

## ARTICLE

# Association of *TCF4* and *CLU* polymorphisms with Fuchs' endothelial dystrophy and implication of *CLU* and *TGFBI* proteins in the disease process

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Fuchs' endothelial dystrophy (FED) is a disease affecting the corneal endothelium. Recent studies reported significant association of polymorphisms in the *TCF4* (*transcription factor 4*) gene, and a borderline association of *PTPRG* (*protein tyrosine phosphatase, receptor type, G*) variants with late-onset FED in Caucasians from the United States. Association of *TCF4* has also been reported in the Chinese population. We aimed to determine association of the reported polymorphisms in *TCF4* and *PTPRG*, and association of polymorphisms in the candidate genes *ZEB1* (*zinc-finger E-box binding homoebox 1*), *COL8A2* (*collagen, type VIII, alpha 2*), *TGFBI* (*transforming growth factor,  $\beta$ -induced*) and *CLU* (*clusterin*) in Australian cases. We also compared the expression of *TGFBI* and *CLU* proteins between FED and normal whole corneas. In all, 30 single-nucleotide polymorphisms (SNPs) from the candidate genes were genotyped in 103 cases and 275 controls. Each SNP and haplotype was assessed for association with the disease. SNP analysis identified an association of *TCF4* (rs613872 ( $P=5.25 \times 10^{-15}$ , OR=4.05), rs9954153 ( $P=3.37 \times 10^{-7}$ , OR=2.58), rs2286812 ( $P=4.23 \times 10^{-6}$ , OR=2.55) and rs17595731 ( $P=3.57 \times 10^{-5}$ , OR=3.79)), *CLU* (rs17466684;  $P=0.003$ , OR=1.85) and one haplotype of *TGFBI* SNPs ( $P=0.011$ , OR=2.29) with FED in Caucasian Australians. No evidence for genetic association of *PTPRG*, *ZEB1* and *COL8A2* was found. Immunohistochemistry showed differential expression of *CLU* and *TGFBI* proteins in FED-affected compared with normal corneas. In conclusion, variation in *TCF4*, *CLU* and *TGFBI*, but not *PTPRG*, *ZEB1* and *COL8A2* genes are associated with FED in Caucasian Australian cases. Differential expression of *CLU* and *TGFBI* proteins in FED-affected corneas provides novel insights into the disease mechanism.

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## INTRODUCTION

Fuchs' endothelial dystrophy (FED, MIM 136800) is a degenerative disease of the corneal endothelium (CE).<sup>1</sup> The earliest clinical feature of FED is progressive formation of microscopic outgrowths (guttæ) in Descemet's Membrane (DM),<sup>2</sup> a collagen-rich basal lamina secreted by the CE.<sup>3</sup> Early stages of FED are characterised by aberrant thickening of DM and gradual loss of the non-regenerative CE.<sup>4</sup> This impairs the ability of the endothelium to pump excess fluid out of the corneal stroma,<sup>1</sup> resulting in corneal oedema, and ultimately leading to vision loss and pain.<sup>2</sup> Corneal transplantation using post-mortem donor tissue is the only effective treatment for FED.<sup>5</sup>

The prevalence of FED varies markedly across the world. It affects ~4% of the USA population over the age of 40 years,<sup>6</sup> but is less frequent in Asian<sup>7</sup> and Middle-Eastern populations.<sup>8</sup> In Australia, corneal grafting for FED accounts for ~6% of all corneal grafts performed annually.<sup>9</sup>

FED is a genetically heterogeneous disease. There are two forms defined by the age of onset. Early-onset FED is rare<sup>10</sup> and is typically inherited as an autosomal dominant disease with high penetrance and almost uniform expressivity.<sup>2</sup> Mutations in the *COL8A2* (*collagen, type*

*VIII, alpha 2*) gene cause some cases of early-onset FED.<sup>11</sup> *COL8A2* is an extracellular matrix protein and is a major component of DM.<sup>12</sup> The more common late-onset FED can either be familial or sporadic, with onset typically after the age of 40 years.<sup>13</sup> The risk of developing the late-onset form increases with age and female sex.<sup>6</sup> Familial late-onset FED shows an autosomal dominant inheritance with high penetrance, but variable expressivity.<sup>6</sup> Mutations in the *ZEB1* (*zinc-finger E-box binding homoebox 1*) gene can cause both sporadic and familial late-onset FED.<sup>6,14</sup> *ZEB1* is expressed in the CE.<sup>15</sup> It regulates cell proliferation and differentiation by inducing epithelial–mesenchymal transition.<sup>16,17</sup> The *SLC4A11* (*solute carrier family 4, sodium borate transporter, member 11*) mutations cause sporadic and familial late-onset FED.<sup>8,18</sup> Mutations identified in the *COL8A2*, *ZEB1* and *SLC4A11* genes account for a small number of FED cases. Furthermore, four chromosomal loci are associated with late-onset FED, but the causative genes remain to be identified.<sup>19</sup>

