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

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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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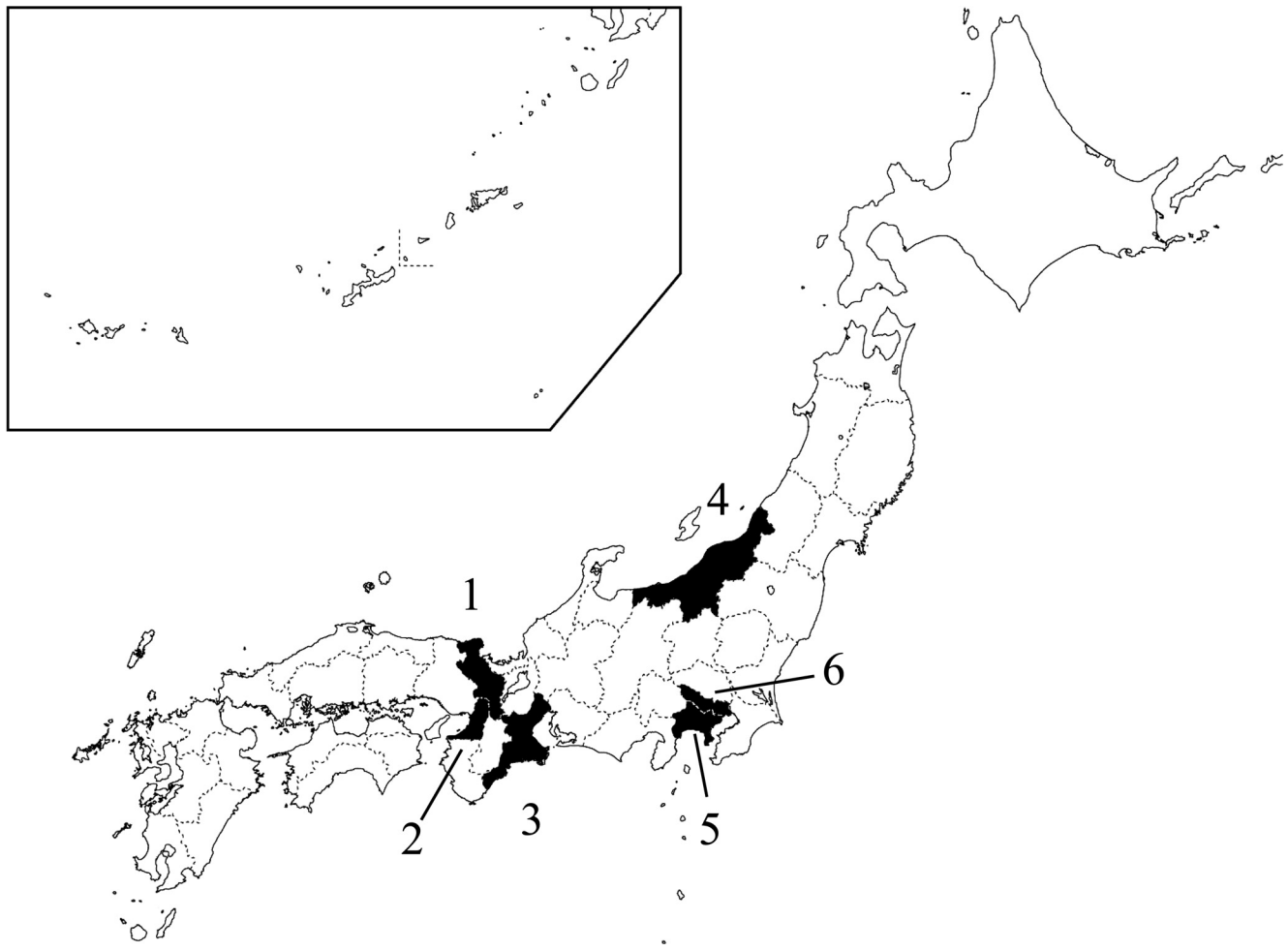

FIGURE 1. Geographic distribution of the residences of the 13 LCD4 patients. Prefectures of residence are shown in *black* and marked as 1, Kyoto; 2, Osaka; 3, Mie; 4, Niigata; 5, Kanagawa; and 6, Tokyo.

