・計画に診断に関する項目を入れておけばよかったのでは?

3. discussion のまとめ

1) 各施設が手持ちの GDLD 患者数は、京都府立医大 20、大阪大 20 以上、順天堂大 14、東京大 20、合計で 100 例に満たないと思われる。

2) 遺伝子解析は施設によって実施の有無に差があり、倫理の問題もある。そう多くはデータとして出せない。

3) 疫学調査は評価に大きく関わることもあり、しっかり行う。調査の項目、フォーマットは府立医大で考えて、各施設に送付する。できるだけ早く行う。中間報告までに各施設の患者について調査する。期間は過去 5〜10 年程度か。

4) 遺伝子治療に関する検討は、症例数が少なく、KO 動物、cell line も確立していない現状では進めることが難しい。また研究期間も短く、遺伝子治療まで踏み込むのは時間的に難しい。動物で検討していない状況では、ヒトに対する試験は不可能である。よって、治療レジメンに新規治療法（遺伝子治療）を盛り込むのは難しい。

5) SCL の効果は臨床で確認されているので、その有用性をしっかり示し、治療レジメンに組み込む。

6) 基礎的な研究は各施設がそれぞれ行う。各施設の手持ちデータは支障が無い範囲で他の施設にも提供する。

平成 22 年度 厚生労働省科学研究費補助金（難治性疾患克服研究事業）
「膠様滴状角膜変性症の標準的治療レジメンの確立と新規治療法の創出」
第 2 回研究班会議 議事録

作成：篠宮 克彦

日時：平成 22 年 11 月 13 日（土）12:30～13:30
場所：神戸ポートピアホテル 本館 2 階 すみれ
参加者：7 名（敬称略）
川崎 諭（京都府立医科大学）
足立 紘子（京都府立医科大学）
篠宮 克彦（京都府立医科大学）
辻川 元一（大阪大学）
相馬 剛至（大阪大学）
臼井 智彦（東京大学）
松田 彰（順天堂大学）

はじめに研究代表者の川崎から、配布資料を用いて本日の議題に関して説明があった。本日の議題は以下のとおりである。

1. 各施設における現在の進捗状況の報告
2. 臨床データ取りの問題点について
3. 来年度継続申請に向けての今後の計画について

1. 各施設における現在の進捗状況の報告

a. 京都府立医科大学

カルテで追跡調査が可能であった GDLD 患者 19 例の背景、術後治療経過、術後 SCL 装着と再発に関するレトロスペクティブ調査の紹介があった（詳細は配布資料を参照）。

b. 大阪大学

過去に遺伝子の positional cloning を行った際の症例が 36 例あるが、そのうち 14 例は他施設へ移っており既に追跡不能で、残り 22 例のうち可能なものを追跡調査する予定とのことであった。現在把握している（通院されている）症例は 6 例とのこと。

c. 順天堂大学

14-15 例の症例を把握しており、すべて遺伝子解析済みということであった。また、村上らはシードと共同で、コンタクトを用いてプラスミド導入ができるかを検討しているとのことであった。プラスミドを荷電でコンタクトレンズに吸着させ、涙液に放出させるというもので、この手法を用いた in vitro、ex vivo 実験のデータが紹介された。SCL をプラスミド溶液に浸漬し、その後 SCL に HCE-T あるいはウサギ摘出角膜を載せ、培養することでプラスミドが細胞内に取り込まれることが確認された。

d. 東京大学

角膜外来で一般カルテを出し、照合中ということであった。現在のところ 9 例あり、8 例は PKP 後 follow up しているとのこと。なお、東京大では以前の症例は SCL 装着を行っておらず、SCL 無しの予後データが得られるかもしれないということであった。

以上のように、現在のところ追跡可能な症例数は 4 施設合計で 60～70 例程度になる。目標とした 100 例にはおそらく届かないであろうとのこと。

2. 臨床データ取りの問題点について

京都府立医大が作成し、各施設に配布したフォーマットがすべて埋められないという意見があった。また、施設によっては資料がしっかり保管されておらず、病変の写真が見つからないということもあるらしい。そのような場合、フォームの記入はできるところだけで良い。継続申請に関わる書類のデッドラインまでにはフォームに記入し、とりあえず府立医大へ送付してもらう。

3. 来年度継続申請に向けての今後の計画

疫学調査はしっかりまとめて資料とする。また、来年度の計画として新たな症例が見つかる可能性があり、SCL の効果が明らかになっていることもあるので、角膜専門でやっている施設に対してアンケートを行うという案が出された。日本角膜学会を通して行えるのではないかということであった。

4. その他

今後の実験に関する案あるいは話題として、以下のようなものが出された。

- SCL を用いてプラスミド導入を行う実験に関しては、村上らに確認する。
- なぜ SCL に効果があるのか、実証する実験が必要。患者の SCL を回収して分析するなど。
- 初期の患者で SCL を載せる前、載せた後でフルオロメトリー計測してみては?
- 遺伝子治療をやるなら、モデル動物を使ったデータが無いと無理。

・理研では受託 KO 動物作製をやってもらえるが、費用は 200 万円程度とのこと。
・GDLD の患者角膜が手に入れば cell line を作ることができ、実験もしやすくなる。大阪大には早期の患者がいるが、まだオペのタイミングではないと。順天堂大ではオペが近々あるかもしれないとのこと。

来年度の継続申請書類は川崎が作成して各施設に回覧する。また、調査の example データを府立医大から各施設へ送付する。次回、第 3 回の班会議は来年 2 月の角膜カンファランスの時に設定する予定。

平成 22 年度 厚生労働省科学研究費補助金（難治性疾患克服研究事業）
「膠様滴状角膜変性症の標準的治療レジメンの確立と新規治療法の創出」
第 3 回研究班会議 議事録

作成：篠宮 克彦

日時：平成 23 年 2 月 17 日（木）12:15～13:15
場所：TKP 品川カンファレンスセンター カンファレンスルーム 7
参加者：10 名（敬称略）
川崎 諭（京都府立医科大学）
稲富 勉（京都府立医科大学）
中川 紘子（京都府立医科大学）
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辻川 元一（大阪大学）
相馬 剛至（大阪大学）
臼井 智彦（東京大学）
宮井 尊史（東京大学）
松田 彰（順天堂大学）

はじめに研究代表者の川崎から、配布資料の説明があった。以下、本日の議題を時系列でまとめた。

1. 実績報告書のフォーマット等について

冊子体で提出を要求されている。総括研究報告は京都府立医大で作成、分担研究報告を各大学から提出していただく。分担研究報告は各大学 2-3 ページ、業績合わせて 4 ページ程度で、患者さんのデータから結果を評価、解析していただくようなもので良い。総括研究報告には関連した論文発表、研究班会議の議事録を加える。事業実績報告書の概要版は府立医大で作成する。

