure contributed by overlying layers of FECD affected cornea.

7.3 Methods developed during the course of this project in the laboratory

- Standardization of EMSA analysis for ZEB1 detection from HCEnC cells.
- Comet-assay from cryogenically preserved blood samples to assess endogenous DNA degradation level.
- Immunohistochemical analysis of paraffin and fresh tissue sections of human corneal endothelial tissue.

7.4 Future Prospective

- Recent literatures suggest that expanded TCF4 CTG repeats cause RNA foci formation which lead to nuclear stress and eventual apoptosis. A drug mediated recovery from RNA foci can be pursued to check if it alleviates nuclear stress and avoids apoptosis of corneal endothelial cells.
- siRNA mediated suppression of COL8A2 by ZEB1 in HEK293 and HCEnC system can be analyzed.
- Allele specific regulation of *FEN1* gene by hsa-miR-1236-3p should be analyzed by modifying its 3'UTR using cell-based CRISPR-Cas9 system.

- Difference in expression of MCTs in control versus FECD cornea can be validated with increased tissue samples. Whether physiologic impact of decreased MCT1 in HCEnC mimics the FECD condition, can be investigated to strengthen its involvement in disease progression.

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