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	CCGGGGCAGACGGAGGTCATC
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	TTTTTTTTTTTTTTTTTTTTTTTTCAGGAACCAGGGAGGTCA
TGFBI_28641	rs17689879	1-Base primer extension	Forward	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTGGCAGGGGATCTAGTGGTTA
TGFBI_28700	rs6871571	1-Base primer extension	Forward	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTCAGCCTGTGTTGGGAGGATT
TGFBI_29257	rs3792900	1-Base primer extension	Forward	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGTGTTAGAGGTTGGTACAGG

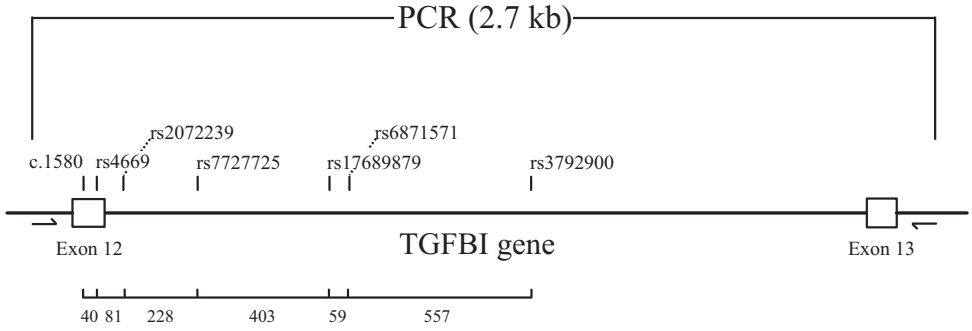

FIGURE 2. The genomic structure for the *TGFBI* gene with sites of mutation and SNPs investigated. *Arrows*: PCR primers that amplify a fragment containing all the SNP and mutation sites. The physical distance between two neighboring SNPs is also indicated in base lengths at the *bottom*.

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.

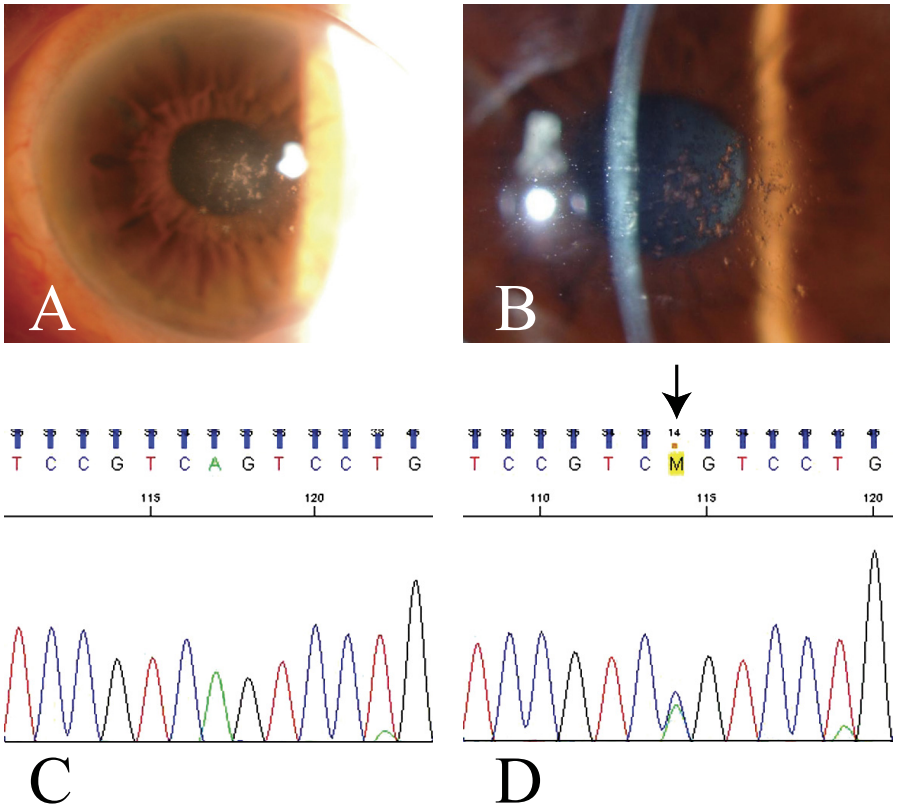

FIGURE 3. Results of the mutation analysis of the 13 LCD4 patients. Scleral scattering (**A**) and iris retroillumination (**B**) demonstrate the corneal opacity in LCD4. The depth of opacity was nearly at the level of Descemet's membrane. Compared with sequence data from normal volunteers (**C**), LCD4 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