（その他）

研究経費、直接経費の証拠書類を各大学から提出していただく。事務局の松尾さんへ。領収書等もそろえて提出いただく。

2. 新規申請の内容に関して

次年度の継続が決定した場合、新規申請書に記載した内容について、平成23年度には何をどの程度実施するかを話し合った。新規申請書には残り 2 年間の研究計画を記載しているが、これらのうち、平成23年度実施分には実現可能性が高いものを選ぶ方向で検討した。以下、各項目の実施可否に関する議論をまとめた。

1. 全国調査

これはメインの課題とも言えるため、平成23年度で実施する。今回は日本角膜学会に協力してもらい、研究班主導で実施する形を取る。

2. GDLD 患者の全身疾患、異常の有無について

大阪大で過去に調べたことがあり、その時は特に何も無かったということ。あまりポジティブなデータは出ないのでは? という意見あり。

レトロスペクティブに調べることは可能、手術既往があれば血液検査のデータは見つかる。また、現在通院している患者のみ問診、血液検査を実施し、異常が見つかれば精密検査を実施するという案も出された。本項目は実施する方向で、詳細は改めて決めるということになった。

3. 角膜バリア機能の測定

4 大学の症例では実施できそうな家系は無さそうとのこと。また、測定する機器が市販されていない。協力してくれる家系があればいいが、やるとなるとかなりのエネルギーが必要、患者限定なら可能かもしれない。まずは協力してもらえる患者、家系があるか、測定機器を使うことができるかを確認してから実施可否の検討をする。

(測定機器は長崎大・ウエマツ Dr)

4. SCL の進行抑制の可能性について

患者左右眼で SCL 装用あり、なしを検討するのは倫理的にも困難。また左右が同じ程度の病変でないことも多く、比較も難しい。府立医大がまとめたレトロスペクティブのデータは移植後再発有無に関してのみ。本項目は内容を練って実施可能かを検討する。

5. メカニズムについて

初発時に SCL 装用する前後で TER を測定できれば、あるいは装用している患者でも SCL を外した後、装用後で測定するなどをやってみたい。

Lactoferin トラップは順天堂大で実施中とのこと。

府立医大には今後オペ予定の症例が 1 例あり、阪大には SCL を嫌がって外した患者がいる。これらで測定できるかもしれない。測定機器は 3. でも触れられたように、使えるか確

認が必要。

6. モデル細胞の確立

府立医大の予備検討で、角膜上皮細胞の不死化は比較的容易に実行可能という結果が出ている。しかし、肝心のGDLD患者の角膜が手に入らない。現時点では少なくともcell lineは存在しない。大阪大で以前に重層化培養ができたとのこと。本項目はGDLD患者のオペがあれば実施可能であり、オペ待ちとなる。

7. SCLプラスミド

順天堂大で検討中。

［VI］

研究成果の刊行に関する一覧表

論文一覧

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15. Sasaki M, Sotozono C, Chihara H, et al. [Characteristic appearance of early-stage Acanthamoeba keratitis]. *Nippon Ganka Gakkai Zasshi.* 114:1030-1035.

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研究成果の刊行物・別刷

Lattice Corneal Dystrophy Type IV (p.Leu527Arg) Is Caused by a Founder Mutation of the *TGFBI* Gene in a Single Japanese Ancestor

Hideki Fukuoka,[1] *Satoshi Kawasaki,*[1] *Kenta Yamasaki,*[1] *Akira Matsuda,*[2] *Akiko Fukumoto,*[1] *Akira Murakami,*[2] *and Shigeru Kinoshita*[1]

Purpose. Lattice corneal dystrophy (LCD) type IV (LCD4) is a late-onset corneal dystrophy with amyloid deposition at the deep stromal layer of cornea. As with other corneal dystrophies, this LCD subtype is also caused by a mutation (p. Leu527Arg) of the transforming growth factor, β-induced (*TGFBI*) gene. Although LCD type I has been reported worldwide, LCD4 has been reported only in the Japanese population. In the present study, a haplotype analysis was performed to investigate whether this LCD subtype is caused by a founder mutation.

Methods. Genomic DNA samples were extracted from 13 unrelated patients with LCD4. As a control, genomic DNA samples from 96 normal volunteers were also analyzed. For the haplotype analysis, the samples were amplified by polymerase chain reaction (PCR), TA-cloned, isothermally amplified, and subjected to a 1-base primer extension assay against a mutation site (c.1580T>G) and six known single-nucleotide polymorphisms (SNPs; rs4669, rs2072239, rs7727725, rs17689879, rs6871571, and rs3792900), which are located adjacent to the mutation site.

Results. The haplotype analysis revealed that all the disease-carrying alleles from the 13 LCD4 patients shared an identical haplotype, whereas non-disease-carrying alleles from the normal volunteers and the LCD4 patients exhibited four haplotypes. There was a statistically significant difference in the haplotype distribution between the disease-carrying and the non-disease-carrying alleles.

Conclusions. The findings of this study strongly indicate that LCD4 was caused by a founder mutation of the *TGFBI* gene that occurred in a single Japanese ancestor. (*Invest Ophthalmol Vis Sci.* 2010;51:4523–4530) DOI:10.1167/iovs.10-5343

Cornea is one of the most transparent tissues in the body, and a substantial number of genes contribute to the attainment and maintenance of the specific properties of this tissue.[1,2] Recent advances in molecular biology have allowed us to understand corneal physiology and disease at the molecular level. One of the prominent events in this research area is the discovery of the transforming growth factor, β-induced (*TGFBI*) gene as a causative gene in five classic autosomal dominant corneal dystrophies.[3] Subsequently, other types of inherited corneal dystrophies, such as Meesmann corneal dystrophy (MECD)[4,5] and gelatinous droplike corneal dystrophy (GDLD),[6,7] have been reported.

Lattice corneal dystrophy (LCD) is characterized by stromal amyloid depositions that typically appear as a network or lattice. LCD type I (LCD1) is one of the five dominant *TGFBI*-related corneal dystrophies with characteristic latticelike refractile lines within the corneal stroma.[8] Other than this common LCD, several minor ones have been reported in the *TGFBI* gene (currently designated as Variant LCD in the IC3D classification) that are caused by different mutations.[9] LCD type IV (LCD4, a variant LCD) is one such corneal dystrophy first reported in 1998 as a late-onset LCD with characteristic amyloid depositions located at the deep stromal layer of cornea.[10] Although LCD1 has been reported world-wide,[3,11] LCD4 has been reported only in the Japanese population.[12–19] Thus, some researchers have theorized that LCD4 may be caused by a founder mutation that occurred in a Japanese ancestor.[20]

In this study, we performed a haplotype analysis on genomic DNA samples obtained from 13 patients with LCD4 to investigate this theory. We found that all the disease-carrying alleles of the investigated 13 LCD4 patients shared an identical haplotype around its causative mutation, whereas healthy alleles exhibited four haplotypes with no apparent preference. These data strongly suggest that all LCD4 mutations descend from a founder mutation that occurred in a single Japanese ancestor.