Recently, single-nucleotide polymorphisms (SNPs) in the *TCF4* (*transcription factor 4*) gene, encoding the E2-2 protein, were reported to be significantly associated with late-onset FED in Caucasian

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Americans.<sup>19</sup> In the same study, SNPs in the *PTPRG* (*protein tyrosine phosphatase, receptor type, G*) gene were nominally associated with FED, but the association did not reach genome-wide significance. Independent replication studies have confirmed association of *TCF4* variants with late-onset FED in Caucasian American<sup>20</sup> and Chinese cohorts,<sup>21</sup> but the association with *PTPRG* remains to be confirmed.

Proteomic analyses have shown overexpression of TGFBI (transforming growth factor,  $\beta$ -induced) and *CLU* (clusterin) proteins in the CE+DM complex from FED patients compared with normal controls.<sup>22</sup> *CLU* is a molecular chaperone and is implicated in protecting cells from effects of physiological stress caused by aging, oxidative stress and apoptosis, which are proposed as potential mechanisms underlying FED pathophysiology.<sup>23–25</sup> TGFBI is a secreted extracellular matrix protein that mediates cell adhesion by interacting with major protein components of DM, including collagens.<sup>22</sup> FED is characterised by breakdown of tight junctions between corneal endothelial cells and transformation into mesenchymal cells, a feature of aberrant cell adhesion. TGFBI is induced by the *TGF- $\beta$*  (*transforming growth factor- $\beta$* ) gene.<sup>26</sup> Interestingly, *TGF- $\beta$*  reportedly regulates both *TCF4* and *ZEB1*, which have been implicated in FED.<sup>27</sup>

This study investigated association of the reported polymorphisms in the *TCF4* and *PTPRG* genes in Australian FED cases. It also investigated association of SNPs in the candidate genes *ZEB1*, *COL8A2*, *TGFBI* and *CLU* in these cases. The *CLU* and *TGFBI* genes were chosen because their function relates to the pathophysiology of FED and the encoded proteins are overexpressed in the diseased DM. FED-associated mutations in *SLC4A11* were reported in four FED-affected Asian cases<sup>8</sup> at the time of this study. However, no disease-associated mutations have been reported in Caucasians; therefore, association of this gene was not assessed. We also performed immunohistochemistry to compare expression and distribution of the TGFBI and *CLU* proteins in sections of FED and normal whole corneas. Our data support genetic association of the *TCF4* gene with FED in Caucasian Australians and show novel but nominal association of *CLU* with FED in these cases. Furthermore, these data show that TGFBI and *CLU* are expressed in the corneal epithelium of FED corneas, but not in control corneas, also a novel finding.

## MATERIALS AND METHODS

### Participant recruitment and sample collection

This study was approved by the Southern Adelaide Health Service/Flinders University Clinical Research Ethics Committee and the Royal Victorian Eye and Ear Hospital Human Research Ethics Committee, in accordance with the requirements of the National Health and Medical Research Council Statement on Ethical Conduct in Human Research (2007). Patients diagnosed with Grade 3–6 advanced FED<sup>28</sup> were recruited and gave written informed consent. Blood samples were collected from 103 participants through the Flinders Eye Clinic (Adelaide, SA, Australia) and the Royal Victorian Eye and Ear Hospital (Melbourne, VIC, Australia). Genomic DNA was extracted using QIAamp DNA Blood Maxi kit (Qiagen Pty Ltd, Doncaster, VIC, Australia), following the manufacturer's protocol. Control genomic DNA samples were from unrelated, unaffected South Australian residents aged over 50 years recruited previously for use as controls in a variety of ocular genetic studies. Two normal and three FED corneas for immunohistochemistry were provided by the Department of Anatomical Pathology (Flinders University, Bedford Park, SA, Australia). FED corneas were obtained for histopathological purposes following corneal transplantation, whereas normal corneas were from deceased donors deemed unsuitable for corneal transplantation and obtained through the Eye Bank of South Australia.