		LCD4 Patients	
Haplotype	Normal Volunteers Healthy Allele	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

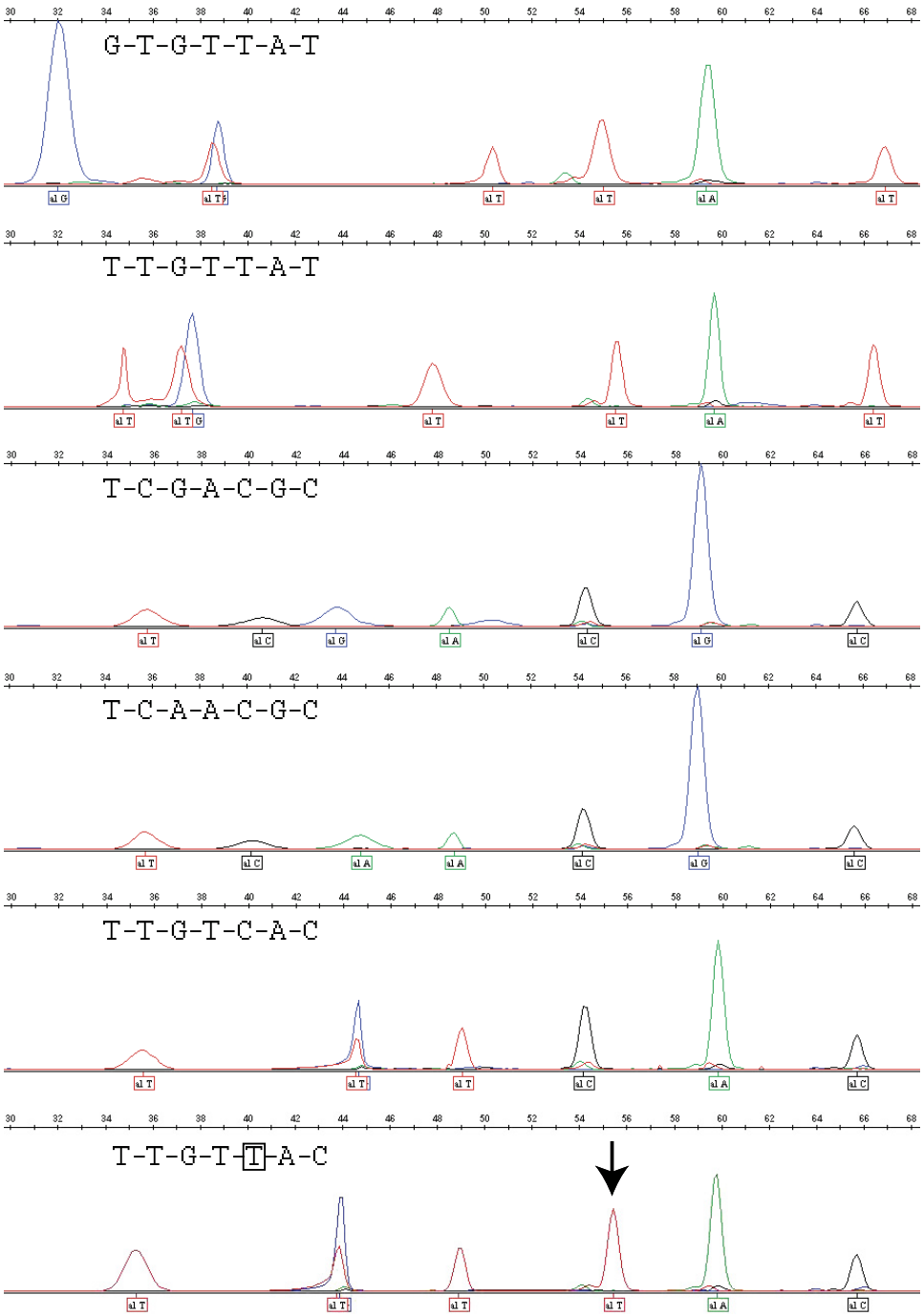

FIGURE 4. Results of the 1-base primer extension assay. Shown are representative chromatogram data for all the observed haplotypes, as indicated at the *top left* corners. The first base (G or T) is of the site of the mutation (c.1580), not of the SNP. The T-T-G-T-T-A-C haplotype is an artificial product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

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