Materials and Methods

Human Samples

Peripheral blood was obtained from 13 patients from 13 unrelated families who had received a clinical diagnosis of LCD4. These 13 patients were 7 men and 6 women, ranging in age from 52 to 83 years (mean age, 69.8). Seven resided in Kyoto, one in Osaka, one in Mie, one in Niigata, one in Kanagawa, and two in Tokyo Prefecture (Fig. 1). Genomic DNA samples from 96 normal Japanese volunteers (48 men and 48 women) were obtained from a research-resource bank (Human Science Research Resource Bank, Osaka, Japan). Written informed consent was obtained from all patients after they were given a detailed explanation of the study protocols. The study adhered to the tenets of the Declaration of Helsinki and was approved by the Institutional Committee for Ethical Issues at Kyoto Prefectural University of Medicine.

Mutation Analysis

Genomic DNA samples were extracted from the peripheral blood of all 13 LCD4 patients by using a commercially available, standard column-

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Disclosure: **H. Fukuoka**, None; **S. Kawasaki**, None; **K. Yamasaki**, None; **A. Matsuda**, None; **A. Fukumoto**, None; **A. Murakami**, None; **S. Kinoshita**, None

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FIGURE 1. Geographic distribution of the residences of the 13 LCD4 patients. Prefectures of residence are shown in *black* and marked as 1, Kyoto; 2, Osaka; 3, Mie; 4, Niigata; 5, Kanagawa; and 6, Tokyo.

based kit (DNeasy Blood & Tissue Kit; Qiagen, GmbH, Hilden, Germany). The samples were then quantitated by the use of a spectrophotometer (NanoDrop; Thermo Fisher Scientific, Inc., Wilmington, DE) and electrophoresed on a 1% agarose gel, to check their integrity. Next, the samples were amplified by polymerase chain reaction (PCR) with primer pairs (Table 1) against the mutation hot spots (exons 4, 11,

TABLE 1. List of the Oligomers

Oligomer	Target	Purpose	Direction	Sequence
Exon4_F	TGFBI	PCR	Forward	CCCCAGAGGCCATCCCTCCT
Exon4_R	TGFBI	PCR	Reverse	CCGGGCAGACGGAGGTCATC
Exon11_F	TGFBI	PCR	Forward	CTCGTGGGAGTATAACCAGT
Exon11_R	TGFBI	PCR	Reverse	TGGGCAGAAGCTCCACCCGG
Exon12_F	TGFBI	PCR	Forward	GACAGGTGACATTTTCTGTGT
Exon12_R	TGFBI	PCR	Reverse	GATCACTACTTTAGAAAAATG
Exon13_R	TGFBI	PCR	Reverse	GCTGCAACTTGAAGGTTGTG
TGFBI_27889	c.1580	1-Base primer extension	Forward	TGCCATCCAGTCTGCAGGAC
TGFBI_27929	rs4669	1-Base primer extension	Forward	TTTTTTTTTTGAAGGAGTCTACACAGTCTT
TGFBI_28010	rs2072239	1-Base primer extension	Forward	TTTTTTTTTTTTTTGTAAAGACCAACTTAAGTACAC
TGFBI_28238	rs7727725	1-Base primer extension	Forward	TTTTTTTTTTTTTTTTTTTTTTTCAGGAACCAGGGAGGTCA
TGFBI_28641	rs17689879	1-Base primer extension	Forward	TTTTTTTTTTTTTTTTTTTTTTTTTTTTGGCAGGGGATCTAGTGGTTA
TGFBI_28700	rs6871571	1-Base primer extension	Forward	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTCAGCCTGTGTTGGGAGGATT
TGFBI_29257	rs3792900	1-Base primer extension	Forward	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGTGTTAGAGGTTGGTACAGG

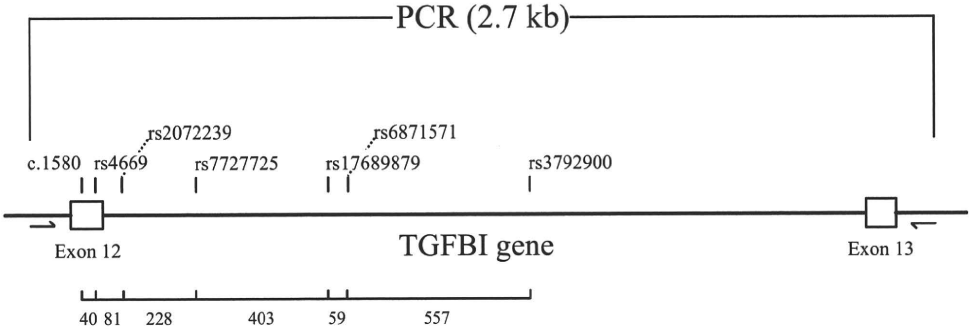

FIGURE 2. The genomic structure for the *TGFBI* gene with sites of mutation and SNPs investigated. *Arrows*: PCR primers that amplify a fragment containing all the SNP and mutation sites. The physical distance between two neighboring SNPs is also indicated in base lengths at the *bottom*.

and 12) of the *TGFBI* gene. The amplified products were then treated with a mixture of exonuclease I and shrimp alkaline phosphatase (ExoSAP-IT; GE Health Care, Ltd., Little Chalfont, UK) to digest residual dNTP and primer. The amplified products were then subjected to sequencing reaction (BigDye Terminator ver. 3.1 Cycle Sequencing Kit; Applied Biosystems, Inc. [ABI], Foster City, CA), and the products were electrophoresed on an automated sequencer (3130xl Genetic Analyzer; ABI). The sequence data were analyzed through the use of commercially available alignment software (Variant Reporter; ABI).

Haplotype Analysis

Genomic DNA samples were amplified by PCR using a primer pair (exon12_F and exon13_R; Table 1) against a genomic region between exon 12 and 13 of the *TGFBI* gene, which harbors the site of the c.1580T>G mutation and six known SNPs (Fig. 2, Table 2). The amplified products were then electrophoresed on a 1% agarose gel, excised, purified with a commercially available column-based purification kit (Wizard SV Gel and PCR Clean-Up System; Promega, Madison, WI), and ligated to a TA-cloning vector (pGEM-T Easy Vector; Promega). The plasmid vector was transformed into chemically competent *Escherichia coli* cells (competent high JM-109; Toyobo Co., Ltd., Osaka, Japan) and seeded on a 1% LB agar plate supplemented with IPTG and X-gal for the standard blue-white selection. After 24 hours' incubation, 16 white colonies were picked from each sample and isothermally amplified overnight with a phi29 polymerase-based plasmid amplification kit (Illustra TempliPhi DNA Amplification Kit; GE Health Care). Each of the amplified products was then subjected to a 1-base primer extension assay (SNaPshot; ABI) with seven pooled primers (Table 1) against the sites of the mutation and the six known SNPs. After treatment with shrimp alkaline phosphatase, the assay products were electrophoresed on the automated sequencer, and the data were analyzed with the use of commercially available software (GeneMapper Software; ABI). Because artificial recombination presumably occurring during the PCR amplification and the bacterial transformation is not negligible in this analysis, a Perl-based program (HapTyper.pl) was created to estimate the most probable haplotype pair from the processed data for each sample.