### Genotyping and data analysis

FED-associated SNPs in *TCF4* (rs613872, rs17595731, rs2286812 and rs9954153) and *PTPRG* (rs7640737 and rs10490775) were selected from the

literature<sup>19</sup> for replication in this study. Tag SNPs in the *ZEB1*, *COL8A2*, *TGFBI* and *CLU* genes were selected on the basis of linkage disequilibrium patterns observed in the Caucasian samples genotyped as part of the International HapMap Project (<http://hapmap.ncbi.nlm.nih.gov/>) using the tagger program implemented in Haploview.<sup>29</sup> Only SNPs conforming to Hardy–Weinberg equilibrium, having no Mendelian genotyping errors and a minimum minor allele frequency of 10%, were included. The linkage disequilibrium ( $r^2$ ) threshold was set to 0.8. All SNPs were genotyped in 103 FED cases and 275 normal controls using iPLEX Gold chemistry (Sequenom Inc., Herston, QLD, Australia) on a MassArray Spectrophotometer (Sequenom Inc.) at the Australian Genome Research Facility (Brisbane, QLD, Australia). Genotypes were analysed using PLINK (<http://pngu.mgh.harvard.edu/~purcell/plink/dataman.shtml>).<sup>30</sup> Chi-square ( $\chi^2$ ) and/or Fisher's exact tests were conducted to assess inheritance models. Significantly associated SNPs were analysed using logistic regression in PLINK to explore the effects of age and sex, although age of recruitment was only available for 258 controls. Analysis of haplotypes with >1% frequency was also performed using PLINK. *P*-values and odds ratios (ORs) were calculated for each haplotype compared with all others. Power calculations were conducted using the Genetic Power Calculator (<http://pngu.mgh.harvard.edu/~purcell/gpc>).<sup>31</sup>

### Immunohistochemistry

Paraffin-embedded sections of FED and normal corneas were immunolabelled for the *CLU* and TGFBI proteins as described previously,<sup>32</sup> except for the following variations.

Citric acid antigen retrieval (0.011 M sodium citrate, pH 6) was performed for immunolabelling with the anti-TGFBI antibody, and alkaline antigen retrieval (1×Dako Target Retrieval Solution, pH 9; Dako Australia Pty Ltd, Scoresby, VIC, Australia) for labelling with the anti-clusterin antibody. Slides were placed in retrieval buffer and heated to boiling point for 4 min in a microwave, before resetting the microwave to low mode and heating the slides for further 10 min. Sections were cooled for 1 h at room temperature, and washed in TBS before incubation with rabbit polyclonal anti-human TGFBI antibody (1:1000; HPA008612; Sigma-Aldrich Inc., St Louis, MO, USA) or anti-human clusterin antibody (1:2000; Sc-8354; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight. The sections were incubated for 1 h at room temperature with the NovoLink Polymer complex reagent (Leica Microsystems, Bannockburn, IL, USA). Primary antibody binding was detected with Liquid DAB+ Substrate Chromogen System (K3468; Dako Australia Pty Ltd). Sections were counterstained with haematoxylin and mounted in DePeX (Merck KGaA, Darmstadt, Germany). Light microscopy and photography were performed on an Olympus BX41 microscope fitted with a digital DP20/DP70 camera using CellSens Standard Photography software (Olympus Corporation, Tokyo, Japan).

## RESULTS

### Genetic analyses

Genotypes of the chosen SNPs were obtained for 103 FED cases and 275 unaffected controls. The mean sex and age distribution of cases and controls is provided in Table 1. Controls were significantly older than cases by design to reduce the likelihood of controls having yet to manifest the disease. The case cohort had a higher percentage of females than the controls consistent with the reported higher prevalence of the disease in females.<sup>6</sup> This study had 80% power to detect a moderate effect size of genotypic relative risk of 1.5 under an

**Table 1** Characteristics of the study population

Variables	Cases	Controls	P-value
Total number of participants	103	275	
Total number of female participants (%)	68 (68%)	138 (53%)	0.01
Average age (SD) (in years)	68.6 (15.5)	76.0 (8.4)	<0.001

Total number of participants with case and control status is given along with sex and age distributions.

additive model, assuming a disease prevalence of 4% and a risk allele frequency of 20%.<sup>31</sup>

Allele frequencies and association results for genotyped SNPs are given in Table 2. We detected significant allelic association between FED and each SNP from the *TCF4* locus. The G allele of the *TCF4* intronic SNP rs613872 showed the most significant association with FED ( $P=5.25 \times 10^{-15}$ ; OR (95% CI)=4.05 (2.82–5.83)). Genotypic analysis indicated significant association of the four *TCF4* SNPs under the dominant model ( $P=4.54 \times 10^{-16}$  at rs613872;  $P=4.39 \times 10^{-7}$  at rs9954153;  $P=8.65 \times 10^{-6}$  at rs2286812 and  $P=4.05 \times 10^{-5}$  at rs17595731). These results replicate the findings of previous studies in Caucasians.<sup>19,20</sup> The rs10490775 and rs7640737 SNPs from the *PTPRG* locus showed a borderline allelic association with FED in this study.

Of the seven tag SNPs from *CLU*, rs17466684 showed significant allelic association with FED ( $P=0.003$ ; OR (95% CI)=1.85 (1.23–2.78)), (Table 2) under a dominant model ( $P=0.006$ ). Logistic regression data revealed that rs17466684 is associated with FED independently of age and sex in this cohort ( $P$ -value=0.0056, OR (95% CI)=1.86 (1.20–2.87)). Haplotype analyses of the genotyped SNPs in *CLU* revealed significant association with FED with an overall  $P$ -value of 0.049 (Table 3). Three specific haplotypes were associated.