Statistical Analysis

For the identification of the statistical significance in the haplotype distribution between the affected alleles and the nonaffected alleles, χ^2 and Fisher's exact tests were performed with commercially available statistical software (SAS ver. 9.1; SAS Institute Inc., Cary, NC). For the calculation of statistical power, R language (R Foundation, Vienna, Austria) was used.

RESULTS

The enrolled 13 LCD4 patients, except for 1 patient, exhibited similar corneal haze composed of isolated or fused refractile opacities, most of them being dotlike, and some being latticelike. Most important, these depositions were mainly located within the deep stromal layer, which seems to be specific to this disease and of great diagnostic value, as reported previously. Sequencing analysis revealed that all the 13 LCD4 patients enrolled in this study exhibited a substitution mutation (T to G; c.1580T>G), resulting in an amino acid transition from leucine to arginine (p.Leu527Arg; Fig. 3). Only one patient (59-year-old woman) was homozygous for the mutation site, and she exhibited a much more severe corneal phenotype than did other patients heterozygous for the mutation, such as another homozygous LCD4 patient detailed in a previous report.[19] One patient had a heterozygous substitution mutation from A to G at a different nucleotide position (c.1631A>G) that results in an amino acid transition from asparagine to serine (p.Asn544Ser), which has already been reported to be causative of another type of variant LCD.[17,20,21] In this patient, these two mutations were located on different alleles from one another, as determined by subsequence haplotype analysis (data not shown).

Haplotype analysis was performed to examine whether the combination of the six SNPs, which are close to the c.1580T>G mutation and hence show a strong linkage disequilibrium to that mutation site, was identical among the disease-

TABLE 2. List of SNPs within the Amplified Region

Region	rs ID	Heterozygosity	Sequence
Exon12	rs4669*	0.494	CCTCAACCGGGAAGGAGTCTACACAGTCTT (C/T) GCTCCCACAAATGAAGCCTTCCGAGCCCTG
Intron12	rs2072239*	0.294	TGAGGGATCACTACTTTAGAAAAATGGAGA (C/T) GTGTACTTAAGTTGGTCTTTACCCAAGAGT
Intron12	rs7727725*	0.494	GGAGGATGAGAGCAGGAACCAGGGAGGTCA (A/T) GAGCCTTGGACAAGGGCACAGAACAGCAGC
Intron12	rs17689879*	0.444	GAGGATGTTTGGCAGGGGATCTAGTGGTTA (C/T) GGGTGGCTAAGAAAAATGAGGAAGGTAAGA
Intron12	rs6871571*	0.494	GAGTATCTTGCAGCCTGTGTTGGGAGGATT (A/G) AATAGGATGCCACACACAGGGCCAGGCAGA
Intron12	rs58761304	N.D.	GCAGGAATGGGAGTTGCAGTGTTTAGCTCA (G/T) ATGCATGCCTGTGAGAGATGCTTCCACTCT
Intron12	rs3792900*	0.453	TGCATGGGATGTCCTTTCAATATCTCTAAC (A/G) CCTGTACCAACCTCTAACACTCTCTGTCCC
Intron12	rs45583534	0.023	ACTGATGTGGGCTGAAAGGAATGCTGAGAC (A/G) TGACGAGGAGAGATGCTGCGGAGGGAATAT
Intron12	rs41502049	0.076	GAAACATGAGTCATACTCACAGAGGAGTAT (C/G) GATTAACTCCTTCTCAGCAGCCAGGGAGCC
Intron12	rs45474493	0.011	AACCCAGAGGCCAACTGACTGCTGGGGCAG (A/T) TTTGTGGTCATGAACATGTGCTTTGTGTCC

The amplified region is illustrated in Figure 2.
* SNPs investigated.

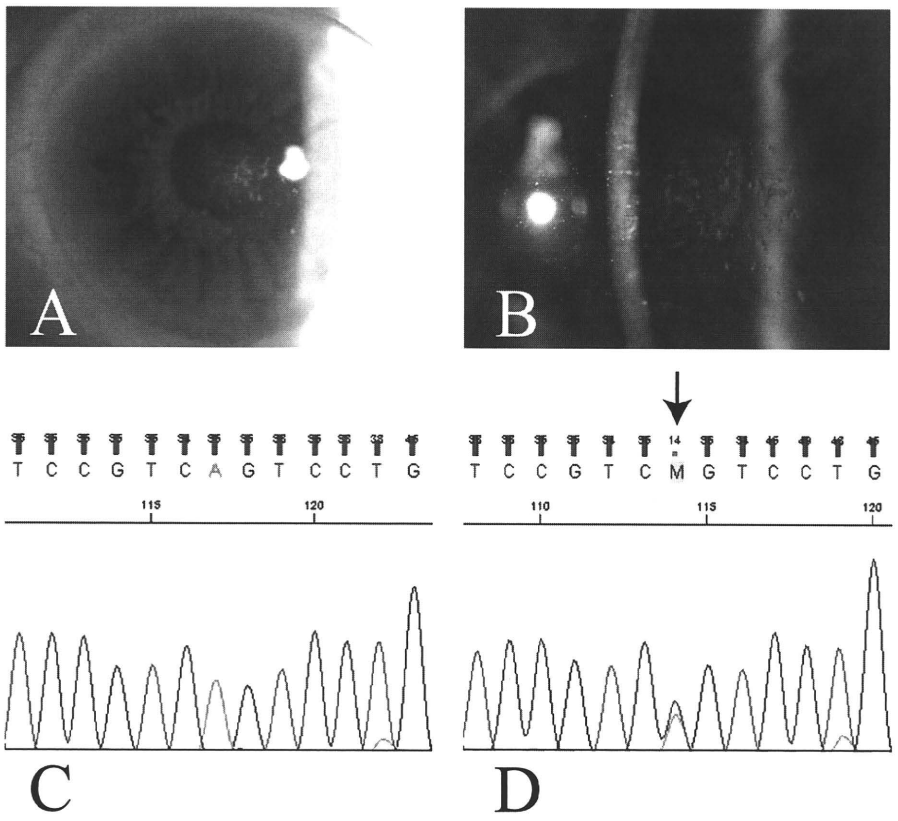

FIGURE 3. Results of the mutation analysis of the 13 LCD4 patients. Scleral scattering (**A**) and iris retroillumination (**B**) demonstrate the corneal opacity in LCD4. The depth of opacity was nearly at the level of Descemet's membrane. Compared with sequence data from normal volunteers (**C**), LCD4 patients carried a heterozygous (**D**) or homozygous T-to-G conversion at c.1580 nucleotide position (*arrow*).

carrying alleles of the 13 LCD4 patients. As expected, all the 14 (12 heterozygous plus 1 homozygous) disease-carrying alleles found in the patients demonstrated an identical SNP-haplotype (Table 3). Two of the 12 healthy alleles from the patients exhibited the same SNP-haplotype as found in the disease-carrying alleles. Three different haplotypes were also found in the healthy alleles. A random cross section of normal volunteers was also examined to investigate which haplotypes are

TABLE 3. Results of the Haplotype Analysis

Patient	Allele	c.1580	rs4669	rs2072239	rs7727725	rs17689879	rs6871571	rs3792900
LCD4_1	Healthy	T	C	G	A	C	G	C
	Disease-carrying	G	T	G	T	T	A	T
LCD4_2	Healthy	T	C	A	A	C	G	C
	Disease-carrying	G	T	G	T	T	A	T
LCD4_3	Healthy	T	T	G	T	C	A	C
	Disease-carrying	G	T	G	T	T	A	T
LCD4_4	Healthy	T	C	G	A	C	G	C
	Disease-carrying	G	T	G	T	T	A	T
LCD4_5	Healthy	T	C	A	A	C	G	C
	Disease-carrying	G	T	G	T	T	A	T
LCD4_6	Healthy	T	T	G	T	C	A	C
	Disease-carrying	G	T	G	T	T	A	T
LCD4_7	Healthy	T	T	G	T	T	A	T
	Disease-carrying	G	T	G	T	T	A	T
LCD4_8	Healthy	T	C	G	A	C	G	C
	Disease-carrying	G	T	G	T	T	A	T
LCD4_9	Healthy	T	C	A	A	C	G	C
	Disease-carrying	G	T	G	T	T	A	T
LCD4_10	Disease-carrying	G	T	G	T	T	A	T
	Disease-carrying	G	T	G	T	T	A	T
LCD4_11	Healthy	T	T	G	T	C	A	C
	Disease-carrying	G	T	G	T	T	A	T
LCD4_12	Healthy	T	T	G	T	T	A	T
	Disease-carrying	G	T	G	T	T	A	T
LCD4_13	Healthy	T	T	G	T	C	A	C
	Disease-carrying	G	T	G	T	T	A	T

c.1580 is the site of causative mutation in LCD4, where T means wild-type and G means mutated-type. All disease-carrying alleles shared an identical SNP haplotype (T-G-T-T-A-T). Note that patient LCD4_10 had c.1580T>G mutations in both alleles.

TABLE 4. Summarization of the Data Obtained by Haplotype Analysis of the 13 LCD4 Patients and the 91 Normal Volunteers

Haplotype	Normal Volunteers Healthy Allele	LCD4 Patients	
		Healthy Allele	Disease-Carrying Allele
C-A-A-C-G-C	37	3	0
C-G-A-C-G-C	23	3	0
T-G-T-C-A-C	57	4	0
T-G-T-T-A-T	65	2	14

dominant in the current Japanese population. Among the 96 normal samples, 91 produced data with sufficient quality for the subsequent analysis, but data from the other 5 samples were omitted due to insufficient quality. In the 91 normal samples, four haplotypes were found, which were identical with those found in the healthy alleles of the LCD4 patients. Of those, the mutation-related haplotype (T-G-T-T-A-T) appeared the most abundant (65/182; 35.7%) in the current Japanese population (Table 4). Statistical significance was found (P = 0.00003, χ^2 test, or P = 0.00002, Fisher's exact test) in difference between the haplotype distribution in the disease-carrying and the healthy alleles. The statistical power for our enrolled samples was 1 at the 0.05 level of significance.

DISCUSSION

Allelic homogeneity is a prominent feature of the TGFBI-related corneal dystrophies.[22,23] This fact can be well explained by two different mechanisms: the first is the presence of mutation hot spots,[24] and the second is a founder mutation. The mutation hot spots are mainly located at cytosine or guanine within CpG dinucleotides. Cytosine within the CpG dinucleotide is frequently modified by methylation (5-methylated cytosine; 5meC) in mammalian cells, mainly for epigenetic regulation.[25] The 5meC can be spontaneously deaminated, thus producing thymine which results in C>T or G>A conversion when the deamination occurs in a sense or antisense strand, respectively.[26] Although a DNA mismatch-repair mechanism normally recognizes and repairs such heteroduplex sites,[27] the conversions are occasionally passed over unnoticed, possibly due to the insufficient stringency of the repair mechanism. Eventually, when such a conversion occurs in a germ-line cell, the C>T or G>A conversions can be inherited over generations.

Within the spectrum of *TGFBI*-related corneal dystrophies, LCD1 and granular corneal dystrophy type 1 (GCD1) are caused by the C>T conversion, granular corneal dystrophy type 2 (GCD2, alternatively designated as Avellino corneal dystrophy) and Thiel-Behnke corneal dystrophy (TBCD)[28] are caused by the G>A conversion, with all these conversions occurring at CpG sites (Table 5). Therefore, it is strongly supposed that these four dominant *TGFBI*-related corneal dystrophies are caused by that mutation mechanism. In actuality, these dystrophies occur at a relatively high frequency in many countries, thus implicating the existence of multiple, independently occurring founders, in different areas of the world.

Apart from the mutation mechanism, other types of mutations, including non-C>T or non-G>A mutations or C>T or G>A mutations occurring at non-CpG sites, sometimes occur. Such types of mutations seem to be mainly caused by the accidentally occurring replication error during cell division that may escape the proofreading function of DNA polymerase, as well as the DNA mismatch-repair process, with a roughly estimated frequency of 1 in 10^9 to 10^{10} base pairs per cell division.[27] Hence, these mutations are predisposed to be much

TABLE 5. Suspected Mutation Mechanism for the *TGFBI*-Related Corneal Dystrophies

Corneal Dystrophy	Nucleotide Change	Protein Change	Mutated at CpG or Non-CpG Site	Mutation Mechanism	Reported Countries
LCD1	c.370C>T	p.Arg124Cys	CpG	Deamination	Brazil, Bulgaria, Chile, China, Czech Republic, France, Germany, Hungary, India, Japan, South Korea, Spain, Switzerland, Thailand, United Kingdom, Ukraine, United States, Vietnam
GCD1	c.1663C>T	p.Arg555Trp	CpG	Deamination	China, Czech Republic, France, Germany, Hungary, India, Japan, Mexico, New Zealand, Poland, Spain, Switzerland, Taiwan, Turkey, United Kingdom, Ukraine, United States, Vietnam
GCD2	c.371G>A	p.Arg124His	CpG	Deamination	China, France, Germany, Hungary, India, Iran, Japan, South Korea, Spain, Switzerland, United Kingdom, United States, Vietnam
TBCD	c.1664G>A	p.Arg555Gln	CpG	Deamination	Brazil, China, Czech Republic, France, Hungary, Japan, New Zealand, Switzerland, United Kingdom, United States
RBCD	c.371G>T	p.Arg124Leu	CpG	Replication error	Brazil, China, Czech Republic, Denmark, France, India, Japan, Switzerland
LCD3A	c.1501C>A	p.Pro501Thr	non-CpG	Replication error	Japan
LCD4	c.1580T>G	p.Leu527Arg	non-CpG	Replication error	Japan
Variant LCD	c.1640T>C	p.Phe547Ser	non-CpG	Replication error	Hungary
Variant LCD	c.1874T>A	p.Val625Asp	non-CpG	Replication error	China