**Table 3 Association between common haplotypes of tag SNPs in the *CLU* gene and FED**

Haplotype	f cases	f controls	Overall $P$ -value=0.049	
			P-value	OR (95% CI)
GAGTCGA	0.30	0.30	0.804	0.95 (0.65–1.40)
GAGCGGA	0.17	0.17	0.992	1.00 (0.67–1.49)
GGACCCA	0.02	0.04	<b>0.035</b>	<b>0.27 (0.06–1.10)</b>
AAACCCG	0.06	0.02	<b>0.017</b>	<b>3.11 (1.22–7.97)</b>
AAACCCA	0.01	0.01	0.748	0.75 (0.13–4.49)
GAGTCGG	0.08	0.08	0.665	0.88 (0.48–1.60)
AAGTCGA	0.04	0.01	<b>0.011</b>	<b>5.20 (1.43–19.0)</b>
GAACCCG	0.05	0.03	0.463	1.41 (0.57–3.45)
GAACCCA	0.02	0.03	0.282	0.51 (0.14–1.86)
GAACCGA	0.04	0.03	0.672	1.21 (0.50–2.92)
GAGCGGG	0.10	0.14	0.148	0.67 (0.39–1.17)
AGACCCA	0.06	0.05	0.565	1.28 (0.56–2.91)
AGACCCG	0.06	0.04	0.369	1.38 (0.69–2.77)
GGACCCG	0.01	0.03	0.068	0.26 (0.05–1.36)

Abbreviations: f, frequency; OR, odds ratio; CI, confidence interval. Significant associations are in bold font.

**Table 2 Results of association tests for SNPs from *TCF4* and *PTPRG*, and tag SNPs from *ZEB1*, *COL8A2*, *TGFBI* and *CLU* loci with FED**

Gene	SNP	Allele 1/2	Allele 1 freq cases	Allele 1 freq controls	P-value	OR (95% CI)
<i>TCF4</i>	Rs613872	G/T	0.42	0.15	<b><math>5.25 \times 10^{-15}</math></b>	<b>4.05 (2.82–5.83)</b>
	Rs9954153	G/T	0.33	0.16	<b><math>3.37 \times 10^{-7}</math></b>	<b>2.58 (1.78–3.74)</b>
	Rs2286812	T/C	0.25	0.12	<b><math>4.23 \times 10^{-6}</math></b>	<b>2.55 (1.70–3.84)</b>
	Rs17595731	C/G	0.10	0.03	<b><math>3.57 \times 10^{-5}</math></b>	<b>3.79 (1.94–7.42)</b>
<i>PTPRG</i>	Rs10490775	A/G	0.11	0.16	0.060	0.62 (0.38–1.02)
	Rs7640737	T/C	0.11	0.16	0.074	0.64 (0.39–1.05)
<i>ZEB1</i>	Rs9299657	A/G	0.26	0.24	0.545	1.12 (0.78–1.62)
	Rs2839657	T/C	0.44	0.45	0.673	0.93 (0.67–1.29)
	Rs2839658	T/C	0.28	0.27	0.906	1.02 (0.71–1.46)
	Rs2839659	T/C	0.37	0.41	0.307	0.84 (0.61–1.17)
	Rs431073	C/A	0.22	0.23	0.892	0.97 (0.66–1.43)
	Rs1618092	C/T	0.44	0.47	0.437	0.88 (0.64–1.22)
	Rs2839666	C/T	0.15	0.15	0.961	0.99 (0.63–1.56)
	Rs11008516	G/T	0.14	0.17	0.318	0.79 (0.51–1.25)
<i>COL8A2</i>	Rs399393	A/G	0.09	0.05	0.066	1.75 (0.96–3.19)
	Rs4652900	A/G	0.23	0.17	0.067	1.45 (0.97–2.15)
	Rs7550047	G/A	0.10	0.07	0.220	1.41 (0.81–2.46)
	Rs7553155	A/T	0.13	0.12	0.573	1.15 (0.71–1.87)
<i>TGFBI</i>	Rs756463	T/C	0.28	0.22	0.090	1.38 (0.95–1.99)
	Rs2073510	G/A	0.27	0.26	0.690	1.08 (0.75–1.55)
	Rs2302038	G/A	0.47	0.45	0.619	1.09 (0.79–1.50)
	Rs4572	C/A	0.31	0.29	0.521	1.12 (0.79–1.59)
	Rs10043360	A/T	0.17	0.17	0.925	0.98 (0.64–1.50)
<i>CLU</i>	Rs17466684	A/G	0.22	0.13	<b>0.003</b>	<b>1.85 (1.23–2.78)</b>
	Rs867231	G/C	0.27	0.30	0.375	0.85 (0.59–1.22)
	Rs3087554	G/A	0.15	0.17	0.497	0.86 (0.55–1.34)
	Rs9314349	G/A	0.35	0.37	0.687	0.93 (0.67–1.31)
	Rs9331888	C/G	0.28	0.27	0.724	1.07 (0.74–1.53)
	Rs1532278	T/C	0.41	0.40	0.747	1.06 (0.76–1.46)
	Rs9331908	A/G	0.31	0.30	0.892	1.02 (0.72–1.45)