LCD1, lattice corneal dystrophy type 1; GCD1, granular corneal dystrophy type 1; GCD2, granular corneal dystrophy type 2; TBCD, Thiel-Behnke corneal dystrophy; RBCD, Reis-Bückler's corneal dystrophy; LCD3A, lattice corneal dystrophy type IIIA; LCD4, lattice corneal dystrophy type IV.

less frequent and tend to become a founder mutation. Other than the four dominant *TGFBI*-related corneal dystrophies described earlier, those including LCD4 clearly meet the criteria for this mutation mechanism (Table 5). In fact, most of these corneal dystrophies occur infrequently and are reported in only one, or at most, only a few countries. For example, in the Japanese population, founder mutations have been reported in corneal dystrophies of LCD type IIIA[23] (currently designated as variant LCD in the IC3D classification), GCD2,[22] and GDLD with a p.Gln118X mutation.[7] Our current haplotype analysis of the 13 LCD4 patients revealed that all their disease-carrying alleles share an identical SNP-haplotype. Since the residences of the 13 patients were not restricted to a small geographic area, but in fact, extended across six different prefectures in Japan (Fig. 1), such biased data seem to be ascribed, not to the preference of a certain SNP-haplotype that was due to a bias toward their place of residence, but rather to the occurrence of a founder mutation in a Japanese ancestor. We imagine that almost all infrequently occurring corneal dystrophies may have been related to the occurrence of founder mutations.

One exception to the hypothesis for the occurrence of a mutation among the *TGFBI*-related corneal dystrophies appears to be Reis-Bücklers corneal dystrophy (RBCD). The mutation of RBCD is located on the same CpG site as that of LCD1 and GCD2 but its substitution is G>T, not C>T or G>A. Therefore, the cause of mutation in RBCD seems to be a simple replication error not a deamination. However, RBCD has been reported in many countries, similar to the other four dominant *TGFBI*-related corneal dystrophies. Therefore, the exact mechanism for the occurrence of the RBCD mutation cannot be

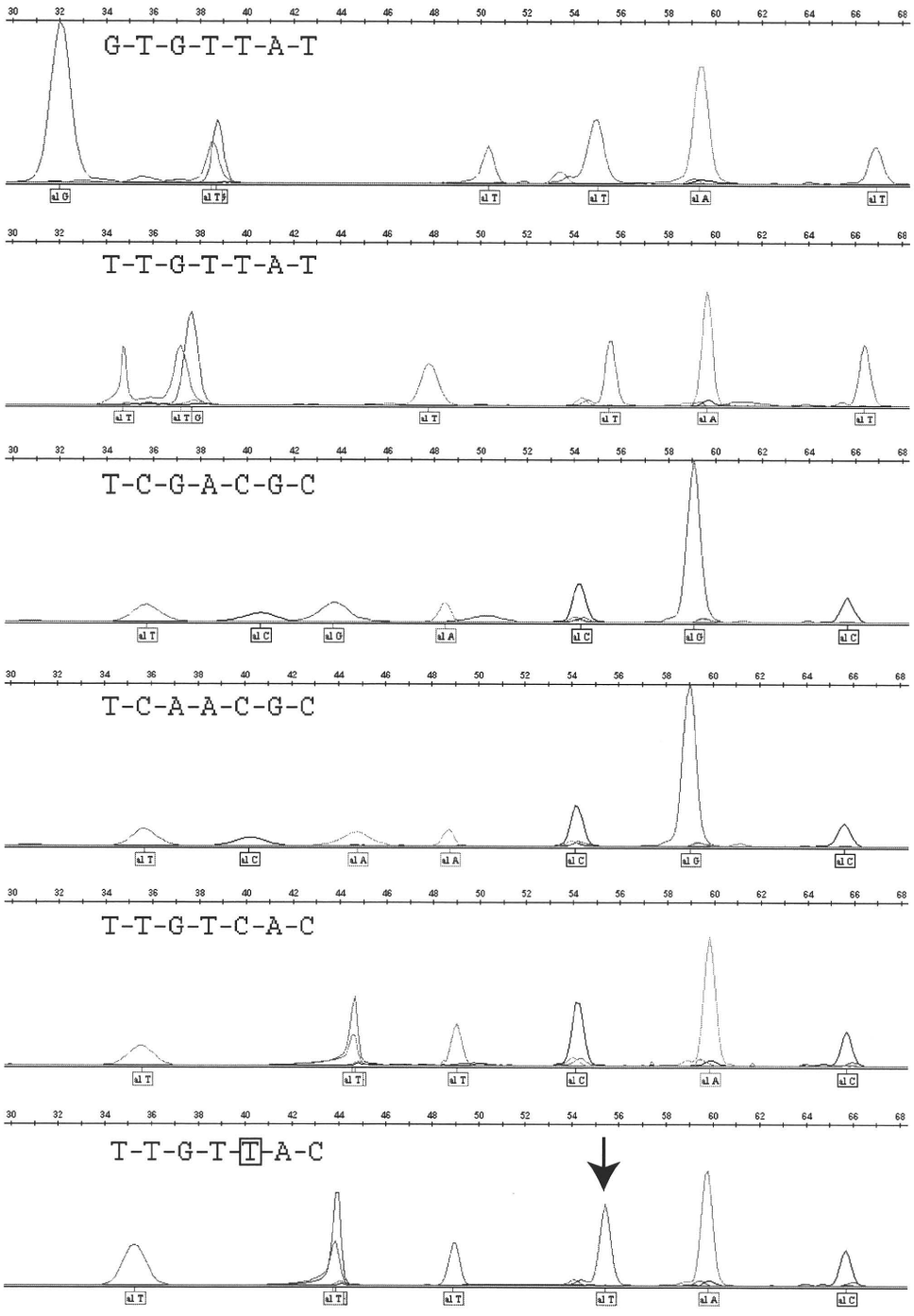

FIGURE 4. Results of the 1-base primer extension assay. Shown are representative chromatogram data for all the observed haplotypes, as indicated at the *top left* corners. The first base (G or T) is of the site of the mutation (c.1580), not of the SNP. The T-T-G-T-T-A-C haplotype is an artificial product by the recombination of the T-T-G-T-C-A-C and the T-T-G-T-T-A-T haplotypes at the indicated site (*square*).

explained by our hypothesis, but it is hoped that it will be elucidated in the future.