Abbreviations: SNP, single-nucleotide polymorphism; 1/2, minor allele/major allele; Freq, frequency; OR, odds ratio; CI, confidence interval. Significant associations are in bold font.

**Table 4** Association between common haplotypes of tag SNPs in the *TGFBI* gene and FED

Haplotype	f cases	f controls	Overall P-value=0.057	
			P-value	OR (95% CI)
TAAAT	0.11	0.05	<b>0.011</b>	<b>2.29 (1.23–4.28)</b>
CAAAT	0.44	0.51	0.083	0.75 (0.54–1.04)
CGGCT	0.29	0.27	0.622	1.09 (0.77–1.55)
TAGAA	0.17	0.17	0.984	1.00 (0.64–1.56)

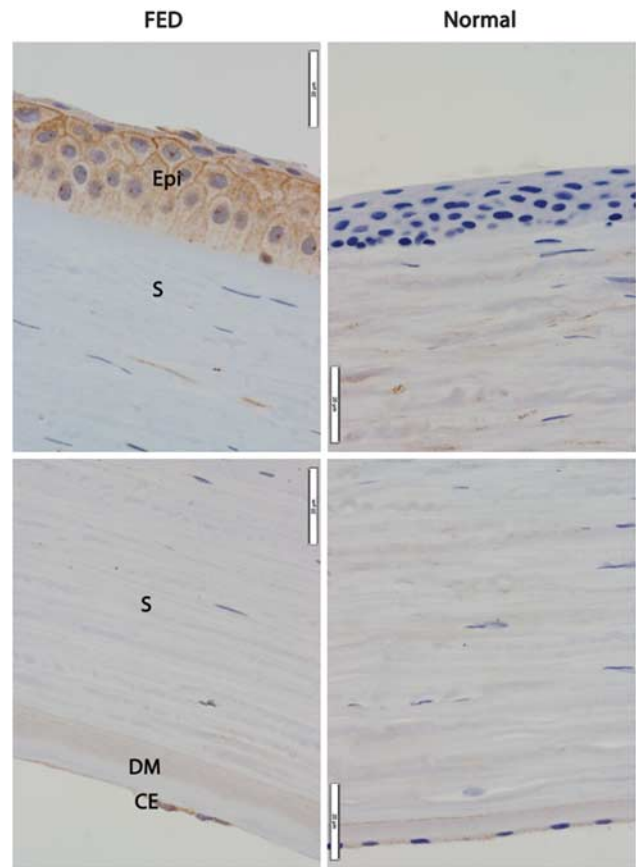
Abbreviations: f, frequency; OR, odds ratio; CI, confidence interval. Significant associations are in bold font.

The GGACCCA haplotype appears to be protective ( $P=0.035$ ; OR (95% CI)=0.27 (0.06–1.10)), while AAACCCG ( $P=0.017$ ; OR (95% CI)=3.11 (1.22–7.97)) and AAGTCGA ( $P=0.011$ ; OR (95% CI)=5.20 (1.43–19)) haplotypes increase the risk of FED. Tag SNPs in the *ZEB1*, *COL8A2* and *TGFBI* genes revealed no allelic association with FED (Table 2). However, haplotype analyses of SNPs in the *TGFBI* gene revealed a borderline association (overall  $P$ -value=0.057; Table 4). The TAAAT haplotype showed significant association ( $P=0.011$ ; OR (95% CI)=2.29 (1.22–7.97)) and remained significant after Bonferroni correction (corrected  $P=0.043$ ). Haplotype analyses performed for SNPs in the *ZEB1* and *COL8A2* genes showed no significant associations (data not shown).

### Immunohistochemistry

Immunohistochemistry was performed on sections of corneas from individuals with advanced FED and normal individuals to investigate differences in distribution of the CLU and *TGFBI* proteins. Immunolabelling results from all three FED corneas showed similar expression patterns for each protein examined. In corneal sections immunolabelled for CLU, weak CLU-positive labelling was observed in the cytoplasm in endothelial cells in normal corneas, but reduced cell numbers made it difficult to ascertain similar labelling in FED-affected corneas (Figure 1, CE, bottom panels). Weak CLU labelling was also seen in the DM in both normal and FED corneas (Figure 1, DM, bottom panels). The protein distributed solely in the stromal aspect of the thickened DM in affected corneas (Figure 1, DM, bottom left panel), whereas uniform distribution was observed in normal corneas (Figure 1, bottom right panel). Similar stromal CLU labelling was observed in both normal and FED corneas (Figure 1, S). No labelling for CLU was observed in normal corneal epithelium (Figure 1, top right panel), but strong cytoplasmic labelling was observed in the corneal epithelium in FED-affected corneas (Figure 1, Epi, top left panel).

Immunolabelling for *TGFBI* did not show detectable protein expression in endothelial cells either in FED-affected or normal corneas (Figure 2, CE, bottom panels). However, *TGFBI* expression was observed in guttae on the posterior face of the DM in FED-affected corneas (Figure 2, DM, bottom left panel). Uniform distribution of the protein was seen in the DM in normal and affected corneas (Figure 2, DM, bottom panels). As expected, the DM was thicker in affected corneas and had guttae (Figure 2, arrow in bottom left panel). *TGFBI* labelling was observed in the stroma in both normal and FED corneas, but the normal stroma showed denser labelling indicating difference in intensity of expression (Figure 2, S). Interestingly, a thickened Bowman's layer devoid of *TGFBI* labelling was observed in FED-affected corneas (Figure 2, BL, top left panel). Comparatively, Bowman's layer was thinner and positively labelled for *TGFBI* in normal corneas (Figure 2, double-headed arrow, top right panel). No



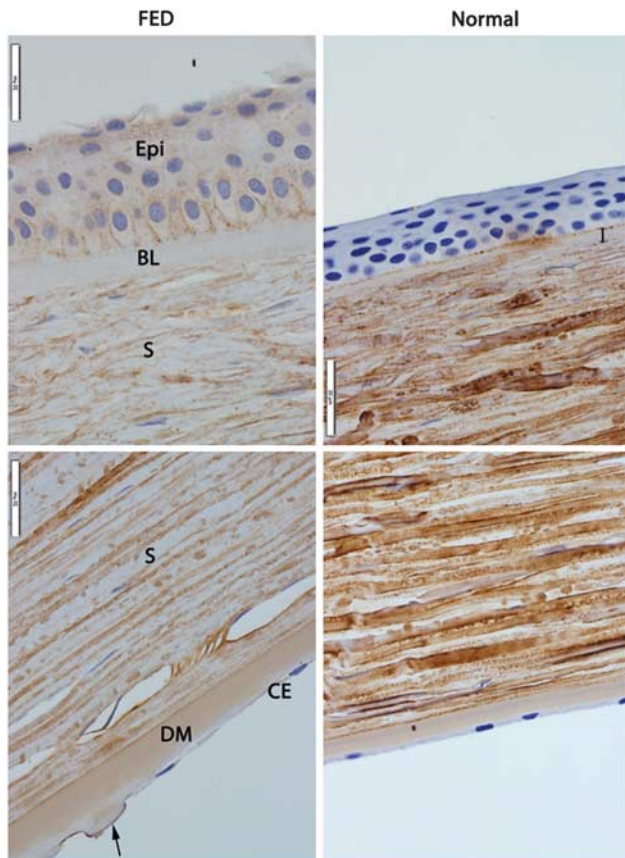
**Figure 1** Immunohistochemical labelling for clusterin (CLU) protein in sections of corneas from FED (left panels) and normal (right panels) individuals. Anti-CLU labelling (brown) can be seen in FED corneal epithelium (Epi), but is absent in normal corneal epithelium. Similar distribution of CLU can be seen in the corneal stroma (S) in both FED and normal corneas. CLU is expressed only along the anterior/stromal face of the DM in FED cornea. In contrast, it is present throughout the DM in normal cornea. CLU is expressed in the CE of both FED and normal corneas. The CE in FED cornea has fewer cells compared with normal cornea, which is consistent with endothelial degeneration in FED. Magnification  $\times 600$ . Representative images from three experiments on independent specimens are shown.

labelling was observed in the epithelium in normal corneas (Figure 2, Epi, top right panel). In contrast, positive immunolabelling for *TGFBI* was observed in the cytoplasm and between adjacent epithelial cells in FED-affected corneas (Figure 2, Epi, top left panel).

### DISCUSSION

The results of this study support recent studies reporting that SNPs in the *TCF4* gene are strongly associated with FED,<sup>19,20</sup> and suggest that *TCF4* contributes to FED pathogenesis in Caucasian Australians. We observed that individuals with the minor allele C at rs17595731, or minor allele G at rs613872 in *TCF4*, are four times more likely to develop FED than the non-carriers. In addition, individuals with allele G at rs9954153 or allele T at rs2286812 have 2.6 times the risk of developing FED than non-carriers. A previous report found that all four *TCF4* SNPs were independently associated with FED,<sup>19</sup> and that a dominant model is most likely. Our data support these findings.