We performed a 1-base primer extension assay for the identification of the SNP-haplotype. The reason we chose that method rather than the standard sequencing analysis is that the former method can be easily multiplexed and hence largely save time and costs compared with the latter method. In addition, a commercially available phi29 polymerase-based, isothermal plasmid amplification procedure has been developed.[29] This procedure is much more time-efficient than the standard plasmid DNA extraction procedure and has been subjected to various applications.[30–33] Thus, we examined whether the phi29 polymerase-amplified plasmid DNA can also be used as the template for the 1-base primer extension assay. As is shown in Figure 4, the chromatogram data of this combined assay was of sufficient quality for the identification of each of the SNPs examined in this study. We think that this combined procedure is quite useful for experimentally determining haplotypes.

We initially expected that data from the 16 plasmids investigated for each sample could be easily divided into one or two haplotype groups without any confusion. However, the actual data were sometimes quite complicated, possibly due to the artificial recombination during the consecutive processes of PCR and bacterial transformation, as reported previously.[34] In that study, a method of eliminating such an artificial recombination was also reported. However, although we performed an additional three-cycle PCR amplification against a 10-times diluted initial PCR product, according to this reconditioning PCR method, the artificial recombination was still observed, possibly due to the insufficiency of the reconditioning PCR. For example, a mutation/SNP-combination T-T-G-T-T-A-C was observed in a normal volunteer who was found to have two haplotypes (T-T-G-T-C-A-C and T-T-G-T-T-A-T; Fig. 4). Therefore, we created a Perl-based software program, to identify the most probable haplotype pair by calculating a score for each of the possible haplotypes estimated from the genotype data of each sample. We carefully reviewed the processed data and found that the software worked properly. We think that this software may be useful for studies conducted in the future that are similar to the present study.

In summary, our findings indicate that LCD4 was caused by a founder mutation that occurred in a single Japanese ancestor. In addition, we have established a time- and cost-efficient new procedure through the combination of an isothermal amplification of plasmid DNA and a 1-base primer extension assay. We created a Perl-based software program that helps estimate the most probable haplotype pair from blended data hampered by randomly occurring artificial recombinations. We hope that the results of this study, as well as the newly developed procedures, will contribute to the further understanding of the etiology, populational genetics, and pathogenesis of inherited dystrophies of the cornea and of other organs.

Acknowledgments

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A novel mutation of the TGFBI gene causing a lattice corneal dystrophy with deep stromal involvement

Lattice corneal dystrophy (LCD) type I is one of the five dominant TGFBI (transformimg growth factor β induced; formerly designated as bigh3 or keratoepithelin)-related corneal dystrophies with characteristic lattice-like refractile lines in the corneal stroma.[1] Other than this common-type LCD, there have also been reported several minor-type LCDs caused by different mutations of the TGFBI gene.[2]

CASE REPORT

An 85-year-old man presented with complaints of bilateral blurred vision. His best-corrected visual acuity was 0.1 in OD and HM/30 cm in OS. He had bilateral corneal haze and cataract. The corneal haze contained many isolated or fused refractile opacities, most of them being dot-like, and some being lattice-like (figure 1A). The opacities were found at all depths of the corneal stroma, but mainly involved the deep stromal layer. The degree of corneal haze was severe in his left eye, but relatively mild in his right eye. His wife and two sons did not show any corneal opacity in their eyes. Cataract surgery was performed on his left eye, but his best-corrected visual acuity in that eye was improved only to 0.02, possibly due to the severe corneal haze.

The sequencing analysis revealed that the patient had a heterozygous c.1486C>T nucleotide change in exon 11, producing a p.Arg496Trp amino acid alteration (figure 2A–C). None of his family members presented the c.1486C>T nucleotide change (data not shown), indicating that the genotype well cosegregates with the phenotype in this pedigree. The splice-donor and acceptor sites for each exon of the TGFBI gene did not present any nucleotide changes. Genomic DNAs from 96 normal Japanese volunteers (48 males and 48 females) did not present the c.1486C>T nucleotide change (data not shown).

COMMENTS

In this case, arginine was substituted for tryptophan at the 496th amino acid of the TGFBI gene. Arginine is a charged amino acid with a basic isoelectric point (10.76), while tryptophan is a polarised but non-charged amino acid with a weak acidic isoelectric point (5.89). In addition, tryptophan has an aromatic residue (indole ring) in its side chain which is bulky, less flexible and thus prone to causing a steric hindrance.[3 4] Therefore,

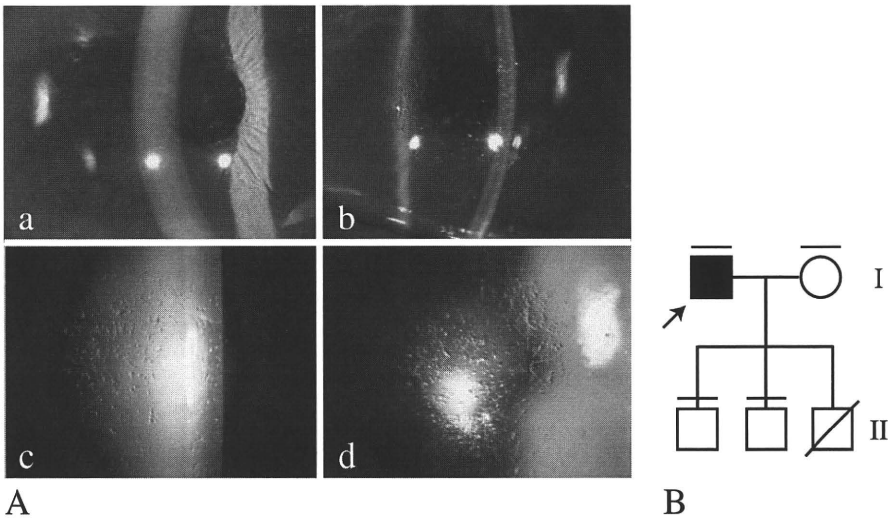

Figure 1 (A) Corneal phenotype of a lattice corneal dystrophy (LCD) patient with a p.Arg496Trp mutation. Photographs demonstrate dot- and lattice-like stromal depositions in the patient's corneas (A, C: right eye; B, D: left eye). (B) Schematic representation of the pedigree of the LCD patient.