*TCF4* encodes the E2-2 protein, which is a member of the ubiquitously expressed class I basic helix-loop-helix transcription



**Figure 2** Immunohistochemical labelling for transforming growth factor  $\beta$ -induced (TGFBI) protein in sections of corneas from FED (left panels) and normal (right panels) individuals. Anti-TGFBI labelling (brown) can be seen in FED corneal epithelium (Epi), but is absent in normal corneal epithelium. TGFBI is not expressed in abnormally thicker Bowman's layer in FED cornea (BL), but is expressed in the thinner Bowman's layer in the normal cornea (double-headed arrow). Expression of TGFBI is less intense in FED corneal stroma (S) compared with normal corneal stroma. Similar distribution of TGFBI can be seen in the DM in both FED and normal corneas. DM in FED cornea is thicker than that in normal cornea, and has guttae (arrow). No TGFBI expression was observed in the corneal endothelium (CE) of both FED and normal corneas. Magnification  $\times 600$ . Representative images from three experiments on independent specimens are shown.

factors that are involved in cellular growth and differentiation.<sup>27</sup> E2-2 is expressed in developing CE.<sup>27</sup> It plays an essential role in developmental processes including EMT (epithelial–mesenchymal transition), a normal aspect of cell migration in embryogenesis and tumor-cell invasion and metastasis.<sup>33</sup> It binds to E-box promoter sequences within target genes and either suppresses or activates tissue-specific gene transcription.<sup>27</sup> E2-2 represses expression of cell-adhesion protein E-cadherin, thereby causing loss of cellular polarity and cell-to-cell contact and hence EMT. ZEB1 protein, which also binds to E-box promoters and is involved in EMT through E-cadherin repression,<sup>34</sup> is upregulated by E2-2. Rare pathological mutations in *ZEB1* reportedly contribute to late-onset FED in Caucasians.<sup>6</sup> These observations suggest that the *TCF4* variants associated with FED could confer the disease risk by altering the expression of *ZEB1*. Loss of function of a copy of E2-2 through coding mutations or genomic deletions leads to Pitt–Hopkins syndrome,<sup>19</sup> which is not known to develop in patients with FED. In addition, *TCF4* maps inside the *FCD2* locus on chromosome 18q.<sup>35</sup> This locus has been linked with

the disease in multiple families, but variation in the *TCF4* gene does not appear to account for the linkage signal.<sup>20</sup>

Association of rs7640737 and rs10490775 SNPs in *PTPRG* with FED was reported, but the result did not meet the requirements for genome-wide significance.<sup>19</sup> The study by Riazuddin and co-workers<sup>20</sup> did not replicate the rs10490775 SNP results. Our study detected a borderline allelic association of rs7640737 and rs10490775 with FED (Table 2). While the direction of association at SNP rs10490775 is consistent between this study and that of Baratz *et al*, at SNP rs7640737 the risk alleles are opposite.<sup>19</sup> Given the inconsistent findings at this locus, we conclude that as yet there is limited evidence for a true association of SNPs from *PTPRG* with FED, although we acknowledge that the power of our study to detect weak associations is limited.

Analysis of tag SNPs from *CLU* revealed a significant association of rs17466684 with advanced disease in the Caucasian Australians (Table 2). This is the first report of genetic association of *CLU* with FED, although further work is required to determine the causative allele. Overexpression of *CLU* has been shown in FED-affected corneas.<sup>22</sup> Its expression is also higher in corneas from normal older individuals compared with younger individuals,<sup>22</sup> which could be relevant for late-onset FED. Our study also shows for the first time the epithelial expression of *CLU* in FED-affected cornea, but not normal controls (Figure 1, Epi, top left panel), implying upregulation of the protein specifically in this cell layer in the disease state. Histologically, the advanced FED-affected cornea is characterised by thickened corneal epithelium, basal corneal epithelial oedema, occasional prominence of Bowman's layer and stromal oedema, and abnormally thickened DM with guttae on its posterior edge.<sup>2</sup> Our study confirms these findings. Furthermore, we observe differential distribution of *CLU* in the DM in FED-affected corneas. *CLU* labelling revealed two distinct layers in the FED DM, with the posterior layer remaining unstained. This pattern was not evident in normal corneas (Figure 1, bottom right panel). Whereas anterior DM is a banded layer of collagen that is laid down before birth, posterior DM is a non-banded collagen layer, which is progressively secreted by normal CE after birth.<sup>36</sup> In FED, endothelial cells secrete an additional banded collagen layer, posterior collagenous layer, in the extra posterior DM.<sup>37</sup> Thus, our data may imply that *CLU* is expressed by corneal endothelial cells, but not secreted in FED cornea. This interpretation is consistent with previous findings, which showed overexpression of *CLU* in endothelial cells, but not throughout the CE+DM complex in FED cornea.<sup>22</sup>

On the basis of these findings, we hypothesise that variants in the *CLU* gene may affect its secretion by corneal endothelial cells, exposing these cells to physiological stresses such as aging, oxidative stress and apoptosis, which may be important in FED pathogenesis.<sup>24,25</sup> Genetic variation at the *CLU* gene is also associated with pseudoexfoliation syndrome, a disease caused by accumulation of extracellular matrix proteins on the surface of the lens and other structures within the anterior chamber.<sup>38,39</sup> FED is also characterised by the accumulation of extracellular matrix proteins, specifically in DM.<sup>40</sup>

Genetic analysis of five tag SNPs in *TGFBI* did not detect any association of individual SNPs with FED, although haplotype analysis revealed that the TAAAT haplotype is significantly associated with carriers being around twice as likely to develop FED. The TAAAT haplotype is possibly tagging FED-associated variants in *TGFBI* not tagged by the individual SNPs. This possibility requires further follow-up. Immunolabelling of TGFBI revealed differential expression and distribution of the protein in FED and normal corneas. Our data show that TGFBI is upregulated in FED corneal epithelium and concentration of immunolabelling between adjacent cells suggests that the protein is secreted by stressed epithelial cells. This assumption is

consistent with previous studies showing that injured/diseased corneal epithelial cells produce and secrete extracellular TGFBI to protect against injury.<sup>41</sup> We also observed that the Bowman's layer in FED-affected cornea was thicker than that in normal cornea and devoid of TGFBI staining (Figure 2). The Bowman's layer is a condensation of the outermost portion of the corneal stroma, which is composed of type I and V collagens interlaced with keratocytes. Stromal keratocytes produce TGFBI that is detectable in Bowman's layer. A recent study demonstrates that 54–63% of anterior keratocytes are depleted in FED,<sup>42</sup> possibly explaining the lack of TGFBI expression in the Bowman's layer of FED cornea in our study. Consistently, our study revealed less intense stromal TGFBI expression in FED corneas, especially in the anterior stroma (Figure 2). Furthermore, TGFBI was present in guttae in FED corneas, consistent with the previous findings.<sup>22</sup> Surprisingly, we did not detect TGFBI expression in endothelial cells in normal or FED corneas. Previous studies reported TGFBI expression and secretion by endothelial cells in normal and FED corneas, with TGFBI overexpression in the latter corneas.<sup>22</sup> These studies used flat mounts of CE+DM complex, whereas we used sections of whole corneas. Therefore, our method would be less sensitive in identifying expression in the CE that could explain the absence of TGFBI labelling in these cells in both normal and diseased corneas.

TGFBI mediates cell adhesion by interacting with collagens, fibronectin and integrins, which are major components of the DM.<sup>43,44</sup> TGF- $\beta$ , which induces the TGFBI protein, has a reported role in the regulation of *TCF4* and *ZEB1* through signaling pathways in EMT.<sup>26</sup> Mutations in *TGFBI* cause some human corneal dystrophies, but have not been reported to cause FED.<sup>2</sup> Our study has revealed the first possible association of *TGFBI* with FED and upregulation of TGFBI in the corneal epithelium in FED. We speculate that TGFBI is overexpressed by injured corneal epithelial cells during the end stage of the disease. TGFBI is known to be expressed in corneas damaged through other mechanisms, including trauma and scarring in addition to dystrophies.<sup>45,46</sup> Thus, it is not possible to distinguish in this study between a causative effect for TGFBI or a responsive one.

Genetic analysis of *ZEB1* and *COL8A2* revealed no significant association of variation in these genes with FED in our study. Mutations in *COL8A2* cause early-onset FED<sup>11</sup> and mutations in *ZEB1* account for a small proportion of late-onset familial FED in Caucasians.<sup>6</sup> The majority of patients in this study had late-onset FED. Thus, a lack of association at *ZEB1* and *COL8A2* is not surprising. Furthermore, any variant with which these tag SNPs are in low linkage disequilibrium are not well tagged and cannot be fully assessed by this methodology. In addition, this study was sufficiently powered to detect an OR of 1.5 or above.<sup>31</sup> A larger number of cases would be required to detect association with OR of <1.5 in any of these genes.

This study has independently replicated genetic association of *TCF4* with advanced FED in Caucasian Australians, confirming that *TCF4* contributes to FED risk. Our study did not support the association of the *PTPRG* gene with FED. However, we did find a novel although nominal association of *CLU* and *TGFBI* with FED. Immunohistochemistry data revealed differential expression and distribution of the *CLU* and TGFBI proteins in FED corneas, supporting a role for these proteins in disease processes. On the basis of these and previous findings in the literature, we propose that aberrant adhesion, mesenchymal transition of CE and physiological stress contribute to FED pathogenesis.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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