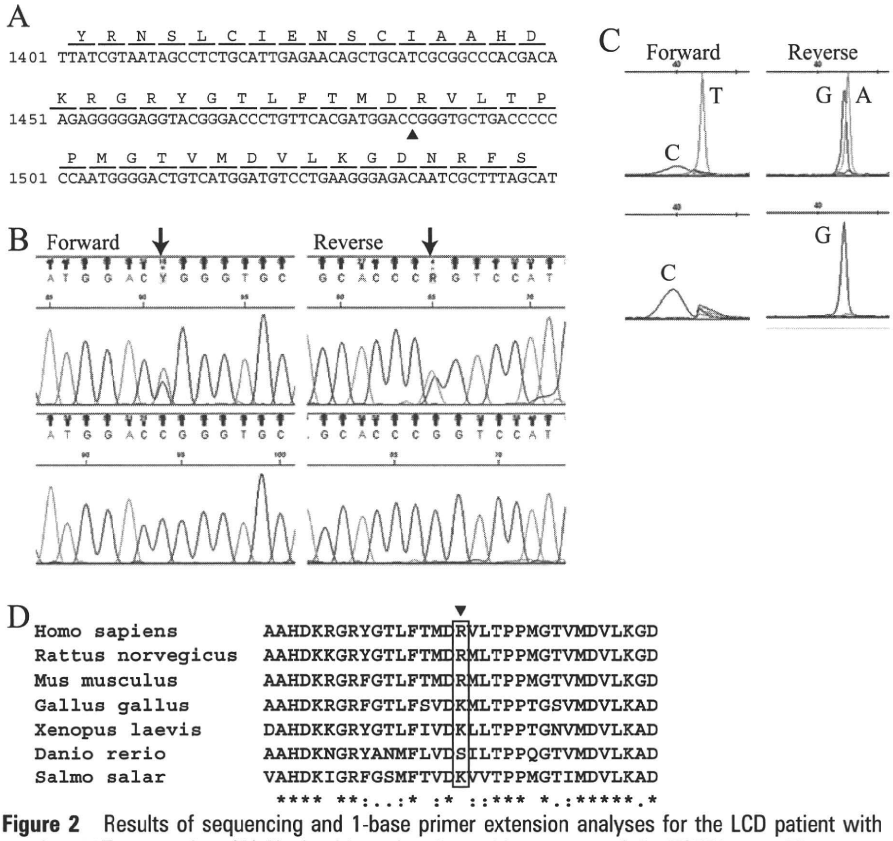

Figure 2 Results of sequencing and 1-base primer extension analyses for the LCD patient with a p.Arg496Trp mutation. (A) Nucleotide and amino acid sequence of the TGFBI gene. The arrowhead indicates the site of the c.1486C>T nucleotide change. (B) Results of sequencing analysis for the exon 11 of the TGFBI gene in the LCD patient (upper) and normal volunteer (lower). Arrows indicate the site of the c.1486C>T nucleotide change. (C) Results of 1-base primer extension analysis for the c.1486 nucleotide of the TGFBI gene in the LCD patient (upper) and normal volunteer (lower). (D) Sequence comparison among seven animals for the TGFBI gene around its 496th amino acid position (arrowhead). Arginine (R) is conserved in the mammalian class but not in other animal classes (birds, amphibians and fish). In these animal classes, except for *Danio rerio* (zebrafish), R is substituted for lysine (K).

amino acid transition from arginine to tryptophan may confer a significant impact on the protein structure and function of the TGFBI gene. Sequence comparison indicates that arginine residue at this amino acid position is conserved in the mammalian class (figure 2D). In most of the other animal classes, arginine is substituted for lysine, an amino acid that has a similar chemical property to arginine. Therefore, the basic amino acid at this amino acid position is presumably required for the proper tertiary structure and the function of the TGFBI protein. Along with the cosegregation between the phenotype and genotype in this pedigree and the absence of this nucleotide change in 96 normal volunteers, we strongly assume that the p.Arg496Trp mutation is pathological.

The corneal phenotype of the LCD patient bearing the p.Arg496Trp missense mutation was similar to that of LCD4.[5] This suggests that the p.Arg496Trp amino acid change confers similar effects on the TGFBI protein characters to those of the p.Leu527Arg amino acid change in LCD4. The p.Arg496 amino acid is located on the forth part of the *Drosophila* fasciclin-I homologous (fasc) domain of the TGFBI protein as in LCD4. Interestingly, a previous study[6] has implied a positional effect of the TGFBI mutations where most of the mutations located on the fasc domain have an amyloidgenic tendency.

In summary, we demonstrate a novel mutation of the TGFBI gene. We hope our current report will contribute to a further understanding of this protein.

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Prospective randomised comparison of external dacryocystorhinostomy with and without silicone intubation: considerations of power

We read with interest the article by Saiju *et al*[1] on a randomised trial comparing the success of dacryocystorhinostomy (DCR) with and without silicone intubation. The authors concluded that there was no statistically significant difference between the two groups and that their study did not support the routine use of silicone tubes. However, we are concerned that the study did not recruit sufficient patients to reach statistical significance.

When a study fails to demonstrate a statistically significant difference, this can be for one of two reasons: (1) that there really is no difference in the wider population between the two arms of the study or (2) that there may be a difference, but insufficient patients were recruited to demonstrate this. To ensure that sufficient patients are recruited, it is necessary to calculate the statistical power of a study.

When setting out to demonstrate that one treatment is better than another, it is necessary to specify the margin of difference, d, above which one treatment will be considered better and the null hypothesis rejected. The value of d will depend on the smallest clinically significant difference considered important. In the case of silicone intubation during DCR, one might adopt the use of tubes if they confer a 10–20% benefit over not using them. This would correspond to a number needed to treat of between 10 and 5 patients (ie, 5–10 patients need to be intubated to achieve one extra successful outcome).

The success of DCR without tubes in this study was 87%. When the expected control success rate is 85%, to be 90% certain that a 10% difference could be detected with $p<0.05$, the required sample size would be 184 patients per group.[2] If 80% power is desired, the sample size is 25% smaller (138 patients per group). If the expected control group success rate is lower, the required sample size increases, becoming a maximum when the control success rate is 50%. With a control success rate of 50%, the required sample size to detect a 10% difference with 80% power would be 405 patients per group.[2]

The authors had 6-month follow-up data for 52 patients (50% were lost to follow-up). By the previously mentioned calculations, the study was underpowered. The difference between the two arms was found to be 3%, implying no difference. However, the 95% CI (−15% to 22%)[3] demonstrates the limited conclusions that can be drawn from the data.

When a negative result is obtained, it is important to consider the power of the study. Otherwise, treatments that may be of benefit may be discarded, and investigators could be unnecessarily put off performing further studies in the same area. Underpowered studies reporting negative results have been noted in other fields.[4–6]

It is worth noting that in this study, the success of surgery without tubes was 87%. With these results, tubes cannot confer more than a 13% benefit (the success rate cannot exceed 100%). Therefore, for the particular population that the authors studied, tubes may indeed not confer a benefit. However, this may not be generally applicable.

To definitively investigate the benefit of tubes in DCR, we would recommend a study that recruits and has follow-up for 138 patients per arm. If, on performing such a study, the control success rate was less than 85%, there would be a loss of power. The control success rate would depend on the case mix—in particular the prevalence of proximal versus distal canalicular block and the presence or absence of a mucocoele.

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